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Abstract

Paramyxida is an order of rhizarian protists that parasitise marine molluscs, annelids and crustaceans. They include notifiable pathogens (*Marteilia* spp.) of bivalves and other taxa of economic significance for shellfish production. The diversity of paramyxids is poorly known, particularly outside of commercially important hosts, and their phylogenetic position is unclear due to their extremely divergent 18S rDNA sequences. However, novel paramyxean lineages are increasingly being detected in a wide range of invertebrate hosts, and interest in the group is growing, marked by the first 'Paramyxean Working Group' Meeting held in Spain in February 2015. We review the diversity, host affiliations, and geographical ranges of all known paramyxids, present a comprehensive phylogeny of the order and clarify its taxonomy. Our phylogenetic analyses confirm the separate status of four genera: *Paramarteilia*, *Marteilioides*, *Paramyxa* and *Marteilia*. Further, as including *M. granula* in *Marteilia* would make the genus paraphyletic we suggest transferring this species to a new genus, *Eomarteilia*. We present sequence data for *Paramyxa nephtys* comb. n., a parasite of polychaete worms, providing morphological data for a clade of otherwise environmental sequences, sister to *Paramarteilia*. Light and electron microscopy analyses show strong similarities with both *Paramyxa* and *Paramyxoides*, and we further discuss the validity of those two genera. We provide histological and electron microscopic data for *Paramarteilia orchestiae*, the type species of that genus originally described from the amphipod *Orchestia*; in situ hybridisation shows that *Paramarteilia* also infects crab species. We present, to our knowledge, the first known results of a paramyxid-specific environmental DNA survey of environmental (filtered water, sediment, etc.) and organismally-derived samples, revealing new lineages and showing that paramyxids are associated with a wider range of hosts and habitat types than previously known. On the basis of our new phylogeny we propose phylogenetic hypotheses for evolution of lifecycle and infectivity traits observed in different paramyxid genera.

Keywords: Paramyxida; *Paramyxa*; *Marteilia*; *Marteilioides*; *Paramarteilia*; *Eomarteilia*; eDNA; 18S rDNA phylogeny

1. Introduction

Paramyxida (Rhizaria, Ascetosporea) are related to haplosporidians, paradinids and mikrocytids (Bass et al., 2009; Hartikainen et al., 2014a,b), although the evolutionary relationships among the five ascetosporean orders are currently unresolved. Paramyxids are apparently exclusively parasites of marine invertebrates – annelids, crustaceans and molluscs. Five genera have been recognised: *Marteilia*, *Paramarteilia*, *Marteilioides*, *Paramyxa* and *Paramyxoides*. However, Feist et al. (2009) suggested that *Marteilioides* and *Paramyxoides* should be suppressed and that *Marteilioides chungmuensis* be reassigned to *Marteilia*, *Marteilioides branchialis* to *Paramarteilia*, and *Paramyxoides* to *Paramyxa*. One of the aims of the present study was to assess this recommendation by applying the first molecular phylogenetic approach to the group as a whole.

Paramyxids are increasingly recognised as pathogens causing economically significant mortalities of bivalves. The best known of these are marteiliosis/Aber disease in the European oyster *Ostrea edulis* and QX disease in the Sydney rock oyster *Saccostrea glomerata*, caused by *Marteilia refringens* and *Marteilia sydneyi,* respectively (Perkins and Wolf, 1976; Berthe et al., 2004; both species are listed as notifiable to the World Organisation for Animal Health (World Organisation for Animal Health (OIE) http://www.oie.int/en/international-standard-setting/aquatic-code/ (2015). Other significant bivalve diseases are caused by *Marteilia cochillia* in cockles (Carrasco et al., 2012, 2013), *Marteilioides chungmuensis* in *Crassostrea gigas* in Korea and Japan (Comps et al., 1986; Itoh et al., 2003), and *Marteilia granula* in the clam *Venerupis philippinarum* in Japan (Itoh et al., 2014).

Paramyxids in crustaceans include *Paramarteilia canceri*, which causes diseases of the edible/brown crab *Cancer pagurus* (Feist et al., 2009), and *Paramarteilia orchestiae* in amphipods, where it has been investigated in relation to modification of their sexual status (Ginsburger-Vogel 1991; Short et al., 2012a,b). However, beyond these very few examples there are so far no other reports of paramyxids causing disease in crustaceans, although more recently copepods have been shown to be vectors in the lifecycle of *M. refringens* (Carrasco et al., 2007; Arzul et al., 2014).

Polychaetes are similarly understudied as potential hosts of paramyxids. Adlard and Nolan (2015) recently demonstrated that *M. sydneyi* cycles through both the polychaete *Nephtys australis* and the oyster *S. glomerata*, providing another example of the complexity of at least some paramyxid lifecycles. Otherwise the only known annelid-infecting paramyxid is *Paramyxa*, of which the only described species, *Paramyxa paradoxa*, was first described in a polychaete larva from Banyuls-sur-Mer on the Mediterranean French coast by Chatton (1911). No similar organisms were reported until a paramyxid parasite of the polychaete *Nephtys caeca* was described by Larsson and Køie (2005) as *Paramyxoides nephtys*, distinguished from *P. paradoxa* on the basis of spore shape and cytology. However, Feist et al. (2009) considered that the characters used to distinguish these two genera were taxonomically invalid and transferred *Paramyxoides* to *Paramyxa*.

Paramyxids are also commonly referred to as paramyxeans. This class/order discrepancy deserves some explanation, to clarify the actual classification of the group and to ground its nomenclature in a robust phylogenetic context, which is an important aim of this study. Like many enigmatic micro-eukaryote groups, paramyxid taxonomy has been historically unstable, partly due to high levels of phenotypic conservation and convergence commonly seen in protists, particularly parasites (Boenigk et al., 2012; Hartikainen et al., 2014b; Neuhauser et al., 2014; Poulin and Randhawa, 2015). The presence of haplosporosome-like bodies provided early evidence that *Marteilia* and *Paramarteilia* were related to haplosporidans (Perkins, 1979), and ultrastructural characteristics supported a relationship between these genera and the first described genus eventually assigned to paramyxids, *Paramyxa* (Chatton, 1911; Desportes and Lom, 1981). *Marteilia* and *Paramarteilia* were described later, in the 1970s (Perkins, 1976; Perkins and Wolf, 1976; Desportes and Ginsburger-Vogel, 1977; Ginsburger-Vogel and Desportes, 1979), as detailed in Desportes and Perkins (1990) and Feist et al. (2009). All three genera are distinguished from haplosporidans by the production of variable numbers of daughter cells endogenously formed within a primary amoeboid stem cell, leading to their characteristic ‘cell within cell’ development. This group has been treated as a class (Paramyxidea Levine, 1980), phylum (Paramyxea Desportes and Perkins, 1990), and most recently as the order Paramyxida in Bass et al. (2009),

which is both the original and most stable taxonomy, concordant with both molecular and morphological analyses (Cavalier-Smith and Chao, 2003a,b; Bass et al., 2009; Feist et al., 2009).

Environmental DNA (eDNA) sequencing studies (i.e. generating and sequencing PCR amplicons or metagenetic fragments from DNA/RNA extracted from environmental samples to assess their biodiversity) are beginning to reveal high levels of diversity within groups of known parasites (Bass et al., 2009, 2015; Hartikainen et al., 2014a,b), providing powerful insights into parasite lifecycles, environmental reservoirs and transmission routes, and previously unknown parasitic lineages. These approaches are seen as increasingly important for disease monitoring and prediction, and policy issues, as described in Stentiford et al. (2014) and Bass et al. (2015). Paramyxid 18S rRNA genes are phylogenetically divergent and therefore usually missed in broadly-targeted 18S sequencing surveys (Bass et al., 2015). In such cases PCR primers designed specifically for the group under study can be very valuable (Hartikainen et al., 2014a,b). One aim of this study was to design and optimise such a primer set to better understand paramyxid diversity and phylogeny.

As well as generating new eDNA-based sequences as described above, we also analyse all available paramyxid 18S rDNA sequences, providing a comprehensive paramyxid phylogenetic tree, in order to rationalise paramyxid nomenclature and determine their evolutionary relationships. We show that *Marteilia*, *Paramarteilia* and *Marteilioides* form highly distinct and robustly supported phylogenetic clades, confirming their validity as separate genera, and that all three genera form a robustly supported clade that also includes *M. granula* (recently described by Itoh et al., 2014), and uncharacterised environmental sequences, confirming the monophyly of the order Paramyxida.

2. Materials and methods

2.1. Sample collection

For invertebrates, 150 mussels, *Mytilus edulis,* were collected from the River Tamar estuary mouth near Cremyll Ferry, Devon, UK in June and July 2013. The June individuals were incubated in sterile artificial sea water (ASW; Culture Collection of Algae and Protozoa (CCAP) recipe (www.ccap.ac.uk/media/documents/ASW.pdf*))* in sets of 10 individuals (clustered according to sampling proximity) for 1 h. Post-incubation, 50-100 ml of water were syringe-filtered through Whatman GF/F filters (GE Healthcare, USA) and filters subsequently fixed in 100% molecular-grade ethanol. A further 150 individuals of *M. edulis* and 222 *Ostrea edulis* were similarly collected from a nearby site, Jupiter Point (River Lynher, Tamar Estuary, UK), in September 2015. All bivalves were dissected and tissue cross-sections including digestive gland and mantle were fixed in Davidson's Solution for histology, glutaraldehyde for electron microscopy (EM), and 100% ethanol (June samples) or flash frozen in liquid nitrogen (July samples) for molecular analyses. Other invertebrates (polychaetes, amphipods, shrimp, barnacles) were also sampled from sediments and under rocks in the mussel sampling areas. Animals were kept intact and preserved in 100% molecular ethanol at -20°C until DNA extraction. Amphipods, *Orchestia gammarellus,* were collected at low tide in the intertidal zone above the high water mark at Castle Cove, Weymouth, England (50°35' 45.6'' N, 2°27' 36'' W; n = 178) between September 2014 and February 2015 and in the Gann Estuary, Dale, Wales (n = 197) during November 2014. For *O. gammarellus,* morphological identity was confirmed, length was measured using calipers, sex was determined and any external abnormalities, i.e. lost limbs or notable markings, were recorded. The *O. gammarellus* were anaesthetised using clove oil (Eugenol 80-90%) at a dilution of 0.2 µl/ml of seawater and were transversely sectioned into three using a stereomicroscope (Leica M125, Leica Microsystems, Germany). One section was placed in 100% ethanol for molecular work; the second section was placed in a 2.5% glutaraldehyde 0.2 M sodium cacodylate buffer for transmission electron microscopy (TEM) and the final section was placed into a cassette in Davidson's Sea Water Fixative for 24 h for histopathology and in situ hybridization (ISH).

Edible crabs, *Cancer pagurus,* were captured in baited traps from the commercial fishery in Weymouth Bay area in January 2004. A total of 30 crabs were transported back to the Weymouth laboratory, where they were anaesthetised on ice for 30 min before dissection. Hepatopancreas, heart, gill, muscle and gonad tissues were fixed in Davidson's sea water fixative for histology and

157 hepatopancreas and gonad samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium
158 cacodylate buffer for EM.

159 Spider crabs, *Maja squinado,* were captured using a Granton trawl on board the Cefas
160 Endeavour from the Cardigan Bay area, Wales, in July 2008. As for edible crabs, 30 spider crabs
161 were anaesthetised on ice for 30 min before dissection; hepatopancreas, heart, gill, muscle and
162 gonad tissues were fixed in Davidson's sea water fixative for histology and hepatopancreas
163 samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for EM.

164 Polychaete worms (100 specimens, mostly *N. caeca*) were collected from the tidal,
165 brackish Fleet lagoon, Weymouth, Dorset, UK (10-30 ppt salinity) on 15 May 2015. Each worm
166 was dissected into three sections in the field and fixed for molecular analyses, histology and EM.

