ABSTRACT
The genus *Pholiota* (Strophariaceae, Basidiomycota) is made up of wood-rotting saprotrophic mushrooms characterized by a yellow or brown pileus with scales and/or slimy, and by a brownish smooth spore with a germ pore. However, these features are not enough to distinguish its species, or separate the genus *Pholiota* from other brown-spored wood-rotting genera such as *Hypholoma* and *Stropharia*. Although internal transcribed spacer (ITS) sequence-based identification has improved identification accuracy for species of *Pholiota*, most *Pholiota* species in Korea are reported based on morphological features. To evaluate the taxonomy of *Pholiota* species, we investigated 62 specimens collected from 1999 to 2019 in Korea using ITS sequence analysis and morphological observation. Twelve of the 16 recorded *Pholiota* species in Korea were identified. While eight species were clearly separated, the ITS analysis did not distinguish three in the *Pholiota adiposa* complex. Therefore, further investigation is required to distinguish these three species. ITS sequences deposited in GenBank confirm that *P. highlandensis* exists in Korea. The presence of the other four *Pholiota* species could not be confirmed through specimens or sequence information in GenBank. A taxonomic key and the ITS sequence data for Korean *Pholiota* species are included and can be good baselines for further research on *Pholiota* taxonomy and diversity.

1. Introduction
The genus *Pholiota* Kummer (1871) is composed of saprotrophic flesh mushrooms in the family Strophariaceae [1]. *Pholiota* is characterized by a yellow or brown pileus with scales, brownish smooth surface spore, and brown spore print [2,3]. *Pholiota squarrosa* is designated as a type species [2]. According to the current overview of Basidiomycota [4], approximately 157 species are recorded in this genus. *Pholiota* species are commonly found in temperate climate regions and they perform important roles in the ecosystem as wood decomposers and soil saprotrophs [2,3].

*Pholiota* species produce a variety of bioactive compounds that can have antitumor and antioxidant effects [5,6]. Activity and application of lignocellulase from *P. adiposa* have been reported in several studies [7,8]. Some *Pholiota* species are edible—e.g. *P. microspora* is well-known for its culinary usage in Asian countries [2,9], whereas *P. squarrosa* is poisonous [10].

The presence or absence of pleurocystidia and cheilocystidia, cystidial incrustation, wall thickness, and coloration have been used as key characteristics to distinguish between *Pholiota* species [2,3]. However, morphological characters are not enough to distinguish the species because macro-morphological characteristics of *Pholiota* species are variable depending on the environmental conditions, and micro-morphological characteristics are often very similar between species. For example, a gelatinous layer can be detected from the fruiting body only during the fresh state, and some species show the gelatinous characteristic only when mature, so it is difficult to identify them when they are collected as immature basidiocarps. Moreover, morphological characteristics can be diverse, even within the same species [2,3]. As such, it is important to proceed with further identification using molecular analysis, which has become an increasingly important tool for accurately identifying fungal species [11,12]. We recently discovered many new fungal species and...
amended misidentified species by reevaluating the other genera using molecular analysis [13–19].

Phylogenetic studies have placed *Pholiota* species within the family Strophariaceae, but they form a paraphyletic clade with *Hypholoma* and *Stropharia* species [20–23]. Recent phylogenetic analysis based on the internal transcribed spacer (ITS) sequence has improved the accuracy at which *Pholiota* species are identified and has led to the recognition of new *Pholiota* species [24–27].

Eighteen species of *Pholiota* have been reported in Korea over the years, but only 16 of these are currently accepted [28–30]. Since *P. adiposa* and *P. squarrosa* were first reported in Korea in 1940 [31], 13 additional *Pholiota* species were reported based on morphology by 2011. Through molecular analysis, *P. abnicola* was transferred to genus *Flammula* in Hymenogastraceae [32], and its name changed to *Flammula abnicola* (Fr.) P. Kumm. [33]. Recently, *P. abietis*, *P. multicingulata*, and *P. limonella* were additionally reported by phylogenetic analysis using ITS sequence [29,30,34]. Later, *P. abietis* was confirmed to be synonymous with *P. limonella* [35]. Most *Pholiota* species were mainly identified based on the features of their basidiocarp, so it is necessary to reevaluate *Pholiota* based on molecular analysis. In this study, we investigate the species diversity of *Pholiota* in Korea based on ITS sequence analysis and morphology.

