이학석사 학위논문

Obstacles in the current identification methods of wood decay fungi: case studies of the genera *Bjerkandera* and *Gloeoporus*

목재부후균 동정 방식에 대한 고찰: 줄버섯속과 무른구멍장이버섯속의 사례연구

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Obstacles in the current identification methods of wood decay fungi: case studies of the genera *Bjerkandera* and *Gloeoporus*

A thesis presented

By

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줄버섯속과 무른구멍장이버섯속의 사례연구

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ABSTRACT

Wood decay fungi, as the name suggests, are a group of fungi which degrade woods in various states. Due to its unique property, wood decay fungi have considerable implications on various aspects of human lives and the global ecosystem. Despite of its significance, taxonomy of wood decay fungi has not yet been settled. Incorrect and arbitrary identification of wood decay fungi, for example, interfere with the optimal application of fungal strains for industrial uses and forest preservation efforts against devastating fungal wood decayers.

Traditional taxonomy of wood decay fungi has been chiefly based on morphological distinctions of fruit bodies. Despite of meticulous observations of

taxonomists, morphological taxonomy often resulted in artificial delimitation constantly overturned by personal tastes of various taxonomists. Advent of PCR for molecular taxonomy provided faster and more objective approach for identification of wood decay fungi; nonetheless, several hindrances remain on the path to discover the true taxonomy of wood decay fungi. This study addresses following problems taxonomists encounter from current identification practices of wood decay fungi: misidentification by incorrectly annotated sequences in public sequence databases and highly variable intraspecific variation among different species, leading to confusion in species identification and delimitation.

Molecular identification based on DNA sequences rely upon reference sequences; thus the integrity of the data available at public sequence databases is fundamental for accurate identification. With ITS and LSU sequences of *Bjerkandera*, this study examines validity of sequences registered at GenBank. Based on the phylogenetic analysis of Korean specimens of *Bjerkandera*, sequences of the genus uploaded on GenBank were cross-checked. Sequences validated as *Bjerkandera* were further compared with other sequences on GenBank by BLAST search to discover any misidentified or unidentified sequence of *Bjerkandera*. Adding all mislabeled sequences, number of *Bjerkandera* sequences available at GenBank nearly doubles.

In case of *Gloeoporus*, its taxonomy is still largely unsettled and sequences of only well-known species are available at GenBank. For the phylogenetic study of this genus, specimens were collected from various countries around the world. Multilocus

phylogeny (ITS, LSU, *tef*, *rpb2*) reveals existence of two new species and biogeographic diversification of *G. dichrous* into three locations, Asia with Alaska, America (except Alaska), and Europe. Such pattern reveals that *G. dichrous* may have dispersed via Beringia, a land bridge once connected two continents, Asia and America. Based on the analyses, I propose *Gloeoporus* s.s. which mostly has clamp connections (except *G. thelephoroides*) and lacks cystidia.

This study presents pitfalls which exist in taxonomic studies of wood decay fungi. As overall taxonomy of this group has not been settled, researchers interested in fungal identification must consider these aspects while they proceed with their studies.

Keywords: Fungal taxonomy, wood decay fungi, misidentification, biogeographic variation, molecular phylogeny

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I. Introduction

Wood decay fungi have been long studied in various cultures due to their unique properties and economic implications. There are numerous historical records which recounts damages caused by wood decay fungi. In England, for instance, damages caused by wood decay fungi in the warships of the Royal Navy are recorded as early as 1684 and emerged as a national concern by the 19th century (Findlay 1974). The forestry industry has long been heavily affected by numerous decay fungi, like *Heterobasidion annosum* which causes pine root rot (Deacon 2009).

While the wood decay fungi initially received attentions due to their pathogenicity and were studied for their identification and preventive methods, they also have numerous benefits in human lives. In Korea, various wood rotting fungi were traditionally prescribed as medicines. Through substance screening and animal tests, we now know scientifically that several wood decay fungi possess potent medicinal properties, including anticancer substances (Sone et al. 1985; Liao et al. 2013). Many research groups around the world are now exploring the possibility of using particular strains of wood decay fungi for various industrial applications, such as biopulping, ethanol production, and bioremediation (Singh and Singh 2014). Ecologically, these fungi perform crucial roles as decomposers in the forest ecosystem and significantly influence global carbon cycle (Singh and Singh 2014; Floudas et al. 2012).

Recognizing the importance, researchers associated with Joint Genome Institute

(JGI) of the US Department of Energy have performed whole genome sequencing of representative wood decayers (Binder et al. 2013). These tremendous amount of data enabled scientists to have better understanding of taxonomy and enzymatic activities of wood decay fungi.

Classifying this extremely diverse group of fungi has always been challenges to past and current fungal taxonomists. One of the broad categorization of the wood decay fungi of Basidiomycota is based on the morphologically and chemically unique types of decay they cause: brown and white rot (Blanchette 1995). Brown rots rapidly depolymerize cellulose of wood cell while leaving lignin intact. On the other hand, White rot fungi have ability to degrade all cell wall components, both lignin and cellulose. White rot fungi produces hydrolases which slowly degrade cellulose while rapidly and completely mineralize lignin. Significant loss of lignin results in zone of white and delignified wood; hence dubbed white rots (Blanchette 1995; Riley et al. 2014).

Recent genomic research on wood decay fungi, however, suggests that the current paradigm of white rot and brown rot categorization may not sufficiently reflect the diversity of this fungal group (Riley et al. 2014). According to the scientists at JGI, some fungal species normally categorized as white rots according to the morphology lacks ligninolytic class II peroxidases (PODs), found in other white rot fungi; these fungi, yet, have ability to degrade all components of woody plant cell walls, exhibiting the typical characteristics of white rot fungi. As genome-wide

research becomes prevalent and our understanding on enzymatic activities of these fungi increases, traditional classification methods based on morphology are challenged and, oftentimes, overturned.

Same pattern is observed in taxonomic aspect of wood decay fungi. Traditionally, taxonomical order of this group had been categorized according to various morphological emphases imposed by different taxonomists. Present-day fungal taxonomists now realize that such groupings were mostly arbitrary as traditional categorization of wood decay fungi largely deviates from the recent molecular classification. Due to such large discrepancy, fungal taxonomists are now in the process of redefining the taxonomic relationship of the group.

While recent technologies in molecular biology have revealed the glimpse of the true relationship of these fungi, there are still numerous problems which must be addressed to approach to the true taxonomy of wood decay fungi. For instance, reliability of fungal identification based on public sequence database is often challenged as reference sequences in the database are occasionally misidentified and unidentified.

Before I further discuss the problems of modern taxonomic techniques, a historical overview of taxonomic study of wood decay fungi will be first presented. I briefly explains how taxonomic study of wood decay fungi has evolved over the past few decades. Afterwards, I specifically delve into the history of taxonomic study on wood decay fungi in Korea. Alongside, the current obstacles in fungal taxonomic

studies in Korea will be discussed. Finally, scope and the aims of this paper will be presented.

1.1 Advancement of Taxonomic Study on Wood Decay Fungi

Identification of the wood decay fungi heavily relied upon analysis of fruit bodies (Breitenbach and Kränzlin 1986) and pure fungal cultures (Stalpers 1978). While morphological identification based on visual analysis of basidiocarps is fast and reliable most of the times, early identification is rarely possible, impeding the forest protection efforts against decay fungi (Nicolotti et al. 2010). Additionally, morphological identification often requires great taxonomic understanding and experiences for accurate identification. Another method, culturing is time-consuming and often suffers contamination which hinders the accurate identification (Nicolotti et al. 2010).

Earlier fungal taxonomists of wood decay fungi had categorized the fungi based on meticulous examination of marco- and micro-morphological characteristics. Nonetheless, recent molecular analysis demonstrates that previous morphological taxonomy largely resulted in artificial and arbitrary grouping of these fungi. Wood decay fungi were once part of the order Aphyllophorales, proposed by Rea, including all miscellaneous fungi not forming gills, *Thelephoraceae*, *Clavariaceae*, *Hydnaceae*, and *Polyporaceae*. While 4 families within the order were grouped together based on hymenophore shape, exhaustive microscopic analysis had already revealed that such groupings are unnatural (Kirk et al. 2008).

Even after the recognition of artificial groupings in wood decay fungi, such groupings continued to be used until the advent of molecular phylogeny in 1990s. As

more DNA sequences of wood decay fungi accumulate, more natural taxonomy based on molecular analysis became possible. Using PCR method, taxonomists now can easily amplify genes of their interest and use them to compare with the corresponding genes of other organisms – the basic concept of DNA barcoding. Numerous DNA regions were proposed as a fungal DNA barcode marker, such as the region of mitochondrial cytochrome c oxidase subunit 1 (COI), the animal barcode marker or the 18S nuclear ribosomal small subunit rRNA gene (SSU). These regions were not as hypervariable or abundant as the internal transcribed spacer (ITS) region which is ultimately selected as a universal barcode marker of fungi by the Fungal Working Group of the Consortium for the Barcode of Life (CBOL) (Schoch et al. 2012). As DNA sequencing becomes more prevalent, scientists also implement multi-gene analysis, combining datasets resulted from analyses of different DNA barcode markers.

Taking the entire research to the next level, scientists now analyze the fungal genome to determine phylogenetic relationships and to understand physiological mechanisms of industrially valuable fungal strains. The 1000 Fungal Genomes (1KFG) project is currently ongoing with collaborations of numerous institutions around the world (Hibbett et al. 2013). In the near future, complete genome sequencing will become more prevalent and facilitate to widen our functional insights of numerous wood decay fungi.

1.2 Taxonomic study of wood decay fungi in Korea

In Korea, the number of fungi forming sporocarp is recorded as 1,600 (Seok et al. 2013) and wood decay fungi comprise more than 220 (Lee and Jung 2005; Lim 2001). The number is anticipated to expand even more as the Korean fungal DNA barcoding project progresses. Such prospect is possibly due to abundance of cryptic species not yet reported and rather short history of Korean mycology. In this section, I describe the history of taxonomic study of wood decay fungi in Korea. Much information has been liberally obtained from previous review on the fungal taxonomic history of Korea by Jung (1990).

The earliest account of mushroom appears in Korean history is *yeongji* (*Ganoderma lucidum*), a medicinal wood decay fungus, recorded as an offering to the palace in 704 (Kim 1145). During Joseon Dynasty (1392–1897), several documents record fungi according to edibility and medicinal property. Early classification of fungi in Korea were, however, principally artificial, accomplished at the convenience of individuals utilizing these fungi.

Scientific reports on Korean native sporocarps were first published by foreign scholars during early 1900s under Japanese rule of Korea. While many of them were simple reports on fungal flora, there were also a number of taxonomic researches, like the study on 11 species of Polyporaceae (Okada 1932).

After the momentary halt in overall academic activities due to social turmoil following the liberation of Korea, fungal research continued to be conducted as

subdiscipline of botany. The first illustrated guide of Korean fungi was published in 1959 (Lee et al. 1959) and subsequently the first thesis on ecological research of wood decay fungi in Gwangneung in 1961 (Lim 1961). After extended absence of meaningful taxonomic research, Lee and Jung (1972) published exhaustive list of Korean Basidiomycota in 1972 as part of the comprehensive survey of Korean biota, reporting total 381 species with photos of specimens and microscopic sketches.

With the establishment of the Korean Society of Mycology in 1972, mycologists in Korea saw expanded opportunities to communicate and disseminate their research outcomes on the journal dedicated for fungal research; however, taxonomic works exclusively on wood decay fungi did rarely appear and only occasionally as part of the extensive flora studies or comprehensive lists of Korean native fungi.

Series of detailed field guides on mushrooms were published since 1980s. The guides include detailed descriptions essential for identifying fungi at the field, including color, taste, size, substrate, and microscopic features for laboratory observation. These descriptions and illustrations are certainly useful in identification. Yet, obstacles remain as numerous variations within the species exist and subjective descriptions, such as color or taste, may not agree among researchers. Another problem was posed by heavy reliance on foreign taxonomic works. Although joining belatedly in the field of mycology gave Korean fungal taxonomists leverage to advance in research with wealth of foreign academic literatures, overdependence on such materials also obstructed the efforts of unrevealing the true taxonomic diversity

of Korean fungal species.

In 1987 and 1991, flora studies on wood-rotting fungi of Jeju (Yang et al.) and Ullung Island (Jung) of Korea were published respectively. Afterwards, a number of taxonomic studies on wood decay fungi were published with detailed microscopic illustrations. These reports were akin to traditional taxonomic papers which detailed macro- and micro-morphological features of basidiocarps; however, even before meticulous morphological studies take firm root in Korea, a new wave of scientific revolution began to sweep the entire field of fungal taxonomy. With the invention of PCR, Korean mycologists joined the worldwide movement toward molecular taxonomy by DNA sequences.

At the dawning of the molecular phylogeny, Korean fungal taxonomists also began to explore the brand-new technique for application in their studies. In 1992, the cytoplasmic 5S rRNA sequence of *Trimorphomyces papilionaceus* (Her et al. 1992) was first published and cytochrome b (*cob*) gene region of mitochondrial DNA (mtDNA) (Hong 1993) in the subsequent year. One of the first analyses of wood decay fungi based on PCR-amplified DNA was conducted for phylogenetic analysis of *Trichaptum* in 1996 with internal transcribed spacer (ITS) 1 and 2 and mitochondrial small subunit (SSU) rRNA gene (Ko and Jung 1996). By late 1990s and early 2000s, several molecular phylogenies of fungal genus, such as *Trametes* (Ko and Jung 1999), *Ceriporia* (Kim and Jung 1999), *Antrodia* (Kim et al. 2001), *Schizopora* (Lim and Jung 2001), *Coprinus* (Ko et al. 2001b), *Hapalopilus* (Ko et al. 2001b) were

conducted, in addition to large-scale papers which propose redefining phylogenetic relationships of the Aphyllophorales (Kim and Jung 2000). Exhaustive phylogenetic studies on wood decay fungi by Korean scholars contributed much to the overall understanding of this complex group of fungi inhabiting on wood.

Now more than two decades have passed since the emergence of PCR method. The process of DNA sequencing has become much cheaper and quicker with technological advances. Numerous Internet databases now host millions of sequences which serve as references for sequence similarity search. Yet, most of the Korean fungal flora studies conducted thus far have heavily depended on morphological observations of basidiocarps. The process of DNA extraction, PCR, and sequencing still requires expertise, time, efforts, and equipment. Such barriers resulted in many ecological reports which significantly depressed the diversity of Korean wood decay fungi and overlooked possible novel species as mere variations of recorded species. As more researchers now incorporate molecular methods to ecological reports, we now recognize that there are many unreported and new species of fungi in Korea and fungal diversity is much greater than previously estimated by morphological observation. Molecular identification method proves to be especially effective in identifying hard-to-distinguish organisms, like wood decay fungi, and to discover diversity within an outwardly single morphospecies.

Molecular identification, however, is not a panacea of all identification problems of the past. This method also has shortcomings despite of its convenience and

apparent objectiveness. For instance, sequencing results may be misleading in a certain species with high intraspecific variation. In such case, morphological observation and meticulous phylogenetic analyses must accompany. Another shortcoming of molecular identification is credibility of public sequence database. Integrity of the data available at DNA sequence databases is the key to DNA barcoding since the sequence of interest will be compared against sequences available at databases for its identification. In public database like GenBank, 20 percent of fungal sequences are estimated to be misidentified (Bridge et al. 2003; Nilsson et al. 2006). This finding suggests that simple identification by BLAST similarity search can be seriously tainted by misidentified sequences at GenBank.

1.3 Scope and aims

This study aims to observe the current problems and obstacles of identifying wood decay fungi with case studies of *Bjerkandera* and *Gloeoporus*. Through the case studies of the two genus, I aim to disclose the shortcomings of morphological observation and simple sequence comparison (i.e. BLAST search) for species identification, especially to researchers who do not specialize in taxonomy of wood decay fungi. Two major problems will be addressed: misidentification by incorrectly annotated sequences in public sequence databases and highly variable intraspecific variation among different species, leading to confusion in species identification and delimitation.

The type species of *Bjerkandera*, *B. adusta* has received attentions for its remarkable ability to degrade various industrial wastes. As a recognition of its economical implication, JGI has completed its whole genome sequences in 2013 (Binder et al. 2013). While many scientists have noticed its physiological property, phylogenetic studies of this particular genus has never been rigorously executed. Since *Bjerkandera* is distributed worldwide and several important strains have been extensively studied, moderate number of sequences are uploaded in the sequence database. In addition, the genus is small and well-defined, thus ideal for confirming the validity of sequences registered at GenBank. Using specimens collected in Korea, DNA sequences were obtained and compared against sequences available at public sequence databases. Through similarity search of *Bjerkandera* sequences via BLAST

search, mislabeled and unlabeled sequences were identified to ultimately estimate the reliability of data in databases.

Gloeoporus has been largely defined by morphological distinctions, but not molecular phylogeny. Public sequence databases also lack sequences of less familiar *Gloeoporus* species, hence collection of specimens is required for phylogenetic studies. *G. dichrous* is well-known species observed in all continents except Antarctica, thus suitable for the study on regional variation of fungi. Its physiological property is still under scrutiny while medical use as an antibiotic has been discussed (Harada et al. 2006). Morphologically (however, not molecularly) related species of *G. taxicola*, was extensively studied for cryptic lineages within the species (Kauserud et al. 2007; Skaven Seierstad et al. 2013). These results revealed that even within a single morphological species, several lineages diverged according to geographic division and substrate. Hence, neither painstaking observation of specimen nor simple sequence similarity search may sufficiently reveal the true diversity of these fungi which sequences are not readily available in the public domain. To understand geographic diversity, specimens of the cosmopolitan species *G. dichrous* were collected from worldwide locations and phylogenetically analyzed for biogeographic pattern. Based on this analysis, phylogenetic and taxonomic studies of the genus *Gloeoporus* were performed to examine validity of the previous morphological delimitation.

