The taxonomic position of *Klosteria bodomorphis* gen. and sp. nov. (Kinetoplastida) based on ultra-structure and SSU rRNA gene sequence analysis

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Summary

A small free-living marine bacteriotrophic flagellate *Klosteria bodomorphis* gen. and sp. nov. was investigated by electron microscopy and molecular methods. This protist has paraxial rods of typical bodonid structure in the flagella, mastigonemes on the anterior flagellum, two nearly parallel basal bodies and discoid mitochondrial cristae. The flagellar pocket and cytostome/cytopharynx complex are supported by two microtubular roots and reinforced microtubular band (mtr). These features confirm that *K. bodomorphis* is a bodonid related to *Bodo*. However, the presence of a layer of dense glycocalyx on the flagella and one battery of cylindrical trichocysts with reticular walls makes it more similar to *Rhynchobodo/Phyllomitus*, although this flagellate characterized by pankinetoplasty. Phylogenetic analysis using the SSU rRNA gene is congruent with the ultrastructural studies and strongly confirms the close relationship of *K. bodomorphis* to the genus *Rhynchobodo* within the order Kinetoplastida.

Key words: Bodonidae, *Klosteria bodomorphis*, evolution, 18S rDNA, phylogeny

Introduction

Free-living kinetoplastids, especially bodonids (Bodonidae Hollande), are an important component of marine ecosystems. In spite of the broad diversity and frequent occurrence of free-living kinetoplastids, their biodiversity and taxonomy are still poorly known. Recently some well-known and new species and genera of kinetoplastid and related flagellates have been reinvestigated by electron microscopy (Brugerolle et al., 1979; Brugerolle, 1985; Mylnikov, 1986; Mylnikov et al., 1998; Elbrächter et al., 1996; Simpson et al., 1997; Frolov et al., 1997, 2001).

The definition of even such a well-known and valid kinetoplastid taxon like *Bodo*, comprising an assemblage of free-living kinetoplastids with a more or less
oval body, apical rostrum, two free heterodynamic flagella, and single kinetoplast (Vickerman, 1991), is unclear. In 1994 one of the authors (A.P. Mylnikov) isolated a small bacterirotroph flagellate from the littoral of the South Baltic. Originally, it was referred to the genus *Bodo* due to its cell shape, arrangement of flagella and behaviour, but subsequent ultrastructural investigation demonstrated that a new genus should be erected for this bodonid (Burzell, 1975; Brugerolle et al., 1979; Brugerolle, 1985).

However, ultrastructural studies did not clarify the taxonomic position of this flagellate within the bodonids as its cell organization combines features of *Bodo* and *Rhynchobodo*.

There is a large SSU rRNA database for representatives of Kinetoplastida. The monophyly of kinetoplastids was confirmed by molecular phylogenetic methods in spite of the high rate of SSU rRNA gene sequence divergence within the group (Dolezel et al., 2000; Maslov et al., 2001; Callahan et al., 2002; Hughes et al., 2003). The high level of genus divergence complicates the phylogenetic comparison of kinetoplastids with other groups of protists including their closest relatives, diplonemids and euglenids. Moreover, there is a problem with the choice of an appropriate outgroup for kinetoplastids, because all available close relatives are highly divergent, and this causes the biases of Long Branch Attraction artifact and partial loss of phylogenetic signal.

In this study, we represent the ultrastructural definition of the new genus and species *Klosteria bodomorphis*. To confirm the phylogenetic position of *K. bodomorphis* we compare its SSU rRNA gene sequence to a representative data set including all genera in the order Kinetoplastida.

**Material and methods**

A clonal culture of *Klosteria bodomorphis* sp. nov. was isolated from littoral samples of the Baltic Sea near the town of Kloster, Germany, in December of 1994. Cultures of *K. bodomorphis* were maintained in a Schmaltz-Pratt medium (add the following to 1 L of water: 28.15 g NaCl, 0.67 g KCl, 5.51 g MgCl₂·6H₂O, 6.92 g MgSO₄·7H₂O, 1.45 g CaCl₂·H₂O, 0.1 g KNO₃, 0.01 g K₂HPO₄·3H₂O) with salinity adjusted to 20‰ and inoculated with bacteria *Aerobacter* (*Klebsiella*) aerogenes as food. Cultures of this flagellate can be obtained from the culture collection maintained at the Institute for Biology of Inland Waters, Russian Academy of Sciences (Borok).

