Ultrastructure and molecular phylogeny of two novel excavate protists

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Ultrastructure and Molecular Phylogeny of Two Novel Excavate Protists

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ABSTRACT

In this thesis, two new excavate flagellates belonging to the Fornicata, a monophyletic group of Excavata containing typical and parasitic members (i.e. *Carpediemonas*, retortamonads, and diplomonads), were investigated for taxonomic and phylogenetic aspects. These undescribed protistan flagellates were isolated from sediment samples collected from sea floor. All flagellates were free-living, heterotrophic, possessing conspicuous ventral groove, and grew under microaerophilic condition. They had two flagella, the posterior flagellum of which lies in a ventral feeding groove, suggesting that these flagellates were excavates.

In phylogenetic analyses of small subunit ribosomal RNA (SSU rRNA) sequences, strong affinity between these flagellates and Fornicata was recovered, thought the internal branching pattern in the Fornicata clade was not resolved with confidence. Two strains (NY0166 and NY0173) were very close and strongly grouped with each other. Another strain (NY0165) was estimated as the shortest branching taxon in the Fornicata. The detailed electron microscopic observations of this protist revealed that NY0165 possesses all the key ultrastructural features characteristic of typical members of the Excavata. Among the ten excavate groups previously recognized, NY0165 and the Fornicata share the arched B fiber as a component of the flagellar apparatus that bridges the right root, a posterior basal body and a left root. Combining both morphological and molecular data analyses, NY0165 is classified as a new excavate in the Fornicata, and described *Dysnectes brevis* gen. et sp. nov., but leaving its taxonomic position within the Fornicata uncertain (*incertae sedis*).

The flagellar apparatus architecture of *D. brevis* was of typical excavates. It is one of the most complex flagellar apparatuses among all eukaryotes so far examined, so

that it would be a good candidate to compare morphological homology of the flagellar apparatus components of eukaryotes. The flagellar apparatus is very diverse at class or phylum level and it is difficult to evaluate homology of components between most eukaryotic taxa. Because, as far as is known, flagella and basal bodies inherit in semi-conservative fashion so that there is a relationship that one flagellum is inherited from the mother cell and the other is newly generated *de novo*. Flagellar transformation is believed as one of principal rules applicable to all eukaryotes, while there has been no report for excavates. I therefore examined the flagellar transformation on *D. brevis* to establish morphological basis of homology estimation of the flagellar apparatus and cell architecture among eukaryotes. It has unfortunately not succeeded yet, however, overall comparison of various eukaryotic groups suggested that the posterior flagellum is older and the anterior flagellum is younger (newly formed). I assumed general rule of flagellar transformation is applicable to the excavates and, based on this assumption, compared the flagellar apparatus components of *D. brevis* with that of other eukaryotes. If the assumption is correct, cell architectures of most eukaryotes can be interpreted under the common morphological basis, which show several clear homology of the flagellar apparatus components between *D. brevis* and other eukaryotes. Flagellar apparatuses of many eukaryotes can be interpreted as reduced forms from that of excavates. This suggests that the excavates have the most primitive cell architecture of the eukaryotes.

The strains NY0166 and NY0173 were also regarded as new members of excavates, because they are very close in SSU rDNA tree and morphologically indistinguishable in light microscopical features. Two strains would be best regarded as the same species. Formal description will be given after ultrastructural examination is

completed. BLAST search indicated that almost the same sequences of SSU rDNA as NY0166 and NY0173 were present in environmental DNA collected from various localities in the world, including Sagami Bay, Japan and California, USA and in the sequences of strain from Halifax, Canada. This indicates that this new excavate species is globally distributed, and suggests that biodiversity of excavates is still in unexplored situation.

Short branching and divergence at the base within the Fornicata clade of the strains examined in this study, especially of *D. brevis*, was discussed. Because the short branch reflects normal evolutionary rate of SSU rDNA, these sequences would be good to use in place of the genes of parasitic members that often cause long branches due to high evolutionary rate. Therefore, special attention should be paid to the free-living Fornicata in future studies. They could play a pivotal role for understanding one of the most important issues in biology, early evolution of eukaryotes.

ABBREVIATIONS

AB, Anterior Basal body; AF, Anterior Flagellum; AR, Anterior Root; B, B fiber; B1, Basal body 1; B2, Basal body 2; C, C fiber; CF, Composite Fiber; DO, Dense organelle; F1, Flagellum 1; F2, Flagellum 2; I, I fiber; IMt, Internal Microtubule; IMtOC, IMt-Organizing Center; IRR, Inner Right Root; LR, Left Root; N, Nucleus; MLS, MultiLayered Structure; ORR, Outer Right Root; PB, Posterior Basal body; PF, Posterior Flagellum; R1, microtubular Root 1; R2, microtubular Root 2; R3, microtubular Root 3; R4, microtubular Root 4; RR, Right Root; SA, Singlet root Associated fiber; SR, Singlet Root; TMR, Transverse Microtubular Root; TMRE, TMR Extension; V, flagellar Vane.

GENERAL INTRODUCTION

Accumulation of ultrastructural data and rapid development of molecular phylogeny have been successfully revealing phylogenetic relationships among dozens of different lineages of eukaryotes, and painting a global picture of eukaryotes is about to accomplish. Several different views of reconstructed eukaryotic phylogeny have been proposed. One of these suggests that eukaryotes are composed of six huge assemblages generally referred to super groups: Opisthokonta, Plantae, Amoebozoa, Chromalveolata, Rhizaria and Exacavata (Simpson and Roger 2004a; Adl et al. 2005). Opisthokonta are an assemblage containing animals and fungi with several other unicellular protists including choanoflagellates, most of which are characterized by possessing posterior flagellum beating behind the cell (Cavalier-Smith 1987a; Cavalier-Smith and Chao 2003). The choanoflagellates are believed to be a close relative of animals (Wainright, P. O. et al. 1993; Karpov, S. A. and Leadbeater, B. S. C. 1998; Lang et al. 2002). Plantae (in a narrow sense) are consisting of three groups of photosynthesizing eukaryotes: green plants (including land plants), red algae and glaucophytes (Archibald and Keeling 2004). These three groups possess the plastid that was derived directly from a cyanobacterium via endosymbiosis (known as primary endosymbiosis), and the glaucophytes still remains cyanobacterial cell wall around their plastids (Aitken and Stanier 1979; Scott et al. 1984). Other four super groups are comprised of protists only. Amoebozoa comprise amoeboid protists represented by slime mold and flagellated amoebae (eg., *Mastigoamoeba*) (Cavalier-Smith 1998). Chromoalveolata are a huge group, comprised of two major groups, Chromista and Alveolata. The Chromista is a taxon proposed as the sixth kingdom of life (Cavalier-Smith 1998). Most members are

characterized by possessing heterokont flagella in swimming stages (the anterior flagellum with tubular flagellar hairs and smooth posterior flagellum), and form a monophyletic group called stramenopiles (Patterson 1989). The heterkokont algae, including brown algae and diatoms, are members of this group together with non-photosynthesizing heterokont protists such as oomycetes, hyphochytrids and labyrinthulids (all used to be treated as fungal taxa), and many other heterotrophic flagellates. Two photosynthesizing groups, cryptophytes and haptophytes, are also included in the Chromista based on similarities in flagellation and/or membrane topology of chloroplast envelope, and molecular phylogenetic analyses (Cavalier-Smith 1998; Yoon et al. 2002). The other group of the Chromalveolata is a monophyletic group, the Alveolata, comprised of ciliates, apicomplexans (Malaria parasites and many sporic protists) and dinoflagellates. Monophyly of Chromalveolata has been proposed based on molecular phylogeny (Patron et al. 2004; Harper and Keeling 2003; Rogers and Keeling 2004). Rhizaria comprise mainly amoeboid protists that are phylogenetically far from those of the Amoebozoa, including the Cercozoa (eg. Foraminifera, chlorarachniophytes) and Radiolaria (Cavalier-Smith 2002). Of six super groups, situation of the Excavata is a little complicated. The Excavata (in a broad sense) comprise flagellates possessing a ventral groove in which posterior flagellum is housed (typical excavates: excavates in a narrow sense), and a monophyletic group called Discicristata containing heterolobose amoebae and Euglenozoa (consisting of kinetoplastids and euglenoids) characterized by disc-like mitochondrial cristae (Cavalier-Smith 2002). Monophyly of excavates is still uncertain, and this is related to the problem of the origin of eukaryotes (see below). The existence of several (six or so) super groups of eukaryotes is now out of question, though to test and refine some

ambiguities is indispensable to construct reliable framework of eukaryotic phylogeny. Despite such successful achievement of painting global picture of eukaryotes, the origin of eukaryotes is still unknown. Several possibilities have been suggested, and it has been discussed that the root of eukaryotes lies in the Excavata.

Patterson (1999) first assigned five groups of protists as excavates: *Carpediemonas*, diplomonads, retortamonads, jakobids and *Trimastix* (See also Simpson & Patterson 1999, Patterson et al. 1999) These excavate protists were recognized recently, but organisms grouped in the excavates have been known for a long time since a parasitic diplomonad *Giardia* was first described by Antoni van Leeuwenhoek (1632-1723) in 1681 as he examined his own diarrheal stools under the microscope (Ford 2005). Ultrastructure of excavates have been studied since 1970's, mostly contributed by Brugerolle (Brugerolle 1991a, 1991b, 2000). He studied many parasitic species of oxymonads (*Monocercomonoides*, *Pyrsonympha* and *Polymastix*) (Brugerolle 1970, 1977, 1980; Brugerolle and Joyon 1973), diplomonads (*Giardia, Enteromonas, Trepomonas* and *Hexamita*) (Brugerolle 1974, 1975a, 1975b, 1975c, 1973a, 1973b), retortamonads (*Retortamonas* and *Chilomastix*) (Brugerolle 1973, 1977) and a parabasalia *Trichomonas* (Brugerolle 1975d, 1976). Detailed comparison of the ultrastructure, particularly the flagellar apparatus, and molecular data started from 1990's. Then, excavates, in a broad sense, are now comprised of ten groups: jakobids, *Malawimonas*, Heterolobosea, Euglenozoa, *Trimastix*, oxymonads, *Carpediemonas*, retortamonads, diplomonads, and parabasalids (Simpson 2003; Simpson and Roger 2004b). Nevertheless, the true diversity of excavate have not been explored yet.

Some members of excavates are thought to be key organisms for understanding eukaryotic cell evolution. Particularly, two parasitic excavates, *Giardia*

intestinalis (a diplomonad) and *Trichomonas vaginalis* (a parabasalid), have been extensively investigated as "primitive" eukaryotic cells that may have diverged from the main trunk of early eukaryotic lineages. They possess no typical mitochondrion, and early phylogenetic studies using small subunit ribosomal RNA (SSU rRNA) and translation elongation factors placed diplomonads and parabasalids at the base of the eukaryotic tree with high statistical supports (e.g., Sogin 1989; Hashimoto et al. 1994). These results were consistent with the "Archezoa" hypothesis. The Archezoa is a kingdom proposed by Cavarier-Smith (1987b) to embrace eukaryotes. He thought the Archezoa, which means ancestral eukaryotes, diverged before acquisition of mitochondria by the endosymbiosis of mitochondrial ancestor from α-proteobacteria, then assembled amitochondriate protist in the Archezoa. Cavalier-Smith included diplomonads, parabasalids, microsporidia, entamoebidae and pelobionta in the Archezoa. However, the genes encoding mitochondria-targeted proteins were found in both diplomonads *Giardia* and parabasalids *Trichomonas* nuclear genomes, clearly indicating that these two protists secondarily lost the typical mitochondrion (reviewed in Embley et al. 2003; van der Giezen and Tovar 2004). In line of these findings, *Giardia* and *Trichomonas* appeared to retain double membrane-bounded organelles, which are most probably a relic of typical mitochondria (Müller 1993; Tovar et al. 2003). Furthermore, recent advances in molecular phylogenetic analyses call the deep branching positions of *Giardia* and *Trichomonas* in early studies into question. *Giardia* and *Trichomonas* genes are generally fast-evolving, and their basal positions (particularly in SSU rRNA trees) are systematic artifacts derived from long-branch attraction (LBA), whereby long-branched ingroup sequences and the long branch leading to the outgroup sequences (those of archaebacteria in most cases) were

artifactually attracted to one another in tree reconstruction (Embley and Hirt 1998; Philippe et al. 2002). Recent multigene phylogenetic studies suggested that these amitochondriate lineages are the closest sisters among eukaryotic lineages, but there is no strong working hypothesis for the positions of the diplomonad plus parabasalid clade in eukaryotic phylogeny (Arisue et al. 2005; Simpson et al. 2006).

Jakobids recently became another candidate for primitive eukaryotic cells. *Reclinomonas americana,* a representative of jakobids, appeared to have the most "ancestral" mitochondrial (mt) genome. *Reclinomonas* mt genome encodes more protein-coding genes than the mt genomes of any other eukaryotes previously studied (Lang et al 1997; Gray et al. 1999; Gray et al 2001). Furthermore, the genes encoding the subunits of bacterial type RNA polymerase (RNA pol) were identified in jakobid mt genomes, whereas all other mitochondrial eukaryotes utilize nuclear-encoded, phage type RNA pol for their mitochondria. Most simply, the evolution of mt RNA pol can be reconciled by the following scenario: (i) The ancestral mt RNA pol was of the bacterial type, inherited from an α-proteobacterium that gave rise to mitochondria, and (ii) then replaced by a laterally acquired phage type enzyme at some time point in early eukaryotic evolution. Under the above scenario, jakobids could be the most primitive lineages, diverging from the main trunk of eukaryotes prior to the transition of mt RNA pol from the bacterial type to the phage type. In a recent unrooted phylogenetic tree, however, jakobids were robustly nested in the clade of Heterolobosea and Euglenozoa, which utilize the phage type RNA pol for their mitochondria (Simpson et al. 2006). If the eukaryotic root does not lie on the jakobid branch, the bacterial type RNA pol genes encoded in jakobid mt genomes may not represent the ancestral character of eukaryotes, and may have been secondarily acquired. Evidently, the character of mt RNA pol alone

is not sufficient to place the root of eukaryotes on the jakobid branch, and the evolutionary position of jakobids should be rigorously explored by rooted phylogenetic analyses.

Although excavates can occupy a key position in eukaryotic evolution, the current data are insufficient to conclude whether the ten excavate groups form a monophyletic assemblage. In the point of the cellular structure, most parasitic and some free-living heterotrophic excavates inhabiting microaerophilic or anaerobic environments lack their Golgi apparatus and classical (typical) mitochondria (Brugerolle 1991b; Brugerolle 1993). On the contrary, aerobic excavates possess both these organelles. Of course, these structures are basic component of eukaryotes. It has been revealed that the excavate lacking classical (typical) mitochondria possesses also two membrane bounded organelle, such as hydrogenosome or mitosome. These are considered as relic of mitochondria and hydorgenosome remains the function for energy production (Müller 1993; Sutak et al. 2004). Mitosome have been reported not to produce the energy but their mitochondrion related genes are coded on the nuclear genome and functioned in mitosome (Tovar et al. 2003). In the oxymonads and retortamonads, mitochondrion related organelles have never been observed (Brugerolle 1991b; Mylinikov 1991; Bernard et al 1997; Simpson et al. 2002a; Simpson and Roger 2004). As a result, excavates are a unique group in comparison with other eukaryotic super groups, since excavates show variety of presence/absence situations of mitochondria and the Gogi body, and possessing of mitochondrion related organelles.

