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A Description of *Cochliopodium megatetrastylus* n. sp. Isolated from a Freshwater Habitat

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Abstract. *Cochliopodium megatetrastylus* n. sp. is described based on light microscopy, fine structure and molecular genetic evidence. Amoebae are broadly oval to somewhat triangular during locomotion with average length of 37 μm and breadth of 50 μm, and surrounded by a hyaloplasm margin, somewhat narrow when at rest but more expanded during locomotion (~ 5–10 μm at the anterior). Sparsely occurring subpseudopodia, barely emergent from the hyaloplasm, are blunt and finger-like, occasionally becoming adhesive laterally or at the posterior. Cysts develop after 2–3 weeks in culture and are round with a distinct margin, decreasing in size from 20 to 5 μm during maturation. The granuloplasm contains refractile crystals. A vesicular nucleus (~ 6 μm), containing a nucleolus (2–3 μm), is variable in shape from somewhat lenticular in section to irregularly rounded with undulating or lobed margins. Surface scales (~ 0.3 μm in height) have an apical deeply concave funnel-like collar (~ 0.15 μm deep), without a spine, composed of radial fine rays and concentric filaments forming a fine mesh, supported on four non-cross-linked styles (~ 0.2 μm apart) attached to a round to broadly angular base plate (0.6–1 μm) with a fine grid texture. Cysts are rounded and enclosed by an organic wall bearing remnants of the scales on its outer surface. Both concatenated analysis of SSU-rDNA and COI genes and comparative morphologies support the designation of *Cochliopodium megatetrastylus* n. sp. as a new species.

Key words: Amoebozoa, comparative morphology, fine structure, molecular genetics, taxonomy.

INTRODUCTION

The amoeboid genus *Cochliopodium* has become of increasing interest due to its rather unique morphology. Although the genus is clearly situated within the Amoebozoa (Discosea, Himatismenida) based on current molecular genetic evidence (e.g. Kudryavtsev *et al.* 2005, Tekle *et al.* 2008, Lahr *et al.* 2011, Smirnov *et al.* 2011), the presence of a flexible surface coat (tectum) composed of complex carbohydrate scales on the upper surface, but open on the basal side without an aperture, presented an organization reminiscent of testate amoebae, thus its placement in the taxonomic hierarchy remained uncertain. When the amoeba floats, it becomes more globose and the enclosing tectum with an open region on one side, where the subpseudopodia emerge, resembles even more a testate amoeba. This may have contributed further to earlier uncertainties about its taxonomic affinities. Page (1987, 1988) placed the cochliopodiums in the phylum Rhizopoda, subclass Testacealobosia (De Saedeleer 1934) and the

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order Himatismenida (Page 1987), a separate group from the then recognized "Gymnamoebae" with variable shape and without a well defined enclosing cuticle or test. Bovee (in Jahn *et al*. 1979) included the Cochliopodiidae in an order Pharopodida that also included, however, some families of the "naked" Gymnamoebae. Although the higher order position of *Cochliopodium* spp. is becoming more clearly established (Lahr *et al.* 2011, Smirnov *et al.* 2011), much remains to be done to clarify their phylogenetic position among the Amoebozoa (e.g. Kudryavtsev *et al.* 2011), and to more fully establish the diversity of species and their relationships at the genus level. Presently, there are 14 published and formally recognized species of *Cochliopodium*, varying in size from < 20 μm to more than 80 μm in length (e.g. Kudryavtsev 2000, 2006; Tekle *et al.* 2013). Prior to molecular genetic taxonomic analyses, the fine structure of the surface microscales was one of the few distinctive features, beyond light microscopic morphology, used to unambiguously describe genera and species, and it remains an important additional source of evidence. Here, we describe a new species *Cochliopodium megatetrastylus* n. sp. based on light microscopic, fine structural, and molecular genetic evidence.

MATERIALS AND METHODS

Laboratory culture

Strain ATCC® 30936, isolated initially from a culture of a freshwater protist identified as *Theratromyxa weberi* obtained at Beltsville, MD (1971), was established in culture at the American Type Culture Collection (Manassas, VA) in ATCC medium 997 for freshwater amoebae, with mixed bacteria as food. The cultures were maintained at Spelman College using the same ATCC agar medium. For light microscopic studies, the amoebae were further subcultured in Petri dishes with distilled water or bottled natural spring water (Deer Park®, Nestlé Corp. Glendale, CA, USA) with added autoclaved grains of rice.

Light microscopy

Observations of amoebae behavior and morphology were recorded over a 6-months time period. Amoeba rate of locomotion and other morphological features were observed using a Zeiss Axiovert 40 CFL inverted light microscope with camera attachment and its photo editing software (Zeiss, Thornwood, NY). Rate of locomotion was measured from amoebae grown in plastic Petri dish cultures.