For electron microscopy a suspension of cells was collected and fixed with a mixture of 2% OsO₄ and 0.6% glutaraldehyde (final concentrations) on Schmaltz-Pratt medium for 15-30 min at 1°C. Then the preparations were treated as in Mylnikov with coauthors (1998).

DNA was extracted using the DNeasy Plant Minikit (Qiagen, Basel, Switzerland). One microlitre (1 µl) of DNA extract was added for each PCR probe. PCR amplifications were done in a total volume of 50 µl with an amplification profile consisting of 40 cycles with 30 s at 94°C, 30 s at 50°C, and 2 min. at 72°C, followed by 5 min. at 72°C for the final extension. The amplified PCR products were purified using the High Pure PCR Purification Kit (Roche, Rotkreuz, Switzerland), then ligated into pGEM-T Vector System (Promega, Wallisellen, Switzerland), cloned in XL-2 Ultra-competent Cells (Stratagene, Basel, Switzerland), sequenced with the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit, and analysed with an ABI-377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to the manufacturer’s instructions. The complete SSU rRNA gene of *K. bodomorphis* was amplified using the universal primers sA (5’ ACCTGGT TGATCCTGCCAGT 3’) and sB (5’ TGATCCTTCT GCAGGTTTCACCTAC 3’). The length of the amplified sequences of SSU rRNA of *K. bodomorphis* was 2072 nucleotides.

The complete SSU rRNA gene sequence from *K. bodomorphis* was manually aligned with sequences from diverse kinetoplastids, diplonemids and euglenids. Preliminary phylogenetic analyses were used to confirm the grouping of *K. bodomorphis* within the bodonids (data not shown). Because of their extremely long branches, the diplonemids, euglenids, *Ichtiobodo* and *Paradobodo* were excluded from the alignment. The final alignment comprised 19 sequences, including the sequence obtained in this study. 1826 unambiguously aligned positions were used in the phylogenetic analyses. The sequence has been submitted to GenBank under accession number AY268046.

Phylogenetic trees were inferred using the maximum likelihood (ML) method (Felsenstein, 1981). The reliability of internal branches was assessed using the bootstrap method (Felsenstein, 1985) with 50 replicates. ML analysis was performed using PAUP (Swofford 1998) under the GTR model of sequence evolution (Lanave et al., 1984; Rodriguez et al., 1990), taking into account a proportion of invariable sites, and a gamma distribution of the rates of substitution for the variable positions, with 8 rate categories. All parameters for ML analysis were estimated from the dataset using Modeltest (Posada and Crandall, 1998). Starting trees were obtained via NJ, and then swapped using the tree-bisection-reconnection algorithm.

**Abbreviations**

af - anterior flagellum, amt - additional micro-
flagellar basal bodies, two flagellar roots can be found surrounded by vesicles (Figs 4, 6, 15, 16). The ventral root of the flagella begins as 6 microtubules near the posterior flagellar basal body and goes behind to form the ventral band of 27 microtubules (Figs 6, 8, 9, 13). Additionally, the two microtubules can be seen between the basal bodies (Fig. 11, arrow). On the cross section of flagellar pocket the band of 4–5 microtubules have been found (Figs 8–10). These microtubules are connected with the surface of the flagellar pocket by fibrillar bridges (Fig. 13). This band forms the so-called MTR band sensu Brugerolle with coauthors (1979) as a reinforced band of microtubules. It goes along the wall of the flagellar pocket and turns to the cytopharynx (Fig. 12), where the band is supplemented with additional microtubules (Figs 14–16). Assemblages of microtubules such as the «microtubular prism» of Burzell (1975) or «nemadesm» of Kivic and Walne (1984) are absent.

