FUSE

Fungal Systematics and Evolution

VOLUME 4
DECEMBER 2019
PAGES 1–12

doi.org/10.3114/fuse.2019.04.01

On the co-occurrence of species of *Wynnea* (*Ascomycota, Pezizales, Sarcoscyphaceae*) and *Armillaria* (*Basidiomycota, Agaricales, Physalacriaceae*)

F. Xu, K.F. LoBuglio, D.H. Pfister*

Harvard University Herbaria and Department of Organismic and Evolutionary Biology, Harvard University, 22 Divinity Avenue, Cambridge, MA 02138, USA

*Corresponding author: dpfister@oeb.harvard.edu

Key words:
Armillaria
symbiosis
sclerotium
Wynnea

Abstract: Species of the genus *Wynnea* are collected in association with a subterranean mass generally referred to as a sclerotium. This is one of the few genera of the *Sarcoscyphaceae* not associated with plant material – wood or leaves. The sclerotium is composed of hyphae of both *Armillaria* species and *Wynnea* species. To verify the existence of *Armillaria* species in the sclerotia of those *Wynnea* species not previously examined and to fully understand the structure and nature of the sclerotium, molecular data and morphological characters were analyzed. Using nuclear ITS rDNA sequences the *Armillaria* species co-occurring with *Wynnea* species were identified from all examined material. These *Armillaria* symbionts fall into two main *Armillaria* groups – the *A. gallica-nabsnona-calvescens* group and the *A. mellea* group. Divergent time estimates of the *Armillaria* and *Wynnea* lineages support a co-evolutionary relationship between these two fungi.

Effectively published online: 9 April 2019.

INTRODUCTION

Sclerotia are dense aggregations of tissue produced by some fungi. The distribution of sclerotia across the Fungi has been reviewed by Smith *et al*. (2015). It is assumed that sclerotia aid in survival of fungi under challenging environmental conditions (Smith *et al*. 2015, Willetts 1971) and in some cases the sclerotia directly or indirectly provide carbohydrates to support subsequent growth of spore producing structures. Sclerotia-forming fungi are found among ecologically and phylogenetically diverse groups of fungi. In the *Pezizales*, only a few species have been reported to produce sclerotia (Smith *et al*. 2015).

Sclerotia of species of the genus *Wynnea* (*Ascomycota, Pezizales, Sarcoscyphaceae*) were first reported by Thaxter (1905), who mentioned that ascomata of species of *W. americana* arose from a mass of fungal tissue that he referred to as a sclerotium. Kar & Pal (1970) and Waraitch (1976) reported that collections from India of *W. macrotis* arose from buried sclerotia. Bosman (1998) described irregular, stellate sclerotia in *W. sparassoides* and Zhuang (2003) mentioned irregularly-shaped sclerotia in some collections of *W. macrospora*. Similar structures were observed in collections of *W. gigantea* from Brazil. All recognized species have been found with sclerotial structure except for *W. sinensis.* In this case the lack of sclerotia is likely due to it being overlooked when collecting apothecia.

Among the *Sarcoscyphaceae* these species of *Wynnea,* along with species of *Geodina,* are the only taxa seemingly not associated with woody plant material or leaves (Denison 1965, Pfister 1979, Li & Kimbrough 1996, Angelini *et al*. 2018). Berkeley listed wood as the substrate in the original description of *W. macrotis* but this likely is the result of faulty collection information (Pfister 1979).

The ecological and life history function of these sclerotia has not been addressed. Thaxter (1905) speculated that its purpose may be to supply moisture and nutriment to the developing apothecia. The nature of the sclerotium has been questioned since Thaxter's day. Nagasawa (1984) first reported that *Armillaria* species were associated with sclerotia of *W. gigantea* (probably either *W. macrotis* or *W. sinensis* based on our studies) and this association was confirmed further by Fukuda *et al.* (2003) who detected *A. cepistipes* in sclerotia of *W. americana* using molecular methods. This refers most probably to *W. macrospora* based on our unpublished phylogenetic study. The co-occurrence of *Armillaria* species with the *Wynnea* species to form these sclerotial masses suggests a complex interaction.

The present study was initiated to determine whether this *Armillaria* – *Wynnea* association was present across all *Wynnea* species. We used nuclear rDNA ITS sequences to identify the *Armillaria* species associated with sclerotia from five *Wynnea* species. The symbiotic relationship between *Wynnea* species and their respective *Armillaria* symbionts was examined using molecular phylogenetic analyses of nuclear 18S, 28S and ITS rDNA sequences for *Wynnea* and ITS for *Armillaria*.

MATERIALS AND METHODS

Sampling

The source of *Armillaria* symbionts (whether from sclerotia, basidiomata or fungal cultures) with voucher information and GenBank accession numbers are given in Table 1. The GenBank sequences of those *Armillaria* species that are not associated with sclerotia that were included in this study are also presented

Table 1. Collections used in the molecular phylogenetic analyses, with voucher information and GenBank accession numbers. The * indicates the DNA sequence was derived from sclerotia of the respective taxa. New GenBank numbers are in bold.

Species	Strain or specimen no.	Origin	Source tissue	ITS GenBank accession number
Armillaria sp.	FH 00445985	West Virginia USA	*Wynnea americana**	**MK271361**
	FH 00445981	New York, USA	*Wynnea americana**	**MK271360**
	CUP 063481	New York, USA	*Wynnea americana**	**MK271359**
	F C0239599	Costa Rica	*Wynnea americana**	**MK271358**
	F C0240179	Pennsylvania, USA	*Wynnea americana**	**MK271357**
	FH s. n.	Brazil	*Wynnea gigantea* *	**MK271362**
	FH ACM624	Brazil	*Wynnea gigantea**	**MK281619**
	NYBG 00449607	Guatemala	*Wynnea gigantea**	**MK281620**
	FH 00445975	Guizhou Prov., China	*Wynnea macrospora**	**MK281621**
	FH 00940720	China	*Wynnea macrospora**	**MK599142**
	CUP 2684	Japan	*Wynnea macrotis**	**MK281622**
	NYBG 02480090	West Virginia USA	*Wynnea sparassoides**	**MK281623**
	JLF1498b	Virginia, USA	*Entoloma abortivum*	FJ940731
A. calvescens	ST17A	Michigan, USA	Basidioma	AY213560
	ST3	Quebec, Canada	Basidioma	AY213559
	ST17B	Michigan, USA	Basidioma	AY213561
	ST18	Michigan, USA	Basidioma	AY213562
A. cepistipes	S20	British Columbia, Canada	Basidioma	AY213582
	M110	British Columbia, Canada	Basidioma	AY213581
	W113	Washington, USA	Basidioma	AY213583
A. gallica	ST22B	Michigan, USA	Basidioma	AY213570
	ST22A	Michigan, USA	Basidioma	AY213569
	ST23	Wisconsin, USA	Basidioma	AY213571
	NA17	Japan	-	AB510872
	NBRC31621	Japan	Galeola sp.	AB716752
	HKAS85517	Xinjiang Prov., China	Basidioma	KT822312
	HKAS51692	Sichuan Prov., China	Basidioma	KT822269
A. gallica (China3)	s. n.	Ji'an, China	*Polyporus umbellatus**	KP162334
A. gallica (China4)	s. n.	Lijiang, China	*Polyporus umbellatus**	KP162319
A. gemina	ST8	New York, USA	Basidioma	AY213555
	ST11	West Virginia, USA	Basidioma	AY213558
	ST9B	New York, USA	Basidioma	AY213557
	ST9A	New York, USA	Basidioma	AY213556
A. mellea	ST5B	Virginia, USA	Multisporous	AY213585
	ST5A	Virginia, USA	Multisporous	AY213584
	ST21	New Hampshire, USA	Multisporous	AY213587
	ST20	Wisconsin, USA	Basidioma	AY213586
	HKAS86588	Japan	Single Spore	KT822246
	TNS-F-70421	Yamagata, Oguni, Japan	-	MF095794
	MEX100	Estado de Mexico, Coatepec Harinas, Mexico	-	JX281808
A. nabsnona	C21	Idaho, USA	Basidioma	AY509175
	ST16	Alaska, USA	Multisporous	AY509178
A. ostoyae	ST2	Washington, USA	Basidioma	AY213553
	ST1	New Hampshire, USA	Multisporous	AY213552
	2002_66_03	Japan	-	AB510896
	89_03B_09	Japan	-	AB510861

