Molecular systematics and ultrastructural characterization of a forgotten species: *Chattonidium setense* (Ciliophora, Heterotrichea)

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Abstract: In the present paper we redescribe the ciliate *Chattonidium setense* Villeneuve 1937 combining morphological observations (live, stained, scanning, and transmission electron microscope) with behavioral notes and molecular data. Ultrastructural analysis revealed remarkable similarities between *Chattonidium* and representative members of the class Heterotrichea in cortical structure and cytoplasmic organization. The most similar genus for these aspects appears to be *Condylostoma*. To verify this relatedness, 18S rRNA genes from *Chattonidium* and from one *Condylostoma* species were sequenced. Phylogenetic analysis indicates *Chattonidium* belongs to the class Heterotrichea defined according to the modern taxonomy, and confirms its relatedness with *Condylostoma* already hypothesized by Villeneuve-Brachon (1940). The presence of the aboral cavity complex, a unique feature never described in other ciliates, and its peculiar organization revealed by ultrastructural analysis fully justify, in our opinion, the maintenance of *Chattonidium* in the separate family Chattonidiidae, established by Villeneuve-Brachon in 1940.

Key words: Protozoa; protist; heterotrichs; SSU rRNA; molecular phylogeny; ultrastructure.

Introduction. Heterotrichs were considered for a long time as typical spirotrichs (Bütschli, 1889); Kahl, 1932; Corliss, 1979). Nevertheless, when the focus on oral structures of ciliates for classification purpose gave way to that on basal body ultrastructural organization (Gerassimova and Seravin, 1976; Lynn, 1976a, b), the existence of a rather close relationship between heterotrichs and karyorelicteans became evident, due to the similarity of their somatic dikinetids (Lynn, 1976c, 1981). On this basis, and supported by the ultrastructural results on the ciliary membrane by Bardele (1980), Small and Lynn (1981) grouped the heterotrichs with Karyorelictea in the subphylum Postciliodesmatophora established by Gerassimova and Seravin (1976) to encompass the species characterized by the hyperdevelopment of postciliary microtubules. Later, the first classification of Ciliates in which heterotrichs were elevated to the class level (Heterotrichea) was officially proposed (de Puytorac et al., 1993); since then, considerable convergence about the validity of this taxon in all the revised classifications of ciliates occurred (see for example Lynn and Small, 1997). Indeed, phylogenetic analyses with molecular markers, which started on protists in the mid-1980s, had supported from the very beginning the separation of heterotrichs from Spirotrichea and their association with Karyorelictea (Baroin-Tourancheau et al., 1992; Hirt et al., 1995). On the other hand, when molecular markers were used to investigate which genera really belonged to this taxon and the phylogenetic relationships among the various species classically included in it, some problems arose, as they often led to very different conclusions from the previous morphological results.

By means of 18S rRNA gene sequencing, in particular, some genera were moved out of Heterotrichea...
erotrichia into other classes in total disagreement with the original morphological findings. This occurred, for example, when Shin et al. (2000) and Lynn and Strüder-Kypke (2002) transferred from Heterotricia to Spirotrichea the species Phacodinium mechtikhoffi and the order Licnophorida respectively. Another example concerns the anaerobic genus Metopus (and the whole order Armophorida), which since the very first molecular studies turned out to be phylogenetically distant from the aerobic heterotrichs, suggesting that heterotrichs, as defined out to be phylogenetically distant from the aerobic which since the very first molecular studies turned out to have as well a solid morphological basis, as the order Licnophorida re-

Portations (van Hoek et al. 2000a, b; Affa’a et al., 2004) and the riboclass Armophorida and Clevelandellida (with the genus Nyctotherus) are no longer included in Heterotricia but in the new class Ar- morphorea (Lynn, 2003a), which is highly supported in 18S rRNA phylogenetic analyses (van Hoek et al., 2000b; Affa’a et al., 2004). Although the kinetids of Metopus and Nyctotherus are not very similar (Lynn, 1981, 1991), the study by Foissner and Agatha (1999) on Metopus hasei and M. inversus highlighted some similarities in the ontogenesis and the ultrastructure between metopids and clevelandellids; moreover, they all presumably possess hydrogenosomes (Fenchel and Finlay, 1991, van Hoek et al., 2000b). Thus, the “riboclass” Armophorea (Lynn, 2003b) might in the future turn out to have as well a solid morphological basis, as Villeneuve-Brachon (1940) already speculated.

