Mycologia, 104(6), 2012, pp. 1490–1501. DOI: 10.3852/12-039

Lactarius subg. *Plinthogalus*: the European taxa and American varieties of *L. lignyotus* re-evaluated

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Abstract: The European species *Lactarius* subg. *Plinthogalus* were subjected to a molecular phylogenetic analysis based on ITS, LSU and *rpb2* sequences. Morphological characters of the species are discussed in the light of the phylogenetic results. In addition to a broad sampling within Europe, some Asian and North American taxa also were included in the analysis. Eight European species are confirmed molecularly: *L. lignyotus*, *L. acris*, *L. azonites*, *L. pterosporus*, *L. ruginosus*, *L. romagnesii*, *L. fuliginosus* and *L. picinus*. Except the sibling species *L. fuliginosus* and *L. picinus*, all are morphologically distinct. Our results suggest that *L. fuliginosus* is associated exclusively with broadleaf trees and *L. picinus* with conifers, but this putative difference in host specificity needs to be investigated further. *Lactarius subruginosus* turns out to be a synonym of either *L. pterosporus* or *L. ruginosus*. The position of *Lactarius terenopus* remains to be clarified. The North American taxa that are closely related to the European *L. lignyotus* (*L. fallax*, *L. lignyotus* var. *canadensis*, var. *nigroviolascens*, var. *marginatus*) are not resolved. Intercontinental conspecificity was demonstrated between Europe and northern Asia but was not found between Europe and southern Asia or between Europe and North America. A taxonomic subdivision of *L.* subg. *Plinthogalus* based on the height of the spore ornamentation should be rejected.

Key words: biogeography, ectomycorrhiza, molecular phylogeny, morphology, Russulales

Introduction

With only 10 species described from Europe, *Lactarius* subg. *Plinthogalus* forms a small but distinct group among the European species of *Lactarius*. The typical characteristics of the group in Europe are the velutinous cap of grayish buff to brown or blackish and the pinkish discoloration of the exposed context or latex. None of the European species possess macrocystidia, but macrocystidia are rare in the subgenus (Hesler and Smith 1979, Verbeken 2000, Le et al. 2007b, Stubbe et al. 2008). Species of *L.* subg. *Plinthogalus* are known for prominent spore ornamentations—in some European species almost 3 µm high. The currently known species in Europe are *L. acris* (Bolton : Fr.) Gray, *L. azonites* (Bull.) Fr., *L. fuliginosus* (Fr. : Fr.) Fr., *L. lignyotus* Fr., *L. picinus* Fr., *L. pterosporus* Romagn., *L. romagnesii* Bon, *L. ruginosus* Romagn., *L. subruginosus* J. Blum ex Bon and *L. terenopus* Romagn. of which the latter five species have spore ornamentations 2 µm high or higher. Bon (1980, 1983) considered those species with high spore ornamentation as a natural group, joining them in *L.* section *Ruginosi* (Bon) Bon while he placed the species with lower spore ornamentation in *L.* section *Plinthogali*. Heilmann-Clausen et al. (1998) disagreed with Bon's classification and regrouped all European species in one section. Verbeken (2000) even stated that the height of the spore ornamentation should not be considered a taxonomically valuable character for infrageneric classification. Modern descriptions and identification keys for the European species were provided by Reil (1997), Verbeken et al. (1998), Heilmann-Clausen et al. (1998) and Basso (1999). Regarding the concepts of *L. ruginosus* and *L. romagnesii*, there are different opinions among various authors. Here we adopt the concepts as defined by Heilmann-Clausen et al. (1998) and Verbeken et al. (1998) and consider both species as having distantly spaced lamellae and spores with high ornamentations that are reticulate in *L. romagnesii* but zebroid in *L. ruginosus*. The argument for these concepts is explained in detail in Verbeken et al. (2001) where the concept of *L. romagnesii* was based on the original description of *L. fuliginosus* f. *speciosus* J.E. Lange (the basionym of *L. romagnesii*). A comprehensive description of *L. subruginosus* is given by Basso (1999), but this species is not treated by the other authors. The most enigmatic species is probably *L. terenopus*. After its description in 1956, no new collections were reported until Moreau and Courtecuisse (2007). They said that the species is close to *L. pterosporus* but differs by bluish gray tinges on the cap, smaller spores and a less developed cellular layer in the pileipellis.

Submittted 30 Jan 2012; accepted for publication 29 Mar 2012.
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Several species have been reported outside Europe. In Japan, China and India *L. acris*, *L. azonites*, *L. fuliginosus*, *L. lignyotus*, *L. pterosporus*, *L. picinus* and *L. romagnesii* have been reported (Imazeki et al. 1988; Li 1991; Ying 1991; Teng 1996; Nagasawa 1998; Das and Sharma 2005). In North America, Hesler and Smith described several varieties of *L. lignyotus* based on differences in context discoloration and pigmentation of the lamella edge: *L. lignyotus* var. *canadensis* (pink discoloration, dark lamella edges), var. *marginatus* (violet discoloration, dark lamella edges) and var. *nigroviolascens* (violet discoloration, plain lamella edges) (Smith and Hesler 1962, Hesler and Smith 1979). The use of European names outside Europe is based on morphological similarities, but the relationship with the European species has not been confirmed molecularly.

Despite being a small group in Europe, with seemingly clear species concepts, we noticed that field identifications often are incorrect. For most species, microscopic characters need to be checked to ascertain the identity. On the other hand, when macroscopic observations are lacking it can be very difficult to distinguish certain species only by their microscopic characters (e.g. *L. picinus* vs. *L. fuliginosus*, *L. subruginosus* vs. *L. ruginosus*, *L. subruginosus* vs. *L. pterosporus*). Some species are distinguished by subtle differences, whereas other species encompass a remarkable variability. *Lactarius azonites* for example can be entirely fuliginous brown to almost completely white (cf. forma *virgineus*). A solution for these problems and an answer to the questions regarding species delimitation is urgently needed. By implementing molecular phylogenetic analyses, we want to reassess the taxonomy of the European species of *L.* subg. *Plinthogalus*, hereby including the North American varieties of *L. lignyotus*, and at the same time test the competing views on the infrageneric classification of this group.

MATERIALS AND METHODS

Specimens.—They were collected by the authors and obtained through loans from different herbaria. Most were collected in Europe, but there are also several Asian and American specimens included in the analysis. To assess the relationship between the European *L. lignyotus* var. *lignyotus* and its varieties from eastern North America, specimens of *L. lignyotus* var. *canadensis*, var. *nigroviolascens* and var. *marginatus* are included in this analysis as are specimens of the closely related species *L. fallax* from western North America, distinguished by a pale vinaceous discoloration, and specimens of *L. atromarginatus* described from Asia and staining violet blue. Nine collections of *L. azonites* made by the first author are included, exhibiting a diverse macromorphology, varying from grayish white to dark brown. In total, the dataset is based on 84 specimens. An overview of specimens used in the analyses and their origins is included herein (TABLE I).

