Asperosporus subterraneus, a new genus and species of sequestrate *Agaricaceae* found in Florida nursery production

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Abstract: We describe a novel sequestrate genus and species, *Asperosporus subterraneus gen. et sp. nov.,* found associated with nursery production of ferns in south Florida. This truffle species has a unique combination of morphological characters among described *Agaricaceae* in that it lacks a stipe or columella, has large, ornamented spores, the fresh sporocarps rapidly stain pink-red when cut or bruised, and they have a rancid smell. Although this fungus does not appear to be a direct plant pathogen, the hyphae of *A. subterraneus* produce a thick hydrophobic mycelial mat that binds the organic matter and therefore prevents water and fertilizer from being absorbed by plants, consequently causing wilting and chlorosis. Using morphological characteristics and phylogenetic reconstruction based on the internal transcribed spacer (ITS), partial large subunit nuclear ribosomal DNA (LSU), second largest subunit of RNA polymerase II (*rpb2*) and translation elongation factor 1-alpha (*tef1*) regions, we describe this taxon as a new genus and species in *Agaricaceae*.

INTRODUCTION

Agaricaceae is a large family of fungi containing species with diverse morphological characteristics, from spore color (white, green, dark brown, black, blue, pale-pink and pale-yellow) to sporocarp type (agaricoid, secotioid, gasteroid) (Lebel *et al.* 2004, Vellinga 2004b). While sporocarps from many genera in *Agaricaceae* bruise or stain red when fresh, this feature can be critical for species level identification in some taxa (Ge *et al*. 2018, Vellinga *et al.* 2010b). Here we refer to *Agaricaceae* as defined in Vellinga *et al.* (2011) but recently a more constrained delimitation of genera in *Agaricaceae s.l.* has been proposed which narrows the number of accepted taxa (Kalichman *et al.* 2020). Recently, several studies have revealed that sequestration is more prolific throughout the family than previously thought and that many of these sequestrate fungi are found in genera with mostly agaricoid fruiting forms (Lebel *et al.* 2004, Kropp *et al*. 2012, Lebel & Syme 2012, Ge *et al*. 2018). Since fungi with gasteroid sporocarp forms are generally hypogeous, finding sufficient collections to describe new taxa is challenging and has resulted in multiple monotypic genera and descriptions based on few collections (Ge & Smith 2013). For instance, genera such as *Amogaster* (gasteroid, now considered in the genus *Lepiota*), and *Barcheria* (gasteroid) are only known from a small number of collections (Lebel *et al.* 2004, Ge & Smith 2013).

In 2017 and 2018, potted Boston fern (*Nephrolepis exaltata*) samples were submitted for disease diagnosis to the University of Florida Plant Diagnostic Clinic (Homestead, Florida). The potting soil contained sequestrate, truffle-like basidiomata that had an unpleasant odor and rapidly stained pink-red when bruised or cut (Fig. 1C). The specimens had highly ornamented brown spores and could not be identified to the genus or species level based on available publications or taxonomic keys of brown-spored truffle-like fungi (Dodge & Zeller 1934, Miller 1999). Preliminary ribosomal sequence data placed the specimens in *Agaricaceae* but with no reliable matches to known species. Based on the unique combination of morphological and molecular characteristics of this truffle we describe this taxon as *Asperosporus subterraneus,* a new genus and species in the *Agaricaceae*. We provide a full description and include sequence data from the internal transcribed spacer (ITS), partial large subunit nuclear ribosomal DNA (LSU), second largest subunit of RNA polymerase II (*rpb2*) and translation elongation factor 1-alpha (*tef1*) regions.

MATERIALS AND METHODS

In 2017 and 2018 potted Boston fern (*N. exaltata*) samples were submitted for disease diagnosis at the University of Florida Plant Diagnostic Clinic (Homestead, Florida) (Fig. 1A). The specimens from 2018 were not dried properly and therefore specimens were not kept nor were DNA sequences obtained. However, photos and notes taken at the time of submission confirm

that the specimens correspond to the same fungus as the ones submitted in 2017. For both the 2017 and 2018 samples, the plants were excavated for root examination and several gasteroid basidiomata were observed (Fig. 1B). Tissues were examined under a light microscope using H_2O, 5 % KOH, and Melzer's reagent. Spore dimensions are given in the following order length, width, and spore Q (length to width ratio) and spore measurements excluded spore ornaments, which were measured separately. All other morphological examinations (such as color and ornamentation) were based on 20–40 individual structures. Dried specimens were deposited at the University of Florida Fungal Herbarium under the accession number FLAS-F-68001.

Small pieces of basidiomata were ground in liquid nitrogen in 1.5 mL Eppendorf tubes and DNA was extracted using the CTAB extraction method (Doyle & Doyle 1987). PCR amplification was completed with the following primer pairs and thermocycler conditions according to each reference: the internal transcribed spacer (ITS) ITS1F/4 (White *et al*. 1990, Gardes & Bruns 1993), large subunit nuclear ribosomal DNA (LSU) LROR/LR6 (Vilgalys & Hester 1990), RNA polymerase II subunit 2 (*rpb2*) bRPB2-6F and bRPB2-7.1R (Matheny 2005), and the translation elongation factor 1-α locus (*tef1*) EF-983F/EF1-1567R (Rehner & Buckley 2005). PCR products were run on a 1.5 % agarose gel using SYBR Green and sequenced at MCLab (San Francisco, CA). Bi-directional reads were trimmed, assembled, and checked for quality using Geneious v. 2020.1.2. Sequences were aligned with MAFFT v. 7.450 (Katoh *et al*. 2002, Katoh & Standley 2013) using default settings.

Three datasets were assembled to infer the phylogenetic position of the unknown fungus. First, an ITS dataset containing 36 sequences was assembled following the taxon sampling in Vellinga *et al.* (2011) as a framework. Additional sequences were added, focusing on taxa with morphological similarities such as the reddening reaction and gasteroid basidiomata. Because some taxa are only represented by their ITS sequence, the objective of this first analysis was to confirm the uniqueness of the new taxon with respect to previously described gasteroid taxa in the *Agaricaceae* (Kropp *et al*. 2012, Lebel & Vellinga 2013, Zhao *et al.* 2016, Ge *et al*. 2018). Second, an LSU dataset containing 47 sequences was assembled based on the family level taxon sampling used in Vellinga *et al.* (2011) and included a wide range of taxa, many of which lack the regions used in the multilocus analysis. This dataset included representatives of many closely related accepted genera in the family. Finally, a combined LSU, *rpb2*, and *tef1* dataset was assembled by mining the NCBI nucleotide database guided by relevant published studies on the family *Agaricaceae* (Kropp *et al*. 2012, Lebel & Syme 2012, Zhao *et al*. 2016). All taxa in the multilocus alignment contained at least LSU and *rpb2* sequences whereas *tef-1* was present in 62 % of the taxa. The ITS region was excluded from the multilocus analysis because incomplete sequence availability across the selected loci resulted in a data matrix with a high percentage of missing data. Three *Limacella* species (*L. glioderma*, *L. guttata*, and *L. delicata*) were used as outgroups for all datasets. Ambiguously aligned regions were excluded from the alignments using Gblocks v. 0.91b (Talavera & Castresana 2007) with reduced stringency settings by allowing gaps within final blocks and less strict flanking positions. Phylogenies were reconstructed using matrices with and without ambiguously aligned regions. Topologies were compared and inspected for conflict. Conflict was assumed to be significant if a group of

taxa was supported at 70 % as monophyletic with one locus but supported as nonmonophyletic by another locus [reciprocal 70 % ML bootstrap support criterion (Reeb *et al.* 2004)].

Maximum Likelihood (ML) phylogenies based on the ITS and LSU regions, and on the concatenated LSU + *rpb2* + *tef1* dataset were constructed using RaxML v. 8.2.10 (Stamatakis 2014) under the GTRGAMMA evolutionary model. Branch support values were estimated using 1 000 bootstrap (BS) replicates. The same alignments were used to infer phylogenies using Bayesian analysis with MrBayes v. 3.2.6 x64 (Huelsenbeck 2004). jModeltest v. 2.1.10 (Darriba *et al*. 2012) was used to estimate the model of evolution for each region and the GTR+I+G model of evolution was assigned to the LSU and *rpb2* regions and the GTR+G model of evolution to the *tef1*. The concatenated matrix was partitioned by locus prior to phylogeny inference. Posterior probabilities (PP) were determined using 10^6 generations with sampling every 1 000 generations. The first 25 % of trees were discarded as the burn in. Sequence data was deposited in NCBI (Table 1), and alignments and trees were deposited on the Open Science Framework (OSF) at https://osf.io/7gn85/?view_only=08df5d71e46b4c08b12388fc775a9c7e.

