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Molecular phylogeny of the Siphonocladales (Chlorophyta: Cladophorophyceae)

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Abstract

The Siphonocladales are tropical to warm-temperate, marine green macro-algae characterized by a wide variety of thallus morphologies, ranging from branched filaments to pseudo-parenchymatous plants. Phylogenetic analyses of partial large subunit (LSU) rDNA sequences sampled from 166 isolates revealed nine well-supported siphonocladalean clades. Analyses of a concatenated dataset of small subunit (SSU) and partial LSU rDNA sequences greatly clarified the phylogeny of the Siphonocladales. However, the position of the root of the Siphonocladales could not be determined unambiguously, as outgroup rooting and molecular clock rooting resulted in a different root placement. Different phylogenetic methods (likelihood, parsimony and distance) yielded similar tree topologies with comparable internal node resolution. Likewise, analyses under more realistic models of sequence evolution, taking into account differences in evolution between stem and loop regions of rRNA, did not differ markedly from analyses using standard four-state models. The molecular phylogeny revealed that all siphonocladalean architectures may be derived from a single *Cladophora*-like ancestor. Parallel and convergent evolution of various morphological characters (including those traditionally employed to circumscribe the families and genera) have occurred in the Siphonocladales. Consequently, incongruence with traditional classifications, including non-monophyly in all families and most genera, was shown.

Keywords: Benthic marine algae; Cladophorales; Convergent evolution; Doublet model; Long branch attraction; Molecular clock rooting; Morphological evolution; Outgroup rooting; Ribosomal DNA; RNA secondary structure; Segregative cell division; Taxonomy; Cladophorophyceae; Molecular systematics; Siphonocladales

1. Introduction

The Cladophorophyceae are green macro-algae found in tropical to cold-temperate coastal waters and freshwater habitats. They have a siphonocladous level of organization, which means that the multicellular thalli are composed of multinucleate cells (van den Hoek et al., 1995). Traditional classifications were largely based on thallus architecture and mode of cell division. Plants consisting of branched filaments were grouped in a large genus *Cladophora* (Fig. 1A) and placed in the order Cladophorales along with the unbranched filamentous genera *Chaetomorpha* and *Rhizoclonium*. The other genera (ca. 20 recognized at present), each characterized by their own typical thallus architecture, were placed in the order Siphonocladales. For example, blade-like thalli were classified in *Anadyomene* (Fig. 1H), plants with strongly inflated branched cells in *Valonia* (Fig. 1J), pseudo-parenchymatous thalli in *Dictyosphaeria* (Fig. 1K) and cushion-like thalli with specialized tenacular cells in *Boodlea*. Family level classification has been highly contentious. Five families are generally recognized (Anadyomenaceae, Boodleaceae, Cladophoraceae, Siphonocladaceae and Valoniaceae) but their boundaries are

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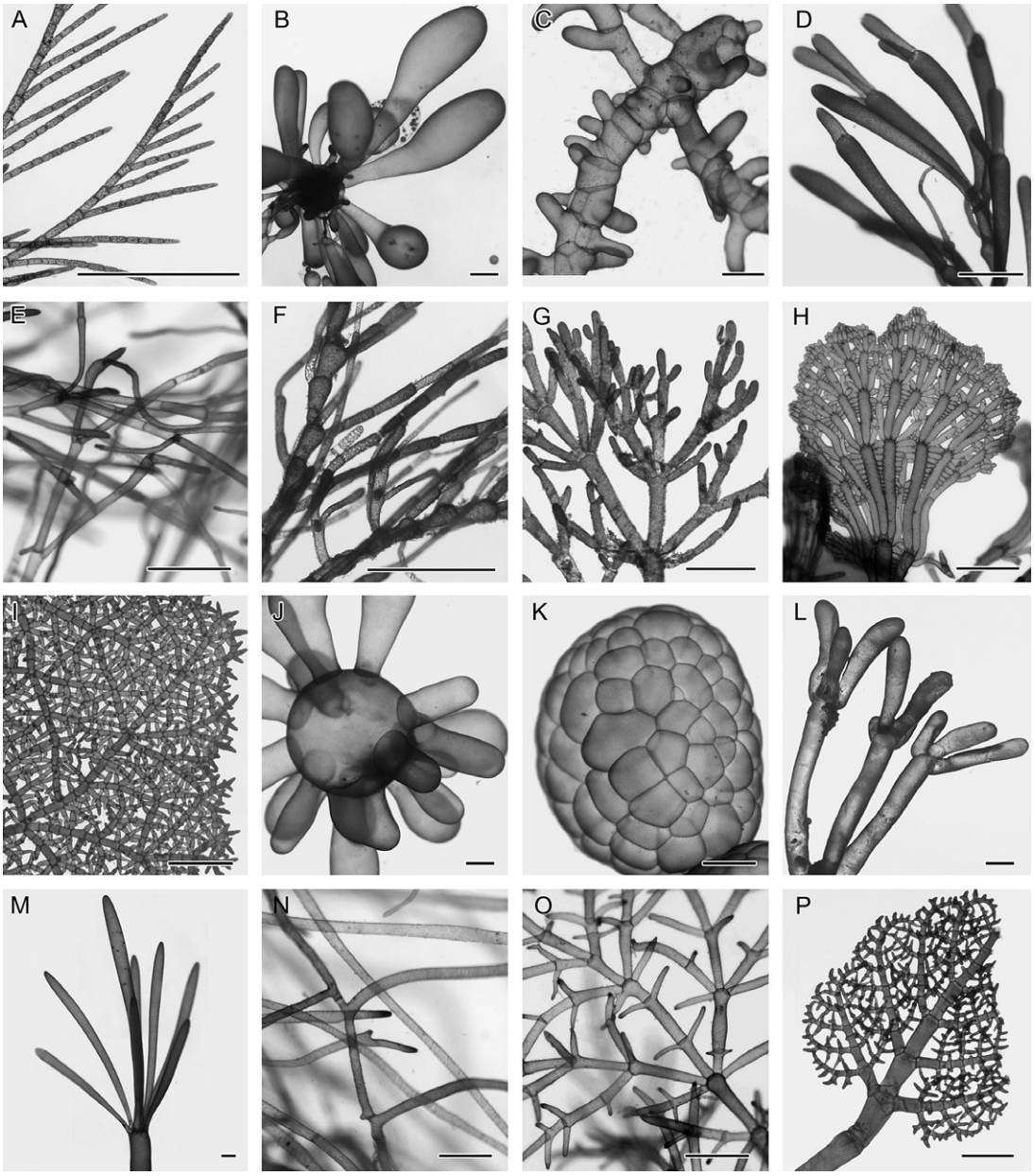


Fig. 1. Morphological variety in the Siphonocladales (A) *Cladophora sericea* (Cladophorales: outgroup), (B) *Boergesenia forbesii* (F252: clade 1), (C) *Siphonocladus pusillus* (F306: clade 1), (D) *Cladophora prolifera* (F280: grade 2), (E) *Cladophora coelothrix* (F275: grade 2), (F) *Cladophora* sp. 1 (F224: "clade" 3), (G) *Cladophora sibogae* (F61: clade 4), (H) *Anadyomene stellata* (F268, clade 5), (I) *Microdictyon krausii* (F2: clade 5), (J) *Valonia utricularis* (F242: clade 6), (K) *Dictyosphaeria cavernosa* (F283: clade 7), (L) *Valoniopsis pachynema* (F24: clade 8), (M) *Apjohnia laetevirens* (F273: clade 9), (N) *Cladophoropsis membranacea* (F295: clade 9), (O) *Phyllodictyon orientale* (F414: clade 9), (P) *Phyllodictyon anastomosans* (F36: clade 9). Scale bars, 1 mm.

rather vague and the genera assigned to them have changed frequently in the course of time (Leliaert, 2004) (Table 1).

Cytokinesis has conventionally been considered to be a key character for ordinal, familial, as well as generic level taxonomy (van den Hoek, 1984). Olsen-Stojkovich (1986) recognized four different modes of cell division within the Cladophorophyceae. Mostly cells divide by centripetal invagination of a primordial septum (CI), a process that is well documented at the light-microscopic (Enomoto and Hirose, 1971) and ultrastructural level (McDonald and Pickett-Heaps, 1976; Scott and Bullock, 1976). Three other specialized modes of cell division occur in various members of the Siphonocladales lineage. In segregative cell division (SCD), the whole protoplasm divides simultaneously into numerous multinucleate aggregates of cytoplasm, which later form walled spheres that remain in the parent cell and expand to form new cells or branches (Børgesen, 1912, 1913; Egerod, 1952). SCD has been described in detail in *Dictyosphaeria* by Enomoto and Okuda (1981); Enomoto et al. (1982) and Okuda et al. (1997). A modified type of segregative cell division (SCDM), in which cytoplasmic spheres are released from the parent cell, settle and form new plants, has been distinguished in *Ventricaria* and *Boergesenia* by Olsen-Stojkovich (1986) and Olsen and

Table 1
Distribution of genera in the five recognized families of the Cladophorophyceae, illustrating the unstable family level classification (based on Børgesen, 1940; Egerod, 1952; Taylor, 1928, 1950, 1960; Womersley and Bailey, 1970; Silva et al., 1996; Kraft, 2000)

	Anadyomenaceae	Boodleaceae	Cladophoraceae	Siphonocladaceae	Valoniaceae	Siphonocladales phylogeny: clade
Anadyomene	•				•	5
Apjohnia			•			9
Boergesenia				•		1
Boodlea		•	•	•	•	9
Chamaedoris				•	•	9
Cladophora			•			2, 3, 4, 5 + Outgroup
Cladophoropsis		•	•	•	•	9
Dictyosphaeria				•	•	7
Ernodesmis				•	•	1
Microdictyon	•	•	•		•	5
Phyllodictyon	•	•		•		9
Siphonocladus				•	•	1
Struvea		•		•	•	9
Struveopsis				•		9
Valonia					•	6
Valoniopsis	•				•	8
Ventricaria				•	•	6

West (1988). In various other members of the Siphonocladales (e.g. *Cladophoropsis* and *Ernodesmis*), cell wounding induces a reaction which closely resembles segregative cell division (La Claire, 1982; O'Neil and La Claire, 1984). In some large-celled taxa, cell division takes place by the formation of a convex septal disk along the cell-wall, followed by the formation of a new lateral; this process has been termed lenticular cell division (LCD). Okuda et al. (1997) demonstrated that in *Valonia*, the process of lenticular cell formation is similar to CI, i.e. by a septum that is produced inwardly from the cell wall. LCD can thus be regarded as a modification of CI in inflated cells, where it is impossible to bridge the large cell diameter by invagination of cell walls (Leliaert et al., 2003).

Early phylogenetic hypotheses based on comparative morphology by van den Hoek (1982, 1984) and Olsen-Stojkovich (1986) were soon followed by studies including immunological distances (Olsen-Stojkovich et al., 1986) and single-copy DNA–DNA hybridization (Bot, 1992). Molecular phylogenetic studies based on gene sequence data of the ribosomal small subunit (SSU: Bakker et al., 1994; Hanyuda et al., 2002) and partial large subunit (LSU: Leliaert et al., 2003) demonstrated that the Cladophorophyceae consists of three main lineages rather than the traditional two orders. Additionally, these studies revealed that the traditional family and genus level classifications did not reflect the phylogenetic relationships. The genus *Cladophora* appeared to be polyphyletic with representatives being distributed in all three lineages. However, all genera with specialized thallus architecture and mode of cell division did clearly group in a single lineage, which was found to correspond largely to the Siphonocladales as traditionally circumscribed, with the exception of a few anomalous *Cladophora* taxa (Leliaert et al., 2003). This lineage exhibits an extremely broad morphological diversity with plants ranging from branched filaments, blade-like, strongly inflated cells to a pseudo-parenchymatous level of organization (Fig. 1). This is in contrast with representatives of the two other lineages, the Cladophorales and the *Aegagropila* lineage, in which morphological variety is basically restricted to very simple, branched or unbranched filaments. Taxa in the Siphonocladales lineage have a mainly tropical to warm-temperate distribution, while many representatives of the Cladophorales and the *Aegagropila* lineage have successfully invaded cold-temperate to even Arctic and Antarctic regions (Wagner and Zaneveld, 1988; Lindstrom, 2001). Likewise, the Siphonocladales are strictly confined to marine environments, whereas several species of the Cladophorales and especially the *Aegagropila* lineage have adapted to freshwater and even terrestrial habitats (Fritsch, 1944; Rindi et al., 2006).

Because of their wide morphological diversity, the Siphonocladales are an excellent group to study the evolutionary mechanisms that underlie morphological diversification. Hence, the need for a robust phylogenetic framework. Previous molecular phylogenetic studies based on SSU and LSU rDNA sequences suffered from two problems. First, most genera were only represented by a single specimen, not taking into account that the morphological characters that define genera and species may have evolved multiple times. In order to fully understand the morphological evolution within the Siphonocladales we here determine phylogenetic relationships among the Siphonocladales based on an extensive taxon sampling of 166 ingroup sequences representing 50 species. A second persistent problem in previous siphonocladalean phylogenies is the lack of overall resolution (using SSU) or resolution in the basal divergences (using LSU). Short and unresolved branches can be attributed to a number of factors, including conflict between characters, lack of phylogenetic informativeness of the markers, insufficient taxon sampling or a historical signal of a rapid evolutionary radiation. In this study we aim to infer the relationships among Siphonocladales with more confidence by increasing the

number of characters (combining SSU and partial LSU sequence data) and by applying more appropriate models of sequence evolution in the phylogenetic analyses. Because the functionality of RNA molecules lies in their secondary structure, which is mediated by base pairing between sometimes distant regions of the RNA molecule, there is a selective pressure for maintenance of the rRNA secondary structure. Substitutions affecting stem nucleotides have a different probability of fixation as compared to a nucleotide in a loop. Considering the widely accepted view that using more realistic models of sequence evolution should lead to more accurate phylogenies, the differences in evolution between stem and loop regions of rDNA should ideally be accounted for (Murray et al., 2005; Telford et al., 2005). Using our molecular phylogenetic results, we aim to assess previous morphology-based hypotheses of siphonocladalean relationships.

2. Materials and methods

2.1. Taxon sampling and morphology

Sample information is listed in Appendix A1 (Supplementary data). Broad taxonomic and geographical sampling was carried out to ensure as complete a representation of the Siphonocladales as possible. We analyzed 166 ingroup specimens belonging to 54 species from all 17 extant genera in the Siphonocladales. Generitypes are included for all but two genera (*Microdictyon* and *Phyllodictyon*). Six representatives of the Cladophorales were selected as outgroup taxa based on existing hypotheses of their affinities with the Siphonocladales (Hanyuda et al., 2002; Leliaert et al., 2003).

To permit direct comparison, morphological characters and their states were collected from specimens also included in the molecular study. Morphological observations were made on specimens preserved in 5% formalin-seawater solution, on rehydrated herbarium specimens or on cultured material. Calcium oxalate crystals were examined using differential interference (Nomarski) contrast. Photographs were taken with an Olympus-DP50 digital camera mounted on a Wild M10 (Leica Microsystems) stereomicroscope.

2.2. DNA amplification and sequencing

DNA was extracted from silica gel-dried specimens, from herbarium material, or from living plants in culture. Total genomic DNA was extracted using a standard CTAB-extraction method and subsequent purification with a Wizard® DNA Clean-Up System (Promega) following the manufacturer's protocol. The SSU rDNA gene (ca. 1700 nucleotides) was amplified as two overlapping products with primer pairs SR1-SS11H and SSU897-18SC2 (Table 2). The partial LSU rDNA gene (ca. 550 nucleotides) was amplified as a single product using primers C1FL and D2FL. For some degraded or contaminated samples, two additional internal primers, specific for the Siphonocladales (LSUintF and LSUintR), were used. PCR conditions of the SSU primer combinations consisted of an initial denaturation step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 s, followed by a final extension of 3 min at 72 °C. For the PCR with the LSU primer combinations, denaturation, annealing and extension steps were reduced to 30 s. Excess primer and dNTP were removed with ExoSAP-IT® (USB Corporation) for 15 min at 37 °C, followed by 15 min at 80 °C to inactivate the enzymes. The resulting products were used for cycle sequencing with the primers of the initial PCR using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit following the manufacturer's instructions. Sequencing products were analyzed with an ABI 3100 Prism Genetic Analyzer (PE Applied Biosystems). Sequences were edited and assembled with Sequencher v4.0.5 software (Gene Codes).