Transmission electron microscopy

The amoeba culture was fixed for transmission electron microscopy as previously published (Anderson *et al.* 1997). A suspension of the amoebae in culture medium at 25°C was placed in a 15-ml graduated conical centrifuge tube and mixed with an equal volume of 4% (w/v) TEM-grade glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, to yield a final fixative of 2% (w/v). The tubes containing the fixed amoebae were plunged in an ice bath, and after 20 min. at 3°C, the glutaraldehyde-fixed cells were gently spun down to form a pellet, the supernatant was removed by aspiration, and 2 ml of 2% (w/v) cold osmium tetroxide solution in 0.2 M cacodylate buffer (pH 7.2) were added and the pellet thoroughly dispersed in the fixative. After 1-h post-fixation at 3°C, the cells were again pelleted and the supernatant removed. The cells were enrobed in 0.4% (w/v) solidified agar. Small cubes (~ 1 mm^3) were cut from the agar block, washed in distilled water, dehydrated in a graded acetone/aqueous series, infiltrated with and embedded in low viscosity epon (Energy Beam Sciences, Agawam, MA), and polymerized at 75°C for 12–18 h. Ultrathin sections were cut with a Porter-Blum MT-2 ultramicrotome (Sorvall, Norwalk, CT) using a diamond knife, collected on uncoated copper grids, and post-stained with Reynold's lead citrate. Scale morphology was observed by depositing small drops of fixed amoebae on a carbon-coated, collodion-covered grid, air dried at room temperature, and shadowed at an angle of 45° with a carbon-platinum source in a vacuum evaporator. Preparations of ultrathin sections or the shadowed grids were viewed with a Philips TEM-201 transmission electron microscope (Einthoven, Netherlands) operated at 60 kV accelerating voltage.

DNA extraction, PCR amplification, alignment and phylogenetic analysis

DNA samples of ATCC® 30936 were extracted using illustra™ DNA Extraction Kit BACC1 (GE Healthcare UK Ltd, Little Chalfont Buckinghamshire HP7 9NA England, Cat. No. RPN8501) per manufacture's instructions and with the addition of a phenol-chloroformisoamyl step using Phase Lock Gel Heavy tubes (Eppendorf AG, Hamburg, Germany, Cat. No. 955154070). Primers for SSU-rDNA genes are from Medlin *et al*. (1988) with three additional primers used to generate overlapping sequences from each clone as described in Snoeyenbos-West *et al*. (2002). Primers for COI gene, approximately 650 bp long, are from Folmer *et al*. (1994). Phusion DNA Polymerase, a strict proofreading enzyme, was used to amplify the genes of interest and Invitrogen Zero Blunt Topo cloning kits were used for cloning. Sequencing of cloned plasmid DNA, was accomplished using vector-specific primers and the BigDye terminator kit (Perkin- Elmer). Sequences were run on an ABI 3100 automated sequencer in Morehouse School of Medicine. We have fully sequenced 2–4 clones of each gene and surveyed up to 8 clones per taxon in order to detect intra-specific variations.

Alignments for SSU-rDNA sequences were constructed in SeaView (Galtier *et al*. 1996, Gouy *et al*. 2010) with alignment algorithm MUSCLE (Edgar 2004) using default settings. Variable regions in the SSU-rDNA alignment that could not be aligned unambiguously were removed manually (1576 bp retained) in Se-Al, Sequence Alignment Editor (Rambaut 1996). Pairwise distances between our sequences were calculated using PAUP* v4.0b10 (Swofford 2002) with uncorrected ("p") distance matrix. The coding region of COI was translated into amino acids using Se-Al. Maximum likelihood phylogenetic trees and bootstrap values for the combined SSU-rDNA and COI data sets were inferred using RAxML BlackBox with default settings and GTR+Γ+I sequence model (Stamatakis *et al*. 2008). A total of ten ingroup taxa including a sequence

represented by '*Oryza sativa*' clone OSIGCRA115O12, previously shown to group within *Cochliopodium,* were analyzed with three outgroup taxa (see Tekle *et al.* in press). In the final concatenated matrix, five of the 13 taxa analyzed were represented with COI sequences.

RESULTS

Light microscopy

Amoebae are broadly oval to somewhat triangular during locomotion (Fig. 1) with average length of 37 µm (27–47, N = 200) and breadth of 50 µm (34–60, N = 200) and move at a rate of ~ 17.1 µm per min. A distinct hyaloplasm with somewhat undulating margin surrounds the amoeba rather narrowly at rest, but becoming relatively broad (~ 5–10 µm at the anterior) during locomotion (Figs 1, 2). The nucleus (5–10 µm) is vesicular. The surrounding cytoplasm contains refractile cell coat crystals and vacuoles distributed throughout the granuloplasm. Occasionally, during locomotion, sparse, blunt pseudopodia emerge from the hyaline margin, migrate posteriorly and become occasionally adhesive to the substrate or form a posterior uroid with adhesive filaments (Fig. 2). During locomotion, the amoebae frequently made contact with one another, aggregated and in some cases fused to form a larger, multinucleated cell (Fig. 3). After 2–3 weeks in culture, amoebae began encysting (Fig. 3, inset); but the overall population still contained many vegetative cells. Cysts are round with a clearly defined margin and sizes ranged from 20 to 5 µm decreasing in size with increasing maturation (note the small cyst attached to the larger one in Fig. 3, inset).

