



A molecular analysis reveals hidden species diversity within the current concept of *Russula maculata* (Russulaceae, Basidiomycota)

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Abstract

The current generally accepted concept of *Russula maculata* defines the species by yellow-brownish spots on the basidiomata, an acrid taste, a yellow spore print and a red pileus. This concept was tested using collections originating from various geographical areas mainly in Europe. Analyses of the ITS region suggested that there were three species within this broad concept. One of them, *R. maculata*, was identified based on the sequence from the epitype. Two other species, *R. nympharum* and *R. sp.*, are described here as newly identified species. The European species *R. maculata* and *R. nympharum* grow in deciduous forests, are similar in their field aspect and are distinctly different in micro-morphological characteristics of spores, pleurocystidia and pileipellis. An Asian species, *R. sp.*, is associated with pine and has smaller basidiomata and spores. These three species form the *R. maculata* complex and represent the sister clade to the *R. globispora* complex. This clade consists of species also characterized by a yellow-brownish context discolouration but with a different type of spore ornamentation. All of the other tested species had an acrid taste and yellow spore print but did not have a conspicuous yellow-brownish context discolouration and were placed in various unrelated clades.

Key words: DNA barcode, ectomycorrhizal fungi, morphology, Pakistan

Introduction

The genus *Russula* Persoon (1796: 100) has more than 200 generally accepted species in Europe (Sarnari 1998, 2005) and more than 750 species worldwide (Kirk *et al.* 2010). Because of this huge taxonomic diversity, current keys to *Russula* frequently use intricate combinations of macro- and micro-morphological characteristics and habitat preferences (e.g., Knudsen *et al.* 2012). Within this complex framework, *Russula maculata* Quélet (1878: 323) is currently considered a well-defined species with striking macro-morphological characteristics, such as its reddish cap, yellow lamellae due to the yellow spore print, the presence of rusty or yellow-brownish spots on the cap and stipe surface and its fairly firm, acrid context. These characteristics are believed to allow for field recognition of this species (Sarnari 1998).

Russula maculata belongs to the generation of early post-Friesian *Russula* names. The original diagnosis (Quélet 1878) is brief and contains only a rudimentary description of the micro-morphological characteristics, as was typical for descriptions of agaricoid fungi in that period. However, the conspicuous spots on the pileus surface, at which the epithet hints, apparently made the species well recognizable, and it became widely accepted. Later interpretations of

Russula maculata were expanded to include some non-spotted russulas with a greyish context discolouration. These are now considered to be separate species but were originally proposed at an infraspecific rank, e.g., *R. maculata* var. *decipiens* Singer (1931: 212) and *R. maculata* f. *paradecipiens* Favre (1992: 19). The introduction of refined microscopic techniques in the first half of the 20th century allowed for the recognition of two, macro-morphologically very similar taxa that were first proposed at a varietal rank. These are currently regarded as separate species, *Russula dryadicola* Fellner & Landa (1993: 34) and *Russula globispora* (Blum 1952: 232) Bon (1986: 55). Both differ from *R. maculata* and have larger, subglobose spores with isolated warts.

As a well-known and almost ‘classical’ species, *Russula maculata* was selected as a type of *Russula* section *Maculatinae* Romagnesi (1962: 173). This section of Romagnesi’s influential systematic framework contains all acrid species with a yellow spore print and reddish pilei (Romagnesi 1967). Sarnari (1998) reconsidered the importance of the pileus colour for infrageneric classification and re-grouped the bulk of the *Maculatinae* species in the broader concept of the *Russula* subsection *Urentes* Maire (1910: 122), which is defined by an acrid taste, yellow spore print, non-incrusted and sulfovanillin positive pileocystidia and the presence of an amyloid suprahilar spot on the spores. Sarnari was well aware of the heterogeneity of this large group of species and further subdivided it, provisionally proposing seven morphological groups ranked as a series. According to this concept, the series *Maculata* n. inval. (incl. *R. maculata*) is characterized by a yellow-brownish context discolouration (and a spotted pileus surface), a fairly dark spore print, large spores and pileocystidia with a few septa.

Russula maculata in its current sense has a wide range of habitats and a wide distribution. It has been reported in various deciduous forest types e.g., in Scandinavia and Germany (Gminder *et al.* 2000, Kauffman 2004), preferring warm, sunny, base rich sites with oak and beech in Germany and Switzerland (Gminder *et al.* 2000, Kränzlin 2005), the Mediterranean *Quercus ilex* forests (Sarnari 1998), and the oak mountain forests of Algeria (Bertault 1978). Gminder and co-workers also reported occurrences in conifer plantations. The species is also known from Asia, but there, it is found in association with conifers as follows: with pines in Pakistan (Sultana *et al.* 2011) and with *Keteleeria evelyniana* (Pinaceae) in China (Ge *et al.* 2012). Das *et al.* (2006) described an Indian species associated with *Quercus*, *R. mayawatiana* Das, Miller & Sharma (2006: 206), and they placed it close to *R. maculata* according to ITS data (the nrDNA internal transcribed spacer region) despite its lack of yellow-brownish spots.

The aim of this study was to determine whether the widely accepted morphological delimitation of *R. maculata* corresponds to a single species. For this purpose, a phylogenetic analysis of the ITS region and morphological studies were performed using material from different geographical areas and habitats.

Materials and methods

Sampling.—Our morphological observations were based on 13 herbarium collections identified as *R. maculata* originating from Europe and one collection from Pakistan. All these collections were also sampled for a phylogenetic analysis of ITS data and were supplemented with other GenBank and UNITE (Kõljalg *et al.* 2013) sequences from Europe and Asia that were retrieved in BLAST searches with a high similarity to the target group. To assess the taxonomic importance of the yellow-brownish context discolouration (i.e., the presence of yellow-brownish to rusty spots on the stipe and pileus surface), we sampled representatives of the *Russula* section *Maculatinae* as defined by Romagnesi (1967) and of the *Russula* subsection *Urentes* as defined by Sarnari (1998) and Knudsen *et al.* (2012). Moreover, *Russula* subsection *Rubrinae* (Melzer & Zvára 1927: 45) Singer (1932: 242) as emended by Sarnari (1998) has also been included because one of its representatives, *R. rutila* Romagnesi (1952: 112), was formerly classified in the section *Maculatinae* (Romagnesi 1967). These groups comprise taxa characterized by medium to large basidiomata with a fairly firm, acrid context, a yellow (or, more rarely, dark ochre) spore print, spores with an amyloid suprahilar spot and pileocystidia with greying contents in sulfovanillin. This definition covers the following species that were included in this study: *R. aurantioflammans* Ruots., Sarnari & Vauras in Sarnari (1998: 717), *R. badia* Quélet (1881: 668), *R. cuprea* (Krombholz 1845: 11) Lange (1926: 47), *R. decipiens* (Singer 1931: 211) Svrček (1967: 228), *R. dryadicola*, *R. formula* J. Schäffer (1940: 111), *R. globispora*, *R. intermedia* Karsten (1888: 38), *R. juniperina* Ubaldi (1985: 25), *R. rubra* (Fries 1821: 58) Fries (1838: 354), *R. rutila* and *R. veternosa* Fries (1838: 354). Our sampling provided a high coverage of the commonly accepted European species in the subsections *Urentes* and *Rubrinae*. Collections sequenced in this study were identified to the species level according to Sarnari (1998) and Knudsen *et al.* (2012). In addition, the phylogenetic analysis included some species with a mild taste or paler spore print that appeared to be closely related to one or more of the 12 target species based on BLAST search results of against GenBank and UNITE or based on a comparison with the ITS *Russula* phylogeny published by Looney *et al.* (2016).

We selected three sequences for each species from reliable sources, preferably from material that had been identified by us and that originated from geographically distant areas. Three sequences of *R. emetica* (Schaeffer 1774: 9) Persoon (1796: 100) were used as an outgroup. All specimens and sequences are listed in the Supplementary Tab. 1. Herbarium material collected by the authors is deposited in the following herbaria: SAV, GENT, STU, M and LAH.

