Focusing on Genera to Improve Species Identification: Revised Systematics of the Ciliate *Spirostomum*

Vittorio Boscaro[a,*], Daniela Carducci[a], Giovanna Barbieri[a], Marcus V.X. Senra[b], Ilaria Andreoli[a], Fabrizio Erra[a], Giulio Petroni[a], Franco Verni[a], and Sergei I. Fokin[a,c]

[a]Università di Pisa, Dipartimento di Biologia, Unità di Zoologia-Antropologia. Via A. Volta 4/6, 56126 Pisa Italy
[b]Universidade Federal de Juiz de Fora, Laboratório de Protozoologia, Brazil
[c]St.-Petersburg State University, Department of Invertebrate Zoology, Russia

Although many papers dealing with the description of new ciliate taxa are published each year, species taxonomy and identification in most groups of the phylum Ciliophora remain confused. This is largely due to a scarcity of surveys on the systematics of immediately higher levels (genera and families) providing data for old and new species together. *Spirostomum* is a common and distinctive inhabitant of fresh- and brackish water environments, including artificial and eutrophic ones, and is a good model for applied ecology and symbiosis research. Despite this, only 3 of the numerous species are commonly cited, and no studies have yet confirmed their monophyly, with the consequence that reproducibility of the results may be flawed. In this paper we present morphological and molecular data for 30 *Spirostomum* populations representing 6 different morphospecies, some of which were collected in previously unreported countries. We performed a detailed revision of *Spirostomum* systematics combining literature surveys, new data on hundreds of organisms and statistical and phylogenetic analyses; our results provide insights on the evolution, ecology and distribution of known morphospecies and a novel one: *Spirostomum subtilis* sp. n. We also offer tools for quick species identification.

Keywords: Feulgen staining; Heterotrichea; ITS; macronucleus type; SSU rRNA; 18S rRNA.

Introduction

Ciliate taxa have been described for almost two centuries, while the theoretical background underlying their understanding changed so much that organisms once described as “perfect” (multicellular) animalcules (Ehrenberg, 1838, Fokin, 2004) are now known to be unicellular and more closely related to some algae than to animals (Adl 2012). Classes and orders were melted, recombined and renamed numerous times, following different criteria (Lynn 2008). The molecular revolution set new standards for characterizations, and a constantly increasing number of papers describing new species are published each year (about 20 new species and 4 new monospecific genera were established only in 2013; e.g. in Chantangsi et al., 2013, Foissner, 2013, Modeo et al., 2013a, Pan et al., 2013a, Pan et al., 2013b, Pan et al., 2013c, Park et al., 2013). Despite this, a huge confusion still reigns in ciliate taxonomy of low-level taxa. Fewer works deal with the redescription of old species (e.g. Modeo et al. 2013b), and even less with the integrated systematics of genera – except for a handful of flagship, intensely studied ones, like *Paramecium*, *Tetrahymena* or *Euplotes* (see for example Boscaro et al., 2012, Chantangsi and Lynn, 2008, Kher et al., 2011, Petroni et al., 2002, Strüder-Kypke et al., 2000). Many old descriptions are so vague that it is virtually impossible to compare new data with the original ones, but because of nomenclatural rules names are hard to dismiss, and pursuing the goal of invalidating them is often not worth the effort. Thus, new species names appear, old ones remain, and confusion increases.

And yet, all applied studies relying on correct species identification (ecology, molecular biology, etc.) would greatly benefit from unambiguous species naming, description and guide to identification. Although the discovery of new species is certainly appealing and informative, the immediately higher hierarchical levels, like genera and families, should be more often

*Corresponding author, fax: +39 502211393 e-mail vboscaro@biologia.unipi.it (V. Boscaro).

reconsidered in order to maintain a holistic view. Integrating new morphological and molecular data from several closely related morphospecies together with a thorough survey of the literature is in fact the best way to detect discrepancies and to better assess biodiversity.

The genus *Spirostomum* Ehrenberg, 1834 is a good example of a weak systematic framework's potential issues. *Spirostomum* representatives are large ciliates of the class Heterotrichea with an eye-catching, distinctive shape; they are common in freshwater and low salinity (brackish) environments, sometimes in high abundances (Bradley et al., 2010, Finlay and Esteban, 1998). They are valuable targets of ecological studies, and some species are often claimed to be good bioindicators for a number of threats to the environment's quality (Nałecz-Jawecki and Sawicki, 1999, Twagilimana et al., 1998). Also, more recently they were investigated as hosts of prokaryotic (Fokin et al., 2005, Schrallhammer et al., 2013, Vannini et al., 2014) and eukaryotic (Esteban et al. 2009) symbionts. Nevertheless, only three species are commonly reported in recent works, against the dozen present in literature. Several papers presented molecular data without morphological descriptions (Hirt et al., 1995, Schmidt et al., 2007b, Schrallhammer et al., 2013, Vannini et al., 2014), or vice-versa (Jang et al. 2012). To our knowledge, only the paper of Fernandes and da Silva Neto (2013) provided linked morphological and molecular data, but only for two species (represented by one population each). Morphological characters employed to define morphospecies are not numerous, and their variability range somewhat differs according to different sources. Finally, *Spirostomum* poses specific obstacles to detailed morphological studies: it is highly contractile and does not always adapt to growth in laboratory conditions, making the culturing of monoclonal strains unpractical.

In this work, we present and discuss morphological and molecular data on 30 *Spirostomum* populations belonging to 5 known morphospecies and a novel species. The populations were collected in several continents, including previously uninvestigated tropical and northern countries. We provide a survey of the genus based on our newly collected data on hundreds of organisms as well as a critical interpretation of literature reports, providing insights on the variability, phylogeny, evolution and distribution of these ciliates. We took particular care to avoid the proliferation of new species names and to revive instead those already present in older literature and reported here again. We also identified a few easily observable key characters for each morphospecies, in order to facilitate identification for non-taxonomists.

Results

Morphological Observations

Twenty two out of the 30 sampled populations analyzed in this work were numerous enough to be subjected to quantitative morphological analysis. Parameters were measured on about 450 living and 420 stained cells (resulting in more than 3,000 and 1,700 raw data, respectively). The main morphological data are summarized in Table 1; a more comprehensive dataset is shown in Supplementary Material Table S1.

In vivo observations. The worm-like appearance typical of *Spirostomum*, with a long peristomial field parallel to the main body axis and a posteriorly located contractile vacuole (CV), is a feature shared by all populations (Fig. 1A-F). Despite the importance sometimes attributed to this character's variability, we observed uniformity in the shape of extremities, the anterior one usually being “rounded” and the posterior one “truncated”. Quantitative parameters generally vary along a smooth gradient between extreme values, that can be very far apart (especially for cell length). Exceptions are the number of kineties, which is significantly higher in population SS4-2 than in the others, and the CV length: cell length ratio, which strongly departs from the average value only in a few populations (PF1a, SS4-1).

