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Ultrastructure and SSU rDNA Phylogeny of *Paraphysomonas vestita* (Stokes) De Saedeleer Isolated from Laguna de Bay, Philippines

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Summary. *Paraphysomonas vestita* is a unicellular, colorless, silica-scaled chrysophyte that plays an important ecological role in freshwater microbial communities as a consumer of prokaryotic and eukaryotic prey. There is little biogeographical information for this minute protist despite its significant role in aquatic food webs. In addition, the phylogenetic relationship of *P. vestita* to other taxa is unclear as *P. vestita* may be polyphyletic or a cryptic species complex. In this study, a clonal isolate from a freshwater sample of Laguna de Bay, Philippines was subjected to morphological study by electron microscopy and its small subunit ribosomal RNA (SSU rRNA) gene was sequenced. Morphological studies showed that the isolate possesses two unequal flagella emerging from the anterior part of the cell. Negative staining revealed the structure of the scales which consist of a baseplate with slightly thickened rim. The narrowing spine arises from the center of the baseplate. These results agree with previously studied isolates of *P. vestita*. The 18S rRNA gene sequence of the isolate had a very high similarity (99%) to *P. vestita* strain PV10. Phylogenetic analysis also showed that the isolate clustered with other *Paraphysomonas* sequences with high bootstrap support. Phylogenetic studies confirmed that *P. vestita* may be polyphyletic. No studies on the ultrastructure and phylogeny of a silica-scaled chrysophyte isolated in the Philippines have been reported so far. Results from this study may contribute to further ultrastructural and phylogenetic studies on aquatic flagellates and specifically to a revision of this potentially polyphyletic species.

Key words: *Paraphysomonas vestita*, chrysophyte, protist, ultrastructure, small subunit ribosomal RNA gene, Philippines.

INTRODUCTION

The chrysophytes *sensu lato* comprise the classes Chrysophyceae and Synurophyceae of Phylum Heterokontophyta (Kristiansen 2000). They are characterized by their heterokont flagella (unequal flagella, a long hairy and short smooth flagellum), and their silicified endogenous resting stages. The Chrysophyceae (= chrysophytes *sensu stricto*) are capable of phagotrophic nutrition and they have a sophisticated capturing mechanism that involves microtubules of the flagellar apparatus (Andersen 2004). They are predominantly freshwater organisms although some chrysophytes are tolerant of significantly wide ranges of salinity. Distribution pattern of most species are poorly known. One of the few exceptions is the species, *Paraphysomonas vestita* (Stokes) de Saedeleer, which occurs over a very

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wide range, being one of the most common species of silica-scaled chrysophytes (Preisig and Hibberd 1982a, Kristiansen 1986, Olsen *et al.* 1999, Kristiansen 2000, Andersen 2004). Species differentiation is based on the fine structure and ornamentation of the scales and spines that cover the cell (Preisig and Hibberd 1982a, b). Due to the small size of these structures, it is insufficient to use light microscopy and electron microscopy (EM) is necessary for study and identification. EM studies facilitated a better way of studying and identifying *Paraphysomonas* spp. (Preisig and Hibberd 1982a, b, 1983; Olsen *et al.* 1999; Kristiansen 2000). The scale-bearing *Paraphysomonas* produces species-specific siliceous scale structures. All of the species are relatively easy to identify using the morphology of their surface scales. *P. vestita* and *P. imperforata* are said to be species that are most frequently recorded globally and therefore most abundant (Preisig and Hibberd 1982a, b; Finlay and Clarke 1999). However, phylogenetic data suggest that *P. vestita* may be polyphyletic, i.e. possibly represent a crytic species complex (Boenigk *et al.* 2005).

Although our understanding in the role of chrysophytes in aquatic ecosystem has improved significantly, we still have insufficient data on the ultrastructure and phylogeny of some members of this group. Currently, rapid identification and classification of microorganisms have been attained by DNA-based methods. A combination of ultrastructural and molecular approaches was used in this study in order to facilitate better understanding on the identity of the isolate. Here, a chrysophyte isolated from Laguna de Bay, Philippines was subjected to morphological evaluation and its phylogeny was determined using the 18S ribosomal RNA (rRNA) gene sequence. No studies on the ultrastructure and phylogeny of a silica-scaled chrysophyte isolated in the Philippines have been reported so far. Results from this study may serve as baseline data for further ultrastructural and phylogenetic studies on aquatic flagellates.

