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Molecular Phylogeny and Surface Morphology of *Colpodella edax* (Alveolata): Insights into the Phagotrophic Ancestry of Apicomplexans

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ABSTRACT. The molecular phylogeny of colpodellids provides a framework for inferences about the earliest stages in apicomplexan evolution and the characteristics of the last common ancestor of apicomplexans and dinoflagellates. We extended this research by presenting phylogenetic analyses of small subunit rRNA gene sequences from *Colpodella edax* and three unidentified eukaryotes published from molecular phylogenetic surveys of anoxic environments. Phylogenetic analyses consistently showed *C. edax* and the environmental sequences nested within a colpodellid clade, which formed the sister group to (eu)apicomplexans. We also presented surface details of *C. edax* using scanning electron microscopy in order to supplement previous ultrastructural investigations of this species using transmission electron microscopy and to provide morphological context for interpreting environmental sequences. The microscopical data confirmed a sparse distribution of micropores, an amphiesma consisting of small polygonal alveoli, flagellar hairs on the anterior flagellum, and a rostrum molded by the underlying (open-sided) conoid. Three flagella were present in some individuals, a peculiar feature also found in the microgametes of some apicomplexans.

Key Words. Alveolates, Apicomplexa, *Colpodella edax*, Dinoflagellata, evolution, phylogeny, small subunit rDNA.

COLPODELLIDS are small predatory flagellates (< 20 μm) with an 'apical complex' that is comprised of rhoptries and an open-sided conoid (syn. pseudoconoid) and is used in the myzocytosis of prey cells (Brugerolle 2002; Mylnikov 1991, 2000; Mylnikov et al. 1998; Simpson and Patterson 1996). An open-sided conoid, which is similar to the closed conoid of apicomplexans (Vivier and Desportes 1990), is also found in the zoospores of perkinsids (*Perkinsus*, *Parvilucifera*, and *Cryptophagus*), suggesting that perkinsids, colpodellids, and apicomplexans are all closely related (Azevedo 1989; Brugerolle 2001; Levine 1978; Norén et al. 1999; Perkins, 1976, 1996). This is supported, in part, by small subunit (SSU) rDNA phylogenies that show colpodellids as the earliest diverging sister group of apicomplexans (Kuvardina et al. 2002). However, phylogenies inferred from multiple gene sequences show perkinsids as the earliest diverging sister group of dinoflagellates (Goggin and Barker 1993; Reece et al. 1997; Saldarriaga et al. 2003; Siddall et al. 1997), the major sister group to apicomplexans (Fast et al. 2002; Gajadhar et al. 1991; Wolters 1991). This phylogenetic topology combined with the ultrastructural similarities of colpodellids and perkinsids provides an ideal framework for making inferences about the last common ancestor of apicomplexans and dinoflagellates (Kuvardina et al. 2002; Leander and Keeling 2003).

Molecular phylogenetic surveys of microeukaryotic diversity in different environments have produced several SSU rDNA sequences with strong affinities to alveolates (ciliates, dinoflagellates, perkinsids, apicomplexans, colpodellids, and a handful of other lineages) (Dawson and Pace 2002; Díez et al. 2001; Edgcomb et al. 2002; López-García et al. 2001; Moon-van der Staay et al. 2001). Many of these sequences are considered novel because they are not closely related to sequences from organisms having well-known morphologies. Consequently, environmental PCR approaches have significant interpretive limitations, as the link between the sequences and the cytological characteristics of the organisms whence the sequences came is difficult to attain (Moreira and López-García 2002). Nonetheless, it is reasonable to anticipate that many of these novel sequences will turn out to come from close relatives of familiar organisms that have yet to be characterized with molecular data. New understanding of colpodellid phylogeny illustrates such an example.

Here, we extend the research of Kuvardina et al. (2002) on the molecular phylogeny of colpodellids, by presenting phylogenetic analyses including SSU rRNA gene sequences from *Colpodella edax* and three uncultured eukaryotes from environmental samples. In addition, we report on the surface details of *C. edax* using scanning electron microscopy (SEM) in order to (1) complement previous ultrastructural investigations of this species and its close relatives using transmission electron microscopy (TEM) (Brugerolle 2001, 2002; Brugerolle and Mignot 1979; Foissner and Foissner 1984; Mylnikov 1988, 1991, 2000; Mylnikov et al. 1998; Simpson and Patterson 1996) and (2) provide morphological context for interpretations about the significance of environmental sequences.

