Running head: New chlorarachnean *Rhabdamoeba marina*

Rhabdamoeba marina is a heterotrophic relative of chlorarachnid algae

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ABSTRACT
Rhabdamoeba marina is a unique and poorly reported amoeba with an uncertain phylogenetic position. We successfully cultured *R. marina* from coastal seawater in Japan and performed a molecular phylogenetic analysis using the small subunit ribosomal RNA (SSU rRNA) gene sequence. Our phylogenetic analysis showed that *R. marina* branched as a basal lineage of Chlorarachnea, a group of marine photosynthetic algae belonging to the phylum Cercozoa within the supergroup Rhizaria. By comparing the ecological and morphological characteristics of *R. marina* with those of photosynthetic chlorarachneans and other cercozoans, we gained insight into the evolution and acquisition of plastids in Chlorarachnida.

Keywords
amoeba; Chlorarachnea; Chlorarachniophytes; flagellate; molecular phylogeny; ptotist; SSU rRNA; ultrastructure

Rhabdamoeba marina is a small marine amoeba described by Dunkerly (1921) from a sample originating from *Trichosphaerium* material of the Plymouth Marine Laboratory, England, UK. However, since it was first documented, *R. marina* has been absent from

taxonomic and ecological studies for more than 70 years. Rogerson et al. (1998) redescribed the amoeba based on a culture established from sand samples of Firth of Clyde, Scotland, and revealed its unique characteristics, such as mostly immotile amoeboid cells with rod-like bodies at the tip of flattened pseudopodia and flagellated cells with two posterior flagella budding from the amoeboid cell. These distinct features have made it challenging to assign *Rhabdamoeba* to any specific taxonomic group, and it has been treated as an amoeba of uncertain affinity (Rogerson et al., 1998). Subsequently, Howe et al. (2011) placed *Rhabdamoeba* within the cercozoan flagellate group Marimonadida owing to the presence of two posteriorly directed flagella, as observed by Rogerson et al. (1998). However, owing to the lack of available cultures of *Rhabdamoeba*, no phylogenetic studies have been performed to validate this taxonomic classification. In the present study, we established a culture of *R. marina* and performed microscopic observations and molecular phylogenetic analysis. Based on the microscopic and phylogenetic data, we discuss the taxonomic position and evolutionary characteristics of *R. marina*.

MATERIALS AND METHODS

Sample collection and culture establishment

Seawater sample was collected from the Tomari fishing port in Tottori, Japan (35.5142 °N, 133.9366 °E) on June 10, 2014. The sample was incubated with ESM medium at 20°C in the dark. After one month of incubation, amoeboid cells of *Rhabdamoeba marina* were detected in the sample. A clonal culture of *R. marina* (SRT404) was established from an amoeboid cell using the micropipetting isolation method. A culture of *Emiliania huxleyi* (CCMP 1516) was added to the isolated cell as prey. The two-member culture was deposited and maintained in the National Institute for Environmental Sciences (NIES) collection, IBARAKI, Japan, as NIES-4232.

Light microscopy

For light microscopy, SRT404 cells were placed in a glass-bottomed dish and observed using an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP73 CCD camera (Olympus).

Electron microscopy

For transmission electron microscopy, SRT404 cells were removed from the bottom of the

culture vessels using a silicone rubber scraper and collected by centrifugation at 2000 × *g* for 5 min. Pelleted cells were suspended in a mixture of 2% (w/v) glutaraldehyde, 0.2 M sodium cacodylate, and 0.25 M sucrose and left for 1 h at room temperature for pre-fixation. The cells were then washed with 0.2 M sodium cacodylate buffer three times. Cells were then post-fixed with 1% (v/v) OsO4 in 0.2 M sodium cacodylate buffer, and dehydration was performed using a graded series of ethanol (30-100%; v/v). After dehydration, the cells were placed in a 1:1 mixture of 100% ethanol and acetone, followed by incubation in pure acetone twice. Resin replacement was performed using a 1:1 mixture of acetone and agar low-viscosity resin R1078 (Agar Scientific Ltd, Stansted, UK) for 1 h at room temperature, followed by pure resin for 2 h at room temperature. Resin was polymerized by heating at 60°C for 12 h. Ultrathin sections were prepared on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria), double-stained with 2% (w/v) uranyl acetate and lead citrate, and observed using a Hitachi H-7650 electron microscope (Hitachi High-Technologies Corp. Tokyo, Japan) equipped with a Veleta TEM CCD camera (Olympus).