Table 1. (Continued).

Species	Strain or specimen no.	Origin	Source tissue	ITS GenBank accession number
	HKAS86580	Jilin Prov., China	Single Spore	KT822311
A. ostoyae (China1)	s. n.	Hailin, China	*Polyporus umbellatus**	KP162333
A. ostoyae (China5)	s. n.	A'ba, China	*Polyporus umbellatus**	KP162328
A. puiggarrii	PPg 85-63.1	Mexico	-	FJ664608
A. sinapina	ST13B	Michigan, USA	Multisporous	AY509170
	ST12	Washington, USA	Basidioma	AY509169
	ST13A	Michigan, USA	Multisporous	AY509169
A. sinapina (Japan1)	ArHA1	Takausutyou Asahikawa, Hokkaido, Japan	*Polyporus umbellatus**	AB300716
A. sinapina (Japan2)	ArHH1	Sanbusigaiti Hurano, Aza Hokkaido, Japan	*Polyporus umbellatus**	AB300717
A. sinapina (Japan3)	ArSF1	Fujinomiya city, Shizuoka Pref., Japan	*Polyporus umbellatus**	AB300718
A. sinapina (China2)	ArHe1	Henan Prov., China	*Polyporus umbellatus**	AB300721
A. sinapina (China6)	ArST1	Shaanxi Prov., China	*Polyporus umbellatus**	AB300719
A. sinapina (China7)	ArSB1	Shaanxi Prov., China	*Polyporus umbellatus**	AB300720
Desarmillaria tabescens	00i-99	Georgia, USA	-	AY213590
	00i-210	Georgia, USA	-	AY213589
	At-Mu.S2	South Carolina, USA	-	AY213588

in Table 1. Eleven herbarium specimens from five *Wynnea* species were included in the molecular phylogenetic analyses. Table 2 lists GenBank numbers for the sequences derived from ascomata.

DNA isolation

To isolate DNA of the symbiont a small piece from the central part of sclerotium was excised from dried herbaria specimen and ground in an Eppendorf tube using a Fastprep FP120 Cell Disruptor (BIO101, CA, USA). Genomic DNA was isolated using the DNeasy Plant Mini kit (Qiagen, Germantown, Maryland) according to the modified protocol of Costa & Roberts (2014). This method also was used for DNA isolation from ascomata of the five *Wynnea* species. An alternative method, as follows, was used to isolate DNA from suspected hyphae of *Armillaria* species. Small pieces of hyphae from the chambers of sclerotia of *W. americana* (F C0239599) and *W. sparassoides* (NYBG 02480090) were picked out under a microscope and placed in PCR tubes. DNA was extracted by an Extract-N-Amp Plant PCR kit (Sigma-Aldrich) following Haelewaters *et al*. (2015).

PCR and sequencing

The ITS rDNA regions of *Armillaria* species were amplified using the *Armillaria*-specific primers ARM1 and ARM2 (Schulze *et al*. 1997) or AR1 and AR2 (Lochman *et al*. 2004). For material that proved to be problematic, internal primers ITS2 and ITS3 (White *et al*. 1990) were employed. Ascomatal samples of *Wynnea* species were amplified for SSU, LSU, and ITS. The SSU region was amplified using primers NS1, NS2, NS4, NS8 (White *et al*. 1990), SL122 and SL344 (Landvik *et al*. 1996). The 5' end of the LSU rDNA region was amplified using the primers LR0R, LR5, LR3 and LR3R (Moncalvo *et al*. 2000; http://www.biology.duke.edu/fungi/mycolab/primers.htm). Amplification of the ITS region used the primers ITS1F (Gardes & Bruns 1993), ITS2, ITS3 and ITS4 (White *et al.* 1990). *Wynnea*-specific ITS primers (forward primer ITS1-WYF (5'-GATCATTRGCCGARAGCG-3') located in ITS1 region and reverse primer ITS4-WYR (5'-CACGGTCGGM CRCGRRCG-3') or ITS4-WYR2 (5'-GATATGCTTAAGTTCAGCGG-3') located in ITS2 region were developed and employed.

For each 25 µL PCR reaction 2 µL of diluted genomic DNA (1/10 and 1/100 dilutions) and 1.25 µL DMSO was included. The SSU, LSU and ITS rDNA were amplified using Econo *Taq* DNA polymerase (Lucigen, Middleton, WI). A modified touch-down PCR program was used with cycling parameters as follows: initial denaturation at 95 °C for 5 min, followed by 10 cycles including denaturation at 95 °C for 60 s, annealing at 62 °C (decreasing 1 °C each cycle or every three cycles) for 60 s and extension at 72 °C for 60 s, then 35 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 60 s, final extension at 72 °C for 7 min and hold at 12 °C. All PCR reactions were done in a Peltier Thermal cycler PTC-200 (MJ research, Watertown, MA). PCR products were purified either directly or after band excision using the QIAquick PCR purification kit (Qiagen, Germantown, Maryland) or Gel Extraction kit (Qiagen, Germantown, Maryland), and then sequenced as described in Hansen *et al*. (2005). Sequencher v. 4.6 (GeneCodes, Ann Arbor, Michigan) was used to edit and assemble the DNA sequences obtained.

Sequence analysis

Alignments of the *Armillaria* and *Wynnea* sequences were done using the MAFFT webserver (http://mafft.cbrc.jp/alignment/server, Katoh & Standley 2013) with the default settings, and then manually optimized in MEGA v. 6.0 (Tamura *et al*. 2013) as necessary. The full alignment is available from TreeBASE under

Table 2. Collections used in the molecular phylogenetic analyses in Fig. 2, with voucher information and GenBank accession numbers.