2. Material and methods

2.1. Sample collection and observation

A total of 62 *Pholiota* specimens were obtained from three herbaria in South Korea: 12 from the National Institute of Biological Resources (NIBR), 28 from the Korea National Arboretum (KA), and 22 from the Seoul National University Fungal Collections (SFC). All samples were collected from 1999 to 2019 in South Korea (Table 1) and were stored dried. Pictures of the fresh fruiting bodies and information on the collection location and date were available, but there was often no accurate ecological data. To observe the microscopic features, the specimens were mounted in 5% (w/v) KOH and 5% (w/v) Congo red solution, and then were observed using an Eclipse 80i light microscope (Nikon, Tokyo, Japan). At least 30 basidiospores, 10 basidia, 10 cheilocystidia, and 10 pleurocystidia were measured per specimen.

2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from the fruiting bodies using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol [36]. The ITS region was amplified using primers (ITS1F/ITS4B) that target the ITS region [37]. PCR amplifications were performed on a thermal cycler (C1000TM; Bio-Rad, Richmond, CA) using the AccuPower PCR premix (Bioneer Co., Daejeon, Korea). The PCR conditions were 95 °C for 5 min; followed by 35 cycles of 95 °C for 40 sec, 55 °C for 40 sec, and 72 °C for 1 min; and finally 72 °C for 5 min. PCR products were loaded on to a 1% agarose gel and purified using the ExpinTM PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea). Samples were sequenced by Sanger sequencing using the aforementioned primers at Macrogen (Seoul, Korea) using an ABI PRISM 3730XL Analyzer (Applied Biosystems, Foster City, CA).

2.3. Phylogenetic analyses

ITS sequences of each sample were proofread using MEGA7 [38] and deposited in GenBank (accession numbers in Table 1). Sequences were aligned using the Multiple Alignment Fast Fourier Transform (MAFFT ver. 7) [39] with ITS sequences of *Pholiota* obtained from GenBank and UNITE. The alignments were checked manually, and upon verification, ambiguous alignments were adjusted. A Neighbor Joining (NJ) Tree was constructed also using MEGA7 with 1000 bootstraps [38]. *Agrocybe* species were selected as outgroups, in accordance with previous studies [22,23].

3. Results

A total of 62 specimens were divided into nine groups based on the ITS analysis. Each group included 1–23 specimens (Table 1). Representative sequences from each group were selected, and phylogenetic analysis was performed together with ITS sequences downloaded from GenBank and UNITE. The nine groups were (Figure 1): *Pholiota adiposa* complex (number of specimens = 23), *P. astragalina* (n = 1), *P. lenta* (n = 2), *P. lubrica* (n = 8), *P. microspora* (n = 2), *P. multicingulata* (n = 11), *P. squarrosa* (n = 6), *P. squarrosoides* (n = 1), and *P. terrestris* (n = 8). The *P. adiposa* complex includes morphologically similar species: *P. adiposa*, *P. aurivella*, and *P. limonella*. Although *P. highlandensis* was not present in our analysis, the ITS sequence of *P. carbonaria* (accession number: AY251301) deposited from Korea is identical to that of *P. highlandensis*.

Pictures of each species are shown in Figure 2. Basidiospores, basidia, and cystidia were well observed from the dried specimens, and their size and shape were in good agreement with previous
reports. Basidiospores were generally elliptical, thick walled with an apical pore, and ranged from 4–9 μm long and 2–6 μm wide. Basidia were clavate in shape and 12–33 μm long. Types of cystidia observed were cheilocystidia, pleurocystidia, and caulocystidia. Cheilocystidia and pleurocystidia were observed in all species except P. microspora. Caulocystidia was only observed in P. squarrosoides and P. terrestris in our specimens. Chrysozystidia were detected in the species of the P. adiposa complex, P. squarrosa, P. squarrosoides, and P. terrestris. The microscopic features of the type species P. squarrosa are shown in Figure 3.

