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Nucleotide sequence determination of the partial SSU rDNA gene and ITS1 region of *Hematodinium* cf. *perezi* and *Hematodinium*-like dinoflagellates

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ABSTRACT: Partial sequences of the small subunit (SSU) rDNA gene of the parasitic dinoflagellates *Hematodinium* cf. *perezi* from the blue crab *Callinectes sapidus* and 3 *Hematodinium*-like organisms from other decapods, *Nephrops norvegicus*, *Chionoecetes bairdi* and *C. opilio*, were compared. The V9 variable domain of this gene showed no nucleotide differences between the 4 parasitic dinoflagellates. Comparison of this region with other protozoans suggested that the 3 *Hematodinium*-like organisms should be considered to be within the genus *Hematodinium*. Differences in nucleotide sequence in the partial ITS1 regions from these 4 parasitic dinoflagellates suggest that there are 2 new species. The *Hematodinium* organism ex *N. norvegicus* warrants the creation of a new species. The *Hematodinium* organisms ex *C. bairdi* and *C. opilio* are probably the same species and also warrant the creation of another new species, while *H.* cf. *perezi* remains distinct from the other isolates.

KEY WORDS: *Hematodinium* · 18S/ITS1 rDNA genes · Molecular taxonomy

INTRODUCTION

Marine crustaceans are parasitized by 2 orders of dinoflagellates: the Blastodiales and the Syndiniales (Shields 1994). In the Syndiniales, the type species, *Hematodinium perezi* Chatton & Poisson, 1931, was described from the portunid crabs *Carcinus maenus* and *Portunus depurator*; the second species, *H. australis* Hudson & Shields, 1994, was described from another portunid, *Portunus pelagicus*. *H.* cf. *perezi* has also been recorded from *Callinectes sapidus*, a decapod of commercial importance (Newman & Johnson 1975, Messick 1994). *Hematodinium*-like dinoflagellates have had severe effects on *Chionoecetes bairdi*, *C. opilio* and *Nephrops norvegicus*, all decapods of commercial importance (Meyers et al. 1987, Field et al. 1992).

Hudson & Shields (1994) have suggested that the taxonomy of the genus *Hematodinium* requires better definition. Taxonomic studies are difficult because electron microscope studies from the type species have not been undertaken and dinospores have not been recorded. The paucity of morphological characters useful for diagnosis of parasites from different hosts further complicates the systematics of the group.

Nucleotide sequence determination of the small subunit (SSU) ribosomal DNA (rDNA) gene and the internal transcribed spacer 1 (ITS1) region has proved a useful diagnostic tool at the levels of genus and species in insects (Porter & Collins 1991), trematodes (Adlard et al. 1993) and protozoans (Cai et al. 1992, Goggin 1994, Diggles & Adlard 1995). Our aim was to determine and compare partial SSU rDNA and ITS1 regions of *Hematodinium* cf. *perezi* and 3 *Hematodinium*-like dinoflagellates. These partial SSU sequences were compared to 6 other dinoflagellates to investigate the systematic distance between members of the genus *Hematodinium* and the organisms classified currently as *Hematodinium*-like, while the partial ITS1 sequences were used to determine the extent of genetic variance between isolates.

