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Nuclear DNA Quantification of Some Ceramialean Algal Spermatia by Fluorescence Microscopic Image Processing and their Nuclear SSU rDNA Sequences

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Nuclear DNA contents of spermatia from eight ceramiacean and four dasyacean algae (Ceramiales, Rhodophyta) and microspores from two land plants were estimated by fluorescence microscopic image processing and their nuclear SSU rDNA sequence data were analyzed. In frequency distribution patterns, the DAPI-stained nuclear volume (NV) of spermatia showed two peaks corresponding to 1C and 2C. Nuclear 2C DNA contents estimated from NV were 0.45-2.31 pg in ceramiacean and 0.40-0.57 pg in dasyacean algae and 8.42-9.51 pg in two land plants, *Capsicum annuum* and *Nicotiana tabacum*. By nuclear patterning of vegetative cells derived from an apical cell, 2C DNA contents of spermatia were 2.31 pg in an alga having uninucleate and non-polyploid nucleus (*Aglaothamnion callophyllidicola*), 0.45-1.94 pg in algae having uninucleate and polyploid nucleus (*Antithamnion* spp. and *Pterothamnion yezoense*), and 0.40-0.62 pg in algae having multinucleate and non-polyploid nuclei (*Griffithsia japonica* and dasyacean algae). Each mature spermatium and microspore (pollen grain) seemed to have a 2C nucleus, which may provide a genetic buffering system to protect the genetic content of a spermatium and microspore from potentially lethal mutations. Nuclear DNA content and SSU rDNA sequence of *Antithamnion sparsum* from Korea were reasonably different from those of *Antithamnion densum* from France. The data did not support the previous taxonomic studies that these two taxa could be conspecific.

Key Words: Ceramiales, DAPI, fluorescence microscopic image processing, genetic buffering system, nuclear DNA contents, nuclear SSU rDNA sequences, Rhodophyta, spermatia

INTRODUCTION

Studies of nuclear DNA content are important in establishing evolutionary relationships (Price 1976; Kapraun and Buratti 1998; Kapraun and Dunwoody 2002) as well as relations of DNA contents with growth rate, habit, generation time and life history (Bennett 1972, 1987; Yamaguchi and Imai 1994; Choi and Lee 1996; Reeves *et al.* 1998; Choi and Kim 2001).

Nuclear volumes (NV) have been shown to be closely correlated with the amount of DNA in higher plants (Sparrow and Mischke 1961; Baetcke *et al.* 1967; Price *et al.* 1973; Sparrow and Nauman 1973) and in some green and red algae (Kapraun and Nguyen 1994; Choi and Lee 1996; Choi and Kim 2001).

We first estimated nuclear DNA content from spermatia, carpogonial branches and tetrasporangia as well as vegetative cells of a red alga, *Dasysiphonia chejuensis* I.K. Lee et West (Dasyaceae, Ceramiales) using a fluorescence microscopic image processor (Choi *et al.* 1994; Choi and Lee 1996; Choi and Kim 2001). Kapraun and his colleagues (Kapraun and Dutcher 1991; Kapraun 1993a, 1993b, 1993c; Kapraun and Buratti 1998; Kapraun and Dunwoody 2002) have estimated nuclear DNA contents in some red and green algae by microspectrophotometry.

Even though microspectrofluorometer and flow cytometer have been adopted broadly for the quantitative studies on the fluorochrome-stained DNA (Coleman *et al.* 1981; Goff and Coleman 1984; Kapraun and Dutcher 1991; Yamaguchi and Imai 1994; Kapraun

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and Buratti 1998; Kapraun and Dunwoody 2002), it is so expensive and not easily available. A fluorescence microscopic image processor is, however, much easier to set up than a microspectrofluorometer and flow cytometer, although the former has rarely been used for the quantitative studies on cytology of algae (Choi *et al.* 1994; Lee *et al.* 1995; Choi and Lee 1996; Choi and Kim 2001).

In this study, the fluorochrome DAPI and the fluorescence microscopic image processor were employed to quantify the nuclear DNA of spermatia in some ceramiacean and dasyacean algae (Ceramiales, Rhodophyta) as well as microspores, from which pollen grains develop, of two land plants as controls. In addition, their nuclear small-subunit (SSU) rDNA sequences were determined to assess phylogenetic relationships of these taxa among the Ceramiales.

MATERIALS AND METHODS

Culture

Unialgal cultures were established from algal samples collected in the field (Table 1). The cultures were incubated at 15 ± 1°C, 12:12 h LD, and 15 μmol photons $m^{-2} \cdot s^{-1}$ provided by cool-white 20W fluorescent lamps. Modified PES medium (Provasoli 1966) without Tris buffer was used in culture (Choi and Lee 1996). Media and dishes were replaced every two weeks.

Tetraspores released from each tetrasporangial plant developed into mature male and female gametophytes after 3-4 weeks. We selected mature male gametophytes from each algal species and mature microsporophytes of two land plants for nuclear DNA quantification.

Nuclear DNA quantification

Culture materials of each mature male gametophyte were prepared by microwave fixation (Goff and Coleman 1987) in 0.5 $\mu g \cdot ml^{-1}$ DAPI in seawater. Algal spermatia were examined by fluorescence microscopic image processor based on an arrangement by Choi and Lee (1996) for measure of DAPI fluorescence. Photographs were made with Kodak Ektachrome 400 film using exposure times of 5-10 s.

DAPI-stained spermatial NV were calculated from the expression:

$$NV = \pi d^3 / 6$$

where d = the average diameter assuming the nucleus to be a sphere (Sparrow and Nauman 1973; Choi and Lee 1996; Choi and Kim 2001). DNA content (pg) was calculated from NV estimates:

DNA content = (NV / 15 μm^3) x 1 pg

(Sparrow and Nauman 1973; Kapraun and Nguyen 1994; Choi and Lee 1996; Choi and Kim 2001).

