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Research Article

Delimitation of *Russula* Subgenus *Amoenula* in Korea Using Three Molecular Markers

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Abstract Distinguishing individual *Russula* species has been difficult due to extensive phenotypic plasticity and obscure morphological and anatomical discontinuities. Due to highly similar macroscopic features, such as the presence of a red-cap, species identification within the *Russula* subgenus *Amoenula* is particularly difficult. Three species of the subgenus *Amoneula* have been reported in Korea. We used a combination of morphology and three molecular markers, the internal transcribed spacer (ITS), 28S nuclear ribosomal large subunit (LSU), and RNA polymerase II gene (RPB2), for identification and study of the genetic diversity of *Russula* subgenus *Amoenula* in Korea. We identified only two species in Korea (*R. mariae* and *R. violeipes*); these two species were indistinguishable according to morphology and LSU, but were found to be reciprocally monophyletic species using ITS and RPB2. The markers, ITS, LSU, and RPB2, have been tested in the past for use as DNA barcoding markers, and findings of our study suggest that ITS and RPB2 had the best performance for the *Russula* subgenus *Amoneula*.

Keywords DNA barcoding, Internal transcribed spacer, RNA polymerase II gene, *Russula mariae*, *Russula violeipes*, 28S nuclear ribosomal large subunit

The genus *Russula* makes a significant contribution to plant host taxa as an ectomycorrhizal symbiont, and is found across a wide range of habitats from tropical to arctic ecosystems [1, 2]. In addition to their ecological roles, many *Russula* species are important food sources for insects [3] and humans [4].

The genus *Russula* is a highly diverse group in the fungal division Basidiomycota. To date, approximately 750 *Russula* species have been reported worldwide [5]. The *Russula* subgenus *Amoenula* Sarnari [6], includes four species (*R. amoena* Quélet [type], *R. amoenicolor* Romagnesi, *R. mariae* Peck, and *R. violeipes* Quélet), and was later amended to include a fifth species (*R. mukteshwarica* [7]).

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Amoenula is characterized by long hyphal ends, absence of dermatocystidia of pileipellis, and bayonet-like hairs in the epicuticular [6]. A phylogenetic analysis based on the internal transcribed spacer (ITS) recovered a well-supported grouping of the two *Amoenula* representatives (*R. amoenicolor*, *R. violeipes*) [8]. Another study based on the 28S nuclear ribosomal large subunit (LSU) showed that *R. amoena*, *R. violeipes*, and *R. bella* Hongo (synonym *R. mariae*) of the subgenus *Amoenula* formed a monophyletic group that included *R. mairei* from the subgenus *Russula* [9].

The individual species within the subgenus *Amoenula* differ in their spore print coloration and the presence and shape of specialized cells in the pileipellis [6]. Despite these distinguishing morphological characteristics, species identification is difficult, as spore prints require fresh specimens and their color is dependent on spore density, while observation of differences in micro-morphological features can be difficult.

ITS, LSU, and RNA polymerase II (RPB2) are three commonly used molecular markers for fungal phylogenetics. The ITS region has the most clearly defined barcode gap between inter- and intraspecific variation across a broad range of fungi; therefore, its adoption as the fungal barcode marker to the Consortium for the Barcode of Life has been formally suggested [10]. The LSU marker is normally more conserved and easier to align than ITS, while RPB2 is also highly conserved among eukaryotes and has proven to be useful for systematics and identification of fungal species [11].

