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ORIGINAL PAPER

The Soil Flagellate *Proleptomonas faecicola*: Cell Organisation and Phylogeny Suggest that the only Described Free-Living Trypanosomatid is not a Kinetoplastid but has Cercomonad Affinities

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The only putative free-living trypanosomatid is *Proleptomonas faecicola* described first by Woodcock in 1916 as a coprophilic flagellate with striking *Leptomonas*-like flagellar movement but lacking a kinetoplast. *P. faecicola* was later identified by Sandon in 1927 as a widespread non-phagotrophic inhabitant of soils. No division stages were seen by either observer. An organism conforming to Woodcock's light microscope description has been isolated from tapwater and cultivated axenically in various serum-containing media. Division has been shown to occur in an aflagellate stage enclosed in a thin cyst wall. Electron microscopy of the flagellate stage reveals that, in addition to the long locomotory flagellum, a second non-motile flagellum is present attached to the body along its entire length. The flagellate's ultrastructure lacks all the major features of the Trypanosomatidae. The several mitochondria of *Proleptomonas* have tubular cristae and lie between intracytoplasmic microtubules originating as a loose cone associated with the flagellar basal bodies. This cytoskeleton is much reduced in the division cyst. A comparable *Proleptomonas*-like flagellate with similar division cysts has been observed in soil samples from farmland. Phylogenetic analysis based on SSU rRNA gene sequences suggests that the cultured organism identified here as *Proleptomonas* is unrelated to the Kinetoplastida and has affinities with the Phylum Cercozoa Cavalier-Smith, even though in morphology, life cycle and mode of feeding it bears little resemblance to any member of that diverse grouping.

Introduction

The kinetoplast-bearing flagellates form a well defined order (Kinetoplastida) or class (Kinetoplastea), now associated with the euglenoid and diplonemean flagellates (classes Euglenoidea and Diplonemea) within the phylum Euglenozoa (Cavalier-Smith 1993). Within the Kinetoplastida, two families are now recognised (Vickerman 1990) – the biflagellate Bodonidae and the uniflagellate Trypanosomatidae. While the former includes free-living as well as parasitic species, all the trypanoso-

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matids that have been described with anything that approaches precision spend their active existence inside another living organism. The only possible exception to this rule is the "acicular flagellate" described by Woodcock (1916) from an infusion of goat dung. It was named by him *Proleptomonas faecicola*, because, he said, "it reminds me of nothing so much as a leptomonad without a kinetonucleus." The 'leptomonad' stage in the life cycles of trypanosomatids is now referred to as the promastigote, and the 'kinetonucleus' as the kinetoplast. He went on to add that "it may well be a present-day representative of the ancestral type from which the genus *Leptomonas* and allied genera have been derived by development of a kinetonucleus." Subsequently, Sandon (1927) found *P. faecicola* to be widespread in soils and assigned it to the Trypanosomatidae. Neither he nor Woodcock observed division or other stages in a life cycle, and soil biologists have added nothing to our knowledge of the nature of this interesting organism since.

Woodcock's views do not accord with present day conclusions on the emergence of the Trypanosomatidae within a paraphyletic Bodonidae (Dolezel et al. 2000; Wright et al. 1999) which already possessed a kinetoplast. Woodcock's failure to detect a kinetoplast may, however, suggest a trypanosomatid comparable to those strains of *Trypanosoma brucei* or *T. evansi* in which the kinetoplast DNA (kDNA) was originally described as missing, but which we now know to display dispersion of the kDNA throughout the single mitochondrion – a condition referred to as dyskinetoplasty (Vickerman 1977). Certainly the occurrence of a dyskinetoplastic free-living trypanosomatid would be of considerable interest – not only because all other trypanosomatids are parasitic, but because viable dyskinetoplastic mutants are known only from mechanically-transmitted bloodstream trypanosomes of the subgenus *Trypanozoon* in which the mitochondrion is permanently repressed (Vickerman 1994).

Here we present an account of the life cycle and ultrastructure of an organism isolated in axenic culture from Antwerp tapwater and identified with near certainty as *Proleptomonas faecicola* Woodcock 1916 or a species closely related to it. In its fine structure the organism bears no resemblance to any trypanosomatid nor indeed to any described protist. Surprisingly, SSU rRNA gene sequencing studies, also reported here, suggest that its nearest relatives lie among the cercomonad flagellates and that it may be assignable to the phylum Cercozoa of Cavalier-Smith (1998a, b).

Results

Light Microscopy

In culture, the organism occurs in two forms – a non-dividing, free-swimming elongate flagellate and a dividing non-flagellated spherical body which will be referred to as the aflagellate or division cyst. The division cysts adhere to one another forming extensive rafts or one continuous veil-like sheet floating in the culture medium. They are the predominant form in cultures,

Under phase or differential interference contrast, the flagellate has an elongate cigar-shaped or slightly curved body 7.0–12 μm long and 1.5–3.0 μm across (n = 50) with a long flagellum 14–29 μm emerging from its anterior extremity. Locomotion is extremely fast with the body vibrating rapidly about a central midpoint. As in trypanosomatids, the flagellar beat passes from base to tip, with occasional reversals to allow the organism to undertake avoidance reactions. During pauses in swimming, the flagellar beat may decrease in frequency and the organism appears to wriggle on the substratum. The nucleus is mid-body and subspherical, but no other prominent inclusions are visible. Flagellates that are not executing rapid swimming movements vary considerably in form. They may be spherical to ovate or elongate, sometimes with a prominent posterior spine and such organisms are believed to be

Figures 2 and 3. Scanning electron micrographs of flagellates and stages in transformation to mature division cysts. **2.** The motile flagellate A shows a long anterior flagellum and the base of the recurrent (posterior) flagellum (arrowhead). Flagellates B and C are in the process of resorbing flagella (B shows seam of intake) into the body by lateral engulfment. Young division cysts such as D show surface pitting while mature ones such as E have a smooth contour. Scale bar 5 μm. **3.** Body of mature flagellate showing base of anterior flagellum (small white arrowhead) and recurrent flagellum attached along entire length of body (large white arrowhead). surface pits (black arrowheads) probably represent endocytosis caveolae. Scale bar 1 μm.

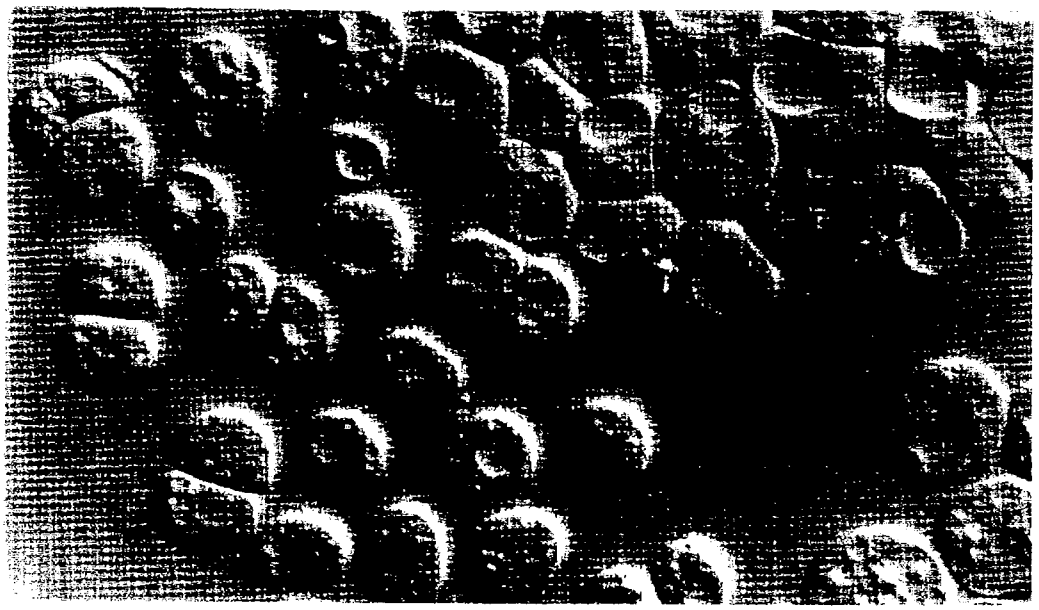
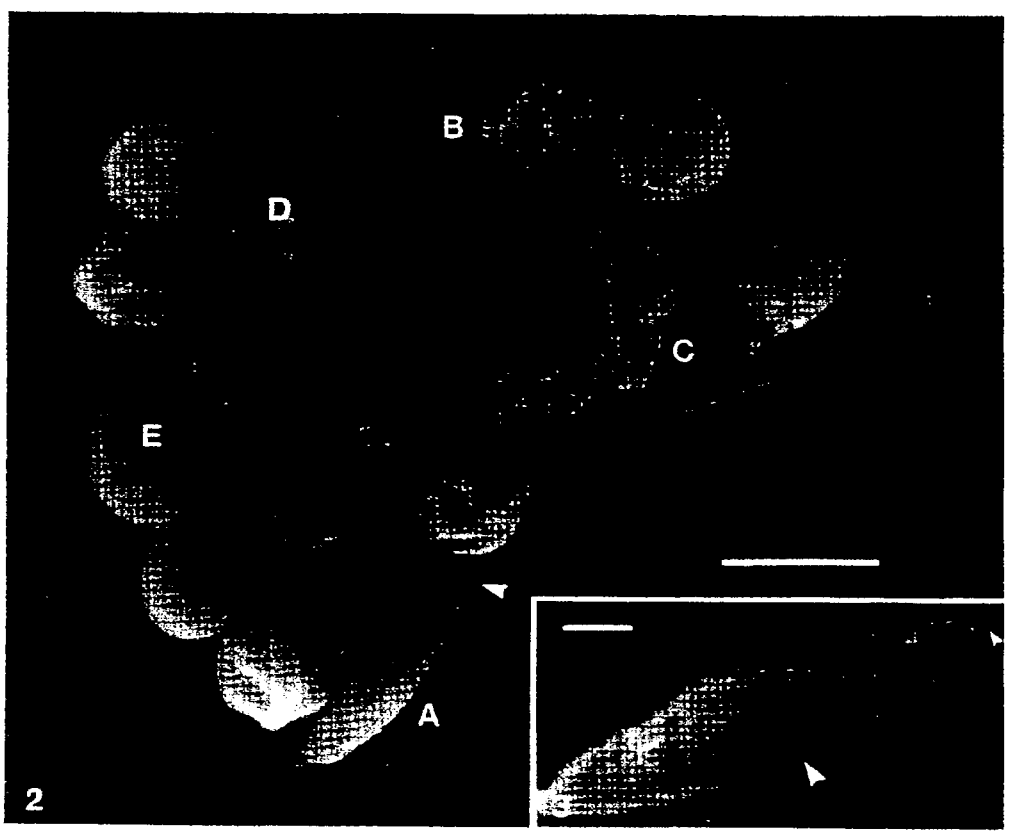


