Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Phylogenetics and Evolution 39 (2006) 542–551
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

New evolutionary lineages, unexpected diversity, and host specificity in the parabasalid genus *Tetratrichomonas*

Ivan Cepicka *, Vladimír Hampl, Jaroslav Kulda, Jaroslav Flegr

Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic

Received 8 September 2005; revised 12 December 2005; accepted 5 January 2006
Available online 10 February 2006

Abstract

We studied morphological and molecular polymorphism of 53 *Tetratrichomonas* isolates obtained from amphibian, reptilian, mammalian hosts, and from a slug with the aid of protargol staining and analyses of ITS1-5.8S rRNA-ITS2, SSU rRNA, and α-tubulin gene sequences. The phylogenetic tree based on the concatenate of all sequences showed the monophyly of the genus *Tetratrichomonas* with respect to the genus *Trichomonas*. Our data suggest that two parabasalid genera, *Pentatrichomonoides* and *Trichomonoides*, may belong to the genus *Tetratrichomonas*. *Tetratrichomonas* isolates were divided into 16 robust host-specific and monophyletic groups that probably represent separate, mostly new, species. As only five *Tetratrichomonas* species were described from the examined host taxa so far, our study uncovered considerable species diversity within the genus. The wide host range, high level of species-specific host specificity, and newly revealed biodiversity make the genus *Tetratrichomonas* a valuable model for studying evolution of parasites.

Keywords: Parabasala; *Tetratrichomonas*; SSU rRNA; ITS; 5.8S rRNA; α-Tubulin; Phylogeny; Host specificity; Molecular polymorphism

1. Introduction

The genus *Tetratrichomonas* is thought to contain approximately 10 valid species and is probably the largest genus among 12 described parabasalid genera parasitizing vertebrates. Tetratrichomonad species can be found mostly in the lower intestine of a broad spectrum of animals including leeches, mollusks, bone fish, and all classes of tetrapods. Little is known about the host specificity of tetratrichomonads and parabasalids in general. It is expected that some tetratrichomonad species can infect a wide range of unrelated host taxa, such as birds and humans in case of *T. gallinarum* (Cepicka et al., 2005; Kutisova et al., 2005; McDowell, 1953), and amphibians and reptiles in case of *T. prowazeki* (Honigberg, 1951), while others are restricted to a single host taxon, e.g., *T. microti* from rodents (Wenrich and Saxe, 1950), *T. limacis* from slugs (Kozloff, 1945; Salleudin, 1972), *T. brumpti* from tortoises (Honigberg, 1951), *T. didelphidis* from marsupials (Andersen and Reilly, 1965; Tasca et al., 2001), *T. buttreyi* from even-toed ungulates (Hibler et al., 1960; Jensen and Hammond, 1964), and *T. ovis* from sheep (Andersen and Levine, 1962). Tetratrichomonads found in fish and leeches (Alexeieff, 1910, 1911) are probably not conspecific with *T. prowazeki* (Brugerolle, 1976).

The genus *Tetratrichomonas* can easily be recognized as it possesses four anterior flagella, a long posterior flagellum with a free distal end and a typically discoid parabasal body, i.e., the golgi complex with adjacent striated fibrils (Brugerolle, 1976; Honigberg, 1963). It closely resembles the genera *Trichomonas*, *Pentatrichomonas*, *Pentatrichomonoides*, *Trichomitopsis*, *Pseudotrypanosoma*, and *Cochlosoma* in ultrastructure of the karyomastigont, specifically by the type of costal fibre and undulating membrane (Brugerolle, 1976; Salleudin, 1972). The close relationship among the seven genera was confirmed also by molecular phylogenetics and together they constitute the family Trichomonadidae (Hampl et al., 2006). Recently,

* Corresponding author. Fax: +420 224919704.
E-mail address: ivan.cepicka@centrum.cz (I. Cepicka).

1055-7903/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2006.01.005

Brugerolle and Bordereau (2004) have established the genus *Trichomonoides*, which also belongs to the family Trichomonadidae.

In the present paper, we analyzed molecular and morphological polymorphism among 53 *Tetratrichomonas* isolates obtained from various hosts on the basis of sequences of the ITS1-5.8S rRNA-ITS2 region and SSU rRNA gene. We also examined the holophyly of the genus *Tetratrichomonas* using both separate and concatenated sequences of the ITS1-5.8S rRNA-ITS2 region, SSU rRNA gene, and α-tubulin gene. The present study represents the first attempt to investigate the intrageneric molecular polymorphism of parabasalid flagellates, and the first attempt to concatenate several gene sequences to obtain a better supported topology of the parabasalian tree. It also combines molecular phylogenetics with biological and morphological approaches.

2. Materials and methods

2.1. Organisms and culture conditions

Information on the origin of isolates included in the study is summarized in Table 1. The isolates, except for SL (Cepicka et al., 2005), PH-KT (Delgado-Viscogliosi et al., 2000), R114 (Tachezy et al., 2002), and 4190 (ATCC Number 50597), were isolated between 1999 and 2004 from faeces or the cecum of mammals, turtles, amphibians, and from hepatopancreases of slugs. The hosts had been kept by private keepers, in zoological gardens, or were captured from wild. The reptiles imported into the Czech Republic were examined immediately after arrival. Flagellates were isolated in Dobell and Leidlaw's biphasic medium (Dobell and Leidlaw, 1926) or in modified TYSGM medium (Diamond, 1982), without mucin and supplemented with rice starch, and maintained in xenic culture. Isolates from mammals were cultured at 37 °C and were subcultured every second or third day; isolates from cold-blooded hosts were cultured at 26 °C and were subcultured every fourth or fifth day. Isolates LMC and KR-PO2 were probably uncultivable in the long term as the trichomonads failed to grow after the tenth passage. *Tritrichomonas muris* isolate MURIS1 was not cultured and DNA was isolated directly from ceacum of a yellow-necked mouse (*Apodemus flavicollis*).

2.2. DNA amplification, cloning, and sequencing

Genomic DNA was isolated using High pure PCR template preparation kit (Roche). Usually, the whole region of 16S rRNA, 5.8S rRNA, ITS1, and ITS2 was amplified with primers 16Sl (TACTTGGTTGATCCTG CC) and ITSF (TTCAGTTCAGCGGGTCTTCC). The ITS1-5.8S rRNA-ITS2 region of isolates IVB, ANOA, GECA5, INDO, KINIX, and GERA3 was amplified using primers ITSF and ITSR (GTAGGTGAACCTG CCGTTGG) that are similar to the primers TFR1 and TFR2 designed by Felleisen (1997). An approximately 1600 bp fragment of the SSU rRNA gene of isolates LMC, SLON, and ZUBR was amplified using primers 16Sl and 16Sr (TGATCCTTCTGCAGGTTCACC). An internal 1130 bp fragment of the α-tubulin gene was amplified using nested PCR. The primary PCR was conducted using primers AtubA (RGTNGGNAAYGCN TGYTGGGA) and AtubB (CCATNCCYTCNCCNAC RTACCA) according to Edgcomb et al. (2001). The secondary PCR was conducted using primers α-tubF1 (TAYTGYYWNGARCAYGGNAT) and α-tubR1 (AC RAANGCNCGYTTNGMRWACAT), similar to the primers used by Moriya et al. (2001).

