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Phylogenetic Relationships of *Sargassum* subgenus *Bactrophycus* (Sargassaceae, Phaeophyceae) inferred from rDNA ITS Sequences

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Phylogenetic relationships of *Sargassum* with the emphasis on subgenus *Bactrophycus* were examined on the basis of internal transcribed spacer (ITS) and 5.8S sequences of nuclear ribosomal DNA (rDNA) for 23 accessions of *Sargassum* representing 18 species, and two outgroups, *Hizikia fusiformis* (Harvey) Okamura and *Myagropsis myagroides* (Mertens ex Turner) Fensholt. Fifteen species were included in the molecular analysis to represent all sections of the subgenus *Bactrophycus* recognized to date (*Spongocarpus, Teretia, Halochloa,* and *Repentia*), and additional three species were analyzed to represent three other subgenera of *Sargassum* (*Sargassum, Schizophycus,* and *Phyllotrichia*) found in Korea. In the resulting phylogenetic trees, *H. fusiformis* was nested in the clade of subgenus *Bactrophycus* as sister group of section *Teretia* with high confidence even though it was designated as one of outgroups in the phylogenetic analysis. Species of section *Teretia* formed a very robust clade. Section *Spongocarpus* was branched off as a sister group of the *Hizikia-Teretia* clade. Members of section *Halochloa* showed very little differences in ITS sequences and formed a very tight clade with *S. yezoense*, which is a member of section *Repentia* (99% in bootstrap analysis). Sequences of ITS of *S. yezoense* was identical with ones of *S. siliquastrum*, although they belong to different sections, *Repentia* and *Halochloa*, respectively. In the phylogenetic analyses of ITS sequences, members of subgenus *Bactrophycus* including the monotypic genus *Hizikia* were clearly separated from other subgenera of *Sargassum* as forming a highly supported monophyletic group. The molecular data strongly claim that *Hizikia* should be treated as a member of the genus *Sargassum*. The molecular study also suggests that sections *Repentia* and *Halochloa* are closely allied, but further analyses with more extensive sampling should be needed to look into taxonomic circum criptions for sections *Halochloa* and *Repentia*, of which taxonomic limitations were not clearly defined in this study.

Key Words: *Hizikia*, ITS, phylogeny, rDNA, *Sargassum* subgenus *Bactrophycus*, sequences

INTRODUCTION

Sargassum, the largest genus in Phaeophyceae with more than 400 described species, is distributed in the tropical to temperate regions of all around the world (Yoshida 1983). Agardh (1889) divided genus *Sargassum* into five subgenera: *Phyllotrichia, Schizophycus, Bactrophycus, Arthrophycus* and *Sargassum* (= *Eusargassum*), and these subgenera are further subdivided into sections, subsections and series (Phillips 1995). Among the subgenera of *Sargassum, Bactrophycus* is known only in the eastern Asiatic region, where it is considered as an ecologically important group by forming massive vegetations at subtidal and lower intertidal zones (Tseng *et al.* 1985).

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On the basis of basal morphology, stem, main branch and reproductive structures, Yoshida (1983) subdivided subgenus *Bactrophycus* into four sections: *Spongocarpus, Teretia, Halochloa,* and *Repentia*. Tseng (1985) added a fifth section *Phyllocystae*, but it was transferred to the subgenus *Sargassum* on the basis of molecular data as well as the morphology of receptacles and basal leaves (Stiger *et al.* 2000). In result, thirty-one species in four sections of subgenus *Bactrophycus* have been described to date (Tseng *et al.* 1985; Stiger *et al.* 2000). Since species of *Bactrophycus* show a wide range of morphological variation like other subgenera of genus *Sargassum*, it is difficult to elucidate phylogenetic relationships with morphological characters (Kilar *et al.* 1992). Therefore, there are a few studies available on phylogenetic relationships in the genus even though extensive studies have been carried out on morphological aspects (Yendo 1907; Setchell 1933, 1936; Okamura 1936; Yoo 1976; Yoshida

1983; Tseng *et al.* 1985; Lee and Yoo 1992).

Recently, molecular analyses have been often utilized to address phylogenetic questions on Fucales group including genus *Sargassum* at family, generic and subgeneric levels (Rousseau *et al.* 1997; Horiguchi and Yoshida 1998; Leclerc *et al.* 1998; Rousseau and Reviers 1999). Stiger *et al.* (2000) analyzed ITS2 sequences to determine taxonomic position of section *Phyllocystae*, which was originally established as a section of subgenus *Bactrophycus* by Tseng *et al.* (1985). In the analysis of ITS2 sequences from 19 species of *Sargassum* representing three subgenera *Phyllotrichia*, *Bactrophycus* and *Sargassum*, Stiger *et al.* (2000) claimed that the section *Phyllocystae* should be transferred from subgenus *Bactrophycus* to subgenus *Sargassum*. However, little discussion was made on the phylogenetic relationships in subgenus *Bactrophycus* because the size of sampling was not large enough for the subgenus.

The ITS sequences of rDNA have been widely used in phylogenetic studies at species level (Olsen *et al.* 1998; Gurgel *et al.* 1999; Kawai *et al.* 2001; Coleman 2001; Coyer *et al.* 2001; Hughey *et al.* 2001; Hughey and Hommersand 1999; van der Strate *et al.* 2002). Rapidly evolving ITS sequences enable to detect genetic differences among isolates or geographical populations even in same species as well as interspecific level (LaJeunesse 2001; Santos *et al.* 2001; van Hannen *et al.* 2000; González *et al.* 2001). Coyer *et al.* (2001) demonstrated that ITS sequences in kelps can reveal local-scale patterns of population changes due to environmental changes, as well as global scale patterns of speciation and biogeography. However, ITS region is not always useful for phylogenetic studies at species level because the divergence of ITS sequences was too high or too low among species in some instances (Leclerc *et al.* 1998; Serrão *et al.* 1999).

The purpose of this study is to assess the interspecific relationships in *Sargassum* subgenus *Bactrophycus* with the sequences of the whole ITS region including both ITS1 and ITS2, and to examine the current taxonomic dispositions of subgenus *Bactrophycus*, which is usually recognized as four sections: *Spongocarpus*, *Teretia*, *Halochloa*, and *Repentia*. We also included in the molecular analyses three other subgenera occurring in Korea (*Phyllotrichia*, *Schizophycus*, *Sargassum*) to test phylogenetic utilities of ITS sequences for the genus *Sargassum*.