MATERIALS AND METHODS

Collection and culture conditions. *Colpodella edax* (Klebs 1892) Simpson and Patterson 1996 was isolated from a freshwater pond near Borok (Yaroslavskaja, Russian Federation) in February 1980. Cultures of *C. edax* were maintained in a Pratt medium (to 1 liter add 0.1 g KNO_3, 0.01 g $MgSO_4 \cdot 7H2O$, 0.01 $K_2HPO_4 \cdot 3H2O$ and 0.001 $FeCl_3 \cdot 6H2O$; pH = 7.2–7.4; 20–22 °C) inoculated with *Klebsiella* sp. and a bacteriotrophic prey organism, *Spumella uniguttata* (chrysophyte).

Light and scanning electron microscopy. Cells were observed under a cover slip fixed in place with "VALAP" (1 vaseline:1 lanolin:1 paraffin wax, Kuznetsov et al. 1992). Light micrographs were produced with a Zeiss Axioplan 2 Imaging microscope (100× Plan-Apochromat, DIC objective lens) connected to a Q-Imaging, Microimager II, black and white digital camera.

A small vol. (10 ml) of cells suspended in medium was transferred into a Petri dish containing filter paper mounted on the inner surface of the lid. The filter paper was saturated with 4% OsO_4 and placed over the dish. The cells were fixed by OsO_4 vapors for 30 min before adding six drops of 4% OsO_4 directly to the medium. After an additional 30 min of fixation, cells were transferred onto a 3-μm polycarbonate membrane filter (Corning Separations Div., Acton, MA), dehydrated with a

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graded series of ethyl alcohol, and critical point dried with CO_2. Filters were mounted on stubs, sputter-coated with gold, and viewed under a Hitachi S4700 Scanning Electron Microscope. Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

DNA extraction, PCR amplification, and sequencing. Genomic DNA was extracted from a pellet of *C. edax* (including *Spumella uniguttata* prey) using a standard hexadecyltrimethylammonium bromide (CTAB) extraction protocol (Zolan and Pukkila 1986). The small subunit (SSU) rRNA gene was amplified as single fragment using universal eukaryotic primers and a standard PCR protocol (Leander et al. 2003). The fragment was gel-isolated with an UltraClean 15 DNA purification Kit (MoBio Laboratories, Inc., Solana Beach, California, USA) and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD, USA). One clone was completely sequenced with ABI big-dye reaction mix using vector primers and four internal primers oriented in both directions.

Alignments and phylogenetic analysis. The new sequence from *C. edax* was added to an existing alignment consisting of diverse alveolates and representative sequences from other major eukaryotic groups—a 69-taxon alignment (Kuvardina et al. 2002). However, we focused on a smaller alignment in order to minimize artifacts of long-branch attraction (LBA) and to perform a more comprehensive analysis of the position of *C. edax* within alveolates. The 69-taxon alignment was trimmed to 36 ingroup taxa (representative alveolates) and 3 outgroup taxa (representative stramenopiles) producing a 39-taxon alignment containing 1,285 sites. Maximum likelihood (ML) and distance methods under different DNA substitution models were performed on the alignments. The alpha shape parameters were estimated from the data using HKY and a gamma distribution with invariable sites and eight rate categories (alpha = 0.31 and Ti/Tv = 1.83 for the 69-taxon alignment and alpha = 0.28 and Ti/Tv = 1.87 for the 39-taxon alignment; fraction of invariable sites was 0.00 for both alignments). Gamma-corrected ML trees (analyzed using the parameters listed above) were constructed with PAUP* 4.0 using the general time reversible (GTR) model for base substitution (Posada and Crandall 1998; Swofford 1999) and tree bisection reconnection (TBR) branch swapping. Gamma corrected ML trees found with HKY and GTR were identical for all relevant nodes. For the 39-taxon dataset, ML bootstrap analyses were performed in PAUP* (Swofford 1999) on one-hundred resampled datasets under an HKY model using the alpha shape parameter and transition/transversion ratio (Ti/Tv) estimated from the original dataset.

