

Craterellus fallax, a Black Trumpet mushroom from eastern North America with a broad host range

Patrick Brandon Matheny · Emily A. Austin ·
Joshua M. Birkebak · Aaron D. Wolfenbarger

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Abstract Phylogenetic analysis of ITS sequences of members of the *Craterellus cornucopioides* complex (Black Trumpet mushrooms) supports the taxonomic separation of *Craterellus fallax* apart from *C. cornucopioides*, with which it has been synonymized in the past. Examination of *Pinus virginiana* ectomycorrhizal (ECM) root tips and sequence comparison with other insufficiently identified environmental sequences from roots of *Tsuga*, *Quercus*, and possibly *Castanea* supports a broad host range in North America for the ECM symbiont *C. fallax*. This is the first molecular confirmation of an ECM symbiont with *P. virginiana*, which associates with a wide diversity of ECM fungi, and the first report of a Cantharellaceae symbiont with this tree, an eastern North American two-needled pine. Three unique species in the *C. cornucopioides* complex are recovered based on phylogenetic analysis: *C. fallax*, *C. cornucopioides*, and an unidentified *Craterellus* species similar to *C. fallax* but smaller in stature with smaller spores.

Keywords Barcoding · Cantharellales · Ecology · Ectomycorrhizas · *Pinus virginiana* · Systematics

Introduction

Black Trumpet mushrooms belong to the *Craterellus cornucopioides* (L.: Fr.) Fr. complex, a group of choice edible wild mushrooms that have a cosmopolitan distribution.

Black Trumpets are particularly esteemed for their culinary qualities in Europe (Pilz et al. 2003) and are a favorite in California (Arora 1991). Members of the *C. cornucopioides* complex are recorded from Europe, North America, Central America, South America, and Asia (Pilz et al. 2003), as well as Australia (Fuhrer 2005; Grgurinovic 1997). Until 1968, the Black Trumpet, also known as the Trumpet of Death or Horn of Plenty, was referred to in eastern North America as *C. cornucopioides* (Hesler 1960; Smith 1964; Smith 1968).

Smith (1968) was the first to distinguish species in the complex based on spore deposit color (salmon vs pale buff) and later by spore size (Smith et al. 1979). These differences led to the description of *Craterellus fallax* A.H. Smith (Figs. 1, 2, and 3), common in deciduous and mixed forests of eastern North America. A later study by Petersen (1975) on cantharelloid fungi reinforced Smith's findings recording the salmon buff to pinkish buff spore print in *C. fallax*, in contrast to the pale cream or ivory colored deposit in European *C. cornucopioides*. Petersen reported that *C. fallax* occurred predominantly in conifer forests from Nova Scotia to Alabama and west to Minnesota. Bigelow (1978), in a study on cantharelloid fungi in the northeast USA and surrounding areas, described the spore deposit of *C. fallax* as "ochraceous-buff" to nearly "ochraceous-orange" when heavy, "light ochraceous-buff" when thin (color designations from Ridgway 1912), and general occurrence in mixed woods or hardwoods. Bigelow also commented that the pileal hyphae of *C. cornucopioides* lack secondary septation and do not disarticulate in contrast to *C. fallax*. Smith (1968) and Smith et al. (1979) reported both *C. fallax* and *C. cornucopioides* in North America, with *C. fallax* common mostly in hardwood forests east of the Great Plains. The distribution of *C. cornucopioides* in North America was not specified in any detail and later reported by Phillips (2005) as "much rarer" on the continent. Since 1968, North

P. B. Matheny (✉) · E. A. Austin · J. M. Birkebak ·
A. D. Wolfenbarger
Department of Ecology and Evolutionary Biology,
University of Tennessee,
569 Dabney Hall,
Knoxville, TN 37996-1610, USA
e-mail: pmatheny@utk.edu

American field guides incorporated the name *C. fallax* and reported general habitat ranges of the species under beech, oak, and other deciduous trees (Lincoff 1981), in deciduous forests (Horn et al. 1993), in hardwoods or mixed woods (Bessette et al. 1997, 2001), in broad-leaved and mixed woods, especially with oak and beech (Roody 2003), and under mixed deciduous trees (Phillips 2005), imparting a rather vague and sometimes conflicting ecological perspective of the species.

