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A RE-ASSESSMENT OF THE INFRA-GENERIC CLASSIFICATION OF THE GENUS *CAULERPA* (CAULERPACEAE, CHLOROPHYTA) INFERRED FROM A TIME-CALIBRATED MOLECULAR PHYLOGENY[1]

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The siphonous green algal family Caulerpaceae includes the monotypic genus *Caulerpella* and the species-rich genus *Caulerpa*. A molecular phylogeny was inferred from chloroplast *tuf*A and *rbc*L DNA sequences analyzed together with a five marker dataset of non-caulerpacean siphonous green algae. Six Caulerpaceae lineages were revealed, but relationships between them remained largely unresolved. A *Caulerpella* clade representing multiple cryptic species was nested within the genus *Caulerpa*. Therefore, that genus is subsumed and *Caulerpa ambigua* Okamura is reinstated. *Caulerpa* subgenus status is proposed for the six lineages substantiated by morphological characters, viz., three monotypic subgenera *Cliftonii*, *Hedleyi*, and *Caulerpella*, subgenus *Araucarioideae* exhibiting stolons covered with scale-like appendages, subgenus *Charoideae* characterized by a verticillate branching mode, and subgenus *Caulerpa* for a clade regarded as the *Caulerpa* core clade. The latter subgenus is subdivided in two sections, i.e., *Sedoideae* for species with pyrenoids and a species-rich section *Caulerpa*. A single section with the same name is proposed for each of the other five subgenera. In addition, species status is proposed for *Caulerpa filicoides* var. *andamanensis* (W.R. Taylor). All *Caulerpa* species without sequence data were examined (or data were taken from species descriptions) and classified in the new classification scheme. A temporal framework of *Caulerpa* diversification is provided by calibrating the phylogeny in geological time. The chronogram suggests that *Caulerpa* diversified into subgenera and sections after the Triassic-Jurassic mass extinction and that infra-section species radiation happened after the Cretaceous-Tertiary mass extinction.

Key index words: ***Caulerpa andamanensis* stat. nov.; *Caulerpa denticulata*; *Caulerpella*; chronogram; group IIA intron; molecular phylogeny; pyrenoid; *rbc*L; relaxed molecular clock; *tuf*A**

***Abbreviations*: AIC, Akaike information criterion; AICc, corrected AIC; *atp*B, beta subunit of the ATP synthase gene; BI, Bayesian Inference; BIC, Bayesian information criterion; BP, Bootstrap Percentage;**

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Ma, Mega-annum; ML, Maximum Likelihood; nt, nucleotide(s); PP, posterior probability; *rbc*L, large subunit of the D-ribulose 1,5-bisphosphate carboxylase-oxygenase gene; *tuf*A, elongation factor Tu gene

The Caulerpaceae Kützing (Bryopsidales, Chlorophyta) is a siphonous green algal family characterized by the presence of ubiquitous trabeculae (i.e., cell wall ingrowths) traversing the cell lumen to provide structural support. The thallus is differentiated into creeping stolons, downward growing rhizophores (with which it can anchor in soft substrate), and upright fronds termed assimilators that bear branchlets termed ramuli of various shapes (Weber-van Bosse 1898, De Senerpont Domis et al. 2003). This cosmopolitan tropical to temperate marine family currently includes two genera, i.e., the species-rich genus *Caulerpa* J.V. Lamouroux and the monotypic genus *Caulerpella* Prudhomme & Lokhorst. The latter genus was created to separate *Caulerpa ambigua* Okamura from the former on the basis of differences in reproductive structures (Prud'homme van Reine and Lokhorst 1992). In *Caulerpa,* the entire content of the vegetative plant divides up into reproductive cells to be released as gametes, resulting in the death of the thallus (i.e., holocarpy). *Caulerpella ambigua* (Okamura) Prudhomme & Lokhorst presumably survives gamete release by forming compound zoidangia on lateral branches cut-off from sterile parts of the thallus by a transverse cell wall (i.e., non-holocarpy). Vegetative, asexual reproduction by detached fragments is considered most common in *Caulerpa* (Prud'homme van Reine et al. 1996, Varela-Álvarez et al. 2012), but is unknown in *Caulerpella.*

Species of the genus *Caulerpa* exhibit a wide array of assimilator morphology and are renowned for their phentotypic plasticity (Peterson 1972, Calvert 1976, Ohba et al. 1992). This plasticity has resulted in an unstable classification of numerous varieties and forms. There are 360 species and infra-specific names in the online database *AlgaeBase* of which 87 species and 117 varieties and forms have been flagged as currently taxonomically accepted (Guiry and Guiry 2013). However, several recent molecular studies by Sauvage et al. (2013) and Belton et al. (2014) have shown the genus to have a taxonomy in need of revision. Species status is proposed for some varieties of taxa in the studies by Belton et al. (2014) and G.S. Belton et al. (unpublished data) although species cannot always be distinguished from each other based on morphology alone, and the authors suggested that it is likely that the best means to distinguish many *Caulerpa* species is through DNA sequence data.

Agardh (1873) subdivided the genus *Caulerpa* into thirteen tribes based on morphological similarities. However, these names were illegitimate because a tribe is a supra-generic rank. Agardh's names were validated by De Toni (1889) who used the rank of section. Weber-van Bosse (1898) recognized twelve of these sections, but considered the Opuntioideae J. Agardh *ex* De Toni as one of four series in the section Sedoideae J. Agardh *ex* De Toni. However, in a molecular phylogenetic study of interspecific relationships in the genus based on the chloroplast-encoded *tuf*A gene, Famà et al. (2002) found that most of these sections are polyphyletic. Their sampled *Caulerpa* species were divided into four clades of which two were monotypic; (i) Australasian endemic *Caulerpa flexilis* J.V. Lamouroux, (ii) *Caulerpa verticillata* J. Agardh, (iii) a clade comprised of species that have a pyrenoid associated with large chloroplasts and vesiculate ramuli with constricted pedicels (i.e., *C. cactoides* [R. Brown *ex* Turner] C. Agardh, *Caulerpa microphysa* [Weber-van Bosse] Feldmann, and *Caulerpa sedoides* C. Agardh [as *C. geminata* Harvey]), and (iv) a clade containing Caribbean *Caulerpa lanuginosa* J. Agardh and *C. paspaloides* (Bory de Saint-Vincent) Greville, and the remaining fifteen sampled *Caulerpa* species which grouped together in an internally largely unresolved crown clade. The crown clade taxa with vesiculate ramuli do not have constricted pedicels and do not contain pyrenoids. The analysis of Stam et al. (2006) revealed the same four *Caulerpa* clades as in Famà et al. (2002), and both studies used *Caulerpella ambigua* as outgroup in their *tuf*A analysis. However, in more recently published multi-locus molecular phylogenies of the Bryopsidales and Dasycladales (Verbruggen et al. 2009a,b), *Caulerpella ambigua* showed conflicting positions with respect to four sampled *Caulerpa* species. The simple diminutive siphon *Pseudochlorodesmis abbreviata* (Gilbert) Abbott & Huisman from Hawaii was revealed by Verbruggen et al. (2009b) as sister to the entire Caulerpaceae, thus representing the closest documented extant lineage to the family. The temperate waters of Southern Australia have been hypothesized to be the geographic origin of the genus (Calvert et al. 1976), but relaxed molecular clock models calibrated with the fossil record (Verbruggen et al. 2009a) indicate that the Caulerpaceae lineage split from the other Halimedineae lineages in the Carboniferous or Permian when southern Australia was still attached to Antarctica (Hommersand 2007). *Pseudochlorodesmis* was, however, not included by Verbruggen et al. (2009a) and would have shortened the branch leading to *Caulerpa.*

This study aims to investigate the deeper phylogenetic relationships within the Caulerpaceae using a wider sampled outgroup and a longer alignment than in Famà et al. (2002) and Stam et al. (2006), as well as a wider sampled ingroup than in Verbruggen et al. (2009a,b), using chloroplast-encoded *tuf*A and *rbc*L gene sequences. In addition it aims to provide for the first time a temporal framework of caulerpacean diversification by calibrating the phylogeny in geological time. The inferred phylogenetic chronogram (i.e., timetree) is subsequently

used to revise the subdivision of the family by giving equal rank to clades equivalent in time. The earlier hypothesized geographic origin of *Caulerpa* is discussed on the basis of the timetree, which may illuminate causal geological events and processes in the history of life (Avise 2009).

MATERIALS AND METHODS

Taxon sampling and sequencing. For this study, diverse caulerpacean collections were gathered mainly from two of the main Caulerpaceae biodiversity centers (Australia and Southeast Asia) and included a number of representatives previously unsequenced (e.g., *C. agardhii* Weber-van Bosse, *C. elongata* Weber-van Bosse, *Caulerpa filicoides* Yamada). Some species are new records for Indonesia, Malaysia, or Palau (indicated in Table S1 in the Supporting Information). The traditional twelve sections and four series are each represented by at least two species, except for the Zosteroideae J. Agardh *ex* De Toni. The Zosteroideae originally contained *Caulerpa filiformis* (Suhr) Hering and *C. flagelliformis* C. Agardh. Newly collected specimens were identified based on references from literature as well as examination of type specimens. Specimen vouchers used in the studies by Stam et al. (2006) and Famà et al. (2002) were also re-examined, although not all specimens of the latter study were available (indicated in Table S1 in the Supporting Information). In addition, new collections of the Caulerpaceae sister-clade *Pseudochlorodesmis* were made.

