The Lycoperdales. A molecular approach to the systematics of some gasteroid mushrooms

Dirk Krüger
Ernst-Moritz-Arndt-Universität Greifswald, Institut für Mikrobiologie und Molekularbiologie, Jahnstr. 15a, D-17487 Greifswald, Germany

Manfred Binder
Clark University, Department of Biology, Sackler Science Center N301, 950 Main Street, Worcester, Massachusetts 01610-1477

Michael Fischer
Universität Regensburg, Institut für Botanik, D-93040 Regensburg, Germany

Hanns Kreisel
Ernst-Moritz-Arndt-Universität Greifswald, Institut für Mikrobiologie und Molekularbiologie, Jahnstr. 15a, D-17487 Greifswald, Germany

Abstract: Based on selected taxa of Lycoperdales and Geastrales, phylogeny and delimitation of several gasteroid mushroom genera were investigated using RFLP and sequence analysis of 18S rDNA and ITS rDNA (ITS1-5.8S-ITS2). Support for an Agaricales-Lycoperdales relationship was found, the Geastrales being clearly separated from this lineage. Within the Lycoperdales, Lycoperdon was found to be polyphyletic, whereas Bovista appeared to be monophyletic.

Key Words: Basidiomycotina, Bovistella, Calvatia, capillitium, gasteromycetes, Geastrum, Lycoperdon, Morganella, Mycenastrum, Myriostoma, Vascellum

INTRODUCTION

Lycoperdales and Geastrales are among the gasteroid representatives of basidiomycetous fungi. Their members have basidiocarps that produce statisomospores. In gasteromycetes, the hymenia cover the inner surfaces of basidiocarps, in contrast to hymenomycetes where they cover outer surfaces (a hymenophore or the entire surface).

It is widely accepted that gasteroid basidiomycetes are derived from hymenoid basidiomycetes with secotoid forms linking both morphological types (Thiers 1984, Singer 1986). However, intermediates between hymenomycetes and gasteroid basidiomycetes are infrequent. For example, Kreisel (1967a) noted similarities in the shape of basidiocarp primordia, cap cuticle, and smell between Lepiota lycoperdoides Kreisel (a synonym to Cystolepiota cystidiosa [A.H. Smith] M. Bon) and Lycoperdon spp., which suggests a possible relationship. The possibility of an Agaricales-Lycoperdales relationship was recently supported by a molecular study (Hibbett et al 1997), in which three puffballs (Calvatia gigantea, Lycoperdon sp., and Tulostoma macrocephalum) were found nested within the euagaric clade, next to Macropleiota procera. This study also demonstrated that Lycoperdales and Geastrales have different phylogenetic origins, and Kreisel’s (1969) separation of the Geastrales based on morphological characters was therefore confirmed.

Capillitial threads are sterile hyphae in the gleba, which provide important characters in the current classification of puffballs and earthstars. In the Geastrales, including Geastrum Pers.: Pers., Myriostoma Desv., and a few other genera, the capillitium is similar to the Lycoperdon type, but shows thick hyphal walls (Sunhede 1989). In contrast to the Lycoperdales, generative hyphae have clamps on nearly all septa and the gleba is supported by at least one sterile columella in all members of the Geastrales.

Within the Lycoperdales, morphological characters such as the capillitium type, presence and structure of a subgleba, opening pattern of the endoperidium, and peridium anatomy are distinctive to the genera (Fischer 1993, Cunningham 1942, Pegler et al 1995, Calonge 1998). The different capillitium types can be described briefly as follows:

The “Lycoperdon type” is composed of long and coherent sclerified hyphae, aseptate or scarcely septate, without main stems, with circular to elliptical pits or without pits. An unpitted true capillitium is characteristic for Lycoperdon pyriforme Schaeff.: Pers., which is the only strictly lignicolous species in Lycoperdon. Capillitium threads here correspond to skeletal hyphae as characterized in the anatomy of poly pores (Cunningham 1946, Pegler et al 1995). This capillitium type is found in all species of Lycoperdon.
in some species of *Bovista*, and in the larger species of *Vascellum* F. Šmarda.