2.3. Sequence alignments, phylogenetic analyses and reconstruction of character evolution

The SSU and partial LSU rDNA sequences were aligned on the basis of their rRNA secondary structure information with DCSE v2.6 (De Rijk and De Wachter, 1993). The rationale for using secondary structure models for aligning rDNA sequences is based on the fact that the conservation of secondary structures exceeds that of nucleotides (Kjer, 1995). The SSU rDNA sequences of several cladophoralean and siphonocladalean representatives incorporated in the European Ribosomal RNA Database (http://www.psb.ugent.be/rRNA/), was used as an initial model for building the SSU alignment. The LSU alignment was based on Leliaert et al. (2003). The alignment of the variable helices 43 and 49 of the SSU gene and helices B15 and C1-1 to C1-5 of the LSU gene [see De Rijk et al. (1999) and Wuyts et al. (2001) for secondary structure nomenclature of the SSU and LSU, respectively] was refined and aided by folding the sequences of each sample using the Mfold software (http://www.bioinfo.rpi.edu/) (Zuker, 2003). Positions with ambivalent homology assignment, mainly situated in the loop regions of the above-mentioned variable helices, were removed prior to phylogenetic analysis. Alignments are available from EMBL-EBI (Accession Nos. ALIGN_001139 and ALIGN_001141 for the SSU and LSU alignments, respectively). The alignments including the secondary structure annotation can be obtained from FL on request.

The amount of phylogenetic signal versus noise in the rDNA data was assessed by three different approaches. First, the measure of skewness [g_1-value calculated by using 10,000 randomly selected trees in PAUP* 4.0b10 (Swofford, 2002)] was compared with the empirical threshold values in Hillis and Huelsenbeck (1992) to verify for non-random structuring of the data. Secondly, the rDNA data were tested for substitutional saturation by plotting the uncorrected distances against corrected distances as determined

Table 2
Primer sequences used for PCR amplification and sequencing

Primer name	Gene	Primer sequence (5′–3′)	Primer direction and position	Reference
SR1	SSU	TACCTGGTTGATCCTGCCAG	F: 1–20[a]	Hanyuda et al. (2002)
SS11H	SSU	CCTTTAAGTTTCAGCCTTGCGACC	R: 1137–1114[a]	This study
SSU897	SSU	GGTGAAATTCTTGGATTTGCGAAAGACG	F: 897–924[a]	This study
18SC2	SSU	TCCGCAGGTTCACCTACGGAG	R: 1781–1761[a]	Bakker et al. (1994)
C1FL	LSU	ACCCGCTGAACTTAAGCATATC	F: 26–47[b]	This study
D2FL	LSU	GGTCCGTGTTTCAAGACGG	R: 651–633[b]	This study
LSUintF	LSU	CGATGAAAAGACCGCTGGC	F: 365–383[b]	This study
LSUintR	LSU	GCCAGCGGTCTTTTCATCG	R: 383–365[b]	This study

F, forward primer; R, reverse primer.
[a] Primer positions numbered according to their respective position in the *Chlamydomonas reinhardtii* SSU rDNA sequence (GenBank Accession No. M32703).
[b] Primer positions numbered according to their respective position in the *Chlorella ellipsoidea* LSU rDNA sequence (GenBank Accession No. D17810).

with the model of sequence evolution yielding the best fit to the data (estimated with PAUP/Modeltest v3.6) (Posada and Crandall, 1998). This was done for the SSU and partial LSU datasets separately, with outgroup taxa in- or excluded. In addition, the I_{ss} statistic, a measure of substitution saturation in molecular phylogenetic data sets, was calculated with DAMBE (Xia and Xie, 2001) for the SSU and LSU data separately as well as for the combined dataset.

Two sets of alignments were considered for the phylogenetic analyses. The first one, consisting of a partial LSU alignment (663 sites) of all 166 ingroup sequences, was used for phylodiversity assessment (i.e. to delimit clusters of sequences that are closely related). Initial analyses of this dataset plus six cladophoralean outgroup sequences showed discordance in the position of the root of the ingroup clade with outgroup rooting when applying different methods of phylogenetic inference (Appendix A2, Supplementary data). A second set of alignments was assembled to assess phylogenetic relationships within the Siphonocladales and determine the position of its root, including a SSU, a partial LSU, and a concatenated SSU + partial LSU alignment of a reduced number of taxa, including representatives of each of the main clades as determined from the phylodiversity assessment (36 ingroup taxa and 6 outgroup sequences, 2368 sites). To exclude incongruent taxon sampling and artefacts associated with it, the two genes were sequenced exclusively from the same isolates. The incongruence length difference (ILD) test (Farris et al., 1995) was used to test for incongruence between the genes. The test was implemented in PAUP (partition homogeneity method with 1000 replicates) and indicated that the SSU and partial LSU rDNA data were not significantly heterogeneous ($P = 0.21$), justifying a combined data approach. The SSU and LSU data were then analyzed separately as well as combined. Because distant outgroups can influence inferred relationships among ingroup taxa (Bergsten, 2005), independent phylogenetic analyses were also conducted on a taxon set composed of the ingroup alone.

Bayesian inference (BI) was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Three approaches of model selection were implemented. Firstly, the most simple model of nucleotide substitution, the Jukes–Cantor (JC) model (Jukes and Cantor, 1969) was chosen for the entire alignment. In the second set of analyses a single, general time-reversible model with rate variation across sites and a proportion of invariable sites (GTR + I + Γ), as estimated by PAUP/MrModeltest 1.0b (Nylander, 2004), was selected for the entire alignment. In the third set of analyses the datasets were partitioned into stem and loop regions. We used the Xstem software (Telford et al., 2005) to extract the RNA secondary structure information from DCSE to a nexus format readable in MrBayes. Different substitution models were then selected for the two partitions. For the loop regions a GTR + I + Γ (a 4-state, single-nucleotide substitution model) was selected by PAUP/MrModelTest, while for the paired stem regions, the doublet model (a 16-state RNA stem substitution model, originally formulated by Schöniger and von Haeseler, 1994) was selected as recommended by Telford et al. (2005). 16-state RNA substitution models consider pairs of nucleotides (16 possible pairs that can be formed with 4 bases) as their elementary states rather than single sites as in 4-state DNA substitution models. Posterior probabilities were calculated using a Metropolis-coupled Markov chain Monte Carlo approach with sampling according to the Metropolis–Hastings algorithm. For all analyses, two independent, simultaneous analyses were run for 3×10^6 generations, each starting from different random trees and sampled every 1000th generation. Each analysis used four chains, one cold and three incrementally heated. Summary statistics and trees were generated using the last 2×10^6 generations. The stationary distribution of the runs was confirmed by the average standard deviations of split frequencies between the two analyses, which approached zero (0.005–0.009, depending on the model selected) after no more than 6×10^5 generations, reflecting the fact that the two tree samples became increasingly similar. The station-

ary distribution of both runs was confirmed by plotting the ln likelihood values of the cold chain against generation numbers, and the burnin value was based on this graph.

Maximum parsimony (MP), minimum evolution (ME) and neighbor-joining (NJ) analyses were performed using PAUP. MP analyses consisted of heuristic searches with 1000 random sequence addition replicates and Tree Bisection Reconnection (TBR) with the option MULTREES and branches being collapsed if it was possible for them to have zero length. MP analyses were performed with or without Goloboff's implied character weighting ($K = 2$, Goloboff, 1993). ME and NJ analyses were performed under a JC model of sequence evolution. Robustness of the inferred MP, NJ and ME trees were tested using non-parametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates.

Pairwise comparison of MP trees from the independent SSU and partial LSU analyses, and combined analyses were undertaken using results from Kishino–Hasegawa tests as recorded using PAUP.

The root of the siphonocladalean tree in all phylogenetic analyses was determined by outgroup rooting with the six cladophoralean taxa mentioned above. Root placement was compared between the separate and combined analyses. Multiple outgroups were used in order to attempt to break up the long naked branches from the ingroup to individual outgroup taxa. Additionally, ingroup sequences were analyzed under a strict molecular clock using MrBayes (concatenated SSU + partial LSU with a clock constrained GTR + I + Γ model, Huelsenbeck et al., 2002). The molecular clock analysis automatically roots the tree along its oldest branch. The fit of the GTR + I + Γ model with clock assumption to the data was assessed by calculating the Bayes factor, i.e. the difference between the logarithms of the harmonic means of the likelihood values of the MCMC samples, and finding the corresponding interpretation in the table of Nylander et al. (2004).

Character evolution was traced along the trees using parsimony as well as maximum likelihood reconstruction (Cunningham et al., 1998) implemented in Mesquite v1.11 (Maddison and Maddison, 2006). For characters where polymorphic states were included, only parsimony reconstruction was employed.

3. Results

3.1. Phylogenetic information and saturation

Specifications of the partial LSU and SSU rDNA datasets used for the phylogenetic analyses, evolutionary models applied, and BI, ME and MP scores are given in Table 3. The SSU fragment was roughly three times as long as the partial LSU fragment but it contained about the same number of variable and parsimony-informative characters. Pairwise sequence divergence in the SSU was found to be considerably lower than in the LSU. The measure of skewness (g_1-value), compared with the empirical threshold values in Hillis and Huelsenbeck (1992) showed that the length distributions of random trees of all data sets were considerably left-skewed, indicating that the alignments were significantly more structured than random data. Saturation plots of the ingroup taxa (Fig. 2A) showed a near-linear correlation of the SSU and LSU data, indicating little saturation. When the outgroup taxa were also considered (Fig. 2B) the saturation plot of LSU was found to level off with increasing genetic distance, indicating saturation between in- and outgroup sequences. The I_{ss} statistic (Xia and Xie, 2001) however, did not reveal significant saturation in any of the datasets.

3.2. Phylogenetic analyses

Phylodiversity assessment performed on the LSU dataset including 166 ingroup sequences revealed 7 well-supported clades, along with a grade of *Cladophora* taxa (grade-2) (Fig. 3A). *Cladophora* sp. 1 (''clade 3'') occupied a separate position in most analyses, except in those under the ME criterion where it clustered with grade-2 (Fig. 3B). Internal branches connecting the main clades were relatively long and supported by high posterior probabilities and bootstrap values.

To determine phylogenetic relationships between the major siphonocladalean clades and to assess the root placement of the ingroup, the second set of alignments, including partial LSU and SSU data of a reduced number of taxa (36 ingroup and 6 outgroup sequences), was analyzed separately as well as combined. Analyses of the LSU data revealed the same main clades as the phylodiversity assessment. Outgroup rooting introduced a long naked branch from the outgroup to the ingroup and showed discordance in the position of the root of the ingroup clade when applying different methods of phylogenetic inference (Fig. 4A and B). Molecular clock rooting positioned the root on yet another branch, separating clades (1-2-3-4-5) from clades (6-7-8-9) (Fig. 4C), with the basal branches being rather weakly supported. The main LSU clades were also revealed in the SSU tree, but here the relationships among and within these clades were largely unresolved (Fig. 4D). The SSU analyses revealed relatively short branches connecting the ingroup and outgroups, positioned on the branch connecting clade 1 (BI and MP analyses) or clade 4 (ME and NJ analyses) with the rest of the ingroup clades (Fig. 4E). Molecular clock rooting altered the ingroup topology (particularly the position of *Cladophoropsis* sp. 4) and placed the root on a branch connecting clades 1 and 4 with the rest of the ingroup (Fig. 4F), again with very weakly supported basal branches.

The combined SSU + LSU analysis was found to perform better than the separate SSU/LSU analyses, both in terms of resolution and the stability of the root placement (as determined by outgroup rooting), which was found to be stable, regardless of the phylogenetic methods employed (Fig. 5A and Table 3). Different phylogenetic methods

Table 3
Specification of data sets, summary of models and model parameters obtained, and details on the BI, ML, MP and ME analyses

	Dataset 1 166 Ingroup taxa	Dataset 2[a] 36 Ingroup taxa + 6 outgroup taxa		
	Partial LSU rDNA	SSU rDNA	Partial LSU rDNA	Concatenated
Alignment length/analyzed	663/663	1724/1632	644/590	2368/2222
Variable sites/parsimony informative sites	169/144	182/122 (142/96)	194/143 (126/89)	376/265 (268/185)
Uncorrected pairwise sequence divergence (max/average)	0.12/0.06	0.08/0.04 (0.03/0.02)	0.20/0.08 (0.09/0.05)	0.08/0.06 (0.04/0.02)
Measure of skewness (g_1-value)	−0.220	−1.211 (−0.559)	−1.529 (−0.419)	−1.291 (−0.702)
I_{ss} statistic ($I_{ss}/I_{ss} \cdot c$, p-value of 32 taxon data subsets)	0.46/0.79, $p < 0.001$	0.52/0.78, $p < 0.001$ (0.47/0.78, $p < 0.001$)	0.55/0.71, $p = 0.040$ (0.37/0.71, $p < 0.001$)	0.67/0.84, $p < 0.001$ (0.62/0.84, $p < 0.001$)
Emperical base frequencies (A/C/G/T)	0.25/0.23/0.32/0.18	0.26/0.22/0.28/0.24	0.25/0.24/0.32/0.19	0.25/0.23/0.29/0.23
Model estimated[b]	TrN + I + G	TIM + I + G (TIM + I + G)	TrN + I + G (TrN + I + G)	TIM + I + G (TrN + I + G)
Substitution rates (A-C/A-G/A-T/C-G/C-T/G-T) [b]	1.00/2.81/1.00/1.00/6.51/1.00	1.00/3.15/1.73/1.73/8.40/1.00	1.00/2.75/1.00/1.00/5.85/1.00	1.00/2.84/1.24/1.24/6.38/1.00
Among-site rate variation: I/G[c]:	0.58/0.70	0.76/0.72 (0.78/0.74)	0.48/0.68 (0.60/0.66)	0.69/0.64 (0.75/0.68)
MP steps/Goloboff fit/# trees	504/−106.88/26	339/−100.25/99	479/−110.70/73	834/−209.94/176
CI/RI	0.49/0.94	0.64/0.79	0.60/0.80	0.61/0.78
ME:score	0.82	0.20	0.77	0.35
BI: estimated marginal likelihood of models (harmonic mean, −ln L)				
JC model	4568.11	4765.68 (4395.41)	3849.55 (2413.50)	8849.94 (6928.80)
GTR + I + Γ model	4151.43	4523.44 (4302.58)	3513.40 (2232.77)	8056.81 (6388.46)
GTR + I + Γ model + doublet model	3856.37	4447.38 (3729.78)	2840.32 (2038.49)	7257.66 (5679.26)
Node resolution[d]				
BI (JC)	38/16/46	62/23/15	69/21/10	74/23/3
BI (GTR + I + Γ model)	33/19/49	46/26/28	59/28/13	62/31/8
BI (GTR + I + Γ//doublet model)	34/19/47	49/26/26	46/41/13	62/31/8
ME	36/19/46	46/26/28	59/18/23	69/28/3
MP (unweighted)	28/14/58	38/21/41	54/23/23	62/15/23
MP (Goloboff weighted)	29/11/59	38/21/41	51/23/26	67/26/8

[a] Values calculated for ingroup taxa only are given between brackets.
[b] Estimated by the Akaike information criterion (AIC) implemented in PAUP/ModelTest.
[c] Proportion of invariable sites (I) and gamma distribution shape parameter (G) as estimated by PAUP/Modeltest.
[d] Percentage of all nodes receiving high (⩾95% PP for BI; ⩾70% BP for ME and MP)/moderate (50–94% PP for BI; 50–69% BP for ME and MP)/low (<50% PP for BI, ME and MP) supported nodes.

