ORIGINAL ARTICLE

Lactifluus piperatus (Russulales, Basidiomycota) and allied species in Western Europe and a preliminary overview of the group worldwide

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Abstract The large, white milkcaps of *Lactifluus* section *Piperati* are well known in the northern hemisphere. Historically, there was extensive debate about the number of European representatives and the diagnostic characteristics that delimit the species. Combining a morphological approach with a phylogenetic study, we aimed to resolve the problems in this section in Europe. Secondly, a molecular analysis of worldwide representatives of *Lactifluus* section *Piperati* was carried out, to verify whether there is intercontinental conspecificity. We compared nuclear ITS and LSU rDNA, nuclear protein-coding *rpb2* and mitochondrial protein-coding *atp6* genealogies to delimit species, using a concatenation of genes, along with Bayesian species delimitation for the European dataset. The phylogenetic analyses show the existence of two species in Europe: *Lactifluus piperatus* and *Lactifluus glaucescens*. Morphologically, the frequently used characteristics of the colouration of the latex and the macrochemical reactions of latex and context appear not to be useful as diagnostic characteristics to discriminate the species, but the microscopical characters of the pileipellis are informative. The preliminary overview of the section worldwide shows that it comprises at least 10 possible species divided over three clades, and that there is no intercontinental conspecificity.

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Introduction

Lactifluus piperatus and allies in Europe

Milkcaps show a striking variability in basidiocarp aspect, ranging from very small to very large, with dry to viscid, smooth to scaly or tomentose caps and different kinds of pigments in the surface structures as well as in the latex. One of the best recognizable and distinct groups commonly occurring throughout Europe is the one with large, white basidiocarps that are not sticky, viscid or bearded, and that have very acrid latex or context. Striking representatives are *Lactifluus piperatus* (L.: Fr.) Kuntze and *Lactifluus glaucescens* (Crossl.) Verbeken. After the recent splitting of the genus *Lactarius* Pers. into three genera, *Lactarius* (subsequently abbreviated as *L.*), *Lactifluus* (Pers.) Roussel (subsequently abbreviated as *Lf.*) and *Multifurca* Buyck & V. Hofst., these species are now situated in *Lactifluus* subg. *Piperati* sect. *Piperati* Verbeken (Buyck et al. 2008; Verbeken et al. 2012). *Lactarius piperatus* (L.: Fr.) Pers. had been chosen as a lectotype for both the genus *Lactarius* and the genus *Lactifluus* (Earle 1909). However, it has recently been accepted to conserve *Lactarius torminosus* (Schaeff.: Fr.) Pers. as type species for the genus *Lactarius* and *Agaricus* sect. *Lactifluus* Pers., the basionym of the genus *Lactifluus*, was automatically typified by *Agaricus lactifluus* L., which applies to the current species *Lactifluus volemus* (Fr.: Fr.) Kuntze (Verbeken et al. 2012; McNeill et al. 2011; Buyck et al. 2010; Norvell 2011; Barrie 2011).

Traditionally, *Lf. piperatus* and its relatives were thought to be related to the group around *Lf. vellereus* (Fr.: Fr.) Kuntze and placed together in *L.* sect. *Albati* (Bat.) Singer (Singer 1962). However, research on a worldwide scale has shown

that the group of white and big milkcaps is artificial and *L.* section *Albati* falls apart in two groups (Eberhardt 2000; Hesler and Smith 1979): *Lf. piperatus* and its relatives in *Lf.* sect. *Piperati* Verbeken, and the group around *Lf. vellereus* in *Lf.* sect. *Albati* (Bataille) Verbeken. The position as sections in two different subgenera is highly supported by morphological characteristics (Heilmann-Clausen et al. 1998; Verbeken 1998a, b), such as pileipellis structures, which are completely different in the two groups.

Lactifluus section *Piperati* has a long history of confusion because of nomenclatural and taxonomical problems and the use of different species concepts.

Nomenclature and taxonomy

Fries (1821) recognised *Lactarius piperatus* and *L. pergamenus* (Sw.: Fr.) Fr. and based the difference between the two mainly on the length and the shape of the stipe, the thickness and the aspect of the cap, and the attachment of the lamellae. Many authors after him did not believe these characters to be relevant, except for the smooth cap in *L. piperatus* versus the more rugulose and irregular cap in *L. pergamenus* (Romagnesi 1956; Bon 1980; Neuhoff 1956; Basso 1999; Heilmann-Clausen et al. 1998). Curiously, none of the original descriptions mentioned the colour change of the latex, even though the greenish discolouration of the latex when drying on the lamellae and the context is often a very striking feature. This brought Crossland (1900) to the publication of *L. glaucescens* Crossl., similar to *L. piperatus* but with distinctly greening latex. In his monograph of Central European milkcaps, Neuhoff (1956) accepts two species: *L. piperatus* without KOH (10 %) reaction of the latex, and *L. glaucescens* with latex that turns yellow in KOH. He further mentions that the latex of both *L. piperatus* and *L. glaucescens* can have a greenish discolouration. According to Blum (1976), there are three species: *L. piperatus* with crowded, pinkish and really decurrent lamellae, unchanging latex and a pileipellis consisting of sphaerocytes covered by a very thin layer of hyphae; and two species with greening latex and a pileipellis consisting of sphaerocytes covered by a thick layer of hyphae: *L. pergamenus* and *L. glaucescens*. He indicates the major differences being the aspect of the pileipellis (rugulose in *L. pergamenus* versus smooth in *L. glaucescens*), the attachment of the lamellae (often almost free in *L. pergamenus* versus decurrent in *L. glaucescens*) and the shape of the stipe (bulbous and swollen at the base in *L. pergamenus* versus tapering downwards in *L. glaucescens*). *Lactarius eburneus* Z. Schaef. was proposed by Schaefer (1979) as a species with a rather long stipe and white, unchanging latex that turns yellow-orange with KOH. However, as the name was already used for an American species (Thiers 1957), Schaefer's name is illegitimate. Romagnesi (1980) proposed *L. spurius* Romagn., a species with a context that turns green, shows no reaction with KOH and has a pileipellis consisting

of globose cells covered by a thin layer of narrow hyphae. This name is invalid since no Latin diagnosis was given. Based on morphological characteristics, most modern revisions (Basso 1999; Heilmann-Clausen et al. 1998; Verbeken et al. 1997) accept two species in Europe: *L. piperatus* and *L. glaucescens*. They all describe *L. piperatus* as a species with white latex that is more or less unchanging on the context (at most somewhat yellowing) and unchanging with KOH. The pileipellis consists of a distinct layer of globose cells, covered with a thin layer of hyaline hyphae (10–30 μm). *Lactarius glaucescens* is characterised by white latex that dries more or less greenish on the context (sometimes very slowly) and turns yellow-orange with KOH. The pileipellis consists of a layer of globose cells, covered with a thick layer of hyaline hyphae (80–120 μm). Verbeken et al. (1997) pointed out that the name *L. pergamenus* has been used for at least two different species and should be better considered as nomen dubium. Romagnesi (1956, 1980), Damblon et al. (1956), Heinemann (1960) and several German authors used the name for a species without greening latex and a pileipellis consisting of globose cells covered by a thin layer of hyphae; whereas Blum (1966), Marchand (1980), Bon (1980) and others used it for a species with greening latex and a pileipellis consisting of globose cells covered by a thick layer of hyphae.

Macromorphology

An important feature that contributed to the confusion is the variation in macrochemical and macromorphological characteristics to distinguish between species of *Lf.* sect. *Piperati*. Verbeken et al. (1997) suggested that too much weight was traditionally given to macroscopical characters, such as the length and the shape of the stipe and the attachment of the lamellae, considering these characters as rather variable in this group. Recently we collected many specimens of this section in Western Europe and we noticed a large morphological variation. Especially the variation in macrochemical reactions was striking and the fact that specimens with different macromorphological characteristics occurred on the same location in the field, as if they were from the same mycelium.

To distinguish between *Lf. piperatus* and *Lf. glaucescens*, most commonly used characteristics are the greening of the latex, and macrochemical tests, such as the reaction of the latex with 10 % KOH (Bataille 1948; Damblon et al. 1956; Neuhoff 1956; Heinemann 1960; Romagnesi 1961; Basso 1999; Blum 1976; Bon 1980; Marchand 1980; Romagnesi 1980; Schaefer 1979; Lecomte 2010) and the reaction of the context with formaldehyde and with a solution further referred to as sulphoformaldehyde (a solution of 50 % formol (at 35 %) and 50 % sulphuric acid (at 70–80 %)) (Bataille 1948; Bon 1980; Marchand 1980; Neuhoff 1956; Romagnesi 1980; Lecomte 2010). Based on carefully executed and standardized macrochemical reactions, together with the colour change of

the latex, we found that we could divide the European collections in four groups, here indicated with provisional names: (1) "*Lf. piperatus*", with no colour change of the latex when drying, no reaction of the latex with KOH and no reaction of the context with (sulpho)formaldehyde; (2) "*Lf. spurius*", with latex that turns green when drying but does not react with KOH and a context that does not react with (sulpho)formaldehyde; (3) "*Lf. glaucescens*", with greening latex that turns pale yellow-orange with KOH and a context that turns blue with (sulpho)formaldehyde; and (4) "*Lf. pergamenus*", with greening latex that turns bright orange with KOH and a context that turns blue after some hours with (sulpho)formaldehyde.

Microscopical features

Several microscopical characteristics can be used to distinguish between the species of *Lf.* sect. *Piperati*. The main characteristics that are used are the shape and ornamentation of the spores, the composition of the lamellar edge, the form of the cheilomacrocystidia and the structure of the pileipellis. The pileipellis structure of this section is rather unique within the genus *Lactifluus*. The pileipellis type is described as a hyphoepithelium (Heilmann-Clausen et al. 1998), with a suprapellis consisting of hyaline hyphae and abundant dermatocystidia, and a subcellular subpellis. Species of this section lack the presence of thick-walled elements in the pellis, which are typical microscopical features for the majority of the genus *Lactifluus* (Verbeken and Walleyn 2010).

