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Unusual position of the genus *Spirotaenia* (Zygnematophyceae) among streptophytes revealed by SSU rDNA and *rbc*L sequence comparisons

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Sexual reproduction by conjugation characterizes the genus *Spirotaenia*, and based on this character alone it has long been considered a member of the class Zygnematophyceae (Streptophyta). The similarity in chloroplast morphology between the unicellular *Spirotaenia* (Mesotaeniaceae) and the filamentous *Spirogyra* (Zygnemataceae) has led to the assumption that the two genera are evolutionarily related, a view apparently supported by *rbc*L sequence comparisons (McCourt *et al.* 2000). We sequenced the nuclear-encoded SSU rDNA and the *rbc*L genes from three *Spirotaenia* species and analysed their molecular phylogeny together with other streptophyte green algae (including representatives of the class Zygnematophyceae) and embryophytes using maximum likelihood, distance, maximum parsimony and Bayesian inference methods. The significance of the results was tested by bootstrap analyses, deletion of long-branch taxa and user-defined tree topologies. The two genes congruently revealed an unexpected position of the genus *Spirotaenia* within the streptophyte algae outside the class Zygnematophyceae, but associated with *Chlorokybus*. In contrast to a previously published *rbc*L phylogeny of conjugating green algae, our results suggest that *Spirotaenia* is not specifically related to *Spirogyra* and may not belong to the class Zygnematophyceae. The absence of the well-known zygnematophycean 1506 group I intron in all SSU rDNA sequences of *Spirotaenia* supports this conclusion.

INTRODUCTION

The taxonomic history of the zygnematophycean genus *Spirotaenia* (Brebisson) Ralfs begins with Ralfs' (1848) publication, but this unicellular alga with parietal helical chloroplasts was first described and depicted by Brébisson in 1846. The peculiar chloroplast morphology, cylindrical or fusiform cell shape, coccoid cell type and sexual reproduction by conjugation are the features that characterize *Spirotaenia* and assign it to the class Zygnematophyceae. As recognized by Mollenhauer (1986), none of these features (except perhaps for the mode of sexual reproduction) can positively identify this genus as a member of the Zygnematophyceae, and the mode of sexual reproduction is known in only a few of its 25 validly described species, with the complete sexual cycle documented in only four taxa (Wawrik 1949; Hoshaw & Hilton 1966; Hilton 1970; Haga & Ehara 1977; Coesel 1981). Although *Spirotaenia* is the most species-rich genus within the Mesotaeniaceae, most of its species are apparently rare and only a few taxa are regularly reported from different parts of the world (see Mollenhauer 1986). Species richness of a genus often indicates its morphological diversity but in *Spirotaenia* this accounts mostly for cell dimensions and chloroplast morphology. Five species are characterized by having an axial chloroplast with cristate longitudinal ridges. This feature was used to classify these species as a separate subgenus *Polytaenia* Lutkemuller (Lutkemuller 1895, 1903), but this was raised later to genus level (Brook 1997) and subsequently renamed *Tortitaenia* Brook (Brook 1998).

Already at Ralfs' time the similarity in chloroplast morphology between *Spirotaenia* and *Spirogyra* Link was recognized. However, due to the difference in their vegetative habit (coccoid vs filamentous), the two genera were placed into different orders; this separation lasted until 1972, when the families Mesotaeniaceae and Zygnemataceae were combined into one order Zygnematales, based on an identical cell wall ultrastructure (Mix 1972). However, even much earlier the two genera were considered as phylogenetically related and often the unicellular *Spirotaenia* was thought of as a kind of ancestral form of the filamentous *Spirogyra* (Randhawa 1959; Yamagishi 1963). This hypothesis was one of the first tested with molecular phylogenetic methods in the Zygnematophyceae (McCourt *et al.* 1995). *Rbc*L sequence comparisons strongly supported a single clade of algae having parietal ribbon-like chloroplasts (the filamentous *Spirogyra* and *Sirogonium* Kutzing, and the unicellular *Spirotaenia;* McCourt *et al.* 1995). Further studies with the same marker supported clades also for unicellular or filamentous taxa with laminate or stellate chloroplasts (Park *et al.* 1996; McCourt *et al.* 2000). However, such clades were not confirmed by small subunit ribosomal DNA (SSU rDNA) phylogenies (Besendahl & Bhattacharya 1999; Gontcharov *et al.* 2003).

To investigate the position of the genus *Spirotaenia sensu lato* (including *Tortitaenia)* in the SSU rDNA phylogeny, sequences from three *Spirotaenia* species were obtained and included in a dataset that comprised 21 sequences from conjugating green algae, 11 from other streptophyte algae and four from embryophytes. Because results clearly contradicting an earlier published *rbc*L phylogeny were obtained, the *rbc*L genes from the same *Spirotaenia* species were also sequenced and incorporated into the *rbc*L dataset presented by McCourt *et al.* (2000). Based on our findings, we conclude that *Spirotaenia* is distinct from any known zygnematophycean taxon and may not belong to the class Zygnematophyceae.

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Table 1. Taxa in the class Zygnematophyceae and other streptophytes with corresponding EMBL/GenBank accession numbers of SSU rDNA and *rbc*L sequences used in this study; newly determined sequences are indicated in bold face, and include the origin of the strain.[1]

