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RESEARCH ARTICLE

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Determination of Diversity, Distribution and Host Specificity of Korean *Laccaria* Using Four Approaches

Hae Jin Cho[a,b], Ki Hyeong Park[a], Myung Soo Park[a], Yoonhee Cho[a], Ji Seon Kim[a], Chang Wan Seo[a], Seung-Yoon Oh[c] and Young Woon Lim[a]

[a]School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul, Korea; [b]Wild Plants and Seed Conservation Department, Baekdudaegan National Arboretum, Bonghwa-gun, Korea; [c]Department of Biology and Chemistry, Changwon National University, Changwon, Korea

ABSTRACT

The genus *Laccaria* (Hydnangiaceae, Agaricales) plays an important role in forest ecosystems as an ectomycorrhizal fungus, contributing to nutrient cycles through symbiosis with many types of trees. Though understanding *Laccaria* diversity and distribution patterns, as well as its association with host plants, is fundamental to constructing a balanced plant diversity and conducting effective forest management, previous studies have not been effective in accurately investigating, as they relied heavily on specimen collection alone. To investigate the true diversity and distribution pattern of *Laccaria* species and determine their host types, we used four different approaches: specimen-based analysis, open database search (ODS), NGS analysis, and species-specific PCR (SSP). As a result, 14 *Laccaria* species have been confirmed in Korea. Results regarding the species distribution pattern were different between specimen-based analysis and SSP. However, when both were integrated, the exact distribution pattern of each *Laccaria* species was determined. In addition, the SSP revealed that many *Laccaria* species have a wide range of host types. This study shows that using these four different approaches is useful in determining the diversity, distribution, and host of ECM fungi. Furthermore, results obtained for *Laccaria* will serve as a baseline to help understand the role of ECM fungi in forest management in response to climate change.

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1. Introduction

Laccaria is a genus of basidiomycete fungi belonging to the family Hydnangiaceae. It is characterized by thick and widely spaced gills with purple-to-flesh color and globose-to-oblong, echinulate, multinucleate basidiospores [1–3]. *Laccaria* species are symbiotic with a variety of trees as ectomycorrhizal (ECM) fungi, and they effectively facilitate nutrient acquisition in their host plants. Their hosts are mostly angiosperms (e.g., *Fagus, Quercus, Betula*, etc.), but some are gymnosperms (e.g., *Abies, Pinus, Larix, Picea, Pseudotsuga*) [4,5]. *Laccaria* species have been used as model species for ECM fungi [6–9].

ECM fungi spend most of their lives underground and only spend a short period making fruiting bodies. In addition, fruiting bodies decay rapidly in the wild, reducing the chance of being detected. With a few exceptions, species in the genus *Laccaria* are small and similar in morphology, which makes accurate identification difficult [10,11]. For this reason, the type species *Laccaria laccata* is known as a deceiver species. Therefore, it is virtually required to conduct molecular analyses in order to accurately identify *Laccaria* species. Clear demarcation of *Laccaria* species in phylogeny has become explicit based on the analysis of multigene sequence data, which include the nuclear rDNA internal transcribed spacer (ITS) region, partial regions of nuclear 28S rDNA (28S), RNA polymerase II subunit 2 (*rpb2*), and translation elongation factor 1-α (*tef1*) [4,5,11–13]. Well-established phylogeny and curated sequence information are highly useful in discovering new species or conducting other molecular-based studies [14,15].

The diversity and distribution of ECM fungi is tightly associated with their host plants; thus, it can be helpful in predicting changes in vegetation. Classically, information on the diversity and distribution of ECM fungi is based on a survey of fungal fruiting bodies [16,17]. We have previously confirmed 13 *Laccaria* species based on specimens collected from fruiting bodies [12,13]. However, this approach may only partially reflect fungal diversity because many species do not produce conspicuous fruiting bodies [18–22]. Soil-based next-generation

CONTACT Young Woon Lim ywlim@snu.ac.kr

sequencing (NGS) techniques seem to overcome the shortcomings of the above method in studies of soil fungal communities [23–25]. However, despite the high potential for detecting soil ECM fungi, methodological biases have been reported in the diversity and distribution analysis of NGS studies [26–28]. Another technique, species-specific PCR, has been used mostly to detect specific species such as pathogens [29,30], mycotoxin producers [31], and food spoilers [32]. There are also studies that distinguish species involved in a specific environment [32,33].

