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A preliminary DNA barcode selection for the genus *Russula* (Russulales, Basidiomycota)

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ABSTRACT

Russula is a worldwid genus which has a high species diversity . Aiming accurate and rapid species identification, candidate genes *nLSU* (28S), *ITS, tef-1a, mtSSU, rpb1*, and *rpb2*, were analysed as potential DNA barcodes. This analysis included 433 sequences from 38 well-circumscribed *Russula* species of eight subgenera. Two vital standards were analysed for success species identification using DNA barcodes, specifically inter- and intra-specific variations together with the success rates of PCR amplification and sequencing. Although the gap between inter- and intra-specific variations was narrow, *ITS* met the qualification standards for a target DNA barcode. Overlapping inter- and intra-specific pairwise distances were observed in *nLSU, tef-1a, mtSSU*, and *rpb2*. The success rates of PCR amplification and sequencing in *mtSSU* and *rpb1* were lower than those of others. Gene combinations were also investigated for resolution of species recognition. *ITS-rpb2* was suggested as the likely target DNA barcode for *Russula*, owing to the two viatal standards above. Since *nLSU* has the lowest minimum of inter-specific variation, and *tef-1a* has the highest overlap between intra- and inter-species variations among the candidate genes, they are disqualified from the selection for DNA barcode of *Russula*.

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Introduction

The genus *Russula* Pers. is a group of gilled mushrooms with brightly coloured pileus and non-lactic fragile basidiocarps. It belongs to the family Russulaceae (Russulales, Agaricomycetes) (Romagnesi 1985; Sarnari 1998, 2005; Li 2014). This genus comprises over 780 species which is the second largest genus among Agaricomycetes. *Russula* species are frequently growing in almost all kinds of forests and is the dominant ectomycorrhizal (ECM) mushrooms, with a geographic range from the arctic tundra to tropical forests (Singer 1986; Buyck et al. 1996; Kirk et al. 2008; Geml et al. 2009, Wang et al. 2009; Li 2014). Although the majority of *Russula* species are edible, a few members are poisonous and some are even lethal (Li et al. 2010a; Chen et al. 2016).

Morphological characters have been regarded as the main criterions for specific identification in *Russula* for a long time in history. The large number of species, high intra-specific variability, and inaccurate descriptions in the literature caused considerable taxonomic inconvenience and confusions (Romagnesi 1985; Sarnari 1998, 2005; Li 2014). For example, *R. virescens* (Schaeff.) Fr. was originally described from Europe, while the illustrations of "*Russula virescens*" in some previous North American field guide books (Metzler and Metzler 1992, Roody 2003, Miller OK and Miller HH 2006, Kuo 2007) have been proved to be *R. parvovirescens* Buyck, D. Mitch. & Parrent; the "*R. virescens-R. crustosa*" group in North America is suggested to be much more complex than suspected, which contains at least a dozen of *Russula* taxa in the eastern US (Buyck et al. 2006; Kuo 2007). Another similar example is "*R. vinosa* Lindblad" in several Chinese fungal monographs (Teng 1963; Tai 1979; Ying et al. 1982, 1987; Wang et al. 2004) should be another species and named as *R. griseocarnosa* X.H. Wang et al. after morphological and *ITS-nLSU* phylogenetic analyses (Wang

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et al. 2009). More recently, the molecular analysis indicated that this "species" has three divergent lineages: one of them represents to *R. griseocarnosa* and the other two possibly correspond to unknown taxa (Li et al. 2010b). The genus *Russula* is easily separated from other genera in morphology; however, morphological distinction at species level within this genus is complicated and time-consuming. A mechanism for the accurate and rapid identification of *Russula* species is, thus, vital and critical for both theoretical and applied research.

DNA barcoding makes use of a short gene sequence as a universal and standard genetic marker for species identification (Hebert et al. 2003; Stockinger et al. 2010). Compared with molecular phylogenetic analyses, DNA barcoding aims to identify unknown samples and cryptic species based on current classifications, rather than elucidating patterns of phylogenetic relationships (Kress et al. 2005). The ideal barcode sequence must be easily amplified and sequenced, conserved within a species, and variable between species (Taberlet et al. 2007). The first attempt at DNA barcoding was to target the mitochondrial gene, cytochrome oxidase I (COI or COX1), for the identification of specific animals and protists (Hebert et al. 2003). However, this gene proved to be too highly conserved and was not suitable for DNA barcoding in the plant kingdom (Ning et al. 2008). Two genes, *rbcL* and *matK*, within the chloroplast coding region and *trnH-psbA*, within the chloroplast noncoding region, together with the *ITS* and *ITS2* regions of ribosomal RNA, were, thus, selected as appropriate DNA barcodes for plants (Hollingsworth et al. 2009; Chen et al. 2010; Li et al. 2011).

DNA barcoding of fungi has only recently been performed. Despite a successful attempt in the genus *Penicillum* (Seifert et al. 2007) and class Oomycetes (Martin 2000; Martin and Tooley 2003; Robideau et al. 2011, Long et al. 2014), the COI gene failed to qualify as a universal fungal target due to unequal intron numbers, an absence of primer commonality, and difficulties in primer design and sequence alignment (Geiser et al. 2007; Gilmore et al. 2009; Vialle et al. 2009). The β-tubulin gene could be used as a suitable DNA barcode for the genera, *Aspergillus* (Geiser et al. 2007; Varga et al. 2011), *Penicillum* (Samson et al. 2004), and *Tuber* (Zampieri et al. 2009), but was not suitable for *Parmeliaceae* and *Sordariomycetes* (Thell et al. 2004; Tang et al. 2007). The gene for transcription elongation factor 1-alpha (*tef-1α*) was suggested as a DNA barcode for the genus *Fusarium* (Geiser et al. 2004), which, along with the second largest RNA polymerase II subunit (*rpb2*), could precisely distinguish the species of genera *Hypocera* (Jaklitsch et al. 2006) and *Neonectria* (Zhao et al. 2011a; b, Zeng et al. 2012). Among the ribosomal RNA genes that are commonly used in molecular phylogenetic analyses, the 18S and 28S rDNA subunits show a high primer commonality; while they were chosen as the DNA barcode for *Glomeromycota* (Schüßler et al. 2001; Schüßler and Walker 2010), they are not appropriate for specific identification because of their low mutation rates (Krüger et al. 2009).