MATERIALS AND METHODS

Twenty-three accessions, representing 18 species of the genus *Sargassum*, were obtained from the fields along the coasts of Korea (Table 1). Among them, 15 species were collected to represent all sections of subgenus *Bactrophycus*, and three species were for subgenera *Phyllotrichia*, *Sargassum* and *Schizophycus* occurring in Korea. To examine the sequence divergence among different populations of the same species, multiple accessions of different geographical origins were compared for *S. horneri*, *S. micracanthum*, and *S. siliquastrum*. *Hizikia fusiformis* and *Myagropsis myagroides* were included as outgroup because they showed the close affinities with the genus *Sargassum* in the previous phylogenetic study of 18S rDNA (Horiguchi and Yoshida 1998).

Prior to DNA extraction, the plants were washed with distilled water and dried on filter paper in air. Total genomic DNA was isolated with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of extracted DNA was determined by comparing the brightness of DNA band with the standard lamda DNA marker band on 0.7% agarose gel stained with ethidium bromide. The ITS regions including 5.8S rDNA were amplified by PCR in 50 μL final volume containing 0.1 to 20 ng template DNA. The primers designed by White *et al.* (1990) were used for PCR amplification and sequencing. In addition, two primers were designed in cases that PCR yields were not ample to carry out sequencing for some species: SAG1, 5'-CAGTGTGGCAGGCTTGG-3', annealed to positions 189-205 in aligned sequences; SAG3, 5'-TGAGTTCACCTAAGCCTAGA-3', annealed to positions 1136-1155 in aligned sequences (see Appendix). Amplification reactions involved 5 min at 94°C for predenaturation, 35 cycles consisting of 30 sec at 94°C for denaturation, 30 sec at 48°C for annealing and 1 min at 72°C for extension, with a final extension of 7 min at 72°C, using a Thermal Cycler 9600 (PE Applied Biosystems, Foster City, CA).

Sequences were aligned using CLUSTAL X (Thompson *et al.* 1999) and the alignment was refined manually. The sequences of ITS regions including ITS1, ITS2, and 5.8S coding region were combined for phylogenetic analyses with PAUP 4.0 (Swofford 2000). *Hizikia fusiformis* and *Myagropsis myagroides* were designated as outgroup to root the trees. Maximum parsimony (MP) analysis was performed using a standard heuristic search with MULPARS and tree-bisection-reconnection (TBR) branch swapping options. All characters were unweighted and unordered. Because the number of near-identical sequences in the alignment precluded

Table 1. Sample information and GenBank accession numbers of ITS sequences for this study. All voucher specimens were deposited in SNU

Taxa		Specimen information	Localities	Date	GenBank Accession number
Genus *Sargassum*					
Subgenus *Bactrophycus*					
Section *Spongocarpus*					
Sargassum horneri (Turner) C. Agardh	(A)*	OAK-77	Anin, Kangneung	27 Oct 1994	AY149998
S. horneri	(B)	OAK-92	Youngheungdo, Inchon	5 Dec 1994	AY149999
Section *Teretia*					
S. confusum C. Agardh	(A)	OAK-68	Eodal, Donghae	23 Sep 1994	AY150000
S. confusum	(B)	OAK-M1	Seopjikoji, Cheju	26 Jan 1999	AY150001
S. pallidum (Turner) C. Agardh		OAK-pa(1)	Yeocha, Geoje	25 Aug 1998	AY150002
S. muticum (Yendo) Fensholt		OAK-75	Jangseungpo, Geoje	7 Oct 1994	AY150003
S. thunbergii (Mertens ex Roth) Kuntze		OAK-98	Seongsan, Cheju	10 Dec 1994	AY150004
S. fulvellum (Turner) C. Agardh		OAK-FUL	Sinsan, Cheju	26 Jan 1999	AY150005
S. hemiphyllum (Turner) C. Agardh		OAK-M4	Hallim, Cheju	27 Jan 1999	AY150006
S. miyabei Yendo		OAK-MY	Anin, Kangneung	8 Nov 1998	AY150007
Section *Halochloa*					
S. coreanum J. Agardh		OAK-M2	Seopjikoji, Cheju	26 Jan 1999	AY150008
S. micracanthum (Kützing) Endlicher	(A)	OAK-95	Seongsan, Cheju	10 Dec 1994	AY150009
S. micracanthum	(B)	OAK-C	Seopseom, Cheju	5 Dec 1998	AY150010
S. macrocarpum C. Agardh		OAK-M6	Sinsan, Cheju	26 Jan 1999	AY150011
S. autumnale Yoshida		OAK-71	Sodol, Jumunjin	31 Aug 1994	AY150012
S. siliquastrum (Mertens ex Turner) C. Agardh	(A)	OAK-M5	Hallim, Cheju	27 Jan 1999	AY150013
S. siliquastrum	(B)	OAK-M8	Sinsan, Cheju	26 Jan 1999	AY150014
S. siliquastrum	(C)	OAK-sq(3)	Seopseom, Cheju	25 Aug 1998	AY150015
S. serratifolium (C. Agardh) C. Agardh		OAK-B	Munseom, Cheju	4 Dec 1998	AY150016
Section *Repentia*					
S. yezoense (Yamada) Yoshida et Konno		OAK-64	Neungpo, Geoje	1 Aug 1994	AY150017
Subgenus *Schizophycus*					
S. patens C. Agardh		OAK-101	Seongsan, Cheju	10 Dec 1994	AY150018
Subgenus *Phyllotrichia*					
S. piluliferum (Turner) C. Agardh		OAK-pf(2)	Yeocha, Geoje	25 Aug 1998	AY150019
Subgenus *Sargassum*					
S. yendoi Okamura et Yamada		OAK-M7	Seopjikoji, Cheju	26 Jan 1999	AY150020
Outgroup					
Hizikia fusiformis (Harvey) Okamura		OAK-M3	Hallim, Cheju	27 Jan 1999	AY150021
Myagropsis myagroides (Mertens ex Turner) Fensholt		OAK-96	Seongsan, Cheju	10 Dec 1994	AY150022

*: Accessions of different geographical origins.

complete analysis in a reasonable amount of time, the maximum tree option was set at 1000. Maximum likelihood (ML) analysis employed the HKY85 model (Hasegawa *et al.* 1985) with transition/transversion ratio estimated from the data set and empirical nucleotide frequencies. Neighbor-joining tree (NJ; Saitou and Nei 1987) was obtained by PAUP (Swofford 2000). Bootstrap analyses (Felsenstein 1985) were used to assess the robustness of the trees with 2000 replicates for MP, ML and NJ analyses.

