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# *Boletus rubricitrinus* belongs in *Pulchroboletus* (*Boletaceae*)

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The boletes are macrofungi which have undergone extensive taxonomic revisions since the advent of molecular tools. This paper provides the first DNA sequences of *Boletus rubricitrinus*, a common Florida bolete often found in lawns under *Quercus*, and likely has a distribution that extends to Texas. Based on ITS and LSU sequences and morphological studies we propose moving it to the genus *Pulchroboletus*. As the holotype is in poor condition, an epitype is established here. A thorough description of macroscopic and microscopic features is also provided for the species. These molecular and morphological data will be useful to further improve our understanding of bolete taxonomy.

**Key words:** Basidiomycota, *Boletales*, phylogeny, taxonomy, epitype.

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Skupina hřibovitých hub prošla od nástupu molekulárních metod rozsáhlými taxonomickými revizemi. Tento článek přináší první sekvence DNA druhu *Boletus rubricitrinus*, běžného floridského hřibu, který často roste v trávě pod duby a jeho rozšíření zasahuje pravděpodobně po Texas. Na základě sekvencí ITS a LSU spolu s morfologickým studiem navrhujeme jeho přesun do rodu *Pulchroboletus*. Jelikož holotyp je ve špatném stavu, je zde vystaven epityp. Práce též přináší podrobný popis makroskopických a mikroskopických znaků tohoto druhu. Uvedená molekulární data i morfologické údaje jsou příspěvkem k lepšímu poznání taxonomie hřibovitých hub.

## INTRODUCTION

The boletes are a polyphyletic assemblage of macrofungi in the *Boletales* which are defined by stipito-pileate basidiomes with tubulose hymenophores. They were first placed in *Boletus* L. (Linnaeus 1753: 1176) and *Boletaceae* (Chevallier 1826: 248), obsolete concepts which both included polypores. The order *Boletales* was later introduced by Gilbert (1931) to exclusively include boletes. Molecular phylogenetic tools (Martin et al. 2011) have expanded the

*Boletales* to include agaricoid, resupinate, and gasteroid fungi (Bruns et al. 1989, Hibbett et al. 1997, Binder & Bresinsky 2002b, Binder & Hibbett 2006). There are over 1,300 species in the *Boletales*, comprised of 17 families and about 100 genera (Kirk et al. 2008). *Boletaceae* sensu stricto now contains about 70 genera and approx. 800 species (Bresinsky et al. 1999, Binder & Bresinsky 2002a, Binder & Hibbett 2006, Drehmel et al. 2008, Desjardin et al. 2009, Orihara et al. 2010, Li et al. 2011, Nuhn et al. 2013, Gelardi et al. 2014, Wu et al. 2014).

There are approximately 300 species of *Boletus* sensu Kirk et al. (2008: 97), although the number is likely to change as more molecular data become available. *Boletus* sect. *Luridi* Fr. (1838: 417), the largest section in *Boletus* sensu Singer (1986: 778), contained 40 species, defined by small, discolouring pores with finely reticulated or furfuraceous stipes. Molecular investigations found *Boletus* sect. *Luridi* to be polyphyletic, resulting in the transfer of species to existing or novel genera within *Boletaceae* (Takahashi et al. 2011, Vizzini 2014a, 2014b, 2014c, Vizzini et al. 2014, Zhao et al. 2014).

*Boletus rubricitrinus* (Murrill) Murrill is a bolete with a brick-coloured pileus, a yellow stipe with red floccules/punctules concentrated at the base, and a usually acidic taste (Murrill 1940). It was described from a collection made on a lawn near *Quercus laurifolia* in Gainesville, Florida, USA and originally placed in *Ceriomyces* Battarra ex Murrill, nom. illeg. (Murrill 1940; see Donk 1958: 167 for interpretation of Battarra names). Singer placed *B. rubricitrinus* in *Boletus* sect. *Luridi* (Singer 1947, Singer 1986).

*Boletus rubricitrinus* has not yet been analysed with molecular tools. In this study, we aim to understand the taxonomic placement of *B. rubricitrinus* in the context of molecular DNA evidence. Also, since this species lacks modern rigorous morphological descriptions, we provide a more detailed microscopic description.

## MATERIAL AND METHODS

Sampling and identification. Specimens examined were collected in peninsular Florida between 2012–2017 and deposited at the University of South Florida Herbarium (USF).

Specimens were identified based on the protologue (Murrill 1940), Murrill's identification keys (Murrill 1972), and examination of the holotype.

Morphological studies. Macroscopic descriptions are based on detailed notes made from fresh basidiomes. Micromorphological features were observed from dried specimens using a compound microscope (AccuScope, Commack, NY, USA); distilled $H_2O$, 5% KOH, and Congo red were used to

rehydrate and stain sections. Measurements were made at 1000× with a calibrated ocular micrometer. Micrographs were taken with a Nikon D3200 camera. Basidiospore dimensions are reported as length by width, with each measurement reported as the minimum, the average minus the standard deviation, the average plus the standard deviation, and the maximum. Measurements are followed by the number of spores counted, and the average quotient Q, where Q = average length divided by average width.

DNA extraction, PCR amplification, and DNA sequencing. Genomic DNA was isolated from dried herbarium specimens (Tab. 1) using a modified CTAB extraction protocol (Doyle & Doyle 1987, Franck et al. 2012); the resulting DNA was diluted in 65 µl of a 10 mM Tris, 1 mM EDTA buffer. Universal primers ITS1/ITS4 were used to amplify ITS1, 5.8S rRNA, and ITS2 (White et al. 1990). The primer pair LR0R/LR7 (Vilgalys & Hester 1990) were used to amplify 28S rRNA (LSU). Amplification reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with 20 µl volumes, using 1 unit ID*Proof*™ Taq Polymerase (Empire Genomics, Buffalo, NY, USA), 2 µl 10× Reaction Buffer, 3 mM $MgCl_2$, 120 ng of each primer, 250 µM dNTPs, and 1 µl of DNA. If amplification failed, serial dilutions were used for additional attempts. Amplification cycle parameters for the ITS region were as follows: 94 °C for 3 minutes for initial denaturation, followed by 40 cycles of 94 °C for 45 s, 51 °C at 45 s for annealing, and an extension at 72 °C for 90 s, with a final extension of 72 °C for 5 minutes. Amplification cycle parameters for the LSU region were as follows: 95 °C for 2 minutes for initial denaturation, followed by 40 cycles of 94 °C for 45 s, 50 °C at 70 s for annealing, and an extension at 72 °C for 120 s, with a final extension of 72 °C for 10 minutes. Samples were visualised in 0.9% agarose using TAE buffer and 1% ethidium bromide to ensure product of expected size was produced. Crude PCR product was purified and sequenced at the DNA Laboratory at Arizona State University with a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) using the same PCR primers and an additional internal primer for LSU, LR5 (Vilgalys & Hester 1990).