Species	Fungarium/Collection	Geographic origin, year and collector	GenBank accession no.		
			SSU	ITS	LSU
Wynnea americana	FH 00445985	USA, WV, 1977, H. Barnhart	MK335789	MK335780	MK335799
	F C0239599	Costa Rica, 1993, G.M. Mueller (4561)	MK335788	MK335779	MK335800
	FH 00445978	NY, USA, no date, K. T. Hodge	MK592785	MK599141	MK599148
	NYBG 02480091	PA, USA, 1907, O.E. Jennings	-	-	MK599149
Wynnea gigantea	FH s. n.	Brazil, 1993, R.T. Guerreiro & R.M.B. Silverira	MK335790	MK335781	MK335801
	FH ACM624	Brazil, 2013, A.C. Magnago	MK335791	MK335782	MK335802
	NYBG 00449607	Guatamala, 1971, A.L. Welden	MK335792	MK335783	-
Wynnea macrospora	FH 00445975	China, Guizhou,1984, M.H. Liu	MK335793	MK335784	MK335803
	FH 00940720	China, Suchuan, 1997, D.S. Hibbet & Z. Wang	MK335794	MK335785	-
		Japan, 1963, K. Tubaki			
Wynnea macrotis	CUP 2684		MK335795	MK33578	MK335804
Wynnea sparassoides	NYBG 02480090	WV, USA, 1982, C.T. Rogerson	MK335796	MK335787	MK335805

accession no. 23751. The best-fit evolutionary model for each dataset was determined using jModelTest v. 0.1 (Posada 2008). Phylogenetic trees and support values were determined from Bayesian inference analyses using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) and Maximum-Likelihood (ML) analyses using RAxML-HPC2 on Abe through the Cipres Science Gateway (www.phylo.org; Miller *et al*. 2010). Clade robustness was assessed using a bootstrap (BS) analyses with 1 000 replicates (Felsenstein 1985). Branches that received Bayesian posterior probabilities (BPP) and bootstrap support for ML (ML-BS) greater than or equal to 0.95 of BPP and 80 % of ML-BS were considered as significant. *Flammulina velutipes* (GenBank #EF595854) and *Hymenopellis radicata* (GenBank #DQ241780) were used as the outgroup species in the phylogenetic analyses of the *Armillaria* species and *Cookeina tricholoma* (SSU:AF006311; LSU:AY945860; ITS:KY649459) and *Microstoma floccosum* (SSU:AF006313; LSU:DQ220370;ITS: AF394046) were used in the *Wynnea* analyses.

Divergence time estimation

Divergence time estimates for the *Armillaria* and *Wynnea* lineages used the ITS DNA sequence data set from the molecular phylogenetic analyses (Table 1, Fig. 1). The secondary calibration strategy implemented by Renner (2005) was applied here to infer the time to Most Recent Common Ancestor (tMRCA). Molecular dating from Koch *et al*. (2017) indicated the initial radiation of the genus *Armillaria* to be 50.81 MYA. We applied this node age to calibrate the same node in our analysis. The BEAST v. 1.8.2 (Drummond *et al*. 2012) software package was used for both initial and secondary tMRCA analyses. The following parameter settings were used: (i) GTR+G model was chosen as the best substitution model by jModelTest; (ii) a relaxed lognormal model (Ree & Smith 2008) was employed for molecular clock analysis; (iii) tree prior was set to Yule speciation; (iv) the length of Markov Chain was set to 50 M generations with parameters sampled every 1 000 generations. A burn-in value of 10 % was selected in Tracer v. 1.6 (Drummond *et al*. 2012) where the Effective Sample Size (ESS) was >200 and convergence among runs was reached. Two independent Markov Chain Monte Carlo (MCMC) analyses were performed for each analysis. After discarding the burn-in and combination using LogCombiner v. 1.8.2 (in BEAST v. 1.8.2 package), Tracer v. 1.6 (Drummond *et al*. 2012) was used to calculate the mean, upper and lower bounds of the 95 % highest posterior density interval (95 % HPD) for divergence times. Tree topologies were interpreted in Treeannotator (in BEAST v. 1.8.2 package) and viewed in FigTree v. 1.4.2 (Rambaut 2008).

Morphological examination of the sclerotia

Dried specimens were prepared for microscopic examination by rehydrating a small portion of sclerotium in water for at least two hours. The sample was sectioned with median sections 25 to 30 µm thick using a freezing microtome. Sections were placed on a microscope slide and mounted in water.

RESULTS

Molecular identification of *Armillaria* sp. associated with *Wynnea* sp.

The ITS rDNA sequences from *Wynnea* sclerotia were identified as species of *Armillaria* based on DNA sequence similarity searching methods with BLAST (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi). As shown in Fig. 1 all of the *Wynnea* symbionts analyzed belong to the genus *Armillaria* and were associated with one of two main *Armillaria* groups: the *A. gallica-nabsnona-calvescens* group or the *A. mellea* group. All symbionts from *W. americana* and *W. macrospora* were placed in the *A. gallica-nabsnona-calvescens* group while those from *W. macrotis* and *W. sparassoides* fell into the *A. mellea* group (supported by 0.99 BPP). Of the three symbionts from *W. gigantea,* FH s.n. from Brazil was associated with the *A. mellea* group but the symbiont from the Guatemala collection belonged to the *A. gallica-nabsnona-calvescens* group (Fig. 1). A second Brazilian collection (FH ACM624) represents an independent lineage outside of the *A. mellea* group.

