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Entamoeba chiangraiensis n. sp. (Amoebozoa: Entamoebidae) isolated from the gut of Asian swamp eel (*Monopterus albus*) in northern Thailand

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Running title: *Entamoeba chiangraiensis* a new species from eel in Thailand

Abstract

The genus *Entamoeba* comprises mostly gut parasites and commensals of invertebrate and vertebrate animals including humans. Herein, we report a new species of *Entamoeba* isolated from the gut of Asian swamp eels (*Monopterus albus*) in northern Thailand. Morphologically, the trophozoite is elongated and has a single prominent pseudopodium with no clear uroid. The trophozoite is actively motile, 30-50 μm in length and 9-13 μm in width. Observed cysts were uninucleate, ranging in size from 12.5-17.5 μm in diameter. Chromatin forms a fine, even lining along the inner nuclear membrane. Fine radial spokes join the karyosome to peripheral chromatin. Size, host and nucleus morphology set our organism apart from other members of the genus reported from fish. The SSU rRNA gene sequences of the new isolates are the first molecular data of an *Entamoeba* species from fish. Phylogenetic analysis places the new organism as sister to *Entamoeba invadens*. Based on the distinct morphology and SSU rRNA gene sequence we describe it as a new species, *Entamoeba chiangraiensis*.

Key words: Archamoebae; intestinal protist; morphology; phylogeny; SSU rRNA

Key findings:

- Description of a new species of *Entamoeba*
- First molecular characterization of an *Entamoeba* species from fish
- Morphological characterization and culturing of the novel *Entamoeba*
- Updated *Entamoeba* phylogeny: four clades containing isolates from ectothermic hosts only

Introduction

Entamoeba is a member of the Entamoebidae, a deep lineage within the Archamoebae (Pánek *et al*. 2016). *Entamoeba* species use pseudopodia for locomotion and lack flagella, a morphologically identifiable Golgi apparatus, peroxisomes, and canonical mitochondria (Loftus *et al*. 2005; Ptáčková *et al*. 2013). *Entamoeba* species have trophozoite and cyst stages. The latter may have one nucleus or as many as eight, each with peripheral chromatin prominently visible. Historically, cyst size and nuclear number and appearance, along with host range information, were considered taxonomically important features and used to identify and group species of *Entamoeba*. However, in recent years it has become obvious that morphological features alone are not sufficient to adequately discriminate species known to be genetically distinct (Clark *et al*. 2006; Stensvold *et al*. 2011). For example, morphology does not distinguish the morphologically identical *E. histolytica* and *E. dispar*, yet only the former is a human pathogen (Gonin *et al*. 2003; Fotedar *el al*. 2007a; Hooshyar *et al*. 2015). The advent of molecular tools has shed light on the taxonomic landscape of *Entamoeba* and clarified several issues associated not only with taxonomy, but also epidemiology and host range (Verweij *et al*. 2003; Fotedar *et al*. 2007b; García *et al*. 2014). Screening of fecal samples from a broad range of hosts using SSU rRNA gene primers has uncovered several new and distinct lineages of *Entamoeba*, indicating a richly diverse genus (Santos *et al*. 2010; Stensvold *et al*. 2011; Jacob *et al*. 2015). Much of this diversity had not been previously recognized.

Members of the genus *Entamoeba* generally inhabit the gastrointestinal tract of vertebrates and invertebrates, but they have also been observed within other protist cells (Ghosh, 1968; Stensvold *et al*. 2011; García *et al*. 2014; Shilton *et al*. 2018). Several *Entamoeba* species are parasitic, but commensals are more common (Hooshyar *et al*. 2015). Uniquely among members of the genus, *E. gingivalis* inhabits the human oral cavity

(Ghabanchi *et al*. 2010; Luszczak *et al*. 2016; Maybodi *et al*. 2016). In addition, a few members of the genus have also been isolated from the environment (Clark and Diamond, 1997; Shiratori and Ishida, 2015).

Most *Entamoeba* gene sequences in public databases originate from species living in endothermic hosts, while relatively few derive from species living in ectotherms. To date, the latter hosts include amphibians, reptiles, and insects (Silberman *et al*. 1999; Garcia *et al*. 2014; Clark and Stensvold, 2015; Jacob *et al*. 2016; Kawano *et al*. 2017). Herein, we report a new species of *Entamoeba*, isolated from the gastrointestinal tract of the fish *Monopterus albus* (the Asian swamp eel) in Chiang Rai, Thailand. We examine its morphological features using light microscopy of living and stained specimens and provide the first SSU rRNA gene sequence of an *Entamoeba* isolated from fish.

