

Universität Regensburg, Fakultät für Biologie und Vorklinische Medizin, Botanisches Institut, Regensburg
Clark University, Department of Biology, Sackler Science Building, Worcester, Massachusetts

M. BINDER & A. BRESINSKY

***Retiboletus*, a new genus for a species-complex in the Boletaceae producing retipolides¹**

With 3 Figures and one Table

Summary

Boletus ornatipes and *B. retipes* are two closely related species in North America, which cannot be differentiated by morphological and anatomical characters. The 25S rDNA was partly sequenced from several isolates of *B. ornatipes*, *B. retipes*, and allied species in the Boletaceae to aid identification. The phylogenetic analysis using maximum likelihood suggests that both species are differently distributed. In addition, our results are supported by a previous study of HELLOWIG (1999), separating three groups in the *B. retipes/ornatipes*-complex based on chemotaxonomical characters. Retipolides form the major pigments in presently analyzed *B. ornatipes* and *B. retipes* collections and are also detected in *B. flavoniger*. This group of secondary metabolites is unique in the Boletales (for distribution of pigments in Boletales see GILL & STEGLICH 1987) and is characteristic of the new genus *Retiboletus*, in which *B. ornatipes* (type), *B. retipes*, and *B. flavoniger* are placed. A positive proof of retipolides is still missing in *B. griseus* and *Tylopilus nigerrimus*. Both species are transferred to *Retiboletus* according to morphological characters and sequence data.

Zusammenfassung

Retiboletus, eine neue Gattung für einen Arten-Komplex mit Retipoliden innerhalb der Boletaceae

Boletus ornatipes und *B. retipes* sind zwei nahe verwandte Arten in Nordamerika, die durch morphologische und anatomische Merkmale nicht unterschieden werden können. Ein Teil der 25S rDNA mehrerer Isolate von *B. ornatipes*, *B. retipes* und verwandten Arten wurde sequenziert, um die Identifizierung zu unterstützen. Die phylogenetische Analyse unter Verwendung von Maximum Likelihood weist auf eine unterschiedliche geographische Verbreitung beider Arten hin. Zusätzlich werden unsere Ergebnisse durch eine vorhergehende Studie von HELLOWIG (1999) unterstützt, in der chemotaxonomische Merkmale zur Abgrenzung dreier Gruppen im *B. retipes/ornatipes*-Komplex verwendet werden. Retipolide bilden die Hauptpigmente in bisher untersuchten Aufsammlungen von *B. ornatipes* und *B. retipes* und können darüber hinaus in *B. flavoniger* nachgewiesen werden. Diese Gruppe von Sekundärmetaboliten ist einzigartig innerhalb der Boletales (zur Verbreitung von Pigmenten in Boletales siehe GILL & STEGLICH 1987) und führt zur Etablierung der neuen Gattung *Retiboletus*, in die *B. ornatipes* (Typus), *B. retipes* und *B. flavoniger* gestellt werden. Ein positiver Nachweis auf Retipolide ist bisher noch ausstehend für *B. griseus* und *Tylopilus nigerrimus*. Beide Arten werden aufgrund morphologischer Merkmale und sequenzanalytischer Daten zu *Retiboletus* gestellt.

Die Gattung *Retiboletus* wird in der vorliegenden Studie neu beschrieben. *Boletus ornatipes*, *B. retipes*, *B. flavoniger*, *B. griseus* und *Tylopilus nigerrimus* werden in diese neu etablierte Gattung *Retiboletus* gestellt und die hierbei notwendigen Neukombinationen durchgeführt.

¹ Dedicated to Professor Dr. Hanns Kreisel, Greifswald on the occasion of his 70th birthday

Introduction

The discovery of retipolides in *Boletus ornatipes* and *B. retipes* (HERMANN 1980) indicated the occurrence of a novel substance group in the Boletaceae. At that time, the Boletaceae were considered to be absolutely homogenous in the production of pigments and other metabolites, which basically consist of pulvinic acids and derivatives. Members of the genera *Boletus* FR., *Boletellus* MURR., *Pulveroboletus* MURRILL, and *Xerocomus* QUÉL. normally produce pulvinic acid derivatives like variegatic acid and xerocomic acid (BESL & BRESINSKY 1978; GILL & STEGLICH 1987). Exceptions are the genera *Austroboletus* (CORNER) WOLFE (not adequately studied), *Gyroporus* QUÉL., *Tylopilus* KARST., and *Xanthoconium* SINGER in which positive proof for pulvinic acid derivatives is still lacking. Instead, an occurrence of cyclopentenones has been demonstrated in case of *Gyroporus* (BRESINSKY & BESL 1978).

Since the description of *B. ornatipes* PECK and *B. retipes* BERK. & CURTIS, it remained uncertain if these are distinct species or not (SINGER 1947, 1986; SMITH & THIERS 1971; BOTH 1993). *B. ornatipes* has a conspicuous but variable habitus (shown in BESSETTE et al. 2000) and is usually easily recognized in the field. *B. retipes* is very similar and differs from *B. ornatipes* only in a yellowish pulverulent pileus. Apparently, this character is not constant and therefore unreliable to distinguish between the two species (BOTH 1993).

