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MOLECULAR PHYLOGENY OF THE GENUS *CAULERPA* (CAULERPALES, CHLOROPHYTA) INFERRED FROM CHLOROPLAST *tuf*A GENE[1]

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The genus *Caulerpa* consists of about 75 species of tropical to subtropical siphonous green algae. To better understand the evolutionary history of the genus, a molecular phylogeny was inferred from chloroplast *tuf*A sequences of 23 taxa. A sequence of *Caulerpella ambigua* was included as a potential outgroup. Results reveal that the latter taxon is, indeed, sister to all ingroup sequences. *Caulerpa* itself consists of a series of relatively ancient and species-poor lineages and a relatively modern and rapidly diversifying clade, containing most of the diversity. The molecular phylogeny conflicts with the intrageneric sectional classification based on morphological characters and an evolutionary scheme based on chloroplast ultrastructure. High bootstrap values support monophyly of *C. mexicana*, *C. sertularioides*, *C. taxifolia*, *C. webbiana*, and *C. prolifera*, whereas most other *Caulerpa* species show para- or polyphyly.

Key index words: ***Caulerpa*; chloroplast DNA; phylogeny; systematics; *tuf*A**

Abbreviation: ***tuf*A, elongation factor TU**

The Bryopsidalean genus *Caulerpa* comprises a group of conspicuous algae distributed in a range of habitats throughout the tropical and subtropical marine realm (Dawson 1966, Hay et al. 1985, Meinesz and Boudouresque 1996). Recently, the genus attracted considerable research interest because species expanded their ranges into more temperate environments (Meinesz and Hesse 1991, Piazzi et al. 1994, Dalton 2000, Kaiser 2000). Most species are well defended against large grazers by a suite of toxic compounds (de Paula and de Oliveira 1982, Paul and Fenical 1986). However, these very grazer deterrents make these plants an ideal substratum for a suite of cryptic meiofauna. Many of these organisms feed on *Caulerpa* despite the toxins (Hay et al. 1994).

Caulerpa belongs to the Bryopsidophyceae (Van den Hoek et al. 1995), a class of algae with a coenocytic thallus organization. Each thallus is essentially a single cell that develops into an elaborate system of branching siphons. *Caulerpa* is defined by the presence of trabeculae: inwardly projecting cylindrical extensions of cell wall material passing through the central lumen of the siphons (Lamouroux 1809, Bold and Wynne 1985). Thalli are composed of a prostrate rhizome (stolon), branched anchoring rhizoids, and upright branches (assimilators) that bear distinctive branchlets and are used in species identification. These units, called metameres (White 1979), can potentially regenerate new ramets after a frond or stipe is cut. Gametogenesis involves migration of cytoplasm into unspecialized gametangia where it is transformed into anisogamous gametes (Goldstein and Morrall 1970, Enomoto and Ohba 1987). Just before dawn, micro- and macrogametes are shed in the water column in species-specific brief release intervals (Clifton 1997, Clifton and Clifton 1999).

Caulerpa includes about 75 species worldwide (Weber-van Bosse 1898, Calvert et al. 1976, Price et al. 1998). Many taxa form discrete well-delimited units with relatively little morphological variability. Yet some taxonomically perceived species exhibit rampant morphological plasticity and ill-defined taxonomic boundaries. Variability in growth forms and in the photosynthetic performance of *Caulerpa* species seem to be related to substrate, light intensity, and water motion (Gacia et al. 1996, Collado-Vides and Robledo 1999). Sectional division among taxa (Agardh 1872, Weber-van Bosse 1898) is predominantly supported by differences in assimilator morphology. These assimilators, however, can be highly plastic and seem under strong control of the environment (Gilbert 1941, Calvert 1976,

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Ohba et al. 1992). Therefore, species boundaries, species relationships, and sectional divisions are questionable.

Ultrastructural traits and DNA sequence differences have been applied to resolve phylogenetic relationships among taxa within *Caulerpa*. A phylogeny of 28 *Caulerpa* species, based on chloroplast ultrastructure (Calvert et al. 1976), reflected an evolutionary trend from putatively ancestral, large, pyrenoid-containing chloroplasts to small chloroplasts lacking pyrenoids. More recently, molecular studies using allozymes (Benzie et al. 1997), chloroplast DNA RFLP (Satoh et al. 1992, Lehman and Manhart 1997), and nuclear rDNA or chloroplast DNA sequences (Pillman et al. 1997, Jousson et al. 1998, 2000, Olsen et al. 1998, Famà et al. 2000, Hanyuda et al. 2000) showed high intraspecific or even intraindividual differences in chloroplast DNA size and nuclear rDNA polymorphism. Such patterns hamper determination of evolutionary relationships in this genus.

The chloroplast gene *tuf*A encodes for elongation factor TU, a molecule that mediates the entry of an amino-acyl-tRNA into the acceptor site of a ribosome during elongation of the nascent polypeptide chain in protein synthesis (Lewin 1997). This gene is encoded by the chloroplast genome of photosynthetic algae but is nuclear encoded in some Charophyceae and in land plants (Baldauf et al. 1990, Bonny and Stutz 1993). The *tuf*A gene is a good candidate for phylogenetic studies above the species level because of its conserved nature across a wide range of organisms. Until recently, however, *tuf*A sequences have been used only to address phylogenetic questions at suprageneric levels (Ludwig et al. 1990, Delwiche et al. 1995, Baldauf et al. 1996).

In this study, we inferred a phylogeny from partial chloroplast *tuf*A sequences among 23 described taxa and a taxon morphologically divergent from all described *Caulerpa* species to test the usefulness of this gene in resolving phylogenetic relationships at the genus level. A sequence of a putative close outgroup, *Caulerpella ambigua* (Prud'homme van Reine and Lokhorst 1992), was also examined to root the obtained phylogeny and to assess the position of this taxon. We also compared the phylogeny with hypotheses of chloroplast ultrastructural evolution (Calvert et al. 1976) and sectional divisions (Weber-van Bosse 1898).

MATERIALS AND METHODS

Taxon sampling. A total of 46 algal specimens was collected from various localities around the world. For identification of *Caulerpa* species, varieties, and forms, the following taxonomic references were used: Weber-van Bosse (1898), Taylor (1960), Womersley (1984), Coppejans and Prud'homme van Reine (1992), and Littler and Littler (2000). Table 1 lists taxa used including their authority, collector, locality description, and EMBL sequence accession number.

