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Phylogeny of trichostome ciliates (Ciliophora, Litostomatea) endosymbiotic in the Yakut horse (*Equus caballus*)

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Abstract

Ciliates of the subclass Trichostomatia inhabit the fermentative regions of the digestive tract of herbivores. Most available small subunit ribosomal RNA (SSrRNA) gene sequences of trichostomes are from species isolated from the rumen of cattle or sheep and from marsupials. No ciliate species endosymbiotic in horses has yet been analyzed. We have sequenced the SSrRNA genes of five ciliate species, isolated from the cecum and colon of four Yakut horses: *Cycloposthium edentatum*, *Cycloposthium ishikawai*, *Tripalmaria dogieli*, *Cochliatoxum periachtum*, and *Paraisotricha colpoidea*.

Based on their morphology, *Cycloposthium*, *Tripalmaria*, and *Cochliatoxum* are classified as Entodiniomorphida, while *Paraisotricha* is considered a member of the Vestibuliferida. Phylogenetic analyses using Bayesian inference, distance, and parsimony methods confirm these placements. The two *Cycloposthium* species cluster together with the published *Cycloposthium* species isolated from a wallaby in Australia. *Tripalmaria* and *Cochliatoxum* branch as a sister group to or basal within the Entodiniomorphida. The Vestibuliferida remain paraphyletic with *Paraisotricha* and *Balantidium* branching basal to all other trichostome species, but not closely related to *Isotricha* and *Dasytricha*.

Keywords: *Cochliatoxum*; *Cycloposthium*; Equine intestine; *Paraisotricha*; SSrRNA; *Tripalmaria*

Introduction

Species of the subclass Trichostomatia (class Litostomatea) inhabit the digestive tract of a variety of hosts. Best known are the rumen ciliates and *Balantidium coli*, the only ciliate that is potentially pathogenic for humans. However, endosymbiotic litostomes are also found in the digestive tracts of horses, zebras, rhinoceroses, hippopotami, rodents, apes, marsupials, and even snakes, frogs, and fish (Corliss 1979). We distinguish three groups within the subclass Trichostomatia: the order Vestibuliferida de Puytorac et al., 1974, the order Entodiniomorphida Reichenow in Doflein and Reichenow, 1929, and the Australian clade (Table 1).

The order Vestibuliferida comprises six families, which are all characterized by holotrichous somatic ciliation and a densely ciliated vestibulum (Lynn and Small 2002). They are endosymbionts of herbivorous placental mammals with the exception of *Balantidium* species, which are parasitic in a variety of insect and vertebrate hosts (Corliss 1979).

The order Entodiniomorphida has reduced somatic ciliature in the form of ciliary tufts or bands and the oral

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Table 1. Updated classification scheme of the Order Trichostomatia

Subclass Trichostomatia
Order Vestibuliferida
Family Balantidiidae
Family Isotrichidae
Family Paraisotrichidae
Family Protocaviellidae
Family Protohalliidae
Family Pycnotrichidae
Order Entodiniomorphida
Suborder Archistomatina
Family Buetschliidae
Suborder Blepharocorythina
Family Blepharocorythidae
Suborder Entodiniomorphina
Family Cycloposthiidae
Family Ophryoscolecidae
Family Parentodiniidae
Family Polydiniellidae
Family Pseudoentodiniidae
Family Rhinozetidae
Family Spirodiniidae
Family Telamodiniidae
Family Troglodytellidae
Australian clade
Family Amylovoracidae
Family Macropodiniidae
Family Polycostidae

area shows functional polykinetids (Lynn and Small 2002). Entodiniomorphids are divided into three suborders. The Archistomatina and Blepharocorythina each include only one family. They are endosymbionts mostly in horses, camels, elephants, and hippopotami, but a few species also occur in cattle and rodents. The third suborder, the Entodiniomorphina, comprises many families, mostly occurring in ruminants and horses, and their relatives. Characteristically, species of this latter suborder possess a thickened pellicle that can have skeletal plates or can be drawn out into spines (Lynn and Small 2002).

Finally, species belonging to the Australian clade inhabit marsupials (Cameron and O'Donoghue 2002a, b 2003 2004; Cameron et al. 2000a, b 2001a, b 2003). Although highly supported by molecular analyses, we find no strong morphological synapomorphies for the three families within this clade (Cameron and O'Donoghue 2004).

Previous phylogenies inferred from small subunit ribosomal RNA (SSrRNA) gene sequence data of litostome ciliates (Cameron and O'Donoghue 2004; Cameron et al. 2001a 2003; Strüder-Kypke et al. 2006; Wright and Lynn 1997a, b; Wright et al. 1997) have clearly confirmed the monophyly of the subclass Trichostomatia. However, most molecular sequence data available for trichostome ciliates derive from species endosymbiotic in cattle, sheep, or marsupials. No taxonomically identified ciliate species from horses have been studied so far. The goal of this study was, therefore, to isolate ciliates from the digestive tract of horses, analyze their SSrRNA gene sequences, and construct a tree to reveal their phylogenetic relationships to the other trichostome species isolated from cattle and marsupials.

