

Molecular phylogeny of the genus *Russula* in Europe with a comparison of modern infrageneric classifications.

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Running title: Molecular phylogeny of *Russula* in Europe

Abstract: Species in the large mushroom genus *Russula* are important ecologically as ectomycorrhizal fungi and economically as comestibles. Most infrageneric classification schemes of this genus have originated in Europe, but these systems are largely arbitrary and incongruent. Using ribosomal DNA sequences for 82 species representing all infrageneric taxa described from Europe, the phylogenetic position and relationships among these species were examined. Cladistic analysis of the ITS1, 5.8S and ITS2 regions showed a cluster of four small to large clades basal in the topology and one large apical clade arising from the deeper nodes, none of which has been previously recognized *in toto* at the subgeneric level. Two of these groups, the *Compactae* and *Lactarioideae*, which have been previously recognized as subsections of section *Compacta*, did not appear to be closely related. Bootstrap support and Bremer decay values indicated that collapse of the tree into monophyly at the deeper nodes would result in two large groups which are consistent with the classical subgeneric concept of the *Eurussulae* and a narrowed *Compacta*-like group. The topology confirmed some previously described infrageneric

taxa at the section level including the *Tenellae* and *Heterophyllae* and at the subsection level including the *Cupreinae*, *Laricinae*, *Lilaceinae*, *Integroidinae*, *Violaceinae*, *Sphagnophilinae*, *Viridantinae*, *Emeticinae*, and portions of the *Polychromae* and *Sardoninae*. The molecular analysis also indicated many interesting new combinations or relationships not previously conceived. Mapping of characters such as spore print color, taste, and presence of acid-resistant incrustations, which have been used to define infrageneric taxa in *Russula*, onto the phylogeny identified some interesting patterns consistent with hypotheses regarding plesiomorphic and apomorphic characters. However, because of potential loss or reversal of character states, this analysis did not support their unequivocal use in infrageneric classification.

INTRODUCTION

Species in the genus *Russula* are conspicuous and important mushroom elements in forest and arctic-alpine ecosystems. They contribute significantly to fungal biomass (Richardson 1970), are important dietary elements for insects and larger animals (Fogel 1975, Fogel & Trappe 1978) and many species are harvested worldwide for human consumption (Hu & Zeng 1992, Guo 1992, Rammeloo & Walley 1993, Buyck 1994 c). As ectomycorrhizal symbionts with forest tree species their importance is well documented (Bills, Holtzman & Miller 1986, Villeneuve, Grandtner & Fortin 1989, 1991, Gardes & Bruns 1996). Unfortunately, the number of species in this genus is large and many are notorious for their intergrading color variation and obscure morphological and anatomical discontinuities. Consequently, it is often difficult for those doing studies of forestry, ectomycorrhizal communities, fungal biodiversity or ethnopharmacology to identify species of *Russula* in most areas with any certainty. A tenable infrageneric classification system is required in order to narrow down taxonomic choices during the identification process.

The genus *Russula* has had a rich taxonomic history in the European mycological literature. The genus was erected by Persoon (1796) during studies of macrofungi in northern Europe. In the ensuing two hundred years, numerous infrageneric classification systems have been proposed. With a few exceptions from North America (Peck 1906, Burlingham 1915, Bills & Miller 1984), Africa (Heim 1937 a, 1938 b, Buyck 1989 a, b, 1990 a, b, 1992, 1993, 1994 a, b, 1997), South America (Singer, Araujo & Ivory 1983) and cosmopolitan treatments such as Singer (1986), most work on *Russula* infrageneric classification has been accomplished in Europe based primarily on European species (Persoon 1801, Fries 1874, Quélet 1888, Barbier 1907, Masee 1907, Bataille 1908, Maire 1910, Ricken 1915, Lange 1926, 1940, Singer 1926, 1932, 1935 a, b, Melzer & Zvára 1927, Crawshay 1930, Schaeffer 1933, 1934, 1935, 1952, Konrad & Josserand 1934, 1935, Romagnesi 1967, 1987, Bon 1988, Reumaux, Bidaud, & Moëgne-Loccoz 1996, Sarnari 1998).

