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Utility of taxon-specific molecular markers for the species identification of herbarium specimens: an example from *Desmarestia japonica* (Phaeophyceae, Desmarestiales) in Korea

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Abstract

Desmarestia japonica (Phaeophyceae, Desmarestiales) was recently established from the Japanese ligulate *Desmarestia* and is morphologically similar to *D. ligulata*. This species has been reported only from Japan. However, the taxonomic reports based on additional regional distributions are needed to clarify this taxonomic entity and its species boundaries. Because *Desmarestia* species have restricted distributions in Korea, we reexamined herbarium specimens of *D. ligulata* deposited at the National Institute of Biological Resources (South Korea). To improve the amplification efficiency of the polymerase chain reaction and avoid contamination by the DNA of other organisms, we developed taxon-specific molecular markers suitable for DNA barcoding of *Desmarestia* species. Nuclear ribosomal small subunit RNA (18S rDNA) and mitochondrial cytochrome c oxidase 1 (*cox1*) regions were selected as target DNA. As a result, both were successfully isolated from herbarium specimens of *D. japonica* acquired over 10 years. These molecular markers provide useful genetic information for herbarium specimens for which conventional molecular analysis is challenging.

Keywords: Nuclear ribosomal small subunit, Mitochondrial cytochrome c oxidase 1, Ligulate *Desmarestia*, Taxon-specific molecular markers

Background

Brown algal species of the genus *Desmarestia* (Desmarestiales) have a worldwide distribution (Guiry and Guiry 2017). *Desmarestia* species inhabit primarily the cold seawater of higher latitudes of both Northern and Southern hemispheres but are rarer in warm seawater (Graham et al. 2009). The genus includes free sulfuric acid-containing species, characterized by many branched or foliose macroscopic thalli with pseudo-parenchymatous cell structures (Yang et al. 2014).

Three species of *Desmarestia* have been reported from Korea, as *D. ligulata* H. Kawai, T. Hanyuda, D.G.Müller, E.C.Yang, A.F.Peters and F.C.Küpper; *D. tabacoides*

Okamura; and *D. viridis* (O.F.Müller) J.V.Lamouroux from Korea (Lee and Hwang 2010). Yang et al. (2014) revised the taxonomic relationship of *Desmarestia* species and suggested new combinations of the subspecies of *D. dudresnayi* and *D. herbacea*. On the species level, they established *D. japonica* from Japanese *Desmarestia* species.

Desmarestia japonica was recently established from Japanese *Desmarestia* species, based on molecular data and morphological characteristics (Yang et al. 2014). This ligulate species had been referred previously to *D. ligulata* in Japan, and its morphology was described by Okamura (1936) and Yoshida (1998) as *D. ligulata*.

Yang et al. (2014) stated that there was no evidence as to whether *D. japonica* occurred in Korea. Thus, there is a need to confirm the taxonomic entity and species boundaries.

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In Korea, *Desmarestia* species show a restricted distribution in terms of ecological habitats. Thus, the National Institute of Biological Resources (Korea) established the scientific project on the distribution and genetic diversity of these rare species, and herbarium specimens of *Desmarestia* species have been deposited since 2007.

Herbarium specimens contain valuable information for genetic investigations (Nicholls 2009). DNA sequences from herbarium specimens can also provide the important molecular evidence to solve taxonomic controversies (Goff et al. 1994; Provan et al. 2008; Hughey and Gabrielson 2012; Saunders and McDevit 2012). However, most herbarium specimens have been found to not be in a suitable condition for molecular biological analyses. DNA degradation and contamination are still major limitations (Taylor and Swann 1994).

Many studies have attempted to overcome the limitations of herbarium specimens as molecular biological materials and to improve molecular tools for DNA extraction and amplification of target DNA regions (e.g., Taylor and Swann 1994; Meusnier et al. 2008; Prosser et al. 2016). Next-generation sequencing (NGS) was recently applied to extract genetic information from old herbarium specimens (e.g., Hughey et al. 2014; Suzuki et al. 2016).