As most modern revisions (Basso 1999; Heilmann-Clausen et al. 1998; Verbeken et al. 1997) accept two species in Europe, we summarize here the main microscopic features of *Lf. piperatus* and *Lf. glaucescens*. The spores of *Lf. piperatus* are subglobose to oblong and slightly larger than those of *Lf. glaucescens*, which are subglobose to ellipsoid. The ornamentation of spores of *Lf. glaucescens* consists of irregular warts that never form a reticulum, while the ornamentation of *Lf. piperatus* spores consists of irregular warts forming an incomplete reticulum. In both species, basidia are cylindric to subclavate and 2–4-spored, and pleuromacrocystidia are abundant. The lamellar edge is heterogeneous in *Lf. piperatus*, while it is almost exclusively formed by cheilomacrocystidia in *Lf. glaucescens*. Cheilomacrocystidia are more emergent in *Lf. glaucescens* than in *Lf. piperatus* (Heilmann-Clausen et al. 1998; Triantafyllou et al. 2011). The pileipellis of both species is a hyphoepithelium (Heilmann-Clausen et al. 1998). The main distinctive characteristic between both species, however, is the structure of this hyphoepithelium, as observed in surface view on mature specimens (in a scalp preparation). This way, the globose cells of the subpellis are clearly observed in between a very thin layer of hyaline hyphae in *Lf. piperatus*, but not in *Lf. glaucescens*, where the covering layer of thin, hyaline hyphae of the suprapellis is much thicker (Heilmann-Clausen et al. 1998).

Lactifluus sect. *Piperati* in a worldwide frame

Outside Europe, species from *Lactifluus* sect. *Piperati*, characterised by their general aspects of white to pale brownish-grey, stout basidiocarps with acrid milk and context, and by their hyphoepithelium pileipellis structure (Heilmann-Clausen et al. 1998), are known to occur in Asia and North America. In Asia, *Lf. dwaliensis* (K. Das, J.R. Sharma & Verbeken) K. Das, *Lf. leucophaeus* (Verbeken & E. Horak) Verbeken, *Lf. novoguineensis* (Henn.) Verbeken, *Lf. olivescens* (Verbeken & E. Horak) Verbeken, *Lf. paleus* (Verbeken & E. Horak) Verbeken, *Lf. roseophyllus* (R. Heim) De Crop and *Lf. subpiperatus* (Hongo) Verbeken are described as morphologically recognisable species within *Lf.* sect. *Piperati*. In addition to these morphologically distinct species, a lot of look-a-likes of the European representatives are found throughout Asia. Up to now they received the same names as their European relatives, without testing whether they are truly conspecific. In North America, Hesler and Smith (1979) recognised four species with several varieties: *Lactarius neuhoffii* Hesler & A.H. Sm., *L. neuhoffii* var. *fragrans* (Burl.) Hesler & A.H. Sm., *L. waltersii* Hesler & A.H. Sm., *L. piperatus* (Fr.) S.F. Gray, *L. piperatus* var. *glaucescens* (Crossl.) Hesler & A.H. Sm. and *L. angustifolius* Hesler & A.H. Sm. The new combinations in the genus *Lactifluus* that were not yet made are proposed here (see Nomenclature of the North American species). Again, some look-a-likes of the European species were given the same names as their European relatives, but it has never been proved for this group that they are conspecific with the European species. Species of *Lf.* sect. *Piperati* are not known to occur in Africa, South America and Australia. The only record of *Lf. piperatus* in Australia concerns probably an introduced species, as it is found under both introduced and native tree species (Fuhrer 2005). Species delimitation worldwide thus remains doubtful and confusing.

Despite the large historical confusion in this group, a targeted phylogenetic study has never been executed. Until now, species delimitation was based on morphological and macrochemical characteristics, without testing if these characteristics are supported by a molecular phylogeny and therefore reliable in delimiting the Western European species within this section. We meet this deficit by using molecular data to delimit species and by comparing the phylogenetic results with information on morphology.

As multiple gene sequence data become increasingly available for Agaricomycotina, more and more studies apply phylogenetic methods on a concatenation of alignments of different genes to reconstruct a species tree. However, research shows that topologies often differ among different genes (Knowles and Carstens 2007). Incongruence can be caused by several evolutionary processes, such as incomplete lineage sorting, hybridisation, gene duplication and horizontal gene transfer (Maddison 1997). In case of incomplete lineage sorting, the

use of concatenated alignments can lead to a poor estimation of the species tree and bootstrap values can provide strong support for this incorrect phylogeny (Kubatko and Degnan 2007). To account for these inconsistencies, new methods have been constructed, such as the hierarchical Bayesian model for species tree inference implemented in *BEAST (Heled and Drummond 2010). *BEAST estimates the species tree directly from the sequence data, and it incorporates uncertainty associated with gene trees, nucleotide substitution model parameters and the coalescent process (Heled and Drummond 2010). Species can be further delimited using Bayesian species delimitation, which accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism (Yang and Rannala 2010). In this study, we will use traditional phylogenetic techniques (maximum likelihood and Bayesian inference) to check for gene-incongruence, and a species tree will be constructed using *BEAST. Additionally, we will use Bayesian species delimitation to delimit species within *Lactifluus* section *Piperati* of Western Europe.

We first studied extensively documented fresh material and herbarium collections from Western European representatives of *Lactifluus* section *Piperati*, using morphological, macrochemical and molecular analyses to test if the above groups of European collections, delimited by macroscopical and macrochemical features, represent phylogenetically distinct species. We then studied fresh material and herbarium collections from European, Asian and North American representatives of *Lactifluus* section *Piperati* using molecular analyses, to verify if there is intercontinental conspecificity in this group.

Material and methods

Sampling

This study is based on Western European, Asian and North American collections of *Lactifluus* section *Piperati* (Table 1). The European collections we used were mainly sampled in Belgium and France, as the herbarium specimens of these regions were provided with comprehensive macroscopic descriptions. The Asian samples were collected in Thailand, India and Vietnam, and the collections from North America mostly are from the state of Tennessee (USA). Two datasets were assembled for further analyses: a European dataset and a worldwide dataset. Two collections of *Lf.* section *Lactifluus* and two collections of *Lf.* subg. *Gerardii* were included as outgroup for the European dataset. For the worldwide phylogeny, the outgroup contains two species from *Lf.* sect. *Albati*.

Morphological analyses

Macromorphological characteristics of material collected by the authors were described in daylight conditions and those of herbarium specimens were based on the notes of the collectors. In order to allow comparison of the macrochemical reactions, the macrochemical tests were standardized. The reagents were recently prepared and were preferably from the same stock. The reagents used are KOH (10 %), formaldehyde (38 %) and sulphoformaldehyde [solution of 50 % formol (at 35 %) and 50 % sulphuric acid (at 70–80 %)]. The tests were carried out on adult specimens that were not too old, fresh and not saturated with water. The reaction of the latex with KOH was tested by isolating a droplet of latex on a glass slide and adding a droplet of the reagent to the latex. The reaction was considered positive when there was a yellow-orange colour change within ten seconds after mixing the latex with KOH. The reaction of the context with (sulpho)formaldehyde was tested on the context of the stipe during a period of 24 h. The reaction was noted as positive when the context colors blue; the time in which the reaction takes place was also recorded.

Micromorphological characters were studied on dried herbarium collections. For general terminology we follow Vellinga (1988) and for terminology concerning pileipellis structures we follow Heilmann-Clausen et al. (1998) and Verbeken (1998a). Line-drawings were made by A. Verbeken. Basidiospores were measured and drawn in side view, in Melzer's reagent, using a Zeiss Axioscop 2 microscope and a drawing tube at a magnification of 6,000×. Measurements were done excluding the ornamentation. Elements of the pileipellis and hymenial elements were measured and drawn halfway the radius of the pileus in Congo-Red in L4, using an Olympus CX31 microscope and drawing tube at a magnification of 1,600×. Basidia length excludes sterigmata length.

DNA extraction, PCR amplification, sequencing and nucleotide alignments

DNA from dry collections was extracted using the protocol described by Nuytinck and Verbeken (2003), with the modifications described in Van de Putte et al. (2010). DNA from fresh material was extracted using the CTAB extraction method described in Nuytinck and Verbeken (2003). Protocols for PCR amplification follow Le et al. (2007). Three nuclear loci and one mitochondrial locus were amplified: (1) the internal transcribed spacer region of ribosomal DNA (ITS), comprising the ITS1 and ITS2 spacer regions and the ribosomal gene 5.8S, using the ITS-1F and ITS4 primers (White et al. 1990; Gardes and Bruns 1993); (2) a part of the ribosomal large subunit 28S region (LSU), using the primers LR0R and LR5 (R. Vilgalys lab 'http://www.biology.duke.edu/fungi/mycolab/primers.htm'); (3) the regions between the conserved domains 6 and 7 of the second largest subunit of the RNA polymerase II (*rpb2*), using the primers bRPB2-6F and fRPB2-7cR (Liu et al. 1999; Matheny 2005) and (4) the mitochondrial ATPase subunit 6 (*atp6*), using primers ATP6-3 and ATP6-2 (Kretzer

Table 1 Specimens and GenBank accession numbers of DNA sequences used in the molecular analyses. All exsiccates are deposited in GENT, except the collections of M. Lecomte, these exsiccates are deposited in his personal herbarium

