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Phylogeny of the Cladophorophyceae (Chlorophyta) inferred from partial LSU rRNA gene sequences: is the recognition of a separate order Siphonocladales justified?

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Phylogenetic relationships within the green algal class Cladophorophyceae were investigated. For 37 species, representing 18 genera, the sequences of the 5′-end of the large subunit rRNA were aligned and analysed. *Ulva fasciata* and *Acrosiphonia spinescens* (Ulvophyceae) were used as outgroup taxa. The final alignment consisted of 644 positions containing 208 parsimony-informative sites. The analysis showed three lineages within the Cladophorophyceae: *Cladophora horii* diverged first, followed by two main lineages. The first lineage includes some *Cladophora* species and genera with a reduced thallus architecture. The second lineage comprises siphonocladalean taxa (excluding part of *Cladophoropsis* and including some *Cladophora* species). From this perspective the Siphonocladales forms a monophyletic group, the Cladophorales remaining paraphyletic.

Key words: Cladophorophyceae, Cladophorales, LSU rRNA, molecular phylogeny, Siphonocladales

Introduction

The Cladophorophyceae nom. nud. (van den Hoek *et al.*, 1995), which includes about 32 genera, comprise a mainly marine class of siphonocladous Chlorophyta with a tropical to cold-water distribution. Thallus organization in the class ranges from branched or unbranched uniseriate filaments to more complex architectural types such as pseudoparenchymatous thalli, thalli composed of inflated cells, stipitate plants, blade-like thalli, and reticulate plants composed of anastomosing filaments. Anastomosis of neighbouring cells is accomplished by four types of tenacular cells (Olsen-Stojkovich, 1986): (1) unspecialized cells with crenulate or annulate apices; (2) minute hapteroid cells formed laterally between adjacent vesicular cells; (3) minute hapteroid cells formed at the distal ends of branches and anastomosing to neighbouring filaments; (4) minute hapteroid cells formed intracellularly between septa. In some *Cladophora* species neighbouring cells occasionally adhere by means of rhizoids sprouting from the basal poles of the cells. Cells in the Cladophorophyceae divide by four modes of cell division (Olsen-Stojkovich, 1986): (1) centripetal invagination (CI): new cross-walls formed by centripetal invagination of a primordial septum (Enomoto & Hirose, 1971); (2) lenticular cell type (LC): a convex septal disc formed along the cell-wall followed by elongation of a new lateral; (3) segregative cell division *sensu stricto* (SDSS): multinucleate aggregates of cytoplasm spontaneously form walled spheres that remain in the parent cell, expand and rupture old parental cell walls; (4) modified segregative cell division (SDM): cytoplasmic spheres are released from the parent cell and grow into new thalli.

The classification of the Cladophorophyceae has been a matter of much confusion and disagreement. The genera presently included have traditionally been placed either in the single order Siphonocladales or in two separate orders, Siphonocladales and Cladophorales. The earliest circumscriptions of the two orders were very vague. The Siphonocladales (type: *Siphonocladus* Schmitz) was created by Oltmanns (1904) to accommodate a rather heterogeneous assemblage of green algae with multinucleate cells. The order was later redefined by Børgesen (1913, 1925), Feldmann (1938*a*, *b*), Egerod (1952) and Jónsson (1962, 1965). The Cladophorales (type: *Cladophora* Kütz-

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ing) was described by Haeckel (1894) to include green algae with multinucleate cells, lacking oogonia, and its circumscription modified by West (1904), Fritsch (1935, 1947) and Papenfuss (1955). The rationale for placing all genera in a single order, Siphonocladales *sensu lato* (Børgesen, 1913, 1925; Feldmann, 1938*b*; Jónsson, 1962, 1965), was the apparent homogeneity of thallus organization, chloroplast morphology and cell wall structure in the group. The separation of the group into the Siphonocladales and the Cladophorales (Børgesen, 1948; Egerod, 1952; Papenfuss, 1955; Womersley, 1984; Bold & Wynne, 1985; Sartoni, 1992) was primarily based on differences in thallus complexity: the Cladophorales *s.s.* comprised taxa with a relatively simple thallus architecture and included *Cladophora* (branched uniseriate filaments), *Chaetomorpha* and *Rhizoclonium* (unbranched uniseriate filaments); the Siphonocladales *s.s.* contained taxa with a more complex morphology. Some authors (Børgesen, 1913; Egerod, 1952) considered segregative cell division to be a principal ordinal character, although they realized that this character does not occur in all genera of the Siphonocladales. We refer to Egerod (1952), Jónsson (1962) and Olsen-Stojkovich (1986) for a more detailed taxonomic history of this complex.

Four families (Anadyomenaceae, Cladophoraceae, Siphonocladaceae and Valoniaceae) are traditionally recognized within the Cladophorophyceae, based on thallus architecture and mode of cell division. Børgesen (1925) recognized a fifth family, Boodleaceae. The boundaries of the families are rather vague and the included genera have changed frequently in the course of time. We refer to Feldmann (1938*b*), Egerod (1952) and Olsen-Stojkovich (1986) for the circumscriptions and taxonomic history of the families.

Within *Cladophora*, the largest genus of the class, 11 different architectural types can be distinguished, representing the sections of *Cladophora* as conceived by van den Hoek (1963, 1982) and van den Hoek & Chihara (2000). Based on a comparison of morphology, van den Hoek (1981, 1982, 1984) hypothesized that numerous reduction and specialization events have occurred independently several times in *Cladophora* sections, resulting in the various reduced (cladophoralean) and specialized (siphonocladalean) morphologies. These reductions and specializations were circumscribed in eight morphological tendencies: (1) the thallus becoming planar (formation of blades), (2) interweaving of filaments by tenacular cells to strengthen the thallus, (3) lateral coalescence of cells to strengthen blades, (4) replacement of successive initiation of laterals by simultaneous lateral formation, (5) increase in the number of laterals per cell, (6) inflation of cells, (7) differentiation between axis and laterals, and (8) reduction of branching. van den Hoek (1984) considered, therefore, that it would be incorrect to range the simpler genera (*Cladophora*, *Rhizoclonium*, *Chaetomorpha*) in one order, Cladophorales, and genera with a more complicated architecture in another order, Siphonocladales. The first molecular evidence supporting van den Hoek's hypothesis was based on immunological distances (Olsen-Stojkovich, 1986) and single-copy DNA–DNA hybridization studies (Bot, 1992). Later, Bakker *et al.* (1994) demonstrated, on the basis of 18S rRNA sequences of 20 species, that neither the Cladophorales nor the Siphonocladales forms a monophyletic group and that there is no basis for the independent recognition of both orders. The 18S rRNA phylogeny supports two lineages, one containing predominantly tropical members including almost all siphonocladalean taxa, the other consisting of mostly warm- to cold-temperate species of *Cladophora*. Hanyuda *et al.* (2002) extended Bakker's phylogeny with 18S rRNA sequences of 21 additional species, including some freshwater representatives of the class. This analysis reveals a new clade, located at the base of the two main lineages, which comprises a mixture of marine and freshwater genera with a simple, *Cladophora*-type architecture. The general consensus today is the recognition of a single order Cladophorales (the choice of name being based on priority) in the class Cladophorophyceae (van den Hoek *et al.*, 1995; van den Hoek & Chihara, 2000).

