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Variability in the *rbcL* Introns of Caulerpalean Algae (Chlorophyta, Ulvophyceae)

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Only 10 examples of introns in the *rbcL* gene have been reported to date. Four new cases from Caulerpales, Ulvophyceae are described here. In the genus *Caulerpa*, the presence of an intron was unstable even in the infraspecific taxa. Based on comprehensive comparisons of the inserted positions, lengths of introns and so on, the presence of at least three kinds of introns, which probably have independent origins, was suggested in Caulerpales.

Key words: Caulerpales — Intron — *rbcL* — Ulvophyceae

The *rbcL* gene encoding the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase is located in the chloroplast genome of photosynthetic eukaryotes as well as that of many prokaryotes. The *rbcL* genes are very stable in their sequence levels, the exons being nearly the same length in all green plants except for a few angiosperm. Thus, there are no gaps in multiple alignments of either nucleotide or amino acid sequences, except for the extreme C terminal end of *rbcL* in green plants. Amino acid sequence similarities are above 80%. The *rbcL* gene is, therefore, useful for phylogenetic analyses across a broad range of taxa (Morden *et al.* 1992, Chase *et al.* 1993, Hasebe *et al.* 1995, Daugbjerg and Andersen 1997).

Although thousands of *rbcL* gene sequences have been accumulated, introns in *rbcL* have rarely been reported. In fact, only 10 cases from Euglenophyta and Chlorophyta have been described to date. Introns (group II) in the *rbcL* gene were first discovered in *Euglena gracilis* of Euglenophyta, which has nine introns (Gingrich and Hallick 1985a, b), and then later reported in several other species of the genus *Euglena* and in the related genus *Astasia longa* (Siemeister and Hachtel 1990, Thomson *et al.* 1995). In the Chlorophyceae, group I introns in the *rbcL* gene have been reported recently in *Pleodorina californica* and *Gonium multicoccum*, which have one and two introns, respectively (Nozaki *et al.* 1998). As for the Ulvophyceae, an intron in the *rbcL* gene has been reported only in *Bryopsis maxima* (Kono *et al.* 1991) and *Codium fragile* (Manhart and Vonderhaar 1991) of Caulerpales.

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Here, we report nucleotide sequences of the *rbcL* genes for 10 Caulerpalean algae and newly discovered introns in 4 of them. Results show that the presence of a *rbcL* intron is unstable in genera and even in species. In addition, phylogenetic analysis based on the nucleotide sequences of the *rbcL* exons suggests multiple instances of intron gains and losses in Caulerpales.

Materials and Methods

Most samples for DNA extraction were collected in the field. A cultivated strain was used for *Bryopsis plumosa*. M. Satoh (Tokushima Univ., Japan) let us use isolated chloroplast DNAs from *Caulerpa brachypus* and *Codium lucasii*. In total, 10 taxa belonging to five of six families of Caulerpales were newly analyzed. In addition, we included previously reported *rbcL* gene sequences, *Bryopsis maxima* and *Codium fragile* in Caulerpales and other species of Chlorophyta, Glaucophyta, and Cyanophyta (Table 1). Hereafter, we abbreviate the genus names within species names as follows, *Bryopsis* (*B.*), *Caulerpa* (*Ca.*), and *Codium* (*C.*). Total DNA was isolated from living tissues using the CTAB method (Doyle and Dickerson 1987, Doyle and Doyle 1987). Total DNAs and chloroplast DNAs were used to amplify *rbcL* sequences by polymerase chain reaction (PCR) using the following 5′ and 3′ primers, respectively: rbc1 or U1-1; U3-2 or 3-2 (Table 2). Fragments were checked on 0.7% agarose gels, and PCR products excised from agarose gels were purified with the Ultra Clean 15 kit (Mo Bio Laboratories). Purified DNAs were ligated into T-vectors using the pGEM T Easy Vector System I kit (Promega) and transformed in DH5α competent cells. Insert sizes were checked by performing PCR with the universal forward and reverse primers. Three clones containing PCR fragments were selected per sample, and their plasmids were isolated using the alkali-SDS method (Sambrook *et al.* 1989). Plasmid DNAs were sequenced with the universal forward and reverse sequencing primers and several internal primers (Table 2). Because the *rbcL* genes of *B. plumosa* and *C. lucasii* are very long, plasmid DNAs containing their *rbcL* genes were first digested