Population name	Cell length (µm)	Cell length/width	Peri./length (%)	CV/length (%)	Kineties number[ab]	CG rows[b]	CG pattern	MAC shape	MAC nodules	MAC Length[c] (mm)	MAC length/width[c]	MIC number	MIC size (µm)	
Mdg2-1	**278** ±32	**8.9** ±1.3	**39** ±4	**16** ±4	**7.0** ±1.2	**3.6** ±0.6	Homogeneous	Ellipsoid	**1.0** ±0.0	**29.2** ±3.4	**4.1** ±1.2	**1.3** ±0.5	**1.9** ±0.2	***Spirostomum***
Seef1	**252** ±30	**8.0** ±1.6	**43** ±5	**17** ±5	**6.1** ±0.5	**3.2** ±0.4	Homogeneous	Ellipsoid	**1.0** ±0.0	**25.9** ±3.3	**3.4** ±0.9	**1.4** ±0.7	**1.9** ±0.3	***Spirostomum***
StMgN	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.
Nor_BG	**315** ±54	**7.9** ±1.8	**44** ±5	**15** ±6	**7.9** ±1.3	**4.3** ±0.6	Homogeneous	Ellipsoid	**1.0** ±0.0	**28.8** ±2.9	**2.2** ±0.5	**1.8** ±0.8	**2.0** ±0.2	***Spirostomum***
Nor_KD	**346** ±55	**11.4** ±2.3	**41** ±5	**17** ±3	**8.2** ±1.5	**2.9** ±0.7	Homogeneous	Ellipsoid	**1.0** ±0.0	**41.1** ±10.3	**4.6** ±2.3	**1.1** ±0.3	**2.0** ±0.4	***Spirostomum***
PFEU3_Sm2	**336** ±64	**10.5** ±2.6	**44** ±7	**15** ±4	**7.4** ±1.3	**3.7** ±0.5	Homogeneous	Ellipsoid	**1.0** ±0.0	**38.5** ±6.2	**3.8** ±1.0	**2.1** ±0.8	**2.1** ±0.3	***Spirostomum***
IP1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.
StJFp2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.
PFEU1_Spte	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.
PF1a	**366** ±39	**9.8** ±1.0	**42** ±4	**27** ±7	**9.1** ±0.8	**2.0** ±0.0	Heterogeneous	Ellipsoid	**1.0** ±0.0	**28.8** ±4.4	**2.8** ±0.6	**1.2** ±0.4	**2.2** ±0.3	***Spirostomum***
LarnCyp	**297** ±36	**7.9** ±1.5	**45** ±5	**16** ±5	**7.4** ±1.0	**4.1** ±0.7	Homogeneous	Elongated	**1.0** ±0.0	**44.7** ±8.5	6.1 ±1.1	**1.3** ±0.6	**1.9** ±0.1	***Spirostomum***
SpirWS	**389** ±73	**10.2** ±2.3	**48** ±4	**12** ±5	**9.8** ±1.1	**2.5** ±0.7	Homogeneous	Elongated	**1.0** ±0.0	**51.0** ±9.9	**6.7** ±1.5	**2.3** ±1.2	**1.8** ±0.3	***Spirostomum***
GNS4	**370** ±41	**9.9** ±1.9	**53** ±4	**16** ±6	**9.4** ±0.5	**3.6** ±0.5	Homogeneous	Elongated	**1.0** ±0.0	**69.8** ±18.2	**9.0** ±3.3	**1.9** ±1.2	**2.0** ±0.4	***Spirostomum***
Pozz	**360** ±64	**12.1** ±1.9	**40** ±4	**17** ±8	**7.4** ±0.9	**3.3** ±0.5	Homogeneous	Elongated	**1.0** ±0.0	**42.6** ±3.7	**5.0** ±1.2	**2.9** ±1.0	**1.7** ±0.3	***Spirostomum***
SAd	**406** ±58	**11.0** ±1.8	**55** ±8	**16** ±6	**9.7** ±1.6	**3.8** ±0.4	Homogeneous	Filiform	**1.0** ±0.0	**137.9** ±20.4	**21.8** ±5.7	**2.9** ±1.5	**1.5** ±0.2	***Spirostomum dharwarensis***
Mdg3	**449** ±92	**12.1** ±3.2	**47** ±5	**17** ±6	**9.2** ±1.9	**4.1** ±0.4	Homogeneous	Ellipsoid	**1.0** ±0.0	**26.8** ±4.7	**2.5** ±0.7	**1.4** ±0.7	**1.8** ±0.2	***Spirostomum***
Mdg2-2	**411** ±58	**9.1** ±1.3	**49** ±4	**13** ±3	**8.7** ±1.1	**3.5** ±0.6	Homogeneous	Moniliform	**15.7** ±2.9	**19.7** ±4.9	**3.4** ±0.7	**5.5** ±1.6	**1.8** ±0.2	***Spirostomum***
Mdg4	**515** ±99	**13.1** ±3.1	**51** ±3	**14** ±3	**8.1** ±1.3	**2.9** ±0.4	Homogeneous	Moniliform	**13.8** ±3.7	**29.9** ±10.8	**4.5** ±1.4	**2.1** ±1.4	**1.8** ±0.2	***Spirostomum***
SmJFp1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.
Thd2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.
Gmn	**518** ±121	**11.0** ±2.1	**44** ±4	**17** ±8	**8.6** ±1.5	**3.0** ±0.5	Homogeneous	Moniliform	**12.8** ±4.4	**28.0** ±3.1	**3.0** ±0.8	**2.2** ±1.2	**2.7** ±0.6	***Spirostomum***
Ind	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.
SAm	**561** ±67	**12.2** ±2.5	**52** ±6	**12** ±2	**9.7** ±1.1	**3.7** ±0.6	Homogeneous	Moniliform	**16.9** ±3.2	**17.3** ±3.8	**4.2** ±1.1	**2.9** ±1.5	**2.7** ±0.5	***Spirostomum***
SmPS	**421** ±47	**9.8** ±2.0	**51** ±4	**16** ±2	**9.4** ±1.4	**3.9** ±0.3	Heterogeneous	Moniliform	**9.4** ±3.4	**29.3** ±6.4	**2.7** ±0.7	**3.2** ±1.1	**1.9** ±0.2	***Spirostomum***
PBG1	**558** ±74	**9.5** ±2.1	**53** ±6	**11** ±3	**10.0** ±1.6	**3.7** ±0.6	Heterogeneous	Moniliform	**13.8** ±2.5	**24.0** ±3.9	**3.2** ±1.0	**1.8** ±1.2	**2.2** ±0.5	***Spirostomum***
SS5	**816** ±86	**13.4** ±2.1	**51** ±4	**11** ±2	**9.9** ±0.9	**3.9** ±0.3	Heterogeneous	Moniliform	**14.2** ±2.7	**25.8** ±5.0	**2.5** ±0.7	**11.5** ±3.2	**2.1** ±0.3	***Spirostomum***
SS4-1	**955** ±132	**20.9** ±3.2	**42** ±4	**25** ±6	**9.5** ±0.6	**1.0** ±0.0	Homogeneous	N/A	N/A	N/A	N/A	N/A	N/A	***tomum subtilis* sp. n.**
Zur3	**837** ±98	**17.3** ±3.3	**53** ±5	**17** ±7	**11.2** ±0.7	**1.0** ±0.1	Homogeneous	Moniliform	**20.4** ±3.0	**17.3** ±4.8	**3.1** ±0.8	**3.1** ±1.5	**2.9** ±0.4	***tomum subtilis* sp. n.**
SS4-2	**1,230** ±180	**12.7** ±2.5	**61** ±5	**9** ±2	**21.5** ±2.5	**4.2** ±0.4	Heterogeneous	Moniliform	**21.0** ±3.1	**35.0** ±7.1	**4.0** ±0.8	**9.4** ±4.7	**1.6** ±0.2	***Spirostomum ambiguum***
Seef2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	*Spirostomum* sp.

Table 1. Selected features of the 30 investigated *Spirostomum* populations.

[a]Inferred from the number of CG stripes

[b]Counted in the middle sector of the cells

[c]Taken for the biggest nodule for moniliform MACs

Peri., Peristome; **CV,** Contractile Vacuole; **CG,** Cortical Granules; **MAC,** macronucleus; **MIC,** micronucleus