This study extends the phylogenies of Bakker *et al.* (1994) and Hanyuda *et al.* (2002), mainly with representatives of species traditionally ascribed to the Siphonocladales *s.s.* including 37 species in 18 genera. The genus *Cladophora* is represented by 10 species, belonging to six sections. The goals of this study are: (1) to compare partial LSU rRNA sequences with the previously published SSU rRNA phylogenies (the LSU is known to be more variable than the SSU (Hassouna *et al.*, 1984; Michot *et al.*, 1984; Rousseau *et al.*, 1997, 2001) and its phylogenetic potential at different taxonomic levels in plants has been demonstrated by Kuzoff *et al.* (1998)); (2) to test van den Hoek's (1982) hypothesis that different genera with complex and simplified thallus architectures represent further specializations of the basic architectural types of *Cladophora*, and that these specialization and reduction events happened several times independently; (3) to examine whether the recognition of a single order Cladophorales is justified and (4) to test the taxonomic significance of morphological characters that were considered to be important in the delineation of the two orders and the different families in the group.

Materials and methods

The specimens used in this study are listed in Table 1. The collected samples were desiccated in silica gel according to Chase & Hills (1991); parts of the same thallus were processed as herbarium specimens and deposited in GENT; for some species, only dried herbarium specimens were available. Although *Boodlea siamensis* is generally regarded as a taxonomic synonym of *B. composita* (Børgesen, 1946: 16), it is treated as a separate entity in this study, based on differences in branching pattern. Morphological characters and their states were collected from specimens also included in the molecular study to permit direct comparison.

DNA was extracted using the DNeasy Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplifications were performed in a Biomed thermocycler with an initial denaturation step of 94°C for 3 min followed by 35 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C, with a final extension step of 3 min at 72°C. The reaction volume was 50 μl and comprised about 0.3 μg genomic DNA, 2 nmol of each dNTP, 30 pmol of each primer, 31 μl H_2O, 5 μl of 10 × reaction buffer, 5 mM $MgCl_2$, 10 μg BSA, 5 μl dimethylsulphoxide (DMSO) and three units of *Taq* polymerase (Goldstar). The approximately 550-nucleotide fragment was amplified using the universal primers C′1 and D2 at positions 25 and 1126 of the complete *Mus musculus* 28S rDNA (Hassouna *et al*., 1984). For some specimens an additional universal primer C′2B at position 383 of the complete mouse 28S rDNA had to be used. Nucleotide sequences of the primers are: C′1 forward (5′-ACCCGCTGAATT-TAAGCATAT-3′), D2 reverse (5′-TCCGTGTTTCAA-GACGG-3′), C′2B forward (5′-GAGTCGGGTYGYT-TGGGAATGCA-3′).

The amplified fragment includes the conserved zones C1 (partial) and C2 and the variable zones D1 and D2 (Michot *et al*., 1984), and comprises about 550 bp. Amplifications were checked for correct length, purity and yield on 1.5% agarose gels and stained with ethidium bromide. Excess primers and nucleotides were removed from PCR products using MnElute (Qiagen) according to the manufacturer's instructions. About 100 fmol of PCR product was used for the sequencing reaction. Both strands of the PCR products were directly sequenced with the PCR primers using the CEQ Cycle Sequencing Kit (Beckman) in the CEQ-2000 DNA Analysis System (Beckman). The final consensus sequence was constructed by means of Sequencher 4.0.5 software (Gene Codes, Ann Arbor, MI).

Alignment of rRNA sequences taking into account the secondary structure is available on the web site http://oberon.rug.ac.be:8080/rRNA/ for LSU sequences (Wuyts *et al*., 2001). The chlorophycean sequences provided in this database were used as a model for building our alignment. Alignment of the highly variable D2 region was aided by constructing the secondary structure of each sample by using the MFOLD software available at http://www.bioinfo.math.rpi.edu (Zuker *et al*., 1999; Mathews *et al*., 1999). The different optimal and suboptimal secondary structures for each species were compared. Compensatory mutations were examined in order to confirm the homologous stem and loop regions, and the sequences were aligned accordingly using the software DCSE v 2.60 (Dedicated Comparative Sequence Editor) (De Rijk & De Wachter, 1993). The underlying principles of using secondary structure models for aligning rRNA sequences have been discussed by Kjer (1995).

The distribution of phylogenetic signal in the data set was explored by comparing the pairwise sequence divergence (minimum, maximum and average) and the number of parsimony-informative sites in the four regions C1, D1, C2 and D2 (Table 2). Both calculations were done after exclusion of sites with ambiguous alignment.

Measure of skewness (g_1 value calculated by using 10 000 randomly selected trees in PAUP*) was compared with the empirical threshold values in Hillis & Huelsenbeck (1992) to verify for non-random structuring of the data.

All phylogenetic analyses were performed using PAUP 4.0* beta test version 10 (Swofford, 2002). *Ulva fasciata* and *Acrosiphonia spinescens* were used as outgroup taxa. Gaps were taken into account as missing characters in all analyses. Maximum parsimony (MP) analyses were carried out using a general heuristic search, with 100 random sequence additions, TBR swapping and MULTREES options; branches were collapsed if it was possible for them to have zero length.

Substitution rates were compared through relative rate tests using the program RRTree (Robinson-Rechavi & Huchon, 2000) (Table 3). The choice of taxa was based on the MP strict consensus phylogram, which showed considerable variation in branch length within certain clades (Fig. 1). A first test was performed using all taxa within clade A4 (between *Cladophora rupestris*, the *Chaetomorpha spiralis* clade and the *Cladophora vagabunda* clade), using clade A3 as outgroup. A second test compared all taxa of the clades B4, B5 and B6, using clade B3 as outgroup. The program MODELTEST version 3.04 (Posada & Crandall, 1998) was used to find the model of sequence evolution that best fits the dataset by a hierarchical likelihood ratio test (LRT) ($\alpha = 0.05$) or the Akaike Information Criterion (minimum theoretical information criterion, AIC). Models were estimated for complete and partial datasets (Table 4). Various preliminary maximum likelihood (ML) analyses indicated that trees were strongly affected by the model chosen. Given that various models were calculated for the different partial datasets (e.g. different models for lineages A and B) (Table 4), and since there were changes in the subsitution rates between lineages (Table 3), it would have been unlikely that the model estimated for the complete dataset would be correct for all sequences in this dataset. Therefore the ML analysis was carried out using the simplest model, a Jukes–Cantor model, as recommended by Takahashi & Nei (2000) and McIvor *et al*. (2002).

Bootstrapping (Felsenstein, 1985) was performed in PAUP* using 1000 replicates for the MP analysis, and 250 replicates for the ML analysis. Decay analysis of MP trees was performed with AutoDecay version 4.0 (Eriksson, 1998).

