Phylogenetic relationships among biological species of *Armillaria* from China

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Highlights

• Phylogenetic relationships among *Armillaria* species from China were determined.

• Four main phylogenetic lineages were identified for Chinese *Armillaria* isolates.

• Unnamed biological species from China grouped in the "*A. gallica* cluster".

Abstract

Fourteen Chinese Biological Species (CBS) of *Armillaria* were previously identified in a collection of Chinese isolates. CBS C, F, G, H, J, L, N and O remained unnamed, while the remaining isolates included *A. borealis, A. cepistipes, A. gallica, A. mellea, A. sinapina* and *A. tabescens.* CBS F was suggested to represent *A. singula* based on basidiocarp morphology. In this study, phylogenetic relationships between Chinese *Armillaria* isolates and those from other parts of the world were determined based on DNA sequence data. Results of this study suggest that CBS F might not represent *A. singula*, and that *A. monadelpha* (a name applied to the North American form of *A. tabescens* by some authors) and *A. tabescens* should be treated as a single species. Four main phylogenetic lineages, referred to as the *A. ostoyae*, *A. gallica*, *A. tabescens* and *A. mellea* clusters, were identified on the phylogenetic trees. The unnamed biological

species grouped within the "*A. gallica* cluster" and were phylogenetically closely related. The results of this study contribute to our current understanding of the systematics of *Armillaria* from South East Asia where these fungi are relatively poorly known.

Keywords: Fungal diagnostics, Intergenic spacer region one (IGS-1), Root rot, Transcription elongation factor one alpha (TEF-1α) gene

1. Introduction

Species of *Armillaria* (Fr.) Staude are well-known in China and other parts of the world where some are important pathogens mainly of woody plants (Shaw and Kile 1991; Baumgartner et al. 2011). Some *Armillaria* species are primary pathogens, causing the disease generally referred to as Armillaria root rot, which is considered amongst the most serious diseases of trees in boreal and temperate forests and various species damage high-value crops. Other species are important components of woody ecosystems by virtue of their saprophytic life strategy, where they contribute significantly to wood degradation (Gregory et al. 1991; Kile et al. 1991). *Armillaria* species also have an important role in the traditions of various Asian cultures as a source of nutrients or linked to traditional medicine (Hobbs 1986). For example, the mushroom fruiting structures of some edible species are utilized as a food source or used in the treatment of hypertension, neurasthenia and epilepsy (Hobbs 1986).

The taxonomy of *Armillaria* is largely based on the morphological and biological species concepts (Baumgartner et al. 2011). As additional species have been described, basidiocarp morphology has provided increasingly limited value and the biological species concept, reliant on reproductive isolation (Mayr 1942), has been increasingly useful (e.g., Morrison et al. 1985; Proffer et al. 1987; Dumas 1988; Coetzee et al. 2003b). This approach gained popularity in the late 1970's with the introduction of mating tests to differentiate *Armillaria* species (Korhonen 1978; Anderson and Ullrich 1979) and it remains a useful method in taxonomic studies. The morphological and biological species concepts have thus been applied to describe various taxa, including *A. mellea* subsp. *nipponica, A. sinapina, A. gallica, A. ostoyae, A. cepistipes, A. ectypa, A. jezoensis, A. singula, A. nabsnona* and various unnamed

biological species from South East Asia (Sung et al. 1989, 1992; Mohammed et al. 1994; Cha and Igarashi 1995; Sung et al. 1995; Ota et al. 1998, 2009).

In a relatively recent study, Qin et al. (2007) expanded current knowledge regarding the *Armillaria* species diversity in China. Using mating studies, fourteen Chinese Biological Species (CBS A to D and F to O) of *Armillaria* were identified among isolates that were collected from 15 provinces of northern and southern China. Eight CBS (C, F, G, H, J, L, N and O) were unnamed, while the remainder included *A. sinapina* (CBS A), *A. gallica* (CBS B), *A. solidipes* (CBS D), formerly treated as *A. ostoyae* (Burdsall and Volk 2008) and pending nomenclatural conservation (Redhead et al. 2011), *A. tabescens* (CBS I), heterothallic *A. mellea* (CBS K), homothallic *A. mellea* (CBS G, suggested to represent *A. mellea* subsp. *nipponica*) and *A. borealis* (CBS M). Based on morphological characteristics, Qin et al. (2007) suggested that CBS F could be *A. singula,* a species that has been reported from Japan (Cha et al. 1994). However, mating tests were not performed to support this assertion.

Mating studies done by Qin et al. (2007) showed that CBS I is compatible with tester strains of *A. tabescens* from Europe. The taxonomy of *A. tabescens* from Asia, Europe and North America is, however, controversial mainly because sexual compatibility studies have provided inconclusive results. Preliminary results of Darmono et al. (1992), based on sexual compatibility tests between North American strains of *A. tabescens* and one strain identified as *A. tabescens* from Italy, suggested that *A. tabescens* from the two continents represent the same taxon. In contrast, Guillaumin et al. (1993) found that strains from Europe identified as *A. tabescens* are intersterile with North American strains of this species. Kile et al. (1994) subsequently proposed that *A. tabescens* from North America should be treated as a distinct species and referred to as *A. monadelpha,* a name that is considered illegitimate by Volk and Burdsall (1995). Ota et al. (1998) reported that Japanese isolates were interfertile with European isolates but intersterile with one North American isolate of this species. Although this would resolve some of these discrepancies, a phylogenetic study of these species has not been undertaken.

On the basis of their basidiocarp and culture morphology, the Chinese biological species were assigned to one of the species clusters introduced by Korhonen (1995). These clusters comprise species that share morphological characteristics and that are

phylogenetically closely related. The clusters were referred to by Korhonen (1995) as the "*A. ectypa* cluster", "*A. gallica* cluster" (including *A. altimontana, A. calvescens, A. cepistipes, A. gallica, A. nabsnona, A. sinapina, A. singula* and *A. jezoensis*), *"A. mellea* cluster", "*A. ostoyae* cluster" (*A. ostoyae, A. borealis* and *A. gemina*) and *"A. tabescens* cluster" (*A. tabescens* and *A. monadelpha*). Based on their morphological characteristics, the unnamed biological species from China (C, F, H, J and L) were suggested to reside in the "*A. gallica* cluster", while CBS N and CBS O were not placed in any of the clusters (Qin et al. 2007). Despite the availability of techniques to resolve such questions, nothing is known regarding the phylogenetic relationships of the unnamed Chinese biological species with those of *Armillaria* spp. from other parts of the world.

Phylogenetic methods utilising DNA sequence data have been widely employed to elucidate the identity of field isolates of *Armillaria* (Coetzee et al. 2003a, b, 2005b; Keča et al. 2006; Sekizaki et al. 2008; Kikuchi and Yamaji 2010; Elías-Román et al. 2013) and to resolve the phylogenetic relationships of *Armillaria* species from various parts of the world (Maphosa et al. 2006; Coetzee et al. 2011). For phylogenetic inference, the internally transcribed spacer regions (ITS) and intergenic spacer region one (IGS-1) have been useful in studies focused on the relationships of taxa from Africa (Coetzee et al. 2005a), South America (Pildain et al. 2009), Australasia (Coetzee et al. 2001), North America (Anderson and Stasovski 1992), Europe (Chillali et al. 1998) and Asia (Terashima et al. 1998; Coetzee et al. 2000). In addition, sequences for part of the transcription elongation factor one alpha (TEF-1α) gene has been used to determine the phylogenetic relationships of taxa from Japan (Hasegawa et al. 2010), Europe (Tsykun et al. 2013) and a global collection of isolates of *Armillaria* species (Maphosa et al. 2006). Despite the importance of *Armillaria* in China, there have not been studies to determine the phylogenetic relationships of Chinese biological species.

The aims of this study were to address some of the unresolved questions that emerged from the research of Qin et al. (2007). The identity of the unnamed CBS F was considered and a species recognition approach based on gene genealogical concordance was followed to assess the suggested differentiation of European and South East Asian *A. tabescens* from its North American counterpart. An additional aim was to determine

the phylogenetic relationships between the Chinese biological species and *Armillaria* species from other regions of the world.

