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Ptolemeba* n. gen., a Novel Genus of Hartmannellid Amoebae (Tubulinea, Amoebozoa); with an Emphasis on the Taxonomy of *Saccamoeba

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ABSTRACT

Hartmannellid amoebae are an unnatural assemblage of amoeboid organisms that are morphologically difficult to discern from one another. In molecular phylogenetic trees of the nuclear-encoded small subunit rDNA, they occupy at least five lineages within Tubulinea, a well-supported clade in Amoebozoa. The polyphyletic nature of the hartmannellids has led to many taxonomic problems, in particular paraphyletic genera. Recent taxonomic revisions have alleviated some of the problems. However, the genus *Saccamoeba* is paraphyletic and is still in need of revision as it currently occupies two distinct lineages. Here, we report a new clade on the tree of Tubulinea, which we infer represents a novel genus that we name *Ptolemeba* n. gen. This genus subsumes a clade of hartmannellid amoebae that were previously considered in the genus *Saccamoeba*, but whose mitochondrial morphology is distinct from *Saccamoeba*. In accordance with previous research, we formalize the clade as distinct from *Saccamoeba*. Transmission electron microscopy of our isolates illustrate that both molecularly discrete species can be further differentiated by their unique mitochondrial cristall morphology.

HARTMANNELLIDAE Volkonsky, 1931 is a family of limax-shaped amoebae within Tubulinea of Amoebozoa (Adl et al. 2012). The family classically includes the genera *Hartmannella*, *Cashia*, *Glaeseria*, and *Saccamoeba* (Page 1974, 1988). These genera are morphologically difficult to ascertain between taxa, and recent molecular data have proven that the lines between the genera are particularly skewed. The most challenging aspect is that hartmannellids look very similar to one another in terms of gross morphology. Generally, the trophozoites are elongate with a high length to breadth of ratio, typically > 4 (Page 1988). They most often move in a monotactic fashion, with non-monotactic pseudopodial extensions resulting in abrupt changes in direction that can be somewhat eruptive in fashion. Cells are uninucleate with a vesicular nucleolus. In addition, molecular phylogenetic trees have shown that even though the hartmannellids are superficially alike at the morphological level they are polyphyletic, comprising at least four different clades in nuclear-encoded small subunit rDNA (SSU rDNA) trees (Brown et al. 2011;

Dyková et al. 2008). The hartmannellids have recently been reviewed and redefined (Brown et al. 2011; Smirnov et al. 2011).

The two most speciose hartmannellid genera are *Hartmannella* and *Saccamoeba*; however, both genera appear to be paraphyletic in molecular phylogenetic reconstructions (Dyková et al. 2008). The fundamental difference between the two genera is that *Saccamoeba* lacks a distinctive hyaline cap on its monotactic pseudopodium, unlike the distinctive hyaline cap observed in *Hartmannella* (Page 1988). *Hartmannella* has been recently redefined according to molecular phylogenetics in Brown et al. (2011) and is beginning to be taxonomically resolved (Smirnov et al. 2011). However, *Saccamoeba* is still greatly in need of revision. The first molecular assignment of *Saccamoeba* was in 2000 from an isolate earlier identified at the American Type Culture Collection (ATCC) as *Saccamoeba limax* (ATCC 30942) (Amaral Zettler et al. 2000). Dykova et al. (2002) isolated an amoeba (strain LOS7N) that is nearly identical in SSU rDNA sequence

(99.58% uncorrected pairwise distance [upd]) to ATCC 30942, *S. limax* sensu (Amaral Zettler et al. 2000). They examined strain LOS7N under transmission electron microscopy (TEM) and found that it has unique tubular mitochondrial cristae that are arranged in an unusual helical pattern that is very uniform (Dyková et al. 2002). This cristal configuration is clearly dissimilar to previous TEM reports of *S. limax* sensu Page, 1985; which has branching tubular cristae that do not appear to have a uniform arrangement (Page 1985). Later, Dyková et al. (2008) isolated a new strain (NTSHR) assigned to *Saccamoeba* that appears to be very similar to Page's concept of *S. limax* in both light and TEM morphology. However, strain NTSHR branches distantly away from *S. limax* sensu Amaral Zettler et al. 2000 (Dyková et al. 2008). This led Dyková et al. to recognize NTSHR as a representative strain of *Saccamoeba* and that *S. limax* sensu Amaral Zettler et al. (2000) should be considered a distinct genus. However, they did not propose a name for that clade of strains. Although helical patterning of mitochondrial cristae is observed in the morphologically similar genus *Cashia* (Page, 1985) as noted by Dyková et al. 2008; the patterning of *Cashia* is much less pronounced and less uniform than in NTSHR (compare fig. 1E–H of Dyková et al. 2008 to fig. 27 and 28 of Page 1985).

