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Morphology and 18S rDNA gene sequence of *Spirostomum minus* and *Spirostomum teres* (Ciliophora: Heterotrichea) from Rio de Janeiro, Brazil

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ABSTRACT. Species of *Spirostomum* Ehrenberg, 1838 are widely used as model organisms in ecological studies of environmental impacts and symbioses between ciliates and human pathogenic bacteria. However, the taxonomy of this genus is confused by the superficiality of the morphological descriptions of its included species, and the use of only a few characters for their differentiation. The present study provides details of total infraciliature, nuclear apparatus, morphometric data and 18S rDNA gene sequences of *Spirostomum teres* Claparède & Lachmann, 1858 and *Spirostomum minus* Roux, 1901, isolated from a sewage treatment plant and a freshwater lake in the city of Rio de Janeiro, Brazil, respectively. For the morphological descriptions of *S. teres* and *S. minus*, living cells were observed using bright-field and differential interference contrast (DIC) microscopy, the total infraciliature and nuclear apparatus were revealed by staining with protargol, and ciliary patterns were observed also with scanning electron microscopy (SEM). The complete sequences of the 18S rDNA of *S. teres* and *S. minus* were obtained using *eukaryotic* universal *primers, and then compared with sequences of other species and populations of Spirostomum deposited in the GenBank database. Living S. minus measured* 400-800 µm in length and 55-115 µm in width, with the following characteristics: adoral zone of membranelles approximately 112 µm long; inconspicuous paroral kinety; 30-40 kineties in somatic ciliature; moniliform macronucleus with 9-25 nodes, approximately 12 micronuclei; single and posterior contractile vacuole; and yellow-brown cytoplasm. Living and fully extended *S. teres* measured approximately 250 µm in length and 65 ìm in width, with the following characteristics: adoral zone of membranelles approximately 92 µm long; approximately 30 somatic kineties; compact macronucleus, approximately five micronuclei; macronuclear groove present; single and posterior contractile vacuole; and colorless cytoplasm. Evidence from 18S rDNA sequences confirms the identification of *S. teres* and suggests the existence of cryptic species closely related to *S. minus*. The use of silver impregnation technique (protargol) allowed the observation and description of a greater number of characters in *S. minus* and *S. teres*, thus assisting the research that require identification of these species.

KEY WORDS. Bioindicators; ciliates; cryptic species; heterotrichs; morphology.

Spirostomum Ehrenberg, 1838 are conspicuous ciliates protists that are easily recognized by their large sizes (500-1000 µm) and elongate bodies, being easily confounded with small helminths. The name *Spirostomum* refers to the ability these ciliates have to contract in a spiral mode. This type of contraction is due to the presence of post-ciliary, sub-pellicular fibers that arise on the anterior end and spiral in a counterclockwise direction toward the posterior end of the body (Ishida *et al*. 1988).

Spirostomum was mentioned for the first time by Ehrenberg (1838) and morphological studies have been subsequently carried out for some species (Packard 1948, Finley *et al*. 1964, Daniel & Mattern 1965, Tuffrau 1967, Kudo 1971). However, these studies were based exclusively on observations of specimens *in vivo*. Repak & Isquith (1974) emphasized the difficulty of separating and identifying species of *Spirostomum* owing to the superficiality of their morphological descriptions and the use of only a few characters for species differentiation. Moreover, most of those characters vary significantly within species (Repak & Isquith 1974), making identification even more difficult. For this reason, the taxonomy of *Spirostomum* has become extremely confusing. More detailed morphological and morphometric studies that increase the number of characters available for species identification are needed to solve this problem. Descriptions should include observations on infraciliary patterns and ultrastructural characters.

Currently, species of *Spirostomum* are identified using only morphological features such as the shape and size of the cell, ratio of the total length/length of the adoral zone of membranelles, location of the contractile vacuole, and configuration of the macronucleus (Foissner *et al*. 1992, Berger *et al*. 1997). However, it should be noted that it is easy to

misidentify some species of *Spirostomum* (Foissner *et al*. 1992). In recent decades, sequences of the 18S small-subunit rRNA gene have been widely used in the phylogenetic study of ciliates, and also in species identification (Lynn & Small 2002, Gong *et al*. 2007, Li *et al*. 2009). The small-subunit rRNA sequences of European populations of *Spirostomum minus* Roux, 1901and *Spirostomum teres* Claparède & Lachmann, 1858 were obtained by Schmidt *et al*. (2007) and have been used in recent phylogenetic studies of the Heterotrichea by several authors.

