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Species Delimitation of Three Species within the *Russula* Subgenus *Compacta* in Korea: *R. eccentrica*, *R. nigricans*, and *R. subnigricans*

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Distinguishing individual *Russula* species can be very difficult due to extensive phenotypic plasticity and obscure morphological and anatomical discontinuities. In this study, we use the internal transcribed spacer (ITS) and 28S nuclear ribosomal large subunit (LSU) markers to identify and study the genetic diversity of species in the *Russula* subgenus *Compacta* in Korea. We focus on two morphologically similar species that are often misidentified for each other: *R. nigricans* and *R. subnigricans*. Based on molecular phylogenetic analyses, we identify three subgroups of *R. nigricans*, with two from Asia and one from Europe/North America. Surprisingly, we find Korean *R. subnigricans* are more closely related to *R. eccentrica* from North America than the type specimen of *R. subnigricans* from Japan. These molecular data, along with habitat data, reveal that Korean *R. subnigricans* had previously been misclassified and should now be recognized as *R. eccentrica*. Both ITS and LSU exhibit high interspecific and low intraspecific variation for *R. eccentrica*, *R. nigricans*, and *R. subnigricans*. These markers provide enough resolutional power to differentiate these species and uncover phylogeographic structure, and will be powerful tools for future ecological studies of *Russula*.

Keywords: DNA barcoding, Internal Transcribed Spacer, 28S Nuclear Ribosomal Large Subunit, *Russula eccentrica*, *Russula nigricans*, *Russula subnigricans*

Introduction

The fungal genus *Russula* is one of the highly diverse groups in Agaricomycetes. *Russula* species play a critical role in maintaining forest biodiversity as ectomycorrhizal fungi and food sources for insects (Yamashita and Hijii, 2007), animals (Fogel and Trappe, 1978) and humans (Guo, 1992; Hu and Zeng, 1992). To date, approximately 750 *Russula* species have been reported worldwide (Kirk *et al.*, 2008), and distributed across a wide range of habitats from the tropics to arctic zones (Bills *et al.*, 1986; Villeneuve *et al.*, 1989; Gardes and Dahlberg, 1996; Buyck and Ovrebo, 2002; Geml *et al.*, 2010). The genus *Russula* is characterized by a basidiocarp with brittle flesh due to the presence of sphaerocysts, lamellae equal or intermixed, and basidiospores with amyloid incrustations (Singer, 1986; Romagnesi, 1996). Numerous infrageneric classifications of *Russula* have been proposed based on macro- and micro-morphological features. However, distinguishing individual species within *Russula* has been very difficult due to extensive phenotypic plasticity and obscure morphological and anatomical discontinuities (Miller and Buyck, 2002).

The *Russula* subgenus *Compacta* is found worldwide and the taxa have a compact structure, cap rim rolled in, and lamellae interspersed by lamellulae (Eberhardt, 2002). Some members within the subgenus *Compacta* are relatively easy to identify because of the dingy color of their fruiting body and tissues, which turn black from cutting, bruising, or aging. Our study focuses on three closely related *Russula* species – *R. nigricans*, *R. subnigricans*, and *R. eccentrica*. Two of these species, *R. nigricans* and *R. subnigricans*, have been recorded in Korea. *R. subnigricans* can be distinguished from *R. nigricans* by (i) no blackening after reddening, (ii) pinkish lamellae, (iii) a slightly tuberculate sulcate pileal margin, and (iv) small warts in the spore ornamentation without a network (Shimono *et al.*, 2004). *Russula eccentrica* reported from North America was confused for and initially identified as *R. subnigricans* (Weber *et al.*, 1985), but features such as pale pink trama, presence of dermatocystidia in a thin pileus cuticle, and larger basidiospores with higher ornamentation (Bills, 1985) can differentiate these two species. Despite distinguishing morphological characters, species identification is difficult, as color change can only be seen in fresh specimens and observing spore ornamentation is not trivial, even under microscopy in the laboratory. Therefore, we explore the use of DNA data to complement morphology-based identification. Correct species identification in this case is not just an exercise in phylogenetics, but is important for human health, as *R. nigricans* is edible, but *R. subnigricans* is notorious for mushroom poisoning (Imazeki and Hongo, 1989; Lee *et al.*, 2001). For these three *Russula* species, molecular data exists from North America, Europe, and Japan, while genetic data are lacking from Korea. We fill this gap by sampling these *Russula* species throughout Korea.

For the kingdom Fungi, the internal transcribed spacer (ITS) region has been formally proposed for the primary fungal barcode gene, as its high sequence variation was shown to resolve closely related species across the fungal tree of life

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(Schoch *et al.*, 2012). The 28S nuclear ribosomal large subunit (LSU) has also proven to be a useful marker in species delimitation. In addition to being easy to amplify, sequence, and align, LSU has been shown to improve phylogenetic resolution, especially for yeast (Fell *et al.*, 2000; Scorzetti *et al.*, 2002) and Glomeromycota (Stockinger *et al.*, 2010). For these reasons, the use of both ITS and LSU needs to be explored for studying the genetic variation of fungal species. Our goals are to determine the intra- and inter-specific variation of ITS and LSU region sequences of representative *Russula* species within the subgenus *Compacta* in Korea. This study is the first step of constructing a comprehensive sequence database and developing molecular markers to study the ecology and evolution of the genus *Russula*.

