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ORIGINAL ARTICLE

Three New Freshwater *Cochliopodium* Species (Himatismenida, Amoebozoa) from the Southeastern United States

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ABSTRACT

Cochliopodium is a lens-shaped genus of Amoebozoa characterized by a flexible layer of microscopic dorsal scales. Recent taxonomic and molecular studies reported cryptic diversity in this group and suggested that the often-used scale morphology is not a reliable character for species delineation in the genus. Here, we described three freshwater *Cochliopodium* spp. from the southeastern United States based on morphological, immunocytochemistry (ICC), and molecular data. A maximum-likelihood phylogenetic analysis and pairwise comparison of COI sequences of *Cochliopodium* species showed that each of these monoclonal cultures were genetically distinct from each other and any described species with molecular data. Two of the new isolates, "crystal UK-YT2" (*Cochliopodium crystalli* n. sp.) and "crystal-like UK-YT3" (*C. jaguari* n. sp.), formed a clade with *C. larifeili*, which all share a prominent microtubule organizing center (MTOC) and have cubical-shaped crystals. The "Marrs Spring UK-YT4" isolate, *C. marrii* n. sp., was 100% identical to "*Cochliopodium* sp. SG-2014 KJ569724." These sequences formed a clade with *C. actinophorum* and *C. arabianum*. While the new isolates can be separated morphologically, most of the taxonomic features used in the group show plasticity; therefore, *Cochliopodium* species can only be reliably identified with the help of molecular data.

THE southeastern United States is known to have some of the most diverse freshwater ecosystems in the United States mostly from a macroscopic perspective (Duncan 2013). Its diversity of microbial eukaryotes has been understudied, especially with a modern molecular approach. Amoebozoa, a eukaryotic supergroup, contains around 2,400 described species (Pawlowski et al. 2012). These organisms are found in a wide range of habitats including marine, soil, and others as forming symbioses with other organisms or as parasites of vertebrates (Anderson 2018). This number is expected to continue to increase with more molecular studies and exploration of diverse habitats (Fučíková and Lahr 2016; Geisen et al. 2014; Nassonova et al. 2010; Tekle 2014).

Cochliopodium Hertwig et Lesser, 1874 sensu Bark 1973 (Himatismenida, Amoebozoa), is a genus of microscopic, amoeboid eukaryotes that inhabit freshwater (Anderson and Tekle 2013; Kudryavtsev 2005, 2006; Page 1988), brackish (Kudryavtsev 2006), and marine environments (Kudryavtsev 2000, 2004; Kudryavtsev and Smirnov 2006; Schaeffer 1926). This genus currently consists of 23 species (Tekle et al. 2015). *Cochliopodium* species are lens-shaped and are round, oval, flabellate, or triangular during locomotion. These amoebae display a variety of shapes and sizes of cytoplasmic inclusions (crystals) and microscales (Anderson and Tekle 2013; Kudryavtsev 1999; Page 1988). Locomotive cells range in size from < 20 μm (Geisen et al. 2014) to over 90 μm (Kudryavtsev 2000; Sadakane et al. 1996) that can reach up to 120 μm (Penard 1890, 1902). *Cochliopodium* spp. are difficult to identify due to the plasticity of the taxonomic features used in the group and cryptic diversity (Geisen et al. 2014; Tekle 2014; Tekle and Wood 2018). Recent studies demonstrate that a morphology-based approach might not capture the full diversity of this genus; however, molecular data such as the mitochondrion-encoded COI barcoding

marker are allowing for a better understanding of this genus (Geisen et al. 2014; Tekle 2014; Tekle et al. 2015).

Cochliopodium has recently been identified as a lineage with at least some sexual species based on physical evidence of plasmogamy to form a multinucleate cell called a plasmodium and data that suggest karyogamy (Tekle et al. 2014). Furthermore, this sexual behavior is supported with genetic evidence that reported nearly complete recombination gene repertoire in some members of this genus (Tekle et al. 2017; Wood et al. 2017). The ploidy formation and depolyploidization process of the genome in the genus is still not completely understood. Discovery of new species and their description in the genus will further our understanding of the nature and mechanisms of sexual reproduction in the group.

Here, we isolated three morphologically distinct *Cochliopodium* species designated as "crystal UK-YT2," "crystal-like UK-YT3," and "Marrs Spring UK-YT4" from freshwater environments in the southeastern United States. The "crystal UK-YT2" and "crystal-like UK-YT3" isolates were sampled from Arabia Lake, Lithonia, Georgia, and "Marrs Spring UK-YT4" was collected from Marrs Spring on the campus of The University of Alabama, Tuscaloosa, Alabama. Morphological data (light microscope), cytological data using an immunocytochemistry (ICC) technique, and molecular data (COI) were used to describe these new isolates.

MATERIALS AND METHODS

Culturing

Freshwater samples were collected from sediment in the littoral zone in Arabia Lake, Lithonia, Georgia (33.671794, −84.127066) (*Cochliopodium* "crystal UK-YT2" and "crystal-like UK-YT3") and from Marrs Spring (33.213610, −87.548340) near the surface containing free-floating green algae on campus at The University of Alabama, Tuscaloosa, Alabama (*Cochliopodium* sp. "Marrs Spring UK-YT4"). The samples were subcultured in Petri dishes with Deer Park® natural spring water (Nestlé Corp., Glendale, CA) and autoclaved rice grains for bacterial growth for food. Single cells of *Cochliopodium* species were isolated for monoclonal cultures on American Type Culture Collection (ATCC) 997 freshwater amoeba agar medium or a plastic Petri dish with natural spring water and rice grains.

Microscopy

Light microscope images were taken on an Axiovert 40 CFL and a Nikon Eclipse E1000 (DIC) with a Zeiss AxioCam ICm1 camera. Images of cells were taken on a glass slide without a coverslip. Morphological data related to size (cell, nucleus, hyaline border, and crystals) of actively locomoting cells were measured with ZEN 2012 lite. Approximately 100 cells from cultures around 2 wk old (peak growth density) were used in the species descriptions of these amoebae. Data related to cytoskeleton (microtubules), DNA, and plasma membrane were collected using ICC methods described in Tekle and Williams (2016).

DNA extraction and molecular analysis

For *Cochliopodium* sp. "crystal UK-YT2," DNA was extracted using illustra™ DNA Extraction Kit BACC1 (GE Healthcare UK Ltd, Little Chalfont Buckinghamshire, U.K., cat. no. RPN8501) per the manufacturer's instructions except with the addition of a phenol–chloroform and isoamyl alcohol step using Phase Lock Gel Heavy tubes (Eppendorf AG, Hamburg, Germany, cat. no. 955154070). Cytochrome oxidase I (COI) was amplified with primers from Folmer et al. (1994). The forward primer was LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′), and reverse primer was HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). The PCR settings were as follows: initial denaturation at 95 °C for 3 min; 35 cycles of 95 °C for 1 min (denaturation), 55 °C for 1 min (primer annealing), and 72 °C for 1 min and 30 s (extension); followed by a final extension at 72 °C for 7 min. PCR amplification of COI was performed with Phusion DNA Polymerase, a strict proofreading enzyme, and cloning was accomplished using Lucigen PCRSmart, Novagen Perfectly Blunt, and Invitrogen Zero Blunt Topo cloning kits. Clones were sequenced using vector-specific primers and the BigDye Terminator kit (Perkin-Elmer, Foster City, CA), and run on an ABI 3100 automated sequencer at Morehouse School of Medicine (Atlanta, GA, USA). To detect intrastrain variations, six COI clones per experiment were fully sequenced.