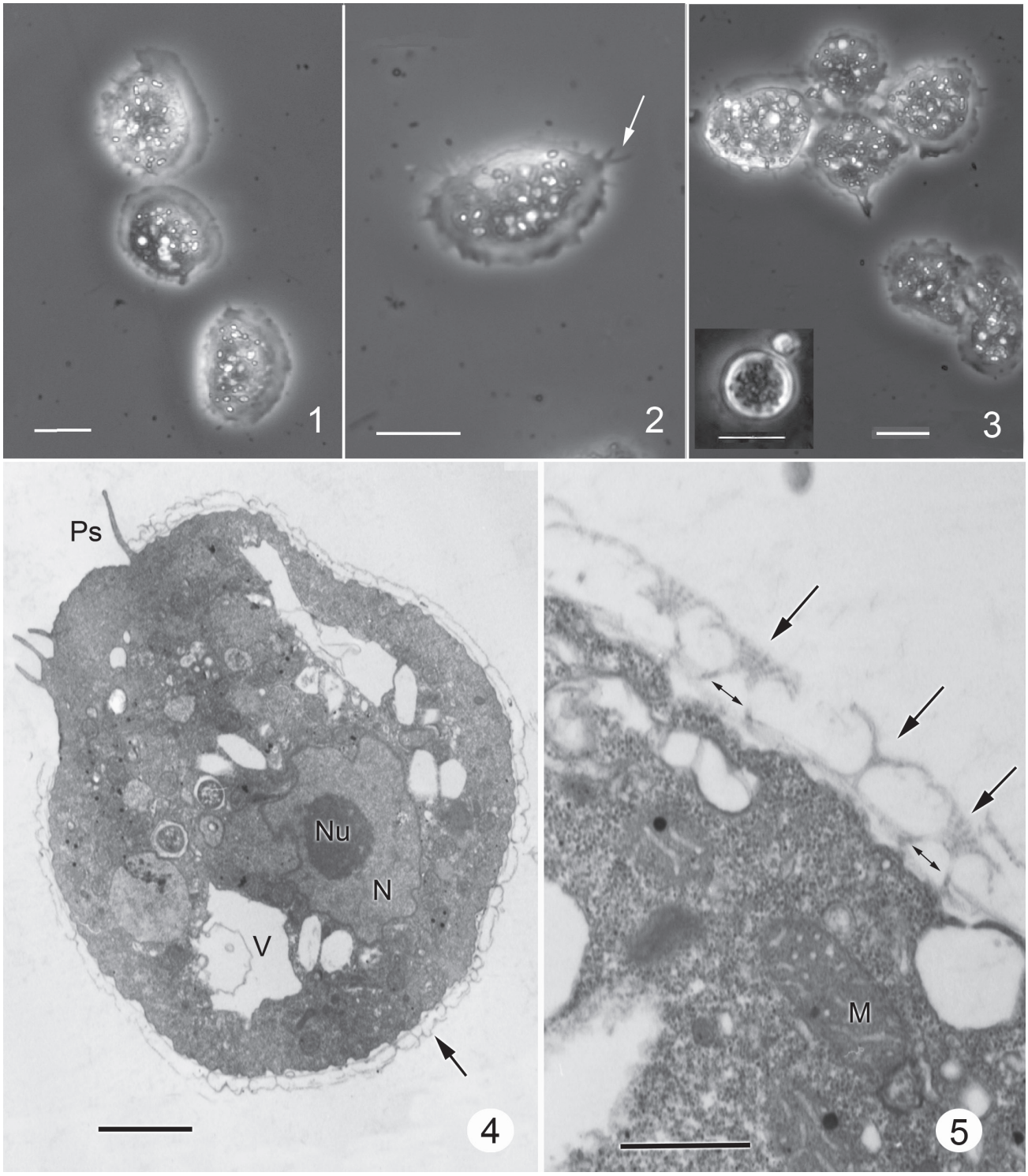
Fine structure

An ultrathin section of an individual *C. megatetrastylus* n. sp. (Fig. 4), extending from the dorsal (scale-bearing) surface to the ventral (pseudopodial-bearing) surface, exhibits the nucleus (~ 5–6 µm), with prominent more electron-dense nucleolus (2–3 µm dia.), and surrounding cytoplasm. Mitochondria (~ 0.4 µm dia. and 1 µm in length) are tubulo-cristate (Fig. 5). More detailed images of the nucleus (Figs 6, 7), show the profile of the nuclear envelope, varying from lenticular in one plane of section, to nearly rounded but with undulating margins, or with lobe-like extensions. Surface scales (mean height 0.3 µm, range 2.0–4.0, N = 30) are tower-like, as observed in other *Cochliopodium* spp. (Fig. 5, arrows). Shadowed preparations (Fig. 8) exhibit the overall perspective as viewed from the top of each scale, consisting of a rounded to angular base plate (arrows), varying markedly in size and shape (~ 0.6–1 µm), and surface views of the round apical collar with radial rays (~ 16). The basal plate has a very fine grid, or sieve-like texture. Four basal styles support a broad, circular, apical funnel-shaped collar (~ 0.5–0.7 dia.) with very thin peripheral rim (Fig. 6). The deeply concave collar with flared perimeter also contains fine, concentrically arranged filaments cross-linking the radial rays, forming a net-like configuration on the walls of the collar, especially visible in vertical sections of the flared collar rims of the towers (Fig. 5, arrows). The distance between the bases of two