Materials and Methods

Materials studies

Fruiting body samples of *R. nigricans* and *R. subnigricans* were collected from locations throughout South Korea between 1992-2012 (Table 1), with dried specimens being deposited in Herbarium Conservation Center of National Academy of Agricultural Sciences (HCCN), Herbarium Conservation Center of National Academy of Agricultural Sciences genebank (HCCNg) and Seoul National University Fungus Collection (SFC). Samples were identified with field guides (Hongo, 1955; Romagnesi, 1996; Kränzlin, 2005; Park and Lee, 2011), a photographic illustration website (http:// www.mtsn.tn.it/russulales-news/), and microscopy. Microscope work was done using a light microscope (Nikon 80i). To allow for comparisons with published data (i.e. Shaffer, 1962), we measure basidia (n=10/sample) and basidiospores (n=20/sample) as length range × width range.

Thirty-three total specimens identified as *R. nigricans* (18 specimens) and *R. subnigricans* (15 specimens) were used in this study (Table 1). Each specimen was re-examined for verification of species identity following three steps: (i) grouping based on basidia and basidiospore sizes, (ii) performing molecular phylogenetic analysis, and (iii) comparing morphological details to published data.

Table 1. Specimens used in this study

Collection No.	Species history	Final ID	Locality	Collection date	Accession number	
					ITS	LSU
HCCN06452	*R. foetans* > *R. nigricans*	*R. nigricans*	Cheongyang-gun, Chungcheongnam-do, Korea	1997-07-11	KC699755	KC699798
HCCN11128	*R. subnigricans* > *R. nigricans*	*R. nigricans*	Jeju-si, Jeju-do, Korea	2003-06-26	KC699765	KC699801
HCCN13850	*R. subnigricans* > *R. nigricans*	*R. nigricans*	Muju-gun, Jeollabuk-do, Korea	2006-07-07	KC699764	KC699799
HCCN16734	*R. nigricans* > *R. nigricans*	*R. nigricans*	Suwon-si, Gyeonggi-do, Korea	2008-08-01	KC699763	KC699800
HCCN18227	*R. nigricans* > *R. nigricans*	*R. nigricans*	Suwon-si, Gyeonggi-do, Korea	2009-07-20	KC699762	KC699802
HCCN18232	*R. nigricans* > *R. nigricans*	*R. nigricans*	Suwon-si, Gyeonggi-do, Korea	2009-07-20	KC699761	KC699803
HCCN18719	*R. nigricans* > *R. nigricans*	*R. nigricans*	Gwacheon-si, Gyeonggi-do, Korea	2009-08-09	KC699766	KC699804
HCCN18875	*R. nigricans* > *R. nigricans*	*R. nigricans*	Gwacheon-si, Gyeonggi-do, Korea	2009-09-17	KC699752	KC699805
HCCN21521	*R. nigricans* > *R. nigricans*	*R. nigricans*	Uijeongbu-si, Gyeonggi-do, Korea	2011-07-27	KC699760	KC699806
HCCN22775	*R. nigricans* > *R. nigricans*	*R. nigricans*	Inje-gun, Gangwon-do, Korea	2012-07-30	KC699753	KC699807
HCCN22804	*R. nigricans* > *R. nigricans*	*R. nigricans*	Yeongju-si, Gyeongsangbuk-do, Korea	2012-07-30	KC699759	KC699808
SFC20120725-47	*R. subnigricans* > *R. nigricans*	*R. nigricans*	Boryeong-si, Chungcheongnam-do, Korea	2012-07-25	KC699754	KC699797
SFC20120905-08	*R. adusta* > *R. nigricans*	*R. nigricans*	Gwanak-gu, Seoul, Korea	2012-09-05	KC699758	KC699809
SFC20120907-22	*R. nigricans* > *R. nigricans*	*R. nigricans*	Jeju-si, Jeju-do, Korea	2012-09-22	KC699757	KC699810
SFC20120919-47	*R. nigricans* > *R. nigricans*	*R. nigricans*	Gongju-si, Chungcheongnam-do, Korea	2012-07-30	KC699756	KC699811
HCCN04639	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Jecheon-si, Chungcheongbuk-do, Korea	1992-08-01	KC699799	KC699782
HCCN14569	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Wonju-si, Gangwon-do, Korea	2006-09-05	KC699773	KC699784
HCCN15792	*R. nigricans* > *R. eccentrica*	*R. eccentrica*		2007-10-24	KC699774	KC699785
HCCN16959	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Gwacheon-si, Gyeonggi-do, Korea	2008-08-17	KC699775	KC699786
HCCN17040	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Cheonan-si, Chungcheongnam-do, Korea	2008-08-18	KC699781	KC699787
HCCN18824	*R. nigricans* > *R. eccentrica*	*R. eccentrica*	Cheonan-si, Chungcheongnam-do, Korea	2009-09-14	KC699768	KC699790
HCCN18746	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Gwacheon-si, Gyeonggi-do, Korea	2009-09-04	KC699769	KC699788
HCCN18766	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Gwacheon-si, Gyeonggi-do, Korea	2009-09-13	KC699767	KC699789
HCCN21694	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Gwacheon-si, Gyeonggi-do, Korea	2011-08-05	KC699780	KC699791
HCCN21739	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Yangju-si, Gyeonggi-do, Korea	2011-08-22	KC699770	KC699792
HCCN23685	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Gwacheon-si, Gyeonggi-do, Korea	2012-09-10	KC699778	KC699783
SFC20120807-09	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Jeju-si, Jeju-do, Korea	2012-08-07	KC699771	KC699793
SFC20120820-39	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Gongju-si, Chungcheongnam-do, Korea	2012-08-20	KC699776	KC699794
SFC20120821-80	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Boryeong-si, Chungcheongnam-do, Korea	2012-08-21	KC699772	KC699795
SFC20120925-03	*R. subnigricans* > *R. eccentrica*	*R. eccentrica*	Gwanak-gu, Seoul, Korea	2012-09-25	KC699777	KC699796
HCCNg289	*R. nigricans* > *Russula* sp.	*Russula* sp.	Wonju-si, Gangwon-do, Korea	1993-07-18		
HCCN16755	*R. nigricans* > *Russula* sp.	*Russula* sp.	Cheonan-si, Chungcheongnam-do, Korea	2008-08-01		
HCCN12317	*R. subnigricans* > *R. lepida*	*R. lepida*	Wonju-si, Gangwon-do, Korea	2004-08-17		