Genomic DNA was extracted from silica dried or herbarium dried algal tissue using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions or was outsourced to AGRF (Australian Genome Research Facility, Adelaide Node, SA, Australia). Double-stranded *tuf*A amplifications were performed in 25 μL following Stam et al. (2006) using the tufAF (5′-TGAAACAGAA MAWCGTCATTATGC-3′; Famà et al. 2002) and tufAR1 (5′-CCATAGGAATTGGACTATCA-3′; Stam et al. 2006) forward and reverse primers. A few samples (indicated in Table S1) were amplified with the newly designed reverse primer tufA652R (5′-GAGTATGGGGTGTAATAGAT-3′) resulting in 164 nucleotides (nt) shorter fragments. Amplification products were purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA), the NucleoSpin Extract II kit (Machery-Nagel, Düren, Germany), or the LaboPass Gel and PCR Clean-up Kit (Cosmo Genetech, Seoul, Korea) following the manufacturer's instructions. Purified PCR products were sent to Macrogen (Seoul, Korea) or First BASE Laboratories Sdn Bhd (Seri Kembangan, Malaysia) for sequencing using the amplification primers. A few samples were extracted, amplified, and sequenced at the Centre for Environmental and Molecular Algal Research (University of New Brunswick, Fredericton, NB, Canada) following Saunders and Kucera (2010). Partial *rbc*L sequences were determined for a subset of the samples. *Rbc*L was amplified as two overlapping fragments of, respectively, 633 and 651 nt using the primer combinations CR-F/CR-mR and CR-mF/CR-R which were designed for this study, the latter fragment on the downstream side of the former. Primer CR-F anneals 26 nt after the intron reported in two *Caulerpa* species in Hanyuda et al. (2000). Primer sequences are CR-F 5′-CTGGWGRSA WAATCARTATATTGC-3′, CR-mF 5′-GGACATTATTTAAAT GCWACTGC-3′, CR-mR 5′-CAATAACAGCATGCATWGCAC G-3′, and CR-R 5′-AGGACTCCATYKAGCAGCATCACG-3′. Both fragments were amplified in 25 μL reaction volumes using the *i*-Taq *plus*DNA Polymerase kit (iNtRON Biotechnology, Seongnam-Si, Korea) and applying the general reaction mixture recommended by the manufacturer. An initial denaturation step of 94°C for 2 min was followed by 10 cycles of 20 s at 94°C 1 min at 45°C, and 2 min at 72°C, and then 25 cycles of 20 s at 94°C, 30 s at 48°C, and 2 min at 72°C. The amplification was ended with a final step of 72°C for 8 min. *Rbc*L PCR products (in case of low yield multiple reactions were pooled) were prepared for sequencing in the same way as the *tuf*A amplifications. The sequence of the two *rbc*L fragments combined was 1,039 nt in length excluding the CR-F and CR-R primers sites and encompasses the nt positions 297–1,335 in a typical green algal *rbc*L gene of 1,428 nt (e.g., GenBank AB260909). For some specimens only the CR-mF/CR-R amplifications were successful (indicated in Table S1). The chromatograms were assembled and edited as described in Draisma et al. (2010a,b).

Dataset assembly and model selection. In addition to 150 newly generated sequences, *tuf*A and *rbc*L sequences representing *Caulerpella* and *Caulerpa* species were downloaded from the GenBank/EMBL database. Only a selection of the sequences representing the *Caulerpa* crown clade was used for analysis to represent high diversity but low sequence redundancy. Some Genbank sequences representing non-crown taxa were also excluded from analyses. The *rbc*L sequences of *Caulerpa brownii* (C. Agardh) Endlicher (GenBank EU380530) and *C. verticillata* (EF583684) were excluded because they were short and largely outside the alignment of this study. The *C. filiformis rbc*L sequence AY004763 was excluded because it is a chimera of *C. filiformis* (nt 1–605) and a member of the angiosperm order Poales (nt 606–1,356). The *Caulerpa flexilis* J.V. Lamouroux *rbc*L sequence AJ512485 was left out because it is identical to that of *Caulerpa okamurae* Weber-van Bosse AB038484. Moreover, these four species were already represented by other specimens. All *Caulerpa, Caulerpella,* and *Pseudochlorodesmis* taxa used in this study are listed in Table S1. *Tuf*A and *rbc*L sequences were aligned separately by eye in the BioEdit Sequence Alignment Editor v.7.2.1 (Hall 1999). Identical or nearly identical sequences were

TABLE 1. Selection of partitioning strategy using the AIC, AICc, and BIC.

lnL	# parameters	# partitions	AIC	AICc	BIC	Partition scheme
−57273.62	227	1	115,001.24	115,020.56	116,505.84	(12345)
−56295.111	454	2	113,498.22	113,578.73	116,507.43	(123) (45)
−54303.861	454	2	109,515.72	109,596.23	112,524.92	(3) (1245)
−53891.12	681	3	109,144.24	109,333.62	113,658.04	(12) (3) (45)
−53635.417	908	4	109,086.83	109,439.71	115,105.24	(1) (2) (3) (45)
−53485.99	1,135	5	109,241.98	109,821.34	116,764.98	(1) (2) (3) (4) (5)

The log-likelihood, number of parameters and the three criterion scores are listed for six partitioning strategies. Lower criterion score values indicate a better fit of the model to the data. Light gray indicates the best scoring for each criterion, darker gray the second best scoring. The best model for all partitions was GTR+G+I. In the partition scheme column 1 = 1st codon position of protein-coding gene, 2 = 2nd codon postition, 3 = 3rd codon position, 4 = 16S cp rDNA, and 5 = 18S nrDNA.

pruned from the dataset (indicated in Table S1). The two aligned markers were then concatenated and incorporated in the five markers (plastid-encoded *tuf*A, *rbc*L, *atp*B, and 16S rDNA and nuclear 18S rDNA) dataset of Verbruggen et al. (2009a; table 1) comprising five Ulvophyceae (outgroup), seventeen Dasycladales, and 34 Bryopsidales. Five *Pseudochlorodesmis* taxa, three *Caulerpella* taxa, and 46 *Caulerpa* taxa were selected to be analyzed together with the five markers dataset of 56 non-caulerpaceans. Five *Caulerpa* taxa were represented by *tuf*A and *rbc*L sequences from different individuals, namely *C. lentillifera* J. Agardh, *C. paspaloides, Caulerpa prolifera* (Forsskål) J.V. Lamouroux, *C. scalpelliformis* var. *denticulata* (Decaisne) Weber-van Bosse, and *C. taxifolia*. Eight taxa were represented only by *tuf*A, namely *C. cactoides, C. fastigiata, C. lanuginosa, C. manorensis* Nizamuddin, *Caulerpella ambigua*-3, and three *Pseudochlorodesmis* spp. Table S1 indicates which *tuf*A and *rbc*Lsequences were used in the analysis with the five markers dataset of non-caulerpaceans.

Model testing was performed in PartitionFinder (Lanfear et al. 2012) to determine the best models and partitioning strategy according to the selection criteria Akaike information criterion (AIC), corrected AIC (AICc) and Bayesian information criterion (BIC). The PartitionFinder analysis pointed to a three partitions scheme: (i) 1st + 2nd codon positions of protein-coding genes, (ii) 3rd codon positions, and (iii) rDNA. A General Time Reversible model (GTR, Yang 1994) along with among-sites rate heterogeneity (G) and an estimated proportion of invariable sites (I) was selected as best model for all three partitions. This partitioning strategy scored very closely to the four partitions scheme adopted in Verbruggen et al. (2009a) in which 1st and 2nd codon positions represented separate partitions rather than a single one. The three partitions scheme was favored here considering the greater support among the three criteria AIC, AICc, and BIC (Table 1).

Phylogenetic analyses. Maximum Likelihood (ML) estimation was performed in RAxML v. 7.2.8 (Stamatakis 2006) with the Ulvophyceae as outgroup and with model and partitioning scheme determined as above. Branch support was assessed with non-parametric bootstrapping of 1,000 replicates (Felsenstein 1985). ML bootstrap percentages (BP) were considered as strong (80%–100%), moderate (70%–79%), weak (50%–69%) or no (<50%) support.