DNA extraction, PCR and sequencing.—DNA extractions were obtained from dried material with the PrepMan® Ultra Sample Reagent kit (Applied Biosystems Inc, Foster City, California). In some cases DNA was extracted with the Gentra Puregene tissue kit (QIAGEN Benelux B.V., Venlo, the Netherlands). Extracts were purified with JetQuick General Clean-up columns (Genomed, Löhne, Germany). A third method for DNA extraction was: 1000 µL extraction-buffer (0.1 M Tris.Cl [pH 8], 0.5 M NaCl, 0.05 M EDTA), 50 µL 10% SDS and 0.774 µL β-mercapto-ethanol were added to the ground material and heated 1 h at 65 C; 2–4 µL proteinase K (20 mg/mL) was added and the mixture kept at 45–50 C overnight; after centrifuging, the supernatant was transferred to a new tube and mixed with an equal volume of cold isopropanol; the mixture was centrifuged and the supernatant disposed of; the remaining DNA pellet was washed with ethanol (70%) was added and dissolved in MilliQ water. If the extract appeared impure, an equal volume of CTAB was added and the tube was heated 15 min at 65 C; 400 µL chloroform/iso-amylalcohol (24 : 1) was added; the mixture was centrifuged and the aqueous phase transferred to a new tube; after adding two volumes of ethanol (96%) the tube cooled at 4 C at least 15 min; after centrifuging, the supernatant was removed; the remaining DNA pellet was washed with ethanol (70%) and dissolved in MilliQ water.

Three loci were amplified and sequenced: (i) the ITS region of the nuc rDNA, comprising ITS1, 5.8S and ITS2, using primers ITS1-F and ITS4 (White et al. 1990), occasionally with intermediate primers ITS2 and ITS3 (White et al. 1990); (ii) part of the LSU nuc rDNA using primers LR0R and LR5 (R. Vilgalys lab http://www.biology.duke.edu/fungi/mycolab/primers.htm); (iii) the region between conserved domains 6 and 7 of the second largest subunit of the RNA polymerase II (*rpb2*), using primers bRPB2-6f and fRPB2-7cR (Liu et al. 1999, Matheny 2005).

The PCR amplification reactions contained 5 µL DNA template, 5 µL amplification buffer, 0.5 µL $MgCl_2$ (25 mM), 1 µL dNTPs (10 mM), 1 µL (4 µL for *rpb2*) each primer solution (10 µM) and 0.3 µL Taq (5 units/µL). MilliQ water was added for 50 µL total. The PCR program started with a 5 min denaturation step at 94 C, followed by 35 cycles of 30 s at 94 C, 30 s at 55 C and 45 s at 70 C; the last cycle ending with an incubation of 7 min at 70 C. In some cases, a touchdown PCR protocol was executed. The PCR reaction was altered by adding only 0.7 µL dNTPs (10 mM), 0.7 µL (3 µL for *rpb2*) of each primer solution (10 µM) and 0.2 µL Taq (5 units/µL). The touchdown PCR profile consisted of 5 min initial denaturation at 95 C; then 10 cycles of 15 s denaturation at 95 C, 20 s annealing at 63 C decreased by one degree each cycle, and 1 min elongation at 72 C. Then followed 35 cycles with the annealing temperature fixed at 53 C, ending with a 2 min elongation after the last cycle.

Purification of PCR products, cycle sequencing reactions and sequencing were performed as described in Le et al. (2007a) or PCR products were sent to Macrogen's sequencing service Europe (Macrogen Europe, Meibergdreef 39,

TABLE I. Specimens and GenBank accession numbers of DNA sequences produced for the molecular analyses. Sequences with accession numbers in italics were retrieved from GenBank. Herbarium abbreviations are adopted from Index Herbariorum

