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Molecular inference, multivariate morphometrics and ecological assessment are applied in concert to delimit species in the *Russula clavipes* complex

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Abstract: Species of *Russula* subsect. *Xerampelinae* are notoriously difficult to identify and name and have not been subject to molecular study. A group of species, referred to here as the *R. clavipes* complex, growing in association with *Salix*, *Betula* and *Populus* as well as coniferous tree species from temperate to arctic and alpine habitats, were examined. Analyses of the nuc rDNA internal transcribed spacer (ITS) region and a numerical analysis of morphological characters were used. The *R. clavipes* complex is a monophyletic group within *Russula* subsect. *Xerampelinae*, according to molecular results. The complex includes three species: *R. nuoljae* is a phylogenetically and morphologically well-supported species while the other two, *R. clavipes* and *R. pascua*, are similar based on ITS data and morphology but separate based on their ecology. *Russula pseudoölivascens* is conspecific with *R. clavipes*. Several combinations of characters traditionally used in the taxonomy of *R.* subsect. *Xerampelinae* are inappropriate for species delimitation in this group and the adequacy of the ITS for species identification in this group is discussed. Detailed microscopic observations on the type collection of *R. nuoljae* are presented and illustrated, along with a key to the European members of *R.* subsect. *Xerampelinae*.

Key words: DNA barcode, ectomycorrhizal fungi, Europe, haplotype analysis, Russula nuoljae, R. pascua

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Introduction

The genus *Russula* Pers. (Russulaceae, Russulales) is a group of ectomycorrhizal (ECM) fungi forming conspicuous and often colorful basidiomata. Members of *Russula* subsect. *Xerampelinae* Singer ("fishy russulas") occur in a variety of habitats in the northern hemisphere, including boreal, alpine and arctic regions. Some species may be circumarctic or circumboreal, but given the taxonomic confusion in this group we here restrict ourselves to Europe.

Members of *Russula* subsect. *Xerampelinae* are defined by the combination of the herring-like odor, browning of flesh after bruising, green $FeSO_4$ reaction of the flesh, presence of a suprahillar amyloid spot on the basidiospores and presence of pileocystidia without acid-resistant incrustation and with weakly graying contents in sulphovanillin. Pileus colors and host association or habitat traditionally are the key characters used in the delimitation of species in this subsection. Some fishy russulas are assumed to be strictly associated with hardwood trees in temperate areas (e.g. *R. faginea* Romagn. with *Fagus sylvatica* and *R. graveolens* Romell with *Quercus* spp.) or coniferous trees (e.g. *R. xerampelina* [Schaeff.] Fr. and *R. favrei* M. Moser; [Romagnesi 1967, Sarnari 2005]). Following this concept *R. clavipes* Velen. is a species associated with conifers, *R. pseudoolivascens* Kärcher is a similar species associated with *Betula* spp. and *Populus tremula* in temperate or boreal areas, *R. subrubens* (J.E. Lange) Bon grows with *Salix* spp. in temperate or arctic-alpine habitats, *R. nuoljae* Kühner once was reported growing with *Salix reticulata* and *Betula nana* above the tree line in Sweden and *R. chamitae* Kühner and *R. pascua* (F.H. Møller and Jul. Schaeff.) Kühner occur in alpine and arctic areas (Romagnesi 1967, Kühner 1975, Kärcher 2002, Sarnari 2005).

In this study we focus on *R. clavipes*, *R. nuoljae*, *R. pascua* and *R. pseudoolivascens*, which occur in a broad range of habitats from temperate regions to the arctic-alpine belt and have inflated cells in pileipellis near the pileus center (Adamčík and Knudsen 2004). Spore ornamentation patterns and ecological preference suggests that the most closely related taxon to the temperate-boreal *R. clavipes* is the arctic-alpine *R. pascua*. *Russula clavipes* is variable in its preference of ectomycorrhizal hosts and *R. pseudoolivascens* and *R. nuoljae* often are treated as synonyms (Adamčík 2004, Adamčík and Knudsen 2004). *Russula subrubens* is recognized as a morphologically distinct species

growing with *Salix* spp. at various altitudes and the alpine *R. chamitae* is considered a synonym of it.

The considerable variability in habitat, pileus color and micromorphological characters observed in species of subsect. *Xerampelinae* encouraged us to study the *R. clavipes* complex and to improve the traditional, morphology-based taxonomy with molecular analyses based on the nuc rDNA ITS region. The aim of the present project is to clarify the taxonomic status of fungi growing in association with deciduous trees in various climatic areas corresponding morphologically to the types of *R. clavipes*, *R. nuoljae* and *R. pascua* as well as to explore the ecological amplitude of *R. clavipes* and its host range. Adamčík (2004) hypothesized that *R. clavipes* is associated with both coniferous and deciduous trees. An alternative hypothesis is that individuals associated with *Betula* and *Populus* belong to the separate species recognized by Kärcher (2002) as *R. pseudoolivascens*.

The nuc rDNA ITS region is a multicopy locus that has been shown to behave like a dikaryotic locus (Aanen et al. 2001, Schnabel et al. 2005, Hughes et al. 2013). ITS sequences of *Russula* species occasionally show evidence of two different ITS variants. While we cannot demonstrate that the ITS region behaves like a dikaryotic locus in the genus *Russula*, the ITS haplotypes from direct ITS sequences of members of *R. clavipes, R. nuoljae* and *R. pascua* were phased out and a minimum spanning tree calculated. A conscious decision was made not to resort to cloning because cloning is prone to recovering rare ITS types instead of haplotypes (Lindner and Banik 2011, Lindner et al. 2013) or pseudogenes (Li et al. 2013) as well as chimeric sequences.

MATERIAL AND METHODS

Sampling.—Herbarium specimens were selected from recently collected material of *Russula* subsect. *Xerampelinae* to cover a variety of geographical origins, habitat preferences, pileus color and micromorphological characters. Before detailed morphological and molecular studies, the collections were identified morphologically (with some exceptions discussed below) following the concepts of Knudsen et al. (2008) and Adamčík and Knudsen (2004). An effort was made to sample collections morphologically similar to or sharing similar ecological preferences with *R. clavipes* and *R. pascua*. Some collections morphologically resemble *R. clavipes*, and by their occurrence in forests dominated by conifers or *Betula* spp., but lack or have only a few inflated terminal cells of the hyphae in the pileipellis. Because the key by Adamčík and Knudsen (2004) does not offer clear identification we tentatively identified them as *R.* cf. *clavipes*. Two additional species sharing the same ecology and habitat with *R. clavipes* also are well represented in the studied material: *R. favrei* associated with conifers (as delimited by Adamčík 2002) and *R. subrubens* associated with *Salix* spp. (as delimited by Adamčík and Knudsen 2004). Collections originating from Sardinia (Italy) identified as *R.* cf. *amoenoides* Romagn. correspond morphologically to the species described by Romagnesi (1967) and were associated with oak (S. Adamčík unpubl). However, because the type description is not sufficiently detailed and there is geographical and climatic disjunction they are treated here as "cf.". All our remaining collections associated with oak are identified as *R. graveolens*.

Specimens collected by the authors are deposited in KRAM, SAV, STU, TU and UPS supplemented by collections loaned from C, FR, G, IB, L, KR and TUB (Thiers continuously updated). Some sequences submitted and identified by U. Eberhardt were taken from the UNITE database (Kõljalg et al. 2013; https://unite.ut.ee). All specimens used for molecular and morphological studies are provided (SUPPLEMENTARY TABLE I).

Selection of specimens used for morphological studies was based on the results of the molecular analysis and delimitation of groups. Only taxa that were molecularly closely related to *R. clavipes* (*R. nuoljae* and *R. pascua)* were analyzed morphologically. These species are referred to here as the *R. clavipes* complex. To critically evaluate the morphological delimitation of the studied taxa, we also included the type specimens of these species in the multivariate morphometric analyses. We refer to assemblages of collections as the clavipes, nuoljae and Pascua clusters (FIG. 1). These clusters were tentatively assigned to the morphologically corresponding type. The groups formed by the members of each cluster including the types were further studied by multivariate morphometric analyses.