Important fact in the excavates is that mitochondria of aerobic excavate is morphologically diverged. Three types of mitochondrial cristae are known in eukaryotes: flat cristae, tubular cristae and discoid cristae. Morphology of mitochondrial

cristae is very stable at higher taxonomic rank (phylum or class) and used as one of principal diagnostic characters of higher taxa. For example, animals, plants and fungi have flat cristae in their mitochondria, and stramenopiles, Alveolata and most other protists possess tubular cristae in their mitochodria (Taylor 1976; Corliss 1984; Page and Blanton 1985; Patterson and Brugerolle 1988). In contrast, all three type of mitochondrial cristae are present in the excavates: flat cristae in *Jakoba* (Patterson 1990), tubular cristae in *Reclinomonas* (O'Kelly 1997) and *Andulcia* (Simpson and Patterson 2001; Lara et al 2006), discoidal cristae in *Malawimonas* (O'Kelly and Nerad 1999), Heterolobosea (Page and Blanton 1985; Fenchel and Patterson 1986; Brugerolle and Simpson 2004) and Euglenozoa (Kivic and Walne 1983; Simpson 1997; Leander et al 2001). Accordingly, it seems that excavates are a super group having chimeric characters in the point of the mitochondrial features.

On the contrary to the dissimilarity of the presence/absence of mitochondrial and characteristics of mitochondrial cristae, the flagellar apparatus of typical excavates is quite similar to each other (Simpson et al 2002a; Simpson 2003; Simpson and Roger 2004b). Pioneering works revealed that excavates possess a unique and very complex cytoskeletal organization associated with the ventral feeding groove. In particular, the cytoskeletal structures in five "typical" excavates—*Carpediemonas*, retortamonads, *Trimastix*, jakobids, and *Malawimonas*—are highly similar to one another (Simpson 2003; Simpson and Roger 2004b). A large number of microtubular root supported the ventral groove. They are composed of most simply the left root (LR), singlet rootlet (SR) and the right root (RR) associated from posterior basal body (PB). The dorsal side of the cell is also supported by anterior root (AR) and cytoskeletal microtubules (dorsal fan) on AR originated from the anterior basal body (AB). Several fibrous materials (A

fiber, B fiber, C fiber and I fiber) bridge between these root systems. It is unlikely that such complex cytoskeletal organization evolved more than once, and, therefore, excavates are likely a natural (monophyletic) group (Simpson 2003; Simpson and Roger 2004b).

In contrast, recovering monophyly for excavates by using molecular data analyses is a much more difficult task. The SSU rRNA phylogenies in which all ten excavate groups are considered have failed to recover the excavate monophyly with high statistical supports (Simpson and Roger 2004b). For protein phylogenetic analyses, taxon sampling is a huge bottleneck: although genomic data are available for pathogenic excavates (e.g., *Giardia*, *Trichomonas*, and the kinetoplastids *Trypanosoma* and *Leishmania*), large scale sequence analyses of other excavate groups are just about to start. The largest protein data set analyzed is comprised of six genes of 3,142 amino acid positions in total, covering all excavate groups except retortamonads (Simpson et al 2006). So far, the ten excavate groups coalesce by molecular data analyses into three assemblages plus *Malawimonas*: (1) jakobids, Heterolobosea, and Euglenozoa; (2) *Trimastix* and oxymonads; and (3) *Carpediemonas*, retortamonads, diplomonads, and parabasalids (Simpson 2003; Simpson and Roger 2004b; Simpson et al. 2005).

Consequently, excavates are uniform in cytoskeletal architecture, though, they have variety of different cellular structures on the other hand. On the molecular data, excavate are not monophyletic. This indicates that these ultrastructural features of excavates could be plesiomorphy of whole eukaryotes. It is possible that some eukaryotic groups still remain characteristics shared with excavates, and some other eukaryotic groups lost their features and developed their own unique characters. Thus, the excavates are an important group for better understanding of the early evolution and

phylogeny of eukaryotes. However, our knowledge on evolution and phylogeny is still fragmentary. One of reasons of uncertainty of excavate evolution is the shortage of both morphology and molecular data. It is required to accumulate such data for many more taxa of the excavates.

Part 1. Phylogenetic analyses of Fornicata protist

INTRODUCTION

One of the main reasons of the stagnancy of studies on excavate evolution would be taxon sampling. Studies on excavates have been undertaken mainly on parasitic taxa. Both the morphological and molecular data are mainly derived from parasitic cells, and thus probably not reflecting excavate diversity. Morphological characters of parasitic excavate are clearly different from those of typical excavates (Friend 1966; Elmendorf et al. 2003), and gene sequences of parasitic organisms are generally more diverged than those of the free-living ones, and can mislead phylogenetic estimates (e.g., LBA artifacts; see general introduction). There are numerous examples to suggest long branch caused by parasitic organisms. Two excavate, parabasalids and diplomonads are animal parasites. They are fast-evolving taxa and their sequences show relatively long branches (Simpson et al 2006). Under such conditions, it is very difficult to reconstruct the true phylogeny (Embley and Hirt 1998; Philippe et al. 2002). Considering the difficulties with parasitic excavates, I am aware of the importance of the free-living lineages, of which both morphological and molecular data may be free from various biases that originate from a parasitic lifestyle.

The ancestral excavate cells should have been free-living, since all lineages (except oxymonads) include free-living members. Fornicata, one of three monophyletic excavate groups, include parasitic protistan group. *Carpediemonas membranifera* is a free-living member of the Fornicata. It has typical ultrastructure of the excavates and its sequence has the shortest branch within the Fornicata (Simpson et al. 2002b). However, many more free-living excavates should be explored from natural environment and the data from free-living organisms are indispensable. Available cultural strains of free-living excavate are limited and more taxa are needed to achieve comprehensive

data sampling. Establishment of new culture strains of free-living excavate would be a great contribution to make a breakthrough for elucidation of early evolution of eukaryotes.

Most excavates inhabit microaerophilic or anaerobic environments. This would be the reason why to culture free-living excavates is difficult. Here I successfully established three new strains of free living excavates that are not described before. Morphology of three novel strains were investigated using light microscope. In addition, the SSU rRNA sequences of these isolated cultures were determined and analyzed.

MATERIALS AND METHODS

Strains. Two strains of NY0165 and NY0166 were collected from the sea floor sediment (~2 m in depth) of Yamakawa Port, Kagoshima Prefecture, Japan on March 15, 2005 during a cruise No. 2004-30, which was operated from March 8 to 16, 2005 by training and research vessel Toyoshio-maru of the Faculty of Applied Biological Sciences, Hiroshima University. The strain of NY0173 was collected from the sea floor sediment at cold seep site of Sagami bay (1174 m, 35 °0.09'N, 139 °13.51'E), during cruise no. NT06-04, using the ROV *Hyper-Dolphin* which was operated on March 12 and 13, 2006 by JAMSTEC (Japan Agency for Marine-Earth Science and Technology). The deep-sea sediment was kindly provided by Dr. Kiyotaka Takishita (JAMSTEC). These sediments were inoculated and maintained as enrichment culture at 15 °C in URO (*Uroglena*) medium including modified TYGM-9 medium (final concentration 5%) under microaerophilic conditions. The modified TYGM-9 medium was prepared in accordance with instructions from American Type Culture Collection (ATCC) with two exceptions: the rice starch solution and bovine serum were replaced by rice grains and horse serum, respectively. The URO medium (Kasai et al. 2004) was prepared with sea water instead of distilled water. A single cells of three strains were isolated from their enrichment cultures by micropipetting. The cell cultures were also maintained at 15 °C in same medium as the enrichment cultures. A little portion of the clonal cultures were put into new media once a week and they were kept as culture strain.

Light Microscopy. Light microscopy was conducted using a Leica DMR light microscope (Leica, Germany) with the images captured by a Keyence VB6010 digital chilled CCD camera (Keyence, Osaka, Japan). To capture video images, we used

a Nikon Optiphoto light microscope (Nikon, Tokyo, Japan) equipped with Sony 3CCD color video camera Exwave HAD (Sony, Tokyo, Japan). The movies were saved as digital files, and frozen frames were clipped.

Amplification, sequencing, and phylogenetic analyses. Genomic DNA was extracted from clonal cells by a phenol/chloroform method (Garriga et al. 1984). Its nearly entire SSU rRNA gene was amplified from genomic DNA by the polymerase chain reaction (PCR) using the primers previously reported in Nakayama et al. (1998). PCR comprised 30 cycles of denaturation at 94 °C for 30 s, annealing at 52-56 °C for 1 min, and extension at 72 °C for 1 min. The amplified DNA fragments were purified from agarose gels using QIAquick Gel Extraction kit (QIAGEN), and then cloned into pGEM-T-easy vector (Promega). The clone was completely sequenced on both strands.

The SSU rRNA sequence of three clones was aligned manually with those of phylogenetically diverged eukaryotes, including representative excavates. The core sequence alignment was kindly provided by Dr. Alastair G. B. Simpson (Dalhousie University, Halifax, Canada). To assess the position of the cultures in the global eukaryotic phylogeny, we prepared a 42-taxon alignment with 943 unambiguously aligned positions. This alignment was subjected to maximum-likelihood (ML) analyses under a GTR model with among-site rate variation approximated by a discrete gamma distribution with four equally probable rate categories plus the proportion of invariable sites (GTR + I + G model). Model parameters were estimated from the data. The optimal tree was heuristically searched for by 10 times of random taxon addition, followed by tree-bisection-reconnection topological rearrangement. ML bootstrap analysis (100 replicates) was conducted under the same setting as described above.

The relationships among three strains, three environenmental DNAs (A3

E026, C1 E027 and C3 E028), strain PPP15C, *Carpediemonas*, two retortamonads, and nine diplomonads were further examined by analyzing a 24-taxon data set with 983 unambiguously aligned positions. The sequence of PPP15C was kindly provided by Mr. Martin Kolisko (Dalhousie University, Halifax, Canada). The ML phylogenetic analyses described above were repeated on this data set with the detailed settings the same as those described above.

PAUP v.4.0b10 was used for all ML phylogenetic analyses described above. GTR + I + G models were selected from 56 models by Modeltest v.3.7 in conjunction with PAUP.

RESULTS

Light microscopy. *Strain NY0165.* The cell of NY0165 was usually pyriform to semi-spherical in shape, measuring 9-14.5 μm long and 4.5-8 μm wide, but sometimes slender, possessing a tapering posterior end (Fig. 1A-B and F). Neither scale nor lorica was present on the cell body. It possesses a conspicuous groove extended down from the part of flagellar insertion to the posterior end of the cell. The face of this groove was regarded as ventral, adopting terminology given by Simpson and Patterson (1999). The cell shape changed depending on cell behavior, such as feeding or attaching to the substratum with the posterior part of the cell. Two flagella are inserted at the anterior part of the cell with wide angle to each other . The anterior flagellum (AF) was nearly the same length as the cell body. During swimming, the AF bent to the right side of the cell and stroked from the dorsal to ventral side (Fig. 1C-E). The cells of NY0165 usually lay on the substratum, moving the cell body back and forth by the AF motion. The posterior flagellum (PF) was three-fourths the length of the AF, and beat in the ventral groove. Sexual reproduction and cyst formation were not observed.

Strains NY0166 and NY0173. NY0166 (Fig. 2) and NY0173 (Fig. 3) are identical and indistinguishable under the light microscope. They are unicellular flagellates possessing a ventral groove (Fig. 2A-D and Fig. 3A-D). The cell body was usually semi-spherical in shape, measuring 10-12 μm long and 5-8 μm wide and was sometimes slender shape with tapered posterior end. The shape of the cell was easily changed by the presence of prey inside the cell or attachment motion on the substrate. Two flagella emerged from the anterior part of the cell. The AF was as nearly the same length as the cell body. It strokes in the left anterior region and beats from dorsal to the left ventral side of the cell. Posterior flagellum (PF), 1.5-2 times length the cell body,

beats in the ventral groove. Cell swam freely in the medium using both flagella or sometimes attached on the substratum and swing back and forth by the anterior flagellar motion. Neither scales nor lorica on the cell was present. Cells reproduced by binary fissions. Sexual reproduction and cyst formation were not observed.

Small subunit rRNA phylogeny. We determined the nearly complete sequence of the SSU rRNA gene of three strains. In the 42-taxon ML analyses, three strains robustly grouped with *Carpediemonas*, retortamonads, and diplomonads (Fornicata) with high bootstrap value (BP = 77 %; Fig. 4). They branched prior to the divergence of the Fornicata in the optimal tree, but this particular tree topology received only BP = 66 %. Generally, taxonomically closely related lineages were recovered as monophyletic clades with moderate to high BP values (Fig. 4). However, any deeper relationships among the major eukaryotic groups were not strongly supported (Fig. 4).

In the 24-taxon analyses, NY0165 recovered the shortest branch in the Fornicata clade and the robust cluster of NY0166, NY0173, three environmental sequences and the strain PPP15C was recovered. These two sequences (NY0166 and NY0173), NY0165 and *Carpediemonas* were placed in the first, second and third deepest positions in the Fornicata clade, respectively. The strong clade of diplomonads and retortamonads was also recovered in the Fornicata clade (Fig. 5). Both the 42-taxon and 24-taxon analysis could not resolve the relationships among the diplomonad + retortamonad clade, *Carpediemonas*, NY0165, and the clade including NY0166, NY0173, three environmental sequences and PPP15C (Fig. 5).

DISCUSSION

Identifications of three strains of flagellates. Three flagellate NY0165, NY0166 and NY0173 were identified as member of the excavates, as they possess ventral feeding groove which is characteristic of excavate under the light microscope. The appearances of these three strains are similar to *Carpedeimonas membranifera*, a free-living member of the Fornicata, (Ekebom et al. 1995/1996; Simpson and Patterson 1999). However, they can be distinguished from one another by the length of PF and behavior. The PF of *Carpedeimonas* is as 3-5 times long as the cell body, but the length of PF in three strains were shorter than that of *C. membranifera*. The PF of NY0165 is shorter than the length of the groove and the PF of NY0166 and NY0173 is as 1.5 - 2 times long as the cell body. *C. membranifera* cells usually swim with the PF and rarely attach to the substratum, while NY0165 cells usually attach to the substratum and sometimes swim mainly using the AF and both NY0166 and NY0173 cells usually swim using the AF and PF and sometimes attach on the substratum. Consequently, new strains established in this study may be related to *Carpediemonas* and moreover, NY0166 and NY0173 are thought to be the same species, and will be described in the near future.

The sequences of two strains, NY0166 and NY0173 were strongly related to the strain PPP15C collected from Halifax, Canada in molecular analysis. Unfortunately, the strain of PPP15C was lost, and only the SSU rRNA sequence is available. Therefore, it is difficult to discuss further about relationships between the three strains, NY0166, NY0173 and PPP15C.

In the 42-taxon ML analyses, three strains robustly grouped with the Fornicata (*Carpediemonas*, retortamonads, and diplomonads) (BP = 77 %; Fig. 3), and

they branched at the base of the Fornicata in the optimal tree, although this particular node received only BP = 66%. In the 24-taxon analyses, the cluster of diplomonads and retortamonads was recovered with BP of 98%, and NY0165, two strains of NY0166 and NY0173, and free-living *Carpediemonas* were placed in the first, second, and third deepest positions in the Fornicata clade, respectively (Fig. 3). However, as the basal position of free-living Fornicata was not strongly supported, it is at present difficult to confirm the correct branching order. Close affinity to the sequences of environmental DNAs. The sequences of NY0166 and NY0173 are very close to the sequences taken from the strain collected from eastern Canada, and to the environmental sequences collected from various site, cold seep site at Sagami Bay, Japan (Takishita et al. in prep), and Guaymas Basin hydrothermal vent, Gulf of California, USA (Edgcomb et al. 2002). The analyses of environmental sequences are powerful tool to investigate the diversity of eukaryotes in certain sampling sites. Most reports have shown that unexpected remarkable diversity of life is present in the natural environment and that large number of unknown protists are included in environmental samples (Díez et al. 2001; Edgcomb et al. 2003; Countway et al 2005; Takishita et al. 2006, 2007), indicating that we are about to start understand true diversity of protists. Therefore, exploring unidentified protists is indispensable step to proceed. A large number of sequences taken from environmental analyses are registered as "uncultured eukaryotes" in GenBank. The fact that the sequences same as newly isolated excavates were found in environmental DNAs is an important result. This strongly suggests that a large number of unknown excavates are present in natural environment, meaning that to explore protistan diversity is essential to understand true eukaryotic diversity.

