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Short title: Australian sequestrate fungi

Australasian sequestrate fungi 18: *Solioccasus polychromus* gen. & sp. nov., a richly colored, tropical to subtropical, hypogeous fungus

James M. Trappe[1]

Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon 97331-5752

Michael A. Castellano

USDA Forest Service, Northern Research Station, 3200 Jefferson Way, Corvallis, Oregon 97331

Roy E. Halling

New York Botanical Garden, 2900 Southern Boulevard, Bronx, New York 10458-5126

Todd W. Osmundson

Forest Pathology and Mycology Laboratory, Department of Environmental Science, Policy and Management, University of California, Berkeley, California 94720-3114

Manfred Binder

CBS Fungal Biodiversity Centre, Evolutionary Phytopathology, Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW), Uppsalalaan 8, 3584 CT, Utrecht, the Netherlands

Nigel Fechner

Queensland Herbarium, Mount Coot-tha Road, Toowong, Brisbane, Queensland 4066, Australia

Nicholas Malajczuk

Treetec Consulting Pty Ltd., P.O Box 1046, Bunbury, Western Australia 6231, Australia

Abstract: *Solioccasus polychromus* gen. & sp. nov., the most brightly colored hypogeous fungus known, is described from Papua New Guinea and tropical northern Australia south into subtropical forests along the Queensland coast and coastal mountains to near Brisbane. Phylogenetic analysis of molecular data places it as a sister genus to *Bothia* in the Boletineae, a clade of predominantly ectomycorrhizal boletes. Ectomycorrhizal trees, such as members of the Myrtaceae (*Eucalyptus*, *Corymbia*, *Lophostemon*, *Melaleuca* spp.) and *Allocasuarina littoralis*, were present usually in mixture or in some cases dominant, so we infer some or all of them to be among the ectomycorrhizal hosts of *S. polychromus*.

Key words: Basidiomycota, Boletales, Boletineae, *Bothia*, DNA, EF1-α, ectomycorrhizae, LSU, ITS, rhizomorphs, tef1

INTRODUCTION

In 1992, three of us (Trappe, Castellano, Malajczuk) along with colleagues and volunteers mounted a fungus-collecting expedition to Papua New Guinea sponsored by the Australian Centre for International Agriculture Research. The two major goals were to (i) collect and identify sporocarps of ectomycorrhizal fungi adapted to tropical habitats and (ii) obtain mycelial isolates that could be tested for inoculating eucalypt seedlings for use in habitat restoration programs in the tropics.

At that time epigeous fungi of the island of New Guinea had received some attention, but hypogeous fungi largely had been ignored. Australian mycologist Dr Jack Simpson had collected *Elaphomyces* and *Descomyces* specimens in Papua New

Guinea and sent them to Trappe, but their identity could not be determined with confidence at that time. Accordingly, we anticipated finding some novel hypogeous fungi, and in *Solioccasus polychromus* our hopes were well rewarded. In subsequent years we found the species in subtropical and tropical Australia as well, in Queensland from near Brisbane north to Cape York and in Northern Territory's Arnhem Land.

MATERIALS AND METHODS

Habitats were sampled for hypogeous sporocarps by raking small patches in the forest floor near ectomycorrhizal hosts and processing collections as described by Castellano et al. (1989). At the end of each collecting day, specimens were sliced vertically in half or, if more than 2 cm broad, into several slabs. After characters of fresh specimens were recorded, the Papua New Guinea collections were placed on trays of a food dehydrator powered by a portable gasoline-powered generator; later collections from Australia were preserved in silica gel. When thoroughly dried, specimens were placed in plastic zipper-sealed bags to prevent them from rehydrating in the 95-plus percent humidity prevalent in that season. Dried specimens were checked daily for mold; if any developed they were further dried and rebagged. Colors were matched to swatches in Kelly and Judd (1989); their color terminology is used and the swatch numbers given in parentheses as "ISCC-NBS #." Herbaria are designated by Index Herbariorum abbreviations (http://sweetgum.nybg.org/ih/).

Microscopic structures were examined with a compound microscope from hand-cut sections mounted respectively in 5% KOH and Melzer's reagent. Meaurements of structural dimensions are given as length × width. Spores also were examined by scanning electron microscopy (SEM).

