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Detection of Laminariaceae Species Based on PCR by Family-specific ITS Primers

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Abstract

To analyze nucleotide sequence encoding internal transcribed spacer (ITS) regions specific to the Laminariaceae family, genomic DNA was isolated from six brown algae species distributed along the east coast of Korea. These included three species from the Laminariaceae family (*Agarum clathratum* Dumortier, *Costaria costata* [C. Agardh] Saunders, and *Saccharina japonica* Areschoug) and two species from the Alariaceae family (*Undaria pinnatifida* [Harvey] Suringer and *Ecklonia cava* Kjellman), both in the order Laminariales, and one species from the family Sargassaceae in the order Fucales (*Sargassum serratifolium*). Based on a sequence analysis of ITS-1 and ITS-2 for *A. clathratum*, *C. costata*, and *E. cava*, oligonucleotides were designed from the regions that showed sequence conservation in Laminariaceae. Following polymerase chain reaction using three sets of primers, amplification of ITS-1 and ITS-2 was detected in reactions using genomic DNA isolated from the species belonging to Laminariaceae, but not from the species belonging to the other families. The results indicate that this method can be used for the detection and identification of Laminariaceae species.

Key words: ITS-1, ITS-2, Laminariaceae, Species detection

Introduction

Algae are photosynthetic organisms found in aquatic environments, and they can be divided into three divisions based on pigment content: Chlorophyta, Pheophyta, and Rhodophyta. The brown algae (Phaeophyta) are a group of mostly marine species found in temperate and colder Northern hemisphere waters. Species belonging to 12 orders in the class Phaeophyceae (Ectocarpales, Ralfsiales, Sphacelariales, Dictyotales, Chordariales, Dictyosiphonales, Scytosiphonales, Cutleriales, Sporochnales, Desmarestiales, Laminariales, and Fucales) are known to be distributed along the coast of Korea (Lee and Kang, 2002). Species belonging to three families (Alariaceae, Chordaceae, and Laminariaceae) in the order Laminariales were found in Korea. The former includes *Undaria pinnatifida*, the most widely cultivated algae in Korea, as well as *Ecklonia cava*, *Ecklonia stolonifera*, and *Eisenia bicyclis*. The Laminariaceae family is distributed along the east coast of Korea and includes *Saccharina japonica*, the second most widely cultivated algae in Korea, along with *Agarum clathratum* and *Costaria costata*.

Commercial production of kelp species has been mostly achieved by mariculture in East Asian countries including China (the largest producer of Laminaria), Korea, and Japan. Korea is the sixth largest macroalgae producer in the world, producing 921,024 MT in 2008, despite the fact that algal species produced in large amounts are mostly cold current-adapted species, meaning that production is restricted during the winter season. For mass production, it is of commercial interest to screen Laminariaceae species in which most of fast-growing algal species belong.

Genes encoding ribosomal RNAs and internal transcribed

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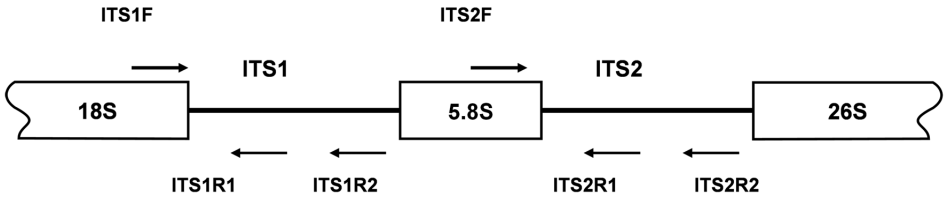

Fig. 1. Diagram showing the locations of oligonucleotides used for the amplification of internal transcribed spacer (ITS)-1 and ITS-2. Figures are not in scale.

spacer (ITS) regions have been used as molecular markers for species identification and phylogenetic analysis of macroalgae (Bird et al., 1992; Ragan et al., 1994). While a rather conserved 18S rRNA sequence provides information for distinguishing evolutionary distant species, a rapidly evolving ITS sequence has been used for the identification of intraspecific variation. In this study, DNA regions encoding ITS-1 and ITS-2 of species belonging to Laminariaceae were analyzed to develop a PCR-based species identification method.

