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**Species of the Parasitic Genus *Duboscquella* are Members of the  
Enigmatic Marine Alveolate Group I**

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Running title: *Duboscquella* phylogeny

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**Small subunit ribosomal RNA gene sequences of *Duboscquella* spp. infecting the tintinnid ciliate, *Favella ehrenbergii*, were determined. Two parasites were sampled from different localities. They are morphologically similar to each other, and both resembling *D. aspida*. Nevertheless two distinct sequences (7.6% divergence) were obtained from them.**

**Phylogenetic trees inferred from maximum likelihood and maximum parsimony revealed that these two *Duboscquella* spp. sequences are enclosed in an environmental clade named Marine Alveolate Group I. This clade consists of large number of picoplanktonic organisms known only from environmental samples from various parts of the ocean world wide, and which therefore lack clear characterisation and identification. We provide morphological and genetic characterization of these two *Duboscquella* genotypes included in this enigmatic clade. *Duboscquella* spp. produce a large number of small flagellated spores as dispersal agents and the presence of such small cells partially explains why the organisms related to these parasites have been detected within**

environmental genetic libraries, built from picoplanktonic size fractions of environmental samples. The huge diversity of Marine Alveolate Group I and the finding that parasites from different marine protists belonging to this lineage suggest that parasitism is a widespread and ecologically relevant phenomenon in the marine environment.

**Key words:** alveolate; *Duboscquella*; environmental clone; *Favella ehrenbergii*; Marine Alveolate Group I; parasitic dinoflagellate.

## Introduction

Analyses of small subunit ribosomal RNA gene (SSU rDNA) sequences from environmental samples ('environmental clones') have revealed an unexpected diversity of picoplankton (<5µm) organisms in marine waters (Diéz et al. 2001; López-García et al. 2001; Moon-van der Staay et al. 2001). Although environmental clones recovered from marine environments include many different taxonomic groups, including prasinophytes, haptophytes and acanthareans, two groups, i.e. stramenopiles and alveolates are particularly

dominant within genetic libraries (López-García et al. 2001; Moon-van der Staay et al. 2001). Such alveolates were further discovered to comprise, in addition to the sequences belonging to ordinary dinoflagellate or ciliate lineages, two distinct lineages with unknown representatives, named the Marine Alveolate Group I and the Marine Alveolate Group II, respectively (López-García et al. 2001). The latter two groups are ubiquitous, being discovered virtually anywhere from tropic to Antarctic regions (López-García et al. 2001; Moon-van der Staay et al. 2001), from coastal and oceanic waters, sediments and hydrothermal vents to permanent anoxic deep waters (Groissillier et al. 2006; Jeon et al. 2006; Stoeck et al. 2006). However, so far, our knowledge of the kind of organisms, which actually represent these lineages is incomplete. Recently, some parasitic dinoflagellates belonging to the subdivision Syndinea of the division Dinoflagellata (Fensome et al. 1993), such as *Syndinium*, *Hematodinium* and *Amoebophrya*, were found to belong to the Marine Alveolate Group II (Groissillier et al. 2006; Skovgaard et al. 2005). The Marine Alveolate Group I still remains 'enigmatic' (Groissillier et al. 2006; Stoeck et al. 2006) with no clear identifications having been made since the recognition of this group (López-García et al. 2001).

Recently Dolven et al. (2007) reported that sequences of some of the 'associates' (probably parasites) of four species of polycystine radiolarians and one phaeodarian species are included in the clade of Marine Alveolate Group I, although no morphological information of these species was provided.

During the course of taxonomic studies on parasitic dinoflagellates along the Japanese coasts, members of the genus *Duboscquella* were often encountered. This genus is known to be parasitic mainly infecting tintinnid ciliates, although other types of hosts are known. The ciliates, *Favella ehrenbergii* (Claparède et Lachmann) Jörgensen, *Tintinnopsis campanula* (Ehrenberg) Dady, and *Codonella galea* Haeckel, were reported to be infected by *Duboscquella tintinicola* (Chatton 1920). However, it later became clear that the flagellated dispersal stage from these different hosts can be distinguished morphologically and that their corresponding trophonts (feeding stage) also have specific characters, meaning that each ciliate species can be infected by a different species. A second species, *D. anisospora* is characterized by its unequal flagellated spore size and unique trophont morphology (Chatton 1952). Cachon (1964) described five new species that were distinguished from each other by

virtue of their trophont morphology. Hosts of these new species include non-tintinnid ciliates and dinoflagellates. Coats (1988) examined the cytology and life history of a parasite in the tintinnid ciliate, *Entintinnus pectinis*, and described it as *D. cachoni* Coats, differing from other members of the genus by the structure of its trophont, the pattern of sporogenesis, and spore morphology.