opposite styles (legs) is ~ 0.2 µm (Fig. 5, double-headed arrows). The distance from the base plate to the bottom of the conical collar is ~ 0.13–0.15 µm, and the depth of the concave apical collar is ~ 0.15 µm, a total scale height of ~ 0.3 µm. There is no evidence of a spine. Cysts, enclosed within a relatively thin, somewhat electron-dense organic wall, also contain remnants of scales on the surface of the wall (Fig. 9). A diagram of a scale (Fig. 10) is based on an interpretation of the fine structure evidence.

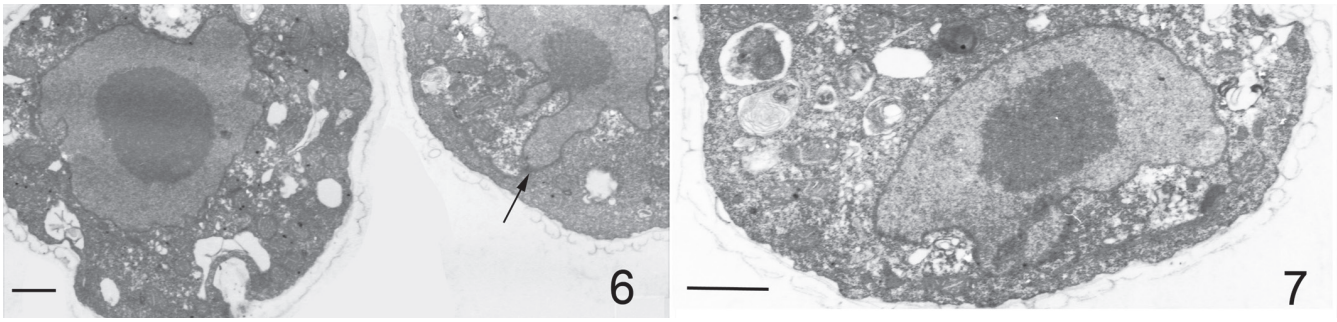
Molecular genetics

Combined SSU-rDNA and COI Phylogeny. RAxML tree of the concatenated SSU-rDNA and COI genes (2398 bp) is well resolved with strong bootstrap supports (Fig. 11). The topology obtained is concordant with previously published data based on SSU-rDNA alone (Tekle *et al.* 2013, Kudryavtsev *et al.* 2011). The genus *Cochliopodium* is recovered as a monophyletic group with full ML bootstrap support (Fig. 11). *Cochliopodium megatetrastylus* n. sp. groups with *Cochliopodium pentatrifurcatum* with full support (Fig. 11). *C. megatetrastylus* n. sp. and *C. pentatrifurcatum* group together to form a sister group relationship with *C. minutoidum* with full support (Fig. 11). These three taxa also form a sister group relationship with a clade consisting of *C. minus* + '*Oryza sativa* CT837767' and *C. actinophorum* with a weak support (45%). Similar tree topologies with similar statistical support were recovered using different models of sequence evolution and probabilistic methods (not shown).

