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1 **A new phylogeny and environmental DNA insight into paramyxids: an increasingly**  
2 **important but enigmatic clade of protistan parasites of marine invertebrates**★

3 Georgia M Ward<sup>a,b</sup>, Martyn Bennett<sup>a,c</sup>, Kelly Bateman<sup>a</sup>, Grant D Stentiford<sup>a,c</sup>, Rose Kerr<sup>a</sup>, Stephen  
4 W Feist<sup>a</sup>, Suzanne T Williams<sup>b</sup>, Cedric Berney<sup>d</sup>, David Bass<sup>a,b,\*</sup>

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8 <sup>a</sup>*Centre for Environment, Fisheries, and Aquaculture Science (Cefas), Barrack Road, The Nothe,*  
9 *Weymouth, Dorset DT4 8UB, UK*

10 <sup>b</sup>*Department of Life Sciences, The Natural History Museum, London SW7 5BD, UK*

11 <sup>c</sup>*School of Biosciences, University of Exeter, Stocker Road, Exeter EX4 4QD, UK*

12 <sup>d</sup>*EPEP team, UMR 7144, CNRS & Sorbonne Universités UPMC Paris 06, Station*  
13 *Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France*

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15

16 \*Corresponding author. David Bass, Tel.: +44 1305 206752.

17 *E-mail address:* [david.bass@cefas.co.uk](mailto:david.bass@cefas.co.uk)

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19 ★Note: Nucleotide sequence data reported in this paper are available in NCBI GenBank under  
20 accession numbers **KX259318-KX259327**.

21

22 **Abstract**

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

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

49 **1. Introduction**

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

60 Paramyxids are increasingly recognised as pathogens causing economically significant  
61 mortalities of bivalves. The best known of these are marteiliosis/Aber disease in the European  
62 oyster *Ostrea edulis* and QX disease in the Sydney rock oyster *Saccostrea glomerata*, caused by  
63 *Marteilia refringens* and *Marteilia sydneyi*, respectively (Perkins and Wolf, 1976; Berthe et al.,  
64 2004; both species are listed as notifiable to the World Organisation for Animal Health (World  
65 Organisation for Animal Health (OIE) [http://www.oie.int/en/international-standard-setting/aquatic-  
66 code/](http://www.oie.int/en/international-standard-setting/aquatic-code/) (2015). Other significant bivalve diseases are caused by *Marteilia cochillia* in cockles  
67 (Carrasco et al., 2012, 2013), *Marteilioides chungmuensis* in *Crassostrea gigas* in Korea and  
68 Japan (Comps et al., 1986; Itoh et al., 2003), and *Marteilia granula* in the clam *Venerupis*  
69 *philippinarum* in Japan (Itoh et al., 2014).

70 Paramyxids in crustaceans include *Paramarteilia canceri*, which causes diseases of the  
71 edible/brown crab *Cancer pagurus* (Feist et al., 2009), and *Paramarteilia orchestiae* in amphipods,  
72 where it has been investigated in relation to modification of their sexual status (Ginsburger-Vogel  
73 1991; Short et al., 2012a,b). However, beyond these very few examples there are so far no other  
74 reports of paramyxids causing disease in crustaceans, although more recently copepods have  
75 been shown to be vectors in the lifecycle of *M. refringens* (Carrasco et al., 2007; Arzul et al., 2014).

76 Polychaetes are similarly understudied as potential hosts of paramyxids. Adlard and Nolan  
77 (2015) recently demonstrated that *M. sydneyi* cycles through both the polychaete *Nephtys australis*  
78 and the oyster *S. glomerata*, providing another example of the complexity of at least some  
79 paramyxid lifecycles. Otherwise the only known annelid-infecting paramyxid is *Paramyxa*, of which  
80 the only described species, *Paramyxa paradoxa*, was first described in a polychaete larva from  
81 Banyuls-sur-Mer on the Mediterranean French coast by Chatton (1911). No similar organisms  
82 were reported until a paramyxid parasite of the polychaete *Nephtys caeca* was described by  
83 Larsson and Køie (2005) as *Paramyxoides nephtys*, distinguished from *P. paradoxa* on the basis  
84 of spore shape and cytology. However, Feist et al. (2009) considered that the characters used to  
85 distinguish these two genera were taxonomically invalid and transferred *Paramyxoides* to  
86 *Paramyxa*.

87 Paramyxids are also commonly referred to as paramyxians. This class/order discrepancy  
88 deserves some explanation, to clarify the actual classification of the group and to ground its  
89 nomenclature in a robust phylogenetic context, which is an important aim of this study. Like many  
90 enigmatic micro-eukaryote groups, paramyxid taxonomy has been historically unstable, partly due  
91 to high levels of phenotypic conservation and convergence commonly seen in protists, particularly  
92 parasites (Boenigk et al., 2012; Hartikainen et al., 2014b; Neuhauser et al., 2014; Poulin and  
93 Randhawa, 2015). The presence of haplosporosome-like bodies provided early evidence that  
94 *Marteilia* and *Paramarteilia* were related to haplosporidans (Perkins, 1979), and ultrastructural  
95 characteristics supported a relationship between these genera and the first described genus  
96 eventually assigned to paramyxids, *Paramyxa* (Chatton, 1911; Desportes and Lom, 1981).  
97 *Marteilia* and *Paramarteilia* were described later, in the 1970s (Perkins, 1976; Perkins and Wolf,  
98 1976; Desportes and Ginsburger-Vogel, 1977; Ginsburger-Vogel and Desportes, 1979), as  
99 detailed in Desportes and Perkins (1990) and Feist et al. (2009). All three genera are distinguished  
100 from haplosporidans by the production of variable numbers of daughter cells endogenously formed  
101 within a primary amoeboid stem cell, leading to their characteristic 'cell within cell' development.  
102 This group has been treated as a class (Paramyxidea Levine, 1980), phylum (Paramyxia  
103 Desportes and Perkins, 1990), and most recently as the order Paramyxida in Bass et al. (2009),

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

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

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

125

## 126 **2. Materials and methods**

### 127 *2.1. Sample collection*

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

153 Edible crabs, *Cancer pagurus*, were captured in baited traps from the commercial fishery in  
154 Weymouth Bay area in January 2004. A total of 30 crabs were transported back to the Weymouth  
155 laboratory, where they were anaesthetised on ice for 30 min before dissection. Hepatopancreas,  
156 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 through 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 Blood 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<sub>2</sub>, 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  $\mu$  l 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*

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

256

### 257 2.5. Histology and in-situ hybridisation (ISH)

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

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

295

## 296 2.6. Transmission Electron Microscopy (TEM)

297 Selected parasite-positive animals were removed from glutaraldehyde and sectioned into 1  
298 mm<sup>3</sup> tissue blocks. The samples were fixed in 2.5% glutaraldehyde 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

