28S rDNA Sequence Data and Chemotaxonomical Analyses on the Generic Concept of *Leccinum* (*Boletales*)

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Dedicated to Prof. Dr. A. Bresinsky on the occasion of his 65th birthday.

Abstract: Following SMITH & THIERS (1968), the most essential characters of the genus *Leccinum* are the whitish context and the darkening in stipe scabrosities. SINGER (1986), however, accepts the genus in a wider sense, including several boletes with a yellow flesh in basidiocarps and a poorly developed, not darkening stipe ornamentation. In this study, a more distinct taxonomic definition for *Leccinum* should be obtained by chemical and molecular investigations. Based on chemical analyses, the presence of caffeic and gallic acid with simultaneous absence of pulvinic acids is proved to be a possible marker for *Leccinum*. Sequence data of the nuc-rLSU (900 b starting from the 5' region of the 28S gene) were generated for 21 *Leccinum* species and for 13 species of the genera *Boletus*, *Tylopilus* and *Xerocomus*. Supported by high bootstrap values, phylogenetic analysis results in a distribution of species with a yellow hymenophore or context and a not darkening stipe ornamentation to several clades. A clear segregation becomes evident between *Leccinum* species with a whitish flesh in basidiocarps (sections *Leccinum* and *Scabra*) and species with a yellowish flesh or hymenophore (sections *Luteoscabra*, *Roseoscabra*, *Eximia* and *Boletus* section *Pseudoleccinum*). Confirmed by the characters of pileus cutis and stipe ornamentation, the remaining critical *Leccinum* species exhibit a close relationship to *Xerocomus*. Based on the results, two new combinations are proposed: *Xerocomus depilatus* and *Xerocomus hortonii*. *Leccinum* is shown to be polyphyletic and has to be restricted to the sections *Leccinum* and *Scabra*. Until additional data are available, species excluded from *Leccinum* should be kept in the genus *Boletus*.

Riassunto: Secondo SMITH & THIERS (1968), i caratteri più essenziali del genere *Leccinum* sono la carne bianca e l'inscurimento delle scabrosità del gambo. Tuttavia, SINGER (1986) accetta il genere in un senso più ampio, includendovi parecchi boleti con carne gialla e ornamentazione del gambo scarsamente sviluppata oppure non inscurente. In questo studio, attraverso indagini chimiche e molecolari, si dovrebbe ottenere una definizione tassonomica di *Leccinum* più chiara. Sulla base di analisi chimiche, la presenza degli acidi caffeico e gallico, con la simultanea assenza di acidi pulvinici, si dimostra essere un possibile marcatore di *Leccinum*. Dati di sequenza rLSU (900 b partendo dalla 5ª regione del gene 28S) sono stati generati per 21 specie di *Leccinum* e 13 specie dei generi *Boletus*, *Tylopilus* e *Xerocomus*. Sostenuta da alti alti valori di bootstrap, l'analisi filogenetica risulta in una distribuzione di specie con imenoforo giallo o carne gialla e ornamentazione del gambo non inscurente in parecchi cladi. Tra le specie di *Leccinum* con carne bianca (sezioni *Leccinum* e *Scabra*) e quelle con carne gialla o imenoforo giallo (sezioni *Luteoscabra*, *Roseoscabra*, *Eximia* e *Boletus* sezione *Pseudoleccinum)* risulta evidente una netta segregazione. Le rimanenti specie critiche di *Leccinum* mostrano una stretta relazione con *Xerocomus* e ciò è confermato dai caratteri della cuticola e dell'ornamentazione del gambo. Sulla base di questi risultati vengono proposte due nuove combinazioni: *Xerocomus depilatus* e *Xerocomus hortonii*. *Leccinum* si dimostra polifiletico e deve essere ristretto alle sezioni *Leccinum* e *Scabra*. Fino a quando non saranno disponibili altri dati, le specie escluse di *Leccinum* dovrebbero essere mantenute nel primitivo genere *Boletus*.

Key Words: *Boletales*, *Leccinum*, *Boletus*, *Tylopilus*, *Xerocomus*, chemotaxonomy, 28S rDNA.

Introduction

The genus *Leccinum* S.F. Gray comprises exclusively obligate ectomycorrhizal fungi, mainly distributed throughout the northern temperate zone (ENGEL 1978). Mycorrhizal partners are deciduous as well as coniferous trees, indicating a strong substrate specificity of some species. For example, the type species *Leccinum aurantiacum* (Bull. ex St.-Amans) S.F. Gray is associated with *Populus tremula* L.

Macroscopically, *Leccinum* can be easily identified by numerous furfuraceous scabrosities or squarrose squamules, covering the stipe, never forming reticulate structures. This distinguishes *Leccinum* from all the remaining genera of *Boletaceae* Chev. The scabrosities consist of fasciculate groups of hyphae, with tips composed by caulohymenial elements, which are typically dark coloured or darkening in late maturity (SMITH & THIERS, 1968).

