Interactions entre ciliés et métazoaires dans deux environnements marins contrastés : les sources hydrothermales et les sédiments anoxiques

Anne-Laure Sauvadet

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Thèse de Doctorat de l'Université Pierre et Marie Curie

Spécialité Biologie marine
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Présentée par
Mlle Anne-Laure Sauvadet

Pour obtenir le grade de
Docteur de l'Université Pierre et Marie Curie

Sujet de la thèse :

Interactions entre ciliés et métazoaires dans deux environnements marins contrastés : les sources hydrothermales et les sédiments anoxiques

Soutenue le Lundi 25 Octobre 2010, devant le jury composé de :

Denis H. Lynn, Professeur University of Guelph, Canada	Rapporteur
Nicole Dubilier, Directeur de recherches Max Planck Institute, Allemagne	Rapporteur
Joseph Schrével, Professeur Muséum National d'Histoire Naturelle/CNRS, Paris	Examinateur
Hervé Moreau, Directeur de recherches Observatoire océanologique de Banyuls/CNRS-UPMC	Examinateur
Pierre De Puytorac, Professeur Université de Clermont-Ferrand	Invité
Laure Guillou, Chargée de recherches Station Biologique de Roscoff/CNRS-UPMC	Directrice de Thèse

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ABBREVIATIONS

ADN	Acide DesoxyriboNucléique
ARN	Acide RiboNucléique
ARNr 18S	Sous-unité 18S de l'ARNr eucaryote
ARNr 28S	Sous-unité 28S de l'ARNr eucaryote
ARNr 16S	Sous-unité 16S de l'ARNr procaryote
BET	Bromure d'éthidium
DAPI	4',6'-diamidino-2-phénylindole
EOL	Encyclopedia Of Life
EPR	East Pacific Rise (dorsale orientale de l'océan Pacifique)
FISH	Fluorescence *in situ* Hybridization
HMMV	Hakon Mosby Mud Volcano
IFREMER	Institut français de recherche pour l'exploitation de la mer
ITS	Internal Transcribed Space
MAR	Mid-Atlantic Ridge (Ride médio-Atlantique)
MEB/T	Microscopie électronique à balayage/transmission (SEM/TEM en anglais)
PCR	Polymerase Chain Reaction
RDA	Redundancy Analysis
RFLP	Restriction Fragment Length Polymorphism
SAR	Stramenopiles-Alveolata-Rhizaria

Introduction Générale : Les eucaryotes unicellulaires

LES EUCARYOTES UNICELLULAIRES

Les eucaryotes que l'on peut voir évoluer, les plantes et les animaux principalement, sont macroscopiques et constitués de millions de cellules individuelles. Ces eucaryotes pluricellulaires représentent une diversité qu'il nous est relativement facile d'apprécier, par opposition aux eucaryotes microscopiques et unicellulaires. Ernst Haeckel en 1866 proposait le taxon Protista pour grouper ces eucaryotes unicellulaires (Haeckel, 1866). Le terme protiste est utilisé aujourd'hui de façon courante.

Les Eucaryotes (*Eukaryota*, *Eukarya* ; Chatton, 1925) sont l'un des trois domaines reconnus du vivant, avec les Archées et les Bactéries (*Archaea*, *Bacteria* ; Woese *et al.*, 1990) (Figure 1). Ils se distinguent principalement par un degré supérieur de complexité et d'organisation. En effet, les eucaryotes possèdent un cytosquelette et un système de compartiments délimités par des membranes. Le terme eucaryote, du grec « eu » : bien/vrai, et « karuon » : grain/noyau, fait référence à l'isolement du matériel nucléaire dans un compartiment – le noyau – délimité par une enveloppe.

Les eucaryotes ont développé des caractères aussi bien structurels que comportementaux. Leur caractéristique la plus significative est la capacité d'absorber et d'internaliser des particules et d'autres cellules, un processus appelé endocytose ou phagocytose. Si les Archées et les Bactéries montrent une diversité importante dans leurs capacités métaboliques, leur diversité morphologique et comportementale reste limitée. Au contraire, bien que les eucaryotes partagent des fonctions métaboliques semblables, ils ont subi une diversification très importante dans leur morphologie et leur comportement. L'apparition de l'endocytose chez la cellule eucaryote ancestrale a généré un nouveau degré de complexité, l'endosymbiose. Il s'agit d'un processus par lequel une cellule est capturée par une autre cellule, et conservée. Les deux cellules vivent alors ensembles, parfois de façon permanente. Les interactions endosymbiotiques se sont généralisées au cours de l'évolution de la cellule eucaryote et de telles associations persistent encore aujourd'hui (Margulis, 1971). Deux cas d'endosymbioses ont marqué l'évolution

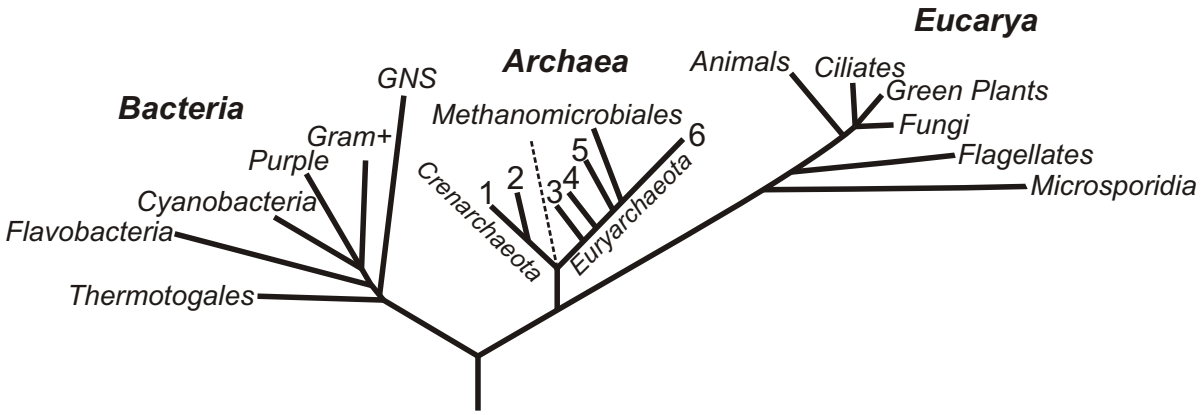

FIG. 1 – Arbre universel du vivant, représentant les trois domaines *Bacteria*, *Archaea* et *Eucarya*, basé sur l'analyse des séquences de la petite sous-unité du ribosome, 18S ARNr (eucaryotes) et 16S ARNr (procaryotes). *Bacteria* : GNS, Green non sulfur bacteria. *Archaea* : 1, genre *Pyrodictium* ; 2, genre *Thermoproteus* ; 3, *Thermococcales* ; 4, *Methanococcales* ; 5, *Methanobacteriales* ; 6, Halophiles extrêmes (d'après Woese *et al.*, 1990).

des eucaryotes : l'origine de la mitochondrie et celle des plastes (voir 3.1).

Les relations entre les différents organismes eucaryotes ont également été déterminées grâce à l'utilisation des données moléculaires (Sogin *et al.*, 1986). La diversité des eucaryotes unicellulaires avait jusqu'alors été sous-estimée et leur filiation avec les eucaryotes multicellulaires étaient difficiles à résoudre (Taylor, 1978). Les premières phylogénies moléculaires basées sur la petite sous-unité de l'ARN ribosomal ont suggéré une cime (crown) de l'arbre phylogénétique composée des groupes multicellulaires (i.e. animaux, plantes et champignons) avec une base d'origine microbienne (Sogin, 1989). La phylogénie des eucaryotes actuelle basée sur les études morphologiques et moléculaires, propose six « super-groupes », chacun composé d'organismes ayant une diversité structurelle, nutritionnelle et comportementale (Adl *et al.*, 2005; Roger and Simpson, 2009) (Figure 2). Les relations entre ces super-groupes (i.e. Opisthokonta, Amoeobozoa, Archaeplastida, Chromalveolata, Rhizaria, Excavata) sont encore mal comprises.

Opisthokonta

Les opisthocontes (Figure 3) regroupent les animaux ou métazoaires et les champignons, avec les organismes unicellulaires qui leurs sont affiliés. En effet, ces deux lignées ont au moins deux taxa unicellulaires qui leur sont proches : les Mesomycetozoea et les choanoflagellés pour les métazoaires, les nucleariides et les microsporidies pour les champignons (pour revue, Adl *et al.*, 2005; Baldauf, 2008). Les opisthocontes ont plusieurs caractéristiques qui leurs sont propres : presque toutes les cellules possèdent des mitochondries à crêtes, et les cellules flagellées ont un unique flagelle inséré dans la partie postérieure de la cellule (unicont ; Cavalier-Smith, 2002).

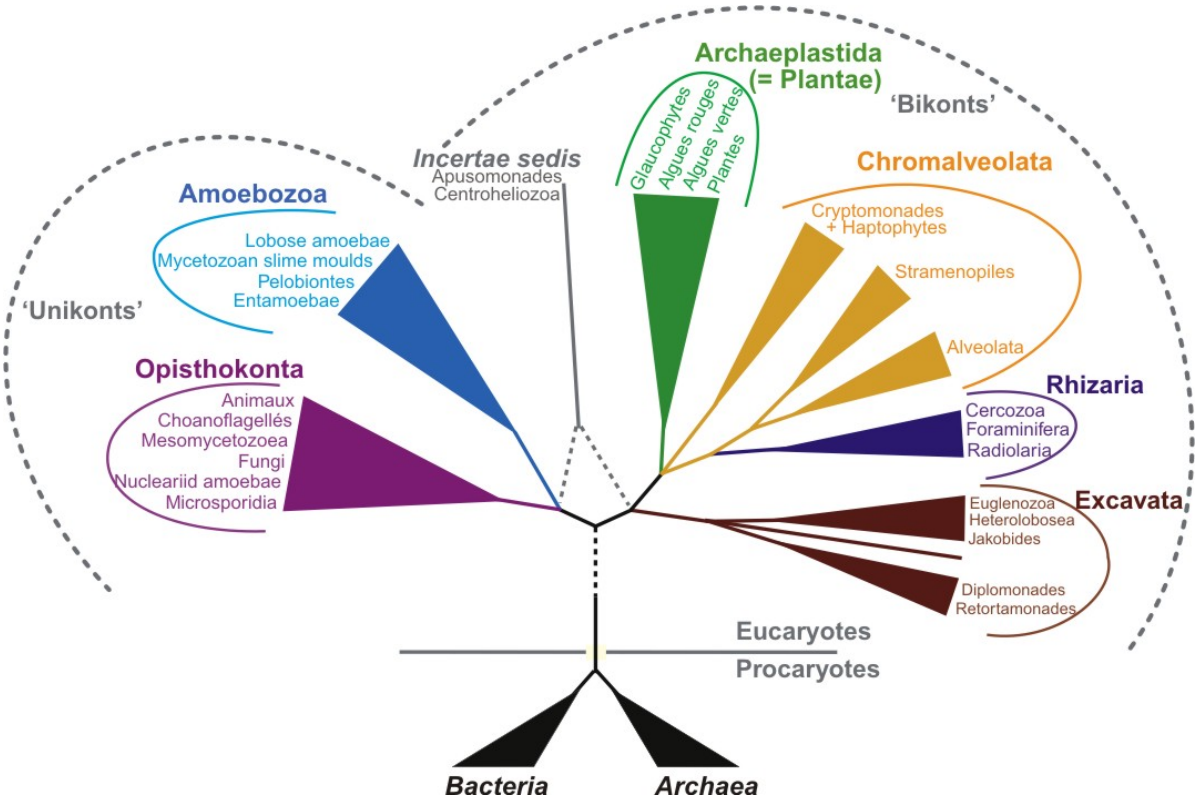

FIG. 2 – Arbre phylogénétique des Eucaryotes. Cet arbre est enraciné selon l'hypothèse des organismes unicontes (cellule eucaryote à un seul flagelle) et bicontes (cellule eucaryote à deux flagelles). Les branches en pointillés indiquent une incertitude quant à la place des groupes (d'après Roger and Simpson, 2009).

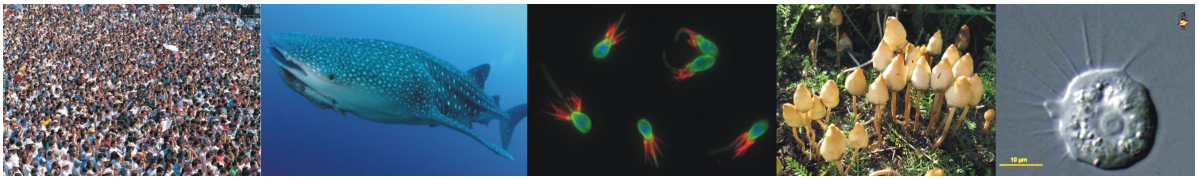

FIG. 3 – Les Hommes ; Requin baleine *Rhincodon typus*, P. Schinck, Fotolia ; Choanoflagellés caractérisés par un flagelle (vert) utilisé pour nager et s'alimenter, entouré par un col de tentacules (rouge) qui piège les proies bactériennes. Le noyau de la cellule est coloré en bleu, N. King lab/UC Berkeley ; Fungi, *Psilocybe semilanceata*, Mycotopia ; Nucleariide, *Nuclearia thermophila*, M. Bahr et D. Patterson, Micro∗scope.

Amoebozoa

Les amoebozoa (Figure 4) sont constitués d'eucaryotes unicellulaires, dont la majorité est sous forme amibienne (i.e. cellule sans flagelle, produisant des pseudopodes) pour partie ou totalité de leur cycle de vie. De nombreux amoebozoa produisent des pseudopodes en forme

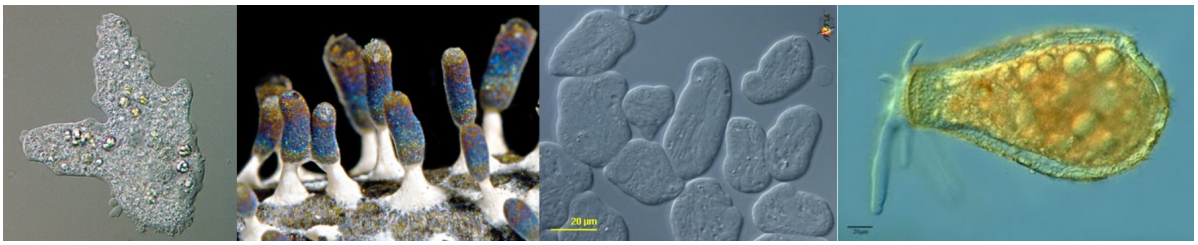

FIG. 4 – *Chaos carolinensis*, MicroscopyU©, Florida State University ; *Diachea leucopodia*, R. Darrah, Collection Eumycetozoan Project Image Gallery ; *Entamoeba* sp., D. Patterson, L. Amaral Zettler, M. Peglar et T. Nerad, American Type Culture Collection, 2001, Micro∗scope ; *Nebela tubulosa*, Arcellinida, amibe à thèque, A. Guillen.

d'éventail, contrastant avec les fins pseudopodes allongés typiques des Rhizaria (voir le paragraphe Rhizaria). Le super-groupe des Amoebozoa inclut les amibes « nues », les amibes à thèques dont la cellule est enfermée dans une sorte de coquille, des moisissures dites glaireuses (i.e. « slime moulds »), les pelobiontes et les entamoeba, qui ont perdu leur mitochondrie. Leur taille peut aller de quelques microns à plusieurs millimètres. Ces amoebozoa sont souvent rencontrés en milieux humides ou aquatiques, et certains sont pathogènes, comme *Entamoeba histolytica* responsable de dysenteries.

Archaeplastida (ou Plantae)

FIG. 5 – *Glaucocystis* sp., `http://www.isufol.net/auras/html/sc_glaucophyta.htm` ; *Callophyllis* sp., Rhodophyta J. Watanabe ; *Acetablaria ryukyuensis*, Chlorophyta, T. Demeulemeester ; Arbres et fougères en forêt.

Les Archaeplastida (Figure 5) comprennent les glaucophytes qui sont des organismes unicellulaires de formes variées (e.g. biflagellé, coccoïde…), les algues rouges ou rhodophytes, les algues vertes, et les plantes. Ils ont pour caractéristique générale de posséder un ou plusieurs plastes, qui ont pour origine une endosymbiose primaire (voir 3.1).

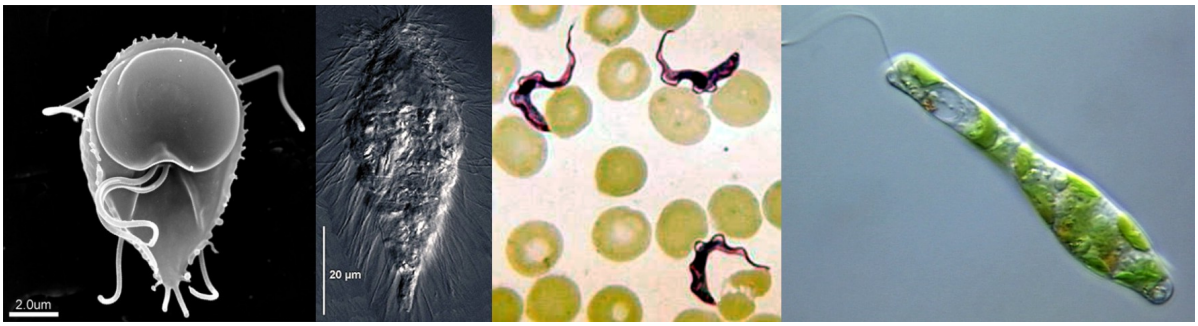

FIG. 6 – *Giardia lamblia*, J. Mancuso ; *Spirotrichonympha flagellata* de la termite *Reticulitermes lucifugus grassei*, G. Brugerolle, Micro∗scope ; *Trypanosoma brucei* parmi des globules rouges, Dr. M. G. Schultz, Encyclopedia of Life (EOL) ; *Euglena gracilis*, bio.rutgers.edu.

Excavata

Le groupe des excavates (Figure 6) est constitué d'organismes unicellulaires très divers qui ont une structure ventrale de nutrition dont il tire leur nom (*excavation*) (Simpson and Patterson, 1999). Certains des organismes de ce groupe ont perdu cette structure mais les analyses phylogénétiques ont démontré leur apparenté (e.g. Simpson *et al.*, 2006). Certains excavates tels que les diplomonades et les parabasalides sont anaérobies ou microaérophiles et possèdent donc des structures spécifiques remplaçant la mitochondrie (voir 3.2). D'autres excavates sont connus comme parasites d'animaux (e.g. Trypanosomes, *Giardia*, *Leishmania*), et la lignée des euglènes contient des organismes photosynthétiques possédant des plastes dérivés d'une algue verte par endosymbiose secondaire (Leander *et al.*, 2007).

Rhizaria

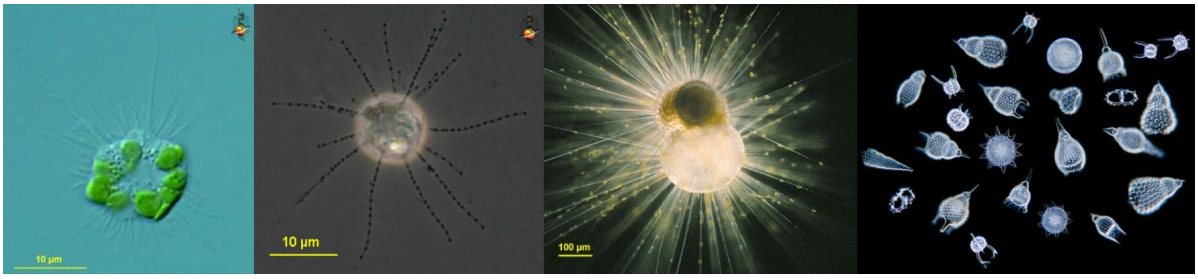

FIG. 7 – *Gymnochlora* sp., Chlorarachniophytes, Cercozoa, D. Patterson et B. Andersen, Micro∗scope ; *Massisteria* sp., Cercozoa, D. Patterson, Massachusetts, 2001, Micro∗scope ; *Globigerinoides* sp., Foraminifère, O. R. Anderson, Micro∗scope ; Radiolaires, Micropolitan Museum.

Les Rhizaria (Figure 7) constituent un groupe important d'organismes ubiquistes, comprenant de nombreux prédateurs. Les principales lignées sont les Cercozoa, les foraminifères et

les radiolaires, dont le regroupement est surtout basé sur les analyses phylogénétiques car les caractéristiques structurales de ces organismes sont très diverses (Nikolaev *et al.*, 2004). Le groupe des Cercozoa contient des organismes différents sans distinction morphologique : on retrouve des flagellés amibiens, des cellules secrétant des écailles de silices à leur surface ou encore des amibes ayant acquis des plastes par endosymbiose secondaire. Les foraminifères ont pour majorité un test d'origine organique ou minérale (calcaire). Ils sont très variés en forme et en taille, et sont distribués dans tous les types d'environnements marins, de la colonne d'eau au sédiment. Les radiolaires sont également des organismes exclusivement marins entourés d'un squelette minéral duquel part de fins pseudopodes. Ils incluent principalement les Acanthaires dont le squelette est composé de sulfate de strontium, et les polycystines dont le squelette minéral varie de simples spicules à des structures complexes. Les tests fossiles des foraminifères et des radiolaires sont utilisés en paléontologie et paléocéanographie.

Chromalveolata

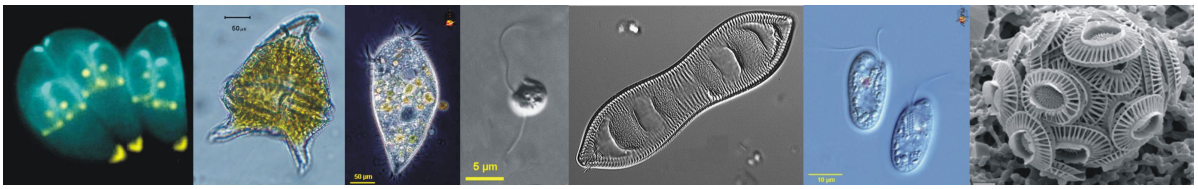

FIG. 8 – *Toxoplasma gondii*, Apicomplexe, K. Hu et J. Murray, PLOS One ; *Peridinium limbatum*, Dinoflagellé, Micro∗scope ; *Stentor* sp., Cilié, D. J. Patterson et A. Ladermann, Collection Cumloden, Micro∗scope ; *Pseudobodo tremulans*, Stramenopile, W. Je Lee, EOL ; *Cymatopleura solea*, Stramenopile, W. Smith, California Academy of Sciences ; *Chilomonas* sp., Cryptomonade, W-S. Feng et D. J. Patterson, Micro∗scope ; *Emiliania huxleyi*, Haptophyte, T. Tyrrell.

Les Chromalveolata (Figure 8) comprennent six groupes : les apicomplexes, les dinoflagellés, les ciliés, les straménopiles, les cryptomonades - flagellés communs des eaux douces et marines - et les haptophytes, producteurs primaires abondants notamment dans les environnements marins (Keeling, 2009). La réunion de ces organismes dans un même groupe est basée sur la présence de plastes dans de nombreux organismes, qui auraient dérivés d'une algue rouge par endosymbiose secondaire (voir 3.1). Les stramenopiles, ou hétéroconthes, forment un groupe divers de parasites, d'algues et d'hétérotrophes retrouvés dans de nombreux environnements. Ce groupe est constitué d'une lignée basale exclusivement composée d'organismes non-photosynthétiques (e.g. oomycètes, bicosoécides), à partir de laquelle toutes les lignées photosynthétiques ont émergé (e.g. diatomées, chrysophytes), avec parfois une perte ultérieure du plaste.

Les ciliés, les dinoflagellés et les apicomplexes s'inscrivent dans le groupe des Alveolata (Figure 9). Les alvéolés sont caractérisés par la présence d'alvéoles corticales, vésicules aplanies empaquetées dans une couche continue soutenant la membrane. Les Apicomplexes sont

des parasites obligatoires qui possèdent un complexe apical utilisé lors de l'infection et/ou l'attachement à la cellule hôte. Ils infectent préférentiellement les cellules animales, le genre le plus connu est *Plasmodium*, l'agent responsable de la malaria chez l'Homme. Au contraire, les dinoflagellés représentent un groupe très divers : beaucoup sont prédateurs ou parasites, certains sont phototrophes et jouent donc un rôle important dans la fixation du carbone, mais d'autres sont responsables d'efflorescences toxiques. Certains dinoflagellés sont protégés d'une thèque rigide formée de plaques cellulosiques. Le groupe des ciliés (i.e. Ciliophora ou Ciliata) est d'après différentes analyses phylogénétiques le groupe le plus divergent des alvéolés (Figure 9) (pour revue, Leander and Keeling, 2003). C'est un groupe important et relativement homogène d'eucaryotes unicellulaires principalement caractérisés par la dualité de leur appareil nucléaire, ainsi que par la présence de cils vibratils à leur surface, à au moins un stade de leur cycle de vie.

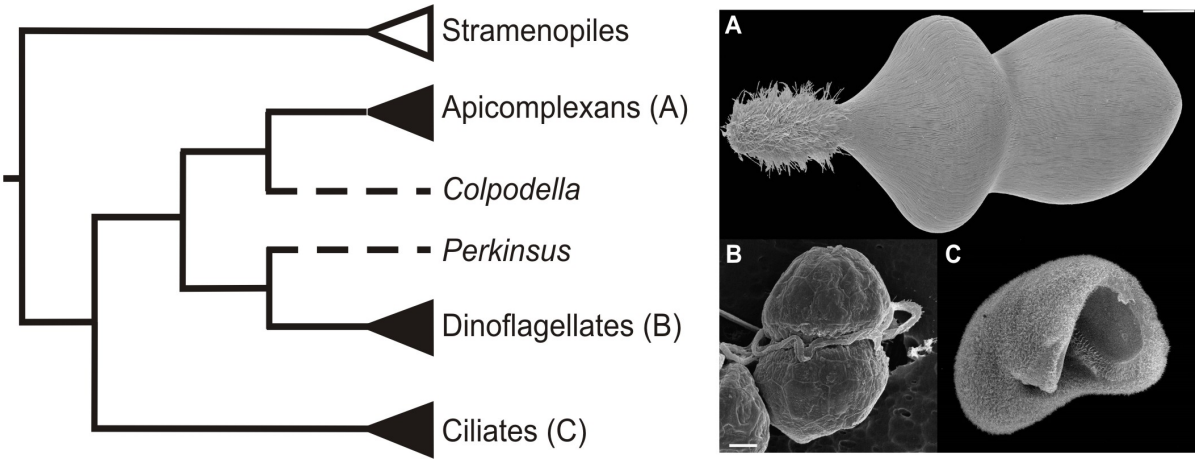

FIG. 9 – Arbre phylogénétique schématisé des alvéolés. Des analyses phylogénétiques indépendantes sur différents jeux de séquences montrent que les ciliés ont divergé avant la radiation des apicomplexes et des dinoflagellés (d'après Leander and Keeling, 2003). **A**, *Trichotokara nothriae* (Rueckert and Leander, 2010) ; **B**, *Pelagodinium béii* (Siano *et al.*, 2010) ; **C**, *Bursaria* sp., Aaron Bell, Ciliate Image Database©, `http://research.plattsburgh.edu/ciliates/`.

Aujourd'hui, les phylogénies moléculaires appuieraient l'hypothèse que les Alveolata et les Stramenopiles seraient groupés avec les Rhizaria pour former le groupe SAR (Stramenopile-Alveolata-Rhizaria). Alors que les cryptomonades et les haptophytes seraient plus proches des Archaeplastida (Hackett *et al.*, 2007; Burki *et al.*, 2007; Burki *et al.*, 2008; Archibald, 2009; Parfrey *et al.*, 2010). Dernièrement, un nouveau taxon nommé *Hacrobia* a été proposé, regroupant les Haptophyta, les Centroheliozoa, les Cryptophyta, les Katablepharida, les Picobiliphyta et les Telonemia (Okamoto *et al.*, 2009).

Partie I
Interactions dans des écosystèmes marins : le cas des eucaryotes unicellulaires

CHAPITRE 1

LES SOURCES HYDROTHERMALES ET LES SÉDIMENTS MARINS : DEUX MILIEUX CONTRASTÉS

Les eucaryotes unicellulaires peuvent coloniser le sol, les sédiments, les milieux marin, lacustre et dulçaquicole, bien qu'ils soient retrouvés essentiellement en milieu aqueux et surtout marin, où ils jouent un rôle important dans les réseaux trophiques océaniques et interviennent dans les différents cycles biogéochimiques. L'environnement marin offre des niches écologiques variés, et deux d'entres elles caractérisées par de forts gradients d'oxydo-réduction ont été sélectionnées pour cette étude : les sources hydrothermales et les sédiments marins côtiers. Ces deux systèmes dépendent de la présence de microorganismes chimiosynthétiques à la base de leur réseau trophique, mais sont également différenciables de par l'origine de la matière organique : inorganique dans le cas des sources hydrothermales et principalement produit par l'activité photosynthétique dans le cas des sédiments côtiers. Ces niches abritent également des métazoaires particuliers, en général endémiques.

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La surface de la Terre est recouverte à 70 % par les océans. Le relief des fonds océaniques est formé par la tectonique des plaques, et se divise en deux grandes parties : la marge continentale et le bassin océanique, caractérisés par de fort taux de sédimentation. La marge continentale correspond à la zone s'étendant du rivage jusqu'à la plaine abyssale. Elle peut-être passive ou active en fonction de l'activité tectonique existante. Le bassin océanique est formé de la plaine abyssale de 4000 à 6000 mètres de profondeur, et des crêtes ou dorsales médio-océaniques où se forment les sources hydrothermales de 2000 à 3000 mètres (Figure 1.1).

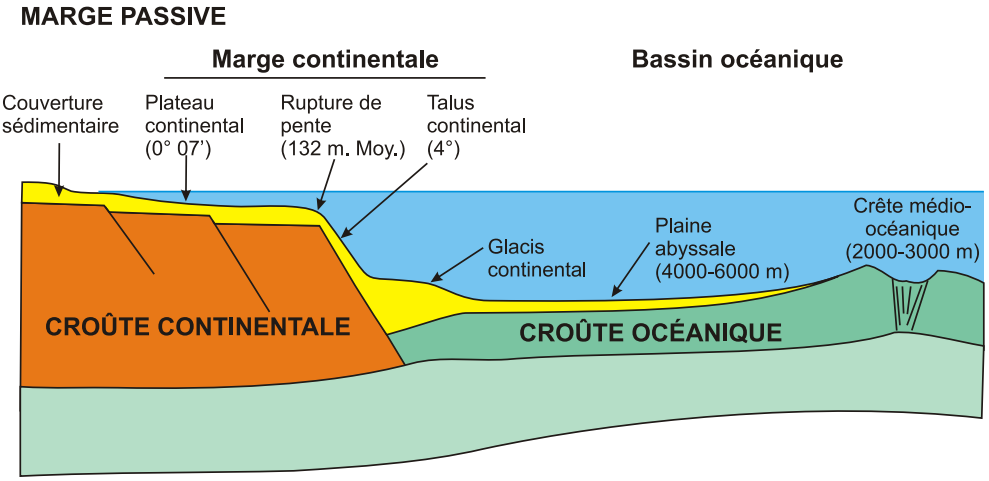

FIG. 1.1 – Schéma du relief des fonds océaniques (Université de Laval, `http://www2.ggl.ulaval.ca/personnel/bourque/intro.pt/planete_terre.html`).

1.1 Les sources hydrothermales

1.1.1 Caractéristiques

La découverte des sources hydrothermales profondes a eu lieu dans les années 1970, lors de l'exploration par le sous-marin américain *Alvin* de la dorsale océanique des îles Galápagos à 2500 m de profondeur. Les scientifiques ont alors découvert une faune abondante répartie autour des sources d'eau chaude, et notamment des communautés riches en bivalves, grands vers et autres métazoaires (Lonsdale, 1977; Corliss and Ballard, 1977; Corliss *et al.*, 1979).

Ces sources sont retrouvées au niveau de zones à forte activité tectonique (Figure 1.2), des zones d'expansion ou d'accrétion du plancher océanique. Ces dorsales océaniques s'étendent sur près de 60 000 km, entre 500 et 4000 m de profondeur, avec une moyenne de 2500 m de profondeur. Ces zones d'activité sont caractérisées par leur vitesse d'expansion qui varie d'un type de dorsale à un autre (Kelley *et al.*, 2002; Edmonds *et al.*, 2003). Elles peuvent être de trois types (Figure 1.3) :

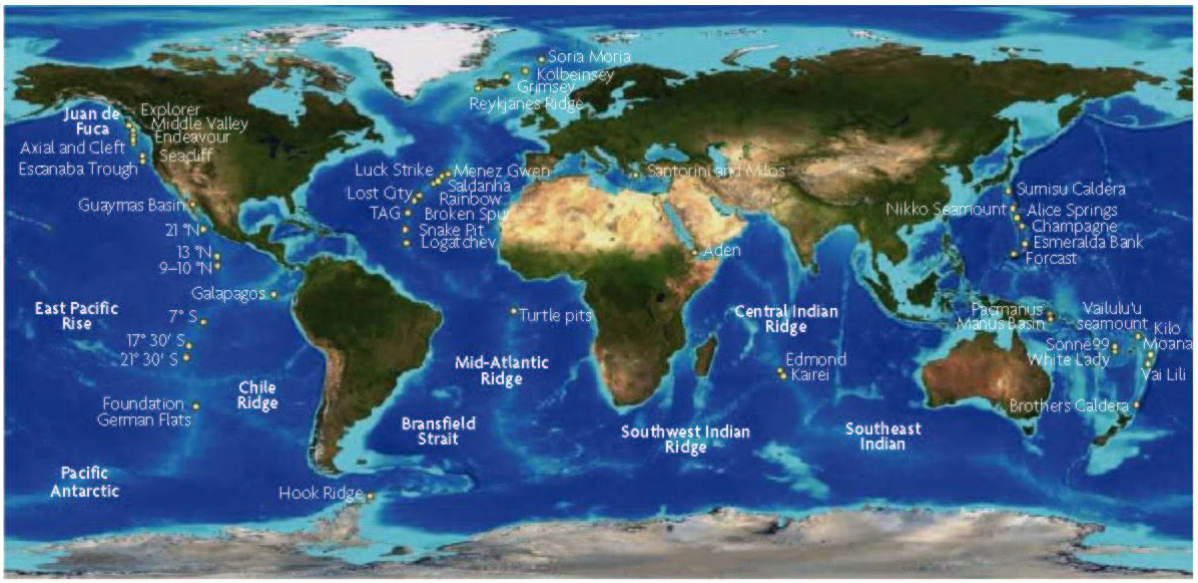

FIG. 1.2 – Localisation des sites hydrothermaux répertoriés (Martin *et al.*, 2008).

– lente, avec une expansion inférieure de 10 à 20 mm/an, sur la Ride Médio-Atlantique ou MAR (Mid Atlantic Ridge) et la ride de l'océan Indien,

– intermédiaire, de 50 à 80 mm/an, sur la Ride Est Pacifique ou EPR (East Pacific Rise) et le rift des Galápagos, et

– rapide, avec une expansion de 110 à 150 mm/an, sur certaines dorsales de l'EPR situées entre 9°N-10°N et 13°N.

Les sources hydrothermales profondes résultent de l'infiltration d'une eau de mer dense et froide (2°C) sur plusieurs kilomètres à travers les fissures de la croûte océanique, rendue fragile par l'activité tectonique (Jannasch, 1995). Il a d'ailleurs été estimé que le volume entier des océans circulait à travers la croûte environ une fois tous les millions d'années (Edwards *et al.*, 2005). A l'approche de la chambre magmatique et au contact des roches en fusion, l'eau s'échauffe et diminue en densité (pour revue, Kelley *et al.*, 2002). Sous l'effet des pressions hydrostatiques et lithosphériques, le fluide surchauffé remonte à la surface en lessivant les roches basaltiques et se charge ainsi en ions métalliques (e.g. Cu^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Si^{+}) et en gaz dissous (e.g. H_2, CH_4, CO, CO_2, H_2S) (Von Damm, 1995). Le fluide peut alors atteindre des températures supérieures à 350°C et jaillir au travers du plancher océanique sous la forme des sources hydrothermales. Les propriétés physico-chimiques de ce fluide hydrothermal anoxique et souvent acide sont sans équivalent dans les milieux côtiers et contrastent fortement avec celles de l'eau de mer caractérisée par un pH à 7,8 et la présence de certains composés comme : O_2, Mg^{2+}, NO_3^-, SO_4^{2-}, PO_4^{3-} (Figure 1.4) (Lilley *et al.*, 1993).

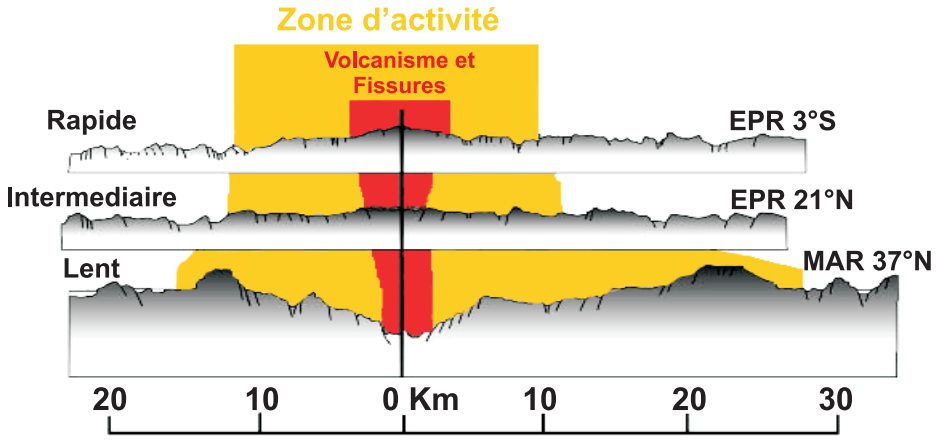

FIG. 1.3 – Les trois types de dorsales océaniques. EPR, East Pacific Rise ; MAR, Mid-Atlantic Ridge (d'après Kelley *et al.*, 2002).

La notion d'extrême, entre minima et maxima

Tous les organismes sont soumis à des contraintes environnementales qui peuvent être :

- physiques : température, pression, irradiation
- biologiques : nutrition, densité de population, parasitisme et prédation
- géochimiques : pH, salinité, fortes concentrations en métaux, produits toxiques, oxygène

Pour la plupart de ces paramètres, il est possible de déterminer des valeurs minimales, optimales et maximales qui conviennent à un organisme donné. La notion de condition extrême n'est relative qu'à l'organisme étudié. A ce jour, il existe une limite au delà de laquelle on ne connaît pas encore d'organisme vivant. Par exemple, jusqu'à présent, 122°C semble être la température maximale à laquelle un organisme peut se développer (Takai *et al.*, 2008). Ces limites ont été encore repoussées par la découverte d'*Archaea* viables dans des sédiments marins profonds âgés de 111 millions d'années à plus de 1600 mètres sous la surface du plancher océanique sous plus de 4000 mètres d'eau (Roussel *et al.*, 2008).

Il n'y a que quelques dizaines d'années que l'exploration d'environnements terrestres, aquatiques, et océaniques a permis de découvrir des organismes adaptés à des conditions particulières.

1.1.2 Oasis de vie

Malgré ces conditions environnementales, la biomasse des systèmes hydrothermaux est 500 à 1000 fois supérieure à celle estimée en milieu abyssal. La faune se distribue selon un schéma concentrique autour des émissions hydrothermales, en fonction de la capacité de résistance et

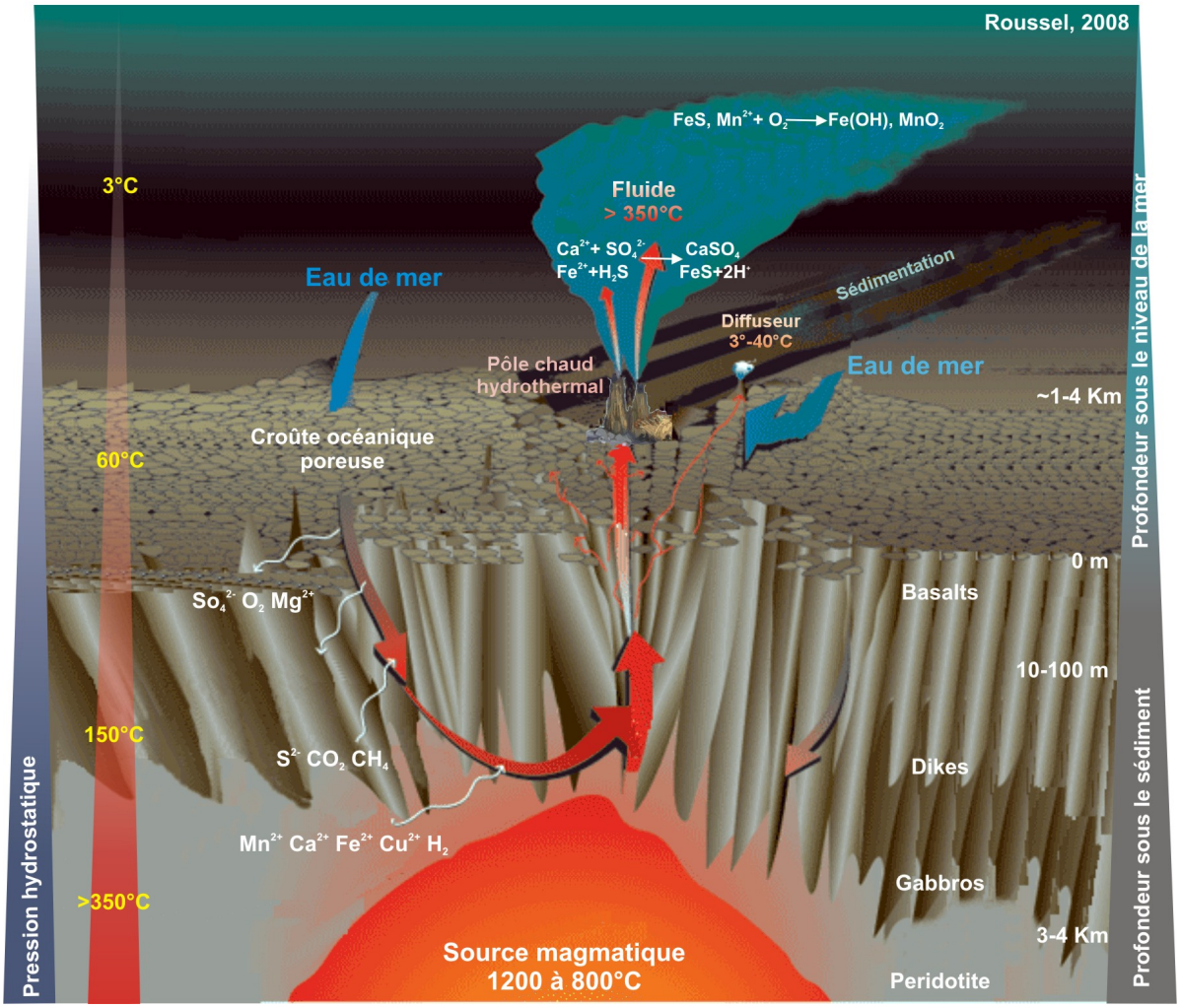

FIG. 1.4 – Caractéristiques physique et chimique des fluides d'un système hydrothermal circulant à travers la croûte océanique (d'après Roussel, 2008; Jannasch, 1995; Kelley *et al.*, 2002).

des besoins trophiques des organismes qui colonisent ces sources.

Les sources hydrothermales sont caractérisées par une chaîne alimentaire basée sur la présence de procaryotes sous formes libres ou symbiotiques. En effet, en absence totale de lumière, le réseau trophique de ces écosystèmes repose presque exclusivement sur la chimiosynthèse microbienne, soit la fixation de CO_2 en absence de lumière (Cf. encadré Définitions). Les organismes procaryotes obtiennent ainsi leur énergie de l'oxydation des composés réduits présents dans le fluide hydrothermal (Lonsdale, 1977; Jannasch and Wirsen, 1979; Jannasch, 1995).

Ces procaryotes forment des associations symbiotiques avec la plupart des métazoaires hydrothermaux (Figure 1.5) (pour revue, Dubilier *et al.*, 2008), plus particulièrement avec les polychètes tels les Alvinellidae (e.g. Cary *et al.*, 1997), les mollusques comme les bivalves Mytilidae et Vesycomyidae (e.g. Childress *et al.*, 1991) ou encore les crustacés avec les crevettes

Rimicaris (e.g. Zbinden *et al.*, 2008).

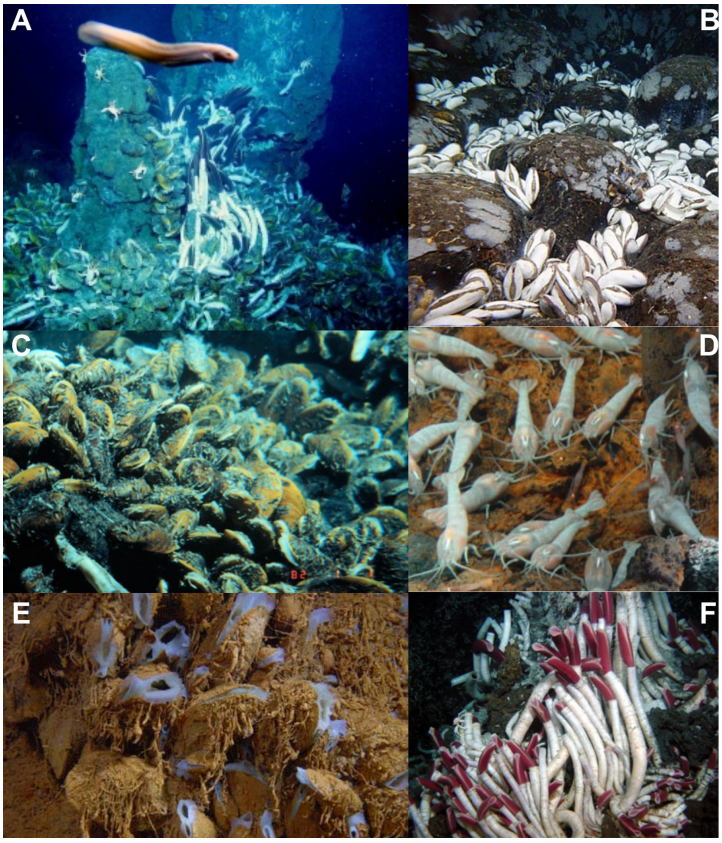

FIG. 1.5 – Exemples de faunes des sources hydrothermales. **A**, Massif de sulfures colonisé par des vers géants, des modioles et des crabes hydrothermaux, EPR, IFREMER. **B**, *Calyptogena magnifica*, Galápagos, T. Shank, Woods Hole Oceanographic Institution. **C**, *Bathymodiolus thermophilus*, EPR, IFREMER. **D**, *Rimicaris exoculata*, Exomar, IFREMER. **E**, *Bathymodiolus azoricus*, site Rainbow MAR, MoMARETO, IFREMER. **F**, Bouquet de *Riftia pachyptila*, Ride du Pacifique oriental, Phare, IFREMER.

Riftia pachyptila, par exemple, est un ver vestimentifère tubicole mesurant jusqu'à 2 mètres de long et de 4 à 5 cm de diamètre. Il s'établit en colonie, pouvant atteindre 100 à 200 individus par m^2, à proximité des cheminées hydrothermales dans des zones de températures allant de 5 à 25°C. *Riftia pachyptila* a été observé le long de la dorsale océanique du Pacifique Est, de la dorsale des Galápagos et dans le bassin de Guaymas. Ce vestimentifère a pour particularité d'être dépourvu de système digestif, mais son tronc loge un organe interne appelé trophosome hébergeant des symbiontes bactériens. Ces symbiontes sont des bactéries sulfo-oxydantes qui transforment les molécules inorganiques d'H_2S, d'oxygène et de CO_2 en sucres assimilables par le ver. L'oxygène et les sulfures nécessaires à la chimiosynthèse des bactéries sont puisés par le ver au niveau de ses filaments branchiaux puis, seraient transportés vers les bactéries grâce à une hémoglobine particulière (Fisher *et al.*, 1988) (Figure 1.6).

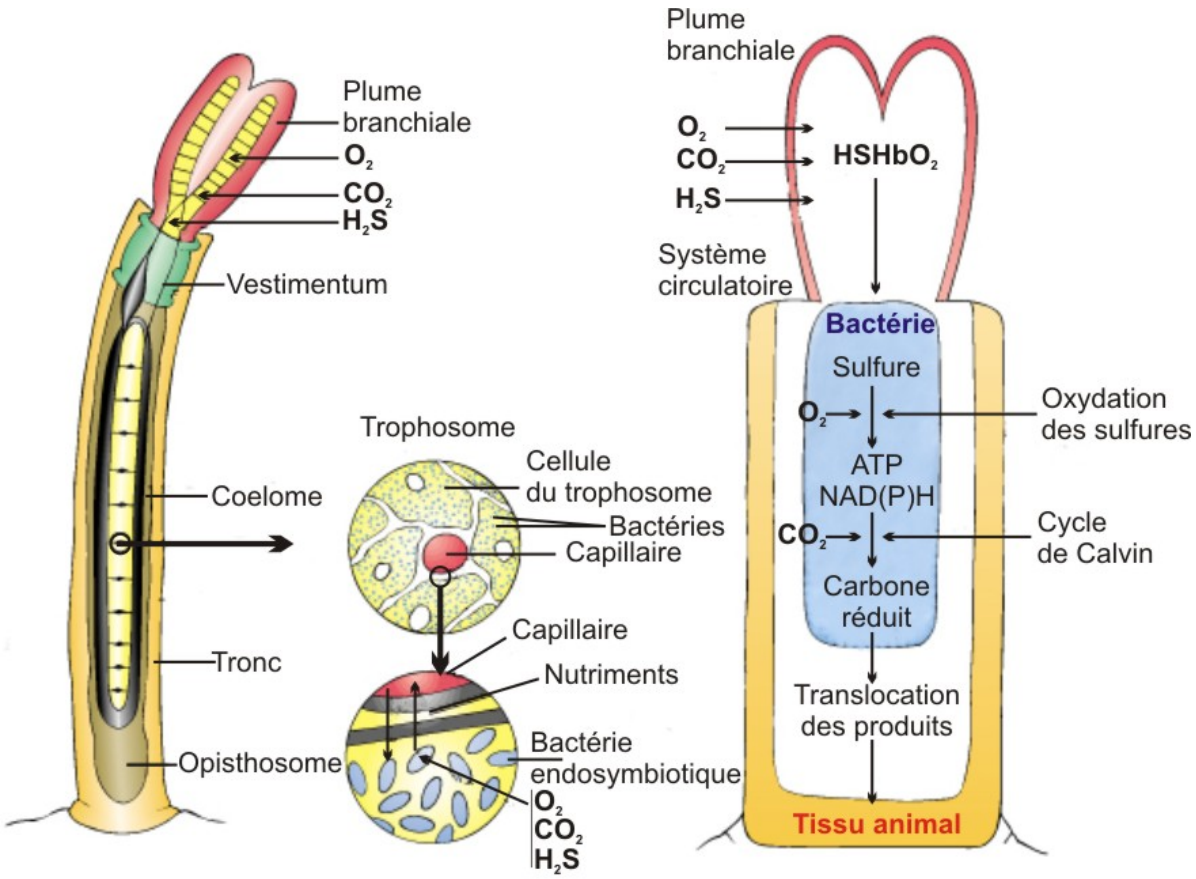

FIG. 1.6 – Schéma d'une coupe transversale du vestimentifère *Riftia pachyptila* illustrant son fonctionnement intracellulaire (d'après `http://cms.daegu.ac.kr/sgpark/life&chemistry/riftia.jpg`).

Les bivalves géants hydrothermaux, formant la sous-famille des Bathymodiolinae, sont un autre exemple d'associations symbiotiques au niveau des sources hydrothermales puisqu'ils abritent également deux types de bactéries chimiosynthétiques. Le métabolisme de ces organismes symbiontes est basé sur l'utilisation de soufre réduit ou de méthane donneur d'électrons, et d'oxygène accepteur d'électrons. Les bactéries soufre-oxydantes (SOB) possèdent le gène *apsA*, qui code pour une enzyme intervenant dans l'oxydation du soufre tandis que les bactéries méthanotrophes (MOB) sont capables d'oxydation aérobie du méthane grâce au gène *pmoA*, et dont l'activité enzymatique a été retrouvée au niveau des branchies des bivalves. Ils possèdent également une RubisCO de Type I qui leur permet une chimioautotrophie (pour revue, Duperron *et al.*, 2009).

Dernièrement, un modèle de réseau trophique a été proposé, basé sur celui obtenu par Tunnicliffe (1991), prenant comme producteur primaire, à la base de ce réseau trophique, les procaryotes libres ou associés, et dans les niveaux supérieurs : des bactérivores, des organismes

symbiotiques, des nécrophages/détritivores et des prédateurs (Figure 1.7 ; Bergquist *et al.*, 2007).

Même si la durée de vie de telles sources n'est pas encore totalement élucidée, elle reste limitée et variable en fonction de leur localisation. En effet, l'âge moyen des sources de la ride Est Pacifique est de quelques décennies (Haymon *et al.*, 1993) alors qu'il peut atteindre quelques milliers d'années pour Lost-City sur la ride médio-Atlantique (Dubinina *et al.*, 2007). Il est également à noter que ce dernier site caractérisé par un pH très alcalin (pH∼11) a très peu de faune associée. Dans tous les cas, si une source hydrothermale s'éteint à la suite de perturbations géologiques, la communauté animale associée disparaît démontrant ainsi la dépendance de ces organismes vis-à-vis de leur niche écologique.

Définitions

Autotrophe (***Autotroph***) Organisme capable de synthétiser tous les constituants cellulaires uniquement à partir de composés inorganiques. Ex : autotrophie au carbone, la source de carbone minéral peut-être le CO_2.

Hétérotrophe (***Heterotroph***) Organisme capable de synthétiser tous les constituants cellulaires uniquement à partir de composés organiques.

Chimio-lithotrophe (***Chemolitotroph***) Organisme qui obtient de l'énergie par oxydation de composés inorganiques (H_2, H_2S, Fe^{2+}, S...), via des réactions endogènes non dépendantes de la lumière.

Chimio-organotrophe (***Chemoorganotroph***) Organisme qui obtient de l'énergie par oxydation de composés organiques (glucose, acétate...).

Mixotrophe (***Mixotroph***) Organisme qui récupère l'énergie de composés inorganiques, mais qui a besoin de composés organiques comme source de carbone.

Phototrophe (***Phototroph***) Organisme qui utilise la lumière comme source d'énergie.

Photoautotrophe (***Photoautotroph***) Organisme phototrophe dont la source de carbone est le CO_2.

Photohétérotrophe (***Photoheterotroph***) Organisme phototrophe dont la source de carbone est organique.

1.1.3 Les eucaryotes unicellulaires dans les environnements hydrothermaux

Les procaryotes et les métazoaires des systèmes hydrothermaux profonds ont été relativement bien étudiés depuis la découverte de ces sources. En revanche, peu d'études décrivent les communautés microbiennes eucaryotes associées aux sources hydrothermales des profondeurs.

Small et Gross (1985) ont observé, pour la première fois, la présence de protistes par microscopie dans des échantillons provenant de la ride Est Pacifique : amibes, chrysophytes, et surtout de nouvelles espèces de ciliés. En effet, six classes de ciliés ont été observées, aussi bien dans des échantillons de substrats artificiels, de roches, que dans l'eau contenue dans les paniers de prélèvements de vers ou de clams. Des forme libres, en conjugaison et enkystées ont été découvertes (Figure 1.8).

Ce n'est que 15 ans plus tard qu'une étude multi-sites est réalisée sur la diversité des

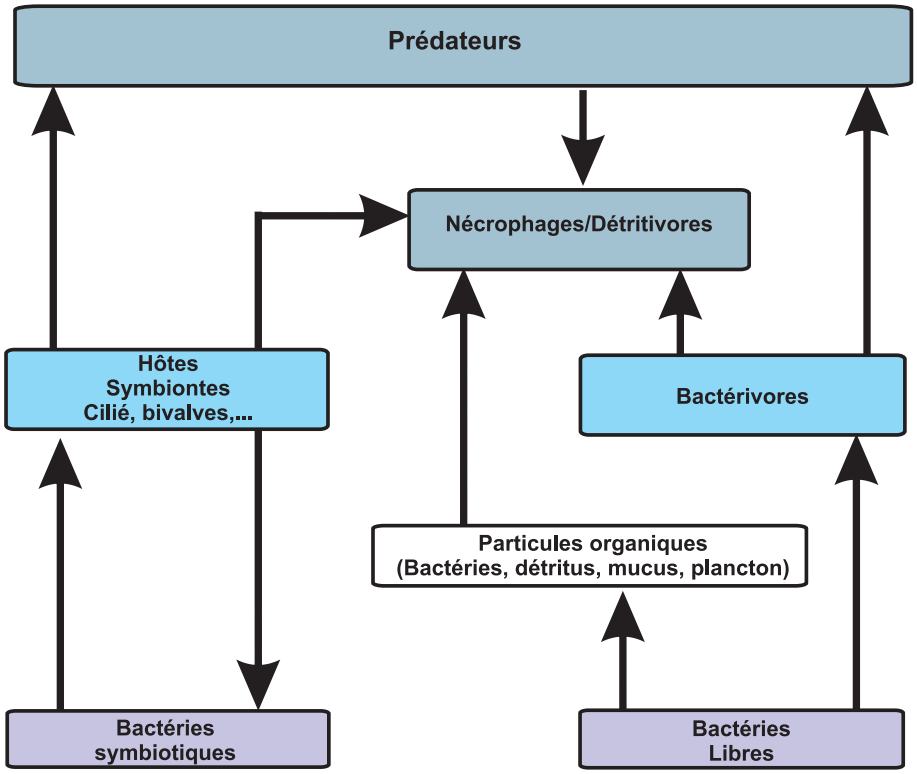

FIG. 1.7 – Exemple de réseau trophique dans un système hydrothermal. Les flèches indiquent la direction du transfert d'énergie (consommation) (d'après Bergquist *et al.*, 2007).

micro-eucaryotes flagellés dans ces environnements (Atkins *et al.*, 2000). Des enrichissements, à partir d'échantillons de quatre sites hydrothermaux de l'océan Pacifique – Juan de Fuca, Bassin de Guaymas, 21°N, et 9°N – ont permis la description morphologique et moléculaire de 18 souches de neuf espèces de flagellés parmi lesquels *Ancyromonas sigmoides* (*incertae sedis* Eukaryota), *Caecitellus parvulus* (Stramenopile) et *Massisteria marina* (Cercozoa). Les études moléculaires ont comparé ces souches « hydrothermales » à des souches témoins de collections ou d'eau de mer côtière, et ont ainsi montré que certaines souches retrouvées au niveau des sources hydrothermales étaient étroitement affiliées à ces souches témoins, suggérant alors une ubiquité et une distribution globale de certaines espèces de flagellés (Atkins *et al.*, 2000).

Quelques études d'isolation et de culture ont permis la description de ciliés anaérobies thermophiles se développant en absence d'oxygène à une température optimale de 48°C jusqu'à une température limite de 52°C (Baumgartner *et al.*, 2002). De même, plus récemment, deux nouvelles espèces d'amibe, *Marinamoeba thermophila* et *Tetramitus thermacidophilus*, ont été isolées et cultivées jusqu'à 50°C et 58°C respectivement, la température optimale de croissance de la première souche étant de 48°C (De Jonckheere *et al.*, 2009; Baumgartner *et al.*, 2009).

Durant les dix dernières années, trois études moléculaires basées sur l'amplification, le clo-

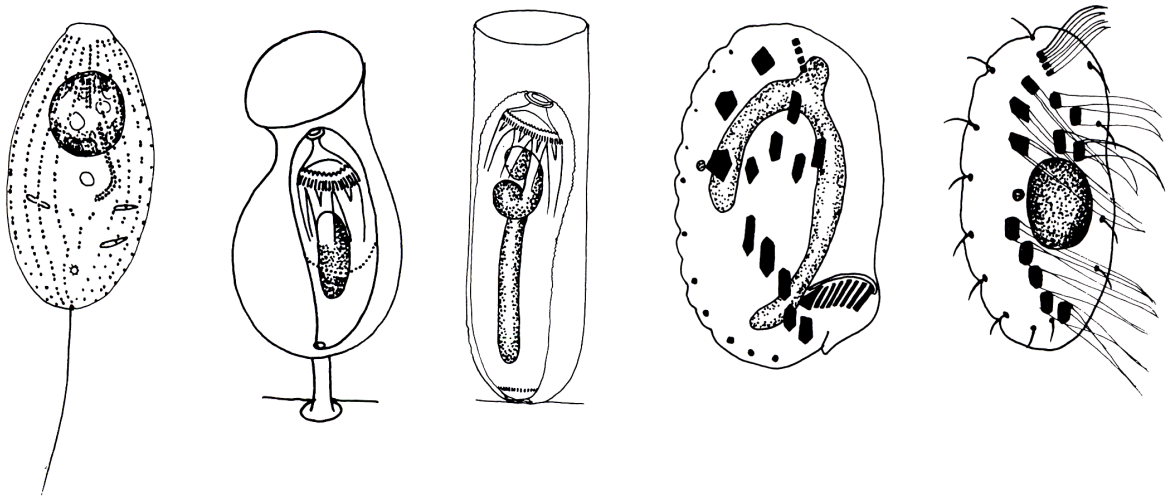

FIG. 1.8 – Dessins de quelques ciliés observés en microscopie sur des échantillons de la ride EPR provenant de *Calyptogena*, *Riftia* ou d'eau hydrothermale (Small and Gross, 1985).

nage et le séquençage du gène codant pour l'ARNr 18S, ont été réalisées dans trois environnements hydrothermaux différents révélant ainsi une étonnante diversité de lignées eucaryotes (Edgcomb *et al.*, 2002; López-García *et al.*, 2003; López-García *et al.*, 2007). La première étude a été effectuée sur les sédiments hydrothermaux du bassin de Guaymas à 2000 mètres de profondeur dans le golfe de Californie, sédiments riches en sulfure et en hydrocarbure. Les analyses ont révélé la présence de lignées *a priori* indigènes et de lignées d'organismes provenant de la couche d'eau supérieure, notamment une grande diversité de lignées photosynthétiques (Edgcomb *et al.*, 2002). La seconde étude décrivait la diversité associée aux sédiments hydrothermaux du site Atlantique Rainbow à 2200 mètres, et la diversité des communautés enrichies sur des substrats artificiels déposés pendant 15 jours dans des colonisateurs placés au niveau des fluides de la cheminée Tour Eiffel du site Lucky Strike à 1600 mètres (López-García *et al.*, 2003). Ces deux sites sont localisés sur MAR, mais les fluides de Rainbow sont très chauds (365°C), acides (pH 2,8) et enrichis en hydrogène, en méthane, et en métaux (Douville *et al.*, 2002). Alors que ceux de Lucky Strike sont généralement moins chauds, environ 200-212°C, mais pouvant aller jusqu'à 333°C pour les fumeurs noirs, et riches en sulfure d'hydrogène (Langmuir *et al.*, 1997). A l'inverse de l'étude des sédiments du bassin de Guaymas, aucune lignée photosynthétique n'a été détectée, suggérant des groupes spécifiques de l'océan profond (López-García *et al.*, 2003).

Une diversité phylogénétique importante a été démontrée au niveau des sédiments, même si les micro-colonisateurs ont permis principalement d'amplifier des séquences de bodonides Excavata et de ciliés. Les Alveolata semblent ainsi dominer l'océan profond en termes de diversité. Les séquences qui leurs sont affiliées sont aussi bien retrouvés dans le plancton océanique profond, les sédiments hydrothermaux, qu'au niveau des cheminées hydrothermales. De plus, comparé aux données du Pacifique dans le bassin de Guaymas, certaines lignées seraient ubi-

quistes dans les systèmes hydrothermaux ; alors que les séquences d'autres groupes, notamment celui des kinetoplastides Excavata ont été détectées en abondance, mais à ce jour uniquement en Atlantique (López-García *et al.*, 2003; Lara *et al.*, 2009).

La troisième et dernière étude a été réalisée au niveau des sources hydrothermales de Lost City qui est un site particulier car excentré par rapport à la dorsale médio-Atlantique (López-García *et al.*, 2007). Il est caractérisé par une forte alcalinité (pH∼11) et une température de fluides relativement basse (90°C). Cette étude était basée sur l'analyse des communautés eucaryotes au niveau de l'interface fluide-eau de mer, et celles associées aux cheminées de carbonate. Des lignées appartenant à huit taxa eucaryotes majeurs ont été détectées : Metazoa, Fungi, Stramenopiles, Alveolata, Radiolaria, Cercozoa, Euglenozoa et Heterolobosea (Excavata) (Figure 1.9). Les banques contenaient à nouveau une majorité de séquences affiliées aux alvéolés. Leur distribution était cependant différente : les séquences de ciliés dominaient dans les fragments de carbonate, alors que la majorité des séquences des banques de clones de fluide-eau de mer était affiliée aux dinoflagellés et aux alvéolés des Groupes I et II (*Syndiniales*). De même, le groupe des Euglenozoa a montré une distribution différente, puisque des séquences de kinetoplastides ont été détectées au niveau des carbonates, et un nouveau groupe de séquences affiliées aux diplonemides a été exclusivement observé à l'interface fluide-eau de mer.

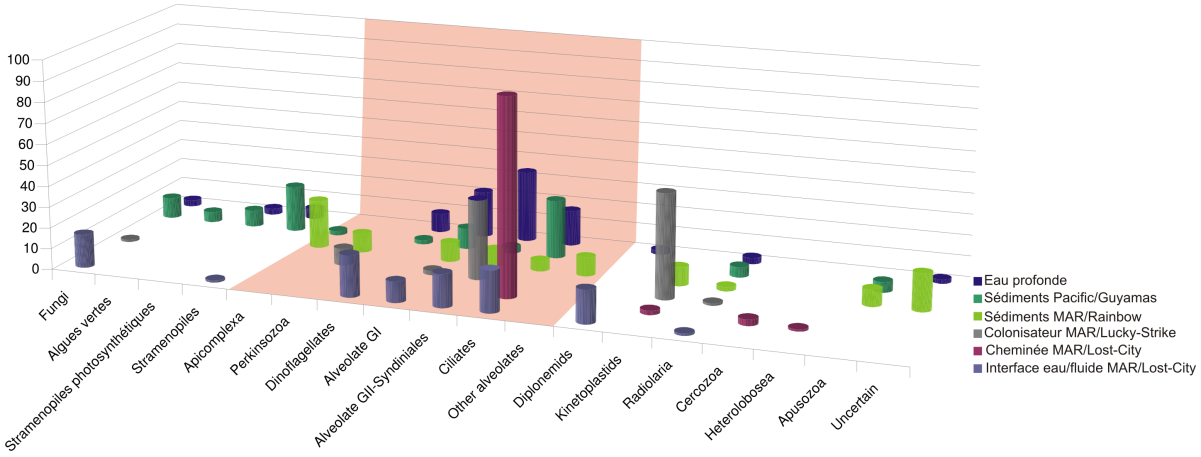

FIG. 1.9 – Abondance relative des clones d'eucaryotes unicellulaires, basée sur le séquençage de l'ARNr 18S à partir d'échantillons de cheminées, de sédiments ou d'eau à l'interface eau de mer/fluide dans des environnements hydrothermaux, comparée à celle d'échantillons de plancton retrouvée dans l'eau de mer profonde. Souligné en rose, le phylum des Alveolata qui semble dominer les librairies génétiques (d'après López-García *et al.*, 2007).

1.2 Les sédiments marins

1.2.1 Caractéristiques

Les sédiments peuvent être définis comme des particules organiques et inorganiques non consolidées qui s'accumulent sur les fonds océaniques. La classification des sédiments marins est basée sur la taille du grain et l'origine de la matière le composant. Les sédiments proviennent de sources diverses comme l'érosion des continents (terrigène), les éruptions volcaniques, l'activité biologique (biogénique), et les processus chimiques (Schulz and Zabel, 2006). L'épaisseur moyenne des sédiments dans les océans est de 500 m, mais localement la couche sédimentaire peut mesurer jusqu'à 10 km (Divins and the NOAA National Geophysical Data Center, 2010).

Les sédiments côtiers et la partie supérieure des marges continentales (Figure 1.1) sont principalement composés de sédiments d'origine terrigène, qui proviennent de l'érosion de roches. Constitués de sable, de boue et de vase dragués par les rivières, leur composition est de façon générale proche des roches ayant permis leur formation (Weltje and von Eynatten, 2004). Les sédiments côtiers et les marges passives sont des environnements dynamiques, puisqu'ils sont fréquemment soumis à des remises en suspension, et caractérisés par de fort taux de sédimentation, produisant l'enfouissement de grandes quantités de carbone organique. Les deltas et les estuaires constituent donc des environnements appropriés pour la génération de gaz biogénique peu profond comme le méthane ou le sulfure d'hydrogène, en raison de l'accumulation rapide de sédiments avec de hautes concentrations de matière organique (Fleischer *et al.*, 2001). Quatre-vingt-quatre pour cent du carbone organique enfoui dans les sédiments est originaire des deltas et des marges, puisque les réserves nutritives terrigènes stimulent la production primaire dans les eaux côtières, tandis que les sédiments pélagiques contiennent moins de 5 % de carbone organique (Baturin, 2006).

Au niveau des marges continentales, des structures géologiques différentes peuvent résulter de la circulation des fluides (Figure 1.10). Cette circulation est liée à la compaction du sédiment ou à la compression tectonique. Par exemple, le volcan de boues Hakon Mosby (HMMV) est un site de suintement froid sur la marge continentale norvégienne, au sud-ouest de la Mer des Barents, caractérisé par l'émission de fluides et de gaz. Le HMMV a une zone centrale composée de sédiments saturés en gaz, entourée par une zone riche en hydrate de gaz et en mate microbienne. Les hydrates de gaz sont des cristaux semblables à de la glace, stables à de hautes pressions et de faibles températures. Ils contiennent de grandes quantités de méthane biogénique et de CO_2.

Un des sites de suintement froid le plus étendu est celui du Golfe du Mexique (pour revue, Cordes *et al.*, 2009). Ces sédiments sont constitués de réservoirs d'hydrocarbures liquides et gazeux qui remontent à la surface du sédiment par des conduits produits par la tectonique (Figure 1.10). Des hydrates de gaz se forment à l'interface eau-sédiment (Sibuet and Olu, 1998).

Les sédiments sont organisés selon des zones successives (Fenchel and Jorgensen, 1977).

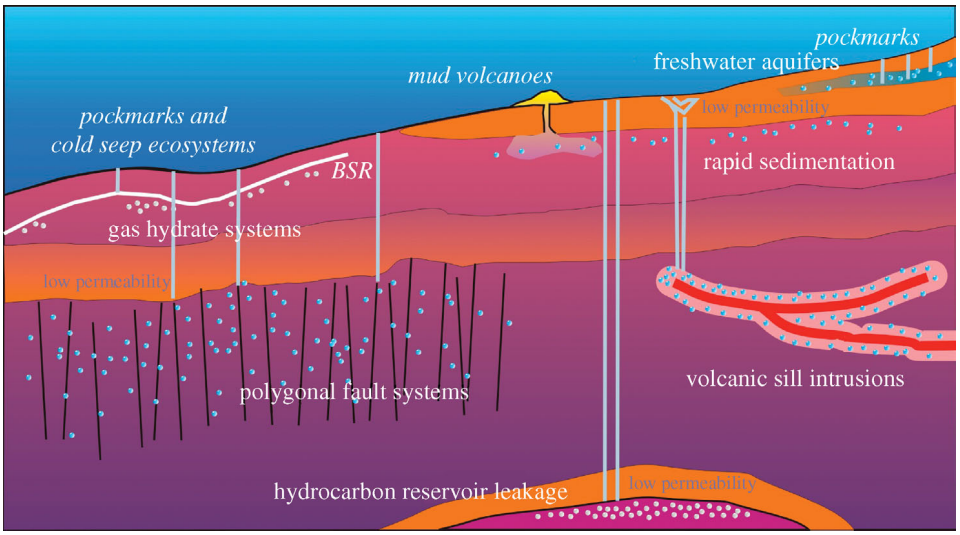

FIG. 1.10 – Schéma des différents systèmes de flux rencontrés sur les marges continentales (Berndt, 2005).

De part leur volume, les sédiments marins jouent un rôle important dans les grands cycles biogéochimiques tels que le cycle du carbone, de l'azote et du soufre. Comme nous l'avons vu plus haut, les sédiments côtiers et les marges sont riches en matière organique. A l'interface oxique entre l'eau de mer et le sédiment, la respiration aérobie est la première voie métabolique réalisée principalement par des bactéries hétérotrophes. Ce métabolisme permet de décomposer la matière organique en substrats solubles en présence d'oxygène. C'est pourquoi cet oxygène est rapidement consommé dans les premiers centimètres rendant le sédiment anoxique plus ou moins rapidement, selon la disponibilité de la matière organique et la concentration en dioxygène de l'eau. Cependant, des épisodes de bioturbation peuvent créer des micro-niches oxiques dans les couches anoxiques et réduites du sédiment ce qui permet de fortes activités métaboliques en raison de la disponibilité d'accepteurs d'électrons favorables (i.e. oxygène, nitrate) (Nielsen *et al.*, 2004).

En général, la profondeur à laquelle l'oxygène diffuse dans les sédiments côtiers n'excède pas un centimètre (Hensen and Zabel, 2000). Au-dessous de cette interface, d'autres cycles se mettent alors en place, avec différents types microbiens. Les organismes procaryotes utilisent des accepteurs terminaux d'électrons autres que l'oxygène et sont retrouvés selon un modèle prévisible avec les organismes dénitrifiants, les organismes sulfato-réducteurs, puis les méthanogènes dans la zone la plus profonde. Le sulfate abondant dans l'eau de mer (∼30 mM) offre une zone de sulfato-réduction particulièrement large (Fenchel, 1987).

1.2.2 Organismes retrouvés dans les sédiments et leur mode de vie

Au niveau côtier, l'environnement sédimentaire est dynamique dans le temps et dans l'espace. La zone dite intertidale ou zone de balancement des marées, est recouverte, au moins en partie, lors des pleines mers, et découverte lors des basses mers. Cette zone appelée également *estran* subit de grandes variations de température quand elle est émergée (i.e. gel en hiver, températures hautes en été). Les populations peuplant cet écosystème vont donc être adaptées à la fois aux conditions maritimes et aériennes, et être capables de résister aux fluctuations physico-chimiques dues aux marées.

La flore comprend des espèces d'algues qui se répartissent sur l'estran en fonction de leur mode de vie et de la nature du substrat. La faune typique inclut des anémones de mer, des coquillages (e.g. moules, berniques), des étoiles de mer, des crabes...

Les sédiments côtiers sont également très riches en annélides. On y retrouve des polychètes sédentaires tels que les Cirratulidae (voir section 4.2), des polychètes formant des galeries qui modifient les caractéristiques chimiques des sédiments tels que les néréis ou *Arenicola marina* (Kristensen, 2001), ou encore des oligochètes particuliers sans bouche tels que le genre *Olavius*. Chez ces derniers, la symbiose avec des bactéries chimioautotrophes a récemment été démontrée (Ruehland *et al.*, 2008).

La matière organique qui se dépose à la surface du sédiment est rapidement enfouie au-dessous de l'interface eau-sédiment par les organismes constituant la macrofaune, et rapidement dégradée par les communautés microbiennes. Les communautés benthiques contribueraient directement au flux de matière à travers l'interface, la re-minéralisation de la matière organique, et le recyclage des substances nutritives (pour revue, Bertics and Ziebis, 2010).

A l'inverse, les sédiments des marges continentales sont peuplés par les mêmes types d'organisme que l'on peut retrouver au niveau des sources hydrothermales. En effet, des mollusques tels que les Mytilidae (e.g. *Bathymodiolus childressi*), ou des vers vestimentifères (e.g. *Lamellibrachia* sp.) colonisent ces écosystèmes. Ces organismes sont aussi associés à des communautés microbiennes permettant la mise en place de métabolisme à base chimiosynthétique (pour revue, Dubilier *et al.*, 2008).

1.2.3 Les eucaryotes unicellulaires dans l'environnement sédimentaire marin

Des études sur les eucaryotes unicellulaires ont été réalisées dans des sédiments anoxiques (Dawson and Pace, 2002; Takishita *et al.*, 2005), et des sédiments marins profonds retrouvés au niveau de suintement froids riches en méthane (Takishita *et al.*, 2007*a*). Ces études révèlent une grande variété de lignées eucaryotes dont certaines sont potentiellement nouvelles. Une proportion non négligeable de ces séquences environnementales est étroitement affiliée à des séquences de parasites appartenant à différentes lignées telles que les Cercozoa, les Stramenopiles, et les alvéolés (Takishita *et al.*, 2005). La présence d'eucaryotes unicellulaires dans la

zone oxique-anoxique des sédiments a été étudiée d'un point de vue écologique, notamment à travers les travaux de Fenchel et collaborateurs qui ont étudié les ciliés dans ces environnements et ont montré entre autre que la présence de ciliés aérobies et micro-aérophiles sous plusieurs centimètres était due à l'existence de micro-habitats oxiques provoqués par les galeries créées par les organismes invertébrés (Fenchel, 1996).

Récemment, une comparaison de la diversité génétique eucaryote obtenue en analysant l'ARNr 18S qui permet de cibler les populations actives, et le gène codant pour l'ARNr 18S estimant les populations totales, a été réalisée sur des mates bactériennes tapissant des sédiments de suintements froids par Takishita *et al.* (2010). Les banques de clones obtenues via l'ARNr 18S montrent une nette dominance de séquences affiliées au groupe de ciliés (en moyenne 84 %), alors que celles obtenues via le gène codant pour l'ARNr 18S sont dominées par un champignon basidiomycète. Huit classes sur les onze décrites chez les ciliés (voir 2.4) ont été retrouvées dans ces banques de clones, suggérant que les ciliés actifs jouent un rôle majeur dans ces environnements, notamment au niveau de la chaîne alimentaire (Takishita *et al.*, 2010).

CHAPITRE 2

LES CILIÉS

Comme abordé dans le Chapitre 1, les eucaryotes unicellulaires ont un rôle non négligeable dans ces environnements particuliers. Parmi ces organismes, les ciliés sont retrouvés dans les environnements marins, dans les sédiments et au niveau des sources hydrothermales. Leur diversité métabolique leur permet d'interagir à différents niveaux et de jouer un rôle significatif dans les cycles biogéochimiques. C'est pourquoi ils seront décrits dans cette partie.

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2.2 Caractérisation des ciliés **31**
2.3 Cycle de vie et génétique **36**
2.4 Taxonomie et phylogénie moléculaire **41**
2.5 Type trophique et écologie **45**

2.1 Historique

La découverte des ciliés remonterait au 17^e siècle et serait attribuée à Antoine van Leuwenhoek. Longtemps appelés infusoires, par leur aptitude à se développer dans des infusions végétales, R. P. Hill leurs donne pourtant le nom de Ciliata dès 1752 (pour revue, Grassé, 1984).

Contrairement aux autres eucaryotes unicellulaires, la majorité des ciliés n'ont aucune structure calcifiée ou rigide permettant la formation de fossile. Cependant, certains Tintinnides sécrètent des loges protéiques rigides en forme de vase ou de tonneau, appelées lorica, qui renferment leur corps cellulaire. Ces lorica peuvent être d'origine minérale ou incorporer de petits morceaux de roches, et peuvent donc être retrouvées sous forme de microfossiles (Figure 2.1). Bien que les Tintinnides se soient principalement développés durant le Jurassique (- 200 à - 145 Ma), les premiers fossiles remontent au moins à l'Ordovicien (- 450 Ma) (Colom, 1948 ; pour revue, Lynn and Small, 1991).

Des études, les premières remontant à la fin du 19^e siècle, ont cependant porté sur la recherche de fossile similaire aux ciliés (pour revue, Schönborn *et al.*, 1999). Certaines pièces d'ambres datant du Triassique (- 230 Ma) pourraient en effet renfermer des espèces de ciliés (Schönborn *et al.*, 1999) (Figure 2.1). De plus, des études phylogénétiques basées sur l'hypothèse de l'horloge moléculaire ont montré que certaines espèces de ciliés dateraient du Paléoprotérozoïque (- 2500 à - 1600 Ma) (Wright and Lynn, 1997).

Les ciliés sont capables au cours de leur cycle de vie de s'enkyster. Ces kystes (Cf. 2.3) de différentes compositions (chitine, calcaire, silice) pourraient avoir formés des microfossiles, mais en l'absence de structures distinctes pour une identification, ils ne sont généralement pas facilement discernables (Foissner *et al.*, 2009).

2.2 Caractérisation des ciliés

Les ciliés sont des cellules complexes avec des morphologies variées en fonction de l'espèce. En 2000, Corliss suggère un total de 8000 espèces décrites en comptant les Tintinnides fossiles ($\sim$200)(Corliss, 2000). Foissner et collaborateurs, dans une revue récente, estiment qu'environ 85 % des ciliés restent encore à décrire, et implémentent jusqu'à 40 000 le nombre d'espèces (biologiques) de ciliés vivant libres (Foissner *et al.*, 2008). Les ciliés sont divers en forme et également en taille ; les cellules font en moyenne entre 30 et 300 μm (Figure 2.2), les plus grands spécimens pouvant atteindre jusqu'à 2 mm (*Stentor* sp.).

Deux principales caractéristiques séparent les ciliés des autres groupes d'eucaryotes unicellulaires :

–**La coexistence de deux types de noyaux** : un macronoyau (le plus volumineux) et un micronoyau (le plus petit). En fonction des espèces, les cellules sont pourvues d'un ou plusieurs de ces deux noyaux (Figure 2.3).

Les micronoyaux sont diploïdes et servent à la recombinaison génétique au cours de la

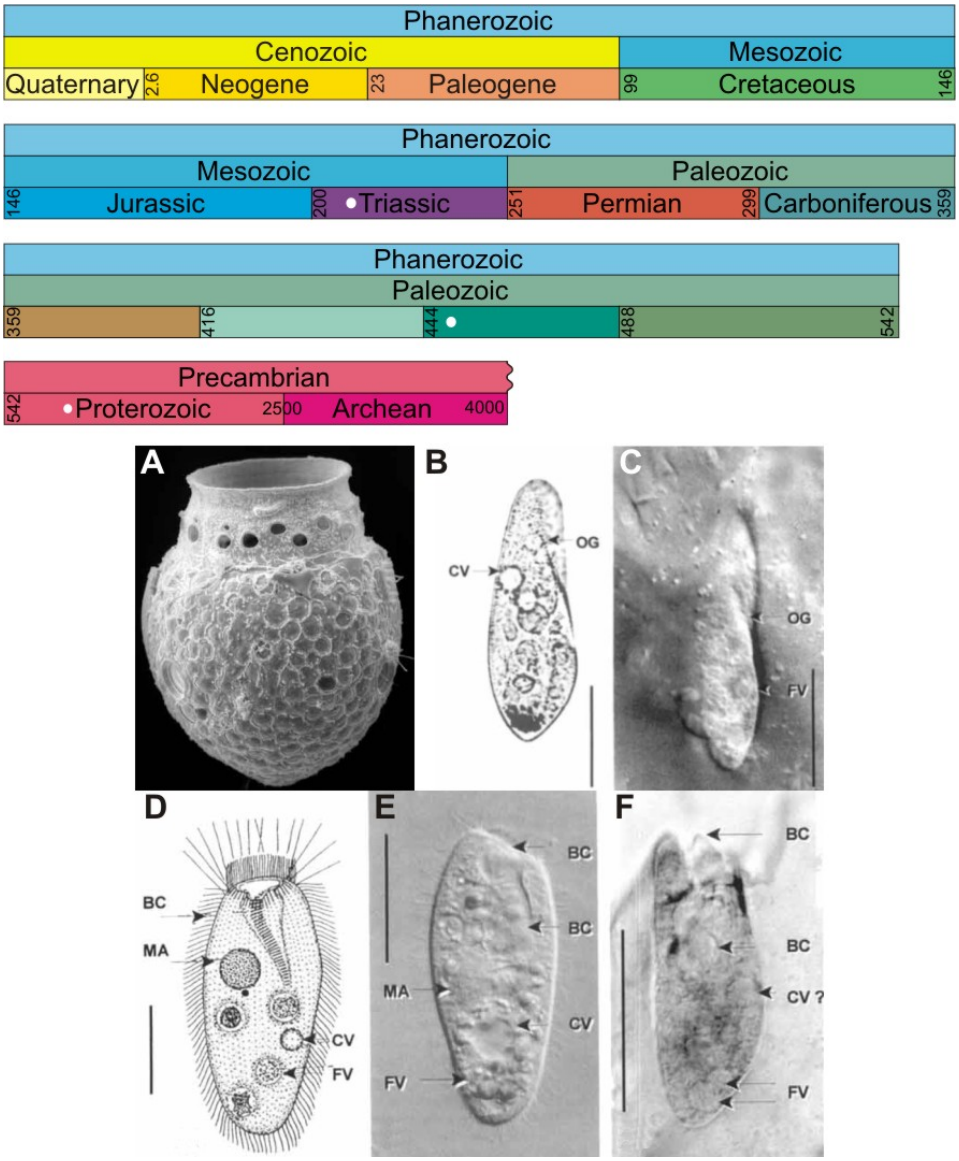

FIG. 2.1 – Echelle géologique (d'après the International Stratigraphic Chart, `http://www.stratigraphy.org/upload/ISChart2009.pdf`) permettant de retracer l'histoire fossile des ciliés. Les points blancs indiquent les périodes probables de l'âge des fossiles retrouvés et du résultat de l'analyse phylogénétique basée sur l'hypothèse de l'horloge moléculaire. **A**, lorica d'un cilié tintinnide, photographie en MEB, Fiona Scott ; **B-C**, *Paramecium aurelia* (B) et le fossile de *P. triassicum* (C). Les deux espèces sont similaires, sauf en taille, *P. aurelia* est d'environ 150×50 μm, alors que *P. triassicum* mesure 42-60×12-17 μm, échelles = 60 μm (B) et 20 μm (C) ; **D-F**, *Paracondylostoma setigerum* (D-E) ressemblant fortement à un organisme fossilisé dans de l'ambre (F). Les cellules ont une taille, une forme et une cavité buccale similaire, échelles = 30 μm. BC : cavité buccale, CV : vacuole contractile, FV : vacuole alimentaire, MA : macronoyau, OG : cannelure orale, PO : ouverture pharyngée (Schönborn *et al.*, 1999).

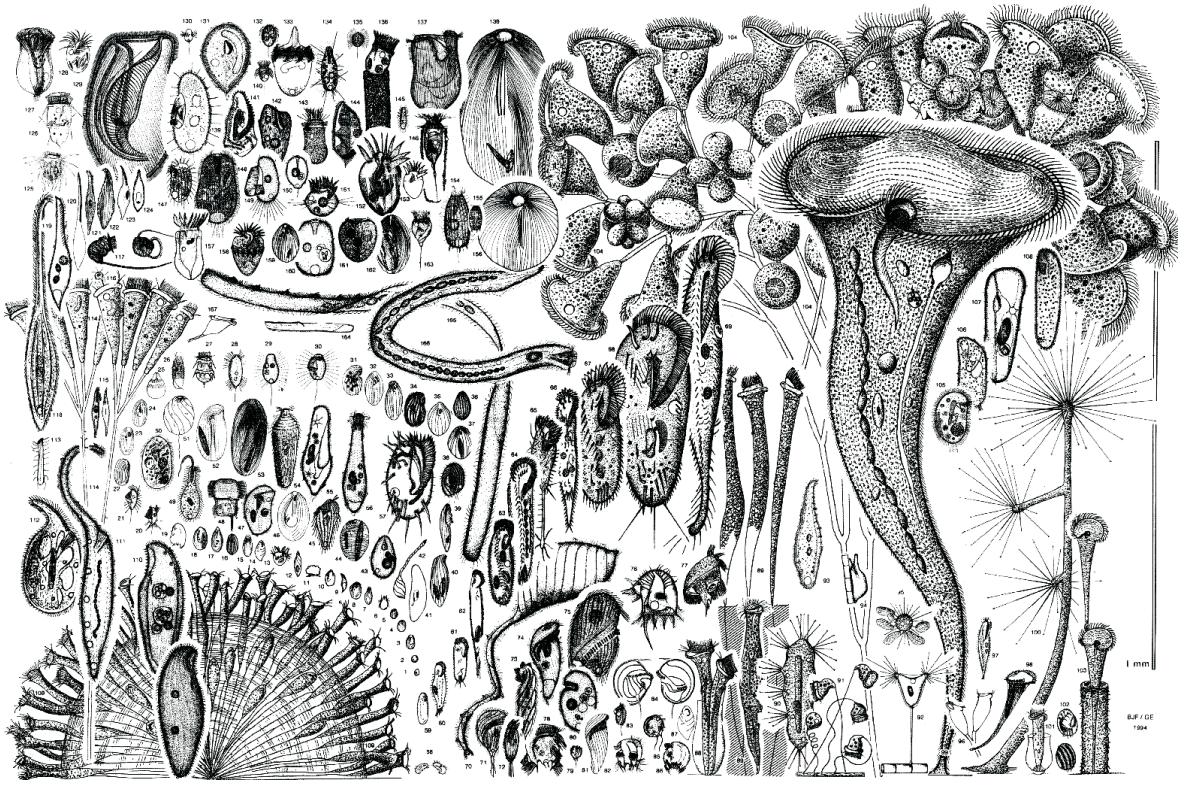

FIG. 2.2 – Dessin illustrant la diversité morphologique des ciliés (B.J. Finlay et G.F. Esteban, Institute of Freshwater Ecology, Windermere Laboratory, UK, `http://members.magnet.at/p.eigner/Diversity.html`). Les dessins sont à l'échelle (1 mm), indiquée à droite de la planche. Les 167 espèces représentées sont listées en annexe.

conjugaison. Ils forment une lignée germinale, et ont donc peu ou pas d'expression génique. Les macronoyaux forment une lignée somatique végétative contrôlant la majeure partie de la machinerie cellulaire notamment la synthèse d'ARN, et déterminent ainsi le phénotype cellulaire (Prescott, 1994). Ils dérivent, pour la majorité des espèces, par amplification de l'ADN micronucléaire par réarrangements des chromosomes du micronoyau (Figure 2.4) (pour revue, Postberg *et al.*, 2008).

Chez certaines espèces, le micronoyau est absent. Elles sont naturellement amicronucléées et dans ce cas, seul le macronoyau assure les fonctions cellulaires (par exemple, certaines espèces de *Tetrahymena*) (Elliot, 1959). Cependant, des études expérimentant des ciliés amicronucléés montrent que les cellules ont alors des structures morphologiques anormales et une viabilité inférieure (Liu *et al.*, 2005).

–**La présence d'une ciliature complexe** (d'où leur nom). En dehors des régions buccales, les cils sont associés à des cinétides, dont la présence et la distribution sont caractéristiques du taxon. Ces ensembles sont organisés en lignes appelées cinéties. Ces cinéties vont d'un pôle à

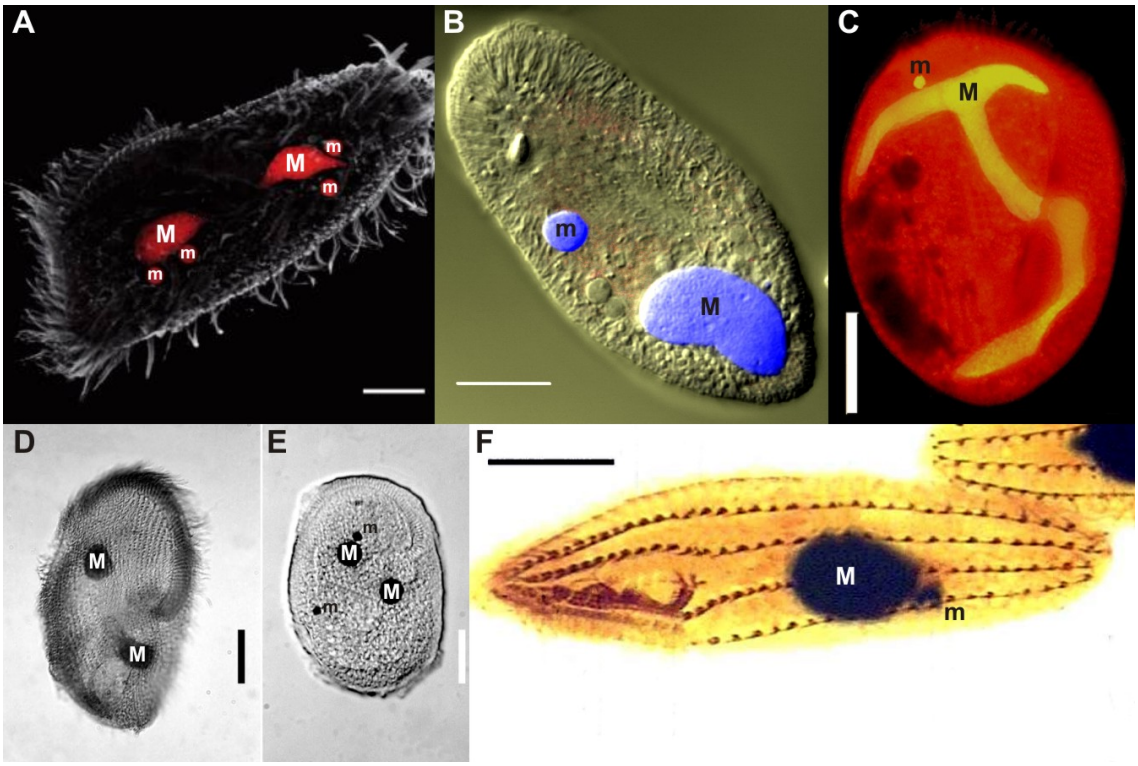

FIG. 2.3 – Micronoyaux (m) et macronoyaux (M) de : *Stylonychia lemnae* (**A**, marqué au To-Pro-3) (Jonsson *et al.*, 2009), *Ichthyophthirius multifiliis* (**B**, marqué au DAPI) (Sun *et al.*, 2009), *Euplotes woodruffi* (**C**, marqué au BET) (Fokin *et al.*, 2008), *Peritromus kahli* (**D** et **E**, imprégnation au protargol et coloration de Feulgen) (Rosati *et al.*, 2004), Scuticociliatia (**F**, imprégnation au Pyridine-silver-carbonate) (Small *et al.*, 2005).

l'autre de la cellule, mais peuvent aussi être positionnées comme une ceinture ciliaire. Les cils d'une cinétie battent les uns après les autres et les différentes cinéties sont synchronisées.