Assuming the minimum DAPI-stained NV of spermatia in the ceramialean algae and microspores in land plants to be 1C (Choi and Lee 1996), we finally determined the polyploidy level of various nuclei.

Nuclear small-subunit rDNA sequence data

To determine SSU rDNA sequences, genomic DNA from eight ceramiacean and four dasyacean algae in the Ceramiales (Table 1) was extracted according to the modified protocol of the CTAB method (Strache-Crain *et al.* 1997). The SSU rDNA was PCR-amplified from total genomic DNA using the primer combinations of Saunders and Kraft (1994, 1996). Direct purification with High Pure™ PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN, USA) and agarose gel purification with Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA) were used to clean PCR products. DNA cleaned by these methods was sequenced with the BigDye™ or dRhodamine Terminator Cycle Sequencing Ready Reaction Kit [PE Applied Biosystems (ABI), Foster City, CA, USA]. Sequence data were collected with the ABI PRISM 3100-Avant Genetic Analyzer or 377 DNA Sequencer. Editing of sequence data were accomplished with the SeqEd DNA sequence Editor (ABI) Software Package.

Edited sequences were aligned relative to one another using the SeqPup multiple alignment program (Gilbert 1995). The final alignment consisted of 13 species, including six previously published sequences (Table 1). The 1812 aligned nucleotide positions of SSU data were edited to move the 5′ and 3′ PCR primers (G01 and G07; Saunders and Kraft 1994), to yield 1761 bp for phylogenetic inference. Sequence data analyses were completed with PAUP* (Swofford 2002) and MacClade (Maddison and Maddison 1999).

Maximum likelihood analysis was used with model for transversions weighted 1.865485 to 1 over transitions (kappa = 3.774786), pre-estimated base frequencies (A = 0.244110, C = 0.207424, G = 0.265463, T = 0.283004; random additions set to 1). For parsimony analyses there were 202 informative sites with gaps included as a fifth

Table 1. Sample information for species investigated in this study

Species collected	Sample location	Sample[a]	GenBank
CERAMIACEAE			
Aglaothamnion callophyllidicola Boo, I.K. Lee, Rueness et Yoshida	Intertidal, Echongdo, Korea, 23 Jun. 1994. H.-G. Choi	CH396	AY643486
Antithamnion aglandum Kim et I.K. Lee	Subtidal, Dokdo, Korea, 21 Jul. 1993. Y.S. Oh	HGC014	-
	Intertidal, Seongsan, Jeju Islands, Korea, 8 Aug. 2001. H.-S. Kim	HSK007	AY643487
A. callocladum Itono	Subtidal, Dokdo, Korea, 21 Mar. 1993. Y.S. Oh	HGC016	-
	Intertidal, Sacheon, Korea. H.-S. Kim	HSK011	AY643488
A. densum Kylin	Intertidal, Kery, France, Jul. 1992. C.A. Maggs	CH008	AY643485
A. nipponicum Yamada et Inagaki	Intertidal, Jeoncheon, Korea, 6 Aug. 1993. Y.S. Oh	HGC018	-
	Intertidal, Chagwido, Cheju Islands, Korea, 21 Feb. 2003. H.-S. Kim	HSK081	AY643489
A. sparsum Tokida	Intertidal, Daecheon, Korea, 23 Apr. 1992. H.-G. Choi and I.K. Lee	CH016	AF236787[b]
Griffithsia japonica Okamura	Intertidal, Soahndo, Korea, 31 May 1996. J.H. Kim and I.K. Lee	CH001	Unpubl.
	Intertidal, Dolsando, Korea, 3 Apr. 1992. Y.S. Oh	HGC021	-
Pterothamnion plumula (Ellis) Nägeli	Intertidal, Zierikzee, Netherlands, 19 Aug. 1997. H-G. Choi	CH028	Unpubl.
P. yezoense (Inagaki) Athanasiadis	Intertidal, Aninjin, Korea, 16 Mar. 1996. E.-Y. Lee	HGC023	-
DASYACEAE			
Dasya collabens Hooker et Harvey	Intertidal, Seogwipo, Jeju Islands, Korea, 7 Aug. 1986. H.-S. Kim	HGC011	-
	Intertidal, Eodal, Korea, 15 Dec. 1996. J.H. Oak and I.K. Lee	CH010	AF488384[c]
D. villosa Harvey	Intertidal, Keomundo, Korea, 21 Jul. 1990. H.-G. Choi	CH018	AF488387[c]
Heterosiphonia japonica Yendo	Intertidal, Mibeop, Korea, 27 Apr. 1994. H.-G. Choi	HGC008	-
	Intertidal, Soahndo, Korea, 31 May 1996. J.H. Kim and I.K. Lee	CH003	AF488394[c]
H. pulchra (Okamura) Falkenberg	Intertidal, Aninjin, Korea, 10 Mar. 1990. H.-G. Choi	HGC009	-
	Intertidal, Wando, Korea, 01 Jun. 1996. J.H. Kim and I.K. Lee	CH004	AF488397[c]
Land plants			
Capsicum annuum Linnaeus	Culture, Lab. of Plant Genetics, SNU. J.Y. Song	HGC051	-
Nicotiana tabacum Linnaeus	Culture, Lab. of Plant Cell Biology, SNU. D.S. Choi	HGC052	-

[a] CH, dried materials and culture by Han-Gu Choi; HGC, culture strains by Han-Gu Choi; HSK, culture strains by Hyung-Seop Kim.
[b] Choi *et al.* (2000).
[c] Choi *et al.* (2002).

base. Parsimony was completed with all changes equally. In both cases 50 random additions of taxa were implemented and the TBR branch swapping option was in effect. To estimate the robustness of internal nodes, bootstrap resampling was completed [100 replicates for maximum likelihood analysis (5 random sequence addition replicates), 1,000 replicates for parsimony (random additions set to 10) and distance analyses (Felsenstein, 1985)]. Unrooted trees were calculated and the ingroup taxa rooted with *Ceramium rubrum* (AF236793) designated as the outgroup (Ragan *et al.* 1994; Saunders *et al.* 1996; Choi *et al.* 2000, 2002).

