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# Derivation of a polymorphic lineage of Gasteromycetes from boletoid ancestors

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***Abstract:*** The phylogeny of selected gasteromycetes and hymenomycetes was inferred from partial nuclear large subunit rDNA (nuc-lsu, 28S) sequences, delimited by primers LR0R and LR5. Taxon sampling with emphasis on relationships within the Boletales further included some gasteroid groups, which obviously have evolved convergent fruiting body morphology, and therefore remained controversial in taxonomy. This study confirms the close relationship of Geastrales, Gauteriales and Phallales and the presumable derivation of Nidulariales and Tulostomatales within the euagarics clade, as widely accepted. In addition, four *Hymenogaster* species investigated were found to be in the euagarics clade and a relationship to the Cortinariaceae was indicated. The gasteroid fungus *Zelleromyces stephensii* is an example for maintaining morphological linkage by a lactiferous hyphal system to the genus *Lactarius* in the Russulales, and this relationship was affirmed in the sequence analysis. Several previously suggested relationships of gasteromycetes and Boletales were reproducible by analyzing nuc-lsu sequences. As a new result, *Astraeus hygrometricus*, the barometer earth star, is an additional representative of the Boletales. Together with *Boletinellus*, *Phlebopus*, *Pisolithus*, *Calostoma*, *Gyroporus*, *Scleroderma*, and *Veligaster*, *Astraeus* forms an unusual group comprising pileate-stipitate hymenomycetes and polymorphic gasteromycetes. This group is a major lineage within the Boletales and we propose the new suborder Sclerodermatineae, including the six families Boletinellaceae fam. nov. (*Boletinellus* and *Phlebopus*), Gyroporaceae (Singer) fam. nov. (*Gyroporus*), Pisolithaceae (*Pisolithus*), Astraeaceae (*Astraeus*), Calostomataceae (*Calostoma*), and the typus subordinis Sclerodermataceae (*Scleroderma* and *Veligaster*). Morphological and ecological characters, and pigment synthesis support the delimitation of the Sclerodermatineae, and indicate the radiation of different lineages in the Boletales originating from fungi with primitive tubular hymenophores. We regard such boletes with gyroid-boletinoid hymenophores, like *Boletinellus*, *Gyrodon*, *Gyroporus*, *Paragyrodon* and *Phlebopus* as key taxa in the evolution of Paxillineae, Sclerodermatineae and Boletineae.

***Key Words:*** *Astraeus*, *Boletinellus*, *Calostoma*, *Gyroporus*, *Pisolithus*, *Phlebopus Scleroderma*, nuc-lsu rDNA, taxonomy

## INTRODUCTION

The Boletales is a predominantly ectomycorrhizal group of homobasidiomycetes and is traditionally characterized by pileate-stipitate fruiting bodies developing tubular hymenophores. The geographical extension of these fungi is worldwide, but main distribution areas are the North American continent and Southeast Asia (Singer 1965, Smith and Thiers 1971, Corner 1972). Several recent approaches have been taken to evaluate the systematic and taxonomic structure of the Boletales, using morphology and anatomy (Watling 1970, Smith and Thiers 1971, Corner 1972, Pegler and Young 1981, Moser 1983, Singer 1986, Agerer 1999), pigment chemistry (Besl et al 1986, Gill and Steglich 1987, Høiland 1987, Besl and Bresinsky 1997), and sequencing analyses (Bruns and Palmer 1989, Bruns et al 1998, Bresinsky et al 1999, Kretzer and Bruns 1999). All of these studies suggested that there is a huge variety in morphology of basidiocarps and hymenophores within the Boletales. For example, the occurrence of pulvinic acids and derivatives, which are the master pigments in the Boletales (Gill and Steglich 1987), in combination with prenylated phenols, and benzoquinones, and grevillins led to the creation of the suborder Suillineae (Besl and Bresinsky 1997), which comprises fungi with lamellate (Gomphidiaceae) or tubular hymenophores (Suillaceae), and gasteromycetes (Rhizopogonaceae). This relationship of fungi with different organized basidiocarps was first shown in a molecular study by Bruns et al (1989).

The discovery of hydroxylated pulvinic acids in *Pisolithus arhizus* (Gill and Watling 1986) and halogenic substituted pulvinic acid derivatives in *Scleroderma sinnamariense* (Arnold et al 1996) suggested a close

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relationship of the Boletales to the gasteroid Sclerodermatales. Additional evidence of this relationship was contributed by the anamorph ascomycete *Sepedonium* (teleomorph *Hypomyces*), which exclusively attacks members of the Boletales (Ammer et al 1997, Besl et al 1998, Sahr et al 1999) and infrequently attacks *Pisolithus* (Gill and Watling 1986). Sequence analyses by Bruns et al (1998), Hibbett et al (1997), and Hughey et al (2000) on three different nuclear and mitochondrial rDNA regions confirmed a close phylogenetic relationship of the Boletales, *Pisolithus*, *Scleroderma*, and *Calostoma*. This result was surprising, since *Calostoma* is placed in the Tulostomales and the gasteroid basidiocarps show a unique morphology. The closest relatives to *Calostoma*, *Pisolithus* and *Scleroderma* within the Boletales are apparently *Boletinellus*, *Gyroporus* and *Phlebopus* (Bruns et al 1998, Hughey et al 2000), which have been placed in the Gyrodontaceae. Interestingly, there is a disagreement about the placement of the Gyrodontaceae between Singer (1986), who merges it in the Boletaceae, and Smith and Thiers (1971), who assume an evolutionary lineage to *Boletinus cavipes* (*Suillus cavipes*) which has a similar hymenophore. So-called gyroid-boletinoid hymenophores with distinct lamellate structures connected by irregular anastomoses, occur in the genera *Gyrodon* and *Boletinellus* and likewise in *Boletinus*. Resembling a honey-comb structure, the gyroid-boletinoid hymenophore has been regarded as precursor of the regular tubular hymenophore (Bresinsky 1996).

Recently, Agerer (1999) introduced several rhizomorph types as conserved characteristics to evaluate higher level relationships of the homobasidiomycetes. Comparing his results with chemical and molecular data, Agerer (1999) transferred the Sclerodermataceae and the Pisolithaceae in the Boletales, based on the boletoid rhizomorph type. Agerer (1999) also regarded the presence of clamps in boletoid rhizomorphs as the plesiomorphic condition, and therefore concluded that the Pisolithaceae is more closely related to the Paxillaceae (incl. Gyrodontaceae) than to the Sclerodermataceae. In addition, he suggested including the Astraeaceae in the Boletales, without providing a precise placement because of insufficient data.

Considering all the evidence, the taxonomic situation is still ambiguous. The Gyrodontaceae, when defined to include *Boletinellus*, *Gyrodon*, *Gyroporus*, *Paragyrodon* and *Phlebopus* is evidently an artificial taxon (Bruns et al 1998) and cannot be supported. Relationships of the polyphyletic Gyrodontaceae to the gasteroid genera *Calostoma*, *Pisolithus* and *Scleroderma* have been disregarded in taxonomy so far. A final question concerns the placement of the barometer earth star *Astraeus hygrometricus* (Astraeaceae), which was placed in the Calostomataceae by Fischer (1933), in the Boletales.