DNA extraction and sequencing

Total DNA from the SRT404 was extracted from the cell pellets collected by centrifugation using a DNeasy Plant Mini Kit (Qiagen Science, Valencia, CA, USA) according to the manufacturer's instructions. The small subunit ribosomal RNA (SSU rRNA) gene fragment of the strain was amplified using PCR using primers 18F and 18R (Yabuki et al., 2010). Amplification involved 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min, followed by an additional extension for 2 min at 72°C at the end of the reaction. Amplified DNA fragments were purified after gel electrophoresis using a QIAquick Gel Extraction Kit (Qiagen Science) and then cloned into the pGEM T-easy vector (Promega, Tokyo, Japan). The inserted DNA fragments were sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Monza, Italy) and BigDye Terminator v3.1 cycle sequencing Kit (Applied Biosystems). The SSU rRNA gene sequence of the SRT404 was deposited in GenBank under accession code xxxx.

Molecular phylogenetic analysis

For the molecular phylogenetic analysis, 79 cercozoan SSU rRNA gene sequences were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). The SSU rRNA gene

sequence of the SRT404 was added to the dataset and aligned using MAFFT v7.520 (Katoh and Standley, 2013) and edited manually by SeaView version 5.0.5 (Gouy et al., 2010). Ambiguously aligned regions were deleted. The dataset comprised 1,571 nucleotide positions. The maximum likelihood (ML) tree was searched using IQ-Tree v.1.6.12 (Nguyen et al., 2015) with the TIM+F+I+G4 model. Branch supports were obtained using an ultrafast bootstrap (BP, 1,000 replicates) with an IQ-Tree (Hoang et al., 2018). Bayesian analysis was carried out using MrBayes v.3.2.6 (Ronquist et al., 2012), with the GTR+Γ model. One cold and three heated Markov chain Monte Carlo simulations with default chain temperatures were run for 2×10^6 generations, sampling the lnL values and trees at 100-generation intervals. Convergence was assessed using the average standard deviation of split frequencies, and the first 25% of the total generations in each analysis were discarded as 'burn-in'. Bayesian posterior probabilities (BPP) and branch lengths were calculated from the remaining trees.

RESULTS AND DISCUSSION

Light microscopic observations showed that the SRT404 exhibited amoeboid and flagellated stages. The amoeboid cells had multiple flattened pseudopodia with rod-like bodies at their tips (Fig. 1A, B). Amoeboid cells showed no obvious motility. A nucleus with a nucleolus was observed in each amoeboid cell (Fig. 1A, B). The flagellated cells were oval with two subequal flagella (Fig. 1C and D). The flagella were inserted from the anterior ventral side of the cell and were directed posteriorly (Fig. 1D). The flagellates exhibited smooth gliding movements while trailing both flagella. Because the light microscopic features of the SRT404 corresponded with the description of *R. marina* by Rogerson et al. (1998), we regarded this strain as *R. marina*.

Transmission electron microscopy revealed that amoeboid cells possessed a nucleus with a nucleolus and food vacuoles (Fig. 1e). The cytoplasm contained many vacuoles of various sizes (Fig. 1e-g). The small Golgi apparatuses, mitochondria with tubular cristae, and microbodies were scattered throughout the cytoplasm (Fig. 1e, g). Large cylindrical extrusomes were observed in flattened pseudopodia (Fig 1E, F). These extrusomes may correspond to rod-like bodies as observed using light microscopy. In the original description, Dunkerly (1921) used a fixed specimen in his light microscopic observations and reported that rod-like bodies slightly project from the surface of pseudopodia like tiny spines. We considered Dunkerly (1921) observed released extrusome

that caused by cell fixation.

In the molecular phylogenetic analysis, *R. marina* grouped with environmental sequences collected from marine lagoon, deep sea, and hydrothermal vent with strong statistical support (BP = 100%, BPP = 1) (Fig. 1i). The *R. marina* and the environmental sequences branched as a sister lineage of a clade comprising Chlorarachnida and Minorisida (i.e., Chlorarachnea) with strong support (BP = 97%, BPP = 1) (Fig. 1I). Based on the molecular phylogenetic analysis, we propose to move *Rhabdamoeba* from Marimonadida to Chlorarachnea.

