

## Morphological and Molecular Characterization of *Hematodinium perezii* (Dinophyceae: Syndiniales), a Dinoflagellate Parasite of the Harbour Crab, *Liocarcinus depurator*

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**ABSTRACT.** *Hematodinium perezii* Chatton and Poisson 1931 (Dinophyceae: Syndiniales) is reported from one of its type hosts, *Liocarcinus depurator*, from Rye Bay in the English Channel, a site in a similar geographical location to that of the type description. The histology and ultrastructure of vegetative trophont stages, and rDNA sequences of the parasite infecting this host are reported for the first time. Ultrastructurally, *H. perezii* was confirmed by the presence of condensed chromatin profiles, trichocysts, an alveolar membrane, and micropores. The pathology of *H. perezii* was similar to other *Hematodinium* descriptions with large numbers of parasites present within the haemolymph and host tissues. No host responses against the parasite were observed. Molecular analysis of the ITS rRNA regions from *H. perezii* infecting *L. depurator* suggests that *Callinectes sapidus* from the United States, and *Portunus trituberculatus* and *Scylla serrata* from China are infected with different genotypes of *H. perezii*. The morphological and molecular characterization of *H. perezii* in one of the type hosts from Europe will allow for a better understanding of the phylogeny of these pathogens of commercially important Crustacea.

**Key Words.** Crustacea, decapod, pathology, portunid, rRNA, taxonomy, ultrastructure.

**H**EMATODINIUM species are parasitic dinoflagellates that are known to infect a growing number of crustacean genera, many of which are exploited as commercial fisheries (reviewed by Stentiford and Shields 2005). Affected hosts undergo dramatic pathological alterations to their organs, tissues, and haemolymph. Respiratory dysfunction results in lethargy, and eventual death. The parasites are identified by their nuclei containing condensed chromatin, the alveolar membrane, cytoplasmic trichocysts, and vacuoles, and, when observed, athenate dinospores. Currently, there are only two formally described species of *Hematodinium*. The type species, *Hematodinium perezii* Chatton and Poisson 1931; was originally described from two portunid crab species, *Carcinus maenas* Linnaeus, 1758, and *Liocarcinus depurator* Linnaeus, 1758, off Luc-sur-Mer and Penpoull, on the English Channel coastline of France, off Arcachon on the Atlantic coast of France, and off Banyuls-sur-Mer on the Mediterranean coast of France (Chatton and Poisson 1931). In the type description, only hand-drawn figures were produced from unstained and stained parasite stages infecting both hosts. A second species, *Hematodinium australis* Hudson and Shields 1994, was described from the Australian sand crab, *Portunus pelagicus* Linnaeus, 1758. It was determined to be distinct from *H. perezii*, on the basis of size of the vegetative stage or trophont, the presence of rounded plasmodial stages, and the austral location of the host. Prior to the current study, no molecular sequence data existed for either of the above *Hematodinium* species from their type hosts.

Following the original description by Chatton and Poisson (1931), *H. perezii* was suggested as infecting additional host species. Gallien (1938) reported observing *H. perezii* infecting *Portunus latipes* Pennant, 1777, from Wimereux on the French coastline, and noted that the slow motion of the plasmodia was in contrast to the rapid torsion of the parasite described by Chatton and Poisson (1931). However, without additional morphological features, a new species was not established. Blue crabs, *Callinectes sapidus* Rathbun, 1896, from the eastern sea-

board of the United States have also been suggested as infected with *H. perezii*. The parasite was discovered infecting blue crabs from North Carolina, Georgia, and Florida (Newman and Johnson 1975), and was subsequently reported from blue crabs from Maryland and Virginia (Messick 1994), and also from New Jersey, South Carolina, Delaware, and Texas (Messick and Shields 2000). Although Newman and Johnson (1975) originally described the parasite as a *Hematodinium* sp., the name *H. perezii* has also been used to describe the parasite infecting *C. sapidus* and other hosts from the Eastern Seaboard of the United States (Lee and Frischer 2004; Messick 1994; Shields 2003; Shields, Scanlon, and Volety 2003; Shields and Squyars 2000) leading to confusion in the parasite's true identity.

*Hematodinium* species have emerged globally as some of the most significant pathogens of commercially exploited, and presumably nonexploited, crustacean hosts (Stentiford and Shields 2005). Although epidemiological studies have been carried out at the regional and national levels (Eaton et al. 1991; Field et al. 1992, 1998; Latrouite et al. 1988; Love et al. 1993; Messick 1994; Meyers et al. 1990; Sheppard et al. 2003; Stentiford, Neil, and Atkinson 2001; Wilhelm and Mialhe 1996), little attempt, until recently, has been made to document the number of *Hematodinium* species, or strains, infecting crustacean hosts, which may shed light upon transmission routes between distant crustacean populations. Such studies have been hampered partially by a lack of specific comparative work on isolates from different geographical regions, but more so by an absence of knowledge on the pathological, ultrastructural, and molecular characteristics of the type species, *H. perezii*, from its type hosts and locations within Europe. The emergence of numerous "*Hematodinium* sp." and "*Hematodinium*-like" descriptions is essentially attributable to the latter. In this article, we report on the rediscovery of *H. perezii* from one of its type hosts, the swimming crab *L. depurator*, from a site in the English Channel adjacent to that in the type description. The morphological and molecular sequence data provided will facilitate future efforts to identify and characterize other *Hematodinium* species and strains.

### MATERIALS AND METHODS

**Collection of animals.** *Liocarcinus depurator* were captured from Rye Bay on the South Coast of England (50°45.988'N,

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Fig. 1. Sample locations of *Liocarcinus depurator* from which *Hematodinium perezii* was detected in the present study (site 1, Rye Bay), and of the locations and host crustaceans from which *H. perezii* was originally described by Chatton and Poisson (1931), sites 2–5). Site 2, Arcachon (*Carcinus maenas*); site 3, Luc-Sur-Mer (*L. depurator* (formerly *Portunus depurator*)); site 4, Banyuls-Sur-Mer (*L. depurator*); and site 5, Penpoull (*C. maenas*).

00°45.035'E) in July 2004 as part of the Centre for Environment, Fisheries, and Aquaculture Sciences (CEFAS) annual Clean Seas Environmental Monitoring Programme (CSEMP) research cruise (Fig. 1). A total of 31 crabs, 17 male and 14 female, were collected from this site using a standard Granton trawl. Live crabs were stored in flowing seawater for a maximum of 2 h prior to anaesthetization by chilling to 4 °C and dissection.

**Histopathology.** The hepatopancreas, gill, gonad, and heart of infected and uninfected *L. depurator* were removed and fixed in Davidson's seawater fixative (Hopwood 1996) for 24 h, and then transferred to 70% ethanol for transport and storage. Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut at 3–5 µm on a rotary microtome, and resulting tissue sections were mounted onto glass slides before staining with haematoxylin and eosin (H&E). Stained sections were examined

using a Nikon Eclipse E800 microscope, and digital images were captured using the Lucia™ Screen Measurement System (Nikon, UK).

**Electron microscopy.** Infected haemolymph or small pieces of tissue from *L. depurator* were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and 1.75% (w/v) sodium chloride for 2 h at room temperature. Fixed samples were washed in 0.1 M sodium cacodylate buffer, pH 7.4, before being postfixed in 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. Samples were washed in three changes of 0.1 M sodium cacodylate buffer. Specimens were embedded in epoxy resin 812 (Agar Scientific, Stansted, United Kingdom) following dehydration through a graded acetone series. Semi-thin sections (1–2 µm) were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections, 70–90 nm thick, of these areas were mounted on uncoated copper grids, stained with uranyl acetate and Reynolds' lead citrate (Reynolds 1963), examined using a JEOL 1210 transmission electron microscope (Jeol Ltd., Tokyo, Japan), and digital images were captured using a Gatan Erlangshen ES500W camera (Gatan Inc., Pleasanton, CA) and Gatan Digital Micrograph™ software (Gatan). Morphometric measurements of parasites were made from ultrathin sections.

**Nucleic acid extraction.** Infected haemolymph and tissue samples from *L. depurator* were preserved in 95% ethanol. Genomic DNA was extracted from preserved samples using a Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA), following the manufacturer protocol. DNA was eluted from the column in 100 µl of elution buffer following a 5-min incubation at room temperature, and stored at –20 °C prior to use in PCR assays.