For *Cochliopodium* spp. "crystal-like UK-YT3" (three isolates) and "Marrs Spring UK-YT4" (one isolate), DNA was extracted using a QIAGEN Blood and Tissue DNA extraction kit (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) was performed with illustra™ PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) to amplify the mitochondrial cytochrome oxidase I (COI) gene with the same primers as above. PCR cleanup was done with GeneJET PCR Purification Kit (Thermo Scientific™, Vilnius, Lithuania). Sanger sequencing was performed at Georgia State's Cell Protein DNA Core Facility (Atlanta, GA, USA). Raw reads were manually edited in Geneious Prime R11 (Kearse et al. 2012) and then aligned with previously published *Cochliopodium* sequences with MAFFT (Katoh et al. 2002). The resulting alignment was 716 base pairs. MEGA7 (Kumar et al. 2016) was used to calculate pairwise distances and run a maximum-likelihood phylogenetic analysis with the K2P model of nucleotide substitution and 1,000 bootstrap replicates. DNA sequences were submitted to GenBank ("crystal UK-YT2": MN389531–MN389537; "crystal-like UK-YT3": MN389538–MN389540; "Marrs Spring UK-YT4": MN389530). The phylogenetic tree was edited in FigTree v1.4 (Rambaut 2012).

RESULTS

Light microscopy observations on morphology and behavior

Cochliopodium sp. "crystal UK-YT2"

The locomotive form of *Cochliopodium* sp. "crystal UK-YT2" had an oval (Fig. 1A) or triangular (Fig. 1B) shape,

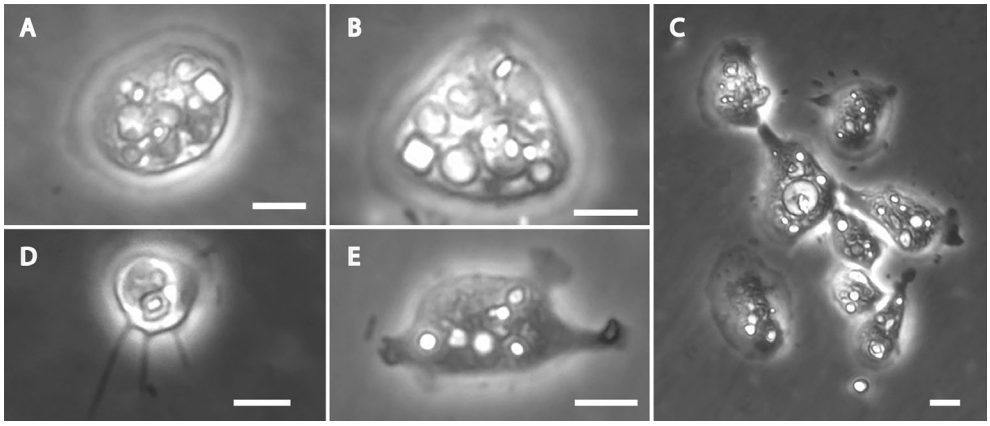

Figure 1 Light microscopic images of *Cochliopodium crystalli* n. sp. were taken on a glass slide without a coverslip. Scale bars = 10 μm. Round (**A**) and triangular (**B**) locomotive amoebas with smooth hyaloplasm and square and round (arrows) cytoplasmic inclusions; (**C**) aggregation of amoebae showing nondirectional movement in early stages of fusion; (**D**) floating amoeba with long, slender pseudopodia; (**E**) amoeba in nondirectional movement displaying multiple cytoplasmic projections.

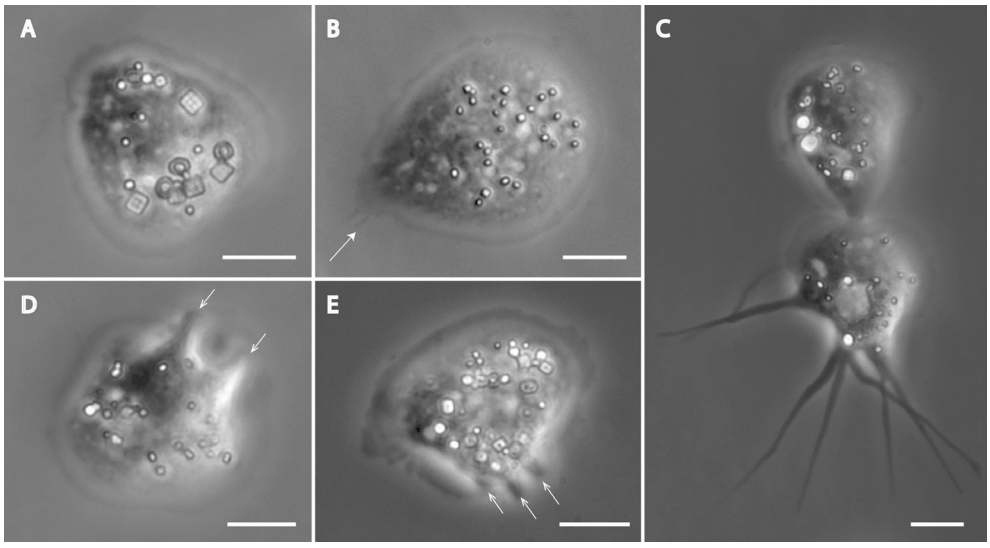

Figure 2 Light microscope images of *Cochliopodium jaguari* n. sp. "crystal-like UK-YT3" taken on a glass slide without a coverslip. Scale bars are 10 μm. Open and closed arrows show granuloplasmic extensions and subpseudopodial extensions of the hyaloplasm, respectively. (**A**) Cell showing spherical and square-shaped crystals; (**B**) cell in locomotion showing only spherical-shaped crystals with subpseudopodial extensions at the posterior end; (**C**) cell in locomotion with spherical and granular crystals, two trailing granuloplasmic extensions; (**D**) single cell in locomotion with square and spherical crystals and three trailing granuloplasmic extensions; (**E**) cells dividing and in the process of attaching to glass slide with long subpseudopodia.

with a mean length of 28 μm (13–45 μm, $n = 112$), a mean width of 27 μm (15–44 μm, $n = 112$) (Table S1), and a length-to-breadth ratio of 1.09 (0.32–2.38, $n = 112$). It moved at an average rate of 28 μm/min. During locomotion, the hyaline margin was smooth, and it did not display any noticeable emerging subpseudopodia (Fig. 1A, B). One or more adhesive uroids often formed, particularly during nondirectional locomotion (Fig. 1E). The floating form of the amoeba was rounded with few long, slender pseudopodia forming toward the distal end of the amoeba (Fig. 1D). The cytoplasm of *Cochliopodium* sp. "crystal UK-YT2" typically contained one or more (up to 8) large crystal-like inclusions or crystals (Fig. 1A–E). The inclusions were round or cubical in shape (Fig. 1A–C, E) and average 4.7 μm in size (1.5–11.3 μm, $n = 33$). In dense cultures, amoebae began to aggregate and fuse, as described in other *Cochliopodium* species (see Tekle et al. 2014) (Fig. 1C); however, fusion frequency was much lower than observed in other cochliopodiums (Tekle et al. 2014). Cysts were not observed in cultures.

