Mycologia, 96(1), 2004, pp. 102–118.

An ITS phylogeny of *Leccinum* and an analysis of the evolution of minisatellite-like sequences within ITS1

Henk C. den Bakker[1]

Nationaal Herbarium Nederland, Universiteit Leiden branch, Phanerogams and Cryptogams of the Netherlands and Europe section, P.O. Box 9514, 2300 RA Leiden, The Netherlands

Barbara Gravendeel

Nationaal Herbarium Nederland, Universiteit Leiden branch, Molecular Systematics taskforce, P.O. Box 9514, 2300 RA Leiden, The Netherlands

Thomas W. Kuyper

Wageningen Agricultural University, Department of Environmental Sciences, Subdepartment of Soil Quality, P.O. Box 8005, 6700 EC Wageningen, The Netherlands

Abstract: Phylogenetic relationships of the European species of *Leccinum* (Boletales, Boletaceae) were investigated by maximum parsimony, Bayesian and likelihood analyses of nrITS1-5.8S-ITS2 and 28S sequences. The separate gene trees inferred were largely concordant, and their combined analysis indicates that several traditional sectional and species-level taxonomic schemes are artificial. In *Leccinum*, the nrITS region ranges in size from 694 to 1480 bp. This extreme length heterogeneity is localized to a part of the ITS1 spacer that contains a minisatellite characterized by the repeated presence of CTATTGAAAAG and CTAATAGAAAG core sequences and mutational derivatives thereof. The number of core sequences present in the minisatellite varied from 12 to 36. Intra-individual sequence variation of the minisatellite was always smaller than between different species, indicating that concerted evolution proceeds rapidly enough to retain phylogenetic signal at the infraspecific level. In contrast, the evolutionary pattern exhibited by the major ITS1 repeat types found was homoplastic when mapped onto the species lineages inferred from the combined 5.8S-ITS2 sequences. The minisatellite therefore appears not to be useful for phylogeny reconstruction at or above the species level.

Key words: Boletales, Fungi, ITS, *Leccinum*, minisatellites, phylogeny, tandem repeats

Accepted for publication June 24, 2004.

[1] Corresponding author. E-mail: bakker@nhn.leidenuniv.nl

INTRODUCTION

Leccinum S.F. Gray is a genus of exclusively obligate ectomycorrhizal boletes with whitish or yellowish flesh and dark(-ening) stipe scabrosities (Engel 1978, Den Bakker 2000). Its species are distributed mainly in the northern temperate zone (Singer 1986), and a few species are described from tropical areas. Mycorrhizal partners are mainly deciduous trees of the families Betulaceae and Salicaceae, but some species are associated with Fagaceae, Ericaceae and Pinaceae (Binder and Besl 2000).

Leccinum species have been traditionally sorted into sections based on a pigment causing discoloration of the context on exposure (present in sections *Leccinum* and *Luteoscabra* Singer, absent in section *Scabra* Smith) and cap cuticle (pileipellis) with palisade trichoderm (present in section *Luteoscabra*; Smith and Thiers 1971, Lannoy and Estades 1995) or cutis-like trichoderm (sections *Scabra* and *Leccinum*). Especially section *Scabra* is defined merely on the absence of characters present in other sections of *Leccinum*. Absence of characters often indicates symplesiomorphy and generally makes the group defined by such a character paraphyletic (Gravendeel et al 2001). Sequences of the nr 28S gene indicate that section *Scabra* is indeed paraphyletic and in need of revision (Binder and Besl 2000).

As with many groups of fungi, the phenotypic variation of possible diagnostic characters in *Leccinum* is poorly understood, as are sexual systems and phylogeographical patterns that can help to facilitate species assessment. As a result, there is considerable variation in the number of species recognized by different authors as different morphological and ecological characters are thought to be of importance for species recognition. In *Leccinum*, ecological characters are mainly restricted to host preferences. It is a matter of opinion whether a morphologically variable taxon growing on a different host is a new species or just an indication of a less stringent host range. Morphological characters in *Leccinum* are mainly based on the microscopical structure of the pileipellis and macroscopical characters such as the colour of the cap and discoloration of the context and the stipe scabrosities (Lannoy and Estades 1995).

The extremes for the European *Leccinum* mycota vary from 13 species according to Watling (1970),

TABLE I. Overview of different European taxa published within *Leccinum* section *Scabra*

Watling (1970)	Engel (1978)	Lannoy & Estades (1995)
L. holopus	*L. holopus*	*L. alboroseolum*
		L. aerugineum
		L. cyaneobasileucum
		L. holopus
		L. nucatum
		L. olivaceosum
L. scabrum	*L. subcinnamomeum*	*L. avellaneum*
		L. brunneogriseolum var. *brunneogriseolum*
		L. brunneogriseolum var. *pubescentium*
		L. brunneogriseolum fo. *chlorinum*
		L. rigidipes
	L. rotundifoliae	*L. rotundifoliae*
	L. scabrum	*L. scabrum* var. *scabrum*
	L. melaneum	*L. scabrum* var. *melaneum*
L. oxydabile	*L. oxydabile*	—
L. variicolor	*L. thalassinum*	*L. variicolor* var. *bertauxii*
	L. variicolor	*L. variicolor* var. *variicolor*
		L. variicolor fo. *atrostelatum*
		L. variicolor fo. *sphagnorum*
L. roseofractum	*L. roseofractum*	*L. roseofractum*
		L. pulchrum
—	—	*L. murinaceum*
—	—	*L. molle*
—	—	*L. umbrinoides*

who mainly based his species on observations of macroscopical characters in combination with host preference, to 36 species according to Lannoy & Estades (1995). The multiplication of the number of species by the latter authors can be explained by their emphasis on the microscopical characters of the pileipellis. Especially in section *Scabra* this has lead to considerable taxonomic disagreement (see TABLE I for the most important views). For example, quite a number of different morphological species and infraspecific taxa are recognized in the *L. holopus* complex. Lannoy and Estades recognise six species and Watling (1970) and Engel (1978) only one. Within the *L. scabrum* complex, Lannoy & Estades recognize many more taxa (five species, four varieties and one forma) than Watling (only one species) and Engel (four species). Finally, *L. variicolor* is divided into two varieties and two formae by Lannoy & Estades, while Engel and Watling recognize only two or one species, respectively.

The nrDNA ITS1-5.8S-ITS2 region is commonly used in molecular phylogenetic studies in Agaricales (Hughes et al 1999, Aanen et al 2000, Peintner et al 2001) and Boletales (Kretzer et al 1996, Binder 1999) and seems to be the ideal marker for studies like this, as it is commonly presented as a straightforward marker for species level phylogenies; Its gene and spacers generally show sufficient variation, are easy to sequence and the results can usually be interpreted without difficulty. However, according to Bruns (2001) ITS has its limitations for phylogenetic analyses, too, due to alignment problems between distantly related species in a genus and occasional lack of variation among closely related species. Moreover, a growing amount of studies indicates considerable length variation in either the ITS1 or ITS2 spacers (Van Herwerden et al 1999, Harris and Crandall 2000, Platas et al 2001, Von der Schulenburg et al 2001, Ko and Jung 2002). In some of these cases (Van Herwerden et al 1999, Platas et al 2001, Von der Schulenburg et al 2001) length polymorphisms are caused by simple repetitive sequence motifs, which prevent unambigous alignment and obscure phylogenetic relationships. Evolution of these repetitive sequences is assumed to be shaped by replication slippage, unequal crossing over and gene conversion, which can lead to the generation of length variation and/or concerted evolution (Dover et al 1993, Lewin 2000). A majority of the species of *Leccinum* also show extreme length variation of the ITS1 spacer (Binder 1999).

We traced the origin of this length variation back to the presence of an irregular repetitive sequence in this spacer, which can be classified as a minisatellite (tandemly repeated sequences in which the motif ranges in size from 10–100 bp), also known as Vari-

able Number Tandem Repeats (VNTR, Nakamura et al, 1987).

The presence of minisatellites in fungal sequences has been discovered only recently (Andersen & Nillson-Tillgren 1997) and until this date has only been reported for Ascomycetes (Giraud et al 1998, Hamann & Osiewacz, 1998, Platas et al 2001, 2002, Wöstemeyer & Kreibich 2002). The only reports of minisatellite-like repetitive sequences in ITS1 are known from Xylariaceae (Platas et al 2001, 2002). As far as we know this is the first basidiomycete minisatellite in ITS1 documented to date.

The predominant way by which minisatellites mutate is gene conversion and unequal crossing over. DNA slippage or slipped strand mispairing (SSM) is thought to be of minor importance (Goldstein & Schlötterer 1999) in the evolution of minisatellites. Giraud et al (1998) and Platas et al (2002), however, assume SSM to be the most important mutational process for the minisatellites they found.

In this paper we focus on how the presence of a minisatellite in our ITS dataset influences our inferences of the phylogeny of *Leccinum*. In addition, we try to detect common patterns in the structure of the minisatellite in order to (i) assess its usefulness as a phylogenetic marker and (ii) elucidate the evolution of this repetitive sequence from a phylogentic perspective.

MATERIALS AND METHODS

Taxon sampling.—Data of all collections used in this study are summarized in TABLE II. Designation (through identification by the first author with reference to the types) of the collections to morphospecies and nomenclature are according to Lannoy & Estades (1995) and Korhonen (1995). If mentioned, fresh material was fixed on CTAB in the field, otherwise herbarium material was used. Of the most common species of section *Scabra* several collections representing populations separated by a considerable geographic distance (mainly Norway/Sweden vs. The Netherlands) were included. Voucher specimens are deposited in L (Nationaal Herbarium Nederland, University Leiden branch, The Netherlands), GENT (Herbarium of the University of Ghent, Belgium), H (Botanical Museum of the University of Helsinki, Finland) and O (Botanical Museum, University of Oslo, Norway). The 28S sequence data of Binder & Besl (2000) were obtained from GenBank. Most material from which the sequences were derived was provided by either the authors of the species or before mentioned authorities on the genus (Korhonen, Lannoy & Estades), which make the identifications of the material, to our opinion, reliable.