**Small subunit (SSU) rRNA gene amplification.** The SSU rRNA gene of the *Hematodinium* sp. infecting *L. depurator* was amplified from two separate genomic DNA samples from different crabs using the forward and reverse primers, H18SF and H18SR3 (Table 1). Amplification reactions were carried out in a DNA Engine thermocycler (MJ Research Inc., Watertown, MA), and each reaction contained 100 ng genomic DNA, 60 mM Tris-SO<sub>4</sub>, pH 8.9, 18 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2mM MgSO<sub>4</sub>, 0.2 mM each dNTP, 2.5 mM each primer, 1 unit Platinum high fidelity Taq (Invitrogen, Carlsbad, CA), and sterilized deionized water to a final volume of 20 µl. Thermal cycling conditions were as follows: denaturation at 94 °C for 1 min; primer annealing at 58 °C for 1 min; extension at 72 °C for 3 min; repeated for 39 cycles, with a final cycle incorporating a 5 min extension at 72 °C.

**Internal transcribed spacer 1 (ITS1) region amplification.** The 3'-end of the SSU rRNA gene and the first internal transcribed spacer (ITS1) region of the *Hematodinium* sp. infecting *L. depurator* were amplified from three separate genomic DNA samples using the forward and reverse primers A and B (Table 1), and the same reagent system and concentrations as above. Thermal cycling conditions were as follows: denatur-

Table 1. Oligonucleotide primers used in this study.

Primer name	Primer sequence (5'–3')	Location	Reference
H18SF	CTGCCAGTAGTCATATGC	5' SSU	This study
H18SR3	CACGGTGAATGTTTGTGTGAA	5' ITS1	This study
A	GTTCCCTTGAACGAGGAATC	3' SSU	Hudson and Adlard (1994)
B	CGCATTTGCTGCGTTCTTC	5.8S	Hudson and Adlard (1994)
H5.8SF1	GCGATGAATGCCTCGGCTCG	5.8S	This study
LSU B	ACGAACGATTTGCACGTCAG	5' LSU	Lenaeers et al. (1989)

ation at 94 °C for 1 min; primer annealing at 52 °C for 1 min; and extension at 72 °C for 3 min; repeated for 35 cycles, with a final cycle incorporating a 7 min extension at 72 °C.

**Internal transcribed spacer 2 (ITS2) and large subunit (LSU) rRNA gene amplification.** The 3'-end of the 5.8S rRNA gene, the second internal transcribed spacer (ITS2), and a portion of the 5'-end of the LSU rRNA gene of the *Hematodinium* sp. infecting *L. depurator* were amplified from three separate genomic DNA samples using the forward and reverse primers, H5.8SF1 and LSU B (Table 1), and the same reagent system and concentrations as above. In addition, the same region was also amplified from three genomic DNA samples from the *Hematodinium* sp. infecting *C. sapidus*. These samples originated from crabs collected from waters adjacent to Wachapreague Inlet, Virginia, United States, in November 2005 (see Small et al. 2007a). Thermal cycling conditions were as follows: denaturation at 94 °C for 1 min; primer annealing at 60 °C for 1 min; and extension at 72 °C for 3 min, repeated for 35 cycles, with a final cycle incorporating a 5 min extension at 72 °C.

**Cloning and sequencing.** Amplification products were visualized using 1.5 or 2% (w/v) agarose gel electrophoresis, stained with ethidium bromide, and viewed under a UV light source. Products of ~1,800 bp for the SSU rRNA gene, 680 bp for the ITS1 region, and ~1,600 bp for the ITS2 region-LSU rRNA gene, were excised from agarose gels and purified using a QIA-quick gel-extraction kit (Qiagen). Cloning and sequencing reactions were performed as previously described (Small et al. 2007a, b). Briefly, adenine (A)-tails were added to purified amplification products to ensure efficient ligation into the plasmid vector pCR®4-TOPO® and transformed into *E. coli* using a TOPO TA Cloning® Kit (Invitrogen) following the manufacturer's protocols. Recombinant plasmids were purified using a miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmid inserts were sequenced bi-directionally using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Norwalk, CT) with M13 sequencing primers using 0.125 × (ITS1 region) or 0.250 × (SSU and ITS2-LSU regions), the recommended amount of Big Dye dictated in the manufacturer's protocols. The sequencing reaction products were precipitated using ethanol/sodium acetate (ABI User Bulletin, April 11, 2002). Precipitated sequencing reactions were re-suspended in 20 µl of Hi-Di formamide (Applied Biosystems) and 10 µl of each were electrophoresed on an ABI 3100 or 3130 Prism genetic analyser (Applied Biosystems). For the SSU rRNA amplifications, three clones from two samples were prepared and sequenced bi-directionally. For the ITS1 and ITS2-LSU amplifications, five clones from each of three samples for each *Hematodinium* spp. were prepared and sequenced bi-directionally.

**Analysis of sequence data.** *Hematodinium* sp. sequences were constructed from the forward and reverse sequencing reactions using Sequencher (version 4.2). Primer sequences were removed from the 5'- and 3'-ends of the sequences. The boundaries of the SSU and 5.8S rRNA genes were identified using data from previous studies (Hamilton, Morrill, and Shaw 2010; Small et al. 2007a, b). The 5'-end of the LSU rRNA gene was identified by comparison to conserved LSU proximal stem sequences (Gottschling and Plötner 2004; Keller et al. 2009), and was defined by the sequence 5'-GGACAT-GAGTCAGGCA-3'.

*Hematodinium* sp. SSU rRNA, ITS, and LSU rRNA sequences obtained from *L. depurator*, and ITS2 and LSU rRNA sequences obtained from *C. sapidus* were compared with those deposited in GenBank using basic local alignment search

tool (BLAST) searches (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI) database. Available SSU rRNA sequences representative of the *Hematodinium* spp. infecting *C. sapidus*, *Nephrops norvegicus* Linnaeus, 1758, *Chionocetes opilio* Frabricius, 1788, *Chionocetes bairdi* Rathbun, 1924, and *C. tanneri* Rathbun, 1893, as well as sequences from species of *Syndinium* and *Amoebophrya*, were downloaded from GenBank for inclusion in phylogenetic analyses (see Table 2). In addition to the above, ITS1 region sequences representative of the *Hematodinium* spp. infecting *C. sapidus*, *Portunus trituberculatus* Miers, 1876, and *Scylla serrata* Forskål, 1775, were also downloaded from GenBank for inclusion in phylogenetic analyses (see Table 2). Multiple alignments of DNA sequences were performed using the CLUSTALW algorithm in MacVector 8.1.2 (MacVector Inc., Cary, NC) with an open gap penalty of seven and an extended gap penalty of three. Genetic distance (uncorrected '-p') calculations were performed using MEGA3 (Kumar, Tamura, and Nei 2004). Parsimony and maximum likelihood analyses were carried out using the PAUP\*4.0b10 (Swofford 2002) plug-in within the Geneious Pro 5.3.6 software package (Biomatter Ltd., Auckland, New Zealand). The nucleotide substitution model for the maximum likelihood analyses was GTR + I + G, as determined by Modeltest vs. 3.7 based on AIC (Akaike information criterion). Heuristic searches were conducted with random seeds and 1,000 bootstrap replicates. Gaps were treated as informative data for the parsimony analysis.

Table 2. GenBank sequences used in this study.