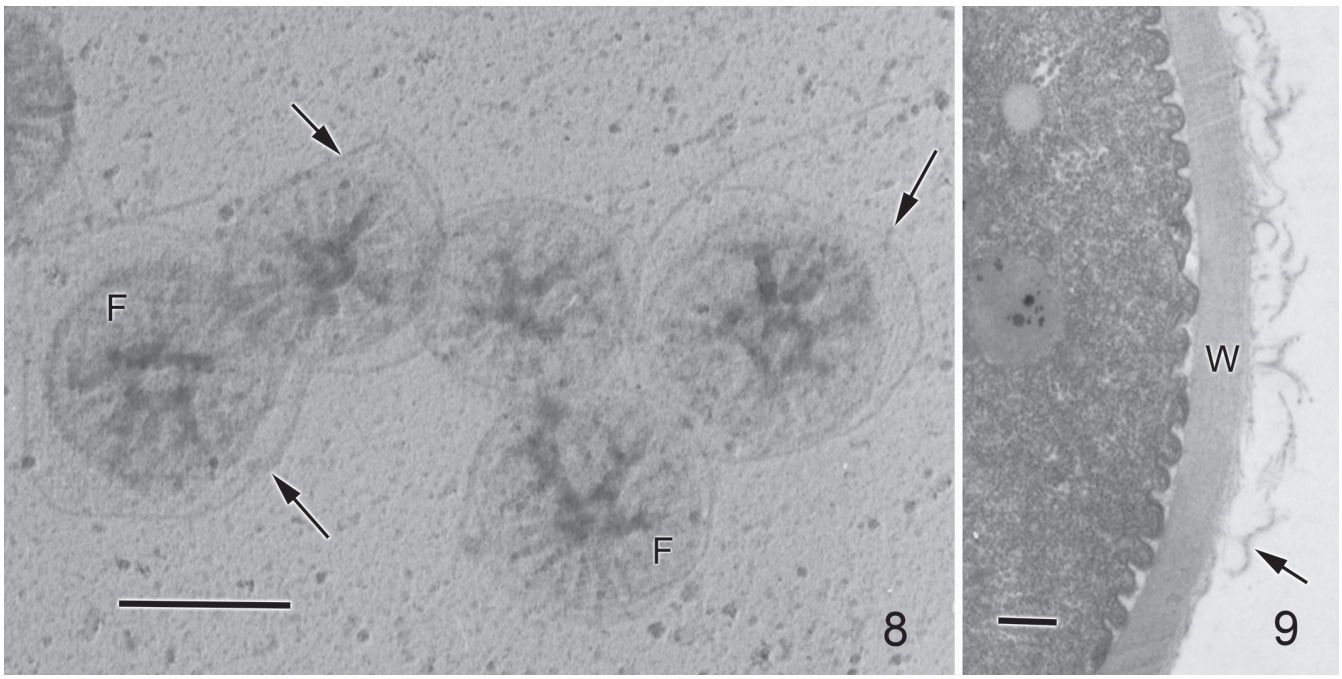
Molecular genetic divergences. Intra-strain variations among the sequenced SSU-rDNA clones were not observed, while we found two copies (alleles) of COI that only differed in a single nucleotide position. The substitution is nonsynonymous and resulted in an amino acid change. The sequence divergence of SSU-



Figs 1–5. Light microscopic and fine structure images of *Cochliopodium megatetrastylus* n. sp. **1–3.** Morphology of living *C. megatetrastylus* n. sp. during locomotion. **1** – locomoting amoebae with anterior hyaloplasm fringe; **2** – morphology of the relatively short subpseudopodia that become adhesive laterally and posteriorly, and attach to the substratum; **3** – aggregation and fusion of amoebae (inset shows a large and nearby small cyst). All scale bars: 20 µm; **4** – fine structure of a cell in cross-section showing the nucleus (N) and prominent nucleolus (Nu), vacuole (V), emergent pseudopodia (Ps) from the ventral surface, and showing scales (arrow) covering the dorsal surface. Scale bar: 2 µm; **5** – tubulo-cristate mitochondria (M) near the cell periphery, coated with surface scales (arrows) shown in profile. Double-headed arrows indicate the distance (~ 0.2 µm) between the bases of a pair of opposite styles that support the apical collar. Scale bar: 0.5 µm.



Figs 6, 7. Fine structure details of the nucleus of *Cochliopodium megatetrastylus* n. sp. **6** – examples of nuclei varying from somewhat rounded with undulating margin to those with lobe-like extensions (arrow); **7** – a lenticular shaped nucleus viewed in one plane of section. Scale bars: 1 µm.



Figs 8, 9. Details of scale structure and fine structure of the cyst wall of *Cochliopodium megatetrastylus* n. sp. **8** – cluster of surface scales viewed from the top of the scale toward the base, showing the basal plates varying in size and shape of the perimeters (arrows), each with an apical, funnel-shaped collar (F) with multiple radial rays emanating from a central core. Scale bar: 0.5 µm; **9** – perimeter of a cyst, showing the organic, finely fibrous wall (W) and an external layer of residual scales (arrow). Scale bar: 0.2 µm.