Sequence alignment, dataset assembly, and phylogenetic analysis. Sequences obtained in this study were run using the BLASTn algorithm (Boratyn et al. 2013) to identify related sequences. These sequences were combined with sequences from the literature (Morris et al. 2008, Smith & Pfister 2009, Gelardi et al. 2014, Frank et al. 2017) for phylogenetic analysis (Tab. 1). Sequences were aligned for ITS and LSU using the Clustal W algorithm (Thompson et al. 1994) in MEGA7 (Kumar et al. 2016) with default parameters. Phylogenetic analyses were run for ITS and LSU separately, as well as a concatenated ITS/LSU dataset.

**Tab. 1.** Sequences used for phylogenetic analyses. Taxon names correspond to those listed in GenBank.

| Taxon | Origin | ITS GenBank No. | LSU GenBank No. | Voucher No. | References |
|---|---|---|---|---|---|
| *Alessioporus ichnusanus* | Corsica, France | KJ729498 | KJ729511 | TO AVX13 | Gelardi et al. 2014 |
| *Alessioporus ichnusanus* | Lazio, Italy | KJ729496 | KJ729509 | MG 420a | Gelardi et al. 2014 |
| *Alessioporus ichnusanus* | Piedmont, Italy | KJ729495 | KJ729508 | RG XER.ICH 6 | Gelardi et al. 2014 |
| *Alessioporus ichnusanus* | Lazio, Italy | KJ729493 | KJ729506 | MG 549a | Gelardi et al. 2014 |
| *Alessioporus rubriflavus* | Suffolk Co., New York, USA | KU736957 | — | ARB 1356 | Frank et al. 2017 |
| *Alessioporus rubriflavus* | Suffolk Co., New York, USA | KC812305 | KC812206 | JLF 2561 | Frank et al. 2017 |
| *Alessioporus rubriflavus* | Oconee Co., South Carolina, USA | KU736958 | — | JLF 2561b | Frank et al. 2017 |
| *Alessioporus rubriflavus* | Elbert Co., Georgia, USA | KT223008 | KT223009 | ARB 1262 | Frank et al. 2017 |
| *Boletus rubricitrinus* | Sarasota Co., Florida, USA | MF193883 | — | USF Franck 3114 | This study |
| *Boletus rubricitrinus* | Hillsborough Co., Florida, USA | MF193884 | MG026638 | USF Farid 335 | This study |
| *Boletus rubricitrinus* | Hillsborough Co., Florida, USA | MF193885 | — | USF Franck 3473 | This study |
| *Boletus rubricitrinus* | Taylor Co., Florida, USA | MF193886 | — | USF Franck 3594 | This study |
| *Boletus* sp. | Guerrero, Mexico | EU569236 | — | UC MHM075 | Morris et al. 2008 |
| *Boletus* sp. | Middlesex Co., Massachusetts, USA | FJ480444 | — | FH MES260 | Smith & Pfister 2009 |
| *Pulchroboletus roseoalbidus* | Sardinia, Italy | KJ729486 | KJ729499 | AMB 12757 | Gelardi et al. 2014 |
| *Pulchroboletus roseoalbidus* | Lazio, Italy | KJ729487 | KJ729500 | MG 532a | Gelardi et al. 2014 |
| *Pulchroboletus roseoalbidus* | Lazio, Italy | KJ729489 | KJ729502 | MG 416a | Gelardi et al. 2014 |
| *Pulchroboletus roseoalbidus* | Emilia Romagna, Italy | KJ729490 | KJ729503 | MCVE 17577 | Gelardi et al. 2014 |
| *Xerocomus depilatus* | — | AY127032 | AF139712 | — | Unpublished |
| *Xerocomus impolitus* | Portugal | HM347651 | — | UF 1464 | Unpublished |
| *Xerocomus impolitus* | Spain | HM347650 | AF139715 | JAM 0585 | Unpublished |
| Uncultured fungus | Ohio, USA | FM999554 | — | isolate S0681 | Unpublished |

Phylogenetic hypotheses were constructed with Bayesian inference (BI) and Maximum parsimony (MP) methods. The best-fit substitution models for both corrected Akaike information criterion (AICc) and Bayesian information criterion (BIC) were determined by jModelTest 2.1.10 (Guindon & Gascuel 2003, Darriba et al. 2012). The BIC model provided for ITS, K80+G, was used for the BI analysis; the BIC model provided for LSU, TrNef+I, was used. BI was conducted with MrBayes version 3.2.6 (Ronquist et al. 2012) with four Markov chain Monte Carlo 10,000,000 generations, sampling trees every 1,000 generations, resulting in 10,001 trees; the first 25% were discarded as burn-in, and a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities (BPP). BPP equal to and above 0.50 were reported. The analysis was also run for

both gene regions with the AICc model provided by jModelTest 2.1.10, and produced the same topology with similar BPP. MP analysis was conducted with PAUP* version 4.0a152 (Swofford 2002) with 1,000 bootstrap replicates (Felsenstein 1985) using a heuristic search; starting trees for branch-swapping were obtained by stepwise addition, and the tree-bisection-reconnection algorithm was used for branch swapping. Bootstrap supports (BS) equal to or greater than 50% were reported. Bayesian consensus trees were visualised in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/), with BPP displayed as node labels. Bayesian consensus trees were then exported as a scalable vector graphic and imported into Inkscape version 0.91 (http://www.inkscape.org) to re-annotate tip labels and add BS. Alignment and phylogenetic trees were uploaded to http://www.treebase.org/ (submission ID 21355).

## RESULTS

### MOLECULAR ANALYSIS

Four ITS sequences and one LSU sequence were obtained from four specimens of *Boletus rubricitrinus* selected for study. The final ITS dataset consisted of our four new sequences and 18 sequences from the literature. These 22 sequences corresponded to six known species, while three environmental sequences from the literature were unidentified members of *Boletaceae*. Both BI and MP produced the same topology. The four newly sequenced *Boletus rubricitrinus* samples clustered as a sister clade to *Pulchroboletus roseoalbidus* (Alessio, Galli & Littini) Gelardi, Vizzini & Simonini with 1.0 BPP and 99.15% BS (Fig. 1). The three environmental sequences formed a sister group to the *Pulchroboletus* clade, with 1.0 BPP and 99.642% BS: EU569236.1, identified as *Boletus* sp., with a voucher collected in Guerrero, Mexico, FM999554.1, an uncultured environmental sequence from Ohio, USA, and FJ480444.1, identified as *Boletus* sp., collected in Massachusetts, USA, with submission notes of the isolation source having a bright orange sclerotium.