Here, we present three novel isolates of freshwater/soil amoebae that were obtained from samples collected from eastern-central Mississippi. Our isolates have a general appearance of saccamoebids, with limax-type morphology and frequently lack a distinctive hyaline cap. We present morphological and ultrastructural data as well as an SSU rDNA phylogeny. One of our strains branches with the taxon "*Saccamoeba limax*" sensu Amaral Zettler et al. 2000 and LOS7N. Our other two strains are virtually identical to one another at the SSU rDNA level except for a 497-bp group I intron in strain Nx13-1c; together they branch as sister to the amoebae with helical cristae, i.e. ATCC 30942 and LOS7N.

MATERIALS AND METHODS

Isolation and culturing

Strains Nx13-1a and Nx13-1c were isolated from a water sample that was collected from Sam D. Hamilton Noxubee National Wildlife Refuge (33.273220°N, 88.790542°W) on September 2, 2013. The water sample was from water that was about 20-cm deep, with a mud sediment bottom. Sediment was also obtained during the water collection. Strain Sk13-4e was isolated from a soil sample that was collected in between the annex and lecture hall of Harned Hall on the Mississippi State University campus, Mississippi State, MS, USA (33.455790°N, 88.788123°W) on September 20, 2013. For the soil sample, a mud slurry was made with ~5 g of soil mixed with 15 ml of sterile ddH₂O. The slurry was then immediately used as an inoculum. For the samples (water and soil/water slurry), three water drops were placed onto the surface of three sterile wMY agar (0.002 yeast extract, 0.002 g malt extract,

0.75 g K₂HPO₄, 15.0 g agar, and 1 liter ddH₂O) Petri plates. The plates were then incubated for 4 d and observed with a 10X objective under a compound microscope. On the edges of the absorbed water droplet amoebae were observed. For all strains, a single amoeba cell was isolated using a flame sterilized 30-gage platinum wire micro loop, where the loop was used to drag a single amoeba along the agar surface to isolate it away from all other eukaryotes on the inoculated plate. Each culture was established as clonal with no other accompanying eukaryotes. Cultures are maintained through monthly passage onto fresh wMY plates inoculated with a streak of *Escherichia coli* (strain K-12 MG1655) as a food source. Cultures Nx13-1a and SK13-4e have been deposited with the ATCC under accession PRA-414 and PRA-415, respectively.

Light microscopy

All light micrographs were taken using a Zeiss AxioSkop 2 (Carl Zeiss Microimaging, Thornwood, NY) connected to an 18-megapixel Canon 650D color digital camera (Canon, Melville, NY). Image capture was performed by use of Canon's image capture software (EOS Utility; Canon). Observations and photomicrographs using differential interference contrast optics were made by inoculating a drop of wMY liquid media with amoebae taken from agar plates onto glass slides covered with a coverslip. Measurements were taken using the measurement function of ImageJ (Schneider et al. 2012).

DNA extraction

For DNA isolation, Nx13-1a, Nx13-1c, and Sk13-4e were cultured with *E. coli* (strain K-12 MG1655) on wMY agar plates at room temperature (ca. 21 °C). DNA was extracted from two plates of dense growth amoebae using a modified salt extraction method (Aljanabi and Martinez 1997) for use as a template in polymerase chain reaction (PCR) amplifications. Briefly, cells were resuspended in 5-ml liquid wMY and collected in a 15-ml conical tube. The cells were pelleted by centrifugation at 3184 g for 2 min, and the cell pellet was suspended in 200 µl of salt homogenizing buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0 and 2 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0). Then proteinase K (20 mg/ml) was added to the solution to a final concentration of 400 µg/ml and sodium dodecyl sulfate was added to final concentration of 2%. The sample was mixed by gentle inverting and then incubated at 56 °C for 1 h. The sample was incubated on ice for 5 min followed by the addition of one volume of 5M NaCl [2.5 M]. The sample was then vortexed lightly and spun 14,000 g at 4 °C for 30 min. The supernatant was moved to a fresh tube and centrifuged again to pellet residual precipitant (14,000 g at 4 °C for 10 min). One volume of 100% isopropanol was added to each supernatant and inverted to mix. The tubes were incubated at –20 °C for 1 h and then spun at 14,000 g at 4 °C for 30 min to precipitate the nucleic acid. To the

pellet, 1 ml of 75% Ethanol was added and spun at as above for 10 min. After drying, the pellet was rehydrated in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