Despite of this unique attributes, the generic limits between *Leccinum* and *Boletus* Dill. : Fr. are still under discussion. ŠUTARA (1989) regards the stipe ornamentation ("Stiellateralstratum") as suitable for a delimitation between the two genera. As a consequence of his study, xerocomoid boletes with patched or granular dotted stipes such as *Boletus depilatus* Redeuilh were placed in *Leccinum*. Another ambiguous case is *B. impolitus* Fr., closely related to *B. depilatus* and placed in section *Luridi* Fr. (*Boletus*) by several authors. This arrangement may be connected with *B. erythropus* (Fr. : Fr.) Krombh., one of the best known fungi of sect. *Luridi*, showing a similar punctate stipe.

In addition, the question whether to keep species with not darkening squamules in *Leccinum* sect. *Luteoscabra* Sing., according to SINGER (1986), or in *Boletus* sect. *Pseudoleccinum* Smith & Thiers is highly controversial. As pointed out by SMITH & THIERS (1968), besides the darkening stipe ornamentation, also the colour of hymenophore and context represents a major characteristic for *Leccinum*. The predominantly whitish flesh of basidiocarps changes to greyish partially progressing to violaceous when cut (sects. *Leccinum* emend. Lan. & Est. and *Scabra* Smith, Thiers & Watl. emend. Lan. & Est.). Yellowish tones in hymenophore and/or flesh are rather exceptional and can only be found in a minority of species (sect. *Luteoscabra*). The majority of species of *Boletaceae* is distinguished by a yellow flesh, turning blue when injured. This discolouration is caused by an enzymatical oxidation of the pulvinic acids variegatic and xerocomic acid (Fig. 1; for details see GILL & STEGLICH 1987). A first screening verified the absence of pigments of the pulvinic acid type in the *Leccinum* species investigated (GABRIEL 1965, BRESINSKY & ORENDI 1970, SCHMITT 1970). The chemical findings in some representatives of *Leccinum* sects. *Luteoscabra*, *Roseoscabra* and *Eximia* (sensu Singer 1986) are in contrast with the above mentioned indications. For *L. extremiorientale* (L. Vass.) Sing., *L. rubropunctum* (Peck) Sing. and *L. subglabripes* (Peck) Sing. xerocomic and variegatic acid were detected. Beyond this, atromentin and traces of cyclopentenone gyroporin were detected in *L. eximium* (Peck) Pomerl., whereas in *L. chromapes* (Frost) Sing. unknown xerocomic acid derivatives were found (BRESINSKY & BESL 1979, SCHMIDT 1990).

Leccinum species are able to synthesize pulvinic acid derivatives and cyclopentenones at least under certain conditions. This is proved by the occurrence of atromentic acid and/or gyroporin in mycelial cultures of *L. scabrum* (Bull. : Fr.) S.F. Gray and *L. aurantiacum* (BRESINSKY ET AL. 1974, BESL ET AL. 1989), and the detection of atromentic and isoxerocomic acid in yellow discoloured areas around insect galleries in infested *L. scabrum* fruitbodies. Gyrocyanin, biogenetically closely connected to gyroporin, could be responsible for the bluish and greenish patches (especially on the stipe base) occurring in several species (see remark in STEGLICH ET AL. 1977).

However, the characteristic discolouration in the context of *Leccinum* basidiocarps by air contact as well as the darkening of stipe scabrosities, is assigned to caffeic acid and gallic aldehyde (Fig. 1; in GILL & STEGLICH 1987 quoted as gallic acid), isolated by EDWARDS & ELSWORTHY (1967) from *L. scabrum* collections. Greenish to bluish colour reactions with iron-(II)-sulfate (compilation in

LANNOY & ESTADES 1995) should be caused by such phenolic compounds, as well. For this reason, a recording of the distribution of caffeic and gallic acid within the *Leccinum* s.l. was one major aspect of this work.

The study was further based on the comprehension of both infrageneric phylogenetic relationships of *Leccinum* and the correlation with closely affiliated genera *Boletus*, *Tylopilus* P. Karst. and *Xerocomus* Quél. Inferred from a comparison of mutational events using ribosomal DNA, recent sequence analyses in various *Basidiomycetes* offered new insights into phylogeny (BRUNS & TAYLOR 1990, VILGALYS ET AL. 1994, KRETZER ET AL. 1996, MONCALVO ET AL. 1997, JOHNSON & VILGALYS 1998). In this study, 900 base pairs originating from the 5' end of 28S rDNA were sequenced for 34 species and analyzed by adequate phylogenetic programs. The composite molecular and chemotaxonomical results should supply more transparency in the generic concept of *Leccinum*.

Materials and methods

Voucher specimens are deposited in the herbarium of the University of Regensburg, Institute of Botany (REG). Concerning the nomenclature of European *Leccinum* species, especially sect. *Leccinum*, we refer to LANNOY & ESTADES (1995).

Table 1: Sources and collection information of taxa included in this study (species, collection number, date, geographic origin, leg./det.):

Not all collections examined are congruent by sequence and chemical analyses. The sequenced species can be recognized by GenBank accession numbers (AF139683 - AF139716) at the end of the collection data; chemically examined species are listed in Table 2. Several collections of a single species are separated by a semicolon.