RESULTS

The sequences of ITS with 5.8S region ranged from 1315 to 1542 bases in 25 sequences from 18 species of *Sargassum* and two outgroup taxa (Table 2). Among them, unavailable were the ITS1 sequence for *S. piluliferum* and the ITS2 sequence for *S. patens* because it was impossible to determine sequences of those regions in spite of repeated experimental trials. For *Sargassum* species included in this study, the ITS1 sequences were

Table 2. The size of ITS1, 5.8S, and ITS2 sequences for taxa included in this study

Taxa		Total	ITS1	5.8S	ITS2
Subgenus *Bactrophycus*					
Section *Spongocarpus*					
Sargassum horneri (Turner) C. Agardh	(A)	1510	769	159	582
S. horneri	(B)	1504	763	159	582
Section *Teretia*					
S. confusum C. Agardh	(A)	1487	742	162	583
S. confusum	(B)	1486	741	162	583
S. pallidum (Turner) C. Agardh		1480	738	159	583
S. muticum (Yendo) Fensholt		1480	738	159	583
S. thunbergii (Mertens ex Roth) Kuntze		1481	739	159	583
S. fulvellum (Turner) C. Agardh		1478	736	159	583
S. hemiphyllum (Turner) C. Agardh		1542	787	162	593
S. miyabei Yendo		1483	741	159	583
Section *Halochloa*					
S. coreanum J. Agardh		1511	768	159	584
S. micracanthum (Kützing) Endlicher	(A)	1510	767	159	584
S. micracanthum	(B)	1509	766	159	584
S. macrocarpum C. Agardh		1509	766	159	584
S. autumnale Yoshida		1511	768	159	584
S. siliquastrum (Mertens ex Turner) C. Agardh	(A)	1511	768	159	584
S. siliquastrum	(B)	1509	766	159	584
S. siliquastrum	(C)	1509	766	159	584
S. serratifolium (C. Agardh) C. Agardh		1509	766	159	584
Section *Repentia*					
S. yezoense (Yamada) Yoshida et Konno		1510	767	159	584
Subgenus *Schizophycus*					
S. patens C. Agardh		887[a]	728	159	-*
Subgenus *Phyllotrichia*					
S. piluliferum (Turner) C. Agardh		649[b]	-*	159	490
Subgenus *Sargassum*					
S. yendoi Okamura et Yamada		1476	733	159	584
Outgroup					
Hizikia fusiformis (Harvey) Okamura		1512	765	159	588
Myagropsis myagroides (Mertens ex Turner) Fensholt		1315	773	159	383

a: The sequence of ITS2 was not included.

b: The sequence of ITS1 was not included.

*: The size is unknown due to incomplete sequences of the region.

728-787 bases in length, and the ITS2 sequences were 490-593 bases. The shortest ITS2 sequence was from *S. piluliferum*, which was shorter than those of other *Sargassum* species by about 100 bases. The ITS1 sequences were always longer than the ITS2 sequences for all taxa included in this study. The size of ITS sequences of *Hizikia fusiformis* fit well in these ranges, but the ITS2 sequence of *Myagropsis myagroides* was 383 bases, which appeared to be much smaller in size than those of *Sargassum* and *Hizikia*. The sequences of 5.8S coding region were 159 bases long for the most species of Sargassum and two outgroup taxa, but the 5.8S sequences of *S. hemiphyllum* and *S. confusum* of subgenus *Bactrophycus* were three bases longer, which was caused by three independent one-base insertions (see Appendix for aligned sequences).

The alignment of ITS sequences from 25 accessions required various indels, of which size ranged from one to 126 bases. Total size of the aligned ITS sequences was 1615 sites. Even though gaps were not included in the phylogenetic analyses, some gaps shared by allied taxa provided useful information for discriminating groups of taxa. Members of section *Teretia* of *Sargassum* subgenus *Bactrophycus* were distinguished by two consider-

Table 3. Pairwise distances between taxa used in this study. Above diagonal is percentage of mean character differences and the below is the number of total character differences

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21[a]	22[b]	23	24	25
1	*S. horneri* A	-	1.2	6.7	6.7	6.7	7.2	6.6	7.5	7.3	7.2	5.8	5.5	5.5	5.5	5.5	5.5	5.4	5.4	5.5	5.4	0.1	0.1	7.6	6.8	12.3
2	*S. horneri* B	18	-	6.0	6.0	6.0	6.6	6.1	6.8	6.8	6.6	4.6	4.3	4.3	4.3	4.3	4.3	4.2	4.2	4.3	4.2	0.1	0.1	7.1	6.3	11.9
3	*S. confusum* A	98	88	-	0.0	0.4	1.4	3.0	2.0	3.0	1.4	4.2	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.1	4.0	0.1	0.1	6.1	4.2	11.2
4	*S. confusum* B	98	88	0	-	0.4	1.4	3.0	2.0	3.0	1.4	4.2	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.1	4.0	0.1	0.1	6.1	4.2	11.1
5	*S. pallidum*	98	88	6	6	-	1.4	2.8	2.0	3.1	1.4	4.2	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.1	4.0	0.1	0.1	5.9	4.2	11.2
6	*S. muticum*	105	96	20	20	20	-	3.3	2.0	3.7	0.4	4.3	3.9	3.9	4.1	4.1	4.1	4.0	4.0	4.2	4.0	0.1	0.1	6.1	4.3	11.2
7	*S. thunbergii*	97	89	44	44	42	48	-	3.7	3.4	3.3	3.8	3.5	3.5	3.6	3.6	3.6	3.5	3.5	3.6	3.5	0.1	0.1	5.9	4.1	11.3
8	*S. fulvellum*	110	100	30	30	30	30	55	-	4.1	2.1	5.0	4.6	4.6	4.8	4.8	4.8	4.7	4.7	4.9	4.7	0.1	0.1	6.8	5.0	11.6
9	*S. hemiphyllum*	107	99	45	45	46	54	51	60	-	3.8	4.8	4.5	4.5	4.5	4.6	4.6	4.4	4.4	4.6	4.5	0.1	0.1	6.9	5.0	11.8
10	*S. miyabei*	105	96	21	21	21	6	49	31	56	-	4.4	4.0	4.0	4.2	4.2	4.2	4.1	4.1	4.2	4.1	0.1	0.1	6.3	4.5	11.4
11	*S. coreanum*	87	69	62	62	62	63	55	73	70	64	-	0.5	0.5	0.5	0.5	0.3	0.4	0.4	0.5	0.4	0.1	0.1	4.3	4.6	10.6
12	*S. micracanthum* A	83	65	58	58	58	57	51	67	66	58	8	-	0.0	0.2	0.2	0.2	0.1	0.1	0.3	0.1	0.1	0.1	4.2	4.3	10.5
13	*S. micracanthum* B	83	65	58	58	58	57	51	67	66	58	8	0	-	0.2	0.2	0.2	0.1	0.1	0.3	0.1	0.1	0.1	4.2	4.3	10.6
14	*S. macrocarpum*	82	64	59	59	59	60	52	70	66	61	7	3	3	-	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	4.1	4.2	10.5
15	*S. autumnale*	82	64	59	59	59	60	52	70	67	61	7	3	3	2	-	0.1	0.1	0.1	0.2	0.1	0.1	0.1	4.1	4.2	10.5
16	*S. siliquastrum* A	82	64	59	59	59	60	52	70	67	61	5	3	3	2	2	-	0.1	0.1	0.2	0.1	0.1	0.1	4.0	4.2	10.3
17	*S. siliquastrum* B	81	63	58	58	58	59	51	69	65	60	6	2	2	1	1	1	-	0.0	0.1	0.0	0.1	0.1	4.0	4.2	10.4
18	*S. siliquastrum* C	81	63	58	58	58	59	51	69	65	60	6	2	2	1	1	1	0	-	0.1	0.0	0.1	0.1	4.0	4.2	10.4
19	*S. serratifolium*	83	65	60	60	60	61	53	71	67	62	8	4	4	3	3	3	2	2	-	0.1	0.1	0.1	4.0	4.3	10.6
20	*S. yezoense*	81	63	58	58	58	59	51	69	66	60	6	2	2	1	1	1	0	0	2	-	0.1	0.1	4.0	4.2	10.4
21	*S. patens* [a]	73	65	59	59	59	62	67	66	69	63	60	61	61	60	60	58	59	59	58	59	-	0.0	0.0	0.1	0.1
22	*S. piluliferum* [b]	58	58	52	52	52	51	46	55	56	52	45	43	43	43	43	43	43	43	44	43	-	-	0.1	0.1	0.1
23	*S. yendoi*	111	103	87	87	85	88	84	97	99	90	63	61	61	60	60	58	59	59	59	59	1	43	-	6.7	11.0
24	*Hizikia*	101	93	62	62	62	63	61	74	74	66	68	64	64	63	63	63	62	62	64	62	63	57	96	-	10.9
25	*Myagropsis*	158	152	141	140	140	141	141	145	149	143	136	135	135	134	134	132	133	133	135	133	101	47	137	139	-

