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Phylogenetic Diversity of Wild Edible *Russula* from Northeastern Thailand on the Basis of Internal Transcribed Spacer Sequence

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ABSTRACT: Phylogenetic relationships among wild edible *Russula* in northeastern Thailand were demonstrated on the basis of the sequence polymorphisms in the internal transcribed spacer (ITS) region of the nuclear ribosomal genes (rDNA). Using PCR-RFLP analysis, they could be divided into three main clusters. ITS sequences of some specimens were also analyzed. The ITS sequences clearly distinguished most specimens from each other, even those sharing similar morphotypes. This approach might be used for further investigation of ectomycorrhizal associations in the forest regeneration programme in Thailand.

KEYWORDS: diversity, *Russula*, Internal Transcribed Spacer (ITS), rDNA, PCR-RFLP.

INTRODUCTION

Mushrooms are regarded as edible and even highly desirable in many areas of the world. In Thailand, local people collect edible mushrooms in fields and forests and purchase them in local markets. In the northeastern part of Thailand, where the forests are still the source of varieties of wild edible mushrooms. Species of *Russula* are among the most popular. *Russula* is a genus of Basidiomycota, belonging to Russulales and distinct from many other edible mushrooms of other orders[1]. The biodiversity of edible *Russula* in Thailand has still not been well investigated due to a lack of involved experts and pure cultures of *Russula* species . Although *Russula* has been widely recorded in Thailand together with references as to the edibility of various species, most of the names reported refer to species collected from the Americas and Europe. The mushrooms available in Southeast Asia are not well defined[2]. The species reported in Thailand include *Russula alboareolata* Hongo, *R. cyanoxantha* Schaeff. Ex Fr., *R. delica* Fr., *R. densifolia* (Secr.) Gill., *R. emetica* (Schaeff. Ex Fr.) Pers. Ex S.F. Gray, *R. foetens* Fr., *R. heterophylla* Fr., *R. nigricans* (Bull.) Fr., *R. rosacea* Pers. Ex S.F. Gray and *R. virescens* Fr[3]. Most of these are edible when cooked[2,4].

The emergence of phylogenetic mycology as a paradigm for fungal biology studies has been greatly accelerated by numerous advancement in phylogenetic methods, especially in the area of molecular systematics. Russulaceae were defined by reticulate spores and heteromerous trama with spherocyst, and were excluded from the order Agaricales along with Boletaceae, when rDNA sequences were analysed[5]. Moreover, *Russula* were also excluded from gilled mushrooms in the euagaries clade by phylogenetic analysis of nuclear and mitochondrial DNA sequences[6]. The internal transcribe spacer (ITS) is one region of the nuclear ribosomal RNA which has been extensively used in molecular systematics. Due to its higher degree of variation than that of the small subunit (SSU) and large subunit (LSU) of rRNA genes, variation among individual rDNA repeats can sometimes be observed within the ITS[7,8]. Thus, it has become one of the most widely used genomic regions for the identification of the biodiversity in various fungal groups, such as shitake mushroom (*Lentinula, Tricholomataceae*), *Ganoderma lucidum* complex, and *Suillus sensu lato*[5,9,10]. Therefore, elucidation of the interspecies biodiversity of edible *Russula* in Thailand by analysis of their ITS region was the focus of this study.

MATERIALS AND METHODS

Sample collection

Fresh specimens of edible *Russula* (n=22) were collected from markets in 17 province in the

northeastern part of Thailand. Common characteristics (e.g. cap colour, rounded to depressed caps, gills that easily break and crumble, adnate and decurrent gills, spore printing and non-latex production property) were investigated. In addition, blue amyloid spore reaction in Melzer's solution and heteromerous trama were also determined at 400x magnification. In order to avoid contamination from the environment, the fresh fruit body tissue, which was removed from the inner part of fruiting body, such as the stalk, was used as specimen. The collected tissue (0.1-0.3 g wet weight) was then kept in the lysis buffer (50 mM Tris–HCl, pH 7.2, 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol) [7]. Specimens could be stored at room temperature for several months.

