Molecular Phylogeny and Biodiversity of the Boletes

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Abstract

We present a phylogenetic analysis of boletes from diverse habitats using both nuclear and mitochondrial ribosomal DNA loci. Our phylogenetic trees demonstrated that the genera *Suillus* and *Leccinum* were well supported. Polyphyly was suggested for other major genera (*Boletus*, *Tylopilus*, *Xerocomus*). We observed a general lack of phylogenetic resolution at the genus and higher level using these two gene regions. Neither of the competing taxonomies proposed by Singer or by Smith was completely supported. Phylogenetic diversity of the boletes was assessed by comparative analyses of branch lengths.

KEYWORDS: Biodiversity, Boletaceae, *Boletus*, *Gyroporus*, *Leccinum*, phylogeny, *Pulveroboletus*, *Suilllus*, *Tylopilus*, *Xerocomus*

Introduction

Boletes are common fleshy mushrooms with a poroid hymenium and ectomycorrhizal habitat. Many mycologists have contributed to current taxonomic concepts in the boletes. Of special interest is the work of Singer (1945), who described the boletes as the Boletineae, which was composed of families including both poroid and lamellate fungi. This expanded the diversity beyond the intuitive notion that boletes were fast-decaying poroid fruiting bodies. In contrast, Smith and Thiers (1971) recognized only a single family, the Boletaceae, comprising ten genera that were entirely poroid. Within the Boletineae *sensu* Singer, there was the family Boletinaceae *sensu* Singer, composed of 18 genera, only seven of which were in common with the genera of Boletinaceae *sensu* Smith. Both the diversity of families and the diversity of genera are quite different between the Singer and Smith classifications. For example, *Pulveroboletus sensu* Smith has only one species (*P. ravenelii*); but *Pulveroboletus sensu* Singer is rich in species and characters.

Because of these differences, there was a problem for this paper to use binomial nomenclature in a consistent manner. Species sequenced for this study are generally named by the genus *sensu* Singer, but in all cases the sense of the species is according to the authority cited in the species list (see Tables 1A and 1B). Species sequenced by others and downloaded from GENBANK are cited by the name given in GENBANK.

With respect to previous work on the molecular phylogeny of the boletes, both nuclear and mitochondrial ribosomal DNA sequences have been studied (Binder and Besl, 2000; Bruns and Szaro, 1992; Bruns et al., 1992; den Bakker et al., 2004; Kretzer et al., 1996; Kretzer and Bruns, 1997). This research has shown the monophyly of the genera *Leccinum* and *Suillus*. In the mitochondrial data set assembled by Bruns et al. (1998), almost all of the boletes are divided between two large clades. One is *Suillus* and related species, and the other is a mix of *Boletus, Leccinum, Tylopilus, Xerocomus,* etc.

One simple measure of the biodiversity of a taxon is simply the number of species. In order to assess the biodiversity of the boletes, however, species concepts in the boletes must be clarified. A promising alternative to a simple species count is to construct the molecular phylogeny of the study group and use various methods to evaluate the diversity represented by the resulting phylogenetic tree (Faith, 1992). This is especially useful if there is uncertainty about limitations of some species. For example, there have been questions about the synonomy of *Boletus ornatipes* and *Pulveroboletus retipes* (Atkinson, 1911; Both, 1993; Bessette et al., 2000). Singer regarded them as one species (Singer, 1947), but Smith and Thiers regarded them as two species (Smith and Thiers, 1971).

This paper attempts to compare diversity estimates for the boletes by species count and by phylogenetic analysis. We also review existing generic concepts in the context of higher level taxonomic groupings which have been previously proposed.

Materials and Methods

Collections and Reference Sequences

Specimens were collected by a number of researchers in many parts of the world although the majority of the collections came from North Carolina and Virginia. Collection numbers are given in Tables 1A and 1B. Table 1A lists the 46 species sequenced for the 25S nuclear RNA genes, and Table 1B lists the 27 species sequenced for the 12S mitochondrial RNA genes. There are 24 collections in common between the two tables. To augment our samples, 63 sequences were obtained from GENBANK. *Limacella glischra* VTGB505 and *Russula virescens* AB154746 were used for the mitochondrial and nuclear outgroup sequences respectively.

