1 Description of *Entamoeba marina* n. sp.

2

3 ***Entamoeba marina* n. sp.; a New Species of *Entamoeba* Isolated From Tidal Flat**

4 **Sediment of Iriomote Island, Okinawa, Japan**

5

6 Takashi Shiratori[a], Ken-Ichiro Ishida[b,1]

7

8 a Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba,

9 Ibaraki 305-8572, Japan

10 b Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki

11 305-8572, Japan

12

[1] **Correspondence**
K. Ishida; Telephone number: +81 298 53 4533 ; Fax number: +81 298 53 4533; e-mail: ken@biol.tsukuba.ac.jp

ABSTRACT

The genus *Entamoeba* includes anaerobic lobose amoebae, most of which are parasites of various vertebrates and invertebrates. We report a new *Entamoeba* species, *E. marina* n. sp. that was isolated from a sample of tidal flat sediments collected at Iriomote Island, Okinawa, Japan. Trophozoites of *E. marina* were 12.8-32.1 µm in length and 6.8-15.9 µm in width, whereas the cysts were 8.9-15.8 µm in diameter and contained four nuclei. The *E. marina* cells contained a rounded nucleus with a small centric karyosome and uniformly arranged peripheral chromatin. Although *E. marina* is morphologically indistinguishable from other tetranucleated cyst-forming *Entamoeba* species, *E. marina* can be distinguished from them based on the combination of molecular phylogenetic analyses using SSU rDNA gene and the difference of collection site. Therefore, we propose *E. marina* as a new species of the genus *Entamoeba*.

Keywords

Amoebozoa; anaerobic protist; Archamoebae; morphology; phylogenetic analysis; SSU rDNA

1 The genus *Entamoeba* is a group of parasitic and free-living anaerobic amoebae. The cells
2 of *Entamoeba* species possess a nucleus with a small nucleolus (karyosome) and peripheral
3 chromatin, but lack flagella and basal bodies, Golgi apparatus, and mitochondria (Pimenta
4 et al. 2002; Ptáčková et al. 2013). The life cycle of most *Entamoeba* species consists of
5 amoeboid trophozoite stage and spherical cyst stage although some species do not form the
6 cysts (Kudo 1954; Ponce-Gordo and Martínez-Díaz 2010). The members of *Entamoeba*
7 form uni-, quadri-, or octo-nucleated cysts with chromatoid bodies (crystalline arrays of
8 ribosomes) (Ponce-Gordo and Martínez-Díaz 2010). The number of nuclei in the cysts and
9 the morphology of karyosome, peripheral chromatin, and chromatoid bodies have been
10 used as important taxonomical traits of this genus (Brooke and Melvin 1984). Molecular
11 phylogenetic analyses with small subunit (SSU) rDNA genes support the monophyly of the
12 genus *Entamoeba* which is known to form a higher clade with other endobiotic and
13 free-living anaerobic amoebae (e.g., *Endolimax*, *Iodamoeba*, *Mastigamoeba*, *Mastigella*,
14 *Pelomyxa*, and *Rhizomastix*), making up Archamoebae (Cavalier-Smith et al. 2004;
15 Ptáčková et al. 2013; Silberman et al. 1999**)**. Most of the *Entamoeba* species parasitize
16 intestines of various vertebrates such as mammals, birds, reptiles, and fish (e.g., *E.*
17 *histolytica*, *E. struthionis*, *E. pyrrhogaster*, *E. testudinis*, and *E. gadi*). (Bullock 1966;
18 Diamond and Clark 1993; Hartmann 1910; Lobeck 1940; Ponce-Gordo et al. 2004). In
19 addition, some species have been reported in other habitats such as the oral cavity of
20 humans (*E. gingivalis*), the gut of the horse-leech (*E. aulastomi*), and inside of the protist
21 cells (*E. paulista*) (Ghosh 1973; Kudo 1945; Nöller 1912). On the other hand, only two
22 species, *E. ecuadoriensis* and *E. moshkovskii*, have been reported from samples of natural

1 environment such as sewage and lake sediment (Clark and Diamond 1997; Heredia et al.
2 2012).
3 In this paper, we report a new *Entamoeba* species (strain SRT209) that was
4 isolated from a tidal flat sediment sample collected at the Iriomote Island, Japan. The
5 isolated strain was studied by light and electron microscopy as well as molecular
6 phylogenetic analyses, in order to determine its taxonomic position.
7

1 **MATERIALS AND METHODS**

2 **Establishment and maintenance of culture**

3 Samples were collected from 30-cm depth of tidal flat sediments near the mouth of the
4 Shiira River in Iriomote Island, Okinawa, Japan (latitude = 24.3241°N, longitude
5 =123.9110°E) on February 5, 2012. A small amount of the sediment was initially cultivated
6 in sealed culture flasks filled with mixture of mTYGM-9 medium (Kasai et al. 2009) and
7 filtered natural seawater (1:20 v/v) under dark conditions at 20°C. A cell of *E. marina* was
8 isolated by a micropipetting method from the culture and then cultivated in a 48-well plate
9 filled with the same medium. The 48-well plate was enclosed within a resealable plastic bag
10 with AnaeroPack™ (Mitsubishi Gas Chemical, Tokyo, Japan) and kept under dark
11 conditions at 20°C. Established culture of *E. marina* (strain SRT209) was maintained in
12 culture flask under dark conditions at 20°C and subcultured monthly.