Table 1. Specimens used in this study

Collection No.	Species history	Final ID	Site	Date	Accession No.		
					ITS	LSU	RPB2
HCCN16834	*R. mariae* > *R. mariae*	*R. mariae*	Wonju-si, Gangwon-do, Korea	Aug 6, 2008	KF361759	KF361809	KF361709
HCCN18725	*R. mariae* > *R. mariae*	*R. mariae*	Gwacheon-si, Gyeonggi-do, Korea	Aug 14, 2009	KF361760	KF361810	KF361710
HCCN18800	*R. violeiceps* > R. mariae	*R. mariae*	Gwacheon-si, Gyeonggi-do, Korea	Sep 14, 2009	KF361761	KF361811	KF361711
HCCN19111	*Russula* sp. > *R. mariae*	*R. mariae*	Cheonan-si, Chungcheongnam-do, Korea	Sep 30, 2009	KF361762	KF361812	KF361712
HCC 20514	*R. mariae* > *R. mariae*	*R. mariae*	Gwanak-gu, Seoul, Korea	Sep 17, 2010	KF361763	KF361813	KF361713
HCCN21685	*R. violeiceps* > *R. mariae*	*R. mariae*	Gwacheon-si, Gyeonggi-do, Korea	Aug 5, 2011	KF361764	KF361814	KF361714
HCCN22939	*Russula* sp. > *R. mariae*	*R. mariae*	Seongnam-si, Gyeonggi-do, Korea	Aug 16, 2012	KF361765	KF361815	KF361715
HCCN23016	*Russula* sp. > *R. mariae*	*R. mariae*	Guri-si, Gyeonggi-do, Korea	Aug 17, 2012	KF361766	KF361816	KF361716
HCCN23379	*Russula* sp. > *R. mariae*	*R. mariae*	Wonju-si, Gangwon-do, Korea	Sep 02, 2012	KF361767	KF361817	KF361717
SFC2012-0724-23	*R. rosea* > *R. mariae*	*R. mariae*	Seosan-si, Chungcheongnam-do, Korea	Jul 24, 2012	KF361768	KF361818	KF361718
SFC2012-0725-45	*R. violeipes* > *R. mariae*	*R. mariae*	Boryeong-si, Chungcheongnam-do, Korea	Jul 25, 2012	KF361769	KF361819	KF361719
SFC2012-0726-37	*R. violeipes* > *R. mariae*	*R. mariae*	Gongju-si, Chungcheongnam-do, Korea	Jul 6, 2012	KF361770	KF361820	KF361720
SFC2012-0804-09	*R. cyanoxantha* > *R. mariae*	*R. mariae*	Ganghwa-gun, Incheon, Korea	Aug 04, 2012	KF361771	KF361821	KF361721
SFC2012-0821-39	*R. alboareolata* > *R. mariae*	*R. mariae*	Boryeong-si, Chungcheongnam-do, Korea	Aug 21, 2012	KF361772	KF361822	KF361722
SFC2012-0825-02	*R. mariae* > *R. mariae*	*R. mariae*	Gwanak-gu, Seoul, Korea	Aug 25, 2012	KF361773	KF361823	KF361723
SFC2012-0831-04	*R. violeipes* > *R. mariae*	*R. mariae*	Gwanak-gu, Seoul, Korea	Aug 31, 2012	KF361774	KF361824	KF361724
SFC2012-0915-10	*Russula* sp. > *R. mariae*	*R. mariae*	Gwanak-gu, Seoul, Korea	Sep 10, 2012	KF361775	KF361825	KF361725
SFC2012-0919-37	*Russula* sp. > *R. mariae*	*R. mariae*	Gwanak-gu, Seoul, Korea	Sep 19, 2012	KF361776	KF361826	KF361726
SFC2012-0922-07	*R. emetica* > *R. mariae*	*R. mariae*	Dongdaemun-gu, Seoul, Korea	Sep 22, 2012	KF361777	KF361827	KF361727
SFC2012-0922-08	*R. rosacea* > *R. mariae*	*R. mariae*	Dongdaemun-gu, Seoul, Korea	Sep 22, 2012	KF361778	KF361828	KF361728
HCCN10627	*R. mariae* > *R. violeipes*	*R. violeipes*	Suwon-si, Gyeonggi-do, Korea	Aug 19, 2002	KF361779	KF361829	KF361729
HCCN11773	*R. mariae* > *R. violeipes*	*R. violeipes*	Seogwipo-si, Jeju-do, Korea	Oct 2, 2003	KF361780	KF361830	KF361730
HCCN16435	*R. rosacea* > *R. violeipes*	*R. violeipes*	Cheonan-si, Chungcheongnam-do, Korea	Jul 5, 2008	KF361781	KF361831	KF361731
HCCN16459	*R. rosacea* > *R. violeipes*	*R. violeipes*	Cheonan-si, Chungcheongnam-do, Korea	Jul 5, 2008	KF361782	KF361832	KF361732
HCCN16735	*R. mariae* > *R. violeipes*	*R. violeipes*	Suwon-si, Gyeonggi-do, Korea	Aug 1, 2008	KF361783	KF361833	KF361733
HCCN16818	*R. mariae* > *R. violeipes*	*R. violeipes*	Wonju-si, Gangwon-do, Korea	Aug 6, 2008	KF361784	KF361834	KF361734
HCCN20149	*R. mariae* > *R. violeipes*	*R. violeipes*	Suwon-si, Gyeonggi-do, Korea	Aug 19, 2010	KF361785	KF361835	KF361735
HCCN21655	*Russula* sp. > *R. violeipes*	*R. violeipes*	Suwon-si, Gyeonggi-do, Korea	Aug 1, 2011	KF361786	KF361836	KF361736
HCCN21858	*R. mariae* > *R. violeipes*	*R. violeipes*	Yeongju-si, Gyeongbuk, Korea	Aug 22, 2011	KF361787	KF361837	KF361737
HCCN22654	*R. mariae* > *R. violeipes*	*R. violeipes*	Wonju-si, Gangwon-do, Korea	Jul 22, 2012	KF361788	KF361838	KF361738
HCCN23030	*R. mariae* > *R. violeipes*	*R. violeipes*	Guri-si, Gyeonggi-do, Korea	Aug 17, 2012	KF361789	KF361839	KF361739
SFC2012-0704-32	*R. mariae* > *R. violeipes*	*R. violeipes*	Inje-gun, Gangwon-do, Korea	Jul 4, 2012	KF361790	KF361840	KF361740
SFC20120719-04	*R. amoena* > *R. violeipes*	*R. violeipes*	Gyeongju-si,, Gyeongbuk, Korea	Jul 19, 2012	KF361791	KF361841	KF361741
SFC2012-0726-33	*Russula* sp. > *R. violeipes*	*R. violeipes*	Gongju-si, Chungcheongnam-do, Korea	Jul 26, 2012	KF361792	KF361842	KF361742
SFC2012-0727-05	*R. rosea* > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Jul 27, 2012	KF361793	KF361843	KF361743
SFC2012-0731-01	*R. emetica* > *R. violeipes*	*R. violeipes*	Yuseong-gu, Daejeon, Korea	Jul 31, 2012	KF361794	KF361844	KF361744
SFC2012-0731-02	*R. emetica* > *R. violeipes*	*R. violeipes*	Yuseong-gu, Daejeon, Korea	Jul 31, 2012	KF361795	KF361845	KF361745
SFC2012-0731-06	*R. mariae* > *R. violeipes*	*R. violeipes*	Yuseong-gu, Daejeon, Korea	Jul 31, 2012	KF361796	KF361846	KF361746