Molecular Techniques

Nucleotide sequences data were produced in the following steps. DNA was isolated from fresh or herbarium material using miniprep procedures with CTAB buffer (Lee and Taylor, 1990).

PCR amplification was accomplished in accordance with standard procedures (Vilgalys and Hester, 1990). Primers used for the amplification of nLSU-rDNA were 5.8SR and LR7. Sequencing primers were LR0R, LR3R, LR5, and LR16 (Hopple and Vilgalys, 1999). Primers for both amplification and sequencing of the mtSSU rDNA were MS1 and MS2 (White et al., 1990). Sequences were derived from fluorescent dye terminator chemistries on an automated ABI 377 sequencer.

Data Analysis

For nLSU-rDNA, the aligned data matrix consisted of 854 characters, 510 characters were constant, 223 variable characters were parsimony informative. For the mtSSU-rDNA, the aligned data matrix had 466 included characters, 278 were constant, and 121 were parsimony informative. Character congruence between the nLSU and mtSSU sequences was evaluated with the incongruence length difference test of Farris et al. (1994) by means of the partition-homogeneity test in PAUP. A third data matrix was assembled using additional sequences from GENBANK to represent taxonomic diversity across the boletes. For this matrix there were 360 constant characters and 331 were parsimony informative. Analyses were conducted using PAUP (Swofford, 1998). Maximum parsimony trees were found using heuristic searching and significance assessed using stepwise-addition bootstrap replicates.

Results

The molecular phylogenetic trees of nuclear and mitochondrial rDNA are shown in Fig. 1 and Fig. 2, respectively (see pages 20 and 21). Both phylogenies share one striking feature which is the large degree of separation between the *Suillus* clade and the rest of the boletes. In Fig. 1, the *Suillus* clade includes *Gomphidius* and *Truncocolumella* with perfect bootstrap support. Other clades with good support are the *Leccinum* clade at 98% and the grouping of

Table 1A. Taxa included in the nLSU-rDNA phylogeny (generated in this study).