Genomic DNA Extraction

To extract the genomic DNA, the collected tissue was ground in liquid nitrogen, then added to 400 ml of lysis buffer and mixed well. The mixture was incubated at 65°C for 1 h followed by phenol-chloroform extraction[7]. DNA was precipitated by cold 95% ethanol and washed with 70% ethanol before air dryinng. The DNA pellet was resuspened in TE buffer containing RNase A (100 mg/ml).

ITS-RFLP Analysis

To compare the relationship between a morphotypic feature (such as cap colour) and ribotyping patterns, PCR-RFLP analysis of ITS was conducted. Genomic DNA was used as a template in the PCR amplification. The PCR solutions included 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 mM of each dNTP, and 2.5 U *Taq* polymerase (Gibco BRL®, Invitrogen, Carlsbad, CA, USA), 0.5 µM each of ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') primers that amplify both ITS regions, including the 5.8 S rDNA[7]. Nucleotide sequences of *R. alboareolata* (700 bp, AF345247), *R. rosacea* (800 bp, AF345249), *R. cyanoxantha* (650 bp, AF345251), *R. densifolia* (700 bp, AF350068), *Russula* RG-2 (750 bp, AF345250), *Russula* RW-3 (800 bp, AF345248), *Russula* RP-3 (700 bp, AF345252), *Russula* RG-5 (750 bp, AF350069), and *Lactarius volemus* (650 bp, AF354455) were deposited in the GenBank. The ITS region was separately digested with *Alu* I, *Hinf* I, *Mbo* I and *Taq* I (Boeringher Mannheim, Indianapolis, IN, USA). The restriction products were electrophoresed in a 2% agarose gel at 80 volts. The gels were documented using a gel documentation system (UVP Corp., Upland, CA, USA).

Data Analysis

The restriction fragments of the different mushrooms were compared manually and then subjected to analysis. Restriction fragments shorter than 50 bp were not visualized on this gel, thus the sum of the restriction fragments may be smaller than that of the undigested fragment. A dendrogram of the ITS-RFLP analysis was constructed from the similarity matrix by the unweighted pair group method with arithmetic