RESULTS

Nuclear DNA quantification

Nuclear DNA contents were estimated from DAPI-stained NV of immature and mature spermatia in male gametophytes of ceramiacean and dasyacean algae (Fig. 1) and microspores in two land plants (Fig. 5). In frequency distribution patterns, NV showed two peaks correspond to 1C and 2C (Figs 2-4 and 6). Nuclear 2C DNA contents were 0.45-2.31 pg in ceramiacean, 0.40-0.57 pg in dasyacean algae, and 8.42-9.51 pg in land plants (Table 2). By nuclear patterning of vegetative cells derived from an apical cell of each species (Goff and Coleman 1990), 2C DNA contents of spermatia were 2.31 pg in alga having uninucleate and non-polyploid nucleus (*Aglaothamnion callophyllidicola*, Fig. 1A and Fig. 4C), 0.45-1.94 pg in algae having uninucleate and polyploid nucleus (*Antithamnion* spp. and *Pterothamnion yezoense*, Fig. 1B-D, Fig. 2A-C and Fig. 3A, B), and 0.40-0.62 pg in algae having multinucleate and non-polyploid nuclei (*Griffithsia japonica* and dasyacean algae, Fig. 1I-L, Fig. 3C and Fig. 4A, B).

Nuclear small-subunit rDNA sequence data

The seven SSU sequences newly completed for this study ranged from 1768 (*Antithamnion densum*) to 1775 (*Griffithsia japonica*) base pairs in length. No ambiguities

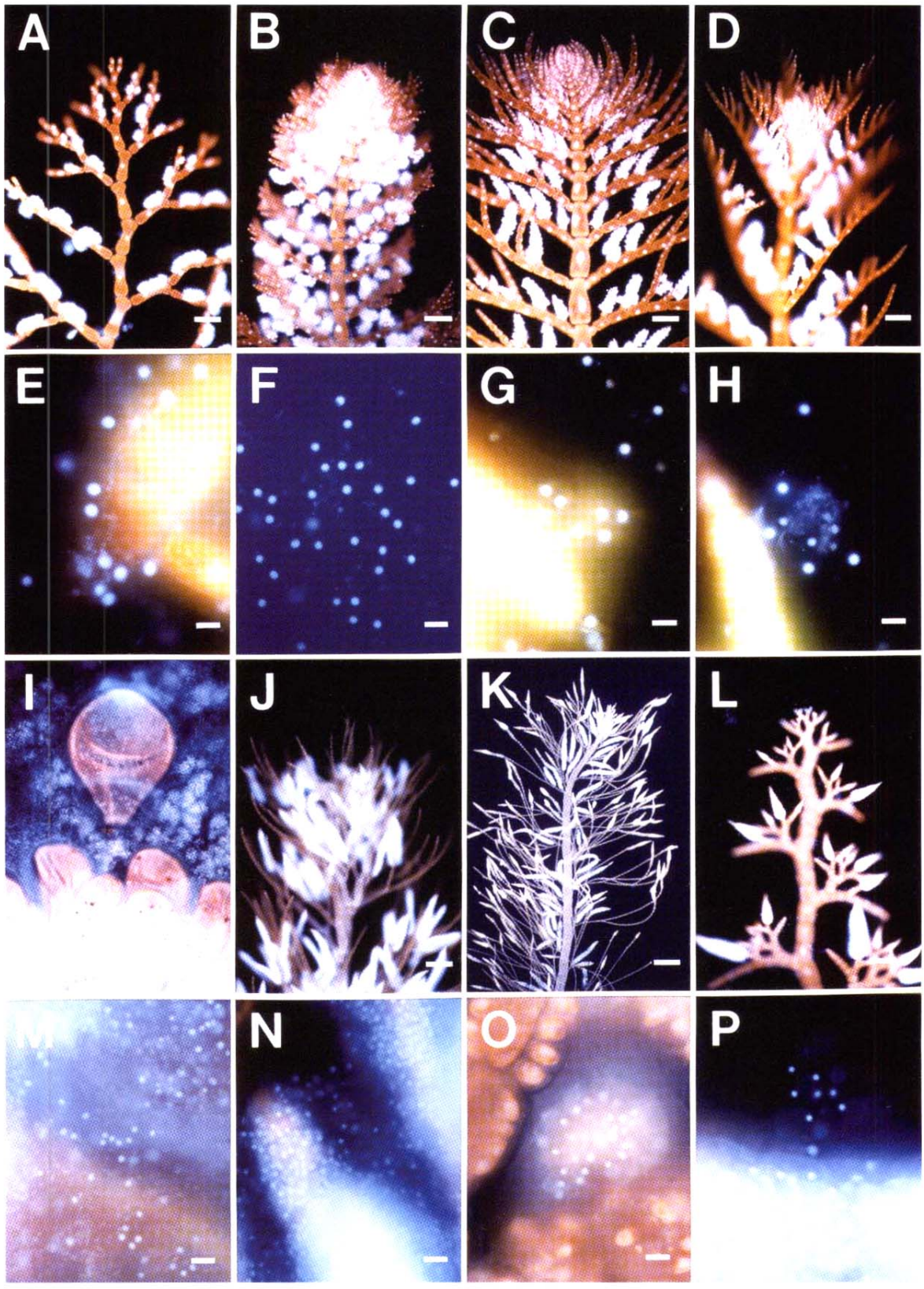

Fig. 1. DAPI-stained male gametophytes and spermatia of eight ceramialean algae. A and E, *Aglaothamnion callophyllidicola*; B and F, *Antithamnion callocladum*; C and G, *A. densum*; D and H, *Antithamnion sparsum*, I and M, *Griffithsia japonica*; J and N, *Dasya collabens;* villosa. K and O, *Dasya villosa*; L and P, *Heterosiphonia pulchra*. Scale bars are 50 μm in A-D and I-L and 10 μm in E-H and M-P.