Distances for both SSU rDNA datasets were calculated with TREE-PUZZLE 5.0 using the HKY substitution matrix (Strimmer and von Haeseler 1996) and with PAUP* using the GTR model. Distance trees were constructed with weighted neighbor joining (WNJ) using Weighbor (Bruno et al. 2000). Five-hundred bootstrap datasets were generated with SEQBOOT (Felsenstein 1993). Respective distances were calculated with the shell script 'puzzleboot' (M. Holder and A. Roger, www.tree-puzzle.de) using the alpha shape parameter and transition/transversion ratios estimated from the original datasets and analyzed with Weighbor.

We also analyzed the 39-taxon dataset with parsimony. Nucleotides were treated as independent, unordered, character states of equal weight and gaps were treated as missing data. A heuristic search was performed using PAUP* 4.0 with ACCTRAN character state optimization, tree bisection reconnection (TBR) branch swapping, random step-wise addition of taxa, and MULTREES on. Bootstrap values from 500 resampled datasets, each with 10 replicates of taxon-resampling, were generated to evaluate the robustness of each node on the most parsimonious tree(s). The tree length, number of most parsimonious trees, number of informative characters, CI and RI were reported.

GenBank accession numbers. (AF069516) *Amoebophrya* sp., (AF274256) *Amphidinium semilunatum*, (U43190) *Axinella polypoides*, (AF158702) *Babesia gibsoni*, (M97909) *Blepharisma americanum*, (AF167154) *Bolidomonas pacifica*, (U82204) *Bursaria truncatella*, (AF174368) *Caecitellus parvulus*, (U97108) *Caenomorpha uniserialis*, (AF060975) *Caryospora bigenetica*, (AY142075) *Colpodella* sp. (American Type Culture Collection 50594), (AY234843) *Colpodella edax*, (AY078092) *Colpodella pontica*, (X53229) *Costaria costata*, (AF080097) *Cryptoperidiniopsis brodyi*, (AF093502) *Cryptosporidium serpentis*, (U37107) *Developayella elegans*, (K02641) *Dictyostelium discoideum*, (U57771) *Didinium nasutum*, (AF239261) *Dinophysis norvegica*, (AF231803) *Durinskia baltica* (formerly *Peridinium balticum*), (AF291427) *Eimeria alabamensis*, (AF372772, AF372785 and AF372786) Environmental sequences from Dawson and Pace (2002), (AJ402327) Eukaryote clone OLI11001, (AJ402349) Eukaryote clone OLI11005, (X65150) *Furgasonia blochmanni*, (X70803) *Glaucocystis nostochinearum*, (L13716) *Gloeodinium viscum*, (U37406) *Gymnodinium beii*, (AF274261) *Gyrodinium dorsum*, (X74131) *Haplosporidium nelsoni*, (AF286023) *Hematodinium* sp., (AF297085) *Hepatozoon* sp., (L26447) *Homalozoon vermiculare*, (X91784) *Jungermannia leiantha*, (AF274268) *Kryptoperidinium foliaceum*, (AF022199) *Lepidodinium viride*, (M87333) *Mallomonas striata*, (U07937) Marine clone misattributed to *Ammonia beccarii*, (AB000912) Marine parasite from *Tridacna crocea*, (AF174370) *Massisteria marina*, (AF457127) *Monocystis agilis*, (M59761) *Neocallimastix* sp., (AF022200) *Noctiluca scintillans*, (AF129883) *Ophryocystis elektroscirrha*, (M14601) *Oxytricha nova*, (AF133909) *Parvilucifera infectans*, (AJ243369) *Pavlova* sp., (AF022201) *Pentapharsodinium tyrrhenicum*, (AF126013) *Perkinsus marinus*, (AF060454) *Platyophrya vorax*, (AF136425) *Porphyra* sp., (Y16239) *Prorocentrum emarginatum*, (AF194409) *Protocruzia* sp., (AF274275) *Pyrodinium bahamense*, (AF310901) *Spongospora subterranea*, (AF236097) *Theileria buffeli*, (AB022111) *Thraustochytrium multirudimentale*, (M97703) *Toxoplasma gondii*, (L31520) *Tracheloraphis* sp., (AF244903) *Trimastix pyriformis*, (AF238264) Unidentified symbiont, (AF255357) *Urocentrum turbo*, (U47852) *Urosporidium crescens*.

