Closteriopsis acicularis (G. M. Smith) Belcher *et* Swale is a fusiform alga closely related to *Chlorella kessleri* Fott *et* Nováková (Chlorophyta, Trebouxiophyceae)

IANA USTINOVA[1], LOTHAR KRIENITZ[2] AND VOLKER A. R. HUSS[1]

[1] *Institute of Botany and Pharmaceutical Biology, University of Erlangen-Nürnberg, Staudtstrasse 5, 91058 Erlangen, Germany*
[2] *Institute of Freshwater Ecology and Inland Fisheries, Alte Fischerhütte 2, 16775 Neuglobsow, Germany*

(*Received 28 October 2000; accepted 20 February 2001*)

Complete nuclear 18S rRNA and chloroplast 16S rRNA coding sequences of the fusiform green alga *Closteriopsis acicularis* (G. M. Smith) Belcher *et* Swale (Chlorophyta) were used to determine its phylogenetic position within the Chlorophyta. Phylogenetic trees inferred from both genes show that *C. acicularis* is most closely related to *Chlorella kessleri* Fott *et* Nováková (Trebouxiophyceae) and does not belong to the family Selenastraceae (Chlorophyceae) as proposed previously. Despite its morphological distinctness, *C. acicularis* has to be placed close to the genus *Chlorella* Beijerinck, and especially to *C. kessleri*. The chloroplast 16S rRNAs of *C. acicularis* and *C. kessleri* possess a unique insertion resulting in an additional AT-rich helix in a length-conserved region of the gene. A close relationship to the genus *Chlorella* is further confirmed by the pyrenoid ultrastructure. The 16S rRNA gene of *C. acicularis* contains a group I subgroup IA3 intron which is most similar to the first intron in the corresponding gene of *Chlamydomonas pallidostigmatica* King (Chlorophyta). In helix P6, the intron carries an additional extension related to intron-encoded endonucleases of the LAGLIDADG family. It is the first group I intron reported in chloroplast 16S rDNA of a species other than *Chlamydomonas*.

Key words: 16S rRNA phylogeny, 18S rRNA phylogeny, *Chlorella kessleri*, Chlorophyceae, chloroplast group I intron, *Closteriopsis acicularis*, LAGLIDADG homing endonuclease, molecular systematics, Selenastraceae, Trebouxiophyceae

Introduction

The genus *Closteriopsis* Lemmermann is characterized by fusiform solitary cells with one chromatophore and many pyrenoids arranged lengthwise. Lemmermann (1899) placed his new genus into the Desmidiales, close to the genus *Closterium* Ralfs. Chodat (1900) suggested that *Closteriopsis* resembles more closely the genus *Raphidium* Kützing in its mode of reproduction, chloroplast shape and lack of end vacuoles. Later, *Closteriopsis* along with other species assigned to *Raphidium* was reclassified with the genus *Ankistrodesmus* Corda (Wille, 1911; Brunnthaler, 1915). Hindák (1970) regarded *Closteriopsis* as a genus of the family Ankistrodesmaceae *sensu* Korshikov consisting of three species: *C. longissima* (Lemmermann) Lemmermann (type species), *C. acicularis* (G. M. Smith) Belcher *et* Swale, and *C. pehri* (Beck-Mannagetta) Hindák. These can be distinguished by cell size, number of pyrenoids in young autospores and by the shape of their chloroplasts. Komárek & Fott (1983) eliminated *C. pehri* from this genus, and Comas (1984, 1992) assigned to it two more species: *C. drepaniformis* Comas and *C. scolia* Comas. Unfortunately, only one strain of *Closteriopsis*, *C. acicularis* SAG 11.86, exists in pure culture and is available for further investigations.

Closteriopsis acicularis differs from other species of *Closteriopsis* by its relatively short cells and smaller number of pyrenoids (Hindák, 1970). The number of autospores correlates with the number of pyrenoids in adult cells. *Closteriopsis acicularis* is a common, though not abundantly occurring, component of the phytoplankton. It is morphologically similar to *Schroederia setigera* (Schroeder) Lemmermann (Characiaceae), but the latter can be distinguished from *C. acicularis* by the possession of only one pyrenoid before protoplast division (Hindák, 1970, 1988). The differences between the genera *Closteriopsis*, *Schroederia* and *Pseudoschroederia* Hegewald *et* Schnepf were further investigated by Hegewald & Schnepf (1986), who compared the morphology and ultrastructure of these algae by electron and light microscopy. They observed that pyrenoids of *C. acicularis* were characterized by a double thylakoid traversing the pyrenoid matrix, in contrast to those of *Schroederia*

Correspondence to: V. A. R. Huss.
e-mail: varhuss@biologie.uni-erlangen.de

and *Pseudoschroederia*. Growth, development and metabolism of *C. acicularis* were also studied for its potential use in biological life support systems (Antonyan *et al*., 1985).

As the systematics and generic composition of Selenastraceae (Blackmann *et* Transley) Fritsch in G. S. West (syn. Ankistrodesmaceae Korshikov) is rather confusing at present, it has been subject to several revisions. However, these were based entirely on morphological and ontogenetic criteria. Marvan *et al*. (1984) suggested a close relationship between *Closteriopsis* and the genera *Chlorolobion* Korshikov, *Monoraphidium* Komarková-Legnerová and *Drepanochloris* Marvan, Komárek *et* Comas, based on selected morphological features in a numerical evaluation of genera belonging to the Selenastraceae. A fast-growing database of small subunit (SSU) rRNA sequences used for inferring phylogenetic relationships in unicellular green algae such as the genus *Chlorella* (Huss *et al*., 1999) still includes only one species of the Selenastraceae: *Ankistrodesmus stipitatus* (Chodat) Komarková-Legnerová (Huss & Sogin, 1990). In agreement with the traditional view, *A. stipitatus* appeared to be closely related to representatives of the family Scenedesmaceae (Chlorophyceae).

Here we present the results of phylogenetic analyses based on nuclear and chloroplast SSU rRNA nucleotide sequences in order to elucidate the phylogenetic position of *C. acicularis* within the Chlorophyta.

Materials and methods

Closteriopsis acicularis SAG 11.86 was obtained from the Sammlung von Algenkulturen at the University of Göttingen (SAG). The strain was cultivated in the recommended 'desmidiacean' medium (Schlösser, 1994). Algae were grown for 1 week at room temperature under aeration with 2 %-CO_2-enriched air and continuous illumination. Cells were harvested and DNA was isolated and purified as described previously (Huss *et al*., 1986). Nuclear and chloroplast SSU rRNA genes were amplified from total genomic DNA by the polymerase chain reaction (PCR) with eukaryote- and chloroplast-specific amplification primers (Giovannoni, 1991; Huss *et al*., 1999). The amplified DNA fragments were either directly sequenced manually using the T7 Sequenase PCR Product Sequencing Kit (Amersham Life Science) or sequenced automatically in an ABI Prism 310 Genetic Analyser (Perkin-Elmer) according to the manufacturer's recommendations. Sequences of both strands of each gene were determined using oligonucleotide primers complementary to conserved regions of the 18S rRNA (Huss *et al*., 1999) and the chloroplast 16S rRNA gene (Table 1). The sequences were manually aligned using the sequence editor program distributed by G. Olsen (Olsen *et al*., 1992) with the following reference sequences taken from GenBank:

18S rRNA: *Pseudendoclonium basiliense* (Z47996), *Gloeotilopsis planctonica* (Z28970), *Chlorella vulgaris* (X13688), *Chlorella lobophora* (X63504), *Chlorella sorokiniana* (X62441), *Chlorella kessleri* (X56105), *Chlorella minutissima* (X56102), *Nanochlorum eucaryotum* (X06425), *Ankistrodesmus stipitatus* (X56100), *Hydrodictyon reticulatum* (M74497), *Scenedesmus vacuolatus* (X56104), *Scenedesmus obliquus* (X56103), *Chlamydomonas reinhardtii* (M32703, J02949), *Volvox carteri* (X53904) and *Dunaliella parva* (M62998).