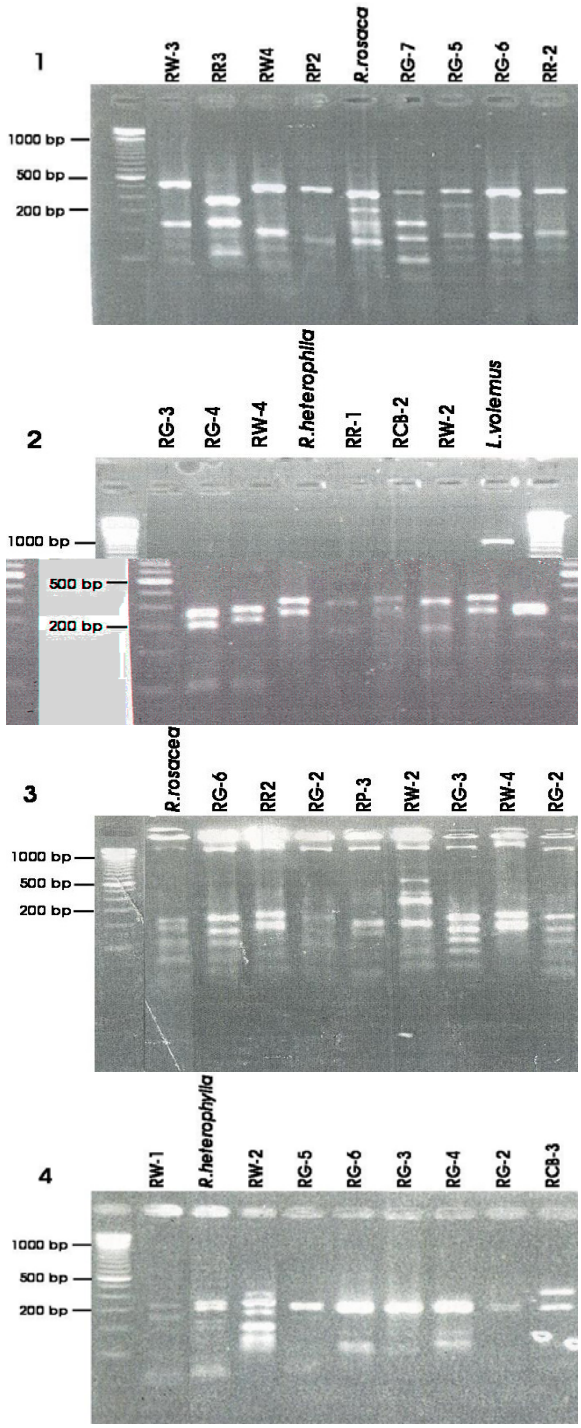

Fig 1. Examples of PCR-RFLP of ITS regions of edible mushroom digested with *Alu* I (1), *Hinf* I (2), *Mbo* I (3) and *Taq* I (4).

Table 1. Restriction fragment sizes of ITS regions of edible *Russula* digested with *Alu* I, *Hinf* I, *Mbo* I and *Taq* I.

Specimen (Herbarium No.)	ITS size (bp)	Fragment size (bp)			
		Alu I (bp)	Hinf I (bp)	Mbo I (bp)	Taq I (bp)
Rd. SUT-1 (B)	700	400, 100	350	300, 200, 100	300, 200
Rc. SUT-1 (P)	650	500, 150	350, 300	300, 200, 150, 100	250, 200
Rp. SUT-2	700	500, 200	380, 320	600, 120, 80	300, 200, 100
Rp. SUT-3	700	500, 200	400, 280	300, 200	280, 220, 150, 100
Rh. SUT-1 (CB)	800	400, 200	400, 200	300, 200, 100	280, 220, 150, 100
RCB. SUT-2	800	400, 200	350, 300	280, 220, 150, 100	300, 200, 150, 100
RCB. SUT-3	800	450, 200	380, 300	300, 200	300, 200
Ra. SUT-1 (A)	700	450, 100	400, 280	300, 200	300, 200, 100
RW. SUT-2	650	420, 300, 150, 120	350, 300	300, 200, 100	280, 200, 150, 100
RW. SUT-3	800	500, 200	350, 300	180, 100 ,50	350, 250, 100
RW. SUT-4	750	500, 200	400, 300	300, 200, 150, 100	350, 250, 100
RW. SUT-5	750	500, 200	380, 320, 100	300, 220, 150	300, 200
Rr. SUT-1 (R)	800	400, 200	380, 300	300, 280	300, 280
RR. SUT-2	700	450, 200	380, 300	320, 208, 100	400, 200
RR. SUT-3	700	350, 200, 150	300, 200, 100	300	400, 200, 150
Rv. SUT-1 (G)	700	450, 200	380, 300, 100	320, 280, 100	400, 250, 100
RG. SUT-2	750	500, 250	380, 300, 100	400, 200	300, 280
RG. SUT-3	750	500, 220	380, 300, 100	400, 250, 100	400, 180, 120
RG. SUT-4	750	400, 200	350, 300, 100	400, 180, 120	200, 100
RG. SUT-5	750	700, 500, 200	380, 320	400, 200, 150	400, 200, 150
RG. SUT-6	700	420, 300, 150, 120	380, 300, 100	400, 200, 150	400, 200, 150
RG. SUT-7	700	450, 200	380, 300, 100	400, 180, 120	400
LC. SUT1	700	420	420, 280	350, 200, 150	320, 280, 100

mean (UPGMA) using the Ntsys program version 2.1 (Exeter Software, Setauket, NY). Alignments and a neighbor-joining phylogenetic analysis were constructed with AliBee-Multiple Alignment Release 2.0[11]. Reproducibility of phylogenetic groups was estimated by bootstrapping with 1,000 replications.