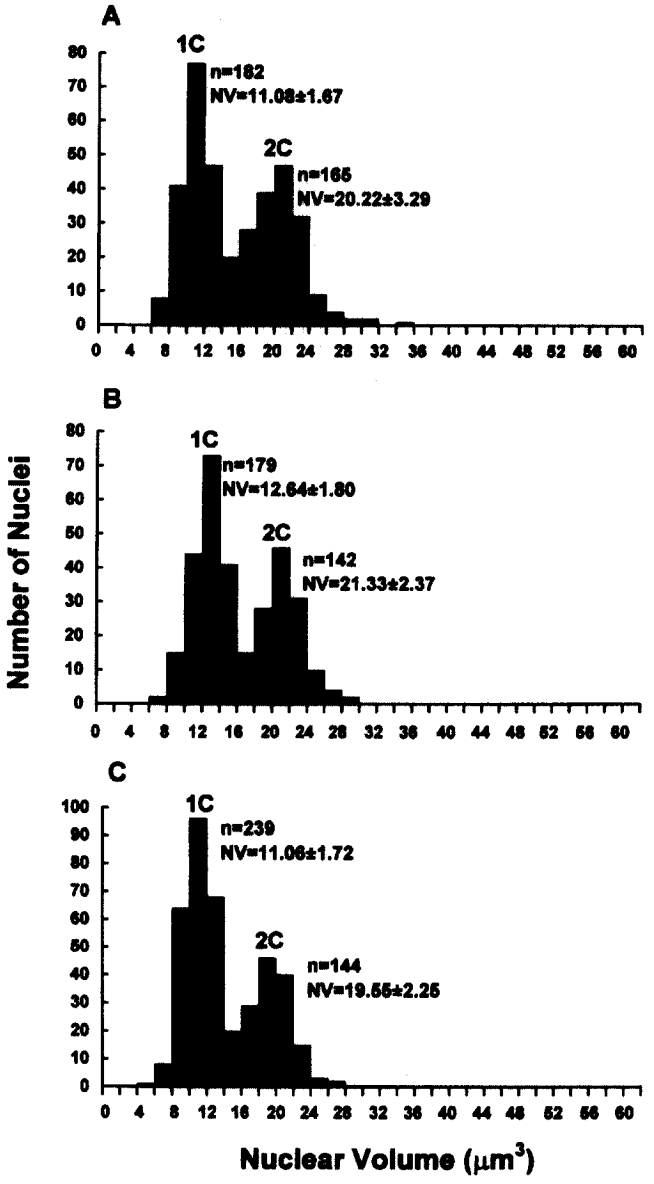

Fig. 2. Frequency distribution histograms of the nuclear volume of DAPI-stained nuclei. Spermatia of male gametophytes in *Antithamnion aglandum* (**A**), *A. callocladum* (**B**), *A. nipponicum* (**C**). C, C-value; n, number of nuclei observed; NV, nuclear volume ± standard deviation.

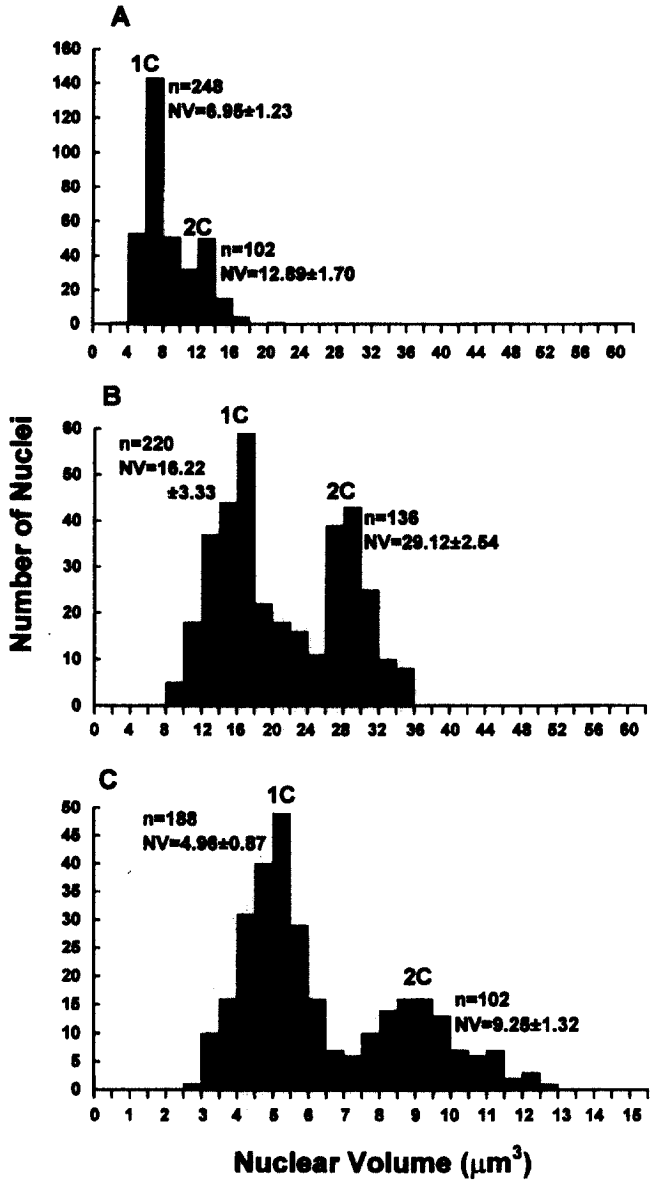

Fig. 3. Frequency distribution histograms of the nuclear volume of DAPI-stained nuclei. Spermatia of male gametophytes in *Antithamnion densum* (**A**), *A. sparsum* (**B**), *Griffithsia japonica* (C). C, C-value; n, number of nuclei observed; NV, nuclear volume ± standard deviation.

were observed in the SSU data. Sequence data have been deposited in GenBank (Table 1) except for those of *Griffithsia japonica* and *Pterothamnion plumula* which will be published in the proceeding papers.