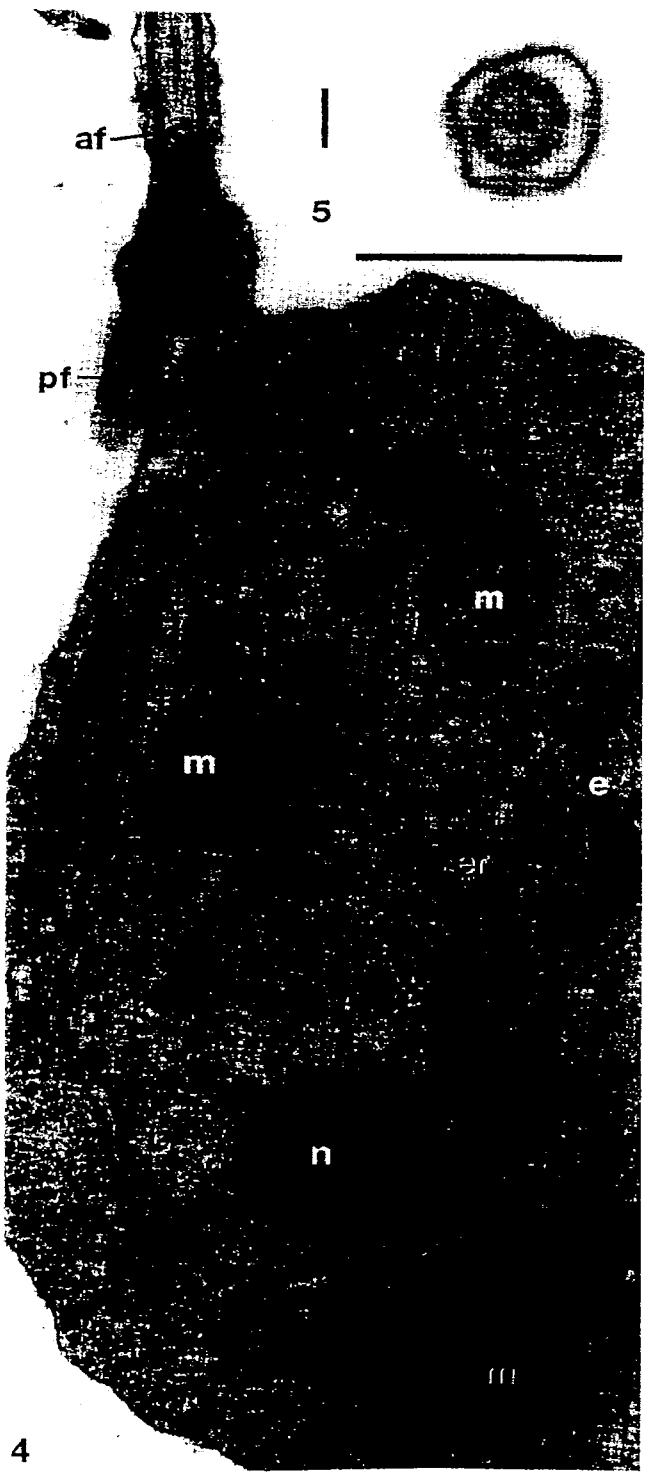
Figure 1. Nomarski image of division cysts of *Proleptomonas faecicola*. Cysts in which one division has been completed are indicated by large black arrows. Encysted forms show the nucleus with large nucleolus (small arrow) and several large vacuoles. Scale bar 10 μm.



in the process of transformation from the dispersive flagellate to the dividing stage. In some of these transforming individuals, a recurrent second flagellum may be seen, attached to the body but becoming free along part or the whole of its length. In addition, the body surface appears to be extremely deformable at either end and sticky cytoplasmic extensions enabling the organism to adhere to the substratum may be present.

The rounded aflagellate is 6–9 μm in diameter (n = 30). It has a central vesicular nucleus and more obvious cytoplasmic inclusions in the form of prominent vacuoles. It undergoes binary fission, the two daughter cells being constrained to adopt hemispherical forms by a thin, surrounding, near-invisible envelope (Fig. 1). Flagella are resorbed before this cyst wall is formed. Each aflagellate may undergo two or more divisions before flagella reappear and the flagellate condition is resumed. In compressed late division cysts, the flagella of daughter organisms are wrapped around the division products and can be seen to commence beating; the motile daughters escape from their straightjacket at staggered intervals by wriggling through a small hole in the cyst wall, deforming the body considerably during the process. Conversely, flagellated forms can be observed to become sluggish and rounded as they settle at the periphery of a raft, withdrawing their flagella in preparation for division. Not all divisions result in the production of flagellates: following some divisions, aflagellate daughter cells are produced and each acquires its own wall. Up to eight daughter cells may be seen inside a common wall, thus resembling the palmella stage of certain algae. In culture stationary phase, flagellate production ceases and many of the division cysts appear to undergo degeneration. Occasionally-observed, more refractile bodies may represent resting cysts, and stationary phase organisms kept in distilled water for up to six months can be used to initiate new cultures.

Continuous cultivation of this isolate over five years has resulted in fewer, less actively motile flagellates being produced.



Figures 4 and 5. Transmission electron micrographs of flagellate stage. **4.** Longitudinal section of anterior half of flagellate showing: bases of anterior (af) and posterior (pf) flagella at anterior tip; numerous elongate mitochondria (m), with tubular cristae, converging on the tip; vacuole (e) possibly containing endocytosed material; nucleus (n) with prominent nucleolus; granular endoplasmic reticulum (er)-rich area of cytoplasm. Scale bar 1 μm. **5.** Transverse section of (anterior) flagellum showing conventional axoneme structure. Scale bar 0.1 μm.

Electron Microscopy

Scanning electron microscopy shows clearly that in addition to the long forwardly directed locomotory flagellum (Fig. 2), a second recurrent flagellum is always present, usually attached to the body along its entire length (Fig. 3) and occasionally extending beyond its posterior extremity. A notable feature of most flagellates is the presence of pits (~125 nm across) scattered over the body surface (Fig. 3); these are believed to represent endocytotic caveolae (see below). During transformation from flagellate to division cyst, the body shortens and the recurrent flagellum sinks into the cytoplasm, followed by the reflexed anterior flagellum (Fig. 3). The smooth-surfaced division cyst occasionally shows clustered pits (Fig. 2).