Dahlman et al. (2000) were the first to perform a molecular phylogenetic estimate of *Craterellus* using a single conserved gene region that encodes the nuclear large subunit ribosomal RNA (LSU or 25–28S rRNA). They utilized between 500 and 600 bp of the 5' end of this gene. Dahlman and colleagues recovered a single clade of North American *C. fallax* sampled from Mississippi, one collection from an undisclosed area of eastern North America (the sequence of which is unavailable on GenBank), black forms of *C. cornucopioides* from North Carolina and California, and European representatives of *C. cornucopioides*, *C. konradii* Bourdot and Maire, and an intermediate form of the latter two. Consequently, *C. fallax* and *C. konradii* were regarded as younger synonyms of *C. cornucopioides*. Moncalvo et al. (2006) reported the clade as the *C. cornucopioides* complex but did not sample multiple individuals. Hansen and Knudsen (1997) reported spore dimensions for *C. cornucopioides* matching that of *C. fallax*, in contrast to Smith et al. (1979). Thus, only subtle distinctions (spore deposit color and characterization of

pileal hyphae) would separate the two species, trait differences disregarded by Dahlman et al. (2000).

Our objectives in this report are to test the hypothesis of conspecificity of *C. fallax* with *C. cornucopioides* comparing ITS sequences produced from fruitbodies and ECM root tips in a phylogenetic framework. We also intend to investigate the range of plant associates of *C. fallax*, which is unclear based on literature reports. Several *Craterellus*-like ITS sequences of ECM root tips are available on GenBank, but none have been reliably identified.

Materials and methods

Fresh materials of *Craterellus* were collected in the field during August 2009 in a mixed forest of *Pinus strobus* L., *Quercus* spp., *Tsuga canadensis* (L.) Carrière, and *Fagus grandifolia* Ehrh., along Chestnut Branch Trail in the Great Smoky Mountains National Park (GSMNP) in North Carolina. These materials were air-dried on food dehydrators and accessioned at the University of Tennessee Fungal Herbarium (TENN). Three collections corresponded to *C. fallax* (TENN064163, TENN064164, TENN064165). A fourth collection, similar to but smaller than *C. fallax*, was also documented (TENN064162), but identification to species based on morphology was unclear.

ECM samples of young saplings of *Pinus virginiana* Mill. (Virginia Pine, est. 3–4 yr old) were collected during October 2009 at 5113 Brown Gap Road in Knoxville,

Fig. 1 Fruitbodies of *C. fallax* (AFTOL-ID 286, PBM 2427, TENN063931) in situ on Mt. Wachusett, Massachusetts. Photo by P.B. Matheny

Fig. 2 Fruitbodies of *C. fallax* (MBP080209b, TENN064163) in situ Chestnut Branch Trail, Great Smoky Mountains National Park, North Carolina. Photo by M.B. Pilkington

Fig. 3 Fruitbodies of *C. fallax* (MGW652, TENN064164) in situ Chestnut Branch Trail, Great Smoky Mountains National Park, North Carolina. Photo by M.G. Wood

Fig. 4 Ectomycorrhizal root tips of a *P. virginiana* sapling. Photo by P.B. Matheny



