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New insights in Russula subsect. Rubrinae: phylogeny and the quest for synapomorphic characters

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	firmula and R. veternosa. Incrustation type is now highlighted as important for the delimitation of species and groups within the Crown clade. Pink or red staining of the incrustations in sulfovanillin is present in all species of the Rubrinae clade and a majority of analysed species of the Integrae clade. The delimitation of the Rubrinae clade and its species circumscriptions are summarised here in a new diagnostic key.

New insights in *Russula* subsect. *Rubrinae*: phylogeny and the quest for synapomorphic characters

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Abstract

Russula is one of the most speciose genera of mushroom-forming fungi, but phylogenetic relationships among species and subgeneric groupings are poorly understood. Our multi-locus phylogenetic reconstruction places *R. firmula*, *R. rubra*, *R. rutila* and *R. veternosa* in a well-supported Rubrinae clade, belonging to the Integrae clade of the Crown clade of the genus *Russula*. Traditional morphology-based classifications placed these four species in two different subsections based on the presence or absence of incrustations on pileocystidia. The Integrae clade also contains *R. integra* and related species that are traditionally placed in other groups based on their mild taste. Ancestral state reconstruction suggests that the common ancestor of the Crown clade and the Integrae clade probably did not have any incrustations in the pileipellis, had a mild taste, yellow spore print and were associated with angiosperms. All four species of the Rubrinae clade are defined by a darker yellow or ochre spore print, acrid taste and incrustations on pileocystidia. This last character contradicts the former splitting of the group because incrustations were apparently overlooked in *R. firmula* and *R. veternosa*. Incrustation type is now highlighted as important for the delimitation of species and groups within the Crown clade. Pink or red staining of the incrustations in sulfovanillin is present in all species of the Rubrinae clade and a majority of analysed species of the Integrae clade. The delimitation of the Rubrinae clade and its species circumscriptions are summarised here in a new diagnostic key.

Keywords: sulfovanillin, incrustations, agarics, multi-locus phylogeny, ancestral state reconstruction

Introduction

Russula Pers. is a species rich genus of ectomycorrhizal fungi with more than 265 morphological species recognised in Europe (Sarnari 1998, 2005) corresponding to 295 molecular operation units (MOTUs) defined by ITS nrDNA sequence data retrieved from GenBank (Looney et al. 2016). The majority of *Russula* species and infrageneric taxa were described based on phenotype (Romagnesi 1967; Sarnari 2005) and only a few studies reconsidered existing morphology-based concepts using molecular data (e.g. Liu et al. 2015). Recent studies suggest that some morphologically defined groups correspond to well-defined phylogenetic lineages (*Russula* subsect. *Xerampelinae* Singer in Adamčík et al. 2016a), however many morphological groups appear to be polyphyletic (*Russula* subsect.

Maculatinae Romagn. in Adamčík et al. 2016b). Adamčík et al. (2016b) demonstrated a high similarity of ITS nrDNA sequences of *R. firmula* Jul. Schäff., *R. rubra* (Lam.) Fr., *R. rutila* Romagn. and *R. veternosa* Fr. These species share several similar morphological characters (yellow spore print, acrid taste of the flesh, amyloid suprahilar spot), but they are traditionally classified in various groups. *Russula rubra* and *R. rutila* are placed within *Russula* subsect. *Rubrinae* (Melzer & Zvára) Singer based on the presence of incrusted pileocystidia, while *R. firmula* and *R. veternosa* are described as lacking such incrustations (Romagnesi 1967; Sarnari 1998). Singer (1986) and Sarnari (1998) placed them in a single group, *R.* subsect. *Urentes* Maire, but Romagnesi (1967) classified *R. veternosa* in *R.* subsect. *Maculatinae* and *R. firmula* in *R.* subsect. *Urentinae* Maire.

The most comprehensive multi-locus *Russula* phylogeny, published by Looney et al. (2016), includes only one of the four above mentioned species, *R. firmula*. According to the authors the species is part of a large phylogenetic lineage called the Crown clade. Based on ITS sequence similarity (Adamčík et al. 2016b) we expect that all four species will be members of the Crown clade. In this study, we present a multi-locus phylogeny of the Crown clade of *Russula* as defined by Looney et al. (2016). Our phylogenetic sampling is based on representation of the majority of morphologically defined sections and subsections recognised by Romagnesi (1967), Singer (1986) and Sarnari (1998) that are, according to Looney et al. (2016), in the Crown clade. We are exploring the phylogenetic support for a lineage that includes *R. rubra*, the type species of subsection *Rubrinae*. We seek to determine whether *R. firmula*, *R. rutila* and *R. veternosa* indeed belong to a single lineage and whether this group can be morphologically recognised. In addition, it is our objective to test the species limits by including material from distant areas of Europe and a variety of habitats. We will also analyse if traditional morphological classification coincides with defined molecular lineages of the Crown clade.

Material and Methods

Sampling

Samples of the four target species- *R. firmula* (11 collections), *R. rubra* (8 collections, including the type), *R. rutila* (15 collections, including the type) and *R. veternosa* (9 collections) were identified based on morphology following Knudsen et al. (2012). Type collections of the target species were sequenced when possible. The neotypes of *R. firmula* designated by Romagnesi (1967) and Sarnari (1998) were not studied because of type material is existing under the form of illustrations cited in the protologue, making the neotypes invalid. We included also the type and two recent collections of *R. quercilicis* Sarnari, a Mediterranean species classified in *Rubrinae* based on its incrusted pileocystidia (Sarnari 1998). All sequences from public databases GenBank (https://www.ncbi.nlm.nih.gov/genbank) and UNITE (https://unite.ut.ee) matching 97% and higher similarity with any of the target species clade were included in the phylogenetic analysis.

For determining the phylogenetic placement of the four target species we used a selection of European species placed in the Crown clade by Looney et al. (2016). We also sought to sample the type species of every section and subsection suggested to be a part of the Crown clade. In addition, we included some well-known and common European species. All collections sequenced in this study originate from various countries of Europe, but a major part of sampling came from Central Europe (Slovakia and Germany) and a majority of them were morphologically identified and sequenced by the authors of this study. This sampling is supplemented by a few sequences published by Looney et al. (2016). Three species of the *Russula* subgenus *Russula* are used as an outgroup. All sequences used in our study are listed in the Supplementary material 1.

Molecular analysis

Total genomic DNA was extracted from dried material using the methods previously described by Adamčík et al. (2016b). We amplified three molecular markers: (1) the internal transcribed spacer regions of the ribosomal DNA (ITS); (2) partial mitochondrial small subunit ribosomal DNA (mtSSU); (3) the region between domains six and seven of the nuclear gene encoding the second largest subunit of RNA polymerase II (*rpb2*). The ITS region was amplified using the primers ITS1F–ITS4 (White et al. 1990; Gardes and Bruns 1993). The mtSSU region was amplified using the primer pair MS1 and MS2 (White et al. 1990). Both molecular markers were amplified with Hot Start Firepol Polymerase (Solis Biodyne, Tartu, Estonia) using the same cycling protocol: 95°C/15 min; 35 repeats (95°C/30sec, 50°C/30 sec, 72°C/1 min); 72°C/10 min; cooling to 4°C. For amplification of *rpb2* we used a new forward primer A-Russ-F (5´-TGTCGGGTCCCATNATYGAA-3´) designed using Primer-Blast (Ye et al. 2012) and a reverse primer frpb2-7CR (Matheny 2005). The *rpb2* was amplified with Hot Start Firepol Polymerase, using this cycling protocol: 95°C/15 min; 35 repeats (95°C/1 min; 58°C/1 min; increasing temperature 59°C/10 sec; 60°C/10 sec ... up to 71°C/10 sec; 72°C/1 min); 72°C/10 min; cooling to 4°C. The PCR products were purified using Exo-Sap enzymes (Thermo Fisher Scientific, Wilmington, Delaware, USA) or Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany). Samples were sequenced directly with BigDye 3.1 technology (Applied Biosystems, now Thermo Fisher Scientific, Wilmington, USA) or sent to Macrogen Europe (Amsterdam, the Netherlands).

Phylogenetic analysis

Sequences were edited in the BioEdit Sequence Alignment Editor version 7.2.5 (Hall 2013) or Geneious version R10 (Kearse et al. 2012). Intra-individual polymorphic sites having more than one signal were marked with NC-IUPAC ambiguity codes. Edited ITS sequences of *R. firmula*, *R. rubra*, *R. rutila* and *R. veternosa* were analysed in BLAST (Kõljalg et al. 2013) and all sequences with high similarity were included in our dataset. Final datasets were aligned by MAFFT version 7 using the strategy E-INS-i (Katoh & Standley 2013) and manually improved in AliView version 1.17.1 (Larsson 2014). All three single-locus datasets were concatenated into one multi-loci dataset using Seaview v. 4.5.1 (Gouy et al. 2010). The concatenated final alignment has been deposited at TreeBASE (XXXX). The multi-locus dataset was analysed using two different methods: Bayesian inference (BI) and the Maximum Likelihood method (ML). For ML analysis, the concatenated alignment was loaded as a phyllip file into the raxmlGUI v. 1.2 (Silvestro et al. 2012) and analysed as a partitioned dataset under the GAMMA+I+G model with 1 000 bootstrap iterations. For BI the dataset was divided into 6 partitions: ITS, mtSSU, intronic region 7 of *rpb2*, and the 1st, 2nd, and 3rd codon positions of *rpb2*. The best substitution model for each partition was computed jointly in PartitionFinder v. 1.1.1 (Lanfear et al. 2012). The BI was computed independently twice in MrBayes version 3.2.6 (Ronquist et al. 2012) with four MCMC chains for 10 000 000 iterations until the standard deviation of split frequencies reached below the 0.01 threshold. The convergence of runs was visually assessed using Trace function in Tracer version 1.6 (Rambaut et al. 2013).

Ancestral state reconstruction

To assess the character evolution of the most important morphological characteristics used for infrageneric classification within the genus *Russula*, we performed ancestral state reconstruction on the dataset used for the phylogenetic analysis. Only species recognised morphologically by authors of this study are included and every species is represented by a single collection. Three morphological characters and the host preference are analysed. The coding of all three morphological characters is based on our own observations. Visibility and red staining of incrustations on pileocystidia and primordial hyphae were scored using both carbolfuchsin and sulfovanillin. Spore print colour is scored in three classes: pale – up to IIb according to Romagnesi (1967), intermediate – IIc to III and yellow – IIId to IVe. The taste of the context is classified in 3 categories: mild, weakly acrid and acrid. The preference of coniferous or angiosperm host was assigned based on published *Russula* monographs (Romagnesi 1967; Einhellinger 1994; Sarnari 1998, 2005; Knudsen et al. 2012) and data from the DEEMY database of ectomycorrhizae (www.deemy.de).

A species tree was inferred using the same procedure for the ML analysis only with species clades pruned to a single representative. Character history was traced across the tree topology using the Trace Character History function using a ML approach with stored probability models implemented in Mesquite v. 2.74 (Maddison and Maddison 2010). Significant support for character states was assessed using a cut-off value of a difference of 2.0 between log-likelihoods of states. The resulting trees were ladderized at the root and displayed as Balls and Sticks graphs. We chose to perform these analyses on the full clade even though the available molecular loci result in poorly resolved relationships throughout most of the Crown clade. We feel this is justified as these outgroup taxa inform the likelihood model with how prevalent these traits are across the relevant clade and how frequent transitions have occurred in the character states. Our interpretation will focus on well-resolved clades associated with the species of interest.

Morphological analysis

The morphological circumscription of the group of *R. rubra* and related species is based on comparisons of our morphological observations of three collections selected for each of the four target species. To narrow the number of micro-morphological characters efficiently, we compared the full detailed descriptions of one collection per species with similar published descriptions (Adamčík and Jančovičová 2012, 2013) to other species of *R.* subsect. *Maculatinae* and R. subsect. *Urentinae* characterised according to Romagnesi (1967) by acrid taste, yellow spore print, spores with an amyloid suprahilar spot and presence of non-incrusted pileocystidia. Characters selected based on this first step comparison were further tested for their taxonomic significance to define the *R. rubra* group from other species of the Crown clade and for differences between species within the lineage.

Micromorphological characteristics were observed using an Olympus CX-41 microscope equipped with Artray Artcam 300MI camera at a magnification of 1000×. Spore were measured using Quick Micro Photo version 2.1 software and spore dimensions exclude ornamentation. 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 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 and Marhold (2000). The cystidia density estimates follow Buyck (1991). 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. Statistics for the measurements of microscopic characteristics were based on 30 measurements per specimen and expressed as the mean ± standard deviation. The classification of spore print colour follows Romagnesi (1967).

Incrustation of pileocystidia is the distinguishing character of *R.* subsect. *Rubrinae* (Romagnesi 1967; Sarnari 1998). For this reason, we paid special attention to the observation of incrustations and contents of pileocystidia using various reagents: Congo red solution, Cresyl blue (Buyck 1989), carbolfuchsin (Romagnesi 1967) and sulfovanillin. In Cresyl blue, the presence of ortho- or metachromatic reactions as explained in Buyck (1989) was examined. Acid-resistant incrustations of the primordial hyphae or pileocystidia were stained with carbolfuchsin and observed in distilled water after incubation for a few seconds in a 10% solution of HCl (following Romagnesi 1967). For observations in sulfovanillin, two techniques were tested: pre-prepared fresh solution of vanillin in sulphuric acid (Moser 1978; Kreisel and Schauer 1987; Adamčík and Knudsen 2004), or crystals of vanillin dissolved in sulphuric acid just before preparation of the object (http://www.deemy.de/). Because the index of refraction of sulphuric acid is different from that of water and the strong acid quickly causes dissolution of cell walls, we used less concentrated sulphuric acid. We dissolved a few crystals of vanillin in one drop of concentrated acid and added one additional drop of distilled water or used a pre-prepared 50% solution of sulphuric acid.