The “*Bovista* type” refers to a composition of several units of sclerified hyphae, mostly aseptate, loosely or densely ramified, with distinct main stems, with or without pits. They may correspond to the binding hyphae in the anatomy of poly pores (Kreisel 1994, 1998). This type occurs in *Bovista plumbea* and many other *Bovista ssp.*, as well as in *Bovistella* Morgan and *Calbovista* Morse. The “transitional type” is intermediate between the *Lycoperdon* type and the *Bovista* type: main stems are discernible, but interconnected by branches. It is typical for several species of *Bovista*, e.g., *Bovista limosa* Rostrup.

The “*Mycenastrum* type” is composed of separate units as in the *Bovista* type, but hyphal ramification is much reduced, and the hyphae are spiny. It is only found in *Mycenastrum corticum* (Guers.) Desv.

The “*Calvatia* type” has a coherent capillitium with loosely ramified hyphae as in the *Lycoperdon* type, but the hyphae are frequently septate. This type may be interpreted as sclerified generative hyphae as distinguished in the polypore anatomy (Kreisel 1994, 1998). It is found in all species of the genus *Calvatia* Fr. including *Langermannia* Rostk. and *Gastropila* A. Czern.

The “*Handkea* type” is similar to the *Lycoperdon* type, but walls exhibit long slit-like pits. Such pits are characteristic for all species of *Handkea* Kreisel (Kreisel 1989), but are typical for *Arachnion lloydianum* Demoulin, too.

In the smaller *Vascellum* species (Kreisel 1993) and in all species of the lignicolous genus *Morganella* (Kreisel and Dring 1966), no true capillitium is found. Instead, the gleba often contains “paracapillitium” (hyaline thin-walled hyphae with frequent clampless septa, also accompanying the capillitium in the other gasteroid taxa, especially *L. pyriforme*). It corresponds to generative hyphae of the poly pores (Kreisel 1993, 1994).

The present study further explores the relationships of gasteroid fungi to agarics, seeks evolutionary lineages within gasteroid orders and genera, and evaluates concepts of current taxonomy using RFLP and sequencing analysis of the nuclear encoded 18S rRNA gene and the ITS spacer region. Our attempts at gathering molecular data focused on inclusion of members of the smaller puffballs in the *Lycoperdaceae* (*Lycoperdon*, *Bovista*, *Bovistella*, *Morganella*, and *Vascellum*) plus the larger puffballs of *Calvatia* (*Lycoperdaceae*) and *Mycenastrum* (*Mycenastraceae*). Taxa were selected to represent several distinct capillitium types. We asked three major questions: i) are lycoperdaceous puffballs derived within the agarics, and what are their closest agaricoid relatives? ii) are *Bovista* and *Lycoperdon* monophyletic genera? and, iii) what is the phylogenetic status of *Lycoperdon pyriforme*?

### Materials and Methods

DNA extraction.—DNA was obtained either from cultures grown on Moser b-medium (Moser 1960), dried herbarium specimens, or fresh basidiocarps, following the extraction protocol of Lee and Taylor (1990) or a CTAB miniprep method (Zolan and Pukkila 1986, Doyle and Doyle 1987). Dried DNA was air-dried and resuspended in 100 μL TE buffer (10 mM Tris HC1, 1 mM Na2EDTA; pH 8.0). DNA was checked on 1% agarose gels (Molecular Biology Certified Agarose, Bio-Rad).

Polymere chain reaction (PCR) and sequencing.—We created two data sets: one for the nuclear small subunit (SSU) 18S rDNA and one for the spacer region (ITS1-5.8S-ITS2). PCR reactions were performed on a Biometra Trio-Thermocycler and included a hot start step (D’Aquilla et al 1991). A droplet of mineral oil (Sigma) covered each 100 μL reaction mixture. Partial SSU rDNA was amplified with primers NS3 and NS8 (White et al 1990) for genera Bovista, Bovistella, Lycoperdon, Morganella, Vascellum, and Lepiota. Lycoperdon pyriforme was represented in the ITS data set with 7 isolates. Reaction parameters mostly correspond to the protocol given above, but differ in annealing temperature (53.5 °C) and extension time (1 min).
TABLE 1. Binary-coded RFLP fragments of ITS1/ITS4 PCR product (undigested 890 bp). Fragment length in bp (read from top to bottom)