Chlorarachnea comprises photosynthetic Chlorarachnida and heterotrophic Minorisida species. Chlorarachnida is a group of marine photosynthetic algae comprising amoebae, coccoids, and flagellates stages (Ishida et al., 2007; Keeling, 2017). The Chlorarachnida plastid, derived from green algal symbionts, is closely related to Bryopsidales through secondary endosymbiosis (Suzuki et al., 2016). Minorisida is a group of heterotrophic bacterivorous uniflagellates that include the sole genus *Minorisa*. (del Campo et al., 2013). Transmission electron microscopy did not reveal any plastids or their possible remnant structures in *R. marina* cells. Because *Minorisa* also lacks plastids, chlorarachnid plastids were probably acquired after the divergence of *Minorisa*. Chlorarachnida has a complex life cycle, comprising amoeba, coccoids, and flagellates (Ishida et al., 2007; Keeling, 2017). In contrast, *Minorisa* and *Rhabdamoeba* have excursive amoeba and flagellate stages (Shiratori et al., in press), suggesting that the common ancestor of Chlorarachnea also possessed these two cell stages and lacked a coccoid stage. In contrast to the bacterivorous *Minorisa*, Rogerson et al. (1998) reported that *R. marina* feeds on small heterotrophic nanoflagellates and autotrophic protists. Our *R. marina* culture also fed on the unicellular alga *E. huxleyi*. Considering plastid acquisition in Chlorarachnida, it is reasonable that ancestral chlorarachneans are algivorous, and *Minorisa* probably lost its algivorous ability. Rogerson et al. (1998) reported that flagellated cells budded off amoeboid cells when localized prey were depleted. Budding flagellated cells from amoeboid cells is reported in the granofilosean amoeba *Clathrulina* and *Reticulamoeba* (Bass et al., 2009, 2012). Although the monophyly and phylogenetic position of Granofilosea in Cercozoa is not clear, it is occasionally positioned as a sister lineage of Chlorarachnea, with insufficient support (Cavalier-Smith et al. 2018). Metromonadea is another group that is possibly a sister group of Chlorarachnea (Bass et al., 2009). Metromonadea comprises small but eukaryovorous flagellates that possess parallel

basal bodies and cylindrical extrusomes, such as *R. marina* (Myl'nikov et al., 1999; Myl'nikova and Myl'nikov, 2011). Further taxonomic sampling and large-scale phylogenetic analyses are required to resolve the relationship between Chlorarachnea and other cercozoan taxa. This study shows that the poorly reported amoeba *R. marina* is a basal lineage of Chlorarachnea. The genomic examination of *Rhabdamoeba* and *Minorisa* may provide important insights into the evolution of Chlorarachnea.

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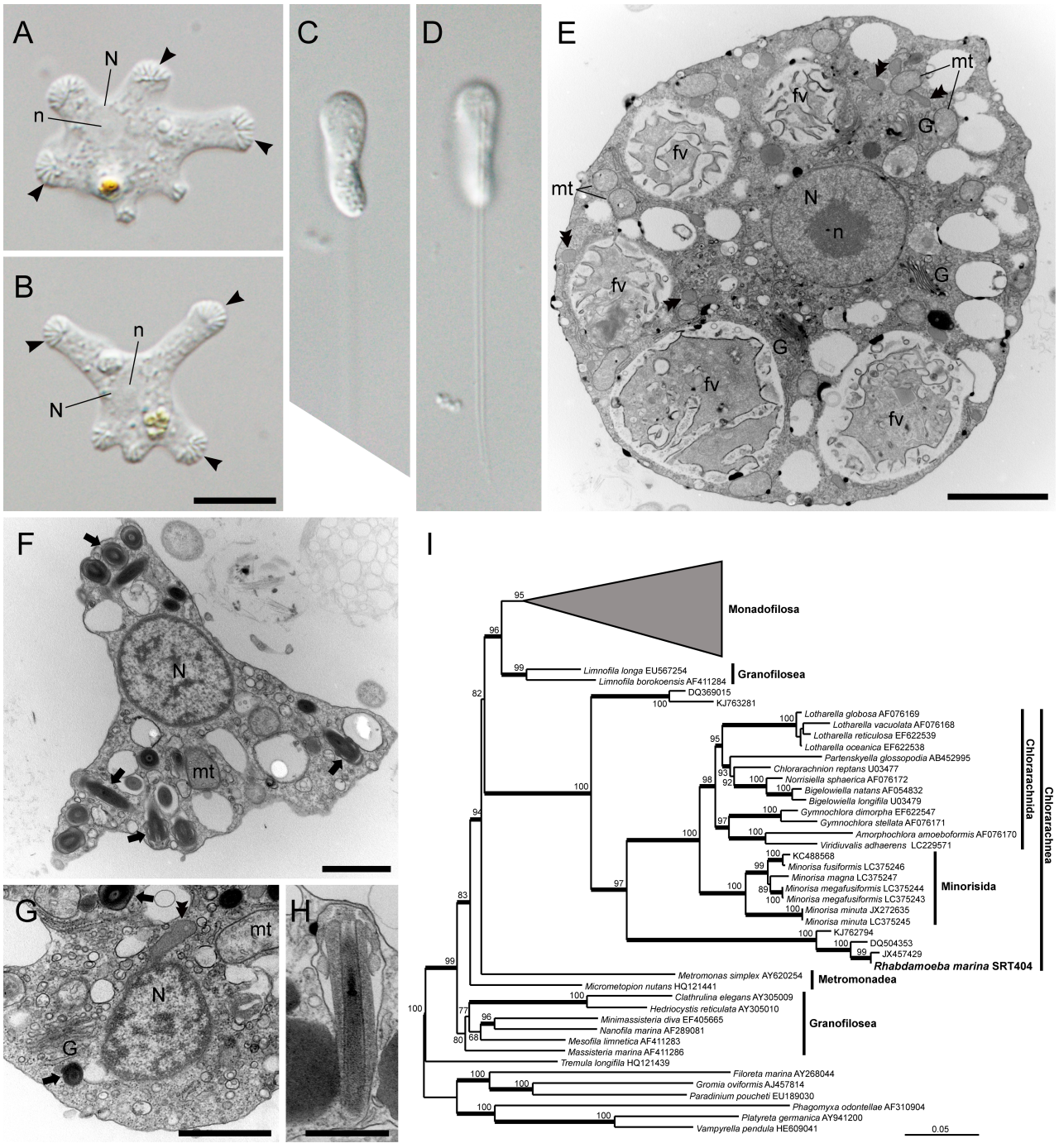
FIGURE LEGENDS