Bayesian inference (BI) was performed with the BEAST package v. 1.4 (BEAST, BEAUti and LogCombiner; Drummond et al. 2006, Drummond and Rambaut 2007), which was also used to produce a time-calibrated phylogeny (chronogram, timetree). Three Markov Chain Monte Carlo chains of 40,000,000 generations (with logging every 4,000 generations) were run independently from a randomly generated starting tree under an uncorrelated lognormal relaxed clock and Yule speciation process. To produce a chronogram, the age (in Ma) of six well supported nodes were input as priors. Ages were set to the mean (μ) and standard deviation (σ) obtained from a normal distribution matching the 95% confidence intervals (CI) reported in Verbruggen et al. (2009a). The six calibrated nodes (indicated in Fig. 1) were (A) the node where the Dasycladales diverge from the Bryopsidales ($\mu = 571$, $\sigma = 30$, 95% CI = 521.7–620.3), (B) the node where Dasycladales diversify ($\mu = 458$, $\sigma = 25$, 95%CI = 416.9–499.1), (C) the node where *Ostreobium* sp. splits from the other Bryopsidales ($\mu = 479$, $\sigma = 20$, 95% CI = 446.1–511.9), (D) the node where the Bryopsidineae diversify ($\mu = 351$, $\sigma = 32$, 95% CI = 298.4–403.6), (E) the node where the Halimedineae diversify ($\mu = 391$, $\sigma = 20$, 95% CI = 358.1–423.9), and (F) the divergence point of the core Halimedineae ($\mu = 303$, $\sigma = 25$, 95% CI = 261.9–344.1). The traces of trees –lnL values from the three independent runs were visualized in Tracer v. 1.5.0 (Rambaut and Drummond 2009) revealing rapid chain convergence, and high run quality (high Effective Sampling Size values). The default 10% burnin period was thus appropriate, and the logs of runs were then combined in LogCombiner, resulting in the exclusion of the first 4,000,000 generations representing the first 1,000 trees from each run. A maximum clade credibility chronogram with mean node heights was calculated from the set of post-burnin trees with TreeAnnotator v.1.6.1 (Rambaut and Drummond 2010). BI posterior probability (PP) values 0.95–1.00 were considered as strong support, values 0.90–0.94 as weak support, and values <0.90 as no support.

Morphological examination. All currently accepted *Caulerpa* species (Table S2 in the Supporting Information) were examined for the presence of pyrenoids associated with the chloroplasts (visible under light microscope after Lugol's iodine stain), assimilators with or without constricted rachis, presence of constricted ramuli pedicels, rhizoids on stolons, and scale-like appendages on stolons. When a species was not available for examination these data were taken, if possible, from the literature description of the species.

RESULTS

Sequence alignment and model selection. EMBL accession numbers of newly generated sequences are given in Table S1. We generated 89 new *tuf*A sequences representing two *Pseudochlorodesmis* spp., three *Caulerpella* spp., and 33 *Caulerpa* spp. (nine representing the crown clade). Alignment was unambiguous for *tuf*A, but gaps to restore alignment were needed in the *tuf*A of *Caulerpa scalpelliformis* (R. Brown *ex* Turner) C. Agardh (three positions), *Caulerpa papillosa* J. Agardh (six positions) and *Caulerpella ambigua* (nine positions). The final *tuf*A alignment was 882 nt in length. We generated 61 new *rbc*L sequences representing one *Pseudochlorodesmis* sp., two *Caulerpella* spp., and 32 *Caulerpa* spp. (ten representing the crown clade). Alignment of the *rbc*L sequences was also straightforward (final alignment was 1,384 nt) after removal of introns found in two specimens. The CR-F/CR-mR PCR fragment of *Caulerpa fergusonii* PERTH 6.10.9.27 contained a 638 nt intron between nt positions 612–613 (based on 1,428 nt complete *rbc*L), which was submitted to EMBL/GenBank separately (accession number FR848361). The secondary structure of the 638 nt intron of *C. fergusonii* G. Murray (specimen PERTH 6.10.9.27) was predicted using the program mfold 3.4 (Zuker et al. 1999) on The mfold Web Server (http://mfold.rna.albany.edu/) of the University at Albany, USA. The predicted secondary structure (Fig. S1 in the Supporting Information) had a group IIA intron structure with six recognizable domains (Bonen and Vogel 2001, Dai et al. 2003). *Caulerpa brownii* specimen L 09.10.057 also contained an intron at the same position, but its sequence was not completely determined because of its great length estimated at ~3,300–3,400 nt by electrophoresis on a 2% agarose gel. Respectively, 693 nt of the 5′-end (FR848362) and 628 nt of the 3′-end (FR848363) were determined. The first 553 nt of the 638 nt *C. fergusonii* intron were alignable with

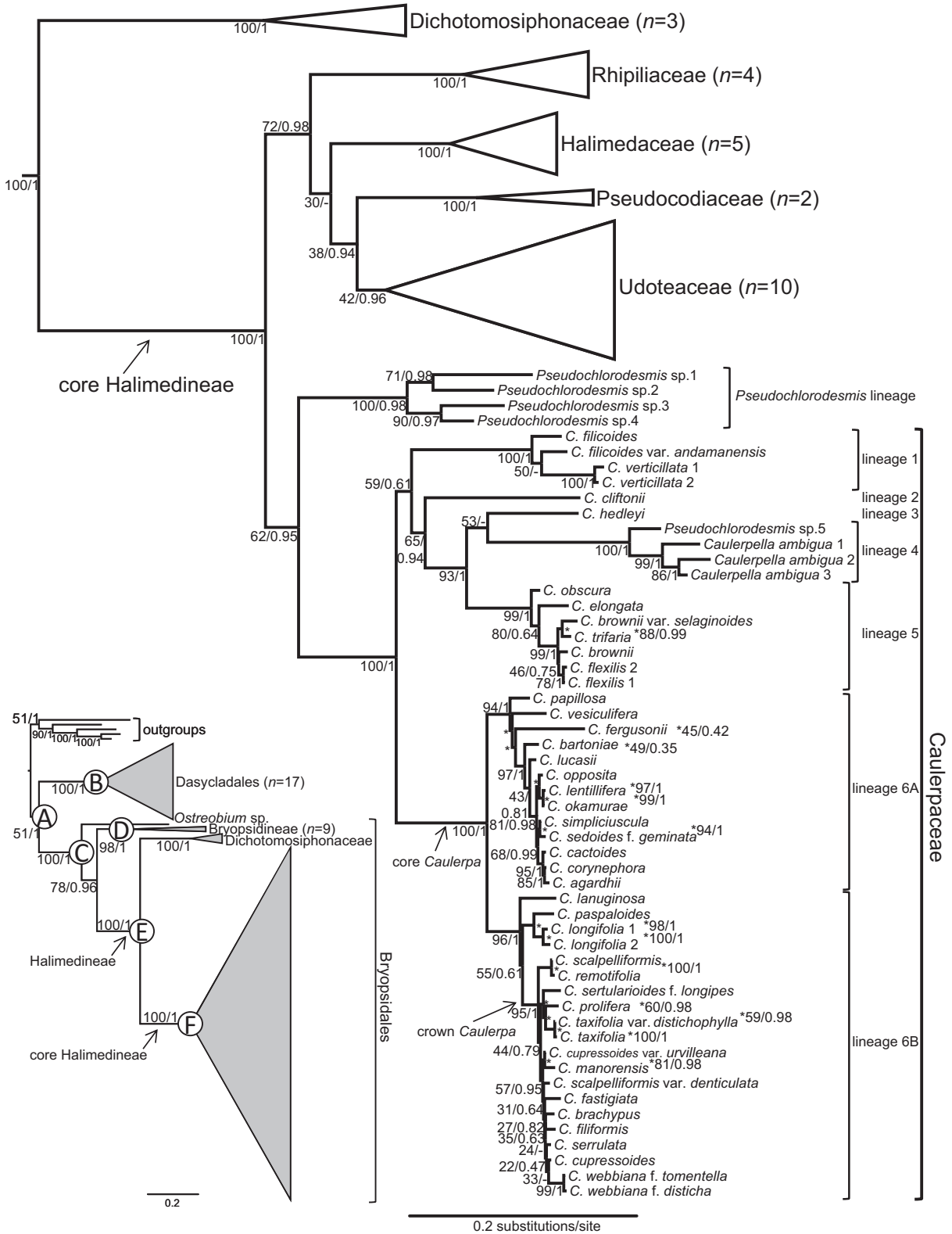

FIG. 1. Five markers Maximum Likelihood (ML) tree of 110 taxa. Only the Caulerpaceae and its sister-clade are shown in detail. The other Halimedineae families are summarized and all other taxa are pruned from the tree. A summary of the complete tree is shown in the lower left corner and the original tree in Figure S3. Branch support values (ML bootstrap percentage/Bayesian Inference [BI] posterior probability) are given near nodes (or right from taxon labels in case of insufficient space near the node, indicated with *). A dash (-) indicates that the branch does not occur in the BI tree. Seven Caulerpaceae lineages (1-6A and B) discussed in the main text are indicated right from the tree. Calibration points (encircled letters A–F) for the chronogram in Figure 2 are indicated in the summarized tree. *C.* = *Caulerpa*.

the 5′-end of the *C. brownii* intron (24 substitutions and three indels) and the last 85 nt (554–638) with the 3′-end (1 substitution). It is also a group IIA intron (Fig. S2 in the Supporting Information).

