ACTA
PROTOZOOLOGICA

Acta Protozool. (2011) 50: 319–326
http://www.eko.uj.edu.pl/ap
doi: 10.4467/16890027AP.11.029.0066

Molecular Phylogeny of *Spirodinium equi*, *Triadinium caudatum* and *Blepharocorys* sp. from the Equine Hindgut

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Summary. Single cell morphotypes of the species *Triadinium caudatum* and *Spirodinium equi*, together with a representative of the genus *Blepharocorys* (*Blepharocorys* sp.) were used for phylogenetic analysis based on their 18S rRNA genes. *Spirodinium equi* clustered with sequences already described for the entodiniomorphs isolated from horses and the *Blepharocorys* sp. also grouped within the Entodiniomorphida clade, although both sequences were distinct from those described from rumen ciliates. *Triadinium caudatum* clustered within the Vestibuliferida, and most closely to that of *Paraisotricha*, only other member of this order which has been described in the horse. It was concluded that although members of the orders Entodiniomorphida and Vestibuliferida are present in the equine gut, and that they share an ancient linage with their rumen counterparts, they are ancestrally different groups.

Key words: Gut ciliates, Entodiniomorphida, horse hindgut, Protista, Vestibulifera

INTRODUCTION

Work on the diversity of ciliates in the hindgut of the horse is a long-established field, with the works of Hsiung (1930a, b) remaining amongst the most comprehensive catalogues of the ciliate species found in the equine hindgut. Although most of the ciliates in the equine hindgut were described as being unique to this environment, it was acknowledged that there was a small degree of overlap between those found in this environment, and those found in the rumen (Becker 1932). More recently ciliates have also been described in the digestive tract of other mammals e.g. chimps (Irbis *et al.* 2008) and elephants (Obanda *et al.* 2007), and when studied as a group, the ciliates of the gastrointestinal tracts of mammals were found to be monophyletic (Moon-van der Staay *et al*. 2004).

The original classification of these organisms relied on their identification by microscopy. More recently, studies have started to rely on the use of 18S rRNA genes for identification of organisms. This has been done, not just for ciliates, but for eukaryotic microorganisms in general. In the case of the ciliates of the equine hindgut, there has been little attention given to combining data

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on cells which have been identified based on morphology with sequence information. To date only a single publication combining these two pieces of information has been published (Strüder-Kypke *et al*. 2007). The current work extends this analysis further.

MATERIALS AND METHODS

Isolation of ciliate DNA

Fresh faecal samples were collected immediately post-defecation from six horses stabled at the Lluest Equestrian Centre, Aberystwyth, which had been on a diet of pasture and formulated rations. Samples were pooled and 5 g was transferred to a centrifuge tube and diluted with 5 ml of Coleman buffer (Coleman 1958). The solution was filtered through 500 µm gauze to remove large fibres and a drop of the filtrate spread evenly onto a 1.35 µm PEN-LPC membrane slide.

Single cell examples were identified on the basis of their morphology (Hsiung 1930a, b) for *Triadinium caudatum*, *Spirodinium equi* and *Blepharocorys* sp. and were harvested using a Zeiss pressure ablation laser microdissection microscope (PALM©) by laser pressure catapulting (LPC) into the cap of a microfuge tube.

DNA extraction was performed on the single cells following the Chelex protocol used in Thomas *et al*. (2005). 50 µl of a 5% w/v solution of Chelex 100 and 2 µl Proteinase K (600 mAu/ml) (QiAamp®, Qiagen) were added to the cap of the microfuge tube, which was inverted and incubated for 30 min. at 37°C. The solution was spun down for 2–3 s at maximum speed (14000 *g*; 13000 rpm) and incubated upright for 10 min. at 98°C and snap cooled on ice. 2 µl of the supernatant was removed and added to 24 µl PCR master mix (GoTaq®, Promega) taking care not to remove any of the Chelex matrix.