Figure 1 Light and electron micrographs of *Rhabdamoeba marina* SRT404 and maximum likelihood tree of Cercozoa. **A, B.** Differential interference contrast (DIC) micrographs of living amoeboid cells of *R. marina*. **C, D.** DIC micrographs of living flagellated cells of *R. marina*. **E-G.** A transmission electron micrograph of *R. marina*. **H.** A transmission electron micrograph of an extrusome of *R. marina*. Arrows indicate extrusomes. Arrowheads indicate rod-like bodies. Double arrowheads indicate microbodies. fv, food vacuole; G, Golgi apparatus; N, nucleus; n, nucleolus; mt, mitochondria. **I.** Molecular phylogenetic tree using 1,571 nucleotide position of 80 cercozoan SSU rRNA gene sequences. Monadofilosean sequences (37 OTUs) are indicated by a triangle. The complete phylogenetic tree is shown in Figure S1. Values at each branch indicate the bootstrap probability (≥50% shown). Bold branches indicate Bayesian posterior probabilities ≥0.95.

SUPPORTING INFORMATION

Figure S1. Maximum likelihood tree of Cercozoa using 1,571 nucleotide position of 80 cercozoan SSU rRNA gene sequences. Values at each branch indicate the bootstrap probability (≥50% shown). Bold branches indicate Bayesian posterior probabilities ≥0.95.