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MATERIALS AND METHODS

Sample collection. A sand crab, *Portunus pelagicus*, infected with *Hematodinium australis* was collected in March 1992, from Moreton Bay, Queensland, Australia. A Norway lobster, *Nephrops norvegicus*, infected with a *Hematodinium*-like dinoflagellate was collected in January 1993, from the Firth of Clyde, on the west coast of Scotland, UK. Two blue crabs, *Callinectes sapidus*, infected with *Hematodinium* cf. *perezi* were collected in November 1993, from the Rappalamoda River, Chesapeake Bay, Virginia, USA. Two Tanner crabs, *Chionoecetes bairdi*, infected with a *Hematodinium*-like dinoflagellate and a Snow crab, *C. opilio*, infected with a *Hematodinium*-like organism were collected in February 1993, near Juneau and Dutch Harbor, Alaska, USA, respectively. *H. australis* from *P. pelagicus* was frozen at –60°C. Hemolymph samples from *Chionoecetes bairdi*, *C. opilio*, and *N. norvegicus* containing *Hematodinium*-like dinoflagellates were collected via syringe and fixed directly in 100% ethanol. *Hematodinium*-infected testes from *Callinectes sapidus* were fixed directly in 100% ethanol and were shaken to produce sediment containing the *Hematodinium* cells. Attempts to obtain samples of the type species, *H. perezi*, from France were unsuccessful.

DNA extraction, purification and amplification. DNA was extracted from the *Hematodinium* organisms using a standard phenol/chloroform method as described by Hudson & Adlard (1994). The first internal transcibed spacer (ITS1) of ribosomal DNA and flanking 3' end of the SSU was amplified by polymerase chain reactions (PCR) using oligonucleotide primers, Primer 1 (forward primer: 5' GTT CCC CTT GAA CGA GGA ATT C) and Primer 2 (reverse primer: 5' CGC ATT TCG CTG CGT TCT TC). PCR amplifications were performed on *Hematodinium* organisms from *Chionoecetes bairdi*, *C. opilio*, *Callinectes sapidus*, *Nephrops norvegicus* and *Portunus pelagicus* as described by Hudson & Adlard (1994). Hudson & Adlard (1994) showed that Primers 1 and 2 favoured the amplification of dinoflagellate DNA. PCR products were purified (Magic PCR Preps, Promega) prior to sequencing. Primers were designed from published sequences available through GenBank: Primer 1, from conserved arthropod sequences; Primers 2 and 3, from conserved protozoan sequences; Primer 4, 100% conserved sequences from yeasts to rabbits; Primer 5, from conserved eukaryotic sequences.

DNA sequencing and analysis. DNA sequencing was by the dideoxy chain termination method (Sanger et al. 1977) using a dsDNA cycle sequencing kit (Gibco/BRL). Five primers were used for sequencing: Primer 1; Primer 2; Primer 3 (forward primer, 5' GTC CCT GCC CTT TGT ACA CA); Primer 4 (forward primer 5' CGT AGG TGA ACC TGC GGA AGG ATC); and Primer 5 (reverse primer 5' GAT CCT TCT GCA GGT TCA CCT AC). Sequencing primers were end-labelled with phosphorus (^{32}P). Both the sense and the non-sense strands were sequenced for the partial SSU, while only the sense strand was sequenced for the partial ITS1 region as Primer 2 failed to produce a readable sequence. However, for the partial ITS1 region Primers 1, 3 and 4 produced identical sequences therefore validating results. Sequencing fragments were electrophoresed at a constant power of 45 W.

DNA sequences were aligned by eye and manipulated with the Eye-ball Sequence Editor (ESEE) software (Cabot & Beckenbach 1989). The partial SSU sequences minus the amplification primer sequence, Primer 1, of *Hematodinium* organisms were compared to known sequences stored in Genbank using the Basic Local Alignment Search Tool (BLAST) routine (Altschul et al. 1990) available through the Australian National Genomic Information System (ANGIS). A total of 6 species from 3 dinoflagellate orders were compared. They were: Order Prorocentrales, *Prorocentrum micans*; Order Phytodiniales, *Gloeodinium viscum*; Order Gymnodiniales, Family Zooxanthellaceae, *Symbiodinium microadriaticum*, *S. corculorum*, *S. pilosum*, *S. meandrinae*. Gaps introduced into the alignment were treated as missing data. Informative sites are bases which are different for 1 sequence but similar in other sequences. A similarity matrix was used to establish relatedness in the V9 domain of the partial SSU region and for the partial ITS1 region.