Molecular analysis.—The total genomic DNA was extracted using a variety of protocols (Eberhardt 2012, Nuytinck & Verbeken 2003, Van de Putte *et al.* 2010). In addition to these cited methods and consumables, the following procedures were used: The ArchivePure DNA yeast and Gram-+kit (5 PRIME, Inc. Hilden, Germany) with a longer incubation time of 0.5 to 12 hours after the addition of Lytic Enzyme Solution or the High Pure PCR Template Preparation Kit for the Isolation of Nucleic Acids from Mammalian Tissue (Roche Applied Science, Indianapolis, USA). The ITS region was amplified using the primers ITS1F–ITS4 or alternatively ITS1F–ITS2 and ITS3–ITS4 (White *et al.* 1990, Gardes & Bruns 1993) and with polymerase PerfectTAQ (5 PRIME, Hilden, Germany) in accordance with the manufacturers recommendation. The PCR amplification followed the protocols of Eberhardt (2012) or Knebelsberger & Stöger (2012) with longer denaturation and annealing times of up to 1 min instead of 30 sec.

The PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) or Exo-Sap enzymes (Thermo Fisher Scientific, Wilmington, Delaware, USA) and directly sequenced with BigDye 3.1 technology (Applied Biosystems, now Thermo Fisher Scientific, Wilmington, USA).

Raw sequences were edited in the BioEdit Sequence Alignment Editor version 7.2.5 (Hall 2013) or Sequencher version 4.8 (Gene Codes Corporation). Intra-individual polymorphic sites having more than one signal were marked with NC-IUPAC ambiguity codes. Edited sequences were aligned by MAFFT version 7 using the strategy E-INS-i (Katoh & Standley 2013).

Phylogenetic analysis.—The final alignment included 78 ITS sequences altogether. Twenty-one sequences corresponded to the current concept of *R. maculata*. Of these, 14 were obtained from herbarium material, while seven additional sequences were retrieved from GenBank or UNITE. Only the ITS1 and partial 5.8S rRNA were obtained from the epitype of *R. maculata*. The aligned data were analysed using Bayesian inference (BI) and the Maximum Likelihood method (ML).

For BI, the dataset was divided into three partitions: ITS1, 5.8S and ITS2. The best substitution model for each partition was computed separately in MEGA (Tamura *et al.* 2013). The following models were chosen for the analysed partitions: K2+G (ITS1), JC (5.8S) and HKY+G+I (ITS2). The Bayesian inference was computed independently twice in MrBayes 3.2.6 (Huelsenbeck *et al.* 2015) in two simultaneous runs with four MCMC chains each run for 10 000 000 iterations. The convergence of runs was visually assessed using Trace function in Tracer version 1.6 (Rambaut *et al.* 2013).

Maximum Likelihood was computed in PhyML using SeaView (Gouy *et al.* 2010) using GTR+GAMMA substitution model with 4 rate classes and 1000 bootstrap replications.

Morphological observations.—Micromorphological characteristics were observed using Olympus CX-41 with oil-immersion lenses at a magnification of 1000×. All drawings of microscopic structures, with the exception of spores, were made with a ‘camera lucida’ using an Olympus U-DA drawing attachment at a projection scale of 2000×. The contents of hymenial cystidia and pileocystidia were illustrated as observed in Congo red preparations from dried material, with the exception of some pileocystidia for which the contents are indicated schematically (dotted). Spores were observed on the lamellae with Melzer’s reagent. All other microscopic observations were made in ammoniacal Congo red, after a short treatment in warm, aqueous KOH solution to dissolve the gelatinous matrix and improve tissue dissociation. All tissues were also examined in Cresyl blue to verify the presence of ortho- or metachromatic reactions as explained in Buyck (1989). Trama and cystidia were examined in a sulfovanillin solution. Acidoresistant incrustations of the primordial hyphae were stained with carbolfuchsin and observed in distilled water after incubation for a few seconds in a 10% solution of HCl (cf. Romagnesi 1967). Spores were scanned with an Artray Artcam 300MI camera and measured by the Quick Micro Photo version 2.1 software. Measurements and line drawings were made using enlarged, scanned pictures of spores with an accuracy of 0.1 µm. The Q value was used to indicate the length/width ratio of the spores. Spore measurements excluded ornamentation. The spore ornamentation density was estimated following Adamčík & Marhold (2000). The cystidia density estimates follow Buyck (1991). Statistics for the measurements of microscopic characteristics were based on 30 measurements per specimen and expressed as the mean ± standard deviation. The species descriptions are based on all studied specimens.

To support the morphological delimitation of a species, we compared the full-detail descriptions (similar to those presented by Adamčík & Jančovičová 2013) of one representative specimen for each clade retrieved in the molecular analysis. The comparison of these representative specimens allowed us to reduce the number of observed characteristics to those showing significant differences in the Tab. 1 (see the three-step procedure in Adamčík *et al.* 2016).

TABLE 1. Comparison of selected characters observed on the studied material of *R. maculata*, *R. nympharum* and *R. sp.* All values of micro-morphological characters are averages of 30 measurements. TC = terminal cells of hyphae in pileipellis. Distinguishing characters are in shaded boxes.

| <i>Herbarium number</i> | <i>R. maculata</i> | | | | | | <i>R. nympharum</i> | | | | <i>R. sp.</i> | |
|---|---------------------------|-----------------|------------|-------------------|------------|------------|---------------------|-------------------|------------|------------|---------------|----------------|
| | PC84521 | FH 2011BT005 | SAV F-933 | 2009BT05 | 2010BT112 | 2010BT184 | SAV F-2130 | FH RUS11121505 | R-9702 | hib10019 | R-0898 | LAH MAM0077 |
| Spores | Size [µm] | 9.1 × 7.5 | 9.3 × 7.8 | 9.2 × 7.6 | 9 × 7.6 | 9.1 × 7.6 | 9.2 × 7.6 | 8.7 × 7.3 | 9.6 × 7.9 | 9 × 7.6 | 9.3 × 7.7 | 8.2 × 6.8 |
| | Q | 1.22 | 1.19 | 1.21 | 1.19 | 1.2 | 1.21 | 1.19 | 1.23 | 1.19 | 1.21 | 1.21 |
| | ornamentation [µm] | 0.6–0.7 | 0.8–1.1 | 0.6–0.9 (–1.1) | 0.6–1 | 0.7–1 | 0.7–0.9 (–1) | 0.3–0.6 | 0.4–0.6 | 0.3–0.6 | 0.4–0.6 | 0.4–0.6 |
| TC near the pileus margin | TC size [µm] | 28.3 × 3.7 | 25 × 3.4 | 21.1 × 3.6 | 18.2 × 2.9 | 21.7 × 3.2 | 26.4 × 3.4 | 21.8 × 2.9 | 26.6 × 2.9 | 21.2 × 2.7 | 23.8 × 2.5 | 28.7 × 3 |
| | attenuated TC [%] | 80 | 72 | 50 | 61 | 75 | 85 | 40 | 37 | 0 | 32 | 66 |
| | difference in width of TC | 1.85 | 0.95 | 1.23 | 1.02 | 1.14 | 1.21 | 0.87 | 0.87 | 0.35 | 0.25 | 1.5 |
| Pileocystidia near the pileus margin | size [µm] | 61.4 × 4.9 | 51.9 × 5.1 | 43 × 6.7 | 63.9 × 5.1 | 59 × 5.5 | 72 × 5.9 | 78 × 10.1 | 64 × 9.1 | 79 × 8.8 | 62.4 × 7.2 | 45 × 6.5 |
| | cell number | 1.3 | 1.3 | 2.5 | 1.6 | 1.3 | 1.5 | 1.7 | 1.2 | 1.5 | 1.3 | 2.7 |
| Pleurocystidia | density | 500–600 | 500–700 | 400–500 | 400–500 | 500–700 | 400–600 | 1000–1100 | 900–1100 | 900–1100 | 800–900 | 300–400 |

Results

Molecular analysis.—The BI majority rule consensus and the ML result did not entirely agree in their gross topology, but corresponded with respect to the clades that include the target species. Figure 1 shows the ML results with both bootstrap and posterior probability support. The disagreement between the results concerns the placement of *R. intermedia*, which is included in a clade with *R. vinososordida* and *R. decipiens* in the BI result. This clade, as well as its sister clade (corresponding topologically to the ML result, but without *R. intermedia*) had borderline posterior probability support. The corresponding clades did not have ML bootstrap support (Fig. 1).