Cosmopolitan diversity of free-living Fornicata. Free-living members of

Fornicata have rarely been studied in detail. Only a few species have been deposited and maintained in culture collections in the world. This would be because it is difficult to find free living excavates, to isolate them, and to keep them as culture strains. Most of excavates inhabit anaerobic environment. Our result showed that these Fornicata flagelates exist as cosmopolitan species in the world. This study clearly demonstrated that why free-living excavate protists were rarely found is due to technical problems. Improvement of culture techniques is essential to reveal true diversity of excavates.

For better understanding of the Fornicata. The Fornicata would be an important group to understand evolution of mitochondria. It is consisting of three taxa: *Carpediemonas*, retortamonads and diplomonads (Simpson 2003), Distribution of mitochondrion-related organelles in the Fornicata is remarkable: *Carpedeimonas* and diplomonads possess hydrogenosome like organelle and mitosome, respectively, but in the retortamonads mitochondria related organelles have not been observed. Parabasalids are closely related to the Fornicata (Simpson et al. 2006), but lacks classical mitochondria and possess hydrogenosome. The presence of varieties of mitochondrion-related organelles in a single clade is surprising so that the Fornicata have received attention with respect to mitochondrial evolution. The mitosome is double-membrane bounded organelle but lacking function of aerobic respiration and its genome is no longer present in it. The mitosome has been investigated intensively in diplomonad *Giardia* for understanding the essential function of mitochondria. The function of the mitosome was recently determined to be maturation of iron-sulfur cluster. *Giardia* codes the *IscS* and *IscU* genes in the nucleus and these proteins mature in mitosome (Tovar et al. 2003).

In summary, I found two novel excavates from natural environments and

successfully established as three new culture strains. SSU rRNA trees demonstrated that they belong to the Fornicata and diverged from near the base of the Fornicata clade. The sequences of two strains (NY0166 and NY0173) strongly clustered with environmental DNAs known as "uncultured eukaryotes" on the database. This study clearly demonstrated that exploring free-living excavates is very important to clarify diversity of Fornicata. Further analyses using strains established in this study would make a great contribution to elucidate true evolution and phylogeny of fast-evolving Fornicata. Moreover, since the Fornicata possesses various degeneration stages of mitochondria, Fornicata protistan group, diplomonads and Fornicata related protistan group, parabasalids are considered as important group to clarify the early evolution of eukaryotes, it is clear that studies of the Fornicata evolution and phylogeny must shed light on the crucial mitochondrial evolution and the early evolution of eukaryotes.

Part 2. Description of a new excavate protist: *Dysnectes brevis* gen. et sp. nov.

INTRODUCTION

The Fornicata consists of three groups of excavates: diplomonads, retortamonads and *Carpediemonas* (Simpson 2003). Each group includes free-living flagellates. Almost all protists within the diplomonads are parasitic. However a few species of diplomonads such as *Trepomonas agilis* or *Hexamita inflata* are known to be free-living and they are nested in the diplomonads clade (Jørgensen and Sterud 2006; Keeling and Brugerolle, 2006). Likewise, some species of *Chilomastix* (retortamonads) are free-living (e.g., *Ch. cuspidate*) (Brugerolle 1991a) but other species of this *Chilomastix* and all other genera of the retortamonads are parasitic. *Carpediemonas* is free-living member of the Fornicata. Two species, *Ca. membranifera* and *Ca. bialata*, have been described. *Ca. bialata* was studied only with the light microscope (Lee and Patterson 2000). Ultrastructure and molecular analyses have been reported for *Ca. membranifera* (Simpson and Patterson 1999; Simpson et al 2002b). The sequence of *Ca. membranifera* are recovered as shortest branch among the Fornicata clade, indicating that it is slow-evolving compared to parasitic members of the Fornicata, diplomonads and retortamonads (Simpson et al 2002b; Keeling and Brugerolle 2006). Therefore, *Ca. membranifera* has been thought as a good representative of the Fornicata since it was described in 1999 (Simpson & Patterson 1999), because it is said that free-living and short branching excavates are important to recover better phylogenetic tree.

In the previous section, I showed that free-living excavate NY0165 was the slowest-evolving excavate within the Fornicata clade. This flagellate is most certainly the best representative of the Fornicata in the SSU rRNA gene phylogeny. It is essential to study their nature of the cell such as flagellar transformation, feeding behavior, cellular structure and flagellar apparatus. Here, I carefully investigated morphology of

this novel flagellate using the light and electron microscopes. NY0165 is a novel excavate and I proposed a new Forncata taxon *Dysnectes brevis* gen. et sp. nov., isolated from the coastal area in the south-western part of Japan. Data consistently indicated that *D. brevis* can be assigned to the Fornicata, as a relative of *Carpediemonas*, retortamonads, and the diplomonads. Moreover it has been proved that *D. brevis* have a quite similar ultrastructure to those of phylogenetically distant group of typical excavate.

MATERIALS AND METHODS

Strain. *Dysnectes brevis* gen. et sp. nov., (NY0165) was maintained at 15 °C in URO (*Uroglena*) medium including modified TYGM-9 medium (final concentration 5%) under microaerophilic conditions as shown in previous section. My strain (NIES-1843) of *D. brevis* was deposited in the Microbial Culture Collection at National Institute for Environmental Studies (NIES, Japan).

Light Microscopy. To capture video images, I used a Nikon Optiphoto light microscope (Nikon, Tokyo, Japan) equipped with differential interference contrast (DIC) optics. The movies were captured by Sony 3CCD color video camera Exwave HAD (Sony, Tokyo, Japan). The movies were saved as digital files, and frozen frames were clipped.

Electron Microscopy. For transmission electron microscopy, the cells of the exponential growth phase after the four days inoculation were used as the specimen. The materials of thin sections were fixed in equal volumes of fixative, which is mixture of 2.5% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide (final concentration) in 0.2 M cacodylate buffer (pH 7.2) at room temperature for 10 min. After centrifugation at 580 *g* (1800 rpm) for 5 min, the pellet was rinsed several times with 0.2 M cacodylate buffer (pH 7.2). This pellet was dehydrated through an ethanol series by keeping it in 30% and 50% ethanol for 1h each, in 75%, 90%, and 95% for 20 min each, followed by four changes in 100% ethanol for 15 min each. The pellet was substituted in 1:1 mixture of propylene oxide and 100% ethanol twice for 10 min each and then to changes to 100% propylene oxide twice for 10 min each. The specimens were embedded in Spurr's resin (Spurr 1969). The resin was polymerized for 10h at 70 °C. Ultrathin sections were cut with a diamond knife and double stained with 2% (w/v) uranyl acetate and lead

citrate (Reynolds 1963). Observations were carried out with a JEOL JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Video Microscopy. Feeding behavior of NY0165 was observed by video microscopy. Prey bacteria were involved in the membrane-like structure moving along the ventral groove (Fig. 6). It moved in a cycle for the frequency of about 10 s from anterior to posterior end of the groove and the speed of the migration is 0.6 μm/s. It was never observed moving backward, from posterior to anterior. Food particle riding the water current produced by the PF was drawn to the posterior part of the ventral groove and was pressed by the membrane-like structure to the posterior end of the groove. Subsequently, the particle was wrapped by the membrane-like structure at the posterior region and then engulfed into the cell body. The membrane-like structure keeps moving at the same speed and pattern even if the bacteria is absent at the groove.

The cells reproduced by cell division (Fig. 7). Initial process for the longitudinal division was duplication of the flagella. Two new flagella emerged on the both side of the AF. The immature flagella sometimes beat with the AF motion. The new groove emerged on the right side of the original groove. The cell having two grooves and two sets of the AF and PF on each groove was observed (Fig. 7B).

Ultrastructure. A spherical nucleus was situated at the anterior ventral side of the cell (Fig. 8B). Two flagella emerged from the apical region of the cell. Two basal bodies were located in the region anterior to the nucleus. No typical mitochondrion was observed. Instead, organelles filled with a dense matrix were frequently observed around the nucleus (Fig. 8B). No Golgi apparatus was observed. Large vacuoles containing bacterial cells were situated at the posterior part of the cell. The ventral groove was supported by microtubules originating from the posterior basal body, while the dorsal side of the cell was free from cytoskeletal microtubules (Fig. 8A and B; see

below for the details). The AF was smooth and no accessory structures, such as hairs or vanes, were observed. The PF had two flagellar vanes extending toward the ventral and dorsal directions (Fig. 8C and D). The ventral vane originated from the proximal end of the flagellum (Fig. 8A-D) and was supported by a dense lamella that was curled at the axoneme side and flattened at the tip of the vane (Fig. 6A and B). The dorsal vane started from a more distal part of the flagellum than the ventral vanes, and was supported by a flattened lamella (Fig. 6E). The transitional plate was situated in the flagellar transition region at the plane of the cell membrane (Fig. 6C). The central doublet microtubules were inserted into the transitional plate (Fig. 6C).

Flagellar apparatus. The overall organization of the flagellar apparatus of *D. brevis* appeared to be homologous to those of typical excavates studied previously. Henceforth, we applied the terminology used in Simpson and Patterson (1999) to *D. brevis*. The flagellar apparatus of *D. brevis* was composed of (i) two basal bodies, (ii) four major microtubular roots, (iii) three major fibers, and (iv) "internal" microtubules (IMt) (Fig. 13).

Basal bodies. The basal bodies associated with the AF and PF were designated as the anterior basal body (AB) and posterior basal body (PB), respectively. Both the AB and PB were approximately 350 nm in length and were arranged at an angle of ca. 120° (Fig. 8A and D). Both basal bodies possessed a cartwheel structure at the proximal end (Fig. 9A, 10A). The IMt originated from the left posterior side of both basal bodies, and radiated along the nucleus in the posterior direction (Fig. 9C, 10, 11A and B, 11D and E).

Anterior root (AR). The AR consisted of a single microtubule and originated from the anterior side of the AB. The AR extended toward the dorsal left side of the cell

under the plasmalemma, and terminated approximately 1 μm from the origin (Fig. 9A-C). No cytoplasmic microtubules (dorsal fan) arose from the AR.

Singlet root (SR). The SR arose between the dorsal left side of the PB and left dorsal side of the right root (RR), and ran posteriorly under the cell membrane (Fig. 10 and 11). At the origin, the SR and the left portion of the RR were connected by the singlet-root associated fiber (SA) (Fig. 10C and D). Posterior to the insertion of the PF, the SR, located between the inner right root (IRR) and left root (LR), runs under the cell membrane and supports the central axis of the ventral groove (Fig. 8B and E , 11G).

Structures supporting the ventral groove. The left root (LR) appeared to support the left half of the ventral groove. The remnant of the B fiber is aligned along the ventral face of the proximal area of the LR (see below for details) and a triple-layered C fiber is aligned along the opposite side (dorsal face) of this root. The LR arises as a single microtubule at the left proximal side of the PB (Fig. 10A), runs posteriorly, progressively adding extra microtubules up to ca. 17, and forms a broad band of microtubules that support the left side of the groove (Fig. 10).

The RR originates from the right-dorsal side of the PB (Fig. 11A and B) and runs posteriorly. At its origin, the RR is a broad band of ca. 18 microtubules. Near the distal end of the PB, the RR splits into two components — the inner right root (IRR) and the outer right root (ORR) comprising 6 and 12 microtubules, respectively. The IRR and ORR are arranged in an L-shape (Fig. 11B and C). A tubular penetration of the cell membrane (gutter) was observed along the ventral side of the corner of the L-shaped arrangement comprising the IRR and ORR (Fig. 10D, 11A and B). The gutter is situated near the proximal portion of the PF, and opens outside the ventral groove at the level at which the PF emerges from the cell body (Fig. 11D and E). Both IRR and ORR have

"comb-like projections" on their dorsal sides (Fig. 8B, 11C-F and H).

At the level of the anterior end of the RR, the I fiber arises as a broad sheet, lying along the entire width of RR and extending posteriorly. The I fiber and microtubules of the RR are connected by fibrous material (Fig. 11C). Posterior to the level at which the RR splits into the two rootlets, the I fiber is reduced in size, covering only the right part of the ORR (Fig. 10E and F, 11C-G).

At the level of the proximal part of the PB, the arched B fiber is situated at the ventral side of the PB, covering the area from the left side of the RR to the single microtubule of LR (Fig. 10A and 11A). Posteriorly, the fiber broadens and covers a broader area, from the vicinity of left tip of the I fiber to the left tips of the LR. On the left side of the groove, the left-most tip of the B fiber curved to the right as a thin sheet attached to the ventral face of the LR. At the region of the insertion of the PF, the B fiber splits into two: the left portion extends along the ventral face of the LR while the right portion extends along the ORR. The left portion is reduced on the left, and remains only at the left tip of the LR (Fig. 10F). The left tip of the B fiber is directed to the ventral side and extends to the right ridge of the groove. At the area below the insertion of the PF, the right portion of the B fiber and I fiber almost connect to each other, and together with ORR, they are arranged in D-shape (Fig. 10E and F, 11F). This complex of fibers and microtubular root is reduced posteriorly and only the ORR remains at the right edge of the ventral groove just under the cell membrane (Fig. 11E-G). Approximately 10 extra microtubules are added consecutively one by one to the right side of the posterior portion of the ORR, so that the number of microtubules comprising the ORR reaches ca. 28 (Fig. 11C-G). Both the B and I fibers entirely disappear in the posterior part of the RR, so that the right half of the ventral groove is supported mainly

by the ORR and IRR (Fig. 8B and 11G).

Composite fiber. At the posterior portion of the cell, the composite fiber (CF) extends along the dorsal side of the ORR (Fig. 8E and 12). The CF appears to be striated (the width of striation was ca. 20 nm) (Fig. 12B).

DISCUSSION

***Dysnectes brevis* gen. et sp. nov. as a novel member of excavates.** Light microscopic features shown in part 1 suggested a taxonomic affinity between *D. brevis* and previously known excavates. Of these, the ventral feeding groove is the hallmark (Simpson and Patterson 1999), and is the most compelling evidence of excavates. At the ultrastructural level, *D. brevis* shares seven features with typical excavates: (i) split RR; (ii) SR; (iii) flagellar vanes; (iv) C fiber; (v) I fiber; (vi) B fiber, and (vii) composite fiber (Table 1). Among eukaryotes, only typical excavates (i.e. *Carpediemonas*, retortamonads, *Trimastix*, *Malawimonas*, and jakobids) possess all these seven ultrastructural features (Simpson 2003; Simpson and Roger 2004b). However, a specific affinity between *D. brevis* and the previously known excavate lineages is still unclear, indicating that while *D. brevis* is a typical excavate, it is nevertheless a novel excavate lineage (Table 2). The point-by-point discussions are provided below.

Flagellar vanes. As found in *D. brevis*, *Chilomastix* possessed curled-up lamella on the ventral part of the paired vanes. Simpson and Patterson (1999) noted that the lamella in *Chilomastix* vanes is homologous to the circlet lamella in the ventral-most vane of *Carpediemonas* and the circular lamella in the *Malawimonas* vane. The ventral curled-up lamella in the *D. brevis* vane is similar to those found in *Chilomastix*. The similarity amongst these structures is evident, and the ventral lamellae of the four lineages, including *D. brevis*, would almost certainly be homologous.