Materials and Methods

Materials

Macroalgal species including *U. pinnatifida, E. cava, A. clathratum, C. costata, S. japonica,* and *S. serratifolium* were collected from the east coast area of Korea. Species collected from at least two different locations were stored at -20°C until DNA isolation was performed. Kits used for plasmid and PCR product purification were purchased from NucleoGen (Seoul, Korea) and pTOP TA V2 cloning vector was obtained from Enzynomics (Daejeon, Korea). Oligonucleotides and a HiQ-PCR mix were obtained from Genotech (Daejeon, Korea). Oligonucleotides used for the initial amplification of ITS-1 and ITS-2 (Fig. 1) and inner regions were designed as described previously (Yotsukura et al., 1999; Kim and Choi, 2010). Oligonucleotides specific to Laminariaceae ITS-1 and ITS-2 were designed from the corresponding regions conserved in *A. clathratum* and *C. costata* but not in *E. cava* (Fig. 2) as follows: 5′-TCCGTAGGTGAACCTGCGG-3′ (ITS1F), 5′-GCTGCGTTCTTCATCGATGC-3′ (ITS1R), 5′-CCAACTTCGCATAACGAATTC (ITS1R1)-3′, 5′-AGGTTGGGGGGGCCGCGGC-3′ (ITS1R2), 5′-TCCTGGGAGCATGCTTGTCG-3′ (ITS2F), 5′-TCCTCCGCTTATTGATATGC-3′ (ITS2R), 5′-CGAGAGTCGCCGCCGAAGCG-3′ (ITS2R1), and 5′-ACGAAAGTGGTACGGTTTCC-3′ (ITS2R2).

Isolation of genomic DNA

Genomic DNA was isolated using previously described nuclei isolation/centyltrimethylammonium bromide (CTAB) methods (Varela-Álvarez et al., 2006). Tissue samples (0.5 g) were 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 ethylenediaminetetraacetic acid (EDTA), 0.2% bovine serum albumin, and 0.2% β-mercaptoethanol). The homogenate was filtered through a 50 μm nylon mesh by squeezing and was subjected to centrifugation at 1,000 *g* for 20 min. The nuclei pellet was resuspended in 50 μL CTAB buffer (2% CTAB, 2% polyvinylpyrrolidone, 100 mM Tris-Cl pH 8.0, 20 mM EDTA, and 1.4 M NaCl) followed by incubation at 65°C for 1 h. The suspension was extracted with the same 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.

Cloning of the genes encoding ITS-1

PCR amplifications of the regions corresponding to the inner segments of ITS-1 and ITS-2 were carried out with sets of primers including ITS1F together with the species-specific primers ITS1R1 or ITS1R2, and ITS2F together with ITS2R1 or ITS2R2, respectively. Amplification of ITS-1 was carried out in a 20 μL PCR mixture including a HiQ-PCR Mix, genomic DNA (0.1 μg), and 1 μM primers. The reaction was carried out with an initial denaturation at 95°C for 5 min followed by 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, together with a final extension at 72°C for 5 min. PCR amplification using ITS2F and ITS2R1 primers was also carried out under the same condition except an annealing temperature at 65°C. Amplified PCR products, resolved upon agarose gel electrophoresis, were purified by gel extraction, cloned into the pTOP TA V2 cloning vector, and then transformed into *E. coli* DH5α as described by Sambrook and Russell (2001). Recombinant plasmids isolated by alkaline lysis were analyzed by *Eco*RI restriction digestion and DNA sequencing.

Sequence analysis

The nucleotide sequences obtained were aligned with other ITS sequences using ClustalW (Thompson et al., 1994). The