In thin sections of flagellates, transmission electron microscopy reveals that the flagella do not arise from a flagellar pocket but from a cytoplasmic promontory at the apex of the cell (Fig. 4). They have conventional axonemal structure and lack the paraflagellar (paraxial) rod characteristic of kinetoplastids (Fig. 5); the recurrent flagellum likewise lacks special junctional complexes for attachment to the body. The two basal bodies each measure ~0.4 µm in length and lie orthogonally or at an obtuse angle to one another; they contain no distinctive internal structures. A dense transverse plate separates basal body and axoneme (Fig. 6) in the active anterior flagellum, but not in the passive posterior flagellum. The marked transitional region between basal body and axoneme, seen in kinetoplastids, is absent. The cytoskeleton of the flagellate consists of a loose cone of microtubules originating from organising centres close to the apposed basal bodies and extending back into the cytoplasm (Fig. 7). Prominent electron-dense masses at the open end of the anterior flagellum basal body and

Figures 6–8. Transmission electron micrographs to show details of flagellar bases and cytoskeleton. **6.** Bases of anterior (af) and posterior (pf) flagella in longitudinal section showing dense plate (arrowed) separating basal body from axoneme (this plate is absent from posterior flagellum), and electron dense material associated with basal bodies. Scale bar 0.5 µm. **7.** Longitudinal section of base of anterior flagellum to show extent of basal body (bb) and longitudinally-orientated loose cone of microtubules (at small arrowheads) of cytoskeleton that arise from electron dense material associated with the basal bodies. Scale bar 0.2 µm. **8.** Longitudinal section through base of posterior flagellum (pf) to show: absence of dense plate at base of axoneme; tenuous association of flagellum with body surface (between arrows); cortical microtubule (small arrowheads) running alongside the junction of body and flagellum; endocytosis caveola (large arrowhead). Scale bar 0.2 µm.

along the inner surface of the basal body of the posterior flagellum (Figs. 6, 7, 8) appear to be the principal microtubule-organising centres; organised flagellar root structures have not been detected. Relatively few microtubules reinforce the plasma membrane (Fig. 8), most pass through the internal cytoplasm converging eventually on the posterior extremity of the flagellate. The several, independent elongate mitochondria are compressed between the cytoskeletal microtubules, most noticably at the anterior end of the body (Fig. 4). The mitochondrial cristae are tubular or shelf-like (Fig. 4) and the mitochondrial matrix exhibits no structures that could be construed as representing a kinetoplast. The surface membrane has many coated pits (Fig. 8) and associated coated vesicles which probably represent stages in endocytosis. Long cisternae of granular endoplasmic reticulum are abundant in the cytoplasm (Fig. 4). Smooth membrane-bound vesicles/vacuoles with clear contents or containing moderately electron dense material (presumably endocytosed) are also common (Fig. 4). One to three prominent Golgi stacks with well-separated cisternae are present alongside the nucleus, usually subtended by a limb of endoplasmic reticulum. Lipid globules are occasionally present.

During transformation of the non-dividing flagellate to the dividing aflagellate stage (Fig. 9), the flagella appear to be withdrawn by fusion of flagellar and plasma membranes so that the axonemes become internalised and then resorbed. In the aflagellate stage the two basal bodies are all that remains

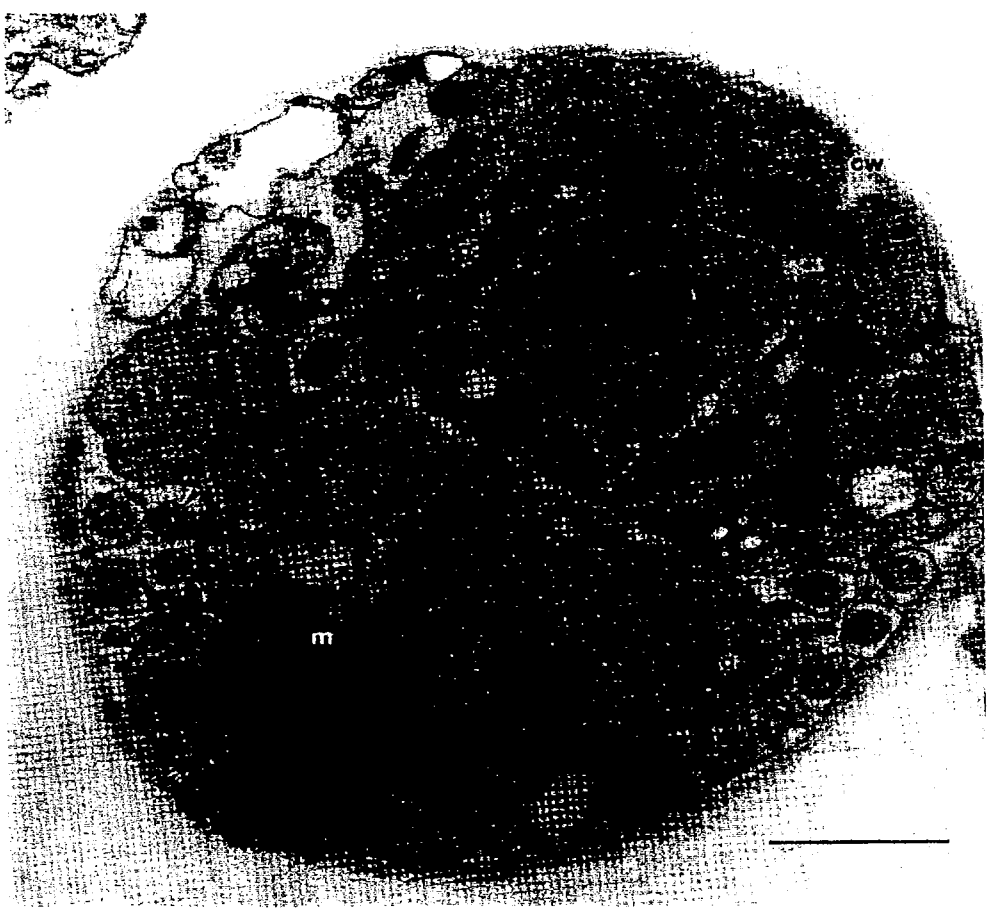


Figure 9. Transmission micrograph of late division cyst with profiles of two individuals enclosed in a common fibrous cyst wall (cw). The flagella are wound around the bodies of the daughter individuals prior to emergence of the mature flagellates through a break in the cyst wall; their tips (small arrowhead), visible on the left, show no central microtubules. In the absence of a restraining cytoskeleton, mitochondria (m) are orientated in all directions. One daughter nucleus (n) has a cytoplasmic intrusion containing a mitochondrion. Endocytotic caveolae (large arrowhead) are still active in the division cyst. Much cytoplasmic debris (d) is excreted into the cyst wall. e – vacuole possibly containing endocytosed material; g – golgi apparatus. Scale bar 1 μm.

of the flagellar apparatus and they sit on the nuclear envelope (Fig. 10) attended by a much-reduced microtubular cytoskeleton. The process of nuclear division has not been studied. The rounded aflagellate appears to continue endocytosing (Fig. 9) avidly, even after surrounding itself with a thin wall composed of fine fibrils (Fig. 9). Thick (10 nm) membrane-bound vacuoles of varying electron density abound in the cytoplasm: those with finely granular contents may represent endocytosed material. In division cysts from aging cultures, clear vacuoles often containing electron-dense inclusion bodies are common. A much-thickened fibrillar wall (up to 3 μm deep) is present around some aflagellate forms and these may correspond to the resting cysts mentioned above.