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

331

### 332 3.2.1. *Marteilioides*

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

338

### 339 3.2.2. *Marteilia* clade

340 The *Marteilia* clade contains sequences which cluster in rough agreement with their  
341 geographical provenance: *M. refringens* and *M. cochillia*, sampled on many independent occasions  
342 from Europe (Kerr et al., unpublished data) and *M. octospora* from Spain (Ruiz et al., 2016) share  
343 very similar 18S sequences, and form a strongly supported clade with the highly distinct *M.*  
344 *sydneyi* sequence from *Saccostrea glomerata* from Queensland, Australia, '*Marteilia* sp. MC' from  
345 *Ruditapes philippinarum* in South Korea (Kang et al., unpublished data; sequence has GenBank  
346 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

373 A paramyxid found in the polychaete *N. caeca* in this study (assigned to *Paramyxa nephtys*  
374 as described in Section 3.3, and labelled as such in Fig. 1A) shares an identical sequence from  
375 0.45 µm-filtered water from Wilcove on the Tamar estuary (not separately shown in Fig. 1A). A  
376 related but clearly distinct sequence was detected in eDNA from an estuarine fish farm in Borneo.  
377 A further two sequences, labelled '*Paramyxa*' to tentatively assign them to this genus pending  
378 ongoing morphological analysis, were detected in DNA extracted from bivalve digestive gland (DG)  
379 samples from the Tamar, UK. One of these was detected at relatively high frequency in *O. edulis*  
380 (62/222; 28%) and *M. edulis* (9/150; 6%), but only in samples taken from Jupiter Point (Tamar) in  
381 September 2015. The other sequence type was detected only in 1/150 *M. edulis* DG from the 2013  
382 Tamar sampling.

383

### 384 3.3. *Paramyxid parasite of N. caeca and other polychaetes*

385 Histological analysis showed that 23/71 *N. caeca* specimens sampled from the Fleet  
386 lagoon (Weymouth, UK) in May 2015 were infected with an unknown paramyxid (Fig. 2). Three of  
387 these exhibited heavy infections of an ellipsoid spore-forming parasite typically restricted to the  
388 intestinal tract of the worm, including the mouth, the intestinal epithelium and lumen along the full  
389 length of the worm. TEM analyses of these heavily infected individuals revealed spore sacs with  
390 striated projections and containing four spores, very similar to those shown for *P. nephtys* in  
391 Larsson and Koie (2005). Also concordant with the description of *P. nephtys*, the developmental  
392 stages of the parasite had penetrated the intestinal epithelium and replicated to replace a large  
393 proportion of the host tissue. Mature stages were released from the intestinal cells into the lumen.  
394 No host response to infection was noted in the epithelium or lumen. Pre-spore stages were also  
395 similar to those described for *P. nephtys*. When all 71 *N. caeca* samples were screened using  
396 paramyxid-specific primers (see Section 2) a further 13 (i.e. total of 36 *Nephtys* individuals) were  
397 PCR-positive. Small samples of other polychaete species were collected from the same site as the  
398 *N. caeca* specimens. DNA from tissue of some of these was also paramyxid PCR-positive and  
399 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 *Paramyxoidea*, 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 haplosporomes and with increased ribosome density. Within the tertiary cell, two spores were

453 present (Fig. 4D). Developmental stages of the parasite were dispersed throughout *O.*  
454 *gammarellus* tissues and organs including the epidermal tissue (Fig. 4C,D), the connective tissue,  
455 heart and ganglia of the nerve cord (Fig. 4A). Furthermore, the cells apparently 'migrate' between  
456 organs and were present in the oocytes of two females (Fig. 4B), which supports the original trans-  
457 ovarial transmission hypothesis (Ginsburger-Vogel, 1979). Although the bi- or tri-cellular stages of  
458 the spore were not observed, the host species, sites of infection and morphology of the parasite  
459 unambiguously confirm this parasite as *Paramarteilia orchestiae*. A total of 369 *O. gammarellus*  
460 were prepared for histology and examined for the presence of *P. orchestiae*; infection was  
461 observed in 12 (3.25%) of these: one from Weymouth and 11 from Dale. A total of 222 of the 369  
462 *O. gammarellus* individuals were screened by PCR using the *Paramarteilia* primers from Short et al.  
463 (2012), including those analysed for histology; 24 of these were positive (10.81%); eight from  
464 Weymouth and 16 from Dale. No obvious pathology was displayed in 15 of these 24 samples.

465 We also present the first known histopathology, ISH and TEM images of *Paramarteilia*  
466 *canceri* in edible crabs and *Paramarteilia* in spider crabs *C. pagurus* and *M. squinado*, respectively  
467 (Fig. 5). The morphology and infection characteristics of *Paramarteilia* in *C. pagurus* were  
468 consistent with those described for *P. canceri* in Feist et al. (2009) and the *Paramarteilia* infection  
469 in *M. squinado* was also very similar structurally. *Paramarteilia canceri* was observed in one of the  
470 30 edible crabs sampled and *Paramarteilia* sp. was observed in two out of the 30 spider crabs  
471 sampled. Developmental stages of the parasite were dispersed throughout the connective tissues  
472 (Fig. 5A), hepatopancreas (Fig. 5B) and gonad (Fig. 5E, G). The parasite is shown to infect the  
473 connective tissues surrounding the oocytes and the oocytes themselves (Fig. 5 E, F) as well as the  
474 testicular follicles (Fig. 5G, H). The parasite in both crabs was similar to that observed in the *O.*  
475 *gammarellus* (see above), and that recorded from *Echinogammarus marinus* by Short et al.  
476 (2012b), the sequence of which is shown in Fig. 1A ([JQ673484](#)). However, more advanced  
477 developmental stages present in the crab species were not observed in amphipods. At present,  
478 based on morphological grounds it is not possible to propose that the same species infects these  
479 hosts. Because no 18S sequence for *P. canceri* exists (although the *P. orchestiae* 18S sequence  
480 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

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

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

525 In the absence of morphological data we refrain from assigning PARAM-1 or -2 to  
526 *Paramyxa*, the characterised genus to which these lineages are most closely and strongly related.  
527 PARAM-1 may turn out to be *P. paradoxa*; however, the relationship between genotypic and  
528 phenotypic distance is not straightforward, and decisions about taxonomic boundaries should be  
529 made taking into account both kinds of evidence (Boenigk et al., 2012). A good illustration of this is  
530 that three morphologically different *Marteilia* spp., *M. refringens*, *M. cochillia* and *M. octospora*, are  
531 extremely similar at the 18S level, yet *Marteilia* has been considered morphologically  
532 indistinguishable from *Marteilioides* and *Eomarteilia* spp., all three genera being very different from  
533 each other in terms of 18S sequence similarity (Fig. 1A). *Marteilia* and the morphologically similar  
534 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



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

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

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

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

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

669 These reports prove that even if some paramyxids are much more geographically localised  
670 than is true for many protists (Bass and Boenigk, 2011), the diversity and distribution of the order  
671 as a whole is greater than shown in Fig. 1 and Table 1. A more complete understanding of the  
672 distributions of these pathogens is increasingly important as new lineages (often with economically  
673 significant effects) are being discovered, and known paramyxids are being found in new hosts  
674 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://paramyxeanworkinggroup.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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1020 **Figure and Table legends**