DNA extraction, PCR amplification, and sequencing

Tissue from fresh fruiting bodies and herbarium material were placed in 2× CTAB buffer and ground with a plastic pestle. Genomic DNA was extracted using a modified CTAB extraction protocol of Rogers and Bendich (1994). The ITS region was amplified using ITS5 and ITS4 (White *et al.*, 1990) and the LSU region was amplified using the forward primers Nig469F or Nig505F with reverse primers LR5 (Vilgalys and Hester, 1990) or Russ3R. Nig469F (GCATTAGTGGGGTC CCCTTTG), Nig505F (TTTCTACGTCTTGGGATTTGCT CTGT), and Russ3R (CCATTAYGCCARCATCCTAAGCA) were newly developed in this study. Each PCR reaction was performed on a C1000^{TM} thermal cycler (Bio-Rad, USA) using the Maxime PCR PreMix-StarTaq (iNtRON Biotechnology Inc., Korea) in a final volume of 20 µl containing 10 pmol of each primer and 1 µl of DNA. PCR conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 40 sec, 55°C for 40 sec and 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed through a 1% agarose gel stained with loading STAR (Dyne Bio, Korea) and purified using the Expin^{TM} PCR Purification Kit (GeneAll Biotechnology, Korea) according to the manufacturer's instructions. Sequencing was done in both forward and reverse directions for each sample using the PCR primers. The DNA sequencing was performed at Macrogen (Korea), using an ABI3700 automated DNA sequencer.

Phylogenetic analyses

Sequences were assembled, proofread, and edited using PHYDIT v3.1 (Chun 1995; available at http://plaza.snu.ac.kr/~ jchun/phydit/). The resulting consensus sequences were deposited in GenBank (accession numbers in Table 1). Outgroups and publicly available sequences for *R. nigricans* and *R. subnigricans* were obtained from GenBank and included in this study (Fig. 1). Different outgroups were selected for each marker based on phylogenetic analyses of the *Russula* genus (Miller and Buyck, 2002; Shimono *et al.*, 2004). Multiple sequence alignments were performed using the default settings of MAFFT v6.903b (Katoh *et al.*, 2002) and the L-INS-algorithm. Alignments were checked by eye and adjusted manually. We determined the most appropriate substitution models using the Bayesian information criterion in jModelTest 2.1.1 (Darriba *et al.*, 2012) for both markers. The K80 + G and K80 + G + I models were selected as the best-fit models for the ITS and LSU regions, respectively. Bayesian inference phylogenetic analyses were performed for each dataset using a Metropolis-coupled Markov chain Monte Carlo algorithm implemented in MrBayes v3.2.1 (Ronquist *et al.*, 2012) with four chains. Two independent searches with random starting trees were run for each dataset for 20 million generations, sampling every 1000th generation. Data from independent runs were combined after removing a 25% burn-in, estimated using Tracer v1.5 (Rambaut and Drummond, 2009). Final consensus trees were constructed using