Phylogeny of the Caulerpaceae. Figure 1 shows the five markers ML tree of the Dasycladales and Bryopsidales with five Ulvophyceae outgroup taxa (110 taxa in total). The outgroup, *Ostreobium* sp., Dasycladales, and Bryopsidineae are pruned from the tree and the Halimedineae families are summarized except for the *Pseudochlorodesmis* clade (*incertae sedis*) and the Caulerpaceae clade, which are shown in detail. The complete tree is summarized in the lower left corner of Figure 1 and shown in full in the Figure S3 in the Supporting Information. ML boostrap percentages (BP) are plotted on the topology of the trees (Fig. 1 and Fig. S3) as well as the BI PP from the BI analysis (not shown) of the same dataset. The ML and BI trees were in general agreement, revealing the same main clades and only differed in a few unsupported topology differences within the main clades. Pair-wise phylogenetic distances, i.e., branch lengths between taxa, were derived from the ML tree and plotted in the Table S3 in the Supporting Information and their frequency distribution is shown in Figure S4 in the Supporting Information. A pilot analyzing the *tuf*A and *rbc*L alignments separately revealed the same Caulerpaceae main clades in the *tuf*A tree and *rbc*L tree (not shown).

Four *Pseudochlorodesmis* specimens consistently formed a sister-clade to the Caulerpaceae. *Pseudochlorodesmis* sp. 5, however, was nested inside the Caulerpaceae and sister to a *Caulerpella ambigua* clade with maximum support. Six main clades can be discerned within the Caulerpaceae and these are indicated as lineages 1-6 in Figure 1. Maximum supported lineage 6 splits into two strongly supported lineages 6A and 6B. Lineage 6B includes a strongly supported *Caulerpa* crown clade. Taxa in Table S1, but not included in Figure 1 and Figure S1, could each be assigned to one of the Caulerpaceae lineages based on the pilot analysis and this is indicated in Table S1. *Tuf*A sequences were not able to differentiate *C. lentillifera* from *C. microphysa* and *C. matsueana* Yamada from *C. opposita* Coppejans & Meinesz (no *rbc*L data of *C. microphysa* and *C. matsueana*). *C. filicoides* var. *filicoides* and *C. filicoides* var. *andamanensis* W.R. Taylor differed by 35 of 744 nt in *tuf*A (4.7%) and 21 of 604 nt in *rbc*L (3.5%). *C. verticillata* 1 and *C. verticillata* 2 differed by a minimum of 12 of 786 nt in *tuf*A (1.5%) and 9 of 604 nt in *rbc*L (1.5%). *Caulerpa scalpelliformis* is clearly not monophyletic. Typical *C. scalpelliformis* and *C. scalpelliformis* var. *denticulata* differ by 23 and a 3 nt indel of 820 nt in *tuf*A (2.9%) and 15 of 663 nt in *rbc*L (2.3%). *Caulerpa brownii* is seemingly not monophyletic. Australian *C. brownii* and New-Zealandish *C. brownii* var. *selaginoides* J. Agardh differ by 17 of 632 nt in *tuf*A (2.7%) and 10 of 604 nt in *rbc*L (1.7%). Sequence divergence within the *Caulerpella ambigua* clade (lineage 4 excluding *Pseudochlorodesmis* sp. 5) is 6.7% in *tuf*A and 4.7% in *rbc*L.

A chronogram of the Caulerpaceae phylogeny with estimated node ages is shown in Figure 2. According to this timetree the Caulerpaceae probably diverged from their sister-clade *Pseudochlorodesmis* during the Paleozoic. The main lineages within the Caulerpaceae were formed in the first half of the Mesozoic and most diversification within these lineages took place during the Cenozoic.

Morphological observations. The morphology of 99 *Caulerpa* species was examined and the observations are reported in Table S2 ordered by phylogenetic lineage.

DISCUSSION

The Caulerpaceae phylogeny. The analysis of the *tuf*A gene and the *rbc*L gene both support the existence of six main lineages in the Caulerpaceae. De Senerpont Domis et al. (2003) mentioned briefly the incongruence between *tuf*A and *rbc*L in *Caulerpa*, but this incongruence was probably caused by the *rbc*L sequence that represented *C. flexilis* (lineage 5) that actually belonged to *C. okamurae* (lineage 6A). The combined analysis of *tuf*A and *rbc*L (in a five marker alignment, Fig. 1 and Fig. S1) resulted in higher support values than when the genes were analyzed separately. Lineages 2 and 3 are both monotypic and revealed here for the first time. Lineages 1, 4, 5, 6A, and 6B were also revealed by Famà et al. (2002) and Stam et al. (2006), but their phylogenies included only a single representative for each of the lineages 1, 4, and 5. Within lineage 6B, *C. lanuginosa*, *C. paspaloides*, and *C. longifolia* do not belong to the strongly supported species-rich *Caulerpa* crown clade. *C. longifolia* was not included in the studies by Famà et al. (2002) and Stam et al. (2006). Relationships between the six lineages are largely unresolved. Lineage 6 is sister to a weakly supported (ML BP 59) or unsupported (BI PP 0.61) clade comprising the other five lineages. The support for the clade comprising lineages 2–5 is weak (ML BP 65, BI PP 0.94). Only the clade with lineages 3–5 gains strong support (ML BP 93, BI PP 1.00). Lineage 3 is sister to lineage 4 in the ML tree (BP 53, Fig. 1) and to lineage 5 in the BI tree (PP 0.53, Fig. 2). The latter hypothesis is most likely on morphological grounds. *Caulerpa hedleyi* (lineage 3) and the members of lineage 5 have stolons covered in scaly appendages. It is clear that more DNA markers need to be added to the *Caulerpa* alignment to resolve phylogenetic relationships between the deeper lineages of the Caulerpaceae as well as relationships within some of these lineages, notably the *Caulerpa* crown clade for which a more variable marker is needed.

The origin of the genus Caulerpa *in place and time.* Calvert et al. (1976; fig. 20) illustrated a hypothetical

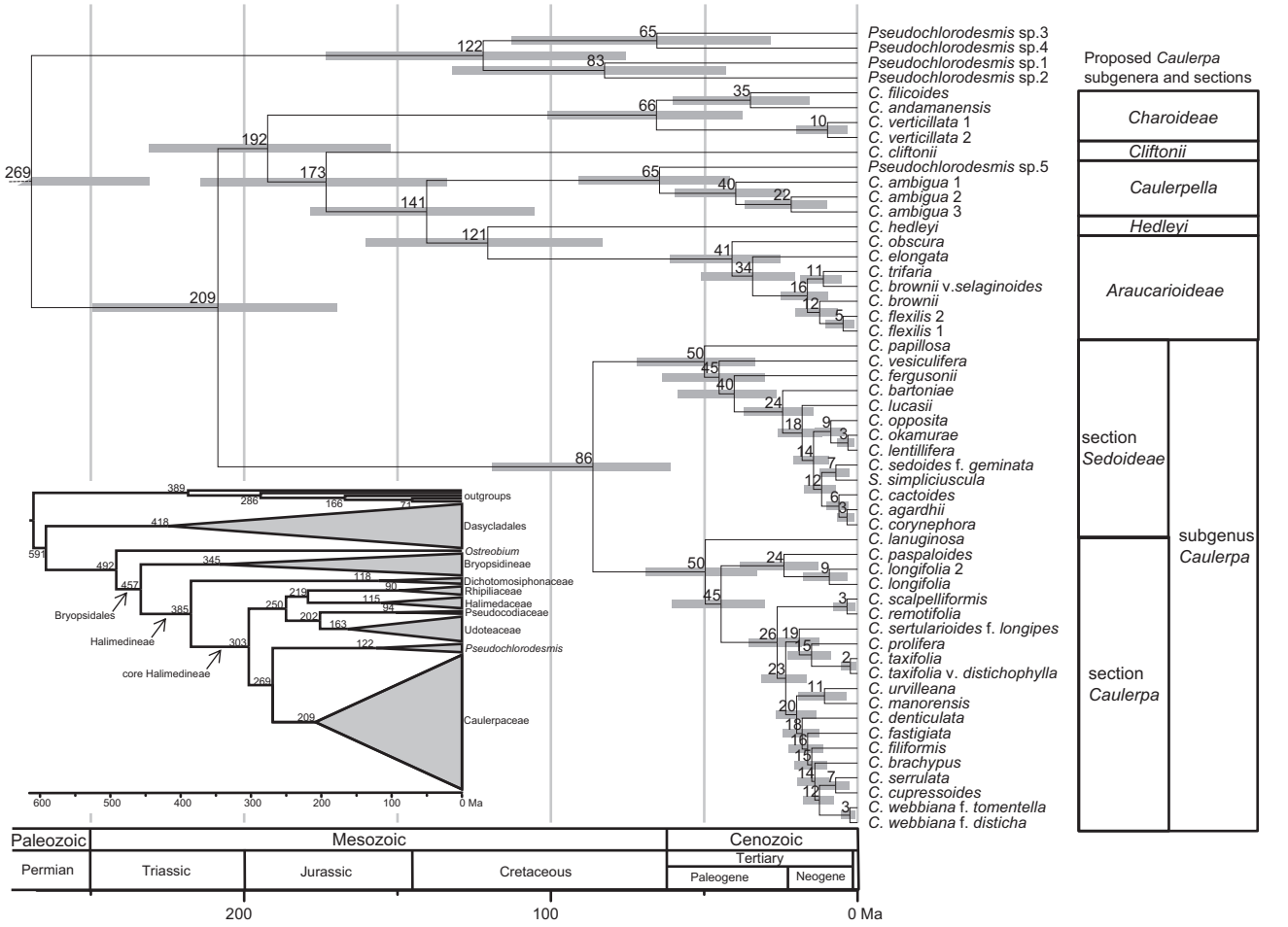

FIG. 2. Chronogram of the Caulerpaceae (all other taxa except for its sister-clade were pruned). Node ages were inferred using Bayesian inference assuming a relaxed molecular clock and a set of node age constraints derived from a chronogram in Verbruggen et al. (2009a) that was calibrated with data from the fossil record. Values at nodes indicate average node ages (in Ma) and gray bars represent 95% confidence intervals. The calibration points used for this analysis are A–F in Figure 1 and explained in the Materials & Methods section. The complete chronogram based on 110 taxa is summarized in the lower left corner including node ages, but without confidence intervals. Major geological eras are indicated along the timescale bar at the bottom. The summarized chronogram has its own timescale. A newly proposed subgeneric classification of the Caulerpaceae is shown right from the chronogram.