RESULTS

The ITS and LSU sequences generated from specimens were compared to the NCBI and UNITE (Abarenkov *et al*. 2010) nucleotide databases, using different BLAST strategies (including/excluding environmental sequences and restricting the search to sequences generated from type specimens). Results based on the ITS sequence (701 bp) yielded top BLAST hits to members of the genus *Coniolepiota* (*e.g.*, HM488757, KC625531, and HM488755) but only with approximately 87 % similarity. Results based on the LSU sequence (868 bp) yielded top BLAST hits to *Chlorophyllum molybdites* (KU049687, KU049686, and KU049684) with approximately 98 % similarity. Since BLAST matches provided limited resolution on the identity and taxonomic placement of this novel truffle, we performed a preliminary phylogenetic analysis based on the ITS and LSU regions.

Phylogenetic reconstruction using the ITS region (909 characters / 36 taxa) placed the novel fungus within the *Agaricus*+satellite clade (*sensu* Vellinga *et al.* 2011), which includes *Agaricus* and several other closely related genera such as *Clarkeinda, Coniolepiota, Eriocybe*, *Heinemannomyces*, and *Hymenagaricus* (BS: 75 %; PP: 0.98). Overall, the resulting topologies from the ML and BI analyses were similar and differences in relationships among taxa were unsupported by BS or PP statistics (Supplementary Fig. S1). The ML analysis placed the novel taxon as sister to *Eriocybe chionea* and the BI placed it sister to *Lepiota* cf. *fuscovinacea*, in both cases with no support.

The phylogenetic tree based on the LSU region (861 characters / 44 taxa) resolved the novel fungus as an unsupported lineage sister to *Agaricus* (Supplementary Fig. S2). The analysis based on LSU provided weak support for most relationships across the family. No conflict was found between the ML and BI analysis. The novel lineage was not recovered as closely related to any of the known sequestrate *Agaricaceae* taxa in either the ITS or LSU analyses.

The concatenated alignment based on LSU, *rpb2* and *tef1* consisted of 2 215 characters and comprised 29 taxa. This dataset included 902 bp for LSU, 766 bp for *rpb2* and 547 bp for *tef1*. ML and BI analyses generated phylogenies with non-conflicting

Table 1. GenBank accession numbers for taxa analyzed in this study. Bold taxa are sequences generated in this study.

Taxon	Collection	Location	ITS	LSU	*rpb2*	*tef1*
Agaricaceae sp.	ecv3807	Thailand	n/a	HM488770	n/a	n/a
Agaricus biberi	LAPAG687	Hungary	KM657919	KR006614	KT951548	KR006642
Agaricus bisporus	AFTOL-ID 448	USA	n/a	MH867670	AF107785	n/a
	n/a	n/a	n/a	MH867670	n/a	n/a
Agaricus campestris	LAPAG370	n/a	KM657927	KR006607	KT951556	KR006636
	n/a	Germany	n/a	AY207134	n/a	n/a
Agaricus comtulus	LAPAG724	Spain	n/a	KT951448	KT951532	KT951593
Agaricus erythosarx	H7638	n/a	JF495069	JF495024	n/a	n/a
	MURU6080	n/a	JF495068	JF495025	n/a	n/a
Agaricus inapertus	ecv2339	USA:California	AF482834	AF482878	n/a	HM488878
Agaricus kerriganii	LAPAG808	Spain	n/a	KT951442	KT951528	KT951589
Agaricus linzhiensis	ZRL2012618	China	KT951378	KT951503	KT951553	KT951582
Agaricus melanosporus	AD9763	n/a	JF495042	JF495027	n/a	n/a
	AD9767	n/a	n/a	JF495026	n/a	n/a
Agaricus subrutilescens	ZRLWXH3276	China	KT951392	KT951522	KT951554	KT951585
Agaricus warritodes	TWM1589	n/a	JF495052	JF495030	n/a	n/a
Asperosporus subterraneus	**FLAS-F-68001**	**USA: Florida**	**SUB9037367**	**SUB9307387**	**MW816922**	**MW816921**
Barcheria willisiana	BA1307	Australia	n/a	AY372212	n/a	n/a
	MEL2177563	Australia	JF495036	AY372216	n/a	n/a
	MEL269353	Australia	JF495035	n/a	n/a	n/a
Chlorophyllum agaricoides	HMAS 71678	China: Nei Mongol	MG742004	MG742021	MG742051	MG742079
Chlorophyllum globosum	PREM 62147	South Africa	MG742002	MG742024	MG742053	MG742081
	n/a	n/a	n/a	MG742023	n/a	n/a
Chlorophyllum molybdites	Z.W.Ge 3377	USA: Florida	MG741992	MG742033	MG742062	MG742090
Clarkeinda trachodes	ecv3550	Thailand	HM488751	KY418837	HM488802	n/a
	ecv3838	Thailand	HM488750	HM488771	n/a	n/a
Coniolepiota sponges	HKAS:60246	China	n/a	KC871015	n/a	n/a
Coniolepiota spongodes	png012	Thailand	HM488756	HM488774	HM488796	HM488796
Coprinus comatus	AFTOL626	n/a	n/a	AF041529	AY780934	AY881026
Coprinus sterquilinus	n/a	n/a	n/a	AF041530	n/a	n/a
Cryptolepiota americana	UTC00143916	n/a	HQ020412	EU130550	n/a	n/a
Cryptolepiota mengei	Trappe 7311	n/a	HQ020413	HQ020417	n/a	n/a
Cryptolepiota microspora	Trappe 17584	n/a	HQ020410	HQ020420	n/a	n/a
Eriocybe chionea	ecv3616	Thailand	HM488753	HM488772	HM488801	n/a
	ecv3560	Thailand	HM488752	HM488773	HM488800	n/a
Heinemannomyces splendidissima	SFSU zrl 3062	n/a	n/a	HM436608	n/a	n/a
	ecv3586	Thailand	HM488760	HM488769	HM488793	KT951657
Hymenagaricus taiwanensis	C.M. Chen 3636	China	n/a	DQ006270	n/a	n/a
Lepiota aff. *fuscovinacea*	mflu 09-0129	Thailand	HM488758	HM488775	n/a	n/a
Lepiota cf. *fuscovinacea*	ecv3556	Thailand	HM488759	HM488776	n/a	n/a
Lepiota omninoflava	KUN-HKAS 106734	China	MN810157	MN810092	MN820951	MN820924

Table 1. (Continued).