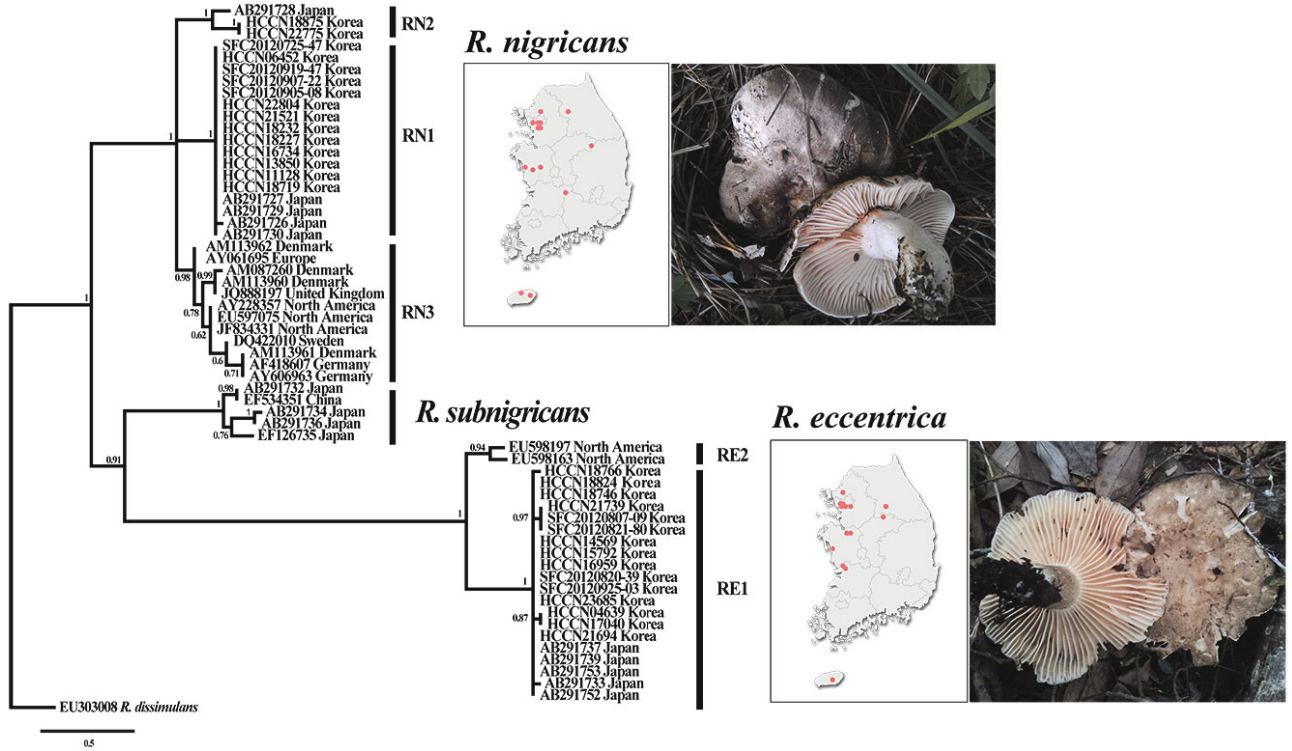

Fig. 1. Bayesian consensus tree (50% majority rule) inferred from sequences of rDNA internal transcribed spacer (ITS) region for 30 Korean *Russula* specimens in the subgenus *Compacta*. Branch support values are given as Bayesian posterior probabilities. The scale bar indicates the number of nucleotide substitutions per site. Each dot on map indicates geographical distribution of specimens used in this study.

the 50% majority rule, with posterior probabilities for each node.

Genetic diversity measures

Maximum intra- and inter-specific sequence dissimilarities of ITS and LSU regions were calculated for (i) each species, (ii) each species subgroup, and (iii) Korean samples in subgroups. For ITS, Korean samples of *R. nigricans* and *R. subnigricans* were supplemented with 17 *R. nigricans*, 10 *R. subnigricans*, and two *R. eccentrica* from GenBank. For LSU, seven *R. nigricans*, seven *R. subnigricans*, and one *R. eccentrica* were included from GenBank. In the case of ITS, we analyze and compare the genetic variation of three fragments (ITS1, ITS2, ITS1-5.8S-ITS2). Maximum intraspecific sequence dissimilarities were calculated using PHYDIT v3.1 (Chun, 1995).

Results

Data preparation

Of the *R. nigricans* and *R. subnigricans* initially selected for this study, 4/18 and 2/15 specimens, respectively, were determined to be incorrectly identified based on microscopic features. Among the four specimens misidentified as *R. nigricans*, two had the same morphology as the 13 *R. subnigricans* specimens while the other two were unidentifiable. Of two specimens misidentified as *R. subnigricans*, one specimen was amended to *R. nigricans* and the other specimen remained unknown. DNA data did not provide definitive results to identify the two specimens initially identified as *R. nigricans*, while the remaining unknown specimen was determined to be *R. lepida* (data not shown). Accordingly, these three specimens were excluded from remaining analyses in the study. Final datasets included 15 specimens each for *R. nigricans*