For the selection of targeted DNA regions, shorter amplicons show higher efficiency in amplification. Thus, the universal DNA mini-barcode (*cox1*) with minimal length has been adopted for biodiversity analysis (Meusnier et al. 2008). However, this short length of the target DNA region could not solve the problem of contamination. During the preparation and conservation of specimens, many sources of contamination can be present. Epiphytic organisms on algal thalli may not be excluded completely during sample preparation. Many algal specimens have such epiphytic organisms, and thus, they may

be included in any DNA analysis. Moreover, fungal and human DNA contamination can occur during conservation in herbaria.

In this study, we developed taxon-specific molecular markers for DNA barcoding of herbarium specimens of *Desmarestia* species deposited in the National Institute of Biological Resources (Korea). The taxon-specific primer pairs were designed for the amplification of DNA barcode regions (18S rDNA and *cox1*). We also report for the first time *D. japonica* from Korea.

Methods

We analyzed herbarium specimens deposited in the National Institute of Biological Resources, Korea (Fig. 1). The morphological characteristics of 21 specimens of Korean *D. ligulata* (Table 1) were measured. Photographs were taken with a digital camera (C-4040 zoom, Olympus, Tokyo, Japan) attached to a light microscope (BX50, Olympus). After the morphological reexamination, we cut a small piece (<0.5 cm²) to minimize the damage from herbarium specimens to be used for DNA analyses. Images of specimens were obtained with a scanner (Epson, Seiko Epson Corp., Japan, Fig. 1).

For the molecular analyses of the specimens, we used the reference sequences of *Desmarestia* species deposited in GenBank (NCBI, National Center for Biotechnology Information). To avoid contamination by fungi and other organisms, we developed taxon-specific primer pairs for the amplification of target DNA regions (Fig. 2, 18S rDNA and *cox1*). We selected a putatively conserved region among reference DNA sequences of *Desmarestia* species. Moreover, the DNA regions conserved with other organisms were excluded for the primer design as much as possible. The universal primer sets for 18S rDNA (A/SSUinR-1 in Lee et al. 2010) and *cox1* (LCO1490/HCO2198 in Folmer et al. 1994) were also tested for comparison.

