

The phylogeny of *Russula* section *Compactae* inferred from the nucleotide sequences of the rDNA large subunit and ITS regions

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Abstract

Phylogenetic relationships within *Russula* section *Compactae* were investigated using sequence data from the nuclear-encoded large subunit ribosomal DNA (n-LSU rDNA) and ITS region, including the 5.8 S rDNA. Forty-two sequences of the n-LSU rDNA and forty-nine sequences of the ITS region with outgroups were used in this study. Analysis of the n-LSU rDNA indicated that the *Compactae* section was divided into three large groups: group A comprised *R. densifolia* and *R. adusta*, group B comprised *R. subnigricans*, and group C comprised *R. nigricans*. The ITS region did not support the monophyly of group B. Subgroup B-5 was sister to all other taxa of the *Compactae* section in the NJ and MP trees, and subgroup B-1 grouped with group C. Consequently, *R. densifolia* and *R. adusta* (group A) and *R. nigricans* (group C) were considered largely monophyletic, but *R. subnigricans* (group B) was not monophyletic. *Russula densifolia* (group A), but not *R. adusta*, were divided into six different subgroups.

Key Words: ITS region, nuclear LSU rDNA, *Russula densifolia*, *Russula nigricans*, *Russula subnigricans*

Introduction

The genus *Russula*, which contains 450 species, represents one of a group of fresh fungi that is distributed throughout the world¹⁴⁾. Most species of *Russula* form obligatory autotrophic mycorrhiza with many types of forest trees of the genera *Pinus*, *Abies*, *Picea*, *Larix*, *Tuga*, *Quercus*, and *Castanopsis*, which are distributed from the tropic to frigid zones. *Russula* species are also commonly found in Japan. Although approximately 70 *Russula* species have been recorded in Japan, three-times more unknown species may exist in this country¹²⁾.

Romagnesi²¹⁾ divided the *Russula* genus into two subgenera: subgenus *Compacta*, which includes the sections *Nigricatinae*, *Archaeinae*, and *Plorantinae*, and the subgenus *Genuinae*. Singer²⁷⁾ divided the *Russula* genus into eleven sections, including the section *Archaeinae*. The subgenus *Compacta* sensu Romagnesi²¹⁾ was divided into three sections, viz. *Archaeinae*, *Plorantes*, and *Compactae*, as by Singer²⁷⁾. Species belonging to the *Compactae* section commonly possess the following characteristics: compact flesh that often becomes pink, bruises, blackens, or at least remains gray; is intermixed with lamellae; and has a suprahilar unamyloid spot on the spores. These characteristics mainly firm the flesh and the intermixed lamellae have been considered primitive^{21, 22)}. Although Singer²⁷⁾ stated that the species of the *Compactae* section are more common in the northern area than in the southern and tropical areas, Buyck³⁾ stated that the *R. nigricans*-group appears to be primarily adapted to warm climates. In Japan, the *Compactae* section comprises six

species, *R. nigricans* Fr., *R. densifolia* Gill., *R. subnigricans* Hongo, *R. adusta* (Pers.) Fr., *R. dissimulans* Shaff., and *R. albonigra* (Krombh.) Fr. based on the color change of the fresh fruit body after cutting or bruising, the width of the lamellae, and the degree of acrid in the lamellae^{12, 13, 20}. Because the fruit body has distinctive variations in color and macro-morphological characteristics, differentiating these species of the *Compactae* section from other *Russula* spp. is easy. However, it is difficult to evaluate the differences among these species at the species or intra-species levels. According to Imazeki and Hongo¹², several *Russula* spp., such as *R. densifolia*, have close lamellae and blacked flesh after cutting. Similarly, *R. subnigricans* is divided into several subspecies based on its poisonousness. Although the species delimitation of ectomycorrhizal mushrooms containing *Russula* has been based on the morphology of the fruit bodies, we cannot exclude the possibility of convergence in morphology. Recent advances in molecular phylogeny using the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA)^{5, 8, 15, 16} and large-subunit (LSU) rDNA^{8, 15, 16, 24, 25, 26} have allowed for investigation of the phylogeny of a variety of organisms at the molecular level. In the present study, we used the nuclear LSU rDNA and ITS region for classification and phylogenetic analyses of the *Compactae* section.

Materials and methods

Sample sources

Sixty-nine samples were used in this study, and 65 of the samples belonged to the *Compactae* section of the *Russula* genus. Collection locations, specimen numbers, and DDBJ accession numbers of the 57 samples that were sequenced in this study are shown in Table 1. Eight samples that were retrieved from databases (DDBJ, EMBL and GenBank) are shown in Table 2.