Taxon	Accession number (strain)	
	SSU rDNA	*rbc*L
Closterium acerosum (Schranke) Ehrenberg *ex* Ralfs	AJ428087	AF203492
Cosmarium botrytis Meneghini *ex* Ralfs	X79498	AF203493
Cosmocladium perissum Roy & Bisset		AF203494
Cylindrocystis sp.		U38695
***Cylindrocystis brebissonii* Meneghini *ex* De Bary**	**AJ549228 (ACOI 55)**	
Desmidium sp.		AF203495
Desmidium swartzii (C. Agardh) C. Agardh *ex* Ralfs	AJ428133	
Euastrum pectinatum Brébisson *ex* Brébisson *in* Ralfs	**AJ549227 (SVCK 203)**	AF20349
Gonatozygon monotaenium De Bary	AJ428084	U71438
Gonatozygon (Genicularia) spirotaenium De Bary	X74753	U71439
Groenbladia undulata (Nordstedt) Förster		AF203498
Hyalotheca dissiliens Brébisson *ex* Ralfs		AF203499
Micrasterias rotata [Greville] Ralfs *ex* Ralfs		AF203500
Mesotaenium caldariorum (Lagerheim) Hansgirg	**AJ549229 (ACOI 898)**	U38696
Mougeotia sp.		U38699
Netrium digitus (Ehrenberg *ex* Ralfs) Itzigsohn & Rothe	AJ428073	U38698
Netrium interruptum (Brébisson *ex* Ralfs) Lütkemüller		
Onychonema sp.	AJ428071	AF203501
Penium margaritaceum (Ehrenberg) Brébisson *in* Ralfs		AF203502
Pleurotaenium trabecula (Ehrenberg) Nägeli	AF115440	AF203503
Roy a anglica G.S. West *in* Hodgetts		
Sirogonium melanosporum (Randhawa) Transeau	AJ428081	LI3484
Sphaerozosma sp.		AF203504
Spirogyra (Sirogonium) stictica (J.E. Smith) Wille	AJ428076	
***Spirogyra* sp.**	**AJ549231 (M 2157)**	**AJ549239 (M 2157)**
Spirogyra sp. M 1810	AJ428074	
Spirogyra maxima (Hassall) Wittrock		LI1057
[Spirotaeniacondensata][2]		U38700
***Spirotaenia alpina* Schmidle**	**AJ549234 (Kies 2686.b)**	**AJ549237 (Kies 2686.b)**
***Spirotaenia condensata* (Brébisson) Ralfs**	**AJ549233 (SVCK 312)**	**AJ549236 (SVCK 312)**
***Spirotaenia obscura* Ralfs**	**AJ549232 (UTEX 1520)**	**AJ549238 (UTEX 1520)**
Staurastrum pingue Teiling		AF203506
Staurodesmus (Arthrodesmus) sp.		AF203491
Teilingia (Sphaerozosma) granulata (Roy & Bisset) Bourrelly	X79496	
Zygnema sp.		
Zygnema peliosporum Wittrock	AJ428077	U38701
Zygnemopsis circumcarinata (Czurda) Krieger		
Zygnemopsis sp.	X79495	AF203508
***Zygogonium tunetanum* Gauthier-Lievre**		**AJ549235 (UTCC 136)**
Xanthidium subhastiferum W West	**AJ549230 (UTCC 136)**	AF203507
Streptophyte algae		
Chaetosphaeridium globosum (Nordstedt) Klebahn	AJ250110	AF408250
Chaetosphaeridium ovalis Smith		AF408251
Chara connivens Salzmann *ex* A. Braun		L13476
Chara globularis Thuillier	Y16465	
Chlorokybus atmophyticus Geitler	M95612	AF408255
Coleochaete irregularis Pringsheim	AF408231	AF408248
Coleochaete orbicularis Pringsheim		LI3477
Coleochaete scutata Brébisson	X68825	
Entransia fimbriata Hughes	**AJ549226 (UTEX 2353)**	AF203496
Klebsormidium nitens (Meneghini *in* Kützing) Lokhorst	AJ250112	
Klebsormidium subtilissimum (Rabenhorst) Silva, Mattox & Blackwell		AF408253
Klebsormidium sp.		L13478
Lamprothamnium macropogon (A. Braun) J.L. Ophel		U27534
Lychnothamnus barbatus (Meyen) Leonhardi		U27533
Mesostigma viride Lauterborn	AJ250109	AF166114
Nitella capillaris (Krocker) J. Groves & Bullock-Webster	AJ250111	
Nitella translucens (Persoon) Agardh		L13482
Nitellopsis obtusa (Desvaux *in* Loiseleur-Deslongchamps) J. Groves	AF408226	U27530
Tolypella intricata Leonhardi		U27532
Tolypella nidifica (O. Müller) A. Braun		U27531
Tolypella prolifera (Ziz *ex* A. Braun) Leonhardi	AF408228	
Embryophytes		
Anthoceros agrestis Paton	X80984	
Anthoceros agrestis Paton	X83522	
Huperzia taxifolia (Swartz) Trevisan		X04465
Marchantia polymorpha Linnaeus		

Table 1. Continued.

Taxon	Accession number (strain) SSU rDNA	*rbc*L
Ophioglossum engelmanii Prantl		LI 1058
Oryza sativa Linnaeus		D00207
Pseudotsuga menziesii (Mirbel) Franco		X52937
Psilotum nudum (Linnaeus) Beauvois		LI 1059
Sphagnum palustre Linnaeus	Y11370	

[1] ACOI. Coimbra Collection of Algae, University of Coimbra, Portugal (http://www.uc.pt/botanica/ACOI.htm); Kies, Prof. Ludwig Kies (University of Hamburg, Germany) research collection; M, Culture Collection Melkonian, Botanical Institute, University of Cologne, Germany; SVCK. Sammlung von Conjugaten-Kulturen, University of Hamburg, Germany (http://www.biologie.uni-hamburg.de/b-online/d44_l/44_l.htm); UTEX. Culture Collection of Algae at the University of Texas at Austin, USA (http://www.bio.utexas.edu/research/utex/); UTCC, University of Toronto Culture Collection of Algae and Cyanobacteria (http://www.botany.utoronto.ca/utcc/). Names of taxa in parentheses correspond to those used in the culture collection catalogue.

[2] *[Spirotaenia condensata]* presumably represents an unknown *Spirogyra* strain.