DNA isolation, amplification, and sequencing. Total DNA was extracted from specimens preserved in either silica gel or in 70% ethanol using guanidine lysis buffer (Maniatis 1982) or the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The Chelex protocol (Goff and Moon 1993) was used to isolate DNA from *C. ambigua* of which only a minute amount of material was available.

Algal specific forward and reverse primers for the *tuf*A gene were designed based on a sequence alignment of 14 algal taxa deposited in GenBank. The forward and reverse primers anneal at position 210 (tufAF 5′-TGAAACAGAAMAWCGTCATT ATGC-3′) and 1062 (tufAR 5′-CCTTCNCGAATMGCRAAW CGC-3′), respectively, of the *Codium fragile* (Suringar) Hariot *tuf*A gene sequence (GenBank accession number U09427). Double-stranded DNAs were amplified using PCR following two protocols.

In the first PCR procedure, reactions were performed in a total volume of 50 μL consisting of 5 mM $MgCl_2$, 0.3 mM each primer, 0.2 mM each dNTP, 0.5 units of Taq DNA polymerase (Roche Diagnostics, Rotkreuz, Switzerland), and 1.0 μL of 10× dilution of template DNA. The reactions were exposed to the following PCR profile: 40 cycles of denaturation (94° C for 1 min), primer annealing (52° C for 1 min), and extension (72° C for 2 min). A 5-min final extension cycle at 72° C followed the 40th cycle to ensure the completion of all novel strands. In the second protocol, a PCR master mix of 13 μL was prepared consisting of 2.5 mM $MgCl_2$, 0.5 mm each primer, 0.2 mM each dNTP, 1.0 M Betaine, 0.5 units of Taq DNA polymerase (PE Applied Biosystems, Foster City, CA, U.S.A.), and 0.5–1.0 μL of 1× or 100× dilution of template DNA. This procedure involved an initial denaturation at 94° C for 3 min, followed by 40 cycles of denaturation (94° C for 1 min), primer annealing (45° C for 1 min), and extension (72° C for 2 min) followed by a final extension step at 72° C for 4 min. In instances in which a very small amount of PCR product was obtained, the faint band was excised from a low melting point agarose gel and used as template in a subsequent amplification with the same primers and PCR conditions described above.

Double-stranded PCR products were cleaned using the High Pure PCR Product Purification Kit (Roche Diagnostics) or excised from a low melting point agarose gel and digested using the GELase™ Agarose Gel-Digesting Preparation (Epicentre Technologies, Madison, WI, U.S.A.) before sequencing. The double-stranded PCR products were used as templates in cycle sequencing reactions. Sequencing primers were the same as those used for amplification. PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Rotkreuz, Switzerland) on an ABI-3100 or an ABI-377 DNA automated sequencer (Applied Biosystems), following manufacturer's instructions.

Molecular data analysis. Sequences were aligned manually using the Genetic Data Environment software, version 2.2 (Larsen et al. 1993). The complete alignment is submitted under EMBL accession number ALIGN−000315. Phylogenetic signal among parsimony-informative sites was assessed by comparing the measure of skewedness (g1-value, PAUP* version 4.0b6, Swofford 2000) with empirical threshold values in Hillis and Huelsenbeck (1992). To determine which model of sequence evolution best fit the data (Huelsenbeck and Rannala 1997), hierarchical likelihood ratio tests were performed using Modeltest version 3.0 (Posada and Crandall 1998). Phylogenetic trees were reconstructed using maximum likelihood (ML) and maximum parsimony (MP) as implemented in PAUP*. ML phylogenies, constrained with obtained Modeltest parameters for the data set, were inferred using the heuristic search algorithm with 10 random taxon orders and the tree bisection-reconnection branch swapping procedure. Nodal support was estimated using bootstrap analyses (100 replicates). Weighted (Goloboff, K = 2) parsimony analyses used heuristic searches with random taxon addition of sequences (10 replicates) and tree bisection-reconnection branch swapping. Bootstrap analyses were performed using heuristic search (10,000 replicates).

To test the significance of suboptimal tree topologies, constraint trees were generated in Treeview (Page 1996). Tree topologies were evaluated with a Kishino-Hasegawa test (Kishino and Hasegawa 1989) under the ML criterion in PAUP*.

Chloroplast ultrastructure. The evolution of four chloroplast characters, considered phylogenetically informative in *Caulerpa* (Calvert et al. 1976), was investigated by mapping the character states onto the MP tree using MacClade 3.03 (Maddison and Maddison 1992).

TABLE 1. Collection data for *Caulerpa* species used for sequence analysis of the *tuf*A gene and EMBL accession number of the sequences.