The Golgi apparatus lies near the flagellar basal bodies (Figs 5, 21). The mitochondria possess discoid cristae and do not have condensed fibrils of DNA like the compact kinetoplast in some bodonids (Figs 4, 7, 14–18). Usually the whole mitochondria is filled by the cristae. Only some parts of mitochondria (for example, in Fig. 16) can be interpreted as a kinetoplast. This characteristic state can be named pankinetoplasty according to Vickerman (1991). A vesicular nucleus with a central nucleolus lies at the level of the bottom of the flagellar pocket and at the end of cytopharynx (Fig. 17). The granules of storage substance are 0.10–0.35 μm in diameter and symbiotic bacteria of 0.3–0.6 μm in length, some of which were dividing, were noted in the cytoplasm (Figs 17, 19). A single battery of 8–9 extrusomes (trichocysts) is situated near the ventral side of flagellar pocket (Figs 4, 5, 8–10, 13, 20). The trichocyst consists of a cylinder of 1.2–1.9 μm in length and 0.15 μm in diameter and an internal rod of 0.6–0.77 μm in length (Fig. 21). Long, hollow cylinders of discharged trichocysts with a distinctive reticular structure can be observed outside the cell (Figs 1, 22). The cylinder of discharged trichocyst is 0.3 μm in diameter. Food vacuoles with captured bacteria are situated in the hind part of the flagellate (Figs 17, 19). Microbody-like bodies have not been found.

**Molecular data**

Analysis of our sequence data clearly shows that *K. bodomorphis* belongs to the order Kinetoplastida (Fig. 23). Within this order, *K. bodomorphis* groups with a strong bootstrap (BP=100) support with the genus

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**Electron microscopy**

*K. bodomorphis* has bean-shaped, slightly flattened cells. The cell body is of 5.4–9.0 μm (usually 6.5–7.0 μm) in length. Two heterodynamic flagella emerge from the shallow flagellar pocket which occupies subapical position. The anterior flagellum is of 12 μm and is directed ahead forming a flexible hook. The posterior flagellum is of 17 μm and is directed backwards. Both flagella do not attach to the cell surface and have short tapering tips (acronemes) (Figs 1, 2). The anterior flagellum bears very fine hairs (mastigonemes) of 2.0–2.5 μm (Fig. 3). The rostrum (apical part of the body anterior to the flagellar insertion) is not prominent. The cytostome as an opening is visible on the tip of the rostrum. The cytostome continues into the tubular cytopharynx. The flagellate ingests bacteria through the cytostome (Fig. 1), forming posterior food vacuoles. Contractile vacuoles are absent. During motion the flagellates jump along the substrate (bottom of Petri dish) with short rapid darts. At high culture density the cells can swim chaotically. In old cultures the cells do not make active motions but only the flagella move slowly. Cysts have not been found.

The cell body is covered by only the unit membrane (plasmalemma) in contrast to the flagella, which are additionally covered by the layer of condensed glycocalyx (Figs 8, 9). Two flagella emerge from the bottom of the small flagellar pocket. The flagellar basal bodies lie nearly parallel to each other and are connected by some fibrils (Figs 5, 11). The flagellar transversal plates are located at the level 0.20–0.25 μm above the cell surface (Figs 4, 5, 7). On cross sections, the paraxial rods of anterior and posterior flagella have circular and trapezium profiles correspondingly (Figs 6, 8–10). Four flagella have been found in the flagellar pocket in some cells (Fig. 10). The cytopharynx, 0.5 μm in diameter, opens roughly on the same level as the aperture of the flagellar pocket (Fig. 4). The cytopharynx continues to the cytopharynx, which is 1.8–2.3 μm in length. The lower part of the cytopharynx is surrounded by vesicles (Figs 4, 6, 15, 16).