Table 1. Specimens used in the phylogenetic analysis with their collecting sites, herbarium number (Voucher), number used in this study (No.), location and EMBL accession numbers

Species	Voucher	No.	Location	EMBL accession number
Anadyomene stellata (Wulfen) C. Agardh	PH 209	F41	Cebu, Philippines	AJ544746
Boergesenia forbesii (Harvey) J. Feldmann	FL 1009	F09	Zanzibar, Tanzania	AJ544742
Boodlea composita (Harvey) Brand	FL 950	F16	Zanzibar, Tanzania	AJ544731
Boodlea montagnei (Harvey ex J. Gray) Egerod	PH 646	F17	Mactan Island, Philippines	AJ544734
Boodlea montagnei (Harvey ex J. Gray) Egerod	FL 958	F18	Zanzibar, Tanzania	AJ544733
Boodlea siamensis Reinbold	FL 999	F35	Zanzibar, Tanzania	AJ544730
Chaetomorpha aerea (Dillwyn) Kützing	FL 998	F04	Zanzibar, Tanzania	AJ544758
Chaetomorpha brachygona Harvey	FL 982	F26	Zanzibar, Tanzania	AJ544759
Chaetomorpha crassa (C. Agardh) Kützing	FL 908	F03	Zanzibar, Tanzania	AJ544767
Chaetomorpha spiralis Okamura	HEC 11621	F83	Weligama, S coast of Sri Lanka	AJ544766
Chamaedoris auriculata Børgesen	SOC 395	F10	Bidhola, S coast of Socotra	AJ544739
Chamaedoris delphinii (Hariot) Feldmann & Børgesen	KZN 2110	F11	KwaZulu-Natal, South Africa	AJ544740
Cladophora capensis (C. Agardh) DeToni	HEC 10900	F80	Cape Peninsula, South Africa	AJ544763
Cladophora coelothrix Kützing	HEC 9394	F58	Mombasa, Kenya	AJ544754
Cladophora coelothrix Kützing	HEC 7418	F62	Malindi, Kenya	AJ544753
Cladophora dotyana Gilbert	HEC 12336	F57	Bulusan, Philippines	AJ544755
Cladophora dotyana Gilbert	KZN 2003	F31	KwaZulu-Natal, South Africa	AJ544756
Cladophora horii van den Hoek & Chihara	HEC 10983	F53	KwaZulu-Natal, South Africa	AJ544728
Cladophora laetevirens (Dillwyn) Kützing	FL 997	F29	Zanzibar, Tanzania	AJ544761
Cladophora montagneana Kützing	FL 900	F30	Zanzibar, Tanzania	AJ544762
Cladophora ordinata (Børgesen) van den Hoek	KZN 2002	F08	KwaZulu-Natal, South Africa	AJ544757
Cladophora rupestris (Linnaeus) Kützing	WIM 01	F44	Boulonais, France	AJ544764
Cladophora sibogae Reinbold	ODC 352	F61	Zanzibar, Tanzania	AJ544752
Cladophora vagabunda (Linnaeus) van den Hoek	FL 1001	F05	Zanzibar, Tanzania	AJ544760
Cladophoropsis herpestica (Montagne) Howe	FL 909	F14	Zanzibar, Tanzania	AJ544751
Cladophoropsis philippinensis Taylor	PH 567	F45	Cebu, Philippines	AJ544735
Dictyosphaeria cavernosa (Forsskål) Børgesen	FL 913	F20	Zanzibar, Tanzania	AJ544745
Ernodesmis verticillata (Kützing) Børgesen	WF 23-3-99	F89	Limon, Costa Rica	AJ544743
Microdictyon kraussii J. Gray	KZN 0272	F02	KwaZulu-Natal, South Africa	AJ544747
Phyllodictyon anastomosans (Harvey) Kraft & Wynne	FL 959	F19	Zanzibar, Tanzania	AJ544729
Phyllodictyon anastomosans (Harvey) Kraft & Wynne	FL 961	F36	Zanzibar, Tanzania	AJ544725
Phyllodictyon orientale (A. Gepp & E. Gepp) Kraft & Wynne	HEC 6173	F64	Bi Ya Doo Island, Maldives	AJ544738
Phyllodictyon papuense nom. prov.	HEC 4548	F68	Madang Prov., Papua New Guinea	AJ544736
Rhizoclonium riparium var *implexum* (Dillwyn) Rosenvinge	HEC 9623	F71	Bretagne, France	AJ544765
Siphonocladus tropicus (P. Crouan & H. Crouan) J. Agardh	Dargent s.n.	F101	Dominican Republic	AJ544744
Struvea elegans Børgesen	HEC 10437	F69	Port Moresby, Papua New Guinea	AJ544737
Struveopsis siamensis (Egerod) P. Silva	FL 916	F15	Zanzibar, Tanzania	AJ544732
Valonia aegagropila C. Agardh	FL 960	F22	Zanzibar, Tanzania	AJ544748
Valonia utricularis (Roth) C. Agardh	FL 922	F23	Zanzibar, Tanzania	AJ544749
Valoniopsis pachynema (G. Martens) Børgesen	FL 1006	F24	Zanzibar, Tanzania	AJ544741
Ventricaria ventricosa (J. Agardh) Olsen & J. West	FL 952	F40	Zanzibar, Tanzania	AJ544750
Outgroup taxa				
Ulva fasciata Delile	KZN 813	F73	KwaZulu-Natal, South Africa	AJ544726
Acrosiphonia spinescens (Kützing) Kjellmann	HEC 9608	F49	Bretagne, France	AJ544727

Table 2. Comparison of the domains C1 (partial), D1, C2, D2: length and position of the regions; number of sites removed prior to phylogenetic analysis; pairwise sequence divergence between ingroup taxa (minimum, maximum and average); number and percentage of parsimony-informative sites with and without outgroup taxa

Domain name	Length and position of the region	No. of sites removed	Pairwise sequence divergence[1]: min. – max. (av.)	No. and percentage of parsimony-informative sites[1]	
				In- and outgroup taxa	Ingroup taxa only
C1 (partial)	56 bp (1 – 56)	0	0 – 0.190 (0.074)	12 (6%)	10 (7%)
D1	153 bp (57 – 209)	9	0 – 0.326 (0.151)	65 (31%)	55 (36%)
C2	143 bp (210 – 352)	0	0 – 0.063 (0.013)	23 (11%)	6 (4%)
D2	292 bp (353 – 644)	94	0 – 0.415 (0.203)	108 (52%)	84 (54%)
Total	644 bp	103	0 – 0.232 (0.120)	208	155

[1]Calculations with ambiguous sites excluded and gaps treated as missing.

Table 3. Relative rate tests of sequences within clade A4, and between clades B4, B5 and B6

	probability[1]
within clade A4	
rupestris vs *spiralis*	p = 0.003
rupestris vs *vagabunda*	p = 0.368
spiralis vs *vagabunda*	p = 0.019
between B4, B5 and B6	
B4 vs B5	p = 0.012
B4 vs B6	p = 0.572
B5 vs B6	p = 0.025

[1]p-levels < 0.05 indicate that two clades evolve at significantly different rates.

Results and discussion

Sequence analyses and phylogeny

The aligned partial rRNA sequences were 644 sites in total. Average base composition was: A, 0.24; U, 0.19; C, 0.23; G, 0.33. The two conserved regions (C1 and C2) were easily aligned but in the divergent domains D1 and D2 numerous gaps had to be introduced, mainly in the loop regions. Site variability (calculated on the basis of pairwise sequence divergence) was greatest in the divergent domain D2 (Table 2). The alignment of this region had been made possible by its common secondary structure in all ingroup taxa: a basal stem, two central loops connected by a central stem, and three peripheral helices with terminal loops of variable size. *Chaetomorpha crassa* and *C. spiralis* possessed an extra helix of 32 nucleotides (positions 497 – 528).

Seventy-one positions with ambivalent alignment (all situated in the loop regions of the divergent domains) and the 32 sites in the extra helix of *Chaetomorpha crassa* and *C. spiralis* were removed prior to phylogenetic analysis. Of the 541 included nucleotide positions, 278 were variable and 208 parsimony-informative. The D2 domain contained the highest number of parsimony-informative sites (Table 2). The skewness value ($g_1 = -0.50$; threshold value $g_1 = -0.12$ ($p = 0.01$) for 25 taxa and 100 characters) indicated that the partial LSU rRNA sequences contained significant non-random structure that probably reflected phylogenetic signal. The average transition/transversion (ti/tv) ratio for the 541 nt alignment was 1.35 for the ingroup taxa alone.