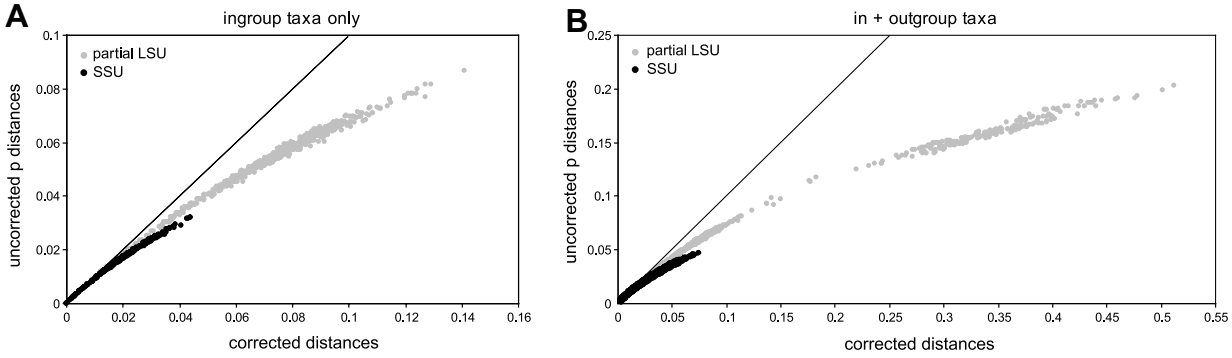


Fig. 2. Analysis of saturation of the SSU (black dots) and partial LSU rDNA (grey dots) sequences by plotting corrected distances versus uncorrected *p*-distances. Corrected distances are calculated using models estimated by PAUP/Modeltest for each specific data partition (Table 3). (A) Analyses of ingroup taxa only. (B) Analyses including outgroup sequences.

(BI/MP/ME) revealed similar tree topologies with comparable internal node resolution (Table 3). The ME tree differed from the BI and MP trees in clustering of taxa of grade-2 and *Cladophora* sp. 1 in a single clade (Fig. 5B). The MP and ME topologies differed from the BI trees in the relationships within clade 5 (Fig. 5C). Kishino–Hasegawa tests revealed that the rooted tree topologies, inferred from SSU and partial LSU separately, and the combined data were significantly different from one another ($P < 0.005$). However, ingroup topologies of separate SSU and partial LSU analyses were not significantly different from those of the combined analyses ($P > 0.05$, up to $P = 0.39$) (Figs. 4B, E and 5D). In the BI analyses, the complexity of the evolutionary models employed was positively correlated with the likelihood of the phylogenetic trees, as expected (Posada and Buckley, 2004). However, all BI analyses yielded similar tree topologies. Furthermore, better fitting (more complex) models did not result in an increase of internal node resolution.

The tree obtained using the clock-constrained GTR + I + Γ model differed from the trees inferred using unconstrained models in the position of the root, which was situated on the branch separating clades (6-7-8-9) from the remainder of ingroup clades (Fig. 5E). Basal branches in this clock-constrained tree were very weakly supported. The estimated marginal ln likelihoods (harmonic means) were -6396.50 with the clock and -6388.46 without the clock, yielding a Bayes factor (B_{10}) of 8.04, indicating positive evidence against the molecular clock assumption. However, Huelsenbeck et al. (2002) found that clock rooting was robust to moderate amounts of rate heterogeneity, meaning that it is still possible to accurately root trees using a molecular clock, even when local clock deviations exist. This leaves us with two different, credible root positions, the first one determined by outgroup rooting, the second one established by a molecular clock. For now, we consider the first one as our working hypothesis, but also take into consideration the alternative root placement. This tree, rooted with the six cladophoralean outgroups, was used as reference topology for ancestral character state reconstruction.

3.3. Evolution of morphological characters

Parsimony and maximum likelihood reconstruction of ancestral states yielded similar results. Fig. 6 illustrates the parsimony reconstruction of the evolution of a number of morphological characters along the SSU + LSU phylogram. Cell division by centripetal invagination (CI) of the cell wall appears to be plesiomorphic in the Siphonocladales, and the three specialized modes of cell division have each evolved several times independently in various clades (Fig. 6A). Fig. 6B shows that tenacular cells (i.e. specialized cells realizing the anastomosis of adjacent cells), evolved in clades 4–9 only. Tenacular cells of type-2 (minute hapteroid cells formed laterally between adjacent vesicular cells, e.g. *Dictyosphaeria* and *Valonia*), type-3 (small hapteroid cells formed at the distal ends of cells, e.g. *Boodlea*) and type-4 (hapteroid cells formed at the base of a cell and attaching to the cell below, e.g. *Apjohnia*) are clearly related and possibly evolved on a single occasion (and were lost secondarily in *Valoniopsis*, *Ventricaria* and some *Cladophoropsis* species). Lateral coalescence of branches (*Anadyomene*) probably evolved as a special form of type-1 tenacular cells (*Microdictyon*). The formation of reticulate thalli is associated with tenacular cells (Fig. 6C). Identical types of tenacular cells however, can be found in different types of reticulate plants, and conversely different types of tenacular cells can produce similar types of net-like plants. For example both tenacular cells type-1 and -3 can generate either flat reticulate blades (e.g. *Microdictyon*, *Struvea*, *Phyllodictyon*) or three-dimensional, cushion-like thalli (*Boodlea*, *Cladophora liebetruthii*). Special, derived types of reticulate plants are found in *Anadyomene* (meshes completely filled with small interstitial cells), and in *Valonia* and *Dictyosphaeria* (net-like nature obscured by the strongly inflated cells). Fig. 6D illustrates that branched thalli with a low number of branches per cell (1–3) emerge as the ancestral state. The increase and the reduction (unbranched thalli) of the number of branches per cell have evolved recurrently in the in- and outgroup. Fig. 6E shows that large cells (diameter more than 800 μm) have evolved at least twice independently and is correlated with the

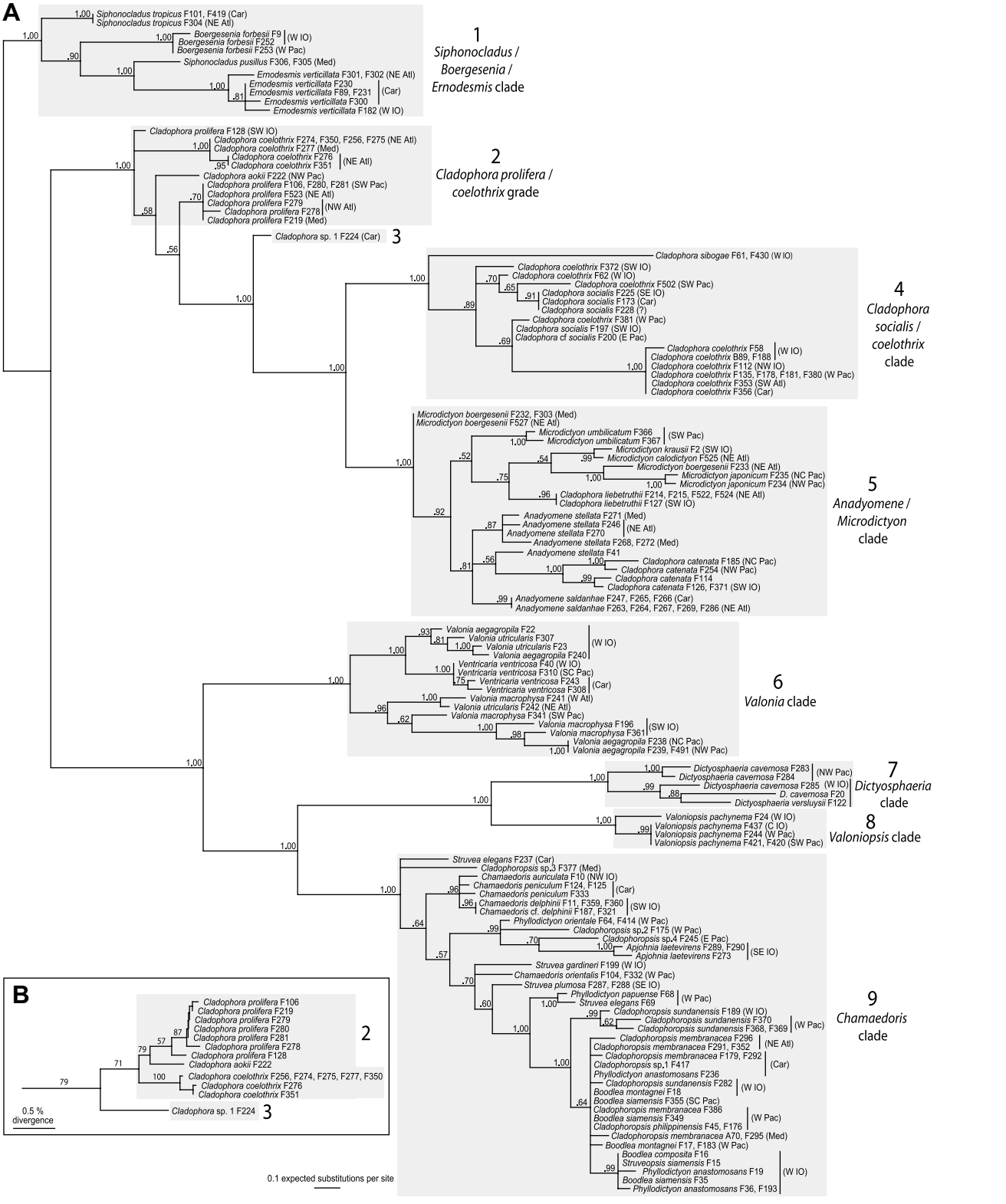
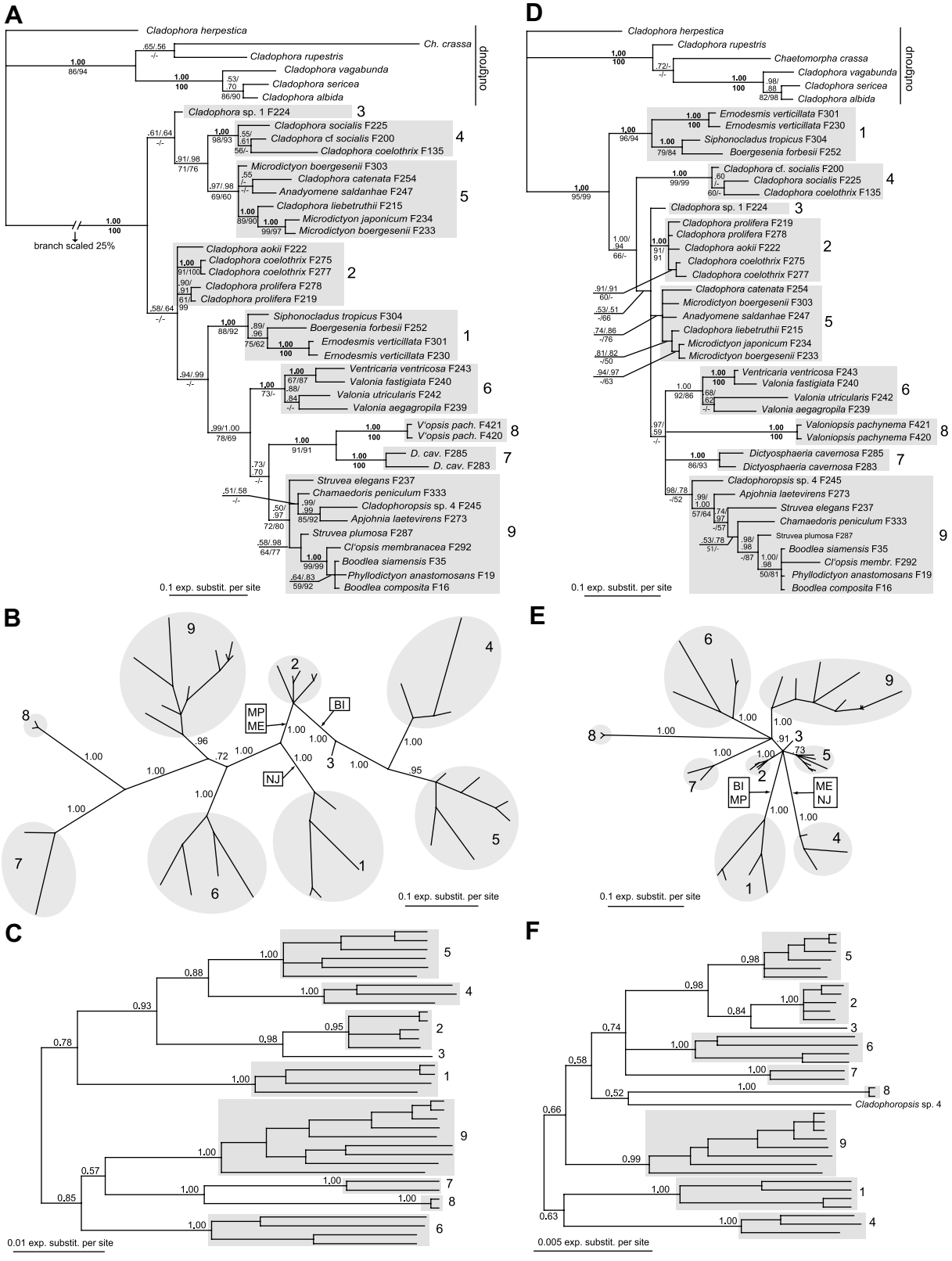


Fig. 3. Phylodiversity of the Siphonocladales based on 166 partial LSU rDNA ingroup sequences. (A) Unrooted BI analysis performed on a partitioned stem:loop dataset using a GTR + I + Γ model for RNA loop regions and a doublet model for RNA stem regions. Values at nodes indicate PP. Geographical regions are indicated next to the taxon names: C IO, central Indian Ocean; Car, Caribbean Sea and the Gulf of Mexico; E Pac, eastern Pacific Ocean; Med, Mediterranean Sea; NC Pac, north-central Pacific Ocean; NE Atl, north-eastern Atlantic Ocean; NW Pac, north-western Pacific Ocean; SC Pac, south-central Pacific Ocean; SE IO, south-eastern Indian Ocean; SW IO, south-western Indian Ocean; SW Pac, south-western Pacific Ocean; SW Pac, south-western Pacific Ocean; W IO, western Indian Ocean; W Pac, western Pacific Ocean. (B) Relationships within grade 2 and "clade" 3 under ME criterion; values above branches indicate BS.

mode of cell division (Fig. 6A): large cells generally divide by LCD, SCD or SCDM, while cells with a smaller diameter (smaller than 800 μm) generally divide by CI. Fig. 6F shows the evolution of different types of rhizoids. All members of the Siphonocladales produce rhizoids in the basal part of the thallus in at least some stage of their development. Two other types of rhizoids have been described; type-2 rhizoids develop at the basal pole of cells in any part



of the thallus and have evolved both in the Siphonocladales and Cladophorales (outgroup); type-3 rhizoids are formed at the apex of cells and have evolved at least twice. Annular constrictions (Fig. 6G), are often found in species with large, club-shaped basal cells or stipe cells (e.g. *Boergesenia*, *Struvea*, *Chamaedoris* and *Apjohnia*) and have clearly evolved recurrently within the Siphonocladales. In most representatives of the Siphonocladales, the formation of a branch is followed by the production of a cross-wall, separating the new lateral from the mother cell. In several taxa this cross-wall formation is markedly delayed (e.g. in *Cladophora coelothrix* and *Boodlea composita*), to such an extent that cross-walls are never formed, resulting in branches that remain in open connection with the mother cells (*Cladophoropsis*, *Chamaedoris*). Delay of cross wall formation has evidently evolved several times in the Siphonocladales as well as in the Cladophorales lineage (*Cladophora herpestica*) (Fig. 6H). Calcium oxalate crystals (Fig. 6I) have been observed in certain species of the Siphonocladales (Leliaert and Coppejans, 2004). Three morphological types are recognized: prismatic or needle-shaped crystals evolved in clade 9; clustered rod-shaped crystals, only found in *Dictyosphaeria* (clade 7); and octahedral crystals present in *Valoniopsis* (clade 8).