SSU rDNA sequencing

The SSU rDNA from Nx13-1a and Nx13-1c was amplified using eukaryotic primers S1 (5'-AACCTGGTTGATCCTGCC-3'), and RibB (5'-GATCCTTCTGCAGGTTACC-3') (Fiore-Donno et al. 2008), GoTaq Green Master Mix (Promega, Madison, WI) following the manufacturer's instructions, and the following cycling parameters: 45 s initial denaturation at 94 °C, followed by 33 cycles of 25 s denaturation at 94 °C, 60 s annealing at 42 °C, and polymerization at 72 °C for 3.5 min. The PCR reactions were analyzed by electrophoresis on 1% agarose gels using Tris-acetate (TA) gel and running buffer (4.84 Tris-base, 1.142 ml Glacial Acetic Acid, filled to 1 liter with ddH₂O). The SSU rDNA of Sk13-4e was amplified as above except with the primers 5'17!SSU (5'-CTGGTTGATCCTGCCAG-3') (Shadwick et al. 2009) and RiBb. For Nx13-1a and Nx13-1c, a 1:100 dilution of the primary PCR reaction was used as a template for nested secondary PCRs using the primer pair 30F (5'-AAAGATTAAGCCATGCATG-3') (Shadwick et al. 2009) and 1492R (5'-ACCTGTTCAGACTT-3') (Lane 1991) and the above cycling parameters. For all strains, the SSU rDNA band (~1,800–2,100 bp) was cut out of the gel under a UV-transilluminator and purified using a 200- μ l barrier pipette tip trimmed to fit into a 1.5-ml microfuge tube. The agarose gel slice containing DNA amplicon was placed on top of the filter in the barrier pipette tip; the pipette tip was then put into a 1.5-ml tube and centrifuged at 17,900 *g* for 10 min to extract the liquid containing the purified PCR product. By using TA as the gel buffer, which does not contain EDTA, the product was directly Sanger sequenced. The PCR products were completely sequenced using the PCR primers and internal sequencing primers. Microheterogeneity was assessed by the presence of mixed peaks in chromatograms. The SSU rDNA sequences are deposited in GenBank, accession numbers KJ542108–KJ542110.

Group I intron prediction

A Group I catalytic intron in the SSU rDNA of *Ptolemeba noxubium* (isolate Nx13-1c) was first identified as a 497-bp insertion in our SSU rDNA alignment. This intron corresponds to position S516 in the *E. coli* 16s rRNA (GenBank accession AB03592), nomenclature of (Johansen and Haugen 2001). The S516 intron was searched for similarity of predicted secondary structure to other Group I Intron sequences using the covariance model (CM) in the package Infernal v1.1 (<http://infernal.janelia.org/>) and a seed alignments from various subgroups of introns from Group I Intron Sequence and Structure Database, the seed alignment of (<http://www.rna.whu.edu.cn/data/alignment/>) (Zhou et al. 2008). The secondary structure was further predicted using the RNAfold server (<http://rna.tbi.univie.ac.at/>) (Mathews et al. 2004) and is presented in Fig. S1.

Molecular phylogenetics

Twenty-nine publicly available SSU rDNA sequences were included with our novel sequences in our phylogenetic analyses. Our new sequences were manually added to an existing seed alignment using Mesquite v2.75 (<http://mesquiteproject.org/>), which was rich in Tubulinea, from Brown et al. (2011). Trees were inferred from an inclusion set of 1,258 unambiguously aligned nucleotide positions using maximum likelihood (ML) and Bayesian inference (BI) methods. A general-time-reversible + gamma distribution + estimation of the proportion of invariant sites (GTR+ Γ +I) was implemented as suggested by the Akaike Information Criterion in ModelTest v3.7 (Posada and Crandall 1998). The ML tree was estimated from 300 tree searches implemented in RAxML v8.0 using 25 discrete gamma rate categories (Stamatakis 2014). Topological support was assessed by ML analyses of 1,000 bootstrap replicates (MLBS) in RAxML. For BI, two independent runs consisting of four Markov Chain Monte Carlo were run in MrBayes v3.12 (Ronquist and Huelsenbeck 2003) for 10,000,000 generations, sampling trees every 100 generations. The "burnin" was set to 2,500,000 generations, by which time all parameters converged as assessed by the "sump" function of MrBayes, an average standard split deviation of < 0.0023, and the potential scale reduction factor convergence diagnostic.

Transmission electron microscopy

For TEM, cells were grown on wMY plates and were harvested into a 15-ml conical tube in the same fashion as DNA. The cells were centrifuged at 4,360 *g* for 2 min. The supernatant was poured off and the cell pellet suspended in a cocktail of culture media containing 2.5% v/v glutaraldehyde and 1% OsO₄ and fixed for 30 min on ice. The fixed cells were then centrifuged at 4360 *g* for 1 min and the supernatant was removed. Cells were then washed once in liquid wMY medium. This process was repeated twice more, with H₂O washes. Cells were again concentrated by centrifugation, and then enrobed in 2.0% (w/v) agarose. Agarose blocks were dehydrated in a graded series of ethanols up to absolute ethanol, and then embedded in SPI-Pon 812 Epoxy resin (SPI Supplies, West Chester, PA). Sections (50 nm) were cut with a diamond knife on a Reichert-Jung Ultracut E Ultramicrotome, mounted on pioloform film in grids using the technique of Rowley and Moran (1975), and were subsequently stained with saturated uranyl acetate in 50% ethanol and with lead citrate. Sections were observed using a JEOL 2100 200 kV TEM electron microscope (JEOL USA, Peabody, MA).

RESULTS

Light microscopy and TEM

Amoebae of Sk13-4e were predominantly monopodial li-max (slug shaped) in appearance (Fig. 1). The lengths of



Figure 1 Differential interference contrast light photomicrographs of *Ptolemeba bulliensis* n. gen. n. sp. amoebae, strain Sk13-4e. Scale bar = 10 μ m.