These infrageneric classification systems, developed from purely morphological criteria, are often complex and largely incongruent, and have been inadequate in resolving arguments regarding evolutionary relationships in *Russula* and important taxonomic characters. Phylogenetic analysis based on DNA sequences of ribosomal genes provides an effective alternative to morphology in reconstructing evolutionary relationships and in evaluating characters useful in taxonomy. Because molecular phylogenetic studies have not previously been aimed specifically at the genus *Russula* and because a majority of the work on *Russula* taxonomy has been accomplished in Europe, a molecular study of proposed infrageneric taxa from Europe is a critical first step to elucidating *Russula* taxonomy worldwide.

In the present study, ribosomal DNA sequences from the ITS1, 5.8S and ITS2 regions were assessed for their usefulness in determining phylogenetic position and relationships among

species representing infrageneric taxa described from Europe. The infrageneric classification systems accepted by Romagnesi (1967, 1987), Bon (1988), and Sarnari (1998), which summarize much of the taxonomic history of *Russula* in Europe, are compared with the molecular results. This research is part of an ongoing project to define infrageneric taxa in the genus *Russula* worldwide. Potential new combinations and circumscriptions of infrageneric taxa will be discussed, however, formal changes and descriptions based on both molecular evidence and morphological analyses will be presented in upcoming manuscripts encompassing infrageneric taxa worldwide.

MATERIALS AND METHODS

Material studied

The ITS1, 5.8S and ITS2 rDNA sequences utilized in this study were obtained from freshly collected specimens or herbarium material. Eighty-two species are included in the present analysis, although many more were sequenced. A hierarchical taxon sampling approach was used; highest priority was given to sequences from European specimens which represent the types for subgenera, sections, and subsections in the genus *Russula* described from Europe. If such specimens were not available or if high quality DNA could not be obtained, alternate species (not the type) widely accepted in the same section or subsection from Europe were used. Finally, specimens from North America representing a particular infrageneric taxon were substituted. The origin of collections, location of voucher specimens (abbreviated according to Holmgren *et al.* 1990) and their infrageneric placement according to Romagnesi (1967, 1985, 1987), Bon (1988), and Sarnari (1998) are presented in Table 1. Classification systems adopted by these authors are summarized in Table 2. Depending on the classification, one to several

species were included in this analysis to represent each infrageneric taxon. A number of taxa were initially evaluated for use in outgroup comparison including *Albatrellus flettii* (Morse) Pouzar, *Boidinia furfuracea* (Bres.) Stalpers & Hjortstam, *Bondarzewia berkeleyi* (Fr.) Sing., *Gloeocystidiellum aculeatum* S. H. Wu, *Hericium erinaceus* Pers., *Lentinellus ursinus* (Fr.) Kühn., and *Heterobasidion annosum* (Fr.) Bref. *Albatrellus flettii* and *Gloeocystidiellum aculeatum* were found to provide the most stable topology and were used in all outgroup comparisons.

Nucleic acid preparation

Tissue from fresh field collected basidiomes and herbarium specimens ground in liquid nitrogen was placed in 2X CTAB buffer and ground with a plastic pestle. Genomic DNA was extracted using CTAB buffers as previously described (Hibbett & Vilgalys 1993) then purified using GENE CLEAN III (Qbiogene, Carlsbad, CA, USA). Aqueous genomic preparations were frozen at - 20 °C until use.

Polymerase chain reaction and sequencing

PCR amplification was performed using Sigma *Taq* polymerase, tailed primer pair M13-ITS5/M13-ITS4 (White *et al.* 1990) in a programmable thermal cycler (Model PTC-100, MJ Research, Inc.). PCR products were electrophoresed through a 1% agarose gel stained with ethidium bromide, excised, and repurified using GENE CLEAN III. The PCR products were further purified using Wizard PCR Preps (Promega, Madison, WI, USA). DNA sequencing reactions were performed using primers M13-Forward (-29) and M13-Reverse labeled with IRD-700 and IRD-800, respectively, in preparation for simultaneous bidirectional sequencing (LI-COR Biotechnology Division, Lincoln, NE, USA). Amplified PCR products were sequenced with the SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Technologies, Madison, WI,

USA) and analyzed on a LI-COR Gene ReadIR 4200-2 automated sequencer. Base ImagIR (vers. 4.0, LI-COR) was used for processing the data. Forward and reverse sequences were compiled, edited, and initially aligned using AlignIR (vers. 1.2, LI-COR) against a large dataset containing *Russula* sequences obtained throughout the world. Final alignment was performed manually.