1021

1022

1023 **Fig. 1.** 18S rDNA phylogenies of paramyxids and relevant GenBank accession numbers. (A)

1024 Phylogeny of paramyxids generated using Bayesian Inference. Values on nodes indicate Bayesian

1025 Posterior Probabilities (BPP); filled circles on nodes indicate maximal support (BPP = 1.0).

1026 Coloured/shaded squares indicate lineages detected in environmental samples, and circles those

1027 detected in host-associated (tissue or incubation) samples. Triangles indicate lineages for which

1028 only environmental sequences exist. Values in shapes indicate prevalence (number of positive

1029 samples/number of samples screened). Where no value is present, the lineage was not detected in

1030 this study. (B) More complete phylogeny of *Marteilia* and *Eomarteilia* with *Paramarteilia orchestiae*

1031 as an outgroup, generated using Bayesian Inference, showing Maximum Likelihood bootstrap and

1032 BPP values.

1033

1034 **Fig. 2.** Transmission Electron Micrographs (TEMs) and supporting light micrographs (H&E), in-situ1035 hybridisation (ISH) of developing and fully matured morphological *Paramyxa nephtys* cells. (A)1036 Developing *P. nephtys* cells detailing the third to sixth nuclei (N3 - N6) of a developing *P. nephtys*1037 spore. TEM. Scale bar = 2  $\mu\text{m}$ . (B) Longitudinal view of two mature *P. nephtys* spores, encased1038 within spore sacs possessing striated projections (SP). TEM. Scale bar = 2  $\mu\text{m}$ . (C) Top-down1039 view of *P. nephtys* spore sac terminal striated projection (SP) showing the projections form a single1040 structure. TEM. Scale bar = 2  $\mu\text{m}$ . (D) Longitudinal view of two mature *P. nephtys* spores. H&E1041 Stain. Scale bar = 25  $\mu\text{m}$ . (E) Longitudinal view of two mature *P. nephtys* spores. ISH. Scale bar =1042 25  $\mu\text{m}$ . (F) Transverse section of four coupled *P. nephtys* spores demonstrating the four1043 tetracellular spore arrangement (C1 - C4). TEM. Scale bar = 2  $\mu\text{m}$ . (G) Transverse section of *P.*1044 *nephtys* spores. H&E Stain. Scale bar = 25  $\mu\text{m}$ . (H) Transverse section of *P. nephtys* spores. Inset1045 ISH. Scale bar = 25  $\mu\text{m}$ .

1046

1047 **Fig. 3.** World map showing distribution of paramyxid clades. Coloured/shaded squares indicate

1048 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

1078 tissue cells (arrow) within the heart. Edible crab. Scale bar = 10  $\mu\text{m}$ . (E) Intracellular *P. canceri*  
1079 infecting the ovary (black arrow) and the connective tissues of the ovary (white arrow). H&E. Edible  
1080 crab. Scale bar = 10  $\mu\text{m}$ . Inset: ISH labelling of *P. canceri* infecting the oocytes themselves (black  
1081 arrow) and the connective tissues of the ovary (white arrow). Edible crab. Scale bar = 10  $\mu\text{m}$ . (F)  
1082 TEM of *P. canceri* within a vitellogenic oocyte. The oocyte is almost completely filled with yolk  
1083 globules (white arrow) the multicellular *P. canceri* parasites (black arrow) are clearly visible within  
1084 the oocyte. Edible crab. Scale bar = 2  $\mu\text{m}$ . (G) *Paramarteilia canceri* infecting the testicular follicles  
1085 (arrow). Edible crab. H&E. Scale bar = 10  $\mu\text{m}$ . Inset: ISH labelling of *P. canceri* infecting the  
1086 testicular follicles. Edible crab. Scale bar = 10  $\mu\text{m}$ . (H) TEM of *P. canceri* within the testicular  
1087 follicles. Parasite appears to be attached to the epithelium of the testicular follicle (arrow). Edible  
1088 crab. Scale bar = 2  $\mu\text{m}$ .

