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SHORT NOTE

The enigmatic truffle *Fevansia aurantiaca* is an ectomycorrhizal member of the *Albatrellus* lineage

Matthew E. Smith · Karlee J. Schell · Michael A. Castellano · Matthew J. Trappe · James M. Trappe

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Abstract *Fevansia aurantiaca* is an orange-colored truffle that has been collected infrequently in the Pacific Northwest of the USA. This sequestrate, hypogeous fungus was originally thought to be related to the genera *Rhizopogon* or *Alpova* in the Boletales, but the large, inflated cells in the trama and the very pale spore mass easily segregated it from these genera. To date, no molecular phylogenetic studies have determined its closest relatives. *F. aurantiaca* was originally discovered in leaf litter beneath Pinaceae, leading Trappe and Castellano (Mycotaxon 75:153–179, 2000) to suggest that it is an ectomycorrhizal symbiont of various members of the Pinaceae. However, without direct ecological or phylogenetic data, it is impossible to confirm the trophic mode of this truffle species. In this study, we combined phylogenetic analysis of the ITS and 28S ribosomal DNA with data on microscopic morphology to determine that *F. aurantiaca* is a member of the *Albatrellus* ectomycorrhizal lineage (Albatrellaceae, Russulales).

Keywords *Albatrellus* · *Albatrellaceae* · Ectomycorrhiza · Russulales · Sequestrate fungi · Truffles

M. E. Smith (✉) · K. J. Schell
Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA
e-mail: trufflesmith@ufl.edu

M. A. Castellano
US Department of Agriculture, Forest Service, Northern Research Station, 3200 SW Jefferson Way, Corvallis, OR 97331, USA

M. J. Trappe · J. M. Trappe
Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97331, USA

Introduction

Sequestrate fungi, including hypogeous truffles and false truffles, have evolved independently within numerous fungal lineages (Trappe et al. 2009). Because they fruit belowground and do not release their spores into the air, truffles usually have reduced morphological features, including a peridium (outer rind) and a gleba (inner tissue with spores) that may be either solid or divided into chambers (Montecchi and Sarasini 2001). However, truffle tissues are often compressed and distorted, thereby making it difficult to determine their closest epigeous relatives based on morphology alone (Ge and Smith 2013; Smith and Healy 2009). The hypogeous nature of truffles can make it challenging to obtain sufficient numbers of collections for detailed taxonomic studies or to determine the trophic mode of individual species. Truffles are most diverse and abundant in forest ecosystems with ectomycorrhizal (ECM) trees, so they are often assumed to form ectomycorrhizae. However, some truffles that fruit in forests with ectomycorrhizal plants are saprobes, so the use of habitat information is not definitive evidence of trophic mode (Tedersoo et al. 2010). Since the ability to form the ECM symbiosis is conserved within fungal lineages, phylogenetic analysis is useful to suggesting the likely trophic mode for a given fungal species (Tedersoo et al. 2010; Albee-Scott 2007).

The rarely encountered truffle *Fevansia aurantiaca* is native to forests of the Pacific Northwest and is morphologically unique, with no obvious morphological affinities with any other described genus or species. Accordingly, Trappe and Castellano (2000) described this truffle in a new genus and named the species for the pale orange hue of its peridium (Trappe and Castellano 2000). They suggested that this truffle had similarities to the genera *Alpova* and *Rhizopogon*

in the order Boletales because of its spores. However, no further studies have focused on the taxonomic placement of this enigmatic truffle. A recent review of ECM fungal lineages by Tedersoo et al. (2010) identified *Fevansia* as one of a few potentially ECM genera for which no molecular data were available and for which the ECM status had not been definitively proven. In order to determine the phylogenetic placement and likely trophic mode of *F. aurantiaca*, we sequenced the internal transcribed spacer (ITS) and the 28S large subunit (LSU) ribosomal DNA regions and subjected them to phylogenetic analysis. Our results indicate that *F. aurantiaca* is member of the *Albatrellus* lineage (Albatrellaceae, Russulales) and probably forms ECM associations with conifers.

Methods

Morphological analysis We examined the morphology of four different collections of *F. aurantiaca* using light microscopy. Dried herbarium specimens are deposited at Oregon State University (OSC) with duplicates deposited at the University of Florida (FLAS). Specimens were hand-sectioned and then mounted in water and Melzer's reagent. We examined, measured, and photographed spores from each specimen and checked for an amyloid response in Melzer's reagent.

