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Lactifluus section *Albati*, The Fleecy milkcaps:

A worldwide exploration

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Introduction

The milkcaps

There has been a revolution in the taxonomical classification of fungi since the rise – and more precisely since the drop in costs – of molecular phylogenetic research. The milkcaps are a particular group of fungi that have experienced major rearrangements.

Traditionally, the genera *Russula* Pers. and *Lactarius* Pers. were seen as very different from other typical mushrooms and were classified in their own order, Russulales Kreisel ex P.M.Kirk, P.F.Cannon & J.C.David, based on the presence of sphaerocytes[1], amyloid[2] spore ornamentation and a gloeoplerous hyphal system[3] (Kreisel 1969; Oberwinkler 1977). The rise of molecular research however, has brought to understanding that historically, too much importance has been put on morphological characteristics; other basidiocarp types had to be included in this order (Donk 1971; Larsson and Larsson 2003; Oberwinkler 1977; Romagnesi 1948). Added to the russuloid clade were fungi with corticoid (forming a crust or patch), resupinate (hymenium on top of fruiting body), discoid, effused-reflexed (partially resupinate, partially pileate), clavarioid (club- or coral-shaped), pileate and gasteroid (with hymenium on the inside of the fruiting body) habits with smooth, poroid (composed of pores), hydnoid (composed of spines), lamellate or labyrinthoid hymenophores, not all sharing sphaerocytes and amyloid spore ornamentation. *Russula*, *Lactarius* and some angiocarpous genera however, still form an important group within the Russulales and are placed in the family Russulaceae Lotsy (Eberhardt and Verbeken 2004; Larsson and Larsson 2003; Miller et al. 2001; Nuytinck et al. 2004).

The Russulaceae, as mentioned above, traditionally consisted of two mainly agaricoid genera: *Russula* and *Lactarius*, popularly known as brittlegills[4] and milkcaps. This group of ectomycorrhizal mushrooms is found in diverse types of vegetations, ranging from boreal to tropical climate regions. The name brittlegills refers to one of the synapomorphic features of this family, which is the occurrence of sphaerocytes. These cells explain the typical brittle consistency of these fungi causing them to break like chalk, which is in contrast with the fibrous texture found in most other fungi (which are completely composed of hyphae, see figure 1.1). Both genera, *Russula* and *Lactarius*, contain these sphaerocytes but in *Russula* they are usually more dominant in the trama of the gills than they are in *Lactarius*[5]. The name "milkcaps" refers to those Russulaceae exuding a latex-like substance (also referred to as latex or milk). This latex is kept in lactiferous hyphae, spreading throughout the trama and with extremities reaching into the hymenium, forming pseudocystidia. These differ from true cystidia by the absence of a septum.

The distinction between both genera used to be simple: brittlegills contain no milk while milkcaps do. Additionally, other features such as brightness and contrast of colours, presence and organization of lamellulae, texture of cap and cap margin usually allow a quick distinction between the traditional genera *Russula* and *Lactarius*.

[1] Round, isodiametric cells spread throughout the pileus, stipe and lamellar trama, occuring intermixed with hyphae.
[2] Colouring dark when reacting with the iodine in Melzer's reagent.
[3] Hyphae with long cells containing oil droplets.
[4] Altough not as commonly used as 'milkcaps', this fits well within this explanation.
[5] At least this was considered a traditional difference before tropical representatives were taken into account.

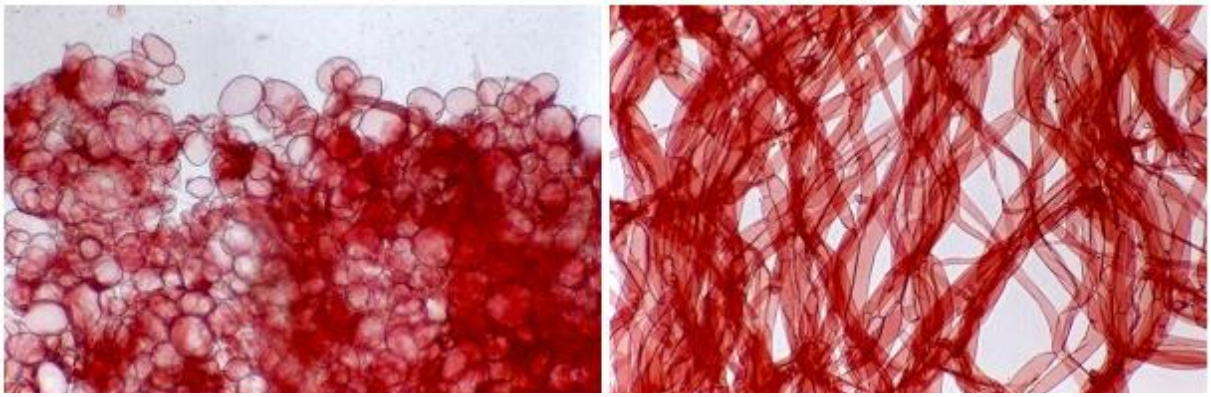

Fig. 1.1: left: gill trama with sphaerocytes, right: gill trama of a non-Russulaceae species. © Giancarlo Partacini, found in Russulales News

Once the study of the Russulaceae also took tropical regions and tropical representatives into account, the distinction between the two genera turned out to be more difficult than previously thought (Buyck 1989; Buyck and Halling 2004; Buyck and Horak 1999; Buyck and Verbeken 1995; Verbeken 1998; Verbeken and Buyck 2002; Verbeken and Walleyn 1999; Verbeken et al. 2000; Verbeken A. & Buyck 2001).

Previously placed within one genus *Lactarius*, the milkcaps have been split up into three genera based on molecular data: *Lactarius*, *Lactifluus* (Pers.) Roussel and *Multifurca* Buyck & Hofst. (Buyck et al. 2008a). The latter genus contains very few representatives, some of them formerly known as *Lactarius*, others as *Russula*. The genus *Russula* was only monophyletic if a small group of species was left out. This small group forms a clade where *Lactarius* and *Russula* are mixed containing the former *Russula* subsect. *Ochricompactae* Bills & O.K. Mill., the Asian *Russula zonaria* Buyck & Desjardin and the American *Lactarius furcatus* Coker (Buyck et al. 2008a). Meanwhile, *Lactarius* and *Lactifluus* can be seen as the two main milkcap genera. To avoid major nomenclatural changes, the proposal to maintain *Lactarius* as a name for the clade containing about 75% of all described milkcap species (Buyck et al. 2010) was approved in 2011 (Barrie 2011; McNeill et al. 2011; Norvell 2011). The consequence was that a new type species needed to be chosen, as the old one – *Lactarius piperatus* (L.: Fr.) Pers. – now belonged to *Lactifluus*. *Lactarius torminosus* (Schaeff.: Fr.) Pers. became the new type species for *Lactarius*. The second, smaller clade was named *Lactifluus* (Pers.) Roussel, as this was the oldest next available name, typified by *Lactifluus volemus* (Fr.: Fr.) Kuntze (Buyck et al. 2010). From now on, *Lactarius* will be referred to as *L*. and *Lactifluus* as *Lf*.

Distinguishing both genera

A clear morphological distinction between the two main milkcap genera has not been found yet. There are some trends however allowing a certain delimitation (Van de Putte et al. 2012b; Verbeken and Nuytinck 2013). From a morphological point of view, *Lactifluus* contains all species with veiled and velvety to tomentose caps and all annulate species. This clearly contrasts with *Lactarius*, where zonate and viscous to glutinate caps are often found. So far, all pleurotoid species also belong within *Lactifluus*, while angiocarpic species are placed within *Lactarius*.

At microscopic level, hymenophoral sphaerocytes, thick-walled cystidia (lamprocystidia) and thick-walled elements in the pileipellis are more typical for *Lactifluus*. Macrocystidia, thin-walled cystidia with a needle-like to granular content, are common in *Lactarius*.

The most striking difference between *Lactarius* and *Lactifluus* is their geographical distribution. Although *Lactarius* does occur in the tropics and subtropics, the genus comprises almost all European milkcaps as well as most other species from boreal and temperate regions. *Lactifluus* only contains a few temperate species and has its main distribution in the tropics and subtropics (De Crop 2016).

Lastly, in contrast to *Lactarius*, *Lactifluus* shows a high genetic diversity but a rather stable morphology. This is reflected in the cryptic species complexes that have recently been uncovered (De Crop et al. 2014; Stubbe et al. 2012a; Stubbe et al. 2010; Van de Putte et al. 2012b; Van de Putte et al. 2015; Van de Putte et al. 2010a). The discovery of *Lf. cocosmus* (Van de Putte & De Kesel) Van de Putte further illustrates this high genetic diversity. This new species seems to occupy an isolated phylogenetic position, representing a distinct, distant clade in the genus (Van de Putte et al. 2009). Multiple other species inhabiting isolated clades have arisen during the past years (Buyck et al. 2007; Morozova et al. 2013; Van de Putte et al. 2009; Wang et al. 2015).

The genus *Lactifluus*

This genus, representing about 25% of all currently known milkcaps, is mainly found in Asia (De Crop 2016; Stubbe et al. 2010; Van de Putte et al. 2010b) and Africa (De Crop 2016; Verbeken and Walleyn 2010) although recently, studies have shown the genus is also significantly represented in South America (De Crop 2016; Henkel et al. 2000; Miller et al. 2002; Sá et al. 2013; Sá and Wartchow 2013; Smith et al. 2011). The genus has been understudied, probably because of its tropical distribution, but recently more species are being discovered and described (De Crop 2016; De Crop et al. 2012; De Crop et al. Under rev.; Kropp 2016; Latha et al. 2016; Maba et al. 2015a; Maba et al. 2014; Maba et al. 2015b; Miller et al. 2012; Sá et al. 2013; Sá and Wartchow 2013; Stubbe et al. 2012a; Van de Putte et al. 2012a; Van de Putte et al. 2010a; Wang et al. 2012; Wang and Verbeken 2006; Zhang et al. 2016). Typical host trees are leguminous trees (Fabaceae), members of the Dipterocarpaceae and the Fagaceae, and of the genera *Uapaca* Baill. (Phyllanthaceae), *Eucalyptus* L'Hér and *Leptospermum* J.R. Forster & G. Forster (Myrtaceae) (De Crop 2016).

Previous studies have questioned the current classification of *Lactifluus* that was mainly morphology-based (Buyck et al. 2008b; Verbeken et al. 2014). Because of this, important changes have been published during the past years concerning the infrageneric classification of the genus. As proposed in a series of three articles on the recombinations needed to accommodate *Lactifluus,* the genus contains six subgenera and one unclassified section (Stubbe et al. 2012b; Verbeken et al. 2011; Verbeken et al. 2012). Big changes are imminent however, as a genus-wide analysis based on both molecular and morphological data conducted by De Crop et al. is accepted for publication (De Crop et al. acpt.). Here, a summarizing overview will be given of the new and adapted classification, proposed by De Crop et al.

Improved infrageneric classification

Traditionally, the genus consisted of six subgenera, only two of them remain in the new classification but are emended. Next to these two subgenera, *Lf*. subg. *Lactariopsis* (Henn.) Verbeken and *Lf*. subg. *Lactifluus,* two new ones are proposed: *Lf*. subg. *Gymnocarpi* (R. Heim ex Verbeken) De Crop and *Lf*. subg. *Pseudogymnocarpi* (Pacioni & Lalli) De Crop. The new classification is fully supported in the concatenated and the individual gene phylogenies (based on ITS-, LSU-, *rpb*2- and *rpb*1-gene sequences). There is one small exception however: *Lf*. sect. *Albati*'s placement in *Lf*. subg. *Lactariopsis* is not supported by the *rpb1* phylogeny. But as this study preferred defining the four largest supported subgenera with an even-balanced diversity and the other individual and concatenated gene phylogenies supported its placement, *Lf*. sect. *Albati* was still included in *Lf*. subg. *Lactariopsis*. The relationships between the subgenera are not yet fully resolved, to fully understand them, more genes will need to be sequenced.

Ten traditional sections are confirmed in their traditional delimitation, others are polyphyletic of which two have been synonymized and seven have been emended. Other clades, not fully supported molecularly, were suspected to represent new sections. However, this study aimed to only assign new sections to fully supported clades characterized by synapomorphic features. Figure 1.2 shows the new arrangement of *Lactifluus* with *Lf*. subg. *Lactariopsis* having four fully supported sections (among which *Lf*. sect. *Albati*) next to eight undescribed clades and two isolated species, *Lf*. subg. *Pseudogymnocarpi* with five sections and two undescribed clades, *Lf*. subg. *Gymnocarpi* with four sections, four undescribed clades and one isolated species, *Lf*. subg. *Lactifluus* with five sections and one isolated species and finally *Lf*. sect. *Allardii* (Hesler & A.H. Smith) De Crop remains unclassified.

As already mentioned above, more genes will need to be sequenced but also a more thorough sampling and search for synapomorphies will be needed to be able to fully resolve infrageneric relationships within this genus.

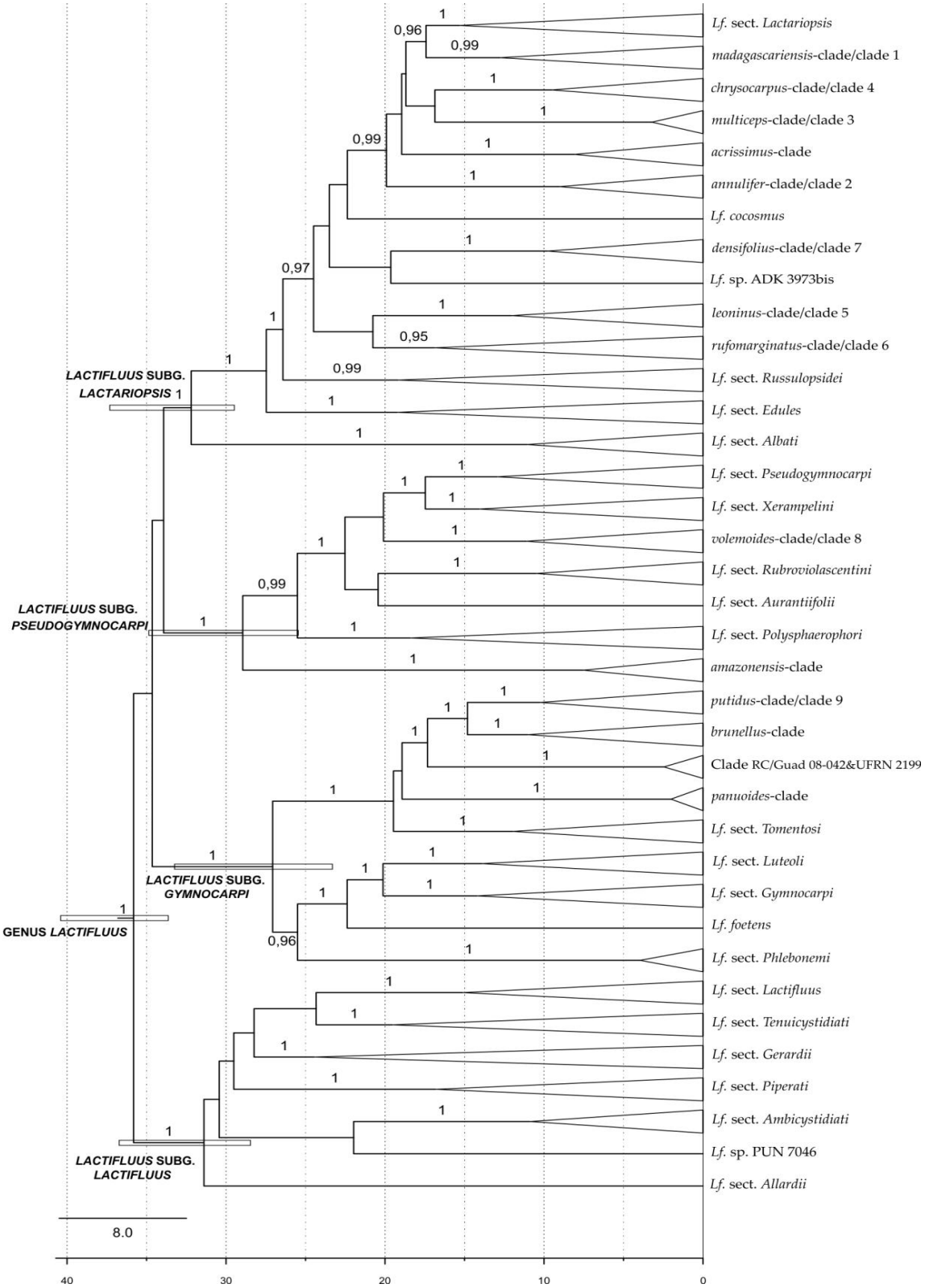

Fig. 1.2: Overview of the genus Lactifluus, inferred from a dated phylogeny (time scale= million years). Adapted from De Crop (2016).

The Fleecy milkcaps

Historical taxonomy

The previous part illustrated that during the past two centuries, the taxonomical landscape within the Russulaceae seriously changed. This thesis will focus on a particular group of milkcaps, known as the Fleecy milkcaps. I will give a brief overview of how the circumscription and systematical placement of this group evolved since the late nineteenth century.

The Latin name '*Albati*' was conceived by Frédéric Bataille (Bataille 1908). He proposed a new classification of fungi, based on the one his predecessor Quélet made (Quelet 1888). Following this classification, he maintained the division of the genus *Lactarius* in three sections: *L*. Sect. *Glutinosi* Quél., *L*. sect. *Velutini* Quél. and *L*. sect. *Pruinosi* Quél., but he emended the subdivisions and groupings. The section *Velutini*, containing species with velutinous, tomentose or pubescent cap cuticles, was subdivided in two subsections based on the colour of the cap: *L*. subsect. *Albati* Bat. (white) and *L*. subsect. *Colorati* Bat. (coloured).

Singer (1942) however, merely divided the genus *Lactarius* in multiple sections and avoided any further hierarchy, arguing previous classifications were too artificial. In this grouping, the section *Albati* contained all milkcaps with firm, white basidiocarps, with short, broad stipes and (slightly) infundibuliform caps.

A third division of this group was made by Heinemann (Heinemann 1948; Heinemann 1960). He followed the works of Bataille and Quélet, and divided the genus *Lactarius* in three sections again: *L*. sect. *Glutinosi*, *L*. sect. *Velutini* and *L*. sect. *Pruinosi*. He also placed the *Albati* as a subsection within *L*. sect. *Velutini*. As research used to be very Eurocentric during the late nineteenth and early twentieth century, all pedigrees were probably based on mainly European species, as is probably the case here.

Later, Hesler & Smith (1979) came up with a new division, based on approximately 250 North-American taxa. They placed all firm, white to pale species with dry and velvety caps within the subgenus *Lactifluus* (Burl.) Hesler & Smith. This subgenus was split into four sections, amongst which the sections *L*. sect. *Albati* (Bat.) Sing and *L*. sect. *Piperati* Fries emend. Species placed in *L*. sect. *Albati* are characterized by completely white caps during juvenile stadia, the stipe being velvety with stipitipellis hairs that are always thick-walled.

Bon (1980) came up with his own genealogy, again based entirely on European species. It was very similar to the one Singer started making in 1942 but had some additions. In his version, the section *Albati* (Bat.) Sing. sensu Bon contained many more large milkcaps. This grouping was split into two subsections: *L*. subsect. *Piperati* (Fr.) Konr. and *L*. subsect. *Velutini* Bat. (which is in accordance with Singer, see below). Subsequently, *L*. subsect. *Velutini* was split into stirps *Vellereus* with latex not reacting with KOH and stirps *Bertillonii* with latex turning yellow with KOH.

Soon after, Singer (1986) published his taxonomy of the milkcaps, based on a worldwide dataset (Singer 1986). Continuing on the work he previously published (Singer 1942), he added some characteristics. Species placed in *L.* sect. *Albati* (Bat.) Sing have spores with a (very) fine ornamentation opposed to other white or whitish *Lactarius* species and specimens react positively with E.P.-reagens (phenolphthalein), turning blue. He divides *L*. sect. *Albati* in a *piperatus*-group (or subsection) and a *vellereus*-group (or subsection) (*L*. subsect. *Piperati* and *L*. subsect. *Velutini*, respectively according to Bon). The distinction between both was based on spacing of the lamellae and the texture of the cap surface.

The lamellae are positioned close to each other for *L*. subsect. *Piperati* and rather widely for *L*. subsect. V*ellereus.* The cap surface is hairless, sometimes with only short hairs on the cap margin for *piperati*-species while it is completely velvety for *vellereus*-species.

Finally, following the discovery that milkcaps actually consist of more than one genus (Buyck et al. 2008a; Buyck et al. 2010), Verbeken recombined *L*. sect. *Albati* and placed the group in the new genus *Lactifluus* (Verbeken et al. 2011). Subsequently, its previous relation with *Lf*. sect. *Piperati* was completely broken up, putting *Lf*. sect. *Albati* closer to African species bearing a ring (Verbeken 1998). As a consequence, *Lactifluus* sect. *Albati* (Bataille) Verbeken is the only section within *Lf*. subg. *Lactariopsis* (Henn.) Verbeken without any African representatives, other sections are mainly found in Africa. Species placed in this section are characterized by firm, white basidiocarps, a lamptrotrichoderm as pileipellis and the very fine spore ornamentation (Verbeken and Vesterholt 1997). What distinguishes this group morphologically from other sections in *Lf*. subg. *Lactariopsis* is the presence of macropleurocystidia and the absence of broad and emergent pseudocystidia (Verbeken 1998).

European species through history

Two species within this section occur in Belgium: *Lf. vellereus* (Fr.: Fr.) Kuntze (including the variety *Lf. vellereus* var. *hometii* (Gillet) Boud.) and *Lf. bertillonii (*Neuhoff ex Z. Schaef.) Verbeken, displayed in figure 1.3. In Dutch they are called 'Schaapje' and 'Vals schaapje' which refers to their large and firm, whitish fruiting bodies and velutinous cap and stipe. *Lactifluus vellereus* is a species showing much variation, reflected in the number of varieties that have been described for this species. The taxonomical value of these varieties however, is being seriously questioned (Verbeken et al. 1997). *Lactifluus vellereus* and *Lf. bertillonii* are look-a-likes in the field, the best way of distinguishing them is the taste of their milk separated from the flesh and its reaction with KOH; *Lf. vellereus* tastes mildly acrid, not reacting with KOH while *Lf. bertillonii* tastes very acrid and turns yellow to orange with KOH (Heilmann-Clausen et al. 1998).

Fig. 1.3 left: Lf. vellereus (AV96-164, picture by A. Verbeken), right: Lf. bertillonii (RW1220, picture by R. Walleyn)

These two European species have had a chequered past though, for a long time they were believed to be one species (Bertillon 1865). Later it was thought *Lf. bertillonii* was an acrid-tasting variety of the more mild-tasting L*f. vellereus* (Neuhoff 1956). The opposite has also been thought however; *vellereus* being the acrid one and *bertillonii* the mild one (Blum 1966). Which species actually was meant when it was first described by Fries will remain a matter of discussion, what is more important is the recognition that these are two separate, closely related species.

The type specimen of *Lf*. sect. *Albati*, *Lf*. *vellereus*[6], was first described by Fries as *Agaricus vellereus* Fr. (Fries 1821). In 1838 he placed it within the genus *Lactarius* (Fries 1838). As in that time, microscopical or chemical characteristics were not yet used for determination, nothing was mentioned about the taste of the flesh or latex.

Bertillon (1865) described *L. vellereus* as a species with acrid tasting milk while introducing *L. velutinus* Bertillon. as an almost identical species but with mild milk and a more velutinous, tomentose cap. He was also the first to mention *L. vellereus* had both acrid and mild tasting types (Neuhoff 1956). It is abundantly clear that without proper microscopical and/or genetic research, there was a lot of confusion about species delimitation in that time.

Almost a century later, Neuhoff (1956) described a new variety of *L. vellereus*: *L. vellereus* var. *bertillonii* Neuhoff with milk that tastes more acrid and reacts positively with potassium hydroxide (KOH), turning yellow. He did add a remark however, stating he believed this to be a separate species. It needed to be handled as a variety awaiting a better systematic placement of the other varieties of this species. Unfortunately he did not add a Latin description of this new species to his publication, making it invalid.