In addition to the fibrillar connection between the flagellar basal bodies, two flagellar roots can be found near the basal bodies. The dorsal root of 2–3 microtubules begins from the basal body of the anterior flagellum and goes up to form the dorsal submembrane band of more than 25 microtubules (Figs 4, 5, 7–10). The dorsal root is associated with the electron dense lamina (Figs 5, 8). The ventral root of the flagella begins as 6 microtubules near the posterior flagellar basal body and goes behind to form the ventral band of 27 microtubules (Figs 6, 8, 9, 13).

Microbody-like bodies have not been found (Fig. 21). The mitochondria possess discoid cristae and do not have condensed fibrils of DNA like the compact kinetoplast in some bodonids (Figs 4, 7, 14–18). Usually the whole mitochondria is filled by the cristae. Only some parts of mitochondria (for example, in Fig. 16) can be interpreted as a kinetoplast. This characteristic state can be named pankinetoplasty according to Vickerman (1991). A vesicular nucleus with a central nucleolus lies at the level of the bottom of the flagellar pocket and at the end of cytopharynx (Fig. 17). The granules of storage substance are 0.10–0.35 μm in diameter and symbiotic bacteria of 0.3–0.6 μm in length, some of which were dividing, were noted in the cytoplasm (Figs 17, 19). A single battery of 8–9 extrusomes (trichocysts) is situated near the ventral side of flagellar pocket (Figs 4, 5, 8–10, 13, 20). The trichocyst consists of a cylinder of 1.2–1.9 μm in length and 0.15 μm in diameter and an internal rod of 0.6–0.77 μm in length (Fig. 21). Long, hollow cylinders of discharged trichocysts with a distinctive reticular structure can be observed outside the cell (Figs 1, 22). The cylinder of discharged trichocyst is 0.3 μm in diameter. Food vacuoles with captured bacteria are situated in the hind part of the flagellate (Figs 17, 19). Microbody-like bodies have not been found.

**Analysis of our sequence data clearly shows that K. bodomorphis belongs to the order Kinetoplastida (Fig. 23). Within this order, K. bodomorphis groups with a strong bootstrap (BP=100) support with the genus**
Figs 1-7. External cell structure and the anterior end of the Klosteria bodomorphis. 1, 2 - The anterior and posterior flagella (af, pf), cytostome (c) and discharged trichocysts are seen. 3 - Fine mastigonemes (mn) cover the surface of anterior flagellum, 4 - 7 - anterior cell end. The anterior and posterior flagella (af, pf), base plate of the flagellum (bp), flagellar pocket (fp), cytopharynx (cp), band MTR (mtr) of microtubules, ventral and dorsal bands of microtubules (vb, db), dorsal microtubular root (dr), vesicles (v), mitochondrion (m) are seen. Bars: 5 μm for figs 1, 2; 1 μm for figs 3-7.
Rhynchobodo within clade 1 (the numbering of the kinetoplastid groups is taken from Simpson with coauthors (2002)). The SSU rDNA-based analysis revealed three strongly supported clades, which fully corroborates earlier data using different phylogenetic markers. The root of the kinetoplastid tree was tentatively attached to group 2 (Cryptobia, Parabodo, B. caudatus, and B. sorokini) according to the hypothesis of Lukeš (Lukeš et al., 2002). Within this clade species of the parasitic genus Cryptobia are mixed with species of the free-living genus Bodo: B. sorokini, B. caudatus.

If this position of the root is confirmed, then group 3 + T with T. cruzi, B. sorokini Petersburg, B. edax, B. uncinatus and B. saltans is related to group 1 with Dimastigella, Rynchomonas, Rhynchobodo, Klosteria, Ornella, B. saliens, B. designis and indetermined kinetoplastid LFS2.

Contrary to the results of the SSU rDNA analysis of Dolezel (Dolezel et al., 2000), these data clearly show
(BP=70) the relative positions of Trypanosomatids (T) and group 3, supporting the hsp 90 based phylogeny of Simpson (Simpson et al., 2002). This phylogenetic reconstruction is consistent with the hypothesis, based on the kDNA organization and compaction, that the representative of group 3 B. saltans is ancestral to the Trypanosomatid T. brucei (Lukeš et al., 2002).