Material and methods

Sampling

The species in this study were isolated in November 2004 from the intestinal digesta of four 7–8-month-old Yakut horses (*Equus caballus*) that were slaughtered. The horses were kept free ranging near the village Yedei in the Namski region of the Republic of Sakha (Yakutia; 62.5° North, 129.5° East). Slaughtering took place at air temperatures of −35 °C. Samples of digesta were taken within 25–30 min after the horse had been slaughtered and were taken from the cecum and the ventral and dorsal ascending colon. Ten ml of digesta were immediately filtered through gauze into a bottle containing warm 95% ethanol, resulting in a final concentration of 75% ethanol. After the ciliate cells had settled, the supernatant was replaced with fresh 95% ethanol to increase the final concentration of ethanol.

Parallel samples fixed with 4% formaldehyde were used to determine the species composition of the individual samples. The samples with the lowest species diversity were chosen and easily identifiable species were picked from the ethanol-fixed aliquots for DNA analysis. One to two milliliters of the sample were pipetted into a Petri dish with 50 ml of distilled water. Individual cells were picked, washed again in water, and finally re-immersed in 95% ethanol until further processing. Each sample contained between 30 and 70 cells.

The names, taxonomic classification, origin of the sample, and the GenBank accession numbers for each of the sampled species are listed in Table 2.

DNA extraction and amplification

DNA was extracted from the ethanol-fixed cells with the MasterPure™ DNA Purification Kit (Epicentre, Madison, WI, USA). Four microliters of the template were used in the subsequent PCR reactions. The PCR amplification was performed under standard conditions in a Perkin-Elmer GeneAmp 2400 thermocycler (PE Applied Biosystems, Mississauga, ON, Canada), using the forward primer 82F (5′-GAAACTGCGAATG GCTC-3′) and the reverse primer C (5′-TTGGTCCG TGTTTCAAGACG-3′; Jerome and Lynn 1996). The

Table 2. List of new trichostome ciliate species sequenced in this report, their taxonomic classification, origin of sampling, and GenBank accession numbers of their small subunit rRNA gene sequences

Species	Order, Family	Sampled from	GenBank Acc. No.
Paraisotricha colpoidea	Vestibuliferida, Paraisotrichidae	Ventral ascending colon Horse no. 1, female	EF632075
Cochliatoxum periachtum	Entodiniomorphida, Spirodiniidae	Dorsal ascending colon Horse no. 1, female	EF632078
Tripalmaria dogieli	Entodiniomorphida, Cycloposthiidae	Dorsal ascending colon Horse no. 2, male	EF632074
Cycloposthium edentatum	Entodiniomorphida, Cycloposthiidae	Cecum Horse no. 4, male	EF632077
Cycloposthium ishikawai	Entodiniomorphida, Cycloposthiidae	Cecum Horse no. 5, male	EF632076

PCR products were purified with the GeneClean kit (Qbiogen, Carlsbad, CA, USA) and sequencing was performed in both directions with a 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA), using an ABI Prism BigDye Terminator (ver. 3.1) and a Cycle Sequencing Ready Reaction kit, using the amplification primers and one additional forward and reverse internal SSrRNA primer (Elwood et al. 1985).

Sequence availability and phylogenetic analyses

The nucleotide sequences obtained from the GenBank/EMBL databases are listed in Table 3. The sequence fragments were imported into Sequencher ver. 4.0.5 (Gene Codes Corp.), trimmed at the ends, assembled into contigs, and checked for sequencing errors. The new sequences were added to our existing DCSE (Dedicated Comparative Sequence Editor; De Rijk and De Wachter 1993) database and automatically aligned against already existing trichostome sequences. Considering secondary structural features of the SSrRNA molecule, we further refined the alignment. Two files were prepared for phylogenetic analyses based on the results of a previous study (Strüder-Kypke et al. 2006). In both files, all positions were used for the analyses with the exception of insertions of one or more nucleotides in a single species (e.g., *Euplotes*). The first file contained the in-group and out-group species and had 1781 positions (data not shown). A second file comprised only litostome species, thus containing only 1654 positions. These alignments are available from the corresponding author upon request. Missing nucleotides at the beginning or end of sequences were treated as missing by MrBayes and PAUP and gaps within the alignment were regarded as a fifth character state.