Finally, sometimes the molecular analysis suggested only the alteration of phylogenetic affiliations within the class Heterotricha: the new genus Maris- tentor, for example, was named after its morphological similarity with stentorids by Lobban et al. (2002) but the molecular characterization lately performed by Miao et al. (2005) showed its closer relatedness to Foliculina.

Nowadays it is particularly useful to include the characterization of a molecular marker in taxonomic description or redescription of genera and species especially in those cases where the studied organisms show a limited number of diagnostic morphological characters sometimes even of difficult interpretation. This situation is extremely common in Protistology due to the small size of organisms and their single-cell nature. As a general rule, within protists the molecular distances between two morphologically related species are much higher than those between morphologically similar metazoan species, especially when the latter belong to groups whose morphology is rich in diagnostic characters like insects, vertebrates, etc. Identification problems related to the absence of a sufficient number of diagnostic morphological features first became evident, for obvious reasons, in prokaryotic microbiology. Nowadays, it is mandatory, for bacterial species description, the characterization of at least one molecular marker, generally the 16S rRNA gene (Stackebrandt and Goebel, 1994; Stackebrandt et al., 2002). Moreover, the knowledge of 16S rRNA gene sequence is a prerequisite to design oligonucleotide species-specific probes that are routinely used, in microbial ecology studies, for in situ recognition of different but morphologically indistinguishable bacterial species (for a review on the topic see Amann et al., 1995). Within ciliates, the 18S rRNA gene sequence is generally sufficient to properly discriminate among morphologically similar species (Bernhard et al., 2001; Petroni et al., 2002) and, in some cases, the retrieved differences have been successfully used to design species-specific probes (Petroni et al., 2003; Schmidt et al., 2006). Obviously, exceptions do exist also among ciliates, like in the case of the genus Tetrahymena, where different morphospecies share identical 18S rRNA gene sequences (Strüder-Kypke et al., 2001). Nevertheless, even in this case these species were successfully discriminated using a faster evolving molecular marker like the mitochondrial cytochrome C oxidase I (Lynn and Strüder-Kypke, 2006). This marker was also successfully used to discriminate among different Paramecium strains (Barth et al., 2006).

From this general picture, it seems evident that, within ciliates, and more generally within protists, the only way to avoid misplacing (or misnaming) of genera and species is the performance of both molecular and morphological characterizations. Indeed, such a multidisciplinary approach has recently been widely applied in Protistology (Baumgartner et al., 2002; Modeo et al., 2003; Foissner et al., 2004; Leander et al., 2004; Rosati et al., 2004; Agatha et al., 2005; Kim et al., 2005; Visvesvara et al., 2005; Fokin et al., 2006; Walker et al., 2006; Vávra et al., 2006; Yoshikawa et al., 2006; Hoppenrath and Leander, 2006). Moreover, the view that both classical taxonomic determination and molecular characterization are required for the clarification of phylogenetic relationships between a species and related genera is now supported.
by scientists all over the world and the need for an interaction between taxonomic specialists (systematists and taxonomists) and ecologists is frequently expressed (Modeo et al., 2003; Gotelli, 2004; Finlay, 2004).

In the present paper we redescribe the ciliate *Chattonidium setense* Villeneuve 1937 combining morphological observations (live, stained, scanning, and transmission electron microscope) with behavioral notes and molecular data. This organism was first collected by Villeneuve in brackish water and described as “le très beau et très curieux Cilié”. Although at first sight the cell resembled in shape and swimming behavior an Oligotrich as *Strombidium* or a Tintinnid without tail, Villeneuve (1937) decided to consider it a heterotrich on the basis of its cilia-ture. The main character on which it was regarded as a new genus, is the existence of a unique huge “posteroaxial cavity” at cell posterior pole covered by six/seven longitudinal rows of membranelles: this peculiarity renders the identification of *Chattonidium* unambiguous allowing the establishment of the new heterotrich family Chattonidiidae (Villeneuve-Brachon, 1940). However, since that original and very enthusiastic description, *C. setense* has not been redescribed with modern morphological techniques. Moreover, as far as we know, it was mentioned in the literature until the work by Corliss (1979) but then it disappeared from most reviews on Ciliates provided over the last 20 years (Small and Lynn, 1985; Margulis et al., 1990; Carey, 1992; Lynn and Small, 2002), the only exceptions being the historical review by Aescht (2001) and Lynn (2003).