Taxon	Locus	GenBank Accession #
<i>Amoebophrya</i> sp.	SSU	AF069516
<i>Amoebophrya</i> sp. 'Dinophysis'	SSU	AF239260
<i>Amoebophrya</i> sp. ex <i>Ceratium tripos</i>	SSU	AY208892
<i>Amoebophrya</i> sp. ex <i>Gymnodinium instriatum</i>	SSU	AF472554
<i>Amoebophrya</i> sp. ex <i>Karlodinium micrum</i>	SSU	AF472553
<i>Amoebophrya</i> sp. ex <i>Prorocentrum micans</i>	SSU	AY208893
<i>Amoebophrya</i> sp. ex <i>Prorocentrum minimum</i>	SSU	AY208894
<i>Amoebophrya</i> sp. ex <i>Scrippsiella</i> sp.	SSU	AF472555
<i>Hematodinium</i> sp. ex <i>Callinectes sapidus</i>	SSU	AF286023
<i>Hematodinium</i> sp. ex <i>Callinectes sapidus</i>	SSU	DQ925237
<i>Hematodinium</i> sp. ex <i>Chionocetes bairdi</i>	SSU	FJ844416
<i>Hematodinium</i> sp. ex <i>Chionocetes opilio</i>	SSU	FJ844422
<i>Hematodinium</i> sp. ex <i>Chionocetes tanneri</i>	SSU	FJ844425
<i>Hematodinium</i> sp. ex <i>Nephrops norvegicus</i>	SSU	FJ844429
<i>Prorocentrum micans</i> (outgroup)	SSU	M 14649
<i>Syndinium</i> sp. ex <i>Corycaeus</i> sp.	SSU	DQ146406
<i>Syndinium turbo</i>	SSU	DQ146403-DQ146405
<i>Hematodinium</i> sp. ex <i>Callinectes sapidus</i>	ITS	DQ925227-DQ925236
<i>Hematodinium</i> sp. ex <i>Callinectes sapidus</i>	ITS	HM067680-HM067683
<i>Hematodinium</i> sp. ex <i>Callinectes sapidus</i>	ITS	FJ844430-FJ844431
<i>Hematodinium</i> sp. ex <i>Portunus trituberculatus</i>	ITS	EF173452-EF173454
<i>Hematodinium</i> sp. ex <i>Scylla serrata</i>	ITS	EF173451

## RESULTS

**Infection prevalence.** The prevalence of *Hematodinium* infection in *L. depurator* from histological samples was 19.4% (6/31). Female crabs had a prevalence of 35.7% (5/14), and male crabs 5.9% (1/17).

**Histopathology and parasite morphology.** In *L. depurator*, uninucleate and multinucleate parasites were observed that are typical of previous histological descriptions of *Hematodinium* spp. infections in crustaceans, with the presence of single or multiple deeply basophilic nuclei surrounded by a strongly eosinophilic cytoplasm. The haemal sinuses of infected *L. depurator* were heavily dilated and were filled with masses of parasite trophonts. Massed parasites were most clearly observed within the haemal sinuses of the heart (Fig. 2, 3), muscle (Fig. 4), and hepatopancreas (Fig. 5). In the latter case, the sinuses were heavily dilated and devoid of the connective tissue cells, reserve inclusion cells, or fixed phagocytes normally observed in uninfected crabs. The claw muscle of heavily infected crabs displayed a characteristic loss of structure, with islands of apparently detached muscle fibres suspended in haemolymph containing masses of parasite cells. Significantly, the ovary of infected female crabs appeared to be arrested in a state of previtellogenesis with parasites in direct confluence with the ovarian cavity and presumed apoptosis of oogonia and early oocytes (Fig. 6). In males, although the testis was coated with friable deposits composed of parasite cells, the collecting ducts contained intact spermatophores containing apparently viable spermatozoa. In such cases, parasites were not confluent with the testicular cavity, but were confined to surrounding haemal sinuses (Fig. 7). Multinucleate trophonts were observed to be attached to the outer wall of haemolymph vessels (Fig. 8). Occasional dividing parasite stages were observed within the haemal spaces of the hepatopancreas (Fig. 9). Haemocyte encapsulation responses were not observed in any of the infected crabs assessed.

Electron microscopy revealed that the haemolymph and tissues of *L. depurator* harboured a parasitic dinoflagellate parasite similar to *Hematodinium* spp. previously described from other marine crustacean hosts. Although the transmission electron microscopy materials represent sections through the parasite life stages, the proposed numbers of nuclei are consistent with observations of similar life stages observed in histological preparations. Based upon the host type and geographical location of collection, the parasite was identified as *Hematodinium perezii*, with EM representing the first ultrastructural representation of the type species of the genus. Stages observed included uninucleate, binucleate, and trinucleate trophonts (Fig. 10–12). Uninucleate trophonts had a mean length of  $6.92 \pm 0.84 \mu\text{m}$  and width of  $5.50 \pm 0.72 \mu\text{m}$  (mean  $\pm$  SD,  $n = 30$ ). Binucleate trophonts had a mean length of  $7.56 \pm 1.47 \mu\text{m}$  and width of  $6.62 \pm 1.39 \mu\text{m}$  (mean  $\pm$  SD,  $n = 15$ ). Trinucleate trophonts had a mean length of  $10.10 \pm 0.95 \mu\text{m}$  and width of  $8.73 \pm 0.58 \mu\text{m}$  (mean  $\pm$  SD,  $n = 5$ ). Each of these stages was characterized by nuclei containing condensed chromatin profiles, cytoplasm containing numerous lipid droplets, trichocysts and mitochondria (Fig. 10–13), and a surrounding alveolar membrane (Fig. 14). Structures resembling the micropores of apicomplexans were also observed in several trophonts (Fig. 15).

**SSU and LSU rRNA genes.** The SSU rRNA amplification product from *H. perezii* infecting *L. depurator* was 1,772 bp in length after removal of primer and flanking ITS1 sequences. Alignment of the six SSU rRNA cloned sequences revealed the presence of two polymorphic sites. The first was at 433 bp from the 5'-end (e.g. two clones, one from each sample, had a

C in place of an A), and the second at 1,442 bp from the 5'-end (e.g. same two clones above had a G in place of an A). Comparison of the SSU rRNA sequences from *H. perezii* to those in GenBank from the *Hematodinium* sp. infecting *C. sapidus* revealed only 2 bp differences between both parasites (alignment length of 1,593 bp). Both maximum likelihood and parsimony (data not shown) analysis produced similar tree topologies and confirmed this close affiliation grouping *H. perezii* from *L. depurator* and the *Hematodinium* sp. from *C. sapidus* together (Clade A, Fig. 16). In our analysis and that of others (Jensen et al. 2010), clade A was sister to a second strongly supported clade (Clade B) containing SSU rRNA sequences from a *Hematodinium* sp. parasitizing a number of other crustacean hosts including *N. norvegicus*, *C. tanneri*, *C. opilio*, and *C. bairdi* (100% support). Both of the above-described clades were sister to SSU rRNA sequences from the genus *Syndinium* (Fig. 16). The *H. perezii* SSU rRNA sequences from *L. depurator* have been deposited in GenBank with accession numbers EF065717–EF065718.

The partial LSU rRNA amplification products from *H. perezii* infecting *L. depurator* and the *Hematodinium* sp. infecting *C. sapidus* were 1,068 bp in length after removal of primer and flanking ITS2 sequences, apart from two cloned sequences from the *Hematodinium* sp. infecting *C. sapidus* that were 1,076 bp in length due to an insertion of an 8 bp repeat (GTTTACTT) at bp 653 from the 5'-end of the LSU rRNA gene sequence. The 15 cloned sequences, five from each sample, from *H. perezii* contained 12 single nucleotide polymorphisms (SNP) between cloned sequences, with the majority (11/12) being transitions. The 15 cloned sequences from the *Hematodinium* sp. infecting *C. sapidus* contained seven SNPs with 5/7 being transitions. Four conserved SNPs (all transitions) were observed between sequences from *H. perezii* infecting *L. depurator* and the *Hematodinium* sp. infecting *C. sapidus*. Consequently, the parasite LSU rRNA sequences from both *L. depurator* and *C. sapidus* had low mean intraspecific genetic distances (' $p$ ' = 0.002 and 0.001 respectively). Similarly, the mean interspecific genetic distance observed between *H. perezii* and the *Hematodinium* sp. from *C. sapidus* was also low (' $p$ ' = 0.006), highlighting the highly conserved nature of the LSU rRNA gene, in addition to the SSU rRNA gene, between these parasites. The LSU rRNA sequences from *H. perezii* infecting *L. depurator* and the *Hematodinium* sp. infecting *C. sapidus* have been deposited in GenBank with accession numbers JN641962–JN641991.