PCR products were either directly sequenced or were subcloned into the pGEM-T EASY vector using the pGEM-T EASY VECTOR SYSTEM I (Promega) and at least two clones obtained from two independent PCR were sequenced. The external primers used for direct sequencing from PCR product were ITSF, ITSR, 16Sl, 16Sr, α-tubF1, and α-tubF2. The external primers used for sequencing from vector were primers SP6 (ATTTAGGTGACACT ATA) and T7 (TAATACGACTCACTATA). The primers used for sequencing of internal regions of the SSU rRNA gene were 514F (GTGCCAGCMGCCGCGG), 1055F (GGTGGTGCATGGCCG), 1385R (GATCCTAACA TTGTAGC), 1055R (CGGCCATGCACCACC), 665R (ATACWCTAAGCGTCCTG), and 295R (AGTCCG ACGGTAACCGC). The primers used for sequencing of internal regions of the α-tubulin gene were TRICHTUBF1 (CTCMTTCGGTGGTGG) and TRICHTUBR1 (KGG GAAGTGGATACG). All genes were sequenced bidirectionally. Sequence data reported in this paper are available in GenBank under accession numbers AY886770–AY886886.

2.3. Phylogenetic analyses

Five data sets containing sequences of ITS1-5.8S rRNA-ITS2 region (two data sets), SSU rRNA, α-tubulin, and the concatenate of the three loci were constructed. Sequences from each locus were aligned using ClustalX 1.81 (Thompson et al., 1997) and alignments were manually edited using the BioEdit sequence editor (Hall, 1999). The concatenated data set was created manually. Alignments are available from the corresponding author upon request. Phylogenetic trees were constructed using Fitch–Margoliash with LogDet distances, maximum parsimony, maximum likelihood, and Bayesian methods. Distance and maximum parsimony trees were constructed in PAUP* 4.0b10 (Swofford, 2002) by 10 replicates of heuristic search. The starting tree was obtained by the stepwise addition procedure with a random order of taxa addition and swapped using the tree bisection–reconnection algorithm. The constant positions were excluded before performing the distance analysis. If two or more sequences were identical at parsimony informative positions, only one of them was retained in the

Table 1
List of trichomonad strains included in the study

Isolate	Host	GenBank accession numbers[b]
Tetratrichomonas isolate from invertebrates		
LMC[a]	*Limax maximux*	AY886801–2, AY886872
Tetratrichomonas isolates from amphibians		
BOMB3[a]	*Bombina bombina*	AY886821, AY886855
COL	*Pleurodeles waltl*	AY886824, AY886858
Tetratrichomonas isolate from lizards		
SL[a]	*Anguis fragilis*	AY886881–4
Tetratrichomonas isolates from turtles		
GECA1[a]	*Geochelone carbonaria*	AY886826, AY886860
GECA5[a]	*Geochelone carbonaria*	AY886778–9, AY886849, AY886850
GEEL1	*Geochelone elegans*	AY886827, AY886861
GEGI1	*Geochelone gigantea*	AY886828
GEPA1	*Geochelone pardalis pardalis*	AY886828
GEPA2[a]	*Geochelone pardalis pardalis*	AY886828
GERA1[a]	*Geochelone radiata*	AY886826, AY886860
GERA2[a]	*Geochelone radiata*	AY886828, AY886860
GERA3	*Geochelone radiata*	AY886780–1, AY886861
CHERS1	*Chersina angulata*	AY886828
CHERS3	*Chersina angulata*	AY886828
INDO[a]	*Indotestudo elongata*	AY886782–5
KAJ	*Macroclemys temminckii*	AY886873–5
KINIX[a]	*Kinixys belliana nogueyi*	AY886790–4, AY886853
KINIX2[a]	*Kinixys belliana nogueyi*	AY886795–6
KOD37	*Geochelone pardalis*	AY886828, AY886860
MALAC1	*Malacochersus tornieri*	AY886828
PYX	*Pyxidea mouhotii*	AY886824, AY886858
TEHE2[a]	*Testudo hermanni*	AY886808–9, AY886870
TENE2M	*Testudo marginata*	AY886839, AY886870
TERA1	*Testudo marginata*	AY886810–3, AY886870
TEST	*Testudo graeca*	AY886814–6, AY886870
THR3	*Testudo horsfieldii*	AY886840
ZS	*Geochelone nigra*	AY886817–9, AY886820, AY886861
Tetratrichomonas isolates from mammals		
ANOA	*Bubalus depressicornis*	AY886770–3, AY886847–8
BON6	*Bos taurus*	AY886822, AY886856
BUVK	*Syncerus caffer caffer*	AY886823, AY886857
BUVP	*Syncerus caffer nanus*	AY886774–7
EQU2	*Equus caballus*	AY886825, AY886859
IVB	*Bison bison*	AY886786–9, AY886851–2, AY886862
KR-PO2	*Bos taurus*	AY886797–8, AY886863
KR-PO3	*Bos taurus*	AY886799, AY886800, AY886854
MANG	*Sus scrofa* f.*domestica*	AY886829, AY886868
PB	*Phacochoerus aethiopicus*	AY886803–4, AY886864
PD22M[a]	*Sus scrofa*	AY886830, AY886865, AY886876–8
PDOU3	*Sus scrofa* f.*domestica*	AY886832
PDOU4	*Sus scrofa* f.*domestica*	AY886833
PDOU7	*Sus scrofa* f.*domestica*	AY886834
PDOU8	*Sus scrofa* f.*domestica*	AY886833, AY886867
PDOU9	*Sus scrofa* f.*domestica*	AY886835
PDOU10	*Sus scrofa* f.*domestica*	AY886831, AY886867
PDOU11	*Sus scrofa* f.*domestica*	AY886829
PDOU12	*Sus scrofa* f.*domestica*	AY886805–6, AY886866
PDOZ1	*Sus scrofa* f.*domestica*	AY886807
PEKB	*Tayassu pecari*	AY886829, AY886868
PEKPR	*Pecari tajacu*	AY886836, AY886866
PVIET	*Sus scrofa* f. *domestica*	AY886837, AY886869
SLON	*Loxodonta africana*	AY886838, AY886861
ZUBR	*Bison bonasus*	AY886841, AY886871, AY886885–6
Tritrichomonas isolates		
T. muris MURIS1[a]	*Apodemus flavicollis*	AY886843–4, AY886846
T. mobilensis TANA	*Tupaia belangeri*	AY886842