Species	Original identification	Voucher	Geographic origin	ITS GenBank accession no.	LSU GenBank accession no.	*rpb2* GenBank accession no.
Lactarius acris	*L. acris*	R. Walleyn 94-801 (GENT)	Belgium	**JQ446085**	—	—
L. acris	*L. acris*	P.A. Moreau 08082502 (GENT)	France	**JQ446084**	**JQ446156**	**JQ446219**
L. acris	*L. acris*	A. Verbeken 97-520 (GENT)	France	*EF560659*	—	—
L. acris	*L. acris*	J. Nuytinck 2001-64 (GENT)	Slovakia	**JQ446082**	—	—
L. acris	*L. acris*	K. Van de Putte 08-094 (GENT)	Slovenia	**JQ446083**	**JQ446155**	—
L. acris	*L. acris*	L.A. Parra 36 (GENT)	Spain	**JQ446081**	**JQ446154**	—
L. acris	*L. acris*	EU 014 (UPS)	(*not specified*)	*DQ421988*	*DQ421988*	*DQ421922*
L. atromarginatus	*L. atromarginatus*	X.H. WANG 1983 (HKAS)	China, Yunnan prov.	**JQ446086**	—	—
L. atromarginatus	*L. atromarginatus*	H.T. Le 314 (CMU)	Thailand, Chiang Mai prov.	*EF560676*	**JQ446158**	**JQ446221**
L. atromarginatus	*L. atromarginatus*	A. Verbeken/R. Walleyn 04-102 (GENT)	Thailand, Chiang Mai prov.	*EF560674*	**JQ446157**	**JQ446220**
L. atromarginatus	*L. lignyotus* var. *marginatus*	E. Nagasawa, TMI18877 (TMI)	Japan, Tottori (Southern Honshu)	**JQ446124**	—	—
L. azonites	*L. azonites*	D. Stubbe 08-512 (GENT)	Belgium	**JQ446097**	**JQ446169**	—
L. azonites	*L. azonites*	D. Stubbe 08-516 (GENT)	Belgium	**JQ446093**	**JQ446165**	**JQ446228**
L. azonites	*L. azonites*	D. Stubbe 08-518 (GENT)	Belgium	**JQ446094**	**JQ446166**	**JQ446229**
L. azonites	*L. azonites*	D. Stubbe 08-519 (GENT)	Belgium	**JQ446095**	**JQ446167**	**JQ446230**
L. azonites	*L. azonites*	D. Stubbe 08-520 (GENT)	Belgium	**JQ446096**	**JQ446168**	**JQ446231**
L. azonites	*L. azonites*	D. Stubbe 08-513 (GENT)	Belgium	—	**JQ446170**	—
L. azonites	*L. azonites*	D. Stubbe 08-514 (GENT)	Belgium	**JQ446098**	**JQ446171**	—
L. azonites	*L. azonites*	D. Stubbe 08-517 (GENT)	Belgium	**JQ446099**	**JQ446172**	—
L. azonites	*L. azonites*	J. Issakainen/J. Vauras 26494 (TURA)	Finland	**JQ446088**	**JQ446160**	**JQ446223**
L. azonites	*L. azonites*	S. Adamčík, SAV F-2225 (SAV)	Slovakia	**JQ446089**	**JQ446161**	**JQ446224**
L. azonites	*L. azonites*	D. Stubbe 08-526 legit M.A. Pérez-De-Gregorio (GENT)	Spain	**JQ446087**	**JQ446159**	**JQ446222**
L. azonites	*L. fuliginosus*	S. Adamčík, SAV F-2229 (SAV)	Slovakia	**JQ446107**	**JQ446176**	**JQ446236**
L. azonites	*L. romagnesii*	A.A. Kiyashko, LE 254459 (LE)	Georgia, Western Caucasus, Abkhaz Republic	**JQ446142**	**JQ446211**	**JQ446266**
L. azonites f. *virgineus*	*L. azonites* f. *virgineus*	D. Stubbe 08-515 (GENT)	Belgium	**JQ446092**	**JQ446164**	**JQ446227**
L. fallax	*L. fallax*	S. Trudell SAT05-267-12 (WTU)	U.S.A., Idaho	**JQ446102**	**JQ446173**	**JQ446232**
L. fallax	*L. fallax*	J. Floberg-F148F (WTU)	U.S.A., Washington	**JQ446103**	**JQ446174**	**JQ446233**
L. fallax	*L. fallax* var. *concolor*	E. Cline-59 (WTU)	U.S.A., Washington	**JQ446104**	—	—
L. fallax	*L. fallax* var. *fallax*	D.E. Desjardin 5543 (SFSU)	U.S.A., California	**JQ446101**	—	—
L. fuliginosus	*L. fuliginosus*	D. Stubbe 06-310 (GENT)	Belgium	**JQ446110**	**JQ446179**	**JQ446239**
L. fuliginosus	*L. fuliginosus*	J. Vauras 16767F (TURA)	Finland	**JQ446108**	**JQ446177**	**JQ446237**

TABLE I. Continued

Species	Original identification	Voucher	Geographic origin	ITS GenBank accession no.	LSU GenBank accession no.	*rpb2* GenBank accession no.
L. fuliginosus	*L. picinus*	R. Walleyn 3703 (GENT)	Czech Republic	*GU258279*	*GU265648*	*GU258388*
L. fuliginosus	*L. fuliginosus*	M.T. Basso 97-24 (GENT)	Sweden	**JQ446111**	**JQ446180**	**JQ446240**
L. fuliginosus	*L. picinus*	P. Ricart, FA-15833 (AMNH)	Iceland	**JQ446131**	**JQ446199**	**JQ446259**
L. fuliginosus	*L. picinus*	D. Stubbe 08-527 legit T. Hering (GENT)	U.K.	**JQ446128**	**JQ446196**	**JQ446256**
L. fuliginosus	*L. romagnesii*	UE 29.09.2002-6 (UPS)	(*not specified*)	*DQ421989*	*DQ421989*	*DQ421923*
L. lignyotus	*L. lignyotus*	UE 06.09.2003-5 (UPS)	(*not specified*)	*DQ421993*	*DQ421993*	*DQ421926*
L. lignyotus	*L. lignyotus*	K. Van de Putte 08-083 (GENT)	Austria	**JQ446113**	**JQ446182**	**JQ446242**
L. lignyotus	*L. lignyotus*	M. Liisa/P. Heinonen 487-2004 (TURA)	Finland	**JQ446115**	**JQ446184**	**JQ446244**
L. lignyotus	*L. lignyotus*	R. Walleyn 1272 (GENT)	France	**JQ446112**	**JQ446181**	**JQ446241**
L. lignyotus	*L. lignyotus*	A.E. Kovalenko, LE 16204 (LE)	Russia, Far East, Primorskiy territory	**JQ446116**	**JQ446185**	**JQ446245**
L. lignyotus	*L. lignyotus*	O.V. Morozova, LE 253465 (LE)	Russia, Moscow region	**JQ446117**	**JQ446186**	**JQ446246**
L. lignyotus	*L. lignyotus*	S. Adamčík, SAV F-2226 (SAV)	Slovakia	**JQ446114**	**JQ446183**	**JQ446243**
L. lignyotus	*L. lignyotus*	R. Halling 8476 (NY)	Russia, Novgorod region	**JQ446126**	**JQ446194**	**JQ446254**
L. aff. *lignyotus*	*L. lignyotus*	S. Trudell 00-223-16 (WTU)	U.S.A., New Hampshire	**JQ446125**	**JQ446193**	**JQ446253**
L. aff. *lignyotus*	*L. lignyotus* var. *canadensis*	J. Nuytinck 2007-001 (GENT)	Canada, Newfoundland	**JQ446123**	**JQ446192**	**JQ446252**
L. aff. *lignyotus*	*L. lignyotus* var. *canadensis*	D.P. Lewis 7259 (GENT)	U.S.A., Mississippi	**JQ446120**	**JQ446189**	**JQ446249**
L. aff. *lignyotus*	*L. lignyotus* var. *lignyotus*	A.S. Methven 9211 (EIU)	U.S.A., North Carolina	**JQ446121**	**JQ446190**	**JQ446250**
L. aff. *lignyotus*	*L. lignyotus* var. *nigroviolascens*	A. Voitk 21-08-2008 (GENT)	Canada, Newfoundland	**JQ446118**	**JQ446187**	**JQ446247**
L. aff. *lignyotus*	*L. lignyotus* var. *nigroviolascens*	A.S. Methven 11828 (EIU)	U.S.A., Michigan	**JQ446119**	**JQ446188**	**JQ446248**
L. aff. *lignyotus*	*L. lignyotus* var. *nigroviolascens*	A.S. Methven 9866 (EIU)	U.S.A., North Carolina	**JQ446122**	**JQ446191**	**JQ446251**
L. picinus	*L. fuliginosus*	O.V. Morozova, LE 215133 (LE)	Russia, Leningradsky Prov.	**JQ446109**	**JQ446178**	**JQ446238**
L. picinus	*L. picinus*	K. Van de Putte 08-077 (GENT)	Austria	**JQ446129**	**JQ446197**	**JQ446257**
L. picinus	*L. picinus*	D. Stubbe 09-616 (GENT)	Italy	**JQ446133**	**JQ446200**	**JQ446260**
L. picinus	*L. picinus*	J. Vauras 97-295 (GENT)	Norway	**JQ446132**	—	—
L. picinus	*L. picinus*	S. Adamčík, SAV F-2223 (SAV)	Slovakia	**JQ446130**	**JQ446198**	**JQ446258**
L. picinus	*L. picinus*	J. Nuytinck 2001-62 (GENT)	Slovakia	**JQ446134**	**JQ446201**	**JQ446261**
L. picinus	*L. picinus*	D. Stubbe 08-525 legit M.A. Pérez-De-Gregorio (GENT)	Spain	**JQ446127**	**JQ446195**	**JQ446255**
L. pterosporus	*L. fuliginosus*	D. Stubbe 08-524 legit M.A. Pérez-De-Gregorio (GENT)	Spain	**JQ446105**	**JQ446175**	**JQ446234**

