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A Proposal for the Lectotype Designation of *Ishige foliacea* (Phaeophyceae, Ishigeaceae) Using DNA Barcoding

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Abstract: Three species of the genus *Ishige* (Phaeophyceae, Ishigeaceae) are known from Korea, Japan and Mexico; they include *Ishige foliacea* Okamura, *I. okamurae* Yendo and *I. sinicola* (Setchell and N.L. Gardner) Chihara. Two species, *I. foliacea* and *I. okamurae*, are present in the algal flora of Korea and Japan. The original description of *I. okamurae* defined two forms of branches, filiform and foliose, but later the foliose branch was recognized as a new species *I. foliacea*, which is epiphytic on *I. okamurae* but can also be free-living. The currently proposed lectotype for *I. foliacea* is based on a free-living form and does not reflect the intent of the original description of the species. In this study, we conducted the DNA barcoding for herbarium specimens to identify *Ishige* species. Additionally, the variation in *cox*3 sequences obtained from *I. okamurae* specimens with two morphological forms collected from waters around the Korean Peninsula was sufficient to separate two species, *I. okamurae* and its epiphyte *I. foliacea*. The epiphytic *I. foliacea* on the lectotype specimen of *I. okamurae* is designated as lectotype *I. foliacea*.

Keywords: *cox*3; DNA barcoding; epiphyte; life form; herbarium specimen

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1. Introduction

The genus *Ishige* Yendo (Phaeophyceae, Ishigeaceae) have three species (*I. okamurae* Yendo, *I. foliacea* Okamura and *I. sinicola* (Setchell and N.L. Gardner) Chihara) reported from Korea, Japan and Mexico [1]. Yendo found two life forms on a single plant body, a filiform frond with abnormal foliose fronds as one of its segments. He described this alga as *I. okamurae* having different morphological forms: the filiform frond (cylindrical or terete) and the foliose frond (flat and leaf-like) [2].

However, Okamura in Segawa [3] treated the foliose form isolated from *I. okamurae* as a new species as *I. foliacea*. Moreover, the foliose type was also placed on the type specimen of *I. okamurae* in the Herbarium, Graduate School of Science, Hokkaido Univ. (SAP) Japan ([4] Figure 17). Additionally, this specimen of *I. okamurae* had a stamp of "TYPUS" indicating a type specimen.

Later, a lectotype specimen of *I. foliacea* was proposed on the basis of a personal communication with Kazuhiro Kogame [5]. However, this specimen was an independent frond that was not epiphytic on the plant body of *I. okamurae* and therefore did not represent *I. foliacea* as originally described [3].

Herbarium specimens include molecular information that helps to analyze taxonomic problems such as the recognition of cryptic species or species boundaries [6,7]. Therefore, DNA barcoding for herbarium specimens can provide valuable information to identify species boundary and to find new species [8].

In the present study, we examined the taxonomic identity of *Ishige* specimens with two life forms on a single plant body of *I. okamurae*. Molecular analyses were conducted to clarify the taxonomic relationships between the two *Ishige* species. In addition, we proposed a new type specimen of *I. foliacea* that shows typical morphological characteristics that fit the original description of *I. okamurae* and *I. foliacea*.

2. Materials and Methods

We examined 1200 sheets of the herbarium specimens of *Ishige* species collected in Korea during 2008–2020 and deposited in the National Institute of Biological Resources (NIBR; KB), Korea. These specimens have the same morphological characteristics as the filiform type of frond with abnormal foliose branches (Table 1, Figure 1), present in *I. okamurae* as described by [2] and in *I. foliacea* following the research in [3]. Morphological analysis was conducted using a light microscope (BX50; Olympus, Tokyo, Japan) with a digital camera (C-4040 Zoom; Olympus). We used a scanner (Epson, Seiko Epson, Nagano, Japan) to obtain images of the herbarium specimens.

Table 1. Sampling information and herbarium specimens of *Ishige* species.