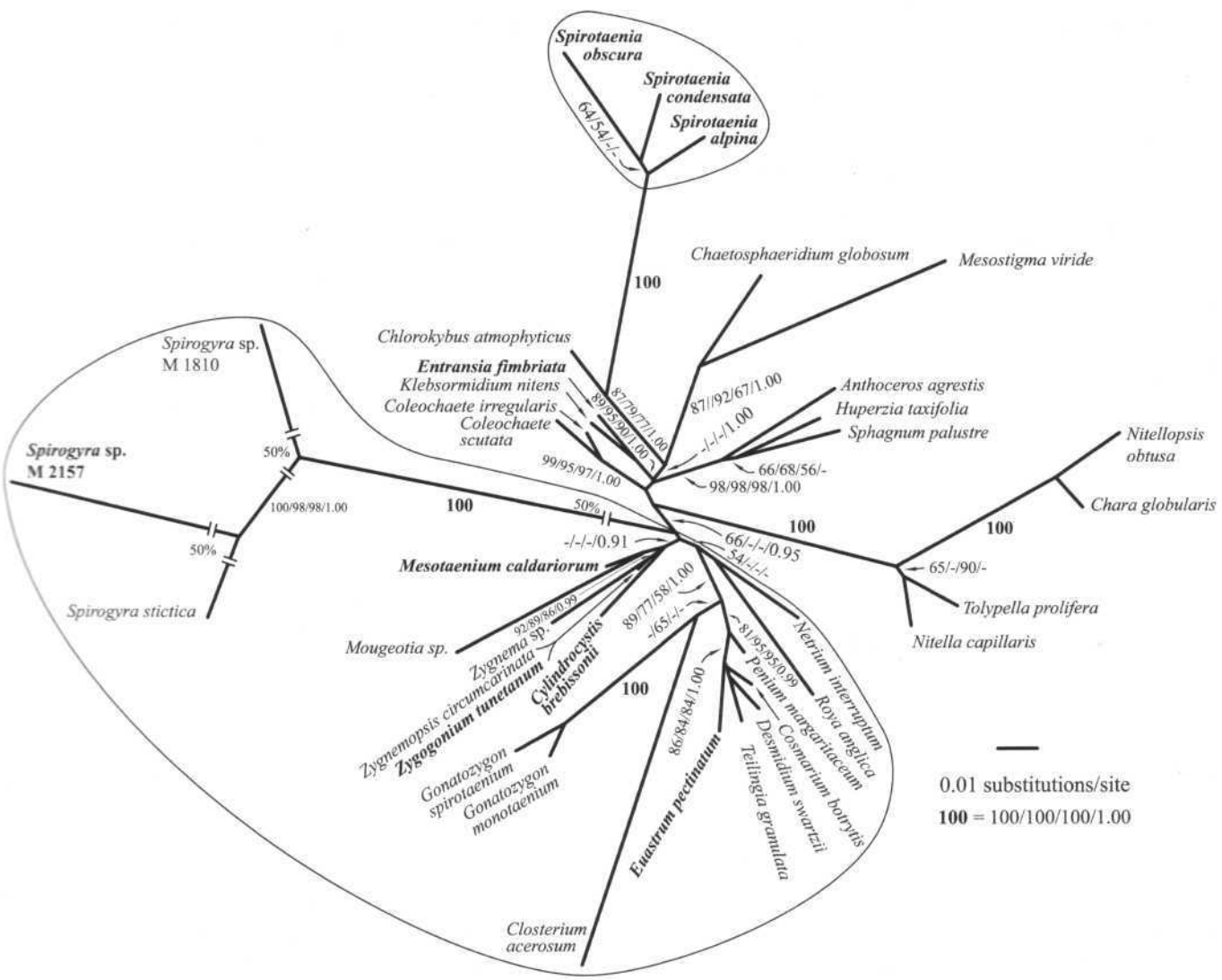

Fig. 1. Unrooted phylogenetic tree showing relationships of conjugating green algae (Zygnematophyceae) and other streptophytes based on comparisons of 36 SSU rDNA sequences (1719 positions). The tree topology shown was inferred by ML using TrN+I+G; corresponding ML, NJ and MP bootstrap percentage values ≥ 50% and PP (BI) ≥ 0.90 are given. New SSU rDNA sequences obtained during this study are indicated in bold (for accession numbers, see Table 1). The two separate clades of conjugating green algae (Zygnematophyceae *sensu stricto* and the three *Spirotaenia* species) are encircled.

MATERIAL AND METHODS

Cultures

Nine strains of streptophyte green algae sequenced for this study were obtained from different culture collections (Table 1) and grown in a modified WARIS-H culture medium (Kies 1967; McFadden & Melkonian 1986) at 20°C with a photon fluence rate of 40 mmol $m^{-2}s^{-1}$ in a 14:10h light-dark cycle.

DNA extraction, amplification and sequencing

Cells were harvested after 2-4 wk of growth and washed several times with distilled water. Total genomic DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the recommendations of the manufacturer. SSU rDNA and *rbc*L were amplified by polymerase chain reactions (PCR) (Saiki *et al.* 1988) using thermocycling protocols and 5'-biotinylated PCR primers as described previously (Marin *et al.* 1998). The primers used to amplify and sequence the newly generated sequences are listed in the following: SSU rDNA (Marin *et al* 1998; Gontcharov *et al.* 2003) and *rbc*L (Gontcharov *et al.*, in press). Prior to sequencing, PCR products were purified with the Dynabeads M-280 system (Dynal Biotech, Oslo, Norway). Sequencing reactions were prepared as described by Hoef-Emden *et al.* (2002) and sequences finally determined with a Li-Cor IR^2 automated sequencer (LI-COR Biosciences, Lincoln, NE, USA).

Sequence alignment and phylogenetic analyses

Sequences were manually aligned using the Olson Multiple Sequence Alignment Editing Program (Olsen 1990). For coding regions of the SSU rDNA of the Zygnematophyceae, the alignment was guided by primary and secondary structure conservation. The alignments are available from the authors on request. Phylogenetic trees were inferred with maximum likelihood (ML), distance (NJ) and maximum parsimony (MP) methods using the PAUP 4.0b 10 program package (Swofford 1998) and Bayesian inference (BI; MrBayes v3.0b3, Huelsenbeck & Ronquist 2001). SSU rDNA (1719 unambiguously aligned positions; only the presumptive coding regions were used) and *rbc*L datasets (1352 nucleotides) were analysed. To decide which evolutionary model (for ML and NJ analyses) fitted the data best, the program Modeltest 3.04 (Posada & Crandall 1998) was used. Distances for NJ analyses were calculated by ML. The robustness of the trees was tested by bootstrap percentages (BP; Felsenstein 1985) using 1000 (NJ) or 100 (ML and MP) replications with a starting tree obtained via stepwise addition (ML and MP) and by posterior probabilities (PP) in BI. In MP, 10 heuristic searches with random taxon input order were used for each bootstrap replicate. In BI, the Markov chains were run for 1 million generations sampling every 100 generations for a total of 10,000 samples. The first 1000 samples from the run were discarded as burn-in. The remaining samples were combined into a single file and analysed using the 'sumt' command in MrBayes. BP < 50% and PP < 0.90 were ignored.

User-defined trees were generated manually by modifying the treefile of the 'best tree' using Tree View 1.6.2 (Page 1996). To compare user-defined tree topologies with the 'best tree', site-wise log-likelihoods were calculated for each topology in PAUP and used as input for CONSEL (Shimodaira & Hasegawa 2001), a program which calculates the probability values according to the Kishino-Hasegawa test (KH; Kishino & Hasegawa 1989), the Shimodaira-Hasegawa test [SH; Shimodaira & Hasegawa 1999; both weighted (w) and unweighted] and the approximately unbiased (AU) test using the multiscale bootstrap technique (Shimodaira 2002).