<table>
<thead>
<tr>
<th>Sample and species</th>
<th>Hin6I</th>
<th>HaeIII</th>
<th>Hpall</th>
<th>MboI</th>
<th>Hinfl</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAW B. radicata</td>
<td>00001</td>
<td>01001</td>
<td>00111</td>
<td>00011</td>
<td>00011</td>
</tr>
<tr>
<td>BNDW B. nigrescens</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>BPRU B. paludosa</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-6 B. plumbea</td>
<td>00001</td>
<td>01000</td>
<td>01000</td>
<td>01000</td>
<td>01000</td>
</tr>
<tr>
<td>DA-54 B. plumbea</td>
<td>00001</td>
<td>01000</td>
<td>01000</td>
<td>01000</td>
<td>01000</td>
</tr>
<tr>
<td>DA-27 B. plumbea</td>
<td>00001</td>
<td>01000</td>
<td>01000</td>
<td>01000</td>
<td>01000</td>
</tr>
<tr>
<td>DA-29 B. polymorpha</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-56 B. polymorpha</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>0462 B. pusilla</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-31 B. pusilla</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>0s22/91 V pratense</td>
<td>11000</td>
<td>00000</td>
<td>11000</td>
<td>00000</td>
<td>11000</td>
</tr>
<tr>
<td>LMAS L. marginatum</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-42 L. mammiforme</td>
<td>10000</td>
<td>10000</td>
<td>10000</td>
<td>10000</td>
<td>10000</td>
</tr>
<tr>
<td>LEVB L. echinatum</td>
<td>00001</td>
<td>01000</td>
<td>10000</td>
<td>01000</td>
<td>01000</td>
</tr>
<tr>
<td>DA-100 L. foetidum</td>
<td>11000</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-23 L. foetidum</td>
<td>11000</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-20 perlatum</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-45 perlatum</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-106 perlatum</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-110 perlatum</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-111 L. perlatum</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-1 L. molle</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
<tr>
<td>DA-16 L. umbrinum</td>
<td>11100</td>
<td>00000</td>
<td>11100</td>
<td>00000</td>
<td>11100</td>
</tr>
<tr>
<td>DA-57 L. lividum</td>
<td>00001</td>
<td>01000</td>
<td>10000</td>
<td>01000</td>
<td>01000</td>
</tr>
<tr>
<td>LLOS L. lividum</td>
<td>00001</td>
<td>00000</td>
<td>00001</td>
<td>00001</td>
<td>00001</td>
</tr>
</tbody>
</table>

20 s). ITS region PCR products were processed for the purpose of preliminary RFLP analyses as well as sequencing analysis.

PCR products serving as templates for sequencing reactions were purified using spin columns (QIAquick PCR Purification Kit, Qiagen), yielding a final volume of approximately 30 μL. ITS PCR products bound for restriction digestion were cleaned by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.7, Biotechnology Grade, Amresco), homogenizing, and centrifugation at 10,600 g for 15 min. Eighty FL of the aqueous phase was removed, mixed with 8 FL sodium acetate and 190 FL ethanol, and chilled at -20 C for several hours. After another centrifugation (15 min, -4 C, 12,900 g) the pellet was washed with 70% ethanol, and then resuspended in TE buffer.

Cycle sequencing products were generated using the ABI Prism BigDye Terminator Ready Reaction Kit (Applied Biosystems) with primers NS5/NS8 and ITS1/ITS4 (White et al 1990). Single stranded products were cleaned (10 μL reaction mixture, 2 μL 3 M sodium acetate at pH 4.8, 55 μL 100% ethanol-centrifuged for 15 min at 21 700 g / 4 C; pellet rinsed twice with 150 μL 70% cold ethanol), dried in a vacuum desiccator for about 1 h, and run on an automated ABI 377 DNA sequencer with a 5.25% acrylamide gel (PAGE-Plus, Amresco).

Restriction fragment length polymorphism (RFLP).—Only isolates with similar sizes of the ITS1/ITS4 PCR product were used in the restriction analyses. After an additional check of concentrations on an agarose gel, 7 to 12 μL of each PCR product was diluted to a final volume of 15 μL. Restriction digests were done with 7 endonucleases: Hinfl I, Mbo I, Hin6 I, Hpa II, Alu I (MBI Fermentas), Hae III (Boehringer Mannheim), and Nla III (New England Biolabs), according to the manufacturer’s instructions.