Anterior root (AR) as the microtubule-organizing center. The AR, which is found in three groups, *Carpediemonas*, *Trimastix*, and *Malawimonas*, is associated with the microtubule-organizing center (MTOC) in all groups. The dorsal fan extends from the AR and supports the dorsal side of the cell (Brugerolle and Patterson 1997; O'Kelly

and Nerad 1999; O'Kelly et al. 1999; Simpson and Patterson 1999; Simpson et al. 2000). The retortamonads and jakobids also have the dorsal fan, which originates from the MTOC located around the AB, and not from the AR (Bernard et al. 1997; Lara et al. 2006; O'Kelly 1993; O'Kelly 1997; Patterson 1990; Simpson and Patterson 2001). In contrast to the typical excavates, the short AR of *D. brevis*, which extends from AB to the left side of the cell, is not associated with either MTOC or dorsal fan. The AR of *D. brevis* could be a degenerated form of the AR with its dorsal fan, which is found in *Carpediemonas*, *Trimastix*, and *Malawimonas*.

Microtubules originated from basal bodies or the adjacent region of basal bodies. In *Trimastix*, the "internal" microtubule (IMt) radiates from the IMt-organizing center (IMtOC), which is located at the left side of the basal bodies (Simpson et al. 2000). Although no IMtOC was identified around the AB in *D. brevis*, the microtubules radiating from the left side of the AB in *D. brevis* are comparable to the IMt of *Trimastix* and could be homologous.

Comb-like A fiber. In jakobids, *Malawimonas*, retortamonads, and *Carpediemonas*, the A fiber is present on the ventral side of the RR and connects this root to the ventral side of the PB. In *Trimastix marina*, the A fiber is replaced by the comb-like projections (Simpson et al. 2006). *Dysnectis brevis* does not possess the A fiber. However, the comb-like projections of in *D. brevis*, situated on the ventral face of the ORR and IRR, are likely homologous to the remnant of the A fiber in *Trimastix* (Simpson et al. 2000). It seems that all typical excavates should possess the A fiber or comb-like projections as a reduced component of the A fiber.

Absence of the typical mitochondrion. Jakobids and *Malawimonas* retain mitochondria while a typical mitochondrion is absent in *Trimastix*, *Carpediemonas*, and

retortamonads. It is generally believed that the typical amitochondriate excavate lineages have lost mitochondria secondarily, since double membrane-bounded organelles — the hydrogenosome-like organelles — are present in *Trimastix* and *Carpediemonas* (Simpson and Patterson 1999; Simpson et al. 2000). Mitochondrion-derived organelles have never been observed in retortamonads. However, a close relative, a diplomonad *Giardia*, retains the remnants of mitochondria (Tovar et al. 2003). In *D. brevis*, no typical mitochondrion was observed, suggesting an evolutionary affinity to the amitochondriate excavate lineages. We observed in *D. brevis* organelles filled with dense matrix, which resemble the hydrogenosomes found in *Carpediemonas* and *Trimastix*, except that the membrane envelope surrounding the organelles cannot be determined due to fixation problems. The organelles could be the remnants of mitochondria, although this must be confirmed by further electron microscopic observations and biochemical, physiological, and molecular biological techniques.

***Dysnectis brevis* belongs to the Fornicata.** Although the study on the morphological data suggested that *D. brevis* is a new member of the typical excavates, the evolutionary affinity of *D. brevis* to the previously identified excavate groups could not be clarified (Table 1). However, the SSU rRNA phylogeny successfully demonstrated a close affinity between *D. brevis* and the Fornicata with a high BP support, although the precise position of *D. brevis* in the Fornicata clade remains unresolved. Our reassessment of the morphological data, considering the results of the SSU rRNA analyses, suggests that *D. brevis* and the Fornicata share a unique structure – the B fiber. In the Fornicata, the B fiber connects the PB and LR and supports the ventral groove (Simpson 2003; Simpson and Roger 2004b). Similarly, the B fiber in *D.*

brevis bridges the RR and LR and supports the ventral groove. Considering the "arch-shaped" B fiber and SSU rRNA phylogeny, I here propose *D. brevis* as a new genus and new species of the Fornicata, though it must be placed as *incertae sedis* is this group because it shows not clear affinity to any other major clade.

Description.

Latin diagnosis of new genus: Dysnectes

Dysnectes Yubuki, Inagaki, Nakayama et Inouye gen. nov. (ICZN and ICBN).

Cellulae semicircularis vel elongatae, biflagellata, phagotrophicis, cum sulco ventrali. Cellulae plerumque ad substratum. Flagellum anticum mobile, abeuns sinistorsum. Flagellum posticum cum biala, pulsus in sulco. Radix antica e corpora basalia anticum exorientia. Sine flabello dorsali. B fibra superpendens radix dextra ad radix sinistra.

Species typifica: *Dysnectes brevis.*

Diagnosis of new genus: Dysnectes

Dysnectes Yubuki, Inagaki, Nakayama et Inouye gen. nov. (ICZN and ICBN).

Cells semicircular or elongate, biflagellate, phagotrophic, possessing a ventral groove. Cells usually attaching to the substratum. Anterior flagellum active, beating back and forth in the vicinity of the left side of the cell. Posterior flagellum possessing two venes, beating in the groove. Anterior root, arising from the anterior basal body. No dorsal fan present. B fiber, covering right root to left root.

Type species: *Dysnectes brevis.*

Latin diagnosis of new species: Dysnectes brevis

Dysnectes brevis Yubuki, Inagaki, Nakayama et Inouye sp. nov. (ICZN and ICBN).

Cellula 9-14.5 μm longa, 4.5-8 μm lata. Flagellum posticum brevis. Cellula ad substratum haerens, interdum reptans vel natans.

Diagnosis of new species: Dysnectes brevis

Dysnectes brevis Yubuki, Inagaki, Nakayama et Inouye sp. nov. (ICZN and ICBN).

Cells 9-14.5 μm long and 4.5-8 μm wide. Posterior flagellum, short. Cells attaching, sometimes gliding or swimming.

Holotype: EM block TNS-AL 161063 deposited in the herbarium, Tsukuba Botanical Garden, National Science Museum, Tsukuba, Japan. Isotype: Fig. 1A

Type locality: Yamakawa port in Kagoshima Prefecture, Japan.

Collection date: March 15, 2005.

Type strain: deposited as NIES-1843 in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES), Japan.

Etymology: The generic name refers to the swimming behavior of the cell (*Dys* = bad, *nectes* = swimmer), and the specific name refers to the length of the posterior flagellum (*brevis* = short).

GENERAL DISCUSSION

Importance of establishment of novel excavates. Free-living protists examined in this study were novel excavates that have not closely related to any previously known excavates. Molecular phylogeny showed phylogenetic distinctness of NY0165. NY0166 and NY0173 are probably the same species but also distinct from previously described excavates. They should be described as new members of excavates. NY0165 was formally described as *Dysnectes brevis* (Dysnectes: bad swimmer, brevis: short). *D. brevis* should be added as a new group of excavates to the ten groups of excavates. Discovery of new species of excavates shows that excavates are more diversified than it is generally thought. Study of free-living excavate is just started and more groups of excavate would be discovered in further.

The shortage of knowledge of excavate diversity has become evident from the fact that the sequences of both NY0166 and NY0173 are clustered with the sequences of environmental DNAs taken form various site of Japan and other countries. Studies about environmental DNAs have clearly shown that a large number of unknown eukaryotes are existing in natural environments (Countway et al. 2005; Takishita et al. 2005, 2007, in prep.) and strongly suggested that undescribed excavates were also included in unknown diversity of eukaryotes. Further establishments of culture strains of excavates should be proceeded to reveal true diversity, distribution and ecological role of excavates.

Morphological characters shared between excavates and other eukaryotes. Information on the ultrastructure have been accumulated for several excavates, including *Carpediemonas membranifera* (Simpson and Patterson 1999), *Trimastix marina* (Simpson et al. 2000), *Andulcia incarcerata* (originally described as

Jakoba incarcerata in Simpson and Patterson 2001). These excavates are phylogenetically diverse, comprising of polyphyletic lineages (Simpson et al 2006). However, cell ultrastructures are surprisingly similar between these different lineages. These facts were interpreted as that excavates remain primitive features of early eukaryotes. Comparison between excavate lineages and comparison between excavates and non-excavate eukaryotes would be essential for comprehensive understanding of cellular evolution of eukaryotes. A huge amount of ultrastructural data has been accumulated for many non-excavate lineages, so that it would be possible to compare the excavates with non-excavate eukaryotes that cover all recognized super groups. Such comparison using morphological data, especially the flagellar apparatus, have rarely been explored. Here, I discuss the flagellar apparatus shared between typical excavates (including *D. brevis*) and various eukaryotic lineages.

The flagellar apparatus comprised of flagella, basal bodies, microtubules and fibrous materials is pivotal structure of the eukaryotic cell. It is probably the most complex structure of the eukaryotic cell, universally distributed, and plays essential roles for sustaining various cell functions, such as flagellar beat (swimming), generation of cytoskeleton and division poles (cell division) and feeding preys (phagocytosis) (Moestrup 1982). Its basic architecture is stable within higher taxa such as classes and phyla and distinct between them (Moestrup 2000). It has been used as a relevant taxonomic marker at higher taxonomic ranks. This crucial architecture of eukaryotes is maintained by a mechanism probably common in all eukaryotes. Eukaryotes most typically possess two flagella that are inherited to daughter cells in semi-conservative fashion. For example, heterokonts possess two heterodynamic flagella. When the cell divides, the short posterior flagellum and long anterior flagellum become the short

posterior flagellum in the next generation, and two newly generated flagella become the long anterior flagellum, that is, for maturation of flagella, several generations are needed. In the example of heterokonts, the posterior flagellum is mature (older generation) and the anterior flagellum is immature (younger generation). Therefore flagellar apparatus can be understood and comparable between eukaryotic groups based on the common and universal numbering system. Moestrup (2000) proposed universal terminology and reviewed the numbering system of most eukaryotes but not that of excavate. Here, terminology of the flagellar apparatus of excavates will be discussed before the detail point by point comparisons between excavate and non-excavate eukaryotes.

Flagellar numbering system in excavates. Simpson (2003) proposed terminology of the flagellar apparatus of excavates referred to the universal flagellar numbering system of Moestrup (2000). In Simpson's definition, the right and left microtubular roots (RR and LR), which are associated with PB, correspond to roots 1 and 2 (R1 and R2), and anterior root (AR) with AB is equivalent to root 4. However, Moestrup (2000) proposed, ' the two roots associated with flagellum 1 are named root 1 and 2, those with flagellum 2 are named roots 3 and 4.' and 'the roots are (usually) named in a clockwise fashion, looking down the basal body from the outside of the cell.' In this situation, excavate numbering system needs some revisions.

In order to apply the universal numbering system to the excavates, understanding of flagellar generation is required. I have been trying to know the fate of two newly generated short flagella in *Dysnectes brevis*, though unfortunately it has not succeeded yet. If we can determine which flagellum is newly generated flagellum, the numbering system of excavate can be correctly applied. Even though, based on

accumulated data for many eukaryote groups, I would be able to designate flagella of excavates. In many groups of eukaryotes, two flagella are inserted as to extend to the anterior and posterior directions. Since the anterior flagellum is immature and posterior flagellum is mature in most cases so far examined, they are designated as F2 and F1, respectively. I therefore tentatively designate the posterior flagellum of *Dysnectes brevis* as F1. Consequently, I propose a new numbering system of excavate flagellar apparatus referred to Moestrup (2000): The short posterior flagellum and long anterior flagellum of *D. brevis* are designated as F1 and F2, respectively, the LR and RR associated with the F1 (PF) and PB (B1) as R1 and R2, respectively and the AR on the F2 (AF) and AB (B2) as R3. Under the new numbering system, excavate flagellar apparatus can be compared with those of other eukaryotic groups. Though the flagellar apparatuses are distinct between higher taxa of eukaryotes, homologous structures can be recognized between flagellar apparatuses of distantly related eukaryotes, if I compare carefully and taking the flagellar apparatus of excavates into account.

Structures associated with R1. In some members of the Prasinophyceae (Viridiplantae) (presumably the most primitive members of green plants), *Halosphaera*, *Pterosperma*, and *Cymbomonas,* and flagellate cells of the Streptophyta (*Klebsormidium*, *Coleochaete*, *Chara* and sperms of liverwort, mosses, ferns, Ginkgo and cycads), R1 (traditionally recognized as root 1d) carries a lamellar object known as the multilayered structure (MLS) (Carothers and Kreitner 1968; Pickett-Heaps 1975; Melkonian 1989; Hori and Moestrup 1987; Moestrup and Hori 1989; Inouye et al. 1990; Moestrup et al. 2003). Wilcox (1989) also reported MLS-like structures on R1 (traditionally recognized as longitudinal microtubular root) in dinoflagellates. The MLS is also present on one of microtubular rootlets in Glaucophyta. Though the root has not

been identified, this root is most probably the R2 (Mignot et al. 1969; Kies 1979, 1989). MLS or MLS like lamellate structure is widely distributed over super groups of eukaryotes, and except a few exception (for example, a prasinophyte *Mesostigma viride* has two MLSs each associated with R1 and R3 and MLS were seen on the R2 in Glaucophyta), MLS (or MLS-like structure) is usually situated on the dorsal side of R1. This is also the case in excavates. The (multilayered) C fiber associated with the dorsal face of R1 (LR), is the characteristics of the typical excavate. The C fiber of these excavates can be considered as homologous with MLS of the green plants and dinoflagellates. Furthermore, the remnant of the B fiber in *D. brevis*, which forms a thin sheet-like structure on the ventral side of R1 (LR), is similar to the plate-like structure in *Crustomastix didiyma* (Nakayama et al. 2000) or keels in *Mesostigma viride* (Melkonian 1989) associated with the ventral side of R1 in prasinophyte. This could also be a homologous structure shared between green plants and excavates.

R2 involved in phagotrophy. The detailed investigations of feeding behavior of a heterokont alga *Epipyxis* (Chrysophyceae, Heterokontophyta) revealed that prey particles are engulfed via the feeding cup, which is formed in the margin of outer and inner microtubules of R2 (R3 in the original description) (Andersen and Wetherbee 1992; Moestrup and Andersen 1991; Wetherbee and Andersen 1992; Wetherbee et al. 1988). R2 associated feeding (R3-feeding sensu Moriya et al. 2002) is widely distributed in the stramenopiles, subgroup of a super group Chromista, to which Heterokontophyta belongs. Colorless flagellate members, bicosoecids and wobllids are thought to use R2 for feeding preys (Moestrup and Thomsen 1976; O'Kelly and Patterson 1996; Fenchel and Patterson 1988; Karpov et al. 1998; O'Kelly and Nerad 1998; Teal et al. 1998; Karpov 2000; Moriya et al. 2000; Karpov et al. 2001). Though

R2 is morphologically distinct from that of *Epipyxis*, they share R2 that split into two bundles of microtubules, and feeding is believed taking place between two split components of R2. So, R2 associated feeding could be a primitive (plesiomorphic) feature of the stramenopiles. In typical excavates, R2 also splits into two bundles, which is one of the seven common features, and particularly, in two excavates *Trimastix* and *Chilomastix*, a cytopharynx is enclosed by the posterior end of split R2 component (RR) (Bernard et al 1997; Weerakoon et al. 1999; Simpson et al. 2000). In *Dysnectes brevis*, unique feeding behavior using the membrane-like structure was observed under the light microscope. I haven't studied yet the ultrastrucrure of the feeding structure, but it is likely that the feeding structures of *D. brevis* are associated with the R2 as in *Trimastix* and *Chilomastix*. Even in the Euglenozoan protists which have distinct flagellar apparatus from those of typical excavates, R2 is associated with the phagotrophic apparatus. Their cytopharynx is clearly associated in the intermediate root (presumably corresponding to the R2). All these characteristic structures appeared in excavates are surprisingly similar to the R2 feeding in the stramenopiles. It is possible that the feeding machinery, in which R2 is involved, evolved in the common ancestor of excavates and stramenopiles. Mechanism of feeding using R2 should be compared between these distant super groups by biochemical and physiological approaches.