DNA extraction and PCR amplification Truffle tissues were ground with liquid nitrogen in 1.5-ml Eppendorf tubes and then DNA was extracted with the DNeasy Plant Mini Kit (QIAGEN, Valencia, California) following the protocols of the manufacturer. The ITS region was PCR-amplified with primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) and the LSU region was PCR-amplified with primers LR0R and LR5 (Vilgalys and Hester 1990). Successful amplicons were electrophoresed on 1.5 % agarose gels and stained with SYBR Green I (Molecular Probes, Eugene, Oregon). Amplicons were cleaned with Exonuclease I and shrimp alkaline phosphatase enzymes (Werle et al. 1994) and sequenced by the University of Florida ICBR (http://www.biotech.ufl.edu/).

Phylogenetic analysis Sequences were compared to GenBank using the BLASTn algorithm (www.ncbi.nlm.nih.gov/) and the 100 closest matches for each gene were retrieved. We generated nucleotide alignments for both the ITS and LSU rDNA regions based on representative GenBank sequences in the software package MESQUITE (Maddison and Maddison 2010). Automated alignment was conducted with the MUSCLE software package (Edgar 2004) followed by manual adjustments.

The final LSU dataset contained 1,080 base pairs and 55 taxa, including 52 species of Russulales as well as two species of Polyporales and one species of Atheliales that served as the out-group taxa. We excluded 69 ambiguously aligned nucleotides from further phylogenetic analysis. We conducted maximum likelihood (ML) analysis with the GTR + I + G algorithmic model in the software package GARLI (Zwickl 2006) and maximum parsimony (MP) analysis with default settings in PAUP* (Swofford 2002). For the ML analysis clade stability was assessed based on 500 bootstrap replicates with default settings in GARLI and for the MP analysis clade stability was assessed based on 1,000 bootstrap replicates with default settings in PAUP*.

The final ITS dataset included 789 base pairs from 41 sequences, including sequences from fruiting bodies of the *Albatrellus* lineage and environmental DNA sequences from ECM root tips and soil clones. Our analysis excluded 305 ambiguously aligned base pairs spread across the ITS1 and ITS2 regions. These ambiguously aligned regions were mostly due to introns present in some *Albatrellus* clade taxa but not in others. ITS analysis was similar to the LSU analysis presented above except that MP bootstrapping was conducted with the rapid bootstrapping method for 500 replicates.

Results

BLASTn analysis indicates that *F. aurantiaca* is related to taxa in the *Albatrellus* lineage (Albatrellaceae, Russulales), including epigeous (*Albatrellus*, *Polyporoletus*), resupinate (*Byssoporia*), and hypogeous (*Leucogaster*, *Leucophleps*, *Mycolevis*) fungal taxa. These include species with highly variable morphological features, including major differences in spore shape and size, spore ornamentation, and sporocarp shape, color, and size (Albee-Scott 2007). Maximum likelihood analysis of LSU generated a tree with likelihood value of –ln 6,320.22660 and MP analysis generated 144 equally parsimonious trees of 864 steps (*CI*=0.388, *RI*=0.672). Both the ML and MP analyses strongly supported a monophyletic *Albatrellus* lineage (Albatrellaceae) within the Russulales (Fig. 1). This clade contained *F. aurantiaca* and species of *Albatrellus*, *Polyporoletus*, *Leucogaster*, and *Byssoporia* that are all considered ECM symbionts of plants (Tedersoo et al. 2010). The two truffle species, *F. aurantiaca* and *Leucogaster citrinus*, are not resolved in the same clade but bootstrap support for relationships within the *Albatrellus* lineage were generally weak. *F. aurantiaca* grouped in a clade with two sequences of *Albatrellus skamanius*, but there was no statistical support for this relationship. Relationships among other taxa within Russulales were neither well resolved nor supported by bootstrapping and several family-level groupings suggest that taxonomic revisions may be necessary in Stereaceae, Hericiaceae, and Echinodontiaceae.