In a recent study, HELLWIG (1999) assumed that previous screenings for chemical compounds in *B. ornatipes* and *B. retipes* (HERMANN 1980; JUSTUS 1993) were based on mixed collections, because both species have been regarded as identical. HELLWIG (1999) accessed material obtained in an extensive collection trip to North America in 1997 undertaken by his colleagues, who kept their collections separately and labelled them as *B. retipes/ornatipes*. In addition, material from Japan and Costa Rica was used for comparison. As a result, HELLWIG (1999) detected traces of xerocomic acid in some samples and a wide distribution of an unidentified compound (Substanz 102) next to the retipolides

already known. According to the presence or absence of particular chemical components, the *B. retipes/ornatipes* collections could be tentatively subdivided in three groups (Gruppe 1–3). Thus, HELLWIG's (1999) findings suggest that *B. retipes/ornatipes* essentially is a species-complex including two or more species.

The goal of our study was to evaluate the results drawn from chemistry with rDNA sequences, using the same set of collections and extending it with other representatives of the Boletaceae. A main objective was to verify possible correlations between geographical distribution and taxonomical implications of the *B. retipes/ornatipes*-complex in North America. To infer phylogenetic relationships we generated a data set of nuc-18S sequences (25S or 28S rDNA gene), a DNA region, which has been successfully used in previous studies (BRESINSKY et al. 1999; BINDER & BESL 2000; BINDER & BRESINSKY 2002).

Material and methods

Fungal material. – Sources, collection information, and GenBank accession numbers (AF456837–AF456825) are listed in Table 1. Voucher specimens are deposited in the Institut für Organische Chemie, Ludwig-Maximilians-Universität München (IOC), the Institut für Botanik, Universität Regensburg (REG) and in The New York Botanical Garden Herbarium (NY). 32 new nuc-18S sequences were generated for this study. We also used six sequences from our previous studies and four other sequences downloaded from GenBank.

DNA isolation and polymerase chain reaction. – 20 mg samples from dried herbarium specimens were ground in liquid nitrogen. Cell lysis proceeded for one hour at 65 °C using 800 µL extraction buffer (50 mM EDTA, 50 mM Tris-HCl, 3% SDS, pH 8.0). The crude preparation (LEE & TAYLOR 1990) was followed by a phenol : chloroform : isoamyl alcohol (25:24:1, Amresco) extraction and an additional step with chloroform. Total DNA was precipitated with 10 µL sodium acetate (3M) and isopropanol (0.54 Vol.%) at –20 °C. DNA pellets were washed three times in 70% ethanol, air dried and resuspended in 100 µL TE buffer.

PCR reactions contained 33 µL DNA solution (adjusted to approximately 5 ng), 10 µL PCR reaction buffer, 2 µL dNTP mix (0.2 mM), 50 pmol each

Table 1
Species, collection data, and GenBank accession numbers of generated nuc-*lsu* sequences

Species	Coll. no.	Host	Date	Locality	Leg./det.	GenBank no.
<i>Boletus appendiculatus</i> SCHAEFF.: FR.	Bap1	mixed forest	10. 08. 1995	Germany	J. SCHREINER	AF456837
<i>Boletus aereus</i> BULL.: FR.	Bae1	mixed forest	08. 08. 1995	Germany	J. SCHREINER	AF456836
<i>Boletus calopus</i> FR.	Bc1	mixed forest	07. 09. 1994	Germany	N. ARNOLD	AF456833
<i>Boletus confiferarum</i> DICKS. & SNELL	7/94	mixed forest	10. 1994	U.S.A.	W. STEGLICH	AF456827
<i>Boletus griseus</i> FROST apud PECK	202/97	mixed forest	30. 08. 1997	MA, U.S.A.	N. ARNOLD	AF456834
<i>Boletus fechtneri</i> VELEN.	Bf1	mixed forest	11. 09. 1994	Germany	M. BEISENHERZ	AF456821
<i>Boletus flavoniger</i> HALLING, G.M.MUELL. & L.D.GÓMEZ	RH7189	mixed forest	24. 11. 1993	Costa Rica	R. HALLING	AF456829
<i>Boletus flavoniger</i> HALLING, G.M.MUELL. & L.D.GÓMEZ	RH7247	mixed forest	08. 06. 1994	Costa Rica	R. HALLING	AF456828
<i>Boletus ornaticipes</i> PECK	Japan1	mixed forest	10. 07. 1997	Japan	H. SHIBATA	AF456807
<i>Boletus flavoniger</i> HALLING, G.M.MUELL. & L.D.GÓMEZ	Japan2	mixed forest	17. 08. 1997	Japan	R. MARUMOTO	AF456806
<i>Boletus reticulatus</i> SCHAEFF. & CURTIS	Ba1 Japan3	<i>Quercus</i> , <i>Corylus</i> mixed forest	27. 08. 1995 n/a	Germany Japan	M. BEISENHERZ R. MARUMOTO	AF456824 AF456822
<i>Boletus retipes</i> BERK. & CURTIS	RH7567	mixed forest	28. 05. 1996	Costa Rica	R. HALLING	AF456808
<i>Boletus retipes/ornaticipes</i>	116/96	<i>Quercus</i>	30. 08. 1996	NC, U.S.A.	N. ARNOLD	AF456823
<i>Boletus retipes/ornaticipes</i>	5/97	<i>Quercus</i>	08. 1997	MA, U.S.A.	*/*	AF456812
<i>Boletus retipes/ornaticipes</i>	11/97	deciduous trees	14. 08. 1997	MA, U.S.A.	*/*	AF456810
<i>Boletus retipes/ornaticipes</i>	22/97	mixed forest	20. 08. 1997	NC, U.S.A.	*/*	AF456831
<i>Boletus retipes/ornaticipes</i>	57/97	mixed forest	21. 08. 1997	NC, U.S.A.	*/*	AF456811
<i>Boletus retipes/ornaticipes</i>	93/97	mixed forest	23. 08. 1997	NC, U.S.A.	*/*	AF456825
<i>Boletus retipes/ornaticipes</i>	94/97	mixed forest	23. 08. 1997	NC, U.S.A.	*/*	AF456814
<i>Boletus retipes/ornaticipes</i>	96/97	mixed forest	23. 08. 1997	NC, U.S.A.	*/*	AF456830
<i>Boletus retipes/ornaticipes</i>	161/97	mixed forest	26. 08. 1997	NC, U.S.A.	*/*	AF456817
<i>Boletus retipes/ornaticipes</i>	186/97	mixed forest	30. 08. 1997	MA, U.S.A.	*/*	AF456819
<i>Boletus retipes/ornaticipes</i>	201/97	mixed forest	30. 08. 1997	MA, U.S.A.	*/*	AF456815
<i>Boletus retipes/ornaticipes</i>	215/97	mixed forest	31. 08. 1997	MA, U.S.A.	*/*	AF456805
<i>Boletus retipes/ornaticipes</i>	219/97	mixed forest	01. 09. 1997	MA, U.S.A.	*/*	AF456809
<i>Chalciponius amarellus</i> (QUEL.) MOSER	Cam1	mixed forest	n/a	Germany	A. BRESINSKY	AF456835