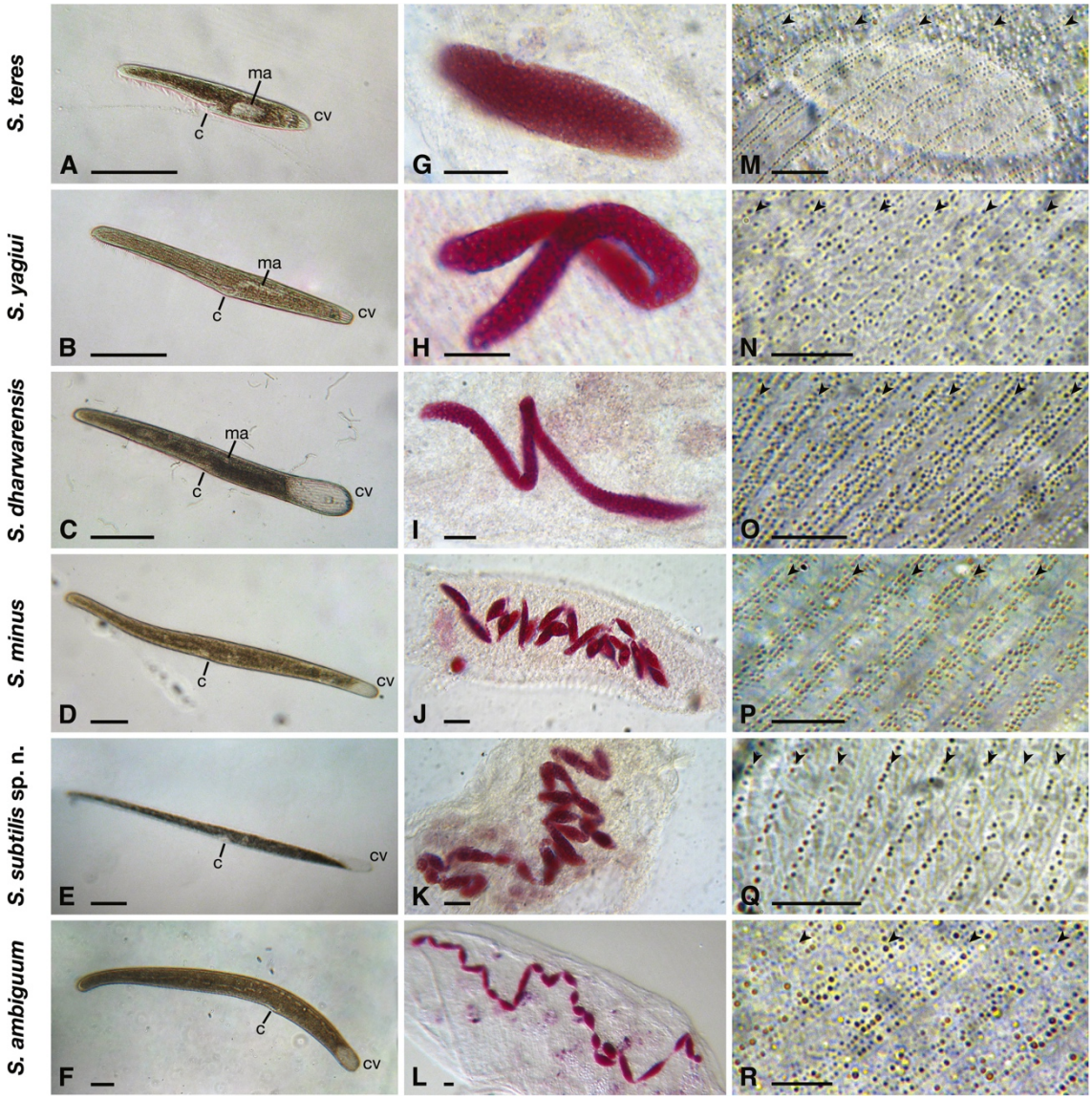

Figure 1. Morphological features of *Spirostomum* morphospecies. For each species, pictures taken with a differential interference contrast microscope show representative living cells (**A-F**), Feulgen-stained macronuclei (**G-L**) and cortical granule (CG) patterns in vivo (**M-R**). The novel morphospecies *Spirostomum subtilis* is particularly thin, often with a large contractile vacuole (E); these characters, and the cytostome position, differentiate it from *S. ambiguum* (F). *S. subtilis* may be distinguished from all other species, and in particular from the longest *S. minus* populations, because of its unique CG pattern (Q). Arrowheads mark the interkinetal CG stripes consisting of several CG rows in all species except *S. subtilis*, where a single row per stripe is visible. *S. teres* is represented by population PFEU3_Sm2, *S. yagiui* by population GNS4, *S. dharwarensis* by population SAd, *S. minus* by population SAm, *S. subtilis* sp. n. by population Zur3 and *S. ambiguum* by population SS4-2. Bars stand for 100 μm (A-F) and 10 μm (G-R). c, cytostome; ma, macronucleus (only "single"-type macronuclei are labeled in living cells); cv, contractile vacuole.

Cortical granules (CG) are arranged in stripes that run parallel to the main body axis. There is always a single stripe between each kinety pair, but it may include one or (more often) several CG rows (Fig. 1M-R); variation in number of rows for each stripe is almost as large within populations as among different populations. This character may vary slightly in different sectors (anterior, median and posterior) as well as different stripes in the same cell (see Supplementary Material Table S1). It is also possible that the CG pattern depends in some degree to the organism's physiological status, because on rare occasions CG rows were almost invisible, despite being clearly detectable in the same population during other observations. Nevertheless, most populations showed 2 or more CG rows in each stripe; the exceptions were populations Zur3 and SS4-1, which virtually never departed from their usual pattern consisting of a single CG row per stripe (Fig. 1Q). The "homogeneous" or "heterogeneous" CG pattern types refer to the occasional presence of granules with different size and/or color in the heterogeneous patterns.

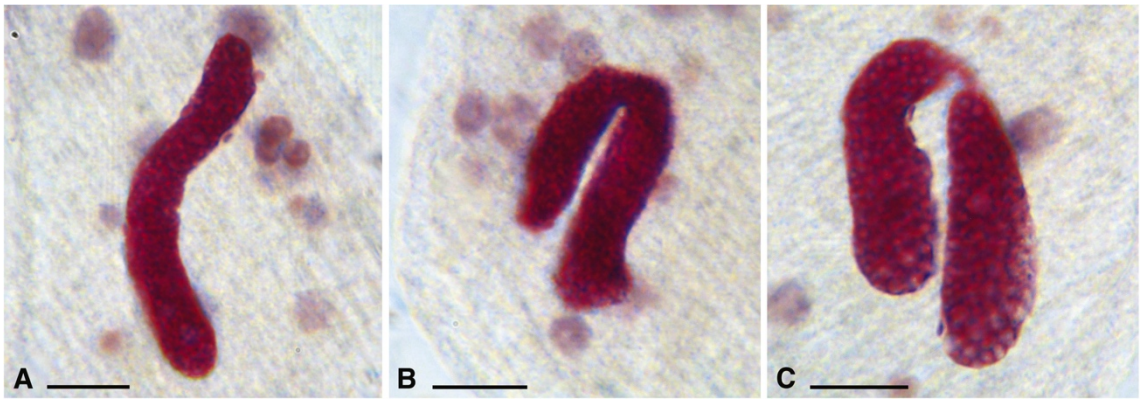

Figure 2. The three variants of the "elongated" type macronuclei. **A,** rod-like; **B,** convoluted (see also Fig. **1H);** C, dividing. Pictures were taken on S. *yagiui* population SpirWS. Bars stand for 10 µm.

Discriminant function analysis performed on living observations strongly differentiated only some of the populations (Supplementary Material Fig. S1A). In particular, SS4-2 on one hand and Zur3 and SS4-1 on another are clearly separated from the bulk of other populations. The remaining 19 are arranged in a continuous multidimensional gradient, with several populations bridging those with more extreme features.

Feulgen observations. Macronuclear (MAC) morphotypes can be divided into two main categories: moniliform (formed by a chain of beads, the MAC nodules; Fig. 1J-L) and single (Fig. 1G-I). The number of nodules in moniliform MACs vary among populations. On the contrary, variation in nodule shape seems to be more related to the organism's condition: ovoid, elongated and spindle-shaped nodules can usually be found in the same population or even cell. The degree of connection between adjacent nodules (from none to thick channels) is also fairly unstable within each population. The "single" MAC morphotype can be further divided in ellipsoid (length: width ratio < 5, Fig. 1G), elongated (length: width ratio > 5, Fig. 1H) and filiform (length: width ratio > 20, Fig. 1I). While the ovoid and filiform MACs show relatively uniform shapes, the elongated MACs are more diverse. This MAC type is only found in the littoral or brackish populations LarnCyp, SpirWS, GNS4 and Pozz, and in all of them it is present in three variants: rod-like, convoluted and dividing (Fig. 2). It is likely that these shapes are associated to different stages of the cell-cycle.

Micronuclei (MICs) vary in number and, to a lesser extent, size. MIC shape is ovoid or ellipsoid, and MICs are always close to MACs; they tend to be located in more or less deep matching depressions in the "single" type MACs. It is important to note that MIC number might be underestimated: it is possible that some get hidden below MACs in Feulgen pictures, because of their smaller size.

Discriminant function analysis on Feulgen observations (Supplementary Material Fig. S1B) differentiated more groups than the analysis of in vivo data. Populations with a moniliform MAC are clustered, with SS4-2 at one extreme of the gradient and SmPS at the other; another group includes populations with ellipsoid or elongated MACs, the latter being more scattered; population SAd is completely separated in virtue of its unique filiform MAC. The two main discriminant functions are both heavily influenced by the number and length of MAC nodules.

Molecular Sequences

The almost complete 18S rRNA gene sequences obtained during this work were 1702 bp long, and did not require the introduction of gaps during alignment; some previously released

Spirostomum sequences slightly differ in this respect, exhibiting a few indels in very short homopolymeric regions. The lowest similarity value shared by all *Spirostomum* sequences is 97.5% (comparing it with those of other heterotrich genera represented by several sequences, the value is similar to the 96.1% of *Stentor* and the 97.3% of *Blepharisma*, but it is higher than the 90.7% of *Condylostoma*).

The ITS1 + 5.8S + ITS2 + 28S region amplified is 662-674 bp long, contains short regions with several indels and it is more variable than the 18S gene region: the lowest identity value for all *Spirostomum* sequences is 89.5%; there is no other extensive set of data for this marker in any other heterotrich genera to compare this value with.

Phylogeny

In all 18S rRNA gene trees, *Spirostomum* strongly clusters within Heterotrichea (Fig. 3). As in other analyses (Miao et al., 2009, Schmidt et al., 2007a), only a few clades above the genus level are resolved, like the "crown-group" formed by *Stentor*, *Blepharisma*, *Maristentor*, *Fabrea* and folliculinids. The relationships among other heterotrich lineages are only weakly supported. Nevertheless, *Spirostomum* sequences never associate with the only sequence of the confamiliar genus *Gruberia* (accession number: L31517; Hirt et al. 1995). The most closely related sequence, instead, belongs to *Anigsteinia* (accession number: HM140405; unpublished), which according to traditional taxonomy should be a close relative of *Blepharisma* (Lynn 2008). However, no morphological observation was provided together with the *Gruberia* and *Anigsteinia* sequences.