Methods

Sample collection and establishment of culture

Two Asian swamp eels were purchased at a local market at Sanpong village, Phan district, Chiang Rai Province, northern Thailand. The eels were obtained at two separate times, in May and July 2018. Colonic contents were placed in modified (no mucin was added) LYSGM medium (Diamond, 1982, http://entamoeba.lshtm.ac.uk/xenic.htm) and incubated at room temperature (25-27 °C). After 24 hours, sediment was transferred to fresh medium and cells were subcultured every two weeks. The culture has been maintained since July 2018.

Light microscopy and staining

A wet mount of live amoebae was prepared and cells were observed using Nikon inverted light microscope. Trophozoites (n=10) and cysts (n=100) were measured using the same microscope. For a more detailed view of the cells, iron hematoxylin staining was performed by the Diagnostic Parasitology Laboratory, London School of Hygiene and

Tropical Medicine. Stained cells were observed with a Leica DMRB microscope fitted with a DFC 420 camera.

DNA extraction, amplification, purification and sequencing

Total genomic DNA was extracted from the culture using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea, catalog No: K-3032) according to manufacturer's specifications. Polymerase chain reaction (PCR) using the broad specificity primers RD5 and RD3 was used to amplify almost the entire SSU rRNA gene (Table 1). Emerald Amp® GT PCR Master Mix for PCR reactions were obtained from TaKaRa Bio USA, Inc. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 40 cycles of: denaturation at 94 °C for 1.3 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, ending with a final extension of 10 minutes at 72 °C.

The resulting PCR products were purified from gels with the GeneJET Gel Extraction Kit (Thermo Scientific; Wardmedic, Thailand) according to manufacturer's specifications. Samples were sequenced with RD5 and RD3 primers, along with ENTAM1, ENTAGENF and ENTAGENR (Table 1).

Phylogenetic analysis

The chromatogram quality of raw reads was checked individually with Sequencher software and ambiguous bases from the ends were removed. Sequences were combined into contigs and checked against the NCBI nr database, where they were identified as *Entamoeba*. A dataset was assembled including the newly derived sequences along with sequences spanning the breadth of molecular diversity of *Entamoeba*. In total, 90 sequences were used. Sequence alignment was performed on the EBI online platform (https://www.ebi.ac.uk/Tools/msa/mafft/) using MAFFT v.7.394 (Katoh and Toh, 2010). Ambiguously aligned positions were removed using Trimal v.1.3 (Capella-Gutierrez et al. 2009) available on the online platform Phylemon 2.0 (http://phylemon.bioinfo.cipf.es). After trimming 1,434 sites remained. Maximum likelihood analysis was conducted using RAxML v.8 (Stamatakis, 2006) on the online platform CIPRES Science Gateway (http://www.phylo.org/index.php/). For ML analysis, the general time reversible+Γ model of nucleotide substitution was employed as dictated by jModelTest v.2.1.10 using the Akaike criterion. Bootstrap support was computed from 1,000 bootstrap replicates.

Results

Culture, light microscopy and phylogenetic analysis

Colonic gut contents were inoculated into modified LYSGM, a medium widely used for xenic cultivation of *Entamoeba* species, and incubated at room temperature overnight. No live amoebae or cysts were observed in any tubes incubated at 37 °C, indicating that this species does not survive at that temperature.

The trophozoite of the amoeba is longer than it is wide (Fig. 1, Fig. 2C, 2D). Length is 40-50 µm (mean 44.31 µm), while width ranges from 9-13 µm (mean 11.18 µm). The cell changes shape slowly while in motion and has a single prominent pseudopodium, while the posterior end is smooth with no obvious uroid (Fig. 1, Fig. 2C, 2D). The granuloplasm has multiple vesicles, while the hyaloplasm is narrow (Fig. 1A). Unstained spherical cysts range from 10.0-17.50 µm in diameter (mean 14.15 µm; ± 1.42 standard deviation; ± 0.13 standard error). Stained cysts range from 10.0-17.50 µm in diameter (mean 13.75 µm; ± 1.54 standard deviation; ± 0.14 standard error). All observed cysts in both live and stained samples were uninucleate (Figs 2A, 2B), with the exception of a single stained example where it looked like there were two nuclei. Large, prominent glycogen vacuoles were present in both live and stained cysts, indicating that all observed cysts were immature (Figs 2A, 2B). Therefore, we cannot state the number of nuclei per cyst definitively, as we were not able to observe mature cysts. Cysts have no distinctive appearance (Figs 2A, 2B).