Leccinum, sect. *Leccinum* emend. Lan. & Est.
Leccinum atrostipitatum Smith, Thiers & Watl., 218/97, 01.09.1997, USA, N. Arnold
L. aurantiacum (Bull.) S.F. Gray [= *Leccinum rufum* (Schaeff.) Kreisel], -, 10.07.97, Germany, H. Besl; La1, 11.09.1994, Germany, M. Beisenherz, AF139689
L. callitrichum Redeuilh ined., GR92103, 03.10.1992, France, C. Berger, AF139690
L. cerinum Korhonen, MK11800, 24.08.1990, Finland, M. Korhonen, AF139692
L. duriusculum (Schulz.) Sing., -, 31.08.1994, Germany, D. Buchmann/A. Bresinsky; 880904/4GL, 04.09.1988, France, Wolfer, AF139695
L. insigne Smith, Thiers & Watl., -, 15.09.1983, USA, H. Thiers/A. Bresinsky
L. nigellum Redeuilh ined., GL4676, 11.09.1979, France, G. Redeuilh, AF139699
L. percandidum (Blum) Lan. & Est., 9210040, 04.10.1992, France, A. Estades, AF139702
L. piceinum Pilát & Dermek, -, 01.09.1987, Germany, N. Luschka
L. populinum Korhonen, MK11247, 19.09.1992, Finland, M. Korhonen, AF139703
L. quercinum Pilát ex Pilát, 10/94, 05.09.1994, Germany, A. Reisinger
L. roseotinctum Watl., -, 23.08.1981, Finland, A. Bresinsky
L. versipelle (Fr.) Snell, -, 10.07.1997, Germany, H. Besl/A. Hagn; Lv2, 06.09.1994, Germany, H. Besl, AF139707

Sect. *Scabra* Smith, Thiers & Watl. emend. Lan. & Est.
Leccinum alaskanum Wells & Kempton, no. 29, 11.09.1983, USA, A. Bresinsky/Ph. Kempton
L. flavostipitatum Dick & Snell, 246/97, 02.09.1997, USA, N. Arnold; 24/98, 23.08.1998, USA, N. Arnold, AF139696
L. holopus (Rostk.) Watl., -, 21.09.1991, Germany, A. Bresinsky; Lh2, 12.09.1995, Germany, M. Fischer, AF139697

L. melaneum (Smotl.) Pilát & Dermek, no. 90, 27.03.1995, New Zealand, A. Bresinsky & B. Wittmann-Bresinsky/A. Bresinsky
L. nucatum Lan. & Est., 9109303, 30.09.1991, France, A. Estades, AF139700
L. palustre Korhonen, MK11107, 02.09.1992, Finland, M. Korhonen, AF139701
L. rotundifoliae (Sing.) Smith, Thiers & Watl., -, 16.08.1981, Finland, A. Bresinsky; MK7676:251, Finland, M. Korhonen, AF139704
L. scabrum (Bull. : Fr.) S.F. Gray, -, 17.09.1988, Germany, A. Bresinsky; 860916/3GL, 16.09.1986, France, G. Lannoy, AF139705
L. subcinnamomeum Pilát & Dermek, -, 09.09.1989, Germany, A. Bresinsky
L. variicolor Watling, 115/79, 18.09.1979, Germany, H. Besl; Lvar1, 19.09.1995, Germany, J. Enzmann, AF139706

Sect. *Luteoscabra* Sing.
Leccinum albellum Sing., 45/97, 20.08.1997, USA, N. Arnold
L. carpini (Schulz) Mos. ex Reid [= *Leccinum griseum* Sing. ?], 103/81, 01.09.1981, Germany, H. Besl; 930808, 08.08.1993, France, G. Lannoy, AF139691
L. corsicum (Roll.) Sing., 931101/1GL, 01.11.1993, France, G. Lannoy, AF139693
L. crocipodium (Letellier) Watl. [= *Leccinum nigrescens* (Richon & Roze) Sing.], 03.09.1997, Germany, L. Krieglsteiner; 930809/1, 09.08.1993, France, G. Lannoy, AF139694
L. extremiorientale (L.Vass.) Sing., -, 10.07.1979, Japan, E. Nagasawa
L. lepidum (Bouchet) Quadraccia, -, 11.06.1984, Italy, H. Besl, AF139698

Sect. *Roseoscabra* Sing.
Leccinum chromapes (Frost) Sing. [= *Tylopilus chromapes* (Frost) Smith & Thiers], 155/97, 26.08.1997, USA, N. Arnold; Lch2, 22.08.1994, USA, W. Steglich, AF139709

Sect. *Eximia* Sing.
Leccinum eximium (Peck) Pomerleau [= *Tylopilus eximius* (Peck) Sing.], 40/97, 20.08.1997, USA, N. Arnold, AF139684

Boletus, sect. *Pseudoleccinum* Smith & Thiers
Boletus hortonii Smith & Thiers [= *Leccinum hortonii* (Smith & Thiers) Hongo & Nagasawa; = *Boletus subglabripes* ssp. *corrugis* Peck], 38/97, 20.08.1997, USA, N. Arnold; 84/94, 23.08.1994, USA, W. Steglich, AF139713
B. longicurvipes Snell & Smith, 23/96, 12.08.1996, USA, N. Arnold, AF139685
B. rubropunctus Peck [= *Leccinum rubropunctum* (Peck) Sing.], TDB-1217, 04.09.1987, USA, T.D. Bruns, AF139687
B. subglabripes Peck [= *Leccinum subglabripes* (Peck) Sing.], 8/97, 14.08.1997, USA, N. Arnold, AF139688