The LSU dataset consisted of one new sequence and 12 sequences from the literature. These 13 sequences corresponded to the same six named species as the ITS tree. For LSU, both BI and MP produced a topology which is congruent with the ITS tree. *Boletus rubricitrinus* formed a sister clade to four *Pulchroboletus roseoalbidus* samples, with 0.9812 BPP and 96.233% BS (Fig. 2). The combined LSU/ITS dataset topology was congruent with the ITS and LSU topologies (Fig. 3). *Boletus rubricitrinus* formed a sister clade to four *P. roseoalbidus* samples, with 1.0 BPP and 100% BS.

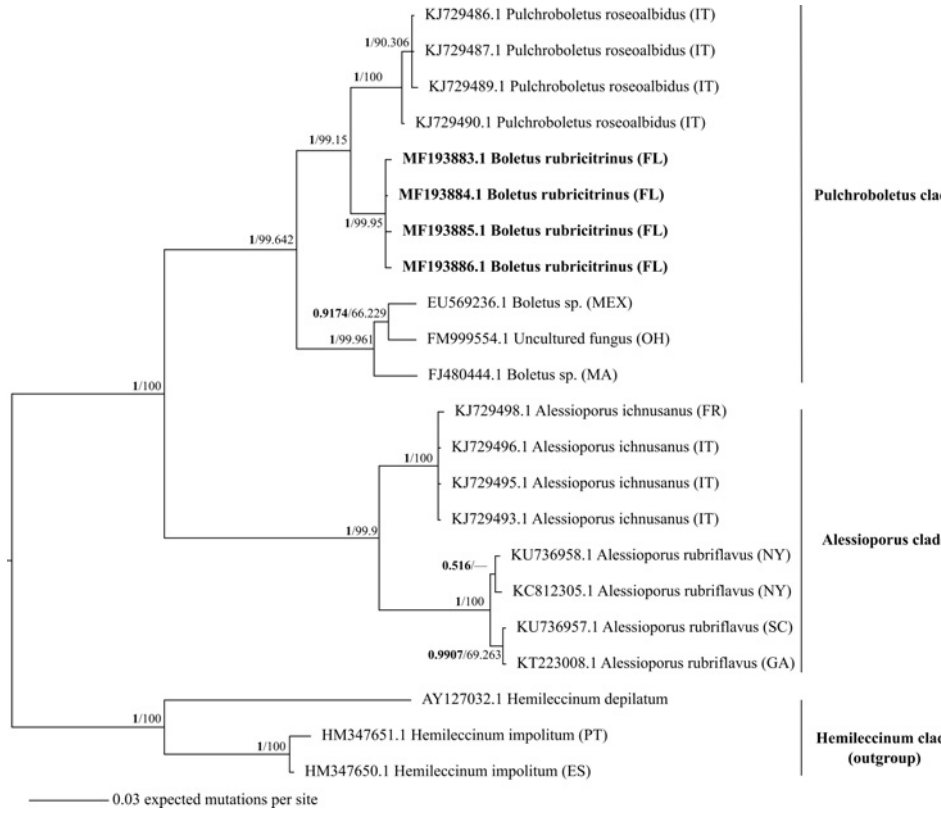


**Fig. 1.** Bayesian tree inferred from ITS sequences. BPP values exceeding 0.5 and ML bootstrap values exceeding 50% are shown adjacent to nodes. GenBank numbers precede the taxon names provided by GenBank, followed by the location of the collection. Novel sequences from this study are in bold.
Abbreviations: IT = Italy, FL = Florida, MEX = Mexico, OH = Ohio, MA = Massachusetts, FR = France, NY = New York, SC = South Carolina, GA = Georgia, PT = Portugal, and ES = Spain; no locality data could be obtained for AY127032, although it is likely from Europe.

## TAXONOMY

### ***Pulchroboletus rubricitrinus*** (Murrill) A. Farid & A.R. Franck, **comb. nov.**

(MycoBank MB 821474) Figs. 4–8

Basionym: *Ceriomyces rubricitrinus* Murrill, Bull. Torrey Bot. Club 67(1): 61 (1940)
≡ *Boletus rubricitrinus* (Murrill) Murrill, Bull. Torrey Bot. Club 67(1): 66 (1940)

Holotype. USA, Florida, Alachua Co., Gainesville, lawn near laurel oak [*Quercus laurifolia*], 2 July 1938, W.A. Murrill s.n. (FLAS F-17321).

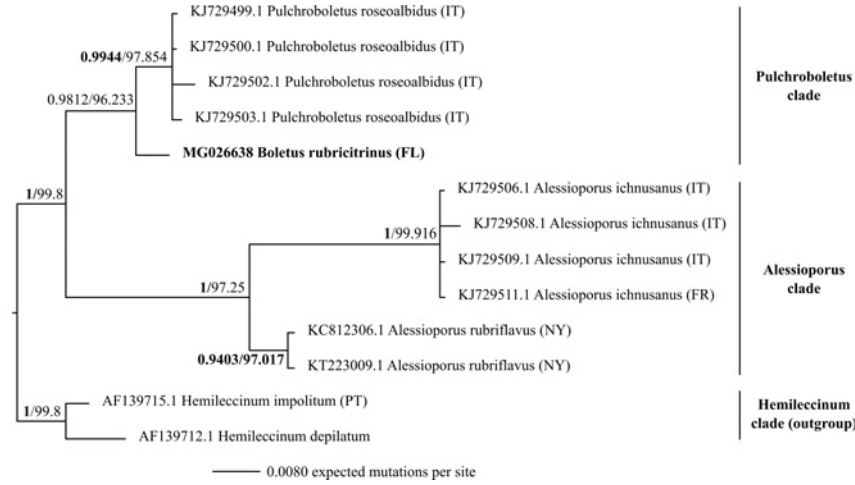


**Fig. 2.** Bayesian tree inferred from LSU sequences. BPP values exceeding 0.5 and ML bootstrap values exceeding 50% are shown adjacent to nodes. LSU GenBank numbers precede the taxon names provided by GenBank, followed by the location of the collection. The novel LSU sequence from this study is in bold.
Abbreviations: IT = Italy, FL = Florida, FR = France, NY = New York, and PT = Portugal; no locality data could be obtained for AF139712, although it is likely from Europe.

