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Re-evaluation of *Armillaria* and *Desarmillaria* in South Korea based on ITS/*tef*1 sequences and morphological characteristics

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Abstract

Fungal species in the genera *Armillaria* and *Desarmillaria* (Physalacriaceae, Agaricales) are well known for their symbiotic relationships with *Gastrodia elata* and *Polyporus umbellatus*, important components of traditional medicine in Asia. In addition, some species in these genera cause Armillaria root disease, which has had a negative economic impact by damaging and destroying urban, horticultural and forest trees. Five species within *Armillaria* and *Desarmillaria* have been previously reported in South Korea, based primarily on basidioma morphology: *A. cepistipes*, *A. gallica*, *A. mellea*, *A. ostoyae* and *D. tabescens* (reported as *A. tabescens*). This study re-evaluated 60 specimens of *Armillaria* and *Desarmillaria* using morphological features and molecular phylogenetic analyses of the ITS and partial translation elongation factor-1α (*tef*1) sequences. In addition, spatial distributions of each *Armillaria* and *Desarmillaria* species in South Korea were determined from combined data based on basidioma collections and environmental DNA (eDNA). Six species (*A. cepestipes*, *A. gallica*, *A. mellea*, *A. nabsnona*, *A. ostoyae* and *D. tabescens*) and three lineages of *A. gallica* (*A. gallica* clade 1–3) were identified in South Korea from both specimens and eDNA. Most of the basidioma specimens used in this study were previously identified as *A. mellea*; however, in this study, no basidioma specimens were identified as *A. mellea*, although this species was detected on Jeju Island from eDNA samples. The detection of *A. nabsnona* was a new report for South Korea. Consistent with previous phylogenetic studies, the *tef*1 analysis had better resolution than the ITS analysis. These distribution data of *Armillaria* and *Desarmillaria* species will contribute to better management of Armillaria root disease as well as cultivation of *G. elata* and *P. umbellatus* in South Korea.

KEYWORDS
Armillaria, *Desarmillaria*, *eDNA*, ITS, Rhizomorph, *tef1*

1 | INTRODUCTION

Fungal species in the genera *Armillaria* (Fr.: Fr.) Staude and *Desarmillaria* (Herink) R. A. Koch & Aime (Physalacriaceae, Agaricales) are necrotrophic pathogens that can cause Armillaria root disease. These fungi spread vegetatively through soil via characteristic rhizomorphs or mycelia, but they can also spread via airborne, sexual basidiospores (Baumgartner, Coetzee, & Hoffmeister,

2011). Armillaria root disease is responsible for significant economic losses through root decay and mortality of more than 600 species of woody plants (Morrison, Williams, & Whitney, 1991). For example, *A. mellea* has caused major yield losses in vineyards of California, USA (Baumgartner, 2004), while *D. tabescens* was responsible for significant yield losses in peach production of Georgia, USA (Williams-Woodward, 2013). Furthermore, Armillaria root disease caused a nearly 27% loss in timber volume of Douglas-fir (*Pseudotsuga menziesii*) (Cruickshank, Morrison, & Lalumière, 2011). Due to these negative economic impacts, several studies have focused on examining and/or reducing dispersal, virulence and pathogenicity of *Armillaria* (e.g., Chapman, Xiao, & Myers, 2004; Hagle & Shaw, 1991; Lung-Escarmant & Guyon, 2004; Raziq & Fox, 2005). These studies indicate that pathogenicity varies significantly among *Armillaria* species; *A. mellea* and *A. ostoyae* are strong pathogens (Burdsall & Volk, 1993; Gregory, 1985; Rishbeth, 1982), while *A. gallica* varies in its pathogenicity (Brazee & Wick, 2009; Bruhn, Wetteroff, Mihail, Kabrick, & Pickens, 2000; Elías-Román et al., 2013; Gregory, 1985; Rishbeth, 1982).

In contrast to being causal agents of root disease, some *Armillaria* species confer beneficial effects to horticultural and related industries. For example, *A. cepistipes*, *A. gallica* and *A. mellea* are used to cultivate *Gastrodia elata* (Guo, Wang, Xue, Zhao, & Yang, 2016; Kusano, 1911; Sung, Jung, Yang, Lee, & Harrington, 1995), while *A. calvescens*, *A. cepistipes*, *A. gallica*, *A. nabsnona* and *A. sinapina* are used to cultivate *Polyporus umbellatus* (Kikuchi & Yamaji, 2010; Xing, Men, & Guo, 2017). Both *G. elata* and *P. umbellatus* are important components of traditional medicine in Asia. However, productivity and cultivation success in *G. elata* and *P. umbellatus* vary depending on the associated *Armillaria* species (Baumgartner et al., 2011; Guo et al., 2016; Kikuchi & Yamaji, 2010; Kusano, 1911; Sung, Jung, Moon, & Kim, 1996).

The genera *Armillaria* and *Desarmillaria* typically have the following characteristics: yellow-brown coloured pileus, stipe with or without annulus, white spore print, black rhizomorph and nutritional status (saprotrophic to parasitic) (Volk & Burdsall, 1995; Watling, Kile, & Burdsall, 1991). At present, approximately 39 species of *Armillaria* (Brazee, Ortiz-Santana, Banik, & Lindner, 2012; Lima, Asai, & Capelari, 2008; Pildain, Coetzee, Wingfield, Wingfield, & Rajchenberg, 2010; Volk & Burdsall, 1995; Watling et al., 1991) and two species of *Desarmillaria* (Koch, Wilson, Séné, Henkel, & Aime, 2017) are recognized worldwide. However, identification based on morphology alone is often unreliable because of a high degree of similarity between some *Armillaria* species (*A. gemina*-*A. ostoyae* and *A. cepistipes*-*A. gallica*) (Antonín, Tomšovský, Sedlák, Májek, & Jankovský, 2009; Bérubé & Dessureault, 1989). In addition, the characteristics of basidiomata can vary depending on environmental conditions (Swift, 1972). Furthermore, identification of biological species based on mating also has limitations (Klopfenstein et al., 2017).

In recent decades, the development of DNA sequence-based analysis has improved the precision of species identification for *Armillaria* and *Desarmillaria*. The ribosomal RNA gene family (rDNA), including the internal transcribed spacers 1 and 2 with the 5.8S (ITS) and the intergenic spacer 1 (IGS1), has been useful in distinguishing some *Armillaria* species (Anderson & Stasovski, 1992; Chillali et al., 1998; Coetzee et al., 2000; Dunne, Glen, Tommerup, Shearer, & Hardy, 2002; Hasegawa, Ota, Hattori, & Kikuchi, 2010; Keča, Bodles, Woodward, Karadžić, & Bojović, 2006; Keča & Solheim, 2010; Kim, Klopfenstein, Hanna, & McDonald, 2006; Lima et al., 2008; Tsykun, Rigling, & Prospero, 2013); however, some species cannot be distinguished based on the rDNA regions. For example, *A. cepistipes*, *A. gallica*, *A. nabsnona* and *A. sinapina* cannot be reliably discriminated on the basis of ITS and/or IGS sequences (Brazee, Hulvey, & Wick, 2011; Kim et al., 2006; Tsykun et al., 2013), nor can *A. borealis*, *A. gemina* and *A. ostoyae* (Hanna, Klopfenstein, Kim, McDonald, & Moore, 2007; Keča & Solheim, 2010; Kim et al., 2006; Pérez-Sierra, Guillaumin, Spooner, & Bridge, 2004). Of late, the translation elongation factor-1 alpha (*tef*1) region has successfully been used to distinguish *Armillaria* species that ITS or IGS could not separate (Antonín et al., 2009; Brazee et al., 2011; Coetzee, Wingfield, Zhao, van Coller, & Wingfield, 2015; Elías-Román et al., 2013; Guo et al., 2016; Hasegawa et al., 2010; Klopfenstein et al., 2017; Maphosa, Wingfield, Coetzee, Mwenje, & Wingfield, 2006; Mulholland et al., 2012; Ota, Kim, Neda, Klopfenstein, & Hasegawa, 2011; Ross-Davis, Hanna, Kim, & Klopfenstein, 2012; Tsykun et al., 2013). On the basis of a recent multilocus phylogenetic study that included *tef*1, the taxonomy of some *Armillaria* species was revised in species that lack an annulus on the stipe. As a result, *A. ectypa* and *A. tabescens* were assigned to the newly described genus, *Desarmillaria* (Koch et al., 2017).