In most cases, species of *Duboscquella* are lethal to their hosts, even having a significant impact on entire populations. This is the case for *Duboscquella cachoni* and the ciliate *Eutintinnus pectinis* in Chesapeake Bay, USA (Coats and Heisler 1989). The genus *Duboscquella* has a characteristic pattern of sporogenesis: there are successive nuclear and cytoplasmic divisions without interruption, termed "palintomy", through which a large number of biflagellate spores are produced. Two types of motile spores have been reported, i.e. the macrospore and the microspore. Both types of spore may be formed by the same species, but only one type is released from a given host (Coats 1988). Ciliates play an important role as predators of microorganisms in the marine food webs (e.g. Johannson et al. 2004), and more attention should be paid to the ecology of their parasites.

The SSU rDNA of several samples of *Duboscquella* spp. was sequenced and the resultant phylogenetic analysis revealed that the genus belongs to the Marine Alveolate Group I clade. This is the first time that this organism has been linked with the ubiquitous, but enigmatic oceanic lineage.

## Results

### Trophont morphology and sporogenesis

Infected tintinnids were collected mainly in summer. Individuals of *Favella ehrenbergii* in the late stage of infection by *Duboscquella* sp. were easily detected because the parasite can be seen through the transparent host lorica (Figs 1, 2). Despite infection, most of the hosts in the collected samples were alive and actively swimming. Sporogenesis (formation of spores) occurred outside the host cell, but within the host lorica. Sometimes *Duboscquella* sporocytes (cells that give rise to spores) were found beyond the confines of the host lorica, spread by the activity of the cilia or by the movement of the tintinnid.



Even here, the parasite continued to exhibit normal cell division. In the sporogenesis stage, a long, rosary-like chain of transparent sporocytes were formed (Figs 1, 2). The process of cell division is very fast (Fig. 2). After 3 hours of this division process, a huge number of motile spores were produced (Fig. 2F). Sporocytes developed flagella when the cell diameter attained a range of 8-15 $\mu$ m, but cell division continued further. The final products of sporogenesis were small bean-shaped flagellated cells, 4.0 – 6.0  $\mu$ m in length and 1.3 – 2.0  $\mu$ m in width (Fig. 2G). Each cell had two flagella, but details of these structures could not be obtained. No dimorphism of motile spores has been observed.

#### Transmission electron microscopy

The specimens used for transmission electron microscopy were obtained from the same sample that contained the specimen designated as *Duboscquella* sp. 2 in our molecular work (see below). It is thus likely that they also belong to *Duboscquella* sp. 2, although no molecular identification was made on the specimens used for ultrastructural investigations. The section of a relatively early

stage of sporogenesis within the host lorica, roughly corresponding to the stage C or D of Figure 2, is presented in Figure 3A. Each parasite cell contains many spherical vesicles. Figure 3B shows a section of a small spherical sporocyte, roughly corresponding to the stage E of Figure 2. The cell possesses a nucleus with dense chromosome-like structures, but these structures do not show features of typical dinokaryotic chromosomes (Fig. 3B). These stages contain small spherical vesicles located at the periphery of the cell, cortical alveoli (= amphiesmal vesicles) (Fig. 3C) and trichocysts (Fig. 3D).

#### Phylogenetic analyses

We successfully amplified and obtained SSU rDNA sequences from 6 *Duboscquella*-like individuals from three localities (Fig. 1) and recognized two different sequences, i.e. *Duboscquella* sp. 1 (Fig. 1A) from Ishikari harbour, Hokkaido (DDBJ/EMBL/GenBank accession number AB295040) and *Duboscquella* sp. 2 from Hamanako Lake (Fig. 1B) and from Kurosaki harbour (Fig. 1C) (DDBJ/EMBL/GenBank accession number: AB295041). Of 1735 bp

(the SSU rDNA sequence length of *Duboscquella* sp. 1), 132 bp differed between sp. 1 and sp. 2 (7.6% divergence). No sequence variations were detected between the Hamana material and the Kurosaki material. The parasites with different SSU rDNA sequences, i.e. sp. 1 and sp. 2, were morphologically similar and it was not possible to distinguish one from another at the light microscopical level. Both sequences were substantially different from that of its host, *Favella ehrenbergii*, and there is no possibility of contamination from the host cell.

Only the ML tree with bootstrap values for both MP and ML is presented (Fig. 4). Tree topologies of both methods were basically the same. In the phylogenetic analyses, in addition to the ciliates, apicomplexa and perkinsozoa, three major groups, corresponding to 1) a typical dinoflagellate clade (subdivision Dinokaryota) (Fensome et al. 1993), 2) Marine Alveolate Group I and 3) Marine Alveolate Group II were recognized (Fig. 4). These clades were supported by high bootstrap values, although support for the Marine Alveolate Group II, including *Syndinium/Hematodinium* clade, in MP method was low (59%/92% = MP/ML). *Duboscquella* species were included in the Marine alveolate Group I

clade with high bootstrap support (100%/100% = MP/ML). The sequences from the two species of *Duboscquella* formed a clade and this clade was supported by high bootstrap values (100%/100% = MP/ML) (Fig. 4). The nearest sister of *Duboscquella* was an environmental clone sequence (OLI11011), which was obtained from seawater collected at a depth of 75 m in the equatorial Pacific Ocean (clones with numbers starting with 'OLI11' are also from the same environment: Moon-van der Staay et al. 2001). The OLI11033 came to sister position to the OLI11011/*Duboscquella* clade, although the bootstrap supports were low. The sister to this clade consists of three sequences, viz. DH147-EKD18, a sample collected at a depth of 2,000 m in the Antarctic polar front (López-García et al. 2001), BL010320.28, a coastal water sample collected in the north west Mediterranean Sea (Massana et al. 2004), and AT4-47, a sample collected from hydrothermal vent sediment from the Mid-Atlantic Ridge Rainbow (López-García et al. 2003). Environmental clones included in this clade are thus closely related to *Duboscquella*, but no perfect matches with our *Duboscquella* sequences were detected among the reported Group I sequences. In addition to this clade, three more clades were recognized in the Marine