This study aims to clarify phylogenetic relationships of gasteromycetes within the Boletales and to evaluate the systematic significance of morphological and chemical characters mentioned above. A representative selection of species was examined using the 5′ portion of the nuclear large subunit rDNA (ca 900 bp), which has been repeatedly used for inferring phylogeny in Boletales and in Agaricales (Binder and Besl 2000, Moncalvo et al 2000).

## MATERIAL AND METHODS

*Fungal material.*—Sources, collection information, and GenBank accession numbers (AF336238–AF336274) are listed in TABLE I. Dried specimens or cultures of species used in this study are deposited in the Institut für Botanik (REG), Universität Regensburg. Taxon sampling was based on an unpublished data set (Binder 1999), including 197 taxa analyzed with neighbor-joining. The selection of 37 species covered a wide spectrum of taxa according to current taxonomy in Boletales and Sclerodermatales s. l., as well as controversial gasteroid genera. *Phlebopus beniensis* was excluded from this study. It is not closely related to other *Phlebopus* species, like *P. portentosus* and *P. sudanicus* (Binder 1999), but to the genus *Pulveroboletus*. We also used 13 sequences from our previous studies and 15 other sequences downloaded from GenBank.

*DNA isolation and polymerase chain reaction.*—150 mg cultured mycelium or 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 and 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 (3 M) 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. Different DNA concentrations in several stock solutions were balanced by adding adequate portions of TE buffer. DNA of dried *Astraeus*, *Scleroderma*, and *Veligaster* species was isolated from mature gleba tissue using the DNeasy Plant Mini Kit (Qiagen), to remove pigments that interfere with DNA amplification.

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 of primers LR0R and LR7 (Vilgalys and Hester 1990), and 1 U *Taq*DNA polymerase (Eurogentec). The final volume was adjusted to 100 μL with sterile $H_2O$. 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 s), extension 72 C (1.5 min). PCR products were purified with the QIAquick PCR cleaning Kit after an examination on agarose gels.

*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 μL BigDye (with *AmpliTaq* polymerase), 8 pmol primer, and 3.5 μL LR0R-LR7 product. The PCR program was: 96 C denaturation (2 min), 47 C annealing (15 s), 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. Sequences were assembled using Chromas 1.43 (http://trishul.sci.gu.edu.au/conor/chromas.html).

*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 (No. S639). 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. The trees were rooted with *Geastrum nanum*, *G. rufescens*, *Gautieria otthii*, and *Hysterangium stoloniferum*

Unconstrained analyses were performed with maximum likelihood (ML) and maximum parsimony (MP) 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 $\alpha = 0.5$. The heuristic search with TBR branch swapping used a starting tree obtained via neighbor-joining. In addition, one hundred bootstrap replicates (Felsenstein 1985) were run with maximum likelihood. The MP analysis using equally weighted parsimony included two steps. One thousand heuristic search replicates were conducted with random addition sequences in the first step, keeping 10 trees per replicate. In the second step, TBR branch swapping was applied on the shortest trees found in step one. One thousand bootstrap replicates were performed with the same settings as in the heuristic MP analysis, but keeping 100 trees per replicate.

Due to different tree topologies (see RESULTS) comparing ML and MP trees, constrained topologies were created using MacClade 3.0 (Maddison and Maddison 1992). Five constrained topologies were introduced forcing hypothesized monophyly of the ambiguous groups, with emphasis on the occurrence of gyroid-boletinoid hymenophores, in each keeping the rest of the tree unresolved. Constrained heuristic searches with maximum parsimony were performed as described above. The ten shortest constrained and unconstrained MP trees were compared with the Kishino-Hasegawa (K-H) test (Kishino and Hasegawa 1989) and the Wilcoxon signed-ranks (WSR) test (Templeton 1983; TABLE II). In addition, a second maximum parsimony analysis was compiled for a subset of 16 species using the branch and bound search method (Hendy and Penny 1982), computing initial upper bound via stepwise, furthest addition sequence, and keeping minimal trees only (FIG. 3).

## RESULTS

The aligned nuc-lsu rDNA data set of 63 sequences covered 948 positions after excluding ambiguous positions 420–430 and 801–804; 534 characters were constant, 99 variable characters were parsimony-uninformative, and 315 characters were parsimony-informative. The phylogenetic analysis with maximum parsimony resulted in 96 equally parsimonious trees in 12 islands, each with a length of 1668 steps (CI = 0.368, RI = 0.650). Tree 1/96 is shown in FIG. 1. There are four major clades supported by higher bootstrap values congruent with the shortest tree found using maximum likelihood (-ln likelihood = 9710.0410), which is presented in FIG. 2: the given outgroup clade (Geastrales, Gauteriales and Phallales), the russuloid clade (Russulales), the euagarics clade (Agaricales incl. Nidulariales, Tulostomatales, and Hymenogastrales pr. p.), and Boletales (incl. Sclerodermatales and Hymenogastrales pr. p.). Besides the Geastrales, Gauteriales, and Phallales, where morphological appearance is usually gasteroid, several relationships of Gasteromycetes and Hymenomycetes are indicated in the remaining clades. In the Russulales, *Zelleromyces stephensii* is apparently closer to *Lactarius* than to *Russula*. Furthermore, different stages of gasteromycetation are evident in the euagarics clade. However, because of limited taxon sampling, relationships can only be described approximately. *Tulostoma brumale* (Tulostomales), which develops a stalk bearing a globular spore sac, shows affiliations to both Agaricaceae and Amanitaceae. *Crucibulum laeve* and *Cyathus striatus* (Nidulariales) with bird nest-like fruiting bodies seem to be close to the Coprinaceae (represented by *Psathyrella gracilis*). *Hymenogaster decorus*, *H. olivaceus*, *H. tener* and *H. vulgaris* (Hymenogastraceae) have reduced stipes and a chambered gleba, and are next to *Hebeloma crustuliniforme*, a member of the Cortinariaceae. These findings suggest that gasteromycetation has occurred repeatedly in the Agaricales, as has been previously suggested (Thiers 1984, Hibbett et al 1997).

The Boletales are supported in the ML and MP analyses with bootstrap values of 89% and 60%, respectively. The suborders Coniophoroineae (resupinate brown rotters) and Tapinellineae (stipiate-pileate brown rotters with gilled hymenophore) could not be separated based on the taxa included in this study (*Coniophora puteana*, *Leucogyrophana olivas-*