<i>Fistulinella viscida</i> (MCNABB) SINGER	238	mixed forest	26. 04. 1995	New Zealand	A. BRESINSKY	AF456826
<i>Phylloporus pelletieri</i> (LÉV.) QUEL.	Pp1	mixed forest	09. 09. 1995	Germany	M. KRONFELDNER	AF456818
<i>Tylophilus indecisus</i> (PECK) MURR.	98/98	deciduous trees	26. 08. 1998	U.S.A	N. ARNOLD, W. HELFER	AF456820
<i>Tylophilus nigerrimus</i> R.HEIM	Tyn1	n/a	15. 07. 1996	Japan	R. MARUMOTO	AF456832
<i>Tylophilus</i> sp.	204/97	mixed forest	30. 08. 1997	U.S.A	N. ARNOLD	AF456813

n/a = not available

/ = N. ARNOLD, W. HELFER, W. STEGLICH, TERPIN/N. ARNOLD
MA = Massachusetts; NC = North Carolina

Sequences obtained from GenBank: *Boletus edulis* BULL.: FR. – AF336240; *Boletus radicans* PERS.: FR. – AF336241; *Boletus retipes* – U11914; *Chalciporus piperatus* (BULL.: FR.) BAT. – AF336244; *Coniophora puteana* (SCHUMACH.) P.KARST. – AF098377; *Suillus cavipes* (OPAT.) A.H. SMITH & THIERS – AF071535; *Suillus luteus* (L.) S.F.GRAY – AF042622; *Tylophilus felleus* (BULL.: FR.) P.KARST. – AF139710; *Xerocomus illudens* (PECK) SINGER – AF139714; *Xerocomus subtomentosus* (L.) FR. – AF139716

of primers LR0R and LR7 (VILGALYS & HESTER 1990), and 1 U *Taq*DNA polymerase (Eurogentec). The final volume was adjusted to 100 μ L with sterile H₂O. The amplifications were run in 37 cycles on a TM3 thermocycler (Biometra) using the following parameters: denaturation 95 °C (1 min), annealing 47 °C (45 sec), extension 72 °C (1.5 min). PCR products were purified with the QIAquick PCR cleaning Kit (Qiagen).

Cycle sequencing and sequence analysis. – Sequencing reactions were set up with primers LR0R, LR3 and LR5 (primer sequences used in this study were obtained from <http://www.botany.duke.edu/fungi/mycolab/primers.htm>) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California). Each reaction mix included 2 μ L BigDye (with *AmpliTaq* polymerase), 8 pmol primer, and 3.5 μ L LR0R-LR7 product. The PCR program was: 96 °C denaturation (2 min), 47 °C annealing (15 sec), and 60 °C extension (4 min) in 35 cycles. Cycle sequencing products were run on an ABI 377 automated DNA sequencer (Applied Biosystems) with a 5.25% polyacrylamide gel (PAGE PLUS, 7M Urea, Amresco) at the Universitätsklinikum Regensburg.

Phylogenetic analyses. – A preliminary alignment with ClustalX (THOMPSON et al. 1997) was manually adjusted in the editor of PAUP* 4.0b4a (SWOFFORD 1998) and submitted to TreeBASE. All trees were rooted with *Coniophora puteana* (SCHUMACH.: FR.) KARST. (Coniophoraceae), *Suillus luteus* (L.) S.F.GRAY, and *S. cavipes* (KLOTZSCH in FR.) SMITH & THIERS (Suillaceae).

Analyses were performed with maximum likelihood (ML) using the complete data set. The ML analysis under the HKY'85 model was performed with 100 heuristic search replicates, transition/transversion ratio = 2, assumed nucleotide frequencies set to empirical frequencies, number of substitution types = 2, rate heterogeneity following the discrete gamma approximation, with four categories and α = 0.5. In addition, one hundred bootstrap replicates (FELSENSTEIN 1985) were run with maximum likelihood. Heuristic searches were performed in PAUP* with the following general settings: MAXTREES set to autoincrease, TBR, random taxa addition sequence, MULTREES on, zero length branches collapsed, gaps treated as "missing", and steepest descent option not in effect. A constrained analysis was performed using the same data set and settings, forcing the hypothetical monophyly of all retipolide producing boletes including *Boletus griseus* FROST in PECK, *Tylophilus* sp., and *T. nigerrimus* R.HEIM. Constrained and unconstrained trees were compared using the Templeton non-parametric test (WSR-test) implemented in PAUP* (TEMPLETON 1983).