All inferred 18S rRNA gene trees displayed 7 highly homogeneous and strongly supported subgeneric clades (Fig. 3). The general topology linking these clades is also constant in all trees with a single exception: the "*subtilis*" clade clusters with the "*ambiguum*" + "*minus* 1" + "*minus* 2" group in Bayesian trees, but it is the sister group of all other *Spirostomum* lineages in Maximum Likelihood trees. Nodes hierarchically above the 7 clades are supported by low statistical values even when they are present in all trees, and are thus relatively unreliable; the noteworthy exception is the association of clades "*teres* 1", "*teres* 2" and "brackish". Sequence identity values within clades are 99.4-100% in the 18S rRNA gene region (*Stentor* morphospecies exhibit similar values: 99.5-100%) and 95.0-100% in the ITS1 + 5.8S + ITS2 + 28S region.

The topology of the Bayesian tree based on the entire rRNA gene cluster sequenced (Fig. 4A) shows similar associations among the populations, but the clade "*teres* 1" is further split in separated lineages. The morphology-based Bayesian tree (Fig. 4B) has a slightly different topology. All populations of the molecular "*teres* 1" and "*teres* 2" clades, plus population Mdg3 of the "brackish" clade, are grouped. Population SS5 shows a substantial divergence from other "*minus* 2" populations, and the "*minus* 1" clade is split; also, the "*minus*" clades do no longer cluster together. Fewer nodes are strongly supported by statistical values in the morphological phylogenesis. The combined tree (Fig. 4C) is generally in accordance with molecular ones, but the position of population SAd within the "brackish" clade is different.

Discussion

Morphospecies Identification

Populations for which we obtained only molecular data were conservatively labeled as "*Spirostomum* sp.". We classified the other 22 populations in morphospecies according to the most recent reviews (Foissner et al., 1992, Repak and Isquith, 1974) and the original descriptions.

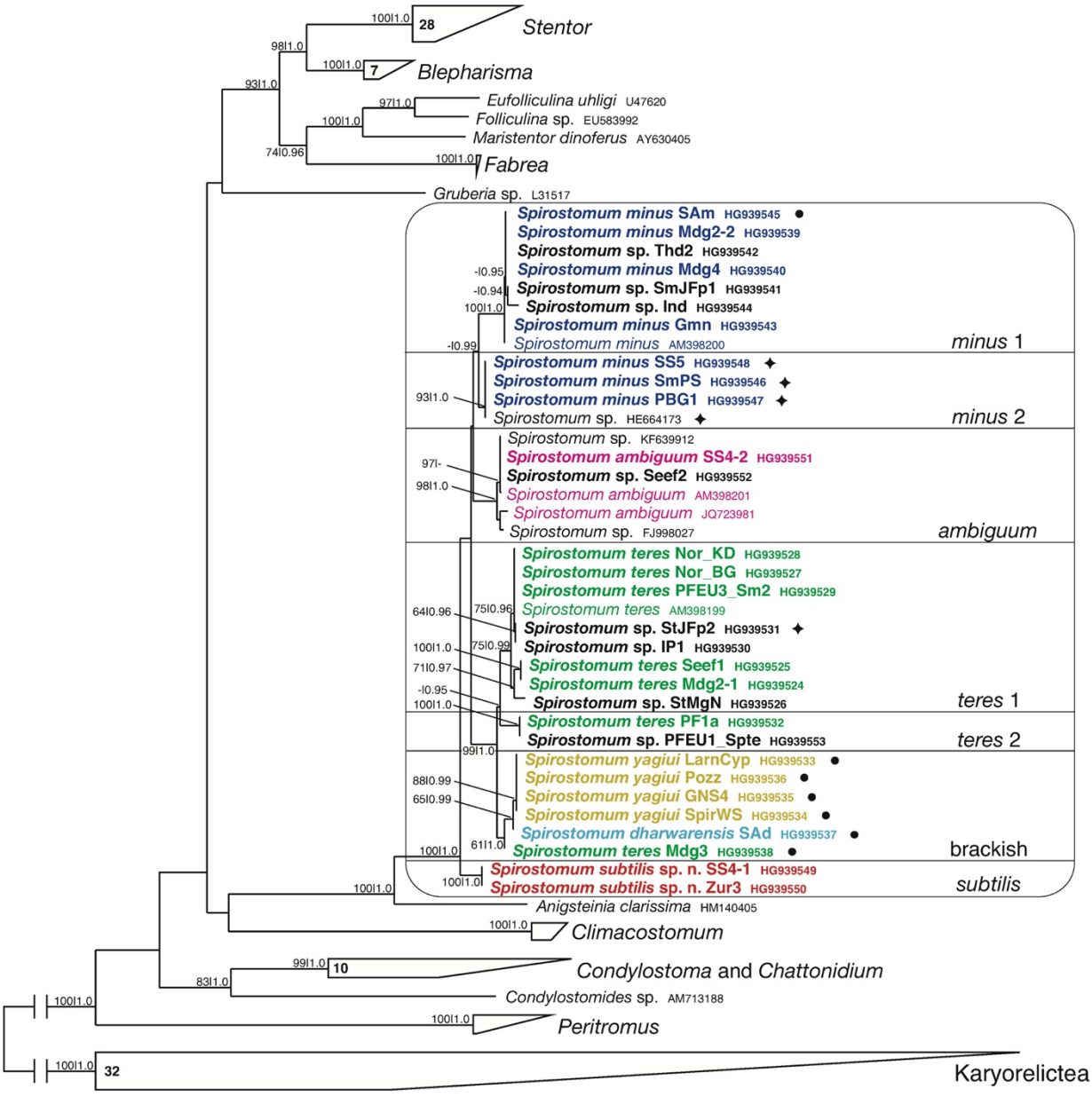

Figure 3. 18S rRNA gene Maximum Likelihood tree of Heterotrichea, with focus on the genus *Spirostomum.* The analysis was performed on character matrix a (see text). Sequences obtained in this work are in bold. Numbers associated with nodes represent bootstrap|posterior probability values; bootstrap values below 60 and posterior probability values below 0.90 were omitted. Numbers in trapezoids show the number of sequences (if more than 2) representing clades collapsed for clarity (see also Supplementary Material Fig. S2). Dark circles indicate populations collected in brackish, littoral or estuarine environments; diamonds indicate populations harboring bacterial symbionts. The bar stands for an estimated divergence of 10%.

Repak and Isquith (1974) recognized 9 valid *Spirostomum* morphospecies. *Spirostomum inflatum*, *Spirostomum loxodes* and Spirostomum *caudatum* are characterized by conspicuous features related to cell shape (respectively, a dilated posterior half, a laterally-oriented anterior "beak" and a long and thin "tail") that were never observed in our populations. Moreover, *S. inflatum* and *S. loxodes* did not appear in published papers after their first description, and are thus either extremely rare, or were originally misidentified (Kahl himself suggested that *S. inflatum*, which he described as a novel taxon in 1932, could be an aberrant form of another species). *Spirostomum intermedium* was synonymized by Foissner et al. (1992) with *S. minus*. We agree with this choice, because no distinctive character was ever described that allowed discrimination between the two morphospecies; the name is virtually absent in recent literature. On similar bases, we would argue that *Spirostomum ephrussi* should be synonymized with *Spirostomum teres*. *S. ephrussi* was never detected after its original description, and a few cells from the *S. teres* populations here characterized actually approaches the peristome: length ratio of 3/5 indicated as the only distinguishing character for *S. ephrussi*.