For molecular analysis, silica gel-preserved basidiome specimens were pulverized with a FastPrep tissue lyser (Qbiogene Inc.) and genomic DNA was extracted by the protocol of Alexander et al. (2007) with minor modifications. Partial sequences were obtained from three nuclear loci: the ribosomal large (25S) subunit (nucLSU), the rDNA internal transcribed spacer (ITS) region and translation elongation factor 1α (tef1 or EF1-α). PCR amplifications were performed in 25 µL volumes consisting of 2.5 µL 10× PCR buffer, 2.5 µL dNTP mix (0.2 mM), 2.5 µL 10× bovine serum

albumin, 1 µL of each primer (10 µmol concentration) 0.2 µL Taq polymerase, 1 µL template DNA and ddH_20 to reach 25 µL total volume. PCR primers were LR0R and LR7 (Vilgalys and Hester 1990) for nucLSU, ITS1 and ITS4 (White et al. 1990) for ITS and 526F + 1567R and 983F + 2218R (Rehner and Buckley 2005) for tef1. Amplification conditions for nucLSU and ITS were (i) initial denaturation at 95 C for 5 min, (ii) 40 cycles of denaturation at 94 C for 60 s, annealing at 56 C for 60 s and extension at 72 C for 60 s, (iii) final extension at 72 C for 5 min. Amplification of tef1 was conducted with a touchup PCR procedure: (i) 95 C for 5 min; (ii) 94 C for 60 s, 56 C for 60 s, increasing 1 C per cycle for the following nine cycles, then 72 C for 60 s; (iii) 30 additional cycles of 94 C for 60 s, 65 C for 60 s and 72 C for 60 s; (iv) final extension at 72 C for 5 min. PCR products were purified with ExoSAP-IT (Affymetrix Inc.), cycle sequenced with the Big Dye terminator sequencing kit 3.1 (Applied Biosystems) and sequenced with an ABI 3700 capillary sequencer at the High Throughput Genomics Facility, University of Washington. Sequencing primers were identical to the PCR primers. Sequences were obtained for two *Solioccasus polychromus* specimens and submitted to GenBank under these accession numbers: J. Trappe 15399, Nhulunbuy, Northern Territory, Australia (nucLSU: GenBank JQ287643; ITS: GenBank JX888459); and R.E. Halling 9417, Frasier Island, Queensland, Australia (nucLSU: GenBank JQ287642 and tef1: GenBank JQ287644).

We initially assessed phylogenetic affinity by submitting similarity searches to GenBank with BLASTn. Because nucLSU, ITS and tef1 sequences showed high sequence similarity to members of the order Boletales, suborder Boletineae, phylogenetic analyses were conducted as described below by adding the *Solioccasus polychromus* nucLSU sequences into the Boletales datasets of Binder and Hibbett (2006) and Desjardin et al. (2009). Because taxon coverage within the Boletales is poor for tef1 and ITS exhibits too much variability to allow alignment across the order, the analysis was conducted only by use of nucLSU sequence data. Sequences were aligned with MAFFT 6 (Katoh et al. 2002, 2005; Katoh and Toh 2007); the alignment was edited with Geneious Pro 4.9.3 (www.geneious.com). The sequence alignment was submitted to TreeBASE under accession number S12026. Nucleotide positions that could not be unambiguously aligned were removed before analysis.