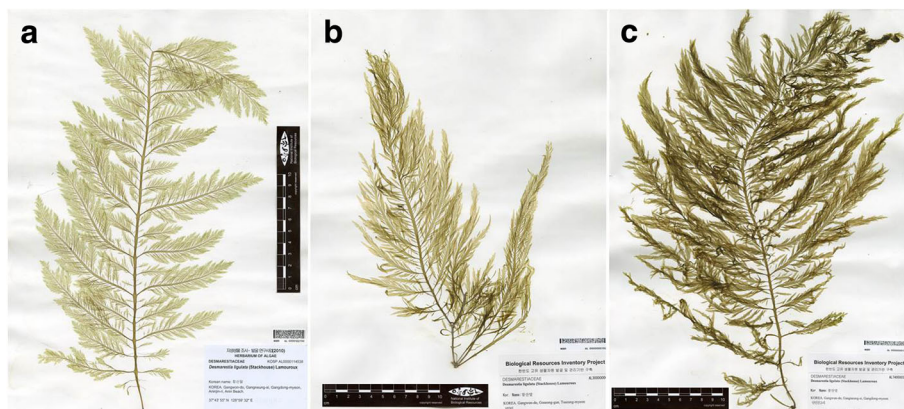


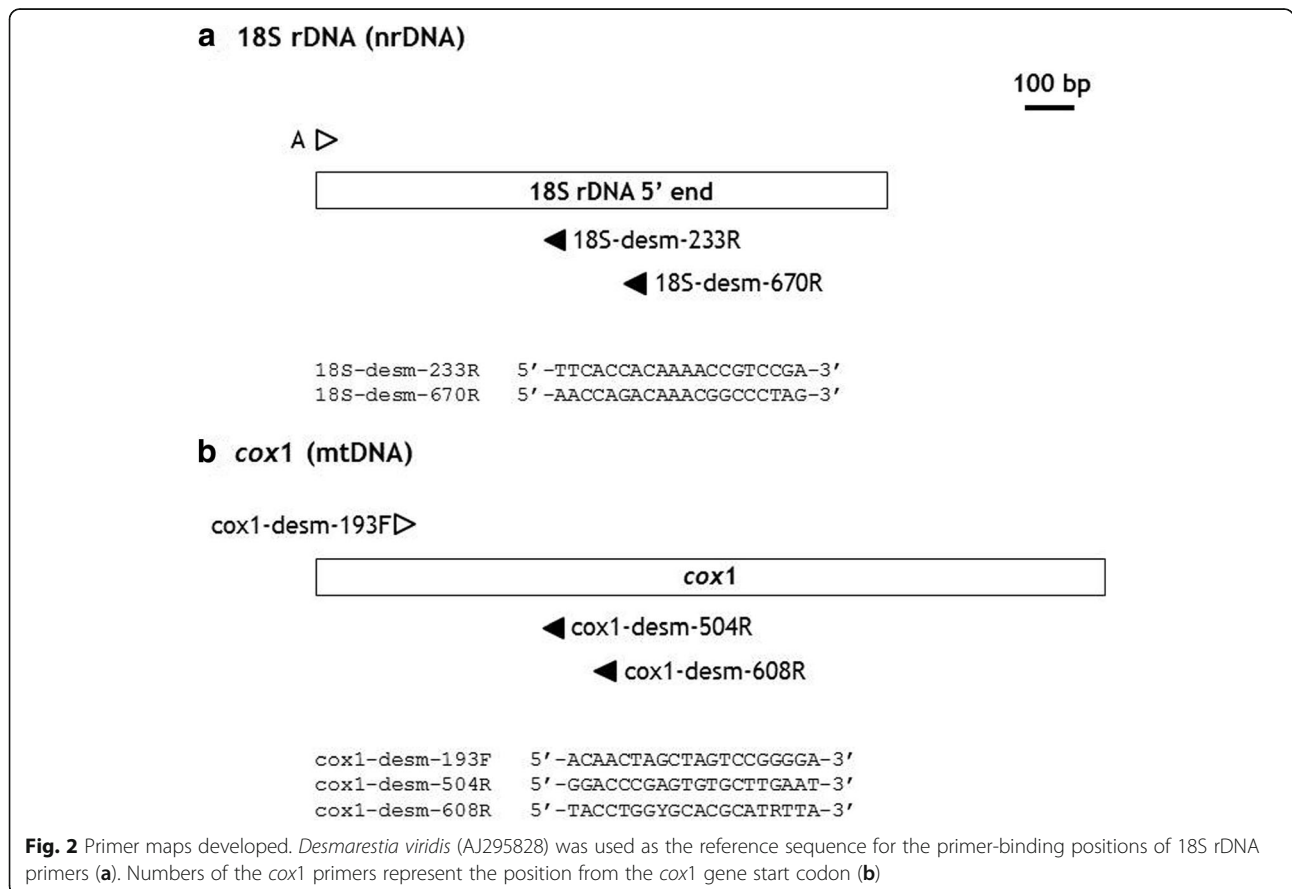
Fig. 1 Herbarium specimens analyzed. These specimens were identified as *D. ligulata* from morphological examinations. **a** NIBRAL0000122790. **b** NIBRAL0000000705. **c** NIBRAL0000000724