RESULTS

Presence of 1506 group I introns

The PCR products resulting from the amplification of nuclear-encoded SSU rDNA in three *Spirotaenia* species were of standard size (c. 1800 bp) and the well-known zygnematophycean 1506 group I intron near the 3' terminus of the coding region (Bhattacharya *et al.* 1994, 1996; Besendahl & Bhattacharya 1999) was not detected. Another taxon sequenced during this study that lacks this intron is *Spirogyra* sp. (M 2157). Its SSU rDNA sequence is very similar to those of *Spirogyra* sp. (strains SVCK 253 and 261; Gontcharov *et al.* 2003), and the three strains presumably belong to the same unidentified *Spirogyra* species, although this would mean that it had a wide geographical distribution (SVCK strains were isolated from two localities in Hamburg, Germany, whereas M 2157 originates from the Far East, Russia).

Phylogenetic analysis of SSU rDNA coding regions

In an unrooted ML analysis, the 36 streptophyte sequences were distributed in seven clades (Fig. 1). Some of the clades were in agreement with an adopted taxonomic subdivision of the Streptophyta and correspond to its taxonomic entities Coleochaetophyceae, Charophyceae and Mesostigmatophyceae. The internal branching order among clades remains unresolved in our phylogeny. Conjugating green algae of the class Zygnematophyceae were distributed in two clades, one included the great majority of the class representatives (19 sequences; 66% ML BP and 0.95 PP) and the other consisted of three *Spirotaenia* sequences with *Chlorokybus atmophyticus* as a sister (87% BP in ML, 77-79% in NJ and MP and 1.00 PP support for their common branch). The Zygnematophyceae cluster is topologically split into three subgroups, a long-branched *Spirogyra* clade diverging first, an unresolved Zygnemataceae—Mesotaeniaceae assemblage and a well-supported Desmidiales clade (89, 77, 58% BP, 1.00 PP) with the mesotaeniacean *Netrium* (Ehrenberg) Itzigsohn & Rothe and *Roya* West & G.S. West as a sister group (54% ML BP). The topology of the Desmidiales clade was basically in agreement with traditional classification of the order, and branches corresponding to the families Gonatozygaceae, Closteriaceae, Peniaceae and Desmidiaceae were distinct from each other. The order of their divergences was well resolved, except for a Gonatozygaceae-Closteriaceae sister group relationship (the latter with no statistical support; Fig. 1). Taxa of the Zygnematales, however, did not split into the traditional families Mesotaeniaceae and Zygnemataceae, nor did they group according to distinct types of chloroplast morphology (see also Gontcharov *et al.* 2003). Moreover, several zygnematalean taxa were loosely associated with the Desmidiales *(Netrium* and *Roya)* or even placed outside the class *(Spirotaenia). Spi-*

Table 2. Comparison of the maximum likelihood trees (Figs 1, 2) with user-defined trees by AU, KH and SH tests (for details, see Results).[1]

Tree topology suggested/dataset	Tree	Diff -lnL[2]	*P*[3]				
			AU	KH test	wKH test	SH test	wSH tets
Spirotaenia-Chlorokybus clade is a sister to the Zygnematophyceae							
SSU rDNA	1	3.0	0.433	0.329	0.329	0.574	0.662
*rbc*L. without *Mesostigma*	2	5.1	0.291	0.278	0.278	0.580	0.524
Spirotaenia-Chlorokybus clade is a sister to *Spirogyra*							
SSU rDNA	3	4.6	0.212	0.262	0.212	0.487	0.434
Spirotaenia is a sister to the Zygnematophyceae							
SSU rDNA	4	31.5	**0.013**	**0.015**	**0.015**	**0.032**	**0.027**
***rbc*L**	5	33.0	**0.004**	**0.006**	**0.006**	**0.009**	**0.009**
*rbc*L. without *Mesostigma*	6	44.6	**0.001**	**0.001**	**0.001**	**0.001**	**0.002**
***rbc*L,** without *Mesostigma* and *Chlorokybus*	7	9.0	0.221	0.158	0.158	0.251	0.302
Spirotaenia is a sister to *Spirogyra*							
SSU rDNA	8	31.6	**0.014**	**0.016**	**0.016**	**0.033**	**0.044**
SSU rDNA, without *Chlorokybus*	9	9.9	**0.034**	**0.044**	**0.044**	**0.046**	0.072
***rbc*L**	10	70.0	**2e-04**	**2e-04**	**2e-04**	**2e-04**	**3e-04**
***rbc*L.** without *Mesostigma*	11	64.4	**5e-07**	**2e-04**	**2e-04**	**2e-04**	**2e-04**
***rbc*L.** without *Mesostigma* and *Chlorokybus*	12	20.9	**0.038**	0.051	**0.049**	0.052	0.078

[1] User defined tree significantly worse than the best tree at $P < 0.05$ is indicated in bold.
[2] Difference in -log-likelihood between the best tree and the user-defined tree.
[3] Probability of obtaining a more extreme T value under the null hypothesis of no difference between the two trees (one-tailed test). AU, the *P* value of the approximately unbiased test calculated from the multiscale bootstrap; KH, the Kishino-Hasegawa test; wKH, the weighted Kjshino—Hasegawa test; SH, the Shimodaira—Hasegawa test; wSH, the weighted Shimodaira-Hasegawa test.

rotaenia and *Spirogyra*, sharing similar chloroplast morphology, were positioned in very different parts of the tree (Fig. 1). Among the *Spirogyra* taxa, strain M 2157 (characterized by the absence of the 1506 group I intron) has the longest individual branch, but was still firmly positioned within the genus.

The accelerated evolutionary rates of some streptophyte sequences or clades could have affected the topology and resolution of the tree. Therefore, the fast-evolving *Spirogyra* and Charophyceae clades were excluded from the analysis. The reduced dataset slightly improved the support for some nodes but did not change the tree topology (results not shown). To test for a possibly artificial attraction of the *Spirotaenia* clade by *Chlorokybus* Geitler, a dataset without the latter sequence was analysed. As a result, *Spirotaenia* was positioned as a sister to the Zygnematophyceae, but without any support for their common branch (tree not shown).