For the Bayesian inference analysis, MrModeltest (Nylander 2004; Posada and Crandall 1998) was employed to find the model of DNA substitution that best fits our data. The General-Time-Reversible (GTR) model for nucleotide substitution, considering invariable sites and gamma distributed substitution rates among sites, was depicted as the best model. This model ($n = 6$, rates = invgamma) was implemented in MrBayes ver. 3.1.1, a phylogenetic program employing Bayesian inference (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), which we used to infer a phylogenetic tree (BI). Two parallel runs were performed and the maximum posterior probability of a phylogeny out of 1,000,000 trees, approximating it with the Markov chain Monte Carlo (MCMC) and sampling every 50th generation, was computed, discarding the first 2000 trees as burn-in. A maximum parsimony (MP) analysis was performed with PAUP* ver. 4.0b10 (Swofford 2002), using 767 and 305 parsimony-informative characters, respectively, and with the tree bisection-reconnection (TBR) branch-swapping algorithm in effect. Species were added randomly ($n = 5$) and the data were bootstrap resampled 1000 times. PHYLIP ver. 3.6a2 (Felsenstein 2004) was employed to construct a distance matrix, using DNADIST to calculate genetic distances with the F84 model assuming gamma distribution (Felsenstein and Churchill 1996; Kishino and Hasegawa 1989). The distance trees, however, were constructed with PAUP, based on the GTR model and assumed gamma distribution of substitution rates, using the neighbor joining (NJ) algorithm (Saitou and Nei 1987). The data were bootstrap re-sampled 1000 times.

Results

Ciliate composition of the digesta samples

Each sample consisted of different ciliate species. While 10–20 species were present in the cecum and the ventral part of the ascending colon, the dorsal colon harbored 20–30 different species. The abundances varied from 34,000 cells/ml in the ventral ascending colon of horse no. 1 up to 120,000 cells/ml in the cecum of horse no. 4.

Table 3. List of small subunit rRNA gene sequences used in this study, including their GenBank/EMBL database accession numbers and reference

Amphileptus procerus (submitted as *Hemiophrys procera*)	AY102175	Zhu, Yu, and Shen, unpubl.
Amylovorax dehorityi	AF298817	Cameron et al. (2001a)
Amylovorax dogieli	AF298825	Cameron et al. (2001a)
Arcuospathidium cultriforme	DQ411456	Strüder-Kypke et al. (2006)
Arcuospathidium muscorum	DQ411456	Strüder-Kypke et al. (2006)
Bandia cribbi	AF298824	Cameron and O'Donoghue (2004)
Bandia smalesae	AF298822	Cameron and O'Donoghue (2004)
Bandia tammar	AF298823	Cameron and O'Donoghue (2004)
Bitricha tasmaniensis	AF298821	Cameron et al. (2001a)
Cycloposthium edentatum	AF042485	Cameron et al. (2003)
Dasytricha ruminantium (strain Guelph)	U57769	Wright and Lynn (1997a)
Dasytricha ruminantium (strain UK)	U27814	Embley et al. (1995)
Didinium nasutum	U57771	Wright and Lynn (1997a)
Diplodinium dentatum	U57764	Wright and Lynn (1997b)
Enchelys polynucleata	DQ411456	Strüder-Kypke et al. (2006)
Entodinium caudatum	U57765	Wright et al. (1997)
Epidinium caudatum	U57763	Wright et al. (1997)
Epispathidium papilliferum (Isolate A)	DQ411456	Strüder-Kypke et al. (2006)
Epispathidium papilliferum (Isolate B)	DQ411456	Strüder-Kypke et al. (2006)
Eudiplodinium maggii	U57766	Wright and Lynn (1997b)
Homalozoon vermiculare	L26477	Leipe et al. (1994)
Isotricha intestinalis	U57770	Wright and Lynn (1997a)
Loxophyllum rostratum	DQ411456	Strüder-Kypke et al. (2006)
Macropodinium ennuensis	AF298820	Cameron et al. (2003)
Macropodinium yalanbense	AF042486	Cameron et al. (2003)
Ophryoscolex purkynjei	U57768	Wright and Lynn (1997b)
Polycosta roundi	AF298819	Cameron and O'Donoghue (2004)
Polycosta turniae	AF298817	Cameron and O'Donoghue (2004)
Polyplastron multivesiculatum	U57767	Wright et al. (1997)
Pseudoamphileptus macrostoma (submitted as *Hemiophrys macrostoma*)	AY102173	Zhu, Yu and Shen, unpubl.
*Siroloxophyllum utricula*riae (submitted as *Loxophyllum utricula*riae)	L26448	Leipe et al. (1994)
Spathidium sp.	Z22931	Hirt et al. (1995)
Spathidium stammeri	DQ411456	Strüder-Kypke et al. (2006)
Teuthophrys trisulca	DQ411456	Strüder-Kypke et al. (2006)