Ecosystem fluctuations caused by climate change will accelerate, and understanding nature from various angles, such as current biodiversity and interliving relationships, is an essential way to respond. Therefore, it is necessary to study the ecology of ECM species for forest health. Describing the biogeography of key ECM species such as *Laccaria* will increase our understanding of fungal-plant interactions and guide forest management and conservation. The main objectives of the current study were to investigate (1) the number of *Laccaria* species in Korea, (2) how these species are distributed, and (3) their host types. Oak and pine are the major tree species in Korea [34], and their ecosystems are affected by climate change. Therefore, we focused our research on *Quercus mongolica* and *Pinus densiflora* as hosts of *Laccaria* species. To meet our goals, we carried out four different approaches: specimen-based analysis, open database search (ODS), NGS analysis, and species-specific PCR (SSP).

2. Materials and methods

2.1. True diversity of Laccaria species in Korea

To determine whether there are more *Laccaria* species present than the 13 already identified, we conducted three advanced methods: ODS, NGS analysis, and the SSP. First, for ODS, we investigated the *Laccaria* sequences in GenBank deposited from Korea using "ITS region," "*Laccaria*," and "Korea" as keywords in the query search and downloaded the resulting items. ITS sequences deposited in our laboratory were excluded, and the remaining sequences were used for further analysis. We determined the correct identification of each *Laccaria* species using phylogenetic analysis. Second, we investigated *Laccaria* OTUs from the soil fungal NGS data. Soil samples from 164 sites (83 pine and 81 oak forests) from 47 localities were used to generate pools of environmental DNA (eDNA) (data not published). All samples were collected in 2013 and 2014 (Figure 1(A)). For NGS, the ITS2 region was amplified using a general fungal primer set, ITS3 and ITS4 [35]. The *Laccaria* sequences were extracted and further analyzed as described above. Finally, we attempted the SSP to confirm the presence of *L. nigra* and *L. ohiensis*. These species were recorded in Korea but were not found in specimens [12,13]. We were able to design a species-specific primer set only for *L. ohiensis* (KH-07192006-1), as reliable *L. nigra* sequences could not be secured. To test whether *L. ohiensis* exists in Korea, we used eDNA obtained for NGS analysis for the SSP. Design of species-specific primers and PCR amplification conditions are described in the section below.

2.2. Distribution of Laccaria species in Korea

To generate a map of *Laccaria* distribution in Korea, information regarding the locations of each species was obtained from the specimen collection records (Figure 1(B)). In addition, species-specific primers for each of the known 14 Korean *Laccaria*

