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Revision of leccinoid fungi, with emphasis on North American taxa, based on molecular and morphological data

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ABSTRACT

The leccinoid fungi are boletes and related sequestrate mushrooms (Boletaceae, Basidiomycota) that have traditionally been placed in *Leccinum*, *Boletus*, *Leccinellum*, and a handful of other less familiar genera. These mushrooms generally feature scabers or scaber-like dots on the surface of the stipe, and they are often fairly tall and slender when compared with other boletes. They are ectomycorrhizal fungi and appear to be fairly strictly associated with specific trees or groups of related trees. In the present study, we investigate the phylogenetic relationships among the leccinoid fungi and other members of the family Boletaceae using portions of three loci from nuc 28S rDNA (28S), translation elongation factor 1-α (*TEF1*), and the RNA polymerase II second-largest subunit (*RPB2*). Two DNA data sets (combined 28S-*TEF1* and 28S-*TEF1*-*RPB2*), comprising sequences from nearly 270 voucher specimens, were evaluated using two different phylogenetic analyses (maximum likelihood and Bayesian inference). Five major clades were obtained, and leccinoid fungi appeared in four of them. Taxonomic proposals supported by our results, representing a broadly circumscribed *Leccinum* that includes several sequestrate genera, along with *Leccinellum*, are made.

INTRODUCTION

The genus *Leccinum* was first recognized by Gray (1821), who used the genus to represent what would correspond, in contemporary terms, to the Boletaceae. Included in *Leccinum* were 10 species. Since Gray listed *Leccinum aurantiacum* first, citing *Boletus aurantiacus* as described by Bulliard (1785) and by Persoon (1801), this species serves as the type species for the genus. The modern concept of *Leccinum* has its origins with Snell (1942), who proposed emending Gray's broad genus to include only "the Versipelles of Fries and Peck," arguing that although "Gray had no such conception of the use of the name *Leccinum*, for in this genus he placed species now found in several of the newer genera," it would be "proper arbitrarily to select the first two presented by Gray under that name," *L. aurantiacum* and *L. scabrum*, to represent the emended genus. Subsequent arrangements of *Leccinum* based on morphology and, to a limited extent, mycorrhizal association were advanced by Smith et al. (1966, 1967, 1968), Smith and Thiers (1971), Singer (1986), Šutara (1989), and Lannoy and Estades (1995).

In an early molecular study, Binder and Besl (2000) used nuc 28S rDNA (28S) sequences and chemotaxonomic analysis to revise *Leccinum*. Their results supported a core clade containing *Leccinum* sections *Leccinum* and *Scabra*, which they proposed as *Leccinum* proper. Sister to this clade was a clade consisting of section *Luteoscabra* and the *Boletus* section *Pseudoleccinum* as described by Smith and Thiers (1971). Sequences representing *L. chromapes*, *B. hortonii*, *B. impolitus*, *B. depilatus*, and *L. eximium* were found to be more distantly located among the bolete genera. Binder and Besl (2000) proposed that all leccinoid fungi not belonging in their more strictly defined *Leccinum*—including those in section *Luteoscabra* and section *Pseudoleccinum* of *Boletus*—should be maintained as species of *Boletus* "[u]ntil more helpful insights can be presented," with the exception of *B. impolitus*, *B. depilatus*, and *B. hortonii*, all of which were placed in *Xerocomus*. However, in a subsequent treatment (Bresinsky and Besl 2003), the genus *Leccinellum* Bresinsky & Manfr. Binder was erected to accommodate the former section *Luteoscabra*, including those species with yellow hymenophores and/or flesh, with *Leccinellum nigrescens* as the type species.

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⊕ Supplemental data for this article can be accessed on the publisher's Web site.

More recent molecular analyses in *Leccinum* were conducted by den Bakker in a series of papers using nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2 (ITS), 28S, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) sequences (den Bakker et al. 2004a, 2004b, 2007; den Bakker and Noordeloos 2005), in which mycorrhizal host specificity was supported as an essential element of *Leccinum* evolution and taxonomy. Den Bakker et al. (2004a) determined that "there seems to be no evolutionary based reason to differentiate between the sections *Leccinum* and *Scabra* as suggested by Smith and Thiers (1971) and Lannoy and Estades (1995)"; although section *Scabra* formed a mostly monophyletic group, it was nested within section *Leccinum*, which was therefore paraphyletic. Den Bakker and Noordeloos (2005) presented taxonomic revisions for *Leccinum* in Europe, recognizing 16 European species; three generic sections were recognized, corresponding to Singer's earlier classification: *Roseoscabra, Luteoscabra*, and *Leccinum*—with the latter section divided into subsections *Leccinum, Fumosa*, and *Scabra*. Additionally, den Bakker and Noordeloos provided support for placement of the sequestrate genus *Chamonixia* within *Leccinum*. Den Bakker's analyses indicated a transcontinental European and North American distribution for some species in section *Leccinum* (den Bakker et al. 2004b) and the presence of closely related sister species between the continents in subsection *Scabra* (den Bakker et al. 2007). The genus *Leccinellum* was not recognized by den Bakker and Noordeloos (2005), since it was paraphyletic both morphologically and phylogenetically.

Šutara (2008) erected the genus *Hemileccinum* to accommodate *Boletus impolitus* and *B. depilatus*, based on the molecular results of previous publications—primarily Binder and Besl (2000), den Bakker and Noordeloos (2005), and Binder and Hibbett (2006, misattributed by Šutara as 2007)—and on morphological characters. More recently, Halling et al. (2012a) established the genus *Sutorius* for *Boletus eximius*, and Halling et al. (2012b) erected *Harrya* for *Leccinum chromapes*. Support for placement of the sequestrate genera *Chamonixia, Octaviania, Turmalinea*, and *Rossbeevera* in or near *Leccinum* has come from multiple studies (e.g., den Bakker and Noordeloos 2005; Binder and Hibbett 2006; Orihara et al. 2010, 2012a, 2012b, 2016; Lebel et al. 2012a, 2012b), increasing the number of taxa in the phylogenetically defined leccinoid group. These phylogenetic studies, in addition to nuclear ribosomal data, also used protein-coding genes (*TEF1, RPB1, RPB2*) or mitochondrial loci (*ATP6*, mtSSU), increasing the understanding of the affinities and evolutionary relationships among leccinoid fungi

and other boletes. Nuhn et al. (2013) placed the Leccinum and Leccinellum clades and the sequestrate fungi mentioned above in the "Leccinoid group," but within this group *Spongiforma* and *Retiboletus* appeared as sisters to the clade that includes the "leccinoid" species.

Hosen et al. (2013) showed that *Spongiforma*, a south Asian genus with epigeous sponge-like basidiomata, was closely related to *Borofutus*, a monotypic genus also from south Asia with a broad-pored hymenophoral surface, and these in turn were close to *Leccinum, Leccinellum*, and *Retiboletus*; similar findings were presented by Orihara and Smith (2017). Wu et al. (2014, 2016) placed the leccinoid species within subfamily Leccinoideae; *Tylocinum* (a monotypic genus from China with a dark color scabrous-like stipe) and *Pseudoaustroboletus* (another Asian genus created to placed *Tylopilus valens*, a species with a distinctly reticulated stipe) were also included within this subfamily. In Vadthanarat et al. (2018), subfamily Leccinoideae also included *Rhodactina*, a sequestrate genus from Asia, which appears as a sister of the Borofutus/ Spongiforma clade. Wu et al. (2018) demonstrated the inclusion of a new monotypic genus from Singapore into the subfamily Leccinoideae, represented by *Spongispora temasekensis*; this species has similar macrocharacters to those of *Leccinum* and *Retiboletus* but with ornamented spores. Molecularly, it appears to be more closely related to *Leccinum* and the sequestrate species than to *Retiboletus*. More recently, Khmelnitsky et al. (2019) described the new genus *Ionosporus* to accommodate *Boletus longipes* from Singapore, along with a new species, *I. australis*, based on *RPB2* sequences; this genus belongs to the subfamily Leccinoideae. Khmelnitsky et al. (2019) also confirm the study of Hosen et al. (2013), which indicated that *B. longipes* was morphologically similar to *Borofutus*.

For the present paper, we studied multiple accessions of leccinoid fungi to determine the most appropriate generic limits of *Leccinum* and related genera. We present molecular results using 28S, *TEF1*, and *RPB2* loci supporting revised circumscription of *Leccinum* to include *Leccinellum* and sequestrate species.