16S rRNA: *Nicotiana tabacum* (Z00044), *Oryza sativa* (X15901), *Chlorella vulgaris* (X16579), *Chlorella kessleri* (X65099), *Chlorella ellipsoidea* (X12742), *Chlorella saccharophila* (D11349), *Nanochlorum eucaryotum* (X76084), *Chlamydomonas reinhardtii* (X03269), *Chlamydomonas pallidostigmatica* (L39865) and *Chlamydomonas moewusii* (X15850).

To improve the alignments, secondary structure models were constructed for all sequences according to Huss & Sogin (1990) and Neefs & De Wachter (1990) for 18S rRNA and according to Gutell (1996) for 16S rRNA. Highly variable regions that could not be aligned unambiguously were excluded resulting in a total of 1724 and 1290 positions, respectively, that were used in the analyses. Phylogenetic trees were inferred by neighbor-joining (NJ), the maximum parsimony (MP) and the maximum likelihood (ML) methods using the PAUP program package 4.0 b3a (Swofford, 2000). For NJ, distance matrices were corrected with the two-parameter method of Kimura (1980). The addition of sequences was jumbled, and a transition/transversion ratio of 2·0 was selected. For MP analyses, the same transition/transversion ratio, full heuristic search with random addition of taxa repeated five times, and tree-bisection-reconnection as a branch-swapping algorithm was used. For ML analyses, the Hasegawa–Kishino–Yano model of evolution and a transition/transversion ratio of 2·0 were selected. Settings for the full heuristic search were the same as for MP analyses. One thousand bootstrap replications for NJ and MP, and 100 replications for ML analyses, were performed to statistically support tree topologies (Felsenstein, 1985). The 18S and 16S rRNA gene sequences of *C. acicularis* were deposited at the EMBL database under the accession numbers Y17470 and Y17632, respectively.

The secondary structure model of the group I intron was constructed according to Michel & Westhof (1990) under consideration of its subgroup. Searches for stacking regions and open reading frames were performed with the MacDNASIS program package (Hitachi).

FASTA3 and BLAST searching algorithms (Altschul *et al*., 1990; Pearson, 1990) were used for DNA and protein sequence similarity searches in the GenBank database. Protein sequences were aligned with the ClustalW 1.8 Multiple Sequence Alignment algorithm (Thompson *et al*., 1994).

Results and discussion

Primary structure of the nuclear and chloroplast SSU rRNA

The 18S rRNA gene sequence of *Closteriopsis acicularis* has a length of 1796 base pairs (bp) and does not contain any introns. A similarity search in GenBank resulted in a best hit of 99·1 % compared

Table 1. Oligonucleotide primers used for PCR and sequencing reactions of chloroplast SSU rRNA genes

Primer name	Annealing site[a]	Sequence[b]	Reference
Cp5′-PCR	8–27	GTGCTGCAGAGAGTTYGATCCTGGCTCAGG[c]	Giovannoni (1991)
Cp3′-PCR	1541–1518	CACGGATCCAAGGAGGTGATCCANCCNCACC[d]	Giovannoni (1991)
227 >	227–243	CTGATTAGCTWGTTGGT	This work
479 >	479–495	GTGCCAGCAGCCGCGGT	Norman Pace[e]
651 >	651–667	AGCGGTGAAATGCGTAG	Giovannoni (1991)
874 >	874–890	ACTCAAAGGAATTGACG	This work
1056 >	1056–1072	GCAACGAGCGCAACCCT	Norman Pace[e]
1288 >	1288–1304	GAATCGCTAGTAATCGC	This work
243 <	243–227	ACCAACWAGCTAATCAG	This work
495 <	495–479	ACCGCGGCTGCTGGCAC	Norman Pace[e]
667 <	667–651	CTACGCATTTCACCGCT	Giovannoni (1991)
890 <	890–874	CGTCAATTCCTTTGAGT	Giovannoni (1991)
1072 <	1072–1056	AGGGTTGCGCTCGTTGC	Norman Pace[e]
1304 <	1304–1288	GCGATTACTAGCGATTC	This work

[a]Annealing positions refer to the SSU rRNA sequence of *E. coli*.
[b]Ambiguous nucleotides are abbreviated according to the IUB standard: W = A/T, Y = C/T, N = A/C/G/T.
[c]Primer contains an additional *Pst*-I restriction site (underlined) at the 5′ end.
[d]Primer contains the additional *Bam*H-I restriction site (underlined) at the 5′ end.
[e] Personal communication.

with *Chlorella kessleri* (Trebouxiophyceae). These sequences differ by only 15 nucleotides, mostly involved in compensating base exchanges in variable regions, as is evident from a secondary structure model (not shown). In comparison, the similarity between *C. acicularis* and *Ankistrodesmus stipitatus* was significantly lower (93·8 %).

The 16S rRNA gene sequence of *C. acicularis* has an unexpected length of 2186 bp due to a single insertion of 612 bp at position 508 of the *E. coli* 16S rRNA (see below). As with the 18S rRNA sequence, after the intron sequence was omitted, the highest similarity (95·0 %) was found to the corresponding gene of *C. kessleri*. Fig. 1 shows a secondary structure model of 16S rRNA of *C. acicularis* in comparison with *C. kessleri*. The two molecules share a unique structural feature, which is neither present in 16S rDNA of the next most closely related *Chlorella* species nor in any other chloroplast or even bacterial rRNA genes sequenced to date. It consists of an unusual AT-rich insertion at a site corresponding to position 747 of the *E. coli* 16S rRNA. A 62 bp long insertion in the 16S rRNA gene of *C. kessleri* SAG 211-11h was reported previously (Huss *et al*., 1992; Oyaizu *et al*., 1993). Moreover, direct sequencing of isolated 16S rRNA of *C. kessleri* with reverse transcriptase showed that the insertion is not spliced during rRNA processing and actually is part of the 16S rRNA molecule (unpublished results). Interestingly, the 16S rDNA of *C. acicularis* also carries a 39 bp long AT-rich insertion at the same site. However, the primary structure of this additional helix 22-1 (Fig. 1) is much less conserved than the rest of the gene. The presence of this specific insertion seems to be unique for *C. acicularis* and *C. kessleri*: among all other chloroplast and bacterial 16S rRNAs investigated, at most 9 nucleotides, which cannot be folded into a helix, were found inserted at this site in *Klebsormidium flaccidum* (Streptophyta, Charophyceae; Huss *et al*., 1993).