Table 1. Taxon sampling, sample information, and DDBJ accession numbers

Taxon	Collection/source of data: collector and date	Accession no.
Chrolophyta		
Ulvophyceae		
Caulerpales		
Bryopsidaceae		
Bryopsis plumosa (Huclson) C. Agardh	Cultivated in Fac. of Sci., Yamagata Univ., Yamagata Pref.: *Hishinuma*. July 2, 1992	AB038480
Bryopsis maxima Okamura	Kono *et al*. (1991)	X55877
Pedobesia ryukyuensis (Yamada et Tanaka) Kobara et Chihara	Kanagawa Pref.: Aburatsubo, Miura city, *Hanyuda*. June 13, 1999	AB038482
Caulerpaceae		
Caulerpa brachypus Harvey	Tokushima Pref.: Uchizuma Beach, Mugi-cho, *Satoh*. June 24, 1997	AB038483
Caulerpa okamurae Weber-van Bosse in Okamura	Kanagawa Pref.: Aburatsubo, Miura city, *Hanyuda*. June 13, 1999	AB038484
Caulerpa rasemosa (Forsskål) J. Agardh var. *clavifera* (Turner) Weber-van Bosse f. *macrophysa* (Kützing) Weber-van Bosse	Okinawa Pref.: Uken, Gushigawa city, *Arai*. August 6, 1999	AB038485
Caulerpa rasemosa (Forsskål) J. Agardh var. *peltata* (Lamouroux) Eubank	Yamaguchi Pref.: Tohwa-cho, *Arai*. July 20, 1999	AB038486
Codiaceae		
Codium fragile (Suringar) Hariot	Manhart and Vonderhaar (1991)	M67453
Codium lucasii Setchell in Lucas	Kohchi Pref.: Murotomisaki-cho, *Satoh*. April 27, 1997	AB038481
Dichotomosiphonaceae		
Dichotomosiphon tuberosus (A. Braun) Ernst	Okinawa Pref.: Ooyama, Ginowan city, *Arai*. October 3, 1997	AB038487
Udoteaceae		
Halimeda discoidea Decaisne	Okinawa Pref.: Uken, Gushigawa city, *Arai*. August 6, 1999	AB038488
Halimeda opuntia (Linnaeus) Lamouroux	Okinawa Pref.: Uken, Gushigawa city, *Arai*. August 6, 1999	AB038489
Charophyceae		
Chara connivens Salzmann ex A. Broun	Manhart (1994)	L13476
Coleochaete orbicularis Prings.	Manhart (1994)	L13477
Spirogyra maxima (Hassall) Wittrock	Manhart (1994)	L11057
Zygnema peliosporum Witter.	McCourt *et al*. (1995)	U38701
Chlorophyceae		
Chlamydomonas reinhardtii Dangeard	Roesler and Ogren (1990)	M62962
Eudorina unicocca G.M. Smith	Nozaki *et al*. (1995)	D63434
Gonium multicoccum Pocock	Nozaki *et al*. (1995)	D63435
Pledorina californica Shaw	Nozaki *et al*. (1995)	D63439
Prasinophyceae		
Cymbomonas tetramitiformis Schiller	Daugbjerg *et al*. (1994)	L34687
Mantoniella squamata (Manton et Park) Desikachary	Daugbjerg *et al*. (1995)	U30278
Nephroselmis minuta (N. Carter) Butcher	Daugbjerg *et al*. (1995)	U30286
Pseudoscourfieldia marina (Throndsen) Manton	Daugbjerg *et al*. (1995)	U30279
Glaucophyta		
Glaucophyceae		
Cyanophora paradoxa Korshikov	Valentin and Zetsche (1990)	X53045
Cyanophyta		
Cyanophyceae		
Anabeana sp.	Curtis and Haselkorn (1983)	L02520
Synechocystis sp.	Kaneko *et al*. (1995)	D64000
Prochlorophyta		
Prochlorothrix hollandica Burger-Wiersma *et al*.	Morden and Golden (1991)	X57359

Table 2. Primer sequences used for PCR amplification and sequencing of *rbcL* regions

Primer name	Primer sequence	Primer position[d]
rbc1[a]	5′-CCA MAA ACW GAA ACW AAA GC-3′	7-26
U1-1[b]	5′-TCC AAA AAC TGA AAC TAA AGC AGG-3′	6-29
1U[b]	5′-TCT TCT ACW GGW ACA TGG AC-3′	181-200
2U[b]	5′-TTG GTW ACW GAA CCT TCT TC-3′	344-325
rbc5[a]	5′-GCT TGW GMT TTR TAR ATW GCT TC-3′	689-667
nP2R[b]	5′-TCA ATA ACC GCA TGC ATT GC-3′	905-886
3U[b]	5′-GGC ATA TGC CAW ACR TGR ATA CC-3′	1163-1141
U3-2[b]	5′-TCT TTC CAA ACT TCA CAA GC-3′	1391-1372
3-2[c]	5′-CCA TAC TTC ACA AGC AGC AGC TAG TTC-3′	1386-1360

[a] These primers were designed by K. Doi (pers. comm.).
[b] These primers were designed in this study.
[c] This primer was designed by Hasebe *et al.* (1992).
[d] Primer positions were numbered according to their position in the *Chlorella vulgaris rbcL* gene (Wakasugi *et al.* 1997).

by several restriction enzymes. They were then cloned and sequenced as mentioned above. Sequencing was performed on an A.L.F. II autosequencing machine (Amersham Pharmacia) using the Thermo Sequenase cycle sequencing kit (USB). Nucleotide sequences of the *rbcL* exons of 28 taxa shown in Table 1 were aligned using Clustal W (Thompson *et al.* 1994). The front and rear regions of alignment were removed because exon sequences are unrevealed in a few or more taxa. The remaining regions (1,133 bp), corresponding to the 27th to 1,159th nucleotides of the *Chlorella vulgaris rbcL* gene sequences (Wakasugi *et al.* 1997), were used for subsequent analyses.

Phylogenetic analyses were performed using maximum likelihood (ML), maximum parsimony (MP), and neighbor joining (NJ). We used Anabaena sp., *Prochlorothrix hollandica*, and *Synechocystis* sp. (Cyanophyta) as the outgroup. NJ trees were constructed using Clustal W. PAUP (Swoford 1993) was used to obtain MP trees, using either character state step matrices (Albert *et al.* 1993) or equal character weighting. The swapping strategy employed was nearest neighbor interchanges branch swapping (NNI), with the MULPARS and Steepest descent options selected and 1,000 random additions. Resulting trees were further analyzed using tree bisection-reconnection branch swapping (TBR) (again employing the MULPARS and Steepest descent options). MOLPHY (Adachi and Hasegawa 1996) was performed to obtain a ML tree. We first did a quick add OTUs search (number of retained top ranking trees by Approx. likelihood is 500). We then performed a local rearrangement search for the resulting topologies of NJ and MP, and a quick add OTUs search of ML, and chose the tree with the highest likelihood score. Based on this topology, we performed further local rearrangement searches to seek a ML tree and to obtain local bootstrap probabilities (LBP). The highest likelihood tree is shown in Fig. 7. Bootstrap values were obtained using PAUP and are based on 1,000 replicates.