scheme for the evolutionary development of the chloroplast in *Caulerpa* and a corresponding phylogenetic tree of the generic sections generated from it. They presumed that the large pyrenoid-containing chloroplast in all but one of their sampled *Sedoideae* was "the most primitive" and speculated that southern Australia, being the apparent center of distribution of pyrenoid-containing species, may also be the geographic origin of *Caulerpa*. However, the pyrenoid-containing chloroplast is not the ancestral type according to the results of this study and appears relatively recent in geological time (nested inside lineage 6A, Fig. 2). The oldest fossil attributed to *Caulerpa* was recovered from the Palo Duro Basin in Texas and dated 280 Ma old (Gustavson and Delevoryas 1992). It resembles the extant species *Caulerpa racemosa* var. *clavifera* (Turner) Weber-van Bosse, but the placement of vesiculate ramuli in the fossil is more regular than in extant *Caulerpa* species with vesiculate ramuli all around the rachis. The phylogenetic chronogram in Figure 2 must be interpreted with great caution as it is calibrated with node ages taken from a timetree that was calibrated with fossils of non-caulerpaceans (fig. 4 in Verbruggen et al. 2009a). The Caulerpaceae tree was essentially grafted into the Dasycladales-Bryopsidales tree. Although the 280 Ma old Palo Duro Basin fossil falls within the 95% confidence interval of the split of the Caulerpaceae from *Pseudochlorodesmis* (indicated by the gray bar around the node at 269 Ma in Fig. 2), the morphology displayed by the fossil seems temporally incongruent since extant *Caulerpa* species with vesiculate ramuli are only found within lineages 6A and 6B, which diversified much later. The validity of this fossil as belonging to the Caulerpaceae is thus questionable, but the possibility that it is indeed a *Caulerpa* cannot be excluded. No other extant macroalgal taxon resembles the morphology of the fossil. Yi et al. (2014) interpreted the

non-calcified thallophytic fossil alga *Menieria minuta* Wang, Jin *et* Zhan from the Lower Silurian (Middle Aeronian, 440 Ma) of eastern Canada (tropical at that time) as *Caulerpa*-like on the basis of branch morphology and attachment structure, but it does not resemble any extant *Caulerpa* species. According to the timetree (Fig. 2), the Caulerpaceae split from the sister-clade *Pseudochlorodesmis* sometime in the late Carboniferous, the Permian, or early Triassic (the 95% confidence interval bar around the node at 269 Ma spans this time-frame). In the Triassic, the supercontinent Pangaea had not yet started to break up, southern Australia was still connected to the Antarctic plate, and the Atlantic Ocean had not yet formed, suggesting a Tethyan origin of *Caulerpa* along the eastern shores of Pangea. The Palo Alto Basin was on the West coast of Pangea. The tropical East Pacific is poor in *Caulerpa* species with only six confirmed species and no endemics (C. Fernández-García et al., unpublished data). During the Triassic, the Tethys Sea was at that time divided by the Cimmerian superterrane into a Paleotethys (North) and a Neotethys (South; Dèzes 1999). Both the Paleotethys and Neotethys were tropical and the extant species of the sister-clade of the Caulerpaceae are only known from the tropics. The other Halimedineae also have a predominantly tropical distribution. The Caulerpaceae lineages 1 and 4 are exclusively tropical, whereas the monotypic lineages 2 and 3 only occur in temperate Australia. Lineage 5 consists of temperate Australasian species with the exception of *C. elongata* which occurs in the tropical Indo-Pacific. Lineages 6A and 6B both contain tropical, temperate, and tropical-temperate species.

A diversification of the genus *Caulerpa* into at least six lineages during the late Triassic to early Cretaceous is congruent with the rediversification of life after the Permian-Triassic (251.4 Ma ago) and Triassic-Jurassic (199.6 Ma ago) mass extinction events in which the majority of marine life on Earth perished (Benton 2003, Tanner et al. 2004). *Caulerpa* may have diverged into more than six lineages during this period, but the extant six *Caulerpa* lineages are the surviving *Caulerpa* lineages of the Cretaceous-Tertiary/Paleogene (K-T) extinction event (65.5 Ma ago). Species radiation within the six lineages took place after the K-T extinction, resulting in the present day *Caulerpa* diversity. Species richness is highest in lineage 6B with more than fifty currently accepted species (Table S2) which is more than 60% of the total number of extant *Caulerpa* species. This is the first study that gives us a sense of the age of the *Caulerpa* lineages. Although, the genus appears to be ancient, most species radiations appear to be of relative recent date. A similar scenario was found by Verbruggen et al. (2009c) in the genus *Halimeda* J.V. Lamouroux (Halimedaceae, Bryopsidales) where five main lineages (given the rank of *Halimeda* sections) evolved during the Cretaceous and diverged within the last 65 Ma. *Halimeda* is probably of tropical origin and in one of the five sections colonized temperate waters multiple times during global cooling in the Paleogene-Neogene. However, it rather seems that in *Caulerpa* lineages 5 and 6 a colonization from temperate to tropical waters happened. The other four lineages are either exclusively tropical (species poor lineages 1 and 4) or exclusively temperate (monotypic lineages 2 and 3). All species in lineage 5 are endemic to temperate Australasia, except for *C. elongata*, which has a tropical Indo-West-Pacific distribution. The eight tropical taxa within lineage 6A are monophyletic and nested within the clade (node age of 14 Ma in Fig. 2) indicating that they evolved from temperate species. Within the *Caulerpa* crown clade the two temperate species *C. scalpelliformis* and *C. remotifolia* are together sister to the rest of the crown clade which contains temperate and tropical taxa. About half of the species of the crown clade occurs in the Indo-Malay archipelago, the biodiversity hotspot of *Caulerpa* diversity, albeit with a low level of endemism (Prud'homme van Reine et al. 1996). Their evolution in the early neogene coincides with the time that this region became a hotspot of marine biodiversity (Renema et al. 2008). Perhaps tropical species moved to lower latitudes during global cooling, whereas extant temperate species are the descendents of species that did not move, but adapted to cooler temperatures.

On the validity of some Caulerpa *species.* Although challenging, the validity of *Caulerpa* species was not the aim of this study, the present authors take the view that a taxonomic revision should be proposed if it is supported by the collected data. Saunders and Kucera (2010) proposed to adopt *tuf*A as the universal DNA barcode marker for marine green macro-algae (with the exception of the Cladophoraceae) because it showed the largest difference between maximum intra- and minimum interspecific divergence of six tested markers. The 3′-end *rbc*L also showed a large barcode gap, but had moderate amplification success, caused, at least in part, by the presence of introns in some taxa, hence reducing its utility as barcode. However, taxon sampling by Saunders and Kucera (2010) focused on the genus *Ulva* L. and only five bryopsidaleans were included in the study (one *Bryopsis* and four *Codium*). Sauvage et al. (2013) used *tuf*A as a barcode to differentiate between species of the *C. racemosa*–*C. peltata* complex, but did not demonstrate a *tuf*A barcode gap for *Caulerpa*. However, if two true biological species are considered to be a single morphological species, then the observed maximum intraspecific variation will be greater than the observed interspecific variation (unless they are sister-species). Within the *Caulerpa* crown clade phylogenetic resolution is limited and the monophyly of some morphologically well-defined species is not resolved. For example, *C. cupressoides* is nested within *C. serrulata* (Forsskål) J. Agardh in Sauvage

et al. (2013; fig. 2), making the latter paraphyletic. In combination with *rbc*L, *tuf*A still cannot resolve relationships within the crown clade (Fig. 1). The fact that DNA sequences could not differentiate morphological species *C. matsueana* from *C. opposita* and *C. lentillifera* from *C. microphysa* suggests that they may be conspecific. This possibility needs further investigation (T. Sauvage, unpublished data). In this study two morphological varieties of a species are considered two distinct species if each variety forms a monophyletic clade by itself and these two clades are not sister-clades. Two genetically and morphologically distinct taxa living in sympatry can be reasoned as additional support for non-conspecificity, especially when one taxon or both taxa remain genetically uniform over large distances. *C. scalpelliformis* (from Australasia) is sister to *C. remotifolia* Sonder with maximum support (Fig. 1) and differs, respectively, 2.9% (*tuf*A) and 2.3% (*rbc*L) from *C. scalpelliformis* var. *denticulata* (from the western Indian Ocean, the Atlantic and the eastern Mediterranean). It is proposed here to reinstate *C. denticulata* Decaisne for the latter taxon. *C. denticulata* (type location the Red Sea) differs from *C. scalpelliformis* f. *typica* (type from southern Australia) in having wider (often overlapping), but less elongate, ramuli with denticulated margins. *Caulerpa scalpelliformis* var. *intermedia* (Decaisne) Weber-van Bosse has ramuli, with often denticulate margins, which are generally longer and less wide than in *C. denticulata*. It also occurs in the western Indian Ocean, therefore, it is considered a variety of *C. denticulata* rather than of *C. scalpelliformis*.