4. Discussion

4.1. Phylogenetic inference and root of the Siphonocladales

Incorrectly rooted trees may result in misleading phylogenetic and taxonomic inferences. It is well documented that spuriously rooted trees may be due to long branches connecting ingroup and outgroup taxa, resulting in long-branch artifacts (Graham et al., 2002). The quality of rooting provided by the outgroup criterion depends on the sampling strategy of the outgroup taxa and of the phylogenetic proximity of the outgroup to the ingroup (Swofford et al., 1996; Wheeler, 1990; Huelsenbeck et al., 2002). It is therefore important to choose outgroup taxa that are closely related to the ingroup in order to reduce artifactual root placement by minimizing the distance between the root node and the first outgroup node (Swofford et al., 1996). In case the sister group of the ingroup has experienced a substantial rate acceleration, more distantly related, but less divergent, outgroups will provide more reliable evidence on ingroup rooting than the sister group (Lyons-Weiler et al., 1998). Less commonly, the inclusion of distantly related outgroup sequences can yield erroneous ingroup topologies (Holland et al., 2003; Bergsten, 2005).

In this study, outgroup taxa were carefully selected based on previous molecular evidence (Hanyuda et al., 2002; Leliaert et al., 2003). Multiple outgroup taxa were selected in order to break up the branch between in- and outgroup, and thus reducing long-branch attraction problem associated with root placement (Maddison et al., 1984). In spite of the fact that these taxa were the closest known, and less divergent, relatives of the Siphonocladales, outgroup rooting in the LSU analyses introduced a long, naked branch, making the phylogenetic analysis prone to long branch attraction. The placement of the root was found to be unstable and dependent on the type of phylogenetic analyses employed. Rooting experiments with artificial, random outgroup sequences (Appendix A2, Supplementary data) revealed identical root positions on long ingroup branches in the ME and MP analyses, suggesting that the root placements in these analyses may indeed have been a consequence of long-branch artifacts. The SSU data provided a lower density of variable and parsimony-informative sites than the LSU data, and suffered less from saturation, even between in- and outgroup taxa. This resulted in a phylogeny in which the outgroup branch was more proportional to the internal ingroup branches. The combined SSU + LSU phylogenetic analyses resulted in phylogenetic analyses with a stable position of the root (as determined by outgroup rooting), placing the *Boergesenia*/*Ernodesmis*/*Siphonocladus* clade (clade 1) sister to the rest of the Siphonocladales.

Outgroup rooting is by far the most common method to determine the root of a phylogenetic tree, although other methods, such as the molecular clock, have been proposed (Huelsenbeck et al., 2002). In the combined SSU + LSU analysis, molecular clock rooting resulted in a different root

Fig. 4. Reconstructed phylogenies of the Siphonocladales based on separate partial LSU and SSU rDNA data. (A) BI 50% majority-rule consensus tree inferred from partial LSU rDNA data, analyzed with a partitioned stem:loop dataset, using a GTR + I + Γ model for RNA loop regions and a doublet model for RNA stem regions; root determined by outgroup rooting with six cladophoralean outgroup taxa. Values above the branches indicate PP (partitioned stem:loop analyses/non-partitioned analyses under a single GTR + I + Γ model); numbers below the branches indicate BS (Goloboff weighted MP/ME). Bold PP or BS values indicate identical, maximum values. (B) Unrooted BI 50% majority-rule consensus tree inferred from partial LSU rDNA ingroup sequences (BI analysis identical as above). Boxes indicate root positions as determined by outgroup rooting, using different phylogenetic inference methods. PP values are indicated for the branches leading to the main clades only. (C) BI 50% majority-rule consensus tree inferred from partial LSU rDNA ingroup sequences, using a clock-constrained GTR + I + Γ model. PP values are indicated for the branches leading to the main clades only. (D) BI 50% majority-rule consensus tree inferred from SSU rDNA data, analyzed with a partitioned stem:loop dataset, using a GTR + I + Γ model for RNA loop regions and a doublet model for RNA stem regions; root determined by outgroup rooting with six cladophoralean outgroup taxa. Values above the branches indicate PP (partitioned stem:loop analyses/non-partitioned analyses under a single GTR + I + Γ model) (E) Unrooted BI 50% majority-rule consensus tree inferred from SSU rDNA ingroup sequences (BI analysis identical as above). Boxes indicate root positions as determined by outgroup rooting, using different phylogenetic inference methods. PP values are indicated for the branches leading to the main clades only. (F) BI 50% majority-rule consensus tree inferred from SSU rDNA ingroup sequences, using a clock-constrained GTR + I + Γ model. PP values are indicated for the branches leading to the main clades only.

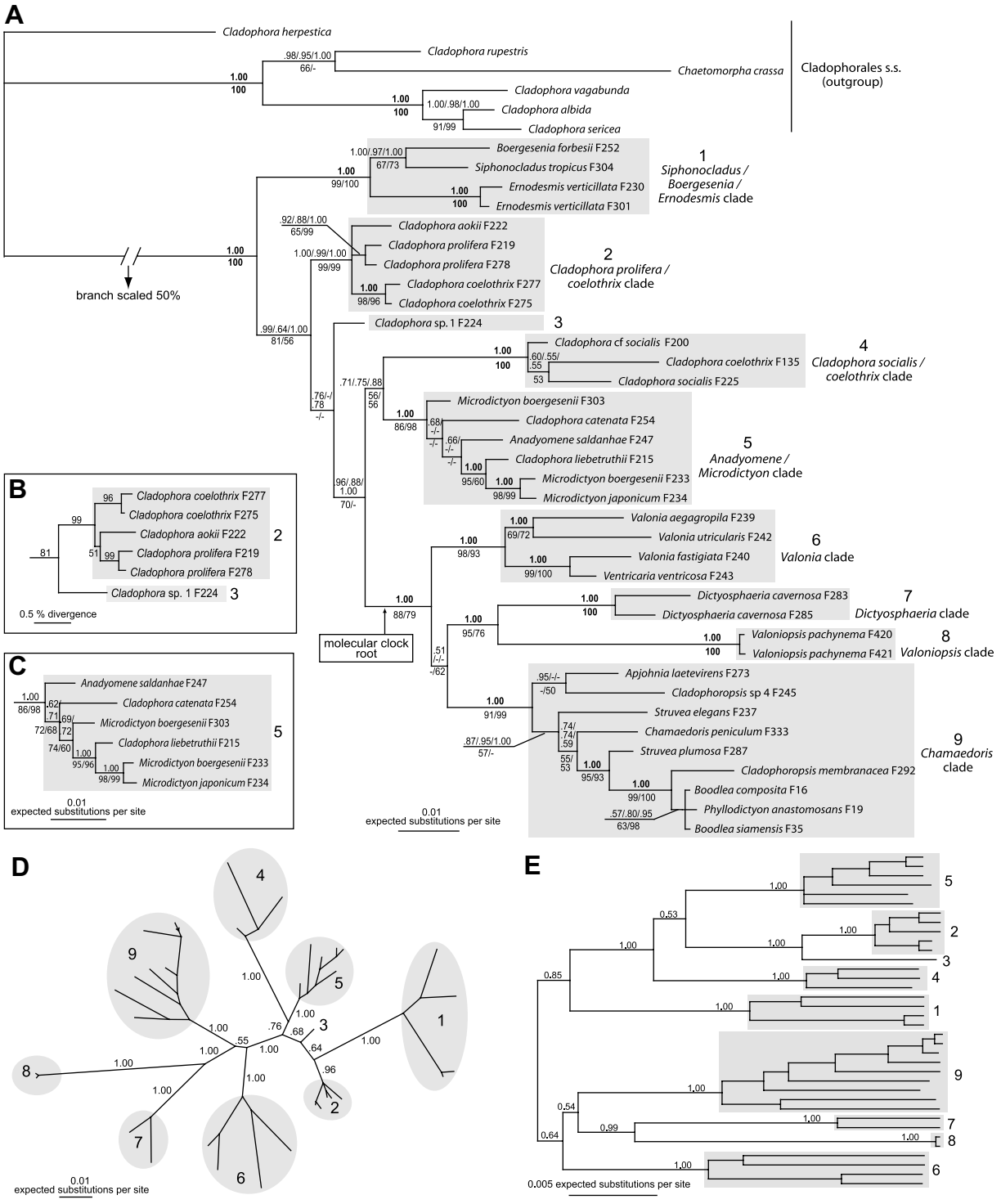


Fig. 5. Reconstructed phylogenies of the Siphonocladales based on combined partial LSU + SSU rDNA data. (A) BI 50% majority-rule consensus tree, analyzed with a partitioned stem:loop dataset, using a GTR + I + Γ model for RNA loop regions and a doublet model for RNA stem regions; root determined by outgroup rooting with six cladophoralean outgroup taxa. Numbers above the branches indicate the PP (partitioned stem:loop analysis/ analysis under a single GTR + I + Γ model/analysis under a single JC model); numbers below the branches indicate BS (Goloboff weighted MP/ME). Bold PP or BS values indicate identical, maximum values. White box with arrow indicates the position of the root as determined by molecular-clock analysis. (B) Relationships within grade 2 and "clade" 3 under ME criterion; values above branches indicate BS. (C) Relationships within clade 5 as determined by BI analysis under a single GTR + I + Γ model. Values above branches indicate PP (GTR + I + Γ model/JC model); values below branches indicate BS (Goloboff weighted MP/ME). (D) Unrooted BI 50% majority-rule consensus tree inferred from ingroup sequences only (BI analysis identical as above). PP values are indicated for the branches leading to the main clades only. (E) BI 50% majority-rule consensus tree inferred from ingroup sequences only, using a clock-constrained GTR + I + Γ model. PP values are indicated for the branches leading to the main clades only.

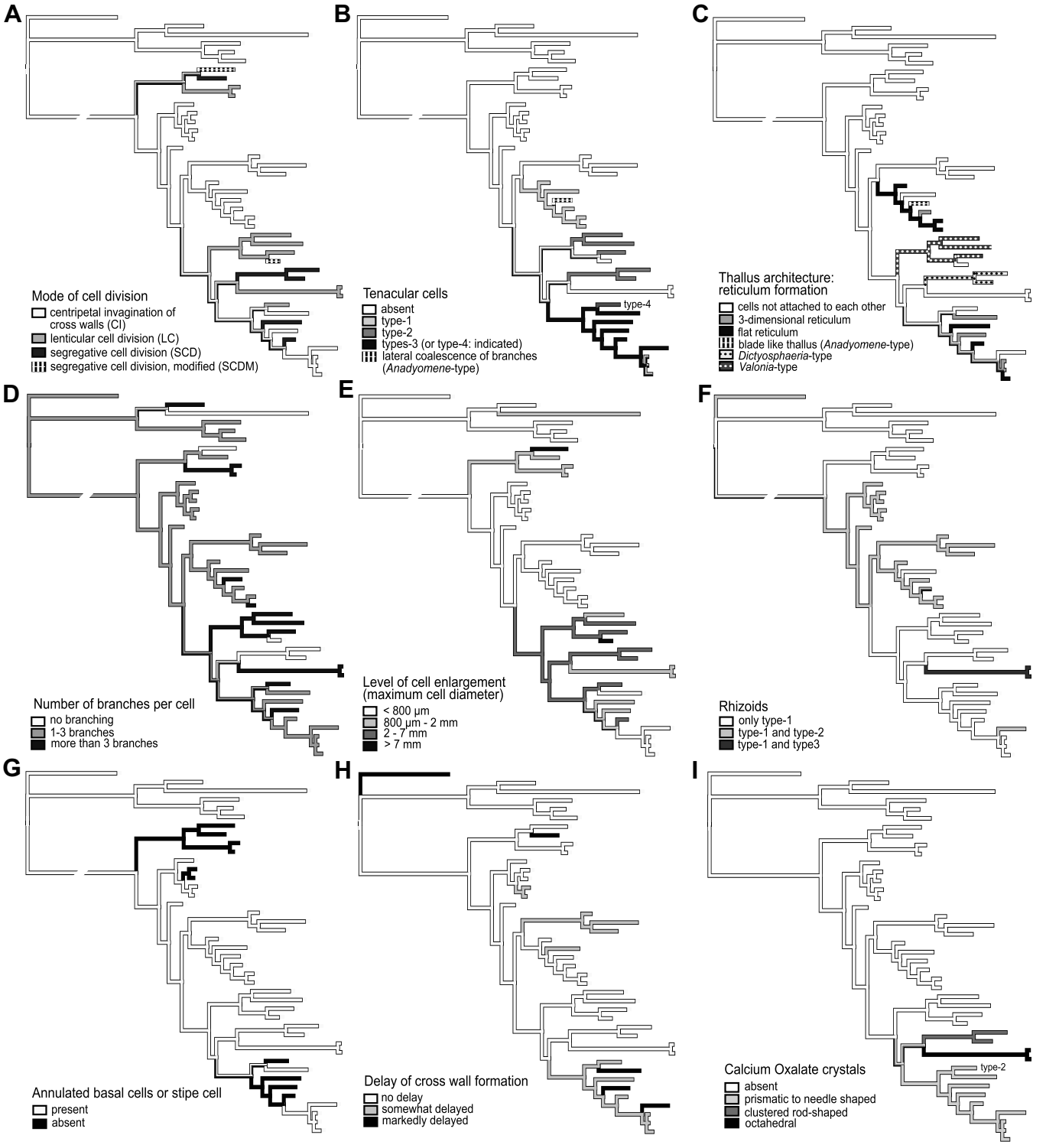


Fig. 6. Morphological character mapping onto the phylogenetic tree (Fig. 5A).

position than in the outgroup rooting analyses, yielding a tree with two main lineages (clades 1-2-3-4-5 and 6-7-8-9) and very weakly supported basal branches (Fig. 5E). Considering the above-mentioned problems with divergent outgroups in this study, the molecular clock root should be regarded as a worthy alternative (Holland et al., 2003). Further studies, including additional molecular markers, will be needed to address the issue of the correct root of the Siphonocladales.

Combining signal from different data sets in phylogenetic analyses has long been debated (Huelsenbeck et al., 1996). Numerous phylogenetic studies have shown that the combination of multiple-gene data sets leads to better resolved and supported trees, compared with single-gene

partitions (Buchheim et al., 2005; Murray et al., 2005; Feau et al., 2006). An additional potential benefit of combination is the appearance of relationships in a simultaneous analysis that does not emerge from the individual partition trees (Gontcharov et al., 2004). However, in a few other studies, combining genes has been shown to reduce the effectiveness of phylogenetic analysis, often because the combined partitions contain contradictory signals (Wortley et al., 2005). In the present study the topological differences between the SSU and LSU data did not represent significant conflict, therefore supporting the usefulness of the combination method. The SSU data contained much less phylogenetic signal than the LSU data to resolve relationships between and within the main siphonocladalean clades (Fig. 4D). The combined SSU + LSU phylogenetic analyses was found to be "superior" to separate analyses, yielding a better resolved tree with more robust support of internal branches. It should be noted however, that it is somewhat erroneous to consider better resolved trees as superior (i.e. closer to the true tree) since there is no a priori knowledge of the true phylogeny and support does not necessarily correlate with accuracy (Gontcharov et al., 2004).

It is well established that phylogenetic methods perform better with models of evolution that are more representative of the actual evolutionary forces affecting the marker in question (Posada and Crandall, 2001). Therefore, when estimating phylogenies based on ribosomal DNA, the differences in evolution between stem and loop regions of the transcribed RNA molecules should ideally be accounted for (Murray et al., 2005; Telford et al., 2005). These studies showed an improvement in the fit of the 16-state models to the evolution of the pairs of stem nucleotides (which is expected given the higher number of parameters) but revealed only minor effects on tree topology and resolution. Similarly, in the present study, our consideration of the phylogeny of the Siphonocladales, analyzed by BI and partitioning stem and loop regions and applying appropriate models (16-state and 4-state, respectively), does only differ slightly from BI analyses using standard 4-state models (including the most simple model, JC), or from phylogenetic analyses using traditional approaches (such as distance and MP methods). This is in agreement with a number of studies, which show that when using data with an appropriate amount of phylogenetic information (in our case the conserved SSU in combination with the more variable LSU), different phylogenetic methods often yield identical trees (Russo et al., 1996).