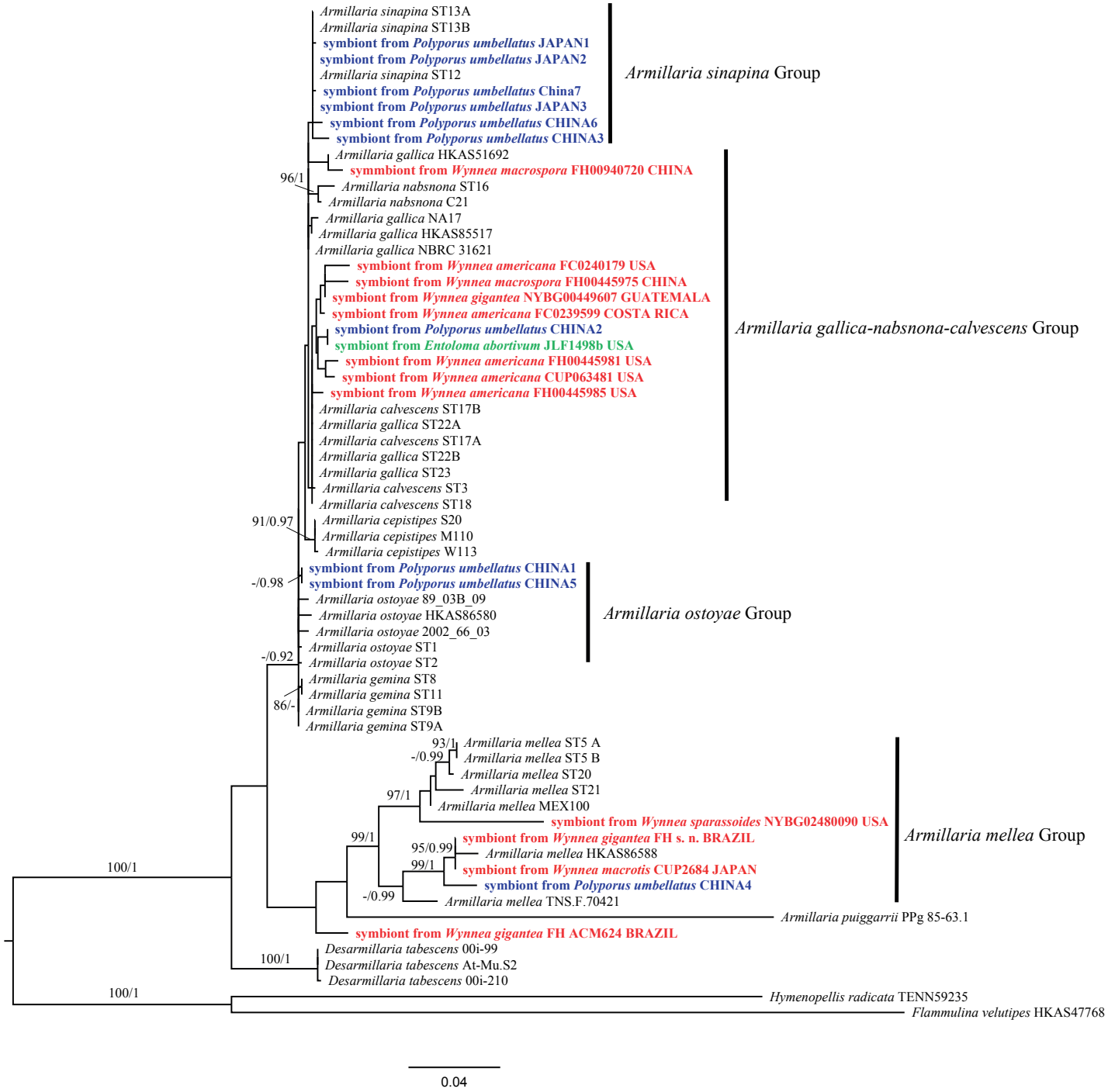

Fig. 1. Phylogenetic relationships among symbionts from sclerotia of *Wynnea* species and species of *Armillaria*. The tree topology was inferred from analysis of ITS rDNA sequence data using Maximum Likelihood (ML) methods. *Hymenopellis radicata* and *Flammulina velutipes* were used as outgroups. The branch support values are indicated on the branches, as ML bootstrap ≥ 80 % and Bayesian posterior probabilities ≥ 0.95 respectively.

There was no correlation between sequence similarity of the *Wynnea* symbionts and their geographic origin (Fig. 1). The symbiont from Eastern Asia *W. macrospora* (FH 00445975, China) showed highest similarity to the symbiont from Eastern North America (symbiont from *W. americana* FC 0240179). The symbiont of *W. macrotis* from Japan (CUP 2684) was more closely related to symbionts of *W. gigantea* from South America (FH s. n. and FH ACM624, Brazil) and *W. sparassoides* from USA (NYBG 02480090).

Symbiotic relationship between *Armillaria* and *Wynnea*

When compared with the phylogenetic tree of *Wynnea* inferred from combined nuclear SSU, ITS and LSU sequences, significant relationships between *Wynnea* and *Armillaria* were revealed (Fig. 2). In the phylogenetic tree of *Wynnea*, the 4 specimens of *W. americana*, from eastern North America and Costa Rica, were sister to the two *W. macrospora* specimens from eastern Asia. The symbionts from each of these species pairs were all shown to belong to the *A. gallica-nabsnona-calvescens* group. The same situation was found in another phylogenetically related species pair, *W. sparassoides* from

Fig. 2. Comparison of phylogenetic trees of *Wynnea* and *Armillaria* species indicating co-evolutionary relationships. Left: Phylogeny of *Wynnea* produced from Bayesian analysis based on combined SSU, ITS and LSU rDNA sequences with *Cookeina tricholoma* and *Microstoma floccosum* as outgroups. Right: Phylogeny of *Armillaria* produced from Bayesian analysis based on ITS rDNA sequences with *Flammulina velutipes* and *Hymenopellis radicata* as outgroups. The branch support values from the analyses are indicated on the branches, as Maximum Likelihood bootstrap ≥ 80 % and Bayesian posterior probabilities ≥ 0.95 respectively. Lines between the two phylogenetic trees indicate the symbiont from a sclerotium and the corresponding apothecium.

eastern North America and *W. macrotis*, from eastern Asia. Symbionts from this species pair belong to the *A. mellea* group. In contrast, the three symbionts from *W. gigantea* were placed in three different lineages; the *W. gigantea* s. n. symbiont from Brazil was placed in the *A. mellea* group, the symbiont from the Guatemalan collection of *W. gigantea* was in the *A. gallica-nabsnona-calvescens* group and the Brazilian collection, FH ACM624 was in an independent lineage (Figs 1 and 2).

Divergence time estimation

The age of the ancestor of the genus *Armillaria* was estimated at 50.81 MYA by Koch *et al*. (2017) for the armillarioid clade. This largely is in agreement with the results of Coetzee *et al*. (2011). Based on this age, an ITS DNA sequence dataset consisting of species within the genus *Armillaria*, including those now placed in the genus *Desarmillaria,* and symbionts from *Wynnea* species was constructed and analyzed for divergence time estimations. The divergence time of the *A. mellea* group was estimated as 43.49 MYA (Fig. 3).

Morphological and molecular examination of the sclerotial structure

Sections were made from the sclerotia of *W. americana* Costa Rica collection (F C0239599) and *W. sparassoides* WV-USA collection (NYBG 02480090) (Figs 4A and 5A) and chambers were observed in sclerotia of these two species (Figs 4B–D, 5B–D). Detailed examination of the structure of the chambers showed that the outer layers (Figs 4E, 5E) resemble the outer excipulum of the apothecia and the inner layers (Figs 4F, 5F) resemble the medullary excipulum of the apothecia, indicating that these two parts of the sclerotia were likely derived from the *Wynnea*.

Within the chamber, there were tangles of thin hyphae, about 1 µm wide and whitish (Figs 4G, 5G). Using the specific PCR primers for *Armillaria* indicated that these hyphae in the chambers were those of *Armillaria* species.

Rhizomorphs were also directly observed in the sclerotia of *W. americana* (NY-USA, CUP 063481 and Costa Rica, F C0239599) and *W. sparassoides* (WV-USA, NYBG 02480090). Figures 4A and 5A show that these rhizomorphs penetrate into the sclerotia. BLAST results of the ITS sequences amplified from rhizomorphs showed these rhizomorphs were from *Armillaria* species.

