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Taku Misonou, Mamiko Seto and Takeshi Nitta: Phylogenetic relationship of three *Bryopsis* species (Derbesiales, Chlorophyta) based on 16S ribosomal RNA sequences

Key Index Words: Bryopsis *(Chlorophyta)—chloroplast 16S rRNA—molecular evolution—phylogeny—sequence divergence.*
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We attempted a molecular phylogenetic approach based on small-subunit rRNA (ribosomal RNA) sequences in three members of genus *Bryopsis*. In this study, we targeted the chloroplast 16S rRNA molecule for the partial sequencing. Their phylogenetic relationship previewed by organelle DNA RFLP (Restriction Fragment Length Polymorphism) analysis (Misonou *et al.* 1989) and some characteristics of *Bryopsis* 16S rRNA were discussed. The algae used were *Bryopsis maxima* Okamura, *B. plumosa* (Hudson) C. Agardh, and *B.* sp. (Misonou *et al.* 1989). The first was collected at Choshi in Chiba, on the Pacific coast, the second at Yokosuka in Kanagawa, and the third at Futtsu in Chiba, from November 1986 to May 1990. The second and third sites are on Tokyo Bay.

Chloroplasts were isolated from algal thalli in an alkaline buffer containing EDTA (Misonou *et al.* 1989, Ishihara *et al.* 1992). Total nucleic acids were extracted by the chloroform/phenol method from this fraction and RNAs were purified with the DNase I treatment. 16S rRNA partial sequences of each species were determined directly with dideoxynucleotide chain termination method using reverse transcriptase accrding to Lane *et al.* (1985). The three primers used are complimentary to the *Escherichia coli* 16S rRNA highly conserved region. Their positions and sequences are I: 357–342 (5′CTGCTGCCTCCCGTAGOH3′), II: 1242–1227 (5′CCATTGTAGCACGTGTOH3′) and III: 1510–1495 (5′GGCTACCTTGTTACGAOH3′) according to *E. coli* nomenclature (Sawada *et al.* in press).

The sequences read with each primer were 466 bases and aligned with the homologous sequence of *Chlorella vulgaris* strain 211-11b 16S rRNA (Neefs *et al.* 1990) as an outgroup in Fig. 1. *Bryopsis* 16S rRNAs show intrageneric sequence divergence between each species (Fig. 1). It might be considered that the speciation of these organisms is an ancient incident or the rate of 16S rRNA molecule evolution in this genus is faster than that of higher plant.

Of the total 466 bases, 443 bases were comparable to each other in sequence. The remaining of 23 bases could not be identified by anomalous bands across the lanes on the gel owing to the nucleotide modification. While the sequences with primer II were homologous, the primer I region of *B. plumosa* has 1 deletion (No. 103 in Fig. 1 I), and sequences with primer III were heterogeneous (Fig. 1). In this region, the chain termination reaction continued only up to 100 bases with 19 anomalous bands. It seems that primer III complimentary to *E. coli* sequence anneals to *Bryopsis* RNAs in low extent due to the sequence heterogeneity, and/or this region is abundant in modified nucleotides.

The genetic distances of these three regions were calculated according to Kimura's 2 parameter method (Kimura 1980) and presented in Table 1. The primer III region of 3 *Bryopsis* is not only heterogeneous each other but also shows low homology with *Chlorella* sequence. Moreover, no homologous sequence with this region was found in GEN-