4.2. Morphological versus phylogenetic species delineation

As in nearly all algal groups, species of Siphonocladales are circumscribed based on the morphological species concept, which recognizes species by discontinuities in morphological characters. A major predicament in doing so lies in the fact that siphonocladalean plants are relatively simple and that there is only a limited range of morphological characters available for delimitation of species. Moreover, many morphological characters have been shown to be highly plastic and subject to environmental conditions.

Table 4
Sequence divergence within a number of monophyletic morphospecies, and within the paraphyletic *Dictyosphaeria cavernosa* and the *Boodlea composita* complex (including the species *Boodlea composita*, *B. montagnei*, *B. siamensis*, *Cladophoropsis membranacea*, *C. philippinensis*, *Phyllodictyon anastomosans* and *Struveopsis siamensis*): maximum and standard deviation of the uncorrected pairwise *p*-distances within each clade

Species	Max	Stdev
Anadyomene saldanhae ($N = 8$)	0	0
Apjohnia laetevirens ($N = 3$)	0.002	0.001
Boergesenia forbesii ($N = 3$)	0.002	0.001
Chamaedoris delphinii ($N = 5$)	0	0
Chamaedoris peniculum ($N = 3$)	0.002	0.001
Cladophora catenata ($N = 5$)	**0.016**	0.007
Cladophora liebetruthii ($N = 5$)	0	0
Cladophora prolifera ($N = 8$)	0.007	0.002
Ernodesmis verticillata ($N = 7$)	0.005	0.002
Microdictyon japonicum ($N = 2$)	0.002	0.001
Siphonocladus tropicus ($N = 3$)	0	0
Valoniopsis pachynema ($N = 5$)	**0.014**	0.007
Ventricaria ventricosa ($N = 4$)	0.004	0.001
Dictyosphaeria cavernosa ($N = 4$)	**0.043**	0.017
Boodlea composita complex ($N = 24$)	0.012	0.003

Values in bold indicate *p*-distances higher than 0.01.

Difficulties of accurate morphospecies delimitations are mirrored in the present molecular phylogeny. Many traditionally circumscribed species are distributed in different clades. The most prominent example is *C. coelothrix*, which was recovered in clades 2 and 4. Other non-monophyletic taxa include *Anadyomene stellata*, *Cladophora socialis*, *Microdictyon boergesenii*, *Struvea elegans*, *Valonia aegagropila*, *Valonia utricularis* and *Valonia macrophysa*. *Dictyosphaeria cavernosa* turns out to be paraphyletic since the isolate from Tanzania is more closely related to *Dictyosphaeria versluysii* than to the *D. cavernosa* isolates from the Seychelles and Japan. On the other hand, several morphologically defined species, do appear as natural groups. This is particularly true for species from the monospecific (and highly distinct) genera *Apjohnia*, *Boergesenia*, *Ernodesmis* and *Valoniopsis*; other examples include *C. liebetruthii*, *Cladophora catenata*, *Siphonocladus tropicus* and *Ventricaria ventricosa*. Pairwise genetic distances within these monophyletic taxa (Table 4) reveal either a low-to-zero genetic distance (e.g. *Anadyomene saldanhae*, *Boergesenia forbesii*, *C. liebetruthii*, *Microdictyon japonicum*, *S. tropicus*), or a higher sequence divergence within species clades, often resulting in distinct sub-clades (e.g. *C. catenata*, *Valoniopsis pachynema* and *Ernodesmis verticillata*).

LSU rDNA sequences, particularly the region encompassing the hyper-variable C1 helices (De Rijk et al., 1999), have been used as a source of diagnostic sequences in several eukaryotic groups, including a number of algal taxa where the information contained in partial LSU data has been shown to be suitable for species level phylogenetic and phylogeographic studies (e.g. Andreakis et al., 2004;

Harvey and Goff, 2006). The genotypic clusters (i.e. groups of closely related or identical sequences preceded by a long, well-supported branch, Mallet, 1995) in the present LSU phylogram (Fig. 3) may correspond to phylogenetic species, where differences in LSU sequences between these genotypic clusters, are clearly greater in magnitude than the differences within the clusters (Verbruggen et al., 2005). Depending on the threshold one uses, 45–65 species can be delimited in the present phylogeny.

An interesting group in this context is the species complex comprising *Cladophoropsis membranacea*, *Phyllodictyon anastomosans*, *Struveopsis siamensis* and several *Boodlea* taxa within clade 9. The observed low sequence divergence within this clade would indicate a single genotypic cluster. However, based on combined evidence from rDNA ITS sequence divergence, differential microsatellite amplification, and data on distribution and thermal ecotypes, van der Strate et al. (2002) demonstrated that *C. membranacea* consists of at least four cryptic species. Increased taxon sampling within this clade reveals at least 12 distinct ITS clades, which could be considered as separate species (Wysor, 2002; Leliaert et al., unpublished data). This level of genetic variability and divergence is clearly not contained in the LSU data, indicating that recently diverged species in the Siphonocladales cannot be distinguished using LSU sequences alone and that cryptic diversity may be much higher than conceived through the present phylogeny.

4.3. Phylogeny and systematics of the Siphonocladales

Few hypotheses regarding the evolution of the Siphonocladales and Cladophorales have been presented in the literature. The first phylogenetic hypotheses, based on comparison of morphological characters, were developed by van den Hoek (1982, 1984) who proposed that different genera in the Siphonocladales and Cladophorales, with more complex or over-simplified architectures, represented further specializations of the basic architectural types found in different *Cladophora* species. For instance, van den Hoek was of the opinion that blade-like *Microdictyon* could have originated from a *Cladophora* species, resembling the present-day *C. liebetruthii*, by planification of the branched filaments. He thought that this process of flattening could have taken place more than once, for example in the presumptive derivation of blade-like *Struvea* from a *C. coelothrix*-like ancestor, through *Cladophoropsis* and *Boodlea*. Other siphonocladalean genera, like *Ernodesmis*, *Valonia* and *Chamaedoris* were thought to have evolved from a *Cladophora pellucida*-like ancestor by inflation of the cells.

The present molecular phylogeny corroborates the principle of van den Hoek's morphology-based evolutionary hypotheses, but suggests that all siphonocladalean architectures may be derived from a single *Cladophora*-like ancestor. This ancestor was presumably characterized by branched filaments and cell division by centripetal invagination of the cell wall. Other siphonocladalean *Cladophora* species, like *C. liebetruthii* and *C. catenata*, have subsequently evolved through secondary reduction events from specialized reticulate (*Microdictyon*) and blade-like (*Anadyomene*) morphologies. Parallel and convergent evolution of various other morphological characters have occurred repetitively in the siphonocladalean tree (Fig. 6). For example, specialized modes of cell division, such as SCD and SCDM, have evolved recurrently, especially in tropical representatives of the Siphonocladales. Segregative cell division and SCD-type wounding response is probably an adaptation of large multinucleate cells to the intensive grazing pressure (mainly by fish and sea urchins) which is very characteristic for shallow tropical rocky shores (van den Hoek and Chihara, 2000). Culture experiments have demonstrated that SCD-type wounding response occurs in a much wider range of siphonocladalean algae than previously conceived, including species of *Boodlea*, *Cladophora*, *Cladophoropsis*, *Ernodesmis*, *Microdictyon*, *Phyllodictyon*, *Valonia* (La Claire, 1982; Felicini and Perrone, 1994; Felicini et al., 1997; Kim et al., 2002; Kim and Klotchkova, 2004; Leliaert, unpublished data). Similarly, the recurrent evolution of intercalary rhizoids and tenacular cells, which promote the formation of cushions or dense turfs loaded with sand that are unattractive to herbivores, can be regarded as an adaptive evolution to tropical environments. The construction of blades can, in some cases, be regarded as an adaptation to low light intensities in deep water or shaded localities (*Phyllodictyon orientale*, *Struvea gardineri*, and several species in *Anadyomene* and *Microdictyon*) (Littler and Littler, 1991; Norris and Olsen, 1991; Leliaert and Coppejans, 2007).

Because several of the morphological characters traditionally employed to circumscribe the families and genera in the Siphonocladales have evolved multiple times independently (Fig. 6), a rearrangement of familial and generic level classification in the Siphonocladales seems inevitable. This is not a new revelation (Bakker et al., 1994; Hanyuda et al., 2002; Leliaert et al., 2003) but previous molecular phylogenies of the Siphonocladales (and Cladophorales) were based on a limited number of taxa and showed weakly resolved relationships among the composing genera. Expanded taxon sampling and an improvement in resolution of the siphonocladalean tree reveals non-monophyly in all families and most genera (*Cladophora*, *Siphonocladus*, *Anadyomene*, *Microdictyon*, *Valonia*, *Phyllodictyon*, *Boodlea*, *Struvea*, *Chamaedoris* and *Cladophoropsis*), with the notable exceptions of the genus *Dictyosphaeria* and, evidently, the monotypic genera *Apjohnia*, *Boergesenia*, *Ernodesmis*, *Ventricaria* and *Valoniopsis*. However, we are of the opinion that the present phylogenetic hypothesis needs to be confirmed with additional, unlinked genetic markers (including organellar genes and non-rDNA nuclear loci) before undertaking drastic taxonomic changes.

4.3.1. Clade 1

The genera *Siphonocladus*, *Boergesenia* and *Ernodesmis* always group together in a well-supported clade. Their sys-

tematic position has been the subject of earlier speculation (Børgesen, 1913, 1940; Oltmanns, 1922; Taylor, 1960; Olsen-Stojkovich, 1986; Leliaert et al., 2003). All three genera are characterized by inflated, club-shaped cells with basal annular constrictions, at least in their early stages of development. When plants grow older, their thallus architectures become considerably dissimilar because of differences in cell division. In *Boergesenia* (Fig. 1B) the single club-shaped cell remains unbranched and cells divide by SCDM, followed by degeneration of the mother cell and the release and settlement of the divided segregative cells. On the other hand, the cells of *Ernodesmis* divide by apical lenticular cells (LCD), resulting in spherical thalli composed of cells with verticillate, apical clusters of branches. In *Siphonocladus* (Fig. 1C), cells divide by SCD, followed by the formation of branches that break through the mother cell and radiate laterally from the club-shaped main axes. *Siphonocladus pusillus*, a species from the Mediterranean Sea and the type of *Siphonocladus*, is apparently more closely related to *E. verticillata* and *B. forbesii* than to the (sub)tropical Atlantic *S. tropicus*.

4.3.2. "Clades" 2-3-4

This group of clades comprises a rather heterogeneous assemblage of *Cladophora* species, which are conventionally ranged in separate sections of the genus (van den Hoek, 1963; van den Hoek and Chihara, 2000). *Cladophora prolifera* (Fig. 1D) and the morphologically allied *C. aokii* are placed in the section *Rugulosae*, based on the acropetal growth, the formation of descending rhizoids at the base of the cells, and the presence of annular constrictions in cells and rhizoids. The phylogenetic affinity between the two taxa is confirmed in the present phylogeny, although only convincingly under the ME criterion; in all other analyses, the Japanese *C. aokii* and a South African isolate of *C. prolifera* failed to group with the main *C. prolifera* clade. *C. coelothrix* (Fig. 1E), *C. socialis* and *Cladophora sibogae* (Fig. 1F) are characterized by cushion-like thalli composed of long cells with laterally inserted branches and rhizoids at their basal cell poles, and are, based on these characteristics, placed in the *Cladophora* section Repentes. The molecular phylogeny supports the monophyletic nature of this morphological group (clade 3), except for the European *C. coelothrix* plants, which are more closely related to *C. prolifera* than to the tropical representatives of *C. coelothrix*, which in their turn form a species complex with *C. socialis* in clade 3. The poorly known, Indo-Pacific *C. sibogae* (Weber-van Bosse, 1913), characterized by typical flabellate branches (Fig. 1G) forms a well separated sister taxon to this *C. coelothrix*/*socialis* complex. The sequence of *Cladophora* sp. 1 is from a Caribbean culture isolate (Fig. 1F) that morphologically resembles the southern European *C. echinus* (Biasoletto) Kützing (a member of the Cladophorales lineage based on unpublished molecular data). Because of the lack of morphological data of this plant in nature, and given the known phenotypic plasticity of *Cladophora* in culture (van den Hoek, 1963), we are unable to assign this isolate to a described taxon.

4.3.3. Clade 5

The genera *Anadyomene* (Fig. 1H) and *Microdictyon* (Fig. 1I), characterized by flat, blade- or net-like thalli, always group together in clade 5, a relationship proposed by Kützing (1843) who established the family Anadyomenaceae for them. *C. liebetruthii* evolved within *Microdictyon* by loss of the planar branching pattern. The morphological similarity between *Anadyomene* and *C. catenata*, which is a member of the *Cladophora* section *Aegagropila*, is much less obvious; however, the molecular phylogeny suggests that this taxon evolved within *Anadyomene* by extreme secondary reduction of branch-systems and loss of the blade-like structure.

4.3.4. Clade 6

All representatives of the morphologically well circumscribed genus *Valonia* (Fig. 1J) group in clade 6. Many workers have commented on the lack of clarity of species concepts within the genus (Børgesen, 1905, 1912, 1913; Egerod, 1952; Olsen and West, 1988) and this is clearly reflected in the present study, which reveals convergence of the limited number of diagnostic characters in *Valonia* (branching pattern, cell dimensions and organization of tenacular cells). Olsen and West (1988) separated *Ventricaria* from *Valonia* based on the evidence of immunological data, mode of cell division (SCDM, as opposed to LCD in *Valonia*) and reduced habit (lack of branches and tenacular cells). The present study clearly shows that *Ventricaria* falls within the *Valonia* clade and that specialized modes of cell division have evolved multiple times independently within various clades of the Siphonocladales. Moreover, segregative cell division, induced by cell wounding in culture, has been demonstrated in several *Valonia* species (Felicini and Perrone, 1994; Felicini et al., 1997; Kim and Klotchkova, 2004).

4.3.5. Clades 7 and 8

The large genetic distances found within pantropical *Dictyosphaeria cavernosa* (Fig. 1K) are in concordance with historic phylogenetic studies based on immunological data (Olsen-Stojkovich et al., 1986), which showed large intraspecific divergence between morphologically identical specimens collected from the Indo-Pacific and the Caribbean. The present study confirms this apparent morphological stasis in *D. cavernosa*. *D. versluysii* (which differs from *D. cavernosa* by the formation of solid thalli) appears to be closely allied to *D. cavernosa* from the Indian Ocean while the *D. cavernosa* isolates from the Pacific form a different sub-clade. More data and wider taxon sampling is needed but, based on the present data, it seems probable that *D. cavernosa* represents a cryptic species complex. The monospecific, Indo-Pacific genus *Valoniopsis* (Fig. 1L) is always revealed as sister to the *Dictyosphaeria*-clade. This relationship is puzzling from a morphological point of view because the two genera hardly have any

characters in common (Fig. 6). A considerable amount of sequence divergence is detected between the East African *V. pachynema* and a clade including plants from the central Indian and western Pacific Oceans, possibly indicating cryptic diversity.

4.3.6. Clade 9

This morphologically diverse clade consists of seven genera. Morphological characters traditionally used to distinguish these genera have focused on modes of branching, types of tenacular cells, presence or absence of annularly constricted cells and mode of cell division (Børgesen, 1905, 1912, 1913, 1940; Egerod, 1952, 1975; Kraft and Wynne, 1996; Leliaert, 2004; Leliaert and Coppejans, 2006, 2007). The molecular data shows that the importance of these characters has been overemphasized. For example, *Phyllodictyon* (Fig. 1O) and *Struvea* are distinguished, based on the different mode of cell division: *Struvea* including those species in which cells divide exclusively by SCD and *Phyllodictyon* encompassing taxa in which cells divide by CI. However, different specialized modes of cell division have obviously evolved multiple times independently (within clade 9, as well as in the whole siphonocladalean lineage) resulting in the non-monophyletic nature of many taxa that are distinguished only on their mode of cell division. Similarly, the morphological feature that differentiates *Chamaedoris*, i.e. the formation of a three-dimensional capitulum, has evolved twice. The reduced branch systems of *Cladophoropsis* (Fig. 1N) can either be regarded as pleisiomorphic in clade 9 or could have evolved multiple times independently. The taxonomic position of the genus *Apjohnia* (Fig. 1M) has long been uncertain. *Apjohnia laetevirens* has been allied with *E. verticillata*, *Cladophora rugulosa* G. Martens and the genus *Anadyomene* based on similarities in branching pattern and thallus architecture (Papenfuss and Chihara, 1975). The phylogenetic relationship of *A. laetevirens* and *P. orientale* in the present study is supported morphologically by the presence of type-4 tenacular cells (Womersley, 1984; Leliaert, 2004; Leliaert and Coppejans, 2007).