DISCUSSION

Ecology of *Armillaria* and relationship with other fungi

Species of the basidiomycete genus *Armillaria* are an important component of the fungi in forest ecosystems worldwide where they are responsible for root rot disease (Tsykun *et al*. 2012). At present, about 70 *Armillaria* species are known (Volk & Burdsall 1995). The individual *Armillaria* species differ in ecological behavior, geographical distribution and host preference (Shaw & Kile 1991). Based on data from France, England and Italy, Guillaumin *et al*. (1993) characterized 142 species from 30 plant families as

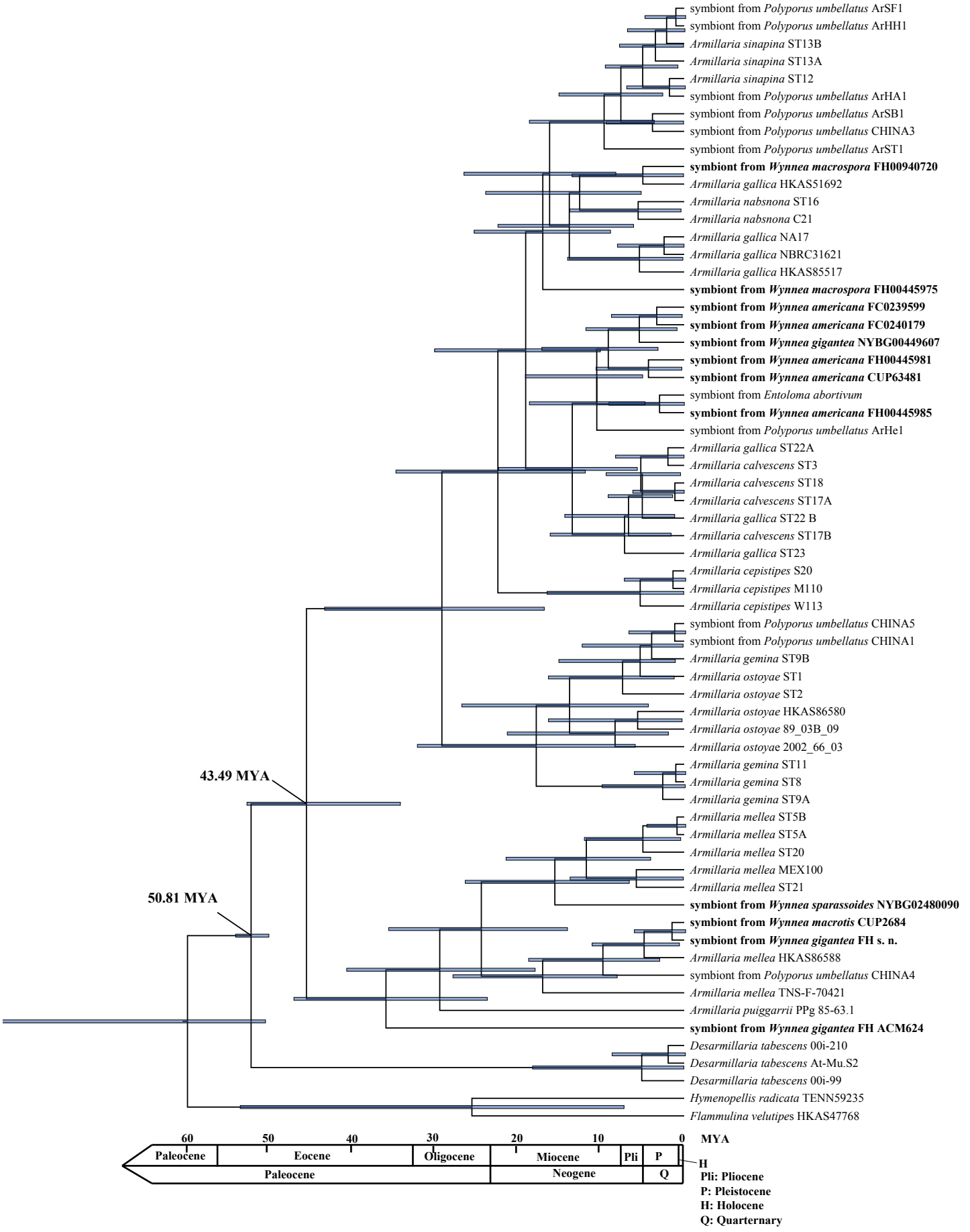

Fig. 3. Chronogram and estimated divergence times of *Armillaria* generated from molecular clock analysis using the ITS DNA sequence data. Chronogram obtained from BEAST using the *Armillaria* divergence time, 50.81 MYA, from Koch *et al.* (2017) as the calibration point. The divergence time of the *A. mellea* group is estimated at 43.49 MYA. Blue bars denote the 95 % highest posterior density (HPD) intervals of the posterior probability distribution of node ages.