TABLE I. Continued

Species	Original identification	Voucher	Geographic origin	ITS GenBank accession no.	LSU GenBank accession no.	*rpb2* GenBank accession no.
L. pterosporus	*L. pterosporus*	K. Van de Putte 08-072 (GENT)	Austria	**JQ446135**	**JQ446203**	**JQ446263**
L. pterosporus	*L. pterosporus*	J. Nuytinck 2001-09 (GENT)	France	**JQ446140**	**JQ446208**	—
L. pterosporus	*L. pterosporus*	D. Stubbe 09-614 (GENT)	Italy	**JQ446138**	*JN389002*	*JN375605*
L. pterosporus	*L. pterosporus*	A.A. Sopina, LE 254462 (LE)	Russia, Western Caucasus, Adygei Republic	**JQ446139**	**JQ446207**	—
L. pterosporus	*L. pterosporus*	K. Van de Putte 08-087 (GENT)	Slovenia	**JQ446136**	**JQ446204**	**JQ446264**
L. pterosporus	*L. subruginosus*	P.A. Moreau 08082501 (GENT)	France	**JQ446152**	**JQ446218**	**JQ446274**
L. pterosporus	*L. subruginosus*	P.A. Moreau 08082503 (GENT)	France	**JQ446151**	**JQ446217**	**JQ446273**
L. pterosporus	*L. subruginosus*	M.T. Basso 95091501 (GENT)	Italy	*EF560661*	—	—
L. pterosporus	*L. terenopus*	P.A. Moreau 06100705 (GENT)	France	**JQ446153**	—	**JQ446275**
L. romagnesii	*L. romagnesii*	R. Walleyn 4024 (GENT)	Belgium	*EF560662*	**JQ446210**	**JQ446265**
L. romagnesii	*L. romagnesii*	R. Walleyn 3272 (GENT)	Slovakia	**JQ446143**	**JQ446212**	**JQ446267**
L. ruginosus	*L. azonites*	R. Watling 29562 (E)	U.K.	**JQ446091**	**JQ446163**	**JQ446226**
L. ruginosus	*L. fuliginosus*	K. Van de Putte 08-092 (GENT)	Slovenia	**JQ446106**	—	**JQ446235**
L. ruginosus	*L. pterosporus*	L. Kosonen/J. Korhonen 04-10-2007 (TURA)	Finland	**JQ446137**	**JQ446205**	—
L. ruginosus	*L. romagnesii*	S. Adamčík, SAV F-88 (SAV)	Slovakia	**JQ446141**	**JQ446209**	—
L. ruginosus	*L. ruginosus*	K. Van de Putte 08-082 (GENT)	Austria	**JQ446144**	**JQ446213**	**JQ446268**
L. ruginosus	*L. ruginosus*	R. Walleyn/A. Verbeken 3147 (GENT)	Czech Republic	*EF560660*	—	**JQ446270**
L. ruginosus	*L. ruginosus*	J. Vauras 99-433 (C)	Denmark	**JQ446146**	—	—
L. ruginosus	*L. ruginosus*	D. Stubbe 09-615 (GENT)	Italy	**JQ446145**	**JQ446214**	**JQ446269**
L. ruginosus	*L. subruginosus*	M. Wilhelm 18-9-1999 (GENT)	Switzerland	**JQ446149**	**JQ446215**	**JQ446271**
L. ruginosus	*L.* cfr. *subruginosus*	D. Stubbe 09-613 (GENT)	Italy	**JQ446150**	**JQ446216**	**JQ446272**
Lactarius sp.	*L. azonites*	A.E. Kovalenko, LE 16493 (LE)	Russia, Far East, Primorskiy territory	**JQ446090**	**JQ446162**	**JQ446225**
Lactarius sp.	*L. azonites*	X.H. Wang 1951 (HKAS)	China, Yunnan Prov.	**JQ446100**	—	—
Lactarius sp.	*L. ruginosus*	X.H. Wang 1954 (HKAS)	China, Yunnan Prov.	**JQ446147**	—	—
Lactarius sp.	*L. ruginosus*	X.H. Wang 1941 (HKAS)	China, Yunnan Prov.	**JQ446148**	—	—

Amsterdam, the Netherlands). Both forward and reverse sequences were produced to resolve undetermined base pairs as much as possible. Contigs were assembled and edited with Sequencher™ 4.8 (Gene Codes Corp., Ann Arbor, Michigan).

Alignment and phylogenetic analysis.—Initial alignments were made with MAFFT 6 (Katoh and Toh 2008a, b) with setting L-INS-i for the ITS alignment and setting FFT-NS-I for the LSU and *rpb2* alignments. Alignments were manually edited further in BioEdit 7.0.9.0 (Hall 1999). The complete dataset was classified in six partitions: ITS1 + ITS2, SSU + 5.8S, LSU, *rpb2*-1 + *rpb2*-2 (first and second codon positions), *rpb2*-3 (third codon positions), *rpb2* intron. Maximum likelihood (ML) analyses were performed with RAxML 7.0.3 (Stamatakis 2006a, b; Stamatakis et al. 2008). A Rapid BS algorithm applying the GTRMIX model was executed for 500 replicates for the separate markers and for 1000 replicates for the combined datasets. Compat.py (Kauff and Lutzoni 2002) was used to detect possible conflicts between the analysis results of different markers. Two combined datasets were analyzed: one containing all three markers and one containing only ITS and LSU data from specimens for which both markers had been obtained. The matrices are submitted at www.treebase.org with accession number S12338.