MATERIALS AND METHODS

Paraphysomonas vestita isolate

The chrysophyte was isolated from Laguna de Bay, Philippines. A clonal culture of the isolate (designated as J1) was established and was maintained in a modified *Panicum* sp. infusion medium (Mitchell 1929) at 20°C under moderate light at the Laboratory of Applied Microbiology, Institute of Biology, University of the Philippines, Diliman, Quezon City. The culture was considered xenic since the bacteria present were consumed by the flagellate for food.

Ultrastructural studies

For transmission electron microscopy (TEM), cultures in late exponential or stationary phases of growth were fixed with glutaraldehyde to a final concentration of 1%. Fixed cells were concentrated to a small volume by centrifugation, and a drop of concentrate was placed on a formva-coated copper grid (400-mesh). After allowing the cells to settle for approximately 10 minutes, excess fluid was removed with a piece of Whatman filter paper and the grid was air-dried. The cells on the grids were then negative-stained with 1% uranyl acetate for 2 minutes. Grids were subsequently rinsed by quickly immersing them in distilled water, and then air-dried. Microscopy was carried out using a JEOL JEM TEM 1010 electron microscope. For the scanning electron microscopy (SEM), the cells were fixed in 2% glutaraldehyde, postfixed in 1% OsO_4 in buffer, rinsed, dehydrated in alcohol and critical-point dried using CO_2. The specimen was viewed under SEM Hitachi S-510 model.

Genomic DNA preparation and amplification

Genomic DNA was extracted using the procedure described by Walsh *et al.* (1991) which utilized Chelex 100 (Sigma). A polymerase chain reaction (PCR) assay employing primers specific to conserved regions of eukaryotic small subunit rRNA (SSU rRNA) genes were used to determine the identity of the chrysophyte. PCR amplification reactions were done in Astec PC707 Programmable Thermal Controller using oligonucleotide primer pairs, ss5 (5'-GGT-TGA TCC TGC CAG TAG TCA TAT GCT TG-3') and ss3 (5'-GAT CCT TCC GCA GGT TCA CCT ACG GAA ACC-3') (Rowan and Powers 1992).

Sequencing

DNA sequencing was performed using an ABI PRISM BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Fister City, CA) on an automated sequencer (ABI PRISM 3100 model; Applied Biosystems). The complete SSU rRNA gene was sequenced using the primers, ss5 and ss3.

Sequence alignment and phylogenetic analysis

The sequences of the isolate obtained were identified through BLAST search in the GenBank by determining the exact match or closest similarity. SSU rRNA gene sequences were aligned using ClustalW function of Mega 3. Gaps and ambiguous sequences were manually identified and removed. The *P. vestita* J1 sequence was deposited in GenBank with the accession number GU220392. This sequence was then aligned with 35 other representative chrysophyte sequences along with *Nannochloropsis granulata* (also a member of the Phylum Heterokontophyta, Class Eustigmatophyceae) that served as an outgroup. These reference sequences are listed in Table 1. A phylogenetic tree was constructed based on 1,454 unambiguously aligned nucleotide sites using the neighbor-joining (NJ) analysis, based on the GTR + Γ + I model, and maximum parsimony (MP) analysis. The analysis was carried out using the program PAUP* Ver. 4.0b10 (Swofford 2000). Clusters in the phylogenetic tree were considered valid if these had a bootstrap support of $\geq$ 50% for at least one of the two analyses used.

Table 1. Reference isolates of chrysophytes used in the phylogenetic analysis.