Sections and species	Region	Geographical location	Legit et det.	EMBL accessions
Caulerpella ambigua (Okamura) Prud'homme van Reine & Lokhorst	GOM	Texas Flower Gardens, USA	B. Wysor	AJ417963
Caulerpa sp.[a]	GOM	Florida Middle Ground, USA	B. Wysor	AJ417962
Araucarioideae				
C. flexilis J.V. Lamouroux	WP	Jervis Bay, Australia	J. Zuccarello	AJ417970
Bryoideae				
C. webbiana Montagne	RS	Dahab, Egypt	A. Meinesz	AJ417958
C. webbiana var. *pickeringii* (Harvey & Bailey) Eubank	WIO	N. Kwa-Zulu Natal, South Africa	S. Fredericq	AJ417966
Charoideae				
C. verticillata J. Agardh	WA	Long Key, Florida, USA	T. Frankovich	AJ417967
Filicoideae				
C. ashmeadii Harvey	WA	Long Key, Florida, USA	B. Wysor	AJ417941
C. mexicana Sonder ex Kützing	CAR	Cuba	J. Montoya	AJ417951
C. mexicana	WA	Content Keys, Florida, USA	B. Wysor	AJ417952
C. mexicana	RS	Dahab, Red Sea	A. Meinesz	AJ417953
C. scalpelliformis (R. Brown ex Turner) C. Agardh	WP	Cape Banks, Australia	J. Zuccarello	AJ417971
C. scalpelliformis var. *denticulata* (Decaisne) Weber-van Bosse	MED	Damour, Lebanon	A. Meinesz	AJ417972
C. selago (Turner) C. Agardh	RS	Abu Dhiab, Egypt	A. Meinesz	AJ417973
C. sertularioides (S.G. Gmelin) M. Howe	CAR	Martinique, Lesser Antilles	F. Sinniger	AJ417944
C. sertularioides	CAR	Colón, Panamá	B. Wysor	AJ417945
C. sertularioides	EP	I. Melones, Panamá	B. Wysor	AJ417946
C. taxifolia (M. Vahl) C. Agardh	WP	Moreton Bay, Australia	T. Pillen	AJ417936
C. taxifolia	RS	Safaga, Egypt	A. Meinesz	AJ417937
C. taxifolia	CAR	Guayacan Island, Puerto-Rico	D. Ballantine	AJ417938
C. taxifolia	WIO	N. Kwa-Zulu Natal, South Africa	S. Fredericq	AJ417939
Lycopodioideae				
C. lanuginosa J. Agardh	WA	Content Keys, Florida, USA	B. Wysor	AJ417959
Paspaloideae				
C. paspaloides (Bory de Saint-Vincent) Greville	WA	Long Key, Florida, USA	B. Wysor	AJ417965
Phyllantoideae				
C. brachypus Harvey	WP	Cangaluyan, Pangasinan	L. de Sénerpont Domis	AJ417934
C. prolifera (Forsskål) J.V. Lamouroux	EIO	Bali	F. Sinniger	AJ417942
C. prolifera f. *zosterifolia* Børgesen	WA	Long Key, Florida	B. Wysor	AJ417943
C. subserrata Okamura	WP	Uken, Japan	T. Hanyuda	AJ417935
Sedoideae				
C. cactoides (Turner) C. Agardh	WP	Jervis Bay, Australia	J. Zuccarello	AJ417969
C. geminata Harvey	WP	Coffs Harbour, Australia	J. Zuccarello	AJ417968
C. geminata	WP	Cape Bank, Australia	J. Zuccarello	AJ417960
C. microphysa (Weber-van Bosse) Feldmann	GOM	Texas Flower Gardens, USA	B. Wysor	AJ417961
C. racemosa (Forsskål) J. Agardh	CAR	Galeta, Panamá	W. Kooistra	AJ417950
C. racemosa var. *lamourouxii* (Turner) Weber-van Bosse	WP	Uken, Japan	T. Hanyuda	AJ417954
C. racemosa var. *macrophysa* (Sonder ex Kützing) W.R. Taylor	CAR	Galeta, Panamá	W. Kooistra	AJ417947
C. racemosa var. *macrophysa*	WA	Long Key, Florida, USA	B. Wysor	AJ417956
C. racemosa var. *occidentalis* (J. Agardh) Børgesen	MED	Livorno, Italy	L. Piazzi	AJ417955
C. racemosa var. *peltata* (Lamouroux) Eubank	CAR	Panamá	B. Wysor	AJ417948
C. racemosa var. *peltata*	EP	Isla Naos, Panamá	B. Wysor	AJ417949
C. racemosa var. *turbinata* (J. Agardh) Eubank	RS	Dahab, Egypt	A. Meinesz	AJ417957

(continued)

RESULTS

Sequence analyses. The length of the 46 *tuf*A partial sequences varied from 811 base pairs in *Caulerpella ambigua* to 820 base pairs in all *Caulerpa* species; 266 sites were variable and 196 were parsimony informative, showing a significant phylogenetic signal ($g_1 = -1.28$). Sequences aligned easily, with no gaps, except for one indel of 9 base pairs to be inserted in the *Caulerpella* sequence. Sequences showed a low GC content (Table 2).

Phylogenetic analyses. To determine phylogenetic relationships among *Caulerpella* and *Caulerpa* species and to root the tree properly, a *tuf*A sequence of *Codium fragile* (GenBank U09427) was included in the alignment and was used as outgroup in an ML analysis (tree not shown). This analysis showed that *Caulerpella* was the most basal taxon to all *Caulerpa* species.

A general time reversible model (GTR, Yang 1994), along with among-sites rate heterogeneity (G) and an estimated proportion of invariable sites (I) was the optimal model on a hierarchical likelihood ratio tests (Table 2). An ML analysis constrained with obtained Modeltest parameter values resulted in the phylogram

TABLE 1. Continued.

Sections and species	Region	Geographical location	Legit et det.	EMBL accessions
Thuyoideae				
C. cupressoides (Vahl) C. Agardh	CAR	St. Barthélemy, Lesser Antilles	O. Jousson	AJ417929
C. cupressoides var. *flabellata* Børgesen	CAR	Cayo Carenero, Bocas del Toro, Panamá	B. Wysor	AJ417930
C. cupressoides var. *lycopodium* Weber-van Bosse	WP	Uken, Japan	T. Hanyuda	AJ417928
C. distichophylla Sonder	EIO	Cottesloe, Australia	A. Millar	AJ417940
C. serrulata (Forsskål) J. Agardh	RS	Dahab, Egypt	A. Meinesz	AJ417931
C. serrulata	WP	Bolinao, Pangasinan	L. de Sénerpont Domis	AJ417932
C. serrulata	CAR	Colón, Panamá	B. Wysor	AJ417933
Vaucherioideae				
C. filiformis (Suhr) K. Hering	WP	Bronte Beach, Australia	J. Zuccarello	AJ417964

Sections follow Weber-van Bosse's (1898) taxonomy.

[a] *Caulerpa* sp. refers to a sample morphologically divergent from all described *Caulerpa* species. This specimen has been deposited at the herbarium of the University of Louisiana at Lafayette (LAF) with the following voucher number: 12.viii.00-1-52.

GOM, Gulf of Mexico; WP, western Pacific; RS, Red Sea; WIO, western Indian Ocean; WA, western Atlantic; CAR, Caribbean Sea; MED, Mediterranean Sea; EP, eastern Pacific; EIO, eastern Indian Ocean.

shown in Figure 1. If *Caulerpella ambigua* was selected as an outgroup, the resulting tree topology showed relatively little phylogenetic resolution among the ingroup taxa. The first taxa to branch off within *Caulerpa* are *C. flexilis* and *C. verticillata*, respectively. These taxa are located on long branches. The remaining diversity is found in two sister clades: one with *C. geminata*, *C. microphysa*, and *C. cactoides* and the other with the remaining taxa.