2. Materials and methods

2.1. Fungal isolates

Isolates included in this study that represent different Chinese Biological Species (Supplementary Table S1) were obtained from the culture collection of Dr. J. Zhao and were previously assigned to biological species in the study by Qin et al. (2007). Additional isolates from other parts of the world were also included to expand the geographical representation of *Armillaria* species in the Northern Hemisphere *Armillaria* phylogeny (Supplementary Table S2). Isolates were grown on malt yeast agar (MYA: 1.5% w/v malt extract, 0.2% w/v and yeast extract 1.5 % w/v agar) medium. Isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

2.2. DNA sequencing

DNA was extracted from isolates representing each of the CBS and other *Armillaria* species following the methods outlined in Coetzee et al. (2005a). PCR reaction conditions and mixtures were the same as those described by Coetzee et al. (2003b) for the IGS-1 region, and Maphosa et al. (2006) for the TEF-1α gene. The IGS-1 region was amplified for all isolates using primers P-1 (Hsiau 1996) and O-1 (Duchesne and Anderson 1990). Amplicons for the partial TEF-1α gene were obtained using primer pair EF595F / EF1160R (Kauserud and Schumacher 2001). Amplicons were purified with a MSB® Spin PCRapace purification kit (Invitek) following the instructions of the manufacturer prior to DNA sequencing. DNA sequences were obtained in both directions for each PCR product with the same primers used for their amplification. Sequencing reactions were done using a BigDye Terminator v3.1 cycle sequencing kit (ABI) following the protocol outlined by the manufacturer. Sequences were determined

on an ABI 3100 DNA automated sequencer. Base calling was visually inspected in CLC Main Workbench (CLC) and forward and reverse strands were assembled into contigs using the same software. The sequences obtained were used in DNA sequence similarity searches against those in GenBank using Blastn to ensure the identity of the isolates.

2.3. Phylogenetic methods

IGS-1 and TEF-1α sequences were obtained from GenBank for *Armillaria* species from other parts of the world (Supplementary Table S2). Sequences selected from GenBank were from well characterised isolates used in previously published studies (see Supplementary Table S2 for references). These sequences together with those for the isolates included in this study formed the IGS-1 and TEF-1α Northern Hemisphere *Armillaria* species matrices, respectively. All multiple sequence alignments (TreeBase Study number: S17215) were done using MAFFT (Katoh et al. 2013) and applying the default settings. Nucleic substitution models were determined with jModelTest (Posada 2008). Phylogenetic trees were generated based on maximum likelihood and Bayesian inference or maximum likelihood and parsimony. In each analysis, the IGS-1 and TEF-1α data were analysed separately. *Armillaria mellea* was used as the outgroup taxon in all analyses.

Maximum likelihood analyses were done using PHYML v. 3.0 (Guindon et al. 2010). The analyses incorporated substitution models that best fitted the individual data sets (Supplementary Table S3) and these were applied using a custom model setting in PHYML. The maximum likelihood trees that were obtained were rooted to *A. mellea*. Confidence levels for the nodes were obtained through a bootstrap analysis (1000 replicates) using the same settings employed to search tree-space for the fundamental maximum likelihood tree.

Bayesian inference of phylogenies was determined using MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001). The likelihood and prior settings were based on the models for each dataset obtained from the analyses using jModelTest (Supplementary Table S3). For analyses of the combined data sets, a model, specific to each data partition was used. Posterior probability distributions were obtained by setting the Markov Chain Monte Carlo (MCMC) function to 4×10^6 generations for each analysis

with a sampling frequency of every 100th tree. Posterior probability values were calculated after excluding (burnin) 25% of the trees generated during the MCMC analysis. ESS (Estimated Sample Size) values for the parameters were subsequently assessed in Tracer v. 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) as a measure of convergence. The trees generated were viewed in FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) to obtain tree topologies and the posterior probability values for their nodes.

Parsimony analyses were conducted using PAUP ver. 4 (Swofford 2002) and employed a heuristic tree search algorithm with 10 replicates of random addition of sequences and TBR branch swapping. Confidence levels at the nodes were determined using bootstrap analyses (1000 replicates) with the same settings but with the addition of sequences set to "closest". Missing, ambiguous and uninformative characters were excluded in all parsimony-based analyses.

2.4. Assessing the conspecificity of CBS F and A. singula

IGS-1 sequences generated for isolates belonging to CBS F were compared with sequences of *Armillaria* in GenBank using Blastn searches. The available IGS-1 sequence (D89926) for *A. singula* in GenBank was downloaded and aligned with sequences of CBS F. Percentage similarity, converted from p-distances, was then determined in MEGA 6 (Tamura et al. 2013) and compared against those obtained from Blastn searches for other *Armillaria* species. In addition, the phylogenetic placement of CBS F and *A. singula* was assessed in a phylogenetic tree generated for the Northern Hemisphere *Armillaria* species.

2.5. Phylogeny of isolates from Europe, North America and South East Asia identified as A. tabescens

In addition to the *A. tabescens* sequences included in Northern Hemisphere *Armillaria* species matrices, other available IGS-1 and TEF-1α DNA sequences for this species were downloaded from GenBank. These were aligned with sequences of *A. tabescens* and *A. mellea* generated in this study (Supplementary Table S1). Phylogenetic trees

were obtained separately for the IGS-1 and TEF-1α data matrices based on parsimony and maximum likelihood. The grouping of isolates representing *A. tabescens* was also assessed in the context of a Northern Hemisphere *Armillaria* species phylogeny.

2.6. Phylogenetic relationships of Chinese Armillaria *species with those from other parts of the world*

Phylogenetic trees were constructed from the IGS-1 and TEF-1α Northern Hemisphere *Armillaria* species matrices separately based on maximum likelihood and Bayesian analyses as described above.

3. Results

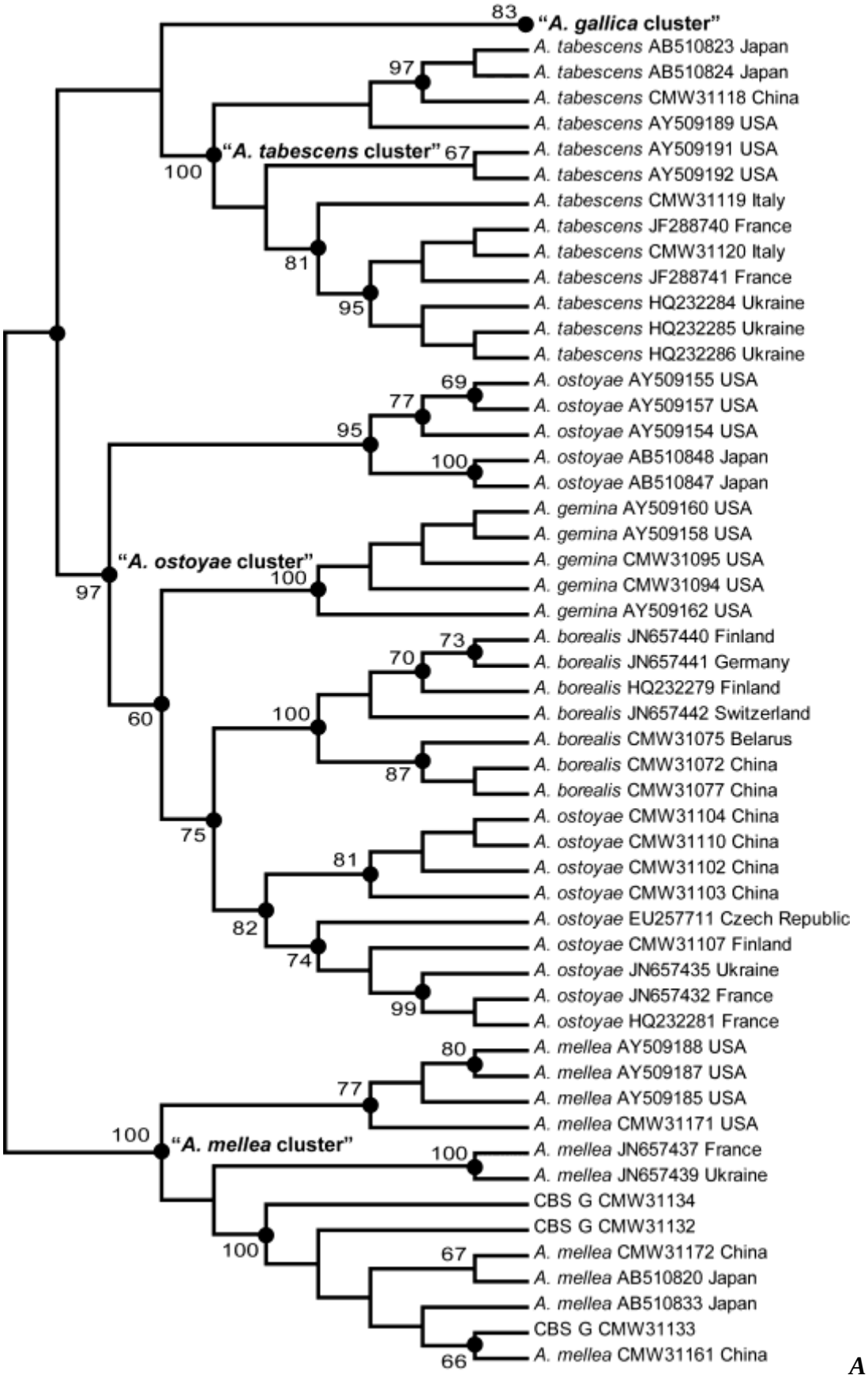
3.1. Amplification of the IGS-1 and TEF-1α regions