13 **Light microscopy**

14 Living cells of *E. marina* were observed on microscope slides under the Axio Imager A2
15 Microscope (Zeiss, Oberkochen, Germany) equipped with the DP71 CCD Camera
16 (Olympus, Tokyo, Japan) or on glass-bottom dishes under the IX71 Inverted Microscope
17 (Olympus) equipped with the DP71 CCD Camera. For detailed observation, the cells were
18 fixed with 2.5% (v/v) glutaraldehyde and stained with the Kohn's Chlorazol Black E (Wako
19 Pure Chemicals, Tokyo, Japan) or the Weigert's Iron Hematoxylin (Wako Pure Chemicals).
20 The stained cells were observed on slides under the Zeiss Axio Imager A2 Microscope
21 equipped with the DP71 CCD Camera.

22 **Transmission electron microscopy**

1 For transmission electron microscopy, the cells were concentrated by centrifugation at 1000
2 *g* for 5 min. The pellets were placed on formvar-coated copper loop and plunged rapidly
3 into liquid propane. The frozen pellets were plunged into liquid nitrogen for several
4 seconds and then placed in 2% osmium tetroxide in acetone at −80°C for 48 h. Then, the
5 fixing solution was kept at −20°C for 2 h and at −4°C for 2 h. The pellets were rinsed with
6 acetone three times and then replaced with Agar Low Viscosity Resin R1078 (Agar
7 Scientific Ltd., Stansted, England). The resin was polymerized by heating at 60°C for 10 h.
8 Ultrathin sections were prepared on the Reichert Ultracut S Ultramicrotome (Leica, Vienna,
9 Austria) and double stained with 2% (w/v) uranyl acetate and lead citrate (Hanaichi et al.
10 1986; Sato 1968) Approximately 20 cells in the specimens were observed under the H-7650
11 Electron Microscope (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with the
12 Veleta TEM CCD Camera (Olympus Soft Imaging System, Munster, Germany).
13 **DNA extraction and amplification, and sequencing of SSU rDNA gene**
14 Total DNA was extracted from centrifuged pellets of SRT209 cells by using the DNeasy
15 Plant Mini Kit (Qiagen, Venlo, Netherlands). To amplify SSU rDNA gene of *E. marina*, we
16 performed polymerase chain reaction (PCR) with SR1-SR12 primers (Nakayama et al.
17 1998). Amplification steps consisted of 30 cycles of denaturation at 94°C for 30 s,
18 annealing at 55°C for 30 min, and extension at 72°C for 2 min. An additional extension for
19 4 min at 72°C was performed at the end of the reaction. Amplified DNA fragments were
20 purified after gel electrophoreses by using the QIAquick Gel Extraction Kit (Qiagen),
21 followed by cloning into the p-GEM® T-easy Vector (Promega, Wisconsin, USA). The
22 insert DNA fragments were completely sequenced by using the 3130 Genetic Analyzer

(Applied Biosystems, Monza, Italy) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The SSU rDNA sequences of the strain SRT209 was deposited as LC031816 in GenBank.

Sequence alignments and phylogenetic analysis

The SSU rDNA sequences of the strain SRT209 was added to an alignment set newly created for this study. The alignment set was automatically aligned with the multiple sequence alignment program MAFFT (Katoh and Toh 2008) and then edited manually by using SeaView (Galtier and Gouy 1996). For phylogenetic analyses, ambiguously aligned regions were manually deleted from each alignment. Finally, SSU rDNA alignment (1,660 positions) was prepared. The alignment files used in the analyses are available on request. The maximum likelihood (ML) tree was heuristically searched by RAxML v.8.0.3 (Stamatakis 2014) under the general time reversible (GTR)+Γ model. Tree searches started with 20 randomized maximum-parsimony trees, and the highest log likelihood (lnL) was selected as the ML tree. An ML bootstrap analysis (1000 replicates) was conducted under the GTR+Γ model with rapid bootstrap option. A Bayesian analysis was run by using MrBayes v. 3.2.2 (Ronquist and Huelsenbeck 2003) with the GTR +Γ model for each dataset. One cold and three heated Markov chain Monte Carlo with default chain temperatures were run for 1×10^6 generations and the sampling lnL values and trees at 100-generation intervals. The first 25% number of generation of each analysis was discarded as "burn-in." The Bayesian posterior probability and branch lengths were calculated from the remaining trees.

1 **RESULTS**

2 **Light microscopy**

3 Locomotive tropozoites of *Entamoeba marina* n. sp. were mostly monopodial and clavate,
4 and had eruptive hyaloplasm at the anterior region (Fig. 1A-D). The living tropozoites were
5 21.3 (12.8-32.1) µm in length and 10.3 (6.8-15.9) µm in width (n = 76), and the fixed cells
6 were 21.6 (11.3-30.65) µm in length and 8.7 (5.46-15.13) µm in width (n = 104). In
7 locomotive tropozoite, hyaloplasm was 1/5-1/10 of the call length (Fig. 1A-D). The
8 locomotive cells frequently change shapes and direction by the eruption of the hyaloplasm.
9 Bulbous uroid (Fig. 1B, C) or adhesive uroidal filament (Fig. 1D) was observed at the
10 posterior end of the locomotive cell. Non-moving oval or round tropozoites were
11 occasionally observed (Fig. Fig. 1E). The tropozoites robustly attached to the substrate and
12 no floating form was observed in the culture. The trophozoites contained several vacuoles
13 in the cytoplasm and one large nucleus (3.1-4.4 µm in diameter, n=20) (Fig. 1). The nucleus
14 was mostly round and occasionally irregular shaped. Small centric karyosome (0.4-0.8 µm,
15 in diameter n=20) and peripheral chromatins were observed in the nucleus (Fig. 1F-I). The
16 peripheral chromatins were arranged mostly uniformly and occasionally randomly around
17 the edge of the nucleus (Fig. 1F-I). Spherical cysts of diameter 10.7 (8.9-15.8) µm (n =
18 157) were observed in aged culture (over one month); these cysts were covered with
19 smooth cell wall (Fig. 2). The stained cysts contained 1-5 nuclei. Among 78 stained cysts,
20 49 (62.8%) contained four nuclei, 14 (17.9%) contained two nuclei, 11 (14.1%) contained
21 three nuclei, 3 (3.8%) contained one nucleus, and only 1 contained five nuclei. The nucleus
22 of the cyst was round in shape, with a small centric karyosome and uniform peripheral

1 chromatin (Fig. 2C). Several large vacuoles (probably glycogen vacuoles) and rod-like
2 chromatoid bodies were frequently observed in the cells (Fig. 2D-F).