Phylogenetic trees constructed with MP and ML methods gave similar topologies. The MP strict consensus tree will be discussed below as will the minor differences in the ML tree. MP analysis of the 43 taxa yielded 24 most parsimonious trees of 795 steps (CI = 0.57, RI = 0.78). The MP strict consensus phylogram with indication of bootstrap and decay index values is shown in Fig. 1. Within the Cladophorophyceae, *Cladophora horii* is placed as the sister taxon of the rest of the group with high bootstrap support. The two main ingroup lineages have long basal branches and are supported by high bootstrap and decay index values. The first lineage (A) consists of four clades with high bootstrap support, and includes the majority of *Cladophora* species, with *Chaetomorpha* and *Rhizoclonium* (three genera traditionally placed in the Cladophorales), and one *Cladophoropsis* species. The second lineage (B) consists of six clades with moderate to high bootstrap support, and contains genera traditionally placed in the Siphonocladales and two *Cladophora* species. The basal branches in both lineages remain largely unresolved. Clade B6 consists of a mixture of species belonging to six genera but the sequences are too conserved to resolve the ultimate polytomies in this clade. The ML differs from the MP consensus tree in some minor aspects: clade A2 forms a sister group to

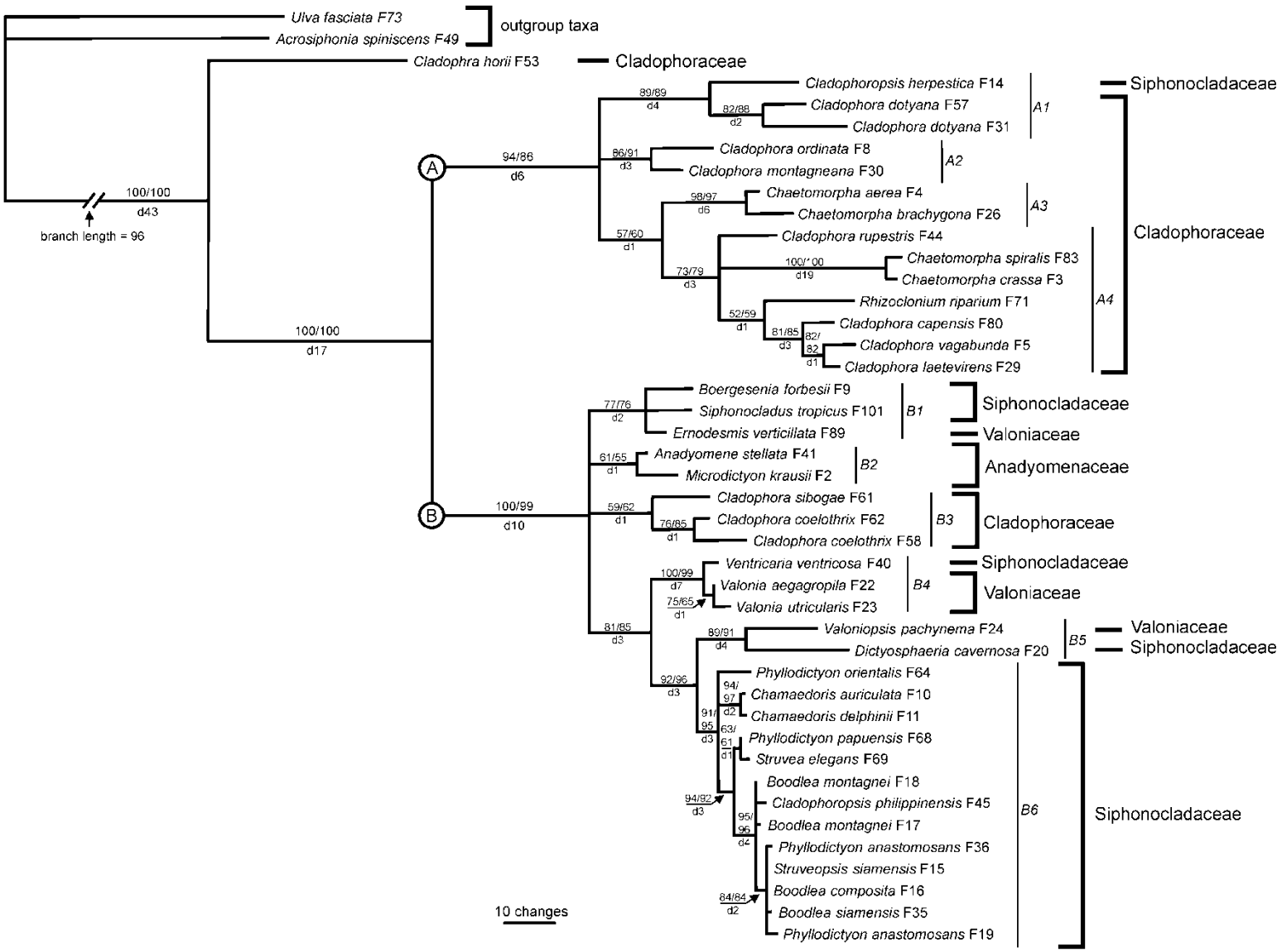


Fig. 1. Strict consensus phylogram of the 24 most parsimonious trees inferred from partial large subunit rRNA sequence data (gaps treated as missing). Tree length = 795 steps, CI = 0.57, RI = 0.78. Bootstrap percentages (MP/ML) are indicated above branches; decay index values are given below branches.

Table 4. Models of DNA substitution estimated for different datasets (ambiguous alignments excluded from the analyses)

Region		all taxa	Ingroup taxa	Lineages A and B	Lineage A	Lineage B
Complete alignment	hLRT	TrNef + G	TIMef + I + G	TIMef + I + G	TrNef + G	TrN + I + G
	AIC	GTR + I + G	GTR + I + G	GTR + I + G	GTR + I + G	TIM + I + G
D1 region	hLRT	K80 + G	K80 + G	K80 + G	K81 + G	K80 + G
	AIC	TVM + G	TVM + I + G	TVM + I + G	TVMef + G	K81uf + I
C2 region	hLRT	F81 + G	JC + I + G	JC + I + G	JC	JC + I
	AIC	TIM + G	GTR + I	GTR + I	TrN	TrN + I
D2 region	hLRT	TrN + G	TrN + G	TIMef + I + G	K80 + G	TIM + G
	AIC	GTR + G	GTR + I + G	GTR + I + G	TVM + G	TIM + G

clades A3 and A4 with low bootstrap support, and in lineage B, clade B1 branches off first, followed by clades B3 and B2 which group together, all with low bootstrap support.

The MP strict consensus phylogram shows considerable variation in branch length within certain clades. For example, within clade A4 the branches leading to *Chaetomorpha spiralis* and *C. crassa* are much longer than the branches leading to other taxa. In lineage B, the branches leading to the species in clade B5 are much longer than the branches in clades B4 and B6. Relative rate tests carried out on all taxa within clade A4 demonstrate that *Chaetomorpha spiralis* and *C. crassa* evolve at a significantly faster rate than *Cladophora rupestris* and the species of the *Cladophora vagabunda* clade. The same tests carried out on all taxa of clades B4, B5 and B6 show that there are significant differences in the substitution rates for species in clade B5 compared with clades B4 and B6.