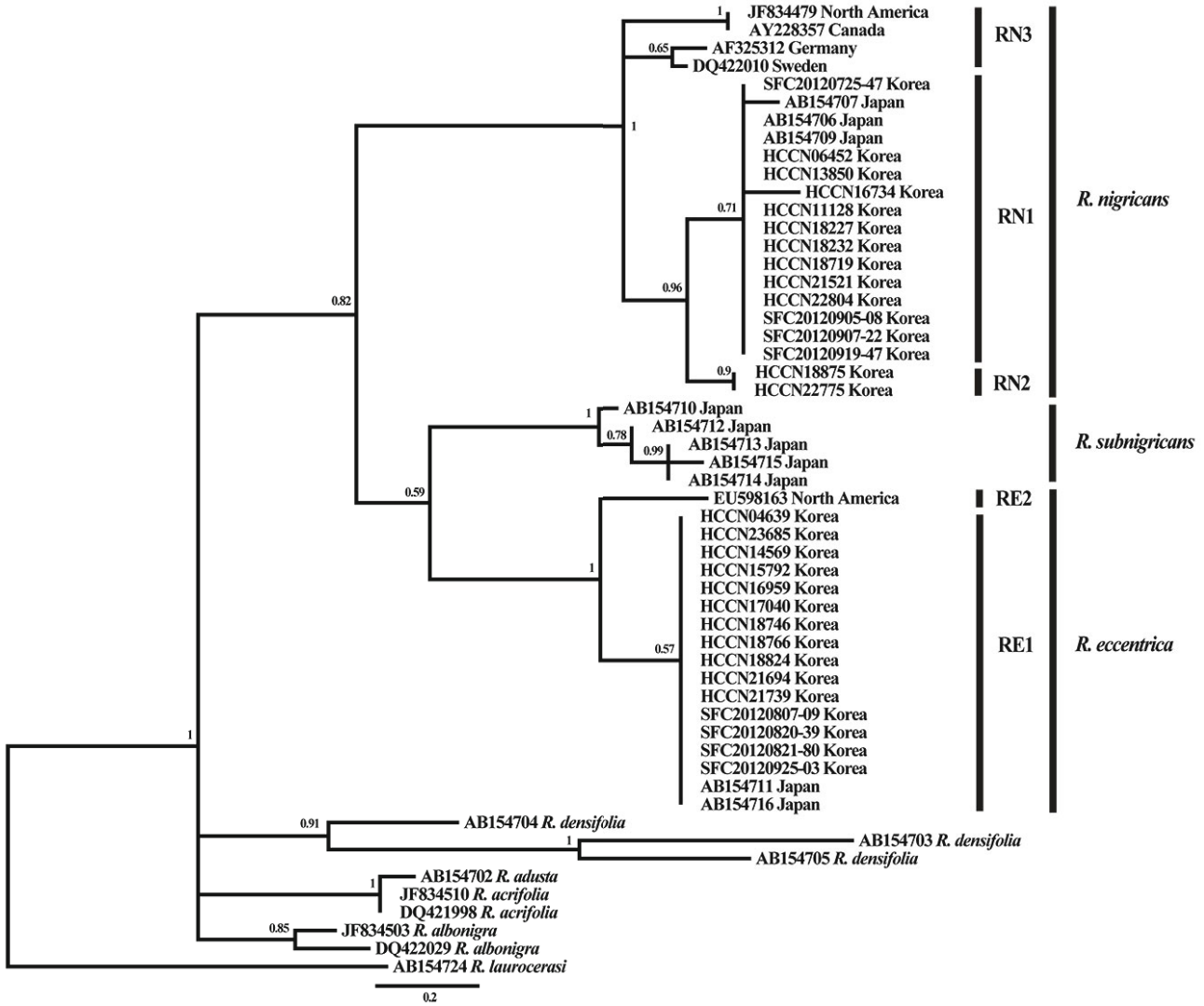

Fig. 2. Bayesian consensus tree (50% majority rule) inferred from sequences of 28S nuclear ribosomal large subunit rRNA gene (LSU) for 30 Korean *Russula* specimens in the subgenus *Compacta*. Branch support values are given as Bayesian posterior probabilities. The scale bar indicates the number of nucleotide substitutions per site.

Table 2. Morphological characteristics of *R. nigricans*, *R. eccentrica*, and *R. subnigricans*

Species	Reference	Basidia size (μm)	Spore size (μm)	Spore ornamentation (μm)
R. nigricans	Shaffer (1962)	40.0-49.0 × 7.3-9.3	6.3-7.9 × 5.3-6.8	0.1-0.3
	Romagnesi (1985)	45.0-55.0 × 6.5-7.5	6.5-8.5 × 5.7-7.0	no data
	Kränzlin (2005)	44.0-55.0 × 8.0-10.0	6.4-8.5 × 5.4-6.6	Up to 0.40
	This study	34.8-41.5 × 6.9-10.1	6.2-7.6 × 5.1-6.1	no data
R. eccentrica	Peck (1911)	no data	6-7	no data
	Bills (1985)	34.0-60.0 × 7.3-9.3	6.5-8.5 × 5.0-6.0	Up to 0.40
	This study	45.7-58.4 × 5.1-8.9	5.4-7.2 × 4.9-6.2	Up to 0.43
R. subnigricans	Hongo (1955)	38.0-50.0 × 8.0-9.5	7.0-9.0 × 6.0-7.0	no data
	Shaffer (1962)	41.0-51.0 × 7.3-9.3	6.1-9.0 × 6.7-8.0	0.1 or less

and *R. subnigricans* from Korea (Table 1) and downloaded sequences from GenBank.