Results

Phylogenetic analysis

The final dataset was composed of 77 specimens belonging to the four studied species and samples with a high sequence similarity, 37 samples of 35 different species of the Crown clade and three of the outgroup. In summary, 117 specimens were included in the analysis. Of these, 46 encompassed only ITS (mainly sequences retrieved from the public databases) and 20 encompassed only two of the three markers. We were able to obtain sequences from the types of *R. rubra* and *R. rutila*, but DNA amplification of the *R. veternosa* type failed. The final topologies of the ML and BI analyses were not congruent, as we expected, given the high sequence variation found between members of the Crown clade. There is no significant clustering across the backbone of the Crown clade. The clade we call Integrae clade, referring to one of the oldest names of an infrageneric *Russula* taxon typified by *R. integra* (L.) Fr. received moderate statistical support (85/1) (Fig. 1). Inside this clade, the four strongly supported subclades concordant with morphologically defined species (called further by the species name), *R. firmula, R. rubra, R. rutila* and *R. veternosa,* are placed in one monophyletic group (100/1) called here the Rubrinae clade. The *Russula rutila* subclade does not include any non-European collections, shows little sequence variation and contains 15 collections sequenced in this study (including the type specimen), a collection TU101893 originally identified as *R. decipiens* (Singer) Svrček retrieved from the UNITE database and an unidentified *Russula* sequence LM5409 originating from an environmental sample. The remaining three subclades of the Rubrinae clade contain some sequences of Asian, or in the case of *R. firmula* subclade also of North American origin. In the *R. veternosa* subclade, there is a little sequence variability and two environmental samples from Iran are nearly identical with one other from Europe. All samples (11 European and two from the Middle East) clustered in the *R. veternosa* subclade probably represent a single species. Our six European collections identified as *R. rubra* and the type collection of the species are clustered in a strongly supported subclade together with another three European collections originating from environmental samples. Sister to this subclade are three sequences from Papua New Guinea that probably represent a closely related undescribed species.

Ten sequences from the collections morphologically assigned to *R. firmula* cluster with the other 17 European collections (73/0.99) and form a moderately supported group (80/1) together with unidentified Asian and North American environmental samples for which conspecificity with this species is unclear. *Russula quercilicis*, the other species classified in the subsect. *Rubrinae* based on incrusted pileocystidia, is placed outside the Rubrinae clade.

Ancestral state reconstruction

After reducing the four species in Rubrinae clade each to a single representative, the topology of the resulting maximum likelihood tree is similar to results of the phylogeny using the complete dataset. The analysis shows good support for the Rubrinae clade, the Integrae clade and the Crown clade (Fig. 2).

The absence of any incrustations on pileocystidia or primordial hyphae is the most probable ancestral state for both the Crown clade and the Integrae clade, but the analyses suggests that within the Integrae clade, most ancestors possessed pink incrustations in sulfovanillin and some members lost this colour change (*R. quercilicis* and *R. cremeoavellanea* Singer) or any incrustation at all (*R. badia* Quél. and *R. tinctipes* J. Blum ex Bon). There is not enough support to resolve relationships for the other members of the Crown clade out of the Integrae clade, but the analysis suggests that the incrustation evolved several times from non-incrusted ancestors.

The analysis of taste of the context shows mild taste as the ancestral state for both the Crown clade and the Integrae clade. The acrid or weakly acrid taste probably evolved more recently and in several lineages within the Crown clade. The Rubrinae clade together with the clade of *R. badia* and *R. quercilicis* represent two of the independent lineages with acrid taste. Acrid species are also found out of the Integrae clade, but the analysis on the current dataset does not allow enough resolution to determine if they share a single origin of acridity with the Rubrinae clade.

The spore print analysis suggests that most of the ancestors in the Crown clade possessed a darker yellow spore print and the paler spore print evolved multiple times. This is especially well demonstrated in the Integrae clade, showing support for isolated positions of *R. decolorans*, *R. rubra* and *R. velenovskyi* Melzer & Zvára with intermediate spore print colour among yellow spore print species. The strong support for a relationship of pale- and dark-spored species is also shown in the species pairs of *R. nauseosa* (Pers.) Fr. – *R. nitida* (Pers.) Fr. and *R. odorata* Romagn. – *R. versicolor* Jul. Schäff.

Ancestral state reconstruction of the host preference demonstrates a similar pattern as the one of the spore print. The majority of ancestors on higher ranks of the phylogeny were probably associated with angiosperms and the high probability for such ancestors is demonstrated for both the Integrae clade and the Rubrinae clade. There is high support for isolated positions of some species preferring coniferous trees that probably evolved from ancestors with preference for angiosperm hosts, including *R. firmula*, *R. badia* and *R. integra*.

In conclusion, the common ancestors of the Crown clade and the Integrae clade probably did not have any incrustations, had a mild taste, a yellow spore print and were associated with an angiosperm host. Taste, spore print and host preference showed only one a unidirectional switch in the Crown clade: from mild to acrid, from yellow to white and from angiosperm to conifer. The only specific change in evolution of the Integrae clade is a dominance of species with pink incrustations in sulfovanillin that probably evolved early in the clade history and some species lost such incrustations later.

Morphological delimitation of the Rubrinae clade

Selected morphological characters further tested for their significance to define the Rubrinae clade are presented in the Table 1. The spores (Figs. 3 and 4), basidia and pleurocystidia of *R. veternosa* are the smallest among the four compared species, but the values of the other three species of *R. rubra* lineage fall within the variation of *R. maculata* Quél. and *R. vinosopurpurea* Jul. Schäff. (Adamčík and Jančovičová 2013). The spore ornamentation shows high variability among the species compared here and cannot define the whole Rubrinae clade, but it may rather serve as a good distinguishing character at the species rank. Hyphal terminations in the pileipellis near the pileus margin show a more or less uniform pattern in all four species: relatively narrow terminal cells that are cylindrical or subulate and usually apically constricted or attenuated and are usually followed by one unbranched subterminal cell or are directly arising from branched hyphae of the subpellis. The terminal cells of *R. rubra* and *R. rutila* are usually more distinctly and frequently attenuated (Fig. 4). The only character that defines the Rubrinae clade perfectly is the presence of incrustations on pileocystidia. The presence of such incrustations is a new discovery of this study for *R. firmula* and *R. veternosa*. These incrustations are sometimes weakly acid-resistant and difficult to see after a carbolfuchsin treatment, but after ca. 30 minutes they stain bright pink in sulfovanillin.

Analysis of incrustations

Because our morphological analysis revealed incrustations for unexpected species of the Rubrinae clade (Table 1, Fig. 5), we analysed their presence for all species accepted in this study. We observed presence and colouring of incrustations using the carbolfuchsin treatment, Cresyl blue and sulfovanillin.

Our observations with carbolfuchsin treatment confirmed in most cases the presence or absence of acid-resistant incrustations in agreement with the literature, the exceptions being incrustations observed in *R. firmula* and *R. veternosa* when none have been mentioned before. The species with incrusted primordial hyphae, traditionally classified within *R.* subgenus

Incrustatula Romagn. (Sarnari 1998), all have abundant acid-resistant incrustations: *R. caerulea* Fr., *R. claroflava* Grove, *R. risigallina* (Batsch) Sacc., *R. turci* Bres., *R. velutipes* Velen., *R. vinosa* Lindblad and *R. zvarae* Velen. Acid-resistant incrustations were also observed in some species with incrusted pileocystidia, which have in addition to incrustations, contents that turn grey in sulfovanillin. The red staining of incrustations on pileocystidia after carbolfuchsin treatment was often weak, instable and restricted to the bases of the pileocystidia and were sometimes unconvincing in: *R. cremeoavellanea*, *R. firmula*, *R. integra*, *R. laeta* F.H. Møller & Jul. Schäff., *R. quercilicis*, *R. rubra*, *R. rutila*, *R. velenovskyi* and *R. veternosa*. The remaining species of the Crown clade included in this study, as well as representatives of *R.* subgenus *Russula* have no acid-resistant incrustations on any cells of the pileipellis.

Russula lepida Fr. is the only species with distinct metachromatic incrustations in pileipellis in Cresyl blue, showing purple incrustations especially in the deeper part of the suprapellis and occurring both on pileocystidia and other undifferentiated hyphae in the pileipellis. We do not think that presence of a weak metachromatic reaction in the pileipellis of some species of the Integrae clade (e.g. *R. laeta*) has taxonomic significance.

The examination of incrustations in sulfovanillin has revealed the most novel information. There is a very quick colour change of incrustations to bright pink or red in *R. caerulea, R. claroflava*, *R. velutipes* and *R. vinosa* (Fig. 5). However, the distribution and the type of incrustations are different and these differences probably correspond also with their different positions in the phylogenetic tree. *Russula velutipes* has very abundant incrustations in the form of droplets but the deep red colouring is usually restricted only to the basal parts of primordial hyphae, the tips of which often bear only hyaline droplets. Large droplets turning quickly to deep and bright red can be found in *R. caerulea*, but the tips of primordial hyphae are usually not incrusted. Primordial hyphae of *R. claroflava* and *R. vinosa* are incrusted on the whole surface with very fine, granulose incrustations that turn quickly bright pink in sulfovanillin. The majority of species of the Integrae clade tend to have incrustations that turn slowly, after ca. 30 minutes, to bright pink: *R. firmula, R. integra, R. laeta, R. rubra, R. rutila, R. velenovskyi* and *R. veternosa*. These incrustations are in the shape of irregular droplets or patches on surface of pileocystidia, usually only near their basal part, but sometimes they cover the whole surface. The colour change is slow and not always convincing, but usually the pink colour shows a very striking contrast especially near the basal septa, where the grey or black colouring of contents is lacking. The intensity of the colour change and the visibility of the incrustations are weak and less conspicuous in *R. rubra*

(Fig. 5b). Incrustations on primordial hyphae of *R. risigallina*, *R. turci*, *R. velutipes* and *R. zvarae* do not change colour (remain hyaline) in sulfovanillin. Incrustations on pileocystidia of *R. cremeoavellanea* do not change colour in sulfovanillin, and only the pileocystidia contents slightly turn grey. The pileocystidia contents of *R. quercilicis* turn dark grey to black, whereas the incrustations are first yellowish and after ca. 60 minutes turn black.

Type studies and species concepts within the Rubrinae clade

Our morphological preliminary identifications were in agreement with the results of phylogenetic analysis. To confirm that the results of this study are in agreement with the original concepts of the studied species, the available type material was included. Information on types of all four species of the Rubrinae clade is summarised in the Table 2. The species concepts of *R. rubra* and *R. rutila* have also been confirmed by successfully sequenced type specimens. The DNA extraction of the *R. veternosa* epitype failed, but the morphological investigation is clearly in agreement with the morphology of recently studied and sequenced collections of this species (Supplementary material 2). We did not study any of the *R. firmula* neotypes proposed by Romagnesi (1967) or Sarnari (1998) since they were not available for loan during our study. In our opinion, both *R. firmula* neotypes are superfluous because the original description (Schaeffer 1940) clearly refer to Schaeffer's description of "*R. nitida* (Pers.) Fr." that cites several illustrations available for the type designation. For the species concept stability, we are designating here Bresadola's plate 458 (Bresadola 1929) that is cited by Schaeffer (1933) as the lectotype of the species and our collection GENT (2010 BT85) as the epitype of *R. firmula* (Supplementary material 3).

Key to species of the Rubrinae clade

Circumscription of the Rubrinae clade: (1) spores with amyloid suprahilar spot; (2) taste of the context strongly acrid; (3) spore print ochre or yellow; (4) pileocystidia turning dark grey to black in sulfovanillin and with incrustations staining pink in sulfovanillin after ca. 30 minutes, that are especially visible at the base

1 Spores with isolated prominent spines

2 Pileus with predominantly pale red, pink, ochre and cream colours; spores mainly up to 8.5 µm long; usually associated with *Fagus* ***R. veternosa***

2* Pileus with predominantly wine-red, blue-red, purple and red-brown colours; spores mainly longer than 8.5 µm; usually associated with coniferous trees ***R. firmula***

1* Spores with warts merged in chains and connected by occasional lines

3 Basidiomata medium sized to large (60–100 mm), with thick context turning slowly grey; pileus cuticle velutinous or matt; spore print ochre (IIIb–IIIc) **R. rubra**

3* Basidiomata small to medium sized (30–70 mm); context soon becoming fragile, thin, and not turning grey; pileus cuticle shiny at least near the pileus margin; spore print yellow (IVb–IVd) **R. rutila**

Discussion

Congruence of morphology based classifications with the Crown clade phylogeny

Our phylogeny is based on altogether 39 species of the Crown clade. The four target species are grouped in the Rubrinae clade which is nested in the larger Integrae clade. In general, the phylogenetic tree often shows strong support for relationships of pairs or triplets of species, but the other nodes on higher ranks usually received weak support, partly explained by incomplete species representation in the Crown clade and a limited number of gene markers.

Looney at al. (2016) analysed a large dataset of ITS *Russula* sequences retrieved from GenBank and identified 1064 MOTUs worldwide, with 295 represented in Europe. The four-locus phylogeny presented by Looney et al. (2016) recognised eight major clades within the genus *Russula*. The clades Farinipes, Nigricans, Archaea, Heterophylla and Compacta are represented by species with a non-amyloid suprahilar spot on their spores. The Delica clade is represented by species with an amyloid suprahilar spot but have frequent short. A majority of species with an amyloid suprahilar spot on spores are in the Russula clade and the Crown clade, but the morphological distinction of these two groups will require further analysis. The Crown clade was represented by nearly of the 50% MOTUs in the total dataset. Some *Russula* groups with a non-amyloid suprahilar spot on the spores, which fall outside the Crown clade, are probably less diverse in Europe than in other continents and therefore we expect that more than half of European MOTUs belong to the Crown clade. The total species number of this clade will certainly be higher than 100 in Europe.