The IGS-1 and EF-1α regions consistently yielded a single band after PCR. The amplicon size for the IGS-1 region varied among the CBS, ranging from 600 bp to 900 bp. Amplification of the EF-1α yielded an amplicon size of approximately 600 bp.

3.2. Assessing the conspecificity of CBS F and A. singula

Isolates belonging to CBS F and considered by Qin et al. (2007) to represent *A. singula* had the highest IGS-1 sequence similarity to those of *A. cepistipes*, *A. gallica* and *A. altimontana* (99% similarity) in GenBank. In contrast, sequence comparisons after aligning the IGS-1 sequence of *A. singula* from GenBank with those for the isolates representing CBS F revealed a 98% sequence similarity.

Phylogenetic trees generated from the IGS-1 data matrix separated the isolates representing CBS F and the sequence of *A. singula* in well supported monophyletic groups (Fig. 1). *Armillaria singula* grouped closest to *A. gallica* from Japan (PP = 1, bootstrap = 91%) within a monophyletic group that included *A. jezoensis* (PP = 0.96, bootstrap = 68%) (Fig. 1). Together this group formed a monophyletic group with *A.*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A. borealis JN657442 Switzerland
A. borealis CMW31075 Belarus
A. borealis CMW31072 China
A. borealis CMW31077 China
A. ostoyae CMW31104 China
A. ostoyae CMW31110 China
A. ostoyae CMW31102 China
A. ostoyae CMW31103 China
A. ostoyae EU257711 Czech Republic
A. ostoyae CMW31107 Finland
A. ostoyae JN657435 Ukraine
A. ostoyae JN657432 France
A. ostoyae HQ232281 France
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A. mellea AY509187 USA
A. mellea AY509185 USA
A. mellea CMW31171 USA
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A. mellea JN657439 Ukraine
CBS G CMW31134
CBS G CMW31132
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CBS G CMW31133
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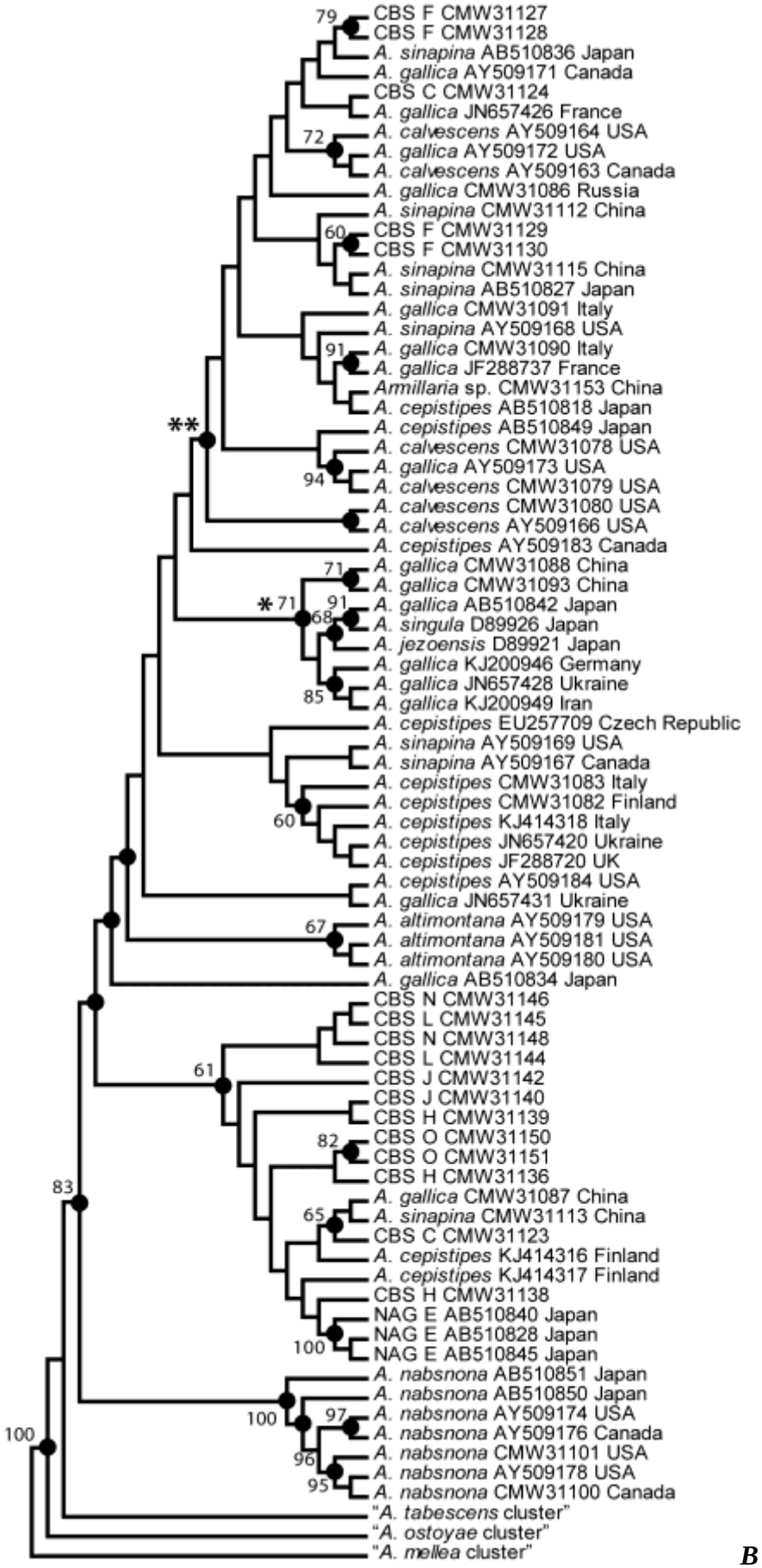
A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CBS C CMW31124
A. gallica JN657426 France
72
A. calvescens AY509164 USA
A. gallica AY509172 USA
A. calvescens AY509163 Canada
A. gallica CMW31086 Russia
A. sinapina CMW31112 China
60
CBS F CMW31129
CBS F CMW31130
A. sinapina CMW31115 China
A. sinapina AB510827 Japan
A. gallica CMW31091 Italy
A. sinapina AY509168 USA
91
A. gallica CMW31090 Italy
A. gallica JF288737 France
Armillaria sp. CMW31153 China
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**
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71
A. gallica CMW31088 China
A. gallica CMW31093 China
*71
91
A. gallica AB510842 Japan
68
A. singula D89926 Japan
A. jezoensis D89921 Japan
A. gallica KJ200946 Germany
A. gallica JN657428 Ukraine
85
A. gallica KJ200949 Iran
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60
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67
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CBS L CMW31145
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CBS L CMW31144
61
CBS J CMW31142
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CBS H CMW31139
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CBS O CMW31150
CBS O CMW31151
CBS H CMW31136
83
A. gallica CMW31087 China
65
A. sinapina CMW31113 China
CBS C CMW31123
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CBS H CMW31138
NAG E AB510840 Japan
100
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97
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96
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95
A. nabsnona CMW31100 Canada
"A. tabescens cluster"
"A. ostoyae cluster"
"A. mellea cluster"