Microscopy.—Pileipellis structures and hymenial elements were observed in Congo red in L4 and in a 10% aqueous potassium hydroxide solution. Basidiospores were observed in Melzer's reagent.

RESULTS

The dataset comprises 83 ITS sequences, 68 LSU sequences and 61 *rpb2* sequences. The concatenated alignment as used for the phylogenetic analysis consists of 2222 characters (1–642: ITS. 643–1503: LSU. 1504–2222: *rpb2*).

The molecular analysis based on ITS, LSU and *rpb2* reveals seven strongly supported European clades (FIG. 1). The analyses of the three separate markers exhibit minor conflicts at a minimum BS support of 65%, but these conflicts always are situated within a strongly supported clade; there are no jumping taxa between these seven clades. Because the *rpb2* sequences were in many cases incomplete and therefore of variable length, an additional analysis was performed using only the ITS and LSU sequences of specimens for which both markers had been obtained. This ITS-LSU analysis retrieved eight European clades with moderate to high BS supports (FIG. 2).

The L. azonites *clade.*—Of the 14 specimens two were misidentified as *L. fuliginosus* and as *L. romagnesii*. Re-examination of the spores confirmed these specimens as *L. azonites*. Dark and pale specimens group together with little genetic variability. The clade contains specimens from Finland, Belgium, Spain, Slovakia and the Caucasus region in Russia. The Chinese specimen identified as *L. azonites* is unrelated to the European species.

The L. acris *clade.*—All specimens morphologically identified as *L. acris* fall into the same clade. Specimens were collected from Slovenia, France, Belgium and Spain. One specimen (*LE16493* identified as *L. azonites*) from the Russian Far East near the Sea of Japan is closely related to *L. acris*. Its spores are different from those of *L. acris*, having more blunt ridges.

The L. pterosporus *clade.*—All specimens in this clade have spores with zebroid ornamentation up to 2.5(3) µm high and crowded lamellae. Several specimens had been identified as *L. subruginosus*, but morphologically we find no difference with the other specimens in this clade. Also the specimen considered to represent *L. terenopus* falls in this clade. One specimen was misidentified as *L. fuliginosus*. Specimen *LE254462* from the Caucasus region is fully conspecific with the specimens from western Europe.

The L. ruginosus *clade.*—The specimens in this clade also have a high, zebroid spore ornamentation, but the lamellae are distantly spaced. Here, two specimens originally were identified as *L. subruginosus* and one as *L. pterosporus*. Three specimens were misidentified as *L. romagnesii*, *L. azonites* and *L. fuliginosus* resp. In the ITS-LSU analysis two specimens (*SAV.F.88*, *D.S.09-615*) are genetically distinctive, but we find no micromorphological features that set them apart. The two Chinese specimens identified as *L. ruginosus* are not related to the European species.

The L. romagnesii *clade.*—Two specimens form a separate clade and fully comply with the concept of *L. romagnesii* as described by Heilmann-Clausen et al. (1998). Three other specimens that were identified as *L. romagnesii* are distributed among the clades of *L. azonites*, *L. ruginosus* and *L. picinus*/*L. fuliginosus*. The morphology of these specimens corresponds with their molecular phylogenetic placement.

The L. fuliginosus/L. picinus *clade.*—Specimens identified as *L. picinus* and *L. fuliginosus* are intermixed in the combined analysis of the three markers. In the ITS-LSU analysis this clade is divided into two subclades with each subclade containing both names. Looking at the exsiccata, pale (i.e. gray) and dark (i.e. blackish gray) specimens are present in both subclades. Microscopically there is no difference between the specimens of the two subclades. Most specimens were collected in mixed forests. Because one clade also contains specimens that have been found in habitats lacking conifers, we designate this