Phylogenetic analysis

Maximum parsimony analyses were performed using PAUP 4.0b5 (Swofford 2001) and gaps in alignment were treated as missing data and excluded. Sequence regions with known nucleotides but which were ambiguously alignable with respect to bordering regions were treated as missing data. Regions longer than four nucleotides with ambiguous alignment were excluded from analysis. A large insertion in *R. olivacea* was coded as a fifth base (Bruns *et al.* 1992, Moncalvo, Wang & Hseu 1995). Due to the large size of the dataset heuristic analysis strategies designed by Maddison, Ruvolo & Swofford (1992) and Olmstead & Palmer (1994) were used to find islands of parsimony. The relative robustness of individual branches and clade stability were estimated by bootstrap analysis (Felsenstein 1985, Hillis & Bull 1993) and decay indices (Bremer 1988). Bootstrap values were generated using the settings 100 replicate searches on all parsimony-informative characters using 5 random sequence addition replications and TBR (tree-bisection-reconnection) branch swapping algorithms in PAUP*. Decay indices were calculated with AutoDecay version 4.0 (Eriksson 1998) with constraints tested in PAUP* and decay values output as a NEXUS tree file displayed with TreeView (Page 1996).

Character mapping

All collections used in the molecular analysis were examined microscopically to verify identification and to evaluate spore and basidium size and presence or absence of incrustations.

The Basic Fuchsin reaction was performed according to Bon (1988). Because many herbarium specimens were used, adequate notes on taste and spore print color were not always available. These characters were recorded from published descriptions of the same taxa (Romagnesi 1985, Bon 1988, Galli 1996, Sarnari 1998). Spore print coloration was evaluated using the coding of Romagnesi (1967).

RESULTS

ITS rDNA amplification products were approximately 660 to 1000 base pairs in length, included two hypervariable regions, one in ITS1 and one in ITS2, and contained about 281 phylogenetically informative positions after alignment. One taxon, *R. olivacea*, contained a 300 base pair insertion in ITS1. This insertion was consistent in this taxon in two collections from Europe and one collection from North America (data not shown). An island of 24 most parsimonious trees of 1860 steps was found. Fig. 1 shows the best most parsimonious tree (likelihood score of $-\ln L = 10899.89008$) based on equally weighted parsimony with a consistency index (CI) of 0.305, retention index (RI) of 0.526, rescaled consistency index (RC) of 0.160 and a homoplasy index (HI) of 0.695. Other equally parsimonious trees in this island differed only in minor rearrangements within terminal clades. Fig. 2 shows a strict consensus of all 24 most parsimonious trees with branch stability statistics.

There was an appropriate level of divergence among ITS sequences for testing hypotheses concerning infrageneric classification in *Russula*. Topology of the phylogram (Figs. 1 and 2) shows four small or large basal clades and one large apical clade that could be delimited at the deeper nodes. Bootstrap support significantly greater than 50% was only observed in four of these deep nodes (Fig. 2). At the mid-level nodes, at least twenty clades could be identified,