Tennessee, and examined for presence of ectomycorrhizas. The root system of one individual was noticeably colonized by small but conspicuous smooth and dichotomously branched ectomycorrhizas, the color of which ranged from beige to tawny to black (Fig. 4). Four samples from a single plant were selected (ECM1–ECM4) after rinsing roots and removing dirt and stored at -20°C in CTAB buffer solution until DNA extraction. Each sample was later thawed and ground with sand in a porcelain mortar. The slurry was transferred to a 1.5-ml microcentrifuge tube and centrifuged, and the supernatant was removed to a new tube. DNA was extracted from the supernatant using an Omega Bio-Tek Fungal DNA extraction kit. DNA extractions of dried specimens followed that as above, except that 10–20 mg of fruitbody tissues was ground to powder in a 1.5-ml microcentrifuge tube with a sterile micropestle and a pinch of sand. PCR was performed on a Bio-Rad C1000 thermocycler using primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990) and genomic DNA samples. Protocols for PCR amplification are specified in White et al. (1990). PCR reagents included $5\times$ colorless GoTaq reaction buffer, GoTaq DNA polymerase, and premixed dNTPs acquired from Promega and prepared as per manufacturer instructions, but dividing reactions in half.

ITS amplicons were purified using a Qiagen PCR purification kit. Sequence reactions were performed using an ABI BigDye 3.1 cycle sequencing kit and primers ITS1F and ITS4. After incorporation of BigDye terminators, sequence products were purified using Sephadex G-50 spin columns. Sequencing was performed on an ABI 3730 48-capillary electrophoresis instrument at the Molecular Biology Resource Facility at the University of Tennessee. Resulting sequence files were edited and assembled into contigs using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA).

To ascertain the autonomous status of *C. fallax* and infer the diversity of its plant associates, we assembled a data matrix of 17 ITS sequences based on blastn results and sequencing of additional species of *Craterellus*, including *C. hesleri* R.H. Petersen (TENN045733, TENN042515) and *C. carolinensis* R.H. Petersen (TENN041586) from North Carolina and Tennessee. To this, we added two European ECM isolates from GenBank (FJ897184 and EU816605, each with 94% sequence similarity and E values of 0.0 to our *C. fallax* query) and the only available *C. cornucopioides* ITS sequence (UDB000053, KF01-46) from the UNITE database (Kõljalg et al. 2005). Five insufficiently identified environmental sequences (100–94% sequences similarity and E values of 0.0) were also added to the data matrix. Sequences were aligned using default parameters in ClustalX 2.0.9 (Larkin et al. 2007) and manually adjusted

in MacClade 4.08 (Maddison and Maddison 2005). An ITS sequence of *Pseudocraterellus sinuosus* (Fr.: Fr.) Corner was provided by K. Hughes (University of Tennessee) for outgroup purposes based on phylogenetic results of Dahlman et al. (2000). Several regions of the *P. sinuosus* and *C. hesleri* TENN045733 sequences exhibited gaps and insertions, which were excluded before phylogenetic analysis (nchar=1023 before character exclusion, nchar=911 after exclusion). GenBank accession numbers are provided in the phylogenetic tree discussed below and in the legend of the tree figure. The ITS alignment is available at (http://www.bio.utk.edu/matheny/Site/Alignments_%26_Data_Sets.html) or can be obtained upon request.

Phylogenetic analysis was performed in PAUP* 4b10 for Unix (Swofford 2003) using maximum likelihood (ML). Likelihood settings were determined from the best-fit model selected by the AIC criterion in Modeltest 3.7 (Posada and Crandall 1998). Starting trees were obtained via stepwise addition, using the as-is addition sequence and the TBR branch-swapping algorithm holding one tree at each step during stepwise addition. Outgroups were ordered last. One thousand bootstrap searches (each replicate limited to 5,000 generations) were conducted in GARLI v0.951 (Zwickl 2006) under the model specified by Modeltest. One thousand bootstrap replicates were also performed under the maximum parsimony (MP) criterion in PAUP* using stepwise addition to obtain starting trees, 10 random replicate searches, and the SPR branch-swapping algorithm with MulTrees turned off. Pairwise “p” (proportional) distances for ITS and nuclear large subunit ribosomal RNA (LSU) sequences were calculated in PAUP* using the “showdist” command. Nine LSU sequences (AY700188, AF105305, AF105301, AF105300, AF105299, AF105298, AF105297, AF105296, and AJ279572) were downloaded from GenBank for LSU distance comparisons using the search term “*Craterellus cornucopioides*.”