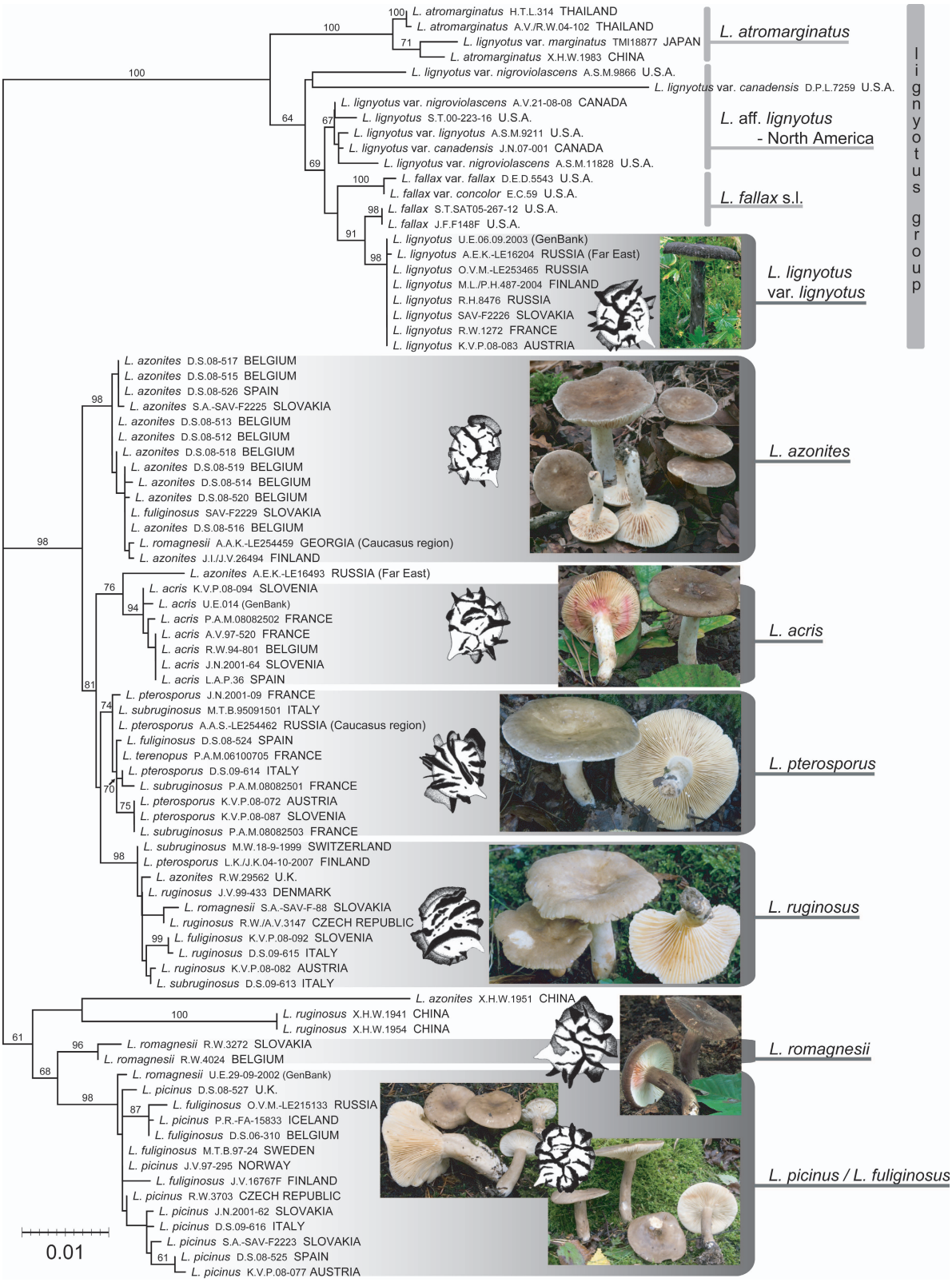

FIG. 1. The best phylogenetic tree resulting from a combined ML analysis based on ITS, LSU and *rpb2* sequences. Bootstrap support values below 60% are omitted. The taxa in the tree have their original identification. Characteristic spores and habitus are provided for each European species.

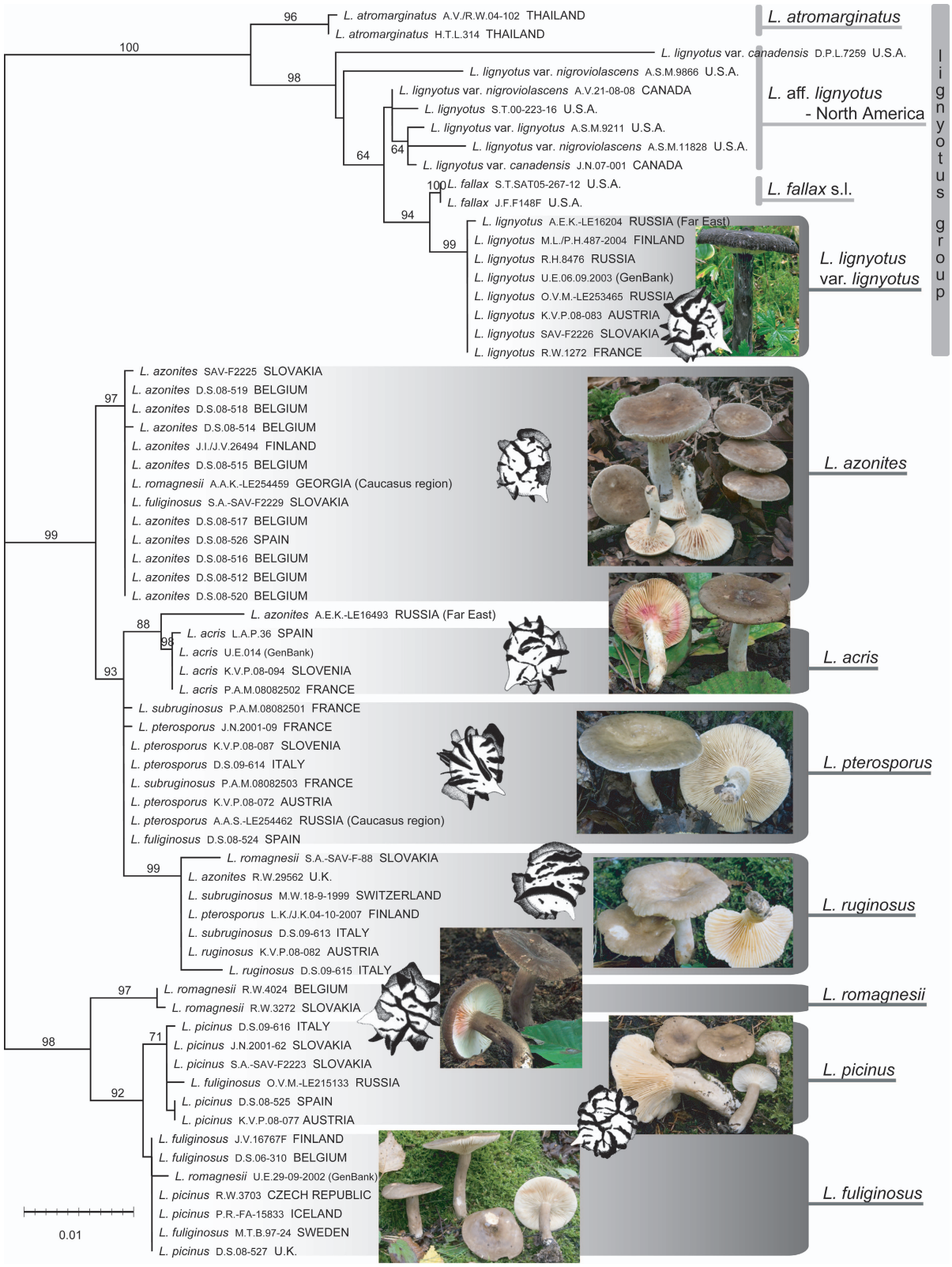

FIG. 2. The best phylogenetic tree resulting from a combined ML analysis based on ITS and LSU sequences. Missing data were reduced to a minimum by including only those specimens for which both markers had been obtained. Bootstrap support values below 60% are omitted. The taxa in the tree have their original identification. Characteristic spores and habitus are given for each European species.

clade as the *L. fuliginosus* clade. The specimen from Iceland (*FA15833*) was found in native *Betula* forest. The other clade contains some specimens from habitats with only conifers, and therefore we name it the *L. picinus* clade.

