Affiliation of *Cochlosoma* to trichomonads confirmed by phylogenetic analysis of the small-subunit rRNA gene and a new family concept of the order Trichomonadida

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The protozoan genus *Cochlosoma* includes parasitic intestinal flagellates of birds and mammals of uncertain taxonomic classification. The presence of an adhesive disc, superficially similar to that of *Giardia*, led to a proposal that *Cochlosoma* should be classified as diplomonads. Careful morphological and ultrastructural observations, however, revealed conspicuous homologies to trichomonads. We addressed the question of classification and phylogenetic affiliation of *Cochlosoma* using the methods of molecular phylogenetics. Analyses based on the 16S rRNA gene sequence of the species *Cochlosoma anatis* very robustly placed *Cochlosoma* in the clade of the parabasalid subfamilies Trichomonadinae, Trichomitopsiinae and Pentatrichomonoidinae of the order Trichomonadida (bootstraps > 94%). The data did not provide robust support for any particular position of *Cochlosoma* within this clade because the sequence suffered from mutational saturation and produced a long branch. The most probable sister taxon of *Cochlosoma* is the genus *Pentatrichomonas*, because their relationship was supported specifically by the slowest-mutating, least-saturated positions as determined using the method slow–fast. Classification of the order Trichomonadida was revised to accommodate knowledge about its phylogeny – the family Cochlosomatidae and subfamilies Trichomitopsiinae and Pentatrichomonoidinae were abandoned, Trichomonadidae was amended and new families Tritrichomonadidae (formerly a subfamily) and Trichomitidae were proposed.

INTRODUCTION

The genus *Cochlosoma* was established by Kotlán (1923) on the basis of the description of the type species *Cochlosoma anatis* from the domestic duck. This species also occurs in wild ducks and geese, coots and domestic turkeys (Kotlán, 1923) and its pathogenicity for turkey poults has been reported (Campbell, 1945; Cooper *et al*., 1995). Other species of *Cochlosoma* have been described from other birds, bats and shrews (Kulda & Nohynková, 1978; Pecka, 1991; Watkins *et al*., 1989). The most prominent structure of *Cochlosoma* is an adhesive disc that superficially resembles that of *Giardia*. This led to speculation that *Cochlosoma* may be related to *Giardia*. However, the presence of a parabasal apparatus, pelta, costa and axostyle point to a relationship with trichomonads (Kulda & Nohynková, 1978). Grassé (1952) placed these parasites provisionally into the order Retortamonadida, possibly because of some morphological similarity of the lateral groove of *Cochlosoma* to a cytostome. A detailed ultrastructural study (Pecka *et al*., 1996) clearly supported the affiliation to trichomonads. Ultrastructural observations showed that, unlike *Giardia*, the adhesive disc of *Cochlosoma* is derived from the typical parabasalian structures costa and pelta. Conspicuous similarities to parabasalids were found in the *Cochlosoma* mastigont that includes the ‘privileged kinetosomes’ of parabasalids with pertinent fibrilar appendages. Of the six flagella of *Cochlosoma*, four are anterior and two recurrent. One recurrent flagellum is associated with a lamelliform-type of undulating membrane. Based on the ultrastructural homologies, Pecka *et al*. (1996) transferred the family Cochlosomatidae Tyzzer 1930 into the phylum Parabasalida, order Trichomonadida. No phylogenetic study using molecular data has been performed with *Cochlosoma*. McElroy *et al*. (2005) sequenced part of the
16S rRNA gene (466 bases) for diagnostic purposes, but did not proceed to a phylogenetic analysis. Here we present an analysis of the phylogenetic position of Cochlosoma using the major part of the 16S rRNA gene.

METHODS

Organism. C. anatis strain AC-2 was isolated from a teal (Anas crecca) in České Budějovice (Czech Republic) in 1989 and subsequently maintained by serial passages in ducklings over 1 year. Intestinal scrapings, from an experimentally infected duck, resuspended in PBS (pH 7.4) were eventually cryopreserved in the presence of 5% (v/v) DMSO and deposited in the culture collection of the Department of Parasitology, Charles University, Prague. To obtain material for DNA isolation, 200 μl of the cryopreserved sample was inoculated into a laboratory-hatched, 7-day-old, protozoan-free duckling. The parasites were harvested 21 days post-inoculation from mucosal scrapings of the intestine of the euthanized duckling.