Our ITS analysis yielded 141 parsimony informative characters and generated 22 equally parsimonious trees of 494

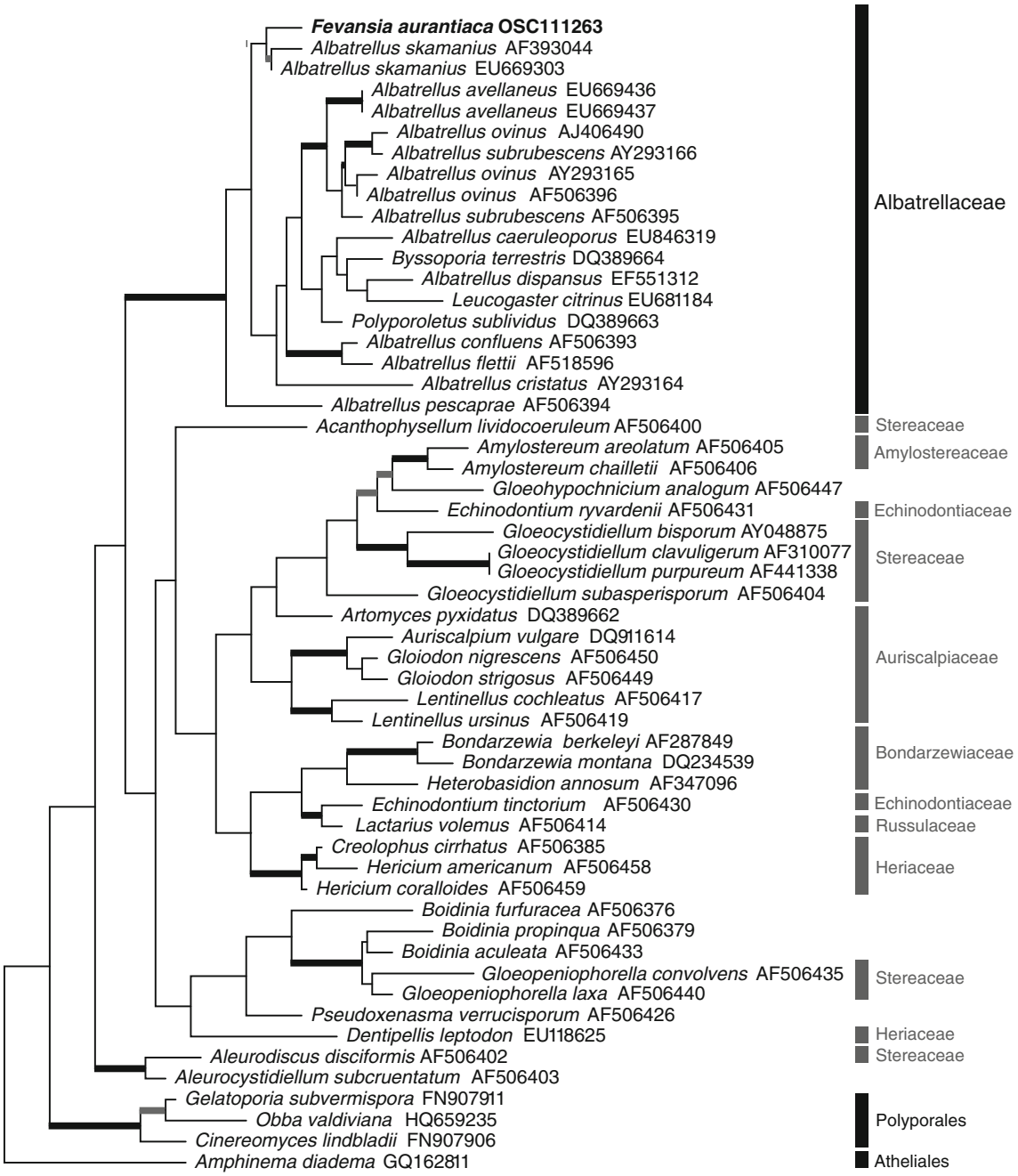

Fig. 1 Maximum Likelihood (ML) phylogeny based on large subunit (LSU) ribosomal DNA shows *F. aurantiaca* nested within the strongly supported *Albatrellus* ectomycorrhizal lineage in the Russulales. Thickened black branches were supported ≥70 % bootstrap values for both ML and maximum parsimony (MP) analyses (Hillis and Bull 1993) whereas thickened gray branches were supported by only one of the two methods. Family names for assorted taxa in Russulales are based on the taxonomy listed by Index Fungorum (www.indexfungorum.org/); taxa without family names are considered incertae sedis