The ITS1-5.8S-ITS2 (*ITS*) region of ribosomal RNA is the most widely analysed for fungal species identification, e.g. *Amanita* and *Cortinarius* of marco-fungi (Zhang et al. 2004, 2010; Frøslev et al. 2007), *Chrysomyxa* and *Melampsora* of smut fungi (Vialle et al. 2009), *Trichoderma* (Druzhinina et al. 2005), Lichenized fungi of *Ascomycota* (Kelly et al. 2011), and Mucorales of *Mucoromycotina* (Schwarz et al. 2006). *ITS* has been suggested to be the universal DNA barcode marker for fungi (Schoch et al. 2012); however, there are multiple paralogous or non-orthologous copies that lead to *ITS* sequence polymorphism (O'Donnell and Cigelnik 1997; Smith et al. 2007; Kovács et al. 2011; Lindner and Banik 2011). It is, thus, necessary to select DNA barcode substitutions to achieve multi-locus fungal identification (Roe et al. 2010).

Several gene makers have been analysed in molecular studies of *Russula*, some of which are phylogenetic analyses, e.g. *nLSU* (28S) analysed by Miller et al. (2001) and Shimono et al. (2004), *ITS* by Miller and Buyck (2002), Li (2014), Zhang (2014), Guo et al. (2014) and Liu et al. (2017), *ITS* and *nLSU* by Eberhardt (2002) and Shimono et al. (2014), *ITS, nLSU*, and *rpb2* by Buyck et al. (2008), *ITS, nLSU, rpb1* and *rpb2* by Looney et al. (2016), and *nLSU*, mtSSU, *tef-1α, rpb1* and *rpb2* by Buyck and Hofstetter (2018). For species delimitation of *Russula*, more analyses focused in *ITS* region (Wang and Sun 2004; Yin et al. 2008; Hampe et al. 2013, Adamčík et al. 2016a; 2016b; Looney 2014). There are relatively fewer researches in which multiple genes were analysed, e.g. *ITS, mtSSU, nLSU* and *rpb2* in Li et al. (2010b), *ITS, nLSU* and *rpb2* in Park et al. (2013), *ITS* and *nLSU* in Park et al. (2014), *ITS, rpb2, atp6, cox3* and *chsi* in Cao et al. (2013) and *ITS, mtSSU* and *rpb2* in Caboň et al. (2017). In the

present study, six genes, namely *nLSU* (28S), *ITS, tef-1α, mtSSU, rpb1*, and *rpb2*, which have been widely analysed in molecular phylogeny, were selected as candidate biomarkers. The efficiency of species identification and the feasibility of these genes to act as DNA barcodes for the genus *Russula* were evaluated.

Materials and methods

Materials

A total of 398 sequences of *ITS, nLSU* (28S), *tef-1α, mtSSU, rpb1* and *rpb2* genes from 59 *Russula* specimens, which represented 27 species, were newly produced from this study. Another 28 sequences of 15 species were retrieved from GenBank (see Table 1 for accession numbers). The total 38 *Russula* species were involved. All of the sampling species can be recognised in morphology and six-gene phylogenetic analyses. For those Chinese specimens under European and North American names, stable morphological resemblance and over 99% ITS sequence identities were regarded as criteria when other genes of other continents were not available. Members of each subgenus in Romagnesi (1985) were representatively sampled.

DNA extraction, PCR amplification, and sequencing

DNA extraction was performed, as per the procedure described by Li et al. (2012). The six candidate genes were amplified and sequenced using the following primer pairs: ITS1/ITS5 (*ITS*, White et al. 1990), LROR/LR5 (*nLSU*, Moncalvo et al. 2000, 2002), EF1-983F/EF1-1567R (*tef-1α*, Morehouse et al. 2003), MS1/MS2 (*mtSSU*, White et al. 1990), RPB1-Ac/RPB1-Cr (*rpb1*, Stiller and Hall 1997; Matheny et al. 2002), and bRPB2-6F/fRPB2-7cR (*rpb2*, Liu et al. 1999; Matheny 2005). PCR was performed in a Techne Prime Thermal Cycler (Cole-Parmer, Staffordshire, UK) using a 50 μL reaction volume composed of 25 μL Biomed 2× Taq Plus PCR MasterMix (Biomed, Beijing, China), 21 μL ddH_2O, 1.5 μL of each primer (10 μmol/L), and 1 μL DNA template. PCR reaction conditions followed those of Li et al. (2012) for *ITS* and *nLSU*, Stenglein et al. (2010) for *tef-1α* and *mtSSU*, and Matheny (2005) for *rpb1* and *rpb2*. PCR products were purified and sequenced by the Biomed Biotech Company (Beijing) using the ABI 3130 DNA sequencer and ABI BigDye 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Comparison of intra- and inter-specific divergence

Sequences were aligned using Mafft 7.311 (Katoh and Standley 2013), and the aligned sequences were manually adjusted in Bioedit 7.0 (Hall 1999). Similarity matrices were calculated using the MegAlign program in DNAStar v7.1 (Lasergene, WI, USA) and the resulting output was analysed and visualised in TaxonGap 2.4.1 (Slabbinck et al. 2008). The intra- and inter-specific pairwise distances were analysed in MEGA 7.0.26 with Kimura's two-parameter (K2P) model (Kumar et al. 2016) and SpeciesIdentifier 1.8 in TaxonDNA (Meier et al. 2006). The DNA barcode gap between the frequency distributions of intra- and inter-specific pairwise distances was calculated using Microsoft Office Excel 2013. The incongruence length difference (ILD) test was carried out to calculate the probability values (*p*-values) in partition homogeneity tests using PAUP 4.0 Beta 10 (Swofford 2004). The *p*-value criterion ($p \geq 0.01$) proposed in Farris et al. (1995) and Cunningham (1997) was followed to test the feasibility that two genes were congruent so they can be analysed together as a combination. Maximum likelihood (ML) phylogenetic analyses of the six genes were carried out using RAxML 8 (Stamatakis 2014) to estimate the intra- and inter-specific genetic distances.