Species	Original identification	Voucher collector	Country	Date	GenBank accession numbers			
					ITS	LSU	rpb2	atp6
Lactifluus section *Piperati*								
GROUP 1 - L.F. GLAUCESCENS GROUP								
Lf. aff. *glaucescens* Asia 1	*L. glaucescens*	*H.T. Le 66*	Thailand	30-08-03	GU258298	GU265639	KF220219	
Lf. aff. *glaucescens* Asia 1	*L. glaucescens*	*H.T. Le 379*	Thailand	16-10-05	KF220019			
Lf. aff. *glaucescens* Asia 1	*L. glaucescens*	*H.T. Le 383*	Thailand	18-10-05	KF220020			
Lf. aff. *glaucescens* Asia 1	*L. glaucescens*	*H.T. Le 237*	Thailand	05-09-04	KF220052	KF220153	KF220238	KF219951
Lf. aff. *glaucescens* Asia 1	*L. glaucescens*	*H.T. Le 241*	Thailand	05-09-04	KF220053	KF220154	KF220239	KF219952
Lf. aff. *glaucescens* Asia 1	*L. glaucescens*	*H.T. Le 244*	Thailand	05-09-04	KF220054	KF220155	KF220240	KF219953
Lf. aff. *glaucescens* Asia 1	*L. leucophaeus*	*H.T. Le 236*	Thailand	05-09-04	KF220060	KF220158	KF220244	KF219957
Lf. aff. *glaucescens* Asia 1	*L.* aff. *piperatus*	*A. Verbeken/K. Das/K. Van de Putte 09-062*	India	15-08-09	KF220096	KF220191	KF220265	KF219990
Lf. aff. *glaucescens* Asia 1	*L.* aff. *piperatus*	*A. Verbeken/K. Das/K. Van de Putte 09-115*	India	03-09-09	KF220097	KF220192	KF220266	KF219991
Lf. aff. *glaucescens* Asia 2	*L. glaucescens*	*H.T. Le 20*	Thailand	19-07-03	KF220018	KF220152		KF219950
Lf. aff. *glaucescens* Asia 2	*L. glaucescens*	*H.T. Le 65*	Thailand	30-08-03	KF220051			
Lf. aff. *glaucescens* Asia 2	*L. glaucescens*	*J. Nuytinck 2011-009*	Vietnam	12-06-11	KF220103	KF220198	KF220272	KF219997
Lf. aff. *glaucescens* Asia 2	*L. glaucescens*	*J. Nuytinck 2011-014*	Vietnam	12-06-11	KF220104	KF220199	KF220273	KF219998
Lf. aff. *glaucescens* USA 1	*L. glaucescens*	*A. Verbeken 04-174*	North America	12-07-04	KF220044	KF220145	KF220231	KF219943
Lf. aff. *glaucescens* USA 2	*L. glaucescens*	*A. Verbeken 04-195*	North America	13-07-04	KF220045	KF220146	KF220232	KF219944
Lf. aff. *glaucescens* USA 2	*L. glaucescens*	*A. Verbeken 05-211*	North America	10-08-05	KF220046	KF220147	KF220233	KF219945
Lf. aff. *glaucescens* USA 2	*L. glaucescens*	*A. Verbeken 05-261*	North America	12-08-05	KF220047	KF220148	KF220234	KF219946
Lf. aff. *glaucescens* USA 3	*L. glaucescens*	*A. Verbeken 05-374*	North America	18-08-05	KF220049	KF220150	KF220236	KF219948
Lf. aff. *leucophaeus* Asia 2	*L. leucophaeus*	*H.T. Le 360*	Thailand	24-07-04	KF220061	KF220159	KF220245	KF219958
Lf. glaucescens Europe	*L. glaucescens*	*J. Nuytinck 2001-02*	France	17-08-01	KF220022			
Lf. glaucescens Europe	*L. glaucescens*	*R. Walleyn 1874*	Belgium	15-08-00	KF220023			
Lf. glaucescens Europe	*L. glaucescens*	*A. Verbeken 97-524*	Belgium	04-08-97	KF220024	KF220128	KF220221	KF219925
Lf. glaucescens Europe	*L. glaucescens*	*R. Walleyn 25-08-92a*	Germany	25-08-92	KF220025			KF219926
Lf. glaucescens Europe	*L. glaucescens*	*A. Verbeken 97-518*	France	25-07-97	KF220026	KF220129		KF219927
Lf. glaucescens Europe	*L. glaucescens*	*R. Walleyn 27-08-92*	Germany	27-08-92	KF220027	KF220130		KF219928
Lf. glaucescens Europe	*L. glaucescens*	*N. Dam 01024*	The Netherlands	06-08-01	KF220028	KF220131	KF220222	KF219929
Lf. glaucescens Europe	*L. glaucescens*	*M. Lecomte 2000 09 17 01*	Belgium	17-09-00	KF220029	KF220132		KF219930
Lf. glaucescens Europe	*L. glaucescens*	*M. Lecomte 2001 08 28 21*	Belgium	28-08-01	KF220030	KF220133	KF220223	KF219931
Lf. glaucescens Europe	*L. glaucescens*	*M. Lecomte 2002 20 09 03*	France	09-02-02	KF220031	KF220134	KF220224	KF219932
Lf. glaucescens Europe	*L. glaucescens*	*M. Lecomte 2008 08 21 01*	Belgium	21-08-08	KF220032	JN388988	JN375591	JN389041

Table 1 (continued)

Species	Original identification	Voucher collector	Country	Date	GenBank accession numbers			
					ITS	LSU	rpb2	atp6
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2001 08 28 14	Belgium	28-08-01	KF220034	KF220136		KF219934
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2001 08 19 35	France	19-08-01	KF220035	KF220137	KF220226	KF219935
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2001 08 28 17	Belgium	28-08-01	KF220036	KF220138		KF219936
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2001 08 28 08	Belgium	28-08-01	KF220038	KF220140	KF220228	KF219938
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2001 08 14 02	Belgium	14-08-01	KF220039	KF220141	KF220229	KF219939
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2001 08 28 18	Belgium	28-08-01	KF220040	KF220142	KF220230	KF219940
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2000 09 23 29	Belgium	23-09-00	KF220041	KF220143		KF219941
Lf. glaucescens Europe	L. glaucescens	M. Lecomte 2002 08 25 01	Belgium	25-08-02	KF220043			
Lf. glaucescens Europe	L. pergamenus	A. Verbeken 93–025 P2	France	25-07-93	KF220062	KF220160	KF220246	KF219959
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 09 23 09	Belgium	23-09-00	KF220063	KF220161		KF219960
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2002 08 25 21	Belgium	25-08-02	KF220064	KF220162	KF220247	KF219961
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2004 08 19 02	Belgium	19-08-04	KF220065	KF220163	KF220248	KF219962
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 05 01	France	05-10-00	KF220066	KF220164	KF220249	KF219963
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 18 79	France	18-10-00	KF220067	KF220165		KF219964
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 02 14	Belgium	02-10-00	KF220068	KF220166	KF220250	KF219965
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 18 87	France	18-10-00	KF220069	KF220167	KF220251	KF219966
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 09 24 01	Belgium	24-09-00	KF220070	KF220168		KF219967
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 1997 07 13 01	France	13-07-97		KF220169		KF219968
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 18 02	France	18-10-00	KF220071	KF220170		KF219969
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 18 108	France	18-10-00	KF220072	KF220171		KF219970
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 09 23 01	Belgium	23-09-00	KF220073	KF220172	KF220252	KF219971
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 18 71	France	18-10-00	KF220074	KF220173		KF219972
Lf. glaucescens Europe	L. pergamenus	M. Lecomte 2000 10 18 01	France	18-10-00	KF220075	KF220174		KF219973
Lf. glaucescens Europe	L. piperatus	M. Lecomte 2000 10 18 52	France	18-10-00	KF220086	KF220181	KF220258	KF219980
Lf. glaucescens Europe	L. piperatus	M. Lecomte 2004 08 19 01	Belgium	19-08-04	KF220094	KF220189	KF220263	KF219988
Lf. glaucescens Europe	L. spurius	M. Lecomte 2002 07 14 01	Belgium	14-07-02	KF220114	KF220207	KF220280	KF220005
Lf. glaucescens Europe	L. spurius	M. Lecomte 2003 06 14 01	Italy	14-06-03	KF220117	KF220210	KF220283	KF220008
Lf. glaucescens Europe	L. spurius	M. Lecomte 2004 08 15 01	France	15-08-04	KF220118	KF220211	KF220284	KF220009
Lf. leucophaeus Asia 1	L. leucophaeus	H.T. Le/A. Verbeken & R. Walleyn 126/04-075	Thailand	23-06-04	KF220056			
Lf. leucophaeus Asia 1	L. leucophaeus	A. Verbeken 97–382 (type)	Papua New Guinea	21-02-97	GU258299	GU265640	KF220241	
Lf. leucophaeus Asia 1	L. leucophaeus	E. Horak 7330	Indonesia	11-01-99	KF220058	KF220156	KF220242	KF219955
Lf. leucophaeus Asia 1	L. leucophaeus	H.T. Le 182	Thailand	05-07-04	KF220059	KF220157	KF220243	KF219956
Lf. roseophyllus Asia 1	L. roseophyllus	J. Nuytinck 2011-076	Vietnam	16-06-11	KF220107	KF220202	KF220276	KF220001

Table 1 (continued)

Species	Original identification	Voucher collector	Country	Date	GenBank accession numbers			
					ITS	LSU	rpb2	atp6
GROUP 2 - Lf. PIPERATUS GROUP								
Lf. aff. *piperatus* Asia 1	*L.* aff. *piperatus*	*A. Verbeken/K. Das/K. Van de Putte 09-008*	India	13-08-09	KF220095	KF220190	KF220264	KF219989
Lf. aff. *piperatus* Asia 2	*L. piperatus*	*H.T. Le 51*	Thailand	29-08-03	KF220076	KF220175	KF220253	
Lf. aff. *piperatus* Asia 2	*L. piperatus*	*H.T. Le 240*	Thailand	08-05-00	KF220077			KF219992
Lf. aff. *piperatus* Asia 2	*L. piperatus*	*H.T. Le 88*	Thailand	15-10-03	KF220098	KF220193	KF220267	KF219993
Lf. aff. *piperatus* Asia 2	*L. piperatus*	*H.T. Le 198*	Thailand	27-07-04	KF220099	KF220194	KF220268	KF219994
Lf. aff. *piperatus* Asia 2	*L. piperatus*	*H.T. Le 242*	Thailand	05-09-03	KF220100	KF220195	KF220269	
Lf. aff. *piperatus* Asia 2	*L.* cf. *piperatus*	*J. Nuytinck 2011-036*	Vietnam	13-06-11	KF220105	KF220200	KF220274	KF219999
Lf. aff. *piperatus* Asia 3	*L. leucophaeus*	*H.T. Le 377*	Thailand	09-08-01	KF220057			
Lf. aff. *piperatus* Asia 3	*L. piperatus*	*H.T. Le 322*	Thailand	27-06-05	KF220078			
Lf. aff. *piperatus* Asia 3	*L. piperatus*	*H.T. Le 293*	Thailand	23-06-05	KF220101	KF220196	KF220270	KF219995
Lf. aff. *piperatus* Asia 3	*L.* cf. *piperatus*	*J. Nuytinck 2011-072*	Vietnam	16-06-11	KF220106	KF220201	KF220275	KF220000
Lf. aff. *piperatus* Asia 4	*L. piperatus*	*H.T. Le 378*	Thailand	08-10-05	KF220102	KF220197	KF220271	KF219996
Lf. aff. *piperatus* Asia 4	*L. subpiperatus*	*H.T. Le 69*	Thailand	30-08-03	KF220112	KF220205		
Lf. aff. *piperatus* Asia 5	*L. subpiperatus*	*H.T. Le/A. Verbeken & R. Walleyn 125/04-072*	Thailand	23-06-04	KF220109			KF220003
Lf. aff. *piperatus* USA 1	*L. glaucescens*	*A. Verbeken 04-202*	North America	14-07-04	KF220021	KF220127	KF220220	KF219949
Lf. aff. *piperatus* USA 2	*L. glaucescens*	*A. Verbeken 05-393*	North America	19-08-05	KF220050	KF220151	KF220237	KF219947
Lf. aff. *piperatus* USA 3	*L. glaucescens*	*A. Verbeken 05-295*	North America	14-08-05	KF220048	KF220149	KF220235	
Lf. piperatus Europe	*L. glaucescens*	*M. Lecomte 2000 10 07 01*	France	07-10-00	KF220033	KF220135	KF220225	KF219933
Lf. piperatus Europe	*L. glaucescens*	*M. Lecomte 2001 08 19 59*	France	19-08-01	KF220037	KF220139	KF220227	KF219937
Lf. piperatus Europe	*L. glaucescens*	*M. Lecomte 2001 10 02 15*	Belgium	02-10-01	KF220042	KF220144		KF219942
Lf. piperatus Europe	*L. piperatus*	*R. Walleyn 3064*	Belgium	08-07-03	KF220079			
Lf. piperatus Europe	*L. piperatus*	*A. Fraiture 2584*	Belgium	27-07-97	KF220080	KF220176	KF220254	KF219974
Lf. piperatus Europe	*L. piperatus*	*J. Vesterholt 96-144*	Denmark	02-09-96	KF220081	KF220177	KF220255	KF219975
Lf. piperatus Europe	*L. piperatus*	*R. Walleyn 25-08-92b*	Germany	25-08-92	KF220082	KF220178	KF220256	KF219976
Lf. piperatus Europe	*L. piperatus*	*J. Vesterholt 96-074*	Denmark	20-08-96	KF220083	KF220179		KF219977
Lf. piperatus Europe	*L. piperatus*	*A. Verbeken 93-023 P1*	France	25-07-93	KF220084	KF220180		KF219978
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte 2007 06 28 01*	France	28-06-07	KF220085		KF220257	KF219979
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte 2002 07 14 02*	Belgium	14-07-02	KF220087	KF220182		KF219981
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte 2001 07 20 01*	France	20-07-01	KF220088	KF220183		KF219982
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte 2003 06 29 01*	France	29-06-03	KF220089	KF220184	KF220259	KF219983
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte 2001 08 19 39*	France	19-08-01	KF220090	KF220185	KF220260	KF219984
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte 2000 09 10 14*	Belgium	10-09-00		KF220186	KF220261	KF219985
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte 2000 08 28 41*	Belgium	28-08-00	KF220091	KF220187	KF220262	KF219986