Table 1 (*continued*)

Isolate	Host	GenBank accession numbers[b]
T. mobilensis 4190 (ATCC 40597)	*Saimiri sciureus*	AY886842
T. nonconforma R114	*Anolis bartschi*	AY886845
Pentatrichomonas hominis isolate PH-KT	*Homo sapiens*	AY886879, AY886880

[a] Isolates obtained from wild animals.
[b] Where more isolates had identical sequences, just one of them was submitted to GenBank.

maximum parsimony analysis to save the computing time. Maximum parsimony and distance trees were bootstrapped with 1000 replicates. Maximum likelihood trees were constructed in the program Phyml (Guindon and Gascuel, 2003). The models of nucleotide substitution for maximum likelihood analyses were chosen by hierarchical nested likelihood ratio tests implemented in Modeltest 3.06 (Posada and Crandall, 1998). Bootstrapping was performed with 100 replicates. Bayesian analyses were performed using the program MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Base frequencies, rates for six different types of substitution, proportion of invariant sites, and shape parameter of the γ correction for the rate heterogeneity with four discrete categories were allowed to vary. Covarion model was used to allow the rate heterogeneity along the tree. The number of generations of Markov chain Monte Carlo depended on particular data set and varied between 1×10^6 and 6.5×10^6 and the trees were sampled every 100th generation. The burn-in was determined from the MS Excel plot of tree log likelihoods against generations. Trees from generations before the plot reached plateau were discarded as burn-in (usually 2,00,000 trees but 28,00,000 trees in case of the α-tubulin gene). Because the polymorphism of amino acids in the α-tubulin sequence was very low, α-tubulin sequences were analyzed at the nucleotide level. In PAUP*, the sequences were designated as coding in the CODONS block and codon model was used in MrBayes analysis. The concatenate was analyzed analogously to the single-locus data sets in PAUP* and Phyml. In MrBayes, gene specific models with independent parameters were set for each partition.

The statistical significance of *Tetratrichomonas* paraphyly was tested by approximately unbiased (AU) test in program Consel 0.1i (Shimodaira and Hasegawa, 2001). In these tests, the overall best tree with was compared with the best trees found under the constraint of *Tetratrichomonas* monophyly. The tree search as well as computation of site likelihoods was performed in PAUP.

2.4. Light microscopy

Moist films spread on coverslips were prepared from pellets of trichomonads obtained from cultures by centrifugation at 600*g* for 10 min. The films were fixed in Bouin–Hollande's fluid for 20 h and stained with 1% protargol (Bayer, Germany) following the Nie's (1950) protocol.

3. Results

3.1. Phylogenetic analyses

A maximum likelihood tree based on the first data set containing ITS1-5.8S rRNA-ITS2 sequences of the genus *Tetratrichomonas* and various other trichomonads is given in Fig. 1A. The family Trichomonadidae (genera *Tetratrichomonas*, *Trichomonas*, and *Pentatrichomonas*) formed a robust clade. The genus *Tetratrichomonas* appeared to be paraphyletic, with genera *Trichomonas* and in some analyses also *Pentatrichomonas* forming its inner branches. However, the crucial nodes were little statistically supported, and the monophyly could not be significantly excluded by approximately unbiased (AU) test ($p = 0.456$). Strains of the genus *Tetratrichomonas* formed 16 well-supported lineages with a high level of host specificity. The lineages formed two large monophyletic groups. The first one, here called the group A, was formed by lineages 1–10 (strains isolated mainly from ruminants and testudinids) and had a moderate statistical support. Lineages 11–16 formed the second group of the genus *Tetratrichomonas* (group B), though with a weak support. Based on uncorrected p distance, the sequentially most similar were lineages 9 and 10 (0.061), and 4 and 5 (0.066). The most divergent tetratrichomonad isolates were SL from lineage 12 and ANOA from lineage 2 (0.278). The interspecific distance between three *Trichomonas* species was in the range of 0.056–0.081; the interspecific distance among five *Tritrichomonas* species ranged between 0.061 and 0.136.

The interrelationships among lineages 1–10 (the group A) were determined in a separate analysis (Fig. 1B). This allowed us to include into the analysis highly variable positions that could not be properly aligned in the broad data set. Following results of the broader analysis, the tree was artificially rooted with representatives of the lineage 1. Lineages 2 and 3 created two branches basal to a cluster of lineages 4–10.

The tree based on SSU rRNA gene sequences is shown in the Fig. 2. The six genera of the family Trichomonadidae formed a well-supported clade. As in the previous analysis, the genus *Tetratrichomonas* was paraphyletic. Genera *Trichomonas*, *Trichomonoides*, and in some analyses also *Pentatrichomonoides* formed its inner separate branches. The crucial nodes were again poorly supported but this time the *Tetratrichomonas* monophyly was