TABLE I. Fungal species, collection information and GenBank accession numbers

| Fungal species | Substrate/host | Country | Leg./det. | Voucher No. | GenBank access. No. |
|---|---|---|---|---|---|
| *Astraeus hygrometricus* (Pers. : Pers.)Morg. | *Pinus* | Switzerland | L. Krieglsteiner | Ashy3 | AF336238 |
| *Boletinellus merulioides* (Schwein.) Murr | mixed forest | U.S.A | N. Arnold, W. Helfer | 22/98 | AF336239 |
| *Boletus edulis* Bull. : Fr. | *Picea* | Austria | W. Seidl | Be2 | AF336240 |
| *Boletus radicans* Pers. : Fr. | *Quercus* | Germany | N. Arnold | Brad1 | AF336241 |
| *Boletus satanas* Lenz | *Quercus, Carpinus* | Germany | L. Krieglsteiner | Bs2 | AF336242 |
| *Calostoma cinnabarina* Desvaux | mixed forest, soil | U.S.A. | N. Arnold | 141/96 | AF336243 |
| *Chalciporus piperatus* (Bull. : Fr.) Bat. | *Picea* | Germany | M. Binder | Cp1 | AF336244 |
| *Chamonixia caespitosa* Roll. | *Picea* | Germany | H. Besl | 92/83 | AF336245 |
| *Crucibulum laeve* (Huds. : Pers.) Pers. | rotten branch | Germany | H. Besl | Crul1 | AF336246 |
| *Cyathus striatus* (Huds. : Pers.) Pers. | rotten branch | Germany | H. Besl | Cyst1 | AF336247 |
| *Gastroboletus turbinatus* (Snell) AH Smith & Thiers | coniferous trees | U.S.A. | N. Arnold/ J. Ammirati | 19/95 | AF336248 |
| *Gautieria otthii* Trog | *Pinus* | Canada | J. Trappe | 636 | AF336249 |
| *Geastrum nanum* Pers. : Pers. | forest glade | Germany | L. Krieglsteiner | WÜ4396 | AF336250 |
| *Geastrum rufescens* Pers. : Pers. | *Quercus* | Germany | L. Krieglsteiner | WÜ2344 | AF336251 |
| *Gyroporus castaneus* (Bull. : Fr.) Quél. | mixed forest | Germany | A. Bresinsky | Gc1 | AF336252 |
| *Gyroporus castaneus* | mixed forest | U.S.A. | N. Arnold | 239/97 | AF336253 |
| *Gyroporus cyanescens* (Bull. : Fr.) Quél. | *Pinus, Betula* | Germany | M. Binder | Gcy2 | AF336254 |
| *Hymenogaster decorus* Tul. | n/a | Germany | L. Krieglsteiner | n/a | AF336255 |
| *Hymenogaster olivaceus* Vitt. | *Tilia* | Germany | H. Besl | Hyoll | AF336256 |
| *Hymenogaster tener* Berk. & Br. | n/a | Germany | L. Krieglsteiner | n/a | AF336257 |
| *Hymenogaster vulgaris* Tul. apud Berk. & Br. | n/a | Germany | L. Krieglsteiner | n/a | AF336258 |
| *Hysterangium stoloniferum* Tul. & Tul. | *Quercus, Pinus* | Germany | L. Krieglsteiner | WÜ3706 | AF336259 |
| *Phlebopus portentosus* (Berk. & Br.) Boedijin | n/a | Africa | K. Wanecek | n/a | AF336260 |
| *Phlebopus sudanicus* (Har. & Pat.) Heinem. | n/a | Africa | D. Thoen | CBS 481.89 | AF336261 |
| *Pisolithus arhizus* (Scop. : Pers.) S Rauschert | mixed forest, soil | U.S.A. | A. Bresinsky | 588 | AF336262 |
| *Scleroderma areolatum* Ehrenb. | *Quercus* | Germany | M. Binder | Sar1 | AF336263 |
| *Scleroderma bovista* Fr. | n/a | Germany | L. Krieglsteiner | WÜ1149 | AF336264 |
| *Scleroderma cepa* Pers. : Pers. | dunes, soil | Tasmania | A. Bresinsky | 184 | AF336265 |
| *Scleroderma citrinum* Pers. : Pers. | *Pinus, Betula* | Germany | H. Besl | Sc1 | AF336266 |
| *Scleroderma dictyosporum* Pat. | Dipterocarpaceae | Malaysia | N. Arnold/H. Besl | MS55 | AF336267 |
| *Scleroderma echinatum* (Petri) Guzmán | Dipterocarpaceae | Malaysia | H. Besl | MS34 | AF336268 |
| *Scleroderma geaster* Fr. | mixed forest | U.S.A. | A. Bresinsky | 594 | AF336269 |

TABLE I. Continued

| Fungal species | Substrate/host | Country | Leg./det. | Voucher No. | GenBank access. No. |
|---|---|---|---|---|---|
| *Scleroderma sinnamariense* Mont. | Dipterocarpaceae | Malaysia | H. Besl, N. Arnold | MS46 | AF336270 |
| *Scleroderma verrucosum* (Bul. : Pers.) Pers. | mixed forest | New Zealand | A. Bresinsky | 5 | AF336271 |
| *Tulostoma brumale* Pers. : Pers. | soil | Germany | M. Binder | Tub2 | AF336272 |
| *Veligaster columnaris* (Berk. & Br.) Guzmán | Dipterocarpaceae | Malaysia | H. Besl | MS43 | AF336273 |
| *Zelleromyces stephensii* (Berk.) AH Smith | n/a | Germany | L. Krieglsteiner | WÜ3706 | AF336274 |

Sequences obtained from GenBank: *Agaricus campestris* L. – U85273; *Amanita citrina* Pers. : Pers. – AF041547; *Coniophora puteana* (Schumach.) P Karst. – AF098377; *Gomphidius glutinosus* (Schaeff.) Fr. – AF071530; *Gyrodon lividus* (Bull.) Fr. – AF098378; *Hebeloma crustuliniforme* (Bull.) Quél. – U11918; *Lactarius corrugis* Peck – U11919; *Leccinum aurantiacum* (Bull.) SF Gray – AF139689; *Leccinum holopus* (Rostk.) Watling – AF139697; *Leucogyrophana olivascens* (Berk. & MA Curtis) Ginns & Weresub – AF167678; *Melanogaster tuberiformis* Corda – AF167679; *Paragyrodon sphaerosporus* (Peck) Sing. – AF071531; *Paxillus filamentosus* Fr. – AF098384; *Paxillus involutus* (Batsch: Fr.) Fr. – AF098385; *Pholiota squarrosa* (Müll. :Fr.) Kumm. – AF056458; *Psathyrella gracilis* (Fr.) Quél. – AF041533; *Rhizopogon subcaerulescens* AH Smith – AF071534; *Russula mairei* Sing. – U11926; *Russula virescens* (Schaeff. : Zanted.) Fr. – AF041548; *Serpula lacrymans* (Wulf.) J Schröt. – AF098402; *Suillus cavipes* (Opat.) AH Smith and Thiers – AF071535; *Suillus sinuspaulianus* (Pomerl. & AH Smith) Sing. – AF071536; *Tapinella panuoides* (Batsch) Gilb. – AF098394; *Xerocomus illudens* (Peck) Sing. – AF139714; *Xerocomus subtomentosus* (L.) Fr. – AF139716.

*cens*, *Serpula lacrymans*; *Tapinella panuoides*). The Suillineae is well confirmed by molecular data (bootstrap = 100%, 98%). This clade includes the ectomycorrhizal species *Suillus (Boletinus) cavipes*, *S. sinuspaulianus* (tubular-boletinoid hymenophore), *Gomphidius glutinosus* (gilled hymenophore) and *Rhizopogon subcaerulescens* (hymenogasteroid). In contrast to the findings of Bruns et al (1998), *Melanogaster tuberiformis* is not in the Suillineae, but in the Paxillineae. The delimitation between the ectomycorrhizal Paxillineae and Boletineae is not resolved due to the polyphyly of the Gyrodontaceae (*Boletinellus*, *Gyrodon*, *Gyroporus*, *Paragyrodon*, *Phlebopus*), which are members of the Paxillineae with gyroid-boletinoid hymenophores. For example, *Gyrodon lividus* is closer to the Boletaceae including *Boletus*, *Chalciporus*, *Gastroboletus*, *Xerocomus*, *Leccinum*, and the gasteroid *Chamonixia*. *Paragyrodon sphaerosporus* is the only gyrodontoid species that is clearly placed in the Paxillineae (Paxillaceae and Melanogastraceae).