We collected *C. setense* twice during sampling in tidal pools, and we took the opportunity to gain familiarity with this fascinating, poorly-known ciliate which can be regarded as an example of the very likely hundreds of formerly described and later neglected protist species. Moreover, by adding the 18S rDNA sequence of this species to the literature molecular data, we intended at improving the general understanding of the intertaxal phylogenetic relationships of the class Heterotrichia. Finally, as Villeneuve-Brachon (1940) considered *Condylostoma* as the heterotrich genus phylogenetically closest to *Chattonidium* with respect to the gross morphology, we aimed at verifying her speculation presenting the first *Condylostoma* 18S rDNA sequence.

**Materials and methods.** Collection and culturing. *Chattonidium setense* was collected during the early springs (April) 1998 and 2001 from the same tidal pools (1–2m deep) near “Torre di Piscinni” (Cagliari, Italy) (38° 54′ 15.57″ N, 8° 46′ 40.67″ E). All the Ciliates were maintained for some time in lab in artificial 33 ppt seawater, at 19°C, on a 12:12h irradiance of 200µmol photons m⁻² s⁻¹. Many attempts were made to establish monoclonal cultures of *C. setense* by weekly addition of autotrophic diatoms (*Pheodactylum tricornutum*), autotrophic flagellates (*Dunaliella tertiolecta*), heterotrophic flagellates (*Chilomonas sp.*), or sterilized rice grain (the latter to support a continuous bacterial growth), which have been useful to obtain cultures of other heterotrichs sampled in the same pools (i.e. *Condylostoma* sp. and *Fabrea salina*). Unfortunately, none of these attempts succeeded with *C. setense* and the species disappeared some weeks later. Moreover, while the other cited heterotrichs were collected again in August and December of the same years, we were unable to find *C. setense* in the pools anymore.

*Condylostoma* sp. strain Poe2.2 was collected during August 2002 from “Poetto” beach (Cagliari, Italy) (39° 11′ 49″ N, 9° 09′ 39″ E). Cells were maintained in lab as previously described. In this case it was possible to establish a monoclonal strain feeding the organisms with *Pheodactylum tricornutum*.

**Live observations.** Ciliates were viewed using differential interference contrast (DIC) microscopy with a Leitz microscope, at 125–1250× magnification. To examine behavior, ciliates were placed in a Petri dish and observed under a Leica MS5 light microscope at 10–64× magnification.

**Fixation, staining, and electron microscopy.** Feulgen staining procedure was used to highlight the nuclear apparatus. For scanning electron microscopy (SEM) the specimens were fixed in 2% OsO₄ in 33 ppt artificial seawater for 10 min, stuck on slides previously covered with poly-L-lysine, and ethanol-dehydrated. Slides were then placed in the critical point drying apparatus and gold coated. For transmission electron microscopy (TEM), cells were fixed in a 1:1 mixture of 2% OsO₄ in 95% ethanol and 2.5% glutaraldehyde in 0.2M cacodylate buffer (pH 7.4) for 45 min; then they were ethanol-dehydrated, transferred to 100% acetone, and embedded in an Epon-araldite mixture. Thin sections were placed on copper grids, and stained with uranyl acetate and
lead citrate.

Measurements. The only literature data available on Chattonidium setense are based on in vivo measurements, while the meristic and morphometric data here presented are from both live and SEM processed specimens. For nuclear apparatus Feulgen stained cells were considered.