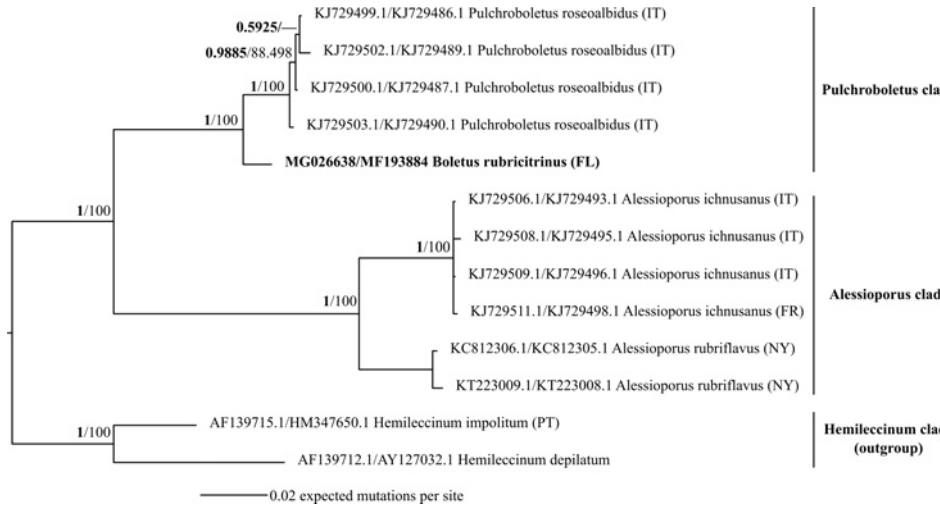


**Fig. 3.** Bayesian tree inferred from combined LSU and ITS sequences. BPP values exceeding 0.5 and ML bootstrap values exceeding 50% are shown adjacent to nodes. LSU/ITS GenBank numbers precede the taxon names provided by GenBank, followed by the location of the collection. The novel LSU/ITS sequence from this study is in bold.
Abbreviations: IT = Italy, FL = Florida, FR = France, NY = New York, and PT = Portugal; no locality data could be obtained for AF139712/AY127032, although it is likely from Europe.

Epitype (designated here, MycoBank MBT 378921). USA, Florida, Hillsborough Co., University of South Florida campus, along S side of sidewalk, N of Alumni Drive and S of Richard Beard garage, lawn, beneath *Quercus virginiana*, 10 June 2016, Arian Farid 335 (USF 288420). GenBank sequences MF193884 (ITS), MG026638 (LSU).

## Examination of holotype

Dried basidiome. Pileus dark brown-olive, occasionally faintly maroon-testaceous in centre, smooth, glabrous. Tubes adnexed-decurrent with a tooth, not separable individually, dark brown, pore mouths subangular. Stipe striate, brownish with a tinge of maroon-red. Mould (*Aspergillus* sp.) damage present on the pileus and stipe of basidiome, and parts of hymenium.

Microscopic features. Basidiospores (12.9)13.4–16.0(18.5) × (3.7)4.3–6.3(6.8) µm (40 spores counted, Q = 2.8), straw-yellow in KOH and water, ellipsoidal to subellipsoidal, sometimes subfusiform, smooth, thin-walled, with a pronounced apiculus and rounded apex, and only rarely with one, two, or three olive-coloured oil droplets (these not lasting over time; Murrill's original protologue defines them as these droplets, which are seen in his drawing alongside the specimen).

Basidia 12.7–25.2 × 10.6–12.2 µm, clavate, subclavate, or cylindrical, smooth, thin-walled, hyaline, yellow-green oil droplets in water and KOH, without basal clamps; sterigmata 1–3 µm long; basidioles clavate to subclavate, size similar to basidia.

Cheilocystidia 19.6–37.5 × 8.4–12.1 µm, light brownish to hyaline in KOH, sometimes encrusted with yellow-green oil droplets, these very small, ventricose to capitulate, clavate, somewhat strangulated at times, apices subclavate to filiform, fusoid. Pleurocystidia shape and size similar to cheilocystidia.

Hymenophoral trama bilateral, boletoid, lateral strata somewhat gelatinised, elements 5–14 µm wide, mediostrata gelatinised, loosely arranged, yellow-brown, hyphae 5–14 µm wide.

The holotype material has sustained much mould damage over time. Although the above features found in the holotype match our other examined collections, other microscopic features could not be discerned through the mould, such as the pileipellis, context, and stipitipellis. Attempts to remove the contaminant mould were attempted, but not successful. Accordingly, we designate an epitype from our sequenced specimens of which we also have photographs.

## Emended description

The description is based only on material examined which was also successfully sequenced: Farid 335, Franck 3114, 3473, 3594 (for details, see Appendix).

**Fig. 4.** Field photograph of *Pulchroboletus rubricitrinus* (Franck 3473). Photograph by A.R. Franck.

Fresh basidiome. Pileus 3–16 cm diameter, at first hemispherical to pulvinate, then becoming convex, then plane, firm when young, becoming soft and fleshy with age; margin involute when young, becoming expanded, uplifted, occasionally lobed, especially when young, occasionally exceeding approx. 1 mm beyond tubes; cuticle somewhat greasy, smooth, occasionally pitted at maturity, glabrous, pink, testaceous, blood-red, with testaceous, vinaceous, or maroon punctules.

Tubes yellow, rounded when young, becoming subangular to angular when mature, adnate, then becoming decurrent with a tooth, tubes separable individually, 0.5–1 cm long tubes, bruising indigo blue at pore mouth and along tubes, 2 pores per mm.

Stipe 5–10 × 2–5 cm, yellow, lacking annulus, cylindrical to clavate, straight, sometimes curving to sinuous, solid, central, base subclavate to fusiform, conspicuous, testaceous, vinaceous, or maroon punctules present on stipe, most frequent at base, becoming large stains on stipe, then becoming longitudinally streaked towards apex of stipe, sometimes becoming finely scabriform or flocciform, especially midway to apex of stipe; upper 1–3 mm of the stipe occasionally reticulate, becoming pronounced at maturity; mycelia below stipe base white.


**Fig. 5.** Field photograph of *Pulchroboletus rubricitrinus* (Farid 335). Photograph by A. Farid.

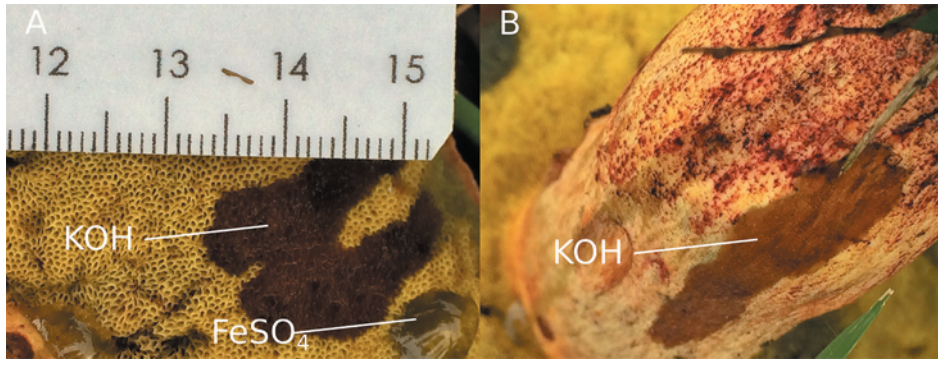


**Fig. 6.** Field photographs of *Pulchroboletus rubricitrinus* (Farid 335). **A** – hymenophore; **B** – flocciform punctuations at stipe base. Photographs by A. Farid.