Taxon	Collection	Location	ITS	LSU	*rpb2*	*tef1*
Leucoagaricus americanus	ecv2454	USA	AY176407	AF482891	n/a	n/a
Leucoagaricus meleagris	n/a	Netherlands	AY176419	AF482890	n/a	n/a
Leucoagaricus sp.	ecv3745	USA: Hawaii	n/a	HM488780	HM488854	n/a
Leucocoprinus cepistipes	ecv3741	USA: Hawaii	n/a	HM488779	HM488844	n/a
Limacella delicata	ZTMyc55818	Switzerland	n/a	KT833807	KT833822	KT833835
Limacella glioderma	MB102389	Germany	n/a	KT833810	KT833825	KT833838
	n/a	Netherlands	n/a	AY176452	n/a	n/a
	HBAU15533	n/a	MZ145070	n/a	n/a	n/a
	GLM:GLM-F51951	Germany	MK412398	n/a	n/a	n/a
	n/a	USA: Michigan	n/a	AY176454	n/a	n/a
Limacella guttata	MB 100157	Germany	n/a	KT833813	KT833828	KT833841
Lycoperdon pyriforme	DSH 96-054	n/a	n/a	AF287873	AY218495	AY883426
Lycoperdon sp.	ecv3825	Thailand	n/a	HM488790	HM488876	n/a
Macrolepiota dolichaula	AFTOL-ID 481	China	DQ221111	DQ411537	DQ385886	n/a
Macrolepiota gasteroidea	H0052	Australia	n/a	JF495031	n/a	n/a
Macrolepiota mastoidea	HKAS 11207	n/a	n/a	JN940272	JN993698	n/a
Macrolepiota procera	KUN: HKAS 8108	China	n/a	AY207233	JN993697	n/a
Macrolepiota turbinata	H0219	Australia	JF495076	JF495032	n/a	n/a
Micropsalliota sp.	Laessoe 6025	Malaysia	n/a	AF482879	n/a	n/a
	ecv3638	Thailand	n/a	HM488768	n/a	n/a
Podaxis pistillaris	n/a	n/a	n/a	AF041539	n/a	n/a
Psuedolepiota zangmui	Z.W.Ge 2175	China: Yunnan	n/a	MG742049	n/a	n/a
Tulostoma kotlabae	n/a	Hungary	n/a	DQ112629	n/a	n/a
Tulostoma squamosum	Mrazek1300	Northern Europe	DQ415732	n/a	n/a	n/a
Xanthagaricus siamensis	MLFU 19-0576	Thailand	MN176993	MN176983	n/a	n/a
Xanthagaricus taiwanensis	n/a	Taiwan	DQ006271	n/a	n/a	n/a

topologies. Bootstrap support values and posterior probabilities were congruent for major nodes and recovered currently accepted phylogenetic relationships among genera within the *Agaricaceae* [for taxonomic revisions of the family see Vellinga (2004b), Vellinga *et al.* (2011), and Kalichman *et al.* (2020)]. The Bayesian analysis recovered (PP: 0.97) the novel taxon as an independent lineage within the *Agaricus*+satellite clade (Fig. 2). Phylogenies based on matrices with and without ambiguously aligned regions resulted in similar topologies. The multilocus phylogeny based on the concatenated alignment with ambiguously aligned regions excluded is shown in Supplementary Fig. S3.

Taxonomy

Asperosporus Karl.-Ayala, Gazis & M.E. Sm., ***gen. nov.*** MycoBank MB 838905.

Etymology: From the Latin "*asper*" (rough) and "*sporus*" (spores), for the highly ornamented spores.
Distinguished from other genera in *Agaricaceae* by a combination

of the following characters: *Basidiomata* hypogeous and sequestrate, globose to subglobose, astipitate, and friable when dry. *Gleba* loculate, brownish, turning pink-red when freshly cut or bruised, hydrophobic, drying powdery and friable. *Basidiospores* subglobose to ellipsoid, pale yellow-orange when young but turning brown with age, thick-walled, ornamented, sterigmal remnants attached, and strongly dextrinoid in Melzer's reagent. *Basidiomata* producing a rancid odor when fresh. *Clamp connections* absent.

Type species: *Asperosporus subterraneus* Karl.-Ayala, Gazis & M.E. Sm.

Asperosporus subterraneus, Karl.-Ayala, Gazis & M.E. Sm., ***sp. nov.*** MycoBank MB 838906. Figs 1, 3A–F.

Etymology: From the Latin "*sub*" (below) and "*terra*" (earth), for the habit of fruiting below the soil

Diagnosis: *Basidiomata* 5–30 mm diam, globose to irregular,

Fig. 1. *Asperosporus subterraneus* specimen FLAS-F-68001 (holotype) **A.** White hydrophobic mycelial mat binding organic matter in one of the potted Boston Fern plants (*Nephrolepsis exaltata*) submitted to the University of Florida Plant Diagnostic Clinic. **B.** Gasteroid basidiomata found in the potting soil. **C.** Red-staining gleba when freshly cut. **D.** Dry powdery gleba in a dried specimen, showing locules and the thin friable peridium. Scale bars: C–D = 1 cm.

astipitate, light tan but drying dark brown and staining pink-red when handled, producing a rancid odor when fresh. *Peridium* thin and friable when dry. *Gleba* loculate, staining pink-red when exposed to air, friable and hydrophobic when dried. *Basidia* 1–3 spored in fresh specimens, collapsing upon drying. *Spores* 16–22 × 12–18 µm, subglobose or ellipsoid, ornamented with warts, thick walled, strongly dextrinoid, often with sterigma remnants remaining attached. The genus is presently monotypic.

Macroscopic features: *Basidiomata* 5–30 mm diam, globose to irregular, lacking a stipe, surface smooth, at first white or light tan but becoming brownish with age, staining pink-red where handled or bruised (Fig. 1C), drying darker brown, particularly where bruised. *Peridium* irregular, friable when dry (Fig. 1D), sometimes sloughing off completely where handled, staining dark brown to black in Melzer's reagent. No response in KOH

when dried. *Gleba* compressed, irregular, loculate (Fig. 3A), lacking a columella, light tan at first with occasional veins of white trama tissue and pockets of darker brown spores, staining pinkish or red when cut and exposed to air, reddish staining more notable in younger specimens, turning dark brown, powdery, delicate and hydrophobic upon drying. *Odor* rancid when fresh, indistinctly fungal when dry. *Taste* not determined.

Microscopic features: *Basidiospores* 16–22 × 12–18 µm (av. 18 × 15 µm) at maturity, globose, subglobose or ellipsoid (Q = 1–1.47 µm, mean Q = 1.21 µm); walls 1–3.5 µm thick (av. =1.8 µm); restricted at the sterigmal attachment point and apiculate, notably ornamented with larger pyramidal to irregular warts, up to 1 µm tall and 1 µm wide. Immature spores are notably smaller, mostly 12–15 × 10–12 µm, and with smaller spines that are 0.5 × 0.5 µm and clearly separated from one another.
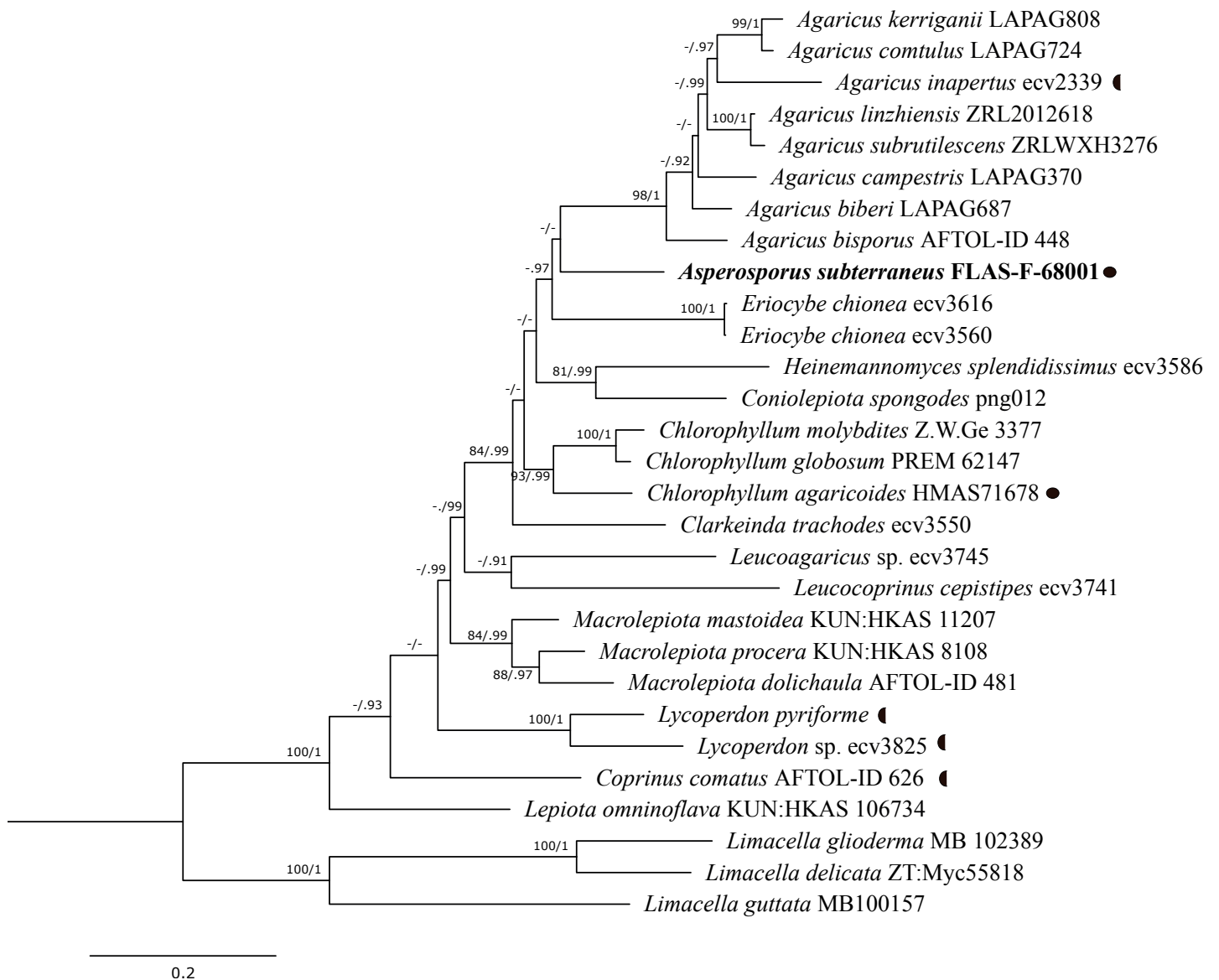
Fig. 2. Maximum likelihood phylogeny based on the combined LSU, *rpb2*, and *tef1* dataset. Branch bootstrap support values >75 % and Bayesian posterior probabilities > 0.90 are indicated. Symbols with closed circles represent gasteroid taxa, symbols with half circles represent secotoid taxa, and all other taxa are agaricoid.