18S rDNA amplification and sequencing. For DNA extraction ~ 5 starved individuals were picked up with a micropipette and repeatedly washed in sterilized 33 ppt seawater to completely remove food contaminants. Specimens were then placed in a 0.2-ml PCR cap and fixed with 70% ethanol. To minimize salt concentration, after centrifugation, ethanol was removed, pellet was rinsed in 70% ethanol, and harvested again; then, ethanol was removed and specimens let dry. Caps were stored at −20°C. PCR mix was added directly into the cap at the moment of use.

The small subunit ribosomal RNA (18S rRNA) genes were amplified in a Primus 96 Plus PCR system (MWG-BIOTECH AG) using the universal eukaryotic primer forward 18S F9 Euro [5'-CTGGTTGTGATCCTGCCAG-3'] (Medlin et al., 1988) and reverse 18S R1513 Hypo [5'-TGATCCTTCYGCAGGTTC-3'] (Petroni et al., 2002). PCR products were purified from primers with Quantum Prep PCR Kleen Spin Columns (BIO-RAD) and directly sequenced in both directions by MWG-BIOTECH AG. To minimize the presence of the inappropriate tool and aligner from the ARB program package (Ludwig et al., 2004). The automated alignment was then corrected by hand to optimize the base-pairing scheme of the rRNA secondary structure using the appropriate tool of ARB package (Kumar et al., 2005). To perform phylogenetic analysis, 18S rRNA gene extremities of the selected species were removed after the shortest sequence (Loxodes magnus). Three filters were constructed to selectively remove or retain the more variable positions before performing the phylogenetic analysis. Filters Cili, nodot (1454 positions) and Cili, nodot (1603 positions) retain only positions conserved in at least 50% and 30% respectively of ciliates within a selection of 40 almost full-length ciliate sequences representative of all groups. Filter Cili, nodot retains all positions (1800). Phylogenetic analyses were performed using DNAPARS program for maximum parsimony (Felsenstein, 1989), DNADIST with Kimura (1980) correction for neighbor joining (Saitou and Nies, 1987), FastDNAML program for maximum likelihood (Felsenstein, 1981), as election of 40 almost full-length ciliate sequences representative of all groups. HKY (Hasegawa et al., 1985) and TN (Tamura and Nies, 1993). All programs are available from the ARB package (Ludwig et al., 2004). For each reconstruction method, the three filters were used. Fifteen trees were thus obtained and their topologies were compared to recognize stable nodes (Ludwig et al., 1998). Maximum parsimony tree stability was also warranted by bootstrap analysis.

Results. General morphology (Fig. 1). Chattonidium setense resembles a little bell (250 × 150μm), with a large, cut off anterior part bearing a huge, circular, slightly open peristome, an enlarged middle region, and a narrower posterior end with the orifice of the posteroaxial cavity we refer to thereafter as aboral cavity (AC) (Fig.1A). The oral ciliature (Fig.1B, C) comprises: 1) the adoral membranelles (AM), inserted all around the peristome at about 1.8μm from each other and consisting of 2 ciliary rows; 2) a long, single-row paraoral membrane (PO) which covers the internal wall of the huge peristome and of a cytoplasmic expansion
Table I. The complete list of the nucleotide sequences used for phylogenetic reconstruction in this study with their accession numbers and references

| Species                     | Accession number | Reference                        |
|-----------------------------|------------------|----------------------------------|
| *Blepharisma americanum*    | M97909           | Greenwood et al. 1991             |
| *Climacostomum virens*      | X65152           | Hammerschmidt et al. 1996        |
| *Chattonidium setense*      | AM295495         | This paper                       |
| *Condylostoma sp.*          | AM295496         | This paper                       |
| *Eufolliculina uhligi*       | U47620           | Hammerschmidt et al. 1996        |
| *Gruberia sp.*              | L31517           | Hirt et al. 1995                 |
| *Loxodes magnus*            | L31519           | Hirt et al. 1995                 |
| *Loxodes striatus*          | U24248           | Hammerschmidt et al. 1996        |
| *Peritomus kahli*           | AJ537427         | Rosati et al. 2004               |
| *Spirostomum ambiguum*      | L31518           | Hirt et al. 1995                 |
| *Stentor coerules*          | AF357145         | Zhu, Yu, and Shen unpublished    |
| *Stentor polymorphus*       | AF357144         | Zhu, Yu, and Shen unpublished    |
| *Stentor roeseli*           | AF357913         | Zhu, Yu, and Shen unpublished    |
| *Tracheloraphis sp.*        | L31520           | Hirt et al. 1995                 |
| *Euplotidium arenarium*     | Y19166           | Petroni et al. 2000              |
| *Lembadion bullinum*        | AF255358         | Strüder-Kypke et al. 2000        |
| *Urocenotrum turbo*         | AF255357         | Strüder-Kypke et al. 2000        |
| *Prorodon teres*            | X71140           | Stechmann et al. 1998            |
| *Pseudoplatyophrya nana*    | AF060452         | Lynn et al. 1999                 |
| *Obertrumia aurea* (as *Obertrumia Georgiana*) | X65149 | Bernhard et al. 1995            |
| *Phacodinium metchnikoffi*  | AJ277877         | Shin et al. 2000                 |
| *Protocruzia sp.*           | X65153           | Hammerschmidt et al. 1996        |