Context firm, whitish to pale yellow, immediately cyanescent, especially in stipe and near tubes, this cyanescence appearing marbled against context, masking pale yellow pigment in context, deep red pigment present at base of stipital context.

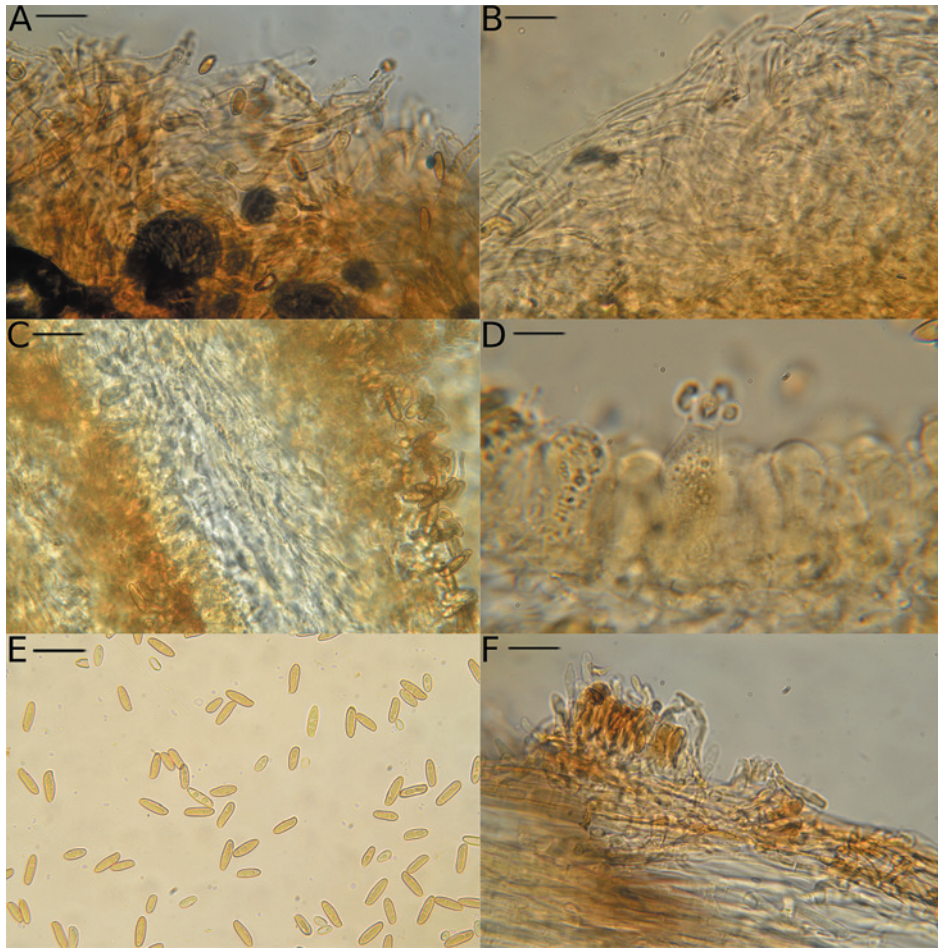


**Fig. 7.** Microscopic features of *Pulchroboletus rubricitrinus*. **A** – pileipellis a trichoderm (Franck 3594); **B** – pileipellis a cutis (Farid 335); **C** – hymenophoral trama (Franck 3473); **D** – basidia and basidioles (Franck 3473); **E** – basidiospores (Farid 335); **F** – fascicles arising from stipitipellis (Farid 335). Scale bars = 15 µm (A–C, E–F), 30 µm (D). Photographs by A. Farid.

Macrochemical reactions. KOH yellow to maroon on pileus, maroon on pores and stipe; $NH_4OH$ yellow to yellow-orange on pileus, stipe, and context, negative on pore mouths (inducing indigo stain, then fading); $FeSO_4$ yellow to olive on stipe, negative elsewhere, bleaching blue stain from hymenophore.

Taste mild to slightly citrusy acidic sour. Odour mild, sometimes faintly fruity or citrusy. Basidiospores olive-brown in fresh deposit.

