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Cryptic Diversity of Free-Living Parabasalids, *Pseudotrichomonas keilini* and *Lacusteria cypriaca* n. g., n. sp., as Inferred from Small Subunit rDNA Sequences

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ABSTRACT. Ultrastructural and molecular phylogenetic evidence indicate that the Parabasalia consists of seven main subgroups: the Trichomonadida, Honigbergiellida, Hypotrichomonadida, Tritrichomonadida, Cristamonadida, Spirotrichonymphida, and Trichonymphida. Only five species of free-living parabasalids are known: *Monotrichomonas carabina*, *Ditrichomonas honigbergii*, *Honigbergiella* sp., *Tetratrichomonas undula*, and *Pseudotrichomonas keilini*. Phylogenetic analyses show that free-living species do not form a clade and instead branch in several different positions within the context of their parasitic relatives. Because the diversity of free-living parabasalids is poorly understood, the systematics of these lineages is in a significant state of disarray. In order to better understand the phylogenetic distribution of free-living parabasalids, we sequenced the small subunit rDNA from three different strains reminiscent of *P. keilini*; the strains were isolated from different geographical locations: (1) mangrove sediments in Japan and (2) sediments in Cyprus. These data demonstrated that the free-living parabasalids *P. keilini* and *Lacusteria cypriaca* n. g., n. sp., form a paraphyletic assemblage near the origin of a clade consisting mostly of parasitic trichomonadids (e.g. *Trichomonas vaginalis*). This paraphyletic distribution of similar morphotypes indicates that free-living trichomonadids represent a compelling example of morphostasis that provides insight into the suite of features present in the most recent free-living ancestor of their parasitic relatives.

Key Words. Excavate, *Honigbergiella*, new species, phylogeny, SEM, ultrastructure.

PARABASALIDS are anaerobic microeukaryotes that lack canonical mitochondria. Over 400 species of parabasalids have been described, and the vast majority of these are intestinal parasites/commensals of insects and vertebrates (Adl et al. 2007; Brugerolle and Lee 2000; Cepicka, Hampl, and Kulda 2010; Yamin 1979). The members of this diverse group of microeukaryotes share several morphological features, such as a parabasal apparatus (i.e. a Golgi complex associated with striated fibers), hydrogenosomes, and a closed mitosis with an external spindle (i.e. cryptopleuromitosis). The Parabasalia has historically been lumped into two main groups: Hypermastigida and Trichomonadida (Brugerolle 1991a; Corliss 1994; Honigberg 1963). The Hypermastigida consisted of species with large, highly multiflagellated cells (e.g. up to a thousand flagella) that thrive within the hindgut of wood-eating termites and cockroaches. The Trichomonadida, by contrast, consisted of much smaller and ultrastructurally simpler species, with six or fewer flagella per mastigont, that are generally beneficial symbionts, commensals, or pathogens in a wide variety of animals, including humans. *Trichomonas vaginalis*, for instance, thrives within the human urogenital tract, *Trichomonas tenax* thrives within the oral cavity of humans, and *Pentatrichomonas hominis* and *Dientamoeba fragilis* inhabit the human intestinal tract (Brugerolle and Lee 2000; Honigberg 1989; Honigberg and Brugerolle 1989; Honigberg, Mattern, and Daniel 1968; Stark et al. 2006).

Improved phylogenetic resolution of a larger sample of parabasalid species has now resulted in the recognition of seven main subgroups rather than two: Honigbergiellida, Trichomonadida, Hypotrichomonadida, Tritrichomonadida, Cristamonadida, Spirotrichonymphida, and Trichonymphida (Cepicka et al. 2010). Only a few free-living parabasalids have been described so far. *Honigbergiella*, *Monotrichomonas*, and *Ditrichomonas* are found in anoxic environments and possess one to three anterior flagella, depending on the genus, and a posterior flagellum (Bernard, Simpson, and Patterson 2000; Farmer 1993; Hampl et al. 2007). Phylogenetic analyses have demonstrated that these three genera cluster as a monophyletic assemblage forming the Honigbergiellidae. *Tetratrichomonas undula*, by contrast, was shown to branch within the Trichomonadida (Cepicka et al. 2010). *Pseudotrichomonas* was the first free-living parabasalid discovered (Bishop 1935, 1939) and is tentatively classified within the Honigbergiellidae based on ultrastructural features (Cepicka et al. 2010).

A culture labeled ''*Pseudotrichomonas keilini*'' is available in the American Type Culture Collection (ATCC 50321), and the small subunit (SSU) rDNA sequence derived from this culture is deposited in GenBank (AY319274). However, the identification of this strain as *P. keilini* was shown to be incorrect (Hampl et al. 2007); this strain lacks an undulating membrane, a diagnostic feature of *P. keilini* (Bishop 1935; Brugerolle 1991b; Lavier 1936a, b). The morphology of ATCC 50321 is identical to *Honigbergiella ruminantium*, and this inference has been confirmed with phylogenetic analyses of SSU rDNA sequences. Accordingly, ATCC 50321 has been renamed as a member of the genus *Honigbergiella* (Hampl et al. 2007). Moreover, while Dufernez et al. (2007) reported an endobiotic *Pseudotrichomonas* from cattle, this flagellate was also subsequently shown to belong to the genus *Honigbergiella* (see Cepicka et al. 2010). Therefore, neither a living culture nor a DNA sequence from a legitimate representative of *P. keilini* was available before this study.

Parasitic parabasalids from several different animal hosts have been characterized at both the morphological and molecular phylogenetic levels. Investigations of free-living parabasalids, by contrast, are scarce, mainly due to the relatively low number of living cultures available and to their low relevance to human health. Because the diversity and evolutionary history of free-living parabasalids is so poorly understood, the systematics of these lineages is in a significant state of confusion. Accordingly, we isolated, cultivated, and sequenced the SSU rDNA from three strains of free-living trichomonads reminiscent of the *P. keilini* morphotype that were isolated from different geographical locations: (1) marine mangrove sediments near Ishigaki Island (Japan) and (2) sediments of uncertain salinity near Voroklini (Cyprus). The main goal of this work was to

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better understand the diversity and phylogenetic distribution of free-living parabasalids relative to parasitic lineages within the group.