167 For environmental samples, 150 L water samples collected at three sites in the Tamar
168 estuary: Cremyll Ferry, Wilcove, and Neal's Point, were passed serially though 50 µm and 20 µm
169 meshes. Material collected on the meshes (filtrand) was transferred to 2 ml cryotubes and fixed in
170 100% ethanol. A 50 L aliquot of water from each site was kept cool and in the dark and transported
171 to the laboratory within 24 h, where aliquots were filtered under pressure onto 142 mm, 0.45 µm
172 cellulose acetate filters (Sartorius, Germany) and immediately stored at -80°C. Littoral sediment
173 samples (0.5 -1 g), from the areas in which mussels were sampled, were taken from the Cremyll
174 site and fixed in 100% ethanol. Water and sediment samples were collected using the same
175 protocols from Newton's Cove and the Fleet lagoon in June and October 2011, and April 2012.
176 Filtered freshwater and littoral marine water were similarly sampled (but without the 0.45 µm-
177 filtering step) and benthic sediments from sites in the Western Cape, South Africa 10x water
178 samples, 14x sediment and sand samples), Sabah, Borneo, Malaysia in December 2011 (38x
179 water samples) and various sites in Florida, USA in June 2014 (47x water samples, 34x
180 invertebrate incubations (as for *M. edulis* incubations, above). Water from shrimp hatchery tanks at
181 the Borneo Marine Research Institute (University of Malaysia, Sabah) was sampled (5x) also as for
182 *M. edulis* incubations.
183

184 *2.2. Sample processing and DNA extraction*

185 Sediment and 50 µm and 20 µm fraction filtrand samples were freeze-dried at -40°C until
186 dry. DNA was extracted from these and the 0.45 µm fraction filtrand using the PowerSoil DNA
187 Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). DNA was extracted from invertebrate (apart
188 from amphipod) tissue from all sites using the DNEasy Blood and Tissue Kit (Qiagen, Germany).
189 Flash frozen mussel tissue was defrosted into RNAlater (Qiagen) before DNA extraction using the
190 96-well DNEasy Bood and Tissue Kit (Qiagen).
191 For the amphipods, the 100% ethanol-preserved samples were suspended in a solution of
192 Lifton's buffer (Sucrose 2.3% w/v, 1M Tris pH 8.0, SDS, 0.5 M EDTA pH 8.0) containing
193 Proteinase K (100 mg/ml). Following incubation overnight at 55°C, DNA was extracted using a
194 phenol chloroform extraction method with ethanol precipitation (Nishiguchi et al., 2002). The
195 resulting DNA was suspended in 40 µl of water and the DNA concentrations of each sample (ng/µl)
196 were quantified via spectrophotometry (NanoDrop ND-1000) and the QuantiFluor®DS-DNA
197 system and Quantus Fluorimeter (Promega, UK) by following the kit manufacturer's instructions.
198 Filters from invertebrate incubation samples were freeze-dried at -40°C for 2 h to remove
199 ethanol. Dried filters were subsequently kept on ice and cut into small pieces using sterile scissors,
200 prior to DNA extraction using the DNEasy Blood and Tissue Kit (Qiagen).
201

202 *2.3. PCR and sequencing*

203 A nested primer set targeting regions V7 and V8 of the paramyxid 18S rRNA gene was
204 designed, based on all available paramyxid sequence data in June 2013. The first round PCR
205 used primers Para1+fN (5'- GCG AGG GGT AAA ATC TGA T -3') and ParaGenrDB (5'- GTG TAC
206 AAA GGA CAG GGA CT-3'). Second round PCR used primers Para3+fN (5'- GGC TTC TGG GAG
207 ATT ACG G -3') and Para2+rN (5'- TCG ATC CCR ACT GRG CC-3') (primer set A). All PCRs
208 were conducted in 20 µl final volumes with 1 µl of template DNA and a final concentration of 0.5
209 µM of each primer, 0.4 mM dNTPs, 2.5 mM of $MgCl_2$, 1x Promega Green Buffer and 0.5 U of
210 Promega GoTaq. Cycling conditions for first round PCR consisted of a 3 min denaturation at 94°C,
211 followed by 42 cycles of 95°C for 30 s, 67°C annealing for 1 min and 72°C for 1 min. Amplicons
212 were extended by final incubation at 72°C for 5 min and stored at 4°C. Second round PCR used 1

213 μl of first round product as template DNA, and the cycling conditions were altered to an annealing
214 temperature of 62°C. These primers were used to screen environmental and invertebrate
215 tissue/incubation samples from the Newton's Cove, Fleet, Tamar estuary, Florida, and Borneo,
216 except those detailed in the following paragraph.

217 Following the publication of sequence data for '*Marteilia*' *granula* (Itoh et al., 2014) primer
218 set A was modified to include this sequence type (primer set B). The resulting hemi-nested PCR
219 protocol used Para1fGW (5'- GGG CGA GGG GTA AAA TCT -3') and ParaGENrGW (5'- GTG
220 TAC AAA GGR CAG GGA CT -3') (first round), followed by Para3fGW (5'- GGC TTY TGG GAG
221 AKT ACG GC -3') and ParaGENrGW (second round). PCR mixtures were prepared as above.
222 Cycling conditions consisted of a 5 min denaturation at 95°C, followed by 30 cycles of 95°C for 1
223 min, 58°C annealing for 1 min and 72°C extension for 1 min. Amplicons were extended by a final
224 incubation at 72°C for 10 min and stored at 4°C. The same cycling conditions were used for both
225 rounds of the hemi-nested PCR. These primers were used to screen the polychaete worms from
226 the Fleet lagoon in May 2015, *O. edulis* and *M. edulis* tissues from Jupiter Point (Tamar, UK)
227 collected in September 2015, and eDNA from South Africa. A panel of samples comprising
228 representatives from each sample set screened using primer set A was screened with primer set B
229 to test for additional diversity not detected by primer set A. No differences between the diversity
230 detected and frequency of paramyxid-positive PCRs were detected in these samples.

231 Fragments were visualised on 1.5% agarose gels stained with GelRed. Amplicons were
232 Sanger sequenced in one direction using primer Para3+fN or Para3fGW. Where direct sequencing
233 produced a mixed product (Cremyll sediment, Wilcove water samples), amplicons were pooled
234 from all PCR-positive samples and clone libraries were prepared using the Stratagene cloning kit
235 (Agilent Technologies, Santa Clara, CA, USA). Eight clones from each sample were sequenced in
236 one direction using the M13R primer.

237

238 *2.4. Phylogenetic analyses*

All available paramyxid sequences were downloaded from National Center for Biotechnology Information, (USA) GenBank, including Blastn searches to identify uncharacterised (including environmental) sequences related to known taxa. These were aligned with sequences generated in this study using Mafft version 7, e-ins-i algorithm (Katoh and Standley, 2013). The resulting alignment, (47 sequences, including haplosporidian outgroup; 1812 positions analysed) was refined manually and analysed using Maximum Likelihood (ML) in RAxML BlackBox version 8 (Stamatakis, 2014) (Generalized time-reversible (GTR) model with CAT approximation (all parameters estimated from the data); an average of 10,000 bootstrap values was mapped onto the tree with the highest likelihood value). A Bayesian consensus tree was constructed using MrBayes v 3.2.5 (Ronquist et al., 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for 2 million generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. Trees were sampled every 1,000 generations. The first 500,000 generations were discarded as burn-in (trees sampled before the likelihood plots reached stationarity) and a consensus tree was constructed from the remaining sample. Sequences generated by this study are available from NCBI GenBank (Accession numbers **KX259318-KX259327**), and are indicated in Fig. 1.

2.5. Histology and in-situ hybridisation (ISH)

Following 24 h fixation, samples were suspended in 70% industrial methylated spirits (IMS) before being dehydrated and infiltrated with paraffin wax using a Vacuum Infiltration processor (Peloris, Leica Microsystems UK). Wax embedded samples were trimmed along the sagittal plane using a rotary microtome (Shandon Finesse 325, Thermo Fisher, UK) to expose tissue. Once trimmed, sections (3-4 µm thick) were mounted onto glass slides and stained using H&E in an auto-stainer (Surgipath, UK) and then cover-slipped (ClearVue, Thermo Fisher, UK). Screening of samples for pathogens was performed using a Nikon Eclipse E800 light microscope (Nikon, UK). Digital images and measurements were captured using the integrated LEICA™ (Leica, UK) camera and LuciaG software (Nikon).

ISH was carried out on *O. gammarellus* slides to localise *P. orchestiae*, and *C. pagurus,* and *M. squinado* slides for *Paramarteilia.* Probes were generated by PCR using *Paramarteilia*-specific primers Porchest298f (5'-CTG ATG AGC CTG GCA AGA CCA C-3') and Porchest396r (5'-TGG GGC ACA CCG ATA CTG GG-3'), producing a 98 bp amplicon specific to the clade marked '*Paramarteilia*' on Fig. 1. The process was also carried out on *N. caeca* slides for *Paramyxa nephtys*; *Paramyxa*-specific probes were generated using primers Paramyxa240f (5'- AGC AGA CCA ATC GCT CGA C -3') and Paramyxa449r (5'- GAC TCA TTC GTG GCG CGT TT -3'), producing a 209 bp amplicon. In each case probes were digoxigenin (DIG)-labelled using digoxigenin-11-dUTP in PCRs of 100 µl volume with a final concentration of 1x Promega colourless buffer, 2.5 mM $MgCl_2$, 20 µM PCR DIG labelling mix (Roche, Switzerland), 0.5 µM of each primer, 0.5 U of Promega GoTaq and 6 µl of template DNA. Amplifications were performed on a Peltier PTC-225 thermal cycler. Cycling conditions consisted of a 5 min denaturation at 94°C, followed by 40 cycles of 95°C for 30 s, taxon-specific annealing temperature for 45 s (60°C for *Paramarteilia*; 55°C for *Paramyxa*), and 72°C for 1 min. Amplicons were extended by final incubation at 72°C for 5 min and stored at 4°C. Tissue sections (4 µm thick) from histologically-positive individuals were mounted on Poly-L lysine slides. These were deparaffinised, rehydrated and then treated with Proteinase K solution (10 mg/ml) for 30 min at 37°C in a humid chamber. Proteolysis was terminated by incubating the slides in 100% industrial methylated spirits for 5 min and rinsing slides with 2x SSC buffer for 5 min at room temperature. Sections were overlaid with a hybridization solution (4 x SSC buffer, 50% formamide, 1 x Denhardt's solution, 10% dextran sulfate, 250 µg/ml Yeast tRNA) containing the probe DNA (50:50 v/v). Slides were heated to 95°C for 5 min and hybridized overnight at 42°C. After hybridization, sections were washed with 1x SSC buffer and 0.5x SSC buffer for 15 min at 42 °C. Slides were blocked with 6% non-fat milk in Tris buffer (pH 7.5) for 1 h at room temperature. The reactions were then developed with anti-DIG antibody conjugated with an alkaline phosphatase, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoylphosphate (X-phos). The sections were counterstained with Nuclear Fast Red and examined under light microscopy. Negative controls lacked the DIG-labelled probe in the hybridization buffer.

296 *2.6. Transmission Electron Microscopy (TEM)*

297 Selected parasite-positive animals were removed from glutaraldehyde and sectioned into 1
298 mm^3 tissue blocks. The samples were fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate
299 buffer (pH 7.4) for 2 h and post-fixed by rinsing them in 1% osmium tetroxide in 0.1 M sodium
300 cacodylate buffer (1 h). The samples received two rinses in 0.2 M sodium cacodylate buffer (10
301 min) before being dehydrated through a graded acetone series (10%, 30%, 50%, 70%, 90% and
302 100%) with 10 min in each solution. Samples were then infiltrated by Agar 100 epoxy resin (Agar
303 Scientific, UK) Agar 100 pre-mix kit medium) and embedded by polymerising the samples at 60°C
304 overnight. Semi-thin sections (0.5 µm – 2 µm) were taken from resulting blocks and stained with
305 Toluidine Blue. Stained semi-thin sections were surveyed using a light microscope to identify target
306 regions, and 70-90 nm ultra-thin sections of these regions were mounted on uncoated copper
307 grids. Finally, the samples were stained with 2% uranyl acetate solution followed by Reynolds'
308 Lead Citrate (Reynolds, 1963) before being examined using a transmission electron microscope
309 (JEOL JEM 1210, Japan). Digital images were obtained using Gatan Digital Micrograph™ software
310 with a Gatan Erlangshen ES500W camera. All raw images files are accessible via Mendeley Data:
311 http://dx.doi.org/10.17632/jvphfxw32t.1.

312

313 **3. Results**

314 *3.1. Paramyxid diversity*

315 Table 1 summarises all paramyxid genera and species for which 18S rDNA sequence data
316 are available in public databases and/or are robustly identified morphologically (as a result of our
317 literature survey), indicating their known host ranges and geographical distributions. The new data
318 generated by the study are also included in this table.

319

320 *3.2. Paramyxid 18S rDNA phylogeny*

Bayesian and ML analyses of all currently known and newly generated paramyxid 18S rDNA data shows that the genera *Marteilia*, *Paramarteilia* and *Marteilioides*, and a newly sequenced parasite of *N. caeca* and other polychaetes, group separately from each other, each in robustly supported clades of congeners and/or environmental sequences (Fig. 1A). *Eomarteilia* (previously *Marteilia*) *granula* does not branch with other *Marteilia* spp, but is sister to all other known paramyxeans with moderate to strong support in ML and Bayesian analyses with maximal taxon sampling (Fig. 1A). We therefore re-assign this to the new genus *Eomarteilia.* Two lineages exclusively comprising environmental sequences are described in section 3.4. Although diversity within each of the genus clades is not high, some other relevant points arise from the phylogenetic analyses.

3.2.1. Marteilioides

The *Marteilioides* clade has two distinct, known sister lineages, one (*M. chungmuensis*) from two *Crassostrea* spp. (*C. gigas* and *C. ariakensis* from Japan and South Korea; a total of five sequences in GenBank), and the other (undescribed *Marteilioides* sp.) from two independent studies in the Manila clam *Ruditapes philippinarum* (two GenBank sequences) (Yanin et al., 2013, first observed by Lee et al., 2001).