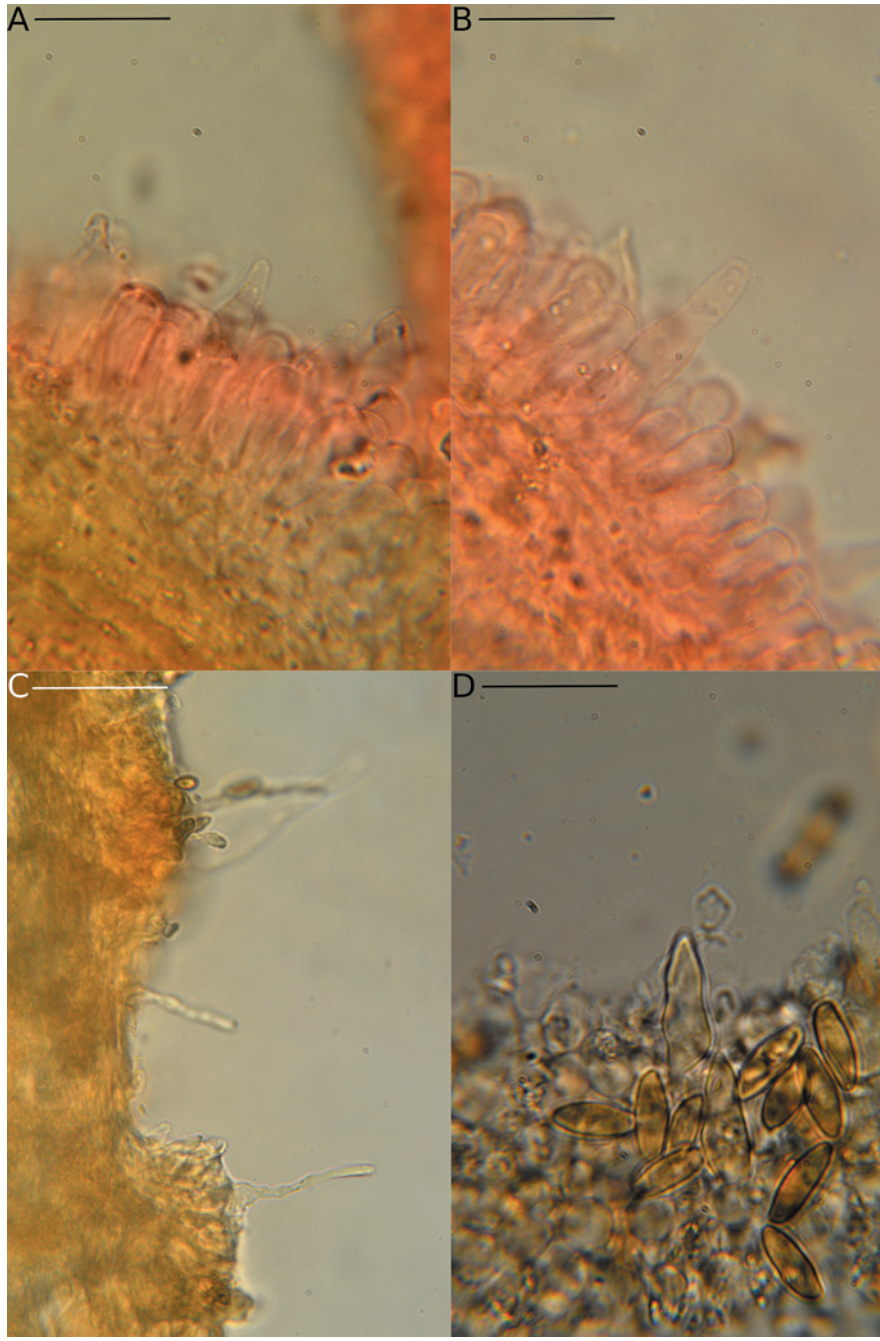


**Fig. 8.** Microscopic features of *Pulchroboletus rubricitrinus*. **A, B** – pleurocystidia; **C** – caulocystidia (Franck 3036); **D** – pleurocystidia (Franck 3473). Scale bars = 30 µm. Photographs by A. Farid.

Dried basidiome. Pileus smooth, glabrous, golden yellow-brown, with brownish red punctules, some becoming black, punctules never more than 0.5 mm diameter, pileus convex. Tubes free from stipe, not separable individually. Stipe golden yellow, sometimes upper portion of stipe reticulate, punctules maroon to black, red colours most prominent at base, scabriform punctules blackish in upper portion.

Microscopic features. Basidiospores (12)13.8–15.9(18) × (4)4.6–5.8(7) µm (48 spores counted, Q = 2.85), straw-yellow in KOH and water, ellipsoidal to subellipsoidal, sometimes subfusiform, smooth, thin-walled, with a pronounced apiculus and rounded apex, having one, two, or three olive-coloured oil droplets.

Basidia 20–30 × 10–16 µm, clavate to subclavate, thin-walled, hyaline, with yellow-green oil guttules in water and KOH, without basal clamps, predominantly four-spored, occasionally two-spored or three-spored; sterigmata 1–3 µm long; basidioles clavate to subclavate, size similar to basidia.

Cheilocystidia 20–32 × 6–8.5 µm, abundant, typically filiform to subclavate, ventricose, sometimes substrangulated, flexuous, cylindrical, apices subclavate to filiform, sometimes aciculate. Pleurocystidia shape and size similar to cheilocystidia, but more commonly ventricose to filiform.

Hymenophoral trama bilateral-divergent appearing subparallel in mature specimens, boletoid, lateral strata somewhat gelatinised, elements 7–15 µm wide, mediostrata gelatinised, loosely arranged, reddish brown, hyphae 7–15 µm wide.

Pileipellis an interwoven trichoderm, sometimes suprapellis collapsing into a cutis, elements filiform, sinuous, not constricting at septa, terminal elements (3)4–9(12) µm wide, some elements pigmented maroon-red, cylindrical, filiform, occasionally clavate, occasionally embedded or encrusted with yellow-green oil guttules, subterminal elements similar in size and shape to suprapellis.

Stipitipellis consisting of parallel to subparallel and longitudinally running, smooth-walled, septate hyphae, 4–6 µm wide, stipitipellis elements occasionally breaking up into pigmented (reddish brown in $H_2O$ and KOH) fascicles arranged in anticlinal bundles, these elements terminating into subclavate to clavate elements, 5–10 µm diameter, 20–30 µm long.

Caulocystidia similar to pleurocystidia, but occasionally filiform, sinuous to flexuous, 50–100 × 5–6 µm; substipitipellis longitudinally interwoven; stipe stratum composed of 4–6 µm diameter septate hyphae, hyaline in $H_2O$ and KOH, with occasional pigmented hyphae (reddish brown in $H_2O$ and KOH) traversing stipe, and occasionally interwoven with stipe stratum, these hyphae 12–15 µm diameter.

Hyphal system monomitic. Clamp connections absent.

Ecology and distribution. Solitary to gregarious, beneath *Quercus* spp., predominantly in disturbed habitats during summer months. Known from peninsular Florida to Texas, common (Fig. 9).

## DISCUSSION

### Phylogenetic position of the genus *Pulchroboletus*

*Boletus rubricitrinus* does not belong to the genus *Boletus*, according to our molecular analyses (Figs. 1–3). It appears that *B. rubricitrinus* is not a member of the subfamily *Boletoideae* (Nuhn et al. 2013, Wu et al. 2014) and is better placed in the genus *Pulchroboletus* Gelardi, Vizzini & Simonini. *Pulchroboletus* is in the *Hypoboletus* group in the subfamily *Xerocomoideae* of *Boletaceae* (Binder and Hibbett 2006, Šutara 2008, Nuhn et al. 2013, Wu et al. 2014).

*Xerocomoideae* contains boletoid and phylloporoid species; most often the pileipellis is a trichoderm. *Xerocomoideae* was erected as a subfamily by Singer (1945b: 279), originally based on the *Phylloporus* Quél. hymenophoral trama. Pegler & Young (1981) raised this subfamily to the family level (*Xerocomaceae*). Molecular evidence has brought this group back again to the subfamily level (Binder & Hibbett 2006, Nuhn et al. 2013, Wu et al. 2014).

*Alessioporus* Gelardi, Vizzini & Simonini and *Pulchroboletus* are two genera erected to accommodate two Mediterranean species formerly placed in *Xerocomus* Quél., *X. ichnusanus* Alessio, Galli & Littini and *X. roseoalbidus* Alessio & Littini, respectively (Gelardi et al. 2014). Recently, Frank et al. (2017) described a novel Eastern North American species in *Alessioporus*, based on ITS sequences. *Hemileccinum* Šutara is a related genus with five species currently known (Šutara 2008, Halling et al. 2015, Wu et al. 2016) and is similar to *Alessioporus* and *Pulchroboletus*, but differs in the presence of very fine scales on the stipe, violet reaction with ammonia on the pileus, and a presence of an iodine-like odour at the base.