Description of species

1. ***Paraisotricha colpoidea*** Fiorentini, 1890 (Fam. Paraisotrichidae) (Fig. 1a)
   Body length, 47–88 μm, body width, 24–56 μm; length/width ratio, 1.1:1.6. Elongated slender forms as well as short and rounded ones occur. The cells are elliptic in shape and circular in cross-section with a slight constriction at the anterior end. A large concrement vacuole ("a subpellicular cytoplasmic inclusion containing refractile grains" Corliss 1979) occupies nearly the entire anterior part of the cell. The oral cavity starts at the posterior end of the concrement vacuole, descending into the cytoplasm half the body length or farther. Somatic ciliation is holotrichous with 28–50 slightly spiraling kineties. Elongated cilia cover the concrement vacuole. One large ovoid macronucleus is mostly centrally located accompanied by a small spherical micronucleus. For a detailed description, see Kornilova (2004) and Wolska (1964).
2. ***Cochliatoxum periachtum*** Gassovsky, 1919 (Fam. Spirodiniidae) (Fig. 1b)
   Body length, 360–510 μm; body width, 180–230 μm; length/width ratio, 1.8:2.1. *C. periachtum* is the largest ciliate in equines. The cells are more or less cylindrical in shape, with both ends rounded. The adoral ciliary zone, consisting of two bands, surrounds a slit-shaped peristome. The somatic ciliature forms an anterior occipital arch and two caudal half-spiral shaped arches posterior. A caudal sheath from which the hindmost conical part of the body protrudes surrounds the posterior end of the cell. One elongated macronucleus lies close to the right surface of the body, accompanied by a small ellipsoidal micronucleus. For a detailed description, see Kornilova (2004) and Senaud and Grain (1972).

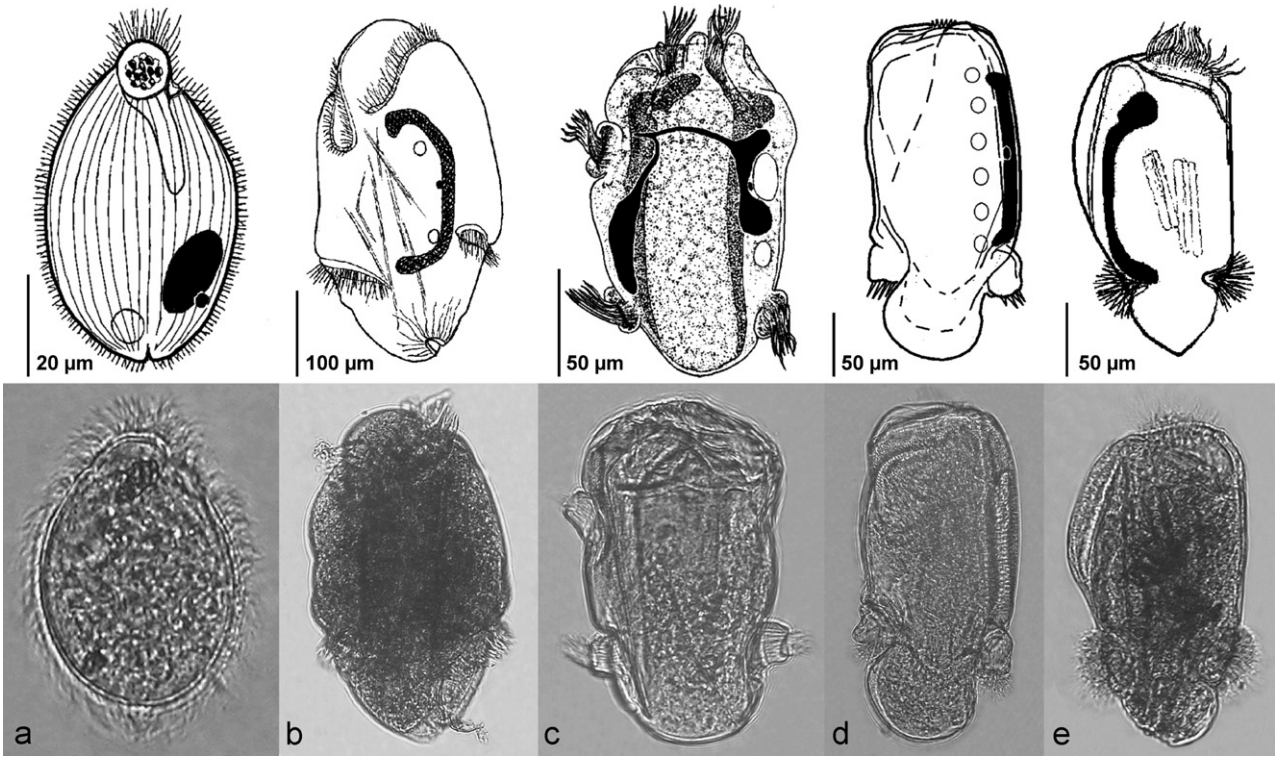

Fig. 1. Schematic drawings (above) and photomicrographs from formaldehyde-fixed cells (below) of the studied species: (a) *Paraisotricha colpoidea*, (b) *Cochliatoxum periachtum*, (c) *Tripalmaria dogieli*, (d) *Cycloposthium edentatum* and (e) *Cycloposthium ishikawai*.