In 1966, Blum described several varieties of *L. vellereus,* acknowledging the variable character of this species. He based this splitting on a combination of two traits: subglobose spores and milk not changing with KOH versus ellipsoid to oblong spores and milk turning yellow with KOH. The former applies to the type of *L. vellereus* along with *L. vellereus* var. *hometii* Gill., var. *odorans* Blum, var. *velutinus* Bert. and var. *fuscecens* Blum. The latter combination of traits applies to *L. vellereus* var. *bertillonii*, var. *boudierii* Blum and var. *quélettii* Blum. Distinctive characteristics are colour, density of the lamellae, smell and flavour. According to him, *L. vellereus* has spaced lamellae and sharp milk, *L. vellereus* var. *hometii* has more dense lamellae, milk that is mildly sharp and sometimes turns violet, *L. vellereus* var. *odorans* also has closer lamellae and smells like geraniums, *L. vellereus* var. *velutinus* has averagely spaced lamellae and milk that is sweetish at first but turns lightly sharp and *L. vellereus* var. *fuscecens* has lamellae standing quite close and milk that tastes sweetish for a long time before turning slightly sharp. In the group with milk turning yellow with KOH, *L. vellereus* var. *bertillonii* has widely spaced lamellae and very sharp milk, *L. vellereus* var. *boudierii* has lamellae standing close, sharp milk and flesh turning brown when exposed and *L. vellereus* var. *quélettii* has averagely spaced lamellae and sharp milk that turns yellow in 3-4 minutes when separated from the flesh (Blum 1966 cit. in; Schaefer 1979).

[6] From now on I will follow the historic placement and hence name giving too (*L*. instead of *Lf*.)

Schaefer (1979) set Neuhoff's mishap right in 1979 by officially describing *L. vellereus* var. *bertillonii* Z. Schaefer accompanied by a Latin description this time. In his handling of the *L. vellereus* vs. *L. bertillonii* case, he too assumed *L. vellereus* had burning milk. He actually used the same characteristics as Blum for describing the varieties. However, he did change some ranks, upgrading varieties to species, which gave rise to: *L. hometii* Gillet and *L. velutinus* with *L. velutinus* var. *fuscecens*. He also described a new species: *L. moravicus* Z. Schaefer sp. nov. with flesh and sometimes also latex turning green(ish) and closely placed lamellae and placed it in the group with milk that turns yellow when reacting with KOH . This positive reaction with KOH and the spores being slightly reticulate would suggest *L. moravicus* to be closely related to the current *Lf. bertillonii* but the closely placed lamellae however rather suggest a resemblance to the American *Lf. subvellereus (*Peck) Nuytinck.

Romagnesi (1980) also described *L. vellereus* as an acrid-tasting species with milk reacting positively to KOH and oblong spores with incomplete reticulate ornamentation. He discovered another species too, *L. albivellus* Romagn., which he described as tasting mildly, not reacting with KOH and with subglobose spores with completely reticulate ornamentation.

Finally, Kytövuori and Korhonen (1990) substantiated the taxonomic status of *L. vellereus* and *L. bertilloni.* By conducting a scrutinous historical/geographical and morphological study they cleared up the debate once and for all. According to them, a lot of taxonomists based themselves on the commonest species in their study area. They decided to perform research were the species Fries (1821) described came from and named that species *L. vellereus*.

They found that when Fries first described *A. vellereus*, he was living in Femsjö, Sweden, where the species with mildly tasting milk is most common so this most probably is the one he had in mind. When writing the new description for *L. vellereus* in his *Epicrisis* (1838) however, he was living in Uppsala, Sweden. In that area, only the sharp tasting species can be found so this description is most probably based on that species. After studying both herbarium specimens and fresh specimens, they decided the mild species Fries started out with, should be named *L. vellereus* and the sharp tasting look-a-like should be named *L. bertillonii*. According them, the species are easy to separate microscopically: *L. bertillonii* has shorter and thinner hairs on the pileus and stipe, and ellipsoid to broadly ellipsoid spores which are finely ornamented but not reticulated (opposed to globose, finely reticulated spores). They also concluded that more research and –more importantly– a better sampling will be needed to clear up the other taxa possibly existing in this group (a lot of varieties have been described, see above).

As an extra proof that the separation of these two species is legit, Hansson et al. (1995) found chemotaxonomic evidence. Commonly used as taxonomic markers, there is a chemical background to the variation in colour (transformation) and taste of the latex. The metabolites responsible for these characteristic differences are formed enzymatically from fatty acid ester precursors as a response to fruit body injuries. Depending on the precursor present and the metabolites affecting it, milkcaps can be divided in three groups. The *Albati* belong to the largest group with white latex becoming pungent after injury due to the formation of bioactive sesquiterpenes. Both *L. vellereus* and *L. bertillonii* contain the same precursor, a sesquiterpene called stearoylvelutinal. In *L. vellereus* this precursor is converted into isovelleral and velleral after injury, opposed to *L. bertillonii,* where metabolites only convert it into velleral.

These primary products are then both reduced to isovellerol and vellerol respectively. In *L. vellereus,* the reaction pathway stops here, in *L. bertillonii* however, vellerol is oxidized to vellerolactone. The latex of both species consequently has a very different chemical profile.

Taking into account the entire taxonomic history of the group surrounding *Lf. vellereus* in Europe, it can clearly be divided into two groups: a *vellereus*-group and a *bertillonii*-group. The *vellereus*-group contains those species with mild milk (wrongly described or interpreted by Blum (1966) and Schaefer (1979)) that does not react with KOH and subglobose spores with a finely reticulate ornamentation. *Lactifluus vellereus* belongs to this group , together with var. *hometii*, var. *odorans*, var. *velutinus* (now a synonym of *Lf. vellereus*) and var. *fuscecens*. The species *L. albivellus* also belongs in this group, but based on its description it can be synonymized with *Lf. vellereus*. The *bertillonii*-group has species with sharp milk that turns yellow to orange with KOH and ellipsoid spores lacking a fully reticulate ornamentation. This group contains *Lf. bertillonii* with var. *boudierii* and var. *quélettii* (now synonym of *Lf. bertillonii*) and *L. moravicus*. *Lactarius moravicus* does differ from *Lf. bertillonii* morphologically; the flesh and latex turning green and the closer lamellae that are not decurrent could be valuable characteristics in delimiting this species. See table 1.1 for a complete summary of the mentioned species and varieties.

Lf. vellereus-group	*Lf. bertillonii*-group
Mild milk not reacting with KOH, spores subglobose with finely reticulate ornamentation	Sharp milk turning yellow with KOH, ellipsoid spores lacking fully reticulate ornamentation
Lf. vellereus spaced lamellae	*Lf. bertillonii* widely spaced lamellae
var. *hometii* closer lamellae, sharper milk that sometimes turn violet	var. *boudierii* closer lamellae, flesh turning brown when exposed
var. *odorans* closer lamellae, smells like G*eranium sp.*	var. *quéletti* widely spaced lamellae, milk turning yellow in 3-4min
var. *velutinus* spaced lamellae, milk sweetish but quickly turning lightly sharp	*L. moravicus* milk and flesh turning green(ish), closer lamellae
var. *fuscecens* closer lamellae, milk sweetish but slowly turning slightly sharp	
L. albivellus no differences with *Lf. vellereus*	

table 1.1: summary of European Albati-species and -varieties

Species outside Europe

Four more species occur outside Belgium: *Lf. deceptivus* (Peck) Kuntze, *Lf. pilosus* (Verbeken, H.T. Le & Lumyong) Verbeken, *Lf. puberulus* (H.A. Wen & J.Z. Ying) Nuytinck and *Lf. subvellereus*.

(1) *Lactifluus deceptivus* is originally described from Earle, Alabama, USA by Peck (1898) and according to Hesler & Smith (1979) it is distributed throughout eastern North America and adjacent southern and western Canada. It has since also been reported from Central and South America (Mexico, Colombia, Costa Rica) and Asia (Vietnam) (based on collections used in this study).

(2) *Lactifluus subvellereus* was originally described from Auburn, Alabama (Peck 1898) and is distributed throughout central and eastern North America according to Hesler & Smith (1979). It has however also been reported in Thailand and Costa Rica (based on collections used in this study). This species has one variety, *Lf. subvellereus* var. *subdistans* Hesler and Smith, described from Eastern North America.

(3) *Lactifluus pilosus* is described from Doi Suthep-Pui National Park, Chiang Mai Province, Thailand (Le et al. 2007b) and has so far also solely been found in Thailand.

(4) *Lactifluus puberulus* is originally described from Daozhen county, Guizhou province, China (Wen and Ying 2005) and has so far not been reported outside of China.

Furthermore, recent expeditions have revealed unknown species from India, Vietnam, Thailand, Russia and North America. Preliminary phylogenetic analyses (De Crop unpubl. res.), place these specimens in *Lf*. sect. *Albati* so we might need to broaden the morphological description of this group to be able to accommodate these species. There are actually several irregularities regarding the taxonomy of this group. In a preliminary tree by De Crop (unpubl. res.) several unknown specimens from Vietnam and others from North-America are completely entangled instead of representing distinct lineages. Although this early analysis was based on just two collections from each locality, this is a strange outcome.

Aims

With this study we aim to clear up any irregularities and review the current placement and definition of *Lf*. sect. *Albati* following the present trend in the mycological world by which the emphasis on morphology is being equally valued as genetics. We aim to achieve this by (1) collecting specimens or at least sequences from species all over the world in order to (2) build a multi-locus phylogeny and (3) study the morphology of newly found phylogenetic species to decide if these represent species complexes or (4) new species that need to be described.

Methodology

Sampling

Fresh material

Multiple sampling expeditions have been conducted, two in Belgium (26/09/2015 and 20/10/2015) and one in Poland (17/09/2015). The latter one was unsuccessful due to bad weather conditions; it was too hot and too dry for most fungi to develop. In Belgium however, both excursions each delivered one collection of *Lf. vellereus*. During an expedition conducted by Jorinde Nuytinck and Quinten Bafort in France (Vosges), two more collections of *Lf. vellereus* were found. Of these collections, macroscopic features were assessed in the field and a small piece of pileus was stored in CTAB-buffer to conserve the genetic material. Hereafter the specimens were dried and stored in the Herbarium Universitatis Gandavensis partim Mycology.

Herbarium material

The group of fleecy milkcaps is already well represented at Ghent in the herbarium (108 collections). These were collected during previous expeditions, mainly throughout South-East Asia, Western Europe and North America. Yet some loans were still requested for *Lf. subvellereus, subvellereus var. subdistans, tomentoso-marginatus, deceptivus* and *vellereus var. virescens* as these species were not represented here in the herbarium. Most of these loans came from North America, those for *Lf. deceptivus* however also came from Central America. For a full overview of all collections used in this thesis, see table 2.1.

Molecular analysis

DNA extraction, amplification and sequencing

Two protocols were followed for extracting DNA from specimens. Genetic material from dried specimens collected after 1980 was extracted using the protocol described by Nuytinck and Verbeken (2003) with modifications proposed by Van de Putte et al. (2010a) (full protocol in appendix A). Older dried specimens or specimens for which the previous protocol proved insufficient, underwent the original protocol by Nuytinck & Verbeken (2003) with slight modifications by Steven Janssens (Plantentuin Meise, see appendix B for full protocol).

After extractions, the DNA quantity was checked using Nanodrop. A concentration of 100-200 ng/µl is recommended. Samples with higher values were diluted accordingly using Milli-Q, samples with lower values but above 25 ng/µl were also tolerated.
PCR amplification and sequencing protocols follow Le et al. (2007a) (see appendix C for full protocol). Three nuclear loci that have already proven to be useful in the Russulaceae (Das et al. 2010; De Crop et al. 2014; Van de Putte et al. 2012a; Van de Putte et al. 2016; Van de Putte et al. 2010a) were amplified and sequenced: the internal transcribed spacer region of ribosomal DNA (ITS) which comprises of spacer regions ITS1 and ITS2 and the ribosomal gene 5.8S; a part of the ribosomal large subunit 28S region (LSU) and the region between the conserved domains 6 and 7 of the second largest subunit of the RNA polymerase II (*rpb2*). The ITS region was amplified using the ITS-1F and ITS-4 primers (White et al. 1990). When this failed, the amplification was divided in two parts using primer ITS-5 with ITS-2 and ITS-3 with ITS-4 (White et al. 1990).

The LSU region was amplified using primers LR0R and LR5 (Moncalvo et al. 2000) and primers b*rpb*2-6F and f*rpb*2-7CR (Liu et al. 1999; Matheny 2005) were used to amplify the *rpb*2 region. Quality of the amplified DNA was checked using gel electrophoresis. Good PCR products showed a clear, single band in their lane on the gel, others were discarded.

The remaining PCR products were cleaned using Exonuclease I and FastAP™ (see appendix D for full protocol), mixed with either the matching forward or reverse primer and sent to Macrogen (Amsterdam, The Netherlands) for sequencing (see appendix D for full protocol). The obtained forward and reverse sequences were assembled into contigs and manually edited based on chromatograms with the SequencherTM v5.0 software (Gene Codes Corporation, Ann Arbor, MI, U.S.A.).

Alignment and phylogenetic analyses

DNA was extracted from 54 collections of which 23 allowed successful amplifications of at least one of the three selected markers. These sequences were supplemented by sequences that were already amplified in the lab here at Ghent, coming from 33 collections. Furthermore, BLAST-searches were done to find GenBank sequences from both identified and unidentified *Albati*-species. The Unite database (Kõljalg et al. 2005) was consulted too for the same reason. These online databases brought 55 extra sequences (either ITS, LSU or rpb2) to the alignment (see table 2.2), bringing the total to 91 ITS-sequences, 36 LSU-sequences and 26 rpb2-sequences. As an outgroup, five species from the group around *Lf. volemus* (Fr.) Kuntze were used because these species also belong to *Lf*. subg. *Lactifluus*. The outgroup consists of two North-American species: *Lf. volemus* sensu lato and *Lf. corrugis* (Peck) Kuntze, two Asian species: *Lf. volemus* sensu lato and *Lf. longipilus* Van de Putte, Le & Verbeken and one European species: *Lf. volemus.*

All sequences were aligned using the online version of MAFFTv7 (Katoh and Standley 2013) on CIPRES Science Gateway V. 3.3 (Miller et al. 2010) with setting E-INS-I, a very slow and accurate method recommended for less than 200 sequences with multiple conserved domains and long gaps. Afterwards, alignments were visually inspected and refined using MEGAv6.06 (Tamura et al. 2013) and BioEdit Sequence Alignment Editor (Hall 1999). The three markers were analysed both separately and concatenated. The ITS gene was partitioned into ITS1 and ITS2 (the spacer regions) and the ribosomal genes 5.8S and 18S. The LSU gene was analysed as a whole. The *rpb*2 gene was partitioned in its intron and the first, second and third positions of both exons. To determine the model that best fits each partition, PartitionFinder (Lanfear et al. 2012) was used analysing the three genes separately with all possible partition combinations.

For reconstructing the phylogeny, maximum likelihood (ML) analyses were conducted using RAxML v8 (Stamatakis 2014) and its rapid bootstrapping algorithm with 1000 iterations and GTRCAT as a model for the bootstrapping phase. Before combining the three markers in one concatenated sequence to construct a multi-locus gene tree, the ML single-locus gene trees were visually compared for compatibility. A significant incongruence would occur if two different relationships for any clades were supported with a ML bootstrap ≥70%.

Species delimitation

The species tree was estimated with *BEAST v2.4.1 (Bouckaert et al. 2014; Drummond and Bouckaert 2015) using a hierarchical Bayesian model. This software conducts multispecies coalescent analyses to estimate the most probable species tree from unlinked multi-locus sequence data. This analysis however, requires at least two specimens per species. So after assigning specimens to taxon subsets, based on the concatenated ML gene tree, singletons were discarded (amounting to 7 sequences). The required substation model by PartitionFinder was not always presented as an option in *BEAST however but there were enough parameters to adjust, allowing the proposed models to be imitated where needed. The analyses were run under a strict clock model because we are working with a low-diversity (partially intra-species) data set with low levels of rate variation and divergence-time estimation (Drummond and Bouckaert 2015). In a data-poor dataset, the fewer parameters that are used, the better. Although the Yule process is proposed as the most simple tree prior, this did not lead to converging runs so instead the birth-death model was chosen under a constant population function with a lognormal population mean. This has been suggested to be an appropriate null-model for phylogenetic diversification (Drummond and Bouckaert 2015). Convergence of the runs was verified by checking the log-likelihoods and effective sample sizes in Tracer v1.6 (Rambaut et al. 2014). Subsequently, the four best converging runs were combined using LogCombiner with a burn-in of 10%. The respective gene trees of those runs were combined using TreeAnnotator v2.3.1. Finally the combined tree-file was visualized and adjusted using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Description of new species

Morphology

Any new phylogenetic species needed to be analysed to assess if it is possible to delineate these species based on morphology. As mentioned above in 'Sampling', macroscopic features were assessed in the field. They are based on all aspects of size, shape, texture, colour (changes) and milk and flesh properties. Colours were described in daylight conditions and follow Kornerup and Wanscher (1978). Lamellar density is given as the sum of the lamellae and lamellulae per centimeter (L+l/cm), measured at 1 cm from the cap margin. Microscopic features were studied from dried material. See Vellinga (1988), Verbeken (1998) and Verbeken & Walleyn (2010) for details on the terminology used. Elements of the pileipellis and hymenial elements were either mounted in 10% KOH (enhances cell expansion), after which congo red was added, or directly mounted in congo red with L4. Basidia length excludes sterigmata. Hairs of the pileipellis were measured from scalps. Line drawings of the pileipellis, however, were made from sections. Spores were studied in Melzer's reagent. They were measured in side view, excluding ornamentation and replicated 20 times for each collection. Spore measurements based on more than one collection are given as (MIN) [Ava-2*SDa] – Ava – Avb – [Avb+2*SDb] (MAX) in which Ava= lowest mean value, Avb= highest mean value, SDa/b= standard deviation of the lowest or highest mean value respectively. MIN is the lowest value measured and MAX the highest value and both are only given if they exceed [Ava-2*SDa] or [Avb+2*SDb] respectively. Q stands for 'quotient length/width' and is given as MINQ – Qa – Qb – MAXQ in which Qa and Qb stand for the lowest and the highest mean quotient respectively. MINQ and MAXQ stand for the lowest and highest value over the quotients of all measured spores.

Spore measurements based on one collection were given as [Ava-2*SD] – Ava – [Ava+2*SD], in which Ava= mean value for the collection and SD= standard deviation. Q is given as MINQ – AvQ – MAXQ, in which AvQ stands for the mean quotient for the collection. Line drawings were made with the aid of a drawing tube at following magnifications: 6000x for spores (Zeiss axioscop 2 microscope), 1000x for individual elements and sections (Olympus cx31 microscope). Comparisons were based on type species if available.

Genus	cf. /aff.	Species epith.	Herbarium no.	Type?	Herbarium	Collection date	Collector	Country	Continent	ITS	LSU	*rpb2*
Lactifluus		*bertillonii*	TURA 3057 FH (MTB)		GENT	24/09/1992	Ruotsalainen J.	Finland	Europe	1	1	
Lactifluus		*bertillonii*	5033/3		GENT	18/09/2010	Hampe F.	Germany	Europe	1	1	
Lactifluus		*bertillonii*	JN 2012-016		GENT	27/08/2012	Nuytinck J.	Germany	Europe	1	1	1
Lactifluus		*deceptivus*	AV 05-249		GENT	12/08/2005	Verbeken A.	North America	North America	1		1
Lactifluus		*deceptivus*	AV 05-325		GENT	15/08/2005	Verbeken A.	North America	North America	1	1	
Lactifluus		*deceptivus*	TENN 065854		GENT	12/08/2011	Rock S.	North America	North America	1		1
Lactifluus		*deceptivus*	JN 2007-012		GENT	26/09/2007	Nuytinck J.	Canada	North America	1	1	1
Lactifluus		*deceptivus*	AV 05-332		GENT	15/08/2005	Verbeken A.	North America	North America	1	1	
Lactifluus		*deceptivus*	AV 05-350		GENT	17/08/2005	Verbeken A.	North America	North America	1	1	1
Lactifluus		*deceptivus*	ASM 11,068		Eastern Illinois - dupl.	12/08/2005	Methven A.	North America	North America			
Lactifluus		*deceptivus*	AV 05-275		GENT	12/08/2005	Verbeken A.	North America	North America			
Lactifluus		*deceptivus*	ASM 13521		Eastern Illinois - dupl.	13/08/2011	Methven A.	North America	North America	1		
Lactifluus		*deceptivus*	AV 04-181		GENT	13/07/2004	Verbeken A.	North America	North America	1		
Lactifluus		*deceptivus*	REH 6064		NY Bot Garden - loan	8/08/1988	Halling R. E.	Colombia	South America			
Lactifluus		*deceptivus*	AEF 523		NY Bot Garden - loan	12/06/1990	Franco-Molano A. E.	Colombia	South America	1		
Lactifluus		*deceptivus*	AEF 555		NY Bot Garden - loan	27/04/1991	Franco-Molano A. E.	Colombia	South America	1	1	

Lactifluus		*deceptivus*	AEF 756		NY Bot Garden - loan	17/06/1991	Franco-Molano A. E.	Colombia	South America	1		
Lactifluus		*deceptivus*	REH 7938		NY Bot Garden - loan	26/06/2000	Halling R. E.	Costa Rica	South America	1	1	1
Lactifluus		*deceptivus*	REH 7993		NY Bot Garden - loan	7/08/2000	Halling R. E.	Costa Rica	South America	1		1
Lactarius		*deceptivus*	NYSf 959	holotype	NY State Museum Herbarium	Aug 1885	C.H. Peck	United States	North America			
Lactifluus		*pilosus*	LTH 227		GENT	5/09/2004	Le T.H.	Thailand	Asia	1		
Lactifluus		*pilosus*	LTH 205	type	GENT	30/07/2004	Le T.H.	Thailand	Asia	1	1	1
Lactifluus		*pilosus*	LTH 204		GENT	28/07/2004	Le T.H.	Thailand	Asia	1	1	1
Lactifluus		*pilosus*	FH12-093		MFLU Herb. - loan	5/07/2012	Hampe F.	Thailand	Asia	1	1	1
Lactifluus		*pilosus*	FH 12-094		GENT		Hampe F.	Thailand	Asia	1	1	1
Lactifluus		*pilosus*	EDC 14-481		GENT	29/07/2014	De Crop E.	Thailand	Asia	1		
Lactifluus		*pilosus*	LTH 349		GENT	16/07/2005	Le T.H.	Thailand	Asia		1	
Lactifluus		*pilosus*	LTH 380		GENT	16/07/2005	Le T.H.	Thailand	Asia			
Lactifluus		*pilosus*	FH12-094		MFLU Herb. - loan	5/07/2012	Hampe F.	Thailand	Asia	1		
Lactifluus		*pilosus*	LTH 56		GENT	30/08/2003	Le T.H.	Thailand	Asia			
Lactifluus	cf.	*piperatus*	KW122		GENT	25/07/2011	Wisitrassameewong K.	Thailand	Asia			
Lactifluus		sect. *Albati*	JN 2011-071		GENT	16/06/2011	Nuytinck J.	Vietnam	Asia	1	1	1
Lactifluus		sect. *Albati*	JN 2011-077		GENT	16/06/2011	Nuytinck J.	Vietnam	Asia	1	1	1
Lactifluus		sect. *Albati*	FH 12-015		MFLU Herb. - loan	21/06/2012	Hampe F.	Thailand	Asia			
Lactifluus		sect. *Albati*	XP1-20120910-01		GENT - gift	10/09/2012	Jiayu C.	China	Asia			
Lactifluus		sect. *Albati*	XP1-20120910-		GENT - gift	10/09/2012	Jiayu C.	China	Asia			