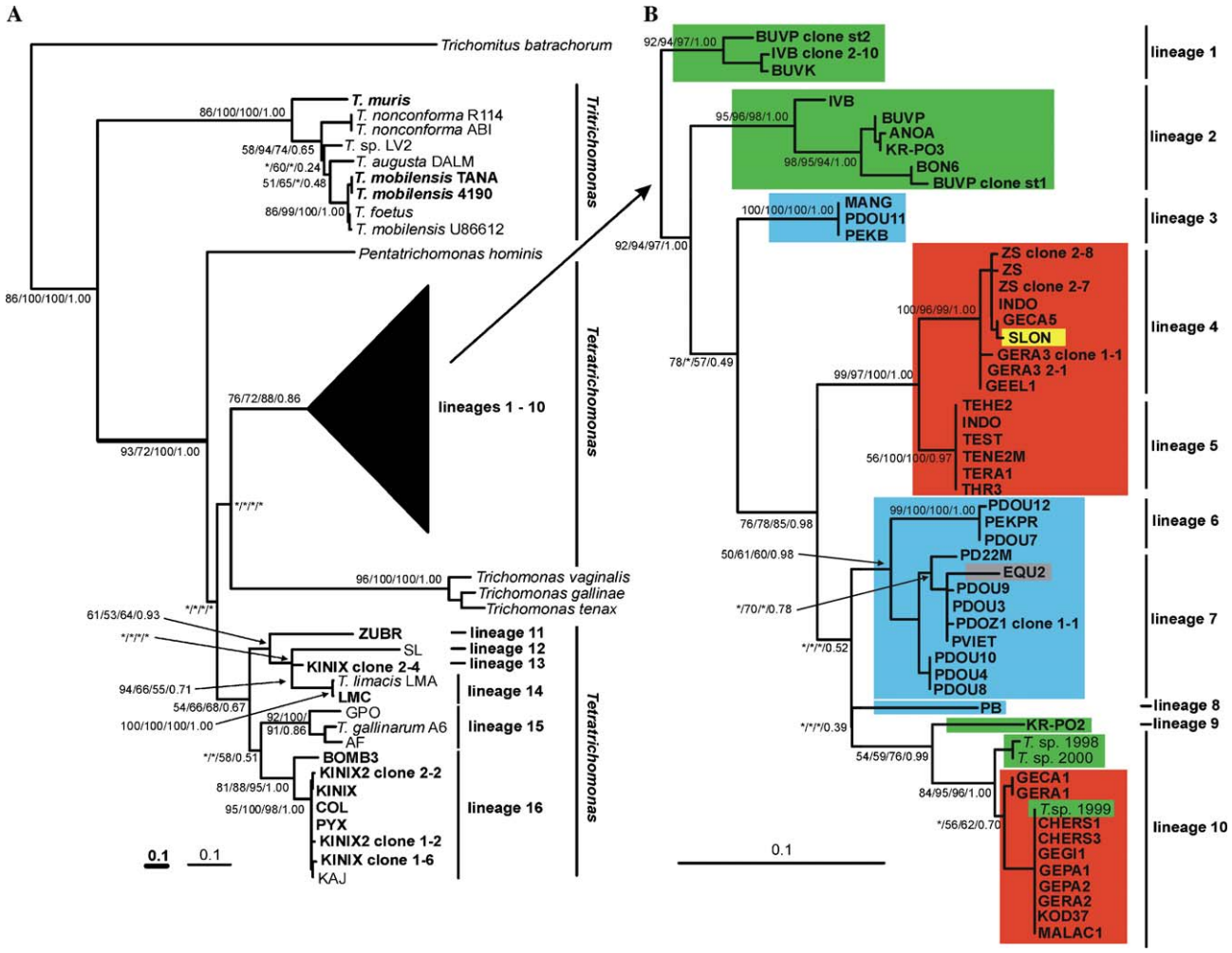

Fig. 1. (A) Phylogenetic tree of trichomonads based on the ITS1-5.8S rRNA-ITS2 region sequence. The tree was constructed by the ML method using GTR + Γ model of substitution and was rooted with *Trichomitus batrachorum*. Bootstrap values from distance, maximum parsimony, maximum likelihood analyses, and Bayesian posterior probabilities, respectively, are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50% or with a different topology. The scale bar indicates the branch lengths corresponding to 10% of sites that underwent substitution event. The branch of the family Trichomonadidae (solid line) has a different scale (50% reduced). Newly determined sequences are in bold. (B) Phylogenetic tree of the *Tetratrichomonas* group A based on the ITS1-5.8S rRNA-ITS2 region sequence. The tree was constructed by the ML method using F81 + Γ model of substitution and was rooted with representatives of the lineage 1. Bootstrap values from distance, maximum parsimony, maximum likelihood analyses, and Bayesian posterior probabilities, respectively, are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50% or with a different topology. The colours indicate origin of isolates: green, bovids (Bovinae); blue, pigs and peccaries (Suoidea); red, tortoises (Testudinidae); yellow, African elephant (*Loxodonta africana*); gray, horse (*Equus caballus*).

rejected by AU test ($p = 0.02$). The above described 16 lineages of the genus *Tetratrichomonas* were recovered, but their interrelationship was, for the most part, poorly supported. One well-supported branch was in conflict with the result of the ITS1-5.8S rRNA-ITS2 analysis—the common branch of lineages 1, 2, and 9. This grouping, however, may be an artifact caused by the long branch of lineage 9. Group A was well supported and, moreover, all sequences in this group contained two common insertions approximately 15 and 20 nucleotides long. Sequences of lineages 11–16 created a paraphyletic group; however, a specific insertion of 10 nucleotides was present in sequences of these lineages. The shortest distance was between lineages 6 and 7 (0.006), and 4 and 5 (0.01). The largest distance was between isolates KAJ from the lineage 16, and clones 2–10 of the isolate IVB from the lineage 1 (0.053). The distance between *Trichomonas vaginalis* and *T. tenax* was 0.021. The distances between four *Tritrichomonas* species were in range 0.015–0.048.

The genus *Tetratrichomonas* was monophyletic and sister to the genus *Trichomonas* in the α-tubulin tree (Fig. 3) in tree reconstructions based on all methods used except Bayesian method. In the Bayesian analysis, the genus *Tetratrichomonas* was paraphyletic having the genus *Trichomonas* as an inner branch sister to the lineage 15, thus at the different position than in ITS1-5.8S rRNA-ITS2 and SSU rRNA trees. The genus *Pentatrichomonas* appeared with a relatively high support at the base of Parabasala causing Trichomonadidae to be polyphyletic. Also the positions of *Trichonympha agilis* and *Hypotrichomonas*