Of all traditional classifications (Singer 1986; Romagnesi 1987; Bon 1988; Sarnari 1998), the one presented by Romagnesi (1987) corresponds best to the currently known phylogeny. Romagnesi defines nine subgenera, of which *R.* subg. *Compacta* (Fr.) Bon, *R.* subg. *Heterophyllidia* Romagn., *R.* subg. *Ingratula* Romagn. and *R.* subg. *Russula* are typified by species out of the Crown clade (Looney et al. 2016). Types of *R.* subg. *Incrustatula* (*R. lilacea* Quél.), *R.* subg. *Tenellula* Romagn. (*R. puellaris* Fr.), *R.* subg. *Polychromidia*

Romagn. (*R. integra*), *R.* subg. *Coccinula* Romagn. (*R. paludosa* Britzelm.) and *R.* subg. *Insidiosula* Romagn. (*R. firmula*) all belong in the Crown clade. The other classifications define groups mixed of species of the Crown clade and members of other *Russula* clades. In the following text, we discuss the morphological delimitation of clades recognised in this study and their nomenclature.

Phylogenetic signal of analysed morphological characters

The phylogeny presented in this study confirms support for the Integrae clade in agreement with Looney et al. (2016). As species with both mild and acrid taste fall within the Integrae clade, this character is not phylogenetically informative for ranks at a high taxonomic level such as *R.* subg. *Insidiosula* defined by Romagnesi (1987) for acrid species. At a lower taxonomic level; we see that within the Crown clade, there are several terminal clades with acrid-tasting species that received high support (Figure 2). Our study does not show support for a larger group defined by spore print, but Looney et al. (2016) reveals good support for a clade that encompass species with incrusted primordial hyphae and white or cream spore print corresponding to *R.* subsect. *Roseinae* Singer and *R.* subsect. *Lilaceinae* Melzer & Zvára (as defined by Romagnesi 1967 and Sarnari 1998). The presence and type of incrustations may have various importances. The primordial incrusted hyphae with both contents and incrustations unchanging in sulfovanillin are probably of polyphyletic origin, e.g. phylogeny in this study shows *R. cremeoavellanea* (primordial hyphae) as closely related to *R. velenovskyi* (pileocystidia) and *R. risigallina* (primordial hyphae) as closely related to *R. puellula* Ebbesen, F.H. Møller & Jul. Schäff. (pileocystidia). On the other hand, species having pileocystida with contents staining grey or black and incrustations pink in sulfovanillin are all grouped in and dominate the Integrae clade.

Classification alternatives

Polyphyletic origin of analysed morphological characters in this study suggests that delimitation of larger phylogenetic lineages within the Crown clade based on morphology will be very hard, and in some cases likely impossible. Morphological recognition of smaller phylogenetic groups of closely related taxa seems to be more realistic, less difficult and particularly important as a start for species identification. Linking back the morphological traits of smaller groups may be an indirect way to circumscribe larger groups. A good example of smaller groups well-supported by both molecular and morphological characters is the Rubrinae clade defined in our study, where we discovered that the pink incrustation of pileocystidia in sulfovanillin is a good synapomorphic character, combined with strongly acrid taste of the context. Other similar examples within the Crown clade are the Maculatinae clade (Adamčík et al. 2016b) and Xerampelinae clade (Adamčík et al. 2016a).

For the rank of infrageneric *Russula* taxa, two models are usually applied. Singer (1986), Bon (1988) and others classified the genus in sections and subsections. Romagnesi (1987), Sarnari (1998) and others have one more rank of subgenus. Considering expected species number the of the Crown clade of nearly 50% of the whole genus diversity worldwide, we think that this phylogenetic lineage deserves to be classified in more than one taxonomic unit, but the current knowledge does not allow us to resolve classification on higher ranks. If we adopt any of already applied classifications systems, the lowest rank of subsection will be appropriate for Rubrinae, Maculatinae, Xerampelinae and other clades of closely related species.

Nomenclature and species circumscription within the Rubrinae clade

Identification of *Russula* species may be very challenging because of a considerable number of nomenclatural and taxonomic problems: published names that are currently not accepted, missing types or a lack of recent type studies (Buyck and Adamčík 2013). In this study, we adopted the concept and names of widely accepted species in the recent literature.

Among the four species of the Rubrinae clade, only *R. rubra* has been consequently reported to have acid-resistant incrustations (Romagnesi 1967). Besides this, the species is also recognizable by field characters (large, thick-fleshed basidiomata, flesh turning grey, pale ochre spore print, bright red cap cuticle with velvety aspect). A similar species, *R. rutila*, has smaller and thin-fleshed basidiomata with discolored pileus cuticle at the disc and a yellow spore print (Fig. 6). *Russula veternosa* has a yellow spore print and larger basidiomata with a pale pink-yellowish pileus. The pileus colour of *R. firmula* is usually dark purple, lilac or with brownish tints towards the disc. What makes the field identification easy is ecology. *Russula firmula* is always associated with conifers and probably has a preference for *Picea* (Supplementary material 1). *Russula veternosa* is considered to be exclusively associated with *Fagus*, *R. rutila* with *Quercus* and while *R. rubra* does not show any preference for a tree genus, it only occurs in deciduous forests. In case the field aspect of basidiomata and host association are ambiguous (e.g. occurrence in mixed forests), spore size and ornamentation the are most useful characters for recognizing species within the Rubrinae clade. *Russula rubra* and *R. rutila* have spores with relatively low warts that are merged and chained (Fig. 4). *Russula firmula* and *R. veternosa* have prominent isolated spines and differ by smaller spores of the latter (Table 1). Sometimes species identification might fail because of not recognizing a proper group and for this reason we provided (see above) a key that defines not only species circumscription but also delimitation of the Rubrinae clade as a whole.

Host preference and geographical distribution

Results of our ancestral state reconstruction suggest that the host plant of the Crown clade ancestors were angiosperms, which is in agreement with results of Looney et al. (2016). The possible driver for species diversification within the genus *Russula* might not be only host or climate switching, but also geographical distance or disjunction. The phylogeny of the Rubrinae clade shows at least one Asian species sister to each European species *R. firmula* and *R. rubra*. The Indian (Kashmir, Himalaya) collection placed in the *R. firmula* clade probably represents a closely related species to *R. firmula* and was also collected under conifers (Itoo et al. 2013). Sequences in the *R. rubra* clade originating from Papua New Guinea (UDB013117, UDB013204, UDB013239) probably represent a separate closely related species to *R. rubra* which is associated with deciduous trees of the genera *Castanopsis* and *Nothofagus* (Tedersoo and Põlme 2012). We think that the placement of the Iranian collections associated with *Fagaceae* in the *R. veternosa* clade but sister to the majority of European collections of *R. veternosa* is probably due to limited sequence data (only ITS) and they probably represent the same species. It would be interesting to study the phylogenetic relationship of European *R. veternosa* with the material reported from Japan (Tsujino et al. 2009), eastern North America (Burlingham 1913) and western North America (Woo 1989). We also hypothesise that host switching is a possible driver for speciation in the Crown clade. For example sister to European species clade of *R. maculata* and *R. nympharum* F. Hampe & Marxm., associated with deciduous trees are Asian collections from Pakistan and China all associated with conifers (Adamčík et al. 2016b).

Incrustations on the hyphae in the pileipellis

Our analysis revealed an important synapomorphic character for *R.* subsect. *Rubrinae*. Besides the acid-resistant incrustations known in *R. rubra* and *R. rutila*, we have demonstrate their presence in *R. firmula* and *R. veternosa* as well. The staining of the incrustations after carbolfuchsin treatment is weak and might be easily overlooked. Staining of incrustations in sulfovanillin is in some species more conspicuous, and this is very convincing in *R. vinosa*, *R. claroflava* and *R. caerulea*. The pink incrustations of Rubrinae clade members and also of some other mild-tasted species of the Integrae clade (e.g. *R. integra*) might be easily overlooked, because it usually becomes visible after ca. 30 minutes. Some publications (e.g. Moser 1978) recommend using concentrated sulphuric acid to prepare sulfovanillin, which is good for observing the dark grey or black colour change of the pileocystidia contents, but this may cause quick dissolution of incrustations as well as rupturing of the cell walls. The presence of pink incrustations in sulfovanillin, is a character so far reported just recently in the North American taxon *R. vinosa* var. *occidentalis* Singer (Adamčík et al. 2015).

In our opinion, the pink incrustations of pileipellis hyphae in sulfovanillin may become a powerful tool for classification in the genus *Russula*. They were observed on both primordial hyphae and pileocystidia with contents darkening in sulfovanillin, but some *Russula* members have acid-resistant incrustations not turning pink, both with primordial hyphae (e.g. *R. turci*, *R. risigallina*) and pileocystidia (e.g. *R. quercilicis*). The intensity and velocity of the pink reaction in sulfovanillin is very different among species, but these differences do not correspond to how they were reported in the literature. The acid-resistant incrustations in the pileipellis were introduced by Melzer and Zvára (1927), but the general utilisation of the character started after the publication of Romagnesi's monograph of the genus *Russula* (Romagnesi 1967). Since then, *R. rubra* has been constantly reported as bearing incrusted pileocystidia and for quite a long time it remains the only member of the *R.* subsection *Rubrinae* defined by incrusted pileocystidia, a yellow spore print and acrid taste. Romagnesi (1967) also mentioned incrusted pileocystidia in *R. rutila*, but he classified this species in the subsection *Maculatinae*. Bon (1988) placed *R. rutila* in *Rubrinae* and Sarnari (1998) expanded the group by including *R. quercilicis* and *R. blumiana* Blum ex Bon. Contrary to the published information, *R. veternosa* and *R. firmula* traditionally reported as having no incrustations, but they have more conspicuous and abundant pink incrustations in sulfovanillin then the other two species of the Rubrinae clade (Fig. 5). *Russula quercilicis* is not a closely related species to Rubrinae clade, and its incrustations in sulfovanillin do not turn pink. During this study we were not able to access material of *R. blumiana* or its type designated by Bon (1986). There is no mentioning of incrustations for *R. veternosa* in the literature, but it was possibly not checked because this is a well-defined species. It is often in the search for unravelling species complexes that additional detailed morphological observations are made. For example, when the taxonomic concept of *R. firmula* was challenged, the authors described incrustations on pileocystidia for related or similar taxa. Marxmüller (2014) described no incrustations in *R. firmula* but reported incrusted pileocystidia for the closely related *R. transiens* (Singer) Romagn. and Singer (1962) described a new species “*R. piceetorum* Singer” (invalid name, no type designated) bearing incrusted pileocystidia and other morphological characters identical with *R. firmula*.

Among fourteen species that form the Integrae clade in our phylogeny, nine have the pink incrustations in sulfovanillin, two have only acid-resistant incrustations and three have no incrustations. Absence of visible incrustations in some species might be caused by a very thick gelatinous-slimy matter in which the hyphal terminations in the pileipellis are embedded and which may form a barrier for contact of the chemical reagent with incrustations. For example, Romagnesi (1967) described weak acid-resistant incrustations on the pileocystidia of *R. paludosa* and *R. tinctipes*, but authors of this study as well as Sarnari (2005) were not able to see any incrustations.

Conclusions

The multi-locus phylogeny of the Crown clade shows strong support for a Rubrinae clade that comprises four European species. This group is morphologically defined by a yellow spore print, acrid taste of the context and pileocystidia with pink incrustations in sulfovanillin. We propose to call the group as *Russula* subsect. *Rubrinae*. *Russula firmula* and *R. veternosa* both have, contrary to traditional opinion, pink incrustations on pileocystidia in sulfovanillin and are new for the subsection, whereas incrustations of *R. quercilicis* do not stain pink in sulfovanillin and accordingly the species is not placed as a close relative of the group in the phylogeny. The Rubrinae clade is placed in the Integrae clade that is dominated by species with pink incrustations in sulfovanillin. Further classification of the Crown clade requires better sampling and more genetic markers. Colour changes not only of the context, but also of the incrustations, are demonstrated to have importance for delimitations of *Russula* taxa on various ranks.

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Tables

Table 1 Comparison of selected characters among four species of *R. rubra* lineage. All values except of the height of spore ornamentation are averages of 20 measurements. TC – terminal cells of hyphae in the pileipellis; T! – type specimen

	R. veternosa PC0124979 T! SAV F-2588 SAV F-3391	*R. rubra* SAV F-914 SAV (HK14300c) SAV F-3229	*R. rutila* SAV F-1564 GENT (2007 BT103) SAV (HK14028)	*R. firmula* GENT (2010 BT85) T! SAV F-2655 SAV (HK14300c)
Spore size	7.2 × 5.8 µm 7.5 × 6 µm 7.2 × 5.7 µm	8.1 × 6.8 µm 8.2 × 6.9 µm 8.4 × 6.9 µm	8.9 × 7.2 µm 8.5 × 6.8 µm 8.7 × 7 µm	8.6 × 7.1 µm 8.3 × 6.9 µm 8.4 × 7 µm
Spore ornamentation	spines 0.7–1.1 µm high, mainly isolated	warts 0.4–0.6 µm high, merged in chains, occasionally connected by fine lines	warts 0.6–0.9 µm high, merged in chains, occasionally connected by short, fine lines	spines 0.8–1.2 µm high, mainly isolated
TC near the pileus margin	29.4 × 2.5 µm 31.1 × 2.6 µm 28.6 × 2.6 µm cylindrical, apically obtuse or constricted to attenuated	33.5 × 3.4 µm 30.1 × 3.1 µm 28.4 × 3.1 µm subulate, occasionally cylindrical, apically usually attenuated or constricted	31.7 × 2.9 µm 24.5 × 2.9 µm 28.1 × 3 µm subulate and apically attenuated	31.1 × 3.1 µm 27.7 × 2.9 µm 26.7 × 3 µm subulate or cylindrical, apically usually attenuated or constricted
Incustations on pileocystidia	weakly acid-resistant after carbolfuchsin treatment, turning slowly to bright pink in sulfovanillin			
Basidia size	33.2 × 11 µm 36.1 × 11.2 µm 35.5 × 10.8 µm	50.3 × 11 µm 54.9 × 11.3 µm 47.8 × 10.9 µm	48 × 11.7 µm 50.4 × 11.9 µm 44.7 × 12 µm	46.5 × 13.1 µm 48.8 × 12.9 µm 41.9 × 12.1 µm
Pleurocystidia size	58.9 × 10.5 µm 59.8 × 10.6 µm 56.5 × 10.6 µm	86 × 11.7 µm 82.5 × 11.5 µm 78.5 × 11.3 µm	65.8 × 9.2 µm 63.1 × 9.3 µm 58.6 × 9.9 µm	72.4 × 10.1 µm 76.1 × 10.3 µm 68.9 × 10.6 µm

Table 2 Overview of the information about types of species within *R. rubra* lineage.