rDNA of *C. megatetrastylus* n. sp., compared to the two sister species, *C. pentatrifurcatum* and *C. minutoidum*, were very small, 0.8 and 0.9%, respectively. The sequence substitutions and an indel were distributed at different positions across the overlapping length of the sequences. COI sequence divergence for the three sister taxa were compared. COI nucleotide level sequence divergence between *C. megatetrastylus* n. sp. and *C. pentatrifurcatum* was lower (4%) compared with *C. minutoidum* (7.7%). We found a number of nonsynonymous substitutions among the COI sequences of the three sister taxa. The total number of amino acids divergences of both *C. pentatrifurcatum* and *C. minutoidum* compared to the new species was the same 3.65% (6 amino acids). These substitutions were rarely shared and found mostly scattered across the length of the sequences. The sequence divergence of *C. minutoidum* and *C. pentatrifurcatum* at an amino acid level

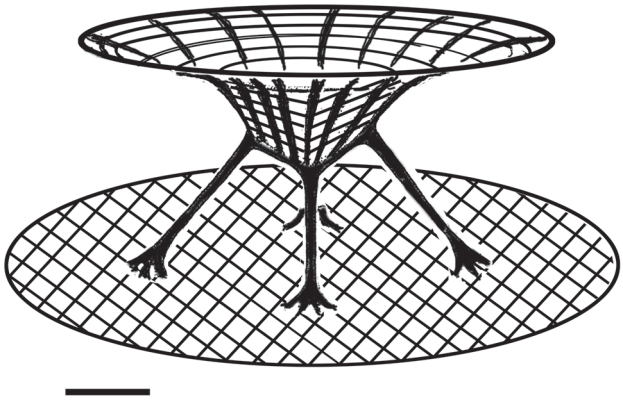


Fig. 10. Diagram of *C. megatetrastylus* n. sp. scale showing the grid-like base plate and the apical conical collar supported on four styles. Scale bar: 0.1 µm.

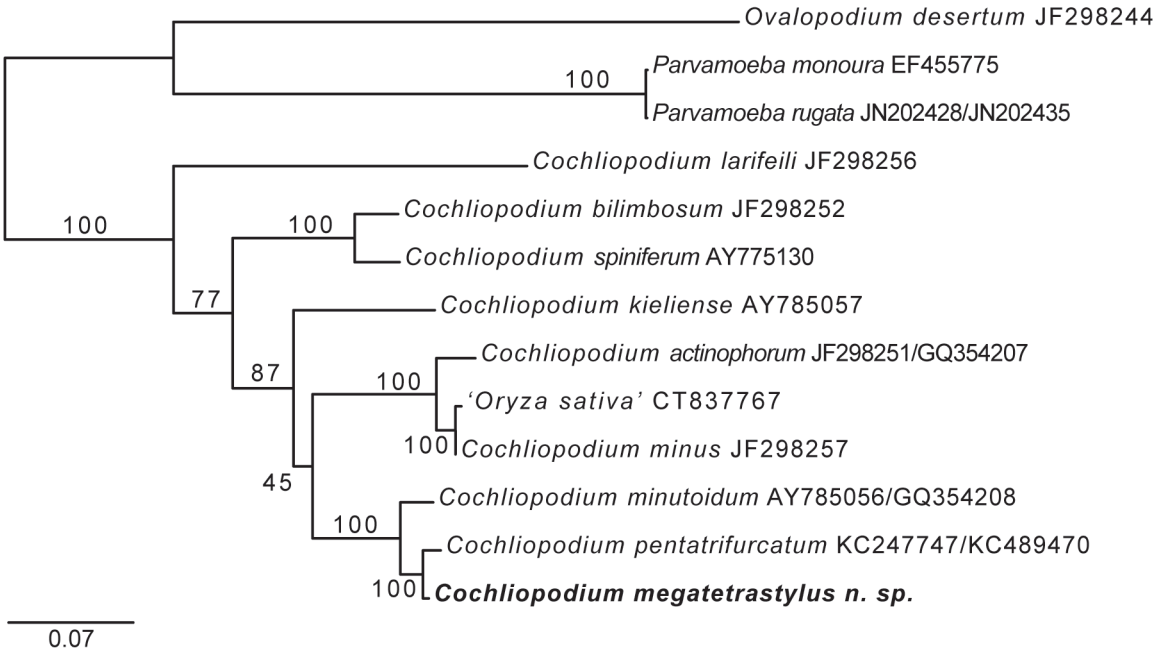


Fig. 11. RAxML tree of combined SSU-rDNA and COI genes showing the placement of *Cochliopodium megatetrastylus* n. sp. within the genus *Cochliopodium*. RAxML bootstrap values are shown at the nodes. The GenBank accession numbers of the respective gene (SSU-rDNA/COI) used in the combined analyses are shown next to the species name. All branches are drawn to scale.

was slightly higher, 4.88%, corresponding to a total of 8 amino acids differences.