Success rates of sequence acquisition

The success rates of PCR amplification and sequencing were calculated and evaluated. In electrophoresis running gel, a single and clear band that fit for the length of target gene can be regarded as the criterion of successful PCR amplification. A chromatogram which has high but not mixed peaks was regarded as the standard of successful sequencing. A success rate of PCR amplification and sequencing is the product of two respective rates.

Results

The overall analysis involved a total of 426 sequences from 38 *Russula* species, targeting six candidate genes, namely *nLSU, ITS, tef-1α, mtSSU, rpb1*, and *rpb2* (Table 1). The sequences were shortened to meet standard DNA barcode requirements.

Table 1. Specimens and sequences in this study.

Taxon name	Herbarium	*LSU*	*ITS*	*tef-1α*	*mtSSU*	*rpb1*	*rpb 2*	Subgenus	Location
Russula acrifolia	HMAS267774	KX441351	KX441104	MF893436	KX441598	KX441845	KX442092	*Compactae*	China Jilin Changbaishan Erdaobaihe
Russula acrifolia	PC 543/BB 08.662	KU237535	NA	KU237965	KU237381	KU237684	KU237821	*Compactae*	Europe
Russula amara	GENT FH12-213	KT933859	KT933998	NA	NA	KT957370	NA	*Incrustatula*	Europe
Russula amara	PC 532/BB 07.782	KU237524	NA	KU237954	KU237370	KU237674	NA	*Incrustatula*	Europe
Russula amoenipes	HMAS263065	KX441319	NA	MF893404	KX441566	KX441813	KX442060	*Polychromidia*	China Yunnan Kunming Qiongzhusi
Russula amoenipes	HMAS263067	MG493214	NA	MG495119	MG518376	MG495099	NA	*Polychromidia*	China Jilin Changbaishan Erdaobaihe
Russula amoenolens	HMAS252622	KX441282	KX441035	MF893367	KX441529	KX441776	KX442023	*Ingratae*	China Jilin Changbaishan Erdaobaihe
Russula amoenolens	HMAS264497	KX441325	KX441078	MF893410	KX441572	KX441819	KX442066	*Ingratae*	China Jilin Longjing Tianfuozhishan
Russula aurea	HMAS250932	KX441261	NA	MF893346	NA	KX441755	KX442002	*Coccinula*	China Jilin Changbaishan Huangsongpu
Russula aurea	HMAS262377	MG493215	NA	MG495120	MG518377	MG495101	MG495109	*Coccinula*	China Jilin Changbaishan Erdaobaihe
Russula aurea	PC 547/BB 07.211	KU237539	NA	KU237969	KU237385	KU237688	NA	*Coccinula*	Europe
Russula brevipes	HMAS252596	KX441277	KX441030	MF893362	KX441524	KX441771	KX442018	*Brevipes*	China Jilin Changbaishan Xizhuxian
Russula brevipes	HMAS252611	KX441280	KX441033	MF893365	KX441527	KX441774	KX442021	*Brevipes*	China Jilin Changbaishan Erdaobaihe
Russula carneipes	HMAS252682	KX441286	KX441039	MF893371	NA	KX441780	KX442027	*Russula*	China Sichuan Dawo Tainingyuke
Russula carneipes	HMAS268187	KX441363	KX441116	MF893448	NA	KX441857	KX442104	*Russula*	China Sichuan Dawo Tainingyuke
Russula changbaiensis	HMAS262355	KX441304	KX441057	MF893389	KX441551	KX441798	KX442045	*Genuina*	China Jilin Changbaishan Erdaobaihe
Russula changbaiensis	HMAS267736	MG493216	MG493202	MG495121	MG518378	MG495106	NA	*Genuina*	China Neimenggu Yakeshi Nanmu
Russula compacta	TENN067133 BPL227	KT933810	KT933952	NA	NA	NA	KT933881	*Malodorae*	North America
Russula compacta	TENN067303 BPL242	KT933819	KT933960	NA	NA	KT957330	KT933890	*Malodorae*	North America
Russula crustosa	TENN067418 BPL265	KT933826	KT933966	NA	NA	KT957338	KT933898	*Malodorae*	North America
Russula crustosa	TENN070180 BPL251	KT933822	KT933963	NA	NA	KT957334	KT933894	*Malodorae*	North America
Russula decolorans	GENT FH12-196	KT933853	KT933992	NA	NA	KT957364	KT933924	*Tenellula*	Europe
Russula decolorans	PC 549/BB 07.322	KU237541	NA	KU237971	KU237387	KU237735	NA	*Tenellula*	Europe
Russula exalbicans	HMAS268774	MG493219	MG493205	NA	NA	NA	MG495110	*Russula*	Sichuan Jiuzhaigou Zhangzha
Russula exalbicans	HMAS269713	KX441408	KX441161	MF893493	NA	NA	KX442149	*Russula*	Sichuan Jiuzhaigou Zhangzha
Russula fellea	GENT FH12-185	KT933850	KT933989	NA	NA	KT957361	KT933921	*Russula*	Europe
Russula fellea	PC 444/BB 07.281	KU237507	NA	KU237936	KU237352	KU237656	KU237793	*Russula*	Europe
Russula firmula	HMAS271096	MG493220	NA	MG495124	MG518381	NA	MG495111	*Russula*	China Sichuan Yajiang Kazilashan
Russula firmula	HMAS271140	KX441459	NA	MF893544	KX441706	KX441953	KX442200	*Russula*	China Sichuan Yajiang Kazilashan
Russula foetens	HMAS271173	KX441470	KX441223	MF893555	KX441717	KX441964	KX442211	*Ingratae*	China Sichuan Litang Cunge
Russula foetens	HMAS271230	KX441476	KX441229	MF893561	KX441723	KX441970	KX442217	*Ingratae*	China Sichuan Litang Cunge
Russula fontqueri	HMAS260632	MG493217	MG493203	MG495122	MG518379	MG495098	NA	*Tenellula*	China Heilongjiang Suifenhe Forest Park
Russula fontqueri	HMAS262398	MG493218	MG493204	MG495123	MG518380	MG495097	NA	*Tenellula*	China Jilin Changbaishan Erdaobaihe
Russula fontqueri	HMAS267744	KX441343	KX441096	NA	KX441590	KX441837	KX442084	*Tenellula*	China Jilin Changbaishan Erdaobaihe
Russula fragilis	GENT FH12-197	NA	KT933993	NA	NA	KT957365	KT933925	*Russula*	Europe
Russula fragilis	PC 443/BB 07.791	NA	NA	NA	KU237351	KU237655	KU237792	*Russula*	Europe
Russula globispora	HMAS269239	KX441383	KX441136	MF893468	KX441630	KX441877	KX442124	*Insidiosula*	China Sichuan Aba S209 Road
Russula globispora	PC 436/BB 07.243	KU237499	NA	KU237929	KU237344	NA	KU237785	*Insidiosula*	Europe
Russula gracillima	GENT FH12-264	KR364226	KR364094	NA	NA	KR364472	KR364342	*Russula*	Europe
Russula gracillima	HMAS262340	MG493221	MG493206	MG495125	MG518382	NA	MG495112	*Russula*	China Jilin Changbaishan Erdaobaihe
Russula gracillima	PC 441/BB 07.785	KU237504	NA	KU237934	KU237349	KU237653	KU237790	*Russula*	Europe
Russula gracillima	PC 584/BB 07.786	KU237568	NA	KU237996	KU237416	KU237712	KU237854	*Russula*	Europe
Russula insignis	HMAS267732	MG493222	MG493207	MG495126	MG518383	NA	NA	*Ingratae*	China Neimenggu Zalantun Xiushui
Russula insignis	HMAS267740	KX441341	KX441094	MF893426	KX441588	KX441835	KX442082	*Ingratae*	China Neimenggu Yakeshi Nanmu
Russula insignis	HMAS267751	KX441346	KX441099	MF893431	KX441593	KX441840	KX442087	*Ingratae*	China Neimenggu Zalantun Xiushui
Russula integra	GENT FH12-172	KT933845	KT933984	NA	NA	KT957356	KT933916	*Polychromidia*	Europe