Cochliopodium sp. "crystal-like UK-YT3"

Cochliopodium sp. "crystal-like UK-YT3" had a similar morphology in size and cell and crystal shape to *Cochliopodium*

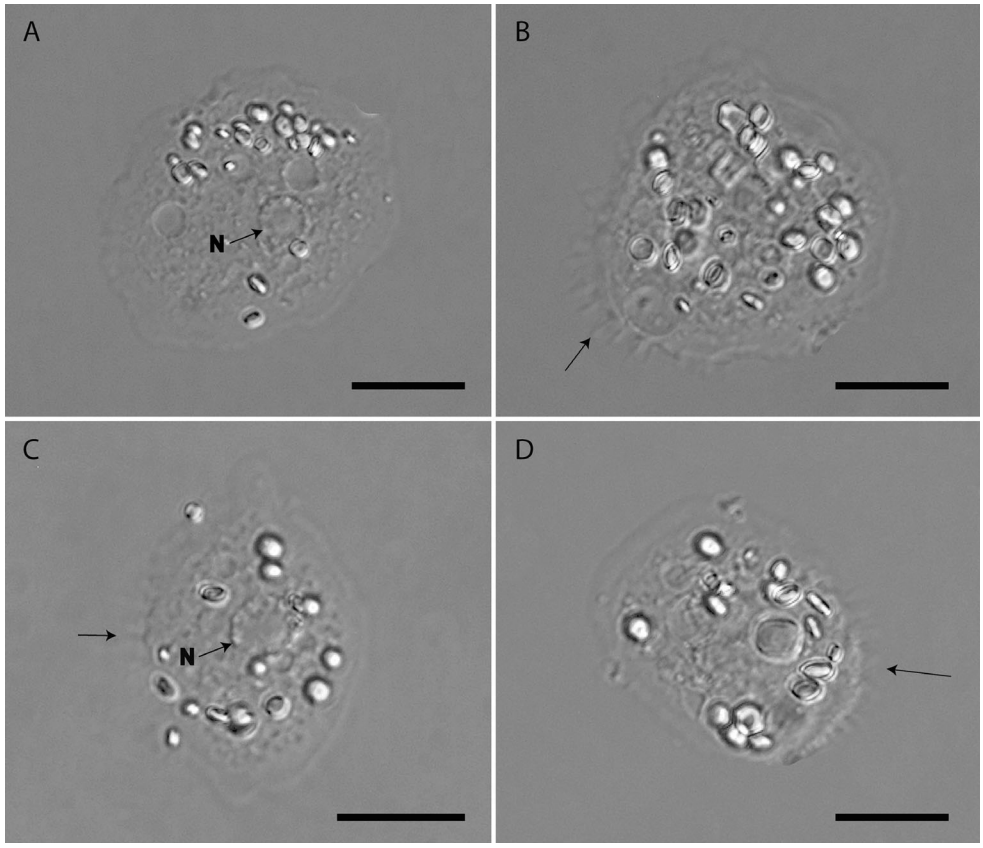

Figure 3 Light microscope imaging with DIC of *Cochliopodium jaguari* n. sp. ("crystal-like UK-YT3") taken on a glass slide without a coverslip. Scale bars are 10 μm. The nucleus is labeled with an "N." Closed arrows show subpseudopodial extensions of the hyaloplasm. (**A**) Nonmotile cell with a single vesicular nucleus, granular crystals, and several vacuoles located in the granuloplasm; (**B**) a cell in locomotion with short subpseudopodial extensions at the posterior end and is filled with spherical crystals and one cubical crystal; (**C**) cell in locomotion with a single vesicular nucleus and having short subpseudopodial extensions from the posterior end; (**D**) cell in locomotion with several large crystals and one large cubical crystal.

sp. "crystal UK-YT2" (Table S1). The "crystal-like UK-YT3" isolate was round to triangular in cell shape that was on average 31.9 μm in length (24.2–45.3 μm, $n = 100$) and 29.7 μm in width (20.3–44.2 μm) (Fig. 2A–E, 3A–D and Table S1). The length-to-breadth ratio ranged from 0.8 to 1.5 (average: 1.1). The hyaline border in "crystal-like UK-YT3" averaged 5.7 μm in width (range: 3.3–8.6 μm). The cells in locomotion sometimes had two to three granuloplasmic extensions or subpseudopodial extensions of the hyaloplasm to form a uroid (Fig. 2D, E; 3B–D). This isolate also contained cytoplasmic inclusions that were cubical or spherical like "crystal UK-YT2." However, the "crystal-like UK-YT3" isolate had more crystals (usually five to 20 and rarely up to 30) that were generally smaller in size than the "crystal UK-YT2" isolate. In "crystal-like UK-YT3," the square crystals ranged from 1.6 to 4.1 μm (average 2.6 μm) (Fig. 2A, 3B, D) (Table S1). The size and shape of the crystals appeared to depend on the age of the culture. Subcultures made from "crystal-like UK-YT3" with cubical crystals often lost their cubical crystals and only had spherical ones (e.g. Fig. 2B), but would usually regain the square crystals in approximately 1–2 wk (e.g. Fig. 2A). The nucleus of "crystal-like UK-YT3" averaged 4.6 μm (range: 4.1–5.4 μm) (Fig. 3A, C and Table S1).

Cochliopodium sp. "Marrs Spring UK-YT4"

Before being isolated into monoclonal culture, "Marrs Spring UK-YT4" was observed engulfing diatoms from the original mixed cultures. Amoebae grew at high densities on ATCC agar plates and Petri dishes with water and rice grains, especially around the rice grains where bacteria were abundant (Fig. 4H). The length of uninucleate cells of "Marrs Spring UK-YT4" in locomotion ranged from 28.0 to 64.1 μm (average 45.1 μm) and the width ranged from 31.1 to 58.9 μm (average 46.4 μm, $n = 100$) (Fig. 4A, B, 5A–D and Table S1). The average length-to-breadth ratio was 1.0, ranging from 0.7 to 1.7. The hyaline border was present around the whole cell and was up to 11.2 μm in width (average: 6.5 μm; range: 3.2–11.2 μm) (Fig. 4A, B, E, 5A–D and Table S1). A uroid was sometimes formed by one to two extensions of the granuloplasm at the posterior end of cells in locomotion (Fig. 4B, 5C). Lateral subpseudopodia were