The D 1 and D 2 region sequences of the LSU rDNA were determined for 35 of the 65 samples that were analyzed in the *Compactae* section¹¹, and three additional sequences derived from fruit bodies (*R. densifolia*; AF 325304, *R. nigricans*; AF 325312 and *R. adusta*; AF 218544) were retrieved from the DDBJ DNA database. The sequences of *R. delica* (AB 154700), *R. chloroides* (AB 154698 and AB 154699), and *R. compacta* (AB 154697) were used as outgroup taxa based on our preliminary analysis²⁵. The ITS sequences were determined for 43 samples, and five additional sequences (*R. nigricans*: AF 418607, AY 061695, AY 228357 and *R. densifolia*; AF 418606, AY 60691) were retrieved from the DDBJ database. The sequences of *R. compacta* (AB 291725) were used as outgroups.

The specimens were dried and kept in the Natural History Museum of Osaka City (OSA-MY), Osaka, Japan. The fungal species were identified based on the Colored Illustration of Mushrooms of Japan II¹², illustrated books of mushrooms from foreign countries^{2, 4, 19}, and monographs of *Russula*^{22, 23}.

DNA extraction and PCR amplification

DNA was extracted from lamellae of the fruit body using the method reported by Suyama et al.²⁸. Dried lamellae (0.5 mg) were placed on a siliconized glass slide and homogenized between two glass slides in 10 μ l of extraction buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% sodium dodecyl sulfate (SDS), and 0.01% proteinase K]. The homogenate was placed in 300 μ l extraction buffer in a 1.5-ml microtube and incubated at 37°C for 1 h and then at 95°C for 10 min. The extract was mixed vigorously and then centrifuged at 15000 g for 5 min. The supernatant was transferred to another tube and used as template DNA.

The LSU rDNA region was amplified two or three times by polymerase chain reaction (PCR) using nested primer sets in 50- μ l volumes, as previously described¹⁰. A negative control that lacked template