Phylogenetic analyses of ITS and LSU regions

The ITS and LSU were successfully amplified and sequenced for all specimens, with approximate sizes of 650 bp and 560 bp, respectively. Phylogenetic relationships inferred from ITS (Fig. 1) and LSU (Fig. 2) were similar, resolving three major groups, generally referring to the three species. Differences between the two phylogenies were minor, being the result of the different sampling available from GenBank. *Russula nigricans* was found to be monophyletic (RN group), with genetic structure in the form of three subgroups. *Russula nigricans* shows some broad geographic variation, with two subgroups (RN1 and RN2) containing specimens exclusively from Korea and Japan, and the third subgroup (RN3) with representatives from Europe and North America (Figs. 1 and 2). *Russula subnigricans* and *R. eccentrica* samples forms two genetically distinct, strongly supported groups with posterior probabilities of 1.0 (Figs. 1 and 2). One group (RS group) contains *R. subnigricans* from Japan and China, including LSU sequences from the type specimen of *R. subnigricans* (AB154714-5, Shimono *et al*., 2004) (Figs. 1 and 2). The second group (RE group) contains the remaining Japanese *R. subnigricans* from GenBank, all Korean *R. subnigricans*, and North American *R. eccentrica*. Geographic structure is present in the RE group, with subgroup RE1 consisting of samples from Japan and Korea, while subgroup RE2 contains North American samples. These results raise the possibility that some *R. subnigricans* from Japan and all *R. subnigricans* from Korea are misclassified, and instead should be recognized as *R. eccentrica*. Due to this uncertainty, we refer to these samples as Korean '*R. subnigricans*' or Korean *R. eccentrica* from this time forth.

Morphological data

To explore a line of evidence independent from ITS and LSU, we compared the sizes of basidia and basidiospores with published data (Table 2). Size variations of the basidia and the basidiospores were observed in both species. Basidia and basidiospores of Korean *R. nigircans* are 34.8–41.5 × 6.5–10.1 μm and 6.2–7.6 × 5.1–6.1 μm, respectively. Basidia and basidiospore measurements of Korean '*R. subnigricans*' are 45.7–58.4 × 5.1–8.9 μm and 5.4–7.2 × 4.9–6.2 μm, respectively. Basidiospore ornamentation of Korean '*R. subnigricans*' is high (0.40 μm) compared published data for *R. subnigircans*, and was more similar to that of *R. eccentrica* (Table 2).

Intra-and interspecific variation of ITS and LSU regions

To investigate the genetic diversity in more detail, we calculated the pairwise dissimilarity for all samples (Table 3). Additionally, boxplots were made to compare intra- and inter-specific dissimilarity values and understand the resolutional power of ITS and LSU (Fig. 3). For most groups, ITS shows higher sequence dissimilarity compared to LSU. Comparing the three ITS regions, ITS1 tends to exhibit higher maximum sequence dissimilarity compared to ITS2 or ITS1-5.8S-ITS2. Of the three *R. nigricans* groups, RN3 shows the highest genetic diversity (ITS=1.34%, LSU=

Table 3. Maximum dissimilarity of the ITS and LSU for DNA barcoding of *R. nigricans* and *R. eccentrica*

Group/subgroup	Maximum dissimilarity (%)			
	ITS1	ITS2	ITS1-5.8S-ITS2	LSU
RN1 subgroup	0	0.46	0.17	0.59
RN2 subgroup	2.43	0.88	1.18	0
RN3 subgroup	2.39	1.78	1.34	1.38
RN group	4.78	5.29	3.36	1.58
Korean specimens in RN1 subgroup	0	0	0	0.4
Korean specimens in RN2 subgroup	0	0	0	0
Korean specimens in RN group	4.31	4.42	3.2	0.99
RE1 subgroup	0.98	0.44	0.34	0
RE2 subgroup	1.48	0.91	0.86	-
RE group	6.83	3.21	3.28	1.19
Korean specimens in RE1 subgroup	0.98	0	0.34	0