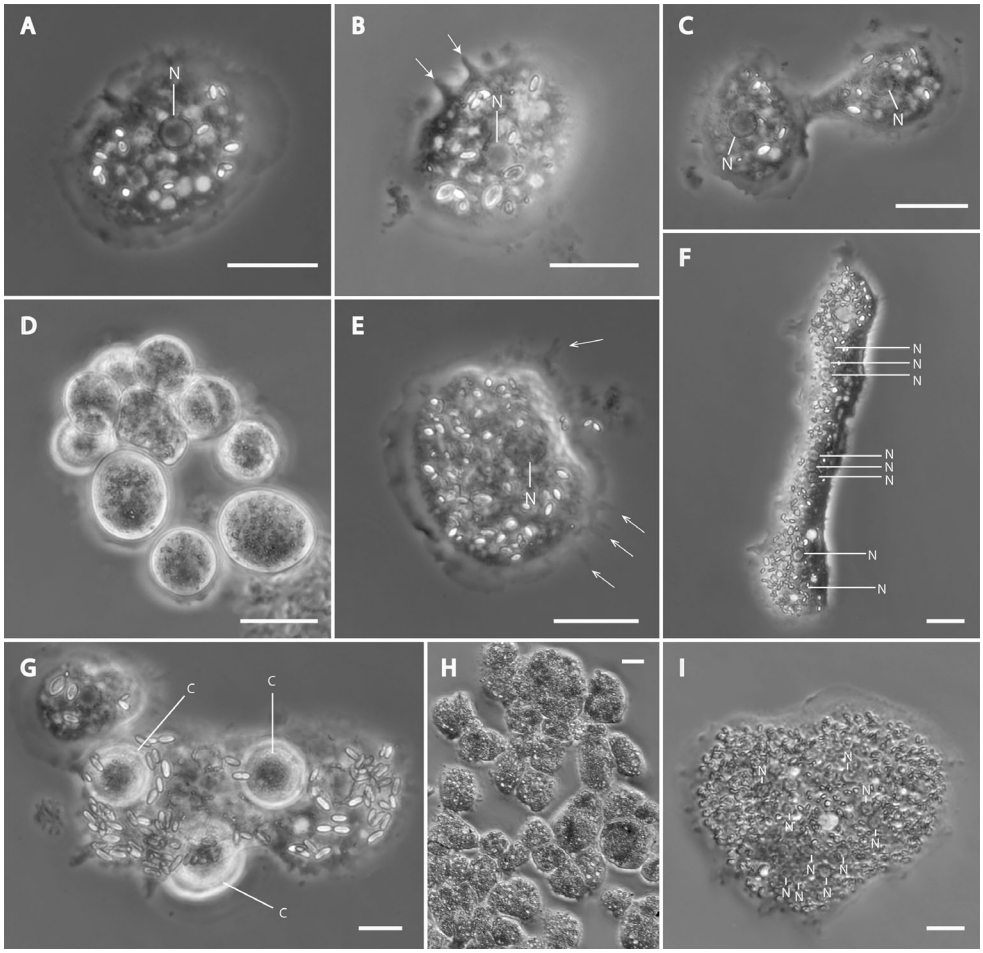

Figure 4 Light microscope images of *Cochliopodium marrii* n. sp. ("Marrs Spring UK-YT4") taken on a glass slide except "H," which was taken on a plastic Petri dish. Scale bars are 20 μm. Nuclei are labeled with an "N." Open and closed arrows show granuloplasmic extensions and subpseudopodial extensions of the hyaloplasm, respectively. (**A**) Cell showing ovoid crystals and a single vesicular nucleus; (**B**) cell in locomotion showing ovoid crystals, a single vesicular nucleus, and granuloplasmic extensions at posterior end; (**C**) cells in division with each part containing a single nucleus; (**D**) cysts; (**E**) cell with fused nucleus and subpseudopodial extensions on lateral sides; (**F**) fused cell containing at least seven nuclei; (**G**) a cell with two cysts inside the cell and engulfing a third cyst; cysts are labeled as "c"; (**H**) culture with a high density of amoebae; (**I**) a large plasmodial cell containing at least ten nuclei.

sometimes present in locomotive cells (Fig. 4E). The unfused cells contained a single vesicular nucleus that was visible under the light microscope (Fig. 4A, B, 5A–D). The average size of the nucleus was 7.6 μm, and it ranged from 6.4 to 8.6 μm (Table S1). Two to 50 crystals were present inside the cells and were ovoid or rice grain-shaped (Fig. 4A–C, E, F, 5A–D) to bipyramidal (Fig. 5D). The average size of the crystals was 4.9 μm and ranged from 2.2 to 10.0 μm (Table S1). Spherical or ovoid-shaped cysts typically formed within 1 wk and were 14–25 μm in diameter (Fig. 4D). Plasmodial cells of "Marrs Spring UK-YT4" sometimes appear to engulf these cysts (Fig. 4G). This isolate was noted for its rapid growth and fusion (Fig. 4E–I). Plasmodial cells grew up to 180 μm in size. These cells were frequently observed to have over five nuclei (Fig. 4F, I) and sometimes contained over 40 nuclei (see Confocal microscopy section).

Confocal microscopy

Immunocytochemistry data showed a dense fibrillary microtubule network throughout most of the cells in *Cochliopodium* spp. "crystal-like UK-YT3" (Fig. 6A, B) and "Marrs Spring UK-YT4" (Fig. 6C, D) with some fibers extending into the hyaloplasm. A prominent microtubule organizing center (MTOC) was only present in "crystal-like UK-YT3," which was similar to *Cochliopodium larifeili* and *Cochliopodium* sp. "crystal UK-YT2" (fig. 2G and H, respectively, in Tekle and Williams 2016). This MTOC was present in most of the cells and was typically located near the center of the cell and the nucleus. A prominent MTOC was absent in the majority of the cells of "Marrs Spring UK-YT4"; however, an MTOC-like structure was rarely present (Fig. 6C, D).

Most of the cells of *Cochliopodium* sp. "crystal-like UK-YT3" were uninucleate, and only a few cells were

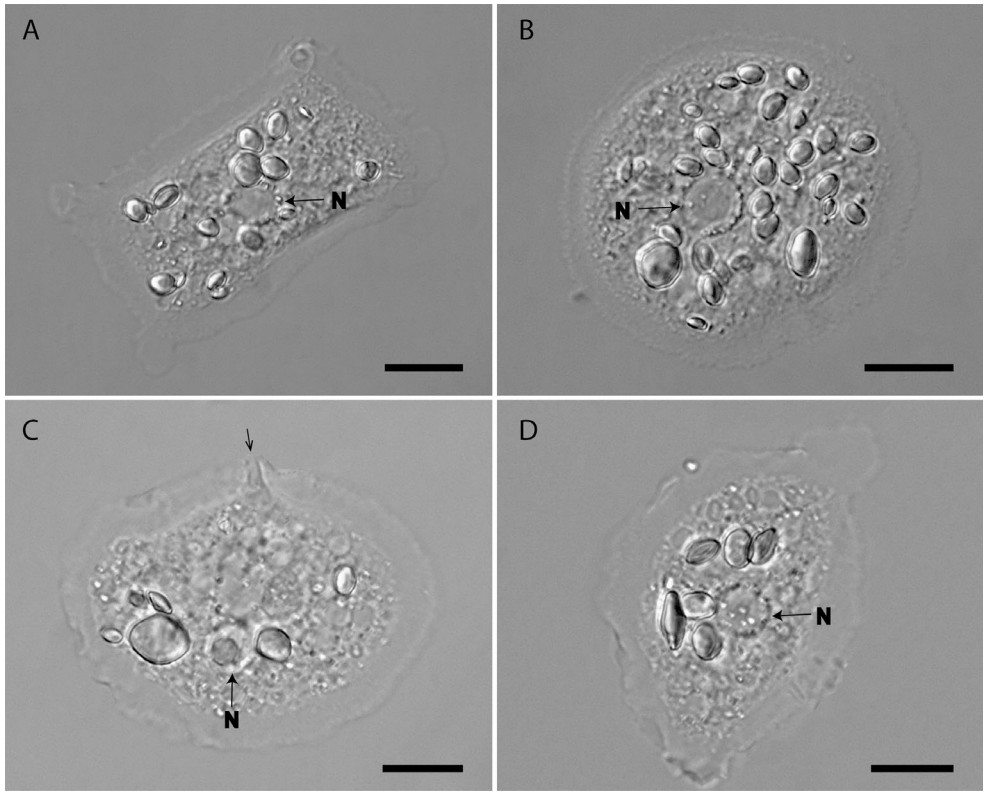

Figure 5 Light microscope imaging with DIC of *Cochliopodium marrii* n. sp. ("Marrs Spring UK-YT4") taken on a glass slide. Scale bars are 10 μm. The nucleus is labeled with an "n." Open arrows show granuloplasmic extensions. (**A**) Rectangular cell showing ovoid crystals, a single vesicular nucleus; (**B**) uninucleate cell not in motion; (**C**) cell in motion showing a granuloplasmic extension and many vacuoles present in the granuloplasm; (**D**) cell in locomotion with ovoid crystals and a single bipyramidal crystal.

binucleate (Fig. S1). It is unclear whether this was a result of cell fusion or a cell undergoing mitosis. Plasmogamy was not observed in these cultures.

Plasmodial cells of *Cochliopodium* sp. "Marrs Spring UK-YT4" were relatively large (up to 180 μm) and could contain over 40 nuclei (Fig. 7C, D). In fused cells, the microtubules were present throughout most of the cell (Fig. 7C, D), but some plasmodial cells displayed small pockets that mostly lacked microtubules but contained multiple nuclei (Fig. 7C, D). These pockets (approximately 20–25 μm in diameter) fall within the size range of the cysts. As previously noted, cells from the culture were observed engulfing cysts under the light microscope (Fig. 4G).

Molecular analysis

Pairwise comparison of COI sequence data of *Cochliopodium* spp. "crystal UK-YT2," "crystal-like UK-YT3," and "Marrs Spring UK-YT4" showed divergences in all cases exceeding 2%, a barcode cutoff (Tekle 2014), compared to any described *Cochliopodium* species (Table 1). The phylogenetic analysis showed that *Cochliopodium* spp. "crystal UK-YT2," "crystal-like UK-YT3," and "Marrs Spring UK-YT4" branched separately as independent lineages (Fig. 8).