Table 1 (continued)

Species	Original identification	Voucher collector	Country	Date	ITS	LSU	rpb2	atp6
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte* 2000 10 02 09	Belgium	02-10-00	KF220092	JN388991	JN375594	JN389043
Lf. piperatus Europe	*L. piperatus*	*M. Lecomte* 2000 08 27 03	Belgium	27-08-00	KF220093	KF220188		KF219987
Lf. piperatus Europe	*L. spurius*	*M. Lecomte* 2001 08 19 65	France	19-08-01	KF220115	KF220208	KF220281	KF220006
Lf. piperatus Europe	*L. spurius*	*M. Lecomte* 2001 08 19 13	France	19-08-01	KF220116	JN388993	JN375596	JN389044
Lf. piperatus Europe	*L. spurius*	*M. Lecomte* 2001 08 19 68	France	19-08-01	KF220119	KF241840	KF241842	KF241841
Lf. piperatus Europe	*L. spurius*	*M. Lecomte* 2001 08 19 23	France	19-08-01	KF220120	KF220212	KF220285	KF220010
Lf. piperatus Europe	*L. spurius*	*M. Lecomte* 2001 08 19 22	France	19-08-01		KF220213	KF220286	KF220011
Lf. piperatus Europe	*L. spurius*	*M. Lecomte* 2001 08 19 55	France	19-08-01	KF220121	KF220214	KF220287	KF220012
Lf. piperatus Europe	*L. spurius*	78111 (type)	France	27-08-78	KF220122	KF220215		KF220013
GROUP 3 - LF. *DWALIENSIS* GROUP								
Lf. aff. *subpiperatus* Asia	*L. subpiperatus*	*H.T. Le* 376	Thailand	08-08-01	KF220110			
Lf. dwaliensis Asia	*L. dwaliensis*	*K. Das* 612 (type)	India	03-10-99				KF219924
Lf. dwaliensis Asia	*L.* sp.	*H.T. Le* 67	Thailand	30-08-03	KF220108	KF220203	KF220277	
Lf. dwaliensis Asia	*L. subpiperatus*	*H.T. Le* 55	Thailand	30-08-03	KF220111	KF220204	KF220278	KF220002
Lf. dwaliensis Asia	*L. subpiperatus*	*H.T. Le* 346	Thailand	02-06-05	KF220113	KF220206	KF220279	KF220004
Lactifluus section *Allardii*								
Lf. allardii	*L. allardii*	*A. Verbeken* 05-286	North America	14-08-05	KF220015	KF220124		
Lf. allardii	*L. allardii*	*J. Nuytinck* 2004-008	North America	13-07-04	KF220016	KF220125	KF220217	
Lf. allardii	*L. allardii*	*A. Verbeken* 05-246	North America	12-08-05	KF220017	KF220126	KF220218	KF219923
Lactifluus subgenus *Gerardii*								
Lf. bicolor	*L. bicolor*	*D. Stubbe* 06-247	Malaysia	19-09-06	JN388955	JN388987	JN375590	JN389040
Lf. cf. ochrogalactus	*L.* cf. *ochrogalactus*	*A. Verbeken/K. Das/K. Van de Putte* 09-120	India	04-09-09	JN388956	JN388990	JN375593	JN389042
Lactifluus section *Lactifluus*								
Lf. crocatus	*L. crocatus*	*K. Van de Putte* 08-034	Thailand	16-07-08	HQ318243	HQ318151	HQ328888	JN389073
Lf. volemus	*L. volemus*	90804-5	Sweden		JN388959	JN389010	JN375612	
Lactifluus section *Albati*								
Lf. leoninus	*L. leoninus*	*D. Stubbe* 07-454	Thailand	04-07-07	KF220055	JN388989	JN375592	KF219954
Lf. vellereus var. *hometii*	*L. vellereus* var. *hometii*	*M.T. Basso* 5231/4	Germany	26-09-10	KF220123	KF220216	KF220288	KF220014

and Bruns 1999). Although all four loci were previously shown to be useful for species delimitation within the genus *Lactifluus* (Stubbe et al. 2010; Van de Putte et al. 2012; Van de Putte et al. 2010), we estimated the evolutionary divergence between the sequences by computing the number of base substitutions per site from averaging over all sequence pairs for each marker in MEGA 5 (Tamura et al. 2011). Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modelled with a gamma distribution (shape parameter=4). Standard error (S.E.) estimates were obtained by a bootstrap procedure (500 replicates). PCR products were sequenced using an automated ABI 3730 XL capillary sequencer (Macrogen). Forward and reverse sequences were assembled into contigs and edited where needed with the Sequencer™ v5.0 software (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). Sequences of both the European and the worldwide datasets were aligned using the online version of MAFFT v6 (Katoh and Toh 2008), with an E-INS-I strategy, and were manually edited in MEGA 5 (Tamura et al. 2011). The alignments can be acquired from the first author and from TreeBASE (S14367).

Phylogenetic analyses

The program Gblocks 0.91b (Castresana 2000) was used to exclude ambiguously aligned positions in the alignments of both datasets, with settings allowing gaps within selected blocks, smaller blocks (minimum 5 bp) and bigger segments with contiguous non-conserved positions (maximum 10 bp). ITS, *rpb2* and *atp6* sequence data were further divided into partitions, while LSU was analysed as a whole. The ITS sequences were partitioned in the partial ribosomal gene 18S, the first spacer region ITS, the ribosomal gene 5.8S and the second spacer region ITS2. The *rpb2* sequences were partitioned into four partitions: the fourth intron of the *rpb2*-gene and the first, second and third codon positions of the exon. The *atp6* sequences were partitioned according to the first, second and third codon positions.

A maximum likelihood (ML) analysis was executed with the program RAxML v7.0.3 (Stamatakis 2006), where a ML analysis was combined with the Rapid Bootstrapping algorithm with 500 replicates (Stamatakis et al. 2008). Bayesian Inference analyses (BI) were executed with MrBayes v3.2.0 (Ronquist et al. 2012) on the high performance computer of Ghent University. MrModeltest v2.3 (Nylander 2004) was first used to determine the model that best fits the data of each partition, using the second order Akaike information criterion (AICc). Five parallel runs, each consisting of one cold and three heated chains, were run for 20 million generations, sampling every 100th generation. Convergence of the different runs was verified by checking the log-likelihoods and the effective sample sizes in Tracer v1.5 (Rambaut and

Drummond 2007). A burn-in was determined in Tracer and a majority rule consensus tree was constructed, using at least three runs that converged to the same likelihood. The ML and BI analyses were performed on each marker separately and on the combination of markers of both the European and the worldwide datasets.

Bayesian species delimitation

Species tree inference

The species tree for the European dataset was estimated using the hierarchical Bayesian model implemented in *BEAST v1.6.2 (Heled and Drummond 2010). *BEAST conducts multispecies coalescent analyses to estimate the most probable species tree directly from the unlinked multi-locus sequence data. *BEAST incorporates the coalescent process, uncertainty associated with gene trees and nucleotide substitution model parameters (Heled and Drummond 2010). To examine the coalescent events for a species, the analysis requires at least two specimens per species. This was not achieved for the worldwide dataset, so Bayesian species delimitation was only used to delimit species within the European representatives of the section. Specimens were assigned to taxon subsets based on the results of the concatenated ML and BI trees. As in the BI analyses, the ITS, *rpb2* and *atp6* sequence data were further divided into partitions, while LSU was analysed as a whole. For each partition, we manually edited the XML file to be able to use the same substitution model as determined for the BI analyses, under an uncorrelated relaxed lognormal clock model (Drummond et al. 2006). We selected the Yule process as a tree prior, with a piecewise linear and constant root population size model. Three independent MCMC analyses were run for a total of 50 million generations, sampling every 100 steps and excluding the first 5 million generations of each run as a burn-in. Convergence was verified by checking the log-likelihoods and the effective sample sizes in Tracer v1.5 (Rambaut and Drummond 2007).