MATERIALS AND METHODS

Sampling and culture conditions. *Pseudotrichomonas keilini* NY0170 (Japan) was collected from mangrove sediments sampled in Ishigaki Island, Okinawa, Japan (24°46′N, 124°12′E) on September 19, 2005. Sediment samples ~2–4 cm deep were collected at low tide. The medium used for enrichment culture and maintenance of *P. keilini* NY0170 (Japan) was 5% modified PYNFH medium (ATCC medium 1034) prepared with sterilized seawater. A single cell was isolated by micropipetting from an enrichment culture and the clonal culture was established and maintained at 22 °C with passages every week. *Pseudotrichomonas keilini* NY0170 (Japan) has been deposited at the ATCC, Manasas, VA, USA (accession number PRA-328).

A different strain of *P. keilini* LIVADIAN (Cyprus) and a novel lineage, namely *Lacusteria cypriaca* n. g., n. sp. (Cyprus), were collected in a single sample of sediments from a lake close to Voroklini village, Cyprus (34°58′N, 33°39′E), on February 26, 2008. The lake was close, but not connected, to the sea; the salinity of the lake was not determined. The sample was transferred to the Czech Republic and stored in a 15-ml tube at room temperature. The two strains were independently cultivated from the original sample at different times: 1 wk after the sampling time for *L. cypriaca*, and 1 mo after the sampling time for *P. keilini*. In both cases 2 ml of the sample were inoculated into Sonneborn's *Paramecium* medium (ATCC medium 802), and the culture was transferred into new medium once a week. However, the number of trichomonads in both the cultures started to decline after a few transfers. They were therefore inoculated into Dobell and Leidlaw's (1926) biphasic medium. *Pseudotrichomonas keilini* LIVADIAN (Cyprus) was able to grow in this medium indefinitely and the medium was used for the routine cultivation of this strain. *Trepomonas* sp. and an unidentified ciliate also persisted in this culture of *P. keilini* LIVADIAN (Cyprus). The strain was cultivated at room temperature with transfers occurring once per week. In contrast, *L. cypriaca* (Cyprus) failed to grow in Dobell-Leidlaw's biphasic medium. The final medium for this isolate consisted of a 9:1 mixture of Sonneborn's *Paramecium* medium and TYSGM-9 (trypticase–yeast extract–serum–gastric mucin) medium without mucin and Tween 80 (Diamond 1982). *Trepomonas* sp. and an unidentified amoeba were also present in the culture of *L. cypriaca* and the density of *L. cypriaca* was usually very low. The strain was cultivated at room temperature with transfers occurring once per week. The strains LIVADIAN containing *P. keilini*, *Trepomonas* sp. and an unidentified ciliate, and LA containing *L. cypriaca* n. g., n. sp. *Trepomonas* sp. and an unidentified amoeba have been deposited in the culture collection of the Department of Parasitology of Charles University in Prague, Prague, Czech Republic.

Light and electron microscopy. Light microscopic observations were performed using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Leica DC500 digital camera (Leica Microsystems, Wetzlar, Germany) or an Olympus Microscope BX51 (Olympus Corporation, Tokyo, Japan) equipped with camera Olympus DP70.

In addition to live cells, the general morphology of *L. cypriaca* n. g., n. sp. (Cyprus) was examined in protargol-stained preparations. Moist films spread on cover slips were prepared from pelleted cultures obtained by centrifugation at 500 *g* for 8 min. The films were fixed in Bouin–Hollande's fluid for 10 h, washed with 70% ethanol, and stained with 1% (w/v) protargol (Bayer, I. G. Farbenindustrie, Frankfurt am Main, Germany) following Nie's (1950) protocol.

For scanning electron microscopy (SEM), cells of *P. keilini* NY0170 (Japan) were mixed with an equal volume of fixative containing 5% (v/v) glutaraldehyde and 0.2 M sucrose in 0.2 M sodium cacodylate buffer (SCB) (pH 7.2) and mounted on glass plates coated with poly-L-lysine at room temperature for 30 min. The glass plates were rinsed with 0.2 M SCB and fixed in 1% (w/v) osmium tetroxide (OsO_4) for 30 min. The fixed cells were then rinsed with 0.2 M SCB and dehydrated with a graded ethanol series from 30% to absolute ethanol. Samples were critical point dried with CO_2 using a Tousimis Samdri Critical Point Dryer (Tousimis Research Corporation, Rockville, MD). Samples were then coated with gold using a Cressington 208HR high-resolution Sputter Coater (Cressington Scientific Instruments Ltd., Watford, UK), and observed with a Hitachi S-4700 field emission SEM (Hitachi Ltd., Tokyo, Japan).

For transmission electron microscopy, cell suspensions of *P. keilini* NY0170 (Japan) were mixed with an equal volume of fixative containing 5% (v/v) glutaraldehyde and 0.2 M sucrose in 0.2 M SCB (pH 7.2) at room temperature for 45 min. Cells were aggregated into a pellet by centrifugation at 1,000 *g* for 5 min and then rinsed with 0.2 M SCB (pH 7.2). The specimens were then fixed in 1% (w/v) OsO_4 in 0.2 M SCB (pH 7.2) at room temperature for 1 h followed by dehydration through an ethanol series, and substitution with acetone. The specimens were embedded in resin (Epon 812). Ultrathin sections were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems) and double-stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963). Ultrathin sections were observed using a Hitachi H7600 TEM (Hitachi Ltd.).

