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Morphological and Molecular Diversity of the Monocercomonadid Genera *Monocercomonas*, *Hexamastix*, and *Honigbergiella* gen. nov.

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The family Monocercomonadidae (Parabasala, Trichomonadida) is characterized by the absence of a costa and in most species also of an undulating membrane; both of which are typical structures of trichomonadids. We have examined 25 isolates of Monocercomonadidae species by sequencing of the SSU rDNA and the ITS region and by light and transmission electron microscopy. The isolates formed three distinct phylogenetically unrelated clades: (1) *Monocercomonas colubrorum*, (2) *Monocercomonas ruminantium* together with a strain ATCC 50321 designated as *Pseudotrichomonas keilini*, and (3) *Hexamastix*. Twenty isolates of *Monocercomonas colubrorum* split into three clades with no host-specificity. The morphological differences among clades were insufficient to classify them as a separate species. Non-monophyly of the cattle commensal *Monocercomonas ruminantium* with the type species *Monocercomonas colubrorum* and absence of *Pseudotrichomonas* characters in the free-living strain ATCC 50321 led to their reclassification into a new genus (*Honigbergiella* gen. nov.). The close relationship of these strains indicates a recent switch between a free-living habit and endobiosis. Two strains of *Hexamastix* represented different species — *Hexamastix kirbyi* Honigberg 1955 and *Hexamastix mitis* sp. nov. Polyphyly of the Monocercomonadidae confirmed that the absence of a costa and an undulating membrane are not taxonomically significant characters and were probably secondarily lost in some or all clades. Our observations, however, indicated that other characters — infrakinetosomal body, comb-like structure, marginal lamella, and the type of axostyle — are fully consistent with the position of Monocercomonadidae species in the parabasalian tree and are, therefore, reasonable taxonomic characters.

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Key words: *Hexamastix*; *Honigbergiella*; *Monocercomonas*; morphology; phylogeny; ultrastructure.

Introduction

Parabasalids are an abundant group of flagellates, mostly intestinal commensals of vertebrates and insects, whose common features include the presence of hydrogenosomes (modified mitochondria), a parabasal apparatus (a Golgi body attached to striated fibers), and nuclear division by a closed pleuromitosis with an external spindle. The cell cycle of parabasalids typically includes only free-swimming trophozoites and true cysts are reported from a few...
species (Brugerolle 1973; Dolan et al. 2004; Farmer 1993).

In a simplified fashion, we can divide parabasalid species into two different types: (1) Ordinary flagellates with a single karyomastigont with up to five anterior flagella and one recurrent flagellum that typically forms an undulating membrane. The undulating membrane is usually underlain by a striated fiber — the costa. A tube-like microtubular sheet — the axostyle — forms the longitudinal axis of the cell. Its opened proximal end extends into a spoon-like capitulum that, together with an adjacent microtubular sheet (the pelta), partially surround the nucleus and perikinetosomal area. All these flagellates are classified as the order Trichomonadida. (2) The second type represents rather extravagant flagellates, whose mastigonts are, with the exception of devescovinids, multiplied and have a group-specific organization and distribution. In calonymphids, the nuclei are also multiplied. All these species inhabit the intestines of insects and constitute the orders Cristamonadida, Trichonymphida, and Spirotrichonymphida.

Based on morphological comparisons, it has been suggested that trichomonadids represent the ancestral morphology of the parabasalids. The basal phylogenetic position of trichomonadids has been ascribed to organisms in the family Monocercomonadae, the simplest trichomonadids, which lack an undulating membrane and costa. Their simple morphology was regarded as a relict of the cell organization of primitive parabasalids (Brugerolle 1976; Honigberg 1963). Molecular phylogenetic studies have cast doubt on this scenario of evolution by showing that monocercomonadids are polyphyletic, forming at least four groups in the tree, with none of them located at the root (Dacks and Redfield 1998; Delgado-Viscogliosi et al. 2000; Edgcomb et al. 1998; Gerbod et al. 2000, 2001, 2002, 2004; Hampi et al. 2004; Keeling et al. 1998; Keeling 2002; Ohkuma et al. 2005). The polyphyly of the monocercomonadids implies that the simple morphology of at least some monocercomonadid clades must have resulted from secondary reduction, unless we accept the independent origin of the undulating membrane and costa in three trichomonadid clades (Hampi et al. 2004). On the other hand, the morphologically derived trichonymphids appeared at the root of parabasalids in analyses of SSU (small subunit) rRNA (Dacks and Redfield 1998; Delgado-Viscogliosi et al. 2000; Keeling et al. 1998; Ohkuma et al. 1998), GADPH, enolase, and \( \gamma \)-tubulin (Gerbod et al. 2004). Although the basal placement of the trichonymphids in the SSU rRNA gene tree was questioned (Hampi et al. 2004), some protein phylogenies and most importantly the concatenation of \( \gamma \)-, \( \beta \)-tubulin, enolase, and GAPDH (Ohkuma et al. 2007) relatively strongly support its correctness.

As the result of unclear phylogenetic relationships, the taxonomy of the parabasalids is in a state of flux. The previous division of the parabasalids into two orders — Hypermastigida and Trichomonadida (Brugerolle 1976; Hollande and Caruette-Valentin 1971; Honigberg 1963) — has gradually been replaced by division into four orders — Trichomonadida, Cristamonadida, Trichonymphida, and Spirotrichonymphida (Adl et al. 2005; Brugerolle and Patterson 2001). This is, however, also incongruent with current phylogenies, because the order Trichomonadida appears polyphyletic with the Cristamonadida and Spirotrichonymphida located inside it (e.g. Hampi et al. 2004, 2006; Ohkuma et al. 2005; Noël et al. 2007). Noël et al. (2007) proposed a return to the two-order division by inclusion of cristamonadids and spirotrichonymphids as families within Trichomonadida. The classification inside the order Trichomonadida was recently revised by us (Hampi et al. 2006) by abolishing the family Cohlosomatidae and the polyphyletic subfamilies within Trichomonadidae and by splitting the polyphyletic family Trichomonadidae into three monophyletic families — Trichomonadidae, Tririchomonadidae, and Trichomitidae. The last family of Trichomonadida, Monocercomonadidae, remained untouched although it is clearly untenable in the long term due to its polyphyletic nature.