II. Case Study of *Bjerkandera*

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

2.1 Introduction

Bjerkandera is a common white-rot fungus found worldwide (Murrill 1905). 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 (Ryvarden and Gilbertson 1993). The two species in this genus, *B. adusta* and *B. fumosa*, are both distributed in North America, Europe, and Asia (Ryvarden and Gilbertson 1993; Gilbertson and Ryvarden 1986; Núñez and Ryvarden 2001). In Korea, *B. adusta* was first reported in 1936 as *Polyporus adustus* (Ueki 1936), and *B. fumosa* officially recorded in 1994 as part of an exhaustive list of Korean wood-rooting fungi (Jung 1994). Systematic taxonomic descriptions of both species were documented in 2010 (Lim et al. 2010).

Bjerkandera plays an ecologically important role in the global carbon cycle by growing on and decomposing dead hardwood trees (Floudas et al. 2012), but also has negative impacts, such as causing timber damage and interfering with the cultivation of culinary mushrooms (Bak et al. 2011). Additional to its effectiveness in decaying lignin, *Bjerkandera* can degrade common anthropogenic pollutants, such as various polycyclic aromatic hydrocarbons (Haritash and Kaushik 2009). Such notable enzymatic activities led scientists to explore industrial application of *Bjerkandera*; *B. adusta* has demonstrated an ability to decolorize synthetic dyes, which can be applied to bioremediation (Choi et al. 2013). 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 1000 fungal genomes project (Binder et al. 2013).

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 (Ryvarden and Gilbertson 1993). 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 incorrect 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 (Hebert et al. 2004) 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 (Bridge et al. 2003; Nilsson et al. 2006).

In this study, I use the genus *Bjerkandera* as an example to quantify, characterize, and correct species misidentifications in GenBank. I choose *Bjerkandera* because 1) there are only two species, 2) the two species are highly similar and easily misidentified by non-specialists despite distinguishing morphological characters,

and 3) the results have implications to genomic and biotechnological research. To complete these goals, I first identify 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), are sequenced since they are the two most common genes used in fungal systematics (Fell et al. 2000; Scorzetti et al. 2002; Schoch et al. 2012). Lastly, all ITS and LSU sequences in GenBank, which have been identified as or show high sequence similarity to *Bjerkandera*, are evaluated against correctly identified *B. adusta* and *B. fumosa* sequences.

2.2 Materials and methods

2.2.1 Specimens and Microscopic Observation

All specimens used in this study were collected throughout the Korean Peninsula between 1989-2013, 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 specimens were characterized in detail.

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

Final ID	Collection No.	Site	Date collected	Accession Number	
				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

2.2.2 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 2X CTAB buffer and ground with a plastic pestle. Genomic DNA was extracted with a modified CTAB extraction protocol (1994). The ITS region was amplified using the primers ITS1F and ITS4-B (Gardes and Bruns 1993) and LSU region was amplified using the primers ITS3 and LR5 (White et al. 1990; Vilgalys and Hester 1990). The 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. (Park et al. 2013). 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).

2.2.3 Sequence Analysis

For all molecular analyses, alignments were performed using MAFFT (Katoh and Standley 2013), and manually adjusted in MEGA5 (Tamura et al. 2011). For the ITS and LSU datasets, neighbor joining (NJ) analyses were performed using MEGA5 and maximum likelihood (ML) analyses were performed using RAxML v8.0.2 (Stamatakis 2006). NJ analyses were performed using p-distances, substitutions including transitions and transversions, pairwise deletion of missing data, and 1000 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 1000 bootstrap replicates. Both rooted and unrooted analyses were performed on the datasets to enhance my 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 (Ko et al. 2001a). Intra- and interspecific pairwise distances were calculated in MEGA5 using the p-distance model, substitutions, including transitions and transversions, and pairwise deletion of gaps.

This 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 variation and high interspecific variation, validating morphological identification. These sequence data and the phylogenetic tree served as the

framework to which I determine whether GenBank sequences are misidentified.

Second, I downloaded all sequences resulting from the search query "*Bjerkandera*" for GenBank. I 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 region, 580-650bp) 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, I validated authenticity of sequences annotated as *Bjerkandera* in GenBank.

Third, I 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. I 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, I downloaded all sequences that had a p-distance of <8% (92% similarity) for ITS and <5% (95% similarity) for LSU. To exclude short sequences, I 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*. I downloaded all ITS sequences labeled as *T. cucumeris* or *R. solani* and determined their phylogenetic relationship with *Bjerkandera* using NJ analysis as describe above. For this analysis, *Waitea circinata* (or anamorphic name *Rhizoctonia zeae*) was used as the outgroup (Toda et al. 2007). The whole process detailed in materials and methods is organized in Fig. 1 as a flowchart.

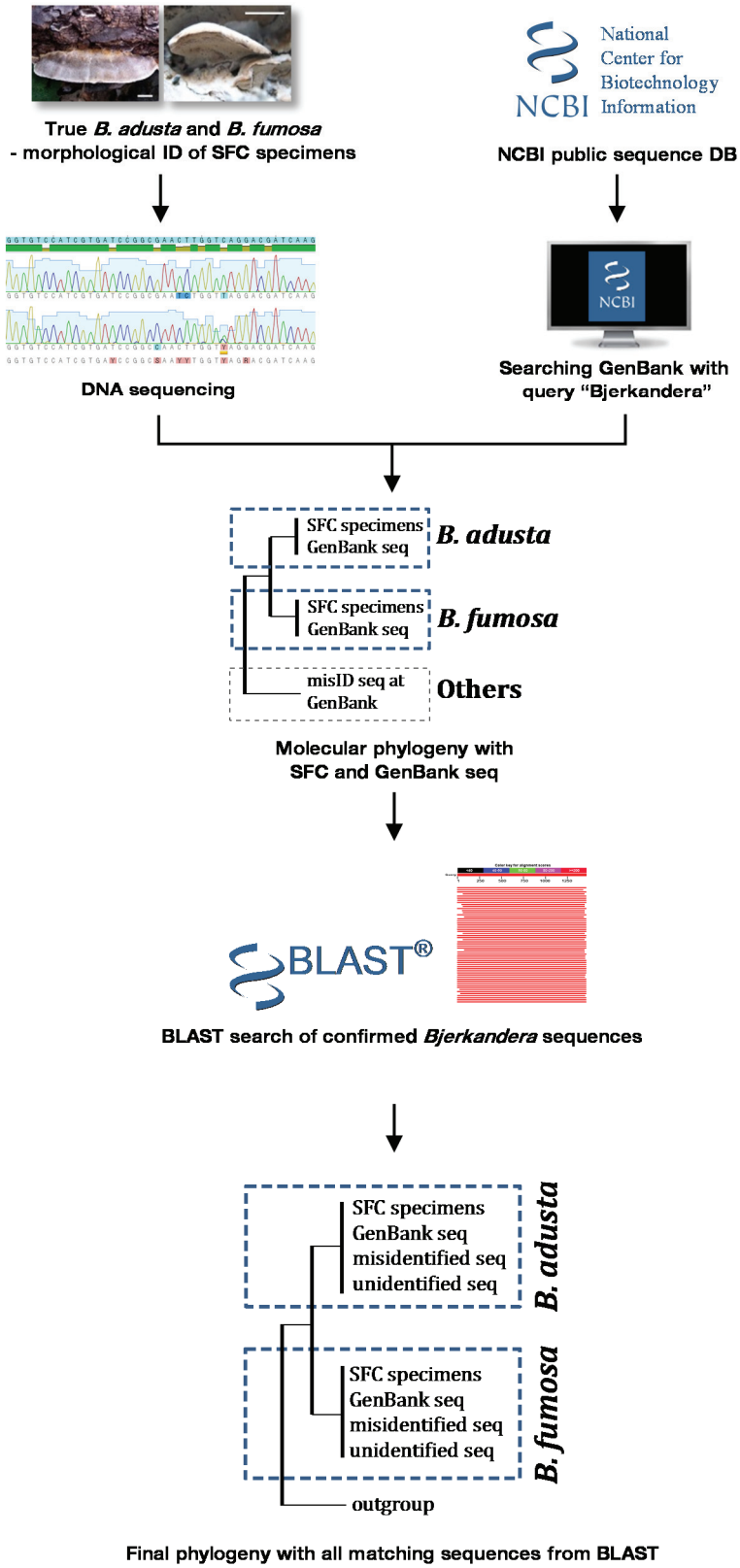


Figure 1. Flowchart of the *Bjerkandera* case study.

2.3 Results

2.3.1 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 compared to published data (Table 2). Clear differences between the two species were observed (Fig. 3). 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.

Due 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 ITS and LSU using both NJ and ML methods were similar and exhibited a clear distinction between the two species (Figs. 4-8). For ITS, intraspecific variation of Korean *B. adusta* and *B. fumosa* was 0.0–0.55% and 0.0%, respectively, while interspecific variation was 5.15–5.89%. For LSU, intraspecific variation of Korean *B. adusta* and *B. fumosa* was 0.0–0.16% for both species, while interspecific variation was 1.44–1.78%.

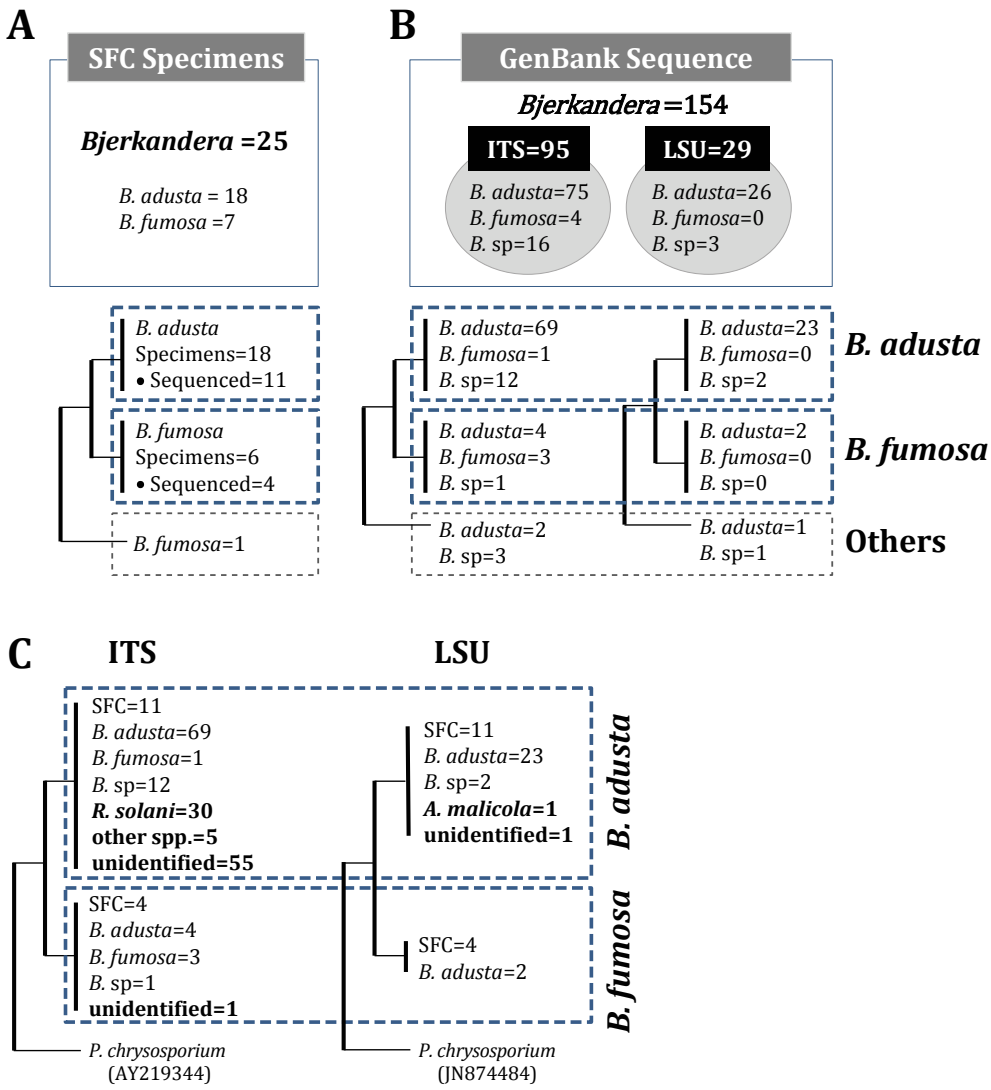


Figure 2. Summary of methodology and misidentifications. (A) Specimens of *Bjerkandera* at SFC. (B) Summary of "*Bjerkandera*" sequences in GenBank (and JGI). Names inside the dashed boxes indicate original names in GenBank. (C) Summary of all *B. adusta* and *B. fumosa* sequences identified in this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

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

	Reference[a]	No. pores (per mm)	Context	Tube	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 (Gilbertson and Ryvarden 1986) and European data from Ryvarden and Gilbertson (Ryvarden and Gilbertson 1993)

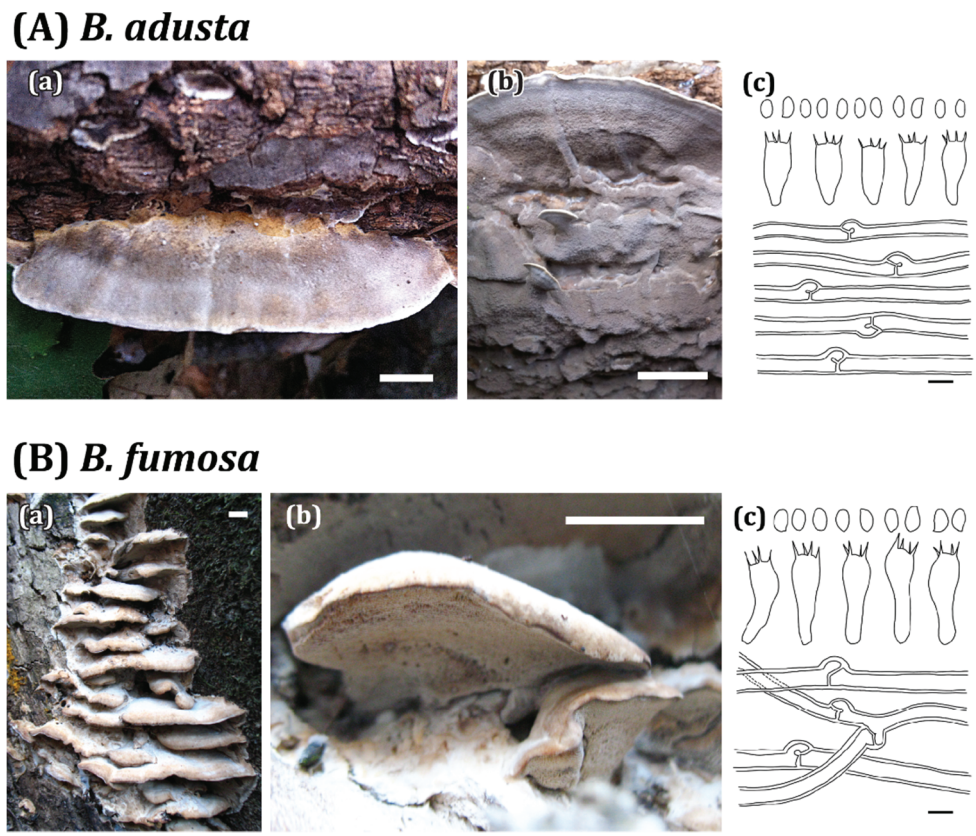


Figure 3. Morphology of (A) *Bjerkandera adusta* and (B) *B. fumosa*. (a) upper surface of basidiocarps, (b) pore surface, and (c) microscopic features. Microscopic features of basidiospores, basidia, and generative hyphae with clamp connection are arranged from top to bottom. Scale bar=1 cm (a, b), 10 μm (c).