Speciation probabilities

Bayesian species delimitation was conducted using Bayesian Phylogenetics and Phylogeography (BPP v2.1a; (Rannala and Yang 2003; Yang and Rannala 2010). This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. The rjMCMC analyses were run for 100.000 generations, sampling each fifth generation, excluding the first 50.000 generations as a burn-in. Each analysis was run twice to confirm consistency between runs. We used algorithm 0, with different fine-tune parameters to confirm stability between runs ($\varepsilon=5$, 10 and 20). As prior distributions on the ancestral population size (θ) and root age (τ_0) can affect the posterior probabilities for models (Yang and Rannala 2010),

we tested three different combinations of priors (Leache and Fujita 2010). The first combination of priors assumes relatively large ancestral population sizes and deep divergences: $\theta \sim G(1, 10)$ and $\tau_0 \sim G(1, 10)$, with both prior means=0.1 and prior variances=0.01. The second prior combination assumes relatively small ancestral population sizes and shallow divergences among species: $\theta \sim G(2, 2000)$ and $\tau_0 \sim G(2, 2000)$, with both prior means=0.001 and variances=5×10^{-7}. The third combination assumes large ancestral populations sizes $\theta \sim G(1, 10)$ and relatively shallow divergences among species $\tau_0 \sim G(2, 2000)$, with prior mean θ=0.1, variance=0.01 and prior mean τ_0=0.001, variance=5×10^{-7}. This is a conservative combination of priors that should favour models containing fewer species. The other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala 2010).

Results

Sequence alignments

In the European dataset, we included 64 European collections of *Lf.* sect. *Piperati* and four outgroup specimens. The worldwide dataset contains 110 collections of *Lf.* sect. *Piperati*, seven collections from sections and subgenera closely related to *Lf.* sect. *Piperati* and two outgroup specimens. After aligning with MAFFT and excluding ambiguously aligned positions with Gblocks, the European dataset contained an ITS alignment with 65 sequences of 726 bases and an overall distance of 0.067 base substitutions per site (standard error (S.E.): 0.010), an LSU alignment with 62 sequences of 910 bases and an overall distance of 0.017 base substitutions per site (S.E.: 0.003), an *rpb2*-alignment with 42 sequences of 695 bases and an overall distance of 0.075 base substitutions per site (S.E.: 0.008), and an *atp6*-alignment with 63 sequences of 622 bases and an overall distance of 0.036 base substitutions per site (S.E.: 0.006) (Table 1). The worldwide dataset included an ITS alignment with 115 sequences of 771 bases and an overall distance of 0.086 base substitutions per site (S.E.: 0.010), an LSU alignment with 103 sequences of 918 bases and an overall distance of 0.022 base substitutions per site (S.E.: 0.003), an *rpb2*-alignment with 80 sequences of 750 bases and an overall distance of 0.079 base substitutions per site (S.E.: 0.006), and an *atp6*-alignment with 98 sequences of 665 bases and an overall distance of 0.036 base substitutions per site (S.E.: 0.005) (Table 1).

Phylogenetic analyses

In the European dataset, the single-locus ML and BI analyses show almost identical topologies, although not every clade is fully supported for each locus. In both multi-locus analyses, each clade is fully supported (Fig. 3). *Lactifluus* sect. *Piperati* consists of two well supported clades, *Lf. piperatus* and *Lf.*

glaucescens, which cannot be further divided into supported subclades. These results thus disagree with the hypothesis of four European species.

The multi-locus ML and BI analyses of the worldwide dataset show almost identical topologies (Fig. 4), with only some minor conflicts (e.g., in clade 1, the relative position of the clades *Lf. leucophaeus* Asia 1 and *Lf. glaucescens* North America 2 differs between both analyses, and the position of some singletons within clade 2 differs, but in neither analyses these positions are supported). These analyses show that worldwide, this section is divided in three clades, which we gave the working names "clade 1 – Glaucescens clade", "clade 2 – Piperatus clade" and "clade 3 – Dwaliensis clade". All three clades are highly supported, but the position of the third clade relative to the two other clades is not resolved. In clade 1 we see some clearly delimited and highly supported subclades, such as at least three Asian subclades (*Lf.* aff. *glaucescens* Asia 1 (bootstrap value only 69), *Lf.* aff. *glaucescens* Asia 2 and *Lf. leucophaeus* Asia), one North American subclade (*Lf.* aff. *glaucescens* North America 2) and one European subclade (*Lf. glaucescens* Europe), although this latter subclade is not supported in the worldwide phylogeny. Likewise, some highly supported subclades could be delimited in clade 2, such as at least three Asian subclades (*Lf.* aff. *piperatus* Asia 2, 3 and 4) and one European subclade (*Lf. piperatus* Europe). The third clade consists of one fully supported subclade (*Lf. dwaliensis* Asia). Additionally, all three clades contain one or more single specimens which do not fall within the subclades discussed above. Further research and additional sampling may point out that they form separate subclades as well. The single-locus ML analyses show different topologies, with a considerable amount of conflict (Fig. 5). Likewise, the BI results show different topologies for each locus, with many conflicting clades. In each gene tree, clade 2 and 3 are monophyletic and well supported. Clade 1, however, is often paraphyletic and not supported. Within each of the three clades, the subclades often switch positions and split up.

Bayesian species delimitation

In the European analysis, the ML and BI analyses clearly showed two monophyletic clades, so we assume two species in Europe. The *BEAST analysis resulted in a species tree that highly supports the same clades and rejects the hypothesis of four European species (Fig. 3). BPP supports the guide tree of two species with a speciation probability of 1.0, and different prior distributions for θ and τ_0 did not affect this outcome (Fig. 3).

Taxonomy of the European species

The molecular results indicate that the current descriptions of *Lactifluus piperatus* and *Lf. glaucescens* require some adjustments. In the following paragraph, we give the new

descriptions of both species, based on literature and own observations on herbarium and freshly collected specimens listed in Table 1.

Lactifluus piperatus (L.: Fr.) Kuntze, Revis. Gen. Pl. 2: 857. 1891. (Fig. 1)

Basionym: *Agaricus piperatus* L., Sp. pl.: 1173. 1753.

≡ *Lactarius piperatus* (L.: Fr.) Pers., Tent. disp. meth. Fung.: 64. 1797.

≡ *Galorrheus piperatus* (L.: Fr.) Fr., Stirp. agri femsion. (III): 57. 1825.

Synonym: *Lactifluus pergamenus* (Sw.: Fr.) Kuntze, Revis. gen. pl. II: 857. 1891. ss. Romagnesi (1956, 1980), Damblon et al. (1956), Heineman (1960), et al.

Neotypus (designated here): Sweden, Uppsala, Nåsten, close to Håga, N 59.84° E 17.57°. *Habitat*: shrubbery at the forest edge, in some places open, but also with some larger trees, many bushes (*Corylus avellana*) cut back earlier the same year; mixed including conifers and birch; with *Quercus* sp., *Corylus avellana* and *Populus* sp. nearby. 9 August 2004, Eberhardt U. 09.08.2004-6 (neotype UPPSALA, isoneotype GENT), GenBank accession numbers: ITS+LSU= DQ422035, *rpb2*=DQ421937. This collection was not included in the Bayesian species delimitation study, but both morphological and molecular studies (based on ITS, LSU and *rpb2*) show that this collection belongs to *Lf. piperatus*.

Pileus 40–120(−160) mm, at first convex with slightly depressed centre and decurved margin, with age expanding and becoming more depressed in the centre; surface smooth, dry, finely cracked, matt or slightly shiny, concentrically wrinkled towards margin, whitish to whitish chrome or cream, typically darkest in the centre, sometimes with buff coloured spots. *Lamellae* at first broadly adnate, then slightly decurrent

Fig. 1 *Lf. piperatus* **a** basidiospores (UE 09.08.2004-6, type), **b** basidia (1–3: AV-RW 93–023, 4–7: JV 96–144), **c** pleuromacrocystidia (JV 96–144), **d** marginal cells (JV 96–144), **e** pleuropseudocystidia (JV 96–144), **f** cheilomacrocystidia (JV 96–144), **g** scalp of the pileipellis, with dermatocystidia (AV-RW 93–023), **h** cross section of the pileipellis, with suprapellis (1) and subpellis (2) (HP 8475)
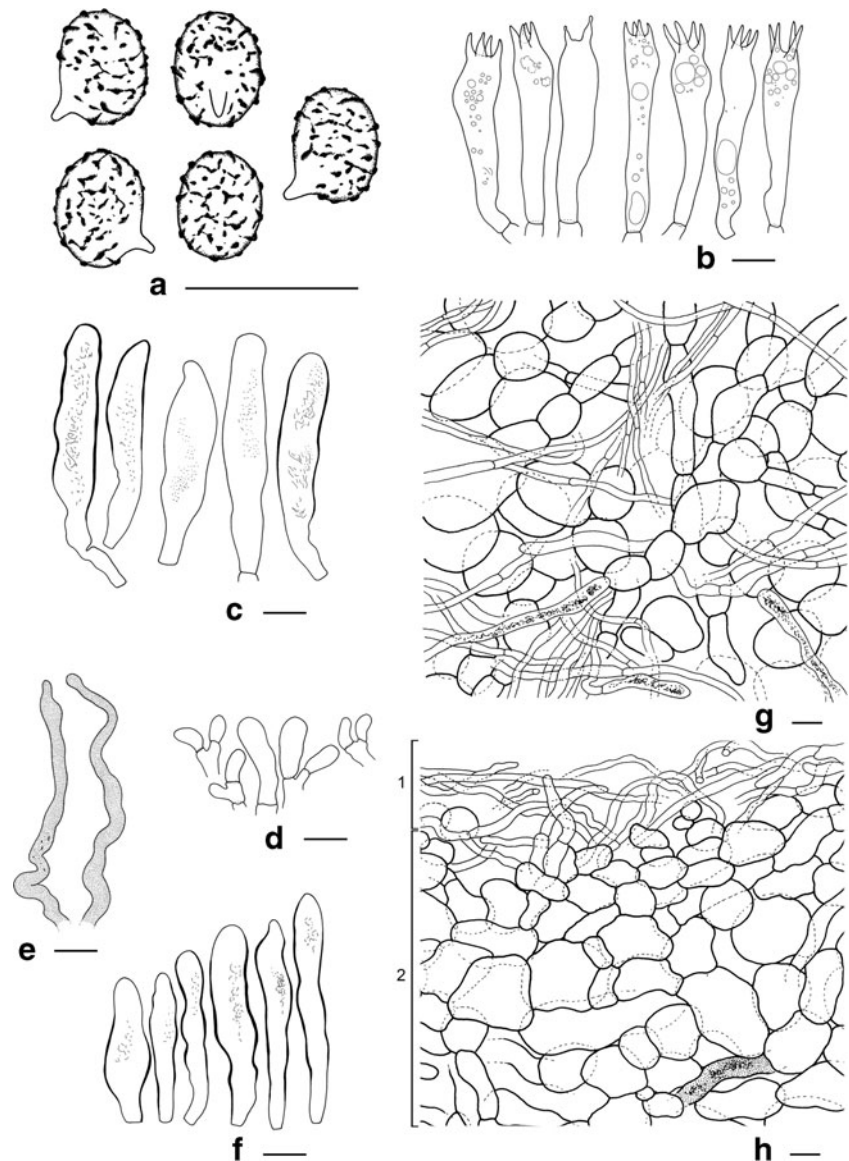
to decurrent, very crowded, very narrow (1.5 mm), with some evenly distributed forkings, pale cream to cream with a pale orange tinge. *Stipe* 40–95×12–30 mm, cylindric or tapering downwards, smooth or uneven, dry, white, tinged whitish chrome or pale cream, becoming buff or brownish from base. *Context* firm to very firm, solid, white, tinged whitish chrome, becoming more yellow when drying, lemon-yellow in the stem base, not reacting with (sulpho)formaldehyde; taste very acrid after a short while; smell slightly acidic, distinctly honey- or apple-like when drying. *Latex* not very abundant, white, drying whitish or greyish green, usually unchanging, but sometimes yellow to orange with KOH, taste becoming very acrid after a while. *Spore deposit* white.

