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Phylogenetic Analysis of *Phyllospadix iwatensis* Based on Nucleotide Sequences Encoding 18S rRNA and ITS-1

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Seagrasses are marine angiosperms of ecological importance in providing shelter and food to aquatic species as well as maintaining the carbon cycle on earth. *Phyllospadix iwatensis* is a seagrass of the family Zosteraceae and is distributed along the eastern coast of Korea. The nucleotide sequences of *P. iwatensis* nuclear genes encoding 18S ribosomal RNA (rRNA) and internal transcribed spacer-1 (ITS-1) were determined for molecular phylogenetic analysis. Genomic DNA was isolated from *P. iwatensis* and used for PCR amplification of 18S rRNA and ITS-1. Examination of the 18S rRNA sequence of *P. iwatensis* showed a close (99% similarity) relationship to *Zostera noltii*, another genus of Zosteraceae, but a distant (84% similarity) evolutionary relationship to other macroalgal Laminariales species. Further discrepancies found in ITS-1 nucleotide sequences between closely related species indicate that the sequence information could be used for species identification.

Key words: 18S rRNA, ITS-1, *Phyllospadix*, Phylogeny

Introduction

Seagrasses are unique marine flowering plants with long, narrow, and very often green leaves growing in marine environments. They are found anchored in sand or mud bottoms in shallow coastal areas and complete their entire life cycle underwater (Phillips and Menez, 1988). Seagrass beds play ecologically important roles by providing crucial habitats and food for various marine organisms from fish to mollusks and microalgae (Turner, 1985). Seagrass is also of interest for its high rates of primary production, contributing up to 15% of the ocean's total carbon storage as well as absorbing almost a quarter of global carbon emission (Williams, 1995). Therefore, from an ecological perspective, it is important to examine the species and populations of seagrasses distributed along coastlines.

Seagrasses belong to one of four plant families, Posidoniaceae, Zosteraceae, Hydrocharitaceae, and Cymodoceaceae, in the order Alismatales (Phillips and Menez, 1988). The family Zosteraceae is closely related to a family of freshwater aquatics, Potamogetonaceae, and contains approximately fourteen species divided into two genera, *Phyllospadix* and *Zostera*. The latter genus is further divided into three subgenera, *Heterozostera*, *Zostera*, and *Zosterella*. The genus *Phyllospadix* has been known as one of the seagrasses found in submerged coastal waters along the temperate North Pacific (Philips, 1979). The genus is morphologically characterized by the rhizome with congested internodes, marginal fin cell of the leaf, and dioecism (Tomlinson, 1982). Two species in the genus, *P. iwatensis* and *P. japonica*, are distributed along the coastline in Korea and Japan. Morphological differences between these two species include the apical shape of the leaf, the number of veins in the leaf, and the color of fibrous remains in the rhizome (Shin et al., 1993). *P. iwatensis* is characterized by a rounded apex, three and five veins in the apical and lower portions of the leaf, respectively, and brown fibrous remains in the rhizome (Kuo et al., 1990; Yabe et al., 1995). Although the morphological characteristics of *P. iwatensis* have been fairly well documented (Shin et al., 1993), little information has been available on its molecular markers and phylogenetic analysis. To perform phylogenetic analysis of *Phyllospadix* using molecular markers, nucleotide sequences of the regions encoding the 18s rRNA and ITS-1 of *P. iwatensis* were determined in this study. Comparisons of the 18S rRNA sequence with that of other species

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provided information on the evolutionary position of *Phyllospadix*, which is distant from other macroalgal species, but close to seagrasses. The nucleotide sequence of the ITS-1 region provides further information for species-specific identification of *Phyllospadix.*

Materials and Methods

Materials

Phyllospadix iwatensis was collected from the coastal areas of Uljin, Korea. Collected samples were stored at -20°C until DNA isolation.

Restriction endonucleases were purchased from Bioneer (Daejeon, Korea). Kits used for plasmid purification, gel extraction, and PCR purification were purchased from NucleoGen (Seoul, Korea). The pGEM®-T Easy Vector system was obtained from Promega Corporation (Madison, WI). Oligonucleotides and a HiQ-PCR mix were obtained from Genotech (DaeJeon, Korea). The oligonucletides used for the amplification of 18s rRNA and ITS-1 were as follows: 5'-TCCGTAGGTGAACCTGCGG (ITS1F); 5'-GCTGCGTTCTTCATCGATGC (ITS1R), 5'-CAA CCTGGTTGATCCTGCCAGT (18SF); 5'-CTGATC CTTCTGCAGGTTCACCTAC (18SR).