Species of *Spirostomum* have been used currently as model organisms in research on human pathogenic bacteria (Fokin *et al*. 2003, 2005). Some species (e.g., *S. minus* and *S. teres*) have also been used in studies of environmental impacts because they are considered good indicators of water quality (Foissner *et al*. 1992, Berger *et al*. 1997, Berger & Foissner 2003) and show sensitivity to certain toxic substances (e.g. heavy metals like nickel, copper, mercury, and zinc; the phenol Na-PCP) (Madoni *et al*. 1992, Madoni 2000). Nevertheless, studies on the morphology and taxonomy of *Spirostomum* species are quite rare (Packard 1948, Finley *et al*. 1964, Daniel & Mattern 1965, Tuffrau 1967, Kudo 1971, Repak & Isquith 1974, Foissner *et al*. 1992).

In the present work, morphological descriptions and morphometric data of *S. teres* and *S. minus* isolated from a sewage treatment plant and a freshwater lake in the municipality of Rio de Janeiro, Brazil, are presented. Descriptions of morphological characters observed on living and protargol-stained organisms, which show details of the nuclear apparatus and infraciliature, are included. Images obtained by electron microscopy (SEM) are presented to complement the descriptions and 3D visualization of characters of the cell surface. The small-subunit 18S rDNA sequences of *S. minus* and *S. teres* from Rio de Janeiro are also presented and characterized.