Results and Discussion

Estimation of genetic similarity/distance of a particular DNA segment with PCR-RFLP is based solely on shared restriction fragments. As a result, the amplification product showing different sizes between taxa (as in the present study) may bias the similarity/distance levels. The use of restriction endonuclease cutting site analysis would provide more accurate data. Using the restriction enzyme *Alu* I produced 15 RFLP patterns, while *Hinf* I, *Mbo* I and *Taq* I generated 11, 10 and 11 patterns, respectively. The restriction fragment sizes of ITS digested with these respective enzymes are summarized in Table 1 and examples of ITS-RFLP profile are depicted in Fig. 1. The combination of profiles of each *Russula* across four restriction enzymes produced 22 ribotypes (Table 2). The goodness of fit test of cluster analysis was carried out by comparing cophenetic value matrices with the original similarity matrix clustered by UPGMA, and was not significantly different ($P>0.05$). From the dendogram in Fig.2, the *Russula* in this study could be divided into 3 main clusters. Cluster I indicated that *R. densifolia* and *Russula* RCB-2 were more closely related to *Lactarious volemus* than other *Russula* strains. Clusters II and III included *Russula* exihibiting a great variety of cap colours. However, most of the green *Russula* appeared in the cluster II with the exception of *Russula* RG-7. In cluster III, *Russula* RW-4, which has a white cap, appeared very closely related to *R. cyanoxantha*, which has a purple cap. These results revealed only partial correlation between *Russula* cap colours and ITS-RFLP patterns. The pictures of wild edible *Russula* grouped with ITS-RFLP are shown in Fig 3.

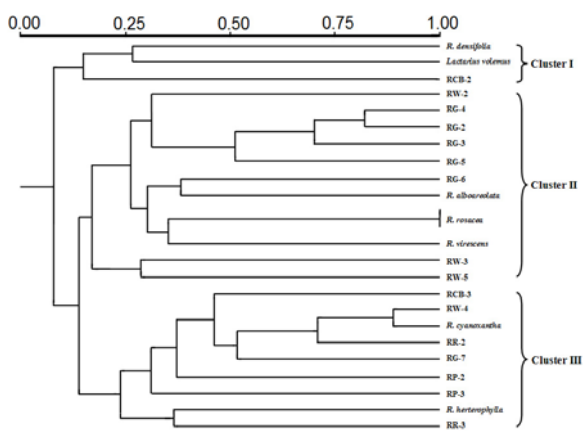

Fig 2. UPGMA dendrogram constructed from similarity indices of various *Russula* collections and an outgroup of *Lactarius volemus* derived from ITS-4/5-PCR-RFLP analysis.

Table 2. Ribotypes and cap colours of edible *Russula* from the Northeast of Thailand.

Cap Colour	Specimen (Herbarium No.)	Identified species/ Code No.	ITS-RFLP group			
			Alu I	Hinf I	Mbo I	Taq I
Black	Rd. SUT-1 (B)	R. densifolia	A-1	H-1	M-9	T-1
Purple	Rc. SUT-1 (P)	R. cyanoxantha	A-12	H-3	M-2	T-8
	Rp. SUT-2	RP-2	A-6	H-2	M-4	T-8
	Rp. SUT-3	RP-3	A-10	H-11	M-8	T-4
Cream to Brown	Rh. SUT-1 (CB)	R. heterophylla	A-15	H-4	M-7	T-3
	RCB. SUT-2	Rcb-2	A-15	H-3	M-5	T-6
	RCB. SUT-3	Rcb-3	A-14	H-7	M-2	T-2
White	Ra. SUT-1 (A)	R. alboareolata	A-3	H-11	M-3	T-9
	RW. SUT-2	RW-2	A-7	H-3	M-5	T-7
	LC. SUT-1	L. volemus	A-16	H-12	M-11	T-9
	RW. SUT-3	RW-3	A-8	H-3	M-3	T-3
	RW. SUT-4	RW-4	A-8	H-9	M-3	T-8
Red	RW. SUT-5	RW-5	A-8	H-3	M-4	T-7
	Rr. SUT-1 (R)	R. rosacea	A-15	H-7	M-2	T-4
	RR. SUT-2	RR-2	A-14	H-7	M-2	T-4
Green	RR. SUT-3	RR-3	A-13	H-5	M-2	T-11
	Rv. SUT-1 (G)	R. virescens	A-14	H-8	M-5	T-2
	RG. SUT-2	RG-2	A-11	H-8	M-5	T-2
	RG. SUT-3	RG-3	A-4	H-8	M-6	T-2
	RG. SUT-4	RG-4	A-5	H-10	M-10	T-2
	RG. SUT-5	RG-5	A-2	H-2	M-1	T-4
	RG. SUT-6	RG-6	A-14	H-8	M-4	T-5
	RG. SUT-7	RG-7	A-9	H-8	M-1	T-10