3.2.2. Marteilia clade

The *Marteilia* clade contains sequences which cluster in rough agreement with their geographical provenance: *M. refringens* and *M. cochillia*, sampled on many independent occasions from Europe (Kerr et al., unpublished data) and *M. octospora* from Spain (Ruiz et al., 2016) share very similar 18S sequences, and form a strongly supported clade with the highly distinct *M. sydneyi* sequence from *Saccostrea glomerata* from Queensland, Australia, '*Marteilia* sp. MC' from *Ruditapes philippinarum* in South Korea (Kang et al., unpublished data; sequence has GenBank accession number **AB823743**), and another distinct sequence derived from a shrimp hatchery tank

347 at the Borneo Marine Research Institute. As noted above, *M. granula* does not belong to this clade.
348 A further sequence (not in GenBank) from *Mytilus* sp. from China was manually copied from Wang
349 et al. (2012) and aligned with the *Marteilia* sequences in Fig. 1A. This is presented separately (Fig.
350 1B) as the 638 bp fragment does not overlap with the Bornean shrimp tank sequence (with which it
351 groups but with no support), but otherwise optimising the alignment between other *Marteilia* clade
352 sequences. This tree does not differ significantly from the comparable part of Fig. 1A but does
353 show that the Chinese *Mytilus*-derived sequence does not group with named *Marteilia* spp. This
354 reduced taxon-sampled tree is also interesting in that, in the absence of other genera, *Eomarteilia*
355 and *Marteilia* form a clade (see Discussion). After our analyses (Fig. 1) had been performed,
356 *Marteilia octospora* was described by Ruiz et al. (2016). The short 18S fragment available for *M.*
357 *octospora* (within **KU641125**), although not in the most variable region of the gene, is almost
358 identical to the corresponding region of *M. cochillia* (Fig. 1).
359
360 *3.2.3. Paramarteilia clade*

361 Sequences in the *Paramarteilia* clade were recovered from mussel-, amphipod- and crab-
362 associated material, and comprise two distinct but closely related sequences types – one only from
363 crustaceans to date (amphipods from the genera *Echinogammarus* (Short et al., 2012a,b, 2014)
364 and *Orchestia* (this study)), and in incubation water from *C. pagurus* and *Cerastoderma edule*. The
365 other 18S-type has to date only been detected in *M. edulis* incubation water. ISH probes designed
366 for the two *Paramarteilia* sequences (it was not possible to design different probes for each
367 sequence type) also hybridised to *Paramarteilia*-infected tissue in crabs *C. pagurus* (connective
368 tissue within hepatopancreas, heart, ovary, testicular follicles) and *M. squinado* (hepatopancreas).
369 The histology and TEM of *Paramarteilia* in *C. pagurus* and *M. squinado* are reported in Section
370 3.6.

371

372 *3.2.4. Paramyxa clade*

A paramyxid found in the polychaete *N. caeca* in this study (assigned to *Paramyxa nephtys* as described in Section 3.3, and labelled as such in Fig. 1A) shares an identical sequence from 0.45 µm-filtered water from Wilcove on the Tamar estuary (not separately shown in Fig. 1A). A related but clearly distinct sequence was detected in eDNA from an estuarine fish farm in Borneo. A further two sequences, labelled '*Paramyxa'* to tentatively assign them to this genus pending ongoing morphological analysis, were detected in DNA extracted from bivalve digestive gland (DG) samples from the Tamar, UK. One of these was detected at relatively high frequency in *O. edulis* (62/222; 28%) and *M. edulis* (9/150; 6%), but only in samples taken from Jupiter Point (Tamar) in September 2015. The other sequence type was detected only in 1/150 *M. edulis* DG from the 2013 Tamar sampling.

3.3. Paramyxid parasite of N. caeca and other polychaetes

Histological analysis showed that 23/71 *N. caeca* specimens sampled from the Fleet lagoon (Weymouth, UK) in May 2015 were infected with an unknown paramyxid (Fig. 2). Three of these exhibited heavy infections of an ellipsoid spore-forming parasite typically restricted to the intestinal tract of the worm, including the mouth, the intestinal epithelium and lumen along the full length of the worm. TEM analyses of these heavily infected individuals revealed spore sacs with striated projections and containing four spores, very similar to those shown for *P. nephtys* in Larsson and Koie (2005). Also concordant with the description of *P. nephtys,* the developmental stages of the parasite had penetrated the intestinal epithelium and replicated to replace a large proportion of the host tissue. Mature stages were released from the intestinal cells into the lumen. No host response to infection was noted in the epithelium or lumen. Pre-spore stages were also similar to those described for *P. nephtys*. When all 71 *N. caeca* samples were screened using paramyxid-specific primers (see Section 2) a further 13 (i.e. total of 36 *Nephtys* individuals) were PCR-positive. Small samples of other polychaete species were collected from the same site as the *N. caeca* specimens. DNA from tissue of some of these was also paramyxid PCR-positive and yielded the same 18S sequence type in 3/5 *Nereis* sp. individuals, 2/14 *Nemertea*-like worms, 1/1

400 *Ophelia*-like worms, and three unidentified polychaete individuals. We refer to this parasite as
401 *Paramyxa nephtys*, rather than *Paramyxoides*, for reasons discussed further in this report.
402

403 *3.4. Paramyxid-specific eDNA analysis*

404 Two lineages in Fig. 1, marked PARAM-1 and -2, have no characterised members, i.e. they
405 have been detected only in eDNA samples. PARAM-1 comprises three identical sequences from
406 marine sites in Florida,USA: one from a littoral filtered water sample and two from filtered water
407 sampled within a *Crassostrea virginica* bed at Seahorse Key. In PARAM-2, two identical
408 sequences (represented by **GU824205** on Fig. 1) were sequenced from the same sample (and
409 possibly the same organism) in a eukaryote-wide survey of filtered water from the Cariaco Bay,
410 Venezuela (Edgcomb et al., 2011). The other very closely related sequence in PARAM-2 was
411 amplified by our paramyxean-specific PCR protocol from 20 µm filtered water from a bed of *C.*
412 *virginica* adjacent to the Whitney Laboratory for Marine Bioscience, Florida, USA. We cannot
413 assume that PARAM-1 or -2 belong to the genus *Paramyxa* as we have no morphological data for
414 them (see Discussion).

415 Some of the other paramyxid clades were also represented in our eDNA screening, as
416 shown in Fig. 3. *Marteilia refringens* 18S rDNA was amplified from sediment and filtered water
417 column samples from Wilcove and Cremyll in the Tamar estuary (but not Neal's Point, furthest
418 from the sea), where it was also detected in *M. edulis* tissue samples (5/144 mantle; 37/287 DG)
419 and filtered incubation water (2/17) samples. However, no sequences corresponding to *M.*
420 *cochillia*, *M. sydneyi, E. granula* or either *Marteilioides* sequence type were recovered from the
421 eDNA screens.

422 The *P. orchestiae* 18S type was detected most frequently in amphipod tissue samples
423 (whole animals) but the same sequence type was also detected in *C. pagurus* incubation water. A
424 closely related sequence (98% similarity) was recovered from *M. edulis* incubation water (1/17
425 samples). In the *Paramyxa* clade, the only PCR amplifications from 'environmental' samples were
426 of the *P. nephtys* 18S-type in *M. edulis* incubation water.

427

428 *3.5. Geographical distribution of paramyxids*

429 Fig.3 suggests strong biogeographical structuring of paramyxid diversity, and that this to an
430 extent reflects the phylogenetic clustering. *Paramarteilia* and *Paramyxa* spp are known only from
431 Europe, *Eomarteilia* from Japan, and *Marteilioides* from the Far East (southern. Korea, Japan,
432 eastern Australia). The *Marteilia* clade is apparently more widely distributed: *M. refringens, M.*
433 *cochillia* and *M. octospora* mostly from Europe (other than one record from the Pacific coast of
434 Mexico (Grijalva-Chon et al., 2015) and another from Kuwait), *M. sydneyi* from Australia, and many
435 additional '*Marteilia* sp.' records unconfirmed by sequencing in the literature therefore not included
436 in Table 1 or Fig. 3. The *Marteilia* clade is also represented in Borneo by an environmental
437 sequence. The environmental clades PARAM-1 and -2 were also only detected in a small number
438 of samples – PARAM-1 in multiple samples from a single site in Florida, USA, and PARAM-2 from
439 low latitude American continent sites (Florida and Venezuela), despite the fact that paramyxid-
440 specific PCR was carried out on eDNA samples from Europe, the Americas, South Africa, and
441 Borneo. None of the South African eDNA samples were paramyxid-positive.

442

443 *3.6. Paramarteilia: confirmation of type species and infections in crab spp.*

444 We present the first known 18S rDNA sequence for the *Paramarteilia* type species *P.*
445 *orchestiae* from the type species host *O. gammarellus*, with histopathology and TEM analyses of
446 the corresponding material (confirmed by *Paramarteilia*-specific ISH) (Fig. 4C inset). Our light and
447 ultrastructural observations were entirely concordant with the original description of *P. orchestiae*
448 (Ginsburger-Vogel and Desportes, 1979). The parasite's primary cells (Fig. 4D) were between 5 -
449 12 µm in diameter and contained multivesicular bodies with spherical vacuoles and electron dense,
450 cylindrical bacilliform haplosporomes (Fig. 4D inset). Up to nine secondary cells (sporonts, C2)
451 were observed, each individually between 3 - 7 µm in diameter, and unlike the primary cells lacking
452 haplosporosomes and with increased ribosome density. Within the tertiary cell, two spores were

present (Fig. 4D). Developmental stages of the parasite were dispersed throughout *O. gammarellus* tissues and organs including the epidermal tissue (Fig. 4C,D), the connective tissue, heart and ganglia of the nerve cord (Fig. 4A). Furthermore, the cells apparently 'migrate' between organs and were present in the oocytes of two females (Fig. 4B), which supports the original trans-ovarial transmission hypothesis (Ginsburger-Vogel, 1979). Although the bi- or tri-cellular stages of the spore were not observed, the host species, sites of infection and morphology of the parasite unambiguously confirm this parasite as *Paramarteilia orchestiae.* A total of 369 *O. gammarellus* were prepared for histology and examined for the presence of *P. orchestiae*; infection was observed in 12 (3.25%) of these: one from Weymouth and 11 from Dale. A total of 222 of the 369 *O. gamarellus* individuals were screened by PCR using the *Paramarteilia* primers from Short et al. (2012), including those analysed for histology; 24 of these were positive (10.81%); eight from Weymouth and 16 from Dale. No obvious pathology was displayed in 15 of these 24 samples.

We also present the first known histopathology, ISH and TEM images of *Paramarteilia canceri* in edible crabs and *Paramarteilia* in spider crabs *C. pagurus* and *M. squinado,* respectively (Fig. 5). The morphology and infection characteristics of *Paramarteilia* in *C. pagurus* were consistent with those described for *P. canceri* in Feist et al. (2009) and the *Paramarteilia* infection in *M. squinado* was also very similar structurally. *Paramarteilia canceri* was observed in one of the 30 edible crabs sampled and *Paramarteilia* sp. was observed in two out of the 30 spider crabs sampled. Developmental stages of the parasite were dispersed throughout the connective tissues (Fig. 5A), hepatopancreas (Fig. 5B) and gonad (Fig. 5E, G). The parasite is shown to infect the connective tissues surrounding the oocytes and the oocytes themselves (Fig. 5 E, F) as well as the testicular follicles (Fig. 5G, H). The parasite in both crabs was similar to that observed in the *O. gammarellu*s (see above), and that recorded from *Echinogammarus marinus* by Short et al. (2012b), the sequence of which is shown in Fig. 1A (**JQ673484**). However, more advanced developmental stages present in the crab species were not observed in amphipods. At present, based on morphological grounds it is not possible to propose that the same species infects these hosts. Because no 18S sequence for *P. canceri* exists (although the *P. orchestiae* 18S sequence was also detected in *C. pagurus* incubation water, and may correspond to *Paramarteilia* infecting

481 edible crabs), and the known sequence variation with the *Paramarteilia* clade is very low (Fig. 1A),
482 we used the same ISH probe as for *P. orchestiae* to successfully probe for *Paramarteilia* in both
483 crab species (Fig. 5 insets).

484

485 **4. Discussion**

486 To our knowledge the phylogenetic analysis in this paper (Fig. 1A) is the first to show the
487 relative branching positions of all paramyxid genera and species for which sequence data are
488 available. The laterally compressed appearance of the tree in Fig. 1 may misleadingly imply low
489 18S sequence differences. In fact all the terminal branches represent distinct lineages, with the
490 possible exceptions of the two bivalve-derived *Paramyxa* sp. sequences at the top of Fig. 1A, and
491 the Venezuelan and Floridean 18S-types in PARAM-2. For example, the clearly distinct *M. cochillia*
492 and *M. refringens* have 99% similar 18S sequences (1733/1742 identical nucleotide positions) and
493 are very obviously different species based on phenotype. Many protistan species are identical, or
494 nearly so, at the 18S level (Bass et al., 2009; Boenigk et al., 2012), yet show very different host
495 associations and sporulation characteristics.

496 Other phylogenetic distinctions in Fig. 1A may reflect different host affiliations – for example
497 the *M. chungmuensis* lineage is to date exclusively associated with the clam *Ruditapes*
498 *philippinarum* and the distinct *Marteilioides* sp. with *Crassostrea* spp. The two closely related
499 *Paramarteilia* 18S types may also have different host associations (molluscs versus crustaceans),
500 although the 'crustacean' sequence has also been detected in *Cerastoderma* incubation water (but
501 may not correspond to a parasite of the cockle), and the *Mytilus*-associated 18S type has to date
502 only been detected once, also in (*Mytilus*) incubation water.