Sterigma remnants often remaining attached and clearly visible in many spores (Fig. 3E) but more common in young spores, most 4–5 μm × 1–2 μm but sometimes up to 9 μm long. Pale yellow-orange when young but becoming darker orange-brown at maturity when observed using KOH or water, strongly dextrinoid in Melzer's reagent with mature spores turning notably darker than younger spores, highly variable in size and shape. *Basidia* 1–3 spored, difficult to find and see, collapsing in mature dried specimens (Fig. 3F). *Cystidia* not observed. *Peridium* (100–)150–250(–350) μm thick, composed of loosely interwoven (Fig. 3B) and irregularly branched and septate, single layered, hyphae 3–5 μm diam, occasionally swelling up to 10 μm; arrangement of hyphae mostly tangled and irregular but occasional bands of hyphae parallel to the exterior near the peridial surface, light yellow-brown, some hyphae strongly dextrinoid. *Trama* tissue 75–200 μm thick, composed of irregularly shaped, elongated and inflated hyaline hyphae, 10–26 μm diam. *Subhymenium* approximately 10–40 μm thick, comprised of densely packed interwoven hyphae with cells 12–14 μm diam that are brown

to orange-brown. *Clamp connections* absent on all hyphae. No response to KOH.

Habitat and distribution: Found in south Florida growing in soil of potted nursery plants with poor drainage. Specimens thus far found only in association with Boston Fern (*N. exaltata*) which was planted in a Canadian peat moss and Florida pine bark mixed potting soil during the winter months.

Typus: **USA**, Florida, Tropical Research and Education Center Plant Diagnostic Clinic, Homestead, Miami-Dade Co., 21 Dec. 2017, *R. Gazis*, MES-3094 (**holotype** FLAS-F-68001).

Additional collection examined: **USA**, Florida, Tropical Research and Education Center Diagnostic Clinic, Homestead, Miami-Dade Co., 4 Dec. 2018, *R. Gazis,* 180871 (Photos deposited in MycoBank, but specimens not dried properly and therefore discarded).
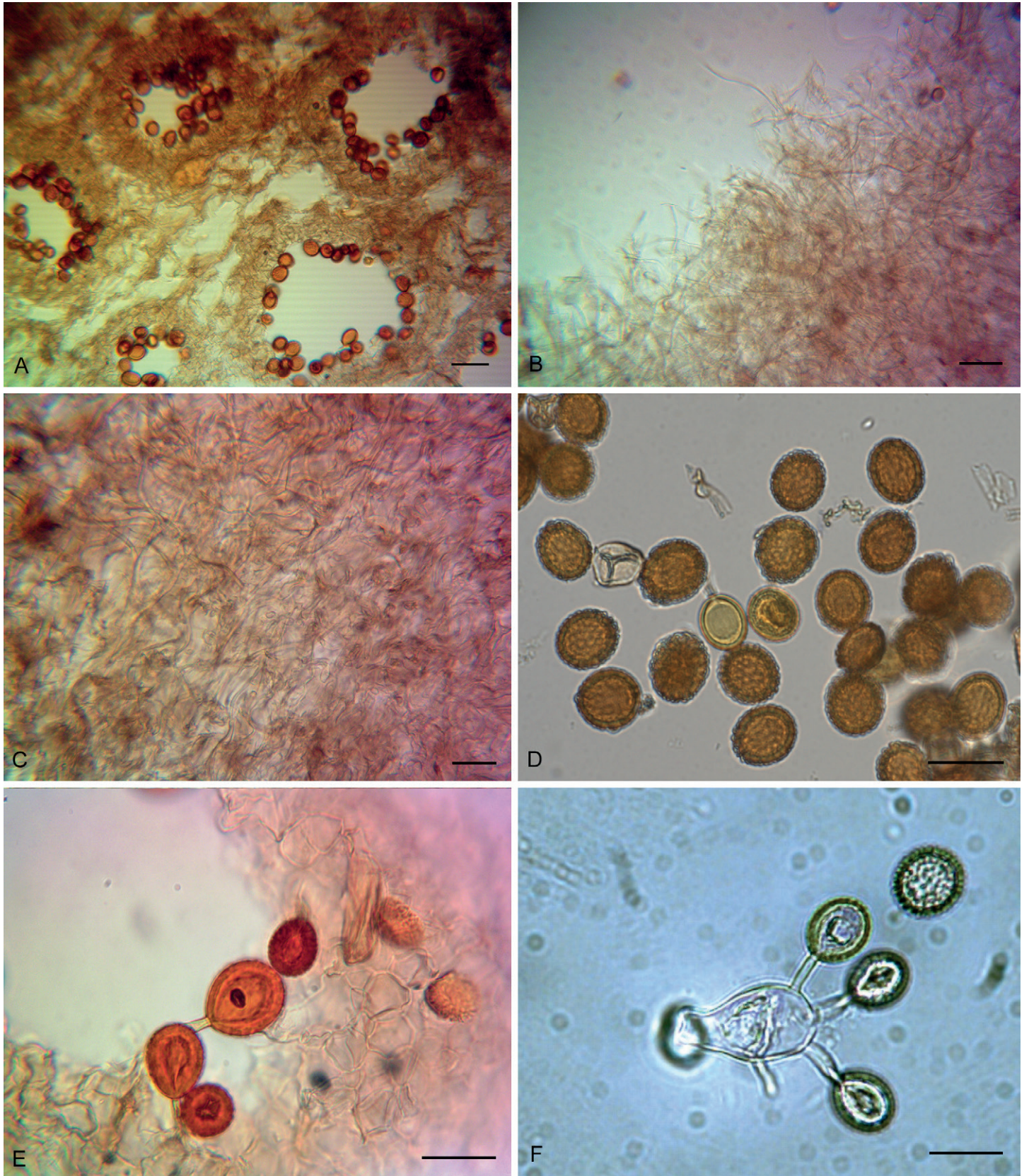
Fig. 3. Microscopic features of *Asperosporus subterraneus* specimen FLAS-F-68001 (holotype) **A.** Loculate gleba **B.** Peridium hyphae. **C.** Tangled peridium hyphae. **D.** Ornamented spores with thick spore walls. **E.** Subhymenium with basidiospores, some of which have retained sterigma remnants (indicated with arrows). **F.** Basidium with basidiospores. Scale bars: A–C = 60 µm, D–F = 20 µm.