In the ML analysis (FIG. 1) *Boletinellus* and *Phlebopus* are the sister group to a group that includes *Astraeus*, *Pisolithus*, *Calostoma*, *Gyroporus*, *Scleroderma* and *Veligaster*. This clade is strongly supported (bootstrap = 100%) and we further call it the new suborder ''Sclerodermatineae'', which will be established in this study (see TAXONOMY and DISCUSSION). In the Sclerodermatineae, *Boletinellus* and *Phlebopus* form the possible starting point for gasteromyceta-

TABLE II. Comparison of unconstrained trees and constrained trees using the Kishino-Hasegawa test and the Wilcoxon signed-ranks test.

| Model | Trees | Steps | Islands | CI | RI | K-H, *P** | WSR, *P*** |
|---|---|---|---|---|---|---|---|
| Unconstrained | 96 | 1668 | 12 | 0.368 | 0.650 | (best) | (best) |
| Constraint 1 | 238 | 1670 | 32 | 0.367 | 0.650 | 0.7239 − 0.7817 | 0.7237 − 0.7866 |
| Constraint 2 | 312 | 1670 | 44 | 0.367 | 0.650 | 0.7520 − 0.7857 | 0.7518 − 0.7903 |
| Constraint 3 | 108 | 1673 | 16 | 0.366 | 0.649 | 0.4661 − 0.5354 | 0.4668 − 0.5335 |
| Constraint 4 | 144 | 1678 | 23 | 0.365 | 0.647 | 0.0489* − 0.1048 | 0.0499** − 0.1013 |
| Constraint 5 | 244 | 1677 | 31 | 0.366 | 0.647 | 0.1172 − 0.2077 | 0.1172 − 0.2128 |

* Approximate probability of getting a more extreme test statistic under the null hypothesis of no difference between the two trees (two-tailed test).

** Wilcoxon signed-ranks test statistic is the smaller of the absolute values of the two rank sums. Asterisked values in table indicate significant difference at $P < 0.05$.

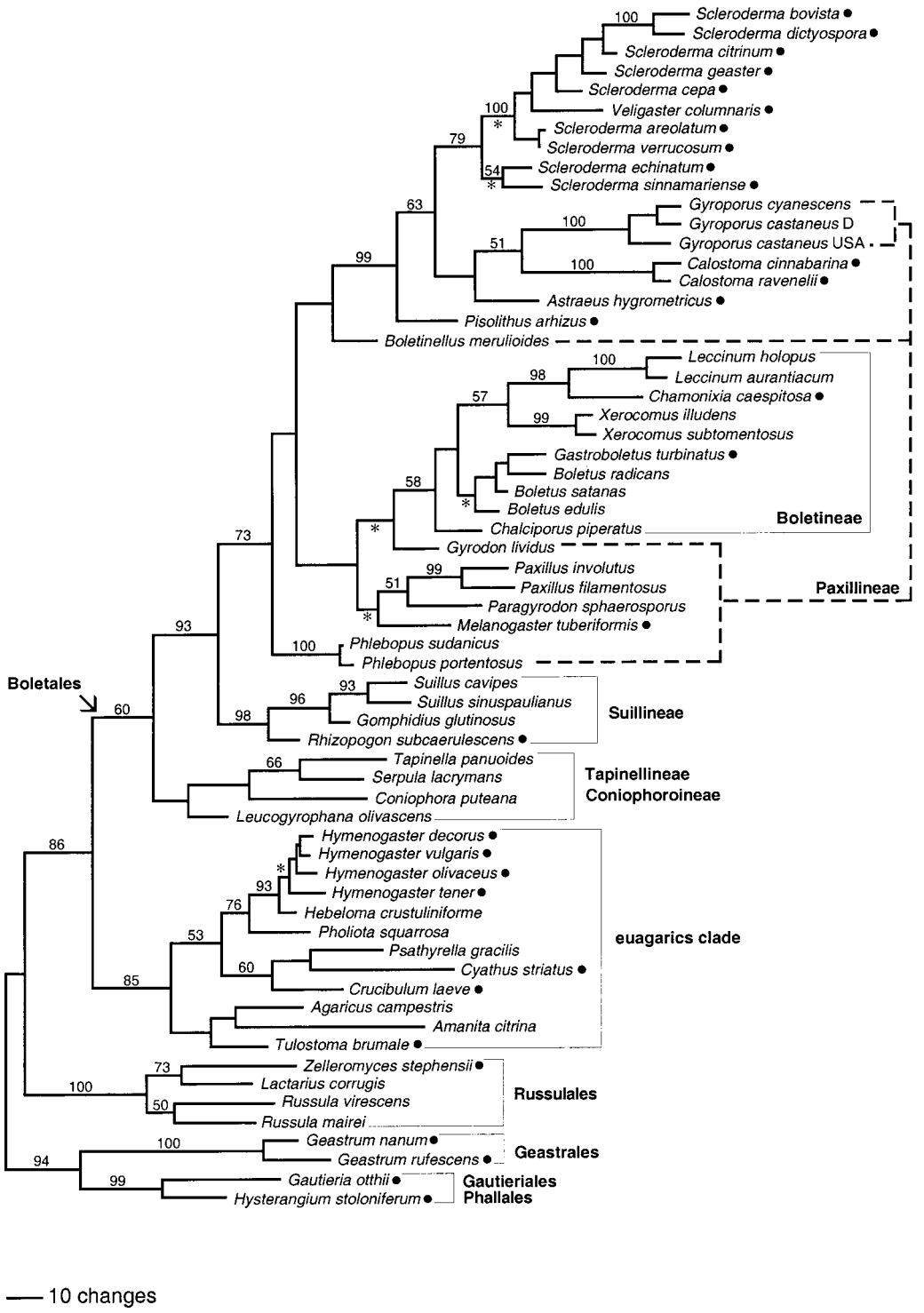


FIG. 1. Tree 1 of 96 equally parsimonious trees inferred from unweighted parsimony analysis using the heuristic search method of the complete 28S dataset. Numbers at nodes indicate bootstrap indices over 50% obtained after 1000 replicates. Branches with asterisks collapse in the strict consensus tree. The dashed line marks off the Paxillineae including the polyphyletic Gyrodontaceae as presented in current taxonomy. Names of clades are indicated on the right side or at arrows on the left. Taxa followed by black dots are gasteromycetes.