(*Continued*)

Table 1. (Continued).

Taxon name	Herbarium	*LSU*	*ITS*	*tef-1α*	*mtSSU*	*rpb1*	*rpb 2*	Subgenus	Location
Russula integra	PC 518/BB 07.198	KU237513	NA	KU237943	KU237359	KU237663	KU237799	*Polychromidia*	Europe
Russula integriformis	HMAS262393	KX441312	KX441065	MF893397	NA	KX441806	KX442053	*Polychromidia*	China Jilin Changbaishan Erdaobaihe
Russula integriformis	HMAS262403	KX441313	KX441066	MF893398	NA	KX441807	KX442054	*Polychromidia*	China Jilin Changbaishan Erdaobaihe
Russula katarinae	HMAS269080	KX441380	KX441133	MF893465	NA	NA	KX442121	*Polychromidia*	China Yunnan Nanhua Zixishan
Russula katarinae	HMAS269755	KX441410	KX441163	MF893495	NA	KX441904	KX442151	*Polychromidia*	China Yunnan Nanhua Zixishan
Russula luteotacta	GENT FH12-187	KT933852	KT933991	NA	NA	KT957363	KT933923	*Russula*	Europe
Russula luteotacta	PC 452/BB 07.188	KU237512	NA	KU237942	KU237358	KU237662	KU237798	*Russula*	Europe
Russula medullata	HMAS251747	KX441268	KX441021	MF893353	NA	KX441762	KX442009	*Heterophyllidia*	China Xizang Mainling Nanyi
Russula medullata	HMAS251761	MG493212	MG493200	MG495118	MG518374	NA	NA	*Heterophyllidia*	China Xizang Mainling Nanyi
Russula medullata	HMAS262348	MG493213	MG493201	NA	MG518375	MG495100	MG495108	*Heterophyllidia*	Jilin Changbaishan Erdaobaihe
Russula murrillii	HMAS271049	KX441438	KX441191	MF893523	KX441685	KX441932	KX442179	*Incrustatula*	China Yunnan Dêqên Baimangxueshan
Russula murrillii	HMAS271144	KX441460	KX441213	MF893545	KX441707	KX441954	KX442201	*Incrustatula*	China Yunnan Dêqên Baimangxueshan
Russula nigricans	PC 429/BB 07.342	KU237495	NA	KU237924	KU237339	KU237643	KU237781	*Compactae*	Europe
Russula nigricans	UPS UE20.09.2004–07	DQ422010	DQ422010	NA	NA	NA	DQ421952	*Compactae*	Europe
Russula ochroleuca	GENT FH12-211	KT933857	KT933996	NA	NA	KT957368	KT933928	*Russula*	Europe
Russula ochroleuca	PC 527/BB 07.303	KU237519	NA	KU237949	KU237365	KU237669	KU237805	*Russula*	Europe
Russula pascua	HMAS252594	KX441276	KX441029	MF893361	KX441523	KX441770	NA	*Polychromidia*	China Jilin Changbaishan Erdaobaihe
Russula pascua	HMAS253222	MG493223	NA	MG495127	MG518384	MG495103	MG495113	*Polychromidia*	China Xizang Mainling Nanyi
Russula pascua	HMAS262382	NA	MG493208	MG495128	MG518385	MG495105	MG495114	*Polychromidia*	China Jilin Changbaishan Erdaobaihe
Russula pseudocyanoxantha	HMAS252849	NA	KX441048	MF893380	KX441542	KX441789	KX442036	*Cyanoxanthinae*	China Yunnan Jingdong Ailaoshan
Russula pseudocyanoxantha	HMAS271691	NA	KX441236	MF893568	KX441730	KX441977	KX442224	*Cyanoxanthinae*	China Yunnan Puer Laiyanghe
Russula pseudograta	HMAS250432	KX441259	KX441012	MF893344	KX441506	KX441753	KX442000	*Ingratae*	China Xizang Nyingchi Nanyi
Russula pseudograta	HMAS251868	KX441273	KX441026	MF893358	KX441520	KX441767	KX442014	*Ingratae*	China Xizang Nyingchi Nanyi
Russula pseudograta	HMAS253194	KX441296	KX441049	MF893381	KX441543	KX441790	KX442037	*Ingratae*	China Xizang Nyingchi Nanyi
Russula pseudopectinatoides	HMAS251523	KX441263	KX441016	MF893348	KX441510	KX441757	KX442004	*Ingratae*	China Xizang Yadong Xiasima
Russula pseudopectinatoides	HMAS251552	MG493224	MG493209	MG495129	MG518386	MG495104	MG495115	*Ingratae*	China Xizang Yadong Xiasima
Russula pseudopectinatoides	HMAS264895	MG493225	MG493210	MG495130	MG518387	MG495102	MG495116	*Ingratae*	China Xizang Yadong Xiasima
Russula pseudopectinatoides	HMAS265020	KX441336	KX441089	MF893421	KX441583	KX441830	KX442077	*Ingratae*	China Xizang Gongbogyamda Cuogaohu