In group 1 there are two well established subgroups, one including two genera group Dimastigella - Rhynchomonas and Rhynchobodo - Klosteria (BP=86%), and the other containing Cruzella, B. saliens, B. designis and indetermined kinetoplastid LFS2 (BP=99%).

**Discussion**

Molecular phylogenetic and ultrastructural analyses together provide the most complete and precise description of an organism. The taxonomic validity of kinetoplastid ultrastructural features is tested by the SSU rDNA-based phylogenetic analysis.

There is a group of ultrastructural features that strongly confirms the relationship of the new genus Klosteria with the order Kinetoplastida. Such features as the presence of paraxial rods in flagella, nearly parallel basal bodies, discoid mitochondrial cristae, and...
the cytopharynx, together with the peculiarities of microtubular rootlet system, including a reinforced microtubular band (mtr), are characteristic for K. bodomorphis and for free-living bodonids, for example Bodo caudatus, B. saltans, B. designis, and B. curvifilus (Burzell, 1975; Eyden, 1977; Brugerolle et al., 1979). Confirming the results of morphological studies, the SSU rDNA analysis puts K. bodomorphis within the bodonids (Bodonidae Hollande) in the order Kinetoplastida.

K. bodomorphis shares some characters with Bodo spp. such as body form, cell motion and a well-developed flagellar pocket and complex cytostome/cytopharinx. It does not possess a proboscis like Dimastigella or Rhynchomonas, the posterior flagellum is not attached to the ventral cell surface as in Procryptobia (Frolov et al., 1997, 2001) and there is no «lip» as in Rhynchobodo/Phyllomitus and Hemistasia (Brugerolle, 1985; Mylnikov, 1986; Elbrächter et al.,

**Fig. 23.** Phylogenetic position of Klosteria bodomorphis (in bold) among eukaryotes, inferred using the maximum likelihood method with the GTR + G + I model. K. bodomorphis clearly belongs to the order Kinetoplastida. Numbers at nodes represent percentages of bootstrap support following 50 data resamplings. The tree was rooted following a recent hypothesis on the position of kinetoplastid origin (Lukeš et al., 2002).
using serial cell sections. But molecular studies strongly refute the monophyly of the genus *Bodo*, and it follows from this that the characters mentioned above appeared independently in different lineages of *Bodo* and in *Klosteria*. The SSU rDNA analysis also confirms that the presence of a proboscis is an apomorphic feature for the clade *Dimastigella - Rhynchomonas*.

*Klosteria* differs from *Bodo* species by the absence of a compact kinetoplast and of the long acronome of the posterior flagellum. In turn, *Klosteria* has cylindrical trichocysts with a reticular envelope and a dense layer of glycocalyx on the flagellar surface. The last two structures are absent in *Bodo* spp. These data are also congruent with the results of SSU rDNA-based phylogenetic analysis where *Klosteria* does not reveal affiliation to any available *Bodo* species.

Thus *Klosteria* is characterized by the presence of cylindrical trichocysts with a reticular envelope of the «lattice tube» type (Elbrächter et al., 1996), a layer of dense glycocalyx on the flagella and pankinetoplasty. The term pankinetoplasty should be distinguished from the term polykinetoplasty. In the case of the latter kDNA forms several or many well-organized agglomerates often localized in swellings of the mitochondrion (Frolov et al., 1997). Obviously, the kinetoplasts in *Klosteria* in a state of eukinetoplasty or of polykinetoplasty are absent because the fibrils of kDNA do not occupy a prominent space in mitochondria. The question of whether *Klosteria* has a single branched mitochondrion, as in usual kinetoplastids (Vickerman, 1991), remains unclear and needs to be investigated using serial cell sections.