Name	*Location*	*Collection*
Austroboletus betula (Schwein.) E. Horak	Orange Co. NC	DD9852
Austroboletus mucosus (Corner) Wolfe	Guyana*	TH6300
Boletellus ananas (M.A. Curtis) Murril	Guyana	TH6264
Boletus bicolor Peck	Watuaga Co. NC	TH6933
Boletus communis Bull.	Watuaga Co. NC	NCJ25
Boletus edulis Bull. : Fr.	Orange Co. NC	HN141
Boletus inedulis (Murril) Murril	Watuaga Co. NC	NCJ14
Boletus subvelutipes Peck	Giles Co. VA	RV98.102
Boletus viridiflavus Coker & Beers	Orange Co. NC	DD972
Gomphidius glutinosus (Schaeff. : Fr.) Fr.	Macon Co. NC	RY1290
Gyrodon merulioides (Schwein.) Singer	Watuaga Co. NC	NCJ12
Gyroporus castaneus (Bull. : Fr.) Quél.	Watuaga Co. NC	NCJ16
Gyroporus cyanescens (Bull. : Fr.) Quél.	Montgomery Co. VA	OKM9827
Leccinum albellum (Peck) Singer	Watuaga Co. NC	TH6968
Leccinum aurantiacum (Bull.) Gray	Giles Co. Va	HN1573
Leccinum rubropunctum (Peck) Singer	Watuaga Co. NC	TH6944
Leccinum rugosiceps (Peck) Singer	Watuaga Co. NC	TH6967
Leccinum scabrum (Bull. : Fr.) Gray	Watuaga Co. NC	NCJ26
Paxillus involutus (Batsch : Fr.) Fr.	Watuaga Co. NC	RV98.135
Phaeogyroporus sp. Singer	Australia**	OKM23801
Phlebopus beniensis (Singer & Digilio) Heinem. & Rammeloo	Puerto Rico	Omon 98.015
Phylloporus rhodoxanthus (Schwein.) Bres.	Durham Co. NC	SAR89
Pulveroboletus auriflammeus (Berk. & M.A. Curtis) Singer	Orange Co. NC	DD973
Pulveroboletus auriporus (Peck) Singer	Orange Co. NC	DD971
Pulveroboletus curtisii (Berk.) Singer	Watuaga Co. NC	TH6943
Pulveroboletus retipes (Berk. & M.A. Curtis) Singer	Giles Co. VA	RV98.127
Rubinoboletus ballouii (Peck) Heinem. & Rammeloo	Guyana	TH6385
Strobilomyces floccopus (Vahl : Fr.) P. Karst.	Orange Co. NC	HN0027
Suillus americanus (Peck) Snell	Giles Co. VA	RV98.116
Suillus grevillei (Klotzsch : Fr.) Singer	Japan***	HN3469
Suillus hirtellus (Peck) Snell	Durham Co. NC	NCJ04
Suillus luteus (L.) Roussel	Durham Co. NC	JM96/41
Suillus pictus (Peck) A.H. Sm. & Thiers	Giles Co. VA	RV98.115
Suillus punctipes (Peck) Singer	Watuaga Co. NC	NCJ17
Truncocolumella citrina Zeller	Payette Nat. For. ID	OKM25732
Tylopilus alboater (Schwein.) Murrill	Durham Co. NC	TH6941
Tylopilus badiceps (Peck) A.H. Sm. & Thiers	Watuaga Co. NC	NCJ20
Tylopilus chromapes (Frost) A.H. Sm. & Thiers	Giles Co. VA	RV98.107
Tylopilus rufonigricans T.W. Henkel	Guyana	TH6376
Tylopilus rhoadsiae (Murrill) Murrill	Giles Co. VA	RV98.261
Tylopilus tabacinus (Peck) Singer	Durham Co. NC	HN2295
Xanthoconium affine (Peck) Singer	Giles Co. VA	RV98.112
Xerocomus amazonicus Singer	Guyana	TH6304
Xerocomus illudens (Peck) Singer	Orange Co. NC	DD9854
Xerocomus sp. Singer	Giles Co. VA	RV98.123
Xerocomus spadiceus (Fr.) Quél.	Clallam Co. WA	OKM25919

* All Guyana collections from the Pakaraima mountains

** Wongamie nature preserve near Toodyay

*** Yamaanashi prefecture near Mt. Fuji

Table 1B. Taxa included in the mitSSU-rDNA phylogeny (generated in this study).

Name	*Location*	*Collection*
Austroboletus mucosus (Corner) Wolfe	Guyana*	TH6300
Boletellus ananas (M.A. Curtis) Murril	Guyana	TH6264
Boletus edulis Bull. : Fr.	Orange Co. NC	HN141
Boletus inedulis (Murril) Murril	Watuaga Co. NC	NCJ14
Boletus ornatipes Peck	Watuaga Co. NC	TYJ15
Boletus viridiflavus Coker & Beers	Orange Co. NC	DD972
Chalciporus piperatus (Bull. : Fr.) Bataille	Watuaga Co. NC	TYJ21
Gyroporus castaneus (Bull. : Fr.) Quél.	Watuaga Co. NC	NCJ16
Leccinum albellum (Peck) Singer	Watuaga Co. NC	TH6968
Leccinum rubropunctum (Peck) Singer	Watuaga Co. NC	TH6944
Pulveroboletus auriflammeus (Berk. & M.A. Curtis) Singer	Orange Co. NC	DD973
Pulveroboletus auriporus (Peck) Singer	Orange Co. NC	DD971
Pulveroboletus curtisii (Berk.) Singer	Watuaga Co. NC	TH6943
Pulveroboletus retipes (Berk. & M.A. Curtis) Singer	Giles Co. VA	RV98.127
Paxillus involutus (Batsch : Fr.) Fr.	Watuaga Co. NC	RV98.135
Rubinoboletus ballouii (Peck) Heinem. & Rammeloo	Guyana	TH6385
Strobilomyces floccopus (Vahl : Fr.) P. Karst.	Orange Co. NC	HN0027
Suillus americanus (Peck) Snell	Giles Co. VA	RV98.116
Suillus granulatus (L.) Roussel	Giles Co. VA	RV98.114
Suillus grevillei (Klotzsch : Fr.) Singer	Japan**	HN3469
Suillus hirtellus (Peck) Snell	Durham Co. NC	NCJ04
Tylopilus badiceps (Peck) A.H. Sm. & Thiers	Watuaga Co. NC	NCJ20
Tylopilus rufonigricans T.W. Henkel	Guyana	TH6376
Tylopilus tabacinus (Peck) Singer	Durham Co. NC	HN2295
Xanthoconium affine (Peck) Singer	Giles Co. VA	RV98.112
Xerocomus amazonicus Singer	Guyana	TH6304
Xerocomus sp.	Giles Co. VA	RV98.123