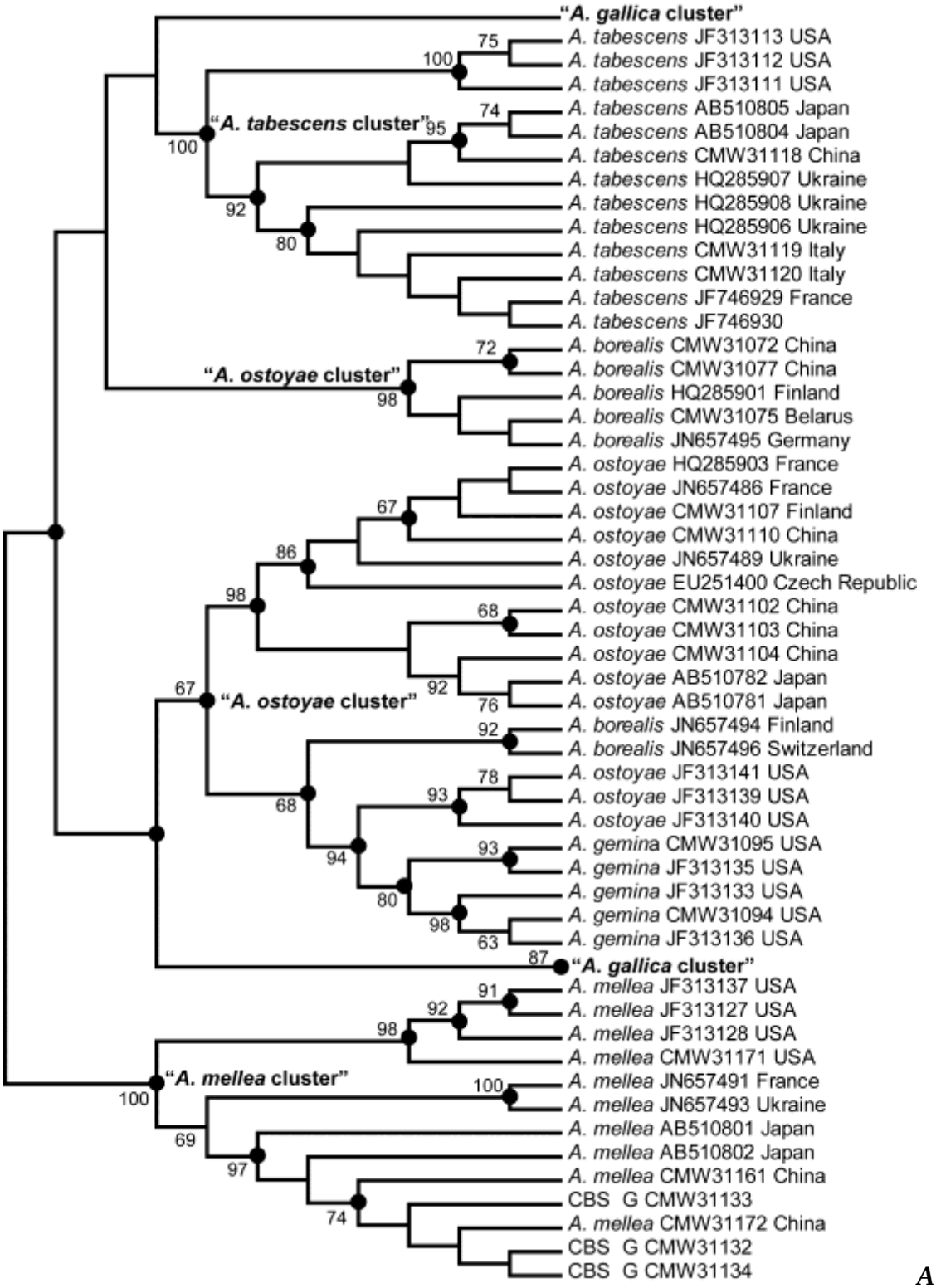
B

Fig. 1 – Phylogenetic tree generated from IGS-1 sequences based on maximum likelihood and converted to a cladogram for a collection of Northern Hemisphere *Armillaria* species. A: Phylogenetic relationships of species in the "*A. mellea", "A. ostoyae"* and "*A. tabescens"* clusters with isolates in the "*A. gallica* cluster" collapsed to a single terminal node. B: Phylogeny of species in the "*A. gallica* cluster" with isolates in the *A. mellea*, *A. ostoyae* and *A. tabescens* clusters collapsed to single terminal nodes. Bootstrap values (≥ 60%) based on maximum likelihood are indicated at the nodes. Posterior probability values (≥ 0.90) are indicated with circles at the nodes. The four main lineages are shown on the branches of the tree. (*) node shared by *A. gallica*, *A. jezoensis* and *A. singula* (PP = 1, bootstrap = 71%). (**) node shared by CBS F, CBS C (CMW31124) *A. calvescens*, *A. cepistipes*, *A. gallica* and *A. sinapina* (PP = 1, bootstrap = 58%).

gallica from Europe and China (PP = 1, bootstrap = 71%). Isolates belonging to CBS F resided in monophyletic group that constituted *A. calvescens, A. cepistipes, A. gallica* and *A. sinapina* (PP = 1, bootstrap = 58%) (Fig. 1).