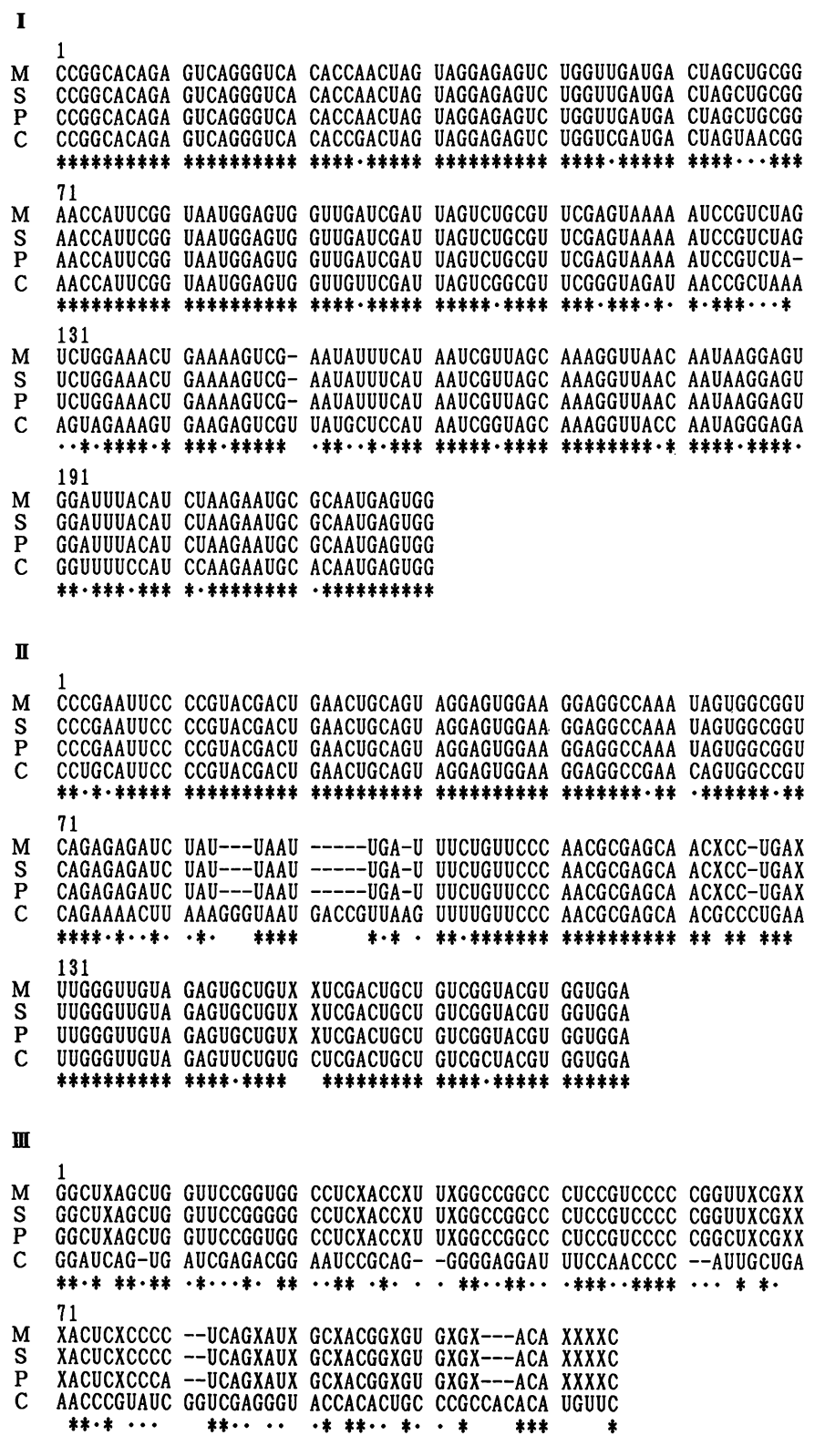
I
1
M CCGGCACAGA GUCAGGGUCA CACCAACUAG UAGGAGAGUC UGGUUGAUGA CUAGCUGCGG
S CCGGCACAGA GUCAGGGUCA CACCAACUAG UAGGAGAGUC UGGUUGAUGA CUAGCUGCGG
P CCGGCACAGA GUCAGGGUCA CACCAACUAG UAGGAGAGUC UGGUUGAUGA CUAGCUGCGG
C CCGGCACAGA GUCAGGGUCA CACCGACUAG UAGGAGAGUC UGGUCGAUGA CUAGUAACGG
71
M AACCAUUCGG UAAUGGAGUG GUUGAUCGAU UAGUCUGCGU UCGAGUAAAA AUCCGUCUAG
S AACCAUUCGG UAAUGGAGUG GUUGAUCGAU UAGUCUGCGU UCGAGUAAAA AUCCGUCUAG
P AACCAUUCGG UAAUGGAGUG GUUGAUCGAU UAGUCUGCGU UCGAGUAAAA AUCCGUCUA-
C AACCAUUCGG UAAUGGAGUG GUUGUUCGAU UAGUCGGCGU UCGGGUAGAU AACCGCUAAA
131
M UCUGGAAACU GAAAAGUCG- AAUAUUUCAU AAUCGUUAGC AAAGGUUAAC AAUAAGGAGU
S UCUGGAAACU GAAAAGUCG- AAUAUUUCAU AAUCGUUAGC AAAGGUUAAC AAUAAGGAGU
P UCUGGAAACU GAAAAGUCG- AAUAUUUCAU AAUCGUUAGC AAAGGUUAAC AAUAAGGAGU
C AGUAGAAAGU GAAGAGUCGU UAUGCUCCAU AAUCGGUAGC AAAGGUUACC AAUAGGGAGA
191
M GGAUUUACAU CUAAGAAUGC GCAAUGAGUGG
S GGAUUUACAU CUAAGAAUGC GCAAUGAGUGG
P GGAUUUACAU CUAAGAAUGC GCAAUGAGUGG
C GGUUUUCCAU CCAAGAAUGC ACAAUGAGUGG
II
1
M CCCGAAUUCC CCGUACGACU GAACUGCAGU AGGAGUGGAA GGAGGCCAAA UAGUGGCGGU
S CCCGAAUUCC CCGUACGACU GAACUGCAGU AGGAGUGGAA GGAGGCCAAA UAGUGGCGGU