DNA extraction, polymerase chain reaction (PCR) amplification, alignment, and phylogenetic analysis. Nearly the entire SSU rRNA genes of three different strains were sequenced at three different institutions: (1) the University of British Columbia, Canada for the 5′-end of the SSU rRNA gene of *P. keilini* NY0170 (Japan), (2) the University of Tsukuba for the rest of the SSU rRNA gene of *P. keilini* NY0170 (Japan), (3) Charles University, Czech Republic for nearly the entire SSU rRNA gene of both *P. keilini* LIVADIAN (Cyprus) and *L. cypriaca* (Cyprus). The 5′-end of the SSU rRNA gene was amplified from genomic DNA extracted from the cells of *P. keilini* NY0170 (Japan) using the MasterPure Complete DNA and RNA purification Kit (Epicentre® Biotechnologies, Madison, WI). The PCR was performed using a total volume of 25 μl and the PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare, Buckinghamshire, UK). Approximately 1,100 bp of the SSU rRNA gene were amplified from the genomic DNA using eukaryotic universal primers (PF1: 5′-GCGCTACC TGGTTGATCCTGCCAGT-3′ and nomet1134R: 5′- TTTAAG TTTCAGCCTTGCG-3′). The PCR protocol had an initial denaturation stage at 95 °C for 2 min; 35 cycles involving 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1.5 min; and final extension at 72 °C for 5 min. The amplified DNA fragments were purified from agarose gels using UltraClean 15 DNA Purification Kit (MO Bio Laboratories Inc., Carlsbad, CA). (2) Nearly the entire SSU rRNA gene was amplified from genomic DNA using eukaryotic universal primers (18sF: 5′-AACCTGGTTGATCCTGCCAG-3′ and 18sR: 5′-CYGCAGGTTCACCTACGGAA-3′). The PCR protocol had an initial denaturation stage at 95 °C for 1 min; 35 cycles involving 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2.5 min; and final extension at 72 °C for 7 min. The amplified DNA fragments were purified from agarose gels using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Both the sequences were determined independently within 2 yr and were ensured to derive from the same strain of *P. keilini* NY0170 (Japan). Two clones were sequenced in total and no polymorphisms were detected.

Genomic DNA of *P. keilini* LIVADIAN (Cyprus) and *L. cypriaca* (Cyprus) was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Nearly the entire SSU rRNA gene was amplified using

trichomonad-specific primers 16Sl: 5′-TACTTGGTTGATCCTGCC-3′ (Tachezy et al. 2002) and 16SRR: 5′-TCACCTACCGTTACCT TG-3′ (Cepicka et al. 2005). The PCR was performed in a volume of 50 µl using *Taq* DNA polymerase (Fermentas) in an appropriate buffer. The PCR protocol had an initial denaturation stage at 94 °C for 4 min; 30 cycles involving 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2.5 min; and final extension at 72 °C for 10 min. The amplified DNA fragments were purified using QIAquick PCR Purification Kit (Qiagen) and were directly sequenced. Both the isolates were sequenced 3 times independently within a year to ensure that the cultures were stable and did not contain more parabasalid species. No intrastrain sequence polymorphism was detected. The three SSU rDNA sequences obtained in this study have been deposited in GenBank under accession numbers HM581663 (*P. keilini*, Japan, NY0170), HM748760 (*P. keilini*, Cyprus, LIVADIAN), HM748759 (*L. cypriaca* n. g., n. sp., Cyprus).

The newly obtained sequences were aligned with those from taxa representing the major lineages of parabasalids, forming a 56-taxon alignment with 1,094 unambiguously aligned positions. PhyML was used for maximum-likelihood (ML) analyses with the GTR models incorporated invariable sites and a discrete gamma distribution (GTR+Γ+I). The model was selected by Modeltest 3.7 (Posada and Crandall 1998) in conjunction with PAUP*. Maximum-likelihood bootstrap analysis (1,000 replicates) was conducted with the same settings.

The alignment was also analyzed with the Bayesian method using MrBayes program 3.0 (Huelsenbeck and Ronquist 2001) under GTR+Γ+I models. The program was set to operate with a gamma distribution and four Monte–Carlo–Markov chains starting from a random tree. A total of 2,000,000 generations was calculated with trees sampled every 50 generations and with a prior burn-in of 100,000 generations with 2,000 sampled trees discarded. A majority rule consensus tree was constructed from 38,000 post-burn in trees. Posterior probabilities corresponded to the frequency at which a given node was found in the post-burn in trees.

RESULTS

Morphology of *Pseudotrichomonas keilini* NY0170 (Japan), *Pseudotrichomonas keilini* LIVADIAN (Cyprus), and *Lacusteria cypriaca* n. g., n sp. (Cyprus). The three strains of free-living trichomonads were similar under the light microscope, except for differences in details of the undulating membrane (Fig. 1–6). Two of the strains, namely *P. keilini* NY0170 (Japan) and *P. keilini* LIVADIAN (Cyprus), corresponded to the diagnostic features of *P. keilini* described by Bishop (1935, 1939) and Brugerolle (1991b), which included three anterior flagella, an undulating membrane, and the absence of a costa. Because the recognition of *P. keilini* in natural samples has been confounded following the deposition of the misidentified ATCC 50321, the morphological features of our three strains will be described briefly here. Although our two strains of *P. keilini* were morphologically identical, they were isolated from very different geographical locations and aquatic habitats: *P. keilini* NY0170 (Japan) was collected from mangrove sediments in Japan and maintained in seawater medium, and *P. keilini* LIVADIAN (Cyprus) was isolated from a pond of uncertain salinity in Cyprus and maintained in freshwater medium. The living cells of *P. keilini* NY0170 (Japan), excluding the extended axostyle, were 10.7 µm (8.5–13.7 µm) long and 7.8 µm (5.3–9.9 µm) wide ($n = 20$) (Fig. 1, 2). The *Trichomonas*-type axostyle extended from the posterior end of the cell and the protruding part was 7.1 µm (4.1–11.9 µm) long. Living cells of *P. keilini* LIVADIAN (Cyprus), excluding the extended axostyle, were 9.4 µm (8.2–10.5 µm) long and 7.1 µm (6.1–8.3 µm) wide ($n = 20$) (Fig. 3, 4). The *Trichomonas*-type axostyle extended from the posterior end of the cell and the protruding part was 5.8 µm (4.9–7.0 µm) long. The cells of both strains were