Dans les régions buccales, la répartition des cils, en structures spécialisées, est variable selon les espèces : ils peuvent être à la surface de la cellule ou dans un renfoncement. Ces structures servent généralement à générer des courants locaux et/ou à la capture de proies (Cf. 2.5).

Une spécialisation est observée pour donner des appareils locomoteurs particuliers en participant à la formation d'aires thigmotactiques (les cils de cette aire s'immobilisent au contact d'un support et deviennent rigides) ou à des appareils fixateurs accompagnés le plus souvent d'une sécrétion de substance gélatineuse (Figure 2.5) (pour revue, Grassé, 1984; Lynn, 2008).

Les cils ont une longueur variable selon les espèces et selon la zone cellulaire. La structure d'un cil est équivalente à celle d'un flagelle. En effet, chaque cil est un ensemble de microtubules appelé axonème, constitué en général de structures de type 9+2, donnant au cil rigidité et flexibilité.

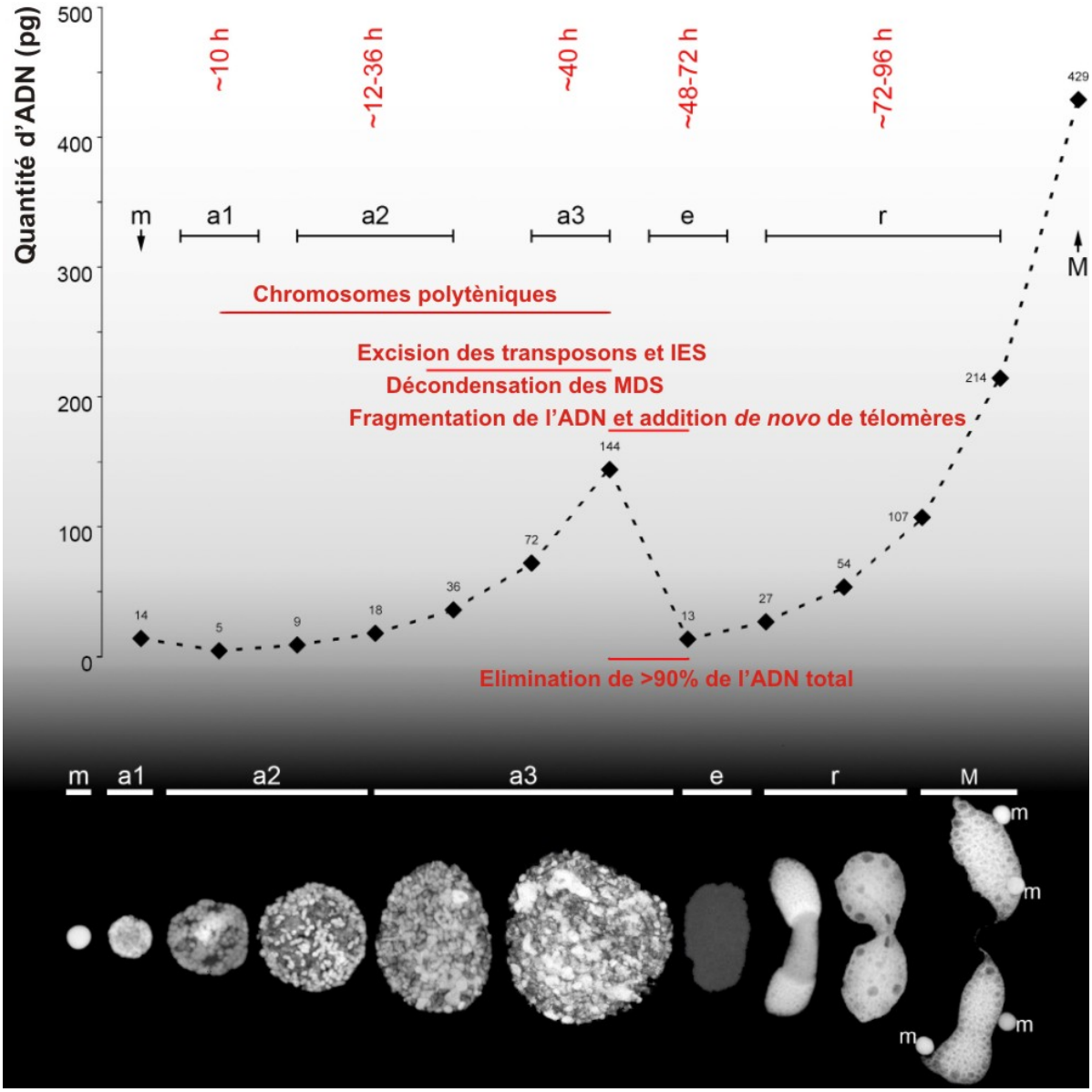

FIG. 2.4 – Contenu en ADN et morphologie liés aux différentes étapes du développement du macronoyau chez *Stylonychia mytilus*. Après la séparation des cellules en division, le macronoyau s'élargit dans une première étape (a1) et devient légèrement plus grand qu'un micronoyau (m). À cette étape, le début de la décondensation de la chromatine est observée. Le développement macronucléaire progresse (a2), des chromosomes spiralés deviennent visibles, et se développent par la suite en chromosomes géants polythènes (a3) pendant les premiers cycles d'amplification d'ADN. Durant cette étape se produit l'excision des séquences internes et des éléments transposons. Après l'étape d'élimination de l'ADN (e), des séquences du micronoyau sont excisées, aboutissant à une étape pauvre en ADN contenant moins de 10 % des séquences micronucléaires. De nouveaux cycles d'amplification de l'ADN ont alors lieu (r), aboutissant à un macronoyau mature (M) (d'après Postberg *et al.*, 2008.)

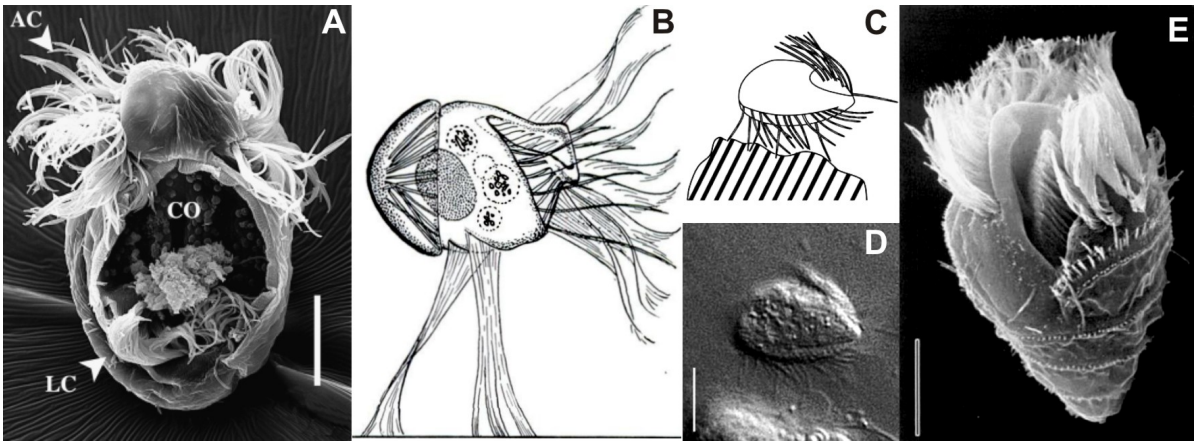

FIG. 2.5 – Exemple de ciliature. **A**, *Troglocorys cava*, une concavité est présente sur une des faces du cilié (CO), dont la partie postérieure est longée par une zone ciliaire (LC). La zone ciliaire adorale (AC) entoure l'ouverture du vestibulum, photographie en MEB, échelle = 10 μm (Tokiwa *et al.*, 2010). **B**, Dessin de *Strombidium calkinsi* fixé sur un substrat (Fauré-Fremiet, 1932). **C-D**, *Larvulina variabilis* attaché au substrat par le champ thigmotactique dorsal, dessin et photographie en DIC, échelle = 10 μm (Bourland, 2009). **E**, *Loboea strobila*, les cils somatiques d'un micron de longueur sont arrangés en une ceinture, photographie en MEB, échelle = 20 μm (Agatha *et al.*, 2004).

2.3 Cycle de vie et génétique

Les ciliés peuvent se reproduire de deux manières différentes : la reproduction dite asexuée ou scission binaire, qui consiste en une division longitudinale permettant d'obtenir deux cellules-filles identiques ; ou la conjugaison, processus sexué mais sans production de gamètes, durant lequel les deux cellules sont accolées via une zone d'adhésion plus ou moins grande (Figure 2.6).

La polarisation des ciliés, avec la présence d'un pôle apical et d'un pôle basal, implique une scission binaire, en général selon un plan équatorial. Ainsi, ce type de division asexuée implique des mécanismes de morphogénèse différents selon les espèces. La partie apicale doit régénérer une partie basale et réciproquement, les organites étant disposés de manière à former des zones dédiées à la nutrition, à la motricité ou à l'excrétion (e.g. Vdacny and Foissner, 2009) (Figure 2.7).

Le micronoyau se divise par mitose, et le macronoyau s'allonge et se divise en deux. Chaque nouvelle cellule obtient une copie du micronoyau et du macronoyau. La division du macronoyau se produit chez tous les ciliés, excepté chez les Karyorelictea (Raikov, 1982), par un processus peu usuel, le processus amitotique durant lequel se produit une ségrégation aléatoire des chromosomes sans condensation de la chromatine (Katz, 2001; Cervantes *et al.*, 2006).

Quand deux ciliés de types sexuels différents se rencontrent, il peut se produire le phénomène de conjugaison. Les types sexuels seront définis par la présence d'allèles distincts dans une région spécialisée du génome des organismes. Selon le principe de la conjugaison, le (ou les) micronoyau

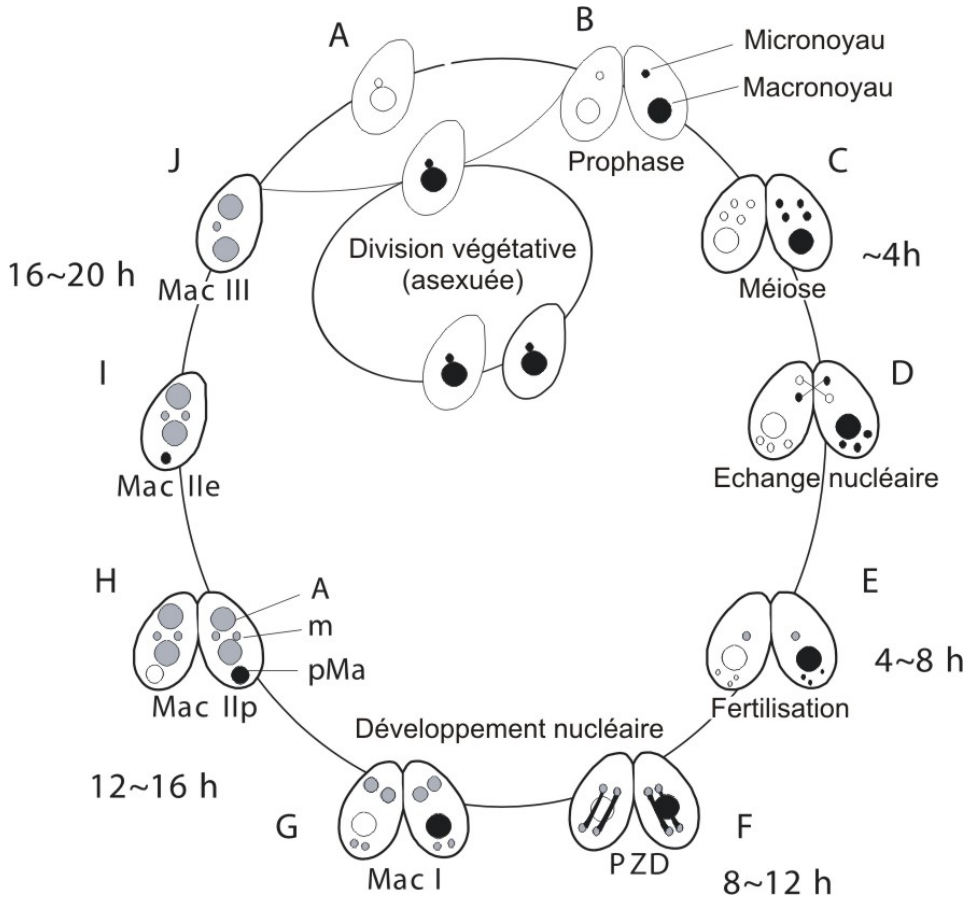

FIG. 2.6 – Exemple du cycle cellulaire de *Tetrahymena thermophila*. **A**, Phase végétative durant laquelle le cilié se divise de façon asexuée. **B**, Deux ciliés de types sexuels différents se préparent à la conjugaison, prophase méiotique. **C**, Méiose des micronoyaux. **D**, Echange nucléaire. Un des quatre micronoyaux se divise par mitose pour former deux pronuclei, et un de ces pronuclei est échangé d'une cellule à l'autre. **E**, Fertilisation, formation d'un syncaryon. **F**, Division post-zygote (PZD). Les noyaux se divisent successivement deux fois. **G**, Mac I. Les noyaux antérieurs se différencient en un nouveau macronoyau, alors que les noyaux dans la partie postérieure forment les futurs micronoyaux. **H**, Mac IIp. Le macronoyau parental (pMA) migre dans la partie postérieure et commence à se détériorer, A : ébauche du nouveau macronoyau. **I**, Mac IIe. Les cellules filles se séparent. **J**, Mac III. Un des deux micronoyaux est éliminé (d'après Akematsu and Endoh, 2010).

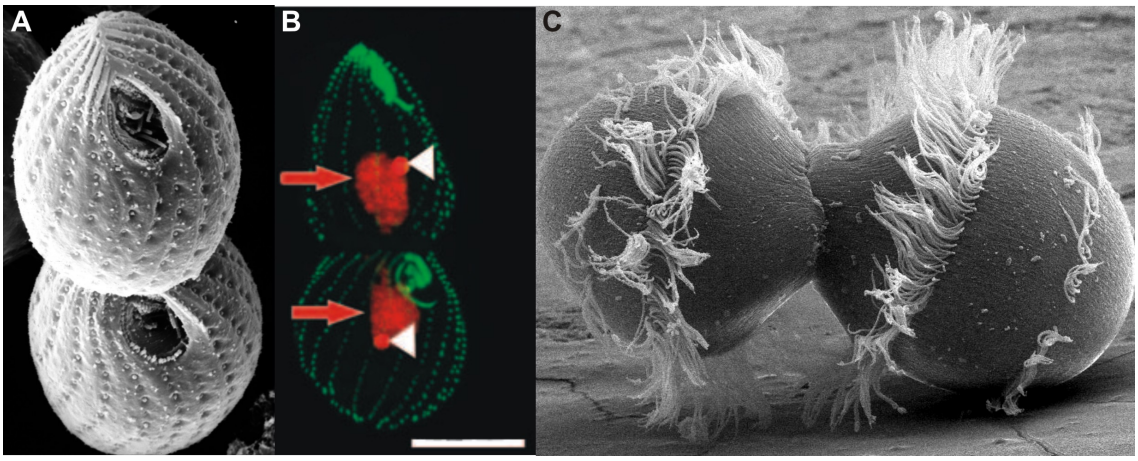

FIG. 2.7 – Division asexuée. **A**, *Tetrahymena thermophila*, photographie en MEB d'une cellule déciliée avec les nouveaux systèmes oraux, photographie en MEB, J. Frankel, Ciliate Image Database©. **B**, Les cellules de *Tetrahymena thermophila* sont marquées avec un anticorps anti-centrine marquant les corps basaux permettant de suivre la genèse des parties orales, et l'ADN des micronoyaux (flèches blanches) et des macronoyaux (flèches rouges) est marqué avec le fluorophore Hoerscht 33342, échelle = 10 μm Pearson and Winey, 2009. **C**, *Didinium* sp., photographie en MEB, Ciliate Image Database©.

subit une division méiotique aboutissant à quatre micronoyaux haploïdes. Trois de ces noyaux se désagrègent et le noyau restant subit une division mitotique. Le noyau dit stationnaire reste dans la cellule, tandis que l'autre migre dans la cellule associée et se fusionne avec le noyau stationnaire de celle-ci pour former un noyau zygote diploïde (Figure 2.6). Le noyau diploïde se divise ensuite de façon mitotique et un des noyaux en résultant devient le nouveau micronoyau, tandis que l'autre se différencie pour donner un nouveau macronoyau (Figure 2.6). L'ADN de l'ancien macronoyau est vraisemblablement éliminé par un mécanisme similaire à celui de l'apoptose (Santos *et al.*, 2000; Jahn and Klobutcher, 2002; Gordeeva *et al.*, 2004).

Le cycle cellulaire des ciliés ne se réduit pas seulement à ces deux modèles bien qu'ils soient prépondérants. Chez certaines espèces, le processus de division sexuée se produit par autofécondation d'un seul organisme, on parle alors d'autogamie ; alors que la division asexuée peut aussi se faire par bourgeonnement ou fissions multiples.

Rappelons que certaines espèces de ciliés sont capables de se différencier en kyste de résistance : une forme cellulaire sphérique complètement exemptée de toute ciliature et protégée par une membrane constituée de plusieurs couches. La formation de ces kystes est une stratégie de survie chez de nombreux ciliés libres et permet la dissémination des cellules (Figure 2.8) (Gutiérrez *et al.*, 2001; Foissner, 2006).

L'enkystement est un processus induit par un état de stress qui permet à la cellule végétative d'échapper temporairement à des conditions environnementales défavorables : le jeûne, la dessiccation, la prédation (Gutiérrez *et al.*, 2003). Le kyste perdure tant que les conditions

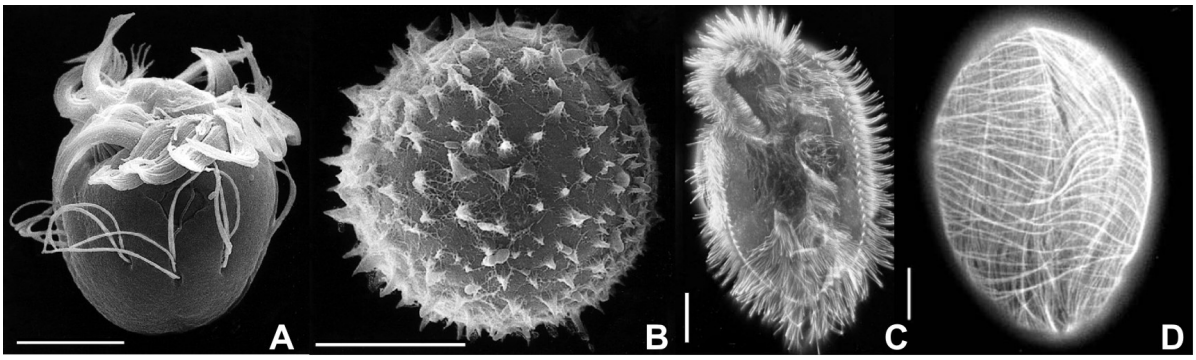

FIG. 2.8 – Kystes. **A**, Cellule mature de *Halteria grandinella*, et **B**, kyste, photographies en MEB, échelles = 10 μm (Foissner *et al.*, 2007). **C**, Cellule de *Sterkiella histriomuscorum* après conjugaison, et **D**, zygokyste formé au cours du cycle de division. Les cellules sont visualisées par immunofluorescence après marquage avec un anticorps anti-alpha tubuline et observées par épifluorescence, échelles = 10 μm (Fryd-Versavel *et al.*, 2010).

sont défavorables. Le retour à des conditions favorables déclenche le processus de désenkystement ; le kyste subit alors un processus de différenciation et une cellule doit complètement se reconstruire sans aucun cytosquelette résiduel apparent. Ce retour à l'état normal peut se réaliser par différents moyens selon les espèces. On peut citer l'émergence de la cellule par un pore spécifique, la rupture de la paroi du kyste par l'ingestion d'eau induisant une pression interne, ou encore la digestion enzymatique de la membrane (Corliss and Esser, 1974).

En raison de la présence de cette membrane résistante, ces kystes sont impossibles à étudier par des méthodes cytologiques, comme l'immunofluorescence. En conséquence, il n'est pas facile de décrire le devenir du cytosquelette pendant le cycle enkystement-dékystement. Cependant, certaines espèces de ciliés subissent, pendant leur cycle sexuel, un état transitoire par la formation d'un zygokyste, une forme cellulaire se rapprochant du kyste mais ne possédant pas de membrane externe, permettant d'étudier les réarrangements lors de ces phénomènes (Figure 2.8) (Fryd-Versavel *et al.*, 2010). Certaines études de biologie moléculaire ont toutefois permis une meilleure compréhension du cycle d'enkystment-désenkystement au moins génétique, en montrant une expression différente dans les transcrits de certains gènes exprimés lors de ce phénomène (Grisvard *et al.*, 2008).

De nombreux sites internet sur les ciliés

Depuis ces dernières années, les sites internet relatifs aux ciliés se sont développés. Certains proposent :

- des présentations généralistes : `http://www.uoguelph.ca/~ciliates/` de Denis H. Lynn présentant une revue de la phylogénie du phylum Ciliophora, avec une description détaillée de certains genres représentatifs ; `http://liv.ac.uk/ciliate/intro.htm` présentant un guide illustré pour identifier des espèces marines principalement sur les côtes anglaises, et les méthodes courantes pour l'étude de ce groupe.
- des présentations génétiques : `http://ciliate.org/index.php/home/welcome`, the TGD Wiki user-updatable database, reprenant les informations sur le génome de *Tetrahymena thermophila* séquencé par le TIGR (Institut pour la Recherche Génomique) ; `http://paramecium.cgm.cnrs-gif.fr/` entièrement dédié au genre *Paramecium*.
- des présentations morphologiques offrant des photographies de ciliés :
  `http://www.micrographia.com/specbiol/protis/cili/cili0100.htm`
  `http://research.plattsburgh.edu/Ciliates/Default.asp` (très belles photographies en MEB)
  `http://protist.i.hosei.ac.jp/PDB/Images/Protista/CiliophoraE.html`
  `http://starcentral.mbl.edu/microscope/portal.php?pagetitle=index` (Micro∗scope)
  `http://www.cerimes.fr/le-catalogue/alimentation-des-infusoires\-cilies.html` hébergé par le CERIMES (le Centre de Ressources et d'Information sur les Multimédias pour l'Enseignement Supérieur) est remarquable. En effet, on peut y voir des vidéos sur la capture de proies par certains ciliés, filmés en 1961 par Dragesco et collaborateurs. Egalement disponibles sur ce site, de nombreux films sur les paramécies (division, nutrition...).

Le cycle de vie relativement court des ciliés ainsi que des mises en culture simple, notamment des paramécies (genre *Paramecium*) et des espèces du genre *Tetrahymena*, offrent de nombreux avantages dans le cadre d'études génétiques. D'ailleurs, la paramécie est souvent comparée aux souris de laboratoire chez les eucaryotes unicellulaires. Ces deux genres représentent dix des espèces dont le séquençage du génome fait partie des programmes de séquençage complet (Table 2.1 ; Cf. encadré Sites internet). De plus, les ciliés sont des modèles intéressants pour les recherches, notamment en génétique. Par exemple, la découverte des capacités catalytiques de l'ARN a été réalisée sur des ciliés du genre *Tetrahymena* dans les années 1980 (Krüger *et al.*, 1982). De même la première découverte de la télomérase a été faite chez l'espèce *Tetrahymena thermophila* en 1985 (Greider and Blackburn, 1985).

Une autre particularité génétique de ce groupe provient de son code génétique. En effet, chez les ciliés, ce code est différent du code standard : les codons UAA et UAG codent pour une glutamine au lieu d'un codon Stop, et spécifiquement chez les *Euplotes*, UGA code pour une cystéine (Figure 2.9) (pour revue, Lozupone *et al.*, 2001). Les études ont montré que ces codes

Organisme	Organelle	Séquence référence	Numéro accession	Longueur (Mpb)	GC (%)	Proteines	ARN	Institution
Tetrahymena pyriformis	mitochondrie	NC_000862	AF160864	0,047	21	44	14	Université de Montreal, Canada
Tetrahymena pigmentosa	mitochondrie	NC_008339	DQ927305	0,047	18	44	14	University of California, Los Angeles
Tetrahymena paravorax	mitochondrie	NC_008338	DQ927304	0,047	18	44	14	University of California, Los Angeles
Tetrahymena malaccensis	mitochondrie	NC_008337	DQ927303	0,048	19	45	14	University of California, Los Angeles
Tetrahymena thermophila	mitochondrie	NC_003029	AF396436	0,048	20	45	14	University of California, Los Angeles
Tetrahymena thermophila	complet	NZ_AAGF00000000	AAGF00000000	104	22	24725		TIGR
Paramecium caudatum	mitochondrie	NC_014262	FN424190	0,044		42	7	University of Leipzig, Germany
Paramecium aurelia	mitochondrie	NC_001324	X15917	0,040	41	46	8	University of Colorado
Paramecium tetraurelia	macronoyau	NC_006058	CR548612	0,98	27	463	1	CNRS, France
Paramecium tetraurelia	complet	NZ_CAAL00000000	CAAL00000000	72	27	39580		Génoscope, France
Sterkiella histriomuscorum		En cours						University of California, Los Angeles
Ichthyophthirius multifiliis		En cours						J. Craig Venter Institute

TABLE 2.1 – Liste des espèces de ciliés dont le génome est séquencé ou en cours de séquençage.

alternatifs se sont développés à partir du code standard par des mutations, mais les mécanismes de cette évolution comme la réassignation des codons ou l'adaptation des organismes à de tels changements, restent encore à définir (pour revue, Kurnaz *et al.*, 2010).

2.4 Taxonomie et phylogénie moléculaire

LA CLASSIFICATION

La *classification* est l'action de distribuer par classe, par catégorie ; système de classement (définition Larousse).

Le but principal des systèmes de classification actuels en science est de rendre compte des liens évolutifs et génétiques entre les organismes ancestraux et actuels. Il existe deux volets à cette classification : (1) la systématique étudiant la diversité des organismes (dénombrement et classement), et (2) la taxonomie représentant la théorie et la pratique de classification des organismes (description et définition).

Publié en 1735 par le naturaliste suédois Carl von Linné (1707-1778), Systema naturae renferme la première classification scientifique des mondes minéral, végétal et animal. Ce système de classification est toujours utilisé aujourd'hui. L'espèce en constitue l'unité de base. Une espèce, au sens biologique, regroupe des individus qui peuvent se croiser pour donner une descendance viable, dans des conditions naturelles.

Le phylum Ciliophora Doflein, 1901 comprend un nombre important d'espèces (∼8000). La classification de ce groupe est complexe, et a connu et connait encore des arrangements continuels par la découverte d'espèces nouvelles.

Une des premières classifications complètes des ciliés initiée par les travaux de Corliss a près de 50 ans (Corliss, 1961; Corliss, 1979). Elle fut d'abord basée sur la morphologie de l'appareil buccal, puis sur l'ultrastructure du cortex somatique qui comprend entre autre l'infraciliature et les constituants du cytosquelette périphérique (Small and Lynn, 1981). La morphogenèse a aussi été utilisée dans la mise en place de certains clades majeurs.

L'analyse des séquences du gène codant pour les sous-unités de l'ARN ribosomique a permit

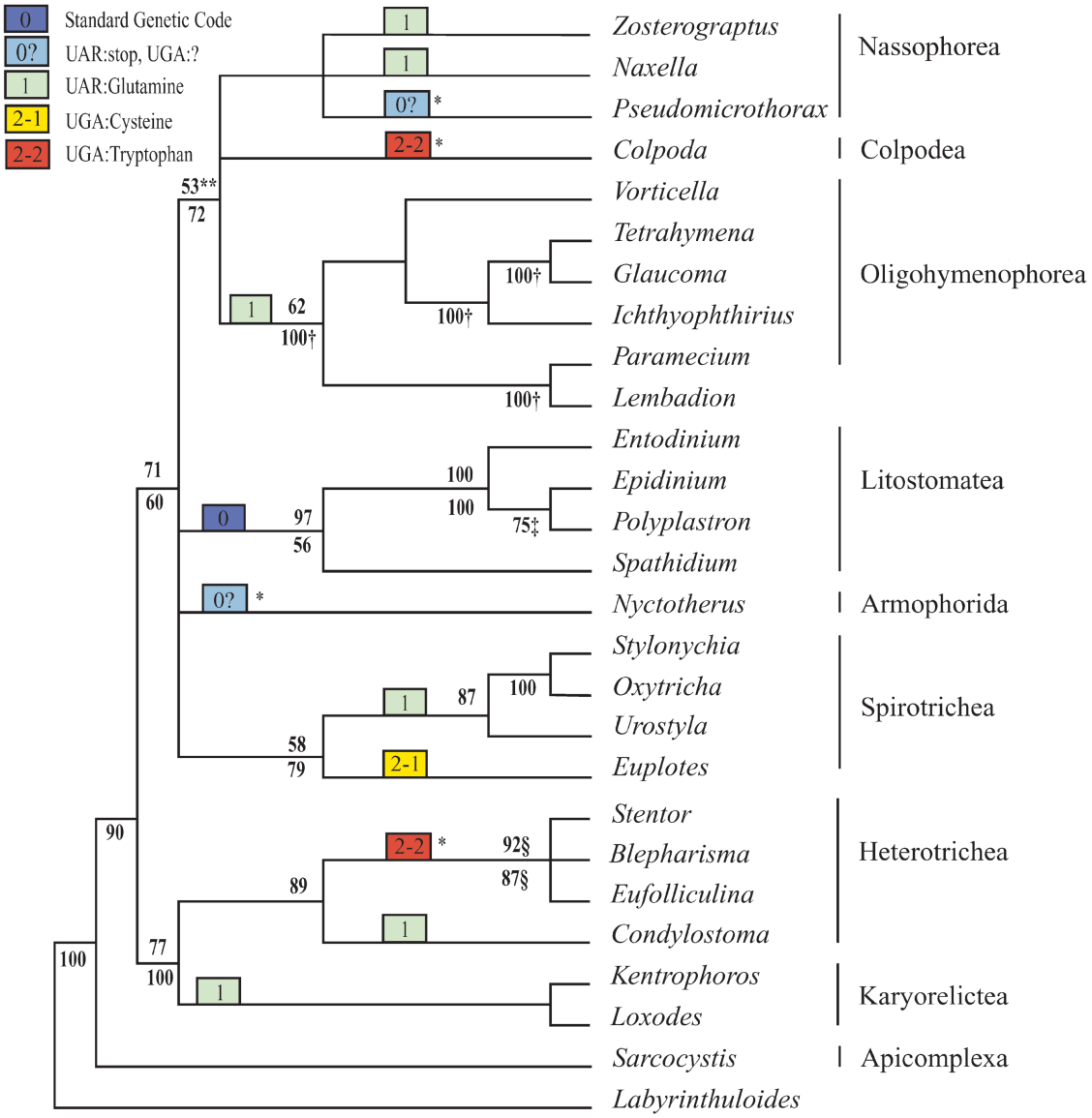

FIG. 2.9 – Arbre phylogénétique des principaux genres de ciliés, basé sur l'analyse des ARNr 18S et 28S, sur lequel est représenté les changements dans le code génétique (Lozupone *et al.*, 2001).

une nouvelle réorganisation de ce phylum des Ciliophora (Lynn and Sogin, 1988). Aujourd'hui, les études taxonomiques et moléculaires sont étroitement liées, et permettent une classification plus précise de ce phylum.

Cette section présente les principales études sur le phylum des Ciliophora. Cependant, de nombreux articles sont paru et continuent de paraitre dans le but d'améliorer la phylogénie de ce groupe aussi bien au niveau de l'espèce, du genre, que de la classe, en croisant les données moléculaires et taxonomiques.

Jusqu'à une vingtaine d'années, deux classifications s'opposaient : l'une « française » apportée par De Puytorac (pour revues récentes, De Puytorac, 1994; De Puytorac *et al.*, 1993) et l'autre « américaine » de Lynn et collaborateurs (Lynn, 2008) ; ce dernier expliquant les différences des deux auteurs dans un article publié en 1997 (Figure 2.10)(Lynn and Small, 1997).

La plus récente phylogénie générale du phylum Ciliophora dans la littérature est celle présentée dans le livre *The Ciliated Protozoa : Characterization, Classification, and Guide to the Literature* (Lynn, 2008). Deux subphyla sont proposés : les Postciliodesmatophora et les Intramacronucleata, comprenant un ensemble de 11 classes : Karyorelictea, Heterotrichea, Litostomatea, Phyllopharyngea, Nassophorea, Colpodea, Spirotrichea, Armophorea, Plagiopylea, Prostomatea, Oligohymenophorea. Récemment, selon D.H. Lynn, les corrélations entre les données moléculaires et taxonomiques montrent que les deux subphyla avec six classes sur les onze (Karyorelictea, Heterotrichea, Phyllopharyngea, Nassophorea, Colpodea, Litostomatea) sont supportés phylogénétiquement (Lynn, 2003*a*; Lynn, 2008).

Les Postciliodesmatophora ont pour principale caractéristique d'avoir des dicinétides somatiques arrangées en rubans de microtubules post-ciliaires juxtaposés (postciliodesmes). Ce groupe comprend deux classes : les Karyorelictea, avec pour particularité un macronoyau qui ne se divise pas ; et les Heterotrichea, ayant un macronoyau qui se divise à l'aide de microtubules extranucléaires. Les Intramacronucleata regroupent quant à eux des cellules dont le macronoyau se divise à l'aide de microtubules intranucléaires. Ils comprennent les neuf classes restantes. Une description détaillée de ces groupes est proposée dans le livre *The Ciliated Protozoa : Characterization, Classification, and Guide to the Literature* (Lynn, 2008), et pour une aide à la description d'espèces de ciliés, le chapitre *Phylum Ciliophora* dans *An Illustrated Guide to the Protozoa* (Lynn and Small, 2000).

L'apport de nouvelles données via l'utilisation d'autres marqueurs nucléaires tels que les ITS ou le gène codant pour la grande sous-unité du ribosome (ARNr 28S), et les marqueurs moléculaires plus discriminants que sont les gènes mitochondriaux (e.g. le gène codant pour la sous-unité 1 de la cytochrome c oxydase ; *cox-1*), permettent à présent d'affiner les relations au sein du phylum Ciliophora. Des études utilisant ces marqueurs ont déjà été réalisées pour déterminer des variations au niveau d'une espèce ou d'un genre (pour exemple, ITS/ARNr 28S/cox : Gentekaki and Lynn, 2009 ; cox : Chantangsi and Lynn, 2008; Chantangsi *et al.*, 2007). Récemment, Strüder-Kypke et Lynn (2010) ont analysé la diversité du gène *cox-1* sur cinq

Phylum CILIOPHORA Doflein, 1901 (d'après De Puytorac, 1994)

Subphylum TUBULICORTICATA de Puytorac et al., 1993
- Superclass POSTCILIODESMATOPHORA Gerassimova & Seravin, 1976
  - **Class KARYORELICTEA** Corliss, 1974
    - Subclass Trachelocercia Jankowski, 1980
      - Order Trachelocercida Kent, 1881
    - Subclass Loxodia Jankowski, 1980
      - Order Loxodida Jankowski in Small & Lynn, 1985
    - Subclass Protocruziidia Jankowski in Small & Lynn, 1985
      - Order Protocruziida Jankowski in Small & Lynn, 1985
    - Subclass Protoheterotrichia Nouzarède, 1977
      - Order Protoheterotrichida Nouzarède, 1977
  - **Class HETEROTRICHEA** Stein, 1859
    - Subclass Heterotrichia Stein, 1859
      - Order Heterotrichida Stein, 1859
      - Order Coliphorida Jankowski, 1967
      - Order Armophorida Jankowksi, 1964
      - Order Phacodiniida Small & Lynn, 1985
      - Order Odontostomatida Sawaya, 1940
      - Order Licnophorida Corliss, 1957
    - Subclass Clevelandellidia de Puytorac & Grain, 1976
      - Order Clevelandellida de Puytorac & Grain, 1976
- Superclass SPIROTRICHA Bütschli, 1889
  - **Class HYPOTRICHEA** Stein, 1859
    - Subclass Euplotia Tuffrau & Fleury in de Puytorac et al., 1993
      - Order Kiitrichida Nozawa, 1941
      - Order Euplotida Small & Lynn, 1985
      - Order Uronychiida Tuffrau & Fleury in de Puytorac et al., 1993
      - Order Gastrocirrhida Tuffrau & Fleury in de Puytorac et al., 1993
    - Subclass Oxytrichia Tuffrau & Fleury in de Puytorac et al., 1993
      - Order Urostylida Jankowski, 1979
      - Order Discocephalida Wicklow, 1982
      - Order Oxytrichida Jankowski, 1979
  - **Class OLIGOTRICHEA** Bütschli, 1887
    - Subclass Oligotrichia Bütschli, 1887
      - Order Oligotrichida Bütschli, 1887
    - Subclass Strobilia Laval-Peuto, Grain & Deroux in de Puytorac et al., 1993
      - Order Choreotrichida Small & Lynn, 1985
      - Order Tintinnida Kofoid & Campbell, 1929
- Superclass TRANSVERSALA de Puytorac et al., 1993
  - **Class COLPODEA** Small & Lynn, 1981
    - Subclass Colpodia Foissner, 1985
      - Order Colpodida de Puytorac et al., 1974
      - Order Grossglockneriida Foissner, 1985
      - Order Bursariomorphida Fernández-Galiano, 1978
      - Order Cyrtolophosidida Foissner, 1978
      - Order Sorogenida Foissner, 1985
      - Order Bryophryida de Puytorac et al., 1979
    - Subclass Bryometopia Foissner, 1985
      - Order Bryometopida Foissner, 1985
  - **Class PLAGIOPYLEA** Small & Lynn, 1985
    - Order Plagiopylida Small & Lynn, 1985

Subphylum FILICORTICATA de Puytorac et al., 1993
- **Class LITOSTOMATEA** Small & Lynn, 1981
  - Order Haptorida Corliss, 1974
  - Order Spathidiida Foissner & Foissner, 1988
  - Order Helicoprorodontida Grain in de Puytorac et al., 1993
  - Order Pleurostomatida Schewiakoff, 1896
  - Order Mesodiniida Grain in de Puytorac et al., 1993
- **Class VESTIBULIFEREA** de Puytorac et al., 1974
  - Order Trichostomatida Bütschli, 1889
  - Order Entodiniomorphida Reichenow in Doflein & Reichenow, 1929
  - Order Blepharocorythida Wolska, 1971

Subphylum EPIPLASMATA de Puytorac et al., 1993
- Superclass CILIOSTOMATOPHORA de Puytorac et al., 1993
  - **Class PHYLLOPHARYNGEA** de Puytorac et al., 1974
    - Subclass Cyrtophoria Fauré-Fremiet in Corliss, 1956
      - Order Chilodonellida Deroux in de Puytorac et al., 1993
      - Order Chlamydodontida Deroux in de Puytorac et al., 1993
      - Order Dysteriida Deroux in de Puytorac et al., 1993
    - Subclass Chonotrichia Wallengren, 1895
      - Order Chilodochonida Batisse in de Puytorac et al., 1993
      - Order Exogemmida Jankowski, 1972
      - Order Cryptogemmida Jankowski, 1975
    - Subclass Rhynchodia Chatton & Lwoff, 1939
      - Order Hypocomatida Deroux, 1976
      - Order Rhynchodida Chatton & Lwoff, 1939
    - Subclass Suctoria Claparède & Lachmann, 1858
      - Order Podophryida Jankowski, 1973
      - Order Exotropida Batisse in de Puytorac et al., 1993
      - Order Entotropida Batisse in de Puytorac et al., 1993
- Superclass MEMBRANELLOPHORA Jankowski, 1975
  - **Class NASSOPHOREA** Small & Lynn, 1981
    - Subclass Prostomatia Schewiakoff, 1896
      - Order Prostomatida Schewiakoff, 1896
      - Order Prorodontida Corliss, 1974
    - Subclass Nassulia Jankowski, 1967
      - Order Nassulopsida Deroux in de Puytorac et al., 1993
      - Order Synhymeniida Deroux, 1978
      - Order Nassulida Jankowski, 1967
      - Order Paranassulida Deroux in de Puytorac et al., 1993
      - Order Parahymenostomatida Grain et al., 1976
      - Order Microthoracida Jankowski, 1967
  - **Class OLIGOHYMENOPHOREA** de Puytorac et al., 1974
    - Subclass Peniculia Fauré-Fremiet in Corliss, 1956
      - Order Peniculida Fauré-Fremiet in Corliss, 1956
      - Order Urocentrida de Puytorac et al., 1987
    - Subclass Scuticociliatia Small, 1967
      - Order Philasterida Small, 1967
      - Order Pleuronematida Fauré-Fremiet in Corliss, 1956
    - Subclass Peritrichia Stein, 1859
      - Order Sessilida Kahl, 1933
      - Order Mobilida Kahl, 1933
    - Subclass Hysterocinetia Diesing, 1866
      - Order Hysterocinetida Diesing, 1866
    - Subclass Astomatia Schewiakoff, 1896
      - Order Hoplitophryida Cheissin, 1930
      - Order Anoplophryida Cépède, 1910
    - Subclass Hymenostomatia Delage & Hérouard, 1896
      - Order Tetrahymenida Fauré-Fremiet in Corliss, 1956
      - Order Ophryoglenida Canella, 1964
    - Subclass Apostomatia Chatton & Lwoff, 1928
      - Order Apostomatida Chatton & Lwoff, 1928
      - Order Astomatophorida Jankowski, 1966
      - Order Pilisuctorida Jankowski, 1966

Phylum CILIOPHORA Doflein, 1901 (d'après, Lynn, 2008)

Subphylum POSTCILIODESMATOPHORA Gerassimova & Seravin, 1976
- **Class KARYORELICTEA** Corliss, 1974
  - Order Protostomatida Small & Lynn, 1985
  - Order Loxodida Jankowski in Small & Lynn, 1985
  - Order Protoheterotrichida Nouzarède, 1977
- **Class HETEROTRICHEA** Stein, 1859
  - Order Heterotrichida Stein, 1859

Subphylum INTRAMACRONUCLEATA Lynn, 1996
- **Class SPIROTRICHEA** Bütschli, 1889
  - Subclass Protocruziidia de Puytorac et al., 1987
    - Order Protocruziida Jankowski in Small & Lynn, 1985
  - Subclass Phacodiniidia Small & Lynn, 1985
    - Order Phacodiniida Small & Lynn, 1985
  - Subclass Hypotrichia Stein, 1859
    - Order Kiitrichida Nozawa, 1941
    - Order Euplotida Small & Lynn, 1985
      - Suborder Discocephalina Wicklow, 1982
      - Suborder Euplotina Small & Lynn, 1985
  - Subclass Oligotrichia Bütschli, 1887
    - Order Strombidiida Petz & Foissner, 1992
  - Subclass Choreotrichia Small & Lynn, 1985
    - Order Tintinnida Kofoid & Campbell, 1929
    - Order Choreotrichida Small & Lynn, 1985
      - Suborder Strombidinopsina Small & Lynn, 1985
      - Suborder Strobilidiina Small & Lynn, 1985
      - Suborder Leegaardiellina Laval-Peuto, Grain & Deroux, 1994
  - Subclass Stichotrichia Small & Lynn, 1985
    - Order Plagiotomida Albaret, 1974
    - Order Stichotrichida Fauré-Fremiet, 1961
    - Order Urostylida Jankowski, 1979
    - Order Sporadotrichida Fauré-Fremiet, 1961
  - Subclass Licnophoria Corliss, 1957
    - Order Licnophorida Corliss, 1957
- **Class ARMOPHOREA** Jankowski, 1964 **
  - Order Armophorida Jankowksi, 1964
  - Order Clevelandellida de Puytorac & Grain, 1976
  - Order Odontostomatida Sawaya, 1940
- **Class LITOSTOMATEA** Small & Lynn, 1981
  - Subclass Haptoria Corliss, 1974
    - Order Haptorida Corliss, 1974
    - Order Pleurostomatida Schewiakoff, 1896
    - Order Cyclotrichida Jankowski, 1980
  - Subclass Trichostomatia Bütschli, 1889
    - Order Vestibuliferida de Puytorac et al., 1974
    - Order Entodiniomorphida Reichenow in Doflein & Reichenow, 1929
      - Suborder Archistomatina de Puytorac et al., 1974
      - Suborder Blepharocorythina Wolska, 1971
      - Suborder Entodiniomorphina Reichenow in Doflein & Reichenow, 1929
- **Class PHYLLOPHARYNGEA** de Puytorac et al., 1974
  - Subclass Phyllopharyngia de Puytorac et al., 1974
    - Order Chlamydodontida Deroux, 1976
    - Order Dysteriida Deroux, 1976
  - Subclass Rhynchodia Chatton & Lwoff, 1939
    - Order Rhynchodida Chatton & Lwoff, 1939
      - Suborder Rhynchodina Chatton & Lwoff, 1939
      - Suborder Ancistrocomina Chatton & Lwoff, 1939
    - Order Hypocomatida Deroux, 1976
  - Subclass Chonotrichia Wallengren, 1895
    - Order Exogemmida Jankowski, 1972
    - Order Cryptogemmida Jankowski, 1975
  - Subclass Suctoria Claparède & Lachmann, 1858
    - Order Exogenida Collin, 1912
    - Order Endogenida Collin, 1912
    - Order Evaginogenida Jankowski in Corliss, 1979
- **Class NASSOPHOREA** Small & Lynn, 1981
  - Order Synhymeniida de Puytorac et al., 1974
  - Order Nassulida Jankowski, 1967
  - Order Microthoracida Jankowski, 1967
- **Class COLPODEA** Small & Lynn, 1981
  - Order Bryometopida Foissner, 1985
  - Order Bryophryida de Puytorac et al., 1979
  - Order Bursariomorphida Fernández-Galiano, 1978
  - Order Colpodida de Puytorac et al., 1974
  - Order Cyrtolophosidida Foissner, 1978
  - Order Sorogenida Foissner, 1985
- **Class PROSTOMATEA** Schewiakoff, 1896
  - Order Prostomatida Schewiakoff, 1896
  - Order Prorodontida Corliss, 1974
- **Class PLAGIOPYLEA** Small & Lynn, 1985**
  - Order Plagiopylida Small & Lynn, 1985
- **Class OLIGOHYMENOPHOREA** de Puytorac et al., 1974
  - Subclass Peniculia Fauré-Fremiet in Corliss, 1956
    - Order Peniculida Fauré-Fremiet in Corliss, 1956
      - Suborder Frontoniina Small & Lynn, 1985
      - Suborder Parameciina Jankowski in Small & Lynn, 1985
  - Subclass Scuticociliatia Small, 1967
    - Order Philasterida Small, 1967
    - Order Pleuronematida Fauré-Fremiet in Corliss, 1956
  - Subclass Hymenostomatia Delage & Hérouard, 1896
    - Order Hymenostomatida Delage & Hérouard, 1896
      - Suborder Tetrahymenina Fauré-Fremiet in Corliss, 1956
      - Suborder Ophryoglenina Canella, 1964
  - Subclass Apostomatia Chatton & Lwoff, 1928
    - Order Apostomatida Chatton & Lwoff, 1928
    - Order Astomatophorida Jankowski, 1966
    - Order Pilisuctorida Jankowski, 1966
  - Subclass Peritrichia Stein, 1859
    - Order Sessilida Kahl, 1933
    - Order Mobilida Kahl, 1933
  - Subclass Astomatia Schewiakoff, 1896
    - Order Astomatida Schewiakoff, 1896

FIG. 2.10 – Description du phylum Ciliophora (De Puytorac, 1994, à gauche ; Lynn, 2008, à droite).

classes de ciliés (Heterotrichea, Spirotrichea, Oligohymenophorea, Nassophorea et Colpodea). L'étude confirme que la résolution intraspécifique obtenue avec ce gène est supérieure à celle obtenue avec n'importe quels gènes nucléaires et montre la possibilité de résoudre des relations au sein de taxa étroitement liés. Aujourd'hui, peu de séquences de ces différents marqueurs sont disponibles en banque (cox : ∼500), à l'inverse des séquences codantes pour le gène de l'ARNr 18S (∼4000).

2.5 Type trophique et écologie

Les ciliés sont des prédateurs, hétérotrophes pour la plupart ; ils se nourrissent de particules organiques, de bactéries, d'algues, d'autres protozoaires voire d'animaux microscopiques. Ils jouent un rôle clé dans le transfert des substances nutritives et de l'énergie via la chaîne alimentaire planctonique. Ils agissent comme des régulateurs sur le pico- et le nanoplancton, comme producteurs primaires dans le cas des espèces autotrophes et mixotrophes (*Myrionecta rubra* par exemple), ou encore comme source de nourriture pour le micro- et le zooplancton (Pierce and Turner, 1992).

Le processus alimentaire principal des ciliés est la phagotrophie, c'est-à-dire l'ingestion de particules alimentaires solides via la formation de vésicules. Le type trophique dépend de la nature et de la dimension des molécules. L'absorption de substances dissoutes peut se faire également soit par diffusion, soit par pinocytose (Lynn, 2008). L'alimentation transmembranaire peut être le seul mode de nutrition chez certains groupes de ciliés comme les astomes par exemple. En effet, ces ciliés, comme leur nom l'indique (*a* : préfixe de négation et *stome* : bouche) n'ont pas de « bouche » et sont retrouvés exclusivement en interactions avec des métazoaires. Ils possèdent en revanche des zones spécifiques pour se fixer aux tissus de leur hôte (De Puytorac, 1954) (Figure 2.11). A ce jour, peu d'informations quant à leur cycle de vie et leur mode d'interaction sont connues, et une seule séquence codant pour l'ARNr 18S est accessible dans les banques de gènes (Affa'a *et al.*, 2004) (Cf. section 4.2).

La structure orale des ciliés se spécialise selon leur régime alimentaire. Les ciliés qui utilisent la phagocytose ont pour la plupart un appareil d'ingestion avec une « bouche » dite cellulaire, le cytostome, flanquée de vésicules digestives (i.e. les lysosomes) ou phagocytaires. La présence de cils dans cette région orale permet chez certaines espèces la formation d'un courant d'eau qui entraîne les particules alimentaires vers le cytostome. Ce cytostome est prolongé par une zone d'ingestion (le cytopharynx) et en sortie, une zone d'excrétion des résidus (le cytoprocte). L'appareil buccale peut-être en surface de la cellule ou former une dépression (Figure 2.12). Par ce processus de phagocytose, les ciliés peuvent se nourrir de proies entières. D'ailleurs, la capture des proies est différente en fonction des espèces de ciliés, et certaines espèces ont développé d'ingénieux système (Dragesco, 1962)(Cf. encadré Sites internet). Par exemple, chez les *Dileptus* (Haptoria, Litostomatea) une protubérance se prolonge de leurs corps (proboscis) et contient à sa surface des organelles appelées toxicystes qui vont libérer des filaments enduits

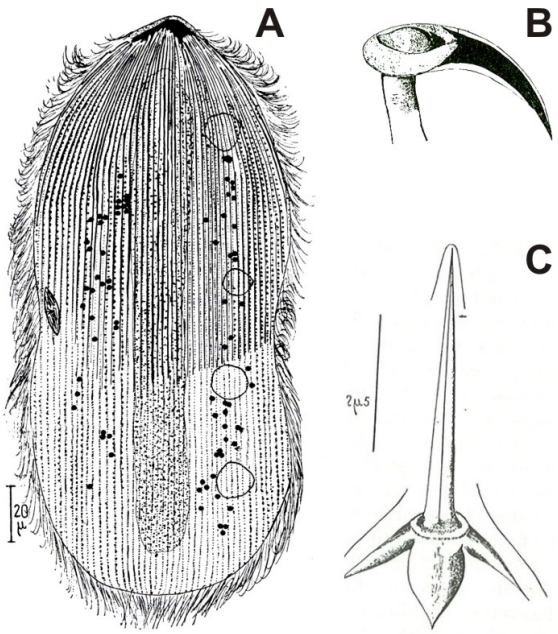

FIG. 2.11 – Les astomes. **A**, Dessin du cilié astome *Durchoniella brasili*. **B**, Dessin de l'appareil fixateur du cilié astome *Metaradiophrya lumbrici*. **C**, Dessin de l'aiguille de l'astome *Maupassella cepedei* (De Puytorac, 1954).

de substances toxiques permettant de commencer à digérer la proie et de la pousser vers le cytostome (Figure 2.12) (Vdacny and Foissner, 2008). D'autres espèces se nourrissant d'algues arrivent en un très court laps de temps à avaler entièrement leur « proie » végétale, ce qui induit une déformation complète de la cellule (pour revue, Grassé, 1984).

Les ciliés sont présents sous diverses formes : libres, fixes, coloniales, parasitaires non pathogènes ou pathogènes, ou encore sous formes symbiotiques. Ils ont pu s'adapter à de nombreuses niches écologiques surtout en milieux humides, parmi les plus étudiées : les eaux douces, saumâtres, marines, le sable, les sols humides...

Leur morphologie, leur cycle de vie (surtout par la formation de kystes), et leur mode trophique expliquent leur ubiquité, la nutrition étant certainement le facteur limitant (Grassé, 1984; Lynn, 2008). Les ciliés sont principalement des espèces libres ; les formes symbiotiques seront abordées plus en détail dans le chapitre suivant. En 2003, sur 1437 espèces décrites, 616 espèces sont décrites en environnement aquatique, et 413 vivent en symbiose (Lynn, 2003*b*).

Les études écologiques impliquant les ciliés ont commencé dès le début des années 1925. Les facteurs influençant la distribution des ciliés dans les eaux douces ont été étudiés, et les résultats montraient qu'il existait des espèces thermophiles et psychrophiles (Noland, 1925). D'autres études montraient également l'importance des ciliés dans les eaux interstitielles et les sédiments (e.g. Fauré-Fremiet, 1950; Dragesco, 1954; Fenchel, 1967) ou dans l'humus recouvrant le sol de forêt (pour revue, Grassé, 1984).

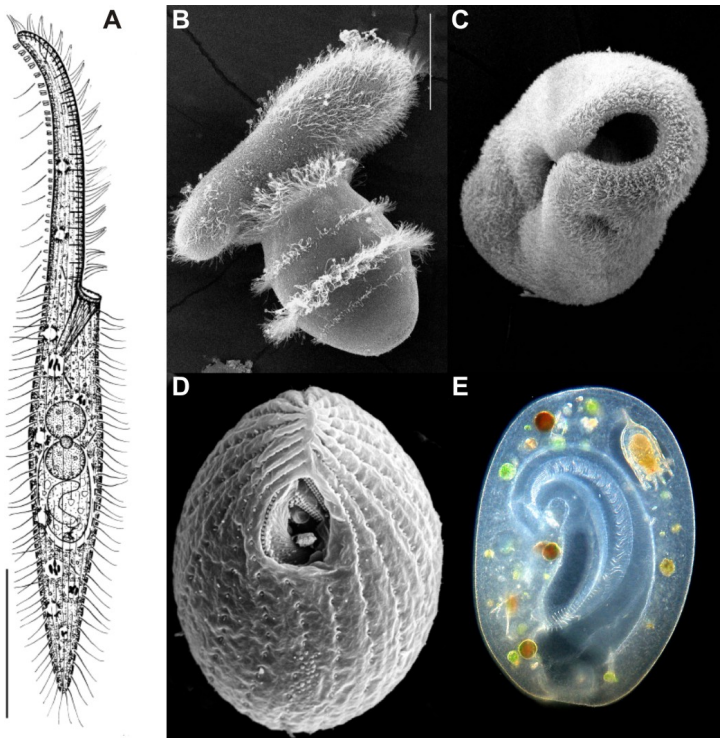

FIG. 2.12 – Nutrition. **A**, Dessin de *Dileptus tirjakovae*, échelle = 50 μm (Vdacny and Foissner, 2008). **B**, *Didinium* mangeant un *Paramecium*, M. Plociniak. **C**, *Bursaria* sp., avec vu sur la cavité orale, G. W. Grimes. **D**, *Tetrahymena thermophila* déciliée avec vu sur l'appareil oral, E.M. Nelsen et J. Frankel. B-D, photographies en MEB, Ciliate Image Database©. **E**, *Bursaria* sp., en microscopie à lumière blanche après un repas (algues, tintinnide...), P. Rotkiewicz, `http://www.pirx.com/`.

La distribution spatiale et saisonnière de la densité et de la biomasse des ciliés suit de façon générale les variations de la production bactérienne et du phytoplancton (e.g. Leakey *et al.*, 1996; Kim *et al.*, 2007). Le type de ciliés et leur taille s'adaptent en fonction des ressources alimentaires (entre autre, la taille des proies) (e.g. Montagnes *et al.*, 1988; Dolan and Marrase, 1995; Johansson *et al.*, 2004). Par exemple, chez les Tintinnides, la taille de l'ouverture de la cavité buccale détermine le spectre et la taille des proies ingérées (Dolan, 2010). La température serait également un aspect physique qui affecte les ciliés, principalement en contrôlant leur taux de croissance (Montagnes, 1996; Montagnes and Lessard, 1999; Santoferrara and Alder, 2009).

Les recherches sur la distribution des ciliés en utilisant les approches morphologiques et moléculaires montrent que les ciliés présentent une distribution différente (i.e. taille, espèce, abondance) entre les océans ouverts et l'environnement côtier (e.g. Moon-van der Staay *et al.*, 2001; Romari and Vaulot, 2004; Countway *et al.*, 2005; Medlin *et al.*, 2006; Not *et al.*, 2007; Dolan *et al.*, 2007; Masquelier and Vaulot, 2008; Santoferrara and Alder, 2009). Des séquences de ciliés sont détectées en nombre importants dans des environnements micro-aérophiles à

anoxiques de l'eau de mer ou du sédiment (e.g. Stoeck and Epstein, 2003; Stoeck *et al.*, 2003; Stoeck *et al.*, 2006; Behnke *et al.*, 2006; Zuendorf *et al.*, 2006; Takishita *et al.*, 2007*b*; Alexander *et al.*, 2009; Behnke *et al.*, 2010). Certains auteurs proposent la présence d'un clade *a priori* limité aux environnements anoxiques, nommé CAR-H (Stoeck and Epstein, 2003). Fenchel et Finlay (1995) proposaient déjà que les ciliés soient les organismes eucaryotes les plus communs dans les environnements anaérobies. Certains ciliés peuvent en outre jouer un rôle important dans ces écosystèmes, puisqu'en excrétant les produits de leur métabolisme anaérobie (e.g. acétate et propionate), ils favoriseraient l'activité microbienne (Biagini *et al.*, 1998).

CHAPITRE 3

LES INTERACTIONS ENTRE ORGANISMES

Les ciliés sont des organismes ubiquistes qui ont donc su s'adapter à des environnements très divers en adoptant des modes de nutrition ou des caractères morphologiques différents, et dans certains cas en mettant en place des relations symbiotiques. Ce cas particulier des symbioses sera abordé dans ce troisième et dernier chapitre.

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3.2 Symbiose et évolution 52
3.3 Symbiose et stratégies écologiques : de nombreux cas chez les ciliés 57

3.1 Généralités

Une interaction biologique désigne un processus impliquant des échanges ou des relations réciproques entre deux ou plusieurs individus d'une même population (relation intraspécifique) ou entre différentes espèces au sein d'une communauté (relation interspécifique). La symbiose, du grec *sym* : et/avec/ensemble et *bios* : vie , définit une association à long terme entre deux ou plusieurs organismes d'espèces différentes intégrés au niveau comportemental, métabolique ou génétique pour une part significative du cycle de vie des partenaires. Ce terme de symbiose à été introduit par Anton De Bary et Bernard Frank à la fin des années 1870 alors qu'ils travaillaient respectivement sur les lichens (résultat d'une symbiose entre un champignon hétérotrophe et une algue verte ou une cyanobactérie, autotrophes) et les mycorhizes (résultat de l'association symbiotique entre des champignons et les racines des plantes) (De Bary, 1879; Frank, 1885).

Selon les effets sur les partenaires de l'interaction symbiotique, les associations tiennent : du parasitisme, symbiose dans laquelle une espèce augmente ses aptitudes aux dépens d'une seconde espèce ; du mutualisme, symbiose dans laquelle les deux espèces augmentent leurs aptitudes ; ou du commensalisme, symbiose dans laquelle un des partenaires augmente ses aptitudes sans pour autant nuir à l'autre. Il existe une graduation entre ces grands types d'interaction et une même association peut démarrer sous une forme mutualisme, pour finir en parasitisme et réciproquement (Figure 3.1).

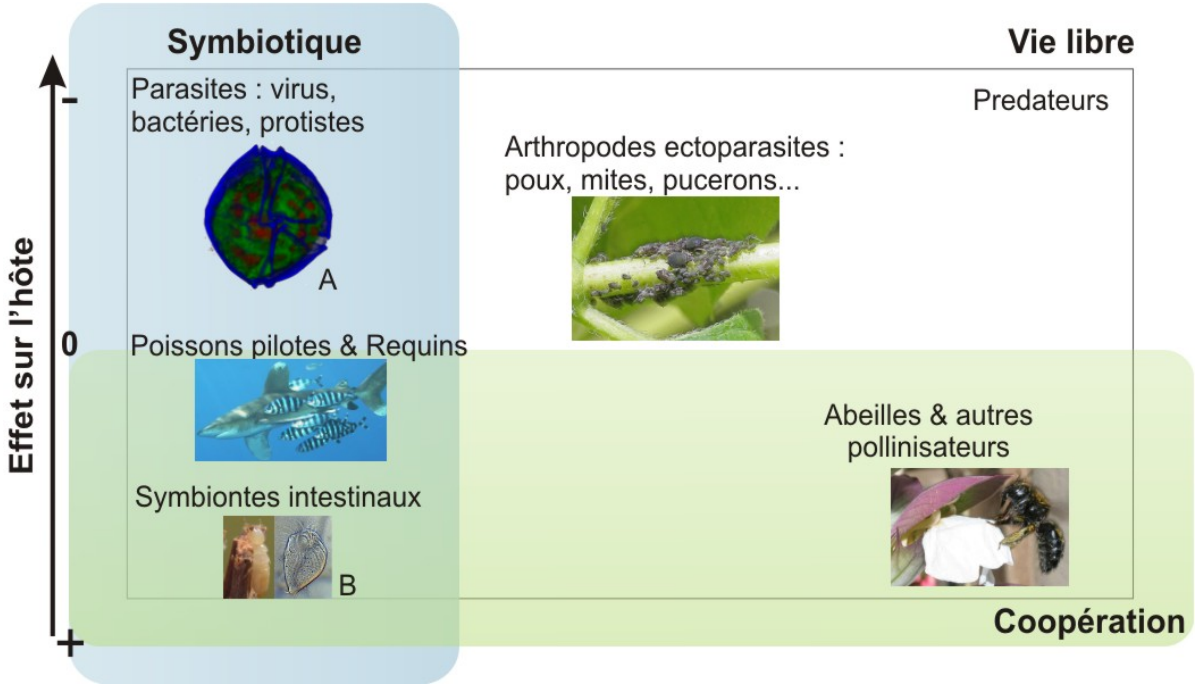

FIG. 3.1 – Représentation des différentes interactions possibles entre deux ou plusieurs organismes (d'après Toft and Mangel, 1991). **A**, *Alexandrium minutum* (bleu) et son parasitoïde (vert) (Chambouvet *et al.*, 2008). **B**, Un termite et son symbionte flagellé.

Il est généralement admis que lorsque les partenaires sont de tailles différentes, le plus grand sera défini comme l'hôte - celui qui héberge - et le symbionte sera défini comme étant l'organisme le plus petit, sans que cela n'ait de conséquence sur la nature de leur relation (Smith and Douglas, 1987). En fonction du niveau de dépendance de l'hôte, la symbiose peut-être obligatoire ou facultative. Selon l'emplacement du symbionte par rapport aux cellules hôtes, on peut affiner le type de relation : on parle alors d'endosymbiose quand le symbionte vit dans l'hôte, et d'ectosymbiose quand les partenaires vivent en association physique proche mais externe, le plus souvent le symbionte est fixé sur son hôte.

Les symbioses intracellulaires présentent deux cas distincts au regard de la dépendance de l'hôte et du symbionte, et de la durée de l'association. On parle alors d'endosymbionte primaire obligatoire, quant l'histoire évolutive entre les partenaires est longue, et d'endosymbionte secondaire facultatif, quant l'association semble être plus récente et que le symbionte conserve sa capacité à recouvrer une forme libre (pour revue, Moya *et al.*, 2008).

3.2 Symbiose et évolution

Pendant plusieurs décennies, l'idée que des associations microbiennes étaient au centre de l'évolution de la cellule eucaryote est restée controversée. Depuis la fin des années 1960, les travaux de Margulis ont contribué à l'établissement d'une théorie associant l'évolution des eucaryotes au phénomène d'endosymbiose (Margulis, 1971). Dans de nombreux phénomènes biologiques d'endosymbiose, des structures cellulaires nouvelles et/ou des capacités métaboliques apparaissent suite à l'évolution de cette interaction, et en favorise son maintien. Aujourd'hui, un consensus sur le rôle essentiel des endosymbioses dans l'origine et l'évolution des cellules eucaryotes est adopté, bien que des controverses sur les détails de ce rôle persistent (De Duve, 2007).

L'acquisition des mitochondries et des plastes a été un événement important dans l'évolution de la cellule eucaryote. La symbiose avec un organisme procaryote, il y a plus d'un milliard d'années, a amorcé ce processus. La physiologie, la phylogénie moléculaire et la biologie cellulaire permettent une meilleure compréhension des événements moléculaires complexes qui ont conduit à l'évolution de l'endosymbionte en organelle spécifique. En perdant leur autonomie, les endosymbiontes ont perdu la plus grande partie de leurs génomes, nécessitant l'évolution de mécanismes complexes pour la biogenèse et l'échange de métabolites.

La chaîne respiratoire mitochondriale est une fonction métabolique acquise par association symbiotique, cependant les détails de cette acquisition sont toujours débattus. En effet, deux types de modèles s'opposent : (1) les modèles proposant à l'origine une cellule avec noyau mais sans mitochondrie, suivie par l'acquisition de mitochondrie dans un hôte eucaryote via la symbiose avec une Alpha-Protéobactérie, et (2) les modèles proposant que la mitochondrie proviendrait d'un hôte procaryote (Alpha-Protéobactérie), qui aurait par la suite acquis les caractéristiques d'une cellule eucaryote (Figure 3.2 ; Embley and Martin, 2006).

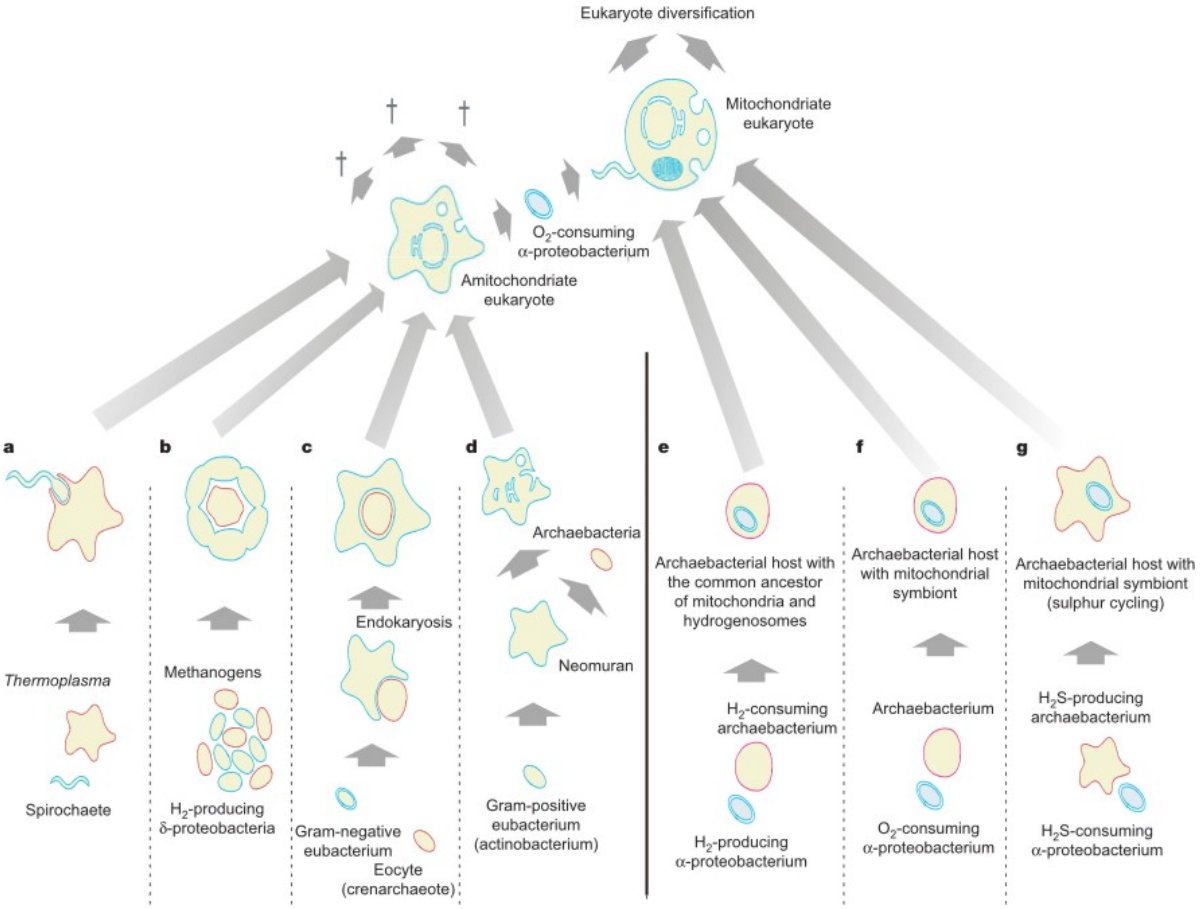

FIG. 3.2 – Les différentes hypothèses de l'origine de la cellule eucaryote basées en général sur des données génomiques. **a-d**, modèles qui proposent à l'origine une cellule avec noyau mais sans mitochondrie, suivie par l'acquisition de mitochondrie dans un hôte eucaryote via la symbiose avec une Alpha-Protéobactérie. **e-g**, modèles qui proposent que la mitochondrie proviendrait d'un hôte procaryote (Alpha-Protéobactérie), qui aurait acquis les caractéristiques d'une cellule eucaryote. L'origine des membranes lipidiques est indiquée par un code couleur : rouge pour une origine archéenne, et bleu pour une origine bactérienne (Embley and Martin, 2006).

Les mitochondries sont des organelles de forme arrondie ou allongée, elles sont le « moteur » de la cellule puisqu'elle renferme tous les composants de la chaîne respiratoire nécessaires à la formation d'ATP. Les hydrogénosomes sont des organelles produisant de l'hydrogène (d'où leur nom) et de l'ATP à partir de l'oxydation anaérobie du pyruvate ou du malate (Figure 3.3) (Müller, 1993). Ils sont retrouvés dans des organismes micro-aérophiles et anaérobies, mais tous les unicellulaires anaérobies ne contiennent pas d'hydrogénosomes. Il a été démontré que les hydrogénosomes sont le produit de l'évolution de la mitochondrie par une perte de leur capacité de respiration et d'une partie de leur génome (Müller, 1993; Fenchel and Finlay, 1995; Palmer, 1997; Martin *et al.*, 2008). Cependant, cette perte d'une partie du génome a été réfutée chez le cilié anaérobie *Nyctotherus ovalis* (Akhmanova *et al.*, 1998). Une étude récente a, en effet, montré que ce cilié possédait le chaînon manquant entre mitochondrie et hydrogénosome : une mitochondrie anaérobie exerçant les activités de la chaîne respiratoire mais ayant aussi la capacité de produire de l'hydrogène (Boxma *et al.*, 2005).

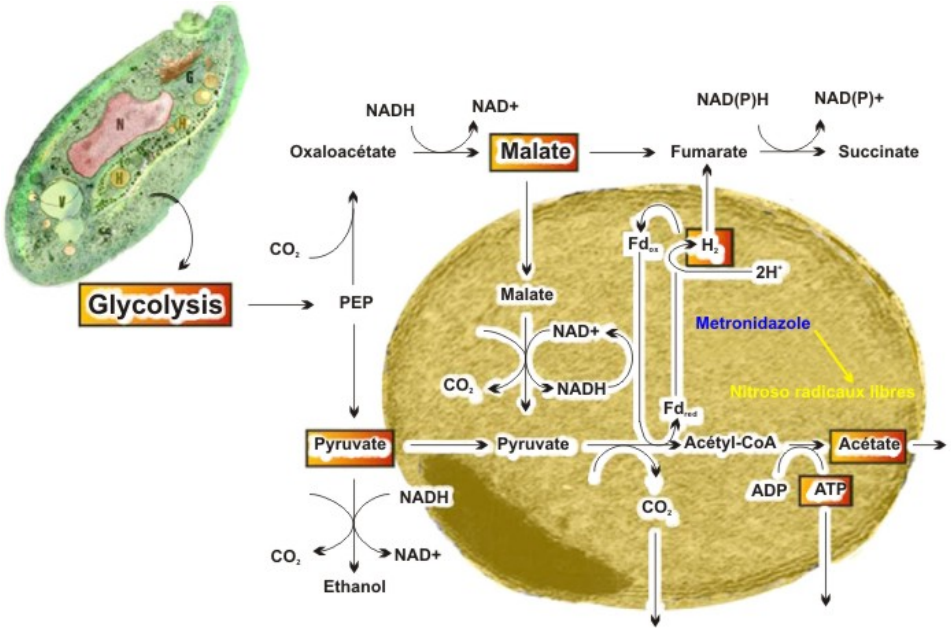

FIG. 3.3 – Métabolisme du pyruvate dans les hydrogénosomes, exemple de *Trichomonas foetus*. La décarboxylation oxydative du pyruvate, contrairement aux autres cellules eucaryotes, est catalysée par une enzyme différente, l'oxydoréductase pyruvate-ferrédoxine. En condition anaérobie, l'hydrogénase catalyse la formation de molécules d'hydrogène via la réduction de protons. Fd : ferrédoxine, PEP : phospho-enol-pyruvate (Benchimol, 2009).

La découverte de cette mitochondrie chez les eucaryotes a renouvelé l'intérêt de l'origine et des aspects évolutifs de la mitochondrie. Deux hypothèses sont retenues pour expliquer l'origine de la mitochondrie et des organelles associées : l'une préférant une descendance verticale à partir de la mitochondrie endosymbionte originale, et la perte d'une partie du génome aboutissant aux hydrogénosomes et aux mitosomes (autre organelle dérivée) ; la seconde proposant deux

événements successifs d'endosymbiose (Figure 3.4), l'acquisition de l'endosymbionte mitochondrial originel, puis sa perte et la formation des organelles dérivées (pour revue, Van der Giezen and Tovar, 2005). La découverte des « mitochondries anaérobies » du cilié *Nyctotherus* soutiendrait l'hypothèse de descendance verticale, et elles pourraient donc être considérées comme des mitochondries dégénérées (Boxma *et al.*, 2005; Van der Giezen and Tovar, 2005).

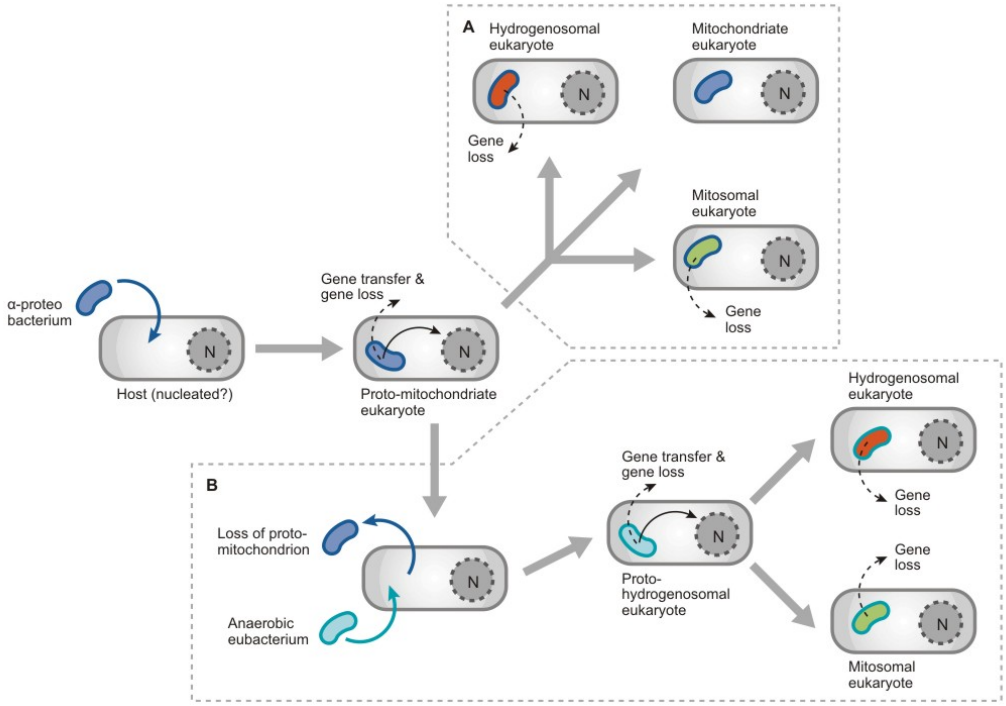

FIG. 3.4 – Evolution de la mitochondrie et des organelles relatifs. **A**, Descendance verticale de l'endosymbionte mitochondrial originel. **B**, Origines des mitosomes et des hydrogénosome via des endosymbioses en série. Les mitochondries et les Alpha-Protéobactéries sont en bleues, les hydrogénosomes en rouge, les mitosomes en vert et les bactéries anaérobies en turquoise. N : noyau (Van der Giezen and Tovar, 2005).

À la différence de l'origine des mitochondries, il n'y a plus de doute sur le fait que les plastes proviennent d'une association entre une cyanobactérie et une cellule hôte eucaryote formée avec un noyau, un cytosquelette et une mitochondrie. Les plastes issus de la symbiose primaire sont caractérisés par la présence de deux membranes provenant de la cyanobactérie, et sont retrouvés dans la lignée des algues rouges, des glaucophytes, et des algues vertes. Ce dernier groupe serait à l'origine des plantes terrestres (Figure 3.5) (pour revue, Archibald, 2009).

Du fait de la proximité régulière des partenaires, la symbiose engendre de nouvelles pressions évolutives impliquant parfois des coévolutions. Ainsi, mitochondries et plastes ont clairement co-évolués avec leurs hôtes depuis leur formation.

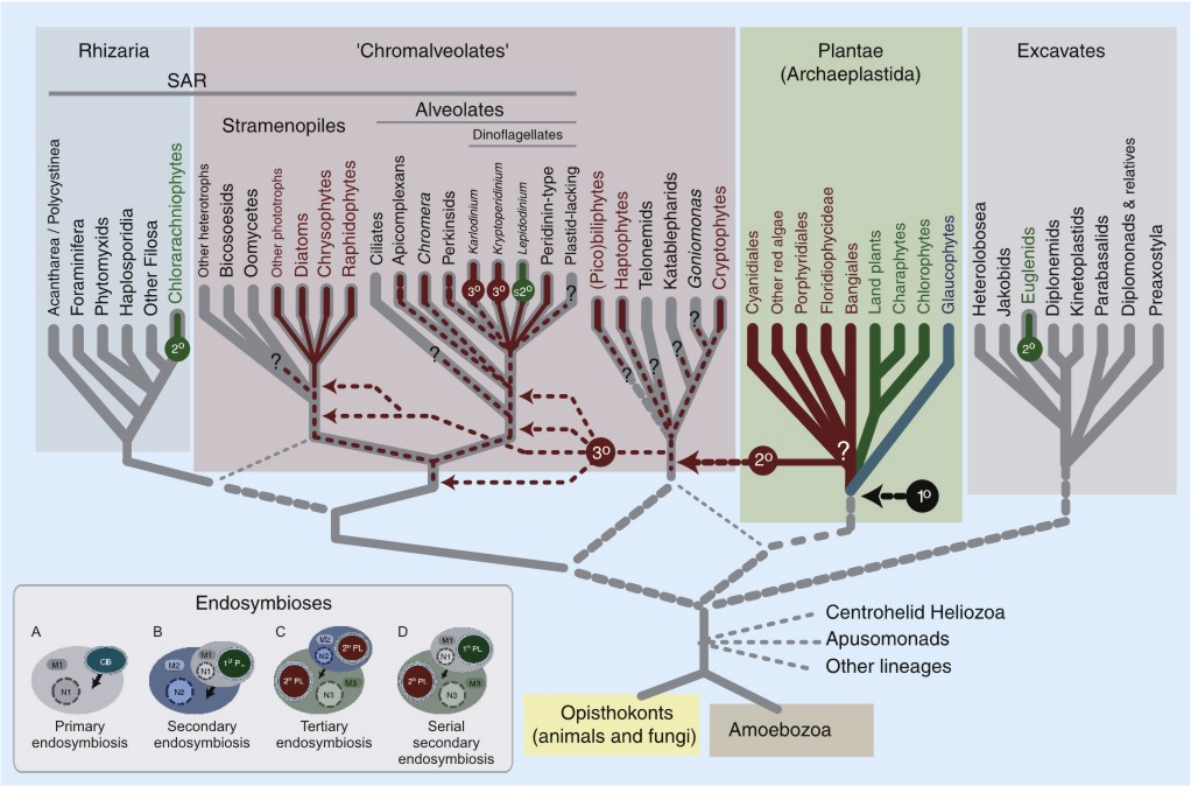

FIG. 3.5 – Hypothèses sur l'origine et la diffusion de la photosynthèse dans les différentes lignées d'eucaryote. Arbre phylogénétique schématisé montrant les six « super-groupes » des eucaryotes. De possibles endosymbioses primaires (1°), secondaires (2°), et tertiaires (3°) intervenues dans les lignées d'algues rouge et verte sont désignées par un code couleur. Les lignes en pointillés indiquent une incertitude quant à la relation entre les organismes et le transfert de plastes. Les points d'interrogations indiquent une incertitude à propos de la présence de plaste et/ou d'un ancêtre photosynthétique, ou un manque de données. **A-D**, Evolution des plastes par endosymbiose primaire, secondaire ou tertiaire. **A**, Endosymbiose primaire à l'origine des plastes, un hôte eucaryote non-photosynthétique conserve la double membrane d'une cyanobactérie ingérée. **B**, Endosymbiose secondaire : un eucaryote non photosynthétique ingère un eucaryote ayant au préalable acquis un plaste par endosymbiose primaire. Tous les plastes d'une symbiose primaire sont entourés de deux membranes. **C**, Endosymbiose tertiaire : un eucaryote possédant ou non un plaste englobe un symbionte résultant d'une endosymbiose secondaire. **D**, l'endosymbiose secondaire « en série » se réalise entre un hôte eucaryote résultant d'une endosymbiose secondaire et un symbionte avec un plaste acquis par endosymbiose primaire. CB : Cyanobactérie, M : mitochondrie, N : noyau, PL : plaste, SAR : Stramenopiles, Alveolata et Rhizaria (d'après Archibald, 2009).

3.3 Symbiose et stratégies écologiques : de nombreux cas chez les ciliés

Ils existent de nombreuses associations aussi bien entre procaryotes et animaux (les bactéries dans le rumen des vaches ou dans l'appareil digestif de l'Homme), qu'entre procaryote et eucaryote unicellulaire (certains lichens par exemple), mais aussi entre eucaryotes (de nombreux parasites unicellulaires chez l'Homme). Des symbioses stables se sont d'ailleurs indépendamment développées plusieurs fois dans les divers groupes d'eucaryotes sachant que pratiquement tous les groupes d'eucaryotes ont la particularité de porter des symbiontes bactériens (Figure 3.6) (pour revue, Moya *et al.*, 2008). Le groupe des ciliés est d'ailleurs un des groupes d'eucaryotes unicellulaires le plus riche en symbiontes bactériens (pour revue, Fokin, 2004).

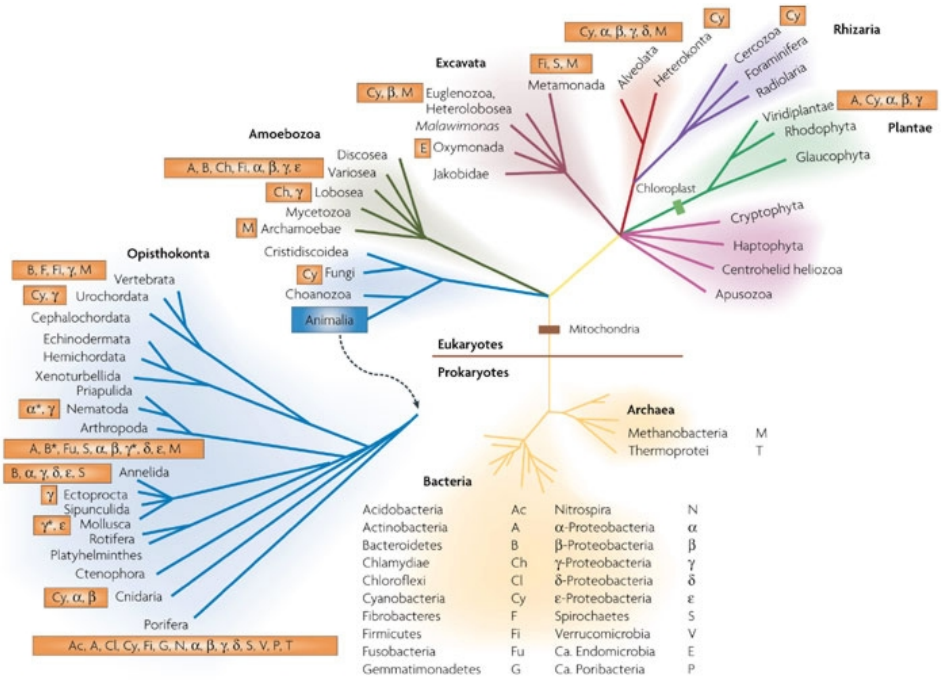

FIG. 3.6 – Distribution phylogénétique des symbiontes procaryotes et de leurs hôtes eucaryotes. La distribution phylogénétique des symbiontes procaryotes indique les classes de Bactéries et d'Archées pour lesquelles des associations avec des hôtes eucaryotes ont été observées. Les données sont le résultat d'études utilisant des approches écologiques, liées au développement, morphologiques, biochimiques et génomiques ayant examiner des cas de symbiose (Moya *et al.*, 2008).

Dans toutes les interactions, il existe un avantage pour l'un ou l'autre des partenaires, ou les deux. En effet, pour l'endosymbionte, le milieu intracellulaire représente un milieu défini et stable. Pour le porteur, quant il peut en tirer avantage, les trois principaux buts à une relation symbiotique stable sont : la nutrition, la défense et l'adaptation à des environnements

particuliers.

Toutes les symbioses mutualistes intracellulaires, analysées au niveau génomique, entre des procaryotes et les eucaryotes sont relatives à la nutrition et au catabolisme (pour revue, Moya *et al.*, 2008). La présence d'un symbionte peut permettre à l'hôte la synthèse de matières carbonées, d'acides aminés, de métabolites secondaires ou encore la fixation de l'azote. Certains protistes vont même jusqu'à « cultiver » leur symbionte pour avoir une réserve de nourriture continue. On peut citer l'exemple du cilié *Kentrophoros* qui ne possède pas de bouche cellulaire et qui porte sur sa face dorsale des bactéries sulfato-réductrice qui seront phagocytées (Figure 3.7) (Fenchel and Finlay, 1989). La forme générale du cilié a été montrée comme adaptée à la « culture » des bactéries par sa forme très plate qui offre donc plus de surface d'adhésion. Ce cilié se développe dans la zone micro-aérophile du sédiment, et possède des chimiorécepteurs permettant de trouver le milieu le plus adéquat (teneur en oxygène) aux bactéries ectosymbiotiques.

Le rôle potentiel de défense du symbionte procaryote n'a été rapporté jusqu'à présent que chez les ciliés (pour revue, Gast *et al.*, 2009). Les ectosymbiontes des espèces d'*Euplotidium*, appelés épixenosomes, protègent le cilié de l'ingestion par d'autres prédateurs en modifiant sa surface pour éviter que le cilié soit « reconnu » et facilement avalé (Figure 3.7) (Rosati *et al.*, 1999; Petroni *et al.*, 2000). D'une autre manière, les endosymbiontes bactériens principalement du genre *Caedibacter*, chez *Paramecium* et *Spirostomum*, favorisent la croissance de leur hôte en leur donnant la possibilité de tuer les cellules de la même espèce dépourvues de ces bactéries (Kusch *et al.*, 2002; Fokin *et al.*, 2003*b*).

Même si la nutrition et la défense relèvent de l'adaptation à des environnements spécifiques, l'interaction entre deux partenaires, ou entre un hôte et plusieurs symbiontes, forme une communauté biologique en constante évolution dans le temps. De fait, les êtres vivants en association symbiotique peuvent s'adapter à des milieux ou environnements qu'ils n'auraient pas toléré seuls ou qui n'auraient pas permis leur développement (pour revue, Gast *et al.*, 2009). Récemment, une symbiose en milieu hydrothermal entre un eucaryote unicellulaire et des bactéries a été démontrée pour la première fois. Il s'agit d'un cilié colonial possédant une lorica, *Folliculinopsis*, formant des bouquets au niveau de la ride Pacifique de Juan de Fuca, et qui abritent des ecto- et des endosymbiontes (Figure 3.8 ; Kouris *et al.*, 2007). Dans les environnements micro-aérophiles (océan profond) ou dans les sédiments anoxiques, les symbiontes sont utilisés principalement pour la réduction ou l'oxydation des sulfates (voir Chapitre 1, Partie 1). De plus, chez les ciliés anaérobies par exemple, la présence d'hydrogène intracellulaire produit par l'hydrogénosome intervient dans la mise en place de symbiose stable avec des archées méthanogènes qui utilisent cette hydrogène dans la réduction du dioxyde de carbone (pour revue, Van Hoek *et al.*, 2000).