Results and Discussion

The lengths of amplified PCR fragments from *Bryopsis plumosa, Caulerpa okamurae, Ca. racemosa* var. *clavifera* f. *macrophysa*, and *Codium lucasii* are larger than those of the other taxa from which only the *rbcL* exon was amplified (Fig. 1). This implies that introns are present in the *rbcL* genes of these four taxa. For the genus *Caulerpa*, the nucleotide sequences of the putative introns were determined based on alignments of *rbcL* sequences between *Caulerpa* species, one of which has long PCR fragments whereas the others have short fragments. Sequences of the putative introns of *B. plumosa* and *C. lucasii* were also determined based on alignments of sequences with *B. maxima* and *C. fragile*, respectively. Alignments of putative exons of these four taxa with the nucleotide sequences of the *Chlorella vulgaris rbcL* gene (Wakasugi *et al.* 1997) show no gaps and over 80% sequence similarity (not shown). In addition, the partial 5′ and 3′ terminal nucleotide sequences of these putative introns (Fig. 2) resemble the consensus sequences of group I introns (Michel *et al.* 1989), and their secondary structure models of domains V and VI (Fig. 3) are similar to consensus secondary structure models of group II introns (Michel *et al.* 1989). Therefore, these introns are considered to be group II introns. In addition, the several features of the *Caulerpa* introns most closely fit those of subgroup IIB introns. The bulged nucleotide A on the 3′ side of hairpin VI is defined as being eight nucleotides upstream from the 3′ intron-exon junction in subgroup IIB introns and seven nucleotides upstream in subgroup IIA introns. The former and latter introns usually end with RAY and YAY, respectively (Figs. 2 and 3) (Michel *et al.* 1989). On the other hand, it was indistinct whether the *Bryopsis* and *Codium* introns belong to subgroup IIA or IIB because their bulging A is seven nucleotides upstream of the 3′ intron-exon junctions, but these introns end with RAY. Figs. 4 and 5 show the aligned sequences with exons and introns. The lengths of the *Ca. okamurae* and *Ca. racemosa* var. *clavifera* f. *macrophysa* introns are 739