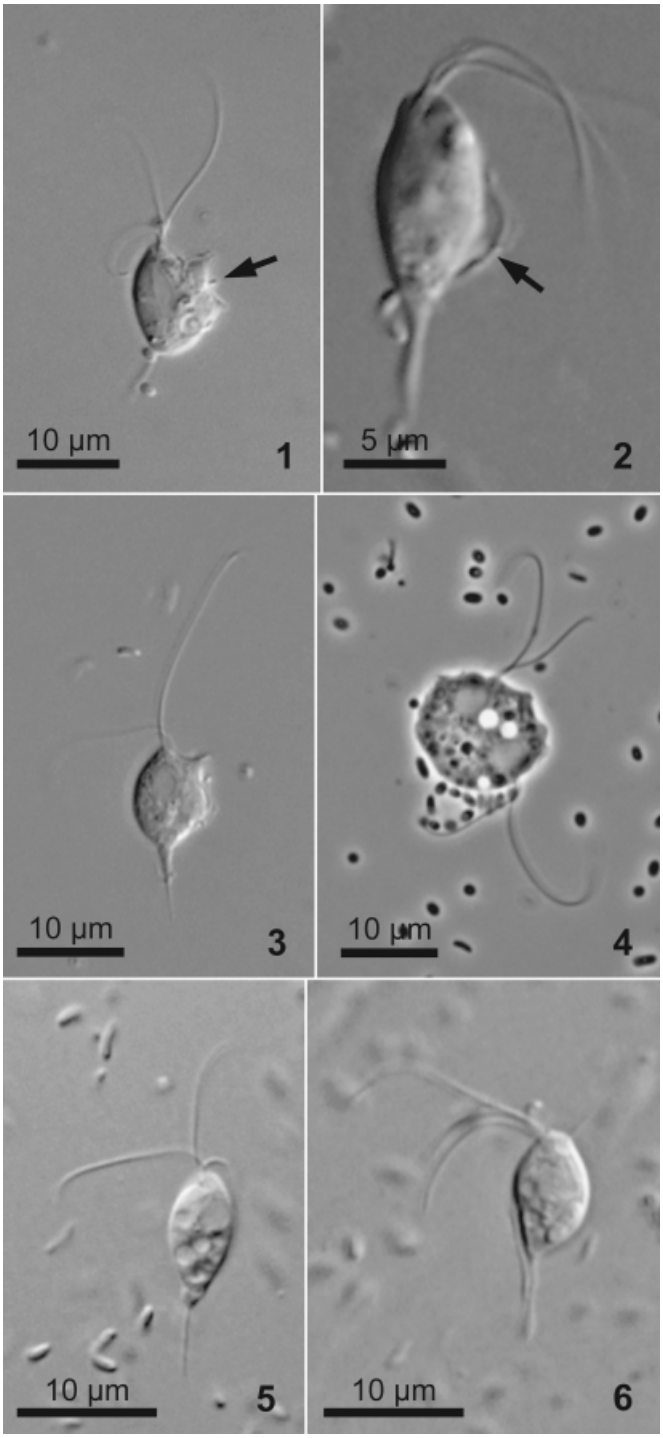

Fig. **1–6.** Light micrographs of *Pseudotrichomonas keilini* NY0170 (Japan), *P. keilini* LIVADIAN (Cyprus), and *Lacusteria cypriaca* n. g., n. sp. (Cyprus). **1.** A cell of *P. keilini* NY0170 (Japan) with three anterior flagella and a posterior flagellum (arrow) along with an undulating membrane. **2.** A cell of *P. keilini* LIVADIAN (Cyprus) with three anterior flagella and a posterior flagellum (arrow) along with an undulating membrane. **3.** An immature cell of *P. keilini* NY0170 (Japan) with two anterior flagella. **4.** A dividing cell of *P. keilini* NY0170 (Japan) with two nuclei and two pairs of anterior flagella. This cell is slightly compressed and enlarged by a cover slip. **5.** A cell of *L. cypriaca* n. g., n. sp. (Cyprus) showing three anterior flagella and an axostyle. **6.** A cell of *L. cypriaca* (Cyprus) showing three anterior flagella and an undulating membrane.