4.4. Biogeography

The present phylogeny confirms the assumption that the Siphonocladales are an originally tropical lineage (Bakker et al., 1994; van den Hoek and Chihara, 2000). Several tropical and subtropical species have successfully invaded the warm-temperate zones in both the northern and southern hemispheres. Based on the present data, cool-temperate representatives seem to be absent in the siphonocladalean lineage.

To establish a credible hypothesis of historical biogeography, one would require a comprehensive species-level phylogeny, knowledge of geographical distributions, and a time scale. The latter is problematic since the age of the Cladophorophyceae remains extremely doubtful. Scarce fossil evidence is provided by Butterfield et al. (1988) who found *Cladophora*-like forms in a submarine Proterozoic shale of Spitsbergen, suggesting that cladophoralean species are 800–700 Ma years old. Younger biosedimentary fossils are reported from the Triassic (245 Ma) and representatives of several genera are recorded from the Jurassic and Lower Cretaceous (135 Ma) (Wray, 1978; Tappan, 1980). The age of the Cladophorophyceae can also be derived indirectly by evaluation of its sister groups, which do have a richer fossil record, Bryopsidophyceae (Bryopsidales) and Dasycladophyceae (Dasycladales) (Zechman et al., 1990; López-Bautista and Chapman, 2003). Both groups are supposed to be Precambrian lineages of tropical marine green algae that have maintained a relatively consistent body plan throughout their 600–570-million year evolutionary history (Berger and Kaever, 1992), and this could therefore be also the minimum age of the Cladophorophyceae (van den Hoek and Chihara, 2000). The old age of the Siphonocladales may explain the lack of signatures of vicariance events in the present phylogenetic tree at the generic level: most of the main clades encompass species from tropical to warm-temperate regions of the world's three major oceans.

Remarkably, this broad spatial scale is also observed down to the level of several genotypic clusters, where identical (or nearly identical) sequences were obtained from plants from distant localities. Examples of clades displaying such biogeographical links include *S. tropicus* and *A. saldanhae* (each occurring on both sides of the Atlantic Ocean), *C. liebetruthii* and *Microdictyon calodictyon/krausii* (each occurring in the NE Atlantic and SW Indian Ocean), *B. forbesii* (W Indian and W Pacific oceans), *C. coelothrix* of clade 4 (W Indian, W Pacific and W Atlantic oceans), *Cladophora prolifera* (Mediterranean Sea, N Atlantic and SW Pacific) and *M. japonicum* (Japan and Hawaii). This could indicate the ability of several siphonocladalean taxa to disperse rapidly over long distances. This is in agreement with the results that were found by van der Strate et al. (2002) who showed that cryptic species of *C. membranacea* have dispersed between both sides of the Atlantic Ocean in a timeframe from thousands to even hundreds of years. Moreover, the present phylogeny suggests the ability of some tropical to warm-temperate siphonocladalean taxa to cross cold temperature barriers (e.g. *C. liebetruthii* and the tropical *C. coelothrix*-clade). Dispersal in many species is accomplished by fragmentation of the thallus or modified segregative cell division, and the ability to float by trapping air in the thallus. For example cushion-like plants of *Boodlea* and the hollow thalli of *Dictyosphaeria* have been observed floating in the open sea (pers. obs.). Also SCDM, along with the dispersal of daughter cells, potentially plays a role in the broad distribution pattern of several species (e.g. *V. ventricosa* and *B. forbesii*). On the other hand, regional endemism is likely to be the case for a number of species, for example *A. laetevirens*, *Stru-*

vea plumosa (S and W Australia) and *Chamaedoris delphinii* (SE Africa). The restricted geographical distribution of these taxa can possibly be explained by their stenothermal nature or the inability to disperse via fragmentation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.04.016.

References

Andreakis, N., Procaccini, G., Kooistra, W.H.C.F., 2004. *Asparagopsis taxiformis* and *Asparagopsis armata* (Bonnemaisoniales, Rhodophyta): genetic and morphological identification of Mediterranean populations. Eur. J. Phycol. 39, 273–283.

Bakker, F.T., Olsen, J.L., Stam, W.T., van den Hoek, C., 1994. The *Cladophora* complex (Chlorophyta): new views based on 18S rRNA gene sequences. Mol. Phyl. Evol. 3, 365–382.

Berger, S., Kaever, M.J., 1992. Dasycladales. Georg Thieme Verlag, Stuttgart.

Bergsten, J., 2005. A review of long-branch attraction. Cladistics 21, 163–193.

Børgesen, F., 1905. Contributions à la connaissance du genre *Siphonocladus* Schmitz. Overs. K. Dan. Vidensk. Selsk. Fosh. 1905, 259–291.

Børgesen, F., 1912. Some Chlorophyceae from the Danish West Indies. II. Bot. Tidskr. 32, 241–273.

Børgesen, F., 1913. The marine algae of the Danish West Indies. Part 1. Chlorophyceae. Dansk Bot. Ark. 1 (4), 158.

Børgesen, F., 1940. Some marine algae from Mauritius. I. Chlorophyceae. Biol. Meddel. Kongel. Danske Vidensk. Selsk. 15 (4), 81.

Bot, P.V.M., 1992. Molecular relationships in the seaweed genus *Cladophora*. Ph.D. Thesis, University of Groningen, The Netherlands, 110 pp.

Buchheim, M., Buchheim, J., Carlson, T., Braband, A., Hepperle, D., Krienitz, L., Wolf, M., Hegewald, E., 2005. Phylogeny of the Hydrodictyaceae (Chlorophyceae): inferences from rDNA data. J. Phycol. 41, 1039–1054.

Butterfield, N.J., Knoll, A.H., Swett, K., 1988. Exceptional preservation of fossils in an Upper Proterozoic shale. Nature 334, 424–427.

Cunningham, C.W., Omland, K., Oakley, T., 1998. Reconstructing ancestral character states: a critical reappraisal. Trends Ecol. Evol. 13, 361–366.

De Rijk, P., Robbrecht, E., de Hoog, S., Caers, A., Van de Peer, Y., De Wachter, R., 1999. Database on the structure of large subunit ribosomal RNA. Nucleic Acids Res. 27, 174–178.

De Rijk, P., De Wachter, R., 1993. DCSE, an interactive tool for sequence alignment and secondary structure research. Comput. Appl. Biosci. 9, 735–740.

Egerod, L.E., 1952. An analysis of the siphonous Chlorophycophyta with special reference to the Siphonocladales, Siphonales, and Dasycladales of Hawaii. Univ. Calif. Publ. Bot. 25, 327–367.

Egerod, L.E., 1975. Marine algae of the Andaman Sea coast of Thailand: Chlorophyceae. Bot. Mar. 18, 41–66.

Enomoto, S., Hirose, H., 1971. On the septum formation of *Microdictyon okamurai* Setchell. Bull. Jap. Soc. Phycol. 19, 90–93.

Enomoto, S., Okuda, K., 1981. Culture studies of *Dictyosphaeria* (Chlorophyceae, Siphonocladales I). Life history and morphogenesis of *Dictyosphaeria cavernosa*. Jap. J. Phycol. 29, 225–236.

Enomoto, S., Hori, T., Okuda, K., 1982. Culture studies of *Dictyosphaeria* (Chlorophyceae, Siphonocladales) II. Morphological analysis of segregative cell division in *Dictyosphaeria cavernosa*. Jap. J. Phycol. 30, 103–112.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1995. Constructing a significance test for incongruence. Syst. Biol. 44, 570–572.

Feau, N., Hamelin, R.C., Bernier, L., 2006. Attributes and congruence of three molecular data sets: inferring phylogenies among *Septoria*-related species from woody perennial plants. Mol. Phyl. Evol. 40, 808–829.

Felicini, G.P., Perrone, C., 1994. Segregative and pseudo-segregative cell division in *Valonia utricularis* (Siphonocladales–Cladophorales complex, Chlorophyta). Giorn. Bot. Ital. 128, 810–812.

Felicini, G.P., Perrone, C., Bottalico, A., 1997. Endogenous and in vitro protoplasts of *Valonia utricularis* and *Valonia aegagropila* (Chlorophyta, Cladophorophyceae). Boll. Museo Region. Sci. Nat. Torino 15, 131–151.

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

Fritsch, F.E., 1944. *Cladophorella calcicola* nov. gen. et sp., a terrestrial member of the Cladophorales. Ann. Bot. 8, 157–171.

Goloboff, P.A., 1993. Estimating character weights during tree search. Cladistics 9, 83–91.

Gontcharov, A.A., Marin, B., Melkonian, M., 2004. Are combined analyses better than single gene phylogenies? A case study using SSU rDNA and rbcL sequence comparisons in the Zygnematophyceae (Streptophyta). Mol. Biol. Evol. 21, 612–624.

Graham, S.W., Olmstead, R.G., Barrett, S.C.H., 2002. Rooting phylogenetic trees with distant outgroups: a case study from the commelinoid monocots. Mol. Biol. Evol. 19, 1769–1781.

Hanyuda, T., Wakana, I., Arai, S., Miyaji, K., Watano, Y., Ueda, K., 2002. Phylogenetic relationships within Cladophorales (Ulvophyceae, Chlorophyta) inferred from 18S rRNA gene sequences, with special reference to *Aegagropila linnaei*. J. Phycol. 38, 564–571.

Harvey, J.B.J., Goff, L.J., 2006. A reassessment of species boundaries in *Cystoseira* and *Halidrys* (Phaeophyceae, Fucales) along the North American west coast. J. Phycol. 42, 707–720.

Hillis, D.M., Huelsenbeck, J.P., 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J. Hered. 83, 189–195.

Holland, B.R., Penny, D., Hendy, M.D., 2003. Outgroup misplacement and phylogenetic inaccuracy under a molecular clock – a simulation study. Syst. Biol. 52, 229–238.

Huelsenbeck, J.P., Bull, J.J., Cunningham, C.W., 1996. Combining data in phylogenetic analysis. Trends Ecol. Evol. 11, 152–158.

Huelsenbeck, J.P., Bollback, J.P., Levine, A.M., 2002. Inferring the root of a phylogenetic tree. Syst. Biol. 51, 32–43.

Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21–123.

Kim, G.H., Klotchkova, T.A., 2004. Development of protoplasts induced from wound-response in fifteen marine green algae. Jap. J. Phycol. 52 (suppl.), 111–116.

Kim, G.H., Klotchkova, T.A., West, J.A., 2002. From protoplasm to swarmer: regeneration of protoplasts from disintegrated cells of the multicellular marine green alga *Microdictyon umbilicatum* (Chlorophyta). J. Phycol. 38, 174–183.

Kjer, K.M., 1995. Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. Mol. Phyl. Evol. 4, 314–330.

Kraft, G.T., 2000. Marine and estuarine benthic green algae (Chlorophyta) of Lord Howe Island, South-western Pacific. Aust. Syst. Bot. 13, 509–648.

Kraft, G.T., Wynne, M.J., 1996. Delineation of the genera Struvea Sonder and Phyllodictyon J.E. Gray (Cladophorales, Chlorophyta). Phycol. Res. 44, 129–143.

Kützing F.T., 1843. Phycologia generalis. Leipzig.

La Claire II, J.W., 1982. Cytomorphological aspects of wound healing in selected Siphonocladales (Chlorophyta). J. Phycol. 18, 379–384.

Leliaert, F., 2004. Taxonomic and phylogenetic studies in the Cladophorophyceae (Chlorophyta). Ph.D. Dissertation, Ghent University, Belgium. 294 pp. (Available from: <http://www.lib.ugent.be/>).

Leliaert, F., Coppejans, E., 2004. Crystalline cell inclusions: a new diagnostic character in the Cladophorophyceae (Chlorophyta). Phycologia 43, 189–203.

Leliaert, F., Coppejans, E., 2006. A revision of *Cladophoropsis* Børgesen (Siphonocladales, Chlorophyta). Phycologia 45, 657–679.

Leliaert, F., Coppejans, E., 2007. Systematics of two deep-water species from the Indo-West Pacific: *Struvea gardineri* A. Gepp & E. Gepp and *Phyllodictyon orientale* (A. Gepp & E. Gepp) Kraft & M.J. Wynne (Siphonocladales, Chlorophyta). Bot. J. Linn. Soc. 153, 115–132.

Leliaert, F., Rousseau, F., de Reviers, B., Coppejans, E., 2003. Phylogeny of the Cladophorophyceae (Chlorophyta) inferred from partial LSU rRNA gene sequences: is the recognition of a separate order Siphonocladales justified? Eur. J. Phycol. 38, 233–246.

Lindstrom, S.C., 2001. The Bering Strait connection: dispersal and speciation in boreal macroalgae. J. Biogeogr. 28, 243–251.

Littler, D.S., Littler, M.M., 1991. Systematics of *Anadyomene* species (Anadyomenaceae, Chlorophyta) in the tropical western Atlantic. J. Phycol. 27, 101–118.

López-Bautista, J.M., Chapman, R.L., 2003. Phylogenetic affinities of the Trentepohliales inferred form small subunit ribosomal DNA. Int. J. Syst. Evol. Microbiol. 53, 2099–2106.

Lyons-Weiler, J., Hoelzer, G.A., Tausch, R.J., 1998. Optimal outgroup analysis. Biol. J. Linn. Soc. 64, 493–511.

Maddison, W.P., Maddison, D.R., 2006. Mesquite: a modular system for evolutionary analysis. Version 1.11. (Available from: <http://mesquiteproject.org>).

Maddison, W.P., Donoghue, M.J., Maddison, D.R., 1984. Outgroup analysis and parsimony. Syst. Zool. 33, 83–103.

Mallet, J., 1995. A species definition for the modern synthesis. Trends Ecol. Evol. 10, 294–299.

McDonald, K.L., Pickett-Heaps, J.D., 1976. Ultrastructure and differentiation in *Cladophora glomerata*. I. Cell division. Am. J. Bot. 63, 592–601.

Murray, S., Jorgensen, M.F., Ho, S.Y.W., Patterson, D.J., Jermiin, L.S., 2005. Improving the analysis of dinoflagellate phylogeny based on rDNA. Protist 156, 269–286.

Norris, J.N., Olsen, J.L., 1991. Deep-water green algae from the Bahamas, including *Cladophora vandenhoekii* sp. nov. (Cladophorales). Phycologia 30, 315–328.

Nylander, J.A.A., 2004. MrModeltest v2. Evolutionary Biology Centre, Uppsala University. (Available from: <http://www.abc.se/~nylander/>).

Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P., Nieves-Aldrey, J.L., 2004. Bayesian phylogenetic analysis of combined data. Syst. Biol. 53, 47–67.

Okuda, K., Mine, I., Morinaga, T., Kuwaki, N., 1997. Cytomorphogenesis in cenocytic green algae: V. Segregative cell division and cortical microtubules in *Dictyosphaeria cavernosa* (Siphonocladales, Chlorophyceae). Phycol. Res. 45, 189–196.

Olsen, J.L., West, J.A., 1988. *Ventricaria* (Siphonocladales–Cladophorales complex, Chlorophyta), a new genus for *Valonia ventricosa*. Phycologia 27, 103–108.

Olsen-Stojkovich, J.L., 1986. Phylogenetic studies of genera in the Siphonocladales–Cladophorales complex (Chlorophyta). Ph.D. Dissertation, University of California, Berkeley, 183 pp.

Olsen-Stojkovich, J., West, J.A., Lowenstein, J.M., 1986. Phylogenetics and biogeography in the Cladophorales complex (Chlorophyta): some insights from immunological distance data. Bot. Mar. 29, 239–249.

O'Neil, R.M., La Claire II, J.W., 1984. Mechanical wounding induces the formation of extensive coated membranes in giant algal cells. Science 255, 331–333.