MATERIALS AND METHODS

Fungal collections.—A total of 124 fresh or dried collections of leccinoid fungi were studied, including 82 collections made by the authors or contributed to the study by private collectors and 42 herbarium collections (SUPPLEMENTARY TABLE 1). Collection and documentation of fresh basidiomes by the authors followed the methods of Kuo and Methven (2014).

Colors were recorded and codified using Kornerup and Wanscher (1978) or HEX codes displayed on a 2013 iMac with Intel Iris Pro graphics, using GNU Image Manipulation Program (GIMP) 2.8.10. Microscopic features were studied using hand sections of fresh material and dried specimens rehydrated in water after immersion in 90% ethanol. Sections were mounted in 2% KOH and in Melzer's reagent and viewed using either a Nikon Alphaphot YS (Tokyo, Japan) or an Olympus BH-2 (Tokyo, Japan) microscope. Specimens collected by the authors or contributed to the study were deposited in the University of Michigan Herbarium (MICH) and the Center for Forest Mycology Research Herbarium (CFMR). Herbaria are cited according to Thiers (continuously updated).

GenBank sequences.—We selected 423 GenBank sequences to expand our analyses and provide context. Some GenBank sequences were included in our data set despite representing collections not reliably vouchered in herbaria because they represent data presented in important previous publications; however, we recommend that future workers discontinue use of these sequences (indicated in SUPPLEMENTARY TABLE 1 in the "Comments" column) because, without support from vouchers, these sequences represent results for experiments that cannot be repeated and tested, violating basic scientific principles. We hope that these sequences will be replaced by future workers with well-documented original sequences backed up by specimens in public herbaria.

DNA isolation, PCR, and sequencing.—DNA extraction and amplification were performed at the Center for Forest Mycology Research (CFMR). Sequencing was performed at the University of Wisconsin Biotechnology Center (UWBC) following Palmer et al. (2008). The 5′ end of the 28S region was amplified with primers LROR and LR5 (Vilgalys and Hester 1990); *TEF1* was amplified with primer pair EF1-983/EF1-1567R (Rehner and Buckley 2005) and *RPB2* with primers bRPB2-6F and bRPB2-7.1R (Matheny 2005). Thermocycler conditions for the 28S region were as follows: initial denaturation at 94 C (2 min), followed by 30 cycles of denaturation at 94 C (40 s), primer annealing at 53 C (40 s), and elongation at 72 C (130 s), and a final extension step of 72 C (5 min). Polymerase chain reaction (PCR) conditions for *TEF1* and *RPB2* differed from those for 28S in 47 cycles of denaturation for 1 min, annealing at 55 C for 1 min, elongation for 100 s, and the final extension

for 10 min. Sequences were edited with Sequencher 5.4.6 (Gene Codes, Ann Arbor, Michigan). Sequences generated in the present study were deposited in GenBank (accession numbers MK601706–MK601823; MK721060–MK721180; MK766269–MK766383), and the alignments were deposited in TreeBASE (S25051, S25052).

Phylogenetic analyses.—DNA sequences were aligned with Clustal W 2.1 through CIPRES Science Gateway 3.3 (Miller et al. 2010). The alignments of the 28S, *TEF1*, and *RPB2* data sets were manually adjusted with AliView 1.18 (Larsson 2014), and a nexus file for each data set was produced in MEGA X (Kumar et al. 2018). The loci were concatenated using SeaView 4 (Gouy et al. 2010), and the two-locus (28S-*TEF1*) and three-locus (28S-*TEF1*-*RPB2*) data sets were partitioned by gene, codon position, and noncoding region. The nexus files for *TEF1, RPB2*, and the concatenated data sets were appended with a MrBayes block that partitioned the data set by codon position (1st, 2nd, 3rd) versus noncoding region (spacers and introns). These nexus files were converted into phylip files and run through jModelTest 2 (Guindon and Gascuel 2003; Darriba et al. 2012) in CIPRES to estimate the best substitution model for each analysis. The best-fit model estimated for these analyses was GTR with GAMMA. Bayesian inference (BI) analyses were performed using MrBayes 3.2.2 (Ronquist et al. 2012) on XSEDE through CIPRES. The parameters used for these analyses were nst = 6, rates = gamma, for 3 000 000 generations in two runs and four chains with trees sampled every 100 generations. The burn-in period was set to 0.25. Maximum likelihood (ML) analyses were performed using RAxML-HPC2 on XSEDE 8.2.10 (Stamatakis 2014) through CIPRES under the GTR model with GAMMA distributed rate heterogeneity and 1000 rapid bootstrap replicates; other parameters were kept at their default settings. Phylogenetic trees for both ML and BI were visualized and edited in FigTree 1.4.4 (Rambaut 2016), and final trees were edited in Adobe Illustrator CC 2018 (San José, California). Strong support values of clades are >90% in ML and >0.95 posterior probabilities (PPs) in BI analyses, whereas moderate support values are >70% and >0.90, respectively. The bootstrap frequencies (>50%) and posterior probabilities (>0.80) are shown on branches.

RESULTS

A total of 118 28S, 121 *TEF1*, and 115 *RPB2* sequences were generated for the present study, whereas 166 28S,

146 *TEF1*, and 111 *RPB2* sequences were retrieved from GenBank. The voucher information for all these sequences and GenBank accession numbers are provided in SUPPLEMENTARY TABLE 1. The trees generated from ML and BI analyses were largely congruent, with exceptions as noted below. The BI tree topology is illustrated for the three-locus analyses (FIG. 1A–E), and the ML tree topology is illustrated for the two-locus analyses (SUPPLEMENTARY FIG. 1). The combined 28S-*TEF*1 data set included 287 ingroup sequences and 1427 characters, whereas the 28S-*TEF1-RPB2* data set included 268 ingroup sequences and 2126 characters. *Chalciporus piperatus* (HKAS 84882) was used as outgroup.