Alveolate Group I lineage, each with high bootstrap values. The sequences from 'associates' of polycystine radiolarians and from a phaeodarian species (Dolven et al. 2007) were also included in the Marine Alveolate Group I clade. One of the four clades was dominated by sequences from these associates (Fig. 4) and associate from *Androccyclas gamphonyca* was included in another clade, which is dominated by environmental clones (Fig. 4). The phylogenetic positions of these 'associates' were quite distant from that of *Duboscquella* spp. (Fig. 4).

## Discussion

### Identity of the organisms

So far, 8 species have been described in the genus *Duboscquella* (Coats 1988).

They have been distinguished from each other based on the trophont and the spore morphologies, the pattern of sporogenesis, and the host specificity. We initially identified all the collected parasites as *Duboscquella aspida*, based on the pattern of sporogenesis and host species (*Favella ehrenbergii*) (see Table 2

in Coats 1988). However, the present study clearly indicates that there are at least two genetically-separated 'species' making it impossible to determine which is the true *D. aspida* Cachon. We therefore identified the two genotypes as *Duboscquella* sp. 1 and sp.2. The purpose of this paper is to report on the phylogenetic position of *Duboscquella* species, and the assessment of their taxonomic entities, including possible presence of cryptic species, should be addressed in future studies.

#### Phylogenetic consideration

In the recent classification systems, the genus *Duboscquella* has been classified in the parasitic order Syndiniales in the subdivision Syndinea of the division Dinoflagellata (e.g. Fensome *et al.* 1993), but its phylogenetic position to this point has been uncertain (Saldarriaga *et al.* 2004) because a molecular phylogenetic study of the genus has never been performed before. This first phylogenetic study clearly revealed that some, if not all, members of *Duboscquella* belongs to the Marine alveolate Group I, while other members of

the order Syndiniales, i.e. *Syndinium*, *Hematodinium* and *Amoebophrya*, belong to the Marine alveolate Group II (Skovgaard et al. 2005). Phylogenetically, Group I is distinct from Group II and the latter is more closely related to the typical dinoflagellates. As pointed out by Saldarriaga et al. (2004), in order to infer the phylogenetic affinities of *Duboscquella* with other members of the alveolates and especially with the typical dinoflagellates and the Marine Alveolate Group II (= part of Syndiniales), it is necessary to study the ultrastructure of *Duboscquella*. We have undertaken the ultrastructural study of *Duboscquella* sp. We were able to demonstrate that the sporocytes of *Duboscquella* possess cortical alveoli (amphiesmal vesicles) as well as trichocysts, both are structures typical for dinoflagellates. However, we did not observe presence of a typical dinokaryon at any stages during sporogenesis. Because of lack of a typical dinokaryon, *Duboscquella* cannot be regarded as typical dinoflagellate (Dinokaryota *sensu* Fensome et al. 1993). Our results indicate that the ultrastructure of *Duboscquella* has some similarities with that of parasitic organisms belonging to the Marine Alveolate Group II, i.e. *Hematodinium* or *Hematodinium*-like organisms (Appleton and Vickerman 1996,

1998; Field et al. 1992; Hudson and Shields 1994; Stentiford et al. 2002), *Syndinium* (Cachon and Cachon 1987; Ris and Kubai 1974) and *Amoebophrya* (Fritz and Nass 1992), including the presence of cortical alveoli, small vesicles, trichocysts and absence of a typical dinokaryon. However, the chromosomes of the latter organisms seem to be much denser than those of *Duboscquella*. The mode of nuclear division in *Syndinium* has been known to be unique and different from that of the typical dinoflagellates (Ris and Kubai 1974). To judge whether *Duboscquella* can be distinguishable ultrastructurally from members of the Marine Alveolate Group II or not, further ultrastructural studies, including mode of nuclear division and details of flagellar apparatuses, are needed.

As already mentioned, all the sequences published to date, which are included in the Marine Alveolate Group I lineage are of unknown organismal origin and no morphological information is available for them. Recently, Dolven et al. (2007) demonstrated that SSU rDNA sequences of some 'associates' of polycystine radiolarians and one phaeodarian species are included in the Marine Alveolate Group I. These 'associates' are probably parasites, but their morphologies have not been studied so far. In this respect, this is the first report



on the taxonomic identity of organisms belonging to the enigmatic lineage,

Marine alveolate Group I.