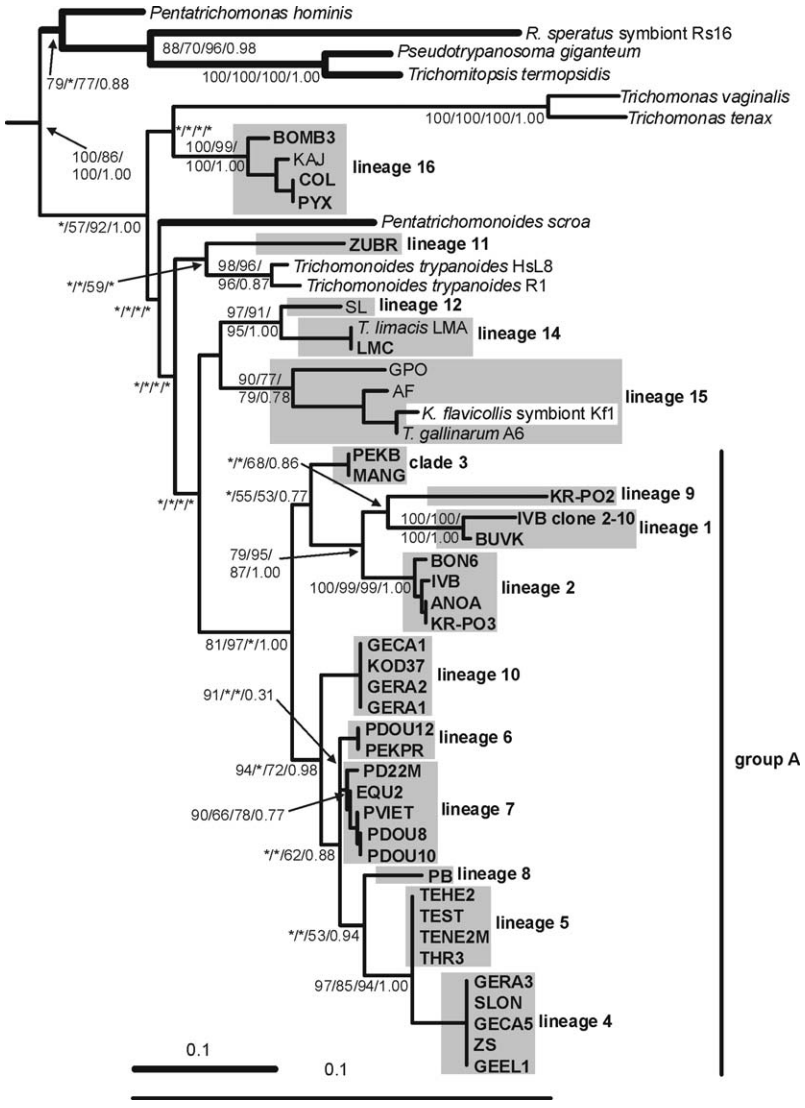

Fig. 2. Phylogenetic tree of trichomonads based on the SSU rRNA gene sequence. The tree was constructed by the ML method using GTR + Γ + I model of substitution and was rooted with genera *Trichomitus* and *Tritrichomonas* (outgroups not shown). Bootstrap values from distance, maximum parsimony, maximum likelihood analyses, and Bayesian posterior probabilities, respectively, are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50% or with a different topology. The branches for *Pentatrichomonas hominis*, *Pseudotrypanosoma giganteum*, *Trichomitopsis termopsidis*, *Kalotermes flavicollis* symbiont Kf1, and *Pentatrichomonoides scroa* (solid lines) have a different scale (66% reduced). The shaded boxes indicate *Tetratrichomonas* clades.

acosta in this analysis were consistent neither with SSU rRNA trees nor with previous α-tubulin analyses (Gerbod et al., 2004).

As inconsistencies among topologies of individual trees were not well supported and there may have been weak but mutually reinforcing phylogenetic signal, we analyzed a concatenate of all the three loci obtained from major *Tetratrichomonas* lineages (i.e., the group A, and lineages 11, 12, 15, and 16), as well as from *Trichomonas vaginalis*, *Trichomitus batrachorum*, *Tritrichomonas foetus*, and *Pentatrichomonas hominis*. Fig. 4 shows the maximum likelihood tree constructed on the basis of this concatenate. This time, the genus *Tetratrichomonas* appeared monophyletic with a moderate statistical support (50–94%). *Trichomonas vaginalis* occupied the position sister to the genus *Tetratrichomonas* and *Pentatrichomonas hominis* was basal to the two genera.

3.2. Morphology

The protargol stained preparations of isolates pertaining to lineages 1 (BUVK and IVB), 2 (IVB and KR-PO3), 4 (ZS, INDO, GECA5 and GEEL1), 5 (TENE2M, THR3, and INDO), 7 (PD22M), 8 (PB), 10 (GECA1, CHERS3, GEPA1, GEPA2, and MALAC1), 11 (ZUBR), 12 (SL), 15 (A6), and 16 (BOMB3, COL, PYX, and KAJ) were examined. Morphological data for lineages 3, 6, 9, 13, and 14 are unavailable. Representatives of all lineages showed most of the typical *Tetratrichomonas* features, namely the shape of parabasal body, well-developed undulating

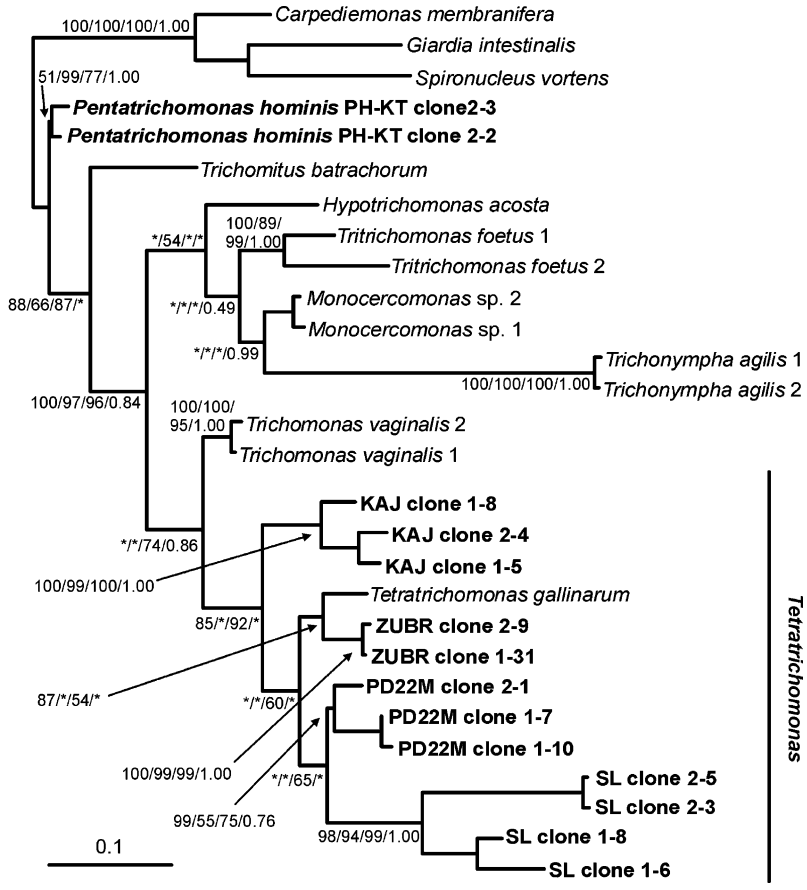

Fig. 3. Phylogenetic tree of trichomonads based on α-tubulin gene sequence. The tree was constructed by the ML method using TRN + Γ model of substitution and was rooted with representatives of Diplomonadida. Bootstrap values from distance, maximum parsimony, maximum likelihood analyses, and Bayesian posterior probabilities, respectively, are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50% or with a different topology.