Species	**Original description**	**Type status**	**Designating publication**	**Country of origin**	**Type condition**
R. firmula	Schaeffer (1940), cited full description in Schaeffer (1933) as "*R. nitida*"	lectotype	designated here	not specified	illustration, Bresadola (1929), pl. 458 (as "*R. badia*")
		epitype	designated here	Germany	herbarium specimen GENT (2010 BT85), sequences ITS KU928142, mtSSU KY471568, *rpb2* KY616675
R. rubra	Fries (1821)	lectotype	Sarnari (1998)	France	illustration, Bulliard (1781), pl. 42, fig. B (as "*Agaric sanguin*")
		epitype	Sarnari (1998)	France	herbarium specimen PC0723456 (Romagnesi n°52-241), sequence ITS (Genbank KY582680)
R. rutila	Romagnesi (1952)	holotype	Romagnesi (1952)	France	herbarium specimen PC0723457 (Romagnesi n°12-IX-45), sequence ITS (Genbank KY582681)
R. veternosa	Fries (1838)	lectotype	Sarnari (1998)	not specified	illustration, Paulet (1855), pl. 74, f. 3 (as "*Agaricus vaternosus*")
		epitype	Sarnari (1998)	France	herbarium specimen PC0124979 (Romagnesi n°53-206), no sequence data

Figures

Fig. 1 Maximum Likelihood phylogeny inferred from three loci (ITS, mtSSU and *rpb2*) with four target species-level clades highlighted as well as the known superclades comprising them. Collection labels are updated with appropriate taxon names except where collector identifications disagree. Basidiomata samples are labelled by herbarium code and collections number in parenthesis, sequences of environmental samples or collections without reference to a herbarium specimen are labelled with accession numbers in italics (sequences from UNITE database start with UDB, others are from GenBank). Countries of origin are included for species in the Rubrinae clade. Bootstrap values followed by Bayesian posterior probabilities are indicated at nodes.

Fig. 2 Maximum Likelihood phylogeny of the Crown clade *Russula* species using three loci (ITS, mtSSU and *rpb2*). Bootstrap labels are displayed for all nodes. Trees are displayed as Balls and Sticks graphs with proportional log-likelihoods of ancestral character states displayed as pie graphs at given nodes.

Fig. 3 *R. veternosa* (epitype, PC0124979). **a** Pileocystidia near the pileus margin. **b** Pileocystidia near the pileus centre. **c** Spores in Melzer's reagent. **d** Hyphal terminations in the pileus margin. **e** Hyphal terminations near the pileus centre. **f** Basidia. **g** Basidiola. **h** Marginal cells. **i** Pleurocystidia. **j** Cheilocystidia. Contents of cystidia are represented as observed in Congo red for some elements only, the others plus sign indicated their contents schematically. Scale bar equals 10 µm, but only 5 µm for spores. Drawings by: S. Jančovičová.

Fig. 4 Microscopic structure of pileipellis and spores of three studied species. *R. firmula* (SAV F-2655). **a** Pileocystidia near the pileus margin. **b** Spores in Melzer's reagent. **c** Hyphal terminations in the pileus margin. *R. rubra* (SAV F-914). **d** Pileocystidia near the pileus margin. **e** Spores in Melzer's reagent. **f** Hyphal terminations in the pileus margin. *R. rutila* (SAV F-1564). **g** Pileocystidia near the pileus margin. **h** Spores in Melzer's reagent. **i** Hyphal terminations in the pileus margin. Contents of cystidia are represented as observed in Congo red for some elements only, the others plus sign indicated their contents schematically. Scale bar equals 10 µm, but only 5 µm for spores. Drawings by: S. Jančovičová.

Fig. 5 Pink incrustations on pileocystidia observed in sulfovanillin. **a** *R. firmula* (SAV F-2137). **b** *R. rubra* (SAV F-4216). **c** *R. rutila* (SAV F-1564). **d** *R. veternosa* (SAV F-1403). **e** *R. caerulea* (SAV F-2151). **f** *R. claroflava* (SAV F-1791).

Fig. 6 Field appearance of four *Russula* species included in this study. **a** *R. firmula* (TU106603), photo by Velo Liiv. **b** *R. rubra* (GENT FH12/227), photo by Felix Hampe. **c** *R. rutila* (SAV F-1497), photo by Per Marstad. **d** *R. veternosa* (SAV F-1403), photo by Per Marstad. Scale bar equals 30 mm.

Supplementary material 1 List of sequences used in this study with associated data. Sequences with accession numbers starting with UDB are retrieved from the UNITE database, all other are from GenBank. EcM – ectomycorrhizal isolates.

Supplementary material 2 Annotated description of the epitype specimen of *R. veternosa*.

Supplementary material 3 Photo of the epitype collection of *R. firmula* (GENT 2010 BT85) photo by Felix Hampe.

Figure 1

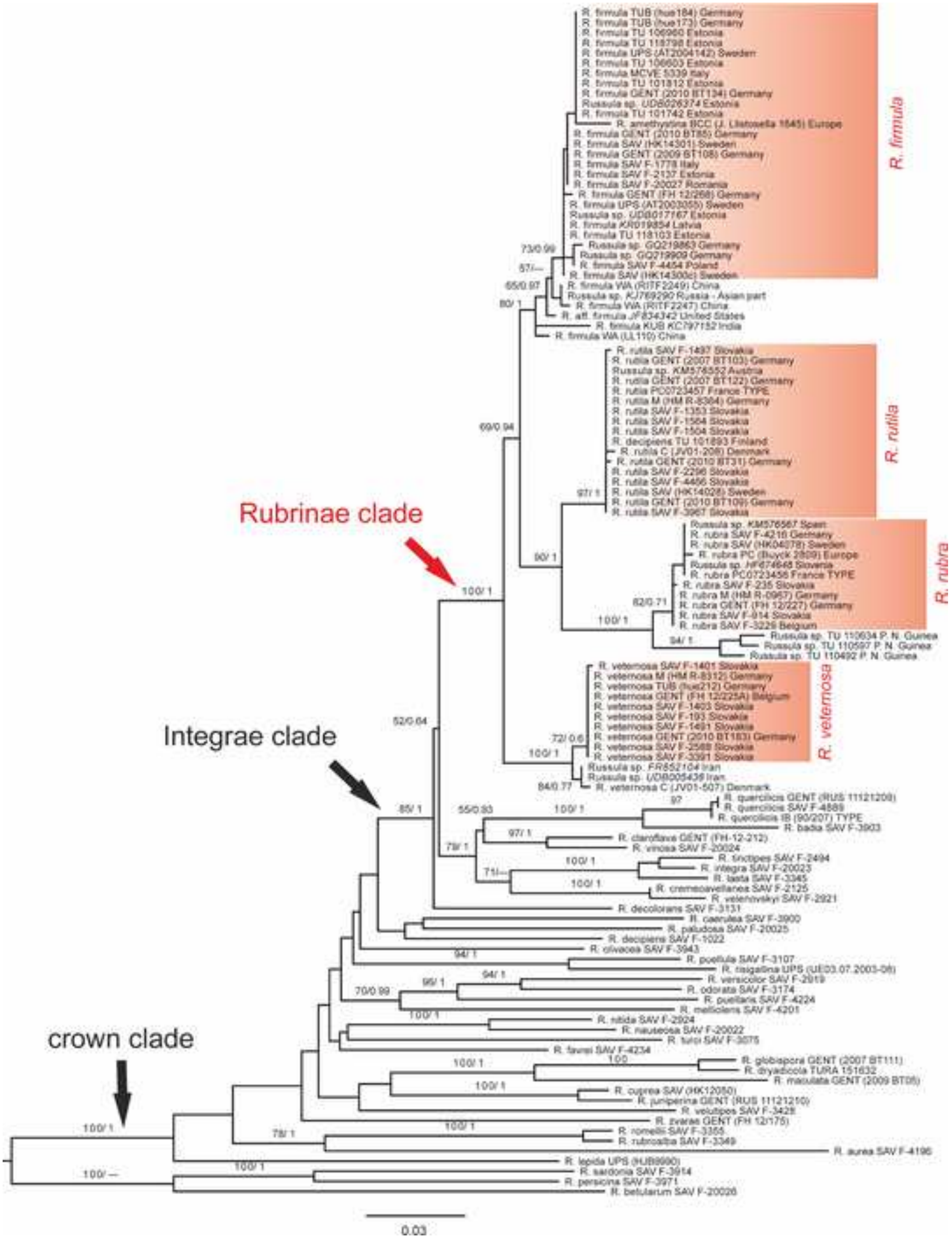

R. firmula TUB (hue184) Germany
R. firmula TUB (hue173) Germany
R. firmula TU 106960 Estonia
R. firmula TU 118798 Estonia
R. firmula UPS (AT2004142) Sweden
R. firmula TU 106603 Estonia
R. firmula MCVE 5339 Italy
R. firmula TU 101812 Estonia
R. firmula GENT (2010 BT134) Germany
Russula sp. UDB026374 Estonia
R. firmula TU 101742 Estonia
R. amethystina BCC (J. Listosella 1645) Europe
R. firmula GENT (2010 BT85) Germany
R. firmula SAV (HK14301) Sweden
R. firmula GENT (2009 BT108) Germany
R. firmula SAV F-1778 Italy
R. firmula SAV F-2137 Estonia
R. firmula SAV F-20027 Romania
R. firmula GENT (FH 12/268) Germany
R. firmula UPS (AT2003055) Sweden
Russula sp. UDB017767 Estonia
R. firmula KR019854 Latvia
R. firmula TU 118103 Estonia
Russula sp. GQ219863 Germany
Russula sp. GQ219909 Germany
R. firmula SAV F-4454 Poland
R. firmula SAV (HK14300c) Sweden
R. firmula WA (RITF2249) China
Russula sp. KJ769290 Russia - Asian part
R. firmula WA (RITF2247) China
R. aff. firmula JF834342 United States
R. firmula KUB KC797152 India
R. firmula WA (LL110) China
73/0.99
57/—
65/0.97
80/ 1
R. firmula
R. rutila SAV F-1497 Slovakia
R. rutila GENT (2007 BT103) Germany
Russula sp. KM576552 Austria
R. rutila GENT (2007 BT122) Germany
R. rutila PC0723457 France TYPE
R. rutila M (HM R-8364) Germany
R. rutila SAV F-1353 Slovakia
R. rutila SAV F-1564 Slovakia
R. rutila SAV F-1504 Slovakia
R. decipiens TU 101893 Finland
R. rutila C (JV01-208) Denmark
R. rutila GENT (2010 BT31) Germany
R. rutila SAV F-2296 Slovakia
R. rutila SAV F-4466 Slovakia
R. rutila SAV (HK14028) Sweden
R. rutila GENT (2010 BT109) Germany
R. rutila SAV F-3967 Slovakia
97/ 1
69/0.94
R. rutila
Russula sp. KM576567 Spain
R. rubra SAV F-4216 Germany
R. rubra SAV (HK04078) Sweden
R. rubra PC (Buyck 2809) Europe
Russula sp. HF674648 Slovenia
R. rubra PC0723456 France TYPE
R. rubra SAV F-235 Slovakia
R. rubra M (HM R-0967) Germany
R. rubra GENT (FH 12/227) Germany
R. rubra SAV F-914 Slovakia
R. rubra SAV F-3229 Belgium
Russula sp. TU 110634 P. N. Guinea
Russula sp. TU 110597 P. N. Guinea
Russula sp. TU 110492 P. N. Guinea
90/ 1
82/0.71
100/ 1
94/ 1
R. rubra
Rubrinae clade
100/ 1
R. veternosa SAV F-1401 Slovakia
R. veternosa M (HM R-8312) Germany
R. veternosa TUB (hue212) Germany
R. veternosa GENT (FH 12/225A) Belgium
R. veternosa SAV F-1403 Slovakia
R. veternosa SAV F-193 Slovakia
R. veternosa SAV F-1491 Slovakia
R. veternosa GENT (2010 BT183) Germany
R. veternosa SAV F-2588 Slovakia
R. veternosa SAV F-3391 Slovakia
Russula sp. FR852104 Iran
Russula sp. UDB005436 Iran
R. veternosa C (JV01-507) Denmark
72/ 0.6
100/ 1
84/0.77
R. veternosa
52/0.64
Integrae clade
85/ 1
55/0.93
100/ 1
97
R. quercilicis GENT (RUS 11121209)
R. quercilicis SAV F-4889
R. quercilicis IB (90/207) TYPE
R. badia SAV F-3903
97/ 1
R. claroflava GENT (FH-12-212)
R. vinosa SAV F-20024
79/ 1
100/ 1
R. tinctipes SAV F-2494
R. integra SAV F-20023
R. lasta SAV F-3345
71/—
100/ 1
R. cremeoavellanea SAV F-2125
R. velenovskyi SAV F-2921
R. decolorans SAV F-3131
R. caerulea SAV F-3900
R. paludosa SAV F-20025
R. decipiens SAV F-1022
R. olivacea SAV F-3943
94/ 1
R. poelluta SAV F-3107
R. risigallina UPS (UE03.07.2003-08)
94/ 1
95/ 1
70/0.99
R. versicolor SAV F-2919
R. odorata SAV F-3174
R. puellaris SAV F-4224
R. melliolens SAV F-4201
100/ 1
R. nitida SAV F-2924
R. nauseosa SAV F-20022
R. turci SAV F-3075
R. favrei SAV F-4234
100/ 1
100
R. globispora GENT (2007 BT111)
R. dryadicola TURA 151632
R. maculata GENT (2009 BT05)
100/ 1
R. cuprea SAV (HK12050)
R. juniperina GENT (RUS 11121210)
R. velutipes SAV F-3428
R. zvarae GENT (FH 12/175)
crown clade
100/ 1
78/ 1
100/ 1
R. romellii SAV F-3355
R. rubroalba SAV F-3349
R. aurea SAV F-4196
R. lepida UPS (HUB9990)
100/ 1
100/ —
R. sardonia SAV F-3914
R. persicina SAV F-3971
R. betularum SAV F-20026
0.03