Five major clades were retrieved from the three-locus analyses (see FIG. 1A–E and SUPPLEMENTARY FIG. 1). Major clade A (FIG. 1A, SUPPLEMENTARY FIG. 1) was not well supported in the analyses of both data sets, but it contains five subclades that received moderate to strong support in the analyses of the three-locus data set. Members of major clade A belong to the *Pulveroboletus* group and subfamily Austroboletoideae as indicated by Wu et al. (2014, 2016). The first subclade includes *Caloboletus*, *Rubroboletus*, *Sutorius*, *Pulveroboletus*, and *Neoboletus*. The close relationship among these genera was well supported in the BI analysis and moderately supported in the ML analysis. The second subclade consisted of species of *Austroboletus*, *Veloporphyrellus*,
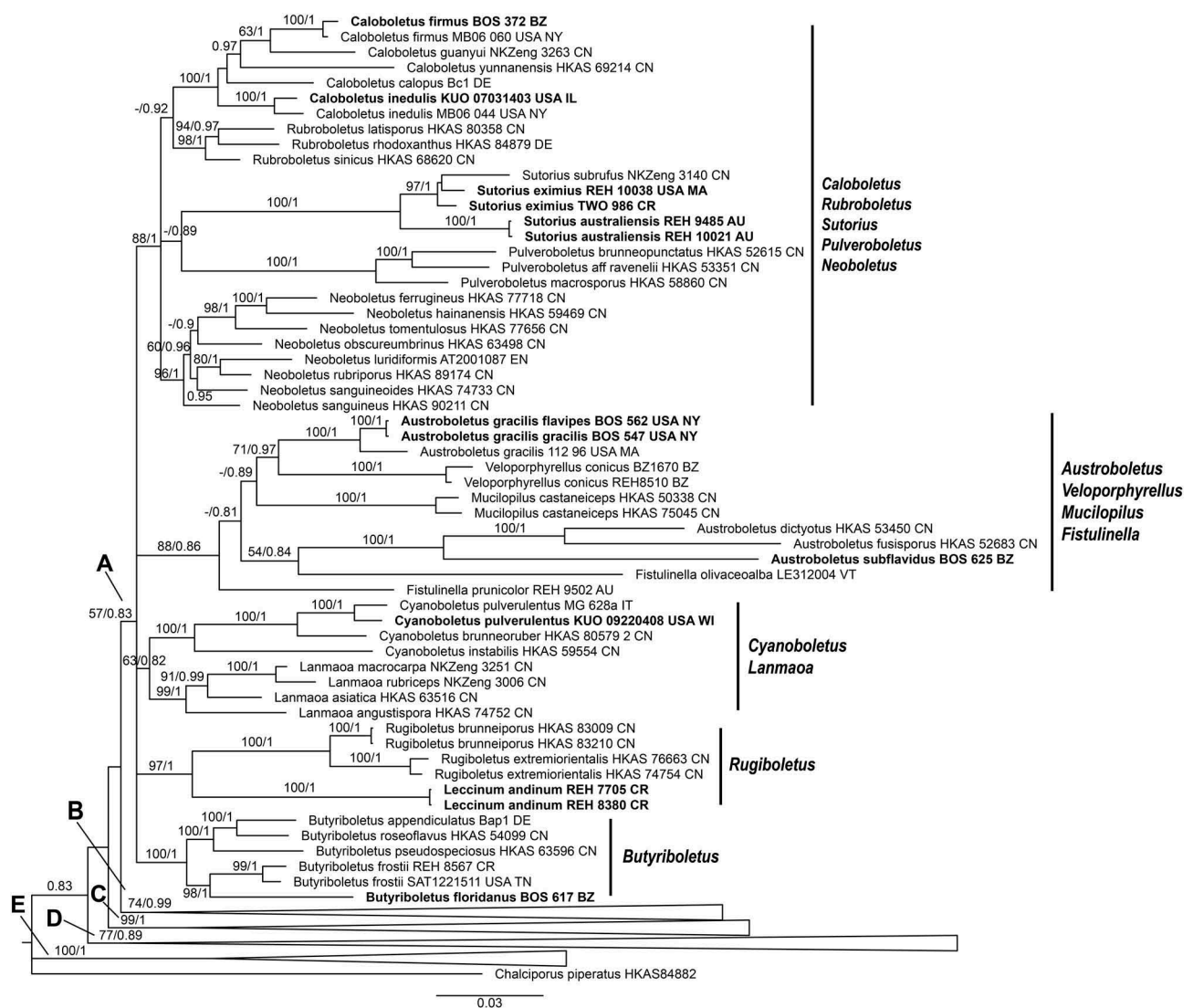
Figure 1. A–E. Phylogenetic relationships among leccinoid fungi and other members of the family Boletaceae inferred from the analyses of the combined data set (28S+*TEF1*+*RPB2*). The BI tree is shown. Support values along branches are from ML bootstrap (≥50%) and Bayesian (PP ≥ 0.80) analyses, respectively. A. Phylogenies of the Austroboletus group and the subfamily Austroboletoideae. B. Phylogeny of the subfamily Boletoideae. C. Phylogeny of the subfamily Xerocomoideae. D. Phylogeny of the subfamily Leccinoideae; the type species of *Leccinum*, *L. aurantiacum*, is highlighted in gray. E. Phylogenies of the subfamily Leccinoideae and the subfamily Zangioideae. Sequences generated in this study are in bold.
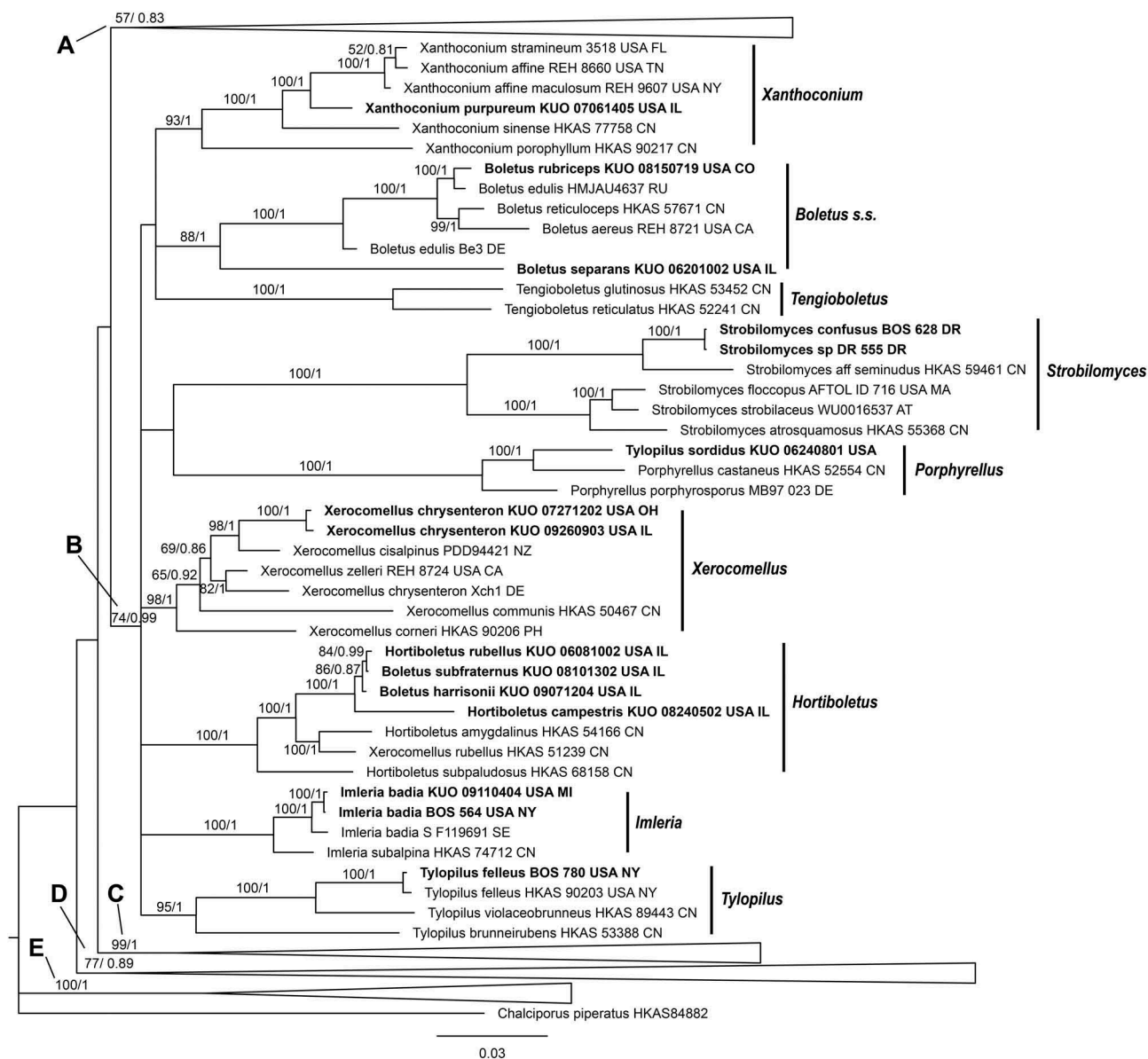
Figure 1. (Continued).

Mucilopilus, and *Fistulinella*. The grouping of these genera was moderately supported in the ML analyses of both data sets. The third subclade includes species of *Cyanoboletus* and *Lanmaoa*; this clade was not strongly supported in the present study. The fourth subclade included species of *Rugiboletus* and *Leccinum*. This subclade was well supported in both analyses of the three-locus data set; here *Leccinum* was recovered as polyphyletic as a result of *L. andinum*, which is sister to species of *Rugiboletus*, placed far from the core *Leccinum* species in major clade D. However, this conflict is resolved with the transfer of *L. andinum* to *Rugiboletus* (see Taxonomy). The fifth subclade includes species of *Butyriboletus*, a well-supported clade in both analyses and data sets.