steps (29 parsimony informative characters, *CI*=0.5735, *RI*=0.742). The midpoint-rooted, majority rule consensus tree is shown in Fig. 2. *F. aurantiaca* is located on a branch between a clade comprised of two sequences of *A. skamanius* and a clade of *Leucogaster* species (and associated ECM root tip sequences), but this relationship did not receive bootstrap support. Similar relationships were suggested by the best ML tree (data not shown) but also received weak support (Fig. 2). The ITS consensus tree highlights the ECM status of members of the *Albatrellus* lineage with both angiosperms and gymnosperms in the northern hemisphere (e.g., *Arbutus*, *Carpinus*, *Fagus*, *Notholithocarpus*, *Pinus*, *Pseudotsuga*, *Tsuga*). Ectomycorrhizal root tip sequences are resolved close to *Mycolevis siccigleba*, *Leucophleps spinispora*, *Byssoporia*

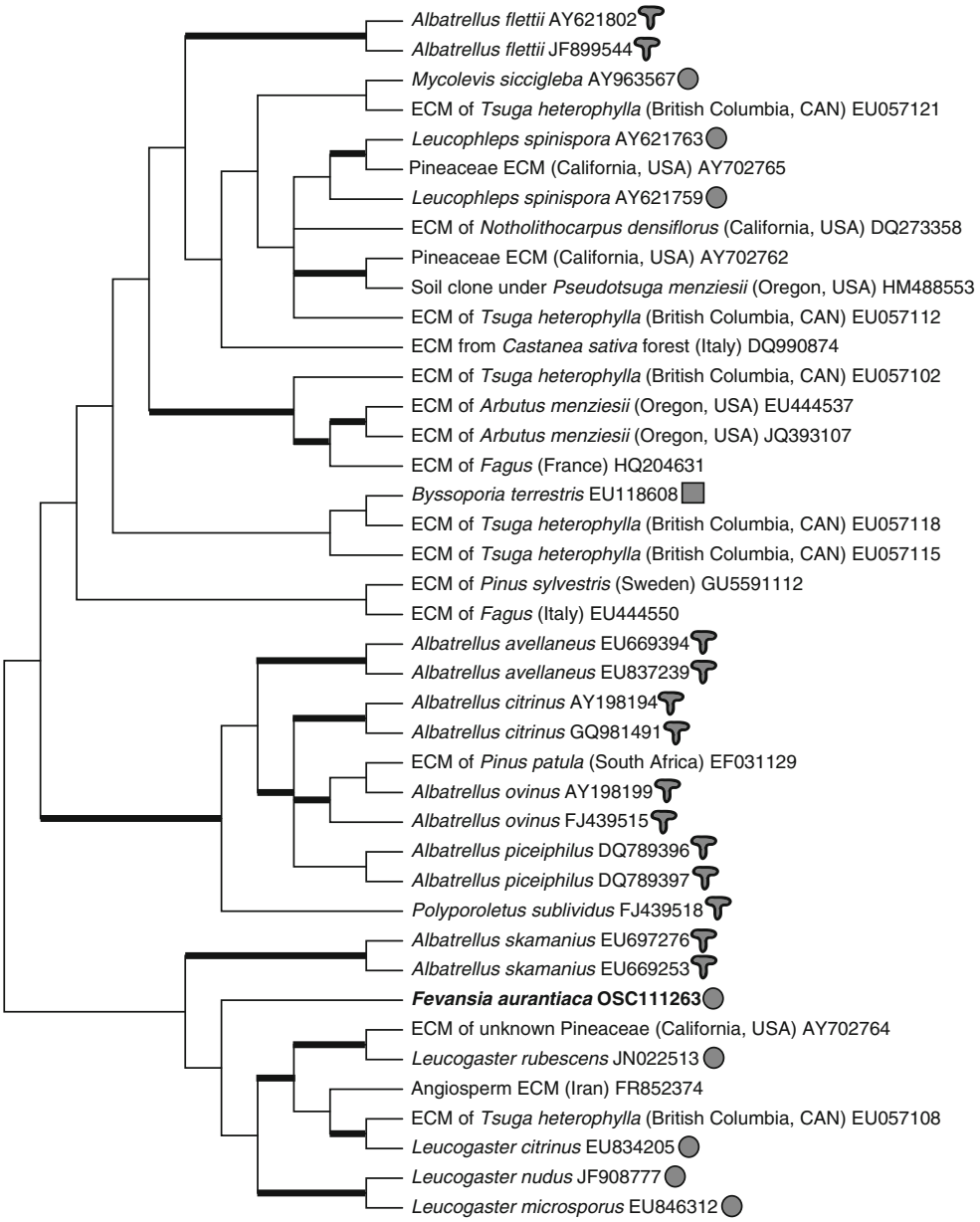

Fig. 2 Midpoint rooted, 50 % majority rule consensus tree generated from parsimony analysis of the ITS ribosomal DNA showing the placement of *Fevansia aurantiaca* among assorted taxa in the *Albatrellus* ectomycorrhizal lineage. Thickened black branches were supported by ≥70 % bootstrap values (Hillis and Bull 1993) for both ML and MP analyses. Stipitate taxa are marked by filled gray mushroom shapes, the resupinate taxon *Byssoporia terrestris* is denoted by a filled gray square, and taxa that form truffles are marked by filled gray circles

terrestris, *Albatrellus ovinus*, *Leucogaster rubescens*, and *L. citrinus*. The ITS analysis also identifies a divergent and well-supported group of sequences (EU057102, EU444537, JQ393107, HQ204631) for which no sporocarps are identified. Root tip sequence EF031129 indicates that *A. ovinus* has been introduced into South Africa with *Pinus patula*.