* All Guyana collections from the Pakaraima mountains
** Yamaanashi prefecture near Mt. Fuji

Table 2. Biodiversity Index Results

Grouping	*Number in Group*	*Index*
All	108	24
Boletoid clade (see note 1)	91	23
Boletus	25	26
Leccinum	13	21
Sulloid clade (see note 2)	13	16
Suillus	8	13
Tylopilus	13	22
Xerocomus	11	19
Basal part of tree (see note 3)	17	27

Note 1: Boletoid clade includes *Austroboletus*, *Boletus*, *Gyroporus*, *Leccinum*, *Melanogaster*, *Paxillus*, *Phaeogyroporus*, *Phlebopus*, *Phylloporus*, *Pisolithus*, *Pulveroboletus*, *Rubinoboletus*, *Scleroderma*, *Strobilomyces*, *Tylopilus*, *Xanthoconium*, and *Xerocomus.*

Note 2: Suilloid clade includes *Chroogomphus*, *Gomphidius*, *Rhizopogon*, *Suillus*, and *Truncoclumella.*

Note 3: Basal portion of the tree includes Sulloid clade, *Coniophora*, Hygrophoropsis, and *Tapinella.*

Gyrodon, Phaeogyroporus, and *Phlebopus* (GPP group) at 79%. Overall, the phylogenetic tree appears to have four major branches:

1) *Phylloporus*, *Pulveroboletus*, and *Xerocomus* (PPX group)
2) *Leccinum* and miscellaneous associates
3) *Boletus, Tylopilus* and miscellaneous
4) *Suillus* and the GPP group noted above

The parsimony analysis of a larger bolete data set (this study and GENBANK sequences) was performed to calculate bootstrap support data on major branches. This nLSU-rDNA phylogeny of boletes including 63 sequences from GENBANK resulted in 98 most parsimonious trees (length=2662). These trees have a consistency index of 0.3509, a homoplasy index of 0.6491, and a retention index of 0.6026. As it was with the smaller data set, this analysis shows large groups dominated by particular genera. For example, there is again a PPX group as noted above. However, in this case there is another cluster of *Xerocomus* outside of the PPX group. Also, as before there are well-supported branches for each of *Leccinum* and *Suillus.*

Unlike the smaller data set, there is not a single branch for most of *Boletus* and *Tylopilus* but rather a series of small groups that are closely related. There appear to be at least three small groups dominated by *Boletus* and two by *Tylopilus.* In addition to the small GPP group that was seen in Fig. 1, there are two other small groups: 1) *Gyroporus* and *Scleroderma*; 2) *Chroogomphus* and *Gomphidius*.