**ITS1 and ITS2 regions.** The ITS1 amplification products from *H. perezii* infecting *L. depurator* were 343 bp in length after removal of primer and flanking sequences. Thirteen of the 15 ITS1 cloned sequences, five from each sample of *H. perezii*, were identical, with the remaining two differing by a single SNP in each sequence – a single transition and a single transversion. BLAST analysis indicated that the ITS1 sequences from *H. perezii* infecting *L. depurator* had a high similarity with the ITS1 sequences of the *Hematodinium* sp. infecting *C. sapidus* (i.e. 94% identity, 98% coverage) and also with partial ITS1 sequences from a *Hematodinium* sp. infecting *P. trituberculatus* and *S. serrata* from China (i.e. 97% identity, 88% coverage). The *Hematodinium* sp. ITS1 sequences from other hosts including *N. norvegicus*, *C. pagurus*, *C. opilio*, *C. tanneri*, and *C. angulatus*, had an 85% identity to *H. perezii*. However, as coverage was greatly reduced to 15% (i.e.; ~ 54 bp), the ITS1 sequences from *H. perezii* infecting *L. depurator* were aligned with ITS1 sequences from the *Hematodinium* spp. infecting *C. sapidus* (16 sequences), *P. trituberculatus* (three sequences), and *S. serrata* (one sequence) only. The resulting partial ITS1 alignment was

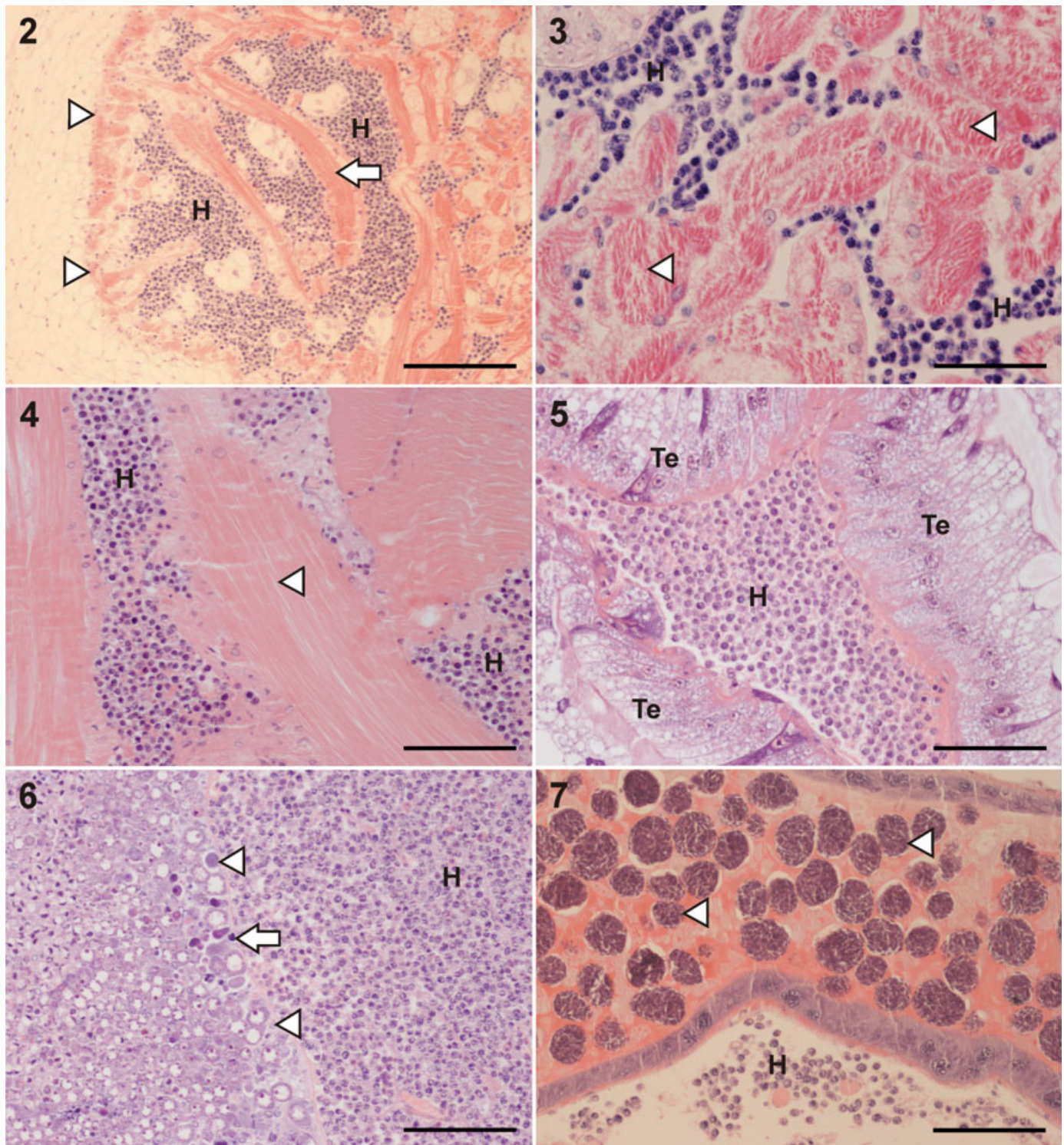


Fig. 2–7. Light micrographs of *Hematodinium perezii* infection in *Liocarcinus depurator*. **2.** Heart showing parasites in haemal sinuses (H). Myocardium (white arrow) and pericardium (white arrowheads) appear normal. Scale bar = 200  $\mu$ m. **3.** Heart showing massed parasites in haemal spaces (H) and relatively normal myocardium (white arrowheads). Scale bar = 50  $\mu$ m. **4.** Skeletal muscle within claw showing massed parasites in haemolymph (H) between muscle fibre blocks (white arrowhead). Scale bar = 100  $\mu$ m. **5.** Hepatopancreas with normal tubule epithelia (Te) and massed parasites within the distended haemal sinuses (H). Scale bar = 100  $\mu$ m. **6.** Ovary (white arrow) showing apparent arrest of oocytes in previtellogenic state and apoptosis of primary oocytes and oogonia (white arrowheads). Parasites are confluent with oocytes throughout much of organ (H). Scale bar = 100  $\mu$ m. **7.** Testis collecting duct with apparently normal spermatophores (white arrowheads) and limitation of parasites to surrounding haemal sinuses (H). Scale bar = 100  $\mu$ m. All H&E staining.

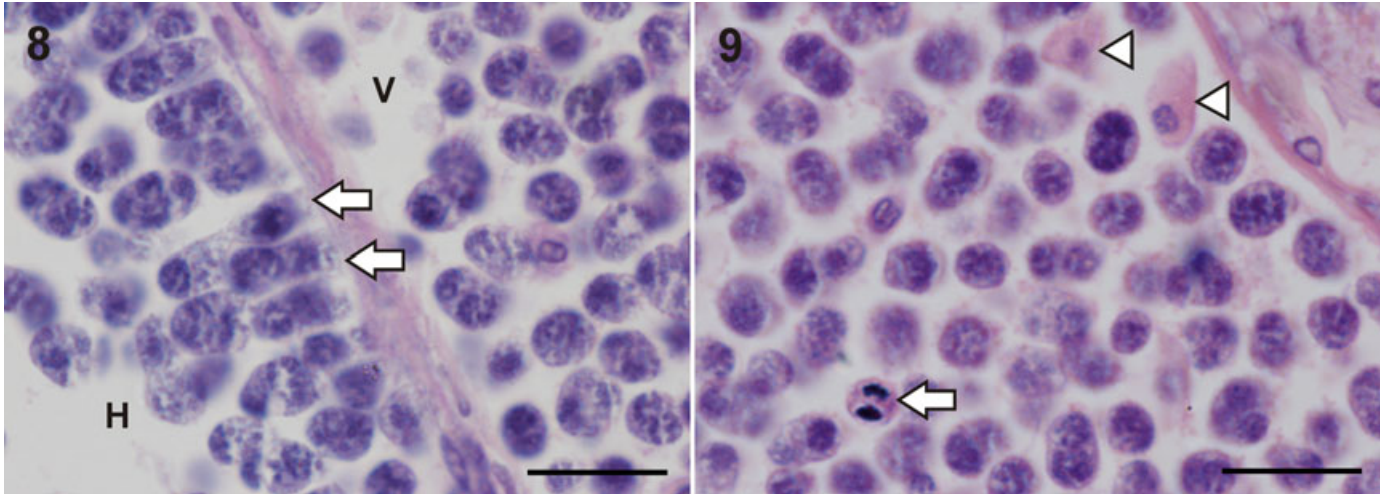


Fig. 8–9. Light micrographs of *Hematodinium perezi* infection in *Liocarcinus depurator*. **8.** Haemolymph vessel with multinucleate trophonts attached to the outer wall (white arrows). Massed circulating parasites are present within the vessel (V) and haemal spaces (H). Scale bar = 25  $\mu$ m. **9.** Haemal space within hepatopancreas containing massed parasites including dividing stages (white arrow) and occasional host haemocytes (white arrowheads). Scale bar = 25  $\mu$ m. Both H&E staining.