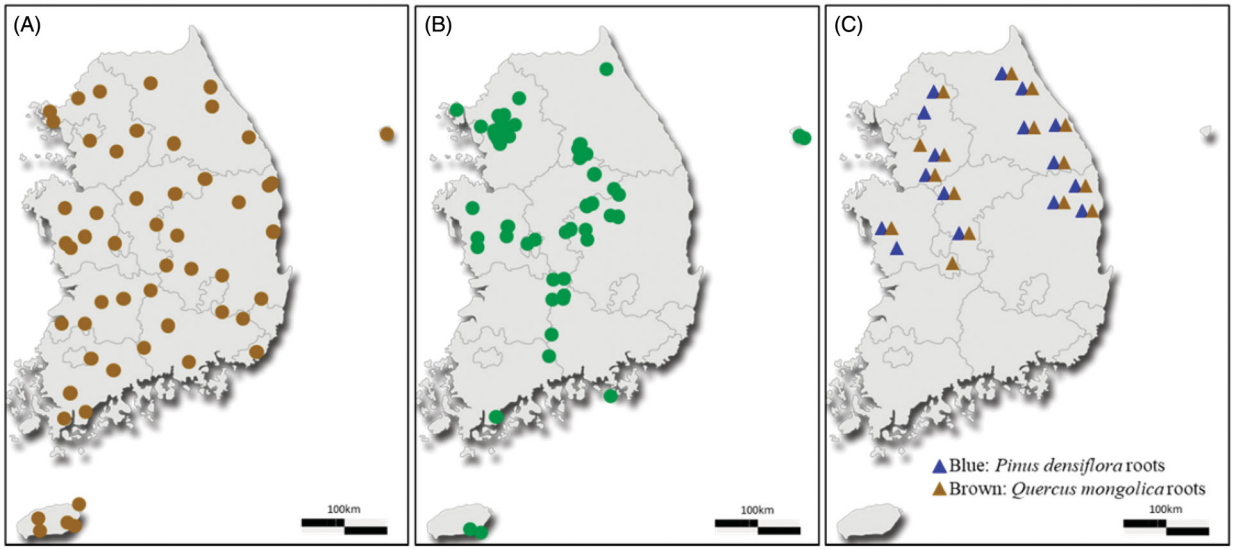

Figure 1. A map showing the sampling sites of (A) soil, (B) specimens, and (C) root of *Pinus densiflora* (blue triangles) and *Quercus mongolica* (brown triangles).

species (including *L. ohiensis*) were designed for the ITS region. They were carefully designed to avoid overlap between *Laccaria* species and other genera in Psathyrellaceae, including *Coprinopsis*, *Lacrymaria*, *Mythicomyces*, and *Psathyrella*. To compare the sequences of all *Laccaria* species and their closely related genera, ITS regions were aligned using MAFFT v7, and interspecific variations were determined using MEGA5. Species-specific regions were selected for construction of species-specific primers. Species-specific primer sets were synthesized by Macrogen (Seoul, Korea), and their specificity was tested.

Two rounds of PCR were performed for sequence amplification. The first round of PCR was conducted using primers ITS1F and ITS4B [36] to amplify the ITS regions of the eDNA, followed by the second round of PCR using each of the *Laccaria* species-specific primer sets. The program for the first PCR was as follows: 5 min at 94 °C; 35 cycles of 40 s at 94 °C, 40 s at 55 °C, and 60 s at 72 °C; and a final extension step of 10 min at 72 °C. The second PCR program was 5 min at 94 °C; 35 cycles of 40 s at 94 °C, 40 s at 60 °C, and 40 s at 72 °C; and a final extension step of 10 min at 72 °C. The succession of PCR was confirmed by running the PCR products on gel electrophoresis. Amplified PCR products for the 14 *Laccaria* species were re-confirmed by sequencing at Macrogen.

2.3. Determining the hosts of Laccaria

To determine the host tree of each *Laccaria* species, we investigated two dominant tree species in Korea: *P. densiflora* and *Q. mongolica*. Root sampling was conducted in 16 pine forests (80 *P. densiflora* trees) and 16 oak forests (80 *Q. mongolica* trees) in Korea in 2019 and 2020 (Figure 1(C)). For each site, we selected five mature host trees with similar DBH (diameter at breast height) that were separated at least 20 m apart to minimize autocorrelation [37]. To track the root directly from the target trees and avoid collecting roots from other tree species, we followed the main roots from the trunk to the lateral roots, to the mycorrhizal root tips after removing litter and upper soil layers. For each tree, two lateral root samples with visible ectomycorrhizal root tips were collected from two different directions. The root samples were stored in plastic bags and transported to the laboratory in an icebox with ice packs. In the laboratory, samples were stored at 4 °C prior to DNA extraction. Sampling was permitted by the National Arboretum Authority.

For DNA extraction, we removed the soil and debris from the roots by gently rinsing in distilled water, then pooling the two root samples from each tree into a single sample. After washing, all fine roots with ectomycorrhizal root tips were isolated using sterilized scissors and surgical gloves. Surface sterilization was conducted with 3% H_2O_2 solution (1 min), and rinsing was performed with sterile distilled water (3 times) to remove possible contaminants from the soil. Sterilized root samples were air-dried on sterilized filter paper inside a clean bench and then finely ground using a mortar, pestle, and liquid nitrogen. Genomic DNA was extracted using a Qiagen PowerSoil DNA extraction kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines. DNA extraction was performed in triplicate. The three separate DNA extracts from each tree were pooled and stored at −20 °C. We investigated the presence of Korean *Laccaria* species on root tips using species-specific primer sets with the method described above.