Table 1. Continued

Collection No.	Species history	Final ID	Site	Date	Accession No.		
					ITS	LSU	RPB2
HCCN16818	*R. mariae* > *R. violeipes*	*R. violeipes*	Wonju-si, Gangwon-do, Korea	Aug 6, 2008	KF361784	KF361834	KF361734
HCCN20149	*R. mariae* > *R. violeipes*	*R. violeipes*	Suwon-si, Gyeonggi-do, Korea	Aug 19, 2010	KF361785	KF361835	KF361735
HCCN21655	*Russula* sp. > *R. violeipes*	*R. violeipes*	Suwon-si, Gyeonggi-do, Korea	Aug 1, 2011	KF361786	KF361836	KF361736
HCCN21858	*R. mariae* > *R. violeipes*	*R. violeipes*	Yeongju-si, Gyeongbuk, Korea	Aug 22, 2011	KF361787	KF361837	KF361737
HCCN22654	*R. mariae* > *R. violeipes*	*R. violeipes*	Wonju-si, Gangwon-do, Korea	Jul 22, 2012	KF361788	KF361838	KF361738
HCCN23030	*R. mariae* > *R. violeipes*	*R. violeipes*	Guri-si, Gyeonggi-do, Korea	Aug 17, 2012	KF361789	KF361839	KF361739
SFC2012-0704-32	*R. mariae* > *R. violeipes*	*R. violeipes*	Inje-gun, Gangwon-do, Korea	Jul 4, 2012	KF361790	KF361840	KF361740
SFC20120719-04	*R. amoena* > *R. violeipes*	*R. violeipes*	Gyeongju-si, Gyeongbuk, Korea	Jul 19, 2012	KF361791	KF361841	KF361741
SFC2012-0726-33	*Russula* sp. > *R. violeipes*	*R. violeipes*	Gongju-si, Chungcheongnam-do, Korea	Jul 26, 2012	KF361792	KF361842	KF361742
SFC2012-0727-05	R. rosea > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Jul 27, 2012	KF361793	KF361843	KF361743
SFC2012-0731-01	*R. emetica* > *R. violeipes*	*R. violeipes*	Yuseong-gu, Daejeon, Korea	Jul 31, 2012	KF361794	KF361844	KF361744
SFC2012-0731-02	*R. emetica* > *R. violeipes*	*R. violeipes*	Yuseong-gu, Daejeon, Korea	Jul 31, 2012	KF361795	KF361845	KF361745
SFC2012-0731-06	*R. mariae* > *R. violeipes*	*R. violeipes*	Yuseong-gu, Daejeon, Korea	Jul 31, 2012	KF361796	KF361846	KF361746
SFC2012-0814-23	*R. mariae* > *R. violeipes*	*R. violeipes*	Ulleung-gun, Gyeongsangbuk-do, Korea	Aug 14, 2012	KF361797	KF361847	KF361747
SFC2012-0817-08	*Russula* sp. > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Aug 17, 2012	KF361798	KF361848	KF361748
SFC2012-0817-09	*Russula* sp. > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Aug 17, 2012	KF361799	KF361849	KF361749
SFC2012-0821-71	*Russula* sp. > *R. violeipes*	*R. violeipes*	Boryeong-si, Chungcheongnam-do, Korea	Aug 21, 2012	KF361800	KF361850	KF361750
SFC2012-0905-07	*R. mariae* > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Sep 5, 2012	KF361801	KF361851	KF361751
SFC2012-0915-16	*R. rosea* > *R. violeipes*	*R. violeipes*	Yeongdeungpo-gu, Seoul, Korea	Sep 15, 2012	KF361802	KF361852	KF361752
SFC2012-0919-27	*R. violeipes* > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Sep 19, 2012	KF361803	KF361853	KF361753
SFC2012-0919-29	*R. cyanoxantha* > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Sep 19, 2012	KF361804	KF361854	KF361754
SFC2012-0919-51	*Russula* sp. > *R. violeipes*	*R. violeipes*	Gongju-si, Chungcheongnam-do, Korea	Sep 19, 2012	KF361805	KF361855	KF361755
SFC2012-0929-05	*Russula* sp. > *R. violeipes*	*R. violeipes*	Seo-gu, Daejeon, Korea	Sep 29, 2012	KF361806	KF361856	KF361756
SFC2012-1005-09	*R. violeipes* > *R. violeipes*	*R. violeipes*	Gwanak-gu, Seoul, Korea	Oct 05, 2012	KF361807	KF361857	KF361757
SFC2012-1010-06	*Russula* sp. > *R. violeipes*	*R. violeipes*	Seosan-si, Chungcheongnam-do, Korea	Oct 10, 2012	KF361808	KF361858	KF361758
HCCN16830	*R. mariae* > *Russula* sp.	*Russula* sp.	Wonju-si, Gangwon-do, Korea	Aug 6, 2008			
HCCN16836	*R. mariae* > *Russula* sp.	*Russula* sp.	Wonju-si, Gangwon-do, Korea	Aug 6, 2008			
SFC20120726-15	*R. mariae* > *Russula* sp.	*Russula* sp.	Gongju-si, Chungcheongnam-do, Korea	Jul 26, 2012			
SFC20120807-06	*R. mariae* > *Russula* sp.	*Russula* sp.	Seogwipo-si, Jeju-do, Korea	Aug 7, 2012			
SFC20120807-27	*R. mariae* > *Russula* sp.	*Russula* sp.	Seogwipo-si, Jeju-do, Korea	Aug 7, 2012			
SFC20120907-07	*R. mariae* > *Russula* sp.	*Russula* sp.	Seogwipo-si, Jeju-do, Korea	Sep 7, 2012			
SFC20120907-21	*R. mariae* > *Russula* sp.	*Russula* sp.	Seogwipo-si, Jeju-do, Korea	Sep 7, 2012			
SFC20120925-10	*R. mariae* > *R. lepida*	*R. lepida*	Gwanak-gu, Seoul, Korea	Sep 25, 2012			
HCCN16625	*R. violeipes* > *R. cyanoxantha*	*R. cyanoxantha*	Pyeongchang-gun, Gangwon-do, Korea	Jul 25, 2008			
HCCN16758	*R. violeipes* > *R. olivacea*	*R. olivacea*	Cheonan-si, Chungcheongnam-do, Korea	Aug 1, 2008			
HCCN17043	*R. violeipes* > *R. olivacea*	*R. olivacea*	Cheonan-si, Chungcheongnam-do, Korea	Aug 18, 2008			
SFC20120807-28	*R. violeipes* > *R. lepida*	*R. lepida*	Seogwipo-si, Jeju-do, Korea	Aug 7, 2012			

HCCN, Herbarium Conservation Center of the National Academy of Agricultural Sciences; SFC, Seoul National University Fungus Collection.

Three species of the *Russula* subgenus *Amoenula*, *R. amoena, R. mariae,* and *R. violeipes,* have been reported from Korea [12]. Each of these has a red-cap and overlapping measurements of basidiospore and basidia size, making morphological identification difficult. Through the *Russula* barcode project, a study initiated by the National Institute of Biological Resources (NIBR) of Korea in an effort to understand the diversity of *Russula* in Korea [13] based on ITS, we found that there were many misidentifications in *Russula*, particularly in the subgenus *Amoenula*. In our study, we clarify the status of *Russula* species of the subgenus *Amoenula* in Korea by analysis of microscopic features and DNA sequences of specimens collected across South Korea. In addition, we evaluate the utility of the three commonly used molecular markers for fungi (ITS, LSU, and RPB2) for species identification in this group.

MATERIALS AND METHODS

Materials studied. Fruiting body samples of *Russula* were collected from locations throughout South Korea between 1985~2012 (Table 1, Figs. 1 and 2); dried specimens were deposited in the Herbarium Conservation Center of the National Academy of Agricultural Sciences (HCCN) or Seoul National University Fungus Collection (SFC). These specimens were identified using field guides [14], a photographic illustration website (http://www.mtsn.tn.it/russulales-news/), and a light microscope (Nikon 80i; Nikon, Tokyo, Japan).

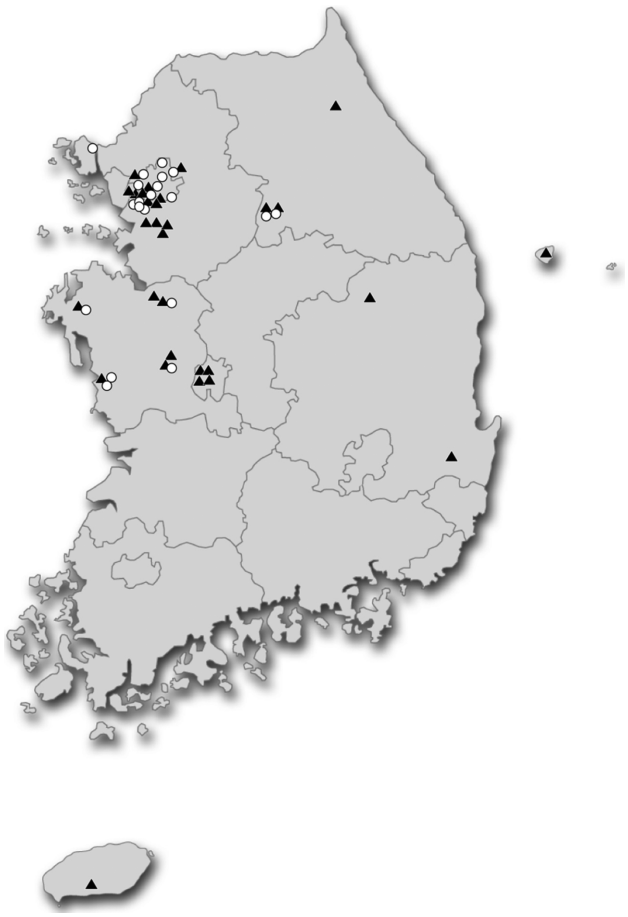

Fig. 1. Geographic distribution of *Russula* specimens used in this study. Black triangles represent sampling sites for *R. violeipes*, and white circles represent sampling sites for *R. mariae*.