Un autre cilié formant des colonies de grande taille, *Zoothamnium niveum* (Oligohymenophorea) (Figure 3.9), est exclusivement retrouvé dans des zones sédimentaires riches en sulfures. Il a été étudié pour son association obligatoire avec des bactéries ectosymbiotiques, qui lui donne

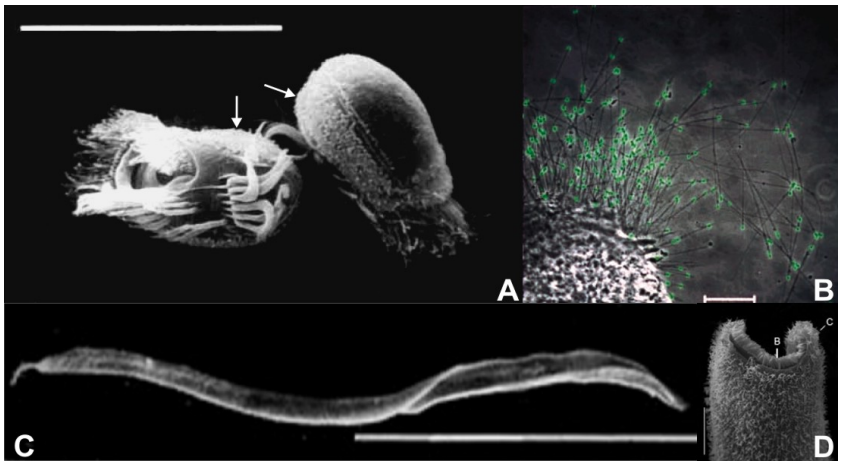

FIG. 3.7 – Symbiose extracellulaire. **A**, Deux *Euplotidium itoi* avec les épixenosomes insérés en bandes latérales (flèches), photographie en MEB, échelle = 100 μm (Rosati *et al.*, 1999). **B**, Détail du processus d'éjection des épixenosomes, ciblés avec des sondes spécifiques, sur *Euplotidium arenarium*, photographie en contraste de phase, échelle = 10 μm (Petroni *et al.*, 2000). **C**, *Kentrophoros* sp., photographie en MEB, échelle = 500 μm. **D**, Coupe horizontale du cilié montrant la distribution des cils (C) et des bactéries (B), photographie en MEB, échelle = 20 μm (Foissner, 1995).

d'ailleurs son aspect blanc et brillant. Ces bactéries chimioautotrophes sont capables d'oxyder les sulfures, et sont de deux types en fonction de la zone qu'elles colonisent sur le cilié, soit des formes de coques près de la partie orale soit des formes en bâtonnets sur le reste de la cellule (Rinke *et al.*, 2006; Rinke *et al.*, 2007). La forme de plume de la colonie de *Zoothamnium niveum* produit un flux unidirectionnel d'eau de mer. Récemment, par des techniques de modélisation numérique, il a été montré que les bactéries à la surface de *Z. niveum* pouvaient supporter des concentrations de sulfures 100 fois plus importantes que des bactéries sur des surfaces plates, comme celles des mates microbiennes (Roy *et al.*, 2009).

Contrairement à la diversité de fonctions occupées par les symbiontes procaryotes, les endosymbiontes eucaryotes sont principalement rapportés comme servant au mécanisme de photosynthèse. Chez les ciliés, le cas le plus documenté dans la littérature est celui du cilié dulçaquicole *Paramecium bursaria* qui contient des algues vertes du genre *Chlorella* (Figure 3.10). Les algues symbiontes approvisionnent l'hôte en carbone, fixé par la photosynthèse, sous forme de maltose, pendant que la paramécie fournit aux algues les composés azotés nécessaires à leur croissance (pour revue, Nowack and Melkonian, 2010). Récemment, un cas intéressant de séquestration d'organites a été trouvé chez un cilié d'eau douce vivant dans des eaux anoxiques. *Histiobalantium natans* est en effet capable de « voler » les mitochondries et les chloroplastes des algues, qu'il ingère pour les utiliser et se développer dans cet environnement sans oxygène (Figure 3.10 ; Esteban *et al.*, 2009).

Dans le milieu marin, de nombreuses associations parasitiques ont été étudiées du fait de

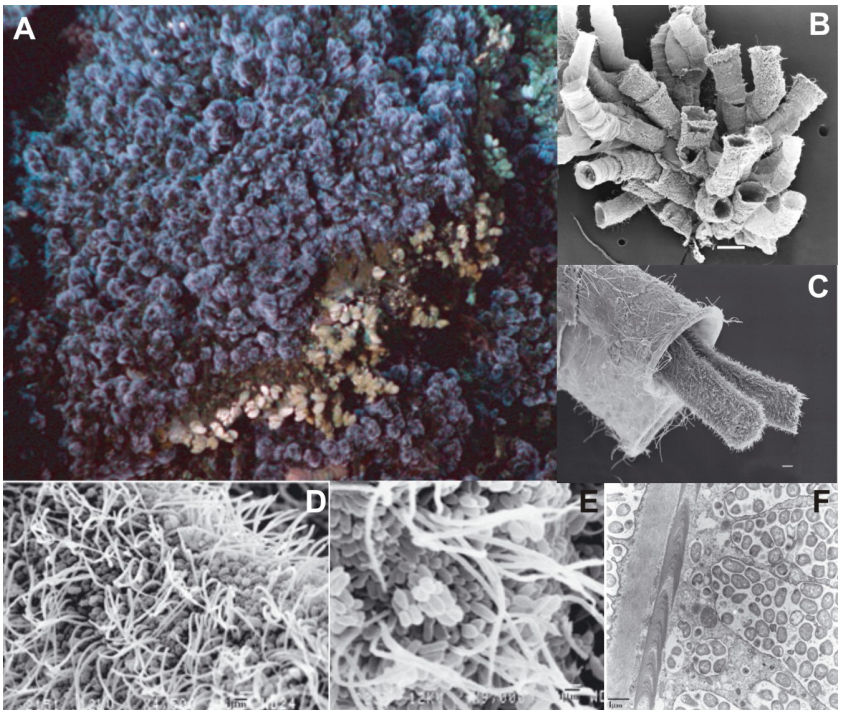

FIG. 3.8 – **A**, Les ciliés *Folliculinopsis* sp. forment des mats de couleur bleue au pied des cheminées hydrothermales, Juan de Fuca. **B**, Détail d'un fragment de mat de folliculinides en microscopie électronique à balayage. **C**, Extension d'un folliculinide. **D**, Les bactéries sont retrouvées entre les cils de l'hôte. **E**, Détail sur les bactéries en forme de coque et de bâtonnet. **F**, Vacuoles chargées de bactéries, photographie en MET (d'après Kouris *et al.*, 2007).

leurs implications économiques et sanitaires. Des études ont été réalisées sur les parasites de poissons ou de crustacés destinés à la consommation. Par exemple, la moule zébrée d'eau douce *Dreissena polymorpha* est un mollusque invasif des côtes européennes et américaines, et des études ont été réalisées sur les ciliés retrouvés en association soit parasitaire soit mutualiste avec cette moule pour essayer de comprendre sa dynamique de développement et d'expansion (e.g. Karatayev *et al.*, 2002; Molloy *et al.*, 2005; Conn *et al.*, 2008). Le cilié *Ichthyophthirius multifiliis* est un parasite obligatoire qui infecte les branchies et la peau de poissons d'eau douce responsable de l'Ichtyophthiriose ou « maladie des points blancs » (bien connue des aquariophiles) et pouvant entrainer la mort (Ewing and Kocan, 1992). Dans ces deux cas, les ciliés ont été retrouvés en association avec des procaryotes, et bien que leur rôle ne soit pas encore défini, les auteurs proposent différentes hyptohèses quant à leur implication dans la virulence ou au contraire dans la régulation de l'infection (Fokin *et al.*, 2003*a*; Sun *et al.*, 2009). L'impact économique et sanitaire en aquaculture a justifié la parution d'une nouvelle revue sur la Scuticociliatose, proposant différents moyens à adopter pour enrayer les infections causées par une vingtaine d'espèces particulièrement virulentes de ciliés, appartenant aux Scuticociliatia (Ciliophora) dont *Miamiensis avidus* et *Uronema marinum* (Harikrishnan *et al.*, 2010).

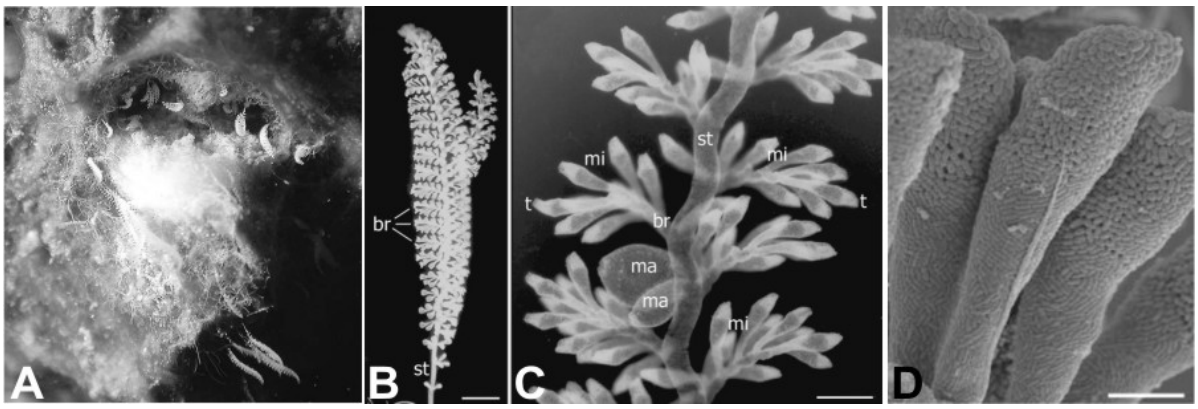

FIG. 3.9 – **A**, Plusieurs colonies de *Zoothamnium niveum* retrouvées au niveau d'une cheminée de sulfures (Roy *et al.*, 2009). **B**, Vue générale d'une colonie entière avec le pied central (st) et les différentes branches (br) portant les cellules, microscopie en lumière blanche, échelle = 0,5 mm. **C**, Détails de la partie moyenne d'une colonie montrant les trois types de cellules de *Z. niveum* : macrozooïdes (ma), microzooïdes (mi), et les zooïdes terminaux (t), microscopie en lumière blanche à contraste de phase, échelle = 50 μm (Rinke *et al.*, 2006). **D**, Bactéries ectosymbiontes à la surface du cilié, en forme de coque au niveau de la partie orale (partie supérieure de la photo) et en forme de bâtonnet du côté aboral des cellules microzoïdes (partie inférieure de la photo), photographie en MEB, échelle = 10 μm (Rinke *et al.*, 2007).

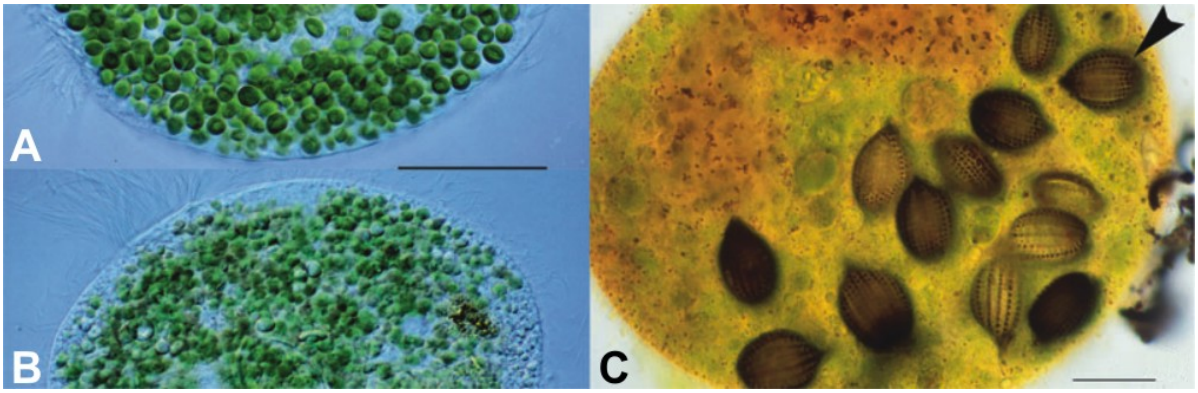

FIG. 3.10 – **A**, *Chlorella* sp., symbiontes du cilié *Euplotes daidaleos*. **B**, Particules vertes à l'intérieur de *Histiobalantium natans*, microscopie à lumière blanche. **C**, De nombreuses cellules de la micro-algue en forme de cœur *Phacus suecicus* sont stockées dans le cytoplasme de *H. natans*. La flèche pointe une de ces algues ingérées. Echelles = 25 μm (Esteban *et al.*, 2009).

OBJECTIFS

Comme nous l'avons exposé dans l'Introduction et la Partie 1, les eucaryotes unicellulaires sont des organismes ubiquistes très diversifiés aussi bien taxonomiquement que physiologiquement. Leur développement dans des environnements contrastés est sans doute lié à leur capacité à interagir avec leur environnement, et avec différents organismes. De plus, la détection récente, par des techniques de biologie moléculaire, de nouvelles lignées eucaryotes (en particulier au sein des Alveolata) dans des environnements encore peu explorés suggère que certains de ces groupes d'eucaryotes unicellulaires seraient inféodés à ces environnements, posant ainsi de nouvelles questions :

- Quels sont les facteurs contrôlant la distribution de ces micro-organismes ?
- Quelles modes d'interaction développent-ils avec leur environnement ?
- Quelles stratégies adaptatives mettent-ils en place ?

Pour essayer de répondre à ces questions, la Partie 2 développe les résultats obtenus au cours de cette thèse, composée de trois études présentées sous la forme d'articles publié, soumis ou en préparation.

Etude 1 Comparative analysis between protist communities from the deep-sea pelagic ecosystem and specific deep hydrothermal habitats.

Anne-Laure Sauvadet, Angélique Gobet and Laure Guillou. Environmental Microbiology, *in press.*

Cette étude est focalisée sur la diversité génétique des eucaryotes unicellulaires détectés au niveau de sources hydrothermales profondes. Elle décrit la diversité des communautés d'eucaryotes unicellulaires au niveau de la ride Est du Pacific (EPR) et de la ride médio-Atlantique (MAR), notamment en association avec les bivalves géants inféodés à ces environnements. Cette diversité a été comparée avec celle observée dans l'océan pélagique profond, afin de déterminer si ces micro-eucaryotes sont spécifiques des environnements hydrothermaux profonds et si certaines niches écologiques sont plus favorables à leur développement.

Etude 2 Tripartite interactions between Cirratulidae (Polychaeta), *Durchoniella* (Ciliophora, Astomatida) and *Bacteria* : A « Russian Doll » complex in anoxic coastal environments.

Anne-Laure Sauvadet,Sophie Le Panse, Erwan G. Roussel, Estelle M-C. Bigeard, Joseph Schrével and Laure Guillou. Protist, *Soumis.*

Cette étude décrit un genre particulier de ciliés appartenant aux Astomes, qui vivent exclusivement en interactions avec des animaux. Ces astomes sont retrouvés dans l'intestin de polychètes vivant dans des sédiments côtiers pauvres en oxygène et riche en matière organique, et composés réduits tels que l'hydrogène sulfuré. L'étude est basée sur la description morphologique et moléculaire de deux espèces de ciliés astomes appartenant au genre *Durchoniella*, que l'on retrouve uniquement en interaction avec des polychètes de la famille des Cirratulidae.

Etude 3 Biogeographical analysis of a tripartite association between Cirratulidae, *Durchoniella* and endocytoplasmic bacteria in tidal sediment of the English Channel.

Anne-Laure Sauvadet, Erwan G. Roussel, Estelle M-C. Bigeard, J. David George, Joseph Schrével and Laure Guillou (En préparation).

Cette étude décrit la distribution biogéographique, basée sur l'analyse de marqueurs phylogénétiques, de chacun des partenaires de l'association tripartite mise en évidence chez certains polychaetes Cirratulidae, et présentée dans l'Etude 2. De plus, afin de définir les facteurs environnementaux pouvant éventuellement contrôler ces interactions, la diversité phylogénétique et la prévalence de chacun des organismes ont été comparées aux données physico-chimiques de chacune des zones de prélèvements le long des côtes française et anglaise de la Manche.

Partie II - Résultats

Les ciliés, des sources hydrothermales aux sédiments côtiers

CHAPITRE 4

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4.1 Comparative analysis between protist communities from the deep-sea pelagic ecosystem and specific deep hydrothermal habitats

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Anne-Laure Sauvadet, Angélique Gobet, Laure Guillou

Les études moléculaires sur les populations d'eucaryotes unicellulaires retrouvées au niveau des sources hydrothermales profondes ont commencé il y a une dizaine d'année. Trois études ont été réalisées et montraient l'importance de certaines lignées dans ces environnements particuliers.

Les deux études de López-García et collaborateurs (López-García *et al.*, 2003; López-García *et al.*, 2007) portaient sur la diversité moléculaire des micro-eucaryotes dans les sédiments, les carbonates (morceaux de cheminées), et à l'interface entre le fluide hydrothermal et l'eau de mer de sites hydrothermaux de la ride médio-Atlantique. Edgcomb et collaborateurs (Edgcomb *et al.*, 2002) se sont intéressés aux sédiments hydrothermaux du bassin de Guaymas dans le golfe de Californie.

Dans cette étude, nous avons recherché les communautés d'eucaryotes unicellulaires associés à l'eau de mer profonde et aux bivalves échantillonnés au niveau de six sites hydrothermaux le long de la ride sud-est Pacifique. Ces communautés ont été caractérisées par microscopie et par l'étude moléculaire du gène codant pour l'ARNr 18S. Les populations ont ainsi pu être comparées aux communautés planctoniques échantillonnées dans l'eau de mer de 500 à 3000 mètres de profondeur de l'océan Pacifique.

A partir de cette eau de mer profonde, deux fractions de taille ont été analysées : les fractions supérieures à 3 μm présentaient principalement des Dinophyceae, alors que les fractions inférieures à 3 μm étaient dominées par des radiolaires et des *Syndiniales*. Au contraire, des dé-

tritivores et des brouteurs opportunistes plus spécifiques ont été détectés dans les échantillons provenant de l'eau dans l'environnement proche des cheminées hydrothermales, appartenant principalement aux groupes des Straménopiles et des Cercozoa. Les communautés observées dans le liquide de la cavité palléale des bivalves hydrothermaux du genre *Bathymodiolus* et *Calyptogena* étaient dominées par des ciliés (Phyllopharyngea, Oligohymenophorea and Oligotrichea) et des Cercozoa. Nous avons également pu comparer ces populations à celles retrouvées dans le liquide palléal de bivalves du genre *Bathymodiolus* échantillonnés au niveau de site hydrothermaux de la ride médio-Atlantique et de suintements froids dans le golfe du Mexique. Contrairement aux populations détectées dans les bivalves le long de la ride sud-est de l'océan Pacifique, ces communautés étaient beaucoup moins homogènes.

Des communautés complexes d'eucaryotes unicellulaires semblent donc être retrouvées de manière spécifique dans les bivalves hydrothermaux, ces derniers pouvant constituer une niche stable pour les micro-eucaryotes, comprenant des brouteurs, des détritivores, des symbiontes mutualistes et potentiellement des parasites. D'ailleurs, de ces communautés, de nouveaux groupes parmi les ciliés semblent se distinguer.

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Comparative analysis between protist communities from the deep-sea pelagic ecosystem and specific deep hydrothermal habitats

Anne-Laure Sauvadet,[1,2]* Angélique Gobet,[1,2†] and Laure Guillou[1,2]

[1]*CNRS, UMR 7144, Groupe Plancton Océanique, Station Biologique de Roscoff, BP 74, 29682 Roscoff, France.*

[2]*UPMC – Université Paris 06, Laboratoire Adaptation et Diversité en Milieu Marin, BP 74, 29682 Roscoff, France.*

Summary

Protist communities associated with deep seawater and bivalves from six hydrothermal sites in the Pacific Ocean were characterized by microscopy and molecular rRNA gene surveys (18S rRNA) and compared with planktonic communities from Pacific deep-pelagic seawater (from 500 to 3000 m in depth). Genetic libraries from larger size fractions (> 3 μm) of deep-pelagic water were mainly dominated by Dinophyceae, whereas small size fractions (< 3 μm) mainly revealed radiolarians and Syndiniales. In contrast, more specific opportunistic detritivores and grazers, mostly belonging to Stramenopiles and Cercozoa, were detected from water surrounding vent chimneys. Protist communities were different in the pallial cavity of the giant hydrothermal bivalves *Bathymodiolus thermophilus* and *Calyptogena magnifica*, dominated by Ciliophora (primarily belonging to Phyllopharyngea, Oligohymenophorea and Oligotrichea) and Cercozoa. Interestingly, protist communities retrieved from the pallial cavity liquid of hydrothermal bivalves were remarkably homogeneous along the Southern East Pacific Rise, in contrast to bivalves collected on the Mid-Atlantic Ridge hydrothermal vents and cold seeps from the Gulf of Mexico. Hence, complex protist communities seem to occur inside hydrothermal bivalves, and these metazoa may constitute a stable micro-niche for micro-eukaryotes, including grazers, detritivores, symbionts and potential parasites. From these communities, new lineages within the ciliates may emerge.

Received 4 December, 2009; accepted 23 April, 2010. *For correspondence. E-mail sauvadet@sb-roscoff.fr; Tel. (+33) 2 98 29 23 23; Fax (+33) 2 98 29 23 24. †Present address: Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany.

Introduction

Ecosystems from hydrothermal vents and hydrocarbon emissions on the continental slope (cold seeps) are based on chemosynthesis. Some of the chemoautotrophic prokaryotic primary producers, capable of oxidizing reduced compounds such as sulfide and methane, are found in symbiotic associations with benthic animals (Sarradin *et al*., 1999; Van Dover, 2000). At hydrothermal vents on the Pacific Rise, the fauna is mainly dominated by patches of giant vestimentiferans (*Riftia pachyptila*), dense populations of large bivalves and tube-building worms (alvinellids) which colonize active smokers. These organisms live under highly unstable environmental conditions, characterized by extreme temperatures, relatively low level of oxygen, the presence of toxic compounds such as hydrogen sulfide (H_2S), and various metals (e.g. iron, zinc, copper; for review see Van Dover, 2000). Although hydrothermal organisms were previously thought to represent ancestral lineages (Newman, 1985), recent analyses of dominant metazoans clearly indicate that hydrothermal fauna is contemporary, originating in the early Miocene (Little and Vrijenhoek, 2003). These organisms are very specialized, as illustrated by the large number of new species, mainly benthic animals and prokaryotes, discovered in these ecosystems during the last 25 years (Van Dover *et al*., 2002). However, studies on protist communities remain relatively sparse. Although all protists isolated from hydrothermal ecosystems are ubiquitous members of the marine environment (Atkins *et al*., 2000), direct microscopic studies suggested that ciliates inhabiting hydrothermal vents represent new communities (Small and Gross, 1985). Among the ciliates, the loricate folliculinids provide clear evidence for endemic and local adaptations. These ciliates form large bright-blue mats at the Juan de Fuca Ridge (Small and Gross, 1985) and have been reported to live with chemolithoautotrophic bacteria (Bergquist *et al*., 2007; Kouris *et al*., 2007).

Our knowledge of protist diversity was deeply modified by the use of culture-independent molecular techniques

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(e.g. Díez *et al*., 2001; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001). First applied on surface and deep oceanic seawater, these approaches were later used on samples collected from deep-sea hydrothermal and cold seep sediments. Two studies performed on sediment, carbonate and fluid–seawater interface of Mid-Atlantic Ridge (MAR) hydrothermal sites (López-García *et al*., 2003; 2007), showed great diversity within the major eukaryotic taxa and high concentrations of alveolates. A third study carried out on the sulfide and hydrocarbon rich hydrothermal sediments of the Guaymas Basin in the Gulf of California (Edgcomb *et al*., 2002) revealed the presence of putative indigenous lineages and typical photosynthetic organisms that may have come from the euphotic zone as suggested by López-García and colleagues (2007). The genetic diversity of microeukaryotes has been also investigated in various oxygen-depleted marine environments, including anoxic sediments (Dawson and Pace, 2002; Takishita *et al*., 2005), deep-sea methane cold seep sediments (Takishita *et al*., 2007), and anoxic deep water off the Venezuelan coast in the Caribbean Sea (Stoeck *et al*., 2003; 2006). These studies likewise revealed great eukaryotic genetic diversity and the presence of novel lineages at a high-taxonomic level, although some of these 'novel lineages' were later considered as artefacts resulting from chimeric and fast evolving sequences (Berney *et al*., 2004). Nonetheless, some sequences retrieved during these studies remain affiliated to unknown taxonomic lineages. In addition, some lineages have so far been retrieved only from anoxic or deep-sea environments, suggesting that very specialized protists could exist, including the novel ciliate class CAR-H (Stoeck *et al*., 2006), the Syndiniales Group II clades 9 and 15 (Guillou *et al*., 2008), and the marine stramenopiles clade 12 (Massana *et al*., 2004). A proportion of these environmental sequences from benthic ecosystems were closely related to known parasites, belonging to different eukaryotic lineages, such as Cercozoa, Stramenopiles, Opisthokonta and alveolates (Takishita *et al*., 2005). These potential parasites are thought to be hosted by the dense benthic animal community associated with hydrothermal vents (Moreira and López-García, 2003).

The aim of the present study was to examine planktonic protist communities from Southern East Pacific Rise (SEPR) hydrothermal sites and to identify potential protists specialized in such ecosystems by comparing the SEPR communities with those from deep Pacific pelagic waters, and other hydrothermal samples from the previous studies. Therefore, a large range of environments were sampled, including deep pelagic seawater (from 500 to 3000 m), water surrounding hydrothermal chimneys and mussel beds, and the liquid from the pallial cavity of bivalves. In addition, protist communities of bivalves associated with MAR hydrothermal environments and deep-sea methane cold seeps (Gulf of Mexico) were investigated. The samples from the Pacific were screened microscopically for the presence of protists, and these direct observations were complemented by molecular and comparative analyses of the phylogenetic community structures based on amplification of the SSU rRNA gene (18S).

Results

Site location and characteristics

The geographic position and identification of stations sampled during this project are shown in Fig. 1, with Table S1 summarizing the general characteristics for each site. Material from the Southern Pacific included deep nutrient-rich water from the Chilean coastal upwelling (UPX1 and UPX3), deep water from moderately oligotrophic stations (EGY1 and EGY6) in the transition zone between the Chilean upwelling and the central Pacific Gyre, and samples from seven stations located along the southern extension of the East Pacific Rise. Samples collected along the SEPR were of five types: (i) deep, oligotrophic water not associated with vents or vent communities (GYR6), (ii) deep water collected 300–900 m below the sea surface, above vent communities (Oasis), (iii) deep water surrounding vent chimneys (Sarah' Spring, Hobbs and Fromveur), (iv) deep water collected 10–20 cm above vent communities and containing dense clouds of small white particles (Oasis), and (v) pallial cavity fluids from mussels, *Bathymodiolus thermophilus*, and clams, *Calyptogena magnifica* (Oasis, Rehu Marka and Lucky Eric). Unlike other vent samples, those from Sarah' Spring were from chimneys that lacked venting. Lucky Eric was a newly discovered hydrothermal site with alvinellid-colonized black smokers surrounded by beds of giant mussels, while Hobbs had alvinellid-covered chimneys. Fromveur was a site with active black smokers and a single colony of *Alvinella* sp., whereas active black smokers covered with *Alvinella* sp. and large patches of mussels, clams and stalked cirripedes were observed on vent chimneys at Oasis and Rehu Marka (Jollivet *et al*., 2004).

Samples from the Northern Atlantic comprised pallial fluid from *Bathymodiolus azoricus* collected at two hydrothermal stations (Menez Gwen and Lucky Strike) on the MAR, and pallial fluid from *Bathymodiolus brooksi* and *Bathymodiolus childressi* collected at deep, cold seep areas at one station (MC853) in the Gulf of Mexico. The Lucky Strike site was characterized by high temperature, active black smokers surrounded by large mussel beds, while the Menez Gwen site had small chimneys surrounded by mussel patches (Sarradin *et al*., 1999). The

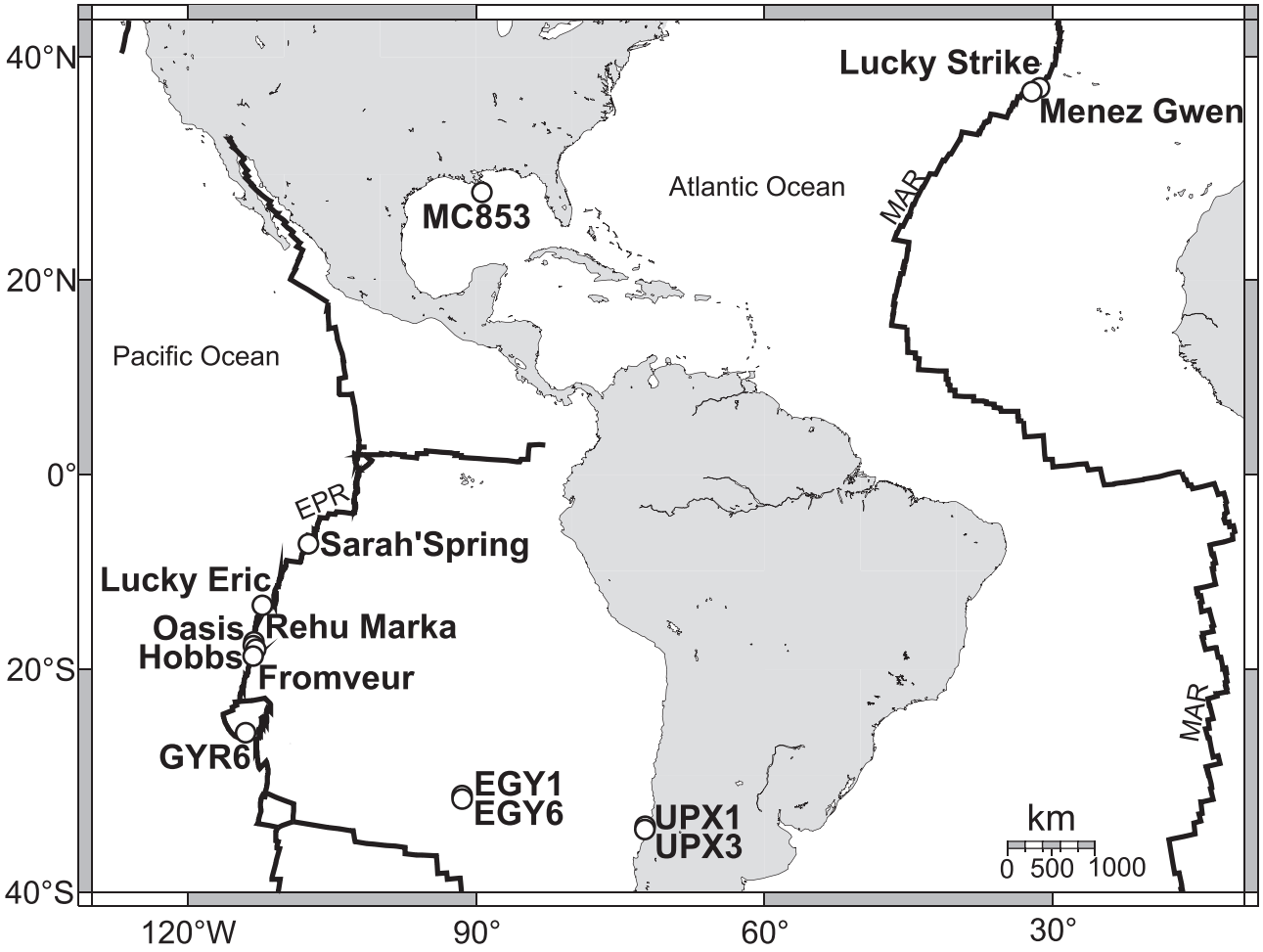

Fig. 1. Location of sampling sites during BIOSPEEDO (SEPR), BIOSOPE (South Pacific Ocean), MoMARETO (MAR) and Expedition to the Deep Slope (Gulf of Mexico) cruises. Both ridges are symbolized by a solid line.

dominant macrofauna at both sites consisted of large mussels (*B. azoricus*) and hydrothermal shrimps (*Rimicaris exoculata*), and giant vestimentiferan and alvinellids were absent. The cold seep areas at MC853 had high methane and hydrocarbon emissions and had siboglinid tubeworms, clams (e.g. *Calyptogena* spp.), and mussels (*Bathymodiolus* spp.) as the dominant, high biomass colonizers (for review see Cordes *et al*., 2009).

Selection and validation of primers

The general eukaryotic primers (Euk328f–Euk329r, see *Experimental procedures*) used to construct the first four genetic libraries obtained during this study (BS1 to BS4) produced a large proportion of metazoan sequences. In particular, sequences generated from water collected 10–20 cm above mussel beds at the Oasis site (libraries BS1 and BS2) were ninety percent metazoan in origin (Fig. 2). Consequently, the reverse primer UNonMet, designed to amplify protists from metazoan tissues (Bower *et al*., 2004; see *Experimental procedures*), was used to target protist communities present in one of the samples collected above mussel beds at Oasis (library designation BS12) and in samples from all other stations.

Comparison of the UNonMet primer motif with 18 215 SSU rRNA gene sequences (> 1300 bp) available in GenBank database and annotated with the KeyDNATools database (Guillou, 2008) is summarized in Table 1. The seven major protistan groups represented in the 18 215 SSU rRNA gene sequences showed sequence matches to the UNonMet primer motif. Over 80% of the sequences available for the Archaeplastida, Chromalveolata, and *incertae sedis* eukaryotes matched the motif. Half or more of the sequences available for the Amoebozoa and Rhizaria were positive for the motif, but only a quarter of the Opisthokonta and Excavata sequences matched the motif. Taxonomic subdivisions within the Amoebozoa, Excavata, Rhizaria and Opisthokonta, however, varied considerably in their potential detection using UNonMet. For example, within the Excavata, none of the 200 plus

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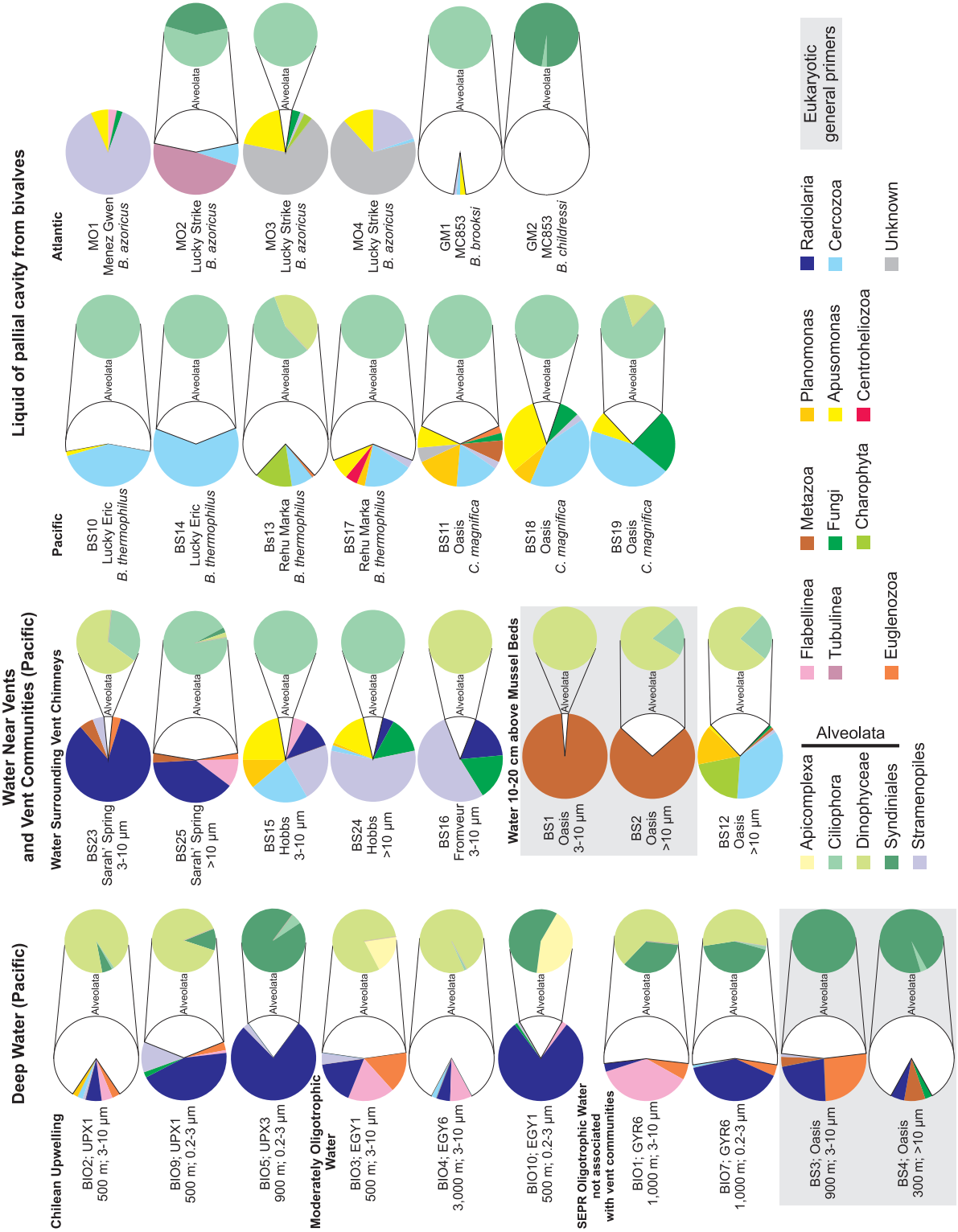

Fig. 2. Relative abundance of protist clones retrieved from deep seawater, from water near vent and vent communities in Pacific Ocean, and liquid of pallial cavity of bivalves from SEPR and Atlantic libraries. The name of the library, the name of the site, the depth and the size fraction or the bivalve species are indicated for each clone library. The different protist divisions were represented by colours in pies, and the Alveolata were separated into four classes in a second pie: Apicomplexa, Ciliophora, Dinophyceae and Syndiniales.

Table 1. Detection of the UNonMet primer inside 18 215 SSU rRNA gene sequences (> 1300 bp) available in GenBank belonging to major eukaryotic lineages.

	Total sequences	Positive match	%[a]
Amoebozoa	386	253	65.54
Amoebozoa *incertae sedis*	49	44	89.80
Archamoebae	30	5	16.67
Flabellinea	122	98	80.33
Mycetozoa	125	52	41.60
Tubulinea	60	54	90.00
Archaeplastida	2002	1826	91.21
Chlorarachniophyta-nucleomorph	13	12	92.31
Chlorophyta	730	698	95.62
Cryptophyta-nucleomorph	91	87	95.60
Rhodophyta	783	677	86.46
Streptophyta	385	352	91.43
Chromalveolata	5143	4264	82.91
Alveolata	3082	2346	76.12
Cryptophyta	132	130	98.48
Haptophyta	171	160	93.57
Stramenopiles	1758	1628	92.61
Excavata	986	259	26.27
Euglenozoa	640	205	32.03
Euglenida	245	160	65.31
Diplonemea	38	25	65.79
Kinetoplastea	394	27	6.85
Fornicata	38	4	10.53
Heterolobosea	35	14	40.00
Jakobida	11	10	90.91
Oxymonadida	57	26	45.61
Parabasalia	205	0	0.00
Incertae sedis	109	99	90.83
Apusomonadida	17	15	88.24
Breviatea	11	11	100.00
Centroheliozoa	50	50	100.00
Planomonadida	13	6	46.15
Telonemia	18	17	94.44
Opisthokonta	8921	2847	31.91
Choanoflagellida	98	97	98.98
Fungi	2813	2507	89.12
Mesomycetozoa	62	58	93.55
Metazoa	5948	185	3.11
with Annelida	452	2	0.44
Arthropoda	2256	4	0.18
Cnidaria	362	0	0.00
Ctenophora	18	0	0.00
Mollusca	542	13	2.40
Myxozoa	179	63	35.20
Nematoda	464	1	0.22
Platyhelminthes	702	8	1.14
Porifera	155	93	60.00
Rhizaria	570	440	77.19
Cercozoa	377	329	87.27
Foraminifera	70	0	0.00
Radiolaria	123	111	90.24

a. % = (Positive match/Total sequences) × 100.

sequences available for the Fornicata and parabasalids showed matches to the UNonMet motif, while well over 80% of the Jakobida sequences were positive. The same observation was made for the Rhizaria, as none of the Foraminifera sequences were revealed by the UNonMet primer. Within the Opisthokonta, only the metazoan sequences presented low frequency of matches, while well over 80% of the sequences for all other taxa representing that group were positive. Of the subdivisions within the Metazoa, only the Porifera and the Myxosoa showed appreciable matches to the UNonMet motif (60% and 35% respectively; Table 1).

Although molecular techniques (PCR and cloning) used to produce clone libraries are known to be inherently biased (Polz and Cavanaugh, 1998; Nocker *et al*., 2007), we assumed that biases were similar for all samples (von Wintzingerode *et al*., 1997), and we chose to express the data as a percentage of clones to compare the distribution of taxa among the genetic libraries. While restriction fragment length polymorphism (RFLP) screening may not be the best technique to assess the full diversity of an environment, we believe its use appropriate for capturing an overview of the dominant members of protist communities represented in our samples from diverse environments. For additional information, coverage values (C_x) calculated from the relative distribution of operational taxonomic units (OTU) for the distinct genetic libraries ranged from 67% to 100% (Table S1). Environmental sequences affiliated with 13 different divisions distributed among six eukaryotic super groups (Table S2), i.e. Amoebozoa, Archaeplastida, Chromalveolata, Excavata, Opisthokonta and Rhizaria (for nomenclature see Adl *et al*., 2005). Some *incertae sedis* eukaryotes with no affiliation to these six eukaryotic super groups were also retrieved, including *Apusomonas*, *Planomonas* (previously assigned to the genus *Ancyromonas*; see Cavalier-Smith *et al*., 2008) and Centroheliozoa.

Comparison of libraries across sample types

Deep oceanic protist communities from the South Pacific were successfully amplified from the 3–10 μm size fractions including 377 clones, obtained at a depth of 500 m from Chilean upwelling station (UPX1; library designation BIO2), at depths of 500 m and 3000 m from oligotrophic transition stations (EGY1 and EGY6; library designation BIO3 and BIO4 respectively), and collected at 1000 m depth from GYR6, a SEPR station not associated with vents or vent communities (library designation BIO1) (Fig. 2). While, the 0.2–3 μm size fractions included 373 clones, collected from Chilean upwelling stations (UPX1 and UPX3) from 500 m (library designation BIO9) and 900 m (library designation BIO5), from the oligotrophic transition station EGY1 obtained from 500 m (library designation BIO10), and from 1000 m depth from GYR6 station (library designation BIO7, Fig. 2). The largest size fraction (> 10 μm) did not allow a correct amplification of these samples.

Clonal libraries developed from water surrounding near vents including 307 clones were from a greater than 10 μm size fractions obtained at the Sarah' Spring site

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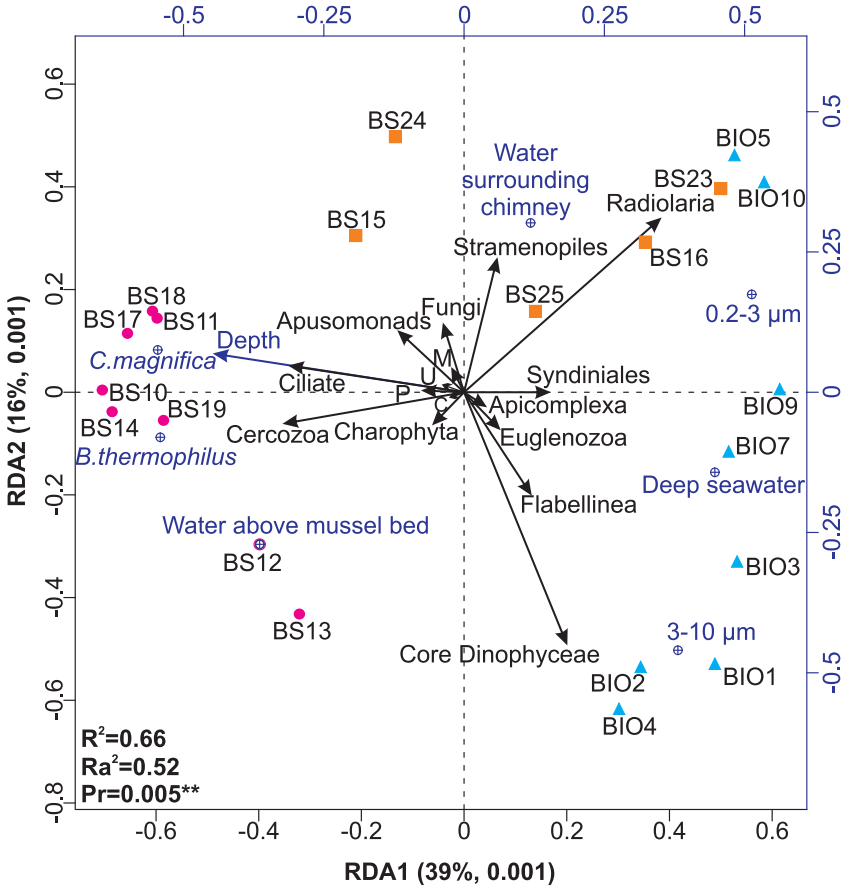

Fig. 3. Plot redundancy analysis (RDA) integrating taxonomic groups, environmental locations and genetic libraries from the Pacific Ocean. The depth and taxonomic groups (continuous variables; C = Centroheliozoa, M = Metazoa, P = *Planomonas*, U = Unknown) are shown as arrows. The vector orientation represents the direction of strongest change; vector length corresponds to the relative abundance. The sample types are illustrated by blue crossed circles. Genetic libraries are represented by solid symbols (blue triangle for deep seawater, yellow square for water surrounding chimneys and pink circle for pallial cavity liquid in bivalves) and a pink empty circle represents water above mussel bed library. The percentage of variability explained by each axis (RDA1 and RDA2) and the *P*-value are shown in brackets. R^2: *R*-squared, Ra^2: adjusted *R*-squared and Pr: *P*-value of the model.

(library designation BS25) and the Hobbs site (library designation BS24), and from the 3–10 µm size fractions from Sarah' Spring (library designation BS23), Hobbs (library designation BS15) and Fromveur chimneys (library designation BS16, Fig. 2). For these samples, the smallest size fractions (0.2–3 µm) were not amplified.

Because of the viscosity of the pallial liquid from bivalves, DNA was extracted on non-filtrated samples. Four genetic libraries developed from pallial cavity fluids retrieved from *B. thermophilus* included 238 clones, from the Lucky Eric site (library designations BS10 and BS14), and from the Rehu Marka site (library designations BS13 and BS17; Fig. 2). Three clonal libraries including 100 clones represented pallial cavity fluids from *C. magnifica* collected from the Oasis site (library designations BS11, BS18 and BS19; Fig. 2). Four supplementary clonal libraries developed from MAR *B. azoricus* fluids included 288 clones, from the Menez Gwen site (library designation MO1), and from three individual mussels collected at the Lucky Strike site (library designations MO2, MO3 and MO4). Two genetic libraries including 127 clones represented pallial cavity fluids from mussels collected from the MC853 site in the Gulf of Mexico, from *B. brooksi* (library designation GM1), and from *B. childressi* (library designation GM2; Fig. 2).

Results of a redundancy analysis (RDA) conducted using the 21 genetic clone libraries corresponding to Pacific locations, excluding the four clone libraries built with the eukaryotic general primer, are shown in Fig. 3. Fifty-five per cent of the variability was explained by the first two axes, with the adjusted *R*-squared value indicating that 52% of the biological variation was significantly explained by our constrained model (Pr = 0.005). This analysis supported three major correlations. The larger size fraction (3–10 µm) from deep water samples, regardless of trophic condition or geographic location, correlated with dinoflagellate sequences ($r = 0.80$, $P < 0.001$), while Flabellinea ($r = 0.48$, $P < 0.01$) and Euglenozoa ($r = 0.50$,

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$P < 0.01$) were also well represented. In contrast, the 0.2–3 μm size fractions were primarily characterized by the presence of radiolarians ($r = 0.68$, $P < 0.001$) and syndinians ($r = 0.69$, $P < 0.001$), with most of the Group II syndinians (77%) detected only in this size fraction. Another significant correlation encompassed all genetic libraries retrieved from the pallial cavity liquid of hydrothermal Pacific bivalves and also included water samples collected 10–20 cm above mussel beds. Ciliophora ($r = 0.88$, $P < 0.001$) and Cercozoa taxa ($r = 0.69$, $P < 0.01$) showed a relationship with all these samples, regardless of site along the SEPR or the species of bivalves sampled (*Calyptogena* and *Bathymodiolus*). The presence of stramenopiles was significantly related to water collected near vent chimneys ($r = 0.78$, $P < 0.001$), regardless of chimney location or sample size fraction.

When a RDA analysis was conducted using the Pacific libraries mentioned above and the genetic libraries corresponding to Atlantic locations (Fig. S1), 50% of the variability was explained by the first three axes, and the adjusted *R*-squared value indicated that 48% of the biological variation was significantly explained by this model (Pr = 0.005). The three-dimensional result is more difficult to interpret, but likely reflects the smaller number of samples obtained from Atlantic locations, none of which comprised small or large size fractions of deep water (only bivalves were sampled). Nevertheless, this analysis revealed no correlation between the majority of Atlantic samples and Pacific samples. Only the genetic library from *B. brooksi* (GM1) was significantly correlated with the four genetic libraries from pallial cavity liquid of Pacific bivalves ($P < 0.001$ with BS10 and BS13, and $P < 0.0001$ with BS17 and BS11). The two genetic libraries retrieved from pallial cavity liquid from *B. azoricus* (MO3 and MO4) were significantly correlated ($P < 0.0001$).

Micro-eukaryote diversity at South East Pacific stations

Microscopic observations. Qualitative observations revealed that seawater sampled near vent chimneys and well above bivalve beds had relatively low densities of micro-organisms, mainly composed of prokaryotes forming aggregates or long-chain colonies (Fig. S2A and B). Water collected 10–20 cm above the mussel bed at Oasis also contained numerous spermatozoa-like cells (i.e. large nucleus, reduced cytoplasm and a single, relatively long flagellum) of various shapes and sizes (Fig. S2C–E), although a morphotype harbouring a large oblong nucleus was the most abundant (Fig. S2D). In contrast, the pallial fluid from the hydrothermal bivalves *C. magnifica* and *B. thermophilus* had dense populations of protists representing a variety of morphotypes (Fig. S2F–K and Fig. 4). Most of these protists swam actively in the viscous pallial liquid, even several hours after sampling. One cell type observed in two *C. magnifica* (No. 9 and 10) and one mussel at the Oasis site had an ovoid body composed of two hemispheres joined at a subequatorial constriction bearing a belt of cilia (Fig. S2F). Another morphotype with an elongate-ovoid shape bearing long cilia (Fig. S2G), occurred in both mussels collected at the Lucky Eric site. A third form detected in a *Bathymodiolus* also collected at the Lucky Eric site (Fig. S2H) had a broadly pyriform body that was truncated at the ciliated anterior end. The fourth morphotype (Fig. S2I–K), only observed in Lugol-fixed pallial fluid from one mussel retrieved from the Oasis site, had a pedicle-like structure located at the aboral end of the lorica (Fig. S2I), and only one nucleus was detected (Fig. S2K). The daughter cells of some species like *Bicosoeca* settle on the parental lorica, as may be the case in Fig. S2J and K.

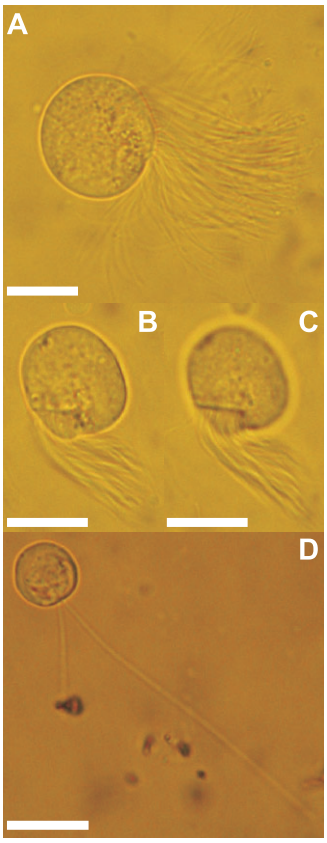

Fig. 4. Microscopic observations of unknown flagellates.
A. One morphotype observed in *C. magnifica* (Oasis).
B and C. Same cell observed under different depth, note the polar insertion of cilia (*C. magnifica*, Oasis).
D. Different flagellate organism (*C. magnifica*, Oasis).
Scale bars correspond to 5 μm.

Figure 4A–D represents unknown flagellate taxa observed in three different clams and two mussels at the Oasis site, three mussels at the Lucky Eric site, and one mussel at the Rehu Marka site. Some of these specimens

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Fig. 5. Bayesian analysis of the representative Ciliophora 18S rRNA gene sequences retrieved from different samples in the study. Study sequences are in bold. Each phylotype from each clone library is represented by one sequence with ≥ 95% similarity grouping. Posterior probability of Bayesian method, Neighbour-Joining and Maximum Parsimony bootstrap values higher than 60% are shown at nodes on the phylogenetic tree. Outgroup sequences are not shown but are composed of 12 sequences among eukaryotic taxa (AF167154, AB011423, EF027355, AF106047, AJ246267, AJ246266, AY705740, X81373, AJ007285, AB017121, Z28974 and AF008242). The number of clones of each representative sequence is indicated in exponent. Triangles symbolize deep seawater, squares water near chimneys, empty circles water above mussel bed and solid circles pallial cavity liquid from bivalves. MAR and Gulf of Mexico sequences are underlined.

exhibited an atypical polar insertion of numerous elongated thin flagella that were deeply rooted inside the cells (Fig. 4A–C). These cells readily lost their flagella with Lugol fixation. Figure 4D likely represents a flagellate having two heterokont flagella.

Molecular diversity at non-vent habitats. Most of the sequences in the 3–10 μm size fractions from deep Pacific water were related to the Dinophyceae (Fig. 2, Table S2), including environmental sequences, *Lepidodinium viride* and *Gymnodinium* sp. (98% similarity, Table S3). A moderate proportion of sequences were affiliated with Flabellinea (two strains of *Vannella*).The Radiolaria were represented by two polycystine genera (*Collodaria* and *Spumellarida*) and a lineage with a 96% similarity to a sequence from the hydrothermal MAR system (López-García *et al*., 2007). The Euglenozoa were predominantly of the Deep Sea Pelagic Diplonemid cluster (DSPD, Lara *et al*., 2009; Fig. S3) while the Syndiniales were Group I. The remaining sequences were allied with Apicomplexa closely related to *Colpodella pontica* (99% similarity), Stramenopiles, cercozoan, *Planomonas*, and ciliates including widespread marine genera such as *Strombidium* and *Novistrombidium* and a MAR environmental sequence retrieved from fluid–seawater mix (LC22-5EP-7, Fig. 5, López-García *et al*., 2007). In contrast, the 0.2–3 μm size fraction libraries were predominantly radiolarian in origin, with the Taxopodia lineage and three polycystine genera, especially *Spumellarida* accounting for most sequences. Dinoflagellates and syndinians (mostly Group II) were second in proportional representation. The remaining clones encompassed the Stramenopiles mostly close to *Caecitellus parvulus*, Apicomplexa, DSPD diplonemids, *Vannella*, fungi, Cercozoa and oligohymenophorean ciliates (Fig. 5) close to MAR environmental sequences retrieved from fluid–seawater mix (LC22-5EP-20; López-García *et al*., 2007).

Two genetic libraries constructed using general eukaryotic primers and water collected well above vents at the Oasis site (BS3 and BS4) were mainly composed of sequences related to syndinians in Group I (44%) and in Group IV (9%). The Euglenozoa (95% as DSPD and 5% close to *Bodo saliens*; Fig. S3), and the polycystine radiolarian were also well represented (Fig. 2, Table S2). Metazoan sequences were mostly related to the hydrozoan (Cnidaria) *Gilia reticulate* (99% sequence similarity, Table S3). No sequence of MArine Stramenopiles (MAST) was detected in the deep seawater libraries, using the both set of primers.

Molecular diversity in water samples near vent and vent communities

- *Water taken near vents/chimney.* The greater than 10 μm size fraction library from Sarah' Spring vents was dominated by ciliate sequences, with a majority close to the phyllopharyngean *Dysteria procera* (95% similarity; Fig. 5). The polycystine radiolaria *Collodaria* and the Flabellinea were second in proportional representation (Table S2). Remaining sequences allied with metazoa (Patellogastropoda), the DSPD diplonemids (Fig. S3), and Sargasso Sea environmental Group I syndinians and dinoflagellates. Most of the sequences in the 3–10 μm size fraction from Sarah' Spring were related to *Collodaria*. The remaining sequences were allied with Patellogastropoda, stramenopiles, dinoflagellates and oligohymenophorean ciliates. Diplonemid sequences were related to the DSPD and the *Diplonemea/Rhynchopus* group (Fig. S3).

  The genetic libraries from Hobbs and Fromveur chimneys were markedly different, comprising half of their sequences related to stramenopiles genera *Caecitellus* and *Cafeteria*. Fungi were mostly related to *Kodamaea* sp., a Saccharomycotina. Apusomonads close to *Amastigomonas debruynei* isolated from surface and deep seawater from the Atlantic and the Sargasso Sea (Scheckenbach *et al*., 2005; Fig. 6), Ciliophora, Cercozoa and polycystine radiolaria were also well represented. The remaining sequences were allied with *Planomonas* (Fig. 6), flabellinean *Vannella* and dinoflagellates.
- *Water taken a few centimetres above bivalve beds.* The two genetic libraries constructed using general eukaryotic primers and water collected 10–20 cm above bivalve beds at the Oasis site (BS1 and BS2) were metazoan in origin, mostly related to the bivalve *B. thermophilus* (99% similarity). Other sequences derived from dinoflagellates related to the Sargasso Sea and the MAR environmental sequence LC22-4EP-19 (98% similarity, López-García *et al*., 2007), and from oligohymenophorean ciliates.

  In contrast, the majority of sequences in the library constructed using the same sample and the UNonMet

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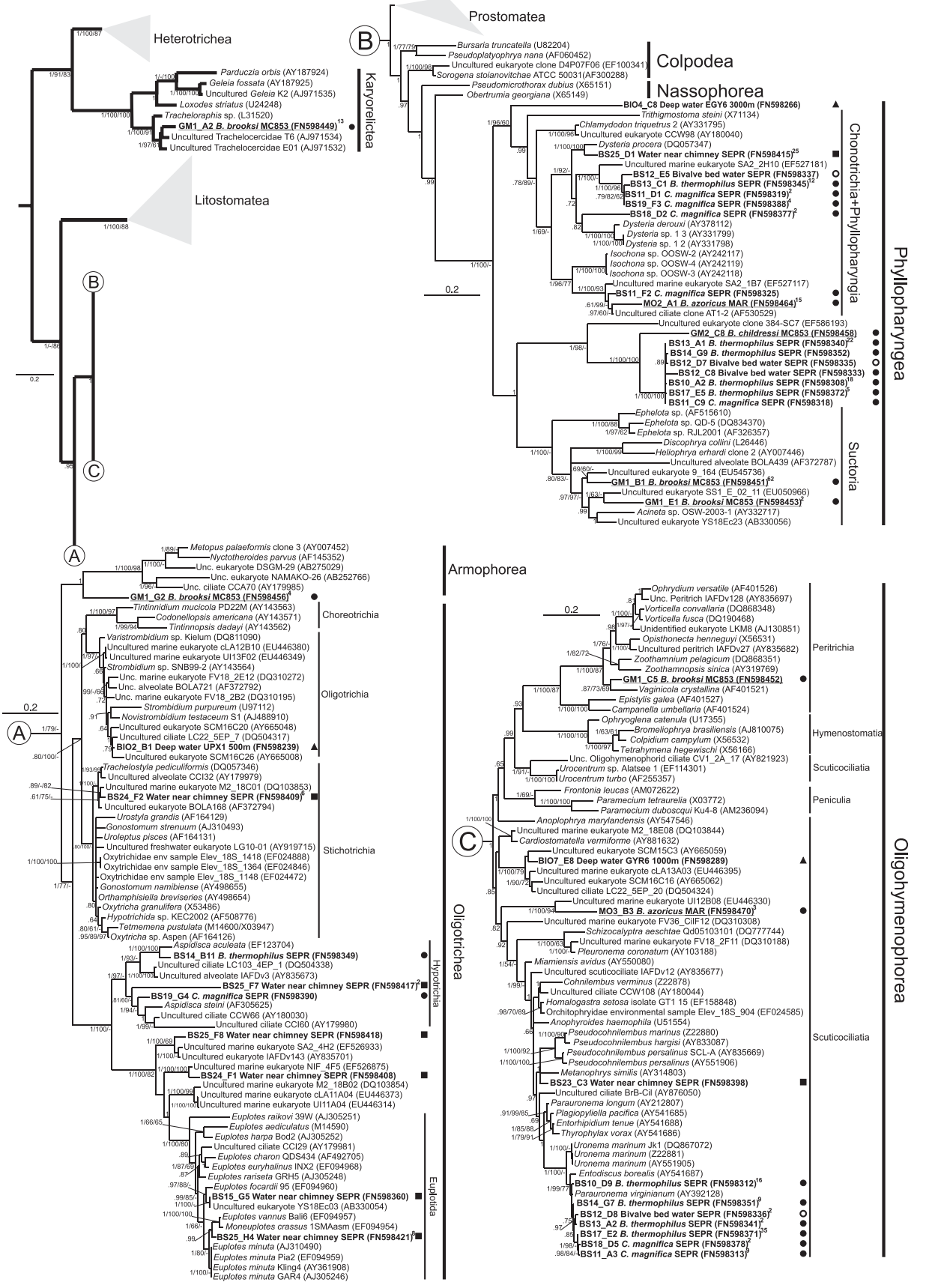

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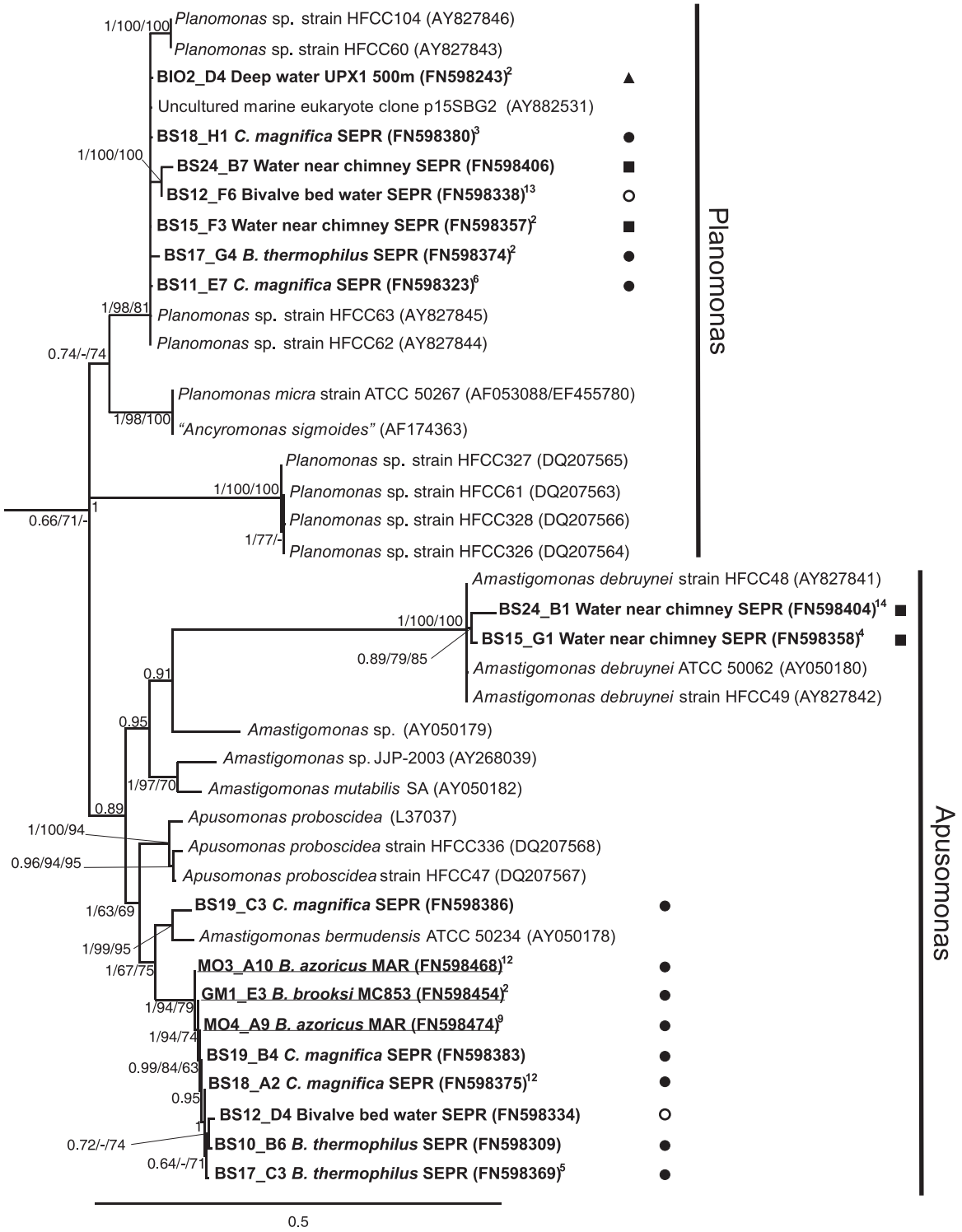

Fig. 6. Bayesian analysis of apusomonads and planomonads environmental 18S rRNA gene sequences retrieved from different samples in the study. Study sequences are in bold. Each phylotype from each clone library is represented by one sequence with ≥ 95% similarity grouping. Posterior probability of Bayesian method, Neighbour-Joining and Maximum Parsimony bootstrap values higher than 60% are shown at nodes on the phylogenetic tree. Outgroup sequences are not shown but are composed of three sequences among Excavata taxon (AF411288, AF053089 and AF119811) and one metazoan sequence (AF174349). The number of clones of each representative sequence is indicated in exponent. Triangles symbolize deep seawater, squares water near chimneys, empty circles water above mussel bed and solid circles pallial cavity liquid from bivalves. MAR and Gulf of Mexico sequences are underlined.

primer (BS12) were Cercozoa primarily related to *Massisteria marina*. Streptophyta and dinoflagellates were second in proportional representation. Planomonads sequences corresponded to environmental sequences from the marine anoxic Cariaco Basin (Stoeck *et al*., 2006) and culture isolates (Fig. 6).The other clones aligned with ciliates, fungi, metazoa (Cnidaria), stramenopiles and apusomonads (Fig. 6).

Molecular diversity of bivalve fluid micro-eukaryotes. Half of the sequences from Lucky Eric mussels were related to ciliate taxa, including Oligohymenophorea close to *Parauronema virginianum*, Phylopharyngea, and oligotrich ciliates linked to *Aspidisca aculeate* (95% similarity; Fig. 5). Environmental cercozoan sequences were second in proportional representation. Sequences from Rehu Marka mussels were also predominantly ciliates in origin, related to Oligohymenophorea close to *P. virginianum* and Phylopharyngea allied with *Ephelota* species (Fig. 5). Dinoflagellates were much better represented in the pallial fluid library from Rehu Marka. Cercozoan sequences related to *M. marina*, while streptophytes were identical to *Luffa quinquefida*. The remaining clones represented apusomonads, environmental centroheliozoa retrieved from marine mud (Cavalier-Smith and von der Heyden, 2007), planomonads, diplonemids and environmental stramenopiles, detected below the chemocline of the anoxic Mariager Fjord (Zuendorf *et al*., 2006).

Most of the sequences from *C. magnifica* were related to the cercozoan family Massiteriidae. Ciliates (Phyllopharyngea and Oligohymenophorea), and apusomonads associated with *Amastigomonas bermudensis* (Fig. 6) were second in proportional representation (Table S2). Fungi belonged to the Chytridiomycota, including sequences close to *Triparticalcar arcticum* (98% similarity), and linked to the basidiomycota agaricomycetes *Cystofilobasidium infirmominiatum* (99% similarity). *Planomonas* sequences were close to environmental sequences from the marine anoxic Cariaco Basin (Fig. 6). The remaining sequences were allied with metazoa, stramenopiles, dinoflagellates and Euglenozoa related to environmental *Prokinetoplastea* sequences retrieved from Atlantic hydrothermal sediments and from the Supersulfidic Anoxic Fjord (López-García *et al*., 2003; Behnke *et al*., 2006).

Micro-eukaryotes diversity associated with bivalves from the MAR and the Gulf of Mexico

Sequences from Menez Gwen mussels were predominantly stramenopiles in origin and were closely associated with *Cafeteria* (Table S2 and S3). The remaining sequences were affiliated with apusomonads, flabellinean *Vannella* and fungi. Most of the sequences in the library from Lucky Strike bivalve (MO2) were related to the Tubulinea (Amoebozoa). Phyllopharyngean ciliates close to the MAR environmental sequence AT1-2 (also from the Lucky strike site, Fig. 5; López-García *et al*., 2003), and Group I syndinians were also well represented. In contrast, most of the sequences in the two other libraries from Lucky Strike bivalves (MO3 and MO4) failed to affiliate with known taxa of eukaryotes. The affiliated sequences were primarily apusomonads close to *A. bermudensis* (92–93% similarity, Fig. 6) and stramenopiles mostly related to *Phaeoplaca thallosa* (95% similarity). The remaining sequences represented fungi, streptophytes, Cercozoa and oligohymenophorean ciliates related to an environmental sequence retrieved from a hypersaline anoxic deep-sea basin (Alexander *et al*., 2009).

Most of the sequences from *B. brooksi* were related to ciliates, primarily the Phyllopharyngea and the Karyorelictea, a taxon only detected in this sample. All these ciliate sequences were very different from those retrieved from SEPR bivalve libraries (Fig. 5). The remaining sequences were allied with the apusomonad *A. bermudensis* (Fig. 6), the cercozoan *M. marina*, and stramenopiles linked to *Cafeteria*. In contrast, the majority of the sequences from *B. childressi* affiliated with the Group II syndinians. The other sequences were allied with phyllopharyngean ciliates.

Discussion

Validation of primers

According to Bower and colleagues (2004), the UNonMet primer specifically amplifies protist communities without confounding amplification of metazoa. Use of this primer has allowed us to describe protist communities otherwise hidden among the numerous metazoan sequences (e.g. from water above mussel beds and from bivalve liquid). The work of Bower and colleagues (2004) and our data, however, indicate that the UNonMet primer may result in the underestimation or absence of some eukaryote groups, particularly in deep-water samples. For example, Euglenozoa, mostly the Kinetoplastea, are underestimated, and Foraminifera, a group known to occur in deep sea (Gooday, 1986), is completely absent from the genetic libraries made with this primer. Our results also indicate that some genera of the phylum Amoebozoa may also be less efficiently amplified using the UNonMet primer. Nonetheless, the genus *Vannella* was often found in our libraries, but has rarely been retrieved from previously developed deep seawater environmental genetic libraries.

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Protists inhabiting the deep ocean

Comprehensive assessment of the molecular diversity of unicellular eukaryotes retrieved from deep seawater has been the focus of several studies over the past 10 years. Reports considering protistan diversity in the aphotic zone (250–3000 m deep) of the Antarctic polar front (López-García *et al*., 2001), at depths up to 3000 m in the Sargasso Sea (Not *et al*., 2007), in abyssal plains of the south-eastern Atlantic Ocean (Scheckenbach *et al*., 2010), and Gulf Stream in the western Atlantic Ocean (Countway *et al*., 2007), and at depths up to 3500 m in the Mediterranean Sea (Alexander *et al*., 2009) have shown much greater diversity of protists in the deep ocean than previously recognized. These studies have also revealed novel lineages of unicellular eukaryotes, including the stramenopiles and the Group I and Group II marine alveolates. In addition, the distribution of lineages and taxa are now known to be heterogeneous relative to depth and size fraction of samples analysed. Our examination of deep water from the Pacific Ocean also revealed differences in protistan communities that were related more to the size fractions analysed, than to sample depth or trophic condition.

Radiolarians and syndinians dominated the small size fraction (0.2–3 μm) of our Pacific seawater samples, while the larger size fraction (3–10 μm) was characterized by core dinophyceans and relatively high proportions of Amoebozoa (Vannellidae) and Euglenozoa (Diplonemea). Dinoflagellates and radiolarians have frequently been reported as important protist lineages in environmental genetic surveys from deep ocean. Radiolarian sequences, particularly from representatives of the Polycystinea, were retrieved in abundance from our 0.2–3 μm size fraction, even though the shells of the described species corresponding to our sequences are larger than 10 μm. Thus, the majority of our radiolarian environmental sequences may come from small reproductive cells (gametes), from specimens ruptured during pre-filtration (Not *et al*., 2007), or from extracellular DNA as previously suggested by Not and colleagues (2009). Sequences affiliated to the Syndiniales (Group I and II) have been detected in diverse habitats including the deep Antarctic polar front, preferentially in the small size fraction (López-García *et al*., 2001), in the deep Sargasso Sea samples passed throughout 2 μm filters (Not *et al*., 2007), and in the whole community from North Atlantic samples (Countway *et al*., 2007). All known species of syndinians are parasites or parasitoids that produce small flagellated cells (dinospores) capable of transmitting infection to new hosts. As for earlier studies, most syndinian sequences encountered in our small size fraction samples likely represent dinospores dispersed into the seawater (Guillou *et al*., 2008).

Sequences affiliated to the genus *Vannella* (Amoebozoa) were a principal component of our Pacific libraries derived from larger size fractions of seawater. Thus, our study shows a probable ubiquity of Amoebozoa, particularly the genus *Vannella* for which representatives have also been detected from terrestrial, freshwater and marine environments (e.g. Sims *et al*., 2002) and even isolated from freshwater and marine fishes (Dyková *et al*., 1999; Dyková and Lom, 2004).

The Euglenozoa encompass a large variety of organisms with very different modes of nutrition, including predation, osmotrophy, parasitism and photoautotrophy. In our study, even the use of the UNonMet primer could underestimate this group, diplonemids represented an important fraction of the deep seawater Pacific samples, as has been observed in environmental studies in other locations (Countway *et al*., 2007; Lara *et al*., 2009; Scheckenbach *et al*., 2010). Thus, DSPD diplonemids are widespread in deep seawater and, as recently suggested by Lara and colleagues (2009), appear to have a more planktonic lifestyle than the *Diplonema/Rhynchopus* group.

Specific protists in hydrothermal environments

Protists from hydrothermal vent environments have only recently gained attention and are thus less known than those inhabiting surface water. Studies began less than a decade ago when Atkins and colleagues (2000) isolated and cultured flagellate strains retrieved from four deep-sea hydrothermal vents: Juan de Fuca Ridge (2200 m), Guaymas Basin (2000 m), and two sites on the North EPR, 21°N (2550 m) and 9°N (2000 m). Since then, hydrothermal sediments from the Guaymas Basin in Pacific (Edgcomb *et al*., 2002) and from the MAR (López-García *et al*., 2003) have been examined, while hydrothermal fluid and seawater mixture, experimental substrates, and chimney carbonates have been sampled on the MAR (López-García *et al*., 2003; 2007). These studies revealed putative indigenous lineages in sulfide and hydrocarbon-rich hydrothermal sediments of the Guaymas Basin, and demonstrated tremendous eukaryote diversity with strong representation of alveolates in the sediment, chimney carbonate and fluid–seawater interface of MAR hydrothermal sites. Our data also demonstrated an important diversity of protists at SEPR and MAR hydrothermal sites.

Most of the protists detected inside carbonate vents and in hydrothermal fluids at the MAR (López-García *et al*., 2007) are probably ubiquitous in the deep ocean, as indicated by our data for diplonemids, radiolarians, Dinophyceae and Syndiniales. Nevertheless, some of the protists encountered at hydrothermal sites, including the stramenopiles (*Cafeteria* and *Caecitellus*), apusozoa,

Fungi and Ciliophora, may be adapted to extreme conditions. The ability of *Cafeteria* and *Caecitellus* to grow in extreme environments is a consequence of their tolerance to high concentrations of sulfide or metal (Atkins *et al*., 1998; 2002). Planomonads and apusomonads also appear well adapted to a wide range of harsh environmental conditions, as they occurred in most of our samples from bivalves and vent chimneys, and have been isolated from sediments associated with vents of the north EPR (Atkins *et al*., 2000) and MAR (López-García *et al*., 2007).

For the Fungi, a potential hydrothermal and/or anaerobic fungal group belonging to the Exobasidiomycetes has been suggested (López-García *et al*., 2007). This cluster also included one sequence retrieved from soil (Moon-van der Staay *et al*., 2006) and probably has a more widespread distribution than previously recognized. Nevertheless, two recent studies based on culture-independent PCR from hydrothermal DNA samples (Le Calvez *et al*., 2009) and culture-dependent diversity of marine filamentous fungi isolated from a deep-sea hydrothermal environment (Burgaud *et al*., 2009) revealed the presence of deep marine fungi and a great diversity of fungal species. Moreover, study on diseased mussels in hydrothermal environments demonstrated the capacity of yeast to infect these bivalves (Van Dover *et al*., 2007). Nonetheless, our fungi sequences from the diverse environments did not show similarity with deep marine fungi, but preferentially close to soil species, and thus they probably demonstrate a wide dissemination.

The ciliates include species that are genetically and morphologically diverse. They are found in a great variety of environments, where they occur as free-living or symbiotic organisms (commensal or parasitic). Although ciliates are ubiquitous members of marine plankton, some isolated sequences or clades may tolerate or be adapted to deep hydrothermal environments. For example, sequences from phyllopharyngean ciliates, including representatives of the subclasses Phyllopharyngia, Suctoria and Chonotrichia, were retrieved from microcolonizer substrates placed near a hydrothermal bivalve bed (López-García *et al*., 2003) and from bivalves (our study). Phyllopharyngea of the family Dysteriidae have been isolated from *Calyptogena* casket water (Small and Gross, 1985) and from our SEPR bivalves. Similarly, one or more sequences in the subclass Scuticociliata (López-García *et al*., 2007, and this study) and in the class Oligotrichea, mostly belonging to the genus *Aspidisca* (Small and Gross, 1985; López-García *et al*., 2007; and this study), have also been detected in hydrothermal environments. As these observations were mainly based on genetic molecular analyses, it is difficult to predict the physiology of these potential hydrothermal protists and to assess whether they really are adapted to these ecosystems, or simply commensals and/or parasites of metazoa.

Protist communities associated with deep hydrothermal and cold seep bivalves

To our knowledge, no earlier study investigated the micro-eukaryote diversity in giant bivalves associated with hydrothermal vent and cold seep environments.

All the species of bivalves examined have different bacterial symbionts (e.g. Duperron *et al*., 2009). *Bathymodiolus thermophilus* has sulfur-oxidizing bacteria, whereas *B. childressi* contains methanotrophic endosymbionts (Childress *et al*., 1986). These two bivalves can also filter seawater to acquire nutrients not supplied by their bacterial symbionts (Page *et al*., 1990). *Bathymodiolus azoricus* and *B. brooksi*, also possible filter-feeders, each contain the two previous types of bacterial endosymbionts. The clam *C. magnifica* exclusively relies on its sulfur-oxidizing bacterial symbionts (Page *et al*., 1991). Protist communities detected in the pallial liquid of these different bivalves differed with sampling site, but not with the bivalve species. Therefore, three communities of protists were retrieved: one associated with Pacific hydrothermal bivalves (*B. thermophilus* and *C. magnifica*), one with Atlantic hydrothermal mussels (*B. azoricus*) and one with cold seep bivalves (*B. brooksi* and *B. childressi*).

The micro-fauna detected in our giant bivalves was clearly different from the protist communities retrieved from the other deep-sea environments (deep seawater and water surrounding chimneys). Nevertheless, some protists detected in the bivalves, for example diatoms, may derive from surface marine waters. Diatoms have been frequently reported from deep oceanic samples, and fragments of frustules were even observed in the gut of *Paralvinella* (Tunnicliffe *et al*., 1985). In addition, the occurrence of sequences related to land plants (e.g. spermatophytes: Streptophyta) and terrestrial fungi (e.g. *Penicillium* sp.) in genetic libraries from deep-sea bivalves suggests a wide dissemination of seeds and/or pollen (Edgcomb *et al*., 2002; Burgaud *et al*., 2009). High densities of spermatozoa were present in seawater collected just above mussel beds and in pallial cavity liquid of our pacific bivalves. The most frequently encountered morphotype was similar to the description of *B. thermophilus* spermatozoon (Le Pennec and Beninger, 1997). Moreover, all male *Bathymodiolus* specimens observed on board ship had mature gonads and were ready to spawn (T. Comtet, pers. comm.). Congruently, the two genetic libraries obtained from mussel bed seawater using the general eukaryotic primers (BS1 and BS2) were dominated by sequences affiliated to *B. thermophilus*. The potential food represented by these numerous gametes may directly benefit suspension feeders, in particular the abundant stalked cirripeds observed in patches at the Oasis site (Sarrazin *et al*., 2006a) and perhaps the heterotrophic protists that colonize bivalves.

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Our genetic diversity and microscopic data suggest that bivalves may represent a relatively stable micro-niche in hydrothermal ecosystems and probably an important source of organic matter for opportunistic micro-detritivorous and grazers such as *Massisteria*, *Cafeteria*, *Caecitellus*, *Amastigomonas*, *Planomonas*, and even some Fungi (especially within Ascomycota). These micro-organisms have been already enriched from organic media, especially several strains isolated from deep-sea sediments (Scheckenbach *et al*., 2005) and from North EPR hydrothermal vents (Atkins *et al*., 2000). Interestingly, some ciliate genera observed particularly in pallial liquid of bivalves (e.g. species of *Aspidisca*, *Dysteria* and *Uronema*) were also detected at the 21°N hydrothermal site (Small and Gross, 1985). These authors also detected *Aspidisca* spp. from a hermetic container containing *C. magnifica* and observed many food vacuoles containing bacteria, suggesting that these ciliates were active bacterivores. Additionally, many sequences retrieved from the SEPR bivalves were closely related to *P. virginianum*, *Uronema marina* and *Entodiscus borealis*, three ciliate species that can be symbionts of Metazoa (Powers, 1933; Lynn and Struder-Kypke, 2005). Hence, ciliates detected both by microscopy and molecular genetics could have different trophic interactions inside bivalves, including bacterivory, symbiosis, parasitism and grazing. Other sequences of putative symbionts/parasites were also detected, such as the kinetoplastid sequences retrieved from bivalves and related to the fish ectoparasite *Ichthyobodo necator* and hydrothermal environmental sequences retrieved from Rainbow sediment (López-García *et al*., 2003). Moreover, one genetic library from cold-seep bivalves (GM2) was completely dominated by Syndiniales Group II, a group probably synonymous with the Amoebophryidae. The only known genus of this family, *Amoebophrya*, infects planktonic species belonging to dinoflagellates, ciliates and radiolarians. The syndinian sequences recovered from cold-seep bivalves may come from infected protists living in the pallial cavity, or may represent parasites of the bivalve. If the latter is correct, then the parasites would likely represent new species, as no syndinian genus is currently known to include species that infect molluscs. *Perkinsus* species, typical parasites of marine bivalves inhabiting the continental shelf, were not detected in our genetic libraries, suggesting that deep-sea bivalves may have a distinct parasite fauna. Thus, as proposed by Moreira and López-García (2003), numerous sequences retrieved from the liquid of the pallial cavity of hydrothermal bivalves appear to result from symbiotic or parasitic associations involving protist–protist and/or protist–metazoan interactions.

While the survey of community structures of deep micro-eukaryotes is far from being complete, most eukaryotic lineages previously retrieved from benthic deep-sea ecosystems, such as hydrothermal vents, probably represent cosmopolitan planktonic organisms. However, some micro-eukaryotes, especially among ciliates, stramenopiles and Cercozoa, may still be considered as potentially restricted to these ecosystems. Molecular surveys and culturing efforts are still required for the identification of unclassified environmental 18S sequences, especially among ciliates. Furthermore, the most important adaptation of hydrothermal metazoans to life in these extreme hydrothermal environments is their symbiotic interactions with prokaryotes. Complementary studies addressing the interaction of micro-eukaryotes with hydrothermal metazoans and their endosymbiotic prokaryotes are needed.

Experimental procedures

Site description and sampling strategy

Samples were collected from different sites (Fig. 1, Table S1) during four different oceanographic cruises: BIOSPEEDO and BIOSOPE were both conducted aboard the oceanographic research vessel *L'Atalante* (South East Pacific Ocean, from 31 March to 13 May 2004 and from 26 October to 11 December 2004 respectively); the Expedition to the Deep Slope was conducted aboard the R/V *Atlantis* (Gulf of Mexico, from 7 May to 2 June 2006). MoMARETO took place on board the oceanographic research vessel *Le Pourquoi Pas?* (Mid-Atlantic Ridge, from 7 August to 6 September 2006). For more details about the BIOSPEEDO cruise transect see Jollivet and colleagues (2004); for MoMARETO cruise see Sarrazin and colleagues (2006b) and BIOSOPE see Claustre and colleagues (2008).

Fragments of chimneys and bivalves were individually collected using the manned submersible *Nautile* during the BIOSPEEDO cruise, the *Alvin* submersible during the Expedition to the Deep Slope and with the aid of the Remotely Operated Vehicle (ROV) *Victor 6000* during the MoMARETO cruise. Samples carefully collected by a manipulator arm were placed in insulated and hermetic containers previously filled with sterilized freshwater to prevent possible pressure problems and contaminations. When opening the container, the difference of water density induced a rapid replacement of the freshwater by surrounding seawater (Fig. S4). On board, only individual unbroken bivalves were retained for further analyses and DNA was directly extracted from 50 ml aliquot of pallial cavity liquid and conserved at −80°C. Seawater samples were collected 10–20 cm above vent mussel beds and in the deep-sea using Niskin bottles manipulated by the *Nautile* close to the hydrothermal sites or from aboard (during the BIOSPEEDO and BIOSOPE cruises; Fig. S4). Once on board, seawater in hermetic container and Niskin bottles (3–5 l) were pre-filtered through several filters assembled in series, i.e. 10 μm and 3 μm pore size polycarbonate filter (47 mm diameter, Nucleopore, Whatman International, Maidstone, UK) and a last 0.2 μm pore size Sterivex® filter under gentle vacuum (10 mm Hg). Polycarbonate and Sterivex® filters were placed in a 5 ml cryovial with 3 ml of DNA lysis buffer (0.75 M sucrose, 40 mM EDTA,

50 mM Tris-HCl, pH 9.0) and stored in liquid nitrogen at −80°C until DNA extraction.

Microscopic observation

Direct microscopic observations using an Olympus BX51 epifluorescence microscope (×40 objective; Olympus Optical CO, Tokyo, Japan), coupled to a Spot RT-slider camera (Diagnostics Instruments, Sterling Heights, MI), were performed on board *L'Atalante* during the BIOSPEEDO cruise, without previous concentration of samples. Living protists were observed from freshly prepared slides or fixed organisms. The fixation was made in Lugol's iodine solution (0.25%) and cells were observed after sedimentation in Utermöhl chambers (50 ml; Utermöhl, 1931).

DNA extractions and PCR amplification

DNA samples were extracted by a modified CTAB (hexadeCylTrimathyl Ammonium Bromide) protocol (Doyle and Doyle, 1987). Polycarbonate and Sterivex® filters (submerged with the DNA lysis buffer) were incubated in a 3% (w/v) CTAB solution preheated at 60°C for at least 1 h with occasional vortexing. Nucleic acids were extracted twice with an equal volume of chloroform-isoamyl alcohol (96:4). After two centrifugations for 15 min at 11 000 *g* (4°C), the aqueous phase was transferred into a clean tube and two volumes of cold isopropanol were added at 4°C for several hours in order to precipitate the nucleic acids. DNA was then subjected to centrifugation (30 min at 11 000 *g* at 4°C), one washing with 70% ethanol (v/v) and re-suspension in sterile distilled water prior to storage at −80°C.

All PCR mixtures (50 µl final volume) contained 2 µM final concentration of DNA template, 1× *Taq* DNA Polymerase buffer, dNTPs (200 µM final concentration each), 2 mM final concentration of $MgCl_2$, 0.2 µM final concentration of each primer and 5 unit·µl^{-1} of *Taq* DNA Polymerase (Promega®, Madison, WI). All amplifications were performed using a 'GeneAmp PCR system' 9700® (Applied Biosystems™). Eukaryotic 18S rRNA gene was amplified with primer sets Euk328f-Euk329r (5′-ACC TGG TTG ATC CTG CCA G-3′; 5′-TGA TCC TTC YGC AGG TTC AC-3′; Moon-van der Staay *et al*., 2000) and Euk328f-UNonMet (5′-TTT AAG TTT CAG CCT TGC G-3′; Bower *et al*., 2004), with the following conditions: an initial incubation step at 95°C during 5 min, followed by 34 cycles with a denaturation step at 95°C during 1 min, an annealing step at 62°C during 2 min, then an extension step at 72°C during 3 min. These cycles were followed by a final extension step at 72°C during 7 min. Amplifications were checked on a 0.8% (w/v) agar gel stained with ethidium bromide.

Cloning and sequencing

PCR products were purified (QIAquick PCR purification kit, Qiagen™) and cloned into *Escherichia coli* using the pCR2.1-TOPO TA vector system (Invitrogen®) following the manufacturer's instructions. The presence of an insert was screened by PCR amplification of white colonies using the vector-specific M13 primers. Positive clones were then screened by RFLP using the restriction enzymes HaeIII and AluI as described by Romari and Vaulot (2004). Clones that produced the same RFLP patterns were grouped together and were considered members of the same OTU (Table S1) and one to several representative clones per RFLP pattern were entirely sequenced using the Big Dye Terminator Cycle Sequencing Kit version 3.0 (PE Biosystems™) and an ABI PRISM model 377 (version 3.3) automated sequencer with the forward and reverse specific primers. Therefore, we determined that sequences belonging to the same RFLP pattern shared sequence similarity ≥ 95%. Six clone libraries (marked with an n*, Table S1) were directly analysed by sequencing because of difficulties to obtain positives clones. OTUs, using a 95% sequence similarity, were thus generated with the DOTUR program (Schloss and Handelsman, 2005).

Phylogenetic analysis

For all genetic libraries, among a total of 2131 screened clones, 477 consensus sequences were obtained. After exclusion of seven *Archaea* sequences and 10 chimeras detected with the KeyDNATools database (Guillou, 2008), 460 suitable-quality full-length sequences with primers used (~1120 pb) corresponding to each OTU detected were obtained. SSU rRNA sequences were then edited in the BioEdit 7.0.5.3 program and aligned using CLUSTALW2 (Hall, 1999; Larkin *et al*., 2007). To determine the first phylogenetic affiliation, each sequence was compared with sequences available in the NCBI database (National Center for Biotechnology Information) using BLAST and KeyDNATools database (Altschul *et al*., 1990; Guillou, 2008).

For the Apusozoa tree (Fig. 6), TrNef+G was selected by hierarchical Likelihood Ratio Tests (hLRT), a submodel of the general time-reversible (GTR+G) with gamma distributed rates and equal base frequencies, selected with ModelTest version 3.7, and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by Bayesian inference (Posada and Crandall, 1998). Bayesian inference was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and started with a random tree, run for one million generations in four chains and burn-in of 2500 generations in order to ensure the use of only stable chains. For the Ciliophora and Euglenozoa trees (Fig. 5 and Fig. S3), a general time-reversible (GTR+I+G) was selected. Bayesian inference started with a random tree, run for two millions generations in four chains and burn-in of 5000 generations. Neighbour-Joining (NJ) and Maximum of Parsimony (MP) trees were inferred using PAUP 4.0b10 via PaupUp graphical interface (Swofford, 2000; Calendini and Martin, 2005). The robustness of inferred topology was supported by bootstrap resampling (1000) with NJ and MP; values over 60% are shown on the trees. The Maximum Likelihood trees were generated and gave the same Bayesian topology.

Good's coverage was used as a coverage index. It is a non-parametric estimator of the proportion of phylotypes in a library of infinite size that would be represented in a smaller library. The coverage index (C_x) of the clone libraries was calculated by Good's method (Good, 1953) as described by Singleton and colleagues (2001), $C_x = 1 - (n_1/N)$ where n_1 is the number of phylotypes appearing only once in a library, and N is the library size.

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Statistical analysis

In order to analyse and interpret the relation between environmental locations and the microbial communities, a canonical redundancy analysis (RDA) was performed (Rao, 1964). RDA was described as a series of multiple regressions followed by a principal component analysis based on linear (Euclidean distance) relationships between variables (Braak, 1995; Vanwijngaarden *et al*., 1995; Legendre and Legendre, 1998). Each variable of a Y table, which contains community data, was regressed on an X table, which contains the explanatory variables (i.e. environmental locations). Variables corresponding to community data were transformed with Hellinger transformation. Legendre and Gallagher (2001) showed that this transformation makes species abundance data amenable to analyses like RDA. Analyses were performed with the statistical software package R Ver.2.7.0 (R Development Core Team, 2009) and the function 'rdaTest' following the algorithm described elsewhere (Legendre and Legendre, 1998). A plot of RDA results was produced, with the function 'graph.rdaTest'. The adjusted coefficient of determination (Ra^2) corrects for the number of explanatory variables in the model and for the number of observations. RDA provides an unbiased estimate of the real contributions of the explanatory variables to the explanation of a community data table (Peres-Neto *et al*., 2006).