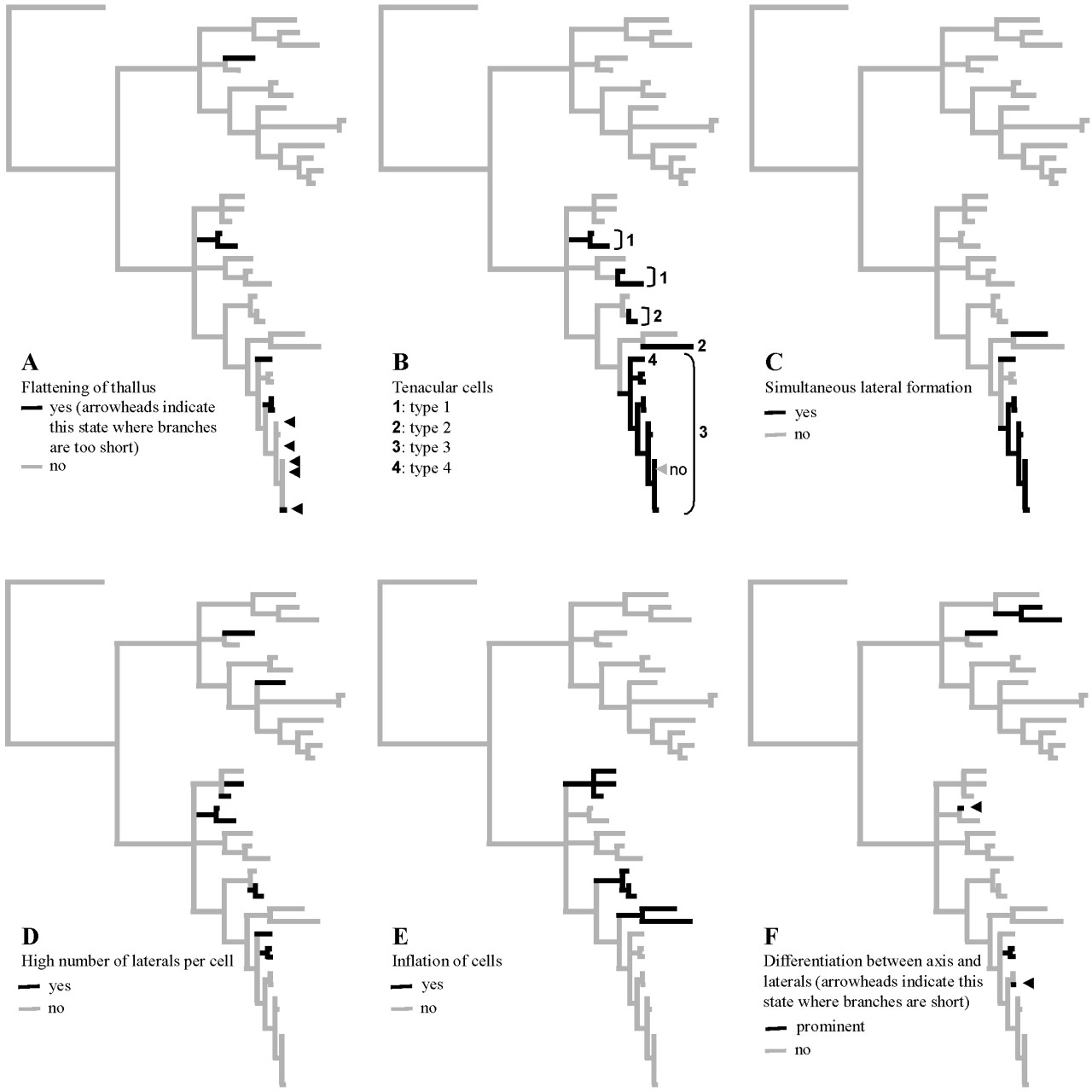
The phylogeny in this study is in general agreement with the 18S rRNA phylogenies of Bakker *et al*. (1994) and Hanyuda *et al*. (2002) but differs in sampling strategy. The two previous studies included mainly *Cladophora* species while the present study focuses on Siphonocladales (*s.s.*). It remains uncertain whether the basal taxon *Cladophora horii*, found in this study, would fit in the basal lineage found by Hanyuda *et al*. (2002).

Phylogeny and morphology

The morphological tendencies generating the morphological variety in the class, as hypothesized by van den Hoek (1982), are mapped on the strict consensus MP tree in Fig. 2. Flattening of the thallus (1) has evolved at least three times independently in the Cladophorophyceae (Fig. 2*A*): in *Phyllodictyon*, *Struvea* and *Boodlea montagnei* (clade B6), *Anadyomene* and *Microdictyon* (clade B2), and *Cladophora ordinata* (clade A2). Tenacular cells (2) are found only in taxa of lineage B (Fig. 2*B*). Tenacular cells of the first type (Olsen-Stojkovich, 1986) occur in *Anadyomene* and *Microdictyon* (clade B2); this type is considered here to be equivalent to van den Hoek's (1982) third morphological tendency, 'lateral coalition of cells'. In *Microdictyon* cell coalition occurs solely at the tips of the apical cells; in *Anadyomene* cells grow close to one another resulting in lateral coalition. The similarity in the mode of cell coalition in the two genera is evident in immature thalli of *Anadyomene*, where the thallus has an open reticulate structure, and anastomosis occurs at the cell apices, as in *Microdictyon* (Littler & Littler, 1991; unpublished personal observation). Tenacular cells of the first type also occur sporadically in *Cladophora coelothrix* (clade B3) (van den Hoek & Chihara, 2000). Tenacular cells (type 2) are found in *Valonia* (clade B4) and *Dictyosphaeria* (clade B5), and most likely evolved twice independently. Tenacular cells (type 3) characterize all taxa in clade B6 (except *Struveopsis*) and may have evolved once in the common ancestor of the clade. The fourth type of tenacular cells were found only in *Phyllodictyon orientalis* (clade B6). Simultaneous lateral formation (4) characterizes the genera *Boodlea*, *Phyllodictyon*, *Struvea* and *Struveopsis* (clade B6), and is also present to some extent in *Valoniopsis* (clade B5) (Fig. 2*C*). High numbers of laterals per cell (5) characterize *Cladophora ordinata* (clade A2), *C. rupestris* (clade A4), *Ernodesmis* (clade B1), *Anadyomene* and *Microdictyon* (clade B2), *Valonia* (clade B4), *Valoniopsis* (clade B5), *Chamaedoris* and *Phyllodictyon orientale* (clade B6) (Fig. 2*D*). This feature may have evolved once in the common ancestor of lineages A and B, or alternatively evolved several times independently. Inflated cells (6) occur in *Boergesenia*, *Ernodesmis* (clade B1), *Valonia*, *Ventricaria* (clade B4), *Valoniopsis* and *Dictyosphaeria* (clade B5) (Fig. 2*E*). Inflation of cells may have evolved once in the common ancestor of lineage B, or alternatively been gained several times independently. Differentiation between axis and laterals (7) is prominent in *Cladophora ordinata* (clade A2), *Cladophora dotyana* (clade A1), *Anadyomene* (clade B2), *Chamaedoris* and *Struvea* (clade B6) (Fig. 3F). Unbranched

thalli (8) originated several times independently in *Chaetomorpha* (clades A3 and A4) and in *Rhizoclonium* (clade A4); the thallus of *Ventricaria ventricosa* (clade B4), consisting of a single cell, can be regarded as an extreme example of branch reduction (Fig. 2*G*).