Isolation of genomic DNA

Genomic DNA was isolated using the nuclei isolation/CTAB methods described in Valera-Alvarez (2006). A 0.5-g tissue sample was ground in a mortar in the presence of liquid nitrogen and homogenized in 5 mL STE buffer (400 mM sucrose, 50 mM Tris. Cl pH 7.8, 20 mM EDTA, 0.2% bovine serum albumin, and 0.2% β-mercaptoethanol). The homogenate was filtered through a 50 μm nylon mesh by squeezing and subjected to centrifugation at 1,000x *g* for 20 min. The nuclei pellet, resuspended in 50 μL of CTAB buffer (2% CTAB, 2% polyvinylpyrrolidone, 1.4 M NaCl, 20 mM EDTA, and 100 mM Tris. Cl pH 8.0), was incubated at 65°C for 1 hr. The suspension was extracted with an equal volume of the mixture containing chloroform:isoamylalcohol (24:1) followed by centrifugation at 14,000 rpm for 3 min. DNA in the supernatant was precipitated by the addition of two volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) followed by centrifugation. The DNA was examined by 0.7% agarose gel electrophoresis.

Cloning of the genes encoding 18S rRNA and ITS-1

Amplification of 18S rRNA and ITS-1 was carried out in 20 μL of PCR mixture including HiQ-Mix PCR mixture, and 0.1 μg of isolated genomic DNA. The reaction was carried out with an initial denaturation at 95°C for 5 min followed by 30 cycles of 45 sec at 94°C, 30 sec at 55°C and 90 sec (18S rRNA) or 45 sec (ITS-1) at 72°C, with a final extension of 5 min at 72°C. Amplified PCR products, resolved upon agarose gel electrophoresis, were purified by gel extraction, cloned into the pGEM®-T Easy vector, and then transformed into *E. coli* DH5α as described by Sambrook et al. (2001). Recombinant plasmids were isolated using alkaline lysis and analyzed by restriction digestion and DNA sequencing.

Sequence alignment and phylogenetic analysis

The obtained nucleotide sequences were aligned with other rRNA and ITS sequences using Clustal W (Thompson et al., 1994). The NCBI GenBank accession numbers of the 18s rRNA sequences examined in this study were as follows: *Phyllospadix iwatensis* (*Phyliwat*, GenBank HQ660595, this study), *Zostera noltii* (AF207058), *Posidonia oceanica* (AY491942), *Potamogeton pusillus* (EF526354), *Ulva pertusa* Kjellman (AB425961), *Ecklonia cava* Kjellman (AF123579), *Agarum clathratum* Dumortier (AF123576), *Costaria costata* Saunders (AB022819), *Porphyra yezoensis* Ueda (DQ666486), and *Gelidium amansii* (Lamouroux) Lamouroux (DQ316994). The NCBI GenBank accession numbers of the ITS-1 sequences examined in this study were as follows: *Phyllospadix iwatensis* (*Phyliwat*, HQ660596, this study), *Zostera noltii* (AF102275), *Phyllospadix torreyi* (AY077985), *Ulva pertusa* Kjellman (HM 485430), *Ecklonia cava* Kjellman (AF319009), *Agarum clathratum* Dumortier (FJ042768), *Costaria costata* Saunders (AF319027), and *Panax japonica* (HQ112433).

A phylogenetic tree was constructed using the neighbor-joining method in MEGA v 4.0 (Tamura et al., 2007). An assessment of tree reliability was conducted using a bootstrap method with 1000 replicates.

Results and Discussion

Aquatic plants and macroalgae distributed along coastlines are important for marine ecology due to their provision of shelter and food for aquatic species as well as their importance in maintaining global carbon storage and emission cycles. *Phyllospadix*, which is distributed along the coast of Korea and Japan, is a perennial seagrass belonging to the family Zosteraceae. Although the morphological charac-

teristics and growth dynamics of *Phyllospadix* have been fairly extensively examined (Shin et al., 1993; Tomlinson, 1982; Park and Lee, 2009), little information is available on its detailed molecular characteristics and phylogenetic analysis. Ribosomal RNA has been used as one of the most common markers for analyzing the molecular phylogeny of macroalgae (Bird et al., 1992; Ragan et al., 1994), although its use has sometimes been known to be hindered by discrepancies due to the presence of duplicate copies. To carry out a molecular-marker-based phylogenetic analysis of *P. iwatensis*, DNA regions encoding 18S rRNA and its neighboring ITS sequences were analyzed for the first time in this study.