Nucleotide sequence accession numbers

The sequences are available from the GenBank database under the following accession numbers: FN598219 to FN598477. All these sequences are in Table S3.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Triplot redundancy analysis (RDA) integrating taxonomic groups, environmental locations and genetic libraries from the Pacific and Atlantic Oceans.
A. The depth and taxonomic groups (continuous variables; A = Apicomplexa, C = Centroheliozoa, M = Metazoa, P = Planomonas, T = Tubulinea) are shown as arrows. The vector orientations represent the direction of strongest change; vector lengths correspond to the relative importance. The sample types are illustrated by blue crossed circles. Genetic libraries are represented by solid symbols (triangle for deep seawater, square for water surrounding chimneys and circle for pallial cavity liquid in bivalves) and an empty circle represents water above mussel bed library. The percentage of variability explained by the two axes (RDA1 and RDA2) and the *P*-value are reported in brackets. R^2: *R*-squared, Ra^2: adjusted *R*-squared and Pr: *P*-value of the model.
B. Three-dimensional plot of the same dataset which allow showing the third axis (RDA3). Binary variables (sample types) that were collinear are not represented on the plot. Genetic libraries represented by solid symbols are plotted using 'h' type, so their *x*–*y* locations can see more clearly (empty symbols).
Fig. S2. Microscopic observations of aggregates (A) or long-chain colonies of procaryotes (B), and spermatozoa-like cells with various shapes and sizes (C–E) fixed with glutaraldehyde, concentrated by filtration and stained with propidium iodide, collected from water above bivalve bed (site Oasis). Scale bars correspond to 5 μm. Microscopic observations of cells from hydrothermal bivalves.
(F) From *C. magnifica*, Oasis, (G and H) from *B. thermophilus*, Lucky Eric. Scale bars correspond to 2 μm.
(I and J) Cells fixed with Lugol (*B. thermophilus*, Rehu Marka). (K) Same organism stained with propidium iodide. Scale bars correspond to 5 μm.
Fig. S3. Bayesian analysis of diplonemids and kinetoplastids environmental 18S rRNA gene sequences retrieved from different samples in the study. Study sequences are in bold. Each phylotype from each clone library is represented by one sequence with ≥ 95% similarity grouping. Posterior probability of Bayesian method and Neighbour-Joining and Maximum Parsimony bootstrap values higher than 60% are shown at nodes. Outgroup sequences were not shown but composed of seven Euglenida sequences (U84733, AJ532400, AF119118, AF090870, AJ532437, AJ532442 and AF283309). The number of clones of each representative sequence is indicated in exponent. Triangles symbolize deep seawater, squares water surrounding chimney and circle pallial cavity liquid in bivalves.
Fig. S4. Sampling of seawater above mussel bed and water surrounding chimney. Seawater sampled at Oasis site, using a Niskin 8 l bottle manipulated by the manned submersible Nautile.
A–C. (A) Opening, immersion, (B and C) and closure of the Niskin bottle.
D–I. Hydrothermal rocks and water surrounding chimney in hermetic container sampled at Hobbs site (D and E) and Sarah' Spring site (F–I).
F. The hermetic container manipulated by the arm was previously filled with sterilized freshwater what created the moiré effect.
All the photos are the property of IFREMER.
Table S1. Sample locations and characteristics of the corresponding genetic libraries.
Table S2. Distribution of the micro-eukaryote phylogenetic community structures based on 18S rRNA gene. The relative abundance of each phylotype retrieved from the different samples was reported in percentage. (*) corresponds to libraries constructed with general eukaryotic primers. B. th: *B. thermophilus*; C. ma: *C. magnifica*; B. az: *B. azoricus*; B. br: *B. brooksi*; B. ch: *B. childressi*.
Table S3. List of sequences deposited in GenBank, with KeyDNATools and BLAST results.

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Table S1 Sample locations and characteristics of the corresponding genetic libraries.

Cruises	Samples type	Date dd/mm/yy	Name of Site (Coordinates)	Depth (m)	Dives No.	Library	n/N[a]	n_1[b]	C_x[c]
Deep water									
BIOSOPE 2004 South Pacific	**CTD samples**								
	Deep water (3-10 µm)	09/12/04	UPX1 (34°51'S,72°42'W)	500	CTD215	BIO2	23/96	14	0.86
	Deep water (0.2-3 µm)	09/12/04	UPX1 (34°51'S,72°42'W)	500	CTD215	BIO9	20/95	12	0.88
	Deep water (0.2-3 µm)	11/12/04	UPX3 (34°69'S,72°49'W)	900	CTD224	BIO5	16/89	8	0.91
	Deep water (3-10 µm)	25/11/04	EGY1 (31°82'S,91°47'W)	500	CTD141	BIO3	15/90	8	0.91
	Deep water (0.2-3 µm)	25/11/04	EGY1 (31°82'S,91°47'W)	500	CTD141	BIO10	13/96	6	0.94
	Deep water (3-10 µm)	30/11/04	EGY6 (31°90'S,91°41'W)	3000	CTD176	BIO4	14/96	6	0.94
	Deep water (3-10 µm)	16/11/04	GYR6 (26°07'S,113°99'W)	1000	CTD118	BIO1	16/95	8	0.92
	Deep water (0.2-3 µm)	16/11/04	GYR6 (26°07'S,113°99'W)	1000	CTD118	BIO7	25/92	13	0.86
BIOSPEEDO 2004 SEPR	**Niskin bottle samples**								
	Water collected 10-20cm above vent mussel bed (>10 µm)	22/04/04	Oasis (17°25'S,113°12'W)	2585	1582	BS12	33*/87	23	0.74
	Water collected 10-20cm above vent mussel bed (3-10 µm)	22/04/04	Oasis (17°25'S,113°12'W)	2585	1582	BS1**	5/80	2	0.98
	Water collected 10-20cm above vent mussel bed (>10 µm)	22/04/04	Oasis (17°25'S,113°12'W)	2585	1582	BS2**	6/37	1	0.97
	Deep water (3-10 µm)	24/04/04	Oasis (17°25'S,113°12'W)	900	1584	BS3**	15/80	4	0.95
	Deep water (>10 µm)	24/04/04	Oasis (17°25'S,113°12'W)	300	1584	BS4**	6/37	3	0.92
	Water surrounding chimneys								
	Hermetic container HC1 (3-10 µm)	09/04/04	Sarah'Spring (7°25'S,107°47'W)	2752	1573	BS23	11/93	5	0.95
	HC1 (> 10 µm)	09/04/04	Sarah'Spring (7°25'S,107°47'W)	2752	1573	BS25	17/85	12	0.86
	HC4 (3-10 µm)	29/04/04	Hobbs (17°35'S,113°14'W)	2593	1588	BS15	11*/18	6	0.67
	HC4 (> 10 µm)	29/04/04	Hobbs (17°35'S,113°14'W)	2593	1588	BS24	16/94	7	0.93
	HC7 (3-10 µm)	21/04/04	Fromveur (18°25'S,113°23'W)	2636	1581	BS16	7*/17	2	0.88

Cruises		Samples type	Date dd/mm/yy	Name of Site (Coordinates)	Depth (m)	Dives No.	Library	n/N[a]	n_1[b]	C_x[c]
Pallial cavity liquid of bivalves										
BIOSPEEDO 2004	**SEPR**	*Bathymodiolus thermophilus* n°959 (male, 19 mm)	24/04/04	Rehu Marka (17°24'S,113°12'W)	2589	1584	BS17	7/64	0	1
		B. thermophilus n°964 (male, 38 mm)	24/04/04	Rehu Marka (17°24'S,113°12'W)	2589	1584	BS13	8/84	1	0.99
		B. thermophilus n°1092 (male, 35 mm)	04/05/04	Lucky Eric (13°59'S,112°28'W)	2623	1592	BS10	5/61	1	0.98
		B. thermophilus n°1094 (female, 31 mm)	04/05/04	Lucky Eric (13°59'S,112°28'W)	2623	1592	BS14	5/29	2	0.93
		Calyptogena magnifica n°9 (sex ND, 27.7 mm)	23/04/04	Oasis (17°25'S,113°12'W)	2585	1583	BS11	12*/36	6	0.84
		C. magnifica n°10 (sex ND, 26.2 mm)	23/04/04	Oasis (17°25'S,113°12'W)	2585	1583	BS18	15*/39	5	0.87
		C. magnifica n°11 (sex ND, 27.6 mm)	23/04/04	Oasis (17°25'S,113°12'W)	2585	1583	BS19	10*/25	4	0.84
MoMARETO	**2006 MAR**	*Bathymodiolus azoricus* n°1 (sex ND, size ND)	27/08/06	Menez Gwen (37°50'N,31°31'W)	814	299-16	MO1	8/91	4	0.96
		B. azoricus n°5 (sex ND, size ND)	24/08/06	Lucky Strike (37°17'N,32°16'W)	1692	296-13	MO2	7/59	2	0.96
		B. azoricus n°2 (sex ND, size ND)	24/08/06	Lucky Strike (37°17'N,32°16'W)	1692	296-13	MO3	6/62	1	0.99
		B. azoricus n°6 (sex ND, size ND)	24/08/06	Lucky Strike (37°17'N,32°16'W)	1692	296-13	MO4	4/76	1	0.98
Gulf of	**Mexico**	*Bathymodiolus brooksi* n°1 (sex ND, size ND)	20/05/06	MC853 (28°38'N, 89°38'W)	1070	4178	GM1	12/86	5	0.94
		Bathymodiolus childressi n°1 (sex ND, size ND)	20/05/06	MC853 (28°38'N, 89°38'W)	1070	4178	GM2	6/41	4	0.90

a. n/N indicates the number (n) of RFLP groups identified in the library composed of (N) clones. (n*) corresponds to a number of OTUs calculated with DOTUR program when libraries were not screened by RFLP.

b. n_1= Number of unique OTU

c. C_x indicates coverage indices, $C_x=1-(n_1/N)$ (see Material and Methods section).

** These genetic libraries were constructed with primer set Euk328f-Euk329r and were not used for statistical analysis.

Table S2 Distribution of the micro-eukaryote phylogenetic community structures based on 18S rRNA gene. The relative abundance of each phylotype retrieved from the different samples was reported in percentage. (*) corresponds to libraries constructed with general eukaryotic primers. B. th: *B. thermophilus*; C. ma: *C. magnifica*; B.az: *B. azoricus*; B. br: *B. brooksi*; B. ch: *B. childressi*

			Deep Pacific Water			Water surrounding chimneys			Water above mussel bed	
						Sarah' Spring		Hobbs/ Fromveur		
SuperGroup	**Division**	**Class**	3-10 µm	0.2-3 µm	3->10 µm*	>10 µm	3-10 µm	3->10 µm	3->10 µm*	>10 µm
Amoebozoa	Flabellinea		16.7	0.8	-	10.6	-	0.8	-	-
	Tubulinea		-	-	-	-	-	-	-	-
Archaeplastida	Charophyta		-	-	-	-	-	-	-	20.7
Chromalveolata	Alveolata	Apicomplexa	2.1	1.9	-	-	-	-	-	-
		Ciliophora	0.5	0.5	0.9	42.4	1.1	6.2	1.7	5.7
		Dinophyceae	58.1	16.1	-	1.2	2.2	1.6	8.5	18.4
		Syndiniales	5.8	13.9	57.3	1.2	-	-	-	-
	Stramenopiles		1.3	3.8	0.7	-	4.3	51.2	-	1.1
Excavata	Euglenozoa		6.1	1.9	17.9	2.4	3.2	-	-	-
Incertae sedis	Apusomonas		-	-	-	-	-	14	-	1.1
	Centroheliozoa		-	-	-	-	-	-	-	-
	Planomonas		0.5	-	-	-	-	2.3	-	14.9
Opisthokonta	Fungi		-	0.8	0.7	-	-	12.4	-	1.1
	Metazoa		-	-	5.1	3.5	5.4	-	89.7	1.1
Rhizaria	Cercozoa		1.1	-	-	-	-	4.7	-	35.6
	Radiolaria		7.7	60.3	17.1	38.8	83.9	7	-	-
Unknown			-	-	-	-	-	-	-	-
		Total clones	377	373	117	85	93	129	117	87

			Pacific bivalves			Atlantic bivalves				
			Rehu Marka	Lucky Eric	Oasis	Menez Gwen	Lucky Strike		MC853	
			B. th		C. ma	B. az	B. az MO2	B. az MO3/MO4	B. br	B. ch
SuperGroup	**Division**	**Class**								
Amoebozoa	Flabellinea		-	-	-	3.3	-	-	-	-
	Tubulinea		-	-	-	-	49.2	-	-	-
Archaeplastida	Charophyta		8.1	-	-	-	-	1.4	-	-
Chromalveolata	Alveolata	Apicomplexa	-	-	-	-	-	-	-	-
		Ciliophora	51.4	50	22	-	25.4	2.2	95.3	2.4
		Dinophyceae	18.9	-	1	-	-	-	-	-
		Syndiniales	-	-	-	-	16.9	-	-	97.6
	Stramenopiles		1.4	-	2	87.9	-	11.6	1.2	-
Excavata	Euglenozoa		0.7	-	1	-	-	-	-	-
Incertae sedis	Apusomonas		3.4	1.1	17	6.6	-	15.2	2.3	-
	Centroheliozoa		2	-	-	-	-	-	-	-
	Planomonas		1.4	-	9	-	-	-	-	-
Opisthokonta	Fungi		-	-	10	2.2	-	1.4	-	-
	Metazoa		-	-	3	-	-	-	-	-
Rhizaria	Cercozoa		12.8	48.9	33	-	8.5	0.7	1.2	-
	Radiolaria		-	-	-	-	-	-	-	-
Unknown			-	-	2	-	-	67.4	-	-
		Total clones	148	90	100	91	59	138	86	41

Table S3 **List of sequences deposited in GenBank, with KeyDNATools and BLAST results**

Name	N°Accession	KeyDNATools Results (->number of matched keys (fragment lenght))				
		Rank 1	Rank 2	Rank 3	Rank 4	Rank 5
BIO1_A11	FN598227	Amoebozoa->87(66-1242)	Lobosa->87(66-1242)	Discosea->87(66-1242)	Flabellinia_Vannellida->87(66-1242)	Vannellidae->86(66-1242)
BIO10_D10	FN598221	Amoebozoa->36(230-1173)	Lobosa->36(230-1173)	Discosea->36(230-1173)	Flabellinia_Vannellida->36(230-1173)	Vannellidae->36(230-1173)
BIO2_F11	FN598246	Amoebozoa->33(233-1179)	Lobosa->33(233-1179)	Discosea->33(233-1179)	Flabellinia_Vannellida->33(233-1179)	Vannellidae->33(233-1179)
BIO3_E2	FN598256	Amoebozoa->36(230-1173)	Lobosa->36(230-1173)	Discosea->36(230-1173)	Flabellinia_Vannellida->36(230-1173)	Vannellidae->36(230-1173)
BIO4_F4	FN598270	Amoebozoa->82(66-1241)	Lobosa->82(66-1241)	Discosea->82(66-1241)	Flabellinia_Vannellida->82(66-1241)	Vannellidae->81(66-1241)
BIO9_F1	FN598305	Amoebozoa->34(215-1173)	Lobosa->34(215-1173)	Discosea->34(215-1173)	Flabellinia_Vannellida->34(215-1173)	Vannellidae->34(215-1173)
BS15_B2	FN598355	Amoebozoa->91(66-1225)	Lobosa->91(66-1225)	Discosea->91(66-1225)	Flabellinia_Vannellida->91(66-1225)	Vannellidae->91(66-1225)
BS25_F2	FN598416	Amoebozoa->85(66-1211)	Lobosa->85(66-1211)	Discosea->85(66-1211)	Flabellinia_Vannellida->85(66-1211)	Vannellidae->85(66-1211)
MO1_C11	FN598461	Amoebozoa->106(66-1226)	Lobosa->106(66-1226)	Discosea->106(66-1226)	Flabellinia_Vannellida->106(66-1226)	Vannellidae->106(66-1226)
MO2_E1	FN598467	Amoebozoa->6(344-1140)	Lobosa->6(344-1140)	Tubulinea->6(344-1140)	Leptomyxida->6(344-1140)	Leptomyxida->6(344-1140)
GM1_A2	FN598449	Chromalveolata->22(55-807)	Alveolata->22(55-807)	Ciliophora->19(55-807)	Karyorelictea->19(55-807)	
GM1_C5	FN598452	Chromalveolata->73(116-1044)	Alveolata->73(116-1044)	Ciliophora->72(116-1044)	Oligohymenophorea->72(116-1044)	Peritrichia->67(117-1044)
BIO7_E8	FN598289	Chromalveolata->33(66-1024)	Alveolata->33(66-1024)	Ciliophora->33(66-1024)	Oligohymenophorea->31(66-1024)	Scuticociliatia->28(66-831)
BS2_B3	FN598423	Chromalveolata->35(32-460)	Alveolata->35(32-460)	Ciliophora->35(32-460)	Oligohymenophorea->35(32-460)	Scuticociliatia->34(32-460)
BS11_A3	FN598313	Chromalveolata->70(107-996)	Alveolata->70(107-996)	Ciliophora->70(107-996)	Oligohymenophorea->70(107-996)	Scuticociliatia->69(107-996)
BS12_D8	FN598336	Chromalveolata->73(38-1064)	Alveolata->73(38-1064)	Ciliophora->71(38-1064)	Oligohymenophorea->71(38-1064)	Scuticociliatia->70(38-1064)
BS13_A2	FN598341	Chromalveolata->73(38-1063)	Alveolata->73(38-1063)	Ciliophora->73(38-1063)	Oligohymenophorea->73(38-1063)	Scuticociliatia->72(38-1063)
BS10_D9	FN598312	Chromalveolata->73(38-1064)	Alveolata->73(38-1064)	Ciliophora->73(38-1064)	Oligohymenophorea->73(38-1064)	Scuticociliatia->72(38-1064)
BS18_D5	FN598378	Chromalveolata->79(38-1064)	Alveolata->79(38-1064)	Ciliophora->79(38-1064)	Oligohymenophorea->79(38-1064)	Scuticociliatia->77(38-1064)
BS14_G7	FN598351	Chromalveolata->79(38-1064)	Alveolata->79(38-1064)	Ciliophora->79(38-1064)	Oligohymenophorea->79(38-1064)	Scuticociliatia->78(38-1064)
BS17_E2	FN598371	Chromalveolata->80(38-1064)	Alveolata->80(38-1064)	Ciliophora->80(38-1064)	Oligohymenophorea->80(38-1064)	Scuticociliatia->79(38-1064)
BS23_C3	FN598398	Chromalveolata->91(82-1028)	Alveolata->91(82-1028)	Ciliophora->90(116-1028)	Oligohymenophorea->90(116-1028)	Scuticociliatia->85(116-1028)
MO3_B3	FN598470	Chromalveolata->9(66-1042)	Alveolata->8(66-827)	Ciliophora->8(66-827)	Oligohymenophorea->8(66-827)	Scuticociliatia->7(66-827)
BS25_H4	FN598421	Chromalveolata->154(57-1070)	Alveolata->154(57-1070)	Ciliophora->153(57-1012)	Oligotrichea->153(57-1012)	Euplotida->144(60-1012)
BS15_G5	FN598360	Chromalveolata->163(57-1132)	Alveolata->163(57-1132)	Ciliophora->163(57-1132)	Oligotrichea->163(57-1132)	Euplotida->151(60-1131)
BS19_G4	FN598390	Chromalveolata->36(126-1047)	Alveolata->36(126-1047)	Ciliophora->36(126-1047)	Oligotrichea->36(126-1047)	Euplotida->17(126-1046)
BS25_F7	FN598417	Chromalveolata->7(180-751)	Alveolata->7(180-751)	Ciliophora->7(180-751)	Oligotrichea->7(180-751)	Euplotida->4(180-751)
BS25_F8	FN598418	Chromalveolata->54(57-1076)	Alveolata->54(57-1076)	Ciliophora->54(57-1076)	Oligotrichea->54(57-1076)	Euplotida->36(60-838)
BS24_F1	FN598408	Chromalveolata->71(57-941)	Alveolata->71(57-941)	Ciliophora->70(57-941)	Oligotrichea->70(57-941)	Euplotida->50(128-941)
BS14_B11	FN598349	Chromalveolata->16(66-996)	Alveolata->16(66-996)	Ciliophora->16(66-996)	Oligotrichea->15(66-772)	Euplotida->8(66-699)
BS4_A1	FN598443	Chromalveolata->56(26-460)	Alveolata->56(26-460)	Ciliophora->55(26-460)	Oligotrichea->52(26-460)	Oligotrichia->12(102-297)
BIO5_A9	FN598274	Chromalveolata->88(120-927)	Alveolata->88(120-927)	Ciliophora->88(120-927)	Oligotrichea->88(120-927)	Oligotrichia->40(187-927)
BIO2_B1	FN598239	Chromalveolata->120(120-1067)	Alveolata->120(120-1067)	Ciliophora->120(120-1067)	Oligotrichea->120(120-1067)	Oligotrichia->54(162-1057)
BS24_F2	FN598409	Chromalveolata->80(63-1056)	Alveolata->80(63-1056)	Ciliophora->80(63-1056)	Oligotrichea->79(63-1056)	Stichotrichia->38(116-1056)
GM1_G2	FN598456	Chromalveolata->19(63-1087)	Alveolata->19(63-1087)	Ciliophora->17(63-1087)	Oligotrichea->15(63-694)	
MO2_A1	FN598464	Chromalveolata->21(74-871)	Alveolata->21(74-871)	Ciliophora->18(102-871)	Phyllopharyngea->17(102-871)	Chonotrichia and Phyllopharyngia->16(102-871)
BS18_D2	FN598377	Chromalveolata->25(63-1017)	Alveolata->25(63-1017)	Ciliophora->23(101-1017)	Phyllopharyngea->23(101-1017)	Chonotrichia and Phyllopharyngia->20(101-804)
BS13_C1	FN598345	Chromalveolata->25(18-750)	Alveolata->25(18-750)	Ciliophora->25(18-750)	Phyllopharyngea->25(18-750)	Chonotrichia and Phyllopharyngia->20(18-750)
BS12_E5	FN598337	Chromalveolata->28(18-758)	Alveolata->28(18-758)	Ciliophora->26(18-572)	Phyllopharyngea->26(18-572)	Chonotrichia and Phyllopharyngia->22(18-572)
BS11_D1	FN598319	Chromalveolata->30(18-1026)	Alveolata->30(18-1026)	Ciliophora->30(18-1026)	Phyllopharyngea->30(18-1026)	Chonotrichia and Phyllopharyngia->25(18-1023)
BS19_F3	FN598388	Chromalveolata->30(18-1026)	Alveolata->30(18-1026)	Ciliophora->30(18-1026)	Phyllopharyngea->30(18-1026)	Chonotrichia and Phyllopharyngia->25(18-1023)
BS25_D1	FN598415	Chromalveolata->31(18-1022)	Alveolata->31(18-1022)	Ciliophora->31(18-1022)	Phyllopharyngea->31(18-1022)	Chonotrichia and Phyllopharyngia->27(18-1022)
BS11_F2	FN598325	Chromalveolata->46(18-886)	Alveolata->46(18-886)	Ciliophora->46(18-886)	Phyllopharyngea->46(18-886)	Chonotrichia and Phyllopharyngia->42(18-886)
BIO4_C8	FN598266	Chromalveolata->12(148-712)	Alveolata->12(148-712)	Ciliophora->12(148-712)	Phyllopharyngea->10(205-682)	Chonotrichia and Phyllopharyngia->7(205-682)
GM1_E1	FN598453	Chromalveolata->19(21-815)	Alveolata->19(21-815)	Ciliophora->17(21-757)	Phyllopharyngea->16(21-757)	Suctoria->14(21-757)
GM1_B1	FN598451	Chromalveolata->28(21-1000)	Alveolata->28(21-1000)	Ciliophora->28(21-1000)	Phyllopharyngea->28(21-1000)	Suctoria->24(21-1000)
GM2_C8	FN598458	Chromalveolata->9(34-772)	Alveolata->8(34-772)	Ciliophora->6(34-772)	Phyllopharyngea->3(34-432)	
BS10_A2	FN598308	Chromalveolata->10(34-783)	Alveolata->10(34-783)	Ciliophora->10(34-783)	Phyllopharyngea->8(34-506)	
BS11_C9	FN598318	Chromalveolata->10(34-783)	Alveolata->10(34-783)	Ciliophora->10(34-783)	Phyllopharyngea->8(34-506)	
BS12_C8	FN598333	Chromalveolata->9(34-807)	Alveolata->9(34-807)	Ciliophora->9(34-807)	Phyllopharyngea->9(34-807)	
BS12_D7	FN598335	Chromalveolata->11(34-782)	Alveolata->11(34-782)	Ciliophora->10(34-782)	Phyllopharyngea->8(34-506)	
BS13_A1	FN598340	Chromalveolata->10(34-783)	Alveolata->10(34-783)	Ciliophora->10(34-783)	Phyllopharyngea->8(34-506)	
BS14_G9	FN598352	Chromalveolata->10(34-782)	Alveolata->10(34-782)	Ciliophora->10(34-782)	Phyllopharyngea->8(34-505)	
BS17_E5	FN598372	Chromalveolata->10(34-783)	Alveolata->10(34-783)	Ciliophora->10(34-783)	Phyllopharyngea->8(34-506)	
BIO10_B6	FN598219	Chromalveolata->10(73-820)	Alveolata->10(73-820)	Apicomplexa->10(73-820)	Colpodellidae->10(73-820)	
BIO3_B2	FN598253	Chromalveolata->10(74-1070)	Alveolata->10(74-1070)	Apicomplexa->10(74-1070)	Colpodellidae->10(74-1070)	
BS4_A6	FN598444	Chromalveolata->119(27-766)	Alveolata->119(27-766)	Dinophyceae->119(27-766)	Syndiniales->119(27-766)	Dino-Group I->118(27-766)
MO2_A3	FN598466	Chromalveolata->123(62-1069)	Alveolata->123(62-1069)	Dinophyceae->123(62-1069)	Syndiniales->120(62-1069)	Dino-Group I->118(62-1069)
BIO2_E9	FN598245	Chromalveolata->26(129-1049)	Alveolata->26(129-1049)	Dinophyceae->25(129-1049)	Syndiniales->22(137-1049)	Dino-Group I->20(137-1049)
BIO9_G10	FN598306	Chromalveolata->29(55-713)	Alveolata->29(55-713)	Dinophyceae->29(55-713)	Syndiniales->27(55-713)	Dino-Group I->26(55-713)
BIO7_G6	FN598292	Chromalveolata->33(448-1049)	Alveolata->33(448-1049)	Dinophyceae->33(448-1049)	Syndiniales->33(448-1049)	Dino-Group I->32(448-1049)
BS25_C6	FN598414	Chromalveolata->38(158-1072)	Alveolata->37(158-1072)	Dinophyceae->37(158-1072)	Syndiniales->34(192-1072)	Dino-Group I->34(192-1072)
BS4_B12	FN598445	Chromalveolata->34(6-718)	Alveolata->34(6-718)	Dinophyceae->34(6-718)	Syndiniales->34(6-718)	Dino-Group I->34(6-718)
BIO1_F7	FN598232	Chromalveolata->57(55-1072)	Alveolata->57(55-1072)	Dinophyceae->57(55-1072)	Syndiniales->57(55-1072)	Dino-Group I->55(55-1072)
BIO7_A9	FN598281	Chromalveolata->77(74-1087)	Alveolata->77(74-1087)	Dinophyceae->77(74-1087)	Syndiniales->65(74-1087)	Dino-Group I->64(74-1087)
BS3_F9	FN598440	Chromalveolata->70(1-600)	Alveolata->70(1-600)	Dinophyceae->70(1-600)	Syndiniales->70(1-600)	Dino-Group I->67(1-458)
BS3_B11	FN598429	Chromalveolata->80(2-933)	Alveolata->80(2-933)	Dinophyceae->80(2-933)	Syndiniales->78(2-915)	Dino-Group I->74(2-915)
BIO7_F6	FN598291	Chromalveolata->77(104-1081)	Alveolata->77(104-1081)	Dinophyceae->76(104-1081)	Syndiniales->76(104-1081)	Dino-Group I->76(104-1081)
BIO2_E4	FN598244	Chromalveolata->77(168-1066)	Alveolata->77(168-1066)	Dinophyceae->77(168-1066)	Syndiniales->77(168-1066)	Dino-Group I->76(168-1066)
BS3_B4	FN598431	Chromalveolata->78(2-956)	Alveolata->78(2-956)	Dinophyceae->78(2-956)	Syndiniales->77(2-956)	Dino-Group I->76(2-956)
BIO1_H6	FN598234	Chromalveolata->82(103-1067)	Alveolata->82(103-1067)	Dinophyceae->82(103-1067)	Syndiniales->82(103-1067)	Dino-Group I->80(103-1067)

Table S3 **List of sequences deposited in GenBank, with KeyDNATools and BLAST results**

Name	N°Accession	KeyDNATools Results (->number of matched keys (fragment lenght))				
		Rank 1	Rank 2	Rank 3	Rank 4	Rank 5
BS3_H4	FN598442	Chromalveolata->82(26-802)	Alveolata->82(26-802)	Dinophyceae->82(26-802)	Syndiniales->82(26-802)	Dino-Group I->81(26-802)
GM2_A1	FN598457	Chromalveolata->104(61-1074)	Alveolata->104(61-1074)	Dinophyceae->104(61-1074)	Syndiniales->104(61-1074)	Dino-Group II->102(61-1074)
BIO9_E2	FN598302	Chromalveolata->121(58-1033)	Alveolata->121(58-1033)	Dinophyceae->121(58-1033)	Syndiniales->121(58-1033)	Dino-Group II->119(58-1033)
BIO5_H9	FN598278	Chromalveolata->125(55-1062)	Alveolata->125(55-1062)	Dinophyceae->125(55-1062)	Syndiniales->125(55-1062)	Dino-Group II->122(55-1062)
BIO7_H4	FN598294	Chromalveolata->124(55-1064)	Alveolata->124(55-1064)	Dinophyceae->124(55-1064)	Syndiniales->124(55-1064)	Dino-Group II->122(55-1064)
BIO7_D12	FN598285	Chromalveolata->132(58-934)	Alveolata->132(58-934)	Dinophyceae->132(58-934)	Syndiniales->132(58-934)	Dino-Group II->130(58-934)
BIO5_C7	FN598275	Chromalveolata->158(67-1093)	Alveolata->158(67-1093)	Dinophyceae->158(67-1093)	Syndiniales->158(67-1093)	Dino-Group II->151(67-1093)
BIO9_A5	FN598296	Chromalveolata->170(61-1093)	Alveolata->170(61-1093)	Dinophyceae->170(61-1093)	Syndiniales->170(61-1093)	Dino-Group II->167(61-1093)
BIO10_B9	FN598220	Chromalveolata->174(61-1096)	Alveolata->174(61-1096)	Dinophyceae->174(61-1096)	Syndiniales->174(61-1096)	Dino-Group II->171(61-1096)
BIO7_E3	FN598288	Chromalveolata->175(61-1096)	Alveolata->175(61-1096)	Dinophyceae->175(61-1096)	Syndiniales->175(61-1096)	Dino-Group II->171(61-1096)
BS3_B3	FN598430	Chromalveolata->196(61-1578)	Alveolata->195(61-1578)	Dinophyceae->195(61-1578)	Syndiniales->195(61-1578)	Dino-Group II->193(61-1578)
BIO10_F8	FN598225	Chromalveolata->29(269-1040)	Alveolata->29(269-1040)	Dinophyceae->28(269-1040)	Syndiniales->25(269-893)	Dino-Group II->21(269-893)
BS3_B10	FN598428	Chromalveolata->61(75-1531)	Alveolata->61(75-1531)	Dinophyceae->61(75-1531)	Syndiniales->61(75-1531)	Dino-Group II->58(75-1426)
BS3_E12	FN598438	Chromalveolata->87(12-900)	Alveolata->87(12-900)	Dinophyceae->87(12-900)	Syndiniales->87(12-900)	Dino-Group II->81(12-900)
BS3_D9	FN598437	Chromalveolata->104(75-1758)	Alveolata->104(75-1758)	Dinophyceae->104(75-1758)	Syndiniales->103(75-1758)	Dino-Group II->96(75-1753)
BS3_E2	FN598439	Chromalveolata->45(64-1772)	Alveolata->45(64-1772)	Dinophyceae->43(64-1772)	Syndiniales->40(64-1772)	Dino-Group IV->20(64-1772)
BS3_D11	FN598433	Chromalveolata->12(144-964)	Stramenopiles->12(144-964)	Bicosoecida->12(144-964)	Bicosoecida->12(144-964)	Bicosoecaceae->5(144-887)
BS15_A1	FN598353	Chromalveolata->18(19-699)	Stramenopiles->18(19-699)	Bicosoecida->17(19-685)	Bicosoecida->17(19-685)	Caecitellaceae->15(52-685)
BS24_A1	FN598402	Chromalveolata->33(90-1035)	Stramenopiles->33(90-1035)	Bicosoecida->33(90-1035)	Bicosoecida->33(90-1035)	Caecitellaceae->27(126-1010)
BS16_H10	FN598366	Chromalveolata->37(66-1000)	Stramenopiles->36(66-1000)	Bicosoecida->36(66-1000)	Bicosoecida->36(66-1000)	Caecitellaceae->29(101-975)
BIO9_E1	FN598301	Chromalveolata->36(66-1001)	Stramenopiles->36(66-1001)	Bicosoecida->36(66-1001)	Bicosoecida->36(66-1001)	Caecitellaceae->29(101-976)
BS11_C8	FN598317	Chromalveolata->37(102-1037)	Stramenopiles->37(102-1037)	Bicosoecida->37(102-1037)	Bicosoecida->37(102-1037)	Caecitellaceae->30(137-1012)
BS24_A2	FN598403	Chromalveolata->37(33-972)	Stramenopiles->37(33-972)	Bicosoecida->37(33-972)	Bicosoecida->37(33-972)	Cafeteriaceae->36(33-972)
MO1_C4	FN598462	Chromalveolata->37(66-1000)	Stramenopiles->37(66-1000)	Bicosoecida->37(66-1000)	Bicosoecida->37(66-1000)	Caecitellaceae->30(101-975)
MO1_A1	FN598459	Chromalveolata->38(66-1000)	Stramenopiles->38(66-1000)	Bicosoecida->38(66-1000)	Bicosoecida->38(66-1000)	Cafeteriaceae->37(66-1000)
BS15_G3	FN598359	Chromalveolata->38(66-1001)	Stramenopiles->38(66-1001)	Bicosoecida->38(66-1001)	Bicosoecida->38(66-1001)	Cafeteriaceae->37(66-1001)
BS16_E11	FN598363	Chromalveolata->38(66-1001)	Stramenopiles->38(66-1001)	Bicosoecida->38(66-1001)	Bicosoecida->38(66-1001)	Cafeteriaceae->37(66-1001)
GM1_A5	FN598450	Chromalveolata->6(69-963)	Stramenopiles->6(69-963)	Bicosoecida->6(69-963)	Bicosoecida->6(69-963)	Cafeteriaceae->5(69-963)
BS16_G10	FN598365	Chromalveolata->7(126-942)	Stramenopiles->7(126-942)	Bicosoecida->7(126-942)	Bicosoecida->7(126-942)	Cafeteriaceae->6(126-942)
BIO5_D11	FN598276	Chromalveolata->45(65-1076)	Stramenopiles->45(65-1076)	Bacillariophyta->45(65-1076)	Centric diatoms_Coscinodiscophyceae->45(65-1076)	Thalassiosira group->12(176-748)
MO3_B1	FN598469	Chromalveolata->16(65-810)	Stramenopiles->16(65-810)	Chrysophyceae and Synurophyceae->16(65-810)	Chrysophyceae and Synurophyceae->16(65-810)	Ochromonadales->7(65-269)
BS12_B5	FN598330	Chromalveolata->35(123-1079)	Stramenopiles->34(123-919)	Chrysophyceae and Synurophyceae->34(123-919)	Chrysophyceae and Synurophyceae->34(123-919)	Paraphysomonadales->6(214-731)
BS17_D2	FN598370	Chromalveolata->40(123-1071)	Stramenopiles->40(123-1071)	Chrysophyceae and Synurophyceae->40(123-1071)	Chrysophyceae and Synurophyceae->40(123-1071)	Paraphysomonadales->10(214-1066)
BIO3_F1	FN598259	Chromalveolata->49(124-1069)	Stramenopiles->49(124-1069)	Chrysophyceae and Synurophyceae->49(124-1069)	Chrysophyceae and Synurophyceae->49(124-1069)	Paraphysomonadales->7(210-1059)
BIO10_F1	FN598224	Chromalveolata->50(123-1072)	Stramenopiles->50(123-1072)	Chrysophyceae and Synurophyceae->50(123-1072)	Chrysophyceae and Synurophyceae->50(123-1072)	Paraphysomonadales->7(214-1062)
MO4_F10	FN598477	Chromalveolata->9(183-913)	Stramenopiles->9(183-913)	Chrysophyceae and Synurophyceae->9(183-913)	Chrysophyceae and Synurophyceae->9(183-913)	
BIO2_G6	FN598249	Chromalveolata->28(261-915)	Stramenopiles->28(261-915)	Labyrinthulea->28(261-915)	Labyrinthulidae->28(261-915)	Labyrinthulidae->28(261-915)
BS18_B5	FN598376	Chromalveolata->41(122-848)	Stramenopiles->40(122-848)	Bacillariophyta->39(204-848)	Pennate diatoms->39(204-848)	
BS23_G1	FN598400	Chromalveolata->15(66-902)	Stramenopiles->15(66-902)	Pirsonia->11(66-902)	Pirsonia->11(66-902)	
BS16_C8	FN598362	Chromalveolata->7(169-974)	Stramenopiles->7(169-974)			
BIO1_A12	FN598228	Chromalveolata->75(67-1071)	Alveolata->75(67-1071)	Dinophyceae->75(67-1071)	Dinophyceae->75(67-1071)	
BIO1_D7	FN598230	Chromalveolata->61(210-1046)	Alveolata->61(210-1046)	Dinophyceae->61(210-1046)	Dinophyceae->59(210-1046)	
BIO1_E10	FN598231	Chromalveolata->77(67-1072)	Alveolata->77(67-1072)	Dinophyceae->77(67-1072)	Dinophyceae->77(67-1072)	
BIO1_H7	FN598235	Chromalveolata->55(59-793)	Alveolata->55(59-793)	Dinophyceae->55(59-793)	Dinophyceae->53(59-793)	
BIO2_A8	FN598238	Chromalveolata->48(55-779)	Alveolata->48(55-779)	Dinophyceae->48(55-779)	Dinophyceae->48(55-779)	
BIO2_B3	FN598240	Chromalveolata->53(67-1073)	Alveolata->53(67-1073)	Dinophyceae->53(67-1073)	Dinophyceae->53(67-1073)	
BIO2_C6	FN598242	Chromalveolata->69(67-1070)	Alveolata->69(67-1070)	Dinophyceae->69(67-1070)	Dinophyceae->69(67-1070)	
BIO3_E3	FN598257	Chromalveolata->73(103-825)	Alveolata->73(103-825)	Dinophyceae->72(117-825)	Dinophyceae->72(117-825)	
BIO3_E4	FN598258	Chromalveolata->55(178-738)	Alveolata->55(178-738)	Dinophyceae->55(178-738)	Dinophyceae->55(178-738)	
BIO4_A6	FN598262	Chromalveolata->63(61-794)	Alveolata->63(61-794)	Dinophyceae->63(61-794)	Dinophyceae->63(61-794)	
BIO4_C10	FN598263	Chromalveolata->31(88-1086)	Alveolata->31(88-1086)	Dinophyceae->31(88-1086)	Dinophyceae->31(88-1086)	
BIO4_C12	FN598264	Chromalveolata->83(67-1075)	Alveolata->83(67-1075)	Dinophyceae->83(67-1075)	Dinophyceae->83(67-1075)	
BIO4_C2	FN598265	Chromalveolata->85(67-1064)	Alveolata->85(67-1064)	Dinophyceae->85(67-1064)	Dinophyceae->85(67-1064)	
BIO4_E12	FN598268	Chromalveolata->86(67-1030)	Alveolata->86(67-1030)	Dinophyceae->86(67-1030)	Dinophyceae->86(67-1030)	
BIO4_G3	FN598272	Chromalveolata->28(101-919)	Alveolata->28(101-919)	Dinophyceae->28(101-919)	Dinophyceae->28(101-919)	
BIO7_A2	FN598280	Chromalveolata->71(67-1071)	Alveolata->71(67-1071)	Dinophyceae->71(67-1071)	Dinophyceae->71(67-1071)	
BIO7_D1	FN598284	Chromalveolata->22(60-1057)	Alveolata->22(60-1057)	Dinophyceae->22(60-1057)	Dinophyceae->22(60-1057)	
BIO7_F2	FN598290	Chromalveolata->56(67-1070)	Alveolata->56(67-1070)	Dinophyceae->56(67-1070)	Dinophyceae->56(67-1070)	
BIO9_D1	FN598298	Chromalveolata->78(67-996)	Alveolata->78(67-996)	Dinophyceae->78(67-996)	Dinophyceae->78(67-996)	
BIO9_E5	FN598303	Chromalveolata->75(67-1071)	Alveolata->75(67-1071)	Dinophyceae->75(67-1071)	Dinophyceae->75(67-1071)	
BS1_G5	FN598394	Chromalveolata->43(30-178)	Alveolata->43(30-178)	Dinophyceae->43(30-178)	Dinophyceae->43(30-178)	
BS12_A3	FN598327	Chromalveolata->70(101-1078)	Alveolata->70(101-1078)	Dinophyceae->70(101-1078)	Dinophyceae->70(101-1078)	
BS12_B11	FN598328	Chromalveolata->68(60-1072)	Alveolata->68(60-1072)	Dinophyceae->68(60-1072)	Dinophyceae->68(60-1072)	
BS12_G11	FN598339	Chromalveolata->81(124-1085)	Alveolata->80(124-1085)	Dinophyceae->80(124-1085)	Dinophyceae->79(167-1085)	
BS13_A9	FN598343	Chromalveolata->19(125-1064)	Alveolata->19(125-1064)	Dinophyceae->19(125-1064)	Dinophyceae->18(125-1064)	
BS13_B7	FN598344	Chromalveolata->69(50-1074)	Alveolata->69(50-1074)	Dinophyceae->69(50-1074)	Dinophyceae->68(50-1074)	
BS16_B9	FN598361	Chromalveolata->90(67-1075)	Alveolata->90(67-1075)	Dinophyceae->90(67-1075)	Dinophyceae->90(67-1075)	
BS19_F5	FN598389	Chromalveolata->19(64-1038)	Alveolata->18(64-1038)	Dinophyceae->17(64-1038)	Dinophyceae->10(64-1038)	
BS2_D11	FN598424	Chromalveolata->42(25-917)	Alveolata->42(25-917)	Dinophyceae->42(25-917)	Dinophyceae->42(25-917)	
BS2_D6	FN598427	Chromalveolata->62(31-999)	Alveolata->62(31-999)	Dinophyceae->62(31-999)	Dinophyceae->62(31-999)	
BS23_A7	FN598396	Chromalveolata->60(50-1084)	Alveolata->60(50-1084)	Dinophyceae->60(50-1084)	Dinophyceae->60(50-1084)	

Table S3 **List of sequences deposited in GenBank, with KeyDNATools and BLAST results**

Name	N°Accession	KeyDNATools Results (->number of matched keys (fragment lenght))				
		Rank 1	Rank 2	Rank 3	Rank 4	Rank 5
BS25_A8	FN598412	Chromalveolata->62(84-1071)	Alveolata->62(84-1071)	Dinophyceae->62(84-1071)	Dinophyceae->61(165-1071)	
BIO7_B10	FN598282	Excavata->103(54-1312)	Euglenozoa->103(54-1312)	Diplonemea->103(54-1312)	Diplonemea->103(54-1312)	
BIO9_B5	FN598297	Excavata->108(54-1315)	Euglenozoa->108(54-1315)	Diplonemea->108(54-1315)	Diplonemea->108(54-1315)	
BS3_D8	FN598436	Excavata->116(54-1320)	Euglenozoa->116(54-1320)	Diplonemea->116(54-1320)	Diplonemea->116(54-1320)	
BIO2_F12	FN598247	Excavata->119(54-1320)	Euglenozoa->119(54-1320)	Diplonemea->119(54-1320)	Diplonemea->119(54-1320)	
BIO2_H4	FN598250	Excavata->122(54-1319)	Euglenozoa->122(54-1319)	Diplonemea->122(54-1319)	Diplonemea->122(54-1319)	
BIO3_F4	FN598260	Excavata->126(100-1320)	Euglenozoa->126(100-1320)	Diplonemea->126(100-1320)	Diplonemea->126(100-1320)	
BIO9_D4	FN598299	Excavata->132(100-1316)	Euglenozoa->132(100-1316)	Diplonemea->132(100-1316)	Diplonemea->132(100-1316)	
BIO2_F3	FN598248	Excavata->132(111-1319)	Euglenozoa->132(111-1319)	Diplonemea->132(111-1319)	Diplonemea->132(111-1319)	
BS3_D7	FN598435	Excavata->133(100-1321)	Euglenozoa->133(100-1321)	Diplonemea->133(100-1321)	Diplonemea->133(100-1321)	
BIO7_E10	FN598287	Excavata->146(54-1320)	Euglenozoa->146(54-1320)	Diplonemea->146(54-1320)	Diplonemea->146(54-1320)	
BS3_G10	FN598441	Excavata->151(54-1319)	Euglenozoa->151(54-1319)	Diplonemea->151(54-1319)	Diplonemea->151(54-1319)	
BS13_H8	FN598347	Excavata->55(54-1286)	Euglenozoa->55(54-1286)	Diplonemea->52(54-1286)	Diplonemea->52(54-1286)	
BS23_H10	FN598401	Excavata->54(54-1301)	Euglenozoa->54(54-1301)	Diplonemea->54(54-1301)	Diplonemea->54(54-1301)	
BS23_B2	FN598397	Excavata->55(111-1296)	Euglenozoa->55(111-1296)	Diplonemea->55(111-1296)	Diplonemea->55(111-1296)	
BS25_B2	FN598413	Excavata->60(54-1312)	Euglenozoa->60(54-1312)	Diplonemea->60(54-1312)	Diplonemea->60(54-1312)	
BS25_H3	FN598420	Excavata->85(54-1307)	Euglenozoa->85(54-1307)	Diplonemea->85(54-1307)	Diplonemea->85(54-1307)	
BIO1_H12	FN598233	Excavata->94(116-1290)	Euglenozoa->94(116-1290)	Diplonemea->94(116-1290)	Diplonemea->94(116-1290)	
BIO3_A2	FN598252	Excavata->98(111-1314)	Euglenozoa->98(111-1314)	Diplonemea->98(111-1314)	Diplonemea->98(111-1314)	
BS3_D4	FN598434	Excavata->402(54-1326)	Euglenozoa->402(54-1326)	Kinetoplastea->402(54-1326)	Metakinetoplastida->402(54-1326)	Neobodonid->139(54-1292)
BS11_C5	FN598316	Excavata->59(50-1296)	Euglenozoa->59(50-1296)	Kinetoplastea->59(50-1296)	Prokinetoplastida->59(50-1296)	Prokinetoplastidae->59(50-1296)
BS17_G10	FN598373	Incertae sedis Eukaryota->69(56-1072)	Centroheliozoa->69(56-1072)	Centroheliozoa->69(56-1072)	Centroheliozoa->69(56-1072)	
BS10_B6	FN598309	Incertae sedis Eukaryota->5(244-1063)	Apusomonadidae->5(244-1063)	Apusomonadidae->5(244-1063)	Apusomonadidae->5(244-1063)	Apusomonadidae->5(244-1063)
BS12_D4	FN598334	Incertae sedis Eukaryota->7(244-1063)	Apusomonadidae->7(244-1063)	Apusomonadidae->7(244-1063)	Apusomonadidae->7(244-1063)	Apusomonadidae->7(244-1063)
BS17_C3	FN598369	Incertae sedis Eukaryota->7(244-928)	Apusomonadidae->7(244-928)	Apusomonadidae->7(244-928)	Apusomonadidae->7(244-928)	Apusomonadidae->7(244-928)
BS18_A2	FN598375	Incertae sedis Eukaryota->8(244-1062)	Apusomonadidae->8(244-1062)	Apusomonadidae->8(244-1062)	Apusomonadidae->8(244-1062)	Apusomonadidae->8(244-1062)
BS19_B4	FN598383	Incertae sedis Eukaryota->8(244-1063)	Apusomonadidae->8(244-1063)	Apusomonadidae->8(244-1063)	Apusomonadidae->8(244-1063)	Apusomonadidae->8(244-1063)
BS19_C3	FN598386	Incertae sedis Eukaryota->8(66-1059)	Apusomonadidae->8(66-1059)	Apusomonadidae->8(66-1059)	Apusomonadidae->8(66-1059)	Apusomonadidae->8(66-1059)
GM1_E3	FN598454	Incertae sedis Eukaryota->8(244-1063)	Apusomonadidae->8(244-1063)	Apusomonadidae->8(244-1063)	Apusomonadidae->8(244-1063)	Apusomonadidae->8(244-1063)
MO3_A10	FN598468	Incertae sedis Eukaryota->8(244-1061)	Apusomonadidae->8(244-1061)	Apusomonadidae->8(244-1061)	Apusomonadidae->8(244-1061)	Apusomonadidae->8(244-1061)
MO4_A9	FN598474	Incertae sedis Eukaryota->7(244-933)	Apusomonadidae->7(244-933)	Apusomonadidae->7(244-933)	Apusomonadidae->7(244-933)	Apusomonadidae->7(244-933)
BS11_E4	FN598322	Incertae sedis Eukaryota->47(66-1139)	Apusomonadidae->47(66-1139)	Apusomonadidae->47(66-1139)	Apusomonadidae->47(66-1139)	Apusomonadidae->47(66-1139)
BS15_G1	FN598358	Incertae sedis Eukaryota->12(100-895)	Apusomonadidae->12(100-895)	Apusomonadidae->12(100-895)	Apusomonadidae->12(100-895)	Apusomonadidae->12(100-895)
BS24_B1	FN598404	Incertae sedis Eukaryota->13(100-894)	Apusomonadidae->13(100-894)	Apusomonadidae->13(100-894)	Apusomonadidae->13(100-894)	Apusomonadidae->13(100-894)
MO1_A3	FN598460	Incertae sedis Eukaryota->16(290-1165)	Apusomonadidae->16(290-1165)	Apusomonadidae->16(290-1165)	Apusomonadidae->16(290-1165)	Apusomonadidae->16(290-1165)
BIO2_D4	FN598243	Incertae sedis Eukaryota->7(105-766)	Planomonadida->7(105-766)	Planomonadida->7(105-766)	Planomonadidae->7(105-766)	
BS11_E7	FN598323	Incertae sedis Eukaryota->8(127-1056)	Planomonadida->8(127-1056)	Planomonadida->8(127-1056)	Planomonadidae->8(127-1056)	
BS12_F6	FN598338	Incertae sedis Eukaryota->6(127-788)	Planomonadida->6(127-788)	Planomonadida->6(127-788)	Planomonadidae->6(127-788)	
BS15_F3	FN598357	Incertae sedis Eukaryota->7(127-788)	Planomonadida->7(127-788)	Planomonadida->7(127-788)	Planomonadidae->7(127-788)	
BS17_G4	FN598374	Incertae sedis Eukaryota->7(127-788)	Planomonadida->7(127-788)	Planomonadida->7(127-788)	Planomonadidae->7(127-788)	
BS18_H1	FN598380	Incertae sedis Eukaryota->8(127-1060)	Planomonadida->8(127-1060)	Planomonadida->8(127-1060)	Planomonadidae->8(127-1060)	
BS24_B7	FN598406	Incertae sedis Eukaryota->6(127-787)	Planomonadida->6(127-787)	Planomonadida->6(127-787)	Planomonadidae->6(127-787)	
BS1_D5	FN598391	Opisthokonta->12(164-904)	Metazoa->12(164-904)	Annelida->12(164-904)	Annelida->12(164-904)	
BS1_H8	FN598395	Opisthokonta->42(34-896)	Metazoa->42(34-896)	Annelida->42(34-896)	Annelida->42(34-896)	
BS1_G3	FN598393	Opisthokonta->14(34-464)	Metazoa->14(34-464)	Annelida->13(34-464)	Annelida->13(34-464)	
BS2_D2	FN598426	Opisthokonta->125(22-1114)	Metazoa->125(22-1114)	Arthropoda->125(22-1114)	Crustacea->125(22-1114)	Maxillopoda->124(22-1114)
BS4_C10	FN598447	Opisthokonta->101(24-932)	Metazoa->100(24-932)	Cnidaria->100(24-932)	Cnidaria->100(24-932)	Hydrozoa->60(26-932)
BS3_B5	FN598432	Opisthokonta->101(24-933)	Metazoa->101(24-933)	Cnidaria->101(24-933)	Cnidaria->101(24-933)	Hydrozoa->59(26-933)
BS12_B6	FN598331	Opisthokonta->5(208-786)	Metazoa->4(330-786)	Cnidaria->4(330-786)	Cnidaria->4(330-786)	
BS4_D6	FN598448	Opisthokonta->26(27-887)	Metazoa->26(27-887)	Ctenophora->26(27-887)	Ctenophora->26(27-887)	
BS1_F5	FN598392	Opisthokonta->25(37-902)	Metazoa->25(37-902)	Mollusca->24(37-902)	Bivalvia->24(37-902)	Mytiloida->9(38-902)
BS2_D12	FN598425	Opisthokonta->20(38-901)	Metazoa->20(38-901)	Mollusca->20(38-901)	Bivalvia->20(38-901)	Mytiloida->8(39-901)
BS23_E2	FN598399	Opisthokonta->36(241-1232)	Metazoa->36(241-1232)	Mollusca->36(241-1232)	Gastropoda->36(241-1232)	Patellogastropoda->36(241-1232)
BS25_G3	FN598419	Opisthokonta->44(238-1039)	Metazoa->44(238-1039)	Mollusca->44(238-1039)	Gastropoda->44(238-1039)	Patellogastropoda->44(238-1039)
BS11_E2	FN598321	Opisthokonta->56(56-1000)	Metazoa->56(56-1000)	Mollusca->56(56-1000)	Gastropoda->56(56-1000)	Patellogastropoda->56(56-1000)
BS2_B11	FN598422	Opisthokonta->59(33-935)	Metazoa->59(33-935)	Mollusca->59(33-935)	Gastropoda->59(33-935)	Vetigastropoda->59(33-935)
BS24_B2	FN598405	Opisthokonta->17(163-1025)	Fungi->17(163-1025)	Ascomycota->16(163-1025)	Saccharomycotina->16(163-1025)	Saccharomycetales->14(163-1025)
BS12_A1	FN598326	Opisthokonta->56(60-572)	Fungi->56(60-572)	Ascomycota->56(60-572)	Pezizomycotina->56(60-572)	Sordariomycetes->26(60-572)
BIO10_E7	FN598223	Opisthokonta->57(119-825)	Fungi->57(119-825)	Ascomycota->57(119-825)	Saccharomycotina->57(119-825)	Saccharomycetales->55(119-825)
BS24_D7	FN598407	Opisthokonta->83(75-1071)	Fungi->83(75-1071)	Ascomycota->82(176-1071)	Pezizomycotina->82(176-1071)	Eurotiomycetes->26(177-1071)
BS19_E3	FN598387	Opisthokonta->12(484-1062)	Fungi->12(484-1062)	Basal fungi->10(484-744)	Basal fungi->10(484-744)	Chytridiomycota->10(484-744)
BS11_B8	FN598315	Opisthokonta->18(215-903)	Fungi->18(215-903)	Basidiomycota->18(215-903)	Agaricomycotina->18(215-903)	Agaricomycetes->13(215-903)
BS18_H2	FN598381	Opisthokonta->18(486-902)	Fungi->18(486-902)	Basidiomycota->18(486-902)	Agaricomycotina->18(486-902)	Agaricomycetes->13(486-902)
BS16_H8	FN598367	Opisthokonta->2(246-935)	Fungi->2(246-935)	Basidiomycota->2(246-935)		
MO1_F5	FN598463	Opisthokonta->22(175-1070)	Fungi->22(175-1070)	Basidiomycota->22(175-1070)	Ustilaginomycotina->22(175-1070)	Exobasidiomycetes->21(175-1070)
MO3_E8	FN598472	Opisthokonta->80(116-1083)	Fungi->80(116-1083)	Basidiomycota->80(116-1083)	Agaricomycotina->80(116-1083)	Agaricomycetes->59(116-1083)
BIO9_E9	FN598304	Opisthokonta->3(126-644)	Fungi->3(126-644)			
BS19_B5	FN598384					
BS4_B4	FN598446					
BS11_B2	FN598314	Rhizaria->12(60-927)	Cercozoa->12(60-927)	Cercozoa->11(60-927)	Chlorarachnida->11(60-927)	Chlorarachniidae->11(60-927)
BS19_B6	FN598385	Rhizaria->14(120-927)	Cercozoa->14(120-927)	Cercozoa->14(120-927)	Cercozoa->14(120-927)	

Table S3 **List of sequences deposited in GenBank, with KeyDNATools and BLAST results**

Name	N°Accession	KeyDNATools Results (->number of matched keys (fragment lenght))				
		Rank 1	Rank 2	Rank 3	Rank 4	Rank 5
MO2_A2	FN598465	Rhizaria->15(64-949)	Cercozoa->15(64-949)	Cercozoa->15(64-949)		
BS15_B5	FN598356	Rhizaria->25(171-1076)	Cercozoa->25(171-1076)	Cercozoa->25(171-1076)	Cercozoa->25(171-1076)	
GM1_F4	FN598455	Cercozoa->26(117-1091)	Cercozoa->26(117-1091)	Cercozoa->26(117-1091)	Cercozoa->26(117-1091)	Massisteriidae->8(117-1091)
BS14_A7	FN598348	Rhizaria->29(127-1090)	Cercozoa->29(127-1090)	Cercozoa->29(127-1090)	Cercozoa->29(127-1090)	
BS10_B7	FN598310	Rhizaria->31(127-1090)	Cercozoa->31(127-1090)	Cercozoa->31(127-1090)	Cercozoa->31(127-1090)	
BS12_B4	FN598329	Rhizaria->39(119-920)	Cercozoa->39(119-920)	Cercozoa->39(119-920)	Cercozoa->39(119-920)	Massisteriidae->5(119-696)
BIO2_A5	FN598236	Rhizaria->58(100-1093)	Cercozoa->58(100-1093)	Cercozoa->58(100-1093)	Cercozoa->58(100-1093)	Thecofilosea->13(232-1087)
BS10_B9	FN598311	Rhizaria->58(111-812)	Cercozoa->58(111-812)	Cercozoa->58(111-812)	Cercozoa->58(111-812)	Massisteriidae->13(111-806)
BS11_E8	FN598324	Rhizaria->64(118-1092)	Cercozoa->64(118-1092)	Cercozoa->64(118-1092)	Cercozoa->64(118-1092)	Massisteriidae->15(118-1092)
BS24_H5	FN598411	Rhizaria->65(120-1094)	Cercozoa->65(120-1094)	Cercozoa->65(120-1094)	Cercozoa->65(120-1094)	Massisteriidae->13(120-1094)
BS13_H1	FN598346	Rhizaria->68(119-1093)	Cercozoa->68(119-1093)	Cercozoa->68(119-1093)	Cercozoa->68(119-1093)	Massisteriidae->16(119-1093)
BS15_A5	FN598354	Rhizaria->68(66-1075)	Cercozoa->68(66-1075)	Cercozoa->68(66-1075)	Cercozoa->68(66-1075)	Massisteriidae->12(111-1075)
BS17_A3	FN598368	Rhizaria->70(119-1093)	Cercozoa->70(119-1093)	Cercozoa->70(119-1093)	Cercozoa->70(119-1093)	Massisteriidae->16(119-1093)
BS19_A3	FN598382	Rhizaria->70(119-1093)	Cercozoa->70(119-1093)	Cercozoa->70(119-1093)	Cercozoa->70(119-1093)	Massisteriidae->15(119-1093)
BIO4_F7	FN598271	Rhizaria->72(111-1092)	Cercozoa->72(111-1092)	Cercozoa->72(111-1092)	Cercozoa->72(111-1092)	Massisteriidae->19(111-1092)
BS18_G1	FN598379	Rhizaria->72(111-1096)	Cercozoa->72(111-1096)	Cercozoa->72(111-1096)	Cercozoa->72(111-1096)	Massisteriidae->16(111-1096)
BS14_G12	FN598350	Rhizaria->73(111-1096)	Cercozoa->73(111-1096)	Cercozoa->73(111-1096)	Cercozoa->73(111-1096)	Massisteriidae->19(111-1096)
BIO7_A1	FN598279					
MO4_C2	FN598475					
BIO3_F9	FN598261	Rhizaria->30(216-969)	Radiolaria->30(216-969)	Acantharea->30(216-969)	Arthracanthida_Symphyacanthida->29(216-969)	
BIO10_G12	FN598226	Rhizaria->111(64-1084)	Radiolaria->111(64-1084)	Polycystinea->111(64-1084)	Spumellarida->111(64-1084)	
BIO9_H1	FN598307	Rhizaria->114(64-1083)	Radiolaria->114(64-1083)	Polycystinea->114(64-1083)	Spumellarida->114(64-1083)	
BIO2_B8	FN598241	Rhizaria->118(66-1083)	Radiolaria->118(66-1083)	Polycystinea->118(66-1083)	Spumellarida->118(66-1083)	
BIO3_A1	FN598251	Rhizaria->120(64-1084)	Radiolaria->120(64-1084)	Polycystinea->120(64-1084)	Spumellarida->120(64-1084)	
BIO1_D11	FN598229	Rhizaria->18(55-1060)	Radiolaria->18(55-1060)	Polycystinea->18(55-1060)		
BS24_F5	FN598410	Rhizaria->20(100-875)	Radiolaria->20(100-875)	Polycystinea->20(100-875)		
BIO3_C6	FN598255	Rhizaria->21(18-1080)	Radiolaria->21(18-1080)	Polycystinea->21(18-1080)	Collodaria->21(18-1080)	Collosphaeridae and Sphaerozoidae->21(18-1080)
BIO4_D2	FN598267	Rhizaria->38(140-1114)	Radiolaria->38(140-1114)	Polycystinea->38(140-1114)	Spumellarida->38(140-1114)	
BIO5_A3	FN598273	Rhizaria->39(18-1080)	Radiolaria->39(18-1080)	Polycystinea->39(18-1080)	Collodaria->39(18-1080)	Collosphaeridae and Sphaerozoidae->39(18-1080)
BIO7_H1	FN598293	Rhizaria->40(120-1078)	Radiolaria->40(120-1078)	Polycystinea->40(120-1078)	Nassellarida->40(120-1078)	Plagoniidae and Pterocorythidae->24(120-1078)
BS16_E9	FN598364	Rhizaria->41(67-1068)	Radiolaria->41(67-1068)	Polycystinea->41(67-1068)		
BIO7_C8	FN598283	Rhizaria->56(30-1065)	Radiolaria->56(30-1065)	Polycystinea->56(30-1065)		
BIO4_E6	FN598269	Rhizaria->6(273-849)	Radiolaria->6(273-849)	Polycystinea->6(273-849)		
BIO3_C3	FN598254	Rhizaria->6(275-852)	Radiolaria->6(275-852)	Polycystinea->6(275-852)		
BIO9_D8	FN598300	Rhizaria->61(18-1082)	Radiolaria->61(18-1082)	Polycystinea->61(18-1082)	Collodaria->61(18-1082)	Collosphaeridae and Sphaerozoidae->61(18-1082)
BIO9_A12	FN598295	Rhizaria->62(50-1071)	Radiolaria->62(50-1071)	Polycystinea->62(50-1071)		
BIO2_A7	FN598237	Rhizaria->69(18-1082)	Radiolaria->69(18-1082)	Polycystinea->69(18-1082)	Collodaria->69(18-1082)	Collosphaeridae and Sphaerozoidae->69(18-1082)
BIO5_D5	FN598277	Rhizaria->74(64-1093)	Radiolaria->74(64-1093)	Polycystinea->74(64-1093)	Spumellarida->74(64-1093)	
BIO7_D2	FN598286	Rhizaria->91(64-1084)	Radiolaria->91(64-1084)	Polycystinea->91(64-1084)	Spumellarida->91(64-1084)	
BIO10_D6	FN598222	Rhizaria->37(25-1061)	Radiolaria->37(25-1061)	Taxopodia->37(25-1061)		
BS12_C1	FN598332	Archaeplastida->54(116-1080)	Charophyta->54(116-1080)	Charophyta->54(116-1080)	Charophyta->54(116-1080)	
MO3_C2	FN598471	Archaeplastida->78(116-1073)	Charophyta->76(116-1073)	Charophyta->76(116-1073)	Charophyta->76(116-1073)	
BS13_A6	FN598342	Archaeplastida->39(602-867)	Charophyta->39(602-867)	Charophyta->39(602-867)	Charophyta->39(602-867)	
BS11_D4	FN598320					
MO3_G3	FN598473					
MO4_C3	FN598476					

Table S3

Name	KeyDNATools Results		Length	Redudancy	BLAST results		
	Rank 6	Rank 7			N°Accession	Identity	Description
BIO1_A11	Vannella->75(66-1242)		1296	35	AY929904	98%	*Vannella* sp. strain S2M2/I
BIO10_D10	Vannella->32(264-1173)		1277	2	AY929912	87%	*Vannella* sp. strain 4354/I
BIO2_F11	Vannella->28(269-1179)		1286	4	AY929911	88%	*Vannella* sp. strain 4354/I
BIO3_E2	Vannella->31(264-1173)		1277	16	AY929911	87%	*Vannella* sp. strain 4354/I
BIO4_F4	Vannella->70(66-1241)		1295	8	AY929904	98%	*Vannella* sp. strain S2M2/I
BIO9_F1	Vannella->30(215-1173)		1277	1	AY929911	88%	*Vannella* sp. strain 4354/I
BS15_B2	Vannella->80(66-1225)		1279	1	AY121853	95%	*Vannella aberdonica* strain ATCC 50815
BS25_F2	Vannella->78(66-1211)		1276	9	AY121853	94%	*Vannella aberdonica* strain ATCC 50815
MO1_C11	Vannella->96(66-1226)		1280	3	AY121853	98%	*Vannella aberdonica* strain ATCC 50815
MO2_E1			1190	29	EU852658	85%	*Flabellulidae* sp. STAR2
GM1_A2			1002	13	AJ971527	94%	Uncultured Trachelocercidae isolate C03
GM1_C5			1094	1	AF401521	93%	*Vaginicola crystallina*
BIO7_E8			1115	1	EU446395	96%	Uncultured marine eukaryote clone cLA13A03
BS2_B3			587	2	AY835669	100%	*Pseudocohnilembus persalinus*
BS11_A3			1046	9	AY392128	98%	*Parauronema virginianum*
BS12_D8			1114	2	AY392128	98%	*Parauronema virginianum*
BS13_A2			1113	2	AY392128	99%	*Parauronema virginianum*
BS10_D9			1114	16	AY392128	99%	*Parauronema virginianum*
BS18_D5			1114	2	AY392128	98%	*Parauronema virginianum*
BS14_G7			1114	9	AY392128	99%	*Parauronema virginianum*
BS17_E2			1114	35	AY392128	99%	*Parauronema virginianum*
BS23_C3			1112	1	AY541685	96%	*Plagiopyliella pacifica*
MO3_B3			1101	3	EU503534	90%	*Gymnodinioides pitelkae*
BS25_H4	Euplotes->21(114-1003)		1182	8	AJ310490	96%	*Euplotes minuta*
BS15_G5	Euplotes->20(114-897)		1194	1	AJ310490	96%	*Euplotes minuta*
BS19_G4			1109	1	AF305625	93%	*Aspidisca steini*
BS25_F7			1070	2	EU880597	89%	*Aspidisca leptaspis* voucher LLQ-07092802
BS25_F8			1158	1	EF526933	95%	Uncultured marine eukaryote clone SA2_4H2
BS24_F1			1147	1	EF526875	96%	Uncultured marine eukaryote clone NIF_4F5
BS14_B11			1079	1	EF123704	95%	*Aspidisca aculeata*
BS4_A1			489	1	DQ918658	97%	Uncultured marine eukaryote clone ENVP223.00271
BIO5_A9			1012	1	EF527106	98%	Uncultured marine eukaryote clone SIF_1F2
BIO2_B1			1125	1	EU371395	98%	Uncultured eukaryote clone NPKS2_101
BS24_F2			1122	6	DQ103853	98%	Uncultured marine eukaryote clone M2_18C01
GM1_G2			1116	4	EU275202	96%	*Caryotricha* sp. *paraconvexa* isolate Qingdao
MO2_A1			1063	15	AF530529	92%	Uncultured ciliate clone AT1-2
BS18_D2			1073	2	DQ057347	88%	*Dysteria procera*
BS13_C1			866	12	EF527181	89%	Uncultured marine eukaryote clone SA2_2H10
BS12_E5			1079	1	EF527181	90%	Uncultured marine eukaryote clone SA2_2H10
BS11_D1			1082	2	EF527181	92%	Uncultured marine eukaryote clone SA2_2H10
BS19_F3			1082	4	EF527181	92%	Uncultured marine eukaryote clone SA2_2H10
BS25_D1			1082	25	DQ057347	95%	*Dysteria procera*
BS11_F2			1024	1	AF530529	97%	Uncultured ciliate clone AT1-2
BIO4_C8			1052	1	AB330055	95%	Uncultured eukaryote clone: YS18Ec14
GM1_E1			1051	2	EU050966	92%	Uncultured eukaryote clone SS1_E_02_11
GM1_B1			1062	62	AY332717	100%	*Acineta* sp. OSW-2003-1
GM2_C8			992	1	AY331805	78%	*Ephelota* sp. OOS-2003 clone 2
BS10_A2			1005	18	DQ834370	79%	*Ephelota* sp. QD-5
BS11_C9			1006	1	DQ834370	79%	*Ephelota* sp. QD-5
BS12_C8			1001	1	DQ834370	80%	*Ephelota* sp. QD-5
BS12_D7			1005	1	DQ834370	79%	*Ephelota* sp. QD-5
BS13_A1			1005	22	DQ834370	79%	*Ephelota* sp. QD-5
BS14_G9			1004	1	DQ834370	79%	*Ephelota* sp. QD-5
BS17_E5			1002	5	DQ834370	79%	*Ephelota* sp. QD-5
BIO10_B6			1132	7	AY078092	99%	*Colpodella pontica*
BIO3_B2			1133	8	AY078092	99%	*Colpodella pontica*
BS4_A6		Clade 1->94(27-494)	836	26	EU793695	97%	Uncultured syndiniales clone PROSOPE.ED-50m.202
MO2_A3		Clade 1->104(62-1069)	1135	10	FJ235531	97%	Uncultured marine eukaryote clone G220G02D
BIO2_E9		Clade 3->5(193-864)	1127	3	FJ440626	88%	*Ichthyodinium chabelardi* isolate PT20
BIO9_G10		Clade 2->17(55-713)	980	1	AF530538	86%	Uncultured alveolate clone AT8-27
BIO7_G6		Clade 1->23(621-1049)	1189	1	EF172996	90%	Uncultured eukaryote clone SSRPD95
BS25_C6		Clade 8->10(460-1064)	1118	1	AY664998	90%	Uncultured eukaryote clone SCM27C35
BS4_B12		Clade 8->13(31-474)	968	4	AY256213	97%	Uncultured eukaryote isolate H48
BIO1_F7		Clade 2->42(55-1069)	1131	15	AF530538	97%	Uncultured alveolate clone AT8-27
BIO7_A9		Clade 2->42(74-1084)	1136	8	DQ916406	97%	Uncultured marine alveolate isolate 11
BS3_F9		Clade 4->38(1-456)	663	4	EU793735	96%	Uncultured syndiniales clone PROSOPE.ED-50m.54
BS3_B11		Clade 3->23(24-915)	945	3	AF290073	97%	Uncultured marine alveolate Group I DH147-EKD18
BIO7_F6		Clade 3->25(257-1071)	1131	2	DQ186536	99%	Uncultured alveolate clone BL010320.28
BIO2_E4		Clade 2->52(168-1063)	1115	1	DQ916406	98%	Uncultured marine alveolate isolate 11
BS3_B4		Clade 2->49(24-956)	956	4	DQ916410	97%	Uncultured marine alveolate isolate 65
BIO1_H6		Clade 3->29(121-1057)	1130	3	AY664995	97%	Uncultured eukaryote clone SCM38C41

Table S3

Name	KeyDNATools Results		Length	Redudancy	BLAST results		
	Rank 6	Rank 7			N°Accession	Identity	Description
BS3_H4		Clade 5->59(26-765)	876	10	EF172816	95%	Uncultured eukaryote clone SSRPB81
GM2_A1		Clade 6->32(168-1052)	1135	40	EF172852	98%	Uncultured eukaryote clone SSRPB11
BIO9_E2		Clade 22->31(112-1033)	1140	2	EU793410	97%	Uncultured syndiniales clone PROSOPE.E5-55m.3
BIO5_H9		Clade 21->16(66-1062)	1133	5	EF172931	98%	Uncultured eukaryote clone SSRPC02
BIO7_H4		Clade 21->18(66-1062)	1132	3	EU793224	95%	Uncultured syndiniales clone PROSOPE.E3-25m.97
BIO7_D12		Clade 10 and 11->25(122-933)	1137	1	AY664988	95%	Uncultured eukaryote clone SCM16C19
BIO5_C7		Clade 1->56(67-1093)	1136	12	EU793221	98%	Uncultured syndiniales clone PROSOPE.E3-25m.52
BIO9_A5		Clade 7->55(67-862)	1136	1	EF172848	96%	Uncultured eukaryote clone SSRPB36
BIO10_B9		Clade 7->44(75-865)	1139	4	EF172848	96%	Uncultured eukaryote clone SSRPB36
BIO7_E3		Clade 7->72(67-866)	1139	7	EF172848	98%	Uncultured eukaryote clone SSRPB36
BS3_B3		Clade 7->48(112-1372)	1823	1	EF172798	95%	Uncultured eukaryote clone SSRPB41
BIO10_F8		Clade 10 and 11->5(269-728)	1139	5	AJ402335	87%	eukaryote clone OLI11023
BS3_B10			1824	1	AY129046	85%	Uncultured marine eukaryote clone UEPACDp4
BS3_E12		Clade 12->24(12-746)	922	1	EU818604	98%	Uncultured marine alveolate clone ZZ0052624
BS3_D9		Clade 9->15(637-1418)	1817	2	AB275016	95%	Uncultured eukaryote clone: DSGM-16
BS3_E2		Hematodinium Group->11(125-1082)	1838	11	EF065718	91%	*Hematodinium perezi* clone Ld18S2
BS3_D11			1023	1	AY520445	99%	*Bicosoeca vacillans* strain ATCC 50063
BS15_A1	Caecitellus->15(52-685)	pseudoparvulus->7(80-685)	792	2	EU106851	93%	*Caecitellus* sp. RCC1078
BS24_A1	Caecitellus->27(126-1010)		1080	28	EF050072	98%	Uncultured picobiliphyte clone He001005.3
BS16_H10	Caecitellus->29(101-975)		1046	2	EF620523	99%	Uncultured bicosoecid clone OC4.7
BIO9_E1	Caecitellus->29(101-976)		1047	11	AY520446	100%	*Caecitellus parvulus*
BS11_C8	Caecitellus->30(137-1012)		1083	1	DQ220715	99%	*Caecitellus paraparvulus* strain HFCC313
BS24_A2	Cafeteria->36(33-972)		1032	25	AM493687	99%	*Cafeteria* sp. CAFSW0510
MO1_C4	Caecitellus->30(101-975)		1046	1	EF620523	100%	Uncultured bicosoecid clone OC4.7
MO1_A1	Cafeteria->37(66-1000)		1062	79	EF027355	99%	*Cafeteria* sp. GOT180
BS15_G3	Cafeteria->37(66-1001)		1063	2	AM493687	99%	*Cafeteria* sp. CAFSW0510
BS16_E11	Cafeteria->37(66-1001)		1063	3	AM493687	100%	*Cafeteria* sp. CAFSW0510
GM1_A5			1050	1	EF620525	93%	Uncultured bicosoecid clone OC4.19
BS16_G10			1028	3	AF174366	95%	*Cafeteria* sp. EPM1
BIO5_D11			1140	2	DQ514865	100%	*Thalassiosira anguste-lineata* strain BEN02-30
MO3_B1			954	1	EF027354	94%	*Spumella* sp. GOT220
BS12_B5			1131	1	DQ103873	96%	Uncultured marine eukaryote clone M3_18G02
BS17_D2			1130	2	DQ103873	97%	Uncultured marine eukaryote clone M3_18G02
BIO3_F1			1129	4	AF109322	98%	*Paraphysomonas bandaiensis*
BIO10_F1			1132	1	AF109322	99%	*Paraphysomonas bandaiensis*
MO4_F10			994	15	AF123296	95%	*Phaeoplaca thallosa*
BIO2_G6			1149	1	FJ800639	97%	Uncultured labyrinthulid clone PJS101305_35
BS18_B5			1059	1	Y10567	94%	*Peridinium foliaceum* endosymbiont
BS23_G1			1149	4	AJ561114	95%	*Pirsonia diadema* isolate P757
BS16_C8			1053	1	AY381186	90%	Uncultured eukaryote clone OR000415.113
BIO1_A12			1140	10	EF527101	99%	Uncultured marine eukaryote clone SIF_1D12
BIO1_D7			1131	1	AF022196	97%	*Gymnodinium* sp. MUCC284
BIO1_E10			1139	4	EF527111	98%	Uncultured marine eukaryote clone SIF_1B11
BIO1_H7			1139	18	AF022199	98%	*Lepidodinium viride*
BIO2_A8			1138	2	EF527108	97%	Uncultured marine eukaryote clone SIF_1G6
BIO2_B3			1142	28	EU371148	99%	Uncultured marine eukaryote clone NPK2_168
BIO2_C6			1139	43	EF527101	99%	Uncultured marine eukaryote clone SIF_1D12
BIO3_E3			1140	10	AY664877	99%	Uncultured eukaryote clone SCM16C67
BIO3_E4			1140	23	AF022196	96%	*Gymnodinium* sp. MUCC284
BIO4_A6			1140	62	AF022199	98%	*Lepidodinium viride*
BIO4_C10			1127	1	AF033869	85%	*Fragilidium subglobosum*
BIO4_C12			1141	8	U52356	99%	Unidentified symbiotic dinoflagellate BBSR 323
BIO4_C2			1141	1	AF022201	98%	*Pentapharsodinium tyrrhenicum*
BIO4_E12			1058	4	EF526903	97%	Uncultured marine eukaryote clone SA1_4C7
BIO4_G3			979	4	DQ500121	93%	*Pyrodinium bahamense* var. *compressum* isolate PG
BIO7_A2			1140	17	EF527101	99%	Uncultured marine eukaryote clone SIF_1D12
BIO7_D1			1135	6	AY803743	92%	*Prorocentrum donghaiense*
BIO7_F2			1139	5	EU371148	99%	Uncultured marine eukaryote clone NPK2_168
BIO9_D1			1137	8	AY664974	99%	Uncultured eukaryote clone SCM28C10
BIO9_E5			1140	24	EF527101	99%	Uncultured marine eukaryote clone SIF_1D12
BS1_G5			717	2	DQ504314	98%	Uncultured alveolate clone LC22_4EP_19
BS12_A3			1142	9	AY664983	98%	Uncultured eukaryote clone SCM38C1
BS12_B11			1136	4	AF022199	88%	*Lepidodinium viride*
BS12_G11			1137	3	AF022201	96%	*Pentapharsodinium tyrrhenicum*
BS13_A9			1129	7	AF022199	94%	*Lepidodinium viride*
BS13_B7			1140	21	AY664983	98%	Uncultured eukaryote clone SCM38C1
BS16_B9			1140	2	EF526903	99%	Uncultured marine eukaryote clone SA1_4C7
BS19_F5			1120	1	EF526792	89%	Uncultured marine eukaryote clone NA1_4G9
BS2_D11			953	4	AY664896	91%	Uncultured eukaryote clone SCM16C59
BS2_D6			1010	4	AY664983	97%	Uncultured eukaryote clone SCM38C1
BS23_A7			1136	2	AF022201	95%	*Pentapharsodinium tyrrhenicum*

Table S3

Name	KeyDNATools Results		Length	Redudancy	BLAST results		
	Rank 6	Rank 7			N°Accession	Identity	Description
BS25_A8			1138	1	AY664924	99%	Uncultured eukaryote clone SCM27C15
BIO7_B10			1341	1	DQ504322	93%	Uncultured diplonemid clone LC22_5EP_18
BIO9_B5			1344	1	DQ504322	95%	Uncultured diplonemid clone LC22_5EP_18
BS3_D8			1349	4	DQ504323	94%	Uncultured diplonemid clone LC22_5EP_19
BIO2_F12			1349	1	DQ504322	94%	Uncultured diplonemid clone LC22_5EP_18
BIO2_H4			1348	1	DQ504322	98%	Uncultured diplonemid clone LC22_5EP_18
BIO3_F4			1349	7	DQ504323	97%	Uncultured diplonemid clone LC22_5EP_19
BIO9_D4			1345	2	DQ504323	97%	Uncultured diplonemid clone LC22_5EP_19
BIO2_F3			1348	1	DQ504322	95%	Uncultured diplonemid clone LC22_5EP_18
BS3_D7			1350	5	DQ504323	95%	Uncultured diplonemid clone LC22_5EP_19
BIO7_E10			1349	3	DQ504322	96%	Uncultured diplonemid clone LC22_5EP_18
BS3_G10			1348	3	DQ504322	96%	Uncultured diplonemid clone LC22_5EP_18
BS13_H8			1331	1	DQ504349	90%	Uncultured diplonemid clone LC22_5EP_32
BS23_H10			1330	2	AY425009	87%	*Diplonema ambulator* strain ATCC 50223
BS23_B2			1325	1	DQ504323	93%	Uncultured diplonemid clone LC22_5EP_19
BS25_B2			1341	1	AY665087	90%	Uncultured eukaryote clone SCM15C6
BS25_H3			1336	1	DQ504322	94%	Uncultured diplonemid clone LC22_5EP_18
BIO1_H12			1319	6	DQ504323	95%	Uncultured diplonemid clone LC22_5EP_19
BIO3_A2			1343	7	DQ504323	95%	Uncultured diplonemid clone LC22_5EP_19
BS3_D4	Neobodo->63(54-1279)	designis->42(178-1279)	1331	9	AF174379	100%	*Bodo saliens*
BS11_C5			1314	1	AF530520	90%	Uncultured kinetoplastid clone AT4-56
BS17_G10			1156	3	AY749538	94%	Uncultured eukaryote clone H22.1
BS10_B6			1144	1	AY050178	92%	*Amastigomonas bermudensis*
BS12_D4			1144	1	AY050178	91%	*Amastigomonas bermudensis*
BS17_C3			989	5	AY050178	91%	*Amastigomonas bermudensis*
BS18_A2			1143	12	AY050178	92%	*Amastigomonas bermudensis*
BS19_B4			1144	1	AY050178	92%	*Amastigomonas bermudensis*
BS19_C3	Amastigomonas->2(66-1059)		1140	1	AY050178	95%	*Amastigomonas bermudensis*
GM1_E3			1144	2	AY050178	93%	*Amastigomonas bermudensis*
MO3_A10			1142	12	AY050178	93%	*Amastigomonas bermudensis*
MO4_A9			1049	9	AY050178	92%	*Amastigomonas bermudensis*
BS11_E4			1179	3	AY050180	75%	*Amastigomonas debruynei*
BS15_G1	Amastigomonas->11(100-895)	debruynei->11(100-895)	1071	4	AY050180	98%	*Amastigomonas debruynei*
BS24_B1	Amastigomonas->12(100-894)	debruynei->12(100-894)	1040	14	AY050180	96%	*Amastigomonas debruynei*
MO1_A3	Amastigomonas->2(538-1165)	debruynei->2(538-1165)	1204	6	AY050180	86%	*Amastigomonas debruynei*
BIO2_D4	Planomonas->7(105-766)		1110	2	AY882531	99%	Uncultured marine eukaryote clone p15SBG2
BS11_E7	Planomonas->8(127-1056)		1072	6	AY882531	99%	Uncultured marine eukaryote clone p15SBG2
BS12_F6	Planomonas->6(127-788)		1130	13	AY882531	98%	Uncultured marine eukaryote clone p15SBG2
BS15_F3	Planomonas->7(127-788)		1049	2	AY882531	99%	Uncultured marine eukaryote clone p15SBG2
BS17_G4	Planomonas->7(127-788)		1032	2	AY882531	99%	Uncultured marine eukaryote clone p15SBG2
BS18_H1	Planomonas->8(127-1060)		1132	3	AY882531	99%	Uncultured marine eukaryote clone p15SBG2
BS24_B7	Planomonas->6(127-787)		1129	1	AY882531	98%	Uncultured marine eukaryote clone p15SBG2
BS1_D5			1065	3	AY364851	99%	*Eurythoe complanata*
BS1_H8			931	1	EU340092	98%	*Aurospio dibranchiata* isolate KP278
BS1_G3			650	1	AM159578	99%	*Amphisamytha galapagensis*
BS2_D2			1115	1	EU082390	100%	*Neolepas zevinae* voucher KACb00420
BS4_C10			956	2	DQ080013	99%	*Gilia reticulata*
BS3_B5			1057	3	DQ080013	99%	*Gilia reticulata*
BS12_B6			1008	1	AF358064	90%	*Haliscera conica*
BS4_D6			953	1	AF293681	98%	Undescribed mertensiid sp. 3
BS1_F5			1022	73	AY649823	99%	*Bathymodiolus aff. thermophilus* WJ-2004
BS2_D12			1015	24	AY649823	99%	*Bathymodiolus aff. thermophilus* WJ-2004
BS23_E2			1294	5	AF308644	93%	*Niveotectura pallida*
BS25_G3			1295	3	AF308644	94%	*Niveotectura pallida*
BS11_E2			1077	3	AF308644	96%	*Niveotectura pallida*
BS2_B11			934	2	DQ093432	99%	*Lepetodrilus elevatus* voucher MCZ DNA100930
BS24_B2	Candida->11(163-1025)		1091	12	GQ120116	99%	Uncultured marine fungus clone FAS_2
BS12_A1			595	1	M83257	97%	*Chaetomium elatum*
BIO10_E7			1132	1	AB013526	99%	*Candida atmosphaerica* strain JCM 9549
BS24_D7			1137	1	AF236103	98%	*Penicillium commune*
BS19_E3			1138	3	DQ536480	98%	*Triparticalcar arcticum* isolate AFTOL-ID 696
BS11_B8			1076	1	DQ645524	98%	*Cystofilobasidium infirmominiatum* isolate AFTOL-ID 1888
BS18_H2			1140	3	DQ645524	99%	*Cystofilobasidium infirmominiatum* isolate AFTOL-ID 1888
BS16_H8			1142	3	GQ995438	77%	Uncultured *Chytridiomycota* clone T3P1AeE01
MO1_F5			1146	2	EF527154	99%	Uncultured marine eukaryote clone NA1_4H10
MO3_E8			1142	2	DQ092911	99%	*Pterula echo* isolate AFTOL-ID 711
BIO9_E9			1062	2	EF526766	88%	Uncultured marine eukaryote clone NIF_4A10
BS19_B5			1009	3	GQ995290	82%	Uncultured *Chytridiomycota* clone T5P2AeE09
BS4_B4			541	1	EU192367	98%	*Malassezia restricta* strain CBS 7877
BS11_B2			1162	1	DQ504353	98%	Uncultured cercozoan clone LC104_3EP_8
BS19_B6			1032	1	AY620300	85%	Uncultured cercozoan clone 13-2.7

Table S3

Name	KeyDNATools Results		Length	Redudancy	BLAST results		
	Rank 6	Rank 7			N°Accession	Identity	Description
MO2_A2			949	5	AF174370	83%	*Massisteria marina* strain GBB2
BS15_B5			1153	2	AF290540	92%	*Cryothecomonas longipes*
GM1_F4			1158	1	AF174370	95%	*Massisteria marina* strain GBB2
BS14_A7			1162	7	AY620345	99%	Uncultured cercozoan clone 7-2.3
BS10_B7			1162	15	AY620345	99%	Uncultured cercozoan clone 7-2.3
BS12_B4			1102	31	AF174370	96%	*Massisteria marina* strain GBB2
BIO2_A5			1154	2	AB275050	98%	Uncultured eukaryote clone: DSGM-50
BS10_B9			1052	11	AY620257	97%	Uncultured cercozoan clone E17
BS11_E8	Massisteria->4(237-1092)	marina->4(237-1092)	1159	5	AF174370	99%	*Massisteria marina* strain GBB2
BS24_H5	Massisteria->2(239-1094)	marina->2(239-1094)	1161	2	AF174370	98%	*Massisteria marina* strain GBB2
BS13_H1	Massisteria->4(238-1093)	marina->4(238-1093)	1160	7	AF174370	99%	*Massisteria marina* strain GBB2
BS15_A5	Massisteria->4(238-1075)	marina->4(238-1075)	1158	2	AF174373	99%	*Massisteria marina* strain CAS1
BS17_A3	Massisteria->4(238-1093)	marina->4(238-1093)	1160	12	AF174370	99%	*Massisteria marina* strain GBB2
BS19_A3	Massisteria->3(238-1093)	marina->3(238-1093)	1160	10	AF174370	99%	*Massisteria marina* strain GBB2
BIO4_F7	Massisteria->4(238-1092)	marina->4(238-1092)	1159	2	AF174370	99%	*Massisteria marina* strain GBB2
BS18_G1			1159	16	AY620257	99%	Uncultured cercozoan clone E17
BS14_G12			1159	11	AY620258	99%	Uncultured cercozoan clone E5
BIO7_A1			1147	1	FJ824126	87%	*Cercozoa* sp. CC-2009a
MO4_C2			671	1	DQ303923	90%	*Ebria tripartita* isolate 2
BIO3_F9			1150	1	AF063239	93%	Arthracanthid 206
BIO10_G12			1140	75	EU287798	98%	Uncultured marine Polycystinea clone OLI011-75m.62
BIO9_H1			1139	34	EU287798	97%	Uncultured marine Polycystinea clone OLI011-75m.62
BIO2_B8			1139	2	EU287798	97%	Uncultured marine Polycystinea clone OLI011-75m.62
BIO3_A1			1140	4	EU287798	97%	Uncultured marine Polycystinea clone OLI011-75m.62
BIO1_D11			1131	3	DQ504355	96%	Uncultured taxopodid-like clone LC22_5EP_23
BS24_F5			1127	2	EU287795	85%	Uncultured marine Polycystinea clone OLI011-75m.50
BIO3_C6			1135	8	AY266295	86%	*Collozoum inerme*
BIO4_D2			1170	4	EU287798	91%	Uncultured marine Polycystinea clone OLI011-75m.62
BIO5_A3			1140	17	AF530524	91%	Uncultured Polycystinea clone AT8-54
BIO7_H1			1138	1	AB246697	92%	*Pterocorys zancleus* isolate 8030
BS16_E9			1140	2	AY665072	99%	Uncultured eukaryote clone SCM27C26
BIO7_C8			1138	2	DQ314831	99%	Uncultured marine eukaryote clone NW414.03
BIO4_E6			1128	1	DQ504355	96%	Uncultured taxopodid-like clone LC22_5EP_23
BIO3_C3			1134	2	DQ504355	98%	Uncultured taxopodid-like clone LC22_5EP_23
BIO9_D8			1132	4	AF530524	94%	Uncultured Polycystinea clone AT8-54
BIO9_A12			1137	2	EF172806	99%	Uncultured eukaryote clone SSRPB27
BIO2_A7			1132	4	AF530524	94%	Uncultured Polycystinea clone AT8-54
BIO5_D5			1149	52	EU287798	94%	Uncultured marine Polycystinea clone OLI011-75m.62
BIO7_D2			1140	33	EU287798	96%	Uncultured marine Polycystinea clone OLI011-75m.62
BIO10_D6			1134	1	DQ314831	98%	Uncultured marine eukaryote clone NW414.03
BS12_C1			1143	18	EU647131	99%	Uncultured streptophyte clone D0810_21_M
MO3_C2			1137	2	AC215459	98%	*Solanum lycopersicum* clone C02SLe0089P21
BS13_A6			1012	12	AF008957	90%	*Luffa quinquefida*
BS11_D4			1169	2	EF023700	78%	Uncultured Closteriaceae clone Amb_18S_970
MO3_G3			908	42	EU143994	73%	Uncultured kathablepharid clone GHB30.9
MO4_C3			904	51	EU143994	73%	Uncultured kathablepharid clone GHB30.9

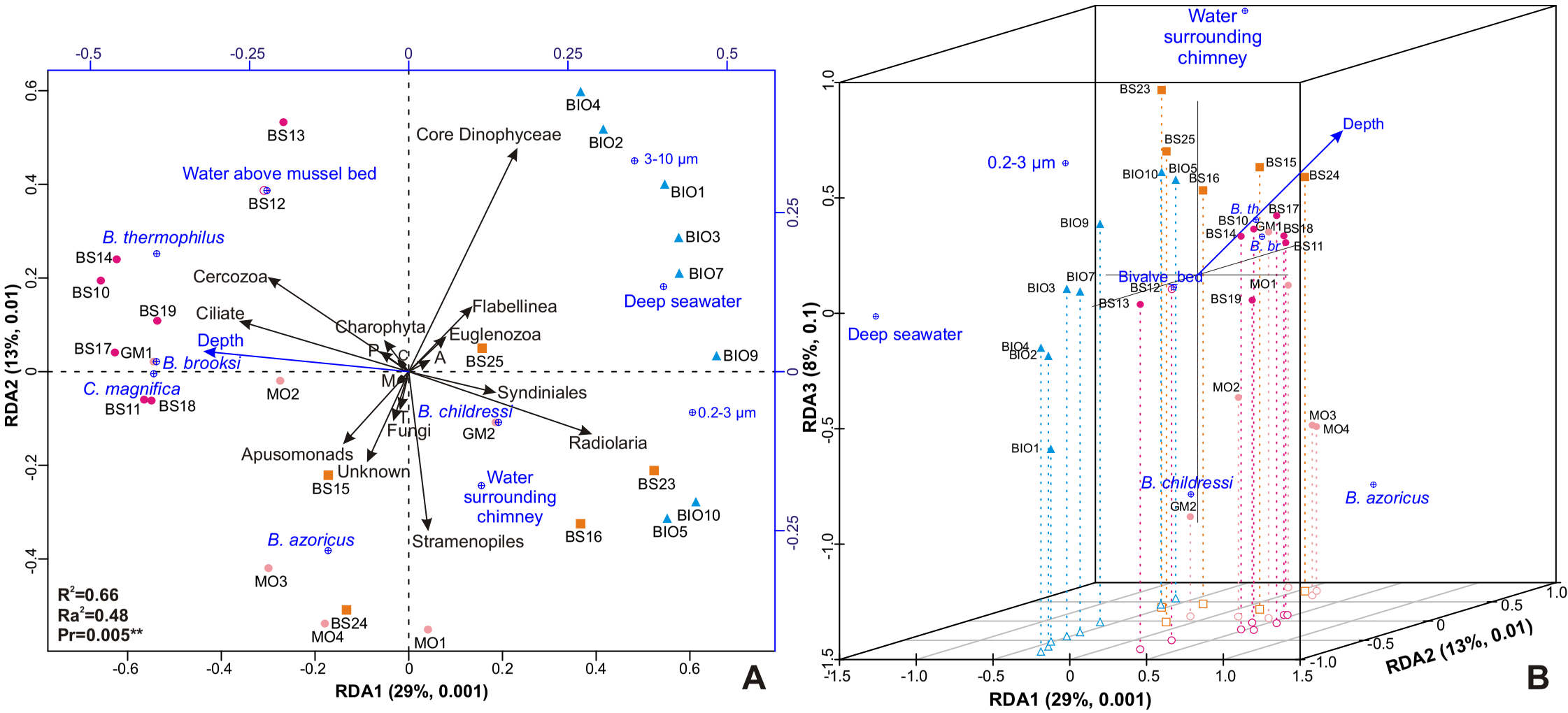

Fig. S1: Triplot redundancy analysis (RDA) integrating taxonomic groups, environmental locations and genetic libraries from the Pacific and Atlantic Oceans. **A.** The depth and taxonomic groups (continuous variables; A= Apicomplexa, C=Centroheliozoa, M=Metazoa, P=Planomonas, T=Tubulinea) are shown as arrows. The vector orientations represent the direction of strongest change; vector lengths correspond to the relative importance. The sample types are illustrated by grey crossed circles. Genetic libraries are represented by solid symbols (triangle for deep seawater, square for water surrounding chimneys and circle for pallial cavity liquid in bivalves) and an empty circle represents water above mussel bed library. The percentage of variability explained by the two axes (RDA1 and RDA2) and the p-value are reported in brackets. R^2: R-squared, Ra^2: adjusted R-squared and Pr: p-value of the model. **B.** Three dimensional plot of the same dataset which allow showing the third axis (RDA3). Binary variables (sample types) that were collinear are not represented on the plot. Genetic libraries represented by solid symbols are plotted using "h" type, so their xy locations can see more clearly (empty symbols).