		02								
Lactifluus	sect. *Albati*	XP2-20120911-04	GENT - gift	11/09/2012	Jiayu C.	China	Asia			
Lactifluus	sect. *Albati*	XP2-20121008-01	GENT - gift	8/10/2012	Jiayu C.	China	Asia			
Lactifluus	sect. *Albati*	KW291	GENT	9/06/2012	Wisitrassameewong K.	Thailand	Asia	1	1	
Lactifluus	*sect. Albati*	Hkas 34181	HKAS	22/09/1999	Yang Z. L.	China	Asia			
Lactifluus	*sect. Albati*	HKAS 39239	HKAS	11/08/2001	Wang X.H.	China	Asia			
Lactifluus	sp.	S 09-059	GENT	15/08/2009	Verbeken A., Das K., Van de Putte K.	India	Asia	1	1	1
Lactifluus	sp.	S 09-063	GENT	15/08/2009	Verbeken A., Das K., Van de Putte K.	India	Asia	1	1	1
Lactifluus	subg. *Lactariopsis*	KW119	GENT	25/07/2011	Wisitrassameewong K.	Thailand	Asia			
Lactifluus	subg. *Lactariopsis*	KW116	GENT	25/07/2011	Wisitrassameewong K.	Thailand	Asia			
Lactifluus	subg. *Piperati*	KW114	GENT	25/07/2011	Wisitrassameewong K.	Thailand	Asia			
Lactifluus	*subvellereus*	AV 05-324	GENT	15/08/2005	Verbeken A.	North America	North America	1		
Lactifluus	*subvellereus*	AV 05-210	GENT	10/08/2005	Verbeken A.	North America	North America	1	1	1
Lactifluus	*subvellereus*	AV13-025	GENT	21/08/2013	Verbeken A.	Canada	North America	1		
Lactifluus	*subvellereus*	TENN 066157	TENN - loan	22/06/2011	Looney BP	North America	North America	1	1	
Lactifluus	*subvellereus*	TENN 065593	TENN - loan	19/07/2011	KWH	North America	North America	1	1	1
Lactifluus	*subvellereus*	ASM 10,383	Eastern Illinois - dupl.	12/07/2004	Methven A.	North America	North America	1	1	
Lactifluus	*subvellereus*	AV 13-025	GENT	21/08/2013	Verbeken A.	Canada	North America	1	1	1

Lactifluus	cf.	*subvellereus*	KW276		GENT	7/06/2012	Wisitrassameewong K.	Thailand	Asia		
Lactifluus		*subvellereus*	ASM 12,075		Eastern Illinois - dupl.	10/08/2008	Methven A.	North America	North America		
Lactifluus	cf.	*subvellereus*	KW385		GENT	31/07/2012	Wisitrassameewong K.	Thailand	Asia		
Lactifluus		*subvellereus*	AV 04-193		GENT	13/07/2004	Verbeken A.	North America	North America	1	
Lactifluus		*subvellereus*	AV 05-326		GENT	15/08/2005	Verbeken A.	North America	North America	1	1
Lactifluus		*subvellereus*	AV 05-288		GENT	14/08/2005	Verbeken A.	North America	North America	1	
Lactifluus		*subvellereus*	AV 05-226		GENT	10/08/2005	Verbeken A.	North America	North America	1	
Lactifluus		*subvellereus*	AV 04-172		GENT	12/07/2004	Verbeken A.	North America	North America	1	
Lactifluus		*subvellereus*	REH 7909		NY Bot Garden - loan	22/10/1990	Halling R. E.	Costa Rica	South America		
Lactifluus		*subvellereus*	ASM 8214		Eastern Illinois - dupl.	12/09/1997	Methven A.	North America	North America	1	
Lactifluus		*subvellereus*	REH 7876		NY Bot Garden - loan	14/07/1999	Halling R. E.	Costa Rica	South America		
Lactifluus		*subvellereus*	ASM 9173		Eastern Illinois - dupl.	7/06/2000	Methven A.	North America	North America		
Lactarius		*subvellereus*	NYSf 3090	holotype	NY State Museum Herbarium	24/07/1897	F.S. Earle	United States	North America		
Lactifluus		*subvellereus* var. *subdistans*	MICH 11220	holotype, part	University of Michigan - loan	8/08/1972	Smith A.H.	North America	North America		
Lactarius		*tomentoso-marginatus*	MICH 11224	holotype, part	University of Michigan - loan	27/08/1973	Nimke C.	North America	North America		

Lactarius		*tomentoso-marginatus*	MICH 37931	paratype, part	University of Michigan - loan	22/09/1975	Smith A.H.	North America	North America			
Lactifluus		*vellereus*	AV 97-586		GENT	13/09/1997	Verbeken A.	Denmark	Europe	1	1	
Lactifluus		*vellereus*	ATHU-M 8075		ATHU-M - loan	1/11/1998	Delivorias P.	Greece	Europe			CONTAM
Lactifluus		*vellereus*	ATHU-M 8076		ATHU-M - loan	8/09/2002	Delivorias P.	Greece	Europe			
Lactifluus		*vellereus*	ATHU-M 8077		GENT	6/11/2010	Delivorias P.	Greece	Europe	1	1	1
Lactifluus		*vellereus*	AV 13-043		GENT	7/11/2013	Verbeken A.	Italy	Europe	1		
Lactifluus	cf.	*vellereus*	AV-KD-KVP 09-102		GENT	31/08/2009	Verbeken A., Das K., Van de Putte K.	India	Asia			
Lactifluus	cf.	*vellereus*	AV-KD-KVP 09-114		GENT	3/09/2009	Verbeken A., Das K., Van de Putte K.	India	Asia			
Lactifluus	cf.	*vellereus*	AV-KD-KVP 09-103		GENT	31/08/2009	Verbeken A., Das K., Van de Putte K.	India	Asia			
Lactifluus		*vellereus*	QB 2015-040		GENT		Q. Bafort	France	Europe			
Lactifluus		*vellereus*	JN 2015-096		GENT		Nuytinck J.	France	Europe			
Lactifluus		*vellereus*	SDW 2015-001		GENT	26/09/2015	Verbeken A.	Belgium	Europe			
Lactifluus		*vellereus*	SDW 2015-002		GENT	20/10/2015	S. De Wilde	Belgium	Europe			
Lactifluus		*vellereus s.l.*	RW 1658		GENT	20/09/1999	Walleyn R.	France	Europe	1	CONTAM	
Lactifluus		*vellereus* var. *hometii*	FH (MTB) 5231/4		GENT	26/09/2010	Hampe F.	Germany	Europe	1	1	1
Lactifluus		*vellereus* var. *hometii*	FH (MTB) 5032/4		GENT	25/09/2010	Hampe F.	Germany	Europe	1	1	1
Lactifluus		*vellereus* var. *virescens*	MICH 11232	holotype, part	University of Michigan - loan	18/08/1961	Smith A.H.	North America	North America			

Table 2.1: used herbarium material

Accession number	Original species name	Corrected species name	country	ITS, LSU or *rpb2*?
HF674650	uncultured *Lactarius*	*Lf. bertillonii*	Slovenia	ITS
KM576556	*Russula* sp.	*Lf. bertillonii*	Romania	ITS
EU598200	*L. deceptivus*	*Lf. deceptivus*	North America	ITS
KF937340	*L. deceptivus*	*Lf. deceptivus*	Colombia	ITS
HQ021852	uncultured *Lactarius*	*Lf. deceptivus 1*	North America	ITS
HQ022196	uncultured *Lactarius*	*Lf. deceptivus 1*	North America	ITS
AY854089	*L. deceptivus*	*Lf. deceptivus 1*	North America	ITS
AY803749	*L. deceptivus*	*Lf. deceptivus 1*	North America	*rpb2*
KJ705226	*L. deceptivus*	*Lf. deceptivus 1*	North America	ITS
KJ705225	*L. deceptivus*	*Lf. deceptivus 1*	North America	ITS
HQ022195	uncultured *Lactarius*	*Lf. deceptivus 1*	North America	ITS
AY631899	*L. deceptivus*	*Lf. deceptivus 1*		LSU
AF218550	*L. deceptivus*	*Lf. deceptivus 1*	Canada	LSU
DQ421935	*L. deceptivus*	*Lf. deceptivus 1*	North America	*rpb2*
JQ396484	uncultured fungus	*Lf. deceptivus 2*	China	LSU
KF937337	*L. deceptivus*	*Lf. deceptivus 3*	Colombia	ITS
KF937339	*L. deceptivus*	*Lf. deceptivus 3*	Colombia	ITS
KF937338	*L. deceptivus*	*Lf. deceptivus 3*	Colombia	ITS
KP348048	uncultured fungus	*Lf. deceptivus 4*	North America	ITS
KP348031	uncultured fungus	*Lf. deceptivus 4*	North America	ITS
KP348038	uncultured fungus	*Lf. deceptivus 4*	North America	ITS
HQ021851	uncultured *Lactarius*	*Lf. deceptivus 4*	North America	ITS
AB509977	*Lactarius* sp.	*Lf. pilosus*	Japan	ITS
AB154758	*L. vellereus*	*Lf. pilosus*	Japan	ITS+LSU
AB509984	*L. piperatus*	*Lf. subvellereus*	Japan	ITS
AB636110	uncultured Russulaceae	*Lf. subvellereus*	Japan	ITS
AY456362	Russula sp.	*Lf. subvellereus*	North America	ITS
AY456366	Russula sp.	*Lf. subvellereus*	North America	ITS
AY456363	Russula sp.	*Lf. subvellereus*	North America	ITS
AY456365	*Russula* sp.	*Lf. subvellereus*	North America	ITS
AY456364	*Russula* sp.	*Lf. subvellereus*	North America	ITS
HM189835	*L. vellereus*	*Lf. subvellereus*	Germany	ITS
DQ422034	*L. vellereus*	*Lf. vellereus*	Sweden	ITS+LSU
DQ421936	*L. vellereus*	*Lf. vellereus*	Sweden	*rpb2*
JN388994	*L. vellereus*	*Lf. vellereus*	Germany	LSU
AY606958	*L. vellereus*	*Lf. vellereus*	Germany	ITS
KT020824	uncultured *Lactarius*	*Lf. vellereus*	Germany	ITS
DQ011144	*L. vellereus*	*Lf. vellereus*	China	ITS
JN375597	*L. vellereus*	*Lf. vellereus*	Germany	*rpb2*
DQ054579	uncultured fungus	*Lf. vellereus*	Italy	ITS+LSU
DQ990841	uncultured *Lactarius*	*Lf. vellereus*	Italy	ITS
DQ054550	uncultured fungus	*Lf. vellereus*	Italy	ITS+LSU
KM576508	*Lactarius* sp.	*Lf. vellereus*	Hungary	ITS
KF220123	*Lf. vellereus* var. *hometii*		Germany	ITS
KF220288	*Lf. vellereus* var. *hometii*		Germany	ITS
KF220216	*Lf. vellereus* var. *hometii*		Germany	LSU
HM639277	*Lactarius* sp.		Honduras	ITS

Table 2.2: list of sequences obtained from GenBank and Unite

Results

Sequence alignments

In total, 168 sequences were used in this thesis. Of this amount,: 96 ITS-sequences ranging from 379 to 973 base pairs (bp) in length (excluding gaps), 41 LSU-sequences ranging from 860 to 1649 bp and 31 *rpb*2-sequences ranging from 556 to 1735 bp. The ITS-alignment had a total length (including gaps) of 1168 bp, the LSU-alignment 2122 bp and the *rpb*2-alignment 1800 bp. The concatenated alignment had a total length of 4034 bp with concatenated sequences varying between 497 and 2498 bp in length[7].

Phylogenetic analyses

Any strange or unexpected outcomes in the phylogenetic analyses may either be explained by a lack of sequences or the sequence coming from GenBank or Unite instead of this lab. This means we cannot know how these sequences have been obtained, if followed protocols were identical to ours and if or how the person cleaned up the raw sequences. Interpreting the chromatograms when cleaning up sequences is a subjective task, every person has his/her own way of doing this. Just a little difference in interpretation may lead to several base pairs differing between two sequences that are actually (almost) identical. Combined with the low number of sequences used here, the smallest difference may already cause divergences in the resulting gene tree. This does not mean we can not draw conclusions from these GenBank- and Unite-sequences (online sequences). If these online sequences accompany our own sequences in a clade, this is good evidence, supporting the identity of that clade. However, a clade purely consisting of online sequences is harder to interpret as we have less information about these sequences and no specimens for morphological analysis.

The single-locus ML analyses (figures 3.1, 3.2 and 3.3) showed no significant conflicts in topology. In the ITS-phylogeny (figure 3.1) *Lf*. sect. *Albati* has a bootstrap (BS) support of 100 and splits up in two large groups. (1) One group consists of specimens representing *Lf. vellereus* and *Lf. deceptivus* with a bootstrap of 100. The group of *Lf. vellereus* has a support of 99, splitting up in two clades (BS: 77 and 82) and one isolated GenBank-sequence (BS: 73). This sequence entirely branches off of the two clades and is coming from Honduras, which is strange as *Lf. vellereus* is distributed throughout Europe. Another sequence within one of the clades, also forms a long and isolated branch. This sequence belongs to a specimen that was found in a Chinese oil field. Both of these isolated specimens in the *Lf. vellereus*-group were obtained from GenBank and did not have a lot of information accompanying them. Another thing catching the eye is he placement of sequences coming from *Lf. vellereus* var. *hometii* specimens. Expected to group together, they are actually divided over both *Lf. vellereus*-clades.The group of *Lf. deceptivus* has a bootstrap value of 99 and splits up in four supported clades (BS: 80, 100, 96 and 100) and four supported, isolated species (BS: 91, 75, 77). Although two isolated species form a clade together, their branches are long enough two consider them as separated. Two of the isolated species are GenBank sequences, both of which were expected to group together with others, based on their origin.

[7] Not every PCR was successful so not every collection was represented by all three markers, explaining those short concatenated sequences.

(2) The other group consists of *Lf. subvellereus, Lf. bertillonii* and *Lf. pilosus* (BS: 91). One sequence, belonging to *Lf. subvellereus*, branches off of the entire group. The branch leading to the rest of Lf. subvellereus has a bootstrap value of 55, meaning it is not supported. However, this unsupported group splits up into four supported clades (BS: 98, 100, 100, 100). Lactifluus bertillonii forms a monophyletic group (BS: 83). One specimen however is put on a separate branch. This sequence, obtained from Unite, was expected to group together with two other Unite-sequences coming from the same location. Lastly, *Lf. pilosus* form one monophyletic clade (BS: 92).

The LSU-phylogeny (figure 3.2) also fully supports *Lf*. sect. *Albati* (BS: 100). Although the major topology does not match the ITS-topology, it mostly has very low support too and smaller groupings that do have full support also do match those of the ITS-phylogeny. One *Lf. bertillonii* sequence branches off at the very beginning, making it a sister to the rest of *Lf*. sect. *Albati*. The other two L*f. bertillonii* sequences group together but with a bootstrap support of 5 so nothing can be said about this. The branch leading to *Lf. pilosus* is not supported (BS: 60) but it splits up in two supported groups (BS: 77 and 96). What is strange however, in one *Lf. pilosus*-group, a GenBank-sequence occurs named *L. vellereus*. The given information tells us this sequence originates from a Japanese museum-specimen. A lot of Asian specimens however, are given European names in lack of better knowledge so this museum specimen most probably actually represents *Lf. pilosus* but was collected and determined before *Lf. pilosus* had been discovered. We can not know for sure unfortunately because we do not have morphological information on this specimen. It would be recommended to contact the owners of this specimen and inform them. The entire group of *Lf. subvellereus* specimens is not supported (BS: 16). It does however consist of three supported clades (BS: 100, 99, 73) that also match the ITS-topology and two species branching off but without support (BS: 24 and 60). The next clade in the LSU-tree consists of *Lf. vellereus* specimens and has full support (BS: 100) but it does not split up as in the ITS-tree. Lastly, the branch leading up to *Lf. deceptivus* specimens has a bootstrap support of 92. It splits up into two clades (BS: 100 and 49) and three separate branches with one specimen (BS: 53, 71, 71). The three supported branches (one group, two single specimens) match the topology of the ITS-tree.

The *rpb*2-phylogeny (figure 3.3) also has full support for *Lf*. sect. *Albati* (BS: 100). It directly splits up into two branches, one leading to *Lf. deceptivus* sequences and one leading to the rest of this section. The branch leading to *Lf. deceptivus* is not supported (BS: 48) but after one specimen branching off, the rest of the group has a bootstrap value of 98 further splitting up into three clades (BS: 82, 90, 53) and two single specimens (BS: 98 and 24). The supported branches match the topologies of both the ITS- and LSU-phylogenies. The *Lf. vellereus*-group is fully supported (BS: 99) and splits up into two clades (BS: 62 and 100). These two clades do not match those found in the ITS-tree however. The branch leading to the one *Lf. bertillonii*-sequence only has a bootstrap of 29. Next, the group of *Lf. subvellereus*-sequences is not supported (BS: 28) but it splits up in two supported clades (BS: 87 and 79) and one separate specimen (BS: 100), matching the subdivision of both previous gene trees. Last, the group of *Lf. pilosus*-specimens is fully supported (BS: 98) and, as in the LSU-tree but with different composition however, it splits up in two supported clades (BS: 99 and 79).

Although the topologies of the single-locus phylogenies do not entirely match, there are no significant conflicts. Any differences were either not supported or occurred in groups with not a lot of specimens. As mentioned above, in case there not much sequences to compare, the smallest difference in sequences can already cause them to diverge.

In the multi-locus ML analyses (figure 3.4), every clade but one has full support and matches the topology of the singe-locus phylogenies. What can be seen is that specimens determined in the field as *Lf. deceptivus* were split up into six different clades, conveniently given the working names *Lf. deceptivus* 1 to 6. *Lf. deceptivus* 1 however, only has a bootstrap of 35. The same goes for *Lf. subvellereus,* being split up into five clades with working names *Lf. subvellereus* 1 to 5. In other words, *Lf. deceptivus* consists of five lineages (with the sixth one not being supported) and *Lf. subvellereus* consists of five supported lineages. Specimens of *Lf. vellereus* group together with a bootstrap of 69. In both the ITS- and *rpb*2-phylogenies and the multi-locus phylogeny, there are some subclades appearing within the *Lf. vellereus*-group. However, a lot of the supposedly concatenated sequences actually consist of two sequences or even just one. Again giving the smallest difference to much weight. Sequences from *Lf. bertillonii* nicely group together in the multi-locus tree with a bootstrap of 92. Sequences of *Lf. pilosus* group together with a bootstrap of 99 with one small exception: there appears to be a specimen identified as *L. vellereus* in this clade. This is not a concatenated sequence however, as it only consists of an LSU-sequence. As already explained above, this Japanese GenBank-sequence most probably represents a *Lf. pilosus*-specimen (we can not know for sure off course unless we have morphological data too).

Species delimitation (fig. 3.5)

At least two sequences are needed for a successful analysis because *BEAST needs to be able to compare intra- and interspecific variability in order to delimit species. Three sequences representing separate phylogenetic species according to the single- and multi-locus phylogenies, *Lf. deceptivus* 5, *Lf. decepticus* 6 and *Lf. subvellereus* 2, were not included in the analysis for this reason. Most of the resulting clades in the tree are not supported, having posterior probabilities (pp) below 0,8. From the root, this tree splits up into a supported branch (pp= 0.86) leading to the four remaining Lf. deceptivus-clades and an unsupported branch leading to the rest of the included specimens. Within the latter group, after *Lf. vellereus* splitting off, the remaining group containing *Lf. bertillonii-i, Lf. subvellereus*- and *Lf. pilosus*-specimens is supported (pp=0,84). Consequently, the branch leading to Lf. pilosus is also supported (pp=0,84). Last, the branch leading up to the coupling of the lineages called *Lf. subvellereus* 1 and 3 is also supported (pp=0.99). To conclude, three lineages are supported with this analysis: *Lf. deceptivus* as a whole, *Lf. pilosus* and the group containing both *Lf. subvellereus* 1 and 3.