All species with yellow-brownish spots included in this study were grouped in a moderately supported clade, referred to here as the *Maculatinae* clade, that includes two well-supported subclades referred to as the *R. globispora* complex (including *R. globispora* and *R. dryadicola*) and the *R. maculata* complex. The latter subclade contains all sequences corresponding to the current morphological concept of *R. maculata*. Within the *R. maculata* complex, all sequences originating from European specimens are clustered in a well-supported subclade that contains two well-supported terminal clades. The first was identified as *R. maculata* because the sequence from the epitype was included there. The second terminal clade represents a new species described below as *R. nympharum* F. Hampe & Marxm. Our collection LAH MAM077 from Pakistan was tentatively identified as *R. maculata* and was placed together with other sequences, one each from China and Papua New Guinea, in a well-supported subclade. Compared to the other subclades of the *Maculatinae* clade, these sequences are on long terminal branches, similar in length to the internal branches of the *R. maculata* or *R. nympharum* clades. This extra-European subclade, together with another singleton sequence of Asian origin, forms a sister cluster to the *R. maculata* / *R. nympharum* subclade.

All other species currently classified in the section *Maculatinae* and the subsection *Urentes*, but not characterized by conspicuous yellow-brownish context discolouration and yellow-brownish spots, are placed in various unrelated clades and sometimes even grouped with mild-tasting species, e.g., *R. decipiens* with *R. vinososordida* Ruots. & Vauras in Vauras & Ruotsalainen (2001: 558), *R. intermedia* with *R. vinosa* Lindblad (1901: 57) or *R. aurantioflammans* with *R. font-queri* Singer (1947: 215).

Morphological differences among the species studied.—The group defined here as the *R. maculata* complex contains three distinct terminal clades. Two of them seem to correspond to only one phylogenetic species each. The third clade contains three sequences of Asian origin and shows a relatively high variability in the ITS region. Of the sequenced collections, only the Pakistani collection LAH MAM077 has been available to us for morphological analysis and is described in full detail as *Russula* sp. in the Taxonomy section below. Selected micro-morphological characteristics of *R. maculata* and the two new species are compared in Tab. 1. We have not found any macro-morphological characteristic that distinguishes *R. maculata* from the second European species *R. nympharum*, but there are four distinct microscopic differences. The latter species has a lower spore ornamentation (up to 0.6 µm), the terminal cells of the hyphae in the pileipellis near the pileus margin are less frequently attenuated and apically constricted, the pileocystidia are wider than 7 µm on average (the individual pileocystidia are occasionally wider than 10 µm) and the pleurocystidia are more densely arranged. On the other hand, the Pakistani species is distinct even in its field aspects (Figs. 2–4). It has smaller and less robust basidiomata. Under the microscope, it is similar to *R. maculata*, but differs from it by its smaller spores and its pileocystidia composed of more cells and more septae.

Discussion

Delimitation of members of *Russula* section *Maculatinae* with yellow-brownish spots.—Our phylogenetic analysis (Fig. 1) suggests that all infrageneric taxa currently used to accommodate *R. maculata* based on the acrid taste of the context and the yellow spore print (i.e., *Russula* section *Maculatinae* and *R.* subsection *Urentes*) are polyphyletic. A posterior probability of 0.94 (Fig. 1) in backbone of the tree is the only statistical support given to any clade retrieved by both BI and ML, and this clade includes both, species that combine an acrid taste of the context and yellow spore print and species with other characteristics (i.e., mild taste or ochre or paler spore print). In spite of its inconsistent support, this result is in line with earlier studies of the genus (Eberhardt 2002, Miller & Buyck 2002). These also suggested that a number of infrageneric taxa including species with a mild-tasting context or species with yellow spore prints irrespective of taste, are unlikely to have phylogenetic support in taxonomically more inclusive studies.

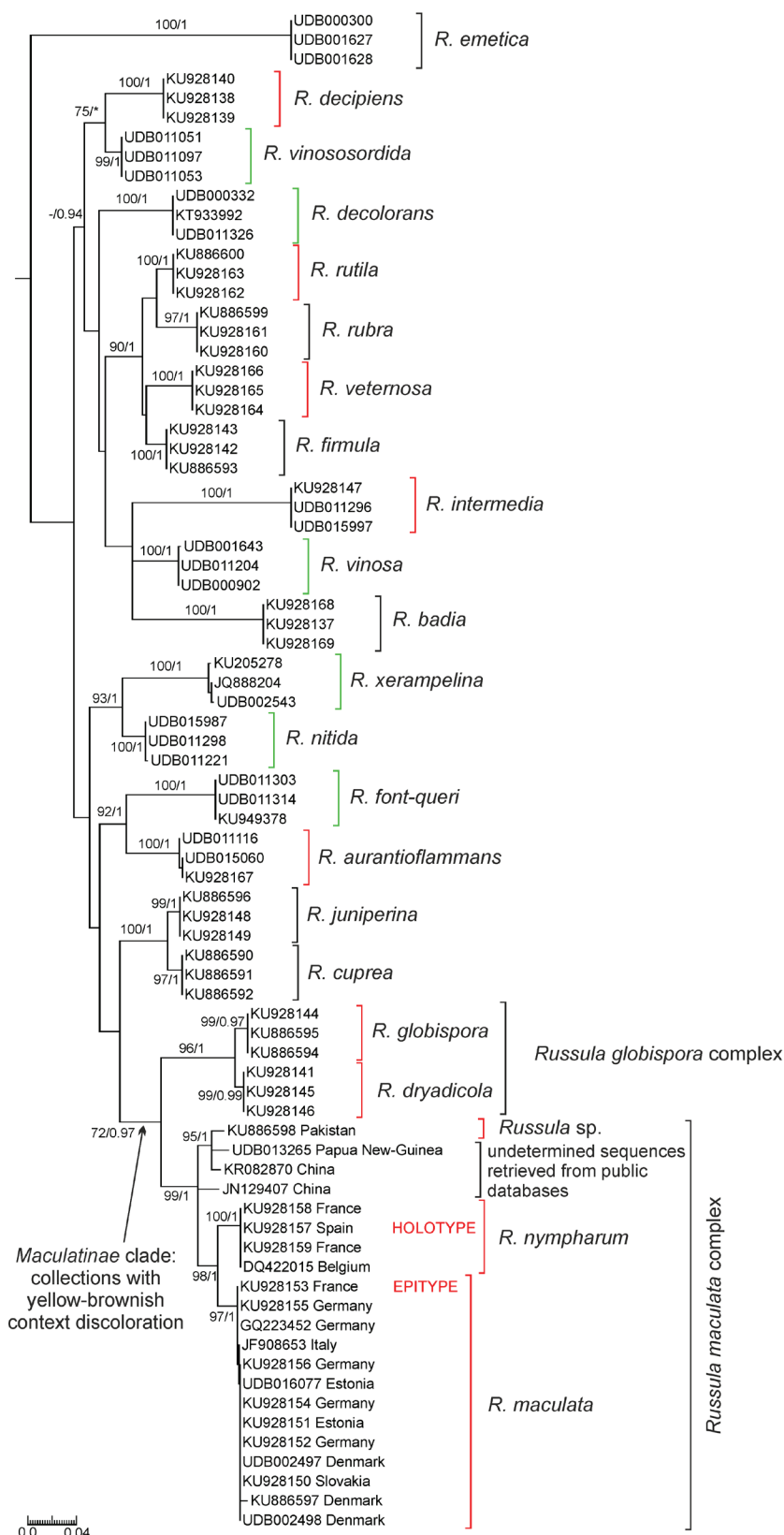


FIGURE 1. Maximum Likelihood phylogeny based on ITS nrDNA region with species-level clades highlighted as well supported clades of species with yellow-brownish context discoloration. Species belonging to *R.* sect. *Maculatinæ* in sense of Romagnesi (1967) are indicated by red brackets and species with mild taste by green brackets. The * indicates a clade which was not retrieved in the BI analyses. Origin of sequences is listed in the Supplementary table 1. Labels of type collections and country of origin are indicated for members from the *R. maculata* complex. Bootstrap values followed by Bayesian posterior probabilities are indicated. Support values are only indicated for nodes with either a bootstrap value above 70 or a posterior probability at or above 0.90.