A total of 62 specimens initially identified as *R. mariae* (24 specimens), *R. violeipes* (11 specimens), *R. amoena* (1 specimen), and other morphologically similar *Russula* species (26 specimens) were examined in this study (Table 1). Each specimen was re-examined for verification of species identity following three steps: i) grouping based on size of basidia and basidiospore, ii) performance of sequence analysis, and iii) comparison of morphological details to published data.

DNA extraction, PCR amplification and sequencing. Genomic DNA from fresh or dried tissues was extracted using the modified CTAB extraction protocol of Rogers and Bendich [15]. The ITS region was amplified using primers ITS1F or ITS5 and ITS4 [16], the LSU region was amplified using primers ITS3 and LR5 [17], with a newly designed primer—Russ3R (CCATTAYGCCARCATCCTA-AGCA), and RPB2 was amplified using primers fRPB2-5F or bRPB2-6F and bRPB2-7R or bRPB2-7.1R [11]. PCR reactions were performed on a C1000 thermal cycler (Bio-Rad, Hercules, CA, USA) using the Maxime PCR PreMix-StarTaq (Intron Biotechnology Inc., Seoul, Korea) in a final volume of 20 μL containing 10 pmol of each primer and 1 μL of DNA. The PCR conditions used 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, Seoul, Korea) and purified using the Expin PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Sequencing was performed in both forward and reverse directions for each sample using the PCR primers. DNA sequencing was performed at the DNA synthesis and Sequencing Facility, Macrogen (Seoul, Korea), using an ABI3700 automated DNA sequencer (PE Applied Biosystems, Foster City, CA, USA).

Sequence analysis. Sequences were assembled, proofread, and edited using PHYDIT v3.1 [18]. The resulting consensus sequences were deposited in GenBank (accession numbers are shown in Table 1). Outgroups and publicly available sequences for *R. mariae* and *R. violeipes* were obtained from GenBank. Different outgroups were selected for each marker based on previous phylogenetic studies of the *Russula* genus [8, 9]. Multiple alignments were performed using the default settings of MAFFT v6.903b [19] and the L-INS-algorithm [19]. Ambiguously aligned positions were checked manually. We determined the most appropriate substitution model using the Bayesian information criterion

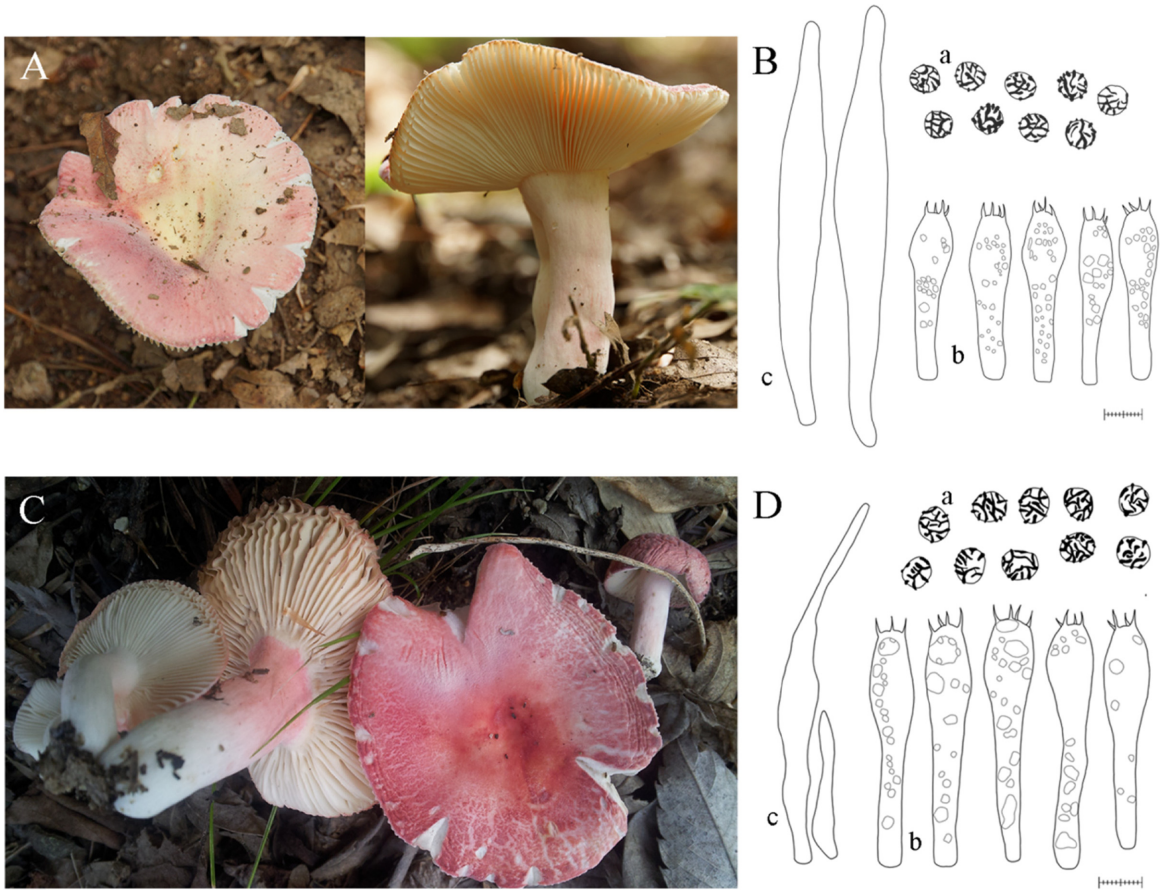

Fig. 2. Morphological features of *Russula mariae* (A, B) and *R. violeipes* (C, D) from Korea. a, basidiospores; b, basidia; c, pleurocystidia (scale bars: B, D = 10 μm).

in jModelTest 2.1.1 [20] for the three markers. The K80 + I, K80 + G, and K80 + G + I models were selected as the best-fit models for ITS, LSU, and RPB2, 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 [21] with four chains. Two independent searches with random starting trees were run for each dataset for 20 million generations, sampling every 100th generation. Tracer v1.5 [22] was used in determining whether runs reached convergence, and 25% of the data were removed as burn-in. Final consensus trees were constructed using the 50% majority rule, with posterior probabilities for each node.