Basidiospores 7.0–10.4×5.2–7.5 μm, av. 8.0–8.5×5.9–6.3 μm, subglobose to oblong, Q=1.10–1.65, av. Q=1.28–1.40; ornamentation up to 0.2 μm high, consisting of irregularly rounded to elongate warts which are aligned or connected by lower lines, forming an incomplete reticulum; plage inamyloid. *Basidia* 40–45×7–9(−10) μm, cylindric to subclavate, (2- or) 4-spored. *Pleuromacrocystidia* abundant, 50–70(−90)×8–11 μm. *Lamellae-edge* heterogeneous. *Cheilomacrocystidia* 35–55×5–10 μm. *Hymenophoral trama* predominantly consisting of hyphae, with many lactiferous hyphae and sometimes sphaerocytes. *Pileipellis* a hyphoepithelium; suprapellis distinct in young specimens, of 2–4 μm broad, hyaline hyphae, becoming very thin when mature (10–30 μm) and clearly showing the underlying cellular layer; subpellis subcellular; dermatocystidia abundant in suprapellis, up to 7 μm broad, cylindric to clavate.

Lactifluus glaucescens (Crossl.) Verbeken (Fig. 2)

Basionym: *Lactarius glaucescens* Crossl., Naturalist, J. Nat. Hist. N. England 1900(516): 5. 1900.

≡ *Lactarius piperatus* var. *glaucescens* (Crossl.) Hesler & A.H. Sm., N. Amer. Species *Lactarius*: 186. 1979

Synonym: *Lactifluus pergamenus* (Sw.: Fr.) Kuntze, Revis. gen. pl. II: 857. 1891. ss. Blum (1966, 1976), Marchand (1980), Bon (1980), et al.

Holotypus: England, West Yorkshire (K), Crossland 1900

Pileus 50–150 mm, convex to plane with a depressed centre; surface smooth, dry, indistinctly velutinous, rather shiny, with irregular dots and darker spots, sometimes slightly wrinkled, white to pale cream. *Lamellae* decurrent, very narrow (2 mm broad), very crowded, whitish, turning greenish by the milk and becoming dirty brownish many hours after bruising. *Stipe* 30–90×10–40 mm, usually shorter than the cap diameter; surface smooth, dry, white to pale cream. *Context* very firm and thick, white, becoming bluish green after hours, sometimes becoming blue with (sulpho)formaldehyde; smell faintly honey-like when drying; taste acrid. *Latex* not very abundant, white, often becoming bluish to greyish green when drying, most often but not always yellow to orange with KOH; taste immediately very acrid. *Spore deposit* white.

Basidiospores 6.5–9.3×5.3–6.9 μm, av. 7.4–8.5×5.8–6.4 μm, subglobose to ellipsoid, Q=1.05–1.45, av. Q=1.26–1.33; ornamentation up to 0.2 μm high, of irregular warts, which are isolated, aligned or connected by lower lines, but never forming a reticulum; plage predominantly inamyloid, occasionally with a slightly amyloid spot. *Basidia* 45–50×7–9 μm, cylindric to subclavate, (2- or) 4-spored. *Pleuromacrocystidia* abundant, originating deep in the trama, mostly strongly emergent, 60–90×7–10 μm. *Lamella edge* almost exclusively with strongly emergent cheilomacrocystidia of 55–70×7–9 μm. *Hymenophoral trama* predominantly consisting of hyphae, with abundant lactiferous hyphae and sometimes sphaerocytes. *Pileipellis* a hyphoepithelium; suprapellis 80–120 μm thick, hiding the underlying cellular layer, consisting of thin, hyaline hyphae, (1–)2–4 μm broad in upper part, 3–5(−6) μm broad in lower part; subpellis almost completely cellular; dermatocystidia abundant in suprapellis, up to 4 μm broad, cylindric to subclavate.

Nomenclature of the North American species

Lactifluus angustifolius (Hesler & A.H. Sm.) De Crop, **comb. nov.**

MYCOBANK 116067

Basionym: *Lactarius angustifolius* Hesler & A.H. Sm., N. Amer. Species *Lactarius*: 190. 1979.

≡ *Lactarius albus* Thiers, Mycologia 49 (5): 712. 1957. (nom. illeg., art. 53.1 ICBN)

Lactifluus neuhoffii (Hesler & A.H. Sm.) De Crop, **comb. nov.**

MYCOBANK 116190

Basionym: *Lactarius neuhoffii* Hesler & A.H. Sm., N. Amer. Species *Lactarius*: 179. 1979.

Lactifluus neuhoffii var. fragrans (Burl.) De Crop, **comb. nov.**

MYCOBANK 117770

Basionym: *Lactarius piperatus* f. *fragrans* Burl., Mem. Torrey Bot. Club 14: 20. 1908.

≡ *Lactarius neuhoffii* var. *fragrans* (Burl.) Hesler & A.H. Sm., N. Amer. Species *Lactarius*: 182. 1979.

Lactifluus waltersii (Hesler & A.H. Sm.) De Crop, **comb. nov.**

MYCOBANK 116132

Basionym: *Lactarius waltersii* Hesler & A.H. Sm., N. Amer. Species *Lactarius*: 183. 1979.

Discussion

Lactifluus *section* Piperati *in Europe*

Our study of *Lf.* sect. *Piperati* shows that the section contains two highly supported species in Europe: *Lf. glaucescens* and

Fig. 2 *Lf. glaucescens*,
a pleuromacrocystidia (AV 93–021), **b** basidiospores (AV-RW 93–025), **c** cheilomacrocystidia (AF 2147), **d** pleuropseudocystidia (AV 93–021), **e** basidia (AV-RW 93–025), **f** scalp of the pileipellis, with dermatocystidia (AV 93–021), **g** cross-section of the pileipellis, with suprapellis (1) and subpellis (2) (AF 1898)
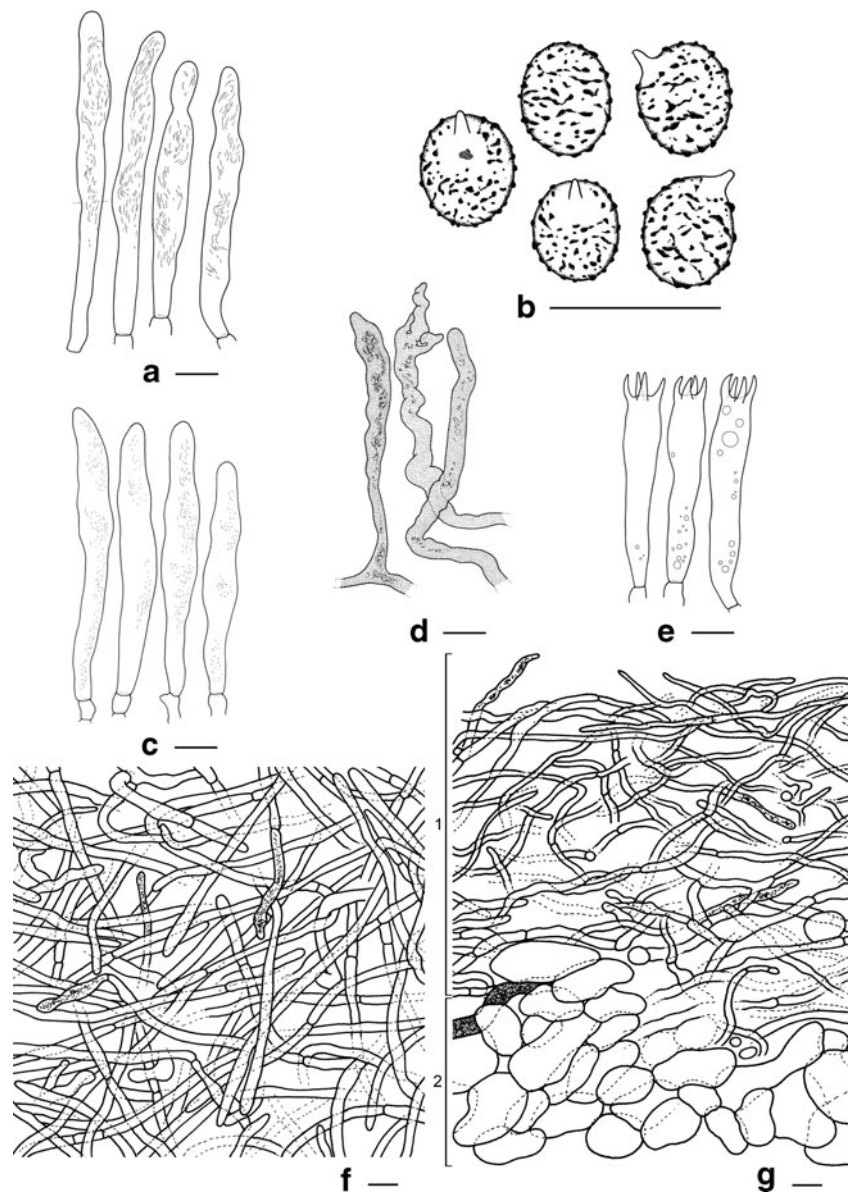
Lf. piperatus (Fig. 3). This result, obtained using molecular data, contradicts our starting hypothesis that this section was possibly represented by four species in Europe, a distinction based on morphological and macrochemical reactions of the latex and the context. Our findings demonstrate that a colour change of drying latex (greenish versus unchanging) is not a diagnostic characteristic. Both *Lf. piperatus* and *Lf. glaucescens* clades contain collections with the latex turning greenish when drying. Our findings reject the diagnostic value of the macrochemical characteristics of the latex and the context to delineate species within this section, since both clades of *Lf. piperatus* and *Lf. glaucescens* contain collections that display a colour reaction of the latex with KOH and the colour reaction of the context with (sulpho)formaldehyde is not a unique characteristic for either one of the species.