Phylogenetic analyses were conducted under a maximum likelihood (ML) optimality criterion with RAxML 7.2.8 (Stamatakis, 2006) implemented on the CIPRES Portal V. 2.2 (http://www.phylo.org/sub_sections/portal/) at the San Diego Supercomputer Center. Two preliminary analyses were conducted, adding the *Solioccasus* nucLSU sequences into the Boletales datasets of Binder and Hibbett (2006; Analysis 1) and Desjardin et al. (2009; Analysis 2). Outgroup taxa for Analysis 1 included four members of the Paxillineae (*Gyrodon monticola*, *G. lividus*, *Paxillus involutus*, *P. vernalis*, *P. filamentosus*). The outgroup taxon for Analysis 2 was *Hydnomerulius pinastri*. The analyses included 50 inferences on the original alignment with distinct randomized starting trees and 1000 bootstrap replicates using a GTR+GAMMA model for both bootstrapping and final ML optimization and allowing RAxML to optimize the number of rate categories and initial rearrangement setting. Most branches in both analyses received poor (< 50%) bootstrap support. The resulting trees (SUPPLEMENTARY FIGS. 1, 2), in addition to the BLASTn query results, were used to guide assembly of a final dataset in which taxa were limited in the interest of generating a tree in which the sister group affinity and broad-scale phylogenetic membership of *Solioccasus* could be shown more clearly. Because no clades having well supported, conflicting arrangements were observed in the two phylograms, the dataset from Analysis 1 therefore was trimmed to produce the reduced dataset by retaining representatives from major Boletineae lineages (SUPPLEMENTARY FIG. 1). To better illustrate the subordinal phylogenetic membership of *Solioccasus*, exemplars of seven additional suborders or major lineages of Boletales (Sclerodermatineae, Suillineae, Hygrophoropsidaceae, Serpulaceae, Tapinellineae, Coniophorineae, Athelioid clade) were added. A RAxML analysis was conducted as previously described, with two members of the Athelioid clade (sensu Binder and Hibbett 2006), *Athelia decipiens* and *Athelopsis subinconspicua* (GenBank accession numbers, FIG. 1) selected as outgroups based on the results of Binder and Hibbett (2006).

RESULTS

Sequence similarity searches in GenBank for LSU, ITS and tef1 sequences support placement of *Solioccasus* within Boletales, suborder Boletineae, family Boletaceae. The nucLSU sequences exhibited highest similarity to *Bothia castanella* (100%

query coverage, 95% maximum identity) for JT 15399 and to *Tylopilus felleus* (100% query coverage, 95% maximum identity) for REH 9417. The latter match is likely a function of the longer length of both the query and top hit sequences, which are longer than many Boletales nucLSU sequences in GenBank; a close relationship between *Solioccasus polychromus* and *Tylopilus felleus* is not supported by phylogenetic analyses, and trimming the REH 9417 sequence to the length of the JT 15399 sequence reveals 100% similarity between the two sequences and yields a best BLAST match to *B. castanella*. The search with the JT 15399 ITS sequence also returned *Bothia* and *Tylopilus* spp. as best hits. A BLAST query by the *tef1* sequence for REH 9417 resulted in a best match to *Boletellus projectellus* (100% query coverage, 86% maximum identity); the weaker similarity exhibited by the tef1 sequence (compared to nucLSU) is likely a function of the lack of *tef1* sequences from Boletineae in GenBank.

The sequence alignment for the reduced dataset (with ambiguously aligned nucleotide positions removed) consisted of 53 taxa and 801 alignment positions (TreeBASE S12026). The RAxML analysis yielded a best tree with a final GAMMA-based likelihood score of −6046.87 (FIG. 1). *Solioccasus* is placed within the Boletineae, strongly supported (bootstrap = 97%) as monophyletic with *Bothia castanella*. The *Solioccasus*/*Bothia* clade appears as paraphyletic with *Tylopilus* in this analysis; however, due to poor bootstrap support for the backbone nodes of the tree, this phylogenetic position as well as the sister groups of this clade cannot be accurately identified from these results.

TAXONOMY

Solioccasus Trappe, Osmundson, Manfr. Binder, Castellano & Halling, gen. nov.

MycoBank MB800824

Basidiomata hypogeous to partly emergent, up to 45 mm broad, subglobose to deeply lobed and furrowed, with robust yellow to orange rhizomorphs emergent from the base and adpressed against the sides. Peridium entire to split open or mostly absent to reveal the gleba locules, ivory in youth but soon developing yellow, pink-orange or reddish orange colors, with or without a suprapellis of a tangled trichodermium. Gleba similarly colored as the peridium, loculate, with a prominent to inconspicuous, dendroid, cartilaginous columella. Spores smooth by light microscopy, minutely roughened by SEM, ellipsoid or rarely subangular to allantoid.

Type species: *Solioccasus polychromus* Trappe, Osmundson, Binder, Castellano, & Halling.

Etymology: Latin *Soli-* (sun) and *–occasus* (setting of heavenly bodies), hence "sunset", in reference to the brilliant yellows, pinks, oranges and reddish oranges of the basidiomata, mimicking the rich colors of Australian sunsets.