DISCUSSION

Morphology

Several puffballs in *Agaricaceae s.l.* (*i.e.*, *Bovista, Calvatia, Lycoperdon*) have features that are similar to those of *A. subterraneus*, such as the highly ornamented, powdery brown spores and a hydrophobic surface. However, these puffballs do not stain red when handled and typically produce aboveground basidiomata (Vellinga, 2004b, Larsson & Jeppson 2008, Jeppson *et al.* 2012). Furthermore, most of these puffball species have spores that are relatively small (*e.g.* less than 10 μm diam) whereas the spores of *A. subterraneus* are much larger (av. 18 × 15 μm) (Miller & Miller 1988). A pink-red staining reaction of the basidiomata is prominent in many genera of the *Agaricaceae*, including in species of *Agaricus, Barcheria, Chlorophyllum, Cystolepiota, Lepiota, Leucoagaricus, Leucocoprinus, Macropsalliota,* and *Melanophyllum* (Pegler 1985, Vellinga 2010, Vellinga *et al.* 2010, 2011, Zhao *et al.*, 2010, 2016, Ge *et al.* 2018). However, the combination of red staining basidiomata and ornamented spores is unique to *A. subterraneus* within *Agaricaceae*. *Leucoagaricus* (*Agaricaceae*) also contains red staining taxa without clamp connections (Vellinga *et al.* 2010a, Dutta *et al.* 2021) but these taxa are all agaricoid. Morphologically, *A. subterraneus* could be mistaken for *Barcheria willisiana* (*Agaricaceae*) which is a fragile, astipitate, sequestrate fungus that contains an enclosed, loculate gleba, stains red when bruised, lacks clamp connections, and contains thick-walled globose spores that are strongly dextrinoid in Melzer's reagent (Lebel *et al.* 2004). However, *Barcheria* is currently known only from Australia and this taxon is easily differentiated from *A. subterraneus* by its smooth spores, scaly peridium, and basidia that remain intact upon drying.

Initial molecular and morphological similarities suggested that *A. subterraneus* was closely related to *Chlorophyllum* (*Agaricaceae*). *Chlorophyllum* is a diverse genus that contains several taxa that are sequestrate and stain upon handling as well as taxa that are associated with disturbed, nutrient-rich landscapes (Vellinga 2004a, Ge *et al.* 2018). Recently, more species of sequestrate *Chlorophyllum* have been discovered, such as *C. lusitanicum* (Carlavilla *et al.* 2018) and *C. levantinum* (Loizides *et al.* 2020). However, sequestrate fungi in this genus typically have smooth spores and a well-defined columella or stipe. Kropp *et al.* (2012) described three new species of sequestrate taxa from western North America and erected a new genus, *Cryptolepiota*, that were later transferred to *Lepiota* (*Agaricaceae*) (Lebel & Vellinga 2013). All of these taxa are subglobose, astipitate, sequestrate fungi that are fragile when dry. *Asperosporus* can be differentiated from species of "*Cryptolepiota*" by the pink-red staining reactions, rancid odor when fresh, ornamented spores and basidia that collapse upon drying. While spore ornamentation can be used to differentiate *Asperosporus* from most *Lepiota* species, there are some sequestrate *Lepiota* species with ornamented spores. However, all of these these taxa (*e.g. L. iberica, L. olbia, L. smurfiorum*, and *L. tulostomoides*) are secotioid and retain their stipe at maturity (Vidal *et al.* 2015). The sequestrate fungus *Neosecotium macrosporum* (*Agaricaceae*) also has ornamented spores and a powdery gleba (Lizárraga *et al.* 2012). While there are currently no publicly available sequence data for *Neosecotium macrosporum*, this sequestrate fungus differs from *A. subterraneus* in that *N. macrosporum* has a columella, a thick peridium, smaller and more globose spores, and flatter ornamentation that give the spores a cracked appearance. *Agaricus* (*Agaricaceae*) is a large genus of saprotrophic fungi containing more than 400 species in six subgenera and 21 sections (Zhao *et al.* 2016, Chen *et al.* 2017). This genus has species with diverse basidiomata types, including many different agaricoid, secotoid, and gasteroid taxa. Newly described sequestrate taxa (Lebel & Syme 2012, Lebel 2013) as well as species that were more recently transferred from other obsolete genera (*e.g. Longula texensis* = *Agaricus deserticola*) have expanded the diversity of this genus. Many taxa in *Agaricus* also stain or bruise red when fresh and all lack clamp connections (Zhao *et al.* 2016). However, species of *Agaricus* can be differentiated from *Asperosporus* by their smooth spores that lack the ornamentation seen in *A. subterraneus*.

Asperosporus subterraneus is characterized by a unique combination of morphological features that also differentiates it from other known sequestrate fungi outside *Agaricaceae*. Sequestrate fungi with fully enclosed basidiomata typically lose their ability to forcibly discharge their spores. Evidence of this can be seen in fungi that have poorly reviving basidia when dried and have spores with sterigma remnants (Trappe *et al.* 2009). Sterigma attachments was seen in *Asperosporus subterraneus* and in other sequestrate fungi such as *Guyanagaster necrorhiza* (*Physalacriaceae*) (Henkel *et al.* 2010). *Guyanagaster necrorhiza* is similar to *A. subterraneus* in that both taxa have strongly dextrinoid, thick-walled, ornamented spores and a well-defined gleba. However, the spores of *G. necrorhiza* are spiny, globose and light tan with pink tones and the peridium is highly warted (Henkel *et al.* 2010). Another genus with similarities to *Asperosporus* is *Octaviania* (*Boletaceae*). *Octaviania* basidiomata have sporocarps that often bruise when handled (blue, green, or black but occasionally red) and have globose to subglobose spores with pyramidal ornamentation and dextrinoid reactions (Orihara *et al.* 2012). However, species of *Octaviania* generally have smaller spores with more conspicuous ornaments and a gleba that never becomes powdery at maturity. The truffle *Xerocomellus macmurphyi* (*Boletaceae*) is also similar to *A. subterraneus* in its powdery gleba and in the dextrinoid spores that are similar in size and ornamentation (Smith *et al.* 2018). However, both *Octaviania* and *Xerocomellus* belong to the *Boletales* and are ectomycorrhizal whereas *A. subterraneus* belongs to the *Agaricaceae* and is likely saprotrophic. Many previously described truffle-like fungi with ornamented brown spores were originally placed within the genus *Hymenogaster*. However, morphological and molecular analyses have shown that *Hymenogaster sensu lato* contains many different unrelated taxa (Bougher & Castellano 1993, Smith *et al.* 2018). We have reviewed the descriptions of taxa in *Hymenogaster sensu lato* (Dodge & Zeller 1934) and were unable to locate any species that match the description of *A. subterraneus*.

Phylogenetic placement

Several challenges arise when attempting to place *A. subterraneus* into a phylogenetic context. No close matches (above 90 %) were found in nucleotide databases (NCBI and UNITE) between ITS sequences of *A. subterraneus* and previously collected specimens or environmental sequences generated through metabarcoding studies. Maximum Likelihood and Bayesian analyses based on the LSU and multilocus datasets placed *Asperosporus subterraneus* as sister to *Agaricus* within the *Agaricus*+satellite genera clade (*sensu* Vellinga *et al.* 2011) without support (Fig. 2, Supplementary Fig.

S2). The phylogenetic tree based on the ITS region also recovered *A. subterraneus* within the *Agaricus*+satellite genera clade, but as an unsupported sister lineage sister to *Eriocybe chionea* in the ML and sister to *Lepiota cf. fuscovinaceae* in the BI analyses (Supplementary Fig. S1). Many of the satellite taxa in the *Agaricus* clade, such as *Coniolepiota*, *Eriocybe*, and *Heinemannomyces* and *Psuedolepiota* are monotypic genera, with limited collections and sequence data, and some of them have been described from a single locality in Asia. *Heinemannomyces* is a monotypic genus but recently has been shown to fall within *Hymenagaricus* (Hussain *et al.* 2018). Moreover, phylogenetic relationships among members of this clade remain unresolved (Watling 1998, Vellinga *et al.* 2011, Ge & Yang 2017) and unsupported nodes may be a reflection of incomplete taxon sampling. Therefore, future efforts to increase taxon sampling may be an important component to improve our accurate phylogenetic placement and understanding of relationships within this group (Som 2015). This includes increasing sequence availability of additional loci from known taxa in this clade together with the description of more unknown taxa.