Figure 1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Granofilosea
DQ369015
KJ763281
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Partenskyella glossopodia AB452995
Chlorarachnion reptans U03477
Norrisiella sphaerica AF076172
Bigelowiella natans AF054832
Bigelowiella longifila U03479
Gymnochlora dimorpha EF622547
Gymnochlora stellata AF076171
Amorphochlora amoeboformis AF076170
Viridiuvalis adhaerens LC229571
Chlorarachnida
KC488568
Minorisa fusiformis LC375246
Minorisa magna LC375247
Minorisa megafusiformis LC375244
Minorisa megafusiformis LC375243
Minorisa minuta JX272635
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Chlorarachnea
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Micrometopion nutans HQ121441
Metromonadea
Clathrulina elegans AY305009
Hedriocystis reticulata AY305010
Minimassisteria diva EF405665
Nanofila marina AF289081
Mesofila limnetica AF411283
Massisteria marina AF411286
Tremula longifila HQ121439
Granofilosea
Filoreta marina AY268044
Gromia oviformis AJ457814
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Vampyrella pendula HE609041
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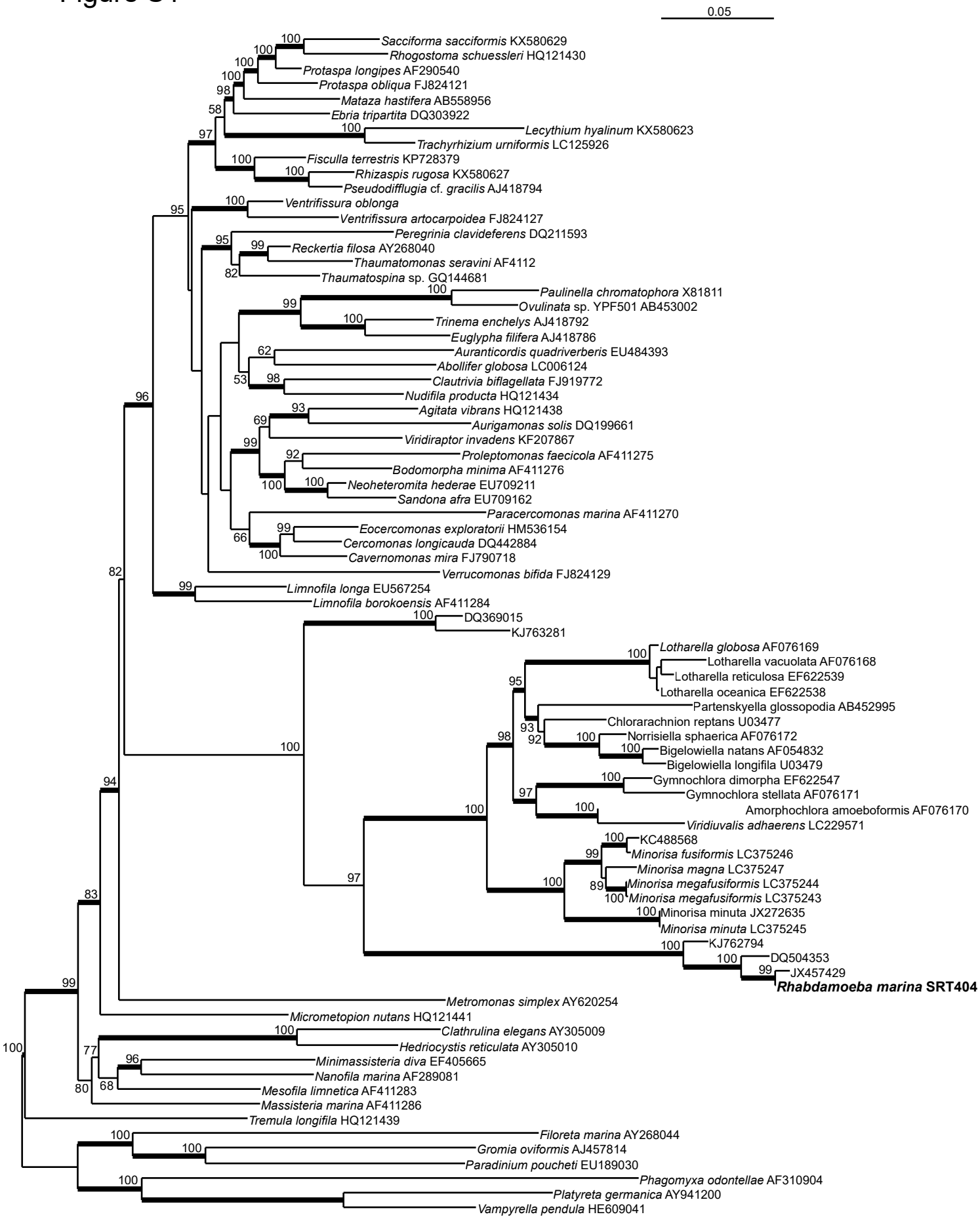
Figure S1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Ebria tripartita DQ303922
Lecythium hyalinum KX580623
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Fisculla terrestris KP728379
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Ventrifissura oblonga
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Paulinella chromatophora X81811
Ovulinata sp. YPF501 AB453002
Trinema enchelys AJ418792
Euglypha filifera AJ418786
Auranticordis quadriverberis EU484393
Abollifer globosa LC006124
Clautrivia biflagellata FJ919772
Nudifila producta HQ121434
Agitata vibrans HQ121438
Aurigamonas solis DQ199661
Viridiraptor invadens KF207867
Proleptomonas faecicola AF411275
Bodomorpha minima AF411276
Neoheteromita hederae EU709211
Sandona afra EU709162
Paracercomonas marina AF411270
Eocercomonas exploratorii HM536154
Cercomonas longicauda DQ442884
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