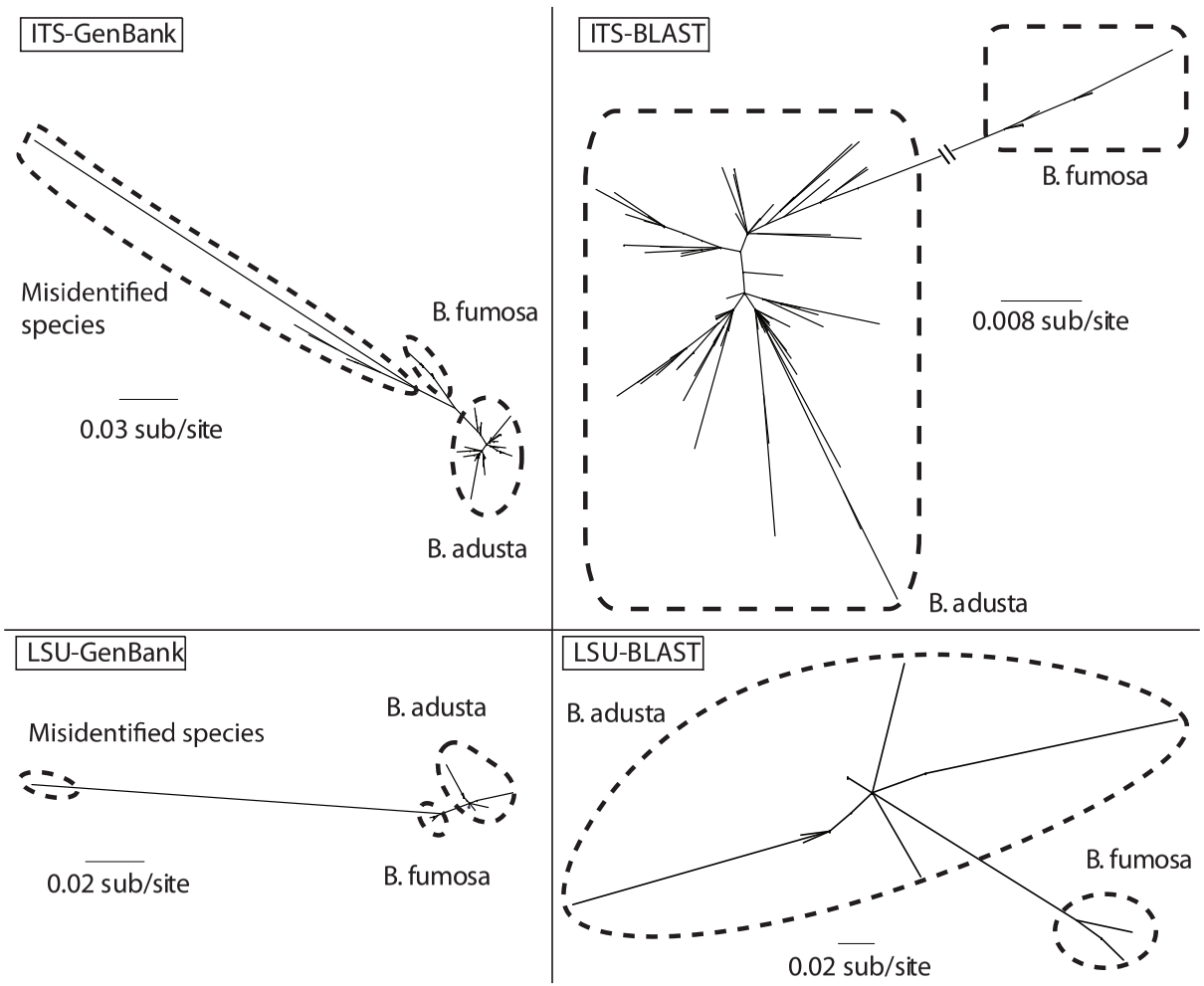


Figure 4. Unrooted maximum likelihood analyses of the four datasets. Clusters for *B. adusta*, *B. fumosa*, and misidentified species are noted with the dotted lines.

2.3.2 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*), while 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 I removed them from subsequent analyses (Figs. 5). Of the *Bjerkandera* sp. sequences, 12 and 1 were identified as *B. adusta* and *B. fumosa*, respectively. Intraspecific variation of ITS for *B. adusta* and *B. fumosa* was 0.0–5.48% and 0.0–1.86 %, respectively, while 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. 6). Intraspecific variation of LSU for *B. adusta* and *B. fumosa* was 0.0–2.45% and 0.0–0.55%, respectively, while interspecific variation was 1.14 – 2.38%.

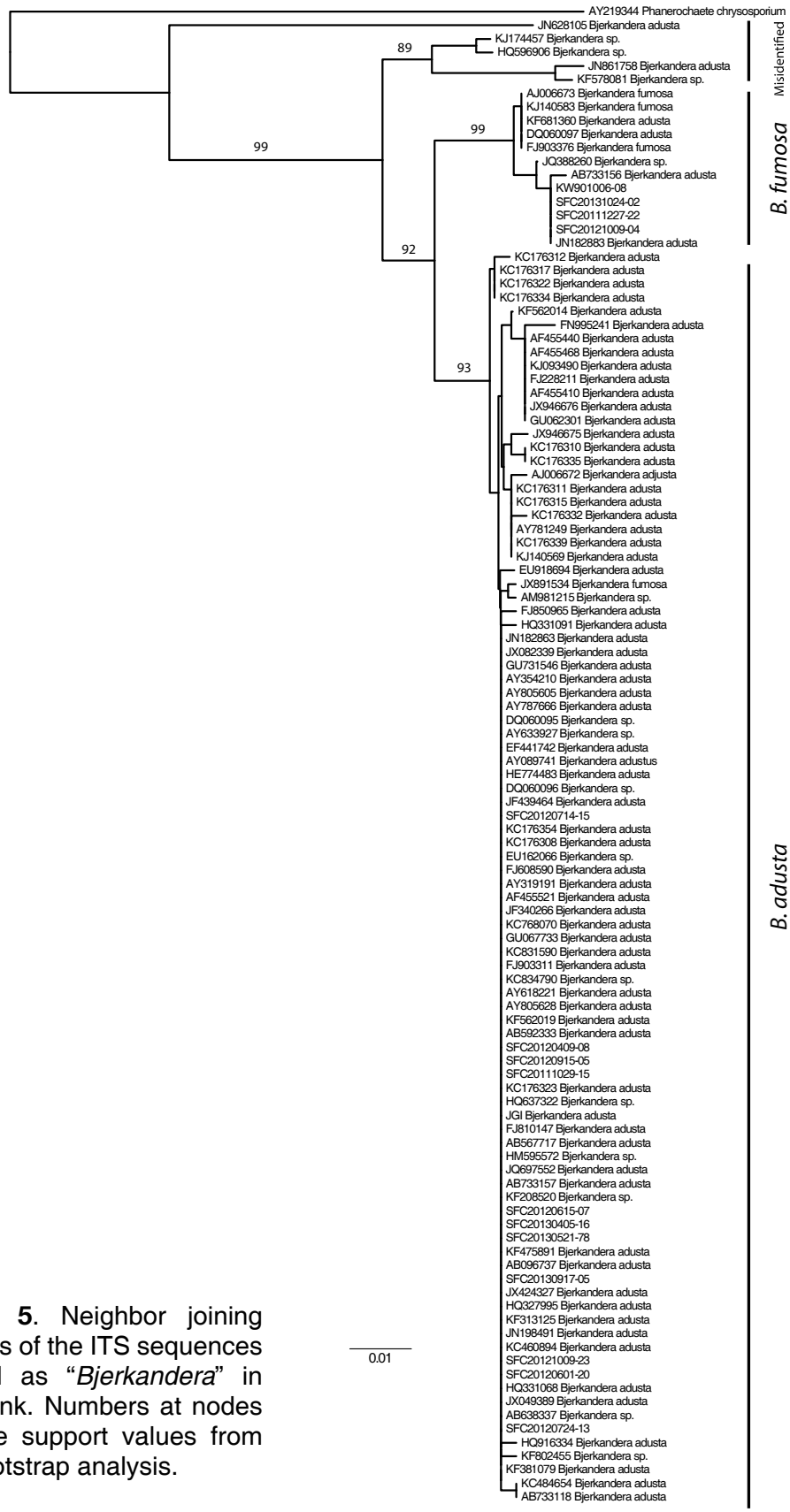


Figure 5. Neighbor joining analysis of the ITS sequences labeled as "*Bjerkandera*" in GenBank. Numbers at nodes indicate support values from the bootstrap analysis.

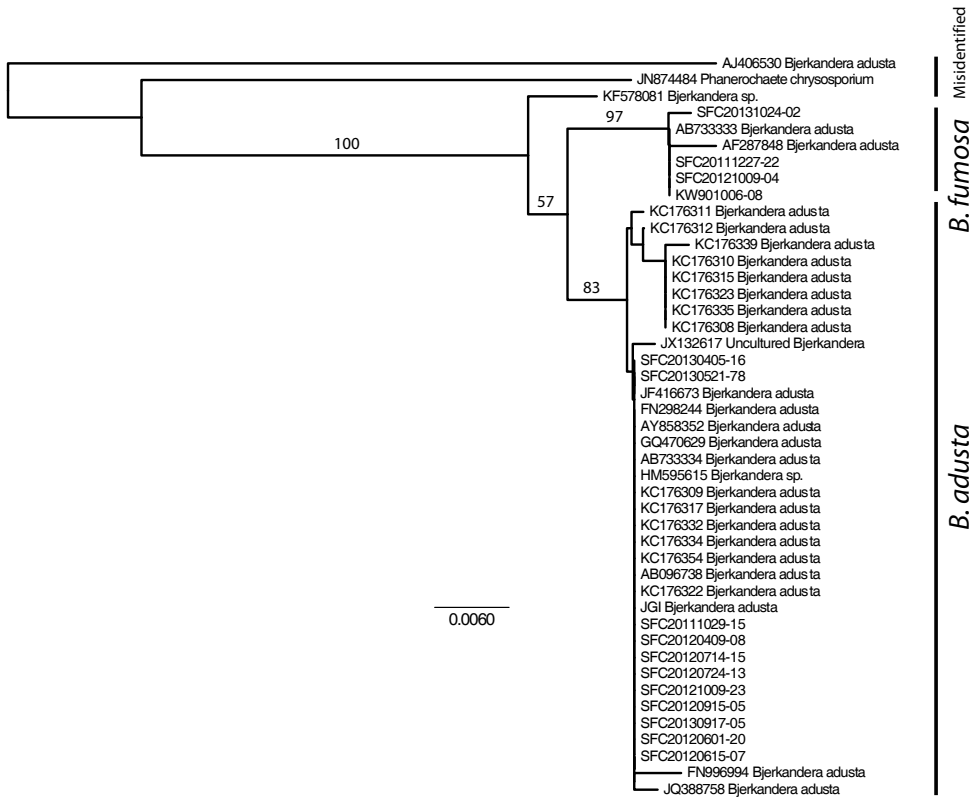


Figure 6. Neighbor joining analysis of the LSU sequences labeled as "*Bjerkandera*" in GenBank. Numbers at nodes indicate support values from the bootstrap analysis.

2.3.3 Misidentified and Unidentified Sequences in GenBank

Based on my 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 (Fig. 7). 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) (Fig. 8). The remaining 13 sequences were not closely related to *Bjerkandera.*

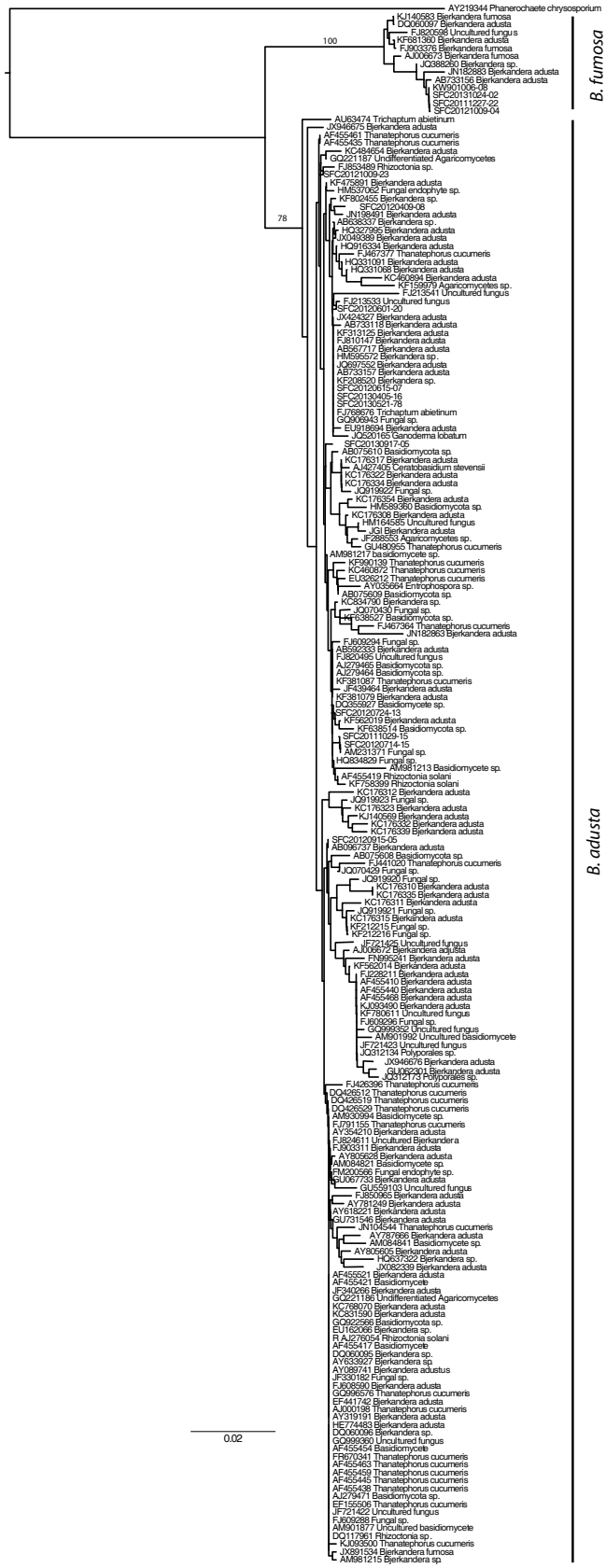


Figure 7. Neighbor joining analysis of the ITS sequences labeled as "*Bjerkandera*", along with highly similar sequences in GenBank identified using BLAST. Numbers at nodes indicate support values from the bootstrap analysis.

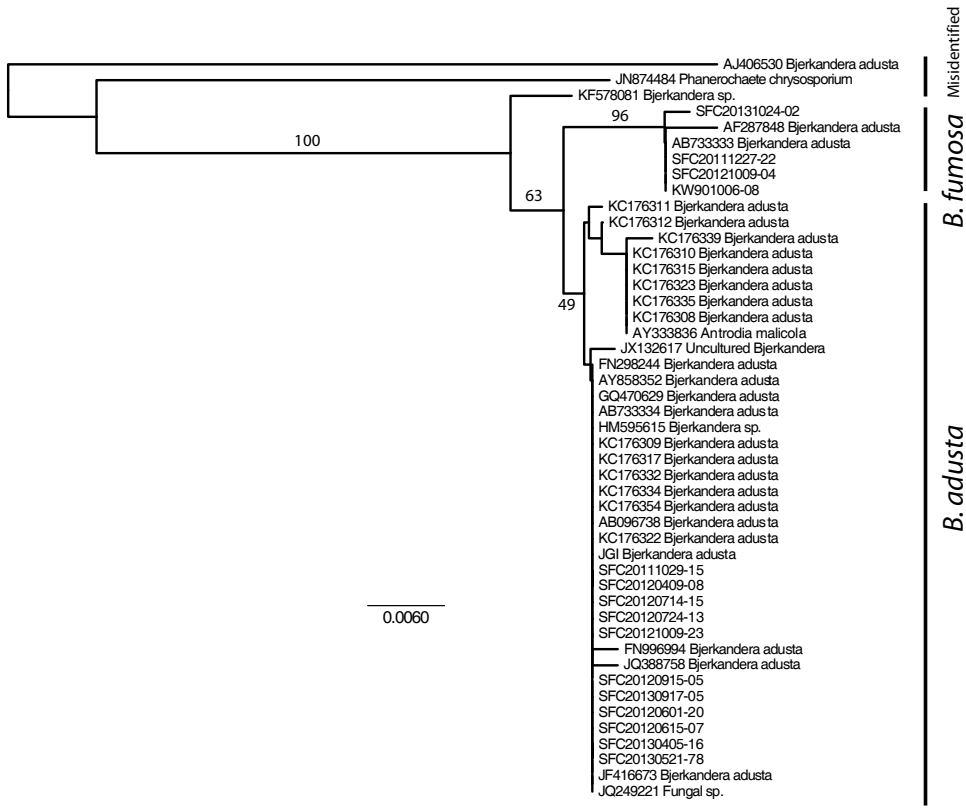


Figure 8. Neighbor joining analysis of the LSU sequences labeled as "*Bjerkandera*", along with highly similar sequences in GenBank identified using BLAST. Numbers at nodes indicate support values from the bootstrap analysis.

2.4 Discussion

The genus *Bjerkandera* can be easily recognized by a blackish to brown tube layer contrasting with a white context (Ryvarden and Gilbertson 1993), while 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 (Núñez and Ryvarden 2001; Ryvarden and Gilbertson 1993; Gilbertson and Ryvarden 1986). In this study, I have rigorously reexamined *Bjerkandera* specimens from Korea and verified the distinguishing morphological characters separating these two species (Fig. 3, Table 2). I 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 (Hebert et al. 2004). However, the efficacy of DNA barcoding depends on public databases having satisfactory taxonomic sampling and sequences that are correctly identified (Nilsson et al. 2006).

I found that the number of misidentified sequences of *Bjerkandera* in GenBank is substantial. More ITS sequences (95 sequences) were present in GenBank compared to LSU (29 sequences), and as such, the problem of misidentification was more evident for ITS sequences. My discussion of misidentification herein focuses on ITS.

The BLAST results revealed that *B. fumosa* was more commonly misidentified as *B. adusta* (n=4) as opposed to the opposite case (n=1). This is likely due to *B. adusta* being more common in the environment compared to *B. fumosa* (Ryvarden and Gilbertson 1993) 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 (Romero et al. 2007). The problem of identification was raised in studies exploring fungal diversity from air, soil, and industrial wastes. Several authors explicitly describe the difficulty distinguishing between *Bjerkandera* and *Thanatephorous* using DNA data due to two highly similar sequences of two

different species uploaded in GenBank (e.g. Romero et al. 2007; Fröhlich-Nowoisky et al. 2009). Other previous studies also raise the problem of identification using environmental DNA data and BLAST for identification (Tringe and Rubin 2005). To clarify the issue, I performed a phylogenetic analysis of my *Bjerkandera* ITS data, adding data from *T. cucumeris*. I found that 1024 sequences of *T. cucumeris* formed a distinct group with high bootstrap support from the 30 sequences re-identified as *B. adusta* (Fig. 9). 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* were found to be misidentified as different wood decay fungi genera (*Antrodia*, *Ganoderma*, *Trichaptum*). While 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).