Major clade B (FIG. 1B, SUPPLEMENTARY FIG. 1) received moderate to strong support in the ML and BI analyses of the three-locus data set, respectively. Members of this clade belong to subfamily Boletoideae as indicated by Wu et al. (2014, 2016). Species of *Xanthoconium*, *Boletus* sensu stricto, *Tengioboletus*, *Strobilomyces*, *Porphyrellus*, *Xerocomellus*, *Hortiboletus*, *Imleria*, and *Tylopilus* were recovered in well-supported clades. Monophyly was confirmed for *Xanthoconium*, *Boletus* sensu stricto, *Tengioboletus*, *Strobilomyces*, *Xerocomellus*, and *Imleria*. Results support placement of *Tylopilus sordidus* in *Porphyrellus*, as well as placement of *Boletus subfraternus* and *B. harrisonii* in *Hortiboletus*, but these taxonomic changes fall outside the focus of the present study.
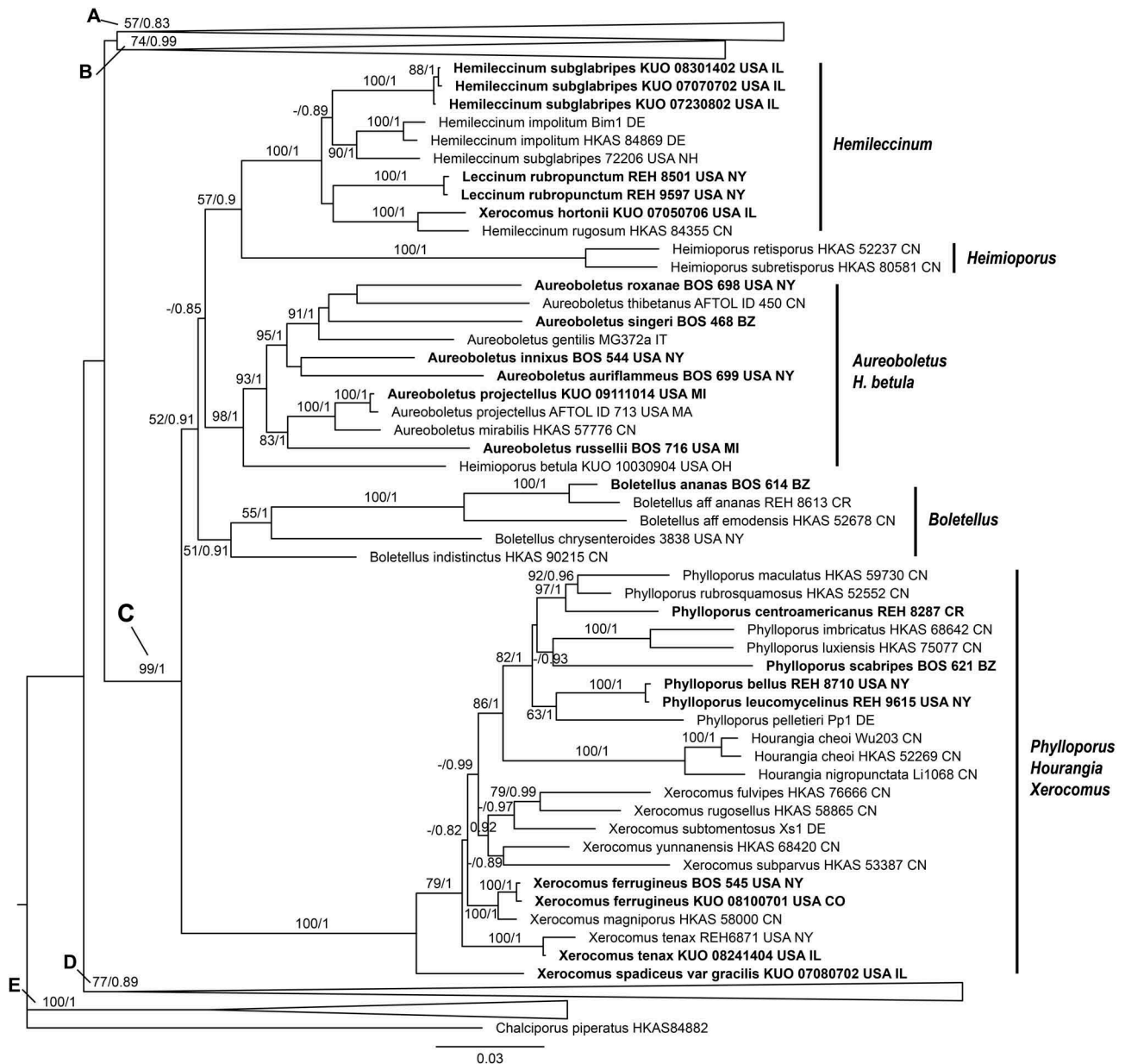
Figure 1. (Continued).

Major clade C (FIG. 1C, SUPPLEMENTARY FIG. 1) received strong support in both analyses of the three-locus data set and in the BI analysis of the two-locus data set. This clade corresponds to the *Hypoboletus* group in Nuhn et al. (2013) and the subfamily Xerocomoideae in Wu et al. (2014, 2016). Here this clade is divided in two subclades. The first subclade was moderately supported in the BI analysis of the three-locus data set and contains species of *Hemileccinum, Leccinum, Xerocomus, Heimioporus, Aureoboletus*, and *Boletellus*. The second subclade was strongly supported in both analyses and data sets, including species of *Phylloporus, Hourangia*, and *Xerocomus*. In the three-locus data set, monophyly was confirmed for *Boletellus, Phylloporus*, and

Hourangia. However, in the case of *Phylloporus*, the monophyletic group is contained within a larger clade that also contains *Hourangia* and *Xerocomus*, with the latter not monophyletic. *Heimioporus* was not monophyletic, with a Chinese collection of the type species, *Heimioporus retisporus*, grouping with a Chinese collection of *Hei. subretisporus*, far from our North American collection of *Hei. betula*, which was placed within *Aureoboletus*. The placement of *Hei. betula* within *Aureoboletus* was strongly supported in both analyses of the three-locus data set and in the BI analysis of the two-locus data set; placement of *Hei. betula* in *Aureoboletus* (see Taxonomy) makes *Heimioporus* monophyletic. *Hemileccinum* contained *Leccinum rubropunctum* and
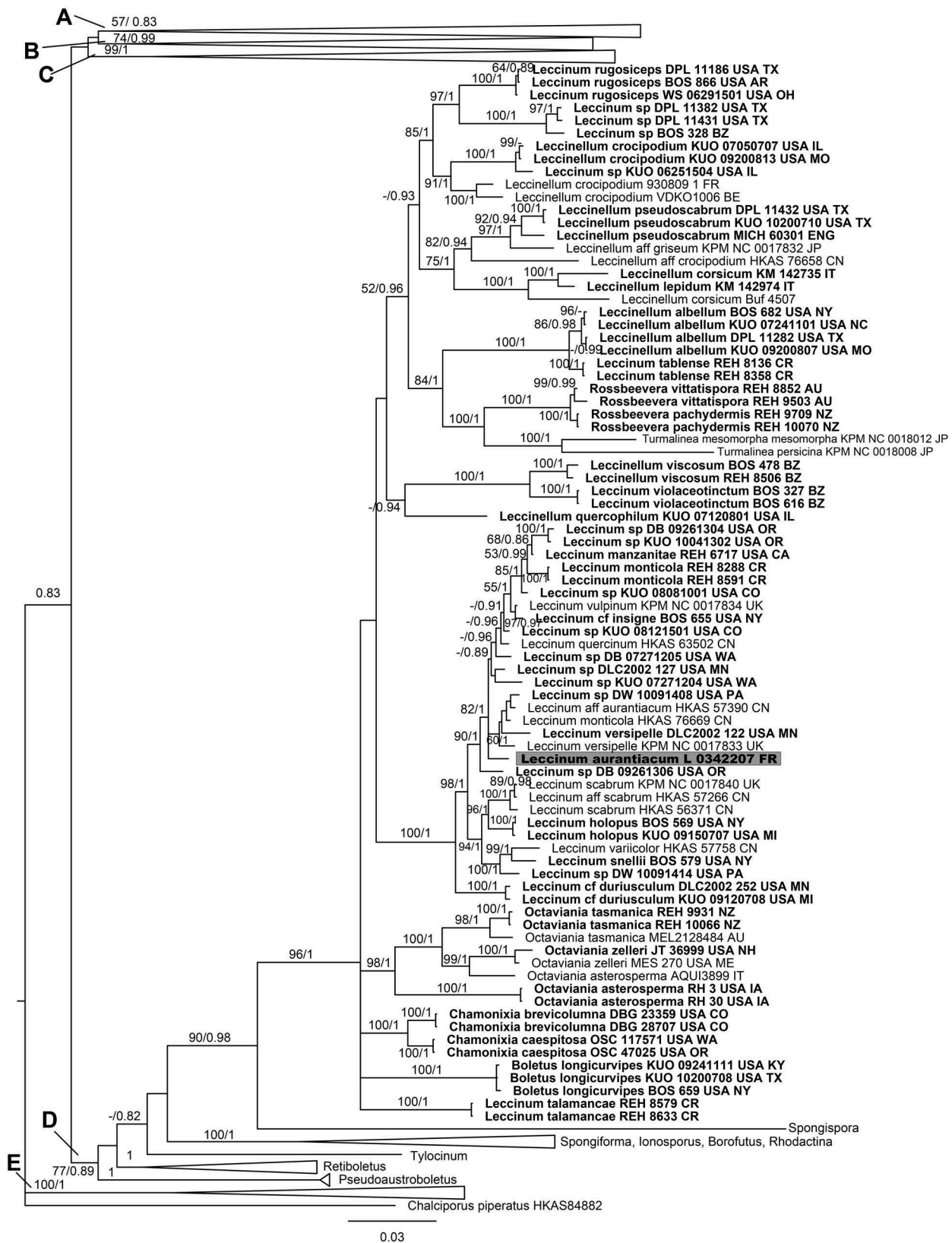
A 57/ 0.83