Molecular methods.—DNA was extracted from dried specimens with the single-tube method described in Eberhardt (2012). We used PureGene yeast/bacteria kit (QIAGEN) with overnight soaking at 37 C and 1 h elution in 300 µL cell lysis solution at 65 C and DNA precipitation overnight or high pure PCR template preparation kit for isolation of nucleic acids from mammalian tissue (Roche Applied Science, Indianapolis, Indiana) following the default protocol. For the majority of samples PCR was done with standard PCR primers (ITS1F, ITS2, ITS3, ITS4; White et al. 1990, Gardes and Bruns 1993) and standard conditions (Eberhardt 2012). For some samples PCR was done with primers ITS1F and LB-W (White et al. 1990, Tedersoo et al 2008). For those samples amplification was done with the PuReTaq ready-to-go kit (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK) with these conditions: 0.5 µL of each primer and 24 µL template DNA, in a 25 µL total volume and a 55 C annealing temperature. PCR products were purified with Exo-Sap enzymes. All sequences were obtained by direct sequencing using the same primers with BigDye 3.1 technology (Thermo Fisher Scientific), edited with Sequencher 4.8 (Gene Codes Corp), and submitted to GenBank (KU205269–KU205350). When sequences showed evidence of dikaryosis in otherwise high quality read regions or phase shifts in the presence of ITS-types of one or a few base pairs length difference (Flot et al. 2006) we attempted to recover the sequence of each of the ITS haplotypes to resolve all ambiguities.

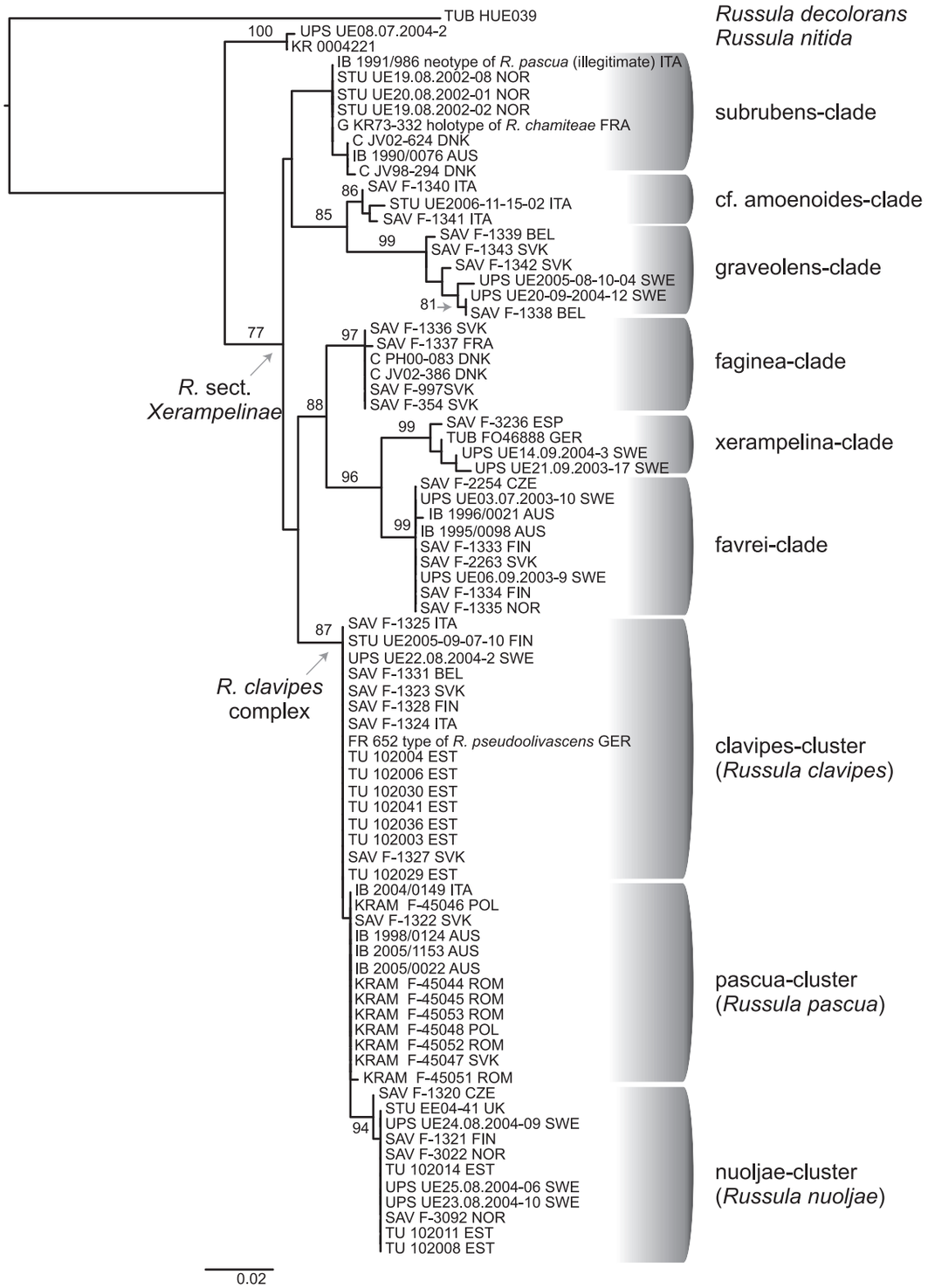

FIG. 1. ML tree with 10 000× bootstrap support of ITS sequences of *Russula* subsect. *Xerampelinae* sequences, rooted with *R. decolorans*. Collections are referred to by their collectors' or herbarium accession numbers (see SUPPLEMENTARY TABLE I). Countries of origin are abbreviated. AUS = Austria, BEL = Belgium, CZE = Czech Republic, DNK = Denmark, ESP = Spain, EST = Estonia, FIN = Finland, FRA = France, GER = Germany, ITA = Italy, NOR = Norway, POL = Poland, ROM = Romania, SVK = Slovakia, SWE = Sweden and UK = United Kingdom.

Sequence alignment was done in MAFFT with the E-INS-i option (Katoh et al. 2005, Katoh and Standley 2013). Maximum-likelihood searches for tree building were carried out locally with 100 replicates with the GTRGAMMA model, selecting the best solution for each analysis in RaXML 8.1.12 (Stamatakis 2014). Fast bootstrap searches were done locally or through the CIPRES Science Gateway (Miller et al. 2010) with 10 000 replicates. Outgroup taxa were chosen based on our ITS analysis (unpubl) including a large proportion of the European *Russula* species. Sequence alignment files were deposited in TreeBASE and are available at http://purl.org/phylo/treebase/phylows/study/TB2:S17208

Haplotype reconstruction from dikaryotic sequences was done by PHASE 2.1.1 (Stephens et al. 2001, Stephens and Donnelly 2003) with the help of the online SeqPHASE service (Flot 2010). Absolute character differences including gaps as a fifth character were calculated in PAUP* (Swofford 2003). A haplotype minimum spanning tree was constructed from these distances with HapStar 0.7 (Teacher and Griffiths 2011).

Morphological observations.—Morphological studies were based exclusively on micromorphological characters because macromorphological characters were not recorded for several specimens, especially those loaned from public herbaria. All micromorphological characters were viewed with an Olympus CX-41 microscope at 1000 ×. All drawings of microscopic structures, with the exception of spores, were made with an Olympus U-DA drawing attachment at a projection scale of 2000 ×. Drawings of basidiospores were scanned with an Artray Artcam 300MI camera and measured by Quick Micro Photo 2.1 software. Enlarged scanned pictures with a 0.1 µm resolution of basidiospores were used for measuring and for making line drawings. Basidiospores were observed in Melzer's reagent on the lamella surface. All tissues were examined in cresyl blue to verify the presence of ortho- or metachromatic reactions (Buyck 1989). Trama hyphae and cystidia were examined in sulphovanillin solution. Acid-resistant incrustation was stained in karbolfuchsin and observed in distilled water after staining for a few seconds in a 10% solution of HCl (cf. Romagnesi 1967). All other microscopic observations were made in ammoniacal Congo red after a short treatment in warm aqueous 10% KOH to dissolve the gelatinous matrix and aid tissue dissociation. The Q value is the length/width ratio of the spores. Spore dimensions exclude ornamentation. Estimates for spore ornamentation density follow Adamčík and Marhold (2000). Density of pleurocystidia is estimated following Buyck (1991). The vertical structure of the pileipellis was observed at the half radius of the pileus. Statistics for the measurements of microscopic characters in the description of *R. nuoljae* are based on 30 measurements observed on the type specimen. Values are recorded as average value plus/minus standard deviation; values in parentheses are measured in 5 or 95 percentiles.