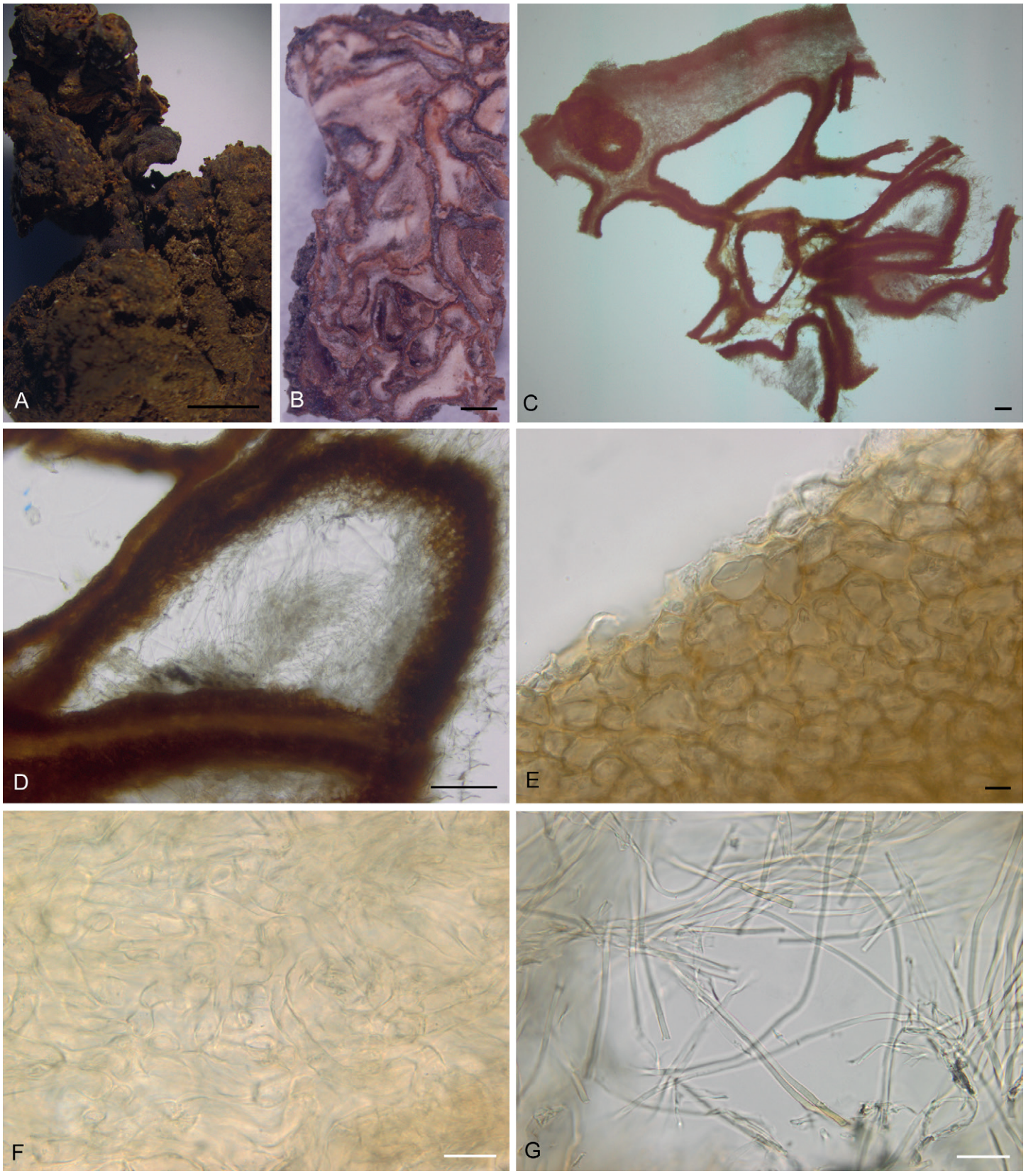

Fig. 4. Morphological characters of *Wynnea americana* (F C0239599). **A.** Partial view of a sclerotium. **B.** Internal part of sclerotium. **C.** Cross section of a sclerotium, freezing microtome section at 30 µm. **D.** Chambered structure. **E.** Outer layer of the chambered structure. **F.** Inner layer of the chambered structure. **G.** Whitish hyphae in the chamber. Bars: A, B = 0.5 mm; C, D = 100 µm; E–G = 10 µm. All mounted in water.

hosts of *Armillaria* species. This indicates both high diversity and interactions of *Armillaria* species.

Armillaria species can act as either host or parasite in interactions with other fungi (Baumgartner *et al*. 2011). Choi *et al*. (2002) reported that the sclerotia of *Polyporus umbellatus* (as *Grifola umbellata*) had a symbiotic relationship with *A. mellea*. Kikuchi & Yamaji (2010) indicated that all the sclerotial samples of *P. umbellatus* had rhizomorphs of *Armillaria* species adherent to and penetrating them. In order to compare our findings with those of *P. umbellatus*, we included ITS sequences

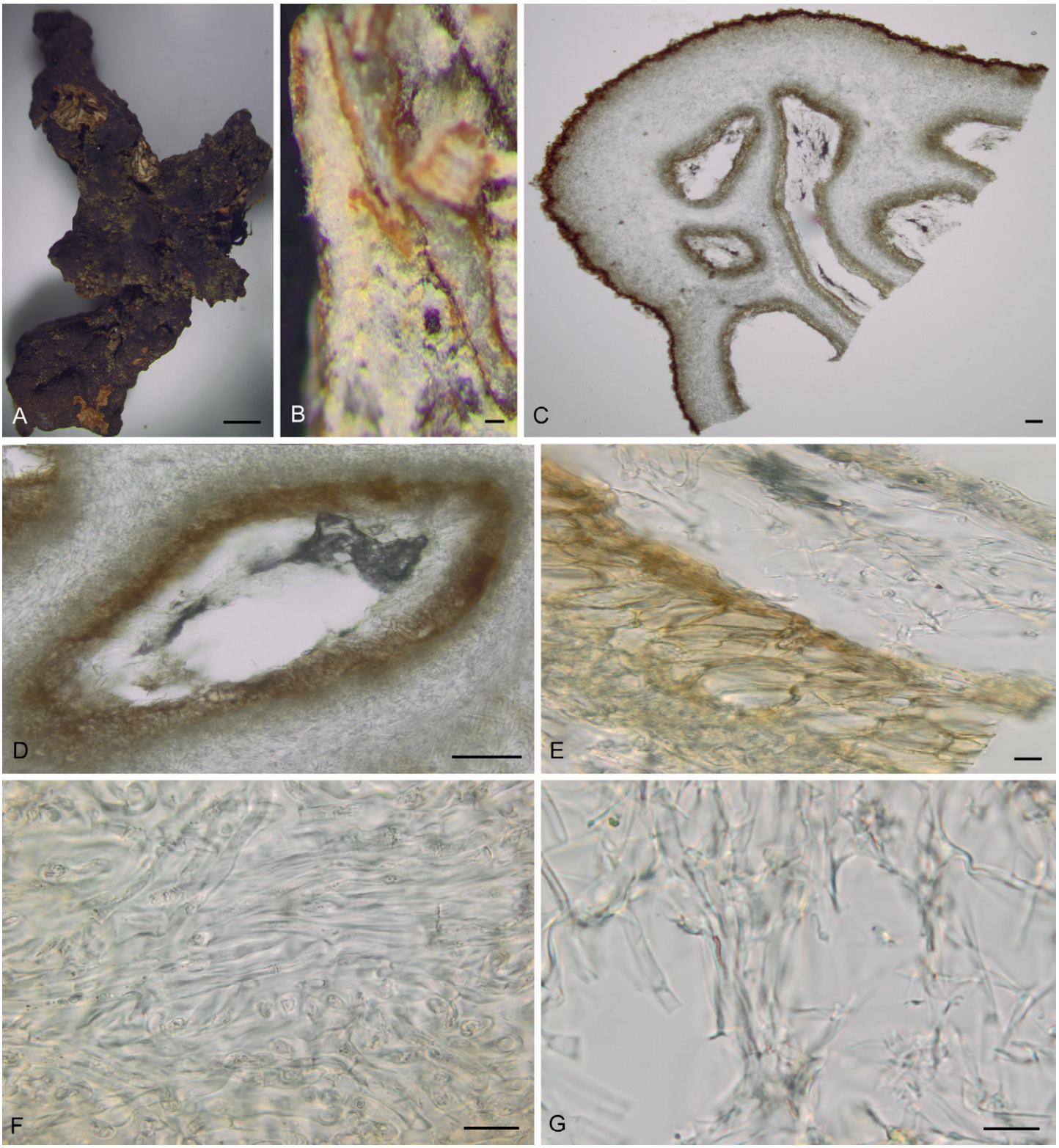

Fig. 5. Morphological characters of *Wynnea sparassoides* (NYBG 02480090). **A.** Partial view of a sclerotium. **B.** Internal part of sclerotium. **C.** Cross section of a sclerotium, freezing microtome section at 30 µm. **D.** Chambered structure. **E.** Outer layer of the chambered structure. **F.** Inner layer of the chambered structure. **G.** Whitish hyphae in the chamber. Bars: A, B = 0.5 mm; C, D = 100 µm; E, F, and G = 10 µm. All mounted in water.

of *Armillaria* sp. (Table 1) from the sclerotia of *P. umbellatus* collected in Japan and China (Kikuchi & Yamaji 2010). As shown in Fig. 1 the *P. umbellatus* symbionts fell into four *Armillaria* groups: *A. sinapina, A. gallica-nabsnona-calvescens*, *A. ostoyae* and *A. mellea*. The symbionts from *W. americana*, *W. macrospora* and the *W. gigantea* the collection from Guatemala (Fig. 1) also fell within the *A. gallica-nabsnona-calvescens* group. Species identification of the symbionts in the *A. gallica-nabsnona-calvescens* group is difficult because these species are morphologically similar and their ITS rDNA sequences are not divergent enough for species assignment (Antonín *et al*. 2009).

