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Sequence Validation for the Identification of the White-Rot Fungi *Bjerkandera* in Public Sequence Databases S

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White-rot fungi of the genus *Bjerkandera* are cosmopolitan and have shown potential for industrial application and bioremediation. When distinguishing morphological characters are no longer present (*e.g.*, cultures or dried specimen fragments), characterizing true sequences of *Bjerkandera* is crucial for accurate identification and application of the species. To build a framework for molecular identification of *Bjerkandera*, we carefully identified specimens of *B. adusta* and *B. fumosa* from Korea based on morphological characters, followed by sequencing the internal transcribed spacer region and 28S nuclear ribosomal large subunit. The phylogenetic analysis of Korean *Bjerkandera* specimens showed clear genetic differentiation between the two species. Using this phylogeny as a framework, we examined the identification accuracy of sequences available in GenBank. Analyses revealed that many *Bjerkandera* sequences in the database are either misidentified or unidentified. This study provides robust reference sequences for sequence-based identification of *Bjerkandera*, and further demonstrates the presence and dangers of incorrect sequences in GenBank.

Keywords: Sequence validation, ITS, 28S nuclear ribosomal large subunit, white-rot fungi, *Bjerkandera*, GenBank

Introduction

Bjerkandera is a common white-rot fungus found worldwide [16]. The genus *Bjerkandera*, erected by Karsten in 1876, is characterized by soft, pileate basidiocarps. The type species, *B. adusta*, exhibits a gray to black tube layer, which contrasts with a white context [22]. The two species in this genus, *B. adusta* and *B. fumosa*, are both distributed in North America, Europe, and Asia [9, 17, 22]. In Korea, *B. adusta* was first reported in 1936 as *Polyporus adustus* [29], and *B. fumosa* was officially recorded in 1994 as part of an exhaustive list of Korean wood-rooting fungi [12]. Systematic taxonomic descriptions of both species were documented in 2010 [15].

Bjerkandera plays an ecologically important role in the global carbon cycle by growing on and decomposing dead hardwood trees [6], but also has negative impacts, such as causing timber damage and interfering with the cultivation of culinary mushrooms [1]. Additional to its effectiveness in decaying lignin, *Bjerkandera* can degrade common anthropogenic pollutants, such as various polycyclic aromatic hydrocarbons [10]. Such notable enzymatic activities led scientists to explore the industrial application of *Bjerkandera*; *B. adusta* has demonstrated an ability to decolorize synthetic dyes, which can be applied to bioremediation [4]. The interest in *Bjerkandera* has been recently renewed, as the whole genome of *B. adusta* has been sequenced by the Joint Genome Institute (JGI) as part of the 1,000 Fungal Genomes project [2].

Superficially, *B. adusta* and *B. fumosa* are similar and are easily confused for each other, especially when basidiocarps are immature, but morphological characters have been identified to distinguish these two species: fruiting body shape, pore size, context and tube thickness, and basidia and spore size [22]. The ease of misidentification is of greater concern for industrially important *B. adusta* strains that are currently preserved as cultures and/or dried specimen fragments; species identification cannot be checked,

as distinguishing morphological characters are no longer present. If the specimens were misidentified, subsequent data, such as DNA sequences, would be incorrectly identified and this problem maintained in public databases and the scientific literature.

DNA barcoding is a useful tool to help classify species and identify cryptic diversity [11] that depends on comparison to public databases. When species identifications in public databases are incorrect, additional samples will be misidentified and the problem perpetuated. In fact, about 20% of species identifications of DNA sequences in public database were estimated to be incorrect or questionable [3, 18].

In this study, we used the genus *Bjerkandera* as an example to quantify, characterize, and correct species misidentifications in GenBank. We chose *Bjerkandera* because (i) there are only two species, (ii) the two species are highly similar and easily misidentified by non-specialists despite distinguishing morphological characters, and (iii) the results have implications to genomic and biotechnological research. To complete these goals, we first identified true *B. adusta* and *B. fumosa* samples through rigorous morphological observation, followed by DNA sequencing to build a framework for comparison. Two molecular markers, the internal transcribed spacer (ITS) and the 28S nuclear ribosomal large subunit (LSU), were sequenced since they are the two most common genes used in fungal systematics [5, 23, 24]. Lastly, all ITS and LSU sequences in GenBank, which have been identified as or show high sequence similarity to *Bjerkandera*, were evaluated against correctly identified *B. adusta* and *B. fumosa* sequences.