Multivariate morphometric analyses and exploratory data analyses.—Morphological data were evaluated with multivariate analyses to obtain better statistical support for species delimitation within the *R. clavipes* complex. Characters used in these analyses were selected by a simple test in two steps: first, the complete set of micromorphological characters used in the modern descriptions (e.g. Adamčík and Jančovičová 2013, Buyck and Adamčík 2013a, Adamčík and Buyck 2014) were observed and compared with a single representative specimen for each species cluster of the *R. clavipes* complex as delimited by the molecular analysis, and, second, characters with distinct differences among the representative specimens were measured on an additional three specimens per species cluster. Characters with significant differences or characters not overlapping in their average value were selected and measured (30/character) on the complete set of all available material belonging to the *R. clavipes* complex according to our molecular analysis and on type specimens.

Multivariate morphometric analyses were performed to elucidate morphological variation within the *R. clavipes* complex. To avoid distortion of the computations in subsequent multivariate analyses (especially in the case of the discriminant analysis), correlation coefficients suitable for detection of highly correlated pairs of morphological characters were computed. In particular the nonparametric Spearman correlation coefficient was selected (Legendre and Legendre 1998). The principal component analysis (PCA) based on a correlation matrix was used for screening the variability of characters in the dataset (Sneath and Sokal 1973, Krzanowski 1990). Canonical discriminant analysis (CDA) was used to test the hypothesis adopted from the molecular analyses (Klecka 1980, Krzanowski 1990). To determine the correct position of the three type specimens not treated by molecular analyses within the morphological space of the multivariate analyses, we adopted a two-step procedure (Slovák and Marhold 2007).

Finally, nearest-neighbor nonparametric classification discriminant analysis (DA) was used for the identification of the percentage of specimens that were correctly identified to morphologically defined species. The basic statistical parameters (average values, standard deviations, medians, 5 and 95 percentiles) and box-plot charts of other statistical parameters (10, 25, 75, 90 percentiles and extreme values) were calculated for all species clusters of the *R. clavipes* complex defined by molecular analyses. Both multivariate morphometric analyses were computed with SAS 8.2 (SAS Institute 2000). The additional statistical parameters were constructed in Statistica 7 (StatSoft 2004).

RESULTS

Molecular analyses.—The ML analysis (FIG. 1) was based on an alignment (509 positions after excluding sites without sequence variation to account for some short sequence reads) of 77 collections of species belonging to *R.* subsect. *Xerampelinae*. *Russula decolorans* (Fr.) Fr. and *R. nitida* (Pers.) Fr. were used as outgroup. Missing data could not be avoided because we obtained only ITS1 sequences for IB 1990/0076 (*R. subrubens*) and IB 1991/986 (the superfluous neotype of *R. pascua*) and only ITS2 for G 73-332, the holotype of *R. chamitae*. For collections with length-variant ITS haplotypes consensus sequences were used.

All members of *R.* subsect. *Xerampelinae* form a weakly supported monophyletic clade (FIG. 1). Collections of the *R. clavipes* complex initially identified as *R. clavipes* or *R. pascua* are included in one bottom clade with intermediate support (87%). The bottom clade includes a well-supported clade (94% support) with collections initially identified as *R. clavipes*, referred to as the nuoljae cluster with an unsupported sister clade with *R. pascua*. The internal branch leading to this monophylum is so short that the pascua cluster appears paraphyletic to the nuoljae cluster. Paraphyletic to this are the remaining *R. clavipes* collections in the clavipes cluster. The latter group also includes the type sequence of *R. pseudoolivascens*. Because the well-supported clade also contains collections from the type locality of *R. nuoljae*, it is referred here as nuoljae cluster.

Collections tentatively identified as *R.* cf. *clavipes*, differing by their low proportion of inflated terminal cells in the pileipellis near the pileus center or the clavate terminal cell near the pileus margin, are included in two well-supported sister clades. Among them the single collection SAV F-3236 from Spain is in the xerampelina clade and the remaining collections originating from Scandinavia are included in the favrei clade.

Sequences of collections assigned to other species of *R.* subsect. *Xerampelinae* form monophyletic groups with or without support (i.e. the subrubens, graveolens and faginea clades. The sequences of the holotype of *R. chamitae* and the neotype of *R. pascua* are included in the subrubens clade. Collections provisionally assigned to *R.* cf. *amoenoides* form a well-supported clade.

An unrooted minimum spanning tree calculated from the observed or reconstructed haplotypes is illustrated (FIG. 2). All but one of the clavipes, nuoljae and pascua cluster haplotype sequences could be obtained or unambiguously reconstructed. Some haplotypes were retrieved from several collections tentatively assigned to the same species (FIG. 2). One collection of nuoljae cluster (STU EE04/41) included two singleton haplotypes. The four most probable haplotypes were included in the tree building analysis. The 16 collections of the clavipes cluster include four different haplotypes, the 13 of the pascua cluster collections 2, and the 11 of nuoljae cluster 9. None of the haplotypes is shared among members of different species clusters and there is no evidence of recombination among haplotypes of different species clades. The minimum difference between clavipes and pascua cluster is 1 bp and between nuoljae cluster and any other taxon 5 bp.

Multivariate morphometric analyses.—Specimens SAV F-1327 (clavipes cluster), TU 102011 (nuoljae cluster) and KRAM F-45045 (pascua cluster) were used as representatives of each of the three species clusters of the *R. clavipes* complex as defined by the molecular analysis of the ITS region. The first comparison of average values revealed differences in spore dimensions, length and shape of terminal cells of hyphae in pileipellis near the pileus margin, width of terminal cells of pileocystidia near the pileus margin, dimensions of marginal cells on the lamellar edge, dimensions of appendages of pleurocystidia and dimensions of basidia. These characters were measured and compared with additional three specimens for each ITS cluster, altogether 12 specimens (values of representative and type specimens are compared in SUPPLEMENTARY TABLE II). This narrowed our selection to seven characters with significant differences (TABLE I). The average values of 30 measurements for these characters were observed on 43 herbarium specimens of the *R. clavipes* complex, all analyzed by DNA sequencing and types of *R. clavipes*, *R. nuoljae* and *R. pascua* (SUPPLEMENTARY TABLE I). Because the highest values of the Spearman correlations coefficients between pairs of morphological characters did not exceed 0.89 all seven characters (TABLE I) were used in further multivariate morphometric analyses. The highest correlations were detected between these pairs of characters: SP length and SP width (correlation coefficient 0.89) and TC length and TC atten (correlation coefficient 0.69).

In the numerical analyses of morphological characters (TABLE I) two data matrices were constructed to recognize the three species clusters defined by the molecular analysis. Matrix 1 comprises 43 analyzed specimens that included 40 specimens analyzed with DNA sequencing and grouped in the three species clusters

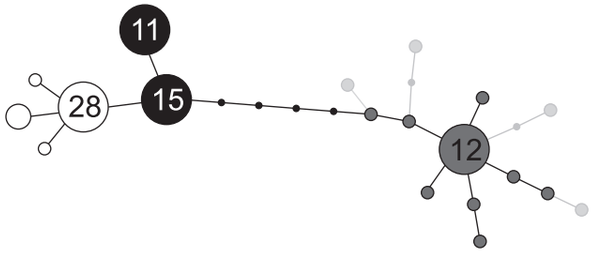

FIG. 2. Unrooted minimum spanning tree of ITS haplotypes of clavipes cluster (*R. clavipes*) (white, online version yellow), pascua cluster (*R. pascua)* (black, online version red) and nuoljae cluster (*R. nuoljae)* (gray, online version orange) clusters. As in haplotype networks, the lines between the circles represent 1 bp difference. For haplotypes retrieved more than twice the number of observations is given within the circle. For circles without number, circle diameter is related to the number of observations, not observed (smallest size), observed once (medium) and observed twice (largest). Two haplotypes of a single collection of *R. nuoljae* could not be unambiguously reconstructed and are represented by four haplotypes, indicated by paler shades.