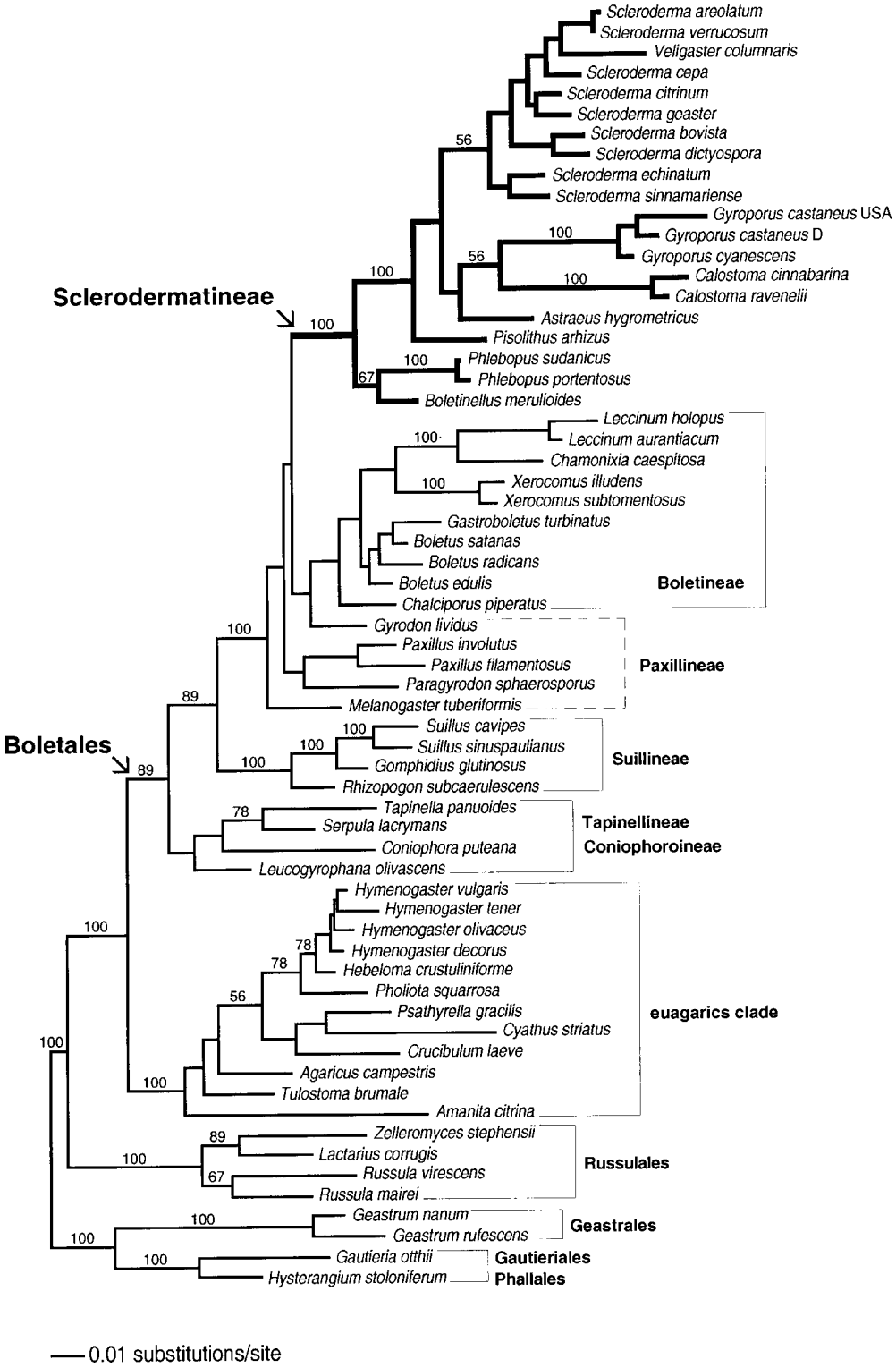


FIG. 2. Shortest tree recovered from the complete 28S dataset with maximum likelihood using the heuristic search method. Bootstrap indices over 50% from 100 replicates are indicated at internodes. The dashed line marks off the Paxillineae, which is still unresolved after the exclusion of *Boletinellus*, *Gyroporus*, and *Phlebopus*. Names of clades are indicated on the right side of at arrows on the left. Bold branches indicate the Sclerodermatineae, the new suborder established in this study.

tion, but the position of *Phlebopus* is ambiguous (see below). *Astraeus hygrometricus* is found to be a new member of the Boletales. The fruiting body morphology of *A. hygrometricus* is therefore a convergent development to the fruiting bodies of the Geastrales. The sister group to *Astraeus* is a weakly supported clade including *Calostoma* and *Gyroporus*, both confirmed by long internodes and 100% bootstrap frequencies. Intraspecific variation occurs in the sequences between European and North American collections of *Gyroporus castaneus*, which only differ in size of the basidiocarps. Another group in the Sclerodermatineae is formed by *Scleroderma* and *Veligaster*, which differs from *Scleroderma* by the development of a stipe and a pseudoveil. The three sections of *Scleroderma* (Guzmán 1970), sect. *Scleroderma* (hyphae with clamps, spores reticulate), sect. *Sclerangium* (hyphae with clamps, spores subreticulate), and sect. *Aculeatispora* (hyphae without clamps, spiny spores) are not resolved in this analysis. The Sclerodermataceae (*Scleroderma*, *Veligaster* and *Pisolithus*) are not supported as monophyletic clade.

The heuristic maximum parsimony search (FIG. 2) does not support the placement of *Phlebopus* in the Sclerodermatineae, and suggests possible relationships to Suillineae and to Paxillineae. To evaluate the conflicting topologies, we tested five constrained topologies forcing hypothesized monophyly of the groupings that come into question.

Constraint 1: *Phlebopus* and *Boletinellus* (forced to form a clade); Constraint 2: *Phlebopus*, *Boletinellus*, *Pisolithus*, *Astraeus*, *Calostoma*, *Gyroporus*, *Scleroderma* and *Veligaster*; Constraint 3: *Phlebopus*, *Gomphidius*, *Suillus* and *Rhizopogon*; Constraint 4: *Phlebopus*, *Gyrodon*, *Paxillus*, *Paragyrodon* and *Melanogaster*; Constraint 5: *Astraeus*, *Calostoma*, *Pisolithus*, *Scleroderma* and *Veligaster*.

Constrained and unconstrained MP trees were compared with the K-H test and the WSR test (TABLE II). In both tests, the monophyly of *Boletinellus* and *Phlebopus*, Sclerodermatineae incl. *Phlebopus*, *Phlebopus* and Suillineae, and the gasteroid genera of the Sclerodermatineae (constraints 1–3, 5) could not be statistically rejected, the monophyly of *Phlebopus* and the Paxillineae (constraint 4) was significantly worse and was partly rejected. To estimate branching order distribution, we used constraints 1–4 to filter 61 859 bootstrap trees obtained by maximum parsimony. Constraint 1 filtered 27 493 trees (1674 steps, 44.5%), constraint 2 filtered 24 111 trees (1674 steps, 38 9%), and constraint 3 filtered 4982 trees (1678 steps, 8%). No tree was recovered using constraint 4 as a filter. To determine the number of bootstrap trees that exclusively support the monophyly of the Sclerodermatineae including *Phlebopus,* 27 493 trees obtained by filtering with constraint 1 were re-filtered with constraint 2. We received 15 128 trees (1674 steps, 55%), which are 24.5% of the bootstrap trees overall. The results suggest that the monophyly of the Sclerodermatineae could not be confirmed in maximum parsimony analyses using a heuristic search. The Gyrodontaceae was polyphyletic in both MP and ML analyses, but a placement of *Phlebopus* could not be assigned with confidence. Focussing on the phylogenetic relationships of *Phlebopus*, we reduced the data set to 16 representative species and performed an additional maximum parsimony analysis using the branch and bound algorithm. This analysis recovered 4 most parsimonious trees (a 100% majority-rule tree is shown in FIG. 3), which confirm *Boletinellus* and *Phlebopus* as sister groups. In agreement with the maximum likelihood analysis, *Boletinellus* and *Phlebopus* are the sister group to *Astraeus*, *Calostoma*, *Gyroporus*, *Pisolithus*, and *Scleroderma*.