Materials and Methods

Specimens and Microscopic Observation

All specimens used in this study were collected throughout the Korean Peninsula between 1989 and 2013, dried, and deposited in the Seoul National University Fungal Collection (SFC) (Table 1). Specimens labeled as *Bjerkandera* were rigorously reexamined based on distinguishing morphological characters to determine their true species identification. Microscopic features were observed using an Eclipse 80i light microscope (Nikon, Japan). After specimen identification was confirmed using DNA sequence analyses (methods below), the macro- and microscopic features of the specimens were characterized in detail.

DNA Extraction, PCR Amplification, and Sequencing

A small piece of fungal tissue from each dried specimen was placed in a 1.5 ml tube containing 2× CTAB buffer and ground with a plastic pestle. Genomic DNA was extracted with a modified CTAB extraction protocol [20]. The ITS region was amplified using the primers ITS1-F and ITS4-B [8], and the LSU region was amplified using the primers ITS3 and LR5 [30, 31]. The

Table 1. Information of *Bjerkandera* specimens used in this study.

Final ID	Collection No.	Site	Date collected	Accession No.	
				ITS	LSU
B. adusta	SFC20111029-15	Pyeongchang-gun, Gangwon-do	29 Oct 2011	KJ704813	KJ704828
	SFC20120409-08	Boryeong-si, Chungcheongnam-do	09 Apr 2012	KJ704814	KJ704829
	SFC20120601-20	Seosan-si, Chungcheongnam-do	01 Jun 2012	KJ704815	KJ704830
	SFC20120615-07	Jeju-do	15 Jun 2012	KJ704816	KJ704831
	SFC20120714-15	Yuseong-gu, Daejeon	14 Jul 2012	KJ704817	KJ704832
	SFC20120724-13	Yesan-gun, Chungcheongnam-do	24 Jul 2012	KJ704812	KJ704827
	SFC20120915-05	Gwanak-gu, Seoul	15 Sep 2012	KJ704818	KJ704833
	SFC20121009-23	Boryeong-si, Chungcheongnam-do	09 Oct 2012	KJ704811	KJ704826
	SFC20130405-16	Sangju-si, Gyeongsangbuk-do	05 Apr 2013	KJ704819	KJ704834
	SFC20130521-78	Taebaek-si, Gangwon-do	21 May 2013	KJ704820	KJ704835
	SFC20130917-H05	Yecheon-gun, Gyeongsangbuk-do	17 Sep 2013	KJ704821	KJ704836
B. fumosa	SFC19901006-08	Anyang-si, Gyeonggi-do	06 Oct 1990	KJ704822	KJ704837
	SFC20111227-22	Chuncheon-si, Gangwon-do	27 Dec 2011	KJ704825	KJ704840
	SFC20121009-04	Boryeong-si, Chungcheongnam-do	09 Oct 2012	KJ704824	KJ704839
	SFC20131024-02	Jeju-do	24 Oct 2013	KJ704823	KJ704838

Specimens identified by morphological observations, but not sequenced:
B. adusta: SFC19891015-20, SFC19900807-21, SFC19950511-07, SFC20010221-25, SFC20011114-06, SFC20030612-01, SFC20030612-04
B. fumosa: SFC19891017-96, SFC19990422-27

amplification was performed in a C1000 thermal cycler (Bio-Rad, USA) using the AccuPower PCR premix (Bioneer Co., Korea) in a final volume of 20 µl containing 10 pmol of each primer and 1 µl of genomic DNA. Thermal cycler conditions for PCR followed Park *et al*. [19]. After verification *via* gel electrophorese on a 1% agarose gel and the PCR product purified using the Expin PCR Purification Kit (GeneAll Biotechnology, Korea), DNA sequencing was performed with an ABI3700 automated DNA sequencer (Applied Biosystems, USA) at Macrogen (Seoul, Korea).