A core group around *R. maculata* had some bootstrap support and good posterior probability support. It consists of species that have an acrid taste, a yellow spore print and a yellow-brownish context discolouration. This discolouration, results in the appearance of yellow-brownish spots on the basidiomata surfaces in early stages of their development, and seems to be a rather good and stable characteristic. Combined with other traditionally used characteristics, it allows the identification of a group of fungi that includes both the *R. maculata* and *R. globispora* species complexes (referred here as the *Maculatinae* clade). Sarnari (1998) was the first author who fully recognized the major taxonomic importance of this characteristic and consequently considered *R. maculata*, *R. dryadicola* and *R. globispora* to form a fairly homogeneous, morphological group within the subsection *Urentes*, which he provisionally referred to as the “series *Maculata*” (nom. inval.). The only validly published name with a defined taxonomic rank for an infrageneric taxon typified by an element contained in the *Maculatinae* clade is the *Russula* section *Maculatinae*. We have not decided on the taxonomic rank of the *Maculatinae* clade primarily because no phylogenetic framework exists upon which a new overall systematic concept of this large and widely distributed genus can be built. Additionally, our sampling for this study was too limited among potentially related infrageneric taxa.

There are two distinct groups distinguishable by their spore ornamentation in the *Maculatinae* clade that correspond to the subclades identified in our molecular analyses. The first, the *Russula maculata* complex, is defined by moderately prominent spore ornamentation to low warts with frequent connectives. The second, the *Russula globispora* complex, has spores with large isolated spines. It is represented in our analysis by *R. dryadicola* and *R. globispora*.

Species nomenclature of the R. maculata complex.—*Russula maculata* is the only species that was recognized in the *R. maculata* complex prior to our study. Recent studies have shown that clearly distinct species are often overlooked, especially in widely recognized species, with conspicuous macro-morphological characteristics. Such assumptions can be a reason for false confidence about a species concept (e.g., Morgado *et al.* 2013, Ainsworth *et al.* 2013). A good example within the family Russulaceae is the European *Lactifluus volemus* (Fries 1821: 69) Kuntze (1891: 857), which was revealed to be a complex of three previously unrecognized species (Van de Putte *et al.* 2016). *Russula maculata* seems to be a similar example.

Most of the characteristics identified here as useful for distinguishing among *R. maculata*, *R. nymphaeum* and *R. sp.* from Pakistan have not been used previously for species delimitation and identification. For this reason, it is difficult to judge if European authors have treated *R. maculata* in a broad sense or if their descriptions can be referred to one of the two European species recognized here. The pileocystidia width is the distinguishing characteristic for *R. maculata* and *R. nymphaeum* (Tab. 1) that is also often included in literature descriptions of *R. maculata*. The measurements given by Romagnesi (1967) for *R. maculata* pileocystidia generally correspond to the typical form (5.2–8.7 µm wide), whereas Einhellinger (1987) and Sarnari (1998) described and illustrated pileocystidia width ranges that may correspond to either species (3–11 µm or 4.2–10 µm, respectively). Marxmüller (2014) provided descriptions and illustrations of two collections: the first (R-8102) probably refers to *R. maculata* sensu stricto (pileocystidia up to 8 µm), but the sequence of the second (collection R-0898) clearly places it in the *R. nymphaeum* clade (Fig. 1). Lejeune (2004) described a discoloured form of *R. maculata* (referred to as the “straminoid form”) and his illustration of the pileocystidia suggests that his description is based on a collection of *R. nymphaeum*.

Our study suggests that at least one species of the *R. maculata* complex occurs in Asia and is associated with conifers; *R. sp.* from Pakistan (KU886598) was associated with *Pinus roxburghii*. Two sequences from outside Europe (UDB013256 from a Papua New Guinea, from a fruitbody collection, recorded with *Castanopsis acuminatissima*, and KR082870 from China with no published ecological details) have the same monophyletic lineage, but sufficient phylogenetic support does not exist to conclude whether they are conspecific with the Pakistani species. The other Asian sequence (JN129407 from China) was generated from a mycorrhizal root tip of *Keteleeria* (*Pinaceae*) (Ge *et al.* 2012) and possibly represents a distinct, undescribed species. Because of geographical distance, ecological difference, ITS variation and paraphyly (Fig. 1), it is possible that the three other sequences retrieved from GenBank (Supplementary Tab. 1) represent species different from *R. sp.* from Pakistan. A previous report of *R. maculata* from Pakistan associated with pines (Sultana *et al.* 2011) probably represents the same species as described here, but we were not able to obtain the corresponding material for confirmation.

Some taxa, either recently described or with a dubious concept, might represent nomenclatural challenges to these new species within the *R. maculata* complex or the wider subsection *Urentes*. The following two names contain a direct reference to the epithet “*maculata*” and should be especially mentioned: *R. maculata* f. *paradecipiens* Favre (1992: 19) and *R. globispora* var. *submaculata* Sarnari (1998: 704). The first, now combined at the species rank [*R. paradecipiens* (Favre) Favre (1999: 30)], was described as having a distinctly greying or even a blackening context (Favre 1992). The second might be distinguished by isolated amyloid spines on spores that clearly matched the *R. globispora* complex as defined above.

Several other species previously classified in the section *Maculatinae* were not included in our study. Although validly published and legitimate, they have not been accepted in most recent studies, e.g., *R. britzelmayrii* Romell in Britzelmayr (1893: 12), *R. cerasina* Martín (1894: 187), *R. poetae* Reumaux *et al.* (1999: 425), *R. papavericolor* Reumaux in Reumaux *et al.* (1996: 286). In our opinion, they do not represent close relatives of *R. maculata* because no conspicuous yellow-brownish spots were mentioned in the descriptions. For the same reason, we are confident that the Indian species *R. mayawatiana* is not a close relative of *R. maculata*. It has no yellow-brownish spots, moreover, the spore ornamentation corresponds more to the *R. globispora* complex. The authors of the Indian species, Das *et al.* (2006), placed it close to *R. maculata* based on ITS data, but the position of both species suggests that the sequence of *R. maculata* used was misidentified (unfortunately the authors do not cite sequence vouchers and the sequences are probably not deposited in public databases).

Distribution and ecology of R. maculata and R. nymphaeum.—Our assessment of the ecological preferences of the two European species is exclusively based on the limited material used in this study. The species descriptions in the literature are difficult to interpret, and therefore, we cannot reliably associate hosts. Our *R. maculata* collections originated from areas as far north as Estonia and as far south as Northern Italy. They were found in association with *Quercus*, *Fagus*, *Carpinus* and *Tilia* in mostly temperate broadleaf forests, but the Slovak collection SAV F-933 originated from a dry, thermophilous, steppe-like habitat with *Quercus cerris*. Our sampling of *R. nymphaeum* suggests a certain preference for Mediterranean evergreen oak forests (collections from Southern France from Val des Nymphes and Mallorca Island), but the collection from Belgium near Brussels is from outside the Mediterranean area. Thus, our data seem to indicate at least a partly overlapping distribution range because the epitype of *R. maculata* originated from Oise region north of Paris and *R. nymphaeum* was collected in Belgium, less than 200 km to the north. It is also very possible that they may share the same type of habitat and even co-occur at a single spot.

Taxonomy

Russula maculata Quél., Bull. Soc. Bot. France 24: 323. 1878. Figs. 2–3

The epitype of the species (PC0084521) was designated, described and illustrated by Adamčík & Jančovičová (2013), and its description is supplemented here by statistical values of micro-morphological characteristics (Tab. 1).

Examined material.—DENMARK. Vejle: Staksrode Skov, N of Vejle Fjord, *Fagus*, 10 August 1999, P.B. Hansen, J. Vesterholt JV99-196 (C); Lolland: Kristianssædevej, S of Kristianssæde Skov, *Tilia*, 6 July 2001, P.B. Hansen & J. Vesterholt JV01-061 (C); Sjælland island: Vejlø Skov S of Næstved, *Fagus*, *Quercus*, 4 September 2001, P.B. Hansen JV01-304 (C); ESTONIA. Saarnaki island: 13 km SEE of Käina, 58°48'6"N 23° 03'88"E, *Tilia*, 23 September 2008, S. Adamčík (SAV F-2130); FRANCE. Oise Dept.: Boran-sur-Oise, *Betula*, *Corylus*, *Quercus*, 14 July 1954, H. Romagnesi 54.43 (PC0084521, epitype); GERMANY. Thüringen: Gustav-Freytag-Weg (Schlossleite) near Arnstadt, 1 June 2009, J. Girwert FH 2009 BT05 (GENT); Rüdigsdorfer Schweiz, near Nordhausen, *Quercus*, 18 September 2010, F. Hampe FH 2010 BT184 (GENT); Gotha palace garden, *Carpinus*, *Fagus*, *Quercus*, *Tilia*, 22 August 2010, F. Hampe FH 2010 BT112 (GENT); Ibid., *Tilia*, *Carpinus*, *Quercus*, 5 July 2011, F. Hampe FH 2011 BT005 (GENT); SLOVAKIA. W Carpathians: Cerová vrchovina Mts., 1.5 km NW of Obručná village, Manický potok, 48°12'15"N 19°51'59"E, *Quercus cerris*, 9 June 2004, S. Adamčík (SAV F-933).