Oltmanns, F., 1922. Morphologie und Biologie der Algen. Ed. 2. vol. 1. Jena.

Papenfuss, G.F., Chihara, M., 1975. The morphology and systematic position of the green algae *Ernodesmis* and *Apjohnia*. Phycologia 14, 309–316.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Posada, D., Crandall, K.A., 2001. Selecting the best-fit model of nucleotide substitution. Syst. Biol. 50, 580–601.

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.

Rindi, F., Lopez-Bautista, J.M., Sherwood, A.R., Guiry, M.D., 2006. Morphology and phylogenetic position of *Spongiochrysis hawaiiensis* gen. et sp. nov., the first known terrestrial member of the order Cladophorales (Ulvophyceae, Chlorophyta). Int. J. Syst. Evol. Microbiol. 56, 913–922.

Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Russo, C.A.M., Takezaki, N., Nei, M., 1996. Efficiencies of different genes and different tree-building methods in recovering a known vertebrate phylogeny. Mol. Biol. Evol. 13, 525–536.

Schöniger, M., von Haeseler, A., 1994. A stochastic model for the evolution of autocorrelated DNA sequences. Mol. Phylogenet. Evol. 3, 240–247.

Scott, J.L., Bullock, K.W., 1976. Ultrastructure of cell-division in *Cladophora*—pre-gametangial cell-division in haploid generation of *Cladophora flexuosa*. Can. J. Bot. 54, 1546–1560.

Silva, P.C., Basson, P.W., Moe, R.L., 1996. Catalogue of the Benthic Marine Algae of the Indian Ocean. University of California Press, Berkeley.

Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Swofford, D.L., Olsen, G.J., Waddell, P.J., Hillis, D.M., 1996. Phylogenetic inference. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics, second ed. Sinauer, Sunderland, MA, pp. 407–514.

Tappan, H., 1980. The Paleobiology of Plant Protists. W.H. Freeman and Co., San Fransisco.

Taylor, W.R., 1928. The marine algae of Florida with special reference to the Dry Tortugas. Publ. Carnegie Inst. Wash. 379, 219.

Taylor, W.R., 1950. Plants of Bikini and Other Northern Marshall Islands. University of Michigan Press, Ann Arbor.

Taylor, W.R., 1960. Marine Algae of the Eastern Tropical and Subtropical Coasts of the Americas. The University of Michigan Press, Ann Arbor.

Telford, M.J., Wise, M.J., Gowri-Shankar, V., 2005. Consideration of RNA secondary structure significantly improves likelihood-based estimates of phylogeny: examples from the bilateria. Mol. Biol. Evol. 22, 1129–1136.

van den Hoek, C., 1963. Revision of the European Species of *Cladophora*. Brill E.J., Leiden.

van den Hoek, C., 1982. A taxonomic revision of the American species of *Cladophora* (Chlorophyceae) in the North Atlantic Ocean and their geographic distribution. Verh. Kon. Ned. Akad. Wetensch., Afd. Natuurk., Tweede Sect. 78, 236.

van den Hoek, C., 1984. The systematics of the Cladophorales. In: Irvine, D.E.G., John, D.M. (Eds.), Systematics of the Green Algae. Academic Press, London, pp. 157–178.

van den Hoek, C., Chihara, M., 2000. A taxonomic revision of the marine species of *Cladophora* (Chlorophyta) along the coasts of Japan and the

Russian Far-east National Science Museum (Tokyo). Monographs 19, 242.

van den Hoek, C., Mann, D.G., Jahns, H.M., 1995. Algae. An Introduction to Phycology. Cambridge University Press, Cambridge.

van der Strate, H.J., Boele-Bos, S.A., Olsen, J.L., van de Zande, L., Stam, W.T., 2002. Phylogeographic studies in the tropical seaweed *Cladophoropsis membranaceae* (Chlorophyta, Ulvophyceae) reveal a cryptic species complex. J. Phycol. 38, 572–582.

Verbruggen, H., De Clerck, O., Kooistra, W.H.C.F., Coppejans, E., 2005. Molecular and morphometric data pinpoint species boundaries in *Halimeda* section *Rhipsalis* (Bryopsidales, Chlorophyta). J. Phycol. 41, 606–621.

Wagner, H.P., Zaneveld, J.S., 1988. The Xanthophyceae and Chlorophyceae of the western Ross Sea, Victoria land, Antarctica and Macquarie Island collected under the direction of Zaneveld, J.S. (1963–1967). Blumea 33, 141–180.

Weber-van Bosse, A., 1913. Liste des algues du Siboga. I. Myxophyceae, Chlorophyceae, Phaeophyceae avec le concours de M. Th. Reinbold. Siboga-Expeditie Monographie 59a. Leiden, 186 pp.

Wheeler, W.C., 1990. Nucleic-acid sequence phylogeny and random outgroups. Cladistics 6, 363–367.

Womersley, H.B.S., 1984. The Marine Benthic Flora of Southern Australia. Part I. Government Printer, South Australia, Adelaide.

Womersley, H.B.S., Bailey, A., 1970. Marine algae of the Solomon Islands. Philos. Trans. R. Soc. Lond. B 259, 257–352.

Wortley, A.H., Rudall, P.J., Harris, D.J., Scotland, R.W., 2005. How much data are needed to resolve a difficult phylogeny? Case study in Lamiales. Syst. Biol. 54, 697–709.

Wray, J.L., 1978. Calcareous algae. In: Haq, B.U., Boersma, A. (Eds.), Introduction to Marine Micropaleontology. Elsevier, New York, pp. 171–187.

Wuyts, J., De Rijk, P., Van de Peer, Y., Winkelmans, T., De Wachter, R., 2001. The European large subunit ribosomal RNA database. Nucleic Acids Res. 29, 175–177.

Wysor, B., 2002. Biodiversity and biogeography of marine green algae of the Republic of Panama. Ph.D. Dissertation, University of Louisiana at Lafayette.

Xia, X., Xie, Z., 2001. DAMBE: data analysis in molecular biology and evolution. J. Hered. 92, 371–373.

Zechman, F.W., Theriot, E.C., Zimmer, E.A., Chapman, R.L., 1990. Phylogeny of the Ulvophyceae (Chlorophyta): cladistic analysis of nuclear-encoded rRNA sequence data. J. Phycol. 26, 700–710.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.

Appendix A1
Specimens used in the phylogenetic analyses with the code used in the present study, collecting data (location, collector, date of collection and voucher information), and EMBL/GenBank accession numbers.

Species	Code	Collecting data	partial LSU rDNA	SSU rDNA
Anadyomene saldanhae A.B. Joly & E.C. Oliveira	F247	North-Star, St Croix (Kooistra, 1988, AsNS / Asal1) [1]	AM503403	AM498745
	F263	Praia, San Tiago, Cape Verde Isl. (Pakker, 1991, AsaTPa23 / Asal2) [1]	AM503404	
	F264	San Tiago, Cape Verde Isl. (Pakker, 1991, AsTCa59 / Asal3) [1]	AM503405	
	F265	Tague Bay, St Croix (Kooistra, 1988, AsTB / Asal4) [1]	AM503406	
	F266	Bonaire (AsBo / Asal6) [1]	AM503407	
	F267	Praia, San Tiago, Cape Verde Isl. (Pakker, 1991, AsTPa29a / Asal7a) [1]	AM503408	
	F269	Punta del Hidalgo, Tenerife, Canary Isl. (1986, AsT / Astel3) [1]	AM503412	
	F286	Praia, San Tiago, Cape Verde Isl. (Pakker, 1991, AsTPa29b / Asal7b) [1]	AM503409	
Anadyomene stellata (Wulfen) C. Agardh	F041	Olango Island, Cebu, Philippines (Leliaert et al., 13.viii.1998, PH 209) [2]	AJ544746	
	F246	Faro de Orchilla, Hierro, Canary Isl. (1991, AsCI2B / Astel1) [1]	AM503410	
	F268	Paliouri, Chaldikidi, Greece (1994, AsGP / Astel2) [1]	AM503411	
	F270	Faro de Orchilla, Hierro, Canary Isl. (1991, AsCI1E / Astel5) [1]	AM503413	
	F271	Heraklion, Kreta, Greece (1994, AsGK / Astel7) [1]	AM503414	
	F272	Stareso, Corsica (Breeman, 1990, AsCOR3 / Astel8) [1]	AM503415	
Apjohnia laetevirens Harvey	F273	Baron Heads, Melbourne, Australia (van Oppen, 1991, ApLB / Apj1) [1]	AM503416	AF510148
	F289	Western Australia (Schils, WA138) [2]	AM503417	
	F290	Western Australia (Schils, WA200) [2]	AM503418	
Boergesenia forbesii (Harvey) Feldmann	F009	Nungwi, Zanzibar, Tanzania (Leliaert, 21.vii.2001, FL1009) [2]	AJ544742	
	F252	Bird Isl., Seychelles (Kooistra, 1993, BOFSey212 / Boerg1) [1]	AM503419	AM498746
	F253	Palau (1992, BOFPalau / Boerg 2) [1]	AM503420	
Boodlea composita (Harvey) F. Brand	F016	Matemwe, Zanzibar, Tanzania (Leliaert, 16.vii.2001, FL950) [2]	AJ544731	AF510157
Boodlea montagnei (Harvey ex J.E. Gray) Egerod	F017	Mactan Island , Philippines (Leliaert et al., 6.viii.1998, PH646) [2]	AJ544734	
	F018	Matemwe, Zanzibar, Tanzania (Leliaert, 16.vii.2001, FL958) [2]	AJ544733	
	F183	SE of Olango Island, Philippines (Verbruggen, 25.i.2004, HV626) [2]	AM503421	
Boodlea siamensis Reinbold	F035	Nungwi, Zanzibar, Tanzania (Leliaert, 21.vii.2001, FL999) [2]	AJ544730	AF510158
	F349	E Mactan Island, Philippines (Verbruggen, 16.ii.2004, HV870) [2]	AM503422	
	F355	Weyalailai Resort, Weyasewa, Yasawas, Fiji (Boedeker 32, 24.i.2005, C29) [3]	AM503423	
Chamaedoris auriculata Børgesen	F010	Nogid, Socotra (Leliaert, 14.iii.1999, SOC395) [2]	AJ544739	
Chamaedoris delphinii (Hariot) Feldmann & Børgesen	F011	Sodwana Bay, KwaZulu-Natal, South Africa (De Clerck et al., 10.ii.2001, KZN2110) [2]	AJ544740	
	F187	KwaZulu-Natal, South Africa (Anderson, s.n.) [2]	AM503424	
	F321	Linkia Reef, KwaZulu-Natal, South Africa (De Clerck et al., 15.viii.1999, KZN694) [2]	AM503425	
	F359	Mzamba, Eastern Cape, South Africa (Stegenga & De Clerck, 21.viii.2005, D53) [3]	AM503426	
	F360	Palm Beach, KwaZulu-Natal, South Africa (Boedeker, 22.viii.2005, D56) [3]	AM503427	
Chamaedoris orientalis Okamura & Higashi	F104	Bulusan, The Philippines (Coppejans, 22.iv.1998, HEC12301A) [2]	AM503428	
	F332	Bulusan, The Philippines (Coppejans, 22.iv.1998, HEC12301B) [2]	AM503429	
Chamaedoris peniculum (J. Ellis & Solander) Kuntze	F124	Puerto Plata, Dominican Republic (Dargent, 8.ii.2002, HODRD2-02-20a) [2]	AM503430	
	F125	Puerto Plata, Dominican Republic (Dargent, 8.ii.2002, HODRD2-02-20b) [2]	AM503431	
	F333	Puerto Plata, Dominican Republic (Dargent, 8.ii.2002, HODRD2-02-20) [2]	AM503432	Z35417
Cladophora aokii Yamada	F222	Hachijo, Japan (van den Hoek, 1990, CryHJ / Cryu1) [1]	AM503434	AM498747
Cladophora catenata (Linnaeus) Kützing	F114	Mabibi, KwaZulu-Natal, South Africa (Coppejans et al., 11.viii.1999, KZN454) [2]	AM503435	
	F126	Rabbit Rock, KwaZulu-Natal, South Africa (Coppejans et al., 13.viii.1999, KZN547) [2]	AM503436	
	F185	Lanikai, Oahu, Hawaii (De Clerck, 25.iv.2003, ODC899) [2]	AM503437	
	F254	Okinawa, Japan (van den Hoek, 1990, CcatOJ / Ccat1) [1]	AM503438	Z35418
	F371	Lala Neck, KwaZulu-Natal, South Africa (PvR, 9.viii.2005, G68) [3]	AM503439	

Appendix A1 (continued)

Species	Code	Collecting data	partial LSU rDNA	SSU rDNA
Cladophora coelothrix Kützing	B89	Isle St Marie, Madagascar (West, 22.v.2004, JW4448)	AM503441	
	F058	Iwatine Bay, Mombasa, Kenya (Coppejans, 11.ix.1992, HEC9394) [2]	AJ544754	
	F062	Malindi, Kenya (Coppejans, 21.iii.1988, HEC7418) [2]	AJ544753	
	F112	Shaghaf Island, Masirah, Oman (Schils, 24.xi.1999, MAS463a) [2]	AM503442	
	F135	Mactan Island, Cebu, Philippines (Leliaert et al., 27.viii.1998, PH568) [2]	AM503443	AM498749
	F178	SE of Olango Island, Philippines (Verbruggen, 25.i.2004, HV622) [2]	AM503444	
	F181	Cabilao, Bohol, Philippines (Verbruggen, 31.i.2004, HV688) [2]	AM503445	
	F188	Mnazi Bay, S of Ras Ruvula, Tanzania (Coppejans, 23.vii.2000, HEC12844) [2]	AM503446	
	F256	unknown locality (culture isolate) [1]	AM503447	
	F274	Praia, San Tiago, Cape Verde Isl. (Pakker, 1991, ClxTPa24 / Ccoel1) [1]	AM503448	
	F275	Roscoff, France (1983, C83.14 / Ccoel2) [1]	AM503449	AM498748
	F276	Candelaria, Tenerife, Canary Isl. (1988, CcCa2 / Ccoel3) [1]	AM503450	
	F277	Corsica (1987, C87.1 / Ccoel4) [1]	AM503451	Z35315
	F350	Roscoff, France (1983, C83.14, A67) [3]	AM503452	
	F351	Punta del Hidalgo, Tenerife (1988, CcCa2, A68) [3]	AM503453	
	F353	Garapuá, Ilha de Tinharé, Bahia, Brasil (Braga, 1.i.2004, JW3735)	AM503454	
	F356	Galeta, Panama (Wysor 866, C98) [3]	AM503455	
	F372	Rocky Bay, KwaZulu-Natal, South Africa (PvR, 17.viii.2005, 2005-29, G71) [3]	AM503456	
	F380	Maratua Island, Indonesia (PvR & de Senerpont Domis, x.2003, 03-435, B14) [3]	AM503457	
	F381	Kakaban Island, Indonesia (PvR & de Senerpont Domis, x.2003, 03-34, B15) [3]	AM503458	
	F502	near Tonkley, N of The Entrance, NSW, Australia (H45) [3]	AM503459	
Cladophora liebetruthii Grunow	F214	Fuerteventura, Canary Isl. (1988, ClieF / Clieb1) [1]	AM503462	
	F127	Palm Beach, KwaZulu-Natal, South Africa (Coppejans et al., 19.viii.1999, KZN802) [2]	AM503461	
	F215	San-Vicente, Cape Verde Isl. (Pakker, 1991, ClieVGa116 / Clieb3) [1]	AM503463	Z35318
	F522	Rais Magos, Madeira (Coppejans & De Clerck, 11.v.2006, HEC15694) [2]	AM503464	
	F524	Rais Magos, Madeira (Coppejans & De Clerck, 11.v.2006, HEC15712) [2]	AM503465	
Cladophora prolifera (Roth) Kützing	F106	Point Lonsdale, Victoria, Australia (De Clerck, 7.vii.1996, ODC519) [2]	AM503466	
	F128	Rabbit Rock, KwaZulu-Natal, South Africa (Coppejans et al., 13.viii.1999, KZN533bis) [2]	AM503467	
	F219	Corsica (1984, Pr84.28 / Cprol1) [1]	AM503468	Z35422
	F278	Fuerteventura, Canary Isl. (1988, CpF / Cprol2) [1]	AM503469	AM498750
	F279	El Medano, Tenerife, Canary Isl. (1988, CproEM / Cprol3) [1]	AM503470	
	F280	Baron Heads, Melbourne, Australia (van Oppen, 1991, CProBA / Cprol4) [1]	AM503471	
	F281	S Australia (1985, Pr85.35 / C prol 5) [1]	AM503472	
	F523	Rais Magos, Madeira (Coppejans & De Clerck, 11.v.2006, HEC15704) [2]	AM503473	
Cladophora sibogae Reinbold	F061	Mangapwani, Zanzibar, Tanzania (De Clerck & Coppejans, 29.viii.1994, ODC352) [2]	AJ544752	
	F430	Msalani, Zanzibar, Tanzania (Leliaert & Coppejans, 11.vii.2001, FL910c) [2]	AM503475	
Cladophora socialis Kützing	F173	Blue Lagoon, Portland, Jamaica (Verbruggen, 22.iii.2003, HV523) [2]	AM503476	
	F197	Mabibi, KwaZulu-Natal, South Africa (De Clerck, 13.ii.2001, KZN2185) [2]	AM503477	
	F225	Cathedral Rocks, Rottnest Isl., Australia (1988, CPS7A / Csoc2) [1]	AM503478	AM498751
	F228	unknown locality (culture isolate) [1]	AM503479	
Cladophora cf. *socialis*	F200	Isla Taboga, Balboa, Panama (Wysor 233, 2.iv.1999) [2]	AM503440	AM498752
Cladophora sp. 1	F224	St Croix, Caribbean (Kooistra, 1988, CPSCr / Csoc1) [1]	AM503480	AM498753