Results

Approximately 1,000 bp-sized amplicons were produced from all our *Craterellus* isolates, as well as the four ECM samples. However, faint shadow bands slightly less than 700 bp were observed in ECM2 and ECM3, and up to three faint bands were visible in ECM4. Negative controls were free of any contaminating DNA. Eleven new sequences have been deposited at GenBank (GU590923–32 and GU982730) for the following species: *C. fallax*, *Craterellus* sp., *C. hesleri*, *C. carolinensis*, and *Pseudocraterellus sinuosus*.

ITS sequences of ECM1, ECM2, and ECM3 were the same and matched the ITS sequence of a GenBank accession (DQ205680, PBM 2427) labeled *C. cornuco-*

pioides with 100% similarity. This accession was originally submitted to NCBI under the name *C. fallax* by one of us (PB Matheny), and the hyperlink to AFTOL-ID 286 provided in the GenBank record specifies the identification as *C. fallax*. This material was collected on Mt. Wachusett in central Massachusetts in a mixed forest composed of *Tsuga*, *Quercus*, *Fagus*, and *Betula* and is shown in Fig. 1. Notes of the collection indicate the fertile surface as cinereous becoming ochre-buff to light ochre-buff or salmon in areas with a pleasant apricot-like odor, features consistent with the description of *C. fallax*. BLAST results also indicated 99–100% similarity to three different environmental isolates from roots of *Tsuga canadensis* in New York (FJ901312), *Quercus* seedlings in North Carolina (AY656927), and an unidentified ECM sample from *Castanea* and *Quercus* plots in New York (DQ424938) (Dulmer and Horton 2005; Walker et al. 2005). The sequence of ECM4 was heterogeneous, but a partial suboptimal sequence from this sample suggests a similarity with *Trichoderma hamatum* (Bonord.) Bainier, a beneficial endophyte of Cacao (Bae et al. 2009). This result requires confirmation by cloning but could confirm the presence of a secondary colonizer on the roots of *P. virginiana* (Rosling et al. 2003).

The model best-fit to the *Craterellus* ITS alignment was a HKY+G model with a gamma distribution shape parameter of 0.3113. This model invoked two substitution types, with a Ti/tv ratio of 1.3017. Three significantly supported clades that we regard as separate species were recovered: *C. fallax*, *C. cornucopioides* (which forms a strongly supported sister group to *C. fallax* in the MP analysis but not in ML analyses), and *Craterellus* sp. (Fig. 5). The *C. fallax* lineage is composed of fruitbody samples from mixed forests in North Carolina and Massachusetts, and several insufficiently identified environmental sequences from eastern North America. Phylogenetic results show that our *C. fallax*–*P. virginiana* ectomycorrhizas are identical to sequences of unidentified *Quercus* ectomycorrhizas from North Carolina, *Castanea* and/or *Quercus* ectomycorrhizas from New York, and *Tsuga* ectomycorrhizas from New York. Samples of *C. cornucopioides* from Europe cluster into a second clade. A third unidentified lineage is also recovered based on two collections from Tennessee and North Carolina. ITS pairwise “p” distance calculations reveal that European isolates of *C. cornucopioides* differ from North American *C. fallax* isolates by 2.1–2.8% with intraspecific variation less than 0.3% in both species. Pairwise LSU “p” distances from nine accessions ranged between 0.0% and 3.5% dissimilarity. If a Tibetan isolate labeled *C. “cornucopioides”* is excluded, then the range of LSU distances between all isolates currently labeled *C. cornucopioides* (sensu lato) falls between 0.0% and 1.1%.