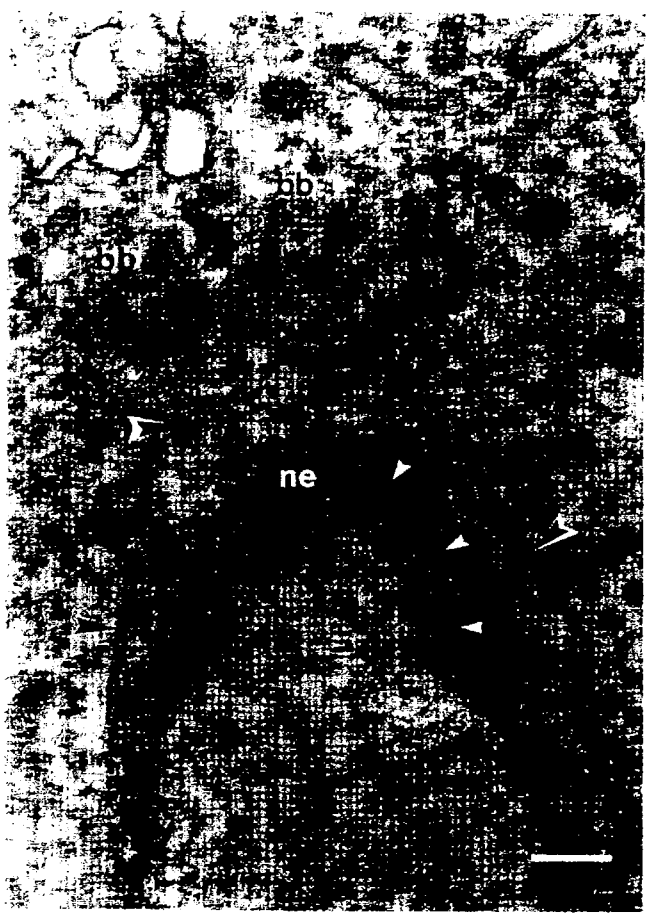


Figure 10. Transmission electron micrograph of nuclear region of *Proleptomonas* following flagellar resorption in the division cyst. The basal bodies (bb) of the flagella now sit close to the nuclear envelope (ne) which shows several nuclear pores (small arrowheads). The cytoskeleton of microtubules (large arrowheads) emanating from the basal bodies is represented by a much reduced cone. Scale bar 0.1 μm.

Soil Examination

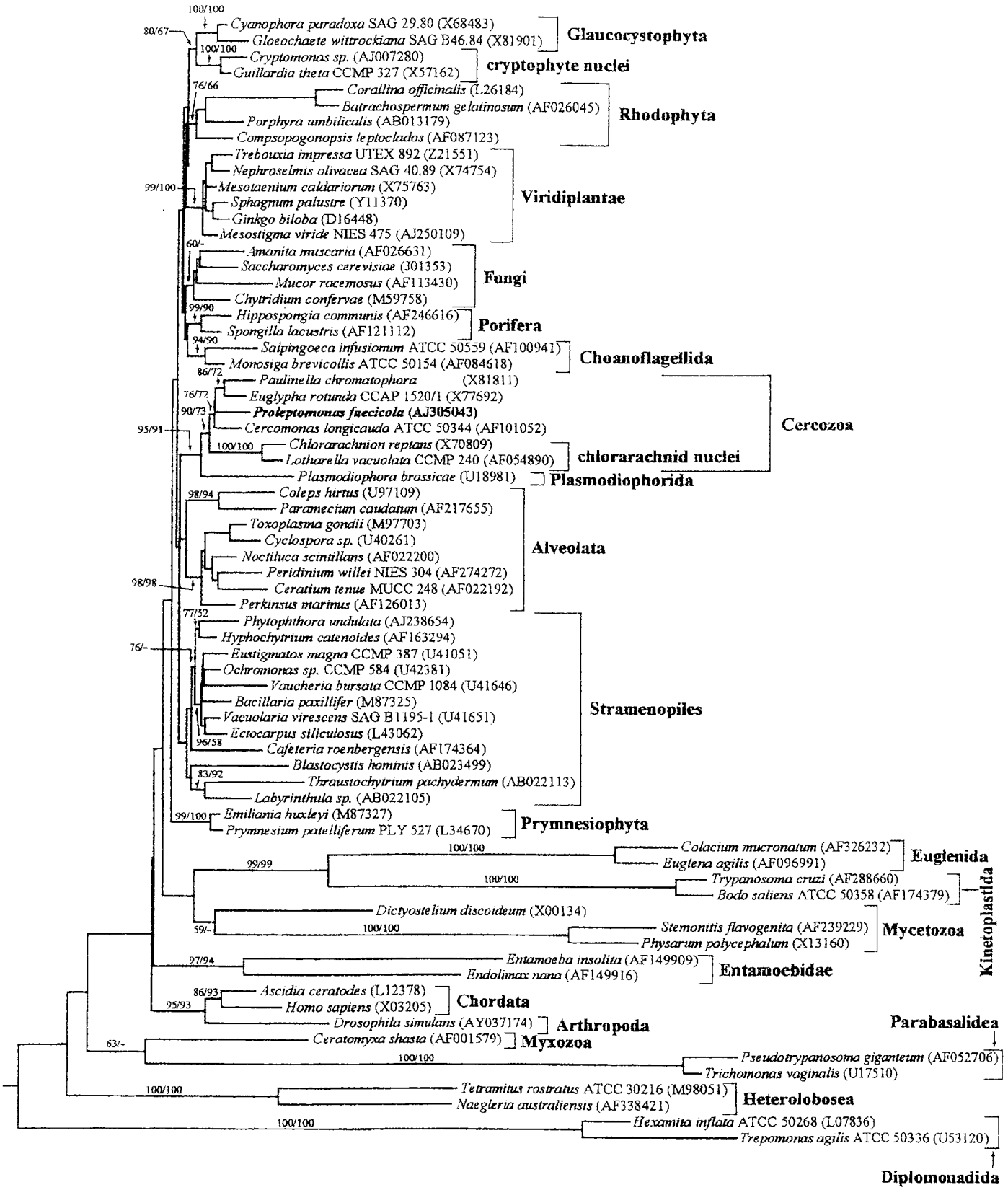
Differential and phase contrast microscopical examination of moistened soil samples for protozoa by the adpressed coverslip method revealed the occasional presence of a flagellate resembling, in both size and characteristic movement, that described above from culture. Flagellates always lacked ingested bacteria and were present at low incidence (26 out of 600 examinations). They were found in both fresh and dried soil samples and were more common in samples two days after moistening than in later examinations (Table 1). In five of the moistened dry samples, the characteristic division cysts were also observed, again similar in size to those produced by the culture forms. Their presence strengthened the view that soil and culture flagellates belong to identical or closely-related species of the genus *Proleptomonas*. In soil forms, two prominent contractile vacuoles, were observed emptying alternately, on either side (anterior and posterior) of the nucleus in both flagellate and division cyst stages. Attempts to cultivate the *Proleptomonas* sp. from soil using the media used for maintenance of the culture version, were unsuccessful due to bacterial overgrowth.

Phylogenetic Analysis

A large eukaryotic crown group alignment and a smaller Cercozoa/Plasmodiophorida alignment (see Methods) provided the datasets for phylogenetic analysis. According to the results of the hierarchical likelihood tests with 'Modeltest', the evolutionary model of Tamura and Nei (1993) with gamma-dis-

Table 1. Incidence of *Proleptomonas* in Sourhope soils as revealed by adpressed coverslip examination of fresh (undried, F) and air-dried (D) samples collected in different months and examined at 2, 4 and 7 days after moistening. Each figure represents the number of positive coverslips out of a total of 25 examined on each occasion.

Collection date	Examination					
	2 days		4 days		7 days	
	F	D	F	D	F	D
03.03.99	1	2	1	0	0	0
04.05.99	2	3	0	3	1	0
06.07.99	1	4	2	1	0	1
12.09.99	3	0	1	0	0	0
Total	7	9	4	4	1	1