Sect. *Luridi* Fr.
Boletus depilatus Redeuilh [= *Leccinum depilatum* (Redeuilh) Šutara], -, 11.09.1994, Germany, A. Bresinsky; Bd1, 03.09.1995, Germany, A. Bresinsky, AF139712
B. erythropus (Fr. : Fr.) Pers., -, 20.07.1997, Germany, M. Binder; Ber1, 01.08.1995, Germany, J. Enzmann/H. Besl, AF139683
B. impolitus Fr. [= *Xerocomus impolitus* (Fr.) Quél.], Bim1, 21.09.1995, Germany, J. Schreiner, AF139715
B. luridus Schaeff. : Fr., Bl2, 14.08.1995, Germany, M. Beisenherz, AF139686

Tylopilus P. Karst.
Tylopilus alboater (Schwein.) Murrill, TDB-1206, 20.09.1989, USA, T.D. Bruns, AF139708
T. felleus (Fr.) Karst., Tf1, 17.09.1994, Germany, M. Binder, AF139710

T. ferrugineus (Frost) Sing., 210/97, 30.08.1997, USA, N. Arnold, AF139711

Xerocomus Quél.
Xerocomus illudens (Peck) Sing., 64/98, 24.08.1998, USA, N. Arnold/W. Helfer, AF139714
X. subtomentosus (Fr.) Quél., Xs1, 10.08.1995, Germany, J. Enzmann/A. Bresinsky, AF139716

Detection of chemical compounds

Whenever possible, fresh basidiocarps were used for extraction. Otherwise, herbarium specimens were accessible.

Crushed material was acidified by small amounts of diluted hydrochloric acid and was extracted several times with acetone. Merged extracts were evaporated and subsequently examined by thin layer chromatography (silica gel F254, Merck Nr. 5715; mobile phase: toluene:ethyl formate:formic acid 10:5:3). Pigments were observed both under daylight and ultraviolet light conditions (366 nm) and were compared to authentic substances. The following RF values were noted: variegatic acid 0,17; xerocomic acid 0,26; gyroporin 0,27; gallic acid 0,27; caffeic acid 0,35.

Total DNA extraction

Approximately 300 mg of fresh basidiocarps or 20 mg of dried herbarium specimens were ground with mortar and pestle under liquid nitrogen. DNA lysis was done for one hour (65°C) using an extraction buffer (50 mM EDTA, 50 mM Tris-HCl, 3% SDS, pH 8.0), applying substantially the procedure of LEE & TAYLOR (1990) with slight modifications (BINDER & FISCHER 1997). The crude preparation was followed by a phenol:chloroform:isoamyl alcohole (25:24:1) extraction and an additional chloroform extraction. Sodium acetate (3 M) was added prior to DNA precipitation with isopropanol. DNA pellets were washed in 70% ethanol, air dried and resuspended in 100 µl TE. Total DNA from older herbarium specimens was isolated with the DNeasy Plant Mini Kit (Qiagen). DNA quality and concentration was examined by electrophoresis on a 1% agarose gel (Molecular Biology Certified Agarose, Biorad).

Polymerase Chain Reaction (PCR) and Cycle sequencing

Primer sequences (5' - 3') used in this work are: LR0R: ACC CGC TGA ACT TAA GC; LR5: TCC TGA GGG AAA CTT CG, LR7: TAC TAC CAC CAA GAT CT (VILGALYS & HESTER 1990, Vilgalys, pers. com.).

A 1500 base pair fragment of the 28S rDNA was enzymatically amplified using the oligonucleotides LR0R und LR7. PCR reactions were run on a T3 thermocycler (Biometra) according to FISCHER (1995). Double stranded PCR products as templates for single stranded amplification were prepared using the QIAquick PCR Purification Kit (Qiagen). Parameters for sequence reactions have been described previously (BINDER ET AL. 1997). Cycle sequencing was performed with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Perkin Elmer). Overall 900 base pairs per species were sequenced in both directions using primers LR0R and LR5 on an ABI 377 automated sequencer (Applied Biosystems).

Phylogenetic analyses

Sequences have been submitted to GenBank (Accession no. AF139683 - AF139716). The automatic alignment with ClustalX (THOMPSON ET AL. 1997) was manually adjusted using MacClade v. 3.04 (MADDISON & MADDISON 1992) and a colour font on a Powermacintosh 7500/100. Gaps, introduced due to insertions and deletions, were treated as missing data. All positions, containing two short ambiguously aligned regions, have been included in the final alignment. Further analysis was conducted on a HP Kayak workstation with components of the PHYLIP 3.5c package (FELSENSTEIN 1995), integrated in ClustalX. The distance matrix was generated using Kimura 2-parameter distances, weighting transition:transversion ratio 2:1. Neighbor-joining (standard parameters) was used to calculate the tree. The appropriate bootstrap analysis (FELSENSTEIN 1985) assessing the confidence in the branches was run with 1000 replicates. These results are presented in Fig. 2 and the alignment can be obtained by the authors on request.