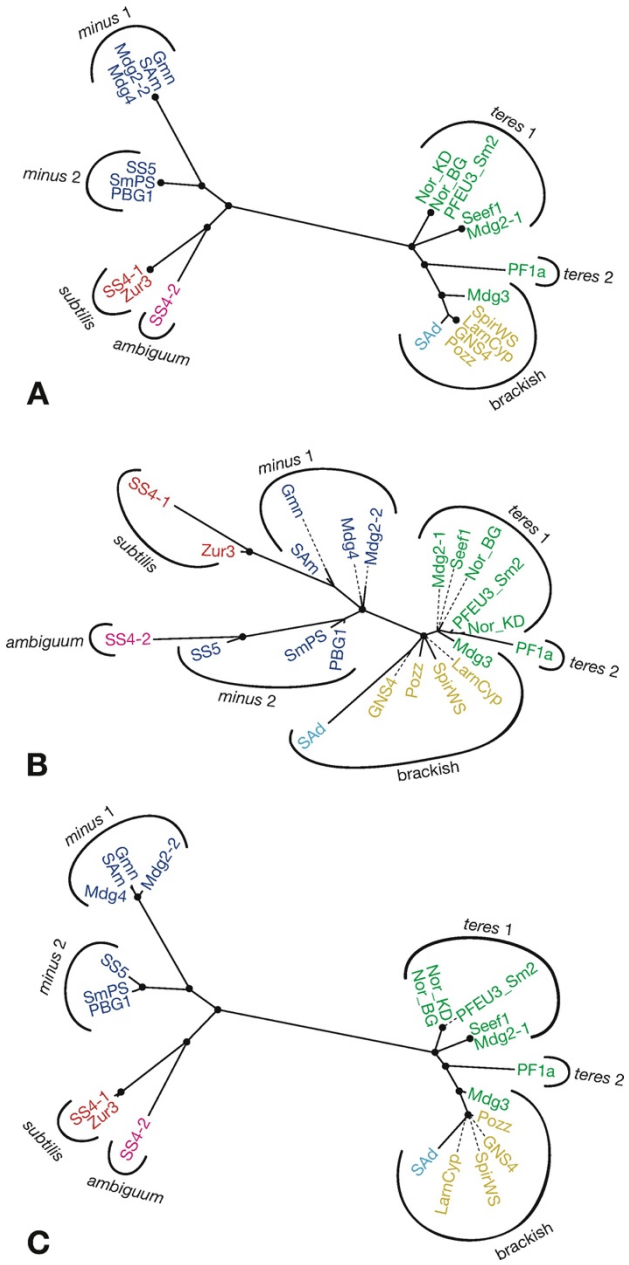

Figure 4. Unrooted Bayesian trees of the 22 populations described both morphologically and molecularly in this work. **A,** based on the 18S +ITS1 +5.8S + ITS2 + 28S region sequence; **B,** based on morphological characters; **C,** based on the mixed character matrix. Circles indicate nodes supported by posterior probability values higher than 0.90. Labels around the trees represent subgeneric clades identified by the 18S rRNA analysis for comparison.

Spirostomum ambiguum, *S. minus* and *S. teres* are by far the most commonly found and studied morphospecies in the genus. According to keys and descriptions consulted, a single population here characterized could be unambiguously assigned to *S. ambiguum*, 7 populations to *S. minus* and 7 to *S. teres*.

Spirostomum yagiui and *Spirostomum dharwarensis* have complex taxonomical histories. In reporting their nomenclatural vicissitudes, Repak and Isquith (1974) lumped together these morphospecies, at that time represented by a single population each and mainly distinguished by the length of their "single"-type MACs. We have found 4 new populations matching the description of *S. yagiui* (Shigenaka 1959) and 1 matching the description of *S. dharwarensis* (Desai, 1966, Seshachar and Padmavathi, 1956), and we can now provide arguments against their synonymization. Although these morphospecies can be unambiguously discriminated only by MAC features, we found that these are quite stable. *S. yagiui* possesses an elongated-type MAC that apparently circles between three different states, all of them unique to this morphospecies. On the other hand, *S. dharwarensis* exhibits a distinctive filiform MAC that is significantly longer and slenderer than all other "single"-type MACs. Since several smaller *S. minus* populations can be reliably distinguished from *S. teres* populations only by MAC type, our opinion is that this character is also sufficient to separate *S. yagiui* and *S. dharwarensis* morphospecies.

Spirostomum semivirescens rarely appears in literature, and is not mentioned in the otherwise comprehensive review of Repak and Isquith (1974). Nevertheless, we agree that it is a valid, probably rare, morphospecies, identifiable mainly by its most distinctive characteristic: the presence of zoochlorellae in the cytoplasm (Esteban et al. 2009). We did not find algae-harboring *Spirostomum* populations in this survey, though.

Spirostomum subtilis sp. n.

Populations Zur3 and SS4-1, despite being *minus*-like in appearance (slender, with a moniliform MAC and a peristome reaching approximately half the length of the body), share features that separate them from all others. They possess the highest length: width ratios (17-21 on average), and their mean length is intermediate between those of *S. minus* and *S. ambiguum*. Statistical analysis of characters collected in vivo shows that the overall morphology of these populations is significantly different from all others' (Supplementary Material Fig. S1A).

Zur3 and SS4-1 additionally present one strong synapomorphy in their unique CG pattern: they are the only populations with CG stripes consisting of a single CG row (Fig. 1Q). Since the number of CG rows in each stripe was never considered before in *Spirostomum* species identification, it is unclear if the description of this morphotype is entirely novel, or if it was lumped by other authors with that of *S. minus*. Our observations, though, provide evidence that the "single row" CG pattern is a distinctive and reliable feature present only in these 2 populations, among those investigated.

The morphological separation is substantiated by molecular data: Zur3 and SS4-1 have identical 18S + ITS1 + 5.8S + ITS2 + 28S sequences, and these differ from those of *S. minus* (2.3-3.4% different sites) and *S. ambiguum* (about 1.9%), the other morphospecies with a moniliform-type MAC. Finally, phylogenetic analyses show that these populations belong to a quite different lineage of uncertain affinity (Figs 3, 4). For all these reasons, we propose to establish a new specific taxon, *Spirostomum subtilis* sp. n., to include populations Zur3 and SS4-1. A detailed description of its features follows at the end of the Discussion, and the tools for its unambiguous discrimination are presented in Figure 1 and the dichotomous key in Supplementary Material Text S1.

Species Phylogeny and Systematics

Our morphological data and statistical analyses clearly show that representatives of the genus *Spirostomum* are distributed along a generally continuous gradient in the morphospace. No single character is able to discriminate among all species. Despite this, a combination of living observations and nuclear features allows unambiguous identification of several groups that correspond to morphospecies (see also below). Molecular phylogeny is largely congruent with this morphological systematics, but there are some telling points in which they disagree.

Spirostomum ambiguum is a well-defined, easily recognizable morphospecies whose monophyly is also strongly supported by molecular sequences. *S. minus*, instead, is morphologically variable and is separated in two clades in molecular trees, here called "*minus* 1" and "*minus* 2". These clades appear to be sister groups in molecular and combined trees (although this fact is undermined by low supporting statistical values), possibly meaning that *S. minus* is indeed a monophyletic group that may include two or more cryptic species. We could not find key characters able to reliably discriminate between subspecific groups in the absence of molecular information; morphological data analyses, both statistical and phylogenetic, also do not recognize them, showing instead a tendency for population SS5 to separate from all others *S. minus*. Hence, we prefer not to create new formal names. It is interesting to notice, however, that all known populations belonging to the "*minus* 2" clade harbor macronuclear bacterial symbionts (Fig. 3), while only one reported population in the "*minus* 1" clade possessed a symbiont (Vannini et al. 2014; Supplementary Material Fig. S2), and it was a cytoplasmic one. Until data on the identity of the bacteria are available, though, this character should not be considered of diagnostic importance. From a molecular point of view, sequences of clade "*minus* 2" present several ambiguous sites, mostly concentrated in a variable region (bases 608-620) of the 18S rRNA gene.

The inclusion of the two populations Zur3 and SS4-1 in the novel species *S. subtilis* is strongly supported by both molecular and morphological trees. The position of *S. subtilis* in phylogenetic trees is not stable: in some analyses it appears to be more closely related to the other morphospecies with moniliform MACs, while in others it is the first-branching lineage in the genus.

Spirostomum yagiui populations share total sequence identity, and they strongly cluster with *S. dharwarensis*. This gives credit to the choice of Repak and Isquith (1974) to unify the two morphospecies; they are distinguishable but closely related, and in our phylogenetic analyses of morphological and mixed data the *S. dharwarensis* population SAd is actually nested within the *yagiui* group (although with very low support).

S. teres sequences are scattered in at least three groups; the interpretation of clades "*teres* 1" and "*teres* 2" mirrors that of the two "*minus*" clades (possibly representing cryptic species in a monophyletic group), but the *S. teres* population Mdg3 is more closely related to *S. yagiui* and *S. dharwarensis* than to other *S. teres*. Also, the structure of "*teres*" clades becomes more complex when fast-evolving sequence regions are introduced in the analyses. Although minor variations in morphological characters can be associated to at least some of the molecularly-identified groups, more populations representing each lineage should be described, before drawing definitive conclusions. On the whole, *S. teres* appears as a relatively well defined but likely non-monophyletic morphospecies that includes several evolutionary lineages.

Character Evolution

The low confidence associated with deeper nodes in *Spirostomum* phylogeny – especially the uncertain positioning of *S. subtilis* – makes most inferences on character evolution unreliable. Nevertheless, something can be said about the evolution of MAC shape, which is arguably the most important diagnostic character separating morphospecies. In fact, the clade including all taxa with a "single"-type MAC (*S. teres*, *S. yagiui* and *S. dharwarensis*) is strongly supported. Our results do not allow assessment of which main MAC morphotype was the ancestral one for the genus, but suggest that the elongated and filiform MAC of *S. yagiui* and *S. dharwarensis*, respectively, are derived from the ellipsoid MAC of *S. teres*. In fact, it seems likely that the morphospecies *S. teres* does not represent a separated evolutionary branch, but defines instead a relatively ancestral set of characters, some of which became differentiated in the derived lineages of *S. dharwarensis* and *S. yagiui*.