## TAXONOMY

Ordo Boletales Gilbert:

**Sclerodermatineae** Binder & Bresinsky subordo nov.

*Typus subordinis:* Sclerodermataceae Corda (1842), Icones Fungorum hucusque cognitorum I-VI, 23.

Carposoma stipitatum pileatum hymenophoro tubuliformi instructum aut habitu gastroideo loco epigaeo vel subepigaeo crescens. Cyclopentenona et acidi pulvinici derivata raro inventa. Fibulae aut adsunt aut rarius desunt. Carposoma si gastroidum est, simile subglobi aut tuberis, perraro pileatum aut multarum radicum, peridio simplici aut non simplici. Cum maturescit, inordinate rumpitur glebamque pulverulentam aut raro pseudoperidiolis emittit. Gleba aut albidi vel flavi aut fusci vel nigri coloris, capillitia plerumque desunt, rarius adsunt. Statismosporae subglobosae vel globosae, lêves, structuris verrucosis echinatesque atque ornamentis subreticulatis aut reticulatis instructae. Carposomatis stipitati pileati stipes saepe vacuus cavernosus, glaber vel leviter fibrillosus, sine ornamentis reticulatis. Hymenophorum tubuliforme merulioideum, boletinoideum, crassis aut tenuibus poris instructum. Carposomatis trama albida vel flavida, plerumque immutabilis, partim color in cyaneum mutatur. Sporae ellipsoideae et lêves; sporarum pulvis flavidus.

Basidiocarps are stipitate-pileate with tubular hymenophore or gasteroid and grow epigeal or subepigeal. Cyclopentenones and pulvinic acid derivates occur sporadically. Clamps are present or exceptionally absent. Gasteroid basidiocarps are subglobose or tuberous, sporadically stipitate or with multiple mycelial rootlets, peridium simple or composed of several layers. At maturity, gasterocarps open irregularly and release the pulverulent gleba; infrequently pseudoperidioles are present. The color of the gleba is whitish to yellow or black brown to black, capillitia are

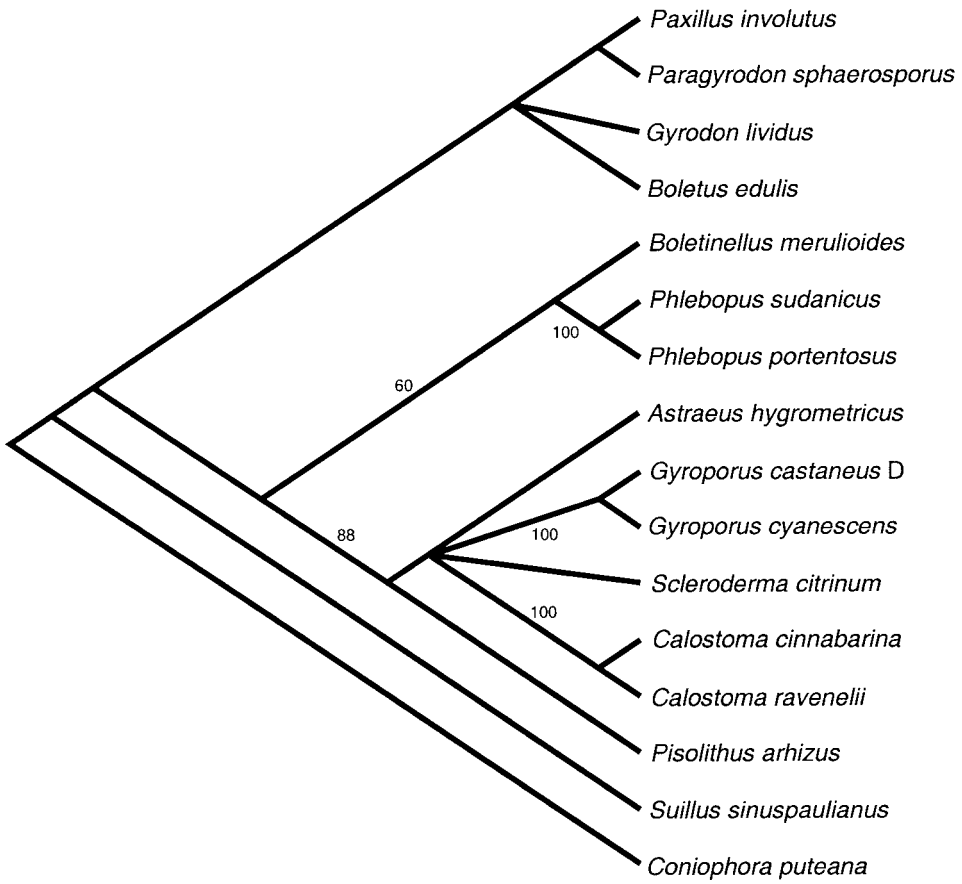


FIG. 3. Maximum parsimony analysis of a subset of 16 species using the branch and bound algorithm. 100% majority-rule tree of 4 equally most parsimonious trees. Bootstrap values over 50% are indicated at internodes.

mostly absent, rarely present. Statismospores are subglobose to globose, smooth or verrucous-spinose, or partly with subreticulate or reticulate ornaments. Stipitate-pileate basidiocarps sometimes with hollow stipes. The stipe surface is glabrous to subfurfuraceous, without reticulate ornamentation. The tubular hymenophore is merulioid, boletinoid, fine-pored or coarse. The context is whitish to yellowish, mostly unchanging or in part turning to blue. Spores are ellipsoid and smooth; spore print is yellow.

**Boletinellaceae** Binder & Bresinsky fam. nov.

*Typus: Boletinellus* Murrill (1909), Mycologia 1: 7.

Carposoma stipitatum pileatum. Pileus nudus et lêvis aut subtiliter tomentosus, olivaceus vel fuscus. Stipes colore pari, basim versus paulatim obscuriore, paene lêvis, sine ornamento reticulato, excentricus aut centralis. Carposomatis excentrico stipite distinctis (*Boletinellus*) hymenophorum est boletinoideum decurrens. Carposomatis centrali stipite insignibus (*Phlebopus*) hymenophorum tubuliforme est poris admodum tenuis instructum ac circum stipitem depressum. Color hymenophori luteus, luteo olivaceus aut luteobrunneus. Caro pallide olivaceo-lutea vel flavida, colore carneo intermixto, immutabilis aut colorem leviter in subcaeruleum mutans; in stipite prope basim saepe caerulea, rufobrunneo aut nigro colore suffoso. Sporae lêves ellipsoideae vel subglobosae, inamyloideae et dextrinoideae. Sporarum pulvis luteolus, luteo-fuscus aut olivaceo-fuscus. Hyphae fibulis exornatae.