many with moderate to high bootstrap values (Figs. 1 and 2). The highest level of support for the topology was found in the shorter terminal or near terminal nodes. Bremer decay indices of between 0 and 13 steps occurred at the deeper nodes so that four to six well separated groups remained after collapse into monophyly at the deepest nodes. The *R. camarophylla*/*R. farinipes* clade received neither strong bootstrap nor high Bremer support values. Sequences from these fungi were the most divergent in the ingroup but the distinction between ingroup and outgroup sequences was highly supported by both bootstrap and Bremer decay values. Only sequences from taxa collected in northern temperate regions of Europe and North America were included in this analysis and taxon sampling included at least one representative from each infrageneric taxon described from Europe. Consequently, not all subgenera, sections, and subsections known in *Russula* worldwide were included in this analysis. Expanding the taxon sampling to include representatives of additional infrageneric taxa from tropical areas might help to reduce some internode lengths and counteract long-branch attraction (Soltis *et al.* 1998), especially in basal nodes such as those containing *R. camarophylla* and *R. farinipes*. Likewise, clade 5, which was consistently delimited during almost all analyses received a bootstrap value less than 50% and only moderate Bremer support.

Mapping of spore print color, taste, and presence of acid-resistant incrustations on cystidia and/or hyphae onto the molecular phylogenies (Fig. 1) indicated that these characters form patterns of occurrence likely to be valuable to the taxonomy of the genus *Russula*. Patterns of spore print coloration (Fig. 1) were evident in the phylogeny and the darkest specific colors (IVa-e according to the system of Romagnesi 1967) as well as the darkest colors overall, occurred in group 6 (shown in Fig. 2). Taxa in groups 1-4 generally had spore print colors ranging from Ia-IIc with the exception of *R. exalbicans*, *R. drimeia*, and *R. sanguinea* with spore

prints as dark as IIIab. Species with acrid or peppery taste in some part of the basidiome occurred throughout the tree, with the largest concentration of acrid or peppery taxa occurring in basal portions of the tree. Specifically, species in clades 6v corresponding to subsection *Cupreinae* Bon, 6m corresponding to subsection *Violaceinae* (Romagnesi) Sarnari, 3a corresponding to subsection *Foetentinae* (Melzer & Zvára) Singer and the large clade 5 in Fig. 2 were acrid, with a number of other taxa recorded as peppery or variable. Acid-resistant incrustations shown in Fig. 1 occurred exclusively in the large more apical group marked 6 in Fig. 2 in clades 6q—t, 6p, and parts of clades 6o and n. The only occurrence of incrustations outside of group 6 was in clade 5c containing *R. atropurpurea*, *R. viscida*, and *R. ochroleuca*, and in *R. pulverulenta* in clade 3a.

DISCUSSION

Creating a tenable system of infrageneric classification in *Russula*, as with any large and complex genus, is a critical first step to help narrow down taxonomic choices during the identification process. A solid infrageneric classification scheme can also help in the identification of characters which are most appropriate and useful for separating taxonomic entities. In cases where a particular species cannot be identified, either because it is undescribed or because there is an inadequate taxonomy for that area, a workable infrageneric classification system based on morphology, ecology, and phylogenetic information would allow an informative taxonomic compromise between "*Russula* sp." and full identification with all the attendant information regardless of where the fungus was collected.

Unfortunately, previous circumscription of infrageneric taxa in *Russula* in Europe, where most of the classification of this genus has originated, has not resulted in a useable

consensus system. For example, the classification system adopted by Romagnesi in his earlier work (Romagnesi 1967) relied on two subgenera, eight sections, and thirty-four subsections for the European *Russula* flora. Romagnesi (1985, 1987) later elevated the sections in his former classification to subgenera and the subsections to sections. Bon (1988) utilized two subgenera in his classification, along with fifteen sections and thirty-nine subsections. Sarnari (1998) included six subgenera, seventeen sections (excluding the extraeuropean *Fistulosae*), and twenty-eight subsections. Although there are some similarities in parts of these classification systems they are largely incongruent.