263 bp. A number of SNPs, deletions and insertions, as well as several repetitive regions (i.e. GGT, CTT, CA) were observed to differ between *Hematodinium* isolate groups (i.e. *H. perezi* from *L. depurator*, the *Hematodinium* sp. infecting *C. sapidus*, and the *Hematodinium* sp. infecting *P. trituberculatus* and *S. serrata*). Consequently, the mean intraspecific genetic distances within the partial ITS1 region were low: with ‘p’ = 0.001 for *H. perezi* infecting *L. depurator*; ‘p’ = 0.003 for the *Hematodinium* sp. infecting *C. sapidus*; and ‘p’ = 0.009 for the *Hematodinium* sp. infecting *P. trituberculatus* and *S. serrata* with both hosts grouped together and the latter figure higher than the rest, probably due to the small sample size (n = 4). The mean interspecific genetic distances observed between the *Hematodinium* sp. from the three host groupings were significantly larger: with ‘p’ = 0.025 between *H. perezi* from *L. depurator* and the *Hematodinium* sp. infecting *P. trituberculatus* and *S. serrata*; and ‘p’ = 0.046 between *H. perezi* from *L. depurator* and the *Hematodinium* sp. from *C. sapidus*. The topologies of trees generated by maximum likelihood (Fig. 17) and parsimony analysis (not shown) were similar, and consistently grouped the ITS1 region sequences into three distinct clades indicative of the geographic location and species of host: Europe as the English Channel from *L. depurator*; east coast North America as Virginia from *C. sapidus*; and Asia as China from *P. trituberculatus* and *S. serrata*.

The ITS2 amplification products from *H. perezi* infecting *L. depurator* were 398 bp in length, apart from two clone sequences that were 400 bp in length due to an insertion of an 2 bp repeat (TA) at bp 396 from the 5'-end of the ITS2 region. The 15 cloned sequences, five from each sample of *H. perezi*, contained six single SNPs between cloned sequences, with four of these represented by transitions observed in a single cloned sequence, and the remaining two transversions observed in 2/15 and 7/15 clone sequences. The ITS2 amplification products from the *Hematodinium* sp. infecting *C. sapidus* were 395 bp in length after removal of primer and flanking sequences. Twelve of the 15 cloned sequences were identical, with the remaining three differing by a single transition in each sequence. The full-length alignment of the ITS2 region from the *H. perezi* infecting *L. depurator* and the *Hematodinium* sp. infecting *C. sapidus* was 402 bp. No ITS2

sequences exist for the *Hematodinium* sp. infecting *P. trituberculatus* and *S. serrata*. Similar to the partial ITS1 region alignment, a number of SNPs, deletions and insertions were observed to differ between *Hematodinium* isolate groups over the ITS2 region. The mean intraspecific genetic distance within the ITS2 region of *H. perezi* from *L. depurator* were low with ‘p’ = 0.004. Likewise, the mean intraspecific genetic distance for the *Hematodinium* sp. infecting *C. sapidus* was also low with ‘p’ = 0.002. The mean interspecific genetic distance observed between *H. perezi* from *L. depurator* and the *Hematodinium* sp. from *C. sapidus* were approximately an order of magnitude greater (‘p’ = 0.035). The ITS1 and ITS2 sequences from *H. perezi* infecting *L. depurator*, and the ITS2 sequences from the *Hematodinium* sp. infecting *C. sapidus*, have been deposited in GenBank with accession numbers EF065708–EF065716, EF153724–153729, and JN641962–JN641991.

## DISCUSSION

Infections caused by *Hematodinium* spp. have been reported from a wide range of geographical locations and host crustaceans, many of which support important commercial fisheries (reviewed by Stentiford and Shields 2005). The definitive identity of the pathogens and number of possible species has received little attention, primarily due to a lack of distinctive characters and molecular sequence data from the type species *H. perezi*. We report the rediscovery of *H. perezi* infecting one of the type hosts, *L. depurator*, from the English Channel, and document the associated histology and ultrastructure of the infecting vegetative trophont stages. Comparison of the parasites SSU rRNA gene and ITS region sequences to those in GenBank indicates that three distinct *H. perezi* genotypes are found infecting several portunid hosts, and substantiates the suggested delineation of *H. perezi* from the *Hematodinium* sp. infecting a number of other crustacean host species from the Northern Hemisphere.

Several morphological features of *Hematodinium* resembling those from the original description by Chatton and Poisson (1931) were observed in infected *L. depurator*. Most notably, parasites with strongly basophilic nuclei were observed in haematoxylin and eosin stained materials. This feature is reported