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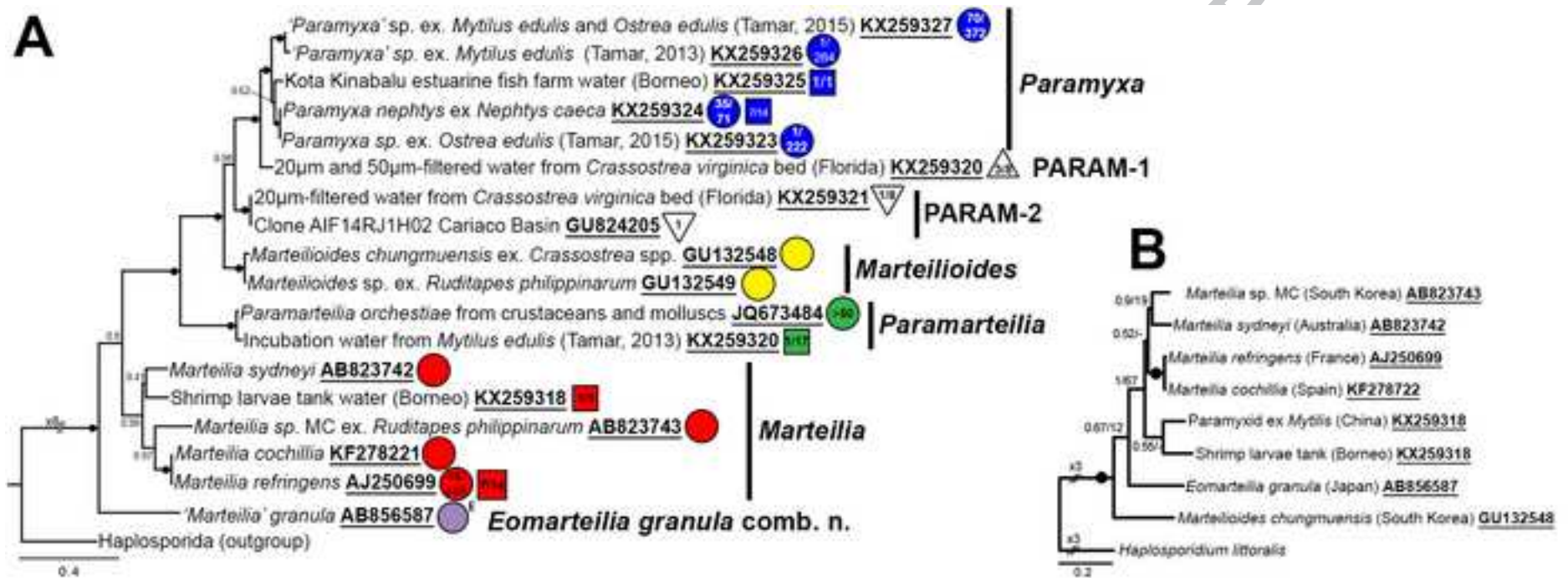
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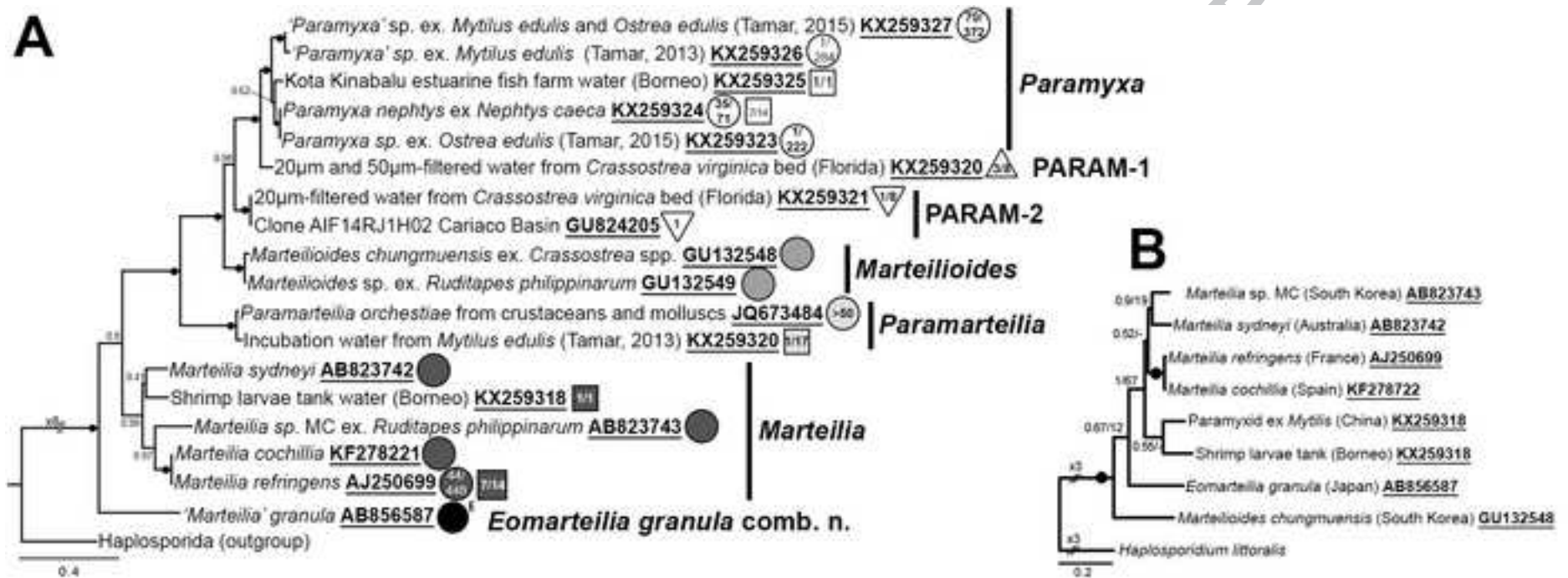
**Table 1.** Review of paramyxids known prior to this study for which sequence data and/or microscopy evidence unambiguously identifies the lineage identity to at least genus level. Where shown in bold, GenBank accession numbers are 18S sequences used in our phylogenetic analyses (Fig. 1). Where not 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.

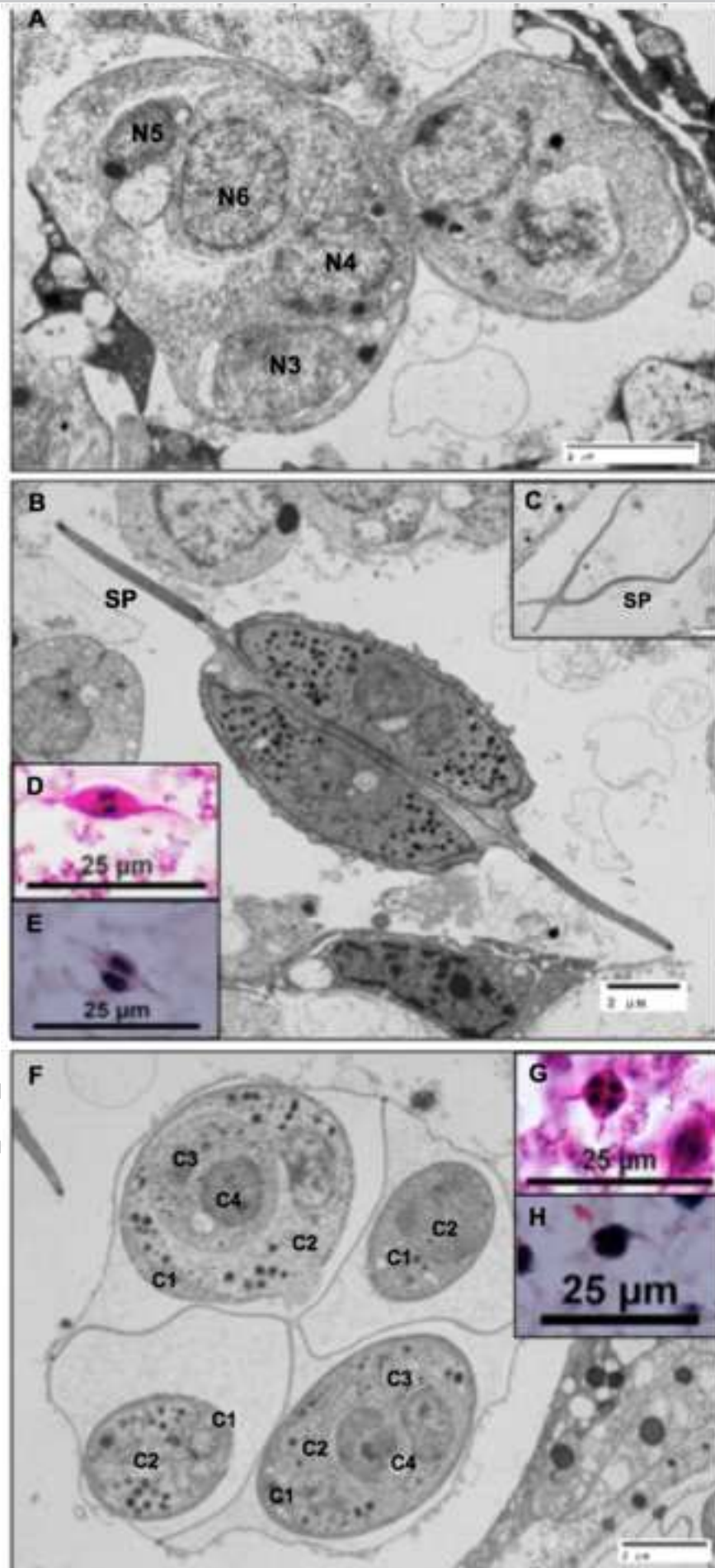
<b>Parasite genus</b>	<b>Species</b>	<b>Host</b>	<b>Geographical Location</b>	<b>Citation</b>	<b>GenBank Accession</b>
<b><i>Paramyxa</i></b>	<i>Paramyxa nephtys</i>	<i>Nephtys caeca</i>	Øresund, Denmark <b><u>Portland, UK</u></b> <b><u>Tamar estuary, UK</u></b>	Larsson and Koie, 2005 <b><u>Ward et al.,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