Russula nymphaeum F. Hampe & Marxm., *sp. nov.* Figs. 4, 6–15

MycoBank no.:—MB 816289.

Etymology.—The species epithet refers to the collection site (Val des Nymphes) of two of the paratypes one of which was illustrated in Marxmüller (2014).

Holotype (designated here).—SPAIN. Mallorca: Bunyola, associated with *Quercus ilex* and *Arbutus unedo*, 15 December 2011, FH11121505 (GENT).

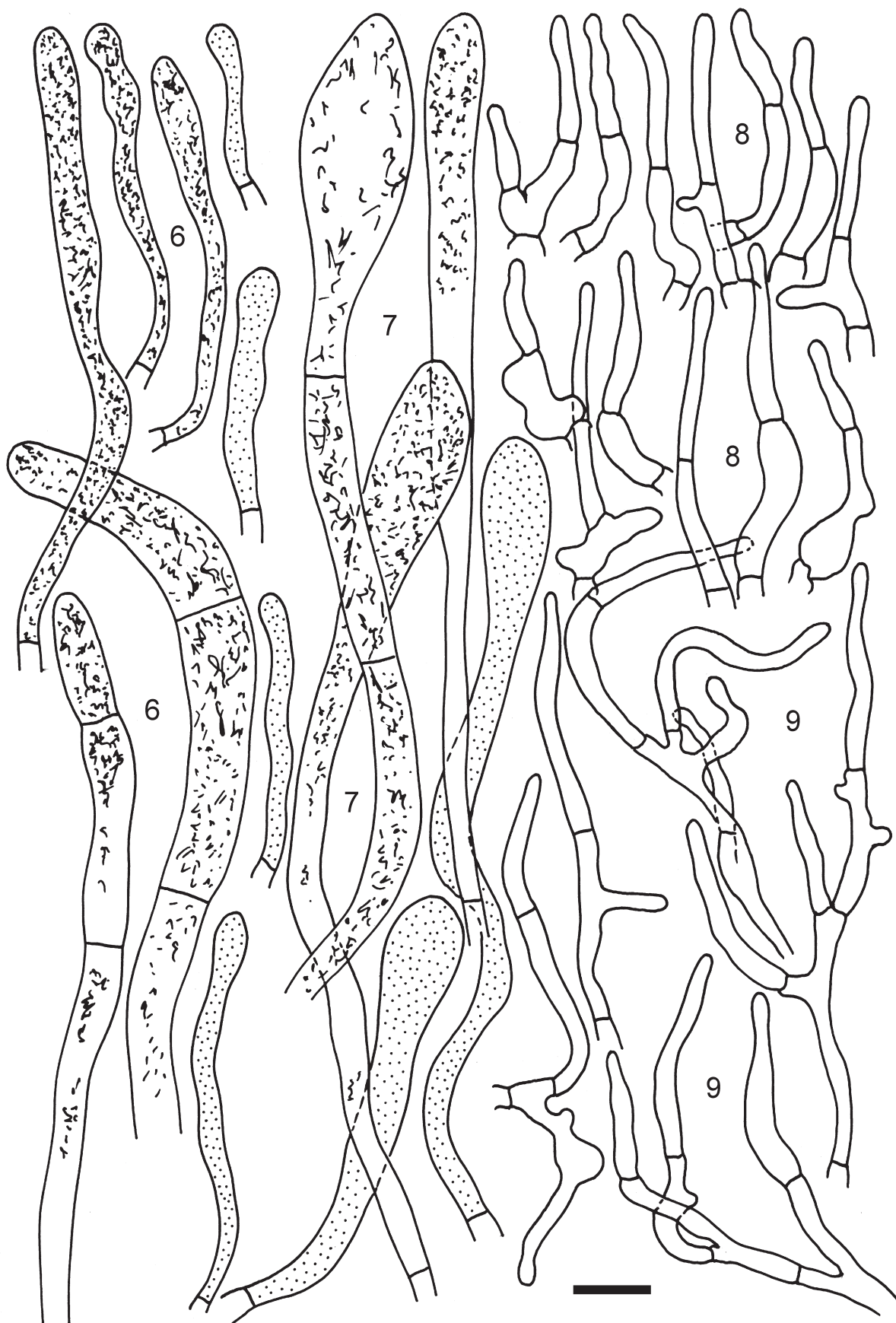
Short diagnosis.—Basidiomata relatively large and with firm, thick context, surface of stipe, pileus and lamellae with yellow-brownish spots, pileus cuticle red or orange and discolouring to cream, taste acrid, spore print yellow, spore ornamentation with low (up to 0.6 µm), amyloid warts often merged or connected by line connections, hymenial cystidia relatively numerous, hyphal terminations in pileipellis near the pileus margin mainly cylindrical, pileocystidia near the pileus margin 6–12 µm wide (on average wider than 7 µm).



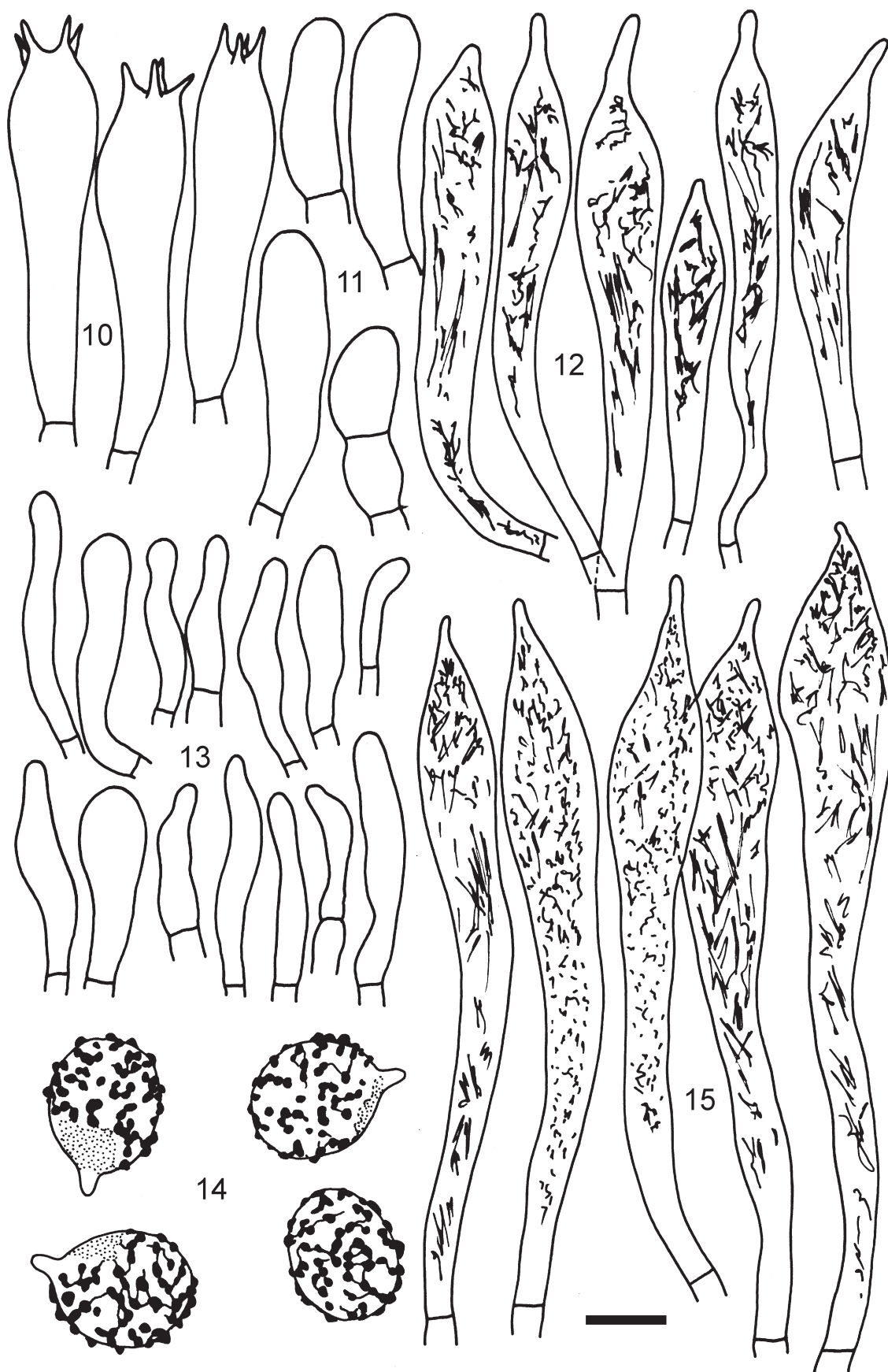
FIGURES 2–5. Field appearance of three *Russula* species included in this study. **2.** *R. maculata* (GENT 2011 BT 005). **3.** *R. maculata* (GENT 2010 BT 184). **4.** Holotype of *R. nymphaeum* (GENT RUS11121505). **5.** *Russula* sp. (LAH MAM 0077). Scale bar equals 30 mm, but only 10 mm for *Russula* sp. Photos by: F. Hampe (Figs. 2–4) and M. Saba (Fig. 5).