The present paper does not aim to revise the higher parabasalian classification but rather further explore parabasalian diversity. Here, we present a morphological, ultrastructural, and phylogenetic analysis of 25 isolates (12 newly reported) with monocercomonadid morphology formerly ascribed to the genera Monocercomonas, Hexamastix, and Pseudotrichomonas. Detailed inspection revealed important structural differences among superficially similar species, which correspond with their phylogenetic nature and together resulted in proposals for taxonomic changes.

**Results**

**Phylogenetic Position and Relationship among Isolates**

Sequences of the SSU rDNA and the ITS region (ITS1, 5.8S rDNA, ITS2, and the adjacent part of
the LSU [large subunit] rDNA) were determined for all strains. Information on the accession numbers of the sequences obtained is given in the Table 1.

A phylogenetic tree of parabasalids, based on the determined SSU rDNA sequences and available sequences from databases (Fig. 1), was constructed by the maximum-likelihood method and the statistical support was calculated by distance, maximum-parsimony, maximum-likelihood, and Bayesian methods. The sequences of our isolates formed three well-supported groups in the tree: (1) Monocercomonas colubrum strains R183, R186, GRIS, PYR-1, LA10, R293, HYDR1, BOA1, BOA4, BOA5, MONT1, CHEL1, BIGA, EUMM, HAD, VAR-1, R208, TSC, and CORON1. These isolates formed a robust clade together with the ATCC strain NS-1PRR. The clade was related to the genera Trichomonas, Dientamoeba, Histomonas, and the orders Cristamonadida and Spirotrichonymphida. (2) Monocercomonas ruminantium strains HER-5 and KOJ-14 that were very closely related to the ATCC strain 50321 designated as Pseudotrichomonas keilini. (3) Two Hexamastix strains CYCL and T that formed a long branch related to termite symbionts ascribed to the genus Tricercomitus.

To further investigate the relationships within the Monocercomonas colubrum clade, we also used for phylogenetic reconstruction, the sequences of the ITS region, which is more polymorphic. The tree in Figure 2 was constructed from the concatenated sequences of SSU rDNA and the ITS region using the same methods as for the whole parabasalian tree. The isolates split into three robust clades. The tree was rooted with the clade 1 (R183, R186, and GRIS) that appeared basal in the analysis with outgroups (not shown). The second clade consisted of isolates TSC, LA10, CORON1, and R293, and 12 remaining isolates formed the third clade together with the ATCC strain NS-1PRR. Some sequences were identical (see zero branch lengths in Fig. 2). Members of clades 1 and 2 differed by 54—62 nucleotides, members of clades 1 and 3 by 57—72 nucleotides, and members of the clades 2 and 3 by 34—45 nucleotides in the concatenated sequences of SSU rDNA and the ITS region. For comparison, two morphologically well-defined species, Trichomonas vaginalis and Trichomonas tenax, differed in the same region in 56 nucleotides. Relationships among isolates did not correspond to their host origin and none of the three clades was host-specific. In some cases, isolates with identical sequences colonized unrelated reptilian hosts, e.g. strains MONT1, CHEL1, EUMM, BIGA, and PYR were isolated from chameleons, blue-tongue, and two unrelated snakes, respectively.

Isolates of Monocercomonas ruminantium and Hexamastix are unrelated to the reptilian strains, and thus were not included in the latter tree, but their genetic distances were also measured. Monocercomonas ruminantium strains HER-5 and KOJ-14 were identical in the concatenated sequences of SSU rDNA and ITS region and differed in 27 positions from the strain ATCC 50321 designated as Pseudotrichomonas keilini. The two Hexamastix strains differed in 101 nucleotides.

Morphology and Ultrastructure of Isolates

The cultured organisms were studied in fresh mounts using phase contrast microscopy, in fixed protargol-stained preparations, and using transmission electron microscopy. Their qualitative morphological characters are summarized in Table 2.

Detailed examination of strain ATCC 50321, deposited in ATCC as Pseudotrichomonas keilini, which is presented below, revealed that it does not possess morphological characters of the genus Pseudotrichomonas. Morphological similarity of this strain and the cattle strains HER-5 and KOJ-14 representing the species Monocercomonas ruminantium together with the phylogenetic positions of these three strains prompted their classification into a new genus Honigbergiella gen. nov.