Table 1 *Russula* species sequenced in this study

Taxon	Collection No.	Extract No.	Origin	Accession No.		Subgroup
				LSU	ITS	
Section <i>plorantes</i>						
<i>R. chloroides</i>	OXA-MY-1710	358	Japan, Minoo city	AB154697	--	
<i>R. chloroides</i>	OXA-MY-1711	343	Japan, Fuchuu city	AB154698	--	
<i>R. delica</i>	OXA-MY-1712	156	Japan, Neyagawa city	AB154700	--	
Section <i>Crassotunicatae</i>						
<i>R. compacta</i>	OSA-MY-1713	206	Japan, Kyoto city	AB154701	AB291725	
Section <i>Compactae</i>						
<i>R. nigricans</i>	OSA-MY-1718	195	Japan, Katano city	AB154706	AB291726	C-1
<i>R. nigricans</i>	OSA-MY-4255	163	Japan, Neyagawa city	AB291708	--	C-1
<i>R. nigricans</i>	OSA-MY-4256	255	Japan, Nagano pref.	--	AB291727	C-1
<i>R. nigricans</i>	OSA-MY-4257	355	Japan, Fuchuu city	--	AB291728	C-2
<i>R. nigricans</i>	OSA-MY-4258	465	Japan, Kouriyama city	--	AB291729	C-1
<i>R. nigricans</i>	OSA-MY-1719	356	Japan, Uji city	AB154707	AB291730	C-1
<i>R. nigricans</i>	OSA-MY-1720	398	Japan, Sendai city	AB154708	--	---
<i>R. nigricans</i>	OSA-MY-1721	4010	Japan, Katano city	AB154709	--	C-1
<i>R. dissimulans</i>	OSA-MY-1727	354	Japan, Saitama pref.	AB154717	AB291731	C-2
<i>R. dissimulans</i>	OSA-MY-1728	399	Japan, Musashimurayama	AB154718	--	---
<i>R. subnigricans</i>	OSA-MY-1722	251	Japan, Uji city	AB154710	AB291732	B-1
<i>R. subnigricans</i>	OSA-MY-1723	252	Japan, Uji city	AB154711	AB291733	B-3
<i>R. subnigricans</i>	OSA-MY-1724	253	Japan, Uji city	AB154712	AB291734	B-1
<i>R. subnigricans</i>	OSA-MY-1725	261	Japan, Shimonoseki city	AB154713	AB291735	B-1
<i>R. subnigricans</i>	TNS-F-237524	391	Japan, Kyoto city	AB154714	AB291736	B-1
<i>R. subnigricans</i>	TNS-F-237524	392	Japan, Kyoto city	AB154715	--	B-1
<i>R. subnigricans</i>	OSA-MY-1726	407	Japan, Mie pref.	AB154716	AB291737	B-3
<i>R. subnigricans</i>	OSA-MY-1729	342	Japan, Fukushima pref.	AB154719	AB291738	B-2
<i>R. subnigricans</i>	OSA-MY-0801	262	Japan, Ootsu city	--	AB291739	B-3
<i>R. subnigricans</i>	OSA-MY-4259	318	Japan, takarazuka city	--	AB291740	B-3
<i>R. subnigricans</i>	OSA-MY-4260	331	Japan, Sendai city	--	AB291741	B-3
<i>R. subnigricans</i>	OSA-MY-4261	332	Japan, Okayama pref.	--	AB291742	B-3
<i>R. subnigricans</i>	OSA-MY-4262	346	Japan, Tokyo to	AB291709	AB291743	B-4
<i>R. subnigricans</i>	OSA-MY-4263	357	Japan, Minoo city	--	AB291744	B-1
<i>R. subnigricans</i>	OSA-MY-4264	376	Japan, Sendai city	AB291710	AB291745	B-5
<i>R. subnigricans</i>	OSA-MY-4265	408	Japan, Kyoto city	--	AB291746	B-1
<i>R. subnigricans</i>	OSA-MY-4266	4202	Japan, Kouriyama city	--	AB291747	B-5
<i>R. subnigricans</i>	-	4203	Japan, Kouriyama city	--	AB291748	B-5
<i>R. subnigricans</i>	-	4205	Japan, Kouriyama city	--	AB291749	B-2
<i>R. subnigricans</i>	OSA-MY-4267	443	Japan, Minoo city	--	AB291750	B-1
<i>R. subnigricans</i>	OSA-MY-4268	446	Japan, Kyoto city	--	AB291751	B-1
<i>R. subnigricans</i>	OSA-MY-4269	454	Japan, Sendai city	--	AB291752	B-3
<i>R. subnigricans</i>	OSA-MY-4270	461	Japan, Kouriyama city	--	AB291753	B-3
<i>R. subnigricans</i>	OSA-MY-4271	472	Japan, Okazaki city	AB291711	--	B-1
<i>R. subnigricans</i>	OSA-MY-4272	515	Japan, Chiba pref.	AB291712	--	B-3
<i>R. subnigricans</i>	OSA-MY-4273	531	Japan, Kouriyama city	AB291713	--	B-5
<i>R. subnigricans</i>	OSA-MY-4275	533	Japan, Nagano pref.	AB291714	--	B-3
<i>R. subnigricans</i>	OSA-MY-4276	535	Japan, Onoda city	AB291715	--	B-1
<i>R. densifolia</i>	OSA-MY-4277	196	Japan, Kyoto city	--	AB291754	A-4
<i>R. densifolia</i>	OSA-MY-4278	155	Japan, Neyagawa city	AB291716	--	A-6
<i>R. densifolia</i>	OSA-MY-1715	294	Japan, Kashihara city	AB154703	AB291755	A-5
<i>R. densifolia</i>	OSA-MY-1716	352	Japan, Yamanashi pref.	AB154704	--	A-3
<i>R. densifolia</i>	OSA-MY-1717	3510	Japan, Minoo city	AB154705	AB291756	A-7
<i>R. densifolia</i>	OSA-MY-4279	359	Japan, Minoo city	AB291717	AB291757	A-4
<i>R. densifolia</i>	OSA-MY-4280	3910	Japan, Musasimurayama c	--	AB291758	A-5
<i>R. densifolia</i>	OSA-MY-4281	403	Japan, Yamaguchi pref.	AB291718	AB291759	A-5
<i>R. densifolia</i>	OSA-MY-4282	404	Japan, Yamaguchi pref.	--	AB291760	A-4
<i>R. densifolia</i>	OSA-MY-4283	405	Japan, Yamaguchi pref.	--	AB291761	A-4
<i>R. densifolia</i>	OSA-MY-4284	441	Japan, Zadaifu city	--	AB291762	A-7
<i>R. densifolia</i>	OSA-MY-4285	442	Japan, Hyougo pref.	AB291719	AB291763	A-6
<i>R. densifolia</i>	OSA-MY-4286	444	Japan, Fukushima pref.	--	AB291764	A-4
<i>R. densifolia</i>	OSA-MY-4287	455	Japan, Sendai city	AB291720	AB291765	A-8
<i>R. densifolia</i>	OSA-MY-4288	456	Japan, Sendai city	AB291721	AB291766	A-8
<i>R. densifolia</i>	OSA-MY-4289	463	Japan, Kouriyama city	AB291722	AB291767	A-8
<i>R. densifolia</i>	OSA-MY-4290	537	Japan, Kouriyama city	AB291723	--	A-4
<i>R. adusta</i>	OSA-MY-1714	351	Japan, Fujiyoshida city	AB154702	AB291768	A-1
<i>R. adusta</i>	OSA-MY-4291	545	Japan, Fujiyoshida city	AB291724	--	A-9

Herbarium collection is listed with collection number OSA-MY, The Natural History Museum of Osaka City.

Subgroups are decided by Fig. 1 and Fig. 2.

--: two specimen of *R. subnigricans* 4203 and 4205 are missing.

---: LSU rDNA and ITS rDNA sequences in several taxa are not gained.

----: two subgroups of *R. nigricans* are not decided.