Cochliopodium sp. "crystal UK-YT2" + (*C.* sp. "crystal-like UK-YT3" + *C. larifeili*) formed a strongly supported clade (93% bootstrap value) in the maximum-likelihood phylogenetic analysis (Fig. 8). Intrastrain variation among the seven clones of COI sequences of the *Cochliopodium* sp. "crystal UK-YT2" was 0.6%. These sequences were 11.5–12.0% and 11.7–12.1% divergent when compared to *Cochliopodium* sp. "crystal-like UK-YT3" and *C. larifeili*, respectively. All COI sequences from the "crystal-like UK-YT3" isolates (4, 5, and 10) were 100% identical to each other. The sister relationship of *C.* "crystal-like UK-YT3" and *C. larifeili* is without bootstrap support (Fig. 8); however, the COI sequences of these two species were the least divergent when comparing all of the sequence data (8.7% divergent; Table 1) from these three isolates with MTOC.

The COI sequence data of *Cochliopodium* sp. "Marrs Spring UK-YT4" and unidentified *Cochliopodium* sp. SG-2014 (KJ569724) clustered together in the phylogenetic tree with full support (Fig. 8). A pairwise analysis showed that these two sequences were 100% identical (Table 1), suggesting that these two isolates are conspecific. Both of these isolates formed a strongly supported (98% bootstrap) sister clade to *C. arabianum* + *C. actinophorum*. The closest COI sequences to "Marrs Spring UK-YT4"

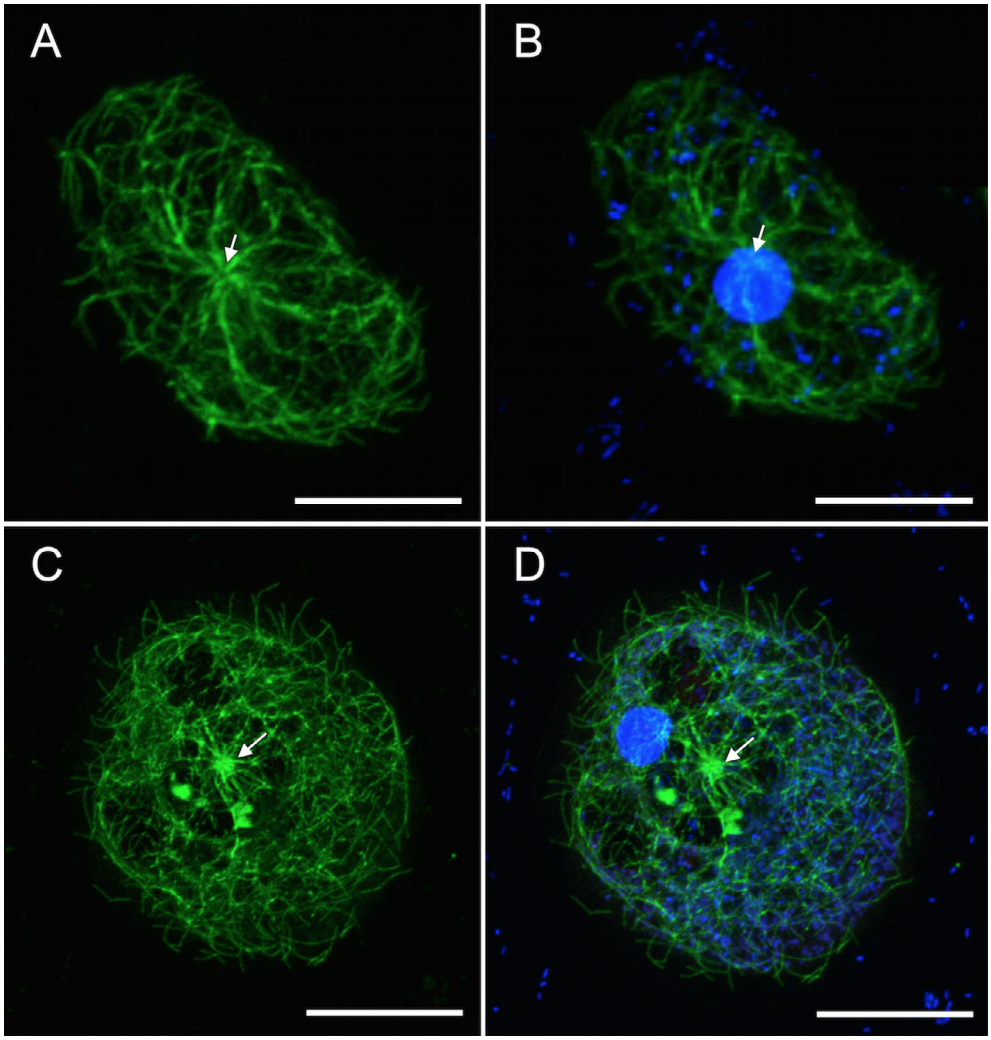

Figure 6 Confocal maximum-intensity projections of the microtubules (green) and DNA (blue; Fig. 6B, D). Scale bars are 10 μm for A, B and 20 μm for C, D. (**A** and **B**) Unfused cell of *Cochliopodium jaguari* n. sp. ("crystal-like UK-YT3"); (**C** and **D**) unfused cell of *Cochliopodium marrii* n. sp. ("Marrs Spring UK-YT4"). Arrows show the microtubule organizing center (MTOC) in A and B, and the MTOC-like organization in C and D.

were 7.9% and 9.4% divergent belonging to *C. arabianum* and *C. actinophorum*, respectively (Table 1).

DISCUSSION

Morphology-based classification challenges in *Cochliopodium*

Cochliopodium species are difficult to identify by morphological characteristics alone due to cryptic diversity and plasticity of some of the key characters. Morphological features (e.g. cell size, nuclear features, cytoplasmic inclusions, microscale morphology) used to circumscribe *Cochliopodium* species usually overlap among species and often cannot be used without molecular data to reliably identify these amoebae (see Fig. 9 and Table S1). For example, cell size can be a difficult diagnostic character to use due to the high variability observed within a population of the same species (Fig. 9, Tekle et al. 2014). A summary of average sizes (length and width) observed among different clades of *Cochliopodium* also show a range that overlaps with other described species (Fig. 9). While some amoebae such as *C. kieliense*, *C. minutoidum*, *C. plurinucleolum*, and *C. gallicum* are generally considered small amoebae (under 20 μm), they do not form a clade in phylogenetic analysis reflecting similarity in size (Fig. 9A, B). In addition to this, determination of cell size in *Cochliopodium* is confounded by the fusion behavior observed in the genus (Tekle et al. 2014).

Similarly, the size of the nucleus in *Cochliopodium* species ranges from as small as 2 μm in *C. gallicum* (Kudryavtsev and Smirnov 2006) and *C. maeoticum* (Kudryavtsev 2006) to as large as 15 μm in *C. gulosum* (Kudryavtsev 2000) and *C. vestitum* (Kudryavtsev 2005). Despite such variance, the reported nuclear size ranges still overlap among different described species of *Cochliopodium*