Genetic diversity measures. In order to assess the variation of base substitution among sites, the number of steps per five successive bases was estimated for the most-parsimonious trees based on each of the three markers using MacClade 4.0 [23]. To determine whether ITS, LSU, and RPB2 are adequate barcoding markers for these species, maximum intraspecific sequence dissimilarity and intra- and interspecific distances were calculated for 1) each species and 2) Korean samples in each species. Maximum intraspecific sequence dissimilarities were calculated

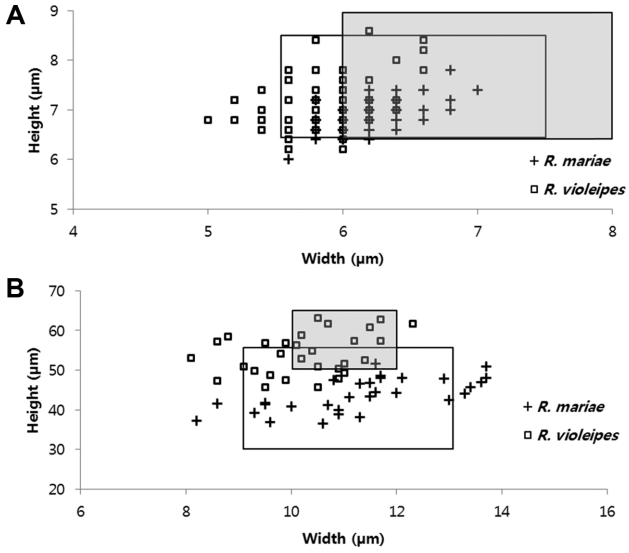

Fig. 3. Size variation of basidiospore (A) and basidia (B) of *Russula mariae* (RM) and *R. violeipes* (RV) from Korea. Plus (+) marks represent *R. mariae* from Korea and square (–) marks represent *R. violeipes* from Korea. White boxes represent previously published data on *R. mariae* from Bill and Miller [24] and grey boxes represent previously published data on *R. violeipes* from Romagnesi [25].

using PHYDIT v3.1 [18], while intra- and interspecific distances were calculated using the Kimura 2-parameter model [26] in MEGA5 [27].

RESULTS

Dataset preparation. Specimens initially identified as *R. mariae* (24 specimens), *R. violeipes* (11 specimens), *R. amoena* (1 specimen), and other morphologically similar *Russula* species (26 specimens) collected from different geographic locations in Korea were selected for this study (Fig. 1). We used a combination of microscopic features and ITS sequence analysis in order to determine which specimens had been misclassified. Of the 24 specimens initially identified as *R. mariae*, four were determined to be true *R. mariae*, 12 as *R. violeipes*, one as *R. lepida*, and seven unknown. Of the 11 specimens initially identified as *R. violeipes*, two were determined to be true *R. violeipes*, five as *R. mariae*, two as *R. olivacea*, and one each of *R. cyanoxantha* and *R. lepida*. Based on morphological and sequence analysis, the single *R. amoena* included in this study was shown to be a misidentified *R. violeipes*. Of the 26 individuals previously identified as other *Russula* species based on macro-morphological characteristics, 11 were re-identified as *R. mariae* and 15 as *R. violeipes*. The final dataset for this study included 20 *R. mariae* and 30 *R. violeipes* from Korea, and zero *R. amoena* (Table 1).

Morphological data. In order to explore morphological characteristics of *R. mariae* and *R. violeipes*, we compared sizes of basidiospore and basidia (Figs. 2 and 3). Basidiospores and basidia of Korean *R. mariae* (RM group) measured 6.0~7.8 × 5.6~7.0 μm and 36.5~51.7 × 8.2~13.7 μm, respectively. These measurements were similar to those reported in previously published results for *R. mariae* [24], however, Korean samples have a wider range in basidia width (Fig. 3A and 3B). Basidiospores and baisidia of Korean *R. violeipes* (RV group) measured 6.2~8.6 × 5.0~6.6 μm and 45.6~63.2 × 8.1~12.3 μm, respectively. Measurements of Korean *R. violeipes* samples were similar to those reported in published records [25], however, basidospores and basidia were generally more narrow (Fig. 3A and 3B). In comparison of the measurements of Korean *R. violeipes* and Korean *R. mariae*, the major difference is that the basidia of Korean *R. violeipes* are slightly longer than those of Korean *R. mariae* (Fig. 3B).