DNA extraction.—Total genomic DNA was obtained from herbarium specimens as follows. Dried tissue of the herbarium specimen was ground to a fine powder with a mortar and pestle. The powder was placed in a 1.5-mL reaction tube and 500 μL CTAB extraction buffer (100 mM Tris-Cl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, pH 8.0) (slightly modified after Zolan and Pukkila 1986) was added and mixed with the material. Samples were incubated at 65 °C for 60 minutes in a water bath and occasionally mixed. An equal volume (500 μL) of chloroform-isoamyl alcohol (24:1) was added to the samples and briefly mixed by vortexing. Tubes were centrifuged at 13 000 rpm for 10 minutes. The upper aqueous layer was removed to a clean and labeled 2-mL reaction tube. Another 450 μL chloroform-isoamyl alcohol was added to the reaction tube and mixed by energetically shaking the solution for 5 to 10 minutes. The samples were again centrifuged for 10 minutes at 13 000 rpm. The 500 μL of the upper phase was then transferred to a new 1.5 mL reaction tube. Precipitation took place by adding 500 μL cold isopropanol to the samples. The samples were then shaken for 5 to 10 minutes at room temperature and subsequently centrifuged for 10 minutes at 5000 rpm. The supernatant was poured off and removed as much as possible by putting the samples upside down on a paper towel. The last isopropanol was then evaporated by drying the samples in a speedvac for 20 minutes on a low temperature. The remaining pellets were dissolved in 300 μL TE. Traces of RNA were removed by adding 3 μL RNAse A (concentration 50–100 units/mL, Sigma) to each sample and incubation for 1 hour at 37 °C. The DNA was precipitated for a second time by adding 150 μL 7.5 M NH4 acetate and 1125 μL 100% ethanol. The samples were then left to precipitate for 30 minutes at −20 °C and subsequently centrifuged for 10 minutes at 13 000 rpm at 4 °C. The ethanol solution was poured off and the remaining pellets were washed twice by addition of 500 μL 76% ethanol-10 mM NH4 acetate, storage for 20 minutes at −20 °C and centrifugation of the samples at 13 000 rpm at 4 °C. The ethanol mix was then poured off and the pellets were dried in a speedvac for 20 minutes. The pellets were dissolved in 50 μL TE and stored at −20 °C. The DNA extraction of the CTAB-fixed material differed only in the grinding step. Sterilized sea sand was added to the samples fixed in a 1.5-mL tube filled with 500 μL CTAB. The samples were ground with a micropestle until the tissue seemed to be homogenized enough for DNA extraction. The rest of the procedure was the same as for the dried material from the 65 °C water bath step onwards. If DNA samples appeared to be contaminated with black or yellow pigments and hampered PCR amplifications, the samples were cleaned using columns of the Dneasy DNA isolation kit (Qiagen, USA).

PCR, cloning and DNA sequencing.—The nr ITS1-5.8S-ITS2 region was amplified with primers ITS5 and ITS4 (White et al 1990) using 1 μL of total genomic DNA in a 75 μL reaction mixture. PCR parameters were 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C. The final extension was 3 min at 72 °C. The PCR products were electrophoresed in a 1.25% agarose gel in 1× TBE (pH 8.3) buffer, stained with ethidium bromide to confirm a single product and cleaned following the Qiagen Qiaquick PCR Cleanup protocol (Qiagen, USA). In cases where cloning was necessary, PCR products were cut out

and subsequently cleaned following the QIAquick Gel Extraction Kit's manual (Qiagen, USA).

For several taxa direct sequencing was not possible. We therefore cloned the PCR products following the pGEM-T Easy Vector protocol (Promega, USA). Per sample, ten transformed bacterial colonies were screened for possible intra-individual length polymorphisms by touching them with a sterile pipette tip and using that as template for PCR. If PCR products of aberrant length were found, two or more transformed colonies representing the length polymorphisms found, were sequenced. When the PCR products appeared to be of the same length, we generally sequenced only one colony. In the case of *L. versipelle*, we sequenced two clones of the same length, to see if these were identical.

The cleaned PCR products or plasmids were sequenced using the ITS2, ITS3, ITS4 and ITS5 primers (White et al 1990) and in some cases standard universal forward and reverse plasmid primers. Samples were sequenced on an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems, USA) using standard dye-terminator chemistry following the manufacturer's protocols.

Sequence similarity, alignment and phylogenetic analysis.—Putatively homologous parts of *Leccinum* nrITS1-5.8S-ITS2 sequences were assessed using Dotplot analyses as implemented in MegAlign version 4.03 (DNASTAR, Inc. 1999) with a windowsize of 10 and a stringency of 90 percent.

Sequences were aligned using the Clustal option of MegAlign. First the whole dataset was aligned with the gap length penalty lowered to 5 instead of 10 to allow for considerable length variation of the ITS1 spacer. *Heterobasidion annosum* (Fr.) Bref. (Kasuga et al 1993, GenBank X70021) and *Boletus mirabilis* Murrill (Berbee et al unpubl, GenBank AF335451) were used to identify borderlines between 18S nrDNA, ITS1, 5.8S, ITS2 and 28S in *Leccinum*. Alignment and trees are made available on TreeBASE (SN1169).

Because the ITS1 spacer in *Leccinum* appeared to consist of up to 70 to 80 percent of an array of tandem repeats (see also Binder 1999), it was necessary to make two separate data sets. One data set consisted of the ITS1 spacer (including an alignable but hardly variable region of 220 bp and a very variable region of a tandemly repeated sequence, differing in length from 130 to 848 bp), the complete 5.8S-ITS2 region and the first 40 bp of 28S gene (hereafter called ITS). In the other data set the tandem repeat was left out of the sequences (this data set is called hereafter ITS without the minisatellite region). Both alignments were fine scaled in MegAlign using the clustal option, with the gap length penalty lowered to 3. Further manual adjustments were made in MacClade version 3.04 (Maddison and Maddison 1992). Sequences are deposited in GenBank (AF454560 until AF454591).

Both data sets were analyzed separately and together and with nr28S sequences collected by Binder and Besl (2000). Results of the separate and combined analyses were compared by eye and Partition Homogeneity tests (Farris et al 1995).

Phylogenetic reconstruction was done using PAUP* version 4.0b10 (Swofford 1999) for the maximum parsimony (MP) and maximum likelihood (ML) analyses and MrBayes 2.01 (Huelsenbeck and Ronquist 2001) for the Baseyian analysis. In all analyses *Leccinum crocipodium* (Letellier) Watling and *L. carpini* (R. Schulz) Moser ex Reid were specified as outgroup as suggested by Binder and Besl (2000), who identified these species to belong to the sistergroup of the sections *Scabra* and *Leccinum* in their phylogeny of Boletales based on nr28S sequences. In the MP analyses 1000 heuristic searches were performed, each with a random addition sequence, MAXTREES set to 10 000, and TBR branch swapping. Bootstrapping in these analyses was performed using 1000 replicates, each with one heuristic search with the addition sequence as given, MAXTREES set to 100, and TBR branch swapping. All characters were assessed as independent, unordered and equally weighted. Gaps were treated as missing characters. For the ML analysis, the appropriate nucleotide substitution model was first determined using Modeltest version 3.06 (Posada and Crandall 1998). A ML analysis was then performed using the substitution model suggested by Modeltest (see results). In the ML analysis 1000 heuristic searches were performed, each with a random addition sequence, MAXTREES set to autoincrease, and TBR branch swapping. For the Bayesian analysis default settings were changed to estimated base frequencies and gamma shape and six substitution types to meet the requirements for the model as determined by Modeltest. The number of generations was set to five million and one tree was saved per 500 generations. The cladogram and posterior credibility values for the clades found are based on the outcome of the last four million generations.

Characterisation of minisatellite.—The boundaries of the irregular tandem repeat array in ITS1 were designated by means of a dotplot analyses as implemented in the alignment program Megalign of the DNAstar package. To visualize the presence of (irregular) tandemly repeats, the dotplot parameters were set to a windowsize of 10 bp (the minimum size of repeats) and a stringency (percent match) of 90 percent. By plotting the sequence against itself under these settings, the repetitive region will become easily visible as a square region composed of short lines parallel to the main diagonal (see FIG. 5 and FIG. 6). To see to what extent the repetition within this region could be explained by direct, regular tandem repetition, we used the program Tandem Repeats Finder Version: 2.02 (Benson 1999), using the default settings for a subset of the sequences.

Conserved regions within the ITS1 spacer were assessed by aligning the ITS1 spacer of *Leccinum* with a number of representatives of the Boletaceae. To facilitate the alignment, the minisatellite in *Leccinum* was left out. Conserved regions as indicated by Van Nues et al (1994) were found by comparison of primary and secondary structures with yeast sequences using secondary structure predictions performed in RNAdraw version 1.1 (Matzura and Wennberg 1996).