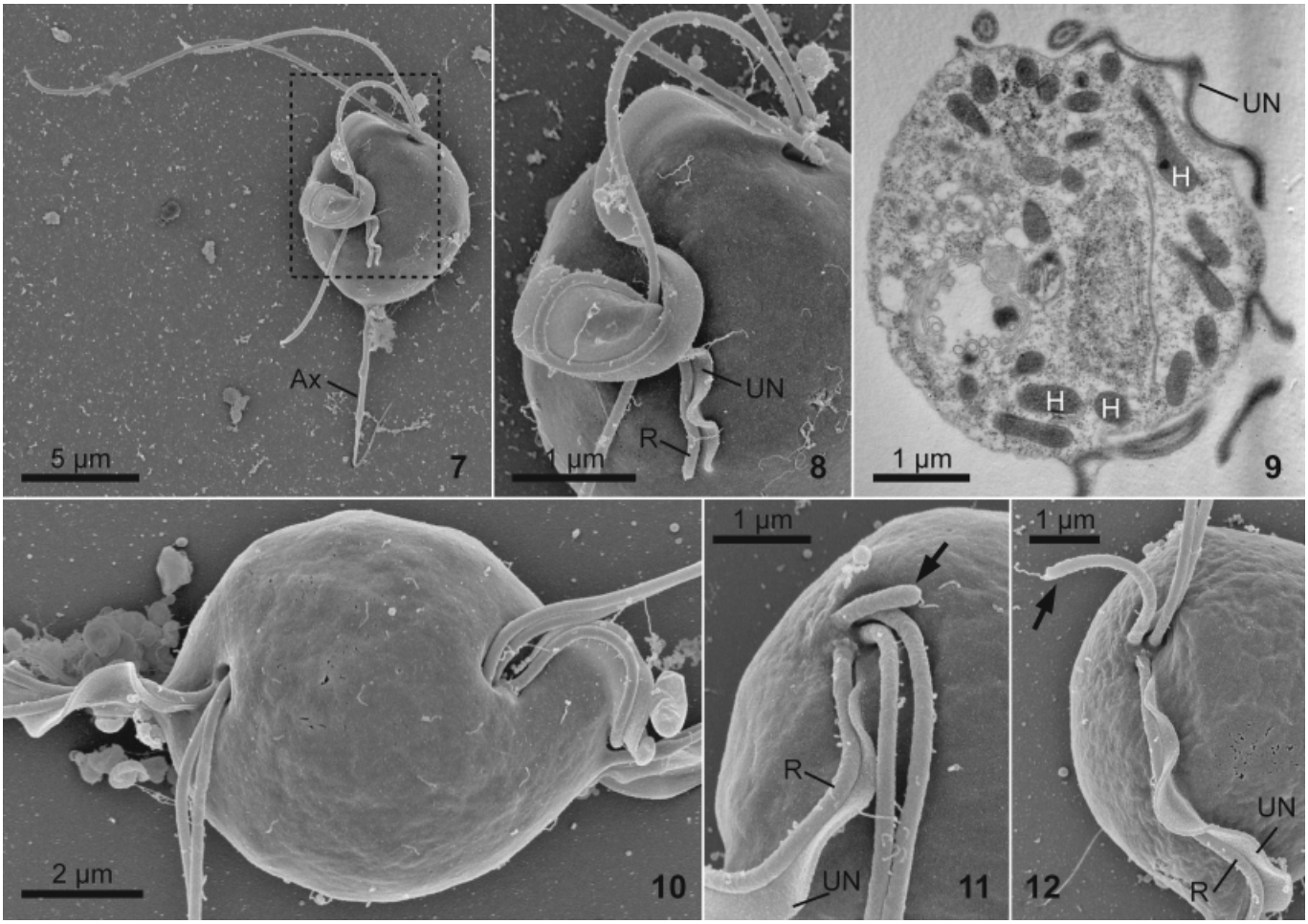

Fig. **7–12.** Electron micrographs of *Pseudotrichomonas keilini* NY0170 (Japan). **7.** A scanning electron micrograph showing three anterior flagella, posterior flagellum with an undulating membrane and an axostyle (Ax). See Fig. 8 for the dotted box. **8.** Detail in the dotted box of Fig. 7 showing the lamelliform undulating membrane (UN) and the termination of the recurrent flagellum (R). **9.** Transmission electron micrograph showing the UN and hydrogenosomes (H). A costa was not observed under the UN. **10.** A dividing cell with two pairs of two anterior flagella and a posterior flagellum. **11.** An immature cell with a short anterior flagellum (arrow) adjacent to two mature anterior flagella. **12.** An immature cell with a short anterior flagellum (arrow) adjacent to two mature anterior flagella.

highly plastic in shape and had three unequal anterior flagella and a posterior recurrent flagellum with a well-developed lamellar-type undulating membrane displaying several waves (Fig. 1–4). The undulating membrane usually reached the posterior end of the cell body. The distal end of the posterior flagellum did not extend beyond the undulating membrane (Fig. 7, 8). The cells contained a large number of elongated hydrogenosomes and lacked a supporting costa beneath the undulating membrane (Fig. 9). Dividing cells and/or immature cells had only two anterior flagella, rather than the normal three (Fig. 3, 4, 10–12). We did not observe a comb-like structure, an infrakinetosomal body, or cyst formation. The strains grew at 16–24 °C and died at 37 °C.

The culture of *L. cypriaca* n. g., n. sp. (Cyprus) consisted of cells that were superficially similar to the morphology of the two *P. keilini* strains (Fig. 5, 6). The living cells of *L. cypriaca*, excluding the extended axostyle, were 8.0 μm (5.0–10.7 μm) long and 5.5 μm (3.6–7.6 μm) wide ($n = 20$). The *Trichomonas*-type axostyle extended from the posterior end of the cell and the protruding part was 4.9 μm (3.1–6.7 μm) long. In contrast to *P. keilini*, which possessed a conventional undulating membrane, the undulating membrane of *L. cypriaca* was weakly developed and usually displayed only a single big wave (Fig. 5, 6). Moreover, the undulating membrane was highly variable in length; sometimes it reached only to one half of the cell whereas in other cases it reached to the posterior end. The distal end of the recurrent flagellum was usually free. However, in some cells the free portion was rather short or could not be detected at all. Protargol-stained preparations allowed us to examine the internal structures of the cells as well. A parabasal body formed a small dark disc close to the nucleus, and a pelta was normally developed. A costa was not observed (Fig. 13–16).

Molecular phylogenetic positions of *Pseudotrichomonas keilini* NY0170 (Japan), *Pseudotrichomonas keilini* LIVADIAN (Cyprus), and *Lacusteria cypriaca* n. g., n. sp. (Cyprus). We determined the nearly complete sequence of the SSU rRNA gene from all three strains and used these to evaluate their phylogenetic positions within the Parabasalia using a 53-taxon alignment. The ML and Bayesian analyses of this alignment resulted in a poorly resolved backbone that gave rise to the following strongly supported clades: the Trichonymphida, Hypotrichomonadida, Cristamonadida, and Spirotrichonymphida. The Honigbergiellida and the Tritrichomonadida formed paraphyletic assemblages. The