3 **Transmission electron microscopy**

4 Trophozoites of *E. marina* contained a nucleus and several food vacuoles in the cytoplasm
5 (Fig. 3A). The nucleus was spherical or slightly irregular in shape and contained an
6 electron-dense small karyosome and peripheral chromatin (Fig. 3B). The distribution of the
7 peripheral chromatin was observed at the edge of the nucleus. Digested bacteria were
8 occasionally observed in the food vacuoles (Fig. 3C). Surface coat (glycocalyx) and scales
9 were not observed on the cell surface (Fig. 3D).

10 **Phylogenetic analysis**

11 We obtained a nearly complete sequence of small subunit (SSU) rDNA gene of *E. marina*
12 (1982 bp) with the GC contents of 29.4% (582/1982 bp). Single nucleotide signature that
13 can exclude other *Entamoeba* species was observed (glycine, position 1,675). Our
14 phylogenetic analysis showed the monophyly of the genus *Entamoeba* (Fig. 4). Within the
15 clade of *Entamoeba*, *E. marina* branched after two basal clades of uni- and octo-nucleated
16 cyst-producing species branched (Fig. 4). *E. marina* did not form a clade with other
17 quadri-nucleated cyst-producing species, unnamed isolates, or environmental sequences
18 (Fig. 4).

19 **Taxonomic Treatment**

20 Phylum: Amoebozoa Lühe, 1913.
21 Infraphylum: Archamoebae Cavalier-Smith, 1983
22 Class: Archamoebea Cavalier-Smith, 1983

1 Order: Pelobiontida Page, 1976

2 Family: Entamoebidae Chatton, 1925

3 Genus: *Entamoeba* Casagrandi and Barbagallo, 1897

4 *Entamoeba marina* n. sp. Shiratori et Ishida, 2014

5 **Description.** Anaerobic lobose amoeba collected from tidal flat sediment. Locomotive
6 trophozites clavate, with eruptive hyaloplasm at anterior region, and 21.3 (12.8-32.1) µm in
7 length and 10.3 (6.8-15.9) µm in width. Nucleus 3.1-4.4 µm in diameter with small centric
8 nuclear karyosome 0.4-0.8 µm in diameter and peripheral chromatin. Peripheral chromatin
9 arranged in mostly uniform and occasionally random. Cysts spherical with smooth cell wall
10 10.7 (8.9-15.8) µm in diameter, with several rod-like chromatoid bodies and a large
11 glycogen vacuole. Mature cysts with four nuclei.

12 **Differential diagnosis.** *E. marina* is different from other *Entamoeba* in having specific
13 substisution (glysine, in position 1,675) of SSU rRNA gene.

14 **Hapantotype**

15 One microscope slide (TNS-AL-58906s) and one EM block(TNS-AL-58906b) deposited to
16 the herbarium, the National Museum of Nature and Science (TNS), Tokyo.

17 **DNA sequence**

18 Small subunit ribosomal DNA, LC031816.

19 **Type locality**

20 Tidal flat sediments (30-cm depth) that appear to be anaerobic near the mouth of Shiira
21 river in Iriomote Island, Okinawa, Japan (latitude = 24.3241°N, longitude = 123.9110°E).

22 **Collection date**

1 February 5, 2012.

2 **Type strain**

3 The strain SRT209, used for describing this species maintained in the National Institute for

4 Environmental Studies (NIES, Tsukuba, Japan) as NIES- 3721.

5 **Etymology**

6 The term species name "*marina*" (marine) derived from Latin refers to the habitat of the

7 isolate.

8

DISCUSSION

Taxonomic justification of *Entamoeba marina* n. sp.

The light and electron microscopic observations showed that *E. marina* share morphological features with genus *Entamoeba*; *E. marina* and other members of *Entamoeba* are anaerobic lobose amoebae that form amoeboid trophozoites and cysts in their lifecycle. They also contain a nucleus that has a karyosome and peripheral chromatin, and lack mitochondria and Golgi apparatus. Our molecular phylogenetic analysis also revealed that *E. marina* belongs to the clade of *Entamoeba*. These morphological and phylogenetic data strongly support that *E. marina* is a member of genus *Entamoeba*.

The phylogenetic analysis showed that *E. marina* is genetically distinguished from other *Entamoeba* species. On the other hand, there are still a lot of *Entamoeba* species that lack the genetic information and some of them (*E. anatis*, *E. aulastomi*, and *E. serpentis*) are morphologically indistinguishable from *E. marina* (Bishop 1937; Ghosh 1968; Morgan and Hawkins 1948; Nöller 1912). Those species are parasites of ducks, leeches, and snakes and reported from samples of their feces and intestines but have not been found from sediment samples (Bishop 1937; Ghosh 1968; Morgan and Hawkins 1948; Noller 1912), whereas *E. marina* was collected from tidal flat sediment and the same sequences have not been reported from any part of animals. Based on the result of the phylogenetic analysis and the difference of collection site, we conclude that *E. marina* should be treated as a new species of *Entamoeba*, although there still be a possibility that *E. marina* can infect an unknown host animal.