Phylogenetic analyses based on nuclear and chloroplast SSU rRNA gene sequences

Fig. 2*A* shows a NJ tree inferred from 18S rRNA gene sequences of selected green algae from both Chlorophyceae and Trebouxiophyceae. MP and ML trees are congruent with the NJ tree. Within the Trebouxiophyceae, *C. acicularis* and *C. kessleri* are the most closely related species supported by a bootstrap value of 100 % in all trees. Both algae form a monophyletic clade with other species of *Chlorella*: *C. vulgaris*, *C. lobophora* and *C. sorokiniana*. This is again statistically very well supported by bootstrap values of 96–100 %. The above *Chlorella* species were recently proposed to constitute the cluster of 'true' *Chlorella* based on 18S rRNA phylogeny, chemotaxonomy and pyrenoid ultrastructure (Huss *et al*., 1999). On the other hand, a member of the Selenastraceae, *Ankistrodesmus stipitatus*, groups with species of *Scenedesmus* and *Hydrodictyon* within the Chlorophyceae. The tree topology agrees well with published data (Huss *et al*., 1999). Fig. 2*B* shows a NJ tree inferred from published chloroplast 16S rRNA gene sequences. The topology of this tree is congruent with the 18S rRNA-based trees: *C. acicularis* and *C. kessleri* appear as the most closely related species within the clade of 'true' *Chlorella*, supported with a bootstrap value of 100 %. In the

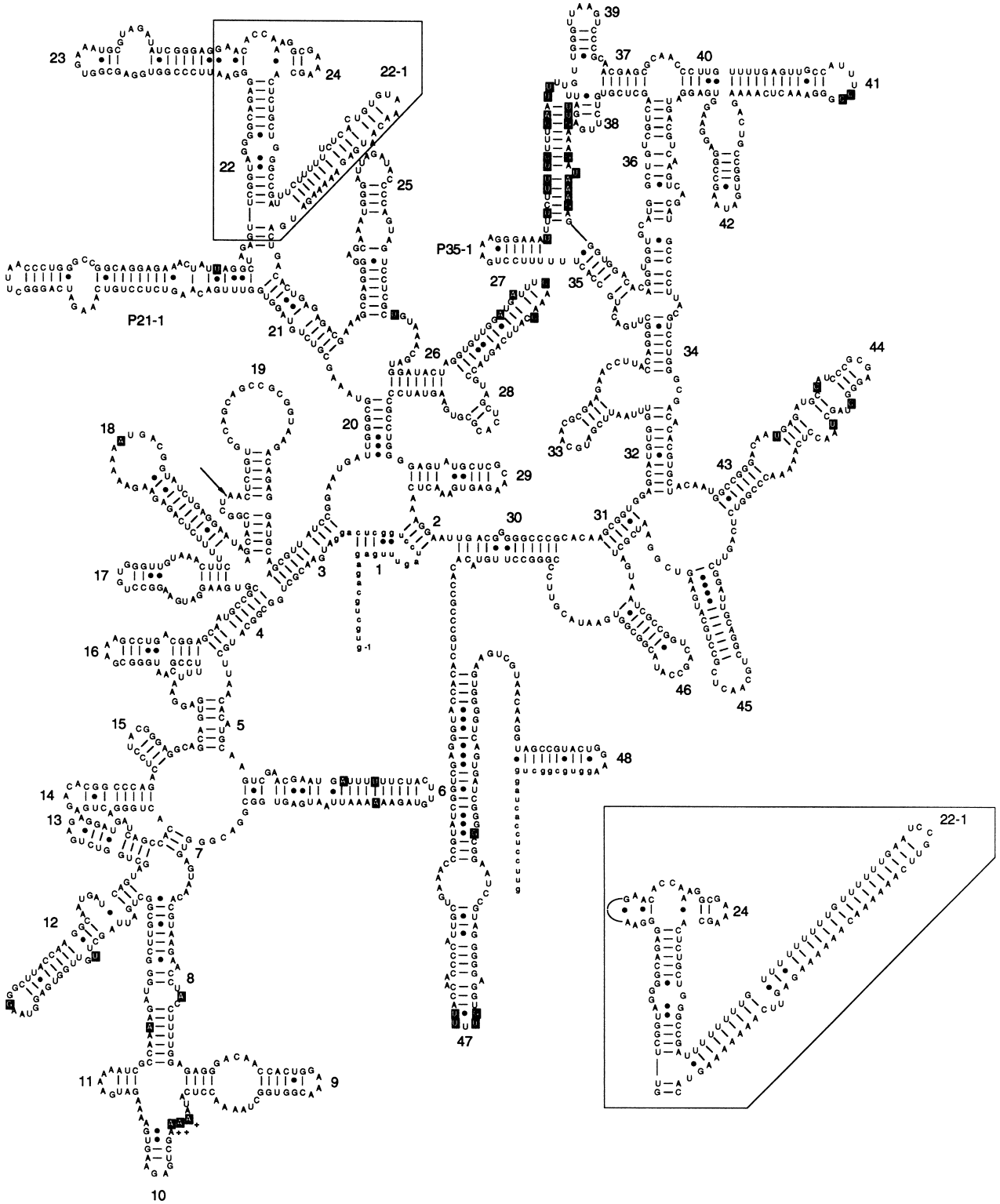

Fig. 1. Secondary structure model of the chloroplast 16S rRNA of *Closteriopsis acicularis* SAG 11·86. The arrow shows the insertion site of a 612 bp long group I intron. White letters on black backgrounds and (+) indicate base substitutions and base insertions compared to *Chlorella kessleri* SAG 211-11g. The additional helix 22-1 is boxed; base substitutions, insertions or deletions in helix 22-1 are not marked. The corresponding helix in *C. kessleri* is shown in the separate box.

MP and ML trees also, both algae group together, although with lower statistical support. In all trees *C. acicularis* appears as a member of the class Trebouxiophyceae. This class is supported by high bootstrap values and is a sister group to the Chlorophyceae, so far represented only by *Chlamydomonas* species. The systematic position of *C. acicularis* within the Trebouxiophyceae inferred from the chloroplast 16S rRNA phylogeny confirms the result of nuclear 18S rRNA analyses. Both analyses show that *C. acicularis* does not belong to the family Selenastraceae. Instead, it is closely associated with the genus *Chlorella sensu* Huss *et al.* (1999).