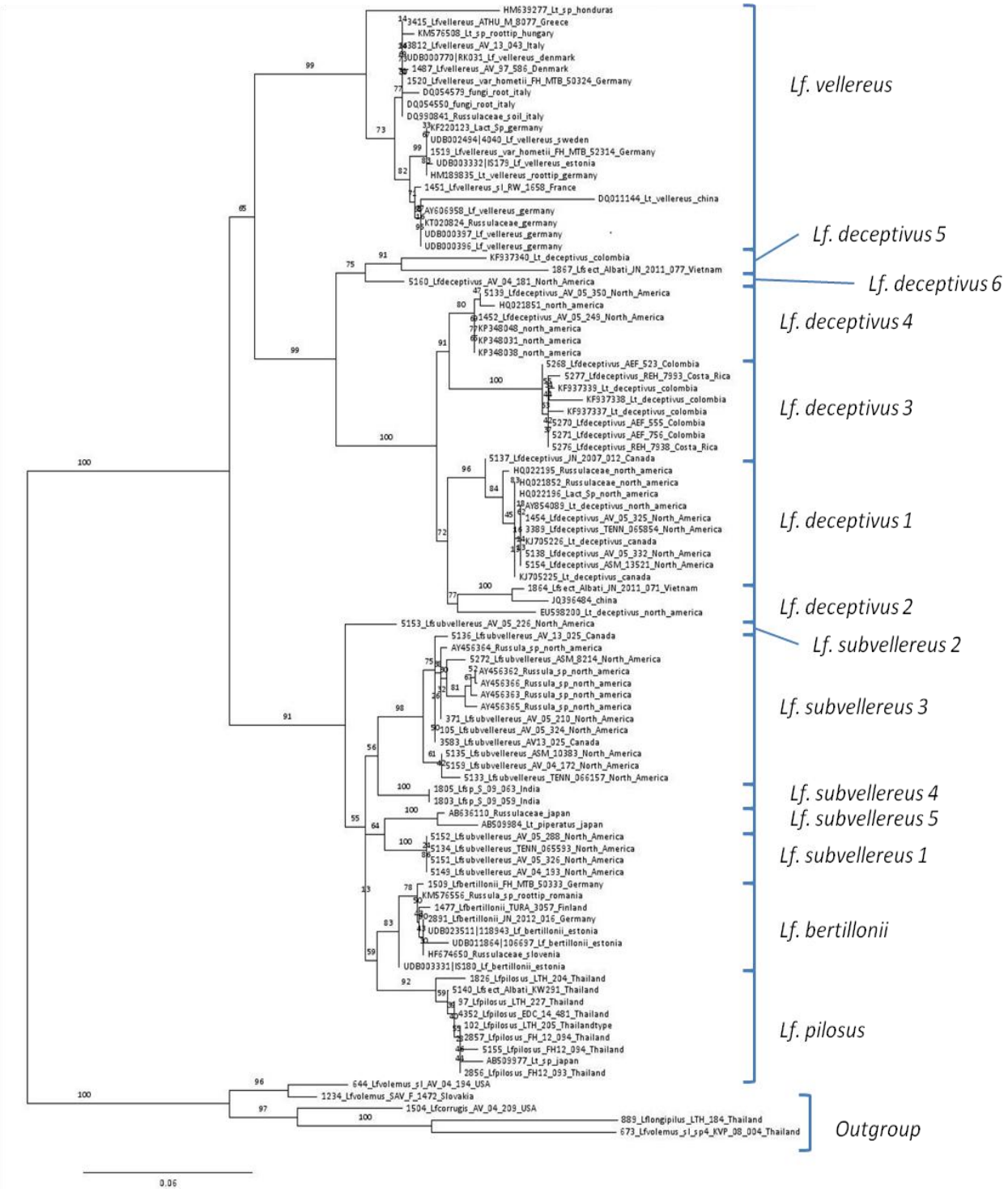

Fig. 3.1: ML single-locus tree of *Lf*. sect. *Albati* based on ITS-sequences

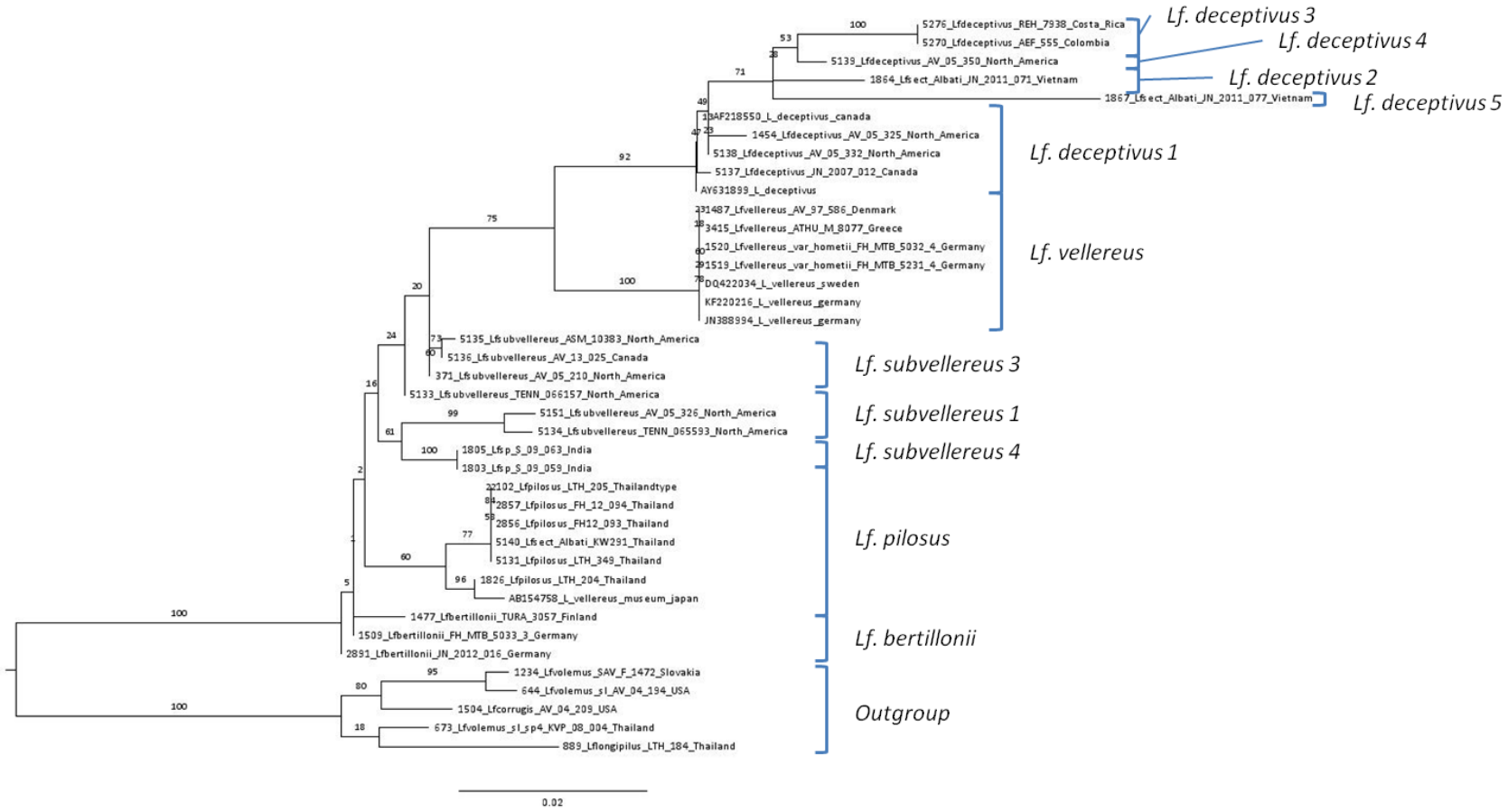

Fig. 3.2: ML single-locus tree of *Lf.* sect. *Albati* based on LSU-sequences

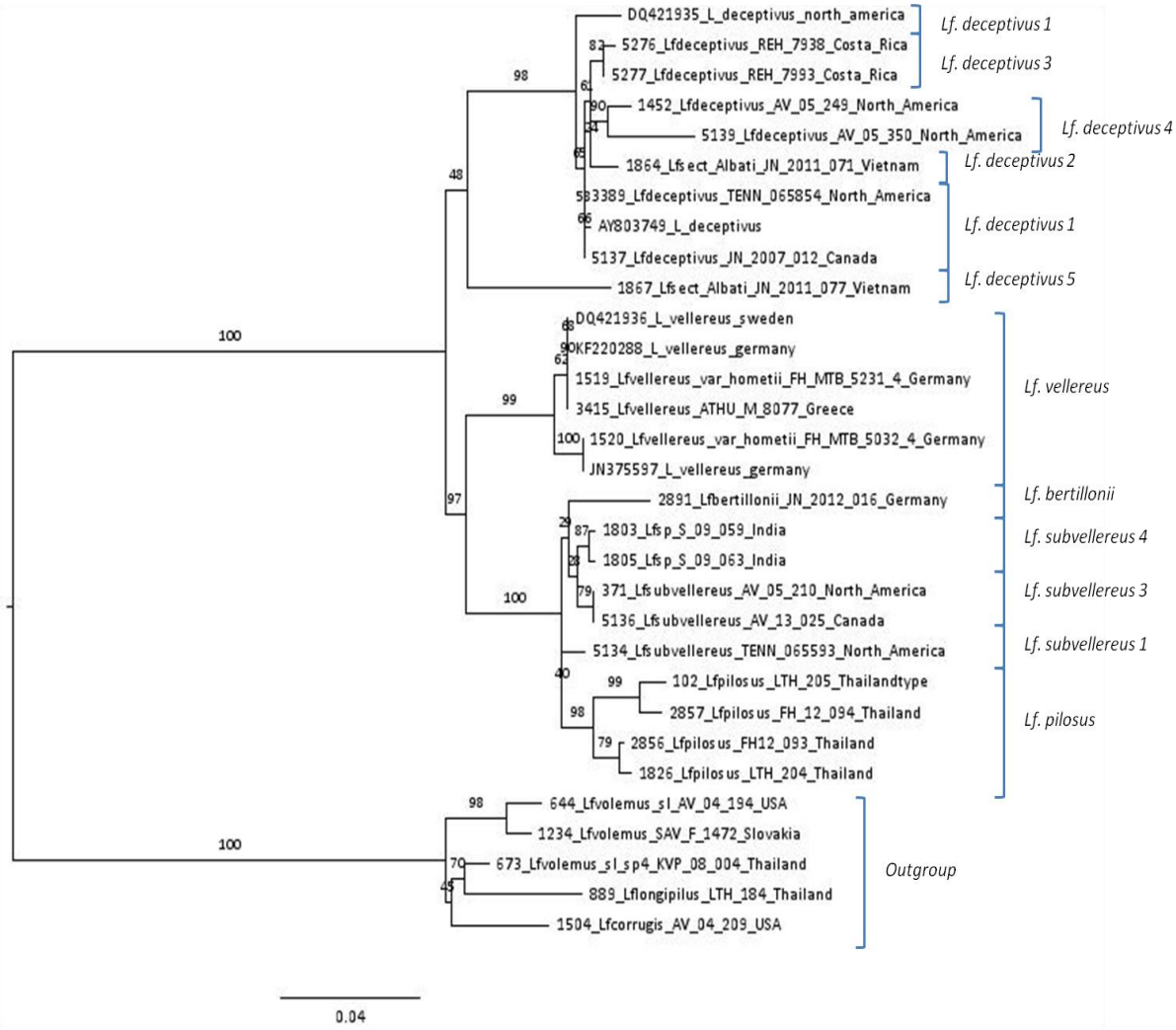

Fig. 3.3: ML single-locus tree of *Lf*. sect. *Albati* based on *rpb2*-sequences

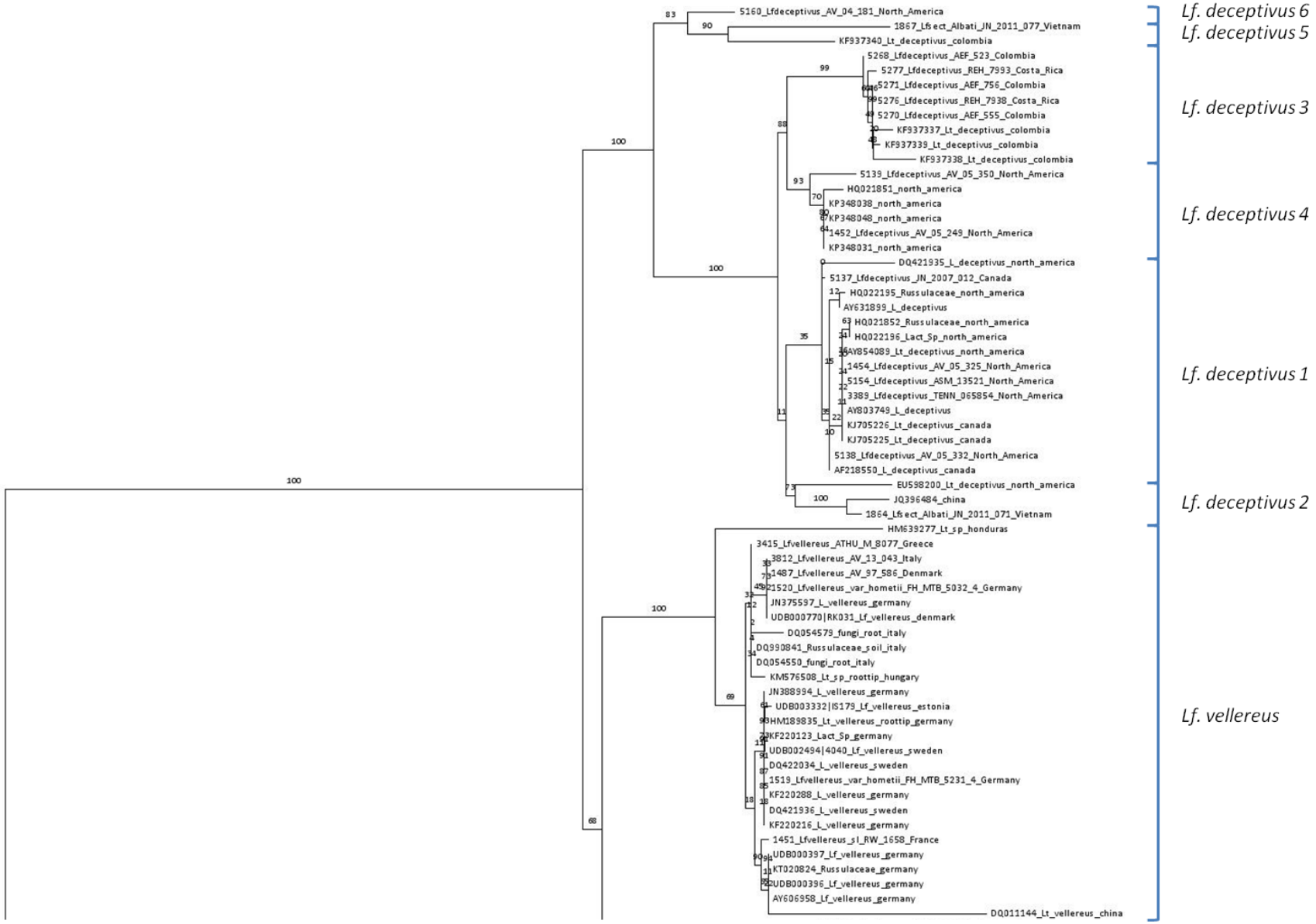

Fig. 3.4a: ML multi-locus tree of *Lf*. sect. *Albati* based on the concatenated data of the ITS-, LSU- and *rpb2*-sequences

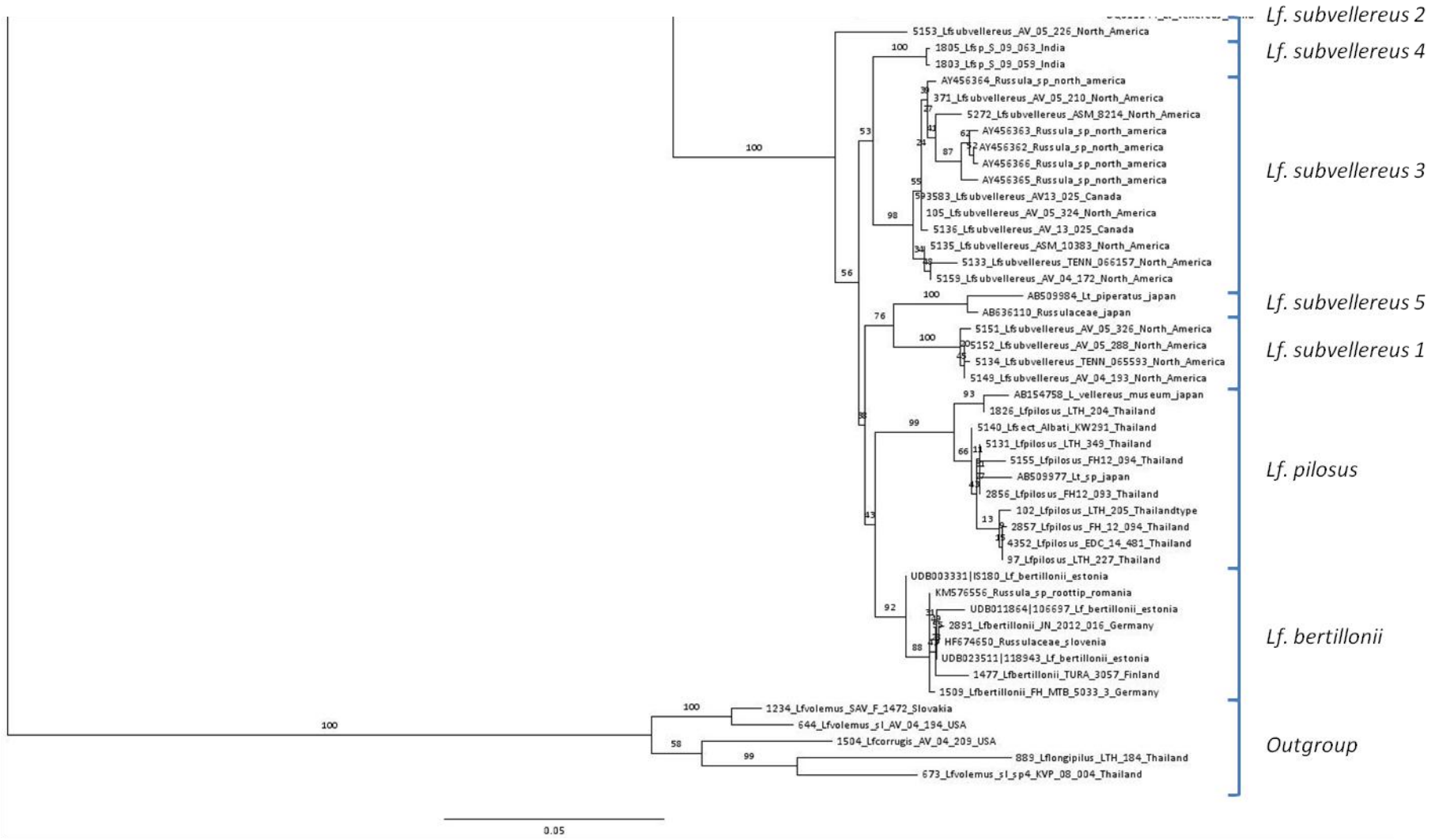

Fig. 3.4b: ML multi-locus tree of *Lf*. sect. *Albati* based on the concatenated data of the ITS-, LSU- and *rpb2*-sequences with bootstrap values on branches

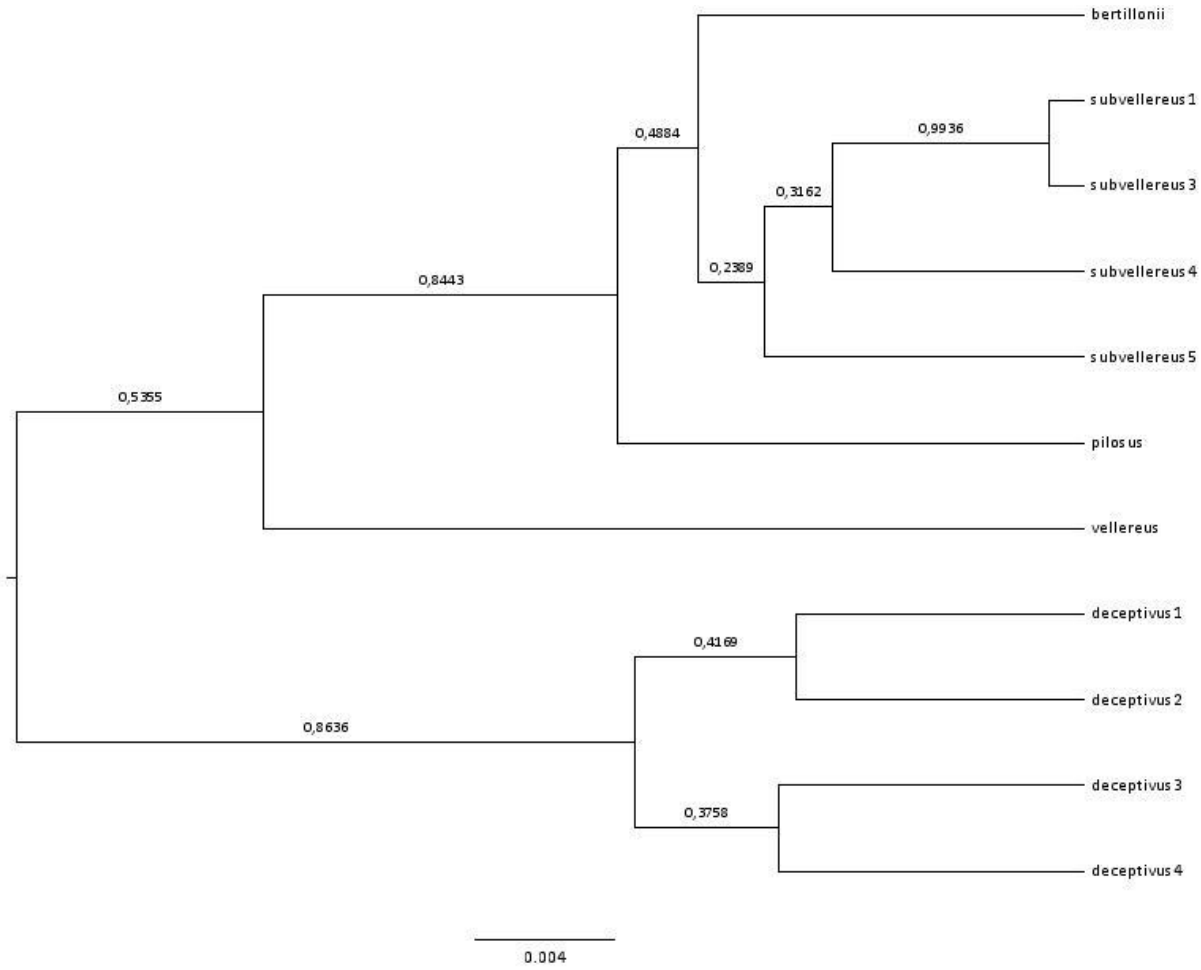

Fig. 3.5: Bayesian species delimitation tree from *BEAST with posterior probabilities on branches

Taxonomy

One collection that came out as a separate phylogenetic species, called *Lf. subvellereus 2*, was not examined because it was composed of a collection of very young specimens (see figure 3.4, herbarium number= AV05-226) that did not have developed spores yet. Another phylogenetic species, *Lf. subvellereus* 5 (see figure 3.4, GenBank-sequences AB509984 and AB636110), was not examined because it was represented by two sequences for which no collections were present to study and no morphological data was available.

Lactifluus deceptivus 1(fig. 3.6, fig. 3.12-a)

Basidiospores broadly ellipsoid to ellipsoid, 6.25–*8.15–10.94*–12.59 × (4.85–)4.93–*6.15–7.83*–8.81 µm (Q= 1.13–*1.30–1.40*–1.62, n=80), ornamentation up to 2 µm high, consisting of small warts, mostly connected by very faint and fine lines, plage not or faintly amyloid, large apiculus, spore deposit color unknown. **Basidia** 28–59 × (5–)11–17 µm, cylindrical to slightly subclavate, thin-walled, 4-spored**. Pleuromacrocystidia** very abundant, 40–63 × 4–12 µm, generally with needle-like contents, sometimes with granular contents or mixed contents, slightly moniliform, tapering upwards, acuminate, originating quite superficially, mostly quite emergent (up to 34 µm). **Cheilopseudocystidia** 3–5 µm wide, elusive (three found through three collections)**. Lamellae-edge** fertile but with few basidia, cheilomacrocystidia same as pleuromacrocystidia**. Gill trama** interwoven, lactiferous hyphae inconspicuous**. Pileipellis** a lamprotrichoderm of interwoven, thick-walled, septate hyphae of which some terminal (hyphoid) elements are slightly uplifted, 35–171(–256) × 4–7 µm**. Stipitipellis** also a lamprotrichoderm, 156–326 x 4–8 µm.

Ecology: Found in mixed forests (growing terrestrial in humus and moss) and *Sphagnum* L. bogs, both with *Betula* L.*, Abies* Mill.*, Picea* A. Dietr.*, Tsuga* (Endl.) Carrière, *Larix* Mill. and *Acer* L. -species.

Distribution: Known from North Carolina and New York, USA and Newfoundland, Canada.

Studied material: NORTH AMERICA – USA – North Carolina, Swain County, Heintoogard, N35°34.78'W83°10.99', alt. 1603 m, 15/08/2005, *A. Verbeken* 05–332 (GENT) – USA – New York, Black Pond, Adirondack Park, Franklin County, N44°25.933'W74°17.841', alt. 600m, terrestrial in humus and moss; *Betula, Abies, Picea, Tsuga* and *Acer* forest, 13/08/2011, *A.S. Methven* 13.521 (Eastern Illinois – dupl.) – CANADA – Newfoundland, Avalon Peninsula, Salmonier road (90), Salmonier NP, N47°15.782'W53°16.928', alt. unknown, *Sphagnum* bog with *Abies balsamea* (L.) Mill., *Larix* and some *Betula* species, 26/09/2007, *J. Nuytinck* 2007–012 (GENT).

Notes: macromorphological descriptions were unavailable

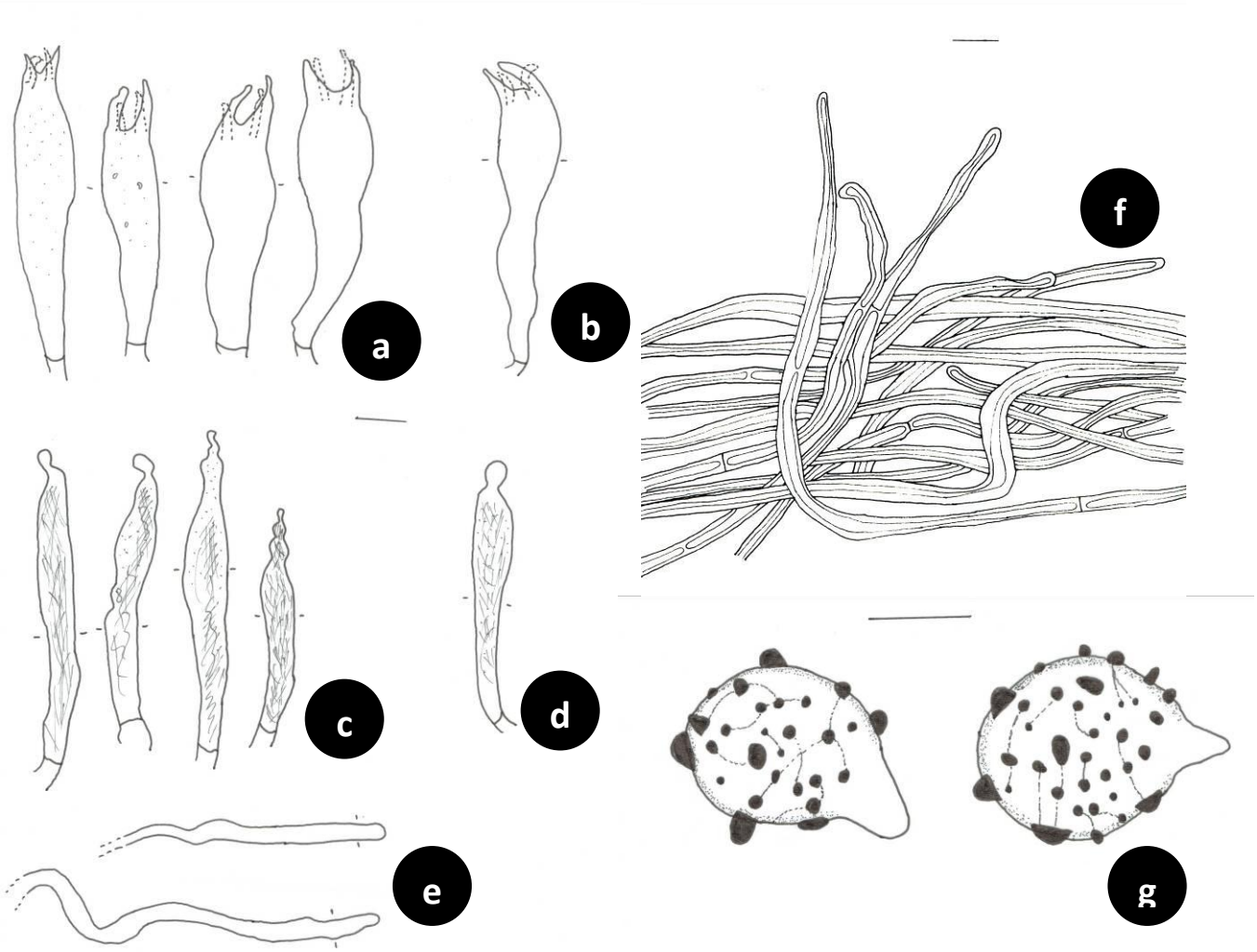

Figure 3.6 – *Lf. deceptivus* 1: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidia e. pseudocystidia f. pileipellis g. spores (scale bar= 10 µm)

Lactifluus deceptivus 2 (fig. 3.7, fig. 3.12-b)

Lamellae distant.