The size of the nucleus in both cysts and trophozoites ranges in diameter from 2.5-7.5 µm (mean 3.97 µm; ± 1.46 standard deviation; ± 0.13 standard error) and is generally found in the anterior half of the trophozoite. The trophozoite nucleus has a karyosome that has the appearance of a cluster of granules (Figs 2C, 2D). Karyosome size is variable depending on how tightly the granules cluster. Chromatin forms a delicate, even lining along the inner membrane of the nucleus (Fig. 2D). Unlike many other *Entamoeba* species, there are no clearly visible clumps of peripheral chromatin. Radial spokes are present in the nucleus joining the karyosome to peripheral chromatin (Figs 2C).

The SSU rRNA gene sequences of the two isolates are nearly complete (1849 and 1856 bp). Both sequences have been deposited in GenBank under accession numbers MK652887 and MK652888. Overall topology of the phylogenetic tree is similar to previous studies (Jacob *et al*. 2015). The tree is artificially rooted to the clade containing the cockroach sequences. These were the earliest diverging *Entamoeba* sequences in the eukaryotic supergroup tree of Kawano et al. 2017. The new SSU rRNA gene sequences are sister to those from *E. invadens* and this relationship has maximum bootstrap support (Fig. 3). The genetic distance between the new sequences and *E. invadens* sequences ranges from 3.4%-3.8% (Table S1). All observed nucleotide differences (including insertion and deletion events) are taxon specific. Intraspecific genetic divergence for the new amoeba and *E. invadens* is 0% and 0.4%, respectively. These sister species are in a clade that also includes *E. ranarum* and an unnamed *Entamoeba* sp., both from amphibian hosts. All members of this clade have been isolated from ectothermic hosts. This clade also has maximum bootstrap support.

Taxonomic Summary

Amoebozoa Lühe 1913, emend. Cavalier-Smith 1998

Archamoebae Cavalier-Smith 1983

Entamoebidae Chatton 1925, emend. Cavalier-Smith 1993

Entamoeba Casagrandi & Barbagallo 1895

Entamoeba chiangraiensis n. sp. Jinatham, Clark & Gentekaki 2019

Diagnosis: Amoeba inhabiting the gut of *Monopterus albus* (Asian swamp eel). Trophozoite is much longer than it is wide; length in motion is 30-50 µm, width 9-13 µm. Trailing end is smooth and devoid of visible uroid processes. Cysts are spherical, appearing smooth and thick-walled. Immature cysts have a single nucleus and a prominent glycogen vacuole, which often obscures the nucleus. Cyst diameter is 10.0-17.5 µm (mean 14.21 µm; ± 1.33 standard deviation; ± 0.12 standard error), nucleus 2.5-7.5 µm (mean 3.97 µm; ± 1.46 standard deviation; ± 0.13 standard error). There is a karyosome composed of granules. Chromatin is evenly distributed around the inner nuclear membrane, forming a thin, uniform lining. Radial spokes connect the karyosome to the peripheral chromatin.

Etymology: the epithet *chiangraiensis* refers to Chiang Rai province, Thailand, in which the organism was isolated

Host: *Monopterus albus*

Type location: isolated from the gut of Asian swamp eel, Sanpong, Phan, Chiang Rai, Thailand

Type material: permanent slide stained with iron-hematoxylin was deposited in the Smithsonian Museum under accession number xxxx.

Type sequence: GenBank accession number MK652887

ZooBank ID: xxxx

Discussion

Like all members of the genus *Entamoeba*, the new species has a nucleus with the characteristic “ring and dot” appearance corresponding to peripheral chromatin and central karyosome (Clark and Stensvold, 2015). *Entamoeba chiangraiensis* n.sp. was isolated twice from the Asian swamp eel, *Monopterus albus*, which inhabits rivers across Southeast Asia. Only a few species of *Entamoeba* from fish have been documented: four from marine hosts and three from freshwater (Table 2 and references therein). Molecular data for any of these species is absent.