Gene amplification and sequencing. DNA was isolated from the suspension of trophozoites with the aid of a High Pure PCR Template preparation kit (Roche Diagnostics) and the 16S rRNA gene was amplified using primers 16SL (5’-TACCTGTTGATCCT-TGCC-3’) and 16SR (5’-GGTCCATGCGATCCTAC-3’) (Tachez et al., 2002). The PCR mixture consisted of 1 mM Tris/HCl (pH 9), 0.01% Triton X-100, 2.5 mM MgCl2, 0.2 mM dNTPs, 1 μM of each primer, 80 pmol DNA μl–1 and 0.1 U Taq polymerase μl–1. The PCR temperature profile consisted of initial denaturation at 92 °C for 4 min, 40 cycles at 92 °C for 30 s, 66 °C for 30 s and 72 °C for 30 s and 90 °C for 30 s and final polymerization at 72 °C for 15 min. The PCR product was purified from the gel and cloned into the vector pCR 2.1-TOPO using a TOPO TA cloning kit (Invitrogen). Two clones were sequenced using the vector and internal sequencing primers. Positions that differed in the sequences of the clones were determined by direct sequence alignment from the PCR products.

Tree construction. Alignments were constructed using the CLUSTAL_X program (Thompson et al., 1997) and manually refined using BioEdit (Hall, 1999). The eukaryotic analysis (1006 positions after editing, included Cochlosoma, 8 parabaladin and 33 other eukaryotic sequences, the broader parabaladin analysis (1155 positions after editing) included Cochlosoma and 77 parabaladin sequences, and the narrower analysis (1552 positions after editing) included Cochlosoma, 12 Trichomonadinae, 2 Trichomitopsiinae, 1 Pentatrichomonoidinae and 4 outgroup sequences. The number of positions in the narrower analysis was reduced in the course of slow-fast analysis (see below). All alignments are available upon request (vlad@natur.cuni.cz).

Phylogenetic trees were constructed using the maximum-likelihood (ML) method in PHYML (Guindon & Gascuel, 2003) (TrN +I+G or GTR+I+G as recommended by MODELLTEST 3.06, parameters optimized by the software), 100 bootstrap replicates in the eukaryotic and the broad parabaladin analyses and 1000 in the narrow parabaladin analysis), the Bayesian method in MrBayes 3.0 (Ronquist & Huelsenbeck, 2003) (iset nst=6 rates=invgamma covarion=yes, temp 0.2, 4 simultaneous MCMC chains, 2000000 generations; trees from generations before the chain reached equilibrium were removed as the 'burn-in'), the maximum-parsimony (MP) method in PAUP 4.0 (Swoford, 1998) (Isearch start = stepwise addseq = random nrep = 10, 1000 bootstrap replicates) and the neighbour-joining method in PAUP 4.0 (LogDet distances, 1000 bootstrap replicates).

RESULTS

The sequenced part of the 16S rRNA gene of C. anatis was 1508 bp long. In the phylogenetic tree (Fig. 1), which was rooted using 30 representatives of various eukaryotic groups, the sequence formed a clade with other parabaladids and the clade received the maximum statistical support. In the parabaladin phylogenetic tree (Fig. 2), the C. anatis sequence robustly branched in the clade of Trichomonadinae, Trichomitopsiinae and Pentatrichomonoidinae (bootstrap values 94–96%).

For a detailed analysis of the position of C. anatis within this clade, a new alignment was created comprising only C. anatis, representatives of the clade and four outgroups (Pseudotrichomonas kelini, Monocercomonas ruminantium, Monotrichomonas carabina and Ditrichomonas honigbergii). In the tree constructed from this alignment (Fig. 3a), Cochlosoma branched with moderate support as a sister taxon to Pentatrichomonoides scroa. The sequence of Cochlosoma produced a relatively long branch, indicating an accelerated mutational rate. Deciphering the phylogenetic relationship of such a sequence is complicated by the fact that certain positions in the sequence might be substitutionally saturated, as they may have undergone more than one substitution, and the information carried by these positions is thus confusing. Consequently, the statistical support for placement of such a sequence in the tree decreases and the sequence may even be misplaced due to artificial attraction to other saturated or highly divergent sequences (Felsenstein, 1978)