3. ***Tripalmaria dogieli*** Gassovsky, 1919 (Fam. Cycloposthiidae) (Fig. 1c)
   Body length, 130–210 µm; body width, 54–91 µm; length/width ratio, 2.1:2.4. The cells are irregularly oviform in shape and laterally flattened. Two caudalia (synciliary tufts) are located posterior and a third one lies anterior to the dorsal posterior caudalium. The proximal part of the caudalia is covered with caudal lips (or "muffs"). The peristome with a retractable adoral ciliary zone is located at the anterior end. The ectoplasm is supported by a skeletal plate, which lies mainly on the right of the cell, but extends beneath the dorsal side and part of the left side. The macronucleus consists of two lobes; its ventral lobe embraces the micronucleus. For a detailed description, see Kornilova (2004).
4. ***Cycloposthium edentatum*** Strelkow, 1928 (Fam. Cycloposthiidae) (Fig. 1d)
   Body length, 100–230 µm; body width, 50–110 µm; length/width ratio, 2.0:2.2. The cell shape is more or less rectangular with a truncated anterior end and a tapered posterior end. The dorsal side is slightly convex while the ventral side is flat. Round caudalia arise on both the dorsal and the ventral bases of the tail flap. Both caudalia possess caudal lips. Two broad skeletal plates extend into the tail. The skeletal plate on the left side of the body has a longitudinal groove. The anterior peristome has a retractable adoral ciliary zone. The elongated macronucleus is situated dorsally, parallel to the long axis of the body, accompanied by an ellipsoidal micronucleus. Six to seven contractile vacuoles are aligned parallel to the macronucleus. For a detailed description, see Fernández-Galiano (1959).
5. ***Cycloposthium ishikawai*** Gassovsky, 1919 (Fam. Cycloposthiidae) (Fig. 1e)
   Body length, 230–280 µm; body width, 110–130 µm; length/width ratio, 2.1:2.2. The cell is obtusely truncated anteriorly and gradually narrowing towards the posterior. The anterior part of the dorsal side is dilated. Caudalia with ciliary arches arise on both the dorsal and the ventral bases of the tail flap. There are no protruding caudal lips around these caudalia. The anterior peristome and adoral ciliary zone are retractable. One elongated macronucleus is dorsally located accompanied by a micronucleus.

SSrRNA gene sequence/primary structure

The number of nucleotides (length) and the GC content (in %) for the SSrRNA are as follows: *P. colpoidea*—1559 nucleotides, 42%; *C. periachtum*—1558 nucleotides, 40%;

T. dogieli—1559 nucleotides, 41%; *C. edentatum*—1559 nucleotides, 41%; *C. ishikawai*—1557 nucleotides, 41%.

All five sequences showed the typical litostome deletions in helices 23_1, 23_8, and 23_9, as well as deletion of the entire helix 23_5 (Leipe et al. 1994; Wright et al. 1997).

Phylogenetic analyses

The phylogenetic positions of the newly sequenced species were determined using three different analyses (Bayesian inference—BI, maximum parsimony—MP, and genetic distances—NJ). As described in the methods section, two datasets were used: the first dataset included all litostome sequences as well as sequences of representatives of each ciliate class. Due to the relatively long branch separating the class Litostomatea from the other classes, we constructed a second dataset including only litostome species. The topology of the in-group was identical for both datasets and, therefore, only the results of the in-group analysis are shown (Fig. 2). The computed trees were re-rooted with *Dileptus* sp. as out-group, since this haptorian species branched basal in the previously computed trees. All three analyses produced congruent phylogenetic trees.

While the subclass Trichostomatia was highly supported as monophyletic (1.0 BI, 99% MP, 99% NJ), the order Vestibuliferida as characterized in Table 1 was paraphyletic. *P. colpoidea* grouped with *B. coli* in the BI and MP analyses, but the branch was not well supported (0.79 BI, 41% MP, 17% NJ). Both species were placed basal to the cluster formed by the entodiniomorphids and the isotrichids in BI (Fig. 2). However, the support values for this node were extremely poor (0.44 BI, 21% MP, 6% NJ). The distance analysis placed *P. colpoidea* basal to all trichostomes and *B. coli* basal to the Australian clade (56% NJ), while the MP analysis grouped *B. coli* and *P. colpoidea* together, but not in a cluster with the entodiniomorphids. Instead, they were grouped with either the Australian clade, or basal to all trichostomes (data not shown). The isotrichids (*Isotricha*, *Dasytricha*) clustered basal to the entodiniomorphids (1.0 BI, 75% MP, 68% NJ).