Although we were only able to generate ITS and LSU sequences from the most recent specimen of *F. aurantiaca* (OSC111263), the four specimens from four different sites in Oregon were morphologically similar to each other and to the description by Trappe and Castellano (2000). The specimens had a dark brownish-orange gleba and spores that were somewhat dextrinoid (but not amyloid) in Melzer's reagent. In water, spores of *F. aurantiaca* were pale grayish-blue in color and slightly refractive (Fig. 3). They were fusoid and ranged 10–13×4–5 μm. When tissues were examined in Melzer's reagent, they exuded droplets of an amorphous, oily substance.

The collection information for examined specimens of *F. aurantiaca* is as follows: Holotype: USA, Oregon, Deschutes County, Deschutes National Forest, Devil's Lake, 3 Aug 1991, *F. Evans*, Trappe 12463, (OSC62081), elev 5,200 ft beneath *Abies lasiocarpa*. Paratype: USA, Oregon, Linn County, Willamette National Forest, Hackleman Creek, 7 Aug. 1969, *J.M. Trappe*, Trappe 1966 (OSC62082), beneath *Pseudotsuga menziesii*. Additional specimens: USA, Oregon, Klamath County, Crater Lake National Park, Picnic Hill, control plot five, 5 Oct. 2004, *M. Trappe*, *M. Hinds*, *J.M. Trappe*, Trappe 30022 (OSC11263), elev 7,150 ft beneath *Abies magnifica* var. *shastensis* and *Tsuga mertensiana* (GenBank accession:

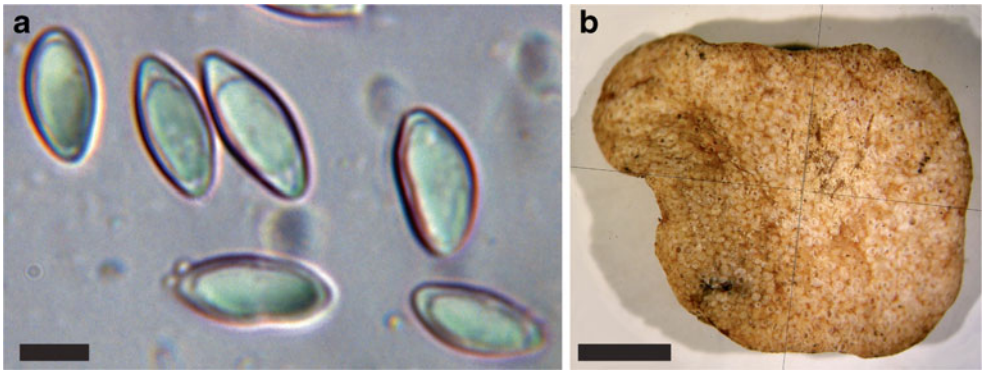

Fig. 3 Morphology of *Fevansia aurantiaca*. **a** Smooth, fusoid spores of *F. aurantiaca* specimen OSC82015 (scale=5 μm). **b** Cross-section of the loculate gleba from fresh *F. aurantiaca* specimen OSC11263 (scale= 5 mm)

KC894736). Clackamas County, Mount Hood National Forest, Still Creek campground, Forest Service Road 2650, 20 Aug. 2001, *A. Beyerle*, Beyerle 1573 (OSC82015) elev 3,800 ft beneath *Abies procera* and *Tsuga* species.