With the scattered appearance of genera such as *Boletus* and *Tylopilus,* the question is whether there is enough evidence to suggest that these groups will never be single, compact groups. In order to check the monophyly of the major genera within the boletes, the large bolete data set (109 taxa) was analyzed using constraints which forced a particular genus to be single compact group. Using distance by the "uncorrected p" method as the optimizing criterion, phylogenies were generated with and

(*Continued on page 22*)

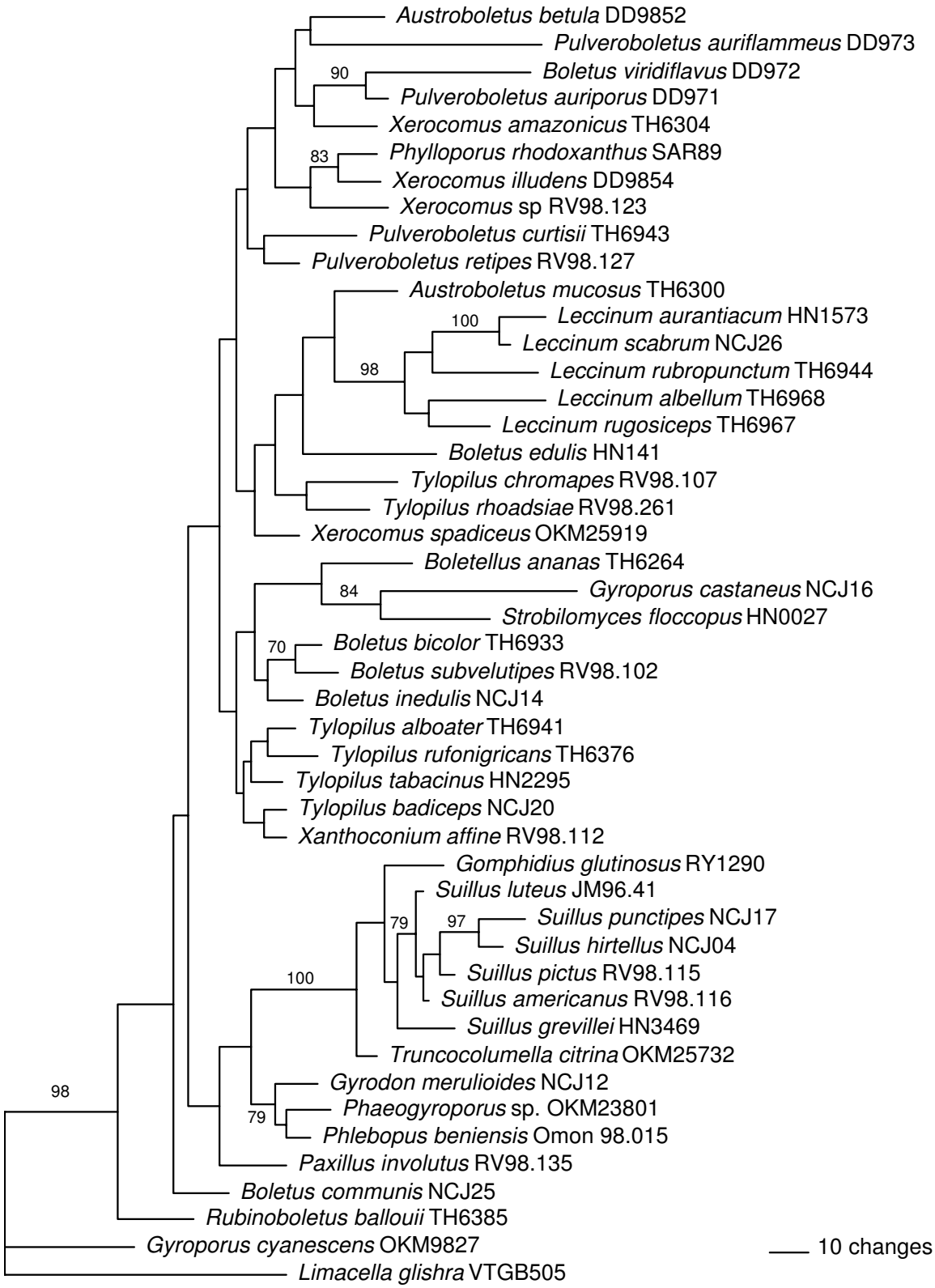

Figure 1. nLSU-rDNA phylogeny of boletes. One of 8 most parsimonious trees (length = 1,314). Shown is the most likely tree determined using a model with 2 substitution types and a trasition to transversion ratio of 2. The tree had a consistency index of 0.4271, a homoplasy index of 0.5729, and a retention index of 0.5132. Above the nodes are bootstrap values generated using 1000 replicates (only values greater or equal to 70% are shown).