The newly determined sequences were added to a multiple alignment modified from Choi *et al.* (2002). The alignment contained five additional sequences from the Ceramiaceae and Dasyaceae (Table 1). A sub-alignment including partial SSU rDNA sequences (positions 651 to 750, complete alignment available on request) was provided (Fig. 7). *Antithamnion aglandum* differ from *A. nipponicum* at only two sites (positions 1598 and 1711; C ↔ T, data not shown) and *A. sparsum* differed from *A. densum* at three sites (Fig. 7; positions 675, 717 and 719; C ↔ T). Actual pairwise distances were computed between these taxa, including all substitutions, deletions and insertions (Table 3). *Antithamnion* species group was highly conserved, ranging from 2 to 18 nucleotide changes between species. *Griffithsia japonica* and *Heterosiphonia pulchra*, however, were very highly divergent, ranging from 164 to 257 nucleotide changes between each and the others.

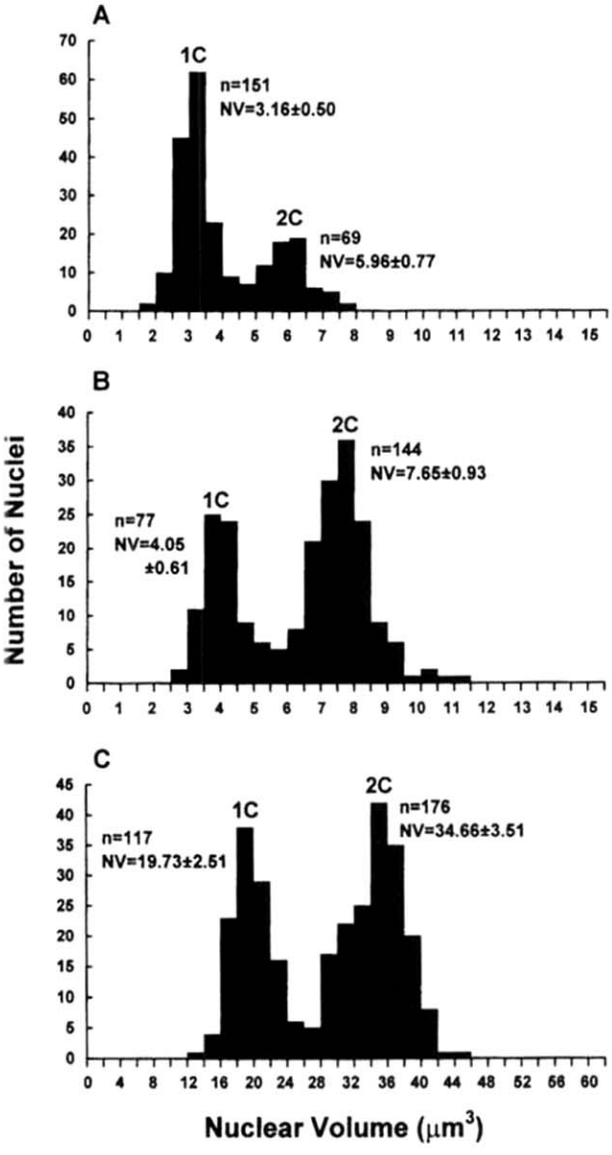

Fig. 4. Frequency distribution histograms of the nuclear volume of DAPI-stained nuclei. Spermatia of male gametophytes in *Heterosiphonia japonica* (**A**), *H. pulchra* (**B**) and *Aglaothamnion callophyllidicola* (**C**). C, C-value; n, number of nuclei observed; NV, nuclear volume ± standard deviation.

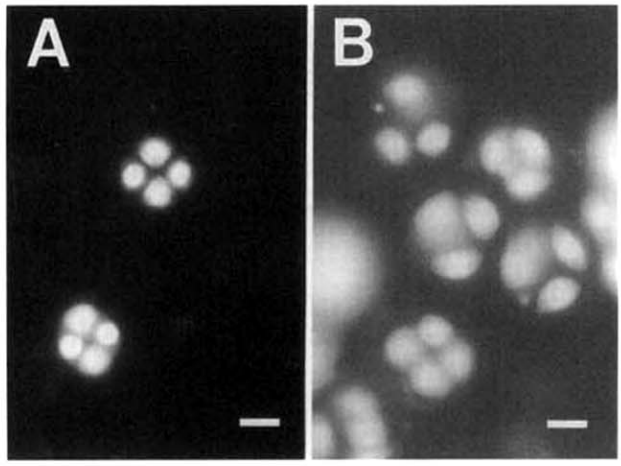

Fig. 5. DAPI-stained microspores of *Capsicum annuum* and *Nicotiana tabacum*. A, *Capsicum annum*; B, *Nicotiana tabacum*. Scale bars are 50 μm.

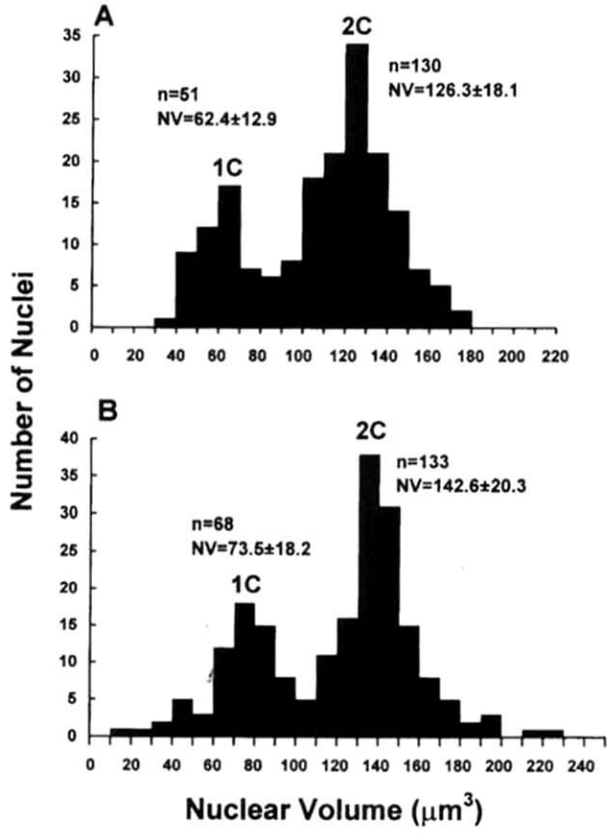

Fig. 6. Frequency distribution histograms of the nuclear volume of DAPI-stained nuclei. Spermatia of male gametophytes in *Capsicum annum* (**A**) and *Nicotiana tabacum* (**B**). C, C-value; n, number of nuclei observed; NV, nuclear volume ± standard deviation.