Pileus up to 100 mm broad, fleshy, firm, first hemispherical, then expanded with depressed centre, extreme margin becoming somewhat sulcate with age, cuticle peeling at margin to 1/3 of pileus radius, glabrous, shining, cream with pale rose to pale orange zones or reddish orange to pinkish red or deep red, often paler to cream at centre, mostly more or less covered with small brownish or reddish spots. *Lamellae* adnate, crowded, more or less furcate towards the stem, slightly anastomosing, fragile, cream, then yellowish, with yellow-brownish spots at age, edge smooth, concolourous or reddish especially towards the pileus margin. *Stem* cylindrical to slightly clavate with rounded base, firm, surface minutely wrinkled, whitish, staining yellow-brownish to brownish when handled or with age, especially towards the base. *Context* firm, whitish, staining yellow-brownish to brownish, taste acrid especially in lamellae, smell fruity or like cedar wood, FeSO_4 and Guaiac very weak or negative. *Spore print* IV d, IV d–e (coded according to Romagnesi 1967).

Spores broadly ellipsoid, $(8\text{--})8.6\text{--}9.8\text{--}(10.8) \times (6.5\text{--})6.9\text{--}7.6\text{--}(7.9) \mu\text{m}$, average $9.2 \times 7.3 \mu\text{m}$, $Q=(1.14\text{--})1.16\text{--}1.23\text{--}(1.27)$, average $Q=1.19$, ornamentation of moderately large and distant: 4–6(–7) amyloid warts in the circle of diameter of $3 \mu\text{m}$ on spore surface, warts $0.3\text{--}0.6 \mu\text{m}$ high, connected with occasional to frequent short or longer fine line connections [0–3 (–4) line connections in the circle], occasionally fused in short chains or crests [0–3(–4) fusions in the circle], chains and crests often branched, but rarely forming a reticulate structure, isolated warts rare. Suprahilar plage amyloid and very distinct. *Basidia* $(39\text{--})45\text{--}55.5\text{--}(62) \times (9\text{--})10.5\text{--}13\text{--}(14) \mu\text{m}$, average $50.3 \times 11.6 \mu\text{m}$, 4-spored, clavate, pedicellate; basidiola first cylindrical or ellipsoid, then clavate, ca. $5\text{--}10 \mu\text{m}$ wide. *Subhymenium* pseudoparenchymatic. *Lamellar trama* mainly composed of large sphaerocytes. *Hymenial cystidia* on lamellar sides moderately numerous, $800\text{--}1100/\text{mm}^2$, fusiform or rarely clavate, pedicellate, acute to acute-pointed on tips and with $2\text{--}7\text{--}(11) \mu\text{m}$ long appendage, thin-walled, measuring $(60\text{--})75\text{--}108.5\text{--}(140) \times (8\text{--})10.5\text{--}13.5\text{--}(15) \mu\text{m}$, average $91.7 \times 11.9 \mu\text{m}$, contents heteromorphous, mostly granular-crystalline, turning brownish-red to almost black in sulfovanilin. Lamellar edges covered with marginal cells, cheilocystidia and dispersed basidia; *marginal cells* on lamellar edges



FIGURES 6–9. *Russula nympharum* (holotype). **6.** Pileocystidia near the pileus centre. **7.** Pileocystidia near the pileus margin. **8.** Hyphal terminations in the pileus centre. **9.** Hyphal terminations near the pileus margin. Contents of cystidia are represented as observed in Congo Red for some elements only, the others are simply filled with dots to indicate their cystidial nature. Scale bar equals 10 μ m. Drawings by: S. Jančovičová.



FIGURES 10–15. *Russula nympharum* (holotype). **10.** Basidia. **11.** Basidiola. **12.** Cheilocystidia. **13.** Marginal cells. **14.** Basidiospores in Melzer's reagent. **15.** Pleurocystidia. Contents of cystidia are represented as observed in Congo red. Scale bar equals 10 μm , but only 5 μm for spores. Drawings by: S. Jančovičová.

not well differentiated, narrower than the basidiola on lamellar sides, mainly narrowly clavate to subcylindrical, often somewhat flexuous or moniliform, with obtuse tips, measuring $(15\text{--}20\text{--}31.5\text{--}33) \times (3\text{--}4\text{--}7\text{--}9) \mu\text{m}$, average $25.9 \times 5.4 \mu\text{m}$; *cheilocystidia* less voluminous than pleurocystidia, clavate or fusiform, pedicellate, mainly with acute tips and usually with $1\text{--}5\text{--}10) \mu\text{m}$ long appendage, thin-walled, measuring $(37\text{--}47\text{--}79\text{--}102) \times (7\text{--}8\text{--}10.5\text{--}12) \mu\text{m}$, average $63.1 \times 9.3 \mu\text{m}$, contents similar as in pleurocystidia. *Pileipellis* orthochromatic in Cresyl blue, $140\text{--}250 \mu\text{m}$ deep, not sharply delimited from the underlying spherocytes of the context; vaguely divided in a $70\text{--}120 \mu\text{m}$ deep, strongly gelatinized suprapellis composed of dense, ascending or erect hyphal ends (trichoderm type) and protruding and near surface repent pileocystidia, sometimes covered with an additional, up to $40 \mu\text{m}$ deep transparent, gelatinous matter that does not colour in Congo red; and a $70\text{--}165 \mu\text{m}$ deep subpellis of less gelatinized, dense, strongly intricate, horizontally oriented, often branched, $2\text{--}5\text{--}7) \mu\text{m}$ wide hyphae. Acidoresistant incrustations absent. Hyphal terminations in pileipellis near the pileus margin with gelatinous coating (not an acidoresistant incrustation) dissolving in KOH, but visible as a hyaline hue in Congo red, terminal cells very variable in size, some very short and comparatively shorter than subterminal cells, mainly cylindrical, sometimes attenuated towards apices, often flexuous, partly moniliform, measuring $(10.5\text{--}17.5\text{--}30.5\text{--}44) \times (2\text{--}2.5\text{--}3.5\text{--}4.5) \mu\text{m}$, average $20.1 \times 2.8 \mu\text{m}$; subterminal cells branched or not, often with lateral branches or nodules, more or less equally wide as terminal cells. Pileipellis near the pileus centre composed of hyphal terminations of one or two cells arising from a dense pseudoparenchymatic subpellis layer, terminal cells not very conspicuous among very numerous pileocystidia and sometimes completely suppressed, cylindrical or occasionally subulate, often flexuous-moniliform, measuring $(11\text{--}16.5\text{--}32.5\text{--}44) \times (2\text{--}2.5\text{--}3.5\text{--}4.5) \mu\text{m}$, average $24.5 \times 2.8 \mu\text{m}$. Pileocystidia near the pileus margin numerous and often very voluminous, narrowly to broadly clavate or fusiform, mainly one-celled, occasionally two or three celled, rarely with more cells, thin-walled or occasionally with slightly (up to $0.5 \mu\text{m}$) thickened walls, obtuse to subacute, usually inflated near apical part, with terminal cells measuring $(26\text{--}41.5\text{--}94.5\text{--}132) \times (4\text{--}6\text{--}11.5\text{--}15.5) \mu\text{m}$, average $68.1 \times 8.8 \mu\text{m}$, contents in Congo red heteromorphous, usually granulose, but sometimes partly crystalline or banded, in sulfovanilin turning dark brownish to blackish. Pileocystidia near the pileus centre very abundant, often clustered and in some spots completely replacing the undifferentiated hyphal terminations, very variable in shape and size, partly narrow and cylindrical, partly similar to those near the pileus margin (clavate or fusiform), but usually smaller, often flexuous and occasionally moniliform, terminal cells measuring $(18\text{--}28.5\text{--}77\text{--}119) \times (3\text{--}4.5\text{--}8.5\text{--}10.5) \mu\text{m}$, average $52.9 \times 6.5 \mu\text{m}$, contents similar to those near the pileus margin, but often with additional yellowish pigments visible in KOH or water. Cystidioid hyphae with heteromorphous contents in subpellis occasional and in the pileus trama dispersed. *Clamp connections* absent in all parts.