Amplification of the 18S rRNA gene and DNA sequence determination

Amplification of the small sub-unit (18S) rRNA gene was performed using the primers used in van Hoek *et al*. (1998); Eukrev (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') and Eukfwd (5'-AAC CTG GTT GAT CCT GCC AGT-3'). PCR was performed using a Biorad MyCycler™, with the following conditions: 94°C for 5 min., then 35 cycles of 94°C for 1 min., 55°C for 1 min. and 72°C for 3 min., followed by a single cycle of 94°C for 1 min., 55°C for 1 min. and 72°C for 10 min. The size of amplicons was checked by electrophoresis on a 1% agarose/TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.0) gel to ensure that they were of the approximate size (1700 bp) anticipated following the use of these primers.

Four microlitres PCR product was combined with 1 µl pCR®4-TOPO® plasmid vector (Invitrogen) and 1 µl salt solution. The reaction was gently mixed and incubated at room temperature for 5 min. 2 µl of the cloning reaction was added to a tube of One Shot® TOP10 chemically competent *E. coli* (Invitrogen). The cells were incubated on ice for 30 min., heat-shocked in a water bath at 42°C for 30 s and immediately transferred back onto ice. 250 µl SOC medium was added to the cells and the tubes were capped tightly and stirred horizontally 175 rpm at 37°C for 1 h.

Following transformation, cells were plated out on LB agar (Sambrook and Russell, 2001) with 50 µg/ml ampicillin and 40 µg/ml X-gal (final concentrations). Transformed colonies (based on blue/white screening) were picked and re-plated on a grid. Verification of an insert in successfully transformed cells was demonstrated by PCR using M13fwd (5'-TGT AAA ACG ACG GCC AGT-3') and M13rev (5'-GGG CAT CAC AGA CCT G-3') primers to check for inserts of the appropriate size. Colonies which had been transformed with a plasmid and which contained an insert of the correct size were picked and cultured overnight in LB broth (Sambrook and Russell 2001) in a shaking incubator (250 rpm) at 37°C.

Cells were harvested in the exponential growth phase (16 h) before being spun down and the supernatant discarded. Plasmid DNA purification was performed using a Qiagen QIAprep® miniprep kit according to the manufacturer's protocol. Sequencing was performed on a Beckman CEQ8000 capillary gel electrophoresis system in both directions using the following primers M13fwd (5'-TGT AAA ACG ACG GCC AGT-3'), M13rev (5'-GGG CAT CAC AGA CCT G-3'), Euk300fwd (5'-AGG GTT CGA TTC TGG AG-3'), Euk690fwd (5'-AGA GGT GAA ATT CT-3') and Euk1200rev (5'-GGG CAT CAC AGA CCT G-3') (Elwood *et al*. 1985) and fragment assembly was performed using the assembly function in Geneious Pro 3.6.2 (Biomatters, 2005–2008) to complete the DNA sequence.

Phylogenetic analysis

Additional related 18S rRNA sequences from litostome ciliates were downloaded from the GenBank database for nucleotide alignment. These are shown in Table 1 and included examples of sequences obtained from the Vestibuliferida and Entodiniomorphida found in the rumen and the hindgut of the horse together with the clade of ciliates found in marsupials. The sequence from *Dileptus* sp. (AF029764) was used as an outlier group, based on the knowledge that it is not found in the digestive tract. Alignment of these sequences was performed using ClustalW ver. 2.00 (Larkin *et al*. 2007). Maximum posterior probability values of clade support (Bayesian Inference) and phylogenetic trees were calculated using the phylogenetic software MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The General Time Reversal (GTR) model was used for nucleotide substitutions with gamma rate variation and four categories. The Markov chain Monte Carlo (MCMC) settings were as follows: number of generations 1,000,000, sub-sampling frequency 50, burn in length 5,000, two parallel runs of three heated chains and one "cold" chain were run until the average standard deviation of the two split frequencies was less than 0.01.