The multiple alignment (13 species with 1761 sites, excluding the 5′ and 3′ PCR primer regions) was converted to a maximum likelihood tree (-Ln = 5903.32278) with bootstrap results from the maximum likelihood, distance and parsimony analyses appended was presented (Fig. 8). In this tree all species of *Antithamnion* formed a solid monophyletic group (92 to 100% bootstrap replicates). Among *Antithamnion* species, two lineages of species were resolved: *A. aglandum-A. callocladum-A. nipponicum* lineage and *A. densum-A. sparsum* lineage, with strong (88 to 99% bootstrap replicates) support in all analyses. *Griffithsia japonica* was moderately to strongly allied to the Dasyaceae and

Table 2. Nuclear DNA contents estimated from nuclear volumes for spermatia of ceramiacean and dasyacean algal species

Species	C-value	*N*	Mean nuclear volume μm^3 (s.d.)	Estimated DNA content pg (s.d.)
CERAMIACEAE				
Aglaothamnion callophyllidicola	1	117	19.73 (2.51)	1.32 (0.17)
	2	144	34.66 (3.51)	2.31 (0.23)
Antithamnion aglandum	1	182	11.08 (1.67)	0.74 (0.11)
	2	144	20.22 (3.29)	1.35 (0.22)
A. callocladum	1	179	12.64 (1.80)	0.84 (0.12)
	2	144	21.33 (2.37)	1.42 (0.16)
A. densum	1	248	6.95 (1.23)	0.46 (0.08)
	2	102	12.89 (1.70)	0.86 (0.11)
A. nipponicum	1	239	11.06 (1.72)	0.74 (0.11)
	2	144	19.55 (2.25)	1.30 (0.15)
A. sparsum	1	220	16.22 (3.33)	1.08 (0.22)
	2	136	29.12 (2.54)	1.94 (0.17)
Griffithsia japonica	1	188	4.96 (0.87)	0.33 (0.06)
	2	102	9.25 (1.32)	0.62 (0.09)
Pterothamnion yezoense	1	152	3.42 (0.84)	0.23 (0.06)
	2	42	6.69 (1.05)	0.45 (0.07)
DASYACEAE				
Dasya collabens	1	49	3.58 (0.51)	0.24 (0.03)
	2	75	6.69 (0.84)	0.45 (0.06)
D. villosa	1	112	4.47 (0.83)	0.30 (0.06)
	2	68	8.56 (1.05)	0.57 (0.07)
Heterosiphonia japonica	1	151	3.16 (0.50)	0.21 (0.03)
	2	69	5.96 (0.77)	0.40 (0.05)
H. pulchra	1	77	4.05 (0.61)	0.27 (0.04)
	2	144	7.65 (0.93)	0.51 (0.06)
Land plants				
Capsicum annuum	1	51	62.44 (12.90)	4.16 (0.86)
	2	130	126.32 (18.13)	8.42 (1.21)
Nicotiana tabacum	1	68	73.49 (18.20)	4.90 (1.21)
	2	133	142.60 (20.32)	9.51 (1.35)

Aglaothamnion callophyllidicola was moderately to strongly (81 to 100% bootstrap replicates) allied to the Dasyaceae/*Griffithsia japonica* clade. Maximum likelihood, distance and parsimony trees differed in the relative positioning of *Heterosiphonia pulchra* and *Griffithsia japonica* within the Dasyaceae/ *Griffithsia japonica* clade, and three species within the *A. aglandum-A. callocladum-A. nipponicum* lineage, but bootstrap support was absent in all cases (Fig. 8).

DISCUSSION

Nuclear DNA contents in ceramialean algal spermatia

Nuclear DNA contents were estimated from DAPI-stained NV of immature and mature spermatia in male gametophytes of eight ceramiacean and four dasyacean algae (Figs 1-4 and Table 2). Nuclear 2C DNA contents varied 5.1-fold, ranging from 0.45 pg (*Pterothamnion yezoense*) to 2.31 pg (*Aglaothamnion callophyllidicola*) in ceramiacean and 2.1-fold, ranging from 0.27 pg (*Dasysiphonia chejuensis*) to 0.57 pg (*Dasya villosa*) in dasyacean algae (Table 2, Choi and Lee 1996). These values are consistent with nuclear 2C DNA content, ranging from 0.32 pg (*Lomentaria baileyana*) to 2.85 pg (*Aglaothamnion boergesenia*), estimates for other red algae (Kapraun 1993a, 1993b; Kapraun and Dunwoody 2002) and are comparable to the range of 0.2-4.9 pg found in marine chlophycean algae (Kapraun 1993c).