RESULTS

Phylogenies.—The aligned complete ITS sequences included 1696 characters, with 437 variable and 287

TABLE II. List of taxa analysed. Nomenclature according to Lannoy & Estades (1995)

Species	Voucher	Collection site	ITS1-5.8S-ITS2-28S(p.p)*	28S**
Section *Scabra*				
Subsection *Scabra*				
Leccinum pulchrum	hdb037	Nieuwkoop, The Netherlands	AF454568	
Leccinum rotundifoliae	hdb086	Jotunheimen, Norway	AF454581	
Leccinum rotundifoliae	mk11429	Kilpisjärvi, Finland	AF454582	AF139704
Leccinum scabrum	hdb022	Borgsjö, Sweden	AF454583	
Leccinum scabrum	hdb053	Hoogeveen, The Netherlands	AF454585	AF139705
Subsection *Pseudoscabra*				
Leccinum brunneogriseolum	hdb039	Schiermonnikoog, The Netherlands	AF454560	
Leccinum rigidipes	hdb042	Voorschoten, The Netherlands	AF454584	
Leccinum variicolor	hdb007	Östavall, Sweden	AF454570	
Leccinum variicolor	hdb031	Amsterdamse Waterleidingduinen, The Netherlands	AF454571	
Leccinum variicolor	hdb051	Erica, The Netherlands	AF454572	AF139706
Subsection *Olivacentes*				
Leccinum holopus	hdb085	Sogndal, Norway	AF454562	AF139697
Leccinum nucatum	hdb040	Nieuwkoop, The Netherlands	AF454561	
Leccinum nucatum	hdb076	Kall, Sweden	AF454563	AF139700
Leccinum nucatum	rw1628	Houwaart, Belgium	AF454564	
Unassigned				
Leccinum palustre	hdb030	Amsterdamse Waterleidingduinen, The Netherlands	AF454586	
Leccinum palustre	hdb015	Borgsjö, Sweden	AF454587	AF139701
Section *Leccinum*				
Subsection *Leccinum*				
Leccinum aurantiacum	rw1656	Ardennes, France	AF454569	AF139689
*Leccinum versipelle****	O64036	Lærdal, Norway	AF454574	AF139692
Leccinum piceinum	men2048	Obertiliach, Austria	AF454578, AF454579	
Leccinum vulpinum	hdb092	Sogndal, Norway	AF454580	
Subsection *Fumosa*				
Leccinum duriusculum	wt001	Wassenaar, The Netherlands	AF454576, AF454577	AF139695
Leccinum roseotinctum	hdb74	Kall, Sweden	AF454575	
Section *Luteoscabra*				
Subsection *Luteoscabra*				
Leccinum crocipodium	rw1659	Ardennes, France	AF454589	AF139694
Leccinum crocipodium	deVries s.n. (1999)	Zeist, The Netherlands	AF454590	
Subsection *Albella*				
Leccinum carpini	hdb065	Breukelen, The Netherlands	AF454588	AF139691
*Leccinum extremioreintale*****		Genbank	AF438582	
*Boletus depilatus*****	RW s.n.	Watermaal-Bosvoorde (Zoniën), Belgium	AY127032	
*Boletus mirabilis*****		Genbank	AF335451	
*Boletus chrysentron*****		Genbank	AF402139	
*Boletus lupinus*****	MA-Fungi 41327	Genbank	AJ296294	

TABLE II. Continued

Species	Voucher	Collection site	ITS1-5.8S-ITS2-28S(p.p)*	28S**
*Boletus aestivalis*****	hdb049	Gieten, The Netherlands	AY130295	
*Boletus edulis*****		Genbank	AF074921.1	

* Genbank accession numbers of sequences collected in this study; ** idem for Binder & Besl (2000); *** *L. cerrinum* and *L. versipelle* are considered as synonyms here.

**** Taxa used in alignment and secondary structure prediction of FIG. 6.

parsimony informative positions (calculated with all sequences included). A branch and bound search based on this alignment yielded 2583 MPTs of 538 steps (CI: 0.885, RI: 0.930, bootstrap consensus shown in FIG. 1). Section *Scabra* and section *Leccinum* are intermixed (56–100% bootstrap support). A total of 13 out of the 19 clades present receive bootstrap support higher than 80%. Composition of most groups of closely related 'species' correlates well with clades found in the ITS dataset without the minisatellite (see below).

The aligned ITS sequences without the minisatellite region included 994 characters, with 254 variable and 147 parsimony informative positions (calculated with all sequences included). A branch and bound search based on this alignment yielded >10 000 MPTs of 306 steps (CI: 0.895, RI: 0.908, bootstrap consensus shown in FIG. 2). Section *Scabra* is nested within section *Leccinum* in all of the more than 10 000 MPTs found. Support for this is very high in all trees found. The bootstrap consensus (FIG. 2) shows most basal clades have a bootstrap support of 70% or more. Moderate support (>70%) is present for the clades existing of clade A, B plus C, and D plus E, respectively. Clade A, B, C, D and E are united in a large, moderately supported (81%) dichotomy, with clade F as sistergroup. Bootstrap support for the individual clades is high (between 99 and 100%), with the exception of clade E, which is weakly supported (55%). Several clades found in the MP analysis con-

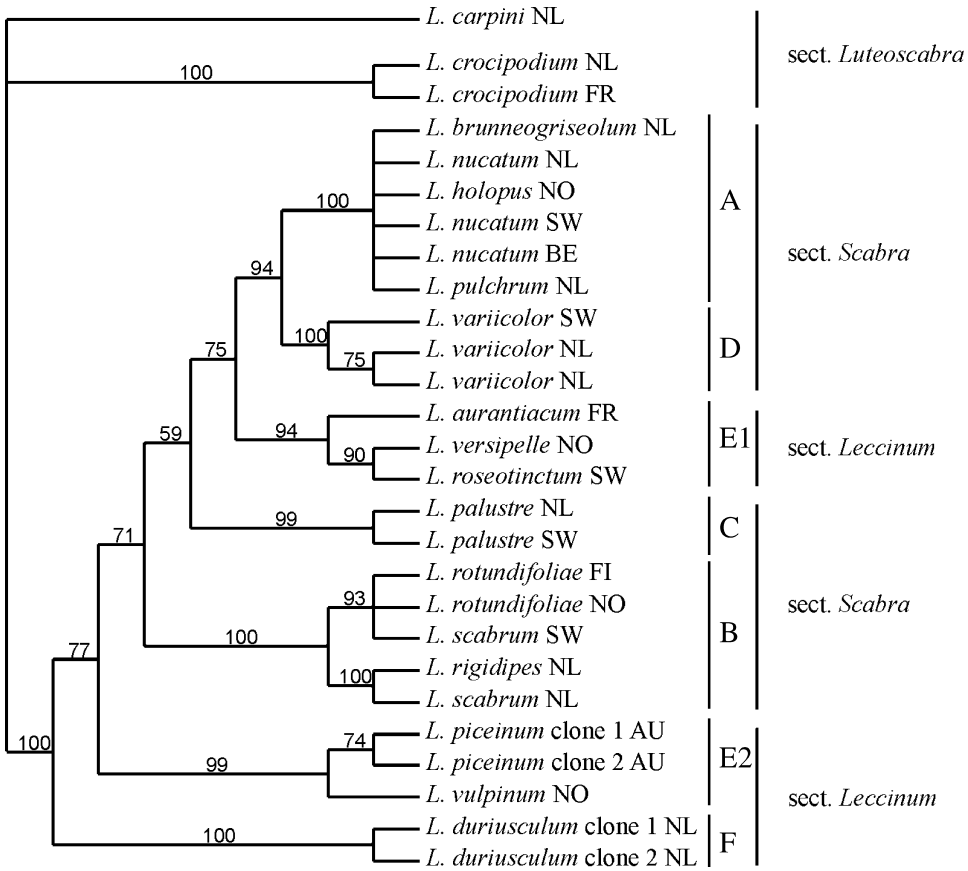

FIG. 1. Bootstrap consensus of complete ITS sequences (branches with <50% BS not indicated).

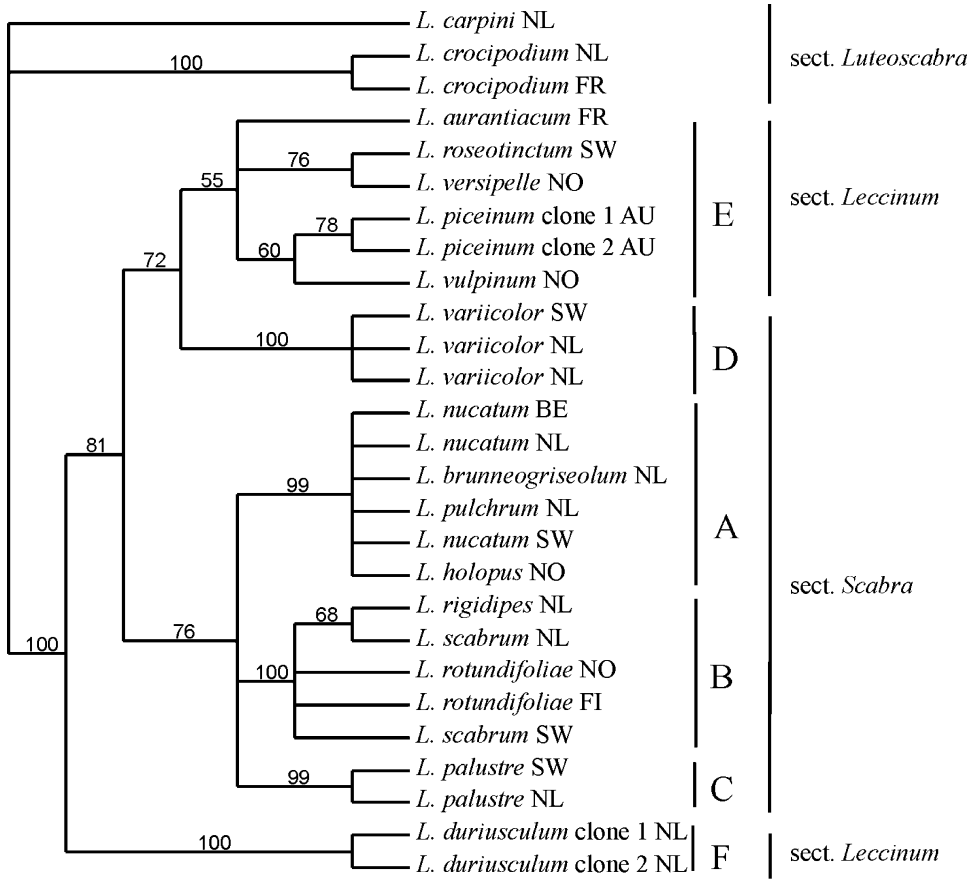

FIG. 2. Bootstrap consensus of ITS sequences without the minisatellite (branches with <50% BS not indicated).

