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

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**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.

**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 the 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. macrospora* 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. macrospora* 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. macrospora* 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.
| 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 CO239599              | Costa Rica            | Wynnea americana*     | MK271358                     |
|             | F CO240179              | 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 macrospora*    | 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                     |
|             | HKAS595517             | Xinjiang Prov., China | Basidioma             | KT822312                     |
|             | HKAS51692              | Sichuan Prov., China  | Basidioma             | KT822269                     |
| A. gallica  | s. n.                  | Ji'an, China          | Polyporus umbellatus* | KP162334                     |
| (China3)    | 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                     |
|             | HKAS586588             | Japan                 | Single Spore          | KT822246                     |
|             | TNS-F-70421            | Yamagata, Oguni, Japan| -                     | MF095794                     |
|             | MEX100                 | Estado de Mexico, Coatepec Harinas, Mexico| - | JX281808                     |
| A. nabsnnona | 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                     |
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 CO239599) 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. (2005). Sequencher v. 4.6 (GeneCodes, Ann Arbor, Michigan) was used to edit and assemble the DNA sequences obtained.

**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’-CAGGTCGGCMCRCGRRC-3’) or ITS4-WYR2 (5’-GATATGC'TTGACCGG-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
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 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).

**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. macrospora 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.
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 symbions 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 symbions from each of these species pairs were all shown to belong to the *A. gallica-nabnsona-calvescens* group. The same situation was found in another phylogenetically related species pair, *W. sparassoides* from...
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.

**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* collection (NYBG 00449607 Guatemala, NY-USA, CUP 063481 and Costa Rica, F C0239599) and *W. sparassoides* (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* collection (NYBG 00449607 Guatemala, NY-USA, CUP 063481 and Costa Rica, F C0239599) and *W. sparassoides* (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*.

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**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...
Co-occurrence of Wynnea and Armillaria

Figure 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.
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. 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.
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

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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.
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-nabsona-calvescens group.

Armillaria mellea was found to be the symbiont 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 (Willett 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 EF1α dataset. This might be because LSU and EF1α 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.

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