Microtubules originated from R3. In stramenopiles, R3, which corresponds to R1 in traditional terminology (Andersen 1987), acts as a microtubule organizing center (MTOC). Cytoplasmic microtubules arise from this root, and run toward the dorsal and posterior direction (Andersen 1991; Karpov et al. 2001; Moriya et al. 2002). Farmer and Roberts (1989) mentioned that this feature of R3 observed in the Chrysophycese (stramenopiles) appeared to be evolutionarily related with TMR/TMRE complex of

dinoflagellate *Amphidinium*. Some dinoflagellates possess TMR (transverse microtubular root) originated from the transverse basal body, which correspond B2. The TMR would be equivalent to the R3 under the Moestrup's terminology (Moestrup 2000). Some species of dinoflagellate such as *A. rhynchocephalum* and *Gymnodinium chlorophorum*, carry cytoplasmic microtubules, termed TMRE (TMR extension), on TMR (Farmer and Roberts 1989; Hansen and Moestrup 2005). Structural similarity between the TMRE on TMR (R3) of dinoflagellate and the cytoplasmic microtubules of the stramenopiles was suggested (Farmer and Roberts 1989). Similar cytoplasmic structures are also present in some excavates. In typical excavates, *Carpediemonas*, *Malawimonas* and *Trimastix*, cytoplasmic microtubules (dorsal fan) arise from R3 and support the dorsal side of the cell (O'Kelly and Nerad 1999; Simpson and Patterson 1999; Simpson et al. 2000). Euglenozoa, a subgroup of the Excavata has the flagellar apparatus distinct from typical excavate, since their architecture is absolutely distinct from those of typical excavates. However, cytoplasmic microtubules named as dorsal band in Euglenozoa are associated with the dorsal root (presumably corresponding to the R3) (Owens et al. 1988). This suggests that the unique flagellar apparatus of the Englenozoa is derived from the typical excavates and retains homologous structures. Consequently, this cytoplasmic microtubules on the R3 seem to be widespread in eukaryotes and the R3 and associated dorsal fan in excavates are probably homologous to those structures of the stramenopiles and alveolates.

Microtubules originated from basal body (or the adjacent region of basal body). Amoebozoa is one of the eukaryotic super groups. Some amoeboid protists are bearing the flagellum, basal body and microtubular rootlet system. Pelobionts (*Mastigamoeba*, *Mastigina* and *Phreatamoeba*) in Amoebozoa possess one single basal

body and it is considered as B1. Their flagellar apparatus is unique in that the MTOC is present at the base of the B1 and a large number of microtubules radiated posteriorly from this MTOC. These microtubules form a corn shaped structure surrounding the nucleus (Brugerolle 1991b). Similar structures were observed in the Cercozoan protists, choanoflagellates (presumably the most primitive members of animal and fungi) and oomycetes (belonging to the stramenopiles). In cercozoan *Cercomonas* and *Hyperamoeba*, MTOC is located in the posterior portion of basal bodies, and many microtubules radiate and form a corn shape structure as in pelobionts (Karpov and Leadbeater 1997; Karpov and Leadbeater 1998). In choanoflagellate *Monosiga* and *Desmarella,* the proximal portion of one of two basal bodies works as MTOC, and microtubules radiate from this center, pass under the cell membrane and support the tentacles (Karpov and Leadbeater 1997, 1998). Chytrid fungi, *Synchytrium* and *Harpochyrium* are observed the microtubules radiated in to the cytoplasm (Barr 1981) An oomycete *Phytophthora* (stramenopiles) also possesses the radiation of microtubules that originate from an adjacent region around two basal bodies and supports the left side of the cell, though no obvious MTOC was identified (Barr and Allan 1985). The radiating microtubules from posterior part of basal bodies were observed in various super groups of eukaryotes. In a typical excavate *Trimastix* (Simpson et al. 2000) and *D. brevis,* 'internal' microtubules (IMt) radiate from an adjacent region around the basal body and extend to the left of the cell (the MTOC in *Trimastix* is originally called as 'internal' microtubule organizing center or 'IMtOC'). Consequently, these characteristic of microtubules of two typical excavates could be assigned to those of Amoebozoa (pelobionts), Rizharia (Cercozoa), Opisthokonta (choanoflagellates and Fungi) and Chromalveolata (stramenopiles).

The presence of various similarities in the flagellar apparatus between the excavates and other phylogenetically distant eukaryotes leads to a hypothesis that the four characters described above have been descended from their 'excavate-like' ancestral cells (i.e. plesiomorphy). More boldly, the universal ancestor of eukaryotic cells might have possessed the morphological characters found in the extent excavates. This scenario requires the specific assumption that excavates are deep or the deepest branches in eukaryotic phylogeny. Unfortunately, there is no data that directly supports the 'deep excavate' or 'deepest excavate' scenarios, so phylogenetic position of excavates (and the issues related this matter) must be examined by both morphology and molecular-based works. For instance, the 'plesiomorphies' identified in this study (see above) should be confirmed by detailed ultrastructural examinations of a larger number of both excavates and non-excavate taxa. In order to settle the monophyly-versus-paraphyly argument over excavates, new multi-gene data sets, which are larger than those used by Simpson et al. (2006), are essential. It would be certainly possible, but extremely challenging, to assess whether excavates are genuine deep branches in eukaryotic tree by molecular data analyses.

Toward a better understanding of Fornicata evolution. A remarkable character of SSU rRNA of new strains are that these sequence are the least divergent of the Fornicata lineages. All molecular sequences of a parasitic member of the Fornicata, such as *Giardia*, are rapidly evolving (Embley and Hirt 1998; Philippe et al. 2002), so that such "long-branched" sequences can introduce various forms of systematic artifacts in tree reconstruction. Thus, the SSU rRNA gene sequences of new strains were less diverged than that of *Giardia*, and it could be a "surrogate" for problematic diplomonads in phylogenetic analyses. If other gene sequences of these strains are

"short-branches," as seen in the SSU rRNA tree, molecular analyses, in which these strains are considered as the representative of the Fornicata lineage, may achieve better phylogenetic estimates. For the same reason, I am anxious to discover unidentified lineages of other excavate groups with less diverged gene sequences, particularly those of parabasalids.

The ancestral excavates cells were most probably free-living, since all lineages, except oxymonads, include free-living members. In theory, to retrace the ancestral morphological characteristics, investigations of free-living Fornicata may be more critical than those of parasitic ones, which are probably biased by their lifestyle. In reality, sampling of free-living Fornicata and the data from these lineages are quite limited, while abundant data are available for parasitic excavates (e.g., *Giardia* and *Trichomonas*). Therefore, isolation and detailed microscopic works of novel free-living Fornicata are essential to study Fornicata evolution.

To summarize, I strongly believe that the search for novel Fornicata and both morphological and molecular works on these members are indispensable for elucidating the evolution of Fornicata and excavates. I worked on the special attention to the free-living excavates and successfully established two novel free-living excavate flagellates. These flagellates are slow-evolving and branched at the base among the Fornicata clade, and possess general morphology of the excavates. Further analyses of these excavates could play a pivotal role for understanding the one of the important issue in biology such as early evolution of eukaryotes.

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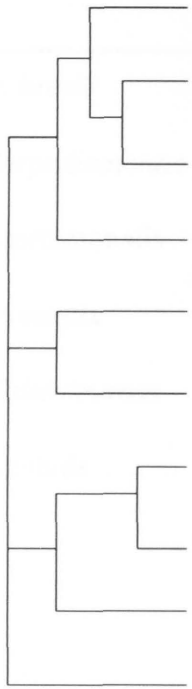
TABLES

Table 1. Distribution of seven ultrastructural features plus ventral groove in the 10 excavates and *Dysnectes brevis* n. gen., n. sp. The excavates possessing all eight morphological features are shaded. Note: (+) = presence; (-) = absence; (?) = arguable; N.D. = no data available. Source of the charcters: adapted from Simpson & Roger (2004). The cladogram is refered from Simpson et al. (2006)



	ventral groove	split RR	SR	flagellar vanes	C fiber	I fiber	B fiber	composit fiber
D. brevis	+	+	+	+	+	+	+	+
Carpediemonas	+	+	+	+	+	+	+	+
retortamonads	+	+	+	+	+	+	+	+
diplomonads	+	+	?	-	-	+	+	+
parabasalids	-	-	-	-	?	-	-	-
Trimastix	+	+	+	+	+	+	+	+
oxymonads	-	-	+	-	+	+	+	-
Euglenozoa	-	-	-	-	-	-	-	-
Jakobids	-	-	-	-	-	-	-	-
Heterolobosea	+	+	-	-	-	+	-	-
Malawimonas	+	+	+	+	+	+	+	N.D.

Table 2. Comparison of ultrastructural features in the five typical excavates with those in *Dysnectes brevis* n. gen., n. sp. The Digits in the column of C fiber show the number of layers. Note: (+) = presence; (-) = absence; BB = basal body; DO[a] = organelle with dense matrix (this study); DO[b] = dense organelle (Simpson & Patterson 1999); H = a double membrane bounded organelle of unknown fanction (presumed hydorogenosome) (Brugerolle & Patterson 1997; Simpson et al. 2000); LR = left root; M = mitochondria; N.D. = no data available; RR = right root.

	number of vane	AR	fan	IMt	Mt addition on LR	gutter	A fiber	B fiber origin	C fiber	Mitochondria
D. brevis	2	+	-	+	left	+	comb-like	LR	3	DO[a]
Carpediemonas	3	+	+	-	-	-	+	LR	2	DO[b]
retortamonads	2/3	-	-	-	left	+	+	LR	2	N.D.
Trimastix	2	+	+	+	right	-	+	RR	2	H
Malawimonas	1	+	+	-	right	-	+	BB	4	M
Jakobids	1	-	+	-	left	-	+	BB	1	M

FIGURES

Fig. **1.** Light micrographs and video images of a novel strain, *Dysnectes brevis* gen. et sp. nov. (NY0165). Arrows and arrowheads indicate the anterior flagella (AF) and the posterior flagella (PF), respectively. **A**. Differential interference contrast microscopy of the living cell. The scale bar represents 5 μm (the bar in Fig. A is applicable for Fig. A & B). **B**. Phase contrast microscopy of the fixed cell. **C-E.** Video images of *D. brevis* (NY0165) showing flagellar behavior. Scale bar represents 10 μm (the bar in Fig. C is applicable for Fig. C-E). **C**, **D**. Video images of the cells viewed from the right side. **E**. Video image of the cell viewed from the dorsal side. **F.** Diagram of light microscopy of *D. brevis* (NY0165).

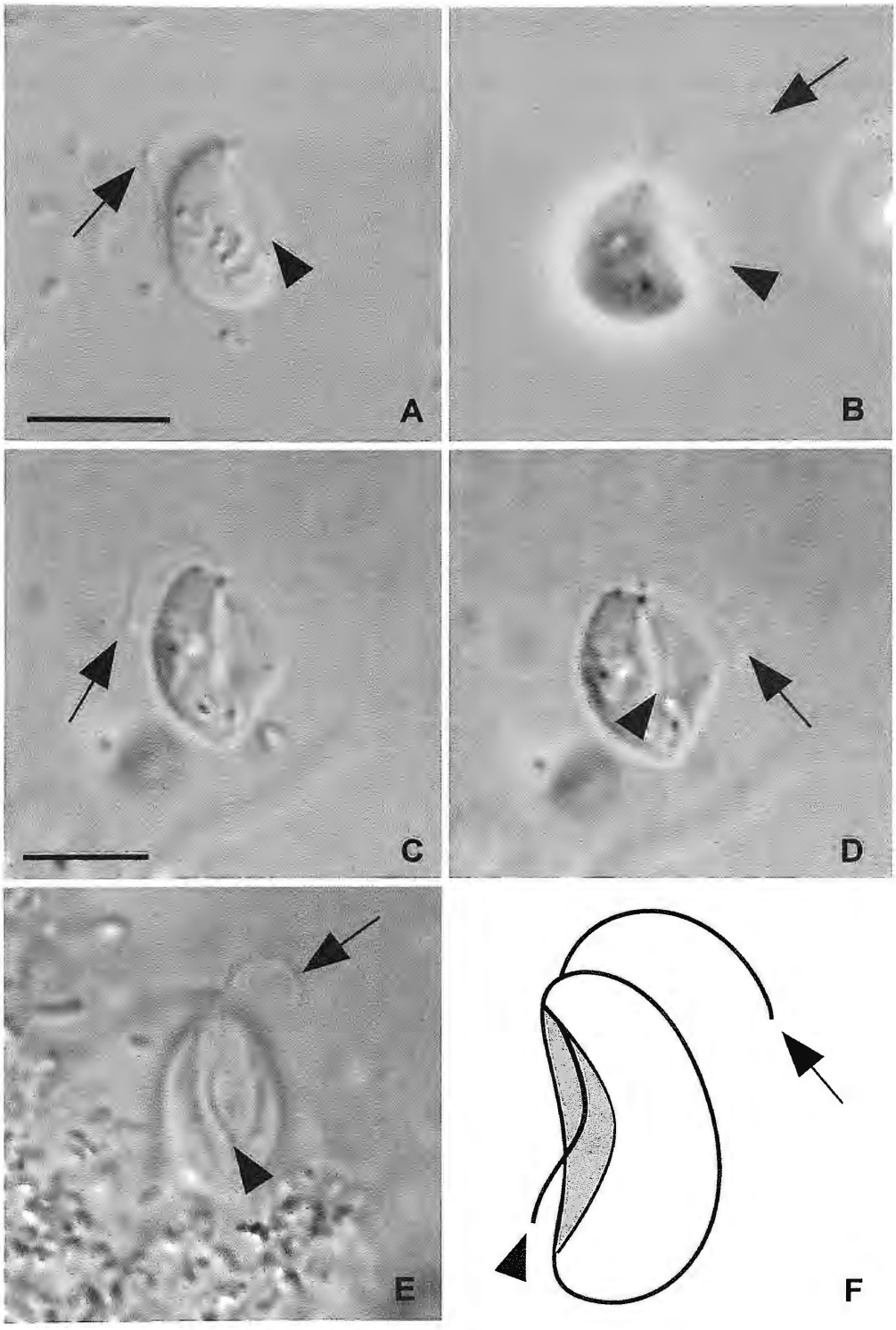


Figure 1

Fig. **2.** Light micrographs of a novel strain, NY0166. Arrows and arrowheads indicate the anterior flagella (AF) and the posterior flagella (PF), respectively. The scale bars represent 10 μm (the bar in Fig. A is applicable for Fig. A and B). **A**. Differential interference contrast microscopy of the living cell. **B**. Phase contrast microscopy of the living cell. **C**. Phase contrast microscopy of the fixed cell. Cell fixed with glutaraldehyde. **D.** Diagram of light microscopy of NY0166.