The group surrounding L. lignyotus.—This clade assembles the European *L. lignyotus* var. *lignyotus*, the Asian *L. atromarginatus*, the western North American *L. fallax* s.l. and the eastern North American varieties of *L. lignyotus*. The European *L. lignyotus* var. *lignyotus* is a phylogenetically strongly supported species. Specimens were examined from France, Slovakia, Finland, Austria, western Russia and the Russian Far East near the Sea of Japan. The lamella edges are mostly pigmented. Even when no pigmentation is visible when viewed with a hand lens, pigmented cheilocystidia usually can be found scattered along the lamella edge viewed under the compound microscope (e.g. in *R.W.1272*, *R.H.8476*). Sometimes pigmentation in the lamella edge is absent (as in *LE16204*, *SAV.F.2226*). All North American specimens of the *lignyotus* group have a pileipellis structure composed of a palisade with slender terminal elements 50–70 µm long. They all have similar spores, clearly discernable from those of the European species, with an ornamentation composed of interconnected, spiny warts or ridges 1–2 µm high. The western North American *L. fallax* s.l. is a paraphyletic species comprising at least two species. The two clades recovered in the analysis cannot be distinguished microscopically. Both clades contain a specimen with pigmented lamella edges (*D.E.D.5543*, *J.F.F-148F*) and a specimen without pigmentation in the lamella edge (*E.C.59*, *S.T.SAT05-267-12*). For the taxa of eastern North America the results are similar; the North American varieties of *L. lignyotus* are paraphyletic and no distinction between clades can be made based on microscopy, pigmentation of the lamella edge or context discoloration. The two specimens that were identified as *L. lignyotus* and *L. lignyotus* var. *lignyotus* are sterile (*S.T.00-223-16*, *A.S.M.9211* resp.). Both have plain lamella edges, although *A.S.M.9211* contains scattered, pigmented cheilocystidia. Also the three specimens identified as *L. lignyotus* var. *nigroviolascens* have pigmented cheilocystidia (*A.V.21-08-2008*, *A.S.M.11828*, *A.S.M.9866*). The Japanese specimen *TMI18877*, identified as *L. lignyotus* var. *marginatus*, groups together with the Thai and Chinese specimens of *L. atromarginatus* in a clade that is sister to all the European and American species of the *lignyotus* group.

DISCUSSION

Molecular phylogenetic analysis confirms the existence of eight European species: *L. lignyotus*, *L. azonites*, *L. acris*, *L. pterosporus*, *L. ruginosus*, *L. romagnesii*, *L. picinus* and *L. fuliginosus*. The two latter species are supported by the ITS-LSU analysis but were not recovered in the combined analysis with *rpb2*, probably due to inconsistent sequence lengths in the *rpb2* alignment. With missing data reduced to a minimum in the ITS-LSU analysis, the two species can be distinguished with moderate support (with species delimitation remaining intact for the other species). The European species are well defined morphologically with exception of the sibling species *L. fuliginosus* and *L. picinus*. *Lactarius fuliginosus* is known as a grayish buff mushroom occurring with broadleaf trees or conifers, while *L. picinus* should be sepia to almost blackish brown and found only near conifers. Although the field identifications probably were based largely on the color of the basidiomata (specimens originally identified as *L. picinus* were consistently darker than those identified as *L. fuliginosus*), our results indicate that pileus color is not reliable as a distinguishing character. Both clades contain specimens with a varying degree of darkness from dull gray (e.g. specimen *LE215133* in the picinus clade) to nearly black (e.g. *R.W.3703* in the fuliginosus clade). A pale *L. picinus* under conifers therefore could have been mistaken for *L. fuliginosus*. The microscopic features are similar in both species. The differences in spore ornamentation, regarding thickness and density of the ridges as mentioned by Heilmann-Clausen et al. (1998), fall within the morphological variability of both species. The host tree seems to be a more reliable feature, with *L. fuliginosus* being associated with deciduous trees and *L. picinus* with conifers. However, this is not always practical because both species also occur in mixed habitats. A study of the ectomycorrhiza dug up below the mushrooms would be useful to verify this supposedly strict separation in host association. Broadleaf trees are the predominant ectomycorrhizal partners for species of *L.* subg. *Plinthogalus*. This is not the case for *L. lignyotus* and its allied species, which are associated with conifers, but phylogenetically they also form a distinct group. It is possible that the separation of *L. fuliginosus* and *L. picinus* is a recent event that was triggered by a host switch to conifers.

A varying degree of darkness of the basidiocarp color also is illustrated strikingly for the species *L. azonites*. Contrary to its macroscopic morphology, the species is uniform genetically. Next to the typical aspect of a brownish gray cap and whitish stipe (*D.S.08-519*), specimens can vary from dirty white (*D.S.08-515*) (as illustrated by the description of the *L. azonites* f. *virgineus* [J.E. Lange] Verbeken) to fuliginous brown in both cap and stipe (*D.S.08-516*) (FIGS. 3, 4). It is nevertheless a species that can be identified correctly by the spores, which often have

thin ridges (a representative drawing is in Heilmann-Clausen et al. 1998). In fresh condition the cap margin usually is bordered by a fine cream-white line, even in the darker specimens, and the latex turns pink with a distinct orange tinge.

According to the original diagnosis by Bon (1985), *L. subruginosus* is characterized by crowded lamellae, a faint coconut odor and mild-flavored flesh that becomes pinkish and ultimately reddish; the winged spore ornamentation is up to 2–3 μm high. Bon further specified the difference with *L. pterosporus*, which has an acrid flavor and strongly crowded lamellae, and with *L. ruginosus*, which has distantly spaced lamellae and a crenulate pileus margin. In this concept *L. subruginosus* morphologically seems to be an intermediate between *L. pterosporus* and *L. ruginosus*. It does not come as a surprise that in the molecular phylogeny the specimens of *L. subruginosus* do not form a separate clade but instead fall either in the clade of *L. pterosporus* or in the clade of *L. ruginosus*. The main characters used to distinguish among *L. pterosporus*, *L. ruginosus* and *L. subruginosus* are density of the lamellae, flavor and odor. Flavor and odor are partly subject to personal interpretation, and the intensity can vary with meteorological conditions. Density of the lamellae, if not expressed in figures, is also susceptible to personal interpretation. Nevertheless, even on the exsiccata, we observed a clear difference in lamella density among all specimens of the *L. ruginosus* clade and those of the *L. pterosporus* clade. When measuring the lamella density by counting the number of lamellae per centimeter at midradius (L/cm), young and small basidiocarps often appear to have denser lamellae than mature basidiocarps of the same species, while in fact the number of lamellae is more or less equal. This method is therefore not ideal for distinguishing species. For a more correct estimation of lamella density, we suggest that lamella density for these species should be measured in relation to cap diameter. By multiplying L/cm with the cap diameter (in centimeters), L becomes an index proportional to the total number of lamellae. A mushroom 5 cm diam and lamella density 7 L/cm then would have a lamella index of 35 (=7 L/cm × 5 cm), which is comparable with a 3 cm diam mushroom and misleadingly higher lamella density of 12 L/cm (lamella index 36). This allows a more objective comparison between specimens of different sizes. Observations on fresh specimens need to be made to obtain and test lamella indices for *L. pterosporus* and *L. ruginosus* and to check whether specimens with intermediate lamella densities exist. This process has been initiated by the authors and is ongoing. Regarding the spores, we have found specimens with those that could be considered typical for either *L. ruginosus* (with spore ornamentation having crenulate edges) or *L. pterosporus* (with strongly curved spore ornamentation up to 3 μm high), but more often there is a strongly overlapping variability. Lamella density is a better character to make the distinction, even when density values for each species have not been established.