3.3. Phylogeny of isolates from Europe, North America and South East Asia identified as A. tabescens

Phylogenetic trees generated for the Northern Hemisphere collection of *Armillaria* species placed all isolates of *A. tabescens* in a strongly supported monophyletic group (both IGS-1 and TEF-1α: PP = 1, bootstrap = 100%) (Figs. 1A, 2A). Phylogenetic trees generated for isolates from China and a larger collection of sequences obtained from GenBank for *A. tabescens* yielded incongruent topologies for the phylogenetic trees generated from IGS-1 and TEF-1α sequences (Supplementary Figure S1). Trees obtained from IGS-1 sequence data placed the isolates from Japan and China in a clade that included sequences for *A. tabescens* from North America. Trees based on TEF-1α sequences grouped the isolates from Asia with sequences of *A. tabescens* originating in Europe. In all phylogenetic trees, the Asian isolates formed a sub-clade with high bootstrap support (IGS-1: maximum likelihood 99%, parsimony 100%; TEF-1α: 86 % for both analyses).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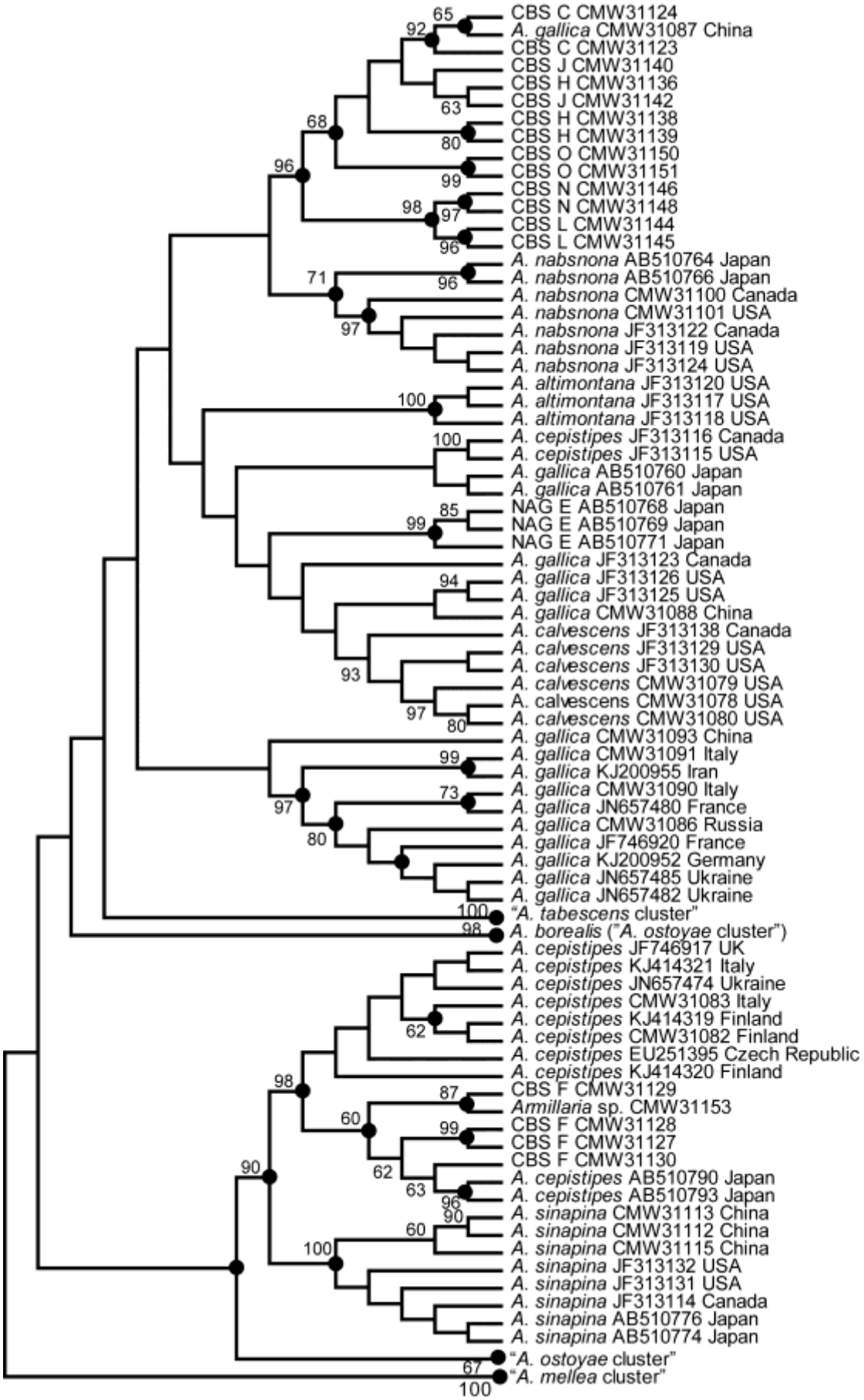
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A. gallica JN657485 Ukraine
A. gallica JN657482 Ukraine
"A. tabescens cluster"
A. borealis ("A. ostoyae cluster")
A. cepistipes JF746917 UK
A. cepistipes KJ414321 Italy
A. cepistipes JN657474 Ukraine
A. cepistipes CMW31083 Italy
A. cepistipes KJ414319 Finland
A. cepistipes CMW31082 Finland
A. cepistipes EU251395 Czech Republic
A. cepistipes KJ414320 Finland
CBS F CMW31129
Armillaria sp. CMW31153
CBS F CMW31128
CBS F CMW31127
CBS F CMW31130
A. cepistipes AB510790 Japan
A. cepistipes AB510793 Japan
A. sinapina CMW31113 China
A. sinapina CMW31112 China
A. sinapina CMW31115 China
A. sinapina JF313132 USA
A. sinapina JF313131 USA
A. sinapina JF313114 Canada
A. sinapina AB510776 Japan
A. sinapina AB510774 Japan
"A. ostoyae cluster"
"A. mellea cluster"

B

Fig. 2 – Phylogenetic tree, based on maximum likelihood analysis of TEF-1α sequences and converted to a cladogram, showing the relationships isolates for a collection of Northern Hemisphere *Armillaria* species. A: Phylogenetic relationships of species in the "*A. mellea", "A. ostoyae"* and "*A. tabescens"* clusters with isolates in the "*A. gallica* cluster" collapsed to a single terminal node. B: Phylogeny of species in the "*A. gallica* cluster" with isolates in the "*A. mellea"*, "*A. ostoyae"* and "*A. tabescens"* clusters collapsed to single terminal nodes. Bootstrap values (≥ 60%) based on maximum likelihood are indicated at the nodes. Posterior probability values (≥ 0.90) are indicated with circles at the nodes.

3.4. Phylogenetic relationships of Chinese Armillaria *species with those from other parts of the world*

Phylogenetic trees generated from the different data matrices differed in their resolution and grouping of *Armillaria* species (Figs. 1, 2). Phylogenetic trees generated from the IGS-1 sequence data generally yielded high bootstrap support at the nodes (Fig. 1). In contrast, trees generated from the TEF-1α sequence data had low resolution at the deeper nodes (Fig. 2). In general, four groups emerged from the IGS-1 sequence matrix (Fig. 1). Following the *Armillaria* cluster names of Korhonen (1995), these groups are referred as the "*A. mellea*", "*A. tabescens*", "*A. ostoye*" and "*A. gallica*" clusters, respectively.

The "*A. ostoyae* cluster" included isolates belonging to *A. borealis*, *A. ostoyae* and *A. gemina*. Isolates representing *A. gemina* clustered in a well supported group in all trees generated from the IGS-1 (Fig. 1A) and TEF-1α (Fig. 2A) sequence data. Isolates belonging to *A. borealis* were placed in a strongly supported group based on IGS-1 sequence data (Fig. 1A). However, isolates of this species from China, Belarus, Germany and Finland were placed distant to isolates belonging to the same species from Finland and Switzerland as well as *A. gemina* and *A. ostoyae* on the trees obtained from the TEF-1α sequence matrix (Fig. 2A). Trees generated from the IGS-1 sequence matrix placed isolates of *A. ostoyae* from China and Europe in two distinct groups, and together they formed a sister group to *A. borealis* (Fig. 1A). Isolates of *A. ostoyae* from Japan and the USA grouped sister to *A. gemina*, *A. borealis* and the cluster that included isolates of this species from Europe and China (Fig. 1A). Trees generated from the TEF-1α sequence matrix grouped isolates of *A. ostoyae* from China, Japan and Europe in a well supported group, while isolates from the USA formed a group with high support and were placed sister to *A. gemina* (Fig. 2A).

The "*A. gallica* cluster" included *A. calvescens*, *A. cepistipes*, *A. gallica*, *A. sinapina*, *A. nabsnona* and the unnamed biological species CBS C, CBS F, CBS H, CBS J, CBS L, CBS N and CBS O (Figs. 1B, 2B). With the exception of some species, most of the isolates could not be separated into monophyletic groups representing their respective species assignments. In this cluster, isolates representing *A. altimontana*, *A. nabsnona* and NAG E were placed in their distinctive species groups with high statistical support on trees generated from the IGS-1 (Fig. 1B) and TEF-1α (Fig. 2B) matrices, respectively. Isolates representing *A. calvescens* formed a strongly supported monophyletic group in phylogenetic trees obtained from the IGS-1 sequence matrix (Fig. 1B), but this was not the case for phylogenetic trees based on the TEF-1α matrix (Fig. 2B). With the exception of CBS J and H, all remaining isolates belonging to CBS grouped together forming their respective species groups based on TEF-1α sequence data (Fig. 2B). *Armillaria nabsnona* formed a sister group with the remaining species having strong bootstrap support and PP = 1 based on the IGS-1 sequences (Fig. 1B). Isolates residing in CBS C (only isolate CMW31123), H, J, L, N, O grouped together with NAG E, *A. cepestipes*, *A. gallica* and *A. sinapina* on the trees generated from IGS-1 sequences (Fig. 1B). The grouping of CBS C, H, J, L, N, O and *A. gallica* (CMW31087) was supported by the trees generated from TEF-1α sequences (Fig. 2B). Within this group, CBS L and CBS N were placed sister to each other with high bootstrap support and posterior probability (Fig. 2B). Chinese biological species F together with an isolate belonging to CBS C (CMW31124) clustered with isolates representing *A. calvescens*, *A. cepistipes*, *A. gallica* and *A. sinapina* based on the IGS-1 sequence matrix (Fig. 1B). CBS F clustered with isolates belonging to *A. cepistipes* (PP = 0.98, bootstrap = 98%) and together, these species were grouped sister to *A. sinapina* based on the TEF-1α sequence data (Fig. 2B).

4. Discussion

Although *Armillaria* spp. are common in China, very little work has been done to identify these fungi. This is the first study to apply DNA sequence analyses to consider the identity of a relatively large collection of isolates from the country, and to assess the phylogenetic relationships among these isolates and those known from other parts of the

world. The specific aims of this study were to determine the identity of the unnamed CBS F, to consider the suggestion that *A. tabescens* from North America should be treated as a species different from its European and Asian counterparts and to determine the phylogenetic relationships of *Armillaria* species from China.