The present study demonstrated that species of *Duboscquella* can produce a large number of dispersal agents, which are small and can nearly reach the size range of picoplankton. When López-García *et al.* (2001) recognized the Marine Alveolate Group I, they analyzed environmental DNA extracted from seawater samples prefiltered on 5µm pore size filters. Since the members of the alveolates, e.g. dinoflagellates and ciliates, are generally large, it was quite surprising that a large number of alveolate species had been detected in this small size fraction. However, if a large number of dispersal agents of parasitic alveolates are distributed widely in the water column, as shown in this study, it is no surprising that the alveolate taxa have been detected in this small size fraction as environmental clones.

In this study, we demonstrated that *Duboscquella* is a member of the Marine Alveolate Group I. This result and the fact that their 'motile spores' are very small suggest that some of the environmental sequences belonging to the Marine Alveolate Group I are actually those from parasites related to *Duboscquella*. In

fact, Coats (1988) reported presence of small size dispersal agents (microspore) in *Duboscquella cachoni* Coats and these can be detected in environmental DNA surveys. In addition, parasites of radiolarian/phaeodarian protists (Dolven et al. 2007) together with some environmental sequences comprise distinct lineages, which are distantly related to *Duboscquella*-containing clade, within the Marine Alveolate Group I (Fig. 4). These facts suggest that many of the members of the Marine Alveolate Group I could be either related to *Duboscquella*-like organisms or parasites of radiolarian/phaeodarian protists. Given that the huge diversity of Marine Alveolate Group I and the finding that parasites from different marine protists belong to this lineage, this suggests that parasitism is really widespread and important in the marine environment. As previous works (e.g. Groisillier et al. 2006) have revealed, the environmental clones belonging to the Marine Alveolate Group I can be detected almost anywhere in the ocean, which means the oceanic waters must be teeming with these small parasitic spores.

## **Methods**

**Sampling:** Water samples were collected with a 40 µm or 100 µm pore size plankton net (NXXX25 or NXX13, RIGOSHA, Saitama, Japan). The samples were kept alive at low temperature and transported to the laboratory.

*Duboscquella* sp. 1 was found infecting the tintinnid ciliate, *Favella ehrenbergii* Claparède et Lachmann at Ishikari Harbour, Hokkaido, Japan on 14 July 2003.

*Duboscquella* sp. 2 was found infecting *F. ehrenbergii* at Hamana Lake, Shizuoka Prefecture, Japan on 9 September 2003 and at Kurosaki Harbour, Kurashiki, Okayama Pref. Japan on 27 June 2005. Note that Hamana Lake is directly connected to the Pacific Ocean through a short channel (at Enshu-nada) and the collection was made near this channel.

**Light microscopy and photographic record:** Individual host organisms infected by the parasites were isolated from field samples with a micropipette using a TS 100 inverted light microscope (Nikon, Tokyo, Japan). They were transferred to the centre of a vinyl tape frame attached to a glass slide (Horiguchi *et al.* 2000) and sealed with a cover glass for photography and observations. Parasites were photographed using a BX-50 light microscope, equipped with Nomarski interference optics (Olympus, Tokyo, Japan). The individual cells used

for PCR were first photographed (Fig. 1); the record of *Duboscquella* sp. 1 (from Ishikari) and sp. 2 (from Hamana Lake) being made while the cells were still inside their hosts but, in the case of *Duboscquella* sp. 2 (from Kurosaki), only cells outside the host lorica were photographed. The sequential photographs showing the development of the sporocytes (Fig. 2) were taken using a specimen collected at Rumoi, Hokkaido, Japan on 19 August 2004. This parasite was not used for sequencing.

**Transmission electron microscopy:** The specimens used for transmission electron microscopy were collected at Kurosaki Harbour, Kurashiki, Okayama Prefecture, Japan on 26 June 2005. Parasites within tintinnids were embedded in 1.5% low-temperature-gelling agarose (Merk, Darmstadt, Germany) made up in seawater and the piece of agarose gel with embedded tintinnids was initially fixed in 2.0% glutaraldehyde made up with 0.1M sodium cacodylate buffer (pH 7.0) with 0.1 M sucrose at room temperature for 2 hours. Then the piece of agarose was briefly rinsed in the same buffer before postfixation in 1% OsO<sub>4</sub> (in DW) at room temperature for 2 hours. After dehydration through an acetone series (30, 50, 70, 80, 90 and 100%), specimens were embedded in Spurr's resin (TAAB Laboratories Equipment Ltd., Berkshire, UK) and sectioned. Sections were placed on Formvar-coated copper grids and double stained with uranyl acetate and lead citrate. Thin sections were examined with a H-7650

transmission electron microscope (Hitachi, Tokyo, Japan) at 80kV.

### **Modified single-cell polymerase chain reaction, amplification and**

**sequencing:** Since extracting DNA of parasite from infected ciliate is difficult, we used the modified single-cell polymerase chain reaction (PCR) method described by Takano and Horiguchi (2004, 2006) to obtain the nucleotide sequences. The procedure for the PCR is as follows: After photography, the cover slip was carefully removed and the sporocytes of *Duboscquella* released from the lorica were transferred through a series of drops of sterile filtrated seawater on a clean glass slide using a micropipette. Several sporocytes (rather than single cells, hence the 'modified' single cell PCR technique) were transferred to a 200µl Perkin-Elmer tube containing 24.5µl PCR reaction mixture with a clean Pasteur pipette for the first round of PCR. Although this method does not amplify the DNA of a single cell, all the sporocytes used originated from a single parasite and are thus clonal.