P CCCGAAUUCC CCGUACGACU GAACUGCAGU AGGAGUGGAA GGAGGCCAAA UAGUGGCGGU
C CCUGCAUUCC CCGUACGACU GAACUGCAGU AGGAGUGGAA GGAGGCCGAA CAGUGGCCGU
71
M CAGAGAGAUC UAU---UAAU -----UGA-U UUCUGUUCCC AACGCGAGCA ACXCC-UGAX
S CAGAGAGAUC UAU---UAAU -----UGA-U UUCUGUUCCC AACGCGAGCA ACXCC-UGAX
P CAGAGAGAUC UAU---UAAU -----UGA-U UUCUGUUCCC AACGCGAGCA ACXCC-UGAX
C CAGAAAACUU AAAGGGUAAU GACCGUUAAG UUUUGUUCCC AACGCGAGCA ACGCCCUGAA
131
M UUGGGUUGUA GAGUGCUGUX XUCGACUGCU GUCGGUACGU GGUGGA
S UUGGGUUGUA GAGUGCUGUX XUCGACUGCU GUCGGUACGU GGUGGA
P UUGGGUUGUA GAGUGCUGUX XUCGACUGCU GUCGGUACGU GGUGGA
C UUGGGUUGUA GAGUUCUGUG CUCGACUGCU GUCGCUACGU GGUGGA
III
1
M GGCUXAGCUG GUUCCGGUGG CCUCXACCXU UXGGCCGGCC CUCCGUCCCC CGGUUXCGXX
S GGCUXAGCUG GUUCCGGGGG CCUCXACCXU UXGGCCGGCC CUCCGUCCCC CGGUUXCGXX
P GGCUXAGCUG GUUCCGGUGG CCUCXACCXU UXGGCCGGCC CUCCGUCCCC CGGCUXCGXX
C GGAUCAG-UG AUCGAGACGG AAUCCGCAG- -GGGGAGGAU UUCCAACCCC --AUUGCUGA
71
M XACUCXCCCC --UCAGXAUX GCXACGGXGU GXGX---ACA XXXXC
S XACUCXCCCC --UCAGXAUX GCXACGGXGU GXGX---ACA XXXXC
P XACUCXCCCA --UCAGXAUX GCXACGGXGU GXGX---ACA XXXXC
C AACCCGUAUC GGUCGAGGGU ACCACACUGC CCGCCACACA UGUUC