RESULTS

Three different ciliate morphotypes were identified unequivocally from fresh faecal samples (Fig. 1); *Triadinium caudatum*, *Spirodinium equi* and *Blepharocorys* sp. The 18S rRNA sequence for each of these organisms was determined and submitted to the EBI DNA database. These sequences were allocated the following accession numbers: *Triadinium caudatum* (FM201782),

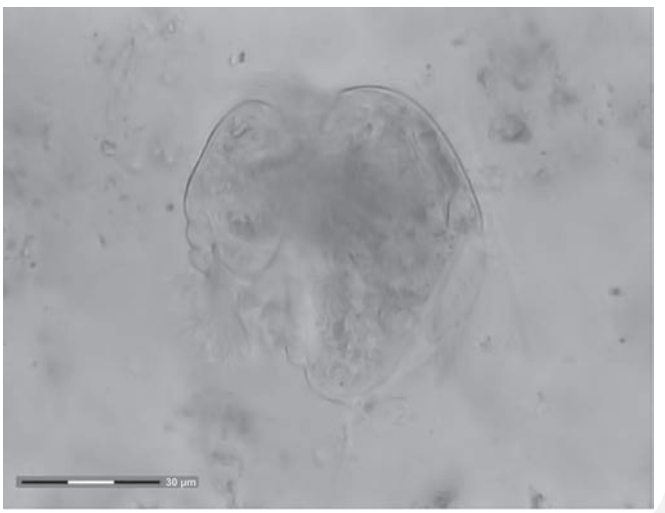
Table 1. Ciliate sources of the DNA sequences used in the phylogenetic analysis, together with their GenBank accession numbers (*Direct Submission).

Species name	GenBank No.	Reference
Amylovorax dehorityi	AF298817	Cameron *et al*. 2001
Amylovorax dogieli	AF298825	Cameron *et al*. 2001
Anoplodinium denticulatum monocanthum	AM158440	Moon-van der Staay *et al.* 2009*
Anoplodinium denticulatum denticulatum	AM158470	Moon-van der Staay *et al.* 2009*
Balantidium coli (Pig)	AM982722	Ponce-Gordo *et al*. 2008
Balantidium coli (Ostrich)	AM982723	Ponce-Gordo *et al*. 2008
Balantidium coli (Gorilla)	AF029763	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*. 2001
Blepharocorys curvigula	AF298821	Ito *et al*. 2010
Blepharocorys uncinata	AB530162	Imai *et al*. 2009*
Bundleia benbrooki	AB555711	Imai *et al*. 2010*
Bundleia nana	AB555712	Imai *et al*. 2010*
Bundleia postciliata	AB555709	Imai *et al*. 2010*
Cochliatoxum periachtum	EF632078	Strüder-Kypke *et al*. 2007
Cycloposthium bipalmatum	AB530165	Imai *et al*. 2009*
Cycloposthium edentatum	EF632077	Strüder-Kypke *et al*. 2007
Cycloposthium ishikawai	EF632076	Strüder-Kypke *et al*. 2007
Cycloposthium sp.	AF042485	Wright and Lynn 1997*
Dasytricha ruminantium	AM158463	Moon-van der Staay *et al.* 2009*
Dasytricha ruminantium strain Guelph	U57769	Wright and Lynn 1997a
Dasytricha ruminantium strain UK	U27814	Embley *et al*. 1995
Dileptus sp.	AF029764	Strüder-Kypke *et al*. 2006
Diplodinium dentatum	U57764	Wright and Lynn 1997b
Diploplastron affine	AM158467	Moon-van der Staay *et al.* 2009*
Enoploplastron triloricatum	AM158461	Moon-van der Staay *et al.* 2009*
Enoploplastron triloricatum	AM158462	Moon-van der Staay *et al.* 2009*
Entodinium bursa	AM158448	Moon-van der Staay *et al.* 2009*
Entodinium nanellum	AM158449	Moon-van der Staay *et al.* 2009*
Entodinium caudatum (4 sequences)	AM158444/5/6/7	Moon-van der Staay *et al.* 2009*
Entodinium caudatum	U57765	Wright *et al*. 1997
Entodinium dubardi	AM158443	Moon-van der Staay *et al.* 2009*
Entodinium furca dilobum	AM158442	Moon-van der Staay *et al.* 2009*
Entodinium simplex	U57765	Wright *et al*. 1997
Epidinium ecaudatum (2 sequences)	AM158474/5	Moon-van der Staay *et al.* 2009*
Epidinium caudatum	U57763	Wright *et al*. 1997
Eremoplastron dilobum	AM158472	Moon-van der Staay *et al.* 2009*
Entodinium furca monolobum	AM158471	Moon-van der Staay *et al.* 2009*
Entodinium furca monolobum	AM158450	Moon-van der Staay *et al.* 2009*
Entodinium longinucleatum	AB481099	Ito *et al*. 2010