Table 1 Comparison of morphological characteristics of *D. japonica*

| Characters | Womersley (1987) (as <i>D. ligulata</i>) | Yoshida (1998) (as <i>D. ligulata</i>) | Yang et al. (2014) (as <i>D. japonica</i>) | Korean specimens (in this study) |
|-------------------|---|--|---|---|
| Color | Medium brown to greenish brown | – | Light olive brown | Light olive brown |
| Height | Usually 20–50 cm tall | 60–100 cm tall | 0.6–1 (– 2) m tall | 40–67 cm |
| Width of branches | With axis and primary laterals mostly 2–5 mm broad | 3–6 mm wide branches | 2–6 (– 20) mm wide branches | (0.7 –) 1.7–4 mm wide branches |
| Holdfast | Discoid holdfast | – | Conical or flattened | Discoid holdfast |
| Branching type | Fronds with frequent opposite branching to 3 orders | Opposite to ca. three times branching | Opposite, branches of limited growth branched to two to three times | Opposite, branched to two to three times |
| Branch morphology | More or less linear but tapering apically and basally, dentate, and with a faint to conspicuous midrib (the axial filament) | Wide linear feather-like branches, tapering apically and basally with midrib | Branches stipitate and attenuate at base and apex | Branches stipitate and attenuate at base and apex |

The DNA extraction, polymerase chain reaction (PCR), and sequencing adopted the methods described in Lee et al. (2011). We isolated total DNAs from the subsampled herbarium specimens. We extended the incubation time of DNA extraction step (1 h). Moreover, the incubation times in washing step were also prolonged to improve the quality of DNA eluents. PCR conditions consisted of 3 min at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C, and a final

7 min extension step at 72 °C. Sequencing was conducted by a commercial service (Genotech, Daejeon, Korea), and the sequencing chromatograms were assembled with Sequencher 5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA). The phylogenetic analyses were constructed using MEGA version 6 (Tamura et al. 2013). The neighbor-joining method and bootstrap analyses (2000 replicates) were used to reconstruct the phylogenetic tree. Molecular study about *Desmarestia* herbarium



specimens had not been carried out in this lab previously. All reagents were in a sterile condition and stored in disposable plastic ware.

Results

Korean *Desmarestia* species showed a restricted distribution pattern, mainly on the northeastern coast mostly in subtidal habits (Lee and Hwang 2010). Because *Desmarestia* species live, herbarium specimens could be effective for the molecular investigation. We examined herbarium specimens deposited in NIBR collected from 10 years ago (Fig. 1). First, we selected samples previously identified as *D. ligulata* according to morphological resemblance (Yang et al. 2014). From the morphological examination, *D. ligulata* has proliferate pinnate-branched thalli and *D. tabacoides* typically has one or two wide-branched or unbranched foliose thalli (Table 1). In the case of *D. viridis*, this species was distinguished by much longer linear branched thalli.

We examined a total of 21 specimens identified as *D. ligulata* from the Korean coast. The thallus is light olive brown in color and, when exposed to air, becomes greenish brown. Korean specimens are up to 67 cm in height and have mostly three orders of branching. In the main axes and primary branches, branches were 2 mm wide, but in tall specimens, they were up to 4 mm wide. Gross morphology, with feather-like pinnate branching, was similar to that of Japanese ligulate *Desmarestia* species. Representative specimens showing morphological differences were also analyzed using molecular methods.

The universal primer set for 18S rDNA (Lee et al. 2010) produced fungal 18S rDNA sequences from the total genomic DNA extracts of herbarium specimens. The 18S rDNA sequenced showed high similarity with *Agaricus bisporus* var. *bisporus* (CP015465, 520/527(99%) from *D. ligulata*). However, we successfully isolated 18S rDNA (MF363011) and *cox1* (MF363010) sequences of *D. japonica* from three specimens, using our taxon-specific primer pairs: NIBRAL0000000724 (Gangneung March 7, 2006), NIBRAL0000122790 (Gangneung May 8, 2009), and NIBRAL0000000705 (Goseong July 23, 2005).