Discussion

This is the first study to examine the evolutionary relationships of the rarely collected truffle *F. aurantiaca*. Although it was originally thought to be related to genera in the Boletales, our results based on DNA sequences of ribosomal ITS and LSU indicate that *F. aurantiaca* is actually a member of the *Albatrellus* lineage. We do not have direct DNA sequence matches from ECM root tips to definitively confirm that *F. aurantiaca* is an ECM symbiont. The most parsimonious conclusion, however, is that *F. aurantiaca* is an ECM fungus because: (1) *F. aurantiaca* is always collected beneath ECM-forming Pinaceae, (2) *F. aurantiaca* is nested within the *Albatrellus* lineage, and (3) all known taxa in the *Albatrellus* lineage form ectomycorrhizas with plants (Tedersoo et al. 2010).

Both ITS and LSU clearly resolved *F. aurantiaca* in the *Albatrellus* lineage but unfortunately provided relatively poor resolution to discern its closest relatives within the group. Despite the poor resolution of our phylogenies, both genes suggest the likelihood that truffles have evolved multiple times within the *Albatrellus* lineage. If we assume that evolution from epigeous to hypogeous truffle form is not a reversible process, we infer that evolution to truffle morphology has probably evolved at least twice and as many as four times in the *Albatrellus* lineage. A similar result was suggested by Albee-Scott (2007) when he resolved *Mycolevis*, *Leucophleps*, and *Leucogaster* into distinct clades based on an ITS rDNA phylogeny of Russulales. However, additional studies with more robust, multi-gene analyses are clearly needed to refine the phylogenetic relationships within the *Albatrellus* lineage.

Although *F. aurantiaca* was associated with *A. skamanius* (Murr.) Pouz. in both the ITS and LSU analyses, this relationship received only weak bootstrap support. Some notable similarities between *F. aurantiaca* and *A. skamanius* are worth mentioning; both are rarely collected, have orangish, reddish, or brownish colored sporocarps that become tough when dried, and are associated with Pinaceae in the Pacific Northwest (Gilbertson and Ryvarden 1986; Trappe and Castellano 2000). Unfortunately, morphological features are not necessarily helpful for additional comparisons within the *Albatrellus* lineage because taxa in this group have an exceedingly wide range of morphological features, including highly variable sporocarp type (stipitate, resupinate, and sequestrate), sporocarp colors (white, yellow, orange, brown, and green, among others) and spore ornamentation (e.g., alveolate in *Leucogaster*, spiny in *Leucophleps* and *Mycolevis*, smooth in *Albatrellus* and *Byssoporia*) (Larsen and Zak 1978; Gilbertson and Ryvarden 1986; Albee-Scott 2007). However, like many species within the *Albatrellus* lineage, *F. aurantiaca* has noticeably large, inflated cells in its tissues and extrudes globules of a pigmented, oily substance when exposed to alkaline solutions (Trappe and Castellano 2000; Albee-Scott 2007; Gilbertson and Ryvarden 1986).

F. aurantiaca is considered rare and was first described as part of an effort to document the fungi of late successional forests in the range of the endangered northern spotted owl (Trappe and Castellano 2000). Several stipitate species within the *Albatrellus* lineage, including *Albatrellus dispanus*, *Albatrellus ellisii*, *Albatrellus flettii*, and several others, have also been considered rare because they are only documented from a few locations across their known ranges (Ginns 1997). However, a recent study of *A. ellisii* used species-specific molecular markers to show that hyphal abundance and distribution remains seasonally stable at several sites across Oregon, even though fruiting bodies are rare or absent at these sites during most years (Gordon and Apple 2011). Based on their findings, Gordon and Apple (2011) suggested the possibility that *A. ellisii* and other putatively rare species within the *Albatrellus* lineage may actually be rare fruiters but more common in hyphal communities.

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