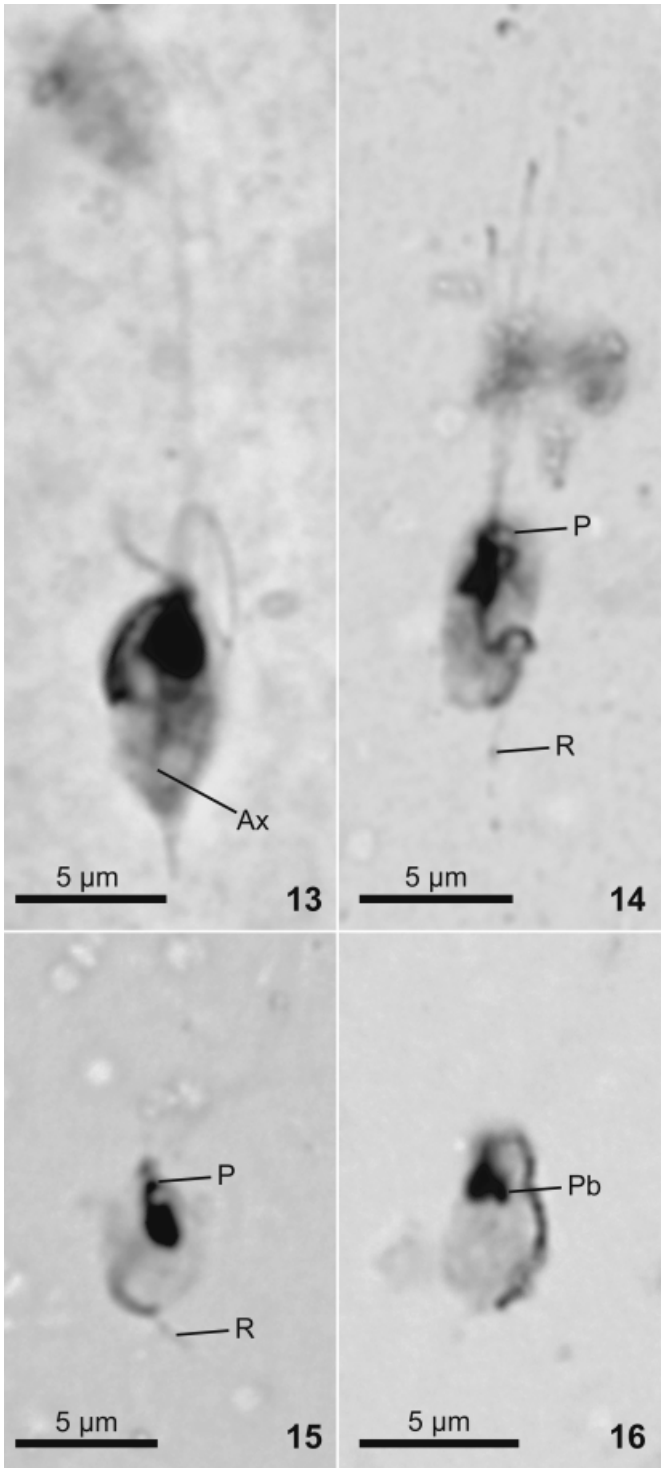

Fig. **13–16.** Protargol-stained specimens of *Lacusteria cypriaca* n. g., n. sp. (Cyprus) showing an axostyle (Ax), a pelta (P), a parabasal body (Pb), and a recurrent flagellum (R).

SSU rDNA sequences from the three different strains did not form an independent clade and did not branch with the free-living *T. undula* or the free-living lineages within the Honigbergiellida. Instead, they formed two independent lineages closely related to the Trichomonadida clade (Fig. 17). The two strains of *P. keilini* formed a robust clade within the Trichomonadida, at the base of the so-called ''*Pentatrichomonas*-group'' (i.e. *Pentatrichomonas*, *Pseudotrypanosoma*, and *Trichomitopsis*) (Cepicka et al. 2010). *Lacusteria cypriaca* n. g., n. sp. was more basal and branched as the sister lineage to the entire trichomonadid clade with high statistical support (Fig. 17).

DISCUSSION

Interpretation of morphology and phylogenetic position of *Pseudotrichomonas* and *Lacusteria* n. g. Following the discovery of *P. keilini* as the first known free-living parabasalid (Bishop 1935, 1939), this species was subsequently reported from several different geographical locations, mainly in freshwater environments (Bernard et al. 2000; Brugerolle 1991b; Lavier 1936a, 1936b). The morphology of our two independent strains of *P. keilini* (NY0170, Japan, and LIVADIAN, Cyprus) was identical to the morphology of *P. keilini* isolated from freshwater ponds by Bishop (1935, 1939) and Brugerolle (1991b). Although Bishop (1935) reported a constant number of anterior flagella, we observed immature cells with fewer anterior flagella in both our strains of *P. keilini*. Because *P. keilini* NY0170 (Japan) was derived from a single diligently washed cell, the possibility that the culture was contaminated by another trichomonad species is highly unlikely. We interpret the presence of cells with a different number of anterior flagella as reflecting different stages in the cell cycle.

Before this molecular phylogenetic study, *P. keilini* was tentatively classified within the Honigbergiellida based on (1) the absence of an infrakinetosomal body, comb-like structure, and costa, and (2) the presence of a lamelliform undulating membrane and three anterior flagella (Cepicka et al. 2010). This preliminary placement of *Pseudotrichomonas* was also supported by the fact that the Honigbergiellida contained the only free-living trichomonads. However, our phylogenetic analyses of the SSU rDNA data clearly demonstrated that these strains robustly clustered within the Trichomonadida instead of the Honigbergiellida.

The SSU rDNA sequences from *P. keilini* NY0170 (Japan) and *P. keilini* LIVADIAN (Cyprus) were similar and differed by only 21 of 1,499 bp. The SSU rDNA sequences from *L. cypriaca* n. g., n. sp. differed from the sequences from *P. keilini* NY0170 (Japan) and *P. keilini* LIVADIAN (Cyprus) by 112 of 1,515 bp and 111 of 1,513 bp, respectively. These differences in the SSU rDNA sequences were reflected in the phylogenetic trees, which showed that *L. cypriaca* and *P. keilini* do not form a clade. Although the two species were similar when observed under the light microscope, the molecular phylogenetic results were consistent with subtle morphological differences in the details of the undulating membrane. *Lacusteria cypriaca* n. g., n. sp. robustly formed a sister lineage to the Trichomonadida; the clade consisting of *P. keilini* NY0170 (Japan) and *P. keilini* LIVADIAN (Cyprus) was specifically related to the *Pentatrichomonas*-group within the Trichomonadida, albeit without strong statistical support. Nonetheless, neither species was closely related to the free-living *T. undula*.