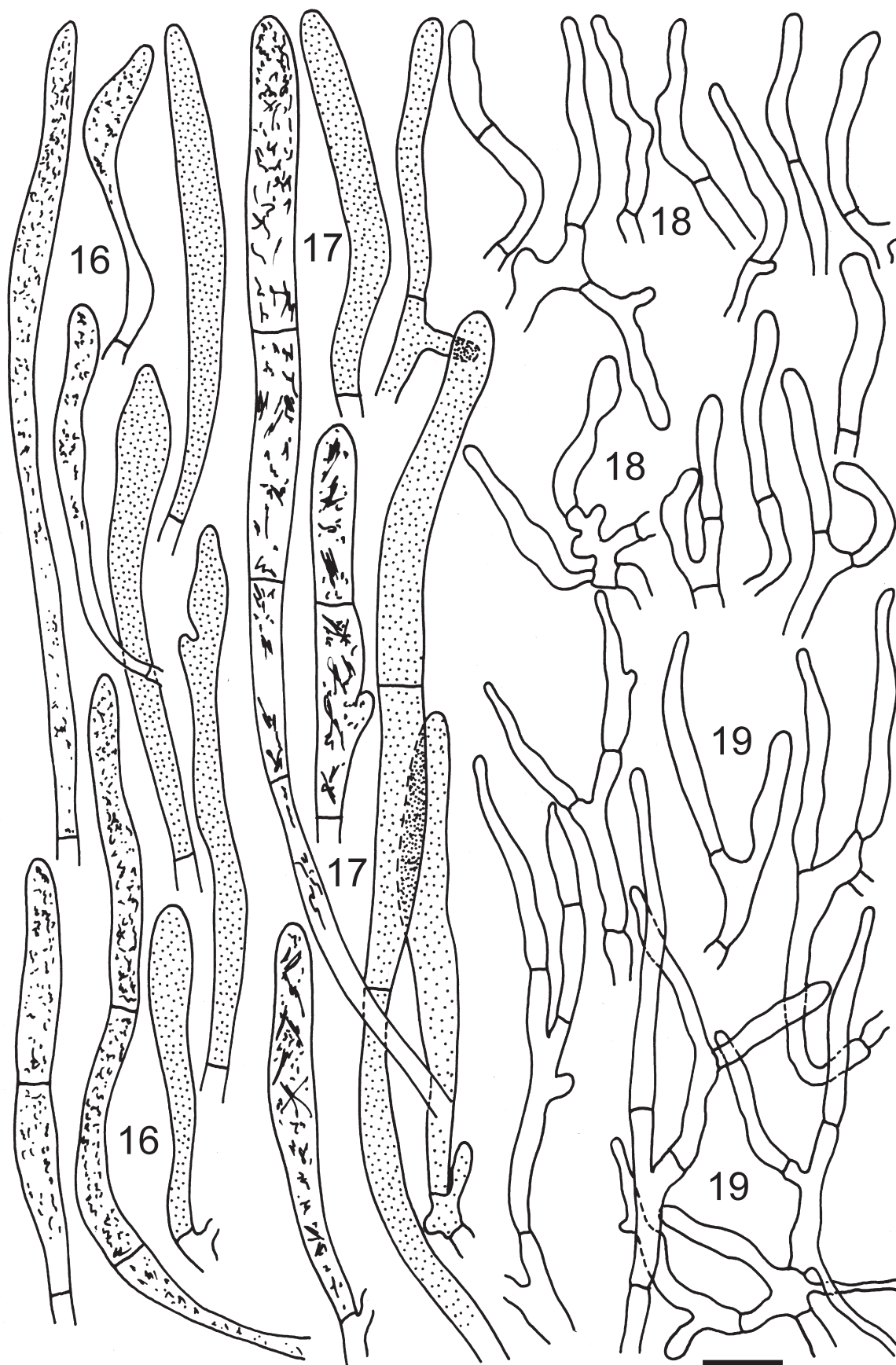
Examined material.—BELGIUM. Forêt de Soignes, 31 August 2000, H.J. Beker *HJB10019* (STU); FRANCE. Drôme Dept.: Gisors, les Chaux, 5 July 1997, H. Marxmüller *R-9702* (M); La Garde-Adhémar, Val des Nymphes, *Quercus ilex*, 30 September 2008, H. Marxmüller *R-0898* (M); SPAIN. Mallorca island: Bunyola, *Quercus ilex*, *Arbutus unedo*, 15 December 2011, F. Hampe & J. Kleine *RUS11121505* (GENT, holotype).

Russula sp. Figs. 5, 16–25

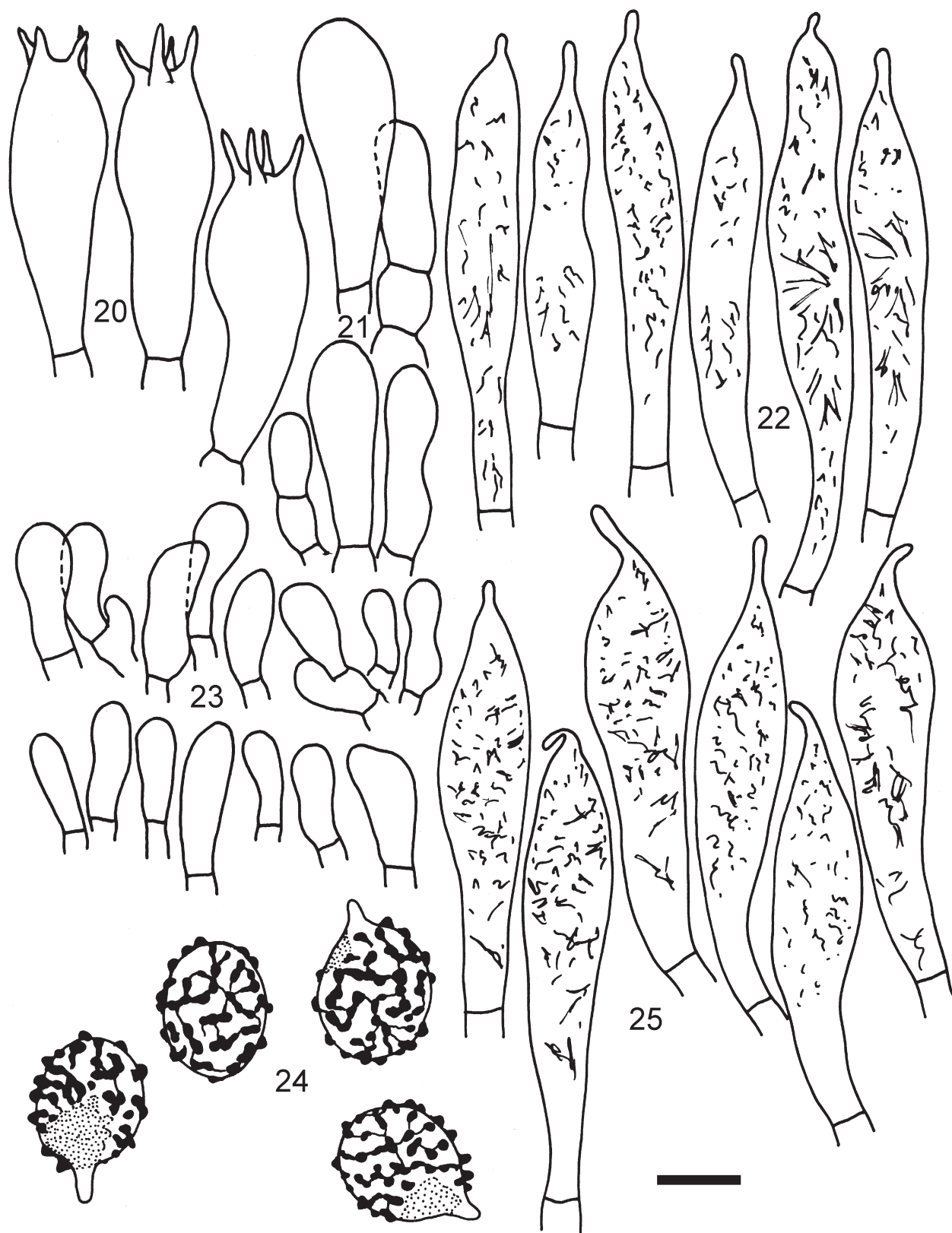
Short diagnosis.—Basidiomata medium to small, pileus with relatively thin context, surface of stipe, pileus and lamellae with yellow-brownish spots, pileus cuticle red and not discolouring distinctly to cream, spore ornamentation with low (up to $0.6 \mu\text{m}$), amyloid warts often merged or connected by line connections, hymenial cystidia dispersed, hyphal terminations in pileipellis near the pileus margin sub-cylindrical or subulate, pileocystidia near the pileus margin frequently with more than two cells and $5.5\text{--}7.5 \mu\text{m}$ wide.