Digests were electrophoresed on 2.5 to 3% agarose gels (twoparts agarose, one part AmpliSize agarose, Bio-Rad) at 50 mA for several hours. Two lanes of standard size marker (MBI Fermentas or Boehringer Mannheim) were run simultaneously. The gels were subjected to 15 min of additional staining in water containing ethidium bromide (Bio-Rad). RFLP patterns were measured, recorded and coded as a binary data matrix corresponding to presence and absence of restriction fragments (Table I). A distance matrix
was calculated with the algorithm of Nei and Li (1979). The distance matrix was used in the neighbor-joining (Saitou and Nei 1987) module of PHYLIP v. 3.57 (Felsenstein 1993), and the output phylogram prepared as an unrooted network.

**Analysis of sequences.**—Complementary sequences were manually edited in SeqEd v. 1.0.3 (Applied Biosystems) and ambiguous base positions were coded in standard nucleotide codes. Four additional sequences drawn from GenBank were marked with GB in the analysis of the 18S data set. Automatically computed alignments using either ClustalW or ClustalX (Thompson et al. 1997) were corrected and manually adjusted in a text editor. Two variable regions in the spacer region data set (positions 140-150, 212-216) were excluded from the alignment. Alignments are available at TreeBASE (http://herbaria.harvard.edu/treebase/; study accession number S601). Sequences have been submitted to EMBL/GenBank/DDBJ databases (http://www.ebi.ac.uk/embl/submission; accession numbers see above). A NEXUS file containing all commands in a PAUP block (Maddison et al. 1997) was generated by aid of MacClade v. 3.04 (Maddison and Maddison 1992) and a text editor. Phylogenetic analyses were performed in PAUP 3.1.1 (Swofford 1990) and PAUP* 4.0 beta version (Swofford 1998) using Fitch Maximum Parsimony (Fitch 1971), in which character states are unordered and equally weighted. Gaps were treated as missing data. Heuristic search was carried out with 1000 replicates, random taxon addition sequence, TBR branch swapping, and MAXTREES set to 10 000. Heuristic bootstrap analyses (Felsenstein 1985) were performed with 100 pseudoreplicates. Trees were exported to graphics programs through TreeView (Page 1996).

To evaluate the results of the ITS region analysis, a constrained tree was created in MacClade, forcing the monophyly of *Lycoperdon* (including *L. echinatum*, *L. foetidum*, *L. mammiforme*, *L. perlatum*, and *L. pyriforme*). The constrained analysis involved a two-step heuristic search with 1000 replicates and MAXTREES set to 10 000. In the second step, branch swapping was performed on the shortest trees found in step 1 (saving a maximum of 100 trees in each replicate). Unconstrained and constrained trees were compared in PAUP* using the Kishino-Hasegawa test (Kishino and Hasegawa 1989) and the Wilcoxon signed-ranks (Templeton 1983).

**Light microscopy.**—Spores and capillitia were examined with a Zeiss Axioskop. Photography was done with a Zeiss MC 80 camera. Examples of capillitia are shown in Figs. 1A–E.

**RESULTS**

SSU rDNA sequence data.—The alignment of the NS5/NS8 sequences resulted in a total of 572 characters, which included 473 constant sites, 39 variable but parsimony-uninformative sites, and 60 parsimony-informative sites. The PAUP* analysis of the SSU data recovered one tree island (Maddison 1991) with 14 equally parsimonious trees with a length of 167 steps and the following scores: CI = 0.731, RI = 0.786, RC = 0.574. Phylogram 1/14, rooted with *Tremella foliacea* as outgroup, is shown in Fig. 2.

Parsimony analysis with increased taxon sampling for the Lycoperdales supports the results of Hibbett et al. (1997), showing the puffballs nested within the euagarics clade, supported by a bootstrap value of...
78%. *Lepiota cristata* (Agaricaceae) was found to be closer to *Mycenastrum corium* (Mycenastraceae) and the true puffballs (Lycoperdaceae) than to *Macrolepiota procera* and *M. konradii*. However, the weakly supported clade including all members of the Agaricales and the Lycoperdales used in this study collapses in the strict consensus tree. The branch leading to the Lycoperdaceae is confirmed with 72% bootstrap support, but neither *Calvatia*, nor *Bovista*, nor *Lycoperdon* appear to be monophyletic. Members of the *Calvatia* segregate *Handkea* could not be successfully amplified and are thus missing in our analysis. Our 18S sequence data did not robustly resolve relationships within a clade consisting of the smaller puffballs (*Bovista*, *Bovistella*, *Lycoperdon*, *Morganella*, *Vascellum*), yielding a polytomy in the consensus of the bootstrap analysis. The SSU data support the separation of the Lycoperdales and the Geastrales as proposed by Kreisel (1969) using morphological and anatomical characters. The earthstars, here represented by *Geastrum triplex*, *G. saccatum*, and *Myriostoma coliforme*, are sister group to the Phallales represented by *Mutinus caninus*, but are not closely related to the Lycoperdales. This result supports the findings of...
Hibbett et al (1997) that Geastrales and Phallales together with Gomphaceae form the gomphoid-phalloid clade (Pine et al 1999), which is separate from the cantharelloid clade represented here by Hydnum repandum.