MATERIAL AND METHODS

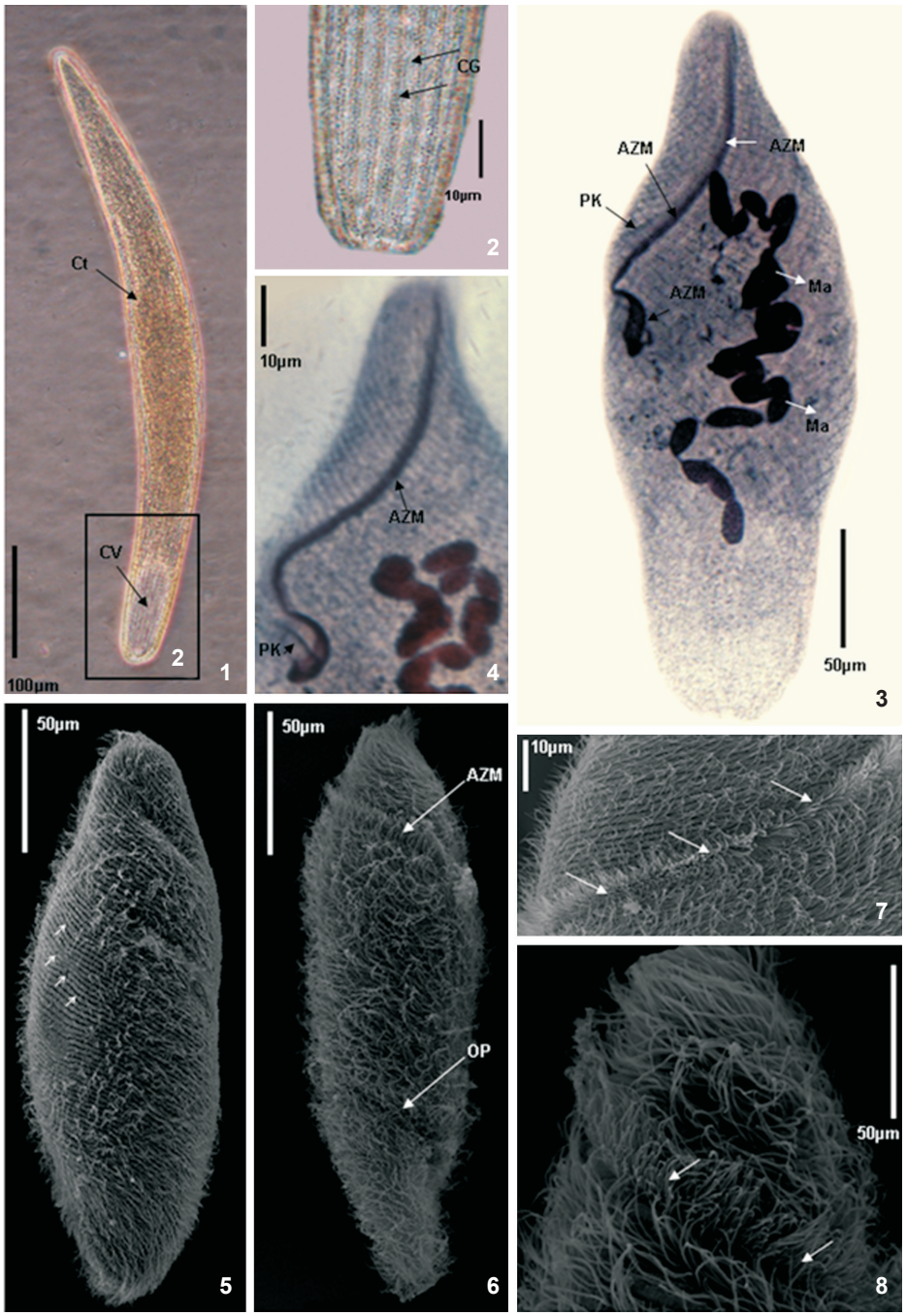
Specimens of *Spirostomum teres* and *S. minus* were isolated from samples collected in a sewage treatment plant in Penha, Rio de Janeiro (22°52'S, 43°13'W), and from a freshwater lake located at the Universidade Federal do Rio de Janeiro (22°51'S, 43°13'W), respectively. Samples from both localities were obtained manually using plastic containers and then maintained in the laboratory for several days at room temperature (about 24°C) as a raw culture for studies as proposed by Foissner (1992). Living cells were picked out from cultures and observed using bright-field and differential interference contrast microscopy. The infraciliature and nuclear apparatus were revealed by staining with protargol according to the method of Dieckmann (1995). Specimens were examined at magnifications of 100-1000x. Patterns of somatic and oral ciliature were observed with scanning electron microscopy according to the methodology of Silva-Neto *et al*. (2012). Measurements were made with Image Pro-plus 5.0® (Media Cybernetics, Inc., Bethesda, MD) using an Olympus BX 51 compound microscope. Slides of protargol-stained *S. minus* and *S. teres* were deposited in collection of the Laboratório de Protistologia (Universidade Federal do Rio de Janeiro), with registry numbers: IBZ-UFRJ0017-X and IBZ-UFRJ0018-X, respectively.

Extraction of genomic DNA from *S. minus* and *S. teres*, PCR amplification of the genes for 18S rRNA, and sequencing of rDNA were performed according to the method described by Schmidt *et al*. (2007). The gene for 18S rRNA was amplified using the *eukaryotic* universal *primers EukA/EukB* (Medlin *et al*. 1988) that extend over the full length of the gene. Sequences of *S. minus* and *S. teres* were edited into contigs using BioEdit (Hall 1999) and deposited at the NCBI/GenBank database with the accession numbers JQ282896 and JQ282897, respectively. These two sequences were compared with the 18S rDNA sequences in the GenBank database of *S. minus* (AM398200), *S. teres* (AM398199) and *Spirostomum ambiguum* Ehrenberg, 1838 (AM398201) obtained by Schmidt *et al*. (2007) using the BLASTN algorithm. The frequencies of individual nucleotides and GC composition of sequences of *S. minus* and *S. teres* from Rio de Janeiro were obtained using CompSeq (Rice *et al*. 2000). The similarity structural matrix and absolute distance matrix between *Spirostomum* species was generated with BioEdit (Hall 1999) and PAUP* 4.0, respectively.