4. Taxonomic key for Korean Pholiota

1. Pileus cuticle lacking any gelatinous layers (surface granulose to fibrillose or scaly, or rarely canescent, glabrous, and hygrophanous) ...............Juk15004

Table 1. Summary and GenBank accession numbers for Pholiota specimens used in this study.

| Species                | Specimen code | Collection Date | Locality                  | GenBank Acc. No. |
|------------------------|---------------|-----------------|---------------------------|------------------|
| P. adiposa complex     | KA14-1545     | 2013-10-14      | Pocheon-si, Gyeonggi-do   | MT879423         |
| (P. adiposa, P. squarrosa) | KA14-1611   | 2014-10-01      | Pocheon-si, Gyeonggi-do   | MT879427         |
| P. limonella           | KA14-1644     | 2014-10-02      | Pocheon-si, Gyeonggi-do   | MT879428         |
| P. squarrosa           | KA15-0760     | 2015-09-23      | Gangneung-si, Gangwon-do  | MT879432         |
| P. terrestris          | KA15-0803     | 2015-10-07      | Pocheon-si, Gyeonggi-do   | MT626084         |

Table 1 continues...
1. Pileus cuticle with a layer of gelatinized hyphae in some part (surface may be glabrous to scaly) ........................................ 2
2. Pileus typically distinctly scaly at the time the veil breaks; chrysocystidia or somewhat similar structures present in hymenium ............................................ 3
2. Pileus fibrillose, scaly or granulose, lacking chrysocystidia in hymenium .......................................................... 7

Figure 1. Phylogenetic trees based on neighbor-joining (NJ) analysis of the ITS region in Pholiota species. Bootstrap support values (1000 replicates) >70% are presented. The recorded Korean Pholiota species are placed in boxes. The shaded boxes represent confirmed species and the dotted boxes represent unidentified species in Korea. The scale bar indicates the number of nucleotide substitutions per site.

1. Gelatinous subcutis evident only with maturity; pileus surface initially densely covered with dry squamules .......................................................... 4
2. Gelatinous subcutis evident throughout development; pileus surface initially viscid and glabrous or more sparsely covered with squamules than above .......................................................... 5
4. Pileus dark grayish brown to dark cinnamon, stipe with scales colored like those on pileus .......... 4. P. terrestris

4. Pileus ground color pallid, inner veil white .......... .......................... P. squarrosoides

5. Strongly glutinous pileus covered with a thick layer of hyaline slime, in the absence of any cystidia........................................ 5. P. microspora

5. Pileus glutinous in moist weather, presence of cheilocystidia .......................................................... 6

6. Spore length shorter than 5 μm ............................... P. flammans

6. Spore length longer than 5 μm ................................ P. adiposa complex

7. Pleurocystidia none. The pileus generally appears dry and appressed fibrillose to echinate-squamulose. ........................................... P. tuberculosa

Figure 2. Fruiting bodies of eight reported Pholiota species in Korea. (A) P. adiposa complex; (B) P. astragalina; (C) P. lenta; (D) P. lubrica; (E) P. multicingulata; (F) P. squarrosa; (G) P. squarrosoides; (H) P. terrestris. Scale bar = 10 mm.
13. Pileus variously colored, marginal area pallid to dark reddish-brown; veil lemon yellow young

7. Pleurocystidia present and prominently projecting .................................................................................................................................................................................. 8

8. Always fruiting on burned ground around charcoal .................................................................................................................................................................................. 9

8. Habitat typically lignicolous, more rarely on soil or humus ................................................................................................................................................................................ 10

9. Stipe 1.5–4 (5) mm thick; veil pallid at first ................................................................. Pholiota highlandensis

9. Stipe 5–10 (5) mm thick; pileus dark yellow-brown; veil lemon yellow young

.......................................................... Pholiota brunnescens

10. Spores 7–10 × 4–6 μm ........................................................................................................ 11

10. Spores smaller 5–7.5 (8) × 3–4.5 (5) μm ....................................................................... 12

11. Pileus 1–3.5 cm wide, stipe 1–3.5 mm thick ........................................................................ Pholiota multicingulata

11. Pileus wider than 3.5 cm, and stipe thicker than 3.5 mm ............................................. Pholiota spumosa

12. Taste bitter and black discoloration .................................................................................. Pholiota astragalina

12. Taste mild to farinaceous ................................................................................................ Pholiota lenta