Overall, results of the molecular analysis presented here suggest that while some infrageneric taxa described previously from Europe and accepted by modern authors are supported, many do not appear to be natural phylogenetically related assemblages of species. Topologically, five to six small to large groups, none of which has been previously recognized *in toto* at the subgeneric level, are evident arising from the deeper nodes and are therefore most likely to represent higher level infrageneric taxa. Two of the groups which have been previously recognized but typically at the section level include the *Compactae* Fr. (= *Nigricantes* Maire, *Nigricantinae* Bataille) and the *Lactarioideae* Maire (= *Plorantes* Bat. ex Singer). Historically these sections have been placed together in the subgenus *Compacta* (Fr.) Bon, however, Miller *et al.* (2001) found in a molecular analysis of the n-LSU, as in the present study, that these groups may not be as closely related as previously believed. The section *Tenellae* Quélet, composed of small species with a tendency for the flesh to bruise yellow or yellow-brown, formed a solid recognizable group, as did the section *Heterophyllae* Fries with a number of recognizable subsections. At the subsection level, several previously described and accepted groups were evident. These included the *Cupreinae* Bon, *Laricinae* (Romagnesi) Bon, *Lilaceinae* (Melzer &

Zvára) J. Schaeffer, *Integroidinae* Romagnesi, *Violaceinae* (Romagnesi) Sarnari, *Sphagnophilinae* (Romagnesi) Bon, *Viridantinae* Melzer & Zvára, *Emeticinae* Melzer & Zvára, and portions of the *Polychromae* Maire and *Sardoninae* Singer. The strength of the relationship in many of these subsectional groups may be slightly exaggerated because multiple representatives were included in this study. Other subsections represented by only one taxon that may be equally strongly delimited include the *Olivaceinae* Singer and the *Maculatinae* Konrad & Jossierand.

Molecular analysis also indicated some interesting new potential combinations or relationships. For example, a small terminal clade was derived containing *R. aurantiaca* from section or subsection *Laetinae* Romagnesi (placed in section *Paraincrustatae* Sarnari), *R. lepida* from section or subsection *Lepidinae* Melzer & Zvára (placed in section *Paraincrustatae* Sarnari), *R. lepidicolor* (placed in section *Paraincrustatae* Sarnari), and *R. rosea* from section or subsection *Roseinae* Singer. Another clade was found containing *R. aquosa*, *R. fellea*, and *R. helodes*, which brings together members from section or subsection *Atropurpurinae* Romagnesi (section and subsection *Russula* of Sarnari), section or subsection *Felleinae* Melzer & Zvára and section or subsection *Sardoninae*.

Examination of overall tree topology and the statistics used to test tree and branch stability resulting from molecular analysis may also be instructive in delimitation of infrageneric taxa in *Russula*. Conceptually, it may be useful to consider break points in the deeper nodes as circumscribing higher level infrageneric taxa such as subgenera, and similar breaks in the mid-level and terminal nodes could be interpreted as sections and subsections, respectively. High levels of branch support or decay values in the deeper nodes could likewise provide an unambiguous delimitation of subgenera, and support at mid-level and terminal nodes could

indicate well delimited sections and subsections. Only four of the five or six groups arising from the deeper nodes appeared to be well supported by bootstrap analysis. Oddly, a bootstrap support only slightly greater than 50% was observed at the deeper node for group 3, the *Heterophyllinae* Fries/*Foetentinae* (Melzer & Zvára) Singer clade, although this topology was observed in virtually every analysis performed regardless of outgroup or exset used. Similarly, a bootstrap value of greater than 50% was not observed for clade 5, which contained members of the *Sardoninae*, *Felleinae* and *Emeticinae*.

Bremer decay analysis indicated that the *Compactae* and *Lactarioideae* have the highest values with the latter group three steps longer than the former. This means that as the trees collapse into monophyly at the deeper nodes during consensus, the *Lactarioideae* would remain distinct for several more steps from all of the other species. At that point there would be strong support for essentially two groups in *Russula*. This conforms well to the classical concept of a delineation between subgenus *Eurussulae* or *Genuinae* (Lange 1940, Melzer & Zvára 1927) and the subgenus *Compacta* which contains the sections *Lactarioideae* (*R. chloroides*, *R. delica*) and *Compactae* (*R. nigricans*, *R. adusta*). Both the *Lactarioideae* and the *Compactae* are composed of species which are mostly white with dense tissues, adnate pileipellis, and somewhat decurrent lamellae which effectively distinguishes the subgenus *Compacta* from all other species of *Russula* in the *Eurussulae* or *Genuinae*, even though members of the *Lactarioideae* and *Compactae* may not be as closely related as previously believed. As with the bootstrap values, Bremer support for the *Heterophyllinae* /*Foetentinae* and *Sardoninae*/*Felleinae*/*Emeticinae* clades is surprisingly moderate.