sist of groups of closely related 'species': Clade A consists of *L. brunneogriseolum*, *L. holopus*, *L. nucatum* and *L. pulchrum*. Sequence divergence in this clade is low (0–1.5%). Clade B consists of *L. rigidipes*, *L. rotundifoliae* and *L. scabrum*. Sequence divergence in this clade is also low (0.5–2.4%). Clade C, D and F consist of *L. palustre*, *L. variicolor* and *L. duriusculum*, respectively. Sequence divergence in all these clades is very low (0.6–1.3%). Clade E consists of *L. aurantiacum*, *L. piceinum*, *L. vulpinum*, *L. roseotinctum* and *L. versipelle*. Molecular divergence in this clade is low again (0.2–2.0%).

A combined analysis of the aligned ITS sequences without the minisatellite and the 28S sequences available on Genbank could only be performed with a subset of accessions (12 of the 27). The combined dataset included 1477 characters, with 203 variable and 131 parsimony informative positions (calculated with all 12 sequences included). A Partition Homogeneity test showed no significant incongruence between the signal of both data sets. A branch and bound search based on this alignment yielded 4 MPTs of 255 steps (CI: 0.878, RI: 0.858). Topology and resolution are almost identical to the MP analysis of the ITS sequences without the minisatellites (see above).

Using Modeltest, the general time-reversible model was chosen, with variable sites assumed to follow a gamma distribution (shape set to 0.6920), nucleotide frequencies set to A:0.2823, C:0.2040, G: 0.2236, T: 0.2900 and substitution rates set to 2.1753 (A↔C), 4.0993 (A↔G), 2.0767 (A↔T), 1.9933 (C↔G), and 5.9494 (C↔T). ML analyses of the ITS sequences resulted in a single tree with log Likelihood score −2975.37353 (see FIG. 3). Topology and resolution are generally congruent with the MP analysis of the ITS sequences without the minisatellite (see above) and indicate that section *Scabra* is nested within section *Leccinum*. However, the position of *L. variicolor* differs in both types of analysis. While *L. variicolor* (clade D) is a sistergroup to clade E in the parsimony analyses, it is placed within clade E in the ML analysis.

The outcome of the Bayesian analysis shows no difference with the topology of the tree found in the ML analysis. Clade credibility values of 95% or higher are generally congruent with clades with a bootstrap support of 70% or higher as found in the MP anal-

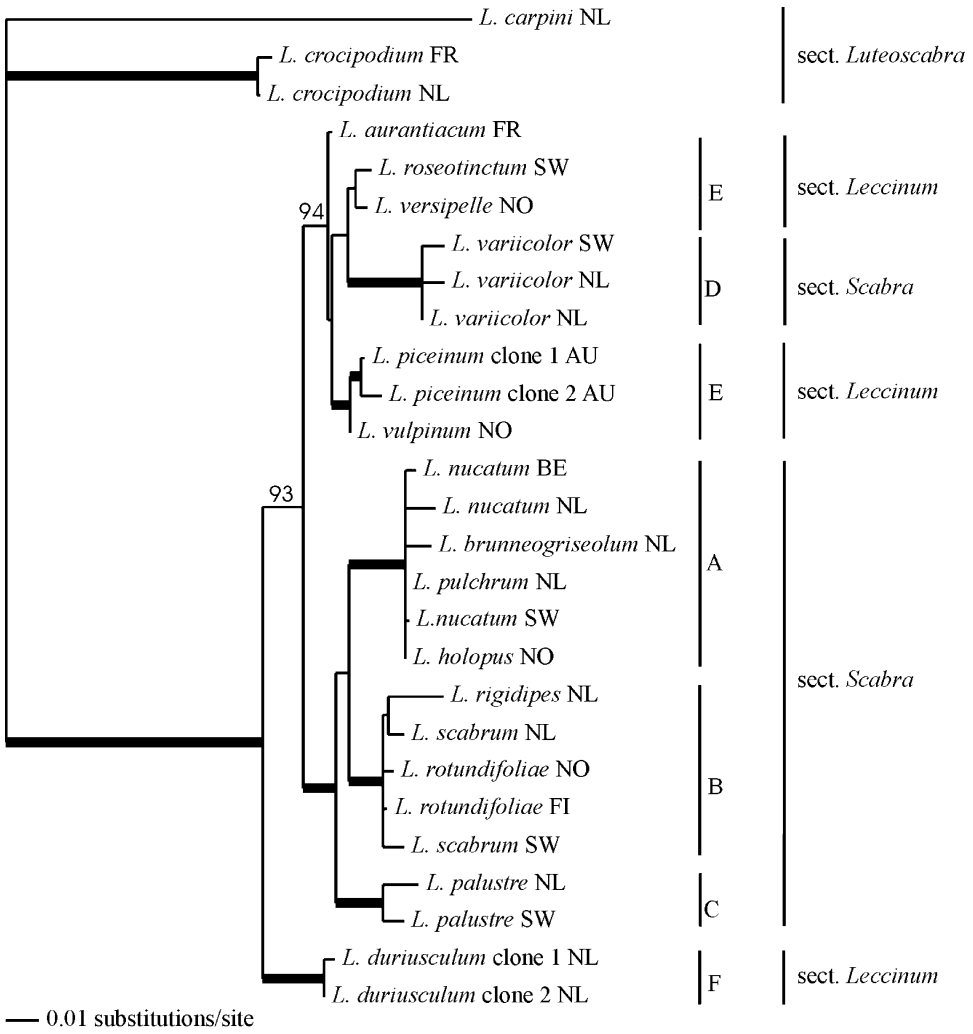

FIG. 3. Phylogram as obtained from ML and Bayesian analyses of the ITS sequences without the minisatellite. Clades printed bold have a posterior probability value of 95 % or more. For clades with a posterior value between the 90 and 95% the % is as indicated.

yses, which is congruent with comparisons made of both types of analyses in recent literature (Leaché & Reeder 2002). A slight exception to this pattern is the clade representing the sistergroup relation of clade F and the other clades, which yields a bootstrap support of 81% in the MP analyses and a clade credibility value of 93% in the Bayesian analyses. The clade which represents the grouping of *L. variicolor* with the clades D and E, respectively gets a bootstrap support of 70% in the MP analyses and a clade credibility value of 94% in the Bayesian analyses. Posterior probability values found for the clades suggesting *L. variicolor* is placed with in clade E instead of being a sistergroup to it are very low (≪95%).

Position and characterization of minisatellite.—PCR products of the ITS region in *Leccinum* ranged from 694 to1480 bp (FIG. 4). ITS1 varied in length from 150 to 868 bp, 5.8S measured ca 158 bp and ITS2 ranged from 270 to 355 bp, with the exception of *L. carpini*, which showed an ITS2 sequence of 574 bp. The length variation observed in the ITS1 spacer in most species of *Leccinum* examined by us is caused by an insert varying in length from 240 to 749 bp. According to Van Nues et al (1994) the ITS1 spacer in yeasts is organised in two functionally and structurally distinct halves with highly conserved nucleotide regions at three different positions. In relation to the position of the A2 processing site, we assume the insert in the ITS1 spacer in *Leccinum* is situated in domain III (see FIG. 5). The ITS1 spacer in Boletaceae inspected by us in general is very short (ca 210 bp) and sequence variation is very low. Variation, consisting of mono- or dinucleotide tandem repeats, is mainly found in two regions associated with processing sites A2 and A3. This variation, that seems to be associated with the processing sites, is virtually ab-