Honigbergiella ruminantium n. comb. — strains HER-5 and KOJ-14

Motile trophozoites and round cysts were observed in cultures of strains HER-5 and KOJ-14. The protargol-stained specimens of strain HER-5 (Figs 3A,B and 4A) essentially corresponded to the morphology of Monocercomonas ruminantium (Braune, 1913) Levine, 1961, as described by Jensen and Hammond (1964). They were typically ovoid or round in shape, the body, not including the axostyle, was 5.2 ± 0.9 (3.7—7.8) μm long and 4.3 ± 0.8 (2.9—6.2) μm wide, had three anterior flagella of slightly uneven lengths and one posterior flagellum that neither formed an undulating membrane nor adhered to the body. An oval nucleus was 1.4 ± 0.2 (0.9—2.1) μm long and 1.0 ± 0.2 (0.5—1.5) μm wide. The parabasal body was disc-shaped and typically situated on the latero-anterior part of the nucleus. The slender axostyle originated at the anterior pole of the cell, extended through
Table 1. Origin of strains included in the study. *Indicates that the host was examined in the wild or immediately after import to the Czech Republic.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Host species</th>
<th>Isolation</th>
<th>GenBank number (SSU rDNA, ITS)</th>
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<tr>
<td>Monocercomonas colubrorum</td>
<td>R183</td>
<td><em>Trophidophis melanurus</em></td>
<td>Kulda (1966)</td>
<td>AY319276</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>TSC</td>
<td><em>Teratoscincus scincus</em></td>
<td>Čepička (2000)</td>
<td>AY319277</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>NS-1PRR (ATCC 50210)</td>
<td><em>Natrix sipedon</em></td>
<td>L.S. Diamond (1959)</td>
<td>AY319266, U17507</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>R208</td>
<td>Anolis equestris*</td>
<td>Kulda (1966)</td>
<td>AY319268</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>PYR-1</td>
<td>Python regius*</td>
<td>Kulda (1998)</td>
<td>AY319270</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>EUMM</td>
<td><em>Eumeces</em> sp.</td>
<td>Čepička (1999)</td>
<td>AY319278, AY319273</td>
</tr>
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<td>Monocercomonas colubrorum</td>
<td>HAD</td>
<td><em>Ahaetula prasina</em></td>
<td>Kulda (1994)</td>
<td>AY319267</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>VAR-1</td>
<td>Varanus exanthematicus*</td>
<td>Kulda (1998)</td>
<td>AY319272</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>R186</td>
<td><em>Trophidophis melanurus</em></td>
<td>Kulda (1966)</td>
<td>DQ174302</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>GRIS</td>
<td>Varanus griseus*</td>
<td>Čepička (2004)</td>
<td>DQ174297</td>
</tr>
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<td>Monocercomonas colubrorum</td>
<td>LA10</td>
<td><em>Lacerta agilis</em></td>
<td>Kulda (1992)</td>
<td>DQ174300</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>R293</td>
<td>Unidentified Cuban lizard</td>
<td>Kulda (1966)</td>
<td>DQ174303</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>BIGA</td>
<td><em>Bitis gabonica</em></td>
<td>Hampl (2004)</td>
<td>DQ174292</td>
</tr>
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<td>Monocercomonas colubrorum</td>
<td>MONT1</td>
<td><em>Chameleo montium</em></td>
<td>Čepička (2004)</td>
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<td>Monocercomonas colubrorum</td>
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<td>DQ174298</td>
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<td><em>Boa constrictor</em></td>
<td>Čepička (2004)</td>
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<td><em>Boa constrictor</em></td>
<td>Čepička (2004)</td>
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</tr>
<tr>
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<td><em>Boa constrictor</em></td>
<td>Hampl (2004)</td>
<td>DQ174294</td>
</tr>
<tr>
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<td>CORON1</td>
<td><em>Coronella austriaca</em></td>
<td>Čepička (2005)</td>
<td>DQ174296</td>
</tr>
<tr>
<td>Honigbergiella ruminantium</td>
<td>KOJ-14</td>
<td><em>Bos Taurus</em></td>
<td>Čepička (2000)</td>
<td>AY319280, AY319271</td>
</tr>
<tr>
<td>Honigbergiella ruminantium</td>
<td>HER-5</td>
<td><em>Bos Taurus</em></td>
<td>Čepička (2000)</td>
<td>AY319279, AY319269</td>
</tr>
<tr>
<td>Hexamastix mitis</td>
<td>CYCL</td>
<td><em>Cyclura nubila</em></td>
<td>Čepička (2003)</td>
<td>AY319275</td>
</tr>
<tr>
<td>Hexamastix kirbyi</td>
<td>T</td>
<td><em>Uromastyx</em></td>
<td>Tachezy (1996)</td>
<td>AY321149</td>
</tr>
<tr>
<td>Honigbergiella sp.</td>
<td>ATCC 50321</td>
<td><em>Free-living</em></td>
<td>Nerad (1987)</td>
<td>AY319274</td>
</tr>
</tbody>
</table>
Figure 1. Polyphyly of Monocercomonadidae. Unrooted phylogenetic tree of parabasalids based on SSU rDNA sequences and constructed by the maximum likelihood method. Values at the nodes indicate statistical support estimated by four methods (distance bootstrap, maximum parsimony bootstrap, maximum likelihood bootstrap, MrBayes posterior probability). GenBank accession numbers are supplied for the termite-symbiont sequences. Shaded boxes indicate the orders Trichonymphida, Spirotrichonymphida, and Cristamonadida; species outside the boxes belong to the order Trichomonadida. Families of Trichomonadida as in Hampl et al. (2006) are indicated. Asterisks indicate that the node was not recovered by a particular method.
the longitudinal axis of the cell, and protruded posteriorly. The length of the free portion of the axostyle varied from 10% to 100% (average 45% ± 20%) of the cell length. Anteriorly, the axostyle formed a narrow capitulum. The pelta was relatively small. Parabasal fibers stained very weakly.

The ultrastructure of the trophozoites of strain HER-5 (Fig. 5) was similar to that of other trichomonads. Four basal bodies were located at the anterior pole of the cell. The basal body of the recurrent flagellum was positioned orthogonally to the basal bodies of the anterior flagella. The recurrent flagellum did not adhere to the cell, did not form an undulating membrane, and the marginal lamella was absent. A costa was also absent. The Golgi body was situated anteriorly to the nucleus and was connected to basal body 2 by a striated parabasal fiber (PF2). Both the infrakinetosomal body and the comb-like structure were absent. A microtubular axostyle overlapped near the basal bodies with microtubules of the pelta that underlay the most anterior part of the cell. The cytoplasm contained food vacuoles, hydrogenosomes, and numerous glycogen granules. The cysts of strain HER-5 were spherical, with a cyst wall separated from the plasma membrane and coated by a glyocalyx. The cytoplasm of the cysts was more granular than that of trophozoites and contained the nucleus, hydrogenosomes, and internalized flagella.

Honigbergiella sp. strain ATCC 50321

The strain ATCC 50321 was deposited in ATCC as *Pseudotrichomonas keilini*, but it does not possess an important character of the genus — the undulating membrane (Figs 3C,D and 6). It shares all characters of *Honigbergiella* as described above. As the phylogenetic analyses revealed its close relationship with strains HER-5 and KOJ-14, there is no doubt that these organisms are congeneric. Strain ATCC 50321 formed true cysts like the strains HER-5 and KOJ-14 but failed to grow at 37 °C. Protargol-stained trophozoites (Figs 3C,D and 4B) were identical in all qualitative characters to the previous strains and only slight differences were observed in

**Figure 2.** Phylogenetic tree of *Monocercomonas colubrorum* strains based on concatenated SSU rDNA and ITS region (5.8S rDNA, ITS1, ITS2, and partial 28S rDNA) sequences. The tree was constructed by the maximum likelihood method. Values at the nodes indicate statistical support estimated by four methods (distance bootstrap, maximum parsimony bootstrap, maximum likelihood bootstrap, Bayesian posterior probabilities). The hosts group (S-snake, L-lizard) is indicated for each strain. The tree was rooted on clade 1. The basal position of this clade was recovered (bootstrap support 94% and above) in separate analyses using *Tritrichomonas foetus* as an outgroup. Asterisks indicate that the node was not recovered by particular method.