Results obtained from ITS-RFLP patterns of *Russula* revealed that it was useful to recognize each collection as distinct taxa. However, the resolution of ITS PCR-RFLP is not great enough to ensure identification of species of the same section or even subgenus of *Lactarius ectomycorrhizae*[12]. The occurrence of varying copies of rDNA within a genome alone is not unusual[13]. This phenomenon was supported by the numerous reports which found that restriction analysis of ITS sequences of arbuscular mycorrhiza fungi have a relatively high level of heterogeneity even within a single spore[14]. In addition, Zézé *et al.*[15](1997) found the heterokaryotic status of the nuclear population within a spore azygospores or chlamydospores. In filamentous fungi, nuclear rDNA genes are present in tandem repeats ranging from about 60 copies in *Coprinus* to 220 in *Neurospora crassa*[16]. This study also found similar results based on the size of digested product and undigested single band of amplified ITS (Table 1). Some specimens showed the sum of fragment lengths greater than the amplified ITS, even when complete digestion was confirmed. This might be due to the difference in ITS copy number in each specimens. This was also found in this study as a distinction was seen between *Russula* RW-3 and *R. alboareolata* by using ITS PCR-RFLP. Nevertheless, both of them indeed appeared to be the same species when nucleotide sequences were compared. The ITS sequence results suggested that sequence analysis yields groups that relate more closely to cap colours and taxonomy than those from PCR-RFLP of the same DNA segment.

The phylogenetic tree constructed only from aligned sequences of 8 *Russula* and 1 *L. volemus* is depicted in Fig. 4. The results obtained from ITS sequence analysis were fundamentally similar to ITS-RFLP analyses. For example, *Russula* RG-2 and *R. alboareolata* belonged to the same group in both analyses. However, *Russula* RP-3 and *R. cyanoxantha* was more closely related to *R. rosaceae* than that indicated in Fig. 2 in which *L. volemus* was in a different cluster from *R. densifolia*. In addition, we found RG-5 as an outgroup when the ITS sequences were considered. This indicated that saturated mutations were found when the ITS gene regions were used for distantly related taxa. When the ITS sequence similarity was compared among the strains which had similar cap colour morphotypes, it was found that the white cap strains *R. alboareolata* and RW-3 shared 100% identical, while RP-3 and *R. cyanoxantha* exhibited 65% similarity. As sequence analysis becomes less expensive and more widely available, the usefulness of labor-intensive ITS-RFLP analysis will decline. However, comparisons of sequences of Thai *Russula* still have not been made with those from North America or Europe. Data and approaches obtained from this study will be useful for further investigation of ectomycorrhizal associations.