3. Results

3.1. The true diversity of Laccaria in Korea

Laccaria ITS sequences were obtained from GenBank (NCBI, January 21, 2021), including eDNA and organism sequences from Korea. A total of 164 sequences were deposited in Korea. Among these, 160 ITS sequences deposited in our laboratory were excluded, and the remaining four sequences were identified. They were labeled as "*L. amethystina* (KR673604 and KF692083)," "*L. bicolor* (KR673539)," and "*Laccaria* sp. (KR673570)." These sequences were identified as one of three species through phylogenetic analysis: *L. japonica* (KR673604 and KF692083), *L. torosa* (KR673539), and *L. vinaceoavellanea* (KR673570) (Table 1). From

Table 1. True diversity of *Laccaria* species in Korea.

Scientific name	Origin of type specimen	Detection methods[c]			
		Specimen	ODS	NGS	SSP
Laccaria alba	China	O	–	–	O
Laccaria araneosa	Korea	O	–	–	O
Laccaria bicolor	Scotland (UK)	O	–	–	O
Laccaria galerinoides[a,b]	Chile	–	–	–	n.d.
Laccaria griseolilacina	Korea	O	–	–	O
Laccaria japonica	Japan	O	O	O	O
Laccaria macrobasidia	Korea	O	–	–	O
Laccaria murina	Japan	O	–	O	O
Laccaria nigra[a,b]	Japan	–	–	–	n.d.
Laccaria ohiensis[a]	North America	–	–	–	O
Laccaria parva	Korea	O	–	O	O
Laccaria proxima	France	O	–	–	O
Laccaria torosa	Korea	O	O	–	O
Laccaria tortilis	Scotland (UK)	O	–	O	O
Laccaria versiforma	Korea	O	–	–	O
Laccaria vinaceoavellanea	Japan	O	O	O	O
Number of detected species		13	3	5	14

[a]The species were reported to Korea but could not be discovered in a previous study.
[b]The species-specific primer set was not designed.
[c]NGS: NGS data; ODS: Open database (GenBank); SSP: species-specific PCR.
n.d.: not determined.