The spatial distribution of *Armillaria* and *Desarmillaria* has proven difficult to determine. Armillaria root disease can sometimes be detected at a site by symptoms, such as chlorotic foliage, crown dieback and/or resinosis; however, these symptoms are often difficult to distinguish from other forest diseases (Morrison et al., 1991). A more accurate assessment of *Armillaria*/*Desarmillaria* distribution can be obtained by surveys for the presence of basidiomata, mycelial fans on attacked trees or rhizomorphs associated with the roots and soil (Morrison et al., 1991). However, *Armillaria*/*Desarmillaria* basidiomata occur only sporadically, mostly in late summer to autumn, and surveys for mycelial fans and rhizomorphs are labour and time intensive and require considerable expertise. In an alternative way, DNA sequence-based methods can detect *Armillaria* directly from soil samples. PCR amplification of environmental DNA (eDNA) with taxa-specific primers has been used to detect and monitor specific fungal species (Bridge & Spooner, 2001; Dauch, Watson, & Jabaji-Hare, 2003; Shukunami et al., 2016; Zambounis, Paplomatas, & Tsaftaris, 2007). Because DNA sequence-based methods can detect *Armillaria* and *Desarmillaria* in soils without extensive surveys, this approach provides more accurate information regarding the distribution and diversity of *Armillaria* and *Desarmillaria* species.

Since *A. mellea* was first reported in Korea in 1940 (Kaburagi, 1940), four *Armillaria*/*Desarmillaria* species have been identified in South Korea: *A. tabescens* s.l. (currently *D. tabescens*) (Lee & Cho, 1977), *A. gallica*, *A. ostoyae* (Sung, Yang, Lee, & Harrington, 1994)

and *A. cepistipes* (Lee, Choi, Kim, & Lee, 2016). Most previous studies were based on macro-morphological, and more recently either ITS- or IGS-based identification (Lee & Cho, 1977; Lee et al., 2016; Oh, Lee, Cheong, & Yoo, 2012; Sung, Yang, Kim, & Harrington, 1997; Sung et al., 1994), and the distribution of *Armillaria/Desarmillaria* in South Korea remains unclear. Therefore, accurate identification of *Armillaria/Desarmillaria* species by DNA sequence-based method is useful and necessary to evaluate the diversity and spatial distribution of *Armillaria/Desarmillaria* in South Korea.

In this study, the main objectives were to (i) evaluate the taxonomic status of *Armillaria/Desarmillaria* species in South Korea using fungal barcode sequence (ITS) and a protein-coding gene (*tef*1) and (ii) determine the distribution of each *Armillaria/Desarmillaria* species using basidioma collection information and molecular detection from eDNA.

2 | MATERIALS AND METHODS

2.1 | Collection of *Armillaria* specimens

A total of 60 *Armillaria* basidioma specimens and isolates were obtained from six South Korean organizations (Seoul National University Fungal Collection, Korea Mushroom Reserve Bank, National Institute of Forest Science, Korea National Arboretum, Gangwon National University, and National Institute of Biological Resources). These specimens were collected across a wide range of South Korea from 2011 to 2016 and were originally identified as four species: *A. gallica*, *A. mellea*, *A. ostoyae* and *D. tabescens* (as *A. tabescens*). Detailed specimen and collection information are provided in Table 1.

2.2 | DNA extraction, PCR amplification, and sequencing

Using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol, genomic DNA was extracted from tissue and mycelium (Rogers & Bendich, 1994). The ITS region was amplified using the primer set ITS1F and ITS4B (Gardes & Bruns, 1993). The partial *tef*1 region was amplified using the primer set EF595F and EF1160R (Kauserud & Schumacher, 2001). PCR amplifications were performed as described in a previous study (Hasegawa et al., 2010). PCR products were visualized on a 1% agarose gel and purified using the Expin™ PCR Purification Kit (GeneAll Biotechnology, Seoul, South Korea). DNA sequencing was performed at Macrogen (Seoul, South Korea) using an ABI PRISM 3730XL Analyzer (Applied Biosystems, CA, USA).

2.3 | Sequence analysis

We used MEGA v. 7 to assemble, proofread and edit DNA sequences (Kumar, Stecher, & Tamura, 2016). The sequences obtained in this study were compared to reference sequences from GenBank. Multiple alignments were performed using the default settings of MAFFT v. 7 (Katoh & Standley, 2013). DNA alignments were checked by eye, and ambiguously aligned positions were manually adjusted. Sequences of three closely related species in the Physalacriaceae, *Guyanagaster necrorhizus* (GenBank accession number for *tef*1: KU289109, KU289110, ITS: KU170949, KU170950), *Oudemansiella cubensis* (GenBank accession number for *tef*1: KU170945, ITS: KU170955) and *Strobilurus esculentus* (GenBank accession number for *tef*1:KF530581, ITS: KF530549) were used as outgroups, based on a previous study (Koch et al., 2017).

A neighbour-joining tree (NJ) was constructed using MEGA v. 7 with the Kimura 2-parameter model (Kimura, 1980). Bootstrap analysis was performed with 1000 replications. In addition, Mr. Bayes version 3.2 was used to determine phylogenies based on Bayesian inference (BI), for 10 million generations, under the Jukes-Cantor model (Ronquist et al., 2012). Trees were sampled every 5,000 generations, and the initial burn-in phase set to 2.5 million generations. Maximum likelihood (ML) analyses were performed using RAxML (Stamatakis, 2006) with the GTR+G model of evolution and 1,000 bootstrap replicates. Trees were viewed in MEGA v. 7 and FigTree v. 1.43 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4 | Morphological observation

Representative specimens used to compare macro- and microscopic features were selected for each species that was identified based on molecular analysis. Macroscopic features of basidiomata were obtained from field data and photographs (in cases with loaned specimens). For observation of microscopic features, slide preparations were made from dried tissue mounted in 3% KOH and stained with Congo red solution and viewed using a light microscope (Nikon 80i; Nikon, Tokyo, Japan). Basidia and basidiospore size were measured from 10 basidia and 20 basidiospores each and organized into boxplot with R 3.3.2 (R Development Core Team 2014).