Cyanophora paradoxa SAG 29.80 (X68483)
Gloeochaete wittrockiana SAG B46.84 (X81901)
Glaucocystophyta
Cryptomonas sp. (AJ007280)
Guillardia theta CCMP 327 (X57162)
cryptophyte nuclei
Corallina officinalis (L26184)
Batrachospermum gelatinosum (AF026045)
Porphyra umbilicalis (AB013179)
Compsopogonopsis leptoclados (AF087123)
Rhodophyta
Trebouxia impressa UTEX 892 (Z21551)
Nephroselmis olivacea SAG 40.89 (X74754)
Mesotaenium caldariorum (X75763)
Sphagnum palustre (Y11370)
Ginkgo biloba (D16448)
Mesostigma viride NIES 475 (AJ250109)
Viridiplantae
Amanita muscaria (AF026631)
Saccharomyces cerevisiae (J01353)
Mucor racemosus (AF113430)
Chytridium confervae (M59758)
Fungi
Hippospongia communis (AF246616)
Spongilla lacustris (AF121112)
Porifera
Salpingoeca infusionum ATCC 50559 (AF100941)
Monosiga brevicollis ATCC 50154 (AF084618)
Choanoflagellida
Paulinella chromatophora (X81811)
Euglypha rotunda CCAP 1520/1 (X77692)
Proleptomonas faecicola (AJ305043)
Cercomonas longicauda ATCC 50344 (AF101052)
Cercozoa
Chlorarachnion reptans (X70809)
Lotharella vacuolata CCMP 240 (AF054890)
chlorarachnid nuclei
Plasmodiophora brassicae (U18981)
Plasmodiophorida
Coleps hirtus (U97109)
Paramecium caudatum (AF217655)
Toxoplasma gondii (M97703)
Cyclospora sp. (U40261)
Noctiluca scintillans (AF022200)
Peridinium willei NIES 304 (AF274272)
Ceratium tenue MUCC 248 (AF022192)
Perkinsus marinus (AF126013)
Alveolata
Phytophthora undulata (AJ238654)
Hyphochytrium catenoides (AF163294)
Eustigmatos magna CCMP 387 (U41051)
Ochromonas sp. CCMP 584 (U42381)
Vaucheria bursata CCMP 1084 (U41646)
Bacillaria paxillifer (M87325)
Vacuolaria virescens SAG B1195-1 (U41651)
Ectocarpus siliculosus (L43062)
Cafeteria roenbergensis (AF174364)
Blastocystis hominis (AB023499)
Thraustochytrium pachydermum (AB022113)
Labyrinthula sp. (AB022105)
Stramenopiles
Emiliania huxleyi (M87327)
Prymnesium patelliferum PLY 527 (L34670)
Prymnesiophyta
Colacium mucronatum (AF326232)
Euglena agilis (AF096991)
Euglenida
Trypanosoma cruzi (AF288660)
Bodo saliens ATCC 50358 (AF174379)
Kinetoplastida
Dictyostelium discoideum (X00134)
Stemonitis flavogenita (AF239229)
Physarum polycephalum (X13160)
Mycetozoa
Entamoeba insolita (AF149909)
Endolimax nana (AF149916)
Entamoebidae
Ascidia ceratodes (L12378)
Homo sapiens (X03205)
Chordata
Drosophila simulans (AY037174)
Arthropoda
Ceratomyxa shasta (AF001579)
Myxozoa
Parabasalidea
Pseudotrypanosoma giganteum (AF052706)
Trichomonas vaginalis (U17510)
Tetramitus rostratus ATCC 30216 (M98051)
Naegleria australiensis (AF338421)
Heterolobosea
Hexamita inflata ATCC 50268 (L07836)
Trepomonas agilis ATCC 50336 (U53120)
Diplomonadida
0.1

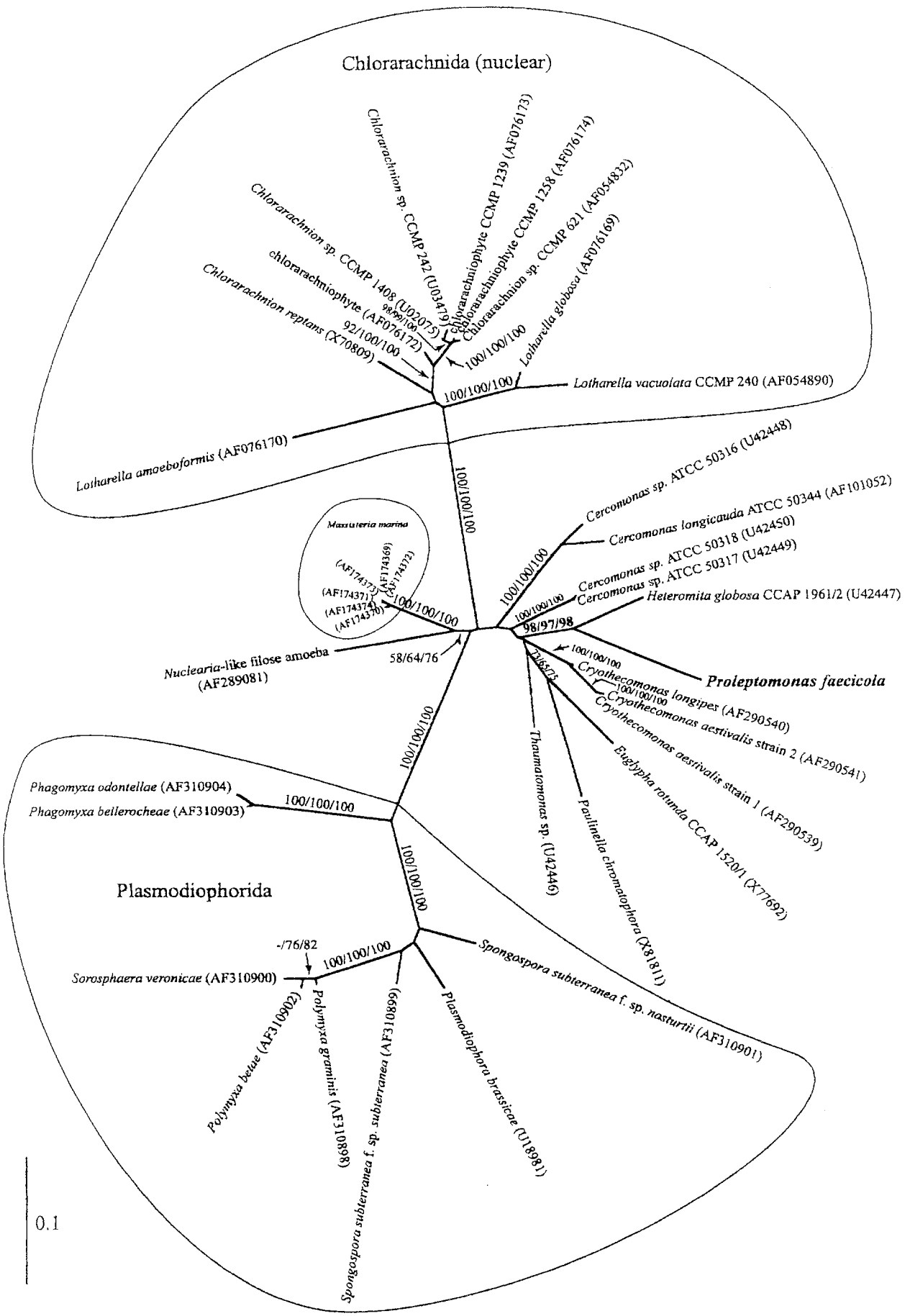
tributed among-site variation (G) and proportion of invariable sites (I) suited both datasets best, and was applied for the distance/neighbor-joining analyses of both alignments and for the maximum likelihood analyses of the smaller alignment.

Figure 11 shows the result of a distance/neighbor-joining analysis of the large alignment, mid-point rooted for better visualisation. Most of the eukaryotic crown groups were supported by high to moderate bootstrap values, although the basal branching order of the tree was disturbed by several long-branch lineages (Diplomonadida, Entamoebidae, Euglenida, Heterolobosea, Kinetoplastida, Mycetozoa, Myxozoa, Parabasalidea) causing a biased tree topology. As an additional indication for highly divergent substitution rates, the alignment also failed the test for homogeneity of base frequencies across sequences (performed only on the 1296 positions used for the analyses; see Methods). A long-branch attraction (LBA) artefact was most obvious in the Metazoa, comprising arthropod, chordate, myxozoan and poriferan taxa: whereas the Porifera clustered with the Choanoflagellida and the Fungi without bootstrap support, the longer branches of the chordate, arthropod and myxozoan lineages were pulled away to join the long-branch taxa at the basal nodes of the tree. Although the tree topology was clearly biased, however, in all analyses *P. faecicola* unambiguously clustered together with the Cercozoa and Plasmodiophorida, but not with the Kinetoplastida (Fig. 11). The bootstrap support for the Cercozoa/Plasmodiophorida-clade, including *P. faecicola* was high: 95% for the distance/neighbor-joining analysis and 91% for the maximum parsimony analysis.

Thus, to find the closest related clade suitable as an outgroup to the Cercozoa/ Plasmodiophorida lineage, the long-branch taxa (Diplomonadida, Entamoebidae, Euglenida, Heterolobosea, Kinetoplastida, Mycetozoa, Myxozoa, Parabasalidea) were omitted from the analyses. The resulting 54-taxa dataset (1403 positions) turned out to be homogeneous with respect to base frequencies across taxa, but the support for the basal branching order did not improve (not shown). The metazoan lineages clustered in a clade with the Fungi and Choanoflagellida without bootstrap support, and only in the maximum likelihood analysis were the Metazoa monophyletic. Presumably the still-included long branches of the chordate and arthropod lineages disturbed the intra-clade structure. In the distance and maximum likelihood analyses, the Cercozoa and Plasmodiophorida clustered with the Stramenopiles and the Alveolata. This association, however, was also not supported.