To verify the neighbor-joining tree, an additional parsimony analysis was conducted with PAUP 3.1.1 (SWOFFORD 1993). A total of 300 random addition replicates were run using heuristic search (TBR branch swapping and DELTRAN active) followed by 50% majority-rule consensus bootstrapping.

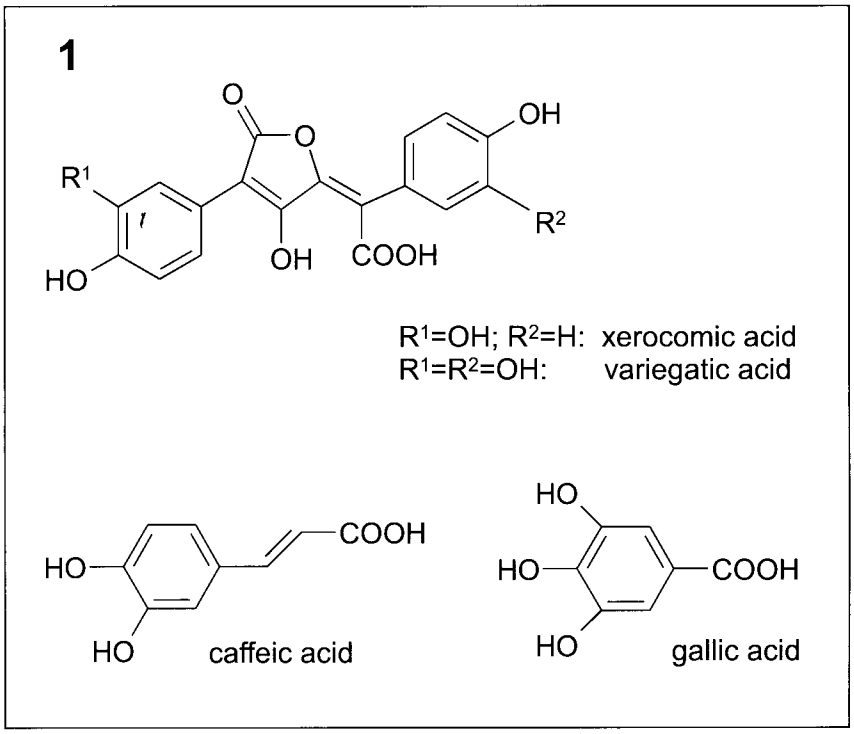
Results

As shown in Table 2, the vast majority of *Leccinum* species (sects. *Leccinum, Scabra* and *Luteoscabra*) is characterized by the absence of the yellow pulvinic acids xerocomic and variegatic acid, and further by the presence of the aromatic carboxilic acids caffeic and/or gallic acid. In contrast, pulvinic acids are present in *Boletus* species, whereas caffeic and gallic acid are not detected.

Caffeic and gallic acid are relatively unstable and easily oxidized substances, accounting for uncertain (*L. alaskanum*) or missing (*L. lepidum, L. melaneum*) evidence in some herbarium specimens. Apart from that, within the *Boletales* they could only be demonstrated for *L. eximium* (see Tab. 2), for *Tylopilus felleus* (in only one collection!) and for *Pisolithus arhizus* (Scop.: Pers.) S. Rauschert (Besl, unpub.) and are absent in all other genera. Therefore, the occurrence of caffeic and gallic acid together with simultaneous absence of pulvinic acids could be an argument for placing corresponding species in the genus *Leccinum*.

Gyroporin appears sporadically in various genera of *Boletales* (see GILL & STEGLICH 1987). The occurrence of this cyclopentenone in some *Leccinum* species is not considered as a distinct character.

Figure 1: Structural formulas of the chemotaxonomically important compounds. Pulvinic acids: xerocomic and variegatic acid. Aromatic carboxilic acids: caffeic and gallic acid.



Sequence analysis

The length of the 28S rDNA sequences of the 34 species listed in Table 1 ranged between 866 and 896 base pairs. The final alignment resulted in 911 sites with 187 variable positions, 158 of which phylogenetically informative.

Analysis with the distance matrix program neighbor-joining (Fig. 2) revealed at least six groups, supported by high bootstrap values and therefore marked with the generic or sectional names *Leccinum* sects. *Leccinum* and *Scabra*, *Luteoscabra*; *Boletus* sects. *Pseudoleccinum*, *Luridi*; *Tylopilus* and a heterogenous clade labeled as xerocomoid boletes. Both *L. chromapes* and *L. eximium* (*Leccinum* sects. *Roseoscabra* and *Eximia*) exhibit an isolated position within the phylogram. The tree is rooted with *L. eximium*.