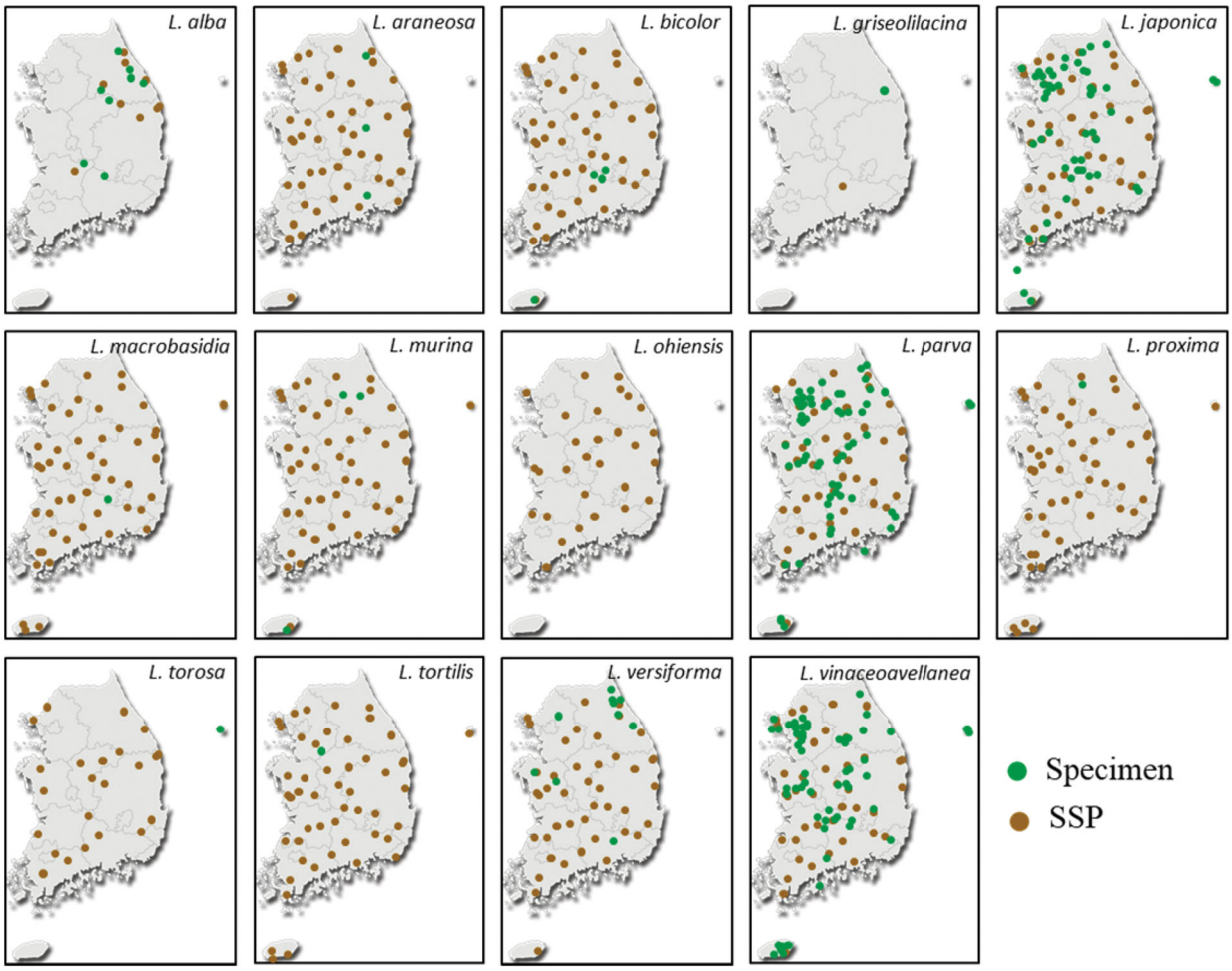

Figure 2. Distribution patterns of the 14 *Laccaria* species in Korea based on collection information of specimens (green circle) and species-specific PCR from eDNA (brown circle).

the fungal NGS data (7,539,877 sequence reads), 34,230 *Laccaria* ITS2 sequences were obtained and identified as five species (*L. japonica*, *L. murina*, *L. parva*, *L. tortilis*, and *L. vinaceoavellanea*) (Table 1). *L. parva* (26,754 sequence reads), *L. vinaceoavellanea* (3,486), *L. japonica* (2,255), and *L. murina* (1,723) were common, whereas *L. tortilis* (12) was found to be very rare. Using the SSP, *L. ohiensis* presented positive bands from 45 sites (18 sites in pine forests and 27 sites in oak forests) out of 164 soil samples (Figure 2). Sequences obtained from the amplified bands were identical to those of *L. ohiensis* (KH-07192006-1).

3.2. Distribution patterns of all Korean Laccaria species

We confirmed the overall distribution pattern of *Laccaria* species based on specimen collection information and the SSP. Analysis of specimen collection information for 13 species was based on specimens collected over 39 years (1981–2019). The collected locations of the specimens were largely limited to accessible areas, such as national parks (Figure 1(B)). In the specimen collection information, *L. japonica*, *L. parva*, and *L. vinaceoavellanea* were found to be evenly distributed across the country, while the other ten Korean *Laccaria* species were collected from restricted areas (Figure 2).

Soil samples were collected more widely throughout Korea than the fruiting bodies of *Laccaria* (Figure 1(A)). The SSP using eDNA allowed us to determine the distribution of *Laccaria* species in Korea. The size of the amplicons for each species-specific primer set ranged from 336 to 529 bp (Table 2). Each species-specific primer set amplified only the corresponding species. The similarity of the amplicons and sequences previously generated from the specimens was over 99% for all species except *L. ohiensis*, in which the fruiting body was not detected, confirming the high specificity of the species-specific primer sets.

The two approaches resulted in three distribution patterns of the 14 *Laccaria* species (Figure 2): (1) nationwide distribution by both methods, (2) limited distribution by the specimen-based method, but even distribution by the SSP, and (3) limited distribution by both methods. The first pattern was observed in *Laccaria parva, L. japonica*, and

Table 2. The species-specific primer sets for 14 Korean *Laccaria* species.