To examine the position of *Proleptomonas faecicola* within the Cercozoa and Plasmodiophorida in greater detail, the phylogenetic analyses on the Cercozoa/Plasmodiophorida alignment (37 taxa) were therefore performed without an outgroup. The alignment passed the test for homogeneity of base frequencies across taxa. The resulting maximum likelihood tree is depicted in Figure 12. In all analyses [distance/neighbor-joining, maximum parsimony (4 equally parsimonious trees), and maximum likelihood], the internal nodes of the trees were not resolved. Three well-supported clades with more than three taxa were present in the trees (Plasmodiophorida, Chlorarachnida, and a clade with almost identical *Massisteria marina* sequences). Most other taxa (except for the "*Nuclearia*-like amoeba" and *Thaumatomonas* sp.) were members of small (two- or three-taxa) clades. *Proleptomonas faecicola* clustered in all analyses with *Heteromita globosa* with high bootstrap support (Fig. 12). Excluding the Chlorarachnida and the Plasmodiophorida from the alignment did not improve the resolution of the inner nodes (not shown). However, an increase of the formerly low to moderate bootstrap values to a significant support (distance: 92%, maximum parsimony: 94%, maximum likelihood: 94%) was found for an association of the "*Nuclearia*-like filose amoeba"

Figure 11. Mid-point rooted distance/neighbor-joining tree with SSU rDNA sequences representing the major eukaryotic lineages. Distance matrix calculated by maximum likelihood; evolutionary model: Tamura-Nei with G + I (Tamura and Nei 1993). Taxa with strain designations, if available (public databases), corresponding accession numbers of DDBJ/EMBL/GenBank in parentheses. *Proleptomonas faecicola* clusters within the Cercozoa. Although the tree topology is biassed due to long branch attraction artefacts (see Results), the Cercozoa/Plasmodiophorida-clade is supported by significant bootstrap values. Bootstrap values on branches: distance/neighbor-joining and unweighted parsimony (1000 replicates) in order from left to right. Except for the Cercozoa/Plasmodiophorida, bootstrap values for terminal clades are not shown. 1296 unambiguously aligned positions; scale bar = substitutions per site.


Chlorarachnida (nuclear)
Chlorarachnion sp. CCMP 242 (U03479)
Chlorarachniophyte CCMP 1239 (AF076173)
Chlorarachniophyte CCMP 1258 (AF076174)
Chlorarachnion sp. CCMP 621 (AF054832)
Lotharella globosa (AF076169)
Chlorarachnion sp. CCMP 1408 (U02075)
chlorarachniophyte (AF076172)
Chlorarachnion reptans (X70809)
Lotharella vacuolata CCMP 240 (AF054890)
Lotharella amoeboformis (AF076170)
Massuteria marina
(AF174373)
(AF174369)
(AF174372)
(AF174371)
(AF174374)
(AF174370)
Nucleariа-like filose amoeba (AF289081)
Cercomonas sp. ATCC 50316 (U42448)
Cercomonas longicauda ATCC 50344 (AF101052)
Cercomonas sp. ATCC 50318 (U42450)
Cercomonas sp. ATCC 50317 (U42449)
Heteromita globosa CCAP 1961/2 (U42447)
Proleptomonas faecicola
Cryothecomonas longipes (AF290540)
Cryothecomonas aestivalis strain 2 (AF290541)
Cryothecomonas aestivalis strain 1 (AF290539)
Euglypha rotunda CCAP 1520/1 (X77692)
Paulinella chromatophora (X81811)
Thaumatomonas sp. (U42446)
Spongospora subterranea f. sp. nasturtii (AF310901)
Plasmodiophora brassicae (U18981)
Spongospora subterranea f. sp. subterranea (AF310899)
Polymyxa graminis (AF310898)
Polymyxa betae (AF310902)
Sorosphaera veronicae (AF310900)
Phagomyxa odontellae (AF310904)
Phagomyxa bellerocheae (AF310903)
Plasmodiophorida
0.1

with *Massisteria marina*, whereas the bootstrap values for *Euglypha* and *Paulinella* remained unaffected.

Discussion

The protist described here from culture as *Proleptomonas faecicola* can be assigned to Woodcock's species with reasonable confidence on account of (1) its size and shape, (2) the extraordinary length of the flagellum and similarity of its movement to that of a promastigote trypanosomatid (leptomonad), (3) lack of any evidence for phagotrophy in both organisms (unusual in coprophilic/soil protozoa), (4) the transient nature of the flagellate phase and lack of observed dividing flagellate forms. Woodcock (1916) observed his flagellate in infusions of goat faeces only over the course of a few days. He could not identify a multiplicative stage and neither could Sandon (1927) in the soil flagellate that he regarded as identical with Woodcock's species. Our finding of division taking place within a thin-walled cyst in both the culture form examined here and that seen in soil samples, explains the inability of both observers to recognise division stages in native material that they examined and suggests that protists from our two sources share a similar and highly unusual life cycle. Flagellate production does not appear to be essential for multiplication of the organism.

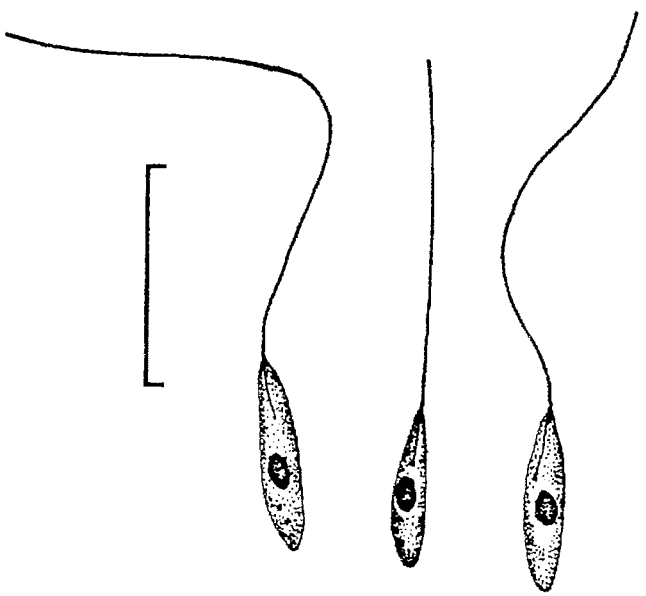


Figure 13. Woodcock's figures of Giemsa-stained *Proleptomonas faecicola*. Note the long flagellum and the line between flagellum base and nucleus interpreted by Woodcock as a "rhizoplast" (flagellar root) but possibly part of a recurrent flagellum. Scale bar = 10 μm.

The presence of a second (recurrent) flagellum on the flagellate stage was not detected by Woodcock, but its presence may be reflected in his statement that "the flagellum is continued backwards for some distance from the anterior end ... probably as a long rhizoplast, and terminates in a prominent basal granule." The recurrent flagellum may have appeared in Woodcock's Giemsa-stained preparations, then, as a rhizoplast; strangely, the "prominent basal granule" referred to in Woodcock's text is not evident in his illustrations (see Fig. 13). Sandon (1927) reported finding flagellates recognised as *P. faecicola* in 17 out of 148 soils "including those from localities as far apart as Greenland and Spitzbergen on the one hand, and India, Sudan and the Argentine on the other." He provided no illustrations, however. His identification of *Proleptomonas* appears to have depended solely upon its pattern of movement: "The movements are quite unmistakable, for it darts forward in a straight line with a spiral movement so rapid that the tip of the body appears blurred like the tip of a vibrating tuning fork: at times it darts backward for a short distance and then resumes its forward motion." This distinctive behaviour in swimming was also used to identify individuals of *P. faecicola* in soil samples taken from the NERC's Soil Biodiversity Programme's Sourhope site in the present work. In addition, the distinctive division cysts have been identified in both the flagellate grown in culture and in Sourhope soil samples. In the intervening years no other authors appear to have added to our knowledge of *P. faecicola* from

Figure 12. Unrooted maximum likelihood tree of 37 cercozoan and plasmodiophorid SSU rDNA sequences. Evolutionary model: Tamura-Nei with G + I. Taxa with strain designations, if available (public databases), corresponding accession numbers of DDBJ/EMBL/GenBank in parentheses. A sister group relationship of *Proleptomonas faecicola* with *Heteromita globosa* is highly supported in all bootstrap analyses. Bootstrap values on branches: distance/neighbor-joining, unweighted parsimony (1000 replicates each), and maximum likelihood (100 replicates) in order from left to right. For the *Massisteria marina* clade terminal bootstrap values are not shown. 1540 unambiguously aligned positions; [-ln L = 12193.59456]. Scale bar = substitutions per site.

soils, and attempts to cultivate *Proleptomonas* from the transient small numbers present in Sourhope soils using the procedure employed for the tapwater flagellate have failed. We tentatively propose that *P. faecicola* is basically a soil flagellate that after heavy rainfall can find its way into tapwater supplies or swim upwards to contaminate the foliage of forage plants to appear eventually in the faeces of animals that feed on these plants. At the moment we have no information on the ultrastructure or phylogeny of the *Proleptomonas* taken from soil, however and it is possible that more than one species is involved in this scenario.