Phylogenetic position and cyst morphology of *E. marina*.

1 Morphological characteristics had been used for important taxonomic traits for *Entamoeba*
2 species as well as their host specificity and pathogenicity (Dobell 1919; Ponce-Gordo and
3 Martínez-Díaz 2010), and especially the number of nucleus in the cyst was considered to
4 reflect phylogenetic relationship (Clark et al. 2006; Silberman et al 1999). However, recent
5 phylogenetic analyses showed that cyst morphologies of *Entamoeba* do not always reflect
6 phylogenetic relationship (Stensvold et al. 2010, 2011). The uninucleated cyst-forming
7 *Entamoeba* species split into three distinctive lineages and the tetranucleated cyst-forming
8 species formed a paraphyletic group including a clade of unnucleated cyst-forming species
9 (Stensvold et al. 2010, 2011). In our phylogenetic analysis, *E. marina* did not closely
10 related to other tetranucleated cyst-forming *Entamoeba* species and branched independently
11 after two basal clades of uni- and octo-nucleated cyst-producing species branched. Our tree
12 suggests possibilities that the tetranucleated cyst was acquired independently in the lineage
13 of *E. marina* or it was acquired in the common ancestor of *E. marina* and the other
14 tetranucleated cyst-producing species, and the cyst morphologies were changed in the
15 lineages of *E. gingivalis* and *E. suis*, and a clade including *E. bovis* respectively.

16 ***Entamoeba* species collected in natural environment.**

17 *E. marina* is the third species of *Entamoeba* that collected from natural environment. *E.*
18 *marina* and the other two species (*E. moshkovskii* and *E. ecuadoriensis*) are
19 morphologically indistinguishable each other (Clark and Diamond 1997; Heredia et al
20 2012) but our phylogenetic analysis showed that *E. marina* distantly positioned from the
21 other two “free living” *Entamoeba* species, which suggest that the adaptation to the natural
22 environment in *E. marina* occurred independently from these two species. It is uncertain

1 whether *E*. *marina* is a free-living species or not since we cannot exclude the possibilities

2 that *E*. *marina* cells in the sample of tidal flat sediment was derived from small animals (e.g.

3 sand worms, crabs, and shellfish) that living in the sediment or feces of animals visited the

4 tidal flat (e.g. bards, and mammals). However, *E*. *marina* lacks the tolerance to low

5 osmolality (fig. S1). It suggests that *E*. *marina* live exclusively in marine environments or

6 in marine animals. Detection of trophozoites from the sample of the tidal flat sediment and

7 experimental infection studies are required to clarify the natural habitat of *E*. *marina*.

1 **ACKNOWLEDGMENTS**

2 We thank Kumiko Nakada-Tsukui (National Institute of Infectious Diseases), Tetsuo
3 Hashimoto (University of Tsukuba), and Keitaro Kume (University of Tsukuba) for aid in
4 osmolality experiments. This work was supported by JSPS KAKENHI Grant Number
5 13J00587 and 23117004.

6

1 **LITERATURE CITED**

2 Bishop, A. 1937. Further observations upon Entamoeba aulastomi Noller. *Parasitology*,

3 29:57-68.

4 Brooke, M. M. & Melvin, D. M. 1984. Morphology of diagnostic stages of intestinal

5 parasites of humans (2nd ed.) US Department of Health and Human Services, Public

6 Health Service, Centers for Disease Control, Atlanta, Ga.

7 Bullock, W. L. 1966. *Entamoeba gadi* sp. n. from the rectum of the pollock, *Pollachius*

8 *virens* (L., 1758), with some observations on its cytochemistry. *J. Parasitol.*

9 52:679-684.

10 Cavalier-Smith, T., Chao, E. E. & Oates, B. 2004. Molecular phylogeny of Amoebozoa and

11 the evolutionary significance of the unikont Phalansterium. *Eur. J. Protistol.*

12 40:21-48.

13 Clark, C. G. & Diamond, L. S. 1997. Intraspecific variation and phylogenetic relationships

14 in the genus *Entamoeba* as revealed by riboprinting. *J. Eukaryot. Microbiol.*

15 44:142-154.

16 Clark, C. G., Kaffashian, F., Tawari, B., Windsor, J. J., Twigg-Flesner, A., Davies-Morel, M.

17 C. G., Blessmann, J., Ebert, F., Peschel, B., Van, A. L., Jackson, C. J., Macfarlane, L.

18 & Tannich, E. 2006. New insights into the phylogeny of *Entamoeba* species

19 provided by analysis of four new small-subunit rRNA genes. *Int. J. Syst. Evol.*

20 *Microbiol.* 56:2235-2239.

21 Diamond, L. S. & Clark, C. G. 1993. A redescription of *Entamoeba histolytica* Schaudinn,

22 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925.