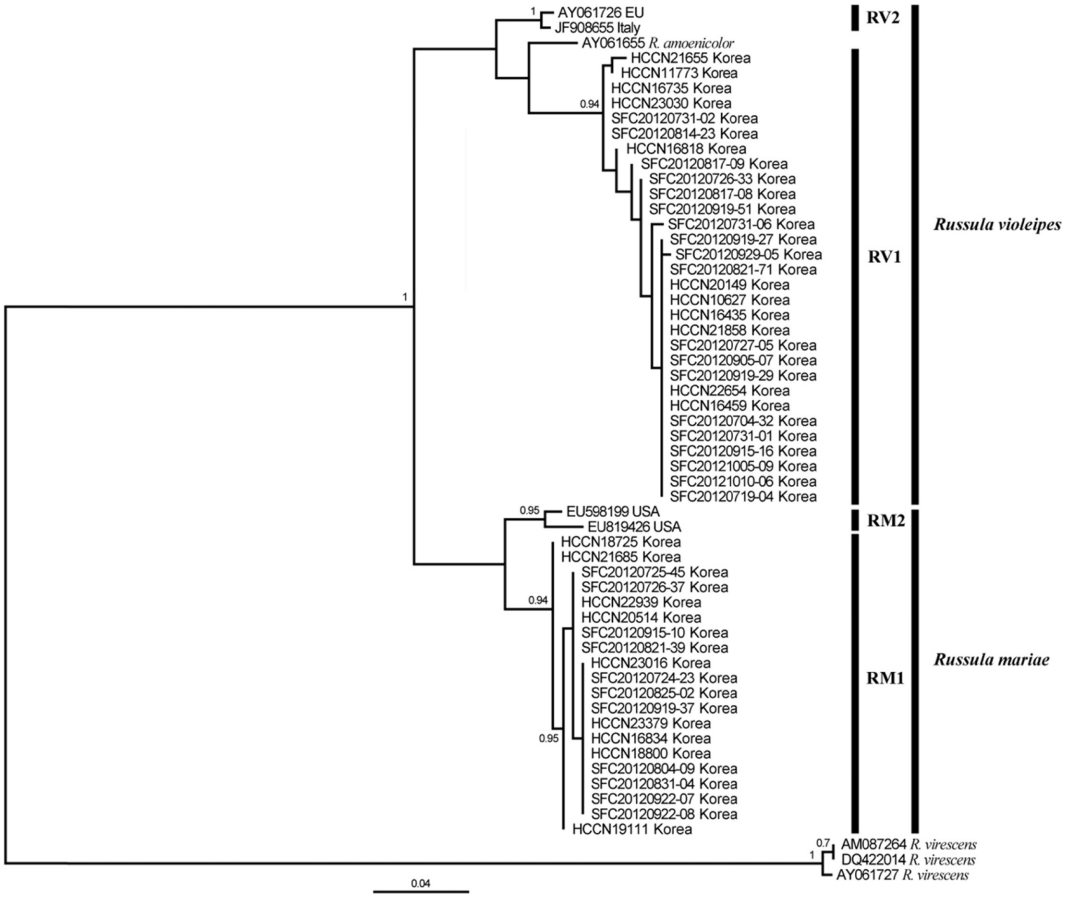

Fig. 4. Bayesian consensus tree (50% majority rule) inferred from sequences of rDNA internal transcribed spacer region for 50 Korean *Russula* specimens in the subgenus *Amoenula*. Bayesian posterior probabilities are only shown for nodes with support > 0.9. The scale bar indicates the number of nucleotide substitutions per site. RV, *R. violeipes*; RM, *R. mariae*.

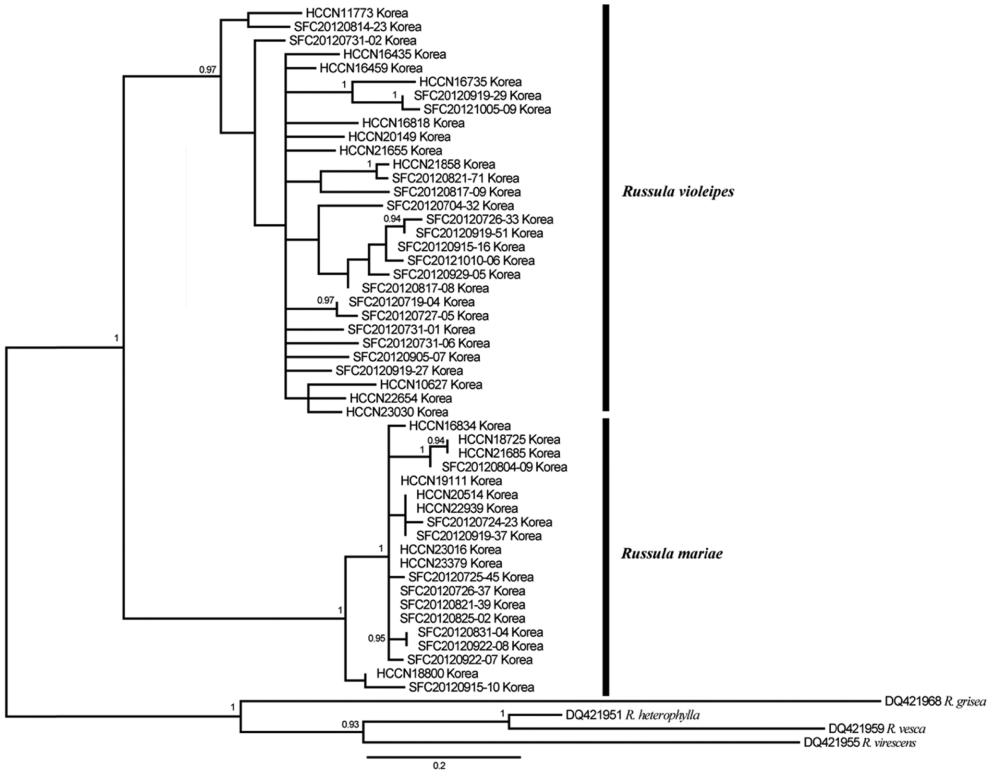

Fig. 5. Bayesian consensus tree (50% majority rule) inferred from sequences of the RNA polymerase II gene (RPB2) for 50 Korean *Russula* specimens in the subgenus *Amoenula*. Bayesian posterior probabilities are only shown for nodes with support > 0.9. The scale bar indicates the number of nucleotide substitutions per site.

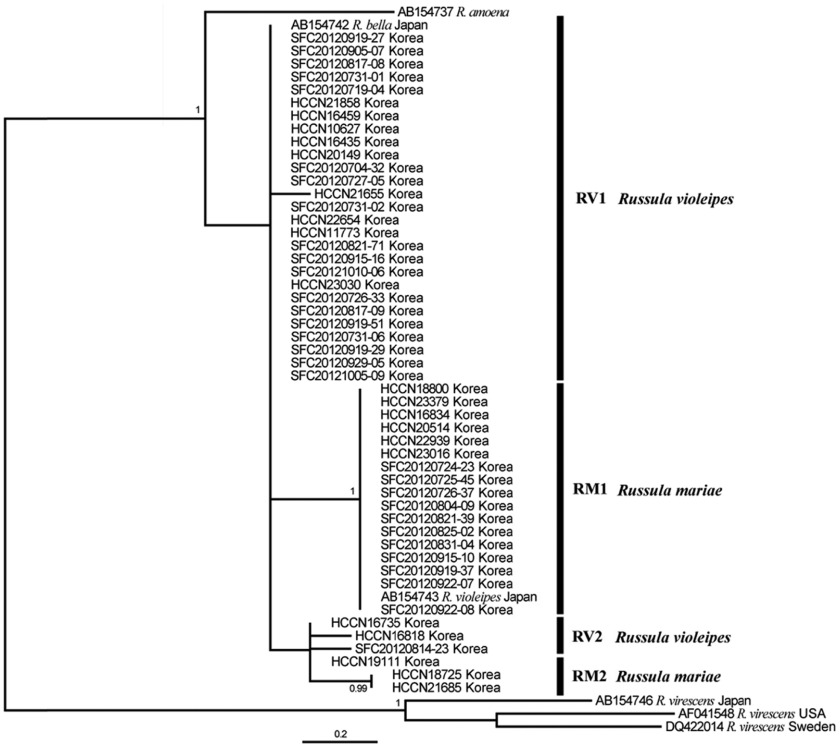

Fig. 6. Bayesian consensus tree (50% majority rule) inferred from sequences of the 28S nuclear ribosomal large subunit rRNA gene for 50 Korean *Russula* specimens in the subgenus *Amoenula*. Bayesian posterior probabilities are only shown for nodes with support > 0.9. The scale bar indicates the number of nucleotide substitutions per site. RV, *R. violeipes*; RM, *R. mariae*.