a, b: Pairwise comparison was made on only either ITS1 sequence or ITS2 sequence because either of them was unavailable for these taxa.

ably large deletions. They were 29 bases and 15 bases in size, and situated in ITS1 at sites 310-338 and 792-806, respectively. The latter was also shared by *S. patens* of subgenus *Schizophycus* and *S. yendoi* of subgenus *Sargassum*. These two species shared an additional deletion of 18 bases at sites 61-78. The 19-base indel at sites 678-696 separated the members of subgenus *Bactrophycus* and *Hizikia fusiformis* from the remaining three subgeneric species of the genus *Sargassum*, *S. patens*, *S. piluliferum*, and *S. yendoi*. The largest deletion among *Sargassum* species was 105-base deletion occurred in ITS2 of *S. piluliferum* at sites 1423-1527. Indels of which size was larger than 80 bases were present in ITS2, while all indels in ITS1 was smaller than 30 bases.

Sequence divergence for 23 accessions of 16 *Sargassum* species and two outgroups ranged from 0.0% to 12.3%. *Sargassum patens* and *S. piluliferum* should be excluded in the discussion on sequence divergence because either of ITS1 and ITS2 sequences was not available for them (Table 3). The highest sequence divergence was observed between *S. horneri* from Anin, Kangneung and *Myagropsis myagroides*, one of outgroups. In general, the sequence divergence between *Myagropsis myagroides* and members of *Sargassum* was considerably high in the range of 10.3-12.3%. However, the sequence divergence between species of *Sargassum* and *Hizikia fusiformis*, which was another presumed outgroup, was relatively low in the range of 4.1-6.8%. The sequence divergence among species of the genus *Sargassum* ranged from 0.0% to 7.6%. The sequence divergence among species was 0.1-7.5% in subgenus *Bactrophycus*. There was a considerable difference in the sequence divergence at sectional level. The sequence divergence was 0.4-4.1% in section *Teretia*, and 0.1-0.5% in section *Halochloa*. In the examination of the sequence divergence at population level, *S. horneri* showed a considerably high divergence of 1.2% between the populations from Anin, Kangneung and from Youngheungdo, Incheon. On the other hand, the sequence divergence was 0.0-0.1% among populations of *S. confusum*, *S. micracanthum*, and *S. siliquastrum*.