**Figure 3.** Protargol-stained specimens of *Honigbergiella ruminantium* strain HER-5 (A, B) and *Honigbergiella* sp. strain ATCC 50321 (C, D). Arrows indicate the parabasal body. Bars = 10 μm.
The cell body, not including the axostyle, was 5.5 ± 0.8 (3.8—7.2) \( \mu \)m long and 4.5 ± 0.9 (3.1—7.2) \( \mu \)m wide, which was on average slightly larger than HER-5, but the difference was not statistically significant. The nucleus was also larger, 1.7 ± 0.3 (1.1—2.5) \( \mu \)m long, and 1.1 ± 0.2 (0.6—1.6) \( \mu \)m wide, and these differences were statistically significant (\( p < 0.001, N = 50 \)). The length of the extracellular part of the axostyle varied from 10% to 60% of the cell length (average 25% ± 10%), which is significantly less (\( p < 0.001, N = 50 \)) than in the Honigbergiella strain HER-5. No difference in the free axostyle length was observed in living cells using phase contrast microscopy.

**Monocercomonas colubrorum** strains

Motile trophozoites and pseudocysts with internalized flagella were observed in cultures of the reptile strains R183, R186, GRIS, TSC, LA10, R293, CORON1, NS-1PRR, R208, PYR-1, EUMM, BIGA, MONT1, CHEL1, HAD, HYDR1, VAR, BOA1, BOA4, and BOA5. Strains R183, R186, TSC, LA10, R293, NS-1PRR, EUMM, HAD, HYDR1, and BOA5 were stained with protargol and representatives of each clade and the strain NS-1PRR that formed a long branch in the tree are given in Figure 7. Their morphology was very similar and corresponded to Monocercomonas colubrorum. Trophozoites had an oblong shape, three anterior flagella of slightly unequal length, and one posterior flagellum. Undulating membranes and costa were not developed and the adherent proximal part of the posterior flagellum trailed above the nucleus in the posterior-right direction. The axostyle was relatively stout. It protruded from the cell posteriorly and gradually narrowed to the tip. Anteriorly, the axostyle formed a spoon-like capitulum covering the nucleus. The pelta formed a scoop-like structure supporting the anterior protrusion of the cell. The parabasal body had the shape of a rod and was located on the right anterior edge of the nucleus, typically under the proximal part of the recurrent flagellum.

The strains differed in the dimensions of the cell, nucleus, and the relative length of the free part of the axostyle (Table 3). Some differences were statistically significant, but, generally, did not correspond with the phylogenetic relationship among strains. For some parameters (cell and nucleus length and width), the variability within clades was higher than among clades and the result of multivariate cluster analysis based on cell dimensions also failed to correspond to their phylogeny (not shown). However, three characters corresponded to the distribution of strains in clades. The cell length-to-width ratio is the smallest in clade 2 and the pool of values of the clade 2 strains differed significantly from the pool of values of the clade 1 strains as well as the clade 3 strains (\( p < 0.001 \)). The difference between clades 1 and 3 was not significant. Analogously, the relative length of the axostyle protrusion was significantly higher in clade 1 than in clades 2 or 3 (\( p < 0.001 \)). Finally, we observed a difference in the shape of the axostyle capitulum that was wider in clade 2 than in the other two clades.

Strains also differed in the capability of phagocytosis. Strains TSC and R293 from clade 2 massively phagocytosed starch granules and bacteria, while trophozoites with engulfed bacteria or starch grains were observed only rarely in the other two strains from clade 2 and in all strains from from clades 1 and 3.

Strain VAR-1 was examined by electron microscopy (Fig. 8). Its internal structure was essentially
identical to that of strain NS-1PRR examined by Mattern et al. (1972). The mastigont consisted of three anterior and one recurrent basal body, infrakinetosomal body, pelta-axostylar complex, and several striated fibers including a comb-like structure. The recurrent flagellum did not form an undulating membrane and was in its proximal part underlain by the marginal lamella. A costa was absent. The Golgi body was located latero-anteriorly from the nucleus and was connected to the basal bodies by the striated parabasal fiber (PF2). The axostyle formed a broad capitulum and sigmoid fibers (F2) connected basal body 2 with the pelta at the pelta-axostylar junction. Pseudo-cysts (Fig. 8C) were enveloped by uncovered plasma membrane and an internalized karyomastigont and internalized flagella were observed inside pseudocysts.

Hexamastix mitis spec. nov. strain CYCL

In the culture of the strain CYCL, we observed only trophozoites; neither cysts nor pseudocysts were present. The morphology of the protargol-stained specimens (Figs 9A—D and 10A,B) did not correspond to any described species, from which it differed by possessing a large disc-shaped parabasal body with a central granule, and a slender axostyle. Trophozoites had round or ovoid cells, the body (axostyle not included) was 7.8 ± 1.1 (5.8—11.3) μm long and 7.1 ± 1.1 (5.3—11.2) μm wide, had six flagella of uneven lengths, and an oval nucleus 2.4 ± 0.3 (1.6—3.2) μm long and 1.6 ± 0.2 (1.1—2.1) μm wide. Neither an undulating membrane nor a costa were developed. The extracellular part of the axostyle was not observed in many specimens, and if observed, was relatively short, extending on average 20%–10% (10—50%) of the cell length. The capitulum of the axostyle was narrow and the pelta was relatively small.