Genbank Accession No.	Organism
AB022864	*Paraphysomonas foraminifera* HT3
AB023070	*Poterioochromonas malhamensis* HT2
AF109325	*Paraphysomonas vestita* PV10
AF123282	*Chromophyton rosanoffii* CCMP260
AF123283	*Chrysocapsa vernalis* CCMP277
AF123284	*Chrysochaete britannica* CCMP280
AF123285	*Chromulina nebulosa* CCMP263
AF123286	*Chrysamoeba pyrenoidifera* CCMP1663
AF123287	*Chrysamoeba mikrokonta* CCMP1857
AF123288	*Lagynion scherffelii* CCMP465
AF123289	*Dinobryon sertularia* CCMP1859
AF123290	*Uroglena americana* CCMP1863
AF123291	*Dinobryon sociale* var. *americana* CCMP1860
AF123292	*Cyclonexis annularis* CCMP1858
AF123293	*Ochromonas tuberculata* CCMP1861
AF123294	*Ochromonas sphaerocystis* CCMP586
AF123296	*Phaeoplaca thallosa* CCMP364
AF123297	*Chrysolepidomonas dendrolepidota* CCMP293
AF123298	*Epipyxis pulchra* CCMP382
AF123299	*Chrysophaera parvula* CCMP293
AF123300	*Chrysosaccus* sp. CCMP295
AF123301	*Epipyxis aureus* CCMP385
AF123302	*Chrysoxys* sp. CCMP591
AF123395	*Poterioochromonas stipitata* CCMP1862
DQ487199	*Synura* sp. HCB-2005
EF165128	*Synura curtispina* CCMP847
EF165129	*Synura petersenii* SAG24.46
U42381	*Ochromonas* sp. CCMP584
U42382	*Ochromonas* sp. CCMP1278
U73226	*Mallomonas splendens* MUCC 294
U73232	*Mallomonas striata* var. *serrata* MUCC 295
Z28335	*Paraphysomonas vestita* SOTON 1
Z38025	*Paraphysomonas foraminifera* SOTON A
AB183852	*Nannochloropsis granulata* MBIC10054

RESULTS

Electron microscopy

The cells were analyzed by TEM to determine the ultrastructure of the clonal isolate, *P. vestita* J1. The cells presented an ovoid circular body. The prominent internal structures of the cell include the nucleus and several vacuoles present nearby (Fig. 1A, B). Golgi complex was also observed (Fig. 1A). Electron micrographs revealed the presence of a number of mitochondria (Fig. 1B, C). No chloroplasts were identified. It can be observed that the nucleus extends to the basal body of the flagella. Both flagella arise from the anterior end of the cell. Vacuoles are present at the periphery. A close view of the two emergent flagella of the isolate exposed the axoneme and the kinetochore (Fig. 1D).

Negative-stained cells of *P. vestita* revealed the scales which consist of a baseplate with slightly thickened rim. The narrowing spine arises from the center of the baseplate (Fig. 2A, B). Fig. 3 shows the scanning electron micrograph of the *P. vestita* scales. On the average, the scales of the isolate measured 1 μm in diameter.

SSU rDNA phylogeny

PCR amplification of the SSU rDNA coding region from *P. vestita* J1 yielded product of approximately 1.8 kb generated using primers ss5 and ss3. Distinct band with the expected size (~1.8 kb) of the amplicon was observed on agarose gel (data not shown). The length of SSU rRNA sequences obtained from the isolate ranged from 1680 bp to 1742 bp. Each sequence showed high similarity to homologous sequences to other chrysophyte isolates reported so far (Table 1). The isolate was 99% similar to the rRNA gene sequence of *P. vestita* PV10 (GenBank accession number AF109325) and clustered with *P. vestita* PV10 with good bootstrap value (Fig. 4). The *P. vestita* strain SOTON 1, however, clustered with *P. foraminifera*.

DISCUSSION

The taxonomic identification of chrysophytes found in environmental water samples can be complicated even for cultured specimens because of the time and experience needed to distinguish behavioral and morphological features characteristic of the different taxa (Lim *et al.* 2001). The use of sequence analysis of amplified SSU rRNA gene for examining phylogenetic relationships among chrysophytes can be helpful in the identification of isolated organisms (Andersen *et al.* 1999, Boenigk *et al.* 2005). In this study, a combination of morphological features using electron microscopy and molecular data was done to better establish the taxo-