RESULTS AND DISCUSSION

Molecular phylogeny of colpodellids. The large number of apparent symplesiomorphies in colpodellids and perkinsids diminishes any expectations that they should form independent monophyletic groups (Siddall et al. 2001). However, molecular phylogenies show that colpodellids and perkinsid not only form distinct clades, but clades positioned on opposite sides of the node shared by apicomplexans and dinoflagellates (Kuvardina et al. 2002; Leander and Keeling 2003). That is, in gamma-corrected ML and gamma-corrected distance trees, colpodellids and perkinsids (*Perkinsus* and *Parvilucifera*) diverge as the earliest sister group to (eu)apicomplexans and dinoflagellates, respectively, albeit with weak statistical support (Fig. 1). The relationships are also supported by parsimony analysis on the 39-taxon alignment, which gave rise to two most parsimonious trees [tree length = 2336, CI (excluding uninformative characters) = 0.36 and RI = 0.43] that differed only in irrelevant parts of the overall topology.

In all phylogenetic analyses, the three known sequences from *Colpodella* spp. formed a well-supported clade with three environmental sequences from anoxic marine sediments published

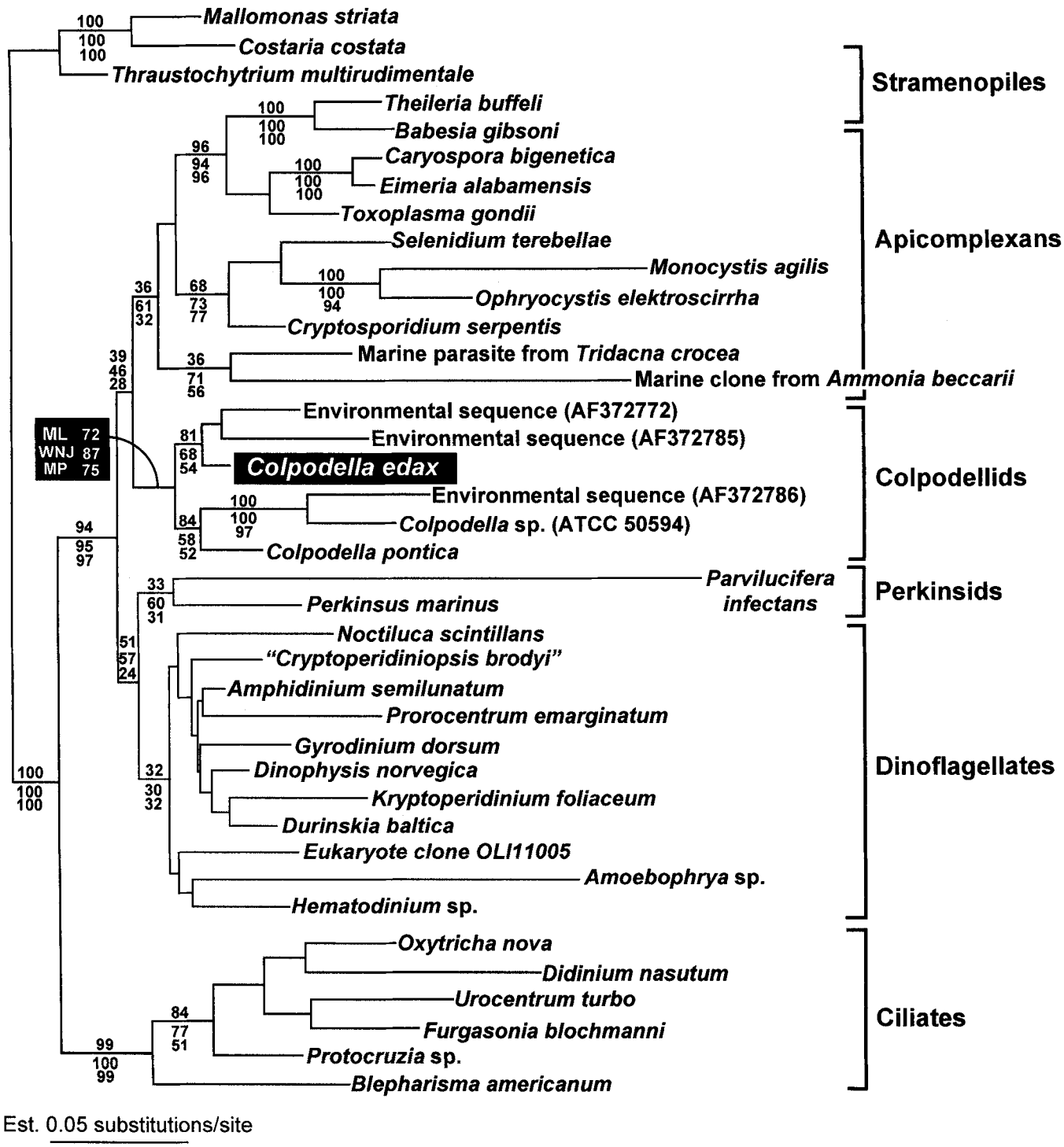

Fig. 1. Gamma-corrected ML tree (− lnL H 12561.004) inferred by using a general time reversible (GTR) model for base substitution on an alignment of 39 small subunit rDNA sequences and 1,285 sites, with stramenopiles as the outgroup, showing the phylogenetic position of *Colpodella edax* and three environmental sequences within alveolates. The numbers at the branches represent gamma-corrected bootstrap percentages using each of the following methods (from top to bottom): maximum likelihood using HKY (ML), weighted neighbor-joining (WNJ) and maximum parsimony (MP). Colpodellids and perkinsids were consistently positioned as the earliest diverging sister lineages to apicomplexans and dinoflagellates, respectively, with weak to moderate support. *Colpodella edax* and the environmental sequences were part of two clades within a well-supported *Colpodella* clade.