Table 2 Accession numbers from database of *Russula* species used in this study

Taxon		Accession No.	
Section	Species	LSU	ITS
Section <i>compactae</i>	<i>R. nigricans</i>	AF325312	-
Section <i>compactae</i>	<i>R. nigricans</i>	-	AF418607
Section <i>compactae</i>	<i>R. nigricans</i>	-	AY228357
Section <i>compactae</i>	<i>R. nigricans</i>	-	AY061695
Section <i>compactae</i>	<i>R. densifolia</i>	AF325304	-
Section <i>compactae</i>	<i>R. densifolia</i>	-	AF418606
Section <i>compactae</i>	<i>R. densifolia</i>	-	AY606961
Section <i>compactae</i>	<i>R. adusta</i>	AF218544	-

-: LSU rDNA and ITS rDNA sequences in several taxa are not found.

DNA was included for each set of reactions. The PCR product was subjected to preparative electrophoresis in a 1.5% agarose gel in TAE buffer. The DNA product was then excised from the ethidium bromide-stained gel and purified using a Jetsorb kit (Genomed, Oeynhausen, Germany) following the manufacturer's instruction. The oligonucleotide primers of the LSU rDNA that were used for amplification were previously described by Mori et al.¹⁷⁾ and Shimono et al.^{24, 25)}. The ITS region was amplified two or three times by PCR using nested primer sets in 50- μ l volumes, similar to the previously method for LSU rDNA. The following four primers were used in this analysis: ITS 1 F, ITS 1, ITS 4 B, and ITS 4^{7, 31)}. To amplify the ITS 1, 5.8 S and ITS 2 regions, the primer set ITS 1 F/ITS 4 B was used for the first amplification, and the partial nested primer set ITS 1/ITS 4 B was used for the second amplification. When sufficient amounts of DNA were not obtained from the second amplification, a third amplification was performed using the partial nested primer set ITS 1/ITS 4.

DNA sequencing and data analysis

Nucleotide sequences of the PCR products were obtained for both strands by direct sequencing with the Genetic Analyzer 373 A (Applied Biosystems, Tokyo, Japan) or DNA sequencer CEQ 2000 XL (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. Primers NL 1, NL 2, NL 3, and TW 14 were used to sequence the LSU rDNA in both directions; primers ITS 1, ITS 3, ITS 2, and ITS 4 were used to sequence the ITS region.

Data analysis

The obtained sequences were initially aligned using the Clustal V package⁹⁾. The alignment was then refined visually using a word processing program with color-coded nucleotides (the data matrix is available upon request from the corresponding author). Phylogenetic trees were obtained from the data using the distance and parsimony methods. For the parsimony analysis, we used the maximum-parsimony (MP) method with a heuristic search using PAUP version 4.0 b 8 a²⁹⁾. This search was repeated 100 times with different random starting points using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. Gaps in the aligned sequences were coded as missing data. The MAXTREES setting was 2000 and tree-bisection-reconnection (TBR) was used as the branch-swapping algorithm. For distance analysis, the most appropriate evolutionary model was determined for a given data set using PAUP* and Modeltest 3.06¹⁸⁾. A starting tree was obtained using the neighbor-joining (NJ) method. With this tree, the likelihood scores were calculated for 56 alternative models of evolution using PAUP*. The output file was then imported to Modeltest to compare the models using Akaike's¹⁾ information criterion (AIC). Once a model of evolution was chosen, it was used to construct phylogenetic

trees with the NJ methods using PAUP*. The strength of the internal branches from the resulting trees was tested by bootstrap analysis using 1000 replications⁶⁾ with the TBR branch-swapping algorithm.

Results

LSU rDNA

Based on 42 LSU sequences that were used in this study, we constructed an alignment dataset of 852 sites that comprised 38 ingroup and 4 outgroup sequences. This dataset was then used for phylogenetic analyses. Of the 852 sites, 34 were variable but uninformative, and 105 sites were phylogenetically informative. An island of the 600 most parsimonious trees of 290 steps, which differed only in the minor branching order of the terminal taxa, was found using MP analysis [consistency index (CI)=0.5345; retention index (RI)=0.8210]. One of the parsimonious trees is shown in Fig. 1. Using Modeltest¹⁸⁾ with Akaike's¹⁾ information criterion (AIC), we concluded that the Tamura-Nei model³⁰⁾ with equal base frequencies, a gamma-distributed rate heterogeneity model (four rate categories, G=0.6360), and an estimated proportion of invariant sites was the most appropriate model of the evolution for this dataset. The NJ tree that was produced using this dataset is not shown because the resulting tree topology was similar to that of the MP tree.