The Trichomonadida includes seven or eight genera, depending on the status of *Trichomonoides trypanoides*, which possess a B-type costa and four or five anterior flagella. Because both *Pseudotrichomonas* and *Lacusteria* n. g. possess three anterior flagella and lack a costa, the close relationship between these lineages and the Trichomonadida was unexpected on the basis of morphological features alone; therefore, the molecular phylogenetic data were necessary to recover this relationship.

There are two types of costae in the Parabasalia: the A type found in the Tritrichomonadidae, *Trichomitus*, and some cristamonadids, and the B type found only in the Trichomonadida. However, several parabasalids lack a costa altogether, such as *Monocercomonas*, *Simplicimonas*, the Dientamoebidae, the

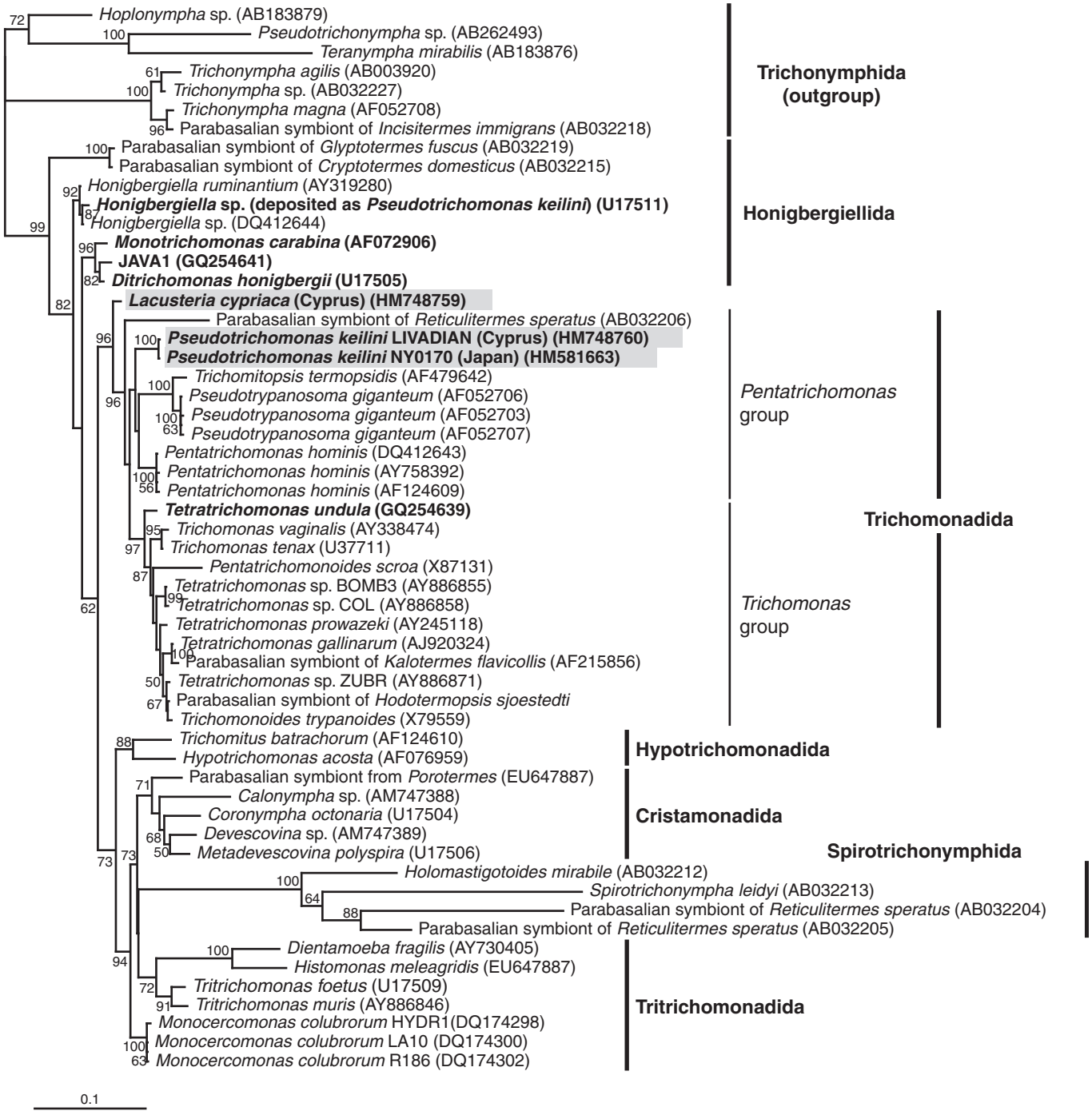

Fig. **17.** Maximum-likelihood (ML) tree inferred from 56 small subunit (SSU) rRNA sequences and the GTR+G+I model. Maximum-likelihood bootstrap values over 50 are shown at the nodes and Bayesian posterior probabilities ≥ 0.95 are represented by thick lines. The sequences recovered from free-living parabasalians are in bold. GenBank accession numbers follow each taxon name. The scale bar represents inferred evolutionary distance in changes/site.

Honigbergiellida, the Spirotrichonymphida, the Trichonymphida, and most cristamonds (Cepicka et al. 2010; Hampl et al. 2004). The mosaic distribution of costate/acostate parabasalids in molecular phylogenetic trees can be explained by either multiple independent origins of a costa, multiple independent losses of a costa, or a combination of independent losses and gains (see Cepicka et al. 2010). The hypothesis that the costa has been lost several times independently has been favored in almost every previous study. In this context, the absence of a costa in both *Pseudotrichomonas* and *Lacusteria* might also represent secondary losses, even though both the *Trichomonas*-group and the *Pentatrichomonas*-group possess a B-type costa. Moreover, the character evolution of fewer flagella in *Pseudotrichomonas* and *Lacusteria* (i.e. whether three anterior flagella represents a shared ancestral state or independent losses from an ancestral state consisting of four to five anterior flagella) remains a

matter of speculation within the current molecular phylogenetic context.