Pathogenicity of the new species is unknown. Only a few species of *Entamoeba* are definitively pathogenic based on histology evidence. These are *E. histolytica*, a human pathogen, *E. nuttalli,* a pathogen of non-human primates, *E. invadens*, a reptile pathogen and *Entamoeba* sp., a toad pathogen (Clark and Stensvold, 2015; Shilton et al. 2018). Microscopic examination of *E. chiangraiensis* cells immediately after sample collection did not reveal ingestion of red blood cells, suggesting that the species is commensal rather than invasive. Nonetheless, to definitively determine pathogenicity further studies will be needed, including histology of infected fish to detect whether *E. chiangraiensis* invades host tissue.

We observed a single nucleus in cysts of the new species. However, the number of nuclei in mature cysts remains undetermined as cysts degenerated before reaching maturity. In the literature, the number of nuclei in cysts of Entamoebae from fish varies from one to four (Table 2 and references within). Species of *Entamoeba* from other ectothermic hosts commonly have four nucleated cysts, although octo-nucleated cysts have been observed in some reptiles, including *E. barreti* from a snapping turtle (Taliaferro and Holmes, 1924).

The host range of our and other species of *Entamoeba* from fish is unknown. We screened a number of fish inhabiting the same environment as the Asian swamp eel (Synbranchiformes) including: *Anabas* sp. (Anabatiformes, n=3), *Tilapia* sp. (Cichliformes, n-5), *Trichogaster* sp. (Anabatiformes, n=3), *Trachinocephalus* (Aulopiformes, n=2) and Siluriformes (Siluriformes, n=4). Our examination included both microscopy and a molecular survey using combinations of the primers described in the methods section. Intestinal contents from all fish were placed in the same culture medium in an attempt to grow amoebae. We were unable to find *Entamoeba* in any of the other hosts using any of the methods described. Although we tried to be as inclusive as possible in our screening, we cannot exclude the possibility that *E. changraiensis* might also inhabit the gut of fish that we have not examined. Host ranges of many *Entamoeba* species remain incompletely known, but they keep expanding. For instance, *E. coli* has traditionally been reported from humans and non-human primates, but is now known in rodents (Clark and Stensvold, 2015). Nonetheless, it seems likely that body temperature will pose a constraint on host range, as Entamoebae from ectotherms have not been found in endotherms and vice versa. *Entamoeba moshkovskii* is a notable exception, having been found in both reptiles and mammals (Garcia et al. 2014); it seems to be the only species of *Entamoeba* that has crossed the ectotherm/endotherm barrier. Within ectotherms, *Entamoeba* species show host specificity at the higher level of classification. Thus, reptilian isolates have never been isolated from amphibians and vice versa.

Entamoeba SSU rRNA gene sequences that have been detected exclusively in ectothermic hosts are diverse and dispersed across the phylogenetic tree, forming four distinct clades. The first clade comprises *E. chiangraiensis*, *E. invadens*, *E. ranarum*, and an unnamed *Entamoeba* sp. (MH890608) from a toad. The latter represents only the second amphibian-derived *Entamoeba* sequence. The SSU rRNA gene sequences from two eels sampled at two separate time points were identical, indicating low intra-specific diversity of this gene in *E. chiangraiensis*. This is similar to *E. invadens,* whose SSU rRNA gene sequences also display a high degree of genetic similarity, even when isolated from different hosts and from different countries (Jacob et al. 2015). The new species groups together with *E. invadens*. When comparing their SSU rRNA sequences, the genetic distance is a little below 4%, almost four-fold than that between *E. histolytica* and *E. dispar*. The second clade contains several variants of *E. terrapinae* derived from aquatic turtles (Garcia et al. 2014).

The third clade contains *Entamoeba insolita*, along with *Entamoeba* RL5 from tortoise and *Entamoeba* RL6 from iguana. These organisms are each represented by a single sequence (Silberman et al. 1999; Stensvold et al. 2011). Finally, the fourth clade consists of numerous sequences of *Entamoeba* from cockroaches (Kawano et al, 2017). In their study, Kawano et al. (2017) examined 186 cockroaches and found Entamoebae in 134. In their phylogenetic analyses, cockroach-derived sequences formed a distinct clade with nine separate groups within. This strongly hints at the presence of a vast diversity of *Entamoeba* that has yet to be uncovered. It seems likely that screening of additional hosts, especially ectotherms, will reveal an ever greater number of novel *Entamoeba* species.

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Conflict of interest

None

Ethical standards

No animals were sacrificed specifically for this work. Asian swamp eel is a popular food in Thailand and can be purchased at local markets. Intestinal contents were obtained from eels that had been purchased for food consumption. Permission and approval for obtaining such contents was obtained from the Mae Fah Luang University Animal Care and Use committee (protocol no. AR01/62).