503 The only morphological characteristics thought to be useful to distinguish between
504 paramyxid genera are the numbers of tertiary cells (C3; which becomes the spore) produced and
505 the number of cells constituting the spores (Feist et al., 2009). However, we show here that these
506 are not taxonomically reliable; the unrelated *Marteilioides* and *Marteilia* both form tri-cellular

spores, although from different numbers of tertiary precursor cells - two in *M. sydneyi*, four in *M. refringens*, six in *M. cochillia*, and eight in *M. octospora*. The only *Marteilioides* sp. for which the number of tertiary cells is known (*M. chungmuensis*) has a single tertiary precursor cell; the possibility that more tertiary cells occur in other *Marteilioides* lineages is too great for this character to be used to distinguish them from each other or from *Marteilia*. Furthermore, *Paramarteilia*, grouping between *Marteilia* and *Marteilioides* (Fig. 1A), forms bi-cellular spores while *Paramyxa* has tetra-cellular spores. Therefore there is no systematic variation in either tertiary cell number or numbers of cells constituting spores with the phylogenetic branching order.

We provide the first known molecular evidence for the phylogenetic position of *Paramyxa*/*Paramyxoides*. The parasite of *N. caeca* that we analysed was ultrastructurally inseparable from the description of *Paramyxoides nephtys* by Larsson and Koie (2005). However, as the only consistent difference between *P. nephtys* and the earlier description of *P. paradoxa* (Chatton, 1911) is the presence of striated radiations on the mature spore tetrads, we agree with Feist et al. (2009) that *Paramyxoides* is a junior synonym of *Paramyxa* and now recognise two species within the latter (original) genus: *P. nephtys* (this study) and *P. paradoxa*, which remains to be sequenced, but we suggest is likely to group with the '*Paramyxa*' sequences from bivalves, or clades PARAM-1/ -2 (Fig. 1A). A morphological description of the *Paramyxa* sp. sequences found in bivalve tissue (Tamar) will be published separately.

In the absence of morphological data we refrain from assigning PARAM-1 or -2 to *Paramyxa*, the characterised genus to which these lineages are most closely and strongly related. PARAM-1 may turn out to be *P. paradoxa*; however, the relationship between genotypic and phenotypic distance is not straightforward, and decisions about taxonomic boundaries should be made taking into account both kinds of evidence (Boenigk et al., 2012). A good illustration of this is that three morphologically different *Marteilia* spp., *M. refringens*, *M. cochillia* and *M. octospora*, are extremely similar at the 18S level, yet *Marteilia* has been considered morphologically indistinguishable from *Marteilioides* and *Eomarteilia* spp., all three genera being very different from each other in terms of 18S sequence similarity (Fig. 1A). *Marteilia* and the morphologically similar but even more basally branching *Eomarteilia* may reflect the ancestral state for the whole order.

535 *Paramyxa* and *Marteilioides* form a robustly supported clade with PARAM-1 and -2,
536 separated by maximal bootstrap support from other paramyxid genera. Therefore the suppression
537 of *Marteilioides* as recommended by Feist et al. (2009) and assumed by Carrasco et al. (2015) is
538 invalid. However, *Marteilioides* remains a poorly sampled genus with only one described species.
539 Sequence data are required to confirm whether '*Marteilioides*' *branchialis* groups with *M.*
540 *chungmuensis* or in the *Paramarteilia* clade, as suggested by Feist et al. (2009). *Paramarteilia*
541 itself is sister to *Paramyxa* + *Marteilioides* with robust support, and is therefore not directly related
542 to *Marteilia*.

543 We provide molecular (ISH) confirmation of *Paramarteilia* infection of edible and spider
544 crabs. The description of *P. canceri* by Feist et al. (2009) is concordant with the parasite cells
545 observed in both edible and spider crabs in this study (two bi-cellular spores). Our ISH results
546 confirm that the parasite infecting both crab species belongs to *Paramarteilia*, and may be the
547 same species of *Paramarteilia*. However, material was not available to generate an 18S sequence
548 for *P. canceri* or the *Paramarteilia* from *M. squinado*, so although the parasite of the latter was
549 morphologically indistinguishable from that in *C. pagurus* (*P. canceri*) we cannot yet confirm it is *P.*
550 *canceri* until sequence data are available. Sequence data are required from both crab-infecting
551 parasites also to confirm whether their sequence is identical to *P. orchestiae*. The role of
552 paramyxids in crab disease has received almost no attention, and will almost certainly reward
553 future research investment.

554 Itoh et al. (2014) and Carrasco et al. (2015) tacitly concur with the Feist et al. (2009) report
555 of suppression of *Marteilioides*, and include '*Marteilia*' *granula* within the genus *Marteilia*. In the
556 Neighbour-Joining (NJ) phylogeny of Itoh et al. (2014) (Fig. 7A) '*M. granula* is separated from
557 other *Marteilia* spp by *Marteilioides*, and in an ML analysis of the same taxon sample in the same
558 paper (Fig. 7B) and Carrasco et al. (2015; Fig. 3A), '*M. granula* forms a weakly supported clade
559 with other *Marteilia* spp. However, our phylogenetic analyses, which additionally include *Paramyxa*
560 (sister to *Marteilioides*) and *Paramarteilia*, show that including '*M. granula* within *Marteilia* would
561 produce a paraphyletic *Marteilia*, as '*M. granula* branches separately with strong support from the
562 clade comprising *M. refringens*, *M. cochillia* and *M. sydneyi* in Fig. 1A. Therefore we suggest that

563 '*M*. *granula* should be assigned to a new genus (*Eomarteilia*). Similarly, *Marteilioides* cannot be
564 considered congeneric with *Marteilia* (see Feist et al., 2009; Carrasco et al., 2015) without
565 incurring paraphyly of *Marteilia* and requiring that all other paramyxid genera be subsumed into
566 *Marteilia*. It would be clearly undesirable and nonsensical to represent such a biological diversity of
567 paramyxids as that illustrated in this study by a single genus.

568 It is worth noting that if an incomplete sampling of paramyxid diversity is used for
569 phylogenetic analyses, the illusion may be given that *Eomarteilia* and *Marteilia* form a holophyletic
570 clade (Fig. 1B); however this has very weak support compared with the more complete taxon
571 sampling in Fig. 1A, emphasising the general desirability of comprehensive taxon sampling as a
572 basis for the best possible phylogenetic interpretation. All of these phylogenetic relationships
573 should be tested further by including more genes in phylogenetic analyses, when available.

574 Using paramyxid group-specific primers to screen e- and organismal DNA samples for
575 'hidden' diversity is a powerful technique, revealing novel lineages and suggesting new
576 ecological/host associations for verification (Moreira and Lopez-Garcia, 2002; Bass et al., 2015).
577 We detected a *Paramarteilia* sequence in *C. pagurus* sampled from the English Channel coast and
578 incubated in sterile ASW, the histopathology and TEM of which was consistent with the description
579 of *P. canceri* from the same site by Feist et al. (2009). We then used ISH to confirm the presence
580 of this sequence type in both *C. pagurus* and *M. squinado*. This approach can be used to
581 determine whether, for example, our detection of *Paramyxa* in *Mytilus* tissue represents actual
582 infection or is more likely trophic passage or accumulated by filtration, and to investigate different
583 lifecycle stages and alternative hosts (Bass et al., 2015). In fact, some aspects of our eDNA results
584 for different paramyxid lineages may be explained by life history traits, for example sites of
585 infection and modes of transmission. *Paramarteilia orchestiae* and *Marteilioides* spp are vertically
586 transmitted (via host eggs); neither has been detected by eDNA methods on environmental
587 samples. On the other hand *P. nephtys*, *M. refringens*, *M. sydneyi*, *M. cochillia* and *Eomarteilia*
588 *granula* infect host digestive gland/gut tissue and are likely or known to be released from these
589 tissues into the environment – the first two taxa in this list have been detected in environmental
590 and well as organismal samples, and *M. refringens* has been shown to use planktonic crustacean

591 hosts. The low current sampling levels preclude any generalisations being made from these
592 observations, but future results and experimental design should take them into account.

593 eDNA methods are also very useful for detecting true geographical range of lineages,
594 which as noted above, is of particular interest in paramyxean studies. However, negative eDNA
595 results are not conclusive, and the fact that we did not detect *M. cochillia*, *M. sydneyi*, *E. granula* or
596 either *M. chungmuensis* 18S types in our screens from the UK, Borneo, South Africa or Florida do
597 not signify that these taxa are not more widely distributed than implied in Fig. 3. Further probing of
598 organismal and environmental samples from more areas is required, ideally using even more
599 tightly lineage-specific primers than we used in this study to maximise detection sensitivity and
600 specificity. In general, the group-specific eDNA results from paramyxeans contrasts with that
601 earlier obtained for Haplosporida (Hartikainen et al., 2014a), which revealed higher levels of
602 diversity and higher detection frequencies from environmental samples. The paramyxid results are
603 perhaps more akin to those for mikrocytids (Hartikainen et al., 2014b), which were more limited in
604 environmental samples and detection was often more strongly associated with potential hosts or
605 particular environmental compartments. It may be that paramyxids and mikrocytids are more tightly
606 host-associated than haplosporidans and/or they less frequently infect small planktonic animals
607 (and are consequently less likely to be captured by sampling of planktonic habitats) either as
608 primary or alternative hosts.

609 A further complexity in the use of eDNA and host screening methods for the detection of
610 novel and existing paramyxid diversity is a lack of understanding surrounding the role played by
611 environmental conditions in the prevalence and pathogenicity of most lineages. Microscopy-based
612 studies suggest the minimum water temperature of 18-20°C necessary for gonad maturation in *C.*
613 *gigas* is similar to that required for development of *M. chungmuensis* within host oocytes (Kang et
614 al., 2000; Ngo et al., 2013), and follow-up studies utilising both microscopy and PCR seem to
615 confirm this (Tun et al., 2008). Temperature has also been shown to be a key parameter in the life
616 cycle of *M. refringens* (Berthe et al., 1998) and *M. sydneyi* (Rubio et al., 2013). Therefore it is
617 essential to account for environmental conditions and seasonality in eDNA (and other) sampling
618 efforts. The 'window of infection' (i.e. the timespan over which infection is observable in the bivalve

host) also varies between taxa, and within the same taxa across several years. Onset of the infection window of *M. sydneyi* in *S. glomerata* often follows a rapid decrease in water salinity, and can last between 8 and 18 weeks, though the exact environmental conditions determining this window remain unknown (Rubio et al., 2013).

Our attempt to collate distribution and host association data from all recorded paramyxid species to date has, in tandem with the phylogenetic analysis, suggested a strong biogeographical structuring of paramyxid lineages. The closely related *M. refringens*, *M. cochillia* and *M. octospora* have only been recorded in Europe (the latter two only from Spain to date), whereas the more distantly related *M. sydneyi* has been reported only from Australia (both east and west coasts when non-sequenced records are considered), suggesting that geographically distant lineages are likely to be more distantly related. Further highly distinct lineages were sampled in Malaysian Borneo (shrimp larvae hatchery tank), South Korea (*Marteilia* sp. MC), and the Yellow Sea and East Sea coasts of China (paramyxid ex. *Mytlius*), where both the native mussel *Perna viridis* and the non-native *Mytilus edulis* were infected (Fig. 1B). The infection of *M. edulis* at these sites suggests that other populations of this mussel are potentially threatened by 'new' parasite lineages in other regions of the world, at least where ecological conditions permit. *Marteilioides* reports confirmed by sequence data are restricted to South Korea and Japan, with non-sequenced records from Australia. *Paramyxa* (including *Paramyxoides*) has only been reported from Europe (Denmark, UK, France), and *Paramarteilia* only from the UK and (the original unsequenced type material) from northern France.

Patchy and low sampling effort explains at least some of these geographical observations. *Paramyxa* and *Paramarteilia* have only been studied at a small number of sites by very few researchers. An informed and more widely distributed sampling effort (including eDNA methods; Bass et al., 2015 and this study), is necessary and very likely to prove broader distributions for many paramyxid lineages. However, lineages that have been more intensively studied, for example *M. refringens* and *M. sydneyi*, are more likely to have been found without and within Europe, respectively, if they were present. Even so, there are many discoveries to be made, as demonstrated by the very recent findings of *M. refringens* in the oysters *C. gigas* and *Crassostrea*

corteziensis in Mexico (Grijalva-Chon et al., 2015), and *M. octospora* in Spain (Ruiz et al., 2016). Limited 18S data show *M. octospora* to be very closely related to *M. cochillia*, and while 18S data are not available for the Mexican *M. refringens*, their IGS rDNA sequences are only 0.3-2.2% dissimilar to *M. refringens* from a Spanish clam, *Chamelea gallina* (**AM292652**); with such similar sequences in the highly variable IGS region their 18S sequences are likely to be extremely similar or identical to the *M. refringens* sequences represented in Fig. 1.

There exist many potential paramyxid lineages for which inadequate microscopic or molecular data are available to establish their taxonomic affinity. For example, a *Marteilia* sp. has been observed at low prevalence (2% of 140 sampled) infecting the digestive gland of cultured rock oysters *Saccostrea forskali* in Chonburi Province, Thailand (Taveekijakarn et al., 2008). Similarly, unidentified *Marteilioides* sp. and *Marteilia* sp. have been observed (in the oocytes and digestive gland, respectively) of the Manila clam, *R. philippinarum,* in Japan (Itoh et al., 2005). These parasites are present at very low prevalence and have yet to be linked to any significant pathogenicity in their hosts. A further undescribed potential *Marteilia* sp. was also observed infecting the digestive diverticulum of the calico scallop, *Argopecten gibbus,* off Cape Canaveral, Florida, USA in 1988-1989 (Moyer et al., 1993). This parasite resulted in the rapid decimation of the scallop population, but further attempts to collect material for ultrastructural analysis were unsuccessful. Although the taxonomic affiliations of this parasite remain unknown, a recent survey of calico scallop abundance in this area and the Gulf of Mexico shelf suggests the parasite is still prevalent at both sites, with late stage infections common (Geiger et al., 2015). Further investigation is needed to ascertain the identity of the parasite and its effect upon the commercial viability of the scallop populations in these areas.