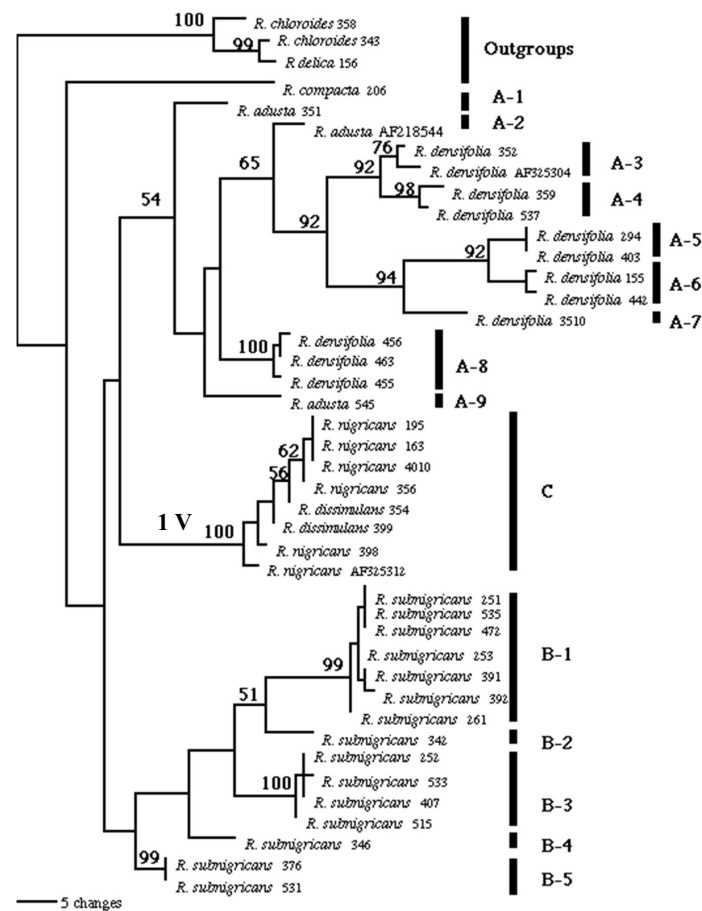


Fig. 1 One of maximum parsimony (MP) trees based on the large subunit rDNA data for 38 samples of the *Compactae* section and four outgroup species. Constant index (CI)=0.5345; retention index (RI)=0.8210. Percent bootstrap support (1000 replications) of more than 50% is shown above the nodes. The symbol (1 V) shows the deletion found between sites 535 and 569 in the *Russula nigricans* group (C)

Three lineages (groups A-C) were found in the *Compactae* section based on the NJ and MP analyses (Fig. 1). Group A comprised *R. densifolia*, which has close lamellae and flesh changes from red to black after cutting, and *R. adusta*, whose flesh changes from pink to gray after cutting, with a low bootstrap value (54%) in the MP tree (Fig. 1). Group B comprised *R. subnigricans*, which has distant lamellae and whose flesh changes to red after cutting, in both NJ and MP trees (Fig. 1), but this group was supported by less than a 50% bootstrap value. Group C, which was supported by a 100% bootstrap value, comprised *R. nigricans*, which has distant lamellae and whose flesh changes from red to black after cutting, and *R. dissimulans*, which has relatively close lamellae, in both NJ and MP trees (Fig. 1).

Fifteen samples belonging to group A that contained *R. adusta* were divided into nine subgroups (A-1-9) in the MP tree (Fig. 1, Table 1). Subgroups A-3 and A-4 grouped with subgroups A-5, A-6, and A-7 with a high bootstrap value (92%) in the MP tree. Subgroup A-8 was composed of a clade with a former middle clade containing five subgroups with a low bootstrap value (Fig. 1). Three *R. adusta*, AF 218544, 545, and 351 were not grouped into one clade. *Russula adusta* AF 218544 grouped with five *R. densifolia* subgroups (A-3-A 7), but *R. adusta* 545 and 351 each formed an independent lineage. Fifteen samples belonging to group B were divided into five subgroups (B-1-5) in the NJ and MP trees. Subgroup B-4 comprised *R. subnigricans* (346); subgroup B-5 comprised *R. subnigricans* (376 and 531); and subgroups B-1, B-3, and B-5 were supported with high bootstrap values in the MP tree (99% or more, Fig. 1). Eight samples of group C (*R. nigricans*), which contained *R. dissimulans*, formed a single monophyletic group in the MP tree with a high bootstrap value (100%).

ITS region

Based on 49 sequences comprising five database sequences and one outgroup sequence, we constructed an alignment dataset of 681 sites. The length of the ITS region, including the 5.8 S rDNA ranged from 606 to 637 bps. We excluded 42 ambiguously aligned sites from the dataset. Of the 639 remaining sites, 58 sites were variable but uninformative, and 183 sites were phylogenetically informative. An island of the 24 most parsimonious trees of 455 steps, which differed only in the minor branching order of the terminal taxa, was found by MP analysis (CI=0.6571; RI=0.9036). One of the parsimonious trees is shown in Fig. 2. Using Modeltest¹⁸⁾ with Akaike's¹⁾ information criterion (AIC), we concluded that the Tamura-Nei model³⁰⁾ with equal base frequencies, a gamma-distributed rate heterogeneity model (four rate categories, G=0.4239), and an estimated proportion of invariant sites was the most appropriate model of evolution for this dataset. The NJ tree is not shown in this paper because it was almost identical to the MP tree.