Russula pseudopersicina	HMAS264484	KX441324	KX441077	MF893409	KX441571	KX441818	KX442065	*Russula*	China Jilin Longjing Tianfuozhishan
Russula pseudopersicina	HMAS267779	KX441352	KX441105	MF893437	KX441599	KX441846	KX442093	*Russula*	China Neimenggu Yakeshi Nanmu
Russula queleti	HMAS271076	MG493226	MG493211	MG495131	NA	NA	MG495117	*Russula*	China Yunnan Dêqên Baimangxueshan
Russula queleti	HMAS271149	KX441462	KX441215	MF893547	KX441709	NA	KX442203	*Russula*	China Yunnan Dêqên Baimangxueshan
Russula rosea	HMAS253340	KX441299	KX441052	MF893384	KX441546	NA	NA	*Incrustatula*	China Yunnan Yulong Botany Garden
Russula rosea	HMAS276801	LT602946	LT602969	NA	NA	KX442534	KX442557	*Incrustatula*	China Fujian Sanming Yangshan
Russula sinica	HMAS271022	KX441433	KX441186	MF893518	KX441680	KX441927	KX442174	*Russula*	China Yunnan Yulong Botany Garden
Russula sinica	HMAS271024	KX441434	KX441187	MF893519	KX441681	KX441928	KX442175	*Russula*	China Yunnan Yulong Botany Garden
Russula turci	HMAS271703	KX441484	KX441237	MF893569	KX441731	KX441978	KX442225	*Incrustatula*	China Yunnan Puer Laiyanghe
Russula turci	HMAS271765	KX441489	KX441242	MF893574	KX441736	KX441983	KX442230	*Incrustatula*	China Yunnan Puer Laiyanghe
Russula turci	HMAS271794	KX441493	KX441246	MF893578	KX441740	KX441987	KX442234	*Incrustatula*	China Yunnan Yiliang Xiaolongmen
Russula zvarae	GENT FH12-175	KT933847	KT933986	NA	NA	KT957358	KT933918	*Incrustatula*	Europe
Russula zvarae	PC 538/BB 08.639	KU237530	NA	KU237960	KU237376	KU237680	KU237816	*Incrustatula*	Europe

Sequence lengths were as follows: 880 bp for n*LSU*, 472 bp for *ITS*, 581 bp for *tef-1α*, 538 bp for *mtSSU*, 918 bp for *rpb1*, and 712 bp for *rpb2*.

The intra- and inter-specific variations are the important standards in determining the feasibility of candidate genes for DNA barcode selection. The resolution of current species, PCR, and sequencing success rates are also essential factors. A clear distinction between intra- and inter-specific divergences is a must for the identification of an ideal specific DNA barcode. Comparisons among sequences of the six candidate genes for each *Russula* species used in this study were analysed with TaxonGap 2.4.1 and the result is presented in Figure 1. *ITS* had the highest minimum of inter-specific variations of 3.2%, followed by *rpb2* (2.2%), *tef-1α* (1.4%), *rpb1* (1.2%), *mtSSU* (1.2%), and *nLSU* (0.7%). It appeared that *rpb2* had a marginally higher resolution than *nLSU, mtSSU, tef-1α*, and *rpb1*. For *rpb2*, all species showed intra-specific variations lower than 2.2%, apart from *R. acrifolia, R. delica*, and *R. queleti*. The minimum inter-specific variation of the six candidate genes also indicated that the ability of *nLSU* to specifically identify *Russula* species was the least among all the genes tested this low ability is due to *nLSU* having the lowest minimum of inter-specific variation. As shown in Figs. 1 and 2, an overlap was observed between the inter- and intra-specific variations in the *tef-1α* (26.3%), *rpb2* (7.9%), *mtSSU* (2.6%), and *nLSU* (2.6%) genes, suggesting these genes were inadequate as individual DNA barcodes for *Russula*. Although no overlap was observed in *rpb1*, the low minimum inter-specific variation (1.2%) hindered its ability to identify *Russula* species (Figure 1). Of all six candidate genes under analysis, *ITS* is most suitable for distinguishing between species. However, it remained restricted by the narrow gap between its intra- and inter-specific variations (Figs. 1 and 2).