These reports prove that even if some paramyxids are much more geographically localised than is true for many protists (Bass and Boenigk, 2011), the diversity and distribution of the order as a whole is greater than shown in Fig. 1 and Table 1. A more complete understanding of the distributions of these pathogens is increasingly important as new lineages (often with economically significant effects) are being discovered, and known paramyxids are being found in new hosts and/or locations (e.g. *M. chungmuensis*, Itoh et al., 2004; *P. canceri*, Feist et al., 2009; *M.*

675 *refringens*, Arzul et al., 2014; *M. sydneyi*, Adlard and Nolan, 2015). Human-mediated transport of
676 these pathogens around the world could introduce them into areas in which they could become
677 active if suitable environmental and/or lifecycle conditions arise. This also applies to other
678 understudied parasites with similar apparently restricted geographical ranges, even though more
679 intensive sampling facilitated by modern molecular screening methods also shows these to be
680 more widespread and diverse than previously thought (e.g. haplosporidians and mikrocytids;
681 Harikainen et al., 2014a,b).

682 Our new data and literature survey indicate that paramyxid lineages are being discovered
683 on a regular basis, and perhaps with increasing frequency as knowledge of the group and methods
684 to detect them improve. Initiatives such as the new Paramyxean Working Group
685 (http://paramyxeanworkingroup.org/) demonstrate that the international community has become
686 more aware and interested in paramyxid research and there is a requirement and appetite for
687 developing this neglected field. However, it remains difficult to estimate the potential emergence
688 and impact of paramyxids on animal hosts of human concern, and their even more hidden roles as
689 parasites in diverse and interacting marine ecosystems because so little is known of their true
690 diversity and distribution. In this study we provide some molecular tools for targeted detection of
691 the full range of known paramyxids in environmental and organismal matrices. Our phylogenetic
692 analysis provides an evolutionary context for understanding how the group has evolved in terms of
693 morphology, distribution and lifecycle. The recent demonstrations that paramyxids occur in a wider
694 range of hosts and environments than previously thought encourage us to study them and their
695 pathogenesis in an ecological context, in addition to their individual effects on key host taxa. There
696 is a need for greater genomic sampling of paramyxids, both to increase the power of phylogenetic
697 (multi-gene) analyses of the group, and to better understand the nature of host-parasite
698 interactions.

699

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Figure and Table legends

Fig. 1. 18S rDNA phylogenies of paramyxids and relevant GenBank accession numbers. (A) Phylogeny of paramyxids generated using Bayesian Inference. Values on nodes indicate Bayesian Posterior Probabilities (BPP); filled circles on nodes indicate maximal support (BPP = 1.0). Coloured/shaded squares indicate lineages detected in environmental samples, and circles those detected in host-associated (tissue or incubation) samples. Triangles indicate lineages for which only environmental sequences exist. Values in shapes indicate prevalence (number of positive samples/number of samples screened). Where no value is present, the lineage was not detected in this study. (B) More complete phylogeny of *Marteilia* and *Eomarteilia* with *Paramarteilia orchestiae* as an outgroup, generated using Bayesian Inference, showing Maximum Likelihood bootstrap and BPP values.

Fig. 2. Transmission Electron Micrographs (TEMs) and supporting light micrographs (H&E), in-situ hybridisation (ISH) of developing and fully matured morphological *Paramyxa nephtys* cells. (A) Developing *P. nephtys* cells detailing the third to sixth nuclei (N3 - N6) of a developing *P. nephtys* spore. TEM. Scale bar = 2 µm. (B**)** Longitudinal view of two mature *P. nephtys* spores, encased within spore sacs possessing striated projections (SP). TEM. Scale bar = 2 µm. (C**)** Top-down view of *P. nephtys* spore sac terminal striated projection (SP) showing the projections form a single structure. TEM. Scale bar = 2 µm. (D**)** Longitudinal view of two mature *P. nephtys* spores. H&E Stain. Scale bar = 25 µm. (E**)** Longitudinal view of two mature *P. nephtys* spores. ISH. Scale bar = 25 µm. (F) Transverse section of four coupled *P. nephtys* spores demonstrating the four tetracellular spore arrangement (C1 - C4). TEM. Scale bar = 2 µm. (G**)** Transverse section of *P. nephtys* spores. H&E Stain. Scale bar = 25 µm. (H**)** Transverse section of *P. nephtys* spores. Inset ISH. Scale bar = 25 µm.

Fig. 3. World map showing distribution of paramyxid clades. Coloured/shaded squares indicate detection of a lineage in environmental samples, and circles those detected in confirmed hosts or

1049 host-associated samples. Triangles indicate lineages for which only environmental sequences
1050 exist. Shapes labelled with asterisks represent lineages detected in this study. The inset shows the
1051 distribution of paramyxids within Europe.
1052
1053 **Fig. 4.** Light and electron micrographs depicting *Paramarteilia orchestiae* cells in *Orchestia*
1054 *gammarellus* tissue. (A) Multiple groups of *P. orchestiae* cells (arrows) within connective tissues
1055 surrounding ganglion of nerve cord. H&E. Scale bar = 10 µm. (B**)** Intracellular *P. orchestiae* in
1056 oocytes (black arrow) and the connective tissue capsule surrounding the oocytes (white arrow).
1057 H&E. Scale bar = 10 µm. **(**C**).** Cluster of *P. orchestiae* cells at different stages of development (*)
1058 within the connective tissue of the leg. H&E. Scale bar = 10 µm. Inset: in situ hybridisation (ISH)
1059 labelling of the *P. orchestiae* cells within the connective tissue of the leg (arrow). Scale bar = 10
1060 µm. (D**)** Individual *P. orchestiae* cell showing the cell-within-cell arrangement of the parasite.
1061 Primary cell (*) contains secondary cells (C2). H&E. Scale bar = 10 µm. Inset: Transmission
1062 Electron Micrograph of *P. orchestiae* cell demonstrating primary cell (C1) and a secondary cell
1063 (C2) containing nucleus (N2). Scale bar = 2 µm.
1064
1065 **Fig. 5.** *Paramarteilia canceri* infecting edible crab (*Cancer pagurus*) tissues and *Paramarteilia* sp.
1066 infecting spider crab (*Maja squinado*) tissues. (A**)** *Paramarteilia canceri* infecting the connective
1067 tissue cells (arrows) within the haemal spaces of the hepatopancreas. Edible crab. H&E. Scale bar
1068 = 25 µm. Inset: in situ hybridisation (ISH) labelling the *P. canceri* cells within the connective tissues
1069 in the hepatopancreas. Edible crab. Scale bar = 25 µm. (B**)** *Paramarteilia sp.* within the
1070 hepatopancreatic tubule epithelial cells (arrows). Spider crab. H&E. Scale bar = 25 µm. Inset: ISH
1071 labelling of *Paramarteilia* sp. (arrow) at the base of the hepatopancreatic tubule epithelium (*).
1072 Spider crab. Scale bar = 10 µm. (C**)** Transmission electron micrograph (TEM) of *P. canceri*
1073 infecting the connective tissue cells within the heart detailing the cell-within-cell arrangement of the
1074 parasite. Primary cell (C1) contains secondary (C2) and tertiary (C3) cells, electron dense
1075 haplosporosomes (black arrows) and multivesicular bodies (white arrows). Edible crab. Scale bar =
1076 1 µm. (D**)** *Paramarteilia canceri* infecting the connective tissue cells (arrow) within the heart.
1077 Edible crab. H&E. Scale bar = 10 µm. Inset: ISH labelling of *P. canceri* infecting the connective

tissue cells (arrow) within the heart. Edible crab. Scale bar = 10 µm. (E**)** Intracellular *P. canceri* infecting the ovary (black arrow) and the connective tissues of the ovary (white arrow). H&E. Edible crab. Scale bar = 10 µm. Inset: ISH labelling of *P. canceri* infecting the oocytes themselves (black arrow) and the connective tissues of the ovary (white arrow). Edible crab. Scale bar = 10 µm. (F**)** TEM of *P. canceri* within a vitellogenic oocyte. The oocyte is almost completely filled with yolk globules (white arrow) the multicellular *P. canceri* parasites (black arrow) are clearly visible within the oocyte. Edible crab. Scale bar = 2 µm. (G**)** *Paramarteilia canceri* infecting the testicular follicles (arrow). Edible crab. H&E. Scale bar = 10 µm. Inset: ISH labelling of *P.canceri* infecting the testicular follicles. Edible crab. Scale bar = 10 µm. (H**)** TEM of *P. canceri* within the testicular follicles. Parasite appears to be attached to the epithelium of the testicular follicle (arrow). Edible crab. Scale bar = 2 µm.

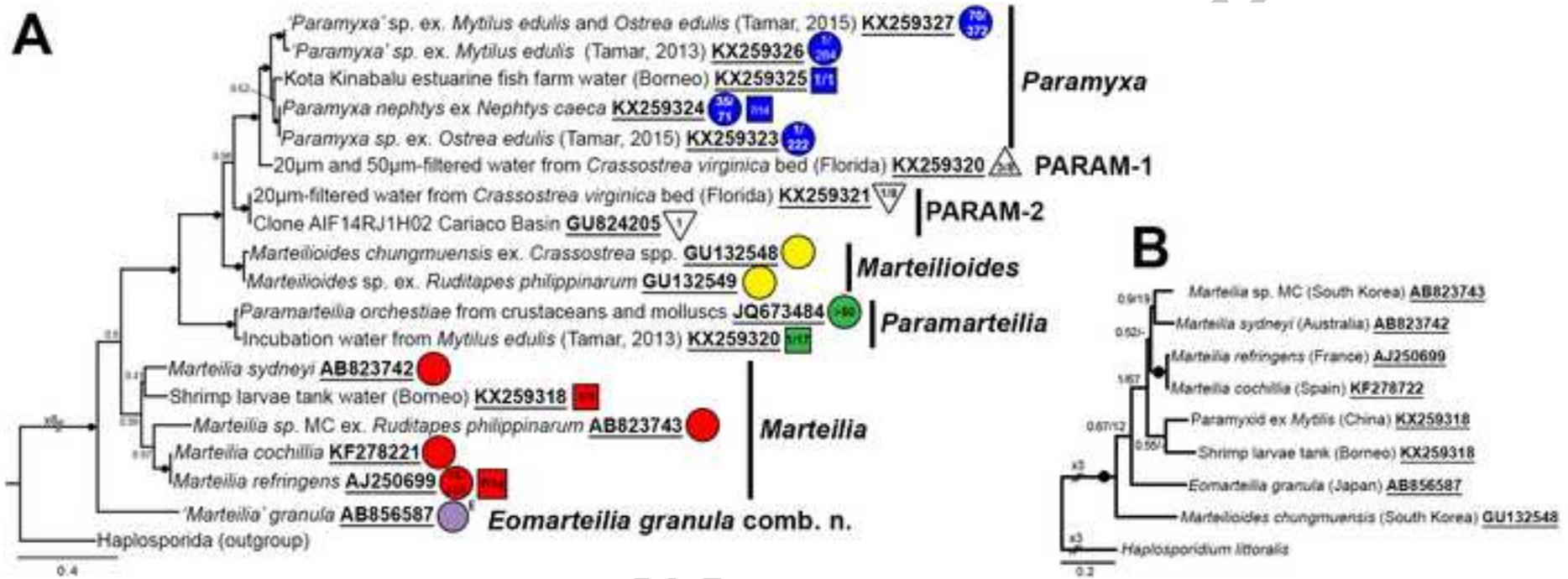
Table 1. Review of paramyxids known prior to this study for which sequence data and/or microscopy evidence unambiguously identifies the lineage identity to at least genus level. Where shown in bold, GenBank accession numbers are 18S sequences used in our phylogenetic analyses (Fig. 1). Where not in bold, sequences are either identical duplicates, or a different region (Internal Transcribed Spacer 1 (ITS1), Intergenic Spacer (IGS) rDNA) from the same lineage, or in a few cases unambiguously identified (e.g. confirmed by subsequent sequencing). Bold geographical locations/citations represent findings confirmed by sequence data. Underlined entries indicate data generated in this study.