Distribution and Environment

An interactive map of geographic locations for many *Spirostomum* spp. samplings presented here and elsewhere can be found online at http://goo.gl/5C10jN. Molecular data are available only for a small fraction of the represented populations, but there is little correlations between sampling location and molecular distance. Even populations from remote areas, like some of those reported here for tropical countries and northern Europe, may share a very high sequence similarity. Nevertheless, one clade – the "brackish" one – is intriguingly defined by an environmental character: it includes all the populations sampled in brackish waters, or at least in littoral zones that experience occasional saltwater inputs. All *S. yagiui* populations and one of the *S. teres* ones (Mdg3) are united in this clade (Fig. 3), thus they probably originate from a common *S. teres*-like ancestor that invaded brackish environments from freshwater ones. *S. dharwarensis* is a more uncertain case: the population described here was found in freshwater, but in an estuarine area where marine and fresh waters likely mix, thus fitting the characteristics of the other "brackish" clade populations. On the other hand, the original Indian populations of *S. dharwarensis* (Desai, 1966, Seshachar and Padmavathi, 1956) were sampled in freshwater environments, distant to the sea. More data are required to establish if this morphospecies is strictly a freshwater inhabitant or if it can be also found in brackish environments.

To support the relevance of water salinity as a diagnostic character, we observed that these 6 populations can survive in freshwater, if gradually acclimated to it, while all those originally sampled in freshwater die when the salinity of their medium increases (data not shown). As a side note, it is to be highlighted that in many old publications, and in some more recent reviews, *Spirostomum* species were considered either "freshwater" or "marine". In our experience, *Spirostomum* is never found in truly marine (above 33‰ salinity) environments, and we suspect that the original samples labeled as "marine" were indeed littoral ones with relatively low salinity. The genus *Spirostomum* likely inhabits only fresh- and brackish-waters.

Revised Systematics of *Spirostomum*

In this paragraph we list schematic descriptions of distinguishable *Spirostomum* morphospecies based on the integration of literature observations and our novel morphological and molecular data. The set of characters sufficient to identify each morphospecies is in bold. This section is also presented as a dichotomous key in Supplementary Material Text S1. We strictly limited our discussion of nuclear features after Feulgen staining, but it is worth to stress that the fundamental MAC type character is usually quite recognizable also in living cells (Fig. 1A-C).

Spirostomum **Ehrenberg, 1834.** Medium to very large ciliates (150 μm – several mm) with a worm-like shape, cylindrical or slightly flattened cell body, length: width ratio ranging from 5 to more than 30. Color varies from faint brown or yellowish to very dark-greenish. A single row of well-developed oral membranelles defines the left side of the long, thin peristomial field, which runs parallel to the main body axis from the anterior end to the cytostome, located at 1/4-2/3 of the body length. Somatic kineties (10-50 in number) homogeneously distributed, parallel to the main body axis, but strongly spiraled when the organism contracts due to myonemes action. One stripe of packed cortical granule rows (1-6) between each kinety pair; cortical granules may be of the same or different size. Contractile vacuole always single and posteriorly located, with a collecting canal reaching the anterior end. Macronucleus single (ellipsoid, elongated or filiform) or moniliform. Micronuclei variable in number but generally small (1-3 μm) and associated with the macronucleus. The monophyly of the genus is strongly supported. Common in fresh water, can be found also in brackish environments. Cosmopolitan.

Spirostomum ambiguum **Ehrenberg, 1834.** [syn: *Trichoda ambiguum* Müller, 1786; *S. ambiguum* var. *major* Roux, 1901] **900 μm – several mm long**. Length: width ratio about 9-17. 15-25 kineties on each side; heterogeneous, numerous (4-5) CG rows per stripe. **Peristome always longer than 1/2 of the body length, often reaching 2/3**. CV much shorter than body length, rarely exceeding 1/10. The color depends on cytoplasmic granules. Moniliform MAC with 12-50 (avg. 15-25) nodules not exceeding 35-45 μm in length when stained by Feulgen reaction. Numerous (up to 100) MICs 1-2 μm long. Monophyletic. Only found in freshwater. Reported in central and northern Europe, England, Russia, central Africa, USA, Jamaica, India and Japan. It sometimes harbors prokaryotic symbionts in the MAC.

Spirostomum caudatum **(Müller, 1786) Delphy, 1939**. [syn. *Enchelis caudata* Müller, 1786; *Uroleptus filum* Ehrenberg, 1833; *Spirostomum filum* Dujardin, 1841; *S. teres* var. *caudatum* Zacharias, 1903] **Tapering, thin posterior tail**. 200-700 (avg. 200-400) μm long. 14-16 kineties on each side. **Peristome about 1/4 of the body length**. Ellipsoid MAC. No molecular sequence available. Found in fresh- and saltwater (?). Reported in central Europe, central Africa and Korea.

Spirostomum dharwarensis **Desai, 1966**. 300-550 (avg. 400) μm long. Length: width ratio about 8-14 (avg. 11). 7-13 (avg. 10) kineties on each side; usually homogeneous 3-4 CG rows per stripe. Peristome variable, from less than 1/2 of the body length to about 2/3. CV usually less than 1/5 of the body length. Dark cytoplasm. **Filiform, convoluted MAC (length: width ratio always > 10, usually > 20; uniform diameter) about 100-200 x 5-10 μm when stained by Feulgen reaction**. Several (1-7) MICs 1-3 μm long. Only one molecular sequence available. Found in freshwater, once in estuarine environment. Reported in southern Africa and India.

Spirostomum minus **Roux, 1901**. [syn: *S. ambiguum* var. *minor* Roux, 1901; *S. intermedium* Kahl, 1932] **350-900 (avg. 450-600) μm long. Length: width ratio about 7-15 (avg. 9-13)**. 6-12 (avg. 8-10) kineties on each side; homogeneous or heterogeneous CG rows, variable in number per stripe (2-4). Peristome about 1/2 of the body length. CV usually less than 1/5 of the body length. **Moniliform MAC** with 5-25 (avg. 10-15) nodules not exceeding 30-40 μm in length when stained by Feulgen reaction. Variable number (up to 20) of 1.5-3.5 μm long MICs. Molecular analyses identify two clades, probably representing cryptic species with no reliable morphological autapomorphy; the morphospecies might be monophyletic. Commonly found in freshwater. Reported in Europe, England, southern and central Africa, Madagascar, USA, Brazil, China, India, possibly Thailand and Australia. It relatively often harbors prokaryotic symbionts in cytoplasm or MAC.

***Spirostomum semivirescens* Perty, 1852**. Numerous zoochlorellae in the cytoplasm. 600-2,000 (avg. 1,200-1,300) μm long; the posterior half is usually encased in a mucilaginous coating. Length: width ratio about 17-40 (avg. 30-40). 14-15 kineties on each side. Peristome about 1/2 of the body length. Moniliform MAC with about 12 nodules. Only found in freshwater. No molecular sequence available. Reported in central Europe, England, Russia and Japan.

***Spirostomum subtilis* sp. n.** 700-1,000 μm long. **Length: width ratio about 14-24**. 9-12 kineties on each side; a **single, homogeneous CG row per stripe**. Peristome about 1/2 of the body length. CV often conspicuous, up to 1/3 of the body length, contrasting with the dark cytoplasm of the anterior part. Moniliform MAC with 15-24 nodules not exceeding 20-25 μm in length when stained by Feulgen reaction. Several (1-6) MICs 2-3 μm long. Monophyletic. Only found in freshwater. Reported in central and northern Europe. Type location and features of the type population Zur3 detailed in Supplementary Material Table S1. **Type materials**: one slide of permanent Feulgen stained specimens collected in Zurich (population Zur3) deposited in the collection of the Museo della Scienza e del Territorio della Certosa di Calci (Calci, Pisa, Italy); extracted genomic DNA available upon request. 18S + ITS1 + 5,8S + ITS2 + 28S region sequence deposited at the ENA database (accession number: HG939550).