The four modes of cell division as defined by Olsen-Stojkovich (1986) are mapped on the strict consensus MP tree in Figs 2*H*–*I*. CI occurs in the basal taxon *Cladophora horii*, in all taxa of lineage A, and in the clades B1 (only in the rhizoids of *Boergesenia*, *Ernodesmis* and *Siphonocladus*), B2, B3, B5 (only in rhizoids of *Valoniopsis*) and B6 (in *Struvea elegans* CI occurs only in the rhizoids) (Fig. 3H). LC occurs in the clades B1 (only *Ernodesmis*), B4 (only *Valonia*) and B5 (only *Valoniopsis*). Okuda *et al*. (1997) demonstrated that in LC division of *Valonia*, protoplasm divides into a lenticular cell by a septum wall which is produced inwardly from the cell wall. This type of cell division can be seen as a modification of CI in taxa with inflated cells, where it is impossible to bridge the large diameter of the cells by invagination of cell walls. The occurrence of LC has co-evolved with the inflation of cells, which in their turn evolved several times independently in lineage B. SDSS and SDM occur only in taxa of lineage B. SDSS occurs in *Siphonocladus* (clade B1), *Dictyosphaeria* (clade B5) and *Struvea* (clade B6); the cells of *Boergesenia*, *Ernodesmis* (clade B1) and *Ventricaria* (clade B4) divide by SDM. In some species of clade B6 (*Phyllodictyon* spp., *Boodlea* spp., *Cladophoropsis philippinensis* and *Chamaedoris* spp.) SDSS occurs only occasionally, for example in



association with a wounding response (La Claire, 1982; van den Hoek & Chihara, 2000; unpublished personal observation). Apparently the mode of cell division is not a clear-cut character in the Cladophorophyceae. Four types are recognized but many species exhibit a mixture of two or three of these types. Moreover, mode of cell division cannot be used to typify the monophyletic clades in the present phylogeny (Fig. 2*I*).

The evolution from *Cladophora*-type architecture (branched filaments with cross-walls at the base of newly formed laterals) to a *Cladophoropsis*-like morphology (mostly unilaterally branched filaments with delay of cross-wall formation) has happened at least twice independently in the Cladophorophyceae (Fig. 2*J*). Delay of cross-wall formation is prominent in *Cladophoropsis* and *Chamaedoris* but also occurs to some extent in *Cladophora coelothrix* (clade B3) and in all other species of clade B6.

Calcium oxalate crystals have been observed in certain species of the Cladophorophyceae (unpublished personal observation) (Fig. 2*K*). Three morphological types have been classified: (1) needle-shaped to hexagonal, (2) clustered rod-shaped and (3) octahedral crystals. Species situated in clade B6 (except *Struvea elegans* and *Chamaedoris*) possess crystals of the first type. Clustered rod-shaped crystals are present in the cells of *Dictyosphaeria* (clade B5). Octahedral crystals have

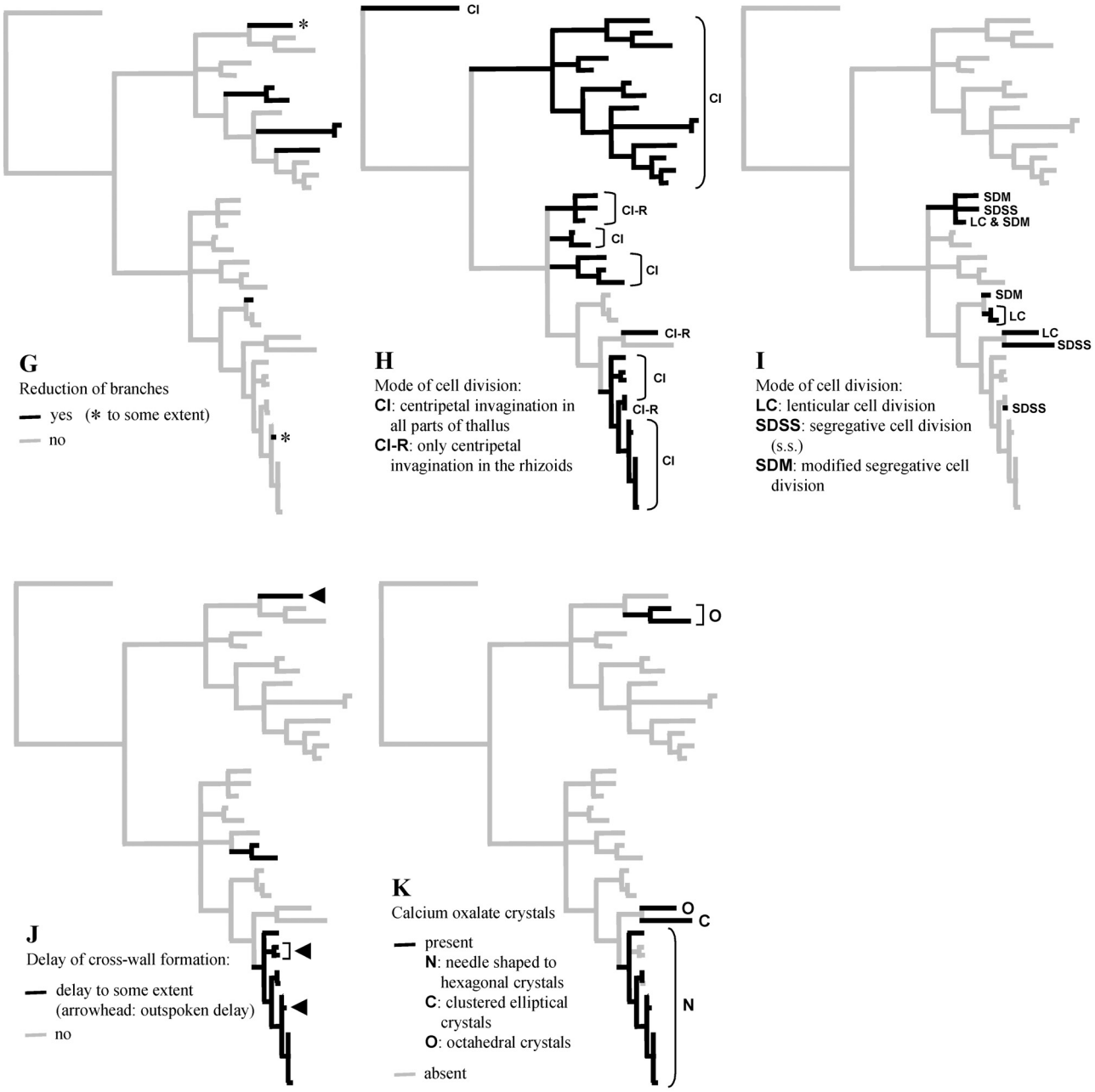


Fig. 2. Character mapping on the strict consensus MP tree.

been found in *Valoniopsis pachynema* (clade B5) and *Cladophora dotyana* (clade A1). Calcium oxalate crystals have evolved at least twice independently, although the crystalline inclusions of the first type may have evolved only once in clade B6.

Phylogeny and taxonomy

The molecular phylogeny of the Cladophorophyceae differs considerably from the traditional classification based on thallus architecture and mode of cell division. Homoplasy caused by convergence, parallel evolution and secondary reduction seems to be an important factor in clouding the evolutionary relationships within the Cladophorophyceae based on morphology. Of the four or five recognized families, only the morphologically well characterized Anadyomenaceae (including *Anadyomene* and *Microdictyon*) appears to form a monophyletic group. The other families are polyphyletic. The species of some genera (e.g. *Valonia*, including *Ventricaria*) seem to form natural groups but other genera are clearly polyphyletic (*Cladophora* and *Chaetomorpha*) or belong to a genus complex (e.g. clade B6).