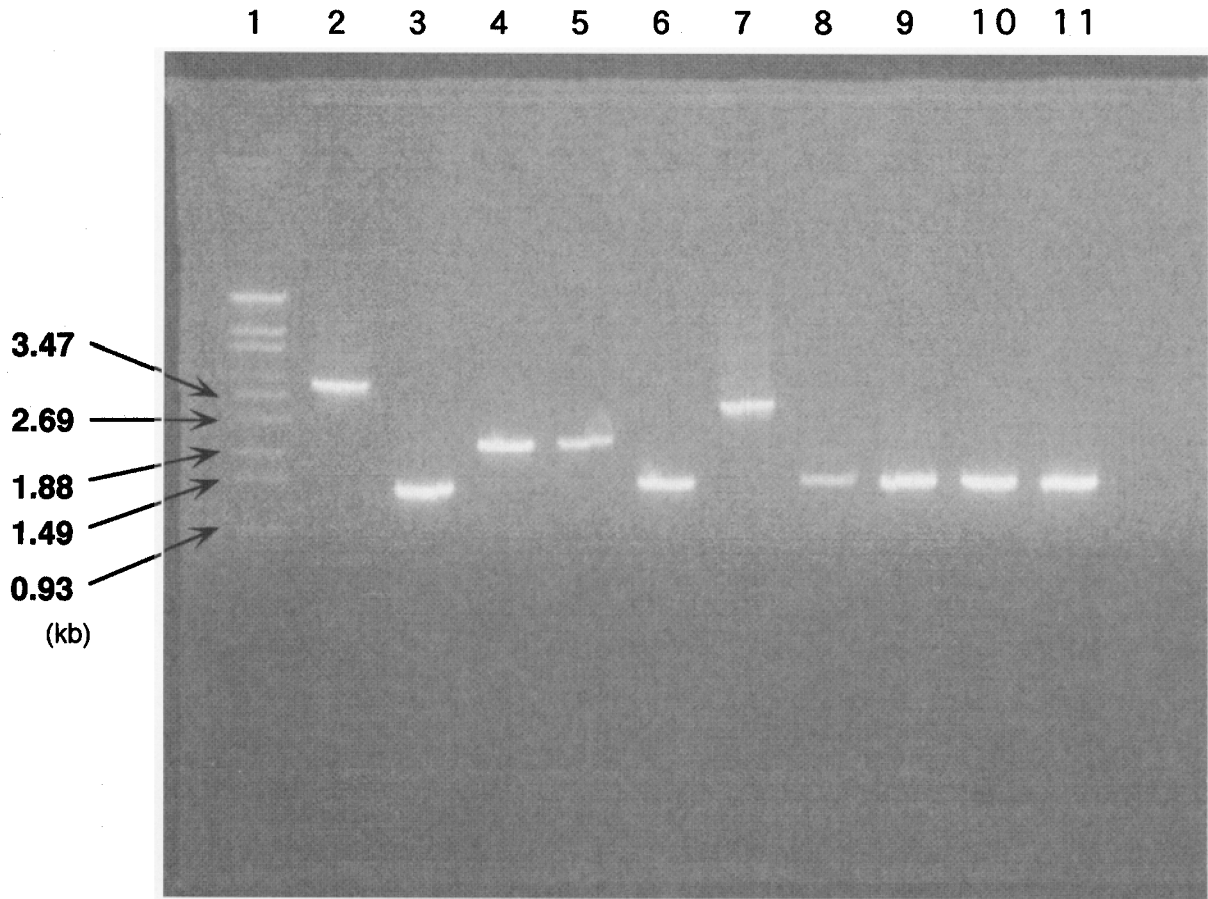

Fig. 1. Agarose gel electrophoretic patterns of PCR products from a partial *rbcL* gene. PCR reaction products were visualized by ethidium bromide staining. Sty I- cut λ DNA size fragments are indicated by numbers (kb). Lane 1=λ standard; lane 2=*Bryopsis plumosa*; lane 3=*Pedobesia ryukyuensis*; lane 4=*Caulerpa brachypus*; lane 5=*Caulerpa okamurae*; lane 6=*Caulerpa racemosa* var. *clavifera* f. *macrophysa*; lane 7=*Caulerpa racemosa* var. *peltata*; lane 8=*Codium lucasii*; lane 9=*Dichotomosiphon tuberosus*; lane 10=*Halimeda discoidea*; lane 11=*Halimeda opuntia*.

II A	GTGCGYC · · · · · · · · · · · · · · ·		YAYYYYAY
II B	GTGYGAY · · · · · · · · · · · · · · ·		YATCYYRAY
Ca-o	GTGCGATCC · · · · ·	739bp · · · ·	TAGTTTAAC
Ca-r	GTGCGATTC · · · · ·	735bp · · · ·	TAGTTTAAC
Br-p	GTGCGACAC · · · ·	2467bp · · · ·	TACCCGAC
Co-l	GTGTGACAC · · · ·	1739bp · · · ·	TACCCGAC

Fig. 2. Alignment of portions of *rbcL* intron sequences (*Caulerpa okamurae* (Ca-o), *Caulerpa racemosa* var. *clavifera* f. *macrophysa* (Ca-r), *Bryopsis plumosa* (Br-p), *Codium lucasii* (Co-l) with consensus sequences for subgroup IIA (IIA) and IIB (IIB) of group II introns (Michel *et al.* 1989). Numbers show the total lengths of introns. Underlines indicate the bulged nucleotide A.

bp and 735 bp, respectively, and no ORF was discovered within either. Likewise, there is no ORF within the intron of *C. lucasii*, which has a length of 1,739 bp. On the other hand, there is an ORF within the *B. plumosa* intron, which has a length of 2,467 bp. This ORF (275 aa) comprises the 1,277th to 2,101st nucleotides of the intron, and its amino acid sequence is very similar to that of *B. maxima* (99% sequence similarity) (Kono *et al.* 1991).

Although introns are present in *Bryopsis plumosa* (Bryopsidaceae), *Codium lucasii* (Codiaceae), one of two varieties of *Caulerpa rasemosa* (Caulerpaceae), and *Ca. okamurae*, there are no introns in *Pedobesia ryukyuensis* (Bryopsidaceae), *Dichotomosiphon tuberosus* (Dichotomosiphonaceae), two species of *Halimeda* (Udoteaceae), one other species of *Caulerpa* and the other variety of *Ca. rasemosa* analyzed here. Thus, the presence of a *rbcL* intron is unstable at the level of genus and even of species.

The *Caulerpa okamurae* and *Ca. racemosa* var. *clavifera* f. *macrophysa* introns are inserted in the same positions, and their nucleotide sequences and secondary structure models of domains are markedly similar (84% sequence similarities) (Figs. 2, 3, 4). This suggests a common origin. Likewise, the congruence of the inserted position and secondary structure models of domains and the high nucleotide sequence similarities (99%) of the *B. maxima* and *B. plumosa*