Sequence Analysis

For all molecular analyses, alignments were performed using MAFFT [13], and manually adjusted in MEGA5 [26]. For the ITS and LSU datasets, neighbor-joining (NJ) analyses were performed using MEGA5, and maximum likelihood (ML) analyses were performed using RAxML ver. 8.0.2 [25]. NJ analyses were performed using p-distances, substitutions including transitions and transversions, pairwise deletion of missing data, and 1,000 bootstrap replicates. ML was performed using the combined rapid bootstrap and search for the best-scoring ML tree analysis, the GTRGAMMA model of sequence evolution, and 1,000 bootstrap replicates. Both rooted and unrooted analyses were performed on the datasets to enhance our ability to identify distantly related species that were mislabeled as *Bjerkandera*. Based on a previous phylogenetic study, *Phanerochaete chrysosporium* was selected as the outgroup for rooted phylogenetic analyses [14]. Intra- and interspecific pairwise distances were calculated in MEGA5 using the p-distance model, substitutions including transitions and transversions, and pairwise deletion of gaps.

Our analysis had three steps. First, phylogenetic trees for ITS and LSU were built using only specimens of *B. adusta* and *B. fumosa* which identities were verified using morphology. Both species were reciprocally monophyletic for both ITS and LSU, with low intraspecific and high interspecific variation, validating the morphological identification. These sequence data and the phylogenetic tree served as the framework to which we determined whether GenBank sequences are misidentified.

Second, we downloaded all sequences resulting from the search query "*Bjerkandera*" for GenBank. We also included ITS and LSU data from the single JGI specimen used in the genome sequencing project. Sequences with over 90% coverage of the ITS region (500–600 bp) and 5′ partial LSU region (including D1 and D2 regions, 580–650 bp) were retained for further analyses. NJ and ML analyses were performed on the ITS and LSU alignments to classify the sequences; if sequences fell within the clades of *B. adusta* or *B. fumosa*, they were classified as such. In the phylogenetic tree, sequences that fell outside clades of the two *Bjerkandera* species were considered misclassified. Through this process, we validated the authenticity of sequences annotated as *Bjerkandera* in GenBank.

Third, we used BLAST to identify sequences highly similar to sequences identified as *B. adusta* and *B. fumosa* from the previous step. This set of sequences represents ones that are unidentified or mislabeled as different genera. We selected sequences based on similarity and coverage. Based on intraspecific p-distances of *B. adusta* and *B. fumosa* from step two (ITS: <6%; LSU: <3%), to be conservative, we downloaded all sequences that had a p-distance of <8% (92% similarity) for ITS and <5% (95% similarity) for LSU. To exclude short sequences, we removed those that had coverage of <80%. As in the previous step, NJ and ML analyses were performed on the two alignments to classify sequences. All work with GenBank was performed on April 2, 2014.

We performed an additional phylogenetic analysis to investigate the relationship between *Thanatephorus cucumeris* (or anamorphic name *Rhizoctonia solani*) and *Bjerkandera adusta*. BLAST search resulted in a substantial number of ITS sequences in GenBank annotated as *T. cucumeris* that were highly similar to *B. adusta*. We downloaded all ITS sequences labeled as *T. cucumeris* or *R. solani* and determined their phylogenetic relationship with *Bjerkandera* using NJ analysis as described above. For this analysis, *Waitea circinata* (or anamorphic name *Rhizoctonia zeae*) was used as the outgroup [27].

Results

Morphological and Molecular Analyses of Korean *Bjerkandera* Specimens

All 25 SFC specimens identified as *Bjerkandera* were used in the preliminary portion of this study. Initial identification of specimens was 18 *B. adusta* and 7 *B. fumosa* (Fig. 2A). Each specimen was reexamined based on distinguishing morphological characters between the two species and

Table 2. Morphological characteristics of *Bjerkandera adusta* and *B. fumosa*.