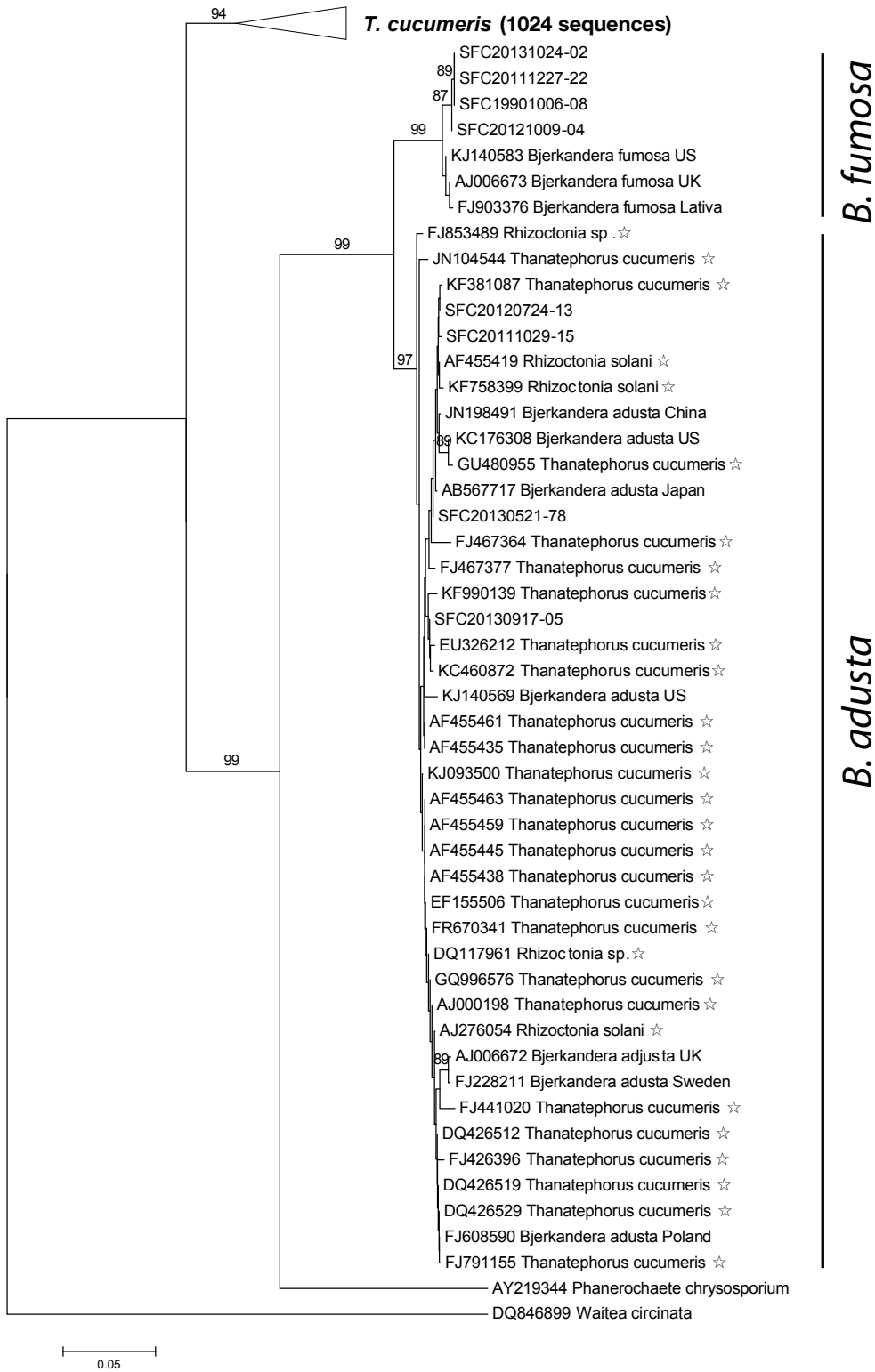


Figure 9. Neighbor joining (NJ) tree identifying *B. adusta* sequences in GenBank incorrectly labeled as *Thanatephorous cucumeris* or anamorphic name *Rhizoctonia solani*. Select ITS sequences of *T. cucumeris* and *Bjerkandera* were compared. Bootstrap values higher than 85 are displayed. *B. adusta* incorrectly labeled as *T. cucumeris* or *R. solani* in GenBank is denoted with star.

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. I 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.

III. Case Study of *Gloeoporus*

Worldwide samples of *Gloeoporus dichrous* reveals biogeographic diversification

3.1 Introduction

Biogeographic studies of fungi have revealed that our current understanding of fungal diversity is significantly depressed (Cai et al. 2014; Skaven Seierstad et al. 2013; Hibbett 2001). Traditionally, taxonomy of basidiomycetous fungi has been dominantly driven by morphological distinctions of fruit bodies. Fungi causing wood decay were also conventionally classified according to the fruit body shape, wood rotting type, and microscopic hyphal structures (Miettinen et al. 2012). Such classifications are, however, now largely considered as artificial as they are inconsistent and conflicting with phylogenetic analysis based on molecular data (Binder et al. 2013; Miettinen et al. 2012). Multi-locus phylogenetic studies of the Polyporales, a morphological group which includes numerous wood decayers, reveal that taxonomy of this particular group is unresolved as numerous genera are shown as polyphyletic (Binder et al. 2013).

Scarcity of studies involving broad sampling adds a layer of confusion in the pursuit of discovering true and natural classification of fungi. While European and North American fungi are relatively well-studied, majority of the counterparts in Africa, Asia and South America are estimated as yet to be accounted (Mueller et al. 2007). Imbalance in available literatures has resulted in partial understanding of fungal diversity outside continental Europe and North America. Recent studies involving broader samplings of wood decay fungi suggest genetic diversification of a morphospeices according to geographic regions (Skaven Seierstad et al. 2013; Vasaitis

et al. 2009).

In this study, the biographical diversity of the wood decay fungi, *Gloeoporus dichrous* was examined by inspecting extensive collection of *G. dichrous*. The species is known to distribute throughout the world as reported in fungal floral studies of various locations around the world, making a suitable candidate for examining the biographical diversification of fungal species. *Gloeoporus taxicola*, morphologically identified as congeneric to *G. dichrous*, has been also previously studied for its biogeographic pattern and host affinity.

Morphologically, the genus *Gloeoporus* is distinct as the gelatinous and elastic tube layer is easily separated from the rest of the fruit body and hymenium is continuously developed over pore mouth (Gilbertson and Ryvarden 1986; Niemelä 1985); however, the taxonomic position of genus *Gloeoporus* is still unsettled. *G. dichrous* and *G. taxicola* are two well-known species of the genus, yet they are not monophyletic according to the molecular phylogenetic analysis (Binder et al. 2013; Jia et al. 2014).

This study aims to analyze the biogeographic pattern of *G. dichrous* and to further contribute to our understanding on the diversity of wood decay fungi. In order to achieve these objectives, worldwide specimens of *G. dichrous* were collected and sequenced for multi-locus analysis. These specimens were analyzed in four different genes and statistically validated for the biogeographic diversity.

3.2 Materials and methods

3.2.1 Specimens

For this study, samples of *G. dichrous* were obtained from various laboratories and herbaria around the world. Each sample was sequenced and compared with reference sequences in GenBank. Any misidentified sample (i.e. not *G. dichrous*) was disregarded from the further analysis. Overall, 61 dried specimens and 8 live cultures were used for this study (Table 3). Alongside, *G. pannocinctus* (FP-135015 from New York, USA) and *G. thelephoroides* (BZ-2896 from Cayo, Belize) were sequenced as outgroups. *G. pannocinctus* is closely related to *G. dichrous* according to the recent studies based on molecular data (Binder et al. 2013) while *G. thelephoroides* is the type species of the genus *Gloeoporus*.

Table 3. Specimens of *Gloeoporus* used for this study

Collection	Country	Code	Locality	Year	ITS	LSU	*tef*	*rpb2*
East Asia								
SFC20111001-71	Korea	KOR	Gangwon-do	2011	○	○	○	○
DY030612-05			Jeollabuk-do	2003	○	○		○
GJ050831-98			Geoje Island	2005	○	○		
NS061014-03			Gangwon-do	2006	○	○	○	○
Yuan 2408	China	CHN	Qinshui, Shanxi	2006	○	○		○
Cui 1320			Huangshan, Anhui	2004	○	○	○	○
Dai 5292			Nanjing, Jiangsu	2003	○	○	○	○
Dai 6932			Shengyang, Liaoning	2005	○	○		○
Dai 10471			Fenyi, Jiangxi	2008	○	○	○	○
Cui 7261			Xiuwu, Henan	2009	○	○	○	○
Dai 11466			Beijing	2009	○	○	○	○
Cui 8853			Shixing, Guangdong	2010	○	○	○	○
Cui 9985			Antu, Jilin	2011	○	○	○	○
Kout 6			Sichuan	2005	○	○	○	○
F25510			Beijing	2009	○	○	○	○
F17257		BER*	Heilongjiang	2004	○	○	○	○
F19830		BER*	Inner Mongolia	2005	○	○	○	○
F10240	Taiwan	TPE	Nantou	1999	○	○	○	
F20513			Nantou	2006	○	○	○	○
F20963			Nantou	2007	○	○	○	○

Collection	Country	Code	Locality	Year	ITS	LSU	*tef*	*rpb2*
F-28839	Japan	JPN	Bonin Islands	2013	○	○	○	○
Europe								
MT ALB	Albania	ALB	Vlorë	2009	○	○	○	○
MT7/11	Czech Republic	CZE	Břeclav	2011	○	○	○	○
BRNU 631507			Tábor	2005	○	○	○	○
BRNU 631521			Brno	2007	○	○	○	○
BRNM 648733			Břeclav	1998	○	○	○	○
Kout 1			South Bohemia	2005	○	○	○	○
Kout 2			South Bohemia	2007	○	○	○	○
Kout 4			Klatovy	2013	○	○	○	○
Kout 5			Nymburk	2014	○	○	○	○
BRNM 705020	Hungary	HUN	Szabolcs-Szatmár-Bereg	2006	○	○	○	○
Kout 3	Russia	RUS	Karelia	2007	○	○	○	○
BRNM 709971	Slovakia	SVK	Pezinok	2004	○	○	○	○
295520	Norway	NOR	Storfjord, Troms	1992	○	○	○	○
65268			Nes, Buskerud	1998	○	○	○	○
64251			Sogndal, Sogn Og Fjordane	2000	○	○	○	○
220192			Tvedestrand, Aust-Agder	2001	○	○	○	○
230773			Trondheim, Sør-Trøndelag	2001	○	○	○	○
68241			Oppegård, Akershus	2004	○	○	○	○
286068			Eidsvoll, Akershus	2005	○	○	○	○
291654			Målselv, Troms	2006	○	○	○	○

Collection	Country	Code	Locality	Year	ITS	LSU	*tef*	*rpb2*
284607	Norway	NOR	Rygge, Østfold	2007	○	○	○	○
286284			Kongsvinger, Hedmark	2007	○	○	○	○
69689			Alta, Finnmark	2008	○	○	○	○
69367			Nesodden, Akershus	2008	○	○	○	○
America								
N.11901	Argentina	ARG	Neuquén	1999	○	○	○	○
18.Mar.02	Chile	CHI	Chaitén	2002	○	○	○	
CBS 446.50[c]	Canada	CAN	British Columbia	1949	○	○	○	○
HHB-15239	United States	BER*	Alaska	1994	○	○	○	○
GAL-3333		BER*	Alaska	1986	○	○	○	○
HHB-15056		BER*	Alaska	1994	○	○	○	
FP-102050[c]		BER*	Alaska	1986	○	○	○	○
HHB-18747		USA	Illinois	2000	○	○	○	○
DL96-261			Michigan	1996	○	○	○	○
DL96-262			Michigan	1996	○	○	○	○
DL96-574			Michigan	1996	○	○	○	○
FP-151129			Michigan	2007	○	○	○	○
HHB-17181			Virginia	1997	○	○	○	○
DLC97-166			Wisconsin	1997	○	○	○	○
FP-102250-Sp[c]			Wisconsin	1987	○	○	○	○
FP-102318-Sp[c]			Wisconsin	1988	○	○	○	○
FP-105267-Sp[c]			Maryland	1958	○	○	○	○

Collection	Country	Code	Locality	Year	ITS	LSU	*tef*	*rpb2*
FP-106899-Sp [c]	United States	USA	Mississippi	1955	○	○	○	○
FP-134973-Sp [c]			New York	1983	○	○	○	○
Oceania								
916456	New Zealand	NZL	Southland	1997	○	○		○
PDD68418			Three Kings Islands	1997	○	○	○	
ICMP 16418 [c]			Stewart Island	1985	○	○	○	○
East Africa								
918063	Uganda	UGA	Rukungiri district	2002	○	○	○	○
918572			Kabale district	2003	○	○	○	○

[c] denotes a live culture

* BER denotes a group of specimens from northeastern China and Alaska, USA.

3.2.2 Molecular analyses

DNA extraction, amplification, and sequencing

A small piece of fungal tissue from each dried specimen was placed in a 1.5 mL tube containing 2X CTAB buffer. For live cultures, mycelial surfaces were scraped off potato dextrose agar plates and placed in a 1.5 ml tube as described above. Both types of samples were ground with a plastic pestle. Genomic DNA was extracted with the modified CTAB extraction protocol (Rogers and Bendich 1994). Four regions were amplified for the multi-locus analysis: internal transcribed spacer (ITS) region, nuclear large subunit ribosomal DNA (LSU), translation elongation factor 1-α (*tef*), and the second-largest subunit of RNA polymerase II (*rpb2*). The ITS region was amplified using the primers ITS1F and ITS4-B (Gardes and Bruns 1993), LSU rDNA region was amplified using the primers LR0R and LR5 (White et al. 1990; Vilgalys and Hester 1990). Genes of *tef* and *rpb2* were amplified using primers EF595F/EF1160R (Kauserud and Schumacher 2001) and RPB2-6F1/bRPB2-7.1R (Matheny 2005) respectively. The PCR amplification was performed in a C1000™ thermal cycler (Bio-Rad, USA) using the AccuPower® PCR premix (Bioneer Co., Seoul, Korea) in a final volume of 20 μL containing 10 pmol of each primer and 1 μL of genomic DNA. Thermocycler conditions for PCR of ITS, LSU, and *tef* followed Park et al. (2013). The condition for amplification of *rpb2* is detailed at http://www.clarku.edu/faculty/dhibbett/rpb2 primers.htm. DNA sequencing was performed with an ABI3700 automated DNA sequencer (Applied Biosystems, Foster

City, CA, USA) at Macrogen (Seoul, Korea).

Molecular phylogeny

For all molecular analyses, alignments were performed with MAFFT online version at http://mafft.cbrc.jp/alignment/server/ (Katoh and Standley 2013) and manually adjusted in MEGA5 (Tamura et al. 2011). In the same program, each gene was analyzed by neighbor-joining (NJ) method using p-distances, substitutions including transitions and transversions, pairwise deletion of missing data, and 1,000 bootstrap replicates. Subsequently, Bayesian and maximum likelihood (ML) analyses were implemented for concatenated dataset. For Bayesian analysis, four genes (ITS, LSU, *tef*, and *rpb2*) were concatenated and partitioned for the inferences. The appropriate model for each gene was selected based on Bayesian information criterion (BIC) values computed from jModelTest 2.1.4. The substitution model HKY+G was used for ITS, K80+I for LSU, K80+G for *tef*, and SYM+I+G for *rpb2*. Bayesian analysis was conducted with MrBayes 3.2.2 (Ronquist et al. 2012) using Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm. Two independent runs were simultaneously executed for 10,000,000 generations with four chains for each run, sampling every 100th generation. Log files generated from two separate runs were checked with Tracer 1.6 (Rambaut et al. 2013), confirming that initial setup of 25% burn-in value was sufficient. ML analysis was performed with RaxML 7.2.6 (Stamatakis 2006) with combined rapid bootstrap and search for best-scoring ML

tree analysis, the GTRCAT model of sequence evolution, and 1,000 bootstrap replicates. Likewise, four genes were concatenated and partitioned for analysis. Trees generated from both analyses were checked and modified in FigTree 1.4 (Rambaut and Drummond 2012).

Intraspecific variation was calculated for datasets of each gene marker. Pairwise distances of sequences were calculated in MEGA5 and exported to Microsoft Excel for data analysis.

Genealogical Sorting Index (GSI)

In order to quantify lineage divergence according to geographical distribution, statistic of genealogical sorting index (GSI) was implemented (Cummings et al. 2008). The phylogenetic tree created from concatenated dataset (without sequences of the new species proposed in this study) by Bayesian analysis was analyzed at the GSI website at http://www.genealogicalsorting.org. Groups were divided according to geographical division, except for Africa which was grouped with Asia. Accordingly, five groups were assigned: America, Asia, Alaska & Northeastern China, Europe, and Oceania. The permutation test was repeated for 10,000 replicates.

3.3 Results

3.3.1 Molecular phylogeny

All specimens of this study were sequenced for molecular phylogeny. ITS and LSU genes are fully sequenced for 69 specimens in the study. A total of 64 *tef* and 64 *rpb2* sequences had been obtained. Phylogenetic analysis of each gene revealed unique lineages which may not considered as same species of *G. dichrous* (Fig. 10). These three lineages are genetically unique and considered as two new species. They will be further explained and discussed separately in Chapter 4 along with the phylogeny of the genus *Gloeoporus*.