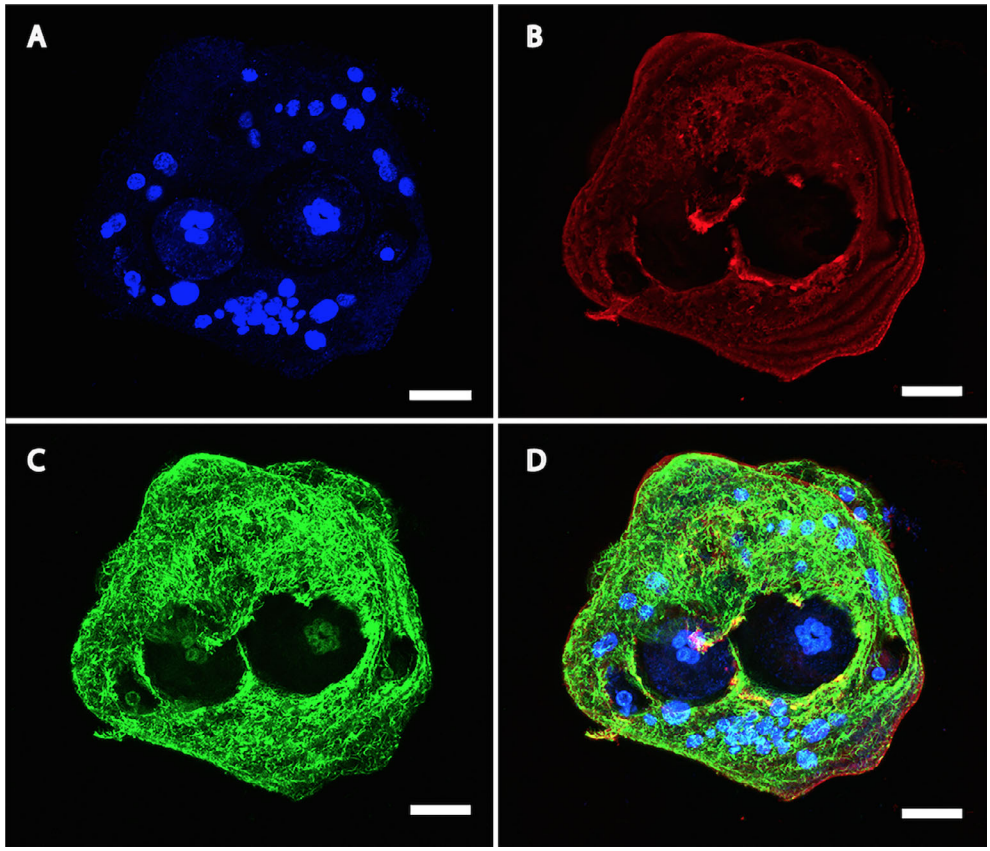

Figure 7 Confocal maximum-intensity projections of a fused cell of *Cochliopodium marrii* n. sp. ("Marrs Spring UK-YT4") containing over 40 nuclei. Scale bars are 20 μm. (**A**) DNA (blue); (**B**) plasma membrane (red); (**C**) microtubules (green); (**D**) overlapping image of DNA (blue), plasma membrane (red), and microtubules (green).

(Table S1) and is not shown to correlate with a phylogenetic tree based on molecular data (Fig. 8).

Crystalline inclusions are present in many genera of Amoebozoa (Bovee 1965; Griffin 1960; Grunbaum et al. 1959). Most *Cochliopodium* species have spherical, granular, ovoid, or bipyramidal cytoplasmic inclusions (crystals) (Table S1). Before this study, cubical-shaped crystals were only known in *C. larifeili* (Kudryavtsev 1999). Here, we identified two new *Cochliopodium* species with cubical-shaped crystals both of which form a clade with *C. larifeili* (Fig. 8). The cubical-shaped crystal might be a shared character in this clade, though more data is needed to confirm this observation. However, the cubical-shaped crystal can no longer be considered as a distinguishing character to identify *C. larifeili*. It is important to note that the cubical-shaped crystals were not permanent structures in "crystal-like UK-YT3" as these amoebae usually lose these types of crystals during subculturing. Some amoeba cells regained these crystals in approximately 1–2 wk of culturing. While the cubical crystals seem to be an important taxonomic feature, their observation requires careful examination of cultures in extended period of time.

The morphology of the microscales that make up the tectum was originally thought to be a delimiting character in the genus (Bark 1973; Kudryavtsev 2004, 2006; Tekle et al. 2013, 2015), but recent studies have questioned its diagnostic value (Geisen et al. 2014; Tekle and Wood 2018). For example, *C. pentatrifurcatum*, a species that was described in part due to the drastically different scale morphology (Tekle et al. 2013) from a closely related species, was recently synonymized under *C. minus* because it could not be separated genetically based on COI (Tekle 2014) and transcriptomic data (Tekle and Wood 2018). Conversely, species that can be separated based on SSU and COI sequence data have been shown to have highly similar scales such as the tower-like scales in *C. minus*, *C. plurinucleolum*, and *C. minutoidum* (Anderson and Tekle 2013; Geisen et al. 2014; Kudryavtsev 2006). Thus far, scale morphology has not been a reliable taxonomic feature to identify some species in this diverse genus, and therefore, it was not examined in this study.

The architecture of the cytoplasmic microtubules has previously been found to be useful character for grouping amoebae (Tekle and Williams 2016). This study reaffirms that the microtubule organization is conserved in certain clades of *Cochliopodium*. In our phylogenetic analysis "crystal UK-YT2" and "crystal-like UK-YT3" grouped with *C. larifeili*, which all have prominent MTOCs located near the center of the cell and typically near the nucleus (Tekle and Williams 2016). The only other *Cochliopodium* species

Table 1. Pairwise distance matrix (%) of the COI barcoding marker of *Cochliopodium crystalli* n. sp. (7 sequences), *C. jaguari* n. sp. (3 sequences), and *C. marrii* n. sp. (2 sequences including "SG-2014 KJ569724") compared to their closest known relatives, *C. larifeili* (1 sequence; KJ781466), *C. arabianum* (2 sequences; KJ781460–KJ781461), and *C. actinophorum* (5 sequences; KJ781462–KJ781465; KJ173779)

		1	2	3	4	5	6
1	*Cochliopodium crystalli* n. sp.	0.0–0.6	–	–	–	–	–
2	*Cochliopodium jaguari* n. sp.	11.5–12.0	0	–	–	–	–
3	*Cochliopodium larifeili* (KJ781466)	11.7–12.1	8.7	–	–	–	–
4	*Cochliopodium marrii* n. sp.	18.3–18.9	17.7	16.8	–	–	–
5	*Cochliopodium* sp. SG-2014 (KJ569724)	18.5–19.0	17.8	16.9	0	–	–
6	*Cochliopodium arabianum* clone YT177 (KJ781460)	19.5–20.1	19	18	7.9	8	–
7	*Cochliopodium actinophorum* strain CCAP 1537/10 (KJ173779)	19.2–19.7	19.4	19.8	9.4	8.8	8.8