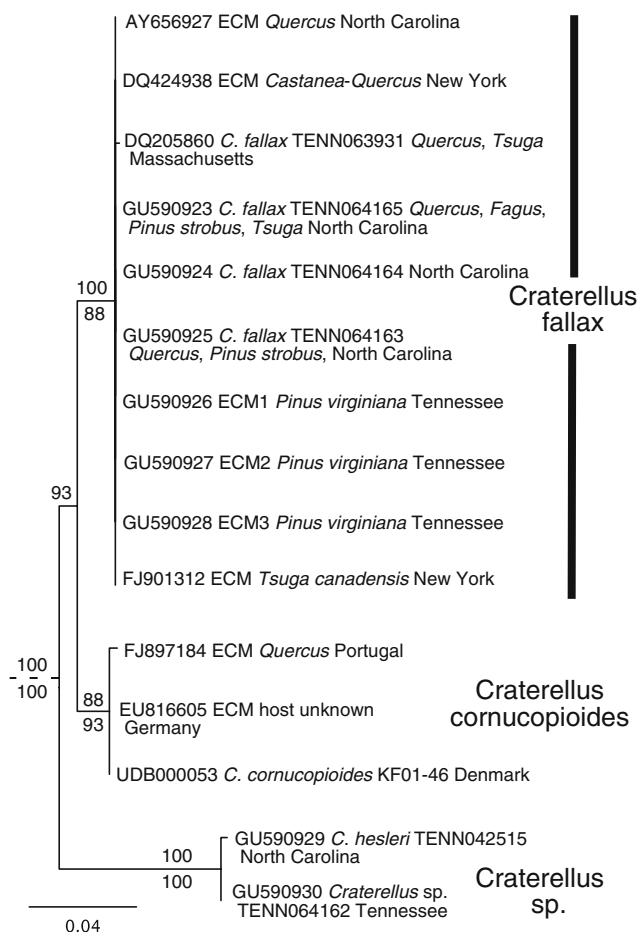


Fig. 5 ML phylogram ($-\ln L=2257.78137$) of the *C. cornucopioides* complex. MP bootstraps are indicated above branches and ML bootstraps below. Three monophyletic groups are recovered: *C. fallax* from eastern North America, *C. cornucopioides* from Europe, and *Craterellus* sp. from the southeast United States. The outgroup ITS sequence, *Pseudocraterellus sinuosus* (GU590932), and a second ITS sequence of *C. hesleri* p.p. (GU590931), are pruned from the tree (indicated by the dashed root branch)

Discussion

This report draws several new conclusions regarding the taxonomy and ecology of *C. fallax*. First, *C. fallax* should be regarded as a species distinct from *C. cornucopioides* based on phylogenetic analysis of the ITS region. In contrast to Dahlman et al. (2000), phylogenetic and taxonomic analysis of which relied on short sequences (c. 580 bp) of the more conserved nuclear LSU rRNA gene region, ITS sequences of *C. cornucopioides* from Europe (Denmark, Portugal, and Germany) form a distinct clade apart from eastern North American *C. fallax* (Fig. 5). The difference in spore deposit color (salmon tinges vs pale cream or ivory) is a suitable trait to distinguish the two species as Smith (1968) described and Petersen (1975) and Bigelow (1978) re-iterated. Differences are reported to exist in pileal hyphal construction as well (Bigelow 1978).