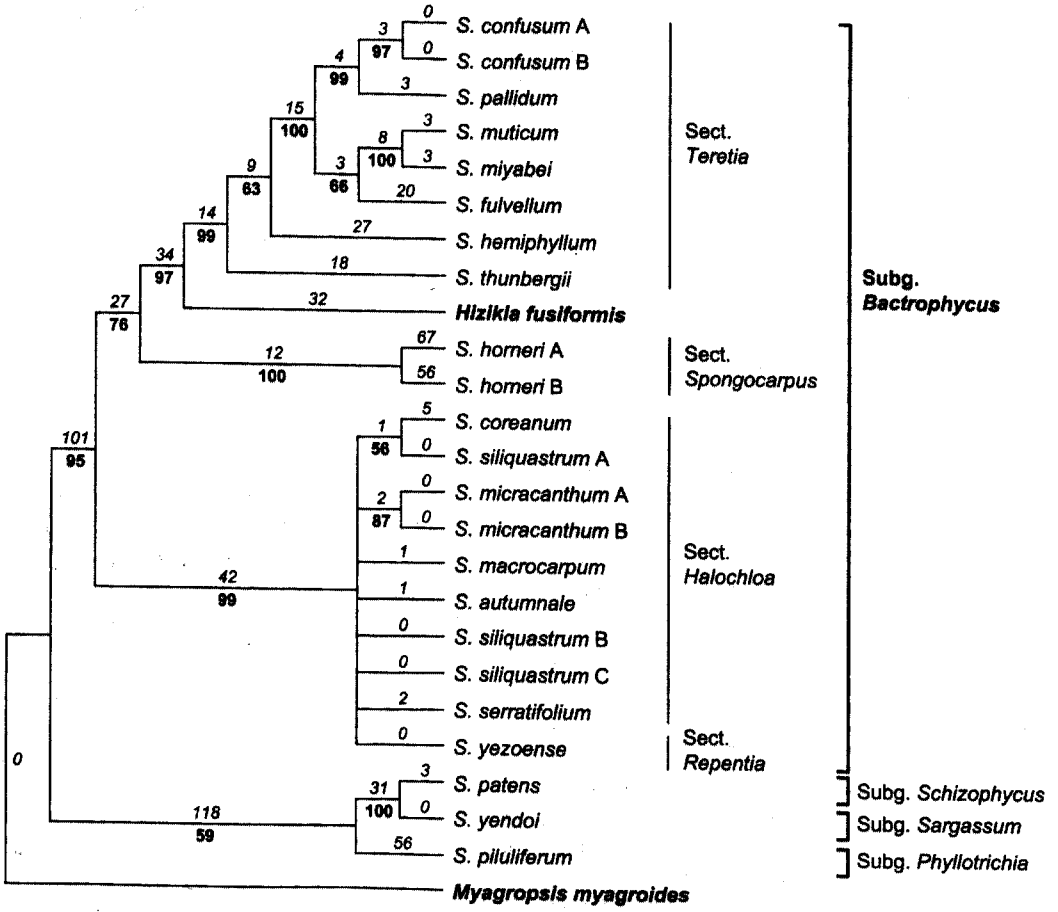

Fig. 1. The strict consensus tree of maximum parsimony analysis inferred from ITS sequences of *Sargassum*. Numbers above the branches indicate branch length, and numbers below the branches indicate bootstrap proportions in 2000 replicates.

In the phylogenetic analysis by PAUP (Swafford 2000), the most branches at various levels were well resolved and supported with high bootstrap values except for the relationships among seven species of sections *Halochloa* and *Repentia*, of which sequences were almost identical (Fig. 1). The relationships among seven species of sections *Halochloa* and *Repentia* were not resolved and the number of maximum parsimony (MP) trees generated by PAUP reached to the value of Maxtree option set by 1000. Neighbor-joining and maximum likelihood analyses also generated the exactly same topology with the MP tree.

Fifteen species of subgenus *Bactrophycus* constituted a very robust monophyletic group supported by 95% bootstrap confidence. To surprise, *Hizikia fusiformis* was nested in the clade of subgenus *Bactrophycus* even though it was designated as outgroup along with *Myagropsis myagroides*. In the subgenus *Bactrophycus* clade, sections *Halochloa* and *Repentia* were clustered together due to the lack of enough sequence variation. The ITS sequence of *S. yezoense* of section *Repentia* was identical with those of *S. siliquastrum* of section *Halochloa*. However, a population of *S. siliquastrum* from Hallim, Cheju showed one-base difference and was separated from them (Table 1, Fig. 1). Sections *Teretia* and *Spongocarpus* were closely allied, and species of section *Teretia* formed a strongly supported monophyletic group with high bootstrap confidence of 99%. *Hizikia fusiformis* was placed as a sister group to section *Teretia*, which was also supported with high bootstrap confidence of 97%. In the remaining three species representing subgenera *Schizophycus*, *Sargassum*, and *Phyllotrichia*, respectively, *S. yendoi* of subgenus *Sargassum* was closely allied with *S. patens* of subgenus *Schizophycus*, which was 100% supported in bootstrap analysis.

DISCUSSION

The size of ITS sequences from *Sargassum* revealed in this study is larger than any ITS sequences reported in

Phaeophyceae to date. The size of ITS1 sequences was 245-704 bases, and ITS2 was 243-525 bases in previous reports (Peters *et al.* 1997; Serrão *et al.* 1999; Coyer *et al.* 2001; Kawai *et al.* 2001; Uwai *et al.* 2001). The largest ITS sequences for both ITS1 and ITS2 were obtained from *S. hemiphyllum*, and their size was 787 bases and 593 bases, respectively.

The size of ITS sequences is considerably different in various algal groups. In Chlorophyceae, it ranges from 416 bases to 531 bases for ITS1 sequences, and from 291 bases to 405 bases for ITS2 sequences (Kooistra *et al.* 1992; Leskinen and Pamilo 1997; Coleman 1999; Fabry *et al.* 1999; Durand *et al.* 2002). On the other hand, the ITS1 is 151-563 bases and ITS2 is 350-799 bases in Rhodophyceae (Goff *et al.* 1994, 1997; Patwary *et al.* 1998). In Rhodophyceae, it is known that the ITS2 is usually larger than ITS1, while the latter is larger than the former in Chlorophyceae and Phaeophyceae. For species of *Sargassum*, ITS1 was always larger than ITS2 by about 160-180 bases. The ratio of ITS1/ITS2 was 1.3 in the genus *Sargassum*, which fit in the range of 1.0-1.6 previously reported for other genera of Fucales (Serrão *et al.* 1999).

In the phylogenetic trees, *Hizikia fusiformis* was always nested in the clade of the genus *Sargassum* as a sister group to section *Teretia* of subgenus *Bactrophycus*, and this placement was highly supported with 97% bootstrap confidence. *Hizikia fusiformis* was described originally as a member of *Cystophyllum* by Harvey (1860), and often considered to be closely allied with *Turbinaria* and *Sargassum*. Okamura (1932) established a monotypic genus *Hizikia* because it was distinguished from *Sargassum* by having no distinctively differentiated blades and vesicles. However, Setchell (1933) emphasized that *H. fusiformis* is very similar with *Sargassum thunbergii*. The plants of *Hizikia* are rather related to those of *Sargassum* in issuing basal blades, forming branches in the axil with a subsympodial mode, and producing receptacles in the axil of second blades (Lee and Kamura 1997). In addition to the close resemblance in morphology, 18S rDNA analysis resulted that *Hizikia fusiformis* was placed between *Myagropsis myagroides* and three species of *Sargassum* subgenus *Bactrophycus*, *S. horneri*, *S. confusum*, and *S. macrocarpum* (Horiguchi and Yoshida 1998). Moreover, Ajisaka (1997) pointed out that *H. fusiformis* (= *Sargassum fusiforme*) should be included in the subgenus *Bactrophycus* next to *S. hemiphyllum* in the cladistic analysis of order Fucales with morphological characters. The phylogenetic analysis of ITS sequences provides additional strong evidence that *H. fusiformis* should be placed in the genus *Sargassum* and it is closely allied with section *Teretia* of subgenus *Bactrophycus*.

In the ITS molecular tree, members of sections *Halochloa* and *Repentia* could not be separated because the sequence divergence was so low. The ITS sequences of *S. yezoense* of section *Repentia* are even identical with those of two Cheju populations of *S. siliquastrum* of section *Halochloa*. On the other hand, the ITS sequences of a Cheju population of *S. siliquastrum* was different from them by one base. *Sargassum siliquastrum*, *S. macrocarpum* and *S. autumnale* have been often regarded as varieties of *S. tortile* until Yoshida (1983) separated them as distinctive species. However, the divergence of ITS sequence was too low to separate them, which may suggest that their taxonomic status as distinctive species should be reconsidered.