According to the results, *Leccinum* has to be estimated as polyphyletic. The majority of the genus is divided into the groups *Leccinum* & *Scabra*, *Luteoscabra* and *Pseudoleccinum*. The first group is composed by sections with a whitish flesh in basidiocarps, sect. *Leccinum* (*L. aurantiacum*, *L. callitrichum*, *L. cerinum*, *L. duriusculum*, *L. nigellum*, *L. percandidum*, *L. populinum*, and *L. versipelle*) and sect. *Scabra* (*L. flavostipitatum*, *L. holopus*, *L. nucatum*, *L. palustre*, *L. rotundifoliae*, *L. scabrum*, and *L. variicolor*). The sequences of *L. populinum* and *L. percandidum* on one hand, and of *L. holopus* and *L. nucatum* on the other hand are identical. The short internal branches within the clade indicate a close relationship between the two sections. The poor resolution is obviously due to the conserved nature of the 28S gene. Consequently, no further descriptions of the intrasectional relations of sects. *Leccinum* and *Scabra* can be given. For this purpose, additional sequence analyses of the more variable ITS spacer regions are in progress.

The next related group is sect. *Luteoscabra* (*L. carpini*, *L. crocipodium*, *L. lepidum* and *L. corsicum*). Species with yellow tones particularly in hymenophore are not forming a consistent narrow clade and are in considerable distance to the first group. The *Luteoscabra* are followed by sect. *Pseudoleccinum* (*B. longicurvipes*, *B. rubropunctus* and *B. subglabripes*) with yellow context and hymenophore and not darkening stipe squamules. *L. chromapes*, however, is not clearly assignable neither to *Leccinum* nor to *Tylopilus* (represented by *T. alboater*, *T. ferrugineus* and *T. felleus*).

Table 2: Chemical compounds of Leccinum and Boletus species examined (inclusive data drawn from literature):

Species	pa	gp	ca	ga	rem.
Leccinum alaskanum	-	-	?	-	
Leccinum albellum	-	-	+	+	
Leccinum atrostipitatum	-	-	(+)	-	
Leccinum aurantiacum	-	+	+	+	
Leccinum carpini	-	-	+	(+)	
Leccinum crocipodium	-	-	+	+	
Leccinum duriusculum	-	-	(+)	-	
Leccinum flavostipitatum	-	-	-	+	
Leccinum holopus	-	-	(+)	-	
Leccinum insigne	-	-	+	+	
Leccinum lepidum	-	-	-	-	
Leccinum melaneum	-	-	-	-	
Leccinum piceinum	-	-	(+)	-	
Leccinum quercinum	-	-	+	-	
Leccinum roseotinctum	-	-	(+)	-	
Leccinum rotundifoliae	-	-	(+)	-	
Leccinum scabrum	-	+	+	+	(1)

Leccinum subcinnamomeum	-	-	+	-	
Leccinum variicolor	-	+	+	-	
Leccinum versipelle	-	+	+	-	
Leccinum chromapes	(+)	-	-	-	(3)
Leccinum eximium	-	(+)	+	-	(2)
Leccinum extremiorientale	+	-	-	-	(3)
Boletus depilatus	+	-	-	-	
Boletus hortonii	+	-	-	-	(2)
Boletus rubropunctus	+	-	-	-	(2)
Boletus subglabripes	+	-	-	-	
Boletus erythropus	+	-	-	-	

Abbreviations: pa = pulvinic acids (xerocomic and/or variegatic acid); gp = gyroporin; ca = caffeic acid; ga = gallic acid; rem. = remarks

Remarks: (1): BESL & BRESINSKY (1977), BRESINSKY & BESL (1979), EDWARDS & ELSWORTHY (1967); (2): BRESINSKY & BESL (1979); (3): SCHMIDT (1990)

The following heterogenous clade consists of the *Xerocomus* species, *X. subtomentosus* and *X. illudens*, and also includes *B. hortonii* (sect. *Pseudoleccinum*), *B. depilatus* and *B. impolitus* (*Boletus* sect. *Luridi*). This group combines essentially boletes with similar habits and a granulate to glabrous stipe, in distant relation to sect. *Luridi*, represented by *B. erythropus* and *B. luridus*. The distribution of boletes with a poorly developed, dotted stipe ornamentation (sect. *Pseudoleccinum*, *L. chromapes*, *L. eximium*, the xerocomoid boletes and *B. erythropus*) among different clades indicates no closer relationship between these fungi. Apparently, this feature has been evolved independently for several times.

Parsimony analysis with PAUP produced 27 most parsimonious trees of 415 steps, consistency index (CI) = 0,578, homoplasy index (HI) = 0,422, CI excluding uninformative characters = 0,557, HI excluding uninformative characters = 0,443, retention index (RI) = 0,824, rescaled consistency index (RC) = 0,477. The resulting 50% majority-rule consensus tree was topologically identical with the neighbor-joining tree except for the arrangement of sects. *Leccinum* and *Scabra*. Except for the internal branches of the *Leccinum* and *Scabra* clade, all branches were supported with 100% (data not shown).

Discussion

Based on the phylogenetic analysis (Fig. 2) and the chemical results (Tab. 2), morphological and anatomical characteristics (according to SMITH & THIERS 1971, SINGER 1986) will be discussed subsequently. For this purpose, we refer to the corresponding numbers 1-4, located on the particular external nodes (Fig. 2).

Although sequence data and chemotaxonomical results correlate partially, the concepts of Smith & Thiers (1971) and Singer (1986) are not entirely supported.