RESULTS

Strong PCR products of approximately 680 base pairs were obtained from the *Hematodinium* spp. and the *Hematodinium*-like organisms (see Hudson & Adlard 1994). These products were then sequenced. Sequences were obtained for 237 bases from the 3' end of the SSU region (Fig. 1) and 278 bases from the 5' end of the ITS1 region (Fig. 2) for *Hematodinium* cf. *perezi* (Isolate designation #1) and the *Hematodinium*-like organisms from *Nephrops norvegicus* (Isolate designation #2), *Chionoecetes bairdi* (Isolate designation #3) and *C. opilio* (Isolate designation #4). There was no nucleotide variation either within isolates of *H.* cf. *perezi* or within isolates of *Hematodinium*-like organisms from *C. bairdi*. No sequence was obtained from *H. australis* because the amplified product was very faint (see Hudson & Adlard 1994) as a result of degraded DNA from the original sample.

Using the BLAST routine on the partial SSU region of the *Hematodinium* organisms, the nucleotide se-

Fig. 1. Alignment of the nucleotide sequence from the 3' end of the small subunit (SSU) region of 4 types of *Hematodinium* (#1 to #4), Order Syndiniales: (1) *H.* cf. *perezi* from *Callinectes sapidus*, (2) *Hematodinium*-like dinoflagellate from *Nephrops norvegicus*, (3) *Hematodinium*-like dinoflagellate from *Chionoecetes bairdi*, (4) *Hematodinium*-like dinoflagellate from *C. opilio*; and 6 species from 3 other dinoflagellate orders: Order Prorocentrales, (5) *Prorocentrum micans*; Order Phytodiniales, (6) *Gloeodinium viscum*; Order Gymnodiniales, Family Zooxanthellaceae, (7) *Symbiodinium microadriaticum*, (8) *S. corculorum*, (9) *S. pilosum*, and (0) *S. meandrinae*. (·) Identical nucleotide

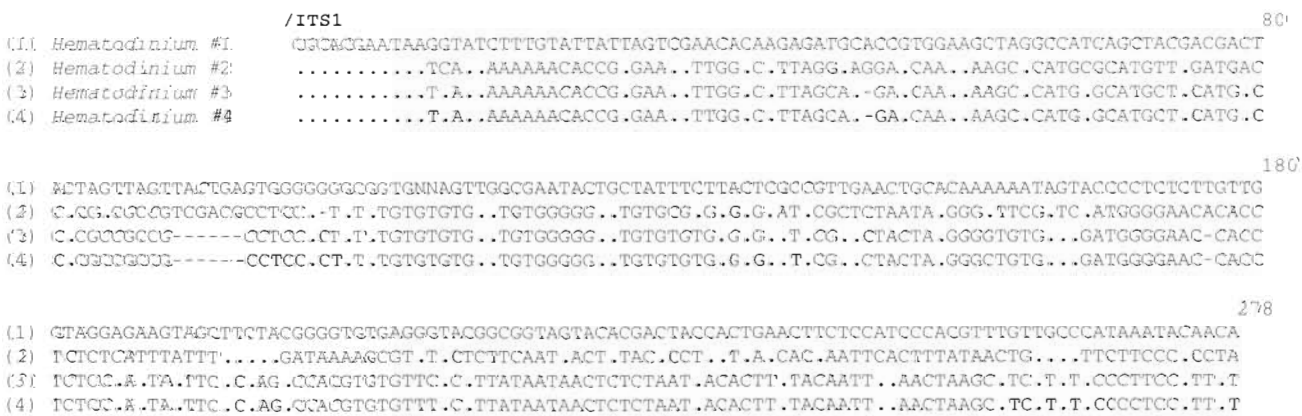

Fig. 2. Alignment of the nucleotide sequence from the 3' end of the small subunit (SSU) region and the 5' end of the ITS1 region of 4 types of *Hematodinium* (#1 to #4): (1) *H.* cf. *perezi* from *Callinectes sapidus*, (2) *Hematodinium*-like dinoflagellate from *Nephrops norvegicus*, (3) *Hematodinium*-like dinoflagellate from *Chionoecetes bairdi*, (4) *Hematodinium*-like dinoflagellate from *C. opilio*. (·) Identical nucleotide; (–) missing nucleotide; (N) indeterminate nucleotide