Parasite genus	Species	Host	Geographical Location	Citation	GenBank Accession
Paramyxa	*Paramyxa nephtys*	*Nephtys caeca*	Øresund, Denmark	Larsson and Koie, 2005	**KX259324 (partial 18S)**
			Portland, UK		
		Environmental (water)	**Tamar estuary, UK**	**Ward et al., 2016**	
	Paramyxa paradoxa	*Poecilochaetus serpens*	Banyuls-sur-Mer, France	Chatton 1911	
	Paramyxa sp.	*Mytilus edulis*	**Tamar estuary, UK**	**Ward et al., 2016**	**KX259326 (partial 18S)**
	'*Paramyxa*' sp.	Environmental (water)	**Malaysian Borneo**	**Ward et al., 2016**	**KX259325 (partial 18S)**
	'*Paramyxa*' sp.	*Ostrea edulis*	**Tamar estuary, UK**	**Ward et al., 2016**	**KX259323 (partial 18S)**
	'*Paramyxa*' sp.	*Mytilus edulis*	**Tamar estuary, UK**	**Ward et al., 2016**	**KN259327 (partial 18S)**
		Ostrea edulis			
Paramarteilia	*Paramarteilia canceri*	Cancer pagurus	Guernsey, UK	Feist et al., 2009	
			Weymouth and Portland, UK		
			South Kimmeridge Bay, UK		
	Paramarteilia orchestiae	*Orchestia gammarellus*	Taulé-Penzé, France	Ginsburger-Vogel et al., 1976	
			Dale, UK	**Ward et al., 2016**	
			Weymouth, UK		
		Echinogammarus marinus	**Inverkeithing, UK**	**Short et al., 2012a**	**JQ673484 (partial 18S)**
			Portsmouth, UK		
		Environmental (mollusc and crustacean incubations)	**Weymouth and Tamar estuary, UK**	**Ward et al., 2016**	
	Paramarteilia sp.	Environmental (*Mytilus edulis* incubation)	**Tamar estuary, UK**	**Ward et al., 2016**	**KX259320 (partial 18S)**
Marteilioides	*Marteilioides branchialis*	*Saccostrea glomerata*	New South Wales, Australia	Anderson and Lester, 1992	
	Marteilioides chungmuensis	*Crassostrea ariakensis*	**Okayama, Japan**	**Itoh et al., 2003**	AB110795 (18S)
			Seomijn River, South Korea	**Yanin et al., 2013**	**GU132548(18S)**

		Crassostrea gigas	**Tongyoung, South Korea**	**Yanin et al., 2013**	GU132457(18S)
		Crassostrea nippona	**Japan**	**Itoh et al., 2004**	
	Marteilioides sp.	*Ruditapes philippinarum*	Hadong/Namhae, South Korea	Lee et al., 2001	
			Tongyoung, South Korea	**Yanin et al., 2013**	**GU132549 (18S)**
Marteilia	*Marteilia christenseni*	*Scrobicularia piperata*	Marennes-Oléron, France	Comps et al., 1983	
	Marteilia cochillia	*Cerastoderma edule*	**Ebro Delta, Catalonia, Spain**	**Carrasco et al., 2013**	KF314809 (IGS)
			Ria de Arousa, Galicia, Spain	**Villalba et al., 2014**	**KF278722 (18S)**
	Marteilia lengehi	*Saccostrea cucullata*	Bandar-Lengeh, Iran	Comps, 1976	
			Qeshm, Iran		
	Marteilia octospora	*Solen marginatus*	**Ría de Arousa, Galicia, Spain**	**Ruiz et al., 2016**	KU641125 (partial 18S) KU641126 (ITS1)
	Marteilia refringens	*Ostrea edulis*	Bassin d'Arcachon, France	Grizel et al., 1974	
			Île d'Oléron, France	**Berthe et al., 2000**	**AJ250699 (18S)**
			Corsica, France	Pichot, 2002	
		Mytilus edulis	Brittany, France	Comps et al., 1975	
			Brittany, France	**Berthe et al., 2000**	**AJ250699 (18S)**
			Tamar estuary, UK	**Bignell et al., 2011**	
		Mytilus galloprovincialis	Venice, Italy	Comps et al., 1982	
			Galicia, Spain	Villalba et al., 1993	
			Istrian Peninsula, Croatia	Zrncic et al., 2001	
			Ebro Delta, Catalonia, Spain	**Carrasco et al., 2008**	
				Carella et al., 2010	AB534169-70 (ITS1)
			Campania, Italy	**Arzul et al., 2014**	
			Corsica, France	**Gombac et al., 2014**	JQ898012-14 (ITS1)
			Slovene Adriatic Sea, Slovenia		
		Ostrea stentina	**Monastir Bay, Tunisia**	**Elgharsalli et al., 2013**	JX119018-22 (IGS)
		Chamelea gallina	**Bay of Palma, Mallorca, Spain**	**Lopes-Flores et al., 2008a**	AM292652 (IGS)
		Solen marginatus	**Huelva, Spain**	**Lopes-Flores et al., 2008b**	AM748037-41 (IGS)
		Ruditapes decussatus	**Thau Lagoon, France**	**Boyer et al., 2013**	
		Xenostrobus securis	Galicia, Spain	Pascual et al., 2010	
		Paracartia grani	**Marennes-Oléron, France**	**Audemard et al., 2001**	
		Paracartia latisetosa	**Diana Lagoon, Corsica, France**	**Arzul et al., 2014**	

		(Crassostrea gigas) *(Crassostrea corteziensis)*	**Sonora, Gulf of California, Mexico**	**Grijalva-Chon et al., 2015**	JQ066723-4 (IGS) JQ066725-6 (IGS)
		(Acartia clausi) *(Acartia discaudata)* *(Acartia italica)* *(Euterpina acutifrons)* *(Oithona* sp.)	Ebro Delta, Catalonia, Spain	Carrasco et al., 2007b	AM504139 (IGS) AM504140 (IGS) AM504141 (IGS) AM504137 (IGS) AM504138, AM504145 (IGS)
		Environmental (water, sediment)	**Tamar estuary, UK**	**Ward et al., 2016**	
	Marteilia sydneyi	*Saccostrea glomerata*	Queensland, Australia	Perkins and Wolf, 1976	AF159248 (ITS1)
			Queensland, Australia	**Kleeman and Adlard, 2000**	
			Pimpama River, Queensland, Australia	**Itoh et al., 2014**	**AB823742 (18S)**
		Nephtys australiensis	**Hawkesbury River, NSW, Australia**	**Adlard and Nolan, 2015**	
	Marteilia sp. MC	*Ruditapes philippinarum*	**South Korea**	**Kang et al., (unpublished)**	**AB823743 (18S)**
	Marteilia sp.	*Mytilus edulis*	**China**	**Wang et al., 2012**	KX259319
	Marteilia sp.	Environmental (*Penaeus* hatchery tank water)	**Malaysian Borneo**	**Ward et al., 2016**	**KX259318 (partial 18S)**
Eomarteilia	*Eomarteilia granula*	*Ruditapes philippinarum*	**Kanagawa, Japan**	**Itoh et al., 2014**	**AB856587 (18S)**
	PARAM-1	Environmental (water)	**Gulf coast, Florida, USA**	**Ward et al., 2016**	**KZ259322 (Partial 18S)**
	PARAM-2	Environmental (water)	**Cariaco Basin, Venezuela**	**Edgcomb et al., 2011**	**GU824205 (18S)**
		Environmental (water)	**Gulf coast, Florida, USA**	**Ward et al., 2016**	**KX259321 (Partial 18S)**

Figure 1 colour



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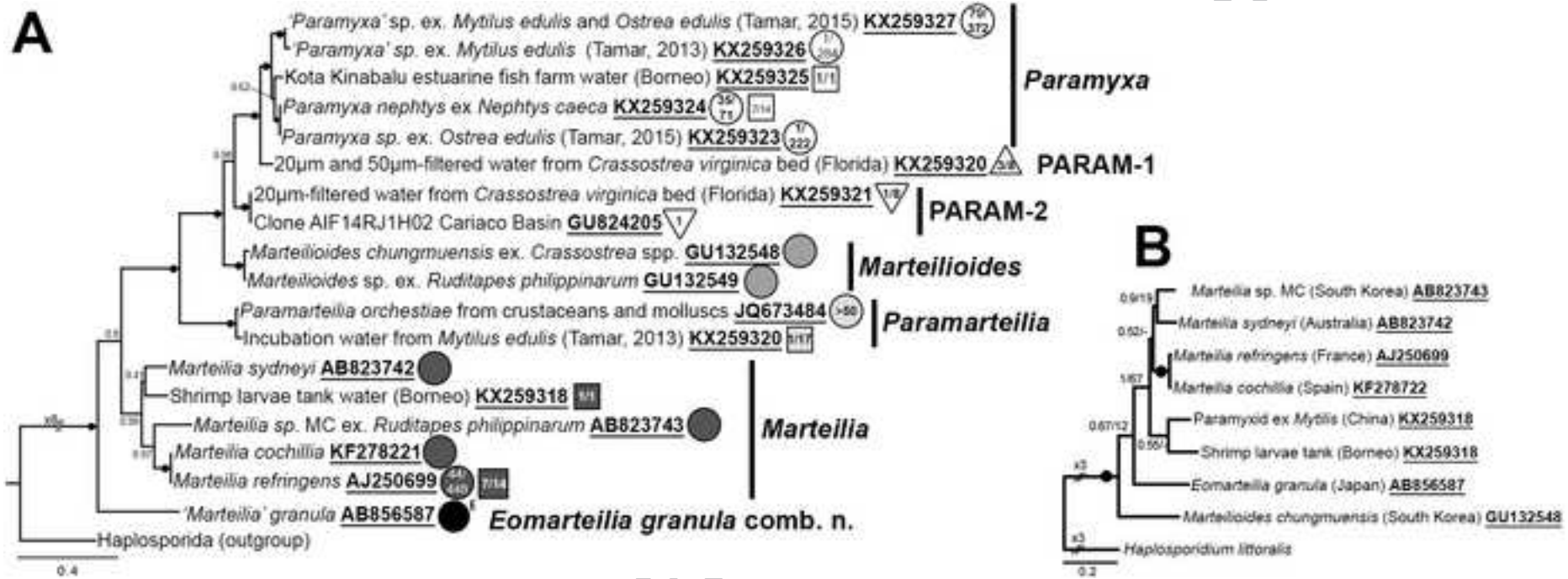

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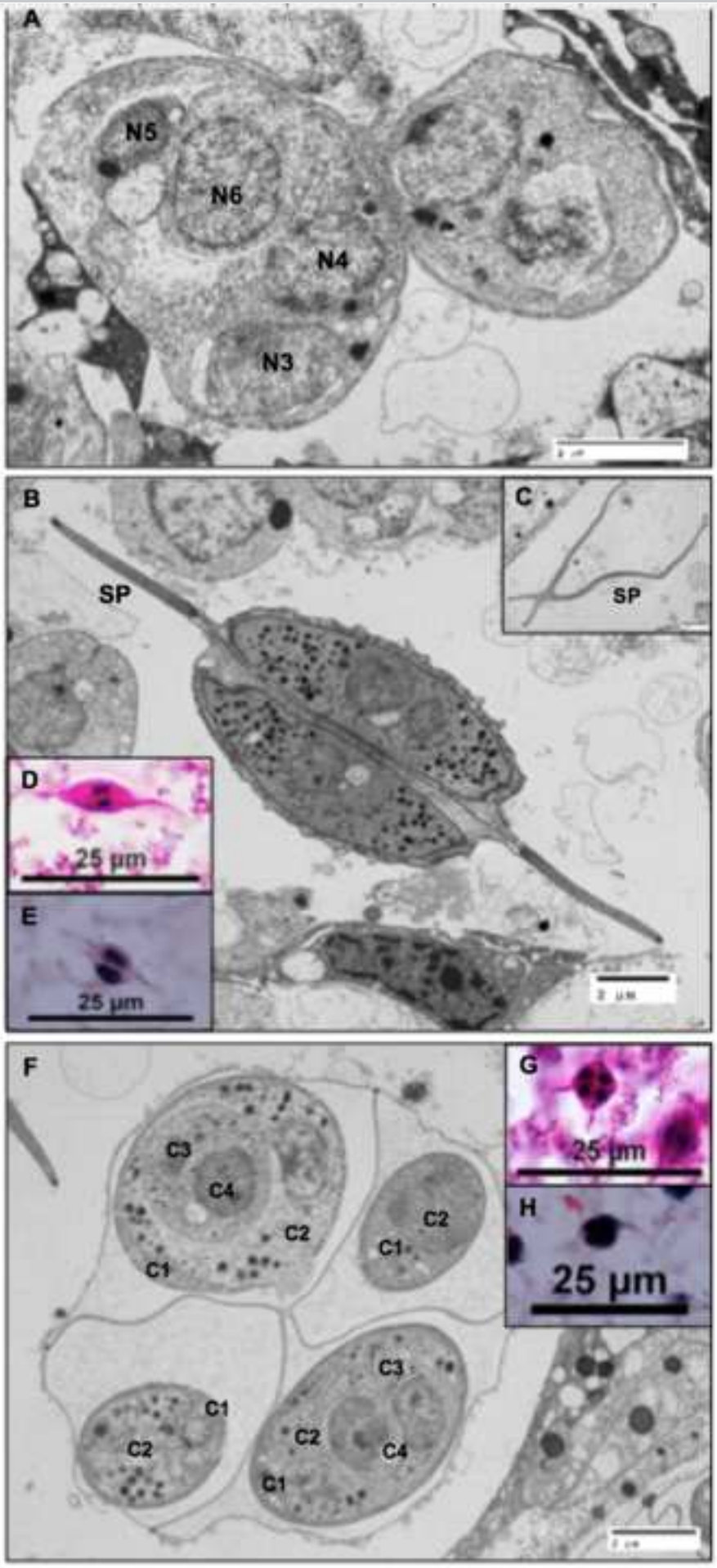