Since symbiosis plays an important role in the biology and ecology of forest trees, affecting growth, water and

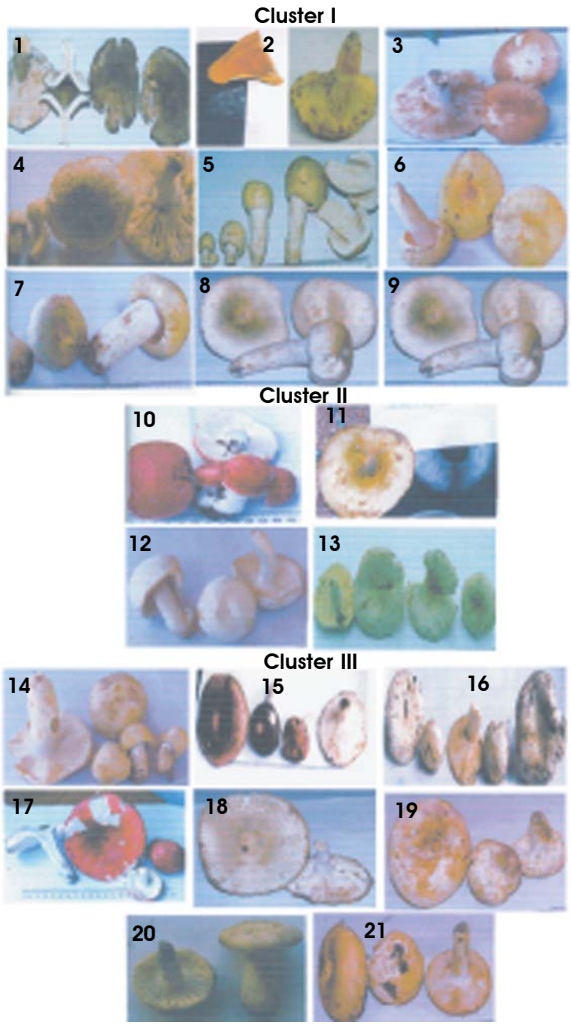

Fig 3. Fresh specimens of edible *Russula* collected from the northeastern part of Thailand; 1) *R. densifolia*, 2) *L. volemus*, 3) RCB-2, 4) RG-4, 5) RG-2, 6) RG-3, 7) RG-5, 8) RG-6, 9) *R. alboareolata*, 10) *R. rosacea*, 11) *R. virescens*, 12) RW-3, 13) RW-5, 14) RCB-3, 15) *R. cyanoxantha*, 16) RW-4, 17) RR-2, 18) RG-7, 19) RP-2, 20) RP-3, and 21) *R. heterophylla* phylogenetically allocated to cluster I (1-9), II (10-13) and III (14-21), respectively.

nutrient absorption, and providing protection from root diseases, this approach may be useful , since it allowed investigation of genetic relationships between genets and within progenies in natural fungal populations with high efficiency[17,18,19]. Several procedures (rDNA typing, RAPD, inter-repeat PCR) allow the rapid identification of ectomycorrhizal isolate using PCR of DNA extracted from vegetative mycelium and fruiting bodies, as well as a single ectomycorrhizal root tips. Therefore, identification of the *Russula* symbionts of indigeneous tree species in Thai forrests, which have been poorly investigated, might be

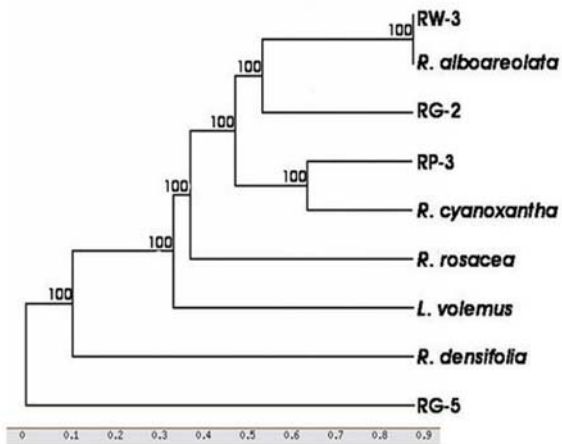

Fig 4. A phylogenetic tree indicating relationships of some *Russula* inferred from ITS nucleotide sequences. Numbers at the nodes indicate the number of bootstrap results that replicated the node determined from the original data out of 1,000 bootstrap replicates.

achievable and of ecological value.

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