In the *Polyporus – Armillaria* association, *Armillaria* was thought to be parasitic on the *Polyporus* host. On the other hand, *Armillaria* was reported to be parasitized by *Entoloma abortivum* (*Basidiomycota, Entolomataceae*), which caused

misshapen *Armillaria* fruiting bodies, carpophoroids (Czederpiltz *et al*. 2001). In our study, we also included one *Armillaria* ITS sequence from the *Entoloma* association and results indicated that the *Armillaria* associated with *Entoloma* was also placed in the *A. gallica-nabsnona-calvescens* group.

Armillaria mellea was found to be the symbionts of *W. sparassoides*, *W. macrotis* and *W. gigantea* sclerotia in the present study and one of the symbionts of *Polyporus umbellatus* (China4). These *Armillaria* taxa were different taxa than those found to be associated with *W. americana* and *W. macrospora, Entoloma* and the nine other *Polyporus* specimens included (Fig. 1). Fukuda *et al*. (2003) reported that *A. mellea* was identified from *W. gigantea* (probably either *W. macrotis* or *W. sinensis* according to our unpublished study) and *A. cepistipes* was identified from *W. americana* (the Japanese material of *W. americana* is now known to be *W. macrospora*), which is in accordance with our results. This broadens our knowledge of the association of *Armillaria* species with other fungi. In this case *Wynnea* species, *Ascomycota*, are associated with members of two *Armillaria* groups and mirrors the situation observed in *P. umbellatus* but in this case the association is across fungal phyla.

Function of sclerotia and its association with *Armillaria*

Sclerotia are reported to help fungi to survive challenging conditions such as extremes of temperature, desiccation, starvation and toxic chemicals (Willetts 1971, Smith *et al.* 2015). Many of the sclerotium-forming fungi are plant pathogens where sclerotia may function in relationship to host-parasite interaction (Coley-Smith & Cooke 1971). Sclerotia are highly variable in their morphology and putatively serve as a resource-storage (Smith *et al*. 2015).

The structure of the sclerotia in *Wynnea* species is fundamentally different than that of most sclerotia. The chambered structure of the sclerotia from *W. americana* was first reported and illustrated by Thaxter (1905). Korf (1972) stated that in *W. americana* the sclerotium was a tangled mass of rhizomorphs. Pfister (1979) also described rhizomorphs on the outside of the sclerotium in some collections of *W. americana*. Nagasawa (1984) presented the structure of *W. gigantea* (possibly *W. sinensis* or *W. macrotis* based on our unpublished phylogenetic study) showing cross sections of the sclerotia. In all of these cases the sclerotium is loosely constructed and composed of two elements. Our molecular results demonstrate that the whitish hyphae inside of the chambers and the rhizomorphic tangle outside the sclerotia are hyphae of *Armillaria* species. The outer surface and the walls of the chambers of the sclerotia are morphologically similar to the excipular tissues of the *Wynnea* ascomata.

To date, all the *Wynnea* species have been collected with sclerotia except *W. sinensis* in which it seems to have been overlooked when the ascomata were collected. In this study, *Armillaria* species were identified from all the sampled sclerotia using molecular phylogenetic techniques. Based on the knowledge obtained to date, we assume that *W. sinensis* also forms sclerotia with *Armillaria* species.

Wynnea gigantea seems to be the only species whose sclerotia have no chambered structure. Amplification of both *Wynnea* and *Armillaria* ITS sequences using specific primers or cloned sequences indicated that the sclerotia of *W. gigantea* also included *Armillaria* hyphae even though chambers were not formed.

Species of *Wynnea* and *Geodina* are the only genera in the family *Sarcoscyphaceae* that are not directly associated with wood or other plant material. The substrate association of *Geodina*, a fungus known from only a few collections, remains a mystery since all collections have been made from soil. The association with *Armillaria* explains the unique habit of *Wynnea* species. But the nature of the symbiosis among the species of *Wynnea* and *Armillaria* is still uncertain. Fresh samples carefully collected will be helpful to illustrate this interesting association. The ecology and life history of *Wynnea* species cannot be fully understood without understanding sclerotial function. We do surmise that the *Wynnea* species are parasitic on *Armillaria* species as seems to be the case in *Polyporus umbellatus* (Xing *et al*. 2017).

Sclerotia are known in other members of the *Pezizomycetes*. These are constructed, so far as is known, of a single fungus and are primarily assumed to be survival structures.

Divergence time estimated

Dating the divergence time of the *A. mellea* group based on ITS sequences was determined to be 43.49 MYA which is older than the 31 MYA divergence time estimated by Koch *et al*. (2017) who used a combined ITS, LSU and *EF1*α dataset. This might be because LSU and *EF1*α genes are more conserved and thus have slower evolutionary rates than that of the ITS rDNA region.

Our divergence time estimate for *Wynnea* species (manuscript in preparation) is 73.23 MYA and predates the 50.81 MYA *Armillaria* ancestor. The divergence times for the *Wynnea* and *Armillaria* species indicate that there is a deep evolutionary relationship between *Armillaria* species and *Wynnea* species. This relationship might be involved in diversification of these species.

CONCLUSIONS

The fact that all species of *Wynnea,* thus far known, are associated with *Armillaria* species points to an interwoven life history and evolution among these fungi. To date, *Wynnea* species, have not been found on wood or plant parts and though seemingly occurring on soil, suggestive of a mycorrhizal habit, no member of the *Sarcoscyphaceae* has been implicated in such mycorrhizal relationships. Although definitive answers to the question of the role of these sclerotia are not at hand, it seems likely that as in the case of *Armillaria* species and *Polyporus umbellatus*, the *Wynnea* species may indeed be parasitic on the *Armillaria* species. The fidelity of the association across taxa and geographical ranges point to a specific dependency on each other.

ACKNOWLEDGMENTS

We gratefully thank the curators of BPI, CUP, F, HKAS, HMAS, NYBG, TNS-F for loan of collections used in this study and Genevieve E. Tocci at FH for dealing with specimens. We are especially grateful to A.C. Magnago for providing Brazilian collections. Funding for this research were provided by an NSF Grant to Donald H. Pfister (DEB-0315940), and a grant from the National Natural Science Foundation of China (NSFC 31401929) and the China Scholarship Council (CSC) to Feng Xu.

REFERENCES

Angellini C, Medardi G, Alvarado P (2018). Contribution to the study of neotropical discomycetes: a new species of the genus *Geodina* (*Geodina salmonicolor* sp. nov.) from the Dominican Republic. *Mycosphere* **9**: 169–177.

Antonín V, Tomšovský M, Sedlák P, *et al*. (2009). Morphological and molecular characterization of the *Armillaria cepistipes – A. gallica* complex in the Czech Republic and Slovakia. *Mycological Progress* **8**: 259–271.