One way of assessing the degree of saturation in a dataset is to plot for each pair of taxa the number of observed differences information noise. In this method, the positions in the alignment were divided into four classes according to their increasing mutation rate. The mutational rate class corresponded to the total number of changes that occurred at the position within the five well-supported clades with robust internal topology: (i) Trichomonas tenax and Trichomonas vaginalis; (ii) Pseudotrichomonas kelini, Monocercomonas ruminantium, Monotrichomonas carabina and Ditrichomonas honigbergii; (iii) Trichomitopsis tenpsidinis, Pseudotryptanosoma gigan- teum and Reticulitermes speratus symbiont; (iv) Tetratrichomonas limacis, Tetratrichomonas prowazeki, Tetratrichomonas gallinarum strain A6, Tetratrichomonas gallinarum strain GPO and Kalotermes flavicollis symbiont; and (v) Trichomonoides trypanoides and Hodotermopsis sjoestedti symbiont. The number of changes was estimated in PAUP 4.0 using the ‘descriptettes’ command in MP mode. New alignments (s0, s1, s2) were created from the original alignment by exclusion of positions with more than zero, one and two changes, respectively. Phylogenetic trees were constructed from these alignments. Average bootstrap values and average posterior probabilities were calculated from the node values in each tree to monitor the change in overall tree robustness. Because the slow-fast method is based on the gradual exclusion of positions carrying information on the internal topology of the above-mentioned five clades, and hence the bootstraps of these nodes decrease as the sequences become identical at the s0 level, these nodes were not included in the calculation of average bootstrap values and average posterior probabilities.

The saturation analysis was performed using the program package MUST (Philippe, 1993).
against the estimate of the ‘true’ number of substitutions. Such a plot for all ingroup pairs of taxa from the narrow dataset is shown in Fig. 3(c). The ‘true’ number of substitutions was estimated from the ML tree constructed in PHYML. If no saturation was present, the number of observed differences would be equal to the number of inferred substitutions (dashed line). In the case of our dataset, the number of observed differences was lower and the difference increased in more distant taxa, indicating that saturation was present. As expected, the highest degree of saturation was detected in pairs including Cochlosoma (open circles).

The influence of substitutional saturation on the topology reconstruction can be reduced by using the slow–fast method. The essence of this method involves estimation of the mutational rate of each position, then step-by-step exclusion of the fastest, and potentially most saturated, positions from the alignment and monitoring how this affects the resulting topology. The positions in our alignment were divided into four classes (0–3) of increasing mutational rate (see Methods). New alignments s2, s1 and s0 were created, from which the positions of rate class 3, 2 mutational rate (see Methods). New alignments s2, s1 and s0 and monitoring how this exclusion of the fastest, and potentially most saturated, positions from the alignment and monitoring how this affects the resulting topology. The positions in our alignment were divided into four classes (0–3) of increasing mutational rate (see Methods). New alignments s2, s1 and s0 were created, from which the positions of rate class 3, 2 mutational rate (see Methods). New alignments s2, s1 and s0 were created, from which the positions of rate class 3, 2

Statistical support for Cochlosoma–Pentatrichomonoides and Cochlosoma–Pentatrichomonas nodes and the change in overall support of the tree in each step of the fast-sites exclusion are depicted graphically in Fig. 4. The overall tree robustness, counted as the average of bootstrap values or of posterior probabilities of the tree nodes, changed only slightly (dashed lines). The support of the Cochlosoma–Pentatrichomonas node gradually increased with fast-sites exclusion in all tree construction methods (solid lines). The most prominent increase took place between the s1 and s0 alignments and was accompanied by a sudden decrease in the support for the Cochlosoma–Pentatrichomonoides node (dotted lines).

**DISCUSSION**

Phylogenetic analysis based on the 16S rRNA gene sequence of C. anatis undoubtedly showed that this protist belongs in the phylum Parabasala. The relationships within Parabasala recovered in our analyses correspond mainly to previously published results (e.g. Keeling, 2002; Gerbod et al., 2002; Hampl et al., 2004; Ohkuma et al., 2005). The observed differences in the shape of deeper nodes can be ascribed to the long-branch attraction or to stochastic forces, as the statistical support for many nodes in all analyses was rather low. The Cochlosoma sequence was robustly placed in the clade consisting of representatives of the subfamilies Trichomonadinae, Trichomitopsiinae and Pentatrichomonoidinae. The classification of Cochlosoma in Parabasala has already been suggested in the ultrastructural study by Pecka et al. (1996) that demonstrated the presence of several parabasalid characters in this protozoan (costa, parabasal body and fibres, axostyle, hydrogenosome-like bodies). The affiliation of Cochlosoma specifically to the subfamily Trichomonadinae is also morphologically reasonable, as the major morphological characters of members of Trichomonadinae (B-type costa and the lamelliform-type of undulating membrane) are present in Cochlosoma.

The 16S rRNA gene phylogeny did not explicitly determine the position of Cochlosoma among genera within the

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**Fig. 1.** Rooted phylogenetic tree of Parabasala and related taxa based on 16S rRNA gene sequences. The tree was constructed by using the ML method in PHYML with the GTR+I+Γ model of substitution. Numbers at nodes indicate statistical support estimated by four methods (distance bootstrap/MP bootstrap/ML bootstrap/MrBayes posterior probability; asterisks indicate that the node was not recovered using that method). To save space, the 30 eukaryotic outgroups are not shown; their position is indicated by the left-most horizontal line. Bar, 10% substitutions.