When observed *G. dichrous* only, numbers of ITS characters slightly vary according to the geographic locations, European specimens have 597, while American and Asian specimens have 598. New Zealand and South American specimens further have an extra character (not at the same location), which sums up to 599 characters. In comparison, new *Gloeoporus* species overall have more ITS characters. Four specimens of *Gloeoporus* sp. 1 have 611 characters, while one specimen, Cui 7261, has 601 characters. Two specimens of *Gloeoporus* sp. 2 have 602 and 603 characters respectively.

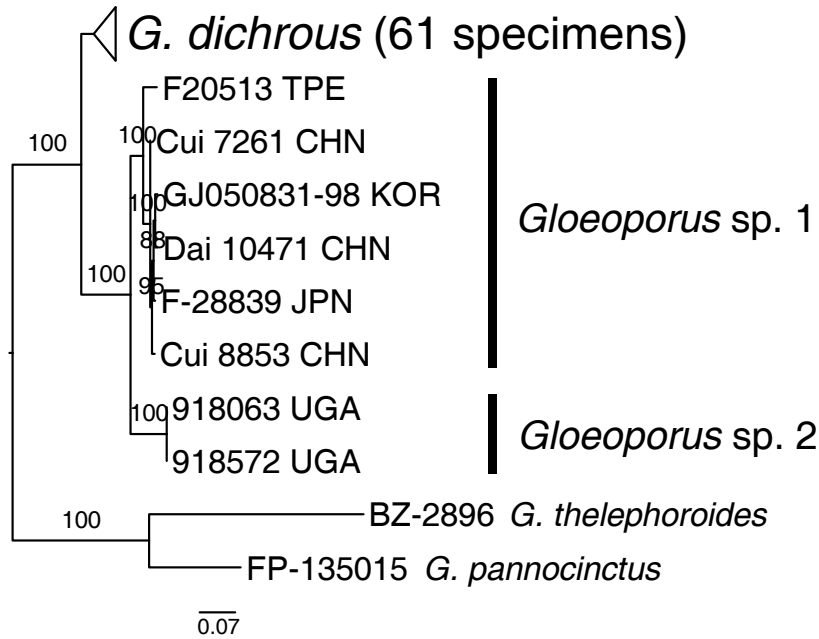


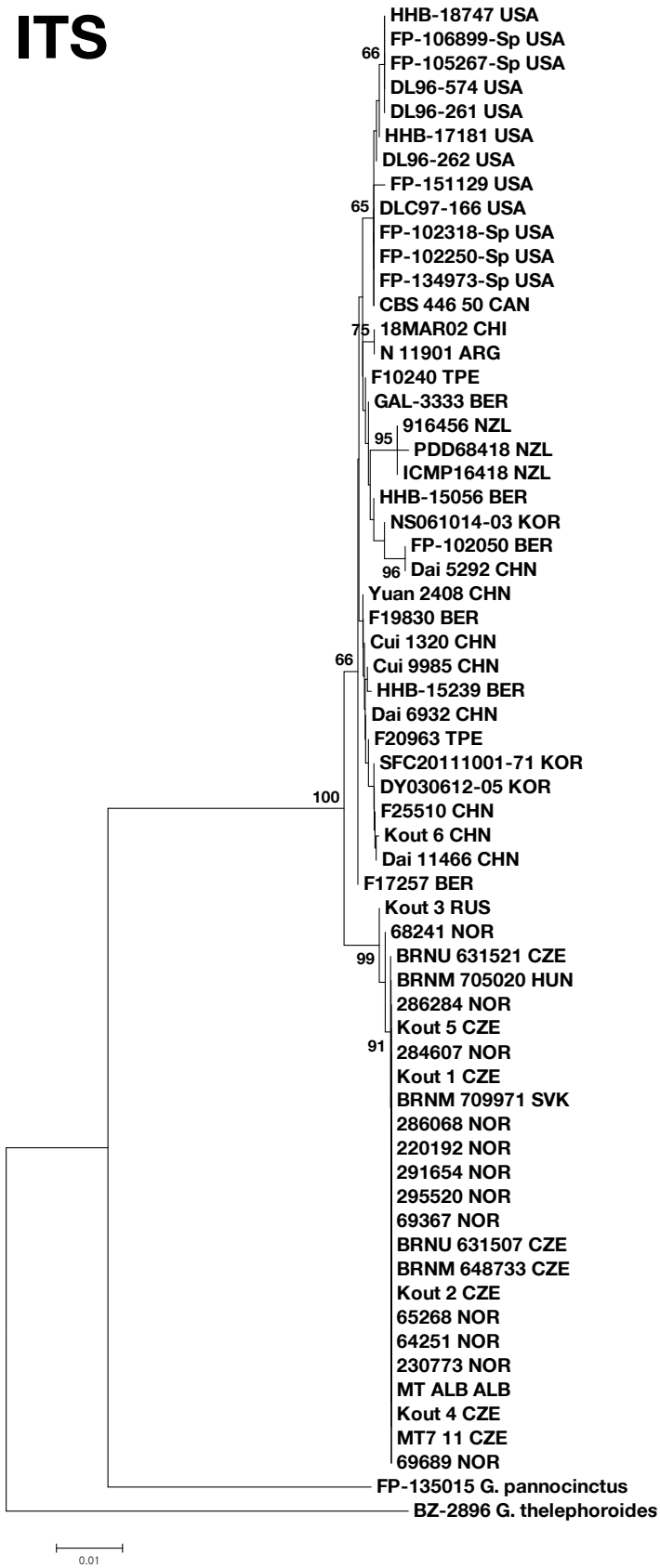
Figure 10. Phylogenetic trees inferred from ML analysis based on the concatenated dataset of ITS, LSU, *tef*, *rpb2.*

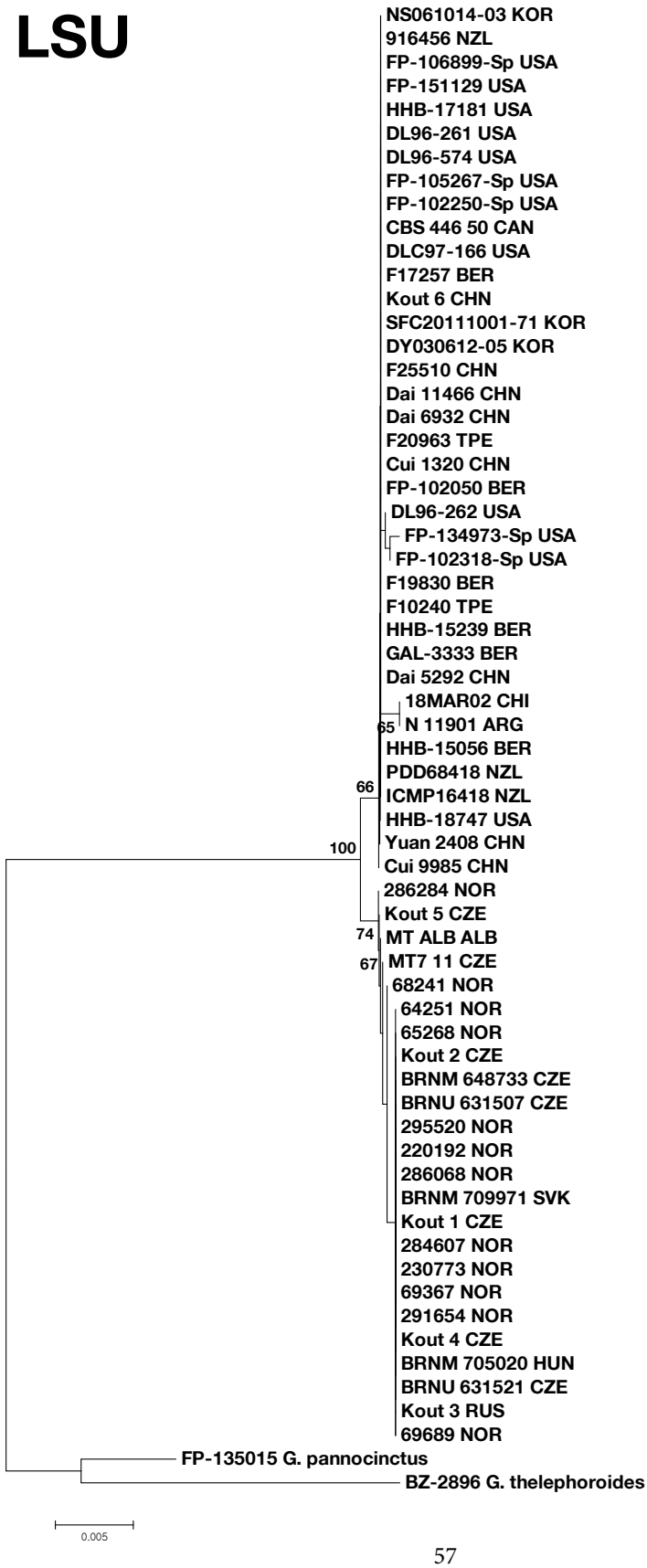
3.3.2 Biogeographic diversification of *G. dichrous*

The phylogenetic trees generated from nuclear genes ITS, LSU, *tef*, and *rpb2* all show clear distinction of European specimens from the rest of the specimens (Fig. 11). The analysis of *tef* gene additionally reveals genetic diversification of European specimens between Northern and Southeastern (including a part of the Central) Europe (Fig. 11). The phylogenetic analysis of LSU gene shows that Asian, American, and New Zealand specimens are altogether grouped in one clade with moderately high bootstrap value. Separation between Asian and American groups are shown in *tef* and *rpb2* phylogenetic trees with fairly robust bootstrap support, while their relationship is not clearly revealed. Interestingly, the *rpb2* analysis shows that Alaskan samples (HHB-15239, FP-102050, GAL-3333) intermingle with Asian samples of *G. dichrous*, instead of American counterparts. The *tef* analysis further illustrates that Northeastern Chinese and Inner Mongolian samples are grouped with specimens from Alaska and British Columbia, Canada and rather have higher affinity with American specimens, a phylogenetic pattern conflicting with the *rpb2* analysis. A sample from British Columbia, northern Canadian state approximated to Alaska, is especially interesting as this specimen is closely related with American samples in all analyses, except for the *tef* analysis which instead shows grouping of specimen with Alaskan and Northeastern Chinese counterparts. From analyses of ITS, *tef*, *rpb2* genes, specimens from New Zealand are clearly distinguished from the rest of the specimens.

The concatenated analyses likewise show clear distinction of European specimen from the rest of the specimens with robust bootstrap support (Fig. 12). Partition of American group from Asian group is also strongly supported by bootstrap. From the concatenated dataset, Alaskan specimens are clearly separated from the rest of the American specimens. Alaskan specimens admix with Asian specimens and evident grouping of Asian specimens is not observed. Specimens of New Zealand, however, are clearly separated from the rest of the specimens with high bootstrap value.

Despite unclear relationships between biogeographic groups, GSI values support the exclusivity of each group. American, Asian, European, and Oceanian groups all have GSI = 1.00, which denotes that the group is monophyletic, with p value <0.0002. The group of Alaskan samples with two Northeastern Chinese samples has GSI = 0.819 with p value of 0.0001. Refer to Fig. 13 for specimen locations and their biogeographic groupings.


ITS
HHB-18747 USA
FP-106899-Sp USA
66
FP-105267-Sp USA
DL96-574 USA
DL96-261 USA
HHB-17181 USA
DL96-262 USA
FP-151129 USA
65
DLC97-166 USA
FP-102318-Sp USA
FP-102250-Sp USA
FP-134973-Sp USA
CBS 446 50 CAN
75
18MAR02 CHI
N 11901 ARG
F10240 TPE
GAL-3333 BER
916456 NZL
95
PDD68418 NZL
ICMP16418 NZL
HHB-15056 BER
NS061014-03 KOR
FP-102050 BER
96
Dai 5292 CHN
Yuan 2408 CHN
F19830 BER
Cui 1320 CHN
66
Cui 9985 CHN
HHB-15239 BER
Dai 6932 CHN
F20963 TPE
SFC20111001-71 KOR
DY030612-05 KOR
100
F25510 CHN
Kout 6 CHN
Dai 11466 CHN
F17257 BER
Kout 3 RUS
68241 NOR
99
BRNU 631521 CZE
BRNM 705020 HUN
286284 NOR
Kout 5 CZE
91
284607 NOR
Kout 1 CZE
BRNM 709971 SVK
286068 NOR
220192 NOR
291654 NOR
295520 NOR
69367 NOR
BRNU 631507 CZE
BRNM 648733 CZE
Kout 2 CZE
65268 NOR
64251 NOR
230773 NOR
MT ALB ALB
Kout 4 CZE
MT7 11 CZE
69689 NOR
FP-135015 G. pannocinctus
BZ-2896 G. thelephoroides
0.01


LSU
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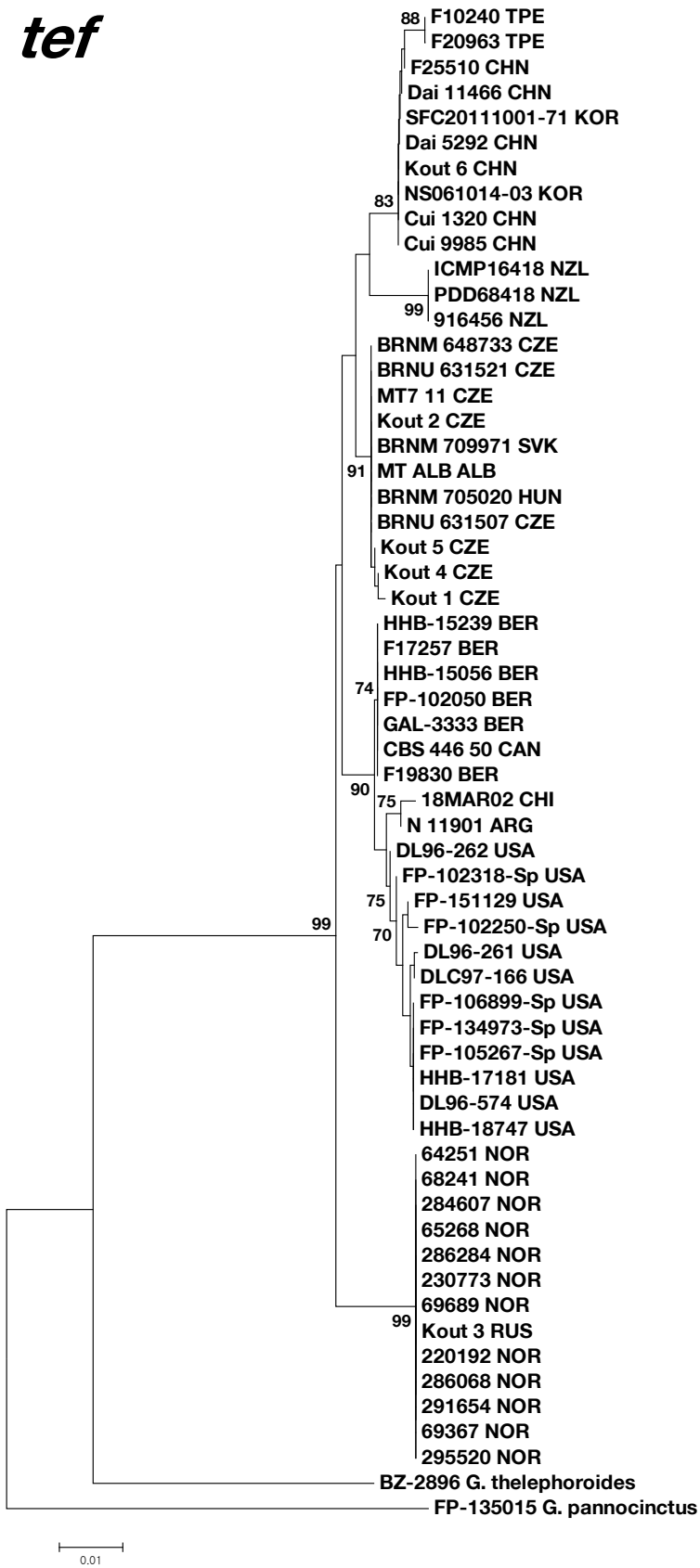
916456 NZL
FP-106899-Sp USA
FP-151129 USA
HHB-17181 USA
DL96-261 USA
DL96-574 USA
FP-105267-Sp USA
FP-102250-Sp USA
CBS 446 50 CAN
DLC97-166 USA
F17257 BER
Kout 6 CHN
SFC20111001-71 KOR
DY030612-05 KOR
F25510 CHN
Dai 11466 CHN
Dai 6932 CHN
F20963 TPE
Cui 1320 CHN
FP-102050 BER
DL96-262 USA
FP-134973-Sp USA
FP-102318-Sp USA
F19830 BER
F10240 TPE
HHB-15239 BER
GAL-3333 BER
Dai 5292 CHN
18MAR02 CHI
65
N 11901 ARG
HHB-15056 BER
PDD68418 NZL
66
ICMP16418 NZL
HHB-18747 USA
100
Yuan 2408 CHN
Cui 9985 CHN
286284 NOR
Kout 5 CZE
74
MT ALB ALB
67
MT7 11 CZE
68241 NOR
64251 NOR
65268 NOR
Kout 2 CZE
BRNM 648733 CZE
BRNU 631507 CZE
295520 NOR
220192 NOR
286068 NOR
BRNM 709971 SVK
Kout 1 CZE
284607 NOR
230773 NOR
69367 NOR
291654 NOR
Kout 4 CZE
BRNM 705020 HUN
BRNU 631521 CZE
Kout 3 RUS
69689 NOR
FP-135015 G. pannocinctus
BZ-2896 G. thelephoroides
0.005


tef
88
F10240 TPE
F20963 TPE
F25510 CHN
Dai 11466 CHN
SFC20111001-71 KOR
Dai 5292 CHN
Kout 6 CHN
NS061014-03 KOR
83
Cui 1320 CHN
Cui 9985 CHN
ICMP16418 NZL
PDD68418 NZL
99
916456 NZL
BRNM 648733 CZE
BRNU 631521 CZE
MT7 11 CZE
Kout 2 CZE
BRNM 709971 SVK
91
MT ALB ALB
BRNM 705020 HUN
BRNU 631507 CZE
Kout 5 CZE
Kout 4 CZE
Kout 1 CZE
HHB-15239 BER
F17257 BER
HHB-15056 BER
74
FP-102050 BER
GAL-3333 BER
CBS 446 50 CAN
F19830 BER
90
75
18MAR02 CHI
N 11901 ARG
DL96-262 USA
FP-102318-Sp USA
75
FP-151129 USA
99
70
FP-102250-Sp USA
DL96-261 USA
DLC97-166 USA
FP-106899-Sp USA
FP-134973-Sp USA
FP-105267-Sp USA
HHB-17181 USA
DL96-574 USA
HHB-18747 USA
64251 NOR
68241 NOR
284607 NOR
65268 NOR
286284 NOR
230773 NOR
69689 NOR
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Kout 3 RUS
220192 NOR
286068 NOR
291654 NOR
69367 NOR
295520 NOR
BZ-2896 G. thelephoroides
FP-135015 G. pannocinctus
0.01