To evaluate the significance of the results obtained, user-defined tree topologies were generated and compared with the ML topology presented in Fig. 1 and with the ML topology obtained when *Chlorokybus* was removed from the analysis, using a number of tests (see Material and Methods). We assessed the likelihood of *Spirotaenia* being a sister to all other members of the class Zygnematophyceae (complete dataset), and also specifically to *Spirogyra* (with or without *Chlorokybus* in the dataset). As seen from the results of the tests, positioning the *Spirotaenia—Chlorokybus* lineage as sister to the Zygnematophyceae (Table 2, tree 1) or *Spirogyra* (tree 3) was not significantly different from the best tree (Fig. 1). However, topologies in which the *Spirotaenia—Chlorokybus* clade was split and only *Spirotaenia* was positioned as a basal divergence of the Zygnematophyceae (tree 4) or as a sister to *Spirogyra* (tree 8) were rejected by all tests (Table 2). With the reduced dataset (with *Chlorokybus* excluded), positioning *Spirotaenia* as sister group to *Spirogyra* (tree 9) is also significantly rejected by most of the tests.

Phylogenetic analysis of *rbc*L

The phylogenetic position of *Spirotaenia* as revealed in the SSU rDNA analyses clearly contradicted results obtained previously using *rbcL* sequence comparisons (McCourt *et al.* 1995, 2000). Therefore, we assessed the phylogenetic position of the three *Spirotaenia* taxa with the same *rbc*L dataset used by McCourt *et al* (2000). The analyses demonstrated that the new sequences formed a robust independent clade outside an otherwise significantly supported Zygnematophyceae (tree not shown) and were not related to a sequence referred to *S. condensata* (accession number U38700).

To test phylogenetic hypotheses arising from the SSU rDNA phylogeny further, an extended *rbc*L dataset was created. In addition to the three *Spirotaenia* species, the following were added to the original dataset: (1) the strain *Spirogyra* sp. M 2157, which is characterized by the absence of the 1506 group I intron in the SSU rDNA (see above); (2) *C. atmophyticus*, which showed the highest affinity to *Spirotaenia* in the SSU rDNA sequence comparisons (see above); and (3) *Mesostigma* Lauterborn, another streptophyte alga for which the *rbc*L sequence recently became available. In an attempt to shorten the relatively long branch (LB) of the species of *Klebsormidium* Silva, Mattox & Blackwell (L13478) that had been excluded from analysis by McCourt *et al.* (2000), one more *Klebsormidium* sequence, *K. subtilissimum* (AF408253), was added to the dataset. Two sequences were excluded from the analysis: *Roya anglica* (U38694), which we have shown to be ambiguous (Gontcharov *et al.*, in press), and *Spondylosium pulchellum* (Archer) Archer, whose GenBank sequence (AF203505) is identical to that of *Sphaerozosma* sp. (AF203504). These changes resulted in a dataset comprising

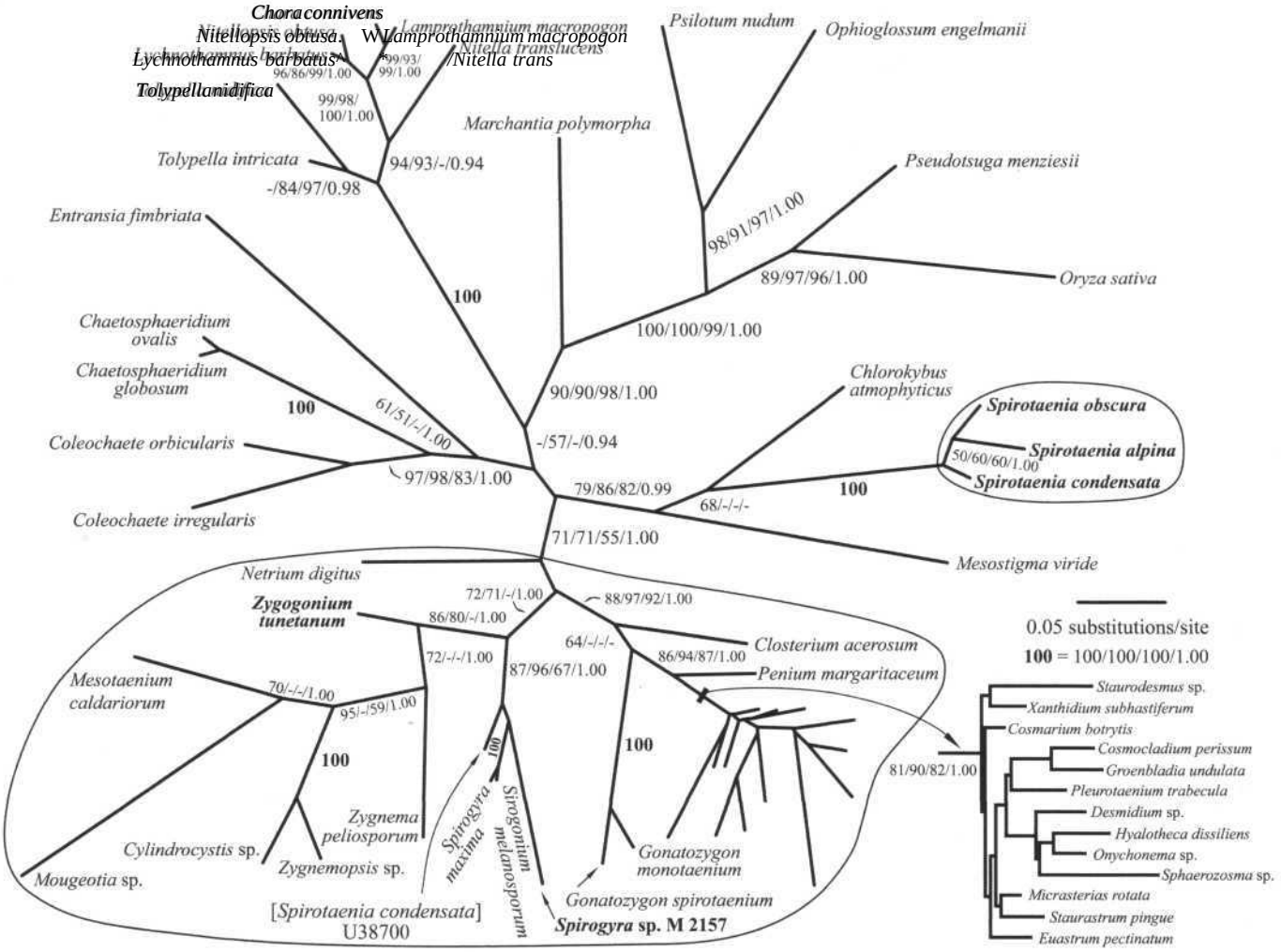

Fig. 2. Unrooted phylogeny of the class Zygnematophyceae and other streptophyte green algae based on comparisons of 50 *rbc*L sequences (1352 nucleotides). The tree shown was constructed with ML (GTR+I+Γ); ML, NJ and MP BP values ≥ 50% and PP ≥ 0.90 (Bayesian inference) are given for all supported nodes except those within the Desmidiaceae clade. New *rbc*L sequences obtained during this study are indicated in bold (for accession numbers, see Table 1). The two separate clades of conjugating green algae (Zygnematophyceae *sensu stricto* and the three *Spirotaenia* species) are encircled. A database sequence (U38700) referred to *S. condensata* (ARL 1300) is placed in brackets (see Discussion for interpretation of this sequence).