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

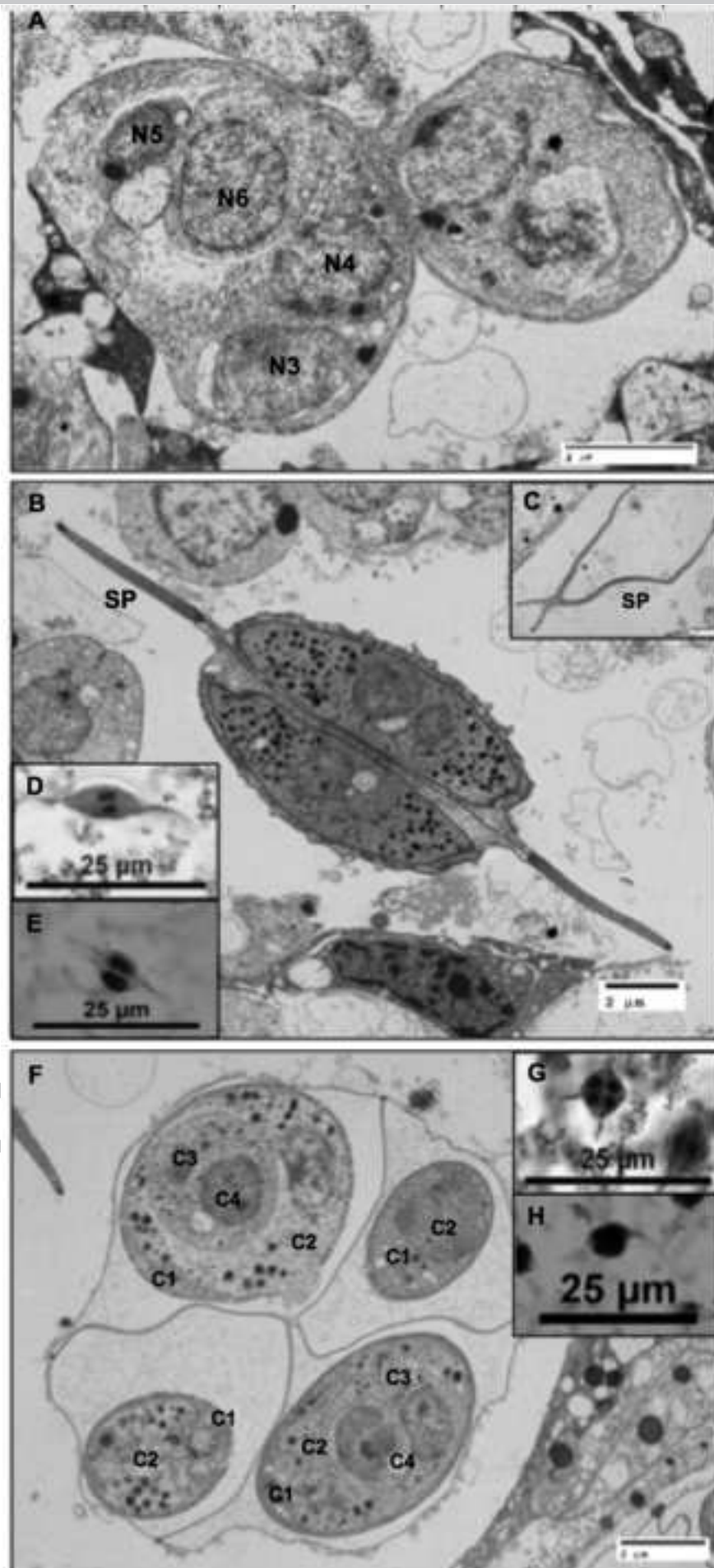
	<i>(Crassostrea gigas)</i>	<b>Sonora, Gulf of California, Mexico</b>	<b>Grijalva-Chon et al., 2015</b>	JQ066723-4 (IGS) JQ066725-6 (IGS)
	<i>(Crassostrea corteziensis)</i>			
	<i>(Acartia clausi)</i>	Ebro Delta, Catalonia, Spain	Carrasco et al., 2007b	AM504139 (IGS)
	<i>(Acartia discaudata)</i>			AM504140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