Species	Forward (5′-3′)	Reverse (5′-3′)	Expected length (bp)
L. alba	TTTTTAGGATCGCCGTGCTGTACAAGTCG	CTGAACTTGATAAAGCTGTTTTTC	450
L. araneosa	GCATGTGCTCGTCTGTCATCTTTATCT	GCAGAACTTCATAAAGCTGCTTCACATC	529
L. bicolor	AGTATGTTTATAGAATGTCATCAATGGGA	GCAGAACTTCATAAAGCTGCTTCACATC	380
L. griseolilacina	ACTCGGATTTTAGGATCGCCGTC	ATTTAAGGAGAGCCGACTTCGTG	442
L. japonica	ATTTTAGGATCGCCGTGGCTTTCC	GGAGCTGAACTTCATATATAGCTGCTT	450
L. macrobasidia	GCTGTGCTGTACAAGCTGGCTTTCC	TATAAAGCTGCTTCACATCCACGGCG	336
L. murina	CTCGTCCGTCATCTTTATATCTCCA	CAATGGACGGTTAGAAGCTGAACTCC	526
L. ohiensis	GACTGTTAGCTGGCTTTCCAAAAG	TATTGATGAAGCCCG	444
L. parva	CTCGGATTTTAGGATYGCCAASTT	TTCATAAAGCTGTTTCACATCCACGG	440
L. proxima	GATAACTCTCGAGGCAACTC	ATAAATGGACGGTTAGAAGCT	490
L. torosa	ATCGCTGTGCTGTACAAGTCAGCTTTCC	CAATGGACAGTTAGAAGCTGAACTTTGT	464
L. tortilis	TGTAGTCTTTCGATATCTCTC	CCAAATTAATGGACGGTTAGAAGCTG	506
L. versiforma	GCACATTTTGTAGTCTTGVATATTGCTT	GCTGAACTTCATAAAGCTGCTTCACATC	437
L. vinaceoavellanea	ACTCGGATTTTAGGATTGYCGTGCTTT	AATGCATTTAAGGAGAGCAGACTAAAT	384

L. vinaceoavellanea, which were confirmed by both methods nationwide. Most of the species exhibited the second pattern. Specimens of nine species (*L. araneosa*, *L. bicolor*, *L. murina*, *L. proxima*, *L. torosa*, *L. tortilis*, *L. macrobasidia*, *L. ohiensis*, and *L. versisforma*) were collected only from a limited area, but were found ubiquitously in Korea when assessed by the SSP. The remaining two species, *L. alba* and *L. griseolilacina*, displayed the third pattern, which were detected only in a limited area by both methods.

3.3. The interaction of Laccaria species with the host plants

We investigated the presence/absence of all 14 Korean *Laccaria* species on 160 root DNA samples using species-specific primer sets (Table 3). *L. ohiensis* was not detected in *Pinus* or *Quercus* roots. *L. versiforma* was detected in the roots of only *Quercus*. The other 12 *Laccaria* species were detected in both *Pinus* and *Quercus* roots. Four species (*L. alba*, *L. griseolilacina*, *L. torosa*, and *L. vinaceoavellanea*) were found to be rare in both tree roots (Table 3).

Table 3. Host specificity of 14 Korean *Laccaria* species using species-specific PCR.

	Number of detection sites (%) using the SSP	
Species	*Pinus densiflora* roots	*Quercus mongolica* roots
L. alba	5 (6.25)	9 (11.25)
L. araneosa	18 (22.5)	29 (36.25)
L. bicolor	11 (13.75)	21 (26.25)
L. griseolilacina	4 (5)	9 (11.25)
L. japonica	9 (11.25)	21 (26.25)
L. macrobasidia	9 (11.25)	33 (41.25)
L. murina	27 (33.75)	57 (71.25)
L. ohiensis	–	–
L. parva	11 (13.75)	20 (25)
L. proxima	62 (77.5)	50 (62.5)
L. torosa	1 (1.25)	5 (6.25)
L. tortilis	15 (18.75)	29 (36.25)
L. versiforma	–	19 (23.75)
L. vinaceoavellanea	2 (2.5)	11 (13.75)

4. Discussion

Fourteen *Laccaria* species were confirmed in Korea using four different methods. Thirteen species were identified in specimens collected over a period of 39 years. One species, *Laccaria ohiensis*, was only detected using the SSP. Analysis of the open database and NGS data revealed three and five *Laccaria* species, respectively, and all of these species were collected as specimens (Table 1). Since NGS technology has several advantages in the study of ECM diversity, it is more widely used than the specimen-based approach [23–26,38]. However, many studies suggest that the NGS approach does not fully cover species diversity in soil [39,40]. Similarly, our results showed that only a small number of *Laccaria* species were detected in the NGS data compared to the specimen-based method. However, the specimens analyzed in this study were collected over a long period of time, whereas the NGS data were obtained from samples collected over a short period (2 years). Furthermore, NGS data were analyzed using fungal general primers (ITS3 and ITS4). If eDNA was collected over a longer period and if *Laccaria*-specific primers were used in the NGS approach, higher species diversity could have been obtained. Regardless, our results show that short-term NGS results do not reflect the true diversity of ECM fungi in a particular region.