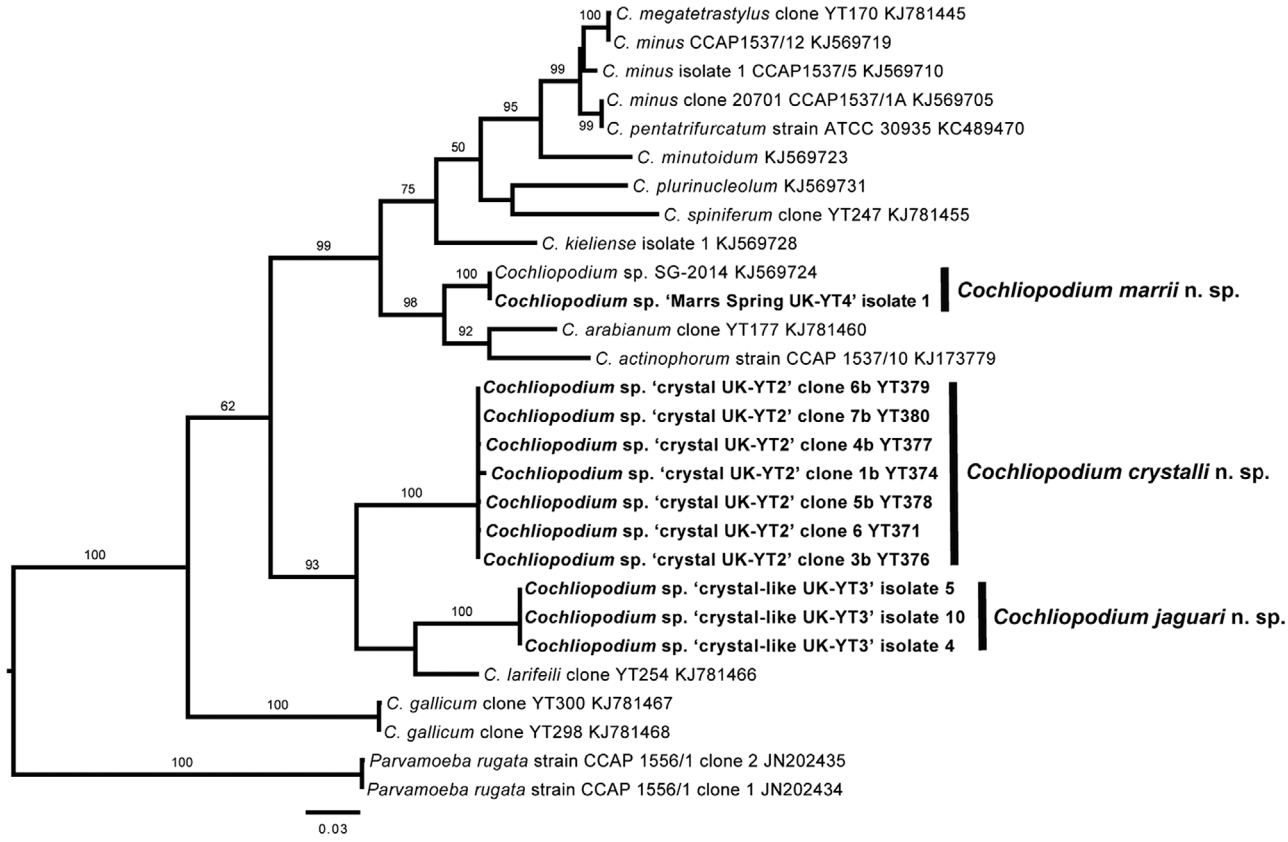

Figure 8 A maximum-likelihood phylogenetic analysis based on the mitochondrion-encoded barcoding marker COI. This analysis was run using the K2P model of nucleotide evolution and 1,000 bootstrap replicates in MEGA 7. Bootstrap values above 50% are shown. Sequences from this study are in bold. This analysis shows the phylogenetic positions of *Cochliopodium crystalli* n. sp. ("crystal UK-YT2"), *Cochliopodium jaguari* n. sp. ("crystal-like UK-YT3"), and *Cochliopodium marrii* n. sp. ("Marrs Spring UK-YT4").

that has a prominent MTOC similar to these three species is *C. gallicum* (Tekle and Williams 2016), a taxon with an ambiguous phylogenetic position. *Cochliopodium gallicum* branched at the base of the tree in this study but was previously reported to form a sister group relationship with *C. larifeili* (Tekle 2014). Variances in phylogenetic positioning of this taxon are likely due to taxon sampling or lack of resolution due to limited genetic signal. Hence, the phylogenetic signal of MTOC in these amoebae requires further analysis using more molecular data. Most other *Cochliopodium* spp. lack prominent MTOC including the new isolate "Marrs Spring UK-YT4" belonging to a clade containing *C. actinophorum* and *C. arabianum*, which all have dense microtubular networks (Tekle and Williams 2016).

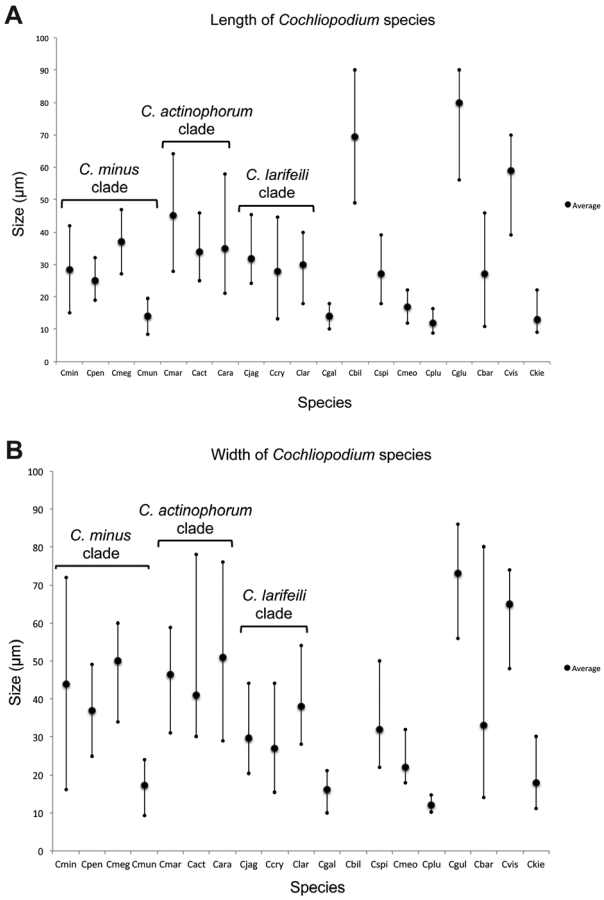

Figure 9 A comparison of the ranges and averages in size of the length (**A**) and width (**B**) of *Cochliopodium* species. The taxonomic names are abbreviated as follows: *C. minus* (Cmin), *C. "pentatrifurcatum"* (Cpen), *C. megatetrastylus* (Cmeg), *C. minutoidum* (Cmun), *C. marrii* n. sp. (Cmar), *C. actinophorum* (Cact), *C. arabianum* (Cara), *C. jaguari* n. sp. (Cjag), *C. crystalli* n. sp. (Ccry), *C. larifeili* (Clar), *C. gallicum* (Cgal), *C. bilimbosum* (Cbil), *C. spiniferum* (Cspi), *C. meoticum* (Cmeo), *C. plurinucleolum* (Cplu), *C. gulosum* (Cgul), *C. barki* (Cbar), *C. vestitum* (Cvis), and *C. kieliense* (Ckie).

Barcoding *Cochliopodium*

The mitochondrion-encoded COI gene has proven to be an important DNA barcode marker for taxonomic delimitation in Amoebozoa (Geisen et al. 2014; Nassonova et al. 2010; Tekle 2014). Particularly this marker has been quite helpful in uncovering cryptic diversity and resolving controversial species identification problems with unusual morphology. The *Cochliopodium* isolates in this study clearly represent three molecularly distinct species. The pairwise distances of the COI sequence data for each of these species were above the barcode cutoff value used in *Cochliopodium* spp. (Tekle 2014), and therefore warrant the description of *Cochliopodium* spp. "crystal UK-YT2," "crystal-like UK-YT3," and "Marrs Spring UK-YT4" as new species.

"Marrs Spring UK-YT4" was found to be genetically identical to a previously published COI sequence data. A *Cochliopodium* species designated as "SG-2014" (KJ569724) had 100% identical COI sequence to "Marrs Spring UK-YT4." The "SG-2014" isolate was sequenced from a DNA sample incorrectly labeled as *C. minus* CCAP 1537/1A at the University of Geneva, Geneva, Switzerland (Geisen et al. 2014). The SSU rDNA (JF298257) and actin gene (JF298270–JF298272) were also previously sequenced from this DNA sample (Kudryavtsev et al. 2011). Geisen et al. (2014) sequenced *C. minus* CCAP 1537/1A and found out that the COI sequence associated with *Cochliopodium* sp. SG-2014 was wrongly attributed to *C. minus* CCAP 1537/1A (Geisen et al. 2014). *Cochliopodium* sp. SG-2014 culture has been reported to be lost and is only known from stored DNA. Here, we isolated a genetically identical isolate to *Cochliopodium* sp. SG-2014 and provide a full description of this species based on molecular and morphological data. This isolate is unique from any described *Cochliopodium* species, and hence, we describe it as new species.

Here, we have described three new freshwater *Cochliopodium* species from the southeastern United States. Additionally, this study rectified the confusion of a mislabeled DNA sample of *C. minus* from the University of Geneva. Working with microscopic organisms such as *Cochliopodium* is difficult due to cryptic diversity, but molecular data such as those based on COI gene (Tekle 2014) or large-scale genomic data (Tekle and Wood 2018) will continue to unravel the hidden diversity of this genus.