Figure 2

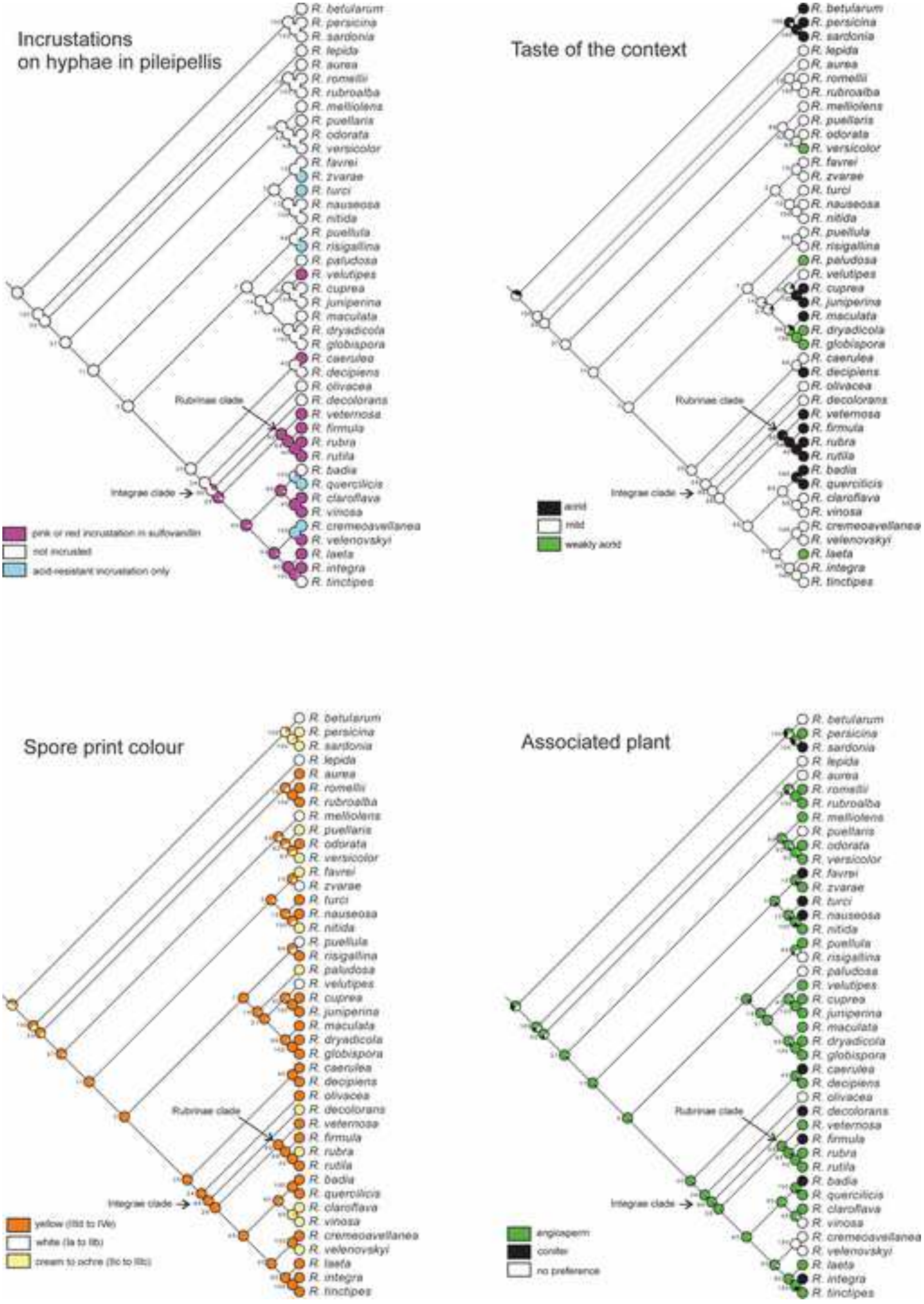
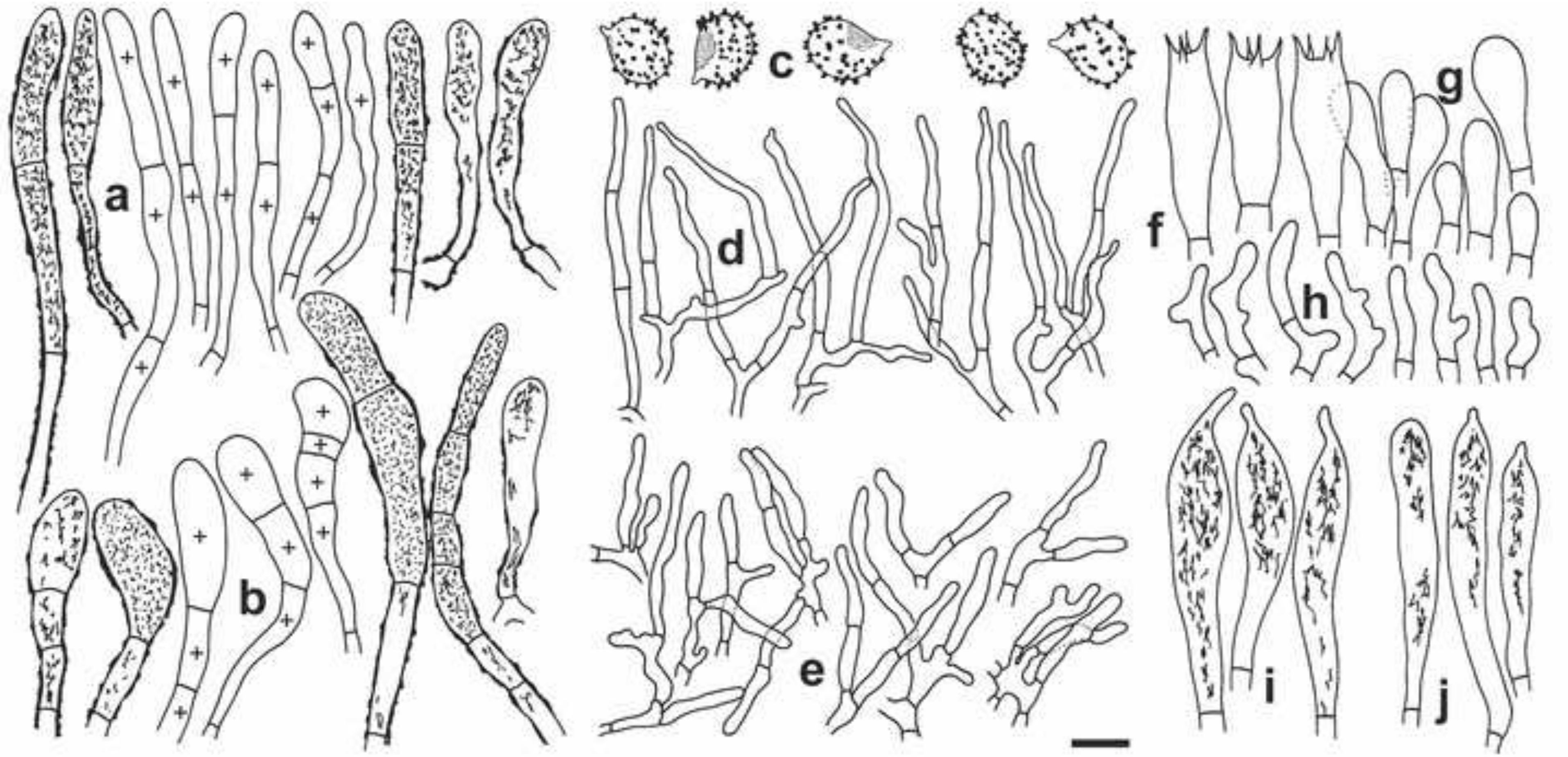

Incrustations on hyphae in pileipellis
R. betularum
R. persicina
R. sardonia
R. lepida
R. aurea
R. romellii
R. rubroalba
R. melliolens
R. puellaris
R. odorata
R. versicolor
R. favrei
R. zvarae
R. turci
R. nauseosa
R. nitida
R. puellula
R. risigallina
R. paludosa
R. velutipes
R. cuprea
R. juniperina
R. maculata
R. dryadicola
R. globispora
R. caerulea
R. decipiens
R. olivacea
R. decolorans
R. veternosa
R. firmula
R. rubra
R. rutila
R. badia
R. quercilicis
R. claroflava
R. vinosa
R. cremeoavellanea
R. velenovskyi
R. laeta
R. integra
R. tinctipes
Rubrinae clade
Integrae clade
pink or red incrustation in sulfovanillin
not incrusted
acid-resistant incrustation only
Taste of the context
R. betularum
R. persicina
R. sardonia
R. lepida
R. aurea
R. romellii
R. rubroalba
R. melliolens
R. puellaris
R. odorata
R. versicolor
R. favrei
R. zvarae
R. turci
R. nauseosa
R. nitida
R. puellula
R. risigallina
R. paludosa
R. velutipes
R. cuprea
R. juniperina
R. maculata
R. dryadicola
R. globispora
R. caerulea
R. decipiens
R. olivacea
R. decolorans
R. veternosa
R. firmula
R. rubra
R. rutila
R. badia
R. quercilicis
R. claroflava
R. vinosa
R. cremeoavellanea
R. velenovskyi
R. laeta
R. integra
R. tinctipes
Rubrinae clade
Integrae clade
acrid
mild
weakly acrid
Spore print colour
R. betularum
R. persicina
R. sardonia
R. lepida
R. aurea
R. romellii
R. rubroalba
R. melliolens
R. puellaris
R. odorata
R. versicolor
R. favrei
R. zvarae
R. turci
R. nauseosa
R. nitida
R. puellula
R. risigallina
R. paludosa
R. velutipes
R. cuprea
R. juniperina
R. maculata
R. dryadicola
R. globispora
R. caerulea
R. decipiens
R. olivacea
R. decolorans
R. veternosa
R. firmula
R. rubra
R. rutila
R. badia
R. quercilicis
R. claroflava
R. vinosa
R. cremeoavellanea
R. velenovskyi
R. laeta
R. integra
R. tinctipes
Rubrinae clade
Integrae clade
yellow (IIId to IVe)
white (Ia to IIb)
cream to ochre (IIc to IIIc)
Associated plant
R. betularum
R. persicina
R. sardonia
R. lepida
R. aurea
R. romellii
R. rubroalba
R. melliolens
R. puellaris
R. odorata
R. versicolor
R. favrei
R. zvarae
R. turci
R. nauseosa
R. nitida
R. puellula
R. risigallina
R. paludosa
R. velutipes
R. cuprea
R. juniperina
R. maculata
R. dryadicola
R. globispora
R. caerulea
R. decipiens
R. olivacea
R. decolorans
R. veternosa
R. firmula
R. rubra
R. rutila
R. badia
R. quercilicis
R. claroflava
R. vinosa
R. cremeoavellanea
R. velenovskyi
R. laeta
R. integra
R. tinctipes
Rubrinae clade
Integrae clade
angiosperm
conifer
no preference