2.5 | *Armillaria* detection from nationwide eDNA using an *Armillaria* specific primer

For *Armillaria/Desarmillaria* detection, 76 eDNA samples deposited at Seoul National University Fungal Collection (SFC) were used. eDNA was extracted from soil samples collected across five sites in 2013 and 33 sites in 2014 using MoBio PowerSoil kit (Table S1). Each site included eDNA from deciduous and coniferous forests. To determine *Armillaria/Desarmillaria* diversity, we designed an *Armillaria/Desarmillaria* genera-specific forward primer in ITS region. Although the ITS region lacks resolution for identification of some *Armillaria* species, it is useful for distinguishing several *Armillaria* species (Chillali et al., 1998; Dunne et al., 2002; Hasegawa et al., 2010; Keča & Solheim, 2010; Keča et al., 2006; Kim et al., 2006; Klopfenstein et al., 2017; Tsykun et al., 2013). Because *Armillaria/Desarmillaria*-specific primers in *tef*1 were unavailable, an *Armillaria/Desarmillaria*-specific forward primer, Armil F1 (5′-TTGGTAGTTRRGTYGGAATAC-3′) in the ITS2

TABLE 1 Collection and associated information of *Armillaria/Desarmillaria* specimens used in this study

Final ID	Collection No.[a]	Original ID	Collection Locality	Latitude	Longitude	GenBank Accession Number	
						tef1	ITS
A. cepistipes	KFRI1616	*A. mellea*	Inje-gun, Gangwon-do	N 38.049280	E 128.425259	MG544785	MG543860
A. cepistipes	KMRB 15072104	*A. gallica*	Inje-gun, Gangwon-do	N 38.049280	E 128.425259	MG544810	MG543885
A. cepistipes	SFC20160927-11	*A. cepistipes*	Ulleung-gun, Gyeongsangbuk-do	N 37.520238	E 130.860045	MG544826	MG543901
A. cepistipes	SFC20160927-39	*A. cepistipes*	Ulleung-gun, Gyeongsangbuk-do	N 37.520238	E 130.860045	MG544827	MG543902
A. gallica clade 1	Ame10	*A. mellea*	Gurye-gun, Jeollanam-do	N 35.202495	E 127.462653	MG544774	MG543850
A. gallica clade 1	F20140902KCM31	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.637373	E 128.553090	MG544778	MG543853
A. gallica clade 1	JS140903-39	*A. mellea*	Boeun-gun, Chungcheongbuk-do	N 36.489457	E 127.729483	MG544781	MG543856
A. gallica clade 1	KFRI3116	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.370474	E 128.389977	MG544795	MG543870
A. gallica clade 1	SFC20130917-33	*A. gallica*	Yecheon-gun, Gyeongsangbuk-do	N 36.723590	E 128.376820	MG544804	MG543879
A. gallica clade 1	SFC20140911-22	*A. tabescens*	Jinan-gun, Jeollabuk-do	N 35.822157	E 127.542886	MG544809	MG543884
A. gallica clade 1	SFC20150903-40	*Armillaria* sp.	Gurye-gun, Jeollanam-do	N 35.241103	E 127.487979	MG544815	MG543890
A. gallica clade 1	SFC20150904-19	*A. tabescens*	Inje-gun, Gangwon-do	N 38.042916	E 128.391330	MG544816	MG543891
A. gallica clade 1	KMRB 15090828	*A. tabescens*	Uiwang-si, Gyeonggi-do	N 37.411858	E 127.034986	MG544817	MG543892
A. gallica clade 1	SFC20150917-16	*A. mellea*	Guri-si, Gyeonggi-do	N 37.622086	E 127.132425	MG544821	MG543896
A. gallica clade 1	SFC20160312-06	*A. gallica*	Goyang-si, Gyeonggi-do	N 37.681560	E 126.769111	MG544824	MG543899
A. gallica clade 1	TPML20120914-60	*A. mellea*	Mt. Jeombong, Inje-gun, Gangwon-do	N 38.049280	E 128.425259	MG544829	MG543904
A. gallica clade 2	KA14-1647	*A. mellea*	Pocheon-si, Gyeonggi-do	N 37.752129	E 127.163461	MG544784	MG543859
A. gallica clade 2	KFRI3123	*A. mellea*	Gunwi-gun, Gyeongsangbuk-do	N 36.016944	E 128.695000	MG544796	MG543871
A. gallica clade 2	SFC20150902-102	*Armillaria* sp.	Inje-gun, Gangwon-do	N 37.997137	E 128.225613	MG544814	MG543889
A. gallica clade 2	KMRB 15091805	*A. mellea*	Jongno-gu, Seoul	N 37.572932	E 126.994352	MG544823	MG543898
A. gallica clade 2	TPML20120913-34	*A. mellea*	Muju-gun, Jeollabuk-do	N 35.860556	E 127.746389	MG544828	MG543903
A. gallica clade 3	Ame7	*A. mellea*	Seogwipo-si, Jeju-do	N 33.254121	E 126.560076	MG544777	MG543852
A. gallica clade 3	KA13-1170	*A. mellea*	Pocheon-si, Gyeonggi-do	N 37.752129	E 127.163461	MG544783	MG543858

(Continues)

TABLE 1 (Continued)

Final ID	Collection No.[a]	Original ID	Collection Locality	Latitude	Longitude	GenBank Accession Number	
						tef1	ITS
A. gallica clade 3	KFRI2393	*A. mellea*	Mungyeong-si, Gyeongsangbuk-do	N 36.586148	E 128.186797	MG544794	MG543869
A. gallica clade 3	SFC20120919-53	*A. tabescens*	Gongju-si, Chungcheongnam-do	N 36.536911	E 127.075351	MG544800	MG543875
A. gallica clade 3	KMRB 15091412	*A. mellea*	Guri-si, Gyeonggi-do	N 37.622086	E 127.132425	MG544818	MG543893
A. nabsnona	Juk14411	*A. mellea*	Jeju-si, Jeju-do	N 33.491405	E 126.594943	MG544782	MG543857
A. ostoaye	Ame5	*A. mellea*	Suwon-si, Gyeonggi-do	N 37.263573	E 127.028601	MG544776	MG543851
A. ostoaye	F20140912KCM03	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.637373	E 128.553090	MG544779	MG543854
A. ostoaye	F20150917KCM08	*A. mellea*	Inje-gun, Gangwon-do	N 38.049280	E 128.425259	MG544780	MG543855
A. ostoaye	KFRI1956	*A. mellea*	Hongcheon-gun, Gangwon-do	N 37.678984	E 128.050328	MG544786	MG543861
A. ostoaye	KFRI1957	*A. mellea*	Hongcheon-gun, Gangwon-do	N 37.678984	E 128.050328	MG544787	MG543862
A. ostoaye	KFRI1959	*A. mellea*	Hongcheon-gun, Gangwon-do	N 37.678984	E 128.050328	MG544788	MG543863
A. ostoaye	KFRI2301	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.500891	E 128.458016	MG544789	MG543864
A. ostoaye	KFRI2302	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.500891	E 128.458016	MG544790	MG543865
A. ostoaye	KFRI2303	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.500891	E 128.458016	MG544791	MG543866
A. ostoaye	KFRI2304	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.500891	E 128.458016	MG544792	MG543867
A. ostoaye	KFRI2317	*A. mellea*	Pyeongchang-gun, Gangwon-do	N 37.500891	E 128.458016	MG544793	MG543868
A. ostoaye	SFC20121010-10	*A. mellea*	Seosan-si, Chungcheongnam-do	N 36.756262	E 126.602820	MG544802	MG543877
A. ostoaye	SFC20130928-03	*A. ostoaye*	Sangju-si, Gyeongsangbuk-do	N 36.421158	E 128.109651	MG544805	MG543880
A. ostoaye	SFC20130928-09	*A. gallica*	Sangju-si, Gyeongsangbuk-do	N 36.421158	E 128.109651	MG544806	MG543881
A. ostoaye	SFC20130928-15	*A. ostoaye*	Sangju-si, Gyeongsangbuk-do	N 36.438889	E 128.096667	MG544807	MG543882

(Continues)

TABLE 1 (Continued)