Caulerpa cupressoides var. *urvilleana* (Mont.) Coppejans & Prud'homme *ex* L.M. Hodgson, P.H. Tri, K. Lewmanomont & K.J. McDermid is sister to *C. manorensis* with strong support (ML BP 81, BI PP 0.98, Fig. 1) and in Sauvage et al. (2013) and Belton et al. (2014) it is sister to *C. chemnitzia* (Esper) J.V. Lamouroux (a taxon not sampled in this study). Typical *C. cupressoides* and *C. cupressoides* var. *urvilleana* occur in sympatry in the Indo-West-Pacific, whereas Caribbean and Indo-Pacific *C. cupressoides* are monophyletic. The present authors are in possession of more (unpublished data) DNA sequences of the variety *urvilleana* from specimens collected in Indonesia, Malaysia, and Palau. It is proposed here to reject *C. cupressoides* var. *urvilleana* (Montagne) Coppejans & Prud'homme *ex* L.M. Hodgson et al. and to reinstate *C. urvilleana* Montagne.

N'Yeurt and Payri (2007) questioned whether *C. elongata* Weber-van Bosse and *C. webbiana* Montagne might be ecomorphs of a single species. This study shows that they are clearly not conspecific and not even closely related. Indo-Pacific *C. elongata* belongs to lineage 5 and *C. webbiana* (from both the Atlantic and Indo-Pacific) belongs to lineage 6B. In *C. webbiana* the apical part of the stolon is distinctly naked, while in *C. elongata* appendages develop close to the growing tip of the stolon. *Caulerpa pickeringii* Harvey & Bailey (flagged as current in *AlgaeBase*) is a synonym of *C. webbiana* var. *pickeringii* (Harvey & Bailey) Eubank, for which there is a *tuf*A sequence available in Genbank (AJ417966). That *tuf*A sequence is identical to that of *C. webbiana* f. *tomentella* (Harvey *ex* J. Agardh) Weber-van Bosse (FM956074), which supports the view that *C. pickeringii* is conspecific with *C. webbiana*. However, there exists no voucher of the specimen that was used to obtain sequence AJ417966 to verify its correct identification.

C. brownii var. *selaginoides* J. Agardh is the sister taxon of *C. trifaria* with strong support (ML BP 88, BI PP 0.99, Fig. 1), not of the Australian *C. brownii*. The two *C. brownii* varieties differ by 2.7% in *tuf*A and 1.7% in *rbc*L, which is more than between many other *Caulerpa* species. According to Chapman (1956) *C. brownii* var. *selaginoides* is endemic to New Zealand including the Chatham Islands and has ramuli more spread out (the distance between the origins of the ramuli is up to twice the diameter of the ramulus) than in *C. brownii* with densely arranged ramuli. Womersley (1956) mentioned that differences are probably due to ecological factors as all grades between the varieties occur, but that the New Zealand forms all fall within var. *selaginoides*. However, we do not yet propose separate species status for *C. brownii* var. *selaginoides*, but recommend to await DNA sequence data of more specimens.

The two *C. filicoides* varieties are paraphyletic with respect to each other in the ML tree (Fig. 1), but monophyletic in the BI tree (BI PP 0.99, Fig. 2). However, their DNA sequences differ enough (4.7% in *tuf*A and 3.5% in *rbc*L) to consider them to be separate species. It is proposed here to give species status to *C. filicoides* var. *andamanensis* which differs from *C. filicoides* var. *filicoides* in having mostly a single whorl of branchlets on a short stipe (up to 2 mm), whereas the latter mostly has 2–3 super-imposed whorls on a longer stipe (5–15 mm).

High sequence divergence in *C. verticillata* (1.5% in *tuf*A as well as in *rbc*L) suggests two species. *C. verticillata* 1 and 2 occur in sympatry, whereas *C. verticillata* 1 specimens from the Caribbean and Indo-Pacific have identical DNA sequences (Table S1). *C. verticillata* specimen FL1148 has not been seen by the present authors. Voucher SGAD1012150 seems to be of *C. verticillata* J. Agardh f. *charoides* (Harvey) Weber-van Bosse. Voucher 03-446 consists of two individual specimens. One possibly represents *C. verticillata* f. *charoides* and the other *C. verticillata* J. Agardh f. *verticillata*, but it is difficult to differentiate between these forms (Thivy and Visalakshmi 1963a,b). Further research is needed before it can be proposed to reinstate *C. charoides* (Harvey *ex* Weber-van Bosse) Thivy & Visalakshmi.

This is the first study that includes more than one *Caulerpella* specimen. *Caulerpella* is nested inside

Caulerpa with strong support (Fig. 1). This supports the opinion of Silva et al. (1996) who retained *C. ambigua* in the genus *Caulerpa* based on the shared internal trabeculate structure and thought that non-holocarpic reproduction should have infrageneric taxonomic value. Therefore, it is proposed to reinstate the binomial *C. ambigua* Okamura. High *tuf*A (6.7%) and *rbc*L (4.7%) sequence divergence between *C. ambigua* specimens suggests multiple species. *C. ambigua* 1 and 2 occur in sympatry (Hawaii), whereas *C. ambigua* 1 from Hawaii and Texas have identical DNA sequences (Table S1). The species status of one or more of the synonymized taxa *Caulerpa vickersiae* Børgesen and *Caulerpa biloba* Kempermann & Stegenga might be restored in the future, but the present data are insufficient. Remarkably, one of the *Pseudochlorodesmis* specimens was also nested within *Caulerpa* ("*Caulerpella*" lineage 4), whereas the other four *Pseudochlorodesmis* specimens formed a strongly supported sister-clade to *Caulerpa* with multiple cryptic species. It is outside the scope of this study to clarify the taxonomy of *Pseudochlorodesmis* any further. In Figure 1, Figure S3 and Table S1 old taxon names are applied, and in Figure 2 and Tables S2 and S3 the newly proposed names are applied.

Inferring a new infrageneric classification of Caulerpa. The traditional *Caulerpa* sections were based on overall thallus morphology, especially of the erect fronds. It has become clear since Famà et al. (2002) that these sections are polyphyletic and do not reflect phylogeny. Vesiculate, terete, and flattened ramuli all evolved multiple times. Only section *Charoideae* J. Agardh *ex* De-Toni remains monophyletic in this study (lineage 1). Nine sections are represented in lineage 6B and five of them also outside lineage 6B (Table S1). Subgenera have also been described in *Caulerpa*. Decaisne (1842) described the *Caulerpa* subgenera *Chauvinia* (Bory) Decaisne (type *Chauvinia paspaloides* Bory = *Caulerpa paspaloides* [Bory] Greville) and *Chemnitzia* Decaisne (type *C. chemnitzia* [Esper] J.V. Lamouroux). The subgenus *Caulerpa* was automatically formed when Decaisne separated these subgenera. The lectotype (*C. prolifera*) was later selected by Eubank Egerod (1952). The subgenus *Eucaulerpa* Endlicher (1843) is a synonym of the subgenus *Caulerpa*, which has priority. The type species of these subgenera all belong to lineage 6B. Although relationships between the six main lineages (Fig. 1) were not unambiguously resolved, the six lineages are clearly distinct clades at the end of relatively long branches. The maximum pair-wise phylogenetic distance within the six lineages is 0.156 (lineage 6) and the minimum pair-wise distance between the six lineages is 0.163 (between lineage 3 and 5; Table S3). The minor gap between these values cannot be discerned in the histogram of Figure S4 where distances are divided in cohorts of 0.005. The low minimum pair-wise distance between lineages can be ascribed to *C. hedleyi* (lineage 3) and would be 0.236 if this species is ignored. The high maximum pair-wise distance within lineages can be attributed to the long branch leading to *C. fergusonii* (lineage 6) and would be 0.125 if this species is ignored. If lineages 6A and 6B are considered separate main lineages, the maximum pair-wise distance within lineages would be 0.099, but minimum pair-wise distance between lineages 0.091 and thus no gap.

In the previous section, the family of the Caulerpaceae has been reduced to a single genus *Caulerpa* when the genus *Caulerpella* was abolished. It is proposed here to ascribe subgenus rank to each of the lineages 1, 2, 3, 4, 5, and 6. The autonym *Caulerpa* is available for lineage 6, because it includes the type. No subgenus names are available for the other lineages, because the types of the other available subgenus names are also included in lineage 6. It is proposed to give subgenus status to the sections *Charoideae* and *Araucarioideae* J. Agardh *ex* De Toni and to apply them to, respectively, lineage 1 and 5. It is proposed to give *Caulerpa* subgenus rank to the genus *Caulerpella* (lineage 4). New *Caulerpa* subgenus names are proposed for monotypic lineage 2 (*Cliftonii*) and lineage 3 (*Hedleyi*). Furthermore, it is proposed to treat the two lineages 6A and 6B of the *Caulerpa* core clade (i.e., subgenus *Caulerpa*) as sections. The other five proposed *Caulerpa* subgenera each contain only a single section bearing the same name as the subgenus. Characteristics of the newly proposed infrageneric taxa are discussed in the next paragraphs and the names are indicated in Figure 2. In Table S2, all the currently accepted *Caulerpa* species names as listed in *AlgaeBase* (searched September 18, 2013) are listed and ordered according to the newly proposed classification.