Both sects., *Leccinum* and *Scabra* (group 1) are forming a close, homogenous clade positioned in considerable distance with respect to all remaining boletes examined. Group 1 is chemically characterized by the synthesis of caffeic and gallic acid and by the simultaneous absence of the pulvinic acids in intact basidiocarps. Stipe scabrosities are strongly developed and darkening, the whitish context changes to greyish or violaceous when cut. Yellowish tones are absent in the flesh of basidiocarps and hymenophore. The pileus epicutis is not fundamentally different from that of *Boletus*. It consists of a trichoderm, composed by predominantly filamentous, partially cylindric hyphae. Globose or inflated end-cells in the pileipellis are not observed.

Species with yellow tones in hymenophore and partly in flesh (group 2) are clearly separated from the first group. In spite of the occurrence of caffeic and gallic acid in some cases and the

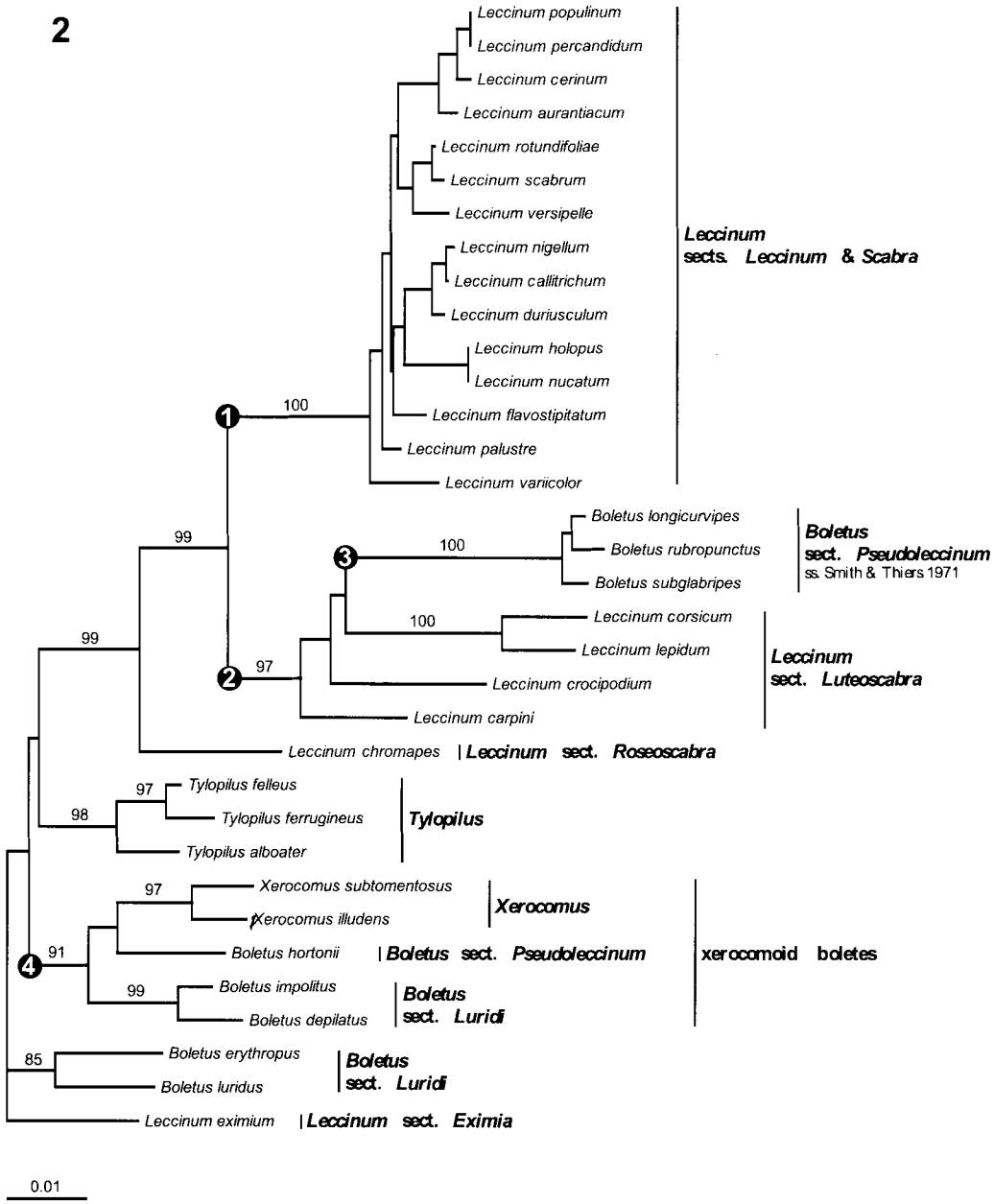


Figure 2: Phylogeny of *Leccinum* s. l. and allied taxa. Phylogram of 34 species based on large subunit rDNA sequences using neighbor-joining method, rooted with *L. eximium*. Bootstrap values (>85%) from 1000 replicates are given above the corresponding branches. Horizontal branch length represent mean distances, vertical branch length is arbitrary. For numbers 1-4 see discussion.

missing of pulvinic acids, these species are not in agreement with the definition of *Leccinum* s. str. given above. *L. corsicum, L. lepidum* and *L. crocipodium* (*Luteoscabra*) show a darkening stipe ornamentation similar to group 1, however, a yellowish hymenophore is found continuously in group 2. The epicutis is mainly formed by cylindrocysts, sporadically interspersed with spherocysts (not in the sense of spherocystes as occurring in the *Russulales*).