quence showed similarities to other dinoflagellates (Fig. 1). *H.* cf. *perezi* (#1) showed a 92.2% (179 bases) similarity to *Gloeodinium viscum* over 194 bases; it also showed a 92.2% (200 bases) similarity to *Prorocentrum micans* over 217 bases and 90.2% (193 bases) similarity for the 3 species of *Symbiodinium* over 214 bases and 89.7% (192 bases) similarity for *S. meandrinae* over 214 bases. Fig. 1 also shows that of the 237 bases obtained from the 3' end of the *Hematodinium* organisms (#1 to #4), the region between bases 110 and 170 (125 to 65 bases upstream from the SSU/ITS1 boundary) was highly variable when compared to the other dinoflagellates. In this region of 60 bases, *H.* cf. *perezi* showed a 78.3% (47 bases) similarity to *G. viscum* with 9 informative sites; *H.* cf. *perezi* showed a 73.3% (44 bases) similarity to *P. micans* with 8 informative sites; and showed a 68.3% (41 bases) similarity to the 4 *Symbiodinium* spp. with 5 informative sites (Table 1). In this variable region there were no differences between the 4 species of *Symbiodinium* and the 4 types of *Hematodinium*. Within the 4 types of *Hematodinium* there was 1 base difference (position 80) between the *Hematodinium*-like dinoflagellates from *Chionoecetes bairdi* and *C. opilo* and the other 2 *Hematodinium* organisms.

In the partial ITS1 region (Fig. 2), *H.* cf. *perezi* (#1) showed a 22.7% (63 bases) similarity with *Hematodinium* #2 with 19 informative sites and 1 deletion over 278 bases; *H.* cf. *perezi* showed a 25.9% (72 bases) similarity for both *Hematodinium* #3 & #4 with 16 informative sites and 3 deletions over 278 bases; *Hematodinium* #2 showed a 66.5% (185 bases) similarity for both *Hematodinium* #3 and #4 with 139 informative sites and 4 deletions over 278 bases; *Hematodinium* #3 showed a 99.3% (276 bases) similarity over 278 bases with *Hematodinium* #4 (Table 2).

Table 2. Similarity matrix for the partial ITS1 region for the 4 *Hematodinium* organisms

	Hemato-dinium #1	*Hemato-dinium* #2	*Hemato-dinium* #3
Hematodinium #1	–		
Hematodinium #2	22.7%	–	
Hematodinium #3	25.9%	66.5%	–
Hematodinium #4	25.9%	66.5%	99.3%

DISCUSSION

Hematodinium australis can be distinguished from *H.* cf. *perezi* by its larger vegetative stage, its round as opposed to vermiform plasmodium, its austral geographic location, and by its different host species. The trophont of *H. australis* differs from the *Hematodinium*-like organisms from *Chionoecetes bairdi* and *C. opilio* in that it is smaller, possesses trichocysts, and has the small form of beaded chromatin. *H. australis* differs from the *Hematodinium*-like organism from *Nephrops norvegicus* in that the trophont stage is larger, it possesses the small form of beaded chromatin, and it does not appear to have the vermiform plasmodium (Hudson & Shields 1994). The *Hematodinium*-like organisms from *N. norvegicus*, *C. bairdi* and *C. opilio* differ from *H.* cf. *perezi* in that they possess the large form of beaded chromatin, while the larger trophont size and absence of trichocysts in the *Hematodinium*-like organisms from *C. bairdi* and *C. opilio* differentiates them from that of *N. norvegicus* (see Table 3).