Figure 3



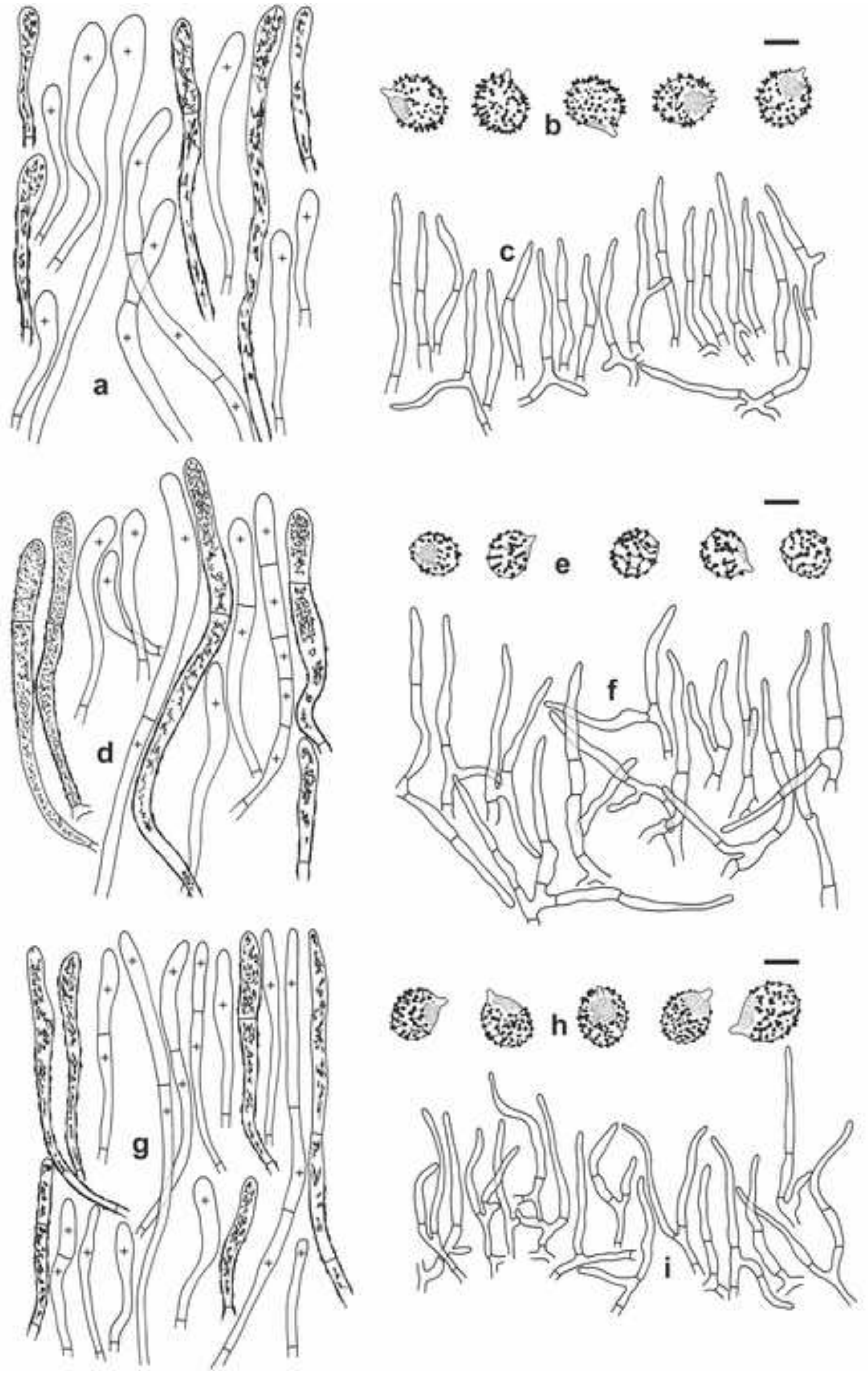

a
b
c
d
e
f
g
h
i

Figure 5

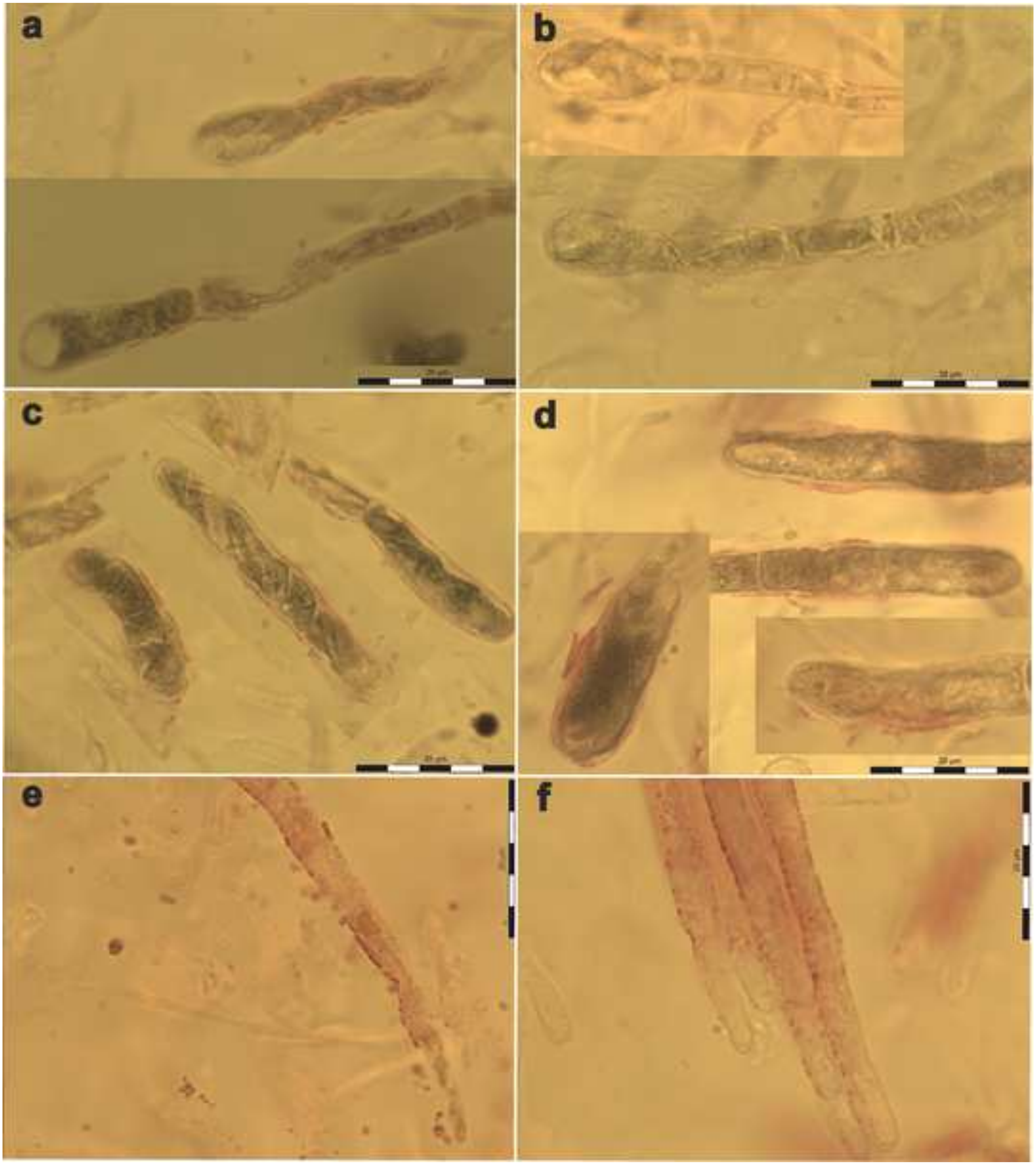


Figure 6

New insights in *Russula* subsect. *Rubrinae*: phylogeny and the quest for synapomorphic characters

Miroslav Caboň, Ursula Eberhardt, Brian Looney, Felix Hampe, Miroslav Kolařík, Soňa Jančovičová, Annemieke Verbeken, Slavomír Adamčík

Supplementary material 1. List of sequences used in this study with associated data. Sequences with accession numbers starting with UDB are retrieved from the UNITE database, all other are from GenBank. EcM – ectomycorrhizal isolates.

Species (orig. identification)	**Herb. acronym (collection number)**	**Country: Collecting area**	**Host**	**Collector**	**Date**	**Sequences source**	**ITS**	**mtSSU**	**RPB2**
R. aurea	SAV F-4196	Slovakia: W Carpathians, Štiavnické vrchy Mts., Kráľovec – Krnišov	*Carpinus*	S. Adamčík	14 Jul 2010	This study	KY582718	KY471610	
R. badia	SAV F-3903	Slovakia: Panonia, Záhorská nížina lowland, Šajdíkove Humence – Horáreň Kalaštov	*Pinus silvestris*	M. Caboň	5 Nov 2012	Adamčík et al. 2016, This study	KU928168	KY471607	
R. betularum	SAV F-20026	Romania: Apuseni Mts., Vladeasa Mts., Poiana Horea – S of the village	*Betula*, *Picea*	M. Caboň	6 Oct 2014	This study	KY582694	KY471585	KY616687
R. caerulea	SAV F-3900	Slovakia: Panonia, Záhorská nížina lowland, Studienka – Tŕnie	*Quercus*, *Pinus silvestris*	M. Caboň	1 Nov 2012	This study	KY582713	KY471606	KY616706
R. claroflava	GENT (FH-12-212)	Germany: Niedersachsen, Hildesheim		F. Hampe	18 Aug 2012	Looney et al. 2016	KT933997		KT933929
R. cremeoavellanea	SAV F-2125	Estonia: Saarema, Põlluküla – Viidumäe Natural Reserve	*Betula*	S. Adamčík	21 Sep 2008	This study	KY582695	KY471586	KY616688
R. cuprea	SAV (HK12050)	Sweden: Gotland, Hangvar – Elingshems oderkyrka	*Quercus*	H. Kaufmann	17 Aug 2012	Adamčík et al. 2016, This study	KU886591	KY471620	KY616721
R. decipiens	SAV F-1022	Slovakia: W Carpathians, Štiavnické vrchy Mts., Nová Dedina – Sovia dolina	*Quercus*	S. Adamčík	28. Jul 2005	This study	KY582683	KY471572	KY616679
R. decolorans	SAV F-3131	Slovakia: W Carpathians, Vysoké Tatry Mts., Ždiar – Trojrohé pleso	*Pinus mugo*	S. Adamčík	16 Sep 2009	This study	KY582705	KY471597	KY616698
R. dryadicola	TURA 151632	Finland: Lapland, Kilpisjärvi – Saana	birch forest	J. Ruotsalainen	16 Aug 1990	Adamčík et al. 2016, This study	KU928141	KY471623	KY616724
R. favrei	SAV F-4234	Poland: W Carpathians, Západné Tatry Mts., Kiry – Koscielicka potok	*Picea*	S. Adamčík	27 Aug 2014	This study	KY582721	KY471614	KY616715
R. firmula	SAV F-2137	Estonia: Hiiumaa, Kõrgessaare – Mägipe	*Picea*, *Pinus silvestris*	S. Adamčík	22 Sep 2008	This study	KY582696	KY471587	KY616689
R. firmula	TU 101742	Estonia: Valga, Palupera vald – Otepää looduspark	spruce forest with *Betula*, *Corylus avellana*	I. Saar	12 Sep 2011	Unite	UDB011315		
R. firmula	TU 101812 (JV26564)	Estonia: Saare, Salme vald – Kaugatoma-Lõo maastikukaitseala Nature Reserve	mixed moist forest	J. Vauras	18 Sep 2008	Unite	UDB016073		
R. firmula	TU 106603	Estonia: Saare, Lümanda vald – Viidu		V. Liiv	16 Aug 2009	Unite	UDB011184		
R. firmula	TU 106960	Estonia: Lääne, Vormsi vald - Hullo	coniferous forest	V. Liiv	15 Sep 2010	Unite	UDB011205		

Species (orig. identification)	**Herb. acronym (collection number)**	**Country: Collecting area**	**Host**	**Collector**	**Date**	**Sequences source**	**ITS**	**mtSSU**	**RPB2**
R. firmula	TU 118103	Estonia: Võru, Rõuge vald – Ruuksu	mixed forest	V. Liiv	10 Sep 2011	Unite	UDB011368		
R. firmula	TU 118798	Estonia: Võru, Varstu vald – Pähni looduskaitseala	mixed forest	V. Liiv	2 Sep 2013	Unite	UDB019573		
R. firmula	GENT (2009 BT108)	Germany: Thuringia, Schellroda – Kaiserwiese	*Picea*	F. Hampe, J. Girwert	14 Nov 2009	This study	KY582670		KY616670
R. firmula	GENT (2010 BT134)	Germany: Thuringia, Riechheim	*Picea*	F. Hampe	26 Sep 2010	This study	KY582672		
R. firmula	GENT (2010 BT85)	Germany: Thuringia, Troistedt		F. Hampe	24 Aug 2010	Adamčík et al. 2016, This study	KU928142	KY471568	KY616675
R. firmula	GENT (FH 12/268)	Germany: Sachsen-Anhalt, Harz, Elend – Kramershai		F. Hampe	3 Oct 2012	This study	KY582675		
R. firmula	TUB (hue 173)	Germany: Baden-Württemberg, Tübingen-Bebenhausen, Schönbuch – Kohlhau	*Picea abies*	U. Eberhardt	22 Oct 1998	Eberhardt 2002	AF418631/UDB000359		
R. firmula	TUB (hue184)	Germany: Baden-Württemberg, Balingen – Plettenberg	*Picea*	U. Eberhardt	22 Oct 1998	Eberhardt and Verbeken 2004	UDB00030/AY606962		
R. firmula	MCVE 5339	Italy: Pordenone	coniferous trees	G. Zecchin	5 Aug 1998	Osmundson et al. 2013	JF908671		
R. firmula	SAV F-1778	Italy: Trento, Sella Valsugana – Bello Valle	*Picea* (*Pinus silvestris*)	S. Adamčík	27 Sep 1997	This study	KY582689	KY471579	KY616682
R. firmula	EcM (H23)	Latvia:			Jul 2013	Gaitnieks et al. 2016	KR019854		
R. firmula	SAV F-4454	Poland: W Carpathians, Západné Tatry Mts., Kiry – Koscielicka potok	*Picea*, *Abies*, *Acer*	M. Caboň	27 Aug 2014	Adamčík et al. 2016, This study	KU886593	KY471616	KY616717
R. firmula	SAV F-20027	Romania: Apuseny Mts., Vladeasa Mts., Doda Pilii – margin of the pasture	*Picea, Abies, Fagus*	M. Caboň	7 Oct 2014	This study	KY582722	KY471615	KY616716
R. firmula	SAV (HK14300c)	Sweden: Angermanland, Adalsliden, Näsaker – Assjoberge	*Picea*	N.O. Nilsson, M. Karlsson	12 Sep 2014	Adamčík et al. 2016, This study	KU928143	KY471622	KY616723
R. firmula	SAV (HK14301)	Sweden: Västernorrlands Län, Avike Bruk – Tyundero		H. Kaufmann	10 Sep 2014	This study	KY582725		KY616677
R. firmula	UPS (AT2003055)	Sweden: Uppsala, Uppsala – Stadskogen	mixed forest	A.F.S. Taylor	31 Jul 2003	This study	KY613995		
R. firmula	UPS (AT2004142)	Sweden: Uppsala, Nåsten	mixed forest	A.F.S. Taylor	24 Jul 2004	GenBank	DQ422017/UDB002501		DQ421958
R. firmula (*R. amethystina*)	BCC (J. Llistosella 1645)	Europe				Miller and Buyck 2002	AY061653		
R. firmula (unid. fungus)	EcM (L9184)	Estonia: Saaremaa , Tuiu kurisud	*Picea abies*		19 Sep 2013	Unite	UDB026374		
R. firmula (unid. *Russula*)	EcM (L323)	Estonia: Saare, Kihelkonna vald – Tagamoisa				Tedersoo et al. 2006	UDB01716/AJ893215		
R. firmula (unid. *Russula*)	soil sample (SC_ITS_060)	Germany: Thuringia, Hainich	*Picea*		Apr 2008	Christ et al. 2011	GQ219863		
R. firmula (unid. *Russula*)	soil sample (SC_ITS_106)	Germany: Thuringia, Hainich	*Picea*		Apr 2008	Christ et al. 2011	GQ219909		
R. globispora	GENT (2007 BT111)	Germany: Thuringia, Gügleben – Werningslebener Wald		F. Hampe	8 Sep 2007	Adamčík et al. 2016, This study	KU928144	KY471564	KY616671