The family Cycloposthiidae was paraphyletic as well. *T. dogieli* did not cluster with the three *Cycloposthium* species. Instead, it formed a highly supported branch with *C. periachtum* (family Spirodiniidae) (1.0 BI, 96% MP, 100% NJ), basal to all entodiniomorphids (1.0 BI, 100% MP, 99% NJ).

C. edentatum isolated from the Yakut horse clustered with *C. edentatum* isolated from the wallaby. However, the latter species showed a rather long branch and the support values differed considerably among the analyses (1.0 BI, 89% MP, 18% NJ). The genetic divergence *C. edentatum* (horse) versus *C. edentatum* (wallaby) was calculated as 1.5%. *C. ishikawai* branched basal to the other two *Cycloposthium* species; its genetic divergence to *C. edentatum* (horse) was calculated as 0.79%, whereas the genetic divergence to *C. edentatum* (wallaby) was 2.11%. The genus itself was highly supported in all three analyses (1.0 BI, 98% MP, 99% NJ).

Both the Australian clade and the order Entodiniomorphida were highly supported as monophyletic (both with 1.0 BI, 100% MP, and >99% NJ). While all three families of the Australian clade (all 1.0 BI, 100% MP and NJ) and the family Ophryoscolecidae (i.e., *Entodinium caudatum*, *Eudiplodinium maggii*, *Diplodinium dentatum*, *Polyplastron multivesiculatum*, *Epidinium caudatum*, and *Ophryoscolex purkynjei*) (1.0 BI, 87% MP, 90% NJ) were highly supported, the support values for the branching pattern within the latter clade were not always high (Fig. 2).

Discussion

It was possible to unambiguously identify the isolated species due to their characteristic morphological features. *T. dogieli* is the only ciliate in horses that has three tufts with caudal lips. It occurs in two different morphotypes: a large (200 μm long) and a small (100 μm long) morphotype. Only cells of the large morphotype were picked. *C. periachtum* is the largest ciliate in the intestine of horses and therefore easy to identify. *Paraisotricha colpoidea* is one of the two *Paraisotricha* species occurring in horses. The second species is *P. minuta*. These species differ in the number of somatic kineties—28–50 in *P. colpoidea* and 17–23 in *P. minuta*. For each picked cell the number of kineties was determined to confirm identity. *C. edentatum* is a well-known species and easy to identify due to its characteristic features (e.g. shape and size of body and macronucleus). It is also very numerous and occurs in three different morphotypes: morphotype *edentatum*, morphotype *scutigerum*, and morphotype *gigas*. Only cells of the morphotype *edentatum* have been picked. *C. ishikawai* has also been identified based on shape and size of cell and macronucleus. The two *Cycloposthium* species differ mostly in size and shape, especially of the posterior end, in the shape of the macronucleus, as well as in the number of contractile vacuoles. Thus, we are confident that the SSrRNA gene sequences reported can be assigned to these taxa. During a previous study from 2001 to 2004 (Kornilova 2006), the digesta of 42 Yakut horses was investigated and *T. dogieli* was found in 73% of the horses, *C. edentatum* in 67%, *C. ishikawai* in 23%, *C. periachtum* in 27%, and *P. colpoidea* in 70% of the horses.

The subclass Trichostomatia has been divided into the three groups, the orders Vestibuliferida and Entodiniomorphida and the Australian clade (Table 1). While the