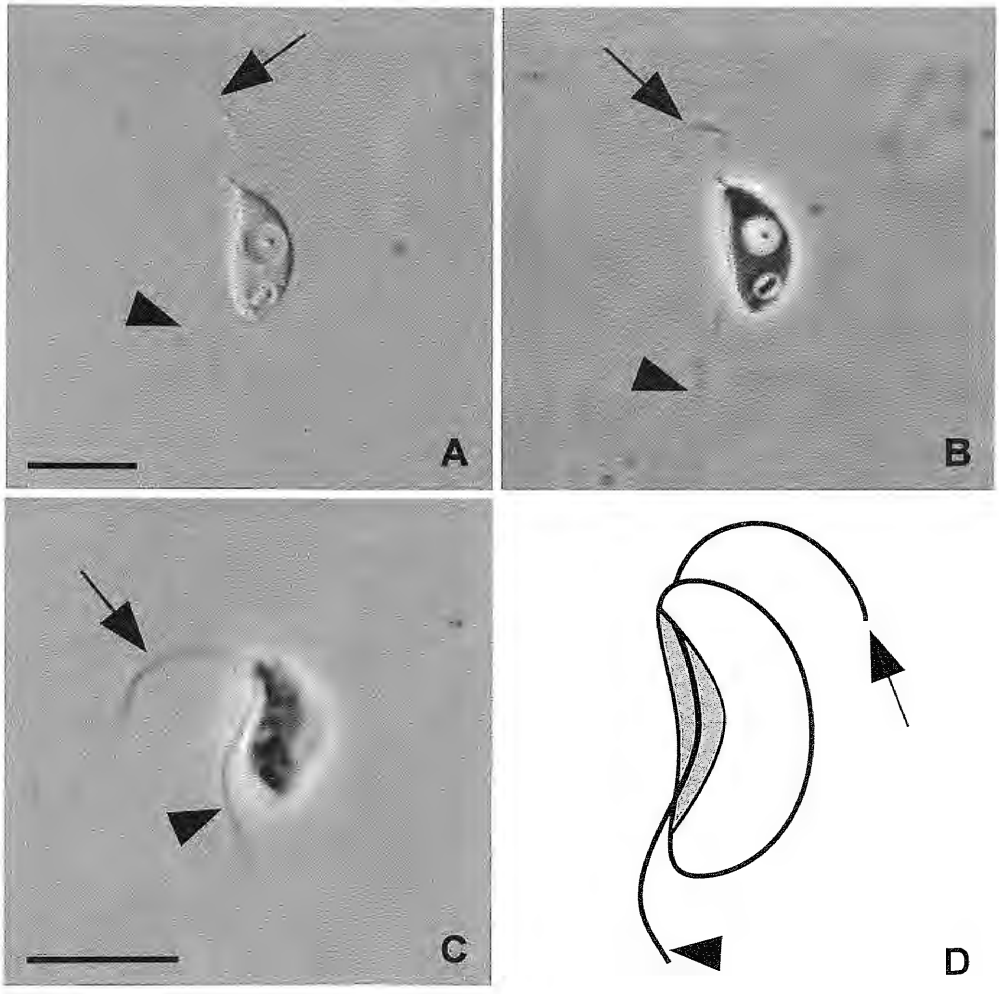


Figure 2

Fig. **3.** Light micrographs of a novel strain, NY0173. Arrows and arrowheads indicate the anterior flagella (AF) and the posterior flagella (PF), respectively. The scale bars represent 10 μm (the bar in Fig. A is applicable for Fig. A-B). **A.** Differential interference contrast microscopy of the living cell. **B.** Differential interference contrast microscopy of the living cell. **C.** Phase contrast microscopy of the fixed cell. Cell fixed with glutaraldehyde **D.** Diagram of light microscopy of NY0173.

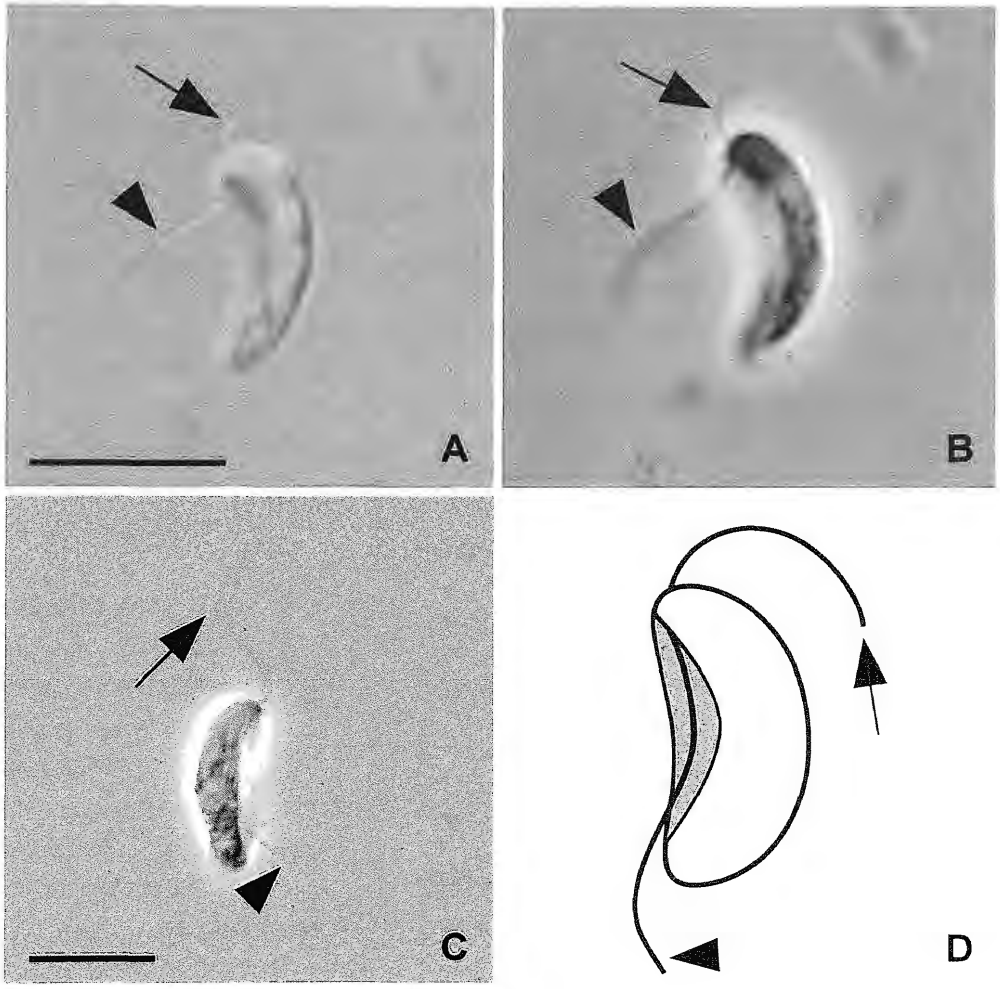


Figure 3

Fig. 4. Phylogenetic tree based on SSU rRNA gene sequences. Maximum likelihood (ML) analysis of 42 taxa sampled from phylogenetically diverged eukaryotes. ML bootstrap values greater than 50% are shown. GenBank accession numbers of the SSU rRNA gene sequences considered in these analyses are shown in parentheses.

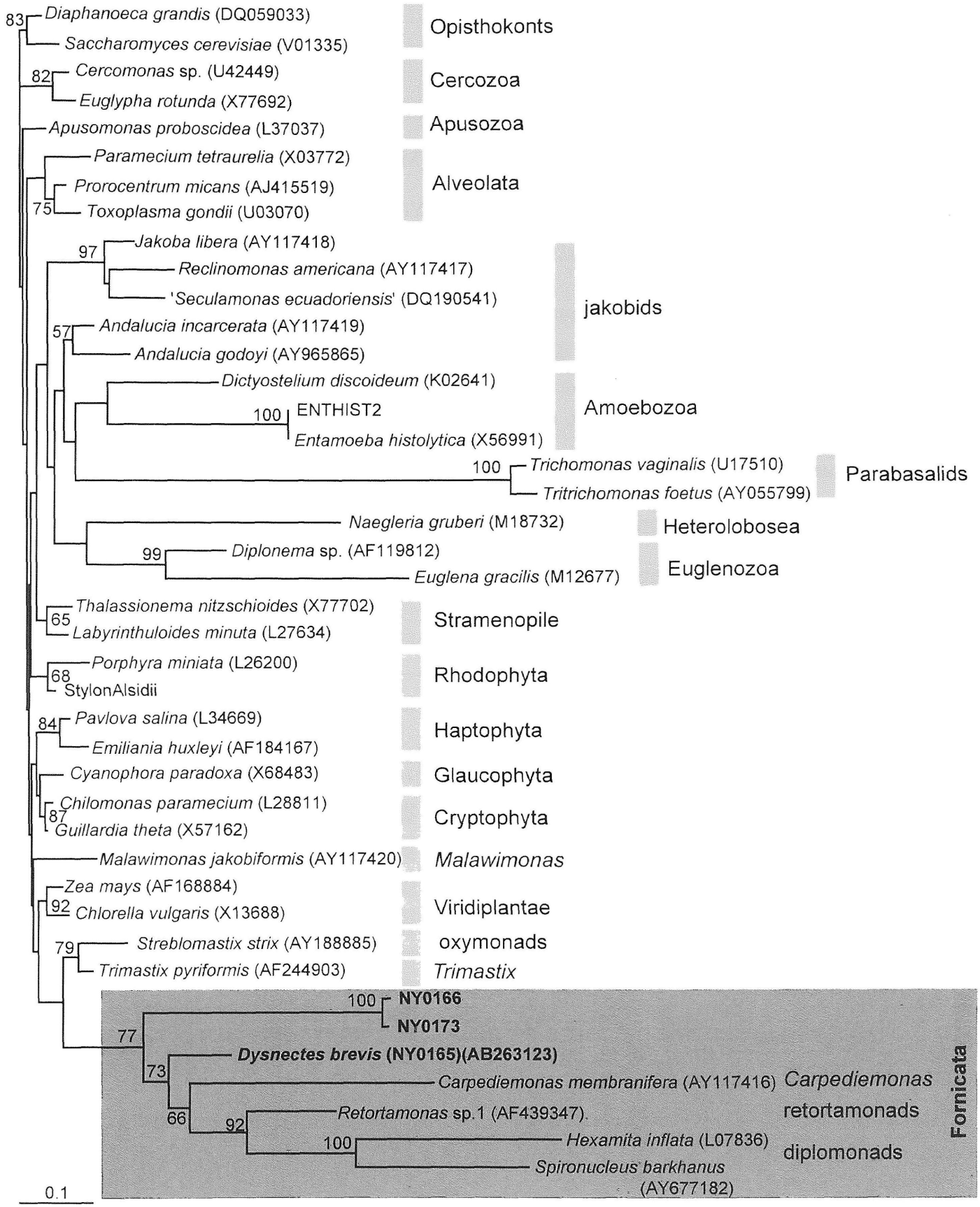

Diaphanoeca grandis (DQ059033)
Saccharomyces cerevisiae (V01335)
Opisthokonts
Cercomonas sp. (U42449)
Euglypha rotunda (X77692)
Cercozoa
Apusomonas proboscidea (L37037)
Apusozoa
Paramecium tetraurelia (X03772)
Prorocentrum micans (AJ415519)
Toxoplasma gondii (U03070)
Alveolata
Jakoba libera (AY117418)
Reclinomonas americana (AY117417)
'Seculamonas ecuadoriensis' (DQ190541)
jakobids
Andalucia incarcerata (AY117419)
Andalucia godoyi (AY965865)
Dictyostelium discoideum (K02641)
ENTHIST2
Entamoeba histolytica (X56991)
Amoebozoa
Trichomonas vaginalis (U17510)
Tritrichomonas foetus (AY055799)
Parabasalids
Naegleria gruberi (M18732)
Heterolobosea
Diplonema sp. (AF119812)
Euglena gracilis (M12677)
Euglenozoa
Thalassionema nitzschioides (X77702)
Labyrinthuloides minuta (L27634)
Stramenopile
Porphyra miniata (L26200)
StylonAlsidii
Rhodophyta
Pavlova salina (L34669)
Emiliania huxleyi (AF184167)
Haptophyta
Cyanophora paradoxa (X68483)
Glaucophyta
Chilomonas paramecium (L28811)
Guillardia theta (X57162)
Cryptophyta
Malawimonas jakobiformis (AY117420)
Malawimonas
Zea mays (AF168884)
Chlorella vulgaris (X13688)
Viridiplantae
Streblomastix strix (AY188885)
oxymonads
Trimastix pyriformis (AF244903)
Trimastix
NY0166
NY0173
Dysnectes brevis (NY0165)(AB263123)
Carpediemonas membranifera (AY117416)
Carpediemonas
Retortamonas sp.1 (AF439347).
retortamonads
Hexamita inflata (L07836)
Spironucleus barkhanus (AY677182)
diplomonads
Fornicata
0.1

Figure 4

Fig. 5. Phylogenetic position of *Dysnectes brevis* gen. et sp. nov. (NY0165), NY0166 and NY0173 based on SSU rRNA gene sequences. ML phylogenetic analysis of 24 taxa focusing on the position of *D. brevis* (NY0165), NY0166 and NY0173 in the Fornicata clade. *Saccharomyces cerevisiae*, *Zea mays*, *Thalassionema nitzschioides*, and *Emiliania huxleyi* are used as the outgroup. GPSGM-5, and three sequences (C1 E027, A3 E016, C3 E028) are environmental sequences collected from Sagami bay, Japan and Guaymas Basin hydrothermal vent, Gulf of California, USA, respectively. PPP15C is the sequences taken from the culture strain from Halifax, Canada. ML bootstrap values greater than 50% are shown. GenBank accession numbers of the SSU rRNA gene sequences are shown in parentheses.