4.1. Identity of CBS F

Qin et al. (2007) suggested that CBS F and *A. singula* are conspecific on the basis of their basidiocarp morphology. *Armillaria singula* was described from Hokkaido (Cha et al. 1994) and it has not been found elsewhere (Ota et al. 1998). There is only one IGS-1 sequence for this species (Terashima et al.1998) and no living cultures are known to exist (Ota et al. 2012).

Results of Blastn searches and phylogenetic analyses of the IGS-1 region revealed that CBS F possibly represents an undescribed *Armillaria* sp. other than *A. singula* but closely related to *A. calvescens, A. cepistipes, A. gallica* and *A. sinapina.* IGS-1 DNA sequences of isolates belonging to CBS F were most similar to those of *A. cepistipes, A. gallica* and *A. altimontana* on GenBank. Phylogenetic trees generated from IGS-1 sequences grouped CBS F distant from *A. singula* and together with *A. calvescens, A. cepistipes, A. gallica* and *A. sinapina* with high bootstrap support and high posterior probability. Phylogenetic trees based on TEF-1α sequences grouped representatives of CBS F and *A. cepistipes* together with high bootstrap support. Isolates of CBS F were, however, sexually incompatible with those of *A. cepistipes, A. gallica* and *A. sinapina* in the study of Qin et al. (2007). Although the results of the current study are not conclusive, given the fact that IGS-sequences could not resolve isolates identified as *A. gallica, A. cepistipes* and *A. sinapina* into their respective species groups on the phylogenetic trees, it suggests that CBS F represents a novel taxon. Future research should focus on obtaining isolates belonging to *A. singula* so that mating tests and phylogenetic studies can be conducted in order to reach a definitive identification.

4.2. Are Armillaria tabescens *isolates from Asia, Europe and North America conspecific?*

Results of this study suggest that *A. tabescens* from Asia, Europe and North America should be treated as a single taxon. Phylogenetic trees grouped isolates of *A. tabescens* from various parts of the world in a strongly supported monophyletic group on trees generated in this study for a large collection of isolates belonging to different *Armillaria* species. Phylogenetic trees placed isolates of *A. tabescens* from China in a monophyletic group with those from Japan. Trees generated from IGS-1 and TEF-1α sequence data were, however, incongruent in the placement of this group relative to isolates of *A. tabescens* from Europe and North America. Although only two loci were used in this study, application of genealogical concordance phylogenetic recognition (Taylor et al. 2000) indicates that these isolates are conspecific. Results of the present study thus support the view that *A. tabescens* from Asia, Europe and North America should be treated as a single taxon. However, further studies including a larger collection and a broader distribution of isolates from the Northern Hemisphere should be undertaken to confirm these results.

4.3. Phylogeny of Armillaria *species from China*

Phylogenetic trees generated from the IGS-1 region and partial TEF-1α gene for the collection of isolates from China used in this study generally resolved four main phylogenetic groups. These are referred to as the "*A. ostoyae*", "*A. gallica*", "*A. tabescens*" and "*A. mellea*" clusters and they are more or less consistent with those defined by Korhonen (1995). The "*A. ostoyae* cluster" included *A. ostoyae* (CBS D), *A. borealis* (CBS M) and *A. gemina*. The "*A. gallica* cluster" formed the largest group and included *A. gallica* (CBS B), *A. sinapina* (CBS A), CBS C, CBS F, CBS H, CBS J, CBS K, CBS L, CBS N and CBS O as well as *A. altimontana*, *A. calvescens*, *A. cepistipes*, *A. nabsnona* and NAG E. The "*A. mellea* cluster" was represented by isolates belonging to *A. mellea* s.s. (CBS K) and CBS G.

The unnamed *Armillaria* Chinese biological species H, J, L, N and O were suggested to be closely related to members of the *A. gallica* cluster based on the

characteristics of their basidiocarps (Qin et al. 2007). This view was supported in the present study where isolates representing these biological species formed a monophyletic group that included *A. gallica* and its closest relatives in the phylogenetic trees generated from IGS-1 and TEF-1α sequences. Trees obtained from the IGS-1 sequences also showed a close relationship between these biological species and NAG E from Japan, although this only had low bootstrap support. The phylogenetic relationships among the Chinese biological species could not be resolved based on IGS-1 sequences due to poor phylogenetic resolution. In contrast, TEF-1α sequences provided better resolution for these biological species. Phylogenetic trees generated from the latter sequences revealed a close relationship between CBS L and CBS N and that they have a sister relationship with CBS C, CBS H, CBS J and CBS O.

Determining the phylogeny of *Armillaria* species from China was complicated by gene trees that differed in their topologies and phylogenetic resolution. Incongruence in the placement of isolates belonging to CBS C, *A. borealis, A. cepistipes, A. gallica, A. sinapina* and *A. ostoyae,* was observed on trees generated respectively from IGS-1 and TEF-1α sequences in this study. Phylogenetic trees obtained from the IGS-1 region grouped the isolates belonging to CBS C in two different clusters, while they grouped together on trees generated from TEF-1α sequences. Similarly, isolates identified as *A. sinapina* were placed at different positions on trees generated from the IGS-1 region, while they formed a monophyletic group on the tree obtained from TEF-1α sequences. Isolates belonging to *A. borealis* formed a monophyletic group in the tree generated from the IGS-1 matrix, while they were separated into distantly related groups on the TEF-1α phylogenetic trees. Isolates belonging to *A. ostoyae* were grouped in two monophyletic groups on trees obtained from the IGS-1 and TEF-1α matrices. Isolates of *A. gallica* and *A. cepistipes* were scattered within the "*A. gallica* cluster" in trees generated for both loci, however, isolates of *A. gallica* from Europe and Iran formed a monophyletic group on the tree obtained from TEF-1α sequences. The discordance between the gene trees could be ascribed to incomplete lineage sorting as result of recent divergence (Maddison 1997). A larger sample size and additional gene regions would be required to resolve this question, but the results are congruent with those of earlier studies (Maphosa et al. 2006; Mulholland et al. 2012; Ross-Davis et al. 2012;

Tsykun et al. 2013) suggesting that the TEF-1α gene will be well- suited for species identification based on sequence comparisons.

The results of this study contribute to our current understanding of the systematics of *Armillaria*, and more specifically *Armillaria* species from South East Asia. With the exception of a few phylogenetic studies that have focused on the species occurring in Japan (Terashima et al. 1998; Hasegawa et al. 2010; Ota et al. 2012), nothing was previously known regarding the phylogeny of Chinese *Armillaria* species prior to this study. This study also expanded the current IGS-1 and TEF-1α DNA sequence database for *Armillaria* species and the data can now be employed in future research to identify field isolates from China using sequence comparisons. Clearly, many questions remain regarding the identity of the genus *Armillaria* from China. In this regard, the most important challenge ahead will be to collect isolates linked to sporocarps and to study these using all available taxonomic tools for *Armillaria*. There are clearly numerous novel species in China and these deserve to be named and studied.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

Acknowledgments

We thank the members of the Tree Protection Co-operative Programme (TPCP), the National Research Foundation (NRF), the THRIP initiative of the Department of Trade and Industry and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) South Africa for financial support. We are sincerely grateful to Dr K. Korhonen for providing us with cultures of *Armillaria*, without which this research would not have been possible.