The *Caulerpa* subgenus *Charoideae* comb. et stat. nov. is proposed for lineage 1 with a single section *Charoideae* for which *C. verticillata* is the lectotype. The unsampled species *Caulerpa kempfii* A.B. Joly & S. Pereira, *Caulerpa murrayi* Weber-van Bosse, and *Caulerpa pusilla* (Kützing) J. Agardh are also assigned to this subgenus. The former two species are only known from northeast Brazil. The last mentioned species has also been found in Brazil as well as in several Caribbean locations. *C. filicoides* and *C. andamanensis* stat. nov. are known only from the tropical Indo-Pacific. Specimens identified as *C. verticillata* are known from both the Indo-Pacific and the Atlantic Ocean. The species in the subgenus and section *Charoideae* are characterized by repeatedly branching ramuli, which are arranged in whorls (i.e., a verticillate branching mode) and stolons, which can be glabrous, densely or sparsely covered by rhizoids or tuberculate.

The *Caulerpa* subgenus *Cliftonii* subgen. nov. is proposed for lineage 2 with a single section *Cliftonii* sect. nov. for which Australian endemic *Caulerpa cliftonii* is the type and currently the only included species. The *Caulerpa* subgenus *Hedleyi* subgen. nov.

is proposed for lineage 3 with a single section *Hedleyi* sect. nov. for which Australian endemic *C. hedleyi* is the type and currently the only included species.

The *Caulerpa* subgenus *Caulerpella* comb. et stat. nov. is proposed for lineage 4 with a single section *Caulerpella* comb. et stat. nov. for which *C. ambigua* is the type and currently the only included species with a cosmopolitan tropical distribution. However, the high DNA sequence divergence between the *C. ambigua* specimens included in this study indicates that the taxon actually comprises multiple (cryptic) species. *Pseudochlorodesmis* sp. 5 should also be included in the subgenus *Caulerpella*. The occurrence of compound zoidangia distinguishes the subgenus *Caulerpella* from the other subgenera, but neither holocarpy nor zoidangia have been reported for *Pseudochlorodesmis* spp. (Abbott and Huisman 2003, 2004). However, compound zoidangia also occur in the halimedinean genera *Halimeda* J.V. Lamouroux and *Chlorodesmis* Harvey & Bailey and thus appear not to be phylogenetically informative in the Bryopsidales (Vroom et al. 1998).

The *Caulerpa* subgenus *Araucarioideae* comb. et stat. nov. is proposed for lineage 5 with a single amended section *Araucarioideae* for which *C. flexilis* is the type. All members of the subgenus and section *Araucarioideae* have conspicuous simple branched or unbranched appendages growing from the surface of the stolon, giving them a scaly or spiny appearance. However, the stolons of *C. webbiana* (lineage 6B) are also covered with outgrowths, but these are identical to the ramuli on the upright assimilators, whereas the stolon appendages in lineage 5 differ from those on the assimilators. *Caulerpa seuratii* Weber-van Bosse is an unsampled species with stolons densely covered by rhizoids, resembling *C. elongata* and *C. webbiana* and is expected to belong to the *Caulerpa* crown clade (lineage 6B). The stolons of *C. lanuginosa* and *C. antoensis* Yamada (both belonging to lineage 6B) are also covered by rhizoids (not by squamulate outgrowths) and so are the stolons of the *Charoideae* species (lineage 1). *C. hedleyi* (lineage 3) has squamulate stolons, but does not belong to lineage 5. However, lineage 3 and 5 might be sister lineages, which would mean that the stolon-covering scale-like appendages could be a synapomorphy. All other *Caulerpa* species have naked (glabrous) stolons, except *Caulerpa heterophylla* I.R. Price, J.M. Huisman & M.A. Borowitzka from West-Australia, which has stolons covered by conical protuberances and is therefore classified here in the *Caulerpa* subgenus and section *Araucarioideae*. *Caulerpa alternans* Womersley has glabrous stolons and is not sampled in this study but is included in the *Araucarioideae* based on unpublished DNA sequence data (G. Belton).

The *Caulerpa* subgenus *Caulerpa* (autonym) is proposed for lineage 6 for which *C. prolifera* is the type. Two *Caulerpa* subgenus *Caulerpa* sections are proposed: an amended section *Sedoideae* J. Agardh *ex* De Toni (lectotype: *C. sedoides*) for lineage 6A and a section *Caulerpa* (autonym) for lineage 6B. Section *Caulerpa* includes the strongly supported *Caulerpa* crown clade (ML BP 95, BI PP 1.00, Fig. 1), as well as *C. longifolia*, *C. paspaloides*, and *C. lanuginosa*. The amended section *Sedoideae* includes *Caulerpa* species that have glabrous stolons. All species in lineage 6A have an Indo-Pacific distribution, except *C. microphysa*, which also occurs in the Atlantic. Several species exhibit assimilators bearing vesiculate (including elongate-ovoid to clavate) ramuli with a constricted pedicel. There are also species without vesiculate ramuli which mostly exhibit a rachis with regularly interspaced constrictions (i.e., annulate). Many species have pyrenoids associated with relatively large chloroplasts, 7–11 μm in length (Calvert 1974, Calvert et al. 1976, Famà et al. 2002, Wynne et al. 2009, present study). In *Caulerpa* species without pyrenoids, chloroplasts are 3–5 μm. No *Caulerpa* species with pyrenoids are known outside lineage 6A. No pyrenoids have been reported for four Australasian species in lineage 6A, i.e., *C. fergusonii*, *C. hodgkinsoniae* J. Agardh, *C. papillosa* J. Agardh, and *C. vesiculifera* (Harvey) Harvey. All have vesiculate ramuli with constricted pedicels, but the rachis is without constrictions in the latter two species. The present authors neither observed pyrenoids when inspecting herbarium vouchers of these species stained with iodine (to make starch around the pyrenoids visible) under the light microscope. However, many chloroplasts in *C. papillosa* showed a 1.5 μm light-colored area. This might be the "presumptive pyrenoid region or pyrenoid-like region" that Borowitzka (1976) reported for the chloroplast of *C. papillosa*. Calvert et al. (1976) did not observe pyrenoids, nor a pyrenoid-like region, in *C. papillosa*, but measured 5–7 μm long chloroplasts which is longer than the 3–5 μm measured in other species without pyrenoids. However, the present authors measured 3–5 μm in voucher material of *C. papillosa* under the light microscope. Hori (1974) stated that pyrenoids usually are recognized by the formation of starch plates and that they are rarely without limiting membranes, but that this is not the case in *C. fergusonii* from Japan. In Japanese *C. fergusonii*, the centrally located matrix of the pyrenoids in the chloroplasts is only set with many small starch grains and is thus less elaborate than the pyrenoid in *C. okamurae*, the other species of the pyrenoid clade studied by Hori by use of an electron microscope. The presence of pyrenoids (observable under the light microscope) might be a synapomorphy within *Caulerpa*. All pyrenoid-containing species form a strongly supported monophyletic clade within lineage 6A (ML BP 81, BI PP 0.98, Fig. 1), except for *Caulerpa bartoniae* G. Murray which is outside this clade, albeit without support (ML BP 43, BI PP 0.81). *C. bartoniae* lacks an annulate rachis and vesiculate ramuli. *C. filiformis* (lineage 6B) has an annulate rachis, but no pyrenoids and neither ramuli with constricted pedicels. Three species without

pyrenoids and for which no DNA sequence data are available, exhibit a rachis with constrictions. The Australian species *C. constricta* I.R. Price, Huisman *et* Borowitzka, lacks ramuli and rachis constrictions are irregularly interspaced. Therefore, it is thought to belong to lineage 6B (section *Caulerpa*). The Australasian species *C. articulata* Harvey and South African *C. holmesiana* G. Murray both have an annulate rachis and ramuli with a constricted pedicel. Therefore, it is proposed to await DNA sequence data before assigning them to one of the two sections of the subgenus *Caulerpa,* although Womersley (1956) considered *C. hodgkinsoniae* to be a synonym of *C. articulata.*

Caulerpa* subgenus *Caulerpa (autonym)

Type: *C. prolifera* (Forsskål) J.V. Lamouroux, lectotypified by Eubank Egerod (1952).

Description: The species have glabrous or pubescent stolons, which in some species are covered by a dense growth of rhizoids. The assimilators with ramuli differ distinctly from the rhizoids or other stolon appendages. Chloroplasts with or without associated pyrenoids, depending on the species.

The subgenus currently includes the sections *Caulerpa* (autonym) and *Sedoideae* J. Agardh *ex* De Toni *emend.* Draisma, Prudhomme, Sauvage & G. Belton.