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**Fig. 3(c).** The ‘true’ number of substitutions was estimated from the ML tree constructed in PHYML. If no saturation was present, the number of observed differences would be equal to the number of inferred substitutions (dashed line). In the case of our dataset, the number of observed differences was lower and the difference increased in more distant taxa, indicating that saturation was present. As expected, the highest degree of saturation was detected in pairs including Cochlosoma (open circles).
Fig. 2. Unrooted phylogenetic tree of Parabasalida based on 16S rRNA gene sequences. The tree was constructed by using the ML method in PHYML with the TrN+I+Γ model of substitution. Numbers at nodes indicate statistical support estimated by four methods (distance bootstrap/MP bootstrap/ML bootstrap/MrBayes posterior probability; asterisks indicate that the node was not recovered using that method). GenBank accession numbers are given for the termite-symbiont sequences. Bar, 10% substitutions.
Fig. 3. Phylogenetic trees and saturation plots for the 16S rRNA gene sequences of Cochlosoma, Trichomonadinae, Trichomitopsiinae and Pentatrichomonoidinae. (a) Phylogenetic tree – complete alignment. The tree was constructed by using the ML method in PHYML with the TrN+I+C model of substitution. Numbers at nodes indicate statistical support estimated by four methods (distance bootstrap/MP bootstrap/ML bootstrap/MrBayes posterior probability; asterisks indicate that the node was not recovered using that method). The tree was rooted with sequences of Pseudotrichomonas keilini, Monocercomonas ruminantium, Monotrichomonas carabina and Ditrichomonas honigbergii. GenBank accession numbers are given for the termite-symbiont sequences. Bar, 10 % substitutions. (b) Phylogenetic tree – alignment s0. Details as for (a). Bar, 1 % substitutions. (c) Saturation plot – complete alignment. For each pair of taxa, the number of observed differences is plotted against the number of substitutions inferred from the ML tree. The dashed line represents the ideal case where the numbers of observed differences and inferred substitutions are equal. The pairs of taxa including Cochlosoma are marked by open circles. (d) Saturation plot – alignment s0. Details as for (c).
In *Pentatrichomonoides*, the sixth flagellum is anterior, with its kinetosome located within the kinetosomal complex in parallel with those of the other anterior flagella (Brugerolle et al., 1994). There is, however, a developmental difference between the mastigonts of *Pentatrichomonas* and *Cochlosoma*. The interphase form of *Pentatrichomonas* possesses five flagella (four anterior, one recurrent). The specimens with the sixth flagellum, although pervasive in the population, apparently represent the early stage of cytokinesis, their sixth flagellum representing a precursor of a new recurrent flagellum of the daughter cell. This is not the case with *Cochlosoma*, where the sixth flagellum appears to be a permanent feature of the non-dividing cell (Pecka et al., 1996). The ultrastructural study of Pecka et al. (1996) revealed a unique structure in *Cochlosoma*, ribbons composed of tiny tubules (8 nm in diameter) attached to the microtubules of the pelta that, in *Cochlosoma*, forms the basis of the adhesive disc cytoskeleton. Although not reported previously in other flagellates, a structure such as this was found later in *Pseudotrypanosoma* (Brugerolle, 1999). *Pseudotrypanosoma* and *Cochlosoma* were both placed in the same part of the tree. However, our analysis undoubtedly rejected the possibility of the two taxa being exclusive sister branches; the closest relative of *Pseudotrypanosoma* was the genus *Trichomitopsis* with 100% bootstrap support. The most probable explanation for the presence of these unique tubules in *Cochlosoma* and *Pseudotrypanosoma* might be secondary loss in the genera *Pentatrichomonas* and *Trichomitopsis*.