somewhat covering part of the peristome right margin. A distinct bundle of cilia (BC) is always present on the external side of this expansion. Somatic ciliation (Fig. 1C) consists of 29–30 kineties (SK) regularly arranged about 6 μm apart from each other. Each kinety bears thick cilia emerging for 5.5 μm out of the cell body. At the posterior end the kineties present 8–10 cilia about 10 μm long (i.e. nearly twice the other cilia) whose combination delimit a ∼32 μm in diameter circular zone, in the middle of which the aboral cavity orifice (AO) is visible surrounded by its own ciliation (Fig. 1D).

*Chattonidium* is highly contractile. The contraction shorten and widen the cell body that, apparently, runs along the spindle shaped, ∼90-μm long aboral cavity, which remains as a rigid, not deformable structure. As a consequence, the circular zone surrounding the aboral cavity orifice appears as sticking out with an ogival shape (Fig. 1E, F). The 10-μm long cilia of the kineties form now a sort of a
Fig. 1. A-G. General morphology of *Chattonidium setense*. A. Picture of a live specimen. Arrow indicates the aboral cavity. In the inset a specimen at an early stage of binary fission. Scale bars = 50 µm. B, C. SEM pictures of the apical region. AM, adoral membranelles; BC, bundle of cilia; PO, paraoral membrane; SK, somatic kineties. Scale bars = 10 µm. D, E. The aboral region in a relaxed state (D) and during contraction (E). AO, aboral orifice. Arrows indicate the crowns of longer cilia. Scale bars = 10 µm. F. A contracted specimen *in vivo*. Scale bar = 50 µm. G. The macronucleus (Ma). Scale bar = 50 µm.
crown at the ogive base, while shorter cilia are visible on its surface (Fig. 1E).

The elongated macronucleus (Ma) starts from the bottom of the cytostome, proceeds with a snake-like course and ends surrounding the aboral cavity (Fig. 1G).

**Fine structure (Figs. 2–4).** The cortex of *C. setense* is covered by the plasmamembrane underlain by thin, membranous alveoli subtended by subcortical microtubules (Fig. 2A). The ectoplasm, well divided from endoplasm by means of a thick layer of fibers, does not contain mitochondria while two kinds of extrusomes are present (see below). In the endoplasm numerous mitochondria and paraglycogen granules are visible, especially in the more external region. More deeply, large vacuoles are common (Fig. 2B).

**Extrusomes (Fig. 2C–F).** Extrusomes of the first type (Fs) are flask-shaped with a thin neck apparently empty and an enlarged belly (about 0.5 µm in diameter). In the belly a material with a foam-like appearance delimits an empty internal region that, according to the reconstruction by serial sections, is in continuity with the neck. These extrusomes are surrounded by a wavy membrane thickened by a dense material (Fig. 2C, D). During the extrusion the expanded foaming material is released (Fig. 2E). Second type extrusomes (Mu) appear as mucocysts and contain a compact dense material (Fig. 2D, F). They are elongated (0.7 µm long and 0.3 µm wide), occurring in the ectoplasm and in the cytoplasmic region, and surrounding the aboral cavity, where they are particularly abundant.