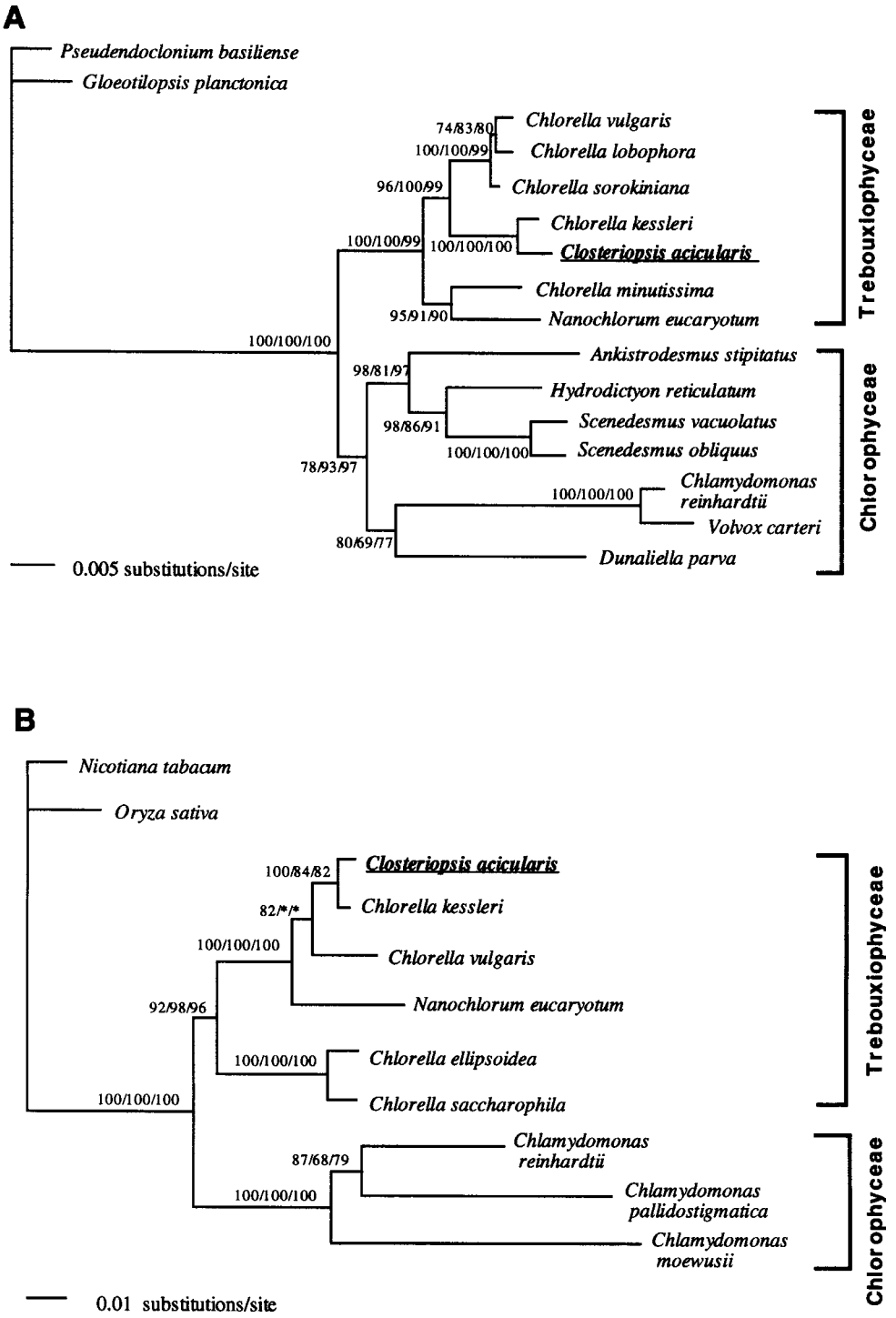

Fig. 2. Neighbor-joining (NJ) trees inferred from nuclear (*A*) and chloroplast (*B*) SSU rRNA gene sequences showing the phylogenetic position of *Closteriopsis acicularis* within the Chlorophyta. The trees are congruent with corresponding ML and MP trees. Bootstrap values (in %) are shown at the internal nodes for NJ and MP (each 1000 replicates), and ML (100 replicates), respectively. Asterisks indicate bootstrap values lower than 50%. Branch lengths correspond to the evolutionary distances indicated by the scales.

A group I intron in the 16S rRNA gene of Closteriopsis acicularis

An AT-rich (63%) insertion of 612 bp at position 508 (*E. coli* numbering) in the 16S rRNA gene of *C. acicularis* can be folded into a secondary structure typical for group I subgroup IA3 introns (Fig. 3*A*). To our knowledge, this is the first group I intron reported in the chloroplast SSU rRNA gene of an organism other than *Chlamydomonas*. In a GenBank search it was found to be most similar in sequence to the group I intron of *Chlamydomonas pallidostigmatica*, which belongs to the same subgroup and interrupts the chloroplast 16S rDNA at the same position. This intron contains an open reading frame (ORF) inserted in helix P6, which codes for a site-specific homing endonuclease (HE), I-*Cpa*II (Turmel *et al.*, 1995*a*, *b*), of the

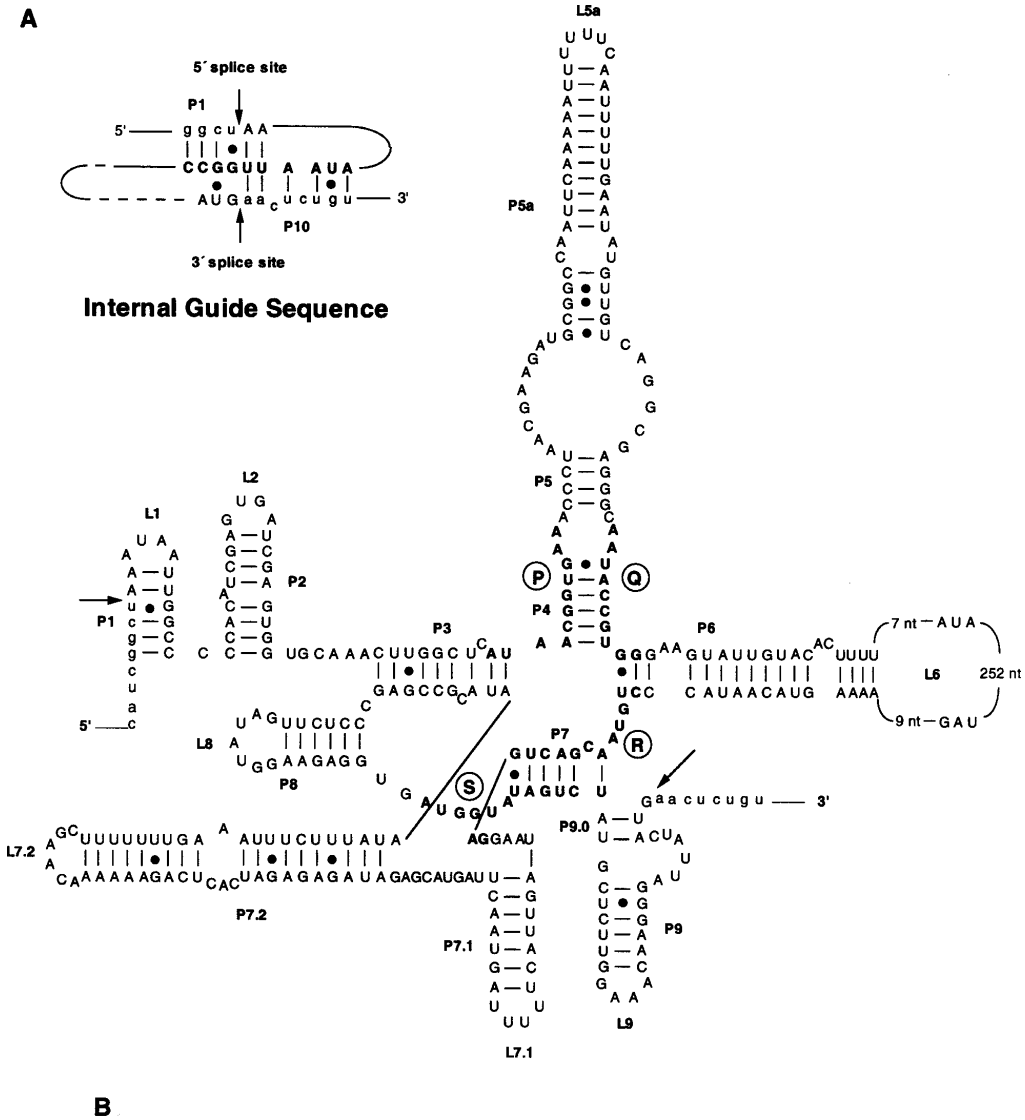

Fig. 3. (*A*) Secondary structure model of the *Closteriopsis acicularis* chloroplast 16S rRNA group I subgroup IA3 intron at position 508 (*E. coli* numbering). Nucleotide residues belonging to the exon and intron are given in lower and upper case letters, respectively. Arrows indicate exon–intron junctions. P, Q, R, S: conserved elements of the catalytic core. P1–P10 and L1–L9.2: helices and terminal loops designated according to current nomenclature. A pseudoknot corresponding to helices P1 and P10 and including the internal guide sequence is shown separately. (*B*) Sequence of the extension in helix P6 and deduced amino acid sequence. The potential initiation codon AUA and the stop codon UAG are underlined.