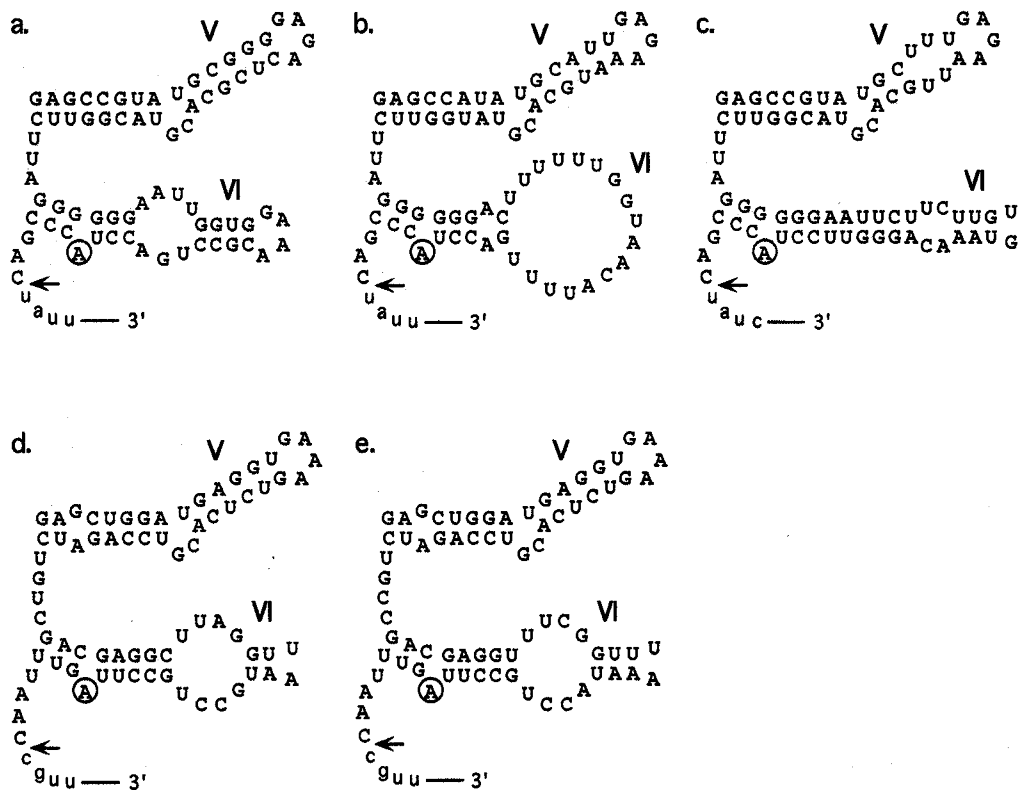

Fig. 3. Secondary structure models of domains V and VI in the *rbcL* introns based on Michel *et al.* (1989) from a) *Bryopsis plumosa* and *Bryopsis maxima*; b) *Codium fragile*; c) *Codium lucasii*; d) *Caulerpa okamurae* and e) *Caulerpa racemosa* var. *clavifera* f. *macrophysa*. The small characters point to exon sequences. Arrows show the intron-exon junctions. Nucleotides in circles indicate the bulged nucleotide A.

introns suggest that they also have a single origin. Although the introns of *C. lucasii* and *C. fragile* are located in the same positions, their secondary structure models of domain VI are different and their nucleotide similarities are lower (66%).

The positions and lengths of the *Caulerpa* introns are considerably different from those of the *Bryopsis* and *Codium* introns and are also different from those of any other reported *rbcL* introns (Fig. 6). The position of insertion into the *rbcL* gene of the *Caulerpa* introns resembles that of one of the introns in *Euglena gracilis* and *Astasis longa*, but the *Caulerpa* introns were located 2 bp further upstream than those of *Euglena gracilis* and *Astasis longa*, and they were about 1.5-2 times longer than the *Euglena gracilis* and *Astasis longa* introns (Fig. 6). In addition, the *Caulerpa* introns have significantly different nucleotide compositions and secondary structure models of the VI domains from the *Bryopsis* and *Codium* introns (Fig. 3). These observations suggest that the origin of the *rbcL* introns in *Caulerpa* is different from that of the *Bryopsis, Codium* and other reported *rbcL* introns.

The phylogenetic tree based on the *rbcL* exon sequences shows the monophyly of the four *Caulerpa* taxa (Fig. 7). In this monophyletic clade, *Caulerpa okamurae* and *Ca. racemosa* var. *clavifera* f. *macrophysa* are not placed together though their *rbcL* introns probably have a common origin. Based on these results, the following two scenarios are inferred to explain the processes of acquisition of these *Caulerpa* introns. (1) Single acquisition: A *Caulerpa* intron was acquired prior to the diversification of all four *Caulerpa* taxa analyzed here, and then spread among *Caulerpa* species via vertical transfer. The intron was then lost in *Ca. brachypus* and *Ca. rasemosa* var. *peltata* or in their ancestors. (2) Double acquisition: *Caulerpa* introns were acquired separately twice, in lineages of *Ca. okamurae* and *Ca. racemosa* var. *clavifera* f. *macrophysa*, via horizontal transfer. In this case, the transfer of introns via infection by a virus or fungus containing introns, or the removal of introns of different genes within the same genome is inferred as the cause of horizontal transfer. Recently, horizontal transfers via virus or lichenization within or among closely related species were suggested for fungus nrDNA group I introns (Bhattacharya *et al.* 1996, Holst-Jensen *et al.* 1999, Friedl *et al.* 2000). In addition, intragenomic horizontal transfer between angiospermous mtDNA group II introns was also suggested (Laroche and Bousquet 1999).

The introns of the *Bryopsis* and *Codium* species have the same inserted positions, but their lengths and secondary structure models of domain VI are significantly different and their sequence similarities are too low for alignment. There-