Final ID	Collection No.[a]	Original ID	Collection Locality	Latitude	Longitude	GenBank Accession Number	
						tef1	ITS
A. ostoaye	KMRB 15072116	*A. mellea*	Inje-gun, Gangwon-do	N 38.049280	E 128.425259	MG544811	MG543886
A. ostoaye	TPML20120926-67	*A. mellea*	Inje-gun, Gangwon-do	N 38.049280	E 128.425259	MG544830	MG543905
A. ostoaye	TPML20130926-03	*A. mellea*	Hongcheon-gun, Gangwon-do	N 37.696952	E 127.888683	MG544831	MG543906
A. ostoaye	TPML20130926-38	*A. mellea*	Hongcheon-gun, Gangwon-do	N 37.696952	E 127.888683	MG544832	MG543907
A. ostoaye	TPML20130926-74	*A. mellea*	Hongcheon-gun, Gangwon-do	N 37.696952	E 127.888683	MG544833	MG543908
A. ostoaye	TPML20130926-84	*A. mellea*	Hongcheon-gun, Gangwon-do	N 37.696952	E 127.888683	MG544834	MG543909
D. tabescens	SFC20120722-04	*A. tabescens*	Gimpo-si, Gyeonggi-do	N 37.604692	E 126.723577	MG544797	MG543872
D. tabescens	SFC20120807-11	*A. tabescens*	Seogwipo-si, Jeju-do	N 33.279914	E 126.720706	MG544798	MG543873
D. tabescens	SFC20120915-07	*A. tabescens*	Gwanak-Gu, Seoul	N 37.462618	E 126.938325	MG544799	MG543874
D. tabescens	SFC20121005-07	*A. tabescens*	Gwanak-Gu, Seoul	N 37.462618	E 126.938325	MG544801	MG543876
D. tabescens	SFC20130807-38	*A. tabescens*	Yecheon-gun, Gyeongsangbuk-do	N 36.685069	E 128.594858	MG544803	MG543878
D. tabescens	SFC20140626-02	*A. tabescens*	Guri-si, Gyeonggi-do	N 37.611560	E 127.139341	MG544808	MG543883
D. tabescens	KMRB 15081103	*A. tabescens*	Guri-si, Gyeonggi-do	N 37.622086	E 127.132425	MG544812	MG543887
D. tabescens	SFC20150812-01	*A. tabescens*	Jongno-gu, Seoul	N 37.574583	E 126.994143	MG544813	MG543888
D. tabescens	KMRB 15091701	*A. tabescens*	Guri-si, Gyeonggi-do	N 37.622086	E 127.132425	MG544819	MG543894
D. tabescens	KMRB 15091706	*A. tabescens*	Guri-si, Gyeonggi-do	N 37.622086	E 127.132425	MG544820	MG543895
D. tabescens	KMRB 15091737	*A. tabescens*	Guri-si, Gyeonggi-do	N 37.622086	E 127.132425	MG544822	MG543897
D. tabescens	SFC20160922-05	*A. tabescens*	Ongjin-gun, Incheon	N 37.262253	E 126.475562	MG544825	MG543900

Note. [a]Seoul National University Fungal Collection (SFC), Korea Mushroom Reserve Bank (KMRB), National Institute of Forest Science (Ame, KFRI), Korea National Arboretum (KA), Kangwon National University (TPML), National Institute of Biological Resources (JS, F, Juk).

region was designed and tested by PCR amplification with DNA obtained from *Armillaria*/*Desarmillaria* species as well as DNA from the family Physalacriaceae, which includes *Cylindrobasidium*, *Cyptotrama*, *Flammulina*, *Hymenopellis*, *Oudemansiella*, *Strobilurus* and *Xerula*.

Two-step PCR amplification was performed using eDNA samples. First PCR was performed with ITS1F and ITS4B primers. Nested PCR was performed using Armil F1 and ITS4 primer (White, Bruns, Lee, & Taylor, 1990). If any amplicons were detected, they were sequenced using same primer set. In cases where the sequence profile was mixed or unclear, PCR products were cloned using a commercial TA cloning kit (Topcloner TA kit; Enzynomics). In a subsequent manner, PCR products were sequenced, and sequences were examined by comparison to the nucleotide database at GenBank using BLAST with 99% identity.

3 | RESULTS

3.1 | Sequence analysis

PCR amplification of the ITS and *tef*1 regions each yielded a single strong band of approximately 600 bp and 450 bp, respectively. In total, 60 ITS and *tef*1 sequences from all basidiomata samples were obtained. ITS sequence analysis separated the *Armillaria* specimens into three groups: *A. gallica* group, *A. ostoyae* group and *D. tabescens*. The *A. gallica* group included *A. cepistipes*, *A. gallica* and *A. nabsnona*. The *A. ostoyae* group included *A. ostoyae*, *A. borealis* and *A. gemina* (Figure 1). Members within each of these two groups could not be distinguished on the basis of ITS sequences. Specimens of *D. tabescens* were contained in a basal clade that was clearly separated from the other two groups.

All *Armillaria*/*Desarmillaria* species formed separate clades in the phylogenetic analysis of the partial *tef*1 sequences. Within the Gallica superclade, the *A. cepistipes* and *A. gallica* clades were well supported in the NJ, ML and BI analyses, while the *A. nabsnona* branch was well supported only in the NJ analysis. These results strongly suggest that these three species exist in South Korea. In addition, *A. gallica* sequences were polyphyletic and separated into four distinct clades. We refer to these five lineages as *A. gallica* clade 1 (*A. gallica* c1), *A. gallica* c2, *A. gallica* c3, European *A. gallica* and North American *A. gallica* clade. *Armillaria gallica* c1 included *A. gallica* from Japan and South Korea, *A. gallica* c2 included *A. gallica* from China and South Korea and *A. gallica* c3 included *A. gallica* from South Korea (Figure 2). Furthermore, *tef*1 sequences within the Solidipes/Ostoyae superclade grouped into a separate clade that was identified as *A. ostoyae*.

From 76 eDNA samples, 35 samples amplified with the *Armillaria*/*Desarmillaria*-specific primer. We detected the *A. gallica* group, *A. mellea*, *A. ostoyae* and *D. tabescens* directly from 31 eDNA samples with *Armillaria*/*Desarmillaria*-specific primers. PCR products from four eDNA samples with heterogeneous ITS sequences were cloned and representative clones were sequenced, which indicated that the ITS sequences from these eDNA samples all belonged to *D. tabescens* (Table S1). Because of the low resolution in the ITS region, sequences within the *A. gallica* group could not be distinguished at the species level; however, the *A. mellea* clade comprised only *A. mellea*, which supports the existence of *A. mellea* in South Korea. In total, six species and three lineages of *A. gallica* group were identified as occurring in South Korea based on ITS and *tef*1 sequence analyses from both specimens and eDNA: *A. cepistipes*, *A. gallica* c1, *A. gallica* c2, *A. gallica* c3, *A. mellea*, *A. nabsnona*, *A. ostoyae* and *D. tabescens*.

3.2 | Morphological observations

Armillaria ostoyae and *D. tabescens* were distinguished, based on the macroscopic features of their stipes; *A. ostoyae* had a darker stipe colour than other species; and *D. tabescens* did not have an annulus on the stipe. However, *A. cepistipes*, *A. gallica* and *A. nabsnona* could not be distinguished on the basis of macroscopic morphological features. Microscopic characteristics, such as morphology of basidia and basidiospores, were not definitive for distinguishing species within the Gallica superclade (Figure 3). Basidia length was generally different among species, but size ranges overlapped between some species (Figure 3a). Length of basidia ranged between 35 and 45 μm for most species, but basidia of *A. ostoyae* was relatively longer (40–55 μm) than others. Basidiospore size overlapped considerably with little distinction among species. Most basidiospores were ellipsoid, and length was within 7–10 μm (Figure 3b), while width was within 4.5–6.5 μm.

3.3 | Distribution of *Armllaria* and *Desarmillaria* species in South Korea

Spatial distribution of *Armillaria* and *Desarmillaria* in South Korea was determined from combined data based on basidioma collections and eDNA sequencing (Figure 4). Based on basidioma collections, *A. gallica* c1 had a wide distribution in South Korea, while *A. gallica* c2 and c3 were collected from a more restricted area (Figure 4b-d). In addition, *A. cepistipes* was only found in north-eastern South Korea (Gangwon province) and on Ulleung Island (Figure 4a), and *A. nabsnona* was found on Jeju Island (Figure 4f). Basidiomata of *A. ostoyae* were collected from the central area of South Korea (Figure 4g), while basidiomata of *D. tabescens* were found within a widely distributed area of South Korea, except in the north-eastern area (Gangwon province) (Figure 4h). In an interesting manner, a basidioma specimen of *A. mellea* was not found.