Because long branches may disrupt relationships in the derived clades, we removed *Caulerpella ambigua*, *C. flexilis*, and *C. verticillata* sequences from the data set before reanalysis. The distribution of random tree lengths was significantly skewed to the left ($g_1 = -1.37$). A new run with Modeltest showed data complexity similar to that in the first run, and GTR was selected as the optimal model. The incorporation of among-sites rate heterogeneity (G) along with the integration of the estimated proportion of sites that are invariable (I) did increase significantly the fit between the GTR model and the data (Table 2). Likelihood analyses on the reduced set and constrained with newly generated Modeltest parameter values resulted in the trees depicted in Figure 2.

TABLE 2. Likelihood parameters obtained from the hierarchical likelihood ratio test as implemented in Modeltest for the complete and partial *tuf*A sequences data sets.

GTR + G + I likelihood model parameters	Complete *tuf*A data set[a]	Partial *tuf*A data set[b]
Base frequencies		
A	0.3587	0.3380
C	0.1286	0.1415
G	0.1702	0.1996
T	0.3425	0.3209
r-matrix		
(AC)	1.1317	1.0650
(AG)	2.4533	2.2611
(AT)	0.3979	0.3933
(CG)	1.9274	1.6103
(CT)	3.3027	3.6189
(GT)	1	1
Gamma shape	0.3192	0.5172
Proportion of invariable sites	0.3518	0.5377
ln likelihood value	−4231.2505	−3184.4138

[a] Includes sequences of all 46 taxa.

[b] Three taxa were excluded: *Caulerpella ambigua*, *Caulerpa flexilis*, and *Caulerpa verticillata*.

The ML tree (Fig. 2), rooted with sequences of *C. geminata*, *C. microphysa*, and *C. cactoides*, consisted of 16 clades with bootstrap values greater than 50%. Within the ingroup, *C. paspaloides* was the sister group to all other taxa, followed by *C. lanuginosa*. The remaining taxa belonged to a clade in which the branching order of a series of clades remained poorly resolved. Nevertheless, *C. cupressoides* and *C. serrulata* were clearly paraphyletic, and two appeared polyphyletic (*C. racemosa* and *C. scalpelliformis*). *Caulerpa taxifolia*, *C. mexicana*, *C. sertularioides*, *C. webbiana*, and *C. prolifera* were monophyletic. Among these five monophyletic taxa, the highest intraspecific genetic distance was observed in *C. mexicana* (0.4%), which was even higher than the distance values found between some species (*C. taxifolia* and *C. distichophylla*, 0.24%; *C. brachipus* and *C. subserrata*, 0.2%).

MP analysis of the reduced data set resulted in one most parsimonious tree of 361 steps (Fig. 3). Most of these clades were the same as in the ML analysis. The main incongruence between MP and ML trees consisted in the different phylogenetic position of *C. webbiana*. In the MP tree *C. webbiana* was sister to the most derived clade (88% bootstrap support), whereas in the ML tree it was sister to *C. cupressoides* and *C. serrulata*, although this relationship lacked good bootstrap support.

A moderate MP bootstrap value (63%) supported the most derived clade, although the branching order of a series of clades within this derived clade did not receive bootstrap support higher than 50% (Fig. 3). In this case, the polyphyly of two morphological sections, Filicoideae and Thuyoideae, and of *C. racemosa* and *C. scalpelliformis* was statistically tested against four