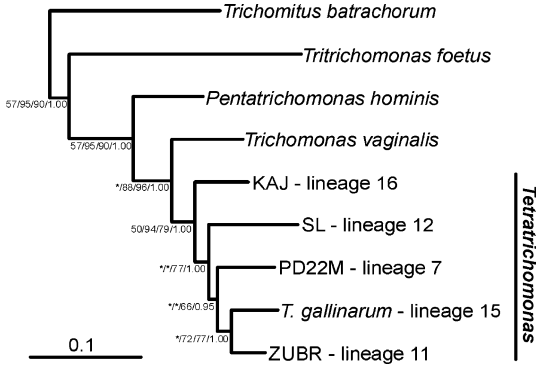

Fig. 4. Phylogenetic tree of trichomonads based on concatenated sequences of α-tubulin, SSU rRNA, and ITS1-5.8S rRNA-ITS2 genes. The tree was constructed by the ML method using TRN + Γ + I model of substitution and was rooted with *Trichomitus batrachorum*. Bootstrap values from distance, maximum parsimony, maximum likelihood analyses, and Bayesian posterior probabilities, respectively, are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50% or with a different topology.

membrane and a free distal portion of the recurrent flagellum.

The morphology of representatives of the lineages 1 and 2 was rather unusual for the genus *Tetratrichomonas*. They possessed only two or three anterior flagella with apparent predominance of biflagellated cells (75% in case of the isolate IVB). The undulating membrane followed a highly spiral course usually extending just about one half of the cell length. In the case of the isolate IVB, the free distal end of the posterior flagellum was approximately as long as the cell body. The pelta and axostyle were weakly developed. The discoid parabasal body was similar to the parabasal body of other *Tetratrichomonas* species.

The morphology of representatives of lineages 4–10 was similar to that of *T. brumpti* and *T. buttreyi* as observed previously (Hibler et al., 1960; Honigberg, 1951; Jensen and Hammond, 1964). We observed slight, but stable morphological differences among lineages 4–10 in cell shape and size, position and size of parabasal body, size and shape of pelta, type of axostyle and thickness of costa. The representatives of the lineages 4 and 5 were morphologically identical and resembled *Tetratrichomonas brumpti* (see Honigberg, 1951) in all important characters (mainly shape and size of nucleus and pelta). Morphology of lineage 7 fully corresponded to *Tetratrichomonas buttreyi* from pigs (Hibler et al., 1960). The isolate PB (lineage 8) from a warthog differed from *T. buttreyi* by its laterally flattened body and by its axostyle morphology that showed a thicker trunk of uniform diameter along its

length protruding posteriorly in a short projection tapering abruptly in a cone-shaped tip.

Representatives of the lineage 10 morphologically resembled isolates from cattle which were determined by Jensen and Hammond (1964) as *T. buttreyi*. The similarity to the cattle tetratrichomonads was confirmed by comparison with protargol stained specimens obtained from a Cuban cattle. Characteristic features were: relatively stout costa, large pelta, and relatively thick trunk of axostyle of a uniform diameter along its length. Neither the members of the clade 10 nor the examined cattle trichomonad conformed to the original description of *T. buttreyi* from pigs (Hibler et al., 1960). The isolate ZUBR (lineage 11) differed in its morphology from all *Tetratrichomonas* species described so far. It is a relatively large trichomonad (mean cell length without protruding part of axostyle 6.8 μm) with a widely oval body and subspherical nucleus, typically possessing three anterior flagella. Axostylar trunk is very thin abruptly extending anteriorly in a spoon-like capitulum closely apposed to the nucleus. The pelta is large but relatively narrow.

The isolate A6 (lineage 15) represented *T. gallinarum* as described by McDowell (1953). Lineage 16 was morphologically identical with *T. prowazeki* as described by Honigberg (1951). Morphology of the isolate SL from the lineage 12, determined previously as *T. prowazeki* (Cepicka et al., 2005; Kutisova et al., 2005), differed from morphology of this species by a fragmented parabasal body present in some fraction (30–60%) of the SL population. The fragmentation resembled that observed in *T. limacis* by Kozloff (1945). Our further observations of tetratrichomonads from several slow worm specimens indicated that it could be a stable character of this species. Although the morphology of lineage 14 was not examined, the localization of parasites in hepatopancreas of *Limax maximus* indicated that the lineage most probably represents *Tetratrichomonas limacis*.

4. Discussion

4.1. Monophyly of the genus Tetratrichomonas and its position within the family Trichomonadidae

Morphological observations showed that our isolates belong to the genus *Tetratrichomonas*, because they share typical characteristics of the genus. On the other hand, our analyses of ITS1-5.8S rRNA-ITS2 and SSU rRNA indicated the possible non-monophyly of the *Tetratrichomonas* isolates, as the genus *Trichomonas* and in some analyses also genera *Trichomonoides*, *Pentatrichomonoides*, *Pentatrichomonas*, and *Hexamastix* formed internal branches of the genus *Tetratrichomonas*.

The position of the genus *Trichomonas*, however, differed in the two gene trees, and nodes causing the paraphyly (or even polyphyly) of *Tetratrichomonas* were not supported by high bootstrap values. Though the genus *Tetratrichomonas* appeared monophyletic in the tree constructed on the basis of the α-tubulin gene, only two of four methods of tree reconstruction gave a strong support for this hypothesis. Concatenated sequences of the three loci showed monophyly of the genus *Tetratrichomonas* with respect to the genus *Trichomonas* with higher support.

In the SSU rRNA tree (Fig. 2), four termite symbionts (*Trichomonoides trypanoides* HsL8 and R1, *Pentatrichomonoides scroa*, and *Kalotermes flavicollis* symbiont Kf1) created internal branches of the genus *Tetratrichomonas*. The sequence of SSU rRNA gene Kf1 (GenBank accession number AF215856) obtained by Gerbod et al. (2000) was believed to originate from *Hexamastix termitis*. As the genus *Hexamastix* differs from the genus *Tetratrichomonas* both in morphology (Honigberg, 1963) and in its phylogenetic position (Hampl et al., 2004), we suppose that the sequence Kf1 does not represent the SSU rRNA gene of *Hexamastix*, but of *T. gallinarum*.

In our analyses, *Pentatrichomonoides scroa* from *Mastotermes darwiniensis* (Berchtold and König, 1995) formed an internal branch of the genus *Tetratrichomonas*. However, this position was not confirmed by all methods used and the bootstrap values were rather low. Moreover, *Pentatrichomonoides scroa* sequence was divergent and formed a long branch. In previous analyses, *Pentatrichomonoides* created a robust clade together with *Trichomonas*, *Tetratrichomonas*, and *Trichomonoides trypanoides* (Delgado-Viscogliosi et al., 2000; Gerbod et al., 2000; Hampl et al., 2004; Keeling, 2002; Viscogliosi et al., 1999). All these studies included only a single tetratrichomonad species (*T. gallinarum*). On the basis of the molecular data obtained in this study we cannot be sure about the true phylogenetic position of the genus *Pentatrichomonoides*. According to the ultrastructural study performed by Brugerolle et al. (1994), the genus *Pentatrichomonoides* shares important morphological characteristics with the genus *Tetratrichomonas* and the described differences (five anterior flagella, unusual organization of axostyle) could be derived.