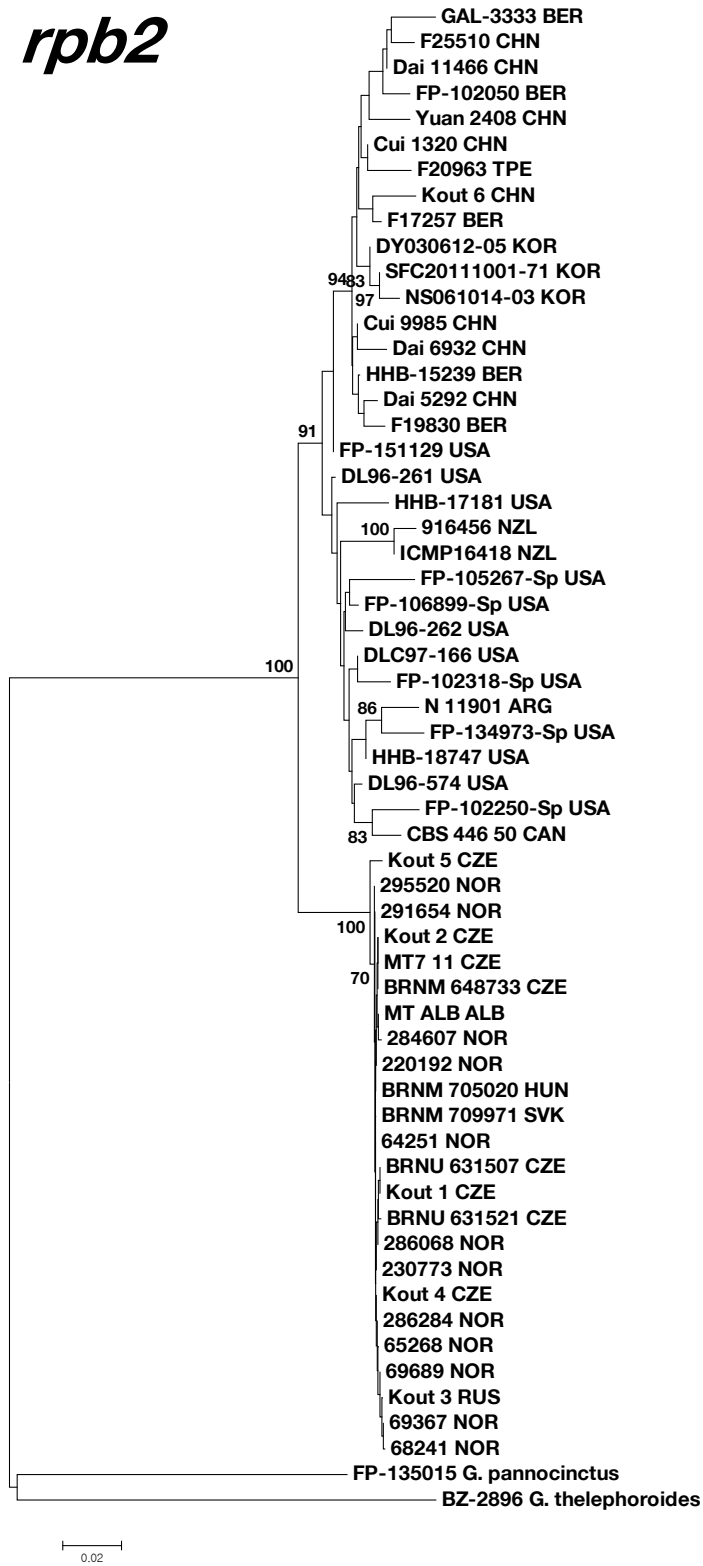

Figure 11. Phylogenetic trees inferred from neighbor joining (NJ) analysis of four datasets, ITS, LSU, *tef* and *rpb2*. Bootstrap scores ≥60 are shown.

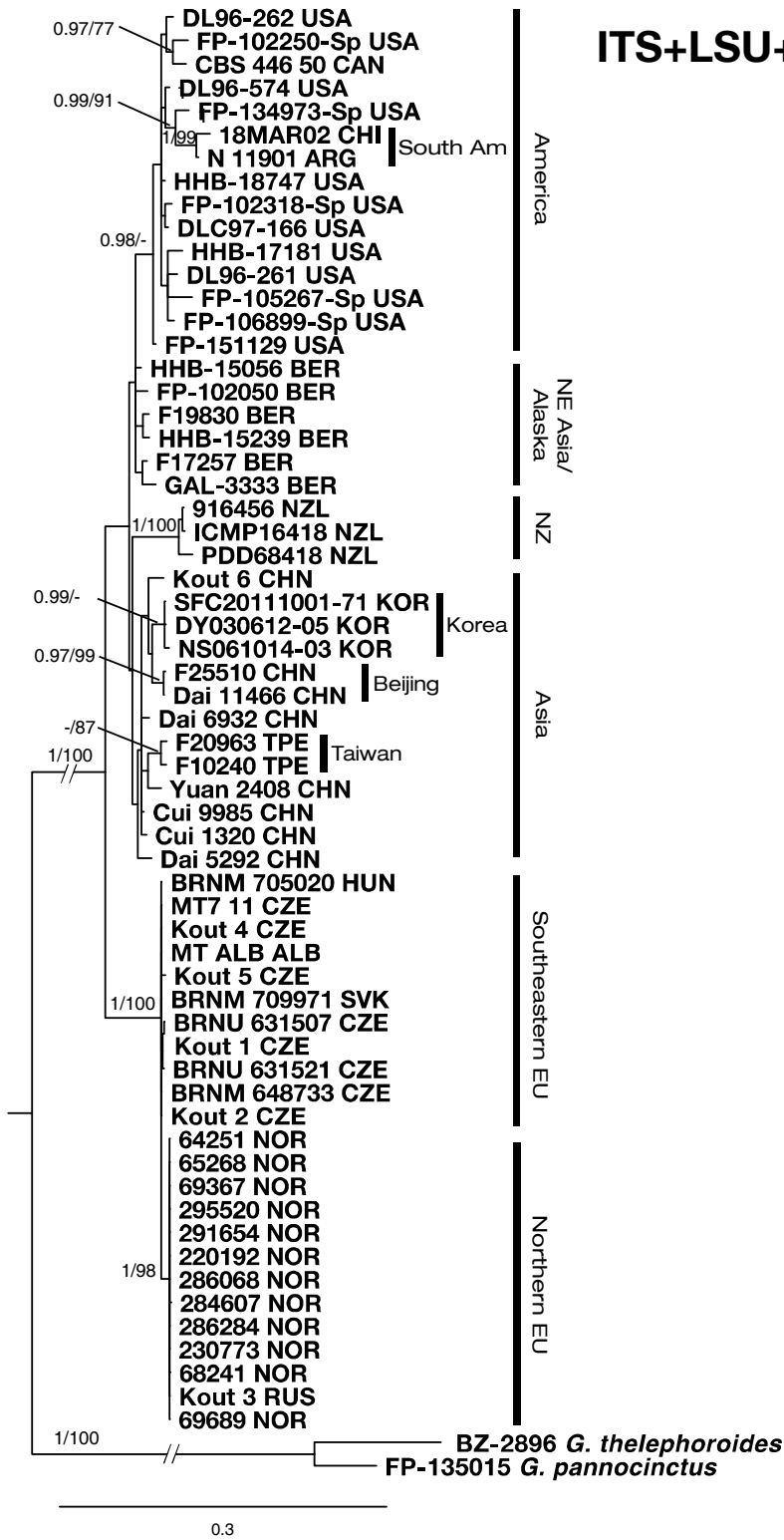

Figure 12. Phylogenetic tree inferred from Bayesian analysis based on the concatenated dataset of ITS, LSU, *tef*, *rpb2* Bayesian posterior probabilities ≥ 0.96 and maximum likelihood bootstrap ≥ 75% are presented on the branches (NZ = New Zealand, EU = Europe).

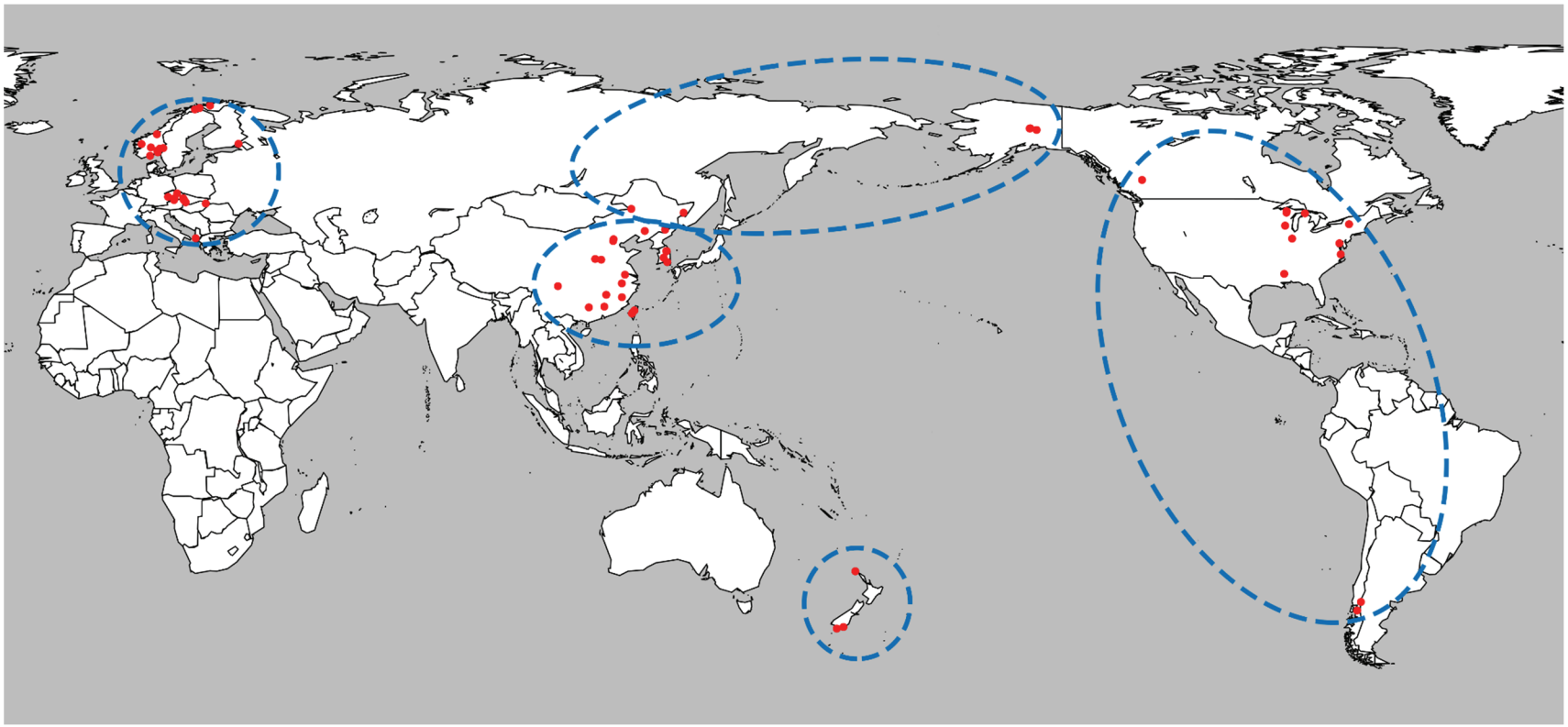

Figure 13. World map with *G. dichrous* specimen locations and geographic groupings.

3.3.3 Intraspecific variation of *G. dichrous*

Throughout all datasets of multi-locus genes used in this study, Korean specimens exhibited low intraspecific variation. The variation was 0.17% for ITS gene, 0.29% for *rpb2*. For LSU and *tef* gene, there was no variation. When all specimens were included for calculation, intraspecific ITS variation was 1.68%. LSU dataset had lower value, 0.48% while *tef* dataset had 2.99%. Among all datasets, *rpb2* showed the highest intraspecific variation, 7.98%. Fig. 14 summarizes intraspecific variation of each gene presented in this study.

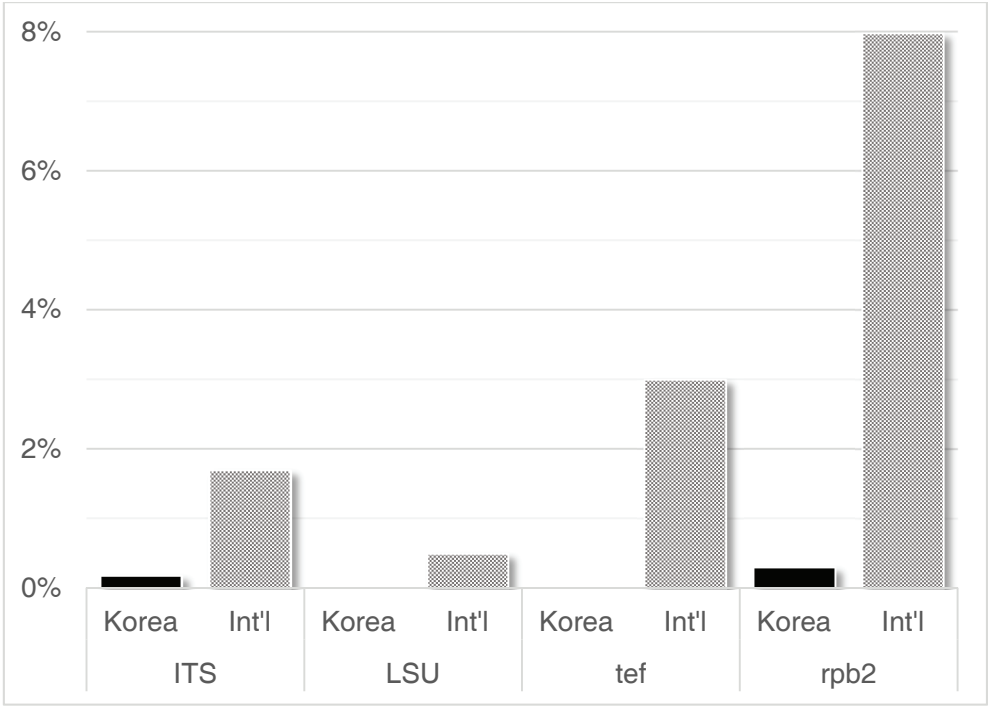

Figure 14. Intraspecific variation of *G. dichrous*. From the datasets of four gene markers, intraspecific variations of Korean specimens and global specimens were calculated.

3.4 Discussion

While fungal species have long been perceived as free of dispersal barrier, recent molecular analyses of various fungal types starkly contrasted with such long-standing belief. Numerous studies have demonstrated the presence of cryptic lineages and biogeographic diversity which conflicts with previous species and genus delimitation (Skrede et al. 2011; Carlsen et al. 2011). In this study, phylogenetic analyses of four loci and GSI were implemented to examine biogeographic diversification of *G. dichrous*. Topology of phylogenetic trees and intraspecific variation values reveal that each geographic clade may not be considered as a separate species; nonetheless, specimens of *G. dichrous* are separated into distinctive groups according to biogeographic division.

While their relationships are not clearly revealed to infer geographical origin of the species and its entire dispersal route, analyses of several datasets coincide with the presence of three major groups: Asia with Alaskan specimens, America (expect Alaska), and Europe. The phylogenetic analysis of LSU (Fig. 11) dataset reveals that Asia and America have almost identical sequence while datasets of fast-evolving protein-coding genes *rpb2* and *tef* illustrate that *G. dichrous* specimens of two continents vary according to geographic location. Diversification of Asian and American specimens is possibly explained by Beringian dispersal. While speciation of numerous organisms were traditionally explained by cross-Beringian connection (Hopkins 1967), similar trends have been observed in several fungal studies which

involve large samplings collected worldwide (Skrede et al. 2011; Sánchez-Ramírez et al. 2015).