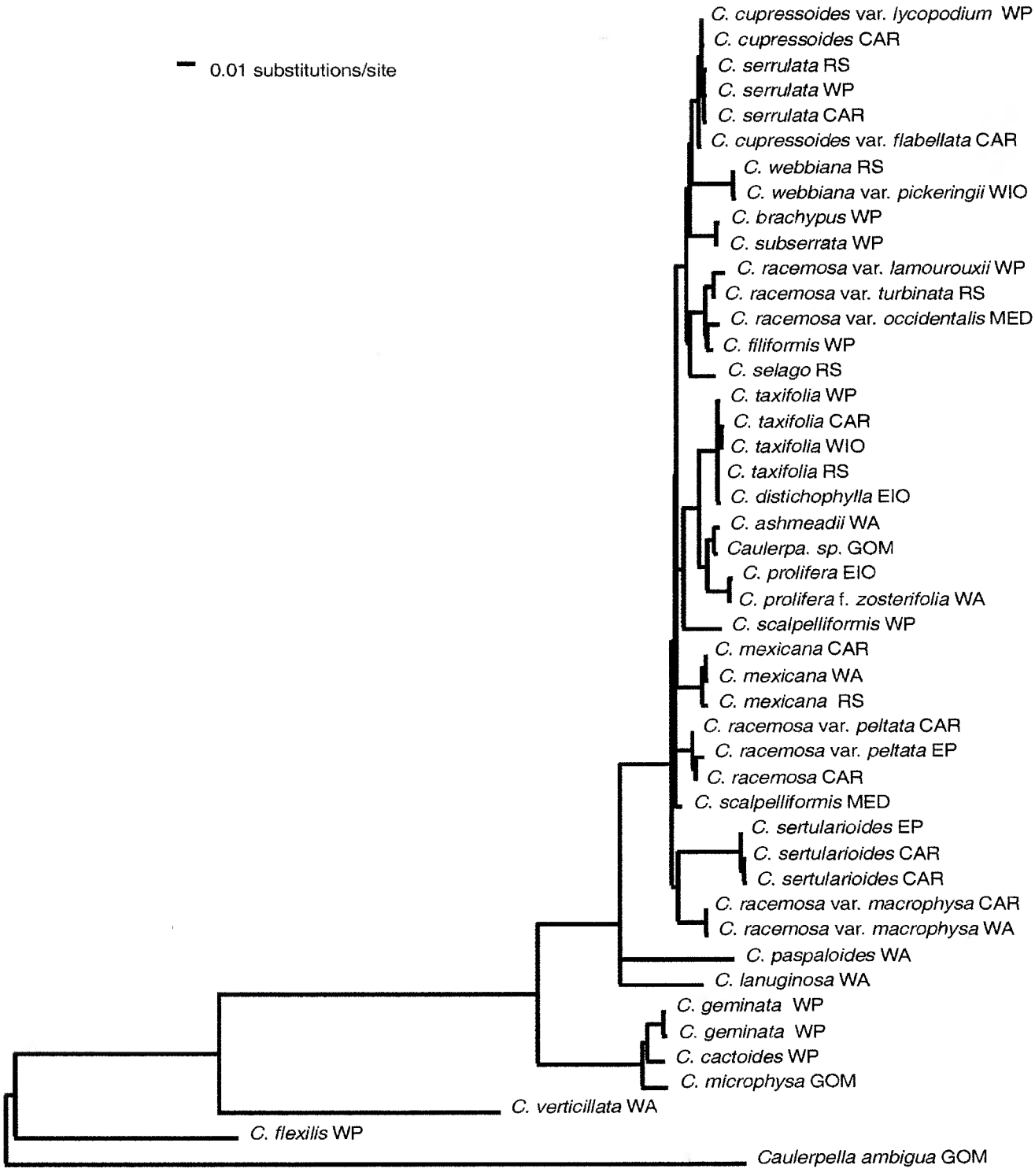

FIG. 1. ML phylogram of the maximum likelihood tree (ln L = −4231.2505) inferred from 46 chloroplast *tuf*A sequences of 23 *Caulerpa* species and one specimen of *Caulerpella ambigua*, used as an outgroup.

other topological alternatives: Filcoideae + *C. distichophylla*, Thuyoideae + *C. taxifolia*, *C. racemosa* + *C. filiformis*, and the monophyly of *C. scalpelliformis* (trees not shown). Kishino-Hasegawa tests results revealed that all four alternative topologies were significantly rejected ($P < 0.05$) (Table 3).

Chloroplast characters—size, occurrences of pyrenoids, number of thylakoids for each granum, and relative amount and length of starch grains (Tables 4 and 5)—were mapped over the obtained *tuf*A phylogeny. Figure 4 shows the distribution of four chloroplast characters, as identified by Calvert et al. (1976), in

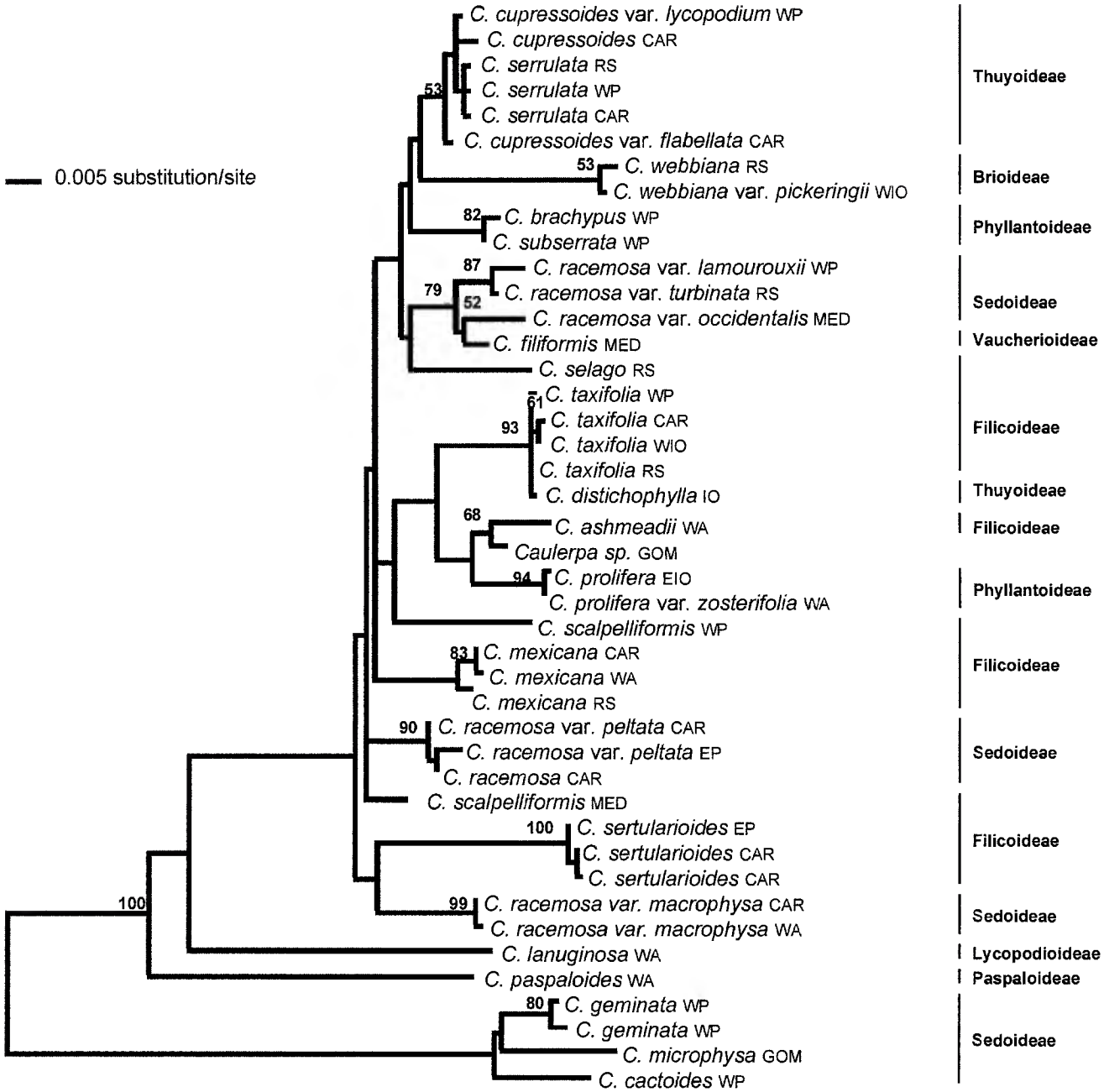

FIG. 2. ML tree (ln L = −3184.4138) resulting from a phylogenetic analysis of 21 *Caulerpa* species. Bootstrap values greater than 50% are reported (100 replicates). Outgroups are *C. geminata*, *C. microphysa*, *C. microphysa*, and *C. cactoides*.

the 18 *Caulerpa* species included in the present phylogenetic study. Presence of pyrenoids is a synapomorphy for the *C. geminata*, *C. cactoides*, and *C. microphysa* clade. Large plastids are also synapomorphies for this lineage. Intermediate size of plastids is a synapomorphy for the *C. lanuginosa* and *C. paspaloides* clade, as well as the number of thylakoids (three to four) per granum (Fig. 4, B and C), although the presence of multiple small starch grains (character D, state 1) seems to have been acquired and lost during the evolution of this genus.