Basidiocarps stipitate-pileate. Pileus glabrous to subtomentose, olive brown to yellow brown. Stipe with same colors, darkening towards the base, almost smooth, without reticulate ornamentation, eccentric or central. Basidiocarps with eccentric stipe (*Boletinellus*) show a boletinoid, decurrent tubular hymenophore. Basidiocarps with central stipe (*Phlebopus*) show a narrow tubular hymenophore, which is depressed around the stipe. Color of hymenophore yellow to olive yellow or yellow brown. Flesh pale olive yellow, with pinkish flush, unchanging or slightly changing to pale bluish green; context of the stipe often turning blue, reddish brown or black towards the base. Spores smooth, ellipsoid to subglobose, inamyloid and dextrinoid. Spore print yellowish, yellow brown to olive brown. Hyphae with clamps.

**Gyroporaceae** (Singer) Binder & Bresinsky fam. nov.

Gyroporaceae Loquin (1981) nom. inval. according to art. 36.1 of the Code of Botanical Nomenclature.

*Basiomym*: Gyroporoideae Singer ex Singer (1986), The Agaricales in modern taxonomy, p. 739. Gyroporaceae Singer (1981) nom. nud. Persoonia 11: 296.

*Typus: Gyroporus* Quélet (1886), Enchiridion fungorum in Europa et praesertim in Gallia vigentium, p. 161.

Carposoma stipitatum pileatum. Pileus subtomentosus vel subsquamosus, coloribus luteis, fuscis vel rubidis tinctus. Stipes lêvis vel leviter fibrillosus, sine ornamento reticulato, partim vascuus cavernosus. Hyphae stipitis exteriores partim et inaequaliter ad axem longitudinalem et decussatim circum eundem crescunt. Hymenophorum tubuliforme circum stipitem depressum, albidum vel stramineum. Caro alba, immutabilis aut colorem in obscure cyaneum mutans (gyrocyaninum). Sporae lêves et ellipsoideae, stramineae, inamyloideae. Sporarum pulvis subflavus. Hyphae fibulis exornatae.

Basidiocarps stipitate-pileate. Pileus subtomentose to subsquamose, with yellowish, brownish or reddish colors. Stipe glabrous to fibrous, without reticulate ornamentation, partly with hollow chambers. Hyphae of the stipe cortex horizontally and irregularly arranged to the longitudinal axis. Tubular hymenophore depressed around the stipe, whitish to straw yellow. Flesh white, unchanging or turning to blue (gyrocyanin). Spores smooth and elliptic, straw yellow, inamyloid. Spore print yellowish. Hyphae with clamps.

## DISCUSSION

The present study and other phylogenetic rDNA analyses (Hibbett et al 1997, Bruns et al 1998, Kretzer et al 1999, Hughey et al 2000) provide evidence of the occurrence of several gasteroid lineages throughout the Hymenomycetes that have convergently evolved. It is likely that earth stars and stinkhorns together form a monophyletic group (Hibbett et al 1997). The closest relatives to the Geastrales, Phallales and Gautieriales are evidently ramarioid and gomphoid fungi as shown in the study of Pine et al (1999), where this group is called gomphoid-phalloid clade. This relationship was confirmed in the study of Humpert et al (2001).

Many gasteromycetes with chambered gleba or similarly organized lacunar cavities have been classified in the Hymenogastrales, however, the monophyly of the Hymenogastrales was soon questioned (Rehsteiner 1892). Instead it was suggested that hymenogasteroid fungi are polyphyletic and related to diverse agaricoid taxa (Bucholtz 1903). In support of this latter view, the gasteroid fungus *Zelleromyces stephensii* is found to be an example for plesiomorphic morphological structures like amyloid spores, sphaerocysts, and a lactiferous hyphal system containing latex (Pegler and Young 1979, Thiers 1984), which is characteristic for the genus *Lactarius* in the Russulales. In addition, phylogenetic relationships between other gasteromycetes and Agaricales become evident in the present analysis. A relationship of the genus *Hymenogaster* and the Cortinariaceae as demonstrated here, was assumed by Oberwinkler (1977), who noticed a similar morphology of basidia and spores. *Hymenogaster* was usually placed in the Hymenogasteraceae along with *Rhizopogon* and *Melanogaster* (Coker and Couch 1928). At the generic level our results are in contrast to the findings of Bruns et al (1998), which show a different species, *Hymenogaster sublilacinus*, in the Boletales close to *Suillus*. These conflicting results between different species suggest that *Hymenogaster* is polyphyletic and application of the generic name cannot be evaluated until the type species, *H. bulliardi*, is sequenced. The development of polymorphic gasteroid basidiocarps in the Agaricales, as displayed by Tulostomatales and Nidulariales, indicates that different modes and mechanisms are involved in gasteromycetation. It is likely that the Tulostomatales have evolved according to the secotioid syndrome hypothesis (Thiers 1984). Fruiting bodies of *Tulostoma brumale* are usually stalked and show a globose peridium, which contains the unchambered gleba (Coker and Couch 1928). However, a close relationship of *Tulostoma* and *Calostoma* has to be rejected according to the present analyses. The Nidulariales exhibit fundamentally different basidiocarps and their formation is not explained by Thiers' theory (1984) without assuming additional steps. *Cyathus striatus* and *Crucibulum laeve* produce cup shaped fruiting bodies and the spores are packed in peridioles, which are dispersed as a whole. The development of peridiole-like structures is convergent in the bird's nest fungi and *Pisolithus*.

In connection with the establishment of mycorrhizal interactions and host specificity, similar patterns of fruiting body diversity can also be observed in the Boletales. The sequence analysis indicates a close relationship between the recently created suborders Coniophoroineae and Tapinellineae (Agerer 1999). Due to the absence of mycorrhizal symbioses, the primitive shape of fruiting bodies and basic secondary metabolism, the Coniophoroineae are regarded as retaining the plesiomorphic morphology and nutritional mode of the Boletales (Besl et al 1986). According to our results, there are at least three ectomycorrhiza forming hymenogasteroid lineages occuring in the Boletales: *Rhizopogon* (Suillineae), *Melanogaster* (Paxillineae) and *Chamonixia* (Boletineae). In addition, *Chamonixia* and *Gautieria* are essentially unrelated; the former being a member of the Boletineae, and the latter being a member of the Gomphales. This placement agrees with other recent studies (Bruns et al 1998, Humpert et al 2001), but dis-

agrees with earlier ideas that were based on similarity of spore morphology (Thiers 1984, Bresinsky 1996). In *Gastroboletus turbinatus*, the process of gastromycetation is still not completed. This secotioid member of the genus *Boletus*, which resembles the habit of its closest relatives, shows a compressed hymenophore and spores are passively dispersed.