Species (orig. identification)	Herb. acronym (collection number)	Country: Collecting area	Host	Collector	Date	Sequences source	ITS	mtSSU	RPB2
R. integra	SAV F-20023	Slovakia: W Carpathians, Západné Tatry Mts., Zakopané – Droga pod Reglami	*Picea*	M. Caboň	25 Aug 2014	This study	KY582682	KY471582	KY616685
R. juniperina	GENT (RUS11121210)	Spain: Mallorca, Valdemossa – Plateau Plan de Pouet	*Quercus ilex*	F. Hampe, J. Kleine	12 Dec 2011	This study	KU886596	KY471571	KY616676
R. laeta	SAV F-3949	Slovakia: W Carpathians, Pohronský Inovec Mts., Hostie	*Quercus*, *Carpinus*	P. Marstad	16 Sep 2013	This study	KY582708	KY471600	KY616709
R. lepida	UPS (HJB9990)	Belgium: Hainaut, Soignes	Broadleaf woodland	H.J. Beker	24 Jul 2004	GenBank, This study	DQ422013	KY471624	DQ421954
R. maculata	GENT (2009 BT05)	Germany: Thuringia, Arnstadt – Gustav-Freytag-Weg, Schlossleite		J. Girwert	1 Jun 2009	Adamčík et al. 2016, This study	KU928155	KY471566	
R. melliolens	SAV F-4201	Slovakia: W Carpathians, Štiavnické vrchy Mts., Sebechleby – Stará hora	*Quercus*, *Carpinus*	S. Adamčík	13 Jul 2010	This study	KY582719	KY471611	KY616712
R. nauseosa	SAV F-20022	Poland: W Carpathians, Západné Tatry Mts., Kiry – Koscielicka potok	*Picea*	S. Adamčík	27 Aug 2014	This study	KY582691	KY471581	KY616684
R. nitida	SAV F-2924	Slovakia: W Carpathians, Volovské vrchy Mts., Predná Hora – recreation area	*Betula*	I. Kautmanová	8 Jul 2009	This study	KY582702	KY471594	KY616696
R. odorata	SAV F-3174	Slovakia: W Carpathians, Tríbeč Mts., Jelenec – N of the autocamp	*Quercus*, *Carpinus*	S. Adamčík	12 Jul 2010	This study	KY582706	KY471598	KY616699
R. olivacea	SAV F-3943	Slovakia: W Carpathians, Tríbeč Mts., Zlatno – Veľký Tríbeč	*Fagus*, *Quercus*, *Carpinus*	S. Adamčík	19 Jul 2013	This study	KY582715	KY471608	KY616708
R. paludosa	SAV F-20025	Poland: W Carpathians, Západné Tatry Mts., Zakopane – Mala Polanka under Grzybowec	*Picea*	S. Adamčík	25 Aug 2014	This study	KY582693	KY471584	KY616686
R. persicina	SAV F-3971	Slovakia: W Carpathians, Pohronský Inovec Mts., Hostie	*Carpinus*, *Fagus*, *Quercus cerris*	S. Adamčík	16 Sep 2013	This study	KY582717	KY471609	KY616711
R. puellaris	SAV F-4224	Slovakia: W Carpathians, Spišská Magura Mts., Vojňany – Dlhá hora	*Abies*	S. Adamčík	26 Aug 2014	This study	KY582720	KY471613	KY616714
R. puellula	SAV F-3107	Slovakia: W Carpathians, Muránska planina National Park, Muráň – Hrdzavá dolina	*Fagus*, *Carpinus*	V. Kautman	6 Jul 2009	This study	KY582704	KY471596	KY616697
R. quercilicis (holotype)	IB (n°90/207)	Italy: Tuscany, Marsiliana	*Quercus ilex*	M. Sarnari	6 Nov 1990	This study	KY582726	KY471570	
R. quercilicis	SAV F-4889	Italy: Tuscany, Marsiliana, Riserva Statale	*Quercus suber*, *Quercus ilex*	L. Michelin	8 Nov 2016	This study	KY613998	KY582728	KY616725
R. quercilicis	GENT (RUS 11121209)	Spain: Mallorca, Valdemossa – Plateau Plan de Pouet	*Quercus ilex*	F. Hampe	12 Dec 2011	This study	KY613996		
R. risigallina	UPS (UE03.07.2003-08)	Sweden: Östagötaland, Linköping in direction to Björkby-Sälja, Ekäng	*Quercus*, *Betula*, *Picea*	U. Eberhardt	3 Jul 2003	GenBank	DQ422022		DQ421961
R. romellii	SAV F-3355	Slovakia: W Carpathians, Malé Karpaty Mts., Kuchyňa – Vývrať, Bučková	*Fagus*, *Quercus*	S. Adamčík	6 Jul 2011	This study	KY582710	KY471602	KY616702
R. rubra	SAV F-3229	Belgium: Walloon, Frasnes	*Quercus*, *Fraxinus*	B. Wasstorp	10 Sep 2010	This study	KY582707	KY471599	KY616700
R. rubra	PC (Buyck 2809)	Europe				Miller and Buyck 2002	AY061717		
R. rubra	GENT (FH 12/227)	Germany: Thuringia, Grosslohra – Friedricslohra		F. Hampe	29 Aug 2012	This study	KY582676		
R. rubra	M (HM R-0967)	Germany: Bayern, München – Grünwald	*Fagus sylvatica*, *Pinus silvestris*	H. Marxmüller	24 Sep 2009	This study	KY582677		

Species (orig. identification)	**Herb. acronym (collection number)**	**Country: Collecting area**	**Host**	**Collector**	**Date**	**Sequences source**	**ITS**	**mtSSU**	**RPB2**
R. rubra	SAV F-4216	Germany: Thuringia, Grosslohra – Friedricslohra	*Fagus*	C. Piuri	29 Aug 2012	Adamčík et al. 2016, This study	KU886599	KY471612	KY616713
R. rubra	SAV F-235	Slovakia: E Carpathians, Vihorlat Mts., Remetské Hámre – Machnatý vrch Nature Reserve	*Fagus*	S. Adamčík	19 Sep 2001	Adamčík et al. 2016, This study	KU928160	KY471589	KY616691
R. rubra	SAV F-914	Slovakia: W Carpathians, Biele Karpaty Mts., Nová Bošáca – Veľký Lopeník	*Fagus sylvatica*, *Quercus*	I. Kautmanová	8 Jul 2002	This study	KY582723	KY471618	KY616719
R. rubra	SAV (HK04078)	Sweden: Bohuslän, Tossene – Hogsams	*Fagus*	H. Kaufmann	15 Sep 2004	Adamčík et al. 2016, This study	KU928161	KY471619	KY616720
R. rubra (epitype)	PC 0723456 (n°52-241)	France: Marne, d´Épernay – Foret de Carnelle		H. Romagnesi	5 Oct 1952	This study	KY582680		
R. rubra (unid. *Russula*)	soil sample (GIS-ABRUSLEP/ 150711)	Slovenia: Istria, Abitanti/Mocunigi	*Fagus sylvatica*	A. Masek	1 Jun 2014	Masek 2016	HF674648		
R. rubra (unid. *Russula*)	EcM (LM196)	Spain	*Quercus petrea*			Suz et al. 2014	KM576567		
R. rubroalba	SAV F-3349	Slovakia: W Carpathians, Štiavnické vrchy Mts., Prenčov – Sitno	*Carpinus*, *Quercus*	S. Adamčík	5 Jul 2011	This study	KY582709	KY471601	KY616701
R. rutila	C (JV01-208)	Denmark: Juelsminde, Palsgård Slotspark	*Fagus*, *Quercus*	J. Vesterholt	25 Aug 2001	This study	KY613997		
R. rutila	GENT (2007 BT103)	Germany: Thuringia, Gutendorf – NSG Diebskammer		J. Girwert	7 Aug 2007	This study	KY582669		
R. rutila	GENT (2007 BT122)	Germany: Thuringia, Gügleben – Werningslebener Wald		J. Girwert	26 Aug 2007	Adamčík et al. 2016, This study	KU928163	KY471565	
R. rutila	GENT (2010 BT109)	Germany: Thuringia, Gotha – Gotha castle park	*Tilia*, *Carpinus*, *Fagus*, *Quercus*	F. Hampe	22 Aug 2010	This study	KY582671		KY616672
R. rutila	GENT (2010 BT31)	Germany: Thuringia, Nohra		J. Girwert	19 Aug 2010	This study	KY582674	KY471567	KY616674
R. rutila	M (HM R-8364)	Germany: Bayern, Starnberg – Seeholz am Ammersee	in decidous-tree forest	A. Einhellinger	3 Oct 1983	This study	KY582679		
R. rutila	SAV F-1353	Slovakia: W Carpathians, Štiavnické vrchy Mts., Obyce	*Quercus*	S. Adamčík	6 Sep 2007	Adamčík et al. 2016, This study	KU928162	KY471573	
R. rutila	SAV F-1497	Slovakia: W Carpathians, Štiavnické vrchy Mts., Jabloňovce – Bohunický Roháč	*Quercus*, *Pinus silvestris*, *Carpinus*	S. Adamčík	8 Sep 2006	This study	KY582686	KY471577	KY616678
R. rutila	SAV F-1504	Slovakia: W Carpathians, Štiavnické vrchy Mts., Jabloňovce – Bohunický Roháč	*Quercus*	S. Adamčík	8 Sep 2006	This study	KY582687		
R. rutila	SAV F-1564	Slovakia: W Carpathians, Štiavnické vrchy Mts., Nová Dedina – Sovia dolina.	*Quercus*	S. Adamčík	4 Sep 2007	This study	KY582688	KY471578	
R. rutila	SAV F-2296	Slovakia: W Carpathians, Štiavnické vrchy Mts., Nová Dedina – Sovia dolina.	*Quercus* (*Carpinus*)	S. Adamčík	21 Jul 2008	This study	KY582697	KY471588	KY616690
R. rutila	SAV F-3967	Slovakia: W Carpathians, Štiavnické vrchy Mts., Ladzany – Buvanec	*Quercus*, *Fagus*, *Carpinus*	S. Adamčík, M. Caboň	17 Sep 2013	This study	KY582716		KY616710
R. rutila	SAV F-4466	Slovakia: W Carpathians, Štiavnické vrchy Mts., Ladzany – Buvanec	*Fagus*, *Quercus*	F. Hampe	9 Sep 2014	Adamčík et al. 2016, This study	KU886600	KY471617	KY616718
R. rutila	SAV (HK14028)	Sweden: Västergötland, Kina	*Quercus*	H. Kaufmann	30 Aug 2014	This study	KY582724	KY471621	KY616722