For the SSU rRNA gene amplification, we used partially modified primers previously described by Nakayama *et al.* (1996). Primers used in this study are shown in Tables 1. The PCR was performed in two steps. In the first round of

PCR, almost the entire SSU rDNA was amplified using the terminal primers SR1 and SR12. In the second round of PCR, 1.0 $\mu$ L of PCR product of the first round of PCR was used as DNA template and the following 3 pairs of primers were used; SR1b & SR5, SR4 & SR9, and SR8 & SR12. The reaction mixture (50  $\mu$ L) of the second round of PCR contained: 36.25  $\mu$ L sterile distilled water, 5  $\mu$ L 10 $\times$  PCR buffer, with MgCl<sub>2</sub>, 2.5 mM dNTPs mixture, 2.5  $\mu$ L DMSO, 0.2  $\mu$ M of each pair of primers, 2.5 units *Taq* polymerase (Bioneer, Seoul, Korea). For the first round of PCR, we used the same mixture as for the second round, but it contained only half the volume.

Amplification reactions were performed using Gene Amp PCR Systems 2400 (Applied Biosystems, Foster City, CA, USA). The PCR conditions for both rounds were one initial cycle of denaturation at 93°C for 1 min, followed by 35 cycles of denaturation at 93°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 45 s. The temperature profile was completed by a final extension cycle at 72°C for 4 min. To check the amplification efficiency, 5  $\mu$ L of PCR products were electrophorized in 1% TAE agarose gels and stained with ethidium bromide. A 55  $\mu$ L aliquot of sterilized distilled water was added to the

PCR products to make the total volume up to 100 $\mu$ L, and 60 $\mu$ L of polyethylene glycol (PEG, 20% PEG6000 powder, 2.5M NaCl) was added to this PCR products and incubated on ice for 1hr to remove the primers and nucleotides excess. The solution was centrifuged at 14,000 rpm for 10 min at 4°C to pellet the purified DNA, which was then rinsed with 70% ethanol and air dried. The pellet was redissolved in sterilized distilled water. The purified PCR products were sequenced directly using the ABI PRISM BigDye terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) and DNA autosequencer ABI PRISM310 Genetic Analyzer or ABI PRISM 3130 Genetic Analyzer (Perkin-Elmer) according to the manufacturer's protocols. Both forward and reverse strands were sequenced.

**Sequencing alignment and phylogenetic analyses:** Acquired sequences were aligned manually based on the secondary structure of the SSU molecule, using sequences of alveolate taxa available on the European rRNA Database website (<http://www.psb.ugent.be/rRNA/index.html>). The names and accession numbers of species used in the SSU rDNA data set are shown in Fig. 4. For the SSU rDNA data set, the ciliate *Colpoda inflata* Stokes, *Favella ehrenbergii*, and

*Eutintinnus pectinis* (Kofoid) Kofoid et Campbell were used as outgroups.

Twenty four environmental samples were also included in the SSU rDNA alignment. Recently published sequences of 'associates' of radiolarian/phaeodarian protists (Dolven et al. 2007) were also included. The phylogenetic analyses were performed using maximum-parsimony (MP) and maximum likelihood (ML) analyses with PAUP version 4.0b10 (Swofford 2002).

The MP analysis was performed using the heuristic search option with the random addition of sequences (1000 replicates) and a branch-swapping algorithm (tree bisection-reconnection). Characters were weighted equally and gaps were treated as missing data. For MP, bootstrap analysis was carried out with 1000 replicates to evaluate statistical reliability of the tree topology (Felsenstein 1985). For ML analysis, we used the program Modeltest 3.06 (Posada and Crandall 1998) to decide which evolutionary model best fits the data by the hierarchical likelihood ratio tests. ML analysis was carried out with the heuristic search option, with a branch-swapping algorithm TBR (tree-bisection-reconnection). As the starting tree, we used the NJ tree. For the SSU rDNA data set, the model selected by hierarchical likelihood ratio tests tree



was the TrN+I+G substitution model. The parameters were as followed:

assumed nucleotide frequencies A = 0.2529, C = 0.2033, G = 0.2551 and T =

0.2887; substitution rate matrix with A-C substitutions = 1.0000, A-G = 2.1097,

A-T = 1.0000, C-G = 1.0000, C-T = 3.0928 and G-T = 1.0000; proportion of sites

assumed to be invariable = 0.2553 and rates for variable sites assumed to follow

a gamma distribution with shape parameter = 0.6219. For bootstrap analyses

with 100 replications for the ML of SSU rDNA data set, we used the heuristic

search option with a branch-swapping algorithm, nearest-neighbor interchange;

NNI, and starting-trees obtained by neighbor joining.