13. Pileus cinnamon to dark cinnamon brown or bay-brown; marginal area not yellow.... Pholiota lenta

13. Pileus variously colored, marginal area pallid to grayish and developing yellow tones in age................................................................. Pholiota lenta

5. Discussion

Pholiota species are often confused with the members of Stropharia and Hypholoma. Species across the three genera can be distinguished by the color of their spore prints. Pholiota species have basidiospores that are dark gray-brown to dark ocher-brown or dark reddish-brown without violet or purple tones, while the latter two have violet- to purple-black basidiospores [40]. In addition to the discernible color of the spores, the presence of scales on a yellowish cap is also a general characteristic that differentiates Pholiota from Stropharia and Hypholoma. However, as the three genera share too many overlapping morphological characteristics and form a paraphyly [20–23], further research is needed to distinguish their relationships.

We identified 12 of the 16 recorded Pholiota species from Korea in this study. Eight species were clearly separated from the ITS tree. On the other hand, three species that grouped into the P. adiposa complex were not distinguished by the ITS analysis. Many mycologists acknowledge the complexity around distinguishing these three species because they share many morphological features [2,35]. Their genetic similarities have also been proven from several other studies. Matsumoto et al. [41] reported that these three species clustered together in an RFLP analysis of ITS, large subunit rDNA, and intergenic spacer (IGS). Papp and Dima [42] grouped P. adiposa, P. limonella, and P. cerifera into the P. adiposa complex as they formed a monophyletic group and was not distinguished by ITS sequence analysis.

The key distinguishable features of P. adiposa, P. aurivella, and P. limonella are the size of the basidiospores and their host preference [2,35,43]. P. limonella has slightly smaller but distinctly narrower spores than do the other two species [43]. The spore size of P. limonella is 6.5–9 × 4–5.3 μm, while those of P. adiposa and P. aurivella are 7.5–9.5 × 5–6.2 μm and 7.5–10.5 × 5–6.5 μm, respectively. In addition, P. aurivella only resides on Salix, while P. limonella prefers Betula, and P. adiposa is found on various deciduous trees, and sometimes even on conifers [43–45]. However, it seems that these differences may be due to environmental or intraspecific variation. In this study, we did not have enough ecological information or consistent ITS sequences for the three species in the P. adiposa complex to distinguish them. To determine whether these three species are of the same or different species, it is necessary to conduct more detailed morphological observations and mating tests, assess their ecological preferences, and compare other genetic markers.

While the ectomycorrhizal species composition in Korea is very different from those of Europe and North America [16,17], there is little difference between saprotrophic fungi compositions between continents [46,47]. Correspondingly, we confirmed that the Korean Pholiota species showed little genetic difference from the European and North American Pholiota species in the ITS neighbor-joining (NJ) phylogeny (Figure 1). Pholiota species seem

Figure 3. Microscopic features of the type species Pholiota squarrosa (SFC20140912-I01). (A) basidia; (B) basidiospores; (C) pleurocystidia; (D) cheilocystidia.
to be distributed over a wide area, which may explain the low genetic variance across continents.

Our specimens did not include five of the previously reported *Pholiota* species (*P. brunescens*, *P. flammans*, *P. highlandensis*, *P. spumosa*, and *P. tuberculosa*) in Korea. However, a *P. carbonaria* (accession number: AY251301) of Korean origin was deposited in GenBank, and was identified as *P. highlandensis*, a pyrophilous species that is synonymous with *P. carbonaria* [48]. Regarding *P. brunescens*, there is an environmental sequence (accession number: LC100010) deposited from Japan [48], and *P. spumosa* (GenBank accession number: JF961346) have been reported from China. Therefore, it is highly possible that these three species also exist in Korea. The presence of *P. flammans* could not be confirmed through specimens, nor through DNA sequence information in any open Database.

In conclusion, we confirmed 12 species of *Pholiota* from Korea based on morphological and sequence analyses. Further investigation is required to distinguish the three species associated with the *P. adiposa* complex. Identification of species in this genus requires a comprehensive consideration of morphological and molecular characteristics. Identification using a BLAST search of ITS sequences has recently become popular because sequencing has become affordable and the available sequences in databases become popular because sequencing has become using a BLAST search of ITS sequences has recently required a comprehensive consideration of morpho-

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**Disclosure statement**

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