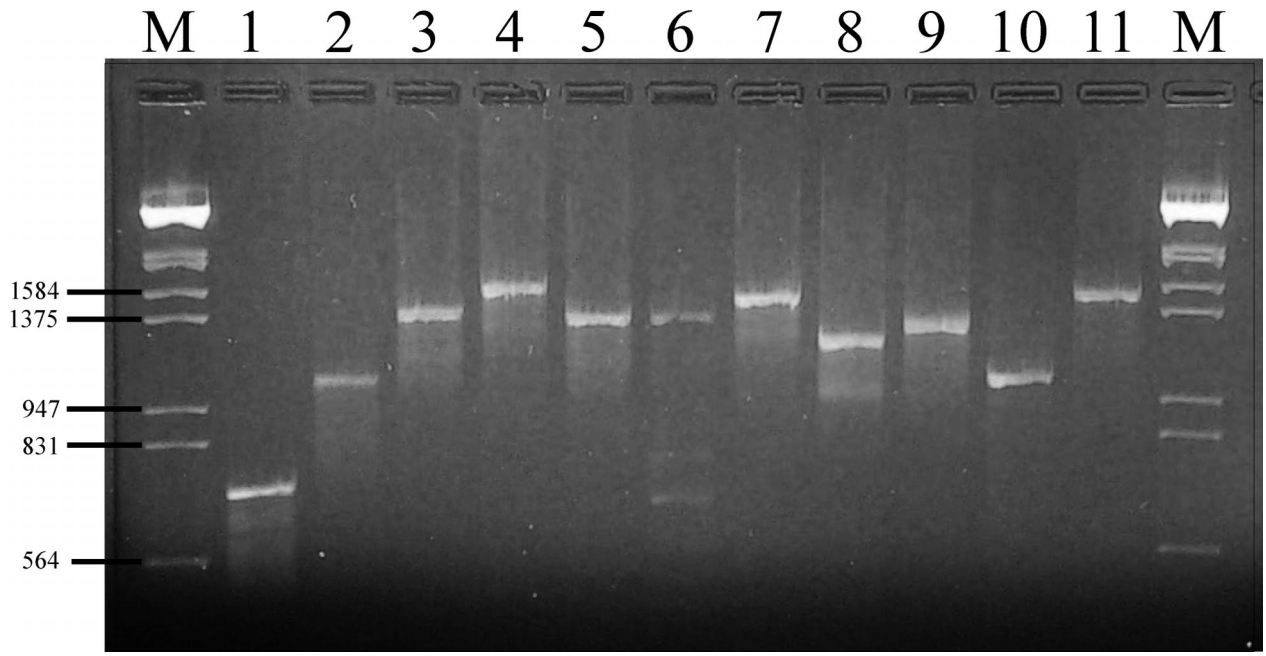

FIG. 4. Amplification of ITS fragment by PCR from genomic DNA of several *Leccinum* species. Lanes: M. *Eco*R1-*Hind*III ladder; 1. *L. crocipodium*; 2. *L. carpini*; 3. *L. duriusculum*; 4. *L. quercinum*; 5. *L. piceinum*; 6. *L. vulpinum*; 7. *L. holopus*; 8. *L. scabrum*; 9. *L. rotundifoliae*; 10. *L. palustre*; 11. *L. variicolor*.

sent in those species of *Leccinum* that contain a minisatellite. For most *Leccinum* species several accessions were sequenced. The length of the ITS1 spacer could vary considerably within one species. When comparing all sequences of one species, indels appeared to be interspersed throughout the whole ITS1 spacer and were not restricted to certain positions (see *L. duriusculum*, *L. piceinum* and *L. variicolor* in FIG. 6).

Dotplot analyses show that all ITS sequences of aberrant length contain an irregularly repeated region. In most species of *Leccinum* analyzed, this region is situated in ITS 1 (FIG. 7), with the exception of *L. crocipodium* and *L. carpini*. In *L. crocipodium*, we found no sign of regions being composed of repetitive sequences of 10 bp or longer. In *L. carpini* however, we found a region of ca 450 bp length that consists of tandem repeats in ITS2 (FIG. 8), which explains the length difference we found in this spacer when compared to other species of *Leccinum*. Both Dotplot analyses and visual inspection of the sequences show two core sequences accounting for a great part of the repetition within this region: a short sequence of approximately 12 bp (CTTATTGAAAAG and derivatives thereof) and a sequence of approximately 11 bp (CTAATAGAAAG see FIG 9). Most variation within these core sequences is caused by point mutations (mostly transitions). The total number of these core sequences found ranges from 12 in *L. piceinum* to 36 in *L. variicolor* and *L. versipelle* (TABLE III). We found an even number of these core sequences and in all but one sample (*L. variicolor* from Sweden). To detect common patterns in presence of these core sequences within the minisatellite, we referred to derivatives of the core sequence as either A (CTTATTGAAAAG or derivatives thereof) or B (CTAATAGAAAG and its derivatives) and used these letters to represent the sequence of core sequences found. We determined these patterns for seven accesions (*L. palustre* NL, *L.variicolor* NL, *L. holopus*, *L. versipelle*, *L. rotundifoliae* FIN, *L. piceinum* clone 1 and *L. duriusculum* clone 1), which represent, in most cases, the longest variant of the minisatellite found in the most important clades. By comparing these patterns in an alignment (see FIG. 10) it becomes clear that *L. duriusculum* clone 1, *L. piceinum* clone 1 and *L. rotundifoliae* share (almost) identical patterns of core sequences, whereas *L. versipelle*, *L. holopus*, *L. variicolor* and *L. palustre* show only partial similarity.

Analyses of the minisatellite sequences with the Tandem Repeat Finder software (summarized in TABLE IV and partly visualized in FIG. 6) shows three of the twelve sequences analyzed contain no direct tandem repeats (*L. duriusculum* clone 2, *L. palustre* and *L. scabrum*). Most of the tandem repeats found in the remainder of the minisatellite sequences show overlapping patterns and do not seem to indicate direct replications. If we compare consensus size and the number of the repeats between the samples an-

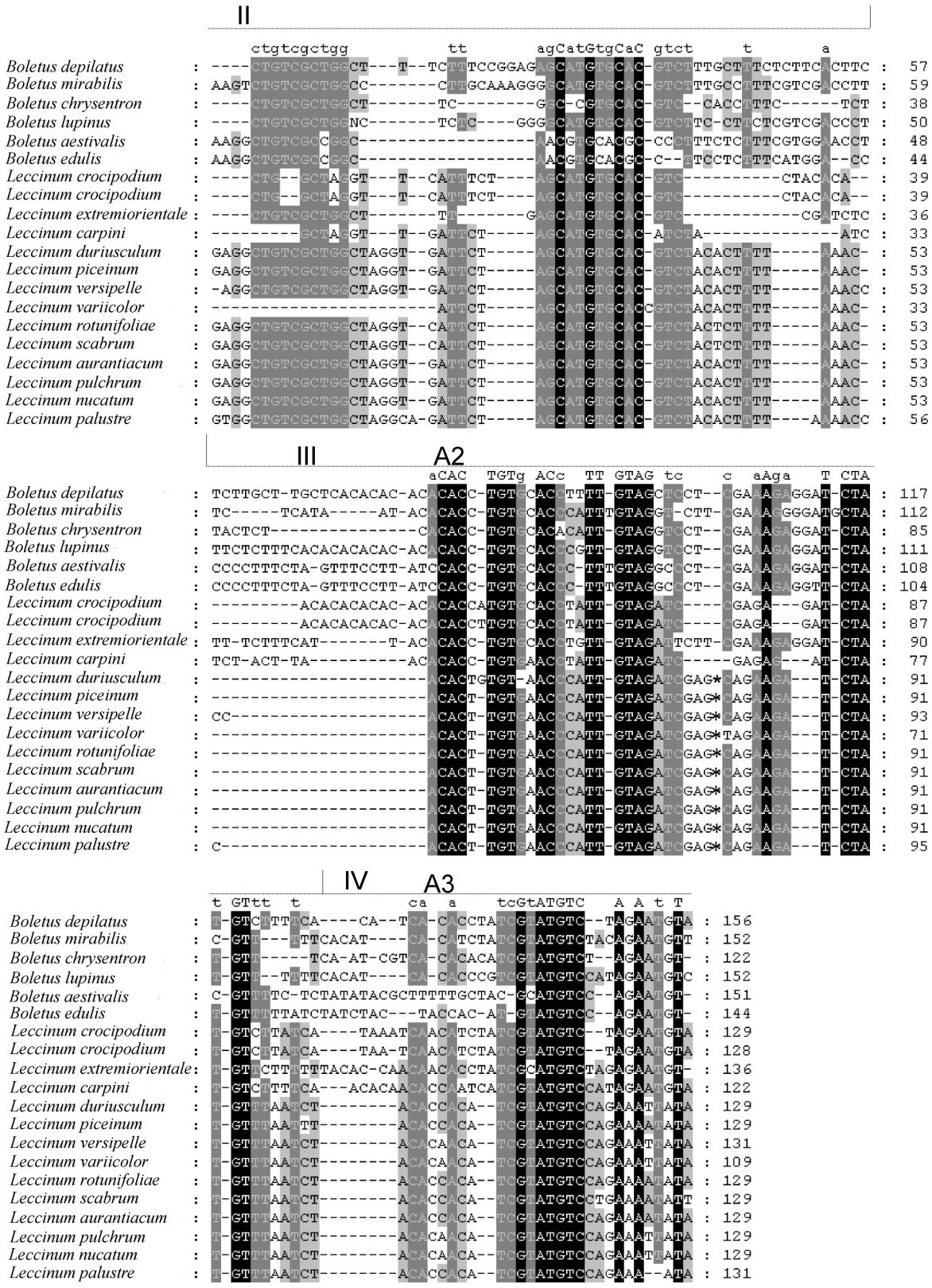

FIG. 5. Partial ITS1 alignment of representatives of *Leccinum* and other Boletaceae. Roman numerals indicate the different domains as indicated by Van Nues et al (1994). A2 and A3 indicate the processing sites essential for 18S and 28S maturation. The arrow and the * in the alignment indicate the position of the minisatellite sequences which are omitted from this alignment. The shading indicates the % conservation: black 100%, dark grey ≥80%, light grey ≥60%.