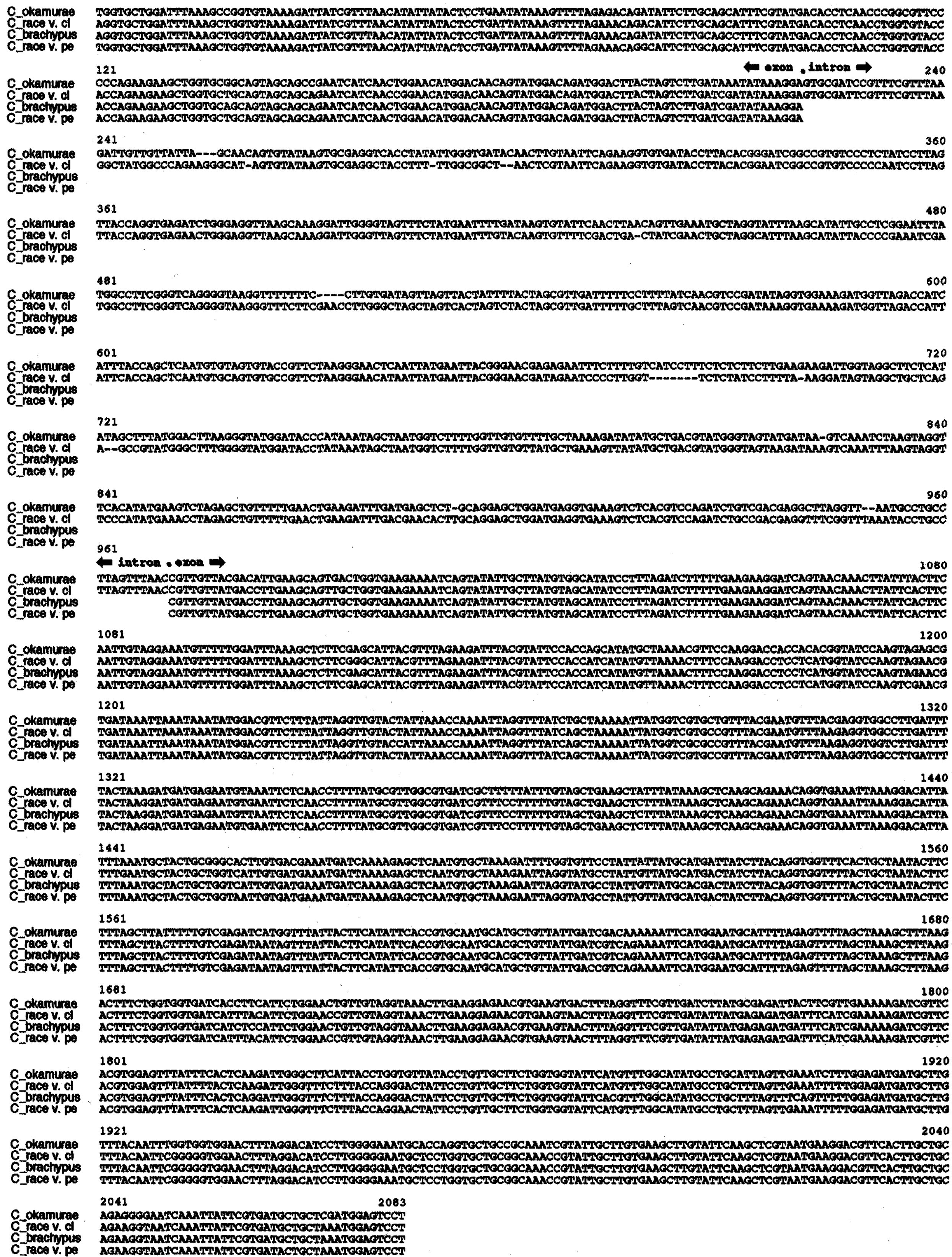

Fig. 4. Alignment of the *rbcL* genes in *Caulerpa okamurae* (C_okamurae), *Caulerpa racemosa* var. *clavifera* f. *macrophysa* (C_race v. cl), *Caulerpa brachypus* (C_brachypus) and *Caulerpa racemosa* var. *peltata* (C_race v. pe). Numbers point to the positions within the alignment.