The applications of *nLSU* and *tef-1α* genes in DNA barcode were not available, because *nLSU* has the low inter-specific variations minimum of (0.7%) and *tef-1α* has an obvious overlap between its inter- and intra-specific variations (26.3%). Combinations of the other genes, *ITS, mtSSU, rpb1*, and *rpb2*, were subsequently analysed. Application of the two-gene combinations provided improved variation compared to that of single genes, with all intra-specific variations being lower than the minimum inter-specific variations (Figs. 3 and 4). The combination of *ITS-mtSSU* and *ITS-rpb2* showed a minimum inter-specific variation of over 4%, which were more appropriate for species identification (Figure 3). The gap between intra- and inter-specific variations of these two combinations was also clear (Figure 4). An alternate combination of *mtSSU-rpb2* was found to be best for its minimum inter-specific variation of 3.8% when commonly used *ITS* sequences were unavailable (Figure 3).

The inter- and intra-specific pairwise distances of the candidate genes were evaluated from their ML trees (Figs 5–10). These results generally agree with those of TaxonGap. Although every species of this study can be well-separated from each other as

Figure 1. Comparisons of intra- and inter-specific variations among *nLSU, ITS, tef-1α, mtSSU, rpb1* and *rpb2* genes of Russula generated by TaxonGap. The inter- and intra-specific variations were presented as the black and grey bars respectively. The minimums of inter-specific variations for each gene were shown as the vertical lines. Taxon names followed the black bars represented the closest species of this analysis.

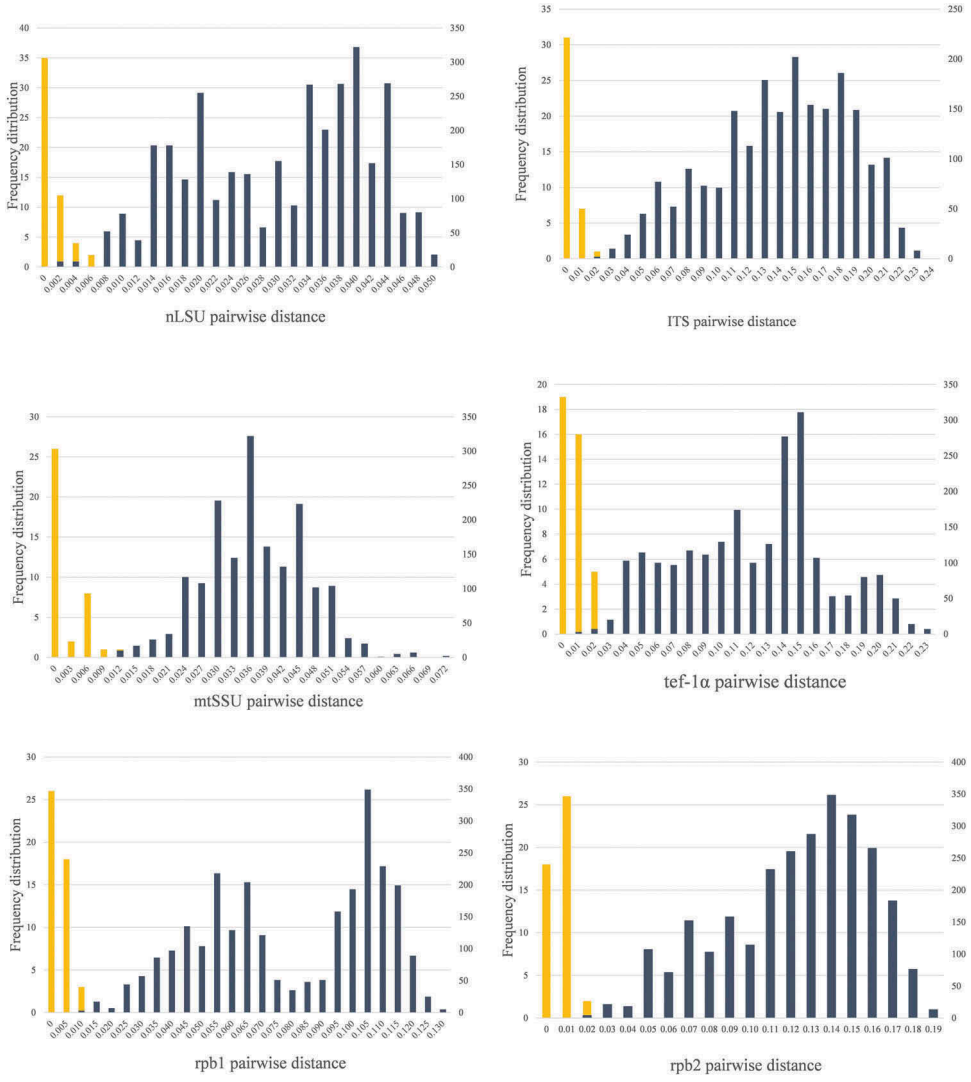

Figure 2. Comparisons of frequency distribution of intra- and inter-specific variation pairwise distances among *nLSU, ITS, tef-1α, mtSSU, rpb1* and *rpb2* genes of *Russula* generated by MEGA and Excel. The interand intra-specific distances are presented as yellow and blue bars respectively.

independent clades with high bootstrap values, overlaps between inter- and intra-specific variations can be observed in phylogenetic topologies of *nLSU* (Figure 5) *tef-1α* (Figure 7), *mtSSU* (Figure 8), and *rpb2* (Figure 10), in contrast, absent in those of *ITS* (Figure 6) and *rpb1* (Figure 9).

Sequence clustering was calculated based on pairwise distances, with the given threshold, using TaxonDNA/Species Identifier 1.8. The intra- and inter-specific divergence of the candidate genes were also evaluated, with the maximum intra-specific distance set as the clustering threshold. Corresponding levels of coincidence between clusters and species for the candidate biomarkers are presented in Table 2. For *tef-1α*, a total of 33 clusters were recognised, suggesting this gene was able to separately identify 33 of the 35 species (94.3%); by contrast *nLSU* was only capable of distinguishing between eight species. The other genes could also successfully distinguish between the *Russula* species used in this analysis.