**ITS region RFLP data.**—Focusing on the phylogeny of *Lycoperdon*, RFLPs of the more variable ITS rDNA region (ITS1-5.8S-ITS2) were used to evaluate the concept of this genus. Unfortunately, a number of key taxa (*Leptioa cristata, Lycoperdon pyriforme, Morganella subincarnata*) had different sized PCR products and were therefore excluded. All isolates included in subsequent analyses had PCR products of 890 bp length. The RFLP neighbor-joining analysis based on the data matrix of TABLE I (FIG. 3) is presented as an unrooted network due to the absence of an appropriate outgroup.

**Bovista** forms a separated group, as well as (i) *L. marginatum* and *L. perlatum* (five specimens from different locations), (ii) *L. echinatum* and *B. radicata*, and (iii) *L. mammiforme, L. molle, L. lividum, L. umbrinum*, and two *L. foetidum* specimens. Restriction digests with two additional enzymes (*Alu I, Nla III*) did not improve resolution concerning the position of *Bovista pusilla, B. polymorpha, B. paludosa*, and *B. nigrescens*.

**ITS region sequence data.**—Based on the RFLP data, species of *Bovista, Bovistella, Lycoperdon, Morganella* and *Vascellum* were selected for a sequencing analysis to compensate for the length heterogeneity of the ITS region PCR products. The chosen outgroup was *Leptioa cristata*. To survey the amount of intraspecific variability in *Lycoperdon pyriforme* we included 7 collections from different origins. The alignment of the spacer region sequences covered a total of 703 characters, including 535 constant characters, 84 variable and parsimony-uninformative characters, and 84 variable and parsimony-informative characters. Two equally parsimonious trees (*CI = 0.713, RI = 0.762, RC = 0.543*) with a length of 286 steps were found in one tree island, one of these is shown in FIG. 4.

Although the collections of *L. pyriforme* originate from different continents (Asia, Europe, and North America), sequences were identical for all strains in-vestigated. This indicates a stagnant rate of evolution in the rDNA spacer region of *L. pyriforme*, which to our knowledge has not been reported within a species so far. Further studies should address the alternative explanation of recent dispersal. A bootstrap value of 41% supports *L. pyriforme* as the sister group to the sparsely resolved genera *Bovista, Bovistella, Lycoperdon, Morganella* and *Vascellum*. The weakly supported *Bovista* clade collapses in the strict consensus tree. Within *Bovista, B. plumbea* and *B. paludosa* (*Bovista type capillitium*), and *B. pusilla* (*Lycoperdon type capillitium*) and *B. polymorpha* (*transitional type capillitium*) form two moderately confirmed groups. The clade including *Morganella subincarnata* (*paracapillitium*), *Lycoperdon perlatum* (*Lycoperdon type capillitium*), and *Vascellum pratense* (*Lycoperdon type capillitium* with abundant paracapillitium) collapses to a trichotomy in the bootstrap analysis. Short branches support a group composed of *Bovista radicata* (*Bovista type capillitium with large pits*), *Lycoperdon foetidum, L. echinatum*, and *L. mammiforme* (each showing the *Lycoperdon* type capillitium) with bootstrap values between 64 and 80%. We conclude that *Lycoperdon* is polyphyletic. A slightly unbalanced selection of specimens did not influence the resolution of the tree. If only one *L. pyriforme* sequence is used, the same topology is found in the most-parsimonious tree. The second most-parsimonious tree (under inclusion of 1 or 7 *L. pyriforme* sequences) only differs in shifting the *Bovista pusilla / B. polymorpha* clade into an unresolved position between *B. plumbea / B. paludosa* and the other puffballs.