Another predominantly gasteroid lineage includes *Pisolithus*, *Astraeus*, *Gyroporus*, *Calostoma*, *Scleroderma* and *Veligaster*, and the sister group to this monophyletic clade are *Boletinellus* and *Phlebopus*. We cannot provide comprehensive characteristics to illustrate this peculiar group. However, a few examples show the nested correlations between these fungi that probably constitute a natural group. *Pisolithus arhizus* develops stipitate gasteroid fruiting bodies with definite peridioles containing the spores (Pegler et al 1995). Both *Scleroderma* and *Pisolithus* spores are enclosed by nutrient hyphae (trophocysts), which stimulate their growth (Guzmán 1970). The residues of the trophocysts represent the characteristic spiny or reticulate spore ornamentation, which does not originate from the eusporial layer (Guzmán 1970). *Astraeus hygrometricus* resembles earth stars (Geastrales) and shows highly specialized peridial layers like *Calostoma* species. According to the sequence analysis, *Calostoma* is closely related to *Gyroporus*, although both genera are strikingly different in appearance. In agreement with Hughey et al (2000), we are not convinced that the interwoven and gelatinized, stalk-like strands of hyphae developed by some *Calostoma* species are homologous to the stipe of a bolete. A similar structure appears in the cortical layer of *Gyroporus* stipes, which could explain this particular formation in *Calostoma* and present an anatomical homology between both genera. In *Gyroporus* species, Corner (1972) and Singer (1986) noticed a stipe layer composed of strongly interwoven hyphal strands, which originate from an external velar layer and the stipe surface. This structure strengthens the solidity of the stipe that becomes more or less hollow, but not gelatinous, and which has to lift the fully expanded pileus from below soil to the surface (pers obs).

We consider *Astraeus*, *Boletinellus*, *Calostoma*, *Gyroporus*, *Phlebopus*, *Pisolithus*, *Scleroderma* and *Veligaster* a natural group, not supported by the maximum parsimony analysis (FIG. 1), but by the maximum likelihood analysis (FIG. 2), and call it the Sclerodermatineae. Hughey et al (2000) demonstrated with a data set including basically the same species that maximum likelihood outperforms maximum parsimony using heuristic search under conditions of unequal rates of nucleotide substitutions. This inference is reinforced by the analysis of a subset of 16 species using the branch and bound algorithm (FIG. 3), which confirms the maximum likelihood tree topology. Within the Sclerodermatineae, well supported internodes and long terminal branches may indicate that this group has a faster rate of evolution than the rest of the Boletales. Interestingly, *Gyroporus cyanescens* and *G. castaneus* form an exlusive group with gasteromycetes. If gasteromycetation occurs in the Sclerodermatineae under the mode as suggested by Thiers (1984), we have to assume that it has happened starting from a *Boletinellus-Phlebopus-Gyroporus* lineage. There is evidence of a relationship between *Phlebopus* and *Gyroporus*, which have identical spore wall ultrastructure (Singer 1986). We continue to assume that the gasteroid taxa in the Sclerodermatineae are monophyletic, a hypothesis which is not rejected by the Kishino-Hasegawa test and the Wilcoxon signed-ranks test (TABLE II). The incidence of several stipitate species in the Sclerodermataceae (Guzmán 1970), e.g., *Veligaster columnaris*, suggests that the hymenophore was enclosed and ballistospory vanished before a reduction of the stipe began. In addition, it is highly unlikely that gasteromycetation has been reversed (Savile 1955, 1968) and *Gyroporus* species have regained the ability of active spore discharge.

However, the position of *Phlebopus* was ambiguous in the heuristic analysis using maximum parsimony (FIG. 1) and we have to discuss possible relationships to *Paxillus* and *Suillus*. Comparisons of constrained and unconstrained trees by the K-H test and by the WSR test suggest that the monophyly of *Phlebopus* and the rest of the Sclerodermatineae, and the monophyly of *Phlebopus* and the Suillineae are likewise possible, whereas the relationship of *Phlebopus* and the Paxillineae was partly rejected (TABLE II). The secondary metabolism of the fungal groups involved is well investigated (Gill and Steglich 1987, Besl and Bresinsky 1997) and provides characteristics to evaluate conclusions drawn from DNA analyses. Besl et al (1996) showed that only *Gyrodon*, *Paxillus* and *Melanogaster* produce (-)-chamonixin and (-)-involutin. The synthesis of both pigments could therefore characterize the Paxillineae chemically. Species of distantly related taxa, like *Chamonixia*, *Leucogyrophana* and *Suillus*, synthesize the right-handed enantiomers, and preliminary screenings on the pigments of *Phlebopus* species solely recovered pulvinic acids (Bresinsky and Besl 1978). The members of the Suillineae characteristically produce prenylated phenols and quinones (Besl and Bresinsky 1997), which are also not found in *Phlebopus* species. Consequently, it is unlikely that *Phlebopus* (and any other member of the Sclerodermatineae) is closely related to the Paxillineae or the Suillineae based on comparison of the production of pigments.

If we consider boletoid rhizomorph subtypes, the

rhizomorphs of Suillaceae and Rhizopogonaceae show significant crystals and pigment droplets (Agerer 1999), which do not occur outside these families. The rhizomorph morphology of the Paxillineae (incl. Gyrodontaceae) and the Boletineae is very similar and therefore difficult to delimit (Agerer 1999). However, Agerer (1999) suggests how to differentiate Paxillineae rhizomorphs by the presence of clamp connections from Boletineae rhizomorphs without clamps. We basically accept this separation, but we cannot agree with Agerer's conclusion to transfer the Pisolithaceae into the Paxillineae and simultaneously the Sclerodermataceae into the Boletineae. In fact, clamps occur in below-ground hyphae of *Pisolithus* and *Scleroderma* (sects. *Sclerangium* and *Scleroderma*, not in sect. *Aculeatispora*) and *Boletinellus*, *Phlebopus*, *Astraeus*, *Gyroporus*, and *Calostoma* (Coker and Couch 1928, Guzmán 1970, Singer 1986). Therefore, we believe it is inappropriate to place *Scleroderma* in the Boletineae, the only suborder of the Boletales where clamps are usually absent.

Moreover, there is no bootstrap support for the monophyly of *Phlebopus* and the Paxillineae or the monophyly of *Pisolithus* and the Paxillineae, but branching order in 8% of the bootstrap trees does not reject relationships of *Phlebopus* and the Suillineae. In spite of a similar hymenophore morphology in *Boletinellus*, *Phlebopus* and *Boletinus* (*Suillus*), the nutritional mode of these fungi shows basic differences and makes a close relationship implausible. The members of the Suillineae form obligate ectomycorrhizal associations with Pinaceae (Singer 1986, Fischer et al 1997), while *Boletinellus* and *Phlebopus* species are root parasites and show mutualistic interactions with root parasitizing insects (Singer 1986, Brundrett and Kendrick 1987). The nutritional mode found in *Boletinellus* and *Phlebopus* species is an exceptional example of a transition between mutualists and parasites in the homobasidiomycetes.

In summary, we suggest that *Boletinellus*, *Gyroporus* and *Phlebopus* should be excluded from the Gyrodontaceae. Based on the limited species sampling in this study, we cannot evaluate the Gyrodontaceae including *Gyrodon* and *Paragyrodon*. We place *Phlebopus* and *Boletinellus* in the new family Boletinellaceae on the basis of morphological characters, similar nutritional requirements, and similar habitat preferences. With respect to the DNA analyses and the lack of distinct morphological transitions between *Pisolithus*, *Astraeus*, *Gyroporus*, *Calostoma*, *Scleroderma*, and *Veligaster*, these genera cannot be joined in a single family. We propose keeping the family status for the genera mentioned above. The Pisolithaceae (*Pisolithus*), the Astraeaceae (*Astraeus*), Calostomataceae (*Calostoma*), the Sclerodermataceae (*Scleroderma* and *Veligaster*), and the new families Gyroporaceae (*Gyroporus*) and Boletinellaceae (*Boletinellus* and *Phlebopus*), form the new suborder Sclerodermatineae.

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