DISCUSSION

Cochliopodium spp. are adapted to widely varied environments (e.g. Page 1988), and have been isolated from soil and aquatic habitats, including freshwater (e.g. Kudryavtsev 1999, 2006; Dyková *et al*. 1998), brackish water (Lesen *et al.* 2010, Schaeffer 1926) and marine habitats (Kudryavtsev and Smirnov 2006). They vary in size from less than 20 µm (e.g. Kudryavtsev 2006) to more than 50 µm (e.g. Archer 1871; Bark 1973; Kudryavtsev 1999, 2000, 2004, 2005; Kudryavtsev and Smirnov 2006; West 1901; Yamaoka and Kunihiro 1985; Yamaoka *et al*. 1991). Among some currently recognized and published species isolated from

soil or freshwater habitats are: *Cochliopodium barki* (Kudryavtsev, Brown and Smirnov 2004), *Cochliopodium kieliense* (Kudryavtsev 2006), *Cochliopodium larifeili* (Kudryavtsev 1999), *Cochlipodium minus* (Page 1988, Kudryavtsev 2006), *C. minutoidum* (Kudryavtsev 2006), and *C. vestitum* (Archer 1871, Kudryavtsev 2006). *Cochliopodium megatetrastylus* n. sp. is also a freshwater amoeba isolated from a culture of the vampyrellid amoeba *Theratromyxa weberi*.

The surface scales of *Cochliopodium* spp. can be categorized into three broad categories based on prior published fine structure evidence (e.g. Tekle *et al*. 2013): *Category 1* are those that are tower-like and possess an apical, funnel-like collar without an emergent spine, *Category 2* are those with tower-like scales, but with an apical spine emerging from the base of the funnel-like collar, and *Category 3* are not tower-like, including those that are plate-like, cube-shaped, or other geometric forms. *Cochliopodium megatetrastylus* n. sp. is assigned to *Category 1*, and described as a new species based on combined discriminative evidence from light microscopy, scale fine structure, and molecular genetics. We adopt the classification scheme of Smirnov *et al.* (2011) that has a detailed analysis down to the level of families, while also recognizing that a higher-level classification scheme (Adl *et al.* 2012) provides a more generalized perspective.

Taxonomic Description

Phylum: AMOEBOZOA
Subphylum: Lobosa
Class: Discosea
Order: Himatismenida
Family: Cochliopodiidae
Cochliopodium megatetrastylus n. sp.

Diagnosis

Amoebae broadly oval to somewhat triangular during locomotion (~ 4 μm per min) with average length of 32 μm (27–47) and breadth of 47 μm (34–60). A distinct hyaloplasm with somewhat undulating margin, narrow when at rest but more expanded during locomotion (~ 5–10 μm at the anterior), surrounds the granuloplasm hump. Sparsely occurring subpseudopodia, barely emergent from the hyaloplasm, are blunt and finger-like, occasionally becoming adhesive latero-posterior. A posterior uroid with adhesive filaments sometimes forms during locomotion. Granuloplasm contains refractile crystals. The vesicular nucleus (~ 5–6 μm), observed in transmission electron microscopic ultrathin sections, is variable in shape from somewhat lenticular in section to irregularly rounded with undulating or lobed margins and includes a distinct nucleolus (2–3 μm). Surface scales (~ 0.3 μm in height) have an apical deeply concave funnel-like collar (~ 0.15 μm deep), without a spine, composed of radial fine rays and concentric filaments forming a fine mesh, supported on four non-cross-linked styles (~ 0.2 μm apart) attached to a round to broadly angular base plate (0.6–1 μm) with a fine grid texture. During locomotion, amoebae frequently make contact, aggregate, and in some cases fuse to form a larger, multinucleated cell. Cysts are rounded, enclosed by an organic wall bearing remnants of the scales on its outer surface.

This diagnosis is according to the *International Code of Zoological Nomenclature* (Article 1.1.1).

Etymology: The distinctive scales with a deep conical apical collar supported on four styles, and the relatively large size of the amoeba compared to others with a similar scale organization of four simple styles, is the basis for the species name: *megatetrastylus*, "mega" for large, and "tetrastylus" for the four styles supporting the apical collar.

Type locality: Unknown, the amoeba was obtained in Beltsville, MD, USA from a freshwater location and was discovered in a culture of a vampyrellid amoeba.

Habitat: Natural body of freshwater.

Type material: A type culture is maintained by the American Type Culture Collection, Manassas, VA (Strain ATCC® 30936), and gene sequences deposited with GenBank, accession numbers KC747718 (SSU-rDNA) and KC747719, KC 747720 (COI).