### Delimitation of *Pulchroboletus* species

*Pulchroboletus* is characterised by a rosy-coloured pileus which is hemispherical and becoming flattened to uplifted at maturity, a yellow tubulose hymenophore which bruises blue, and a smooth to fibrillose yellow-orange stipe with basal maroon punctuations. Both species of *Pulchroboletus* can be found in warm climates, and while both are associated with *Quercus* spp., *Pulchroboletus roseoalbidus* also associates with *Castanea* and *Cistus*. *Pulchroboletus roseoalbidus* tends to grow in caespitose clusters, while *P. rubricitrinus* tends to grow gregariously.

Morphological similarities exist between *P. roseoalbidus* and *P. rubricitrinus*. Both have a pinkish red cuticular colour on the pileus, but *P. roseoalbidus* exhibits a much paler pink pileus. The pileus diameter in both species overlap, with *P. rubricitrinus* occasionally expanding to 15 cm diameter; both are hemispherical to convex, becoming applanate to somewhat uplifted at maturity. Both

pileus cuticles are subtomentose to glabrous, non-viscid, dry, and somewhat greasy with moisture. The tubes of both species are depressed, then become decurrent with a tooth. The spore print of both species is olive-brown. Basidiospores of both species exhibit similar shapes, and are one-, two-, or three-guttulate. Singer (1986) reported the KOH reaction as deep red on the pileus and brown elsewhere in *P. rubricitrinus*; our observations indicate a reddish brown on the pileus and a deep (maroon) red on the pore surface. Application of KOH to *P. roseoalbidus* results in a pinkish colour on the pileal context, orange on the stipe context, and reddish brown at the base of the stipe.

The main distinguishing morphological feature between these two species are the maroon floccules present on the stipe of *P. rubricitrinus*, which are present as mere punctules in *P. roseoalbidus*. Another distinguishing feature is the context colour; the pileus context of *P. roseoalbidus* is lilac-pinkish while the pileal and stipe contexts in *P. rubricitrinus* are whitish yellow, and maroon red at the base of the stipe. The granular pseudoannular zone on the stipe of *P. roseoalbidus* is not present in *P. rubricitrinus*. Cystidia in *P. rubricitrinus* are generally shorter in length than *P. roseoalbidus*.

The reaction of $NH_4OH$ differs between the two species. It is rusty brown on the hymenophore, orange on the stipe, and negative elsewhere (bleaching lilac-pink context colour away) on *P. roseoalbidus*; $NH_4OH$ on *P. rubricitrinus* reacts yellow to orange on the pileus, pores and context, and brown on the stipe. *Pulchroboletus roseoalbidus* exhibits olive colours with the application of $FeSO_4$ on all tissues; *P. rubricitrinus* exhibits a yellow colour on the stipe, negative elsewhere, and bleaching blue colour from stained hymenophore.

While *P. roseoalbidus* is found in the Mediterranean, data from mycoportal.org (Fig. 9, Appendix) show that *P. rubricitrinus* is distributed from Florida to Texas. We have not verified these identifications from mycoportal.org, although a photograph from Texas in Metzler & Metzler (2010: 209) is consistent with the diagnostic macromorphological features of *P. rubricitrinus*.

Most specimens on mycoportal.org were found beneath *Quercus virginiana*, *Q. laurifolia*, or *Quercus* spp. One collection was beneath *Pinus* as well as *Quercus* spp. (H. Luke, s.n., 11 June 2000). *Pulchroboletus rubricitrinus* is likely mycorrhizal with *Quercus virginiana* and *Q. laurifolia*. Our observations (records included in Appendix) indicate that *P. rubricitrinus* is typically found in lawns beneath or near *Quercus* spp., and not in treeless lawns. Gelardi et al. (2014) considered both *Alessioporus* and *Pulchroboletus* to be mycorrhizal.

**Potentially related species**

Five specimens collected by Rolf Singer in Miami-Dade Co. and originally identified as *Boletus rubricitrinus* were excluded from our analyses as these

likely represent collections of *B. fairchildianus* (Singer) Singer. *Boletus fairchildianus* was first described as *B. rubricitrinus* var. *fairchildianus* Singer (Singer 1945a) and later elevated to the species level (Singer 1977). Although *B. fairchildianus* is similar to *P. rubricitrinus*, we cannot be certain if *B. fairchildianus* is closely related to it, especially without DNA sequences. Singer (1945a) notes that it differs from *P. rubricitrinus* by its red pore mouths. However, photographs identified by Bessette et al. (2016: 104) as *B. fairchildianus* show a redder stipe which is less floccose and less reticulated, exhibits darker bruising, and a more variable colour of red in the pileus.

This study has also identified three unknown environmental bolete sequences from GenBank which may belong in *Pulchroboletus* (Tab. 1, Fig. 1). The sequence EU569326.1 was from a specimen found in a cloud forest in tropical Mexico (Morris et al. 2008). The sequence FM999554.1 was from an uncultured environmental sample from a beech-maple forest in Ohio, USA (Burke et al. 2009). The sequence FJ480444.1 was from a bright orange sclerotium collected in Massachusetts, found near the sclerotia of a *Boletus rubropunctus* Peck specimen (Smith & Pfister 2009); Smith and Pfister postulated that despite being present in ancestral bolete lineages, sclerotium growth was lost by many taxa in the *Boletales*, and has resurfaced as a convergent trait in the suborders *Boletineae* and *Suillineae*. This indicates the first-known sclerotium-forming species in the *Hypoboletus* group.

## CONCLUSION

This paper updates our understanding of the taxonomy of *Pulchroboletus rubricitrinus* in the light of DNA phylogenetics and provides the first sequences of this bolete. A thorough morphological description is now also available, and an epitype has been established. These molecular and morphological data will be useful to further improve our understanding of taxonomic groups during this period of rapid bolete reclassification.

## APPENDIX

Data download from Mycoportal.org. Specimens without GPS coordinates were georeferenced using Geocoder (version 1.22.4) with Google set as the geocoding service, and a custom Python script (2.7.10). If locality data could not be obtained, municipality level data were obtained, up to county level. One specimen only had state-wide level data entered (Texas, D.P. Lewis, 5060), and was excluded from the map. Two specimens (BPI 781720, NCU-F-0002363) were annotated as pieces of Murrill's type collection, and were excluded from the visualisation.