**Somatic ciliature (Fig. 2G–I).** The somatic kineties consist of dikinetids. Only the anterior kinetosome bear a cilium (Fig. 2G). The infraciliary pattern is as follows: a divergent, well developed postciliary ribbon (Pc), which extends posteriorly, and a laterally-directed fiber (LF), which apparently connects with the postciliary ribbon of the preceding dikinetid, are associated with the posterior kinetosome; a tangentially oriented transverse ribbon (T) is associated with the anterior kinetosome (Fig. 2H, I). A microtubular ribbon connects the two kinetosomes. (Fig. 2I). Very likely posterior and transversal ribbons together make up the kinetodesma (Kd) and through it are continuous with the subpellicular microtubules (Fig. 2A).

**Oral ciliature (Fig. 2J, K).** The membranellar dikinetids have a convergent postciliary ribbon, a tangential transverse ribbon, and fibers connecting the two kinetosomes (Fig. 2J). These ribbons apparently form bundles connecting all the kinetosomes of each membranelle and the membranelles one with the other. At the base of the kinetosomes a dense plate is present. From the plates of both kinetosomes of each dikinetid a ribbon of microtubules arises forming nematodesmata which join to nematodesmata of the adjacent membranelles. Nematodesmata also arise from the BC kinetosomes (Fig. 2K).

We could not definitely establish whether the nematodesmata join to form the conspicuous bundle of microtubules going, with a spiral-like course, toward the posterior end of the cell (Figs. 2B, 3A).

**Reserve substances and macronucleus (Fig. 3).** Polysaccharide reserves are in the form of paraglycogen granules of different size (Fig. 3A). In stationary phase, macronuclear chromatin forms a dense meshwork of thin, branched bodies, in which numerous, conspicuous nucleoli (Nu) are dispersed (Fig. 3B).

**The aboral cavity complex (Fig. 4).** The aboral cavity complex consists of the aboral cavity and the surrounding cytoplasm delimited by microtubules and fibers. The aboral cavity is closed at its apical end like a “cul de sac” (Fig. 4A) and its continuous wall consists of the whole cortex. The cross section of the cavity is roughly exagonal and each vertex is occupied by membranelles of two thick rows of cilia (Fig. 4B). Bundles of microtubules originate from the ciliary bases and surround the cavity (Figs. 3A, 4C). At the orifice level the cilia are closer to each other and are inserted following a spiral pattern (Figs. 1D; 4C, D). A layer of fibrils (F) separates the thin cytoplasm surrounding the aboral cavity from the remaining cytoplasm (Fig. 4B–D). This cytoplasmic region contains many mitochondria, paraglycogen granules, and extrusomes, mainly of the second type (Mu). On the whole, as it is clearly visible in semithin (2 µm-thick) sections at the optical microscope, the aboral cavity complex appears as a distinct body with a well defined shape (Fig. 4D inset).

**Behavioral notes.** The ciliates spend most of time swimming but, occasionally, they tend to stick to the substrate by their posterior end, from which they appear to spread “mucus”. Often, the substrate is represented by a single floating detritus particle to which 3–4 cells adhere forming small groups. This behavior lasts for a few minutes, then ciliates separate, going back to swimming.
SSU rRNA gene sequences and phylogenetic analysis (Figs. 5, 6). The almost complete 18S rRNA gene sequence of *Chattonidium setense* and *Condylostoma* sp. strain Poe2.2 were determined through the direct sequencing of PCR products; excluding primers, they are 1659 and 1652 bp long respectively. Their GC content is 46% and 47% respectively. In *C. setense* 18S rRNA sequence, one polymorphic site was observed in position 1275. In this position a U/C double pick was unambiguously present on both forward and reverse electropherograms.

In a preliminary phylogenetic analysis, we added
Fig. 3. A-B. Fine structure by TEM. A. Cytoplasmic features. Arrows indicate the numerous paraglycogen granules. Scale bar = 0.5 μm. B. Macronuclear organization. Nu, nucleoli. Scale bar = 1 μm.
C. setense and Condylostoma sp. strain Poe2.2 sequences to an ARB database comprising all available 18S rRNA ciliate sequences using interactive parsimony (Ludwig et al., 2004). As expected, both sequences branched within the Heterotrichia. A deeper phylogenetic analysis focused on Heterotrichia was then performed with a dataset of sequences comprising all Postciliodesmatophora (Heterotrichia plus Karyorelictea) and, as outgroup, a selection of Intramacronucleata belonging to different classes. To reduce the possibility of long-branch attraction artifacts, slow-evolving outgroup was selected (Philippe, 2000).