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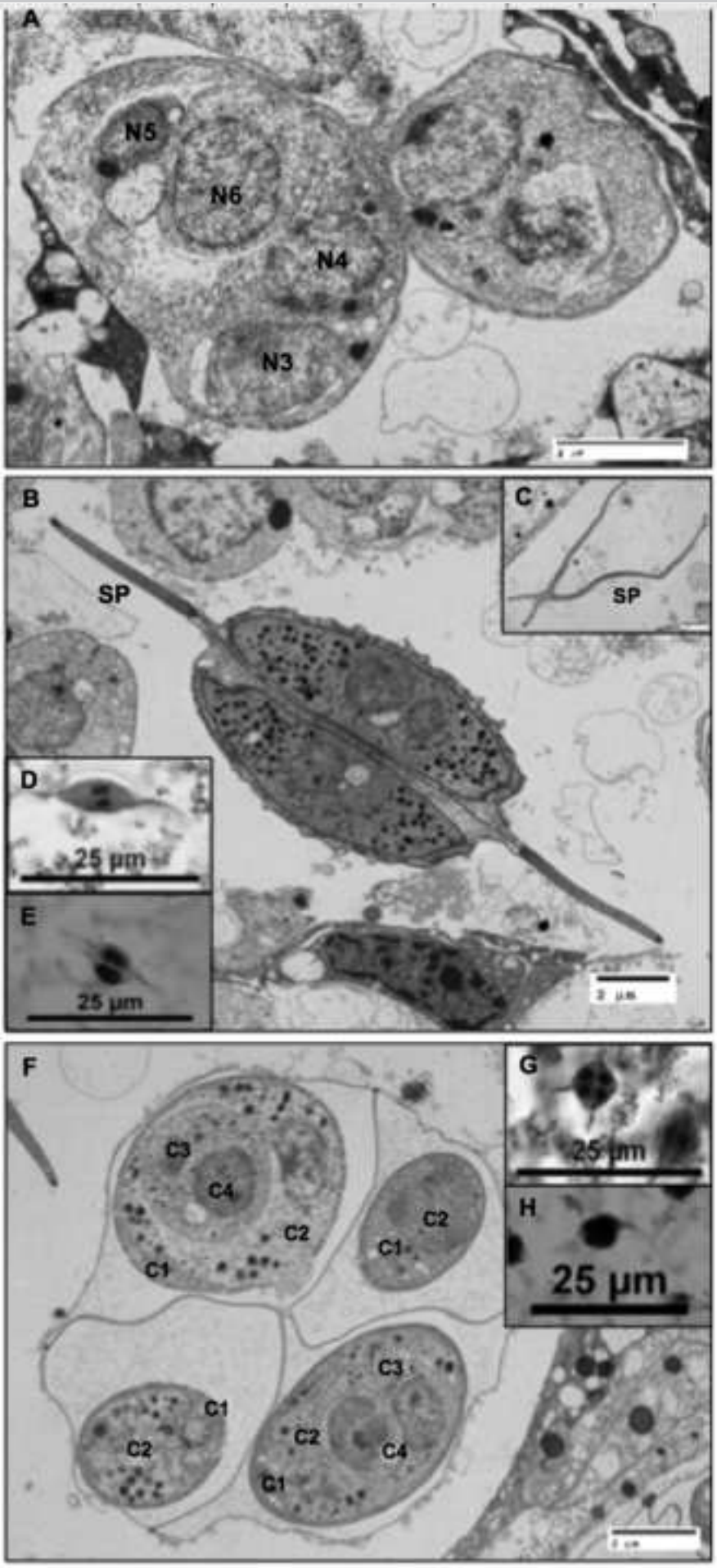

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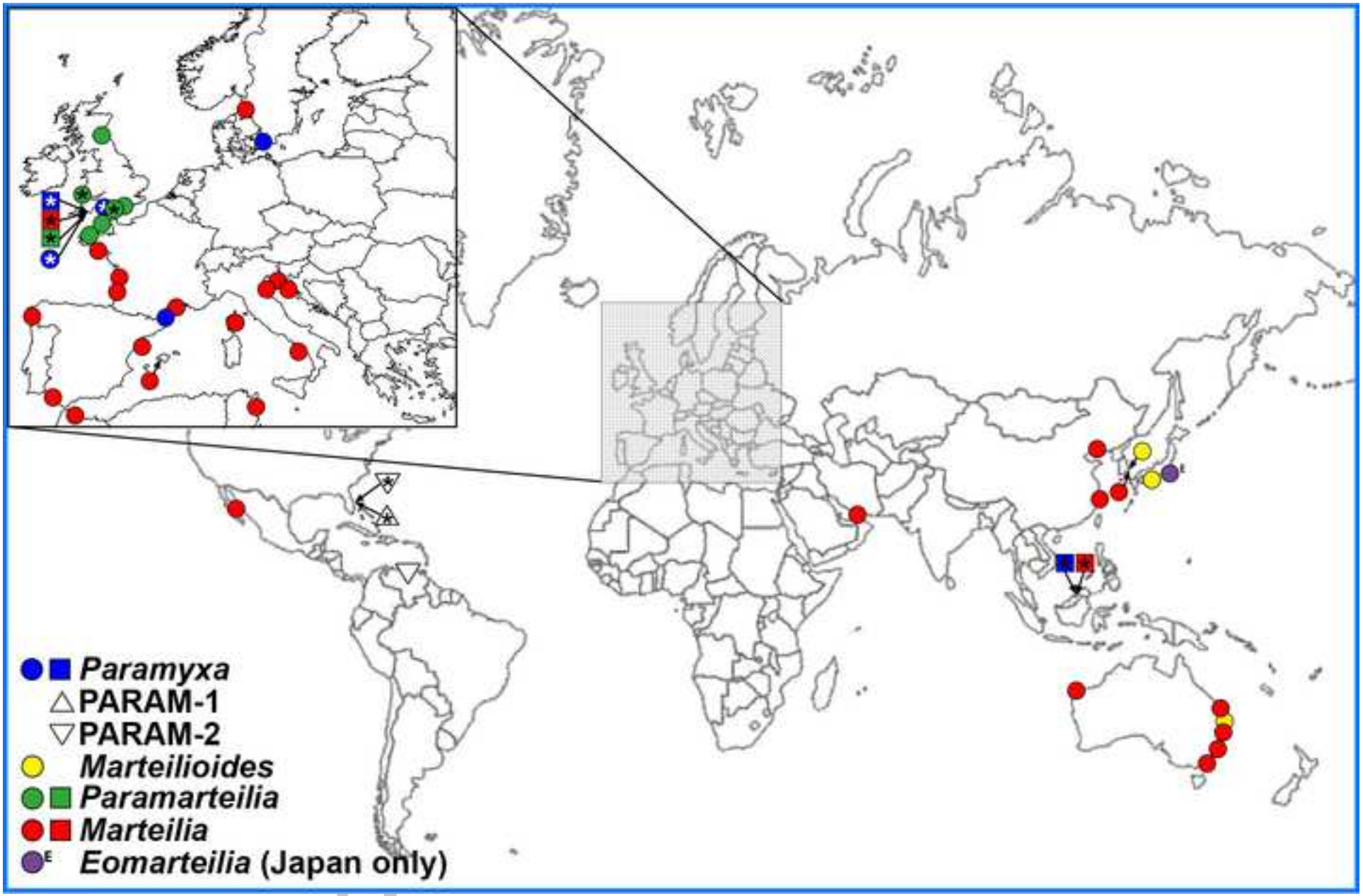
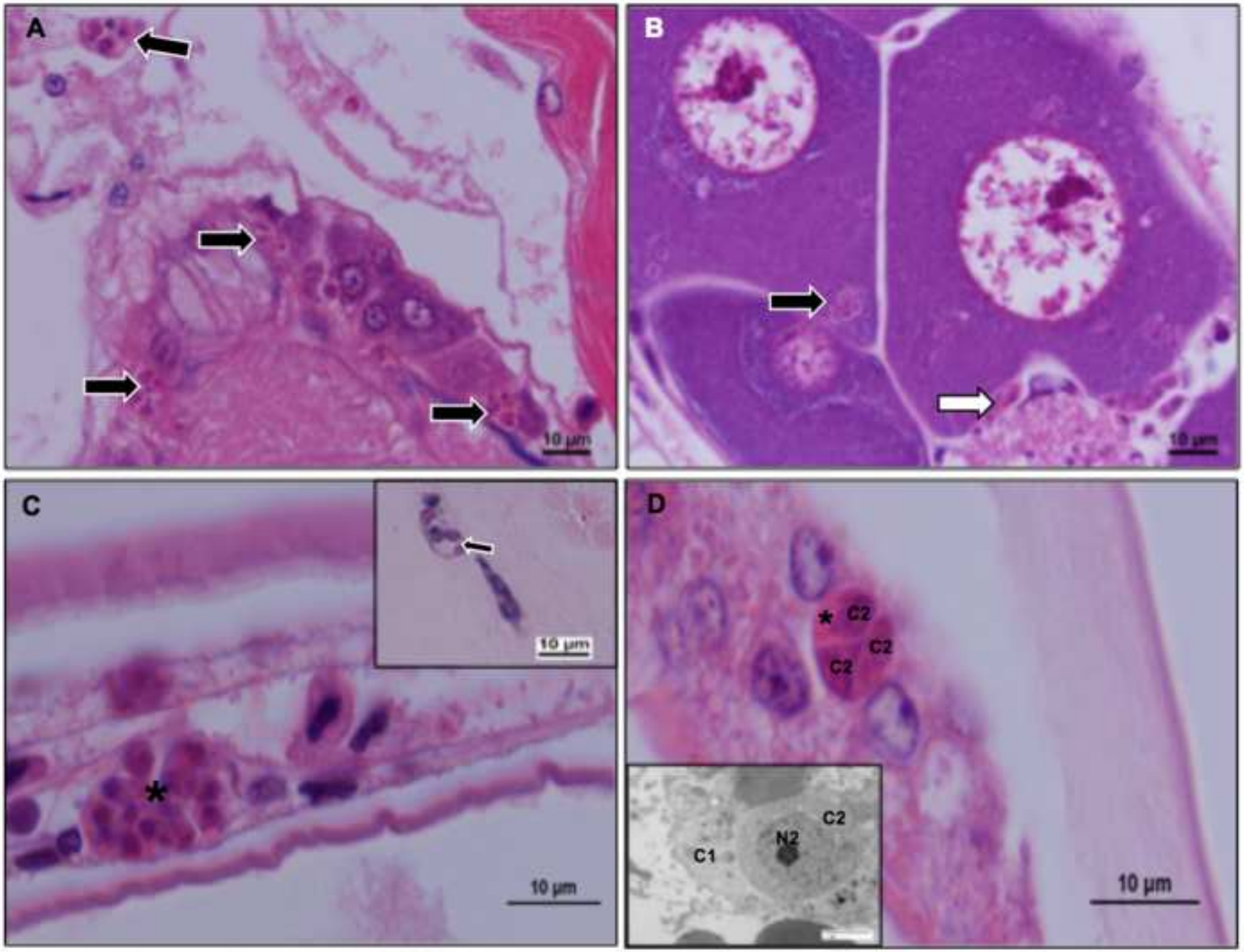