Changes in morphological/biological characters can be induced either by the immediate environment of the parasite, e.g. the host and its own environment, or by the direct expression of different genomes. To test whether the morphological differences that exist for *Hematodinium* organisms are phenotypic or genotypic responses we examined the partial nucleotide sequence of the 3' end of the SSU gene from 2 isolates of *H.* cf. *perezi* and *Hematodinium* ex *C. bairdi* and 1 isolate of the other 2 *Hematodinium* organisms. One of the criteria for selecting a tandemly repeated unit such as the rDNA gene is that these units are subject to concerted evolution which tends to homogenize sequences among individuals and among populations (Dowling et al. 1990). Therefore, only a small number of isolates are required to type genetically each organism. Sogin & Elwood (1986) stated that in the SSU gene there were 2 regions that drift very rapidly in all eucaryotic rDNA and one of these is situated in the 3' end of the SSU gene. This region, the V9 domain (see Neefs et al. 1990 for nomenclature) is situated approx-

Table 1. Similarity matrix showing relatedness (% homology) in the V9 domain of the partial small subunit (SSU) region for the 4 *Hematodinium* organisms (*Hem* #1–4) and the 6 dinoflagellates: *Prorocentrum micans* (*P. mic.*); *Gloeodinium viscum* (*G. vis.*); *Symbiodinium microadriaticum* (*S. mic.*); *S. corculorum* (*S. cor.*); *S. pilosum* (*S. pil.*); and *S. meandrinae* (*S. mea.*)

	Hem #1–4	*P. mic.*	*G. vis.*	*S. mic.*	*S. cor.*	*S. pil.*	*S. mea.*
Hematodinium organisms #1–4	100%	73.3%	78.3%	68.3%	68.3%	68.3%	68.3%

Table 3. Characteristics of vegetative stages of different species and forms of Hematodinium from various hosts. V: veriform; R: round; N.O.. not observed

Species: / Host:	*H. perezi* / *C. sapidus*	*H. australis* / *P. pelagicus*	*H*-like / *N. norvegicus*	*H*-like / *C. bairdi*
Average size (µm)	6.4–10.4	9.9–11.9	6–10	12.8–15.6
Plasmodium form	V	R	V & R	R
Presence of trichocysts	Yes	Yes	Yes	No
Chromatin pattern	Small	Small	Large	Large
Dinospores present	N.O.	N.O.	Yes[a]	Yes
Water temp.[b]	Jan–Jul 4–26°C	Jul–Dec[c] 17–26°C	Mar–Apr 10–13°C	Jun–Aug 6.2°C

[a] R. Field pers. comm.; [b] water temperature when host collected; [c] Shields & Wood (1993)

imately between 125 and 65 bases upstream of the SSU/ITS1 boundary. The V9 domain was present in the partial sequence of the 3' end of the SSU gene from the 4 *Hematodinium* organisms, and the analysis of its sequence provided a clear indication of the genetic status of the *Hematodinium*-like organisms.

Differences exist between other orders of dinoflagellates in the V9 domain. We found between 24 and 34% difference in nucleotide sequence in the V9 domain when we compared 2 free-living orders of dinoflagellates (1 species in each) and 1 symbiotic order of dinoflagellate (1 family, 4 species) to a parasitic order containing *H.* cf. *perezi*. Differences have also been observed between families in the same order in the V9 domain. Nucleotide sequence differences of 70% occurred in the V9 domain of 2 hypotrich ciliates, *Oxytricha granulifera* (Oxytrichidae) (Schlegel et al. 1991) and *Euplotes aediculatus* (Euplotidae) (Sogin & Elwood 1986). Furthermore, comparison of the V9 domain of 2 genera, *Onychodromus quadricornutus* and *Oxytricha granulifera*, within the same family (Oxytrichidae) showed 18.3% difference in nucleotide sequence (Schlegel et al. 1991). Species level comparison within the V9 domain of the free-living ciliate genus *Tetrahymena* showed 13 species that fell into 2 major homology groups with 10% difference in nucleotide sequence over this region (Sogin et al. 1986). However, no differences occurred between 4 species of symbiotic dinoflagellate *Symbiodinium* spp. In 2 congeneric species of parasitic protozoa, the coccidian *Cryptosporidium* displayed a 99% similarity over the SSU gene, but none of these differences occurred within the V9 domain (Cai et al. 1992). From this data there were sequence differences in the V9 domain between representatives of the same order but 2 different families (holotrichous ciliates), and between representatives of the same family but 2 different genera (*Onychodromus* and *Oxytricha*). Between congeneric species, there were both differences (*Tetrahymena*) and no differences (*Symbiodinium* and *Cryptosporidium*).