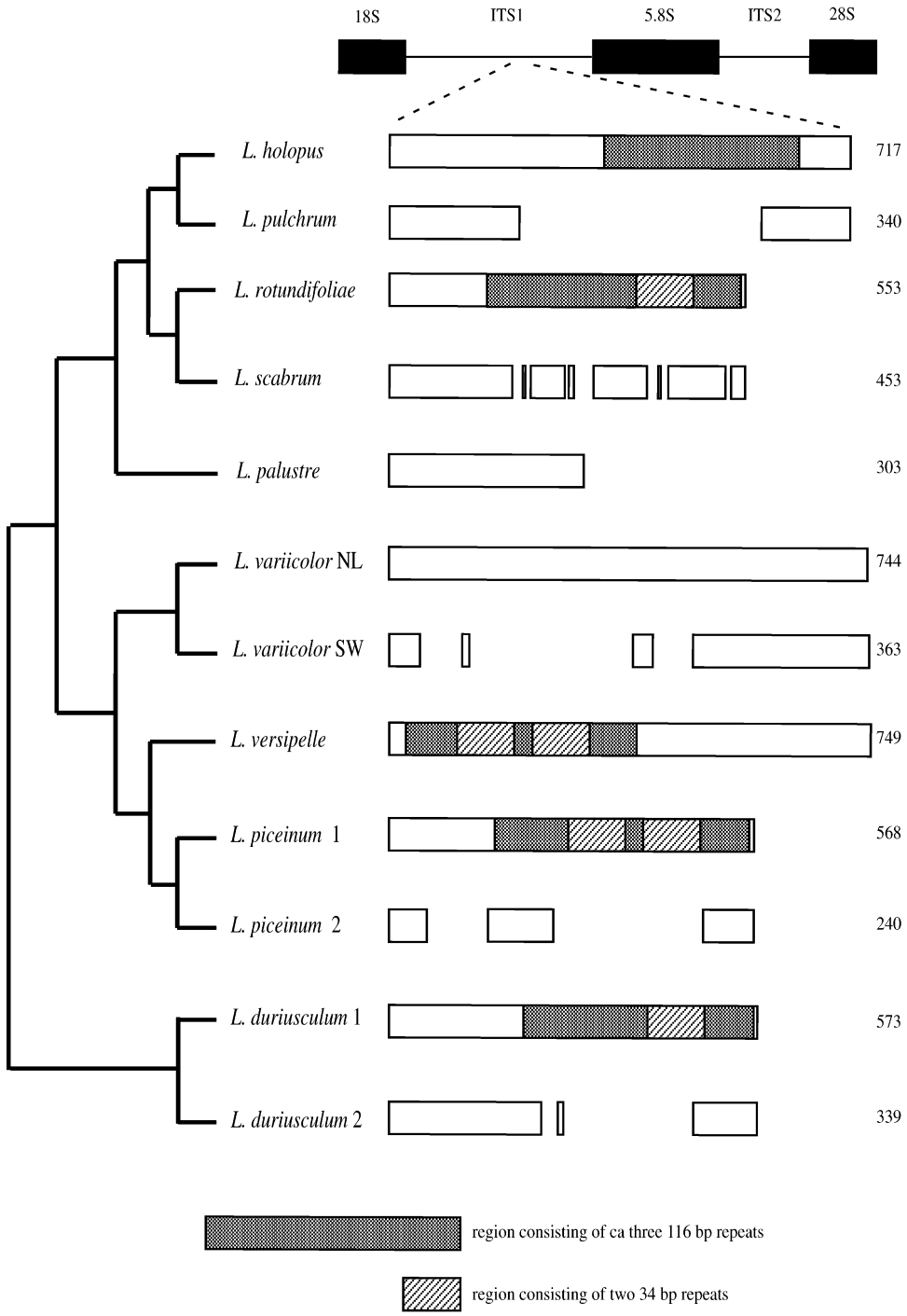

FIG. 6. Schematic overview of minisatellite structure in *Leccinum* as found with the Tandem Repeats finder software. Shaded areas indicate the presence of tandem repeats. Horizontal bars are in proportion to the lengths of the complete minisatellite region. The size of the minisatellite region is given in bp. The phylogenetic tree of the taxa included is a schematic representation of the ITS tree in FIG. 2.

alyzed, we can detect several common patterns. Five taxa (*L. duriusculum* clone 1, *L. holopus*, *L. piceinum* clone 1, *L. rotundifoliae* and *L. versipelle*) contain a tandemly repeated sequence that consists of circa three repeats of approximately 116 bp. A tandemly repeated sequence composed of two repeats of 34 bp size is found in five of the samples analyzed. In *L. piceinum* clone 1 and *L. versipelle* this sequence is present in twofold, in *L. rotundifolia*, *L. piceinum* clone 1 and *L. duriusculum* clone 1 it is only present once

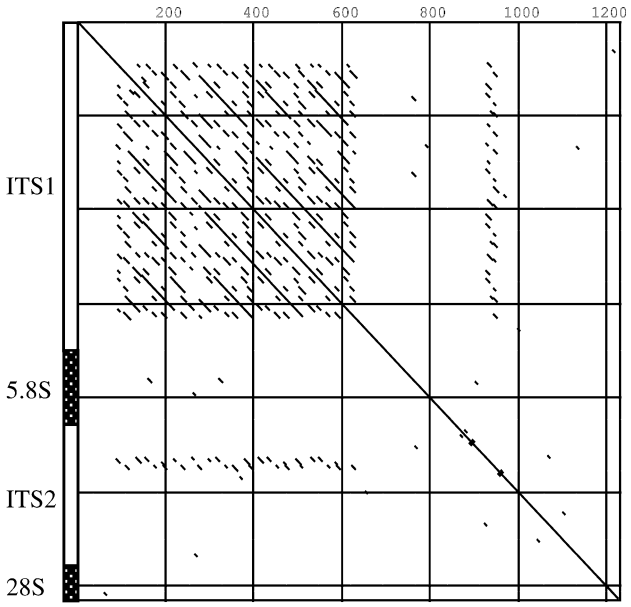

FIG. 7. Dotplot of ITS sequence of *L. rotundifolia* (windowsize 10, 90% similarity). The (upper) x-axis indicates the position within the sequence, the y-axis the different regions within the ITS-sequence. The presence of lines parallel to the main diagonal indicate repeats within the sequence. In this case it shows the presence of a (imperfectly) tandemly repeated sequence in the 3′ end of ITS1.

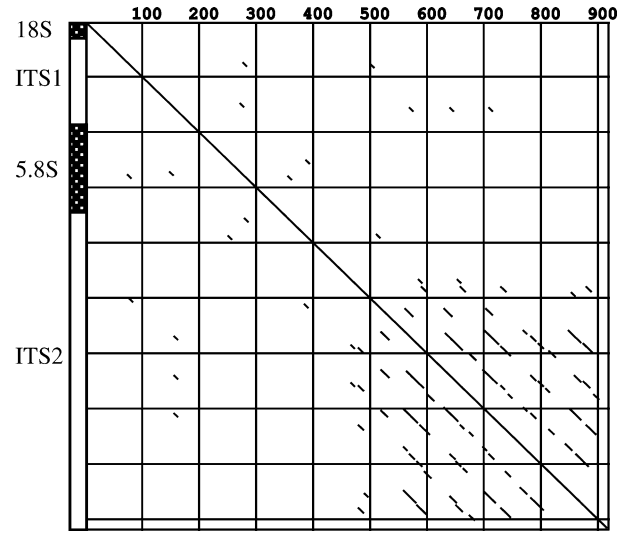

FIG. 8. Dotplot of ITS sequence of *L. carpini* (windowsize 10; 90% similarity). The (upper) x-axis indicates the position within the sequence, the y-axis the different regions within the ITS-sequence. The presence of lines parallel to the main diagonal indicate repeats within the sequence. In this case it shows the presence of a (imperfectly) tandemly repeated sequence in the 3′ end of ITS2.

(see FIG. 6). Remarkable is that this tandem repeat of two times 34 bp is positioned in the sequences that consists of three repeats of 116 bp. In four samples (*L. piceinum* clone 1, *L. piceinum* clone 2, *L. variicolor* NL and *L. variicolor* SW) a small tandemly repeated sequence consisting of two times 17 bp is found. In the samples of *L. variicolor* this tandem repeat partly overlaps with a two times 36 bp tandem repeat, in *L. piceinum* clone 1 it overlaps with a two times 34 bp repeat.

A BLAST search on Genbank of the complete minisatellite showed that it is unique for *Leccinum*. When individual repeats were subjected to a BLAST search, these repeats matched with small pieces of genes sequenced in whole genome projects, such as of *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Arabidopsis thaliana*.

Although minisatellites usually are so variable that they can even be used in forensic research (Meyer and Mitchell 1995), the minisatellite found in *Leccinum* displays considerably less allelic variation. For instance, all species of clade A in FIG. 3 show only two alleles (a long and a short variant), irrelevant of their geographical origin. The same holds for *L. piceinum* and *L. vulpinum*. Although the accessions of the latter were collected at a geographical distance of at least 1600 kilometers, the long length variant of the minisatellite of both species is identical. In contrast, the minisatellites found in the species of clade B in FIG. 3 are less homogeneous. Although we have sampled only five specimens, we can discriminate between a long allele present in Scandinavian accessions of *L. scabrum* and *L. rotundifoliae* and a shorter allele present in Dutch accessions of *L. scabrum* and *L. rigidipes*.

DISCUSSION

Phylogenies.—A MP analyses of the complete ITS sequences results in a completely resolved phylogeny with high bootstrap support (FIG. 1). However, this phylogeny seems to represent more the history of the minisatellite (see below). The phylogenetic signal present in ITS minus the minisatellite seems to be swamped by the phylogenetic signal of the not orthologously evolving minisatellites.

Taxonomic implications.—Separate and combined analyses of sequences of the nr28S gene (Binder and Besl 2000) and the ITS sequences analyzed here indicate that there seems to be no evolutionary based reason to differentiate between the sections *Leccinum* and *Scabra* as suggested by Smith and Thiers (1971) and Lannoy and Estades (1995). Most of the species belonging to section *Scabra* form a monophyletic

FIG. 9. Sequence of the minisatellite found in ITS1 of *L. rotundifoliae*. Core sequences are underlined. A and B indicate the two types of core sequences. Numbers above the sequence indicate the positions within the sequence.

clade. This clade is nested within section *Leccinum*, thereby making the latter section paraphyletic. Moreover, *L. variicolor* seems not related to the other members of section *Scabra*, making this section polyphyletic. Our results indicate that *L. variicolor* represents a lineage that has diverged from section *Leccinum* and subsequently lost the pigments causing blackish discoloration.