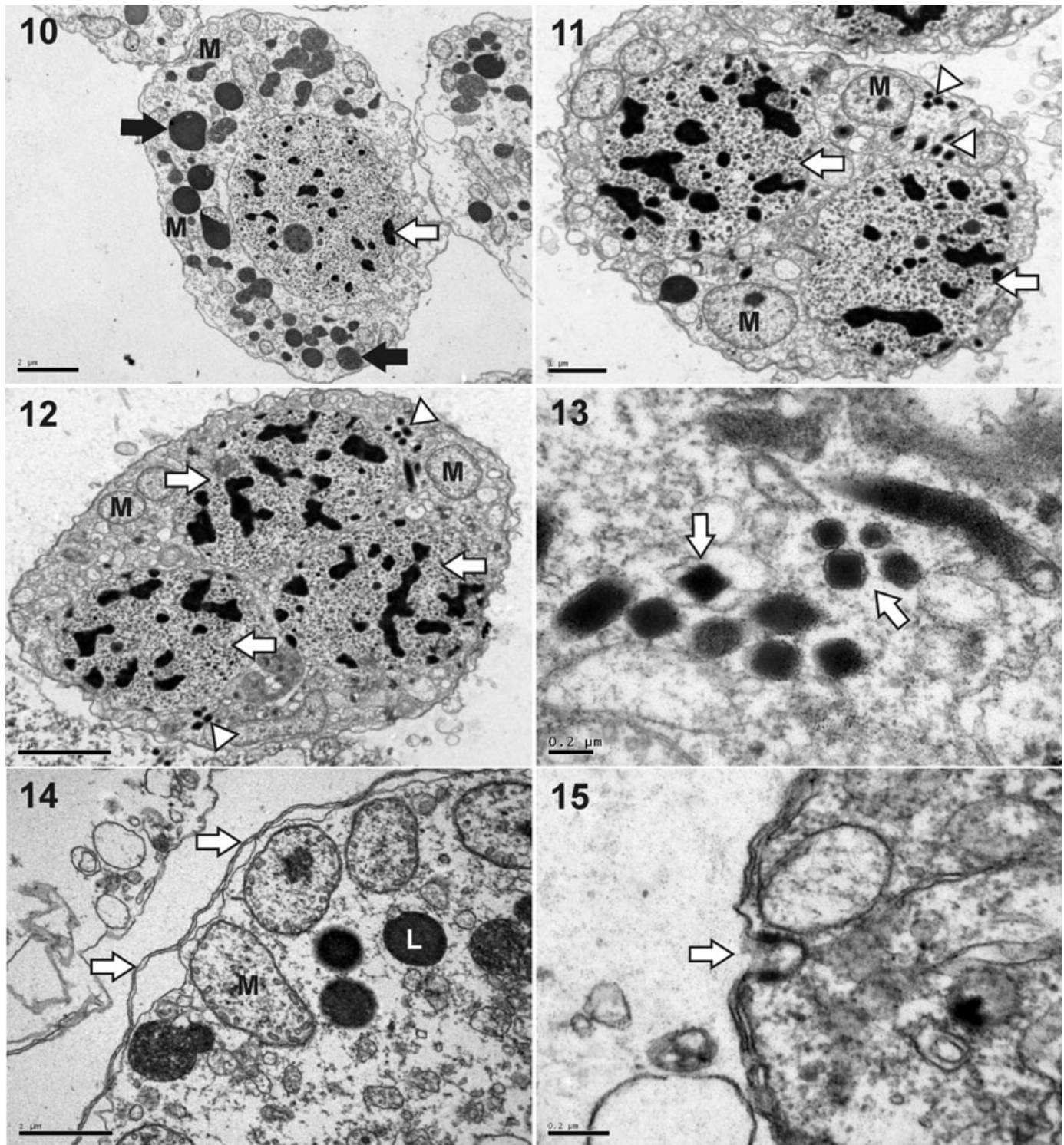


Fig. 10–15. Transmission electron micrographs of *Hematodinium perezi* infection in *Liocarcinus depurator*. **10.** Uninucleate trophont with mitochondria (M), lipid droplets (black arrows), and single nucleus containing condensed chromatin profiles (white arrow). Scale bar = 2  $\mu\text{m}$ . **11.** Binucleate trophont. Mitochondria (M), nuclei (white arrows), and trichocysts (white arrowheads). Scale bar = 1  $\mu\text{m}$ . **12.** Trinucleate trophont. Mitochondria (M), trichocysts (white arrowheads), nuclei (white arrows). Scale bar = 2  $\mu\text{m}$ . **13.** Increased magnification of trichocysts (white arrows) in trophont. Scale bar = 200 nm. **14.** Periphery of trophont showing alveolar membrane (white arrows) and peripherally aligned mitochondria (M) and lipid droplets (L). Scale bar = 2  $\mu\text{m}$ . **15.** Micropore in alveolar membrane of trophont (white arrow). Scale bar = 200 nm.

in all *Hematodinium* histological studies and, along with its presence in a crustacean host, is routinely considered as diagnostic for this genus (Stentiford and Shields 2005). Parasite

stages were identified as uninucleate and multinucleate trophonts containing condensed chromatin profiles, lipid droplets, mitochondria, and a surrounding alveolar membrane.

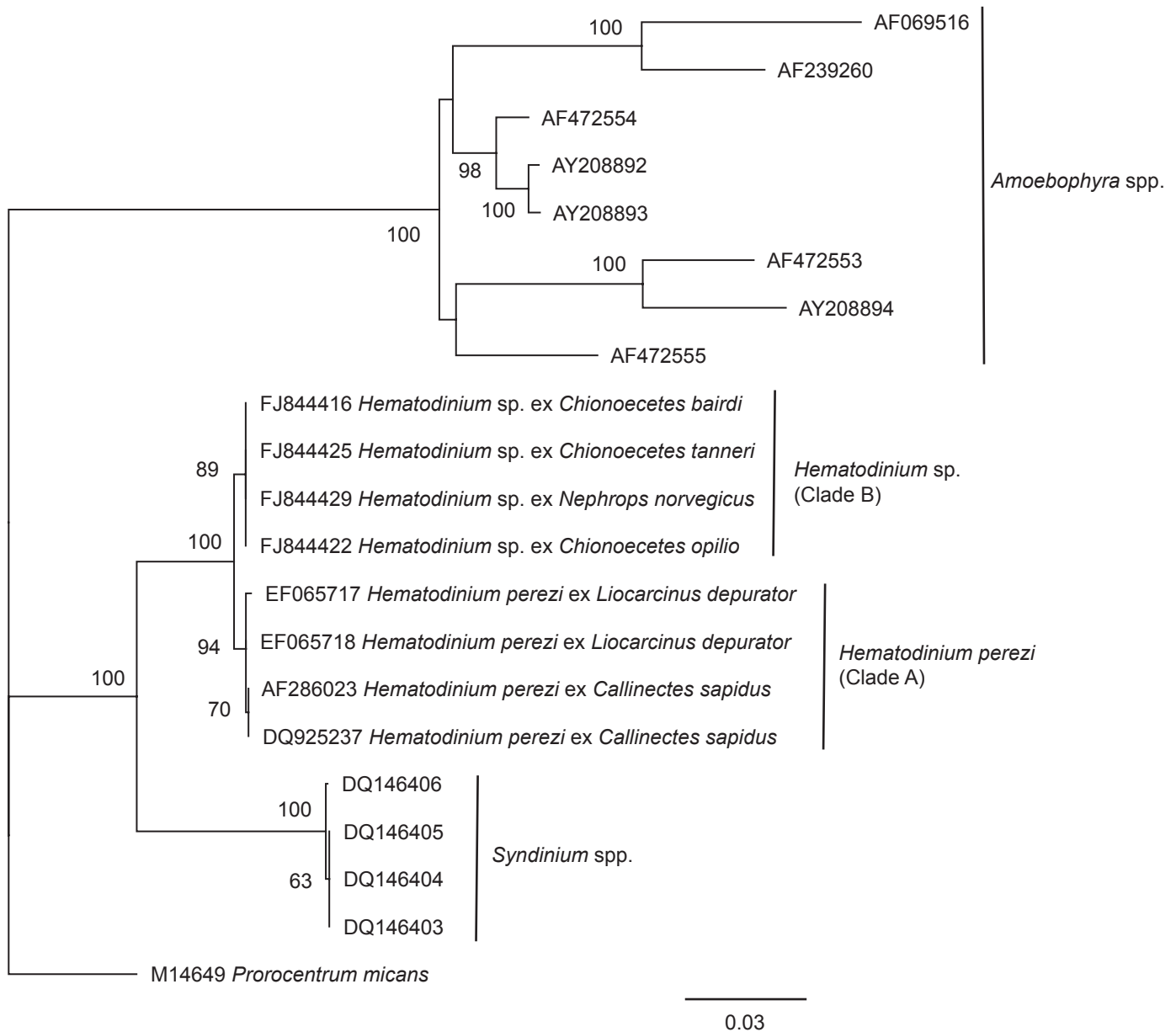


Fig. 16. Maximum likelihood (ML) tree resulting from analysis of the small subunit (SSU) rRNA gene sequences from *Hematodinium* spp. and other members of Syndinea, with *Prorocentrum micans* used as the outgroup. Numbers at nodes represent Bootstrap support values for each clade. Clade A (*Hematodinium perezii*) and clade B (*Hematodinium* sp.) designations are from Jensen et al. (2010).

Trichocysts were also observed in the cytoplasm of the trophonts, and were described by Chatton and Poisson (1931) from nonmotile *H. perezii* stages that were “teardrop-like” in shape and also containing refringent granules. Although Chatton and Poisson (1931) only observed trichocysts in these cells, they were probably present in the other stages examined, as they are difficult to observe via light microscopy. Micropores were also observed in several trophonts and have previously been documented from in vitro and in vivo forms of the *Hematodinium* sp. infecting *N. norvegicus* (Appleton and Vickerman 1996). The trophonts were of comparable size and morphology to other vegetative *Hematodinium* spp. described from crustaceans (Field et al. 1992; Hudson and Adlard 1996; Stentiford and Neil 2010; Stentiford and Shields 2005).

The above findings, together with the host species and geographical location of collection adjacent to the type location, led us to believe that we had rediscovered *H. perezii* in one of its type hosts.

Subsequent to the original description of *H. perezii* by Chatton and Poisson (1931), *Hematodinium* infections have also been reported from the type hosts *C. maenas* and *L. depurator* from several other locations throughout Europe. Hamilton, Morrill, and Shaw (2007) described a *Hematodinium* sp. infecting *C. maenas* from the Clyde Sea on the West Coast of Scotland. In this study, the parasite from *C. maenas* was reported as morphologically similar to *H. perezii* and other reported *Hematodinium* spp. descriptions. However, it was > 97% similar in the ITS region to the *Hematodinium* sp.