High levels of homology within the V9 domain of these protistans appear indicative of 'generic' status when compared with classical morphological systematics. No differences occurred in the V9 domain of the 4 *Hematodinium* organisms, and only 1 site varied (99.6% similarity) in the remaining partial SSU sequence. As only a partial SSU sequence was investigated, it is possible that nucleotide differences between *Hematodinium* and *Hematodinium*-like organisms could occur elsewhere in the SSU gene. Rowan & Powers (1992) showed that in the partial SSU of 2 dinoflagellates, *Symbiodinium microadriaticum* and *S. pilosum*, there were only 2 nucleotide differences in 478 bases, even though they are very different from each other by morphological, biochemical, physiological and behavioral criteria. However, we believe that the evidence derived from the highly variable V9 domain strongly suggests that the *Hematodinium*-like organisms should be considered to be within the genus *Hematodinium*. It is worthy of note that without investigation of confamilial genera no definitive placement of cryptic species within a genus can be made.

Assuming the *Hematodinium*-like dinoflagellates from *Chionoecetes bairdi*, *C. opilio* and *Nephrops norvegicus* are in the same genus as *H.* cf. *perezi*, we then compared the partial ITS1 sequence to determine the extent of genetic variance between the 4 *Hematodinium* organisms. ITS regions evolve fast and may vary among species within a genus or among populations (Lee & Taylor 1992). The large variation of the partial ITS1 region between *H.* cf. *perezi* and *Hematodinium* #2, #3 and #4 is consistent with morphological characters. *Hematodinium* #2, #3 and #4 have round plasmodia and large beaded chromatin, whereas *H.* cf. *perezi* has vermiform plasmodia and small beaded chromatin. Even with the morphological similarities between #2, #3 and #4, #2 varied substantially from #3 and #4, whereas there was only 2 base differences between #3 and #4 in the partial ITS1 region and no differences in the partial SSU gene.

Comparison of 2 congeneric species of coccidian parasites, *Cryptosporidium parvum* and *C. muris*, revealed only 1 major difference between the species over the complete ITS1 region of 381 bases (Cai et al. 1992). Goggin (1994) concluded that 2 species of apicomplexan protists, *Perkinsus atlanticus* and *P. olseni*, belonged to a single species as they were similar over the complete ITS1 region of 196 bases and the 5.8S and the ITS2 regions, while both showed a 23% sequence difference in the ITS1 region when compared to a third species. Differences in nucleotide sequence in the partial ITS1 regions from these 4 *Hematodinium* organisms suggest that there are 2 new species. The *Hematodinium* organism ex *N. norvegicus* warrants the creation of a new species. The *Hematodinium* organisms ex *C. bairdi* and *C. opilio* are probably the same species residing in different hosts and warrant the creation of another new species, while *H.* cf. *perezi* remains distinct from the other isolates. However, we stop short of erecting new species until sequence data becomes available for confamilial genera within the Order Syndiniales (sensu Taylor 1987), e.g. *Syndinium* and *Trypanodinium* which both have representatives parasitic in crustacea. Only then can a new diagnosis of the genus *Hematodinium* be made.

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