Specimen No.	Collection Site	Collection Date	Accession No.
NIBRAL0000142110	Seongsan-ri, Seongsan-eup, Seogwipo-si, Jeju-do, South Korea	23 July 2005	*I. foliacea* (MW221457) *I. okamurae* (MW221460)
NIBRAL0000157450	Uisin-myeon, Jindo-gun, Jeollanam-do, South Korea	08 May 2009	*I. foliacea* (MW221458) *I. okamurae* (MW221461)
NIBRAL0000157676	Songjeong-ri, Mijo-myeon, Namhae-gun, Gyeongsangnam-do	08 May 2009	*I. foliacea* (MW221459) *I. okamurae* (MW221462)

Among 21 sheets of *I. okamurae* specimens with epiphytic *I. foliacea*, three specimens from different collection sites were selected for molecular analysis (Table 1, Figure 1). Each sample of the *I. okamurae* and the epiphytic *I. foliacea* on it was cut from a single plant body and three identical processes were conducted using three NIBR herbarium specimens.

We also re-examined the specimens of *I. okamurae* and *I. sinicola* analyzed by the authors in [4]. A molecular analysis of herbarium specimens was conducted as a follow-up to previous studies [8]. We isolated and separated the two life forms of specimens obtained from a single herbarium specimen and removed a small piece (<0.5 cm^2) for molecular phylogenetic analysis. DNA extraction, polymerase chain reaction (PCR), and sequencing were conducted following the protocols reported by Lee and Lee (2018).

We selected *cox*3 region to compare with the DNA sequences of the *Ishige* species previously reported [5]. We designed PCR primers to amplify the *cox*3 region from *Ishige* samples (forward primer, cox3-Ish-44F, 5′-TAGTTTCTCGAAGCCCTTGG-3′; reverse primer, cox3-Ish-641R, 5′-TGGAAGCCRTGRAAACCTGT-3′), using the *cox*3 DNA sequences deposited in NCBI GenBank (www.ncbi.nlm.nih.gov/Genbank).

The PCR conditions were as follows: 3 min at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C, with a final 7 min extension at 72 °C. A commercial sequencing service (Macrogen, Seoul, Korea) was used to determine DNA sequences and Sequencher 5.4.6 (Gene Codes, Ann Arbor, MI, USA) was used to assemble the chromatograms. Phylogenetic analysis of the taxonomic relationships among *Ishige* species was performed using MEGA ver. 6 [9].