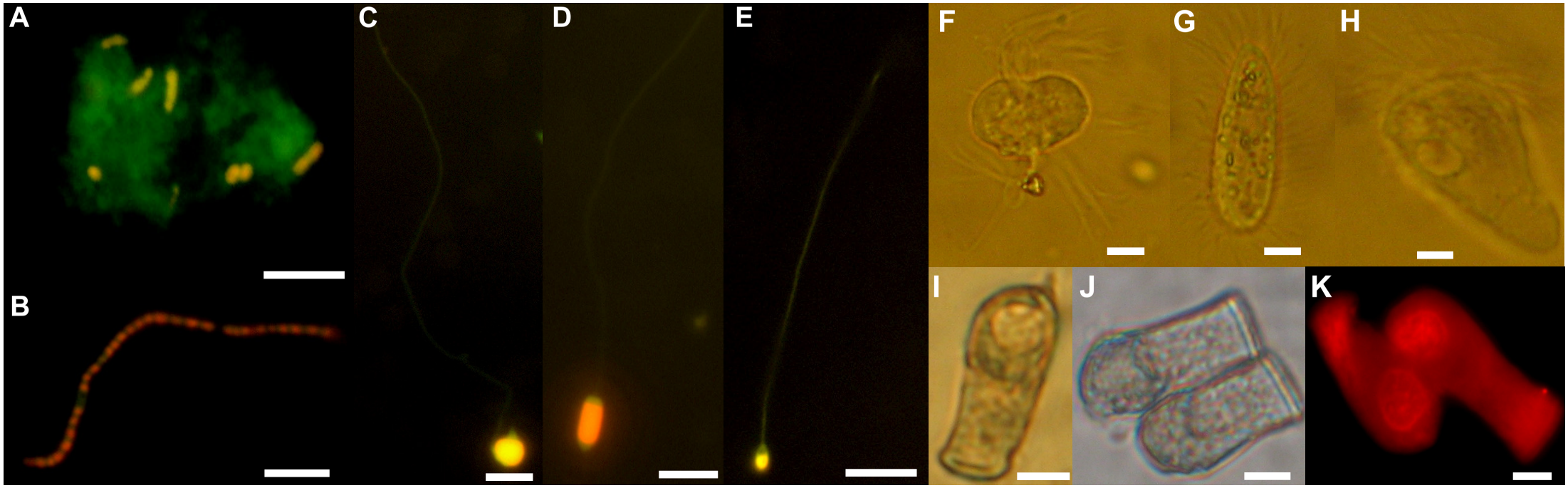

Fig. S2 Microscopic observations of aggregates **(A)** or long chain colonies of procaryotes **(B)**, and spermatozoa-like cells with various shapes and sizes **(C-E)** fixed with glutaraldehyde, concentrated by filtration and stained with propidium iodide, collected from water above bivalve bed (site Oasis). Scale bars correspond to 5 µm. Microscopic observations of cells from hydrothermal bivalves **(F)** from *C. magnifica*, Oasis, **(G-H)** from *B. thermophilus*, Lucky Eric. Scale bars correspond to 2 µm. **(I-J)** Cell fixed with Lugol from *B. thermophilus*, Rehu Marka. **(K)** Same organism stained with propidium iodide. Scale bars correspond to 5 µm.

Fig. S3 Bayesian analysis of diplonemids and kinetoplastids environmental 18S rRNA gene sequences retrieved from different samples in the study. Study sequences are in bold. Each phylotype from each clone library is represented by one sequence with ≥95% similarity grouping. Posterior probability of Bayesian method and Neighbour-Joining and Maximum Parsimony bootstrap values higher than 60% are shown at nodes. Outgroup sequences were not shown but composed of seven Euglenida sequences (U84733, AJ532400, AF119118, AF090870, AJ532437, AJ532442 and AF283309). The number of clones of each representative sequence is indicated in exponent. Triangles symbolize deep seawater, squares water surrounding chimney and circle pallial cavity liquid in bivalves.

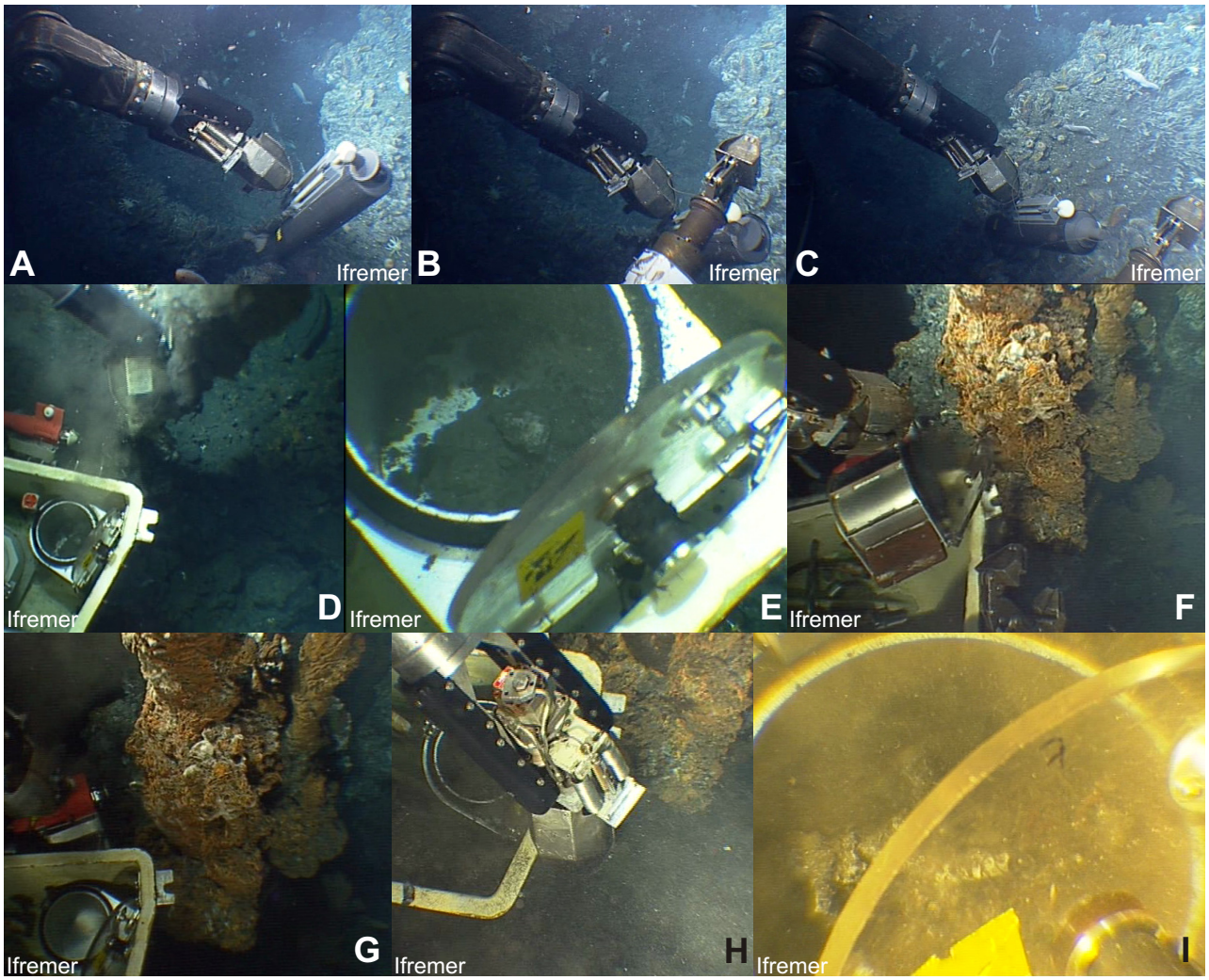

Fig. S4 Sampling of seawater above mussel bed and water surrounding chimney.
Seawater sampled at Oasis site, using a Niskin 8L bottle manipulated by the manned submersible Nautile. **A**. Opening, immersion, **B/C**. and closure of the Niskin bottle. Hydrothermal rocks and water surrounding chimney in hermetic container sampled at Hobbs site **(D/E)** and Sarah' Spring site **(F-I)**. **F**. The hermetic container manipulated by the arm was previously filled with sterilized freshwater what created the moiré effect. All the photos are the property of IFREMER.

4.2 Tripartite interactions between Cirratulidae (Polychaeta), *Durchoniella* (Ciliophora, Astomatida), and *Bacteria* : A « Russian Doll » complex in anoxic coastal environments

PROTIST
(SOUMIS)

Anne-Laure Sauvadet, Sophie Le Panse, Erwan G. Roussel, Estelle M-C. Bigeard, Joseph Schrével, and Laure Guillou

Les polychètes qui font partis des Annélides sont des animaux essentiellement marins et sont retrouvés dans différents environnements. Les Cirratulidae sont une famille de polychètes dépositivores que l'on retrouve dans les systèmes sédimentaires côtiers. Ces vers captent les particules alimentaires à la surface du sédiment grâce à leur paire de palpes cannelées et leurs nombreux filaments tentaculaires. Ceux sont des vers lents, qui se déplacent peu, et leur corps est enterré au-dessous de la surface du sédiment dans les 15 premiers centimètres. Seules leurs longues branchies et leurs nombreux filaments tentaculaires sortent de la couche sédimentaire. Ces vers ont un corps épais, demi-cylindrique, à segments très courts et très serrés, ils peuvent atteindre 20 cm avec 300 segments. Ils ont une coloration jaune-orange, brun-rougeâtre ou vert-bronze, et leurs branchies et les tentacules sont d'une couleur rouge-sang (Day, 1967).

La distribution des Cirratulidae est mondiale ; ils ont été reportés dans les océans Pacifique, Indien, Atlantique, en mer Méditerranée et mer Noire, en Manche, en mer du Nord, à Skagerrak et Kattegat (Pettibone, 1982). Deux espèces de vers Cirratulidae sont retrouvées le long des côtes européennes : *Cirratulus cirratus* Müller et *Cirriformia tentaculata* Montagu (anciennement *Audouinia tentaculata*).

Ces polychètes sont retrouvés dans divers environnements, sous des roches ou dans un sédiment très grossier avec de nombreux cailloux, jusqu'à des sédiments noir et glaiseux. Ces sédiments sont souvent riches en sulfure d'hydrogène et constituent donc un environnement contenant très peu d'oxygène. La provision en oxygène est entretenue grâce aux filaments branchiaux qui s'étendent à la surface du sédiment. Les polychètes font également face aux périodes de marée qui induisent une hypoxie. De plus, en laissant ses branchies à l'extérieur,

ils sont soumis à une prédation surtout en basse mer ; ils s'enfoncent donc plus profondément et rétracte leurs branchies (Bestwick *et al.*, 1989). Ils se retrouvent donc en totale anaérobiose. Le métabolisme énergétique anaérobie est amorcé à une pO_2 inférieure à 30 % de saturation, et génère notamment l'accumulation de succinate, d'alanine et d'acides gras volatiles.

Ces Cirratulidae ont également été observés depuis la fin du 19^e siècle, en raison de la présence dans leur tube digestif d'un genre particulier de ciliés endocommensaux, les Astomes. Ces ciliés sont retrouvés principalement dans le tractus digestif des annélides et chez quelques batraciens (Table 4.1). Ils sont caractérisés par l'absence de bouche (d'où leur nom) et se fixent donc aux tissus de leur hôte via des zones définies appelées thigmotactiques et/ou des appareils squelettiques de type crochet ou ventouse (Figure 2.11). Ils ont été observés pour la première fois en 1788, mais c'est en 1910 que Cépède développe ses recherches principalement sur leur habitat et leur anatomie (Cépède, 1910). Dans les années 1950, De Puytorac consacre également ces travaux aux astomes, et précise notamment un genre d'astome retrouvé chez les Cirratulidae, les *Durchoniella* (De Puytorac, 1954). Cependant, les études sur les astomes restent très anatomiques, portant surtout pas la suite sur l'analyse de leur teneur en ADN (nombre de micronoyaux, de macronoyaux...). Leur cycle de développement et leur transmission reste, à ce jour, indéterminés.

Ordre	Famille	Genre	Hôte
Anoplophryida	Anoplophryidae		**Oligochètes terricoles**
		Almophrya	Glossoscolecidae
		Anoplophrya	Lumbricidae, Glossoscolecidae, Megascolecidae
		Corlissiella	Glossoscolecidae
		Lomiella	Megascolecidae
		Lubetiella	Octochaetidae
		Sigmophrya	Megascolecidae
	Buetschliellidae		**Polychètes limicoles**
		Anoplophryopsis	Eunicidae
		Butschliella	Opheliidae
		Herpinella	Cirratulidae
		Rhizocaryum	Spionidae
Haptophryida	Clausilocolidae		**Gastropodes et oligochètes**
		Clausilocola	Clausiliidae
		Haptophryopsis	Megascolecidae
		Proclausilocola	Clausiliidae
	Haptophryidae		
		Annelophrya	Triclades
		Cepedietta	Batraciens
		Haptophrya	Planaires d'eau douce
		Lachmannella	Planaires d'eau douce et marine
		Steinella	Planaires d'eau douce et marine
Hoplitophryida	Hoplitophryidae		**Oligochètes limicoles principalement**
		Akidodes	Enchytraeidae
		Buetschliellopsis	Enchytraeidae
		Delphyella	Enchytraeidae
		Hoplitophrya	Lumbriculidae, Enchytraeidae, Eudrilidae, Glossoscolecidae, Tubificidae
		Jirovecella	Enchytraeidae
		Juxtamesnilella	Lumbriculidae essentiellement
		Juxtaradiophrya	Lumbriculidae, Enchytraeidae, Naididae, Tubificidae
		Mesnilella	Lumbriculidae, Enchytraeidae, Naididae, Tubificidae
		Mixtophrya	Naididae
		Protoradiophryopsis	Glossoscolecidae
		Radiophryoides	Naidinae
	Contophryidae		**Oligochètes terricoles**
		Contophrya	Glossoscolecidae
		Dicontophrya	Glossoscolecidae
	Intoshellinidae		**Oligochètes et polychète limicoles**
		Intoshellina	Tubificidae
		Monodontophrya	Tubificidae
		Spirobuetschliella	Serpulidae
	Maupasellidae		**Oligochètes terricoles et hirudinées**
		Acanthophrya	Hirudinées
		Buchneriella	Glossoscolecidae, Megascolecidae
		Georgevitchiella	Lumbricidae
		Maupasella	Lumbricidae
	Radiophryidae		**Polychètes limicoles et terricoles**
		Acanthodiophrya	Glossoscolecidae
		Anthonyella	Lumbriculidae,
		Cheissinophrya	Glossoscolecidae
		Coelophrya	Glossoscolecidae
		Desmophrya	Lamellibranches
		Dicoelophrya	Glossoscolecidae
		Durchoniella	Polychètes Cirratulidae
		Eudrilophrya	Eudrilinae
		Helella	Tubificidae
		Hovasseiella	Polychètes Cirratulidae
		Metaracoelophrya	Glossoscolecidae
		Metaradiophrya	Megascolecidae, Lumbriculidae, Glossoscolecidae
		Mimophrya	Megascolecidae, Eudrilidae
		Mrazekiella	Tubificidae
		Ochridanus	Tubificidae
		Paracoelophrya	Glossoscolecidae
		Radiophrya	Enchytraeidae, Naididae, Tubificidae
		Radiophryopsis	Tubificidae

TABLE 4.1 – Phylogenie des Astomes et leurs hôtes (d'après De Puytorac, 1994).

TRIPARTITE INTERACTIONS BETWEEN CIRRATULIDAE (POLYCHAETA), *DURCHONIELLA* (CILIOPHORA, ASTOMATIDA), AND *BACTERIA* : A « RUSSIAN DOLL » COMPLEX IN ANOXIC COASTAL ENVIRONMENTS

Anne-Laure Sauvadet[a,b], Sophie Le Panse[c], Erwan G. Roussel[d], Estelle M-C. Bigeard[a,b], Joseph Schrével[e], and Laure Guillou[a,b]

[a]CNRS, UMR 7144, Groupe Plancton Océanique, Station Biologique de Roscoff, 29682 Roscoff, France.
[b]UPMC - Université Paris 06, Laboratoire Adaptation et Diversité en Milieu Marin, 29682 Roscoff, France.
[c]CNRS, FR 2424, Service Informatique et Génomique, Station Biologique de Roscoff, 29682 Roscoff, France.
[d]School of Earth and Ocean Sciences, Cardiff University, Main Building, Park Place, Cardiff, Wales, UK.
[e]Muséum National d'Histoire Naturelle, Département RDDM, CNRS FRE 3206, CP 52, 61 Rue Buffon, 75231 Paris Cedex 05, France.

Corresponding author. E-mail : sauvadet@sb-roscoff.fr ; Tel. (+33) 2 98 29 23 23 ; Fax (+33) 2 98 29 23 24.

Abstract

An update of the taxonomic description of two endocommensal astome ciliates, *Durchoniella brasili* De Puytorac, 1954 and *Durchoniella legeri-duboscqui* De Puytorac, 1954 was performed by combining morphological observations (live, stained, scanning, and transmission electron microscopy) with molecular genetic analyses. These astomes were retrieved from the middle intestine of a marine cirratulid worm, *Cirriformia tentaculata*, collected in the bay of Roscoff (Northwest French coast). This study supports the initial diagnosis performed by De Puytorac (1954). The apical apparatus, usually used by astomes to attach to their host, was observed by scanning electron microscopy offering new details of this characteristic structure. Reproduction of astomes occurred by binary fission, although a rarely described conjugation event was also observed for *Durchoniella brasili*. The 18S rRNA gene of both species showed 98% identity and phylogenetic analyses positioned these *Durchoniella* species at the base of Scuticociliatia (Oligohymenophorea), with *Anoplophrya marylandensis*, an astome from a lumbricid annelid. Moreover, ultrastructural examination by transmission electron microscopy and fluorescence *in situ* hybridization analysis revealed the presence of living endocytoplasmic cocci and rod-shaped bacteria that were not associated with any of the host organites or specialized structures. In both species of astomes, genetic libraries based on bacterial 16S rRNA genes were dominated by Chlamydiae and *Epsilon-Proteobacteria*.

Introduction

Unicellular ciliates are genetically and morphologically diverse (∼8000 described species) (Lynn, 2008). These microorganisms are mainly characterized by two nuclei, the micro- and macronucleus, and the presence of cilia at least once during their life cycle. Their diversification probably allowed their adaptation to various environments, such as soil, freshwater, and marine habitats. Ciliates are mainly free-living predators that generally have an oral cavity used during phagotrophy, although ciliates can also perform pinocytosis and cell surface absorption (Corliss, 1979). Most of the ciliates that live in association with metazoans are mutualists, although parasitic interactions were also clearly established for some species (e.g. Ewing and Kocan, 1992). Moreover, ciliates hosting prokaryotes are widespread associations (for review, Fokin, 2004; Görtz, 2001), which seem to be frequent in environments such as hydrothermal vents (Kouris *et al.*, 2007) or anoxic environments (for e.g. Ott *et al.*, 2005). Prokaryotic symbionts could play a nutritive or defensive role, or could also be used to modify the environmental conditions (for review, Gast *et al.*, 2009).

Astomes, a particular group of ciliates belonging to the Subclass Astomatia, Order Astomatida Schewiakoff, 1896 are endocommensals living in association with annelids and batrachians. No free-living astome specimens have been described so far. Cells are all mouthless, and a thigmotactic field or specialized structures in the form of hooks, spines or suckers are used

to attach to the tissue of their host. As astomes do not seem to damage the host's tissues, it has been suggested that the intestine of their host could represent a stable ecological niche that concentrates specific nutrients (De Puytorac, 1954). However, their ecology is still poorly understood. Observed for the first time in 1788, it is only at the beginning of the 20th century that the anatomy and habitat of astomes were described (Cépède, 1910; De Puytorac, 1954). Based upon morphological criteria, astomes are commonly placed within the Oligohymenophorea (De Puytorac, 1954; De Puytorac *et al.*, 1979). This statement was recently confirmed by the analysis of a 18S rRNA sequence of *Anoplophrya marylandensis* retrieved from the intestine of a lumbricid annelid (Affa'a *et al.*, 2004).

Durchoniella brasili and *Durchoniella legeri-duboscqui*, two species of astomes first described by De Puytorac (1954), are only found in the intestine of *Cirriformia tentaculata* Montagu, a cirratulid polychaete widespread over European coasts (previously *Audouinia tentaculata*). This marine worm is commonly found in sediments characterized by reducing conditions and sometimes by a strong hydrogen sulphide odour (George, 1964). *Cirriformia tentaculata* is found in the upper sediment layers and traps food particles falling onto the sediment surface with its tentacular filaments (Fauvel, 1927). These filaments are also used to acquire oxygen, exposing these worms to predation during low tide. Hence, worms bury themself deeply into the sediment retracting their tentacles, and therefore encountering chronic anoxic conditions (Bestwick *et al.*, 1989).

The aim of the present study, based on De Puytorac's initial work (1954), was to give a comprehensive description of the *Durchoniella* species retrieved from the intestine of *Cirriformia tentaculata* by combining microscopical and molecular genetic analyses. Furthermore, as endocytoplasmic bacteria were detected in these astomes, an additional level of association in this particular marine model has been also described.

Results

Distribution of *Cirriformia tentaculata* and *Durchoniella* spp.

Cirriformia tentaculata specimens (Figure 4.1A) were retrieved from anoxic sediments between 3 to 15 cm depth. The sediments were characterized by a black colour (Figure 4.1B) and sometimes a strong hydrogen sulphide odour. Six samples (20 grams each) from the sediments and the interstitial water surrounding the worms were analysed by microscopy. Amoebas, copepods, eggs resembling those retrieved from *Cirriformia*, as well as unicellular cells were observed in the interstitial waters and in the sediment matrix. However, no *Durchoniella* cells were identified.

When worms were dissected in small pieces and then individually observed, both *Durchoniella brasili* and *Durchoniella legeri-duboscqui* were found in the middle region of worm intestine. Direct fixation of the astomes to host tissues was not observed in our preparations as *Durchoniella* spp. were always located outside the gut content (Figure 4.1C and D). Af-

ter dissection, the majority of *Durchoniella* swam vigorously in the surrounding seawater, *D. legeri-duboscqui* revolving faster than *D. brasili*. However, some cells remained stuck in a viscous gel-like liquid. In order to try and maintain astomes in culture, different conditions were tested by incubating the isolated astomes in sterile seawater, rice medium (see Roscoff Culture Collection website), and F/2 medium (Guillard, 1975). The addition of worm extracts (50 mg of tissue boiled in 40 mL of seawater for 3 hours) to the different media was also tested. All cultures were performed in triplicates and incubated in the dark at 15°C. *Durchoniella brasili* and *D. legeri-duboscqui* stopped swimming as soon as they were transferred to the F/2 medium. Although seawater without worm extract was the only condition where living astomes could be observed after 24 hours, they could only be maintained for a maximum of four days in this condition.

Distribution of astomes assessed on 34 *Cirriformia tentaculata* collected from June to September 2009 showed : 4 worms without any astome, 16 containing only *D. brasili*, 13 with both species, and one with only *D. legeri-duboscqui*. *Durchoniella brasili* represented 88% (n=5752) of the total number of astomes counted (n=6547). On average, the intestine of *C. tentaculata* harboured 150 astomes and has 3.5 times more *D. brasili* than *D. legeri-duboscqui*.

Morphology and ultrastructure of *Durchoniella brasili* and *Durchoniella legeri-duboscqui*

Durchoniella brasili and *Durchoniella legeri-duboscqui* cells were observed using light and electronic microscopy (Figure 4.2 to 4.5). Although intracellular infrastructures of both species of *Durchoniella* were similar, they could be differentiated by their general morphology. *Durchoniella brasili* was characterized by a flat body truncated at the posterior end (Figure 4.2A and C), that was on average twice as long as it was wide, and ranged in vivo from 75 to 170 μm length by 29 to 103 μm width (n=34). The shape of the cell was slightly concave on its ventral part (Figure 4.2B). By contrast, cells of *D. legeri-duboscqui* were more elongated, 2-3 times longer than *D. brasili* (Figure 4.2E-G) ; ranging *in vivo* from 230 to 400 μm length and 68 to 183 μm width (n=21).

The cytoplasm was colourless, containing numerous refracting globules notably in *D. brasili* (Figure 4.2C), suggesting lipid granules as they were also easily observable with a vital Cresyl Blue staining (Figure 4.3G). In *D. brasili*, two to six contractile vacuoles could be observed using a Neutral Red coloration (Figure 4.2D), whereas *D. legeri-duboscqui* had six to fifteen contractile vacuoles (Figure 4.2H). In both cells, vacuoles were distributed in a single row always positioned on the opposite side to the micronucleus (Figure 4.2C and D).

Both species were holotrichous (Figure 4.2), and had cilia that were on average 10 μm long. At the extreme anterior part of both species, a non-ciliated zone harboured a skeletal apparatus on the cell surface (Figure 4.3). The apparatus of *D. brasili* was hardly observable among the cilia on the scanning electron microscopy (SEM) micrographs (Figure 4.3A and B), whereas the apical point of *D. legeri-duboscqui* was more prominent and pyramid-shaped, with at least

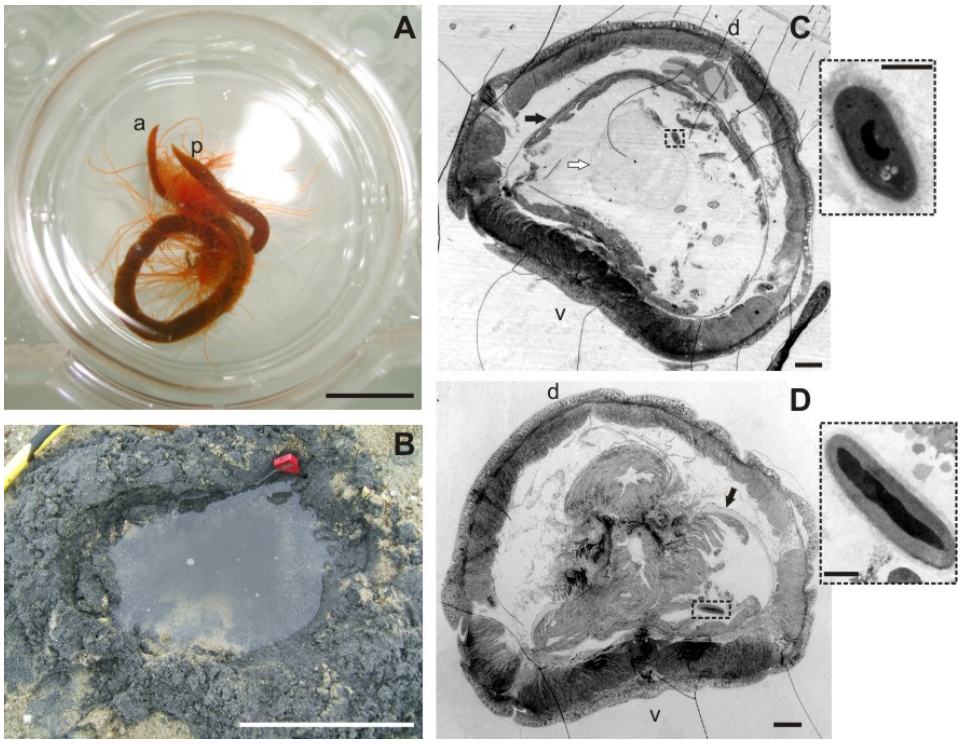

FIG. 4.1 – Localisation of *Durchoniella* species from *Cirriformia tentaculata*. **(A)** *Cirriformia tentaculata*, with « a », the anterior part and « p », the posterior part. Scale bar = 1 cm. **(B)** Example of a sampling zone at Roscoff. Scale bar = 25 cm. **(C-D)** Semi-thin sections of *C. tentaculata* with dorsal (d) and ventral (v) sides, limits of digestive tract and gut content are represented by arrows respectively full and empty. Scale bars = 100 μm. *Durchoniella brasili* (top) and *D. legeri-duboscqui* (bottom) were enlarged in parts of semi-thin sections. Scale bars = 20 μm.

two visible edges (Figure 4.3C and D). Transmission electron (TEM) micrographs revealed more details : the apical structure had a massive base inserted in the cytoplasm (example of *D. brasili*, Figure 4.3E). Numerous electron dense bodies were distributed around the apical base (Figure 4.3E and Figure 4.5A). In both species, Cresyl Blue coloration clearly revealed the edges of an apical point (Figure 4.3F-I). The base of the apical structure was a truncated cone, between 10-20 μm diameter at the base and 5-7 μm high, which displayed an homogeneous slightly fibrous substructure (Figure 4.3F-I). The different kineties clearly observable on *D. brasili* (Figure 4.3F) and *D. legeri-duboscqui* (Figure 4.3H), converged at this apical apparatus.

The two typical nuclei of ciliates were observed in both *D. brasili* and *D. legeri-duboscqui*. The micronucleus, always located close to the macronucleus in the middle of the cell, was ovoid to fusiform shaped (Figure 4.4A-C) and was 9-20 μm long and 3-9 μm wide in *D. brasili*(n=15), and 17-25 μm long by 7-11 μm wide in *D. legeri-duboscqui* (n=8). The fusiform micronucleus was due to a division stage, characterized by filamentous structures corresponding to chromosomes (Figure 4.4A-C). The macronucleus had a gutter-like shape, more irregular in *D.*

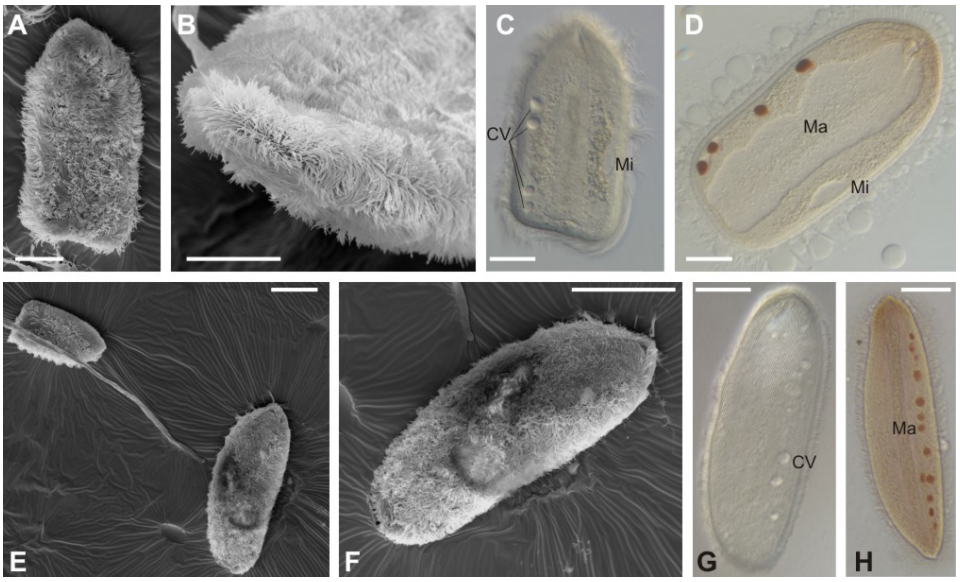

FIG. 4.2 – Scanning electron (SEM) and light microscopy micrographs of *Durchoniella brasili* (**A-D**, scale bars = 20 μm), *Durchoniella legeri-duboscqui* (**F-H**, scale bars = 50 μm) and both (**E**, scale bar = 50 μm) from *Cirriformia tentaculata*. **(A-B-E-F)** SEM views. **(C-D-G-H)** DIC Nomarski observation of live cells. Mi : Micronucleus, Ma : Macronucleus, CV : Contractile Vacuoles, with Neutral Red colouring (D and H).

legeri-duboscqui, extending along the total length of both cells, without however reaching the extremities (Figure 4.4A and B). The size of the macronucleus was 65-144 μm long by 10-33 μm wide in *D. brasili* (n=39) and 90-340 μm by 49-97 μm in *D. legeri-duboscqui* (n=12). The macronucleus could only be partially observed using TEM, as only sections were visible presenting large rounded chromatin granules randomly distributed, and numerous smaller chromatin granules (Figure 4.4D). In *Durchoniella* species, vegetative divisions took place by binary fission (e.g. *D. brasili*, Figure 4.4E and F ; *D. legeri-duboscqui*, Figure 4.4G and H). As the fission furrow was perpendicular to the cell axis, and equatorial, the division process ended in two cells of equivalent size (Figure 4.4E and G).

Conjugation process was only observed in *D. brasili* (Figure 4.4I-N). This rarely described event was only observed once overall *Durchoniella* cells analysed for more than a year. Both pairing cells, always motile, were of equal size and attached at the same level by their apical extremities, forming an acute angle (Figure 4.4I-K). Four micronuclei per cell were identified by DAPI coloration (Figure 4.4L-N), and grouped together in the cell on the left of Figure 4.4L (zoom on Figure 4.4M). By contrast, in the cell on the right of Figure 4.4L (zoom on Figure 4.4N), micronuclei were more disseminated, in particular with one of them located close to the apical extremity of the cell. In both cells, one of the four micronuclei was more diffuse, probably reflecting deterioration (Figure 4.4L-N).

Different shapes of contractile vacuoles were identified by TEM (Figure 4.5B and C). One

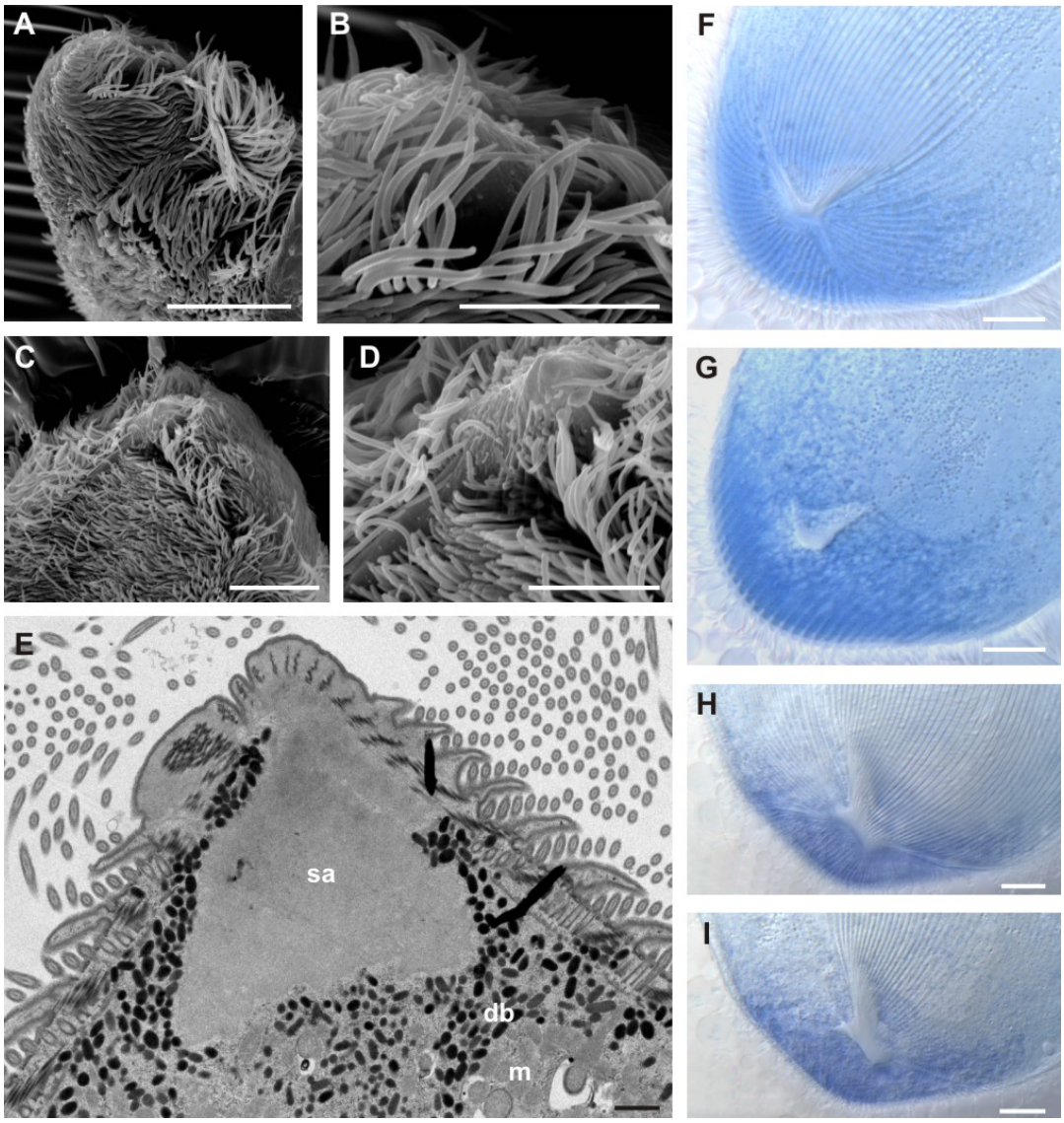

FIG. 4.3 – Scanning electron, transmission electron (TEM), and light microscopy micrographs of the apical structure of *Durchoniella brasili* (A, B, E, F, G) and *Durchoniella legeri-duboscqui* (C, D, H, I). **(A)** SEM of anterior part of *D. brasili*, with a zoom on the skeletal apparatus **(B)**. **(C)** SEM of anterior part of *D. legeri-duboscqui*, with a zoom on the skeletal apparatus **(D)**, scale bars A-C = 10 μm, scale bars B-D = 5 μm. **(E)** TEM of the apical structure of *D. brasili*, sa : apical structure, db : dense body, m : mitochondria, scale bar = 1 μm. **(F-G)** DIC Nomarski observations of the apical structure after a Cresyl Blue staining of *D. brasili* and **(H-I)** *D. legeri-duboscqui* cells, scale bars = 10 μm.

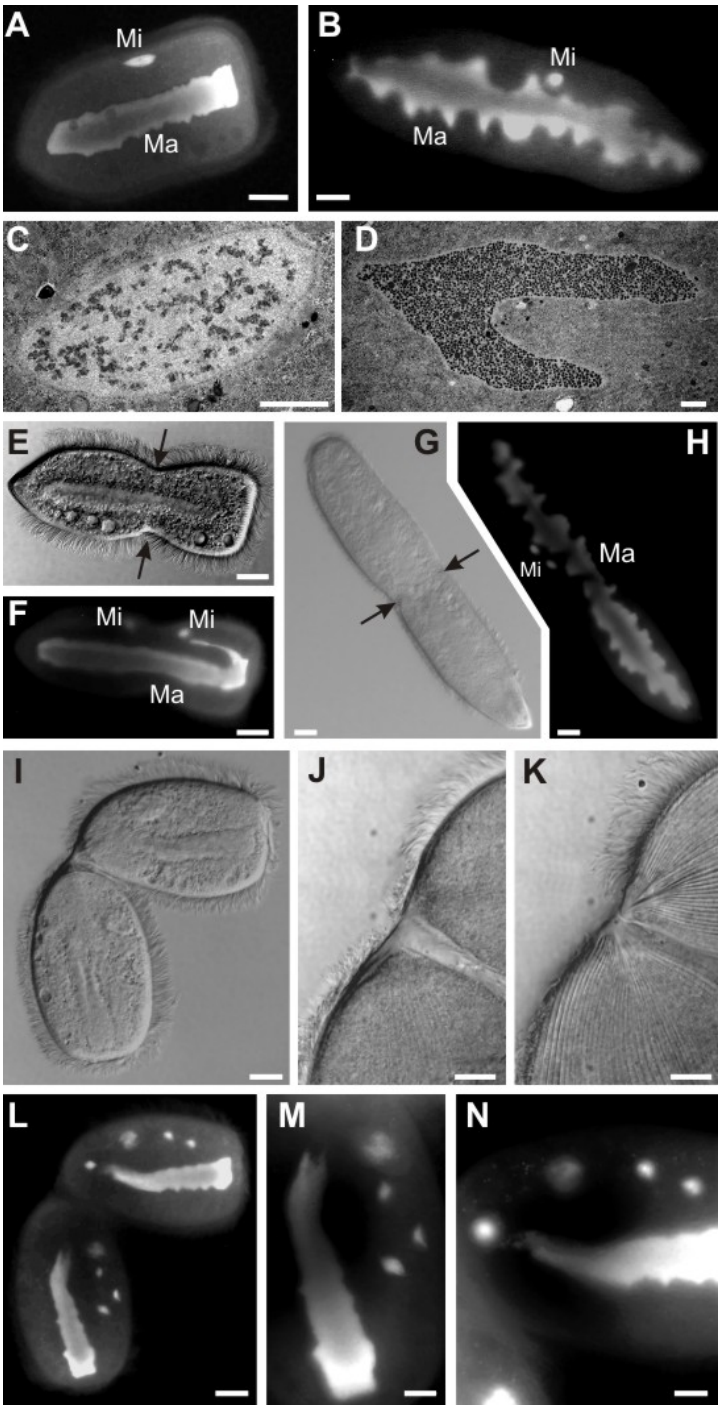

FIG. 4.4 – Nuclear content of *Durchoniella brasili* **(A)** and *Durchoniella legeri-duboscqui* **(B)** fixed cells stained with DAPI, Mi : Micronucleus, Ma : Macronucleus. TEM micrographs of micronucleus **(C)** and a section of macronucleus **(D)**, scale bars = 2 μm. Binary fission for *D. brasili* **(E-F)** and *D. legeri-duboscqui* **(G-H)**, arrows corresponding to the plane of constriction. Scale bars = 20 μm. Conjugation process of *D. brasili* **(I-N)**, with zoom on adhesion part **(J-K)**, and nuclear content of the two conjugating cells **(M-N)**, scale bars I-L = 20 μm, J-K-M-N = 10 μm.

type of vacuole was circular and surmounted by a channel with numerous emerging radiant fibres (Figure 4.5B), while another type was characterized by a discharge canal inserted in the membrane (Figure 4.5C).

The structure of cilia and insertion pattern in the membrane of *Durchoniella* were arranged, as for ciliates, having nine doublets of microtubules surrounding a central pair (Figure 4.5D and E). Somatic dikineties bordered by dense ectoplasmic fibres were inserted in a narrow furrow (4.5 μm deep) down to the myoneme-like fibres on the two-thirds basal part of the cell (Figure 4.5F), whereas only monokinety was observable near the apical point (Figure 4.5G). Microscopic observations using Cresyl Blue staining also showed these numerous longitudinal furrows ($\sim$70 per cell) at the surface of the cell (Figure 4.3F). Mitochondria, homogeneously distributed in the whole cell, were ovoid to elongate. TEM micrographs also revealed numerous dense bodies in the cytoplasm usually found near the apical apparatus or cell membrane (Figure 4.3E, Figure 4.5A and H).

Phylogenetic analysis of *Durchoniella brasili* and *Durchoniella legeri-duboscqui* based on 18S rRNA gene

To date, morphological criteria were used to describe astomes, and as their phylogenetic position remains poorly unknown, a 18S rRNA gene survey was performed. Fifty-four living *Durchoniella* cells were isolated from 19 different *Cirriformia tentaculata* worms. DNA was extracted, amplified by PCR, and sequenced from each single-cell. The 18S rRNA gene sequences of *D. brasili* (n = 38) and *D. legeri-duboscqui* (n = 16) shared 98% similarity, based on 1630 bp. Using Maximum Likelihood and Bayesian phylogenetic methods, *D. brasili* and *D. legeri-duboscqui* were shown to form a unique cluster with the only other sequence of an astome available in GenBank, *Anoplophrya marylandensis* retrieved from the intestinal content of a lumbricid annelid (AY547546 ; Affa'a *et al.*, 2004)(Figure 4.6). The 18S rRNA gene sequences from *D. brasili* and *D. legeri-duboscqui* were 92% similar to *A. marylandensis*. The relationship between these three astome species was supported by a posterior probability of one, Maximum Likelihood (ML) bootstrap of 99%, Neighbour Joining (NJ) bootstrap of 100%, and Maximum of Parsimony (MP) bootstrap of 96%. They formed a clade placed at the base of the Subclass Scuticociliatia within the Class Oligohymenophorea, a position supported by a posterior probability of 0.90, 73% ML bootstrap, 86% NJ bootstrap, and 90% MP bootstrap (Figure 4.6).

Microscopic identification of *Bacteria*

TEM revealed numerous prokaryotic-like bodies that were not observed using SEM and light microscopy. DAPI coloration and fluorescence *in situ* hybridization (FISH) analysis using a general bacterial probe (EUB338) confirmed the presence of living bacteria in the cytoplasm of all *D. brasili* and *D. legeri-duboscqui* cells observed. No cells were observed using the general

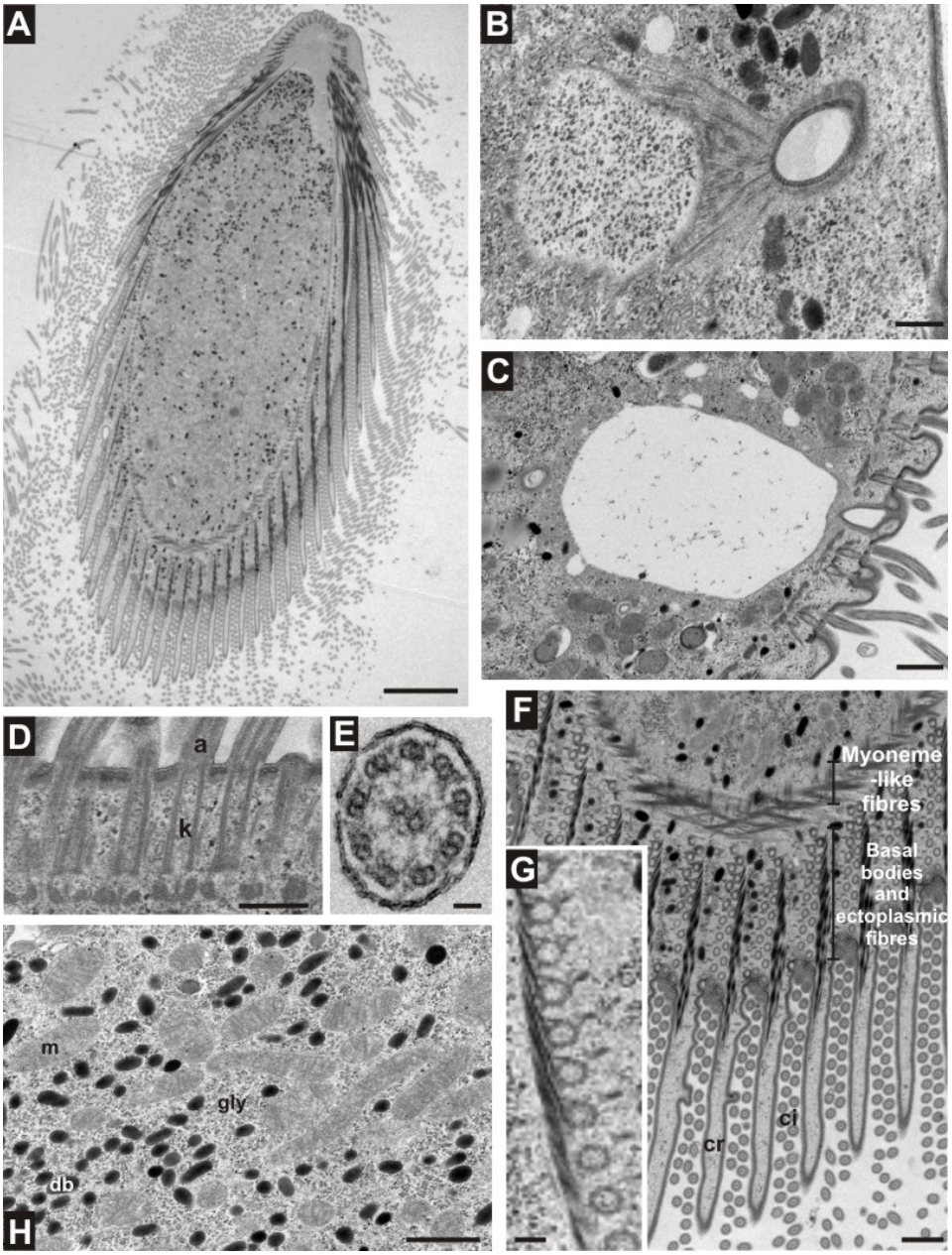

FIG. 4.5 – Transmission electron micrographs of *Durchoniella brasili*. **(A)** *Durchoniella brasili*, scale bar = 5 μm. **(B-C)** Two contractile vacuoles (B, scale bar = 500 nm, E = 1 μm). **(D)** Longitudinal section of a part of the cortex with a : axoneme and k : kinetosome of cilia, scale bar = 200 nm. **(E)** Cross section of a cilium, 9+2 typical pattern of microtubules, scale bar = 10 nm. **(F)** Basal part of *D. brasili* with the somatic dikineties lined by dense ectoplasmic fibres and separated by narrow crests (cr), and underlain by myoneme-like fibres, ci : cilia, scale bar = 1 μm. **(G)** Detail of monokinety from the apical third part of the cell, scale bar = 500 nm. **(H)** Detail of a middle cytoplasm area richs in mitochondria (m), dense bodies (db), and glycogen particles (gly), scale bar = 100 nm.

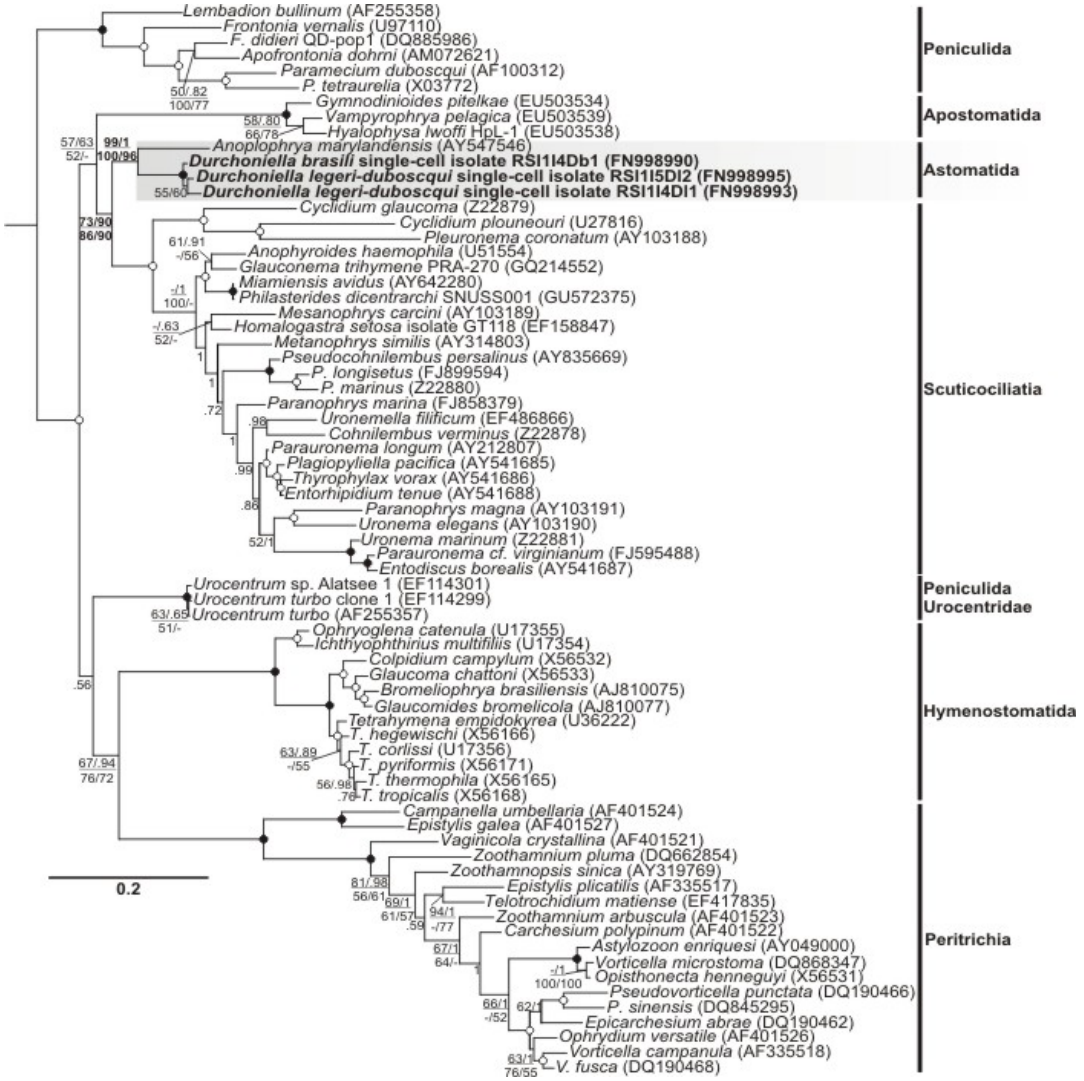

FIG. 4.6 – Bayesian analysis of the 18S rRNA gene sequences of representatives of the Class Oligohymenophorea. *Durchoniella* sequences are in bold. Each astome isolate was named RSIxIyDb/Dlz, where RSIx represents the site number (R for Roscoff and SI for Souris Island), Iy the worm number, and Dbz or Dlz represents the astome number (Db for *Durchoniella brasili* and Dl for *Durchoniella legeri-duboscqui*). Maximum Likelihood bootstrap, posterior probability of Bayesian method, and Neighbour-Joining and Maximum Parsimony bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. Black dots correspond to values of one (posterior probability) and 100% (ML, NJ, MP). White dots correspond to values between 80 and 100% (or 0.80 and one). Outgroup sequences not shown are composed of three ciliophora sequences (Phyllopharyngea, AY378112 ; Colpodea, M97908 ; and Karyorelictea, GQ16715).

archaeal probe ARCH915. These numerous endocytoplasmic bacteria were freely distributed throughout all the cytoplasm of the *Durchoniella* cells (Figure 4.7A). Although bacteria were not incorporated in food vacuoles or in association with nuclei or eukaryote organelles, all the bacteria observed using TEM were surrounded by an electron-translucent halo delimited by a very thin membrane (Figure 4.7B, white arrowhead and Figure 4.7C black arrowheads). All bacteria observed had two membranes (Figure 4.7B, black arrowheads), contained granular cytoplasm and nucleoid-like structures (Figure 4.7B-E). Rod-shaped and cocci bacteria, respectively 2-3 μm long and 0.5-0.8 μm of diameter, were observed by TEM (Figure 4.7B-E) and FISH (Figure 4.7F and G). Endocytoplasmic bacterial division was suggested by some observations (Figure 4.7E). Bacteria were not motile inside *Durchoniella* cells, and no flagella were observed.

Phylogenetic analysis of *Bacteria* of *D. brasili* and *D. legeri-duboscqui* based on 18S rRNA gene

According to the previous microscopic observations, two endocytoplasmic bacterial 16S rRNA gene clone libraries were constructed from DNA extracted from cells of *D. brasili* (n=54) and *D. legeri-duboscqui* (n=7), representing a total of 151 clones for *D. brasili* (clone library named Db) and 90 clones for *D. legeri-duboscqui* (clone library named Dl). No PCR product was obtained, even by nested PCR amplification, using general archaeal primers, confirming the FISH microscopic observations.

In order to prevent and control contamination by extracellular bacteria from the environment, worms and astomes were rinsed, under sterile conditions, three times with sterilized/filtered/sterilized seawater. The rinse water from the astomes was used as negative control, and after DNA extraction a very faint PCR product was amplified and cloned. Only 12 clones had an insert with the expected size. The majority of sequences from the rinse water clone library (named Water control) were related to the firmicutes *Geobacillus* species (n=4) and to Chlamydiae (n=3). The other sequences were related to Actinobacteria *Microccocus* sp. (n=1), *Gamma-Proteobacteria Psychrobacter* sp. (n=2), and to bacteroidetes (n=2) linked to the endocytoplasmic bacteria clone DlC9 (Figure 4.8).

Whole bacterial 16S rRNA gene sequences of Db and Dl clone libraries were respectively assigned to 14 and 17 OTUs, based on 800 bp and 95% genus level for phylotype differentiation (Schloss and Handelsman, 2004). The coverage values for Db and Dl 16S rRNA gene clone libraries were respectively 97% and 87%. Rarefaction curves were only strongly curvilinear for both clone libraries below 92% similarity, probably due to strong intra-lineage diversity in some phylotype (data not shown). Clone library from *D. brasili* was dominated by sequences related to Chlamydiae (53%) and Proteobacteria (46%), especially *Epsilon-Proteobacteria* (33%, Figure 4.8). The *Epsilon-Proteobacteria* sequences formed a unique cluster only distantly related to to environmental sequences retrieved from gorgonian and sunken-wood gastropods (i.e. highest similarity to pure culture, 88%). Chlamydiae genetic diversity was relatively high, and

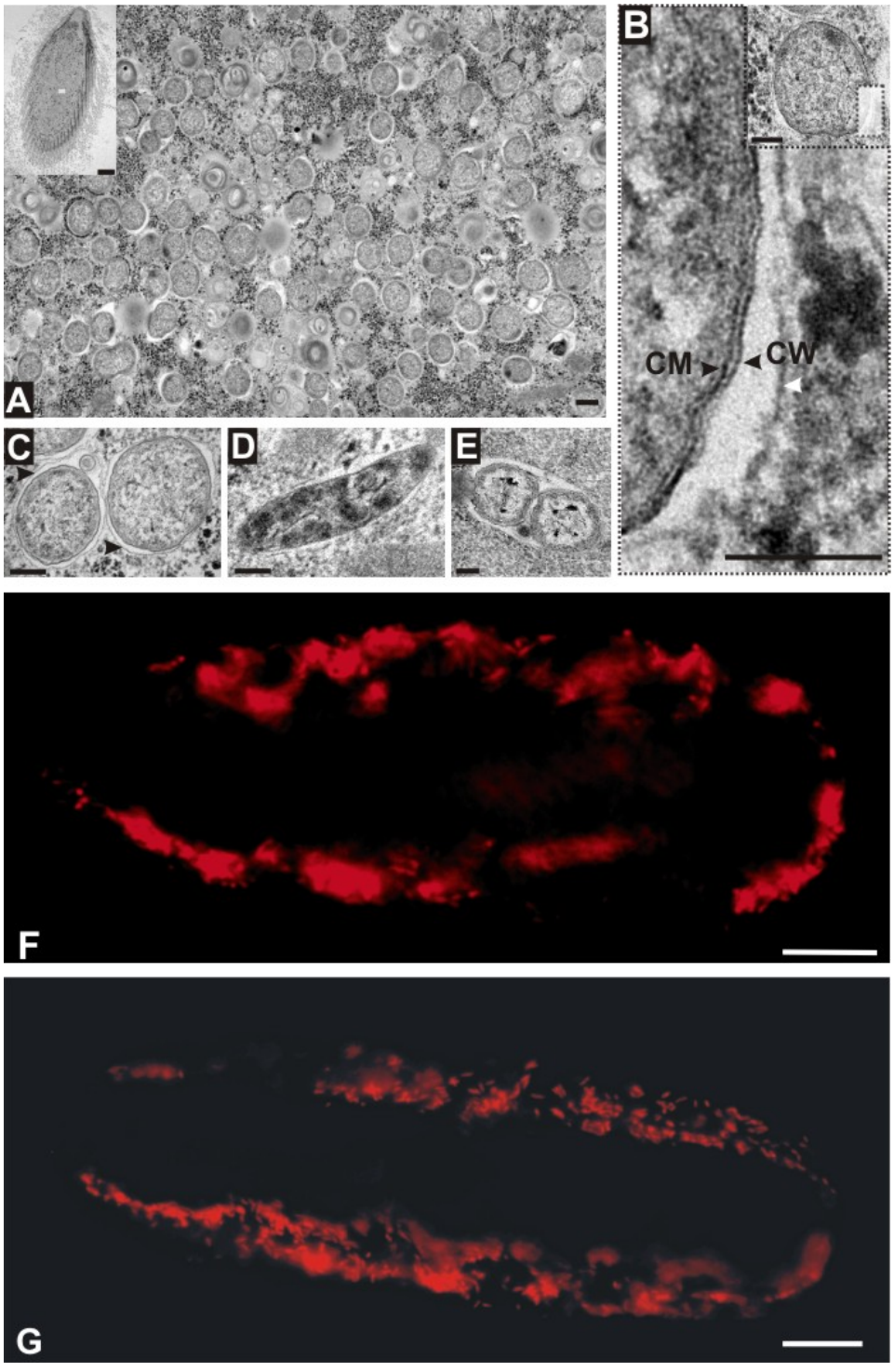

FIG. 4.7 – Transmission electron and light microscopy micrographs of endocytoplasmic bacteria. **(A)** White square on entire cell shows where the zoom was made (scale bar = 5 μm). Note the numerous bacteria-like organites, scale bar = 500 nm. **(B)** Single bacterium (scale bar = 200 nm) with an enlarged view of the boxed region, CM : inner cytoplasmic membrane, CW : outer envelope (black arrowheads), and host membrane (white arrowhead) ; scale bar = 100 nm. **(C)** Detail of bacteria-like cells in cocci-shaped. Note the electron-translucent halo delimited by a thin membrane around bacteria (arrowheads), scale bar = 200 nm. **(D)** Rod-shaped bacterium, scale bar = 200 nm. **(E)** Dividing cells of endocytoplasmic bacteria, scale bar = 200 nm. **(F-G)** Results of FISH with EUB338 general bacterial probe on *D. brasili* (F, scale bar = 10 μm) and *D. legeri-duboscqui* (G, scale bar = 20 μm).

was represented by two groups of sequences (group 1 and 2, Figure 4.8A). Group 1 was related to chlamydial symbionts retrieved from other marine organisms (i.e. fish and deuterostomia) and insects (Figure 4.8) and was the most abundant Chlamydiae group in Db clone library, representing 29% of the Db sequences. Bacterial diversity in *D. legeri-duboscqui* was composed mainly of sequences related to the Chlamydiae group 2 (76%) and *Proteobacteria* (19%).

To characterize bacterial activity, PCR amplifications using primers specific to methyl-coenzyme M reductase (*mcrA*), adenylylsulfate reductase (*APS*), and dissimilatory sulphite reductase (*dsrAB*) genes, were performed on extracted DNA from both *Durchoniella* species ; however no PCR product was detected.

Discussion

Amendments to previous descriptions

The initial diagnoses of *Durchoniella* species, *D. brasili* and *D. legeri-duboscqui*, are available in *Contribution à l'étude cytologique et taxonomique des infusoires astomes* De Puytorac, 1954, and are summarized by De Puytorac in *Traité de Zoologie : Infusoires Ciliés* (1994).

The present study of *D. brasili* and *D. legeri-duboscqui* collected from *Cirriformia tentaculata* from the bay of Roscoff based on electronic microscopy and phylogenetic analysis supports the initial diagnosis performed by De Puytorac. Only slight differences in the size of the cells were observed, as *D. brasili* was smaller and *D. legeri-duboscqui* was bigger than initially observed (factor 1.2 in both cases). SEM micrographs confirmed the shape and morphology of both *Durchoniella* species, and confirmed the characteristics of the apical apparatus. The apical point in both *Durchoniella* appeared much less prominent and developed than in other astome species with hook-shaped and needle-shaped structures, such as astomes in the earthworm, *Metaradiophrya lumbrici* and *Maupasella cepedei*, respectively. These structures do not seem essential to attach to the host tissues, as most astomes can use their thigmotactic field for attachment (De Puytorac, 1954). In *Durchoniella* species, this thigmotactic field could be delimited by dikinetids area and used to attach to the intestine of *C. tentaculata*. The structure and shape of these astomes were provided by ectoplasmic fibres found along the somatic kineties, and by myoneme-like cortical fibres that could provide both elasticity and resilience to the cell (De Puytorac, 1954). Moreover, the dense bodies observed in the apical part and beneath the cell surface could be storage organelles, or could also contain enzymes used for defence or phagocytosis.
The structural and cytoplasmic characteristics of *D. brasili* and *D. legeri-duboscqui* seem to reflect morphological and physiological adaptations to their host *C. tentaculata*, suggesting host-symbiont interactions.

Only a single event of conjugaison of *D. brasili*, only previously described by Collin (1911), was observed by DAPI coloration, reflecting the difficulty in observing conjugation in astomes. Conjugation in *D. brasili* does not seem related to low nutrient conditions, as incubation of *C.*

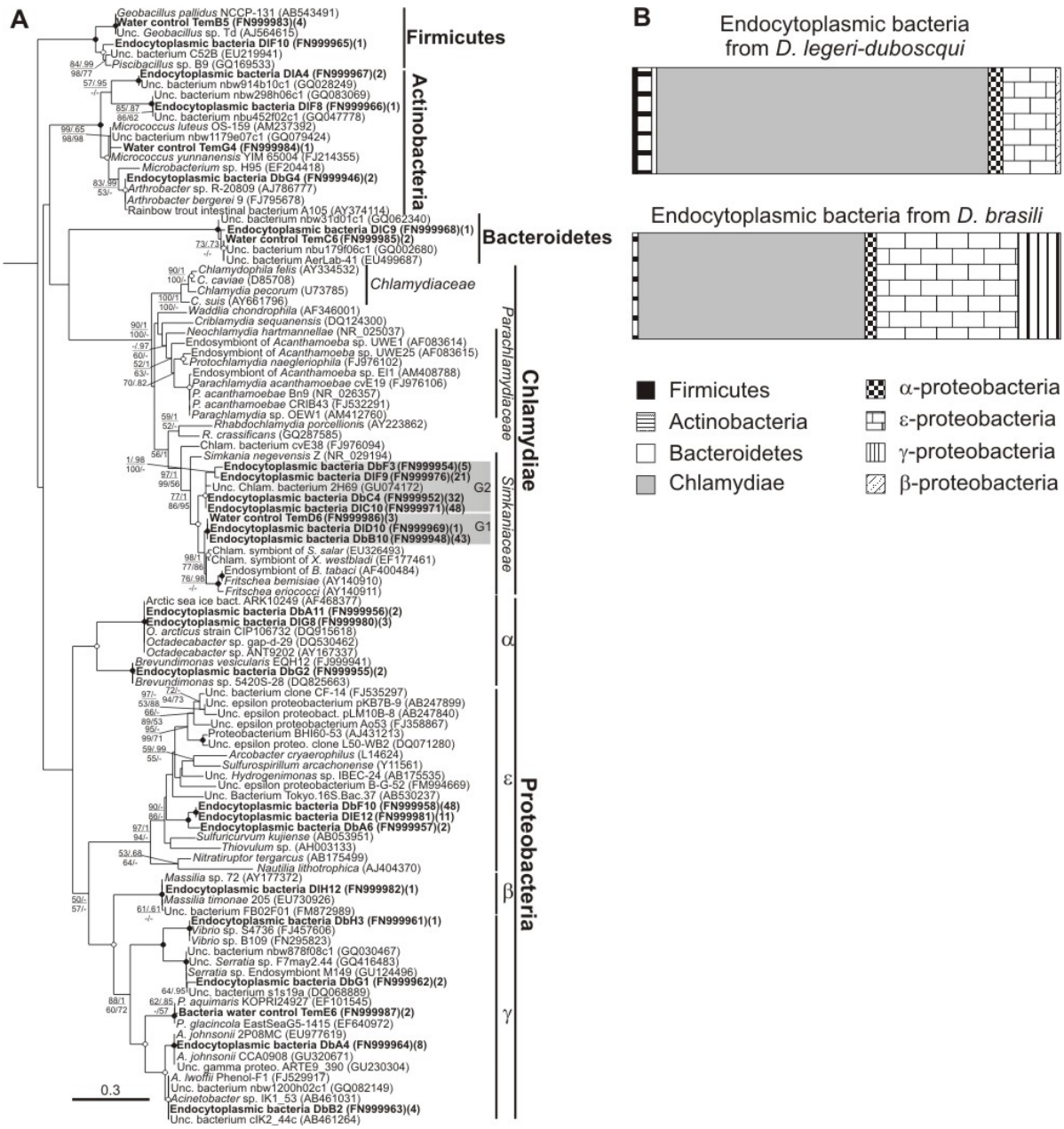

FIG. 4.8 – **(A)** Bayesian analysis of bacterial 16S rRNA gene sequences retrieved from *Durchoniella brasili* and *Durchoniella legeri-duboscqui*. Study sequences are in bold. Each phylotype from each clone library is represented by one sequence with ≥98% similarity grouping. Maximum Likelihood bootstrap, posterior probability of Bayesian method, Neighbour-Joining, and Maximum Parsimony bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. Black dots correspond to values of one (posterior probability) and 100% (ML, NJ, MP). White dots correspond to values between 80 and 100% (or 0.80 and one). The outgroup (not shown) is an archaeal sequence of *Pyrococcus abyssi*. The number of clones of each representative sequence is indicated in brackets. G1 : Chlamydiae group 1, G2 : Chlamydiae group 2. **(B)** Distribution of the bacterial phylogenetic community structure based on 16S rRNA from both astomes. The relative abundance of each phylotype was calculated and represented in column diagram.

tentaculata in seawater without nutrient addition up to eight weeks did not stimulate conjugation of intestinal *Durchoniella* cells. The single pair of conjugants observed during this study was retrieved from a worm with an abundant intestinal astome community (n = 203), suggesting that the *Durchoniella* cell density could be one of the key factors for conjugation stimulation. Although conjugating *Durchoniella* cells described by Collin (1911), also collected at Roscoff, were adhering along $^{2}/_{3}$ of their total length, we only observed adhesion at the apical region, as described for *Maupasella herculei* by De Puytorac (1954).

The life cycle of *D. brasili* and *D. legeri-duboscqui* remains unclear. Although *Durchoniella* abundance decreases with incubation period of *C. tentaculata*, no cyst form, a stage improving resistance and dispersal mechanisms, was ever observed. Moreover, *Durchoniella* spp. do not seem to be free-living ciliates, as they were not detected in the surrounding seawater, sediment or water used after worms were removed, suggesting these astomes completely depend of their host.

Systematic position

Systematic position of astomes is still controversial, as De Puytorac considered the group as polyphyletic (De Puytorac, 1954; De Puytorac *et al.*, 1979), whereas Lynn classified all astomes within the order Astomatida (Lynn, 2008). *Durchoniella brasili* and *D. legeri-duboscqui* were initially placed by De Puytorac (1954) within the Class Oligohymenophorea, Subclass Astomatia, Order Hoplitophryida, Family Radiophryidae and Subfamily Durchoniellinae. Based on the analysis of the thigmotactic field and apical structure, all astomes probably share a common ancestor with the Thigmotrichidae, a family belonging to the Subclass Scuticociliatia (De Puytorac, 1954; De Puytorac *et al.*, 1979).

To date, the only astome gene sequence available in public databases suggested that astomes were oligohymenophorean ciliates, placed at the basal node of the Scuticociliatia (Affa'a *et al.*, 2004). The topology of our phylogenetic tree built from ciliate and *Durchoniella* 18S rRNA gene sequences, supported by high statistical confidence levels, showed that *D. brasili* and *D. legeri-duboscqui* were also in the class Oligohymenophorea. Moreover, the phylogenetic analyses showed that the 18S rRNA gene sequences from *D. brasili* and *D. legeri-duboscqui* group into distinct clusters, confirming the initial morphological diagnosis of two distinct species. This conclusion was also supported by the analysis of sequences of the *cox-1* gene (Sauvadet *et al.*, in preparation). However, as 18S rRNA gene sequences of thigmotrich ciliates are not available, it is still not possible to decide conclusively on the phylogenetic origin of astomes.

A third « Russian doll » : the bacteria

The presence of endosymbiotic bacteria in astomes was first reported in specimens retrieved from the oligochaete *Lumbriculus variegatus* by Hovasse (1945). Although several astomes were also shown to harbour bacteria (Hovasse, 1945; Hovasse, 1946), the endosymbiotic association

does not seem to be required for all astomes as several were shown to be free from bacteria, such as *Spirobütschiella chattoni* and *Cepedella hepatica* (Hovasse, 1946). Bacteria were never previously observed in *Durchoniella* from *C. tentaculata* sampled at Roscoff using a acid fuchsin-azure II coloration (Hovasse, 1946), suggesting a technical bias as the present study describes endocytoplasmic bacteria in both *D. brasili* and *D. legeri-duboscqui* by combining stained and transmission electron microscopy with molecular genetic analysis. Moreover, *Durchoniella* species could contain up to eight different phylotypes of bacteria, in opposition to the hypothesis of a single bacterial species per astome initially suggested by Hovasse (1945 ; 1946). Based on the analysis of clone libraries, the general bacterial population structure was similar between both *Durchoniella* species, as 16S rRNA sequences were mainly related to the phylum Chlamydiae and class *Epsilon-Proteobacteria*.

Epsilon-Proteobacteria are known to play an important role in the biogeochemical sulphur cycle and in chemosynthetic symbioses with different metazoans, notably in hydrothermal environments (for review, Campbell *et al.*, 2006). The *Epsilon-Proteobacteria* detected in *Durchoniella* may represent a new host-specific symbiont, as they do not have any known close relative. *Epsilon-Proteobacteria* are usually metabolically versatile, suggesting they could adapt to the rapidly changing geochemical conditions encountered by *C. tentaculata*. Therefore, these previously unknown *Epsilon-Proteobacteria* endosymbionts may be in symbiotic association with their endocommensal astomes, and/or indirectly interact with the cirratulids retrieved from chemically-reduced sediments.

Members of the phylum Chlamydiae are described as obligate intracellular bacteria, commonly found in many different organisms, such as mammals, fish, insects, and unicellular eukaryotes (for review, Horn, 2008). To date, among unicellular eukaryotes, Chlamydiae were only detected in amoebas. Chlamydiae have a characteristic life cycle, comprising a metabolically inert infectious form called an elementary body (EB), and a reticulate non-infectious intracellular body (RB). These cells are usually grouped in single-cell inclusions or multiple larger inclusions (Horn, 2008). Sequences affiliated to Chlamydiae retrieved from *Durchoniella* were in the Family Symkaniaceae and were related to symbionts designated as EB and RB1 in the deuterostomian *Xenoturbella* (Israelsson, 2007). The RB1 symbionts were large rounded cells, with similar form and size ($\sim$0.6 μm) to the endocytoplasmic bacteria from *Durchoniella* observed using TEM. Although Chlamydiae from *Xenoturbella* accumulated in large numbers in inclusions, the bacteria observed in *Durchoniella* were only found individually in inclusions homogeneously distributed in the host's cytoplasm. Chlamydiae retrieved from *Xenoturbella* were also found in association with *Gamma-Proteobacteria* (Kjeldsen *et al.*, 2010), which were different from the genotypes derived from *Durchoniella*. These bacteria moreover were absent from the food vacuoles or specialized organelles of *Durchoniella*, suggesting bacteria did not represent a nutrient reserve. Therefore, bacterial symbionts may be used by their host for defence or more likely for environmental adaptation.

Microscopic observation using DAPI showed that the endocytoplasmic bacteria were ho-

mogeneously distributed in the *Durchoniella* cells during conjugation. The same observation was also made during binary fission using SYBR Green coloration and FISH analysis (data not shown), suggesting that the bacterial symbionts were acquired by vertical transmission. However, further molecular genetic analyses of the biogeographic distribution would be required to show any co-speciation mechanisms. Although the different partners of this marine tripartite association have now been described, the interactions and ecological strategies remain to be explored.

Materials and methods

Site description and sampling

Intestinal contents of *Cirriformia tentaculata* collected between March 2009 and May 2010 from several different sites from Roscoff (03°59'W, 48°43'N ; France) were examined. Cirratulids were meticulously cleaned with 0.2 μm-filtered seawater by removing all grains of sand with forceps. Each worm was then placed individually in a 6-well culture plate filled with 10 mL filtered seawater, and maintained up to eight weeks at room temperature with a daily seawater renewal without nutrient addition. Dissections were performed after worms were incubated for at least 30 min in 7% (v/v) $MgCl_2$ in filtered seawater, prepared at least two hours in advance and stored at 4°C.

Fixation and Staining

All solutions and rinsing were performed with filtered/sterilized/filtered (F/S/F) seawater. DAPI (0.225 μg/mL final concentration) stains were performed on glutaraldehyde-seawater (0.25%, v/v) or paraformaldehyde-seawater (4%, w/v) fixed cells in AF1 solution (Glycerol + PBS, CytiFluor, London, UK). From a 2 g/L stock solution of Neutral Red, cells were incubated 15 min in a 50 mg/L solution at room temperature, and rinsed twice. Cresyl Blue coloration was performed by incubating cells in a F/S/F seawater droplet with one grain of dye, and rinsed once in F/S/F seawater.

Bacterial FISH

General probe Eub338 (Amann *et al.*, 1990) was used to observe bacteria by *in situ* hybridization in both *Durchoniella* species. Non-sense probes (EUBN) were also used as negative controls and no signal was detected. All probes were synthesized with Cyanine-3 at the 5'-end (Thermo Fisher Scientific GmbH, Ulm, Germany). The probes, received in a lyophilized form, were dissolved in sterile deionized water purified with the MilliQ system from Millipore (resistivity 18 M) to a final concentration of 50 ng/μL and aliquots stored in dark at −80°C.

Cells were individually collected with a drawn pipette and were placed on the coated slide (Polysine® slides, Menzel GmbH, Brainschweig, Germany). Cells were fixed for 10 min in 4%

(w/v) paraformaldehyde solution and dehydrated with 80% ethanol for 10 min. Cells were then incubated with proteinase K (5 μg/mL final concentration) and lysozyme (2 mg/mL final concentration) at 37°C for one hour. Rinsing and pre-hybridization were peformed 10 min at 46°C in hybridization buffer (40% v/v formamide in 5X SSC, where 5X SSC = 83 mM NaCl, 83 mM sodium citrate, pH 7.0). Hybridizations with each fluorescent probe were performed on slides in a hybridization chamber (Evergreen Scientific, Los Angeles, USA) at 46°C for 10 min in 300 μL of hybridization buffer containing the probes at a final concentration of 5 ng/μL. After hybridization, cells were washed twice in a bath of 5X SSC at 48°C for 10 min. Slides were mounted in anti-fading reagent AF1 containing DAPI (see fixation and staining section), and stored at 4°C in dark until they were observed.

Light, epifluorescence and electron microscopy

Living or fixed material extracted from worms was examined on freshly prepared slides and photographed with an Olympus BX51 epifluorescence microscope (Olympus Optical CO, Tokyo, Japan), coupled to a Spot RT-slider camera (Diagnostics Instruments, Sterling Heights, MI). For scanning electron microscopy (SEM), cells were fixed for 4 h at room temperature in a fixative containing 4% (v/v) glutaraldehyde, 0.25 M sucrose, and 0.1 M sodium cacodylate pH 7.4. Samples were then rinsed in a series of buffer solutions containing graded concentrations of sucrose and post-fixed for one hour at 4°C in 1.5% (w/v) OsO_4 in Cacodylate 0.2 M, pH 7.4/NaCl 0.33 M. Dehydration was carried out in a graded alcohol series and finally dried using critical point dryer (CPD 030 Bal-Tec, Balzers, Lichtenstein). The dried cells were coated with gold in a sputter coater before observation with a JSM 5200 SEM (Jeol Ldt, Tokyo, Japan). Fixation for transmission electron microscopy (TEM) was performed as described above. After dehydration, samples were embedded in Epon™. Sections were made using a diamond knife on a Leica ultracut UCT ultramicrotome (Leica Mikrosysteme Gmbh, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and observed with a JEM 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) or a Quanta 200 environmental SEM (FEI, Hillsboro, Oregon, USA).

DNA extraction

Before DNA extraction, worms were rinsed three times, all material (dissection tools, dissection boxes, and jar) were incubated in DNA AWAY® (Molecular BioProducts™), ethanol, and sterilized for 30 min at 121°C. Single-cells of *Durchoniella* spp. were then isolated with sterilized drawn pipettes and rinsed individually three times in F/S/F seawater. DNA on single-cells was extracted by a modified Guanidinium Isothiocyanate (GITC) protocol (Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006). Each fresh single-cell was ground in 50 μL of the GITC extraction buffer, and crushed with tube-adapted piston pellet (Kimble Chase®) for at least one minute. Tubes were incubated for 20 min at 72°C, and quickly centrifuged. One

volume of cold isopropanol was added at -20°C overnight. After centrifugation 15 min at 14,000 g at 4°C, one washing with 70% (v/v) ethanol, DNA was hydrated in sterile distilled water and stored at 4°C. The extraction product was used to amplify 18S and 16S rRNA genes.

PCR and sequencing

All PCR mixtures (30 μL final volume) contained 1X Taq DNA Polymerase buffer, 1μL of dNTPs (20 mM each), 3 μL of $MgCl_2$ (25 mM), 0.1 μL of each primer (100 μM) and 0.25 μL of Taq DNA Polymerase (5 unit/μL, Promega®, Madison, Wisconsin). All amplifications were performed using a GeneAmp PCR system 9700® (Applied Biosystems™) or MJ Mini Cycler (Biorad™). Negative controls were also carried out with DNA extractions performed with no sample, with and without piston pellet. For all controls, no PCR products were detected.

Eukaryotic 18S rRNA gene was amplified with primers EukMK-63F/EukMK-1818R (5'-ACG CTT GTC TCA AAG ATT A-3' ; 5'-ACG GAA ACC TTG TTA CGA-3' ; M. Kawachi, unpublished) with the following conditions : one cycle of 5 min at 95°C, 30 cycles of 1 min at 95°C, 1.5 min at 57°C, and 1.5 min at 72°C, and one cycle of 10 min at 72°C. Bacterial 16S rRNA gene amplification was conducted by PCR with primers E8f/U1492r (DeLong, 1992; Eden *et al.*, 1991). PCR cycles were as follows : one cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at 49°C, and 2 min at 72°C, and one cycle of 6 min at 72°C. Three independent PCR products were pooled and purified (QIAquick PCR purification Kit ; Qiagen™) and cloned into *Escherichia coli* (One Shot F10', Promega™) using the pCR2.1-TOPO TA vector system (Invitrogen®) following the manufacturer's instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Positive PCR products were purified (ExoSAP-IT® For PCR Product Clean-Up, USB™) and sequenced in both directions with specific primers using the Big Dye Terminator Cycle Sequencing Kit version 3.0 (PE Biosystems™) and an ABI PRISM model 377 (version 3.3) automated sequencer.

Phylogenetic analysis

Available sequences were edited in the BioEdit 7.0.5.3 program and aligned using CLUSTALW2 (Hall, 1999; Larkin *et al.*, 2007). To determine the first phylogenetic affiliation, each sequence was compared with sequences available in the NCBI database (National Center for Biotechnology Information) using BLAST (Altschul *et al.*, 1990). For the Oligohymenophorea tree (Figure 4.6), TIM2+I+G was selected by hierarchical Likelihood Ratio Tests (hLRT) with gamma distributed rates and a proportion of invariable sites, via jModeltest 0.1.1 (Posada, 2008) and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by Maximum Likelihood and Bayesian inference (Posada and Crandall, 1998). Maximum Likelihood was conducted using PhyML 3.0 (Guindon *et al.*, 2005) and the robustness of the inferred topology was supported by bootstrap resampling (100). Bayesian inference was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and started with a random

tree, run for 344 000 generations in four chains (Standard deviation = 0.007) and burn-in of 860 generations in order to ensure the use of only stable chains. For the bacterial tree (Figure 4.8), a general time-reversible (GTR+G) model with gamma distributed rates was selected. Maximum Likelihood was supported by bootstrap resampling (100), and Bayesian inference run for two millions generations in four chains (Sd = 0.010) and burn-in of 5000 generations. For the two phylogenetic analyses, Neighbour Joining (NJ) and Maximum of Parsimony (MP) trees were inferred using PAUP 4.0b10 via PaupUp graphical interface (Calendini and Martin, 2005). The robustness of inferred topologies was supported by bootstrap resampling (1000) with NJ and MP ; values over 50% are shown on the trees. The Maximum Likelihood trees gave the same topologies than Bayesian trees for both ciliates and bacteria. Trees were visualised and labelled with TreeDyn (Chevenet *et al.*, 2006).

Nucleotide sequence accession numbers

The sequences are available from the GenBank database under the following accession numbers : FN998987 to FN999022 for 18S rRNA gene sequences and, FN999946 to FN999987 for 16S rRNA gene sequences.

Acknowledgements

The authors are deeply indebted to Professor Pierre De Puytorac for his pioneering works on these astome species. We also sincerely thank Philippe Crassous (IFREMER) for his help with scanning electron microscopy. We thank Morgan Perennou and Gwenn Tanguy (Ouest-Genopole platform®) for sequencing operations. This work is supported by the ANR Deep Oases. A.L. Sauvadet is supported by a grant from the Ministère de la Recherche.

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Résulats complémentaires : Descriptive note on *Durchoniella cirratuli* from *Cirratulus cirratus*.

Anne-Laure Sauvadet, Sophie Le Panse, J. David George, Joseph Schrével, and Laure Guillou

Durchoniella cirratuli is a third species in the genus *Durchoniella*, originally described by De Puytorac (1954). This astome was retrieved from a cirratulid polychaete, *Cirratulus cirratus*, collected in muddy sediment or under stones. It was characterized by the presence of two rows of eyes on its head, contrary to *Cirriformia tentaculata*, harboring *D. brasili* and *D. legeri-duboscqui* (Sauvadet *et al.*, Sub.).

Materiel and methods

See page 126-129.

Results and discussions

Cirratulus cirratus specimens were retrieved from anoxic sediments in the first ten centimetres depth. The sediment was very muddy and characterized by a black colour (Figure 4.9A). As with *C. tentaculata*, *C. cirratus* was also characterized by an orange color and numerous tentacles (Figure 4.9B). The principal difference was the presence of two rows of eyes on its head (white arrowheads, Figure 4.9C) whereas adult *C. tentaculata* didn't have eye.

The astome, *Durchoniella cirratuli*, was found in the middle region of the worm intestine (Figure 4.9D). After dissection, *D. cirratuli* swam vigorously in the surrounding water. *Durchoniella cirratuli* were observed and counted in four *Cirratulus cirratus* collected in October 2009 from Plymouth (Stonehouse Pool site, 4°9.87'W 50°21.92'N). The intestines of the four *C. cirratus* harbored three, 28, 101, and 381 astomes respectively.

Durchoniella cirratuli was characterized by an elongated and thin body (Figure 4.9D), ranging *in vivo* from 207-339 μm length and 11-67 μm width (n=20). Contrary to *D. brasili* and *D. legeri-duboscqui*, *D. cirratuli* had two rows of contractile vacuoles that could be observed using a Neutral Red coloration (Figure 4.9E). One was positioned on the opposite side of the micronucleus with four to nine vacuoles (n=11), whereas the second was close to the micronucleus, with two to five vacuoles (n=11).