Sk13-4e amoebae were 17.1–37.2 μ m (mean = 25.6 μ m, SD = 4.2, n = 52), with breadths of 3.5–8.1 μ m (mean = 5.5 μ m, SD = 1.1, n = 52). The average length to breadth ratio is 4.7 (Fig. 1). Cells possess a single nucleus that is 2.4–4.8 μ m in diam. (mean = 3.4 μ m, SD = 0.57, n = 44) with a prominent nucleolus that is 2.7–1.2 μ m (mean = 1.9 μ m, SD = 0.37, n = 44) in diam. (Fig. 1A, B, E, G, H). Locomotive amoebae move in a monotactic fashion and can abruptly change direction with semi-eruptive pseudopodial extensions (Video S1). In locomotive forms, there is often a prominent hyaline cap at the anterior end. This hyaline cap may however be absent (Video S1). On the posterior end, there is a villous-bulbous uroid (Fig. 1) (Video S1). Over 6 mo of culture, no cysts were observed.

Amoebae of Nx13-1a and Nx13-1c were predominantly monopodial limax in appearance (Fig. 2). The lengths of the amoebae were 11.9–29.8 μ m (mean = 18.8 μ m, SD = 3.62, n = 40), with breadths of 4.3–8.1 μ m (mean = 6.0 μ m, SD = 0.96, n = 40). The mean length to breadth ratio is 3.2 (Fig. 2). Cells possess a single nucleus

that is 2.6–5.5 μ m in diam. (mean = 3.9 μ m, SD = 0.73, n = 22) with a prominent nucleolus that is 1.1–3.6 μ m (mean = 2.3 μ m, SD = 0.66, n = 22) in diam. (Fig. 2A, B). There is sometimes a thin crescent-shaped hyaline cap on the anterior end of amoebae, and on their posterior, there is a morulate-bulbous uroid (Fig. 2). Locomotive amoebae move in a monotactic fashion and can abruptly change direction with semi-eruptive pseudopodial extensions (Video S2). In locomotive forms, there is often a prominent hyaline cap at the anterior end (Video S2). Over 7 mo of culture, no cysts were observed.

The mitochondrial cristal configuration varies between strains Sk13-4e and Nx13-1a. Both strains have tubular mitochondrial cristae, but Sk13-4e has a unique morphology where the tubular cristae are arranged in a uniform fashion that appears to be a helical patterning (Fig. 3A, S3). Strain Nx13-1a is much less uniform, with tubular cristae that are not arranged in a helical pattern (Fig. 3B). Additional TEM micrographs of both taxa are available in Fig. S2–S5.

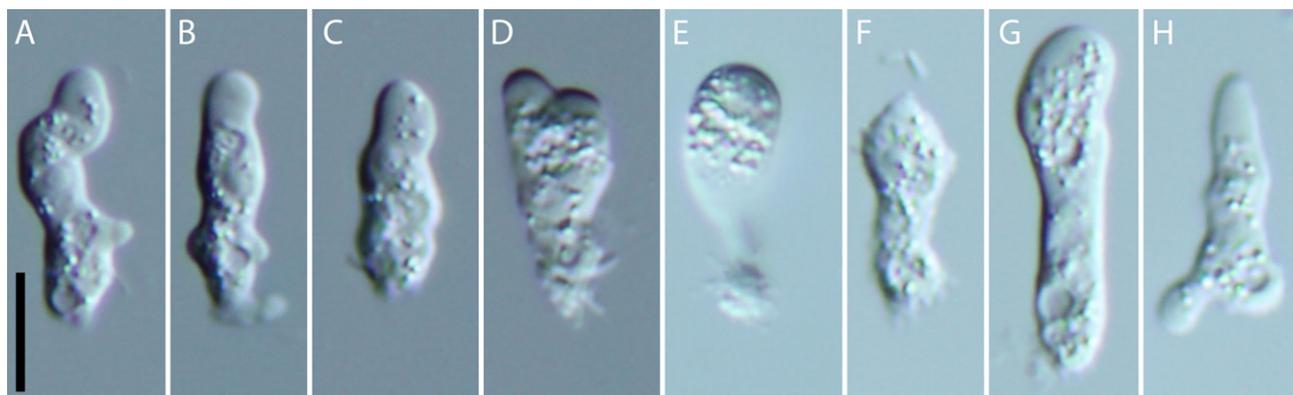


Figure 2 Differential interference contrast light photomicrographs *Ptolemeba noxubium* n. gen. n. sp. amoebae, strains Nx13-1a (A–D) and Nx13-1c (E–H). Scale bar = 10 μ m.