Ecology of *Asperosporus subterraneus*

Asperosporus subterraneus has thus far only been found in association with potted Boston Fern plants submitted to the University of Florida Plant Diagnostic Clinic in Homestead, Florida in 2017 and 2018. The hyphae of *A. subterraneus* extensively colonize the substrate, thereby generating a hydrophobic mat which binds the organic matter and prevents the plant's roots from accessing water and nutrients. There is no evidence to suggest that *A. subterraneus* is a plant pathogen because there was no obvious root contact with the fungus. However, *A. subterraneus* can cause plants to develop wilting and chlorosis that make them unmarketable. The dense hyphal mats and ample basidiomata found within the substrate suggest that this fungus can become prolific in nursery settings and that the source of inoculum is likely the potting soil. Thus far basidiomata have not been found in soil or pots without plants. The south Florida ornamental industry generally grows plants in outdoor shade houses that allow ambient rainfall, temperature, and humidity to impact the growth of plants. The planting containers likely create suitable conditions for this fungus to fruit during plant production. *Asperosporus subterraneus* has been a recurrent problem in ornamental nurseries in south Florida and is thought to be associated with potting soil containing a mix of peat moss and pine bark (R. Gazis, pers. obs.). A robust understanding of the lifestyle, growth requirements, and dispersal of this fungus will help develop effective management recommendations for nurseries to prevent financial losses due to unmarketable plants. There are several fungi in the *Agaricaceae* that are common in nursery settings or in indoor potted plants. For example, *Leucocoprinus birnbaumii*, commonly called the "yellow house plant mushroom" is frequently found in indoor potted houseplants (Vellinga 2004a). Some disturbance-adapted *Agaricaceae* species have been found far outside their known natural distributions. For example, *Smithiomyces mexicanus,* known from tropical regions in Florida and Brazil, was found growing in Belgium around swimming pools with a sub-tropical landscape (Vellinga 1999). The nutritional mode for *Asperosporus subterraneus* remains unknown but we can infer it as being saprotrophic because it falls within the *Agaricaceae*, close to the *Agaricus* clade, which only contains saprotrophic

species (Vellinga 2004b). Although this newly described fungus was not found in a natural environment and we do not know the geographic origin of this fungus, we still introduce this taxon as novel based on morphological characteristics and molecular evidence. Furthermore, the morphological characteristics and molecular placement of this truffle highlight the high diversity and unique combinations of morphological characteristics of sequestrate fungi within the *Agaricaceae*.

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REFERENCES

Abarenkov K, Nilsson RH, Larsson KH, *et al*. (2010). The UNITE database for molecular identification of fungi-recent updates and future perspectives. *New Phytologist* **186**: 281–285.

Bougher NL, Castellano MA (1993). Delimitation of *Hymenogaster sensu stricto* and four new segregate genera. *Mycologia* **85**: 273–293.

Carlavilla JR, Moreno G, Mohedano JM (2018). *Chlorophyllum lusitanicum*, a rare species from the Iberian Peninsula. *Boletín de la Sociedad Micológica de Madrid* **42**: 99–105.

Darriba D, Taboada GL, Doallo R, *et al*. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772–772.

Dodge CW, Zeller SM (1934). *Hymenogaster* and related genera. *Annals of the Missouri Botanical Garden* **21**: 625–708.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Dutta AK, Stallman JK, Bera S, *et al*. (2021). Lepiotaceous fungi of West Bengal, India: two new species of *Leucoagaricus*. *Mycological Progress* **20**: 493–507.

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.

Ge ZW, Jacobs A, Vellinga EC, *et al.* (2018). A multi-gene phylogeny of *Chlorophyllum* (*Agaricaceae*, *Basidiomycota*): new species, new combination and infrageneric classification. *MycoKeys* **32**: 65–90.

Ge ZW, Smith ME (2013). Phylogenetic analysis of rDNA sequences indicates that the sequestrate *Amogaster viridiglebus* is derived from within the agaricoid genus *Lepiota* (*Agaricaceae*). *Mycological Progress* **12**: 151–155.

Henkel TW, Smith ME, Aime MC (2010). *Guyanagaster*, a new wood-decaying sequestrate fungal genus related to *Armillaria* (*Physalacriaceae, Agaricales, Basidiomycota*). *American Journal of Botany* **97**: 1474–1484.

Huelsenbeck JP, Larget B, Alfaro ME (2004). Bayesian phylogenetic model selection using reversible jump Markov chain Monte Carlo. *Molecular Biology and Evolution* **21**: 1123–1133.

Hussain S, Ahmad H, Sher H, *et al*. (2018). *Xanthagaricus pakistanicus sp. nov.* (*Agaricaceae*): first report of the genus from Pakistan. *Turkish Journal of Botany* **42**: 123–133.

Jeppson M, Larsson E, Martín MP (2012). *Lycoperdon rupicola* and *L. subumbrinum*: two new puffballs from Europe. *Mycological Progress* **11**: 887–897.

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

Katoh K, Misawa K, Kuma, *et al*. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30***: 3059–3066.

Kropp BR, Albee-Scott S, Castellano MA, *et al*. (2012). *Cryptolepiota*, a new sequestrate genus in the *Agaricaceae* with evidence for adaptive radiation in western North America. *Mycologia* **104**: 164–174.

Larsson E, Jeppson M (2008). Phylogenetic relationships among species and genera of Lycoperdaceae based on ITS and LSU sequence data from north European taxa. *Mycological Research* **112**: 4–22.

Lebel T (2013). Two new species of sequestrate *Agaricus* (section Minores) from Australia. *Mycological Progress* **12**: 699–707.

Lebel T, Syme A (2012). Sequestrate species of *Agaricus* and *Macrolepiota* from Australia: new species and combinations and their position in a calibrated phylogeny. *Mycologia* **104**: 496–520.

Lebel T, Thompson DK, Udovicic F (2004). Description and affinities of a new sequestrate fungus, *Barcheria willisiana gen. et sp. nov.* (*Agaricales*) from Australia. *Mycological Research* **108**: 206–213.

Lebel T, Vellinga EC (2013). Description and affinities of a sequestrate *Lepiota* (*Agaricaceae*) from Australia. *Mycological Progress* **12**: 525–532.

Lizárraga M, Esqueda M, Vargas-Luna M, *et al.* (2012). First record of the sequestrate fungus *Neosecotium macrosporum* (*Agaricales, Lepiotaceae*) from Mexico. *Mycotaxon* **120**: 437–441.

Loizides M, Alvarado P, Polemis E, *et al*. (2020). Multiple evolutionary origins of sequestrate species in the agaricoid genus *Chlorophyllum*. *Mycologia* **112**: 400–422.

Matheny PB (2005). Improving phylogenetic inference of mushrooms with *RPB1* and *RPB2* nucleotide sequences (*Inocybe*; *Agaricales*). *Molecular Phylogenetics and Evolution* **35**: 1–20.

Miller SL (1999). Field key to common genera of hypogeous and gasteroid basidiomycetes of North America. *McIlvainea* **14**: 8–29.

Miller OK, Miller H (1988). *Gasteromycetes: morphological and developmental features, with keys to the orders, families, and genera*. Mad River Press, Eureka, CA, USA.

Orihara T, Smith ME, Shimomura N, *et al*. (2012). Diversity and systematics of the sequestrate genus *Octaviania* in Japan: two new subgenera and eleven new species. *Persoonia* **28**: 85–112.

Pegler DN (1985). The genus *Clarkeinda* (*Basidiomycotina*: *Agaricaceae*). *Botanical Journal of the Linnean Society* **91**: 245–252.

Reeb V, Lutzoni F, Roux C (2004). Contribution of *RPB2* to multilocus phylogenetic studies of the euascomycetes (*Pezizomycotina, Fungi*) with special emphasis on the lichen-forming *Acarosporaceae* and evolution of polyspory. *Molecular Phylogenetics and Evolution* **32**: 1036–1060.

Rehner SA, Buckley E (2005). A *Beauveria* phylogeny inferred from nuclear ITS and *EF1-α* sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* **97**: 84–98.