Basidiomata small to medium sized, $40\text{--}45 \text{ mm}$ tall. *Pileus* $27\text{--}34 \text{ mm}$ in diameter, convex, centrally slightly depressed, surface dry, smooth, matt, vivid red or strong red with centre reddish orange and rusty spotted with spots sometimes concentrically arranged; margin even, or slightly involute, without striations. *Lamellae* regular, adnate, crowded, light yellow, pale yellow or light orange yellow, brittle, edge entire, concolourous. *Stipe* $35\text{--}40 \times 8\text{--}10 \text{ mm}$, central, cylindrical to subcylindrical, stuffed, slightly longitudinally wrinkled, white, towards base with light yellow-brownish or moderate yellow-brownish spots, without pinkish shades. *Context* compact, not firm, smell and taste not recorded.

Spores $(7.4\text{--}7.8\text{--}8.6\text{--}9.4) \times (6.2\text{--}6.4\text{--}7.2\text{--}7.7) \mu\text{m}$, average $8.2 \times 6.8 \mu\text{m}$, $Q = (1.13\text{--}1.17\text{--}1.24\text{--}1.29)$, average 1.21, ornamentation consisting of $(4\text{--}5\text{--}7)$, moderately large and distant amyloid warts in the circle of diameter of $3 \mu\text{m}$ on spore surface, warts $0.4\text{--}0.6 \mu\text{m}$ high, connected with occasional to frequent short or longer fine line connections [(0–)1–3(–5) line connections in the circle], occasionally fused in short or longer chains [(0–)1–3(–4) fusions in the circle], chains and crests often branched, but rarely forming a reticulate structure, isolated warts rare. Suprahilar plage amyloid, large. *Basidia* $(30\text{--}33\text{--}37.5\text{--}40) \times (10\text{--}11\text{--}13) \mu\text{m}$, average $35.4 \times 12.1 \mu\text{m}$, 4-spored, clavate, sometimes pedicellate;



FIGURES 16–19. *Russula* sp. from Pakistan (LAH MAM 0077). **16.** Pileocystidia near the pileus centre. **17.** Pileocystidia near the pileus margin. **18.** Hyphal terminations in the pileus centre. **19.** Hyphal terminations near the pileus margin. Contents of cystidia are represented as observed in Congo Red for some elements only, the others are simply filled with dots to indicate their cystidial nature. Scale bar equals 10 μ m. Drawings by: S. Jančovičová.



FIGURES 20–25. *Russula* sp. from Pakistan (LAH MAM 0077). 20. Basidia. 21. Basidiola. 22. Cheilocystidia. 23. Marginal cells. 24. Basidiospores in Melzer's reagent. 25. Pleurocystidia. Contents of cystidia are represented as observed in Congo red. Scale bar equals 10 μm , but only 5 μm for spores. Drawings by: S. Jančovičová.

basidiola first cylindrical or ellipsoid, then clavate, ca. 5–10(–12.5) μm wide. *Subhymenium* pseudoparenchymatic. *Lamellar trama* mainly composed of large sphaerocytes. *Hymenial cystidia* on lamellar sides widely dispersed to dispersed, 300–400/mm², fusiform or rarely clavate, pedicellate, thin-walled, measuring (49–)52–65(–72) \times (10–)10.5–14(–16) μm , average 58.6 \times 12.3 μm , apically acute to acute-pointed and with (1–)2–7 μm long appendage, contents heteromorphous, granular-banded, yellowish, turning brownish-red to almost black in sulfovanillin. Lamellar edges covered with abundant marginal cells, occasional cheilocystidia and dispersed basidia; *marginal cells* not well differentiated, similar to the basidiola on lamellar sides, but smaller, mainly clavate to subcylindrical, sometimes constricted in half length, with obtuse tips, measuring (9–)12–17.5(–19) \times (4–)4.5–7(–7.5) μm , average 15 \times 5.8 μm ; *cheilocystidia* narrower than pleurocystidia, clavate or fusiform, pedicellate, thin-walled, measuring (42–)48–64.5(–73) \times 8–10.5(–12.5) μm , average 56.5 \times 9.3 μm , apically with mainly acute tips and usually with 1–6 μm long appendages, contents similar as in pleurocystidia. *Pileipellis* orthochromatic in Cresyl blue, 115–135 μm deep, sharply delimited from the underlying sphaerocytes of the context; distinctly divided in a 60–75 μm deep, strongly gelatinized suprapellis of loose, erect or ascending hyphal terminations and, near surface, with some repent, longer pileocystidia; and a 55–65 μm deep subpellis of less gelatinized, dense, irregularly, but near the trama horizontally oriented, intricate, branched, 2–5 μm wide hyphae. Acidoresistant incrustations absent. Hyphal terminations in pileipellis near the pileus margin slender and branched, thin-walled, with terminal cells measuring (14–)20.5–37(–48) \times 2.5–3.5 μm , average 28.7 \times 3 μm , mainly narrowly subulate or fusiform, partly subcylindrical, usually apically attenuated or constricted, often moniliform; near the pileus centre with mainly cylindrical, often flexuous terminal cells, measuring (13–)16.5–27(–31) \times (2–)2.5–4(–4.5) μm , average 21.7 \times 3.2 μm , apically obtuse; subterminal cells mainly branched or not, often with lateral branches or nodules, equally wide as terminal cells. Pileocystidia near the pileus margin numerous, narrowly clavate or fusiform, mainly two or more-celled [1–5(–6) celled], thin-walled, with terminal cells measuring (18–)33.5–57(–67) \times (4–)5.5–7.5(–8.5) μm , average 45 \times 6.5 μm , apically obtuse, subterminal cells equally wide or narrower, often shorter, contents in Congo red heteromorphous, granulose or crystalline, turning dark reddish-brown to black in sulfovanillin; near the pileus centre smaller and narrower, with terminal cells measuring (30–)34.5–59.5(–81) \times (3.5–)4–6(–7) μm , average 47.1 \times 5.1 μm , more frequently one-celled, with more granular and yellow-coloured contents. Cystidioid hyphae in subpellis and pileus trama dispersed, with heteromorphous-granulose, yellowish, often oleiferous contents. *Clamp connections* absent in all parts.

Examined material.—PAKISTAN. Khyber Pakhtoon Khaw, Mansehra, Batrasi, under *Pinus roxburghii*, 3 August 2014, Malka Saba MAM 0077 (LAH, duplicate FH00304559).

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