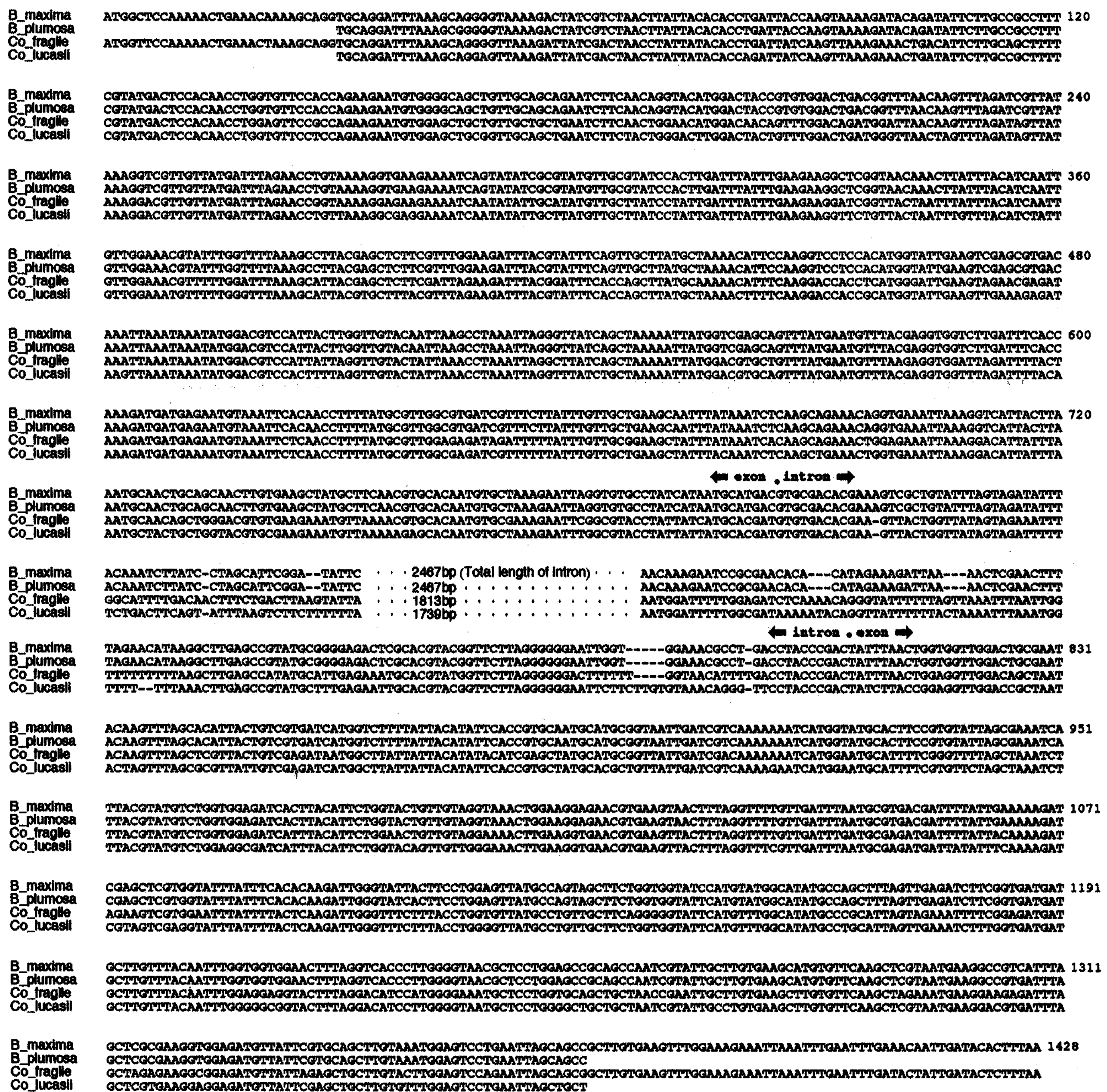

Fig. 5. Alignment of the *rbcL* exons and 5′ and 3′ terminal portions of introns in *Bryopsis maxima* (B_maxima), *Bryopsis plumosa* (B_plumosa), *Codium fragile* (Co_fragile) and *Codium lucasii* (Co_lucasii). Numbers in the right margin show the exon positions corresponding to the *Bryopsis maxima rbcL* exons (Kono *et al.* 1991).

fore, the *Bryopsis* and *Codium* introns have different origins and were inserted independently. These observations and phylogenetic relationships among *Bryopsis, Codium*, and *Pedobesia* (Fig. 7) suggest that *Codium* introns were gained after the divergence of *Pedobesia ryukyuensis* and *Bryopsis* introns were inserted into a common ancestor of *B. fragile* and *B. maxima*.

Finally, it was suggested that *rbcL* introns were acquired separately at least three times in Caulerpales.

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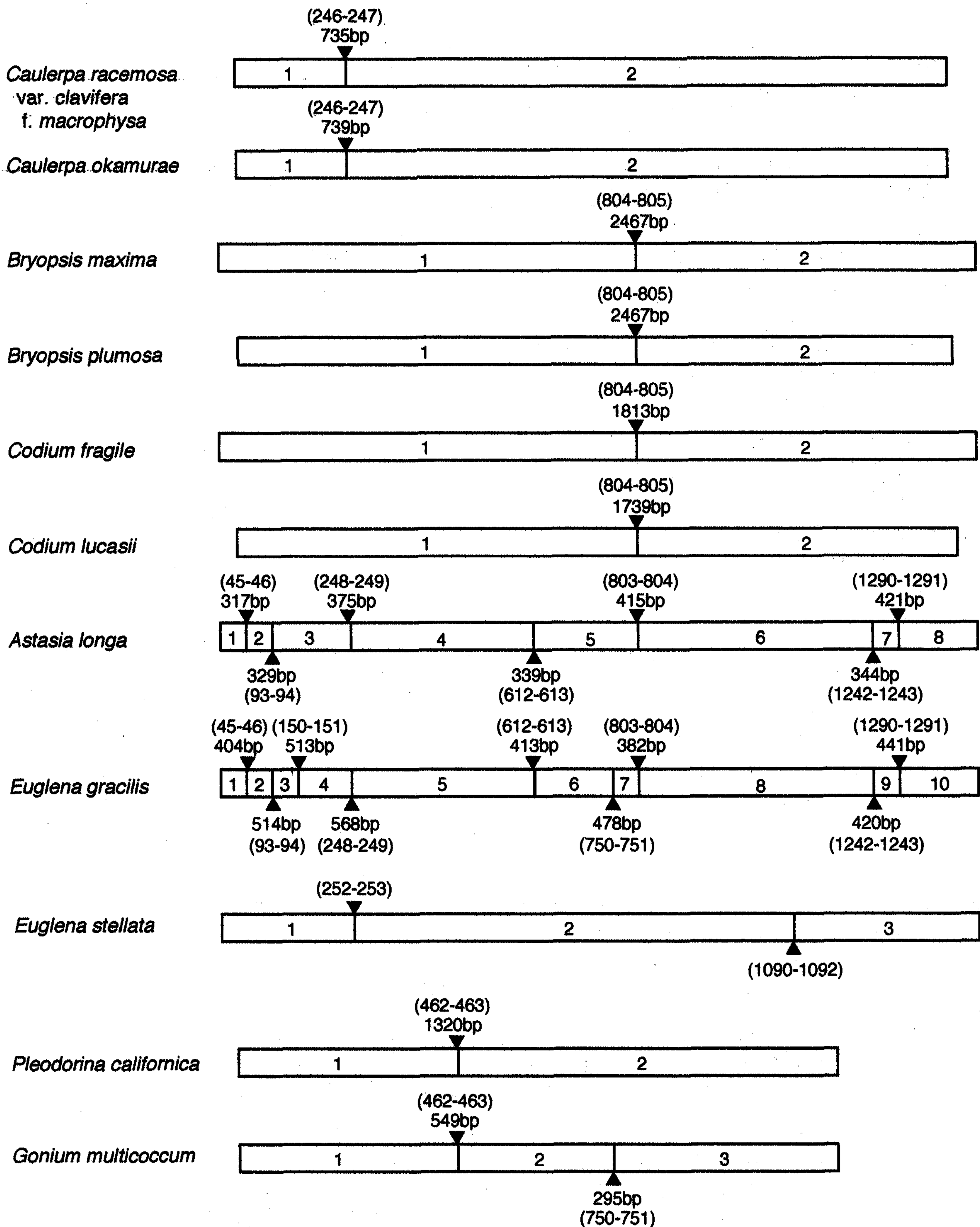