TAXONOMIC APPENDIX BASED ON ADL ET AL. (2019)

AMORPHEA Adl et al. 2012
Amoebozoa Lühe 1913 emend. Cavalier-Smith 1998
Discosea Cavalier-Smith 2004, sensu Smirnov et al. 2011
Centramoebia Cavalier-Smith et al. 2016
Himatismenida Page 1987
Cochliopodium Hertwig et Lesser, 1874 sensu Bark, 1973

Cochliopodium crystalli Wood & Tekle n. sp.

Diagnosis. Amoebae with features of the genus, such as smooth hyaloplasmic margin surrounding a granular hump. The granuloplasm of the amoeba often contains one or more large crystalline inclusions, size 1.5–11.3 µm (mean 4.7 µm) and round or square in shape, which are characteristic for the species. During locomotion, the amoeba is oval to triangular in shape with a smooth hyaloplasmic margin which never shows emerging subpseudopodia, sometimes with an adhesive uroid. Length of locomotive form 13–45 µm (mean 28 µm), width 15–44 µm (mean 27 µm), and length–breadth ratio 0.32–2.38 (mean 1.09).

Etymology. The species name is derived from the large, crystal-like inclusions present in the granuloplasm of the amoeba.

Type locality. Arabia Lake, located in Lithonia, DeKalb County, GA, USA (N 33.6703869, W −84.1279724); elevation 232 m above sea level.

Habitat. Natural body of freshwater. The sample was taken from sediment in the littoral zone.
Type material. COI (accession number: MN389531–MN389537) sequences have been deposited in GenBank, and this amoeba is represented by light microscopic images in Fig. 1.
Differential Diagnosis. In size and shape of the locomotive form, this species is most similar to *C. jaguari* n. sp. and *C. larifeili* (Kudryavtsev 1999). However, the granuloplasmic crystals of *C. crystalli* n. sp. were fewer in number and much larger than those of *C. jaguari* n. sp. and *C. larifeili*, and posterior granuloplasmic projections during locomotion seen in *C. larifeili* and *C. jaguari* n. sp. were not observed in the new species. Molecularly, *C. crystalli* n. sp. is unique compared to the other *Cochliopodium* spp. with published genetic data. The cytochrome oxidase I (COI) sequence of this species has a divergence of 11.7–12.1% and 11.5–12.0% from its closest known relatives, *C. larifeili* and *C. jaguari* n. sp. (Table 1).

Cochliopodium jaguari Melton & Tekle n. sp.

Diagnosis. Cells round or triangular; lens-shaped. Cells range from 24.2 to 45.3 μm (average 31.9 μm) in length and 20.3–44.2 μm (average 29.7 μm) in width ($n = 100$). Average length-to-breadth ratio 1.1 (range: 0.8–1.5). Granuloplasm typically contains 5–20 (up to 30) granular, spherical (1–3 μm), or square-shaped crystals (1.6–4.1 μm). Cells surrounded by hyaloplasmic margins ranging from 3.3 to 8.6 μm. Uroid formed by two to three granuloplasmic extensions or subpseudopodial extensions of the hyaloplasm during locomotion. Nucleus ranging from 4.1 to 5.4 μm (average 4.6 μm). Nucleolus round and central under the light microscope. Cells typically uninucleate and sometimes binucleate. No fusion of cells observed. Most cells contain a prominent MTOC that is close to the nucleus.
Etymology. This species is named after the mascot of Spelman College, jaguars, and its rosetted skin spots reminiscent of the crystals found in the new species of amoeba.
Type Locality. Arabia Lake, Lithonia, GA, USA (33.671794, −84.127066); elevation 232 m above sea level.
Habitat. Natural body of freshwater. The sample was taken from sediment in the littoral zone.
Type material. A type culture will be kept in Tekle laboratory cryotank storage; COI GenBank Accession number (accession number: MN389538–MN389540)
Differential Diagnosis. *Cochliopodium jaguari* n. sp. most closely morphologically resembles *C. larifeili* and *C. crystalli* n. sp. in the cell shape and size, crystal shape, nucleus size, microtubule organization. This clade of three *Cochliopodium* spp. can be distinguished from other known *Cochliopodium* spp. by cubical crystals. While species in this clade can be difficult to identify on morphology alone, *C. jaguari* n. sp. and *C. larifeili* (Kudryavtsev 1999) typically have more crystals that are smaller in size compared to *C. crystalli* n. sp. Additionally, posterior granuloplasmic projections have been noted in *C. larifeili* (Kudryavtsev 1999) and *C. jaguari* n. sp., but were not observed in *C. crystalli* n. sp. A differential diagnosis can more easily be made with the COI barcoding marker. When compared to *C. larifeili* and *C. crystalli*, *C. jaguari* n. sp. was 8.7% and 11.5–12.0% divergent, respectively (Table 1).

Cochliopodium marrii Melton & Tekle n. sp.

Diagnosis. Cells round or oval; lens-shaped. Average length during locomotion 45.1 μm (range: 28.0–64.1 μm; $n = 100$); Average width during locomotion 46.4 μm (range: 31.1–58.9 μm; $n = 100$). Average length-to-breadth ratio 1.0 (range: 0.7–1.7). Cells with a single vesicular nucleus easily visible with light microscopy. Average nucleus size 7.6 μm (range: 6.4–8.6 μm; $n = 10$). Nucleolus round and central under the light microscope. Fusion of cells common and up to around 180 μm in size; over 40 nuclei can be present in a single fused cell. Amoebae containing a few to over 50 crystals in the granuloplasm from ovoid to bipyramidal in shape. Average size of the crystals was 4.9 μm (range: 2.2–10 μm; $n = 100$). Hyaloplasm present around the whole cell up to 11.2 μm (average 6.5 μm; range 3.2–11.2 μm; $n = 100$). Uroid formed by one to two granuloplasmic extensions sometimes present in locomotive cells. Spherical to ovoid cysts 14–25 μm in diameter. Fusion of cells is common. Dense microtubules. Most cells lack a clear MTOC; MTOC-like structure only sometimes present.
Etymology. This species is named after the type locality of "Marrs" Spring on the campus of The University of Alabama.
Type Locality. Marrs Spring located on the campus of The University of Alabama, Tuscaloosa, Alabama, USA (33.213613, −87.548335); elevation 61 m above sea level.
Habitat. Freshwater natural spring. The sample was taken from green algae floating near the top of the water.
Type material. A type culture will be kept in Tekle laboratory cryotank storage; COI GenBank Accession number (accession number: MN389530)
Differential Diagnosis. When comparing uninucleate locomotive cells, *Cochliopodium marrii* n. sp. is a medium sized species that falls within the size range of other closely related and described *Cochliopodium* species such as *C. actinophorum* (Kudryavtsev 2014) and *C. arabianum* (Tekle et al. 2014). This cell size alone can differentiate *C. marrii* n. sp. from the small *Cochliopodium* species < 20 μm (i.e. *C. gallicum*, *C. kieliense*, *C. maeoticum*, *C. minutoidum*, *C. plurinucleolum*) (Geisen et al. 2014; Kudryavtsev 2006; Kudryavtsev and Smirnov 2006) and large species such as *C. bilimbosum* (Sadakane et al. 1996) and *C. gulosum* (Kudryavtsev 2000) that can be up to 90 μm and *C. granulatum* that can reach 120 μm (Penard 1890, 1902). *C. marrii* n. sp. can more easily be distinguished from other *Cochliopodium* species by the COI barcoding marker. *C. marrii* n. sp. was 9.4% and 7.9% divergent from *C. actinophorum* and *C. arabianum*, respectively (Table 1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Confocal maximum-intensity projection of *Cochliopodium jaguari* sp. nov. ("crystal-like UK-YT3") displaying the DNA (blue) and the plasma membrane (red).
Table S1. A morphological comparison of *Cochliopodium* spp.