Solioccasus polychromus Trappe, Osmundson, Manfr. Binder, Castellano & Halling, sp. nov. FIGS. 2, 3

MycoBank MB800825

Macrocharacters: Basidiomata gregarious, 8–32 × 10–45 mm, subglobose to turbinate or irregularly lobed and furrowed, on larger specimens the base deeply indented; base with appressed to emergent, yellow to orange rhizomorphs up to 1.7 mm broad. Peridium continuous and pallid-pubescent in youth, dry, with age the pubescence remaining only in depressions and the peridium split in places or reduced to remnant patches to expose the gleba, where present ≤ 0.5 mm thick and sordid yellowish white to olive but orange in cross section, soon becoming moderate

yellow (ISCC-NBS 87) to strong pink (ISCC-NBS 2), then deep pink (ISCC-NBS 3) mottled and streaked with light orange-yellow (ISCC-NBS 70) to moderate orange-yellow (ISCC-NBS 71) and at full maturity variously vivid orange (ISCC-NBS 48), vivid orange-yellow (ISCC-NBS 66) and vivid reddish orange (ISCC-NBS 70). Rhizomorphs up to 0.8 mm thick, scattered to abundant, concolorous with the peridium, emergent from the base and appressed on bottom and sides of basidiomata. Gleba rubbery-cartilaginous, with globose to elongate locules 0.1–1.5 mm broad, ivory to pale yellow in youth, soon developing the same range of colors as the peridium, with an inconspicuous to robust gray-translucent to yellow or red, dendroid, cartilaginous columella up to 1 mm broad at the base, some branches percurrent and becoming pink to orange where emerging at the basidiome surface. Spores lining the locules moderate greenish yellow (ISCC-NBS 102) in mass, becoming cinnamon to brown in patches in fully mature specimens. Odor pleasant to pungent or fungoid. Flavor not distinctive.

Microcharacters: Spores ellipsoid to broadly ellipsoid or occasionally subangular to subfusoid or allantoid in some specimens, with a sterigma attachment 1 × 1–2 µm; (8–)10–14(–17) × (5–)6–8(–9) µm (Q = 1.4–2.6), the lower Qs predominant in some specimens, the higher Qs in others, the walls up to 0.8(–1) µm thick at maturity, appearing smooth by light microscopy or faintly roughened by Nomarski imaging but distinctly minutely roughened by SEM; light yellow in KOH and Melzer's reagent, deep blue in cotton blue. Basidia clavate, 32–40 × 7–10 µm, with 2(–4) sterigmata ± 4–6 × 1 µm. Peridium 200–300 µm thick, with a suprapellis, pellis and subpellis; suprapellis a tangled trichodermium of hyaline, obtuse hyphal

tips 3–5 μm broad; pellis tightly constructed of periclinal, hyaline, highly gelatinized hyphae 2–5 μm broad; subpellis of similar but tightly interwoven hyphae. Gleba trama 100–250 μm thick, of sinuous-subparallel, hyaline, highly gelatinized hyphae 2–4 μm broad. Subhymenium of interwoven hyphae otherwise similar to those of the trama. Clamp connections lacking.

Etymology: Greek, *poly-* (many) and *-chromus* (colored), "many colored".

Distribution, *habitat and season:* Australia (Northern Territory and Queensland) and Papua New Guinea, in lowland tropical and subtropical forests with various mixtures of ectomycorrhizal *Allocasuarina littoralis*, *Corymbia dichromophloia*, *C. erythrophloia*, *C. polycarpa*, *Eucalyptus pellita*, *E. pilularis*, *E. racemosa*, *E. tetrodonta*, *Leptospermum* spp., *Lophostemon sp*. and *Melaleuca spp*., often in sandy soil. February, March.

Holotype: PAPUA NEW GUINEA. Kirrawa-Meru Track near Morehead River Crossing under *Melaleuca* and *Lophostemon* spp., *J. Trappe*, *G. Tubeg*, *N. Malajczuk*, *R. Young H5569*, 19 Feb 1992 (BRI; isotypes LAE, MEL, NY, OSC 130764, PERTH).