Using forward primer A (Medlin et al. 1988; Lee et al. 2010), two reverse primers (Fig. 2a) produced PCR bands from DNA extracts of *D. ligulata*. The combination A/18S-desm-233R produced 213 bp, and A/18S-desm-670R amplified 650 bp of 18S rDNA without primer-binding sites. The three 18S rDNA sequences had the same sequence and 100% similarity with *D. japonica* (HE866912-HE866915, Yang et al. 2014). However, these 18S rDNA regions also had identical sequences with *D. aculeata* (HE866893-4), *D. distans* (HE866923), *D. latifrons* (HE866916), *D. ligulata* (HE866917-22), and *D. muelleri* (HE866924-5). Thus,

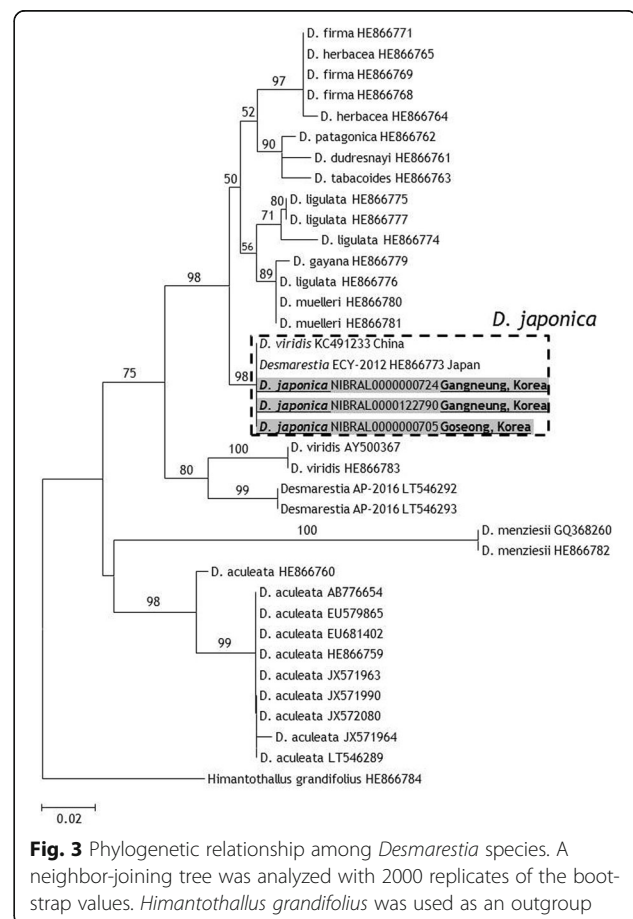
these 18S rDNA sequences alone could not provide sufficient genetic information to discriminate interspecific relationships among *Desmarestia* species.

For the amplification of *cox1* sequences (Fig. 2b, one forward and two reverse primers), the combinations of *cox1*-desm-193F/*cox1*-desm-504R and *cox1*-desm-193F/*cox1*-desm-608R successfully amplified the *cox1* region of *Desmarestia* species. The primer pair of *cox1*-desm-193F/*cox1*-desm-504R showed high efficiency in amplification (272 bp excluding primer-binding sites). Thus, we used this combination to amplify *cox1* from *Desmarestia* specimens.

Korean *D. japonica* samples had the same *cox1* sequence with Japanese *D. japonica* (HE866773 in Yang et al. 2014). A *cox1* sequence reported from China as *D. viridis* (KC491233) also had 100% similarity with *D. japonica*. Because *D. japonica* showed below 97.4% similarity with other *Desmarestia* species deposited in the GenBank, this Chinese sample was likely misidentified (Fig. 3).

Discussion

The herbarium specimens examined from the Korean coasts have feather-like features and were smaller than



the taller samples described by Yoshida (1998) as *D. ligulata* and Yang et al. (2014) as *D. japonica* (Table 1). However, they were similar in the color, branching pattern, and height of Japanese plants as well as Australian plants (Womersley 1987). Lamouroux's (1813) illustration of *D. ligulata* showed that some primary laterals of the frond were dichotomous and some secondary laterals did not branch oppositely. However, we did not find such dichotomous branches within our Korean specimens whereas we did mostly observe opposite branching in secondary laterals.