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## References

**Appleton PL, Vickerman, K** (1996) Presence of apicomplexan-type micropores in a parasitic dinoflagellate, *Hematodinium* sp. *Parasitol Res* **82**: 279-282

**Appleton PL, Vickerman, K** (1998) *In vitro* cultivation and developmental cycle in culture of a parasitic dinoflagellate (*Hematodinium* sp.) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters. *Parasitology* **116**: 115-130.

**Cachon J** (1964) Contribution a l'étude des Péridiniens parasites. Cytologie, cycles évolutifs. *Ann Sci Nat* **12** ser **VI**: 1-158

**Cachon J, Cachon M** (1978) Parasitic dinoflagellates. In Taylor FJR (ed) *The Biology of Dinoflagellates*. Blackwell Scientific Publications, Oxford, pp 571-610

**Chatton É** (1920) Les Périidiniens parasites: morphologie, reproduction, éthologie. Arch Zool Exp Gen **59**:1-475

**Chatton É** (1952) Classe des Dinoflagellés ou Périidiniens. In Grassé PP (ed) Traité de Zoologie. 1(1), Masson, Paris, pp 309-390

**Coats DW** (1988) *Duboscquella cachoni* n. sp., a parasitic dinoflagellate lethal to its tintinnine host *Eutintinnus pectinis*. J Protozool **35**: 607-617

**Coats DW, Heisler JJ** (1989) Spatial and temporal occurrence of the parasitic dinoflagellate *Duboscquella cachoni* and its tintinnine host *Eutintinnus pectinis* in Chesapeake Bay. Mar Biol **101**: 401-409

**Diéz B, Pedros-Alió G, Massana R** (2001) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene

cloning and sequencing. *Appl Environ Microbiol* **67**: 2932-2941

**Dolven JK, Lindqvist C, Albert VA, Bjørklund KR, Yuasa T, Takahashi O,**

**Mayama S** (2007) Molecular diversity of alveolates associated with neritic North

Atlantic radiolarians. *Protist* **158**: 65-76

**Felsenstein J** (1985) Confidence limits on phylogenies: an approach using the

bootstrap. *Evolution* **39**: 783-791

**Fensome R A, Taylor FJR, Norris G, Sarjeant WAS, Wharton DI, Williams GL**

(1993) A Classification of Living and Fossil Dinoflagellates. *Micropaleontology*,

Special Publication Number 7, Sheridan Press, Hanover, Pennsylvania: 1-351

**Field RH, Chapman CJ, Taylor AC, Neil DM, Vickerman K** (1992) Infection of

the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of

dinoflagellate on the west coast of Scotland. *Dis Aquat Org* **13**: 1-15.

**Fritz L, Nass M** (1992) Development of the endoparasitic dinoflagellate

*Amoebophrya ceratii* within host dinoflagellate species. J Phycol **28**: 312-320.

**Groisiller A, Massana R, Valentin K, Vaulot D Guillou L** (2006) Genetic

diversity and habitats of two enigmatic marine alveolate lineages. Aquat Microb

Ecol **42**: 277-291

**Horiguchi T, Yoshizawa-Ebata J, Nakayama T** (2000) *Halostylodinium*

*arenarium*, gen. et sp. nov. (Dinophyceae), a coccoid sand-dwelling

dinoflagellate from subtropical Japan. J Phycol **36**: 960-971

**Hudson DA, Shields JD** (1994) *Hematodinium australis* n. sp., a parasitic

dinoflagellate of the sand crab *Portunus pelagicus* from Moreton Bay, Australia.

Dis Aquat Org **19**: 109-119

**Jeon SO, Bunge J, Stoeck T, Barger KJA, Hong SH, and Epstein SS** (2006)

Synthetic Statistical Approach Reveals a High Degree of Richness of Microbial

Eukaryotes in an Anoxic Water Column. *Appl Environ Microbiol* **72**: 6578-6583

**Johannsen M, Gorokhova E, Larsson U** (2004) Annual variability in ciliate community structure, potential prey and predators in the open northern Baltic Sea proper. *J Plankton Res* **26**: 67-80

**López-García P, Rodríguez-Valera F, Pedrós-Alió C, Moreira D** (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* **401**: 603-607

**López-García P, Philippe H, Gail F, Moreira D** (2003) Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the mid-Atlantic ridge. *Proc Natl Acad Sci USA* **100**: 697-702

**Massana R, Balagué V, Guillou L, Pedrós-Alió C, Moreira D** (2004) Picoeukaryotic diversity in an oligotrophic coastal site studied by molecular and culturing approaches. *FEMS Microbiol Ecol* **50**: 231-243

**Moon-Van der Staay SY, DeWachier R, Vaultot D** (2001) Oceanic 18S rDNA sequences picoplankton reveal unsuspected eukaryotic diversity. *Nature* **401**: 607-610

**Nakayama T, Watanabe S, Mitsui K, Uchida H, Inouye I** (1996) The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. *Phycol Res* **44**: 47-55

**Posada D, Crandall KA** (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818

**Raush H, Larsen N, Schmitt R** (1989) Phylogenetic relationships of green algae *Volvox carteri* deduce from small-subunit ribosomal RNA comparison. *J Mol Evol* **29**: 255-265

**Ris H, Kubai DF** (1974) An unusual mitotic mechanism in the parasitic

protozoan *Syndinium* sp. J Cell Biol **60**: 702-720

**Saldarriaga JF, Taylor FJR, Cavalier-Smith T, Menden-Deuer S, Keeling PJ**

(2004) Molecular data and the evolutionary history of dinoflagellates. Eur J

Protistol **40**: 85-111

**Skovgaard A, Massana R, Balagué V, Saiz E** (2005) Phylogenetic position of the copepod-infecting parasite *Syndinium turbo* (Dinoflagellata, Syndinea).