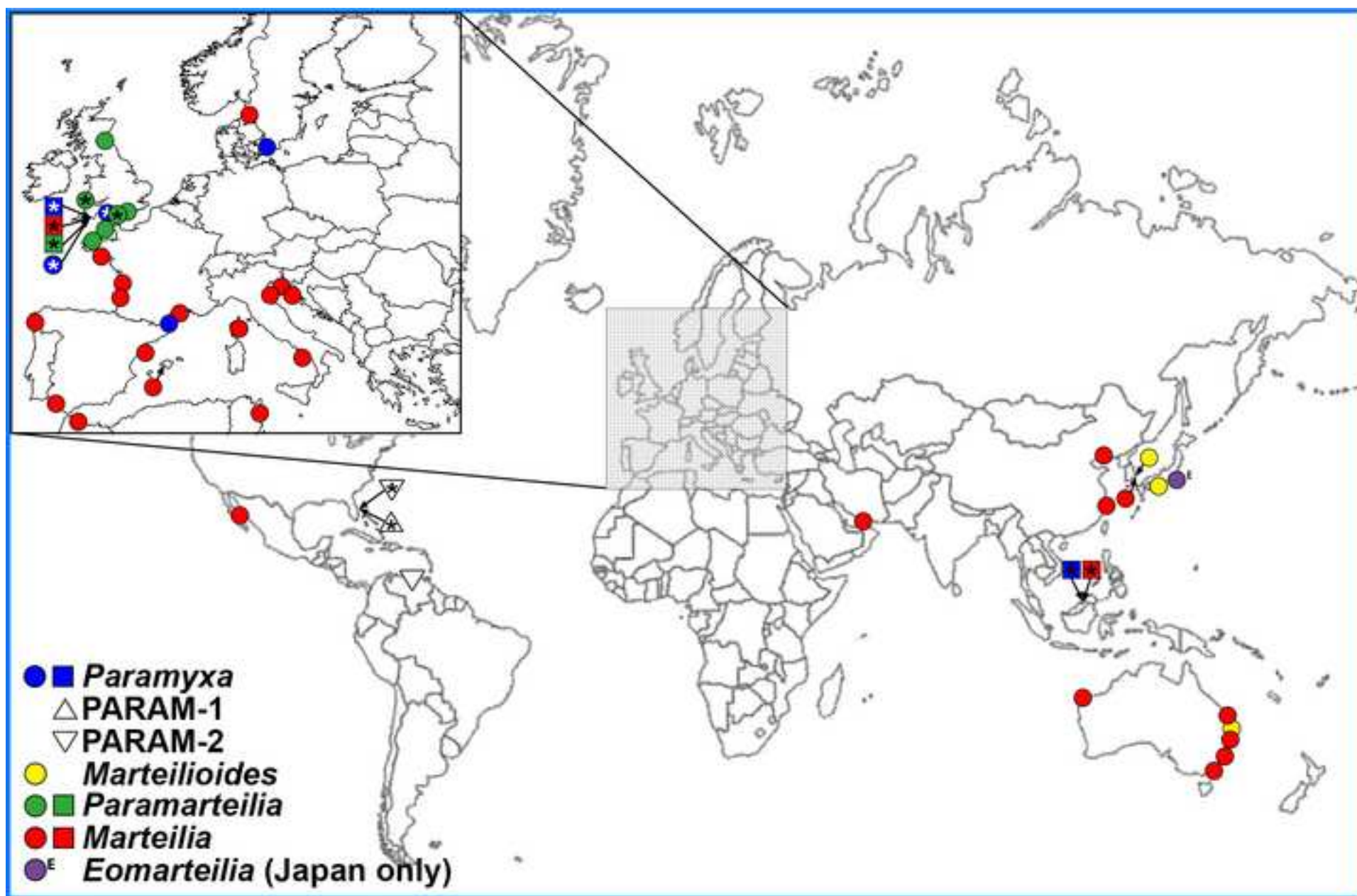


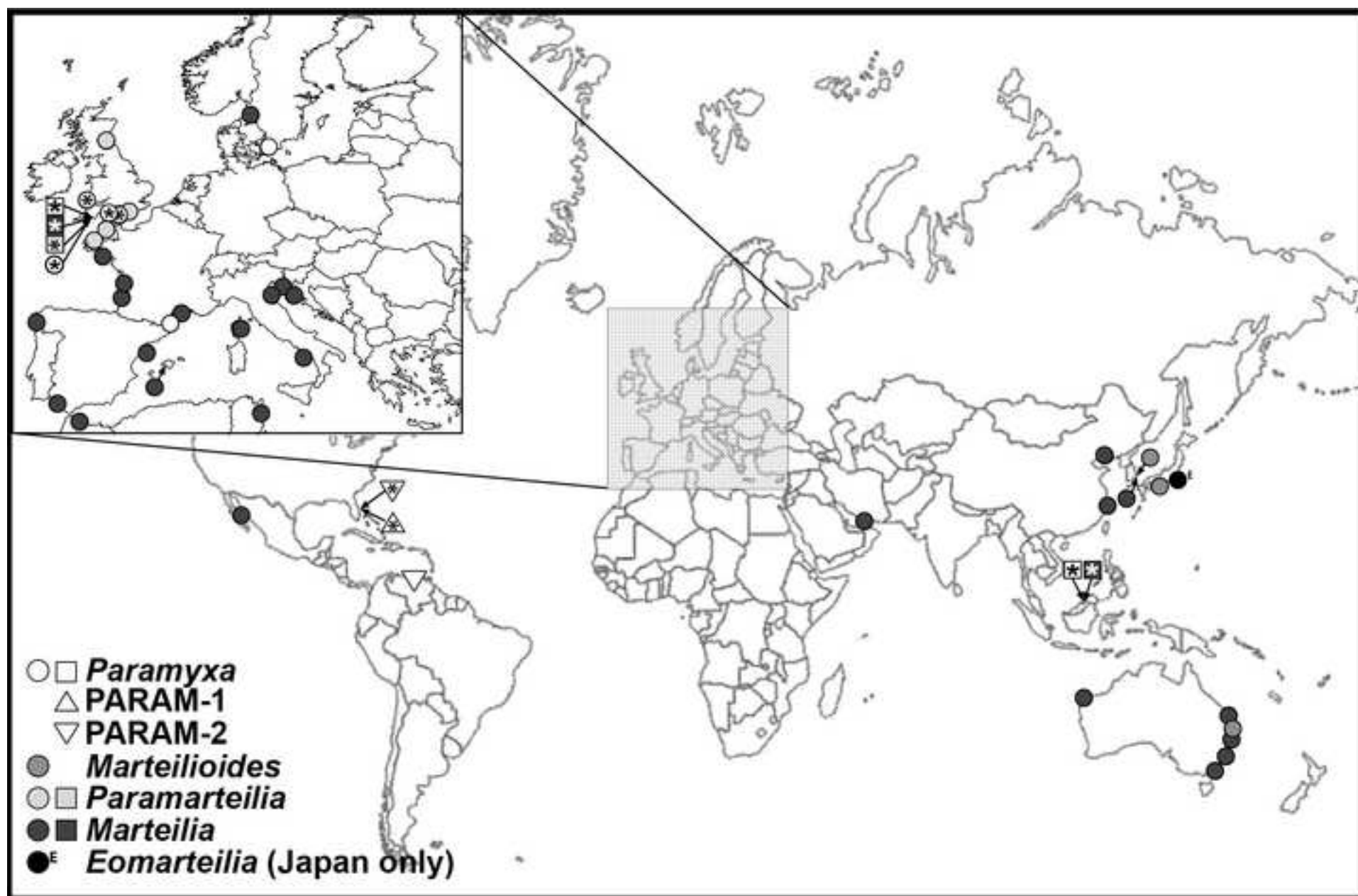


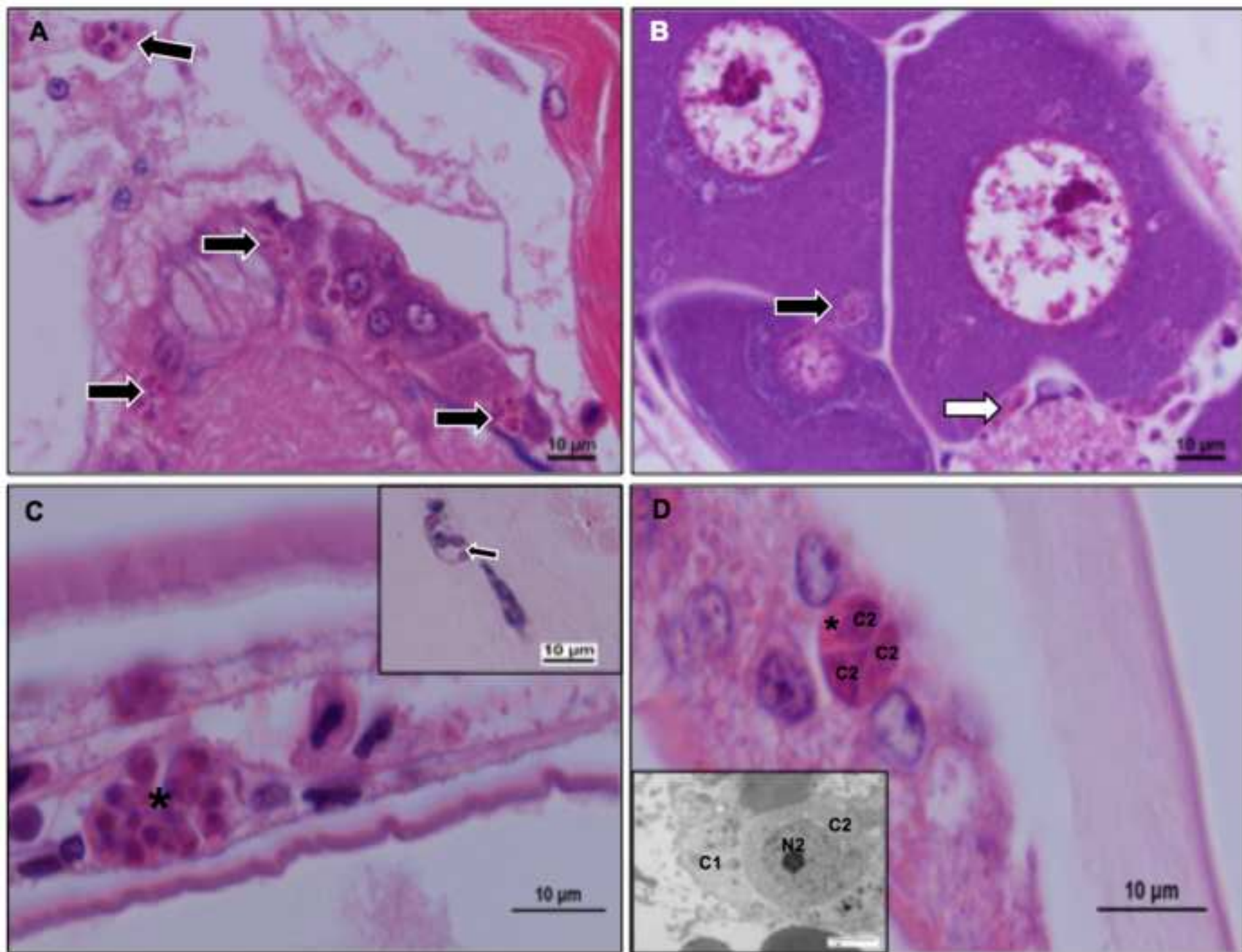