B 74/0.99

C 99/1

64/0.89 **Leccinum rugosiceps DPL 11186 USA TX**
100/1 **Leccinum rugosiceps BOS 866 USA AR**
97/1 **Leccinum rugosiceps WS 06291501 USA OH**
97/1 **Leccinum sp DPL 11382 USA TX**
100/1 **Leccinum sp DPL 11431 USA TX**
85/1 **Leccinum sp BOS 328 BZ**
99/- **Leccinellum crocipodium KUO 07050707 USA IL**
100/1 **Leccinellum crocipodium KUO 09200813 USA MO**
Leccinum sp KUO 06251504 USA IL
91/1 Leccinellum crocipodium 930809 1 FR
100/1 Leccinellum crocipodium VDKO1006 BE
-/0.93 100/1 **Leccinellum pseudoscabrum DPL 11432 USA TX**
92/0.94 **Leccinellum pseudoscabrum KUO 10200710 USA TX**
97/1 **Leccinellum pseudoscabrum MICH 60301 ENG**
82/0.94 Leccinellum aff griseum KPM NC 0017832 JP
75/1 Leccinellum aff crocipodium HKAS 76658 CN
100/1 **Leccinellum corsicum KM 142735 IT**
100/1 **Leccinellum lepidum KM 142974 IT**
Leccinellum corsicum Buf 4507
52/0.96 96/- **Leccinellum albellum BOS 682 USA NY**
86/0.98 **Leccinellum albellum KUO 07241101 USA NC**
Leccinellum albellum DPL 11282 USA TX
-/0.99 **Leccinellum albellum KUO 09200807 USA MO**
100/1 **Leccinum tablense REH 8136 CR**
84/1 **Leccinum tablense REH 8358 CR**
99/0.99 **Rossbeevera vittatispora REH 8852 AU**
100/1 **Rossbeevera vittatispora REH 9503 AU**
100/1 **Rossbeevera pachydermis REH 9709 NZ**
100/1 **Rossbeevera pachydermis REH 10070 NZ**
100/1 Turmalinea mesomorpha mesomorpha KPM NC 0018012 JP
Turmalinea persicina KPM NC 0018008 JP
100/1 **Leccinellum viscosum BOS 478 BZ**
-/0.94 **Leccinellum viscosum REH 8506 BZ**
100/1 **Leccinum violaceotinctum BOS 327 BZ**
Leccinum violaceotinctum BOS 616 BZ
Leccinellum quercophilum KUO 07120801 USA IL
100/1 **Leccinum sp DB 09261304 USA OR**
68/0.86 **Leccinum sp KUO 10041302 USA OR**
53/0.99 **Leccinum manzanitae REH 6717 USA CA**
85/1 **Leccinum monticola REH 8288 CR**
100/1 **Leccinum monticola REH 8591 CR**
55/1 **Leccinum sp KUO 08081001 USA CO**
-/0.91 Leccinum vulpinum KPM NC 0017834 UK
-/0.96 97/0.97 **Leccinum cf insigne BOS 655 USA NY**
Leccinum sp KUO 08121501 USA CO
-/0.96 Leccinum quercinum HKAS 63502 CN
-/0.89 **Leccinum sp DB 07271205 USA WA**
Leccinum sp DLC2002 127 USA MN
Leccinum sp KUO 07271204 USA WA
82/1 **Leccinum sp DW 10091408 USA PA**
Leccinum aff aurantiacum HKAS 57390 CN
90/1 Leccinum monticola HKAS 76669 CN
60/1 **Leccinum versipelle DLC2002 122 USA MN**
Leccinum versipelle KPM NC 0017833 UK
Leccinum aurantiacum L 0342207 FR
98/1 **Leccinum sp DB 09261306 USA OR**
89/0.98 Leccinum scabrum KPM NC 0017840 UK
100/1 Leccinum aff scabrum HKAS 57266 CN
96/1 Leccinum scabrum HKAS 56371 CN
100/1 **Leccinum holopus BOS 569 USA NY**
100/1 **Leccinum holopus KUO 09150707 USA MI**
94/1 99/1 Leccinum variicolor HKAS 57758 CN
Leccinum snellii BOS 579 USA NY
100/1 **Leccinum sp DW 10091414 USA PA**
100/1 **Leccinum cf duriusculum DLC2002 252 USA MN**
Leccinum cf duriusculum KUO 09120708 USA MI
100/1 **Octaviania tasmanica REH 9931 NZ**
98/1 **Octaviania tasmanica REH 10066 NZ**
100/1 Octaviania tasmanica MEL2128484 AU
98/1 100/1 **Octaviania zelleri JT 36999 USA NH**
99/1 Octaviania zelleri MES 270 USA ME
Octaviania asterosperma AQUI3899 IT
100/1 **Octaviania asterosperma RH 3 USA IA**
Octaviania asterosperma RH 30 USA IA
96/1 100/1 **Chamonixia brevicolumna DBG 23359 USA CO**
100/1 **Chamonixia brevicolumna DBG 28707 USA CO**
Chamonixia caespitosa OSC 117571 USA WA
100/1 **Chamonixia caespitosa OSC 47025 USA OR**
90/0.98 100/1 **Boletus longicurvipes KUO 09241111 USA KY**
Boletus longicurvipes KUO 10200708 USA TX
Boletus longicurvipes BOS 659 USA NY
100/1 **Leccinum talamancae REH 8579 CR**
Leccinum talamancae REH 8633 CR