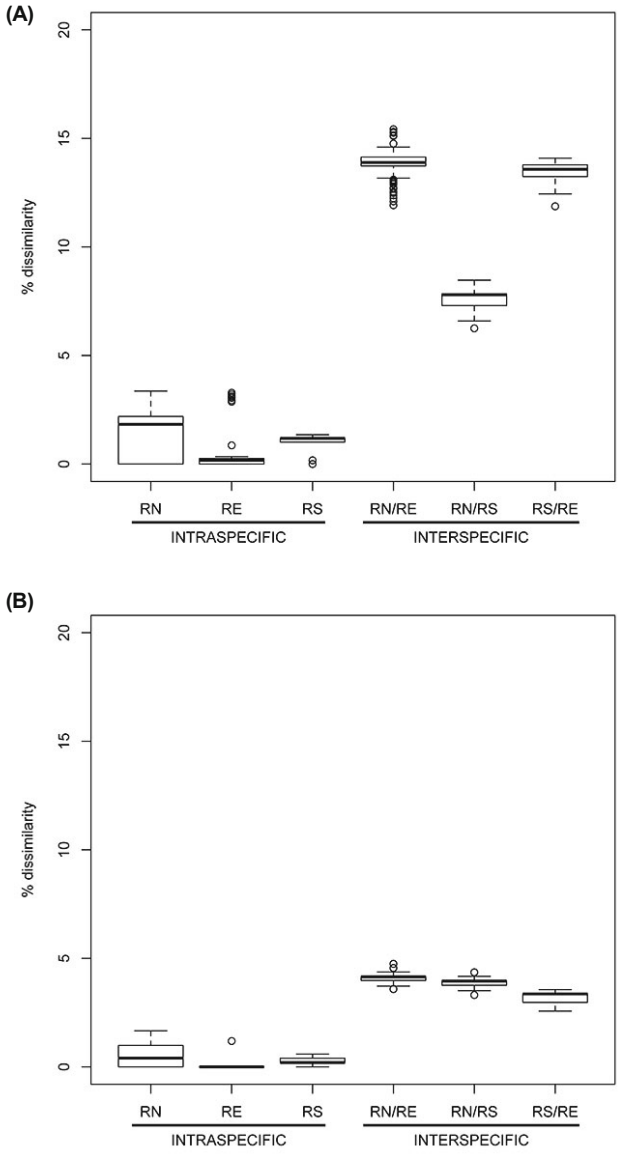

Fig. 3. Boxplots of intra- and inter-specific variation of three *Russula* species in the subgenus *Compacta*. Pair-wise comparisons are made of % dissimilarity of the barcoding markers (A) Internal transcribed spacer (ITS) and (B) 28S nuclear ribosomal large subunit rRNA gene (LSU). RN, *R. nigricans*; RE, *R. eccentrica*; RS, *R. subnigricans*.

1.38%). Korean *R. nigricans* (ITS=3.2%, LSU=0.99%) contain levels of genetic diversity similar to the total RN group (ITS=3.36%, LSU=1.58%). In contrast, Korean *R. eccentrica* exhibit much lower genetic diversity (ITS=0.34%, LSU=0%) compared to the total RE group (ITS=3.28%, LSU=1.19%).

Discussion

In Korea, *R. nigricans* and '*R. subnigricans*' are commonly found during the summer and fall. These two species are important to the ecosystem, as they have ectomycorrhizal associations with numerous hardwood and coniferous species (Bok and Shin, 1986; Park and Lee, 2011). Our molecular study highlights a possible species misclassification, with Korean '*R. subnigricans*' being more closely related to *R. eccentrica* from North America than *R. subnigricans* from Japan. A second line of evidence, habitat-type of collected samples in Korea, corroborated this misclassification. *Russula subnigricans* can be found associated with broad-leaved evergreen trees (Hongo, 1955; Shaffer, 1962), while *R. eccentrica* is usually in deciduous forests (Shaffer, 1962). Korean '*R. subnigricans*' were collected from deciduous forests in the central region of the Korean peninsula (Fig. 1), matching descriptions of *R. eccentrica* habitat. Broad-leaved evergreen forest habitat is present and restricted to the southern area of Korea (Yun *et al.*, 2011), and thorough surveys of this region need to be undertaken to determine if true *R. subnigricans* exists in Korea. The results from morphological features, phylogenetic analysis, and habitat data all indicate that '*R. subnigricans*' from Korea (and Japan) have been incorrectly identified and are in fact *R. eccentrica*. *Russula subnigricans* is notorious for mushroom poisoning (Imazeki and Hongo, 1989; Lee *et al.*, 2001), and the misidentification of a non-poisonous mushroom (*R. eccentrica*) as a poisonous one (*R. subnigricans*) is expected to go undetected longer as it is not fatal, while the converse situation would immediately be known. This report represents the first record of *R. eccentrica* in Asia, and the identity of the two representative *Russula* subgenus *Compacta* species in Korea should now be known as *R. nigricans* and *R. eccentrica*. Due to this misidentification, results from previous research on *R. subnigricans*, such as on its chemical compounds (Kwon and Bae, 2010) and fungal diversity (Bok and Shin, 1986) need to be carefully reconsidered.