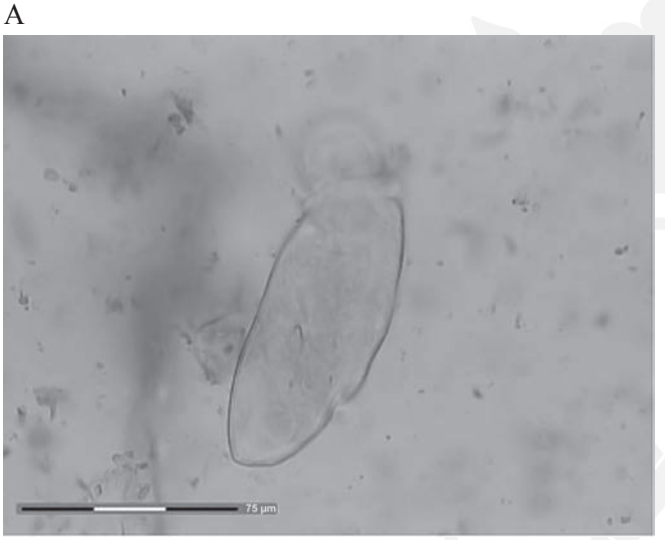
Species name	GenBank No.	Reference
Entodinium simplex	AM158466	Moon-van der Staay *et al.* 2009*
Epidinium caudatum	ECU57763	Wright *et al*. 1997
Epidinium caudatum	AM158474	Moon-van der Staay *et al.* 2009*
Epidinium fasciculus	AM158465	Moon-van der Staay *et al.* 2009*
Eremoplastron dilobum	AM158472	Moon-van der Staay *et al.* 2009*
Eremoplastron neglectum	AM158473	Moon-van der Staay *et al.* 2009*
Eremoplastron rostratum	AM158469	Moon-van der Staay *et al.* 2009*
Enoploplastron tricloricatum	AM158461	Moon-van der Staay *et al.* 2009*
Eudiplodinium maggii	U57766	Wright and Lynn 1997b
Eudiplodinium maggii	AM158451	Moon-van der Staay *et al.* 2009*
Eudiplodinium maggii	AM158452	Wright and Lynn 1997b
Isotricha intestinalis	AM158441	Moon-van der Staay *et al.* 2009*
Isotricha intestinalis	AM158453	Moon-van der Staay *et al.* 2009*
Isotricha intestinalis	IIU57770	Wright and Lynn 1997a
Isotricha prostoma	AF029762	Strüder-Kypke *et al*. 2006
Isotricha prostoma (3 sequences)	AM158454/5/6	Moon-van der Staay *et al.* 2009*
Macropodinium ennuensis	AF298820	Cameron *et al*. 2001*
Metadinium medium	AM158464	Moon-van der Staay *et al.* 2009*
Metadinium medium	AB535215	Ito *et al*. 2010
Ophryoscolex caudatus	AM158467	Moon-van der Staay *et al.* 2009*
Ophryoscolex purkynjei	U57768	Wright and Lynn 1997b
Ostracodinium clipeolum	AB536717	Ito *et al*. 2010
Ostracodinium dentatum	AM158460	Moon-van der Staay *et al.* 2009*
Ostracodinium gracile	AM158468	Moon-van der Staay *et al.* 2009*
Ostracodinium gracile	AB535662	Ito *et al*. 2010
Ostracodinium trivesiculatum	AB536718	Ito *et al*. 2010
Paraisotricha colpoidea	EF632075	Strüder-Kypke *et al*. 2007
Polycosta roundi	AF298819	Cameron and O'Donoghue 2004
Polycosta turniae	AF298817	Cameron and O'Donoghue 2004
Polyplastron multivesiculatum (2 sequences)	AM158458/9	Moon-van der Staay *et al.* 2009*
Polyplastron multivesiculatum	PMU57767	Wright *et al*. 1997
Polyplastron multivesiculatum	PMU27815	Embley *et al*. 1995
Raabena bella	AB534183	Ito *et al*. 2010
Tripalmaria doglieli	EF632074	Strüder-Kypke *et al*. 2007
Triplumaria selenica	AB533538	Ito *et al*. 2010
Troglodytella abrassarti	EU680308	Modry *et al.* 2009