Some ultrastructural characters of *Klosteria* are shared with *Rhynchobodo armata*, *Phyllomitus apiculatus*, *Hemistasia phaeocystica* and *Postgaardi mariagerensis* (Brugerolle, 1985; Mylnikov, 1986; Mylnikov et al., 1988; Elbrächter et al., 1996; Simpson et al., 1997). Anaerobic *Postgaardi mariagerensis* is a recently described organism with a covering of rod-shaped bacteria and with two thickened flagella inserted into an anterior pocket. This flagellate does not have mitochondria but has the typical bodonid arrangement of two microtubular flagellar roots, MTR band and additional extrusomes, which resemble the trichocysts of *K. bodomorphis*. The latter differs from *Postgaardi mariagerensis* by the presence of normal mitochondria. It seems that these ultrastructural features carry a true informative phylogenetic signal as can be deduced from the molecular studies where *K. bodomorphis* unambiguously groups with *Rhynchobodo*, forming a sister clade to the *Dimastigella - Rhynchomonas* clade.

Another flagellate with trichocysts is *Hemistasia phaeocystica*. The mitochondria of this organism are of the polykinetoplast type (Vickerman, 1991). They contain dense, irregular clusters of DNA, which are scattered all over the mitochondrion and not concentrated near the flagellar bases. The trichocysts have an internal crest structure which is absent in *Klosteria* and they are of 4 µm in length which is twice longer than in *K. bodomorphis*. *K. bodomorphis* differs from *Hemistasia* by the absence of the cell «lip» and has only one row of extrusomes in the battery as opposed to four rows in *Hemistasia*.

The other extrusome-containing flagellates *Rhynchobodo armata*, *Phyllomitus apiculatus*, *P. amylophagus* (the latter two are often considered to belong to the genus *Rhynchobodo* according to Brugerolle, 1985) have a remarkable cell body «lip», similar trichocysts with a reticular envelope, a microtubular prism, two microtubular flagellar roots and band MTR, a dense layer of glycocalyx on both flagella and the cell surface, and mitochondria of eukinetoplast or polykinetoplast type. In contrast to these organisms *Klosteria bodomorphis* does not possess a body «lip», nor a microtubular prism as multi-microtubular formation, nor dense glycocalyx on the body surface, and has mitochondria without remarkable kinetoplast. In addition, in *K. bodomorphis* the battery of extrusomes consists of only one row of trichocysts, not several as in *Rhynchobodo* and *Phyllomitus*. Probably the presence of trichocysts of similar structure and a layer of dense glycocalyx demonstrates the resemblance between *K. bodomorphis* and *Rhynchobodo/Phyllomitus*. Additionally, *Phyllomitus apiculatus* and *K. bodomorphis* have symbiotic cytoplasmic bacteria. The absence of a microtubular prism and dense glycocalyx on the cell surface in *Klosteria* shows a more simple cell structure compared to Rhynchobodo and *Phyllomitus* and presumes possible reduction of these two structures in *Klosteria*. Thus, ultrastructural and molecular phylogenetic approaches applied together demonstrate the relationship of *K. bodomorphis* to *Rhynchobodo* sp. within the order Kinetoplastida.

**Taxonomic appendix**

*Klosteria* Mylnikov et Nikolaev gen. nov.


**Remarks.** This genus resembles the genus *Bodo* in body shape, differing from it by the presence of trichocysts and absence of visible compact kinetoplast.
Etymology. Name Klosteria is formed from the town of Kloster, Germany, where the flagellate was found.

Klosteria bodomorphis. Mylnikov et Nikolaev sp. nov.

Diagnosis. Flagellates have two long heterodinamic flagella. Anterior flagellum 12-13 µm, posterior one, 16-18 µm. Slightly flattened cell body is 5.4-9.0 µm (usually 6.5-7.0 µm) in length. Nucleus in anterior cell part. Battery of cylindrical trychocysts positioned around flagellar pocket. Flagella covered with thickened glycocalyx. No contractile vacuoles. Food vacuole located posteriorly. Moving by jumping on the substrate; rarely by swimming. Cysts unknown.

Remarks. This species resembles Bodo curvifilus and B. designis (Burzell, 1975; Eyden, 1977) in body shape, differing from them by more flexible anterior flagellum and flattened cell body, as well as by the presence of trichocysts.

Ethymology. Name bodomorphis means resembling Bodo.

Occurrence. Marine. 9‰ in habitat, South Baltic. 20‰ in cultures.

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