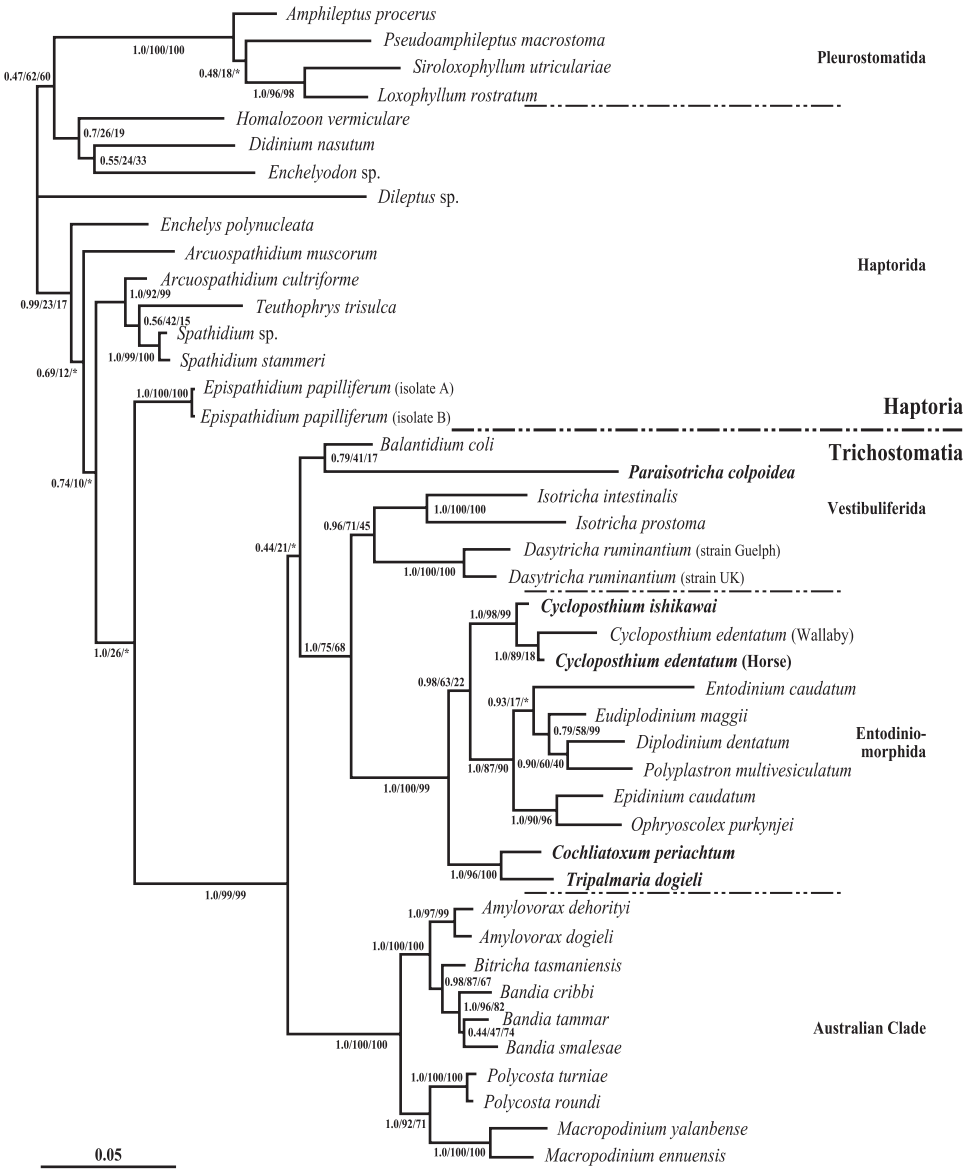

Fig. 2. Maximum likelihood tree computed with MrBayes ver. 3.1.1 (Ronquist and Huelsenbeck 2003), based on the General-Time-Reversible (GTR) model with gamma-distribution and an estimate of invariable sites, determined by MrModeltest (Nylander 2004). The first numbers at the nodes represent the posterior probability values of the Bayesian analysis and the second and third numbers represent bootstrap values (percent out of 1000 replicates) for maximum parsimony (Swofford 2002) and neighbor joining (Saitou and Nei 1987), respectively. An asterisk indicates bootstrap values of less than 10%. The scale bar represents 5 changes per 100 positions. New sequences appear in bold face.

latter two groups are clearly monophyletic assemblages, the Vestibuliferida cannot be identified as monophyletic by SSrRNA gene sequences. The branching pattern can either represent "true" paraphyly, or it can be due to a mix of long-branch attraction (LBA) artifact, based on the fast evolution of single clades of vestibuliferids, and undersampling. *B. coli* has been isolated from a gorilla, *Isotricha* spp. and *Dasytricha ruminantium* from cattle and sheep, and *P. colpoidea* from the Yakut horse. The support values for the node of the cluster of entodiniomorphids and vestibuliferids are extremely poor (0.44 BI, 21% MP, 6% NJ) and the support for a branching of the isotrichids with the entodiniomorphids is only high in BI (1.0) but lower in MP (75%) and NJ (68%).

Based on their holotrichous somatic ciliation and their occurrence in hosts that represent a large variety of classes, we could assume that the vestibuliferids are the most ancestral trichostomes. Therefore, the taxa of the order Vestibuliferida have been separated longer from each other than from the other species of trichostomes. If we further consider that (a) they inhabit a large variety of hosts and (b) that the higher divergence rate

represents periods of rapid evolution associated with the invasion of new host species (Dykhuizen 1990), then this may explain why our SSrRNA gene sequence analyses failed to depict the vestibuliferids as a monophyletic assemblage.