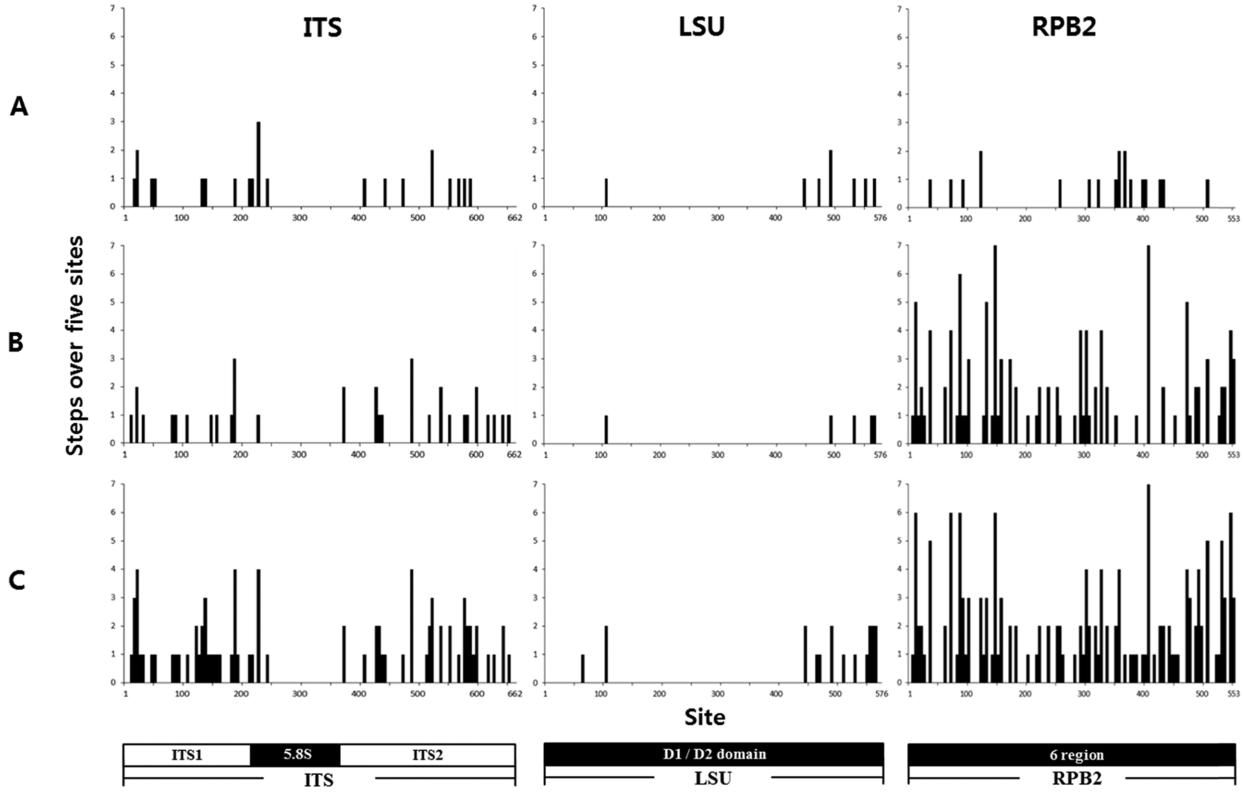

Fig. 7. Site variation of the internal transcribed spacer (ITS), 28S nuclear ribosomal large subunit rRNA gene (LSU), and RNA polymerase II gene (RPB2) sequence of *R. mariae* and *R. violeipes*. Variation in base substitution numbers among sites across the length of the genes was assessed using a window size of five consecutive bases from *R. mariae* (A), *R. violeipes* (B), and all Korean specimens of *R. mariae* and *R. violeipes* (C).

Sequence analyses of ITS, LSU, and RPB2. In order to infer the phylogenetic position of *R. mariae* and *R. violeipes*, the ITS (630~650 bp), LSU (570 bp), and RPB2 (600 bp) regions were amplified and sequenced for all specimens. In general, the ITS and RPB2 phylogenies were similar and resolved the relationship between *R. mariae* and *R. violeipes* (Figs. 4 and 5). In contrast, the LSU phylogeny was not well resolved, with no separation between the two species (Fig. 6). For RPB2, *R. violeipes* and *R. mariae* were found to be reciprocally monophyletic (Fig. 5). The relationship between Korean samples and their conspecifics in other countries cannot be evaluated because no sequences were available in Genbank. For ITS, all Korean samples were divided into two monophyletic groups. One of these groups clustered with known sequences of *R. mariae* from North America, while the other group clustered with known sequences of *R. violeipes* and *R. amoenicolor* (Fig. 4). The monophyletic *R. mariae* group showed geographic variation, with one subgroup (RM1) containing specimens exclusively from Korea and the second subgroup (RM2) including representatives from North America (Fig. 4). The second group contained Korean *R. violeipes* (RV1) and European *R. violeipes* (RV2), and was rendered paraphyletic by a single *R. amoenicolor* from Europe, but with low support (posterior probability = 0.59) (Fig. 4).

Intra- and interspecific variation of ITS, LSU, and RPB2. In examination of the distribution of variable sites of each gene, the number of parsimony steps over five base windows is shown in Fig. 7. From the diagram, we can see that sequence variation was highest in RPB2, followed by ITS, then LSU. RPB2 showed a relatively even distribution of variation across the gene, and the high rate of change was driven primarily by high variation in the *R. violeipes* group (RV group). ITS showed moderate variation across the marker, except in the intervening region of ITS consisting of the conserved 5.8S rRNA gene. LSU exhibited the lowest variation of the three markers; sequence variation for the D2 region was slightly greater than that of the D1 region (Fig. 7).

In addition, boxplots were made for comparison of intra- and interspecific dissimilarity and to understand the resolutional power of ITS, LSU, and RPB2 (Fig. 8). Intra- and interspecific variation for both ITS and RPB2 showed a clear pattern of non-overlap, whereas LSU showed a pattern of overlapping of intra- and interspecific variation.

DISCUSSION

In this study, we re-evaluate the status of *Russula* species of the subgenus *Amoenula* in Korea using microscopic features