Basidiospores subglobose to ellipsoid, 8.66–*10.09*–11.53 × 6.63–*7.60*–8.58 µm (Q= 1.06–*1.33*–1.52, plage not amyloid, n=20), ornamentation up to 1.76 µm high, consisting of small to medium–sized warts and spines, some connected by very faint and fine lines, large apiculus, spore deposit color unknown. **Basidia** 33–49 × 11–13 µm, cylindrical to slightly subclavate, exceptionally large sterigmata, up to 18 µm, thin-walled, 4-spored**. Pleuromacrocystidia** 27–51 × 5–9 µm, generally with needle-like contents, sometimes with granular or oil-like contents and sometimes with mixed contents, slightly moniliform, tapering upwards, acuminate, not abundant**. Pleuroseudocystidia** 2–4 µm wide, typical pseudocystidia**. Lamellae-edge** fertile but with few basidia, basidioles abundant, cheilomacrocystidia same as pleuromacrocystidia**.**

Pileipellis a lamprotrichoderm of interwoven, thick-walled, septate hyphae of which some terminal (hyphoïd) elements are slightly uplifted 184–213 x 5–6 µm**.**

Ecology: Found in *Pinus kesiya* Royle ex Gordon dominated forest.

Distribution: Known from Vietnam.

Studied material: ASIA – VIETNAM – Bi Dup Nui Ba National Park, Huyen Lac Duong, Dalat city, near Tram Kiem Lam Giang Ly, N12°10.480'E108°41.469', alt. 1474 m, *Pinus kesiya* dominated forest, 16/06/2011, *J. Nuytinck* 2011–071 (GENT).

Notes: macromorphological descriptions were unavailable, no stipe present in collection in good enough state to analyze stipitipellis. Exceptionally large sterigmata.

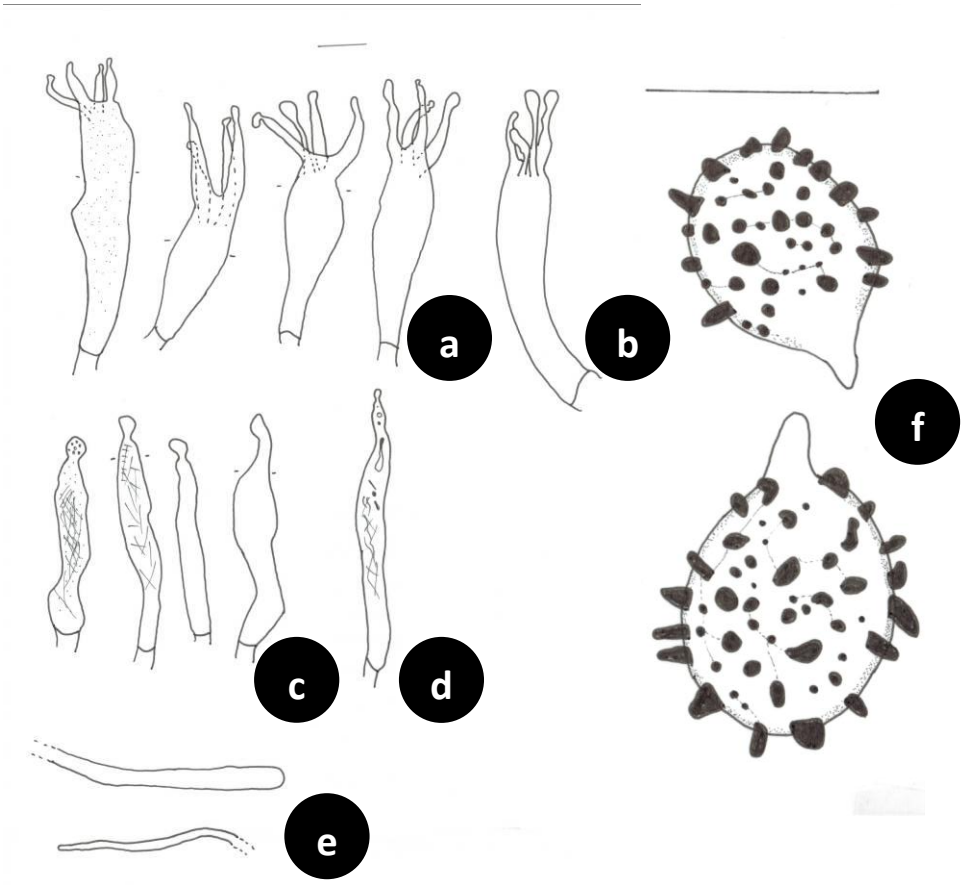

Figure 3.7 – *Lf. deceptivus* 2: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidium e. pseudocystidia f. spores (scale bar= 10 µm)

Lactifluus deceptivus 3 (fig. 3.8, fig. 3.12-c)

Lactifluus hallingi De Wilde *nom. prov.*

Diagnosis: a large-sized white species, greatly resembling *Lf. deceptivus*. Macroscopically it is defined by a white, firm pileus with dry surface, pale orange a first, turning brownish orange at the center and a white stipe also turning brownish. The milk sometimes stains tissues pinkish. Microscopically this species has a lamprotrichoderm as pileipellis with longer hairs than *Lf. deceptivus*, (broadly) ellipsoid spores consisting of warts of which some are finely connected.
Etymology: a reference to R.E. Halling, the first collector of this species.
Holotypus: SOUTH AMERICA – COLOMBIA – DEPT. Antioquia: Municipo de Santa Rosa de Osos, vereda La Pulgarina, coordinates unknown, alt. unknown, Woods; *Quercus humboltii*, 27/04/1991, A.E. Franco–Molano 555 (NY BOT GARDEN)

Pileus 4-12(24) cm broad, infundibuliform, margin involute, surface dry, matted tomentose at first, eventually fibrillose to recurved scaly at disc, cream or pale orange (5A3) at first, then browner near brownish orange (6C6) at disc and paler (whitish) toward margin, cottony roll of tissue at margin. **Lamellae** subdistant, subdecurrent to decurrent, edge sharp and even. **Stipe** 3-5 x 1,1-3,5 cm, white, staining brownish where injured, sometimes curved, surface dry, centrally attached to pileus. **Context** firm and thick, very hard but brittle. **Latex** abundant, white, staining tissues pinkish to brownish eventually, taste very acrid.
Basidiospores broadly ellipsoid to ellipsoid, (7.90–)7.91–*9.55*–*10.12*–11.56(–12.06) × 5.96–*7.07*–*8.05*–9.26(–9.34) µm (Q= 1.13–*1.26*–*1.36*–1.61, n=60), ornamentation up to 1.39 µm high, consisting of small warts, mostly isolated but some connected by very faint and fine lines, plage not to very faintly amyloid, large apiculus, spore deposit color unknown. **Basidia** 36–59 × 8–13 µm, cylindrical to slightly subclavate, thin-walled, 4-spored**. Pleuromacrocystidia** 36–68 × 4–10 µm, generally with needle-like contents, sometimes with granular contents or mixed contents, sometimes slightly moniliform (mostly one constriction at apex), tapering upwards, acuminate, originating quite superficially, mostly quite emergent (up to 25 µm). **Pleuropseudocystidia** 2–4 µm wide, typical pseudocystidia, rather abundant**. Lamellae-edge** fertile but mostly consisting of basidioles and cheilomacrocystidia, same as pleuromacrocystidia**. Gill trama** interwoven, lactiferous hyphae slightly inconspicuous**. Pileipellis** a lamprotrichoderm of interwoven, thick-walled, septate hyphae of which some terminal (hyphoïd) elements are slightly uplifted, 98–306 × 3–6 µm**. Stipitipellis** also a lamprotrichoderm, 209–284 x 6–8 µm.
Ecology: Found on soil in woods with *Quercus humboltii* Bonpl.*, Q. seemanii* Liebm. & *Q. copeyensis* C.H. Mull.

Distribution: Known from Colombia and Costa Rica (very common in Talamanca mountains).
Studied material: SOUTH AMERICA – COLOMBIA – DEPT. Antioquia: Municipo de Santa Rosa de Osos, vereda La Pulgarina, coordinates unknown, alt. unknown, Woods; *Quercus humboltii*, 27/04/1991, A.E. Franco–Molano 555 (NY BOT GARDEN – loan) – COLOMBIA – DEPT. Antioquia: Municipio de San Pedro, vereda El Chaquiro, finca la Espanola, coordinates unknown, alt. 2700 m, Woods; *Quercus humboltii*, 12/06/1990, A.E. Franco–Molano 523 (NY BOT GARDEN – loan) – COLOMBIA – DEPT. Antioquia: Municipia Santa Rosa de Osos,corregimiento de Aragon, vereda El Quince, N0°49’ W75°8.2’, alt. 256 m, Woods; *Quercus* species, 17/06/1991, A.E. Franco–Molano 756 (NY BOT GARDEN – loan) –

CENTRAL AMERICA – COSTA RICA – San José: Canton Dota, San Gerardo. Albergue de la Montaña, Savegre, 5km SW of Cerro de la Muerte, N 9°33.036' W 83° 48.450', alt. 2200 m, *Quercus seemanii* & *Q. copeyensis*; On soil, 26/06/2000, R.E. Halling 7938 (NY BOT GARDEN – loan) – COSTA RICA – San José: Canton Dota, Jardin, 3,5km W of Empalme, N 9°42.864' W 83°58.464', alt. 2220 m, 7/08/2000, R.E. Halling 7993 (NY BOT GARDEN – loan)

Notes: the picture was not taken from a collection examined here but from a morphologically similar collection from same location (REH 4977). Latex staining tissues pinkish not observed in other clades. Pileipellis hairs grow longer (longest hairs at least 40 µm longer) than in any other clade. Central/South American distribution unique for *Lf*. sect. *Albati*, possibly isolated.

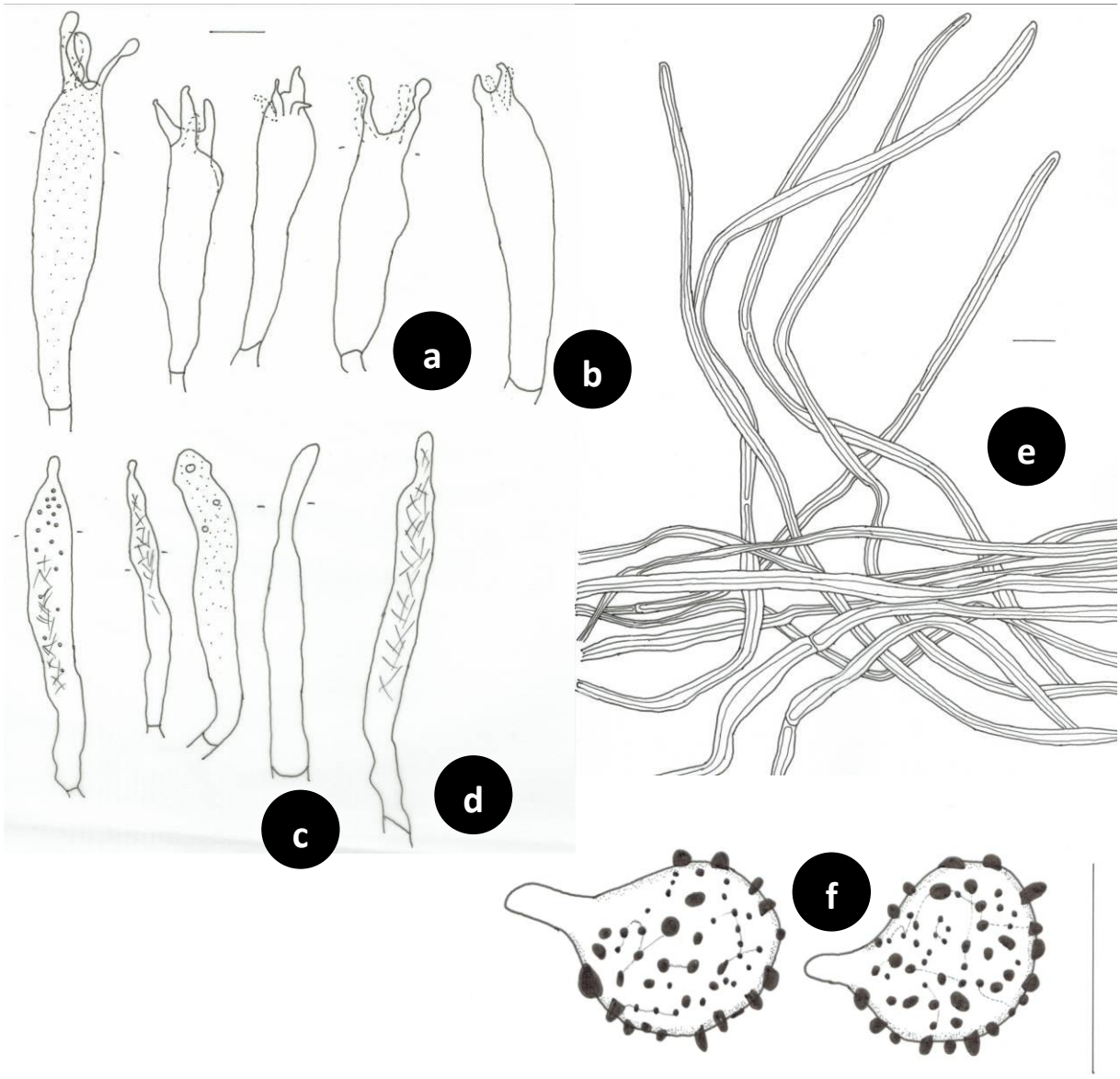

Figure 3.8– *Lf. hallingi*: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidia e. pileipellis f. spores (scale bar= 10 µm)

Lactifluus deceptivus 4 (fig. 3.9, fig. 3.12-d)

Pileus 7–9cm broad, convex to infundibuliform, cream-coloured, locally warm beige or pale yellow, feeling cottony soft, (becoming) locally fibrillose or torn into patches, entirely covered by a spider web-like cottony layer but not always visible, locally appearing smooth and felty, margin involute with a cottony layer which may partially cover the lamellae, peels off up to center. **Lamellae** white (3AZ or even whiter), medium tan when dried, adnate to decurrent, dense (11L+l/cm), sometimes forked, lamellulae of different lengths but mostly one between every two lamellae. **Stipe** 5–6 × 2–2.5 cm, white, pale brown at base, staining locally yellowish when bruised, smooth, cylindrical, square basis. **Context** firm and thick, compressible in pileus, white, slightly yellowish when cut, tastes very acrid, smell agreeable, fruity, lemon-like, immediately turning blue with gaiac, immediately turning salmon with $FeSO_4$. **Latex** abundant, white, unchanging but staining lamellae dark buff to pale brown, unchanging with KOH, taste acrid.

Basidiospores broadly ellipsoid to ellipsoid, 8.78–*10.17*–*10.83*–11.98(–12.09) × 6.02–*7.32*–*8.16*–8.93(–9.18) µm (Q= 1.22–*1.33*–*1.40*–1.64, n=40), ornamentation up to 1.6 µm high, consisting of small to medium-sized warts and spines, mostly isolated, few connected by very faint and fine lines, plage (very faintly) amyloid, large apiculus. **Basidia** 34–53 × 10–15 µm, mostly cylindrical, seldom slightly subclavate, thin-walled, 4-spored**. Pleuromacrocystidia** very abundant 35–68 × 5–13 µm, generally with needle-like contents, sometimes with granular contents or mixed contents, sometimes slightly moniliform (mostly one constriction at apex), tapering upwards, acuminate, originating quite superficially, mostly quite emergent (up to 25 µm)**. Cheilopseudocystidia** 2–5 µm wide**. Lamellae-edge** fertile but mostly consisting of basidioles and cheilomacrocystidia, basidia slightly shorter, cheilomacrocystidia same as pleuromacrocystidia**. Gill trama** interwoven, lactiferous hyphae slightly inconspicuous**. Pileipellis** a lamprotrichoderm of interwoven, thick-walled, septate hyphae of which some terminal (hyphoïd) elements are slightly uplifted, 156–266 × 4–6 µm**. Stipitipellis** also a lamprotrichoderm, 233–400 x 5–6 µm.

Ecology: Unknown

Distribution: Known from North Carolina, USA.

Studied material: NORTH AMERICA – USA – North Carolina, Cataloochee, Caldwell Fork Trail, N35°37.89' W83°05.31', alt. 807 m, 12/08/2005, *A. Verbeken* 05–249 (GENT) – USA – North Carolina, Swain County, Kephart Prong Trail, N35°35.14' W83°21.51', alt. 869 m, 17/08/2005, *A. Verbeken* 05–350 (GENT)

NOTES: no information available regarding ecology.

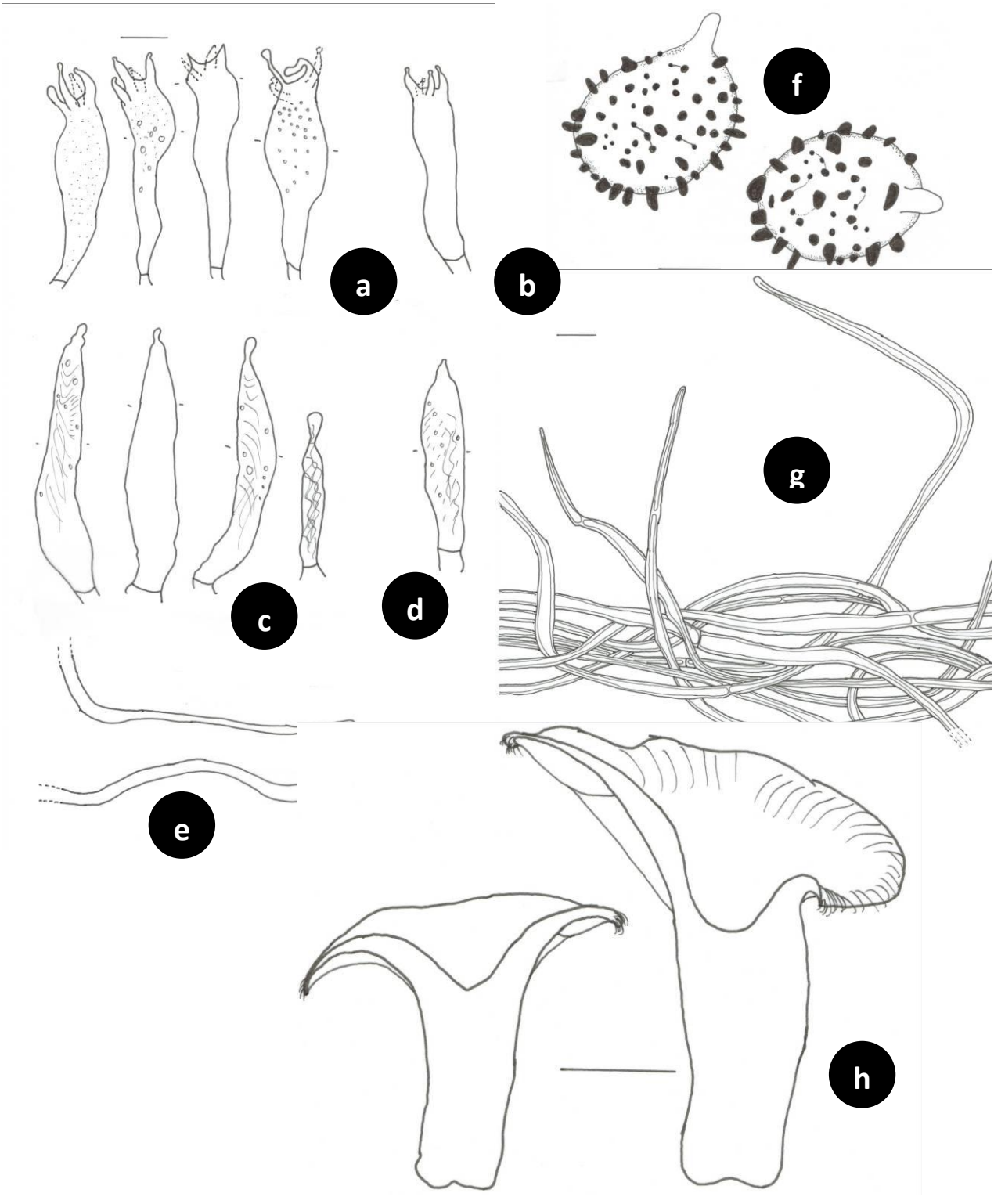

Figure 3.9 – *Lf. deceptivus* 4: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidia e. pseudocystidia f. spores g. pileipellis (scale bar= 10 µm) h. basidiocarps (scale bar= 2,5 cm)

Lactifluus deceptivus 5 (fig 3.10, fig. 3.12-e)

Basidiospores 6.39–*7.75*–9.13 × 5.20–*6.42*–7.64 µm (n=20), subglobose to ellipsoid, Q= 1.11–*1.21*–1.37, plage not amyloid, ornamentation up to 1.59 µm high, consisting of small to warts and spines, some connected by very faint and fine lines, large apiculus, spore deposit color unknown. **Basidia** 32–47 × 9–13 µm, cylindrical to slightly subclavate, thin-walled, 4-spored**. Pleuromacrocystidia** 43–53 × 6–8 µm, generally with needle-like contents, sometimes with granular or oil-like contents and sometimes with mixed contents, slightly moniliform, tapering upwards, acuminate**. Pleuropseudocystidia** 3–4 µm wide, typical pseudocystidia, hard to find**. Lamellae-edge** fertile but with less, slightly shorter basidia, basidioles abundant, cheilomacrocystidia same as pleuromacrocystidia but slightly shorter. **Pileipellis** a lamprotrichoderm of interwoven, thick-walled, septate hyphae of which some terminal (hyphoid) elements are slightly uplifted, 198–257 × 6–7 µm**. Stipitipellis** also a lamprotrichoderm, 184–254 x 5–7 µm.
Ecology Found in *Pinus kesiya* dominated forest.
Distribution: Known from Vietnam.
Studied material: ASIA – VIETNAM – Bi Dup Nui Ba National Park, Huyen Lac Duong, Dalat city, near Tram Kiem Lam Giang Ly, N12°10.480'E108°41.469', alt. 1474 m, *Pinus kesiya* dominated forest, 16/06/2011, *J. Nuytinck* 2011–077 (GENT).
Notes: macromorphological descriptions were unavailable.