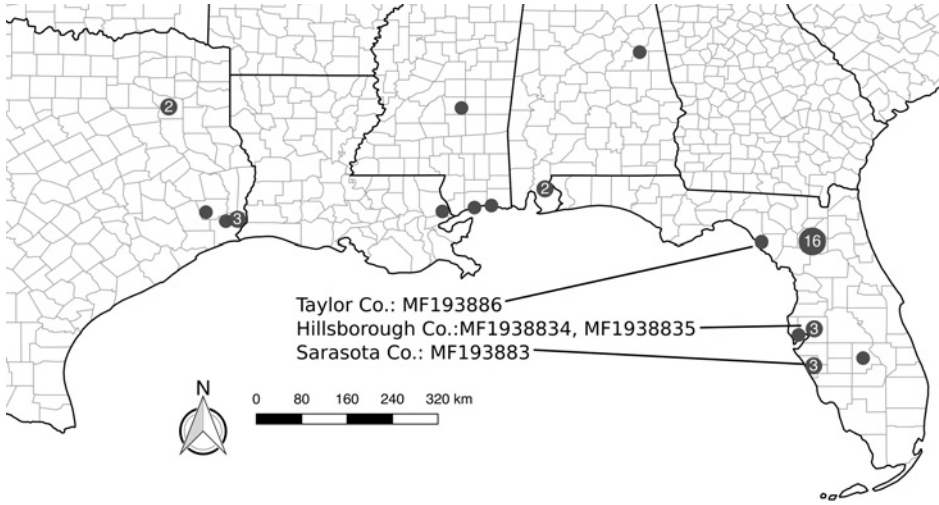


**Fig. 9.** Map generated from Mycoportal.org data download using QGIS (version 2.18.2). Counties with more than one collection are shown with numbers indicating the number of collections reported, and visualised as county centroids. Coordinate reference system: EPSG:54032.

United States. Alabama. Baldwin Co., vic. Spanish Fort, Meaher State Park, pine plantation, 22 July 2005, J.L. Mata 1681 (USAM 00121). – Baldwin Co., 21 July 1982, D.P. Lewis 3201 (F C0223076). – Cleburne Co., Cheaha State Park, Cheaha Lake Trail, 3 August 2005, J.L. Mata 1768 (USAM 00207). – Florida. Alachua Co., 27 June 1943, W.A. Murrill F 2380 (FH00489330). – Alachua Co., Gainesville, 26 May 1943, R. Singer 2130 (FH 00489180); ibid., 26 May 1943, R. Singer 2133 (FH 00489331); ibid., 26 May 1943, R. Singer 2135 (FH 00489181); ibid., 28 June 1943, R. Singer 2123a (FH 00489324); ibid., s.d., Murrill (FLAS 15864); ibid., September 1954, W.A. Murrill (BPI 781645); ibid., 936 NW 30th Avenue, 29 July 1982, G. Benny (FLAS 53093); ibid., 1202 NW 16th Avenue, lawn near oaks, 30 July 1982, J. Gibson (FLAS 53107); ibid., at 1401 NW 61st Terr., on the lawn beneath oaks and pines, 11 June 2000, H. Luke (FLAS 57598); ibid., at the entrance of Austin Cary Forest, off of Hwy 24, beneath live oak trees, 14 July 1998, J. Kimbrough (FLAS 56762); ibid., near Fifield Hall, on the lawn beneath *Quercus laurifolia*, 8 July 1998, S. Angels & A. Berry (FLAS 56758); ibid., near Fifield Hall, Hull Rd., beneath live oak tree, 24 July 1997, S. Chandler (FLAS 56570); ibid., Newnan's Lake, edge of pond near Lake, open grass, 8 October 1943, W.A. Murrill (FLAS 19503); ibid., off of NW 4th St. near intersection with NW 10th Avenue, under live oak on median, 29 July 1988, J. Benny (FLAS 55454); ibid., Sugarfoot Hammock, beneath laurel oaks [*Quercus laurifolia*] near open field, 23 July 1969, J. Kimbrough (FLAS 48650). – Highlands Co., 2 September 1942, R. Singer, F181a (FH 00489328). – Hillsborough Co., University of South Florida campus, just N of CCT building, lawn under *Quercus virginiana*, 16 August 2014, A.R. Franck 3473 (USF 275174, USF 275175, USF 275176, USF 275198); ibid., along S side of sidewalk, N of Alumni Drive and S of Richard Beard garage, lawn, beneath *Quercus virginiana*, 10 June 2016, Arian Farid 335 (USF 288420); ibid., along N side of sidewalk, S of Alumni Drive and S of Richard Beard garage, lawn, beneath *Quercus virginiana*, 29 Jun 2017, Arian Farid 575 (USF 293750). – Pinellas Co., St. Petersburg, NW corner of 36th Avenue NE and 1st Street NE, lawn under *Quercus virginiana*, 7 November 2015, A.R. Franck 3970 (USF 282763). – Sarasota Co., Lake Sarasota, 0.2 km S of Bee Ridge Road, 2.2 km E of I-75, under *Quercus laurifolia*, 22 June 2012, A.R. Franck 3036 (USF 273129); ibid., 27 August 2012, A.R. Franck 3114 (USF 273128); ibid., 22 August 2012, A.R. Franck 3112 (USF 273130). – Taylor Co., South side of FL 51, N bank of

Steinhatchee River, Steinhatchee, roadside under *Quercus* sp., 27 September 2014, A.R. Franck 3594 (USF 276072). – Louisiana. St. Tammany Par., Slidell, 8 September 1998, S. Horsch 1780 (F C0223079). – Mississippi. Jackson Co., Gulf Coast Research Lab, scattered to gregarious under *Quercus virginiana*, 25 July 1982, D. Guravich 1523 (MICH 61387). – Long Co., University of Southern Mississippi, Gulf Park Campus, 17 July 1993, W.G. Cibula 1639 (F C0223078). – Texas. Hardin Co., Big Thicket National Preserve, Lance Rosier Unit, 23 July 1983, D.P. Lewis 3544 (F C0223082). – Jefferson Co., Beaumont, Pietsch School, 26 June 1983, D.P. Lewis 3535 (F C0223086). – Orange Co., Vidor, Catholic Church grounds, 28 July 1992, D.P. Lewis 4760 (F C0223084); ibid., near residence, 26 September 1987, D.P. Lewis 4083 (F C0223081). – Tyler Co., Big Thicket National Preserve, Beech Creek Unit, 4 August 1982, D.P. Lewis 3249 (F C0223080). – Tyler Co., Forest Lake Experimental Forest, plot 44, 25 July 1992, D.P. Lewis 4742 (F C0223085).

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