Proleptomonas faecicola is unusual among heterotrophic soil flagellates in being non-phagotrophic. It also has a somewhat unusual developmental cycle with division taking place only in the temporarily encysted aflagellate form, and not in the flagellated stage which is presumably dispersive. A similar cycle has not been reported for any kinetoplastid. A thick-walled cyst which would enable the flagellate to endure drying out of the soil (or survive digestion in the ruminant stomach) has occasionally been observed in the cultured organism. Sedimented culture forms can survive in distilled water for at least six months and in the present work *Proleptomonas* has been recovered from dried and frozen soil.

A schematic diagram of the fine structure of the flagellate phase of *Proleptomonas* is given in Figure 14. Despite its superficial trypanosomatid appearance by light microscopy, especially in rapid locomotion, the presence of several mitochondria with cristae of unusual form and lacking kinetoplasts, and the absence of a cortical microtubular cytoskeleton, of a flagellar pocket and of paraxial rods in the flagella, all suggest that *P. faecicola* is not a trypanosomatid, a conclusion confirmed by the phylogenetic data which tentatively suggest its placement within the Phylum Cercozoa Cavalier-Smith 1998.

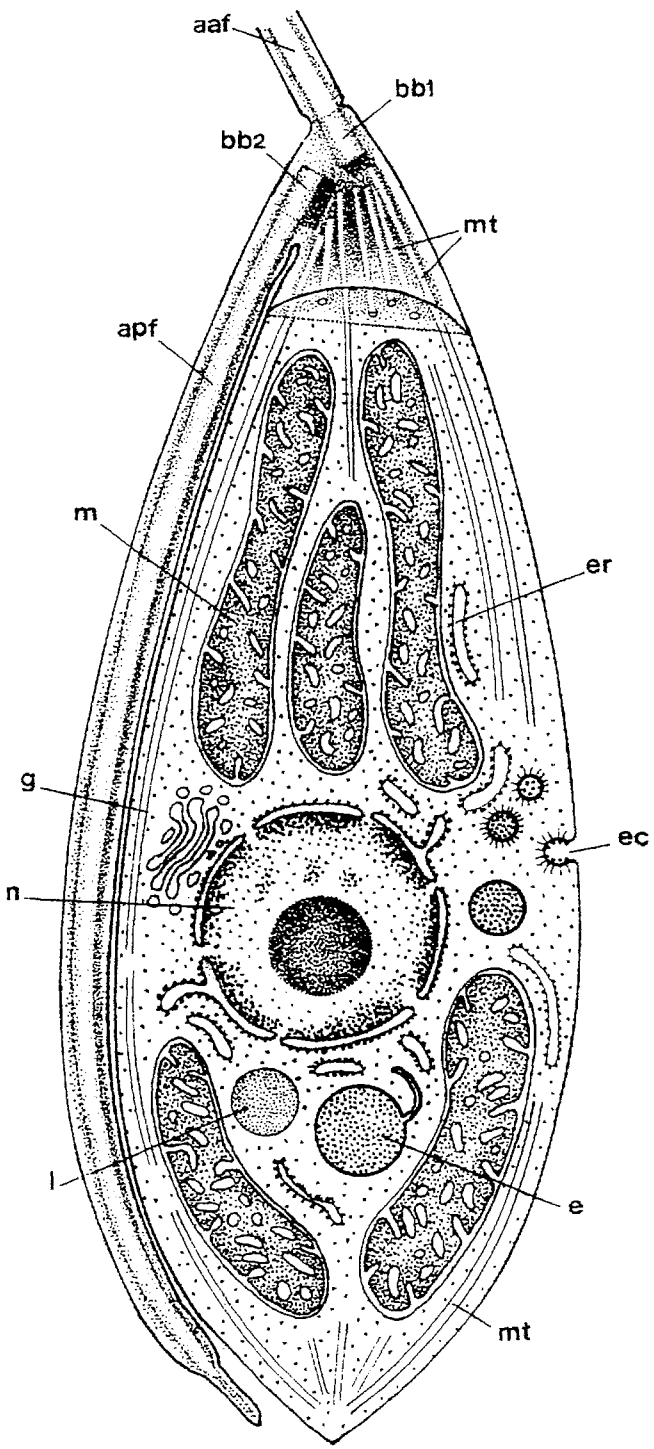


Figure 14. Schematic diagram of organisation of flagellate phase of *Proleptomonas faecicola*. The body is seen in longitudinal section except for its anterior tip which, along with the flagella are viewed in semi-transparency to show the basal bodies and the proximal part of the cone of microtubules that constitutes the cytoskeleton. Unlike in other Cercozoa, the nucleus and Golgi apparatus are divorced from the flagellar apparatus in the flagellate phase. The longitudinally-orientated intracytoplasmic microtubules reconverge at the posterior end of the body.
aaf: axoneme of anterior flagellum; apf: axoneme of posterior (recurrent) flagellum; bb1, bb2: basal bodies of anterior and posterior flagella, respectively; ec: endocytosis caveola; e: vacuole of endocytosed material; er: granular endoplasmic reticulum; g: Golgi apparatus; l: lipid; m: mitochondrion; mt: microtubules of cytoskeletal cone; n: nucleus.

In this phylum erected largely on the basis of SSU rDNA sequence data, Cavalier-Smith (1998a) included a rag bag of amoeboid organisms: the plant-parasitic subphylum Phytomyxa (which included the Plasmodiophorida), the subphylum Reticulofilosa (or Chlorarachnida, whose members exist as photosynthetic amoeboid plasmodia, their chloroplast-bearing cells linked by a network of reticulopodia), and the very diverse subphylum Monadofilosa. This last group embraced the non-flagellated filose amoebae including testate members such as *Euglypha* and *Paulinella* (Testaceofilosida), the silica scale-producing Thaumatomonadida (e.g. *Thaumatomonas*), and the Cercomonadida. To this original assemblage, has now been added (Kühn et al. 2000) on the strength of SSU rDNA sequencing, the order Cryothecomonadida Cavalier-Smith 1993 which contains ice-dwelling, theca-bearing amoeboid biflagellates of the genus *Cryothecomonas*.

As pointed out by Karpov (2000), compared with almost every other flagellate order, the Cercomonadida are singularly lacking in unique characteristics. They are naked biflagellates with heterodynamic flagella, the recurrent flagellum usually serving as a skid for creeping or gliding on the substratum. They feed by producing temporary pseudopodia that engulf bacteria or other prey, and include the genera *Cercomonas* Dujardin (frequently synonymised with *Cercobodo* Krassilstschick) and *Heteromita* whose species are extremely common in soil; the marine flagellate *Massisteria marina* has also been assigned to this order (Patterson and Zölffel 1991). In its cellular organisation, *Proleptomonas* with its naked surface, heterodynamic flagella and cytoskeletal cone of microtubules bears some structural similarities only to the Cercomonadida (see MacDonald et al. 1977; Mignot and Brugerolle 1975; Mylnikov 1986; Schuster and Pollak 1978), but its nucleus is divorced from the flagellar bases and the organism is neither amoeboid nor phagotrophic. *Heteromita globosa* – which from the phylogenetic analysis recounted above is nearest in SSU rDNA sequence to *Proleptomonas* – is reputed to form temporary as well as more permanent cysts (Sandon 1927), but division does not occur within them. Another soil flagellate, *Dingensia angusta* (Dujardin) Patterson et Zolffel (formerly "*Spiromonas angusta*", see Patterson and Zölffel 1991 for history), has division cysts but neither its ultrastructure nor its phylogeny has been studied.

Cavalier-Smith and Chao (1996/7) found much uncertainty over relationships within the Cercozoa depending on the phylogenetic analysis method employed and the organisms included. This uncertanty may be due to highly divergent evolutionary rates or to problematic taxon sampling (see e.g. Philippe 2000). Both conditions may cause long branch attraction artefacts or destabilise the internal nodes of a tree by introducing homoplasies, especially when inappropriate evolutionary models are applied (Yang 1996). In the present study, to determine first the approximate position of *Proleptomonas faecicola*, a large alignment of the eukaryotic crown group lineages including the long-branch taxa was undertaken. The resulting tree (Fig. 11) showed a biassed branching order at the basal nodes presumably due to LBA artefacts. However, *Proleptomonas* was neither extraordinarily long-branched (relative to the other sequences in the large alignment) nor found in a basal, and therefore unreliable, position, but clustered unambiguously with high bootstrap support within the Cercozoa. In contrast to the findings of Kühn et al. (2000), but in agreement with Cavalier-Smith and Chao (1996/7), Van de Peer et al. (2000) and Bulman et al. (2001), this clade also included the Plasmodiophorida.