Results

The nuc-lsu rDNA data set of 42 sequences included 925 aligned positions. 689 characters were constant, 85 variable characters were parsimony-uninformative, and 151 characters were parsimony-informative. The genera *Boletus* and *Tylopilus* are polyphyletic in both maximum likelihood and maximum parsimony analyses, whereas the Boletaceae is confirmed as monophyletic clade. The shortest tree (Fig. 1) found using maximum likelihood ($-\ln$ likelihood = 4357.71191) resulted in three major clades for the North American *B. retipes/ornatipes* collections, corresponding to chemotaxonomical groups 1–3 detected by HELLOWIG (1999).

Group 1 includes collections from Massachusetts and North Carolina and receives strong support by a bootstrap value of 100%. The nine sequences in group 1 only differ in seven base positions, which suggests that the isolates are conspecific. Collection *B. retipes/ornatipes* 93/97 shows an additional five base pairs deletion at position 510–514. Nested between groups 1 and 2 are *Boletus griseus*, *Tylopilus nigerrimus*, and *Tylopilus* sp., which have not been screened for chemical compounds in HELLOWIG'S study. Except for different colors, *B. griseus* and *T. nigerrimus* strongly resemble the habitus of *B. ornatipes* and *B. retipes* and a close relationship is not surprising. Though appearing to be close to *B. griseus* based on morphology, *Tylopilus* sp. was not unambiguously identified.

Group 2, also supported by 100%, contains two subclades. The Japanese collections, identified as *B. retipes* and *B. ornatipes*, are putatively conspecific and differ in two base pair positions. They form the sister group to two identical collections from North Carolina and a *B. retipes* collection from Costa Rica. Together group 2 and group 3, which exclusively includes collections from North Carolina, are supported by a bootstrap value of 83%. Another group consists of two *B. flavoniger* isolates from Costa Rica, which produce the retipolides found in groups 1–3, and additionally larger amounts of unidentified metabolites (HELLOWIG 1999). Supported by morphology and chemotaxonomy, a phylogenetic relation-

ship between *B. flavoniger* and groups 1–3 is indicated.

The phylogenetic analysis using maximum parsimony (MP) recovered 216 trees of 560 steps (CI = 0.555, RI = 0.712) in seven islands (MADDISON 1991). There was no conflict comparing MP and ML topologies, however, the clade including all retipolides producing boletes collapses in the strict consensus tree (not shown). In addition, the position of *B. griseus* and *Tylopilus* sp., and *B. flavoniger* remained unresolved. The constrained analysis designed to test support for the monophyly of groups 1–3 including *B. griseus*, *Tylopilus* sp., *T. nigerrimus*, and *B. flavoniger* recovered 100 trees of 562 steps (two steps longer than the unconstrained trees). None of the trees ($p = 0.6698–0.8378$) was statistically rejected by the WSR-test.