The congruence of the ITS data with the 28S data (and not the minisatellite data found in ITS1) makes it clear that the minisatellite is unsuitable for phylogenetic analysis of relations above the species level in *Leccinum*. However, processes like concerted evolution make it an ideal marker to reveal relations at and below the species level, as homogenization seems to take place in its tandem repeat arrays.

Leccinum rigidipes and *L. rotundifoliae* share almost identical ITS sequences with *L. scabrum* populations which were collected on locations nearby. *Leccinum rigidipes* is distinguished from *L. scabrum* by the presence of cylindrical elements in the pileipellis and a rigid, clavate stipe. However, *L. scabrum* often has a clavate stipe, too, and cylindrical elements in the pileipellis are difficult to distinguish, as this character seems to be not discrete. Transitions between cylindrical and other elongate elements appear to be rather common. *Leccinum rotundifoliae* was considered 'a truly distinctive species' according to Smith and Thiers (1971). We think that it might just be an ecotype associated with the dwarf shrub *Betula nana* and that it owes its distinctive smaller size to the mountainous and tundra environments it grows in. A similar case was found for *Hebeloma alpinum* by Aanen et al (2000).

TABLE III. Overview of the number of core sequences found in the minisatellite region and the ratio of the two types recognized

Accession	Total core sequences	cttattgaaaag: ctaatagaggg
L. duriusculum clone 1	30	15:15
L. duriusculum clone 2	18	09:09
L. holopus	34	17:17
L. palustre NL	14	07:07
L. piceinum clone 1	30	15:15
L. piceinum clone 2	12	06:06
L. pulchrum	16	08:08
L. rotundifoliae Fin	28	14:14
L. scabrum NL	20	10:10
L. variicolor NL	36	17:19
L. variicolor SW	17	08:09
L. versipelle Norway	36	18:18

The second interesting group consists of the *L. holopus* complex (clade A in FIGS. 2 and 3). This clade of diverse, morphologically clearly circumscribed species is very homogenous in its ITS sequences if we do not take into account the length variations of the ITS1 spacer. Many species recognized by Lannoy & Estades (1995) within this group are distinguished by the presence or absence of cylindrical elements in the pileipellis and the absence or presence of pigment in the pileipellis and the stipe. As already mentioned, the presence or absence of cylindrical elements is not discrete. Nr ITS1-5.8S-ITS2 sequences indicate that recognition of *L. brunneogriseolum*, *L. holopus*, *L. nucatum*, and *L. pulchrum* as separate species may not be warranted. However, we think it would be premature to consider this entire group conspecific as lack of ITS variation not necessarily provides evidence of conspecifity (Bruns 2001). Even if we do consider albinism and pileipellis characters invalid for species delimitation, there is still enough morphological variation to distinguish at least two species or species groups. More accessions of additional localities need to be studied and the outcomes of additional molecular markers have to be obtained, before decisions about definitive species delimitations can be made. The current ITS perspective

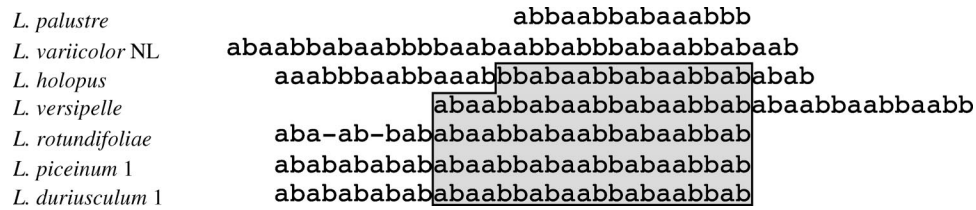

FIG. 10. Alignment of the two core sequence patterns displayed in a number of representative *Leccinum* species. The grey boxes indicate a putative homologues pattern and correspond to the dark grey areas in FIG. 6.

agrees reasonably well with Watling's (1970) conservative view of species, but whether additional molecules or increased sampling will distinguish more taxa remains to be investigated.

Functionality of ITS1 spacer.—The presence of a large and heterogeneous insert in the ITS1 spacer could indicate a loss of function for this region in *Leccinum*. Muir et al (2001) have shown that multiple ITS variants in one genome can indicate some of these variants have actually lost their function. They also show that these non-functional ITS regions are characterized by the presence of more mutations, especially in the more conserved regions. According to Van Nues et al (1994) deletions in domain III severely reduce the formation of 17S rRNA. Given the fact that, in those samples in which we found the insert, no other sequences lacking the insert were present, we have to conclude the ITS1 region is likely to be still functional. It appears that insertions within this domain are tolerated. Moreover, sequence divergence between *Leccinum* and several *Boletus* species in regions immediately adjacent to the insert (see FIG. 5) is virtually absent, indicating that the adjacent regions are relatively conserved, also in the samples containing the minisatellite.

Characterization of minisatellite.—The minisatellite found in the ITS1 spacer of *Leccinum* is AT-rich (59–64%). Other minisatellites reported in fungi are either GC-rich (Anderson & Torsten 1996) or AT-rich (Giraud et al. 1998). AT-richness is a character shared with the ITS-region in which the minisatellite is positioned.

TABLE IV. Overview of the most important results of the Tandem Repeats Finder analyses

Sample	Size minisatellite region	Consensus size	Copy Number	% Matches	Position tandem repeat
L. duriusculum clone 1	573	34	2.0	90	401–467
		115	3.1	83	209–567
L. duriusculum clone 2	339	No repeats found			
L. holopus	717	27	2.1	90	89–145
		108	2.8	87	31–335
		116	2.6	87	349–652
L. palustre	303	No repeats found			
L. piceinum clone 1	568	34	2.1	89	281–352
		116	3.4	89	165–561
		34	2.2	90	397–470
		17	1.9	93	454–486
L. piceinum clone 2	240	34	2.1	87	64–135
L. pulchrum NL	340	27	2.1	90	89–145
L. rotundifoliae Fin	553	117	3.4	90	152–547
		35	2.1	85	384–457
L. scabrum NL	453	No repeats found			
L. variicolor NL	744	36	1.9	97	620–689
		17	1.9	93	676–708
L. variicolor SW	363	36	1.9	94	241–310
		17	1.9	100	296–329
L. versipelle	749	34	2.1	87	106–177
		34	2.1	92	223–294
		116	3.0	91	26–375
		161	1.9	89	225–529
		20	2.1	90	677–716

Both the dotplot analysis and the mapping of the core sequence variants show that the minisatellite region containss a high level of repetition. A search for tandem repeats however shows some minisatellite regions to contain no such repeats, while others can only partly be explained by direct repetition. An explanation for this result is almost certainly the fact that repetition within a sequence is only detected with the Tandem Repeats Finder software when repeats are rather perfect (>80% match) copies of each other. Repeats therefore have to be relatively unaffected by mutations to be detected with this kind of software. However, the patterns found with the tandem repeat software are, in addition to the patterns found when mapping the core sequences, not completely meaningless. One of the common patterns found with the tandem repeat software is the presence of a region consisting of three tandem repeats of ca 116 bp (see FIG. 6). Comparison of these regions with the patterns found when mapping the core sequences show that this 116 bp tandem repeat region is also (almost) identical in its core sequence pattern. We conclude that both methods to analyze the patterns of repetition point towards the same homologous regions in several species.

A remarkable feature of the minisatellite here is the fact that we found the number of core sequences to be even in all but one sample (*L. variicolor* SW). According to Platas et al (2001) inverted repeats are associated with stable folding positions of a tandem repeat array. Although we could not find inverted repeats of considerable size within the minisatellites examined, a secondary structure prediction (data not shown) of the minisatellite region with the Mfold 3.0 software (Mathews et al 1999, Zucker et al 1999) shows this region to fold in such a way that most of the core sequences form a stem-like structure. These stem-like structure consist either of an AA, AB or BB pair of core sequences. This property of the core sequences might enable the minisatellite region to fold into a stable secondary structure within the ITS spacer, which may explain why it can be situated in a rather conservative part of this spacer. It is probably also putting a strong constraint on the minisatellite variants that can persist within the organism, since badly folding mutants can have a deleterious effect on the organism.

Mechanisms causing length polymorphisms.—Mapping of the core sequences shows the minisatellites found can be divided into two groups. One group consists of *L. rotundifoliae*, *L. piceinum* and *L. duriusculum*, three phylogentically rather unrelated species, with a minisatellite size of 550 to 575 bp, and a rather identical core sequence pattern. The other group comprises *L. variicolor*, *L. versipelle*, *L. holopus* and *L. palustre*, in which the minisatellite is considerably shorter or longer in size. Although some of these species (*L. versipelle* and *L. holopus*) show pieces of their minisatellite to be homologous to patterns found in the other group, they are all characterized by patterns that indicate a rather drastic expansion or contraction of the region. Assuming the longest minisatellite is to be the primitive state of the sequence, length polymorphisms found in closely related accessions (*L. rotundifoliae* and *L. scabrum*, *L. variicolor* NL and *L. variicolor* SW) or within one individual (*L. piceinum*, *L. duriusculum*) can best be explained by simple deletion processes.