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Table 1. Primers used to amplify and sequence *Entamoeba chiangraiensis*

Primer name	**Primer sequence (5’—3’)**	**References**
RD5	ATCTGGTTGATCCTGCCAGT	Clark et al (2006)
RD3	ATCCTTCCGCAGGTTCACCTAC	
ENTAGEN_F	ACTTCAGGGGGAGTATGGTCAC	Stensvold et al (2011)
ENTAGEN_R	CAAGATGTCTAAGGGCATCACAG	
ENTAM1	GTTGATCCTGCCAGTATTATATG	Verweij et al (2001)

Table 2. Species of *Entamoeba* isolated from fish

Species	Host-salinity, location	# of cyst nuclei	Cyst diameter (µm)	References
Entamoeba chiangraiensis	Asian swamp eel (*Monopterus albus*), freshwater, Thailand	Uncertain	10.0-17.5	This report
Entamoeba ctenopharyngodoni	Carp, freshwater, China	1-4	7.8-10.4	Chen (1955)
Entamoeba gadi	Pollock (*Pollachius virens*), marine, USA	1-2	6.0-11.8	Bullock (1966)
Entamoeba molae	Ocean sunfish (*Mola mola*), marine, USA	1	Not observed	Noble and Noble (1966)
Entamoeba nezumia	Macrourid fish (*Nezumia bairdi*), marine, Greenland	1-4	7.7	Orias and Noble (1971)
Entamoeba pimelodi	Catfish (*Pimelodus clarias*), freshwater, Brazil	1	Not mentioned	Cunha and Penido (1926)
Entamoeba salpae	Fish (*Box salpa* syn. *Sarpa salpa*), marine, France	4	Not mentioned	Alexeieff (1912)
*Entamoeba synodontis**	Catfish (*Synodontis schall*), freshwater, Egypt	Uncertain	Uncertain	Imam *et al*. (1987)

* Description is incomplete in the original text

FIGURE LEGENDS

Fig. 1. Light micrographs of living trophozoites of *Entamoeba chiangraiensis* n. sp. Arrowhead indicates the nucleus. Scale bar = 25 µm.