Among *Antithamnion* species 2C DNA contents of *A. aglandum, A. callocladum* and *A. nipponicum*, varied 1.1-

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                    660        670        680        690        700
                     .          .          .          .          .
Ceram.rub    AGT-CGGCCTCACGGTCGATCTTGTTGG-CGGCCGCCTTTGTGGAGGGGG  [700]
Anti.calo    G.G-..T.T......AT.T.....C-..-..............T....  [700]
Anti.agla    G.G-..T.T......AT.T.....C-..-..............T....  [700]
Antit.nip    G.G-..T.T......AT.T.....C-..-..............T....  [700]
Antith.sp    G.G-T.T.T......ATAT.....C-..-..............T....  [700]
Antit.den    G.G-T.T.T......ATAT.....-..-..............T....  [700]
Pterotham    G.G-..T........AT.T.....C-..-..............T....  [700]
Aglao.cal    G.A-.AT....G...AT...T...C-.C-T..G..............  [700]
Griff.jap    GAA-GCT..G..A..AGCT..GATGCCC-T.TT..T..C.....TT..  [700]
Heter.jap    G.C-..T..G.GA..A..CGTC.AG...-TT.A..............  [700]
Das.colla    G.C-..T..GA.A..A..CGTC.AG-.TGTT.A..............  [700]
Dasya.vil    G.C-..T..GTGA..A...A....C-.T-.T.A..............  [700]
Heter.pul    G..G..TT.G..A.AA..T.GC...-CC-.TTG.A.........TT..C  [700]

                    710        720        730        740        750
                     .          .          .          .          .
Ceram.rub    GCTTAGGTCGGTGCTTTATTGTCACTCCTTTGTCGCTGCCACCGTTTACT  [750]
Anti.calo    ...CG.--T.......C.C..C.T.G..G-A..............  [750]
Anti.agla    ...CG.--T.......C.C..C.T.G..G-A..............  [750]
Antit.nip    ...CG.--T.......C.C..C.T.G..G-A..............  [750]
Antith.sp    ...CGA--T.......C.C..C.T.GT.G-A..............  [750]
Antit.den    ...CGA--T.............C.T.GT.G-A..............  [750]
Pterotham    ...CG.--T.......C....C.T.G..G-A..............  [750]
Aglao.cal    ....G.--T.............C.T.G..A-A..TC..........  [750]
Griff.jap    ..A.T.--T.............CAT.GTAG-..CAA.C.........C...  [750]
Heter.jap    ....G.--T.......CGC..CAT.A..A-A..............  [750]
Das.colla    ....G.--T........GC..CAT.A..A-A..............  [750]
Dasya.vil    ....G.--T.......C.C..CATTGT.A-A..............  [750]
Heter.pul    .G.AGT-CT...T........GAT.GGGC-.A.T.........T......  [750]
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Fig. 7. A sub-alignment (positions 651 to 750) for 13 species of the Ceramiales in the multiple alignment. A dash (-) indicates a gap in the multiple alignment; a dot (.) that a species has the same character state for that site as in *Ceramium rubrum* (*Ceram.rub*). Numbers to the right indicate nucleotide position in the multiple alignment. Bold characters (positions 675, 717 and 719) indicate three substitutions (C ↔ T) between *Antithamnion sparsum* (*Antith.sp*) and *A. densum* (*Antit.den*).

Table 3. Actual pairwise distances (nucleotide changes) between the species included in the SSU alignment. Bold numbers indicate nucleotide changes between five species of *Antithamnion*

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Ceramium rubrum*	-												
2. *Antithamnion callocladum*	74	-											
3. *Antithamnion aglandum*	76	**5**	-										
4. *Antithamnion nipponicum*	76	**5**	**2**	-									
5. *Antithamnion sparsum*	80	**15**	**12**	**12**	-								
6. *Antithamnion densum*	77	**18**	**15**	**15**	**3**	-							
7. *Pterothamnion plumula*	61	46	43	43	43	44	-						
8. *Aglaothamnion callophyllidicola*	150	135	135	133	143	142	134	-					
9. *Griffithsia japonica*	255	244	244	242	248	246	244	257	-				
10. *Heterosiphonia japonica*	139	124	121	121	123	125	112	168	243	-			
11. *Dasya collabens*	143	128	125	125	127	127	120	164	240	21	-		
12. *Dasya villosa*	147	129	126	126	125	128	123	172	256	60	68	-	
13. *Heterosiphonia pulchra*	203	201	203	201	207	204	198	223	273	178	174	182	-