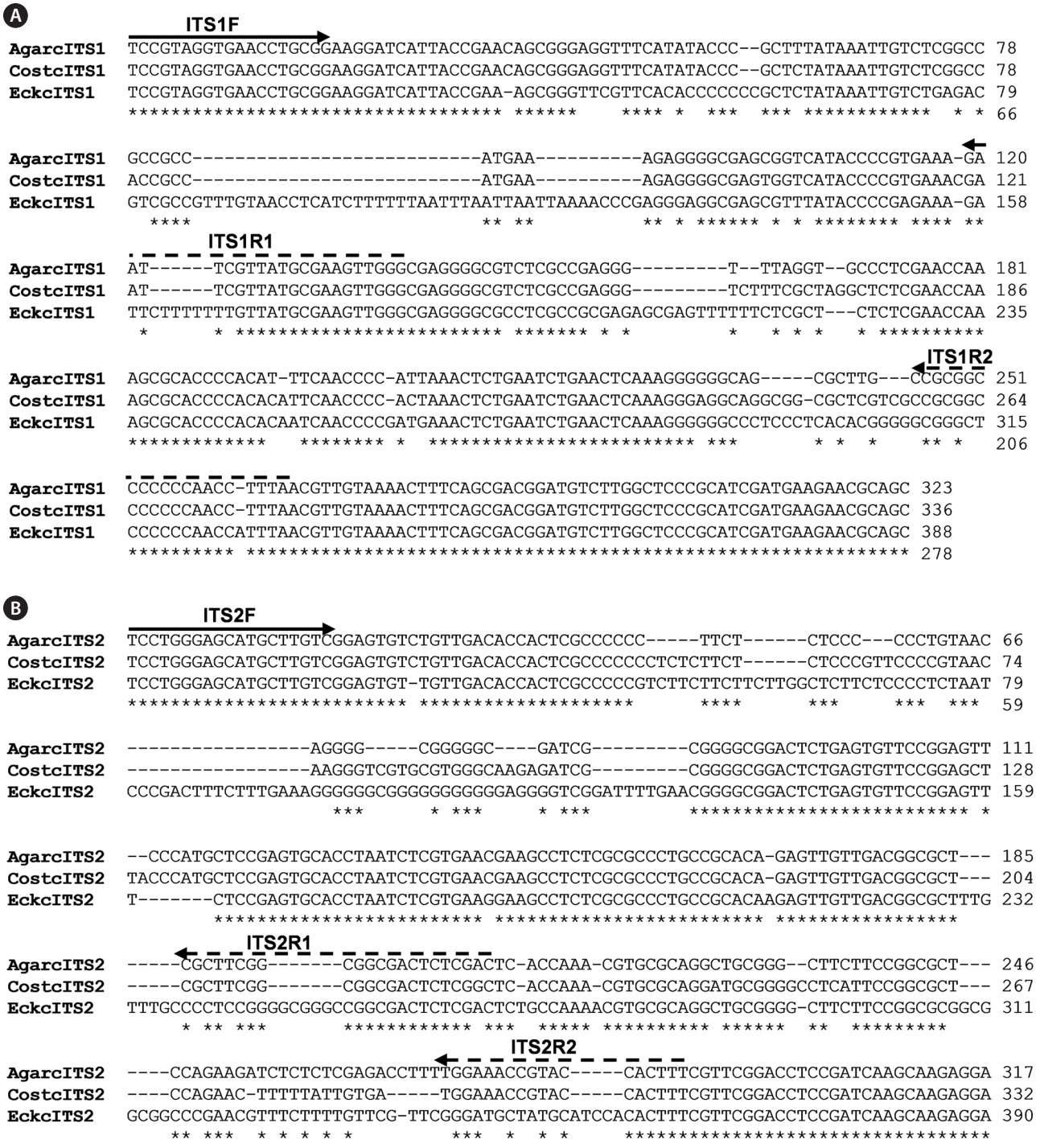

Fig. 2. Comparison of internal transcribed spacer (ITS)-1 (A) and ITS-2 (B) sequences of *Agarum clathratum* (AgarcITS1 and AgarcITS2), *Costaria costata* (CostcITS1 and CostcITS2), and *Ecklonia cava* (EckcITS1 and EckcITS2) using Clustal W. Nucleotide sequences at the ends were trimmed to make the same length for comparison and the broken arrows indicates the positions of ITS-specific primers used for PCR amplification.

accession numbers of ITS-1and ITS-2 sequences, acquired from the NCBI GenBank database (Guiry and Guiry, 2011) were as follows: *E. cava* Kjellman (AF319009), *A. clathratum* Dumortier (FJ042768), and *C. costata* Saunders (AF319027).

Results and Discussion

The production of macroalgae has been increasing in recent years as its demand for commercial application has increased.