Sections *Halochloa* and *Repentia* are not separable according to the ITS sequence analyses. In fact, the taxonomic boundary between these two sections has been obscure. *Sargassum okamurae* and *S. yamadae*, currently recognized as members of section *Repentia*, were originally separated from *S. sagamianum* Yendo of section *Halochloa*. In addition, *S. yezoense* was also transferred from *S. sagamianum* var. *yezoense* Yamada of section *Halochloa* (Yoshida and Konno 1983; Yoshida 1983). The ITS sequence analysis generated a tight clade of sections *Halochloa* and *Repentia*, of which sequence divergence was very low among them. The ITS data strongly suggest the alliance between sections *Halochloa* and *Repentia*. The morphological character such as decumbent stem, secondary attaching discs and shape of receptacles, separating these sections, should be reexamined to evaluate proper taxonomic prospects in future.

The discussion on phylogenetic relationships among three subgerera *Schizophycus*, *Sargassum*, and *Phyllotrichia* should be limited because the size of sampling for these subgenera was not large enough for phylogenetic analysis. However, taken the phylogenetic relationships among these three subgenera constructed on the basis of ITS sequences, the ITS molecular tree of this study utilizing both ITS1 and ITS2 is different from one based on only ITS2 sequences by Stiger *et al.* (2000). In the ITS2 tree, species of subgenus *Sargassum* were allied with the clade of subgenus *Bactrophycus*. In their ITS2 tree, *S. yendoi* was not included. However, in their unpublished results submitted to GenBank database, the ITS2 sequence of *S. yendoi* (GenBank accession no. AB043667)

was almost identical with one of *S. piluliferum* (GenBank accession no. AB043617). Therefore, further studies are demanded for molecular characters as well as morphological features to clarify taxonomic position of *S. yendoi*. In this study, the ITS sequences have been proved to be useful to clarify phylogenetic relationships in the genus *Sargassum*. As expanding the size of sampling to represent the broad spectrum of the genus, the analysis of ITS sequences will certainly provide an enhanced view on the phylogeny of *Sargassum*.