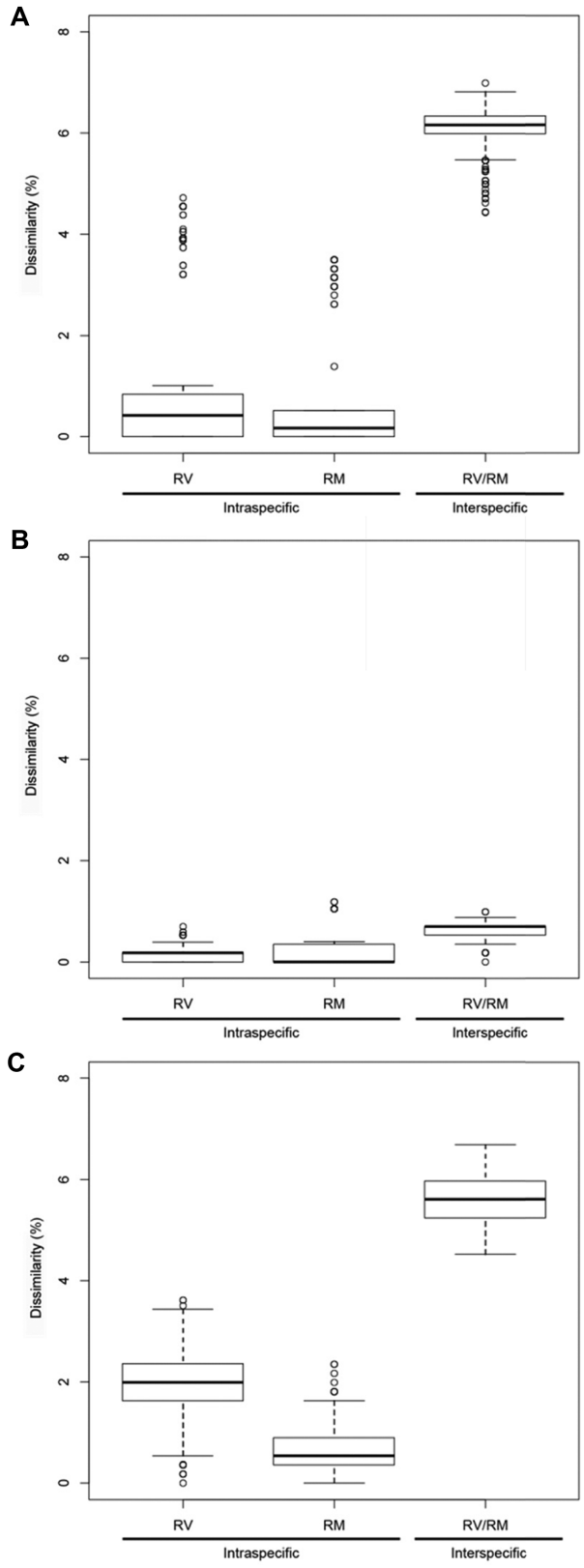

Fig. 8. Boxplots of intra- and interspecific variation of *Russula mariae* (RM) and *R. violeipes* (RV). Pair-wise comparisons are made of % dissimilarity of the barcoding markers (A) internal transcribed spacer, (B) 28S nuclear ribosomal large subunit rRNA gene, and (C) RNA polymerase II gene.

and DNA sequence analyses. *Russula amoena*, *R. mariae*, and *R. violeipes* of the subgenus *Amoenula* have been previously reported from Korea [12]. Due to similar macroscopic characteristics, such as red/pink cap coloration, differentiation of *Russula* species is difficult without the aid of microscopic or DNA data. *Russula mariae* and *R. violeipes* are particularly prone to misidentification because in addition to similar morphology and coloration, micro-morphological features overlap. This strong potential for misidentification should be considered carefully when evaluating previous research on fungal diversity that relies on macro-morphological characteristics for identification [28]. In our careful evaluation of the species identification of several *Russula* species using microscopic characteristics and DNA data, we found that misidentification was common. Of the specimens used in this study, it was found that 56 specimens out of 62 (90%) were incorrectly identified. Focusing on the subgenus *Amoenula*, our final dataset was comprised of 20 *R. mariae*, 30 *R. violeipes*, and zero *R. amoena*. Our study raises the possibility that *R. amoena* is not distributed in Korea. Additional sampling across the Korean peninsula and comparison with *R. amoena* from Europe using morphological and sequence analyses will be necessary in order to evaluate the status of *R. amoena* in Korea.

The phylogenetic results of the three molecular markers (RPB2, ITS, and LSU) varied, with RPB2 and ITS recovering two species groups corresponding to *R. violeipes* and *R. mariae*, while LSU provided no resolution between the two species. The ITS dataset included samples from outside of Korea and showed that there is phylogeographic structure in both *R. mariae* and *R. violeipes*. *Russula mariae* has distinct groups from Korea (RM1) and North America (RM2) (Fig. 4), while *R. violeipes* has distinct groups from Korea (RV1) and Europe (RV2). This genetic structure might be related to regional differences as a result of allopatry. Previous studies have shown that geographic separation can result in genetic divergence of mushroom populations [29]. This may also explain the reasons behind genetic differences between these two species in Korea and their conspecifics in Europe and North America.

Russula violeipes, in the ITS phylogeny, is paraphyletic due to a single *R. amoenicolor* specimen from Europe, but with low support (posterior probability = 0.59). It should be noted that the microscopic measurements of RV1 closely resembled those of previously reported data from Europe (Fig. 3) and presence of globose basal cells in the pileipellis of *R. violeipes* has been shown to differentiate it from *R. amoenicolor*, which does not have this trait [24]. We believe that the paraphyly of *R. violeipes* and *R. amoenicolor* is erroneous due to the low posterior probability support and distinguishing morphological characteristics, however additional sampling will be necessary in order to clarify this relationship.

The amount and spatial position of variation in the three markers (ITS, LSU, and RPB2) differ between markers and between groups (Fig. 6). For ITS, the ITS1 and ITS2

regions show similar levels of variation in both *R. mariae* and *R. violeipes*. LSU shows overall low levels of sequence variation across the marker and across two species, but with more changes in the D2 region. Among the three markers, RPB2 shows the highest level of variation, and this variation is evenly spread across the marker. Of particular interest, the high level of DNA variation is driven by differences in *R. violeipes*. However, in order to fully understand the driving force behind this sequence variation, conduct of additional studies of *R. violeipes* and *R. mariae* at the genomic level will be necessary.

Intra- and interspecific pairwise sequence comparisons among all samples used in this study were performed for each of the three datasets. The magnitude of sequence dissimilarities varied according to locus. In comparison of the sequence variation of the two *Russula* species, we observed clear sequence dissimilarity between intra- and interspecific variation for both ITS and RPB2, whereas LSU showed overlapping inter and intra specific variation (Fig. 8). ITS and RPB2 of these *Russula* species conform to the requirements as optimal barcoding markers, being easily amplifiable using a single set of primers, and should provide sufficient resolution for identification of species [30]. ITS and RPB2 exhibited low intraspecific variation and high interspecific variation in our dataset and specimens collected in Korea could be distinguished.

In conclusion, we used microscopic characteristics and DNA sequences in order to find misidentified *Russula* specimens in herbarium collections. In particular, *R. mariae* and *R. violeipes* were previously misidentified as five different species (Table 1) when classification was based solely on macroscopic characteristics. In addition, our study raises the possibility that *R. amoena* does not exist in Korea, although additional work will be necessary in order to verify this. Identification in the field is normally based on macroscopic traits such as shape and color, however, we show that such characteristics are inadequate for classification and often lead to incorrect identification. LSU proved to be ineffective for use of DNA markers in closely related *Russula* phylogenetics and barcoding, while both ITS and RPB2 markers allowed for clear discrimination of *R. mariae* and *R. violeipes*, as well as distinguishing broad geographic patterns (i.e., Korea vs. Europe/North America) within them. *Russula* species have important roles in the environment in mutualistic relationships with plants and ITS and RPB2 markers will be powerful tools in elucidating the ecology and evolution of *Russula* in Korea and worldwide.

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