Sequences R1 (GenBank number X79559) and HsL8 (AB032234) of the SSU rRNA gene from *Reticulitermes santonensis* and *Hodotermopsis sjoestedti* symbionts (Berchtold and König, 1995; Ohkuma et al., 2000), believed to represent the species *Trichomonoides trypanoides* (Brugerolle and Bordereau, 2004), were in our analysis of the SSU rRNA gene placed, albeit with a weak support, into the genus *Tetratrichomonas*. It was sister to the lineage 11 but it did not contain the insertion common to lineages 11–16. Brugerolle and Bordereau (2004) refer *Trichomonoides* to be closely related to *Pentatrichomonoides*, but it is apparently morphologically closer to *Tetratrichomonas* differing from it only by possessing a paraxonemal fibre in the recurrent flagellum.

4.2. Sixteen host-specific Tetratrichomonas species instead of five?

According to our morphological observations, some tetratrichomonad lineages represent already described species *Tetratrichomonas brumpti* (lineages 4 and 5), *T. buttreyi*

(lineage 7), *T. limacis* (lineage 14), *T. gallinarum* (lineage 15) and *T. prowazeki* (lineage 16). Morphologically, lineages 1, 2, 8, 10, 11, and 12 represent still undescribed tetratrichomonad species. Their formal description will be the subject of another study.

Some lineages seem to be generally restricted to one particular taxonomic group of hosts—lineages 1 and 2 to bovine ungulates, lineages 6 and 7 to pigs and peccaries, lineages 4 and 5 to land tortoises, and lineage 14 to slugs. The infection of a horse and an African elephant in the lineages 7 and 4, respectively, could be accidental. The interesting exceptions of the narrow host range are lineages 15 and 10.

Tetratrichomonas gallinarum from birds and humans (lineage 15) is the only tetratrichomonad species which has already undergone a detailed phylogenetic study (Cepicka et al., 2005; Kutisova et al., 2005). RAPD and sequence data showed that tetratrichomonad isolates from birds could represent at least three species, one of them being possibly also a species complex itself.

Most isolates of lineage 10 were obtained from tropical land tortoises. Surprisingly, sequences of tetratrichomonads isolated from the preputial cavity of North American bulls (Walker et al., 2003) were also placed into the lineage 10. Morphologically, the lineage 10 might represent bovine trichomonads described as *Tetratrichomonas buttreyi* by Jensen and Hammond (1964). Common diet of cattle and testudinid tortoises, i.e., herbivory, and coprophagy (and thus potentially similar intestine physiology), could explain the curious relationship between tetratrichomonads from cattle and tortoises. As isolates from suid and bovid ungulates formed different branches and differ also morphologically, our findings throw doubt on the conspecificity of the bovine and the porcine tetratrichomonads, as suggested by Jensen and Hammond (1964).

Lineages 4 and 5 from testudinid tortoises morphologically correspond to *Tetratrichomonas brumpti*, which is thought to be tortoise-specific (Honigberg, 1951). The lineages are morphologically identical but differ in their hosts and in geographic distribution. All isolates of the lineage 5 except for the isolate INDO were isolated from the palearctic genus *Testudo*, while most isolates of the lineage 4 were obtained from the tropical genus *Geochelone*. The genetic distance between the two lineages is comparable to the distance of species *Trichomonas vaginalis* and *Trichomonas tenax* and they could, in fact, represent two cryptic allopatric species.

As the question of trichomonad sexuality has not been satisfactorily answered yet, the biospecies concept cannot be applied. The only widely accepted species concept for trichomonads is that of morphospecies. A species taxon is then recognized as such monophyletic assembly of lineages (i.e., individuals sharing a common history) which can be distinguished from other lineage assemblies on the basis of a significant phenotypic marker. These markers have morphological, ecological, etological, sometimes even purely molecular character. Usually, the host specificity is considered as a significant phenotypic marker. The lineages recovered by our analysis are holophyletic and possibly more or less host-specific. At least 10 of them represent different morphospecies. The genetic distances between these morphospecies and the remaining six lineages are comparable with interspecific distances in the genera *Trichomonas*, *Tritrichomonas*, and *Tetratrichomonas*. We therefore suggest that they may also represent independent species. Our data show that the genus *Tetratrichomonas* is much more diverse than previously thought.

Acknowledgments

We wish to express our thanks to J. Bulantová for kind providing the material from most of the tortoises. We thank Joel B. Dacks for critical reading of the manuscript. This work was supported by Grants MSM0021620828 and GAUK 269/2004/B-BIO/PřF.

References

Alexeieff, A., 1910. Sur les flagellés intestinaux des poissons marins. Arch. Zool. Exp. Gén. 6, 1–20.

Alexeieff, A., 1911. Sur la spécification dans le genre *Trichomonas* Donné. C.R. Soc. Biol. (Paris) 71, 539–541.

Andersen, F.L., Levine, N.D., 1962. The morphology of *Trichomonas ovis* from the cecum of domestic sheep. J. Parasitol. 48, 589–595.

Andersen, F.L., Reilly, J.R., 1965. The anatomy of *Tetratrichomonas didelphidis* (Hegner and Ratcliffe, 1927) comb. n. from the opossum. J. Parasitol. 51, 913–921.

Berchtold, M., König, H., 1995. Phylogenetic position of the two uncultivated trichomonads *Pentatrichomonoides scroa* Kirby and *Metadevescovina extranea* Kirby from the hindgut of the termite *Mastotermes darwiniensis* Froggatt. Syst. Appl. Microbiol. 18, 567–573.

Brugerolle, G., 1976. Cytologie ultrastructurale, systematique et evolution des Trichomonadida. Annales de la Station Biologique Besse-en-Chandesse 10, 1–57.

Brugerolle, G., Bordereau, C., 2004. The flagellates of the termite *Hodotermopsis sjoestedti* with special reference to *Hoplonympha*, *Holomastigotes* and *Trichomonoides trypanoides* n. comb. Eur. J. Protistol. 40, 163–174.

Brugerolle, G., Breunig, A., König, H., 1994. Ultrastructural-study of *Pentatrichomonoides* sp., a trichomonad flagellate from *Mastotermes darwiniensis*. Eur. J. Protistol. 30, 372–378.

Cepicka, I., Kutišová, K., Tachezy, J., Kulda, J., Flegr, J., 2005. Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Vet. Parasitol. 128, 11–21.