The monophyly of *Lycoperdon echinatum, L. foetidum, L. perlatum, L. mammiforme, and L. pyriforme* was forced in a constrained analysis, leaving the rest of the tree unresolved. The shortest constrained tree required 9 additional steps, but is not rejected in the Kishino-Hasegawa test and in the Templeton test (TABLE II).

To illustrate the influence of the agaricoid outgroup...
**Lepiota cristata** on tree topology and possibly long branch effects, a heuristic search was performed under exclusion of **Lepiota**, and exclusion of 6 of the 7 **L. pyriforme** sequences. The resulting phylogram is shown as an unrooted network [(Fig. 5; 1 of 3 similar, equally parsimonious trees (209 steps, CI = 0.718, RI = 0.535, RC = 0.384) found in one tree island)]. **Lycoperdon pyriforme** is positioned on the longest branch. There is a **Bovista** group, and a group of **L. echinatum / L. mammiforme / L. foetidum / Bovistella**. The latter is the sister group to **Morganella / Vascellum / L. perlatum**, all three sitting on relatively long branches.
Light microscopy.—Spores and capillitia have been observed and in some cases documented with photographs. Capillitium threads of Geastrales (Figs. 1A, 1B) and Lycoperdales (Fig. 1C–E) differ in the width of the hyphal walls, elasticity, branching pattern, occurrence of pits, septa, and pointed appendages.

**DISCUSSION**

The phylogenetic origins of the Geastrales and the Phallales remain unresolved in this study (Fig. 2). The earthstars represent a separate lineage of gasteromycetes and have evolved independently of the Lycoperdales. In traditional taxonomy, Geastrales and Lycoperdales are basically distinguished by a different habitus and by distinct capillitial characters. Earthstar capillitia do not exhibit any pores or pits and are very thick-walled, leaving a narrow lumen in the hypha (Sunhede 1989).

The development of hyphal networks or capillitia during the formation of the gleba structure might be regarded as a convergent process in different groups of gasteroid fungi and therefore provide a way to detect major groups. *Myriostoma coliforme* (Geastrales) features a thick-walled capillitium forming thorns (Fig. 1B) similar to that of *Mycenastrum corium* (Lycoperdales), which is composed of thin-walled hyphae and organized in separate units (Fig. 1C). Capillitium types that are obviously similar may have resulted from convergent evolution, but in some cases they can provide evidence of relationships within definite gasteroid groups. SSU rDNA data (Fig. 2) show *Mycenastrum corium* as a true representative of the Lycoperdales. In addition, a moderate bootstrap value of 78% supports *M. corium* and *Lepiota cristata* as sister groups. This result confirms a close relationship of Lycoperdales and Lepiotaceae, which has not previously been resolved with confidence (Hibbett et al 1997). An extended analysis using additional sequence data from Johnson and Vilgalys (1998) corroborates the close relationship of the Lycoperdales and the Lepiotaceae (phylogram not shown). According to the molecular data obtained in this study as well as from the analyses of Hibbett et al (1997), Johnson and Vilgalys (1998), and Pine et al (1999), we suggest the inclusion of the Lycoperdales in the Agaricales as Lycoperdaceae, close to Lepiotaceae.

Within the Lycoperdaceae, an uncertainty remains with respect to the boundaries between *Lycoperdon* and *Bovista*. In contrast to the SSU data (Fig. 2), ITS region sequences (Fig. 4) suggest that *Bovista* is monophyletic as delimited by Kreisel (1967b). In *Bovista*, the type of capillitium (Kreisel 1964, 1967b) has been used as a taxonomic character. A transitional connection of *Bovista* species with *Bovista* type capillitium by species with *Lycoperdon* type capillitium (*B. pusilla*) and transitional type capillitium (*B. polymorpha*) to *Lycoperdon* could not be supported by RFLP analysis (Fig. 3). Next to the *Bovista* type capillitium (*B. paludosa, B. plumbea, B. nigrescens*), both the transitional type and the *Lycoperdon* type occur within *Bovista*, which makes capillititial characters...
equivocal to separate *Bovista* and *Lycoperdon*. Important characters in *Lycoperdon* are a cellular subgleba and a gleba with pseudocolumella (Pegler et al. 1995). In *Bovista* spp. with a developed subgleba, it is always compact; the gleba has no distinct pseudocolumella.