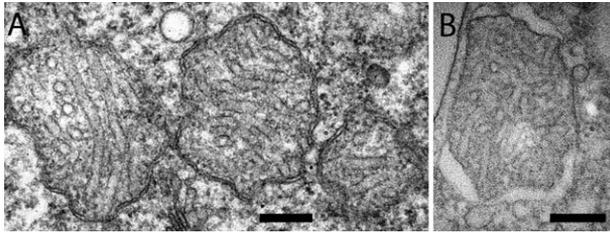


Figure 3 Transmission electron micrographs of mitochondria in (A) *Ptolemeba bulliensis* n. gen. n. sp. strain Sk13-4e and (B) *Ptolemeba noxubium* n. gen. n. sp. strain Nx13-1a. Scale bar = 200 nm.

SSU rDNA data and phylogenetic analyses

The SSU rDNA sequences obtained from our new strains are 1,727, 2,226, and 1,927 bp, for Nx13-1a, Nx13-1c, and Sk13-4e, respectively. The 497 bp difference between strains Nx13-1c and Nx13-1a is attributed to the group I intron present in Nx13-1c (see below). Besides the group I intron, these strains differ by 2 bp (upd 0.00119, Table 1). Phylogenetic analyses of SSU rDNA data (Fig. 4) show that strain Sk13-4e branches with full support with three other publicly available SSU rDNA sequences attributed to *Saccamoeba* (Amaral Zettler et al. 2000). Strain Sk13-4e is highly similar to sequences AF293902 (strain ATCC 30942) and AY145442 (strain LOS7N), with an upd of 0.00645 and 0.00822, respectively, based on an alignment of 1,716 bp. These sequences are very similar to the SSU rDNA sequence GU5690162 (strain T2), with an average upd of 0.07. However, data from strain T2 is only available as a truncated SSU rDNA sequence. Strains Nx13-1a and Nx13-1c are fully supported as the sister to the clade of Sk13-4e + ATCC 30042 + LOS7N + T2 (Fig. 4). The maximum upd in the clade is between Nx13-1c and T2, with an upd of 0.1977. The average upd between Sk13-4e and strains Nx13-1a and Nx13-1c is 0.136 (Table 1). Our phylogenetic results show that these taxa branch robustly within the Tubulinea clade (Fig. 4) and with high support (96% MLBS/1.0 BI) as sister to a clade of *Amoeba* + *Chaos* + *Copromyxa* + *Saccamoeba* sensu stricto + *Glaeseria* (Fig. 4). Our phylogenetic analyses show that the clade that our strains are in is unique from the *Saccamoeba* clade, which is also fully supported (Fig. 4).

Group I Intron in Nx13-1c

As noted above, strain Nx13-1c has a 497-bp insertion that is unique from its sister strain Nx13-1a. The insertion sequence was searched for RNA secondary structural homology using a CM in Infernal, which yielded an expected value of $4.8e-37$ to the IE3 subgroup of group I introns, suggesting that the secondary folding motif is highly similar to these introns. Subsequently, the secondary structure predicted in RNAfold of this group I intron is available in Fig. S1.

DISCUSSION

Trophozoites of the Hartmannellidae have a limax morphology that most often move in a monoaxial fashion (Page 1988). Depending on the genus, they may or may not have a distinct hyaline cap or uroid and their nucleus morphology is always vesicular (Page 1985). The Hartmannellidae is historically a problematic taxonomic group within the Tubulinea; because, their generic features make them difficult to assign a genus. *Saccamoeba* is a prime example of the problematic nature of hartmannellid taxonomy (Smirnov et al. 2011).

Saccamoeba is a genus of amoebae found mainly in freshwater and soil. The amoebae are characterized by having a thin hyaline cap at the leading edge of an actively moving trophozoite. *Saccamoeba* was first described in 1897 (Frenzel 1897), and later emended (Bovee 1972). In 1974, Page reevaluated the status of *Saccamoeba* noting that the original diagnosis of *Saccamoeba* "was so vague as to be of little use," because the drawing could be construed as any number of amoeboid taxa. Nonetheless, our current concept of *Saccamoeba* is most adequately diagnosed in Bovee 1972 (Page 1974). The genus consists of amoebae that often lack a hyaline cap, and have villous-knob uroid and a vesicular nucleolus. Bovee considered the type species of the genus to be *S. lucens* (Bovee 1972; Page 1974). Of the available data on isolates attributed to the genus *Saccamoeba*, *S. limax* is probably the most robust and therefore, stable (Page 1974). In accordance with the well-discussed argument in Dyková et al. 2008; we recognize *S. limax* strain NTSHR (EU869301) as an exemplar strain of the genus *Saccamoeba*. Briefly, NTSHR has tubular mitochondrial

Table 1. Uncorrected pairwise distances between SSU rDNA sequences of the genus *Ptolemeba*

Taxon	Strain	Nx13-1a	Nx13-1c	Sk13-4e	30942	LOS7N
<i>P. noxubium</i>	Nx13-1a	–				
<i>P. noxubium</i>	Nx13-1c	0.00119	–			
<i>P. bulliensis</i>	Sk13-4e	0.13675	0.13555	–		
<i>P. bulliensis</i>	30942	0.13615	0.13615	0.00645	–	
<i>P. bulliensis</i>	LOS7N	0.13793	0.13794	0.00822	0.00411	–
<i>P. sp.</i>	T2	0.19767	0.19770	0.06595	0.07442	0.07314

Pairwise distances among the coding regions of the SSU rDNA sequences were computed using an inclusion set of 1,716 nucleotide characters except for *Ptolemeba* sp., strain T2 where only a truncated sequence is available for analyses.