RESULTS

Morphology of *Spirostomum minus* Roux, 1901

Living and fully extended organisms have vermiform, elongated bodies, tapered at the ends and with a total length of 400-800 µm (Fig. 1). Contracted organisms have ellipsoid bodies (Figs 3, 4 and 6), a total length of 175-262 µm (mean = 206 µm) and a width of 55-115 µm. During contraction, specimens of *S. minus* twist their oral and somatic ciliature in a counterclockwise direction (Fig. 5). The adoral zone of membranelles (AZM) was 112µm (64-142 µm) long and extended from the anterior end of the ciliate to a cytostome located approximately at the midpoint of the body (Figs 3, 4 and 6). In contracted specimens, the AZM made a counter-clockwise turn around the body (Figs 3, 4 and 6-8). Inconspicuous paroral kinety located to the left of the AZM (Figs 3 and 4). Somatic ciliature composed of an average of 35 (range 30-40) kineties (Fig. 5). In contracted individuals, torsion of the cell caused somatic kineties to be oriented obliquely in relation to the central axis of the body (Fig. 5). Moniliform macronucleus, with 9-25 nodes connected by nuclear bridges (Fig. 3); approximately 12 micronuclei (mean diameter 1.8 µm) near or overlapping the macronucleus (Fig. 3). Single contractile vacuole located at the posterior end of the body (Figs 1 and 2). Living organisms were yellow-brown in color when observed under low magnification (Fig. 1). This coloration was due to the presence of pale brown cortical granules (approx. 0.5 µm) that were densely packed between the kineties (Fig. 2). Individuals moved by gliding slowly over the substrate or by swimming freely above it (Table I).



Figures 1-8. Morphology of *Spirostomum minus*: (1) image obtained from phase contrast microscopy of the living organism showing general shape of the cell, cytostome, and single contractile vacuole in posterior end of the body; (2) detail of posterior end of cell showing cortical granules; (3) ventral view of *S. minus* after staining with protargol showing nuclear apparatus, somatic kineties, and oral infraciliature; (4) closeup of oral region showing the arrangement of adoral zone of membranelles and paraoral kinetie; (5) scanning electron micrography showing general shape of the cell in dorsal view and somatic kineties (arrows) obliquely twisted in relation to the central axis by contraction; (6) dividing cells showing morphogenesis of the oral primordial of daughter cells; (7-8) scanning electron micrography showing the general morphology of the oral region and adoral zone of membranelles (arrows). (AZM) Adoral zone of membranelles, (PK) paroral kinety, (CV) contractile vacuole, (Ma) macronucleus, (Ct) cytostome, (CG) cortical granules, (OP) oral primordium.

Table I. Morphometric data of *Spirostomum minus* and *S. teres* from sewage treatment system by activated sludge in Rio de Janeiro. All measures were performed on specimens protargol impregnated and are given in micrometer (µm). (SD) standard deviation, (SE) standard error, (CV) coefficient of variation, (n) number of specimens investigated, (AZM) adoral zone of membranelles.

Characters	Mean	Median	Minimum	Maximum	SD	SE	CV (%)	n
Spirostomum minus								
Body length	206.0	200.0	175.0	262.0	23.5	4.7	0.1	25
Body width	82.0	82.0	55.0	115.0	15.1	3.0	0.1	25
Length of AZM	112.0	111.0	64.0	142.0	19.3	3.8	0.1	25
Total length of macronucleus	232.0	228.0	125.0	362.0	60.9	12.1	0.2	25
Width of macronucleus	8.0	8.0	5.0	12.0	1.8	0.3	0.2	25
Diameter of micronuclei	1.8	1.9	1.3	3.0	0.3	0.0	0.1	25
Number of micronuclei	12.0	8.0	3.0	30.0	3.8	2.7	0.3	25
Number of macronuclear nodes	17.0	17.0	9.0	25.0	4.4	0.8	0.2	25
Number of somatic kineties	35.0	34.0	30.0	40.0	1.1	0.6	0.1	25
Spirostomum teres								
Body length	165.0	167.0	93.0	196.0	25.4	4.6	0.1	30
Body width	66.0	61.0	38.0	96.0	15.5	2.8	0.2	30
Length of AZM	92.0	93.0	56.0	114.0	15.3	2.8	0.1	30
Total length of macronucleus	38.0	38.0	20.0	52.0	6.2	1.1	0.1	30
Width of macronucleus	12.0	13.0	7.0	16.0	2.6	0.4	0.2	30
Diameter of micronuclei	2.0	2.0	1.0	2.9	0.4	0.0	0.2	30
Number of micronuclei	5.0	5.0	1.0	11.0	2.9	0.5	0.5	30
Number of macronuclear nodes	1.0	1.0	1.0	1.0	0.0	0.0	0.0	30
Number of somatic kineties	30.0	40.0	22.0	37.0	5.9	0.1	0.1	30