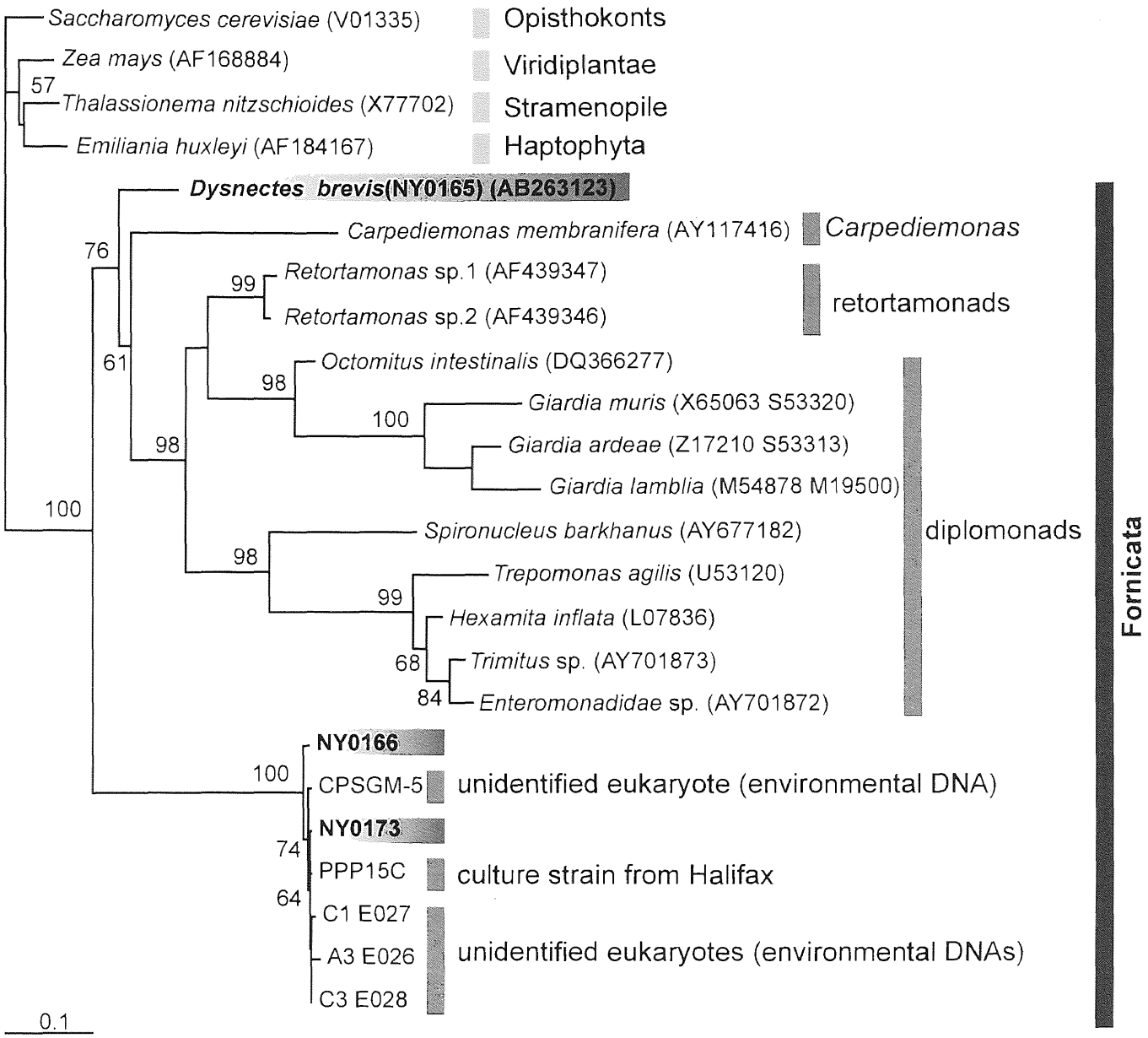

Saccharomyces cerevisiae (V01335)
Zea mays (AF168884)
57
Thalassionema nitzschioides (X77702)
Emiliania huxleyi (AF184167)
Opisthokonts
Viridiplantae
Stramenopile
Haptophyta
Dysnectes brevis(NY0165) (AB263123)
Carpediemonas membranifera (AY117416)
Carpediemonas
76
Retortamonas sp.1 (AF439347)
99
Retortamonas sp.2 (AF439346)
retortamonads
61
Octomitus intestinalis (DQ366277)
98
Giardia muris (X65063 S53320)
100
98
Giardia ardeae (Z17210 S53313)
Giardia lamblia (M54878 M19500)
100
Spironucleus barkhanus (AY677182)
diplomonads
98
Trepomonas agilis (U53120)
99
Hexamita inflata (L07836)
68
Trimitus sp. (AY701873)
84
Enteromonadidae sp. (AY701872)
Fornicata
NY0166
100
CPSGM-5
unidentified eukaryote (environmental DNA)
NY0173
74
PPP15C
culture strain from Halifax
64
C1 E027
A3 E026
C3 E028
unidentified eukaryotes (environmental DNAs)
0.1

Figure 5

Fig. 6. Video microscopy of *Dysnectes brevis* gen. et sp. nov. (NY0165) showing the membrane-like structure moving from anterior end to posterior end along the ventral feeding groove. Arrows and arrowheads indicate the membrane-like structure and bacteria, respectively. AF, Anterior Flagellum; PF, Posterior Flagellum. Scale bar represents 5 μm (the bar in Fig. A is applicable for Fig. A-H).

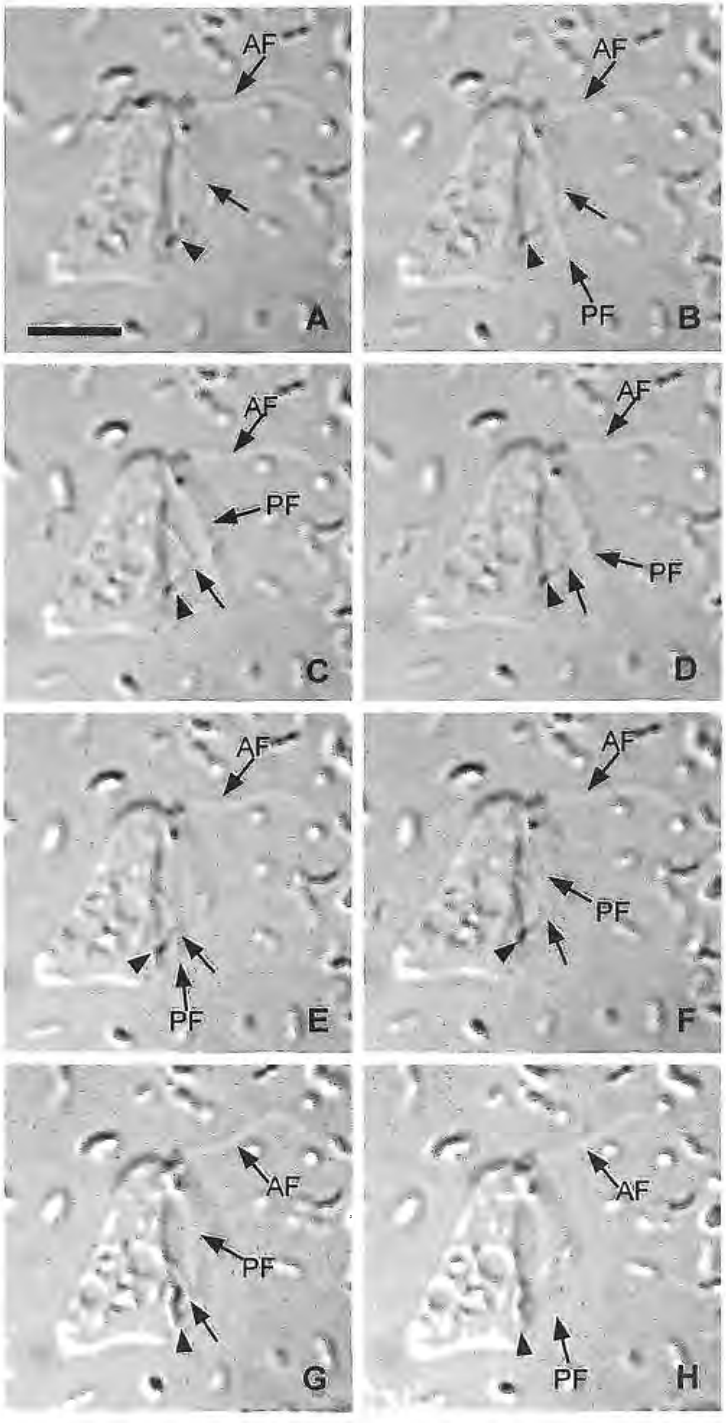


Figure 6

Fig. 7. Video microscopy of *Dysnectes brevis* gen. et sp. nov. (NY0165). Showing the transformation of flagella. **A**. The newly inserted flagella (arrowheads) appear on the both side of anterior flagella (AF) at the anterior end of the cell. **B**. A organism having two feeding groove and two sets of anterior and posterior flagella (AF and PF) at the anterior end of the both groove. Scale bar represents 5 μm that is applicable for both figures.

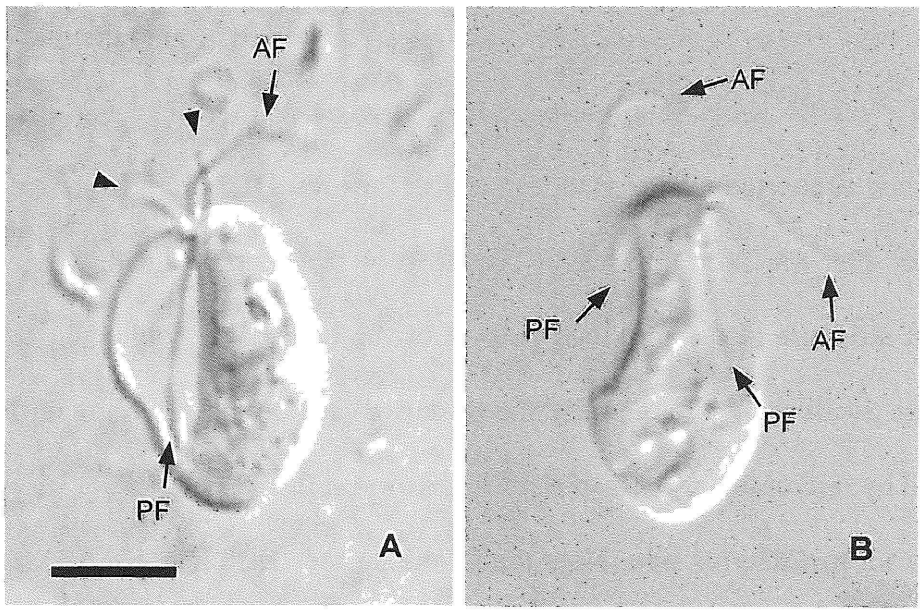
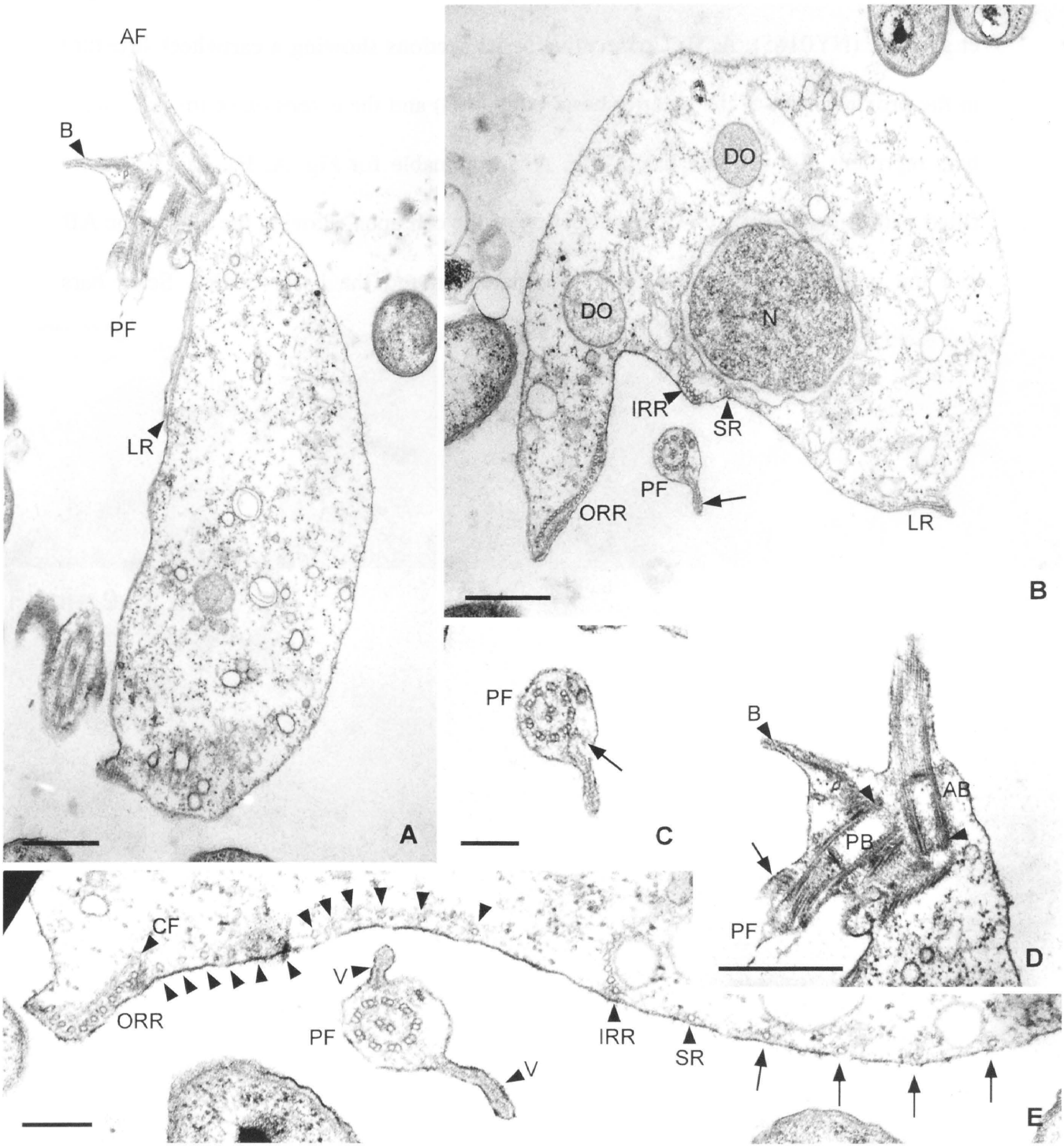


Figure 7

Fig. 8. Transmission electron micrographs of general morphology of *Dysnectes brevis* gen. et sp. nov. (NY0165). AB, Anterior Basal Body; AF, Anterior flagellum; B, B fiber; CF, composite fiber; DO, organelles filled with dense matrix; IRR, inner right root; LR, Left Root; ORR, outer right root; PB, Posterior Basal body; PF, posterior flagellum; SR, singlet root. **A**. Longitudinal section of a cell, as viewed from the left side. AF and PF are inserted from anterior end of the cell. **B**. Transverse section of a cell, as viewed from the anterior side. The DO are observed around the nucleus. Arrow indicates a ventral vane. **C**. High-magnification view of the PF of Fig. 8B showing a ventral vane supported by a curled-up lamella (arrow). **D**. Extreme anterior portion of the cell of Fig. 8A. Note the lateral striations of the ventral lamella (arrow) and the cartwheel structure at the proximal portion of the both basal bodies (arrowheads). **E**. Transverse section of the area near the posterior part of the cell showing the groove supported by microtubular roots of the LR (arrows), SR, IRR and ORR (arrowheads), as viewed from the anterior side. Note the CF along the ORR. The PF possesses ventral and dorsal vanes (V). Scale bars in A, B and D represent 500 nm, and those in C and E represent 200 nm.


AF
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DO
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ORR
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C
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PB
PF
D
CF
ORR
V
PF
IRR
SR
V
E

Figure 8

Fig. 9. Transmission electron micrographs of anterior root (AR) in *Dysnectes brevis* gen. et sp. nov. (NY0165). **A, B.** Consecutive serial sections showing a cartwheel structure in the proximal part of the anterior basal body (AB) and the extension of the AR. Scale bars represent 200 nm (the bar in Fig. A is applicable for Fig. A, B). DO, orgenelle filled with dense matrix. **C.** Oblique section of the cell apex showing the AR on the AB and the internal microtubules (IMt) originated around the basal bodies. Scale bars represent 200 nm. B, B fiber; PF, Posterior flagellum.