Table 1. Genetic distances ($\times 10^{-3}$ Knuc) of three regions between each of three *Bryopsis* and *Chlorella* 16S rRNA.

region	M-S	M-P	S-P	M-C	S-C	P-C
I	0.0	0.0	0.0	171.4	171.4	166.1
II	0.0	0.0	0.0	113.5	113.5	113.5
III	12.5	25.1	12.3	681.5	673.0	756.5
total	2.3	4.5	6.8	213.9	213.7	217.4

M: *Bryopsis maxima*; S: *B.* sp.; P: *B. plumosa*; C: *Chlorella vulgaris*.

BANK database. These results show that *Bryopsis* 16S RNA sequences around the position 1400-1500 are not conservative even in the primer III region considered homologous from bacteria to higher plant chloroplast. This may suggest that these algae occupy a unique position in plant kingdom.

From these genetic distances, a phylogenetic tree was inferred in Fig. 2 by UPGMA (Sokal and Michener 1958), and NJ method (Saitou and Nei 1987) with bootstrap confidence limits using the Clustal V computer program (Higgins 1991). In both trees, *B.* sp. is distinguished from other 2 species. It is shown that this alga, having regarded as an intraspecific variation of *B. plumosa*, is an independent species and has rather far relationship with *B. plumosa*. In the UPGMA tree, *B.* sp. is clustered with *B. maxima* as previous RFLP analysis (Misonou *et al.* 1989). On the other hand, although the bootstrap confidence interval is not sufficiently high, *B.*. sp. is separated from *B. maxima*—*B. plumosa* cluster in the NJ tree. In the case of this study, UPGMA tree seems to be more reliable for the close relationship of these algae. More sequence informations may be required for further phylogenetic analyses of *Bryopsis*.

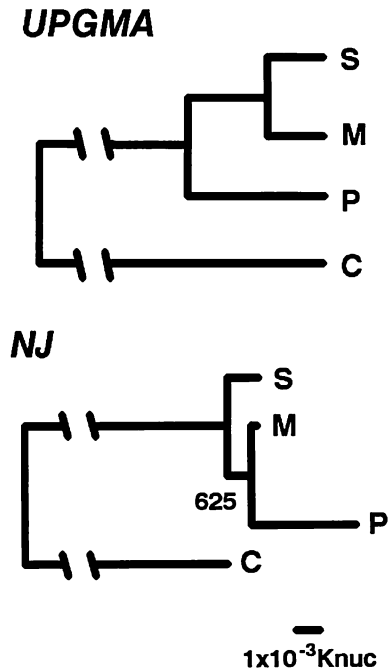

Fig.2. Phylogenetic trees drawn with genetic distances of three *Bryopsis* and *Chlorella* 16S rRNA partial sequences using 2 tree construction methods. M, *Bryopsis maxima*; S, *B.* sp.; P, *B. plumosa*; C, *Chlorella vulgaris* 211-11b. The number under the branch in NJ tree is the confidence interval for nodes based upon 1,000 bootstrap samples.

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Fig. 1. Partial sequences of three *Bryopsis* 16S rRNA. The sequences determined with three primers are aligned. M, *Bryopsis maxima*; S, *B.* sp.; P, *B. plumosa*; C, *Chlorella vulgaris* 211-11b. Hyphens and Xs show deletions and uncleotides that could not be identified, respectively. Asterisks are the homologous nucleotides with three *Bryopsis* and *Chlorella*. Dots are the nucleotides homologous within three *Bryopsis* while not with *Chlorella*.

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御園生　拓*・瀬戸麻美子*・新田　毅**：16S リボソーム RNA 塩基配列による 3 種のハネモ属藻（ツユノイト目，緑藻植物門）の系統関係

大型緑藻ハネモ属の系統を明らかにするために，葉緑体 16S rRNA（リボソーム RNA）の塩基配列を比較し解析することを試みた。逆転写酵素を利用したサンガー反応のプライマーには 16S rRNA に特異的な保存配列を 3 種類用い，計466塩基を読んだ。ミナトハネモ (*Bryopsis* sp.) は独立した種であり，ハネモ (*B. plumosa*) やオオハモネ (*B. maxima*) とは遺伝的に離れているという結果を得た。また，これらのハネモ 16S rRNA の塩基 No. 1400～1500に相当する部分は既知の生物の配列との相同性が低く，これらが他の植物と系統的に離れている可能性が示唆された。（*400 甲府市武田 4　山梨大学教育学部生物学教室，**183 府中市幸町 3　東京農工大学一般教育部生物）

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