Morphology of *Spirostomum teres*

Fully extended living organisms had a rounded posterior end and tapered anterior end. They were 150-250 µm in length and approximately 65 ìm in width (Fig. 9). Contracted cells had an ellipsoid body, with slightly tapered ends (Figs 10, 12 and 13). The oral and somatic ciliature of *S. teres* twisted in a counterclockwise direction during contraction (Fig. 10). The adoral zone of membranelles (AZM) was approximately 92 µm (56-114 µm) long and extended from the anterior end of the ciliate to the cytostome, which was located in the anterior third of the body (Figs 10-14). In contracted cells, the AZM made a counterclockwise turn around the body (Figs 10-14). Inconspicuous paroral kineties were located to the left of the AZM (Fig. 14). Somatic ciliature composed of about 30 kineties (Fig. 10). In contracted individuals, torsion of the cell caused somatic kineties to be oriented obliquely in relation to the central axis of the body (Fig. 10). Nuclear apparatus composed of single, compact macronucleus located approximately in the center of the body (Fig. 12 and 13). In some cells, macronuclear grooves that hold micronuclei were observed (Fig. 15). Approximately 5 micronuclei (5 µm in diameter) were dispersed throughout the cytoplasm or overlapping the macronucleus. A single, conspicuous contractile vacuole occupied almost the entire posterior third of the body (Fig. 9). Live cells were colorless (Fig. 9), and moved slowly, gliding over the substrate (Table I).