Based on the RFLP data, *Lycoperdon* appears to be polyphyletic, which leads to the problem of redefining the generic limitations between *Lycoperdon*, *Bovistella*, *Morganella*, and *Vascellum*. *Lycoperdon pyriforme* has abundant capillitium and paracapillitium, all *Morganella* spp. have only paracapillitium, *Vascellum pratense* has a much reduced capillitium and abundant paracapillitium while most other *Vascellum* spp. have only paracapillitium. Combining both morphological and our current DNA data, we can not provide a conclusive solution. Molecular studies on an increased number of taxa are needed to understand the phylogeny of *Lycoperdon* and allies. Preliminary RFLP data suggested that *Vascellum*, *Morganella*, and *Lycoperdon pyriforme* are distinct from the other *Lycoperdon* spp. However, *Morganella* and *L. pyriforme* had to be excluded from the RFLP analysis due to the different size of the PCR product. *Lycoperdon pyriforme* is the only *Lycoperdon* species growing strictly on wood and woody debris. Species of *Morganella* are also lignicolous (Kreisel and Dring 1966); the specimens used here, however, as one of the only two samples of the genus from Europe, was found in a *Sphagnum* bog (Besl et al. 1982). *Lycoperdon perlatum* sometimes is found on very decomposed wood (as were samples DA-20, DA-110, DA-111). *Vascellum* has no lignicolous representatives (Kreisel 1993). Members of *Bovista* section *Xyloperdon* (Kreisel 1967b) grow on the bark of living trees, not on dead wood. *Xyloperdon* samples were not available for this study.

The genus *Lycoperdon* is polyphyletic in all analyses shown in the present study. The topology of the unconstrained ITS phylogram (Fig. 4) differs from the RFLP analysis (Fig. 3) in the isolated position of *Lycoperdon perlatum*, while a clade including *L. maminforme*, *L. echinatum* and *L. foetidum* is confirmed with a bootstrap value of 80%. In the RFLP analysis (Fig. 3), *Lycoperdon lividum* specimens (from Scotland and Germany) are next to *L. molle*. The RFLP data suggest additionally that *L. foetidum*, *L. umbrinum*, *L. lividum*, *L. molle* and *L. maminforme* are not closely related to *L. perlatum*, the type species of *Lycoperdon*, or *L. echinatum*. Five samples of *L. perlatum* from different locations and *L. marginatum* cluster together in the RFLP analysis. The samples identified as *L. perlatum*, including DA-111 *L. perlatum* var. *bonordenii*, all yielded exactly equal restriction patterns. Another group combines *L. echinatum* and *Bovistella radicata*, in spite of considerably different morphological characters.

*Lycoperdon pyriforme* does not cluster with any other *Lycoperdon* species and could be segregated from the genus by additional characters. It is isolated within *Lycoperdon* not only for its obligate lignicolous habitat, but also for a few morphological characters. The subgleba remains white even in mature specimens, pits are lacking in the capillitium, and spherocysts in the outer layer of the exoperidium have a unique form (Demoulin 1972). The pear-shaped basidiocarps grow in clusters and are uniquely attached with conspicuous white mycelial strands. However, after the segregation of *L. pyriforme*, *Lycoperdon* remains polyphyletic in each analysis presented here. Therefore, we will not establish a new segregate genus to accommodate *Lycoperdon pyriforme* or transfer it to *Morganella* (see about similarities above) before more molecular data on species of Lycoperdaceae are available.

ACKNOWLEDGMENTS

DK wishes to thank Prof. Andreas Bresinsky, Dr. Helmut Besl, and other colleagues at Regensburg University for supporting his thesis. DNA sequencing was facilitated by the University Clinics of Regensburg. Mr. Günter Kolb (Regensburg, Germany) and Prof. Gabriel Moreno (Alcalá de Henares, Spain) helped us with spore microscopy. Thanks to Drs. Markus Scholler and Annemarthe Rubner (West Lafayette, Indiana), and Erika Retzlaff (Greifswald, Germany) for advice and encouragement in the course of this study. Also thanks are directed to collectors of specimens. Drs. Randy Small and Rick Weinstein (Knoxville, Tennessee), and the reviewers are thanked for helping improve earlier drafts.