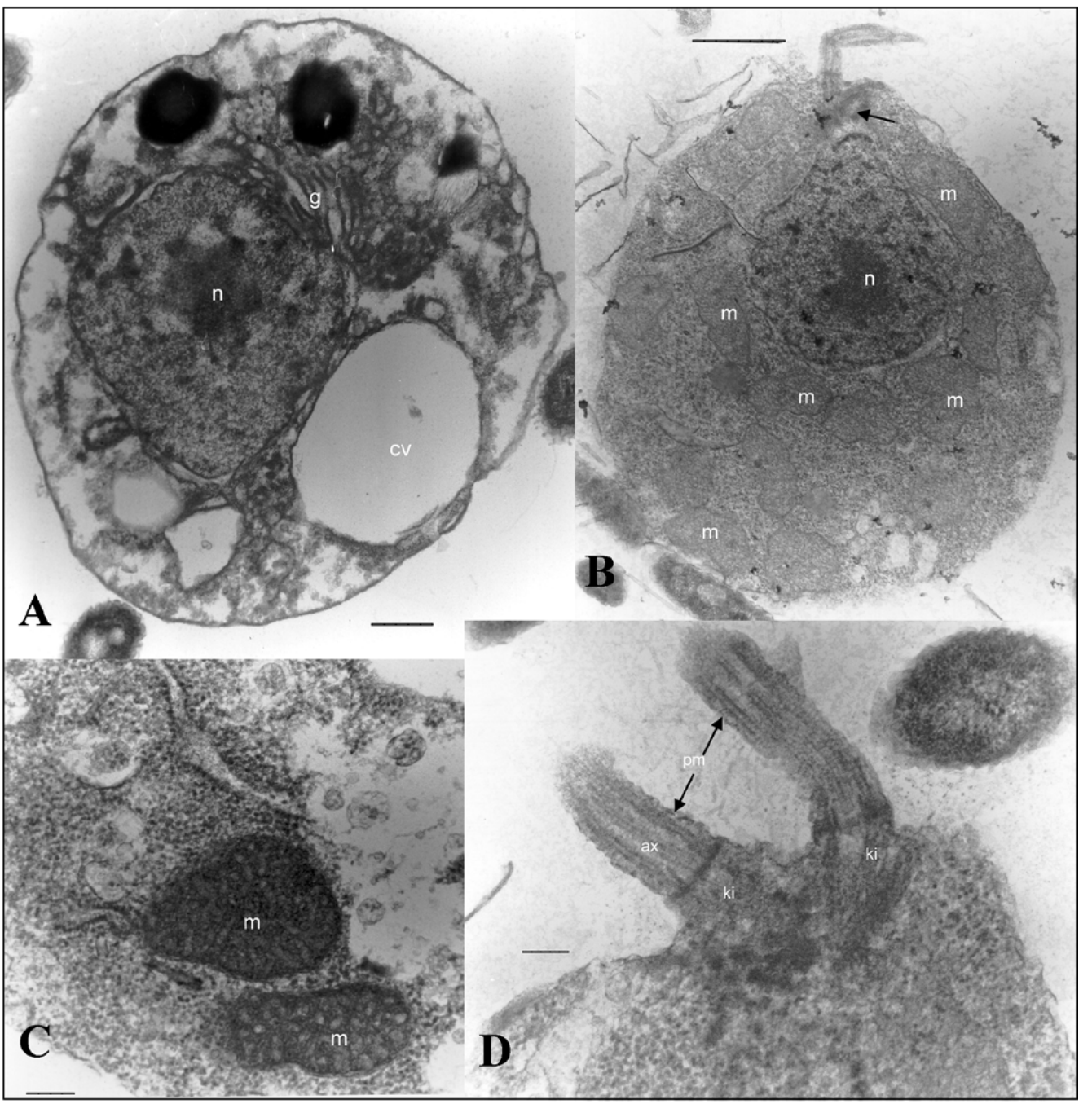

Fig. 1. A – Cross section of the isolate under TEM. Vacuoles including a contractile vacuole (cv), the nucleus (n), and the Golgi complex (g) are present. Bar: 400 nm; **B** – longitudinal section of the cell showing the two flagella on the anterior end. Arrow indicates the presence of the smaller flagellum. The nucleus (n) and several mitochondria (m) are prominent. Vacuoles (v) are also present. Bar: 850 nm; **C** – thin section of the cell showing a closer view of the mitochondria (m). Bar: 200 nm; **D** – a close view of the two emergent flagella of the isolate. The axoneme (ax), kinetochore (ki) and plasma membrane (pm) are observed. Bar: 200 nm.

nomic roots of the isolate. The results of the sequence analysis of the SSU rDNA confirmed the identity of the isolate as *P. vestita* but also confirmed a potential polyphyly of the species.