Delgado-Viscogliosi, P., Viscogliosi, E., Gerbod, D., Kulda, J., Sogin, M.L., Edgcomb, V.P., 2000. Molecular phylogeny of parabasalids based on small subunit rRNA sequences, with emphasis on the Trichomonadinae subfamily. J. Eukaryot. Microbiol. 47, 70–75.

Diamond, L.S., 1982. A new liquid medium for xenic cultivation of *Entamoeba histolytica* and other lumen-dwelling protozoa. J. Parasitol. 68, 958–959.

Dobell, C., Leidlaw, P.P., 1926. On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. Parasitology 18, 283–318.

Edgcomb, V.P., Roger, A.J., Simpson, A.G.B., Kysela, D.T., Sogin, M.L., 2001. Evolutionary relationships among "jakobid" flagellates as indicated by alpha- and beta-tubulin phylogenies. Mol. Biol. Evol., 514–522.

Felleisen, R.S., 1997. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer regions (ITS) of trichomonadid protozoa. Parasitology 115, 111–119.

Gerbod, D., Edgcomb, V., Noël, C., Delgado-Viscogliosi, P., Viscogliosi, E., 2000. Phylogenetic position of parabasalid symbionts from the

termite *Calotermes flavicollis* based on small subunit rRNA sequences. Int. Microbiol. 3, 165–172.

Gerbod, D., Sanders, E., Moriya, S., Noël, C., Takasu, H., Fast, N.M., Delgado-Viscogliosi, P., Ohkuma, M., Kudo, T., Capron, M., Palmer, J.D., Keeling, P., Viscogliosi, E., 2004. Molecular phylogenies of Parabasalia inferred from four protein genes and comparison with rRNA trees. Mol. Phylogenet. Evol. 31, 572–580.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Hampl, V., Cepicka, I., Flegr, J., Tachezy, J., Kulda, J., 2004. Critical analysis of the topology and rooting of the parabasalian 16S sRNA tree. Mol. Phylogenet. Evol. 32, 711–723.

Hampl, V., Vrlík, M., Cepicka, I., Pecka, Z., Kulda, J., Tachezy, J., 2006. Affiliation of *Cochlosoma* to trichomonads confirmed by phylogenetic analysis of the small-subunit rRNA gene and a new family concept of the order Trichomonadida. Int. J. Syst. Evol. Microbiol. 56, 305–312.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hibler, C.P., Hammond, D.M., Caskey, F.H., Johnson, A.E., Fitzgerald, P.R., 1960. The morphology and incidence of the trichomonads of swine, *Tritrichomonas suis* (Gruby & Delafond), *Tritrichomonas rotunda* n. sp. and *Trichomonas buttreyi* n. sp. J. Protozool. 7, 159–171.

Honigberg, B.M., 1951. Structure and morphogenesis of *Trichomonas prowazeki* Alexeieff and *Trichomonas brumpti* Alexeieff. Univ. Calif. Publ. Zoöl. 55, 337–394.

Honigberg, B.M., 1963. Evolutionary and systematic relationships in the flagellate order Trichomonadida Kirby. J. Protozool. 10, 20–63.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Jensen, E.A., Hammond, D.M., 1964. A morphological study of trichomonads and related flagellates from the bovine digestive tract. J. Protozool. 11, 386–394.

Keeling, P.J., 2002. Molecular phylogenetic position of *Trichomitopsis termopsidis* (Parabasalia) and evidence for the Trichomitopsiinae. Eur. J. Protistol. 38, 279–286.

Kozloff, E.N., 1945. The morphology of *Trichomonas limacis* Dujardin. J. Morphol. 77, 53–61.

Kutisova, K., Kulda, J., Cepicka, I., Flegr, J., Koudela, B., Tachezy, J., 2005. Tetratrichomonads from oral cavity and respiratory tract of humans. Parasitology 131, 309–319.

McDowell, S., 1953. A morphological and taxonomical study of the caecal protozoa of the common fowl, *Gallus gallus*. J. Morphol. 92, 337–399.

Moriya, S., Tanaka, K., Ohkuma, M., Sugano, S., Kuod, T., 2001. Diversification of the microtubule system in the early stage of eukaryote evolution: elongation factor 1α and α-tubulin protein phylogeny of termite symbiotic oxymonad and hypermastigote protists. J. Mol. Evol. 52, 6–16.

Nie, D., 1950. Morphology and taxonomy of the intestinal protozoa of the guinea pig, *Cavia porcella*. J. Morphol. 86, 381–493.

Ohkuma, M., Ohtoko, K., Iida, T., Tokura, M., Moriya, S., Usami, R., Horikoshi, K., Kuod, T., 2000. Phylogenetic identification of hypermastigotes, *Pseudotrichonympha*, *Spirotrichonympha*, *Holomastigotoides*, and parabasalian symbionts in the hindgut of termites. J. Eukaryot. Microbiol. 47, 249–259.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Salleudin, A.S.M., 1972. Fine structure of *Tetratrichomonas limacis* (Dujardin). Can. J. Zool. 50, 695–701.

Shimodaira, H., Hasegawa, M., 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17, 1246–1247.

Swofford, D.L., 2002. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4.0b10., Sinauer Associates, Sunderland, MA, USA.

Tachezy, J., Tachezy, R., Hampl, V., Šedinová, M., Vaňáčová, Š, Vrlík, M., van Ranst, M., Flegr, J., Kulda, J., 2002. Cattle pathogen *Tritrichomonas suis* (Riedmüller, 1928) and pig commensal *Tritrichomonas suis* (Gruby & delafond, 1843) belong to the same species. J. Eukaryot. Microbiol. 49, 154–163.

Tasca, T., De Carli, G.A., Glock, L., Jeckel-Neto, E.A., 2001. Morphologic aspects of *Tetratrichomonas didelphidis* isolated from opossums *Didelphis marsupialis* and *Lutreolina crassicaudata*. Mem. I. Oswaldo Cruz 96, 265–271.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Viscogliosi, E., Edgcomb, V.P., Gerbod, D., Noël, C., Delgado-Viscogliosi, P., 1999. Molecular evolution inferred from small subunit rRNA sequences: what does it tell us about phylogenetic relationships and taxonomy of the parabasalids? Parasite 6, 279–291.

Walker, R.L., Hayes, D.C., Sawyer, S.J., Nordhausen, R.W., Van Hoosear, K.A., BonDurant, R.H., 2003. Comparison of the 5.8S rRNA gene and internal transcribed spacer of trichomonadid protoza recovered from the bovine preputial cavity. J. Vet. Diagn. Invest. 15, 14–20.

Wenrich, D.H., Saxe, L.H., 1950. *Trichomonas microti*, n. sp. (Protozoa, Mastigophora). J. Parasitol. 36, 261–269.