Seventeen samples belonging to group A, which contains *R. adusta*, were divided into seven subgroups (A-1, A-3-A-8) in the MP tree with a moderate bootstrap value (64%)(Fig. 2). In addition, five subgroups (A-3-A-7) that were found in the LSU rDNA tree (Fig. 1, with 92%) also comprised one large clade in the ITS tree with a high bootstrap value (99%)(Fig. 2). Subgroup A-8 formed a clade with the former middle clade that contained five subgroups with a low bootstrap value (Fig. 2). Fourteen samples belonging to group B were divided into five subgroups (B-1-B-5) in the MP tree (Fig. 2, Table 1). Subgroup B-1 was sister to group C with a moderate bootstrap value (73%). *Russula subnigricans* 376, 4202, and 4203 formed an independent subgroup, B-5, that was sister to all taxa of the *Compactae* section that were used in this study. Subgroups B-2, B-3, and B-4 grouped together with a high bootstrap value (97%), and group B was polyphyletic in the ITS tree. Three subgroups (C-1, C-2, C-3) were found in the ITS tree (Fig. 2, Table 1) with high or moderate bootstrap values (98%, 83%, and 60%, respectively), and subgroup C-2 comprised *R. nigricans* and *R. dissimulans*. The monophyly of group C was also supported in the ITS tree with a high bootstrap value (100%).

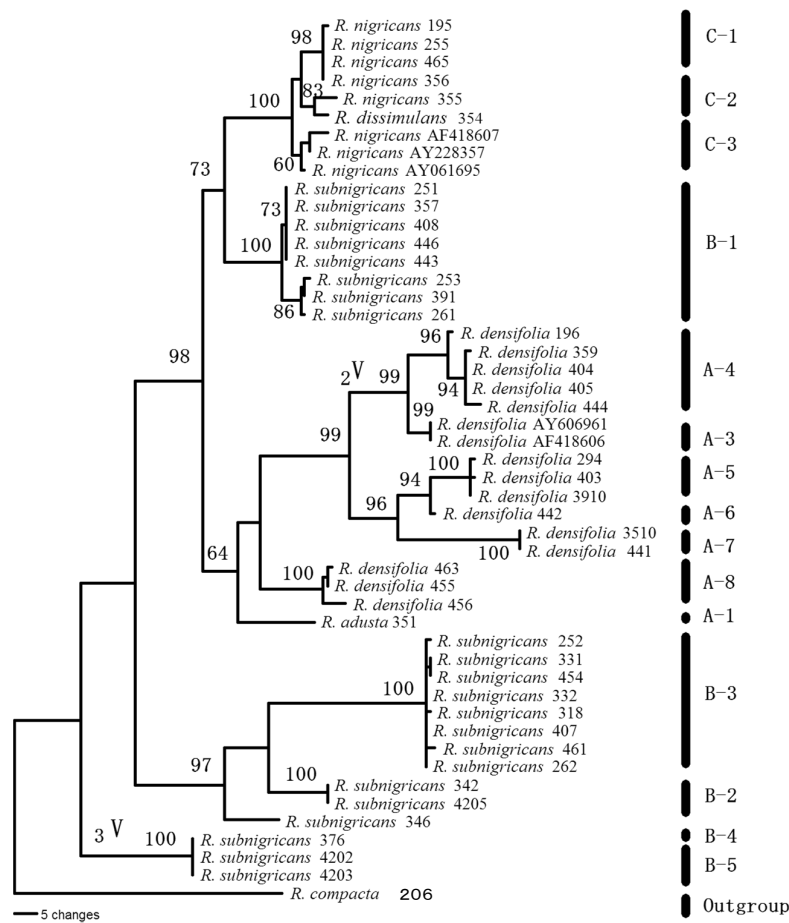


Fig. 2 A maximum parsimony (MP) tree based on the ITS region in the rDNA data for 48 samples of the *Compactae* section and one outgroup species. Constant index (CI)=0.6571; retention index (RI)=0.9036. Percent bootstrap support (1000 replications) equal to or more than 60% is shown above the nodes. The symbol (2 V) shows the deletion found between sites 224 and 245 in *Russula densifolia* (A-3 and A-4). The symbol (3 V) shows the insertion found between sites 172 and 177 in *R. subnigricans* (B-5)

Indels in the LSU rDNA and ITS region

Some deletions and insertions (indels) were found in the LSU rDNA and ITS region, and three long indels were found in the LSU rDNA and ITS region. The indels in the LSU rDNA and ITS region are shown in Figs. 3 and 4, respectively. A 30-bp deletion in the LSU rDNA sequences from samples of *R. nigricans* was observed (group C, Fig. 3). Similarly, a 20-bp deletion in the ITS 1 sequences, which was not found in the other subgroups of *R. densifolia* (A-5, A-6, A-7, and A-8), from subgroups A-3 and A-4 of *R. densifolia* was observed (Fig. 4). These deletions in *R. nigricans* and *R. densifolia* were found in European and Japanese specimens. In addition, 6 bps were inserted into the ITS 1 sequences of subgroup B-5 of *R. subnigricans* (data not shown).