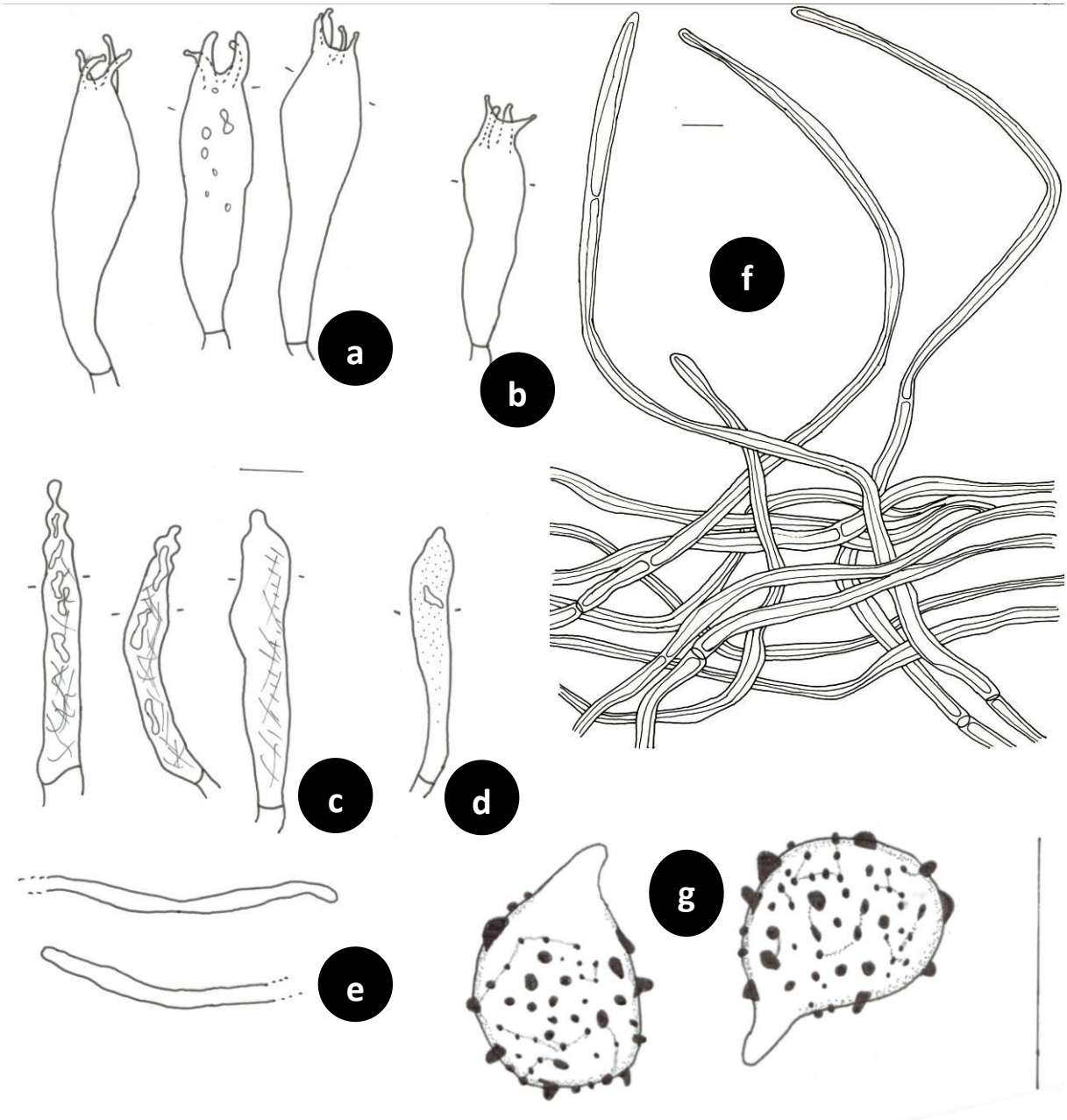

Figure 3.10 – *Lf. deceptivus* 5: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidium e. pseudocystidia f. pileipellis g. spores (scale bar= 10 µm)

Lactifluus deceptivus 6 (fig. 3.11)

Pileus 6–10 cm broad, slightly infundibuliform, pale yellow to dirty brownish yellow, locally whitish, smooth, soft, irregularly fissured when older (even somewhat squamulose), margin white, cottony soft, peeling off, showing a striate underlaying part, pilangiocarpic when young (not growing into stipitipellis but touching it). **Lamellae** white, staining dirty pinkish by latex, moderately distant, 9 L+l/cm, furcations rather common but especially in half closer to stipe, edge entire, concolorous. **Stipe** 4–7 x 2–3 cm, white, slightly cottony, smooth**. Context** white, thick, fleshy and firm in pileus, solid in stipe, taste acrid, smell sweetish acrid, staining salmon orange with $FeSO_4$. **Latex** white, abundant, acrid, unchanging with KOH, drying pinkish**.**

Basidiospores 5.94–*6.99*–8.04 × 4.57–*5.32*–6.07 µm (n=20), subglobose to ellipsoid, Q= 1.13–*1.32*–1.64, plage not amyloid, ornamentation very low, consisting of small warts and spines, many connected by fine lines forming an incomplete reticulum, spore deposit color unknown. **Basidia** 39–53 × 8–10 µm, cylindrical to slightly subclavate, thin-walled, 4-spored**. Pleuromacrocystidia** 46–77 × 5–7 µm, generally with needle-like contents, sometimes with granular or oil-like contents, slightly moniliform, tapering upwards, acuminate**. Pleuropseudocystidia** very hard to find, one was observed**. Lamellae-edge** fertile cheilomacrocystidia same as pleuromacrocystidia**. Pileipellis** a lamprotrichoderm of interwoven, thick-walled, septate hyphae of which some terminal (hyphoïd) elements are slightly uplifted, 250–269 × 7 µm. **Stipitipellis** also a lamprotrichoderm, 353–431 x 7 µm.

Ecology Found in mixed forest.

Distribution: Known from Tennessee, USA.

Studied material: NORTH AMERICA – USA – Great Smoky Mountains National Park, Serier County, near Cosby, Greenbrier section, Cascade trail, N35°41.22'E83°23.86', alt. 709 m, mixed forest, 13/07/2004, *A. Verbeken* 04–181 (GENT).

Notes: longest stipitipellis hairs at least 31 µm longer in comparison with other clades, very small spores: 1µm less in both length and width compared to second smallest collection (may be due to young age of specimens however) with most connective lines, almost forming a reticulum. No pictures available.

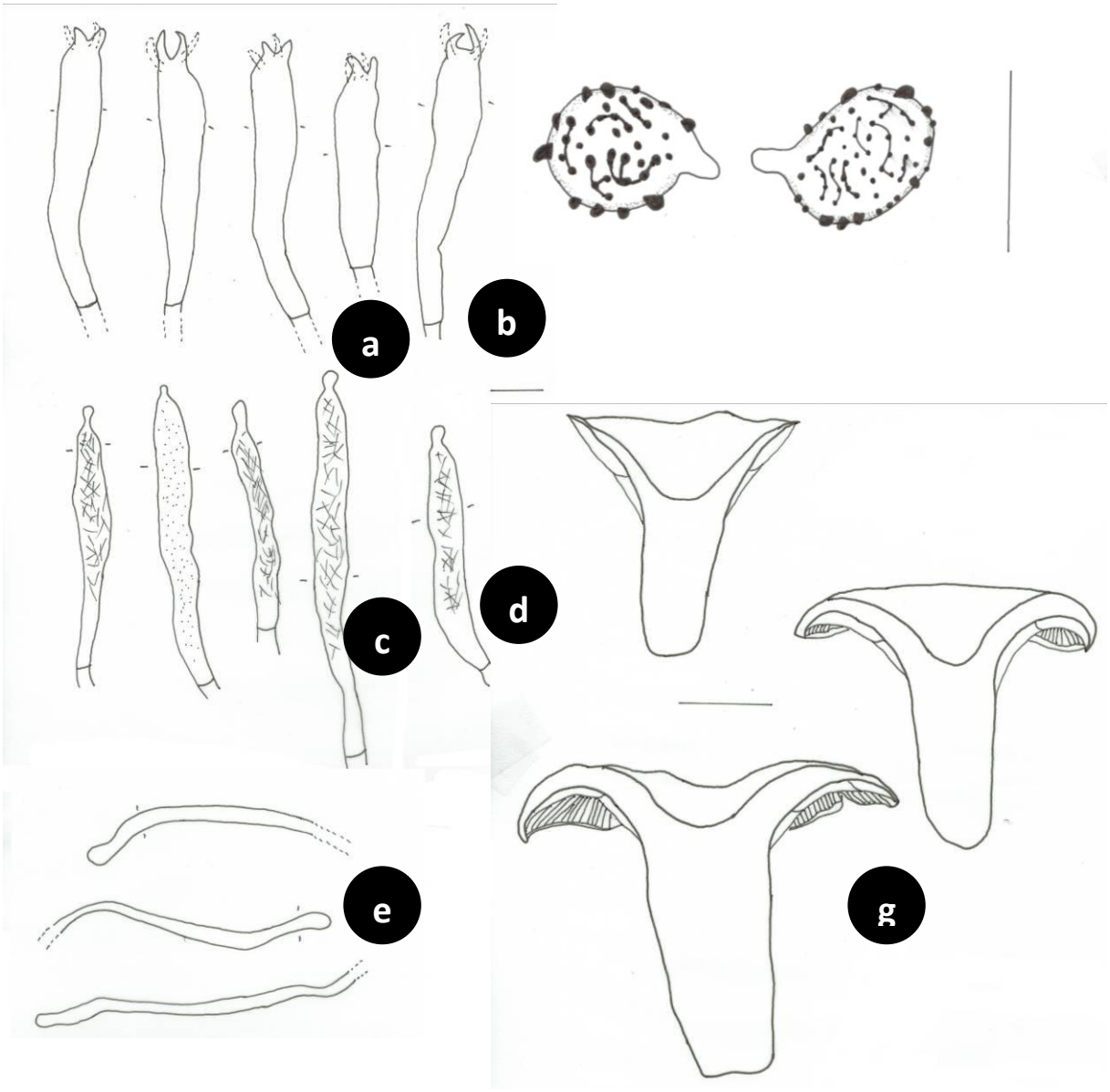

Figure 3.11 – *Lf. deceptivus* 6: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidium e. pseudocystidia f. spores (scale bar= 10 µm) g. basidiocarps (scale bar= 2,5 cm)

Fig. 3.12 Basidiocarps of *L.* sect. *Albati* species. a: *deceptivus* 1 (AV 05-332), b: *deceptivus* 2 (JN 2011-071), c: deceptivus 3 (REH 4977), d: *deceptivus* 4 (AV 05-249), e: *deceptivus* 5 (JN 2011-077), f: *subvellereus* 3 (AV 13-025)

Lactifluus subvellereus 1(fig. 3.13)

Pileus 7.5–10.5 cm broad, slightly infundibuliform, irregularly curving margin, surface soft, woolly, cream-coloured to pale yellow with locally some shades of pinkish grey (not much)**. Lamellae** sometimes branching, strongly intervenose veins. **Stipe** 5–6 × 1.5–2 cm**. Context** turning light lemony yellow when cut, smells fruity, acidic, like *Melissa officinalis* L. (balm mint) or rotting lemons, turning immediately blue with gaiac, turning salmon with $FeSO_4$. **Latex** white, drying pale to hardly yellow when isolated, distinctive yellow when drying on lamellae, unchanging with KOH, burning acrid.
Basidiospores broadly ellipsoid to ellipsoid, 5.92–*7.02–8.24*–9.81 × 4.34–*5.42–5.88*–7.14(–7.34) µm (Q= 1.09–*1.27–1.42*–1.62, n=80), ornamentation up to 1.13 µm, consisting of fine, small warts, some isolated, some connected by faint and fine lines, plage inamyloid, spore deposit color unknown, small apiculus. **Basidia** 36–65 × 6–12 µm, mostly cylindrical, some slightly subclavate, some basidioles with refractive edge, thin-walled, 4-spored**. Pleuromacrocystidia** with coarse, needle-like and/or granular contents, mostly deeper in hymenium, best seen near edge, fusoid to acuminate with constrictions near apex, sometimes slightly moniliform**. Pseudocystidia** 2.5–4 µm wide, typical pseudocystidia, very few (one found in hymenium, one near edge through three collections)**. Lamellae-edge** fertile but with less basidia, cheilomacrocystidia same as pleuromacrocystidia but sometimes slightly more emergent and present in larger numbers. **Gill trama** mostly consisting of hyphae, no rosettes observed, lactiferous hyphae conspicuous especially near edge. **Pileipellis** a lamprotrichoderm of narrow, interwoven, thick-walled hyphae sometimes ending in more or less erect, thick-walled, septate, unbranched hyphoïd elements, 182–306 × 3–4 µm, sometimes accompanied by shorter elements containing a granular content (ends of lactiferous hyphae/pseudocystidia).
Ecology: Found in mixed forests.
Distribution: Known from Tennessee and North Carolina, USA.
Studied material: NORTH AMERICA – USA –Tennessee, Serier County, GSMNP, near cosby, Greenbrier section, Cascade trail, N35°41.22'W83°23.86', alt. 709 m, mixed forest, 13/07/2004, *A. Verbeken* 04–193 (GENT) – Tennessee, Cocke County, Madron Bald Trail, N35°46.17' W83°16.01', alt. 564 m, 14/08/2005, *A. Verbeken* 05–288 (GENT) – USA –North Carolina, Swain County, Heintoogard, N35°34.78'W83°10.99', alt. 1603 m, 15/08/2005, *A. Verbeken* 05–326 (GENT) – USA – North Carolina, standing indian campground, Kimsey creek trail, Franklin, Macon county, N 35°4.550' W 83°31.702', alt. 1100 m, 19/07/2011, K.W. Hughes, TENN 065593 (HERBARIUM TENN – loan).
Notes: spore ornamentation slightly higher (up to 0.50 µm) than *subvellereus* 3. No pictures available.

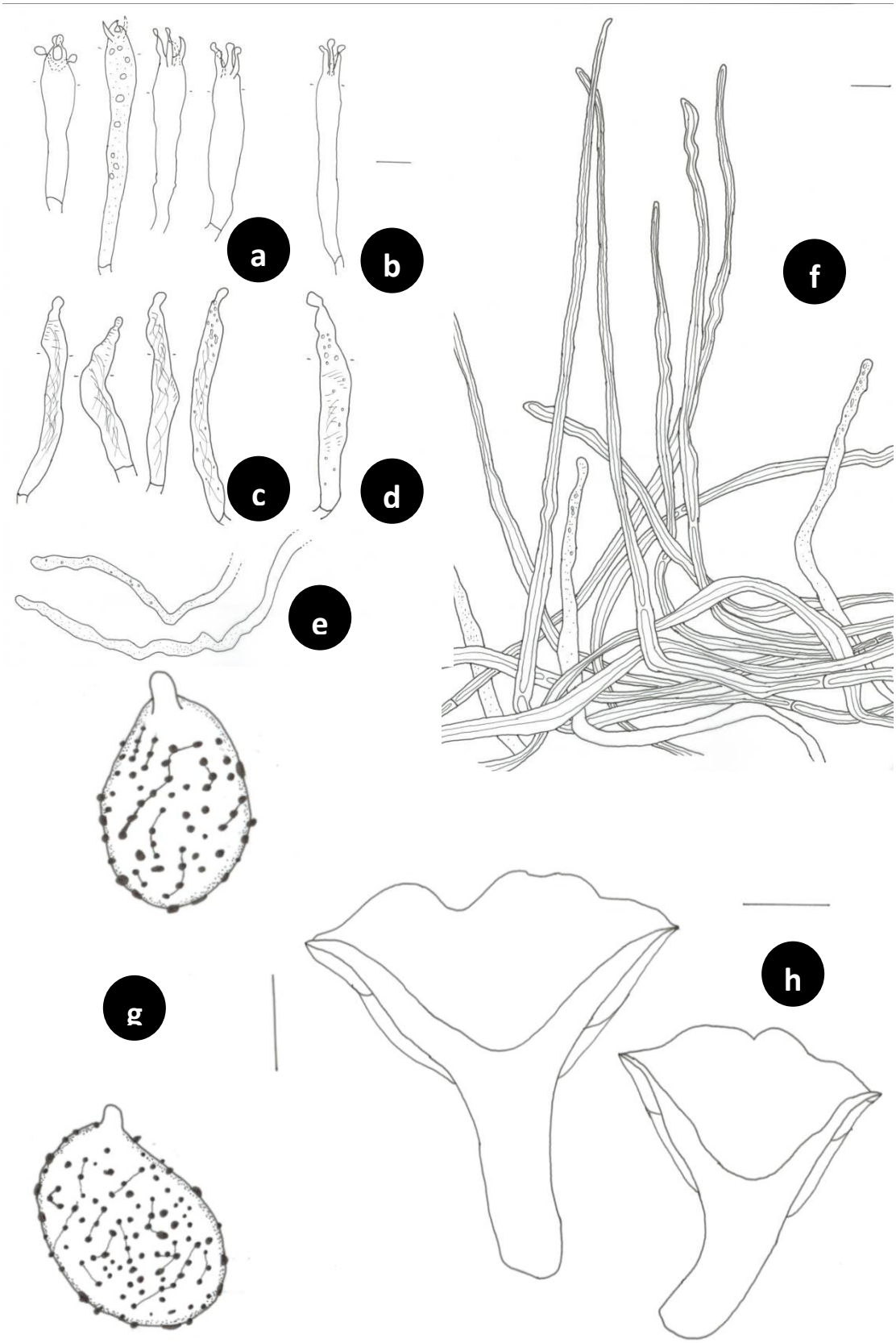

Figure 3.13 – *Lf. subvellereus* 1: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidium e. pseudocystidia f. pileipellis g. spores (scale bar= 10 µm) g. basidiocarps (scale bar= 2,5 cm)

Lactifluus subvellereus 3 (fig. 3.12-f, fig. 3.14)

Pileus 6.5–17 cm broad, firm, irregularly depressed to planoconvex, widely infundibuliform, margin irregularly bent downwards or involute then widely V-shaped but becoming straighter, sometimes locally grooved or crenulate. White to cream-coloured (4AZ), pruinose, velvety, (finely) woolly, dry, feels like chamois-leather, sometimes with aerolate pustules at margin, locally with round, woolly hairs, locally more beige or with grayish pink or flesh coloured areas, sometimes woolly areas almost pure white. **Lamellae** abundant to very distant, often regular long-short pattern, slightly decurrent to broadly adnate sometimes abruptly ending with the cottony cover of the stipe taking over, in others where the stipe is smoother subdecurrent, brittle and thick, cream-coloured (3AZ), staining yellowish to pale brownish by milk or after bruising, edge same colour, 3–8 L+l/cm (halfway).
Stipe 3.5–6 x 1.5–3 cm, short, cylindrical, centrally attached, sometimes tapering downwards, smooth, velvety and white to cream-coloured like pileus but with more buff spots, also grayish lilac spots in older specimens, yellowish zone right under lamellae in younger specimens. **Context** thick and firm, white but turning pale to bright sulfurish yellow when cut, taste burning acrid, smells fruity, almost lemony, turning bright to golden yellow with KOH, turning salmon with $FeSO_4$, immediately dark blue with gaiac. **Latex** abundant, white, turning cream to (pale) yellow when dried, taste burning acrid when isolated from flesh, no reaction with KOH when isolated from flesh. **Basidiospores** broadly ellipsoid to ellipsoid, 5.72–*6.85–7.64*–8.63(–8.64) × 4.09–*5.02–5.78*–6.77(–6.87) µm (Q= 1.13–*1.33–1.44*–1.74, n=100), ornamentation up to 1.4 µm, consisting of isolated fine, small warts, some connected by faint and fine lines, plage mostly inamyloid, sometimes with a very faint round marking, small apiculus, spore deposit color unknown. **Basidia** 46–69 × 8–11 µm, often subclavate, four-spored, thin-walled**. Pleuromacrocystidia** 38–106 × 4–9 µm, with coarse, needle-like contents, sometimes also granular and/or oil-like, mostly deeper in hymenium, best seen near edge, fusoid to acuminate with constrictions near apex, slightly moniliform**. Pleuropseudocystidia** 4µm wide, extremely elusive (only one was found throughout five collections)**. Lamellae-edge** fertile but with few and slightly smaller basidia, cheilomacrocystidia same as pleuromacrocystidia but sometimes slightly more emergent and present in larger numbers. **Gill trama** mostly consisting of hyphae, no rosettes observed, lactiferous hyphae inconspicuous. **Pileipellis** a lamprotrichoderm of narrow, interwoven, thick-walled hyphae with some ending in more or less erect, thick-walled, septate, unbranched hyphoïd elements, 181–391(–450) × 3–5 µm, sometimes accompanied by shorter elements containing a granular content (ends of lactiferous hyphae/pseudocystidia).
Ecology: Found in mixed forests with *Quercus* L., *Pinus* L., *Pseudotsuga* Carrière, *Acer* and *Liriodendron* L. species
Distribution: Known from Tennessee and Nort Carolina, USA and Montréal, Canada.
Studied material: NORTH AMERICA – USA –Tennessee, Cock County, GSMNP, Maddron Bald Trail, between Gabes Mountain Trail & Albright Grove, N35°45.35'W83°16.32', alt. 777m, mixed forest with *Quercus*, *Pinus*, *Pseudotsuga*, *Acer*, *Liriodendron* species etc., 12/07/2004, *A. Verbeken* 04–172 (GENT) – Ibidem, mixed deciduous–coniferous forest; scattered to gregarious; terrestrial in humus, 12/07/2004, *A. S. Methven* 10,383 (GENT, Eastern Illinois – dupl.) – USA – North Carolina, around the greenbrier field station, N35°44.38'W83°25.45', alt. 488 m, 10/08/2005, *A. Verbeken* 05–210 (GENT) – North Calorina, Swain County, Heintoogard, N 35°34.78'. W 83°10.99', alt. 1603 m, 15/08/2005, *A. Verbeken* 05–324 (GENT) – CANADA – Prov. Québec, Montréal, Arboretum Morgan, N 45°26.139' W 73° 56.898', 21/08/2013, *A. Verbeken 13–025* (GENT)
Notes: both hairs on pileipellis (85 µm) and stipitipellis (100 µm) longer than those of *subvellereus* 1.

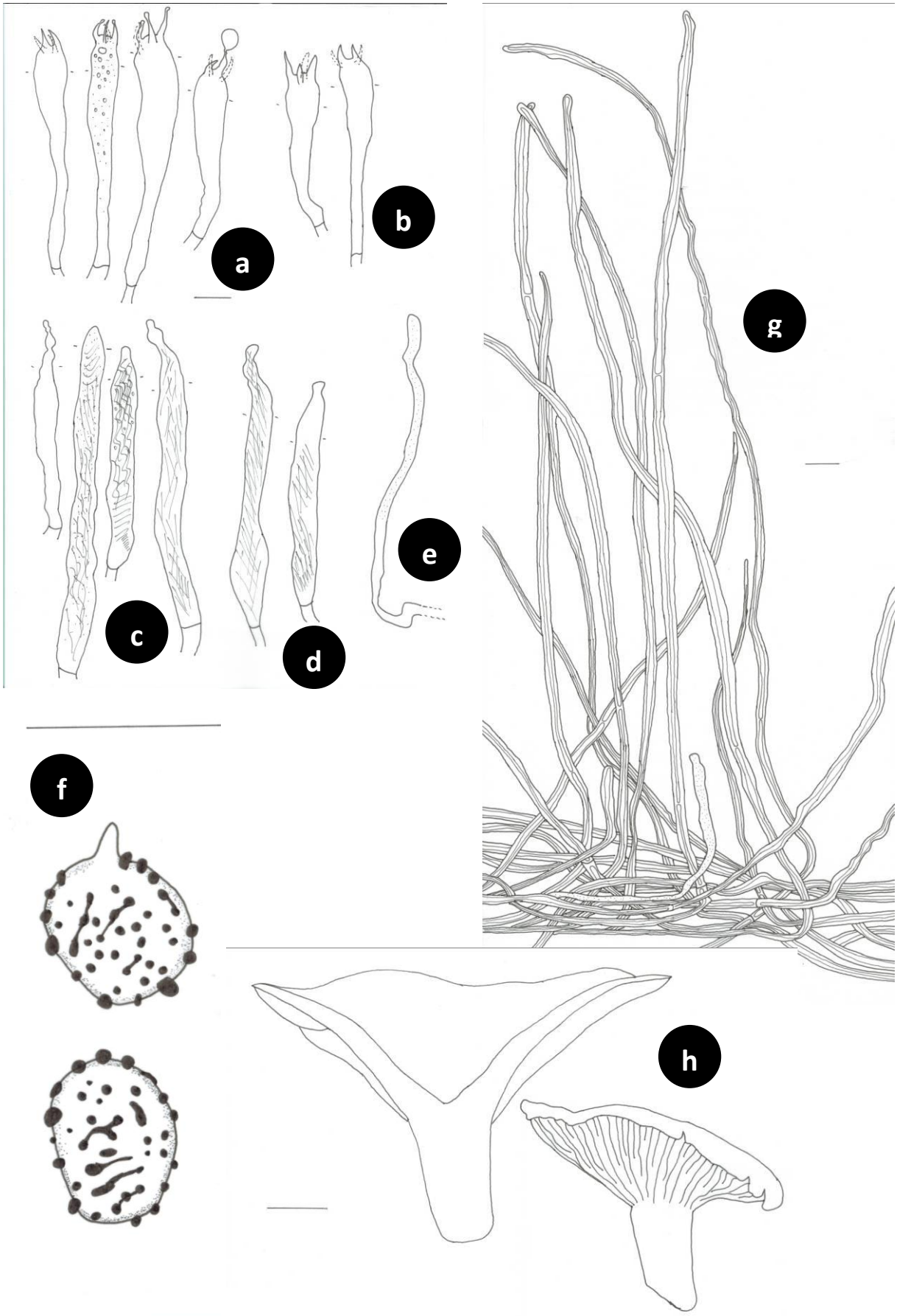

Figure 3.14 – *Lf. subvellereus* 3: a. basidia b. marginal basidium c. macropleurocystidia d. macrocheilocystidium e. pseudocystidia f. spores g. pileipellis (scale bar= 10 µm) h. basidiocarps (scale bar= 2,5 cm)

Discussion

Sampling, lab work

The European specimens used in this study, mainly came from the western half of Europe. The eastern half however, was only represented by two specimens from Slovakia but unfortunately, DNA extractions were unsuccessful. Next to this lack of geographical spread in the European sampling, there was also some lack of taxonomical spread. Both *Lf. bertillonii* and *Lf. vellereus* were represented, but despite the great number of known varieties of *Lf. vellereus*, only *Lf. vellereus* var. *hometii* was represented in the dataset. Clearing up the debate regarding these varieties will require a more thorough sampling throughout Europe, preferably by people able to correctly identify these varieties. Notwithstanding there generally is a large need to mainly conduct expeditions in the tropics, this particular case nicely shows that even in Europe, with a long and ongoing history of taxonomy (see introduction), work is never finished.