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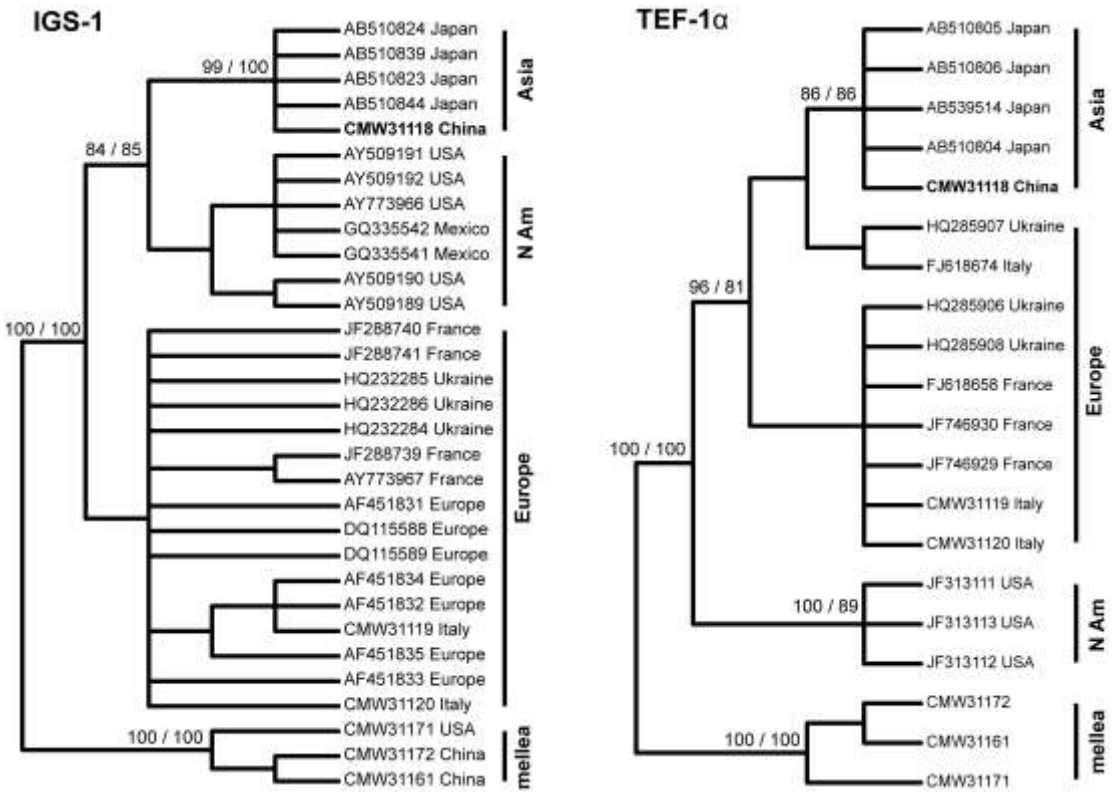
Supplementary data



Supplementary Fig. S1 – Cladograms showing the relationship of isolates belonging to *A. tabescens* that originated from China, Japan, Europe and North America. Parsimony analysis yielded 26 most parsimonious trees for the IGS-1 matrix with tree lengths (TL) of 164 steps, consistency index (CI) = 0.96 and retention index (RI) = 0.98. The TEF-1α matrix yielded four most parsimonious trees with TL = 69 steps, CI = 0.96 and RI = 0.98. Bootstrap values obtained from parsimony are shown at the nodes followed by those determined through maximum likelihood. *Armillaria mellea* was used as the outgroup taxon to root the trees.

Supplementary Table S1 – Isolates from China used to determine the phylogenetic species among biological species of *Armillaria* from China.

Species	**CMW[a] culture no.**	**Alternative no.**		**GenBank accession no.**	
		KK[a]	Qin[a]	IGS-1	TEF-1α
Armillaria borealis	CMW31072	3348/5	01015	KM205304	KM205251
	CMW31077	3354/1	01013	KM205306	KM205253
A. gallica	CMW31087	3399/1	96027	KM205313	KM205260
	CMW31088	3374/1	02147	KM205314	KM205261
	CMW31093	3395/1	96011	KM205317	KM205264
A. mellea	CMW31161	3336/5	00109	KM205320	KM205267
	CMW31172	3329	00017	KM205322	KM205269
A. ostoyae	CMW31102	3412/1	97058-B	KM205325	KM205272
	CMW31103	3403/1	96043	KM205326	KM205273
	CMW31104	3404/1	96044	KM205327	KM205274
	CMW31110	3401/1	96035	KM205329	KM205276
A. sinapina	CMW31112	3393/1	93012	KM205330	KM205277
	CMW31113	3397/1	96015	KM205331	KM205278
	CMW31115	3425/1	99104	KM205332	KM205279
A. tabescens	CMW31118	3480/16	99022	KM205333	KM205280
Armillaria sp.	CMW31153	3344	01009	KM205336	KM205283
CBS[b] C	CMW31123	3428/8	99110	KM205337	KM205284
	CMW31124	3409/2	97047	KM205338	KM205285
CBS F	CMW31127	3355/1	01107	KM205339	KM205286
	CMW31128	3424/1	99102	KM205340	KM205287
	CMW31129	3405/1	96060	KM205341	KM205288
	CMW31130	3426/1	99107	KM205342	KM205289
CBS G	CMW31132	3345/1	01010	KM205343	KM205290
	CMW31133	3422/1	99044	KM205344	KM205291
	CMW31134	3356/1	02001	KM205345	KM205292
CBS H	CMW31136	3419/1	99012	KM205346	KM205293
	CMW31138	3320/6	00006	KM205347	KM205294
	CMW31139	3328/1	00015	KM205348	KM205295

Supplementary Table S1 (continued)

Species	**CMW[a] culture no.**	**Alternative no.**		**GenBank accession no.**	
		KK[a]	Qin[a]	IGS-1	TEF-1α
CBS J	CMW31140	3154/1		KM205349	KM205296
	CMW31142	3333/1	00101	KM205350	KM205297
CBS L	CMW31144	3342/1	00125	KM205351	KM205298
	CMW31145	3343/1	00126	KM205352	KM205299
CBS N	CMW31146	3365/3	02068	KM205353	KM205300
	CMW31148	3363/3	02066	KM205354	KM205301
CBS O	CMW31150	3369/2	02072	KM205355	KM205302
	CMW31151	3369/13	02072	KM205356	KM205303

[a] Culture collection abbreviations: Culture M. Wingfield (CMW), K. Korhonen (KK) and G.-F. Qin (Qin).

[b] Chinese Biological Species

Supplementary Table S2 – Additional isolates used in this study and sequences obtained from GenBank.

Species	Isolate	Country	IGS	TEF-1α	Reference [a]
Armillaria altimontana	837	Idaho, USA	AY509179	JF313120	Kim et al. (2006), Ross-Davis et al. (2012)
	D82	Idaho, USA	AY509180	JF313118	Kim et al. (2006), Ross-Davis et al. (2012)
	POR100	Idaho, USA	AY509181	JF313117	Kim et al. (2006), Ross-Davis et al. (2012)
A. borealis	A1	Finland	JN657440	JN657494	Tsykun et al. (2013)
	A5	Germany	JN657441	JN657495	Tsykun et al. (2013)
	A2	Finland	HQ232279	HQ285901	Tsykun et al. (2013)
	A618	Switzerland	JN657442	JN657496	Tsykun et al. (2013)
	CMW31075, KK0124/1	Belarus	KM205305	KM205252	This study
A. calvescens	ST3	Quebec, Canada	AY509163	JF313138	Kim et al. (2006), Ross-Davis et al. (2012)
	ST17	Michigan, USA	AY509164	JF313130	Kim et al. (2006), Ross-Davis et al. (2012)
	ST18	Michigan, USA	AY509166	JF313129	Kim et al. (2006), Ross-Davis et al. (2012)
	CMW31078, KK3437/1	USA	KM205307	KM205254	This study
	CMW31079, KK3438/1	USA	KM205308	KM205255	This study
A. calvescens	CMW31080, KK3456/1	USA	KM205309	KM205256	This study
A. cepistipes	BRNM706814	Czech Republic	EU257709	EU251395	Antonın et al. (2009)
	SY1Ra	UK	JF288720	JF746917	Mulholland et al. (2012)

Supplementary Table S2 (continued)

Species	Isolate	Country	IGS	TEF-1α	Reference [a]
A. cepistipes	B2	Finland	KJ414316	KJ414319	Tsykun et al.(2013), Keča et al. (2014)
	EB3	Finland	KJ414317	KJ414320	Mulholland et al. (2012), Keča et al. (2014)
	B5	Italy	KJ414318	KJ414321	Tsykun et al. (2013), Keča et al. (2014)
	C13AE	Ukraine	JN657420	JN657474	Tsykun et al. (2013)
	94_46_01	Fukushima, Japan	AB510849	AB510793	Hasegawa et al. (2010)
	90-10-12	Niigata, Japan	AB510818	AB510790	Hasegawa et al. (2010)
	S20	British Columbia, Canada	AY509183	JF313116	Kim et al. (2006), Ross-Davis et al. (2012)
	W113	Washington, USA	AY509184	JF313115	Kim et al. (2006), Ross-Davis et al. (2012)
	CMW31082, KK9908/2	Finland	KM205310	KM205258	This study
	CMW31083, KK3160/2	Italy	KM205311	KM205258	This study
A. gallica	86-016/3	Munchen, Germany	KJ200946	KJ200952	Keča et al. (2014)
	86-032/1	Iran	KJ200949	KJ200955	Keča et al. (2014)
	Y7C-S1	Ukraine	JN657431	JN657485	Tsykun et al. (2012)
	E5	France	JF288737	JF746920	Mullholland et al. (2012)
	E6	France	JN657426	JN657480	Tsykun et al. (2013)
	HY2a	Ukraine	JN657428	JN657482	Tsykun et al. (2013)
	NA13	Japan	AB510842	AB510760	Hasegawa et al. (2010)
	NA4	Japan	AB510834	AB510761	Hasegawa et al. (2010)