C. periachtum and *T. dogieli* are strongly supported as sister taxa within the entodiniomorphids. This grouping contradicts the classifications based on morphological characters that assign *C. periachtum* to the family Spirodiniidae and *T. dogieli* to the family Cycloposthiidae (Lynn and Small 2002). Ultrastructural and morphological studies (Senaud and Grain 1972) found *C. periachtum* to show similarities with both the Cycloposthiidae and the Ophryoscolecidae (e.g., the presence of somatic syncilia and a pharyngeal basket), but also distinct differences. Both Ophryoscolecidae and Cycloposthiidae commonly possess retractable oral or adoral synciliary tufts and skeletal plates, while the Spirodiniidae lack skeletal plates and have somatic ciliary bands. Earlier morphological and ultrastructural studies (Wolska 1978a, b) showed a great similarity in the infraciliature of *Cycloposthium* and *Tripalmaria*, but distinct differences in their ultrastructure. A comparison between the three genera, *Tripalmaria*, *Cycloposthium*, and *Cochliatoxum*, indicated that the ectoplasm in *T. dogieli* is different from other cycloposthiids and ophryoscolecids: the cell membrane in unciliated areas is covered by an amorphous substance similar to the mucoidal layer in *Cochliatoxum* (Senaud and Grain 1972; Wolska 1978b). Furthermore, in the non-ciliated regions of both species, short, non-ciliated kinetosomes underlie the dense net of the ectoplasm (Wolska 1978b). Based on these studies, Grain (1994) suggested assigning *Tripalmaria* to a separate family – the Tripalmariidae. Although the sister-group relationship between *Tripalmaria* and *Cochliatoxum* is highly supported in all analyses, the possibility that this branch is an artifact due to taxon undersampling has to be considered, since two trichostome suborders (Blepharocorythina and Archistomatina) and five families of the order Entodiniomorphida are not represented in this analysis.

C. edentatum isolated from the Yakut horse is closely related to *C. edentatum* from the wallaby. The branch of the wallaby endosymbionts is long, suggesting a faster rate of evolution, probably related to the invasion of a new host.

However, the genetic divergence of the two *C. edentatum* species is extremely large when compared to general genetic distance of other ciliate taxa. The genetic distance between them is $d = 0.015$, representing a 1.5% divergence. If the theory that a host change of *C. edentatum* took place through transfaunation, probably when horses were introduced in Australia – a fairly recent event (ca. 200 years ago) – then this would mean a divergence rate of 1.5% in roughly 200 years. This estimate is incredibly high, compared with the evolutionary divergence rate of 1% in 72–80 My (million years) estimated by Wright and Lynn (1997c) for *Ichthyophthirius* and even compared with the divergence rate of the rumen ciliates with 1% every 8–11 My. Opposed to this, the divergence rate between *C. edentatum* from the Yakut horse and *C. ishikawai* is only 0.0078 (0.78%). This leaves three interpretations: (1) The majority of horse ciliate species (84%) occur only in horses and close relatives; therefore, a host change might have triggered fast evolution, resulting in extreme divergence rates as shown here. (2) Genetic divergence in *C. edentatum* may have already occurred within the different horse species. Horses originally introduced to Australia came from England and, due to the geographical isolation, no contact with Yakut horses existed for several thousand years. (3) What we define as *C. edentatum* might actually include several cryptic species. Cameron et al. (2000b) described *C. edentatum* from the wallaby with species-typical features, but significantly smaller than previously described specimens. Although the general morphological features are identical, our species is almost twice as large as the species isolated from the wallaby. Additional support for cryptic species is given by the fact that we distinguished three morphotypes in *C. edentatum*, which may, in fact, represent different species. To support any of the three theories, the genetic variability of *C. edentatum* from different horse species across the world needs to be analyzed.

C. ishikawai groups basal to the other two *Cycloposthium* species, and the genus is well supported as monophyletic. As *C. ishikawai* differs morphologically from the other *Cycloposthium* species (i.e., caudalia without caudal lips compared with all other *Cycloposthium* species that possess caudalia with caudal lips), the question arises whether it should be transferred to a different genus. Da Cunha (1938) transferred another *Cycloposthium* species, *Cycloposthium vorax* from the intestine of the capybara, into the new genus *Toxodinium*, characterized by caudalia without caudal lips. However, at this point we cannot recommend a transfer based only on the molecular data of two *Cycloposthium* species. At a minimum, we will need to obtain the SSrRNA gene sequence of *Toxodinium* to confirm that it is a sister species to *C. ishikawai*.

Perissodactyls evolved earlier than ungulates and this is reflected in the topology of the Entodiniomorphida where the horse ciliates branch basal to the other species that inhabit the rumen. Overall, 84% of the ciliate species and 67% of the ciliate genera in the intestine of equids are unique for this host. Sixty-nine species (trichostomes and allantosomatids) inhabit exclusively equid hosts (horses, kulans, asses, zebras) and only 13 species live in equids as well as in other hosts (rhinoceroses and elephants). Generally, host specificity is high among the entodiniomorphids with different families being associated with a certain range of hosts (Dehority 1996; Van Hoven et al. 1987). Only the family

Cycloposthiidae is found in a number of hosts. Cameron and O'Donoghue (2004) discussed this topic comprehensively, especially in relation to ciliates found in marsupials.

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