1 *J. Eukaryot. Microbiol.* 40:340-344.
2 Dobell, C. 1919. The Amoebae Living in Man. A Zoological Monograph. J. Bale, Sons, and
3 Danielson, London.
4 Galtier, N. & Gouy, M. 1996. SEAVIEW and PHYLO_WIN: two graphic tools for
5 sequence alignment and molecular phylogeny. *Comput. Appl. Biosci.* 12:543-548
6 Ghosh, T. N. 1968. Observations on the type specimen of *Entamoeba serpentis* (Cunha and
7 Fonseca, 1917). *J. Protozool.* 15:164-166.
8 Ghosh, T. N. 1973. Studies on the Genus *Entamoeba*: I. *E. paulista* (Carini) Stabler & Chen,
9 1936. *Arch. Protistenk.* 115:162-167.
10 Hanaichi, T., Sato, T., Hoshino, M. & Mizuno, N. 1986. A stable lead stain by modification
11 of Sato's method. *J. Electron. Microsc.* 35:304-306.
12 Hartmann. M. 1910. Ueber eine neue Darmamoebe, *Entamoeba testudinis*, n. sp. *Mem. Inst.*
13 *Oswaldo. Cruz.* 2:3-10.
14 Heredia, R. D., Fonseca, J. A. & Lopez, M. C. 2012. *Entamoeba moshkovskii* perspectives
15 of a new agent to be considered in the diagnosis of amebiasis. *Acta. Tropica.*
16 123:139-145.
17 Kasai, F., Kawachi, M., Erata, M., Mori, F., Yumoto, K., Sato, M. & Ishimoto, M. 2009.
18 NIES-Collection. List of strains (8th ed.) *Jpn. J. Phycol. (Sôrui)* 57:1-350.
19 Katoh, K. & Toh, H. 2008. Recent developments in the MAFFT multiple sequence
20 alignment program. *Brief. Bioinform.* 9:286-298.
21 Kudo, R. R. 1954. Protozoology (4th ed.) Charles Thomas, Springfield, p. 446-459.
22

Lobeck, E. A. 1940. *Entamoeba pyrrhogaster* n. sp., with notes on other intestinal amoebae from salamanders. *J. Parasitol.* 26:243-272.

Morgan, B. B. & Hawkins, P. A. 1948. Veterinary Protozoology. Burgess Publishing Co., Minneapolis. p. 101.

Nakayama, T., Marin, B., Kranz, H. D., Surek, B., Huss, V. A. R., Inouye, I. & Melkonian, M. 1998. The basal position of scaly green flagellates among the green algae (Chlorophyta) is revealed by analyses of nuclear-encoded SSU rDNA sequences. *Protist* 149:367-380.

Nöller, W. 1912. *Entamoeba aulastomi* nov. spec., eine neue parasitische Amöbe aus dem Pferdeegel, (Aulastomum gulo Moq.-Tand.) *Arch. Protistenk.* 24:195-200.

Pimenta, P. F. P., Diamond, L. S. & Mirelman, D. 2002. *Entamoeba histolytica* Schaudinn, 1903 and *Entamoeba dispar* Brumpt, 1925: Differences in their cell surfaces and in the bacteria-containing vacuoles. *J. Eukaryot. Microbiol.* 49:209-219.

Ponce-Gordo, F. & Martínez-Díaz, R. A. 2010. Taxonomía y filogenia del género *Entamoeba*, Una revisión histórica. *Rev. Ibero-latinoam. parasitol.* 69:5-37.

Ponce-Gordo, F., Martinez, D. R. A. & Herrera, S. 2004. *Entamoeba struthionis* n. sp. (Sarcomastigophora: Endamoebidae) from ostriches (Struthio camelus). *Vet. Parasitol.* 119:327-335.

Ptáčková, E., Kostygov, A., Chistyakova, L., Falteisek, L., Frolov, A., Patterson, D., Walker, G. & Cepicka, I. 2013. Evolution of Archamoebeae: morphological and molecular evidence for pelobionts including *Rhizomastix*, *Entamoeba*, *Iodamoeba*, and *Endolimax*. *Protist* 164:380-410.

1 Ronquist, F. & Huelsenbeck, J. P. 2003. MRBAYES 3: Bayesian phylogenetic inference
2 under mixed models. *Bioinformatics* 19:1572-1574.
3 Sato, T. 1968. A modified method for lead staining of thin sections. *J. Electron. Microsc.*
4 17:158-159.
5 Silberman, J. D., Clark, C. G., Diamond, L. S. & Sogin, M. L. 1999. Phylogeny of the
6 genera *Entamoeba* and *Endolimax* as deduced from small-subunit ribosomal RNA
7 sequences. *Mol. Biol. Evol.* 16:1740-1751.
8 Stamatakis, A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis
9 of large phylogenies. *Bioinformatics* 30:1312-1313
10 Stensvold, C. R., Lebbad, M. & Clark, C. G. 2010. Genetic characterisation of uninucleated
11 cyst-producing *Entamoeba* spp. from ruminants. *Int. J. Parasitol.* 40:775-778.
12 Stensvold, C. R., Lebbad, M., Victory, E. L., Verweij, J. L., Tannich, E., Alfellani, M.,
13 Legarraga, P. & Clark, C. G., 2011. Increased sampling reveals novel lineages of
14 *Entamoeba*: consequences of genetic diversity and host specificity for taxonomy
15 and molecular detection. *Protistology* 162:525-541

FIGURE LEGEND

Fig. 1. Light micrographs of living and stained trophozoites of *Entamoeba marina* n. sp. K, karyosome; N, nucleus. Arrowheads indicate the peripheral chromatin. **A-F.** Living trophozoites. **G-I**. Iron-hematoxylin-stained trophozoites. Scale bar = 10 μm.

Fig. 2. Light micrographs of living and stained cysts of *Entamoeba marina* n. sp. GV, glycogen vacuole; K, karyosome; N, nucleus. Arrows indicate chromatoid bodies. Arrowheads indicate the peripheral chromatin. **A.** Living cyst. **B.** Chlorazol Black-stained cyst. **C-F.** Iron-hematoxylin-stained cysts. Scale bar = 10 μm.