PCR and sequencing success rates are another standard requirement of eligible DNA barcode genes. *ITS, nLSU*, and *tef-1α* could be easily amplified and sequenced with success rates of over 90%. On the other hand, the *mtSSU* gene had a relatively low PCR and sequencing success rate (78.3%) (Table 3). The primers commonly used in phylogenetic analysis of Basidiomycota were suitable for most species of the *Russula* genus.

Congruencies of individual partitions were calculated using the partition homogeneity test. The *p*-values of the gene combinations were *ITS-mtSSU* (0.20), *ITS-rpb1*

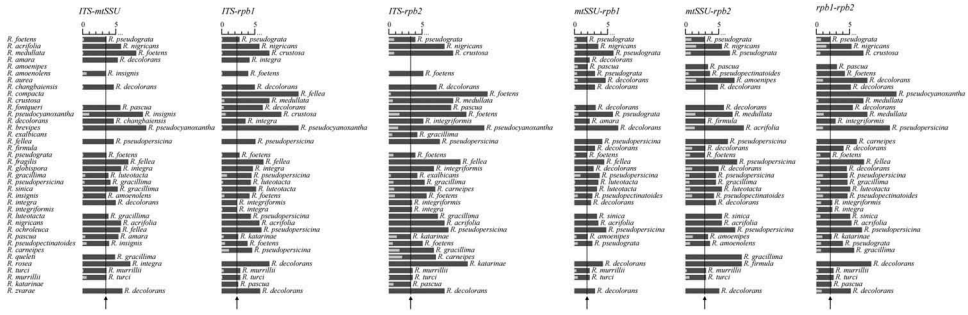

Figure 3. Comparisons of intra- and inter-specific variations among ITS-mtSSU, ITS-rpb1, ITS-rpb2, mtSSUrpb1, mtSSU-rpb2 and rpb1-rpb2 gene combinations of Russula generated by TaxonGap. The inter- and intra-specific variations were presented as the black and grey bars respectively. The minimums of interspecific variations for each gene were shown as the vertical lines. Taxon names followed the black bars represented the closest species of this analysis.

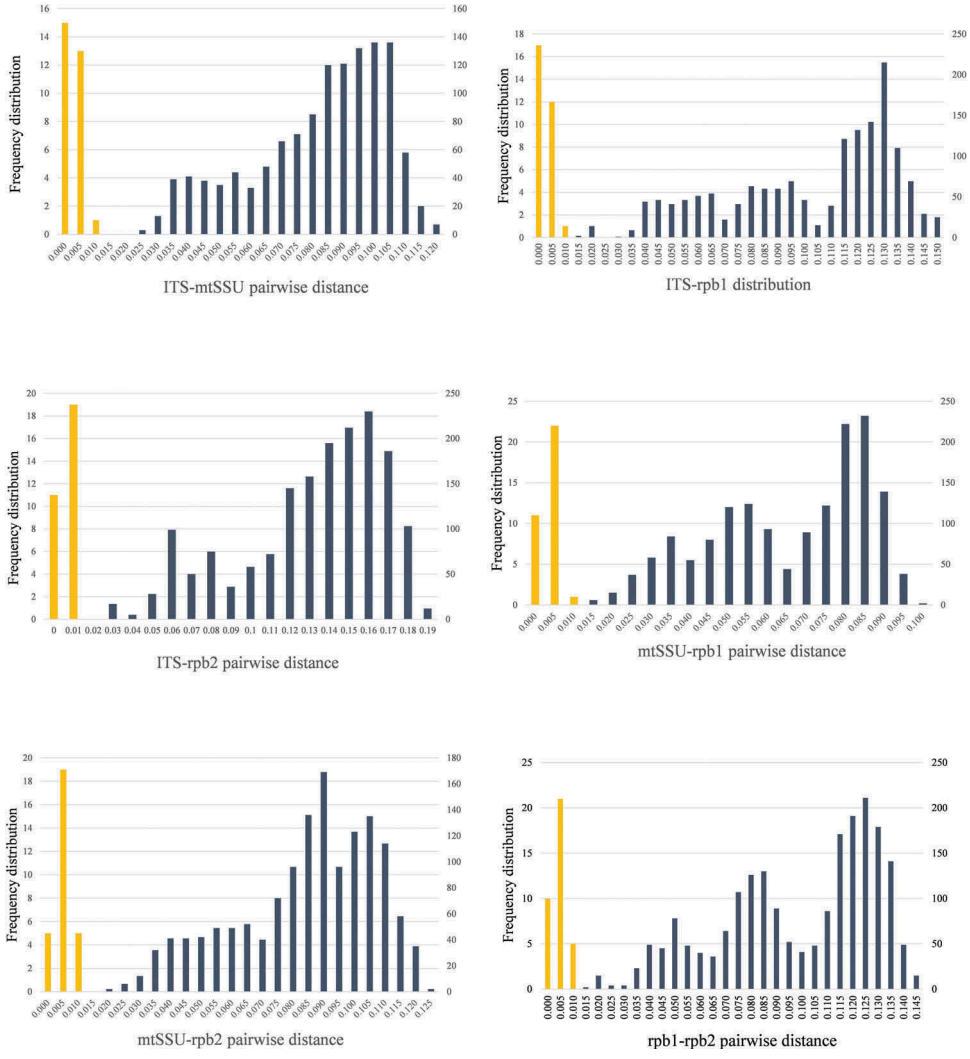

Figure 4. Comparisons of frequency distribution ofintra- and inter-specific variation pairwise distances among *ITS-mtSSU, ITS-rpb1, ITS-rpb2, mtSSU-rpb1, mtSSU-rpb2* and *rpb1-rpb2* gene combinations of *Russula* generated by MEGA and Excel. The inter- and intra-specific distances are presented as yellow and blue bars respectively.

Table 2. Clustering at a given threshold of the candidate genes of *Russula* DNA barcode derived using TaxonDNA/species identified.