Spirodinium equi (FM201781) and *Blepharocorys* sp. (FM201780).

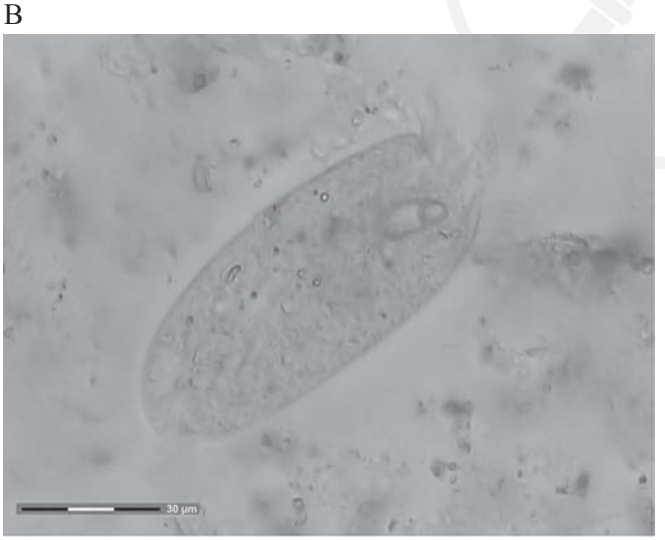
As can be seen from Fig. 2, there is a relationship between Entodiniomorph ciliates from the rumen and the equine ciliates, whereby there is a major clade which includes organisms from both environments. However, it is also clear that within this clade, there is a subdivision, whereby the organisms from the two different sources do not cluster on the same branch, meaning that although their origins are monophyletic, the rumen ciliates and the horse hindgut ciliates are clearly two different groups.



A



B



C

Fig. 1. The three different organisms used for study in this work. **A** – *Triadinium caudatum*; **B** – *Spirodinium equi*; **C** – *Blepharocorys* sp.; Scale bars: A – 30 µm, B – 75 µm, C – 30 µm.

DISCUSSION

Different ciliated protozoa have been found to have a predilection for different parts of the equine hindgut (Adam 1951) with different species inhabiting the caecum and colon. Since the cells used in the current work were from faecal material they must be counted as including representation of the distal fauna, and so may not be representative of those found in the more proximal areas of the hindgut of these animals (e.g. caecal ciliates). However, given their distribution within the dendrogram seen in Fig. 2, it does demonstrate that the samples studied here do include a diverse group of species, with the three new sequences showing representation at three different positions within the dendrogram.