Paratypes: AUSTRALIA: NORTHERN TERRITORY: Nhulunbuy, *V. Gordon*, *Trappe 15398*, 16 Mar 1995 (DNA); Nhulunbuy, Nambra Arts and Crafts Center, *J. Trappe*, *M. Castellano & V. Gordon*, *Trappe 15399*, 16 Mar 1995 (BRI, OSC 130891); Nhulunbuy, Yirrkala site, *V. Gordon*, *Trappe 15384*, 16 Mar 1995 (DNA, OSC 130889); Nhulunbuy, 0.3 km up Rainbow Cliff Road, *J. Trappe*, *M. Castellano*, *V. Gordon*, *Trappe 15391*, 16 Mar 1995 (BRI, DNA, OSC 130774); 30 km S of Nhulunbuy, *J. Trappe 15377*, 15 Mar 1995 (DNA, OSC 130896). QUEENSLAND: Cape York, 6 km W of Scherges RAAF Base, *J. Trappe 15348*, 11 Mar 1995 (BRI, PERTH); Cape York, 15 km from Weipa on Weipa-Archer River Road, *J. Trappe & P. Reddell*, *Trappe 15320*, 10 Mar 1995 (MEL, OSC 130769) and *P. Reddell*, *Trappe 15359*, 12 Mar 1995 (BRI, OSC 130768); Cooloola, Wide Bay District, Freshwater Road, *N. Fechner & R. Halling*, *AQ795158*, 21 Feb 2011 (BRI); Moreton District, pedestrian track parallel to Old Logan Road, Camira, *N. Fechner & K.*

Querengasser, *AQ795159*, 15 Apr 2009 (BRI); Fraser Island, Great Sandy National Park, Northern Road 10.5 km from Central Station toward Kingfisher Resort, *M. Castellano*, *Trappe 34636*, 24 May 2010 (MEL, OSC 130892); Fraser Island, Pile Valley-Kingfisher Bay Rd, 25°24′30.5″S, 153°3′25.5″E, 62 m, *R. Halling*, *REH9290*, 28 Mar 2010 (BRI, NY) and 26°27′26.3″S, 153°4′32.7″E, 34 m, *N. Fechner & R. Halling*, *REH9417*, 16 Feb 2011 (BRI, NY); Fraser Island, Wide Bay District, Pile Valley-Kingfisher Bay Road, *N. Fechner & R.E. Halling*, *AQ795156*, 16 Feb 2011 (BRI); Fraser Island, Wide Bay District, Pile Valley-Lake McKenzie Road, *N. Fechner & R.E. Halling*, *AQ795157*, 16 Feb 2011 (BRI); Paluma Range National Park, Hidden Valley Rd. 6.2 km W of Talvare Road, *M. Castellano*, *Trappe 13415*, 3 Mar 1994 (BRI, OSC 130893). PAPUA NEW GUINEA: WESTERN PROVINCE: Near Kiriwo on track to Meru, *N. Bougher & R. Young H5554*, 16 Feb 1992 (OSC 130766, PERTH, PNG); Kiriwo, 3 km on track to Meru, *J. Trappe*, *G. Tubeg*, *N. Malajczuk*, *K. Bisam*, *R. Young H5551*, 16 Feb 1992 (BRI, LAE, OSC 130767, PERTH) *& H5552*, 16 Feb 1992 (CANB, MEL, NY, OSC 130765, PERTH, PNG); near Meru on track from Kiriwo, *R. Young H5553*, 16 Feb 1992 (LAE, MEL, OSC 130886, PERTH) and *Katawe Baku H5557*, 16 Feb. 1992 (LAE, OSC 130887, PERTH).

DISCUSSION

Sequestrate fungi have evolved in many forms and lineages of the Boletales (Grubisha et al. 2001; Binder and Bresinsky 2002; Binder and Hibbett 2006; Desjardin et al. 2008, 2009; Lebel et al. 2012). Both DNA sequence similarity and phylogenetic analyses strongly support the placement of *Solioccasus* within order Boletales, suborder Boletineae, Boletaceae. *Solioccasus* is strongly supported as monophyletic with *Bothia castanella*, the sole described species in *Bothia* and a species with a combination of features that creates uncertainty in determining its taxonomic placement on morphological grounds (Halling et al. 2007). Low bootstrap support for backbone branches in both Halling et al. (2007) and the present study preclude identifying the closest relatives of the *Solioccasus*/*Bothia* clade; at

present it is possible to conclude with reasonable confidence only that placement of *Solioccasus* within Boletineae and a close relationship to *Bothia* are likely. It is clear that systematic studies of boletes will benefit greatly from availability of sequences for additional loci and additional taxa.