Nonetheless, we have established *L. cypriaca* as a new genus and species because the morphological and molecular phylogenetic data presented here demonstrate that these free-living trichomonadids constitute a separate and distinct lineage within the Parabasalia.

TAXONOMIC SUMMARY

Phylum Parabasalia
Class Trichomonadea
Order Trichomonadida
Family Trichomonadidae
Pseudotrichomonas Bishop, 1939

We transfer the genus *Pseudotrichomonas* into the order Trichomonadida and the family Trichomonadidae based on the phylogenetic relationships.

Lacusteria n. g.

Description. Three unequal anterior flagella. Recurrent flagellum forms a weakly developed undulating membrane of variable length with a single wave. The distal end of the posterior flagellum free at least in some cells. Costa absent. Parabasal body ellipsoidal. Axostyle of *Trichomonas*-type. Phylogenetically distinct from other undulating-membrane-bearing parabasalid genera.

Type species. *Lacusteria cypriaca* sp. n.

Other species. None.

Etymology. A Latin adjective *lacuster* has meaning of ''inhabiting lakes'' in English and *-ia* is a Latin diminutive. This generic name refers the habitat of the organism. The name *Lacusteria* is considered to be of feminine gender.

Lacusteria cypriaca n. sp.

Description. Characteristics of the genus. Body size, without the axostyle protrusion, 8.0 ± 1.3 (5.0–10.7) μm long and 5.5 ± 0.9 (5.0–7.6) μm wide. Protruding part of axostyle measuring 4.9 ± 0.8 (4.9–6.7) μm.

Type material. One hapantotype slide (6/69) and three paratype slides (6/43, 6/44, 6/45) with protargol-stained preparations of *L. cypriaca* are deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Type culture. Strain LA deposited in culture collection of the Department of Parasitology of Charles University in Prague, Prague, Czech Republic.

Type habitat. Pond near Voroklini village, Cyprus (34°58′N, 33°39′E).

Habitat. Low oxygen sediments.

Etymology. The specific name *cypriaca* refers to the geographical location where the type strain was collected. The species epithet agrees with the feminine gender of the generic name *Lacusteria*.

Gene sequence. Nearly complete sequence of SSU rRNA gene of the type isolate LA is deposited in GenBank under accession number HM748759.

The diversity of free-living parabasalids. One arrangement of four or five basal bodies, so-called ''privileged basal bodies'' consisting of parallel basal bodies of anterior flagella plus one orthogonal basal body of the recurrent flagellum, is inferred to be a synapomorphy for the Parabasalia. This basic cytoskeletal system is best reflected in free-living trichomonads, like the three strains described here, and symbiotic lineages of trichomonadids, tritrichomonadids, and hypotrichomonadids. In some symbiotic lineages, like the Trichonymphea and a few others, parts of this basic system can be repeated many times within a single, highly complex cell. Molecular phylogenetic analyses based on several different molecular markers have shown that the Trichonymphea form the sister group to the rest of the parabasalids (Gerbod et al. 2004; Keeling, Poulsen, and McFadden 1998; Ohkuma et al. 2007). Our hypothesis is that parabasalids containing one system of privileged basal bodies represent the morphology and lifestyle of the most recent parabasalid ancestor.

Molecular phylogenetic data suggest that the nearest sister lineage to the Parabasalia is the Fornicata, which includes diplomonads (e.g. *Giardia*, *Spironucleus*, *Octomitus*, and *Enteromonas*) and retortamonads (*Retortamonas* and *Chilomastix*) (Hampl et al. 2009). In addition, a large number of free-living fornicates from anaerobic environments have been recognized with culture-dependent surveys and environmental PCR surveys (e.g. *Carpediemonas*, *Dysnectes*, *Hicanonectes*, and *Kipferlia*), and these lineages branch near the origin of the more inclusive group (Kolisko et al. 2010; Park et al. 2009; Simpson et al. 2002; Yubuki et al. 2007). This phylogenetic distribution of free-living lineages strongly suggests that the most recent ancestor of the Fornicata was free-living. Although parabasalids also evolved from free-living ancestors at some point in their evolutionary history, it is currently unclear what this common ancestor was like. Our study sheds some light on this question by demonstrating a phylogenetic pattern of free-living *Lacusteria*/*Pseudotrichomonas*-like lineages that form a paraphyletic stem group from which parasitic lineages of trichomonadids (e.g. *Trichomonas*, *Tetratrichomonas*, *Pentatrichomonas*, *Cochlosoma*, *Pseudotrypanosoma*, and *Trichomitopsis*) might have evolved. Nonetheless, current molecular phylogenetic evidence does not allow us to confidently infer whether different free-living parabasalids reflect ancestral modes of life (i.e. morphostasis), reflect lineages derived from endobiotic ancestors, or reflect some combination of both possibilities.

Nonetheless, before this study, only two lineages of free-living parabasalids, namely the Honigbergiellida and *T. undula*, were known. The three free-living strains we describe here branch separately from the previously known free-living lineages, which indicates that free-living parabasalids are more diverse and phylogenetically interspersed than recognized previously. There are now at least four independent free-living lineages that are distributed throughout the Parabasalia: Honigbergiellidae (*Honigbergiella*, *Monotrichomonas*, and *Ditrichomonas*), *T. undula*, *P. keilini*, and *L. cypriaca* n. g., n. sp. These lineages likely represent only a very small fraction of the total number of free-living parabasalids on earth. Accordingly, continued exploration of the overall diversity of free-living parabasalids is expected to significantly improve our understanding of the origins and early evolution of their important parasitic relatives and the group as a whole.

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[Correction made after publication September 28, 2010: Fig.17 has been replaced because the posterior probabilities were missing in the the phylogenetic tree.]

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