We detected the *A. gallica* group, *A. mellea*, *A. ostoyae* and *D. tabescens* from the eDNA analysis. The *A. gallica* group was detected only in Gangwon province and northern Gyeongsangbuk-do (Figure 4a-d, f), and *A. mellea* was detected from eDNA on Jeju Island (Figure 4e). Because the *A. gallica* group could not be identified at the species level, it is probable that it contains multiple species or lineages within the Gallica superclade (i.e., *A. cepistipes*, *A. gallica* c1, *A. gallica* c2, *A. gallica* c3 and *A. nabsnona*). Detection of *A. ostoyae* and *D. tabescens* in eDNA showed a similar distribution pattern as that found using the basidioma specimens (Figure 4g, h).

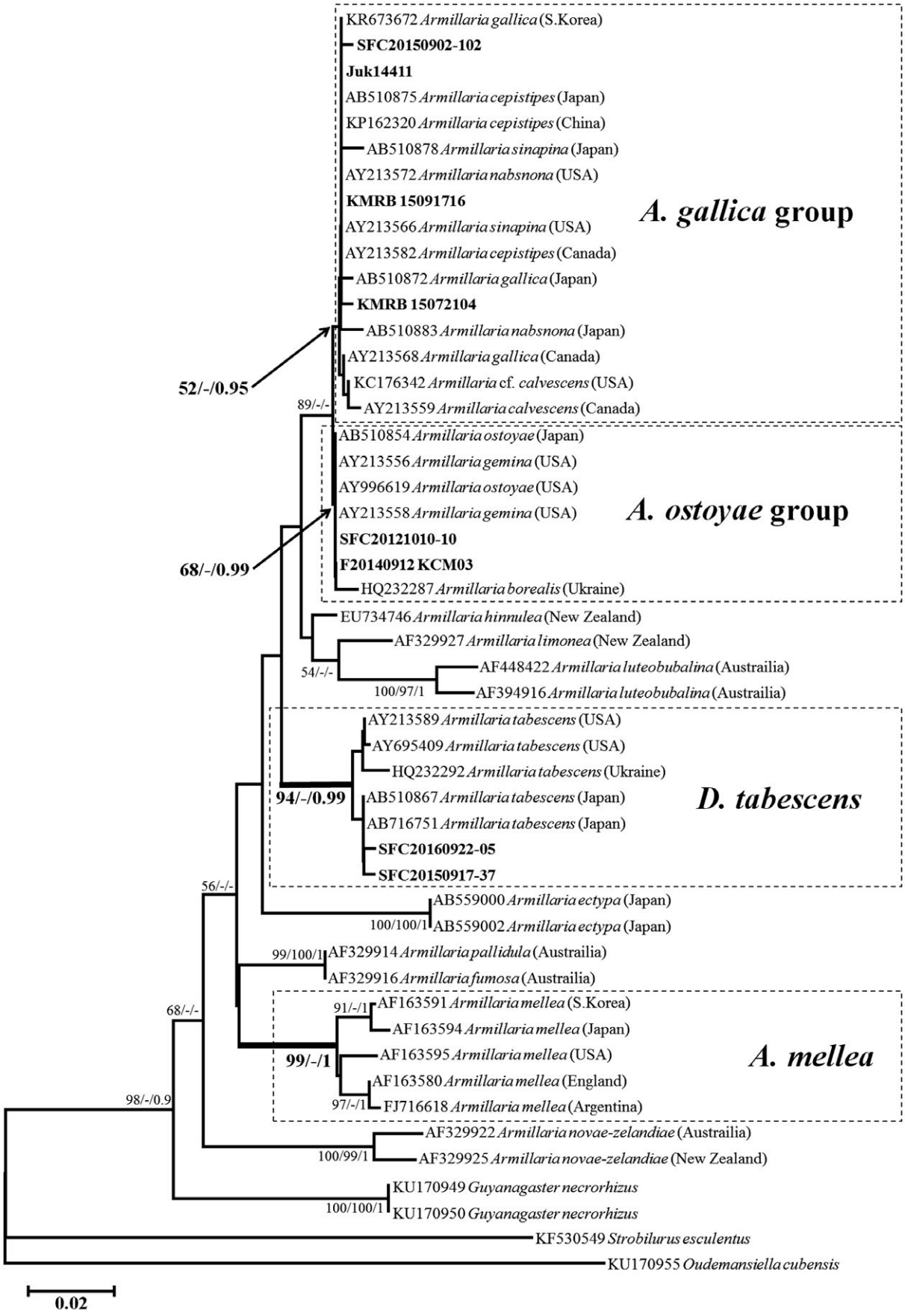
KR673672 Armillaria gallica (S.Korea)
SFC20150902-102
Juk14411
AB510875 Armillaria cepistipes (Japan)
KP162320 Armillaria cepistipes (China)
AB510878 Armillaria sinapina (Japan)
AY213572 Armillaria nabsnona (USA)
KMRB 15091716
AY213566 Armillaria sinapina (USA)
AY213582 Armillaria cepistipes (Canada)
AB510872 Armillaria gallica (Japan)
KMRB 15072104
AB510883 Armillaria nabsnona (Japan)
AY213568 Armillaria gallica (Canada)
KC176342 Armillaria cf. calvescens (USA)
AY213559 Armillaria calvescens (Canada)
A. gallica group
52/-/0.95
89/-/-
AB510854 Armillaria ostoyae (Japan)
AY213556 Armillaria gemina (USA)
AY996619 Armillaria ostoyae (USA)
AY213558 Armillaria gemina (USA)
SFC20121010-10
F20140912 KCM03
HQ232287 Armillaria borealis (Ukraine)
A. ostoyae group
68/-/0.99
EU734746 Armillaria hinnulea (New Zealand)
AF329927 Armillaria limonea (New Zealand)
54/-/-
AF448422 Armillaria luteobubalina (Australia)
100/97/1
AF394916 Armillaria luteobubalina (Australia)
AY213589 Armillaria tabescens (USA)
AY695409 Armillaria tabescens (USA)
HQ232292 Armillaria tabescens (Ukraine)
94/-/0.99
AB510867 Armillaria tabescens (Japan)
AB716751 Armillaria tabescens (Japan)
SFC20160922-05
SFC20150917-37
D. tabescens
56/-/-
AB559000 Armillaria ectypa (Japan)
100/100/1
AB559002 Armillaria ectypa (Japan)
99/100/1
AF329914 Armillaria pallidula (Australia)
AF329916 Armillaria fumosa (Australia)
68/-/-
91/-/1
AF163591 Armillaria mellea (S.Korea)
AF163594 Armillaria mellea (Japan)
99/-/1
AF163595 Armillaria mellea (USA)
AF163580 Armillaria mellea (England)
97/-/1
FJ716618 Armillaria mellea (Argentina)
A. mellea
98/-/0.9
AF329922 Armillaria novae-zelandiae (Australia)
100/99/1
AF329925 Armillaria novae-zelandiae (New Zealand)
KU170949 Guyanagaster necrorhizus
100/100/1
KU170950 Guyanagaster necrorhizus
KF530549 Strobilurus esculentus
KU170955 Oudemansiella cubensis
0.02

FIGURE 1 Phylogenetic trees based on neighbour-joining (NJ) analysis of the ITS region in *Armillaria* and *Desarmillaria* species worldwide. Branch support values are shown as percentages of bootstrap support (NJ), maximum likelihood (ML) bootstrap values and Bayesian posterior probabilities. Percentage of bootstrap support (1,000 replicates) greater than 50%, ML bootstrap values over 90 and Bayesian posterior probabilities over 95 are shown above tree branches. The scale bar indicates the number of nucleotide substitutions per site

4 | DISCUSSION

4.1 | Taxonomic status of *Armillaria* and *Desarmillaria* in South Korea

The genera *Armillaria* and *Desarmillaria* are found in soil, tree roots and dead wood, as basidiomata, mycelial fans and rhizomorphs. However, *Armillaria* and *Desarmillaria* can be difficult to identify at the species level because some species share similar morphological characteristics. Some species, such as *A. ostoyae*, can be distinguished from other *Armillaria* species in South Korea by the slightly longer basidia (Figure 3a) and dark colour of stipe. In addition, *D. tabescens* is distinguishable by the lack of annulus on its stipe; however, it is unknown if exannulate *D. ectypa* also occurs in South Korea.