We often spotted colonies of *Solioccasus* because one or more specimens were partly emergent, so their bright colors might attract insect or bird mycophagists. However, most basidiomata were covered by litter, sand or soil; mammals likely detect them by odor. Papua New Guinea has diverse populations of native rodents and other potential mycophagists (Rowe et al. 2008), and Australian mycophagists include numerous marsupials as well as native rodents (Claridge and May 1994). An estimated nine or more rodent dispersal events have occurred between New Guinea and Australia over the past 5 000 000 or more years, and sea-level fluctuations during the Pleistocene have created a series of land bridges between the two land masses (Rowe et al. 2008). Dispersal of *Solioccasus* across the presently shallow Torres Straight can be reasonably attributed mostly to mammals and possibly birds.

The fresh appearance of *Solioccasus polychromus*, with its many rhizomorphs, split peridium with exposed locules, and cartilaginous gleba with a prominent dendroid trama/columella, led us to suppose it was a brightly colored *Gautieria* sp. in the field, and our notes designated it as the "many colored Gautieria." When we later had access to a microscope and saw what we interpreted as boletoid spores, we supposed it to be a new genus and species in the Boletaceae. The molecular data support that hypothesis.

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LEGENDS

FIG. 1. Single-locus phylogram of representatives of Boletales based on maximum likelihood analysis of nuclear ribosomal large subunit DNA sequences by RAxML-HPC 7.2.8. Final GAMMA-based score of best tree = −6046.87. Bootstrap percentage values (based on 1000 bootstrap replicates) greater than 50 appear above branches. *Solioccasus* is strongly supported (bootstrap = 97%) as monophyletic with *Bothia castanella*. *Athelia decipiens* and *Athelopsis subinconspicua* were used as outgroup taxa.

FIG. 2. *Solioccasus polychromus* basidiomata. a. Cross section showing gleba and prominent columella (co). b. Surface with appressed concolorous rhizomorphs (ar) and exposed glebal locules (gl). Bars = 10 mm.

FIG. 3. *Solioccasus polychromus* photomicrographs. a. Sinuous-interwoven trama hyphae; bar = 20 µm. b. Peridium suprapellis (su) and pellis (pe); bar = 20 µm. c. Single basidium (ba) with four spores attached to sterigmata (st); bar = 5 µm. d. Basidiospores in cross section; bar = 10 µm. e. Scanning electron micrograph of basidiospores showing the faintly roughened surface; bar = 5 µm. f. Scanning electron micrograph of basidiospores showing point of sterigmal attachment; bar = 5 µm.

FOOTNOTES

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[1]Corresponding author. E-mail: trappej@onid.orst.edu