Fig. 6. Physical maps of the *rbcL* gene inserted into the intron(s). Exons are open boxes and numbered from left. Triangles indicate the inserted positions of introns in the *rbcL* gene, and the number above or below each triangle shows the length of the intron. Numbers in parentheses show the exon position coordinates to the *rbcL* gene of *Chlorella vulgaris* (Wakasugi *et al*. 1997).

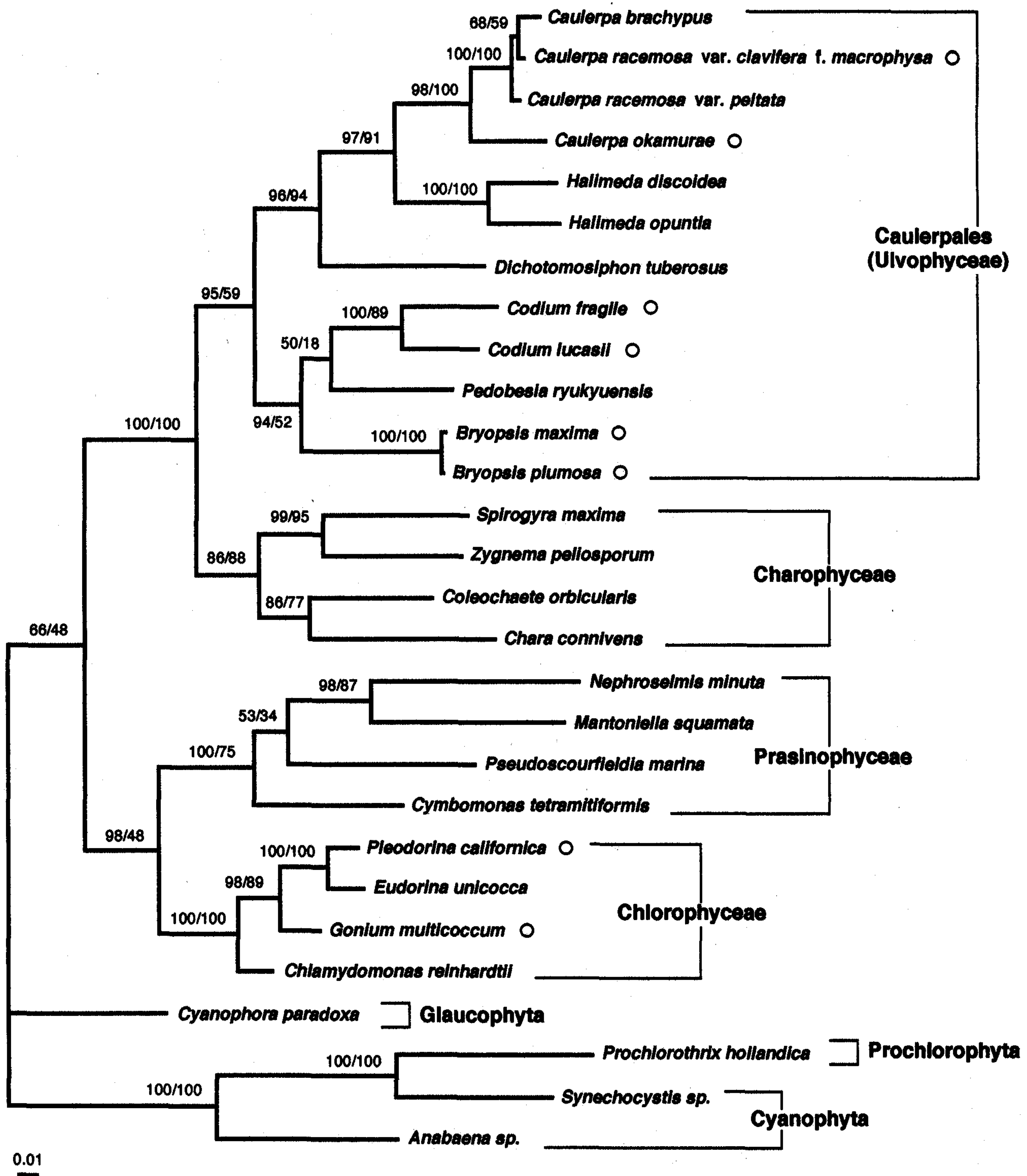

Fig. 7. Maximum likelihood tree of 28 OTU based on *rbcL* exon sequences (1133 bp). Numbers indicate local bootstrap probability (left) obtained by MOLPHY and bootstrap probability (right) obtained by PAUP for the same topology. Open circles show the existence of *rbcL* introns. Scale indicates base substitution rate.

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