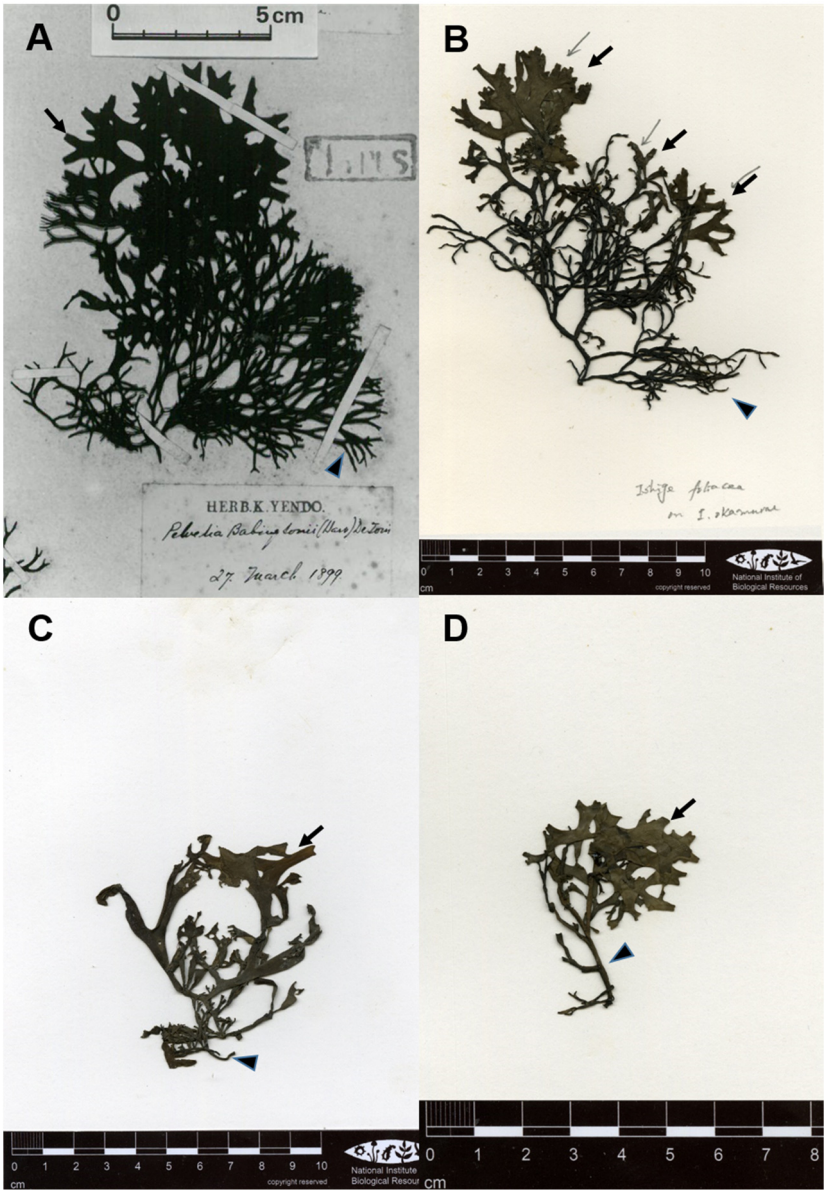

Figure 1. Herbarium specimens of *Ishige foliacea* on branches of *I. okamurae*. Arrows indicate *I. foliacea* and arrowheads indicate *I. okamurae* (**A**) Lectotype specimen of *I. okamurae* (SAP, photocopy image). (**B**–**D**) Two forms of *Ishige* species collected from Korea: (**B**) *Ishige* samples collected from Jindo-gun, Korea (NIBRAL0000157450), (**C**) *Ishige* samples collected from Seongsan-eup, Jejudo, Korea (NIBRAL0000142110) and (**D**) *Ishige* samples collected from Namhae-gun, Korea (NIBRAL0000157676).

3. Results

3.1. DNA Analyses for Herbarium Specimens

Twenty-one sheets of *I. okamurae* specimens with two life forms on a single plant body following the original morphological description of [2] are studied from the NIBR herbarium (Figure 1). The nucleotide sequence variation of *cox*3 showed the sufficient genetic information to differentiate *I. okamurae* and *I. foliacea* (19.6–21.4% of nucleotide sequence difference between the two species) in [5]. In the present study, we discriminated two forms (filiform and foliose frond) isolated from a thallus (Figure 1), in which the two parts belonged to two species (*I. okamurae* and *I. foliacea*). We successfully amplified 598 bp of PCR products and obtained 558 bp of the *cox*3 region. using a primer pair (cox3-Ish-44F/cox3-Ish-641R) (GenBank accession number MW221457-MW221462, Table 1).

The similarity among *cox*3 sequences of the filiform parts of *I. okamurae* in this study was 99.5% (three base differences). From the results of BLAST searching in GenBank, those *cox*3 sequences of the filiform parts showed 96.6–100% similarity with *I. okamurae* deposited in GenBank (Figure 2).