TABLE I. Selected characters used in numerical analyses of 42 specimens of *R. clavipes* complex

Abbreviation	Description
SP length	Length of spores (μm)
SP width	Width of spores (μm)
TC length	Length of terminal cells of the hyphae in the pileipellis near the cap margin (μm)
TC width	Width of terminal cells of the hyphae in the pileipellis near the cap margin (μm)
TC atten	Proportion of attenuated terminal cells of the hyphae in the pileipellis near the cap margin (%)
TC diff	Difference in width of terminal cells of the hyphae in the pileipellis near the cap margin at the widest part and the apical part (μm)
PC width	Width of terminal cells of the pileocystidia near the cap margin (μm)

of the *R. clavipes* complex from the ML analysis and three type specimens (of *R. clavipes*, *R. nuoljae*, *R. pascua*). Matrix 2 differs by not including the three type specimens mentioned above.

Matrix 1 was analyzed only by principal component analysis (PCA). PCA did not reveal a clear pattern, only a tendency to separate the analyzed specimens into three groups corresponding with the species clusters (FIG. 3). Specimens of the clavipes and nuoljae clusters were fairly well separated along the first ordination axis. In contrast specimens of the pascua cluster were placed in the PCA diagram upward along the second ordination axis, overlapping both of the other groups but especially with the clavipes cluster. All type specimens were placed together with the species clusters they had been tentatively assigned to, except for the type of *R. pseudoolivascens*, which is grouped in clavipes cluster. Characters contributing to separation along the first ordination axis are TC atten, SP width and SP length; along the second ordination axis TC width, PC width and TC diff.

The canonical discriminant analysis (CDA1) performed on Matrix 2 shows the nuoljae cluster well separated from the other two species clusters along both canonical axes (data not shown). Specimens of the clavipes and pascua cluster overlap along both canonical axes. CDA2 (Matrix 1) shows essentially the same pattern detected by CDA1. The type specimens of *R. clavipes*, *R. nuoljae* and *R. pascua* were unambiguously placed in corresponding species clusters (FIG. 4) that are in the text below called by a species name. CDA2 supports the inclusion of *R. pseudoölivascens* type in clavipes cluster, although it is on the margin of the group and isolated from other group members along the first canonical axis. The characters contributing most

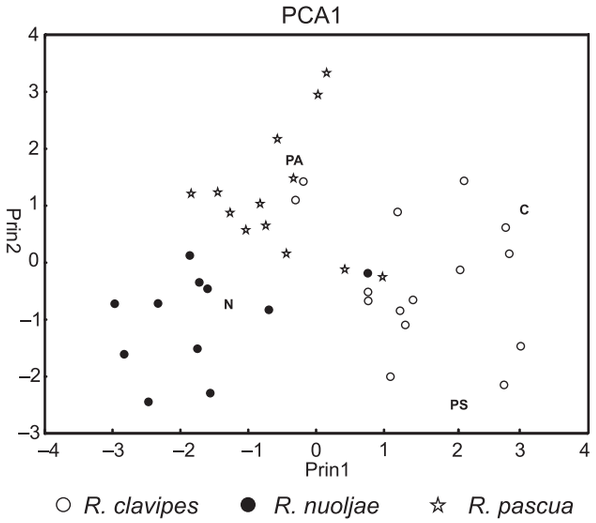

FIG. 3. Principal component analysis (PCA) of the *R. clavipes* complex based on 43 specimens and seven morphological characters. Different species clusters resulting from the ITS ML are recognized by shapes, empty circle = clavipes cluster, full circle = nuoljae cluster, star = pascua cluster. Positions of type specimens are indicated by initials of epithets: C = *R. clavipes*, N = *R. nuoljae*, PA = *R. pascua* and PS = *R. pseudoölivascens*. The first two ordination axes show 43.5% and 27.4% of total variation, respectively.

distinctly to the separation along the first axis are PC width, TC atten, SP width and TC diff (TABLE I, SUPPLEMENTARY TABLE III). For the second canonical axis the contribution of TC length is dominating.

Because PCA, CDA1 or CDA2 analyses did not reveal distinct differences between specimens of *R. clavipes* and *R. pascua* we performed an additional CDA3 analysis using Matrix3 with 29 specimens of both species clusters, not including type specimens of *R. clavipes* and *R. pascua*, to search for better support for their morphological delimitation. CDA3 does not support clear separation of the groups although they overlap slightly (FIG. 5). The variability along the canonical axis is mainly contributed by TC atten, TC length and SP width (SUPPLEMENTARY TABLE IV).

Classificatory discriminant analyses (DA1) on Matrix 1 (k = 8) revealed a high percentage of correctly identified specimens: 82.4% of *R. clavipes*, 100 % of *R. nuoljae* and 92.9% of *R. pascua*. Classificatory discriminant analyses (DA2) computing data only on *R. clavipes* and *R. pascua* (k = 20) produce similar results: 88.2% of correctly classified specimens of *R. clavipes* and 92.9% of *R. pascua*.

Exploratory data analyses.—The largest contribution to the separation of *R. nuoljae* along the first canonical axis in CDA2 revealed characters PC width and TC atten. PC width is the only character that shows a clear

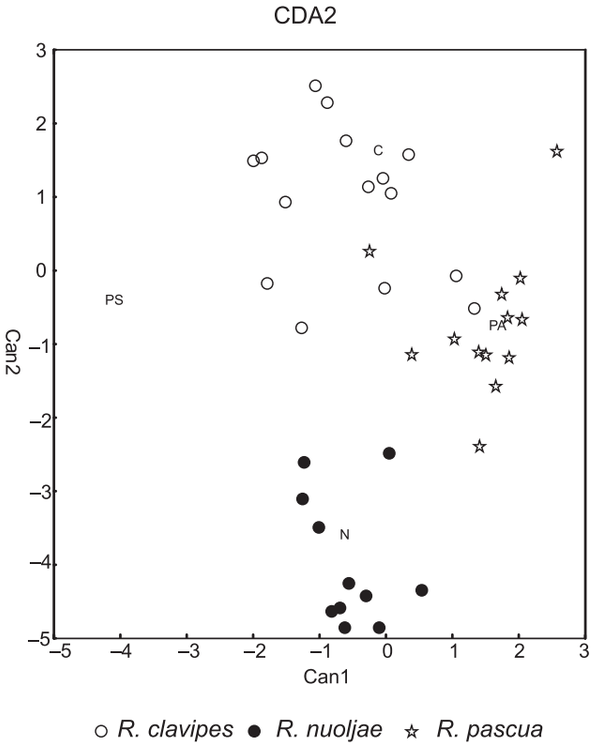

FIG. 4. Canonical discriminant analysis (CDA2) of *R. clavipes* complex based on 43 specimens and seven morphological characters. Different species clusters resulted in the molecular analysis are recognized by shapes, empty circle = clavipes cluster, full circle = nuoljae cluster, star = pascua cluster. Positions of type specimens are indicated by initials of epithets: C = *R. clavipes*, N = *R. nuoljae*, PA = *R. pascua* and PS = *R. pseudoölivascens*.