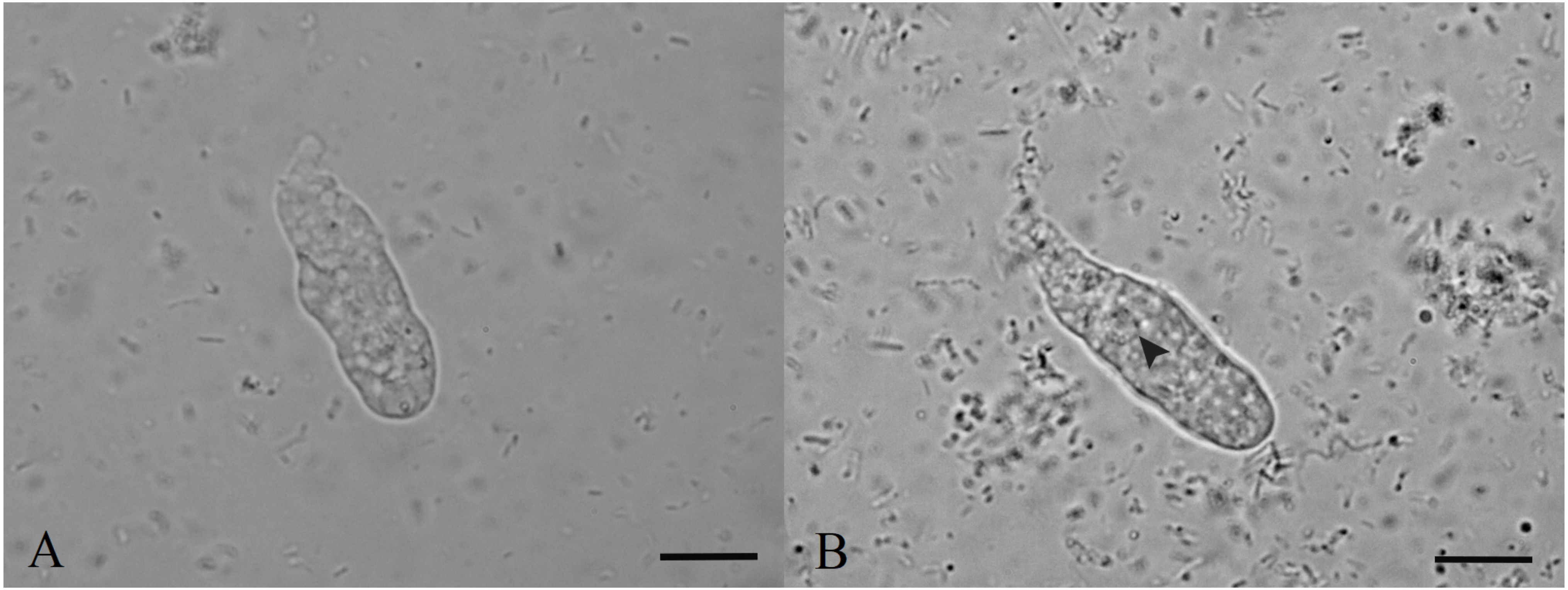


Fig. 2. Light micrographs of trophozoites and cysts stained with iron hematoxylin. **A-B**. Stained cysts. N = nucleus; G = glycogen vacuole; CW = cyst wall. **C-D**. Stained trophozoites. RS = radial spokes connecting karyosome to peripheral chromatin; Chr = peripheral chromatin forming an even fine lining around nuclear membrane; K = karyosome consisting of granules. Scale bar = 10 μm.