Basidia of *R. nigricans* collected in Korea were slightly shorter than those of previously reported from Europe and North America (Table 2). The measurements of Korean *R. eccentrica* differed compared to North American individuals, as basidia were longer and basidiospores were smaller (Bills, 1985). These difference may be an artifact of imprecise measurements using old microscopes or a true difference resulting from geological separation and genetic divergence (Vasaitis *et al.*, 2009; Kleine *et al.*, 2013).

Different levels of variation in ITS are not uncommon, as some species show low intraspecific ITS variation (*Boletus edulis* - 0.3%, *Amantia muscaria* - 0.9%), while others show relatively high variation (*Pisolithus tinctorius* - 24.7%, *Tricholoma robustum* - 35.3%) (Nilsson *et al.*, 2008). Due to this, intraspecific variation of molecular markers should be determined before performing phylogenetic analysis or barcoding. Intraspecific ITS sequence variations of *R. nigricans* (3.36%) and *R. eccentrica* (3.28%) are similar to those reported from other basidiomycetes species (Anderson and Stasovski, 1992; Vasiliauskas *et al.*, 1999; James *et al.*, 2001; Lickey *et al.*, 2002; Nilsson *et al.*, 2008). For Korean samples, the patterns for these two species are quite different, with Korean *R. nigricans* capturing a majority of this genetic variation (3.2%) and Korean *R. eccentrica* showing very low variation (0.34%). This can be explained by the fact that Korean *R. nigricans* samples are present in two of the three subgroups, which accounts for a majority of the variation. For *R. eccentrica*, it is possible that colonization and subse-

quent isolation in Korea and Japan happened relatively recently, resulting in low genetic variation. However, more sampling in North America and more surveys throughout Asia are needed to clarify the distributions and genetic diversity of *R. eccentrica* and *R. subnigricans*.

Intra- and inter-specific pairwise sequence comparisons among all samples used in this study were performed for each of the two datasets. The magnitude of the pairwise dissimilarity varied by locus. In comparing the sequence variation of the three *Russula* species, we see a clear pattern of non-overlapping intra- and interspecific variation for both ITS and LSU, with ITS having a much larger gap (Fig. 3). In fact, interspecific variation is more than one order of magnitude greater than intraspecific variation in ITS. For these three species, ITS performs much better in distinguishing species, but LSU is also effective and should still be considered when accounting for the ease in amplifying, sequencing, and aligning. In all, these results indicate that ITS and LSU have potential use as barcoding markers for Korean *Russula* species.

The ITS marker consists of two ITS regions (ITS1, ITS2) that surround 5.8S rRNA. Some researchers prefer using only ITS1 as a marker due to high sequence variation (Chen *et al.*, 2001; Hinrikson *et al.*, 2005). We compared sequence variation in individual ITS1 and ITS2 regions to understand whether genetic variation in the ITS regions is comparable. Our results show that even within the same species, some groups have higher variation in ITS2, while others have higher variation in ITS1 (Table 3). For example, in *R. nigricans*, two of the subgroups (RN2, RN3) show higher variation in ITS1, however the third group (RN1) is extreme in showing no variation at ITS1 (Table 3). To avoid potential problems of measuring genetic diversity with ITS, we suggest using the complete ITS1-5.8S-ITS2 region. For LSU, intraspecific genetic variation is generally low, and lower for most groups when compared to ITS. This is expected, as ITS is non-functional and its rate of evolutions should be higher than the more conserved LSU. However, there are some exceptions, as seen in subgroups RN1, RN3, and Korean RN1. In these cases, variation is generally low, but slightly higher in LSU than ITS. These differences can be attributed to two factors: (i) different sampling available in GenBank (RN1 and RN3 subgroups), and (ii) ITS and LSU markers are 500–600 bp and slight differences in variation are driven by 1–2 base pair changes (RN1 and Korean RN1 subgroups). Despite the overall lower variation in LSU, our results show that it can still be a useful genetic marker, as its conserved nature reduces ambiguity in sequence alignment and provides the comparable phylogenetic results to ITS (Figs. 1 and 2).

In our study, both ITS and LSU markers showed low intra- and high interspecific variation, allowing for the clear discrimination of three *Russula* species in the subgenus *Compacta*, as well as of broad geographic patterns (i.e. Asia versus Europe/North America) within them. In fact, it is molecular data that lead towards identifying a misclassification of *Russula* in Korea. What was previously believed to be *R. subnigricans* in Korea is actually *R. eccentrica*. Molecular results were subsequently corroborated by morphological and habitat data. The misidentification of Korean *R. eccentrica* was unexpected, but reflects the difficulty in distinguishing *Russula* species based on morphology. Although we did not find *R. subnigricans* in Korea, it is still possible that this species does exist. The distribution of *R. subnigricans* includes Japan and Mainland China, which geographically surround Korea. The next step will be to thoroughly survey broad-leaved evergreen tree habitats in southern South Korea for the presence of *R. subnigricans*. *Russula* species have important roles in the environment in mutualistic relationships with plants, and ITS and LSU markers will be powerful tools in elucidating the ecology and evolution of *Russula* in Korea and worldwide.

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