Fig. 3. Transmission electron micrographs of *Entamoeba marina* n. sp. Arrowhead indicates a food vacuole containing digested bacteria. B, bacteria that digested in a food vacuole. FV, food vacuole; K, karyosome; N, nucleus. **A.** Approximately longitudinal section of the cell. Scale bar = 2 μm. **B.** A rounded nucleus with a small centric karyosome. Scale bar = 1 μm. **C.** A food vacuole containing digested bacteria. Scale bar = 1 μm. **D.** High magnification view of cell surface. Scale bar = 500 nm.

Fig. 4. Maximum likelihood tree of Archamoebae and *Dictyostelium discoideum* (out group) using 1,660 positions of small subunit (SSU) rDNA gene. Nucleus number in mature cysts are labeled as shown in Stensvold et al. (2011). Dashed branches are shortened to quarter their original length. Unnamed sequences are shown by sequence ID and accession number. Values on each nodes are bootstrap percentage (BP, left) and Bayesian

1 posterior probabilities (BPP, right). Only BP ≥50% and BPP ≥0.5 are shown.

SUPPORTING INFORMATION

***Entamoeba marina* n. sp.; a New Species of *Entamoeba* Isolated From Tidal Flat Sediment of Iriomote Island, Okinawa, Japan** by Takashi Shiratori & Ken-Ichiro Ishida

Fig. 1S. Effect of osmolality on the growth of *Entamoeba marina* n. sp. The y-axis shows cell density of trophozoites on the bottom of culture flask. For adjustment of osmolality, mTYGM-9 was diluted with NaCl solutions with different concentrations instead of natural seawater. *E. marina* was subcultured into culture flasks filled with mTYGM-9 diluted with NaCl solutions and incubated under dark conditions at 20°C. Trophozoites were counted under CKX41 inverted microscope (Olympus, Tokyo, Japan).