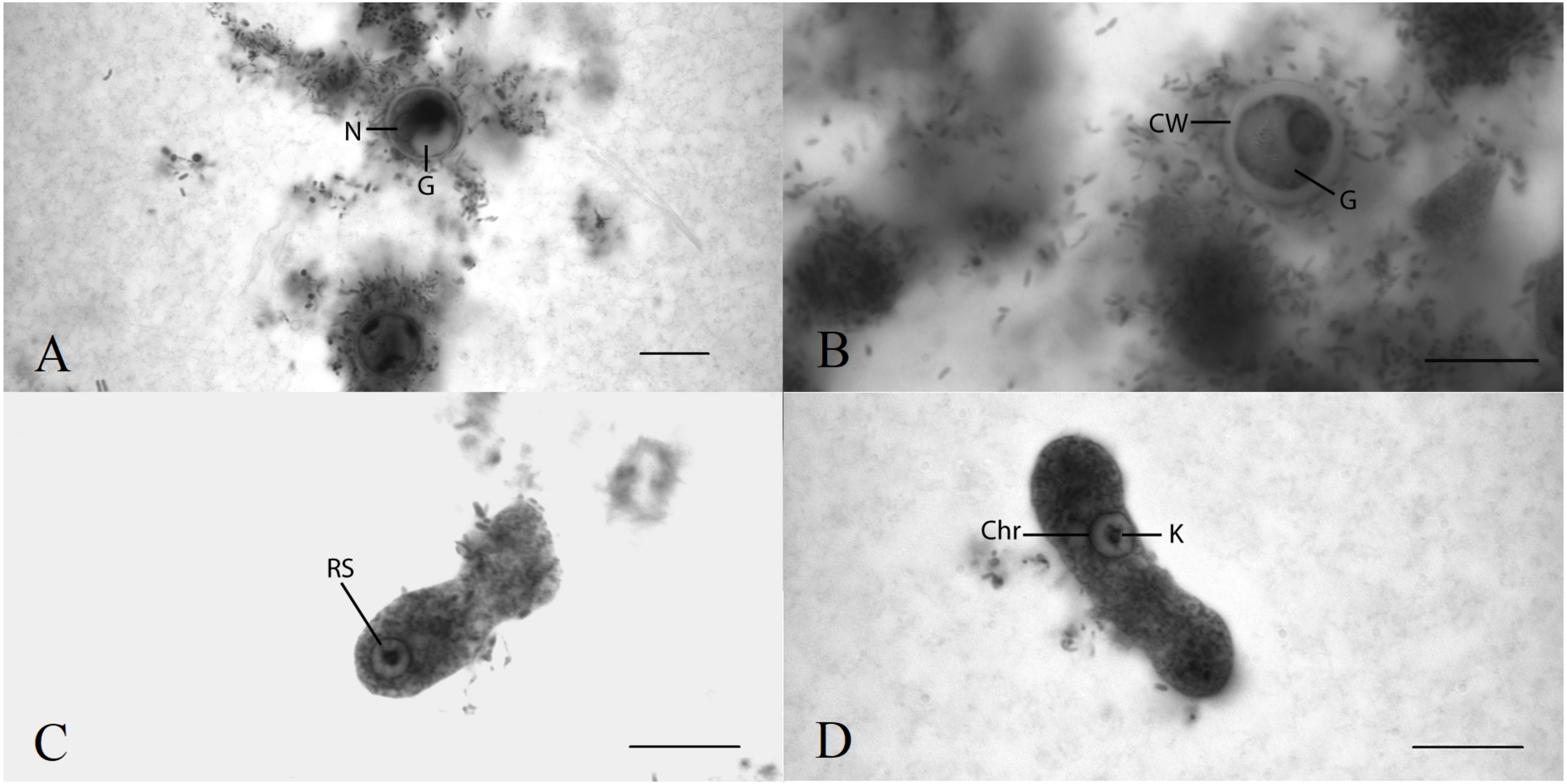
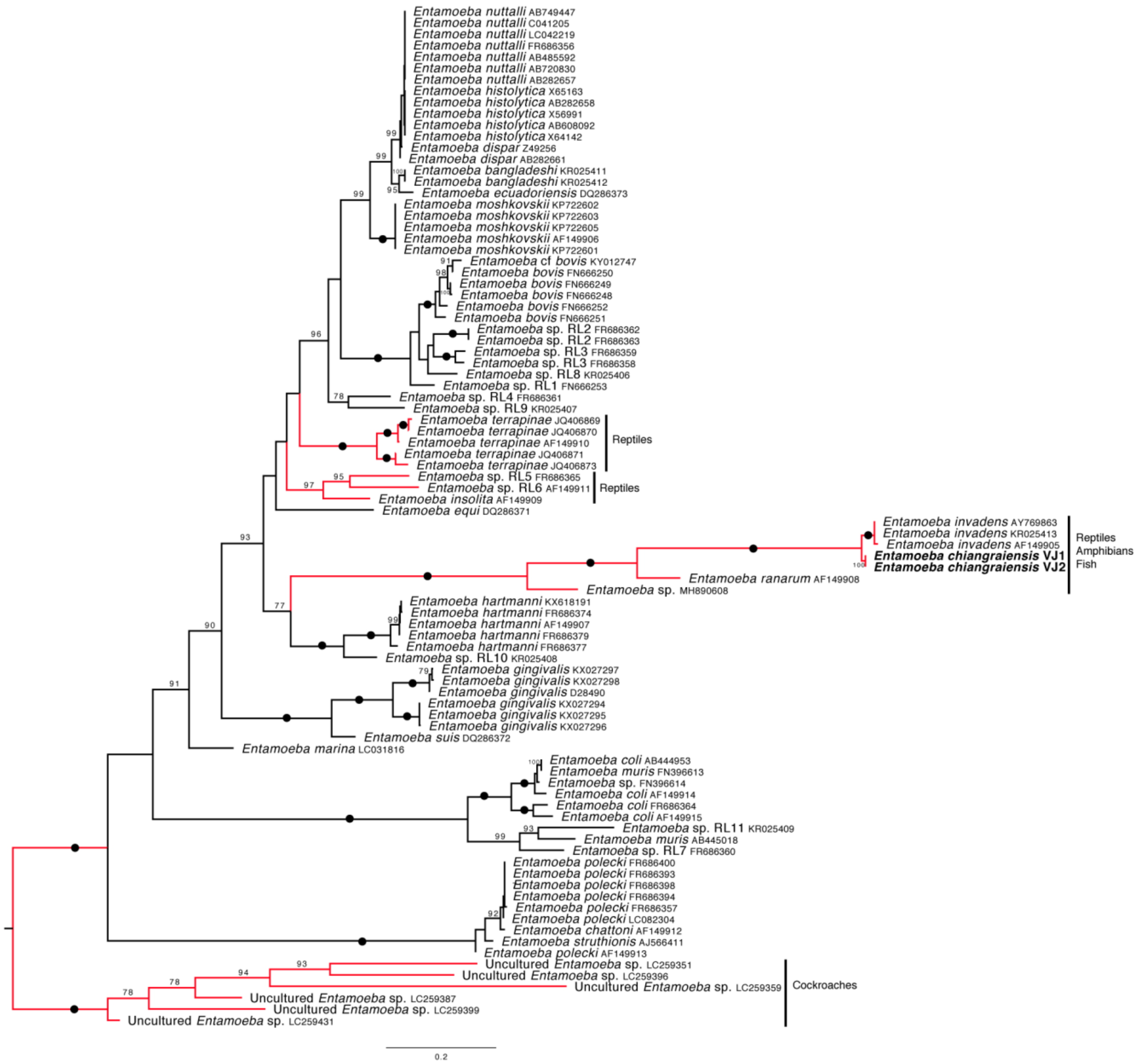