Discussion

Although the topologies between the ITS region and LSU rDNA trees were similar, a few inconsistencies existed. For instance, all taxa of the *Compactae* section that were used in this study were divided into three large clades (A, B, and C) in the LSU rDNA tree. In contrast, group B was divided into five subgroups that did not group into a clade in the ITS tree.

Russula densifolia (group A), including *R. adusta*, grouped into a large clade for the LSU rDNA and ITS region; however, the bootstrap supports of the clade were low (54%–64%). The group A based on the LSU rDNA was divided into nine subgroups (A-1-A 9) (Fig. 1). According to the ITS analyses, group A was divided into seven subgroups (A-1, A-3-A-8) (Fig. 2). However, for *R. adusta* (A-1), six subgroups (A-3-A-8) were supported by high bootstrap values (99, 96, 100, -, 100, 100, respectively) in the ITS tree (Fig. 2). In these subgroups, A-3 formed a monophyletic group with A-4 with high bootstrap values in both trees. The sequences belonging to these subgroups shared a 20-bp deletion in the ITS region (Fig. 4,

	[520 530 540 550 560 570 580]
	[. ]
<i>R. delica</i> 358 (outgroup)	TCAGCACGCGCCCTCGGGTGTGTCGGGGCCTC-GGCCACGCC-ACGTGCTTAGGA TG
<i>R. compacta</i> 206 (outgroup)	TCAGCACGCG-----CCTC--GGTG---TCGGGGCCTTTTGGCTCACGCT-ACGTGCTTAGGATG
<i>R. adusta</i> 351 (A-1)	TCAGCACGCG-----CCTTTTGGTG---TCGGGGC-TTC-GGCCTACGCT-ACGTGCTTAGGATG
<i>R. densifolia</i> 352 (A-3)	TCAGCACGCG-----CCTTTTGGCG---TCGGGGCTTCGGCCTACGTTT-ACGTGCTTAGGATG
<i>R. densifolia</i> 294 (A-5)	TCAGCACGCA-----CCTTTTGGTG---TCGGGGCTTC-GGCCTACGTTT-ACGTGCTTAGGATG
<i>R. densifolia</i> 3510 (A-7)	TCAGCATGCG-----CCTTTTGGCG---TCGGGGCTTAGGCCTACGTTT-ACGTGCTTAGGATG
<i>R. densifolia</i> 456 (A-8)	TCAGCACGCG-----CCTTTTGGTG---TCGGGGCTTT-GGCCACGCT-ACGTGCTTAGGATG
<i>R. nigricans</i> 195 (C)	TCGGCACGTA---CC-----GTGCTTAGGATG
<i>R. nigricans</i> AF325312 (C)	TCGGCACGTA---CC-----GTGCTTAGGATG
<i>R. subnigricans</i> 251 (B-1)	TCAGCACGCG-----CCTCTCGGCG---TCGGGGCTTC-GGCCACGCT-ACGTGCTTAGGATG
<i>R. subnigricans</i> 252 (B-3)	TCAGCACGCA-----CCTTTTGGTG---TCGGGGCCTT--GGCCACGCC-ACGTGCTTAGGATG
<i>R. subnigricans</i> 346 (B-5)	TCAGCATGTG---CCTTTTGGTG---TCGGGGCCTC--GGCCACGCT-ACGTGCTTAGGATG

Fig. 3 Insertion/deletion (indels) found between sites 535 and 569 of the large subunit rDNA in the *Russula nigricans* group (C). The presence or absence of the indels is consistent with the respective clades

	[220 230 240 250]
	[. . . .]
<i>R. compacta</i> (outgroup)	GAATGTT-CTTGCAA- TGACT- TGCAATAT AAATA
<i>R. nigricans</i> 195(C-1)	GAATGTCATTCATTTGCGATAACACGCAATCAATA
<i>R. adusta</i> 351(A-1)	GAATGTCATTCATTTGCGATCATGCGCAATCAATA
<i>R. densifolia</i> AY606961(A-3)	GAATATCA-----ATCAATA
<i>R. densifolia</i> 196(A-4)	GAATATCA-----ATCAATA
<i>R. densifolia</i> 294(A-5)	GAATGTCATTCATTTGCGATAACACGCAATCAATA
<i>R. densifolia</i> 3510(A-7)	GAATGTCATTCATTTGTGCTAACGCGCAATTAATA
<i>R. subnigricans</i> 251(B-1)	GAATGTCATTCATTTGCGATCACACGCAATCAATA
<i>R. subnigricans</i> 342(B-2)	GAATGTCATTAGTTTTCGATCACACGCAACCAATA
<i>R. subnigricans</i> 346(B-4)	GAATGTCATTCGTTTTCGATCACACGCAATCAATA
<i>R. subnigricans</i> 376(B-5)	GAATGTCATTCATTTGCGATCACACGCAATCAATA