Microscopic features (basidia and basidiospores) of our samples were similar to previous species descriptions, except for *A. ostoyae* (Antonín et al., 2009; Lee & Cho, 1977; Sung et al., 1994; Volk & Burdsall, 1995); basidiospores of *A. ostoyae* in our collection were slightly larger than those of previous reports in South Korea (Sung et al., 1994). In addition, neither the size nor shape of basidiospores was useful for identification purposes, as shapes are similar within the genus, and the size range of basidiospores from different species frequently overlapped. Basidia tended to exhibit different sizes among species, but it was difficult to base identification solely on this character, because the basidia sizes frequently overlapped with another species. Similar morphology between some species was previously reported for some *Armillaria* species (Bérubé & Dessureault, 1989). These results demonstrate that *Armillaria* identification in South Korea cannot be based solely on microscopic features (Figure 3).

For species that share similar morphology, sequence-based analysis can facilitate species-level identification. Consistent with previous studies (Chillali et al., 1998; Dunne et al., 2002; Hasegawa et al., 2010; Keča & Solheim, 2010; Keča et al., 2006; Kim et al., 2006; Tsykun et al., 2013), the ITS marker is useful to identify *A. mellea* and *D. tabescens* in South Korea. In addition, most *Armillaria* species can be distinguished on the basis of *tef*1 sequences (Elías-Román et al., 2013; Guo et al., 2016; Hasegawa et al., 2010; Klopfenstein et al., 2017; Maphosa et al., 2006; Ross-Davis et al., 2012; Tsykun et al., 2013). Using ITS and *tef*1 sequences, previous reports of five *Armillaria*/*Desarmillaria* species (*A. cepistipes*, *A. gallica*, *A. mellea*, *A. ostoyae* and *D. tabescens*) in South Korea were all confirmed (Maphosa et al., 2006; Oh et al., 2012; Seok et al., 2013; Sung et al., 1994, 1997). The detection of *A. nabsnona* was a new report for South Korea.

Armillaria specimens originally identified as *A. gallica*, *A. mellea*, *A. ostoyae* and *D. tabescens* (as *A. tabescens*) were validated by ITS and *tef*1 sequences analysis (Table 1). Although *A. mellea* was frequently reported in South Korea (Maphosa et al., 2006; Oh et al., 2012; Seok et al., 2013; Sung et al., 1994, 1997), in this study, specimens previously identified as *A. mellea* were assigned to other *Armillaria* species (Table 1); however, *A. mellea* was detected from eDNA samples. These results demonstrate that morphological characters are insufficient for accurate identification of all *Armillaria* spp. in South Korea.

Previous studies suggest that *A. ostoyae* comprised two distinct clades, and the clade containing North American isolates was provisionally assigned to *A. solidipes* based on phylogenetic analysis (Guo et al., 2016). Similar to that, *A. gallica* is polyphyletic and probably comprises cryptic species (Antonín et al., 2009; Elías-Román et al., 2013; Guo et al., 2016; Keča, Klopfenstein, Kim, Solheim, & Woodward, 2015; Klopfenstein et al., 2017; Qin, Zhao, & Korhonen, 2007). The current study showed similar results, with *A. gallica* clearly separated into five lineages: *A. gallica* c1-c3, European, and North American *A. gallica* clade. South Korean *Armillaria* samples were placed into *A. gallica* c1-c3 (Figure 2). This suggests that distinct clades of *A. gallica* could represent different species. However, further studies, such as mating tests, phylogenetic analyses of multiple loci and/or other approaches, are needed to determine whether the *A. gallica* lineage in South Korea comprises multiple cryptic species.

4.2 | Distribution of *Armillaria* species in South Korea

Our inferences on the distribution of all *Armillaria* species in South Korea are limited by the small number of specimens that were observed. However, this study does provide preliminary distribution data based on available specimens and eDNA from soil samples. The *Armillaria*/*Desarmillaria*-specific ITS primer was useful for selective detection of *Armillaria*/*Desarmillaria* species in soil even in the absence of basidioma. In an interesting manner, *A. mellea* was not found among the basidioma specimens and only found in eDNA samples. This might be due to rarity, restricted habitat or sparse/sporadic fruiting during surveys for *A. mellea* in South Korea. In our study, *A. gallica* c1, *A. ostoyae* and *D. tabescens* had a wide distribution in South Korea, while *A. cepistipes*, *A. gallica* c2, *A. gallica* c3, *A. mellea* and *A. nabsnona* were found in more restricted areas. Differences in distribution of *Armillaria*/*Desarmillaria* species are likely related to the abiotic and biotic environment, such as climate, soil, microbial communities, or vegetation and host species. Further studies with more detailed sampling are needed to better understand the relationship among environmental factors and *Armillaria*/*Desarmillaria* species.

Armillaria/*Desarmillaria* can spread vegetatively via growth of rhizomorphs and/or mycelia (Baumgartner et al., 2011); however,