Fig. 1

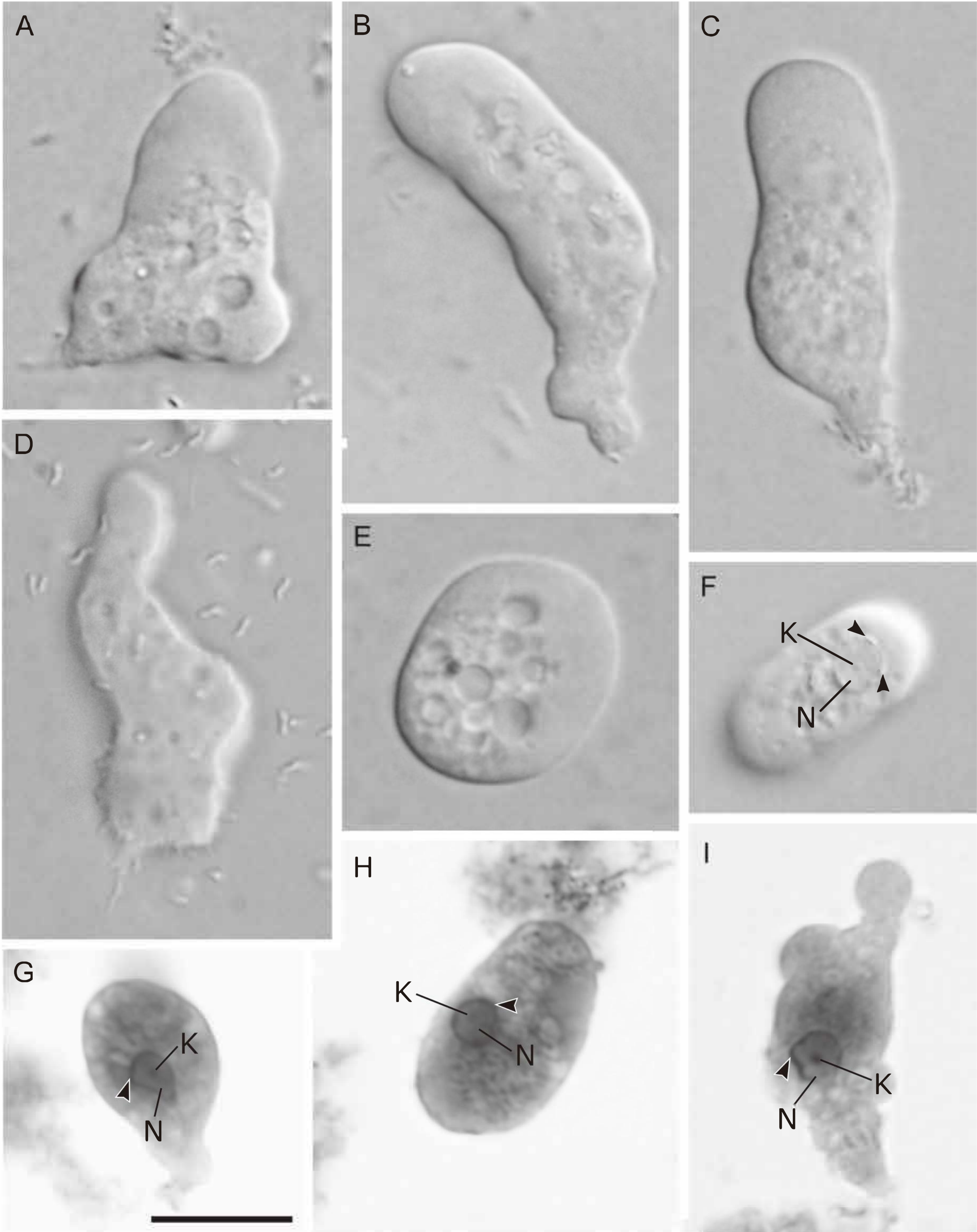

Fig. 2

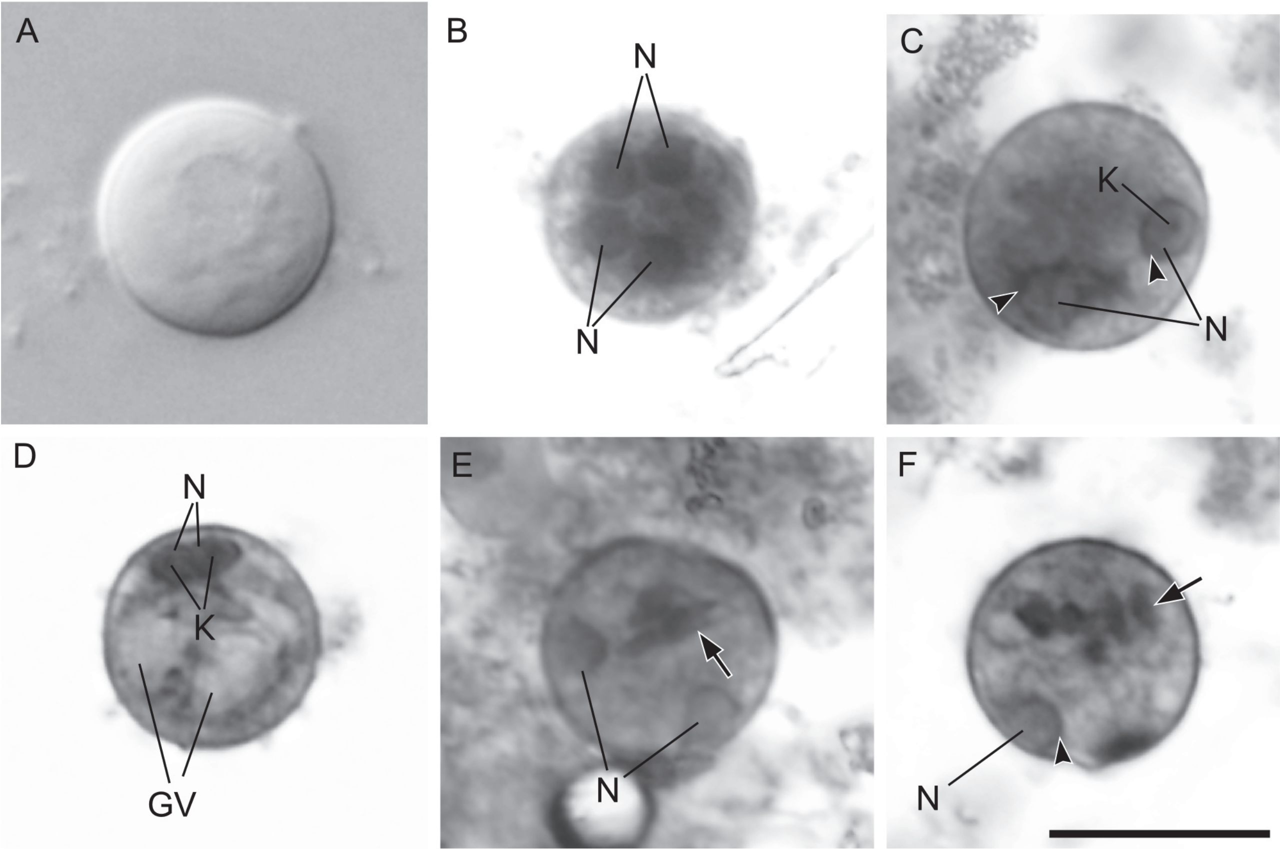