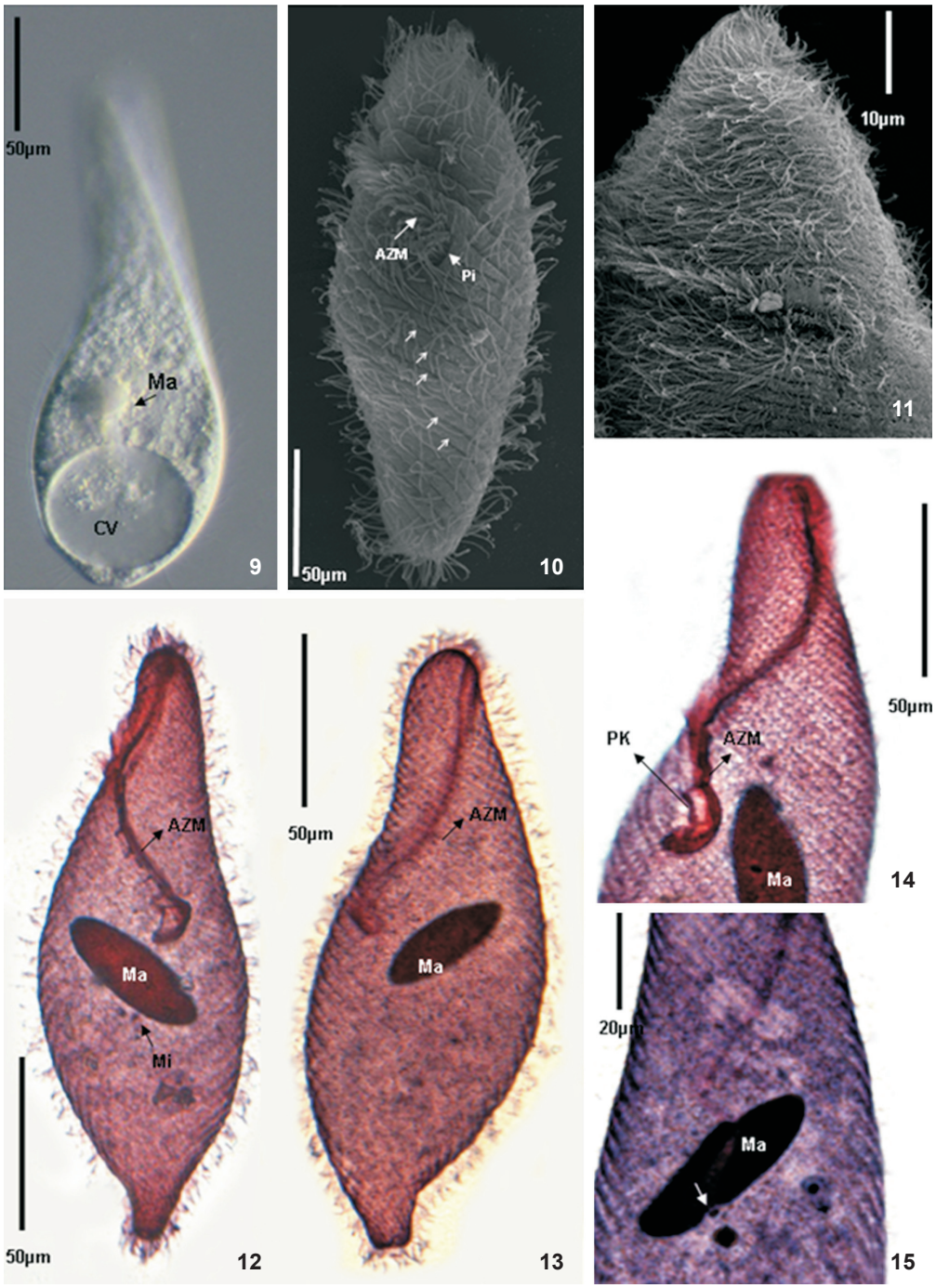
Molecular characterization

The nucleotide composition and GC content of the 18S rDNA gene sequences of the *S. minus*, *S. teres* and *S. ambiguum* obtained by Schmidt *et al.* (2007) and the sequences of *S. minus* and *S. teres* obtained in this study are presented for comparison in Table II. The 18S rDNA sequences confirmed the morphological identification of *S. teres* collected in Brazil. This sequence is composed by 1,558 bp being more similar to that of *S. teres* (98.7%), obtained by Schmidt *et al.* (2007), differing from it by 10 nucleotides (Table III).

Table II. Nucleotidic composition and GC content of *S. minus*, *S. teres* and *S. ambiguum* by Schmidt *et al.* (2007) and *S. minus* and *S. teres* from Rio de Janeiro. Data obtained from an alignment of 1312 bp. All values are given in percent.

Species	Adenine	Cytosine	Guanine	Thymine	GC content
S. minus	26.91	19.21	27.67	26.22	46.88
S. minus RJ	26.96	19.35	27.65	25.97	46.99
S. teres	26.98	19.28	27.82	25.91	47.10
S. teres RJ	26.84	19.26	27.98	25.93	47.23
S. ambiguum	26.98	19.36	27.52	26.14	46.88

The sequence from *S. minus* obtained in the present study consisted of 1,348 bp and 46.99% of the GC content. This se-



Figures 9-15. Morphology of *Spirostomum teres* (9) image obtained with differential interference contrast (DIC) microscopy of the living organism showing general shape of the cell, compact macronucleus and single contractile vacuole; (10) scanning electron micrography in ventral view showing adoral zone of membranelles, peristomial infundibulum and somatic kineties (arrows) obliquely twisted; (11) closeup of anterior end showing part of the adoral zone of membranelles. (12-15) Protargol-stained cells: (12) ventral view showing adoral zone of membranelles and nuclear apparatus; (13) dorsal view; (14) closeup of oral ciliature showing adoral zone of membranelles and paroral kinety; (15) macronuclear groove and micronuclei (arrow). (AZM) Adoral zone of membranelles, (PK) paroral kinety, (CV) contractile vacuole, (Ma) macronucleus, (Mi) micronuclei, (Pi) peristomial infundibulum.

quence showed 97.3% of similarity to the sequence from *S. minus* obtained by Schmidt *et al*. (2007) and 31 different nucleotide sites (Table III). The relatively low percentage of similarity and the high number of nucleotide differences between these two sequences suggest that they are cryptic species. The pairwise distances (absolute distance) and differences between 18S rDNA sequences of *Spirostomum* populations/species are given in Table III.