In the analysis of the concatenated dataset, Asian specimens of *G. dichrous* and Alaskan specimens admix despite of clear division of American group from Asian group. Independent analysis of *tef* gene, however, shows that Alaskan specimens along with Northeastern Chinese samples are grouped separately from the Asian. Alaskan group clearly diverge from rest of the American samples, suggesting the group may have been separated by vicariance. Conceivably, the population of *G. dichrous* dispersed to the American Continent (or vice versa to the Asian Continent) through Beringia, a land bridge which once connected Asia and America. By the end of the Pliocene (about 2.4 Myr ago), arctic ice cap was established as the global temperature plummeted (Hewitt 2000) and eventually separated Alaska from the rest of the American continent. Blockage by glaciation may have affected the dispersal route and resulted in current genetic diversification.

Such biogeographic pattern does not completely match with the equivalent analysis of *Gloeoporus taxicola* (Skaven Seierstad et al. 2013). Even though they are currently in the same genus, recent researches suggest that *G. taxicola* and *G. dichrous* are not as closely related (Jia et al. 2014; Binder et al. 2013) as previously considered by morphological taxonomists. *G. dichrous* of this study displays geographically unique and exclusive genetic variations while *G. taxicola* has two main lineages which is not specific to geographical locations. One of the lineages is a widespread group

which distributes throughout Eurasia to North America and other group which are only found in parts of the European continent and associate almost exclusively with *Pinus sylvestris* as a substrate. Wood rotting fungi *Serpula himantioides* exhibits similar pattern as *G. taxicola* as one of the lineages has wider distribution when other lineages have stricter host preferences (Carlsen et al. 2011).

Conversely, numerous other species- and genus-level studies show geographically unique clades without a prevalent or dominant type (Moncalvo and Buchanan 2008; Cai et al. 2014; Sánchez-Ramírez et al. 2015; Skrede et al. 2011). Yet, these researches all differ in explanation of spreading mechanism of fungi, either by vicariance, long distance dispersal, or a combination of both mechanisms. A wide spectrum of biogeographic patterns of fungi suggests that fungal diversification is a very unique and complex process for each species. Thus previously long-held belief of barrier-free proliferation of fungi – so they must be everywhere – must be reconsidered.

G. dichrous is rather well-known species of wood rotting fungi due to its unique morphology and worldwide distribution. This study, however, verifies that even within the distinctive morphological species, biogeographic diversity does exist. Along with other fungal taxonomic studies which involves a large of number of specimens, results of the study calls for a scrutiny of familiar species identified based on its distinctive morphology. Wood rotting fungi have been extensively studied for the possibility of industrial application and understanding their genetic diversity is crucial for discovery of novel substances and exceptional strains for practical applications.

IV. Case Study of *Gloeoporus*

Phylogeny and taxonomy of the genus *Gloeoporus* (Polyporales, Basidiomycota)

4.1 Introduction

Gloeoporus Mont. is morphologically defined group with easily separated gelatinous hymenophore and a continuous hymenium over the pore mouth (Ryvarden and Johansen 1980). The genus was first established in 1842 by Montagne to describe a subtropical species *G. conchoides* (syn. *G. thelephoroides*). Species of *Gloeoporus* have pore surfaces of pinkish white, cream, or orange to deep reddish color with small pores (Gilbertson and Ryvarden 1986). Fresh fruiting bodies have gelatinous and elastic hymenophore, a distinguishing feature of *Gloeoporus*, which becomes resinous and cartilaginous when dry.

Currently, *Gloeoporus* includes about 12 accepted species based on morphological characters (Coelho et al. 2006), including a recent addition of *G. guerreroanus* which grows on bamboo trees. Among these species, two different hyphal systems are observed, either simple septate or clamped hyphae (Ryvarden 1991). For example, *G. dichrous* and *G. pannocinctus* have generative hyphae with clamps while *G. taxicola*, and *G. thelephoroides*, and *G. guerreroanus* have simple-septate generative hyphae (Gilbertson and Ryvarden 1986; Coelho et al. 2006; Niemelä 1985). Inconsistent hyphal system has long been perceived as a possible phenomenon in the Corticiaceae s.l. (Ryvarden 1991).

Recent molecular taxonomic and phylogenetic researches, however, suggest that previous delimitation of *Gloeoporus* needs considerable revision. Numerous studies on the Polyporales show that grouping of *G. dichrous* and *G. pannocinctus* is well

supported while *G. taxicola* has affinity with different genera (Binder et al. 2013; Jia et al. 2014). Due to lack of comprehensive phylogenetic studies of *Gloeoporus*, artificial grouping of the genus still remains and exacerbates confusion in determining taxonomic position of the genus. As a result, species in *Gloeoporus* still undergo constant reposition. Currently, Index Fungorum (http://www.indexfungorum.org) lists *G. dichrous* as *Gelatoporia dichroa* (Fr.) Ginns, unlike MycoBank (http://www.mycobank.org) where the species is registered under *Gloeoporus*. Such disagreement calls for a need to define taxonomic position of *Gloeoporus*.

In this study, I perform multi-locus phylogeny of *Gloeoporus* to define taxonomic position and relation of the genus. Worldwide specimens of *Gloeoporus* were collected and molecularly analyzed. In the course of analysis, two undescribed species of *Gloeoporus* were identified and presented as new species with morphological descriptions.

4.2 Materials and methods

4.2.1 Specimens

11 dried specimens of *Gloeoporus*, including *G. dichrous* and two new species were selected from the Table 3 of the previous chapter. DNA sequences of related genera were obtained from Floudas and Hibbett (2015). Microscopic features of the specimens were observed with Eclipse 80i light microscope (Nikon, Japan). Slides were prepared in Melzer's reagent for measurement. For the description of spore sizes, at least 30 spores were measured.

4.2.2 Molecular analyses

DNA extraction, amplification, and sequencing

A small piece of fungal tissue from each dried specimen was placed in a 1.5 mL tube containing 2X CTAB buffer and ground with a plastic pestle. Genomic DNA was extracted with the modified CTAB extraction protocol (Rogers and Bendich 1994). Four regions were amplified for the multi-locus analysis: internal transcribed spacer (ITS) region, nuclear large subunit ribosomal DNA (LSU), and the second-largest subunit of RNA polymerase II (*rpb2*). The ITS region was amplified using the primers ITS1F and ITS4-B (Gardes and Bruns 1993), LSU rDNA region was amplified using the primers LR0R and LR5 (White et al. 1990; Vilgalys and Hester 1990). The *rpb2* gene was amplified using primers RPB2-6F1/bRPB2-7.1R (Matheny 2005). The PCR amplification was performed in a C1000™ thermal cycler (Bio-Rad, USA) using the AccuPower® PCR premix (Bioneer Co., Seoul, Korea) in a final volume of 20 μL containing 10 pmol of each primer and 1 μL of genomic DNA. Thermocycler conditions for PCR of ITS and LSU followed Park et al. (2013). The condition for amplification of *rpb2* is detailed at http://www.clarku.edu/faculty/dhibbett/rpb2 primers.htm. DNA sequencing was performed with an ABI3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) at Macrogen (Seoul, Korea).

Molecular phylogeny

For all molecular analyses, alignments were performed with MAFFT online version at http://mafft.cbrc.jp/alignment/server/ (Katoh and Standley 2013) and manually adjusted in MEGA5 (Tamura et al. 2011). In the same program, ITS gene was analyzed by neighbor-joining (NJ) method using p-distances, substitutions including transitions and transversions, pairwise deletion of missing data, and 1,000 bootstrap replicates. With concatenated dataset of four genes, ML analysis was performed by RaxML 7.2.6 (Stamatakis 2006) with combined rapid bootstrap and search for best-scoring ML tree analysis, the GTRCAT model of sequence evolution, and 1,000 bootstrap replicates. Trees generated from the analysis was checked and modified in FigTree 1.4 (Rambaut and Drummond 2012).

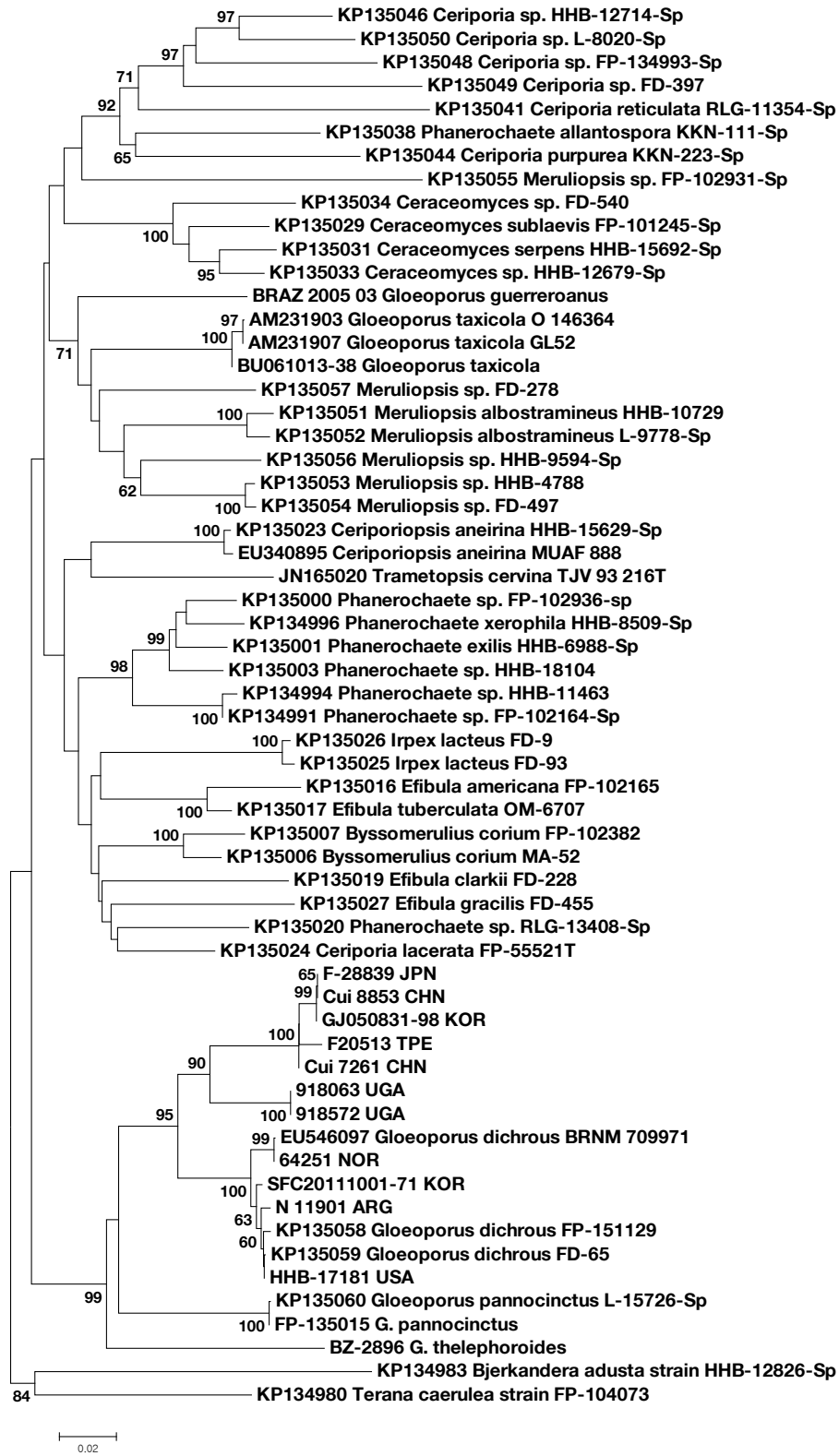

Figure 15. Phylogenetic tree inferred from neighbor joining analysis based on the dataset of ITS. Bootstrap scores ≥60 are shown.

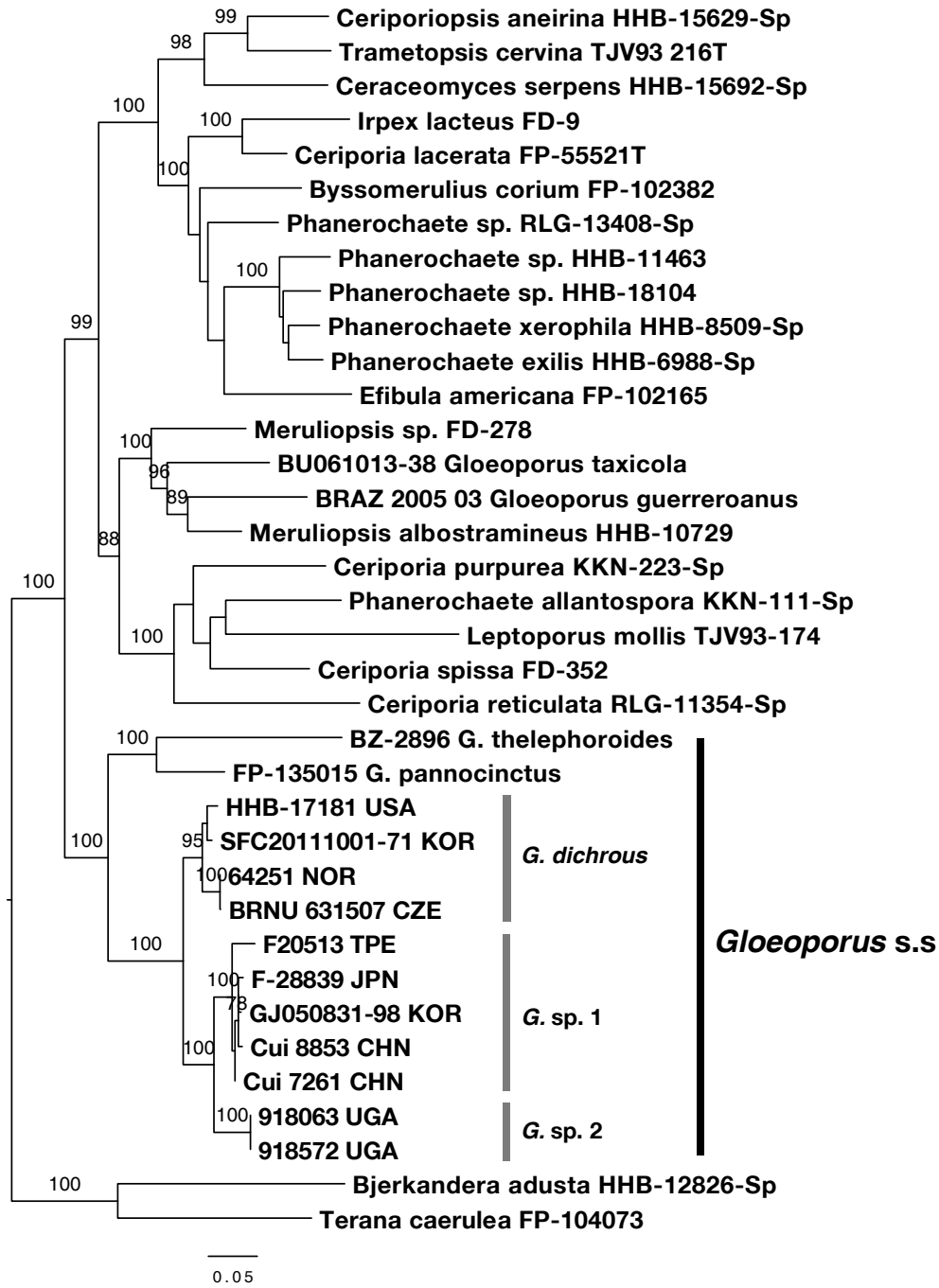

Figure 16. Phylogenetic tree inferred from ML analysis based on the concatenated dataset of ITS, LSU, and *rpb2.* Likelihood bootstrap ≥ 70% are presented on the branches.

4.3 Results

Phylogenetic analysis based on ITS gene (Fig. 15) clearly supports a monophyletic clade including two clamped species, *G. dichrous*, *G. pannocinctus*, and one clampless species, *G. thelephoroides*, with strong bootstrap support. Two species without clamps, *G. taxicola* and *G. guerreroanus*, are not part of this clade, but intermingle with *Meruliopsis*. Similar taxonomic pattern is observed in the analysis implemented with concatenated dataset (Fig. 16).

All analyses furthermore concur with the existence of new species which are closely related to *G. dichrous* but molecularly and morphologically distinct. These two species also exhibit hyphal system with clamps, likewise other two species within the clade. Fig. 17 illustrates the distinctive microscopic features of the new species presented in this study. Noticeably shorter basidia distinguish these species from other *Gloeoporus* species (Table 4). These specimens were originally identified as *G. dichrous* morphologically as their macro-morphology is identical to *G. dichrous*. *Gloeoporus* sp. 1 distributes throughout Four East Asian countries, Korea, China, Taiwan, and Japan, geographically wider than another new species. *Gloeoporus* sp. 2 observed in Uganda, Africa. While some of the new species appear in remote locations, such as Bonin Island of Japan, others share geographic locations with *G. dichrous* (Fig. 18).

The monophyletic clade of *Gloeoporus* overall includes four species with clamped hyphae, *G. dichrous*, *G. pannocinctus*, *Gloeoporus* sp. 1, and *Gloeoporus* sp. 2 and one

species with simple septa, *G. thelephoroides*. Based on the ITS phylogenetic tree, the type species of *Gloeoporus*, *G. thelephoroides*, forms the basal branch. *G. pannocinctus* and *G. dichrous* branch off subsequently. Two new species diverge most recently and form a sister group to *G. dichrous*. Geographic grouping is observed in *G. dichrous* which European specimens are grouped together against a set of Asian and American specimens.