Baumgartner K, Coetzee MP, Hoffmeister D (2011). Secrets of the subterranean pathosystem of *Armillaria*. *Molecular Plant Pathology* **12**: 515–534.

Bosman TE (1998). What does the red-backed vole have to do with *Wynnea sparassoides* and *Wynnea americana* in North America? *McIlvainea* **13**: 69–80.

Choi KD, Kwon JK, Shim JO, *et al.* (2002). Sclerotia development of *Grifola umbellata*. *Mycobiology* **30**: 65–69.

Coetzee MP, Bloomer P, Wingfield MJ, *et al.* (2011). Paleogene radiation of a plant pathogenic mushroom. *PloS One* **6**: e28545.

Coley-Smith JR, Cooke RC (1971). Survival and germination of fungal sclerotia. *Annual Review of Phytopathology* **9**: 65–92.

Costa CM, Roberts RP (2014). Techniques for improving the quality and quatity of DNA extracted from herbarium specimens. *Phytoeuron* **48**: 1–8.

Czederpiltz DL, Volk TJ, Burdsall HJ (2001). Field observations and inoculation experiments to determine the nature of the carpophoroids associated with *Entoloma abortivum* and *Armillaria*. *Mycologia* **93**: 841–851.

Denison WC (1965). Central American *Pezizales*. I. A new genus of the *Sarcoscyphaceae*. *Mycologia* **57**: 649–656.

Drummond AJ, Suchard MA, Xie D, *et al*. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**: 1969–1973.

Felsenstein J (1985). Confidence-delimitation on phylogenies - an approach using the bootstrap. *Evolution* **39**: 783–791.

Fukuda M, Nakashima E, Hayashi K, *et al*. (2003). Identification of the biological species of *Armillaria* associated with *Wynnea* and *Entoloma abortivum* using PCR-RFLP analysis of the intergenic region (IGR) of ribosomal DNA. *Mycological Research* **107**: 1435–1441.

Gardes M, Bruns TD (1993). ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Guillaumin JJ, Mohammed C, Anselmi N, *et al.* (1993). Geographical distribution and ecology of the *Armillaria* species in western Europe. *European Journal of Forest Pathology* **23**: 321–341.

Haelewaters D, Gorczak M, Pfliegler WP, *et al.* (2015). Bringing *Laboulbeniales* into the 21st century: enhanced techniques for extraction and PCR amplification of DNA from minute ectoparasitic fungi. *IMA Fungus* **6**: 363–372.

Hansen K, Lobuglio K, Pfister DH (2005). Evolutionary relationships of the cup-fungus genus *Peziza* and *Pezizaceae* inferred from multiple nuclear genes: RPB2, beta-tubulin, and LSU rDNA. *Molecular Phylogenetics and Evolution* **36**: 1–23.

Kar AK, Pal KP (1970). The *Pezizales* of Eastern India. *Canadian Journal of Botany* **48**: 145–146.

Katoh K, Standley DM (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.

Kikuchi G, Yamaji H (2010). Identification of *Armillaria* species associated with *Polyporus umbellatus* using ITS sequences of nuclear ribosomal DNA. *Mycoscience* **51**: 366–372.

Koch RA, Wilson AW, Séné O, *et al.* (2017). Resolved phylogeny and biogeography of the root pathogen *Armillaria* and its gasteroid relative, *Guyanagaster*. *BMC Evolutionary Biology* **17**: 33.

Korf RP (1972). Synoptic key to the genera of the *Pezizales*. *Mycologia* **64**: 937–994.

Landvik S, Shailer NFJ, Eriksson OE (1996). SSU rDNA sequence support for a close relationship between the *Elaphomycetales* and the *Eurotiales* and *Onygenales. Mycoscience* **37**: 237–241.

Li LT, Kimbrough JW (1996). Spore ontogeny in species of *Phillipsia* and *Wynnea* (*Pezizales*). *Canadian Journal of Botany* **74**: 10–18.

Lochman J, Sery O, Mikes V (2004). The rapid identification of European *Armillaria* species from soil samples by nested PCR. *FEMS Microbiology Letters* **237**: 105–110.

Miller MA, Pfeiffer W, Schwartz T (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees, in Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA: 1–8.

Moncalvo JM, Lutzoni FM, Rehner SA, *et al.* (2000). Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic Biology* **49**: 278–305.

Nagasawa E (1984). Sclerotia of *Wynnea gigantea*. *Reports of the Tottori Mycological Institute* **22**: 80–81.

Pfister DH (1979). A monograph of the genus *Wynnea* (*Pezizales, Sarcoscyphaceae*). *Mycologia* **71**: 144–159.

Posada D (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253–1256.

Rambaut A (2008). FigTree 1.4.2. Institute of Evolutionary Biology, Univ. Edinburgh, Edinburgh. http://tree.bio.ed.ac.uk/software/

Ree RH, Smith SA (2008). Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic Biology* **57**: 4–14.

Renner SS (2005). Relaxed molecular clocks for dating historical plant dispersal events. *Trends in Plant Science* **10**: 550–558.

Ronquist F, Huelsenbeck P (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Schulze S, Bahnweg G, Moller, *et al*. (1997). Identification of the genus *Armillaria* by specific amplification of an rDNA-ITS fragment and evaluation of genetic variation within *A. ostoyae* by rDNA-RFLP and RAPD analysis. *Forest Pathology* **27**: 225–239.

Shaw CG, Kile GA (1991). *Armillaria root disease*. Agricultural Handbook No. 691. USDA Forest Service, Washington DC.

Smith ME, Henkel TW, Rollins JA (2015). How many fungi make sclerotia? *Fungal Ecology* **13**: 211–220.

Tamura K, Stecher G, Peterson D, *et al*. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Thaxter R (1905). A new American species of *Wynnea*. *Botanical Gazette* **39**: 241–247.

Tsykun T, Rigling D, Nikolaychuk V, *et al*. (2012). Diversity and ecology of *Armillaria* species in virgin forests in the Ukrainian Carpathians. *Mycological Progress* **11**: 403–414.

Volk TJ, Burdsall HH (1995). A nomenclatural study of *Armillaria* species. *Synopsis Fungorum* 8. Fungiflora: Oslo, Norway.

Waraitch KS (1976). New species of *Aleuria* and *Wynnea* from India. *Transactions of the British Mycological Society* **67**: 533–536.

White TJ, Bruns T, Lee S, *et al*. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocol: a guide to methods and applications* (Innes MA, Gelfand DH, Sninsky JJ, eds). Academic Press, USA: 315–322.

Willetts HJ (1971). The survival of fungal sclerotia under adverse environmental conditions. *Biological Reviews* **46**: 287–407.

Xing X, Men J, Guo S (2017). Phylogenetic constraints on *Polyporus umbellatus* - *Armillaria* associations. *Scientific Reports* **7**: 4226.
Zhuang WY (2003). Notes on *Wynnea* (*Pezizales*) from Asia. *Mycotaxon* **87**: 131–136.