Hexamastix kirbyi strain T

In the culture of the strain T, we observed only trophozoites and neither cysts nor pseudocysts were present. The morphology of protargol-stained specimens (Figs 9E—H and 10C,D)
corresponded to *Hexamastix kirbyi* Honigberg, 1955. Trophozoites were ovoid, oval, or round and the body without the axostyle was 7.7 ± 1.0 (6.1—9.9) μm long and 6.0 ± 0.9 (4.2—8.5) μm wide. Cells had six flagella of uneven length and an oval nucleus 2.1 ± 0.3 (1.4—2.9) μm long and 1.3 ± 0.2 (0.9—1.9) μm wide. Undulating membranes and costa were not developed. The axostyle was slender, expanding abruptly into a broad capitulum in the nuclear area. Its distal part stretched out 30% ± 10% (10—50%) of the cell length. The pelta was relatively large. The parabasal body had a shape of a small ring or a tear with a dark-stained outline and was located at the anterior part of the nucleus or above the nucleus close to the basal bodies. Parabasal fibers were not visible by light microscopy. The ultrastructure of strain T (Fig. 11) corresponded to that of *Hexamastix* in Brugerolle (1976). The basal body of the recurrent flagellum was positioned orthogonally to the five basal bodies of anterior flagella. Basal body 2 bore the parabasal fibers (PF) and the sigmoid fibers (F2) directed to the pelta-axostylar junction. Basal bodies 1 and 3 bore hooked laminae F1 and F3. The Golgi body, associated with the parabasal fibers (PF2), was located anterolaterally to the nucleus. A comb-like structure, infrakinetosomal body, and marginal lamella were not present (Table 2).

**Discussion**

Our isolates formed three distinct and unrelated clades in the parabasalian tree. The most species-rich clade was formed by the *Monocercomonas* isolates from reptiles. The taxonomy of the reptile species of *Monocercomonas* is rather confusing (see discussion in Honigberg 1963). Up to 30 species were described, but descriptions are mostly insufficient and based on the assumed host specificity. Moskowitz (1951) and Honigberg (1963) recognized only two valid species, *Monocercomonas colubrorum* (Hammerschmidt, 1844) Doflein, 1901 and *Monocercomonas moskowitzi* Honigberg, 1963. The morphology of our strains corresponded with the description of *Monocercomonas colubrorum* by Moskowitz (1951) and...
differed from Monocercomonas moskowitzi in their longer cells, shorter adherent part of recurrent flagellum, and the shape of axostyle that had a longer external part which narrowed continuously to the tip, and not abruptly as in Monocercomonas moskowitzi. The clade of Monocercomonas colubrorum appeared in our tree as a sister to a large clade of cristamonads, spirotrichonymphids, and tritrichomonads, although the statistical support for such a position was low. The morphology of Monocercomonas can hardly be directly compared with spirotrichonymphids or cristamonadids, whose cell morphology is rather derived, probably as a result of accelerated evolution triggered by the selection pressure in the insect gut environment. However, Monocercomonas shares several characters with Tritrichomonas: the rigid shape of the cell, a stout tube-like axostyle, a rod shaped parabasal body, and the presence of a marginal lamella, a comb-like structure, and an infrakinetosomal body (Mattern et al. 1972).

In the analyses based on concatenated sequences of SSU rDNA and the ITS region, strains of Monocercomonas colubrorum split into three robust clades. The nucleotide differences among the clades were higher or similar to the difference between the two valid species Trichomonas vaginalis and Trichomonas tenax. Distribution of strains in clades corresponded neither to their capability for phagocytosis nor to the host taxon. Protargol-stained specimens of different strains exhibited differences in their morphology, but only two of these parameters, cell length-to-width ratio and relative length of axostyle protrusion, seemed to be clade-specific. These characters could, however, be affected by culture conditions and fixation. The only qualitative character that was potentially clade-specific was the shape of the axostyle capitulum which was wider in clade 2. Although genetic distances suggested that the three clades might represent separate species, we did not find any firm diagnostic characters for these species, and therefore, for the present regard all examined strains as belonging to a single species, namely Monocercomonas colubrorum.

The only species described from cattle that possesses three anterior flagella and a free recurrent flagellum is Monocercomonas ruminantium (Braune, 1913) Levine, 1961. The strain HER-5 shared most morphological characteristics with Monocercomonas ruminantium as described by Jensen and Hammond (1964) — size and shape of the cell, slender axostyle, disc-shaped parabasal body, and formation of true cysts. It differs in the relative length of the extracellular part of the axostyle, which was longer in our isolate. This difference could be ascribed to the fixation conditions and to the fact that Jensen and Hammond (1964) prepared smears directly from the hosts, while we stained specimens from culture. As the essential characters of the strain HER-5 agreed with the description, we assume that HER-5 and KOJ-14 (whose sequence was identical to HER-5) correspond to the species described by Jensen and Hammond (1964) as Monocercomonas ruminantium. In the phylogenetic tree, these strains did not form a clade with isolates from reptiles representing Monocercomonas colubrorum, the type species of the genus Monocercomonas, but branched inside the free-living trichomonad clade close to ATCC isolate 50321. Generic classification of Monocercomonas ruminantium is thus not valid and is revised here.
<table>
<thead>
<tr>
<th>Taxon</th>
<th>Trunk of axostyle</th>
<th>Capitulum of axostyle</th>
<th>Pelta</th>
<th>Parabasal body</th>
<th>Um</th>
<th>Ib</th>
<th>Co</th>
<th>ML</th>
<th>Costa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honigbergiella ruminantium</td>
<td>Slender</td>
<td>Narrow</td>
<td>Small</td>
<td>Disc with dark central granule</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Honigbergiella sp.</td>
<td>Slender</td>
<td>Narrow</td>
<td>Small</td>
<td>Disc with dark central granule</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hexamastix mitis</td>
<td>Slender</td>
<td>Narrow</td>
<td>Small</td>
<td>Disc with dark central granule</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hexamastix kirbyi</td>
<td>Slender</td>
<td>Scoop-like, abruptly widening</td>
<td>Large, wavy</td>
<td>Tear or ring with dark outline</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Monocercomonas colubrorum</td>
<td>Stout, tapering continuously to the tip</td>
<td>Spoon-like, slowly widening</td>
<td>Large, scoop-like</td>
<td>Rod</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Trichomonadidae</td>
<td>Slender</td>
<td>Various</td>
<td>Various</td>
<td>Various</td>
<td>Lamelliform</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Type B</td>
</tr>
<tr>
<td>Tririchomonadidae</td>
<td>Stout with short projection tapering abruptly to the conical tip</td>
<td>Spoon-like, slowly widening</td>
<td>Various</td>
<td>Rod</td>
<td>Railform</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Type A</td>
</tr>
<tr>
<td>Trichomitidae</td>
<td>Moderate diameter, long gradually tapering projection</td>
<td>Spatulate or spoon-shaped with lateral extensions, slowly widening</td>
<td>Large, crecent-shaped</td>
<td>Biramous</td>
<td>Lamelliform</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>Type A</td>
</tr>
</tbody>
</table>
The affinity of strains HER-5 and KOJ-14 to the free-living species and the Trichomonadidae sensu Hampl et al. (2006) corresponded with their morphology and ultrastructure — a slender axostyle, smaller cells with less rigid shape, and the absence of a comb-like structure and infrakinetosomal body.