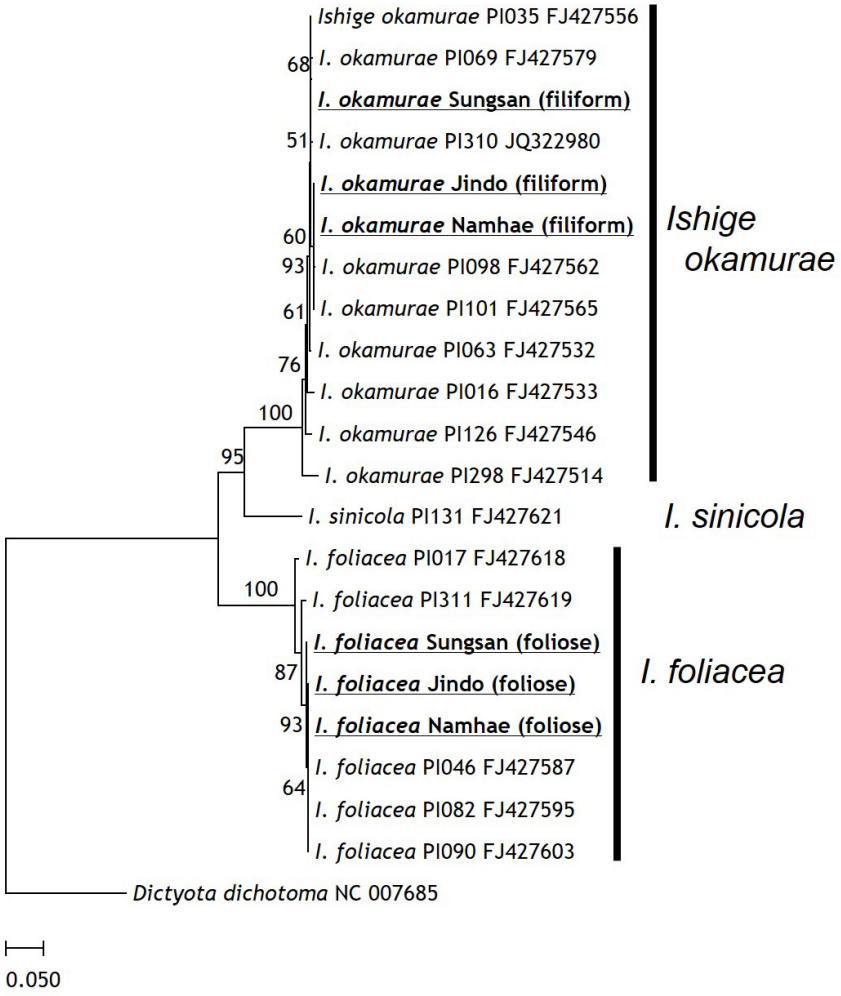

Figure 2. Phylogenetic relationships among the *Ishige* species. The neighbour-joining tree of *cox*3 sequences was constructed using 2000 bootstrap replicates.

The sequences obtained from the foliose parts epiphytic on the branches of *I. okamurae* (Figure 1, putative *I. foliacea*) showed 97.5–100% similarity with *I. foliacea* sequences available in GenBank, and one base difference (99.8% similarity) was present among specimens under examination. Moreover, the *cox*3 sequence of the foliose plant collected from Seongsan (Jejudo, Korea) confirmed it is a new haplotype (one base difference with previously reported *cox*3 sequences). Therefore, the foliose plant epiphytic on the branch of *I. okamurae* was identified as *I. foliacea*. These results corroborated the original description of Yendo (1907), who cited two life forms on a single plant body of *I. okamurae*. Sequence similarity between the two life forms was within the range reported between species of *Ishige* (79.6–79.7%).

3.2. Typification of Lectotype Specimen

Ishige foliacea Okamura. Segawa. Sci. Pap. Inst. Alg. Res., Fac. Sci., Hokkaido Imp. Univ. 1: 66. 1935.

Lectotype (designated here): Shimoda, Japan. 27 March 1899. (TI, lectotype) (see Figure 1A for an image of the lectotype. The lectotype is only the epiphytic foliose specimen

marked with an arrow; not the filiform specimens representing *Ishige okamurae* Yendo marked with an arrowhead).

4. Discussion

Ishige okamurae was described as two life forms (the filiform type (cylindrical or terete) and the foliose type) on one plant body by [2]. However, this type of *I. okamurae* was rarely collected from Korea. Among 1200 sheets of *Ishige* specimens collected from around the Korean peninsula during 2008-2020, only fifteen specimens had two life forms. Moreover, [5] taxonomically treated Korean and Japan *I. foliacea* (*I. sinicola* auct. japon. and corea.) based on free-living specimens and not with epiphytic form on *I. okamurae*, and designated the free-living form of the specimen as the lectotype for *I. foliacea* [5].

In the original description of the new species, *Ishige okamurae* and *I. foliacea*, indicated the presence of epiphytic foliose fronds and filiform fronds as the main distinguishing traits [2,3]. The study in [4] recognized the absence of the type specimen of *I. okamurae* and designated a lectotype of *I. okamurae* consistent with Yendo's original description and drawings; this typification was later confirmed by the authors in [5].

Okamura in [3] examined the form of two individuals adhering together among Yendo's specimens, and recognized them as different species, with one attached to the other. He described the foliose form as a new species, *I. foliacea*. Therefore, the lectotype of *I. foliacea* should be a specimen in which two individuals are attached and corresponds more closely to the original description and drawings by the author in [2] and Okamura in [3].