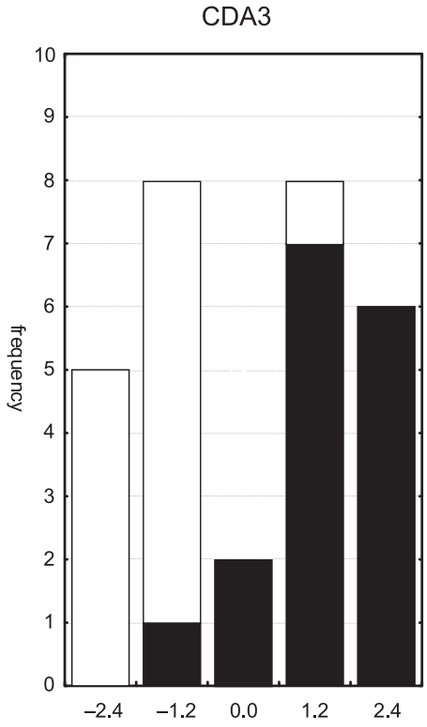

FIG. 5. Histograms of the canonical discriminant analysis (CDA3) of clavipes cluster (black columns) and pascua cluster (white columns) based on 29 specimens and seven morphological characters.

distinction between *R. nuoljae* and the other two species. It does not overlap with other species in 25–75th percentiles or in average values per individuals (SUPPLEMENTARY FIG. 1). The highest average value of this character for *R. nuoljae* is 5.5 µm (SAV F-1321, Finland), and the lowest for two other species is 5.6 µm (*R. clavipes* SAV F-1331, Belgium). TC atten reveals a clear difference between *R. nuoljae* and *R. clavipes* but not a distinct difference from *R. pascua* (SUPPLEMENTARY FIG. 2). The other two characters contributing to the separation of the *R. nuoljae* in CDA1 are SP width and TC diff. The *R. nuoljae* values for these characters partly overlap with *R. clavipes* values but are similar to *R. pascua* values (SUPPLEMENTARY FIG. 1).

According to CDA3, the best characters for the delimitation of *R. clavipes* and *R. pascua* are TC atten, TC length and SP width with the first two characters revealing distinct differences (SUPPLEMENTARY FIGS. 1, 2). Only one isolated specimen of *R. pascua* (SAV F-1322, Slovakia) reaches the minimum average value of TC atten observed for *R. clavipes* (SUPPLEMENTARY FIG. 2). Eleven of 14 *R. pascua* specimens have lower TC length value than 30 µm while for *R. clavipes* specimens values always exceed 30 µm.

In summary *R. nuoljae* differs from the other two species by narrower pileocystidia (PC width) and typically cylindrical not attenuated and moderately long terminal cells of the hyphae in the pileipellis near the pileus margin (TC atten, TC length). The latter two characters distinguish also *R. pascua* from *R. clavipes*, including the type of *R. pseudoölivascens*; the former has usually shorter and not distinctly attenuated terminal cells in the pileipellis near the pileus margin. The differences are illustrated (FIG. 6).

TAXONOMY

Russula nuoljae Kühner, Bull. Soc. Mycol. France 91:388, 1975. FIGS. 7–8

Typification: SWEDEN, LAPLAND, Abisko surroundings, toward the Nuolja mountain station, 14 Aug 1967, *Kühner No. 67.129* (holotype G00059394, designated by Kühner 1975).

Description of micromorphological characters of the type. Basidiospores (7.6)8.1–8.7–9.4(–10) × (6.2–)6.4–6.9–7.4(–7.8) µm, Q = (1.15–)1.18–1.25–1.32(–1.37),

ornamented with relatively dense, (4–)5–7(–8) in a 3 µm diam circle on the spore surface, amyloid warts, 0.6–0.8 µm high, occasionally interconnected by fine lines, 0–2(–3) line connections in the circle, often fused into short chains, (0–)1–3(–4) fusions in the circle, isolated warts occasional; suprahilar spot amyloid. Basidia (43–)44–48.5–53(–58) × (10–)10.5–11.5–12.5(–13) µm, four-spored, clavate; basidiola first cylindrical, then clavate, ca 5–11 µm wide. Subhymenium pseudoparenchymatic. Lamellar trama mainly composed of large sphaerocytes. Pleurocystidia widely dispersed to dispersed, ca. 300–400/mm^2, (63–)69–77.5–85.5(–96) × (7–)8–9.8–11.5(–12) µm on the lamellar sides, fusiform to clavate, pedicellate, apically acute or obtuse, mainly with 1–8(–15) µm long appendage, thin-walled, in Congo red with heteromorphous (sometimes in part of the volume), crystalline-banded contents that weakly react in sulphovanillin. Marginal cells not well differentiated, (14–)17–21.3–25.5(–28) × (3–)3.5–5.1–6.5(–8.5) µm, usually narrower than basidioles on the lamellar sides, cylindrical or clavate, rarely fusiform, usually obtuse, often flexuous and sometimes slightly moniliform. Cheilocystidia in shape similar to the pleurocystidia but smaller, (44–)46.5–54.6–62.5(–67) × (6–)6.5–7.7–9(–10) µm, with only a few yellowish inclusions. Pileipellis orthochromatic in cresyl blue, not sharply delimited from the underlying sphaerocytes of the context, 90–110 µm deep, near surface with up to 15 µm deep gelatinous matter; vaguely divided into 45–55 µm deep suprapellis of ascending to repent, relatively dense, strongly gelatinized hyphae and 45–55 µm deep subpellis of dense, less gelatinized, horizontally oriented, intricate, 2–5 µm wide hyphae; acid-resistant incrustations absent. Hyphal endings of the suprapellis thin-walled, near the pileus margin with mainly cylindrical, occasionally or rarely slightly attenuated terminal cells, (21–)26.1–34.8–43.4(–51) × (3–)3.7–4.5–5.4(–6) µm, apically obtuse and usually not moniliform; subterminal cells mainly not branched, frequently shorter and wider, branching usually at the third cell rank. Terminal cells of the hyphae near the pileus center distinctly shorter and wider, (16–)20–25.6–31(–36) × 4–5.6–7.5(–11.5) µm, mainly cylindrical but often also inflated-lageniform (wider than 6 µm), rarely fusiform, subterminal cells not branched (branching mainly at the third cell rank) but usually equally long and wide as terminal cells. Pileocystidia inconspicuous and narrow, in Congo red with few yellowish inclusions mostly restricted to the terminal cells, in sulphovanillin negative, cylindrical, apically sometimes moniliform, one- or multicelled, often occurring in small fascicules, terminal cells near the pileus margin (37–)44–59.2–74(–99) × (4–)4.2–5–5.9(–6.5) µm, near the pileus center smaller and (22–)29–40.6–52.5(–71) × (4–)4.5–5.1–6(–6.5) µm. Cystidioid hyphae in subpellis and trama absent. Clamp connections absent in all parts.

Distribution and ecology.—Russula nuoljae has been known only from the type locality (Kühner 1975) and was not accepted in recent literature. The origin of specimens studied here (FIG. 1, SUPPLEMENTARY TABLE I) suggests that *R. nuoljae* has a wide distribution. We report it from the type locality (northern Sweden), from Scotland eastward to Estonia and from Finland southward to the Czech Republic. We did not collect it in dry pine forests like *R. clavipes*, but it occurs in a variety of habitats from montane-boreal mixed forests dominated by *Betula* spp. and *Picea abies* to deciduous subarctic to subalpine forests usually dominated by *Betula* spp. All collections were associated with *Betula,* but the question whether *R. nuoljae* is a strict associate of this tree has to be verified in further studies.

Notes: Betula and *Picea* also are associated frequently with *R. clavipes*, and in our experience it is impossible to distinguish this species from *R. nuoljae* in the field. The latter typically has a bright red pileus (SUPPLEMENTARY FIG. 3) that is rare but also is observed in *R. clavipes*. In addition, some collections of *R. nuoljae* had green-dominated pilei similar to the typical aspect of *R. clavipes*.

Our results clearly demonstrate that *R. nuoljae* is a distinct species. Kühner (1975) described *R. nuoljae* as species with all typical *Xerampelinae* characters except for the absence of pileocystidia. This study confirmed the observation by Adamčík and Knudsen (2004) that Kühner overlooked the inconspicuous pileocystidia. Detailed descriptions of other two species in the complex, *R. clavipes* and *R. pascua*, are provided by Adamčík (2004) and Adamčík and Knudsen (2004).