LAGLIDADG family (see below). The *C. acicularis* intron carries an approximately 270 bp long extension at the same site (Fig. 3). This extension does not contain any significantly long ORF that starts with an AUG or GUG initiation codon. However, a protein of 85 amino acid residues (Fig. 3*B*) could be potentially translated from an AUA codon which was shown to be recognized in some cases as an initiation codon in organelles of eukaryotes (Folley & Fox, 1991; Mulero & Fox, 1994; Nickelsen *et al.*, 1999). 'Stop-to-stop' translation extends this putative ORF into helix P5 over the conserved core elements of the intron. Examples of other intron encoded proteins, whose ORFs do not start with AUG and extend into the upstream exon, have been reported previously (reviewed in Lambowitz & Belfort, 1993; Johansen *et al.*, 1996).

Nucleotide and amino acid sequence-based similarity searches showed that the putative ORF in *C. acicularis* is homologous to C-terminal parts of

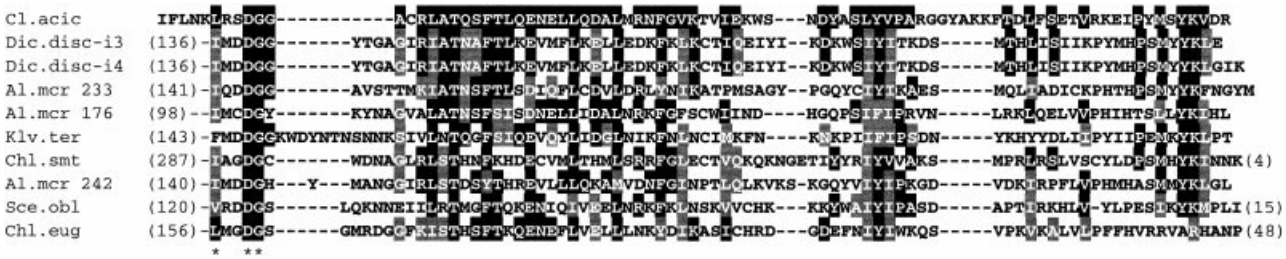

Fig. 4. Alignment of a putative ORF encoded in a group I intron in chloroplast 16S rDNA of *Closteriopsis acicularis* and C-terminal parts of most similar eukaryotic intron-encoded ORFs related to the LAGLIDADG family of homing endonucleases. Numbers in parentheses at the beginning/end of lanes correspond to number of amino acids not used in the alignment. Black/grey boxed letters show amino acids that are identical/chemically similar to those of *C. acicularis* in at least four more sequences. The following ORFs (with protein accession numbers in parentheses) were used in the alignment: Cl.acic: ORF in *C. acicularis* (no AUG codon); Dic.disc-i3: ORF in intron 3 in mitochondrial (mt) *cox*1/2 gene of *Dictyostelium discoideum* (CAA57470); Dic.disc-i4: ORF in intron 4 in mt *cox*1/2 gene of *Dictyostelium discoideum* (BAA21127); Al.mcr 233: ORF 233 in intron 3 in mt *cob* gene of *Allomyces macrogynus* (S63640); Al.mcr 176: ORF 176 in intron 7 in mt *cox*1 gene of *Allomyces macrogynus* (S63653); Klv.ter: ORF in intron in mt 21S rDNA of *Kluyveromyces* (CAA24978); Chl.smt: partially intronic ORF in mt *cob* gene of *Chlamydomonas smithii* (codes for a homing endonuclease) (CAA39012); Al.mcr 242: ORF 242 in intron 5 in mt *cob* gene of *Allomyces macrogynus* (S63641); Sce.obl: ORF in intron 2 in mt 21S rRNA gene of *Scenedesmus obliquus* (AAF72046); Chl.eug: ORF145 in intron 2 in mt *nad*5 gene of *Chlamydomonas eugametos* (T11047). Asterisks indicate conserved amino acid residues belonging to the second LAGLIDADG repeat according to Dalgaard *et al.* (1997).

mitochondrial intronic ORFs that code for proteins related to the LAGLIDADG family of HEs (Belfort & Roberts, 1997; Dalgaard *et al.*, 1997). Among the proteins with most similar sequences, at least one, from the mitochondrial apocytochrome *b* gene of *Chlamydomonas smithii*, is an active HE (Colleaux *et al.*, 1990). Some of the other most similar ORFs were identified as members of this protein family based on sequence homology (see Dalgaard *et al.*, 1997). At the nucleotide level, the highest similarity was found between the putative ORF in *C. acicularis* and the mitochondrial intronic ORF of *Chlamydomonas eugametos*, which stays in frame with the upstream *nad*5 exon and does not start with the AUG initiation codon either (Denovan-Wright *et al.*, 1998). At the protein level, the *C. acicularis* ORF is most similar (48% identity, 65% positives in a 40 amino acid overlap) to the Omega ORF encoded by a group I intron in the *cox*1 gene of the chytridiomycete *Allomyces macrogynus* (Paquin *et al.*, 1995). However, alignment of homologous domains of *C. acicularis* and other ORFs suggests that these domains do not include a complete LAGLIDADG motif itself but just a part of the second LAGLIDADG repeat (Fig. 4). This conserved motif is believed to take part in both target DNA recognition and cleavage and constitutes the active site of HEs (Belfort & Roberts, 1997).