The highest levels of bootstrap and decay support for the topology were mostly found in the terminal or near terminal nodes. These values are somewhat artifactual as the highest values

generally corresponded to relationships between multiple taxa representing the same section or subsection according to previous classifications. On the other hand, the high level of support for these relationships confirms the circumscription of these groups at the sectional or subsectional level.

It should also be noted that the greatest overall support, especially bootstrap values at the deeper and mid-level nodes, was found in the more basal clades (clades 1-5 and the lower portion of clade 6 in Fig. 2). Many of these groups, including the sections *Compactae*, *Foetentinae*, and *Heterophyllinae* are well represented and highly diverse in tropical areas and are potentially quite ancient. It is possible that the taxa contained in most of clade 6 represent taxa which are more derived, having recently radiated following the last glaciation. This view is further supported by the consensus topology observed in clades 6 k and o.

Throughout the taxonomic history of the genus *Russula* in Europe, several characters have been consistently relied upon to form the bases for infrageneric classification and taxonomy. Persoon (1801) provided a classification scheme based on the color of the pileus. Fries (1874) produced the first roughly natural classification based primarily on tissue texture, lamellae morphology, and lamella and spore color. The classification of Quélet (1888) delimited two sections in *Russula*, the *Xanthosporae* with strongly pigmented spores and the *Leucosporae* with weakly pigmented spores. Masee (1902) categorized species in *Russula* based on a combination of the acrid or mild taste of the flesh and the color of the lamellae. Although Konrad & Jossierand (1934) considered this classification entirely artificial, the characters of taste and spore print color continued to be elements in almost all classification schemes that followed. Melzer & Zvára (1927) employed a new class of character, macro- and microchemical reactions, especially iron sulfate and Basic Fuchsin. This paved the way for exploration of a plethora of macro- and

microchemical tests designed to separate *Russula* species by observing color changes after addition of gum guaiac, sulfovanillin, formaldehyde, aniline oil or phenol, among others (Singer 1986). Romagnesi (1967) delimited one section and parts of several others based on the presence of primordial hyphae which are incrustated hyphae or cystidia that become deeply magenta-colored after treatment with Basic Fuchsin, and remain colored or acid-resistant in weak hydrochloric acid.

Although not all characters which have been used to classify species of *Russula* were evaluated in the present study, mapping of spore print color, taste, and presence of acid-resistant incrustations onto the molecular phylogeny was enlightening. These characters formed patterns of occurrence which were somewhat more natural than previously believed, and are confirmed as valuable to the taxonomy of *Russula*. Patterns of spore print coloration were evident in the phylogeny and the lightest specific colors as well as the lightest overall colors occurred in the more basal clades while the darkest colors occurred primarily in the apical clades. This pattern, if consistent after additional taxon sampling, suggests that darker spore print colors may be apomorphic in northern temperate regions.

Species with at least some acrid or peppery taste occurred throughout the tree, in subsections *Cupreinae*, *Violaceinae*, *Foetentinae*, and the large clade 5 comprised of species in the *Sardoninae*/*Felleinae*/*Emeticinae* clades, with typically a reddish or yellowish pileus and light colored spore print. Acrid or peppery tasting species occupied most of the basal clades, while mild tasting species occurred in more apical clades. Acrid taste in *Lactarius* and *Russula* has been shown to result from a number of sesquiterpenoid lactone compounds and their oxidative states (Hansson & Sterner 1991, Daniewski *et al.* 1992 a, b, 1993, Berhardi *et al.* 1993,

Wang *et al.* 1994) and more critical biochemical research is necessary to further elucidate taste as a character in *Russula* taxonomy.