As ECM fungi live symbiotically with various plant species, their presence is highly dependent on vegetation changes. Therefore, the distribution map of each ECM species may be an important indicator of climate change as well as helpful for forest management. The distribution of the 14 *Laccaria* species was confirmed by tracking the specimen collection information and using the SSP. These two methods displayed different distribution patterns for each species, and the results disagreed to a great extent. The specimen collection information showed that the distribution of each *Laccaria* species was biased to places that were easily accessible. On the other hand, the SSP showed that all species, except two, were evenly distributed below-ground across the country (Figure 2). These results are generally consistent with previous studies on ECM fungal

diversity: ECM fungi such as Sebacinales or Thelephorales are rarely collected in the field, but are found to be quite abundant underground [38,41–43]. Two species, *L. alba* and *L. griseolilacina*, have been shown to have limited distribution in both specimen-based analysis and soil SSP results. They have been mainly detected in areas of high altitude or at relatively cool temperatures. Similar results have been reported for *L. alba* in China; the distribution pattern was localized in mixed forests of over 750 m in altitude in Yunnan and Jilin provinces [44]. For ECM fungi, fruiting body production is greatly dependent on temperature, precipitation, elevation, latitude, and interaction with their host plants [45,46]. It can therefore be assumed that *L. alba* and *L. griseolilacina* are associated with plant communities at high altitudes.

According to Wilson [5], *Laccaria* taxa in the Northern Hemisphere interact with both angiosperms and gymnosperms. Approximately 3700 species of vascular plants have been reported in Korea (http://www.nature.go.kr/kpni/index.do.2021.03), and oak and pine make up approximately 70% of individual plants in the country. However, it is difficult to determine the host of *Laccaria* taxa based on the collection information of the fruiting bodies, because most oak and pine trees share mixed forests with several other plant species in Korea. Therefore, implementing the SSP using fungal DNA extracted from a specific tree root can improve the accuracy of host determination. Through this method, we confirmed that many *Laccaria* species are generalist ECM fungi that interact with both conifers and hardwoods (Table 3). *L. versiforma* was previously reported to fruit on the ground surrounding *Quercus* spp. and *P. densiflora* [12]. However, in the present study, it was only detected in the roots of *Q. mongolica*. Although *L. ohiensis* has been collected near a *Quercus* species in the USA [47], it has not been found in oak or pine in Korea. However, *L. ohiensis* has been detected in several soil eDNA samples (Figure 2), indicating that it is possible to determine its exact host using the SSP in the area where it was present. Four species (*L. alba*, *L. griseolilacina*, *L. torosa*, and *L. vinaceoavellanea*) were found infrequently in the roots of both tree species, so it is possible that they interact with other host plants and were only detected by chance. Further studies are needed to determine their hosts.

In conclusion, we used four methods to determine the true diversity of *Laccaria* species in Korea. ECM fungi form fruiting bodies for a short period of time and are mainly found in the form of a mycelial network associated with below-ground root tips. To investigate the true diversity of ECM fungi, it is recommended to consider both the use of specimen information (fruiting body collection) and NGS technology. For species that were not confirmed by either of these two approaches, the SSP was efficient. Specific markers for each *Laccaria* species were easily determined through ITS sequence analysis, and SSP was also effective in determining the distribution and host specificity of all *Laccaria* species. This study indicates the true diversity and distribution of *Laccaria* species in Korea. Host specificity was investigated for only two tree species in this study, but by gradually expanding to other tree species, types of host species will be clarified for all *Laccaria* species. Our data for *Laccaria* may be useful for forest conservation and management in response to climate change.

Disclosure statement

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ORCID

Hae Jin Cho http://orcid.org/0000-0003-3041-5824
Young Woon Lim http://orcid.org/0000-0003-2864-3449

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