The genus ***Cladophora*** as presently conceived is polyphyletic, corresponding with the results of Hanyuda *et al*. (2002). Ten species of *Cladophora* are included in the study, representing six sections: Rugulosae, Glomeratae, Longi-articulatae, Repentes, Rupestres and Willeella. The species of the first section are placed in lineage B while all other *Cladophora* species fall within the basal lineage or lineage A.

The section **Repentes** is characterized by cushion-like growth forms and the development of rhizoids sprouting from the basal poles of cells in any part of the thallus. The two species included in this study, *C. coelothrix* and *C. sibogae*, are sister taxa in clade B3. Based on morphological characters, van den Hoek (1982) suggested possible relationships between the *Cladophora* section Boodleoides and the genus *Microdictyon*, and between the sections Boodleoides, Aegagropila and Repentes. There is no support for this hypothesis in the present study but the 18S rRNA phylogeny of Bakker *et al*. (1994) confirmed the affinity between *Microdictyon boergesenii*, *Cladophora liebetruthii* (section Boodleoides) and *Cladophora catenata* (section Aegagropila), and indicated a possible relationship of the species above with two *Cladophora* species of the section Repentes (*C. coelothrix*, *C. socialis*) and *C. prolifera* (section Rugulosae).

Thallus architecture in the section **Glomeratae** *s.l.* (van den Hoek & Chihara, 2000) is relatively heterogeneous and varies from strictly acropetal to irregular; thallus growth takes place by apical or intercalary cell division. Based on the four species included in this study this section is polyphyletic: three species (*C. capensis*, *C. laetevirens* and *C. vagabunda*) are grouped in clade A4 with *Rhizoclonium riparium* var. *implexum*; the fourth species, *C. montagneana*, is located in clade A2 together with *C. ordinata* (section Willeella, see below). The section **Longi-articulatae**, characterized by acropetally organized branch systems, growth by apical cell division and thalli with a conspicuous basal stipe, is represented here by a single species, *C. dotyana*. *Cladophora dotyana* from the Philippines and South Africa always clade together, with *Cladophoropsis herpestica* forming a sister taxon (clade A1). The grouping of *C. herpestica* with *C. dotyana* is unexpected from a morphological viewpoint, as these taxa have very few characters in common. The section **Rugulosae** is characterized by dark green, broom-like thalli composed of acropetally organized branch systems, growth mainly by apical cell division, and attachment by numerous stipes producing descending rhizoids. The section is represented in this study by a single recently described species, *C. horii* (van den Hoek & Chihara, 2000), which forms the basal branch of the Cladophorophyceae. In the 18S rRNA phylogeny of Bakker *et al*. (1994), *C. prolifera*, another representative of the section Rugulosae (not included in this study), grouped with *C. coelothrix* and *C. socialis* in a clade corresponding to clade B3 in the present study. The section **Rupestres** (*s.s.*) (van den Hoek & Chihara, 2000) is also characterized by dark green, broom-like thalli, but they are composed of dense, irregular branch-systems with distinct main axes bearing several short laterals. *C. rupestris*, the only species of the section, groups together in clade A4 with the species of the section Glomeratae, *Rhizoclonium* and two *Chaetomorpha* species. According to van den Hoek & Chihara (2000), *C. rupestris* fits perfectly in the section Glomeratae from a morphological viewpoint, but is placed in a different section because of its position in the 18S rRNA phylogenetic tree of Bakker *et al*. (1994).

The section **Willeella** is characterized by fan-like thalli composed of regular, oppositely branched laterals in a single plane. The section is represented by a single species, *C. ordinata*, the type and only species of *Willeella* (Børgesen 1930). Its distinct morphology has led to this taxonomic viewpoint being still widely accepted today (van den Hoek & de Rios, 1972; Silva *et al*., 1996). *C. ordinata* groups together with *C. montagneana* (section Glomeratae) in clade A2. The two species have a number of characters in common: similar general architecture; apical cells with an obtuse tip and an apical thickening of the cell wall; and opposite and equal

laterals (less frequent in *C. montagneana*). van den Hoek (1982: 124) suggested a close relationship between *C. ordinata* and *C. rupestris* based on similarities in branch-pattern, but in our phylogeny these species do not group together. Based on the present results the genus *Willeella* could either be reduced to a section of *Cladophora* (*s.l.*, comprising all taxa of lineage A), or alternatively the genus *Willeella* could be maintained and *C. montagneana* should then be transferred to this genus.

The genus ***Chaetomorpha*** is characterized by unbranched filaments growing exclusively by intercalary cell division. Species are distinguished by only a few characters such as cell shape and diameter, thallus morphology, and mode of attachment (thalli are either basally attached and erect, or unattached and form entangled, free-floating masses). Four *Chaetomorpha* species are included in this study: two attached and two unattached species. *Chaetomorpha aerea* (attached) and *C. brachygona* (unattached) always group together in clade A3. The other *Chaetomorpha* species (the attached *C. spiralis* and unattached *C. crassa*) are located in clade A4, together with some *Cladophora* species and *Rhizoclonium*. As already suggested by van den Hoek (1982), *Chaetomorpha* can be regarded as a reduced form of *Cladophora* and not the primitive sister genus of *Cladophora*. The genus is polyphyletic and evolved at least twice independently within lineage A. Bakker *et al.* (1994) already showed that *Chaetomorpha* falls within a *Cladophora* clade on the basis of one (unidentified) *Chaetomorpha* species. Hanyuda *et al.* (2002) found *Chaetomorpha okamurae* in the basal clade, but this species may belong to *Cladophora* (van den Hoek, 1963). Since all *Cladophora* species in this study have attached thalli, it is likely that unattached forms of *Chaetomorpha* are derived from attached forms; this event took place at least twice independently. Moreover juvenile plants of *C. crassa* have been observed attached as epiphytes; older plants soon come loose and are able to grow further as unattached forms (E. Coppejans, unpublished data).

Rhizoclonium also falls within *Cladophora*, and can be regarded as a reduced form in lineage A. Hanyuda *et al.* (2002) have already demonstrated that the evolution from *Cladophora*-type architecture to *Rhizoclonium*-like morphology (unbranched filaments with rhizoidal laterals) has taken place several times independently.

The monotypic genera ***Ernodesmis*** and ***Boergesenia***, and ***Siphonocladus tropicus***, always group together in clade B1. Their systematic positions were the subject of earlier speculation (Papenfuss & Chihara, 1975; Olsen-Stojkovich, 1986). Some authors considered *Ernodesmis* to be a member of the Siphonocladaceae based on its annular constrictions (Børgesen, 1913, 1940), while others (Oltmanns, 1922; Taylor, 1960) allied *Ernodesmis* with *Valonia* and placed it in the Valoniaceae based on lenticular and tenacular cells. *Boergesenia* has been allied with both *Ventricaria ventricosa* and *Siphonocladus*, based on the mode of cell division and presence of basal annular constrictions, respectively (Olsen-Stojkovich, 1986). Olsen-Stojkovich (1986) considered *Siphonocladus* to be related to *Dictyosphaeria* based on immunological and morphological evidence. *Ernodesmis*, *Boergesenia* and *Siphonocladus* are all characterized by inflated, club-shaped cells with basal annular constrictions but differ in their mode of cell division, and consequently in their thallus architecture. *Ernodesmis* forms spherical thalli composed of cells with verticillate, apical clusters of branches formed by LC (SDM occurs only occasionally). The branches in *Siphonocladus tropicus* are formed by SDSS and radiate laterally from the club-shaped main axes. In *Boergesenia* the club-shaped cells remain unbranched and cell division occurs by SDM.