Acid-resistant incrustations occurred primarily in a large group of closely related species. The only occurrence of incrustations outside of this group was in a clade containing *R. atropurpurea*, *R. viscida*, and *R. ochroleuca*, and in *R. pulverulenta*. These incrustations, however, differ in that they are not acid-resistant and the magenta color resulting from the Basic Fuchsin staining process fades quickly. It is not known whether acid-resistant and nonacid-resistant incrustations are slightly or greatly different in biochemical composition. Incrustations in *Russula* hyphae or cystidia appear to be the result of differential wall composition, with greater amounts of acid-resistant material deposited near the surface of the hyphae in highly incrustated species and possibly less or different material in nonincrustated species. In some cases nonincrustated species may develop with acid-resistant material in internal wall layers resulting in acid-resistant coloration but no surface relief which can be interpreted as incrustations.

Taxonomic treatments of the genus *Russula* have often used combinations of characters to group species into more or less distinct groups. Crawshaw (1930), Lange (1940), Blum (1962) and more recently Thiers (1997) for example, followed the approach of Masee (1902) and divided species into groups based on spore print color (white or yellow) and taste (acrid or mild). The taxonomic infrastructure proposed by Bon (1988) for the subgenus *Russula* Romagnesi *s. str.* (= *Eurussulae* Melzer & Zvára or *Genuinae* Lange) placed all species with incrustated cuticular elements into three closely related sections, and separated species with nonincrustated dermatocystidia into mild or acrid groups.

Molecular analysis suggests that with few exceptions neither of these approaches consistently delimits natural phylogenetically distinct groups. If light spore print color is defined

as III or less according to the system of Romagnesi (1967), the large clade 5 composed of section *Russula* Bon (= *Fragiles* Fr.) with subsections *Felleinae* Melzer & Zvára, *Citrinae* (Romagnesi) Bon, *Emeticinae* Melzer & Zvára, *Atropurpurinae* (Romagnesi) Bon, together with section *Firmae* Fries containing subsections *Exalbicantinae* Singer and *Sanguininae* Melzer & Zvára, is morphologically and phylogenetically distinct. Likewise, except for the acrid or peppery taste in *R. rubra* and *R. aurantiaca*, all of the taxa in clades 6r-t would be mild tasting with acid-resistant incrustations. Unfortunately, at least with this taxon sampling and analysis, evolution of these characters with potential loss or reversal of character states appears to be too variable to allow clearcut natural infrageneric groups to be drawn.

In addition to incrustations on hyphae and cystidia, a number of other microscopic characters have also been used in the taxonomy of *Russula* but remain to be fully explored for their contribution to an infrageneric classification system. Elements of the pileipellis including presence and morphology of dermatocystidia, their reaction in aldehyde compounds, general nature of base tissues and anatomy of the pileus trama have been generally used in the delimitation of *Russula* species. Although some of these characters are more difficult to map onto a molecular phylogeny they may provide additional natural characters useful in the circumscription of infrageneric taxa.

Crawshay (1930), in examining spore ornamentation in *Russula* using a relatively new iodine reagent (Melzer 1924), proclaimed that the difficulties in the taxonomy of *Russula* would be solved by a classification based on spore ornamentation types. Although these expectations were never fully realized, spore morphology, ornamentation characteristics, and size remain useful taxonomic features. Likewise, Buyck (1991) demonstrated that examination of basidia provides many characteristics which may be useful in infrageneric classification. Unfortunately,

neither spore nor basidium characteristics have been critically examined using morphometric and statistical approaches.

If the molecular analysis presented here is an accurate depiction of the true phylogeny for *Russula* in Europe, groups which have been thought to possess primitive characters do indeed seem to occupy a more basal position in the tree topology. These plesiomorphic characters (Romagnesi 1967) include pale or dull colored pileus, white spore prints, adnate cuticle, firm acrid flesh, presence of lamellulae, weak spore ornamentation, elongated spores, and an epicutis with dermatocystidia. The more apomorphic characters include bright pileus coloration, colored spore prints, separable cuticle, more tender mild flesh, absence of lamellulae, well developed spore ornamentation, subglobose spores, and epicutis with specialized base tissue and incrustated primordial hyphae. These plesiomorphic and apomorphic characters are reflected by molecular analysis of European species.