Our phylogenetic results support the species recognised by modern revisions (Heilmann-Clausen et al. 1998; Basso 1999; Verbeken et al. 1997), who based their conclusions mainly on the microscopical characteristics of the pileipellis (Figs. 1g–h, 2f–g). Likewise, the differences in composition of the lamella edge and the length of the cheilomacrocystidia remain good diagnostic characteristics. Contrary to the descriptions of Heilmann-Clausen et al. (1998), Basso (1999) and Verbeken et al. (1997), we show that the macrochemical reactions are not useful as a diagnostic characteristic. Our experience in determining milkcap species from both *Lactarius* and *Lactifluus* taught us that the colour change of the latex in contact with KOH is largely depending on the time interval between isolating the latex and bringing it in contact with the solution. To accommodate to this effect, we used a strict
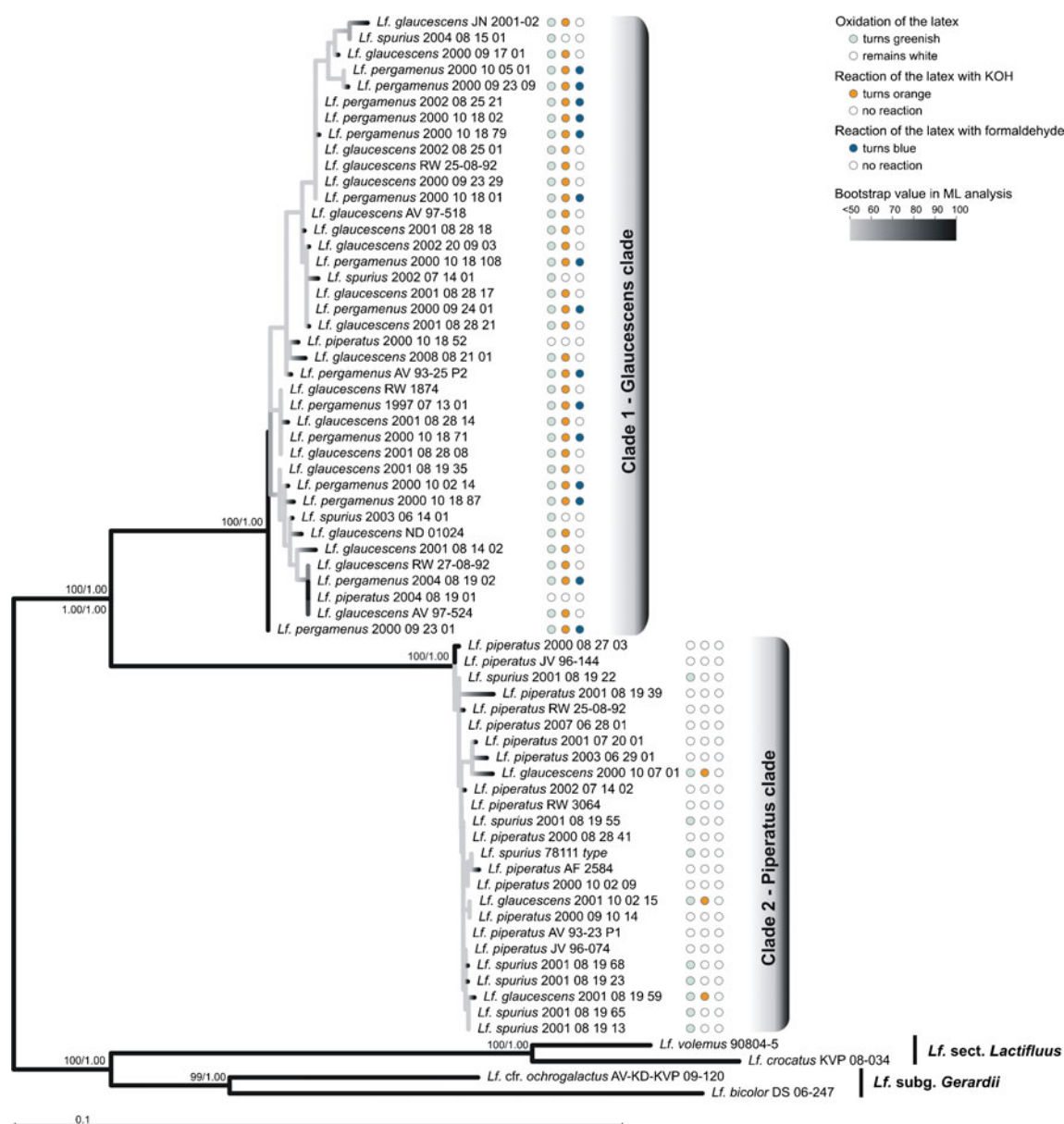
Fig. 3 ML tree of the European dataset, based on the concatenated data of ITS, LSU, *rpb2* and *atp6* sequences. Voucher names given in the tree are the provisional names as explained in the Introduction. *Branch colours* indicate statistical support of the clades: *black branches* are strongly supported, branches in *light grey* are poorly resolved.

Intermediate *shades of grey* represent intermediate support (see *gradient legend*). Bootstrap values above 50 and posterior probabilities exceeding 0.95 are shown above branches. Posterior probabilities from the *BEAST analysis and the species probabilities from the BPP analysis are plotted below the branch of the split between *Lf. glaucescens* and *Lf. piperatus*

protocol for applying the chemicals, as described in the materials and methods section. Additionally, the reaction with the chemicals often varies with the age and the condition of the specimens. This is in accordance with the observations within the genus *Lactarius*, subsection *Triviales*. One of the characteristics often used to distinguish between *L. trivialis* and *L. utilis* is the reaction of the latex with KOH, which turns orange-yellow in *L. trivialis* and is unchanging in *L. utilis* (Heilmann-Clausen et al. 1998). However, this reaction appears to be strongly dependent on the time between isolating the latex and bringing it in contact with the KOH-solution. For

both species, the reaction turns out more positive when the KOH is added on dry latex (unpubl. data). Romagnesi (1980) further indicated that the reaction of the context of species

Fig. 4 ML tree of the worldwide dataset, based on the concatenated data ▶ of ITS, LSU, *rpb2* and *atp6* sequences. Voucher names given in the tree are the revised identifications as explained in the results section. *Branch colours* indicate statistical support of the clades: *black branches* are strongly supported, branches in *light grey* are poorly supported. Intermediate *shades of grey* represent intermediate support (see *gradient legend*). Bootstrap values above 50 and posterior probabilities exceeding 0.95 are shown
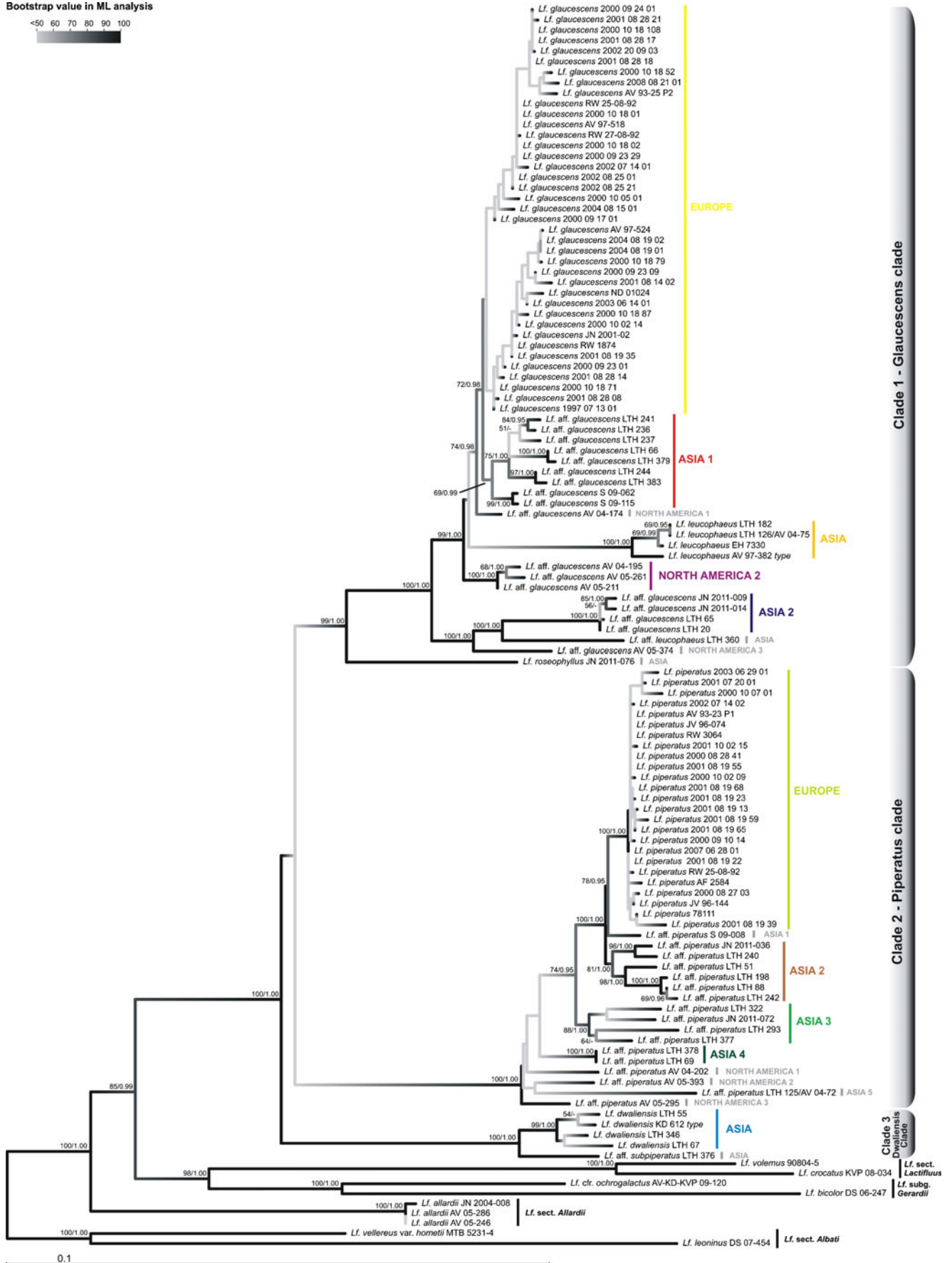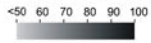
from *Lf.* sect. *Piperati* with sulphoformaldehyde is strongly dependent on the stage of development of the specimen.

During our European study, we predominantly focused on collections from Belgium and France, as the herbarium specimens of these regions are provided with comprehensive macroscopic descriptions and macrochemical tests according to our protocol. We realize that this is a rather limited distribution, but until now, all the samples from other European countries that we included in the study fall within one of the two European clades, so we assume that there are two species within *Lf.* section *Piperati* in Europe. However, we cannot completely rule out the possibility of another species from East or South Europe, therefore additional sampling in those regions is needed.