Specimen *P.A.M.06100705*, identified as *Lactarius terenopus*, turns out to belong in the clade of *L. pterosporus*. The spores of this specimen indeed are small for *L. pterosporus* (Moreau and Courtecuisse 2007) but still fall within the variability of the species. The bluish gray tinge of the cap and the slightly less developed subpellis of the pileipellis must be regarded in the same way. With only one specimen examined here, it is premature to draw conclusions on the status of *L. terenopus* as a species. The spores of the type specimen (*H. Romagnesi 51-242* [PC]) bear a reticulate ornamentation up to 2 μm high, reminiscent of *L. romagnesii*, but *L. terenopus* is a much paler species and has smaller spores, averaging only 7.6 × 6.7 μm compared to 8.0–8.7 × 7.0–7.4 μm in *L. romagnesii* (Heilmann-Clausen et al. 1998). We do not reject *L. terenopus* as a species, but its position remains to be confirmed and more material certainly is needed. So far, there are no more collections known in addition to the type material.

Lactarius romagnesii, next to *L. pterosporus* and *L. ruginosus*, is the third species with high spore ornamentation confirmed by this study. It is a dark brown species and, as opposed to *L. pterosporus* and *L. ruginosus*, the spore ornamentation is clearly reticulate with ridges often having an angular aspect (representative spores are shown in Heilmann-Clausen et al. 1998). The two specimens in this study that match the descriptions given by Heilmann-Clausen et al. (1998) and Verbeken et al. (1998) form a distinct, monophyletic clade. The other specimens fall in the clades of the other European species. These erroneous identifications must be due to the different species concepts in literature. Our results confirm the species concept and the argumentation given by Heilmann-Clausen et al. (1998) and Verbeken et al. (1998, 2001), which is based on the interpretation of the original description of *L. fuliginosus* f. *speciosus* (basionym of *L. romagnesii*).

No misidentifications have been encountered for *L. acris*. The species indeed is readily recognizable in the field by its bright pink latex and slightly viscid, pale buff cap. The spore ornamentation consists of a broken reticulum of acute ridges, less than 2 μm high. Specimen *LE16493*, collected in the Russian Far East near the Sea of Japan, is closely related to *L. acris* but represents an undescribed species with spores ornamented with blunt ridges.

FIGS. 3, 4. Photographs of *Lactarius azonites* illustrating the varying degree of darkness of the basidiocarp color. 3. *L. azonites* f. *virgineus*, *D.S.08-515*. 4. *L. azonites*, *D.S.08-516*.

Lactarius lignyotus var. *lignyotus* is another unmistakable European species, with its black-brown cap and stipe, and contrasting white lamellae. The species is found all over Europe and even in the Russian Far East. Its distribution covers the entire temperate and boreal belt of the Old World, however, without making the leap to the New World. The pigmentation of the lamella edge (and hence of the cheilocystidia) is clearly a variable character in this species. This is also the case in the North American species of the *L. lignyotus* group. Our results indicate that coloration of the lamella edge should not be used as a taxonomic character. None of the North American taxa (*L. lignyotus* var. *canadensis*, *L. lignyotus* var. *nigroviolascens*, *L. fallax* var. *fallax*, *L. fallax* var. *concolor*) were resolved in the phylogeny. The genetic diversity is much greater than in Europe (or Asia as far as we can tell). Because context discoloration is the other main character used for differentiating the taxa in North America, it might prove useful to observe this more objectively and more elaborately. Some discolorations may appear only after several hours (in *A.V.21-08-2008* violet appeared overnight). It thus is highly probable that for some specimens the observations of the color reaction were incomplete and therefore misleading. Also host specificity should be studied in detail because in North America *L.* aff. *lignyotus* may grow with several conifer genera while in Europe *L. lignyotus* is strictly associated with *Picea*. With *L. fallax* as the closest relative of the European *L. lignyotus* var. *lignyotus*, none of the other North American taxa can be considered varieties of *L. lignyotus*. What is misapplied in Japan as *L. lignyotus* var. *marginatus* is in fact *L. atromarginatus*, described from Papua New Guinea (Verbeken and Horak 2000) but also reported from Thailand and China (Stubbe et al. 2008, Wang 2008).

Intercontinental conspecificity is demonstrated between Europe and northern Asia. This can be explained by the continuous boreal forest stretching from northern Europe to far eastern Russia. With few samples to test intercontinental conspecificity between Europe and North America and between Europe and southern Asia, the supposed intercontinental conspecificity was rejected. Sampling should be made more elaborate to further substantiate the absence of conspecificity, but this preliminary conclusion is in line with other transcontinental studies of *Lactarius* and *Lactifluus* (Nuytinck et al. 2007, Stubbe et al. 2010).

The European species do not form a monophyletic group but are distributed over three clades. *Lactarius lignyotus* is nested within an intercontinental clade with American and Asian species. *Lactarius pterosporus* and *L. ruginosus* group together with *L. acris* and *L. azonites*. The third clade is composed of *L. romagnesii*, *L. picinus* and *L. fuliginosus*. This also demonstrates that the sectional classification as proposed by Bon (1980, 1983), in which species with low spore ornamentation are separated from species with high spore ornamentation, should be abandoned. We refrain from proposing an alternative subdivision for *L.* subg. *Plinthogalus* because this is best done after a study that comprises species from all continents.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to everyone who sent specimens for this study: Slavomir Adamčík, Pierre-Arthur Moreau, Jorinde Nuytinck, Kobeke Van de Putte, Luis Parra, Huyen Than Le, Xiang-Hua Wang, Jukka Vauras, Roy Watling, Dennis Desjardin, Olga Morozova, David P. Lewis, Andrew Methven, Eiji Nagasawa, Anrus Voitk, Markus Wilhelm, Vladimír Antonín, Tuula Niskanen, Guðríður Gyða Eyjólfsdóttir, Joe Ammirati, Roy Halling, Greg Mueller, Omer Van de Kerckhove,

Etienne Vanaelst, Peter Verstraeten and André de Haan. Many thanks to Felix Hampe for the photographs of *Lactarius acris* and *L. romagnesii*, to Ruben Walleyn for his photograph of *L. ruginosus* and to Kobeke van de Putte for her photograph of *L. lignyotus*. The first author was financially supported by a scholarship of the Institute for the Promotion of Innovation through Science and Technology (IWT-Vlaanderen).

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