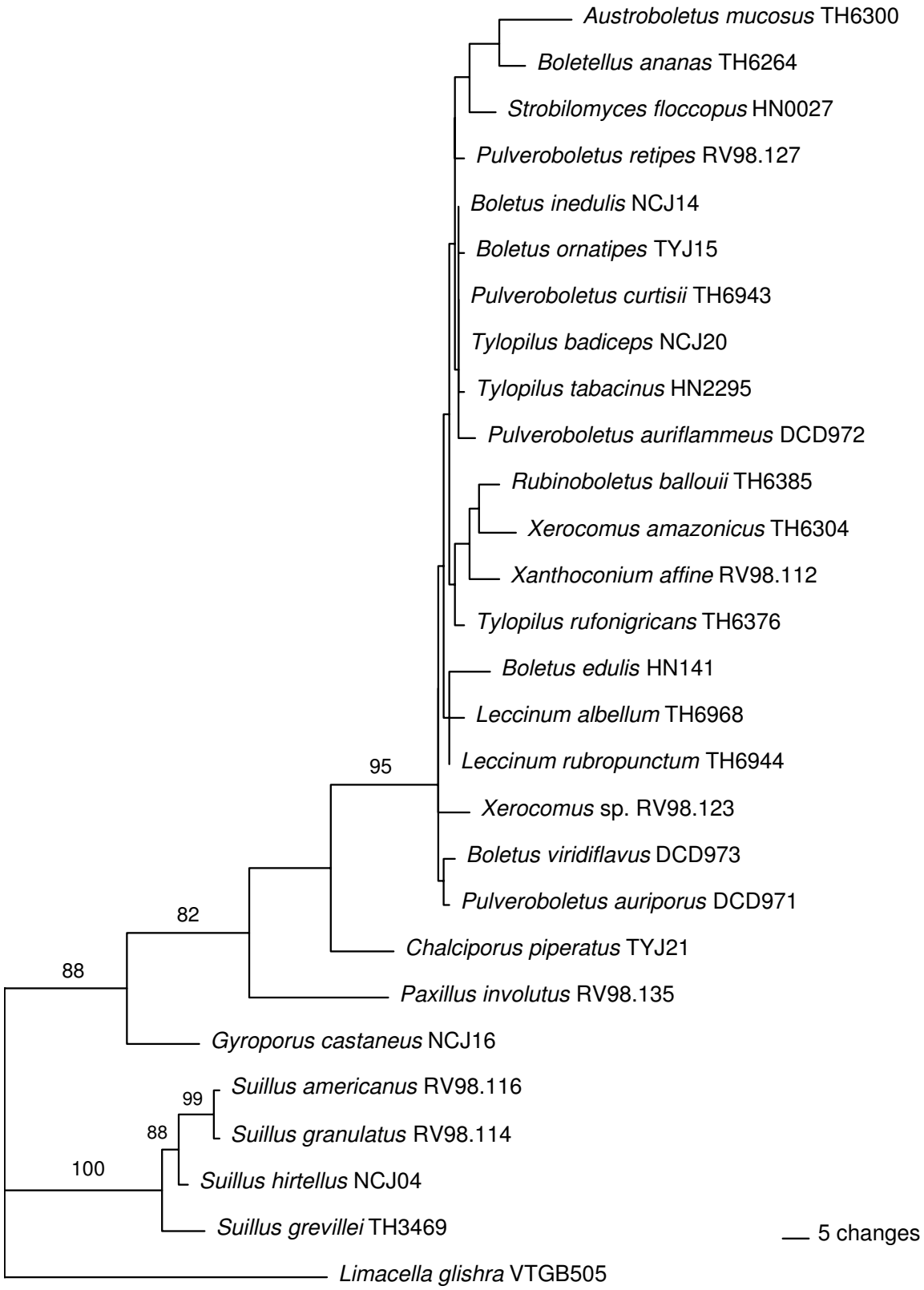

Figure 2. mtSSU-rDNA phylogeny of boletes. There were 54 most parsimonious trees (length = 345). Shown is the most likely tree with a consistency index of 0.7014, a homoplasy index of 0.2986, and a retention index of 0.7785.