Supplementary Table S2 (continued)

Species	Isolate	Country	IGS	TEF-1α	Reference [a]
A. gallica	M70	British Columbia, Canada	AY509171	JF313123	Kim et al. (2006), Ross-Davis et al. (2012)
	ST22	Michigan, USA	AY509172	JF313126	Kim et al. (2006), Ross-Davis et al. (2012)
	ST23	Wisconsin, USA	AY509173	JF313125	Kim et al. (2006), Ross-Davis et al. (2012)
	CMW31086, KK5298/2	Russia	KM205312	KM205259	This study
	CMW31090, KK8104/3	Italy	KM205315	KM205262	This study
	CMW31091, KK3090/2	Italy	KM205316	KM205263	This study
A. gemina	ST8	New York, USA	AY509158	JF313136	Kim et al. (2006), Ross-Davis et al. (2012)
	ST9	New York, USA	AY509160	JF313135	Kim et al. (2006), Ross-Davis et al. (2012)
	ST11	West Virginia, USA	AY509162	JF313133	Kim et al. (2006), Ross-Davis et al. (2012)
	CMW31094, 3443/1	USA	KM205318	KM205265	This study
	CMW31095, KK3454/1	USA	KM205319	KM205266	This study
A. mellea	D1	France	JN657437	JN657491	Tsykun et al. (2013)
	HY3	Ukraine	JN657439	JN657493	Tsykun et al. (2013)
	94_5	Japan	AB510833	AB510802	Hasegawa et al. (2010)
	A_12	Japan	AB510820	AB510801	Hasegawa et al. (2010)
	ST5	Virginia, USA	AY509185	JF313137	Kim et al. (2006), Ross-Davis et al. (2012)

Supplementary Table S2 (continued)

Species	Isolate	Country	IGS	TEF-1α	Reference [a]
A. mellea	ST20	Wisconsin, USA	AY509187	JF313128	Kim et al. (2006), Ross-Davis et al. (2012)
	ST21	New Hampshire, USA	AY509188	JF313127	Kim et al. (2006), Ross-Davis et al. (2012)
	CMW31171, KK3441	New Hampshire, USA	KM205321	KM205268	
A. nabsnona	C21	Idaho, USA	AY509174	JF313119	Kim et al. (2006), Ross-Davis et al. (2012)
	M90	British Columbia, Canada	AY509176	JF313122	Kim et al. (2006), Ross-Davis et al. (2012)
	ST16	Alaska, USA	AY509178	JF313124	Kim et al. (2006), Ross-Davis et al. (2012)
	NB4	Aomori, Japan	AB510851	AB510764	Hasegawa et al. (2010)
	00-3-1	Aomori, Japan	AB510850	AB510766	Hasegawa et al. (2010)
	CMW31100, KK3458/1	Canada	KM205323	KM205270	This study
	CMW31101, KK3459/1	USA	KM205324	KM205271	This study
A. solidipes	BRNM 706815	Czech Republic	EU257711	EU251400	Antonin et al.(2009)
	C5	France	HQ232281	HQ285903	Tsykun et al. (2013)
	C2	France	JN657432	JN657486	Tsykun et al. (2013)
	HpAg1	Ukraine	JN657435	JN657489	Tsykun et al. (2013)
	NC8	Aomori, Japan	AB510848	AB510782	Hasegawa et al. (2010)
	2002_66_03	Tochigi, Japan	AB510847	AB510781	Hasegawa et al. (2010)
	ST1	New Hampshire, USA	AY509154	JF313141	Kim et al. (2006), Ross-Davis et al. (2012)
	ST2	Washington, USA	AY509155	JF313139	Kim et al. (2006), Ross-Davis et al. (2012)

Supplementary Table S2 (continued)

Species	Isolate	Country	IGS	TEF-1α	Reference [a]
A. solidipes	P1404	Idaho, USA	AY509157	JF313140	Kim et al. (2006), Ross-Davis et al. (2012)
	CMW31107, KK1066/3	Finland	KM205328	KM205275	This study
A. sinapina	M50	British Columbia, Canada	AY509167	JF313114	Kim et al. (2006), Ross-Davis et al. (2012)
	ST12	Washington, USA	AY509168	JF313132	Kim et al. (2006), Ross-Davis et al. (2012)
	ST13	Michigan, USA	AY509169	JF313131	Kim et al. (2006), Ross-Davis et al. (2012
	96-7-1	Hokkaido, Japan	AB510827	AB510774	Hasegawa et al. (2010)
	05-13-2	Hokkaido, Japan	AB510836	AB510776	Hasegawa et al. (2010)
A. tabescens	HAt1S5	Ukraine	HQ232284	HQ285906	Tsykun et al. (2013)
	HAt2S5	Ukraine	HQ232285	HQ285907	Tsykun et al. (2013)
	HAt5S3	Ukraine	HQ232286	HQ285908	Tsykun et al. (2013)
	ET3	France	JF288740	JF746929	Mulholland et al. (2012)
	ET4	France	JF288741	JF746930	Mulholland et al. (2012)
	96-3-3	Japan	AB510824	AB510805	Hasegawa et al. (2010)
	96-1-8	Japan	AB510823	AB510804	Hasegawa et al. (2010)
	AT-MU-S2	South Carolina, USA	AY509189	JF313113	Kim et al. (2006), Ross-Davis et al. (2012)
	OOI-99	Georgia, USA	AY509192	JF313112	Kim et al. (2006), Ross-Davis et al. (2012)
	OOI-210	Georgia, USA	AY509191	JF313111	Kim et al. (2006), Ross-Davis et al. (2012)

Supplementary Table S2 (continued)

Species	Isolate	Country	IGS	TEF-1α	Reference [a]
A. tabescens	CMW31119, KK3380/1	Italy	KM205334	KM205281	This study
	CMW31120, KK9083/4	Italy	KM205335	KM205282	This study
NAG E	NE4	Tottori, Japan	AB510828	AB510771	Hasegawa et al. (2010)
	94-2-1	Nagano, Japan	AB510840	AB510768	Hasegawa et al. (2010)
	96-37-1	Kanagawa, Japan	AB510845	AB510769	Hasegawa et al. (2010)
A. singula	HUA9101	Japan	D89926		Tersahima et al. (1998)
A. jezoensis	HUA9116	Japan	D89921		Tersahima et al. (1998)

[a] Antonín et al. (2009) Mycological Progress 8: 259–271.; Hasegawa et al. (2010) Mycologia 101: 898–910.; Keča et al. (2014) Forest Pathology: online; doi:10.1111/efp.12135.; Kim et al. (2006) Forest Pathology 36: 145–164.; Mulholland et al. (2012) Forest Pathology 42: 229–238.; Ross-Davis et al. (2012) Mycoscience 53: 161–165.; Terashima et al. (1998) European Journal of Forest Pathology 28: 11–19.; Tsykun et al. (2013) Mycologia 105: 1059–1076.

Supplementary Table S3 – Data matrices and nucleotide substitution models employed in maximum likelihood and Bayesian analyses.

Matrix	**Number of characters**	**Model**	**α shape parameter**	**Portion of invariable sites**
***Armillaria tabescens* dataset**				
IGS-1	608	GTR+I	0.1030	
TEF-1α	447	TIM1ef+G	0.2480	
Northern Hemisphere *Armillaria* phylogeny dataset				
IGS-1	787	TVM+G	0.9730	
TEF-1α	594	TIM3ef+G	0.8530	0.3850