The most basal group in this analysis is a small clade which includes *R. camarophylla*, which has been placed in the section *Archaeinae* Heim ex Buyck & Sarnari, and *R. farinipes*, which has classically been placed by most authors in subsection *Foetentinae* because of its dull brownish yellow pileus color and acrid taste. Singer (1986), however, placed this species in the subsection *Farinipedes* Singer because of the abundant dermatocystidia, white spore print, and fruity rather than disagreeable odor. Although these taxa have not been previously classified together, Buyck (1998) discussed similarities in the characters of the *Archaeinae* and some members of section *Heterophyllae* Fries especially subsections *Foetentinae* and *Cyanoxanthinae* Singer. Characters in common in these groups include small spores with low and weakly amyloid ornamentation and pale or white spore print. Members of the *Archaeinae* along with the *Compactae* have diagnostically narrow basidia. Because sequences of these two species were the

most divergent in the dataset, there was initial concern that the weakly supported relationship observed was due to long-branch attraction. However, molecular analyses which included additional tropical and therefore putatively more primitive members of the *Archaeinae*, *Foetentinae*, *Cyanoxanthinae*, and *Compactae* (not shown) indicated that the relationship between *R. camarophylla* and *R. farinipes* is supportable. Additional sampling in these groups will elucidate the relationships of plesiomorphic *Russula* species.

The choice of an outgroup for the analysis in this study was derived from a related analysis (E. Larsson, unpublished) of the n-LSU from nearly a hundred taxa in the Russuloid lineage. In addition, *Gloeocystidiellum aculeatum*, a corticioid taxon with amyloid ornamented spores worked well for outgroup comparison in previously published molecular studies (Miller *et al.* 2001). There is controversy over how best to choose outgroup taxa and whether to choose one or multiple taxa in the outgroup (Kitching *et al.* 1998). A number of analyses (not shown) were performed with alternative members of the large and diverse Russuloid clade (Hibbett & Donoghue 1995, Hibbett *et al.* 1997), alone and in various combinations, as outgroups including *Bondarzewia berkeleyi*, *Heterobasidion annosum*, *Hericium erinaceus*, *Lentinellus ursinus*, and *Boidinia furfuracea* in addition to *G. aculeatum* and *Albatrellus flettii*. For the most part, overall topology of the molecular trees generated were unaffected by choice of outgroup. However using *B. furfuracea* as an outgroup resulted in pulling the subsection *Sphagnophilinae* into a basal position for unknown reasons. Other more distant choices for outgroup resulted in minor rearrangements in some of the terminal clades such as placement of *R. maculata* within a clade containing *R. adulterina* and *R. cuprea* or placing the *Lactarioideae* and *Compactae* together in the same clade. Often these rearrangements occurred in terminal or near terminal clades with low branch stability.

Taxon sampling in this study concentrated on one or a few collections from northern temperate biogeographical regions representing each infrageneric taxon rather than several of each species. It should be noted that population level genetic variation and phenotypic plasticity must be considered before circumscription of infrageneric taxa can be completed. A much clearer picture of natural infrageneric groups and their relationships will be found with wider sampling of more taxa within each group and critical evaluation of their characteristics.

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Fig. 1. Best (-ln likelihood) of 24 equally most parsimonious trees of 1860 steps inferred from equally weighted parsimony analysis of ITS1, 5.8S and ITS2 nrDNA sequences depicted as a phylogram. Characters mapped onto the phylogeny include spore print color, taste, and presence of acid-resistant incrustations.

Fig. 2. Strict consensus of 24 most parsimonious trees of 1860 steps resulting from equally weighted parsimony analysis of ITS1, 5.8S and ITS2 nrDNA sequences. Numbers above branches are bootstrap values above 50% from 100 replicate-heuristic searches on all parsimony-informative characters using 5 random sequence addition replications and TBR branch swapping algorithms in PAUP*. Decay indices (Bremer support values) are shown below branches. Deeper nodes are denoted with arrows.