DISCUSSION

This study represents an estimate of phylogenetic relationships within the genus *Caulerpa*, based on the analysis of chloroplast *tuf*A sequences. Clades obtained did not support morphological sections, as proposed by Weber-van Bosse (1898), because none of the four sections for which more than one representative species was analyzed formed a monophyletic group. Although several *Caulerpa* species still have to be added to better define the phylogenetic relationships in the genus, *tuf*A phylogenetic analyses of 23 taxa of *Caulerpa* already re-

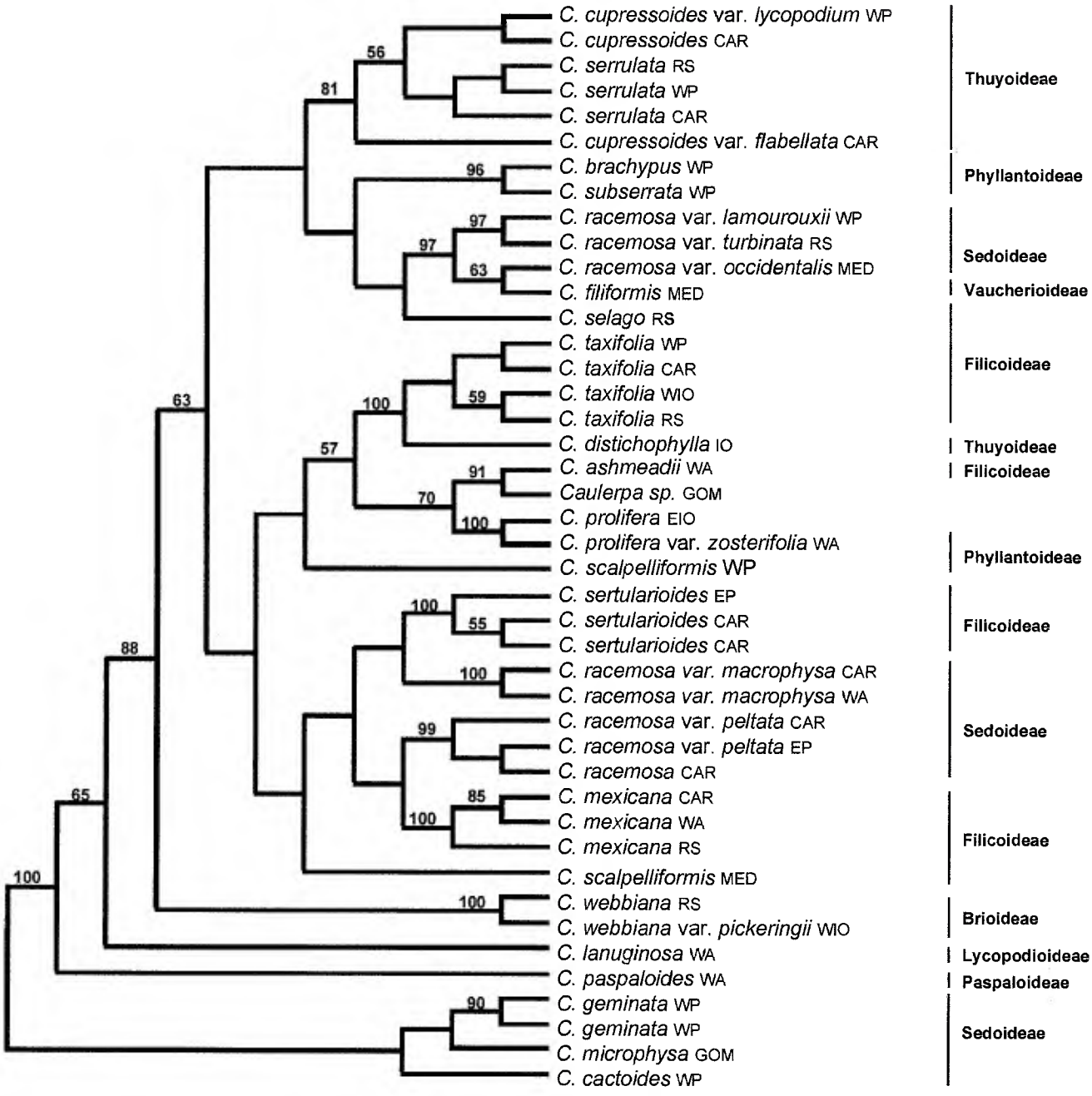

FIG. 3. Topology of the single most parsimonious tree from the MP analysis of the chloroplast *tuf*A sequences of the reduced *Caulerpa* data set (length = 361 steps, Consistency index = 0.637, Retention index = 0.806 and Rescaled consistency index = 0.514). Clades receiving 50% or greater bootstrap support are indicated above branches.

veal the existence of species-poor ancient lineages and a rapidly diversifying clade.

The highest genetic divergence between *Caulerpella ambigua* and all *Caulerpa* species supports the taxonomic distinction of *Caulerpella* as proposed by Prud'homme van Reine and Lokhorst (1992). *Caulerpella ambigua* differs from *Caulerpa* by its nonholocarpic mode of reproduction, although it shares most anatomical characters with its sister genus (e.g. presence of trabeculae, coenocytic thalli, stoloniferous habit with rhizoids, and branched vertical axes).

Caulerpa flexilis and *C. verticillata*, which possibly represent an ancestral species-poor lineage, share a smaller chloroplast type with the most derived species. Moreover, the presence of dense appendages covering the stolons of *C. flexilis* has been reported as a constant morphological character in this species (Weber-van Bosse 1898, Price et al. 1998). However,

TABLE 3. Evaluation of the four alternative tree topologies of *Caulerpa* relationships, using Kishino-Hasegawa test (Kishino and Hasegawa 1989).