	Reference[a]	No. pores (mm)	Context thickness	Tube thickness	Basidia size (µm)	Spore size (µm)
B. adusta	This study	5–8	up to 5 mm	up to 1.5 mm	10.4–13.4(14.8) × 4.5–6.1	3.0–5.0 × 1.2–2.2
	America	6–7	up to 6 mm	up to 1 mm	22–25 × 5–6	5–6 × 2.5–3.5
	Europe	6–7	up to 6 mm	up to 1 mm	10–14 × 4–5	4.5–6 × 2.5–3.5
B. fumosa	This study	4–5	up to 14 mm	up to 2 mm	16.8–21.6 × 5.4–6.7	4.2–5 × 2.4–3.4
	America	2–5	up to 15 mm	up to 4 mm	12–14 × 4–5	5–5.5 × 2–3.5
	Europe	2–5	up to 15 mm	up to 4 mm	20–22 × 5–7	5.5–7 × 2.5–3.5

[a]North American data from Gilbertson and Ryvarden [9] and European data from Ryvarden and Gilbertson [22].

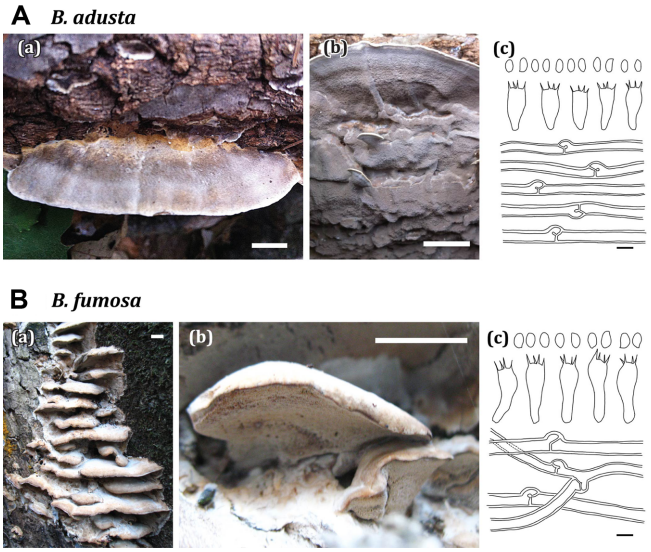

Fig. 1. Morphology of (**A**) *Bjerkandera adusta* and (**B**) *B. fumosa*. (a) Upper surface of basidiocarps, (b) pore surface, and (c) microscopic features. Microscopic features of basidiospores, basidia, and generative hyphae with clamp connection are arranged from top to bottom. Scale bar = 1 cm (a, b), 10 μm (c).

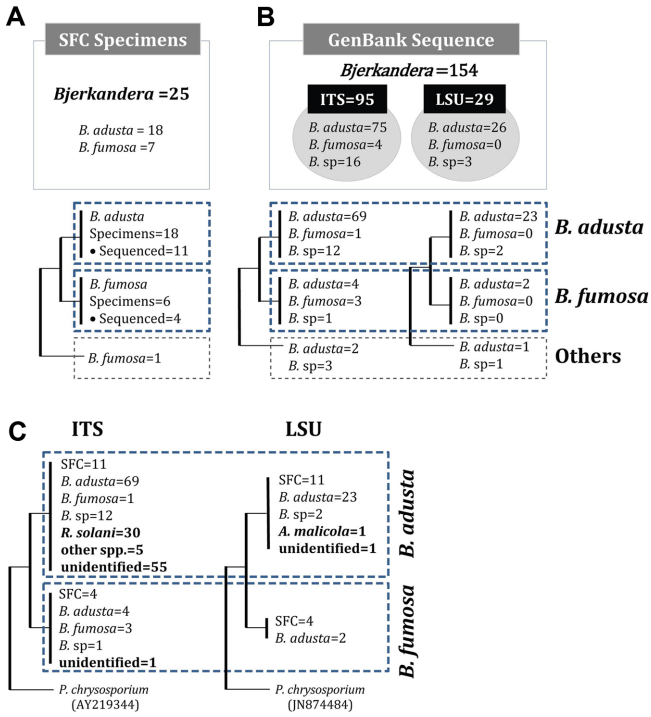

Fig. 2. Summary of methodology and misidentifications. (**A**) Specimens of *Bjerkandera* at SFC. (**B**) Summary of "*Bjerkandera*" sequences in GenBank (and JGI). Names inside the dashed boxes indicate original names in GenBank. (**C**) Summary of all *B. adusta* and *B. fumosa* sequences identified in this study. Names inside the dashed boxes indicate the original identifications in GenBank.

compared with published data (Table 2). Clear differences between the two species were observed (Fig. 1). The final identification recognized 18 *B. adusta* and 6 *B. fumosa*. One specimen of *B. fumosa* proved not to be *Bjerkandera* and was excluded from the study.