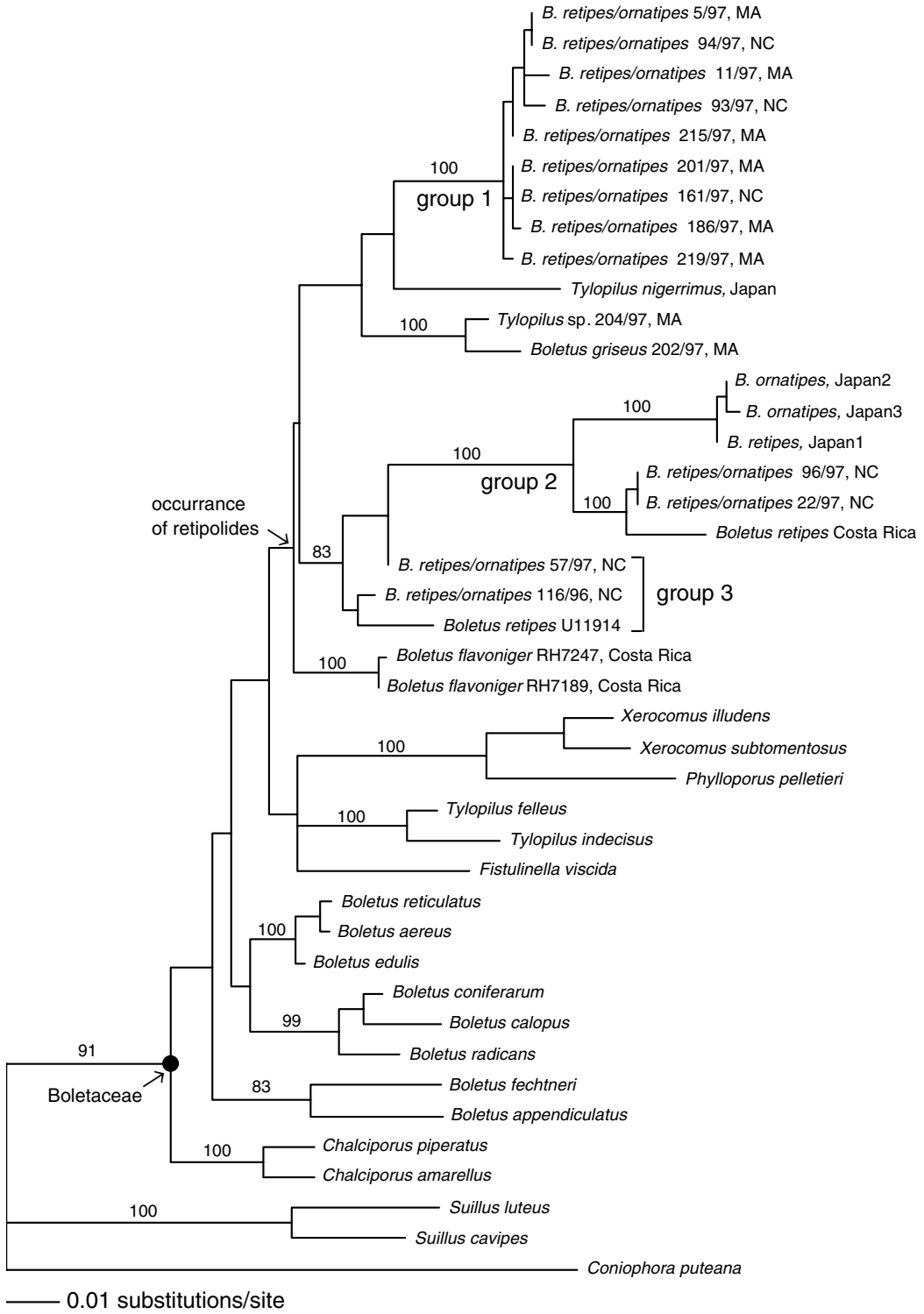
Discussion

Nuc-lsu sequence data indicate that the *B. retipes/ornatipes*-complex is distinct from other groups in the Boletaceae, specifically the genus *Boletus*. In addition, the results of this study confirm the findings of HELLOWIG (1999), who showed that different patterns of retipolides and other chemical components occur in *B. ornatipes* and *B. retipes* collections. Both sequence data and chemotaxonomical data allow to differentiate between groups in this puzzling species-complex for the first time.

Still, there are no morphological or anatomical characters available to distinguish *B. ornatipes* from *B. retipes*. The number of collections investigated, however, provides evidence of a vicariant geographical distribution. *B. retipes* was described from North

Fig. 1

Phylogenetic relationships of *Retiboletus* inferred from nuc-lsu (LR0R-LR5) sequences using maximum likelihood (tree score: $-\log L = 4357.71191$). Numbers above branches are bootstrap frequencies, values below 50% are not shown. Groups 1–3 are the groups proposed by HELLOWIG (1999), based on the distribution of retipolides. The common node, of which retipolides producing boletes arise, is indicated by an arrow



Carolina by BERKELEY & CURTIS in 1872. PECK (1878) described *B. ornatipes* six years later in Michigan, based on the absence of a pulverulent pileus. Unfortunately, PECK did not designate a type, but an authentic collection is available (BOTH 1993). Our own observations from Massachusetts and several other observations from Michigan and New York State (SMITH & THIERS 1971; BOTH 1993) suggest that *B. retipes* is not occurring in the northern part of Eastern North America. All collections made in Massachusetts (MA) are in group 1, lack a pulverulent pileus and stipe, and can be assigned to *B. ornatipes*. In addition, our results suggest that *B. ornatipes* occurs in North Carolina (NC) as well. For example, sequences of collections 5/97 (MA) and 94/97 (NC), respectively, 201/97 (MA) and 161/97 (NC) are identical.

B. retipes (groups 2, 3) is apparently more widely distributed than *B. ornatipes*. North Carolina is most likely the main distribution area of *B. retipes* in North America, where it overlaps with the distribution area of *B. ornatipes*. SINGER (1947) also reports *B. retipes* from South Carolina. For this reason, we conclude that *B. retipes* is limited to the southern part of Eastern North America. In addition, *B. retipes* occurs in the Neotropics and East Asia based on the collections from Costa Rica and Japan used in this study. A further separation of *B. retipes* into species originating from different continents is difficult with the data at hand. According to HELLWIG (1999), neither Substanz 102 nor xerocomic acid is detected in groups 2 and 3. In contrast, both components were constantly traced in group 1 (*B. ornatipes*). Group 3 is different from group 2 in producing retipolides B and D (HELLWIG 1999). In regard of missing morphological and anatomical characters, which could be used to separate group 2 and group 3, we cannot confirm a correlation between chemical and morphological evidence in this case.

Boletus griseus and *Tylopilus nigerrimus* are nested between *B. ornatipes* and *B. retipes* in our study. In some essential characters *B. griseus* resembles *B. ornatipes*, which led SMITH & THIERS (1971) to create a new subsection for both species, subsect. *Reticulati* SMITH & THIERS in the genus *Boletus*. Preliminary results of chemical screenings do not

point at the presence of retipolides (Arnold, pers. comm.) or pulvinic acid derivatives (BRESINSKY & BESL 1978) in *B. griseus*. The occurrence of so-called “chemical albinos” is not exceptional in the Boletales (e.g. *Tylopilus*) and explains the different colours in hymenophore and stipe of *B. ornatipes* and *B. griseus*, which are used for differentiation between the two species. *T. nigerrimus* (shown in IMAZEKI et al. 1988) is very similar to *B. griseus* and is distributed in East Asia. This species is not yet scanned for chemical compounds and has black tones in pileus and stipe. *B. flavoniger* is another species similar to *B. ornatipes* and *B. retipes*. It has recently been described from Costa Rica and differs from the previously mentioned species in an orange brown staining hymenophore and context and extremely bitter taste (HALLING & MUELLER 1999). The two collections of *B. flavoniger* RH7247 (Holotype) and RH7189 show differing chemical components (HELLWIG 1999). Both collections lack xerocomic acid, Substanz 102 is present. Retipolide B and retipolide D are only present in RH7247, which questions the taxonomical use of both metabolites on the species level again. The nuc-1su sequences of RH7247 and RH7189 only differ in one position and the collections are putatively conspecific.