The two American species, *Lf. deceptivus* and *Lf. subvellereus*, were well sampled throughout their known range. However, as will be discussed further, *Lf. deceptivus* (or at least closely related look-a-likes) apparently also occurs in the North of South America (Colombia, Costa Rica) and is even reported from south-east Asia (Vietnam). Some further sampling in these areas would be advised for future research, especially in Asia as there were only two Asian specimens. During recent communications with prof. dr. R.E. Halling, a *Lf. subvellereus* look-a-like was also reported from the north of South America and has been sent to us on loan (too late to be included in this thesis unfortunately). Consequently, for this species, sampling campaigns through this region would also be advised.

Only one of the two Asian species of *Lf*. sect. *Albati* was included in this study: *Lf. pilosus*, known as a recently discovered species from Thailand. As will be discussed below however, there were also two Japanese sequences obtained from GenBank that grouped within the *Lf. pilosus*-clade. This could mean this species has a wider distribution than is currently known., the other Asian species, *Lf. puberulus* was not represented in this study, not by herbarium specimens, nor by GenBank- or Unite-sequences. for this species, a much greater effort in sampling throughout its known region and maybe even beyond is recommended.

Next to the sampling being somehow insufficient, the molecular lab work was also unsuccessful at times. Of the 54 DNA extractions that succeeded (having attempted this on more than 70 collections), only 23 turned out at least one PCR product (some needing three attempts) that was successfully sequenced and processed to a usable DNA sequence. Off course, despite molecular methods having been around long enough to become affordable, we will always be limited in some way. There will always be a threshold in DNA quality or quantity, under which extraction and/or amplification will not be successful. Still, there are ways of anticipating for these limitations. By making sure collected specimens are dried in the best possible way (not dried too fast or for too long, not letting the specimens burn...) the circumstances allow and preserving them in the best possible way, more collections would still allow DNA analyses. Preservation methods that involve drying specimens have been proven to cause considerable degradation to fungal DNA (Bainard et al. 2010).

The best way to preserve specimens would actually not be achieved by drying them. According to Bainard et al.(2010) the least amount of damage occurs when preserving samples in freezed conditions. In case field work does not allow this, storing specimens in CTAB-buffer is also preferred over drying methods. In conclusion, drying fungi with the goal of preserving them for future molecular analysis is not recommended. The best solution is to freeze the specimens or store them (or a small part) in CTAB-buffer.

Only one type collection was included in the phylogenetic analyses as most type collections are rather old. The one included type, for *Lf. pilosus* (LTH-205), was collected in 2004. The other type collections for *Lf. vellereus, Lf. deceptivus* and *Lf. subvellereus* however, were collected in 1961, 1885 and 1897 respectively. Having been stored in dried conditions for multiple decennia, the DNA in these specimens is too fragmented (Bainard et al. 2010) for a PCR to be successful. Because of this, if one species splits up into multiple clades, it will be hard to tell at first which of those clades still represents the already existing species. We will need to compare morphological, ecological and geographical data between the description of the type species and the resulting description of each clade.

More sampling campaigns would lead to more recent collections of species, making the use of older collections (of which the DNA is most probably already useless) unnecessary for building phylogenies. These collections remain useful for morphological analysis however. It would be recommended to add epitypes or isotypes of recently collected specimens in order to have a type specimen that also allows successful DNA analysis.

Phylogenies, species delimitation

Despite the sampling and lab work not going entirely as planned, the phylogenetic analyses turned out some interesting results. One species proved to be correctly delimited: *Lf. bertillonii* formed monophyletic clades in both the ITS- and multi-locus gene trees (figures 3.1, 3.4 respectively). In the LSU- and *rpb*2-gene trees, it was only represented by three and one specimens respectively, explaining the low bootstrap values (5 and 29).

Another species, *Lf. pilosus*, showed dichotomies in some phylogenies. The clade is monophyletic in the ITS-phylogeny (figure 3.1). In both the LSU-, *rpb2*- and concatenated phylogenies (figures 3.2, 3.3, 3.4 respectively) however, the clade splits up in two groups (not always supported however). One of these subgroups always contains the specimen LTH-204 , while the other subgroup always contains LTH-205, the type specimen. LTH-204 is accompanied by a Japanese GenBank-sequence (wrongly) named *Lf. vellereus* in the LSU- and the concatenated phylogeny and accompanied by FH 12-093 in the *rpb*2-phylogeny. This last grouping is strange because FH 12-093 is found in the other subgroup in the other phylogenies. Because of the low number of sequences representing *Lf. pilosus* and the one sequence switching between subgroups, no conclusions can be made regarding the validity of *Lf. pilosus* as one species. Following Dettman et al. (2003) however, we could argue that monophyly at each of the sampled loci is an unreasonably strict criterion for species delimitation, a growing number of taxonomists agree with this (Eberhardt et al. 2015; McKay et al. 2010; Van de Putte et al. 2016). In two of the three single-locus phylogenies, *Lf. pilosus* splits up in two subgroups with one always containing the type specimen (LTH-205) and the other always containing LTH-204. We see the same thing in the multi-locus phylogeny but one of the groups only has a BS of 66. Some more sampling and amplification of other loci would be recommended to clear this out.

These outcomes may be due to differences in DNA-sequences being artefacts or the species just showing greater intraspecific variation, so for now, *Lf. pilosus* stands as one species.

Another species showing similar results is *Lf. vellereus*. In the ITS-phylogeny (figure 3.1), its clade splits up into two subgroups, each supported by significant bootstraps. In the *rpb2*-phylogeny (figure 3.3), it also splits up, however the two groups do not show the same composition and one is not supported (BS: 62). In the concatenated phylogeny (figure 3.4), there is also a split but not supported at all and in the LSU-phylogeny (figure 3.2) there is no dichotomy. Because of the smaller number of LSU- and *rpb2*-sequences available, the bootstrap support that is lacking at times, the composition of both subgroups not being the same and the two *Lf. vellereus* var. *hometii*-sequences always being placed in opposite groups, no conclusions can be made about this either. It does however show some more thorough study would be recommended in which more extensive sampling is done and more molecular markers are used.

Although not always having full support (mainly because of the low number of sequences), *Lf. deceptivus* consistently splits up into five different lineages/clades with a sixth one appearing in the ITS-phylogeny. Based on morphology and distribution, one species, *Lf. deceptivus 3 (*named *Lf. hallingi)*, differed enough from the others to be given the status of separate species. Following the General Lineage Concept of species (De Queiroz 2007), the only necessary property of species is existing as a separately evolving metapopulation lineage. Being fully supported as a separate clade in each of the phylogenies, *Lf. hallingi* certainly accounts for this criterion. Secondly, there are also some properties providing extra evidence of lineage separation. Different species concepts (biological, phylogenetic, morphological, geographical...) are now used as additional proof for species delimitation. The specimens of *Lf. hallingi* were unique in some characteristics. Macromorphologically, the latex staining tissues pinkish has not been observed in other clades. Micromorphologically, *Lf. hallingi* has longer hairs on the pileipellis (306 µm) than any other clade (up to 260 µm) that was studied here. In the original description by Hesler & Smith (1979) however, pileipellis hairs are described up to 300 µm so this will need to be further investigated. The most striking characteristic is the range of this species. Described by Hesler & Smith (1979) as a purely North American species, the Central American distribution of this species is a valuable piece of evidence of lineage separation (De Queiroz 2007). One could argue that from a political point of view, Colombia lies in South America. However, the collections used here were found north west from the Andes mountains. These mountains form a natural barrier between the north west of Colombia and the rest of South America so from a geographical/biological point of view, this area belongs more with Central America.
Two other clades, *Lf. deceptivus* 2 and 5, did have different distributions compared to the other clades but were each only represented by one collection that could be studied (and one GenBank sequence for *Lf. deceptivus* 2). So even if differences were found, this would not be significant from a statistical point of view. Delimiting a species based on one collection seems quite hard to approve of.

Despite finding no morphological or geographical differences supporting the split into one or more new species, *Lf. subvellereus* did consistently split up into multiple phylogenetic lineages/clades. Five supported phylogenetic lineages appear in the ITS- phylogeny and the concatenated phylogeny, four lineages of which three supported appear in the LSU-gene tree and three supported lineages appear in the *rpb2*-phylogeny with topologies always matching.

Two clades, *Lf. subvellereus* 4 and 5 looked promising, having a separate geographical distribution (India and Japan respectively) but the collections were either in too bad a state to be studied microscopically (*Lf. subvellereus* 4) or not present here as the sequences were obtained from GenBank (*Lf. subvellereus* 5). So again, more sampling and the use of more and/or other molecular markers would be recommended for future research.

The species delimitation with *BEAST did not turn out any definitive results, only supporting three lineages: *Lf. deceptivus* as a whole, *Lf. pilosus* and the group containing both *Lf. subvellereus* 1 and 3. One explanation might we did not assign the right taxon sets to certain lineages. In *BEAST, when setting up the analysis, based on the phylogenetic analyses, you need to manually assign lineages to a certain taxon, in other words, already delimit the species as expected by giving them a working name. The assignment of different taxa however, was based on the previous phylogenetic analyses where the several subclades were supported. Another, more probable, explanation might be the lack of sequences in general and for some specific clades. There is no proper balance between the subclades, some are represented by several sequences from several loci but others have but few sequences and/or few different loci. For future research, again, a more thorough sampling is recommended but also the use of other, possibly more informative markers. ITS, LSU and *rpb*2 have already proven to be useful in delimiting species in the Russulaceae (Das et al. 2010; De Crop et al. 2014; Van de Putte et al. 2012a; Van de Putte et al. 2016; Van de Putte et al. 2010a). However, within species complexes, some markers like LSU and *atp6* may prove to be too conservative (De Crop et al. 2014) while others like *tub, hsp,* and *tif* (Balasundaram et al. 2015) can be even more or at least as informative as ITS, the standard barcoding marker. For future research, using Bayesian Phylogenetics & Phylogeography v2.1 (Rannala and Yang 2003; Yang and Rannala 2010) next to *BEAST is also recommended. Different parameters are used in BPP and more emphasis is put on prior distribution and root age also allowing to adjust for smaller datasets.

Lf. sect. *Albati* species

This group of milkcaps, commonly reffered to as the Fleecy milkcaps, is defined by species with firm, white basidiocarps, a lamprotrichoderm as pileipellis and fine spore ornamentation (Verbeken et al. 1997). What distinguishes this group from other sections within *Lf*. subg. *Lactariopsis* is the presence of macropleurocystidia, the absence of broad and emergent pseudocystidia and no species occurring in Africa (Verbeken 1998). The type species of this section, *Lf. vellereus*, is defined by a velutinous cap, mild to slightly bitter milk, subglobose to ellipsoid spores and a European distribution (Heilmann-Clausen et al. 1998). Its variety, *Lf. vellereus* var. *hometii* differs in the lamellae standing closer and the milk sometimes turning violet. The validity of this variety however is questionable (Verbeken et al. 1997). Just as has been done for a number of other varieties of *Lf. vellereus* and *Lf. bertillonii*, *Lf. vellereus* var. *hometii* may need to be synonymized with *Lf. vellereus*. Based on the results of this study, no definitive conclusion can be made but the opinion of Verbeken et al. (1997) remains plausible as in the phylogenies (figures 3.1, 3.2, 3.3, 3.4) the two sequences from this variety mixed in with the other sequences of *Lf. vellereus* instead of separating themselves (even dividing themselves over both subgroups at times). The other European species, *Lf. bertillonii*, is also defined by firm white basidiocarps with a velutinous cap but the lamellae are more crowded, the spores are globose to ellipsoid and the milk is very acrid (Heilmann-Clausen et al. 1998).

Hesler & Smith (1979) described *Lf. deceptivus* as a North American species with white basidiocarps, turning brownish to yellowish with age, broadly ellipsoid spores with isolated ornamentation and very acrid milk that does not change colour. In this study, six different clades occurred for *Lf. deceptivus.* Three of these clades could possibly represent the 'real' *Lf. deceptivus: Lf. deceptivus 1, 4 and 6*. As the type specimen was not included in the phylogenies, we can not know for sure but based on their description and North American distribution, these clades match the official description best. *Lf. deceptivus* 1 and 4 do not differ much from each other morphologically – except for the stipitipellis hairs being up to 100 µm longer in *Lf. deceptivus* 4 – and they also match the official description – except for the spores exhibiting very fine connective lines and the basidia being up to 6 µm broader. Still, most probably one of these clades represents the real *Lf. deceptivus*. The third clade, *Lf. deceptivus* 6 does differ slightly with the milk sometimes turning pink as the most striking difference. Further, the spores of this clade are smaller (up to 1 µm for both length and width) and have very fine yet distinctive connective lines. Two clades were found in Vietnam so they either represent a new species based on their distribution or the range of *Lf. deceptivus* needs to be expanded. Morphologically, both clades slightly differ from the official description. First, *Lf. deceptivus* 2 has distant lamellae, opposed to the officially described close lamellae and slightly broader basidia with exceptionally long sterigmata (up to 18 µm). Second, *Lf. deceptivus* 5 also has more distant lamellae and slightly broader basidia but also has smaller spores (6.39-*7.75*-9.13 x 5.20-*6.42*-7.64 µm versus 9-12(-13) x 7.5-9 µm) and slightly broader stipitipellis hairs (up to 4 µm). The last clade, *Lf. deceptivus* 3, is treated as a new species named *Lf. hallingi,* based on its consistent monophyly in the phylogenies, the latex staining tissues pink sometimes, the pileipellis hairs growing up to 40 µm longer than any other clade and the unique Central American distribution[8].

The other North American species in this study, *Lf. subvellereus*, is defined by white, firm basidiocarps turning yellow with age, crowded lamellae, small ellipsoid spores with low ornamentation (up to 0.2 µm) and pale yellow latex tasting very acrid (Hesler and Smith 1979). Hesler & Smith (1979) also described a variety, *Lf. subvellereus* var. *subdistans*, of which the most striking difference is the lamellae standing subdistant to distant with age. DNA amplification however, was not successful on this specimen. Microscopically, this variety has longer basidia (up to 10 µm), longer cystidia (up to 20 µm) and longer pileipellis hairs (up to 50 µm). Five different clades appeared for *Lf. subvellereus* in this study. Not much can be said about three of them; *Lf. subvellereus* 2 consisted of one North American collection that was way too young, *Lf. subvellereus* 4 consisted of two collections from India but in too bad a state to be studied microscopically and *Lf. subvellereus* 5 consisted of two Japanese GenBank-sequences. This does suggest *Lf. subvellereus* may have a larger range than is currently accepted, also appearing throughout Asia. The two other clades, *Lf. subvellereus* 1 and 3, both North American, were studied microscopically. The difference between *Lf. subvellereus* 1 and the official description is that we found longer stipitipellis (up to 100 µm) and pileipellis hairs (up to 100 µm) and higher spore ornamentation (up to 1.3 µm). For *Lf. subvellereus* 3, the differences with the description by Hesler & Smith (1979) lies in the lamellae standing distant, the pleuro- and cheilomacrocystidia both being up to 20 µm longer, the stipitipellis hairs (up to 200 µm) and pileipellis hairs (up to 200 µm) also being longer and the spore ornamentation being slightly higher (up to 1 µm).

[8] This is even unique for the entire *Lf*. sect. *Albati*!

Included in this study but not studied microscopically as this species did not consistently divide in multiple subgroups[9], *Lf. pilosus* is a species from Thailand. It is defined by yellowish white basidiocarps with white latex turning yellowish and tasting very acrid. Microscopically the very long hairs on the pileipellis and stipitipellis (up to 320 µm and 360 µm respectively) are also defining (Le et al. 2007b). Although being described from Thailand, the distribution of this species may need to be broadened. In the phtlogenies, two GenBank-sequences coming from Japanese specimens were consistently placed within the *Lf. pilosus*-clade. The other Asian species, *Lf. puberulus* was not accounted for in this study. This species, however, is defined by small (1.4-4 cm versus 4-17(-20) cm for all other species), white basidiocarps, turning (pinkish) cinnamon, lamellae with a tint of pale olivine and a mild taste and is described from China (Wen and Ying 2005).

Future perspectives

During the course of this thesis, the title was adjusted from 'a worldwide assessment' to 'a worldwide exploration'. And that is exactly what this research can be called, an initial exploration leading to more research. Three items can certainly be improved: sampling, molecular markers and software use. As was already clear quite early in this study, *Lf*. sect. *Albati* could be better represented in herbaria worldwide. Even in Europe there is a lack of specimens both geographically (Eastern Europe is undersampled) as taxonomically (the varieties of *Lf. vellereus*). Next, based on this study, more sampling is needed throughout Asia. The Asian species, *Lf. puberulus* was not represented at all. More so, *Lf. deceptivus* clades two and five, were collected in Vietnam, both clades were only represented by one specimen however. Even *Lf. subvellereus* had Asian collections, *Lf. subvellereus* clades four and five were from India and Japan respectively but both clades were badly represented. Next, more molecular markers could be amplified to make the phylogenies more informative, even if some taxa would still be undersampled. Even based on the dataset of this thesis, more information could be extracted. Last, the software that is used could be enhanced. As mentioned above, especially the species delimitation could have been done better, by including BPP and other programs for example.

[9] It did form two subgroups sometimes but never with the same composition + only based on 4-5 sequences.

Conclusion

By conducting sampling campaigns, requesting loans and consulting online databases GenBank and Unite, we collected specimens and sequences from all over the world. Based on both single- and multi-locus phylogenetic trees, we mainly found cryptic diversity within *Lf*. sect. *Albati*. Multiple species (*Lf. subvellereus, Lf. deceptivus, Lf. vellereus*) consistently divided into supported subgroups in the gene trees but were too similar in appearance to be delimited as species. Except for one subclade of *Lf. deceptivus*: based on its Central American distribution and some clear morphological differences, this clade was given the rank of new species, called *Lf. hallingi*. The greatest conclusion of this worldwide exploration however, is that there remain some unresolved questions. For future investigations, more thorough sampling would be recommended, especially focusing on neglected regions like eastern Europe, Asia and Central America. With a bigger dataset, more evenly spread both geographically as taxonomically (including all species and varieties), more of this cryptic diversity would be resolved and the questionable status of some varieties too.

English recap

Traditionally, the agaricoid genera *Russula* and *Lactarius* were considered as different from other typical mushrooms so, based on the presence of sphaerocytes, amyloid spore ornamentation and a gloeoplerous hyphal system, they were placed in their own order, Russulales. Molecular research however, learned us that too much focus had been put on morphology historically. Next to the classic agaricoid fungi with lamellae, a lot of other basidiocarp types and hymenophore types were included in the Russulales. The genera *Russula* and *Lactarius* were consequently put together in the family *Russulaceae.*

The distinction between the two genera *Russula* and *Lactarius* used to be simple. *Lactarius* species exude a milky substance, called latex, which is kept in lactiferous hyphae. Next to this obvious difference, colours, organization of lamellulae and the texture of the cap were also useful features. The discovery of tropical *Russulaceae* with mixed features however, thought us that the distinction is not as easy as it seemed at first. In addition, molecular research including these tropical specimens, lead to the discovery that the genera *Russula* and *Lactarius* needed to be split up in four genera. *Russula* was only monophyletic if a small group of species was left out. Together with some former *Lactarius*-species, this group formed the new genus *Multifurca*, mainly inhabiting some species with mixed features. Next, the genus *Lactarius* needed to be split up, forming the new genus *Lactifluus.*

A proper morphological distinction between *Lactarius* and *Lactifluus* does not exist, there are some trends however. *Lactifluus* contains all species with veiled and velvety to tomentose caps and all annulate species, contrasting *Lactarius*, where zonate and viscous to glutinate caps are often found. So far, all pleurotoid species also belong within *Lactifluus*, while angiocarpic species are placed within *Lactarius*. The most striking difference between *Lactarius* and *Lactifluus* is their geographical distribution. Although *Lactarius* does occur in the tropics and subtropics, the genus comprises almost all European milkcaps as well as most other species from boreal and temperate regions. *Lactifluus* only contains a few temperate species and has its main distribution in the tropics and subtropics. Last, another contrast between both genera is that *Lactifluus* shows a high genetic diversity with a stable morphology, opposed to *Lactarius*. This is reflected in the high number of cryptic species complexes and species on long, isolated phylogenetic branches in *Lactifluus*.

Recently, following the general trend in the mycological world, the morphology-based infrageneric classification of the genus Lactifluus has been put into question. Next to small adjustments already having been published in the previous years, a genus-wide molecular and morphological analysis has led to a new classification of the genus. Instead of six subgenera, the genus is now divided in four subgenera: Lf. subg. *Lactariopsis*, *Lf*. subg. *Lactifluus, Lf*. subg. *Gymnocarpi* and *Lf*. subg. *Pseudogymnocarpi.* The subgenus *Lf*. subg. *Lactariopsis*, defined by mainly African species bearing a ring, inhabits the temperate section *Lf*. sect. *Albati* also called the Fleecy milkcaps. This section differs from other sections in this subgenus because of the presence of macropleurocystidia and the absence of broad and emergent pseudocystidia and is characterized by species with firm, white basidiocarps, a lamptrotrichoderm as pileipellis and very fine spore ornamentation.

Consisting of six species in total, two *Lf*. sect. *Albati*-species occur in Belgium and are distributed throughout Europe: *Lf. vellereus* (including the variety *Lf. vellereus* var. *hometii*) and *Lf. bertillonii*. In Dutch they are called 'Schaapje' and 'Vals schaapje' which refers to their large and firm, whitish fruiting bodies and velutinous cap and stipe.

Lactifluus vellereus is a species showing much variation, reflected in the number of varieties that have been described for this species. The taxonomical value of these varieties however, is being seriously questioned.

Two other species have a North American distribution (although, as can be read further, this it not entirely right), *Lf. deceptivus* and *Lf. subvellereus*. Lastly, *Lf. pilosus* and *Lf. puberulus* are described from Asia (Thailand and China respectively).

Recently, expeditions have brought unknown specimens from India, Vietnam, Thailand, Russia, North America and South America. Preliminary molecular analyses placed these specimens within *Lf*. sect. *Albati*. Based on these results and some other irregularities, we wish to clear up any issues regarding the delimitation of species within this section by building a multi-locus phylogeny based on worldwide sampling. By subsequently studying the morphology of any discovered (cryptic) species complex, we will be able to adjust the current placement and definition of *Lf*. sect. *Albati*.

After sampling specimens and sequences in multiple ways by collecting fresh specimens, looking for specimens in the Herbarium Gandavensis, requesting loans from other herbaria and consulting online databases GenBank and Unite, molecular lab work was conducted. This consisted of extracting DNA, conducting PCR's with either ITS-, LSU- or *rpb2*-primers, checking the quality of PCR products by gel electrophoresis and preparing successfully amplified samples for sequencing by an external company. In total, 168 sequences were used in the alignment (representing all species except *Lf. puberulus*), amounting to 96 ITS-sequences, 41 LSU-sequences and 31 *rpb*2-sequences. The outgroup was made up of five species from the group around *Lf. volemus*. In order to build a maximum likelihood (ML) multi-locus gene tree, we first built ML single-locus trees to assert if any conflicts arised between the different tree topologies. Once all conflicts were worked out, we built a ML multi-locus phylogeny and conducted a Bayesian species delimitation. Based on the results of the molecular analyses, collections of interest were then studied microscopically, measuring and drawing elements of the hymenium, the spores, the pileipellis and the stipitipellis.

Despite some minor conflicts, the single-locus ML analyses showed congruent topologies so a multi-locus ML phylogeny was also built. This phylogeny showed us that *Lf*. sect *Albati* had full bootstrap support. The only species consistently forming a monophyletic clade was *Lf. bertillonii*.
In some of the single-locus trees, *Lf. pilosus* split up into two subclades, including Japanese GenBank-sequences. In the multi-locus gene tree it did too but not supported by bootstrap values. Some more research into this is recommended. It is already convincing however the range of *Lf. pilosus* will need to be expanded.
Similarly, *Lf. vellereus* also splits up in some of the ML phylogenies, sometimes supported and sometimes not. No real conclusions can be drawn for this species. We do suspect *Lf. vellereus* var. *hometii* to be questionable as a variety based on the fact the varieties do not group together and/or separate themselves.
For *Lf. deceptivus*, we consistently found five different subclades with a sixth one appearing in the ITS-phylogeny. Based on the subsequent morphological study of the specimens in these subclades, on subclades was found to differ enough morphologically and geographically to be given the rank of species. Distributed through Colombia and Costa Rica with a latex turning pink sometimes and very long hairs on both pileipellis and stipitipellis it was named *Lf. hallingi*. Despite also differing in multiple features, the other subclades were not found to represent new species.