The characteristic feature of the genus *Pseudo-trichomonas* is the presence of a lamelliform undulating membrane (Brugerolle 1991). We showed clearly that no undulating membrane is present in the isolate ATCC 50321, so the original identification of this isolate as *Pseudotrichomonas keilini* was incorrect. The morphology of this isolate closely resembled that of isolate HER-5. The only difference that we detected was the relative length of the extracellular part of the axostyle and the size of the nucleus in fixed specimens. These differences can, however, be ascribed to variations in fixation conditions. Isolates HER-5, KOJ-14, and ATCC 50321 formed true cysts but unlike HER-5 and KOJ-14, ATCC 50321 failed to grow at 37°C. Morphological similarities between the isolates HER-5 and ATCC 50321 corresponded to the very high similarity in their SSU rDNA and ITS region sequences. They differed in 27 nucleotides (2% divergence), which is certainly within the range of intrageneric differences in parabasalids.

Based on our findings, we establish a new parabasalid genus, *Honigbergiella* gen. nov., that contains strain ATCC 50321 and the species formerly classified as *Monocercomonas ruminantium*. Strain ATCC 50321 differs from *Monocercomonas ruminantium* only slightly in morphology and sequence. However, because they differ also in ecology — free-living versus intestinal commensal — and in temperature tolerance, and because we consider these ecological characters significant, we keep them as separate species.

**Figure 8.** Ultrastructure of *Monocercomonas colubrorum* strain VAR-1. **A**, longitudinal section of a trophozoite with mastigont; **B**, transversal section of trophozoite; **C**, pseudocyst. 1, 2, 3, R — basal bodies, Ax — axostyle, Co — comb-like structure, Gb — Golgi body, H — hydrogenosome, Ib — infrakinetosomal body, Ifl — internalized flagellum, N — nucleus, MI — marginal lamella, Pe — pelta, Pf — parabasal fiber, F2 — sigmoid fiber.
Honigbergiella sp. and Honigbergiella ruminantium, respectively. The species status of Honigbergiella sp. needs to be resolved in the future.

The position of the commensal species Honigbergiella ruminantium in the clade of free-living species and its remarkable similarities to Honigbergiella sp. indicate that Honigbergiella ruminantium probably switched very recently from a free-living to a commensal way of life. An alternative explanation for this pattern would be two independent switches to a free-living habit in Honigbergiella sp. and the ancestor of Ditrichomonas honigbergii and Monotrichomonas carabina. This alternative, however, we regard as less probable.

On the other hand, it cannot be excluded that Honigbergiella sp. was isolated from a creek that was contaminated by cattle feces and that Honigbergiella sp. is in fact a commensal able to survive for certain period in freshwater. The true cysts with a cyst wall, which we reported for both species, would make survival in harsh external conditions easier.

The sequences of the two Hexamastix isolates differed in approximately two times as many nucleotides compared to the species Trichomonas vaginalis and Trichomonas tenax, indicating that these isolates very probably represent two distinct species.

**Figure 9.** Protargol-stained specimens of Hexamastix mitis strain CYCL (A–D) and Hexamastix kirbyi strain T (E–H). Ax — expanded capitulum of axostyle, Pb — parabasal body, Pe — pelta. Bars = 10 μm (in A, B, C, G, H) and 5 μm (in D, E, F).

**Figure 10.** Line drawings of Hexamastix mitis strain CYCL (A, B) and Hexamastix kirbyi strain T (C, D). Ax — axostyle, Pb — parabasal body, Pe — pelta, Pf — parabasal fiber.
The morphology of isolate T corresponded to the description of *Hexamastix kirbyi* in every essential detail (ring-shaped parabasal body, slender axostyle, the cell dimensions, relatively large pelta). The second isolate, CYCL, differed by the combination of a slender axostyle and a large disc-shaped parabasal body with central granule from all described species — *Hexamastix caviae* and *Hexamastix termitis* (stout axostyle), *Hexamastix batrachorum* (biarmed parabasal body), *Hexamastix crassus*, and *Hexamastix robustus* (twisted axostyle) (Honigberg 1955; Honigberg and Christian 1954; Nie 1950). We regard the isolate CYCL as a new species, *Hexamastix mitis* sp. nov.