52 taxa. A test for homogeneity of base frequencies across the taxa revealed no GC bias in the complete dataset, as well as in alignment with the third codon positions only. Among the sequences added, only *K. subtilissimum* (AF408253) was distinct in elevated GC content in the third codon positions (31% vs the 15-19% which is typical for *Spirotaenia, Chlorokybus, Mesostigma* and most other streptophyte green algae).

The added sequences (except for *Spirogyra* sp. M 2157, see below) were not positioned in any of the clades resolved by McCourt *et al.* (2000), but formed two new assemblages. *Klebsormidium* was placed outside the Zygnematophyceae; however, there was no support for the very short node separating *Klebsormidium* from the Zygnematophyceae. Our *Spirotaenia* spp. were again found in a clade that included *Chlorokybus,* but also *Mesostigma,* and this lineage branched prior to *Klebsormidium* and the Zygnematophyceae (tree not shown). Removal of *Klebsormidium* spp. from the analysis resulted in increased significance for the Zygnematophyceae (71% BP in ML and NJ, 55% in MP and 1.00 PP) with the *Mesostigma—Chlorokybus—Spirotaenia* clade (79-86% BP, 0.99 PP) as a sister to the Zygnematophyceae (Fig. 2). For all further analyses, *Klebsormidium* was omitted from the dataset. When *Mesostigma* was excluded from the analysis, *Netrium* occupied a more internal position in the Zygnematophyceae, diverging on the branch leading to the Desmidiales (see also McCourt *et al.* 2000); however, *Chlorokybus-Spirotaenia* was even more distant from the Zygnematophyceae, and was positioned as a sister to the Charophyceae (tree not shown). Removal of both *Chlorokybus* and *Mesostigma* from the dataset did not alter the position of *Spirotaenia* with respect to the Charophyceae (tree not shown). Depending on the method of analysis and the dataset, *Spirogyra* sp. M 2157 branched either before or after the GenBank sequence U38700 designated *[Spirotaenia condensata]* within an otherwise well-supported *Spirogyra* clade (Fig. 2).

To investigate a possible effect of degenerated and putatively homoplasious third codon positions (Nickrent *et al.*

2000. Nozaki et al., 2000) on the tree topology, a dataset with only the first and second positions (901 nucleotides) was also analysed. The three *Spirotaenia* sequences again formed a robust clade topologically associated with *Chlorokybus* and *Mesostigma.* or the Charophyceae, depending on the method of analyses, and were not positioned within the Zygnematophyceae (tree not shown). The Zygnematophyceae clade obtained no statistical support, but its internal topology was nearly identical to that depicted in Fig. 2. The reduced dataset thus lowered phylogenetic resolution but changed neither the composition of the clades nor the tree topology.

Tests of user-defined *rbch* tree topologies with several datasets were performed to evaluate a possible sister group relationship of *Spirotaenia* with the Zygnematophyceae or with *Spirogyra* (Table 2). The results showed that positioning of rhe clade comprising *Spirotaenia* and *Chlorokybus (Mesostigma* was excluded from the dataset) as a sister to the Zygnematophyceae (tree 2) was not significantly different from the best tree; however, placing *Spirotaenia* alone in the same position (trees 5, 6) obtained no support in all analyses (Table 2 Positioning *Spirotaenia* as sister to the Zygnematophyceae could be enforced only when both *Mesostigma* and *Chlorokybus* were excluded from the analyses (tree 7). An affiliation of *Spirotaenia* to *Spirogyra* (trees 10-12) was rejected in all datasets and with most tests (only when both *Mesostigma* and *Chlorokybus* were removed, did the *P* values for the KH, SH and wSH tests range between 0.051 and 0.078; Table 2).

DISCUSSION

Our phylogenetic analyses of 36 SSU rDNA and 52 *rbc*L sequences revealed an unexpected position of the genus *Spirotaenia* among streptophyte green algae. Both genes are consistent in placing *Spirotaenia* separate from all other members of the class Zygnematophyceae, instead placing it with the genus *Chlorokybus* (Chlorokybophyceae). Based on a comparison of the newly obtained *rbch* sequence of *S. condensata* and the outcome of our phylogenetic analyses, we conclude that the previously published sequence (U38700) assigned to the same species and shown to be a close relative of *Spirogyra* (McCourt *et al* 1995, 2000) was most likely derived from an unknown *Spirogyra* strain and presumably the result of either a mix up of strains or DNA samples. Therefore, the present stydy is the first in which the phylogenetic affiliation of the genus *Spirotaenia* is assessed with molecular tools.

In both (SSU rDNA and *rbc*L) trees, the three *Spirotaenia* species formed a strongly supported clade with a relatively long common branch (Figs 1,2). We think it is unlikely that the unusual position of the *Spirotaenia* sequences was caused by LB attraction because *Spirotaenia* was not attracted to the extremely LB of the *Spirogyra* sequences in the SSU rDNA phylogeny (Fig. 1). Instead, a likely LB attraction was observed between the Charophyceae (characterized by a branch of a similar length to that of *Spirotaenia)* and *Spirogyra,* and this apparently resulted in only moderate support for the monophyly of the Zygnematophyceae in the SSU rDNA phylogeny. With the *rbc*L dataset having no obvious LB taxa and functioning under different evolutionary constraints, the support for the position of *Spirotaenia* outside the Zygnematophyceae is even stronger than in the SSU rDNA dataset (Fig. 2).