Smith ME, Castellano MA, Frank JL (2018). *Hymenogaster macmurphyi* and *Splanchnomyces behrii* are sequestrate species of *Xerocomellus* from the western United States. *Mycologia* **110**: 605–617.

Som A (2015). Causes, consequences and solutions of phylogenetic incongruence. *Briefings in Bioinformatics* **16**: 536–548.

Stamatakis A (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312–1313.

Talavera G, Castresana J (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56**: 564–577.

Trappe JM (2009). *Diversity, ecology, and conservation of truffle fungi in forests of the Pacific Northwest* (Vol. 772). US Department of Agriculture, Forest Service, Pacific Northwest Research Station.

Vellinga EC (1999). An American in a Belgian swimming pool. *Mycologist* **2**: 50–53.

Vellinga EC (2004). Ecology and distribution of lepiotaceous fungi (*Agaricaceae*) – A review. *Nova Hedwigia* **78**: 273–300.

Vellinga EC (2004). Genera in the family *Agaricaceae*: evidence from nrITS and nrLSU sequences. *Mycological Research* **108**: 354–377.

Vellinga, EC (2010). Lepiotaceous fungi in California, USA *Leucoagaricus* sect. *Piloselli*. *Mycotaxon* **112**: 393–444.

Vellinga EC, Contu M, Vizzini A (2010). *Leucoagaricus decipiens* and *La. erythrophaeus*, a new species pair in sect. *Piloselli*. *Mycologia* **102**: 447–454.

Vellinga EC, Sysouphanthong P, Hyde KD (2011). The family *Agaricaceae*: phylogenies and two new white-spored genera. *Mycologia* **103**: 494–509.

Vidal JM, Juste P, García F, *et al*. (2015). Hongos secotioides del género *Lepiota*: dos nuevas especies, dos nuevas combinaciones y reevaluación del género *Cribrospora*. *Boletín Micológico de FAMCAL* **10**: 47–71.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

Watling R (1998). *Heinemannomyces*: a new lazuline-spored agaric genus from South-East Asia. *Belgian Journal of Botany*, 133–138.

White TJ, Bruns T, Lee S, *et al*. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics In: Innis MA, Gelfand DH, Sninsky JJ and White JW (eds): *A Guide to Molecular Methods and Applications*. Academic Press, New York: 315–322.

Zhao R, Karunarathna S, Raspé O, *et al*. (2011). Major clades in tropical *Agaricus*. *Fungal Diversity* **51**: 279–296.

Zhao RL, Desjardin DE, Soytong K, *et al*. (2010). A monograph of *Micropsalliota* in Northern Thailand based on morphological and molecular data. *Fungal Diversity* **45**: 33–79.

Zhao RL, Zhou JL, Chen J, *et al*. (2016). Towards standardizing taxonomic ranks using divergence times–a case study for reconstruction of the *Agaricus* taxonomic system. *Fungal Diversity* **78**: 239–292.
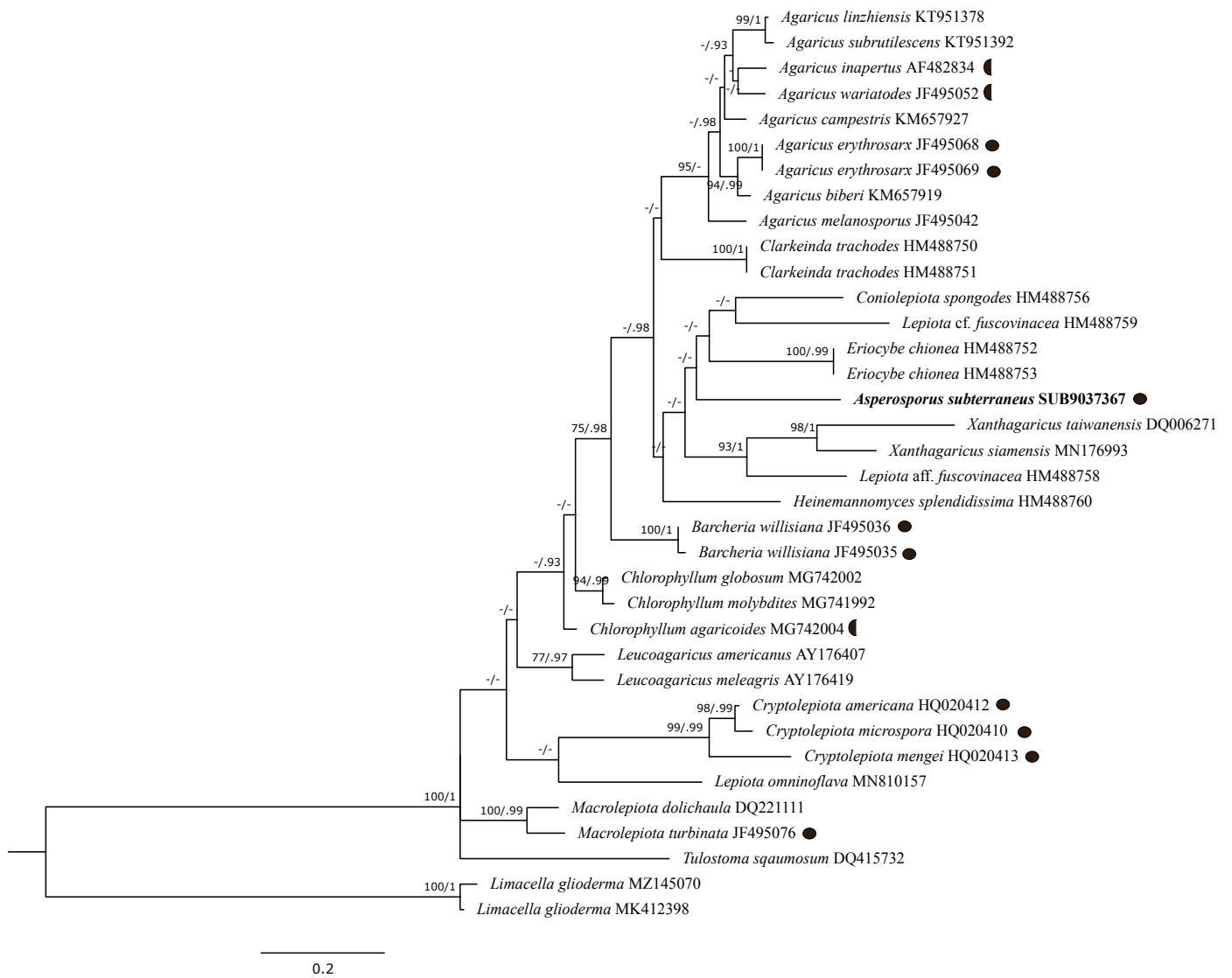
Supplementary Material: http://fuse-journal.org/

Fig. S1. Maximum likelihood tree based on ITS dataset. Branch support values >75 % and Bayesian posterior probabilities > 0.90 are indicated. Symbols with closed circles represent gasteroid taxa, symbols with half circles represent secotoid taxa, and all other taxa are agaricoid. *Limacella glioderma* was used as an outgroup.