Fig. 3. Maximum likelihood phylogenetic tree inferred from 90 SSUrRNA sequences and 1434 sites. Tree is artificially rooted to cockroach derived *Entamoeba* sequences. Newly generated sequences are depicted in bold lettering. Numerical values indicate bootstrap support. Only values above 70 are shown. Full circles represent maximum bootstrap support. Clades in red consist of sequences exclusively from ectothermic hosts.


Entamoeba nuttalli AB749447
Entamoeba nuttalli C041205
Entamoeba nuttalli LC042219
Entamoeba nuttalli FR686356
Entamoeba nuttalli AB485592
Entamoeba nuttalli AB720830
Entamoeba nuttalli AB282657
Entamoeba histolytica X65163
Entamoeba histolytica AB282658
Entamoeba histolytica X56991
Entamoeba histolytica AB608092
Entamoeba histolytica X64142
Entamoeba dispar Z49256
Entamoeba dispar AB282661
Entamoeba bangladeshi KR025411
Entamoeba bangladeshi KR025412
Entamoeba ecuadoriensis DQ286373
Entamoeba moshkovskii KP722602
Entamoeba moshkovskii KP722603
Entamoeba moshkovskii KP722605
Entamoeba moshkovskii AF149906
Entamoeba moshkovskii KP722601
Entamoeba cf bovis KY012747
Entamoeba bovis FN666250
Entamoeba bovis FN666249
Entamoeba bovis FN666248
Entamoeba bovis FN666252
Entamoeba bovis FN666251
Entamoeba sp. RL2 FR686362
Entamoeba sp. RL2 FR686363
Entamoeba sp. RL3 FR686359
Entamoeba sp. RL3 FR686358
Entamoeba sp. RL8 KR025406
Entamoeba sp. RL1 FN666253
Entamoeba sp. RL4 FR686361
Entamoeba sp. RL9 KR025407
Entamoeba terrapinae JQ406869
Entamoeba terrapinae JQ406870
Entamoeba terrapinae AF149910
Entamoeba terrapinae JQ406871
Entamoeba terrapinae JQ406873
Reptiles
Entamoeba sp. RL5 FR686365
Entamoeba sp. RL6 AF149911
Entamoeba insolita AF149909
Reptiles
Entamoeba equi DQ286371
Entamoeba invadens AY769863
Entamoeba invadens KR025413
Entamoeba invadens AF149905
Entamoeba chiangraiensis VJ1
Entamoeba chiangraiensis VJ2
Reptiles
Amphibians
Fish
Entamoeba ranarum AF149908
Entamoeba sp. MH890608
Entamoeba hartmanni KX618191
Entamoeba hartmanni FR686374
Entamoeba hartmanni AF149907
Entamoeba hartmanni FR686379
Entamoeba hartmanni FR686377
Entamoeba sp. RL10 KR025408
Entamoeba gingivalis KX027297
Entamoeba gingivalis KX027298
Entamoeba gingivalis D28490
Entamoeba gingivalis KX027294
Entamoeba gingivalis KX027295
Entamoeba gingivalis KX027296
Entamoeba suis DQ286372
Entamoeba marina LC031816
Entamoeba coli AB444953
Entamoeba muris FN396613
Entamoeba sp. FN396614
Entamoeba coli AF149914
Entamoeba coli FR686364
Entamoeba coli AF149915
Entamoeba sp. RL11 KR025409
Entamoeba muris AB445018
Entamoeba sp. RL7 FR686360
Entamoeba polecki FR686400
Entamoeba polecki FR686393
Entamoeba polecki FR686398
Entamoeba polecki FR686394
Entamoeba polecki FR686357
Entamoeba polecki LC082304
Entamoeba chattoni AF149912
Entamoeba struthionis AJ566411
Entamoeba polecki AF149913
Uncultured Entamoeba sp. LC259351
Uncultured Entamoeba sp. LC259396
Uncultured Entamoeba sp. LC259359
Cockroaches
Uncultured Entamoeba sp. LC259387
Uncultured Entamoeba sp. LC259399
Uncultured Entamoeba sp. LC259431
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