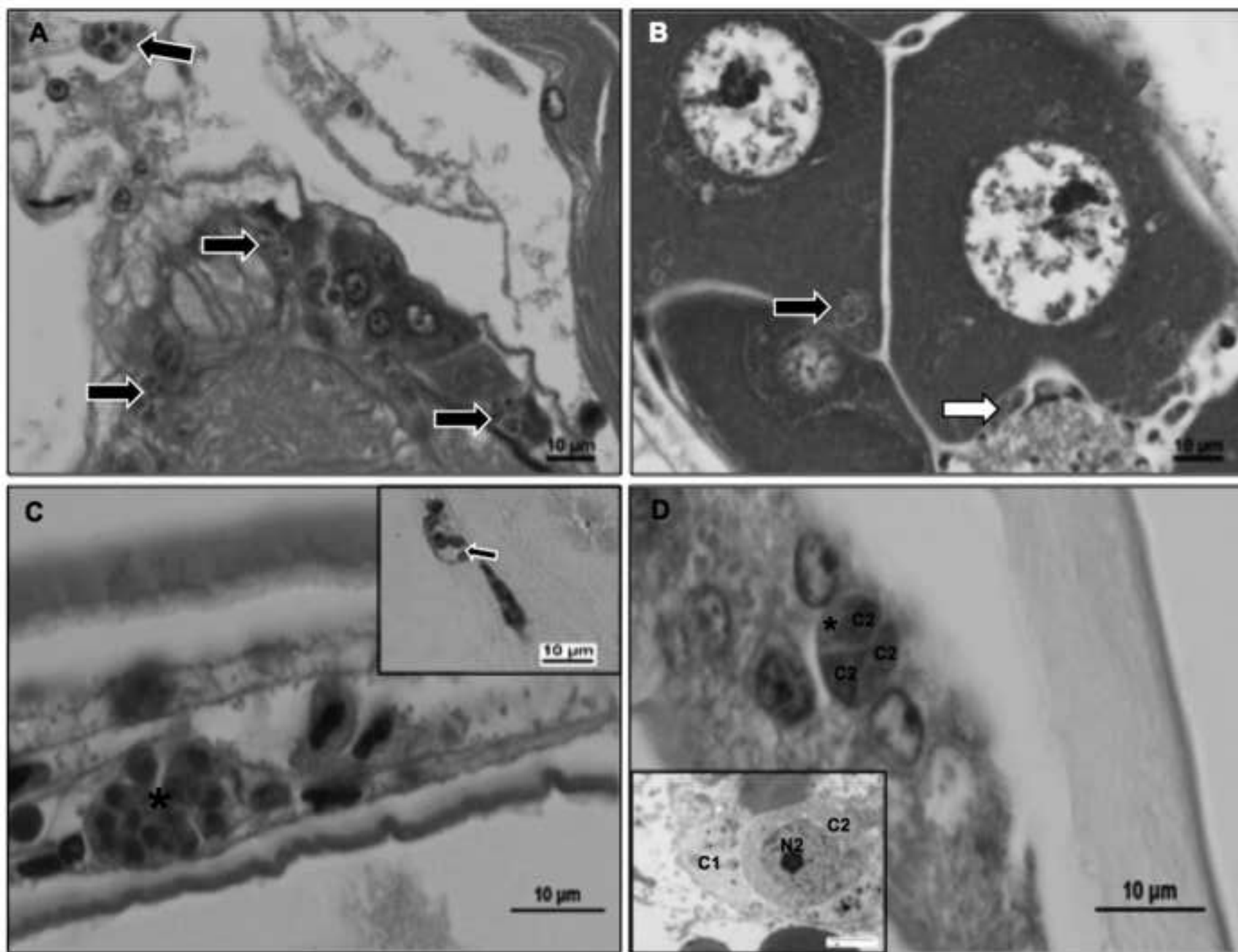


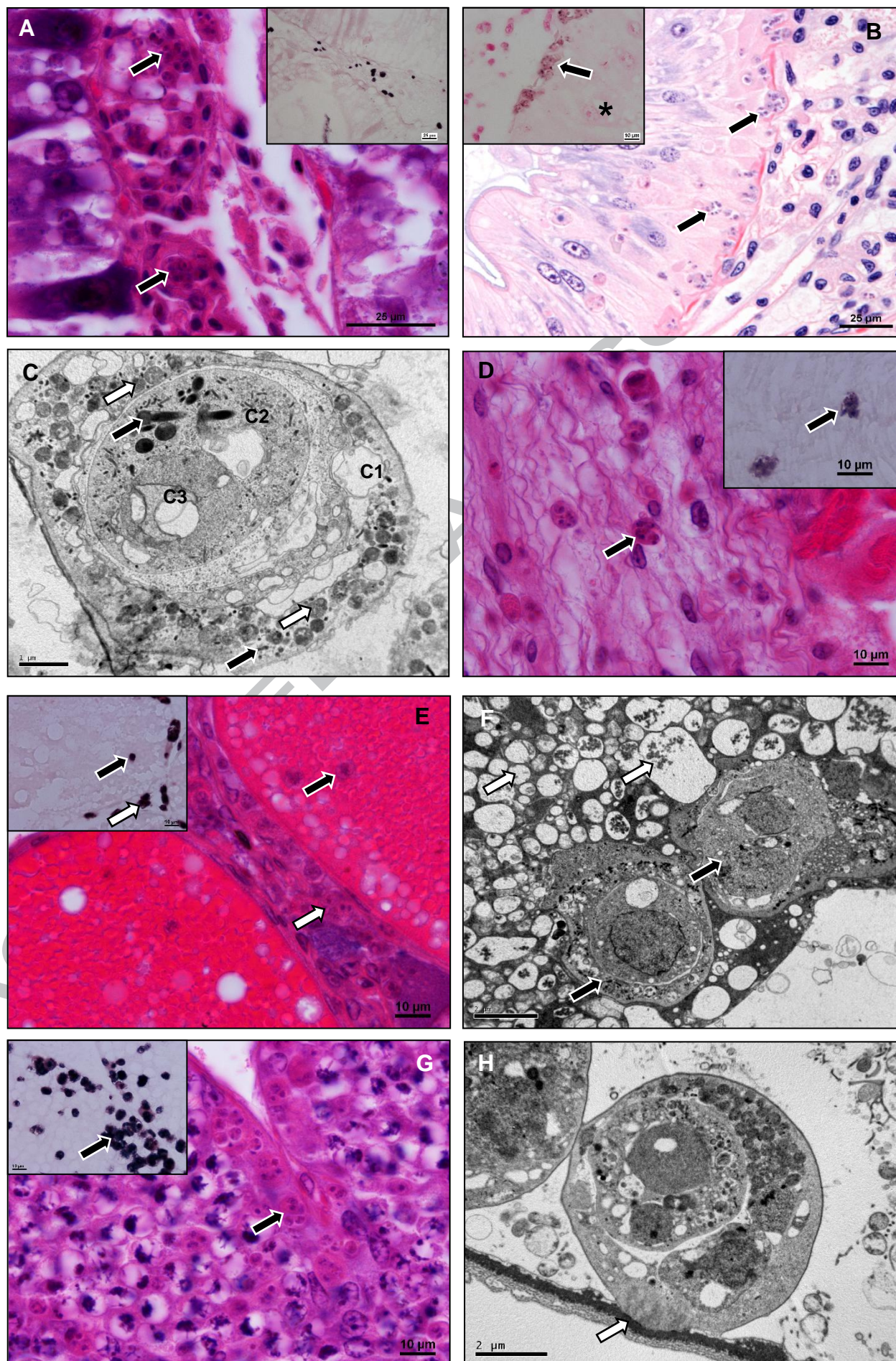


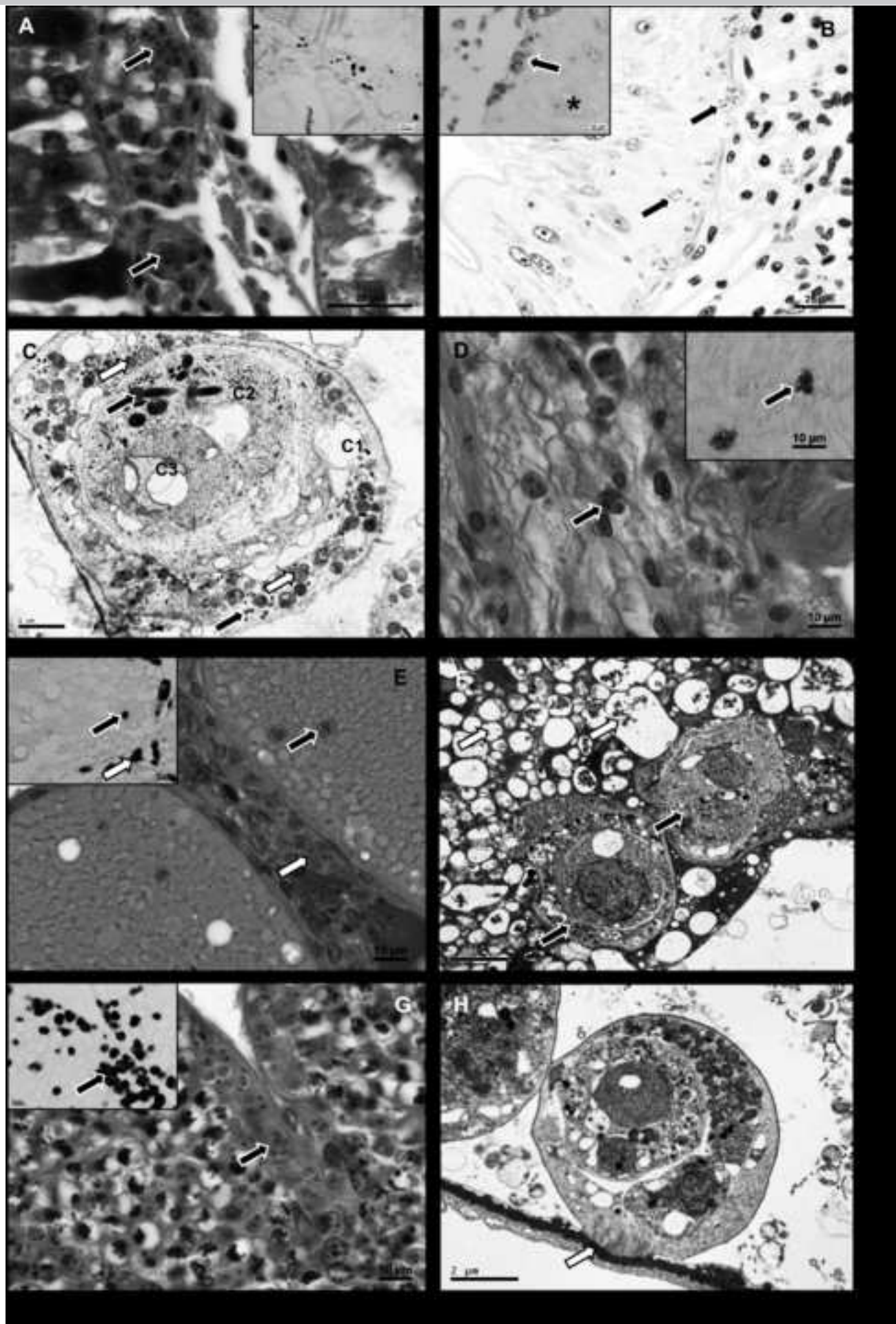








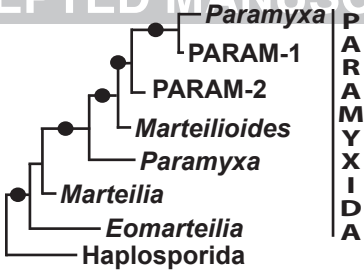




PARAMYXIDA

(Ascetosporea, Rhizaria)

ACCEPTED MANUSCRIPT



0.1 subs/site



## 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*