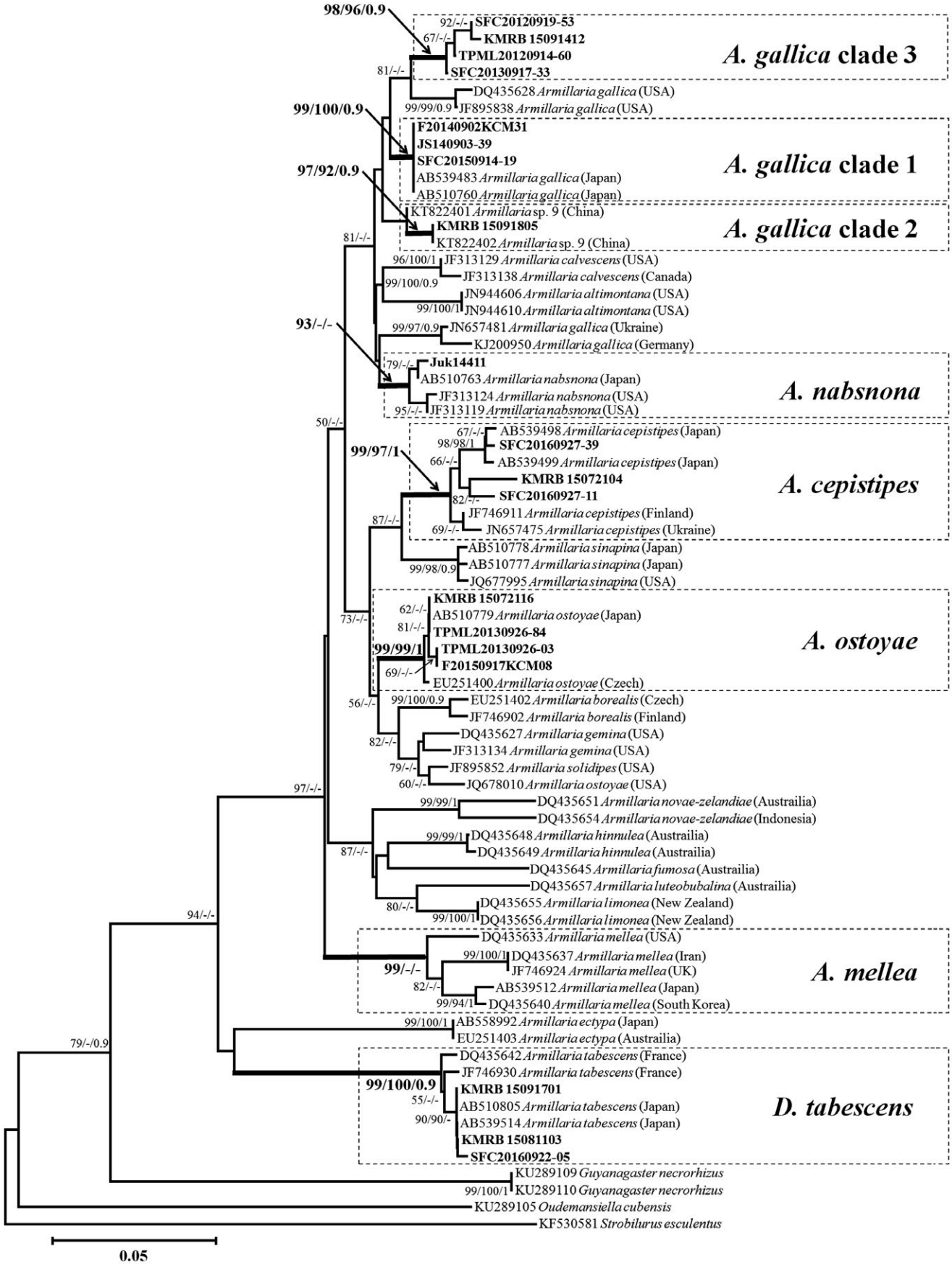
A. gallica clade 3
A. gallica clade 1
A. gallica clade 2
A. nabsnona
A. cepistipes
A. ostoyae
A. mellea
D. tabescens
SFC20120919-53
KMRB 15091412
TPML20120914-60
SFC20130917-33
DQ435628 Armillaria gallica (USA)
JF895838 Armillaria gallica (USA)
F20140902KCM31
JS140903-39
SFC20150914-19
AB539483 Armillaria gallica (Japan)
AB510760 Armillaria gallica (Japan)
KT822401 Armillaria sp. 9 (China)
KMRB 15091805
KT822402 Armillaria sp. 9 (China)
JF313129 Armillaria calvescens (USA)
JF313138 Armillaria calvescens (Canada)
JN944606 Armillaria altimontana (USA)
JN944610 Armillaria altimontana (USA)
JN657481 Armillaria gallica (Ukraine)
KJ200950 Armillaria gallica (Germany)
Juk14411
AB510763 Armillaria nabsnona (Japan)
JF313124 Armillaria nabsnona (USA)
JF313119 Armillaria nabsnona (USA)
AB539498 Armillaria cepistipes (Japan)
SFC20160927-39
AB539499 Armillaria cepistipes (Japan)
KMRB 15072104
SFC20160927-11
JF746911 Armillaria cepistipes (Finland)
JN657475 Armillaria cepistipes (Ukraine)
AB510778 Armillaria sinapina (Japan)
AB510777 Armillaria sinapina (Japan)
JQ677995 Armillaria sinapina (USA)
KMRB 15072116
AB510779 Armillaria ostoyae (Japan)
TPML20130926-84
TPML20130926-03
F20150917KCM08
EU251400 Armillaria ostoyae (Czech)
EU251402 Armillaria borealis (Czech)
JF746902 Armillaria borealis (Finland)
DQ435627 Armillaria gemina (USA)
JF313134 Armillaria gemina (USA)
JF895852 Armillaria solidipes (USA)
JQ678010 Armillaria ostoyae (USA)
DQ435651 Armillaria novae-zelandiae (Austrailia)
DQ435654 Armillaria novae-zelandiae (Indonesia)
DQ435648 Armillaria hinnulea (Austrailia)
DQ435649 Armillaria hinnulea (Austrailia)
DQ435645 Armillaria fumosa (Austrailia)
DQ435657 Armillaria luteobubalina (Austrailia)
DQ435655 Armillaria limonea (New Zealand)
DQ435656 Armillaria limonea (New Zealand)
DQ435633 Armillaria mellea (USA)
DQ435637 Armillaria mellea (Iran)
JF746924 Armillaria mellea (UK)
AB539512 Armillaria mellea (Japan)
DQ435640 Armillaria mellea (South Korea)
AB558992 Armillaria ectypa (Japan)
EU251403 Armillaria ectypa (Austrailia)
DQ435642 Armillaria tabescens (France)
JF746930 Armillaria tabescens (France)
KMRB 15091701
AB510805 Armillaria tabescens (Japan)
AB539514 Armillaria tabescens (Japan)
KMRB 15081103
SFC20160922-05
KU289109 Guyanagaster necrorhizus
KU289110 Guyanagaster necrorhizus
KU289105 Oudemansiella cubensis
KF530581 Strobilurus esculentus
0.05

FIGURE 2 Phylogenetic trees based on neighbour-joining (NJ) analysis of the partial translation elongation factor-1α region in *Armillaria* and *Desarmillaria* species worldwide. Branch support values are shown as percentages of bootstrap support (NJ), maximum likelihood (ML) bootstrap values and Bayesian posterior probabilities. Percentage of bootstrap support (1,000 replicates) greater than 50%, ML bootstrap values over 90 and Bayesian posterior probabilities over 95 are shown above tree branches. The scale bar indicates the number of nucleotide substitutions per site

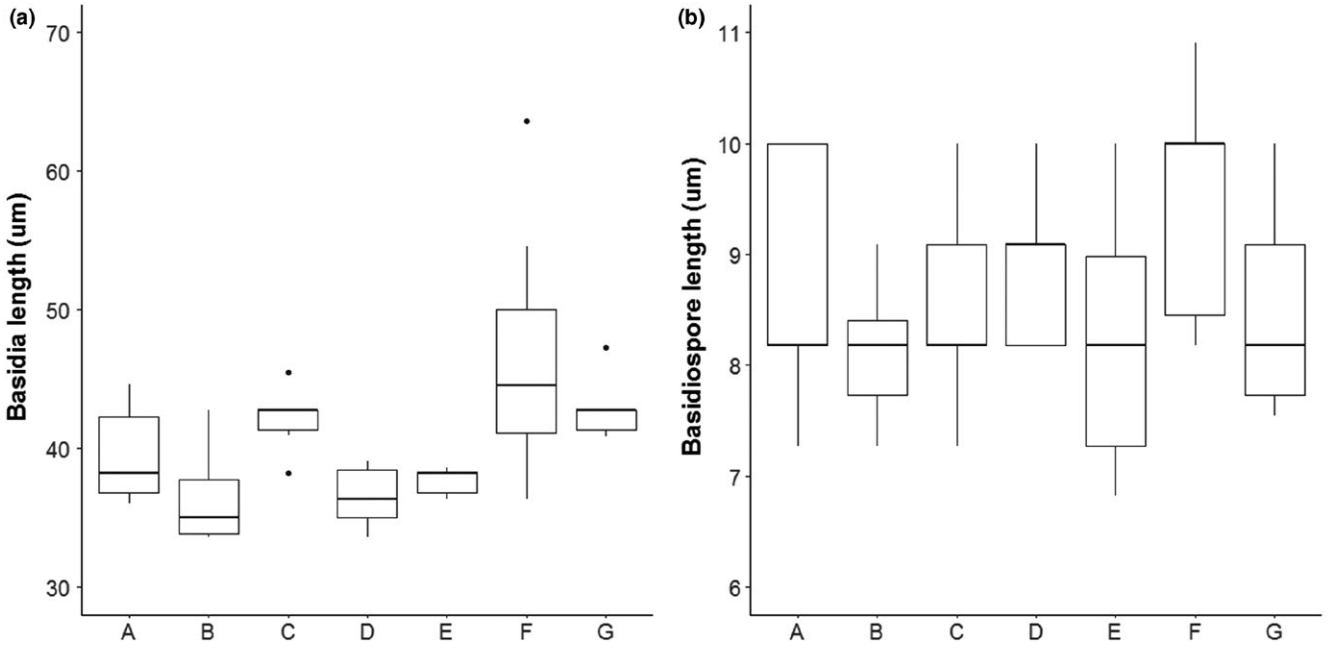

FIGURE 3 Comparison of microscopic feature of *Armillaria* and *Desarmillaria* in Korea. (a) Basidia length. (b) Basidiospore length. A: *A. cepistipes*, B: *A. gallica* clade 1, C: *A. gallica* clade 2, D: *A. gallica* clade 3, E: *A. nabsnona*, F: *A. ostoyae*, G: *D. tabescens*