The results of our analyses compared with the results of HELLWIG (1999) suggest that all retipolide producing fungi, including *T. nigerrimus*, *B. griseus*, and *B. flavoniger*, form a natural and unique group within the Boletaceae. This relationship is partly resolved in the maximum likelihood analysis and is not rejected by the WSR-test using maximum parsimony. We create the new genus *Retiboletus* to separate the species mentioned above from *Boletus* and *Tylopilus*, respectively, and five new combinations are made.

***Retiboletus* BINDER & BRESINSKY gen. nov.**

Retipolida inventa aut partim adsunt, acidi pulvinici derivata rarius desunt. Carposoma stipitatum pileatum, hymenophoro tubuliformi. Pileus siccus aut subtiliter tomentosus, niger, fusco-griseus vel olivaceus. Color hymenophori pallidus, griseolus vel flavum, brunnescens, aurantio-brunnescens, partim immutabi-

lis. Cystidia praesentibus. Stipes centralis, reticulatus, basim versus paulatim obscuriore. Contextus pallidus, flavidus vel luteus, plerumque in aurantio-brunneum mutatur, aut partim vix immutabilis. Fibulae nullae. Sporarum pulvis olivaceo-fuscus aut luteo-fuscus. Sporae leves, ellipsoideae vel subfusoidae, inamyloideae, vix dextrinoideae.

Typus: *Boletus ornatipes* PECK, Ann. Rept. N. Y. State Mus. 29: 67. 1878.

Retipolides are frequently occurring or exceptionally missing, in addition, pulvinic acid derivatives sporadically occur. Basidiocarps are stipitate-pileate with a tubular hymenophore. Pilei are convex to plane, dry to subtomentose, black, dark gray, mustard yellow to olive brown. The color of the hymenophores ranges from pallid, grayish to yellow, changing to brown or orange brown when bruised, sometimes not changing. Cystidia are present. A central stipe shows reticulate ornamentation, the base is usually darker than the apical region. The context is pallid, yellow or vivid yellow, changing to orange brown or slightly changing. Clamp connections are absent. Spore deposit is olive brown to yellow brown. The smooth spores are ellipsoid to subfusoid, inamyloid and partly dextrinoid.

New combinations

Retiboletus ornatipes (PECK) BINDER & BRESINSKY comb. nova

Basionym: *Boletus ornatipes* PECK, Ann. Rept. N. Y. State Mus. 29: 67. 1878.

Retiboletus griseus (FROST in PECK) BINDER & BRESINSKY comb. nova

Basionym: *Boletus griseus* FROST in PECK, Ann. Rept. N. Y. State Mus. 29: 45. 1878.

Retiboletus nigerrimus (R.HEIM) BINDER & BRESINSKY comb. nova

Basionym: *Boletus nigerrimus* R.HEIM, Rev. Mycol. Paris 28: 281. 1963.

Retiboletus retipes (BERKELY & CURTIS) BINDER & BRESINSKY comb. nova

Basionym: *Boletus retipes* BERKELEY & CURTIS, Grevillea 1: 36. 1872.

Retiboletus flavoniger (HALLING, G.M.MUELL. & L.D.GÓMEZ) BINDER & HALLING comb. nova

Basionym: *Boletus flavoniger* HALLING, G.M.MUELL. & L.D.GÓMEZ, Mycologia 91(5): 893–899. 1999.

Annotations to *Retiboletus ornatipes* (PECK) BINDER & BRESINSKY and *R. griseus* (FROST in PECK) BINDER & BRESINSKY

Retiboletus ornatipes, the type species of the genus, is one of the most showy representatives within the boletes, quite spectacular in its deep yellow colours shown in the flesh as well as in the other parts of the fruit bodies, painting the fingers yellow if touched. Since pulvinic acid derivatives are missing or at least only present in traces, no part of the fruiting body will stain blue after bruising. Instead, the colours will turn to grayish black or partially even black when the fungus is dried. The taste of the flesh is somewhat bitter.

As already stated by SMITH & THIERS (1971), the cystidia of *R. ornatipes* (as well as those of *R. griseus*) exhibit some special features not to be found in other boletes. In their monograph it is indicated that the colour of the cystidia in KOH and in Melzer's reagent is a valuable aid to identify herbarium specimens as members of the subsection *Reticulati* including the two species (*R. ornatipes*, *R. griseus*) mentioned here. The cystidia are characterized as being "hyaline in KOH when fresh, however, when dried material is revived in KOH it shows strongly pigmented, ochraceous to bister, amorphous masses occupying most of the ventricose part of the cystidia. The same colored substances turn to dark red brown in Melzer's reagent" (the cystidia therefore are said to be dextrinoid).