Boletus reticulatus AF456824
Boletus edulis AF462356
Boletus satanas AF071528
Boletus appendiculatus AF456837
Gastroboletus turbinatus AF336248
Chalciporus piperatus AF336244
Chalciporus amarellus AF456835
Xerocomus rubellus AF050649
Xerocomus chrysenteron AF514808
Boletus inedulis AY612803
Retiboletus retipes AF456823
Retiboletus ornatipes AF456805
Retiboletus griseus AF456834
Boletus luridus AF139686
Boletineae
Tylopilus balloui EU430734
Tylopilus oradivensis EU430731
Tylopilus felleus AF139710
Tylopilus alboater AF139708
Tylopilus plumbeoviolaceus AF457405
Solioccasus polychromus Trappe 15399
Solioccasus polychromus Halling 9417
Bothia castanella DQ867119
Boletus gertrudiae AF457407
Chamonixia caespitosa AF336245
Leccinum rotundifoliae AF139704
Leccinum duriusculum AF139695
Leccinum aurantiacum AY612810
Leccinum scabrum AY612814
Xerocomus subtomentosus AF139716
Phylloporus pelletieri AF456818
Phylloporus rhodoxanthus U11925
Heimiella retispora AF050650
Boletellus ananas AY612799
Paxillus involutus AF167682
Gyrodon monticola AF352040
Paxillineae
Phlebopus portentosus AF336260
Boletinellus merulioides AF336239
Sclerodermatineae
Scleroderma verrucosum AF336271
Gyroporus cyanescens AF336254
Calostoma cinnabarinum AF336243
Pisolithus arhizus AF336262
Rhizopogon luteolus AF098400
Suillus luteus AF042622
Chroogomphus rutilus AF098399
Gomphidius oregonensis AF071530
Suillineae
Hygrophoropsis aurantiaca AF042007
Leucogyrophana mollusca AF098381
Hygrophoropsidaceae
Serpula lacrymans AF098402
Austropaxillus statuum AF098390
Serpulaceae
Tapinella panuoides AF287879
Tapinellineae
Coniophora marmorata AF352038
Coniophorineae
Athelopsis subinconspicua AY586634
Athelia decipiens AY586632
Athelioid clade
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84
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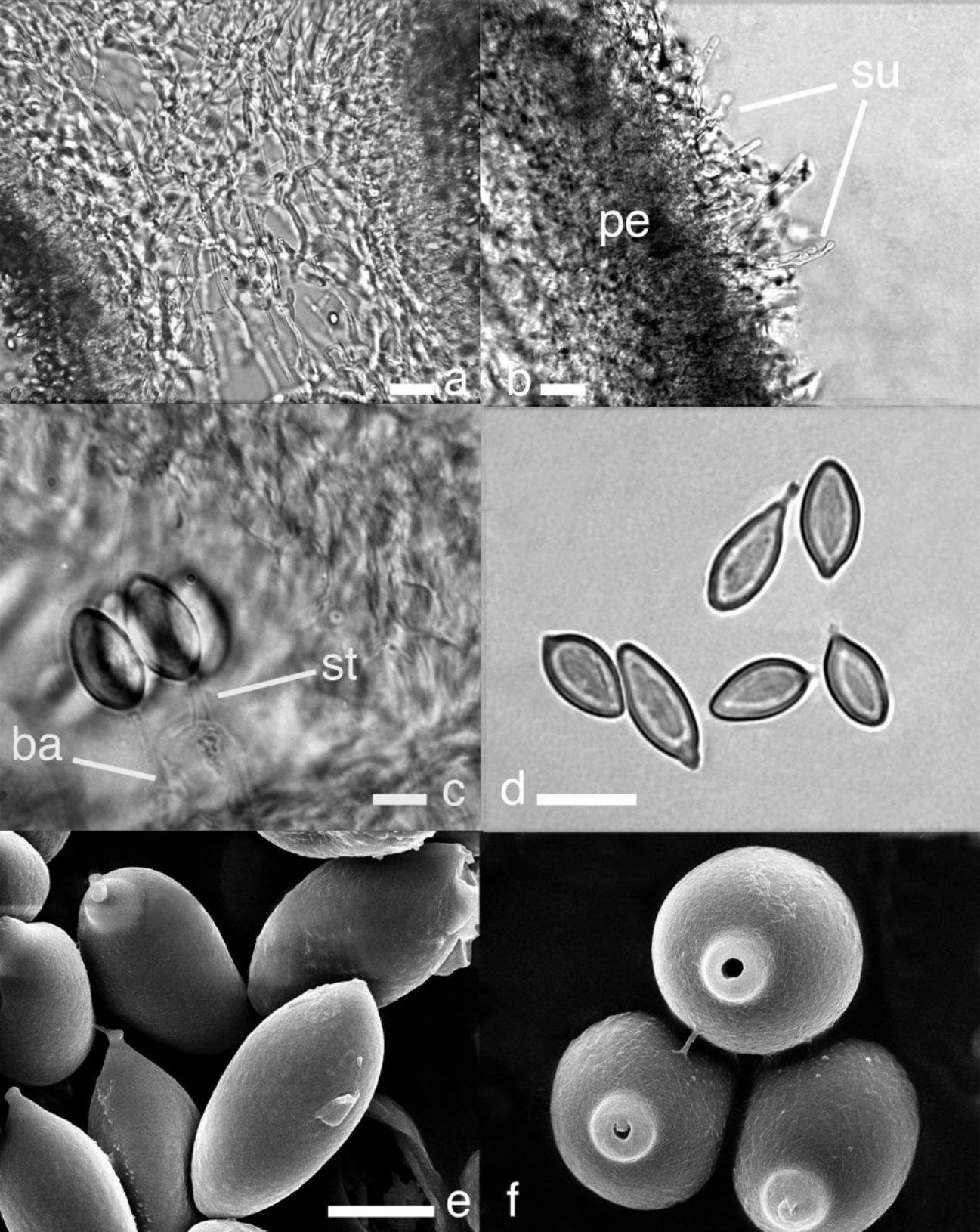
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