The North American distribution of *Lf. deceptivus* will probably need to be expanded however as some subclades contained specimens from Vietnam.
The last species of *Lf*. sect *Albati* that was studied, *Lf. subvellereus*, also consistently split up into multiple subclades. Of these five groups, three of them could not be studied microscopically. One subclade consisted of one collection of specimens that were way too young, another one consisted of two Indian collections that were in too bad a state to be studied and the third one, consisted of two Japanese GenBank sequences. It is probable however, the North American distribution of *Lf. subvellereus* may need to be expanded to India, Japan and Vietnam. The two other subclades, both from North America, were also studied microscopically but no significant differences with the official description of the species were found.

The species delimitation did not turn out any definitive results, probably because of the relatively small dataset that it was based on. This was a general issue throughout this study. For future research I would recommend a more thorough sampling campaign, focussing on the areas that turned out some interesting results in this study (India, Vietnam, Japan, Central America) and also Europe as *Lf. vellereus* and its varieties also need some thorough investigation.

Nederlandse samenvatting

Traditioneel gezien werden de agaricoide genera *Russula* en *Lactarius* apart ingedeeld in hun eigen orde Russulales op basis van de aanwezigheid van sphaerocyten, de amyloide sporenornamentatie en het gloeoplere hyfensysteem. Moleculair onderzoek heeft echter aangetoond dat men historisch te veel nadruk legde op de morfologie. Naast de agaricoide paddenstoelen met plaatjes, moesten ook andere basidiocarp en hymenofoor types toegevoegd worden aan de Russulales. Bijgevolg werden de genera *Russula* en *Lactarius* in hun eigen familie geplaatst: de *Russulaceae*.

Aanvankelijk was het onderscheid tussen Russula en Lactarius simpel. Lactarius-soorten, ook melkzwammen genoemd, scheiden een melkachtige substantie af bij beschadiging, de latex, die in lacticiferen wordt bewaard. Naast dit duidelijk verschil waren de kleuren, organisatie van de lamellulae en de textuur van de hoed bruikbare kenmerken ter onderscheid. De ontdekking van tropische *Russulaceae* met gemengde kenmerken daarentegen, leerde ons dat het onderscheid tussen beide genera toch niet zo simpel is. Daarnaast heeft moleculair onderzoek met inbegrip van deze tropische exemplaren aangetoond dat de genera *Russula* en *Lactarius* moesten opgesplitst worden. *Russula* was enkel monofyletisch als een kleine groep ervan werd afgesplitst. Samen met enkele *Lactarius*-soorten, vormde deze groep het nieuwe genus *Multifurca*. Verder moesten de resterende melkzwammen ook nog opgesplitst worden in enerzijds *Lactarius* en anderzijds *Lactifluus*.

Een deftig morfologisch onderscheid tussen beide genera bestaat niet, er zijn wel trends waar te nemen. Lactifluus heeft alle soorten met gesluierde en viltige tot tomentose hoeden en alle geringde soorten in tegenstelling tot *Lactarius*, waar vooral soorten met zonate en slijmerige tot plakkerige hoeden gevonden worden. Tevens zijn er microscopische trends. Het opvallendste verschil is de geografische distributie van beide genera. Ondanks dat Lactarius ook in de tropen en subtropen voorkomt, bevat het genus bijna alle Europese melkzwammen en die uit gematigde en boreale streken. *Lactifluus* bevat maar enkele gematigde soorten en wordt vooral in de tropen en subtropen gevonden. Een laatste contrast is dat *Lactifluus*, in tegenstelling tot *Lactarius*, een zeer hoge genetische diversiteit vertoont maar een eerder stabiele morfologie. Dit wordt weerspiegeld in het groot aantal cryptische soortscomplexen en geïsoleerde soorten op een lange fylogenetische tak die het genus herbergt.

De algemene trend in de mycologische wereld volgend, is recent de infragenerische indeling van *Lactifluus* in vraag gesteld aangezien deze zwaar op morfologie is gebaseerd. In de voorbije jaren zijn daardoor al kleine aanpassingen voorgesteld geweest in die indeling. Daarnaast is nu echter een volledig vernieuwde indeling voorgesteld, gebaseerd op een genuswijd moleculair en morfologisch onderzoek. In deze nieuwe indeling bestaat *Lactifluus* maar uit vier subgenera meer in plaats van zes: *Lf*. subg. *Lactariopsis*, *Lf*. subg. *Lactifluus*, *Lf*. subg. *Gymnocarpi* en *Lf*. subg. *Pseudogymnocarpi*. Het subgenus *Lactariopsis*, gedefinieerd door geringde soorten met een grotendeels Afrikaanse verspreiding, bevat ook de gematigde sectie *Lf*. sect. *Albati.* Deze verschilt van andere secties in dit subgenus door de aanwezigheid van macropleurocystidia, de afwezigheid van brede, emergente pseudocystidia en wordt zelf gedefinieerd door soorten met witte, stevige vruchtlichamen, een lamprotrichoderm als pileipellis en zeer fijne sporenornamentatie.

Bestaande uit zes soorten, bevat deze sectie twee soorten die ook in België voorkomen en een Europese verspreiding kennen: *Lf. vellereus* (en *Lf. vellereus* var. *hometii*) en *Lf. bertillonii*, respectievelijk ook Schaapje en Vals schaapje genoemd door de grote witte vruchtlichamen met viltige hoed en steel. *Lactifluus vellereus* is een variabele soort, gereflecteerd in het aantal variëteiten die ervoor beschreven zijn. De taxonomische waarde ervan wordt echter in vraag gesteld.

Twee andere soorten zijn gekend van Noord-Amerika: *Lf. deceptivus* en *Lf. subvellereus* en nog twee andere van Azië: *Lf. pilosus* uit Thailand en *Lf. puberulus* uit China. Recente expedities hebben echter ook onbekende soorten opgeleverd uit India, Vietnam, Thailand, Rusland, Noord-Amerika en Zuid-Amerika. Een voorbereidende moleculaire analyse plaatste deze specimens binnen *Lf*. sect. *Albati*. Door dit en andere onregelmatigheden willen we de afbakening van soorten binnen deze sectie en van de sectie zelf aan een grondig onderzoek onderwerpen. Dit willen we bereiken door een multi-locus fylogenie op te stellen, gebaseerd op collecties van over heel de wereld. Door vervolgens de morfologie van ontdekte (cryptische) soorten te bestuderen, zullen we de omschrijving van deze sectie en de soorten erin kunnen aanpassen.

Collecties van *Lf*. sect. *Albati* of minstens DNA-sequenties werden op meerdere manieren verzameld: verse exemplaren zoeken, het Herbarium Gandavensis afzoeken, loans aanvragen van andere herbaria en de online databanken GenBank en Unite raadplegen. Voorafgaand aan de moleculaire analyse kwam dan het labowerk: DNA extracties uitvoeren, PCR's met primers van het ITS-, LSU- of *rpb2*-gen, gelelectroforeses om de kwaliteit van de PCR-producten na te gaan en ten slotte het opkuisen en voorbereiden van de goedgekeurde PCR-producten om deze dan door een extern bedrijf te laten sequeneren. In totaal werden 168 sequenties (komend van alle soorten behalve *Lf. puberulus*) gebruikt in het alignement: 96 ITS-sequenties, 41 LSU-sequenties en 31 *rpb2*-sequenties. De outgroup was samengesteld uit vijf soorten uit de groep rond *Lf. volemus*. Om een maximum likelihood (ML) multi-locus genenboom te mogen bouwen, moesten we eerst nagaan of er geen conflicten waren tussen de toplogiëen van de single-locus fylogenieën. Na dit te controleren en eventuele conflicten op te lossen, hebben we dan de multi-locus fylogenie gegenereerd en een Bayesiaanse soortsafbakening uitgevoerd. Gebaseerd op de resultaten van deze moleculaire analyses hebben we dan de collecties die dit eisen, microscopisch bestudeerd. Daarbij hebben we elementen van het hymenium, de sporen, de pileipellis en de stipitipellis gemeten en getekend.

Ondanks enkele kleinere conflicten, toonden de single-locus ML genenbomen analoge topologiëen dus is er ook een multi-locus ML fylogenie opgesteld. In deze fylogenie kreeg *Lf*. sect. *Albati* volledige ondersteuning. De enige soort die telkens een monofyletische clade vormde, was *Lf. bertillonii*.
In sommige van de single-locus bomen, splitste Lf. pilosus zich op in twee subgroepen, met inbegrip van twee Japanse GenBanksequenties. In de multi-locus boom gebeurde dit ook maar niet ondersteund. Verder onderzoek, liefst gebaseerd op meer exemplaren, is hier zeker aangeraden. Het lijkt wel zeer waarschijnlijk dat de verspreiding van *Lf. pilosus* zal moeten uitgebreid worden naar Japan.
De resultaten voor *Lf. vellereus* waren vergelijkbaar. De soort splitst zich op in sommige bomen, al dan niet ondersteund. Opnieuw is hiervoor verder, meer uitgebreid onderzoek aan te raden. Wel lijkt het uit deze analyse dat de taxonomische waarden van *Lf. vellereus* var. *hometii* terecht in vraag wordt gesteld. De sequenties van deze variëteit splitsen zich niet af van de rest en zitten telkens verdeeld over de subgroepen.

Voor *Lf. deceptivus* vonden we consistent zes verschillende subclades. Gebaseerd op de navolgende morfologische studie van deze subclades, hebben we één clade gevonden die genoeg afweek van de rest en van de soortsbeschrijving om als nieuwe soort beschreven te worden. Gevonden in Colombia en Costa Rica, heeft deze soort latex die soms roze kleurt en zeer lange haren op de pilei- en stipitipellis en kreeg deze de naam *Lf. hallingi*. Ondanks dat de andere clades ook verschillen vertoonden (zowel morfologisch als geografisch), verschilde geen enkele andere genoeg om als nieuwe soort beschreven te worden. De verdeling van *Lf. deceptivus* zal wel moeten uitgebreid worden daar er ook twee subclades waren met Vietnamese exemplaren.
De laatste bestudeerde soort, *Lf. subvellereus*, splitste zich ook telkens op in meerdere subclades. Van deze vijf groepen, konden er drie niet morfologisch bestudeerd worden: een clade bestond uit en collectie van te jonge exemplaren, een andere clade met Indische exemplaren was in te slechte staat en nog een andere clade bestond uit twee Japanse GenBanksequenties. Hierop gebaseerd kunnen we wel zeggen dat de verspreiding van *Lf. subvellereus* mogelijk naar India en Vietnam moet worden uitgebreid. De twee Noord-Amerikaanse clades die wel konden bestudeerd worden, vertoonden niet veel afwijking van de officiële soortsbeschrijving.

De Bayesiaanse soortsafbakening leverde niet veel resultaat op, waarschijnlijk door de relatief kleine dataset waarop deze gebaseerd was. Dit was ook een algemeen ongemak doorheen de studie. Voor verder onderzoek van deze sectie zou ik een grondige verzamelcampagne aanraden die zich richt op die gebieden die in deze studie merkwaardige resultaten opleverden: India, Vietnam, Japan en Centraal Amerika en Europa omdat de variëteiten van Lf. vellereus ook verder moeten aan de tand gevoeld worden.

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Appendices

Appendix A: normal DNA extraction protocol

DNA extraction from DRIED SPECIMENS (herbarium material)

(Matt Sauer + optional CTAB extraction)

Solutions needed:

Extraction buffer:

0.1 M Tris.Cl (pH=8)
0.5 M NaCl
0.05 M EDTA
(0.01 M β-mercapto-ethanol)
10% SDS
Isopropanol (= 2-propanol)
70% EtOH
MilliQ H_2O

- Take 0.5 – 1 g of the herbarium specimen (e.g. part of lamella) and put it together with two 2 glass beads in a 2 ml eppendorf tube (mark tubes, flame tweezers in between two specimens!)
- Freeze tubes in liquid nitrogen
- Tubes in bead beater: 3 runs of 1 min 30 sec at speed 30

- in separate vial:
  - Pipet amount extraction-buffer needed in separate vial

- Add 1000 µl extraction-buffer and 50 µl 10 % SDS to each sample
- Vortex
- Leave it for 1 h at 65°C and occasionally vortex to dissolve most of the material
- Add 2 µl proteinase K, mix and leave over night at 50-55°C

- Centrifuge for 10 min at max speed (13200-14000 rpm)
- Transfer supernatant to a new 2 ml eppendorf tube
- Add an equal volume (~ 1000 µl) of Iso-propanol, mix by inverting the tube
- Centrifuge for 10 min at max speed (13200-14000 rpm)
- Pour off the supernatant (not in sink!)

- Wash the DNA pellet by adding 200 µl 70 % EtOH, leave it for 20 min
- Centrifuge for 10 min at max speed (13200-14000 rpm)
- Use a pipette to clear away the supernatant and air dry the DNA pellet
- Dissolve the DNA pellet in 100 µl milliQ H_2O, pipette up and down until DNA is dissolved
- Store samples at -5°C

Appendix B: DNA extraction for older specimens or specimens for which normal protocol failed

DNA extraction from DRIED SPECIMENS (herbarium material)

(Plantentuin Meise, Steven Janssens style (last update 4/8/2014))

Grinding

1. Take 0.5 – 1 g of the herbarium specimen (e.g. part of lamella) and put it together with two 2 glass beads in a 2 ml eppendorf tube (mark tubes, flame tweezers in between two specimens!)
2. Freeze tubes in liquid nitrogen
3. Tubes in bead beater: 3 runs of 1 min 30 sec at speed 30

Lysis (CTAB2x-buffer)

(2% CTAB and 1% PVP-40) + β-mercapto-ethanol 0.3% (=30 µl / 10ml).

Step *Action*

1 Pre-heat lysis buffer at 60 °C.
2 Add 800 µl lysis buffer to each extraction tube + 5µl β-mercapto-ethanol .
3 Vortex shortly, make sure to suspend all the sample (break up clumps of material)
4 Incubate the tubes at 60 °C for 1 or 2 hours (e.g. over lunch) or over night.
5 invert now and then to homogenise (at least 3 times in the total time)
5b Optional: when material is not nicely ground.(and the metal beads were not gone) grind (hot 60°C) with pre-heated grinding blocks

Extraction (under fume hood)

Step Action

1 Let the tubes cool down to 22°C (=room temperature).
2 Add an equal volume of (chloroform/isoamylalcohol 24:1) to each extraction tube.
3 vortex 2x + 2 min shaking to keep chloroform in suspension
4 Centrifuge for 10 min. at 11 000 rpm (13 000 rcf) (22°C)
5 Carefully transfer 700 µl of the upper aqueous phase to a new 1,5 ml tube.
6 add an equal volume of chloroform/isoamylalcohol 24:1. (second purification)
7 2x vortex + 2 min shaking. (second purification)
8 Centrifuge 10 min. at 11 000 rpm (22°C) (13 000 rcf). (second purification)
9 Carefully transfer 600 µl of the upper aqueous phase to a new 1,5 ml lo-bind tube. (second purification)
9b Alternative step 9 : if dirt has been transferred.
-Take the maximum possible amount (not 600).
-Centrifuge for 5 min at 14 000 rpm. (22°C)
-Carefully transfer 600 µl of the upper aqueous phase to a new 1,5 ml lo-bind tube, without disturbing the pellet (=third purification)

Isopropanol - Precipitation

Step Action

1 Add 0.8 volumes of isopropanol (=480 µl for 600 µl or 400µl for 500µl). Shake gently (= invert the tubes 50 times) to obtain a homogenous solution.
2 Store 20 min or overnight at –20 °C for maximal precipitation.
3 Centrifuge 10 min at 14 000 rpm (20 000 rcf) (4°C). Remove supernatant, take care of the pelleted DNA. Place inverted for a few minutes.
4 Add 600 µl 70% ethanol. Loosen the pellet (if possible).
5 Store during 20 min at -20°C.
6 Centrifuge again at 10 000 rpm for 10 min (4°C). Remove supernatant, take care of the pelleted DNA. Place inverted for a few minutes.
7 Put the tubes horizontal, air dry (+/- 1 hour).
8 Dissolve pellet in 100 µl of ddH2O (pH 8,5) at 60°C.
9 Cool to room temperature. (Add 2 µl RNAse A (1/10) per tube, mix and incubate 2 minutes at room temperature.) Store the DNA in the DNA stock: 4°C short term storage, 20°C long term storage.

Appendix C: PCR protocol

What you need:

ingredients

- Primer1 (forw. primer)
- Primer2 (rev. primer)
- dNTP's
- Taq Polymerase
- MgCl2
- amplification buffer
- MilliQ water
- DNA-preparations

disposables

- ice
- 1.5ml-tube
- 200µl-tubes or strips

materials

- pipettes: 0.5-10µl, 10-100µl, 100-1000µl
- centrifuge
- PCR thermocycler
- Vortex

Master mix recipe:

- ITS / LSU / GPD - for 30 µl reactions

Master mix (per sample)		Master mix (for __ samples **+ 1 blanco +** 10%)	
Amplification buffer	3 ul	Amplification buffer	__ ul
MgCl2 (25mM)	0.3 ul	MgCl2	__ ul
dNTPs (10mM)	0.6 ul	dNTPs	__ ul
Primer 1 (10µM)	**0.6** ul	Primer 1	__ ul
Primer 2 (10µM)	**0.6** ul	Primer 2	__ ul
H2O MilliQ	21.72 ul	H2O MilliQ	__ ul

Taq (5u/µl)	0.18 ul	Taq	__ ul
Total volume:	27 µl	**Total volume:**	__ µl
DNA volume	3 µl		

- RPB2 - *for 30 µl reactions*

Master mix (per sample)		Master mix (for __ samples **+ 1 blanco +** 10%)	
Amplification buffer	3 ul	Amplification buffer	__ ul
MgCl2 (25mM)	0.3 ul	MgCl2	__ ul
dNTPs (10mM)	0.6 ul	dNTPs	__ ul
Primer 1 (10µM)#	**2.4** ul	Primer 1	__ ul
Primer 2 (10µM)#	**2.4** ul	Primer 2	__ ul
H2O MilliQ	18.12 ul	H2O MilliQ	__ ul
Taq (5u/µl)	0.18 ul	Taq	__ ul
Total volume:	27 µl	**Total volume:**	__ µl
DNA volume	3 µl		

#primers with degenerate sites

How to make the master mix:

- remove Taq only from freezer when needed
- centrifuge Taq down before use

- Get every ingredient out of the freezer and put on ice, **(!) except Taq (!)**
- Work in the laminar flow bench, treat bench and pipette ends with 70% ethanol, DNA erase (+ clean with ddH_2O) and everything with UV light before starting (leave out primers, dNTP's, Taq and DNA extractions)
- Mark PCR-tubes + blanco
- Pipette calculated amounts of master mix ingredients into a 1.5 or 2 ml-tube, finish with adding Taq, immediately place Taq back in the freezer – everything on ice (!)
- Mix gently with pipette
- Centrifuge master mix down
- Add 45 µl of master mix in each PCR-tube (in cooled plates)

- Add 5 µl of DNA and mix by gently pipetting
- Close all lids securely

- **PCR programs:**
- **- ITS / LSU / RPB2-**
- -program name: -
- ~~*Lid at 105°C*~~
- ~~1. (preheating) **94°C -- 10 sec**~~
- ~~2. (pause – place samples – press enter to proceed)~~
- 3. (initial denaturation) **94°C -- 1-5 min.**
- 4. (denaturation) **94°C -- 30 sec.**
- 5. (annealing) **55°C -- 30 sec.**
- 6. (extension) **70°C -- 30-60 (45) sec.**
- ***7. step 4.-6.: 25-35 cycles (= 34 repeats)***
- 8. (final extension) **70°C -- 5-10 (7) min.**
- 9. (end) (**4°) 20°C -- forever**

Checking PCR success: gel-electrophoresis

What you need:

Ingredients

Agarose, 1xTAE-buffer

markers

- DNA molecular weight marker-ladder

materials

- Bottle + other gel equipment
- (scale)
- micro-wave oven
- electophoresis equipment
- ethidium bromide-room

casting the gel:

- agarose gel: 2,5 g agarose (use scale) + 200 ml 1xTAE for small gel; 3 g agarose (use scale) + 250 ml 1xTAE for large gel
- When bottle of TAE is empty >> refill by 20 ml of 50xTAE and add 980 ml of BiDi

- put 2,5 or 3 g agarose in an bottle
- add 200 or 250 ml of 1xTAE and shake

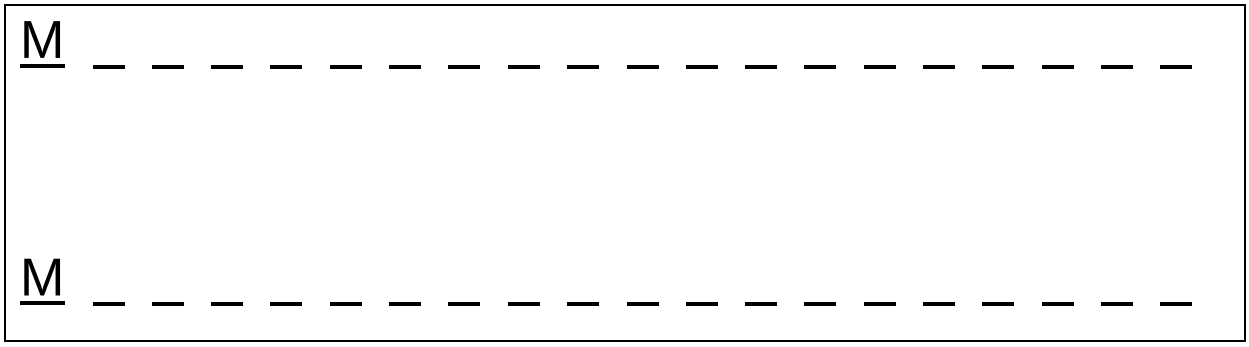
- heat in microwave until solution is clear (200 ml: 3 x nr.8-60sec. + stir + extra time might be needed)
- let bottle cool down (under streaming water)
- set the mold in the holder
- place spacers/combs
- pour cooled agarose mixture in cast (± 55°C - no air bubbles!)
- let it rest
- remove spacers and place gell in correct position

PS: for genomic DNA gel (small): 0,8% agarose = 0,56g agarose + 70ml TAE, at 100mV for 45 min.

loading the gel:

- load 3 µl of molecular weight marker (one slot/row) GeneRuler 1 kb (store at -20°C)
- load 3-5 µl of PCR products into gel slots
- run gel: **120 V - 400mA - 30 min for small gel, 50 min for large gel.**

20 slots



photographing the gel in the ethidium bromide (EtBr) room:

- introduction to room by Pieter or colleague
- always put coat & orange gloves on before entering the EtBr room
- everything **in** the EtBr room **= dirty** / everything **out** the EtBr room **= clean --- don't mix !**
- leave gloves and all contaminated material in the EtBr room
- transfer gel on aluminium foil
- wear extra coat and gloves
- fill out form in the EtBr-room
- place gel in EtBr bath: **30 min.**
- put gel in BioRad machine and switch UV light on
- open the program to take photo of gel (login: biorad, password: biorad):

  file – geldox – auto – manual – decrease exposure until no more red spots – save – image – crop – save – file – print – print settings – actual size – print

  --- don't leave the room with contaminated gloves ---

- after photographing, discard gel

throw away the gloves before leaving the EtBr-room

Appendix D: Macrogen protocol

PCR product purification with Exonucleas I + fastAP™ (www.fermentas.com)

Make stock solution:

- 100µl Exonuclease I (4000units, 65 euro)
  - ➔ Removes residual oligonucleotides and single-stranded DNA from the PCR product
- 200µl fastAP thermosensitive alkaline phosphatase (1000units, 55 euro) (successor of CIAP)
  - ➔ Catalyzes the removal of 5' phosphate groups from DNA, thus treating unincorporated dNTPs and preventing self ligation
- 30µl buffer (is supplied with both exonuclease and fastAP)
- 270µl H_2O

→ Add 1µl of this stock with 5µl PCR product

→ Mix, spin down and incubate 15 minutes at 37°C, followed by 15 minutes at 85°C to inactivate the enzymes

→ This product can be used as the purified PCR product in the next steps

Preparing for Macrogen

Forward: 5µl sample + 5 µl primer Forw (5µM)

Reverse: 5µl sample + 5 µl primer Rev (5µM)