Isolation of high-quality genomic DNA from macroalgae and its use in PCR amplification have previously been hindered by the intractability of various algae to many DNA extraction procedures and the coextraction of polysaccharide and polyphenolic compounds, which can inhibit the processes (Varma et al., 2007; Koonjul et al., 1999). Methods have been described for the successful isolation of genomic DNA from various macroalgal species (Nakajima et al., 2000; Snirc et al., 2010; Hoarau et al., 2007). In this study, genomic DNA was isolated from ground *Phyllospadix* using the STE and CTAB buffer method (Valera-Alvarez, 2006). The quality of the isolated genomic DNA was confirmed by the presence of high-molecular-weight genomic DNA upon agarose gel electrophoresis (data not shown). PCR amplifications of the target genes were carried out using genomic DNA templates and primers deduced from the conserved sequences (Yotsukura et al., 1999). Amplification of the regions corresponding to 18S rRNA and ITS-1 results in the synthesis of approximately 1.8 kb and 300 bp, respectively, fragments which are similar to the sizes of the corresponding regions in other macroalgal species including *Ecklonia cava* and *Costaria costata* (data not shown). Amplified fragments were purified from agarose gel, cloned into a pGEM-T vector, and then transformed into *E. coli* DH5α (Sambrook et al., 2001). Recombinant plasmids were isolated from transformants and analyzed by *Eco*RI restriction digestion followed by agarose gel electrophoresis (data not shown).

Sequencing analysis of 18S rRNA and ITS-1 clones showed the presence of 1,824- and 289 bp fragments, including the regions corresponding to the primers (Fig. 1 and Fig. 2). Nucleotide sequences were obtained from at least two independent clones in which the sequences were confirmed by analysis from both directions. Analysis of *P. iwatensis* 18s rRNA using BlastN indicated the highest similarity to the18s rRNA of other seagrasses including *Zostera noltii* and *Posidonia oceanica*. *P. iwatensis* 18S rRNA showed a 99% similarity to that of *Z. noltii,* another genus in Zosteraceae, and 98% similarity to that of *P. oceanica*, belonging to the family Posidoniaceae in the same order. Comparison of the 18s rRNA sequences of *Agarum clathratum* Dumortier and *Costaria costata* belonging to the same family, Laminariaceae, and *Ecklonia cava* Kjellman of the Alariaceae in the same order, Laminariales, showed almost completely identical 1.8 kb sequences, with divergence at only one position (data not shown). In contrast, the 18s rRNA sequence of *P. iwatensis* showed only 84% similarity with those of macroalgal species belonging to Laminariceae. The results indicate a clear evolutionary distinction between seagrasses and other macroalgal species.

To further examine the phylogenetic differences among closely related species, DNA sequences encoding *P. iwatensis* ITS-1 with high variability were determined and compared with those of other members of the family Zosteraceae (Fig. 2). In contrast to the relatively highly conserved 18s rRNA sequences, the ITS-1 fragments showed higher sequence variability in the region, as shown by an 87% similarity to that of *P. torreyi* and 70% similarity to that of *Z. noltii.* This provides useful information, not only for the detailed evolutionary analysis of *P. iwatensis* but also for its species-specific identification among closely related species.

Phylogenetic trees were constructed based upon 18S rRNA and ITS-1 sequences of *P. iwatensis* (Fig. 3 and Fig. 4) compared with those of other macroalgae and seagrasses. Species belonging to the family Laminales were hardly distinguished by their almost identical 18srRNA sequences, as shown by *Costaria costata*, *Agarum clathratum*, members of the family Laminariaceae, and even *Ecklonia cava*, belonging to the neighboring family, Alariaceae, in the same order, Laminariales (Fig. 3). However, *P. iwatensis* 18S rRNA showed the closest, but distinguishable, evolutionary relationship with *Z. noltii*, another member of Zosteraceae. This was followed by *Posidonia oceanica* in the same order, Alismatales, and *Potamogeton pusillus*, a closely related but freshwater aquatic. These results were consistent with previous morphology-based classifi-cations and indicated that 18S rRNA can be used to analyze the evolutionary relationships of *Phyllospadix*. The results also indicated that seagrasses are