It is worth noting that both *Spirodinium equi* and *Blepharocorys* sp. lie within the Entodiniomorphida clade, but that they are found on separate branches, neither of which group within the main cluster of Entodiniomorphida found within the rumen cluster. *Blepharocorys* sp. lies on its own as a single branch within the clade, whereas *Spirodinium equi* lies on a branch containing *Cochliatoxum periachtum* and *Tripalmaria dogieli*; two other ciliates isolated from the hindgut of the horse (Strüder-Kypke *et al*. 2007). Neighbouring this *Spirodinium* / *Cochliatoxum* / *Tripalmaria* branch is another branch which contains the only other representatives of the equine Entodiniomorphidae (*Cycloposthium edentatum* and *Cycloposthium ishikawai*. Thus, it is clear that although all six of the equine Entodiniomorphidae which have thus far been characterised at the molecular level are genuine members of this order, they are distinct from those members which have been described from the rumen. It is also worth noting that the work of Davis (1941) discussed *Spirodinium equi* in the context of members of the genus *Cycloposthium* as well as *Tripalmaria dogieli*, supporting the observation that not only do these organisms share DNA sequence similarity, but also that they have similarity at the level of cell morphology.

The third novel sequence reported here is an example from *Triadinium caudatum* and this group with *Paraisotricha colpoidea*, the only previous example of a member of the order Vestibuliferida described in the equine hindgut for which sequence information has been previously determined (Strüder-Kypke *et al*. 2006). They also sit within a larger cluster containing representatives of the species *Balantidium coli* from three different vertebrate sources: pig (Ponce-Gordo