Owing to the old age of many specimens, DNA was not successfully sequenced for all samples. The ITS and LSU regions were successfully amplified and sequenced for 11 *B. adusta* and 4 *B. fumosa*. Phylogenetic relationships inferred from the ITS and LSU, using both NJ and ML methods, were similar and exhibited a clear distinction between the two species (Figs. S1–S5). For ITS, the intraspecific variation of Korean *B. adusta* and *B. fumosa* was 0.0–0.55% and 0.0%, respectively, whereas the interspecific variation was 5.15–5.89%. For LSU, the intraspecific variation of Korean *B. adusta* and *B. fumosa* was 0.0–0.16% for both species, and the interspecific variation was 1.44–1.78%.

Validity of *Bjerkandera* Sequences in GenBank

The query for ITS and LSU sequences labeled as *Bjerkandera* in GenBank (including JGI sequences) recovered 95 and 29 sequences, respectively. Of the 95 *Bjerkandera* ITS sequences, 75 were labeled as *B. adusta*, 4 as *B. fumosa*, and 16 as *Bjerkandera* sp. For the *B. adusta* records, one sequence used an old name (*B. adustus*), and one was misspelled (*B. adjusta*). Based on the phylogenetic analyses, 10.5% (10/95) of the sequences were shown to be misidentified (Fig. 2B). Five of these misidentified sequences (*B. adusta*: JN861758, JN628105; *Bjerkandera* sp.: HQ596906, KF578081, KJ174457) fell outside the clades of *B. adusta* and *B. fumosa*, so we removed them from subsequent analyses (Figs. S2–S3). Of the *Bjerkandera* sp. sequences, 12 and 1 were identified as *B. adusta* and *B. fumosa*, respectively. The intraspecific variation of ITS for *B. adusta* and *B. fumosa* was 0.0–5.48% and 0.0–1.86%, respectively, and the interspecific variation was 3.53–7.85%.

Of the 29 *Bjerkandera* LSU sequences, 26 were initially identified as *B. adusta*, zero as *B. fumosa*, and 3 as *Bjerkandera* sp. Based on phylogenetic analyses, 13.8% (4/29) of the sequences were shown to be misidentified (Fig. 2B). Two sequences (*B. adusta*: AJ406530; *Bjerkandera* sp.: KF578081) were inferred to be unrelated to *Bjerkandera* and removed from subsequent analyses (Figs. S4–S5). The intraspecific variation of LSU for *B. adusta* and *B. fumosa* was 0.0–2.45% and 0.0–0.55%, respectively, whereas the interspecific variation was 1.14–2.38%.

Misidentified and Unidentified Sequences in GenBank

Based on our search criteria (see Materials and Methods section), a total of 121 unique ITS and 15 unique LSU sequences were identified to be highly similar to *B. adusta* and *B. fumosa* and included in the final phylogenetic analyses. For ITS, 90 sequences were shown to be *B. adusta* and 1 *B. fumosa* (boldface in Fig. 2C). The remaining 30 sequences were not *Bjerkandera*. For *B. adusta*, 30 sequences were previously identified as *T. cucumeris* (or anamorphic name *R. solani*), 2 *Trichaptum abietinum* (FJ768676, U63474), 1 *Entrophospora* sp. (AY035664), 1 *Ceratobasidium stevensii* (AJ427405), 1 *Ganoderma lobatum* (JQ520165), and 55 unidentified sequences. For *B. fumosa*, one sequence was an unidentified species (FJ820598). For LSU, two sequences were misidentified and shown to be *B. adusta*: *Antrodia malicola* (AY333836) and an unidentified fungal species (JQ249221). The remaining 13 sequences were not closely related to *Bjerkandera.* All GenBank sequences used in this study (retrieved on April 2, 2014), their database identification, and corrected species information are listed in the Table S1.