Protist **156**: 413-423

**Stentiford GD, Green M, Bateman K, Small HJ, Neil, DM, Feist SW** (2002)

Infection by a *Hematodinium*-like parasitic dinoflagellate causes Pink Crab

Disease (PCD) in the edible crab *Cancer pagurus*. J Invert Pathol **79**: 179-191

**Stoeck T, Hayward B, Taylor TG, Varela R Epstein SS** (2006) A multiple

PCR-primer approach to access the microeukaryotic diversity in environmental

samples. Protist **157**: 31-43



**Swofford DL** (2002) PAUP\*: Phylogenetic Analysis Using Parsimony (\*and Other Methods), Version 4.0b10. Sinauer, Sunderland, MA

**Takano Y, Horiguchi T** (2004) Surface ultrastructure and molecular phylogenetics of four unarmored heterotrophic dinoflagellates, including the type species of the genus *Gyrodinium*. *Phycol Res* **52**: 107-116

**Takano Y, Horiguchi T** (2006) Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell. *J Phycol* **42**: 251-256

## Figure legends

**Figure 1.** *Duboscquella* sp. 1 and sp. 2 infecting tintinnid ciliate, *Favella ehrenbergii*. The micrographs illustrates the cells used for single cell PCR. A: *Duboscquella* sp. 1 from Ishikari Harbour, Hokkaido, Japan. The host cell (H) and many spherical parasitic sporocysts (P) can be seen within the lorica (L). B: *Duboscquella* sp. 2 from Hamana Lake, Shizuoka, Japan. Both host (H) and parasites (P) can be seen within the lorica (L). C: *Duboscquella* sp. 2 from Kurosaki Harbour, Okayama, Japan.

**Figure 2.** Different stages of maturation (A-F) of *Duboscquella* sp. from Rumoi, Hokkaido, Japan (same individual) inside its host. The numbers on the right corner represent the time (minutes) elapsed since the beginning of the observation. At stage 'A', the host is still visible, while at stage 'B', it already collapsed. G: Motile spore with the two flagella marked with arrows. H: host cell, P: parasite cell.

**Figure 3.** Transmission electron micrographs of *Duboscquella* sp. from Kurosaki

Harbour, Kurashiki, Okayama Pref., Japan (most probably *Duboscquella* sp. 2).

A: The host *Favella ehrenbergii* and its parasite *Duboscquella* sp. in relatively early stage of sporogenesis. Note that parasite cells (P) contain many spherical vesicles. H: host cell, P: parasite cell, B: Section through a small sporocyte at late stage of sporogenesis, showing a nucleus (N) containing dense chromosome-like structures (Ch) and spherical vesicles (V). C: Close up of a sporocyte, showing cortical alveoli (arrows). D: Close up of a sporocyte, showing a trichocyst (T).

**Figure 4.** Phylogenetic tree inferred from SSU rRNA gene sequences. The tree was constructed using ML method. Bootstrap values higher than >50% from both MP and ML analyses (MP/ML %) are given at each node. *Duboscquella* sp. 1 and sp. 2 (in shaded square) are included in the Marine Alveolate Group I clade. . DDBJ/EMBL/GenBank accession number of each taxon is indicated in brackets. *Duboscquella* sp. 1 was registered as *Duboscquella* sp. 'Ishikari/2003', while sp.2 was registered as *Duboscquella* sp. 'Hamana/2003' The operational taxonomic units starting with 'Associate' represent associates of polycystine

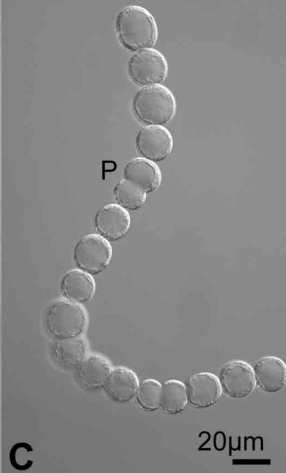
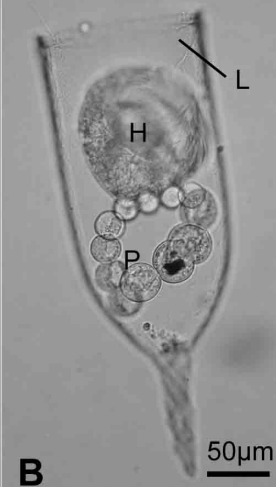
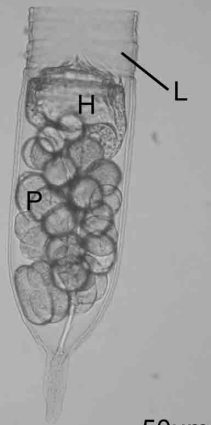
radiolarians and one phaeodarian protist (*Challengeron diodon*) published by

Dolven et al. (2007).