Appendix A1 (continued)

Species	Code	Collecting data	partial LSU rDNA	SSU rDNA
Cladophoropsis membranacea (Hofman Bang ex C. Agardh) Børgesen	A70	Latakia, Syria (collector unknown, 1991, CloMT-188) [3]	AM503482	
	F179	Drax Hall, St. Ann Parish, Jamaica (Verbruggen, 7.iii.2003, HV387) [2]	AM503483	
	F291	B. de Gattas, San Vicente, Cape Verde Isl. (Pakker, 1991, CloVGb83 / Csmem1 / CmCVI SanV BdG) [1]	AM503484	
	F292	Willemstad, Curacao (1991, CloCW / Csmem2 / CmCurW) [1]	AM503485	Z35322
	F295	Latakia, Syria (1991, CloMT / Csmem5 / CmMedSL) [1]	AM503486	
	F296	Punta del Hidalgo, Tenerife, Canary Isl. (CloPH / Csmem6 / CmCITFPdH) [1]	AM503487	
	F352	Fuerteventura (1988, CloF1, A77) [3]	AM503488	
	F386	Kakaban Island, marine lake, Indonesia (PvR 03- 25, ix.1999, B16) [3]	AM503489	
Cladophoropsis philippinensis W.R. Taylor	F045	Mactan Island, Philippines (Leliaert et al., 27.viii.1998, PH567) [2]	AJ544735	
	F176	SW Panglao Island, Philippines (Verbruggen, 1.ii.2004, HV710) [2]	AM503490	
Cladophoropsis sp. 1	F417	Key West, Florida, USA (Humm, 15 ix 1964, MO 390, West4296) [4]	AM503491	
Cladophoropsis sp. 2	F175	Looc, N side of Cabilao, Philippines (Verbruggen, 29.i.2004, HV670) [2]	AM503492	
Cladophoropsis sp. 3	F377	Panjang Island, Indonesia (PvR & de Senerpont Domis, x.2003, 03-391, B11) [3]	AM503493	
Cladophoropsis sp. 4	F245	Puerto Penasco, Baha California, Mexico (VPP794 / Vopsis4) [1]	AM503494	AM498754
Cladophoropsis sundanensis Reinbold	F189	Mnazi Bay, Mtwara area, Tanzania (Coppejans et al, 29.vii.2000, HEC12976)	AM503495	
	F282	Poivre, Seychelles (Kooistra, 1993, ClosuSey616E / Cssun 1) [1]	AM503496	
	F368	Damar kecil, Indonesia (Draisma & PvR, 8.ix.2005, 509.051, G50) [3]	AM503497	
	F369	Ayer besar, Indonesia (Prud'homme van Reine, 10.ix.2005, 509.099, G52) [3]	AM503498	
	F370	Onrus, Indonesia (Cleary, 7.ix.2005, 509.532, G59) [3]	AM503499	
Dictyosphaeria cavernosa (Forsskål) Børgesen	F020	Mbudya Island, Tanzania (Leliaert, 11.vii.2001, FL913) [2]	AJ544745	
	F283	Shimoda, Japan (van den Hoek, 1990, D.cavSJ25b / Dcav4) [1]	AM503500	AM498755
	F284	Okinawa, Japan (van den Hoek, 1990, D.cavOJ12B / Dcav5) [1]	AM503501	
	F285	Poivre Atoll, Seychelles (Kooistra, 1993, DISSey486 / Dcav7) [1]	AM503502	AM498756
Dictyosphaeria versluysii Weber-van Bosse	F122	Pongwe, Tanzania, Zanzibar (Leliaert, 20.vii.2001, FL993) [2]	AM503503	
Ernodesmis verticillata (Kützing) Børgesen	F089	Limon, Costa Rica (Freshwater, 23.iii.1999)	AJ544743	
	F182	Plage de Libanona, Fort Dauphin, Madagascar (Coppejans et al., 2.ix.2002, HEC15268) [2]	AM503504	
	F230	Fuerteventura, Canary Isl. (1988, EvF / Erno1) [1]	AM503505	AM498757
	F231	Grapetree Bay, St Croix (Kooistra, 1988, EvGTB / Erno6) [1]	AM503506	
	F300	Lagun, Bonaire (Kooistra, 1991, EvBL / Erno8) [1]	AM503507	
	F301	El Medano, Tenerife, Canary Isl. (1988, EvEM / Erno9) [1]	AM503508	AM498758
	F302	Punta del Hidalgo, Tenerife, Canary Isl. (1988, EvPH / Erno10) [1]	AM503509	
Microdictyon boergesenii Setchell	F232	Corsica (1990, MbCORa / Mboerg1) [1]	AM503510	
	F233	B. de Gattas, San Vicente, Cape Verde Isl. (Pakker, 1991, MbVGa122 / Mboerg5) [1]	AM503511	AM498759
	F303	N-Atlantic, Bahamas (Mb1557 / Mboerg2) [1, 4]	AM503512	Z35324
	F527	Island of Porto Santos, Madeira (Coppejans & De Clerck, 14.v.2006, HEC15756) [2]	AM503513	
Microdictyon calodictyon (Montagne) Kützing	F525	Rais Magos, Madeira (Coppejans & De Clerck, 11.v.2006, HEC15713) [2]	AM503514	
Microdictyon japonicum Setchell	F234	Shimoda, Japan (van den Hoek, 1990, MjSJ / Mjap1) [1]	AM503515	AM498760
	F235	Hawaii (West, Mj1554 / M jap 2) [1, 4]	AM503516	
Microdictyon kraussii J.E. Gray	F2	Sodwana Bay, KwaZulu-Natal, South Africa (Coppejans et al., 9.viii.1999, KZN272) [2]	AJ544747	
Microdictyon umbilicatum (Velley) Zanardini	F366	Whangarei, Urquhart Bay, New Zealand (Nelson & Heesch, UPN 462, 21.viii.2005, G12) [3]	AM503517	
	F367	Tutuhaha, North Island, New Zealand (Nelson & Heesch UPN 445, 20.viii.2005, G21) [3]	AM503518	
Phyllodictyon anastomosans (Harvey) Kraft & M.J. Wynne	F019	Matemwe, Zanzibar, Tanzania (Leliaert, 17.vii.2001, FL959) [2]	AJ544729	AF510159
	F036	Chwaka Bay, Zanzibar, Tanzania (Leliaert, 17.vii.2001, FL961) [2]	AJ544725	
	F193	Mtwara, Tanzania (Coppejans & Verbruggen, 27.vii.2001, HEC14579) [2]	AM503519	
	F236	Malta Baths, St Croix (Kooistra, 1988, SaMB / Sana1) [1]	AM503520	

Appendix A1 (continued)

Species	Code	Collecting data	partial LSU rDNA	SSU rDNA
Phyllodictyon orientale (A.Gepp & E.S.Gepp) Kraft & M.J. Wynne	F064	Bi Ya Doo Island, Maldives (Coppejans, 8.iv.1986, HEC6173) [2]	AJ544738	
	F414	Grande Comoro I., Comoros (Earle, 1977, West 1631 / Struv1) [4]	AM503521	
Phyllodictyon papuense nom. prov.	F068	Laing Island, Papua New Guinea (Coppejans, vi.1980, HEC4548) [2]	AJ544736	
Siphonocladus pusillus (C. Agardh ex Kützing) Hauck	F305	Stareso harbour, Corsica (Breeman, 1993, StCOR3 / Siph4) [1]	AM503522	
	F306	Osceluccia, Corsica (Breeman, 1993, StCOR1 / Siph5) [1]	AM503523	
Siphonocladus tropicus (P.L. Crouan & H.M. Crouan) J. Agardh	F101	Dominican Republic (Dargent, s.n.)	AJ544744	
	F304	Las Americas, Tenerife, Canary Isl. (1991, StCID / Siph1) [1]	AM503524	AM498761
	F419	Lagun, Bonaire (1991, culture: St-BL / Siph6) [1]	AM503525	
Struvea elegans Børgesen	F069	Loloata Island, Papua New Guinea (Coppejans, 5.viii.1994, HEC10437) [2]	AJ544737	
	F237	Bahamas (West, SE1572 / Sele1) [1, 4]	AM503526	AF510149
Struvea gardineri A. Gepp & E. Gepp	F199	Plate Island, Seychelles (Coppejans et al., 7.i.1993, SEY771a) [2]	AM072287	
Struvea plumosa Sonder	F287	Western Australia (Schils, WA221) [2]	AM503527	AF510161
	F288	Western Australia (Schils, s.n.) [2]	AM503528	
Struveopsis siamensis (Egerod) P.C. Silva	F015	Mbudya Island, Tanzania (Leliaert, 11.vii.2001, FL916) [2]	AJ544732	
Valonia aegagropila C. Agardh	F022	Chwaka Bay, Zanzibar, Tanzania (Leliaert, 17.vii.2001, FL960) [2]	AJ544748	
	F238	Hawaii (VAJOS1 / Vaeg3) [1]	AM503529	
	F239	Okinawa, Japan (van den Hoek, 1990, VA1a3OJ / Vaeg7) [1]	AM503530	AM498762
	F491	Kitayama, Japan (25.ii.2001, herbarium of the Natural History Museum (BM))	AM503531	
Valonia fastigiata Harvey ex J. Agardh	F240	Desroches Atoll, Seychelles (Kooistra, 1993, VFSey563 / Vfas1) [1]	AM503532	AM498763
Valonia macrophysa Kützing	F196	Sodwana Bay, KwaZulu-Natal, South Africa (De Clerck et al. 11.ii.2001, KZN2153) [2]	AM503533	
	F241	Brasil (VM2628 / Vmac3) [1, 4]	AM503534	
	F341	Sylphs Hole, Lord Howe Island (Millar, 26.x.2005), as *Ventricaria nutrix*	AM503535	
	F361	Palm Beach, KwaZulu-Natal, South Africa (Boedeker, 22.viii.2005, D62) [3]	AM503536	
Valonia utricularis (Roth) C. Agardh	F023	Matemwe, Zanzibar, Tanzania (Leliaert, 14.vii.2001, FL922) [2]	AJ544749	
	F242	Fuerteventura, Canary Isl. (1988, VUF / Vutric19) [1]	AM503537	Z35323
	F307	Mare Anglaise, Mahe, Seychelles (Kooistra, 1993, VU70A / Vutric16) [1]	AM503538	
Valoniopsis pachynema (G. Martens) Børgesen	F24	Nungwi, Zanzibar, Tanzania (Leliaert, 21.vii.2001, FL1006) [2]	AJ544741	
	F244	Hainan, China (Bartsch, 1991, VPP-C018 / Vopsis3) [1]	AM503539	
	F420	Alma Bay, Magnetic I., Queensland, Australia (4.vi.1987, West 2827 / Vopsis7) [1, 4]	AM503540	AM498764
	F421	Yule Point, Queensland, Australia (West, 12.vi.1987, West 2834 / Vopsis8) [1, 4]	AM503541	AM498765
	F437	Dondra, Matara, Sri Lanka (Coppejans, 10.ix.2005, HEC15633b) [2]	AM503542	
Ventricaria ventricosa (J. Agardh) J.L. Olsen & J.A. West	F40	Matemwe, Zanzibar, Tanzania (Leliaert, 16.vii.2001, FL952) [2]	AJ544750	
	F243	St Croix (Kooistra, 1988, VV8 / Ventr5) [1]	AM503543	AM502590
	F308	San Blas Isl., Panama (Kooistra, 1998, VVPanF2 / Ventr3) [1]	AM503544	
	F310	Punaauia, Tahiti (N'Yeurt, 2005, Ventr12) [1]	AM503545	
Outgroup taxa				
Chaetomorpha crassa (C. Agardh) Kützing	F003	Mbudya Island, Tanzania (Leliaert, 11.vii.2001, FL908) [2]	AJ544767	AB062701
Cladophora albida (Nees) Kutzing	F516	W. Hokkaido, Japan (1985, A85.101 / Calb3) [1]	AM503433	Z35421
Cladophora herpestica (Montagne) Kützing	F517	Shimoda, Japan (van den Hoek, 1990, Cloz20b3SJ / Cherp3) [1]	AM503460	Z35419
Cladophora rupestris (Linnaeus) Kützing	F044	Boulogne, France (Leliaert, 2001) [2]	AJ544764	Z35319
Cladophora sericea (Hudson) Kützing	F518	Roscoff, France (1984, S84.35 / Cser1) [1]	AM503474	Z35320
Cladophora vagabunda (Linnaeus) van den Hoek	F519	Roscoff, France (1983, V83.17 / Cvaga3) [1]	AM503481	Z35316
Alignment			SSU: ALIGN_001141	LSU: ALIGN_001139

[1] Culture isolate from the algal culture collection of the University of Groningen (Netherlands), now maintained in the University of Ghent (Belgium).
[2] Voucher specimen deposited in the herbarium of the University of Ghent (Belgium) (GENT).
[3] Voucher specimen deposited in the National Herbarium of the Netherlands (Leiden University branch) (L).
[4] Culture isolate from the algal culture collection of John West (University of Melbourne, Australia), duplicates in the University of Ghent (Belgium).

Appendix A2

Illustration of the unstable root positions as determined by outgroup rooting in the partial LSU analyses.

Phylogenetic analyses performed on the partial LSU dataset including 166 ingroup plus 6 outgroup sequences showed that the branches connecting the outgroup taxa to the ingroup clade were extremely long compared to the interior ingroup branches, and that the position of the root of the ingroup clade was unstable and highly subject to the employed method of phylogenetic inference (BI, ME, NJ or MP) (Figs A-D). Rooting experiments with random outgroup sequences revealed identical root placements in the NJ, ME and unweighted MP analyses respectively (gray boxes in Fig. e), indicating that this unstable root positions could be attributed to a long branch artefact (Graham 2002). In the BI analyses, artificial (random) outgroups rooted the tree along the long branches of clades-1 or -9, depending on the model selected. In all analyses, ingroup topologies remained nearly unaltered by the inclusion of random outgroup sequences. Fig. E shows the internal phylogeny of the Siphonocladales (based on 166 partial LSU rDNA ingroup sequences) with indication of the different root positions as determined by outgroup rooting. The dark grey ellipses indicate the seven main clades; grade-2 is indicated by a lighter ellipse; number 3 indicates *Cladophora* sp. 1. White boxes show the different positions of the outgroup rooting with "real" outgroup sequences. Grey boxes indicate the root positions as determined by artificial, random sequences. PP are indicated along the branches.