0.83

L
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D -/0.82
1
100/1 Spongispora
Spongiforma, Ionosporus, Borofutus, Rhodactina
Tylocinum
Retiboletus
E 77/0.89
1
100/1 Pseudoaustroboletus
Chalciporus piperatus HKAS84882
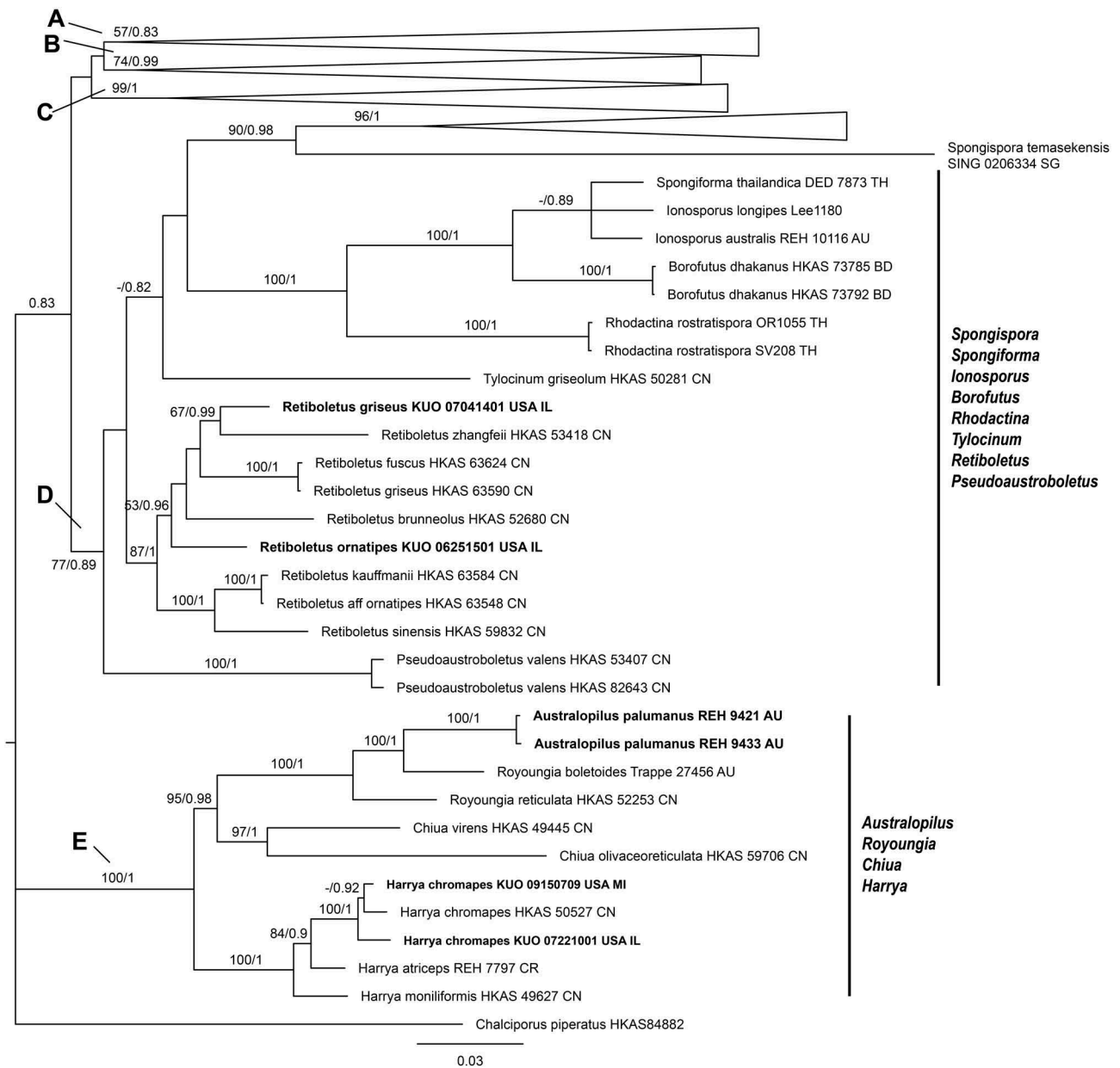
0.03

Figure 1. (Continued).

Figure 1. (Continued).

Xerocomus hortonii; monophyly is accomplished with the transfer of both species to *Hemileccinum* (see Taxonomy).

Major clade D (FIG. 1D–E), representing the primary focus of the present work, was moderately supported in both analyses of the three-locus data set but not supported in the analyses of the two-locus data set. Members of this major clade belong to subfamily Leccinoideae as indicated by Wu et al. (2014, 2016, 2018) and Vadthanarat et al. (2018). Based on the three-locus analyses (FIG. 1D), this major clade consists of six subclades. The first subclade contained species of *Leccinum, Leccinellum, Rossbeevera, Turmalinea, Octaviania, Chamonixia,* and *Boletus* sensu lato; this subclade was strongly supported in the analyses of both data sets. The second subclade is composed of *Spongispora temasekensis*, which appears as a sister lineage to the first subclade. The third subclade included species of *Spongiforma, Ionosporus, Borofutus,* and *Rhodactina*. The fourth subclade corresponded to the lineage of *Tylocinum griseolum*. The fifth subclade contained species of *Retiboletus*, and the sixth subclade corresponded to the lineage of *Pseudoaustroboletus valens*. Monophyly was confirmed for several genera here, including *Spongispora, Borofutus, Rhodactina, Tylocinum, Retiboletus,* and *Pseudoaustroboletus*. However, *Leccinum* and *Leccinellum* were recovered as polyphyletic, closely related to each other and to the sequestrate genera *Rossbeevera, Turmalinea, Octaviana,*

and *Chamonixia*, along with *Boletus longicurvipes* and *L. talamancae*. Since our results provide statistical support in the analyses of both data sets for a common ancestor for *Leccinum, Leccinellum, B. longicurvipes, L. talamancae*, and the sequestrate species included in this study, we propose, with reference to the guidelines for establishment of genera set forth by Vellinga et al. (2015), a broadly defined genus *Leccinum* (see discussion under *Leccinum* in Taxonomy).

Major clade E (FIG. 1E, SUPPLEMENTARY FIG. 1) received strong support in the analyses of both data sets and was recovered as basal to the remaining major clades in the three-locus phylogeny. It consisted of two subclades, the first one containing species of *Australopilus, Royoungia*, and *Chiua*, and the second with species of *Harrya*. Our results confirmed monophyly for *Australopilus, Chiua*, and *Harrya* but not for *Royoungia*. Members of this major clade have been placed in subfamily Zangioideae by Wu et al. (2014, 2016).

TAXONOMY

Aureoboletus betula (Schwein.) M. Kuo & B. Ortiz, comb. nov.
MycoBank MB832333
≡ *Boletus betula* Schwein., Schriften Naturforsch Gesellschaft Leipzig 1:94. 1822 (basionym).
≡ *Heimioporus betula* (Schwein.) E. Horak, Sydowia 56:239. 2004.
Specimen examined: USA. OHIO: Adams County, 3 Oct 2009, *KUO 10030904* (MICH).

Hemileccinum hortonii (A.H. Sm. & Thiers) M. Kuo & B. Ortiz, comb. nov.
MycoBank MB829998
≡ *Boletus subglabripes* var. *corrugis* Peck, Bull N Y St Mus 2:112. 1897.
≡ *Boletus hortonii* A.H. Sm. & Thiers, Boletes Mich:319. 1971 (basionym).
≡ *Leccinum hortonii* (A.H. Sm. & Thiers) Hongo & Nagas., Rep Tottori Mycol Inst 16:50. 1978.
≡ *Xerocomus hortonii* (A.H. Sm. & Thiers) Manf. Binder & Besl, Micologia 2000:85. 2000.
Specimen examined: USA. ILLINOIS: Coles County, 5 Jul 2007, *KUO 07050706* (MICH).
Comments: The basionym, *Boletus hortonii* A.H. Sm. & Thiers, was published as a *nomen novum* for *B. subglabripes* var. *corrugis* Peck.

Hemileccinum rubropunctum (Peck) Halling & B. Ortiz, comb. nov.
MycoBank MB829999

≡ *Boletus rubropunctus* Peck, Ann Rep N Y St Mus Nat Hist 50:109. 1897 (basionym).
≡ *Leccinum rubropunctum* (Peck) Singer, Am Mid Nat 37:117. 1947.
Specimens examined: USA. NEW YORK: Bronx County, 17 Sep 2003, *R.E. Halling 8501* (NY 00792788); Bronx County, 18 Aug 2011, *R.E. Halling 9597* (NY 1193924).

Leccinum Gray, Nat Arrangement Br Plants 1:646. 1821.
Type: Leccinum aurantiacum (Bull.) Gray, Nat Arrangement Br Plants 1:646. 1821.
= *Octaviania* Vittad., Monogr Tuberacearum:15. 1831.
= *Krombholzia* P. Karst., Revue Mycol 3:17. 1881.
= *Chamonixia* Rolland, Bull Soc Mycol Fr 15:76. 1899.
= *Trachypus* Bataille, Bull Soc d'Hist Nat Doubs 15:32. 1908.
= *Leccinellum* Bresinsky & Manfr. Binder, Regensb Mykol Schriften 11:231. 2003.
= *Rossbeevera* T. Lebel & Orihara, Fungal Diversity 52:73. 2011.
= *Turmalinea* Orihara & N. Maek., Persoonia 37:186. 2016.
Description (emended): Basidiomata either boletoid or sequestrate; ectomycorrhizal; solitary to gregarious; North America, Central America, Europe, Asia, Australasia. Boletoid basidiomata with pileus glabrous, tomentose, or fibrillose, convex becoming planoconvex, smooth or rugulose; pores round to angular, pore surface whitish to yellowish, brownish, or grayish, bruising brownish to bluish, or not bruising, often depressed at the stipe; stipe usually scabrous; context white or yellow, sometimes changing to gray, pink, or blue when sliced; basidiospores fusiform and smooth, inamyloid; hymenial cystidia present; pileipellis an ixocutis, cutis, or trichoderm; clamp connections absent. Sequestrate basidiomata hypogeous or emergent; small (mostly under 5 cm across); globose to subglobose, pyriform, ellipsoid, or reniform; sessile or with a pseudostipe; surface glabrous, tomentose, or scabrous, often bruising; context of peridium often bruising; gleba chambered, whitish young, maturing to dark brown, reddish brown, or black; columella absent or present; basidiospores globose to ellipsoid, ovoid, or fusoid, ornamented, inamyloid or dextrinoid; hymenial cystidia present or absent; clamp connections absent.
Comments: Leccinum is emended here to include the boletoid genus *Leccinellum* and the sequestrate genera *Octaviania, Chamonixia, Rossbeevera*, and *Turmalinea*. We considered three taxonomic alternatives to represent

our results: (i) the creation of new genera to reflect clades within major clade D (see FIG. 1D and SUPPLEMENTARY FIG. 1); (ii) no taxonomic maneuvers within major clade D; and (iii) broad circumscription of *Leccinum* to include *Leccinellum* and the sequestrate genera found in major clade D.