The classification of *Cochlosoma* in the separate family *Cochlosomatidae* (Pecka et al., 1996) conflicts with the
phylogenetic affiliations of *Cochlosoma* revealed in our analysis, because *Cochlosoma* was placed in the robust clade comprising exclusively representatives of the family Trichomonadidae. However, the easiest solution, reclassification of Cochlosomatidae as a subfamily of Trichomonadidae, is problematic. The tree shown in Fig. 3(b) represents the most reliable tree of the clade, because it is based on slow-mutating positions and is in agreement with results of other analyses (Keeling, 2002; Gerbod et al., 2002; Hampl et al., 2004; Ohkuma et al., 2005). If we consider the tree to be correct, establishing the subfamily Cochlosomatinae and keeping the recognized subfamilies Trichomitopisiinae Brugerolle 1977 (*Trichomitopsis, Pseudotrypanosoma*) and Pentatrichomonoidinae Honigberg 1963 (*Pentatrichomonoides*) would inevitably mean that Trichomonadinae must be divided into two or even three subfamilies: (i) *Pentatrichomonas*; (ii) possibly *Tetrastrichomonas* strain KA; and (iii) the remaining taxa. Such overcomplicated subdividing of the relatively small group of organisms into three subtaxa, which would not be easy to define morphologically, in our opinion would be inappropriate; moreover, some relationships in the tree are too poorly supported to serve as a base for revision. Another and our preferred approach as to how to harmonize the classification with the tree would be to join all taxa in the clade – Cochlosomatidae, Trichomitopisiinae, Pentatrichomonoidinae and the genera *Trichomonas, Trichomonoides, Tetrastrichomonas* and *Pentatrichomonas* – into the single amended family Trichomonadidae. The family can be well defined by the B-type of costa, the lamelliform undulating membrane and by the absence of both a comb-like structure and infrakinetosomal body in the mastigont. Remaining taxa of the former Trichomonadidae, i.e. the genera *Trichomitus* (formerly part of the subfamily Trichomona- dinae) and *Tririchomonas* (formerly the subfamily Tritrichomonadinae), are not monophyletic with the amended Trichomonadidae. We propose that the genera *Trichomitus* and *Tririchomonas* should form the independent families Trichomitidae and Tritrichomonadidae. The family Trichomitidae, which includes a single genus, *Trichomitus*, is characterized by the A-type of costa, by a lamelliform undulating membrane and by the presence of a comb-like structure in the mastigont. The family Tritrichomonadidae, which includes the single genus *Tririchomonas*, is characterized by the A-type of costa, by a flagellar system undulating membrane and by the presence of both a comb-like structure and infrakinetosomal body in the mastigont.

Our new classification is closer to the natural system because it reorganizes the highly polyphyletic former Trichomonadidae together with Cochlosomatidae into three morphologically well-defined monophyla. This revision, however, does not solve all the problems of parabasalian systematics, because other higher taxa are still non-monophyletic, namely the family Monocercomonadidae and order Trichomonadida. Solving these issues is beyond the scope of the present paper and can only be achieved together with a general revision of the phylum Parabasala. It is to be expected that some taxa in the future will be incorporated into the newly established families or vice versa, e.g. the genus *Hypotrichomonas* to Trichomitidae, free-living genera and the genera *Hexamastix* and *Tricercomitus* to Trichomonadidae and Tritrichomonadidae to Cristamonadida.

**Taxonomic summary**

**Trichomonadida Kirby 1947**

Diagnosis: parabasalids with a single karyomastigont, one to five anterior flagella or flagellar system absent, costa present or absent, cresta absent.

**Monocercomonadidae Kirby 1944**

Diagnosis: one to five anterior flagella or flagellar system absent, undulating membrane present or absent, costa absent, comb-like structure and infrakinetosomal body in mastigont present or absent, parabasal body of various shapes.

Type genus *Monocercomonas* Grassi 1879.

**Trichomonadidae Chalmers & Pekkola 1918 emend.**

Diagnosis: four or five anterior flagella, undulating membrane of lamelliform-type, costa of B-type present, neither comb-like structure nor infrakinetosomal body present in mastigont, parabasal body of various shapes.

Type genus *Trichomonas* Donné 1836.

Other genera: *Tetrastrichomonas* Parisi 1910; *Pentatrichomonas* Mesnil 1914; *Pseudotrypanosoma* Grassi 1917; *Trichomitopsis* Kofoid & Swezy 1919; *Cochlosoma* Kotlán 1923; *Pentatrichomonoides* Kirby 1931; *Trichomonoides* Brugerolle & Bordereau 2004.

**Tritrichomonadidae Honigberg 1963 emend.**

Diagnosis: three or four anterior flagella, undulating membrane of rail-type, costa of A-type present, comb-like structure and infrakinetosomal body present in mastigont, parabasal body rod-shaped. Previously held the rank of subfamily.

Type genus *Tririchomonas* Kofoid 1920.

No other genera.

**Trichomitidae fam. nov.**

Diagnosis: three anterior flagella, undulating membrane of lamelliform-type, costa of A-type present, comb-like structure, but not infrakinetosomal body, present in mastigont. Parabasal body biramous.

Type genus *Trichomitus* Swezy 1915.

No other genera.
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