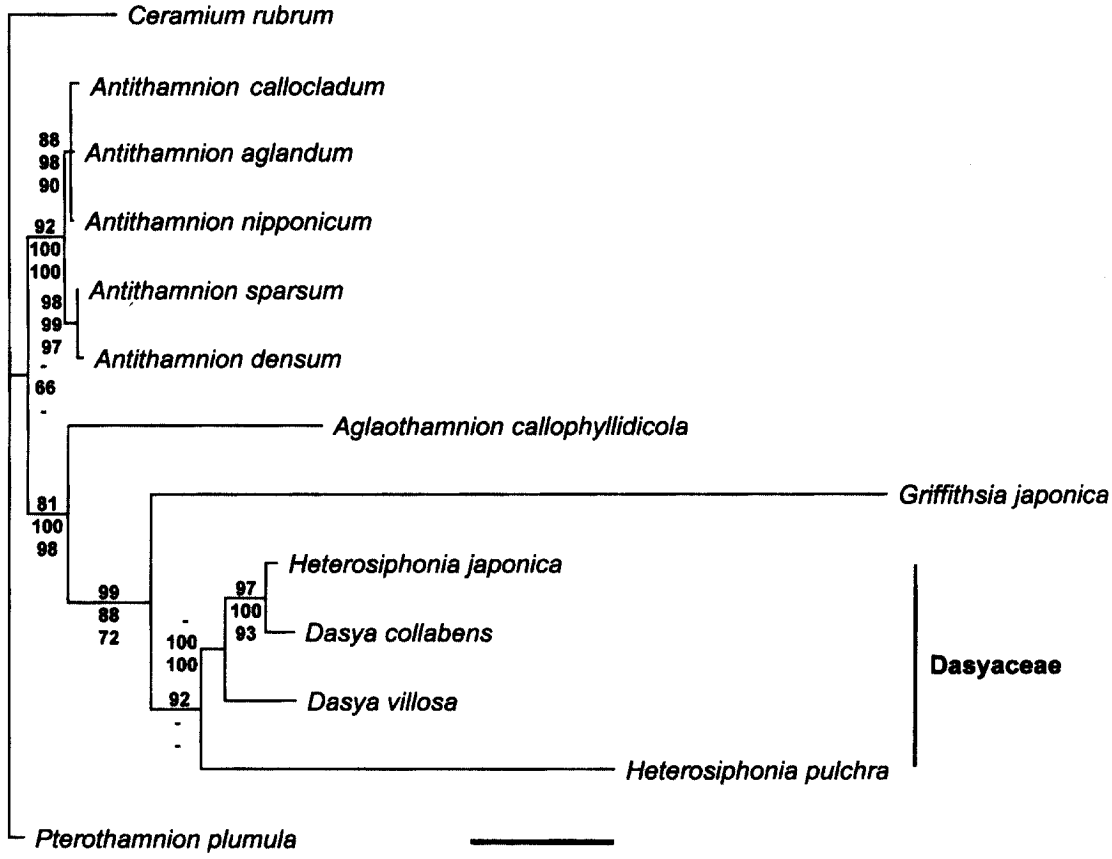

Fig. 8. Tree constructed with maximum likelihood for the SSU alignment. Values at branches represent percentage of 100 bootstrap replicates for maximum likelihood analysis (top value) and of 1,000 bootstrap replicates for distance (middle value) and parsimony (bottom value) analyses. Scale bar = 0.05 substitutions per site.

fold, ranging from 1.30 pg (*A. nipponicum*) to 1.42 pg (*A. callocladum*) (Fig. 2A-C, Table 2). Those of *A. densum* (0.86 pg) and *A. sparsum* (1.94 pg), however, varied 2.3-fold (Fig. 3A and B, Table 2).

Fluorescence microscopy gave no indication of cytoplasmic DNA in pollen or pollen tubes of two orchid species, while the vegetative and the generative nuclei both showed bright DAPI fluorescence (Johansen *et al.* 1995). We, however, could estimate NV of DAPI-stained microspores in two land plants. Nuclear DNA contents of *Capsicum annuum* and *Nicotiana tabacum* (Fig. 5 and 6, Table 2) calculated from NV were 2C = 8.42 and 9.51 pg, respectively. The values were similar to those estimated by flow cytometer (5.5-7.51 pg in *Capsicum annuum*, Arumuganathan and Earle 1991; 8.75-9.63 pg in *Nicotiana tabacum*, Arumuganathan and Earle 1991; 9.67 pg in *Nicotiana tabacum*, Galbraith *et al.* 1983).

Each mature spermatium of ceramialean algae and microspore (pollen grain) of two land plants seem to have a 2C nucleus, which may provide a genetic buffering system to protect the genetic content of a spermatium and microspore from potentially lethal mutations.

Nuclear small-subunit rDNA sequence data

In our SSU rDNA sequence data, *Antithamnion* species group was highly conserved, ranging from 2 to 18 nucleotide changes between species. *Antithamnion sparsum* differed from *A. densum* at three sites (Fig. 8; positions 675, 717 and 719; C ↔ T). The SSU rDNA sequences from three species of *Heterocladia* (Rhodomelaceae, Ceramiales), which had been identified as *H. australis* Decaisne, *Trigenea australis* Sonder (= *H. caudata* L. Phillips, H.-G. Choi, G.W. Saunders et Kraft) and *T. umbellate* J. Agardh [= *H. umbellate* (J. Agardh) L. Phillips, H.-G. Choi, G.W. Saunders et Kraft], were virtually identical (Phillips *et al.* 2000). Therefore, one or more nucleotide changes in the SSU rDNA sequences observed between two taxa in a highly conserved group such as *Antithamnion* species, indicate that they could not be conspecific.

Consistent with previous SSU results (Saunders *et al.* 1996; Choi *et al.* 2000, 2002), our SSU data indicate that the Ceramiaceae is paraphyletic. In our SSU tree, one

strongly supported group and three single-taxon lineages were resolved within the Ceramiaceae: (1) *Antithamnion* species group; (2) *Aglaothamnion*; (3) *Griffithsia*; and (4) *Pterothamnion*. Within the *Antithamnion* species group, two lineages of species were resolved: *A. aglandum-A. callocladum-A. nipponicum lineage* (subgenus *Pteroton*) and *A. densum-A. sparsum lineage* (subgenus *Antithamnion*) in all analyses, consistent with the tree topology inferred from *rbc*S data (Lee *et al.* 2001).

Taxonomic issues of *Antithamnion sparsum* and *A. densum*

Athanasiadis (1990) placed *A. defectum* Kylin (Type locality: Friday Harbor, Canoe Island, Wash., USA) and *A. sparsum* Tokida (Type locality: Lake Tobuchi, Saghalien, Japan) in synonymy with *A. densum* (Type locality: Peru) after investigation of the relevant types. Incomplete interfertility by hybridization experiments between isolates of *A. sparsum* from Korea and an isolate of *A. defectum* from California suggested that these taxa were under speciation (Boo and Lee 1983).

However, our nuclear DNA content and SSU rDNA sequence data of *A. sparsum* from Korea are reasonably different from those of *Antithamnion densum* from France. The data do not support the previous taxonomic studies that these two taxa could be conspecific (Yoshida 1981; Athanasiadis 1990, 1996; Guiry and Maggs 1991; Yoshida *et al.* 1998). In addition, chromosome studies have been reported a diploid number of ca. 24 chromosomes in *A. densum* from France, ca. 21 chromosomes in *A. defectum* from California and ca. 44 chromosomes in *A. sparsum* from Korea (G.H. Kim pers. comm.). *Antithamnion sparsum* in the north-west Pacific may be an independent identity at the species level separate from *A. densum* in the north Atlantic and the south-east Pacific and *A. defectum* in the north-east Pacific, but we lack adequate morphological and molecular data for *A. densum, A. defectum, A. sparsum* and their relatives from each type locality and, therefore, postpone formal taxonomic proposals.

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