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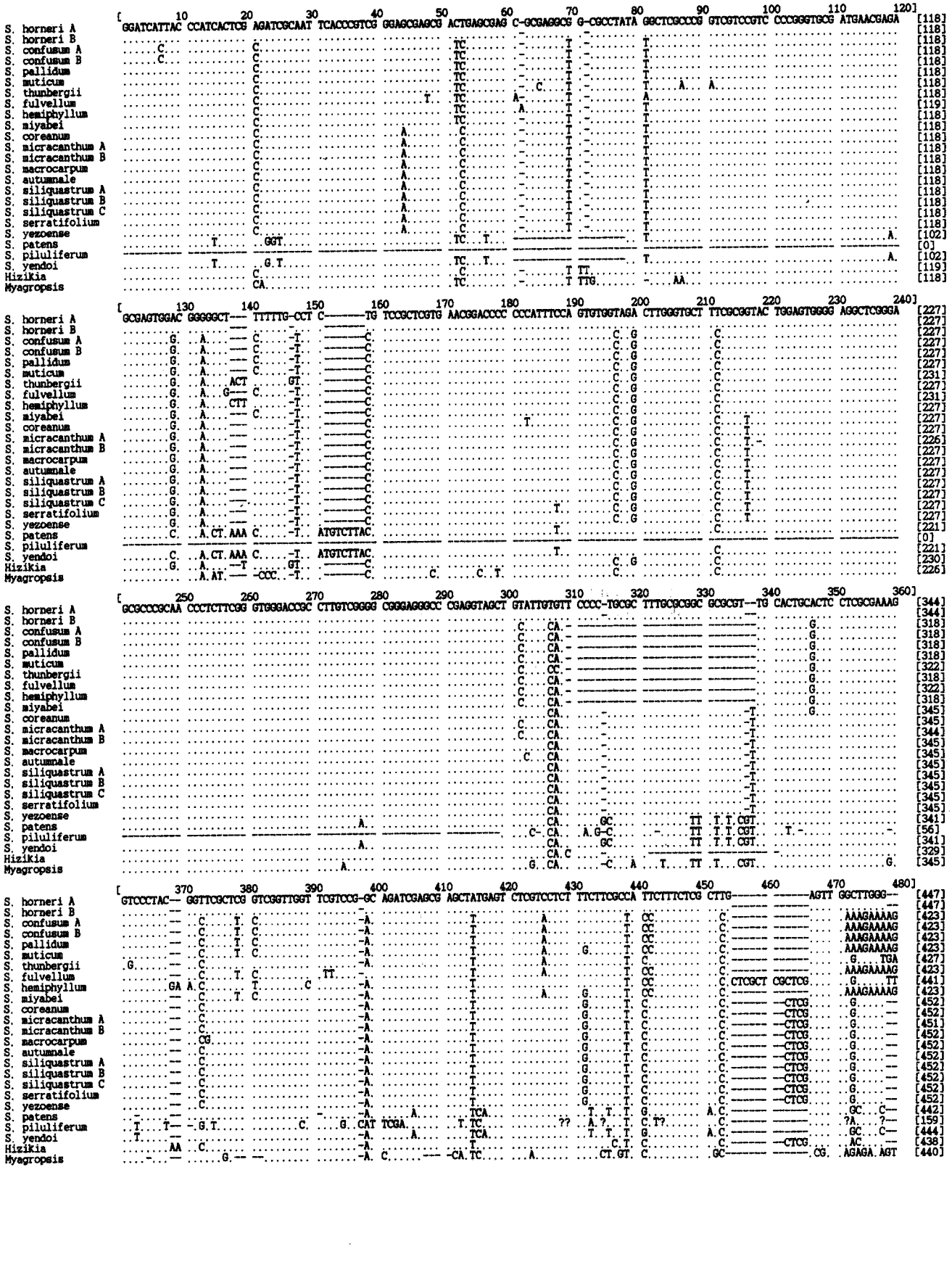
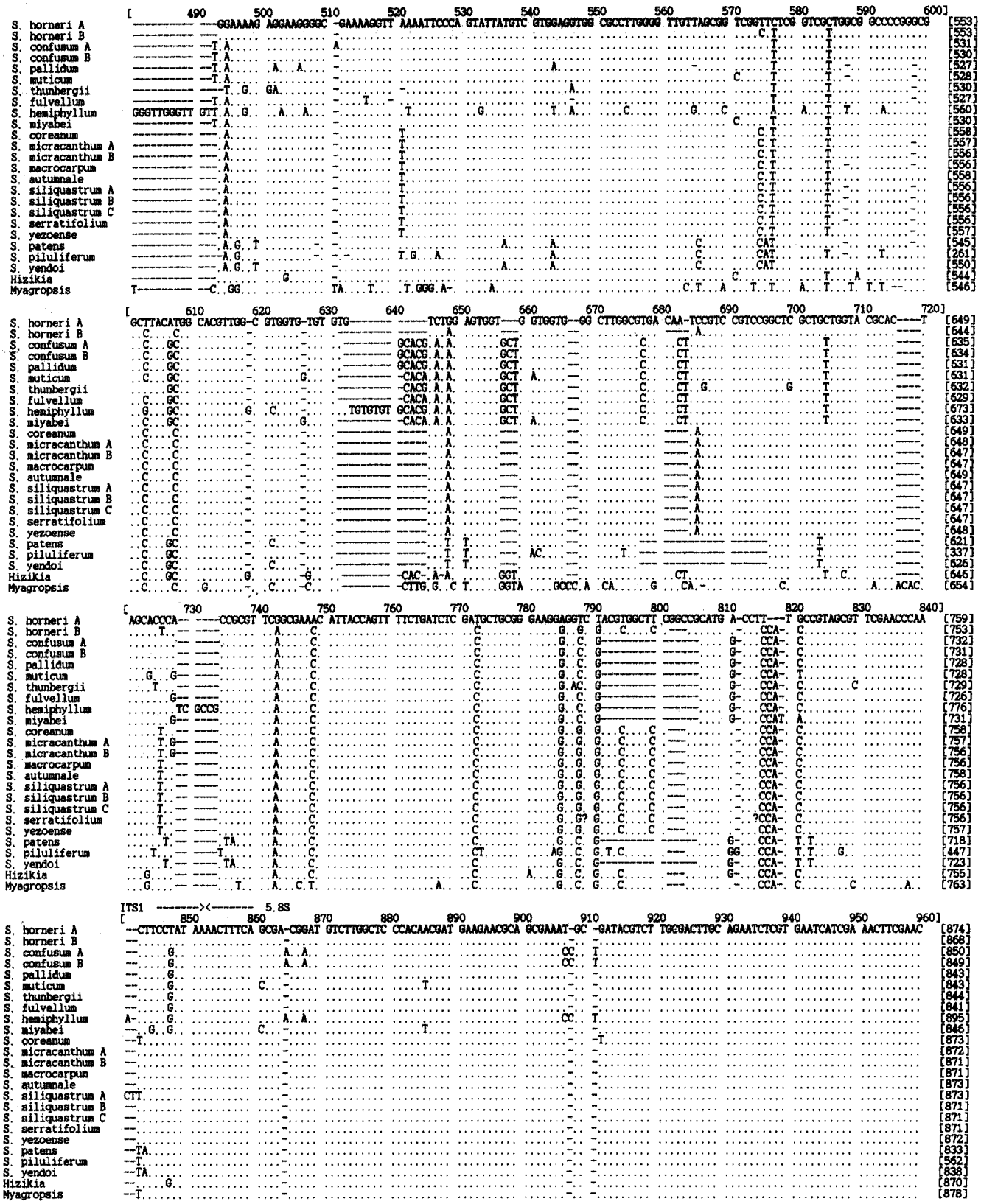
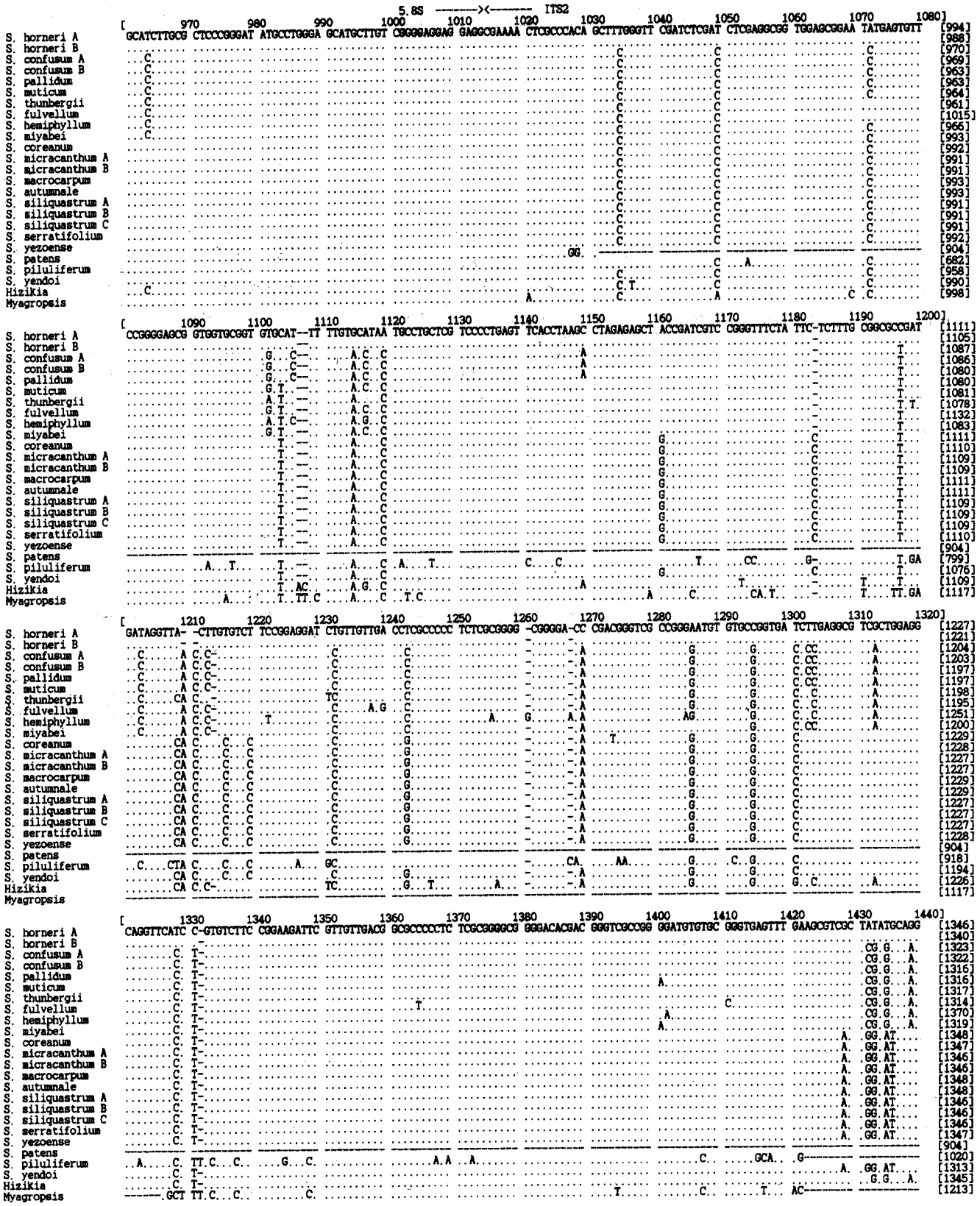
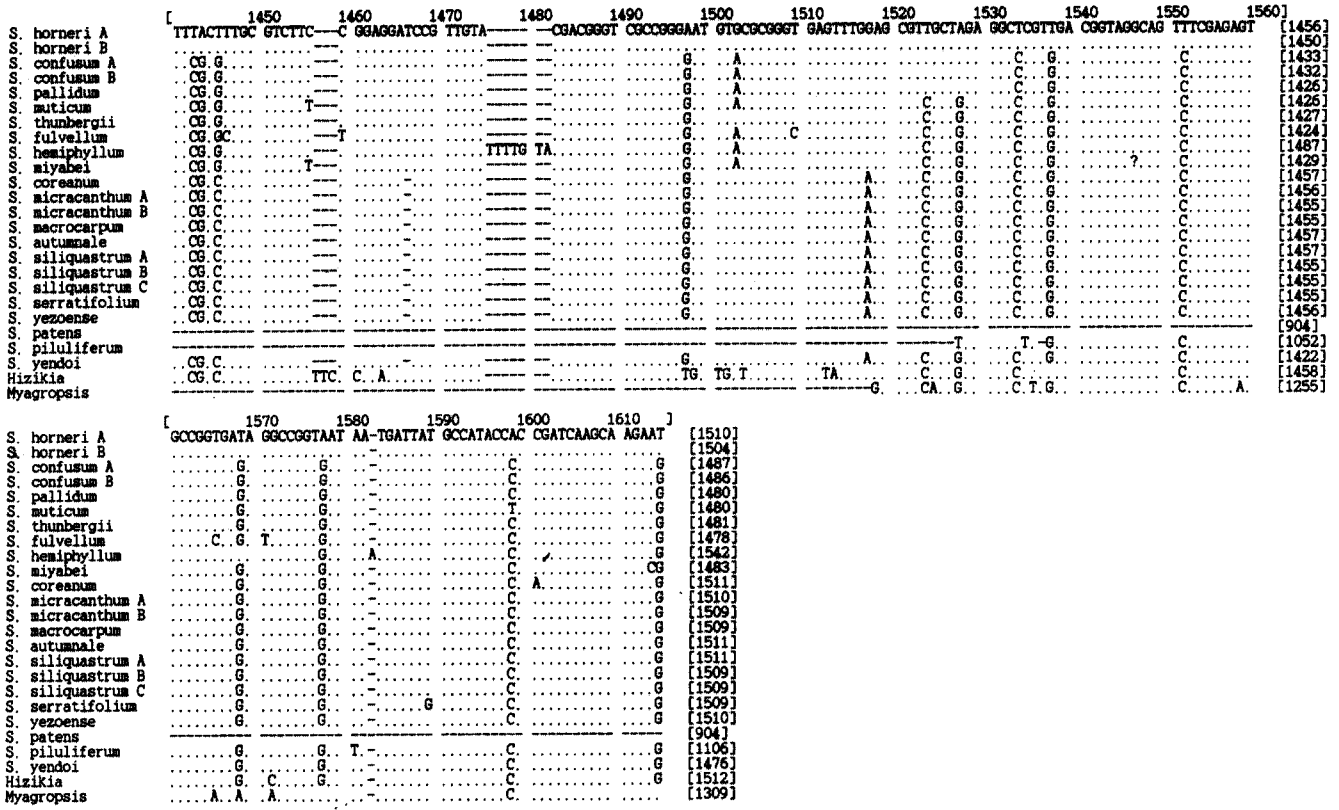
Appendix: Aligned ITS sequences from 25 accessions of 18 species of *Sargassum* and two outgroup species.