Table 4. Comparison of microscopic features of *Gloeoporus dichrous* and *Gloeoporus* new species

	Specimen	No. pores (mm)	Basidia size (μm)	Spore size (μm)
Gloeoporus sp. 1	F-28839 JPN	7-9	10.1–13.3 × 2.9–3.9	3.2–5.0 × 0.8–1.8
Gloeoporus sp. 2	918063 UGA	7-9	10.4–13.9 × 2.9–3.7	3.0–4.8 × 0.8–1.6
G. dichrous	HHB-17181 USA	4-6	13.1–16.9 × 3.7–4.1	3.6–5.2 × 0.6–1.2
	916456 NZL	4-5	15.6–17.6 × 3.3–4.3	4.0–5.6 × 1.2–1.8
	F20963 TPE	4-6	15.6–16.9 × 3.2–4.0	(3.6–4.8 × 1.0–1.4)
	286284 NOR	4-6	(14.0–18.0 × 3.0–4.0)	(3.5–5.5 × 0.7–1.5)

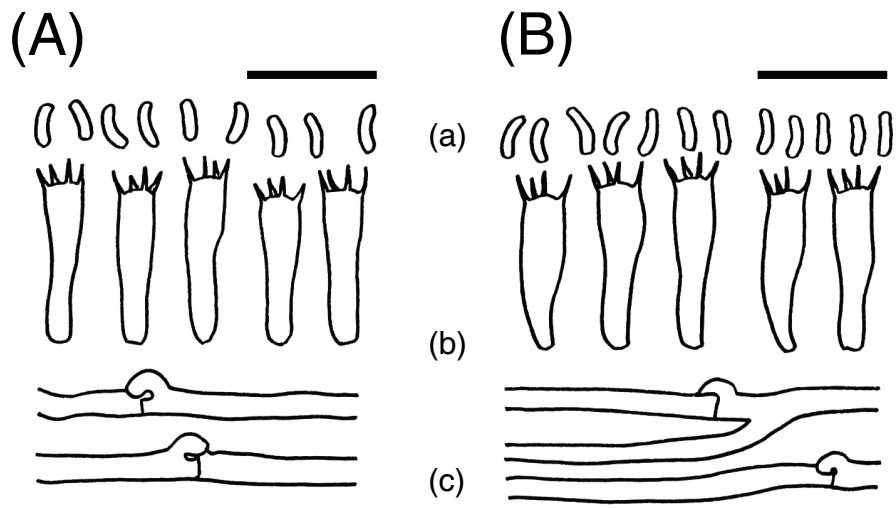

Figure 17. Microscopic features of (A) *Gloeoporus* sp. 1 and (B) *Gloeoporus* sp. 2. (a) basidiospores, (b) basidia, (c) generative hyphae with clamp connections. Scale bar = 10 μm.

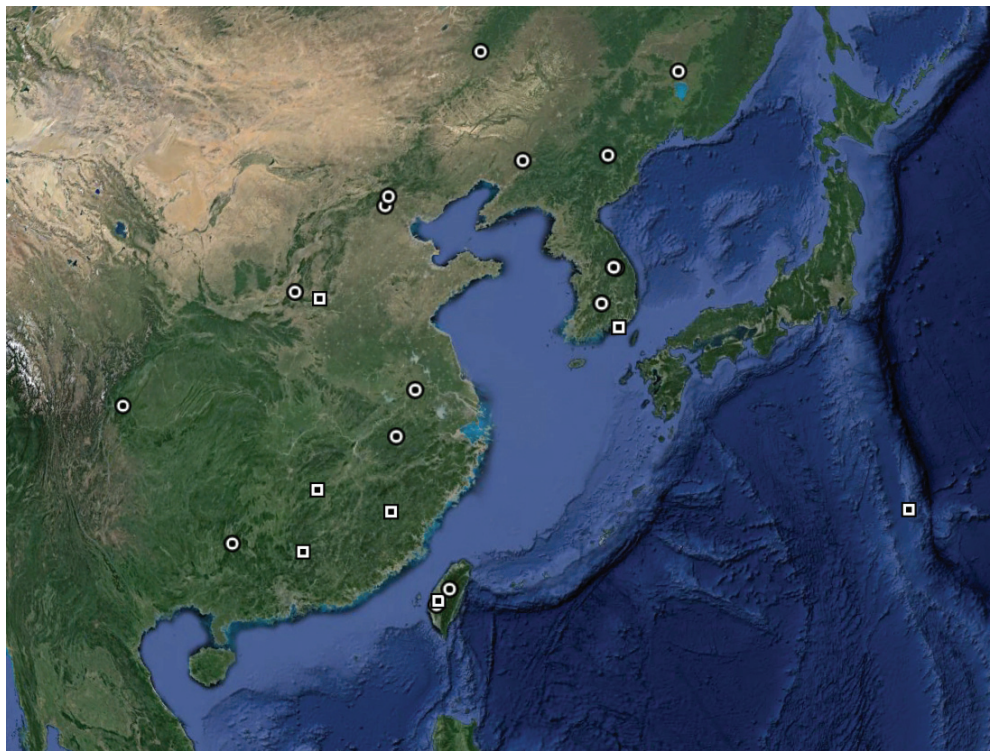

Figure 18. Sympatric distribution of *G. dichrous* (⊙) and *Gloeoporus* new species (⊡) in East Asia. Note that some of the icons are overlapped (source: Google Earth).

4.4 Discussion

4.4.1 *Gloeoporus* sensu stricto

Taxonomic relationship of two well-known *Gloeoporus* species, *G. dichrous* and *G. taxicola*, has long been unsettled and constantly altered by numerous taxonomists. Based on their morphology, *G. dichrous* and *G. taxicola* were often considered congeneric. Recent taxonomic studies suggest that they are both positioned in the phlebioid clade of the Polyporales, but not as closely related as previous taxonomists had believed (Binder et al. 2013).

Current morphological grouping of *Gloeoporus* is polyphyletic according to molecular phylogenetic analyses of this study. When the clade with the type species of *Gloeoporus* is considered as *Gloeoporus* sensu stricto, species within mostly have clamp connections, except *G. thelephoroides*, and lack cystidia. While mixed hyphal system (e.g. species with and without clamps) of *Gloeoporus* may appear somewhat counterintuitive, some genera of the phlebioid clade indeed exhibit various hyphal systems within a genus. *Phanerochaete* and related genera are such examples. Greslebin et al. (2004) erected *Rhizochaete* which includes both clamped and clampless species. In recent studies, Floudas and Hibbett (2015) also state that existence of clamped species in *Phanerochaete* which mostly encompass species lacking clamp connections should not sound peculiar as they define *Phanerochaete* sensu stricto. The clade of *Gloeoporus* s.s. is robustly supported by all phylogenetic analyses. When I define this morphologically and molecularly supported clade with

the type species as the genus *Gloeoporus*, *Gloeoporus* s.s. becomes a small and well defined group mostly with clamped species.

Two species of *Gloeoporus* forming simple septa, *G. taxicola* and *G. guerreroanus*, have closer affinity with *Meruliopsis*. Two species form cystidia, microscopic feature not observed in *Gloeoporus* s.s. *G. guerreroanus* was published as a new species in 2007 without submission of nucleic acid sequences. Phylogenetic analyses of this study, however, reveal that the species is not part of the monophyletic clade of *Gloeoporus* s.s. Another cystidium-forming species, *G. taxicola*, demonstrates the same phylogenetic result. With progress on phylogenetic studies of related genera of *Gloeoporus*, these species should be considered for renaming.

The genus *Gloeoporus* s.l. includes several species which are found in tropical/subtropical regions and have not been sequenced for molecular analyses. For example, *G. longisporus*, the recent new species published in 2010 is reported from Costa Rica. Mata and Ryvarden (2010) describe the species as lacking both cystidia and clamps; thus phylogenetic assessment is required to verify the taxonomic position of this species. Extensive flora studies of tropical regions, based on molecular taxonomic method may facilitate uncovering the diversity of this genera.

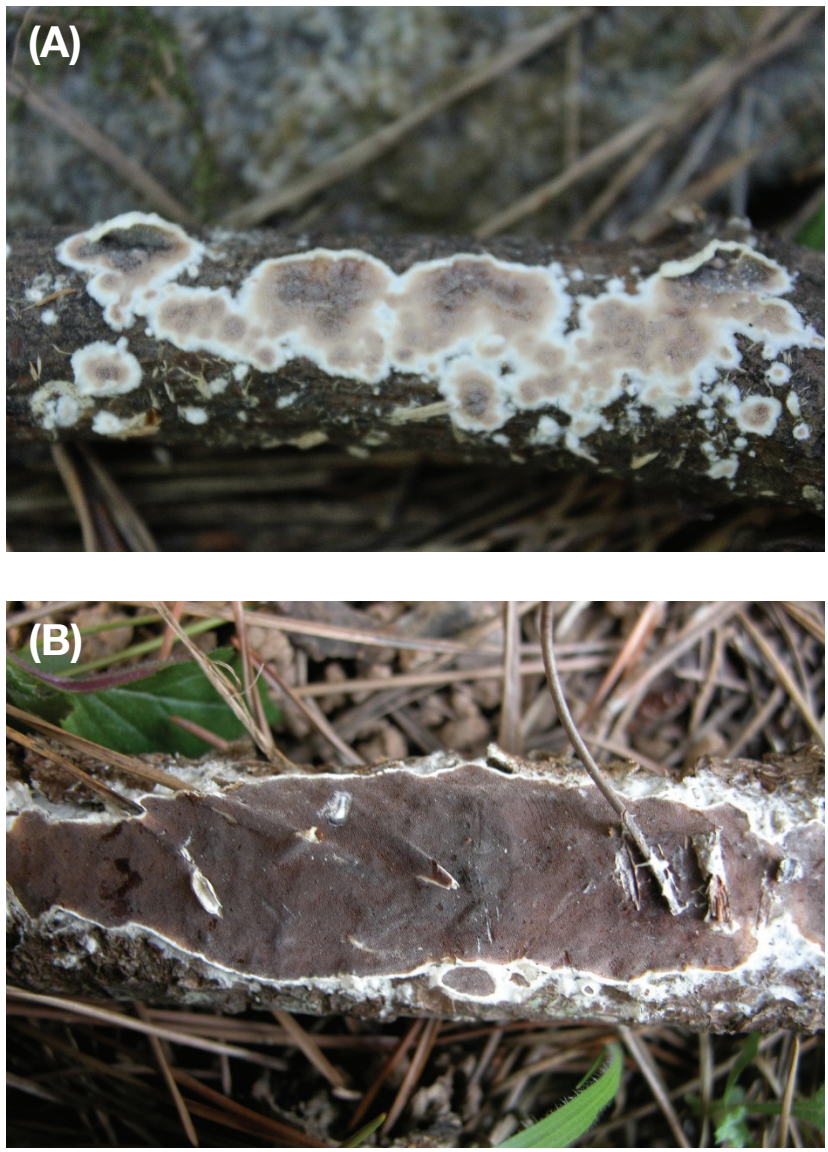

Figure 19. Comparison of *Gloeoporus dichrous* and *Gloeoporus* sp. 1. (A) Korean specimens of *G. dichrous* (SFC20111001-71) (B) *Gloeoporus* sp. 1 (GJ050831-98).

4.4.2 New species of *Gloeoporus*

G. dichrous and the new species of *Gloeoporus* have nearly identical macro-morphology. Fig. 19 presents Korean specimens of *G. dichrous* (SFC20111001-71) and *Gloeoporus* sp. 1 (GJ050831-98), which display similar color and shape. Both specimens display white cottony (byssoid) margin which sharply contrast with the dark pore surface. The pore surface of *G. dichrous* has varying color from light reddish to dark purplish and brown depending on the degree of senescence. While the specific *G. dichrous* in Fig. 19 has reddish brown color which is typical at its earlier stage, *Gloeoporus* sp. 1 has dark purplish color, which may easily be considered as *G. dichrous* at its mature stage.

Due to remarkably similar morphology to *G. dichrous*, new species of *Gloeoporus* may have been repeatedly identified as *G. dichrous* based on its physical traits. Moreover, sympatric distribution of the new species with *G. dichrous* may have hindered mycologists to discover these species (Fig. 18). *Gloeoporus* sp. 1 distribute throughout Korea, China, Taiwan, and Japan and *Gloeoporus* sp. 2 in Uganda. While more extensive sampling is required to understand their ecology and distribution pattern, these species may possibly be rare and endemic species. *Gloeoporus* sp. 1 is found from sites where anthropogenic disturbances are small, such as a remote island of Japan and natural reserves of China. *Gloeoporus* sp. 2 is found from Bwindi Impenetrable Forest, a primeval forest in Uganda. If their distribution range is limited to specific areas, it is possible that these species may become extinct if their habitats

are endangered.

It is also noteworthy that two new species are distributed in Asian and African countries. In the estimation of undiscovered fungal species, mycologists assume that majority of the corticoid fungi, wood rotters forming smooth and patch-like fruit bodies on tree branches, have already discovered in the European continent. In contrast, numbers of the unknown species are exceptionally larger than the known species in Asia, Africa, and Oceania. For instance, tropical Asia alone is estimated to have more than 1,000 species of corticoid fungi yet to be discovered (Mueller et al. 2007). Thus fungal surveys must be proceeded prudently in these areas in order to understand true diversity of the wood decay fungi.

V. Conclusion

Wood decay fungi have been long studied in various cultures due to their unique properties and economic implications. This study examines the taxonomic problems which plague identification process of wood decay fungi.

As DNA barcoding has become prevalent, accuracy of sequences registered at GenBank was tested to estimate the reliability of public sequence database. The result showed that misidentified sequences at database can confuse researchers who use BLAST search for identification; thus meticulous phylogenetic studies must accompany prior to identification of wood decay fungi.

Biogeographic diversification of wood decay fungi is often depressed as wood decay fungi were long considered to be free of barrier. The study on *Gloeoporus* revealed geographic grouping of *G. dichrous* largely based on continents and two new species from East Asia and Africa. Such finding reminds taxonomists that neither simple morphological examination nor similarity search of DNA sequence may sufficiently reveal the true diversity of wood decay fungi.

Any scholar interested in identification of fungi are advised to consider these hindrances in their research process. Scholars must be prudent in identification and publication of their sequences for greater understanding of taxonomy of wood decay fungi.

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VII. Abstract in Korean 국문초록

목재부후균은 다양한 상태의 목재를 분해하는 균류를 지칭한다. 이러한 독특한 특성을 바탕으로 목재부후균은 인류의 문화적, 경제적, 과학적인 측면에 다양한 영향을 끼쳐왔으며 더 크게는 탄소순환 등 지구 생태적인 측면에서도 끼치는 영향이 크다. 이러한 중요성에도 불구하고 목재부후균의 분류체계는 아직도 많은 부분 정립되지 못한 상태이다. 잘못된 동정은 목재부후균을 이용한 산업 활용 혹은 산림피해를 야기하는 목재부후균종을 방제하기 위한 노력에도 큰 걸림돌이 된다.

과거에는 균 자실체의 형태적 특징을 바탕으로 한 동정방식이 주로 이루어져 왔으나, 분류학자마다 선호하는 특징이 달라 잦은 분류체계 변경과 더불어 인위적인 분류방식이라는 문제가 야기되었다. PCR의 등장과 함께 제시된 분자 동정 방식은 빠르고 객관적인 동정을 가능케 하였다. 하지만 분자 분석에도 한계와 문제점이 존재한다. 이 연구는 목재부후균을 동정하는 과정에서 연구자들이 맞닥트리는 두 문제점에 대해 논한다. 첫 번째는 염기서열 데이터베이스에 산재하는 잘못된 서열을 통한 오동정 가능성이며 두 번째는 종내 변이가 종별로 달라 종을 구분 짓는 확실한 기준이 없어 야기되는 혼란이다.

DNA염기서열을 바탕으로 한 분자 분석은 정확한 비교서열을 필요로 한다. 그러므로 염기서열 데이터베이스에 등록된 데이터의 정확도가 동정을 좌지우지 할 수 있다. 이 논문에서는 줄버섯속의 ITS와 LSU 서열을 통해 GenBank에 등록된 서열의 정확도를 측정한다. 한국의 줄버섯속 표본으로 계통연구를

실시하여 GenBank에 업로드된 줄버섯속 서열을 검사한다. 줄버섯속으로 검증된 서열을 BLAST로 비교 검색하여 줄버섯속이지만 오동정 혹은 미동정된 채 등록된 서열을 찾는다. 이러한 과정을 거쳐 발견된 서열을 현재 GenBank에 등록된 줄버섯 염기서열들과 합치면 그 수가 거의 두 배로 증가한다.

무른구멍장이버섯속의 경우 분류체계가 아직 정립되지 않았으며 유명한 몇 종을 제외하고는 GenBank 상에 서열이 등록된 종이 많지 않다. 따라서 계통연구를 위해 전 세계의 표본을 수집하였다. 다중유전자(ITS, LSU, *tef*, *rpb2*)를 통한 계통연구는 두 개의 신종과 겹무른구멍장이버섯(*G. dichrous*)내에 생물 지리학적 다양성이 존재함을 보여주었다. 아시아와 알라스카, 알라스카를 제외한 아메리카 대륙, 유럽이라는 세 가지 그룹을 통해 겹무른구멍장이버섯이 과거 아시아와 아메리카 대륙 가운데 존재했던 베링 육교를 통해 전파되었을 가능성이 드러났다. 이러한 결과를 바탕으로 좁은 의미(狹義)의 무른구멍장이버섯속을 제안한다. 속내 *G. thelephoroides*를 제외한 다른 종은 모두 꺾쇠연결(clamp connection)구조가 관찰되며 낭상체(cystidium)가 없는 종들을 포함한다.

이 연구는 목재부후균의 동정을 할 때 연구자들이 대면하는 문제점을 짚어보았다. 목재부후균은 분류체계가 정립되지 않았으므로, 균류 동정을 하고자 하는 연구자들은 이러한 한계와 문제를 정확히 인식한 후 임해야 할 것이다.