General features

Chrysophytes and other related flagellates can be found in marine or freshwater environments as suggested by several authors (Landry *et al.* 1991, Lim *et*

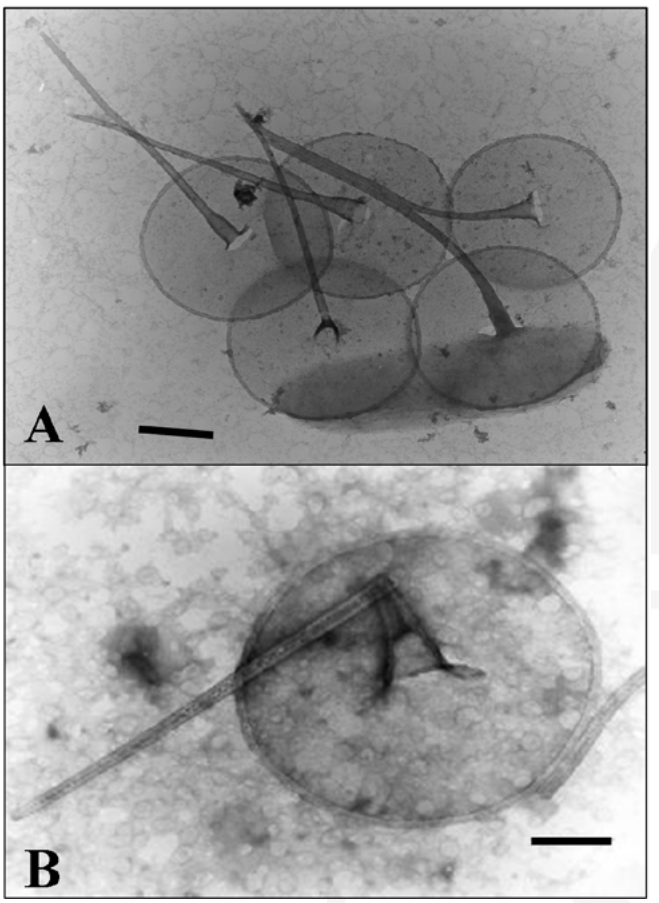

Fig. 2. Negative-stained *P. vestita* scales. **A** – The group of scales shows the slightly thickened rim of the spines and the spines arising from the center of the baseplate. Bar: 380 nm; **B** – *P. vestita* scale consisting of a baseplate with slightly thickened rim (140,000 ×). A tapering spine with an acute tip arises from the center of the baseplate. Bar: 200 nm.

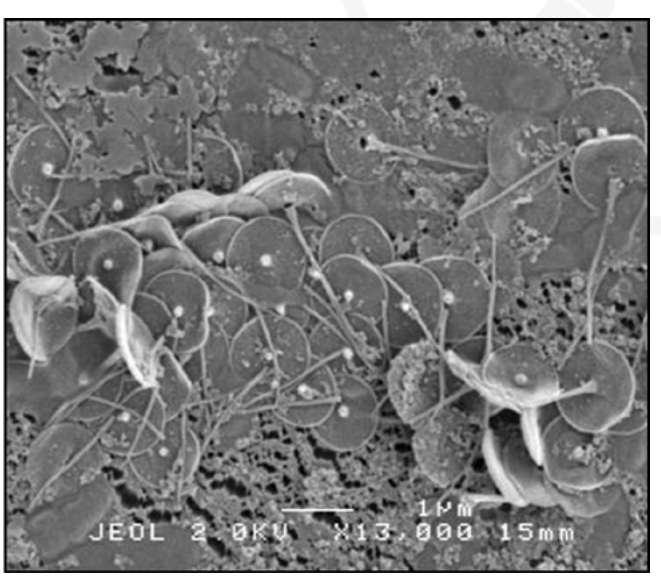

Fig 3. Scanning electron micrograph of *P. vestita* scales. Bar: 1 μm.