In all calculated trees, a steady association of C. setense and Condylostoma sp. strain Poe2.2 is observed. This clade represents an independent evolutionary path within Heterotrichia; associations with already characterized organisms are not unambiguously resolved as evidenced by the multifurcation present in TREEPUZZLE trees (e.g. Fig. 6) and the low bootstrap values of parsimony trees (e.g. Fig. 5). Other stable, highly supported associations among different genera are represented by: 1) Maristenor and Eufolliculina; 2) Maristenor, Blepharisma, Stentor, and Eufolliculina. Also the association between Blepharisma and Stentor is generally recovered. These results are in accordance with previous publications (Rosati et al., 2004; Miao et al., 2005). Although Peritromus kahli was generally branching basally within Heterotrichia, this position was not recovered in all calculated trees and sometimes it branched within the multifurcation (data not shown). The positioning of remaining species, Climacostomum virens, Spirostomum ambiguus, and
Fig. 5. Phylogenetic relationships within the class Heterotricha based on 18S rRNA gene sequences. Phylogenetic analysis performed by DNAPARS program for maximum parsimony (Felsenstein 1989) and limited to 1454 positions. A proper selection of slow evolving 18S rRNA gene sequences belonging to the subphylum Intramacronucleata was chosen as outgroup (dashed box). Numbers at nodes represent the bootstrap values out of 1000 replicates. Lengths of the branches are informative and calculated using the “calculate branch lengths” option in the ARB software package (Ludwig et al. 2004). The scale bar corresponds to 10 substitutions per 100 nucleotide positions. The association of *Chattonidium* and *Condylostoma* is highly supported.

*Gruberia* sp. was even more ambiguous. The association between *C. virens* and *S. ambiguum* was observed only in few TREEPUZZLE trees (Fig. 6).

**Discussion.** To take up the recognition or the redesription of a ciliate species by the modern techniques involves a certain amount of problems. Indeed many species have been described during the first half of the last century by light microscopic observation and only illustrated by more or less defined drawings. Moreover the same parameters have not always been considered by different authors and often some likely corresponding features have been reported with different names or differently interpreted. Thus, misinterpretation are possible: the same species can be reported with different names or different species can be interpreted as a single one. In any case, these old studies, based on a patient, descriptive work, have to be always taken into consideration and represent a priceless starting point for modern systematic and phylogenetic analyses. Nevertheless, some species, probably rare or with specific ecological niches, once described in the old literature have then been almost completely neglected in successive studies. This is the case of *Chattonidium setense* described for the first time by Villeneuve in 1937 (in Fig. 7 the original drawing by Villeneuve, 1937 is reported like it was later reissued in the paper Villeneuve-Brachon, 1940). The peculiar characteristic of this species, namely the aboral cavity (posteroaxial cavity according to Villeneuve, 1937), that led the author in 1940 to establish the family Chattonidiidae with a single genus and a single species, excludes the possibility of an erroneous determination of the species already by *in vivo* observation. The multidisciplinary study we performed by morphological and molecu-
lar approaches confirms the inclusion of *Chattonidium* in Heterotrichia. Similarities in cortical structures and cytoplasmic organization, between *Chattonidium* and representative members of the class are remarkable. The kinetid organization of *Chattonidium* is in accordance with the general scheme reported by Lynn (1981, 1991) for heterotrichs. *Condylostoma* (Bohatier, 1978) presents the organization of infraciliary structures most similar to that of *Chattonidium*, but differs from the latter as in its dikinetids both kinetosomes are ciliated. Indeed, a certain intertaxal variability within this general scheme does exist in Heterotrichia (Rosati et al., 2004). Also the cytoplasmic organization, with a well distinct ectoplasm devoid of mitochondria and the endoplasm with an internal vacuolized region are very similar in the two species. In a number of heterotrich ciliates the elements of contraction and relaxation are represented by the overlapping ribbons of postciliary microtubules (Km fiber) and by the subcortical fibrillar network, also reported as myoneme (Yogosawa-Ohara and Shigenaka, 1985). In *Chattonidium* a typical subcortical myoneme is not visible; the fibrillar network separating the ectoplasm from the endoplasm is probably involved in contraction.