Comparative diagnosis

Morphology and fine structure. Among the *Cochliopodium* spp. with tower-like scales and four styles supporting the flared apical collar (*Category 1*), there are two that are comparable to the scales of *C. megatetrastylus,* i.e. *C. barki* and *C. minutoidum*. All three, including *C. megatetrastylus*, have scales with a deeply concave, fine mesh apical collar. However, there are differences in morphology and fine structure that differentiate them from *C. megatetrastylus*. *Cochliopodium barki* is smaller than *C. megatetrastylus*. The morphology of *C. barki* as reported by Kudryavtsev *et al*. (2004) is as follows: mean length in locomotion 27 μm; breadth 32–33 μm; length: breadth ratio 0.82–0.87. The nucleus as viewed in transmission electron microscopic ultrathin sections is round to somewhat oval with a prominent central nucleolus.

C. minutoidum is much smaller (Kudryavtsev 2006): length in locomotion 14 µm; breadth 17.3 µm; length: breadth ratio 0.83. The subpseudopodia are prominent up to 5–6 µm in length. The morphology of *C. megatetrastylus* differs substantially from the former two. It is larger than *C. barki* and *C. minutoidum*. Its mean length is 37 µm and breadth 50 µm, with a broad anterior hyaloplasm up to 10 µm wide. It lacks the prominent subpseudopodia of *C. minutoidum*. The nucleus in ultrathin sections varies from lenticular to lobate, and appears markedly different from those published for *C. barki* or *C. minutoidum*. The scale of *Cochliopodium minus* (e.g. Dykova *et al.* 1998) also has a fine, mesh-like conical collar, but the collar is much more pronounced in depth and width compared to that of *C. megatetrastylus*, and the collar is supported on a broad base of eight short styles according to the interpretation of Kudryavtsev (2006). *C. megatetrastylus* (~ 40 µm) is also larger than *C. minus* (15–42 µm) (Kudryavtsev 2006).

The scales of our new species also resemble those of previously published, but un-named species. The fine structure of a scale with four styles and similar conical collar to *C. megatetrastylus* was reported by Yamaoka *et al.* (1984) for an amoeba isolated from the sediments of an aquarium and designated as NYS strain (Nagatani *et al.* 1981). However, the scale differs from our isolate in having much longer styles relative to the depth of the collar (twice as long as the depth of the collar) as evidenced from an inspection of their vertical ultrathin sections through the height of the scales, whereas the scales of *C. megatetrastylus* have styles that are about the same length as the depth of the collar. The scale of the NYS strain is 0.7 µm tall (Nagatani *et al.* 1981) compared to 0.3 µm for *C. megatetrastylus.* The surface of NYS strain is covered by an amorphous coat (~ 50 nm) underlying the scales, a feature not visible in our amoeba.

Based on the molecular genetic evidence (Fig. 11), *C. megatetrastylus* branches closely with a recently described new species *Cochliopodium pentatrifurcatum* (Tekle *et al.* 2013). However, the scale of *C. pentatrifurcatum* is very different, including a collar composed of five radially arranged spokes that are trifurcate where they join the rim of the collar, and it bears a short terminal spine. The mean size of *C. pentatrifurcatum* (25 µm, range 19–32) is smaller than that of *C. megatetrastylus* (32 µm).

Molecular genetic evidence. Sequence divergence comparisons and the phylogenetic tree (Fig. 11) inferred from concatenated SSU-rDNA and COI genes demonstrates that the new species is a member of the genus *Cochliopodium* and sufficiently different from other described species to suggest that it is a valid new species. The combined SSU-rDNA and COI phylogeny shows the new species is sister to *C. pentatrifurcatum.* Sequence divergence of analyses of SSU-rDNA among the three sister taxa ((*C. megatetrastylus* + *C. pentatrifurcatum*) *C. minutoidum*) is very small (0.8–0.9%) falling in the range of intra-strain variability observed in some members of Amoebozoa (Nassonova *et al*. 2010). Analysis of SSU-rDNA alone was unable to provide a conclusive relationship among the three species due to high sequence similarities (not shown). More particularly, however, the sequence divergences observed in COI were sufficient to distinguish the three sister taxa. At the nucleotide level, *C. pentatrifurcatum* has less COI sequence divergence (4%) than *C. minutoidum* (7%) when compared to the new species. However, most of these differences are synonymous substitutions and at the amino acid level, both sister taxa are only 6 amino acids (3.4%) divergent from the new species.

Most of the substitutions (nucleotide or amino acid), and an indel among the three taxa are distributed at different positions spanning the sequences thus making each species distinguishable from each other. Moreover, the sequence divergences observed among the three species, both at the amino acid and nucleotide levels, of the COI gene are well above the proposed conservative threshold (~ 2.7%) for species designation used in birds (Hebert *et al*. 2003).

Final comment. The above combined evidence from light microscopic morphology, fine structural features, and molecular phylogenetic analyses provide consistent and sufficiently strong evidence to propose that *C. megatetrastylus* is a new species. It falls within a clade that includes closely related sister taxa and begins to define a subgroup of cochliopodiums that may yield some interesting insights into their molecular phylogeny and ecophysiological relationships.

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