Table III. The structural similarities (%) of the 18S rDNA gene sequences of *S. minus* and *S. teres* from Rio de Janeiro (RJ), and others species/populations of *Spirostomum* species. sequenced. Absolute distance (number of nucleotidic differences) is shown in parenthesis.

Species	*S. minus*	*S. minus* RJ	*S. ambiguum*	*S. teres*
S. minus	–			
S. minus RJ	0.973 (31)	–		
S. ambiguum	0.982 (23)	0.970 (35)	–	
S. teres	0.978 (28)	0.972 (32)	0.975 (32)	–
S. teres RJ	0.973 (28)	0.964 (36)	0.971 (31)	0.987 (10)

DISCUSSION

Studies of the morphology of species of *Spirostomum* are rare. The most recent and complete work was presented by Foissner *et al*. (1992). In it, aspects of the ecology and morphology of *S. ambiguum*, *Spirostomum caudatum* Delphy, 1939, *S. minus* and *S. teres* were described. The reviews of Repak & Isquith (1974) and Foissner *et al*. (1992) were used by us as a basis for species identification.

Of the nine species that currently comprise *Spirostomum*, five (including *S. minus*) possess a moniliform macronucleus. These species are distinguished mainly by the shape of the body extremities and the size of the peristome (Repak & Isquith 1974). The other species of moniliform macronucleus differ from *S. minus* in the following characteristics (Table IV): *Spirostomum inflatum* Kahl, 1932 is a marine organism with a wider or "inflated" posterior end, whereas *S. minus* is a freshwater ciliate with a slightly tapered posterior end. Specimens of *Spirostomum loxodes* Stokes, 1885 have a tapered anterior end, with a peristome that occupies one-third of the total length of the body (vs. 1/2 in *S. minus*). *Spirostomum intermedium* Kahl, 1932 has a smaller body (300-400 µm) than other species. The species that most resembles *S. minus* is *S. ambiguum* (Table IV). Those two species are the only ones that have yellow pigments, and the major difference between them is size. Individuals of *S. ambiguum* are larger, reaching a length of up to 4 mm (Table IV), and have a larger peristome and a greater number of macronuclear nodules, somatic kineties, and micronuclei than *S. minus* (Table IV). The strain of *S. minus* described in the present study is very similar to the strains described by Repak & Isquith (1974) and Foissner *et al*. (1992) (Table IV). All morphological characteristics overlap in these three populations, except the number of somatic kineties, which is greater in the Brazilian population (30-40 vs. 20-24). However, according to Repak & Isquith (1974), the number of somatic kineties is a quite variable characteristic among morphospecies of *Spirostomum*. Thus, based on observation of morphological characters as compared to previous populations described by Repak & Isquith (1974) and Foissner *et al*. (1992), and also by comparison with similar species, we conclude that the Brazilian species described is *S. minus*.

Based on molecular data obtained for *S. minus* in the present study and by comparison with 18S rDNA gene sequence of *S. minus* provided by Schmidt *et al*. (2007) (single sequence available in GenBank database for this species), we observed a relatively low percentage of similarity (97.3%) between these two populations and 31 different nucleotide sites. This fact suggests the possibility of cryptic species within the *S. minus* complex. However, morphological characters of *S. minus* were not presented by Schmidt *et al*. (2007), therefore it is not possible to compare both populations morphologically. There is an evident need for sequencing a greater number of *S. minus* morphotypes in order to ascertain the existence of cryptic species.

Among all species of *Spirostomum*, only *S. teres* and *Spirostomum ephrussi* Delphy, 1939 have a compact macronucleus. These two species also share other similar characteristics such as length, number of kineties and number of micronuclei (Table V). However, there have been no new observations or new reports on *S. ephrussi* since its original description. Thus, based on morphological similarity Repak & Isquith (1974) considered *S. ephrussi* as junior synonym of *S. teres*. The population of *S. teres* described in the present study is very similar to the population studied by Foissner *et al*. (1992), but the number of micronuclei is greater in the former (Table V). All other morphological characteristics observed for the Brazilian strain of *S. teres* overlap with the population described previously (Table V) and confirm the identity of the species.