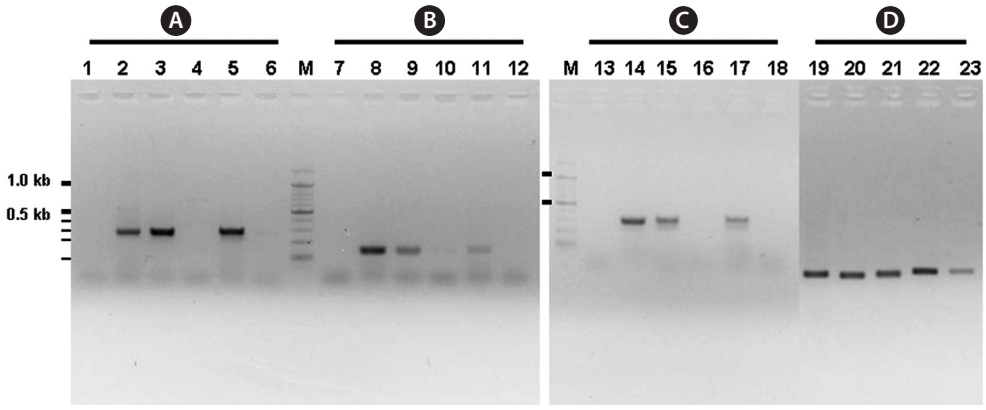

Fig. 3. PCR amplification of the regions encoding internal transcribed spacer (ITS)-1 and ITS-2. PCR was carried out with sets of oligonucleotdes ITS1F together with ITS1R2 (A) or ITS1R1 (B), and ITS2F together with ITS2R2 (C), or ITS2R1 (D). Each lane includes PCR products amplified by using genomic DNA isolated from *Ecklonia cava* (1, 7, and 13), *Agarum clathratum* (2, 8, 14, and 19), *Saccharina japonica* (3, 9, 15, and 20), *Undaria pinnatifida* (4, 10, 16, and 21), *Costaria costata* (lanes 5, 11, 17, and 22), and *Sargassum serratifolium* (lanes 6, 12, 18, and 23). PCR were carried out with annealing temperature at 60°C (a-c) and 65°C (d) and products were analyzed by 1.5% agarose gel electrophoresis. Lanes M include 100 bp size markers and thick bars indicate 1.0 and 0.5 kb, respectively.

In Korea, the production of major algal species including *Undaria*, *Saccharina*, and *Porphyra* becomes restricted in the winter season. To extend the seaweed cultivation industry to meet an increasing demand, it is necessary to find a species that can be produced throughout the year or that can grow during the high temperature season with a fast rate of productivity. Species belonging to Laminariaceae are candidates for this screening as the family includes species such as *Macrocystis,* the largest known seaweed, and *Saccharina,* which is produced in large amounts in Korea.

Algal species are classified by phenotypic characteristics as well as genetic markers (Yoon et al. 2001; Saunders, 2005). Differences in the morphological characteristics including the presence of mucilaginous organs in the sporophyte, lack of an eyespot in meiospores, and uniflagellation of the sperm exist among the families in the order Laminariales (Kawai and Sasaki, 2000). The Laminariaceae family is characterized by the presence of a distinct stipe, paraphyses without hyaline appendages, and the absence of outgrowths or splitting of the transition zone. In particular, *S. japonica* has a single, cylindrical stipe and a holdfast with multibranched haptera. The thallus of *C. costata* has a branched holdfast, a flattened and finely grooved stipe, and an elliptical blade up to 2 m long and 30 cm wide, with parallel ribs running along its length. The thallus of *A. clathratum* has a branched holdfast, a stipe up to 30 cm long, a stiff blade riddled with small but distinctive holes, and a wide midrib. To perform a detailed classification of Laminariaceae species and to develop a genetic marker-based detection method, nucleotide sequence-encoding ITS regions were analyzed for six brown algae species collected from coastal areas of Korea. These included five species from the order Laminariales and one species from the order Fucales. The former included three species of Laminariaceae (*A. clathratum*, *C. costata*, and *S. japonica*) and two species of Alariaceae (*U. pinnatifida* and *E. cava*). A relatively distant member belonging to the family Sargassaceae of the order Fucales, *Sargassum serratifolium,* was also included.