The *Spirotaenia—Chlorokybus* and *Spirotaenia—Chlorokybus-Mesostigma* clades (in the SSU rDNA and *rbc*L analyses, respectively) were supported nearly similarly in both phylogenies, but their position in the trees was not defined. These clades could well be sisters of the Zygnematophyceae according to tests with user-defined trees (Table 2, trees 1,2). However, placement of *Spirotaenia* alone into the Zygnematophyceae or as a sister group to it (trees 4-6, 8, 10, 11) was rejected by all analyses when *Chlorokybus* was retained in the dataset. Could the unusual position of *Spirotaenia* be due to homoplasy of gene sequences between this taxon and *Chlorokybus?* Comparison of phylogenies with or without *Chlorokybus* suggests that this taxon affects the position of *Spirotaenia* in the SSU rDNA and *rbch* trees, and the user-defined topology tests support this conclusion (Table 2, trees 7, 9, 12). Because *Chlorokybus* is not a LB taxon in both the SSU rDNA and *rbc*L phylogenies, homoplasy may be responsible for the attraction of *Chlorokybus* to *Spirotaenia.* However, for the *rbc*L dataset, neither an unusual GC bias nor saturation in the third positions of *rbch* codons was observed upon the inclusion of the *rbch* sequences of *Mesostigma* and *Chlorokybus.* Thus, although we cannot exclude attraction between sequences of *Chlorokybus* and *Spirotaenia* due to homoplasy, we can almost definitely rule out a sister group relationship between *Spirotaenia* and *Spirogyra,* as is often suspected and was previously deduced from molecular phylogenetic analyses (McCourt *et al.* 2000). Based on the present analysis, we may conclude either that *Spirotaenia* is a sister to all Zygnematophyceae or that it is perhaps not even monophyletic with the Zygnematophyceae.

The lack of the 1506 group I intron in the three *Spirotaenia* SSU rDNA sequences is another indication of the distinctness of the genus. Within the Zygnematophyceae, absence of this intron has so far only been demonstrated in three closely related *Spirogyra* isolates (among more than 100 zygnematophycean SSU rDNA sequences known to date, unpublished observations). It is very likely that absence of the intron in three strains of *Spirogyra* is due to a single loss event (Gontcharov *et al* 2003). The loss of the 1506 group I intron in *Spirogyra* was apparently associated with an acceleration of evolutionary rates in the SSU rRNA gene because these strains have the most divergent SSU rDNA sequences of the Zygnematophyceae. Although secondary loss of the 1506 intron cannot be ruled out for *Spirotaenia* as well, the phylogenetic position of this genus among streptophyte green algae makes this scenario unlikely.

The presence in our dataset of *Spirotaenia* taxa with different chloroplast morphology [a character previously used to separate *Spirotaenia* into two genera (Brook 1997, 1998)] allowed us to test validity of this taxonomic rearrangement. The SSU rDNA phylogeny revealed that the two species with *Tortitaenia*-type chloroplasts could not be separated from *S. condensata,* thus questioning the concept of the new genus. The *rbch* phylogeny is less conclusive in this respect because the branching pattern within *Spirotaenia* was dependent on taxon sampling and method of analysis. Thus, a first assessment of the genus *Tortitaenia* with molecular markers suggests that the morphological character used to separate it from *Spirotaenia* may not adequately reflect the evolutionary process and that further analyses are required.

Spirotaenia is assigned to the Zygnematophyceae on the

basis of the presence of sexual reproduction by conjugation (documented in four species, two of which were studied here: *S. condensata* and *S. obscura)* and its helical chloroplasts resembling those of *Spirogyra.* A similar chloroplast type, however, occurs in other green algae (e.g. in the genera *Koliella* Hindäk, *Elakatothrix* Wille, *Gloeotila* Kützing and *Closteriospira* Reverdin), and this character should not be used as evidence of common origin. In fact, the helical chloroplasts ascend in opposite directions in *Spirotaenia* (from left to right) and *Spirogyra* (from right to left), and there are some differences in their fate during the sexual cycles (Hoshaw & Hilton 1966).

The process of conjugation is rather diverse among the Zygnematophyceae (Brook 1981), and *Spirotaenia* differs from all other conjugating green algae in producing no conjugation tube or vesicle. Instead, paired cell walls gelatinize completely prior to gamete fusion (Hoshaw & Hilton 1966; Biebel 1975). Another distinct character of sexual reproduction in *Spirotaenia* is the division of the conjugating cell into two gametes that fuse with the respective gametes of the opposite cell to yield a pair of zygospores (Hoshaw & Hilton 1966; Haga & Ehara 1977). Conjugation resulting in the formation of two zygotes has been reported in natural populations of a few desmid taxa [some species of *Closterium* Nitzsch *ex* Ralfs and *Actinotaenium* (Nägeli) Teiling] but is not known in the Zygnematales (Růžicka 1977), in which *Spirotaenia* is currently placed.

In conclusion, the present study provides evidence that the genus *Spirotaenia,* previously regarded as a close relative to *Spirogyra,* occupies an unusual position among streptophyte green algae. More specifically, *Spirotaenia* is not a sister of *Spirogyra* and arguably does not belong to the Zygnematophyceae *sensu stricto.* It would be of great interest to study this largely neglected green algal genus in much more detail in the future, especially its life history, ultrastructure and its biochemical and molecular properties.

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REFERENCES

BESENDAHL A. & BHATTACHARYA D. 1999. Evolutionary analyses of small-subunit rDNA coding regions and the 1506 group I introns of Zygnematales (Charophyceae, Streptophyta). *Journal of Phycology* 35: 560-569.

BHATTACHARYA D., SUREK B., RÜSING M., DAMBERGER S. & MELKONIAN M. 1994. Group I introns are inherited through common ancestry in the nuclear-encoded rRNA of Zygnematales (Chlorophyta). *Proceedings of the National Academy of Sciences* 91: 9916—9920.

BHATTACHARYA D., DAMBERGER S., SUREK B. & MELKONIAN M. 1996. Primary and secondary structure analysis of the rDNA group I introns of the Zygnematales. *Current Genetics* 29: 282—286.

BIEBEL P. 1975. Morphology and life cycles of saccoderm desmids in culture. *Nova Hedwigia, Beiheft* 42: 39—57.

BROOK A.J. 1981. *The biology of desmids.* Blackwell Scientific, Oxford, UK. 276 pp. *[Botanical Monographs* 16.]

BROOK A.J. 1997. The proposed establishment of a new desmid genus *Polytaenia,* previously the sub-genus *Polytaenia* of the genus *Spirotaenia,* and a description of a new species, *P. luetkemuelleri. The Quekett Journal of Microscopy* 38: 7-14.

BROOK A.J. 1998. *Tortitaenia* nom. nov. pro *Polytaenia* Brook, a name of a genus of saccoderm desmids. *The Quekett Journal of Microscopy* 38: 146.