Molecular phylogenetic studies of *Desmarestia* species were conducted to establish new species and to reconstruct the phylogenetic relationships (Tan and Druehl 1996; Yang et al. 2014). As a result, the key reference sequences of the 18S rDNA and *cox1* region are available in GenBank. Thus, we selected these DNA sequences as the target regions for taxon-specific molecular markers of *Desmarestia* species.

DNA degradation in dried algal specimens and contamination are the major reasons for the failure of DNA analyses. The universal *cox1* primer pair could not amplify the *cox1* region from herbarium specimens of *Desmarestia*. In the case of 18S rDNA, fungal DNAs were amplified. Thus, a primer pair is required having high specificity and efficiency in amplifying the target DNA region from herbarium samples. In this study, we developed new primer pairs having short fragments of PCR to enhance the efficiency of amplification (Meusnier et al. 2008) and the specificity for the target plant samples (Fig. 2).

The primer pairs developed could successfully amplify 18S rDNA and *cox1* regions from specimens of *Desmarestia* species. When the universal primers were used in the analyses, the samples showed no PCR band (*cox1*) or amplified fungal 18S rDNAs. The 18S rDNA and *cox1* region could provide robust results for the finding of taxonomic entities of *D. japonica*. This report of *D. japonica* is the first regarding the distribution of *D. japonica* after the establishment of this species based on Japanese specimens (Yang et al. 2014).

The isolated 18S rDNA sequence of *Desmarestia* species could not provide a taxonomic resolution at the interspecific level and was not a suitable marker to analyze the taxonomic entities of Korean samples. The *cox1* region has been selected frequently as a standard marker for algal DNA barcode use (Lane et al. 2007). In this study, the *cox1* region provided suitable genetic information to examine the taxonomic entity of *D. japonica* from Korea. Yang et al. (2014) also found effective taxonomic resolution of the *cox1* region, reflecting species delimitations among *Desmarestia* species and proposed the *cox1* region as a potential barcode marker for the genus *Desmarestia*.

The overall morphologies of *D. japonica* specimens were variable in branching and branch width (Fig. 1, Table 1). Moreover, their morphologies were similar to those of *D. ligulata*.

In this study, we found *D. japonica* from herbarium specimens in NIBR using the taxon-specific primer pair (Fig. 2). These specimens were collected over 10 years ago and were first identified as *D. ligulata* based on morphological characteristics (Fig. 1). A Chinese *cox1* sequence (KC491233) of *D. viridis* also showed 100% similarity in the *cox1* region with Japanese *D. japonica*. These results indicate an extended distribution in Korea and China for *D. japonica* (Fig. 3). Consequently, a molecular taxonomic reexamination of the morphological resemblance among *D. japonica*, *D. ligulata*, and *D. viridis* is needed in future studies.

Conclusions

We developed taxon-specific primer sets to amplify the 18S rDNA and *cox1* regions without contaminants (e.g., fungi and epiphytic organisms) and successfully isolated DNA regions from herbarium specimens over 10 years old. From these results, we confirmed the presence of *D. japonica* from Korea and China. We believe that the new molecular markers we have developed also provide useful information for DNA barcoding species of the economic seaweed *Desmarestia*.

Abbreviations

cox1: Mitochondrial cytochrome c oxidase 1; NCBI: National Center for Biotechnology Information; NGS: Next-generation sequencing; PCR: Polymerase chain reaction; rDNA: Ribosomal DNA

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Availability of data and materials

All datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SRL conducted the research, analyzed the molecular result, and prepare the draft. EYL designed the research, analyzed the herbarium specimens, and prepared the draft in part. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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