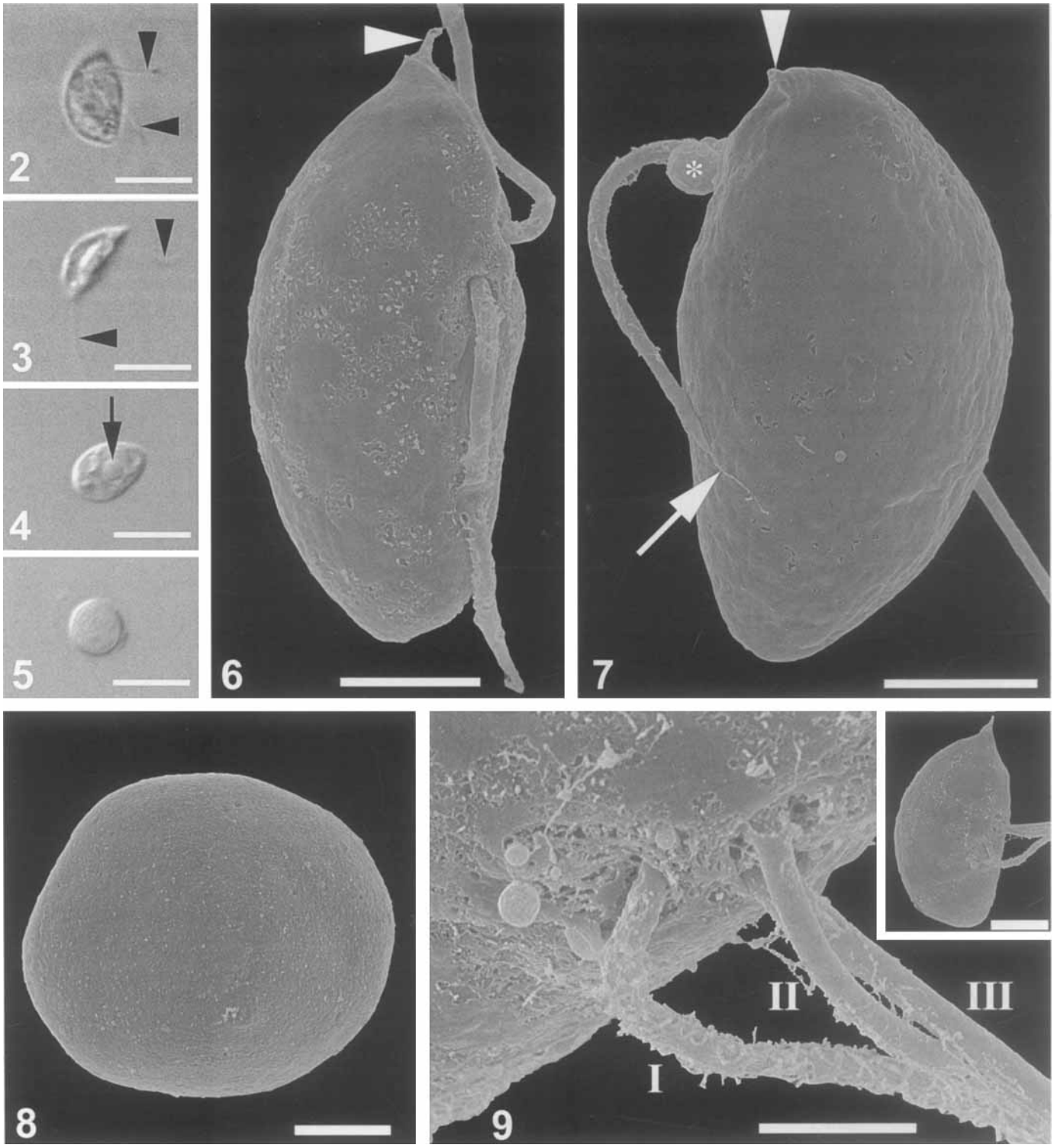

Fig. **2–9**. General morphology of *Colpodella edax*. **2–4**. Light micrographs of zoospores showing cell shape, two flagella (arrowheads) and a centrally positioned nucleus (arrow) (Bar = 10 μm). **5**. Light micrograph of a resting cyst (Bar = 10 μm). **6**. Scanning electron micrograph (SEM) showing the apical rostrum (arrowhead) and the lateral insertion of two flagella on the right-hand side of a cell (Bar = 1.5 μm). **7**. SEM of the left-hand side of a cell showing the apical rostrum (arrowhead). In many cells, an amorphous protuberance (asterisk) was present near the flagellar insertions, and the anterior flagellum tapered to a fine thread (arrow) (Bar = 1.5 μm). **8**. SEM of a resting cyst showing scattered minute pores (arrows) (Bar = 1.5 μm). **9**. High magnification SEM showing the insertion of three flagella (Roman numerals) found in an otherwise normal looking cell (inset, Bar = 1.5 μm) (Bar = 1 μm).