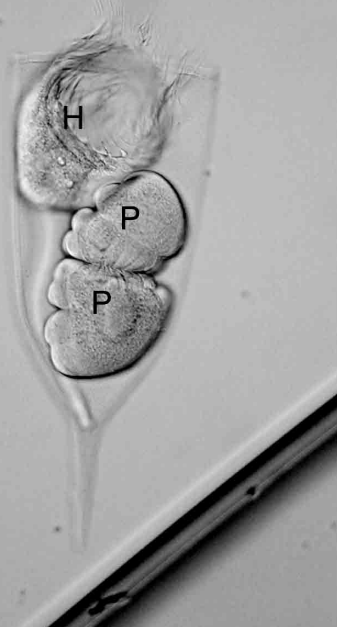
**Table 1.** Oligonucleotide primers used for SSU rDNA amplification and sequencing

| Code   | Synthesis direction | Sequence                   | Anneals to * |
|--------|---------------------|----------------------------|--------------|
| SR1    | Forward             | 5'-TACCTGGTTGATCCTGCCAG-3' | 1-10         |
| SR1b   | Forward             | GATCCTGCCAGTAGTCATATGCTT   | 10-33        |
| SR2TAK | Forward             | CATTCAAATTTCTGMCCTATC      | 293-313      |
| SR3    | Reverse             | AGGCTCCCTGTCCGGAATC        | 394-376      |
| SR4    | Forward             | AGGGCAAGTCTGGTGCCAG        | 548-566      |
| SR5TAK | Reverse             | ACTACGAGCTTTTTAACTGC       | 630-611      |
| SR8TAK | Forward             | GGATTGACAGATTGAKAGCT       | 1224-1243    |
| SR9    | Reverse             | AACTAAGAACGGCCATGCAC       | 1286-1267    |
| SR12   | Reverse             | CCTTCCGCAGGTTTCACCTAC      | 1781-1762    |

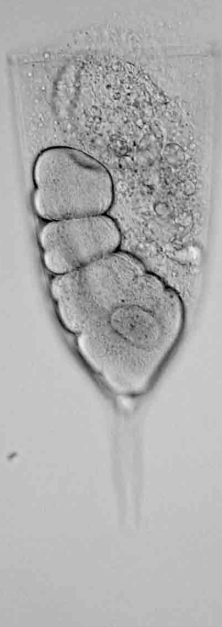
\* Annealing site in the 18s rDNA of *Volvox carteri* (Rausch *et al.* 1989)



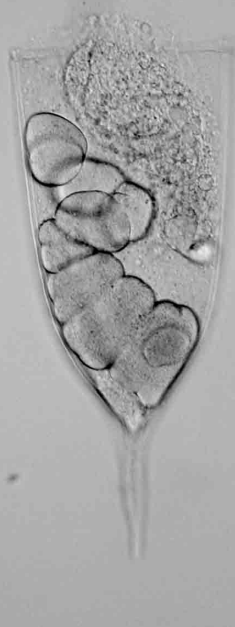
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**A**

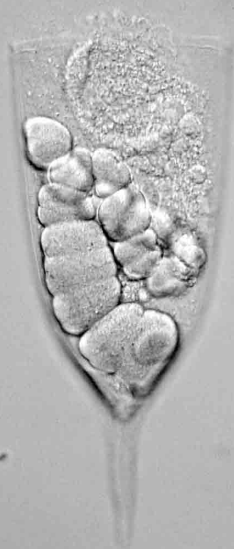
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**B**

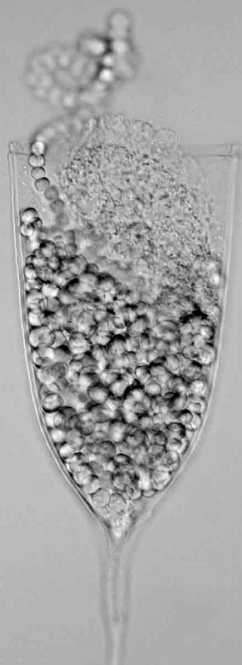
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**C**

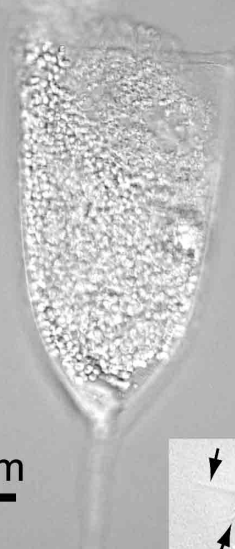
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**D**

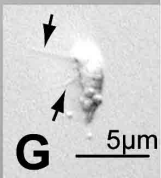
74

**E**

172



50µm

**F****G**