Also the organization of the macronuclear chromatin shows remarkable similarities in *Chattonidium* to that in other heterotrichs, and polysaccharidic reserve substances are in the form of paraglycogen as in other members of the class analyzed so far (Rosati et al., 2004).

**Extrusomes.** Simple extrusomes in the form of subcortical vesicles or granules are very common and diversified in Heterotrichia where, however, they play different roles in relation to the different content, while differentiated extrusomes are rare (Rosati and Modeo, 2003). Only in *Condylostoma magnum* rod-shaped bodies, with a complex structure (Rosati and Verni, 1984) and in *Peritromus kahli*
small trichocyst-like bodies (Rosati et al., 2004) have been reported. The two kinds of extrusomes found in Chattonidium can, in our opinion, be both included in the heterogeneous group of extrusomes commonly referred to as “mucocysts”. They differ from each other in dimension, morphology, appearance of their content, and distribution in the cell; thus, very likely, they meet different requirements of the organism.

**Aboral cavity complex.** This structure, well described at the light microscopical level by Villeneuve (1937) and Villeneuve-Brachon (1940), remains a unique feature of Chattonidium.

Not only do electron microscopical data confirm morphological observations of the previous author, but they also support her functional considerations. The aboral cavity has nothing to do with the contractile vacuole of other ciliates. It is delimited by the whole cortex and is surrounded by a particular cytoplasmic region which contains mitochondria (like the endoplasm) and extrusomes (like the ectoplasm). These extrusomes, mainly Mu extrusomes, probably provide the apparently mucous substance by which the ciliates adhere to the substrate or temporary form little groups. It can be hypothesized that the movement of the six membranelle-like ciliary structures in the cavity lumen propels the mucus toward the orifice, where the closer cilia condense the substance forming the “long fil” described by Villeneuve-Brachon (1940). Very likely the subcortical microtubules, originated by the ciliary bases, contribute to maintain open the lumen of the cavity and rigid the cytoplasm of the aboral cavity complex, while the fibers separating this cytoplasm from the remaining cytoplasm, contract the latter. Thus, the aboral cavity complex maintains its shape independently from cell contraction.

**Phylogenetic analysis.** The comparative analysis of the 18S rRNA gene sequences indicates that Chattonidium and Condylostoma actually belong to Heterotricha and are related to each other as correctly supposed by Villeneuve-Brachon (1940) on a morphological basis. The association between the two organisms is present, and highly supported, in all calculated trees (they branch together in all calculated trees forming a highly supported clade). Despite the increased species sampling, phylogenetic resolution within Heterotricha does not increase respect to previous studies (Rosati et al., 2004; Miao et al., 2005). In detail, herein we reconfirm the associations of: 1) Stentor and Blepharisma; 2) Maristensor and Eufolliculina; 3) Stentor, Blepharisma, Maristensor, and Eufolliculina (referred to as “stentorid clade” in Miao et al., 2005). Also the basal positioning of Peritromus is generally retrieved (Rosati et al., 2004). On the other side, branching positions close to the base of Heterotricha remain poorly resolved as graphically evident from TREEPUZZLE tree reconstructions, where a multifurcation generally occurs (Fig. 6). Although the association of Spirostomus and Climacostomum is observed in some trees (TREEPUZZLE program with more conservative filters and e.g. Fig. 6), in others they branch independently (DNADIST, DNAPARS and FastDNAni programs, and e.g. Fig. 5). In general, phylo-
genetic resolution of branching patterns at the base of Heterotrichea will need a wider sampling of taxa and/or the use of a different molecular phylogenetic marker.

Conclusions. Ultrastructural and molecular results here presented indicate that *Chattonidium* belongs to the class Heterotrichea defined according to the modern taxonomy, and confirm its re-

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