The two typical nuclei of ciliates were observed in *D. cirratuli*. The micronucleus, always located close to the macronucleus, was ovoid to fusiform shaped (Figure 4.9F) and was 10-26 μm long and 7-10 μm wide (n=10). The macronucleus had a gutter-like shape, with very irregular contours, and was extended along the length of cells, without however reaching the extremities (Figure 4.9F). The size of the macronucleus was 202-267 μm long by 25-39 μm wide

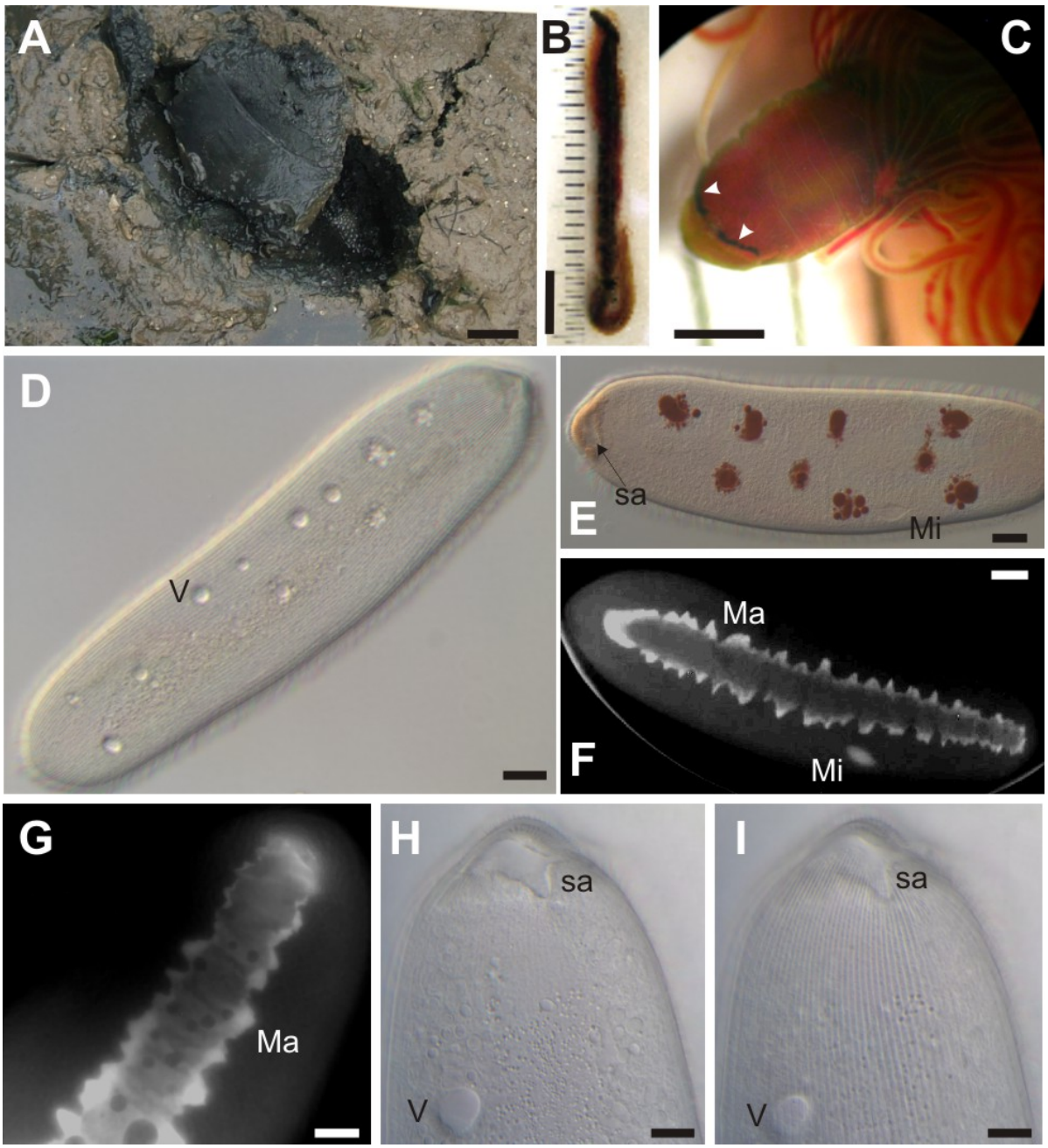

FIG. 4.9 – **(A)** Example of a sampling zone at Stonehouse Pool (Plymouth), scale bar = 5 cm. **(B-C)** *Cirratulus cirratus*, the white arrowheads show the two rows of eyes. Scale bars = 1 cm and 1 mm. **(D-E)** DIC Nomarski observation of live cells of *Durchoniella cirratuli*. Contractile vacuoles were observed with Neutral Red colouring. Mi : Micronucleus, sa : apical structure, V : Contractile vacuoles, scale bars = 20 μm. **(F-G)** Nuclear content of a *D. cirratuli* fixed cell stained with DAPI with zoom on the macronucleus **(G)**, Mi : Micronucleus, Ma : Macronucleus, scale bars = 20 μm and 10 μm. **(H-I)** DIC Nomarski observations of the apical structure after a Cresyl Blue staining of *D. cirratuli*, sa : apical structure, V : Contractile vacuoles, scale bars = 10 μm.

(n=5). The macronucleus showed dense round marks, which were distributed along its length (Figure 4.9G).

At the extreme anterior part of the species, a non-ciliated zone was underlain by the same base observed in *D. brasili* and *D. legeri-duboscqui*. The base of the apical structure was a truncated cone, 10-20 μm diameter at the base and 5-7 μm high (Figure 4.9E and Figure 4.9H-I).

Conjugation was observed in *D. cirratuli* (Figure 4.10A-E). Both pairing cells, always motile, were of equal size and attached at the same level along 2/3 of the length with a clearly visible weld at the apical end (Figure 4.10A). Eight micronuclei could be identified by DAPI coloration in the cell on the left of Figure 4.10B, and were grouped together except one in the posterior part of the cell (arrowhead). In the cell on the right of Figure 4.10B (zoom on Figure 4.10C), six micronuclei were grouped in the apical end. Two and four micronuclei, in the cell on the right and on the left respectively, were more diffuse, probably reflecting deterioration. Conjugation was observed in a population with fewer cells than the number of *D. brasili*. Binary fission was not seen.

DNA was extracted from four *D. cirratuli*, amplified by PCR, and sequenced from each single-cell. The 18S rRNA gene sequences of *D. cirratuli* (n = 4) shared 98% similarity with the other two *Durchoniella* species, based on 1630 bp. Based on Maximum Likelihood and Bayesian phylogenetic methods, the *D. cirratuli* sequences formed a unique cluster with the other sequence of *Durchoniella* species (Sauvadet *et al.*, Sub.) and the sequence of *Anoplophrya marylandensis* retrieved from the intestinal content of a lumbricid annelid (AY547546 ; Affa'a *et al.*, 2004) (Figure 4.11). the sequence from *D. cirratuli* was 92% similar to *A. marylandensis*. The relationship between these astomes species was supported by a posterior probability of one, Maximum Likelihood (ML) bootstrap of 100%, and Neighbour Joining (NJ) bootstrap of 100%. They formed a clade placed at the base of the Subclass Scuticociliatia whithin the Class Oligohymenophorea, a position supported by a posterior probability of 0.96, 80% ML bootstrap, and 92% NJ bootstrap (Figure 4.11). It is interesting to note that one of the two sequences from *D. legeri-duboscqui* was more closely related to sequences from *D. cirratuli* than sequences of the other *D. legeri-duboscqui*.

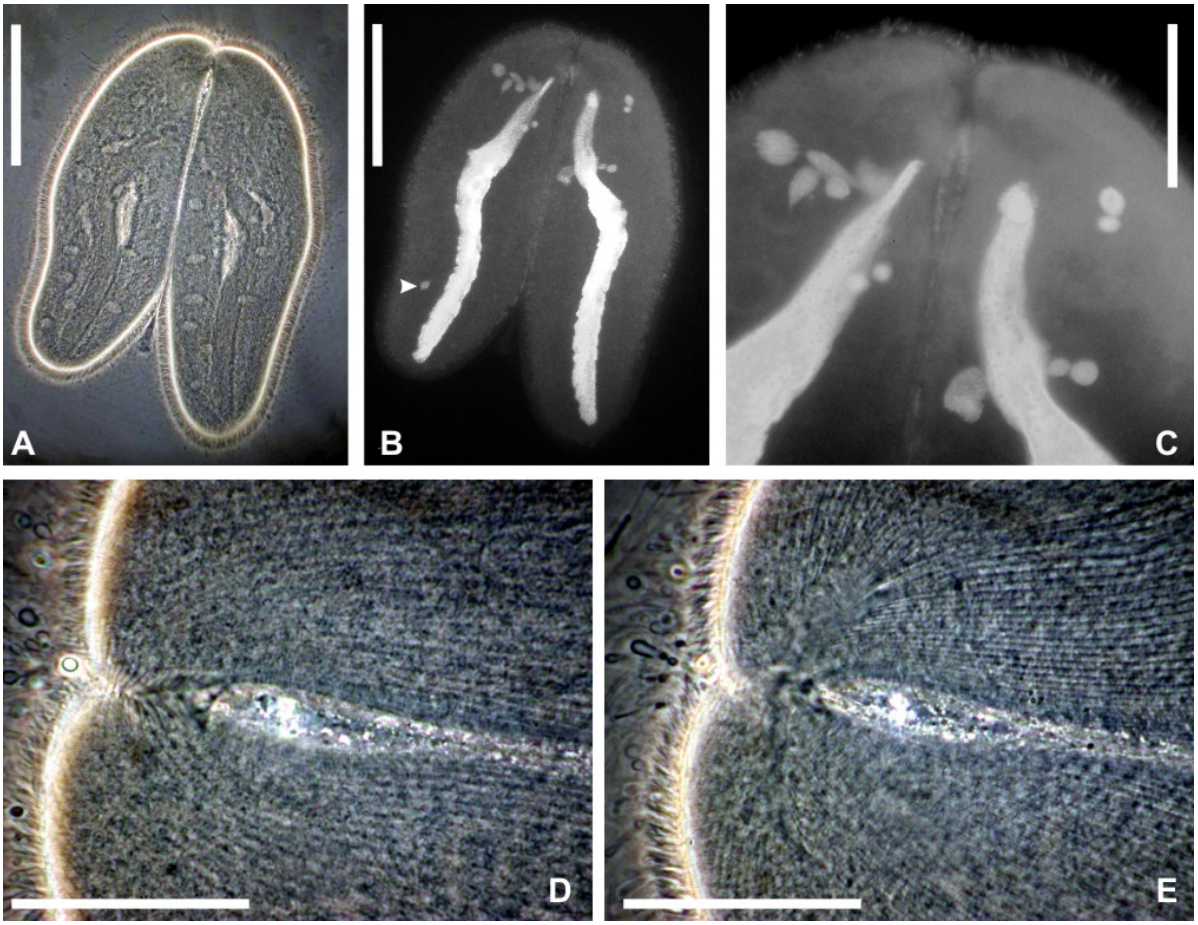

FIG. 4.10 – Conjugation of *Durchoniella cirratuli* on fixed cells stained with DAPI **(A-B)** with zoom on nuclear content of the two resulting cells **(C)** and zoom on adhesion part **(D-E)**, scale bars A-B = 100 μm, C-D-E = 50 μm.

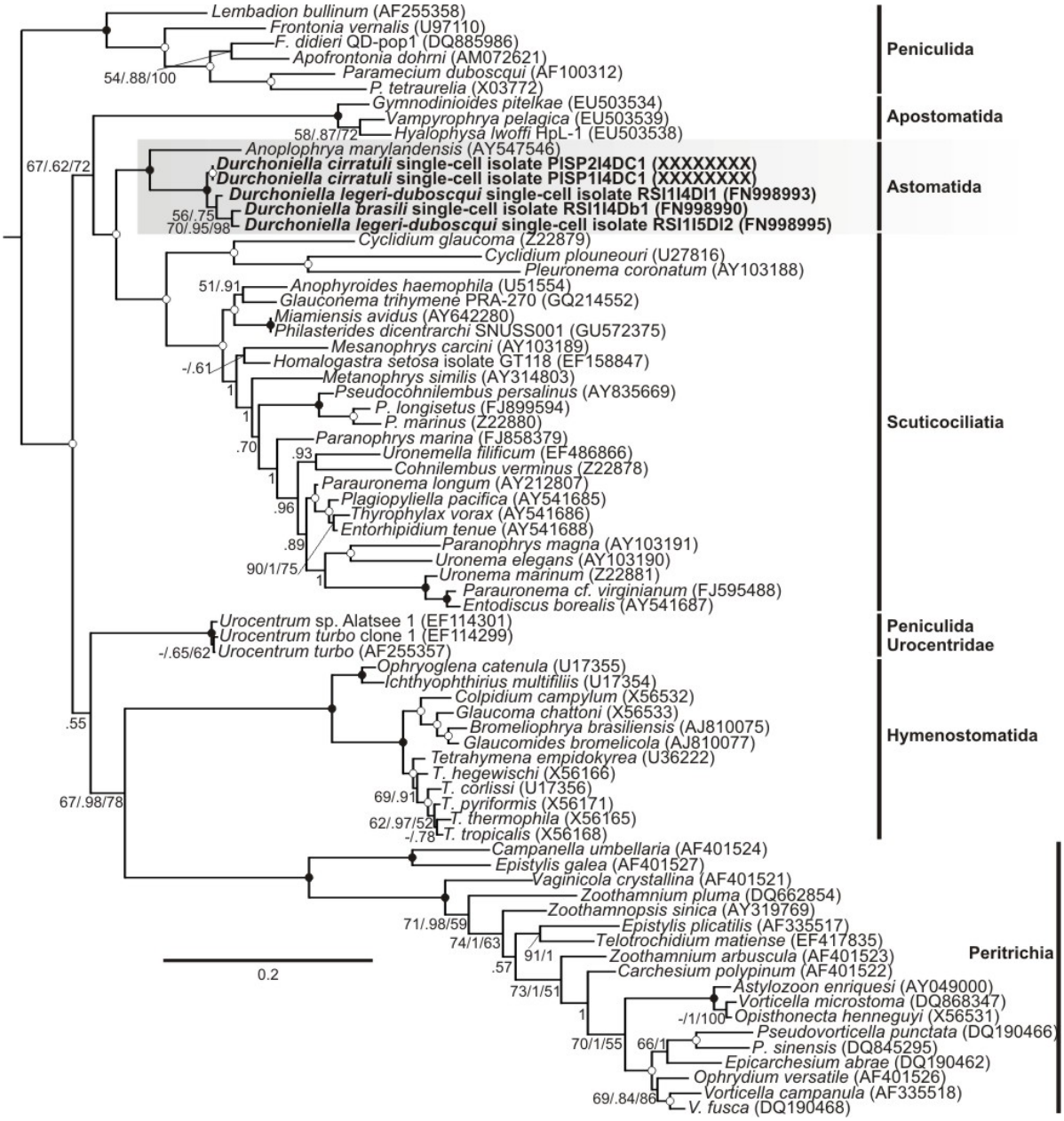

FIG. 4.11 – Bayesian analysis of the 18S rRNA gene representative sequences of the Class Oligohymenophorea. *Durchoniella* sequences are in bold. Each astome isolate was named with the initial of the city (R for Roscoff and Pl for Plymouth), the initial and the number of the site (SI for Souris Island and SP for Stonehouse Pool), the worm number (Iy), and the initial and the number of the *Durchoniella* species (Db : *D. brasili* , Dl : *D. legeri-duboscqui* and DC : *D. cirratuli*). Maximum Likelihood bootstrap, posterior probability of Bayesian method, and Neighbour-Joining bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. Black dots correspond to values of one (posterior probability) and 100% (ML, NJ). White dots correspond to values between 80 and 100% (or 0.80 and one). Outgroup sequences not shown were three ciliate 18S rRNA sequences : Phyllopharyngea, AY378112 ; Colpodea, M97908 ; and Karyorelictea, GQ16715.

4.3 Biogeographical analysis of a tripartite association between Cirratulidae, *Durchoniella*, and endocytoplasmic bacteria in tidal sediments of the English Channel

En préparation

Anne-Laure Sauvadet, Erwan G. Roussel, Estelle M-C. Bigeard, J. David George, Joseph Schrével, and Laure Guillou

La diversité et la distribution biogéographique de chaque partenaires de l'association tripartite entre les polychètes cirratulides (*Cirriformia tentaculata* et *Cirratulus cirratus*), les ciliés astomes (*Durchoniella brasili*, *D. legeri-duboscqui* et *D. cirratuli*), et leur bactéries endocytoplasmiques, ont été caractérisés en combinant les observations morphologiques aux analyses de génétiques moléculaires. Les vers ont été collectés sur 16 sites répartis le long des côtes française et anglaise de la Manche.

Bien que l'abondance des *Durchoniella* par polychète (n=82) était hétérogène et spécifique du site de prélèvement, l'intestin de *C. tentaculata* contenait au total une moyenne de 100 *D. brasili* (84%) et *D. legeri-duboscqui* (16%). L'intestin de *C. cirratus* contenait exclusivement *D. cirratuli*. La faible diversité génétique observée chez *Durchoniella* et les espèces de cirratulides, basée sur le gène codant pour la sous-unité 1 de la cytochrome oxidase et le gène codant pour l'ARNr 18S, n'a pas montré de distribution biogéographique ou biochimique significative.

Treize librairies génétiques, basée sur le gène codant pour l'ARNr 16S, ont été réalisés sur les bactéries endocytoplasmiques des cellules de *Durchoniella* associées aux différents sites. Il s'est avéré que ces librairies génétiques étaient dominées par trois nouveaux clades parmi les Mollicutes et les *Proteobacteria*. De plus, la présence de Mollicutes et de Chlamydiae dans les cellules d'astomes semble être relative à des évènements épisodiques, alors que les clades de *Proteobacteria* pourraient représenter des symbiontes obligatoires puisqu'ils sont retrouvés chez toutes les espèces de *Durchoniella* dans des populations géographiquement distinctes.

BIOGEOGRAPHICAL ANALYSIS OF A TRIPARTITE ASSOCIATION BETWEEN CIRRATULIDAE, *DURCHONIELLA*, AND ENDOCYTOPLASMIC BACTERIA IN TIDAL SEDIMENTS OF THE ENGLISH CHANNEL

Anne-Laure Sauvadet[a,b], Erwan G. Roussel[c], Estelle M-C. Bigeard[a,b], J. David George[d], Joseph Schrével[e], and Laure Guillou[a,b]

[a]CNRS, UMR 7144, Groupe Plancton Océanique, Station Biologique de Roscoff, 29682 Roscoff, France.
[b]UPMC - Université Paris 06, Laboratoire Adaptation et Diversité en Milieu Marin, 29682 Roscoff, France.
[c]School of Earth and Ocean Sciences, Cardiff University, Main Building, Park Place, Cardiff, Wales, UK.
[d]Natural History Museum, Department of Zoology, Cromwell Road, London, SW7 5BD, UK.
[e]Muséum National d'Histoire Naturelle, Département RDDM, CNRS FRE 3206, CP 52, 61 Rue Buffon, 75231 Paris Cedex 05, France.

Corresponding author. E-mail : sauvadet@sb-roscoff.fr ; Tel. (+33) 2 98 29 23 23 ; Fax (+33) 2 98 29 23 24.

Abstract

The diversity and biogeographical distribution of each partner of a tripartite association between cirratulid polychaetes (*Cirriformia tentaculata* and *Cirratulus cirratus*), ciliated astomes (*Durchoniella brasili*, *Durchoniella legeri-duboscqui* and *Durchoniella cirratuli*) and their endocytoplasmic bacteria, collected from tidal sediments on 16 sites along the English and French coasts of the English Channel, were characterized by combining morphological observations and molecular genetic analyses. Although the abundance of *Durchoniella* cells per polychaete (n=82) was heterogeneous and site specific, the intestine of *C. tentaculata* harboured on average a total of ∼100 *D.brasili* (84%) and *D. legeri-duboscqui* (16%). The gut of *C. cirratus* exclusively contained *D. cirratuli*. The low genetic diversity of *Durchoniella* and cirratulid species, based on the mitochondrial cytochrome oxidase subunit 1 gene (*cox-1*) and the 18S rRNA gene, did not show any significant biogeographical or biogeochemical distribution. Thirteen genetic libraries, based on bacterial 16S rRNA genes performed on the endocytoplasmic bacteria of *Durchoniella* cells from different sites, were dominated by three new clades within Mollicutes and *Proteobacteria*. Moreover, the occurrence of Mollicutes and Chlamydiae in *Durchoniella* cells seems to be linked to episodic events, whereas the *Proteobacteria* clades could represent permanent symbionts as they were retrieved from all *Durchoniella* species in geographically separate populations.

Introduction

Symbiotic associations have important ecological and evolutionary implications (nutrition, defence, speciation and reproduction) on the different associated organisms. Long-term associations usually imply co-evolution and a high degree of specialization of each partner (for review, Thrall *et al.*, 2007). The most common examples of a long-term association between two partners in eukaryotic cells are mitochondria and plastid Embley and Martin, 2006, for review. Although, tripartite associations are also probably widespread, such as the interaction between some termites, protists, and their bacteria (e.g. Noda *et al.*, 2005), they remain poorly studied.

A tripartite association in tidal sediments was recently discovered between Cirratulidae polychaetes (*Cirriformia tentaculata* and *Cirratulus cirratus*) and endocommensal astomes harbouring endocytoplasmic bacteria (Sauvadet *et al.*, Sub.). These cirratulids are widespread over European coasts, especially along the English Channel, and are found in different environments, from gravel and stones to fine black mud sediments characterized by reducing conditions and sometimes by a strong hydrogen sulphide odour (George, 1964). The decrease in the organic carbon content and the increase in particle size of the substrata may also negatively control the abundance of *C. tentaculata* (George, 1964). These worms are found in the upper sediment layers and they trap food particles falling onto the sediment surface with their tentacular filaments (Fauvel, 1927). These filaments are also used to acquire oxygen, exposing worms to

predation during low tide. Since these worms bury themselves deeply into the sediment, retracting their tentacles to avoid predation during low tide, they encounter chronic anoxic conditions and they produce volatile fatty acids (Bestwick *et al.*, 1989).

Astomes are a group of endocommensal ciliates, living in association with batrachians and invertebrates such as annelids. Astomes were identified for the first time in 1788, but it was not until the beginning of the 20^{th} century that their anatomy and habitat were described (Cépède, 1910; De Puytorac, 1954). Like most ciliates, astomes have two nuclei and cilia on their cell surface, but also have an unique attachment apparatus and/or thigmotactic field that can be used for interactions with their host De Puytorac, 1954. Cirratulid worms harboured astomes within the genus *Durchoniella* : *Durchoniella brasili* and *Durchoniella legeri-duboscqui*, which are specifically associated with the polychaete *Cirriformia tentaculata*, and *Durchoniella cirratuli*, which is specifically associated with the polychaete *Cirratulus cirratus*. These astomes were described by De Puytorac (1954), but Sauvadet *et al.* (Sauvadet *et al.*, Sub.) only recently demonstrated the presence of endocytoplasmic bacteria in *D. brasili* and *D. legeri-duboscqui*.

The aim of this study was to compare the respective phylogenetic diversity and biogeographical distribution of *Durchoniella* cells and their endocytoplasmic bacteria found in *C. tentaculata* and *C. cirratus*, along the English and French coasts of the English Channel. The biogeochemical habitat of these communities was also analysed in order to identify possible geographical and/or environmental factors controlling the tripartite association.

Results

Site description

Sixteen sites were sampled along the English coast of the English Channel between Plymouth and Portsmouth, and along the French coast between Wimereux and Roscoff. The geographic position names and geochemical characteristics of each site are presented (Figure 4.12, Table 4.2 and Table S1).

Polychaetes from Plymouth (Pl) were collected from three different sites, Admirals Hard (AH), Stonehouse Pool (SP), and Mount Batten (MB), separated by 120 m and 2644 m, respectively. For each site two duplicate sub-sites were sampled (PlAH1/PlAH2 separated by 11 m, PlSP1/PlSP2 separated by 19 m, and PlMB1/PlMB2 separated by 18 m). All the sediments collected from Plymouth had a black colour, were muddy, and contained some gravel (Figure 4.12A-B), except for PlMB, which was sandier, covered by seaweed, and had a hydrogen sulphide odour (Figure 4.12C).

Two sites were sampled at Portsmouth. One site was located in the mouth of an old landfill, Horsea Island (HI), characterized by very muddy sediment as found at AH and SP sites (Figure 4.12B). Polychaetes from HI were only found under large stones. The second site analysed at Portsmouth, Langstone Harbour (LH), separated from HI by 7 km, was on a small beach. Two duplicate sub-sites, separated by 8 m, were sampled and were characterized by a sandy

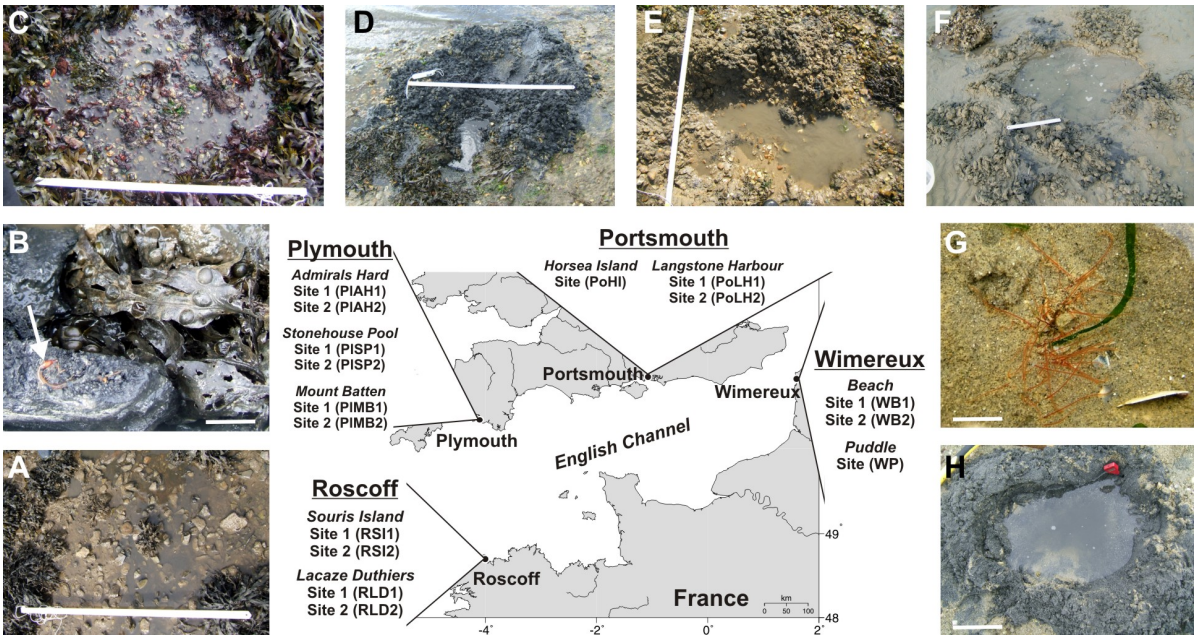

FIG. 4.12 – Location of sampling sites along the English and the French coasts of the English Channel. **A**, Type of substratum on the surface at Admirals Hard, Stonehouse Pool (Plymouth) (scale bar = 1 m). **B**, Muddy consistency of the sediment at Admirals Hard, Stonehouse Pool (Plymouth), and Horsea Island (Portsmouth). The arrow show one cirratulid (scale bar = 10 cm). **C**, Type of substratum on surface under the seaweed layer at Mount Batten (Plymouth) (scale bar = 1 m). **D-E**, Type of substratum observed at Langstone Harbour sites, LH1 and LH2 respectively (scale bar = 1 m). **F**, Sandy substratum containing the cirratulids collected at Wimereux Beach sites (scale bar = 20 cm). **G**, Tentacles of cirratulid polychaetes in a puddle sampling at Wimereux (scale bar = 10 cm). **H**, Type of substratum at Roscoff sites (scale bar = 10 cm).

substratum with many rocks, and showed black sediment patches heterogeneously distributed (PlLH1, Figure 4.12D ; PlLH2, Figure 4.12E).

On the French coast, sites at Wimereux were on a large sandy beach, and worms were collected from a fine grained sand with very small black traces (WB1/WB2, distant of 16 m ; Figure 4.12F), and in a small puddle near the shore (WP separated by 250 m ; Figure 4.12G). At Roscoff, polychaetes were sampled at two sites separated by 230 m, in duplicate sub-sites (RSI1/RSI2 separated by 13 m, and RLD1/RLD2 separated by 26 m). The sediment was sandy with reduced conditions and larger size particles than at Wimereux (Figure 4.12H).

Geochemical analyses of sediments were performed on a reference site located between each duplicate sub-site. The overall geochemical data, although quite heterogeneous, was characteristic of marine environments. Phosphate concentrations were very low or were not detected from the sediments from the English sites, whereas values were between 10 and 260 μM for sites at Roscoff and Wimereux (Table 4.2). High methane concentrations were detected over most sites but were heterogeneously distributed, with very high concentrations for PlSP and

PoHI sites (i.e. 259 and 553 μmol/g of wet sediment), whereas the other English and French sites were below 75 μmol/g of wet sediment (Table 4.2). The sediments from Wimereux had the lowest methane concentrations ($<$ 1.2 μmol/g of wet sediment). The chloride concentrations were in the range of seawater for most sites, except for the sediments from Wimereux and Roscoff, which showed lower concentration of chloride (i.e. 370-487 mM, Table 4.2). The analysis of sulphide was only performed for four sites in France. Although a sulphidic odour could be detected from these sites, no sulphide was found in the sediment pore waters, suggesting that the sulphide was probably too volatile to be measured in the pore waters.

Sample	PlAHg	PlSPg	PlMBg	PoHI	PoLHg	WB1	WB2	WP	RSI1	RLD1	RLD2
Methane*	4.8	259.7	3.6	553.6	-	1.0	-	ND	16.0	74.7	6.3
Carbon dioxide*	11329.6	17157.2	7226.4	11356.1	-	4538.6	-	2186.5	12715.1	14920.4	5561.5
Hydrogen*	ND	3.8	ND	1.8	-	1.0	-	11.4	1.5	ND	1.4
Acetate (µM)	9.0	3.2	15.3	22.3	79.3	38.4	21.8	20.6	77.0	99.9	18.5
Lactate (µM)	5.3	4.2	7.2	2.4	2.5	2.2	3.6	5.3	4.1	12.6	2.4
Formate (µM)	11.8	10.1	12.9	7.3	6.1	14.2	11.0	11.7	6.1	25.6	8.9
Propionate (µM)	ND	ND	ND	1.7	6.8	4.8	2.9	4.7	12.1	24.7	2.4
Chloride (mM)	495.1	515.2	535.4	535.4	520.7	370.6	474.5	481.1	426.7	487.7	560.2
Sulphate (mM)	20.4	22.0	23.3	22.4	22.5	16.4	23.8	20.5	22.0	31.1	30.8
Phosphate (µM)	ND	ND	ND	13.3	ND	70.0	100.0	ND	105.0	260.0	10.0
Nitrate (µM)	ND	ND	2.5	ND	6.7	20.0	10.0	ND	140.0	ND	10.0
Thiosulphate (µM)	ND	25.6	4.8	2.1	ND	ND	ND	ND	ND	6.4	3.6
Sulphide (µM)	-	-	-	-	-	ND	-	ND	ND	-	ND
Ammonium (µM)	179.9	226.7	90.6	229.2	124.6	208.1	209.5	8.6	602.3	485.1	54.7
Magnesium (mM)	47.1	50.3	52.6	59.5	60.7	34.7	21.7	56.6	52.4	48.9	53.8
Calcium (mM)	8.1	8.7	9.4	10.3	10.8	5.9	3.8	9.6	9.2	8.5	8.9

Pl: Plymouth, Po: Portsmouth, W: Wimereux, R: Roscoff, AH: Admirals Hard, SP: Stonehouse Pool, MB: Mount Batten, HI: Horsea Island, LH: Langstone Harbour, B: Beach, P: Puddle, SI: Souris Island, LD: Lacaze Duthiers, g: geochemistry site, *: µmol.g^{-1} wet sediment, ND: Not detected, -: Not mesured

TABLE 4.2 – Geochemical data from sediments from reference sub-sites.

Cirriformia tentaculata and *Cirratulus cirratus*

A total of four *C. cirratus* and 78 *C. tentaculata* were analysed during this study (Table S1). Specimens of *C. cirratus* could be distinguished from *C. tentaculata* by the presence of two rows of eyes on their head and because they harbour only the astome *Durchoniella cirratuli* (Fauvel, 1927; De Puytorac, 1954). Although *C. cirratus* was searched for at all sites, they were only found at Stonehouse Pool in very low abundance. Very low density of *C. tentaculata* was observed on Horsea Island compared to all the other sites, as only two worms were collected

despite sampling of a large area, and only one worm harboured *Durchoniella* cells.

The phylogenetic diversity of both Cirratulidae was analysed based on the mitochondrial gene coding for the cytochrome oxidase subunit I (*cox-1*, 620 bp, Figure S1). Tree topologies using Maximum Likelihood (ML), Bayesian inference (BI), Maximum Parsimony (MP), and Neighbour Joining (NJ) methods were very similar. Nine clades based on a 100% identity cut-off, were detected after the analysis of the 82 sequences with these four phylogenetic methods. Two sequences from worms harbouring *D. cirratuli* and corresponding to a morphological description of *C. cirratus* (PlSP1I4 and PlSP2I4, Figure S1) were affiliated with the clade 5 of *C. tentaculata*. In contrast, the worm PlMB1I5 harbouring *D. brasili* and *D. legeri-duboscqui* was affiliated with two sequences of *C. cirratus*. Sequences from *C. tentaculata* grouped in two major clades (clade 5 and clade 6). All the sequences of *C. tentaculata* from PlAH (n=10) grouped in clade 5, which also included a majority of worm sequences retrieved from the English coast, whereas sequences from the French coast were mainly placed in clade 6. Moreover, three polychaete sequences from Roscoff formed two clusters (clade 3 and 4) well supported by the four phylogenetic methods (RSI1I4, RSI1I1, RSI1I3, Fig. S1). Based on the phylogenetic diversity of *cox-1* gene sequences and redundancy analysis, no statistically significant biogeographical distribution was found for worms from Plymouth, Portsmouth, Wimereux, and Roscoff. Moreover, no distinct monophyletic clade was formed by polychaete sequences from each side of the English Channel.

Durchoniella species

Sixty four percent of the *C. tentaculata* analysed harboured at least one *D. brasili* and/or *D. legeri-duboscqui*, and only a very low abundance (n=3) of *D. cirratuli* was retrieved from one of the four *C. cirratus* (PlSP2I4) whereas between 28 and 381 cells of *D. cirratuli* were detected in the three others (Figure 4.13 ; Table S1). Moreover, the average number of astomes (n=92) per worm from Plymouth was lower than for worms from Portsmouth and Roscoff (n=310). Only 50% of worms from AH sites harboured ciliates, and no evidence of astomes was found from all cirratulids collected from Wimereux.

Furthermore, the relative abundance of the two *Durchoniella* species in *C. tentaculata* was different between sites, although on average *D. brasili* (84%) was more abundant than *D. legeri-duboscqui* (16% ; Figure 4.13). *Durchoniella brasili* dominated the gut of worms from Roscoff (85% from 100% of cells), except for two worms which harboured 50% and 70% of *D. legeri-duboscqui* (sites RSI1 and RLD2). At Portsmouth, *D. brasili* was more abundant in worms from PoHI and PoLH1 (64% from 100% of cells), while 50% of the *C. tentaculata* from PoLH2 harboured 40% to 97% of *D. legeri-duboscqui*. The worms from Plymouth sites had a majority of *D. legeri-duboscqui* (55 from 77% of cells).

However, the distribution of the different *Durchoniella* per Cirratulidae between sites was not significantly explained by any statistical correlation (redundancy analysis) using the astome abundance and geochemical data (Ra^2 : 0.36, 45% of the biological variation significantly

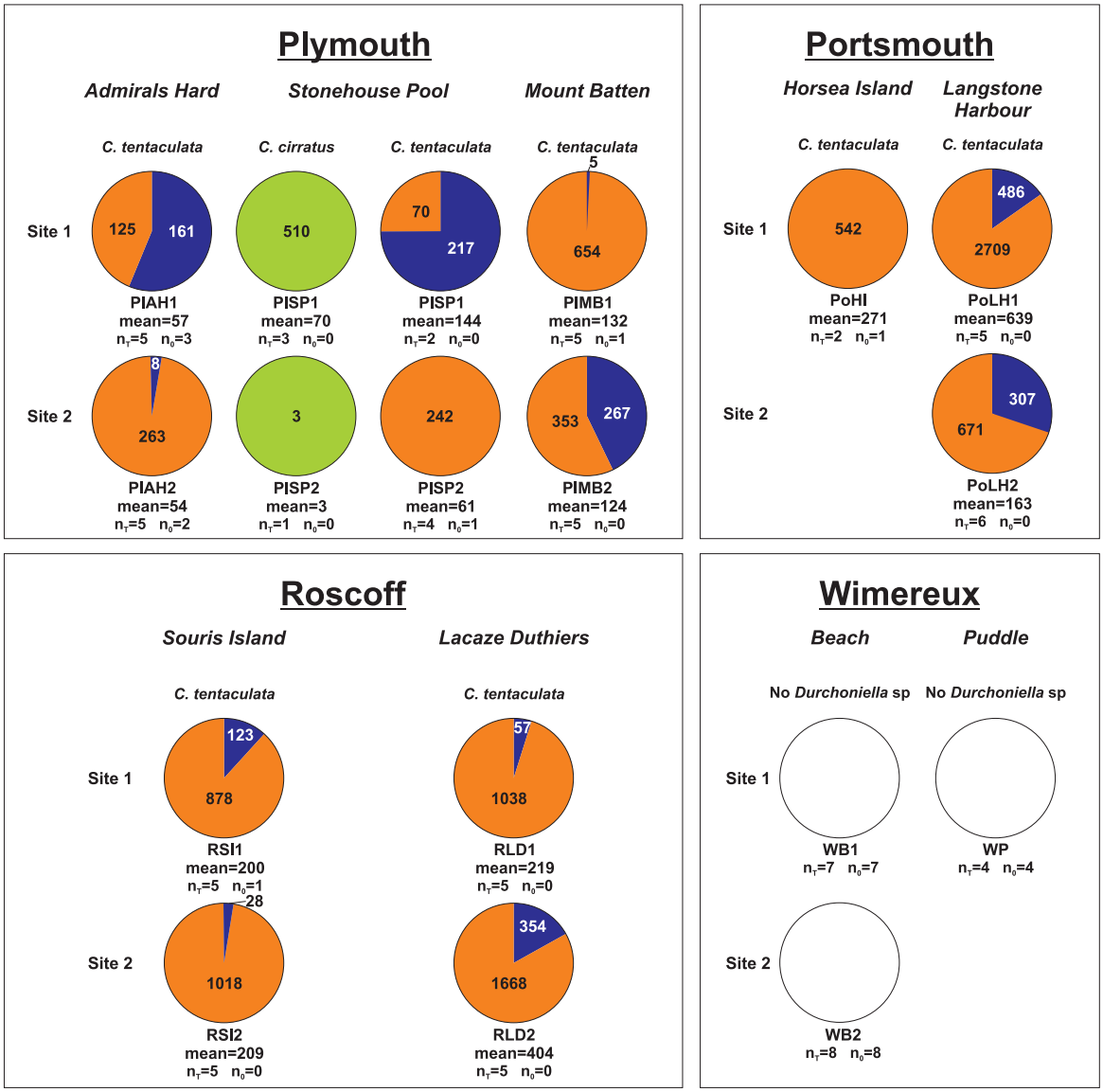

FIG. 4.13 – Relative abundance of *Durchoniella* species retrieved from cirratulids collected at each site. The three *Durchoniella* species are colour coded : orange for *Durchoniella brasili*, blue for *Durchoniella legeri-duboscqui* from *Cirriformia tentaculata*, and green for *Durchoniella cirratuli* from *Cirratulus cirratus*. (n_T) corresponds to the total number of worms analyzed, and (n_0) corresponds to the number of worms without astomes.

explained by the model ; data not shown).

The phylogenetic analysis of the 18S rRNA gene (Figure 4.14) and mitochondrial cytochrome oxidase subunit I gene sequences (*cox-1* ; Figure S2) from 119 individual endocommensal *Durchoniella* cells was performed using ML, BI, MP, and NJ methods. All tree topologies were identical for the 18S rRNA gene. Although up to 5 *Durchoniella* single-cells were isolated per worm for phylogenetic analysis of the 18S rRNA, 20% could not be amplified. Except for some cells, *Durchoniella* cells from the same species isolated from the same worm had identical gene sequences. The 67 *Durchoniella* sequences analysed with the four phylogenetic methods grouped in four clades based on 1630 bp and 100% identity cut-off (Figure 4.14). Clade 1 corresponded to *D. cirratuli* and the clade 4 to *D. brasili*. *Durchoniella legeri-duboscqui* species grouped into two different clades based on a 100% identity cut-off : clade 2 had three representative sequences from Roscoff and Portsmouth, and clade 3 had 16 representative sequences distributed between different sites. The *D. legeri-duboscqui* clade 3 was affiliated to the *D. brasili* clade 4, while the *D. legeri-duboscqui* clade 2 was related to the *D. cirratuli* clade 1, although this topology was not supported by high statistical confidence levels.

The *cox-1* gene tree topologies, using ML, BI, MP, and NJ methods, were identical. Although up to 5 *Durchoniella* single-cells were isolated per worm, the *cox-1* gene could only be amplified in 50% of them, notably from *D. legeri-duboscqui*, as only one cell was amplified (Clade 3, Figure S2). The 62 *cox-1* gene sequences of *Durchoniella* cells analysed with the four phylogenetic methods grouped into four clades based on 630 bp-length and 100% identity cut-off (Figure S2). The *cox-1* gene clades were identical to those observed in the 18S rRNA gene analysis : clade 1 corresponded to *D. cirratuli* ; clade 3 to *D. legeri-duboscqui* ; and *D. brasili* grouped into two different clades, clade 4a with eight representative sequences from Roscoff and Portsmouth, and clade 4b with 48 representative sequences from all sites. The affiliation of clade 4a and clade 4b showed high support values for the four phylogenetic methods (Figure S2).

Clade 1 (*D. cirratuli*) and clade 3 (*D. legeri-duboscqui*) were found with both *cox-1* and 18S rRNA genes, whereas clade 4 (*D. brasili*) formed two clades with *cox-1* gene. Clade 2 (*D. legeri-duboscqui*) was only observed using the 18S rRNA gene, as a probable consequence of the low number of *D. legeri-duboscqui cox-1* gene sequences analysed. However, the distribution of sequences in clades 2 and 3, or in clades 4a and 4b did not seem to be related to any geographical or geochemical factor. Moreover, no statically significant (p=0.05) cospeciation events were found between *Durchoniella* and their hosts (data not shown).

Phylogenetic analysis of *Bacteria*

Microscopic observations using DAPI staining of *Durchoniella* cells from different sites always showed the presence of prokaryotes (data not shown). Out of the 26 possible representative combinations between sites and *Durchoniella* sp., 23 DNA extractions were performed from *Durchoniella* cells stored in Lugol or glutaraldehyde. However, only 13 bacterial 16S rRNA gene

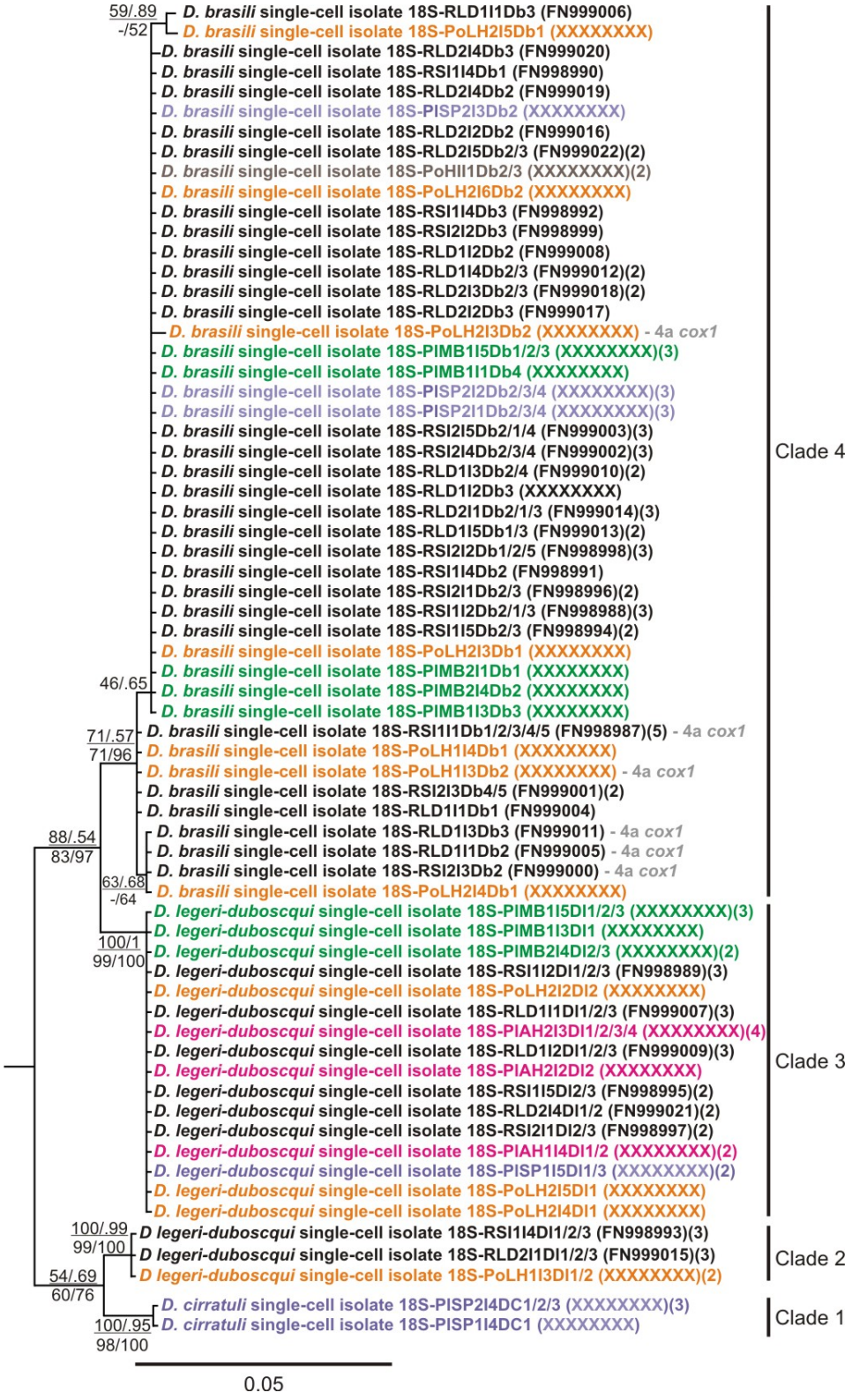

FIG. 4.14 – Maximum Likelihood (ML) tree based on 18S rRNA gene sequences of *Durchoniella* cells isolated from cirratulid polychaetes. ML bootstrap value, bayesian posterior probability (BI), Maximum Parsimony (MP) and Neighbour Joining (NJ) bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. The colours indicate the geographical origin : Pink for AH1/AH2 (Plymouth), purple for SP1/SP2 (Plymouth), green for MB1/MB2 (Plymouth), brown for HI (Portsmouth), orange for LH1/LH2 (Portsmouth), blue for WB1/WB2 and WP (Wimereux), and black for RSI1/RSI2 and RLD1/RLD2 (Roscoff). The number in brackets after the GenBank Accession Number corresponds to the number of identical sequences based on the total length of the gene sequence. The tree is rooted with the sequence of *Anoplophrya marylandensis* (AY547546).

PCR amplifications were obtained, as a probable consequence of too low bacterial DNA concentrations. Endocytoplasmic bacterial 16S rRNA gene clone libraries were constructed from the positive PCR amplifications, representing a total of 304 clones (Figure 4.15). Three to eleven OTUs were assigned per clone library based on 900 bp and 99% identity cut-off (Schloss and Handelsman, 2004). The coverage values for the 16S rRNA gene clone libraries ranged between 88% and 100%. Nine bacterial phylotypes were identified and heterogeneously distributed among the 13 clone libraries (Figure 4.15). No statistically significant distribution or correlation was found between the relative abundance or presence of bacterial phylotypes and the geographic or geochemical factors (RDA analysis, data not shown).

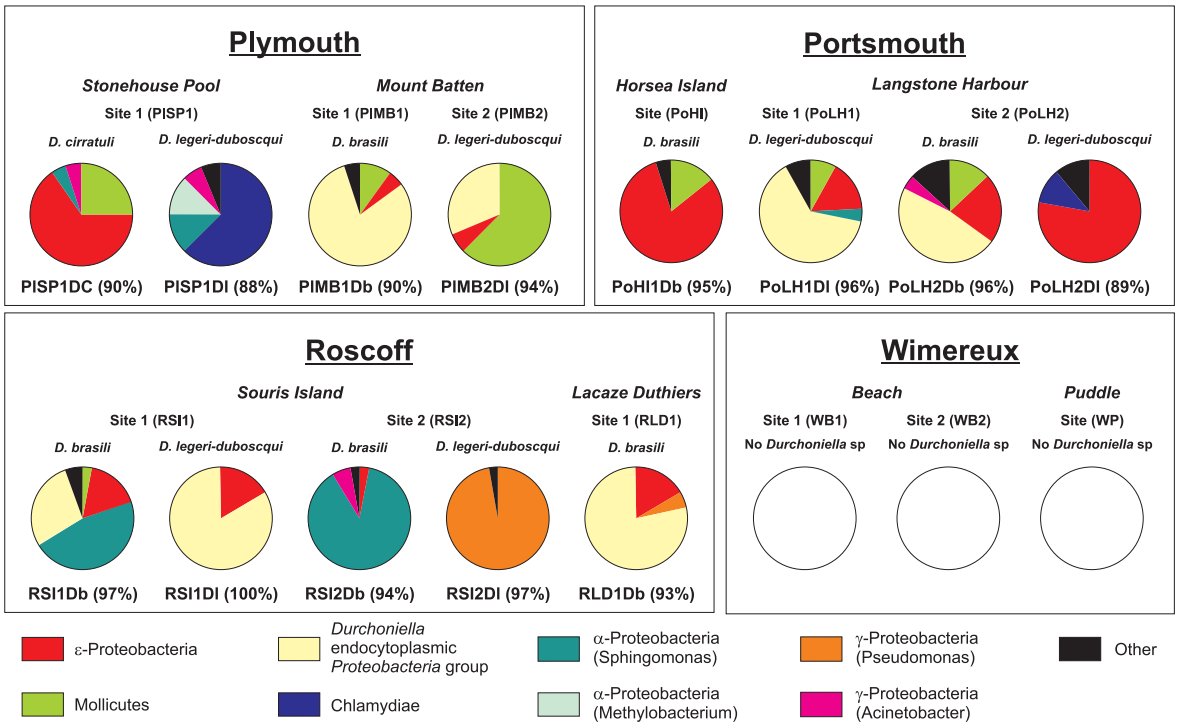

FIG. 4.15 – Distribution by sites of bacterial community structure based on 16S rRNA from the three *Durchoniella* species . The relative abundance of each phylotype was calculated and colour coded in the pie charts. The name of the genetic library is indicated under each pie with the coverage value indicated in brackets. At Wimereux sites, no *Durchoniella* was observed so no bacterial genetic library was constructed.

The 16S rRNA gene clone libraries were dominated by three phylotypes related to Mollicutes (Figure S3), *Epsilon-Proteobacteria* (Figure 4.16), and unclassified *Proteobacteria* (Figure 4.17). Sequences related to Mollicutes were mostly detected in clone libraries from Plymouth and Portsmouth. All sequences related to Mollicutes grouped within a single cluster only very distantly affiliated with its closest relatives (88% identity), a group of uncultured Mollicutes symbionts retrieved from isopods, fish and shrimps (Figure S3). Sequences related to *Epsilon-*

Proteobacteria were retrieved from 92% of the clone libraries and formed a unique cluster supported by high statistical confidence levels by the four phylogenetic methods (Figure 4.16). These *Epsilon-Proteobacteria* grouped with sequences previously retrieved from *D. brasili* and *D. legeri-duboscqui* (FN999957, FN999958, and FN999981 ; Figure 4.16) and were only distantly affiliated to environmental sequences retrieved from gorgonian and sunken-wood gastropods. As this independent *Epsilon-Proteobacteria* cluster from *Durchoniella* was only very distantly related to cultured relatives (i.e. highest similarity to pure culture, 88%), it was named Marine Group III by analogy to Marine Group I and II described by Campbell *et al.* (2006).

A group of sequences affiliated to unclassified *Proteobacteria* was well represented or dominated the seven 16S rRNA gene clone libraries (27% to 83%). The closest affiliated sequence, a *Gamma-Proteobacteria*, shared only 80% identity with the sequences from this new group named *Durchoniella* endocytoplasmic *Proteobacteria* group (DEPG, Figure 4.17).

Chlamydiae, retrieved in abundance in the previous genetic libraries constructed on *Durchoniella* collected from Roscoff (Sauvadet *et al.*, Sub.), were only detected in PlSP1Dl and PoLH2Dl clone libraries, respectively with 63% and 11% of the sequences. These sequences were related to the Group 2 defined in the family Simkaniaceae (Sauvadet *et al.*, Sub.).

In order to identify possible evolutionary relationships between the astomes and their endocytoplasmic bacteria, a 16S rRNA gene phylogenetic consensus tree of *Epsilon-Proteobacteria* and DEPG bacterial symbionts isolated from *Durchoniella* species was compared to the tree based on 18S rRNA gene of their *Durchoniella* hosts, and showed statistically significant congruency ($p = 0.001$, for a null hypothesis of host switches ; Figure S4).

Discussion

Distribution of cirratulids and their endocommensal *Durchoniella*

Although the factors controlling the distribution of Cirratulidae in tidal sediments remain unclear, it has been previously suggested that the size of the grain and the presence of nutrients in the sediment have an effect (George, 1964). The higher hydrocarbon pollution levels that may occur in small urban harbours, such as Admirals Hard and Stonehouse Pool, apparently did not affect the cirratulid distribution as it has been previously shown that cirratulids could resist oil pollutions (George, 1971).

Some studies suggest that the worm's age and the specific environmental conditions might control the distribution of endocomensal astomes (De Puytorac, 1994). As age could be a potential factor controlling the astome distribution, all the cirratulids analysed in this study had the same range of size in order to compare specimens of approximately the same age (Wilson, 1936). However, no statistically significant environmental factor was found to clearly control the abundance of endocomensal *Durchoniella*. Wimereux was one of the most remarkable sampling sites as none of the worms (n=19) analysed harboured *Durchoniella* cells. The *C. tentaculata* at Wimereux did not appear to be a cryptic cirratulid species as the phylogenetic analyses

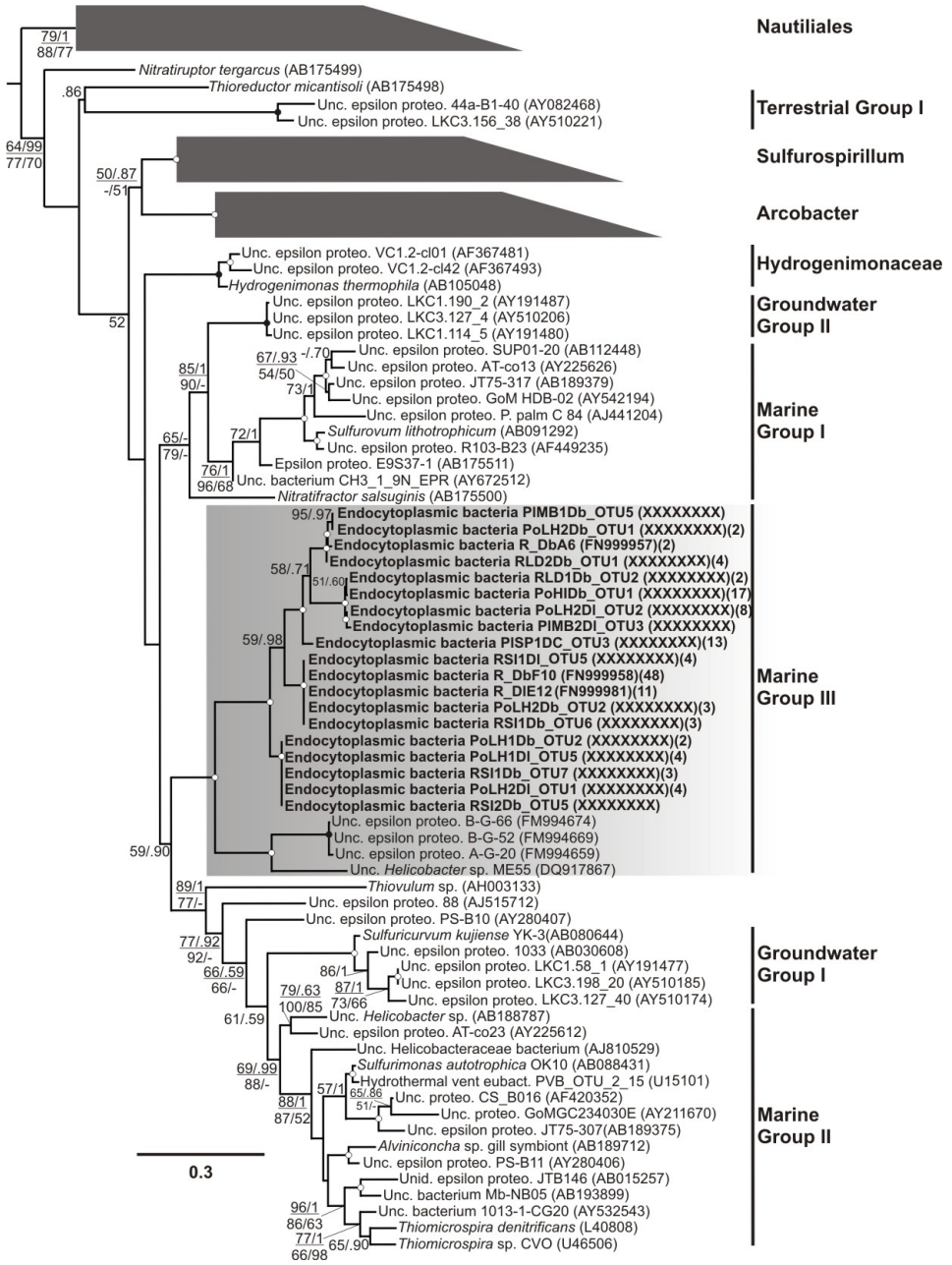

FIG. 4.16 – Maximum Likelihood (ML) tree based on 900 bp of the 16S rRNA gene sequences of *Epsilon-Proteobacteria* retrieved from *Durchoniella* cells isolated from cirratulid polychaetes. Study sequences are in bold. Each OTU from each clone library is represented by one sequence with 99% similarity grouping. ML bootstrap value, bayesian posterior probability (BI), Maximum Parsimony (MP) and Neighbour Joining (NJ) bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. Black dots correspond to values of one (posterior probability) and 100% (ML, NJ, MP). White dots correspond to values between 80 and 100% (or 0.80 and one). The numbers of clones of each representative sequence are indicated in brackets. The outgroup (not shown) is the sequence of the *Gamma-Proteobacteria Escherichia coli* (X80725).

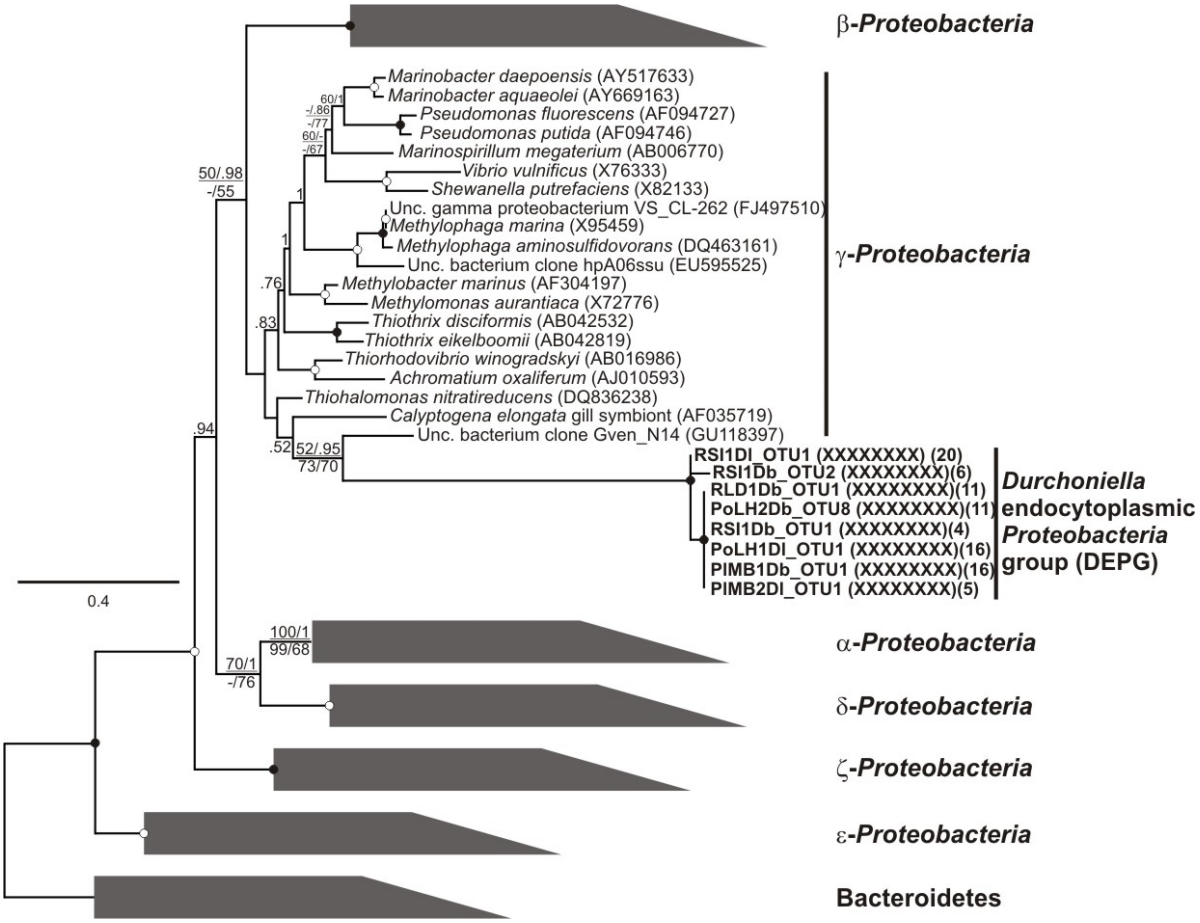

FIG. 4.17 – Maximum Likelihood (ML) tree based on 900 bp of the 16S rRNA gene sequences of *Proteobacteria*. The different classes are detailed in Emerson *et al.* (Emerson *et al.*, 2007). Sequences retrieved from *Durchoniella* cells isolated from cirratulids polychaetes are in bold. Each OTU from each clone library is represented by one sequence with 99% similarity grouping. ML bootstrap value, bayesian posterior probability (BI), Maximum Parsimony (MP) and Neighbour Joining (NJ) bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. Black dots correspond to values of one (posterior probability) and 100% (ML, NJ, MP). White dots correspond to values between 80 and 100% (or 0.80 and one). The numbers of clones of each representative sequence are indicated in brackets. The outgroup is composed of Bacteroidetes sequences (M58770, M59051, L11703).

based on *cox-1* gene did not separate these worms in a distinct clade. Hence, absence of detectable astomes in the cirratulids from Wimereux may be controlled by environmental factors, as the chloride and sulphate concentrations in the pore waters were lower than the average concentrations measured from all other sites, suggesting freshwater fluxes in the sediment. The sediment from Wimereux was also less reduced and had lower methane concentrations than the other sites harbouring cirratulids with astomes. Admirals Hard was the only other site besides Wimereux harbouring a large proportion of worms without astomes (50%) and low astome abundance per worm (56 astomes). Hence, cirratulids in specific environmental conditions seemed able to live without astome, suggesting there is no mandatory requirement for cirratulids to harbour astomes.

Phylogenetic diversity of *Durchoniella* and cirratulids

The initial designing of primers for the amplification of the *cox-1* gene has been difficult due to the low number of sequences available in the databases (Gentekaki and Lynn, 2009). Hence, the amplification of the *cox-1* gene from *Durchoniella* was also difficult, suggesting that the primers were not adapted to this genus. However, both 18S rRNA and *cox-1* genes discriminated the three species of *Durchoniella* (i.e. *D. brasili*, *D. legeri-duboscqui* and *D. cirratuli*), confirming the morphological descriptions (De Puytorac, 1954) and a previous molecular genetic analysis (Sauvadet *et al.*, Sub.). Although the *Durchoniella cox-1* and 18S rRNA genes seemed to share the same evolutionary history (tree topology), the *cox-1* gene showed higher degrees of resolution as the *D. brasili cox-1* gene clade 4 clearly formed two sub-clades in opposition to the 18S rRNA gene. Nevertheless, a slight incongruence was noticed between the *cox-1* and 18S rRNA gene tree topologies for *Durchoniella* PoLH2I3Db2, suggesting a divergence in the evolutionary history of the mitochondrial marker and the nuclear marker that could have occurred during significant events, such as conjugation.

Although the *cox-1* gene has a high rate of evolution, and was therefore previously used to successfully investigate intraspecific variation in ciliate populations (Barth *et al.*, 2006; Gentekaki and Lynn, 2009), both 18S rRNA and *cox-1* genes showed no or a very low genetic diversity at the isolate level for *C. cirratus*, *C. tentaculata*, *D. brasili*, *D. legeri-duboscqui*, and *D. cirratuli*, suggesting either (i) wide dissemination events or (ii) no specific genetic drift and speciation events have occurred recently. However, *D. brasili*, *D. legeri-duboscqui*, and *D. cirratuli* were host-specific, as the majority of the 56 endocomensal astome genera found over 20 different host families (De Puytorac, 1994). One of the worms morphologically identified as *C. cirratus* and harbouring only *D. cirratuli* cells was phylogenetically affiliated to *C. tentaculata*, suggesting the occurrence of selection mechanisms between the cirratulids and *Durchoniella*. This phylogenetic incongruence between the morphological description and the phylogenetical marker *cox-1* gene could also be the consequence of different evolutionary histories between the mitochondrial genes and the nuclear genome. However, no geographical or geochemical pattern could explain the phylogenetic distribution of *Durchoniella*. Although the explanation of

the biogeographical distribution of ciliates remains controversial (for review, Foissner, 2008), it seems to be linked to the diversity and gene fluxes that occur in the ecosystem studied (Katz *et al.*, 2005). With other ciliates, coastal marine environments seem to be stable evolutionary environments with high gene flow (Katz *et al.*, 2005). Hence, the low phylogenetic diversity and wide distribution of cirratulid and *Durchoniella* species suggest a co-dispersion of cirratulid and *Durchoniella* during the worm hypothetic pelagic stages. However, more discriminating markers, such as allozymes or microsatellites, and broader geographical sampling would be required to confirm the lack of intraspecific diversity and biogeographical distribution.

Durchoniella endocytoplasmic bacterial symbiont distribution

Nine different bacterial phylotypes were heterogeneously distributed among *Durchoniella* cells retrieved from *C. tentaculata* or *C. cirratus*. The occurrence of multiple bacterial phylotypes confirmed the previous observations on *Durchoniella* cells from Roscoff (Sauvadet *et al.*, Sub.), suggesting there was no specific bacterial phylotype associated with each *Durchoniella* species. The presence of several endosymbiotic bacterial phylotypes is quite common in ciliates, and is usually constrained to specific compartments of the ciliate (for review Fokin, 2004). Three major phylotypes dominated >54% of the endocytoplasmic clone libraries (i.e. *Epsilon-Proteobacteria*, DEPG and Mollicutes) ; other phylotypes, such as Chlamydiae, were only found in 15% of the clone libraries and were probably related to punctual invasion or contamination.

Mollicutes are widespread commensals or pathogens of vertebrates, plants, and various arthropods (for review Razin and Hayflick, 2010). They rarely cause the death of their hosts, although they can produce chronic infections (Wang *et al.*, 2007). Moreover, these microorganisms have a reduced genome that does not allow a large range of metabolic activities (Razin and Hayflick, 2010). Mollicutes found in the midgut crustaceans, notably in isopods, might reduce the risk of infection by more parasitic or pathogen microorganisms (Wang *et al.*, 2007). The presence of Mollicutes in *Durchoniella* cells may be linked to opportunistic events or very specific environmental conditions, as Mollicutes were never detected previously (Sauvadet *et al.*, Sub.). This also suggests that these obligate intracellular bacteria were not required by *Durchoniella* and therefore could be infectious bacteria.

Epsilon-Proteobacteria were detected in a majority of the genetic clone libraries (85%). As previously suggested (Sauvadet *et al.*, Sub.), *Epsilon-Proteobacteria* may represent a host-specific symbiont (i.e. *Durchoniella*-specific) as they were retrieved from the three distinct *Durchoniella* species in geographically separated populations, and grouped within the same cluster without any known close relative (*Epsilon-Proteobacteria* Marine Group III). While MGIII did not appear to be specific to any *Durchoniella* sp., there was no obvious biogeographical distribution either. However, the presence of these putative chemolithotrophes was probably linked to the environmental conditions. Hence, the worm may channel oxygen from the sediment surface, and reduced compounds (i.e. methane and sulphide) present in the deeper sediment layer, to the potential chemolithotrophic bacteria of the endocommensal astome.

The bacterial communities could therefore fix carbon autotrophically and transfer synthesized organic compounds to the astome, a mouthless ciliate with an unknown nutritional mode.

The presence of a second group (DEPG) affiliated to *Proteobacteria* could also be a permanent endocytoplasmic event as this group of sequences was observed in 54% of clone libraries regardless of geographic location. The DEPG sequences strongly diverge phylogenetically within the *Proteobacteria*, suggesting an ancient co-speciation event with the genus *Durchoniella*. Moreover, statistically significant (p=0.001) cospeciation analyses between *Durchoniella* hosts and their endocytoplasmic bacteria suggested that *Proteobacteria* could have been acquired by an ancestral protist. Co-evolution events allowed by vertical transmission (i.e. binary fission and conjugation) could have therefore favoured the strong divergence observed within the Marine Group III and DEPG. This hypothesis would also need to be confirmed by an extensive sampling of different astome species harbouring *Proteobacteria*.

Additional data using different molecular marker with *Durchoniella* and cirratulids from very distinct geographical locations and other species of astomes could help us resolve their distribution and validate the possible hypothesis of a bacterial co-speciation event. Furthermore, the use of genomic approaches may help to understand the potential metabolic roles of these endosymbiotic bacteria and their capacity to interact with other prokaryotes, astomes, worms, and environment.

Materials and methods

Source and location of *Durchoniella* spp.

Intestinal contents of *Cirriformia tentaculata* and *Cirratulus cirratus* collected between Septembre 9^{th} 2009 and October 6^{th} 2009 from different sites from Plymouth, Portsmouth, Wimereux, and Roscoff was examined. All the characteristics of the sites were available in (Figure 4.12) and table S1. Cirratulids were meticulously cleaned with 0.2 μm-filtered seawater and by removing all grains of sand with forceps. Each worm was then placed individually in a 6-well culture plate filled with 10 mL 0.2 μm-filtered seawater. Dissections occured within week following the sampling in the natural environment, after incubation of worms for at least 30 minutes in a 7% (w/v) $MgCl_2$ filtered seawater solution. The total number of each *Durchoniella* species per worm was counted following the morphological descriptions given by De Puytorac (1954).

Chemical analysis of sediment pore water

Sediment samples were centrifuged for 15 min at 2300 g in a Hettich Rotanta 460R centrifuge at 10°C with oxygen-free nitrogen. The pore water supernatant was then removed and analysed for anions, cations, and suphide. Chloride, sulphate, nitrate, volatile fatty acid (VFA) concentrations, and other anions were determined using an ICS-2000 ion chromatography sys-

tem with an AS50 autosampler (Dionex Ltd, Surrey, UK) fitted with two Ionpac AS15 columns in series, and an anion self-regenerating suppressor (ASRS®-ULTRA II 4mm) in combination with a DS6 heated conductivity cell (Dionex Ltd, Surrey, UK) under the conditions described previously (Webster *et al.*, 2009). Ammonium and other cations were analysed using a DX-120 ion chromatography system with an AS40 autosampler (Dionex Ltd, Surrey, UK) fitted with an Ionpac CS16 and a cation self-regenerating suppressor (CSRS®-300 4mm) in combination with a DS4-1 heated conductivity cell (Dionex Ltd, Surrey, UK), and using 32 mM methanesulphonic acid as an eluent. Methane, hydrogen, and carbon dioxide were analysed by adding 2.5 cm^3 of sediment to 5 mL of KCl (10 mM) in 20 mL volume gas-tight serum bottles and headspace gas analysed by GC using a modified Perkin Elmer/Arnel Clarus 500 Natural Gas Analyser fitted with a flame ionization detector and a thermal conductivity detector. Headspace gas from sediment slurries was analysed by GC directly. The sulphide analysis was performed as described by Cline (1969).

DNA isolation

DNA on worms was extracted by a modified CTAB (hexadeCylTrimethyl Ammonium Bromide) protocol Doyle and Doyle, 1987. A small fresh piece or ethanol-preserved sample dried off were incubated in a 2% (w/v) CTAB solution with 2% (w/v) polyvinylpolypyrrolidone (PVP), preheated at 60°C for six hours with occasional vortexing. Nucleic acids were extracted with an equal volume of chloroform-isoamyl alcohol (24 :1). After centrifugation for 15 min at 15,000 *g* at 4°C, the aqueous phase was transferred into a clean tube and 2/3 volumes of cold isopropanol were added at -20°C overnight in order to precipitate the nucleic acids. DNA was then centrifuged for 15 min at 14,000 *g* at 4°C, washed with 70% (v/v) ethanol and hydrated in sterile distilled water prior to storage at 4°C.

DNA of single-cells was extracted by a modified Guanidinium Isothiocyanate (GITC) protocol (Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006). Each fresh single-cell was ground in 50 μL of the GITC extraction buffer, and crushed with tube-adapted piston pellet (Kimble Chase®) for at least one minute. Tubes were incubated for 20 min at 72°C, and quickly centrifuged. One volume of cold isopropanol was added at -20°C overnight. After centrifugation for 15 min at 14,000 *g* at 4°C, one washing with 70% (v/v) ethanol, DNA was hydrated in sterile distilled water prior to storage at 4°C. Each extraction product was used to amplify eukaryotic 18S rRNA gene and cytochrome oxidase (*cox-1*) gene. DNA used to amplify prokaryotic 16S rRNA gene was extracted on 100 cells (except for RSI2Dl library, 28 cells) of astomes fixed in Lugol or glutaraldehyde. Before prokaryotic DNA extraction, fixed astomes were rinsed five times in filtrated/sterilized/filtrated seawater, and all materials (i.e. dissection tools, dissection boxes and jar) were incubated in DNA AWAY® (Molecular BioProducts), ethanol, and sterilized for 30 min at 121°C.

PCR and sequencing

All PCR mixtures of 30 μL final volume contained 1X Taq DNA Polymerase buffer, 1 μL of dNTPs (20 mM each), 3 μL of $MgCl_2$ (25 mM), 0.1 μL of each primer (100 μM), and 0.25 μL of Taq DNA Polymerase (5 unit/μL, Promega®, Madison, Wisconsin). All amplifications were performed using a GeneAmp PCR system 9700® (Applied Biosystems™) or MJ Mini Cycler (Biorad™). Negative controls were also carried out with DNA extractions performed with no sample, with and without piston pellet. For all controls, no PCR products were detected.

The eukaryotic 18S rRNA gene was amplified with primers EukMK-63F/EukMK-1818R (5'-ACG CTT GTC TCA AAG ATT A-3' ; 5'-ACG GAA ACC TTG TTA CGA-3' ; M. Kawachi, unpublished) with the following conditions : one cycle of 5 min at 95°C ; 30 cycles of 1 min at 95°C, 1.5 min at 57°C and 1.5 min at 72°C ; and one cycle of 10 min at 72°C. The ciliate *cox-1* gene was amplified with primers coxFper/339R (Gentekaki and Lynn, 2009) and the following inversed touchdown PCR protocol : 5 min at 94°C ; followed by 5 cycles of 30 sec at 94°C, 1 min at 46°C (increasing 1.8°C every cycle), and 1.5 min at 72°C ; then followed by 30 cycles of 30 sec at 94°C, 1 min at 55°C, and 1.50 min at 72°C ; and a final amplification step of 10 min at 72°C.

The polychaete *cox-1* gene was amplified by nested PCR with primers M1/M2 and 1 μL of diluted DNA (1/10) in the first round. For the second round, 1 μL of the first product was amplified with primers LCO1490/HCO2198 (Folmer *et al.*, 1994). The two rounds were realized under the following conditions : one cycle of 5 min at 95°C ; followed by 35 cycles of 1 min at 95°C, 45 sec at 50°C and, 1 min at 72°C ; and then one cycle of 10 min at 72°C.

Bacterial 16S rRNA gene amplification was conducted by PCR with primers E8f/U1492r (DeLong, 1992; Eden *et al.*, 1991). PCR cycles were as follows : one cycle of 5 min at 94°C ; 30 cycles of 1 min at 94°C, 1.5 min at 49°C and, 2 min at 72°C ; and one cycle of 6 min at 72°C. Three independent PCR products were pooled and purified (QIAquick PCR purification Kit ; Qiagen™) and cloned into *Escherichia coli* (One Shot F10', Promega™) using the pCR2.1-TOPO TA vector system (Invitrogen®) following the manufacturer's instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Positive PCR products were purified (ExoSAP-IT® For PCR Product Clean-Up, USB™) and sequenced using the Big Dye Terminator Cycle Sequencing Kit version 3.0 (PE Biosystems™) and an ABI PRISM model 377 (version 3.3) automated sequencer with specific primers.

Phylogenetic analysis

Available sequences were edited in the BioEdit 7.0.5.3 program and aligned using CLUSTALW2 (Hall, 1999; Larkin *et al.*, 2007). To determine the first phylogenetic affiliation, each sequence was compared with sequences available in the NCBI database (National Center for Biotechnology Information) using BLAST (Altschul *et al.*, 1990). For the worm tree based on

cox-1 gene sequences (Figure S1), TPM2uf+I was selected by hierarchical Likelihood Ratio Tests (hLRT) with equal distributed rates and a proportion of invariable sites, via jModeltest 0.1.1 Posada, 2008 and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by Maximum Likelihood (ML) and Bayesian inference (BI) (Posada and Crandall, 1998). Maximum Likelihood was conducted using PhyML 3.0 (Guindon *et al.*, 2005) and the robustness of inferred topology was supported by bootstrap resampling (100). Bayesian inference was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and started with a random tree, run for 3 million generations in four chains (Standard deviation = 0.010) and burn-in of 7500 generations in order to ensure the use of only stable chains. For the *Durchoniella* trees based on 18S rRNA gene (Figure 4.14) and *cox-1* gene sequences (Figure S2), TrN+G was selected by hLRT with gamma distributed rates, via jModeltest 0.1.1 (Posada, 2008) and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by ML and BI (Posada and Crandall, 1998). Maximum Likelihood was conducted using PhyML 3.0 (Guindon *et al.*, 2005) and the robustness of inferred topology was supported by bootstrap resampling (100). Bayesian inference started with a random tree, run for 1,956,000 and 3 millions generations, respectively for 18S and *cox-1* set, in four chains (Standard deviations = 0.009) and burn-in of 5000 and 7500 generations in order to ensure the use of only stable chains. For the bacterial 16S rRNA trees (Figure 4.16, Figure 4.17, Figure S3), TIM1+I+G or TIM2+I+G (Mollicutes) with gamma distributed rates and a proportion of invariable sites were selected via jModeltest 0.1.1. Bayesian inference started with a random tree, run for 1,300,000 generations for the Mollicutes and the *Proteobacteria* sequences, and 2,715,000 for the *Epsilon-Proteobacteria*, in four chains (Sd = 0.004, 0.006, and 0.010 respectively) and burn-in of 3250 or 6800 generations respectively. Neighbour Joining (NJ) and Maximum of Parsimony (MP) trees were inferred using PAUP 4.0b10 via PaupUp graphical interface (Calendini and Martin, 2005; Swofford, 2000). The robustness of inferred topology was supported by bootstrap resampling (1000) with NJ and MP ; values over 50% are shown on the trees. The ML analysis gave the same topology as BI. Trees are visualised and noted with TreeDyn (Chevenet *et al.*, 2006).

Good's coverage was used to calculate the coverage index. It is a nonparametric estimator of the proportion of phylotypes in a library of infinite size that would be represented in a smaller library. The coverage index (C_x) of the clone libraries was calculated by Good's method (Good, 1953) as described by Singleton and colleagues (Singleton *et al.*, 2001), C_x = 1-(n_1/N) where n_1 is the number of OTUs appearing only once in a library, and N is the library size.

Reconciliation analysis with Jungles analysis (Charleston, 1998) as implemented in TreeMap 3.0 was used to assess whether species of bacteria and ciliates have undergone parallel diversification. TreeMap (Page, 1994) uses reconciled trees to compute the fit between the host and symbiont phylogenies, allowing the incorporation of host-switching events and considering all potentially optimal solutions. TreeMap allows a graphic display of the results and therefore the identification of coevolutionary events.

Nucleotide sequence accession numbers

The sequences are available from the GenBank database under the following accession numbers : XXXXXXXX to XXXXXXXX.

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Supplementary material

Table S1 Sample locations and number of *Durchoniella* cells counted for each cirratulids. Except labeled individuals, all the worms were *Cirriformia tentaculata*.

Sample	City	Location	Site	Individual	Coordinates	Temperature	Size (mm)	Sampling date	Dissection date	Dl	Db	DC
PlSPS1l1	Plymouth	Stonehouse Pool	S1	Cc-l1	4°9.87'W 50°21.92'N	19.1°C	50	07/09/2009	09/09/2009	0	0	101
PlSPS1l2	Plymouth	Stonehouse Pool	S1	l2			50	07/09/2009	09/09/2009	209	60	0
PlSPS1l3	Plymouth	Stonehouse Pool	S1	Cc-l3			66	07/09/2009	09/09/2009	0	0	381
PlSPS1l4	Plymouth	Stonehouse Pool	S1	Cc-l4			55	07/09/2009	10/09/2009	0	0	28
PlSPS1l5	Plymouth	Stonehouse Pool	S1	l5			60	07/09/2009	09/09/2009	8	10	0
PlSPS2l1	Plymouth	Stonehouse Pool	S2	l1	4°9.90'W 50°21.92'N	19.1°C	52	07/09/2009	10/09/2009	0	19	0
PlSPS2l2	Plymouth	Stonehouse Pool	S2	l2			80	07/09/2009	10/09/2009	0	205	0
PlSPS2l3	Plymouth	Stonehouse Pool	S2	l3			58	07/09/2009	10/09/2009	0	18	0
PlSPS2l4	Plymouth	Stonehouse Pool	S2	l4			48	07/09/2009	10/09/2009	0	0	3
PlSPS2l5	Plymouth	Stonehouse Pool	S2	l5			51	07/09/2009	09/09/2009	0	0	0
PlMBS1l1	Plymouth	Mount Batten	S1	l1	4°7.67'W 50°21.47'N	17.7°C	180	07/09/2009	10/09/2009	0	182	0
PlMBS1l2	Plymouth	Mount Batten	S1	l2			70	07/09/2009	10/09/2009	0	309	0
PlMBS1l3	Plymouth	Mount Batten	S1	l3			53	07/09/2009	11/09/2009	2	154	0
PlMBS1l4	Plymouth	Mount Batten	S1	l4			62	07/09/2009	11/09/2009	0	0	0
PlMBS1l5	Plymouth	Mount Batten	S1	l5			90	07/09/2009	11/09/2009	3	9	0
PlMBS2l1	Plymouth	Mount Batten	S2	l1	4°7.67'W 50°21.45'N	17.7°C	49	07/09/2009	11/09/2009	0	9	0
PlMBS2l2	Plymouth	Mount Batten	S2	l2			55	07/09/2009	11/09/2009	0	136	0
PlMBS2l3	Plymouth	Mount Batten	S2	l3			73	07/09/2009	11/09/2009	139	113	0
PlMBS2l4	Plymouth	Mount Batten	S2	l4			65	07/09/2009	11/09/2009	14	95	0
PlMBS2l5	Plymouth	Mount Batten	S2	l5			55	07/09/2009	11/09/2009	114	0	0
PlAHS1l1	Plymouth	Admirals Hard	S1	l1	4°9.80'W 50°21.87'N	17.0°C	55	07/09/2009	12/09/2009	0	1	0
PlAHS1l2	Plymouth	Admirals Hard	S1	l2			84	07/09/2009	12/09/2009	0	0	0
PlAHS1l3	Plymouth	Admirals Hard	S1	l3			83	07/09/2009	12/09/2009	0	0	0
PlAHS1l4	Plymouth	Admirals Hard	S1	l4			63	07/09/2009	13/09/2009	161	124	0
PlAHS1l5	Plymouth	Admirals Hard	S1	l5			58	07/09/2009	13/09/2009	0	0	0
PlAHS2l1	Plymouth	Admirals Hard	S2	l1	4°9.78'W 50°21.87'N	17.0°C	50	07/09/2009	12/09/2009	0	68	0
PlAHS2l2	Plymouth	Admirals Hard	S2	l2			53	07/09/2009	12/09/2009	4	195	0
PlAHS2l3	Plymouth	Admirals Hard	S2	l3			78	07/09/2009	12/09/2009	4	0	0
PlAHS2l4	Plymouth	Admirals Hard	S2	l4			54	07/09/2009	13/09/2009	0	0	0
PlAHS2l5	Plymouth	Admirals Hard	S2	l5			70	07/09/2009	13/09/2009	0	0	0
PoHIl1	Porstmouth	Horsea Island		l1	1°1.90'W 50°50.38'N	19.7°C	65	14/09/2009	15/09/2009	0	542	0
PoHIl2	Porstmouth	Horsea Island		l2			70	14/09/2009	15/09/2009	0	0	0
PoLHS1l1	Porstmouth	Langstone Harbour	S1	l1	1°1.90'W 50°47.78'N	19.0°C	120	14/09/2009	15/09/2009	264	487	0
PoLHS1l2	Porstmouth	Langstone Harbour	S1	l2			70	14/09/2009	15/09/2009	0	116	0
PoLHS1l3	Porstmouth	Langstone Harbour	S1	l3			110	14/09/2009	16/09/2009	222	1092	0
PoLHS1l4	Porstmouth	Langstone Harbour	S1	l4			110	14/09/2009	16/09/2009	0	505	0
PoLHS1l5	Porstmouth	Langstone Harbour	S1	l5			60	14/09/2009	17/09/2009	0	509	0
PoLHS2l1	Porstmouth	Langstone Harbour	S2	l1	1°1.90'W 50°47.80'N	19.0°C	100	14/09/2009	15/09/2009	0	75	0
PoLHS2l2	Porstmouth	Langstone Harbour	S2	l2			80	14/09/2009	15/09/2009	211	86	0
PoLHS2l3	Porstmouth	Langstone Harbour	S2	l3			107	14/09/2009	16/09/2009	0	21	0
PoLHS2l4	Porstmouth	Langstone Harbour	S2	l4			68	14/09/2009	16/09/2009	47	1	0
PoLHS2l5	Porstmouth	Langstone Harbour	S2	l5			70	14/09/2009	16/09/2009	49	72	0
PoLHS2l6	Porstmouth	Langstone Harbour	S2	l6			85	14/09/2009	17/09/2009	0	416	0
WB1l1	Wimereux	Beach	S1	l1	1°35.98'E 50°45.82'N	12.6°C	68	21/09/2009	23/09/2009	0	0	0
WB1l2	Wimereux	Beach	S1	l2			58	21/09/2009	23/09/2009	0	0	0
WB1l3	Wimereux	Beach	S1	l3			70	21/09/2009	24/09/2009	0	0	0
WB1l4	Wimereux	Beach	S1	l4			55	21/09/2009	24/09/2009	0	0	0
WB1l5	Wimereux	Beach	S1	l5			55	21/09/2009	24/09/2009	0	0	0
WB1l6	Wimereux	Beach	S1	l6			58	21/09/2009	24/09/2009	0	0	0
WB1l7	Wimereux	Beach	S1	l7			50	21/09/2009	24/09/2009	0	0	0
WB2l1	Wimereux	Beach	S2	l1	1°35.98'E 50°45.82'N	12.6°C	100	21/09/2009	23/09/2009	0	0	0
WB2l2	Wimereux	Beach	S2	l2			50	21/09/2009	23/09/2009	0	0	0
WB2l3	Wimereux	Beach	S2	l3			83	21/09/2009	24/09/2009	0	0	0
WB2l4	Wimereux	Beach	S2	l4			50	21/09/2009	24/09/2009	0	0	0
WB2l5	Wimereux	Beach	S2	l5			90	21/09/2009	24/09/2009	0	0	0
WB2l6	Wimereux	Beach	S2	l6			58	21/09/2009	24/09/2009	0	0	0
WB2l7	Wimereux	Beach	S2	l7			56	21/09/2009	24/09/2009	0	0	0
WB2l8	Wimereux	Beach	S2	l8			50	21/09/2009	24/09/2009	0	0	0
WPl1	Wimereux	Puddle		l1	1°36.37'E 50°46.00'N	13.8°C	51	21/09/2009	23/09/2009	0	0	0
WPl2	Wimereux	Puddle		l2			110	21/09/2009	24/09/2009	0	0	0
WPl3	Wimereux	Puddle		l3			50	21/09/2009	24/09/2009	0	0	0
WPl4	Wimereux	Puddle		l4			50	21/09/2009	24/09/2009	0	0	0
RSl1l1	Roscoff	Souris Island	S1	l1	3°59.30'W 48°43.70'N	13.9°C	62	30/09/2009	01/10/2009	0	348	0
RSl1l2	Roscoff	Souris Island	S1	l2			71	30/09/2009	01/10/2009	68	400	0
RSl1l3	Roscoff	Souris Island	S1	l3			115	30/09/2009	01/10/2009	0	0	0
RSl1l4	Roscoff	Souris Island	S1	l4			62	30/09/2009	01/10/2009	6	82	0
RSl1l5	Roscoff	Souris Island	S1	l5			78	30/09/2009	01/10/2009	49	48	0
RSl2l1	Roscoff	Souris Island	S2	l1	3°59.31'W 48°43.71'N	13.9°C	54	30/09/2009	05/10/2009	28	93	0
RSl2l2	Roscoff	Souris Island	S2	l2			58	30/09/2009	05/10/2009	0	159	0
RSl2l3	Roscoff	Souris Island	S2	l3			80	30/09/2009	05/10/2009	0	300	0
RSl2l4	Roscoff	Souris Island	S2	l4			58	30/09/2009	05/10/2009	0	59	0
RSl2l5	Roscoff	Souris Island	S2	l5			64	30/09/2009	05/10/2009	0	407	0
RLD1l1	Roscoff	Lacaze Duthiers	S1	l1	3°59.13'W 48°43.67'N	13.9°C	60	30/09/2009	02/10/2009	26	176	0
RLD1l2	Roscoff	Lacaze Duthiers	S1	l2			92	30/09/2009	02/10/2009	31	242	0
RLD1l3	Roscoff	Lacaze Duthiers	S1	l3			78	30/09/2009	02/10/2009	0	232	0
RLD1l4	Roscoff	Lacaze Duthiers	S1	l4			55	30/09/2009	05/10/2009	0	320	0
RLD1l5	Roscoff	Lacaze Duthiers	S1	l5			68	30/09/2009	05/10/2009	0	68	0
RLD2l1	Roscoff	Lacaze Duthiers	S2	l1	3°59.11'W 48°43.67'N	13.9°C	57	30/09/2009	06/10/2009	352	119	0
RLD2l2	Roscoff	Lacaze Duthiers	S2	l2			95	30/09/2009	06/10/2009	0	516	0
RLD2l3	Roscoff	Lacaze Duthiers	S2	l3			90	30/09/2009	06/10/2009	0	309	0
RLD2l4	Roscoff	Lacaze Duthiers	S2	l4			90	30/09/2009	06/10/2009	2	227	0
RLD2l5	Roscoff	Lacaze Duthiers	S2	l5			86	30/09/2009	06/10/2009	0	497	0

Dl: *Durchoniella legeri-duboscqui*, Db: *Durchoniella brasili*, DC: *Durchoniella cirratuli, Cc: Cirratulus cirratus*

FIG. S1. Maximum Likelihood tree based on *cox-1* gene sequences of the 82 provide species of cirratulid polychaetes collected in this study. The code characterizing the sites in Figure 1 is used to name each individual worm, with Ix the number of the specimen. Maximum Likelihood (ML) bootstrap value, posterior probability of Bayesian inference (BI), Maximum Parsimony (MP), and Neighbour-Joining (NJ) bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. The tree is rooted with two Cirratulidae. The colours indicate the geographical origin: pink for AH1/AH2 (Plymouth), purple for SP1/SP2 (Plymouth), green for MB1/MB2 (Plymouth), brown for HI (Portsmouth), orange for LH1/LH2 (Portsmouth), blue for WB1/WB2 and WP (Wimereux) and black for RSI1/RSI2 and RLD1/RLD2 (Roscoff). The white dot indicates the presence of *Durchoniella brasili* and/or *D. legeri-duboscqui*; and the black dot, the presence of *D. cirratuli*.