Discussion

The genus *Bjerkandera* can be easily recognized by a blackish to brown tube layer contrasting with a white context [22], whereas the two species *B. adusta* and *B. fumosa* can be distinguished by pore size, thickness of context and tube layer, and size of basidia (Table 2). Despite the presence of distinguishing morphological characters for *B. adusta* and *B. fumosa*, misidentification is common, especially for those not specializing in taxonomic classification of fungi. This problem of misidentification is made worse since both species are sympatric and have a global distribution [9, 17, 22]. In this study, we have rigorously reexamined *Bjerkandera* specimens from Korea and verified the distinguishing morphological characters separating these two species (Fig. 1, Table 2). We also found that DNA data are useful to distinguish between *B. adusta* and *B. fumosa*, as phylogenetic analyses of ITS and LSU both recovered reciprocally monophyletic groups; thus molecular identification based on either of these two DNA markers is sufficient to distinguish *Bjerkandera* species.

DNA data are a powerful tool to aid in species identification. An approach such as DNA barcoding has become popular for species identification because it is easy and straightforward for a non-specialist to use [11]. However, the efficacy of DNA barcoding depends on public databases having satisfactory taxonomic sampling and sequences that are correctly identified [18]. We found that the number of misidentified sequences of *Bjerkandera* in GenBank is substantial. More ITS sequences (95 sequences) were present in GenBank compared with LSU (29 sequences), and as such, the problem of misidentification was more evident for ITS sequences. Our discussion of misidentification herein focuses on ITS.

The results revealed that *B. fumosa* was more commonly misidentified as *B. adusta* ($n = 4$) as opposed to the opposite case ($n = 1$) (Fig. 2B). This is likely due to *B. adusta* being more common in the environment compared with *B. fumosa* [22], and *B. adusta* being the focus of more academic and industrial research. In addition to misidentified sequences, there were many unidentified sequences that, through the phylogenetic analyses, were shown to be *B. adusta* or *B. fumosa*. Recognition of these previously misidentified and unidentified sequences of *B. adusta* (90 sequences) and *B. fumosa* (1 sequence) nearly doubles the number of *Bjerkandera* ITS sequences in GenBank.

Of the misidentifications between genera, some sequences originally identified as *T. cucumeris* (or anamorphic name *R. solani*) were later re-identified as *B. adusta*. Morphologically, these two species are different in culture morphology, with *B. adusta* possessing hyaline hyphae with conidia, and *T. cucumeris* having brownish hyphae without conidia [21]. The problem of identification was raised in studies exploring fungal diversity from air, soil, and industrial wastes. Several authors explicitly described the difficulty distinguishing between *Bjerkandera* and *Thanatephorous* using DNA data, due to the highly similar sequences of the two different species uploaded in GenBank [*e.g.*, 7, 21]. Other previous studies also raise the problem of identification using environmental DNA data and BLAST for identification [28]. To clarify the issue, we performed a phylogenetic analysis of our *Bjerkandera* ITS data, adding data from *T. cucumeris*. We found that 1,024 sequences of *T. cucumeris* formed a distinct group with high bootstrap support from the 30 sequences re-identified as *B. adusta* (Fig. S6). These results indicate that *T. cucumeris* and *B. adusta* are distinguishable with molecular data, and the problem was due to misidentified sequences.

For a small subset of sequences, *Bjerkandera* species were found to be misidentified as different wood decay fungi genera (*Antrodia*, *Ganoderma*, *Trichaptum*). Although the basidiocarps of *Bjerkandera* are morphologically distinct from these wood decay fungi, such misidentification may occur in the absence of fungal taxonomic expertise or apparent morphological distinctions (*e.g.*, working with cultures, immature basidiocarps, or environmental samples).

These scenarios exemplify the importance of thorough

morphological observation and correct identification of specimens/cultures before uploading associated DNA data to GenBank. Misidentification in groups such as *Bjerkandera* can have important implications to biotechnological research. Considering the interest *Bjerkandera* has attracted for various industrial applications, it is necessary that *Bjerkandera* cultures and stocks are molecularly verified for potential misidentification. For accurate comprehension of the evolution and mechanisms underlying enzymatic activities and optimum application of strains, precise taxonomy is paramount. This problem of misidentification perpetuated through public databases and future studies are not confined to *Bjerkandera* or wood-rotting fungi. We hope that researchers understand the responsibility of using a public database, and are prudent in accurate species identification and annotation before submitting sequence data for public use.

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