al. 1999, Kristiansen 2000, Lim *et al.* 2001, Christensen-Dalsgaard and Fenchel 2003, Pichrtová *et al.* 2007, Boenigk 2008). The flagellate herein described presented ultrastructural features coincident with previous *Paraphysomonas vestita* descriptions (Lim *et al.* 2001, Vigna and Siver 2003). Silica-scaled flagellates can be easily distinguished for species identification. An important character in chrysophyte taxonomy is its scale morphology. The scaled of *Paraphysomonas* species are more or less spherical and approximately 1 μm in size (Lim *et al.* 2001). Same sizes of scales were also observed in *P. vestita* J1. Within the chrysophyte genus, *Paraphysomonas* species are distinguished from the ultrastructure of silica scales which cover the cells (Preisig and Hibberd 1982a, Olsen *et al.* 1999, Lim *et al.* 2001). The scales of the *P. vestita* consist of a baseplate with a slightly thickened rim and a narrowing spine with an acute tip arises from the center of the base plate. The spine is normally distinctly longer than the diameter of the disc (Preisig and Hibberd 1982a, Lim *et al.* 2001). Very similar features of the scales were observed in the isolate (Fig. 2A, B). Chloroplasts were not observed in this organism since *Paraphysomonas* has unpigmented plastids called leucoplasts in place of chloroplasts (Preisig and Hibberd 1982c, Andersen 2004). Negative staining revealed the structure of the scales and the spines (Fig. 2A, B). These results were true also with the reported freshwater *P. vestita* isolates of Lim *et al.* (2001). The scales of this species consist of a baseplate with slightly thickened rim. A tapering spine with an acute tip arises from the center of the baseplate (Preisig and Hibberd 1982a). The spines are generally longer relative to the baseplate than the spines of *P. imperforata* (Lim *et al.* 2001).

Cells of the genus *Paraphysomonas* possess a long hairy flagellum and a second short, smooth flagellum visible in EM preparations. Species of this genus are surrounded by siliceous scales (Preisig and Hibberd 1982a, Lim *et al.* 2001). Even in light microscopy, the possession of two flagella in the isolate is evident. Transmission electron micrographs verified these results (Fig. 1B, D). Species of the ubiquitous genus *Paraphysomonas* use the longer of their two flagella to draw a considerable amount of current of water toward the cell surface near to the base of this flagellum (probably with the aid of the shorter flagellum), where bacteria or food particles are taken into a feeding basket near the flagellar bases at the anterior end of the cell (Ishikagi and Sleigh 2001, Andersen 2004).