Additional material of *R. ornatipes*, checked by us (No. 86-399, USA, North Carolina, Macon County, Ball Creek area, Coweeta Hydrologic. Lab, Highway 441 near Franklin, 04.09.1986, leg. W. STEGLICH & A. BRESINSKY, specimens deposited in the Botanische Staatssammlung München (M), exhibited the microscopical characters (Fig. 2) described by SMITH & THIERS (1971). The rather slender, bottle like to fusoid cystidia were filled with yellow

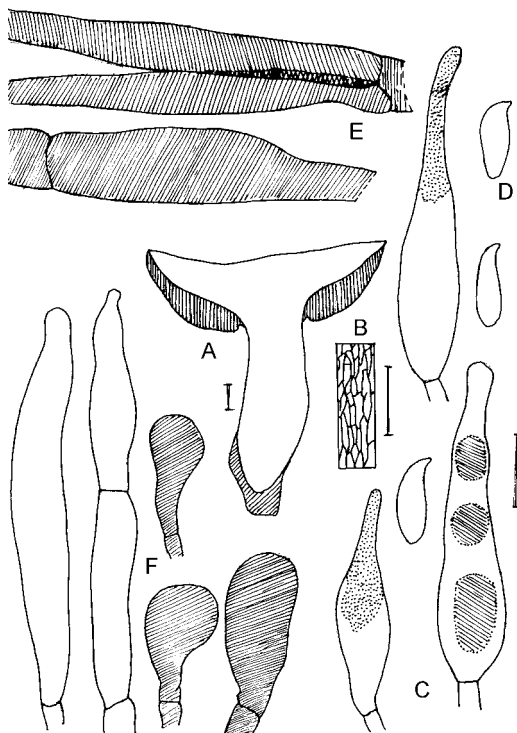


Fig. 2
Retiboletus ornatipes (M, No. 86-399, leg.
W. STEGLICH & A. BRESINSKY)

A — cross section through carpophore; B — net on the surface of upper part of the stipe; C — cystidia from the hymenium; D — spores; E — hyphae of pileipellis; F — hyphae and balloon like cells from cortical layer of stipe

Hatching applies to colour in Melzer's (C, E, F), dotted parts show colour reaction in KOH

Scale bar: A, B = 1 cm; C–F = 10 μ m

coloured contents distributed partially in the slender neck, partially also, as stated by SMITH & THIERS (1971), in the ventricose part of the cystidia. Pieces of the hymenophore mounted in Melzer's solution turned their dirty brownish colour to a very vivid red brown. In this condition the contents of the cystidia, rather big globular masses and smaller grains, are stained deeply orange brown. A similar (more or less dextrinoid) colour change in Melzer's could be observed in case of the hyphae composing the cortical layers of the cap and of the stipe as well. Here the contents of the hyphae turned in Melzer's to a vivid yellow colour. In the cortex of the stipe, besides sometimes branched lon-

gitudinal hyphae also balloon-like elements have been found showing the same colour change in Melzer's. These elements may be interpreted as the remnants of a reduced sterile hymenium on the surface of the stipe, however, the basidia of the hymenophore are slender and not globose. The spores are not clearly dextrinoid. In Melzer's the wall of the spores appears to be coloured a little bit darker, turning to brownish as compared to spores mounted in KOH. According to SMITH & THIERS (1971), the spores are dingy pallid yellow in KOH or a few ochraceous, in Melzer's pale orange tan.

Retiboletus griseus lacks the vivid and deep yellow colors of *R. ornatipes*. As an exception, a more or less faint yellow pigmentation may be observed. According to SMITH & THIERS (1971), there is some risk in such rare cases and *B. griseus* may be confused with *R. ornatipes*. However, the distinction between the two species is usually possible without any problem. The pallid context of *R. griseus* does not turn to blue; only a colour change to dingy vinaceous tones may be observed if exposed to the air. Microscopical characters have been reinvestigated in a collection from U.S.A. (No. 86-301, Tennessee, Smoky Mountains National Park, Cades Cove, 28.08.1986, leg. A. BRESINSKY, specimens deposited in M). The bottle like shaped cystidia (Fig. 3) exhibited yellowish brown or brown contents when observed in KOH and vivid yellow to brownish yellow masses in Melzer's reagent. The same colours in KOH, and in Melzer's respectively, have been seen in the hyphae composing the cortical layer of the pileus and of the stipe as well. FLORES ARZÙ & SIMONINI (2000) made similar observations on a *R. griseus* collection from Guatemala; as a difference to our material, the pleurocystidia showed tobacco brown contents in KOH and blackish brown contents in Melzer's reagent.

Similar to *R. ornatipes*, the stipe surface of *R. griseus* is composed of rather broad clavate to balloon shaped elements appearing in nest like clusters among longitudinal hyphae which partially are very slender. Most of all these elements show yellow colours in Melzer's, sometimes in form of globular masses (so especially within the balloon cells; Fig. 3). The majority of spores is nearly hyalin in Melzer's; a few spores, however, appear to be completely dextrinoid.

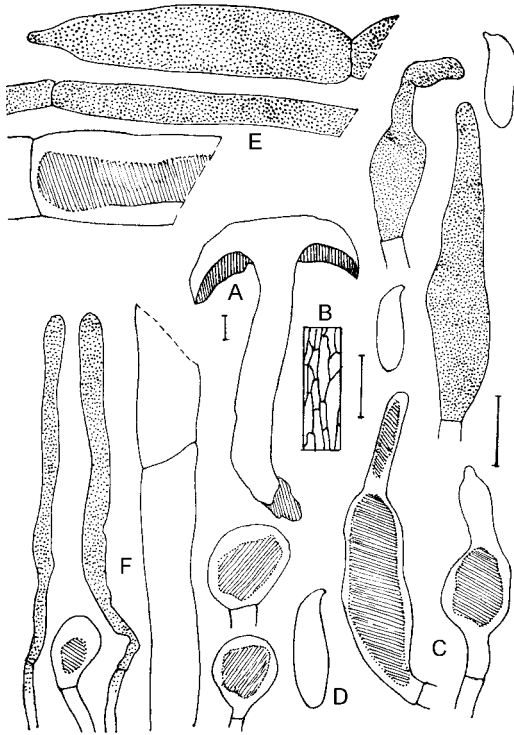


Fig. 3
Retiboletus griseus (M, No. 86-301, leg. A. BRESINSKY)