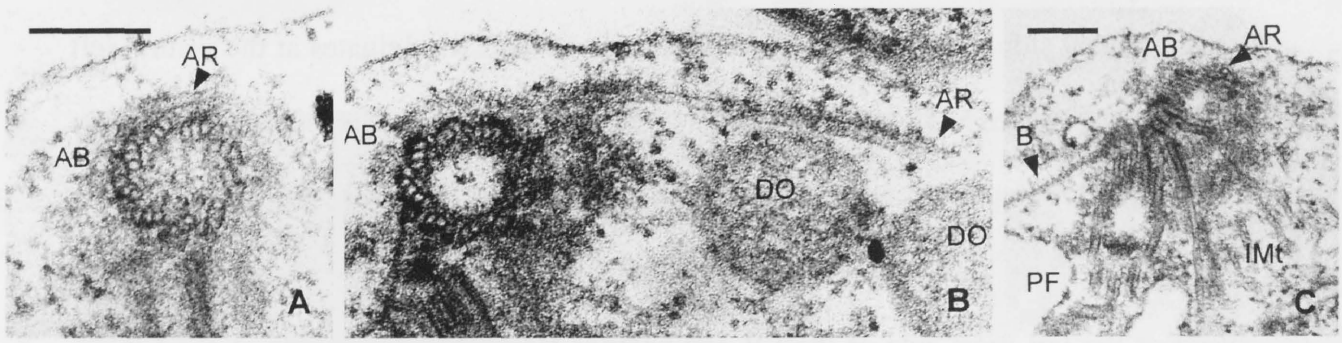
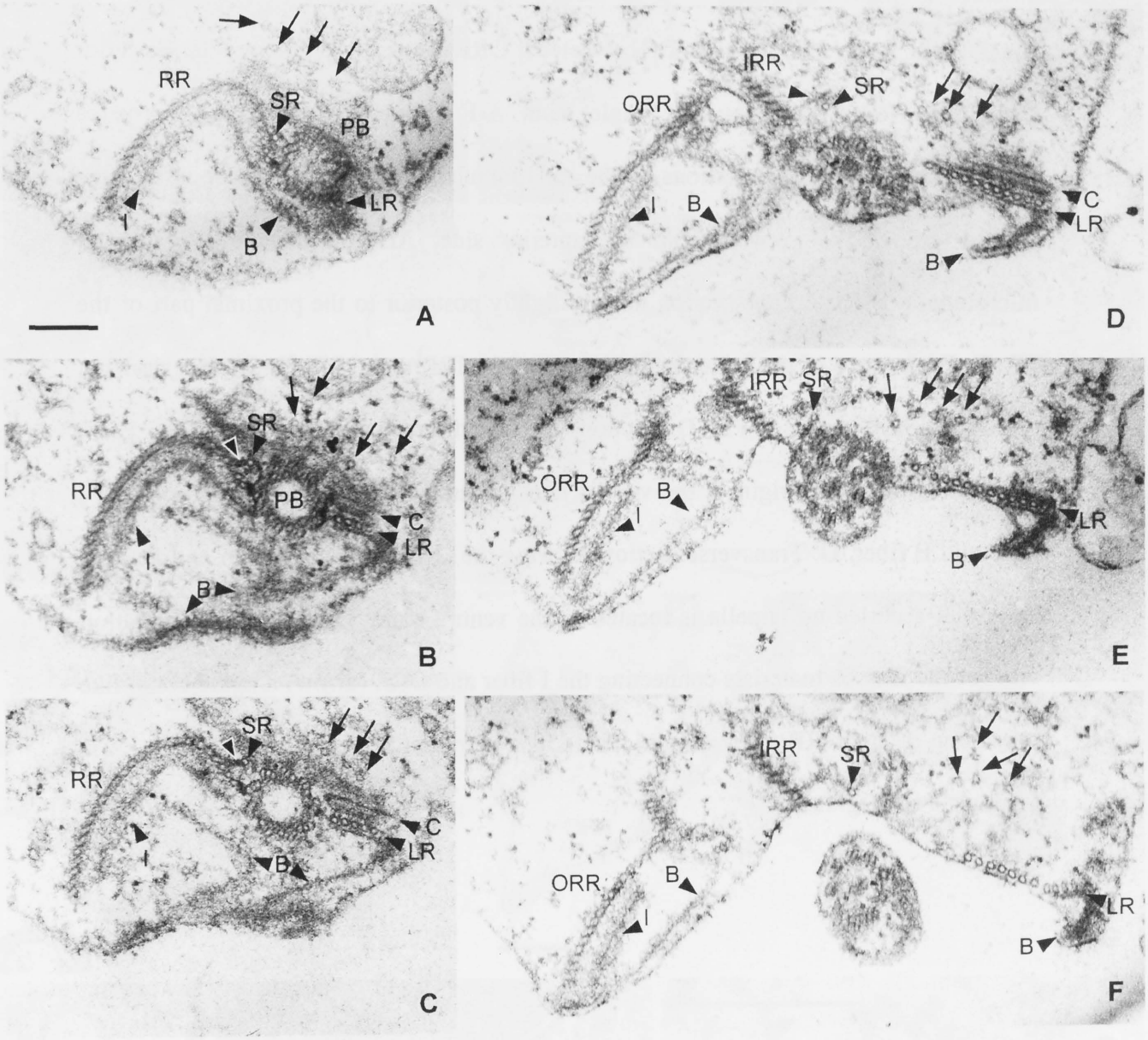


Figure 9

Fig. **10**. Non-consecutive serial transverse sections of *Dysnectes brevis* gen. et sp. nov. (NY0165) showing the orientations of the microtubular roots and fibers supporting the ventral groove, as viewed from the anterior side. Arrows indicate the internal microtubule (IMt). B, B fiber; C, C fiber; I, I fiber; IRR, Inner Right Root; LR, Left Root; ORR, Outer Right Root; PB, Posterior Basal body; RR, Right Root; SR, Singlet Root. **A**. Proximal sections of the PB close to the cartwheel structure. The SR located on the dorsal side of the RR. Single microtubule of the LR originates at the ventral left side of PB. The arched B fiber (B) extends between the LR and left side of the RR via the posterior side of PB. **B**. Sections slightly posterior of the emergence of the C fiber (C) on the dorsal side of the LR consisted of four microtubules. Note that the B fiber turns at the left-most side of the LR and extends along the ventral side of the LR. **C**. The SR is close to the RR with a SR-associated fiber (SA). **D**. The IRR and ORR are dissociated from the RR. The B fiber also detaches and supports the left and right wall. **E**. The B fiber runs along the LR and reduces to the left side. **F**. The B fiber on the left wall reduces to the tips of the LR. Scale bar represents 200 nm (the bar in Fig. A is applicable for Fig. A-F).


RR
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RR
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IRR
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Figure 10

Fig. **11**. Transmission electron micrographs of *Dysnectes brevis* gen. et sp. nov. (NY0165). B, B fiber; C, C fiber; DO, organelle filled with dense matrix; I, I fiber; IRR, Inner Right Root; LR, Left Root; N, Nucleus; ORR, Outer Right Root; PB, Posterior Basal body; RR, Right Root; SR, Singlet Root. **A-F**. Non-consecutive serial transverse sections showing the orientations of the microtubular roots and fibers supporting the ventral groove, as viewed from the anterior side. Arrows indicate the internal microtubule (IMt). **A**. The section that is slightly posterior to the proximal part of the PB. **B**. Emergence of the gutter at the corner of the IRR and ORR. **C**. Section showing the transitional region of PB. **D**. Section showing the reduction of the B fiber. **E, F**. Section showing the origin of the ventral lamella (arrowhead) and orientation of the I fiber and B fiber. **G**. Transverse section showing the coalescence of I fiber and B fiber. Note that a curled-up lamella is located in the ventral vane. **H**. A high-magnification view of the fibrous materials connecting the I fiber and ORR (arrow) and the comb-like projection on the ORR (arrowheads). Scale bars represent 200 nm (the bar in Fig. A is applicable for Fig. A-F).

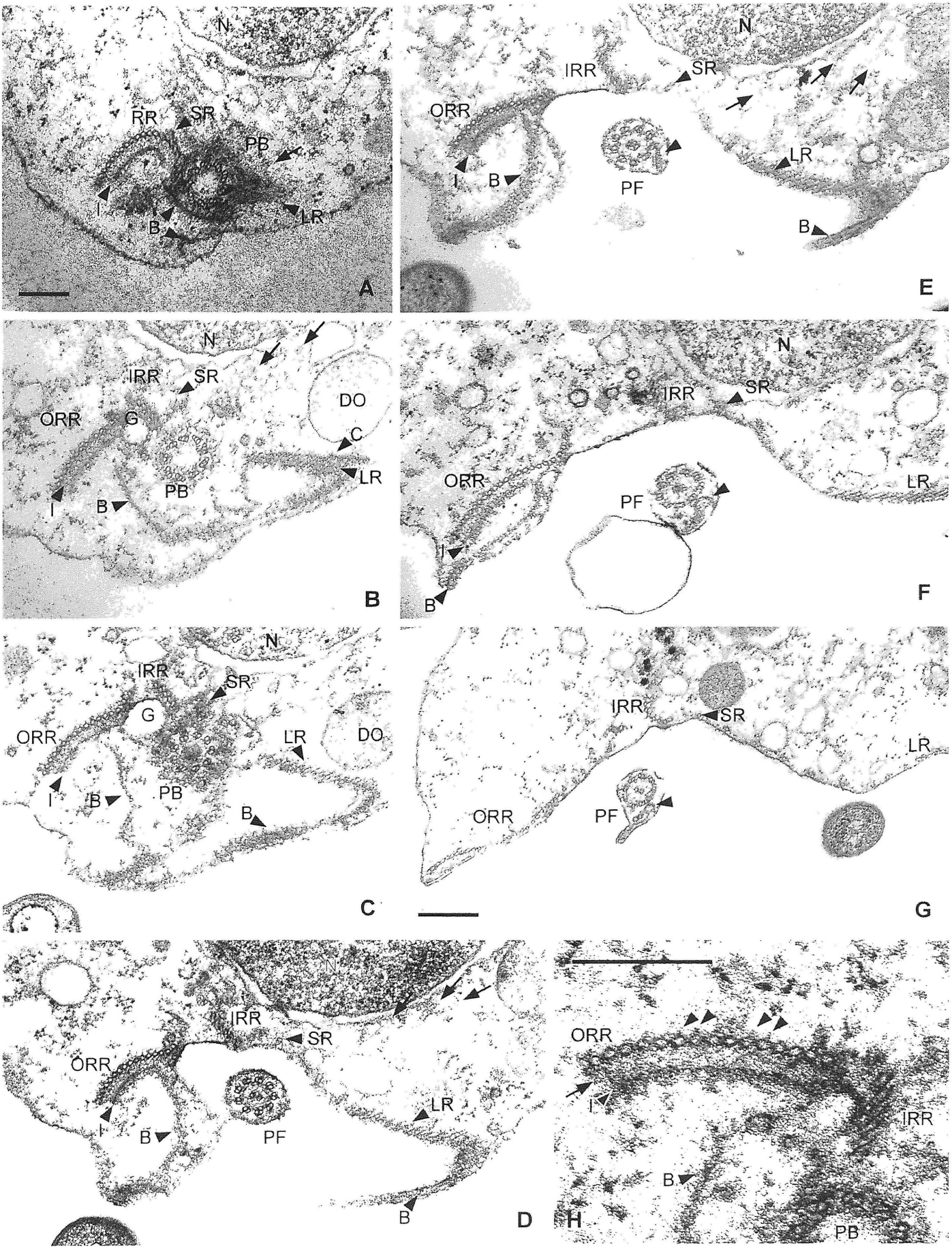

Figure 11

Fig. **12**. Transmission electron micrographs showing the composit fiber (CF) of *Dysnectes brevis* gen. et sp. nov. (NY0165). **A**. Grazing section showing the merging of the inner right root (IRR) and outer right root (ORR). **B**. A high magnification of Fig. 12A showing the striated band of the CF. Scale bars in Figs. A and B represent 500 nm and 200 nm, respectively.

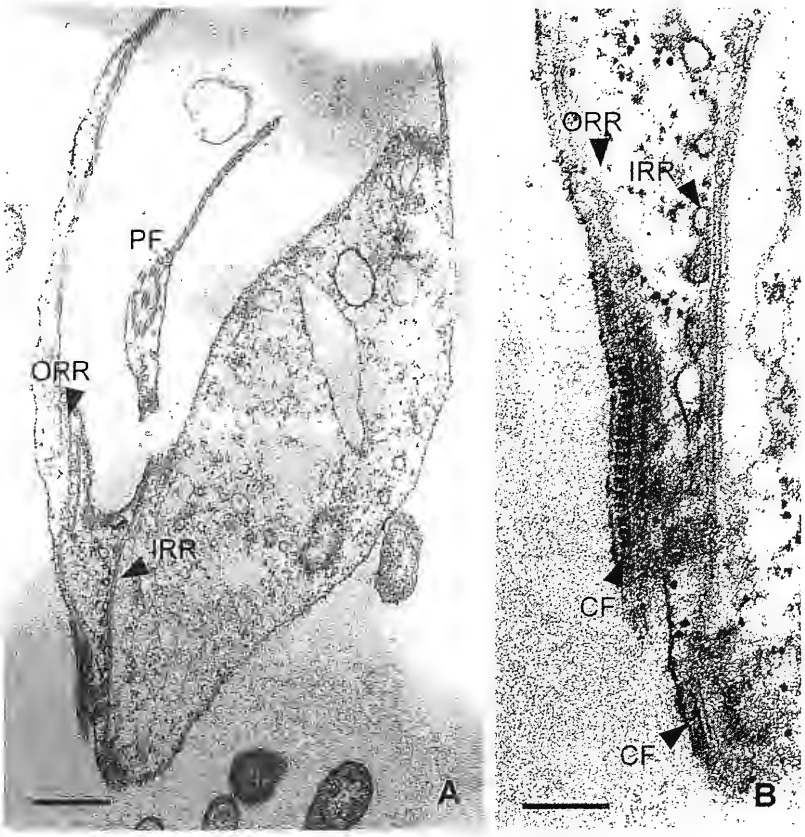

Figure 12

Fig. 13. Diagram of the flagellar apparatus of *Dysnectes brevis* gen. et sp. nov. (NY0165) based on the electron micrographs illustrated in the preceding plates. AB, Anterior Basal body; AR, Anterior Root; B, B fiber; B1, Basal body 1; B2, Basal body 2; C, C fiber; I, I fiber; IMt, Internal Microtubule; IRR, Inner Right Root; LR, Left Root; MLS, MultiLayered Structure; ORR, Outer Right Root; PB, Posterior Basal body; R1, microtubular Root 1; R2, microtubular Root 2; R3, microtubular Root 3; R4, microtubular Root 4; SR, Singlet Root.

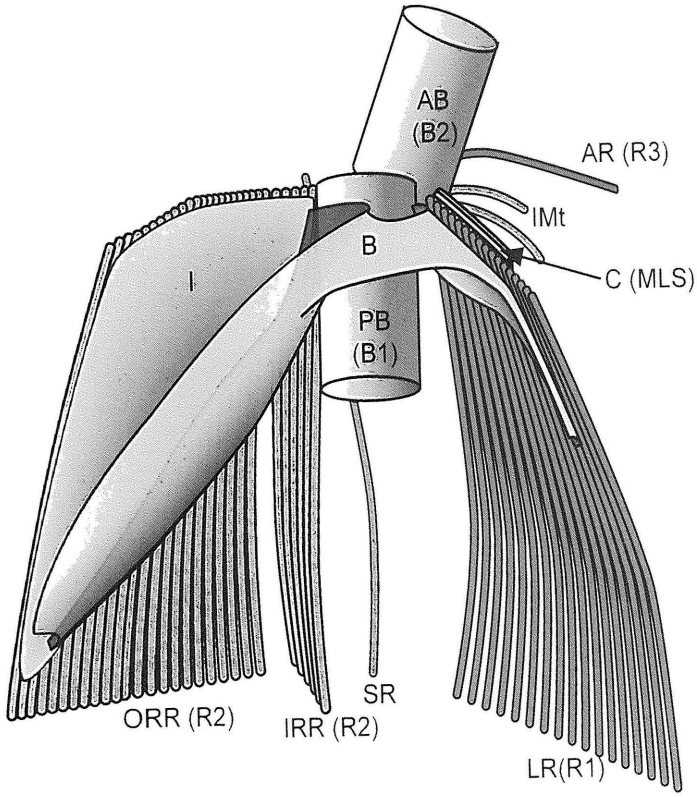

Figure 13