Fig. S2. Maximum likelihood tree based on LSU dataset. Branch support values >75 % and Bayesian posterior probabilities > 0.90 are indicated. Symbols with closed circles represent gasteroid taxa, symbols with half circles represent secotoid taxa, and all other taxa are agaricoid. *Limacella glioderma* was used as an outgroup.
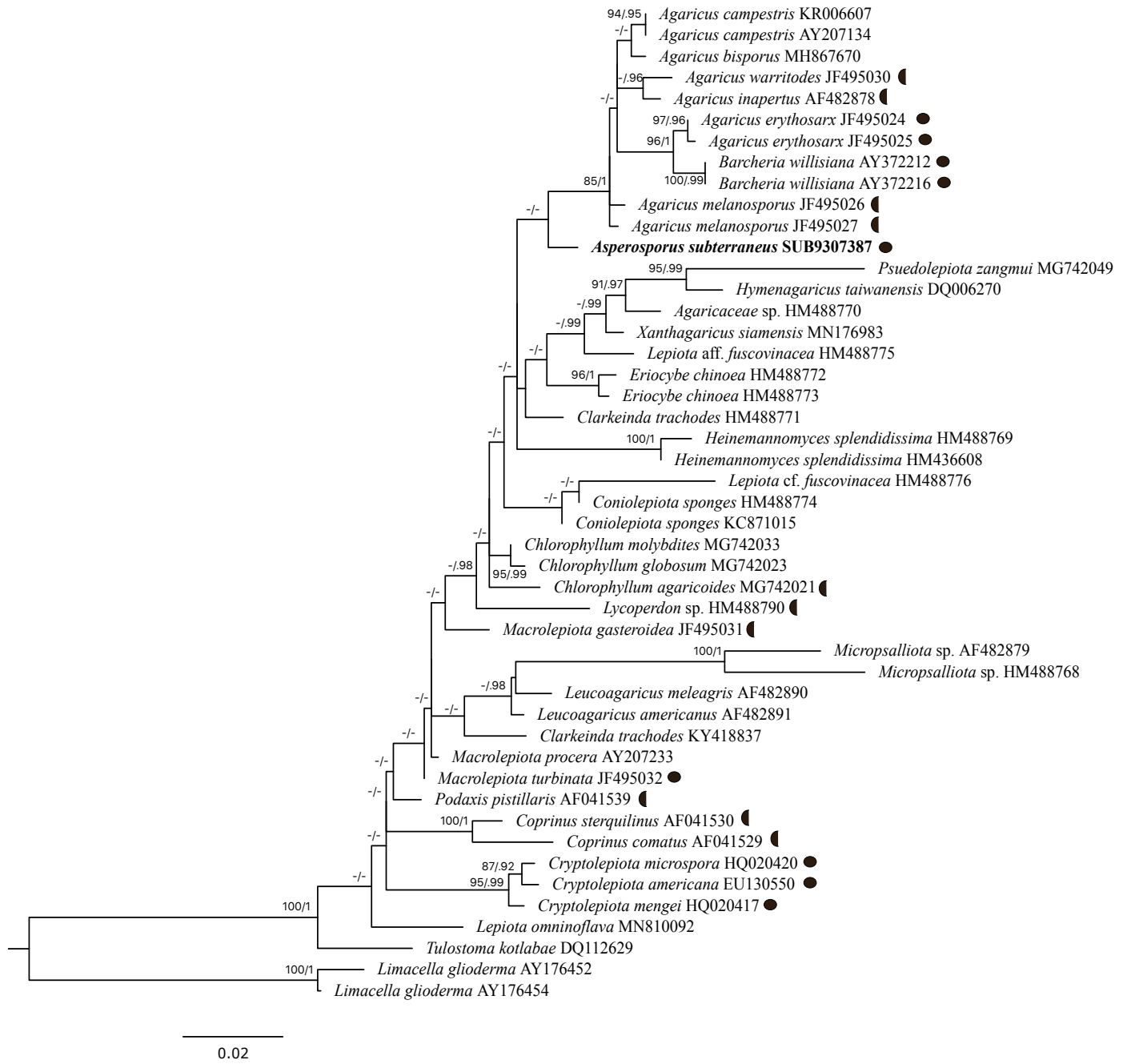
Fig. S3. Maximum likelihood tree based on concatenated LSU, *rpb2*, and *tef1* dataset with ambiguously aligned regions excluded. Branch bootstrap support values >75 % and Bayesian posterior probabilities > 0.90 are indicated. *Limacella delicata, Limacella glioderma* and *Limacella guttata* were used as an outgroup
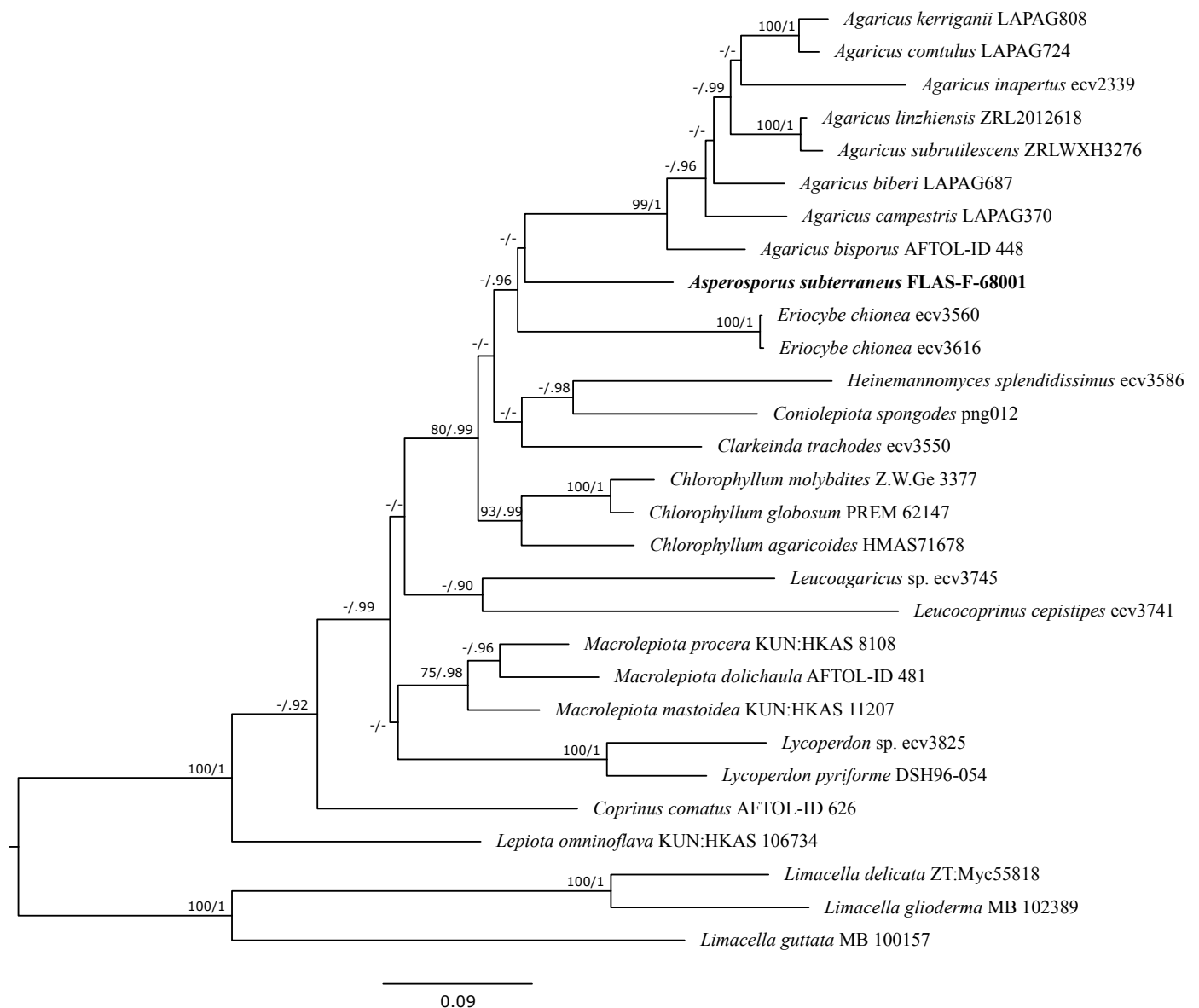
Figure S1

Supplementary Fig. S1. Maximum likelihood tree based on ITS dataset. Branch support values >75 % and Bayesian posterior probabilities > 0.90 are indicated. Symbols with closed circles represent gasteroid taxa, symbols with half circles represent secotoid taxa, and all other taxa are agaricoid. *Limacella glioderma* was used as an outgroup.

Figure S2

Supplementary Fig. S2. Maximum likelihood tree based on LSU dataset. Branch support values >75 % and Bayesian posterior probabilities > 0.90 are indicated. Symbols with closed circles represent gasteroid taxa, symbols with half circles represent secotoid taxa, and all other taxa are agaricoid. *Limacella glioderma* was used as an outgroup.

Figure S3

Supplementary Fig. S3. Maximum likelihood tree based on concatenated LSU, *rpb2*, and *tef1* dataset with ambiguously aligned regions excluded. Branch bootstrap support values >75 % and Bayesian posterior probabilities > 0.90 are indicated. *Limacella delicata, Limacella glioderma* and *Limacella guttata* were used as an outgroup.