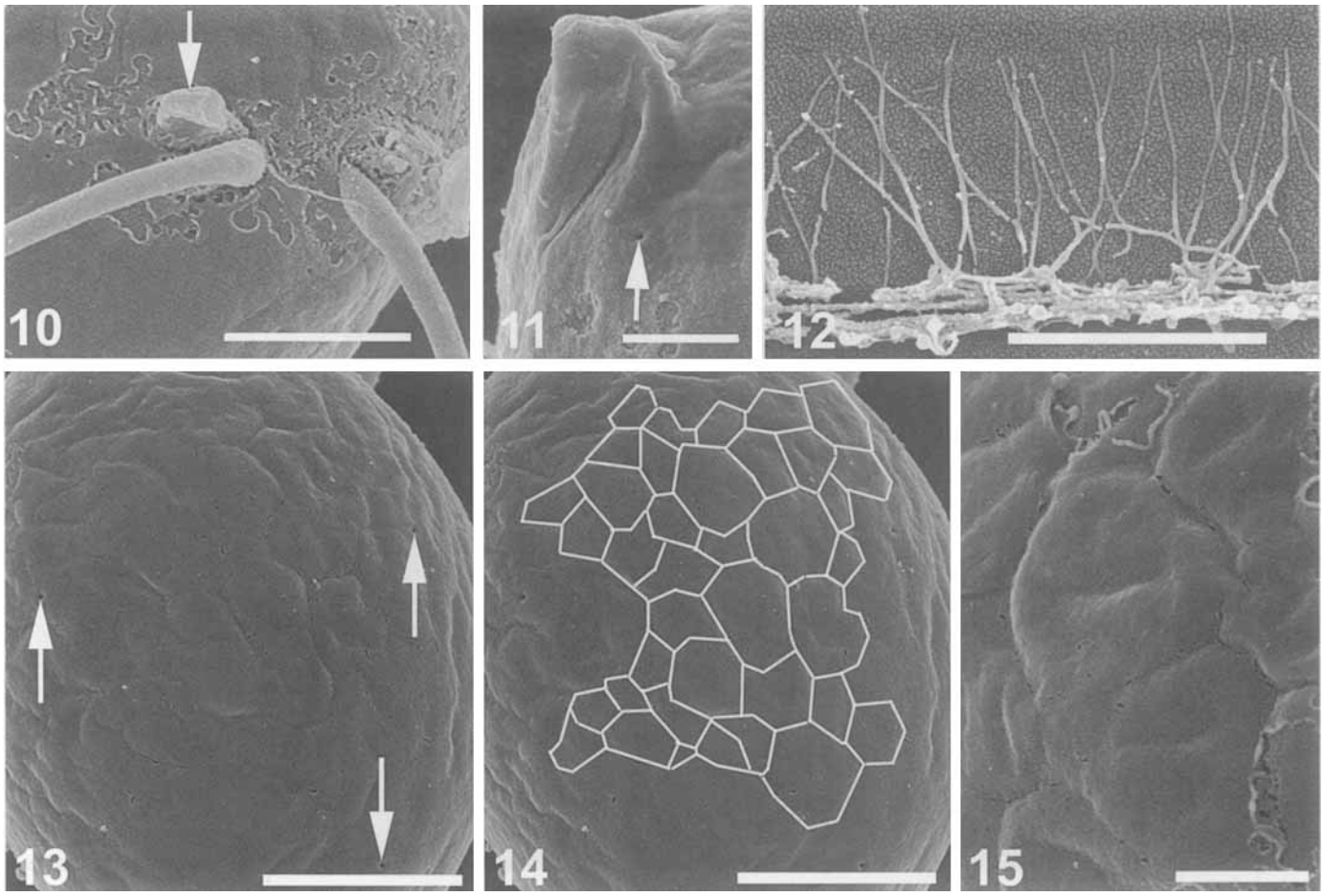

Fig. **10–15**. Scanning electron micrographs (SEM) showing surface features of *Colpodella edax*. **10**. A ventrolateral view showing an amorphous protuberance (arrow) emerging near the insertion of the posterior flagellum (the apical rostrum is oriented to the upper right-hand corner of the micrograph) (Bar = 1 μm). **11**. High-magnification view of the rostrum and a pore (arrow). The inverted U-shaped ridge is inferred to be a manifestation of the underlying open-sided conoid (Bar = 0.5 μm). **12**. Hairs on the anterior flagellum were occasionally, albeit poorly, preserved (Bar = 1 μm). **13**. Indentations on the cell surface demarcate the boundaries of alveoli. Minute pores were scattered sparsely across the cell surface (arrows) (Bar = 1 μm). **14**. The same image as in Fig. 13 except with alveolar boundaries highlighted in order to show the pattern of polygonal shaped alveoli (Bar = 1 μm). **15**. Higher magnification view of the boundaries between the underlying alveoli (Bar = 0.5 μm).

previously by Dawson and Pace (2002) (Fig. 1). *Colpodella edax* diverged as the earliest lineage within one of the two subclades (Fig. 1). These results illustrate how sequences identified as 'novel' in environmental PCR studies may come from familiar organisms that have yet to be sampled with molecular sequence data. Environmental PCR surveys, nonetheless, have demonstrated that our understanding of colpodellid diversity is far from complete. For instance, this