Alternative (i) would have required the segregation of five new genera from *Leccinum* and *Leccinellum* in order to accommodate a genus for the *Ll. pseudoscabrum, Ll. griseum, Ll. corsicum,* and *Ll. lepidum* clade; a genus for *Ll. albellum* and *L. tablense*; a genus for the *Ll. viscosum, L. violaceotinctum,* and *Ll. quercophilum* clade; a genus for *B. longicurvipes*; and a genus for *L. talamancae*. The principal advantages of this alternative would include the preservation of sequestrate genus names, clarification of *Leccinellum* (which has presented precise delineation challenges since its inception), and a narrowly focused *Leccinum*. Disadvantages, however, would include a proliferation of genera, a large number of name changes for established fungi, and, most importantly, lack of backbone support for individual genera within major clade D; although the clades are clearly recovered and well supported individually in our results, backbone support is low, making their precise positions unresolved and potentially subject to change with additional taxon sampling.

Alternative (ii), in which no taxonomic maneuvers are proposed, has the advantage of not necessitating any name changes among the fungi in major clade D but has the major disadvantage of not using the present results to support taxonomic decisions. Additionally, *Leccinum* and *Leccinellum* would remain nonmonophyletic.

Alternative (iii), broad circumscription of *Leccinum*, would require the transfer of species of *Chamonixia, Rossbeevera, Turmalinea, Octaviania,* and *Leccinellum* into *Leccinum*. The basics of this alternative were proposed by den Bakker and Noordeloos (2005). Advantages to this alternative include simplicity, a comparatively low number of name changes (which would occur primarily among the sequestrate fungi), good backbone support, clarification of *Leccinellum* and *Leccinum*, and, importantly, a taxonomic scheme in which the shared genus name enables nonspecialists to recognize the closely shared evolution of the taxa. Disadvantages include the potential for a large genus containing hundreds of species and the loss of generic names for sequestrate fungi.

Applying the guidelines for the establishment of new genera proposed by Vellinga et al. (2015), we note the authors' expressed concern that "[i]n several groups, the translation from a phylogenetic tree into a classification is taken into extremes, where every single clade is recognized as a separate genus. This does not increase insight in the evolutionary history of the group in question, [sic] only inflates the taxonomic framework." This concern, combined with the five guidelines proposed by the authors—especially guideline 3 ("[t]he branching of the phylogenetic trees should have sufficient and strong statistical support")—leads us to prefer alternative (iii), in which *Leccinum* is broadly circumscribed to include sequestrate species, as is currently done in *Agaricus* (Lebel and Syme 2012), *Amanita* (Justo et al. 2010), *Cortinarius* (Peintner et al. 2001), and *Russula* (Lebel and Tonkin 2007). Additionally, use of the genus name *Leccinum* for sequestrate leccinoid fungi provides instant recognition of the shared evolutionary history in a way that is accessible to nonspecialists and the general public; this evolutionary story is lost when obscured by a proliferation of genus names. We agree with Justo et al. (2010) that although "it is an unfortunate paradox of modern taxonomy that improvements in understanding of phylogeny can cause the loss of names that highlight unique clades with distinguishing morphological features," ultimately "the primary (if not the sole) organizing principle for biological classifications should be phylogeny."

Leccinum asterospermum (Vittad.) M. Kuo & B. Ortiz, comb. nov.
MycoBank MB832336
≡ *Octaviania asterosperma* Vittad., Monogr Tuberacearum:17, t. 3:7. 1831 (basionym).

Comments: Our results support placement of species of *Octaviania* within a broadly circumscribed *Leccinum*. Here we transfer the type species of *Octaviania, O. asterosperma,* which we have included in our analyses.

Leccinum caespitosum (Rolland) M. Kuo & B. Ortiz, comb. nov.
MycoBank MB832334
≡ *Chamonixia caespitosa* Rolland, Bull Soc Mycol Fr 15:76. 1899 (basionym).

Comments: Our results support placement of species of *Chamonixia* within a broadly circumscribed *Leccinum*. Here we transfer the type species of *Chamonixia, C. caespitosa,* which we have included in our analyses.

Leccinum longicurvipes (Snell & A.H. Sm.) M. Kuo & B. Ortiz, comb. nov.
MycoBank MB832345

≡ *Boletus longicurvipes* Snell & A.H. Sm., J Elisha Mitchell Sci Soc 56:325. 1940 (basionym).

Specimens examined: USA. KENTUCKY: Wolfe County, 24 Sep 2011, *KUO 09241111* (MICH); NEW YORK: Erie County, 29 Aug 2004, *BOS 659* (CFMR); TEXAS: San Jacinto County, 20 Oct 2007, *KUO 10200708* (MICH).

Leccinum pachydermum (Zeller & C.W. Dodge) M. Kuo & B. Ortiz, comb. nov.
MycoBank MB832337

≡ *Hymenogaster pachydermis* Zeller & C.W. Dodge, Ann Missouri Bot Gard 21:637. 1934 (basionym).

≡ *Rossbeevera pachydermis* (Zeller & C.W. Dodge) T. Lebel, Fungal Diversity 52:64, 73. 2012.

Comments: Our results support placement of species of *Rossbeevera* within a broadly circumscribed *Leccinum*. Here we transfer the type species of *Rossbeevera, R. pachydermis*, which we have included in our analyses.

Leccinum persicinum (Orihara) M. Kuo & B. Ortiz, comb. nov.
MycoBank MB832335

≡ *Turmalinea persicina* Orihara, Persoonia 37:188. 2016 (basionym).

Comments: Our results support placement of species of *Turmalinea* within a broadly circumscribed *Leccinum*. Here we transfer the type species of *Turmalinea, T. persicina*, which we have included in our analyses.

Leccinum quercophilum (M. Kuo) M. Kuo, comb. nov.
MycoBank MB832338

≡ *Leccinellum quercophilum* M. Kuo, Mycotaxon 124:327. 2013 (basionym).

Comments: Our results support placement of species of *Leccinellum* within a broadly circumscribed *Leccinum*. Most species of *Leccinellum* do not require new combinations, having been placed in *Leccinum* by previous workers. Here we transfer *Leccinellum quercophilum*, which we have included in our analyses but which has never been placed in *Leccinum*.

Rugiboletus andinus (Halling) Halling & B. Ortiz, comb. nov.
MycoBank MB830007

≡ *Leccinum andinum* Halling, Mycotaxon 34:106. 1989 (basionym).

Specimens examined: COSTA RICA: San José, Dota, 8 Jun 1997, *R.E. Halling 7705* (NY 181460); San José, Dota, 11 Jun 2003, *R.E. Halling 8380* (NY 00796145).

Comments: Our results strongly support placement of *L andinum* in *Rugiboletus*. Comparing morphological characters of *Rugiboletus* with those of *L. andinum*, we find several similarities, specifically with *R. brunneiporus*, including the rugulose to wrinkled pileus, the yellow hymenophore that stains blue after bruising, the scabrous stipes, and association with members of the Fagaceae. However, *L. andinum* has larger spores than other *Rugiboletus* species.