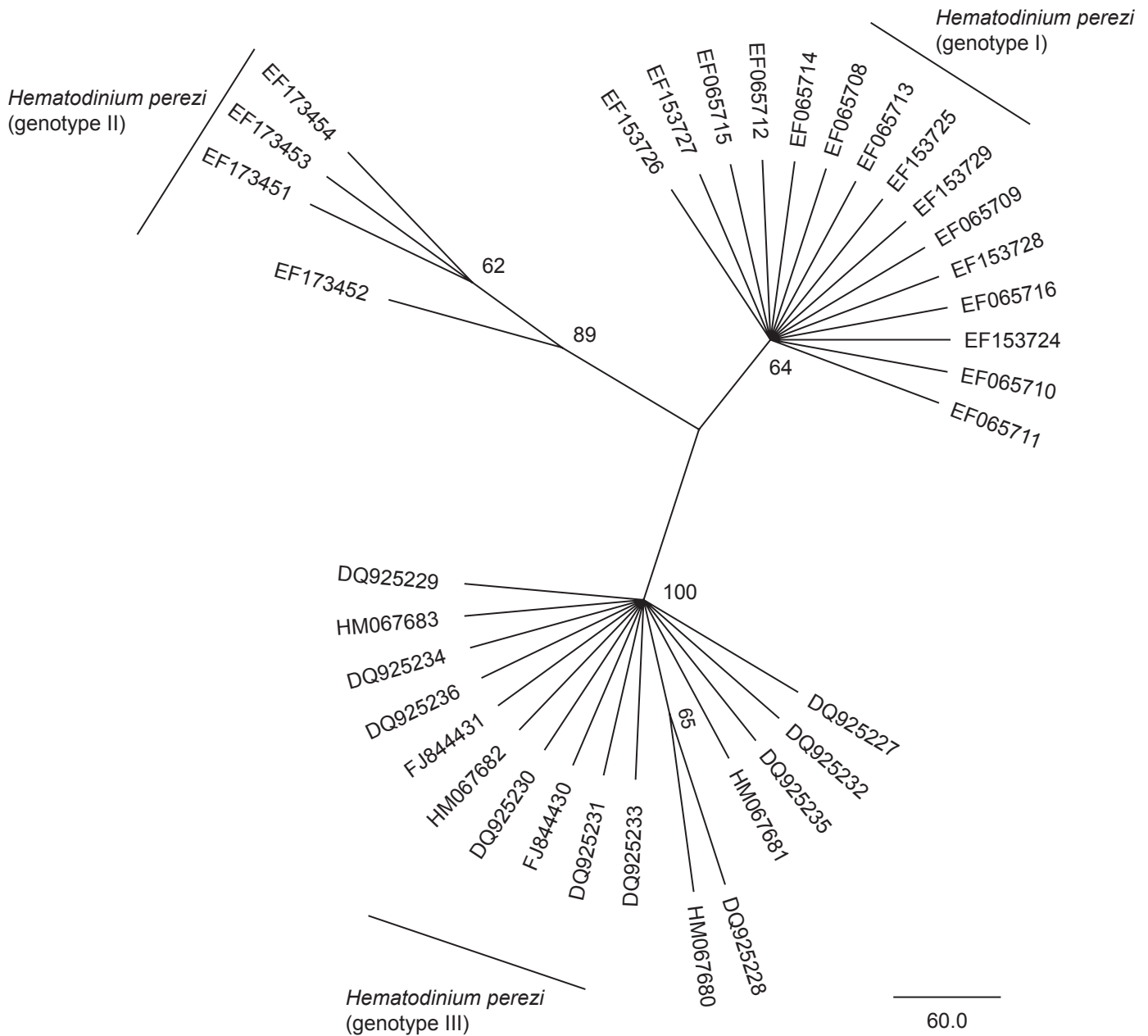


Fig. 17. An unrooted maximum likelihood (ML) tree resulting from analysis of the ITS1 rRNA region from *Hematodinium perezii* infecting *Liocarcinus depurator* (genotype I), *Portunus trituberculatus* and *Scylla serrata* (genotype II), and *Callinectes sapidus* (genotype III). Numbers at nodes represent Bootstrap support values for each clade.

known to infect *N. norvegicus* from this location, indicating infection by the same species (Field et al. 1992; Small et al. 2007b). Likewise, Eigemann, Burmeister, and Skovgaard (2010) also reported a *Hematodinium* sp. infecting *L. depurator* from Danish waters, and describe that its ITS1 sequences were identical to a *Hematodinium* sp. infecting *P. bernhardus* Linnaeus, 1758, *C. opilio*, and *N. norvegicus*. The *Hematodinium* species infecting these hosts (and others), primarily from boreal locations, is an apparent generalist, capable of infecting many different crustaceans (see Hamilton et al. 2007, 2010; Jensen et al. 2010; Small et al. 2006, 2007b). Critically, however, the above examples were from host geographic locations distant to those reported by Chatton and Poisson (1931). Prior to the

current study, only one other paper has reported a *Hematodinium* infection in one of its type hosts in, or near, to its type location. Stentiford and Feist (2005) observed a parasite putatively identified as *H. perezii* in histological sections of the hepatopancreas of a single *C. maenas* from the South English coastline. Unfortunately, the infection stage was very light, and we were unable to obtain molecular sequence data from this sample (HJS., pers. observ.). Clearly, the absence of unambiguous morphological features as well as any DNA sequences from *H. perezii* has facilitated the potential misidentification of this parasite, and has, in part, resulted in numerous *Hematodinium* sp. or *Hematodinium*-like descriptions now present in the literature.

Few, if any, morphological characters have been identified that can help to delineate between *Hematodinium* isolates. It is also problematic to obtain and definitively identify similar life stages for comparative ultrastructural studies. Factors such as host, season, or availability of nutrients may also influence cell morphology, as has been shown to occur in *Perkinsus* spp. (Bushek, Ford, and Allen. 1994; Goggin and Lester 1995; La Peyre, Faisal, and Bureson 1993). In addition, only one *Hematodinium* species, that from *N. norvegicus*, has had its in vitro life cycle documented (Appleton and Vickerman 1998), and even then this may be different from the in vivo life cycle, or modulated by media components or culturing techniques. Molecular sequence data are increasingly used to supplement morphological taxonomic studies, or provide clarification and resolution in cases where morphological characters alone cannot differentiate between parasites (see Bureson, Reece, and Dungan 2005). For example, the ITS regions of the rRNA complex have previously been used to examine relationships between *Hematodinium* isolates (Eigemann et al. 2010; Hamilton et al. 2007, 2010; Hudson and Adlard 1996; Jensen et al. 2010; Small et al. 2007a, b; Xu et al. 2010), and are routinely used to discriminate between species and strains of other important dinoflagellates and aquatic parasites (Dungan and Reece 2006; Goggin 1994; Hill et al. 2010; Litaker et al. 2007; Small et al. 2009). Although the ITS regions of the rRNA complex are transcribed, they are not translated into functional protein sequences, and are therefore often found to be much more variable than the adjacent rRNA genes. The variation observed within the ITS regions offer an increased resolution of the potential relationships that are not readily apparent in analysis of the more conserved rRNA sequences, such as the SSU rRNA gene, which are generally used for higher level classification studies. This trend is observed in our analysis of *H. perezii* from *L. depurator*, as the SSU rRNA and 5'-end of the LSU rRNA gene sequences were closely related to those from the *Hematodinium* sp. infecting *C. sapidus*.

However, comparison of the ITS1 region sequences from *H. perezii* infecting *L. depurator* to those in GenBank indicated that several other portunid hosts were also infected with *H. perezii*, albeit subtly different forms. In the absence of a comprehensive morphological study of the type species, and agreement of phylogenies derived from multiple independent loci, we suggest that the ITS regions be used to designate different *H. perezii* genotypes infecting the following hosts in an order of sequence similarity to the *H. perezii* sequences from *L. depurator* (genotype I): *P. trituberculatus* and *S. serrata* (genotype II), and *C. sapidus* (genotype III) (Fig. 17). *Hematodinium* infections have recently emerged in China, and have been found infecting several different cultured hosts. *Hematodinium* has been reported as the causative agent of a "milky blood disease" in *Portunus trituberculatus* and *Scylla serrata* (Li et al. 2008; Xu et al. 2007) and recently in the ridgetail prawn, *Exopalaemon carinicauda* Holthuis, 1950 (Xu et al. 2010). In their preliminary phylogenetic analysis of the partial ITS1 region, Xu et al. (2010) suggest that the parasite infecting *E. carinicauda* is closely related to the parasite species infecting *P. trituberculatus* and *S. serrata*, because only two nucleotide insertions are noted between *Hematodinium* sequences from all three hosts. As variation in the ITS regions has been documented from other *Hematodinium* isolates (Hamilton et al. 2010; Small et al. 2007a, b, and observed in *H. perezii* in the current study), it is likely that these hosts are infected with the same genotype. However, as only four partial ITS1 region sequences representing *H. perezii* genotype II are deposited in GenBank (i.e. one sequences from *S. serrata*,

and three sequences from *P. trituberculatus*), additional sequencing efforts directed towards the ITS1 region and other informative regions are required to fully ascertain the sequence variation within this genotype, its relationship with other *H. perezii* isolates, and other *Hematodinium* spp.