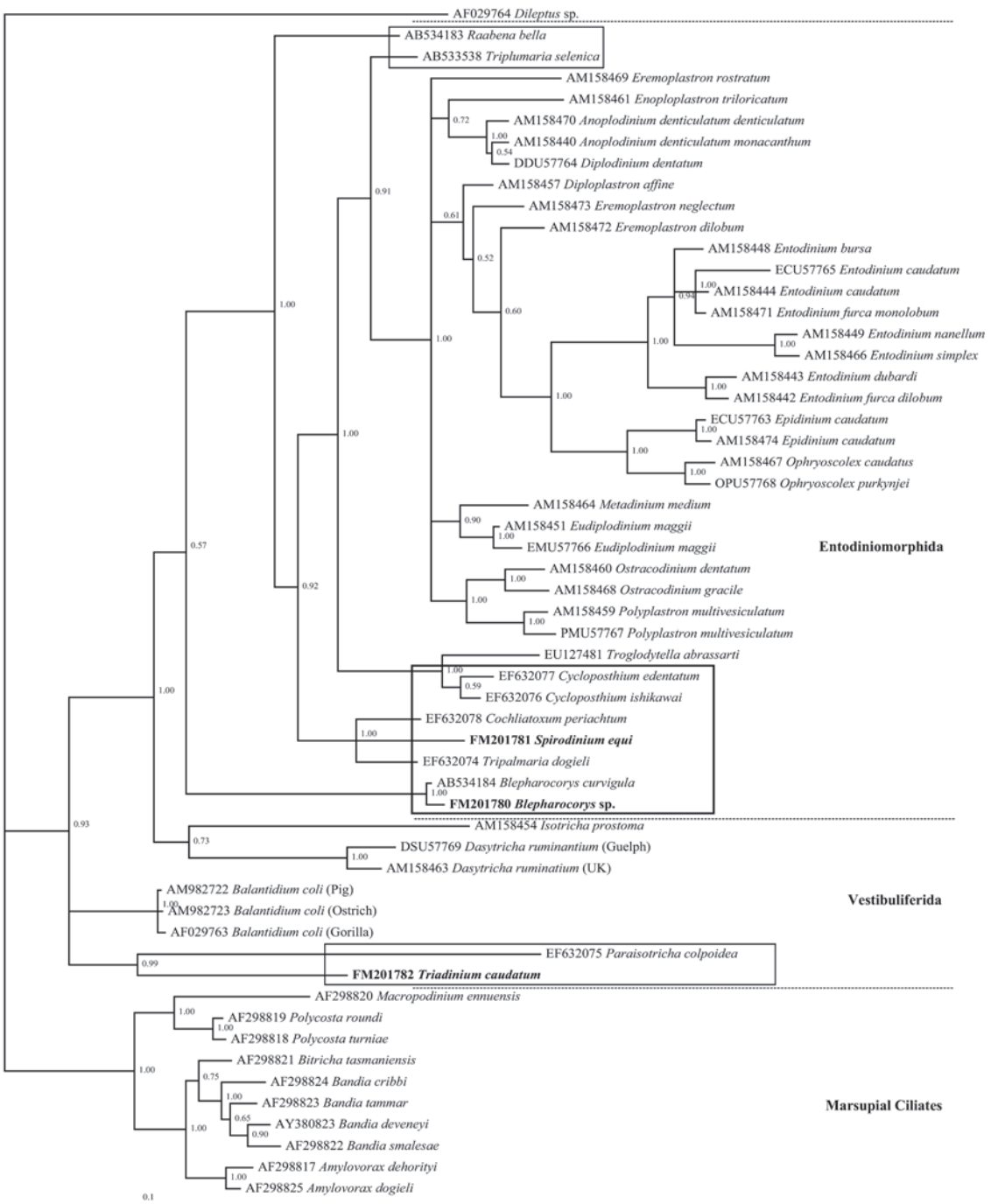


Fig. 2. A Bayesian inference dendrogram showing the relationship between ciliates from the digestive tract of mammals. The three new sequences described in the current work – *Triadinium caudatum*, *Spirodinium equi* and *Blepharocorys* sp. are shown in bold and those from horses are shown in boxed areas.

et al. 2008), gorilla (Strüder-Kypke *et al*. 2007) and ostrich (Ponce-Gordo *et al*. 2008), and have a branch neighbouring this containing the only other members of the order Vestibuliferida within the dendrogram: *Dasytricha ruminantium* and *Isotricha prostoma* (examples of Vestibuliferida isolated from the rumen). However,

as with the Entodiniomorphida example, the Vestibuliferida being described here as being from the horse are discrete from those found in the rumen.

Additional sequences into the phylogenetic tree of the Trichostomatia have helped in part to resolve the grouping of ciliates found in the equine hindgut. Some of the Blepharocorythidae have formed a strongly supported group basal to the rest of the equine Entodiniomorphs. However, *Raabena bella*, another member of this family is placed outside these as the most basal equine ciliate species. The family Cyclopothiidae, including all the *Cycloposthium* species as well as *Tripalmaria dogieli* and *Tripulmaria selenica* remains paraphyletic inferring the genetic diversity in this clade had been previously underestimated. This effect is even more noticeable in the Vestibuliferida group that contains both *Triadinium caudatum* sequenced in this study and three *Bundleia* species. These are all members of the order Entodiniomorphida and present a significant incongruence in the tree. The branch lengths for *Triadinium caudatum* and *Paraisotricha colpoidea* together with the *Bundleia* species are amongst the longest seen on this dendrogram, demonstrating that although they are related, there is still considerable sequence diversity between them. It is also possible that there still may be an effect of undersampling causing long branch attraction leading to artifactual groupings that were previously mentioned in the study by Strüder-Kypke *et al*. (2007).

In both the orders Entodiniomorph and Vestibuliferida, the equine ciliate species are basal to the ruminant species and form separate host-specific groups. This reflects the evolution of perissodactyls in respect to artiodactlyls and could indicate a degree of co-evolution between endosymbiotic microbes and their hosts. However, inference of timescales on phylogenetic trees containing ciliates should be made with some caution due to the irregularities in the rate of their molecular evolution (Wright *et al.* 1997).

In conclusion, the three novel sequences described here support the observation that the equine hindgut contains representatives of both order Vestibuliferida and order Entodiniomorphida, and that while they cluster with sequences from other organisms within their respective orders, it is clear that in both cases the equine members of these orders cluster within their own distinct branch which has diversified from those organisms isolated from the digestive tract of ruminants.

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Received on 22nd July, 2011; revised on 3rd October, 2011; accepted on 4th October, 2011