***Spirostomum teres* Cláparéde and Lachmann, 1858-1859.** [syn: *S. ephrussi* Delphy, 1939] 150-650 (avg. 250-450) μm long. Length: width ratio about 5-16 (avg. 8-12). 5-15 (avg. 7-10) kineties on each side; usually homogeneous CG rows, variable in number per stripe (2-4). Peristome from 1/3 to slightly more than 1/2 of the body length. CV usually less than 1/5 of the body length. Often brownish. **Ellipsoid MAC (length: width ratio < 5) in the middle sector of the body, about 20-50 x 5-20 μm when stained by Feulgen reaction**. A few (1-3) MICs 1-2 μm long. Molecular analyses suggest that this morphospecies include phylogenetically diverse lineages, some of which are more closely related to *S. yagiui* and *S. dharwarensis*; no reliable morphological autapomorphy has yet been detected for these lineages. Found in both fresh- and brackish-water environments. Reported in Europe, central Africa, Madagascar, USA, Brazil, Caspian Sea, India, China and Korea. It sometimes harbors cytoplasmic prokaryotic symbionts.

***Spirostomum yagiui* Shigenaka, 1959**. 240-500 (avg. 300-400) μm long. Length: width ratio about 6-17 (avg. 8-14). 6-12 (avg. 7-10) kineties on each side; usually homogeneous CG rows, variable in number per stripe (2-4). Peristome variable, from about 1/3 of the body length to more than 1/2. CV less than 1/5 of the body length. Usually brownish. **Elongated MAC (length: width ratio > 5) exhibiting different shapes during cell cycle, about 35-90 x 5-15 μm when stained by Feulgen reaction**. A few (up to 6) MICs about 2 μm long. Likely monophyletic. **Only found in brackish water, or in close proximity to saltwater basins**. Found in Mediterranean Sea islands (Sicily and Cyprus), northern Europe, Russia and Japan.

Concluding Remarks

We have presented the first systematic work on the ubiquitous genus *Spirostomum* based on both morphological and molecular characters. According to literature and our results, there are 8 valid morphospecies, one of which is novel, although some may be complexes of sibling species. Morphological and molecular data do agree to a large extent, and a small set of unambiguous characters is sufficient to discriminate among taxa. New data on S. *caudatum* and *S. semivirescens*, the morphospecies still lacking a molecular characterization, should be welcomed in the future to complete this survey.

In addition to our conclusions on the genus *Spirostomum*, we provided a case for the relevance of genera as valuable targets for systematics studies. A lot of ciliate genera are easy to discriminate, and many of them are quite common, but species identification is often a bigger issue. Whenever

possible, we encourage taxonomists to present updated, multidisciplinary and quantitative data on several species at the same time and perform analyses aiming to organize the extant knowledge, with the goal of investigating biodiversity while providing non-taxonomists with the most practical tools for recognizing species.

Methods

Samples and cultures: Table 1 and Supplementary Material Table S1 list all investigated *Spirostomum* populations and sampling metadata. Aliquots of sediment and water from each sample were observed under the dissecting microscope in order to detect organisms belonging to the genus *Spirostomum* and divide them according to their main morphotype. The resulting populations were then maintained at 18-20 °C in their original medium, periodically enriched with rice grains, lettuce medium and/or modified Cerophyl medium (Boscaro et al. 2013) inoculated with *Raoultella planticola* (*Gammaproteobacteria*).

Morphological data: Single *Spirostomum* cells were harvested from the culture medium and observed with a Leitz (Weitzlar, Germany) differential interference contrast microscope equipped with a digital camera (Canon PowerShot S45). The device developed by Skovorodkin (1990) was employed to stop the organisms' movement without altering their shape. Feulgen staining was performed to observe the features of the nuclear apparatus. Length measures, on both living and fixed cells, were taken on collected pictures with the software Macnification v2.0.1 (Orbicule bvba). Pictures are available upon request; fixed slides were deposited in the collection of the Museo della Scienza e del Territorio della Certosa di Calci (Calci, Pisa, Italy).

Discriminant function analyses were performed with the software Statistica v6 on 10 characters from in vivo observations, and 5 characters from Feulgen observations (Supplementary Material Table S1). Only cells with at least 70% of the parameters measured were employed, resulting in two character matrices of 1,960 and 1,395 data (196 and 279 cells) for in vivo and Feulgen analyses, respectively. Missing data (less than 5% in the in vivo matrix and less than 2% in the Feulgen matrix) were substituted with mean values.

Molecular data: About 30-50 cells from each well-growing population were individually picked with a glass micropipet, washed several times in sterile water and fixed in EtOH 70%. 18S rRNA gene and a sequence containing the complete ITS1 + 5.8S + ITS2 region and part of the 28S rRNA gene were amplified and sequenced as described previously (Boscaro et al. 2012). When members of a population were not numerous enough, only a few organisms were collected, and PCR mixtures were directly applied on the exsiccated cells as described in Andreoli et al. (2009); these amplicons did not require hemi-nested PCR reactions to be sequenced. Standard ambiguity letters were associated to sites that showed double peaks in electropherograms despite repeated sequencing attempts.

All sequences were deposited at the European Nucleotide Archive (ENA) database (accession numbers: HG939524-53).

Phylogenetic analyses: Phylogenetic analyses on the 18S rRNA gene sequence were performed on 127 homologous sequences of Heterotrichea and Karyorelictea, trimmed at the ends in order to obtain a rectangular matrix (matrix *a*, 1,680 characters) and additionally excluding columns containing only one non-gap character (matrix *b*, 1,660 characters) and columns in which the most represented base was present in less than 20% of taxa (matrix *c*, 1,629 characters). Unless where differently specified, similarity values were calculated on unmodified matrices, ignoring ambiguous sites. A 131-sequence matrix was built in order to include some available *Spirostomum* sequences that were too short, or contained too many ambiguous characters, to be

employed in the main analysis; missing data were then treated as gaps. Phylogenetic analyses were also performed on the subset of 22 populations studied in more detail, employing the entire 18S + ITS1 + 5.8S + ITS2 + 28S region (2,379 characters), morphological data (18 characters), and a mixed character matrix. Morphological characters employed in the phylogenetic analysis are shown in Supplementary Material Table S1; qualitative characters were coded in unordered categories, and each quantitative, continuous characters were coded in 6 ordered discrete categories.

Maximum Likelihood analyses were performed with PHYML (Guindon and Gascuel 2003) as implemented in the ARB software package (Ludwig et al. 2004); 100 pseudoreplicates were used for bootstrapping. Bayesian Analyses were performed with MrBayes v3.2.2 (Ronquist and Huelsenbeck 2003), employing 3 independent runs with 1 cold and 3 heated chains each; runs were iterated for 1,000,000 generations. The best-fitting model for phylogenetic inferences on molecular data was chosen among 88 candidate models according to jModelTest v2.1.4 (Darriba et al. 2012). The AIC criterion always selected the GTR + I + G model; the continuous gamma function was approximated with 4 discrete categories.

Acknowledgements

The authors wish to thank Michela Balestri, Marta Barresi, Marco Campera, Filipe Chetverikov, Roberto JP Dias, Natalia A Lebedeva, Alessia Rossi, Valentina Serra, Cristiana Sigona and Ilya Skovorodkin for providing some of the samples, Claudia Vannini and Letizia Modeo for advices and corrections on manuscript drafts and the editor and the two anonymous reviewers for their careful revisions and advices. Simone Gabrielli is gratefully acknowledged for help with graphic artwork. Financial support for this research was provided by CAPES (project 006995/2011-51), CNPq (project 484005/2013-8), European Commission FP7-PEOPLE-2009-IRSES project CINAR PATHOBACTER (project number 247658) and the Fondazione Cassa di Risparmio di Pistoia e Pescia.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.Org/10.1016/j.protis.2014.05.004.

References

Adi SM, Simpson AGB, Lane CE, Lukeš J, Bass D, Bowser SS, Brown MW, Burki F, Dunthorn M, Hampl V, Heiss A, Hoppenrath M, Lara E, Le Gall L, Lynn DH, McManus H, Mitchell EAD, Mozley-Stanridge SE, Parfrey LW, Pawlowski J, Rueckert S, Shadwick L, Schoch CL, Smirnov A, Spiegel FW (2012) The revised classification of eukaryotes. J Eukaryot Microbiol 59:429-493

Andreoli I, Mangini L, Ferrantini F, Santangelo G, Verni F, Petroni G (2009) Molecular phylogeny of unculturable Karyorelictea (Alveolata, Ciliophora). Zool Scr 38:651-662

Boscaro V, Fokin SI, Verni F, Petroni G (2012) Survey of *Paramecium duboscqui* using three markers and assessment of the molecular variability in the genus *Paramecium.* Mol Phylogenet Evol **65:1004-1013**

Boscaro V, Fokin SI, Schrallhammer M, Schweikert M, Petroni G (2013) Revised systematics of *Holospora*-like bacteria and characterization of *"Candidatus* Gortzia infectiva", a novel macronuclear symbiont of *Paramecium jenningsi.* Microb Ecol 65:255-267

Bradley MW, Esteban GF, Finlay BJ (2010) Ciliates in chalkstream habitats congregate in biodiversity hot spots. Res Microbiol 161:619-625