Our results also suggest that phylogenetic analysis of LSU sequences alone should be interpreted cautiously when considering species recognition. Barcode analysis of the *C. cornucopioides* complex will not be fruitful if relying on short LSU sequences only. However, ITS pairwise comparisons reveal between 2.1% and 2.8% “p” distances between European isolates of *C. cornucopioides* and what we consider North American isolates of *C. fallax* with negligible intraspecific variation recorded (<0.3%). Concerns have been expressed about the limits of ITS as a marker to delimit species (Nilsson et al. 2006; Smith et al. 2007), but analysis of mitochondrial large subunit (mtLSU) sequences might be worth exploration. In *Craterellus*, a pairwise comparison between two mtLSU sequences (AF185977 sampled from Massachusetts, and AF185976 produced from a yellow mutant form labeled Danell43, geographic origin unknown) (Pine et al. 1999) reveals nine site differences, including two single gapped positions, from an aligned length of 489 bp (“p” distance 1.8%). Thus, one might predict that mtLSU–rRNA sequences could be useful to distinguish *C. fallax* from *C. cornucopioides*.

An LSU sequence (AF105299) of Californian *C. cornucopioides* is identical to a sequence of *C. fallax* from Mississippi (AF105305), but differs at three positions (all gaps) compared to material from Massachusetts (AY700188). However, according to Arora (1986), *C. cornucopioides* from California has a whitish to pale yellow spore print and smaller spores than *C. fallax* from eastern North America. Careful morphological scrutiny and ITS or mtLSU sequence analysis should be helpful to determine the taxonomic status of *C. cornucopioides* in California and southern Oregon, where it is common and harvested in large quantities as food, and in areas north of southern Oregon, where it is rare or less frequently encountered (Pilz et al. 2003).

Species of chanterelles and allied fungi are thought to exhibit a very broad host range (Pilz et al. 2003). Our results demonstrate that *C. fallax* exhibits a broad host range that includes members of Pinaceae (*Pinus* and *Tsuga*) and Fagaceae (*Quercus* and/or *Castanea*). *Craterellus fallax* may also serve an important symbiont for very young trees, where it forms relatively abundant ectomycorrhizas judging by our molecular identification of three different samples from a single sapling of *P. virginiana*. The only molecularly confirmed European plant associate for *C. cornucopioides* at this time is *Quercus*. The extent of host associations in other *Craterellus* species is generally not well known. Trappe (2004), however, showed that *C. “neotubaeformis”* nom. prov. of Pilz et al. (2003) forms ectomycorrhizas with three different genera of Pinaceae (*Tsuga*, *Picea*, *Pseudotsuga*) in Oregon.

This is the first report of molecular confirmation of an ECM symbiont with *P. virginiana* from the field and the first record of a Cantharellaceae symbiont with this plant species. *Pinus virginiana* is a two-needled pine with a distribution north to New York and New Jersey, west to Pennsylvania, Ohio, and Indiana, and south to northern parts of South Carolina, Georgia, Alabama, and Mississippi (Elias 1980). Virginia Pine forms ECM associates with numerous species of Agaricomycetes and one ascomycete: *Rhizopogon roseolus* (Corda) Th. Fr., *Pisolithus tinctorius* (Pers.) Coker and Couch (current name: *P. arhizus* (Scop.) Rauschert), *Astraeus hygrometricus* (Pers.) Morgan, *Thelephora terrestris* Ehrh., nine species of *Amanita* Pers., 13 species of *Boletus* L.: Fr. sensu lato, *Scleroderma aurantium* (L.) Pers., *Chroogomphus vinicolor* (Peck) O.K. Mill., *Russula emetica* (Schaeff.: Fr.) Pers., one species of *Inocybe*, and *Cenococcum* Moug. & Fr. (HacsKaylo 1955, 1965; Hepting 1971; Melin et al. 1958; Miller and Rudolph 1986; Schramm 1966). *Lepiota rhacodes* (Vittad.) Quél. (current name: *Chlorophyllum rhacodes* (Vittad.) Vellinga) was reported to form an ECM association with *P. virginiana* based on a pure culture synthesis experiment (HacsKaylo 1955). However, species of Agaricaceae are not mycorrhizal (Vellinga 2004), so a *Chlorophyllum* association is highly dubious. Nevertheless, *P. virginiana* is of ecological importance on restoration sites contaminated with heavy metals or waste by-products of mining (Schramm 1966) and is a primary invader of rare plant communities such as alkaline prairies and serpentine barrens of eastern North America (Cumming and Kelly 2007; Hilgartner et al. 2009; Thiet and Boerner 2007). It has been hypothesized that the ECM physiology of *P. virginiana* suppresses heavy metal toxicity symptoms when growing on serpentine soils (Miller and Cumming 2000; Wilkins 1991). While pure synthesis experiments have demonstrated ECM formation on short roots of *P. virginiana* (HacsKaylo 1955), no species of fungal symbionts have been identified by molecular identification, a method generally considered a reliable means to determine the mycorrhizal status of a fungus (Rinaldi et al. 2008).