LITERATURE CITED


Doyle JJ, Doyle JL. 1987. A rapid isolation procedure from
small quantities of fresh leaf tissue. Phytochem Bull 19:
11–15.
Felsenstein J. 1985. Confidence limits on phylogenies: an
Felsenstein J. 1993. PHYLIP (Phylogeny Inference Package) v.
Fischer E. 1933. Reihe Gasteromyceteae. In: Die Natürlichen
Pflanzenfamilien. 2nd ed. Vol. 7a. Leipzig, Germany:
Engelmann. 122 p.
Fitch WM. 1971. Toward defining the course of evolution:
Minimal change for a specific tree topology. Syst Zool
Hibbett DS, Pine EM, Langer E, Langer G, Donoghue MJ.
1997. Evolution of gilled mushrooms and puffballs inferred
Johnson J, Vilgalys R. 1998. Phylogenetic systematics of Le-
piota sensu lato based on nuclear large subunit rDNA
likelihood estimate of the evolutionary tree topologies
from DNA sequence data, and the branching order of
Feddes Repertorium 69:196–211.
Kreisel H. 1967a. Die Großpilze des Greifswalder Botanischen
Gartens. Wiss Z Ernst-Moritz-Arndt-Univ Greifswald,
Kreisel H. 1967b. Taxonomisch-pflanzengeographische Revi-
sion der Gattung Bovista. Beih Nova Hedwigia 25:1–
244.
Kreisel H. 1969. Grundzüge eines natürlichen Systems der Pil-
ze. Jena, Germany: Gustav Fischer Verlag/Cramer. 245
p, 8 pl.
Kreisel H. 1899. Studies in the Calvatia complex (Basidiomyc-
Kreisel H. 1993. A key to Vascellum (Gasteromycetes) with
Kreisel H. 1994. Studies in the Calvatia complex (Basidiomy-
etes) 2. Feddes Repertorium 105:369–376.
Kreisel H. 1998. Die Gattungen Calvatia und Handkea in Eu-
Kreisel H. 1966. An emendation of the genus Mor-
ganella Zeller (Lycoperdaceae). Feddes Repertorium
Lee SB, Taylor JW. 1990. Isolation of DNA from fungal my-
celium and single cells. In: Innis MA, Gelfand DH, Snin-
sky JJ, White TJ eds. PCR protocols, a guide to methods
and applications. San Diego, California: Academic
Maddison DR. 1991. The discovery and importance of mul-
tiple islands of most-parasimonious trees. Syst Zool 40:
315–328.
Maddison DR, Maddison WP. 1997. NEXUS: An ex-
tensible file format for systematic information. Syst Biol
46:590–621.
Maddison WP, Maddison DR. 1992. MacClade: analysis of
phylogeny and character evolution. v3. Clade: analysis of
phylogeny and character evolution. v3: Clade: analysis of
phylogeny and character evolution. v3. Sunderland,
Massachusetts: Sinauer Associates.
Bad Heilbrunn, Germany: J. Klinkhardt. 440 p.
Nei M, Li WH. 1979. Mathematical model for studying ge-
netic variation in terms of restriction endonucleases.
Proc Natl Acad Sci USA 76:5269–5273.
Page RDM. 1996. TREEVIEW: an application to display phy-
logenetic trees on personal computers. Comp Appl
Biosci 12:357–358.
Pegler DN, Lassøe T, Spooner BM. 1995. British puffballs,
earthstars and stinkhorns. Kew, England: Royal Botanic
Gardens. 255 p.
relationships of cantharellloid and clavarioid Homobas-
idiomycetes based on mitochondrial and nuclear rDNA
method for reconstructing phylogenetic trees. Mol Biol
Singer R. 1986. The Agaricales in modern taxonomy. König-
Swofford DL. 1990. PAUP: phylogenetic analysis using par-
simony. Version 3.1.1. Washington DC: Smithsonian In-
stitution.
Swofford DL. 1998. PAUP*: phylogenetic analysis using pars-
imony (*and other methods). Version 4.0. Sunderland,
Massachusetts: Sinauer Associates.
Templeton AR. 1983. Phylogenetic inference from restric-
tion endonuclease cleavage site maps with particular
reference to the evolution of humans and the apes.
Evolution 37:221–244.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Hig-
gins DG. 1997. The CLUSTAL_X windows interface:
flexible strategies for multiple sequence alignment aid-
4882.
and direct sequencing of fungal ribosomal RNA genes
for phylogenetics. In: Innis MA, Gelfand DH, Sninsky
JJ, White TJ eds. PCR protocols, a guide to methods
and applications. San Diego, California: Academic
Zolan M, Pukkila PJ. 1986. Inheritance of DNA methylation