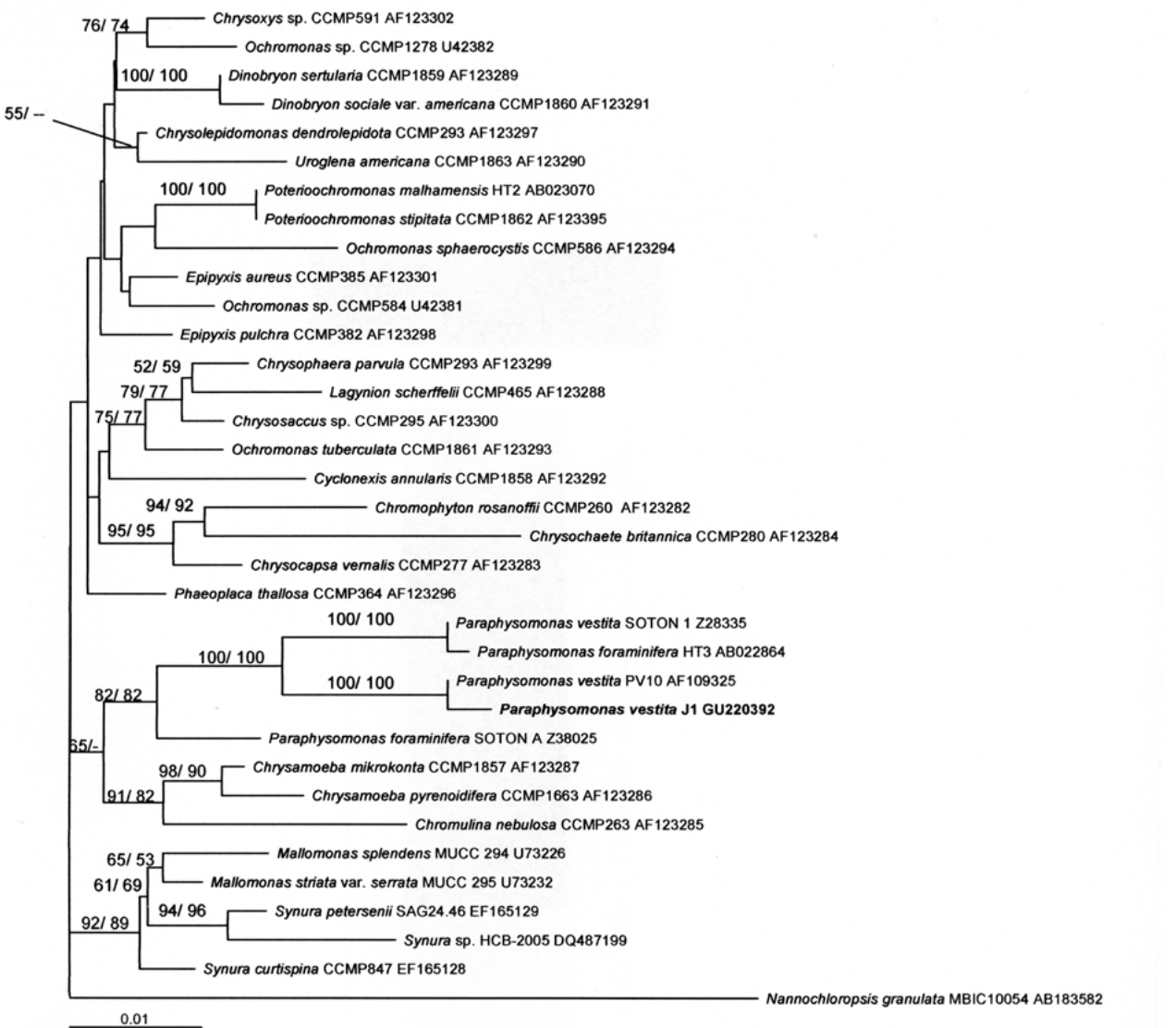

Fig. 4. Phylogenetic tree based on 1,454 unambiguously aligned nucleotide sites of 18S rDNA of chrysophytes. The phylogenetic tree was constructed using the neighbor-joining GTR + Γ + I model and was rooted to *Nannochloropsis granulata*. The values on each node represent bootstrap supports from neighbor-joining and maximum parsimony analyses, respectively. Bootstrap supports < 50% are not shown. The scale bar on the lower left side represents one substitutional change per 100 nucleotide positions.

SSU rDNA phylogeny

The phylogenetic relationships among some of chrysophyte species and their affinities to other protistan taxa are unclear. These conditions are largely a consequence of the fact that small protists possess few readily apparent morphological features on which to base taxonomic and phylogenetic schemes and with which to identify them in natural assemblages (Caron *et al.* 1999). As a substitute approach for dealing with these issues, the SSU rRNA gene of the isolate was sequenced. Thus, a phylogenetic analysis based on that sequence information was performed. Results show that the *P. vestita* J1 in this study belongs to the same monophyletic group with the other *P. vestita* isolates previously studied with high bootstrap support (Fig. 4). Moreover, it also had a 99% sequence similarity with *P. vestita* strain PV10 (GenBank accession no. AF109325). This confirmed the identity of the isolate as well as supported the phylo-

genetic grouping of this species based on the morphology of its scales (Caron *et al.* 1999, Lim *et al.* 2001). However, the phylogenetic data hint to a polyphyly of *P. vestita*, specifically the position of *P. foraminifera* HT3 within the *P. vestita* cluster is suspicious.

Conclusion

Identification of organisms from environmental samples by morphological data alone can sometimes be misleading. The use of molecular tools (e.g. PCR and DNA sequencing technology) can be helpful in identifying such cases. Combination of both morphological data and molecular data can promote better classification of heterotrophic flagellates.

In this study, the fine structures of the chrysophyte isolate was evaluated and its phylogeny was determined. The isolate was identified at the species level by morphological and molecular data. The cell morphology of the isolated chrysophyte was determined by TEM and revealed the presence of two flagella inserted at the anterior part of the cell, perpendicular to each other; a single pear-shaped nucleus extending at the direction of the basal bodies, Golgi body, and contractile vacuoles. Phylogenetic analysis of the isolate using the 18S rRNA gene sequence revealed that the isolate was *Paraphysomonas vestita*.

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