In our analysis, the *Hexamastix* strains formed a long branch with a poorly supported position. The most probable sister clade to *Hexamastix* are the three termite symbionts ascribed to the genus *Tricercomitus*. In Figure 1, *Hexamastix* and *Tricercomitus* branch together with Trichonymphida. However, we have shown previously (Hampl et al. 2004) that this position is probably a result of long-branch attraction (Felsenstein 1978) and the most probable position of *Hexamastix* and *Tricercomitus* is next to the clade of *Ditrichomonas*, *Monotrichomonas*, and *Honigbergiella*. This position would correspond also to a *Hexamastix* morphology — non-rigid cell shape and a slender axostyle, in contrast to a tube-like stout axostyle.
Table 3. Dimensions (in μm) of protargol-stained specimens of *Monocercomonas colubrorum* strains. Average of 50 specimens ± standard deviation (smallest-largest value). Representatives of clades are separated by a line.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell length</th>
<th>Cell width</th>
<th>Cell length/cell width</th>
<th>Nucleus length</th>
<th>Nucleus width</th>
<th>Nucleus length/nucleus width</th>
<th>Free portion of axostyle/cell length</th>
</tr>
</thead>
<tbody>
<tr>
<td>R183</td>
<td>9.4 ± 1.2</td>
<td>4.1 ± 0.6</td>
<td>2.3 ± 0.5</td>
<td>2.1 ± 0.4</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(7—12.2)</td>
<td>(2—5.3)</td>
<td>(1.6—4.4)</td>
<td>(1.4—2.8)</td>
<td>(0.9—1.8)</td>
<td>(1—2.1)</td>
<td>(0.2—1)</td>
</tr>
<tr>
<td>R186</td>
<td>9 ± 1.2</td>
<td>4.5 ± 0.8</td>
<td>2.1 ± 0.4</td>
<td>2 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(6.6—11.8)</td>
<td>(2.2—6.1)</td>
<td>(1.3—3.6)</td>
<td>(1.3—2.7)</td>
<td>(0.8—1.6)</td>
<td>(1.2—2.7)</td>
<td>(0.3—1.2)</td>
</tr>
<tr>
<td>LA10</td>
<td>9.7 ± 1.3</td>
<td>5.3 ± 0.7</td>
<td>1.8 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(6.7—11.6)</td>
<td>(4.1—6.6)</td>
<td>(1.3—2.3)</td>
<td>(1.6—2.9)</td>
<td>(0.8—1.8)</td>
<td>(1.3—2.5)</td>
<td>(0.2—0.6)</td>
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<tr>
<td>R293</td>
<td>8.8 ± 1.0</td>
<td>4.6 ± 0.5</td>
<td>1.9 ± 0.2</td>
<td>2 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(6.3—11.8)</td>
<td>(3.6—5.8)</td>
<td>(1.4—2.4)</td>
<td>(1.4—2.8)</td>
<td>(0.7—1.7)</td>
<td>(1.2—3.9)</td>
<td>(0.2—0.5)</td>
</tr>
<tr>
<td>TSC</td>
<td>9.5 ± 1.1</td>
<td>6.2 ± 0.8</td>
<td>1.6 ± 0.2</td>
<td>2.2 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(6.8—12)</td>
<td>(4.6—7.6)</td>
<td>(1.1—2.2)</td>
<td>(1.2—3.5)</td>
<td>(0.8—2.1)</td>
<td>(1.2—2.5)</td>
<td>(0.1—0.5)</td>
</tr>
<tr>
<td>NS-1PRR</td>
<td>8.2 ± 0.8</td>
<td>4.2 ± 0.4</td>
<td>2 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>0.3 ± 0.1</td>
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<tr>
<td></td>
<td>(6.5—10)</td>
<td>(3.5—4.9)</td>
<td>(1.6—2.4)</td>
<td>(1.5—2.5)</td>
<td>(0.8—1.6)</td>
<td>(1.3—2.8)</td>
<td>(0.1—0.4)</td>
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<td>EUMM</td>
<td>11.4 ± 1.4</td>
<td>5.6 ± 0.9</td>
<td>2.1 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>2 ± 0.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(8.8—15.2)</td>
<td>(4.1—8.5)</td>
<td>(1.3—3.1)</td>
<td>(1.8—3.8)</td>
<td>(0.9—2.7)</td>
<td>(1.4—2.7)</td>
<td>(0.2—0.9)</td>
</tr>
<tr>
<td>HAD</td>
<td>9.4 ± 0.8</td>
<td>4.2 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(7.4—11.3)</td>
<td>(3.1—5)</td>
<td>(1.7—2.9)</td>
<td>(1.7—2.7)</td>
<td>(0.9—1.7)</td>
<td>(1.3—2.5)</td>
<td>(0.2—0.5)</td>
</tr>
<tr>
<td>HYDR1</td>
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<td>4.3 ± 0.4</td>
<td>2.2 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(7.5—12.2)</td>
<td>(3.3—5.7)</td>
<td>(1.8—3)</td>
<td>(1.7—2.8)</td>
<td>(1—1.7)</td>
<td>(1.2—2.4)</td>
<td>(0.1—0.4)</td>
</tr>
<tr>
<td>BOA5</td>
<td>9.3 ± 1.0</td>
<td>4.3 ± 0.4</td>
<td>2.2 ± 0.2</td>
<td>2 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>0.3 ± 0.1</td>
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<td></td>
<td>(7.1—12)</td>
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<td>(1.3—2.7)</td>
<td>(0.9—2.1)</td>
<td>(1.3—2.4)</td>
<td>(0.1—0.4)</td>
</tr>
</tbody>
</table>
and rigid oblong shape of tritrichomonadids and Monocercomonas colubrorum. Hexamastix also does not possess a comb-like structure, an infrakinetosomal body, or a marginal lamella. The position of the Hexamastix clade should be confirmed in the future using another gene.

The polyphyletic nature of the family Monocercomonadidae, derived from our analyses, has also been previously recognized (Dacks and Redfield 1998; Delgado-Viscogliosi et al. 2000; Edgcomb et al. 1998; Gerbod et al. 2000, 2001, 2002, 2004; Hampl et al. 2004; Keeling et al. 1998; Keeling 2002; Ohkuma et al. 2005). The present study, however, shows that characteristics of species, other than the absence of a costa and an undulating membrane, correspond well with their position in the tree. These include the shape of the axostyle and parabasal body, rigidity of the cell, and the presence or absence of an infrakinetosomal body, comb-like structure, and marginal lamella. Molecular phylogeny is, therefore, not in conflict with the morphology, but only with the classification, which in this case has been based on non-relevant characters.

TAXONOMIC SUMMARY

Honigbergiella gen. nov. Hampl, Čepička and Kulda

Type species — Honigbergiella ruminantium n. comb.

Etymology — Named after outstanding protozoologist Bronislaw M. Honigberg.