without constraints. The resultant tree scores were compared by the Templeton test and the Kishino-Hasegawa test. Those genera which showed a significant effect of the constraint were *Boletus, Pulveroboletus, Tylopilus,* and *Xerocomus.* This suggests that these genera are not monophyletic groups.

Results from the biodiversity analysis are shown in Table 2. These results are based on the larger bolete data set. Distance derived branch lengths (by the "uncorrected p" method) were totaled for major clades, major genera, and a few minor genera. The biodiversity index was evaluated as the total branch length for a chosen group divided by the total number of taxa for that group. Table 2 show that there is a significant difference in the biodiversity index ranging from a low of 13 to a high of 27. In other words, the biodiversity estimates given by biodiversity estimates from genetic analysis could differ from simply counting species by as much as a factor of two.

Discussion

The analyses reported here provide new insights into the relationships and diversity of bolete taxa. These results are consistent with previous estimates of the phylogeny of the Boletales, suggesting a deep divergence between suilloid fungi and *Boletus* and allies and a distinct *Leccinum* clade. Though lacking resolution, the mitochondrial rDNA data also gave a tree with distinct clades for *Suillus* and for a mix of other major genera. In order to compare the topologies of the nLSU-rDNA and mtSSU-rDNA genes, the data for species common to both data sets were combined and subject to the partition homogeneity test. The p value was 0.01 indicating that the data supported significantly different topologies. This means that the different genes give significantly different trees and the phylogenies are not congruent.

The tree resulting from the combined bolete data set with bootstrap support analysis provides information regarding questions about groupings of species. For a particular genus, the species of that genus may be found in only one group, in two groups, in many groups, or scattered throughout the phylogenetic tree. For the following genera, their representative species are found in only one group which is well supported by bootstrap analysis: *Chroogomphus, Coniophora, Gyroporous, Leccinum, Phlebopus, Phylloporus,* and *Suillus.* However, species of *Xerocomus* are in two distinct groups and groupings of *Boletus, Pulveroboletus,* and *Tylopilus* are scattered throughout the tree. As noted in the Results section, constraint analysis argues against monophyly of *Boletus, Pulveroboletus, Tylopilus,* and *Xerocomus*.

A tree branch dominated by *Leccinum* is interesting because it contains *Boletus longicurvipes* and *B. subglabripes,* which were part of a group labeled by Smith as pseudoleccinum. A detailed analysis of *Leccinum sensu stricto* and related species is given by Binder and Besl (2000). They limit *Leccinum* to sections Leccinum and Scabra, which are characterized by whitish flesh. Others with yellowish flesh may be excluded from genus *Leccinum.* Nevertheless, the pseudoleccinums are very closely related to genus *Leccinum* by the 98% bootstrap support for the clade containing the pseudoleccinums, the *Leccinum sensu stricto,* and the other leccinums.

Many of the genera that Singer included in the Boletaceae were not included in this analysis. However, our results did support the inclusion of *Phlebopus* and *Phaeogyroporus* in this group. In addition, the data suggest that the lamellate forms (*Paxillus* and *Phylloporus*) are derived from poroid boletes. Our phylogenies supported the inclusion of *Boletellus* and *Strobilomyces* within the Boletaceae as posited by Smith and Thiers. Neither the Singer nor the Smith Boletaceae is monophyletic; both are rather unwieldy and would benefit by further taxonomic divisions.

With respect to bolete biodiversity, most of the larger genera have diversity in line with the total number of taxa in that genus. However, the genus *Suillus* showed only two thirds of the diversity of most other groupings. Until our sampling can include additional taxa from western North America and Europe, our results on biodiversity must be considered preliminary.

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