KEY TO EUROPEAN SPECIES OF *RUSSULA* SUBSECT. *XERAMPELINAE*

1. Terminal cells of hyphae in the pileipellis near the pileus margin clavate, lanceolate or fusiform; basidiospores on average longer than 9.5 µm; growing in coniferous, mixed montane or boreal forests . . . . . *R. favrei*

1′. Terminal cells of hyphae in the pileipellis near the pileus margin attenuated, cylindrical or lageniform; basidiospores usually smaller 2

2. Inflated terminal cells of hyphae in the pileipellis frequent near the pileus center 3

2′. Inflated terminal cells of hyphae in the pileipellis absent or rare near the pileus center 6

3. Terminal cells of hyphae in the pileipellis near the pileus margin often lageniform-inflated and short (<25 µm long), hardwood species usually

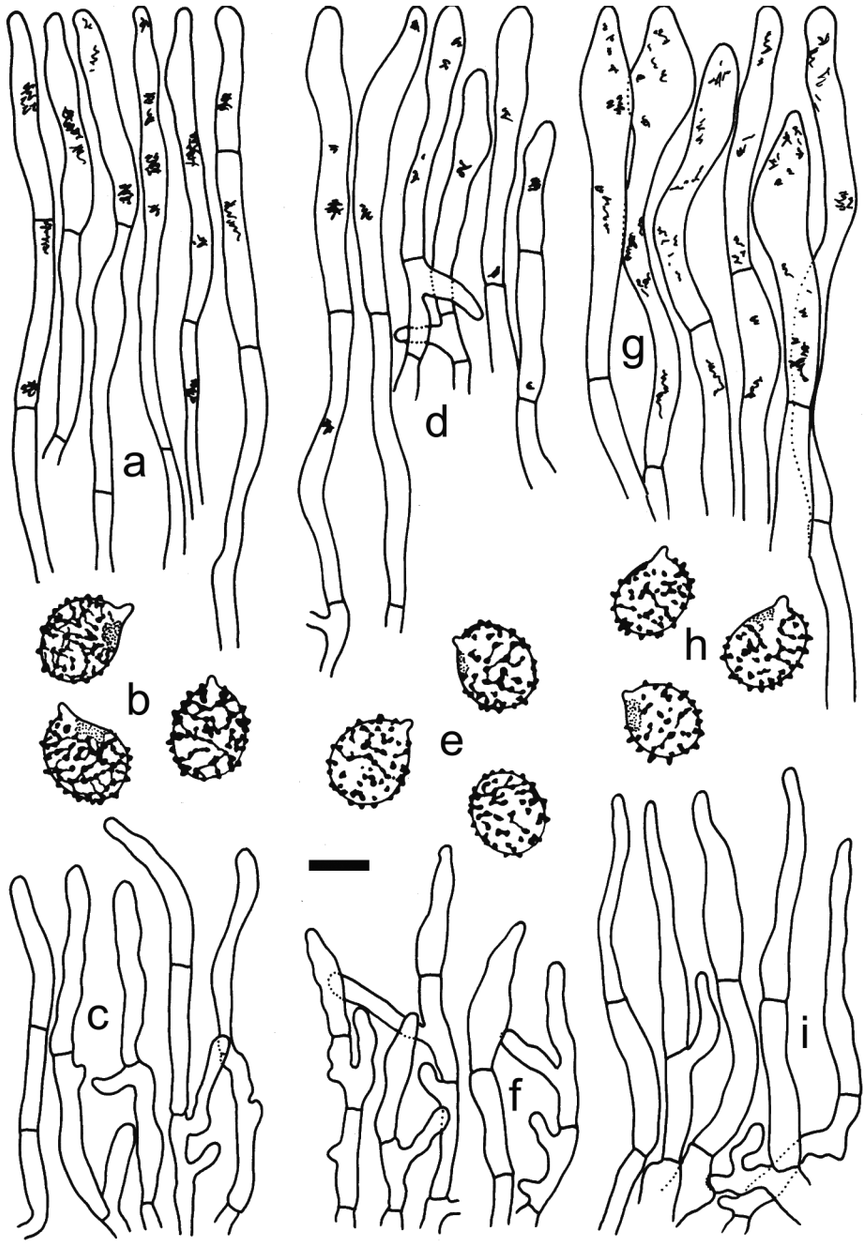

FIG. 6. Comparison of the microscopic structures showing distinct differences among studied species. *Russula nuoljae* (UPS UE23.08.2004-10): A. Pileocystidia. B. Basidiospores. C. Terminal cells of hyphae in the pileipellis near the pileus margin. *Russula pascua* (KRAM F-45045): D. Pileocystidia. E. Basidiospores. F. Terminal cells of hyphae in the pileipellis near the pileus margin. *Russula clavipes* (SAV F-1327). G. Pileocystidia. H. Basidiospores. I. Terminal cells of hyphae in the pileipellis near the pileus margin. Bars = 10 µm.

associated with *Fagus* but also with *Quercus* and *Carpinus* . . . . . *R. faginea*

3′. Terminal cells of hyphae in the pileipellis near the pileus margin not lageniform-inflated or growing in alpine-arctic areas 4

4. Pileocystidia on average < 6 µm wide, terminal cells of hyphae in the pileipellis near the pileus margin mostly cylindrical, growing in boreal, montane forests to arctic-alpine habitats, often associated with *Betula* . . . . . *R. nuoljae*

4′. Pileocystidia on average > 6 µm wide, terminal cells of hyphae in the pileipellis near the pileus margin mostly subulate and attenuated, growing in coniferous, mixed or deciduous

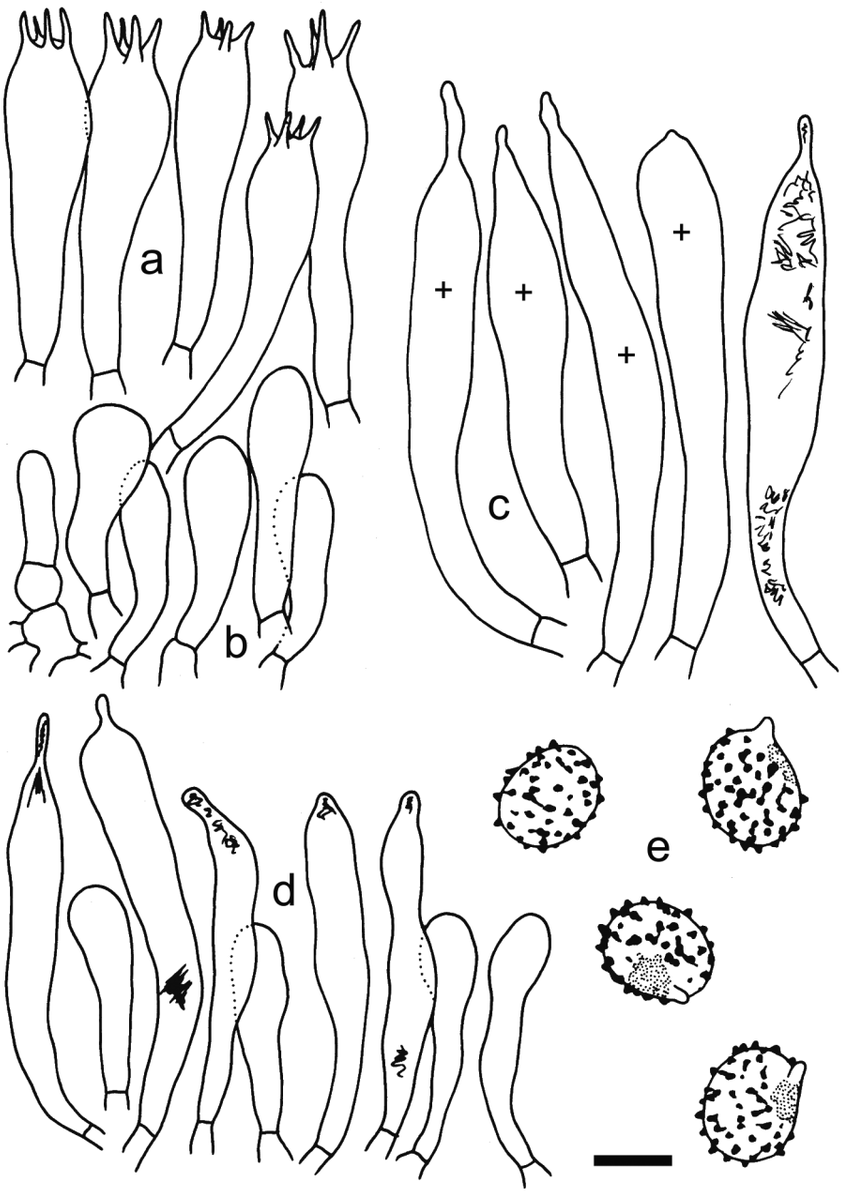

FIG. 7. *Russula nuoljae*, holotype (G00059394). A. Elements in the pileipellis: pileocystidia. B. Terminal cells of hyphae in the pileipellis near the pileus margin. C. Terminal cells of hyphae in the pileipellis near the pileus center. Bars = 10 μm.