Fig. 4 Insertion/deletion (indels) found between sites 224 and 245 of the rDNA ITS region in *Russula densifolia* (A-3 and A-4). The presence or absence of the indels is consistent with the respective clades

and 2 V shown in Fig. 2). This deletion represented a synapomorphy in A-3 and A-4, which strongly supported the monophyly of the subgroup. Imazeki and Hongo¹²⁾ suggested that *R. densifolia* is divided into several small species based on their macroscopic features, and this suggestion was supported by the present phylogenetic analyses. In the *Compactae* section of Europe, two species were similar to *R. densifolia* by having close lamellae and changing in color after cutting of the flesh: *R. acrifolia* Romagnesi, *R. anthracina* Romagnesi. Kibby¹³⁾ stated that Rayner²⁰⁾ and Phillips¹⁹⁾ recorded *R. acrifolia* as *R. densifolia* and *R. anthracina* as *R. albonigra*. It is possible that the six phylogenetic subgroups of *R. densifolia* that are shown in this study (Figs. 1-2), including *R. albonigra*, *R. anthracina*, and *R. acrifolia* have been reported in Europe^{13, 19, 20)}. However, further investigations of macro- and micro-morphological characteristics (fruit body, spores, and cystidia, particularly the conditions of the lamellae and stipe) are required to clarify the species within *R. densifolia* in Japan. *Russula subnigricans* (group B) is similar to *R. nigricans* by having thick and distant lamellae, and Shimono et al.²⁵⁾ reported that *R. subnigricans* should be divided into three subgroups based on their fruit body characteristics, surface ornamentation of spores, and LSU rDNA information. Shimono et al.²⁶⁾ reported the ecological fruiting sites, spore ornamentation, and texture of the cap for the four subgroups of *R. subnigricans* that were used in this study. Several differences among these subgroups (B-1, B-2, B-3, B-5) were observed. In the future, we will report the several subgroups of *R. subnigricans* as new species or varieties. The current analyses using 28 samples of group B showed that group B was divided into five subgroups (B-1-5). *Russula subnigricans* (B-1), including the specimen type (TNS-F-237524; National Museum of Nature and Science, Tsukuba, Japan), grouped with *R. nigricans* (group C) in the ITS tree (Fig. 2), and subgroup B-5 was sister to all other taxa of the *Compactae* section. The sequences of subgroup B-5 (*R. subnigricans*) had a 6-bp insertion (TTTTAT) in the ITS region (3 V shown in Fig. 2), and this insertion supports the monophyly of subgroup B-5. *Russula nigricans* (group C), including the European specimens, formed a clade with high bootstrap values (100%) in the LSU rDNA and ITS trees (Figs. 1-2). The sequences belonging to this subgroup shared a 30-bp deletion in the LSU DNA sequence (Fig. 3 and 1 V shown in Fig. 1), and this deletion was not found in the sequences of other groups (groups A and B). Therefore, the deletion was a synapomorphy of group C, which strongly supports the monophyly of group C. This result was also supported by the characteristics of *R. nigricans*, in having thick and distant lamellae and strong reddening before blackening after flesh cutting.

These results indicate that the three groups (A-C) of the *Compactae* section are divided into further phylogenetic subgroups. Further investigations of the macro- and micro-morphological characteristics, such as morphological characteristics of fruit bodies, spore ornamentation, and cystidia of the cap are required for taxonomic revision of the *Compactae* section. Buyck³⁾ described the conspicuous diversity of African "*Compactae*", which is currently divided into many subsections. Further analyses using many more specimens from Africa, the tropics, and Asia are required to clarify the phylogenetic relationships within the *Compactae* section.

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リボソーム DNA の大サブユニット領域および ITS 領域の塩基配列にもとづくベニタケ科クロハツ節の分子系統

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要 約

リボソーム DNA 28 S 領域 (LSU rDNA) および 5.8 S を含む ITS 領域の塩基配列に基づいて、ベニタケ属クロハツ節の分子系統解析を行った。外群を含めて 28 S 領域の解析には 42 個、ITS 領域では 49 個のシーケンスを用いた。最節約法 (MP) に基づく、リボソーム DNA の 28 S 領域の解析では、クロハツ節は大きな 3 群 (group A-C) に別れた。Group A はクロハツモドキ (*Russula densifolia*) とコゲイロハツ (*R. adusta*)、Group B はニセクロハツ (*R. subnigricans*)、Group C はクロハツ (*R. nigricans*) で構成された。ITS 領域の解析では Group B の単系統を支持しなかった。MP および近隣結合法 (NJ) では Group B-5 は今回の研究で用いたすべてのクロハツ節の個体と姉妹群を形成し、Group B-1 は Group C と同群に属した。これらのことから、クロハツモドキとコゲイロハツを含む Group A とクロハツを含む group C はともに単系統であると推測されたが、ニセクロハツ (group B) は単系統ではないと考えられた。コゲイロハツを除くとクロハツモドキの特徴を示す仲間は 6 小群に別れた。