DISCUSSION

This study presents the phylogenetic relationship among "leccinoid" fungi and other members of the family Boletaceae using 28S, *TEF1*, and *RPB2* sequences. Overall, in terms of the segregation of leccinoid fungi and certain sequestrate species within this family, we obtained similar results to those presented by Nuhn et al. (2013), Wu et al. (2014, 2016, 2018), and Orihara and Smith (2017). Here, however, more than 40 species representing *Boletus, Leccinum, Leccinellum*, and several sequestrate fungi have been compared for the first time using these molecular markers, providing a better understanding of the circumscription of these genera and other members of the Boletaceae. Although the focus of our study was the delimitation of the genera *Leccinum* and *Leccinellum*, other members of the family Boletaceae were included because of the recent placement of species originally classified as "leccinoid" into other genera in this family. Here we tried to select species represented with the three markers under study, although there are cases in the two-locus data set where only 28S was available and a few cases where only *TEF1* was available, as well as some cases in the three-locus data set where *RPB2* or *TEF1* was unavailable.

The first subclade in major clade A (FIG. 1A, SUPPLEMENTARY FIG. 1), which includes *Caloboletus, Rubroboletus, Sutorius, Pulveroboletus*, and *Neoboletus*, was also presented by Wu et al. (2014, 2016) as part of the Pulveroboletus group. More recently, Chai et al. (2019), using 28S, ITS, *TEF1*, and *RPB2* data, studied species of *Neoboletus, Sutorius, Costatisporus*, and *Caloboletus*, demonstrating the close evolutionary relationship among them. The second subclade, containing *Austroboletus, Veloporphyrellus, Mucilopilus*, and *Fistulinella*, represents a well-supported clade that was also recovered by Wu et al. (2014, 2016) under the subfamily Austroboletoideae. The clade containing *Cyanoboletus* and *Lanmaoa* was not supported in the present study or in Wu et al. (2016); however, the close relationship between these genera was well

supported in the study of Chai et al. (2019) using 28S, ITS, and *TEF1* data. The fourth subclade, containing *Rugiboletus* and *L. andinum*, is well supported in the three-locus tree; therefore, we transferred *L. andinum* to *Rugiboletus*. The fifth subclade includes species of *Butyriboletus*; the monophyly of this genus was also supported by Chai et al. (2019).

The genera grouped within major clade B (FIG. 1B, SUPPLEMENTARY FIG. 1), representing members of subfamily Boletoideae, were also included in the studies of Wu et al. (2014, 2016). Here the relationships among these genera were supported in the three-locus analyses but not in the 28S-*TEF1* analyses, where the placement of *Porphyrellus* was not resolved.

In major clade C (FIG. 1C, SUPPLEMENTARY FIG. 1), we obtained strong support for placement of the studied species within subfamily Xerocomoideae, similar to the results of Wu et al. (2014, 2016). Although we only included representative species within this subfamily, our results support a close relationship among *Hemileccinum, Heimioporus, Aureoboletus*, and *Boletellus*. The relationship among these genera was also studied by Halling et al. (2015), but here we present new findings: *Leccinum rubropunctum* and *Xerocomus hortonii* belong to *Hemileccinum* and *Hei. betula* belongs to *Aureoboletus*. The placement of *Hei. betula* was not resolved previously, probably because only 28S data were used. The present study also confirms the placement of *A. singeri* within *Aureoboletus* as suggested by Takahashi et al. (2016). Our study supports the strong relationship among *Phylloporus, Hourangia*, and *Xerocomus* previously indicated by Zhu et al. (2015) and Wu et al. (2016), where *Phylloporus* and *Hourangia* are closer to each other than to *Xerocomus*. However, here *Xerocomus* was not recovered as monophyletic. Additionally, although we obtained strong support for the monophyly of *Phylloporus*, our results are somewhat different from those of Neves et al. (2012) and Wu et al. (2016), probably because of the use of different loci and species. However, here, *P. bellus* and *P. leucomycelinus* grouped together—a scenario that was considered by Neves et al. (2012) as a possible contamination, since most of their *P. leucomycelinus* sequences cluster with *P. caballeroi*. This issue clearly requires further evaluation.

Major clade D (FIG. 1D, SUPPLEMENTARY FIG. 1), based on the three-locus analyses, consists of *Leccinum* as emended here, which is related to the other five subclades with moderate support. The relationship between *Leccinum* and the genera in the other subclades within subfamily Leccinoideae has been reported in previous studies (Hosen et al. 2013; Wu et al. 2014, 2016, 2018; Orihara and Smith 2017; Vadthanarat et al. 2018; Khmelnitsky et al. 2019).

However, the relationship among these subclades has not always been well supported, and statistical support appears to vary depending on the outgroup and genera included. In Wu et al. (2014, 2016), using a more distant outgroup and fewer representatives of *Leccinum/Leccinellum*, support for the relationship between these groups was strong. The same is true in Wu et al. (2018); using *Boletus edulis* as the outgroup, the relationship among all the six subclades appeared well supported. In this last study, the authors also included the genus *Binderoboletus*, which also appears to be part of the subfamily Leccinoideae. In our study, we ran preliminary analyses including *Binderoboletus*, but its placement was never resolved; most of the time it appeared at a basal position, close to the outgroup but never close to subfamily Leccinoideae; therefore, we excluded it for the final analyses. Using *Chalciporus piperatus* as the outgroup in the three-locus tree, we obtained moderate support for the grouping of all six clades. With respect to *Leccinum* as emended, including species of *Leccinum, Leccinellum*, and the sequestrate genera, we obtained strong support in the analyses of both data sets. *Leccinum* was also well supported in the analyses we ran separately for each locus (not presented here). The inclusion of sequestrate species of *Rossbeevera, Turmalinea, Octaviania*, and *Chamonixia* within *Leccinum* has been demonstrated in several studies, using ITS, 28S, *TEF1, ATP6, RPB1*, and *RPB2* (Orihara et al. 2010, 2012a, 2012b, 2016; Lebel and Syme 2012; Wu et al. 2014, 2016, 2018; Orihara and Smith 2017; Vadthanarat et al. 2018), also supporting the transfer of these genera into *Leccinum*.

In our results, a close relationship was demonstrated among *L. rugosiceps* (from North America), several *Leccinum* species from North and Central America, and *L. crocipodium* (including multiple samples of the latter from Europe and North America, which appeared to represent different species). Additionally, our results indicate a close relationship among *L. pseudoscabrum* (samples from North America and Europe), *L. corsicum* (Europe), and *L. lepidum* (Europe). Sequences of *L. corsicum* were not monophyletic in our results, raising the possibility that one of the collections may be misidentified. The group containing *L. albellum* (North America), *L. tablense* (Costa Rica), *Rossbeevera* (Australia and New Zealand), and *Turmalinea* (Japan) was well supported. Collections of *L. viscosum* and *L. violaceotinctum* from Belize appeared to be related to *L. quercophilum* from Illinois. We found a close relationship between *L. manzanitae* (California) and *L. monticola* (Costa Rica); also, *L. insigne* from North America appears to be related to *L. vulpinum* from Europe. European and Asian samples of *L. scabrum* and North American *L. holopus* are

closely related to each other, as well as North American *L. snellii* and Chinese *L. variicolor*. It appears that several collections were misidentified from analysis primarily of 28S sequences: *L. manzanitae* TDB969 did not group with *L. manzanitae* REH6717, and *L. monticola* HKAS 76669 from China did not match other collections of *L. monticola* from Costa Rica. Additionally, collections *L.* cf. *duriusculum* from North America did not group with *L. duriusculum* from Europe; collection *L.* aff. *aurantiacum* HKAS 57390 appears closely related to *L. versipelle*; *B. rubropunctus* TH 6944 and TBD 1217 represent *B. longicurpives*; and *C. caespitosa* 92-83 appears to represent *C. brevicolumna*. In the *Hemileccinum* clade, *H. subglabripes* 72206 from New Hampshire does not cluster with the isolates from Illinois; it appears to represent *H. impolitum* instead.

Major clade E (FIG. 1E, SUPPLEMENTARY FIG. 1) includes the leccinoid species of *Harrya* and was well supported in the present study. The relationship between *Harrya* and the other genera in this group has been shown in previous studies. Wu et al. (2014, 2016) placed them in subfamily Zangioideae.

The present study represents a significant step in defining relationships among leccinoid fungi, and the taxonomy proposed here clarifies several previously troublesome areas. Broad circumscription of *Leccinum* will allow workers to proceed with infrageneric and species-level studies that include additional taxa and loci in further assessing the diversity and evolutionary relationships within the leccinoid fungi. Our further studies will also include the description of possible new species of *Leccinum* in North America.

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