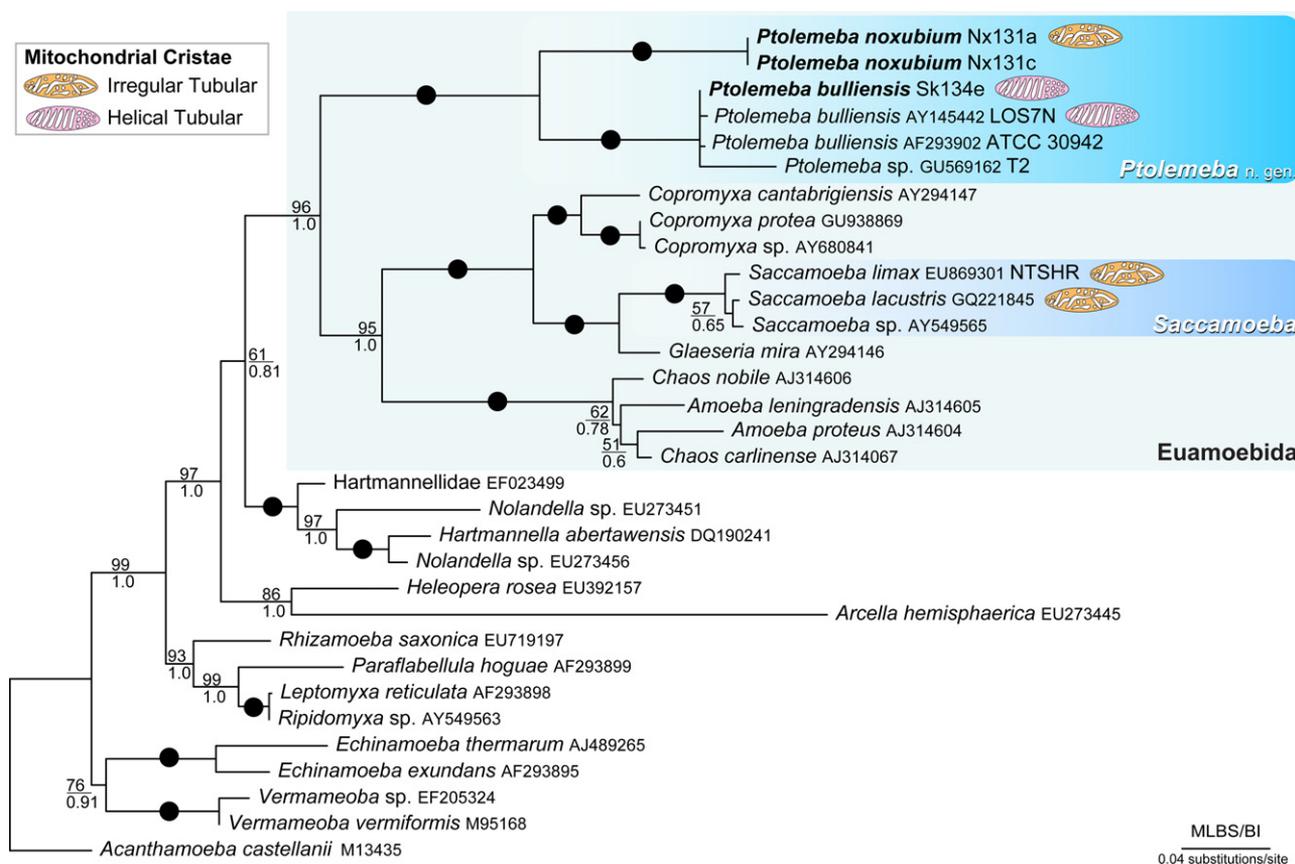


Figure 4 Maximum likelihood tree of 32 SSU rDNA sequences inferred under the GTR+I+Γ model inferred in RAxML. Support values at each node are presented for RAxML ML bootstrapping and Bayesian Inference posterior probabilities (MLBS/BI). Bootstrap values and BI posterior probabilities of 100%/1.0, respectively, are represented by a black dot. Support values less than 50% or 0.50 for the respective method are not presented. GenBank accession numbers of SSU sequences used in analyses are listed next to the taxon names. The scale bar represents evolutionary distance in changes per site. Cartoon schematics of mitochondrial cristal configurations for important taxa to this study are shown next to the taxa names.

cristae that are irregularly patterned, whereas other strains attributed to *Saccamoeba* (i.e. LOS7N, sister to ATCC 30942) has tubular cristae arranged in a helical pattern (Dyková et al. 2002). Further, given the size measurements of NTSHR, the isolate fits within the morphological species concept of *S. limax* (Page 1974) and should be treated as such (Dyková et al. 2008). Moreover, the most recently described species of *Saccamoeba*, *S. lacustris*, falls within a clade with *S. limax* NTSHR (Corsaro et al. 2010). Taking these points into account, the most appropriate taxonomic action is to accept the genus concept of *Saccamoeba* sensu Dyková et al. (2008); and provide a new genus concept for the clade of other amoebae previously recognized as *Saccamoeba* in SSU rDNA sequences, such as ATCC 30942 of Amaral Zettler et al. (2000). Here, we name that clade as the new genus *Ptolemeba* n. gen., which herein contains two described species.