Species (orig. identification)	**Herb. acronym (collection number)**	**Country: Collecting area**	**Host**	**Collector**	**Date**	**Sequences source**	**ITS**	**mtSSU**	**RPB2**
R. rutila (*R. decipiens*)	TU 101893 (JV24034F)	Finland: Southwest Finland, Turku – Ruissalo	forest margin with *Quercus robur*, *Betula*, *Tilia cordata*, *Corylus avellana*	K. Kokkonen, J. Vauras	7 Sep 2006	Unite	UDB016087		
R. rutila (epitype)	PC 0723457 (n°12-IX-45)	France: Val-d'Oise, Asniéres sur Oise	*Quercus*	H. Romagnesi	12 Sep 1945	This study	KY582681		
R. rutila (unid. *Russula*)	EcM (LM5409)	Austria: South-East Austria	*Quercus petrea*			Suz et al. 2014	KM576552		
R. sardonia	SAV F-3914	Slovakia: W Carpathians, Záhorská nížina lowland, Malacky - Pekyna	*Quercus*, *Pinus silvestris*	M. Caboň	1 Nov 2011	This study	KY582714	KY582727	KY616707
R. tinctipes	SAV F-2494	Slovakia: W Carpathians, Tríbeč Mts., Súľovce – Betlehem	*Quercus*, *Carpinus*	S. Adamčík	23 Jul 2008	This study	KY582698	KY471590	KY616692
R. turci	SAV F-3075	Norway: Nord-Trøndelag, Steinkjer – Noem	*Picea*	S. Adamčík	2 Sep 2009	This study	KY582703	KY471595	
R. velenovskyi	SAV F-2921	Slovakia: W Carpathians, Volovské vrchy Mts., Predná Hora – recreation area	*Betula*	V. Kautman	8 Jul 2009	This study	KY582701	KY471593	KY616695
R. velutipes	SAV F-3428	Slovakia: E Carpathians, Bukovské vrchy Mts., Nová Sedlica – Stužica Natural Reserve	*Fagus*, *Abies*	S. Adamčík	26 Sep 2011	This study	KY582711	KY471604	KY616704
R. versicolor	SAV F-2919	Slovakia: W Carpathians, Volovské vrchy Mts., Predná Hora – recreation area	*Betula*	P. Marstad	8 Jul 2009	This study	KY582700	KY471592	KY616694
R. veternosa	GENT (FH 12/225A)	Belgium: Foret de Soignes, Brussels	*Fagus*	F. Hampe		Adamčík et al. 2016, This study	KU928165	KY471569	
R. veternosa	C (JV01-507)	Denmark: East Jutlands, Horsens – Elbæk Skov	*Fagus*, *Quercus*	J. Vesterholt	23 Sep 2001	Unite	UDB000115		
R. veternosa	GENT (2010 BT183)	Germany: Niedersachsen, Hildesheim – Röderhof	*Fagus*	F. Hampe	4 Sep 2010	This study	KY582673		KY616673
R. veternosa	M (HM R-8312)	Germany: Bayern	*Fagus sylvatica*	H. Marxmüller	24 Sep 1983	This study	KY582678		
R. veternosa	TUB (hue212)	Germany: Baden-Württemberg, Tübingen - Pfrondorf – Schönbuch	*Fagus sylvatica*	U. Eberhardt	22 Jul 1997	Eberhardt 2002	AF418630/ UDB000304		
R. veternosa	SAV F-1401	Slovakia: W Carpathians, Pohronský Inovec Mts., Malá Lehota – Dudkov vrch	*Quercus*, *Fagus*	S. Adamčík	6 Sep 2006	Adamčík et al. 2016, This study	KU928164	KY471574	KY616680
R. veternosa	SAV F-1403	Slovakia: W Carpathians, Pohronský Inovec Mts., Malá Lehota – Dudkov vrch	*Fagus*	S. Adamčík	6 Sep 2006	This study	KY582684	KY471575	
R. veternosa	SAV F-1491	Slovakia: W Carpathians, Pohronský Inovec Mts., Malá Lehota – Dudkov vrch	*Fagus* (*Quercus*)	P. Marstad	6 Sep 2006	This study	KY582685	KY471576	KY616681
R. veternosa	SAV F-193	Slovakia: E Carpathians, Vihorlat Mts., Hliviština	*Fagus*	S. Adamčík	13 Jul 2001	This study	KY582690	KY471580	KY616683
R. veternosa	SAV F-2588	Slovakia: W Carpathians, Kremnické vrchy Mts., Badín – Badínsky prales Nature Reserve	*Fagus*	S. Adamčík	28 Sep 2009	This study	KY582699	KY471591	KY616693
R. veternosa	SAV F-3391	Slovakia: W Carpathians, Malé Karpaty Mts., Lozorno - Bukovina	*Fagus*	S. Adamčík	7 Jul 2011	Adamčík et al. 2016, This study	KU928166	KY471603	KY616703
R. veternosa (unid. fungus)	EcM (B674_Rus_Nowshr)	Iran: Mazandaran, Noshahr, Nowshahr	*Fagaceae*			Unite	UDB005438		

Species (orig. identification)	**Herb. acronym (collection number)**	**Country: Collecting area**	**Host**	**Collector**	**Date**	**Sequences source**	**ITS**	**mtSSU**	**RPB2**
R. veternosa (unid. fungus)	soil sample (Ir11)	Iran: N Iran				Bahram et al. 2011	FR852104		
R. vinosa	SAV F-20024	Poland: W Carpathians, Západné Tatry Mts., Zakopane – Mala Polanka under Grzybowec	*Picea*	M. Caboň	25 Aug 2014	This study	KY582692	KY471583	
R. zvarae	GENT (FH-12-175)	Germany: Thuringia, Erfurt – Aspenbusch		F. Hampe	1 Aug 2012		KT933986		KT933918
Russula sp.	TU 110492	Papua New Guinea: Eastern Highlands Prov., Daulo Pass	*Nothofagus*	L. Tedersoo		Tedersoo and Põlme 2012	UDB013117		
Russula sp.	TU 110597	Papua New Guinea: Eastern Highlands Prov., Mt Michael NP – E Houmare	mixed *Castanopsis*, *Nothofagus* forest	L. Tedersoo		Tedersoo and Põlme 2012	UDB013204		
Russula sp.	TU 110634	Papua New Guinea: Eastern Highlands Prov., Mt Michael NP	*Castanopsis acuminatissima*	L. Tedersoo		Tedersoo and Põlme 2012	UDB013239		
Russula sp. (*R.* aff. *firmula*)	(r-03038)	United States: California		R.M. Davis		GenBank	JF834342		
Russula sp. (*R. firmula*)	WA (LL110)	China: Zhagana, Tewo County, Gansu			Aug 2015	Kang et al. 2016	KX008985		
Russula sp. (*R. firmula*)	WA (RITF2247)	China: Jilin, Changbai Mountain, lushui River				GenBank	KJ867372		
Russula sp. (*R. firmula*)	WA (RITF2249)	China: Jilin, Changbai Mountain, lushui River				GenBank	KJ867373		
Russula sp. (*R. firmula*)	KUB	India: Himalaya, Kashmir, Darksum forest	*Pinus wallichiana*	Z.A. Itoo	18 Sep 2012	Itoo et al. 2013	KC797152		
Russula sp. (unid. *Russula*)	EcM (clone 65)	Russia – Asian part: Sikhote Alin Reserve	*Pinus koraiensis*			Malysheva et al. 2014	KJ769290		

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New insights in *Russula* subsect. *Rubrinae*: phylogeny and the quest for synapomorphic characters

Miroslav Caboň, Ursula Eberhardt, Brian Looney, Felix Hampe, Miroslav Kolařík, Soňa Jančovičová, Annemieke Verbeken, Slavomír Adamčík

Supplementary material 2. Annotated description of the epitype specimen of *Russula veternosa*

***Russula veternosa* Fr.**, Epicr Syst Mycol: 354. 1838.

MycoBank no.: MB 203741.

Lectotype (designated by Sarnari 1998): Paulet, Traité des champignons, Iconographie des champignons, pl. 74, f. 3, 1808–1835.

Epitype (designated by Sarnari 1998): France. Oise. Forêt de Compiègne, Hêtraie, 29. Août 1953, *H. Romagnesi 53-206*, as “*Russula schiffneri* Singer” (PC0124979).

Original description:

15. R. veternosa, acris, pileo vesiculoso-carnoso plano-depresso polito, pellicula tenui adnata expallente, margine membranaceo laevi, stipite e spongioso cavo molli aequali laevi fragili albo, lamellis adnatis angustis antice latioribus inaequalibus stramineis. Paul. t. 74 f. 3. descr. optima! In silvaticis; etiam Upsaliae rarius obvia. Carne alba spongiosa ad R. fragiles valde accedit. Pileus 2—3 unc. l. e roseo l. incarnato mox disco albicat l. lutescit.

Spores broadly ellipsoid, (6.5–)6.9–7.6(–8) × (5.2–)5.5–6(–6.3) µm, av. 7.2 × 5.8 µm, Q=(1.13–)1.22–1.3(–1.35), Q=1.26, ornamentation of moderately distant, fine, 4–6(–7) amyloid spines in the circle of diameter of 3 µm on spore surface, spines 0.7–1.1(–1.3) µm high, mainly isolated, line connections very rare and short (0–1 in the circle), occasionally merged in pairs or triplets [0–2(–3) fusions in the circle]. Suprahilar plage large, amyloid. **Basidia** (29–)30.5–36(–37) × (7.5–)9.5–12.5(–13) µm, av. 33.2 × 11 µm, 4–spored, clavate; basidiola first cylindrical, then clavate, ca. 5.5–11 µm wide. **Subhymenium** pseudoparenchymatic. **Lamellar trama** mainly composed of large sphaerocytes. **Pleurocystidia** dispersed, ca. 400–700/mm^2, fusiform or clavate, pedicellate, acute on tips and with 3–9 µm long appendage, thin-walled, measuring (48–)52–66(–70) × (8.5–)9.5–11.5(–12.5) µm, av. 58.9 × 10.5 µm, contents heteromorphous-banded, slowly turning red-brown in sulfovanillin. Lamellar edges (damaged in the type specimen, marginal cells and cheilocystidia observed on specimen SAV F-1503) covered by marginal cells, cheilocystidia and dispersed basidia; marginal cells on lamellar edges small and narrow, cylindrical, flexuous, often with lateral nodes or branches, measuring (14–)15–23(–28) × 3–4(–4.5) µm, average 19.1 × 3.6 µm; cheilocystidia narrower than pleurocystidia, clavate or narrowly fusiform, often pedicellate, with obtuse or acute tips and usually with 1–3(–5) µm long appendage, thin-walled, measuring (40–)49–61(–67) × (6–)5.5–7.5(–8) µm, average 55 × 6.9 µm, contents similar as in pleurocystidia. **Pileipellis** orthochromatic in Cresyl blue, 130–165 µm deep, not sharply delimited from the underlying spherocytes of the context, strongly gelatinized in all parts; vaguely divided in 70–100 µm deep suprapellis composed of relatively dense, erect or ascending, branched hyphal ends and pileocystidia; gradually passing to 50–65 µm deep subpellis of dense, branched, irregularly and near trama horizontally oriented, 2–5(–8) µm wide hyphae. Acid-resistant incrustations present but sometimes stain inconspicuously pink are present only near bases of pileocystidia. **Hyphal terminations in pileipellis** near the pileus margin slender often flexuous, sometimes slightly moniliform; terminal cells cylindrical or attenuated, obtuse or constricted near apices, measuring (19–)21.5–37(–48) × 2–3 µm, av. 29.4 × 2.5 µm; followed by 0–2 unbranched cells, subterminal cells usually equally wide and long, often with lateral nodules or branches. Hyphal terminations near the pileus centre composed of shorter cells, terminal cells short cylindrical and wider, with obtuse or rarely constricted apices, measuring (6.5–)12–22(–35) × (2–)2.5–4 µm, av. 17.1 × 3.2 µm. **Pileocystidia** near the pileus margin numerous, cylindrical or clavate, mainly 2–3(–5) celled, occasionally one-celled, thin-walled, obtuse, terminal cells measuring (15–)19.5–41.5(–50) × (4.5–)5–7(–8) µm, av. 30.5 × 6 µm; contents in Congo red heteromorphous-granular or banded, turning slowly grey in sulfovanillin and after ca. 30 minutes with distinct pink incrustation near bases; subterminal cells usually narrower, equally wide or sometimes longer. Pileocystidia near the pileus centre with more inflated and shorter cells, terminal cells measuring (11.5–)15–30(–44) × (5.5–)7–10.5(–12) µm, av. 22.8 × 8.8 µm. **Cystidioid hyphae** with heteromorphous contents observed only in subpellis, probably absent in the cap trama. **Clamp connections** absent in all parts.

Morphological delimitation of *R. veternosa*

Characters observed on the type of *R. veternosa* were compared with available published detailed type studies of *R. badia* Quél., *R. globispora* (Blum) Bon, *R. maculata* Quél., *R. vinosopurpurea* Jul. Schäff. (Adamčík & Jančovičová 2013), *R. decipiens* (Singer) Svrček and affiliated taxa (Adamčík & Jančovičová 2012) and *R. nympharum* F. Hampe & Marxm. (Adamčík et al. 2016). We identified morphological characters specific for *R. veternosa* observed on spores, hyphal terminations in pileipellis near the pileus margin, pileocystidia, basidia and pleurocystidia.

Spores showing a combination of small size and isolated fine spines are unique for *R. veternosa*, particularly the small spore width is unique within the analysed dataset. Hyphal terminations in the pileipellis near the pileus margin are variable in shape, cylindrical or attenuated terminal cells reminds those of *R. decipiens* or *R. badia*, but they are very narrow in *R. veternosa* (only 2.5 µm wide in average and maximum up to 3.5 µm). Mainly two and more-celled pileocystidia (near the pileus margin) with relatively short terminal cells are similar to those of *R. vinosopurpurea*. There is not a single species within *R.* subsect. *Maculatinae* in the sense of Romagnesi that has incrusted pileocystidia. In the literature, *R. veternosa* is consistently described as having no incrustations in the pileipellis. However, we observed acid-resistant incrustations that turn pink in sulfovanillin in the type specimen and verified their consistent presence in other studied material of the species. Basidia (33.2 × 11 µm) and pleurocystidia (58.9 × 10.5 µm) are the smallest among the compared species.

In conclusion, the type specimen of *R. veternosa* differs from other analysed species descriptions by the small (particularly narrow) spores, narrow terminal cells of hyphae in pileipillis near the pileus margin of variable shape, pileocystidia with acid-resistant incrustations turning pink in sulfovanillin and small (in particular short) basidia and pileocystidia.

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New insights in *Russula* subsect. *Rubrinae*: phylogeny and the quest for synapomorphic characters

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Supplementary material 3. Photo of the epitype collection of *R. firmula* (GENT 2010 BT85). Photo by Felix Hampe.