The lectotype of *I. okamurae* (in [4]) selected among Yendo specimens is consistent with the original description of Okamura in [3] and that of the study in [2]. Therefore, the foliose part of the herbarium sheet, i.e., the epiphytic specimen living on the filiform specimen already designated as the lectotype of *I. okamurae* should also be the lectotype of *I. foliacea*. That is, the collection item in which two species are attached to each other must also be the type of *I. foliacea* to match the original description and drawings of the two species. This lectotype designation fits the original description and drawings of the two species and is therefore in accordance with Art. 9.3 and Art. 9.4 of the International Code of Nomenclature (ICN) for algae, fungi, and plants [10]. Moreover, the lectotype designation should reflect on the species description, including original materials (e.g., hand drawing, messages in notes) and carefully selected among syntypes, if possible [e.g., typification of brown alga *Laminaria rodriguezii* in [11]). However, the previous proposed lectotype specimen of *I. foliacea* in [5] could not satisfied for the species description in [2,3].

The lectotype of *I. foliacea* was designated on the base of the annotation of Yendo's specimen by Tadao Yoshida [5]. However, this annotation by Yoshida did not mean any indication of intent by the original author who named the species. The lectotype designation should be based on the original materials, which were associated with the preparation of description, diagnosis, or illustration with analysis (Art. 9.3 and Art. 9.4 in the ICN). Therefore, the lectotype of *I. foliacea* should follow the original description by [2] and Okamura in [3]. In addition, the study in [5] analyzed only those samples in which *I. foliacea* and *I. okamurae* were growing independently and designated a specimen of *I. foliacea* showing the free-living form as a lectotype. This is clearly inconsistent with [2] and Okamura's original description [3]; that is, the foliose fronds of *I. foliacea* were epiphytic on the branch of *I. okamurae*.

The foliose branch of filiform *I. okamurae* was identified as *I. foliacea*. The *cox*3 sequences of the foliose fronds in the herbarium specimens showed 97.5–100% sequence similarity with individuals of *I. foliacea* in free-living specimens and formed a clade apart from *I. okamurae* and *I. sinicola*. The filiform *I. okamurae* with foliose branches showed high similarity with *I. okamurae* reported from Japan and Taiwan (96.6–100%). Therefore, the rarely reported individuals with two life forms consisted of two species (*I. foliacea* is epiphytic on *I. okamurae*). These results are congruent with the morphological descriptions reported by the authors in [2]. Moreover, a molecular taxonomic study based on the *cox*3 showed high sequence similarity between the free-living and epiphytic *I. foliacea* [5].

This finding is interesting because it confirms that *I. foliacea* is present in two life forms (independent and epiphytic on *I. okamurae*). The *cox*3 gene region was taxonomically sufficient to classify the three species of the genus *Ishige*.

To confirm the taxonomic relationship among the three species of the genus *Ishige*, molecular taxonomic analyses should be conducted on type specimens of the three species: *I. foliacea*, *I. sinicola*, and *I. okamurae*. Studies on the taxonomic status of *Ishige sinicola* in Korea should be on conducted because many algae show biogeographical distribution on both the coast of California and the coast of Northeast Asia. For example, *Padina durvillei* was reported originally from Chile; however, it is distributed in cosmopolitan regions, including Asia and Africa. The type of localities of *Erithro tetraseriata and Rhodymenia californica* are the coast of California; they were also discovered on the Asian coast [1]. Even though no DNA sequence of the genus *Ishige* was successfully produced from the type of specimen, the exact lectotype designation is required to prevent taxonomic confusion.

The original description given by the authors in [2] should be a key reference for determining the type specimen. In this study, we analyzed DNA sequences from two life forms of morphotypes, including *I. okamurae* and *I. foliacea*. This generic information presents a useful taxonomic guideline for further studies. Moreover, the specimen of *I. foliacea* epiphytic on *I. okamurae* deposited at the Hokkaido University Herbarium (SAP) was observed only from the photocopy of the specimen provided by SAP. Thus, the analysis of the Japanese samples of epiphytic *I. foliacea* on *I. okamurae*, including the lectotype, need to be further studied.

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Conflicts of Interest: The authors declare no conflict of interest.

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