Tree topology	Length[a]	Length difference[b]	P[c]
Filicoideae + *C. distichophylla*	386	25	0.0013*
Thuyoideae + *C. taxifolia*	386	25	0.0013*
C. racemosa + *C. filiformis*	386	25	0.0013*
C. scalpelliformis WP + *C. scalpelliformis* MED	386	25	0.0013*

[a]Length represents the total number of evolutionary changes on the tree.

[b]Length difference represents additional steps to most parsimonious not constrained tree topology (length, 361; Fig. 3).

[c]Probability to obtain a more extreme t value under the null hypothesis of no difference between the two trees (two-tailed test). *Significant difference at $P < 0.05$.

similar appendages or protuberances have been described in other eight *Caulerpa* species, including *C. webbiana*, which belongs to the most derived clade.

Caulerpa verticillata is among the most diminutive *Caulerpa* species. Smith and Walters (1999) showed marked differences in fragmentation success among thalli of three *Caulerpa* species using laboratory-based bioassays. Unlike thalli of *C. taxifolia* and *C. prolifera*, thalli of *C. verticillata* seem to possess a very limited capacity to regenerate after fragmentation. Furthermore, this species has distinct photosynthetic and morphological traits such as a high chl content, probably related to the density of rhizoid clusters and upright branches (Collado-Vides and Robledo 1999). These physiological differences appear to be consistent with our *tuf*A sequence data. However, regenerative capacity data across all species are required to confirm this possibility.

Our phylogeny based on *tuf*A sequences does not support the evolutionary scheme proposed by Calvert et al. (1976) in which chloroplasts of *Caulerpa* evolved from a large and complex pyrenoid containing organelle to a smaller and structurally simple one. Indeed, although *C. geminata*, *C. microphysa*, and *C. cactoides* possess a complex chloroplast structure, they do not represent the most ancient lines in the phylogenetic tree. Nevertheless, *C. cactoides*, *C. microphysa*, and *C. geminata* are united by the presence of pyrenoids and large chloroplasts (Calvert et al. 1976). The putative phylogenetic importance of pyrenoid presence should be confirmed by a sequence analysis of *C. okamurae*, *C. fergusonii*, and *C. lentillifera*, which also possess pyrenoids.

TABLE 4. Chloroplast structural characters and character states considered being of systematic value among *Caulerpa* species according to Calvert et al. (1976).

Character symbol	Character description	State 0	State 1	State 2
A	Pyrenoid	Present	Absent	
B	Chloroplast size	3–5 μm	5–7 μm	9–11 μm
C	Number of thylakoid in each granum	1–2	3–4	
D	Number and length of starch grains	1–2, 1–1.5 μm	1 to several, ≤0.5 μm	

TABLE 5. Matrix of chloroplast character states considered being of systematic value in 18 *Caulerpa* species (Calvert et al. 1976).

	Character			
Taxon	A	B	C	D
C. cupressoides	1	0	0	0
C. serrulata	1	0	0	0
C. webbiana	1	0	0	0
C. filiformis	1	0	0	0
C. taxifolia	1	0	0	0
C. ashmeadii	1	0	0	0
C. prolifera	1	0	0	0
C. mexicana	1	0	0	0
C. racemosa	1	0	0	0
C. scalpelliformis	1	0	0	0
C. sertularioides	1	0	0	0
C. lanuginosa	1	1	1	1
C. paspaloides	1	1	1	1
C. geminata	0	2	0	1
C. microphysa	0	2	0	1
C. cactoides	0	2	0	1
C. verticillata	1	0	0	0
C. flexilis	1	0	0	0

For characters and states see Table 4.

ML and MP trees have very similar topologies, although MP analysis provides a stronger support for some major clades. This includes support for the basal phylogenetic position of *C. paspaloides* and *C. lanuginosa* and the placement of *C. webbiana* as the sister taxon of the most derived clade.

Some monophyletic species emerge consistently from all analyses, with strong support (*C. taxifolia*, *C. prolifera*, *C. sertularioides*, *C. webbiana*, and *C. mexicana*). In particular, our results confirm the taxonomic distinction between *C. taxifolia* and *C. mexicana*, identified by Olsen et al. (1998) on the basis of rDNA ITS sequence data. In the present phylogenetic study *C. distichophylla* is sister taxon of *C. taxifolia*. Between these two morphological similar taxa the sequence divergence is only slightly greater (0.24%) than the genetic divergence found within *C. taxifolia* (0.2%), suggesting that they may represent morphotypes of a single species. However, unequivocal conspecific identification will require molecular analyses from a more variable genetic region and additional specimens of *C. distichophylla* from the Indian Ocean, where it seems to be confined (Silva et al. 1996, Huisman 2000). The *tuf*A sequence data indicate that *C. cupressoides* and *C. serrulata* are paraphyletic, a result also supported by distance analysis of allozyme data (Benzie et al. 1997).

Caulerpa racemosa comprises a complex of varieties and forms that are still poorly understood. Varieties are known to change morphology from one type to another (Calvert et al. 1976, Ohba and Enomoto 1987). Nevertheless, Benzie et al. (1997) showed that allozyme variation within *C. racemosa* varieties was comparable with that found between other *Caulerpa* species. Recently, Famà et al. (2000) reported high intraindividual internal transcribed sequence polymorphism in *C. racemosa*. The possible causes of this variation could