Phylogenetic position of Closteriopsis acicularis

The molecular analyses of SSU rRNAs (Fig. 2) demonstrate that *Closteriopsis acicularis* does not belong to the family Selenastraceae (Chlorophyceae) as proposed previously, but to a different class of green algae, the Trebouxiophyceae. Within this class it is most closely related to a clade that includes *Chlorella vulgaris*, *C. lobophora*, *C. sorokiniana* and *C. kessleri*. Based on 18S rRNA analyses it was suggested that the genus *Chlorella* be restricted to these four species and that they be referred to as the group of 'true' *Chlorella* (Huss *et al.*, 1999). Other algae previously assigned to *Chlorella* were shown either to belong to different genera or to constitute separate lineages within both Chlorophyceae and Trebouxiophyceae (Hanagata & Chihara, 1997; Huss *et al.*, 1999). In all phylogenetic trees inferred from both nuclear and chloroplast SSU rRNAs, *C. acicularis* always groups with *C. kessleri* in a basal position of the *Chlorella* clade. However, the morphology of the two algae is very different. The cells of *C. kessleri* are coccoid and possess only one pyrenoid, while *C. acicularis* has needle-like cells with many pyrenoids arranged lengthwise in the chromatophore. In classical systematics the form of the cell and number of pyrenoids is thought to be one of the most important morphological criteria. However, in the case of *C. acicularis* we are confronted with the fact that molecular and classical systematic approaches are not congruent. The observation that *C. acicularis* is not related to the morphologically similar family Selenastraceae but to the morphologically different genus *Chlorella*, convincingly demonstrates that applying classical morphology-based criteria to such simply organized organisms can be misleading. It is well known that closely related algae may have a very different morphology. The 18S rRNA sequence of the colony-forming, coenobic green alga

Volvox carteri differs by only 27 positions from that of the unicellular *Chlamydomonas reinhardtii* which was placed in a different family of the order Volvocales (Rausch *et al*., 1989). The example of *C. acicularis* is even more extreme, as its 18S rRNA sequence differs from that of *C. kessleri* by only 15 positions. On the other hand, examples of morphologically similar but evolutionarily distant algae are numerous, especially among species previously assigned to the genus *Chlorella* (Huss *et al*., 1999).

Although morphological characteristics are not helpful in demonstrating the relationships of *C. acicularis* with the genus *Chlorella*, other criteria might be applied that proved to be useful for the systematics of *Chlorella* (Huss *et al*., 1999). The pyrenoid ultrastructure of different *Chlorella* species supported the separation of *Chlorella* into two groups, in agreement with investigations of the cell wall chemistry (Takeda, 1993*a*, *b*; Ikeda & Takeda, 1995). Species with glucose/mannose-containing cell walls exhibited quite diverse pyrenoid structures indicating that these species might not be closely related. Species with glucosamine-containing cell walls (*C. kessleri*, *C. vulgaris*, *C. sorokiniana*) showed identical pyrenoid structures suggesting a closer evolutionary relationship. In contrast to all other '*Chlorella*' species, their pyrenoids were traversed by a double-layered thylakoid. Hegewald & Schnepf (1986) reported the same pyrenoid structure for *C. acicularis*. Thus, molecular and ultrastructural data are in agreement and both demonstrate a close phylogenetic relationship of *C. acicularis* to the genus *Chlorella*.

The unique insertion which forms an additional AT-rich helix at the same site in the chloroplast SSU rRNA of both *C. acicularis* and *C. kessleri* (Fig. 1) shows that these two species belong to a separate evolutionary lineage within the *Chlorella* clade. As other *Chlorella* species do not possess this insertion, one can conclude that *C. acicularis* acquired its morphological distinctness even after it and *C. kessleri* diverged from other *Chlorella* species. The origin of this extraordinary insertion at a site that corresponds to nucleotide 747 of *E. coli* 16S rRNA is not clear. Most likely, it was acquired by the common ancestor of *C. acicularis* and *C. kessleri* and not by the common ancestor of the genus *Chlorella* itself, as in the latter case we would have to assume a multiple loss in the chloroplast SSU rRNA of all other *Chlorella* species. The insertion could originate from mobile elements such as transposons, or it could be a degenerated remnant of a group I intron. The latter hypothesis is not very likely, as position 747 is not known as an intron insertion site (Johansen *et al*., 1996). However, the insertion may represent the result of an unspecific homing event, whereby the mobile intron was accidentally inserted at a heterologous site and later could not be spliced out from the RNA. The evolution of such an unspliced insertion would then probably go towards maximal reduction. Some other short insertions which could be folded into two helices were found in SSU rRNA genes and were proposed to be degenerated group I introns (Grube *et al*., 1996). However, the insertions in chloroplast SSU rRNA of *C. acicularis* and *C. kessleri* do not show any structural features reminiscent of group I introns. It is rather unlikely that an accidentally acquired sequence that is not removed from the RNA transcript could be inserted at a site involved in critical interactions with other components of the translational machinery without being deleterious. Thus, the existence of this helix in the rRNA agrees with the observation that position 747 in 16S chloroplast rRNA is not involved in such important interactions (Triman, 1995; Triman & Adams, 1997). The question then remains of why this site is so strictly length-conserved otherwise.

A unique feature that distinguishes *Closteriopsis acicularis* from all Trebouxiophyceae is the presence of a group I intron in its chloroplast 16S rRNA gene. Prior to our study, there were only few known group I introns in chloroplast 16S rRNA, all occurring in different species of the genus *Chlamydomonas* (Chlorophyceae) (Durocher *et al*., 1989; Turmel *et al*., 1995*a*). Some of them carry ORFs that code for proteins related to the LAGLIDADG family of homing endonucleases (HEs). At least one protein, I-*Cpa*II, which is encoded by the first intron in 16S rRNA of *C. pallidostigmatica*, was proved to be an active endonuclease *in vitro* (Turmel *et al*., 1995*b*). HEs are proteins which are often encoded in group I introns and perform single- or double-strand breaks in intronless DNA alleles near an intron insertion site, thus promoting 'intron homing' (Belfort & Perlman, 1995; Belfort & Roberts, 1997). From the four known families of HEs, the largest and most widespread is the LAGLIDADG family, which also includes proteins encoded by organellar group I introns in eukaryotes (Dalgaard *et al*., 1997).

The intron coding for I-*Cpa*II is inserted at the same site 508 as the intron of *C. acicularis*. The two introns are very similar in sequences of the conserved core elements and share the same structural features. At the insertion site of the ORF coding for I-*Cpa*II, the intron of *C. acicularis* carries an extension with sequence similarities to C-terminal halves of some LAGLIDADG proteins, which may code for a protein of about 90 amino acids, although no typical start-codon is evident. Surprisingly, the extension itself does not show sequence similarity to the I-*Cpa*II protein. Instead, it is more similar to mitochondrial algal and fungal LAGLIDADG ORFs. This fact is remarkable: along with other lines of evidence, similar observations of closely

related introns carrying different ORFs helped to establish the hypothesis that HEs are independent invasive genetic elements that are able to colonize pre-existing introns via homing (Lambowitz & Belfort, 1993; Dalgaard *et al*., 1997). The degenerated ORF in the intron of *C. acicularis* may represent a new example of invasion of this type. It remains to be determined whether this ORF-like insertion may be translated into a protein with endonuclease activity. However, its small size, the absence of the initiation codon and the fact that the sequence, although homologous to LAGLIDADG ORFs, does not include the LAGLIDADG motif itself suggest that the insertion may be the non-functional relic of an ORF that once encoded a homing endonuclease.