Figure 3 black and white

Figure 4 colour



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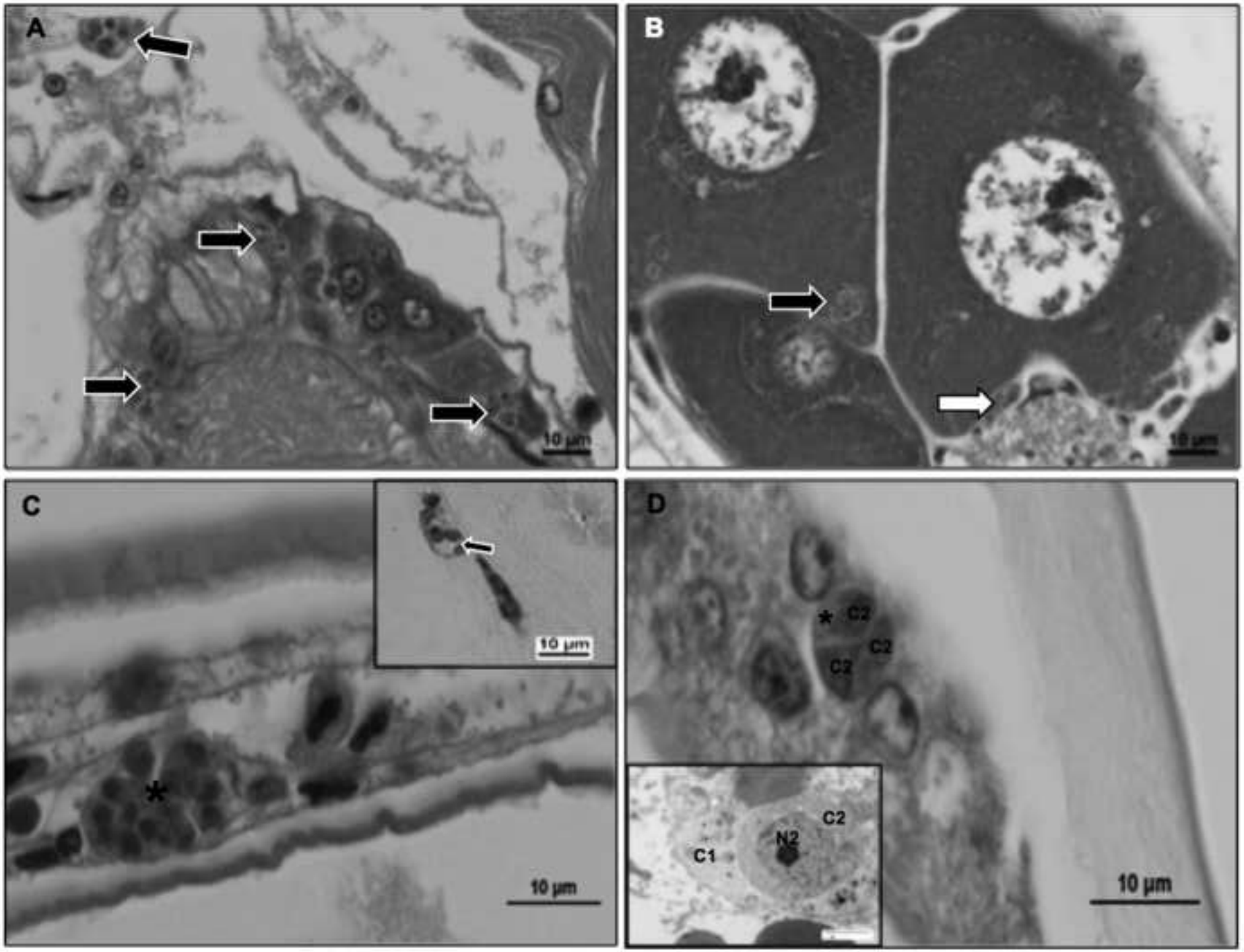
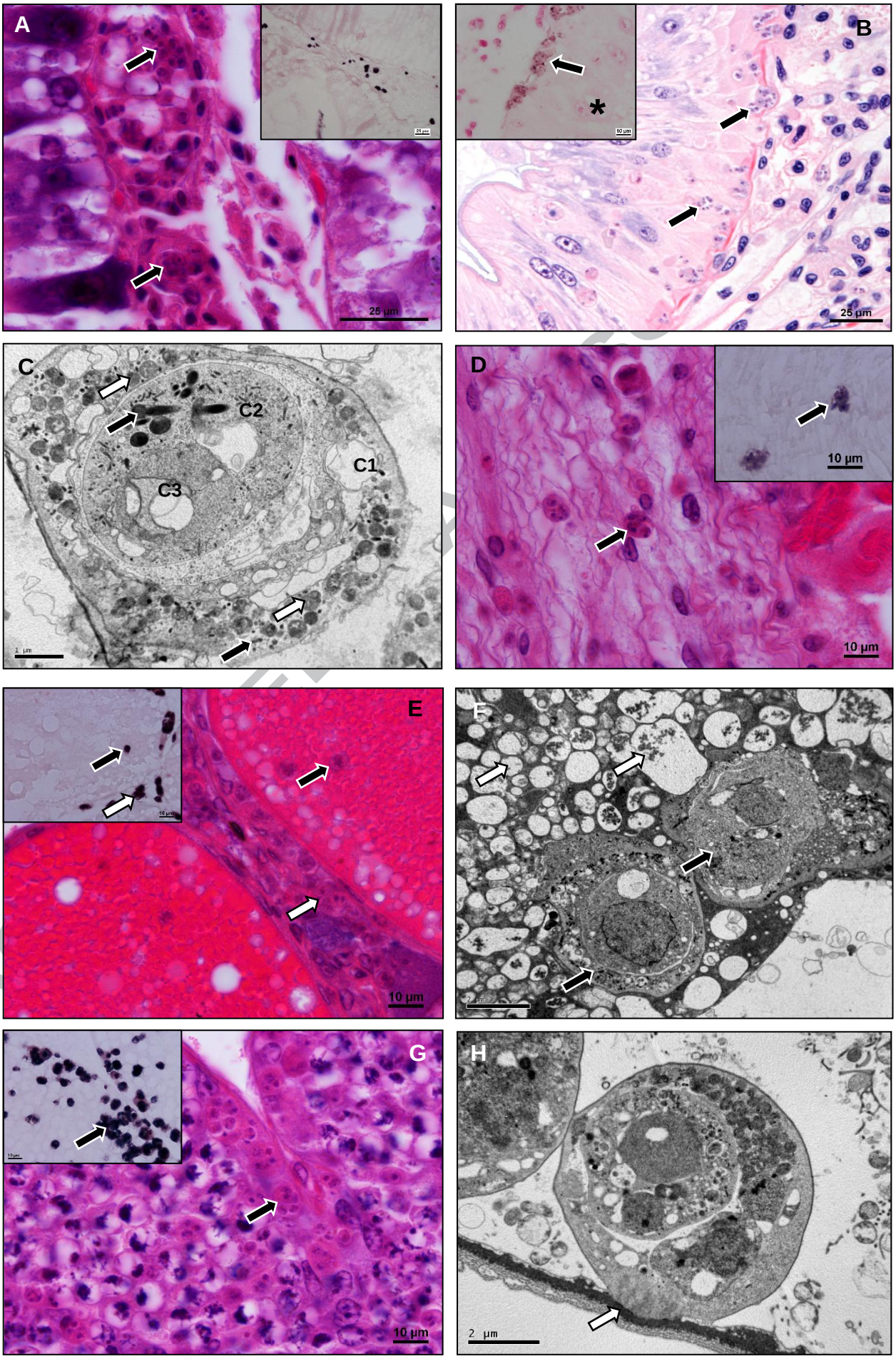


Figure 5 colour



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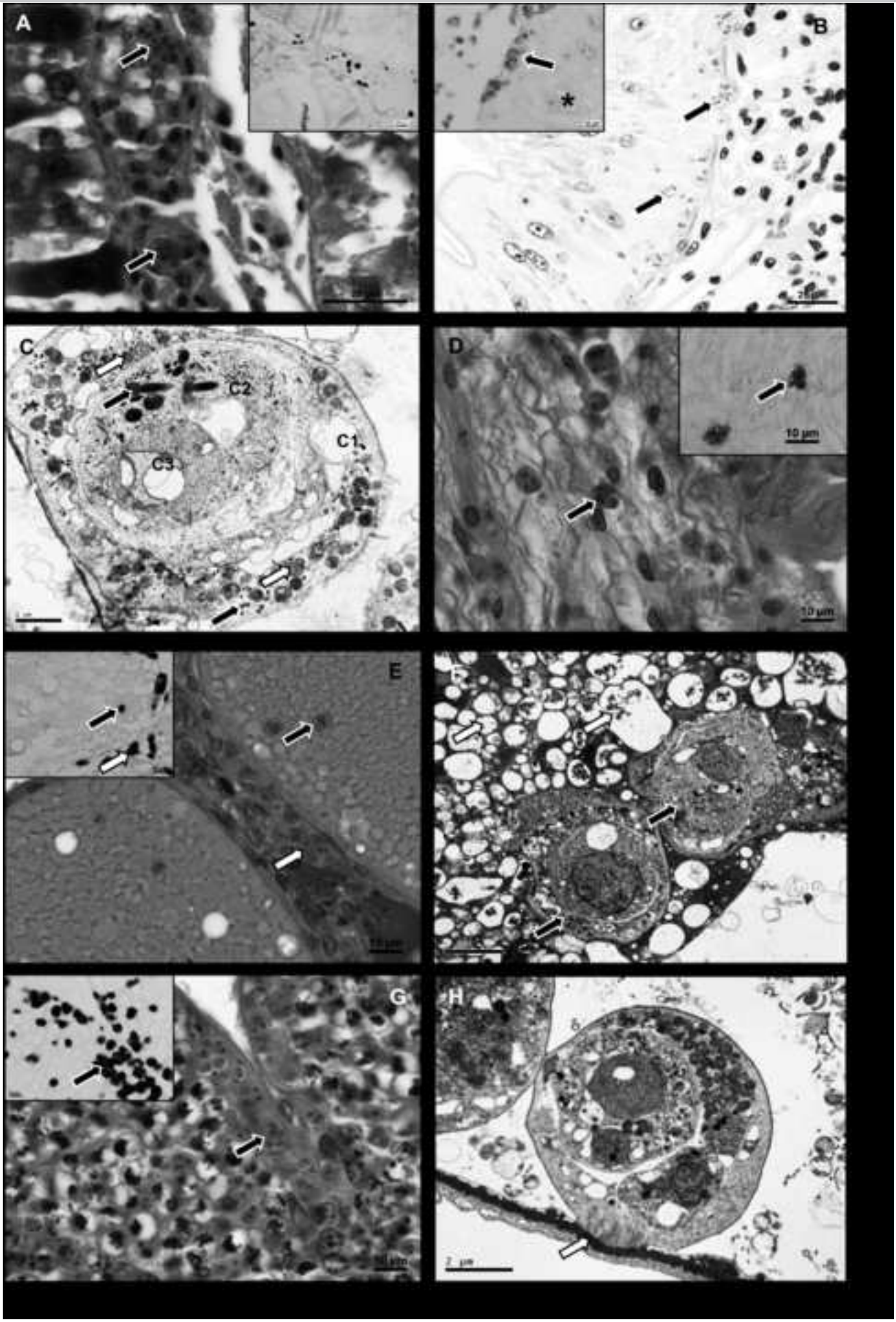

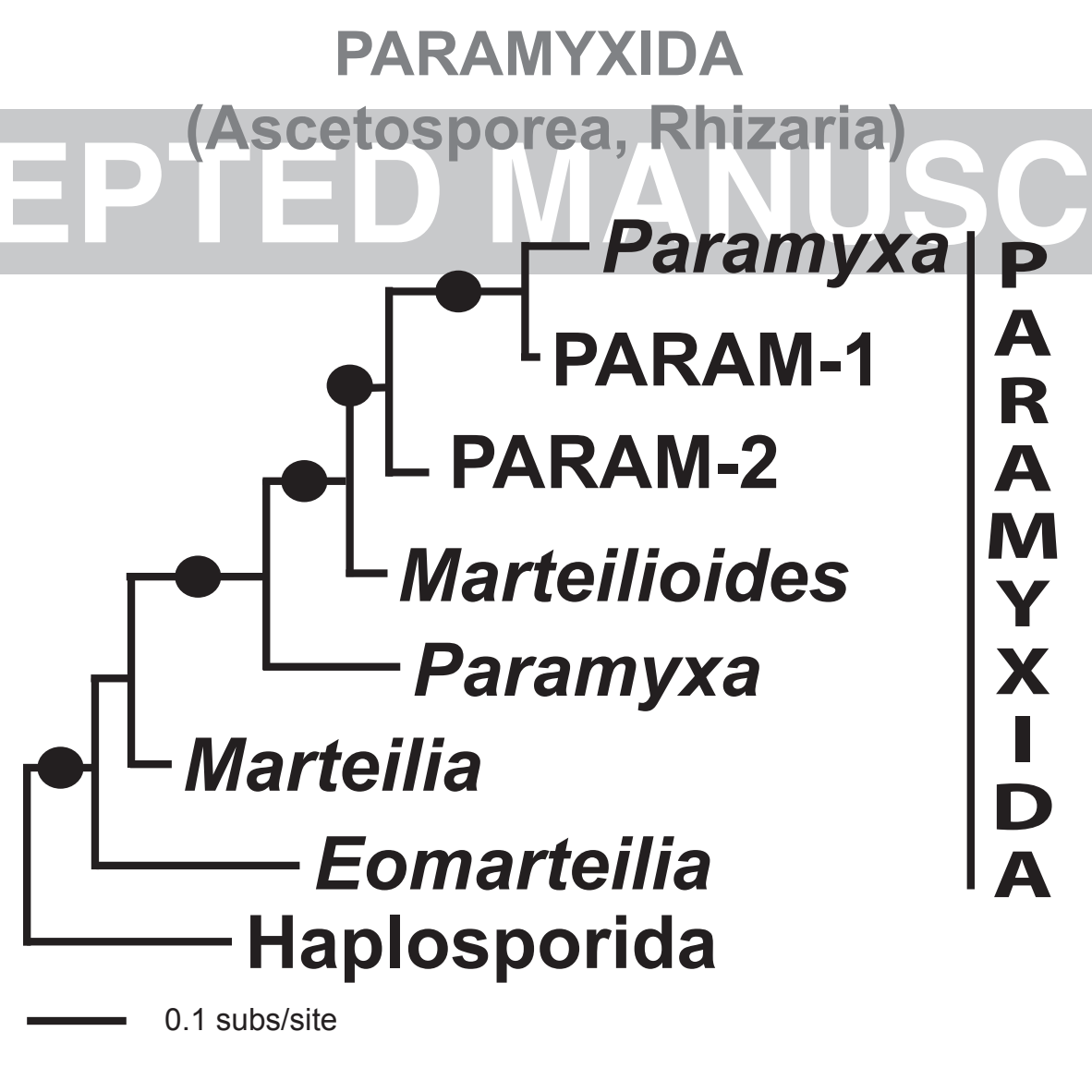

Highlights

- Phylogeny of the Paramyxida confirms five genera: *Marteilia*, *Eomarteilia*, *Paramarteilia*, *Paramyxa*, and *Marteilioides*

- Specific primers and environmental DNA approaches reveal novel diversity and distribution of paramyxids

- Paramyxids are parasites of diverse marine molluscs, crustaceans and polychaetes

- *Paramyxa nephtys* was identified in *Nephtys caeca* and the first 18S rDNA sequence reported for this genus

- *Paramarteilia* was identified in amphipods *Orchestia*, *Echinogammarus*, and crabs *Cancer*, *Carcinus*, *Maja*