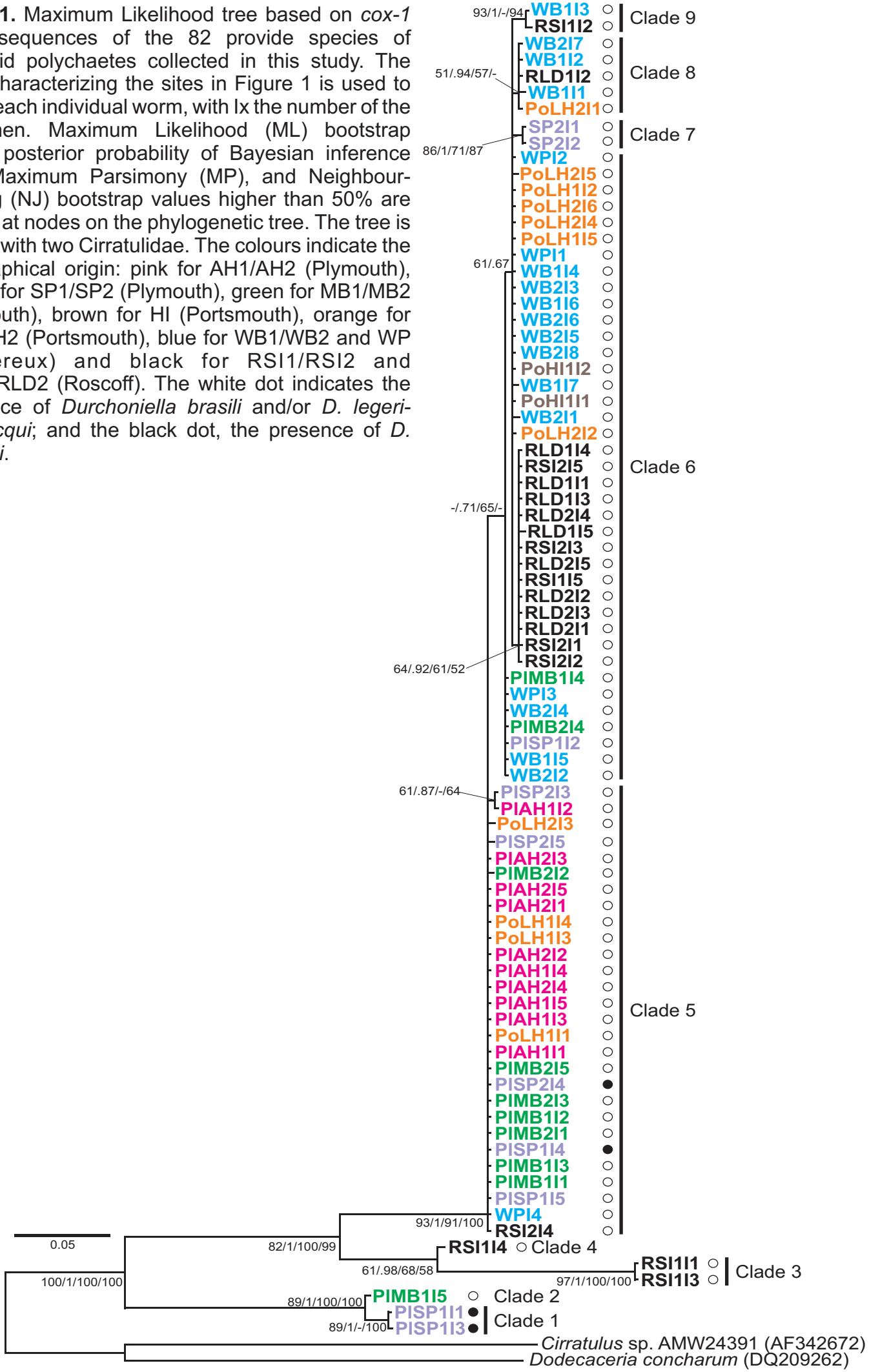
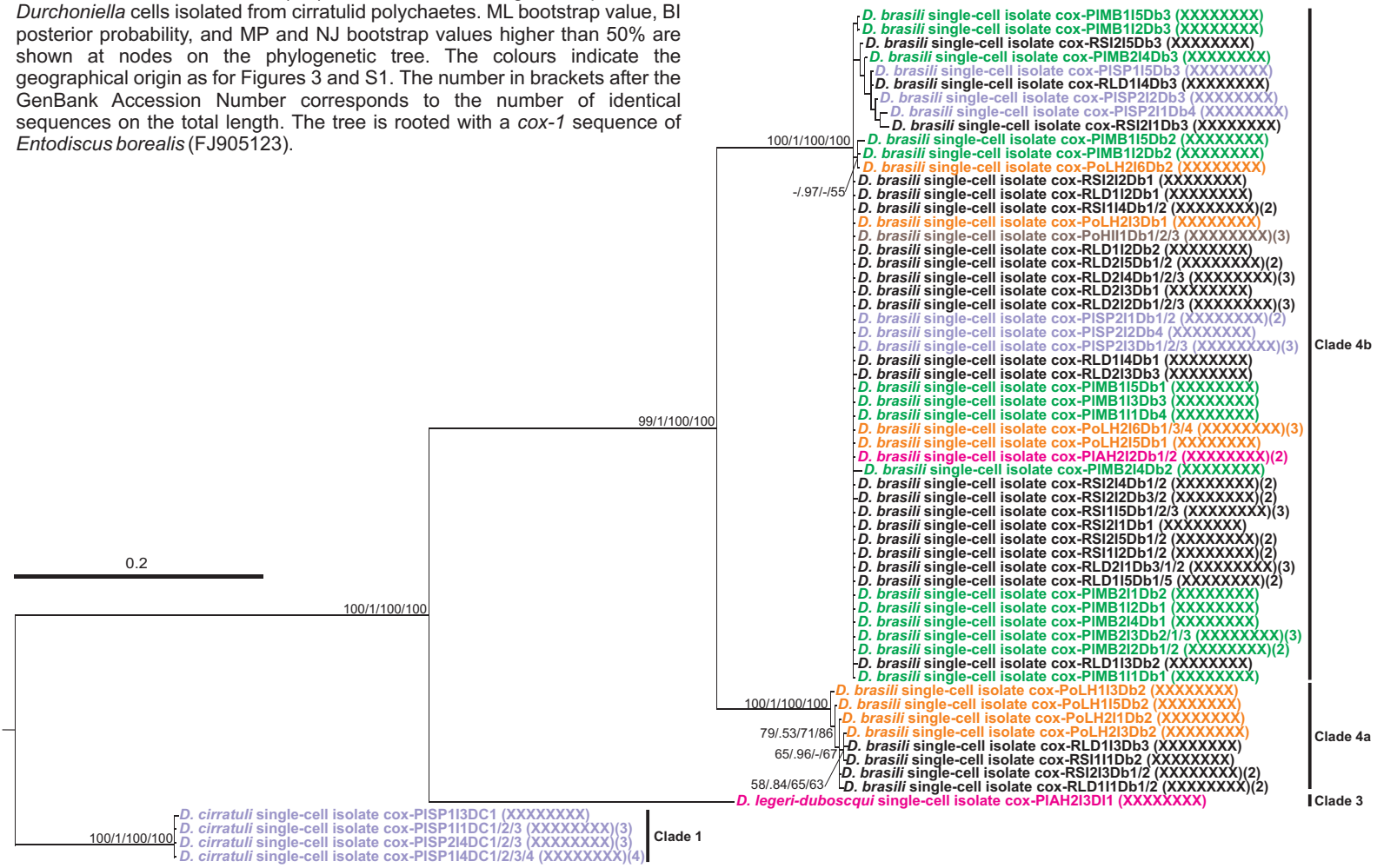


FIG. S2. Maximum Likelihood (ML) tree based on *cox-1* gene sequences of *Durchoniella* cells isolated from cirratulid polychaetes. ML bootstrap value, BI posterior probability, and MP and NJ bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. The colours indicate the geographical origin as for Figures 3 and S1. The number in brackets after the GenBank Accession Number corresponds to the number of identical sequences on the total length. The tree is rooted with a *cox-1* sequence of *Entodiscus borealis* (FJ905123).



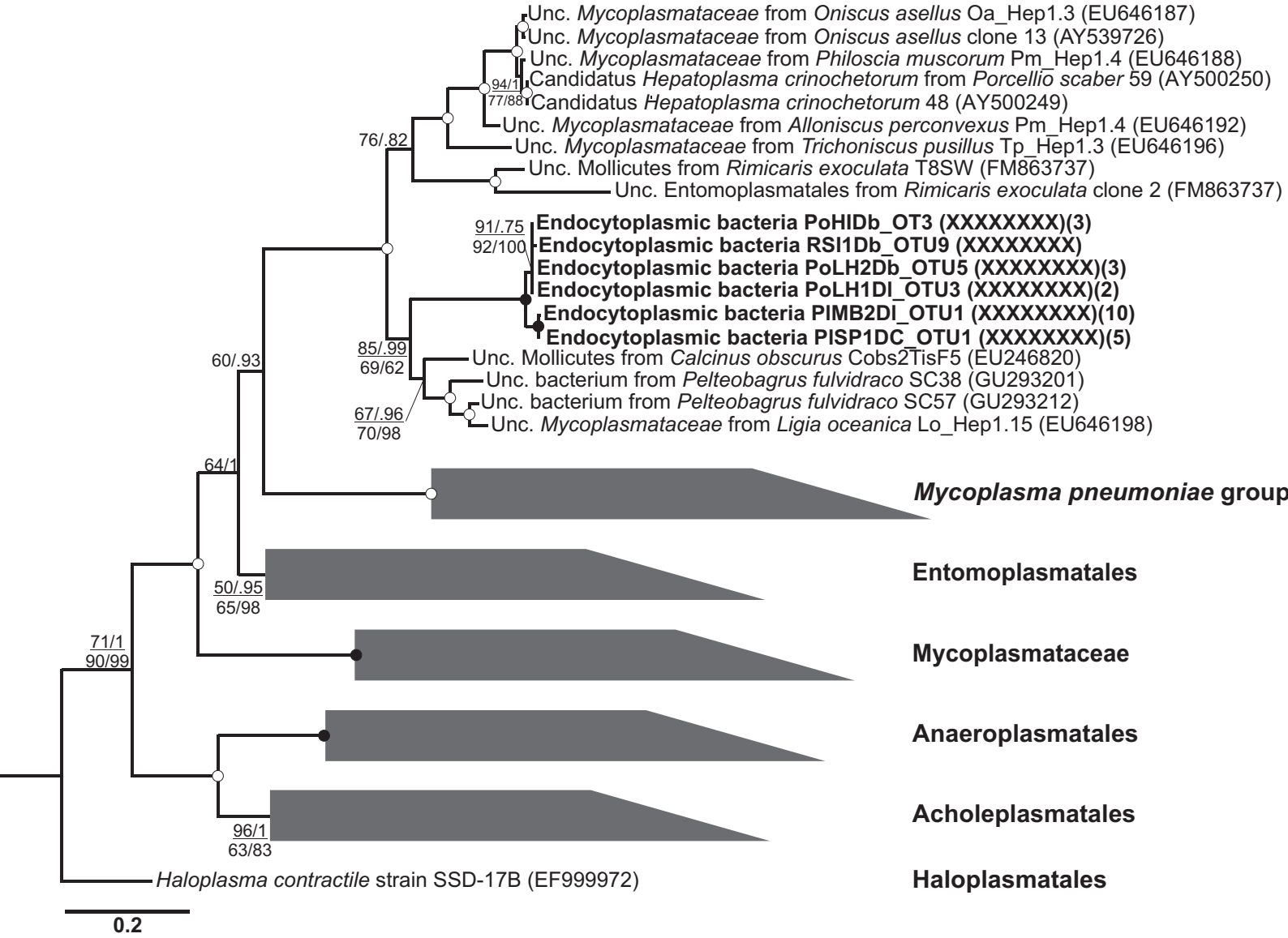


FIG. S3. Maximum Likelihood (ML) tree based on 16S rRNA gene sequences of Mollicutes retrieved from *Durchoniella* cells isolated from cirratulid polychaetes. Study sequences are in bold. Each OTU from each clone library is represented by one sequence with 99% similarity grouping. ML bootstrap value, BI posterior probability, and MP and NJ bootstrap values higher than 50% are shown at nodes on the phylogenetic tree. Black dots correspond to values of one (posterior probability) and 100% (ML, NJ, MP). White dots correspond to values between 80 and 100% (or 0.80 and one). The number of clones of each representative sequence is indicated in brackets after the GenBank Accession Number. The outgroup (not shown) is composed of two sequences of *Proteobacteria* (AB071665 and L14627).

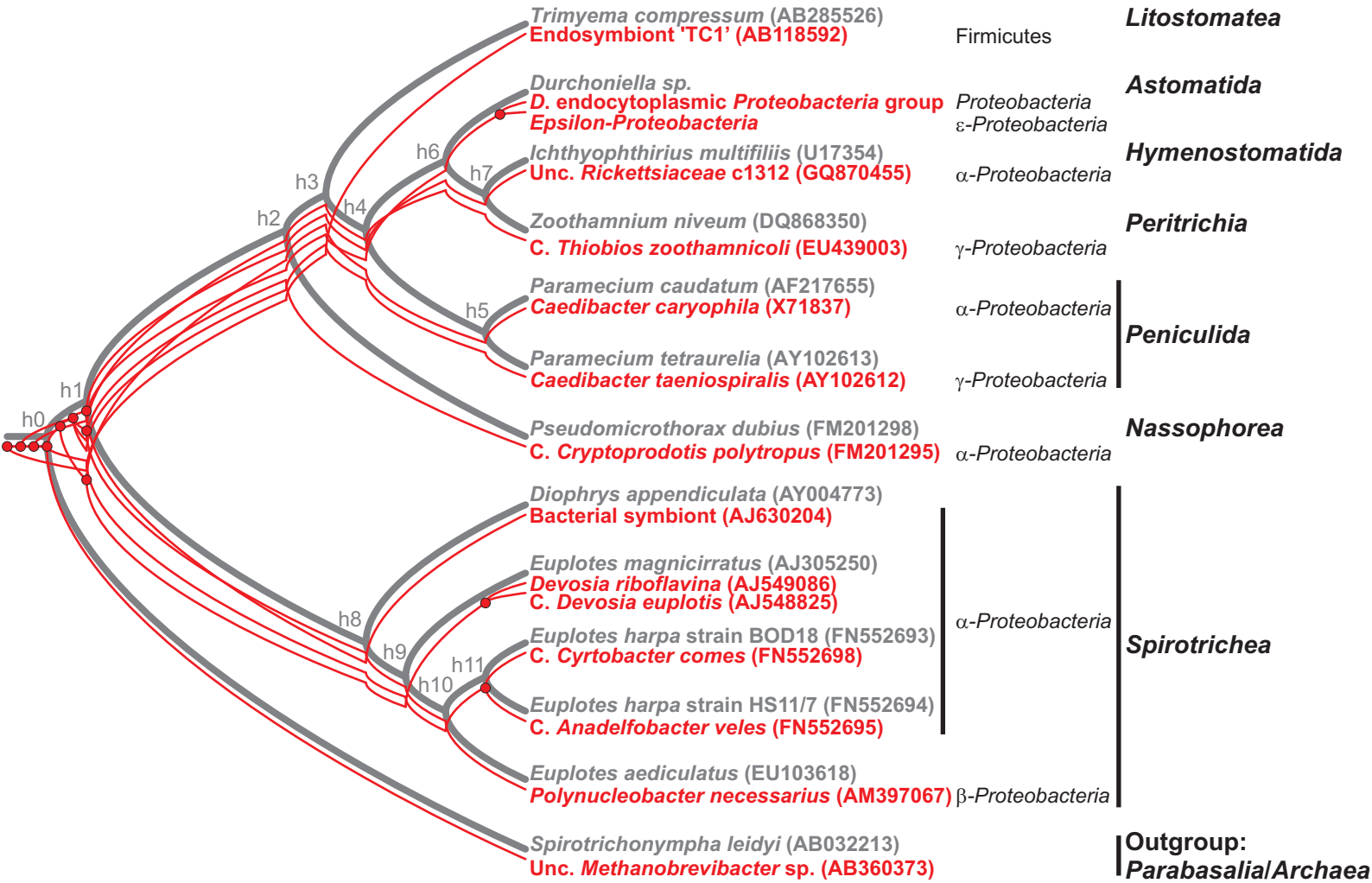


FIG. S4. Treemap 3.0 tanglegram of endosymbiotic bacteria and their ciliated host (p=0.001, for a null hypothesis of host switches). Grey and red lines indicate the phylogenies of the ciliates and their bacterial endosymbionts, respectively. The closed circle represents codivergence events. Each node on host tree is designated hx. The outgroup corresponds to a Parabasalia (Excavata) and its archaean endosymbiont.

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Partie III - Conclusions et Perspectives

CHAPITRE 5

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5.1 Spécificité à l'environnement ou niche écologique stable ?

5.1.1 « *Everything is everywhere...* but, *the environment selects.* »

Cette hypothèse de Baas Becking, en 1934 (Baas Becking, 1934; De Wit and Bouvier, 2006), a été développée sur des espèces cosmopolites de bactéries étudiées en culture. Elle se fonde sur le fait que les organismes microscopiques représentent des populations tellement denses et étendues, que chaque espèce peut être retrouvée partout, mais l'environnement local sélectionne si une espèce se développera à un site particulier.

Aujourd'hui, deux écoles débattent sur la phylogéographie des microorganismes eucaryotes, notamment celle des ciliés. La première maintient que tous les microorganismes ont une distribution globale, avec un faible degré d'endémisme, et un faible nombre d'espèces en raison du manque d'événement de spéciation. Finlay et Fenchel, adeptes de la proposition de Baas Becking, ont étendu l'étude aux petits animaux aquatiques et aux protistes sur deux sites distincts, puis l'ont généralisée à l'ensemble des petits eucaryotes aquatiques et terrestres. Ils ont démontré que des organismes eucaryotes de petites tailles, vivant libres, présentent une distribution mondiale sans corrélation géographique et génétique (Fenchel, 1997; Finlay and Fenchel, 2002; Fenchel and Finlay, 2004). La deuxième école propose un degré plus important d'endémisme, considérant que plus de la moitié du monde microbien est toujours non décrite et spécialement les espèces rares (Foissner, 2006). Ces auteurs se basent sur l'observation d'espèces dites « flagship », qui auraient une distribution géographique limitée, et sur le nombre toujours en augmentation de descriptions de nouvelles espèces (Foissner, 2006; Foissner, 2008).

5.1.2 L'identification des organismes par les études moléculaires

A l'inverse des premières études de biogéographie, basées uniquement sur les aspects morphologiques des espèces, qui pouvaient s'avérer parfois trompeurs (cas des espèces cryptiques), les études moléculaires ont permis certains postulats favorisant l'une ou l'autre des écoles (López-García *et al.*, 2003; Katz *et al.*, 2005; Richards and Bass, 2005; Weisse, 2008). Les études de diversité moléculaire sur les micro-eucaryotes ont commencé bien plus tard que celles effectuées sur les procaryotes, le nombre des séquences dans les banques de données publiques l'atteste. En effet, environ deux millions de séquences du gène codant pour l'ARNr 16S des procaryotes sont aujourd'hui accessibles en banques, contre moins de 300 000 séquences du gène codant pour l'ARNr 18S des eucaryotes.

Les premières études dans les années 2000 ont révélé la présence de trois catégories de séquences dans les données obtenues : les séquences étroitement affiliées à des espèces ou des genres connus, les séquences formant des groupes divergeant dans les taxa connus, et les séquences sans affiliation directe avec des eucaryotes déjà décrits. Ces dernières séquences étant plus favorablement retrouvées dans des environnements anoxiques (sédiments et colonne d'eau) (pour revue, Epstein and López-García, 2008). Cependant, ces études restreintes à la détection de séquences d'ADN ne prennent pas systématiquement en compte les cellules vivantes des orga-

nismes, puisque l'ADN extracellulaire peut également être amplifié. Ces études sont également biaisées par un ensemble de facteurs, entre autres l'utilisation des techniques de PCR induisant des chimères et une sélection des séquences d'organismes ne reflétant pas obligatoirement la diversité réelle *in situ*. Il est à noter que ces analyses prises avec minutie permettent d'accéder à une large diversité génétique d'organismes unicellulaires qui n'aurait pas été découverte par des analyses culturales ou morphologiques.

5.1.3 Les sources hydrothermales profondes et les sédiments anoxiques

Ces environnements particuliers ont tout pour fasciner les protistologues. En effet, les conditions retrouvées au niveau des sources hydrothermales notamment, sont proches de celles proposées comme étant liées à l'apparition de la vie sur Terre (Baross and Hoffman, 1985; Reysenbach and Cady, 2001). Les organismes vivent dans des conditions instables, caractérisées par des températures fortes, de faibles concentrations en oxygène et la présence de composés toxiques. Il est apparu cependant que les métazoaires dominants ces écosystèmes étaient contemporains, mais avaient certainement un haut degré de spécialisation comme l'illustre le nombre important de nouvelles espèces découvertes aussi bien chez les métazoaires que chez les procaryotes (Little and Vrijenhoek, 2003; Van Dover *et al.*, 2002). Mais qu'en est-il des organismes eucaryotes unicellulaires ?

Les études de diversité sur les micro-eucaryotes unicellulaires dans ces environnements ont permis la découverte de nouvelles lignées potentielles de haut rang taxonomique. Notre étude sur les micro-eucaryotes associés aux environnements hydrothermaux de l'océan Pacifique comparés à l'océan profond (Article 1) apporte deux nouvelles évidences dans la répartition possible de ces eucaryotes. La première évidence est favorable à la théorie « *Everything is everywhere...* » puisque la comparaison des données obtenues sur les différents environnements a révélé la présence de probables communautés cosmopolites, détectées aussi bien au niveau des substrats hydrothermaux que dans les eaux pélagiques, et par les autres études hydrothermales des systèmes de l'océan Atlantique. La seconde évidence est plus favorable à la deuxième partie de cette théorie « ...but*, the environment selects.* », puisque la présence de potentiels organismes inféodés a probablement été mise en évidence notamment dans le cas des Straménopiles, des champignons ou des ciliés. Cependant, dans ce dernier groupe, de potentiels organismes inféodés ont été retrouvés que par l'étude d'un nouveau substrat, les bivalves géants. Ces bivalves pourraient être une niche écologique stable permettant l'endémisme de certains protistes, notamment de ciliés, qu'ils soient parasites ou mutualistes.

Cependant, les études moléculaires permettent difficilement de préciser la physiologie des ces micro-organismes potentiellement inféodés et de savoir s'ils ont ou non acquis des mécanismes d'adaptation, ou s'ils sont en interaction avec les métazoaires. Des études supplémentaires restent à mener, en complétant les données moléculaires de diversité aussi bien sur des bases ADN que ARN, que par des essais de cultures et des descriptions morphologiques comme nous avons commencé à réaliser sur les échantillons de bivalves (Article 1). Des études biochimiques

et génomiques sur ces micro-eucaryotes pourraient nous renseigner sur les adaptations possibles engendrées par ces environnements particuliers, par exemple la réduction de leur génome comme certains parasites obligatoires (e.g. Katinka *et al.*, 2001). Ces champs d'analyses permettraient de mettre en relation l'environnement et l'organisme, et de confirmer des interactions possibles entre métazoaires, protistes et certainement procaryotes, comme c'est le cas pour les nombreux métazoaires *hydrothermaux*, dont les bivalves.

5.2 Les interactions entre organismes : le cas des ciliés astomes

5.2.1 Commensalisme, mutualisme ou parasitisme ?

Les études de biogéographie distinguent généralement les organismes vivant libres de ceux vivant en relation symbiotique avec d'autres organismes, car ces micro-organismes symbiotiques suivraient probablement la distribution de leurs hôtes et non pas une distribution globale. Cependant, étant donné les nombreuses interactions existantes entre les organismes, existe-t-il un organisme que l'on pourrait vraiment considérer totalement libre sans aucune interaction ?

Les ciliés Astomes sont le contre exemple de cette vie à l'état libre puisqu'ils ont toujours été observés chez un hôte. Les 56 genres répertoriés sont retrouvés dans une vingtaine de familles hôtes entre oligochètes terricoles et polychètes côtiers, en passant par des batraciens ou des planaires (De Puytorac, 1954). Ces ciliés ont probablement évolué au-delà du parasitisme puisqu'ils ne semblent pas causer la mort de leurs hôtes et sont limités dans leur habitat à des zones précises du ver allant de l'intestin antérieur à l'intestin moyen (De Puytorac, 1954). Cette limitation à une région définie de l'hôte suggère que des caractéristiques cellulaires ou biochimiques sont nécessaires à la survie et/ou à la présence des symbiontes. Une étude sur le ver *Allolobophora savignyi* a montré que les valeurs de pH, variant en fonction de la zone de l'intestin (même faiblement), délimitaient trois zones distinctes colonisées par trois espèces d'astomes différentes (De Puytorac and Mauret, 1956). Une autre étude sur une espèce d'astome colonisant des salamandres retrouvées en altitude (jusqu'à 1400 m) a montré que leur prévalence était inversement relative à l'altitude, certainement en raison des faibles températures (Powders, 1970). De plus, l'absence de bouche de ces ciliés amène à s'interroger sur le type d'interaction qui est mis en place, puisqu'ils ne sont plus capables de capturer des proies.

Des études sur les hôtes des ciliés astomes *Durchoniella* ont montré que les Cirratulidae étaient capables de vivre dans des milieux pauvres en oxygène car ils possèdent une hémoglobine extracellulaire combinant une forte affinité à l'oxygène avec un effet Bohr prononcé, c'est-à-dire une libération optimale de l'oxygène quand la pression partielle en CO_2 augmente dans les tissus (Dales and Warren, 1980; Warren, 1981). Cette hémoglobine permet ainsi le transport de l'O_2 et son stockage en condition d'hypoxie. Les astomes pourraient donc avoir un apport en oxygène continu, apport semble-t-il vital puisqu'ils possèdent *a priori* uniquement des mitochondries classiques, à l'inverse en général des ciliés retrouvés dans les sédiments anoxiques possédant des mitosomes ou des hydrogénosomes.

La notion de spécificité envers leurs hôtes semble complexe. En effet, la présence des ciliés astomes est selon les espèces décrites, d'une spécificité stricte, c'est-à-dire associée à un seul hôte, ou plus étendue avec la possibilité pour une espèce donnée de coloniser une variété d'hôte, de genres différents ou non. Il est intéressant de noter que, comme l'a montré De Puytorac (1954), certains vers au voisinage de ceux trouvés infectés vont être exempts de ces ciliés qu'ils soient d'une même espèce ou d'espèce différente (Articles 2 et 3). Se pose alors la question de la reconnaissance de l'hôte par le symbionte ou inversement. La mise en place de chimiotactisme dans la reconnaissance des partenaires à un moment du cycle de vie du ver pourrait être envisagée, comme pour les nombreux cas répertoriés entre les plantes et leurs bactéries symbiotiques (pour publication récente, Gurich and Gonzalez, 2009; Bright and Bulgheresi, 2010). Des études basées sur la reproduction *in vitro* de leur microcosme pourraient alors permettre d'isoler des molécules agissant comme signaux attracteurs.

5.2.2 Transmission et cycle de vie

Le cycle de vie des astomes reste indéterminé. La formation de kystes notamment n'a pas été identifié chez les astomes, hormis une possible forme de résistance dans une gaine visqueuse observée par De Puytorac (1954). Si les kystes ne sont pas utilisés comme vecteur de dissémination et donc de transmission, comment peut se réaliser le passage du symbionte dans les populations de vers ?

La transmission des symbiontes d'une génération à l'autre peut s'effectuer soit horizontalement soit verticalement. Les symbiontes acquis de l'environnement proviennent de population d'organismes libres et génétiquement diversifiés. Ces symbiontes versatiles vont rentrer en compétition avec le reste de la communauté microbienne, et de ce fait favoriser le parasitisme. La transmission verticale concerne des organismes symbiontes transférés à la génération suivante qui seront sélectionnés à partir d'une population génétiquement restreinte. Ce type de transmission est donc plus favorable à l'installation d'interaction mutualiste. L'absence apparente de virulence chez les astomes est donc en faveur d'une transmission verticale.

Un exemple intéressant d'une possible transmission verticale, révélée par les travaux de De Puytorac (1954), est celui de la sangsue *Helobdella stagnalis* qui porte ses jeunes larves sous son ventre, et qui a pu être observée en train de régurgiter ses astomes endocommensaux (*Acanthophrya* sp.), permettant ainsi un contact avec la nouvelle génération. Des astomes englobés dans un mucus dense ont également été observées chez *C. tentaculata* (Article 2), indiquant peut-être un système équivalent de transmission de l'adulte vers les larves (Figure 5.1). Si on prend pour autre exemple le ver *Riftia pachyptila*, les symbiontes bactériens sont transmis de façon horizontale. En effet, les symbiontes présents dans l'environnement vont s'accumuler dans une sorte de mucus sécrété à la surface de la larve de l'animal, envahir le tissu épithélial, puis migrer vers des cellules en différenciation qui formeront le trophosome (Nussbaumer *et al.*, 2006). Il est reconnu que dans le cas de *Cirriformia tentaculata*, le ver sécrète à sa surface un mucus malodorant permettant d'échapper à la prédation de certains poissons (George, 1964). Il serait

envisageable qu'une telle sorte de mucus puisse également servir, comme dans le cas de *Riftia*, à concentrer les astomes pour qu'ils aillent coloniser l'intestin des jeunes larves. Cependant, les premières études de De Puytorac révèlent (1954), par l'observation de trois espèces de vers différents, que de jeunes individus nouvellement éclos ne présentaient pas d'astomes ; la même constatation étant faite sur des vers âgés, sauf s'ils étaient collectés dans une population à forte prévalence d'infection. Ainsi, il est possible qu'une combinaison des deux exemples précédents puisse avoir lieu dans le cas des Cirratulidae : les vers fortement colonisés pourraient répandre dans l'environnement des astomes enfermés dans un mucus qui entrerait par la suite en contact avec les individus de la population (à un stade larvaire avancé ou non), transmettant ainsi les symbiontes ciliés.

A ce stade, le cycle de vie de l'hôte, et dans les cas qui nous intéressent de *C. tentaculata* et *C. cirratus*, semble intéressant à développer (Figure 5.1). Les deux polychètes présentent des cycles de vie longs (annuels, au moins). Les males et les femelles ont un dimorphisme basé sur la couleur, et la taille chez *C. cirratus*, les femelles étant plus grosses que les males (Olive, 1970). Le sexe ratio est équivalent chez *C. tentaculata* mais plus discuté chez *C. cirratus*, une prépondérance des femelles pouvant être observée (Stephenson, 1950; Olive, 1970; Gibbs, 1971). Contrairement à *C. tentaculata*, il n'existe pas de développement saisonnier des gamètes chez *C. cirratus*, tous les stades de maturation peuvent être trouvés toute l'année (George, 1964; Gibbs, 1971).*Cirratulus cirratus* dépose ses œufs dans un mucus qui adhère au sédiment, puis ces masses d'œufs sont rapidement couvertes de particules de sédiment, et difficiles à détecter (Gibbs, 1971). Au contraire, *C. tentaculata* éjecte simplement ses gamètes dans le milieu (George, 1964). Un stade planctonique très court dans les premiers stades larvaires a été proposé (George, 1964), mais aucune forme décrite n'a été retrouvée dans le plancton (George, 1964). Le cycle de vie long de ces hôtes pourrait donc faciliter la mise en place d'interaction à long terme avec les symbiontes astomes. De plus, les astomes pourraient être dispersés en même temps que les gamètes, et les coloniser ou rester à proximité protégés par un mucus en attendant la colonisation de la nouvelle génération à un stade propice.

La mise en évidence de ces systèmes de transmission ainsi que du cycle de vie des astomes pourrait être envisagée par l'observation de nombreuses populations d'astomes sur des hôtes à différents stades de leurs cycles de vie. Une autre possibilité serait la mise en culture en laboratoire de ces ciliés astomes. En effet, une étude a montré que chez le cilié parasite *Ichthyophthirius multifiliis* qui normalement a besoin de son hôte pour se développer et accomplir son cycle, l'induction de la transformation d'un stade à un autre pouvait être réalisée *in vitro* en utilisant une lignée cellulaire de tissu de poisson (Nielsen and Buchmann, 2000).

5.3 Les bactéries : une stratégie adaptative ?

Les microorganismes procaryotes sont largement répandus dans tous les environnements. Etant donné leur ubiquité, il n'est pas surprenant de trouver de nombreuses espèces de pro-

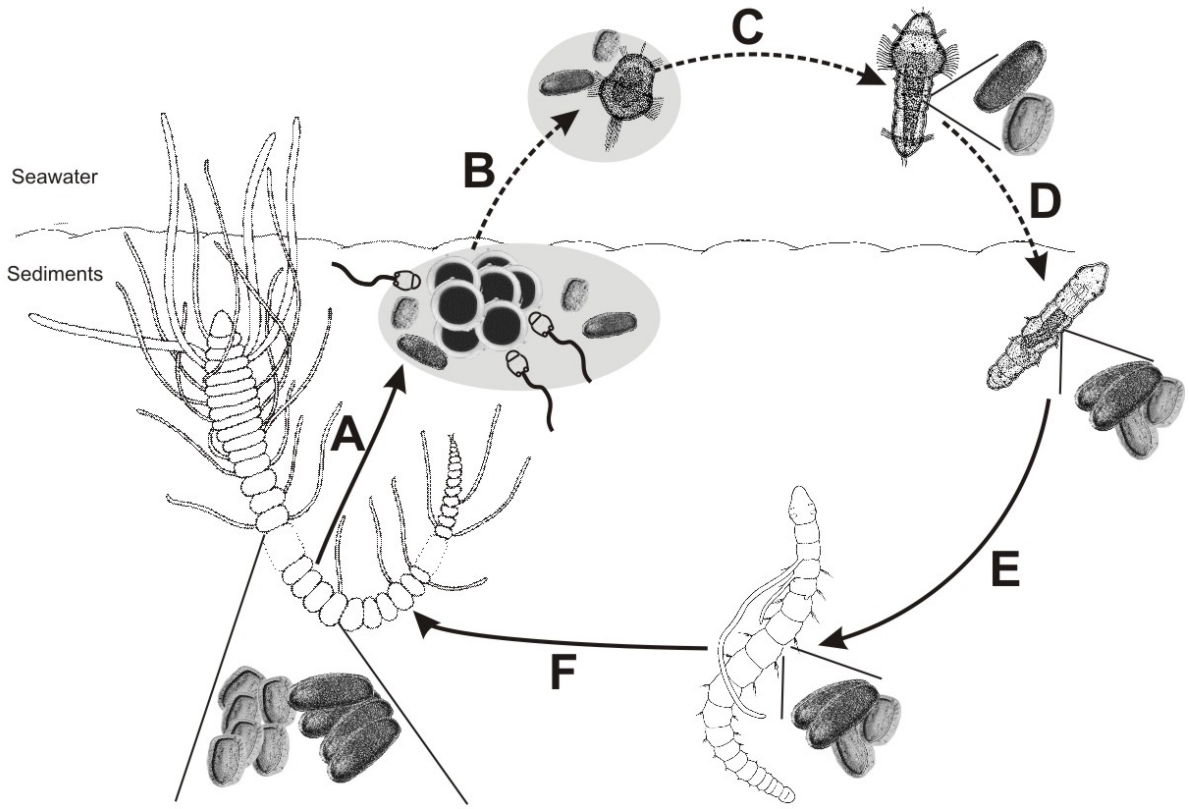

FIG. 5.1 – Cirratulidae-*Durchoniella* hypothetical life cycle. **A**, *Durchoniella* and eggs are released at the same time by the adult Cirratulidae. Gametes could be protected by a mucus also containing *Durchoniella*. **B**, *Durchoniella* cells stay in contact with the larvae until they can start colonizing the immature gut of the swimming trochosphere ($<$ 24 hours). The complex could therefore be dispersed in the environment. **C**, The fully formed larva harbours endocommensal *Durchoniella*. **D**, Juvenile polychaete starts a sedimentary lifestyle. **E**, *Durchoniella* starts fully colonising the gut of their host. **F**, Fertile adult cirratulids harbors a dense *Durchoniella* community before starting a new cycle.

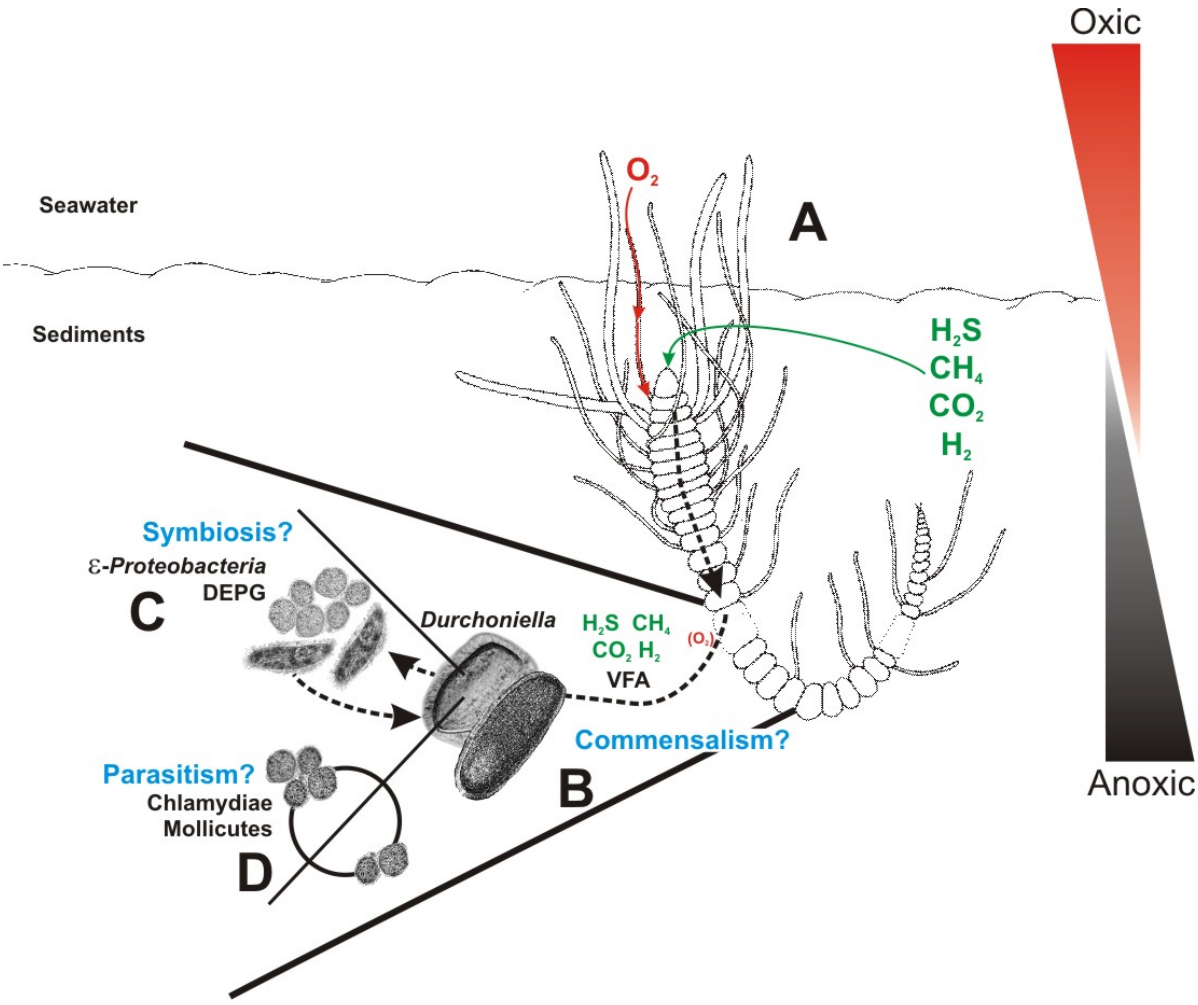

FIG. 5.2 – Hypothetical interactions occurring in Cirratulidae from tidal sediments. **A**, Cirratulids traps food particles falling onto the sediment surface with their tentacular filaments. These filaments are also used to acquire oxygen that can be stored in the tissues. During low tide or to escape predation, the worm buries itself deeply into the sediment retracting its tentacles, therefore encountering anoxic conditions. **B**, During hypoxia, the worm switches to an anaerobic metabolism producing volatile fatty acids (VFA). In the reduced sediment zone, the worm could uptake compounds, such as sulphide, methane, and carbon dioxide. The endocommensal *Durchoniella* could survive using the oxygen reserves of cirratulids, and/or by interacting with its endocytoplasmic bacteria. **C**, The gut of the worm may represent an optimal environment to sustain microaerophilic metabolism, such as those of chemolithotrophic endocytoplasmic bacteria (e.g. Sulphur-oxidizing and Methane-oxidizing *Proteobacteria*). The bacterial communities could therefore fix carbon autotrophically and transfer synthesized organic compounds to the astome. **D**, Episodic events from potentially parasitic bacteria (e.g. Chlamydiae and Mollicutes) could also probably occur.

caryotes en interaction avec d'autres organismes. Les bénéfices dérivés de ces interactions sont variés et incluent une influence mutuelle sur la nutrition, la défense, la reproduction et le développement. Ces associations vont également permettre l'exploitation de niches écologiques particulières. Les symbioses chimiosynthétiques sont le meilleur exemple de ces adaptations, permettant aussi bien de coloniser des environnements hydrothermaux que des zones côtières spécifiques (Dubilier *et al.*, 2008). Dans ces environnements réduits, les sulfites ou le méthane sont la source d'électrons, mais ils sont spontanément oxydés en présence d'oxygène (Zhang and Millero, 1993) et sont donc accessibles dans la zone anoxique ou micro-oxique (sédiments). A l'inverse, l'oxygène, accepteur d'électrons, est contenu dans la colonne d'eau. Les animaux contenant des symbiontes chimio-autotrophes doivent être capables d'acquérir aussi bien l'un et l'autre des composés en jouant sur leur comportement, leur morphologie ou encore via des adaptations métaboliques (Chaston and Goodrich-Blair, 2010).

Ainsi les *Durchoniella* peuvent être une niche écologique idéale pour des bactéries. La présence des astomes dans les cirratulids permet probablement aux symbiontes bactériens d'obtenir les composés nécessaires à leur croissance : l'oxygène via la circulation sanguine du ver qui circule des tentacules vers le corps de l'animal comme nous l'avons décrits dans la partie 5.2.1 ; et les sources d'électrons, présentes soit dans les sédiments réduits et absorbées par le polychète en même temps que les particules ingérées (e.g. CH_4, H_2S), soit sous forme de composés produits par le ver (Figure 5.2). En effet, le métabolisme anaérobique des polychètes résulte de la production d'alanine, de succinate et d'acides gras volatils (Bestwick *et al.*, 1989). Ces produits seraient absorbés par les ciliés astomes, et les bactéries détectées à l'intérieur utiliseraient ces produits pour fournir les composés nécessaires à la croissance des *Durchoniella*. Ce mode d'interaction pourrait également expliquer le fait que les vers collectés à Wimereux dans des sédiments sableux avec une importante circulation d'eau et un faible état réduit, n'aient pas été trouvés en interaction avec des ciliés astomes, puisqu'ils sont peu voir pas du tout en anaérobiose (Article 3).

Les types bactériens que nous avons majoritairement détectés, notamment les Proteobacteria, ont des métabolismes variables et s'adaptent à de nombreuses conditions physico-chimiques (Campbell *et al.*, 2006). Des techniques basées sur l'utilisation d'isotopes marqués pourraient nous renseigner quant aux produits utilisés par les différentes cellules. Et si de nouveaux essais de cultures des *Durchoniella* s'avéraient fructueux, l'utilisation d'antibiotiques pourrait nous permettre de définir les relations existantes entre les bactéries et les astomes.

Les interactions à long termes produisent différentes sortes de réarrangements chez les partenaires. Les changements pouvant survenir chez l' hôte comprennent le développement de structures spécialisées où les bactéries sont concentrées, ainsi que la modification de leur système immunitaire face au symbionte. Dans le cas des *Durchoniella*, les bactéries sont observées individuellement dans des vacuoles pouvant servir d'une part à réguler la population en ne permettant pas un développement aléatoire des symbiontes dans le cytoplasme, et d'autre part à préserver ces bactéries endosymbiontes du système immunitaire et enzymatique de leur hôte

(Fokin, 2004). Chez les endosymbiontes obligatoires dont l'interaction est bien établie, il est observé une réduction du génome par rapport à des organismes du même type vivant libres. Les gènes impliqués dans des mécanismes fondamentaux tels que la réplication de l'ADN ou la transcription, sont plus conservés que d'autres classes de gènes, et peuvent d'ailleurs représenter plus d'un tiers du génome (pour revue, Moya *et al.*, 2008). La perte de gènes métaboliques est également observée en présence d'un symbionte secondaire facultatif qui compense les déficiences métaboliques du premier symbionte ou de l' hôte. Bien qu'initialement, les relations entre le premier symbionte et un symbionte facultatif soient transitoires, une nouvelle interaction stable peut s'établir, et dans ce cas les deux espèces co-évoluent. Nos études moléculaires montrent la présence de groupes bactériens *a priori* distincts des groupes déjà étudiés ou retrouvés dans l'environnement (Article 3), qui pourraient avoir co-évolués avec leurs hôtes. Des analyses supplémentaires sur un plus grand nombre d' hôtes et dans des environnements variés seront nécessaires pour confirmer la spéciation de ces phylotypes.

La multiplicité des symbiontes dans un même hôte n'est pas rare et le degré de spécificité taxonomique varie en fonction des associations (Chaston and Goodrich-Blair, 2010). Dans les *Bathymodiolus* ou les oligochètes, la présence de jusqu'à six symbiontes différents a été démontrée (Dubilier *et al.*, 2008). Certains eucaryotes unicellulaires peuvent également abriter plusieurs genres bactériens (Fokin, 2004; Gast *et al.*, 2009). Les méthodes d'acquisition des symbiontes par leurs hôtes ont un effet majeur sur la diversité, la phylogénie et l'évolution de ces symbiontes (Bright and Bulgheresi, 2010).

Les astomes ont deux modes de division, par scission binaire et par conjugaison. Dans les deux cas, l'observation de bactéries dans les cellules filles (FISH et/ou coloration DAPI) fait penser à une transmission de type vertical. Les études sur différentes symbioses impliquant des procaryotes, montrent que les symbiontes obligatoires sont transmis de façon verticale et le plus souvent regroupés dans des structures spécialisées. Au contraire, les symbiontes facultatifs sont transmis horizontalement, et retrouvés plus en périphérie des structures spécialisées (Bright and Bulgheresi, 2010). Ainsi, les types bactériens retrouvés plus majoritairement semblent plutôt provenir d'une transmission verticale. Cependant, la transmission verticale n'est pas stricte, et les analyses phylogénétiques montrent des transferts horizontaux occasionnels entre les espèces, ou provenant de l'environnement. Ainsi, la détection sporadique de séquences relatives à des bactéries intracellulaires obligatoires telles que les Chlamydiae ou les Mollicutes (Articles 2 et 3), pourrait être due à cette dualité dans le type de transmission.

Une étape essentielle dans la compréhension d'une association symbiotique est l'identification des partenaires et de la nature de l'interaction. Dans la plupart des cas, la relation entre hôte et le symbionte est tellement étroite, que la culture des microorganismes n'est pas possible, les rendant difficiles à étudier. Cependant, les techniques émergeantes de génomique et de transcriptomique offrent de nouvelles opportunités pour l'étude de ces interactions. Une étude basée sur ces nouveaux outils nous aiderait à comprendre les mécanismes engendrés par l'association tripartite entre polychète, astomes et bactéries.

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Annexes

ANNEXE A

LÉGENDE DÉTAILLÉE DE LA FIGURE 2.2

1. *Cinetochilum margaritaceum* 2. *Halteria* n. sp. 3. *Cyclidium citrullus* 4. *Urotricha* sp. 5. *Thigmogaster* sp. 6. *Mesodinium* sp. 7. *Philasterides* sp. 8. *Chilodonella uncinata* 9.*Litonotus uninucleatus* 10. *Cyclidium plouneouri* 11. *Aspidisca cicada* 12. *Lacrymaria* sp. 13. *Halteria grandinella* 14. *Trimyema* sp. 15. *Zosterodasys* sp. 16. *Glaucoma scintillans* 17. *Ctedectoma wilberti* 18. *Calyptotricha lanuginosa* 19. *Saprodinium* sp. 20. *Cristigera setosa* 21. *Cyclidium* sp. 22. *Pseudomicrothorax dubius* 23. *Sathrophilus muscorum* 24. *Paranophrys* sp. 25. *Askenasia* sp. 26. *Metacystis tesselata* 27. *Hastatella radians* 28. *Balanonema biceps* 29. *Uronema marinum* 30. *Cyclidium glaucoma* 31. *Colpoda cucullus* 32. *Dexiotricha media* 33. *Colpidium campylum* 34. *Placus luciae* 35/36. *Tetrahymena pyriformis*-complex 37. *Placus* sp. 38. *Cinetochilum margaritaceum* 39. *Blepharisma hyalinum* 40. *Loxodes* sp. 41. *Paramecium aurelia*-complex 42. *Lacrymaria olor* 43. *Tetrahymena vorax* 44. *Entosiphon* sp. (euglenid) 45. *Spathidium sulcatum* 46. *Pleuronema coronatum* 47. *Chilodonella* sp. 48. *Urocentrum turbo* 49. *Ileonema dispar* 50. *Nassula tumida* 51. *Sagittaria poligonalis* 52. *Trithigmostoma cucullulus* 53. *Frontonia leucas* 54. *Lacrymaria elegans* 55. *Paramecium caudatum* 56. *Lacrymaria* sp. 57. *Euplotes* sp. 58. *Drepanomonas revoluta* 59. *Tachysoma* sp. 60. *Tachysoma pellionella* 61. *Stylonychia putrina* 62. *Urosoma cienkowski* 63. *Holosticha* sp. 64. *Keronopsis monilata* 65. *Ancystropodium maupasi* 66. *Urosoma cienkowski* 67. *Gastrostyla steinii* 68. *Stylonychia mytilus* 69. *Uroleptus piscis* 70. *Lacrymaria olor* 71. *Metopus striatus* 72. *Metopus undulans* 73. *Tropidoatractus acuminatus* 74. *Metopus* sp. 75. *Brachonella spiralis* 76. *Caenomorpha* sp. 77. *Caenomorpha uniserialis* 78. *Saprodinium dentatum* 79. *Isocyclidium globosum* 80. *Plagiopyla nasuta* 81. *Metopus es* 82. *Saprodinium* sp. 83. *Cyclidium porcatum* 84. *Discomorphella pectinata* 85. *Mylestoma uncinatum* 86. *Saprodinium difficile* 87. *Trimyema compressum* 88. *Vaginicola crystallina* 89. *Ophrydium versatile* 90. *Trichophrya epistylidis* 91. *Vorticella natans* 92. *Acineta* sp. 93. *Loxophyllum helus* 94. *Chilodonella* sp. 95. *Sphaerophrya magna* 96. *Epistylis* sp. 97. *Paruroleptus caudatus* 98. *Stentor* sp. 99. *Stentor polymorphus* 100. *Dendrosoma radians* 101. *Stylocola striata* 102. *Aspidisca costata* 103. *Stentor roeseli* 104. *Epistylis flavicans* 105. *Nassula picta* 106. *Paramecium*

bursaria 107. *Frontonia* sp. 108. *Deltopylum rhabdoides* 109. *Ophrydium eichornii* 110. *Loxodes striatus* 111. *Dileptus* sp. 112. *Trachelius ovum* 113. *Arcuospathidium vermiforme* 114. *Epistylis plicatilis* 115. *Kahlilembus attenuatus* 116. *Endosphaera terebrans* 117. *Vorticella mayeri* 118. *Litonotus cygnus* 119. *Amphileptus* sp. 120. *Litonotus cygnus* 121. *Litonotus fasciola* 122. *Loxophyllum helus* 123. *Litonotus fasciola* 124. *Loxophyllum* sp. 125. *Hastatella aesculacantha* 126. *Hastatella radians* 127. *Phascolodon vorticella* 128. *Strombidium velox* 129. *Bursaria truncatella* 130. *Urozona bütschlii* 131. *Disematostoma gyrans* 132. *Urotricha furcata* 133. *Monodinium balbiani* 134. *Actinobolina* sp. 135. *Urotricha furcata* 136. *Tintinnopsis* sp. 137. *Bursaridium pseudobursaria* 138. *Prorodon palustris* 139. *Histiobalantium majus* 140. *Balanion planctonicum* 141. *Hypotrichidium conicum* 142. *Disematostoma* (Leucophrys) *tetraedrica* 143. *Tintinnopsis lacustris* 144. *Lembadion* sp. 145. *Enchelyomorpha vermicularis* 146. *Tintinnidium fluviatile* 147. *Platynematum sociale* 148. *Holophrya ovum* 149. *Pleuronema* sp. 150. *Urotricha* sp. 151. *Halteria* sp. 152. *Strombidium* sp. 153. *Strombidium sulcatum* 154. *Coleps* sp. 155. *Coleps hirtus* 156. *Prorodon discolor* 157. *Codonella cratera* 158. *Strombidium viride* 159. *Prorodon* sp. 160. *Prorodon* sp. 161. *Holophrya gargamellae* 162. *Frontonia acuminate* 163. *Strobilidium adherens* 164. *Spirostomum* sp. 165. *Spirostomum minus* 166. *Homalozoon vermiculare* 167. *Vorticella* sp.

ANNEXE B

ARTICLE ANNEXE - MICROBIAL-SIZE SSU RDNA ENVIRONMENTAL LIBRARIES PUZZLES THE ECOLOGY AND GENETIC DIVERSITY OF RADIOLARIA

Microbial-Size SSU rDNA Environmental Libraries Puzzles the Ecology and Genetic Diversity of Radiolaria (En revision)

Manon Viprey ; Fabrice Not ; Ramon Massana ; Anne-Laure Sauvadet ; Seung Yeo Moon-Van Der Staay, Daniel Vaulot and Laure Guillou

VIPREY ET AL.---ENVIRONMENTAL SSU RDNA SEQUENCES OF RADIOLARIANS

Microbial-Size SSU rDNA Environmental Libraries Puzzles the Ecology and Genetic Diversity of Radiolaria

MANON VIPREY[a], FABRICE NOT[a,b], RAMON MASSANA[b], ANNE-LAURE SAUVADET[a], SEUNG YEO MOON-VAN DER STAAY[c], DANIEL VAULOT[a] and LAURE GUILLOU[a]

[a] CNRS and Université Pierre et Marie Curie (Paris 06), UMR7144, Station Biologique de Roscoff, *BP74, 29682 Roscoff Cedex, France*

[b] Institut de Ciències del Mar, CSIC, *Passeig Marítim de la Barceloneta 37--49, 08003 Barcelona, Catalonia, Spain*

[c] *Huelmer Str. 68, 47574 Goch, Germany*

Corresponding author: L. Guillou, Station Biologique de Roscoff, BP74, 29682 Roscoff Cedex, France---Telephone number: 33 2 98 29 23 79 ; FAX number: 33 2 98 29 23 24 ; e-mail: lguillou@sb-roscoff.fr

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ABSTRACT. Radiolaria play important roles in marine ecosystems, they are active plankton predators, many species bear endosymbiotic algae, and exhibit mineral skeletons. Here we carried out a global analysis of radiolarian's genetic diversity using small subunit (SSU) rDNA gene sequences retrieved from the GenBank database and obtained from environmental surveys. Out of the 44,017 SSU rDNA gene sequences screened, 1,529 belonged to the Radiolaria, among which 1,473 corresponded to environmental sequences. Radiolaria account for 10.7 % of sequences retrieved from planktonic environments. Phylogenies performed on full-length SSU rDNA gene sequences revealed six main clades. The Acantharea holding a basal position among the Radiolaria and five clades affiliated to the Polycystinea. The later including the Spumellarida, a clade comprising the Nassellarida and Collodaria, and three clades largely represented by environmental sequences (RAD A, B, and C) all together being monophyletic. Overall, relative contribution of Radiolaria to environmental genetic libraries is prominent in the aphotic zone of open oceans, in particular for the picoplanktonic (< 3 µm) size fraction. Sequences from this size fraction may originate from cell debris, but we can not rule out the possible existence of very small species not yet described and/or the production of very small life stages at depth.

Key Words. Radiolaria, Polycystinea, Acantharea, molecular ecology, 18S rDNA, picoplankton, biogeography

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In his illustrated monograph of plankton samples collected during the Challenger expeditions (1873-1876), Haeckel used the term “Radiolaria” to refer to several types of beautifully shaped protists including the Acantharea, Polycystinea, and Phaeodarea (Haeckel 1887). Radiolaria are marine protists playing an important role in marine food webs as predators of a wide variety of zoo- and phytoplankton preys (Caron and Swanberg 1990; Swanberg and Anderson 1985). Many radiolarian species have endosymbiotic algae belonging to various taxa, e.g. Haptophyta, Dinophyceae, or Chloroplastida (Anderson 1983; Gast and Caron 2001; Gast et al. 2000; Stoecker et al. 2009). The cell size of Radiolaria ranges from ten micrometers to several centimeters for the largest colonial species. Solitary Radiolaria are roughly spherical planktonic organisms with mineral skeleton. Acantharea, Polycystinea and Phaeodarea differ by their skeleton composition, composed of strontium sulphate ($SrSO_4$), opaline silica (SiO_2), and silica plus organic matter, respectively. Whereas the skeleton of Acantharea is rapidly dissolved in sea water after death, both polycystinean and phaeodarean siliceous skeletons contribute substantially to the microfossil record in marine sediments and are largely used in geology and paleo-oceanography.

Despite the fact that Radiolaria have been described for more than a century, not a single species has been cultivated to date. Consequently, molecular information have been traditionally obtained from individual specimen collected in situ (Amaral-Zettler et al. 1997; Kunitomo et al. 2006). After a decade of debate on the monophyly or polyphyly of Radiolaria (Amaral-Zettler et al. 1997; López-García et al. 2002), and the introduction of sequences from isolated species (Nikolaev et al. 2004; Polet et al. 2004; Yuasa et al. 2005; Yuasa et al. 2009) the currently accepted situation recognizes Phaeodarea as members of Cercozoa, clearly separated from the rest of Radiolaria composed of Acantharea, Polycystinea (including Spumellarida, Nassellarida and Collodaria), and Taxopodida. Together with Cercozoa and Foraminifera, Radiolaria form a super-group called the Rhizaria (Cavalier-Smith 2002),

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MATERIALS AND METHODS

Sampling and SSU rDNA genetic libraries. SSU rDNA sequences reported in the present study were obtained from genetic libraries performed on samples collected worldwide (i.e. Mediterranean Sea, Atlantic, Indian and Pacific Ocean). Details of experimental procedures according to each sampling location are provided for each cruise separately in the following sections and in Table 1 and Table S1.

PROSOPE cruise (Mediterranean Sea and Atlantic Ocean). Sampling was carried out in September and October 1999. Samples were collected in the Morocco upwelling (Atlantic Ocean) and across a transect from the strait of Gibraltar through the Ionian Sea, to the Ligurian Sea (Mediterranean Sea). Several stations and depths were sampled, information on sampling procedures and clone libraries construction were described in Viprey et al. (2008).

VANC10MV cruise (Indian Ocean). Sequences from the Indian Ocean were obtained from the surface and deep chorophyll maximum at stations 1, 9, 18 and 23 of a transect performed across the Indian Ocean. Details of sampling procedures and clone libraries constructions are provided in Not et al. (2008). Representative clones identified as Radiolaria by BLASTn searches were selected for full-length sequencing and included in the present study.

BIOSOPE cruise (South-East Pacific Ocean). Sequences were retrieved from ten South-East Pacific samples collected from the aphotic zone (between 500 and 3,000 m) at six sampling stations selected along a transect from the Marquesas Islands to the Chilean coast, through the central Pacific gyre (Claustre et al. 2008). Eight liters of water were harvested using 12 L Niskin bottles fitted on a Rosette sampler equipped with conductivity, temperature, and depth sensors. Seawater samples were pre-filtered through 200 µm and 3 µm successively. Picoplanktonic cells were collected on a 0.2 µm pore size Sterivex (Millipore, Molsheim, France) unit. After filtrations, Sterivex units and cryovials containing the > 3 µm filter were filled with lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris pH 9.0)

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Table 1. Radiolarian sequences in environmental genetic libraries. Number of genetic libraries analyzed, total number of clones screened, percentage of clones belonging to Radiolaria, size fraction and primer set used during the PCR amplification are reported. “na” means that the corresponding information is not available. When the number of radiolarian clone is not available (na), the number of sequences related to Radiolaria is given within brackets. Genetic libraries performed with similar primers set and directly comparable are indicated in bold. Some datasets reported here are not included in the phylogenies (Fig. S1--5) due to short length sequences and low overlap with sequences from other datasets. More details on datasets used are provided in table S1.

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Marine Environment	Number of libraries	Total number of clones	Percentages of Radiolarian	Size fraction	PCR primer set	Reference
Coastal Euphotic						
Blanes Bay	4	339	**1**	< 3µm	**EukA-EukB**	Massana *et al.* (2004
Gibraltar Strait	2	204	**6**	< 3µm	**Euk328f-Euk329r**	This study
Atlantic Ocean	1	126	**2**	< 3µm	**Euk328f-Euk329r**	This study
China Sea	2	323	8	-	18N1-18N11R	Yuan *et al.* (2004)
Gibraltar Strait	2	441	47	< 3µm	Euk328f-CHLO02r	This study
Atlantic Ocean	1	53	17	< 3µm	Euk328f-CHLO02r	This study
Coastal Aphotic						
South Pacific	1	96	47	> 3µm	Euk328f-UNonMet	This study
South Pacific	2	192	54	< 3µm	Euk328f-UNonMet	This study
Open Sea Euphotic						
Equatorial Pacific	1	103	**18**	< 3µm	**Euk328f-Euk329r**	Moon-van der Staay *et al.* (2001) and this study
Indian Ocean	6	456	**13**	< 3µm	**EukA-EukB**	Not *et al.* (2008) and this study
Sargasso Sea	3	465	**12**	-	**EukA-EukB'**	Countway *et al.* (2007)
Sargasso Sea	4	78	**3**	< 2µm	**Euk328f-Euk329r**	Not *et al.* (2007)
Mediterranean Sea	12	1,338	**3**	< 3µm	**Euk328f-Euk329r**	This study
Canadian Basin	4	na	**na (18)**	< 3µm	**EukA-EukB**	Lovejoy *et al.* (2006)
Sargasso Sea	na	na	na (13)	na	na	Armbrust *et al.* unpublished
Mediterranean Sea	12	1,818	34	< 3µm	Euk328f-CHLO02r	This study
Open Sea Aphotic						
Sargasso Sea	3	458	**44**	-	**EukA-EukB'**	Countway *et al.* (2007)
Sargasso Sea	2	151	**29**	< 2µm	**Euk328f-Euk329r**	Not *et al.* (2007)
South Pacific	3	287	6	> 3µm	Euk328f-UNonMet	This study
South Pacific	4	478	35	< 3µm	Euk328f-UNonMet	This study
Antarctic	4	na	na (1)	< 5µm	EK-1A-18S-1520R	López-García *et al.* (2001)
Hydrothermal vents						
Guaymas Basin	7	276	6	-	Mix of euk primers	Edgcomb *et al.* (2002)
Mid-Atlantic ridge	8	266	0.4	-	Mix of euk primers	López-García *et al.* (2007)
South East Pacific rise	2	189	22	> 10 µm	Euk328f-UNonMet	This study
South East Pacific rise	1	94	13	3-10 µm	Euk328f-UNonMet	This study
Other Anoxic						
Cariaco Basin	3	500	**na (7)**	-	**EukA-EukB**	Stoeck *et al.* (2003)
Cariaco Basin	3	497	na (8)	-	na	Stoeck *et al.* (2006)

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and frozen in liquid nitrogen. DNA from both size fractions (< and > 3 µm) was extracted using a 3 % CTAB (Cetyltrimethylammonium bromide) extraction procedure (Doyle and Doyle 1987). PCR products obtained using primers Euk328 (Romari and Vaulot 2004) and UNonMet (Bower et al. 2004), were cloned using the TOPO-TA cloning kit (Invitrogen, Cergy Pontoise, France). The primer set used for PCR allow the detection of most of eukaryotic lineages, including Radiolaria, with the exception of Metazoa, and some Amoebozoa and Excavata. Genetic polymorphism among clones retrieved was assessed by RFLP (restriction fragment length polymorphism): PCR products were digested with 1 Unit μL^{-1} of restriction enzyme HaeIII (Gibco BRL, Invitrogen) for 6 to 12 h at 37 °C. The digested products were separated by electrophoresis at 80 V for 2 to 3 h in a 2.5 % low-melting-point agarose gel. One clone per Operational Taxonomic Unit (OTU) was sequenced entirely using a ABI Prism 3100 (Applied Biosystems, Courtaboeuf, France).

OLIPAC cruise (Equatorial Pacific Ocean). Sampling procedures and genetic library construction of the Equatorial Pacific Ocean dataset are described in Moon-van der Staay et al. (2001).

BIOSPEEDO (South East Pacific Rise). Sequences were obtained from samples collected at hydrothermal vents along the South East Pacific Rise (7° 24'S to 21° 33'S). Sampling was performed with the submersible Nautile from IFREMER. Vent fragments were collected and stored in situ with surrounding water into hermetic containers. Back onboard, water from sterile containers was filtered successively through 10, 3, and 0.2 µm. Environmental sequences have also been recovered from two additional samples collected using Niskin bottles fired at 300 and 900 m depth, above the Rehu Marka hydrothermal field. Samples were handled as those obtained from the containers. DNA extraction, PCR amplification, and screening of clones were performed as for the BIOSOPE cruise.

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Development of sequence database and identification of Radiolarian sequences. The SSU rDNA database used in our analysis was built with all sequences deposited in GenBank up to December 2007, and sequences from genetic libraries generated herein. The software package KeyDNAtools (http://KeyDNAtools.com) was used to i) detect sequences belonging to Radiolaria within the database including more than 44,000 sequences, ii) identify potential chimeras, and iii) automatically assign sequences to a specific taxa (Guillou et al. 2008).

Phylogenetic analyses. Alignment and preliminary neighbor joining phylogenetic analysis of SSU rDNA radiolarian sequences was done using the Mafft 5.8 (Katoh et al. 2007) and Mega (Tamura et al. 2007) software. This allowed selection of representatives for the different genetic clades observed. One representative clone per genetic libraries was selected for groups of sequences exhibiting more than 98 % identity. Final phylogenies were inferred from a SSU rDNA sequence alignment (accession numbers available in Table S3) containing full-length sequences belonging to all radiolarian taxonomic groups. *Amoebophrya* sp., *Gymnodinium catenatum* and *Alexandrium tamarense* (Alveolata) were used to root the trees. Sequences were aligned using the slow and iterative refinement method FFT-NS-i of the Mafft 5.8 software (Katoh et al. 2007). The secondary structures were deduced by visualizing the alignment with the BioEdit editor (Hall 1999) and using previously published studies (Lange et al. 1996). Beside the use of KeyDNAtools, chimeras were also checked by BLASTn analysis by submitting different regions of the sequences and confronting alternative phylogenetic trees using 300-bp pieces from the 5' and 3' ends and from the middle part of the gene. Poorly aligned and very variable regions of the alignment were automatically removed with Gblocks (Castresana 2000) using the following parameters: allowing gaps in half position and minimum length of a block equal to five. Different nested models of DNA substitution and associated parameters were estimated using Modeltest (Posada and Crandall 1998). Settings given by Modeltest were used to perform maximum-likelihood (ML) and

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which belong to an assemblage (called 'SAR'), including Stramenopiles, Alveolata and Rhizaria, based upon a multigene phylogeny approach (Burki et al. 2007).

During the last decade, the number of SSU rDNA radiolarian sequences deposited in GenBank has increased drastically, in relation with the development of cloning-sequencing approaches from environmental DNA. Radiolarian sequences are consistently retrieved in several distinct marine habitats and from various size fractions down to picoplanktonic (cells < 3 µm) communities, raising questions regarding their diversity and ecology (Countway et al. 2007; Not et al. 2007; Not et al. 2008; Stoeck and Epstein 2003; Viprey et al. 2008). Recently, environmental survey focused on pico-size fraction unveiled five unexpected clades; RAD I to RAD V (Not et al. 2007). Three of these clades included exclusively environmental sequences among which many could not be clearly assigned to any morphologically defined taxa, suggesting that further lineages may exist.

The present study aimed at (i) provide an overview of the radiolarian genetic diversity using SSU rDNA sequences both from single cells and environmental clone libraries (ii) seek global patterns on the biogeographical distribution and habitat preferences of radiolarians assessed by their genetic signatures.

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neighbour-joining (NJ) analyses. NJ, ML and maximum parsimony (MP) analyses were performed using Paup 4.0b10 (Swofford 2002). Bootstrap values for NJ and MP were obtained from 1,000 replicates. For MP, the number of rearrangements was limited to 5000 for each bootstrap replicate. An additional analysis using Bayesian inference was performed with MrBayes v.3.0b4 (Huelsenbeck and Ronquist 2001). The GTR model of substitution was used, taking into account a gamma-shaped distribution of the rates of substitution among sites. The chains were run for 1,000,000 generations. Trees were sampled every 100 generations. The first 5,000 sample trees, corresponding to the initial phase before the chains became stationary were discarded (burn in). All sequences generated in the present study have been deposited in the GenBank database under accession numbers XXXXXXXX-XXXXXXXX.

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RESULTS

Overview of the SSU rDNA sequences dataset used. To complement the existing dataset for radiolarian sequences available in GenBank, we constructed additional environmental genetic libraries from a broad range of oceanic ecosystems: the Mediterranean Sea, the Atlantic Ocean, the Indian Ocean, the Equatorial and South East Pacific Ocean. We screened the entire SSU rDNA databases (i.e. 44,017 sequences), including a total of 526 SSU rDNA environmental libraries (i.e. 22,226 sequences obtained from marine and continental samples) and 21,791 sequences obtained from cultures or isolated cells (Table 2). Altogether, 1,529 radiolarian sequences have been detected, among which 56 were from uncultured isolated cells and 1,473 from environmental genetic libraries, representing 0.3 % and 8.9 % of the sequences, respectively. Most radiolarian sequences from environmental libraries belonged to

Table 2. Occurrence of radiolarian SSU rDNA gene sequences across marine (water and sediment) and continental (freshwater, biofilm and soil) ecosystems. Env. stands for environmental sequences. The number of genetic libraries screened for each environment is given into brackets in the first column. In the third column brackets indicate the contribution of radiolarian sequences to the total number of sequences retrieved in a particular environment.

Origin of the sequences and number of clone libraries considered	**Number of SSU rDNA sequences screened**	**Number of sequences belonging to Radiolaria**	**Number of sequences per radiolarian group**				
			Acantharea	**Spumellarida**	**Nassellarida and Collodaria**	**RAD A**	**RAD B**
Cultures and Isolates*	21,791	56 (0.3 %)	14	15	24	0	3
Env. Continental (150)	5,711	0	0	0	0	0	0
Water column Plankton (42)	1,213	0	0	0	0	0	0
Biofilm / microbial mats (16)	392	0	0	0	0	0	0
Soil (39)	3,361	0	0	0	0	0	0
Sediment (30)	438	0	0	0	0	0	0
Others** (23)	307	0	0	0	0	0	0
Env. Marine (376)	16,515	1,473 (8.9 %)	305	652	166	90	260
Water column Plankton (277)	13,573	1,449 (10.7 %)	300	650	156	88	255
Sediment (46)	1,607	17 (1.1 %)	4	2	9	1	1
Others** (53)	1,335	7 (0.5 %)	1	0	1	1	4
Total	**44,017**	**1,529**	**319**	**667**	**190**	**90**	**263**

* Including PCR analysis of uncultivated single cell. Sequences belonging to Radiolaria are all from uncultivated single cells.

** Others correspond to aerosol, bioreactors, gut communities, etc…

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Spumellarida, Acantharea, and RAD B clade as defined in our analysis (652, 305, and 260 sequences, respectively).

Phylogenetic diversity of Radiolaria. To obtain an overview of the radiolarian phylogeny we selected 119 near full-length Rhizaria sequences among which half of them were retrieved from microbial size (< 5 µm) environmental genetic libraries. Based upon Maximum-Likelihood (ML) and Bayesian analyses, Radiolaria are composed of two main lineages (Fig. 1). The Acantharea strongly supported by all analyses, and a second monophyletic group supported by Bayesian posterior probabilities only (Fig. 1). This later group encloses five main clades, each of them supported by strong bootstrap values: the Spumellarida, the closely related Nassellarida and Collodaria, and three novel clades named RAD A, B, and C. Besides environmental sequences, RAD B includes a sequence of *Sticholonche* sp. (Taxopodida) and two sequences obtained from single cell PCR-amplifications of putative Spumellarida (isolates 7039 and 7017, Kunitomo et al. 2006), suggesting that these five groups all belong to Polycystinea. Detailed phylogenetic analysis of each radiolarian taxa identified (including partial length sequences) and taxonomical framework for further examination of environmental sequences occurrences are provided as supplementary material (Fig. S1--5 and Table S4, S5).

Biogeography of radiolarian sequences. In the present study we assessed the distribution patterns and habitat preferences of radiolarians based on their environmental sequences' occurrences. Radiolaria were recovered from all marine environments investigated (e.g. eutrophic to oligotrophic, euphotic and aphotic, including sub-oxic to anoxic, Table 1, 2). Radiolaria relative contribution ranged from < 1 % of the clones in Mid-Atlantic ridge hydrothermal vent environments up to 54 % in the aphotic coastal waters of the South Pacific (Table 1, S1). In order to interpret the relative occurrences of radiolarian sequences according to environmental settings, only genetic libraries constructed with the general eukaryotic

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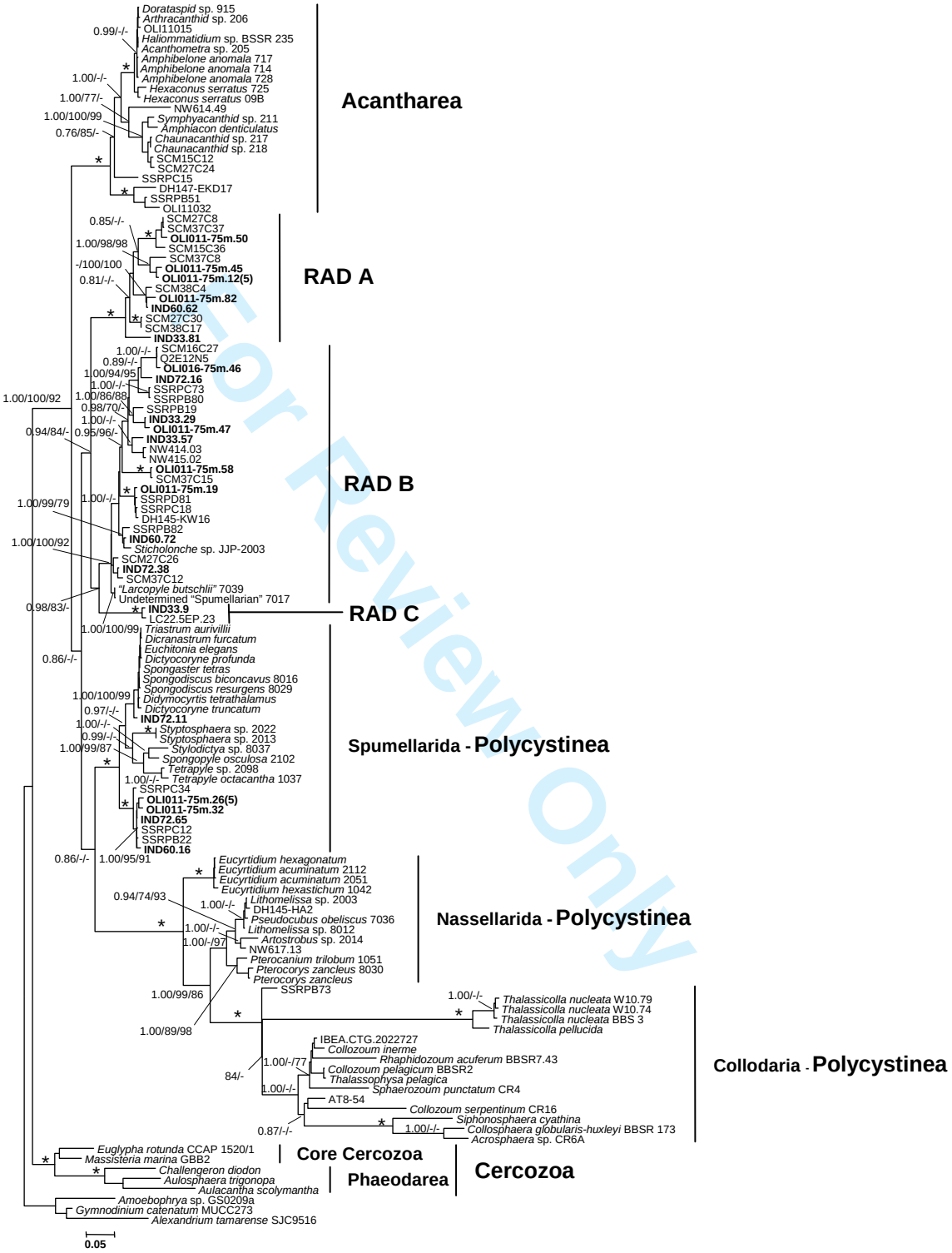
Acantharea
RAD A
RAD B
RAD C
Spumellarida - Polycystinea
Nassellarida - Polycystinea
Collodaria - Polycystinea
Core Cercozoa
Phaeodarea
Cercozoa
0.05

Figure 1

primer sets Euk328/Euk329, EukA/EukB, and EukA/EukB', which are quite similar in their position and base composition (Table S2), can be compared. Indeed, the use of a primer biased towards Chloroplastida (CHLO02r), but presenting only a single mismatch to Radiolaria led to an increased recovery of radiolarian sequences (Viprey et al. 2008). For instance, Radiolaria represented 47 % of the total number of clones from the Strait of Gibraltar using the Euk328f/CHLO02r primer set, vs. 6 % with the Euk328f/Euk329r primer set (Table 1). Therefore, our analysis of comparable datasets suggests that the proportion of radiolarian sequences increases from coastal to open ocean waters at surface (< 6 % up to 18 %) and from euphotic to deeper waters (Table1, Fig. 2).

Vertical distribution across euphotic waters and size-fractionation. The relative contribution of radiolarian groups obtained from size fractionated samples (< 3 µm) along the euphotic zone of the Mediterranean Sea (5-110 m) and the Indian Ocean (5-85 m) is presented in Fig. 2. All radiolarian lineages were retrieved in those samples, except for Collodaria and Acanth-I which were not detected at all. Qualitatively, the taxonomic composition of most radiolarian assemblages was quite similar along the depth profiles except for RAD B environmental sequences which were more diversified at depth. Despite the use of different primer sets in the Mediterranean Sea (Euk328/CHLO02r) and in the Indian Ocean (EukA/EukB), both datasets showed a significant increase in the relative contribution of Radiolarian sequences with depth. The pattern observed is mainly driven by the increasing contribution of the Spumellarida, Nassellarida, and to a lesser extent of RAD B lineages.
In aphotic waters of the South Pacific Ocean, relative contributions of radiolarians were more important below the 3 µm size fraction than above (41 % and 16 %, respectively Fig. 3). Taxonomic assemblages were slightly different between the two size fractions analyzed since only Collodaria and Spumellarida were present in both size fractions. Spumellarida was the most represented taxa in the less than 3 µm size fraction. Acantharea and RAD C contributed

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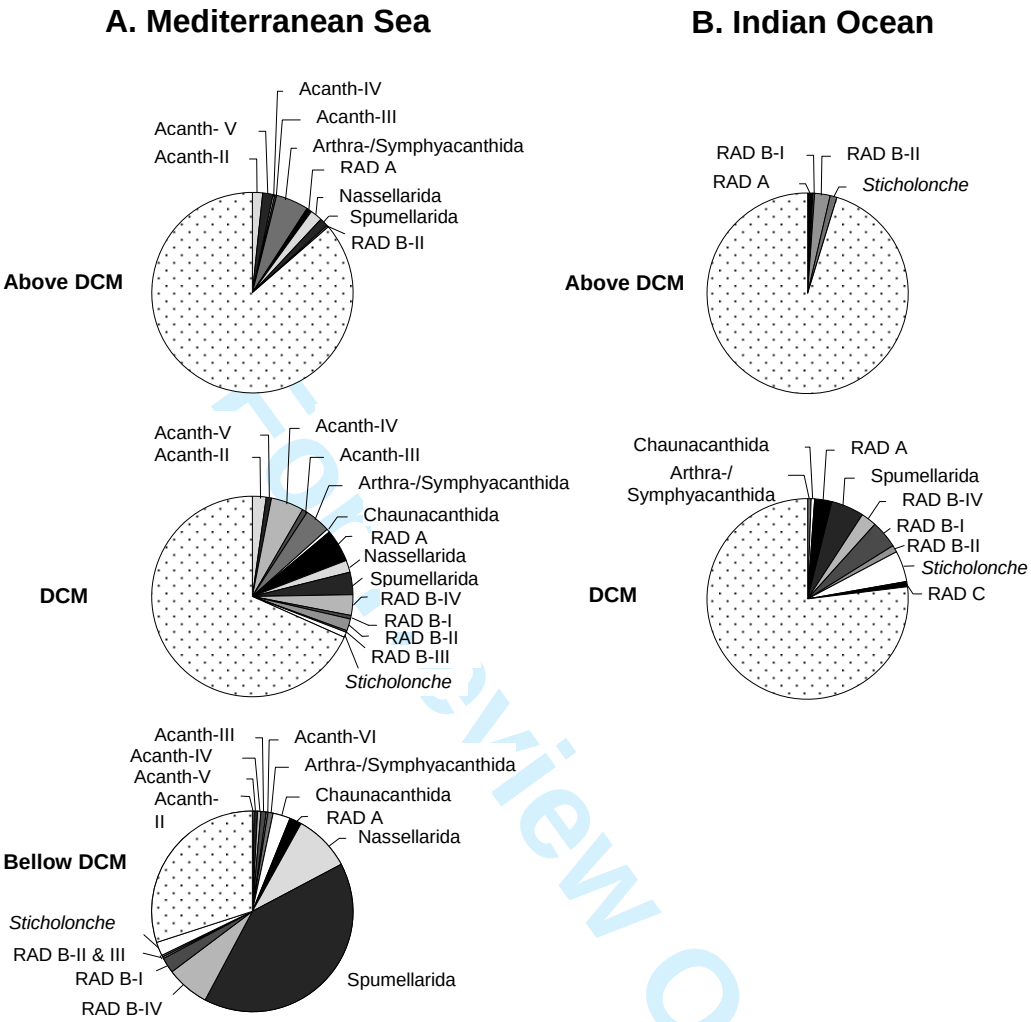

Figure 2

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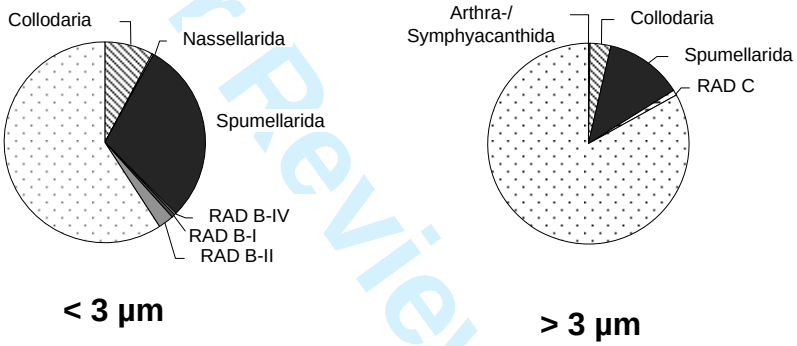

Figure 3

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only to the fraction above 3 µm while Nassellarida, RAD B-I, RAD B-II and RAD B-IV were recovered only from picoplankton.

DISCUSSION

Overall Radiolarian phylogeny. Our phylogenetic reconstruction, built using 21 new full-length environmental sequences, supports a common ancestor to the group formed by Acantharea and Polycystinea. Morphological description of polycystinean swarmers belonging to the order Spumellarida demonstrated they contain a large strontium sulphate crystal, similar in composition to the acantharean skeleton (Hollande and Martoja 1974). This observation stands as an additional argument for the common origin of Acantharea and Polycystinea. Regarding polycystinean phylogeny, all recent studies argued for the polyphyly of this taxa with the Spumellarida being closer to the taxopodida *Sticholonche* and the Acantharea than to other Polycystinea (i.e. Nassellarida and Collodaria, Kunitomo et al. 2006; Not et al. 2007; Takahashi et al. 2004; Yuasa et al. 2005). In contrast, the present reconstruction suggests the monophyly of Polycystinea, the Acantharea being in a basal position within Radiolaria and the Polycystinea being composed by five distinct lineages (Fig. 1).

Most radiolarian clades identified and well supported in our phylogenetic analyses are composed of environmental sequences only (Fig. S1--5). Radiolarian taxonomy remains largely under-sampled from a molecular point of view. For instance, from the 12 families described within Spumellarida (Table S4), SSU rDNA sequences of identified isolates are available for five families only, all represented in this study (i.e. Lithellidae, Coccodiscidae, Pyloniidae, Ethmosphaeridae and the polyphyletic Spongodiscidae). Such observation highlights the critical need to obtain sequence data from well characterized organisms through

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morpho-molecular approaches on single cells. Ideally the taxonomic information should be collected along with environmental (sampling depth, physico-chemical characteristics) and ecological data (presence of symbionts and/or parasites and their taxonomic affiliation, presence and content of digestive vacuoles), which would certainly contribute to understand better the biology of these organisms.

Ecological perspectives. Radiolaria are strictly restricted to marine environments and therefore differ from other Rhizaria (Cercozoa and Foraminifera) reported to inhabit both marine and freshwater ecosystems (Bass and Cavalier-Smith 2004; Holzmann et al. 2003). With 10.7 % of sequences, Radiolaria consistently represent the third most abundant group of organisms (after Alveolates and Stramenopiles) in environmental surveys of plankton samples. Radiolaria are widely distributed, but were not detected from salt marsh (Stoeck and Epstein 2003) and estuarine genetic libraries so far (Romari and Vaulot 2004). Even though both Nassellarida and Spumellarida have been observed in Norwegian fjords (Swanberg and Bjørklund 1987), radiolarian sequences are preferentially recovered from open-sea where they reached on average ca 20 % of the clones, similar to those observed in oceanic systems for MArine STramenopiles (MAST) and Syndiniales (Massana and Pedrós-Alió 2008). In coastal settings, Radiolaria have a lower contribution (usually < 6 %) compared to MAST and Syndiniales which contribute up to 10 % and 40 % of the clones, respectively (Guillou et al. 2008; Massana et al. 2004; Not et al. 2009). Coastal genetic libraries containing Radiolaria come from waters that are either oligotrophic (Blanes Bay, China Sea) or under oceanic influence (Morocco upwelling, Chilean upwelling, Strait of Gibraltar) and in a lesser extant from shallow temperate water (Marie et al. 2010; Medlin et al. 2006; Romari and Vaulot 2004; Savin et al. 2004). Several clades among Acantharea (Acanth-I to V), Spumellarida (Spum-II) and RAD B (RAD B-III and the “Sticholonche” cluster) are recovered only from oligotrophic waters (Fig S2--3, and S5). All other groups are present at least in two contrasted

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marine systems and display always representatives in oceanic waters, confirming that Radiolaria are foremost blue-water organisms as previously reported from plankton net sampling (Anderson et al. 2000; Febvre et al. 2000). Colonial association or mixotrophy in association with photosynthetic symbionts have emerged in several radiolarian lineages and could be a key adaptation to these low nutrient environments (Norris 1996).

The Mediterranean and Indian Ocean datasets highlight an increase of the relative contribution and genetic diversity of radiolarian environmental sequences with depth along the euphotic layer (Fig. 2). Two recent works in the Sargasso Sea (Countway et al. 2007; Not et al. 2007) have shown a more important contribution of Radiolaria within genetic libraries in aphotic waters (from 500 to 3,000 m). This trend is confirmed by the present work but is in contradiction with the ecology of most Radiolaria as the main factors advanced to explain their vertical distribution are prey abundances, temperature, salinity and dissolved oxygen content (Ishitani and Takahashi 2007; Klaas 2001). According to Swanberg and Caron (1991) Acantharea get their resources in a large part from photosynthetic symbionts and thus need light to thrive. Many Collodaria such as *Thalassicolla*, *Collozoum*, or *Sphaerozoum* genera, bear also phototrophic symbionts (Gast et al. 2003; Takahashi et al. 2003). High representation of collodarian environmental sequences in aphotic waters (Fig. S4) does not fit with the vertical distribution of colonial Collodaria directly observed from the central North Pacific where they are always more abundant above the DCM (Dennett et al. 2002). Overall, living Polycystinea are more abundant in surface euphotic waters or at least in the upper 300 m of the column water in many environments (e.g. Itaki et al. 2003; Kling and Boltovskoy 1995; Yamashita et al. 2002). Only some rare living polycystinean specimen belonging to *Cycladophora davisiana*, *Larcopyle butschlii*, *Pterocorys zancleus* or *Dictyocoryne profunda* have been recovered from deep waters (below 500 m, Abelman and Gowing 1997; Itaki et al. 2003; Yamashita et al. 2002). Several non exclusive reasons may explain these discrepancies

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between observations and representation in genetic libraries. PCR artifacts are probably important as we observed an absence of widespread rhizarian taxa such as Foraminifera and Phaeodarea, from the 22,226 environmental sequences dataset screened in this study. This observation illustrates the fact that closely related lineages are differentially amplified by PCR using the current protocols. Additionally, the major contribution of environmental sequences within genetic libraries from deep waters may simply reflect the relative rarefaction of other eukaryotic lineages, leading to the overrepresentation of radiolarian sequences. Additionally, environmental radiolarian sequences from depth may not necessarily result from living organisms. Since many Radiolaria bear a fragile skeleton, a large fraction of environmental sequences detected from the picoplanktonic fraction could arise from cell breakage during sample collection while sequences from larger size fractions could originate from intact cells (Not et al. 2009). Predominance of Polycystinea sequences at depth, for which the siliceous skeleton can persist longer after death in comparison to strontium sulfate of Acantharea, argues in favor of this hypothesis. However, the presence of several radiolarian lineages in the picoplanktonic size fraction and not detected in the larger size fractions from deep waters of the Pacific Ocean is puzzling, as fragments of larger cells are not expected to be all calibrated to less than 3 µm after cell breakage. Alternatively, these environmental sequences could originate from small picoplanktonic species and/or smaller stages of the life cycle. Radiolarian reproduction is not well elucidated and published works describing these spores are rare. For instance, flagellated spores of *Sphaerozoum neapolitanum* and *Thalassicolla nucleata* are quite large, with 15 µm in long and 6 µm in diameter (Anderson 1978; Hollande 1974) and we cannot exclude that even smaller gametes are produced in other radiolarian species. Acantharea are known to produce small biflagellated isogametes during sexual reproduction (Febvre et al. 2000). Radiolarian reproductive strategies could be similar to that of some planktonic foraminifera, which produced hundreds to thousands of small gametes per

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parent cell. For instance, *Globigerina sacculifer*, a foraminifera bearing photosynthetic symbionts is classically observed near the sea surface during its vegetative stage, whilst the reproduction preferentially occur in deeper layers, around 400 m and is synchronized with moon phases (Bijma et al. 1994; Erez et al. 1991). Direct observation of radiolarian picoplanktonic communities using SSU rDNA probes detected by fluorescent in situ hybridization becomes feasible since a good set of sequences is now available for probe design. Such morpho-molecular, quantitative, approaches would certainly help to better understand radiolarian ecology in future investigations.

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FIGURE LEGENDS

Fig 1. SSU rDNA phylogenetic analysis of Radiolaria inferred from 1,252 homologous position of an alignment of 122 nearly full-length sequences (three Alveolata and five Cercozoa sequences used as outgroup). Sequences obtained in this study are in bold. GenBank accession numbers are provided in Table S3. The tree was inferred by the ML method based on a TrN equal base frequencies model of DNA substitutions with a gamma distribution shape parameter of 0.5748, a proportion of invariable sites of 0.1148 and substitution rates of R(b) [A-G] = 2.5115, R(e) [C-T] = 3.8639, and 1.0 for all other substitution rates. Total number of rearrangements tried = 102,661. Bootstrap values greater than 70 (percentage of 1,000 replicates) obtained from neighbor-joining and maximum parsimony methods are reported (NJ/MP). A * indicates 100 / 100 bootstrap values. Scale bar corresponds to 5 % divergence. Posterior probabilities superior to 0.8 obtained in an additional analysis by Bayesian inference are reported for the major nodes.

Fig 2. Taxonomic composition of the radiolarian euphotic communities in the < 3 µm size fraction from Mediterranean Sea (A) and the Indian Ocean (B) based on identifications of partial SSU rDNA gene sequences using the KeyDNAtools software. Taxa names are given following detailed phylogenies provided as supplementary material (Fig. S1--5). Clone libraries were performed using the Euk328f/CHLO02r and the EukA/EukB primer sets, respectively. DCM stands for Deep Chlorophyll Maximum. Libraries from nearly same depth (above DCM, DCM and below DCM) are grouped together. Dotted pies correspond to the non-radiolarian sequences.