The presence of homologous group I introns at the same site in chloroplast 16S rRNA genes of the distantly related *C. acicularis* and *C. pallidostigmatica* may be explained by lateral transfer of this intron via intron homing promoted by HEs that specifically recognize site 508. This hypothesis is supported by the fact that ORFs that belong to the LAGLIDADG family are present in both introns. In contrast, the hypothesis that both algae inherited the intron from a common ancestor is less plausible, as in this case one should assume a multiple loss of corresponding introns at the same position in all other green algae. Numerous examples of lateral transfer of introns between divergent species were reported for bacteria, fungi, vascular plants and algae (Bhattacharya *et al*., 1996; Cho & Palmer, 1999; Holst-Jensen *et al*., 1999; Paquin *et al*., 1999) as well as between different subcellular compartments (chloroplasts and mitochondria) of algae and fungi (Dürrenberger & Rochaix, 1991; Turmel *et al*., 1995*a*). However, the exact mechanism of how group I introns are transferred between distantly related organisms remains an open question, as horizontal transfer requires intron-containing and intronless alleles to occur in the same genetic environment. This is particularly difficult to imagine for those organisms that are not able to reproduce sexually, such as *Closteriopsis acicularis*. To explain this phenomenon, scenarios involving virus-, phagocytosis- or symbiosis-mediated horizontal intron transpositions were previously proposed (Johansen *et al*., 1996; Einvik *et al*., 1998; Nishida *et al*., 1998; Holst-Jensen *et al*., 1999).

The taxonomic conclusions drawn from our rDNA sequence analyses may not be applied to the whole genus *Closteriopsis*, but only to *C. acicularis*. It remains unclear whether other species of *Closteriopsis* also belong to the Trebouxiophyceae. It would be particularly important to include the type species *C. longissima* in the molecular analyses. Unfortunately, no cultures of *Closteriopsis* species other than *C. acicularis* are available from culture collections. Our finding that a morphologically very different alga is genetically closely related to a species of *Chlorella* is not unique. An even closer relationship of *Micractinium pusillum*, a bristle- or 'spine'-bearing chlorococcal alga, with *C. sorokiniana* has been found (D. Hepperle and E. Hegewald, personal communication). The 18S rRNA sequences of *M. pusillum* and *C. sorokiniana* strain Baslerová Prag A14 differ by only 5 nucleotides. Although their and our results would justify inclusion of *M. pusillum* and *C. acicularis* in the genus *Chlorella* on a genetic basis, the problems of combining such morphologically different species in one genus are obvious. In agreement with Hepperle and Hegewald, we suggest that further studies, and discussions, are needed to elaborate a new taxonomic concept for these algae that respects both morphological and molecular data, in as much as more such cases are expected to be discovered.

Closteriopsis acicularis has turned out to be interesting in two aspects. First, its close phylogenetic relationship to *Chlorella kessleri* despite its morphological distinctness demands new discussions of generic concepts in green algae. And second, it enlarges the repertoire of introns and LAGLIDADG ORFs in chloroplast SSU rRNAs that prior to our study were found only in species of *Chlamydomonas*.

Acknowledgements

We thank T. Rohr for sequencing part of the chloroplast 16S rRNA of *C. acicularis*. This work was supported by the Deutsche Forschungsgemeinschaft (Hu 416/6-4).

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol*., **215**: 403–410.

Antonyan, A.A., Levinskikh, M.A. & Sukhova, N.I. (1985). Study of growth, development and metabolism of algae *Closteriopsis acicularis* during cell limitation with nitrogen as applied to the human biological life support system. *Kosm. Biol. Aviakosm. Med*., **19**: 69–73.

Belfort, M. & Perlman, S. (1995). Mechanism of intron mobility. *J. Biol. Chem*., **270**: 30237–30240.

Belfort, M. & Roberts, R.J. (1997). Homing endonucleases: keeping the house in order. *Nucleic Acids Res*., **25**: 3379–3388.

Bhattacharya, D., Friedl, T. & Damberger, S. (1996). Nuclear-encoded rDNA group I introns: origin and phylogenetic relationships of insertion site lineages in the green algae. *Mol. Biol. Evol*., **13**: 978–989.

Brunnthaler, J. (1915). Chlorophyceae II. In *Süsswasserflora Deutschlands, Österreichs und der Schweiz* (Pascher, A., editor)., **5**: 53–205.

Cho, Y. & Palmer, J.D. (1999). Multiple acquisitions via horizontal transfer of a group I intron in the mitochondrial *cox*1 gene during evolution of the Araceae family. *Mol. Biol. Evol*., **16**: 1155–1165.

Chodat, R. (1900). Sur trois genres nouveaux de Protococcoidées et sur la florule planktonique d'un étang du Danemark. *Mem. Herb. Boiss*., **5**: 289–314.

Colleaux, L., Michel-Wolwertz, M.R., Matagne, R.F. &

Dujon, B. (1990). The apocytochrome *b* gene of *Chlamydomonas smithii* contains a mobile intron related to both *Saccharomyces* and *Neurospora* introns. *Mol. Gen. Genet.*, **223**: 288–296.

Comas, A. (1984). Chlorococcales (Chlorophyceae) de algunos acuatorios de Pinar del Río, Cuba. *Acta Bot. Cubana.*, **17**: 1–60.

Comas, A. (1992). Taxonomische Beiträge zur Grünalgenflora (Chlorellales) Kubas. *Algol. Stud.*, **65**: 11–21.

Dalgaard, J.Z., Klar, A.J., Moser, M.J., Holley, W.R., Chatterjee, A. & Mian, I.S. (1997). Statistical modeling and analysis of the LAGLIDADG family of site-specific endonucleases and identification of an intein that encodes a site-specific endonuclease of the HNH family. *Nucleic Acids Res.*, **25**: 4626–4638.

Denovan-Wright, E.M., Nedelcu, A.M. & Lee, R.W. (1998). Complete sequence of the mitochondrial DNA of *Chlamydomonas eugametos*. *Plant Mol. Biol.*, **36**: 285–295.

Durocher, V., Gauthier, A., Bellemare, G. & Lemieux, C. (1989). An optional group I intron between the chloroplast small subunit rRNA genes of *Chlamydomonas moewusii* and *C. eugametos*. *Curr. Genet.*, **15**: 277–282.

Dürrenberger, F. & Rochaix, J.-D. (1991). Chloroplast ribosomal intron of *Chlamydomonas reinhardtii*: *in vitro* self-splicing, DNA endonuclease activity and *in vivo* mobility. *EMBO J.*, **10**: 3495–3501.

Einvik, C., Elde, M. & Johansen, S. (1998). Group I twintrons: genetic elements in myxomycete and schizopyrenid amoeboflagellate ribosomal DNAs. *J. Biotechnol.*, **64**: 63–74.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**: 783–791.

Folley, L.S. & Fox, T.D. (1991). Site-directed mutagenesis of a *Saccharomyces cerevisiae* mitochondrial translation initiation condon. *Genetics.*, **129**: 659–668.

Giovannoni, S. (1991). The polymerase chain reaction. In *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt, E. & Goodfellow, M., editors), 175–201. J. Wiley, Chichester.

Grube, M., Gargas, A. & De Priest, P.T. (1996). A small insertion in the SSU rDNA of the lichen fungus *Arthonia lapidicola* is a degenerate group-I intron. *Curr. Genet.*, **29**: 582–586.

Gutell, R.R. (1996). Comparative sequence analysis and the structure of 16S and 23S rRNA. In *Ribosomal RNA: Structure, Evolution, Processing, and Function in Protein Biosynthesis* (Zimmermann, R.A. & Dahlberg, A.E., editors), 111–128. CRC Press, Boca Raton.

Hanagata, N. & Chihara, M. (1997). Concordance between phylogenetic data and ultrastructural features in the classification of *Chlorella* and related taxa. *Phycologia.*, **36** (Suppl.): 38.

Hegewald, E. & Schnepf, E. (1986). Zur Struktur und Taxonomie spindelförmiger Chlorellales (Chlorophyta): *Schroederia, Pseudoschroederia* gen. nov., *Closteriopsis*. *Algol. Stud.*, **42**: 21–48.