(with *Betula*, *Populus*, *Salix*) forests from temperate to arctic-alpine areas 5

5. Terminal cells of hyphae in the pileipellis near the pileus margin mostly <30 μm, growing in arctic-alpine habitats above tree line, typically on calcareous bedrock . . . . . *R. pascua*

5′. Terminal cells of hyphae in the pileipellis near the pileus margin mostly >30 μm, growing in deciduous (with *Betula*, *Populus* or *Salix*), mixed and coniferous forests in temperate to boreal climates, typically on peaty, acidic and moist soil . . . . . *R. clavipes*

6. Terminal cells of hyphae in the pileipellis near the pileus margin cylindrical and narrow, ca. 3–5 μm wide, associated with *Salix*, from lowlands of temperate belt to arctic-alpine areas . . . . . *R. subrubens*

6′. If terminal cells of hyphae in the pileipellis near the pileus margin cylindrical, than often >5 μm wide, associated with coniferous trees or with *Quercus,* possibly *Tilia* and *Carpinus* . . .7

7. Basidiospores with spines connected by numerous lines, pileus typically dark pur-

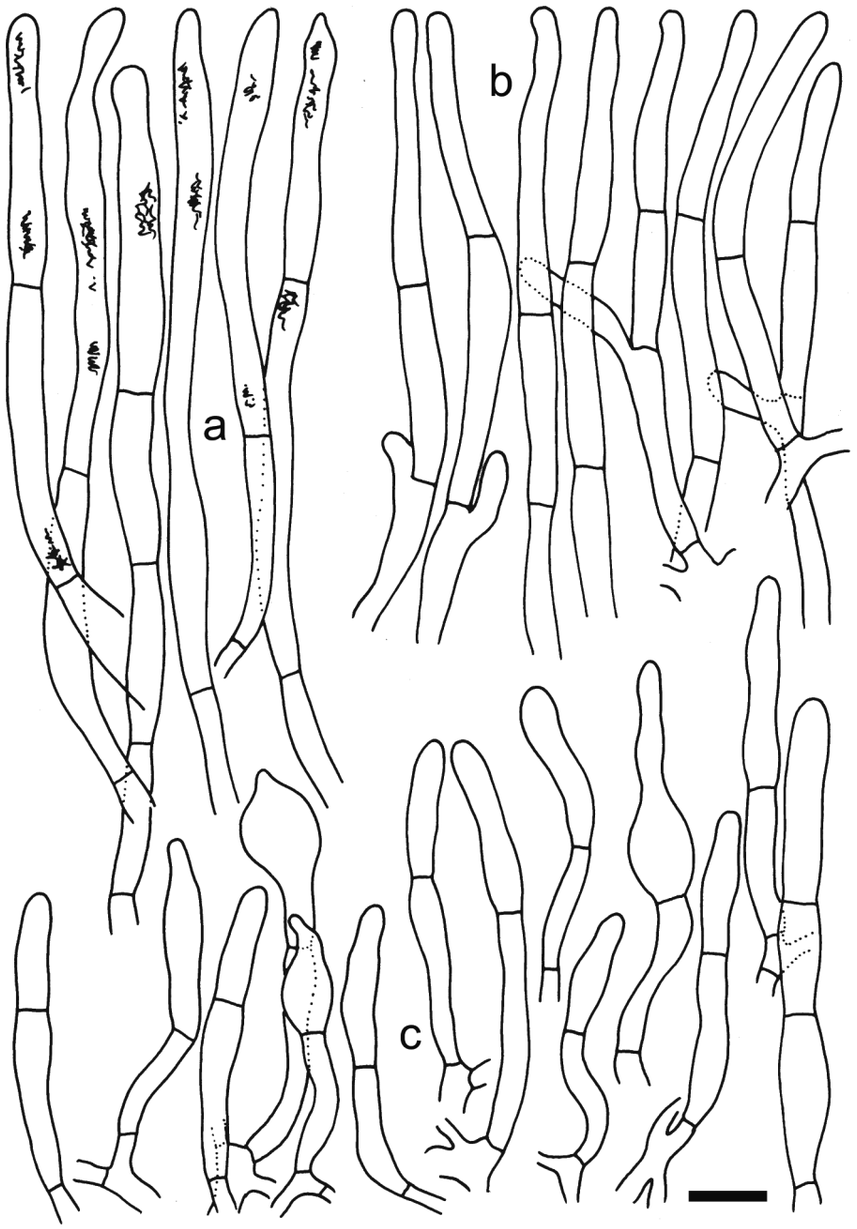

FIG. 8. *Russula nuoljae*, holotype (G00059394). A. Elements of the hymenium: basidia. B. Basidiola. C. Pleurocystidia. D. Marginal cells on the lamellar edge and cheilocystidia. E. Basidiospores (E). Bars = 10 μm.

ple with black center, associated with conifers . . . . . *R. xerampelina*

7′. Basidiospores with occasional to rare line connections, color of pileus variable, associated with hardwood trees (mostly *Quercus*) 8

8. Terminal cells of hyphae in the pileipellis near the pileus margin apically attenuated, subulate, fusiform or lageniform, associated with *Quercus*, widely distributed in Europe . . . . . *R. graveolens*

8′. Terminal cells of hyphae in the pileipellis near the pileus margin short (often up to 30 μm), cylindrical and apically usually obtuse, associated with *Quercus* in the Mediterranean area . . . . . *R.* cf. *amoenoides*

DISCUSSION

Species delimitation in the R. clavipes *complex.—Russula* subsect. *Xerampelinae* has not been analyzed with

molecular data before. Our study focuses mainly on *R. clavipes* and its distinction from *R. nuoljae* and *R. pascua*, species for which we have a reasonably good sampling. Collections tentatively assigned to other species of *R.* subsect. *Xerampelinae* were included to illustrate relationships and delimitation of the *R. clavipes* complex.

We tested previous morphology-based hypotheses of species concepts and delimitation based on molecular analysis of the ITS region. Specimens identified morphologically with the keys of Adamčík and Knudsen (2004) and Knudsen et al. (2008) form monophyletic clades in the ITS topology that correspond to *R. subrubens*, *R. graveolens*, *R. faginea*, *R. xerampelina*, *R. favrei*, *R. pascua* and, with the reservations outlined under *Sampling* above, to *R. amoenoides*. The ITS variation within some clades suggests they may be species complexes rather than species.

Collections initially identified as *R. clavipes* morphologically do not form a monophyletic clade. Some collections are included in the well-supported clade that morphologically corresponds to the type specimen of *R. nuoljae* while other collections are included in the unresolved paraphyletic part of the *R. clavipes* complex clade and correspond morphologically to the type of *R. clavipes*. Morphological delimitation of *R. nuoljae* is clearly supported by narrow pileocystidia (< 6 µm wide on average) and mainly cylindrical terminal cells of hyphae in the pileipellis near the pileus margin (FIG. 6). In their descriptions and morphological analyses of *R. clavipes* Adamčík (2004) and Adamčík and Knudsen (2004) also included morphological variability contributed by *R. nuoljae*. The latter was treated as a synonym of *R. clavipes* (e.g. compare the wide basidiospores measured on Greenland collections by Adamčík and Knudsen 2004 with those in SUPPLEMENTARY FIG. 1).