Candidate genes	Largest intra-specific distance	Number of cluster	Corresponding to species taxa
ITS	1.06%	35	35(100%)
nLSU	2.95%	8	36 (22.2%)
tef-1α	2.58%	33	35(94.3%)
mtSSU	1.30%	32	32(100%)
rpb1	1.09%	36	36(100%)
rpb2	2.02%	37	37 (100%)
ITS-mtSSU	0.59%	32	29 (100%)
ITS-rpb1	0.79%	33	33 (100%)
ITS-rpb2	0.76%	36	34 (100%)
mtSSU-rpb1	0.89%	31	31 (100%)
mtSSU-rpb2	1.44%	31	31 (100%)
rpb1-rpb2	1.23%	35	35 (100%)

Table 3. PCR and sequencing successful rate of the candidate genes.

Candidate genes	PCR	Sequencing	PCR and sequencing
ITS	98.3%	89.6%	88.1%
nLSU	100%	94.9%	94.9%
tef-1α	100%	93.2%	93.2%
mtSSU	94.9%	84.0%	79.7%
rpb1	93.2%	87.1%	81.2%
rpb2	93.2%	94.5%	88.1%

(0.08), *ITS-rpb2* (0.02), *mtSSU-rpb1* (0.05), *mtSSU-rpb2* (0.01), and *rpb1-rpb2* (0.90). All of these results are equal or greater than 0.01. So it is suggested that the individual partitions of these gene combinations were congruent.

Discussion

The two vital conditions for DNA barcode evaluation are sufficient intra- and inter-specific variation, as well as high PCR and sequencing success rates (Zhao et al. 2011a, 2011b; Zeng 2012; Zhu et al. 2014). Taking both these standards into consideration, the use of *ITS* was considered to be an adequate primary *Russula* DNA barcode *in situ*ations of single gene analysis. We found that *ITS* had relatively high PCR and sequencing rates (Table 3), and that all the species used in this analysis could be recognised, when this gene was targeted (Table 2). Targeting *ITS* as the universal fungal DNA barcode has also been previously suggested (Seifert 2009; Schoch et al. 2012). Although no overlap was observed between the intra- and inter-specific distances in *ITS* (Figs. 1 and 6), the gap between the two variations was narrow (Figure 2). Gene combinations were, thus, considered necessary to get sufficient resolution at the species level.

Our analyses showed that the *ITS-rpb2* combination could act as a suitable DNA barcode for the genus *Russula*, demonstrating the best performance as a DNA barcode for various *Russula* species. First, there were suitable intra- and inter-specific variations (Figs. 3 and 4) with the DNA barcode gap being the largest among all candidate genes and gene combinations analysed. In addition, this gene combination recognised all 34 *Russula* species. This conclusion was also supported by the analysis using TaxonGap (Slabbinck et al. 2008) and SpeciesIdentifier in TaxonDNA (Meier et al. 2006), as shown in Table 2. Second, the PCR amplification and sequencing success rates were relatively higher in *ITS* and *rpb2* (88.1% in Table 3). This combination was, thus, recommended as the primary DNA barcode for the genus *Russula in situ*ations where multigene analysis may be performed. Our analyses also suggested that the combination of *mtSSU-rpb2* was the best DNA barcode substitute for identifying *Russula* when PCR or sequencing targeting *ITS* was unsuccessful because of the gap between intra- and inter-species variation (Figs. 3 and 4).

The nuclear large subunit ribosomal RNA gene (*nLSU*) has often been analysed to elucidate the phylogenetic relationships of fungal groups at the generic or higher taxonomic ranks (Johnson and Vilgalys 1998). It has also been suggested to be the most appropriate DNA barcode for yeast-like fungi (Kurtzman and Robnett 1998; Fell et al. 2000; Ninet et al. 2003). Of the 36 species involved in this study, only six were recognised as a single cluster when analysed through TaxonDNA (Table 2). Although targeting *nLSU* had the highest PCR and sequencing success rates (Table 3), our analyses indicated that *nLSU* was not a suitable DNA marker because of its inability to specifically recognise *Russula* species (Figs. 1, 2 and 5). *nLSU*, thus, failed to act as the target DNA barcode for this genus.

Another gene often used in fungal phylogenetic analyses is *tef-1α* (Jaklitsch et al. 2006; Stenglein et al. 2010; Zhao et al. 2016, Zhao et al. 2017; He et al. 2017), which had the second highest PCR and sequencing success rates (Table 3). This gene has previously been regarded as the target DNA barcode in certain groups (Geiser et al. 2004; Druzhinina et al. 2005; Li et al. 2013); however, our analyses showed that *tef-1α* the occurrence of overlap between intra- and inter-species

variation among the candidate genes (Figs. 1, 2 and 7) was the highest for this gene. For this reason, *tef-1α* was excluded as the target DNA barcode for *Russula*.

The genes of the first and second largest RNA polymerase II subunits (*rpb1* and *rpb2*) and the mitochondrial small subunit (*mtSSU*), which have been commonly analysed in fungal phylogeny (Matheny et al. 2007; Nordin et al. 2010; Stenglein et al. 2010; Sekimoto et al. 2011; Chen et al. 2012), were also employed as candidate biomarkers for this study. Overlap between intra- and inter-species variation was detected in both *mtSSU* and *rpb2* (Figs. 1, 2, 8 and 10). For *rpb1*, although no overlap was observed (Figs. 1, 2 and 9), the low minimum inter-specific variation (1.2%) made the gap between the two variations too narrow (Figs. 1 and 2). The gene *rpb1* also had relatively low PCR and sequencing success rates (81.2%, Table 3), which further hampered its practicality as an eligible DNA barcode.

Our results indicate that *ITS-rpb2* combination meets the requirements for a good DNA barcode for *Russula*. The barcode gap of this combination is visible in Fig. 4. It is much wider than that of *ITS* in Fig. 2, which is invisible in the same abscissa axis. For single genes, *ITS* and *nLSU* possessed high PCR and sequencing rates, but the gap between inter- and intra-specific variations of *ITS* was narrow, *nLSU* is inefficient in specific recognition. Overlapping occurred between the two variations in *tef-1α, rpb2, mtSSU*, and *nLSU*, which may lead to misidentification. PCR and sequencing success rates are relatively low in *mtSSU* and *rpb1*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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