Chantangsi C, Lynn DH (2008) Phylogenetic relationships within the genus *Tetrahymena* inferred from the cytochrome *c* oxidase subunit 1 and the small subunit ribosomal RNA genes. Mol Phylogenet Evol 49:979-987

Chantangsi C, Lynn DH, Rueckert S, Prokopowicz AJ, Pan ha S, Leander BS (2013) *Fusiforma themisticola* n. gen., n. sp., a new genus and species of apostome ciliate infecting the hyperiid amphipod *Themisto libellula* in the Canadian Beaufort Sea (Arctic Ocean), and establishment of the Pseudocolliniidae (Ciliophora, Apostomatia). Protist 164:793-810

Claparède E, Lachmann J (1858-1859) Études sur les infusoires et les rhizopodes. Mém Inst Nat Genévois 5:1-260

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9:772

Delphy J (1939) Sur les Spirostomes. Archs néerl Zool **3:141-145**

Desai RN (1966) Nuclear apparatus and binary fission in *Spirostomum dharwarensis* n. sp. Experientia **22:35-36**

Ehrenberg CG (1838) Die Infusionsthierchen als vollkommene Organismen. Leopold Voss, Leipzig

Esteban GF, Bradley MW, Finlay BJ (2009) A case-building *Spirostomum* (Ciliophora, Heterotrichida) with zoochlorellae. Eur J Protistol 45:156-158

Fernandes NM, da Silva Neto ID (2013) Morphology and 18S rDNA gene sequence of *Spirostomum minus* and *Spirostomum teres* (Ciliophora: Heterotrichea) from Rio de Janeiro, Brazil. Zoologia 30:72-79

Finlay BJ, Esteban GF (1998) Freshwater protozoa: biodiversity and ecological function. Biodiversity Conserv 7:1163-1186

Foissner W (2013) Description of *Glaucomides bromelicola* n. gen., n. sp. (Ciliophora, Tetrahymenida), a macrostome forming inhabitant of bromeliads (Bromeliaceae), including redescriptions of *Glaucoma scintillans* and *G. reniformis.* J Eukaryot Microbiol 60:137-157

Foissner W, Berger H, Kohmann F (1992) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems - Band II: Peritrichida, Heterotrichida, Odontostomatida. Informationsberichte des Bayerisches Landesamtes für Wasserwirtschaft, Deggendorf, 502 p

Fokin Sl (2004) A brief history of ciliate studies (late XVII - the first third of the XX century). Protistology 3:283-296

Fokin SI, Schweikert M, Brümmer F, Görtz HD (2005) *Spirostomum* spp. (Ciliophora, Protista), a suitable system for endocytobiosis research. Protoplasma 225:93-102

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696-704

Hirt RP, Dyal PL, Wilkinson M, Finlay BJ, Roberts DM, Embley TM (1995) Phylogenetic relationships among karyorelictids and heterotrichs inferred from small subunit rRNA sequences: resolution at the base of the ciliate tree. Mol Phylogenet Evol 4:77-87

Jang SW, Kwon CB, Shin MK (2012) First records of two *Spirostomum* ciliates (Heterotrichea: Heterotrichida: Spirostomidae) from Korea. Anim Syst Evol Divers **28:29- 35**

Kahl A (1932) Urtiere oder Protozoa. I: Wimpertiere oder Ciliata (Infusoria). 2. Spirotricha. In Dahl F (ed) Die Tierwelt Deutschland. Gustav Fischer, Jena, pp 437-40

Kher CP, Doerder FP, Cooper J, Ikonomi P, Achilles-Day U, Küpper FC, Lynn DH (2011) Barcoding *Tetrahymena*: discriminating species and identifying unknowns using the cytochrome *c* oxidase subunit I (cox-1) barcode. Protist 162: 2-13

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Kumar Y, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363-1371

Lynn DH (2008) The Ciliated Protozoa. Characterization, Classification, and Guide to the Literature. 3rd edition Springer, Dordrecht, 605 p

Miao M, Song W, Clamp JC, Al-Rasheid KAS, Al-Khedhairy AA, Al-Arift S (2009) Further consideration of the phylogeny of some "traditional" heterotrichs (Protista, Ciliophora) of uncertain affinities, based on new sequences of the small subunit rRNA gene. J Eukaryotic Microbiol 56:244-250

Modeo L, Petroni G, Lobban CS, Verni F, Vannini C (2013a) Morphological, ultrastructural, and molecular characterization of *Euplotidium rosati* n. sp. (Ciliophora, Euplotida) from Guam. J Eukaryotic Microbiol 60:25-36

Modeo L, Fokin SI, Boscaro V, Andreoli I, Ferrantini F, Rosati G, Verni F, Petroni G (2013b) Morphology, ultrastructure, and molecular phylogeny of the ciliate *Sonderia vorax* with insights into the systematics of order Plagiopylida. BMC Microbiol 13:40

Nałecz-Jawecki G, Sawicki J (1999) Spirotox - a new tool for testing the toxicity of volatile compounds. Chemosphere 38:3211-3218

Pan H, Li L, Al-Rasheid KAS, Song W (2013a) Morphological and molecular description of three new species of the cyrtophorid genus *Chlamydodon* (Ciliophora, Cyrtophoria). J Eukaryot Microbiol 60:2-12

Pan H, Gao F, Lin X, Warren A, Song W (2013b) Three new *Loxophyllum* species (Ciliophora: Pleurostomatida) from China with a brief review of the marine and brackish *Loxophyllum* species. J Eukaryot Microbiol 60:44-56

Pan Y, Li J, Li L, Hu X, Al-Rasheid KAS, Warren A (2013c) Ontogeny and molecular phylogeny of a new marine ciliate genus, *Heterokeronopsis* g. n. (Protozoa, Ciliophora, Hypotricha), with description of a new species. Eur J Protistol 49:298-311

Park KM, Jung JH, Min GS (2013) Morphology, morphogenesis, and molecular phylogeny of *Anteholosticha multicirrata* n. sp. (Ciliophora, Spirotrichea) with a note on morphogenesis of *A. pulchra* (Kahl, 1932) Berger, 2003. J Eukaryot Microbiol 60:564-577

Perty M (1852) Zur Kenntniss kleinster Lebensformen nach Bau, Funktionen, Systematik, mit Specialverzeichniss der in der Schweiz beobachteten. Jent & Reinert, Bern, 228 p

Petroni G, Dini F, Verni F, Rosati G (2002) A molecular approach to the tangled intrageneric relationships underlying phylogeny in *Euplotes* (Ciliophora. Spirotrichea). Mol Phylogenet Evol 22:118-130

Repak AJ, Isquith IR (1974) The systematics of the genus Spirostomum Ehrenberg, 1838. Acta Protozool 12:325- 333

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574

Roux (1901) Faune infusorienne de eaux stagnantes des environs de Genève. Kündig, Genève, 148 p

Schmidt SL, Foissner W, Schlegel M, Bernhard D (2007a) Molecular phylogeny of the Heterotrichea (Ciliophora, Postciliodesmatophora) based on small subunit rRNA gene sequences. J Eukaryot Microbiol 54:358-363

Schmidt SL, Treuner T, Schlegel M, Bernhard D (2007b) Multiplex PCR approach for species detection and differentiation within the genus *Spirostomum* (Ciliophora, Heterotrichea). Protist 158:139-145

Schrallhammer M, Ferrantini F, Vannini C, Galati S, Schweikert M, Görtz HD, Verni F, Petroni G (2013) *"Candidatus* Megaira polyxenophila" gen. nov., sp. nov.: considerations on evolutionary history, host range and shift of early divergent rickettsiae. PLoS ONE 8:e72581

Seshachar BR, Padmavathi PB (1956) The cytology of a new species of *Spirostomum.* J Protozool 3:145-150

Shigenaka Y (1959) A new marine ciliate, *Spirostomum yagiui* n. sp. Zool Mag Tokyo 68:368-372

Skovorodkin IN (1990) A device for immobilizing biological objects in the light microscope studies. Tsitologiya 32:301- 302

Strüder-Kypke MC, Wright ADG, Fokin SI, Lynn DH (2000) Phylogenetic relationships of the genus *Paramecium* inferred from small subunit rRNA gene sequences. Mol Phylogenet Evol **14:122-130**

Twagilimana L, Bohatier J, Groliere CA, Bonnemoy F, Sargos D (1998) A new low-cost microbiotest with the protozoan *Spirostomum teres*: culture conditions and assessment of sensitivity of the ciliate to 14 pure chemicals. Ecotoxicol Environ Safety 41:231-244

Vannini C, Boscaro V, Ferrantini F, Benken KA, Mironov Tl, Schweikert M, Görtz HD, Fokin SI, Sabaneyeva EV, Petroni G (2014) Flagellar movement in two bacteria of the family *Rickettsiaceae*: a re-evaluation of motility in an evolutionary perspective. PLoS ONE 9:e87718