We have also documented a third unidentified species of the *C. cornucopioides* complex. The taxonomic designation of *Craterellus* sp. (TENN064162) is not clear, but it resembles a small form of *C. fallax*. The former has smaller spores (7.0–9.0×4.0–6.0 μm) than *C. fallax* (10.0–13.0×7.0–9.0 μm), thus affirming their genetic differences. *Craterellus hesleri* is similar in size and was described from the GSMNP in Tennessee (Petersen 1975). ITS sequences of the collection and “*C. hesleri*” TENN042515 collected in 1980 at a North American Mycological Association foray in North Carolina are identical. However, the ITS sequence of a second collection of *C. hesleri* (TENN045733) is not the same as the previous two and clusters towards the root in our

phylogenetic tree (not shown). An examination of the holotype of *C. hesleri* (TENN037838, the packet of which is labeled “*Pseudocraterellus hesleri*”) shows a minute species (pileus originally described up to 7 mm broad) with a light grayish brown or ochraceous yellow hymenophore, traits of which are inconsistent with our smaller version of *C. fallax*, which has a drab-gray hymenophore and dark gray to black stipe (ostensibly hollow) with a pallid point of attachment. *Craterellus carolinensis* R.H. Petersen also lacks a perforate pileus, and the ITS1 sequence of TENN041586 (collected and determined by R.H. Petersen) indicates it is more closely related to *Pseudocraterellus sinuosus* (GU590532) and *C. hesleri* (GU590531) (not shown in Fig. 5). Several small to tiny species of *Craterellus* (or *Pseudocraterellus*) are enumerated in Bigelow (1978) and Petersen (1969, 1975). The description of *C. subundulatus* (Peck) Peck provided in Bigelow (1978) records a dark brown to gray brown small pileus, similarly colored stipe (“blackish”), and spore dimensions not inconsistent with our unclarified *Craterellus* sp.; however, the pileus of the latter is clearly perforate in contrast to the shallowly depressed and imperforate pileus of *C. subundulatus* fide Bigelow. An examination of material labeled *Pseudocraterellus subundulatus* (Peck) D.A. Reid from Tennessee (TENN037839) shows an “Avellaneous” to “Wood Brown” hymenophore with a shallowly depressed pileus that contrasts with the “Drab-Gray” to “Light Drab” hymenophore and perforate pileus of *Craterellus* sp. *Craterellus calyculus* (Berk. and M.A. Curtis) Burt lacks a perforate pileus and has larger spores ($9.5\text{--}12.0 \times 7.0\text{--}9.0 \mu\text{m}$) (Petersen 1969). Thus, none of these previously described species match the third unidentified lineage of the *C. cornucopioides* complex. A complete description, including photographs and spore deposit color, are needed before fully addressing the taxonomic status of this species.

In summary, both ITS and morphological features can be used to distinguish *C. fallax* from European isolates of *C. cornucopioides*. The ITS phylogeny is consistent with differences in spore deposit color between the two species, a difference first noticed by Smith (1968), and pileal hyphal construction may differ as well (Bigelow 1978). We suggest these lines of evidence support a reasonable assessment and resuscitate *C. fallax* as an autonomous species, and that at least three unique lineages constitute the *C. cornucopioides* complex.

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