Fig. 3

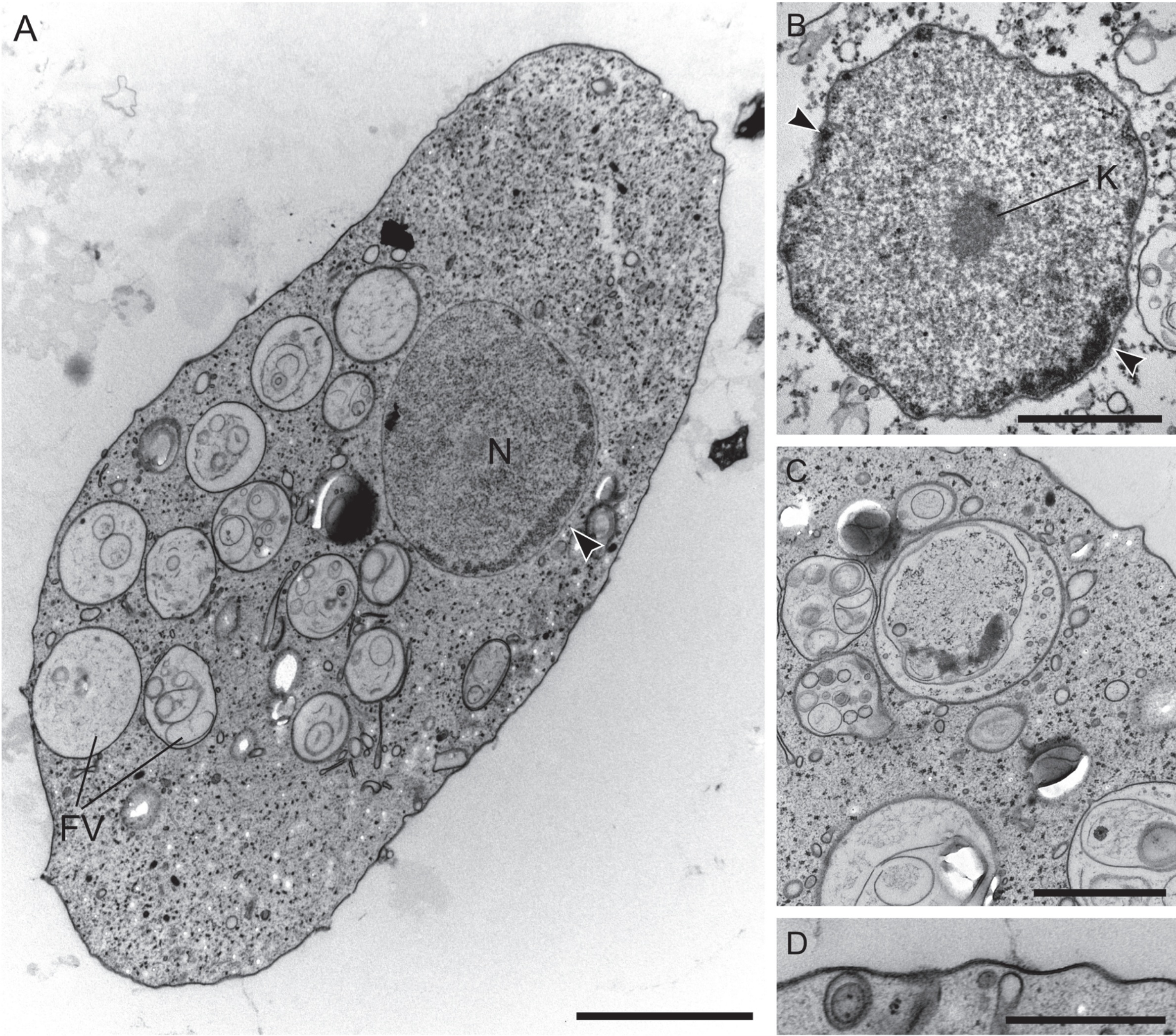

Fig. 4

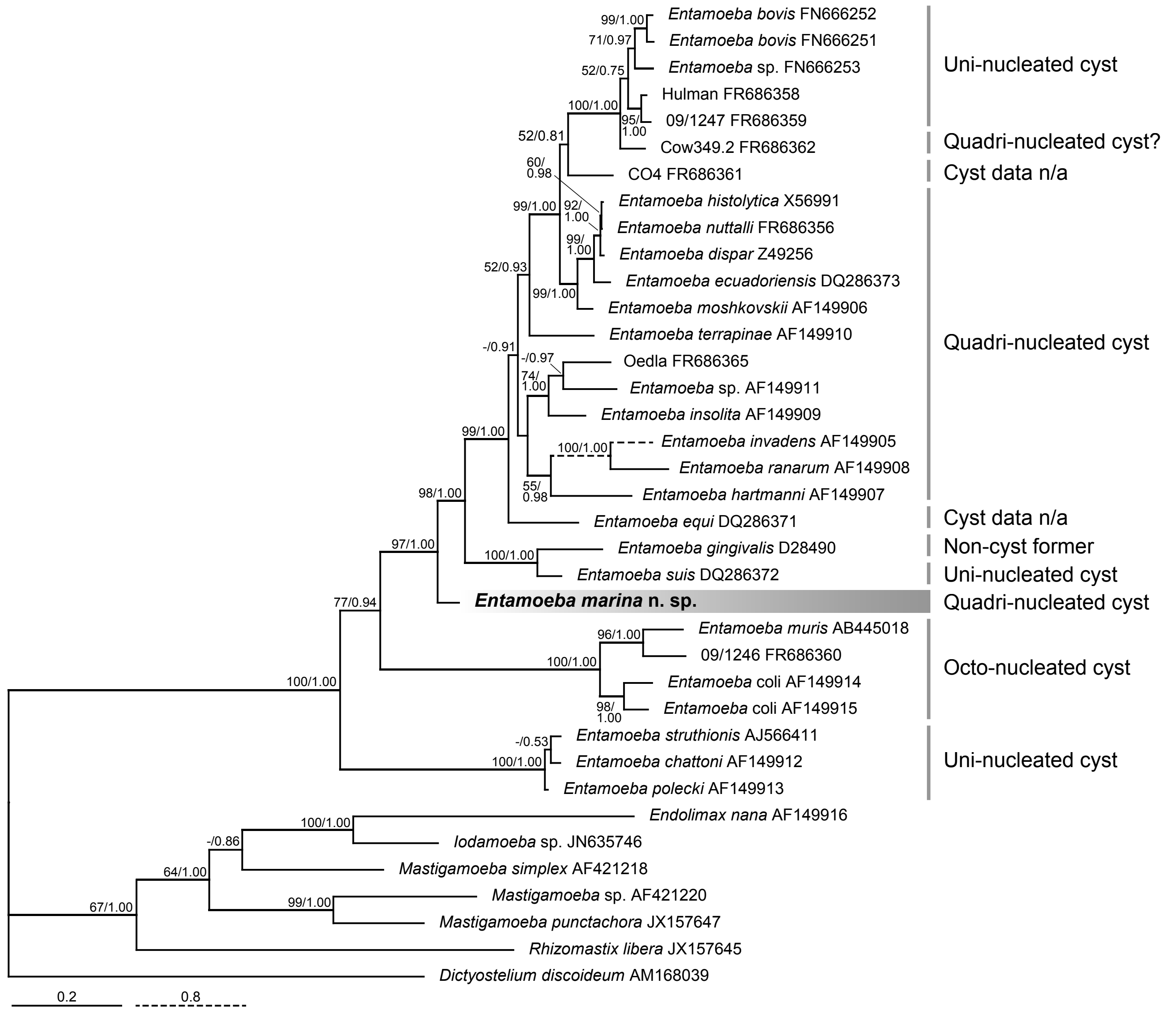

Fig. S1