Molecular and multivariate morphometric analyses do not clearly delimit *R. clavipes* from *R. pascua*. Adamčík and Knudsen (2004) distinguished these species based on characters of the terminal cells of the hyphae in the pileipellis near the pileus margin. These cells are longer and have higher length/width ratio in *R. clavipes* compared to *R. pascua*. Our sampling includes collections of *R. pascua* originating from the Carpathians (Polish, Slovak and Romanian collections; see SUPPLEMENTARY TABLE I), an area not sampled in previous studies, and these collections account for the morphological overlap between *R. pascua* and *R. clavipes*. Despite weak support for the delimitation of *R. pascua* from *R. clavipes* in molecular and morphological analyses, *R. pascua* clearly differs in ecology. All collections originating from the Alps and Carpathians occurred in calcareous areas with *Dryas octopetala*, *Polygonum viviparum* and dwarf *Salix* spp. *Russula clavipes* has not been found in such habitats. Because all collections with a typical field aspect and habitat for *R. pascua* are supported as *R. pascua* by molecular results and because there is apparent morphological support for the recognition of more than 80% of the collections of both *R. clavipes* and *R. pascua* we follow the current opinion in the literature and accept both taxa at the species rank.

The haplotype analysis of ITS sequences (FIG. 2) reveals that collections assigned to *R. clavipes*, *R. nuoljae* or *R. pascua* do not share ITS haplotypes. Despite low interspecific ITS variation the results do not suggest incomplete lineage sorting or recent or current sexual crossing. This supports the species delimitations used here but not the weak monophyly of the pascua cluster in the ML results. Maximum intraspecific variation in all taxa is at least equal to the minimum interspecific variation so that barcode gap analyses (Meyer and Paulay 2005) would fail. However, given that ITS normally lumps rather than splits species in higher fungi (Schoch et al. 2012), we expect that other loci will properly resolve the three species.

Russula pseudoölivascens *is a synonym of* R. clavipes.—Kärcher (2002) recognized *Russula* collections with green pilei strictly associated with deciduous trees as the new species *R. pseudoölivascens* and distinguished it from *R. clavipes* by the presence of more numerous inflated elements in the pileipellis. Adamčík (2004) demonstrated that the type of *R. clavipes* collected with *Picea* has the same morphological characters as collections originating from deciduous forests. Further the type of *R. pseudoölivascens* and another collection from exclusively deciduous forest (SAV F-1331 from Belgium) occur in the same species cluster as typical *R. clavipes* in our molecular analyses (FIGS. 1, 2). Although the position of the *R. pseudoölivascens* type in CDA2 is isolated from all other specimens, it is closest to other specimens of *R. clavipes*.

Delimitation of R. clavipes *complex from other members of* R. subsect. Xerampelinae.—The recognition of the *R. clavipes* complex within *R.* subsect. *Xerampelinae* is relatively easy. Members of this complex are characterized by the presence of inflated cells in the pileipellis that are wider than 6 µm. This character also occurs in *R. faginea* and *R. graveolens*, but these species are associated with *Fagus* or *Quercus*, respectively, whereas members of the *R. clavipes* complex are mycorrhizal with hosts typical for the boreal, alpine or arctic belt (e.g. *Betula*, *Dryas*, *Picea*, *Pinus*, *Populus*, *Salix*). Moreover, *R. faginea* has large basidiomata with hard flesh, the terminal cells of the hyphae in

the pileipellis near the pileus margin are short and also frequently inflated and basidiospores are ornamented with large and prominent spines (Adamčík 2003). In *R. graveolens* inflated terminal cells of the hyphae in the pileipellis always are restricted to the pileus margin (S. Adamčík unpubl). Specimens of *R. graveolens* form a well-supported clade but there is apparent variability in the ITS that corresponds to morphology, suggesting this should be referred to as a species complex until further studies are completed. Other characters useful for the delimitation of the *R. clavipes* complex are the contents of pileocystidia, which are dispersed and inconspicuous in Congo red. In contrast other species of the subsection have the contents usually consistently heteromorphous and conspicuous in Congo red.

Here we differentiate the *R. clavipes* complex from *R. subrubens* by the presence of inflated terminal cells near the pileus center. Sarnari (2005) distinguished *R. subrubens* from other alpine and arctic species of the subsection, including *R. pascua*, by the blackening of pileocystidia in sulphobenzaldehyde and its larger spores, following the original concept of the species (Kühner 1975). However, the superfluous neotype Sarnari (2005) designated for *R. pascua* is not included in the *R. clavipes* complex but placed within the *R. subrubens* clade in the ITS analysis (FIG. 1). The lack of support for subrubens clade (FIG. 1) is probably a result of missing data (incomplete ITS sequences with only ITS1 or ITS2 present) in three of eight sequences, including the *R. chamitae* type and the neotype of *R. pascua*. The position of *R. chamitae* type in the subrubens clade corresponds to the conclusions of Adamčík and Knudsen (2004).

Traditional species concept based on pileus color and habitat preference is unreliable.—At least 59 validly published taxa are in subsect. *Xerampelinae* from Europe (Buyck and Adamčík 2013b). The traditional concepts applied to members of this subsection rely heavily on a combination of habitat preferences and pileus color and in some taxa another single or a few other distinctive morphological characters are used. The widely accepted species are those included in the European monograph by Romagnesi (1967). Following the same philosophy of species delimitation several authors interpret deviations from the commonly accepted species concepts as new species, *R. citrinocincta* Reumaux and *R. cookeiana* Reumaux (Reumaux et al. 1996) and *R. tarda* Jurkeit, Grauwinker and Albers (Jurkeit et al. 2011).

Our results cast doubt on the reliability of these traditional characters for species delimitation. It appears that not only the strictness of the host association but also the importance of the pileus color have been overestimated. Some collections that we initially identified as *R.* cf. *clavipes* had green pilei but lacked inflated cells in the pileipellis near the pileus center or had clavate terminal cells in pileipellis near the pileus margin and grew in coniferous forests. These specimens could be interpreted as *R. pseudoolivascens*, *R. citrinocincta*, *R. cookeiana* etc. based on the literature. The collections of *R.* cf. *clavipes*, originating from boreal forests in Scandinavia (FIG. 1, SUPPLEMENTARY TABLE I), are included in the *R. favrei* clade and the single collection of *R.* cf. *clavipes* from Spain (SAV F-3236) is in the *R. xerampelina* clade, irrespective of pileus color. Our morphological observations suggest that some Scandinavian collections (SAV F-1333, SAV F-1334, SAV F-1335) are merely green forms of *R. favrei*, which is supported also by microcharacters. However, if the pileus color is discounted as a reliable character for the recognition of *R. clavipes R. favrei* and *R. nuoljae*, then we also can expect to come across a green form of *R. xerampelina*, a taxon now defined by its brilliantly reddish purple pileus. Unfortunately the sampling within the *R. xerampelina* clade is insufficient for a conclusion about the taxonomic status of the single collection with green pilei included in the xerampelina clade.

The R. clavipes *complex and the DNA barcode.*—Occurrence of fungal basidiomata is seasonal and thus molecular data have replaced morphology for the identification of fungi in many contexts such as ecology or biogeography. The ITS, the recognized DNA barcode marker of fungi, is the most important locus for species identification (Schoch et al. 2012). Several studies of ectomycorrhizal fungal communities of arctic and alpine habitats (i.e. Geml et al. 2009, Timling et al. 2012, Tedersoo et al. 2013, Morgado et al. 2014) used ITS for species identification for investigating ecological and biogeographical questions. According to current practice, ITS sequences of 97% similarity or higher were recognized as OTUs in lieu of species (Lindahl et el. 2013, Tedersoo et al. 2014). Although ITS variation in none of the species clades exceeds 3%, we do not know whether additional species are hidden within some of these clades. A 97% cut-off would recognize the seven main clades (FIG. 1) but not recognize species within the *R. clavipes* complex. The results presented here are consistent with other studies (e.g. den Bakker et al. 2007, Liimatainen et al. 2014, Eberhardt et al. 2015). The *R. clavipes* complex is yet another example of cold-adapted species where a metabarcoding approach will not pick up ecological and biogeographical patterns that are most likely to have been influenced by quaternary environmental changes.

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