Caulerpa* section *Caulerpa (autonym)

Type: *C. prolifera* (Forsskål) J.V. Lamouroux, see subgenus *Caulerpa.*

Description: The species have glabrous or pubescent stolons which in some species are covered by a dense growth of rhizoids. The assimilators with ramuli differ distinctly from the rhizoids or other stolon appendages. Chloroplasts 3–5 μm long (5–7 μm in *C. paspaloides*) without associated pyrenoids.

Caulerpa* section *Sedoideae J. Agardh *ex* De Toni *emend.* Draisma, Prudhomme, Sauvage *et* G. Belton.

Basionym: Sectio *Sedoideae* J. Agardh *ex* De Toni (1889) in G.B. De Toni: *Sylloge chlorophycearum omnium* p. 473.

Type: *C. sedoides* C.A. Agardh.

Description: The species have glabrous stolons. Some species have a constricted rachis. Pedicels of ramuli in most species constricted. Chloroplasts 9–11 μm long (3–7 μm in *C. papillosa*) with associated pyrenoids. Four species without pyrenoids are added based on molecular evidence. Two species (*C. articulata* and *C. holmesiana*) without pyrenoids, but with constricted pedicels and an annulate rachis may be added in the future if molecular evidence becomes available.

Caulerpa* subgenus *Araucarioideae (J. Agardh *ex* De Toni) Draisma, Prudhomme, Sauvage *et* G. Belton **comb. nov. et. stat. nov.**

Basionym: Sectio *Araucarioideae* J. Agardh *ex* De Toni (1889) in G.B. De Toni: *Sylloge chlorophycearum omnium* 469.

Type: *C. flexilis* C.A. Agardh.

The subgenus currently includes a single section *Araucarioideae* J. Agardh *ex* De Toni *emend.* Draisma, Prudhomme, Sauvage *et* G. Belton.

Caulerpa* section *Araucarioideae J. Agardh *ex* De Toni **emend**. Draisma, Prudhomme, Sauvage *et* G. Belton.

Basionym: Sectio *Araucarioideae* J. Agardh *ex* De Toni (1889) in G.B. De Toni: *Sylloge chlorophycearum omnium* p. 469.

Type: *C. flexilis* C.A. Agardh.

The species have pubescent stolons that are covered by small branched or unbranched scales or conical protuberances with the exception of *C. alternans* which has glabrous stolons, but is added here based on DNA sequence data. Chloroplasts 3–5 μm long without associated pyrenoids.

Caulerpa* subgenus *Charoideae (J. Agardh *ex* De Toni) Draisma, Prudhomme, Sauvage *et* G. Belton **comb. nov. et stat. nov.**

Basionym: *Caulerpa* sectio *Charoideae* J. Agardh *ex* De Toni (1889) in G.B. De Toni: *Sylloge chlorophycearum omnium* p. 470.

Type: *C. verticillata* J.G. Agardh.

The species have thin, pubescent stolons, a verticillate branching mode, and thin, much branched ramuli. Chloroplasts 3–5 μm long without associated pyrenoids.

The subgenus currently includes a single section *Charoideae* J. Agardh *ex* De Toni.

Caulerpa* subgenus *Caulerpella (Prud'homme *et* Lokhorst) Draisma, Prudhomme *et* Sauvage **comb. nov. et stat. nov.**

Basionym: *Caulerpella* Prud'homme *et* Lokhorst (1992) in W.F. Prud'homme van Reine & G.M. Lokhorst: *Caulerpella* gen. nov. a non-holocarpic member of the Caulerpales (Chlorophyta). *Nova Hedwigia* 54, pp. 114–115, figs 1–4.

Type: *C. ambigua* Okamura.

The species are non-holocarpic and form zoidangia that are separated from the sterile part of the thallus by a cell wall.

The subgenus currently includes a single section *Caulerpella* Draisma, Prudhomme *et* Sauvage.

Caulerpa* section *Caulerpella (Prud'homme *et* Lokhorst) Draisma, Prudhomme *et* Sauvage **comb. nov. et stat. nov.**

Basyonym: *Caulerpa* subgenus *Caulerpella* (Prud'homme *et* Lokhorst) Draisma, Prudhomme *et* Sauvage (2014) in Draisma et al.: DOI: 10.1111/jpy.12231

Type: *C. ambigua* Okamura.

Description as for the *Caulerpa* subgenus *Caulerpella.*

Caulerpa* subgenus *Cliftonii Draisma, Prudhomme *et* G. Belton **subgen. nov.**

Type: *C. cliftonii* Harvey.

Description: With glabrous stolons and thin irregularly branched terete radially arranged laterals and a much thicker rachis. The laterals are covered from their base on with irregularly placed ramuli, which are alternately branched in their lower half. Chloroplasts 3–4 μm long without associated pyrenoids.

The subgenus currently includes a single section *Cliftonii* Draisma, Prudhomme *et* G. Belton.

Caulerpa* section *Cliftonii Draisma, Prudhomme *et* G. Belton **sectio nov.**

Type: *C. cliftonii* Harvey.

Description as for the *Caulerpa* subgenus *Cliftonii*.

Caulerpa* subgenus *Hedleyi G. Belton **subgen. nov.**

Type: *C. hedleyi* Weber-van Bosse.

Description: Stolons covered with branched spines. Assimilators irregularly branched with two opposite rows of laterals densely covered with repeatedly bifurcating ramuli ending in tiny spines. Chloroplasts 3–5 μm long without associated pyrenoids.

The subgenus currently includes a single section *Hedleyi* G. Belton.

Caulerpa* section *Hedleyi G. Belton **sectio nov.**

Type: *C. hedleyi* Weber-van Bosse.

Description as for the *Caulerpa* subgenus *Hedleyi*.

Caulerpa andamanensis (W.R. Taylor) Draisma, Prudhomme *et* Sauvage **comb. nov. et stat. nov.**

Basionym: *C. filicoides* var. *andamanensis* W.R. Taylor (1965), An interesting *Caulerpa* from the Andaman Sea, *J. Phycol.* 1: 154–156, fig. 1.

Type locality is northeast of Ritchie's Archipelago, Andaman Islands.

Holotype: In US (isotype in MICH).

Occurrence: Known from Tanzania, India, Sri Lanka, Andaman Islands, Palau, Micronesia, Papua New Guinea, Australia, Fiji, and also Hawaii (H. Spalding, unpublished).

Rbc*L introns*. The two newly discovered introns in *C. fergusonii* and *C. brownii* were located at exactly the same position as the fourth and fifth intron in the *rbc*L of, respectively, the euglenids *Euglena longa* (Pringsheim) Marin & Melkonian (GenBank AJ294725) and *Euglena gracilis* Klebs (Genbank M12109). However, their sequences differed significantly and were unalignable. They were also unalignable with the downstream located (outside the alignment of this study) group II introns that were identified by Hanyuda et al. (2000) in two *Caulerpa* species. No Open Reading Frame (ORF) was detected in domain IV in *C. fergusonii*. The length difference between the *C. fergusonii* and the *C. brownii* intron is located in domain IV and the latter may have an ORF. The ORF in *C. fergusonii* was probably lost recently, because the sequences of the two introns are so similar. The introns are mobile DNAs when they encode the ORFs, but after they lose the ORFs, they are presumably immobile (Bonen and Vogel 2001, Dai et al. 2003). The ORF is required for mobility of the introns and for splicing. Without an ORF, the introns still have to be spliced efficiently, because they are in housekeeping genes. The splicing factors in *Caulerpa* are currently unknown, but it seems that they were already present when the intron was inserted. Therefore, additional group II introns may be expected elsewhere in the chloroplast genome of *Caulerpa*. The *rbc*L of *C. obscura* L 09.10.052 may also contain an intron about one hundred nt longer than the one in *C. fergusonii* based on the estimated size of a CR-F/CR-mR PCR fragment, which could not be sequenced successfully. The presence of a large intron may also be the reason why amplification of the CR-F/CR-mR fragment failed for several other specimens.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Secondary structure of the Group IIA intron (638 nt) found in *Caulerpa fergusonii* PERTH 6.10.9.27 (Genbank FR848361) determined with the program mfold 3.4 on The mfold Web Server (http://mfold.rna.albany.edu/).

Figure S2. Secondary structure of the incompletely determined Group IIA intron (Genbank FR848362 [5′-end] and FR848363 [3′-end]) found in *Caulerpa brownii* L 09.10.057 determined with the program mfold 3.4 on The mfold Web Server (http://mfold.rna.albany.edu/).

Figure S3. Five markers Maximum Likelihood (ML) tree of 105 Dasycladales and Bryopsidales and five Ulvophyceae (outgroup).

Figure S4. Histogram with frequency distribution of pairwise phylogenetic distances listed in Table S3 and derived from the phylogeny in Figure 1.

Table S1. Caulerpaceae and *Pseudochlorodesmis* specimens and sequence data used in the present study.

Table S2. All currently accepted Caulerpaceae species according to www.algaebase.org (searched 18 September 2013) ordered by the infrageneric classification proposed in the present study and by alphabet.

Table S3. Pairwise distances (branch lengths) of the Caulerpaceae derived from the Maximum Likelihood phylogeny in Figure 1.