is the first evidence that colpodellids inhabit anoxic environments. However, it is impossible to know from environmental PCR surveys alone whether the sequences came from organisms that actually live in the sampled environment or came from cell debris that migrated there haphazardly (e.g. sedimentation of colpodellid cysts from higher in the water column).

Surface morphology of *Colpodella edax*. The life cycle of *C. edax* consisted of two main stages: flagellated trophozoites (Fig. 2–4 and 6–7) and resting cysts (Fig. 5 and 8). The cysts were simple spheres and aside from some sparsely distributed pores, were completely devoid of any surface features (Fig. 8). In most trophozoites (23 of 30 cells observed), two flagella were inserted laterally on the right-hand side of the cell (Fig. 6 and 7). The anterior flagellum was shorter than the posterior one, which is consistent with previous reports on *C. edax* (Mylnikov 1988, 1991; Mylnikov et al. 1998; Simpson and Patterson 1996). Like in perkinsids (Azevedo 1989; Blackbourn et al. 1998), the distal ends of the flagella in *C. edax* tapered to a narrow tip and both flagella emerged from shallow pockets (Fig. 6 and 7). The simple hairs on the anterior flagellum of *C. edax* reported by Mylnikov (1991) were occasionally evident when the flagella were oriented so that the hairs laid flat against the filter paper (Fig. 12). The flagellar hairs were similar to those described in *Perkinsus marinus* (Perkins 1996) and *P. qugwadi* (Blackbourn et al. 1998).