Hindak, F. (1970). A contribution to the systematics of the family Ankistrodesmaceae (Chlorophyta). *Algol. Stud.*, **1**: 7–32.

Hindak, F. (1988). Studies on the chlorococcal algae (Chlorophyceae): IV. *Biol. Pr.*, **34**: 1–263.

Holst-Jensen, A., Vaage, M., Schumacher, T. & Johansen, S. (1999). Structural characteristics and possible horizontal transfer of group I introns between closely related plant pathogenic fungi. *Mol. Biol. Evol.*, **16**: 114–126.

Huss, V.A.R. & Sogin, M.L. (1990). Phylogenetic position of some *Chlorella* species within the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. *J. Mol. Evol.*, **31**: 432–442.

Huss, V.A.R., Dörr, R., Grossmann, U. & Kessler, E. (1986). Deoxyribonucleic acid reassociation in the taxonomy of the genus *Chlorella*. *I. Chlorella sorokiniana*. *Arch. Microbiol.*, **145**: 329–333.

Huss, V.A.R., Hirsch, E. & Giovannoni, S.J. (1992). *C. kessleri* plastid DNA for 16S-like small subunit rRNA. GenBank accession number X65099.

Huss, V.A.R., Straub, S. & Kranz, H.D. (1993). *Klebsormidium flaccidum* gene for plastid 16S ribosomal RNA. GenBank accession number X75522.

Huss, V.A.R., Frank, C., Hartmann, E.C., Hirmer, M., Kloboucek, A., Seidel, B.M., Wenzeler, P. & Kessler, E. (1999). Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). *J. Phycol.*, **35**: 587–598.

Ikeda, T. & Takeda, H. (1995). Species-specific differences of pyrenoids in *Chlorella* (Chlorophyta). *J. Phycol.*, **31**: 813–818.

Johansen, S., Muscarella, D.E. & Vogt, V.M. (1996). Insertion elements in ribosomal DNA. In *Ribosomal RNA: Structure, Evolution, Processing, and Function in Protein Biosynthesis* (Zimmermann, R.A. & Dahlberg, A.E., editors), 89–108. CRC Press, Boca Raton.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**: 111–120.

Komárek, J. & Fott, B. (1983). *Das Phytoplankton des Süsswassers* 7, 1. *Chlorophyceae* (*Grünalgen*). *Ordnung: Chlorococcales*. Schweizerbart, Stuttgart.

Lambowitz, A.M. & Belfort, M. (1993). Introns as mobile genetic elements. *Annu. Rev. Biochem.*, **62**: 587–622.

Lemmermann, E. (1899). Das Phytoplankton sächsischer Teiche. *Forschungsber. Biol. Stat. Plön.*, **7**: 96–135.

Marvan, P., Komarek, J. & Comas, A. (1984). Weighting and scaling of features in numerical evaluation of coccal green algae (genera of the Selenastraceae). *Algol. Stud.*, **37**: 363–399.

Michel, F. & Westhof, E. (1990). Modelling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis. *J. Mol. Biol.*, **216**: 585–610.

Mulero, J.J. & Fox, T.D. (1994). Reduced but accurate translation from a mutant AUA initiation condon in the mitochondrial COX2 mRNA of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **242**: 383–390.

Neefs, J.-M. & De Wachter, R. (1990). A proposal for the secondary structure of a variable area of eukaryotic small ribosomal subunit RNA involving the existence of a pseudoknot. *Nucleic Acids Res.*, **18**: 5695–5704.

Nickelsen, J., Fleischmann, M., Boudreau, E., Rahire, M. & Rochaix, J.-D. (1999). Identification of *cis*-acting RNA leader elements required for chloroplast *psb*D gene expression in *Chlamydomonas*. *Plant Cell.*, **11**: 957–970.

Nishida, K., Suzuki, S., Kimura, Y., Nomura, N., Fujie, M. & Yamada, T. (1998). Group I introns in *Chlorella* viruses: biological implications. *Virology.*, **242**: 319–326.

Olsen, G.J., Overbeek, R., Larsen, N., Marsh, T.L., McCaughey, M.J., Maciukenas, M.A., Kuan, W.-M., Macke, T.J., Xing, Y. & Woese, C.R. (1992). The ribosomal database project. *Nucleic Acids Res.*, **20**: 2199–2200.

Oyaizu, H., Matsumoto, S., Inoue, H. & Takeda, H. (1993). Polyphyletic origins of chlorophyte chloroplasts. *J. Gen. Appl. Microbiol.*, **39**: 313–319.

Paquin, B., Forget, L., Roewer, I. & Lang, B.F. (1995). Molecular phylogeny of *Allomyces macrogynus*: congruency between nuclear ribosomal RNA- and mitochondrial protein-based trees. *J. Mol. Evol.*, **41**: 657–665.

Paquin, B., Heinfling, A. & Shub, D.A. (1999). Sporadic distribution of tRNA(Arg)CCU introns among alpha-purple bacteria: evidence for horizontal transmission and transposition of a group I intron. *J. Bacteriol.*, **181**: 1049–1053.

Pearson, W.R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol.*, **183**: 63–98.

Rausch, H., Larsen, N. & Schmitt, R. (1989). Phylogenetic relationships of the green alga *Volvox carteri* deduced from small-subunit ribosomal RNA comparisons. *J. Mol. Evol.*, **29**: 255–265.

Schlösser, U.G. (1994). SAG–Sammlung von Algenkulturen at the University of Göttingen. Catalogue of strains 1994. *Bot. Acta.*, **107**: 113–186.

Swofford, D.L. (2000). Paup*. Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, MA.

Takeda, H. (1993*a*). Chemical composition of cell walls as a taxonomical marker. *J. Plant Res.*, **106**: 195–200.

Takeda, H. (1993*b*). Taxonomical assignment of chlorococcal algae from their cell wall composition. *Phytochemistry.*, **34**: 1053–1055.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL

W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., **22**: 4673–4680.

Triman, K.L. (1995). Mutational analysis of 16S ribosomal RNA structure and function in *Escherichia coli*. *Adv. Genet*., **33**: 1–39.

Triman, K.L. & Adams, B.J. (1997). Expansion of the 16S and 23S ribosomal RNA mutation databases (16SMDB and 23SMDB). *Nucleic Acids Res*., **25**: 188–191.

Turmel, M., Mercier, J.-P., Côté, V., Otis, C. & Lemieux, C. (1995*a*). The site-specific DNA endonuclease encoded by a group I intron in the *Chlamydomonas pallidostigmatica* chloroplast small subunit rRNA gene introduces a single-strand break at low concentrations of Mg^{2+}. *Nucleic Acids Res*., **23**: 2519–2525.

Turmel, M., Côté, V., Otis, C., Mercier, J.-P., Gray, M.W., Lonergan, K.M. & Lemieux, C. (1995*b*). Evolutionary transfer of ORF-containing group I introns between different subcellular compartments (chloroplast and mitochondrion). *Mol. Biol. Evol*., **12**: 533–545.

Wille, N. (1911). Chlorophyceae. In *Natürliche Pflanzenfamilien*, vol. 1, part 2 (Engler, A. & Prantl, K., editors), 1–136. Engelmann, Leipzig.