COESEL P.F.M. 1981. *Spirotaenia diplobelica* spec. nov. (Chlorophyta, Mesotaeniaceae). *Acta Botanica Neerlandica* 30: 433-437.

FELSENSTEIN J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

GONTCHAROV A.A., MARIN B. & MELKONIAN M. 2003. Molecular phylogeny of conjugating green algae (Zygnematophyceae, Streptophyta) inferred from SSU rDNA sequence comparisons. *Journal of Molecular Evolution* 56: 89-104.

GONTCHAROV A.A., MARIN B. & MELKONIAN M. Are combined analyses better than single gene phylogenies? A case study using SSU rDNA and rbcL sequence comparisons in the Zygnematophyceae (Streptophyta). *Molecular Biology and Evolution.* In press.

HAGA M. & EHARA J. 1977. Observations on vegetative cells and the process of sexual reproduction in *Spirotaenia obscura* Ralfs. *Bulletin of the Japanese Society of Phycology* 25: 45—54.

HILTON R.L. 1970. *A cytological and morphological study of the alga* Spirotaenia condensata *Brebisson.* PhD thesis. University of Arizona. 87 pp.

HOEF-EMDEN K., MARIN B. & MELKONIAN M. 2002. Nuclear and nucleomorph SSU rDNA phylogeny in the Cryptophyta and the evolution of cryptophyte diversity. *Journal of Molecular Evolution* 55: 161-179.

HOSHAW R.W. & HILTON R.L. 1966. Observation on the sexual cycle of the saccoderm desmid *Spirotaenia condensata. Journal of the Arizona—Nevada Academy of Sciences* 4: 88—92.

HUELSENBECK J.P. & RONQUIST F. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.

KIES L. 1967. Über Zellteilung und Zygotenbildung bei *Roya obtusa* (Breb.) W. & G.S. West. *Mitteilungen aus dem Institut für Allgemeine Botanik und Botanischer Garten Hamburg* 12: 35—42.

KISHINO H. & HASEGAWA M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of the Hominoidea. *Journal of Molecular Evolution* 29: 170-179.

LÜTKEMÜLLER J. 1895. Über die Gattung *Spirotaenia* Breb. *Österreichische botanische Zeitschrift* 45: 1-6, 51-57, 88-94.

LÜTKEMÜLLER J. 1903. Über die Gattung *Spirotaenia* Breb. II. *Österreichische botanische Zeitschrift* 53: 396-405, 483-488.

MARIN B., KLINGBERG M. & MELKONIAN M. 1998. Phylogenetic relationships among the Cryptophyta: analyses of nuclear-encoded SSU rRNA sequences support the monophyly of extant plastid-containing lineages. *Protist* 149: 265-276.

MCCOURT R.M., KAROL K.G., KAPLAN S. & HOSHAW R.W. 1995. Using *rbc*L sequences to test hypotheses of chloroplast and thallus evolution in conjugating green algae (Zygnematales, Charophyceae). *Journal of Phycology* 31: 989-995.

MCCOURT R.M., KAROL K.G., BELL J., HELM-BYCHOWSKI K.M., GRAJEWSKA A., WOJCIECHOWSKI M.F. & HOSHAW R.W. 2000. Phylogeny of the conjugating green algae (Zygnemophyceae) based on *rbcI* sequences. *Journal of Phycology* 36: 747-758.

MCFADDEN G.I. & MELKONIAN M. 1986. Use of Hepes buffer for microalgal culture media and fixation for electron microscopy. *Phycologia* 25: 551-557.

Mix M. 1972. Die Feinstruktur der Zellwände bei Mesotaeniaceae und Gonatozygaceae mit einer vergleichenden Betrachtung der verschiedenen Wandtypen der Conjugatophyceae und über deren systematischen Wert. *Archiv für Mikrobiologie* 81: 197-220.

MOLLENHAUER D. 1986. Contribution towards a revision of the genus *Spirotaenia* (Mesotaeniaceae). *Nova Hedwigia, Beiheft* 56: 61-90.

NICKRENT D.L., PARKINSON C.L., PALMER J.D. & DUFF R.J. 2000. Multigene phylogeny of land plants with special reference to bryophytes

and the earliest land plants. *Molecular Biology and Evolution* 17: 1885-1895.

NOZAKI H., MISAWA K., KAJITA T., KATO M., NOHARA S. & WATANABE M.M. 2000. Origin and evolution of the colonial Volvocales (Chlorophyceae) as inferred from multiple, chloroplast gene sequences. *Molecular Phylogenetics and Evolution* 17: 256—268.

OLSEN G.J. 1990. *Multiple Sequence Alignment Editing Program.* University of Illinois, Urbana, Illinois.

PAGE R.D.M. 1996. Tree View: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357-358.

PARK N.E., KAROL K.G., HOSHAW R.W. & MCCOURT R.M. 1996. Phylogeny of *Gonatozygon* and *Genicularia* (Gonatozygaceae, Desmidiales) based on *rbch* sequences. *European Journal of Phycology* 31: 309-313.

POSADA D. & CRANDALL K.A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

RALFS J. 1848. *The British Desmidieae.* Reeve, Benham and Reeve, London. 226 pp.

RANDHAWA M.S. 1959. *Zygnemaceae.* Indian Council of Agriculture Research, New Delhi. 478 pp.

RUZICKA J. 1977. *Die Desmidiaceen Mitteleuropas.* Band 1, 1 Lieferung. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany. 299 pp.

SAIKI R.K., GELFAND D.H., STOFFEN S., SCHARF S.J., HIGUCHI R., HORN G.T., MULLIS K.B. & ERLICH H.A. 1988. Primer-directed enzymatic amplification of DNA with thermo-stable DNA polymerase. *Science* 239: 487-491.

SHIMODAIRA H. 2002. An approximately unbiased test of phylogenetic tree selection. *Systematic Biology* 51: 492-508.

SHIMODAIRA H. & HASEGAWA M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* 16: 1114-1116.

SHIMODAIRA H. & HASEGAWA M. 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. *Bioinformatics* 17: 1246-1247.

SWOFFORD D.L. 1998. *PAUP* Phylogenetic analysis using parsimony (*and other methods).* Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

WAWRIK F. 1949. Neue Algen aus den Grundwasserweihern bei Kienberg-Gaming, N-Österreich. *Österreichische botanische Zeitschrift* 96: 1-4.

YAMAGISHI T. 1963. Classification of the Zygnemataceae. *Science Report of the Tokyo Kyoiku Daigaku, Section B* 11: 191—210.

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