Some trophozoites of *C. edax* had three flagella (7 of 30 cells observed), and many trophozoites with two flagella (18 of 23 observed) possessed an amorphous protuberance near one of the flagella (Fig. 7 and 10). The third flagellum was always closely associated with the anterior-most flagellum (Fig. 9). The protuberance, on the other hand, was associated with the anterior and posterior flagellum with equal frequency (Fig. 7 and 10). It seems unlikely that the presence of either a third flagellum or the protuberance indicates a predivisional stage in these trophozoites, because (four-way) cell multiplication occurs within the cysts of colpodellids (Brugerolle 2002; Mylnikov

1991; Simpson and Patterson 1996). We are unaware of any reports of a colpodellid trophozoite dividing outside of the cyst.

Even though our observations suggest that the trophozoites of *C. edax* have either two or three flagella, we cannot determine, in the absence of corroborating TEM data, whether the flagellum-associated protuberance is somehow associated with this characteristic or simply a fixation artifact. Nonetheless, it is interesting to note that the male microgametes of some apicomplexans (e.g. coccidians and gregarines) have one, two or three flagella (Brugerolle and Mignot 1979; Cheissin 1964; Mylnikov et al. 1998; Reger and Florendo 1970; Scholtyseck 1965; Schrével 1969, 1970; Schrével and Besse 1975). Because the occurrence of three flagella in eukaryotic microbes is very rare, one possible inference given the phylogenetic topology in Fig. 1 is that the occasional presence of three flagella in swimming stages is homologous in colpodellids and apicomplexans.

Observations with light microscopy indicated that trophozoites have a flattened ventral side, which has been referred to as a 'ventral gutter' (Simpson and Patterson 1996) (Fig. 3). The ventral surface was more flattened than the concave dorsal surface, however, no distinct gutter was observed with the SEM (Fig. 6, 7 and 9). A short rostrum (0.6 μm) that functions in myzocytosis was always present at the anterior end of trophozoites; a feature shared with most other colpodellid species (Simpson and Patterson 1996). The rostrum took the form of an inverted U-shaped ridge (Fig. 11), which is inferred to be a manifestation of the underlying open-sided conoid (Mylnikov 1991; Mylnikov et al. 1998). This inference is consistent with TEM studies of other related species that show the open-sided conoid within the rostrum (Brugerolle 2002; Brugerolle and Mignot 1979; Mylnikov 2000; Simpson and Patterson 1996).

Patterns on the cell surface of the trophozoites were associated with minute pores and underlying alveolar sacs. The pores were sparsely scattered over the cell surface and are inferred to be the openings of micropores (Brugerolle 2002; Mylnikov et al. 1998) (Fig. 11 and 13). These openings almost certainly correspond to the pores reported in the previous TEM study of this species (Fig. 3a in Mylnikov et al. 1998). Although trichocysts have been observed in *C. edax* with TEM (Mylnikov et al. 1998), no evidence of ejected trichocysts were observed with SEM. TEM studies of *Colpodella* (Brugerolle 2002; Brugerolle and Mignot 1979) and *Perkinsus* (Blackbourn et al. 1998) demonstrate three membranes around the cell, but offer little insight into the overall pattern of alveoli. SEM micrographs of cells that experienced shrinking during chemical fixation and dehydration revealed the pattern of underlying alveoli of the amphiesma (Fig. 13–15). The abutting alveoli of *C. edax* took the form of small polygons ranging in size from 0.5 to 0.1 μm across (Fig. 14–15). This pattern of small polygonal alveoli is also found in *C. pontica* (Mylnikov 2000), *Oxyrrhis*, and several dinoflagellates (Dodge and Crawford 1971), suggesting that it may be a symplesiomorphic character of both dinoflagellates and apicomplexans.

Phylogenies derived from analyses of SSU rDNA suggest that the last ancestor of dinoflagellates and apicomplexans possessed all of the characteristics shared by colpodellids and perkinsids (Kuvardina et al. 2002; Leander and Keeling 2003). This ancestor is inferred to have possessed at least two laterally inserted flagella with tapered ends, flagellar hairs, an open-sided conoid, rhoptries, micronemes, trichocysts, sparsely distributed micropores, an amphiesma of small polygonal alveoli and a myzocytosis-based mode of feeding. Potential synapomorphies that unite colpodellids with (eu)apicomplexans include a four-way divisional cyst (shared by colpodellids, cryptosporidians, and archigregarines, Leander et al. 2003) and perhaps the presence of three flagella at some stage in the lifecycle (e.g. microgametes and trophozoites).

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