The genera ***Microdictyon*** and ***Anadyomene***, characterized by unistratose, blade-like thalli and similar modes of cell coalition, always group together in clade B2, a relationship proposed by Kützing (1843: 302, 311) who established the family Anadyomenaceae for them. The family has been placed either in the Siphonocladales (*s.s.*) because of the blade-like thalli (Børgesen, 1942; Egerod, 1952) or in the Cladophorales (*s.s.*) based on the mode of cell division (Papenfuss, 1955). In the present study the family is positioned among the siphonocladalean taxa.

Valonia has traditionally been allied with *Dictyosphaeria* and placed in the Valoniaceae because its thalli lack a central axis and are composed of inflated cells (Feldmann, 1938*b*; Egerod, 1952). Olsen & West (1988) linked *Valonia* to *Ernodesmis* and *Valoniopsis* on the basis of lenticular cell division. They erected *Ventricaria* based on *Valonia ventricosa* on the evidence of immunological data, mode of cell division and reduced habit, and suggested that it had phylogenetic alliances with *Siphonocladus* and *Dictyosphaeria*. *Valonia*, in contrast, seemed to be closely related to *Valoniopsis*, *Chaetomorpha* and *Cladophora vagabunda*. In the present study the *Valonia* species were placed in clade B4, very closely related to *Ventricaria* (divergence of only 0.86%), questioning whether the recognition of a separate genus is warranted. The three species form a sister group to clades B5 and B6 (see below).

Valoniopsis has been considered to be related either to *Anadyomene*, based on similarities in branching pattern (Børgesen, 1934), or *Valonia* and

Ernodesmis in the Valoniaceae because of lenticular cell division and inflated cells (Papenfuss & Egerod, 1957). This study shows neither position to be correct, instead linking *Valoniopsis* with ***Dictyosphaeria***, although the two genera differ in thallus architecture and mode of cell division.

The tight clustering of ***Boodlea, Chamaedoris, Cladophoropsis*** (*p.p.*), ***Phyllodictyon, Struvea*** and ***Struveopsis*** in clade B6 is not surprising given that only *Chamaedoris* can be easily distinguished (Børgesen, 1913; Egerod, 1952; Kooistra *et al.*, 1993; Leliaert *et al.*, 1998). Based on ITS sequences, Kooistra *et al.* (1993) suggested the very close affinity between *Boodlea coacta* (a taxonomic synonym of *B. composita*), *Phyllodictyon anastomosans* and *Cladophoropsis membranacea* (not included in this study). Immunological results (Olsen-Stojkovich, 1986) indicated that *Chamaedoris* is closely related to *Cladophoropsis membranacea*, but to a lesser extent to *Boodlea* and *Phyllodictyon*.

The two ***Cladophoropsis*** species included in the present study emerge in the two main lineages: *C. philippinensis* falls within the *Struvea* complex (clade B6), while *C. herpestica* groups together with *Cladophora* species of the section Longiarticulatae in lineage A. Non-monophyly of *Cladophoropsis* has also been demonstrated by Bakker *et al.* (1994), based on 18S rRNA sequences of *C. membranacea* and *C. zollingeri*. Two important characters distinguishing the *Cladophoropsis* species from both lineages are the mode of cell division and calcium oxalate crystals. In the *Cladophoropsis* species of lineage B segregative cell division occasionally occurs, especially in response to wounding (La Claire, 1982), and the cells contain calcium oxalate crystals (unpublished personal observation). The *Cladophoropsis* species of lineage A divide by centripetal invagination and lack calcium oxalate crystals. The *Cladophoropsis* species in lineage A can be regarded as reduced forms of a *Cladophora*-type architecture. The *Cladophoropsis* species in clade B6 can either be seen as reduced forms of a *Boodlea*/*Struvea*-type architecture, or the *Cladophoropsis*-type morphology can be considered as an ancestral state from which the *Boodlea*/*Struvea*-type architecture has evolved.

Conclusions

The partial LSU rRNA sequences contain the appropriate amount of variation to resolve the basal divergences within the Cladophorophyceae. The present study in combination with previously published 18S rRNA phylogenies (Bakker *et al.*, 1994; Hanyuda *et al.*, 2002) reveals three or four lineages within the Cladophorophyceae: one or two basal lineages comprising taxa with a *Cladophora*-type architecture, and two main lineages. The first lineage (A) includes most *Cladophora* species and several taxa with a reduced thallus architecture. The second lineage (B) comprises taxa with specialized morphologies and a few *Cladophora* species with some unique characters (e.g. rhizoids in the apical regions of the thallus) not found in the species of lineage A. The basal lineage comprising *Cladophora horii* suggests that the siphonocladalean morphologies arose as specialized forms from a *Cladophora*-like ancestor. The present study partially confirms van den Hoek's (1982) hypothesis. Different reduction events have indeed occurred several times independently. All taxa with specialized thalli, however, are grouped in one lineage, with the first divergences being unresolved. This either indicates a single evolutionary event of an ancestor with a *Cladophora*-type architecture giving rise to the siphonocladalean taxa, or a number of independent evolutionary events which took place in a relatively short period of time and therefore cannot be revealed in the present phylogeny. The grouping of all siphonocladalean taxa (excluding part of *Cladophoropsis* and including some *Cladophora* species) in one lineage, separated by long branches from the cladophoralean lineages, clearly supports the recognition of a separate order Siphonocladales with the Cladophorales (*s.s.*) remaining paraphyletic. This is in contrast to the viewpoint of Bakker *et al.* (1994) who regarded the order Siphonocladales as polyphyletic and stated that there is no basis for independent recognition of the Cladophorales (*s.s.*) and Siphonocladales (*s.s.*).

In order to construct a classification that reflects the evolutionary history of the Cladophorophyceae, dramatic taxonomic changes have to be made. The polyphyletic genus *Cladophora* could be split into different genera as already pointed out by Bakker *et al.* (1994) and van den Hoek & Chihara (2000), or the other genera in the lineage (*Chaetomorpha*, *Cladophoropsis* (*p.p.*) and *Rhizoclonium*) could be transferred to *Cladophora*. The very close relationship between *Boodlea*, *Chamaedoris*, *Cladophoropsis* (*p.p.*), *Phyllodictyon*, *Struvea* and *Struveopsis*, in combination with fuzzy morphological boundaries between these genera, would favour the recognition of a single genus. Merging other closely related genera (e.g. *Valoniopsis* and *Dictyosphaeria*) would be more problematic from a morphological viewpoint since both genera are characterized by distinct characters. In general, the essential difficulty arising from reforming the cladophorophycean taxonomy is finding apomorphic morphological characters for the monophyletic groups. Before undertaking

radical taxonomic and nomenclatural changes, further morphological and molecular research is needed. Ultrastructural and chemical studies might provide useful characters to delimit natural groups in the rest of the Cladophorophyceae (Hanyuda *et al.*, 2002). In future extended phylogenies, the different genera should be represented by additional species, including their types. This is essential because the simple morphological characters that provide generic concepts in the class could easily have evolved multiple times. The relationship between taxa in the terminal clade B6 requires further investigation using more variable molecular markers.

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