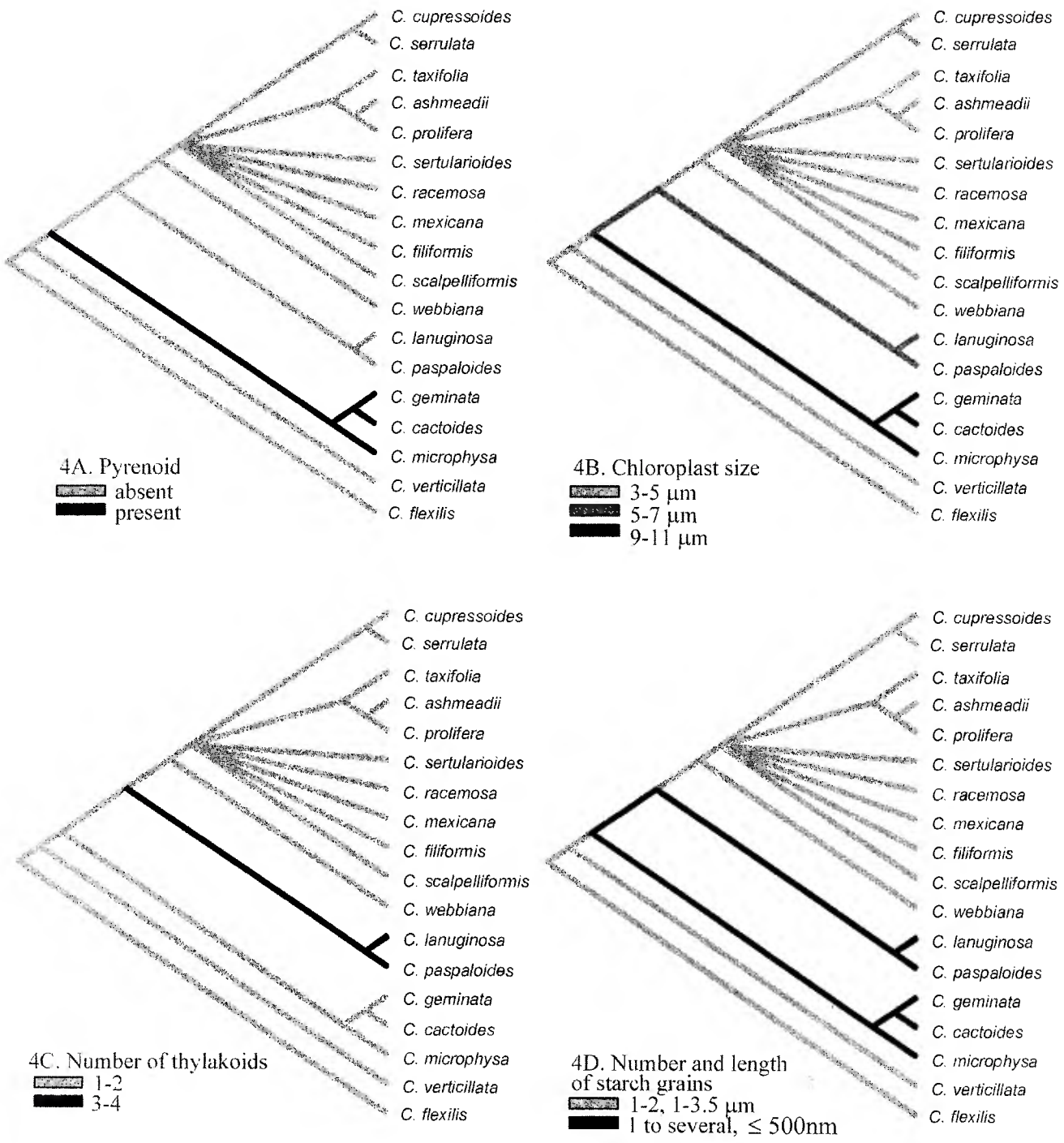

FIG. 4. Cladograms derived from chloroplast *tuf*A sequences. The branches of different gray-scaled colors represent the distribution of chloroplast character states (Table 4) among 18 *Caulerpa* species. (A) Pyrenoid, (B) chloroplast, (C) thylakoids, and (D) starch grains.

be attributed to the maintenance of ancestral polymorphism and/or incomplete lineage sorting, which may be associated to other factors such as asexual reproduction, polyploidy, the presence of ribosomal cistrons on multiple chromosomes, and hybridization. Unlike nuclear genes, plastid genes are effectively reproducing clonally, because of the typically uniparental mode of inheritance. However, lack of recombination of chloroplast DNA (Birky 1995) could give rise to distinct types that can in theory be encountered even within clusters of populations.

Delwiche et al. (1995) showed that incongruence exists between trees constructed from plastid *tuf*A and *rbc*L/*rbc*S sequences. Possible explanations of incongruence between these two genes include horizontal gene transfer of the RUBISCO operon or the presence in the ancestral proteobacteria and cyanobacteria of two sets of RUBISCO genes. Alternative expla-

nations are noisy data and/or one or both data sets are not producing the correct tree.

The analyses of *tuf*A sequences from six varieties of *C. racemosa* show that this species is polyphyletic, confirming earlier conclusions based on allozyme data (Benzie et al. 1997). Furthermore, in *C. racemosa* congruence exists among major lineages (e.g. *C. racemosa* var. *lamourouxii* from the western Pacific and *C. racemosa* var. *turbinata* from the Red Sea, *C. racemosa* var. *macrophysa* from the Caribbean and Gulf of Mexico), identified by nrITS1, *rbc*L (Famà et al., unpublished data), and *tuf*A phylogenies.

One specimen included in this study does not conform to any recognized species description. The complete absence of assimilators distinguishes this unidentified species from most other described species of *Caulerpa*, with the exception of some varieties of *C. racemosa* (e.g. *C. racemosa* var. *lamourouxii* f. *requienii* and *C. racemosa* var. *simplicima*). Based on *tuf*A sequence data, *C. ashmeadii* is the closest relative to this unidentified specimen. Sequence divergence between this unidentified species and *C. ashmeadii* is 1%, suggesting it is a distinct species. To establish the validity of this taxon as a new species or as a morphological variant of *C. ashmeadii*, *tuf*A sequences of additional samples are required.

In conclusion, although complete clarification of *Caulerpa* systematics will necessarily require the examination of representatives of other species together with the use of additional genes, the *tuf*A phylogenetic results reveal that *Caulerpa* itself consists of a series of relatively ancient and species-poor lineages and a relatively modern and rapidly diversifying clade containing most of the morphological and species diversity. These discrete lineages are in disagreement with the taxonomy of *Caulerpa* inferred from chloroplast and morphological features, and many species do not appear to be monophyletic. A well-supported molecular based phylogeny, of which this is a start, may aid in the discovery of additional morphological characters that will define evolutionary species.

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