A — cross section through carpophore; B — net on the surface of upper part of the stipe; C — cystidia from the hymenium; D — spores; E — hyphae of pileipellis; F — hyphae and balloon like cells from cortical layer of stipe

Hatching applies to colour in Melzer's (C, E, F), dotted parts show colour reaction in KOH

Scale bar: A, B = 1 cm; C–F = 10 µm

Acknowledgements

We are grateful to Ernst E. Both for helpful comments on the *Boletus retipes/ornatipes*-complex. Special thanks are due to Norbert Arnold, Roy Halling, and all others who provided their collections for sequencing. The senior author is indebted to Prof. Dr. Wolfgang Steglich for so many inspiring suggestions; the detection and isolation of retipolides and the elucidation of their structure have been carried out by him and his co-workers. Our study was supported by a grant (BR 217/12-2 to A. Bresinsky and M. Fischer) of the Deutsche Forschungsgemeinschaft (DFG).

References

- BESSETTE, A. E.; ROODY, W. C. & BESSETTE, A. R. 2000: North American Boletes. A color guide to the fleshy pored mushrooms. – Syracuse University Press.
- BINDER, M. & BESL, H. 2000: 28S rDNA sequence data and chemotaxonomical analyses on the generic concept of *Leccinum* (Boletales). A.M.B., Italy. *Centro Studi Micologici*. – *Micologia* **2000**: 1–82.
- BINDER, M. & BRESINSKY, A. 2002: Derivation of a polymorphic lineage of Gasteromycetes from boletoid ancestors. – *Mycologia* **94**: 83–96.
- BOTH, E. E. 1993: The Boletes of North America. A compendium. 436 pp. – Buffalo, New York.
- BRESINSKY, A. & BESL, H. 1978: Notizen über Vorkommen und systematische Bewertung von Pigmenten in Höheren Pilzen (3). Untersuchungen an Boletales aus Amerika. – *Z. Mykol.* **45**: 247–264.
- BRESINSKY, A.; JAROSCH, M.; FISCHER, M.; SCHÖNBERGER, I. & WITTMANN-BRESINSKY, B. 1999: Phylogenetic relationships within *Paxillus* s.l. (Basidiomycetes, Boletales): Separation of a Southern Hemisphere genus. – *Plant. Biol.* **1**: 327–333.
- FELSENSTEIN, J. 1985: Confidence limits on phylogenies: an approach using the bootstrap. – *Evolution* **39**: 783–791.
- FLORES ARZÚ, R. & SIMONINI, G. 2000: Contributo alla conoscenza delle Boletales del Guatemala. – *Riv. Micologia* **2**: 121–145.
- GILL, M. & STEGLICH, W. 1987: Pigments of fungi (Macromycetes). – *Prod. Chem. Org. Nat. Prod.* **51**: 1–17.
- HALLING, R. E. & MUELLER, G. M. 1999: New boletes from Costa Rica. – *Mycologia* **91**: 893–899.
- HELLWIG, V. 1999: Isolierung, Strukturaufklärung und chemotaxonomische Untersuchung von Sekundärmetaboliten aus Pilzen. – Univ. München, Fak. Chemie und Pharmazie, Diss.
- HERMANN, R. 1980: Untersuchungen zur Konstitution, Synthese und Biosynthese von Pilzfarbstoffen. – Univ. Bonn, Diss.
- IMAZEKI, R.; OTANI, Y. & HONGO, T. 1988: Fungi of Japan. – Tokyo.
- JUSTUS, K. 1993: Untersuchungen über die Inhaltsstoffe des nordamerikanischen Röhrlings *Boletus retipes*. – Univ. Bonn, Diss.
- LEE, S. B. & TAYLOR, J. W. 1990: Isolation of DNA from fungal mycelia and single cells: 282–287. – In: M. A. INNIS; D. H. GELFAND; J. J. SNINSKY & T. J. WHITE (eds.), PCR protocols, a guide to methods and applications. – San Diego, California.

- MADDISON, D. R. 1991: The discovery and importance of multiple islands of most-parsimonious trees. – *Syst. Zool.* **40**: 315–328.
- SINGER, R. 1947: The Boletoidae of Florida. The Boletineae of Florida with notes on extralimital species III. – *Amer. Midl. Nat.* **37**: 1–135.
- SINGER, R. 1986: The Agaricales in Modern Taxonomy. 4. ed. – Königstein.
- SMITH, A. H. & THIERS, H. D. 1971: The Boletes of Michigan. – Ann Arbor, Michigan.
- SWOFFORD, D. L. 1998: PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0. – Sunderland, Massachusetts: Sinauer Associates.
- TEMPLETON, A. R. 1983: Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. – *Evolution* **37**: 221–244.
- THOMPSON, J. D.; GIBSON, T. J.; PLEWNIAK, F.; JEANMOUGIN, F. & HIGGINS, D. G. 1997: The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. – *Nucl. Acids. Res.* **24**: 4876–4882.
- VILGALYS, R. & HESTER, M. 1990: Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. – *J. Bacteriol.* **172**: 4238–4246.

Addresses of the authors:

Dr. Manfred Binder, Clark University, Department of Biology, Sackler Science Building, N-301, 950 Main Street, Worcester, MA 01610, U.S.A.;
Prof. Dr. Andreas Bresinsky, Universität Regensburg, Fakultät für Biologie und Vorklinische Medizin, Botanisches Institut, Postfach, D-93040 Regensburg, Germany.

Manuscript received: December 12th, 2001.