In the smaller dataset comprising only cercozoan and plasmodiophorid sequences, the Chlorarachnida and Plasmodiophorida were potentially disturbing long-branch taxa, and in our maximum parsimony analysis of this alignment, a long branch attraction artefact could indeed be observed: the chlorarachnid and plasmodiophorid sequences clustered together as sister clades (not shown), an effect not observed in the distance and maximum likelihood analyses. Apart from the influences of these two clades, the internal branching order of the tree was dominated by short internal but comparably long terminal branches. Several genera were represented by only one sequence (*Thaumatomonas*, *Paulinella*, *Euglypha*, *Heteromita*, and the "*Nuclearia*-like filose amoeba", also *Proleptomonas*). Of the taxa examined in this study, *Heteromita globosa* can be considered with high probability to be the closest relative to *Proleptomonas faecicola*, but to determine the position of *Proleptomonas* within the Cercozoa with higher accuracy, taxon sampling needs to be improved significantly.

The effects of a problematic taxon sampling in combination with an inappropriate evolutionary model became obvious in an analysis of the phylogenetic position of *Cryothecomonas* by Kühn et al. (2000). Although the more robust maximum likelihood method was used to construct an SSU rDNA phylogeny with several crown group lineages (see Fig. 1 in Kühn et al. 2000), due to an LBA artefact *Plasmodiophora brassica* branched off basally. The authors used *Trypanosoma* sequences – known to be highly divergent (see also Fig. 11, this study) – as an outgroup and used a simple evolutionary model

not taking into account among-site rate variation. In the study of Bulman et al. (2001), a complex evolutionary model (substitution rate calibrated distance matrix) was applied and taxon sampling was improved by using short-branched and closely related taxa as an outgroup. Further, new plasmodiophorid sequences were added to the alignment and broke the long branch of *Plasmodiophora brassica*.

The SSU rDNA analysis does not as yet give a clear-cut answer to the exact position of *Proleptomonas* within the Cercozoa. Despite its failure to emerge as a genuine trypanosomatid, *Proleptomonas faecicola* is clearly a highly unusual organism.

Methods

Isolation and cultivation: The organism whose ultrastructure and phylogeny are described here was isolated by D. Le Ray and E. Willaert in January 1972 during a surveillance project of free-living amoebae (*Naegleria* spp.) in chlorinated tapwater. Three litres of tapwater from the Antwerp urban network were drawn through a Millipore 0.22 µm filter which was then transferred to a bacterised (*Escherichia coli*) non-nutrient agar plate for incubation at 26 °C. The outgrowing spherical organisms were cultivated axenically in the casitone-glucose-fetal calf serum medium (CGVS) of Willaert (1971), initially without the 5% fetal calf serum but with penicillin and streptomycin. Fast development of flagellate forms was observed over 48h after subculture, then abundant production of division cysts. The derived organism was cloned and cryopreserved with 5% dimethyl sulphoxide (cooling rate of –1 °C/min to –70 °C, then 5 °C/min to –100 °C before transfer to liquid nitrogen. (–196 °C). After tentative identification as "*Monas* sp." by Prof. A. Hollande (Paris), the cryostabilate (ITMAP 422) was maintained for many years in the collection of the Prince Leopold Institute of Tropical Medicine, Antwerp. The organism was reidentified as *Proleptomonas faecicola* Woodcock by KV when DLR brought it to Glasgow in 1975 for ultrastructural studies. *Proleptomonas* was maintained on CGVS or modified Chang's serum-casein-glucose-yeast extract medium (SCGYEM, De Jonckheere 1977) adjusted to pH 5.0 and with 10% fetal calf serum When grown in 15 ml medium in 50 ml Nunclon flasks at 25 °C, subculture was undertaken at two weekly intervals. Cultures remained viable for one month.

The *Proleptomonas* sp. cryostabilate ITMAP 422 was brought back into culture in January 1975 by DLR in Glasgow and again in February 1997 by J De J on disposal of the Antwerp cryostabilate collection. Cultures of the organism are available on request from KV.

Soil examination: Soil samples from each of 25 plots were obtained on four occasions (March 2, May 4, July 7 and September 7, 1999) from the NERC Soil Biodiversity Thematic Programme experimental site at the Macaulay Land Use Research Institute's Sourhope Research Station, near Kelso in Southern Scotland (Grid reference NT 854196). Livestock had been excluded from the site since April 1998. Full details of the shallow brown forest soils at the site and methods of sampling are given by Finlay et al. (2000); the adpressed coverslip technique used for examining soil flagellates was a modification of that described by Ekelund and Patterson (1997). Briefly, samples taken to a depth of 5 cm and consisting primarily of the upper soil organic layer were packed into 5 cm plastic petri dishes and moistened to a thick slurry with Cerophyl infusion medium (Page 1988). After covering with a disc of lens tissue, a No. 1 coverslip was adpressed to the damp soil. It was removed and examined microscopically after 2, 4 and 7d incubation at 18 °C. Both fresh and air-dried samples of soil were examined.

Electron microscopy: CGVS-grown organisms were pelleted by centrifugation at 1000 *g* for 10 mins and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min. After washing (3 × 30 min) in buffer with 1% sucrose, and postfixation in buffered 1% OsO_4 for 1h, pellets were dehydrated in alcohol with staining in 1% uranyl acetate in 70% alcohol. Pellets were embedded in Spurr's resin and sections stained with uranyl acetate and lead citrate for viewing in an AEI EM801 transmission electron microscope. For scanning electron microscopy, the postfixed organisms were retained on 1.0 µm pore-size nucleopore filters and dehydrated in acetone before critical point drying, coating with gold and examination in a Phillips PSEM scanning microscope.

Phylogenetic analysis: For phylogenetic analysis, DNA was isolated directly from thawed cryostabilate ITMAP 422 taken out of liquid nitrogen without culturing the organism. The SSU rDNA was amplified using universal eukaryotic primers in the polymerase chain reaction (PCR). The amplification conditions were 1 min at 94 °C, 1.5 min at 55 °C and 2 min at 72 °C for 30 cycles using Taq DNA polymerase (Boehringer Mannheim, Germany). The PCR products were sequenced using the PCR product sequencing kit (Amersham Life Science Inc., Cleve-

land, Ohio, USA) employing the SSU rDNA amplification primers and conserved internal primers. Reaction products were separated on 6% acrylamide-urea sequencing gels and autoradiographed overnight at room temperature.

From the amplified SSU rDNA of the Antwerp *Proleptomonas* strain, 1601 bp were sequenced and submitted to DDBJ/EMBL/GenBank (acc. no. AJ305043). Sequences around the 5′- and 3′-primers were not obtained.

Two different alignments with manually aligned sequences were prepared using the multiple sequence editor Seaview (Galtier et al. 1996); for species included and their accession numbers, see Figs. 11 and 12. First, a large alignment with 70 SSU rDNA sequences representing the major eukaryotic lineages was performed. 1296 unambiguously aligned positions were used for the phylogenetic analyses. The second, smaller, alignment comprised 37 SSU rDNA sequences exclusively from the Cercozoa (including Plasmodiophorida and Chlorarachnida) and yielded 1540 unambiguously aligned positions. The nuclear sequence of the chlorarachnid *Gymnochlora stellata* was excluded from the alignment due to ambiguities. Phylogenetic analyses were performed with PAUP* 4.0b8 (portable version under Debian GNU/Linux 2.2 or PPC version; Swofford 1998). The homogeneity of base frequencies was tested using the command "basefreqs" implemented in PAUP 4.0. Prior to the phylogenetic analyses, for each of the datasets the evolutionary model fitting the data best was determined by using Modeltest 3.06 (hierarchical likelihood ratio tests; Posada and Crandall 1998). All datasets were subjected to maximum parsimony (Fitch 1977; heuristic search, 10 random addition replicates), to distance/neighbor-joining (Saitou and Nei 1987) and to bootstrap analyses under both optimality criteria (1000 replicates; Felsenstein 1985). The distances for the neighbor-joining trees were calculated using maximum likelihood and the evolutionary model proposed by Modeltest. The smaller dataset with 37 taxa was also analysed using the maximum likelihood criterion (Felsenstein 1981) with the proposed evolutionary model for a single heuristic search and for a bootstrap analysis (100 replicates). The sequence alignment is available on request from K H-E.

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