The isolation of genomic DNA from macroalgae and its subsequent use in PCR are known to be hindered by polysaccharides and phenolic compounds (Koonjul et al., 1999; Varma et al., 2007). Among the methods used for genomic DNA isolation from macroalgae (Varela-Álvarez et al., 2006; Hoarau et al., 2007; Snirc et al., 2010), the STE and CTAB buffer method was used for the species tested in this study. Upon confirming the integrity of genomic DNA by agarose gel electrophoresis (data not shown), DNA was used as a template for amplification of the regions corresponding to ITS. The typical ribosome in algae is composed of four rRNAs of which the rRNA cluster of 18S-5.8S-26S is transcribed from a single transcriptional unit (Coleman and Mai, 1997; Yotsukura et al., 1999; Torres-Machorro et al, 2010). In this study, PCR amplification of regions encoding ITS-1 and ITS-2 using a genomic DNA template and primers (ITS1F/ITS1R and ITS2F/ITS2R) resulted in 250-300 bp ITS-1 and ITS-2 fragments depending on the species, similar to the sizes reported from other macroalgal species (data not shown). At least three independent clones were analyzed to confirm the ITS sequences presented as many copies in the genome. A sequence comparison of ITS-1 and ITS-2 was first performed with three closely related species, *C. costata*, *A. clathratum*, and *E. cava*, to detect the regions that have differentiated between the species (Fig. 2). The former two, *A. clathratum* and *C. costata* belonging to the family Laminariaceae, and *E. cava* belonging to the neighboring family Alariaceae, were barely distinguishable, with almost identical 18s rRNAs except for a single divergence at one position (data not shown). The sequence analy-

sis of DNA fragments encoding the ITS-1 region obtained in this study showed 100% identity with the sequence reported from *C. costata* (AF319027) and *A. clathratum* (FJ042768), and 97% identity with the sequence reported from *E. cava* (AF3190009). Analysis of ITS-2 sequences also showed complete identity with those reported from the corresponding species, *C. costata* (AF319027), *A. clathratum* (FJ042768), and *E. cava* (AF319009). Sequence comparison among the species indicated that *A. clathratum* and *C. costata*, showing 91% and 89% identity with ITS-1 and ITS-2, respectively, are more conserved when compared to *E. cava*. Based on this result, Laminariaceae-specific oligonucleotides were designed from the regions conserved in *A. clathratum* and *C. costata* but not in *E. cava* (Fig. 2).

The inner segments of ITS-1 and ITS-2 were amplified using primers specific to the Laminariaceae family designed in this study. In PCRs using three sets of primers, amplified products were detected in reactions using genomic DNA isolated from *A. clathratum*, *S. japonica*, and *C. costata,* all of which are members of Laminariaceae (Fig. 3A-3C). The sizes of the PCR products were 262 bp, 139 bp, and 292 bp, respectively, as expected from the sequences. No amplified products were observed in reactions using genomic DNA templates isolated from *U. pinnatifida* and *E. cava* belonging to Alariaceae, and the relatively distantly related species *Sargassum serratifolum*. This suggests Laminariaceae-specific ITS-1 and ITS-2 amplification only in a PCR using three sets of ITS primers including ITS1R1, ITS1R2, and ITS2R2. For PCR amplification with ITS2F and ITS2R1, amplified products of 200 bp were observed in all samples under the same reaction conditions (data not shown). PCR amplification was also carried out at an annealing temperature of 65°C to exclude the possibility of nonspecific amplification due to the lower annealing condition (60°C) used above. The result also showed amplification of the fragments in all samples under the same reaction conditions although the intensity of the fragment in *Sargassum* was slightly lower than in the others tested (Fig. 3D). This suggests that the region corresponding to the ITS2R1 primer is more conserved in brown algae than the other ITS regions. Therefore, the ITS2R1 primer can be used for ITS-2 amplification in most of Phaeophyceae species but may not be a good primer for specific detection of Laminariaceae species.

The present study clearly showed that only genomic DNA isolated from the family Laminariaceae could be amplified by PCR, indicating that PCR using the primer sets of ITS1F together with ITS1R1 or ITS1R2, and ITS2F together with ITS2R1, can be used for the specific detection of Laminariaceae. This principle can be applied to develop other family-specific detection methods.

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