The mechanism causing length polymorphisms in fungal minisatelitte-like sequences mentioned in literature is slipped strand mispairing (SSM) (Giraud et al 1998, Platas et al 2002). This process is mostly considered to be responsible for repetitive sequences consisting of repeats of 10 bp or less (microsatellites). The main reasons for Giraud et al (1998) to assume SSM as the main mutational mechanism are the particular structures of the minisatellites and the absence of evidence for recombination between alleles of the flanking regions. We did not find any evidence to assume allelic recombination in the flanking region. However, since the flanking regions seem to be relatively conserved, there are not much variable sites found in these regions anyway. Moreover, unequal crossing over does not necessarily imply recombination in the flanking regions (McAllister and Werren 1999). Neither the patterns found by mapping the core sequences, nor the Tandem Repeats Finder analyses, revealed patterns that lead to the conclusion that SSM is not the most probable process responsible for the length polymorphisms found. Gene conversion and unequal crossing over therefore seem to be better candidates to explain the processes involved in expansion of the minisatellite region.

Phylogenetic and systematic utility of minisatellite.—The structure and length of the minisatellite region appear not to be phylogenetically informative, as they do not correlate well with main monophyletic clades in the molecular phylogenies reconstructed. This is probably because the mutational processes responsible for the structure and size of the minisatellites act under strong structural constraints. Although these characteristics make the ITS1 spacer unsuitable as a phylogenetic marker at or above the species level in *Leccinum*, it does not mean that large parts of ITS1 are completely useless from a systematic point of view. Combined with morphological and ecological data ITS1 sheds new light on the status of many morphologically defined species. The minisatellite in

ITS1 seems to unique for *Leccinum* and is specific in its length and composition for most clades which coincide with morphologically circumscribed species or species-groups, making it an ideal marker for PCR based further ecological and evolutionary investigations of traditionally controversial species complexes in *Leccinum*.

ACKNOWLEDGMENTS

The authors would like to thank Machiel Noordeloos and Else C. Vellinga for reviewing earlier drafts of the manuscript and Marcel Eurlings for technical support and guidance during the design and execution of the experiments. Mauri Korhonen and Ruben Walleyn are thanked for the supply of Scandinavian and Belgian collections and sharing knowledge in the field. This research was supported by a Kits van Waveren Foundation training grant.

LITERATURE CITED

Aanen DK, Kuyper TW, Mes TH, Hoekstra RF. 2000. The evolution of reproductive isolation in the ectomycorrhizal *Hebeloma crustuliniforme* aggregate (Basidiomycetes) in northwestern Europe: a phylogenetic approach. Evolution 54:1192–1206.

Andersen TH, Nilsson-Tillgren T. 1997. A fungal minisatellite. Nature 386:771.

Benson G. 1999. Tandem Repeats Finder: a program to analyze DNA sequences. Nucleic Acid Research. 272:573–580.

Binder M. 1999. Zur molekularen Systematik der Boletales: Boletineae und Sclerodermatineae subordo nov. [PhD Dissertation]. Regensburg, Germany: University of Regensburg. 148 p.

———, Besl H. 2000. 28S rDNA sequence data and chemotaxonomical analyses on the generic conept of *Leccinum* (Boletales). In: Associazone Micologica Bresadola, ed. Micologia 2000. Brescia Italy: Grafica Sette. p 75–86.

Bruns TD. 2001. ITS reality. Inoculum 52(6):2–3.

Den Bakker HC. 2000. Hoe raak ik thuis in de Boleten. 3. Het geslacht *Leccinum* (Berken- of Ruigsteelboleten). Coolia 43(4):206–219.

Dover GA, Ruiz Linares Bowen AT, Hancock JM. 1993. Detection and quantification of concerted evolution and molecular drive. In: Abelson JN ed. Methods in enzymology, New York: Academic Press. p 525–541.

Engel H. 1978. Rauhstielröhrlinge—die Gattung *Leccinum* in Europa. Coburg, Germany: Hilmar Schneider. 76 p.

Farris JS, Kallersjo M, Kluge AG, Bult C. 1995. Testing significance of incongruence. Cladistics 10:315–319.

Gravendeel B, Chase MW, de Vogel EF, Roos MC, Mes THM, Bachmann K. 2001. Molecular phylogeny of Coelogyne (Epidendroideae; Orchidaceae) based on plastid RFLPs, *mat*K, and nuclear ribosomal ITS sequences: evidence for polyphyly. Am J of Bot 88(10): 1915–1927.

Giraud T, Fortini D, Levis C, Brygoo Y. 1998. The minisatellite MSB1, in the fungus *Botrytis cinerea*, probably mutates by slippage. Molec Biol Evol 15(11):1524–1531.

Graur D, Li W-H. 2000. Fundamentals of molecular evolution. Sunderland, Massachusetts: Sinauer. 443 p.

Hamann A, Osiewacz HD. Genome analysis of filamentous fungi: identification and characterization of an unusual GT-rich minisatellite in the ascomycete *Podospora anserine*. Curr Genet 34:88–92.

Harris DJ, Crandall KA. 2000. Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies. Molec Biol Evol 17:284–291.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–5.

Hughes KW, McGhee LL, Methven AS, Johnson JE, Petersen RH. 1999. Patterns of geographic speciation in the genus *Flammulina* based on sequences of the ribosomal ITS1–5.8S-ITS2 area. Mycologia 91:978–986.

Kasuga T, Mitchelson K. 1993. Determination of the DNA sequence of the 5.8S ribosomal gene of *Heterobasidion annosum* and *Heterobasidion araucariae*. Nucleic Acids Res 21:1320.

Kretzer A, Li Y, Szaro T, Bruns TD. 1996. Internal transcribed spacer sequences from 38 recognized species of *Suillus sensu lato*: phylogenetic and taxonomic implications. Mycologia 88(5):776–785.

Ko KS, Jung HS. 2002. Three nonorthologues ITS1 types are present in a polypore fungus *Trichaptum abietinum*. Mol Phylogenet Evol 23:112–122.

Korhonen M. 1995. New Boletoid fungi in the genus *Leccinum* from Fennoscandinavia. Karstenia 35:53–66.

Lannoy G, Estades A. 1995. Monographie des *Leccinum* d'Europe. La Roche-sur-Foron, France: Fédéderation Mycologique Dauphiné-Savoie. 229 p.

Lewin B. 2000. Genes VII. New York: Oxford University Press Inc. 990 p.

Maddison WP, Maddison DR. 1992. MacClade version 3.04. Sunderland, Massachusetts: Sinauer.

Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol 288:911–940.

Matzura O, Wennberg A. 1996. RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. CABIOS 12: 247–249.

McAllister BF, Werren JH. 1999. Evolution of tandemly repeated sequences: what happens at the end of an array? J Mol Evol 48:469–481.

Metzenberg AB, Wurzer G, Huisman THJ, Smithies O. 1991. Homology requirements for unequal crossing over in humans. Genetics 128:143–161.

Meyer W, Mitchell TG. 1995. Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: strain variation in *Cryptococcus neoformans*. Electrophoresis 16:1648–1656.

Muir G, Fleming CC, Schlötterer C. 2001. Three divergent rDNA clusters predate species divergence in *Quercus*

petraea (Matt.) Liebl. and *Quercus robur* L. Molec Biol Evol 18:112–119.

Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E, et al. 1987. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235(4796): 1616–1622.

Peintner U, Bougher NL, Castellano MA, Moncalvo JM, Moser MM, Trappe JM, Vilgalys R. 2001. Multiple origins of sequestrate fungi related to *Cortinarius* (Cortinariaceae). Am Journ of Botany 88(12):2168–2179.

Platas G, Acero J, Borkowski JA, Gonzalez V, Portal MA, Rubio V, Sanchez-Ballestros J, Salazar O, Pelaez F. 2001. Presence of a simple tandem repeat in the ITS1 region of the Xylariales. Curr Microbiology 43:43–50.

Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14(9):817–818.

Singer R. 1986. The Agaricales in modern taxonomy. 4th ed. Koenigstein, Germany: Koeltz Scientific Books. 981 p.

Smith AH, Thiers HD. 1971. The Boletes of Michigan. Ann Arbor, Michigan: The University of Michigan Press. 417 p.

Swofford DL. 1999. PAUP*—Phylogenetic analysis using parsimony (* and other methods), version 4.0b10. Sunderland, Massachusetts: Sinauer.

Van Herwerden L, Blair D, Agatsuma T. 1999. Intra-and interindividual variation in ITS1 of *Paragonimus westermani* (Trematoda: Digenea) and related species: implications for phylogenetic studies. Mol Phylogenet Evol 12(1):67–73.

Van Nues RW, Rientjes JMJ, Van der Sande CAFM, Zerp SF, Sluiter C, Venema J, Planta RJ, Raué HA. 1994. Separate structural elements with internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA. Nucl Acids Res 22(6):912–919.

Von der Schulenburg JH, Hancock JM, Pagnamenta A, Sloggett JJ, Majerus MEN, Hurst GDD. 2001. Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Molec Biol Evol 18:648–660.

Watling R. 1970. Boletaceae, Gomphidiaceae: Paxillaceae. In: Henderson DM, Orton PD, Watling R, eds. British Fungus Flora. Agarics and Boleti 1. Edinburgh, United Kingdom: Royal Botanical Gardens. p 45–57.

White TJ, Bruns TD, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfland DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego: Acadamic Press. p 315–322.

Wöstemeyer J, Kreibich A. 2002. Repetitive DNA elements in fungi (Mycota): impact on genomic architecture and evolution. Curr Genet 41:189–198.

Zolan ME, Pukkila PJ. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Mol Cell Biol 6:195–200.

Zuker M, Mathews DH, Turner DH. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. In: Barciszewski J, Clark BFC, eds. RNA biochemistry and biotechnology. NATO ASI Series. Dordrecht: Kluwer Academic Publishers. p 11–43.