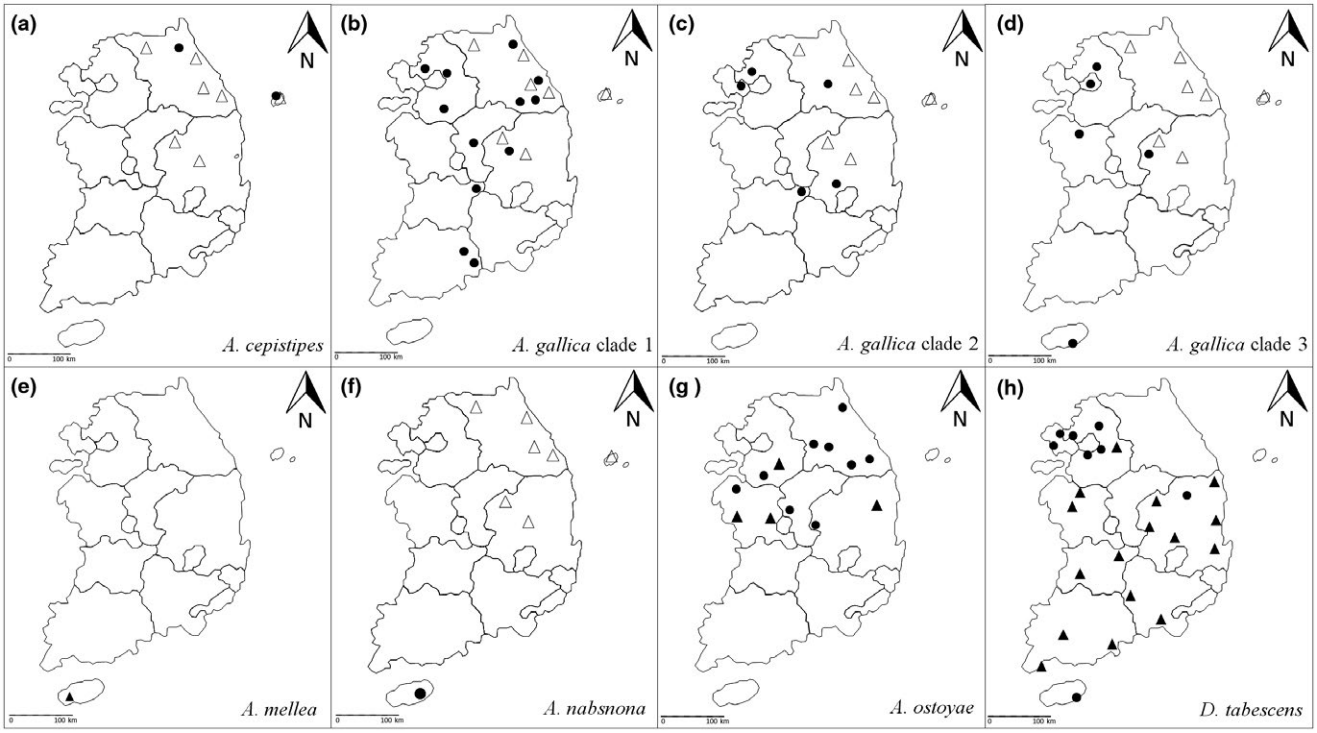

FIGURE 4 Spatial distribution of *Armillaria* and *Desarmillaria* in South Korea from fruiting bodies and eDNA. Fruiting body samples (closed circles), eDNA samples (closed triangles) and eDNA samples identified as *A. gallica* group (open triangles)

individual trees were not inspected for rhizomorphs and mycelial fans. Thus, it is likely that the distributions of species in these genera were underestimated because some localized infections and infestations likely escaped detection in areas where basidiomata were not found. In situations where *Armillaria*/*Desarmillaria* species did not form extensive rhizomorph networks, the probability of detection in soil via eDNA would likely decrease. For example, growth pattern of rhizomorphs varies among *Armillaria*/*Desarmillaria* species, and some species are less likely to form rhizomorphs (Heinzelmann, Prospero, & Rigling, 2017; Labbé et al., 2017; Mihail, Bruhn, & Leininger, 2002; Morrison, 2004; Redfern & Filip, 1991; Rishbeth, 1985). Nevertheless, *Armillaria*/*Desarmillaria* can be detected from eDNA if it is present in the soil as basidiospores, mycelia or rhizomorphs.

Root diseases caused by *A. mellea*, *A. ostoyae* and *D. tabescens* cause significant damage to woody plants globally (Baumgartner et al., 2011; Hood, Redfern, & Kile, 1991; Williams-Woodward, 2013). In our study, *A. ostoyae* and *D. tabescens* were widely distributed in South Korea. However, severe forest damage has not been reported in South Korea despite reports of Armillaria root disease caused by *A. ostoyae* in Gangwon province and other regions (Nam, Choi, Ko, & Lee, 2016). Reports of minor to mild damage in South Korean forests might be due to the presence of fewer susceptible tree species in these locations (e.g., Hood et al., 1991; Morrison & Mallett, 1996), a high ratio of adapted, mixed forests in South Korea or lack of close inspection; however, Armillaria root disease can also cause reduced forest growth in the absence of readily observable symptoms (Cruickshank et al., 2011; Morrison, Pellow, Norris, & Nemec, 2000). Both *A. ostoyae* and *D. tabescens* represent potential threats to South Korean forests, and these threats could be exacerbated by climate change (Klopfenstein, Kim, Hanna, Richardson, & Lundquist, 2009; Sturrock et al., 2011). For this reason, continued study and monitoring of *Armillaria*/*Desarmillaria* are warranted in South Korea.

In conclusion, we detected six species of *Armillaria*/*Desarmillaria* (*A. ostoyae*, *A. mellea*, *A. gallica*, *A. nabsnona*, *A. cepistipes* and *D. tabescens*), and three lineages of *A. gallica* in South Korea based on basidiomata (using ITS and *tef*1 sequence analysis) and eDNA from soil samples (using *Armillaria*/*Desarmillaria*-specific ITS primer). *Armillaria*/*Desarmillaria* identification could not be confirmed using morphological features alone. Identification was validated by *tef*1 sequence analysis, which found three clades that may represent cryptic species in the *A. gallica* complex. As characteristics of *A. gallica* may differ according to clade, further investigation is required to confirm the cryptic species occurring within this taxon and to determine their respective roles in Armillaria root disease and in symbioses with *G. elata* and *P. umbellatus*. The distribution of *Armillaria* species in South Korea was estimated using basidiomata and eDNA extracted from soil samples. Detecting *Armillaria* species with eDNA has its limitations in that small soil samples may not reflect the site and closely related species cannot always be differentiated. However, our data show that eDNA can provide a rapid detection method for monitoring and managing Armillaria root disease, while also contributing to cultivation of *G. elata* and *P. umbellatus*. The development of *Armillaria*/*Desarmillaria* species-specific primers facilitates more extensive sampling of basidiomata, soil, mycelial fans and rhizomorphs for systematic approaches in future studies.

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