Fig 3. Taxonomic composition of deep radiolarian communities (500 to 3,000 m) from the South Pacific, based on identifications of partial SSU rDNA gene sequences using the KeyDNAtools software, and presented according to their size fraction (< and > 3 µm). Taxa

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names are given following detailed phylogenies provided as supplementary material (Fig. S1--5). Dotted pies correspond to the non radiolarian sequences.

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VIPREY ET AL.---ENVIRONMENTAL SSU RDNA SEQUENCES OF RADIOLARIANS

Microbial-Size SSU rDNA Environmental Libraries Puzzles the Ecology and Genetic Diversity of Radiolaria

MANON VIPREY[a], FABRICE NOT[a,b], RAMON MASSANA[b], ANNE-LAURE SAUVADET[a], SEUNG YEO MOON-VAN DER STAAY[c], DANIEL VAULOT[a] and LAURE GUILLOU[a]

Supplementary Information

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Material and methods

Phylogenetic analyses of radiolarian taxa based on partial length SSU rDNA sequences.

Alignments and preliminary neighbor joining phylogenetic analysis of SSU rDNA radiolarian sequences was done using the Mafft 5.8 (Katoh et al., 2007) and Mega (Tamura et al. 2007) software. This allowed selection of representatives for the different genetic clades observed. As a general rule, a representative clone per genetic libraries was selected for groups of sequences exhibiting more than 98 % identity. Final phylogenies were inferred from six different SSU rDNA sequence alignments (GenBank access numbers of published sequences available in Table S3). The alignments were based on partial length sequences of the specific taxonomic groups: Acantharea, Spumellarida, Collodaria/Nassellarida, RAD A, RAD B, and Rad C. *Amoebophrya* sp., *Gymnodinium catenatum* and *Alexandrium tamarense* (Alveolata) were used to root the trees. Sequences were aligned using the slow and iterative refinement method FFT-NS-i of the Mafft 5.8 software (Katoh et al. 2007). Poorly aligned and very variable regions of the alignments were automatically removed with Gblocks (Castresana 2000) using the following parameters: allowing gaps in half position and minimum length of a block equal to five. Different nested models of DNA substitution and associated parameters were estimated using Modeltest (Posada and Crandall 1998). Settings given by Modeltest were used to perform maximum-likelihood (ML) and neighbour-joining (NJ) analyses. NJ, ML and maximum parsimony (MP) analyses were performed using Paup 4.0b10 (Swofford 2002). Bootstrap values for NJ and MP were obtained from 1,000 replicates. For MP, the number of rearrangements was limited to 5000 for each bootstrap replicate.

As a taxonomic framework, families considered in this study within Polycystinea and Acantharea are provided in tables S4 and S5, respectively. We used roman numbering to labeled

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subdivisions within RAD B, Spumellarida, and Acantharea lineages (Fig. S1--5). For instance, six clades exclusively composed by environmental sequences have been detected within Acantharea. They have been labeled Acanth-I to Acanth-VI to study their environmental distribution and help discussions. Concordance with previous published names is also provided in supplementary figures (Fig. S1--5). For instance, Acanth-I, Acanth-II, Spum-I, RAD B-I, and RAD B-II, are considered to be synonymous to RAD I and RAD II, published by Not et al. (2007).

Results

Sequences belonging to RAD A (Fig. S1A) were first recovered from 100 m depth off Nansha Islands (South China Sea) by Yuan et al. (2004) who described this group as "a cluster […] between Polycystinea and Acantharea". Clones NS51C262, NS51B282, NS371B04, NS371B39 and NS51D167 from the mentioned study were not included in the present analysis due to the short length of sequences. In our analysis we introduced 31 new sequences belonging to RAD A from a variety of ecosystems (open-ocean, coastal and anoxic). Most sequences came from the euphotic layer, excepted two sequences retrieved from 300 m in the Pacific Ocean (clone BS4.C6) and from 340 m in the anoxic Cariaco Basin (clone CAR-E.187, not included in the present analysis due to the low overlap with the other sequences, Stoeck et al. 2003). Environmental sequences belonging to RAD A have been retrieved both from picoplanktonic and larger size fractions. RAD A is composed of five distinct and well supported clusters that probably will require further separations. Clade RAD C (Fig. S1B) displays a relatively low genetic diversity since it is composed of five closely related sequences (96.8 % similarity). Three sequences are from deep waters of the central South Pacific gyre (between 500 and 3,000 m), one is from a mix of hydrothermal fluid and seawater collected from the mid-Atlantic Ridge, and

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the last one is from the euphotic layer of the Indian Ocean. RAD C sequences originate from both picoplanktonic and larger size fractions.

RAD B includes several well differentiated clades supported by all statistical analyses (Fig. S2). The “Sticholonche” clade encloses a representative of the genus *Sticholonche* and environmental sequences from oceanic and euphotic waters, with the exception of two sequences from deep Sargasso Sea samples ENVP366.00116 and SSRPB82, not included here (Countway et al. 2007; Not et al. 2007). RAD B-I and II were previously defined by Not et al. (2007) as RAD IV and V, respectively. RAD B-I, II, as well as the newly described clade RAD B-III are only composed of environmental sequences. RAD B-I and II originate from diverse oceanic or coastal samples and from both euphotic and aphotic waters. RAD B-I displays higher genetic variability than previously reported including a sub-cluster restricted to deep waters (BIO9_A12, BIO9_B10, and SSRPB19). RAD B-III sequences are all from euphotic and oceanic waters (Mediterranean Sea, Sargasso Sea, and Pacific Ocean). The RAD B-IV clade includes two sequences obtained from isolates (7039 and 7017) by single cell PCR (Fig. S2). The relationship between these isolates and *Sticholonche zanclea* was reported previously (Kunitomo et al. 2006). The RAD B-IV clade is not supported by the ML topology resulting from the near full-length sequence analysis (Fig. 1) and will probably be split into different clusters (at least two) in the future. RAD B-IV is the only cluster within RAD B containing sequences retrieved from hydrothermal environments.

Among Polycystinea, Spumellarida comprises sequences from morphologically described isolates belonging to different families, i.e. Ethmosphaeridae, Pyloniidae, Spongodiscidae, and Coccodiscidae (Fig. S3). All these families are monophyletic except for the Spongodiscidae with two sequences (*Stylodictya* sp. 8037 and *Spongopyle osculosa* 2102) which are more related to

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the Pyloniidae than to other Spongodiscidae, as reported previously (Kunitomo et al. 2006; Not et al. 2007). Spongodiscidae and Coccodiscidae are present in environmental libraries from diverse systems (oceanic, coastal and anoxic) and from both euphotic and aphotic waters. In addition to these families, Spumellarida displays three distinct environmental clades named Spum-I, II and III (Fig. S3). Spum-I had been previously described as RAD III by Not et al. (2007), while Spum-II and III emerge from the present analysis. As already noticed previously by Not et al. (2007), clade Spum-I contains two subgroups observed both in our full-length and partial analyses (named in this study Spum-I-1 and I-2). These new sequences confirm the aphotic origin of Spum-I-1 as well as the widespread distribution of Spum-I-2. Spum-II and III clades contain sequences from euphotic waters only and are strongly associated with the Spongodiscidae and Coccodiscidae families. Sequences belonging to Spum-III were restricted to Indian Ocean, Mediterranean Sea and South China Sea (clone NS371C36, not includes in this phylogenetic analysis, Yuan et al. 2004), while Spum-II comprises only two sequences from Mediterranean Sea.

Several polycystinean environmental sequences belong to the Nassellarida and the Collodaria that are strongly associated when considering nearly full-length SSU rDNA gene sequences (Fig. 1). The Collodaria appear as a monophyletic group, while the Nassellarida are paraphyletic (Fig. 1 and S4). The Nassellarida are divided into two clades, one composed of the genus *Eucyrtidium* (Theoperidae) and the second containing the others Nassellarida. Within the Nassellarida, only the Plagoniidae family has representatives from environmental genetic libraries. Plagoniidae sequences came mainly from euphotic oceanic waters except for one sequences from deep water (clone DH145-HA2 from 500 m in the Antarctic Ocean, López-García et al. 2002). All but one Collodaria environmental sequences were recovered from aphotic waters (hydrothermal and

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anoxic waters, 4 clones from the anoxic Cariaco Basin, not included in the present study, Stoeck et al. 2003; Stoeck et al. 2006), while the isolates from this group were from the euphotic zone. Acantharea are composed of 8 distinct clades supported by bootstrap values higher than 80 % (Fig. S5). Two clades include described species (Chaunacanthida, and a complex assemblage including members of Arthracanthida and Symphyacanthida), and six are exclusively composed of environmental sequences (Acanth-I to VI). While the Chaunacanthida isolates come all from oceanic euphotic waters, environmental sequences are also recovered from oligotrophic coastal waters (clone NS51B291 from Nansha Islands and two clones from Blanes Bay BL010320.09 and BL010320.10, Massana et al. 2004; Yuan et al. 2004) or anoxic hydrothermal sediments as well (clones C3_E016 and E010 from Edgcomb et al. 2002). The Arthracanthida/Symphyacanthida complex is made of several families belonging to Arthracanthida (Doratapsidae, Acanthometridae and Hexalapsidae) and Symphyacanthida (Pseudolithidae and Amphilithidae). It is composed of both isolates and environmental sequences. In agreement with the nearly full-length analysis (Fig. 1) and previously published work (Kunitomo et al. 2006), the Hexalapsidae have a basal position among the Arthracanthida/Symphyacanthida complex. Acanth-I and Acanth-II correspond respectively to RAD I and RAD II described by Not et al. (2007). Based on environmental sequences obtained from at least two distinct genetic libraries, the present study describes four additional clades (Acanth-III, IV, V and VI, Fig. S5). Acanth-I is composed of sequences from a unique habitat (i.e. deep Sargasso Sea waters from 500 m and 3,000 m) as previously noted by Not et al. (2007) and Countway et al. (2007). Acanth-III to V environmental sequences are from the Mediterranean Sea, except for one sequence of Acanth-IV that come from the euphotic water of the Sargasso Sea (clone ENVP21819.00358, not included in this analysis, Countway et al. 2007)

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and one sequence of Acanth-III (BS3-G1) from the deep Pacific waters. Other acantharean environmental clusters exhibit sequences from diverse origins. The most striking example is probably clade Acanth-VI recovered from the Mediterranean Sea and Arctic euphotic waters (C9-65 and NW614.49, respectively), but also from anoxic sediments collected close to hydrothermal vents (clones C3_E029 and C3_E013, not included in the phylogenetic analysis, Edgcomb et al. 2002).

Discussion

Acantharea. They are divided into four orders: Arthracanthida, Chaunacanthida, Holacanthida and Symphiacanthida (Table S5). Members of Holacanthida are missing in the present analysis (no isolates have been sequenced to date) and some novel lineages recovered within Acantharea may belong to this order. Chaunacanthida are well defined, whereas boundaries between Arthracanthida and Symphyacanthida are overlapping, as previously observed by Yuasa et al. (2005). Symphyacanthida are polyphyletic, and the genus *Haliommatidium* (Symphyacanthida) is closer to the arthracanthid *Acanthometra* sp. than other Symphyacanthida. Furthermore, the Hexalapsidae (Arthracanthida) diverges prior to the group formed by both Arthracanthida and Symphyacanthida. This fact is quite surprising as both orders are clearly distinct based on morphological data. First there is only one nucleus in the central capsule of the Symphyacanthida, a unique feature within Acantharea. Second, Arthracanthida are characterized by a thick capsular wall, composed of a large inner fibrillar layer, separating the endoplasm and the periplasmic cortex. Structures equivalent to this capsular wall exist in other orders, but consist in thinner and more superficial inner coats. Finally, Arthracanthida is the only order within Acantharea for which gametogenesis occurs in a gamont which keeps the aspect of the vegetative cell, whereas other orders encyst themselves just before gametogenesis. For the isolate

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"*Haliommatidium* BSSR 235", the criteria used to identify this cell prior to DNA extraction are not provided in the original publication (Amaral-Zettler et al. 1997). The isolate "Symphyacanthid 211" (Amaral-Zettler and Caron 2000) is clearly assigned to the Chaunacanthida in the present analysis. The Genbank sequences of *Amphibelone* and *Hexaconus* isolates are not associated with any published work and therefore there is no way to test the validity of their taxonomical assignment.

Polycystinea. Based on morphological data, Polycystinea are composed of three main orders: the Collodaria, the Spumellarida, and the Nassellarida (Table S4). Dichotomy between Spumellarida and Nassellarida is based on the skeleton plan (spherical in Spumellarida, not spherical in Nassellarida), the capsular wall perforation (round pores uniformly distributed in Spumellarida, pores only in the polar region in Nassellarida) and the radiation of the actinopodia (from all over the surface of the central capsule in Spumellarida, arising from a pore plate at one pole of the central capsule in Nassellarida). Collodaria contain skeleton-less or spicular polycystinean, such as members of Sphaerozoidae able to form colonies, and the Thalassicollidae and Thallasosphaeridae composed of solitary organisms. Recently Collosphaeridae were annexed to Collodaria based upon SSU rDNA phylogenies (Kunitomo et al. 2006; Yuasa et al. 2005). Collosphaeridae contain also colonial species, but display a well developed internal skeleton with a radial symmetry, a spherical cell body and a central capsule perforated uniformly, and therefore is morphologically more similar to Spumellarida than other Collodaria. These features likely argue for the classification of all collodarians inside Spumellarida. On the contrary, all phylogenies based upon molecular data, including the present analysis (Fig. 1), place Collodaria close to Nassellarida (Not et al. 2007; Yuasa et al. 2005). However, it is important to note that Collodaria are mostly composed of colonial organisms, a life mode that could have deeply

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modified their morphology. Furthermore, Collodaria display quite long branches in phylogenetic reconstructions and have already been proposed as a fast evolving group (López-García et al. 2002; Polet et al. 2004). Therefore this group can be partially affected by long branch attraction artefacts (Felsenstein 1978), especially in the case of the inclusion of Collosphaeridae within Collodaria.

In addition to these polycystinean lineages (Spumellarida, Collodaria, and Nassellarida), the present study suggests the inclusion of several clusters mainly composed of environmental sequences (RAD A, B and C) within the Polycystinea. The taxonomic affiliation of RAD A, B and C is quite complex since RAD B is composed of both putative Spumellarida and Taxopodida (*Sticholonche*), and no sequence from described isolates is available within RAD A and C (Fig. S1--2). However, several reasons lead us to think that RAD A, B and C are probably all members of the Polycystinea. First, sequences of the isolates 7039 and 7017 belonging to RAD B were previously published by Kunitomo et al. (2006) as "*Larcopyle butschlii"* and "undetermined spumellarian", respectively. Based on published photographs, isolates 7039 and 7017 display the morphological characters of Spumellarida (spherical body plan, axopodia radiating uniformly from all over the surface of the central capsule). Furthermore, skeleton of cell 7039 presents a spiral structure, a character shared by all Litheliidae (Spumellarida) where *Larcopyle butschlii* is actually described. Skeleton morphology of isolate 7017 cannot be observed preventing further resolution of its taxonomic position within Spumellarida. RAD B includes also *Sticholonche zanclea* an atypical genus described as a pelagic organism with rows of motile oar-like pseudopodia, a bilateral symmetry and a heart shape body, and is therefore apparently quite different from Polycystinea. In fact, the taxonomy of *Sticholonche* is quite uncertain: initially described within the Taxopodida (Fol 1883), the genus was later included within Radiolaria

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sensu Haeckel (Hollande et al. 1967), then moved to the polyphyletic Heliozoa (Cachon and Cachon 1978), and recently transferred back to the emended Radiolaria based on morphological and molecular data (Cavalier-Smith 1998; Nikolaev et al. 2004). The placement of *Sticholonche* within the Radiolaria has been confirmed by a combined LSU and SSU rDNA analysis (Moreira et al. 2007). From a morphological point of view, *Sticholonche* lacks one of the most important features of the Radiolaria: the central capsule. However, this feature is also present in Phaeodarea which are now considered to be Cercozoa. Furthermore, the presence in the *Sticholonche* cytoplasm of numerous tangential and long external radial siliceous spicules could be related to the polycystinean siliceous skeleton. In agreement with previous studies (Kunitomo et al. 2006; Not et al. 2007), our analyses highlight the relatedness between *Sticholonche* and the Spumellarida, which would probably lead to the demise of the Taxopodida and the inclusion of *Sticholonche* within Polycystinea.

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Figure legends

Figure S1 . **A.** SSU rDNA phylogenetic analysis of RAD A clade (Radiolaria) inferred from 740 homologous position of an alignment of 39 partial sequences (including 3 Alveolata and 5 Cercozoa sequences as outgroup, not shown). The tree was inferred by the ML method based on a TrN equal base frequencies model of DNA substitutions with a gamma distribution shape parameter of 0.3309 and substitution rates of R(b) [A-G] = 2.1510, R(e) [C-T] = 3.4259, and 1.0 for all other substitution rates. Total number rearrangements tried = 18,510. **B.** SSU rDNA phylogenetic analysis of RAD C clade (Radiolaria) inferred from 882 homologous positions of a 18S partial sequences alignment (including 3 Alveolata, 5 Cercozoa, and 5 other radiolarian taxa sequences as outgroup, not shown). The tree was inferred by the ML method based on a TrN equal base frequencies model of DNA substitutions with a gamma distribution shape parameter of 0.4161 and substitution rates of R(b) [A-G] = 2.1430, R(e) [C-T] = 3.5264, and 1.0 for all other substitution rates. Total number rearrangements tried = 1,911. Bootstrap values greater than 70 (percentage of 1,000 replicates) obtained from neighbour-joining and maximum parsimony methods are reported (NJ/MP). A * indicates 100 / 100 bootstrap values. Scale bar corresponds to 5 % divergence.

Figure S2. SSU rDNA phylogenetic analysis of the novel clade named RAD B inferred from 747 homologous position of an alignment of 75 partial sequences (including 3 Alveolata and 5 Cercozoa sequences as outgroup). The tree was inferred by the ML method based on a TrN equal base frequencies model of DNA substitutions with a gamma distribution shape parameter of 0.5119 and substitution rates of R(b) [A-G] = 2.2962, R(e) [C-T] = 3.3385, and 1.0 for all other substitution rates. Total number rearrangements tried = 147,991. For reference, the previous nomenclature from Not et al. (2007) is given into brackets. New sequences obtained in this study

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are in bold. Bootstrap values greater than 70 (percentage of 1,000 replicates) obtained from neighbour-joining and maximum parsimony methods are reported (NJ/MP). A * indicates 100 / 100 bootstrap values. Scale bar corresponds to 5 % divergence.

Figure S3. SSU rDNA phylogenetic analysis of Spumellarida (Polycystinea, Radiolaria) inferred from 714 homologous position of an alignment of 76 partial sequences (including 3 Alveolata and 5 Cercozoa sequences as outgroup). Legend as in figure S2 except for the following parameters: gamma distribution shape parameter of 0.4573 and substitution rates of R(b) [A-G] = 1.9920, R(e) [C-T] = 4.1501, and 1.0 for all other substitution rates. Total number rearrangements tried = 105,115. Bootstrap values greater than 70 (percentage of 1,000 replicates) obtained from neighbour-joining and maximum parsimony methods are reported (NJ/MP). A * indicates 100 / 100 bootstrap values. Scale bar corresponds to 5 % divergence. Scale bar corresponds to 2 % divergence.

Figure S4. SSU rDNA phylogenetic analysis of Collodaria and Nassellarida (Polycystinea, Radiolaria) from 700 homologous position of an alignment of 59 partial sequences. Legend as in figure S2 except for the following parameters: gamma distribution shape parameter of 0.5924 and substitution rates of R(b) [A-G] = 2.1666, R(e) [C-T] = 3.5276, and 1.0 for all other substitution rates. Total number rearrangements tried = 66,408. Scale bar corresponds to 5 % divergence.

Figure S5. SSU rDNA phylogenetic analysis of Acantharea (Radiolaria) inferred from 700 homologous position of an alignment of 90 partial sequences. Legend as in figure S2 except for the following parameters: gamma distribution shape parameter of 0.5748 and substitution rates of R(b) [A-G] = 2.1047, R(e) [C-T] = 3.6922, and 1.0 for all other substitution rates. Total number rearrangements tried = 273,048. Scale bar corresponds to 5 % divergence.

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Table S1. Description of genetic libraries built for this study, or partially published in other work. Accession numbers correspond to radiolarian sequences only.

Environment and Cruise	Station and Site	Coordinates	Date	Library code	Depth (m)	Primers	Collection procedure and size fraction	# of clones screened
Mediterranean Sea PROSOPE See also Viprey et al. (2008) Accession numbers XX to XX	St 1 Strait of Gibralatar	38° 08' N 05° 18' W	14-Sep-99	C1-30	30	Euk328f-CHLO02r	Niskin bottle, <3µm	246
				E1-30	30	Euk328f-Euk329r	Niskin bottle, <3µm	135
				C1-80	80	Euk328f-CHLO02r	Niskin bottle, <3µm	195
				E1-80	80	Euk328f-Euk329r	Niskin bottle, <3µm	69
	St 3 Algerian Basin	37° 98' N 03° 83' E	16-Sep-99	C3-5	5	Euk328f-CHLO02r	Niskin bottle, <3µm	165
				E3-5	5	Euk328f-Euk329r	Niskin bottle, <3µm	46
				C3-25	25	Euk328f-CHLO02r	Niskin bottle, <3µm	23
				E3-25	25	Euk328f-Euk329r	Niskin bottle, <3µm	56
				C3-95	95	Euk328f-CHLO02r	Niskin bottle, <3µm	173
				E3-95	95	Euk328f-Euk329r	Niskin bottle, <3µm	27
	St 5 Strait of Sicily	36° 47' N 13° 32' E	18-Sep-99	C5-25	25	Euk328f-CHLO02r	Niskin bottle, <3µm	151
				E5-25	25	Euk328f-Euk329r	Niskin bottle, <3µm	168
				C5-55	55	Euk328f-CHLO02r	Niskin bottle, <3µm	131
				E5-55	55	Euk328f-Euk329r	Niskin bottle, <3µm	185
	St MIO Ionian Basin	33° 98' N 22° 02' E	20-Sep-99	CM-5	5	Euk328f-CHLO02r	Niskin bottle, <3µm	173
				EM-5	5	Euk328f-Euk329r	Niskin bottle, <3µm	62
				CM-50	50	Euk328f-CHLO02r	Niskin bottle, <3µm	171
				EM-50	50	Euk328f-Euk329r	Niskin bottle, <3µm	102
				CM-110	110	Euk328f-CHLO02r	Niskin bottle, <3µm	134
				EM-110	110	Euk328f-Euk329r	Niskin bottle, <3µm	115
	St 9 Tyrrhenian Basin	41° 88' N 10° 43' E	28-Sep-99	C9-5	5	Euk328f-CHLO02r	Niskin bottle, <3µm	101
				E9-5	5	Euk328f-Euk329r	Niskin bottle, <3µm	144
				C9-65	65	Euk328f-CHLO02r	Niskin bottle, <3µm	223
				E9-65	65	Euk328f-Euk329r	Niskin bottle, <3µm	119
	St DYF Ligurian Basin	43° 38' N 07° 82' E	30-Sep-99	CD-15	15	Euk328f-CHLO02r	Niskin bottle, <3µm	172
				ED-15	15	Euk328f-Euk329r	Niskin bottle, <3µm	118
				CD-50	50	Euk328f-CHLO02r	Niskin bottle, <3µm	201
				ED-50	50	Euk328f-Euk329r	Niskin bottle, <3µm	196
Atlantic Ocean PROSOPE See also Viprey et al. (2008) Accession numbers XX to XX	St UPW Morocco upwelling	31° 02' N 10° 03' W	09-Sep-99	CU-30	30	Euk328f-CHLO02r	Niskin bottle, <3µm	53
				EU-30	30	Euk328f-Euk329r	Niskin bottle, <3µm	126
Indian Ocean VANC10MV See also	St 1	35° 03' S 23° 44' E	May-June 2003	IND1	5	EukA-EukB	Niskin bottle, <3µm	13
				IND2	25	EukA-EukB	Niskin bottle, <3µm	33
	St 9	31° 49' S	May-June	IND31	5	EukA-EukB	Niskin bottle, <3µm	139

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Not et al.(2008)		52° 36' E	2003	IND33	74	EukA-EukB	Niskin bottle, <3µm	74
Accession numbers for	St 18	17° 10' S	May-June	IND58	5	EukA-EukB	Niskin bottle, <3µm	62
complete sequences		83° 40' E	2003	IND60	85	EukA-EukB	Niskin bottle, <3µm	70
XXX to XXX	St 23	12° 13' S	15 May-13	IND70	5	EukA-EukB	Niskin bottle, <3µm	78
		96° 47' E	June	IND72	75	EukA-EukB	Niskin bottle, <3µm	66
			2003					
Equatorial Pacific Ocean	St 2	11° 30' S	7-Nov-94	OLI011	75	Euk328f-Euk329r	Niskin bottle, <3µm	115
OLIPAC		150° 00' W						
See also Moon van-der Staay	St 3	10° 00' S	8-Nov-94	OLI016	75	Euk328f-Euk329r	Niskin bottle, <3µm	5
et al. (2001)		150° 00' W						
Accession numbers								
XX to XX								
South East Pacific Ocean	GYR2 and GYR6	26° 00' S	12-Nov-04	BIO8	500	Euk328f-UnonMet	Niskin bottle, <3µm	95
BIOSOPE	Gyre	113° 99' W	16-Nov-04	BIO7	1000	Euk328f-UnonMet	Niskin bottle, <3µm	93
Accession numbers		26° 07' S		BIO1	1000	Euk328f-UnonMet	Niskin bottle, >3µm	95
XX to XX		113° 99' W						
	EGY1 and EGY6	31° 81' S	25-Nov-04	BIO10	500	Euk328f-UnonMet	Niskin bottle, <3µm	96
	East of Gyre	91° 46' W		BIO3	500	Euk328f-UnonMet	Niskin bottle, >3µm	90
				BIO6	1000	Euk328f-UnonMet	Niskin bottle, <3µm	96
				BIO4	3000	Euk328f-UnonMet	Niskin bottle, >3µm	96
	UPX1 and UPX3	34° 54' S	9-Dec-04	BIO9	500	Euk328f-UnonMet	Niskin bottle, <3µm	95
	Chilean upwelling	72° 39' W		BIO2	500	Euk328f-UnonMet	Niskin bottle, >3µm	96
				BIO5	900	Euk328f-UnonMet	Niskin bottle, <3µm	89
South East Pacific Rise	Oasis	17°25'S	30-Apr- 04	BS16	2585	Euk328f-UnonMet	Hermetic container with hydrothermal vent chimney fragments, <3µm	17
BIOSPEEDO		113°12'W						
Accession numbers								
XX to XX								
	Sarah'Spring	7°25'S	09-Apr-04	BS23	2752	Euk328f-UnonMet	Hermetic container with hydrothermal vent chimney fragments, 3-10µm	93
		107°47'W						
				BS25	2752	Euk328f-UnonMet	Hermetic container with hydrothermal vent chimney fragments, >10µm	85
	Hobbs	17°35'S	28/29-Apr-	BS15	2593	Euk328f-UnonMet	Hermetic container with hydrothermal vent chimney fragments, 3-10µm	18
		113°14'W	04					
				BS24	2593	Euk328f-UnonMet	Hermetic container with hydrothermal vent chimney fragments, >10µm	94
	Rehu Marka	17°24'S	24-Apr-04	BS3	900	Euk328f-Euk329r	Niskin bottle, 3-10µm	80
	(water column)	113°12'W		BS4	300	Euk328f-Euk329r	Niskin bottle, >10µm	37
Total number of clones								**6000**

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Table S2. Primer sequences used or cited in this study.

Primer name	Target taxa	Sequence (5'-3')	References
Euk328 (forward)	Eukaryotes	ACCTGGTTGATCCTGCCAG	(Romari and Vaulot, 2004)
Euk329 (reverse)	Eukaryotes	TGATCCTTCYGCAGGTTCAC	(Romari and Vaulot, 2004)
EukA (forward)	Eukaryotes	AACCTGGTTGATCCTGCCAGT	Medlin et al. (1988)
EukB (reverse)	Eukaryotes	TGATCCTTCTGCAGGTTCACCTAC	Medlin et al. (1988)
EukB' (reverse)	Eukaryotes	GATCCTTCTGCAGGTTCACCTAC	Medlin et al. (1988), Countway et al. (2007)
Euk528 (forward)	Eukaryotes	GCGGTAATTCCAGCTCCAA	Helwood et al. (1985)
18N1 (forward)	Eukaryotes	GGATCAGAATTCTATCTGGTTGATCCTGCAG	(Shao *et al*., 2002)
18N11R (reverse)	Eukaryotes	CTCAGTAAGCTTGATCCTTCCGCAGGTTCACC	(Shao *et al*., 2002)
EK1A (forward)	Eukaryotes	CTGGTTGATCCTGCCAG	López-García et al. (2001)
18S1520 (reverse)	Eukaryotes	CYGCAGGTTCACCTAC	López-García et al. (2001)
CHLO02 (reverse)	Chloroplastida	CTTCGAGCCCCCAACTTTC	Zhu et al. (2005)
UNonMet (reverse)	Eukaryotes except metazoans	TTTAAGTTTCAGCCTTGCG	Bower et al. (2004)

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Table S3. List of the 18S rRNA gene sequences available from Genbank used in this study. “na” means that the corresponding information is not available

Taxonomic affiliation	Taxon name	Sample localisation	Accession number	Publication
Acantharea	*Acanthometra* sp. 205	Bermuda, Atlantic Ocean	AF063240	(Amaral-Zettler and Caron, 2000)
	Amphiacon denticulatus	na	AB178585	Oka, unpublished
	Amphibelone anomala 714	na	AB178582	Oka, unpublished
	Amphibelone anomala 717	na	AB178583	Oka, unpublished
	Amphibelone anomala 728	na	AB178584	Oka, unpublished
	Amphibelone cultellata 628	na	AB178581	Oka, unpublished
	Arthracanthid sp. 206	Bermuda, Atlantic Ocean	AF063239	(Amaral-Zettler and Caron, 2000)
	Chaunacanthid sp. 217	Bermuda, Atlantic Ocean	AF063241	(Amaral-Zettler and Caron, 2000)
	Chaunacanthid sp. 218	Bermuda, Atlantic Ocean	AF018158	(Amaral-Zettler *et al.*, 1997)
	Dorataspid sp. 915	na	AB178586	Oka, unpublished
	Haliommatidium sp. BSSR 235	Bermuda, Atlantic Ocean	AF018159	(Amaral-Zettler *et al.*, 1997)
	Hexaconus serratus 09B	na	AB178587	Oka, unpublished
	Hexaconus serratus 725	na	AB178588	Oka, unpublished
	Symphyacanthid sp. 211	Bermuda, Atlantic Ocean	AF063242	(Amaral-Zettler and Caron, 2000)
Polycystinea	*Acrosphaera* sp. CR6A	Bermuda, Atlantic Ocean	AF091148	(Amaral-Zettler *et al.*, 1999)
	Artostrobus sp. 2014	Izu Peninsula, Pacific Ocean	AB246685	(Kunitomo *et al.*, 2006)
	Collosphaera globularis BBSR 173	Bermuda, Atlantic Ocean	AF018163	(Amaral-Zettler *et al.*, 1997)
	Collozoum inerme	Villefranche/Mer, Mediterranean Sea	AY266295	(Polet *et al.*, 2004)
	Collozoum pelagicum BBSR2	Bermuda, Atlantic Ocean	AF091146	(Amaral-Zettler *et al.*, 1999)
	Collozoum serpentinum CR16	Bermuda, Atlantic Ocean	AF018162	(Amaral-Zettler *et al.*, 1997)
	Dicranastrum furcatum	Okinawa Island, Pacific Ocean	AB179733	(Yuasa *et al.*, 2005)
	Dictyocoryne profunda	Okinawa Island, Pacific Ocean	AB101540	(Takahashi *et al.*, 2004)
	Dictyocoryne truncatum	Okinawa Island, Pacific Ocean	AB101541	(Takahashi *et al.*, 2004)
	Didymocyrtis tetrathalamus	na	AB193605	(Yuasa *et al.*, 2004)
	Euchitonia elegans	Okinawa Island, Pacific Ocean	AB179732	(Yuasa *et al.*, 2005)
	Eucyrtidium acuminatum 2051	Izu Peninsula, Pacific Ocean	AB246687	(Kunitomo *et al.*, 2006)
	Eucyrtidium acuminatum 2112	Izu Peninsula, Pacific Ocean	AB246690	(Kunitomo *et al.*, 2006)
	Eucyrtidium hexagonatum	Okinawa Island, Pacific Ocean	AB179735	(Yuasa et al. 2005)
	Eucyrtidium hexastichum 1042	Izu Peninsula, Pacific Ocean	AB246681	(Kunitomo *et al.*, 2006)
	‘*Larcopyle butschlii*’ 7039	Izu Peninsula, Pacific Ocean	AB246693	(Kunitomo *et al.*, 2006)
	Lithomelissa sp. 2003	Izu Peninsula, Pacific Ocean	AB246683	(Kunitomo et al. 2006)
	Lithomelissa sp. 8012	Izu Peninsula, Pacific Ocean	AB246694	(Kunitomo et al. 2006)
	Pseudocubus obeliscus 7036	Izu Peninsula, Pacific Ocean	AB246692	(Kunitomo et al. 2006)
	Pterocanium trilobum 1051	Izu Peninsula, Pacific Ocean	AB246682	(Kunitomo et al. 2006)
	Pterocorys zancleus	Okinawa Island, Pacific Ocean	AB179736	(Yuasa et al. 2005)
	Pterocorys zancleus 8030	Izu Peninsula, Pacific Ocean	AB246697	(Kunitomo et al. 2006)
	Rhaphidozoum acuferum BSR7.43	Bermuda, Atlantic Ocean	AF091147	(Amaral Zettler et al. 1999)
	Siphonosphaera cyathina	Bermuda, Atlantic Ocean	AF091145	(Amaral Zettler et al. 1999)
	Sphaerozoum punctatum CR4	Bermuda, Atlantic Ocean	AF018161	(Amaral-Zettler et al. 1997)
	Spongaster tetras	Okinawa Island, Pacific Ocean	AB101542	(Takahashi et al. 2004)
	Spongodiscus biconcavus 8016	Izu Peninsula, Pacific Ocean	AB246695	(Kunitomo et al. 2006)
	Spongodiscus resurgens 8029	Izu Peninsula, Pacific Ocean	AB246696	(Kunitomo et al. 2006)
	Spongopyle osculosa 2102	Izu Peninsula, Pacific Ocean	AB246689	(Kunitomo et al. 2006)
	Stylodictya sp. 8037	Izu Peninsula, Pacific Ocean	AB246698	(Kunitomo et al. 2006)

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	Styptosphaera sp. 2013	Izu Peninsula, Pacific Ocean	AB246684	(Kunitomo et al. 2006)
	Styptosphaera sp. 2022	Izu Peninsula, Pacific Ocean	AB246686	(Kunitomo et al. 2006)
	Tetrapyle octacantha 1037	Izu Peninsula, Pacific Ocean	AB246680	(Kunitomo et al. 2006)
	Tetrapyle sp. 2098	Izu Peninsula, Pacific Ocean	AB246688	(Kunitomo et al. 2006)
	Thalassicolla nucleata BBS 3	Bermuda, Atlantic Ocean	AF018160	(Amaral-Zettler et al. 1997)
	Thalassicolla nucleata W10.74	North Pacific Gyre, Pacific Ocean	AF057743	(Amaral-Zettler *et al.*, 1998)
	Thalassicolla nucleata W10.79	North Pacific Gyre, Pacific Ocean	AF057744	(Amaral-Zettler *et al.*, 1998)
	Thalassicolla pellucida	Villefranche/Mer, Mediterranean Sea	AY266297	(Polet *et al.*, 2004)
	Thalassophysa pelagica	Villefranche/Mer, Mediterranean Sea	AY266296	(Polet et al. 2004)
	Triastrum aurivillii	Okinawa Island, Pacific Ocean	AB179734	(Yuasa et al. 2005)
	'Undetermined Spumellarian' 7017	Izu Peninsula, Pacific Ocean	AB246691	(Kunitomo et al. 2006)
Taxopodida	*Sticholonche zanclea* JJP-2003	Villefranche/Mer, Mediterranean Sea	AY268045	(Nikolaev *et al.*, 2004)
Cercozoa	*Aulacantha scolymantha*	Villefranche/Mer, Mediterranean Sea	AY266294	(Polet et al. 2004)
	Aulosphaera trigonopa	Villefranche/Mer, Mediterranean Sea	AY266292	(Polet et al. 2004)
	Challengeron diodon	na	AB218765	(Yuasa *et al.*, 2006)
	Euglypha rotunda CCAP 1520/1	na	X77692	(Bhattacharya *et al.*, 1995)
	Massisteria marina GBB2	na	AF174370	(Atkins *et al.*, 2000)
Alveolata	*Alexandrium tamarense* SJC9516	Chinhae Bay, South Korea	AB088307	(Kim *et al.*, 2004)
	Amoebophrya sp. GS0209a	Gunsan, Yellow Sea	AY775285	Kim, unpublished
	Gymnodinium catenatum MUCC273	na	AF022193	(Saunders *et al.*, 1997)

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Table S3 continued

Taxonomic affiliation	Taxon name	Sample Localisation	Accession Number	Publication
Environmental samples	AT8-54	Mid-Atlantic Ridge	AF530524	(López-García *et al.*, 2003)
	DH145-HA2	Drake passage, Antarctic Ocean	AF382824	(López-García *et al.*, 2002)
	DH145-KW16	Drake passage, Antarctic Ocean	AF382825	(López-García *et al.*, 2002)
	DH147-EKD17	Drake passage, Antarctic Ocean	AF290072	(López-García *et al.*, 2001)
	IBEA.CTG.2022727	Sargasso Sea	2022727	(Venter *et al.*, 2004)
	LC22.5EP.23	Mid-Atlantic Ridge	DQ504355	(López-García *et al.*, 2007)
	NW414.03	Canadian Basin, Artic Ocean	DQ314831	(Lovejoy *et al.*, 2006)
	NW415.02	Canadian Basin, Artic Ocean	DQ314830	(Lovejoy *et al.*, 2006)
	NW614.49	Canadian Basin, Artic Ocean	DQ314821	(Lovejoy *et al.*, 2006)
	NW617.13	Canadian Basin, Artic Ocean	DQ314838	(Lovejoy *et al.*, 2006)
	OLI11015	Equatorial Pacific Ocean	AJ402332	(Moon-van der Staay *et al.*, 2001)
	OLI11032	Equatorial Pacific Ocean	AJ402342	(Moon-van der Staay *et al.*, 2001)
	Q2E12N5	Sargasso Sea	EF173011	(Not *et al.*, 2007)
	SCM15C12	Sargasso Sea	AY665098	Armbrust et al., unpublished
	SCM15C36	Sargasso Sea	AY665017	Armbrust et al., unpublished
	SCM16C27	Sargasso Sea	AY665035	Armbrust et al., unpublished
	SCM27C24	Sargasso Sea	AY665095	Armbrust et al., unpublished
	SCM27C26	Sargasso Sea	AY665072	Armbrust et al., unpublished
	SCM27C30	Sargasso Sea	AY665067	Armbrust et al., unpublished
	SCM27C8	Sargasso Sea	AY665010	Armbrust et al., unpublished
	SCM37C12	Sargasso Sea	AY665077	Armbrust et al., unpublished
	SCM37C15	Sargasso Sea	AY665078	Armbrust et al., unpublished
	SCM37C37	Sargasso Sea	AY665011	Armbrust et al., unpublished
	SCM37C8	Sargasso Sea	AY665076	Armbrust et al., unpublished
	SCM38C17	Sargasso Sea	AY665073	Armbrust et al., unpublished
	SCM38C4	Sargasso Sea	AY665068	Armbrust et al., unpublished
	SSRPB19	Sargasso Sea	EF172805	(Not *et al.*, 2007)
	SSRPB22	Sargasso Sea	EF172808	(Not *et al.*, 2007)
	SSRPB51	Sargasso Sea	EF172802	(Not *et al.*, 2007)
	SSRPB59	Sargasso Sea	EF172842	(Not *et al.*, 2007)
	SSRPB70	Sargasso Sea	EF172841	(Not *et al.*, 2007)
	SSRPB73	Sargasso Sea	EF172833	(Not *et al.*, 2007)
	SSRPB80	Sargasso Sea	EF172807	(Not *et al.*, 2007)
	SSRPB82	Sargasso Sea	EF172835	(Not *et al.*, 2007)
	SSRPC12	Sargasso Sea	EF172891	(Not *et al.*, 2007)
	SSRPC15	Sargasso Sea	EF172908	(Not *et al.*, 2007)
	SSRPC18	Sargasso Sea	EF172906	(Not *et al.*, 2007)
	SSRPC34	Sargasso Sea	EF172903	(Not *et al.*, 2007)
	SSRPC52	Sargasso Sea	EF172930	(Not *et al.*, 2007)
	SSRPC73	Sargasso Sea	EF172907	(Not *et al.*, 2007)
	SSRPD81	Sargasso Sea	EF172984	(Not *et al.*, 2007)

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Table S4: Polycystinea taxonomy at the family level based on morphological characters. Families for which at least one SSU rDNA sequence of identified isolate is available are marked by an asterisk (*).

Class	Order	Family	Authority
Polycystinea			Ehrenberg 1838
	Spumellarida		Ehrenberg 1875
		Litheliidae *	Haeckel 1862
		Actinommiidae	Haeckel 1862
		Coccodiscidae *	Haeckel 1862
		Spongodiscidae *	Haeckel 1862
		Pyloniidae *	Haeckel 1881
		Tholoniidae	Haeckel 1887
		Phacodiscidae	Haeckel 1881
		Sponguridae	Haeckel 1862
		Ethmosphaeridae *	See Takahashi 1991
		Myelastridae	Riedel 1971, see Takahashi 1991
		Larnacillidae	Haeckel 1887
		Porodiscidae	See Takahashi 1991
	Nassellarida		Ehrenberg 1875
		Cannobotryidae	Haeckel 1881
		Spyridae	Ehrenberg 1847 emend. Petrushevskaya 1971 (=Trissocyclidae and Acanthodesmiidae)
		Theoperidae *	Haeckel 1881
		Carpocaniidae	Haeckel 1881
		Pterocorythidae *	Haeckel 1881
		Artostrobiidae	Riedel 1967
		Plagoniidae *	Haeckel 1881
	Collodaria		Haeckel 1881
		Thalassosphaeridae	Haeckel 1862
		Sphaerozoidae * (incl. Collozoidae)	Haeckel 1862
		Thalassicollidae *	Haeckel 1862
		Collosphaeridae *	Müller 1858 (add within collodaria based on molecular analysis, previously in Spumellarida)

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Table S5: Acantharea taxonomy at the family level based on morphological characters. Families for which at least one SSU rDNA sequence of identified isolate is available are marked by an asterisk (*).

Class	Order	Family	Authority
Acantharea			Haeckel 1881
	Arthracanthida		Schewiakoff 1926
		Doratapsidae *	Haeckel 1887
		Phractopeltidae	Haeckel 1887
		Diploconidae	Haeckel 1887
		Lithopteridae	Haeckel 1887 emend. Popofsky 1904
		Hexalpsidae *	Haeckel 1887
		Acanthometridae *	Haeckel 1887 emend. Schewiakoff 1926
		Phyllostauridae	Schewiakoff 1926
		Dictyacanthidae	Schewiakoff 1926
		Stauracanthidae	Schewiakoff 1926
	Symphyacanthida		Schewiakoff 1926
		Amphilithidae *	Haeckel 1887 emend. Schewiakoff 1926
		Pseudolithidae *	Schewiakoff 1926
		Astrolithidae	Haeckel 1887 emend. Schewiakoff 1926
	Chaunacanthida		Schewiakoff 1926
		Stauraconidae *	Schewiakoff 1926
		Gigartaconidae	Schewiakoff 1926
		Conaconidae	Schewiakoff 1926
	Holacanthida		Schewiakoff 1926
		Acanthocollidae	Schewiakoff 1926
		Acanthoplegmidae	Schewiakoff 1926
		Acanthochiasmidae	Haeckel 1962

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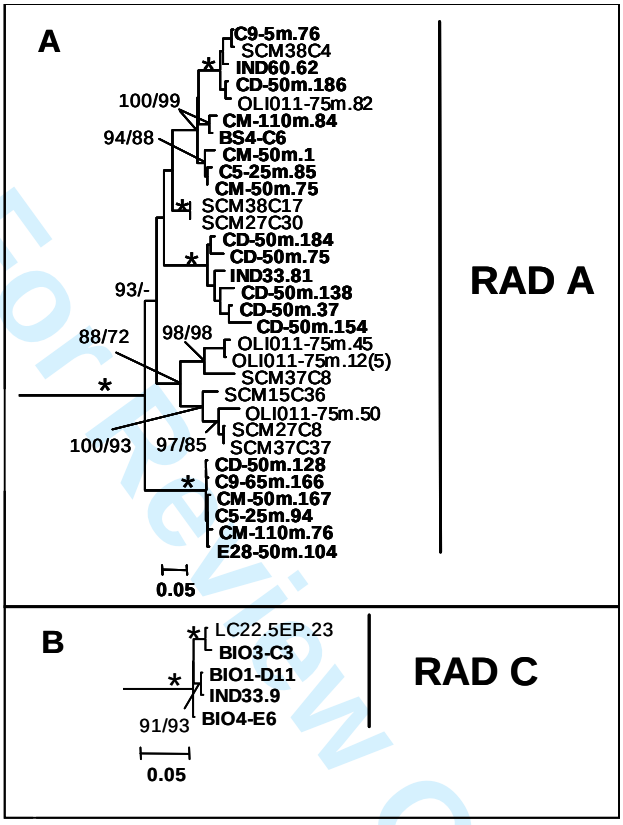

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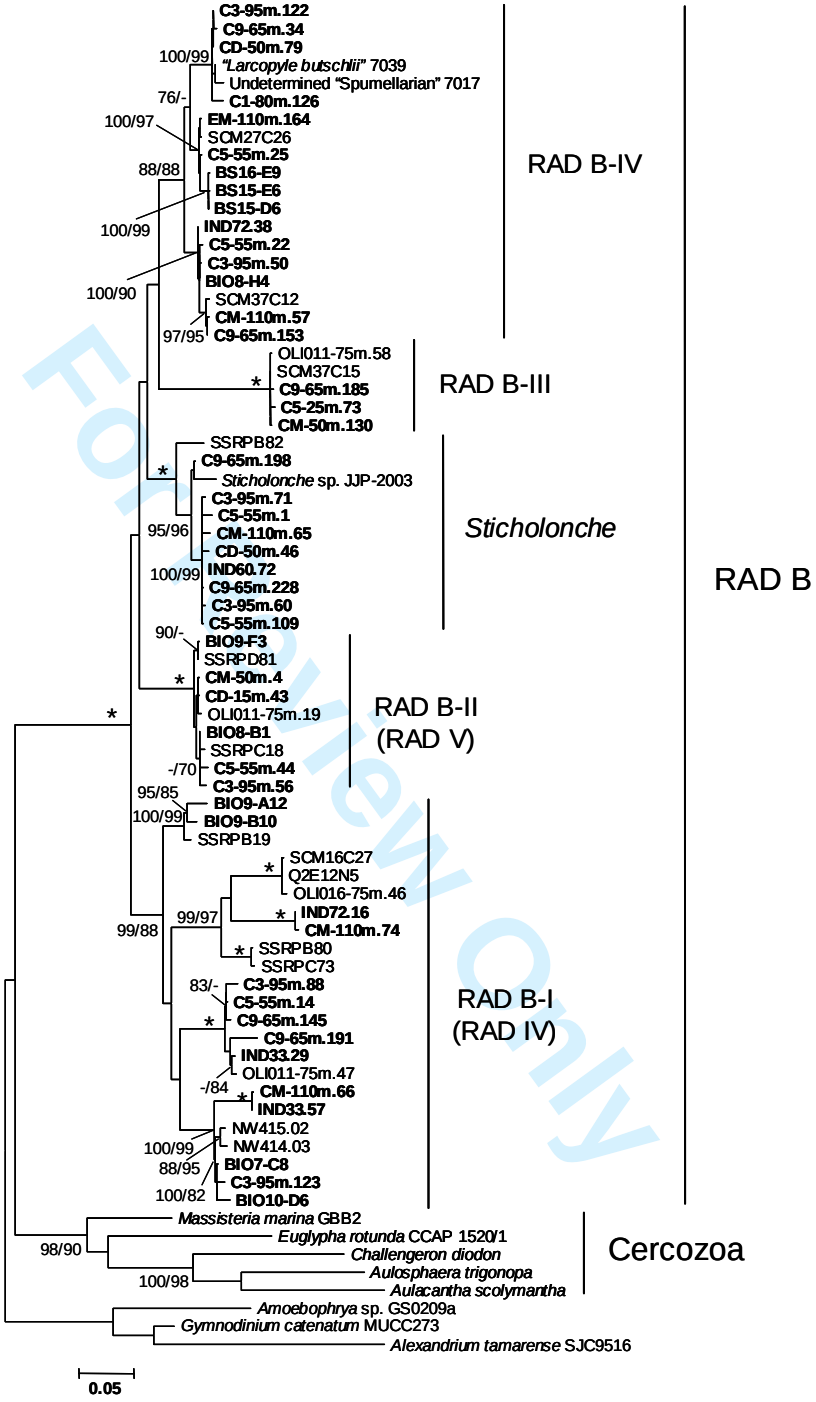
Figure S1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100/99
"Larcopyle butschlii" 7039
Undetermined "Spumellarian" 7017
76/-
C1-80m.126
100/97
EM-110m.164
SCM27C26
C5-55m.25
88/88
BS16-E9
BS15-E6
BS15-D6
100/99
IND72.38
C5-55m.22
C3-95m.50
100/90
BIO8-H4
SCM37C12
CM-110m.57
97/95
C9-65m.153
RAD B-IV
OLI011-75m.58
SCM37C15
*
C9-65m.185
C5-25m.73
CM-50m.130
RAD B-III
SSRPB82
C9-65m.198
*
Sticholonche sp. JJP-2003
C3-95m.71
C5-55m.1
95/96
CM-110m.65
CD-50m.46
Sticholonche
100/99
IND60.72
C9-65m.228
C3-95m.60
C5-55m.109
RAD B
90/-
BIO9-F3
SSRPD81
*
CM-50m.4
CD-15m.43
OLI011-75m.19
*
BIO8-B1
SSRPC18
-/70
C5-55m.44
C3-95m.56
RAD B-II
(RAD V)
95/85
BIO9-A12
100/99
BIO9-B10
SSRPB19
*
SCM16C27
Q2E12N5
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99/88
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SSRPC73
83/-
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*
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-/84
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(RAD IV)
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NW414.03
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Euglypha rotunda CCAP 1520/1
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Challengeron diodon
Aulosphaera trigonopa
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Aulacantha scolymantha
Cercozoa
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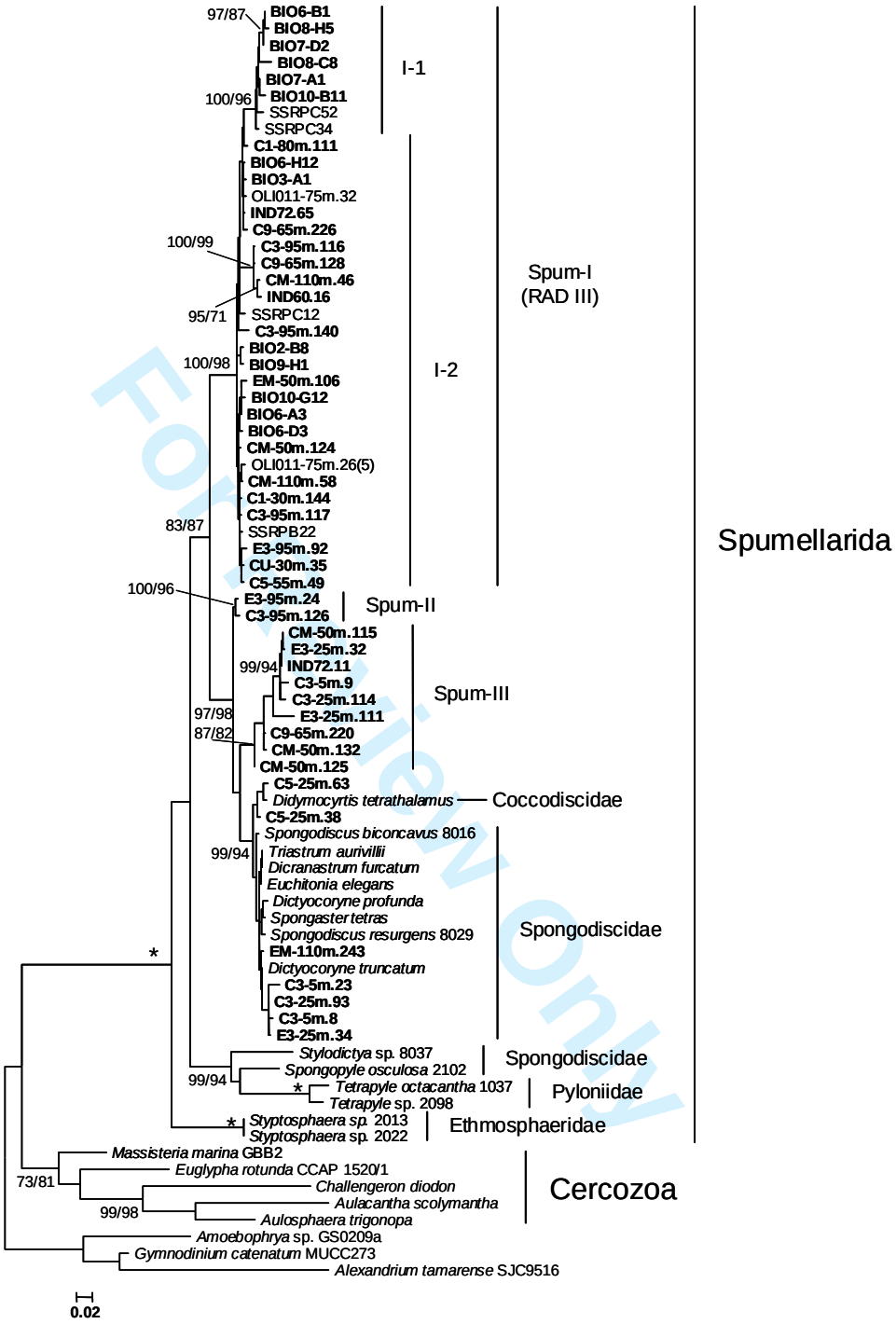
Figure S2


BIO6-B1
BIO8-H5
BIO7-D2
BIO8-C8
BIO7-A1
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SSRPC52
SSRPC34
C1-80m.111
BIO6-H12
BIO3-A1
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IND72.65
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C3-95m.116
C9-65m.128
CM-110m.46
IND60.16
SSRPC12
C3-95m.140
BIO2-B8
BIO9-H1
EM-50m.106
BIO10-G12
BIO6-A3
BIO6-D3
CM-50m.124
OLI011-75m.26(5)
CM-110m.58
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C3-95m.117
SSRPB22
E3-95m.92
CU-30m.35
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C3-5m.9
C3-25m.114
E3-25m.111
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CM-50m.132
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Dicranastrum furcatum
Euchitonia elegans
Dictyocoryne profunda
Spongaster tetras
Spongodiscus resurgens 8029
EM-110m.243
Dictyocoryne truncatum
C3-5m.23
C3-25m.93
C3-5m.8
E3-25m.34
Stylodictya sp. 8037
Spongopyle osculosa 2102
Tetrapyle octacantha 1037
Tetrapyle sp. 2098
Styptosphaera sp. 2013
Styptosphaera sp. 2022
Massisteria marina GBB2
Euglypha rotunda CCAP 1520/1
Challengeron diodon
Aulacantha scolymantha
Aulosphaera trigonopa
Amoebophrya sp. GS0209a
Gymnodinium catenatum MUCC273
Alexandrium tamarense SJC9516
97/87
100/96
100/99
95/71
100/98
83/87
100/96
99/94
97/98
87/82
99/94
99/94
73/81
99/98
I-1
I-2
Spum-I
(RAD III)
Spum-II
Spum-III
Coccodiscidae
Spongodiscidae
Spongodiscidae
Pyloniidae
Ethmosphaeridae
Cercozoa
Spumellarida
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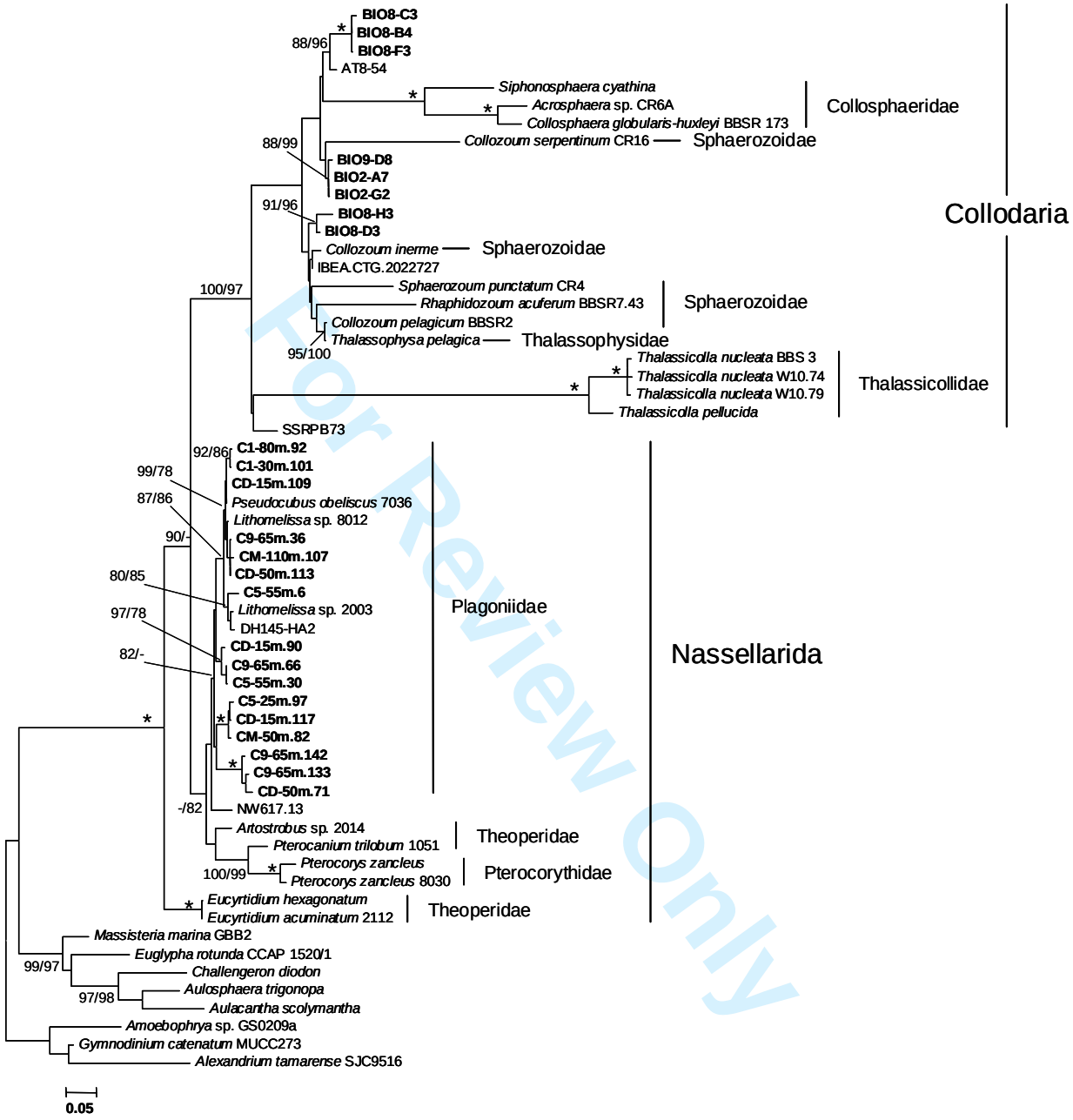

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Figure S4

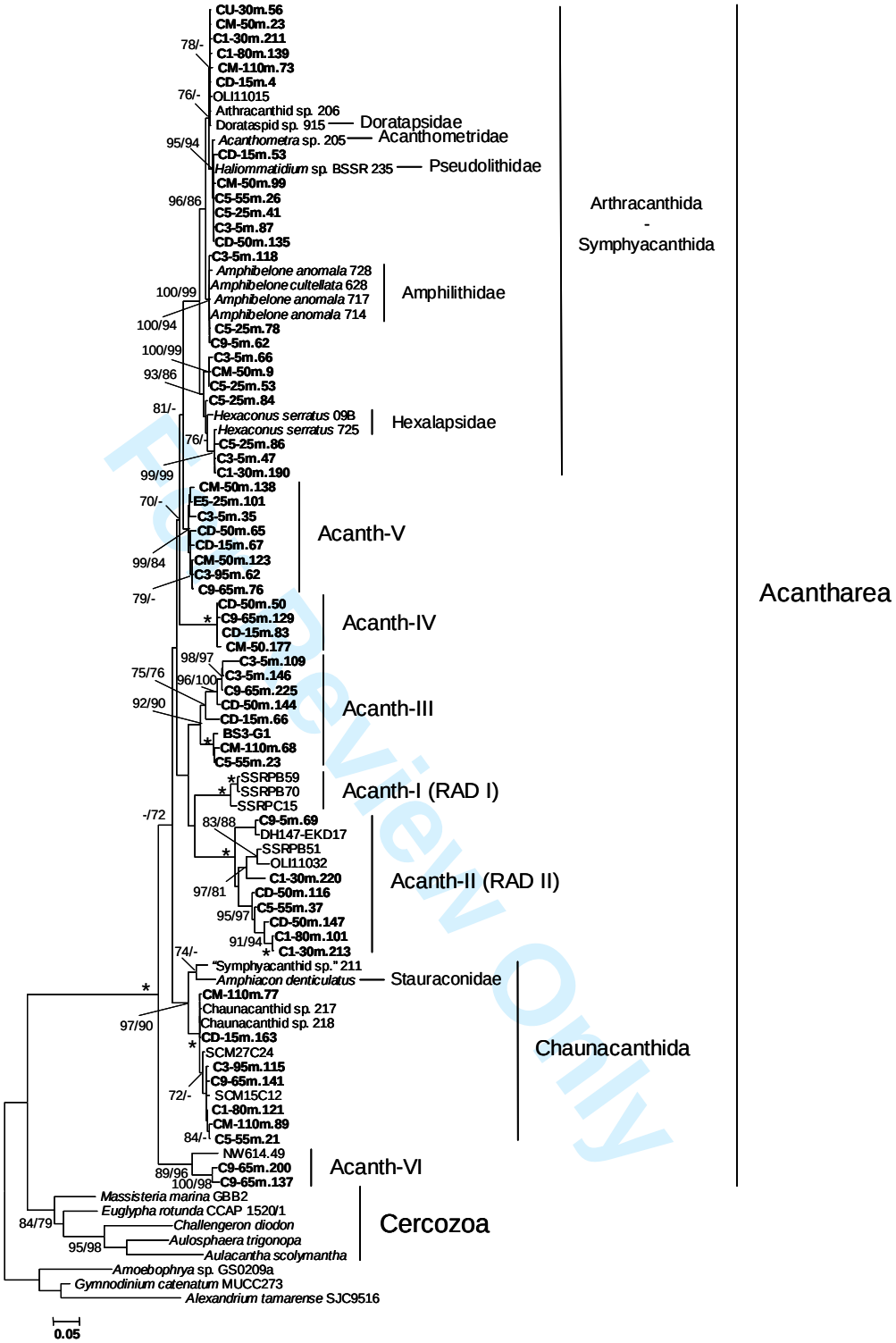

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Figure S5

ANNEXE C

POSTER 1 – LES MICRO-ORGANISMES MARINS EXTREMOPHILES : L'ORIGINE DE LA VIE ?

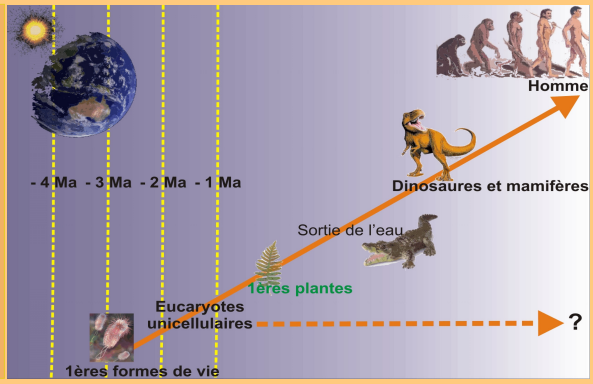
Doctoriales des Stations ; France, Villefranche sur mer, 2008

A.L. Sauvadet, A. Gobet, J. Winkler, D. Vaulot and L. Guillou

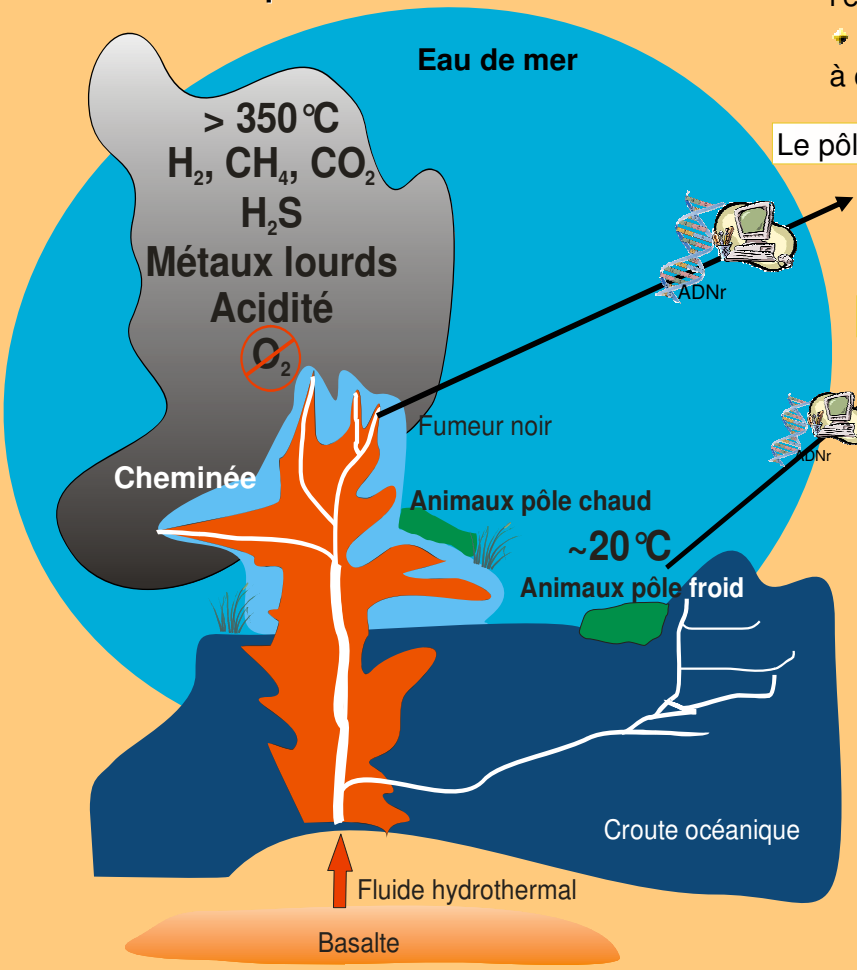
LES MICRO-ORGANISMES MARINS EXTREMOPHILES : L'origine de la vie ?

Anne-Laure Sauvadet, A. Gobet, J. Winkler, D. Vaulot, L. Guillou
UMR 7144 Adaptation et diversité en milieu marin - Équipe Plancton océanique, Station biologique de Roscoff, Roscoff

La vie est apparue dans les océans il y a un peu plus de 3,5 milliards d'années sous forme de **micro-organismes unicellulaires**.
L'atmosphère terrestre était alors **dépourvue d'oxygène**, pauvre en matière organique, et **riche en hydrogène sulfuré** (H_2S).
Dans les années 70, des environnements présentant des conditions similaires, les **sources hydrothermales océaniques,** ont été découverts. Ces sources pourraient être des refuges pour des **organismes primitifs**.



Un exemple d'écosystème hydrothermal : la ride est-Pacifique



- Diversité de micro-organismes supérieure à celle de l'eau de mer (à partir de 300 mètres sous la surface)
- Majorités de micro-organismes ubiquistes à de nombreux écosystèmes

Le pôle chaud : les cheminées hydrothermales…

- Micro-organismes inféodés aux sources hydrothermales

Le pôle froid : les moules…

- Majorité de ciliées dont un nouveau groupe
- Micro-organismes également présents dans des organismes marins (oursins, algues)

- Découverte de micro-organismes **spécifiques** de l'écosystème hydrothermale…sont-ils primitifs ?

A suivre …

- Étude de la répartition **biogéographique** des micro-organismes, par comparaison avec des écosystèmes hydrothermaux de la **dorsale Atlantique**.
- Étude d'**autres modèles** animaux, des vers hydrothermaux, et comparaison avec des vers côtiers.

sauvadet@sb-roscoff.fr

ANR Financement ANR Deep Oases

ANNEXE D

POSTER 2 – PROTIST COMMUNITIES ASSOCIATED WITH BIVALVES IN DEEP HYDROTHERMAL VENTS

Gordon-Marine microbes ; Italie, 2008

A.L. Sauvadet, A. Gobet, L. Guillou

Protist communities associated with bivalves in deep hydrothermal vents

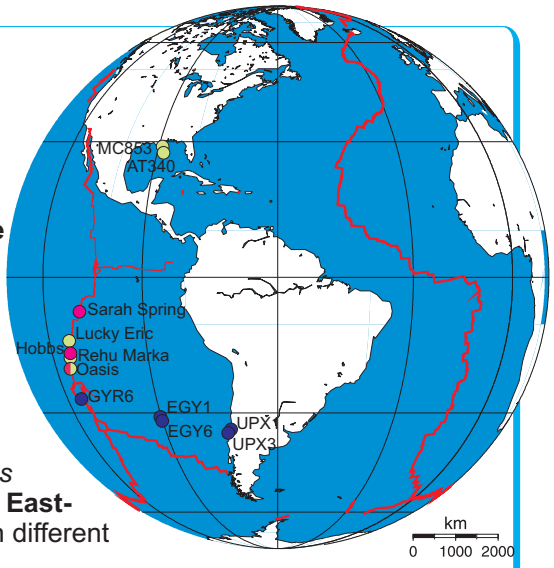
Anne-Laure SAUVADET, Angélique GOBET and Laure GUILLOU.

UMR 7144 - Adaptation and diversity in the marine environment - Diversity of oceanic plankton team, CNRS, station biologique de Roscoff, Place Georges Teissier, Roscoff, France.
sauvadet@sb-roscoff.fr

Deep marine hydrothermal vents are characterized by:

- high temperatures (50-300°C) and pressures (3,000-4,000 atm)
- high pH and salinity gradients
- as well as high concentrations of toxic chemicals such as hydrogen sulphide

As these extreme physico-chemical conditions are similar to those of the **early life on earth**, these ecosystems were considered as potential refuges for "primitive" organisms or living forms still retaining ancestral characteristics.
Although hydrothermal metazoans are in fact modern animals which have secondarily re-colonized deep hydrothermal vents(1), this hypothesis may still have some relevance for very **small eukaryotes** like protists.



In this study, using microscopic observations and SSU 18S rRNA PCR survey, we compared the protist communities from several species of bivalves (*Bathymodiolus thermophilus and Calyptogena magnifica*) collected from different locations on the **East-Pacific Rise (EPR).** These communities were then compared to those collected in different habitats:

- along the deep aphotic water column
- in the surrounding waters of hydrothermal vent chimneys
- in water above bivalve bed

Results: Microscopy and statistical analysis

Microscopic observations showed that, close to these hydrothermal vents, protists were relatively rare, except for dense communities inhabiting the palleal liquid of bivalves. In fact, different species of ciliates (Fig.1, all photos except 6, 8, 9) dominated microscopic observations. Note the remarkable ciliate insertion of species on photos 2 and 3. None of these protists have been formally identified but the organism on first picture looks like the photosynthetic ciliate *Myrionecta rubra* (photo.1'), which can colonize coastal mussels .

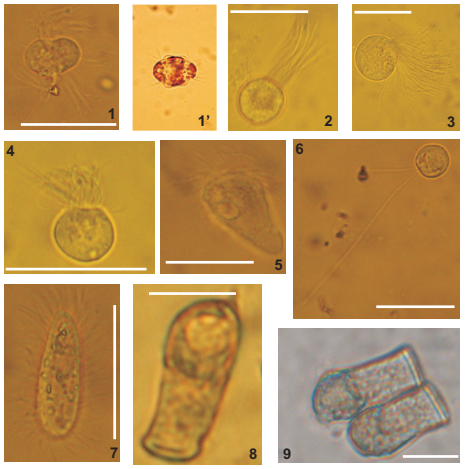


Fig. 1: Microscopic observations of living protists (1-7) or fixed with lugol (8 and 9) (obj*40) collected from bivalves (Biospeedo cruise, EPR, 2004). Scale bar = 10µm

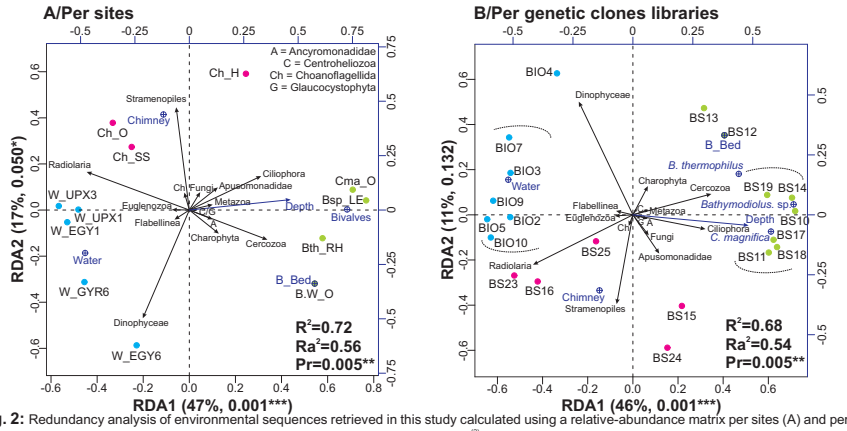


Fig. 2: Redundancy analysis of environmental sequences retrieved in this study calculated using a relative-abundance matrix per sites (A) and per genetic clones libraries (B). Sequences were grouped at the eukaryotic division level(2). Samples (empty circle), binaries variables (full blue circle) and continuous variable (arrow) were plotted using RDA1 and RDA2 (percentage of variability explained and p-value are reported in brackets). In the bottom right of the RDA are represented the R-squared, the adjusted R-squared(3) and p-value of the model.

- W_UPX1: Water 500mbsl UPX1 site (BIO2, BIO9)
- W_UPX3: Water 900mbsl UPX3 site (BIO5)
- W_EGY1: Water 500mbsl EGY1 site (BIO3, BIO10)
- W_EGY6: Water 3000mbsl EGY6 site (BIO4)
- W_GYR6: Water 1000mbsl GYR6 site (BIO7)
- Ch_O: Chimney Oasis site (BS16)
- Ch_SS: Chimney Sarah Spring site (BS23, BS25)
- Ch_H: Chimney Hobbs site (BS15, BS24)
- B.W_O: Water above bivalves' bed Oasis site (BS12)
- Bsp_LE: *Bathymodiolus* sp. Lucky Eric site (BS10, BS14)
- Cma_O: *Calyptogena magnifica* Oasis site (BS11, BS18, BS19)
- Bth_RH: *Bathymodiolus thermophilus* Rehu Marka site (BS13, BS17)

20 genetic clone libraries are spread over 11 geographical sites (see earth illustration) which enclose to 3 bivalve species (in green), 1 sample of water above bivalve bed (B.W_O), 3 hydrothermal vent sites (Sarah Spring, Oasis and Hobbs; in red) and 5 sites in Equatorial Pacific ocean (BIOSOPE cruise) corresponding to deep water samples (in blue).
Amplification was conducted by PCR with primers set Euk328f (eukaryotic general) and UNonMet(4) (non metazoa specificity). All samples representing a total of 1399 clones. Sequencing was carried out either on the entire clone library or after an RFLP.

Eukaryotic lineage commonly found in **deep pacific ocean waters** (500 mbsl to 3,000 mbsl), as Dinophyceae and Euglenozoa (Fig. 3, blue) were ubiquitous, whereas **Radiolaria**(5) (Pearson:0.57, p-value:0.009; Fig. 2A,2B) were only detected in the deep water column and in two hydrothermal chimney samples (BS23/25).

Sequences affiliated to **Cercozoa** (P: 0.88; p: 0.001, with water sample: P: -0.54; p: 0.01) and **Stramenopiles** (P: 0.77, p: 0.003; Fig. 2A,2B) were specifically retrieved from **hydrothermal habitat**, respectively bivalve and chimney. Moreover, sequences affiliated to **Ciliates** are detected mostly to **bivalve libraries** (40 % of the total number of clones in these libraries; P:0.88, p: 0.0002).

mbsl: meters below sea level
P: Pearson's coefficient correlation, p: associated p_value

Marine Microbes, Barga, Italy
07/13/2008 - 07/18/2008

Results: Phylogenetic analyses

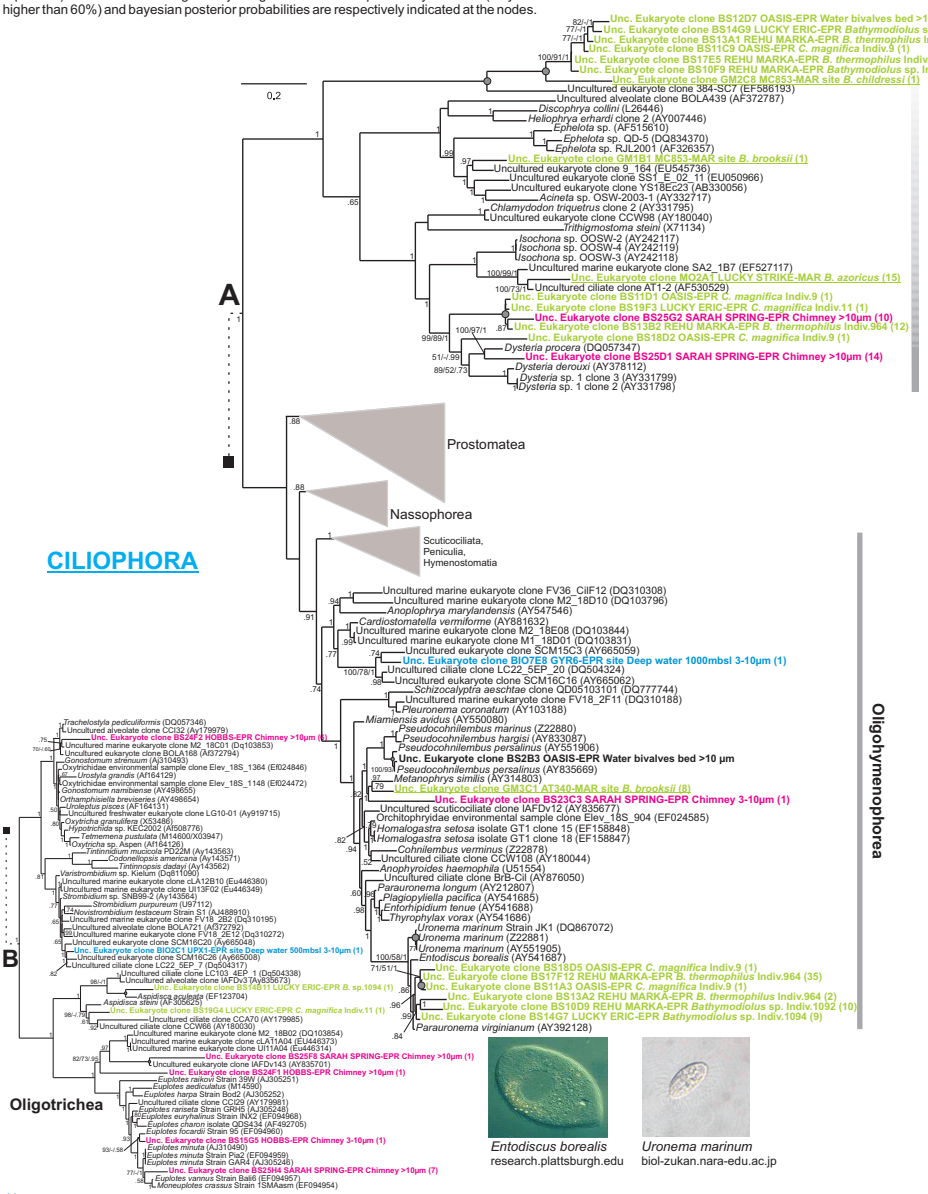


Fig. 5: Bayesian analysis of representative Ciliophora retrieved in this study (only the Phyllopharyngea, Oligohymenophorea (A) and Oligotrichea (B)). Bootstrap values (1,000 replicates) obtained with a neighbour-joining and a maximum parsimony methods (only for values higher than 60%) and bayesian posterior probabilities are respectively indicated at the nodes.

Entodiscus borealis
research.plattsburgh.edu

Uronema marinum
biol-zukan.nara-edu.ac.jp

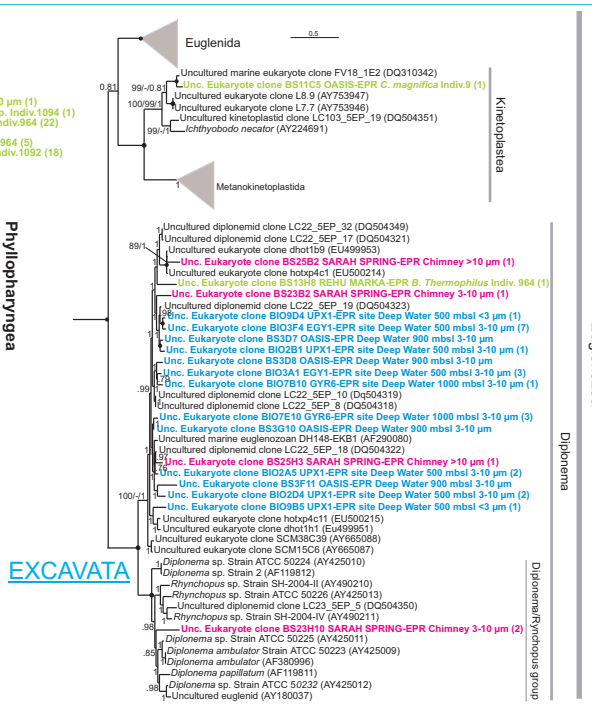


Fig. 3: Bayesian analysis of representative Excavata retrieved in this study (only the Euglenozoa). Bootstrap values (1,000 replicates) obtained with a neighbour-joining and a maximum parsimony methods (only for values higher than 60%) and bayesian posterior probabilities are respectively indicated at the nodes.

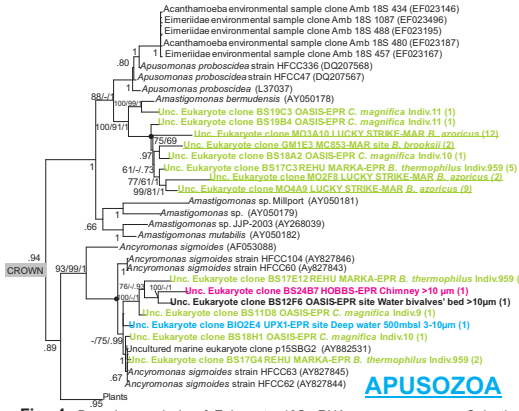


Fig. 4: Bayesian analysis of Eukaryote 18S rRNA gene sequences. Only the Apusomanads and Ancyromonads are represented. Bootstrap values (1,000 replicates) obtained with a neighbour-joining and a maximum parsimony methods (only for values higher than 60%) and bayesian posterior probabilities are respectively indicated at the nodes.

Most Euglenozoa sequences are close to **Diplonemea** (Fig. 3). In particular, the majority are associated with environmental sequences (called Deep-Sea diplonemid group) retrieved from **fluid-seawater mix from Lost City (MAR)** by Lopez-Garcia *et al.*[6].

Incertea Sedis such as **Apusomonads** and **Ancyromonads** (Fig. 4), and **Excavata** (Fig. 3), have also been detected in hydrothermal samples, mostly representative of the Apusozoa. Sequences related to Apusomonads form a cluster which is close to *Apusomonas berludensis*[7].

We have detected 3 different orders within Ciliophora (Fig. 5A, 5B). A cluster exclusively composed of sequences retrieved from bivalve sample is closely related to **symbiotic or parasitic** ciliates of other metazoans (Fig. 5A). Similarly, a new cluster belonging to **Phyllopharyngea** is also detected and closely related with a sequence found in environmental biofilms from stony streams[8] (96% of sequence identity).

An important eukaryotic micro-fauna is detected in association with hydrothermal bivalves by microscopic observations. Although reasons for such protist concentration are still unclear, it seems evident that this protect environment form by metazoans represents a relatively stable micro-niches propitious to their growth.
A majority of these protists are ciliates, mainly grouped into two putative new clusters within Phyllopharyngea and Oligohymenophorea. These communities seem very different from protists inhabiting the rest of the water column.

Additional studies are needed to elucidate the type of relationships between these protists and metazoans. Preliminary FISH analysis showed the presence of prokaryotes located inside vesicle in protists.

Comparative analysis is also being investigated on protist communities in hydrothermal bivalves located on Middle Atlantic Ridge with those collected from East Pacific Rise.

(1) Little, C.T.S., and Vrijenhoek, R.C. (2003) Are hydrothermal vent animals living fossils? Trends in Ecology & Evolution 18: 582-8.
(2) Javier del Campo *et al.* (2008) In press.
(3) Legendre, P. and Legendre, L. 1998. Numerical Ecology. 2nd English ed. Elsevier, Amsterdam
(4) Bower, S.M., Carnegie, R.B., Goh, B., Jones, S.R.M., Lowe, G.J., and Mak, M.W.S. (2004) Preferential PCR amplification of parasitic protistan small subunit rDNA from metazoan tissues. Journal of Eukaryotic Microbiology 51: 325-32.
(5) Viprey, M., Not, F., Massana, R., Moon-van der Staay, S.Y., Vaulot, D., and Guillou, L. (2008) Genetic diversity of Radiolaria assessed by environmental microbial-size 18S rDNA libraries. In press.
(6) Lopez-Garcia, P., Vereshchaka, A., and Moreira, D. (2007) Eukaryotic diversity associated with carbonates and fluid-seawater interface in Lost City hydrothermal field. Environmental Microbiology 9: 546-554.
(7) Cavalier-Smith, T., and Chao, E.E.Y. (2003) Phylogeny of choanozoa, apusozoa, and other protozoa and early eukaryote megaevolution. Journal of Molecular Evolution 56: 540-63.
(8) Dopheide, A., Lear, G., Stott, R., and Lewis, G. (2008) Molecular characterization of ciliate diversity in stream biofilms. Applied and Environmental Microbiology 74: 1740-7.

The authors are deeply indebted to Didier Jollivet, chief of the operation during BIOSPEEDO cruise, Hervé Claustre, chief scientist during BIOSOPE cruise on board the oceanographic research vessel "L'Atalante" and to Stéphane Hourdez to Gulf of Mexico samples. We also sincerely thank Mr. Pierre Legendre for his help in providing statistical work. We are also grateful Ouest-Génopole for sequencing operations. This work is supported by the ANR Deep Oases. A.L. Sauvadet is supported by a grant from the Ministère de la Recherche.

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ANR Deep Oases

Marine Microbes, Barga, Italy
07/13/2008 - 07/18/2008

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Abstract

Cilliates (Ciliophora) are defined as one of the main groups within the eukaryotic systematic. These ubiquitous unicellular microorganisms have adapted to a wide range of habitats (terrestrial, marine and freshwater habitats) using different morphological and physiological strategies, some of which are based on symbiosis. Out of the numerous ecological niches found in the marine environment, two were selected in this study : hydrothermal vents and coastal marine sediments. Morphological observations (live, stained, scanning, and transmission electron microscopy) and molecular genetic analyses (PCR, cloning, sequencing, *in situ* hybridization) were combined in order to characterise the unicellular eukaryote communities associated with endemic metazoans from these two contrasted environments (hydrothermal bivalves and intertidal polychaetes).

The present study (i) compared the distribution of unicellular eukaryotes associated with different deep-sea marine environments (seawater, hydrothermal vents, Bathymodiolus), (ii) revealed new clades within the ciliates detected in hydrothermal bivalves, (iii) redescribed and compared the distribution of astomes, a specific group of ciliates, only found associated with metazoans, (iv) defined endocytoplasmic bacteria as a third partner in this tripartite association.

Here we show that a majority of unicellular eukaryotes detected in hydrothermal environments are also widespread in oceanic waters. However, distinct and stable ecological niches, such as endemic bivalves, harbour more specific ciliate communities, either symbiotic and/or parasitic. Moreover, Cirratulidae polychaetes from anoxic coastal sediments are part of a tripartite association with endocommensal ciliates harbouring endocytoplasmic bacteria. The endosymbiotic bacteria, mainly affiliated to *Epsilon-Proteobacteria* and to a new *Proteobacteria* clade named *Durchoniella* Endocytoplasmic *Proteobacteria* Group (DEPG), were phylogeneticaly distant from any previously described Proteobacteria, suggesting a common evolutionary history. These new models may help to better understand the interactions between eukaryotes and bacteria.

Keywords : Ciliophora ; Astome ; Bivalves ; Polychaetes ; *Bacteria* ; *Proteobacteria* ; Pacific ocean ; English Channel ; Deep hydrothermal vents ; Coastal marine sediments ; Molecular genetic analyses ; Microscopy ; Phylogeny ; 18S rRNA ; 16S rRNA ; TEM ; SEM.

Résumé

Les ciliés (Ciliophora) sont un groupe majoritaire dans la systématique des eucaryotes unicellulaires. Ces organismes ubiquistes se sont adaptés à différents environnements - terrestres, marins et lacustres – en adoptant des modes de nutrition ou des caractères morphologiques diversifiés, et dans certains cas en mettant en place des relations symbiotiques.
L'environnement marin présente des niches écologiques variées et deux d'entre elles ont été sélectionnées pour cette étude : les sources hydrothermales et les sédiments marins côtiers. Des techniques moléculaires (PCR, clonage, séquençage, hybridation *in situ*) et microscopiques (MET, MEB, coloration DAPI) ont été utilisées pour caractériser les communautés d'eucaryotes unicellulaires associées à des métazoaires endémiques de ces deux environnements contrastés (bivalves hydrothermaux et polychaetes sédimentaires).
Ce travail de recherche a permis (i) de caractériser et de comparer la distribution des communautés d'eucaryotes unicellulaires associées à différents environnements marins profonds (eaux profondes, cheminées hydrothermales, Bathymodiolus), (ii) de mettre en évidence de nouveaux clades, notamment chez les ciliés détectés dans les bivalves hydrothermaux, (iii) de redéfinir et de comparer la distribution d'un groupe particulier de ciliés, les astomes, retrouvé uniquement en interaction avec des métazoaires, (iv) de mettre en évidence dans ces astomes endocommensaux, un troisième degré d'interaction représenté par des bactéries endocytoplasmiques.
Ainsi, cette étude montre qu'une majorité d'eucaryotes unicellulaires détectés au niveau des sources hydrothermales sont des organismes cosmopolites des océans. Cependant, des niches écologiques distinctes et stables, représentées par les bivalves endémiques, abritent des communautés de ciliés plus spécifiques, probablement de type symbiotique ou parasitique. De plus, les polychètes Cirratulidae retrouvés dans les sédiments côtiers réduits abritent un modèle d'interaction tripartite composé d'astomes endocommensaux associés à des bactéries endocytoplasmiques. Ces endosymbiontes, principalement affiliés à des *Epsilon-Proteobacteria*, et un nouveau groupe de Protéobactéries, nommé *Durchoniella* Endocytoplasmic *Proteobacteria* Group (DEPG), sont phylogénétiquement très éloignés des organismes déjà décrits, et pourraient être ainsi les témoins d'une histoire évolutive commune. Ces nouveaux modèles d'étude ouvrent le champ des interactions entre eucaryotes et bactéries.

Mots Clés : Ciliophora ; Astome ; Bivalves ; Polychaetes ; *Bacteria* ; *Proteobacteria* ; Océan Pacifique ; Manche ; Sources hydrothermales marines ; Sédiments côtiers réduits ; Analyse moléculaire ; Microscopie ; Phylogénie ; ARNr 18S ; ARNr 16S ; MET ; MEB.