S. horneri A
S. horneri B
S. confusum A
S. confusum B
S. pallidum
S. muticum
S. thunbergii
S. fulvellum
S. hemiphyllum
S. miyabei
S. coreanum
S. micracanthum A
S. micracanthum B
S. macrocarpum
S. autumnale
S. siliquastrum A
S. siliquastrum B
S. siliquastrum C
S. serratifolium
S. yezoense
S. patens
S. piluliferum
S. yendoi
Hizikia
Myagropsis
ITS1
5.8S


5.8S
ITS2
S. horneri A
S. horneri B
S. confusum A
S. confusum B
S. pallidum
S. muticum
S. thunbergii
S. fulvellum
S. hemiphyllum
S. miyabei
S. coreanum
S. micracanthum A
S. micracanthum B
S. macrocarpum
S. autumnale
S. siliquastrum A
S. siliquastrum B
S. siliquastrum C
S. serratifolium
S. yezoense
S. patens
S. piluliferum
S. yendoi
Hizikia
Myagropsis


[1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560]
S. horneri A TTTACTTTGC GTCTTC—C GGAGGATCCG TTGTA———— —CGACGGGT CGCCGGGAAT GTGCGCGGGT GAGTTTGGAG CGTTGCTAGA GGCTCGTTGA CGGTAGGCAG TTTCGAGAGT [1456]
S. horneri B [1450]
S. confusum A [1433]
S. confusum B [1432]
S. pallidum [1426]
S. muticum [1426]
S. thunbergii [1427]
S. fulvellum [1424]
S. hemiphyllum [1487]
S. miyabei [1429]
S. coreanum [1457]
S. micracanthum A [1456]
S. micracanthum B [1455]
S. macrocarpum [1455]
S. autumnale [1457]
S. siliquastrum A [1457]
S. siliquastrum B [1455]
S. siliquastrum C [1455]
S. serratifolium [1455]
S. yezoense [1456]
S. patens [904]
S. piluliferum [1052]
S. yendoi [1422]
Hizikia [1458]
Myagropsis [1255]
[1570 1580 1590 1600 1610]
S. horneri A GCCGGTGATA GGCCGGTAAT AA-TGATTAT GCCATACCAC CGATCAAGCA AGAAT [1510]
S. horneri B [1504]
S. confusum A [1487]
S. confusum B [1486]
S. pallidum [1480]
S. muticum [1480]
S. thunbergii [1481]
S. fulvellum [1478]
S. hemiphyllum [1542]
S. miyabei [1483]
S. coreanum [1511]
S. micracanthum A [1510]
S. micracanthum B [1509]
S. macrocarpum [1509]
S. autumnale [1511]
S. siliquastrum A [1511]
S. siliquastrum B [1509]
S. siliquastrum C [1509]
S. serratifolium [1509]
S. yezoense [1510]
S. patens [904]
S. piluliferum [1106]
S. yendoi [1476]
Hizikia [1512]
Myagropsis [1309]