Lactifluus *section* Piperati *worldwide*

The worldwide phylogeny presented here suggests at least ten potential species within *Lf.* section *Piperati*, divided over three clades (Fig. 4). The actual number of species is likely to be higher, since by analysing the Asian and North American collections, the variation amongst those collections appeared to be much larger than previously thought by field determinations. This led to an undersampling of certain potential species, since they were only represented by one or two collections. Consequently, it was not possible to carry out a *BEAST analysis to construct a species tree, as that method requires more than one specimen per species to calculate the coalescent event for that extant species. The information on the coalescent event is needed to estimate the population size, which is in turn needed to infer speciation times and species topology (Heled and Drummond 2010). Deleting these singletons would lead to a reduction of the Asian and North American datasets and consequently to an underestimation of the actual number of species within this section. We can conclude that additional sampling is needed to get a better view on the actual species composition of *Lf.* sect. *Piperati* and to be able to construct a species tree using *BEAST, to correct for the potential amount of incomplete lineage sorting present in these data (Fig. 5).

Our preliminary analyses of the concatenated dataset denote that the European species are not found in North America or Asia and vice versa, so there is no intercontinental conspecificity. The first clade within the section worldwide contains three strongly supported subclades: *Lf. leucophaeus* Asia, *Lf.* aff. *glaucescens* North America 2 and *Lf.* aff. *glaucescens* Asia 2 (Fig. 4). The *Lf. leucophaeus*-clade is positioned on a long branch, which may indicate that this species underwent many changes since its split from the most recent common ancestor which it shares with *Lf.* aff. *glaucescens* North America 1, *Lf.* aff. *glaucescens* Asia 1 *and Lf. glaucescens* Europe. Morphologically, this species, with latex that changes from white to bluish green, differs from all European representatives of *Lf. glaucescens* by

darker pileus colours (greyish brown) and a thinner layer of hyphae in the pileipellis (Verbeken and Horak 1999). So far, the other well-supported clades have not been morphologically investigated. The clade *Lf.* aff. *glaucescens* Asia1 is weakly supported (BS: 69, PP: 0.99) and the *Lf. glaucescens* clade from Europe is not supported at all (BS: 44, PP: 0.48). The latter can indicate that some other processes are going on here, such as hybridisation or a recent divergence between the European clade, the Asia 1 clade and the North America 1 clade. To elucidate this, a more thorough sampling is needed, especially from the Asian and North American representatives. There are also four singletons within clade 1 (AV 04–174 and AV 05–374 from North America, LTH 360 and JN 11–076 from Asia). Only one of these singletons is morphologically identified as a separate species, namely *Lf. roseophyllus* (JN 11–076), which differs from the remainder of clade 1 by its pink salmon and creamy coloured latex, and by its salmon orange to pale orange-brownish coloured lamellae (Heim 1966 and field observations). These morphological differences and its distant position support the delimitation of *Lf. roseophyllus*.

The second clade shows four highly supported subclades: one European and three Asian subclades (*Lf.* aff. *piperatus* Asia 2, 3 & 4; Fig. 4). Until now, none of those Asian subclades have been morphologically investigated. One of these subclades, *Lf. piperatus* Asia 3, contains four specimens from Thailand and Vietnam on rather long branches. Further morphological examination and Bayesian species delimitation might clarify if the specimens from this subclade really belong to the same species, or if this subclade needs to be split into separate smaller subclades. This clade also includes five singletons (AV 04–202, AV 05–393 and AV 05–295 from North America, S 09–008 and LTH 125/AV 04–072 from Asia) and none of them were previously described as a separate species.

Finally, the third clade consists of one well defined subclade, together with one singleton (LTH 376), and all collections are from Asia. The clade differentiates morphologically from the rest of the section by its distant cream-coloured lamellae. Most of the specimens in this clade were originally identified in the field as *Lf. subpiperatus*, but after microscopical examination, Le (2007) found that these specimens have bigger and more globose spores than *Lf. subpiperatus*. Additionally, she found that these specimens have distinct pleuromacrocystidia, while these are absent in *Lf. subpiperatus*. We also succeeded to sequence the *atp6*-region for the type specimen of *Lf. dwaliensis* and it falls within this clade. This might lead to the conclusion that all those specimens are representatives of *Lf. dwaliensis*. Although the third clade itself is well supported, its position relative to the other two clades is still uncertain. In the different gene trees, this clade jumps from being a sister clade to clade 1, to being a sister clade to both clade 1 and clade 2.
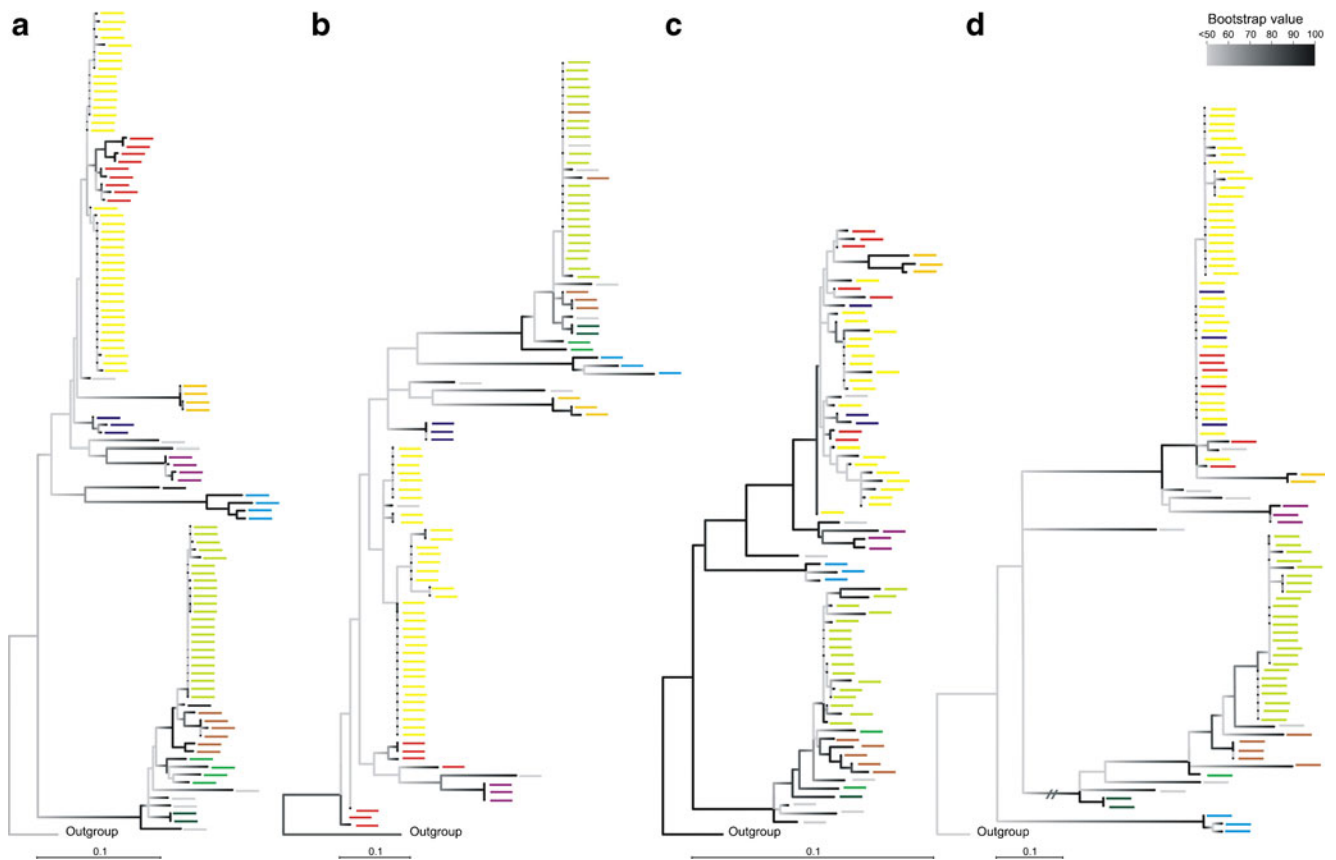
Fig. 5 ML gene trees for **a** ITS, **b** LSU, **c** *rpb2* and **d** *atp6*, with the colour code of the provisional species as in Fig. 4, showing lack of monophyly for certain clades. Bootstrap values are shown by the *grey scale* (see *gradient legend*)

Additional sampling and the sequencing of more markers may elucidate the position of this third clade within *Lf.* sect. *Piperati*.

The genus *Lactifluus* is known to contain species complexes with cryptic and semi-cryptic diversity. Explicit examples are *Lf.* subg. *Gerardii* and *Lf.* sect. *Lactifluus*. Stubbe et al. (2010) uncovered at least 30 strongly supported clades in *Lf.* subg. *Gerardii*, of which only 18 are morphologically identifiable species. In their study of *Lf.* sect. *Lactifluus* from Thailand, Van de Putte et al. (2010) elucidated 18 phylogenetic species, where of six species are also morphologically distinguished; and in their study of *Lf.* sect. *Lactifluus* in India, Van de Putte et al. (2012) showed the existence of six species, of which three were newly described based on phylogeny and morphology. Preliminary studies on African *Lactifluus* sections also suggest the presence of cryptic and/ or semi-cryptic diversity in *Lf.* sect. *Pseudogymnocarpi* (unpubl. data). In accordance with those results, our preliminary worldwide study suggests that *Lf.* section *Piperati* may contain cryptic and/or semi-cryptic species. To clarify this assumption, a more thorough sampling is needed, especially in Asia and North America, where a lot of countries and states are underexplored.

Because of the rather cryptic morphology and the low support in the worldwide gene trees, we will not describe the non-European clades as new species yet. First, the sampling should be increased, and the problem of contradicting and poorly supported gene trees should be treated in detail. In contrast to results from other studies within the genus *Lactifluus* (Stubbe et al. 2010; Van de Putte et al. 2012, 2010), the phylogenetic markers used here appear not to be as effective to strongly support species within *Lf.* sect. *Piperati*. As indicated by their low evolutionary divergence, LSU and *atp6* are too conservative and therefore contain not enough phylogenetic signal to delimit species within this section. ITS and *rpb2* are informative, but the amplification of *rpb2* failed for many collections. This can be explained by the fact that the majority of the herbarium specimens used for this study are between 10–20 years old. Both more recent material (preferably stored on CTAB buffer) and more informative markers could be helpful in improving these results.

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