Cashia and *Glaeseria* are other hartmannellid amoeba genus that are morphologically similar to our *Ptolemeba* strains and *Saccamoeba* (Page 1985, 1988). For example, both *Cashia* and *Glaeseria* often lack a conspicuous hyaline cap similar to *Ptolemeba* and *Saccamoeba*. *Ptolemeba*

is distinct from *Glaeseria* at the molecular level (Fig. 4) as well as *Glaeseria* forms cyts. Unlike *Glaeseria* and *Saccamoeba*, there are no molecular data attributed to *Cashia*. However, our results suggest that *Ptolemeba* is a distinct genus from *Cashia*. *Cashia* is reported to have unbranched helical mitochondrial cristae. Although our isolate Sk13-4e has unbranched tubular mitochondrial cristae arranged in a uniform configuration like that of LOS7N (Dyková et al. 2008), this configuration is dissimilar in pattern to that of *Cashia* (see fig. 27 and 28 of Page 1985). Furthermore, *Cashia* does not form a villous-knob uroid structure (Page 1988). Our strains Sk13-4e, Nx13-1a, and Nx13-1c, as well as LOS7N, almost always have a conspicuous villous knob (Fig. 1, 2) (also see fig. 1B of Dyková et al. 2002).

Although the various strains of *Ptolemeba* represent significant genetic distances at the SSU rDNA level (average intrageneric upd of 0.13), they form a fully supported clade given the SSU rDNA data currently available (Fig. 4). Genetic distances in other genera have been reported to be on the magnitude of our genus concept of *Ptolemeba*; for example, the heterolobosean genus *Acrasis* has upd of greater than 0.15 (Brown et al. 2012). Moreover, these

strains of *Ptolemeba* are morphologically similar at the light microscopic level. Therefore, for name stability, we have decided to consider these taxa the same genus. Our results suggest that there are at least two species, in the genus *Ptolemeba*, *Ptolemeba bulliensis* n. sp. and *P. noxubium* n. sp. The unpublished strain T2 (GU569162) probably represents another species, but unfortunately only a truncated portion of the SSU rDNA sequence (241 bp) is available and the ends are somewhat divergent from the other *Ptolemeba* species, which may be the result of sequencing error. However, *Ptolemeba* sp. strain T2 is almost certainly a unique species and should be analyzed with full length SSU rDNA sequence data and morphological characterization. In addition, it would be interesting to examine the mitochondrial cristal configuration to see if it also possesses helical patterning, like *P. bulliensis*. It should be noted that mitochondrial cristae might be a variable character in the hartmannellid amoebae. It is variable in the closely related euamoebid, *Chaos carolinense*, in which the cristal morphologies changed from unbranched tubular to a cubic morphology that could be interpreted as helical under starvation conditions (Deng et al. 1999). More studies on cristal variability should be conducted with the hartmannellid amoebae to test for variability in this character.

Our data provide taxonomic clarification of the hartmannellid genus *Saccamoeba*, which was previously shown to be paraphyletic (Brown et al. 2011; Corsaro et al. 2010; Dyková et al. 2008; Smirnov et al. 2011). The data presented warrant the description a new genus that is morphologically similar to *Saccamoeba*, but branches as sister to a clade that contains *Saccamoeba* sensu stricto in addition to other euamoebids (*Amoeba*, *Chaos*, *Copromyxa*, and *Glaeseria*). This study further illustrates the necessity of molecular data in combination with morphological data to properly resolve taxonomic issues with amoeboid protists.

TAXONOMIC SUMMARY

Amoebozoa Luhe 1913, emend. Cavalier-Smith 1998
Tubulinea Smirnov et al. 2005
Euamoebida Lepsi 1960, emend. Smirnov et al. 2011

Ptolemeba Brown et al. n. gen

Diagnosis. Small bacterivorous, limax-shaped amoeba. Cells typically form broad lobose pseudopodia as wide as the cell proper often with a thin hyaline cap, but a prominent hyaline cap may or may not be present. Locomotive trophozoites most commonly with a villous uroid. Mitochondrion with tubular cristae. Cysts not observed.

Type species. *Ptolemeba bulliensis* n. sp.

Etymology. The genus name is derived from the name of the first mascot for Mississippi State University, a bulldog named Ptolemy, who in turn was named after the Greco-Roman mathematician, Claudius Ptolemy. We chose this name because of type locality of the type isolate (Sk13-4e), which was the Mississippi State University campus. We chose to end the genus name with “-eba” to make the word sound like “amoeba.”