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   1 CAACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTG
  61 CAAGTATGAACTAATTCAGACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTT
 121 GTTTGATGGTACCTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCA
 181 AACCCCGACTTCTGGAAGGGATGCATTTGTTAGATAAAAGGTTGACACGGGCCTTGTGCC
 241 TGTTGCTCTGATGATTCATGATAACTTGACGGATCGCATGGCCTTTGTGCCGGCGACGCA
 301 TCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGGGGCCTACCATGGTGGTG
 361 ACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACA
 421 TCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACGGGGAGGTAGTGACAAT
 481 AAATAACAATACCGGGCTCTTTGAGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTT
 541 AACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAAT
 601 AGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGACCTTGGGTTGGGTCGGC
 661 CGGTCCGCCTTATGGTGTGCACCGACCGTCTCGTCCCTTCTGCTGGTGATGCGTTCCTGT
 721 CCTTAGTTGGTCGGGTCGTGCCTCCGGCGCTGTTACTTTGAAAGAAATTAGAGTGCTCAA
 781 AGCAAGCCTATGCTCTGTATACATTAGCATGGGATAACATCACAGGATTTCGATCCTATT
 841 TTGTTGGCCTTCGGGATCGGAGTAATGATTAAAAGGGACAGTCGGGGGCATTCGTATTTC
 901 ATAGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACAACTGCGAAAGCATTTGCCA
 961 AGGATGTTTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGACGATCAGATACCGTCC
1021 TAGTCTCAACCATAAACGATGCCGACCAGGGATTGGCGGATGTTGCTTTTAGGACTCCGC
1081 CAGCACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGA
1141 AACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACT
1201 CAACACGGGGAAACTTACCAGGTCCAGACATAGTAAGGATTGACAGATTGAGAGCTCTTT
1261 CTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTT
1321 AATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGCGGAGGTGACCCTTCGT
1381 GGCCAGCTTCTTAGAGGGACTATGGCCGCCTAGGCCACGGAAGTTTGAGGCAATAACAGG
1441 TCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGTATTCAACGAGT
1501 TTATAACCTTAGCTGATAGGCTTGGGTAATCTTTGAAAATTTCATCGTGATGGGGATAGA
1561 TCATTGCAATTGTTGGTCTTCAACGAGGAATTCCTAGTAAGCGTGAGTCATCAGCTCGCG
1621 TTGACTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAATGGTCCG
1681 GTGAAGTGCTCGGATTGTGGCGACGTTAGTGGTCTGCCGCTGGCGACGTCGTGAGAAGTC
1741 CACTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCT
1801 GCAGAAGGATCAG
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Fig.1. Nucleotide sequence of *P. iwatensis* 18S rDNA. Sequences corresponding to the primers used for PCR amplification are underlined.

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Phyliwa     ACCAAGAACAAGTGAAC--TTGTCCACAGG--CC ---------TTTGCCTGAAGGACGCCCGTCGTTTTA   57
Phyltor     ACCGCGAACAAGTGAACATTGTCGT--AGGGCCTC----- TTTGCCTGAAGGACGCCCATCGTTTGA   60
Zostnol     ACCACGAACAAATTA --ATTTGTATGAAGGATCCACATTTTGATGTAAC---------- ATTTTTGTA   56
            ***   ******* * *     ****     ***  **    ****     **              **  *

Phyliwa     ACTCCGTTGGGTGCTCCATAACCAATCCACGGCACGA-TCGTTGCCAAGGCAAA — CGGA  114
Phyltor     ACTTCGTCGGGCGCTCCGCAACCAATCCACGGCACGA-TCGTTGCCAAGGCAAA —CGGA  117
Zostnol     ACTTCATTGTGTGCTCCATAACAAAAAAACGGCACAAATTGTTGCCAAGGCAAAAATGTA  116
            *** * * * * ***** *** **    *******       **************   * *

Phyliwa     AGGACGATTCGGTGGTGGTGC--CCAATGTGCAT-- -CATCCACGATCGTCCATGTATCT-  169
Phyltor     AAGATGATTCCGTGGTGGTGC –CCAAA-TGCATT- -CATCCACGATCGTCTAAGTATCTC  173
Zostnol     AGGAGTATTTTGAGATGATATGTTGAAATGCATTTTCATCAAAGATATCTTCATTATCTT  176
            *  **  ***   *    ** *       ** *****   **** * ***          ****

Phyliwa     --AATCCTCATGGATTGC       185
Phyltor     TAAATCCTCATGGATTGC       191
Zostnol     --AATCCTCATGGATAGC       192
              ********** **
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Fig. 2. Comparison of ITS-1 sequence of *P. iwatensis* to those of *P. torreyi* and *Zostera noltii* using Clustal W. Nucleotide sequences at the ends were trimmed to the same length for comparison.

phylogenetically closer to the species in Chlorophytae (*Ulva pertusa*) than to the species in Divisions Rhodophyta and Phaeophyta.