The phase of the life cycle must be taken into account in comparative studies of *Spirostomum* species. Packard (1948) performed a morphological study of the nuclear apparatus of *S. teres* and observed a relationship between age since division and number of macronuclear nodules. Specimens that have recently gone through division or conjugation have fewer nodules, and micronuclei with smaller diameters compared with more mature cells. Internal factors (e.g., cytoplasmatic pressure) or external (e.g., chemical composition) can also alter the shape of the macronucleus in *S. teres* (Packard 1948). In the present study, the presence of a macronuclear groove housing one micronucleus was observed in some specimens of *S. teres* (Fig. 15). This characteristic was also observed in populations of *S. teres* by Packard (1948).

Many authors state in their descriptions that *Spirostomum* species lack a paroral membrane (Tuffrau 1967, Repak & Isquith 1974, Ishida *et al*. 1988). However, Fernandez-Leborans (1985)

Table IV. Morphological characteristics of populations of *Spirostomum minus* and *S. ambiguum*.

	S. ambiguum	*S. ambiguum*	*S. minus*	*S. minus*	*S. minus*
Total length (µm)	1000-4000	1000-4000	500-800	300-800	400-800
Length of peristome	2/3 of body	–	1/2 of body	–	1/2 of body
Color	yellow	yellow	yellow	yellow	yellow
Macronuclear shape	moniliform	moniliform	moniliform	moniliform	moniliform
Number of macronuclear nodes	12-50	10-50	24	8-50	9-25
Number of micronuclei	12-100	–	4-20	–	3-30
Number of kineties	46	70-90	20-24	20-24	30-40
Reference	Repak & Isquith (1974)	Foissner *et al. (*2002)	Repak & Isquith (1974)	Foissner *et al.* (2002)	Present study

Table V. Morphological comparisons between similar species and populations of *Spirostomum teres*.

	S. ephrussi	*S. teres*	*S. teres*	*S. teres*
Total lenght (µm)	450	150-400	150-600	150-250
Lenght of peristoma	3/5 of body	1/2 of body	1/2 of body	1/2 of body
Color	–	colorless	colorless	colorless
Macronuclear shape	compact	ellipsoid	ellipsoid	ellipsoid
Number of macronuclear nodes	1	1	1	1
Number of micronuclei	–	–	1-2	1-11
Number of kineties	–	14-24	25-30	22-37
Reference	Repak & Isquith (1974)	Repak & Isquith (1974)	Foissner *et al.* (2002)	Present study

used silver-staining to confirm the presence of a paroral kinety consisting of a single row of cilia on the right margin of the peristome in *S. teres*. A paroral kinety was visible in cells of *S. minus* and *S. teres* stained with protargol in the present study (Figs 4 and 14), corroborating that observation. This paroral kinety may correspond to the paroral membrane of other heterotrichs, but a morphogenetic study is needed to verify their homology with one another.

Spirostomum species are excellent model organisms and suitable bioindicators for microbiological, ecological, environmental, and ecotoxicological analyses (Madoni *et al*. 1992, Berger *et al*. 1997, Madoni 2000, Berger & Foissner 2003). Fast, accurate characterization of species for environmental analyses requires molecular approaches that can complement or even completely replace traditional morphological methods, which are often resource- and time-consuming. However, the gene coding the 18S rRNA has been sequenced for only three species of *Spirostomum*, which emphasizes the need for molecular characterization of more *Spirostomum* species to be used in studies of environmental impact.

The use of silver-staining allowed the observation and description of a greater number of characters in *S. minus* and *S. teres*. The 18S rDNA sequences confirm the identification of *S. teres* from Brazil and suggest the existence of cryptic species for Brazilian *S. minus* and European population presented by Schmidt *et al*. (2007). The present study contributed to a better understanding of the morphology of these species, which are widely used as models in ecological studies of environmental impact and in studies of symbiosis between ciliates and human pathogenic bacteria.

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