Ptolemeba bulliensis Brown et al. n. sp

Diagnosis. Species with characteristics of the genus. Freshwater inhabiting monotactic naked amoeba. Length in locomotion 25.6 μm (37.2–17.1), breadth 5.5 μm (L/B ratio: 4.7). Thin hyaline crescent-like cap at the anterior of the cell, and most often with a spineolate to villous-bulbous uroid. Amoebae uninucleate with vesicular nucleus 4.9–2.5 μm (mean = 3.4 μm) in diam., central nucleolus, 2.7–1.2 μm (mean = 1.9 μm) in diam. Mitochondria with straight, nonbranching, tubular cristae arranged in a uniform helical pattern. No distinct floating form observed. No cyst observed.

Type location. The type isolate of this species was isolated from topsoil collected in between the annex and lecture hall of Harned Hall on the Mississippi State University campus, Mississippi State, MS, USA (33.455790°N, 88.788123°W).

Type material. A fixed and embedded resin TEM block of Sk13-4e was deposited in the Smithsonian Museum under accession 1231517. This permanent physical specimen is considered the hapantotype (name-bearing type) of the genus and species (see Art. 73.3 of the International Code for Zoological Nomenclature, 4th Edition). The type culture (Sk13-4e) has been deposited with the ATCC under accession PRA-414.

Gene sequence data. The nearly complete SSU rRNA gene of the type isolate (Sk13-4e) is deposited in GenBank under accession no. KJ542108.

Etymology. Similar to the genus' etymology, the species name *bulliensis* refers to the nickname of the mascot for Mississippi State University, “Bully,” spelled here as “bulli.” The species name is ended with “-ensis,” which means “from a place,” referring to the type locality.

Ptolemeba noxubium Brown et al. n. sp

Diagnosis. Species with characteristics of the genus. Freshwater inhabiting monotactic naked amoeba. Length in locomotion 18.8 μm (29.8–12.0), breadth 6.0 μm (L/B ratio: 3.2). Thin hyaline crescent-like cap at the anterior of the cell, and frequently a villous-bulbous uroid that may be fasciculate. Amoebae uninucleate with vesicular nucleus 5.5–2.6 μm (mean = 3.9) in diam., central nucleolus, 3.5–1.1 μm (mean = 2.2 μm) in diam. Mitochondria with tubular cristae. No distinct floating form observed. No cyst observed.

Type location. The type isolate of this species was isolated from freshwater/sediment sample collected from a bald cypress swamp, Bluff Lake in the Sam D. Hamilton Noxubee National Wildlife Refuge (33.273220°N, 88.790542°W).

Type material. A fixed and embedded resin TEM block of the type isolate Nx13-1a was deposited in the Smithsonian Museum under accession 1231518. This permanent physical specimen is considered the hapantotype (name-bearing type) of the species. The type culture (Nx13-1a) has been deposited with the ATCC under accession PRA-415.

Gene sequence data. The nearly complete SSU rRNA gene of the type isolate (Nx13-1a) is deposited in GenBank under accession no. KJ542109.

Etymology. The species name *noxubium* refers to the type locality of the species, the Sam D. Hamilton Noxubee National Wildlife Refuge, often referred to as “Noxubee.”

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. RNAfold predicted RNA secondary folding structure of the predicted group I intron in Nx13-1c. The figure depicts the minimum free energy (MFE) secondary structure with base-pair probabilities (color scale). The minimum free energy is predicted as –122.60 kcal/mol.

The RNAfold command line was "RNAfold -p -d2 -noLP < in > out."

Figure S2. Whole cell section transmission electron micrographs of *Ptolemeba bulliensis* n. gen. n. sp. strain Sk13-4e. Scale bar of *A* = 2 μ m. Scale bar of *B* = 2 μ m.

Figure S3. Transmission electron micrographs *Ptolemeba bulliensis* n. gen. n. sp. strain Sk13-4e showing mitochondria (**A**) and the cell's membrane (**B**). Scale bar of *A* = 1 μ m. Scale bar of *B* = 500 nm.

Figure S4. Whole cell section transmission electron micrographs of *Ptolemeba noxubium* n. sp. strain Nx13-1a. Scale bar of *A* = 2 μ m. Scale bar of *B* = 1 μ m.

Figure S5. Transmission electron micrographs of *Ptolemeba noxubium* n. sp. strain Nx13-1a showing mitochondria (**A**) and the cell's membrane (**B**). Scale bar of *A* = 1 μ m. Scale bar of *B* = 500 nm.

Video S1. Real-time differential interference contrast microscopical video of *Ptolemeba bulliensis* strain Sk13-4e.

Video S2. Real-time differential interference contrast microscopical video of *Ptolemeba noxubium* strain Nx13-1a and Nx13-1c. The first half of the video is strain Nx13-1a and the latter half is Nx13-1c.