Our analysis also revealed that the *H. perezii* isolate infecting *L. depurator* was found to be closely related, but again distinct, from the *H. perezii* isolate infecting *C. sapidus* from the East Coast of the United States (genotype III). Newman and Johnson (1975) were the first to describe a *Hematodinium* sp. infecting *C. sapidus* from North Carolina, Georgia, and Florida in the United States. It was subsequently reported as infecting *C. sapidus* from other states including Maryland and Virginia (Messick 1994), and from New Jersey, South Carolina, Delaware, and Texas (Messick and Shields 2000). American researchers studying the disease in *C. sapidus* from the above locations have intermittently described the infective parasite as *H. perezii* or *Hematodinium* sp., probably after MacLean and Ruddell (1978) described three new Eastern Seaboard hosts for *H. perezii*, and erroneously stated that *H. perezii* was identified in *C. sapidus* (Newman and Johnson 1975; originally described a *Hematodinium* sp. resembling *H. perezii*). In our analysis, *H. perezii* genotype III is composed of ITS1 sequences generated from three independent studies in which isolates of *H. perezii* from Virginia were sequenced, including infected *C. sapidus* haemolymph samples and water samples containing the parasite (Jensen et al. 2010; Li et al. 2010; Small et al. 2007a), demonstrating reproducible unique sequences within this genotype. However, because of the lack of sequence information on *H. perezii* from states other than Virginia, and the fact that the early reports in the literature of its presence in *C. sapidus* were based on blood smears or histological analysis, it is therefore unclear for most reports (see below), if the same *H. perezii* genotype (or even species) infects *C. sapidus* from these different locations on the Eastern Seaboard, and this needs to be addressed. The only exception may be hosts from Georgia, as an ITS1-targeted Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay developed by us gave similar banding patterns when applied to *Hematodinium* DNAs from blue crabs from Virginia and Georgia (Small et al. 2007a), tentatively indicating that these are infected with the same genotype. However, as recommended by Small and Pagenkopp (2010), sequencing of the parasite's ITS regions is needed to confirm this suggestion.

Likewise, *Hematodinium* infections have been reported from a number of other crustaceans from the East Coast of the United States, and include *C. maenas*, the spider crab *Libinia emarginata* Leach, 1815, the stone crab *Menippe mercenaria* Say, 1818, the lesser blue crab *Callinectes similis* Williams, 1966, the portunid crab *Ovalipes ocellatus* Herbst, 1799, the rock crabs *Cancer irroratus* Say, 1817 and *Cancer borealis* Stimpson, 1859, various xanthid crabs, and amphipods (Johnson 1986; MacLean and Ruddell 1978; Messick and Shields 2000; Sheppard et al. 2003). Much like the situation with *C. sapidus*, it remains unclear if these hosts are infected with the same *H. perezii* genotype (or even species), and future studies should be directed towards clarifying this issue.

The substantial variation between the nonalignable ITS1 sequences from *H. perezii* and what is probably a second *Hematodinium* sp. (clade B, Jensen et al. 2010) is somewhat surprising, especially considering that the SSU rRNA gene sequences were well conserved between *H. perezii* and those representing clade B. However, the secondary structures of the divergent ITS1 clades were remarkably similar, suggesting a similar functional role (Jensen et al. 2010). Jensen et al. (2010)

hypothesize that the ITS region may have evolved rapidly in connection with functional diversification during a host switch. Support for this hypothesis may come from the present study in which we have documented three different *H. perezii* ITS1 genotypes infecting different portunid hosts from distant geographical locations (i.e. Europe, USA, and Asia). Invasive crustaceans have received significant attention in the last 10 yr, primarily due to their deleterious effects upon indigenous hosts and ecosystems. *Carcinus maenas* is reported to have been introduced to the east coast of the USA around 1817 by human-mediated transport mechanisms (i.e. hull fouling and solid ballast, see Carlton and Cohen 2003), concurrent with trans-Atlantic trade in goods between Europe and the USA. Conversely, *C. sapidus* has also been documented regularly along the French Atlantic coastline since 1900 (Goulletquer et al. 2002; Nehring, Speckels, and Albersmeyer 2008). In addition, *C. maenas* has been recently found in Japanese waters in 1994 (see Carlton and Cohen 2003) and it is now likely that it is also present, albeit currently unreported, in Chinese waters, as is predicted to happen in invasion models for this crab species (Compton, Leathwick, and Inglis 2010). It is therefore possible that *H. perezii* may have been introduced to the United States from Europe (i.e. France), or vice versa, via an infected host, and may have spread to China during a second introduction event. This would account for the subtly different *H. perezii* ITS1 sequences presented in genotypes I, II, and III, presumably rapidly evolving due to a host switch, and, in particular, the closer affinity that genotype II in hosts from China has with genotype I in *L. depurator* from Europe, rather than with genotype III in *C. sapidus* from the United States. Other dispersal hypotheses include global transport of infectious stages via oceanic currents or ballast water. However, as dinospores, which are thought to be the infective stage of *H. perezii* infecting *C. sapidus*, are detectable for only a short time (i.e. up to 7 d) after being released into the seawater from infected crabs, this short survival time may limit its transmission potential (Li et al. 2010).

*Hematodinium perezii* was originally reported as a rare parasite of *C. maenas* and *L. depurator*. In the original description by Chatton and Poisson (1931), C. Perez observed the parasite in only one of 1,000 *C. maenas* from Arcachon in 1905, whereas R. Poisson observed the parasite in 3 of 470 *L. depurator* from Luc-Sur-Mer and Banyuls-Sur-Mer in 1925 and 1927, respectively, and M. Prenant observed the parasite in only one of 3,000 *C. maenas* from Penpoull near Roscoff in 1929. Chatton and Poisson (1931) suggested that the parasite is infrequently observed because it undergoes rapid proliferation and may kill its host quickly, as is the case for the *H. perezii* infecting *C. sapidus*, which in laboratory-based challenge experiments induced a median time to death of approximately 30 d (Shields and Squyars 2000). However, the rarity of the parasite may also be a function of seasonality as other *Hematodinium* spp. show consistent seasonal peaks in prevalence (Briggs and McAliskey 2002; Eaton et al. 1991; Field et al. 1998; Hamilton, Morrill, and Shaw 2009; Messick and Shields 2000; Meyers et al. 1990; Stentiford et al. 2001). Alternatively, Rye Bay may contain physiographical and hydrographical features or substrate types that support concentration of infective stages and thus support the higher prevalence observed in *L. depurator*.

Several PCR-based diagnostic assays are now available for detection and quantification of *Hematodinium* infections in crustaceans and environmental samples (Eigemann et al. 2010; Frischer et al. 2006; Greubl et al. 2002; Hamilton et al. 2009; Hudson and Adlard 1994; Li et al. 2010; Small et al. 2006, 2007a, b). We have previously designed a set of diagnostic

primers to detect *H. perezii* genotype II infecting *C. sapidus*, and have shown that the same primer set can also be used to detect genotypes I and III infecting *L. depurator* and *P. trituberculatus*, respectively (Small et al. 2007a). Cross-species amplification was the result of high sequence similarity at primer annealing sites preventing single-round (PCR) identification; however, the particular genotypes infecting each host could be identified by performing a restriction enzyme digestion of the partial ITS1 amplification product. The primers were originally designed to target the ITS1 region sequences of *H. perezii* infecting *C. sapidus*, and a nucleotide mismatch occurs in the reverse priming region in *H. perezii* infecting *L. depurator*. Although the forward and reverse primers are still capable of binding to the *Hematodinium* ITS1 regions of this genotype, we suggest that if this assay is used to detect *H. perezii* in *L. depurator*, the reverse primer would be redesigned to the following, to ensure 100% sequence identity with target *H. perezii* genotype I ITS1 rRNA sequences - reverse primer 5'-CTAGTCATACGTTTGAAAAAAGCC-3'.

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