Hexamastix mitis spec. nov. Hampl, Čepička and Kulda
Diagnosis — Body (without axostyle) 7.8 ± 1.1 (5.8—11.3) μm long and 7.1 ± 1.1 (5.3—11.2) μm wide, six flagella of uneven lengths, oval nucleus 2.4 ± 0.3 (1.6—3.2) μm long and 1.6 ± 0.2 (1.1—2.1) μm wide, parabasal body disc-shaped, axostyle slender with relatively short protruding part extending on average 20% ± 10% (10—50%) of the cell length, capitulum of axostyle narrow, pelta relatively small.

Type host — Cyclura nubila.

Habitat — Isolated from feces. Presumably lives in the lower intestine.

Type locality — Prague, Czech Republic. The type host species naturally occurs in Cuba.

Syntype slides — Protargol preparations of the isolate CYCL, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic.

Etymology — mitis [Latin] — fine, gentle, mild, soft. The axostyle and pelta of this species are small and fine.

METHODS

Organisms: Information on the origin of strains included in this study is summarized in Table 1. All organisms, except strain CYCL, are deposited in the culture collection of the Department of Parasitology, Charles University in Prague, Czech Republic and are available upon request. Strain CYCL was not cultivable long term.

Cultivation: Reptile strains R183, R186, GRIS, TSC, LA10, R293, CORON1, NS-1PRR, R208, PYR-1, EUMM, BIPA, MONT1, CHEL1, HAD, HYDR1, BOAS, VAR-1, BOA1, and BOA4 were maintained as axenic cultures in TYM medium (Clark and Diamond 2002) supplemented with 10% heat-inactivated horse serum. The pH of the medium was adjusted to 7.2. Strains HER-5, KOJ-14, CYCL, and T were maintained as xenic cultures with admixed bacteria in modified TYSGM-9 medium without mucin and tween, and with rice starch (Clark and Diamond 2002).

The strains HER-5 and KOJ-14 were cultivated at 37°C and the other strains, mostly reptilian isolates, at 26°C. The cultures maintained in TYM were grown with three transfers before harvesting in a medium without agar.

Light microscopy: Moist fixed films spread on coverslips were prepared from soft pellets of protists obtained by centrifugation (500 g) of cultures. The films were fixed in Bouin-Hollande’s fluid and stained with protargol (Bayer AG, Germany) according to the protocol recommended by Nie (1950). Trichomonads were observed and photographed with an Olympus BX51 microscope using immersion objective 100 × UPPlanApo. Fifty specimens of each strain were measured from digital photographs using Grimage software calibrated with objective micrometer. Only non-dividing cells stretched in horizontal plane were measured.

Statistical tests: The significance of differences in cell dimensions among strains was tested using ANOVA and the post-hoc test Scheffé in Statistica (StatSoft). Multivariate cluster analysis was also performed in Statistica (StatSoft).
Transmission electron microscopy: Pellets of protists, obtained by centrifugation (500 g) of cultures, were fixed in 2.5% (w/v) glutaraldehyde and 5 mM CaCl₂ in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature for 2 h. After washing three times in PBS, the cells were post-fixed in 1% (w/v) osmium tetroxide, 0.8% potassium ferrocyanide, and 5 mM CaCl₂ in 0.1 M Na-cacodylate buffer. After washing in excess of PBS, the fixed samples were dehydrated in an ethanol series, transferred to acetone, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Phillips EM 420 electron microscope.

PCR amplification: DNA was isolated using a high pure PCR template preparation kit (Roche, Indianapolis, USA). The 5.8S rRNA gene with the flanking areas ITS1 and ITS2 was amplified by PCR using trichomonad-specific primers designed according to the TFR1 and TFR2 primers described by Felleisen (1997): forward primer — ITSF (TTCAGTTACCGTCCCTCC); reverse primer ITSR (GTAGGTGAACCTGCCGTTGG). The reaction mixture consisted of 75 mM Tris—HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween-20, 1.9 mM mixture consisted of 75 mM Tris—HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween-20, 1.9 mM MgCl₂, 0.1 mM each dNTP, 0.25 μM both primers, 0.05 U/μl Taq polymerase, and 0.5—5 ng/μl DNA. The reaction conditions were as follows: 39 cycles of 90°C (0.5 min), 66°C (1 min) and 72°C (1 min) and ended by a final extension step at 72°C (15 min).

The SSU rDNA was amplified using trichomonad-specific primers 16SI (TACCTTGGTGATCCTGCC) and 16Sr (TCACCTACCGTT-20). The reaction mixture consisted of 75 mM Tris—HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween-20, 2.5 mM MgCl₂, 0.04 mM each dNTP, 0.001 mM both primers, 0.05 U/μl Taq polymerase, and 0.5—5 ng/μl DNA. The reaction conditions were as follows: 39 cycles of 90°C (0.5 min), 66°C (1 min) and 72°C (1.5 min) and ended by a final extension step at 72°C (15 min).

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In MrBayes, the base frequencies, rates for six different types of substitutions, number of invariant sites, and shape parameter of the gamma correction for rate heterogeneity were allowed to vary, covarian was used to model the rate heterogeneity along the tree. 2,000,000 generations of the Markov Chain Monte Carlo for the large analysis and 3,500,000 generations for the Monocercomonas colubrorum analysis were run with default settings (4 simultaneous chains, heating temperature 0.2). The first 2000 trees for the large analysis and 15,000 trees for the Monocercomonas colubrorum analysis were
removed as the “burn-in”. In the *Monocercomonas colubrorum* analysis, the data set was constructed by concatenating of SSU rDNA, ITS1, 5.8S rDNA, ITS2, and partial LSU rDNA, and specific models with independent parameters were assigned to each gene.

Maximum likelihood trees were constructed in PhyML using the GTR+I+Γ model with parameters estimated by the software. Maximum parsimony and distance trees were constructed in PAUP4.0 (Swofford 1998). For maximum parsimony tree construction, heuristic searching with ten replicates and random taxa addition was used. The neighbor-joining method with LogDet distances and estimated proportion of invariable sites was used to construct the distance trees. The proportions of invariable sites, 0.40 for the larger and 0.91 for the smaller analysis, were estimated in PAUP 4.0. The node support was assessed by bootstrapping (100 replicates for maximum parsimony and 1000 replicates for maximum likelihood and distance methods).

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