Phylogenetic analysis was also carried out using ITS-1 as a molecular marker (Fig. 4). The results showed the closest relationship to another member in

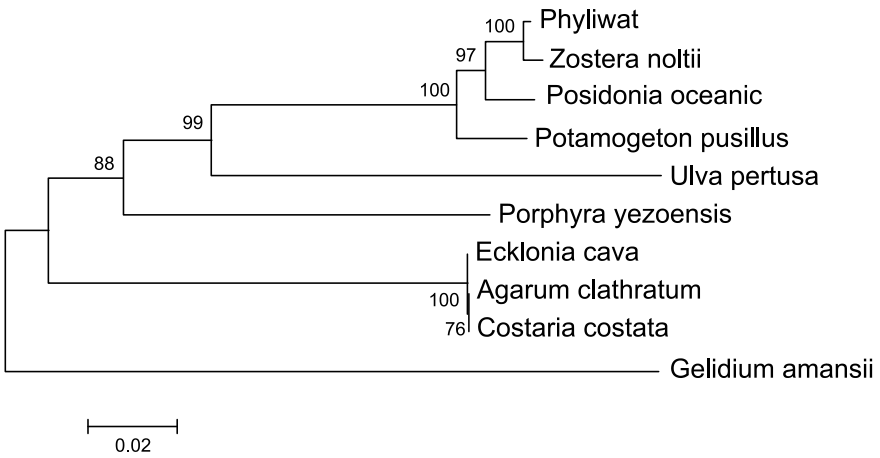

Fig. 3. Phylogenetic analysis of macroalgal species by using 18S rDNA nucleotide sequence. Compared sequences include 18S rDNA of *P. iwatensis* (*Phyliwat,* HQ660595, this study*),* Zostera noltii (AF207058), Posidonia oceanica (AY491942), *Potamogeton pusillus* (EF526354), *Ulva pertusa* Kjellman (AB425961), *Ecklonia cava* Kjellman (AF123579), *Agarum clathratum* Dumortier (AF123576), *Costaria costata* Saunders (AB022819), *Porphyra yezoensis* Ueda (DQ666486), and *Gelidium amansii* (Lamouroux) Lamouroux (DQ316994).

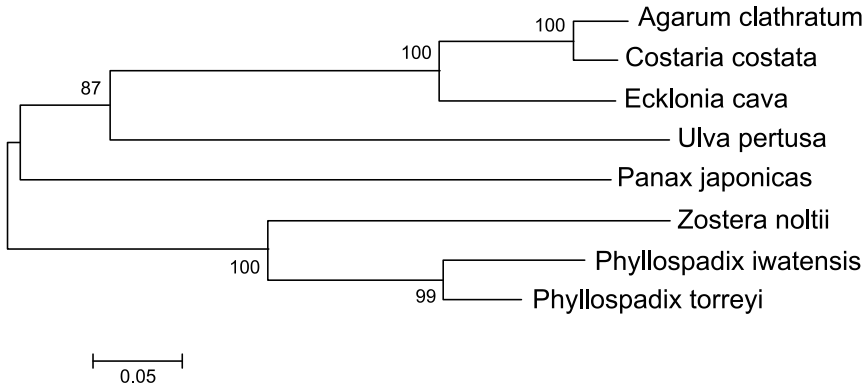

Fig. 4. Phylogenetic analysis of macroalgae using nucleotide sequences encoding ITS-1. Accession numbers of sequences corresponding to ITS-1 regions searched from NCBI GenBank were as follows: *P. iwatensis* (*Phyliwat*, HQ660596, this study*), Zostera noltii* (AF102275), *Phyllospadix torreyi* (AY077985), *Ulva pertusa* Kjellman (HM485430), *Ecklonia cava* Kjellman (AF319009), *Agarum clathratum* Dumortier (FJ042768), *Costaria costata* Saunders (AF319027), and *Panax japonica* (HQ112433).

the genus, *P. torreyi*, followed by *Z. noltii*, another member of the same family. In particular, the evolutionary relationships among members of the order Laminariales (*E. cava*, *A. clathratum*, and *C. costata*), which were indistinguishable using 18S rRNA markers, were clearly differentiated using ITS-1 comparison. In addition, the evolutionary position of *P. iwatensis* was further distinguished from *P. torreyi*, in the same genus, and *Zostera noltii*, in the same family, showing a clear distinction between *P. iwatensis* and other seagrasses.

In summary, nucleotide sequences encoding 18s rRNA and ITS-1 of *P. iwatensis* were obtained and used for a molecular phylogenetic analysis of *P. iwatensis*. The results provide information on the phylogenetic relationship between *P. iwatensis* and species of evolutionarily distant macroalgae, as well as closely related seagrasses. The information obtained will also be helpful for species-specific identification.

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