Leccinum carpini (*Luteoscabra*, subsection *Albella*) differs from the species mentioned above by a more distinct stipe ornamentation and chains of spherocysts in the pileipellis. It is an interesting question, why *L. carpini* was placed in *Luteoscabra*, since it exhibits brownish, at most dirty yellowish tones in the hymenophore at late maturity. Except for the anatomy of the epicutis it is similar to the members of group 1. Supported by a high bootstrap value *L. carpini* is within group 2 in the phylogram, but it displays the shortest distance of all species included to sections *Leccinum* and *Scabra*. An exclusion of *L. carpini* from *Leccinum* is hardly to recognize and therefore an unsolved problem.

Except *Boletus hortonii* (see below), *B. longicurvipes*, *B. rubropunctus* and *B. subglabripes*, placed in sect. *Pseudoleccinum* within *Boletus* by SMITH & THIERS (1971), form a well defined group in the phylogram. This group (3) is distinguished by the occurrence of pulvinic acids, the missing of caffeic and gallic acid, furfuraceous to subglabrous stipe, yellow flesh and hymenophore, and not darkening stipe ornamentation. The epicuticular end-cells are inflated, partially showing isodiametrical dimensions.

The remaining sections *Roseoscabra* und *Eximia* are clearly isolated and are in great distance to the other clades. *L. chromapes* (unknown xerocomic acid derivatives) and *L. eximium* (synthesis of atromentin and traces of gyroporin) are also chemical outliers. *L. chromapes* has been transferred from *Leccinum* (SMITH & THIERS 1968) to *Tylopilus*, which is hardly to characterize chemically. A relation of *L. chromapes* to *Tylopilus* cannot be disclaimed, even though not confirmed by sequence data. *L. eximium*, however, is definitely to be placed within *Boletus*.

The last group 4 is very heterogenous, with *X. subtomentosus* as the type species of *Xerocomus*. It comprises exclusively boletes with a xerocomoid habit (hymenophore and flesh yellow to lemon yellow, stipe glabrous, granulate to punctate, ornamentation sometimes striate, but never reticulate and not darkening). All representatives of this clade produce pulvinic acids, whereas caffeic and gallic acid are missing. In contrast to the view of SMITH & THIERS (1971) *B. hortonii* is not related to *B. subglabripes*, but shows a close relationship to the *Xerocomus* species. As noticed by SMITH & THIERS (1968), the progression to inflated epicuticular hyphae can be observed in sect. *Pseudoleccinum* as well as in the *X. subtomentosus* complex, including *X. illudens*. Since this tendency also shows up in *B. depilatus* and *B. impolitus*, a more accurate examination of this feature seems necessary. *Boletus depilatus*, *B. impolitus* and *B. hortonii* don't belong to *Leccinum* or to *Boletus* sect. *Luridi*. On the basis of the molecular results they have to be placed in *Xerocomus*.

As a consequence of our results, *Leccinum* could be interpreted as a single heterogenous clade (except *L. eximium*). However, molecular delimitation between the species with yellowish flesh and/or hymenophore (2, 3 and 4) and the species with whitish flesh and hymenophore (1) is quite clear and substantiated by additional features. Based on the combination of characteristics mentioned above the genus *Leccinum* should be restricted to the sections *Leccinum* and *Scabra*. The remaining boletes in discussion have to be removed from *Leccinum*. Until more helpful insights can be presented, the relevant species should be kept in the genus *Boletus*. A final re-evaluation of *Leccinum* towards a natural classification should be based on additional sequence data and chemical analyses of the affiliated representatives of *Austroboletus* (Corner) Wolfe and the gastroid genus *Chamonixia* Roll.

Species excluded from *Leccinum*: *Boletus carpini* (Schulz) Pears., *Boletus corsicus* Rolland, *Boletus crocipodius* Letellier, *Boletus eximius* Peck, *Boletus lepidus* Bouchet, *Boletus rubropunctus* Peck, *Boletus subglabripes* Peck, *Tylopilus chromapes* (Frost) Smith & Thiers, *Xerocomus impolitus* (Fr.) Quél.

New combinations:

Xerocomus depilatus (Redeuilh) Binder & Besl comb. nov.
Basionym: *Boletus depilatus* Redeuilh, Bull. Soc. Mycol. Fr. 101: 389, 1985
Synonym: *Leccinum depilatum* (Redeuilh) Šutara, 1989

Xerocomus hortonii (Smith & Thiers) Binder & Besl comb. nov.
Basionym: *Boletus hortonii* Smith & Thiers, The Boletes of Michigan, p. 319, 1971
Synonyms: *Boletus subglabripes* var. *corrugis* Peck, 1889
Leccinum hortonii (Smith & Thiers) Hongo & Nagasawa, 1978

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