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## Molecular phylogeny of Russulaceae (Basidiomycetes; Russulales) inferred from the nucleotide sequences of nuclear large subunit rDNA

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**Abstract** Phylogenetic relationships of the genera *Russula* and *Lactarius* were investigated using sequence data from the nuclear-encoded large subunit ribosomal DNA (LSU rDNA). Ninety-five sequences belonging to the genera *Russula* and *Lactarius*, including 31 sequences from the databases, were used in this study. Analysis of the LSU rDNA region indicated that Russulaceae was divided into six groups (group A–F) in the neighbor-joining (NJ) tree. *Lactarius* consisted of one large clade (group A). Therefore, this genus was found to be monophyletic. However, the monophyly of genus *Russula* remained unclear. The genus *Russula* consisted of five groups in the NJ tree. Group B includes sects. *Plorantes* and *Archaeinae* (Heim), and group C includes sects. *Delicoarchaeae* and *Russula* in the NJ tree. Neither of the two groups formed a single clade in the most parsimonious (MP) tree. Group D includes many taxa having colored spore prints and amyloid in suprahilar plage of spores in sect. *Russula* and sect. *Rigidae*. Group E consists of only sect. *Compactae* and is further divided into three subclades, represented by *R. densifolia*, *R. nigricans*, and *R. subnigricans*, respectively. Group F contains sects. *Rigidae*, *Ingratae*, and *Pelliculariae*. Sect. *Compactae* and sect. *Plorantes* should not be as closely related as previously supposed. *Russula earlei* may be placed in sect. *Archaeinae* Heim. *Russula flavida* (subsect. *Amoeninae*) is placed in sect. *Russula* with *R. aurea* with a high bootstrap value (99%). The nuclear LSU rDNA region is a useful tool in recognition of species of Russulaceae and may provide information concerning phylogenetic relationships between the genera *Russula* and *Lactarius*.

**Key words** *Lactarius* · *Russula earlei* · *Russula subnigricans* · Sections *Compactae* and *Plorantes*

### Introduction

Russulaceae consists of the genera *Russula* and *Lactarius*, which are worldwide groups of fresh fungi with 750 and 350 species, respectively (Kirk et al. 2001). These taxa are found from tropical to frigid regions and mostly form obligately ectotrophic mycorrhiza with many kinds of forest trees of the genera *Pinus*, *Abies*, *Picea*, *Larix*, and *Tsuga*. Differences between *Russula* and *Lactarius* include the presence or absence of latex and whether they have spherocysts in the trama of the lamellae. However, identification of these taxa based on latex is difficult with dried specimens. Recently, *L. compinensis* was transferred to the genus *Russula* based on its strong taste, true sulfovanillin-positive dermatocystida, lack of latex in a fresh sample, and a molecular phylogenetic analysis (Henkel et al. 2000). Monographs giving partial or regional descriptions of *Russula* have been published by many researchers (Singer 1932; Heim 1937), particularly in Europe (Romagnesi 1967; Bon 1987; Sarnari 1998). However, phylogenetic relationships of the sections, subsections, and species have not been fully determined.

Climates in Japan range from subtropical to subfrigid. Okinawa-urajirogashi (*Quercus miyagii*) and spruce forests, respectively, grow in these regions. In these forests, a large number of *Russula* and *Lactarius* form ectotrophic mycorrhizae with many trees. The western district of Japan has a very hot summer and a humid rainy season, which corresponds to a subtropical region. Many tropical taxa of Russulaceae grow in this area: *R. eburneoareolata* of section *Pelliculariae*, *R. japonica* of section *Delicoarchaeae*, *R. castanopsidis* of section *Pachycystides*, and the pleuoleid *L. uyedae* of section *Panuoidei*. Miller and Buyck (2002) stated that the classification by Romagnesi (1996) and Sarnari (1998) was consistent with their molecular results. Because taxa from tropical and Asian districts are not included in the

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taxonomic system of Romagnesi and Sarnari, we used the classification system of Singer (1986), in which he created several tropical sections that included Japanese taxa: sections *Pelliculariae*, *Delicoarcheae*, and *Pachycystidia* in *Russula* and section *Panuoidei* in *Lactarius*. Although about 70 species have been identified in Japan, three times that number remain unidentified.

Recent advances in molecular techniques using internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) (Kretzer et al. 1996; Michell and Bresinsky 1999; Aanen et al. 2000; Sanchez-Ballesteros et al. 2000) and large subunits (LSU) (Drehmel et al. 1999; Hopple and Vilgalys 1999; Thorn et al. 2000; Humpert et al. 2001; Moncalvo et al. 2002) have made it possible to investigate the phylogeny of a variety of organisms at the molecular level. There are also a few reports in Russulaceae (Henkel et al. 2000; Miller et al. 2001; Miller and Buyck 2002). Because identification to date has been based on the morphology of fruiting bodies, we cannot exclude circumstances of convergent evolution. Therefore, we used the LSU of the rDNA for the classification and phylogenetic analysis of Russulaceae.

The present study aims to use the nuclear LSU of the rDNA to identify species of Russulaceae and to provide information about phylogenetic differences between the genera *Russula* and *Lactarius*.

## Materials and methods

### Sample sources

Sixty-four taxa of Russulaceae belonging to the genera *Russula* and *Lactarius* were used in this study. Their collection locations, specimen numbers, and accession numbers in the nucleotide sequence databases (DDBJ) are provided in Table 1. Of the 64 taxa determined in this study, 62 were found to be in the genus *Russula* and 2 in the genus *Lactarius*. Thirty-one taxa and 3 outgroup taxa from the database are shown in Table 2; these include 16 *Russula*, 14 *Lactarius*, and 1 *Macowanites americanus*. The data set consisted of 95 ingroup and 3 outgroup sequences. The outgroup taxa comprised *Boletus edulis* (AF071457), and two sequences of *Bondarzewia berkeleyi* (AF218563, AF287849) were used as outgroup taxa based on our preliminary analysis.

To cover all major lineages of *Russula* proposed by Singer (1986), we selected at least 1 taxon from each of the eight major lineages, 17 subsections and 45 species. We used 18 taxa in section *Compactae*, 7 in *Ingratae*, 7 in subsection *Amoenae*, and 5 in subsection *Virescentinae*. We also used a few taxa in each of the other sections and subsections.

Fungal DNA was extracted from fresh materials or dried specimens and remaining specimens were dried and kept at the Natural History Museum of Osaka City. The fungal species were identified based on the work of Imazeki and Hongo (1989), some illustrated books of mushrooms from foreign countries (Bon 1987; Phillips 1991; Courtecuisse and Duhem 1995), and monographs of *Russula* (Romagnesi 1996; Sarnari 1998).

### DNA extraction and PCR amplification

DNA was isolated from the lamellae of the fruiting body using the method reported by Suyama et al. (1996). Dried lamellae (0.5 mg) were placed on a siliconized glass slide and homogenized between two glass slides in 10  $\mu$ l extraction buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% sodium dodecyl sulfate (SDS), and 0.01% proteinase K]. The homogenate was placed in 300  $\mu$ 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 15000g 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 and the PCR reactions conducted in 50- $\mu$ l volumes as previously described (Hirata and Takamatsu 1996). A negative control that lacked template DNA was included for each set of reactions. The PCR product was subjected to preparative electrophoresis in 1.5% agarose gel in a TAE buffer. The DNA product from each amplification was then excised from the ethidium bromide-stained gel and purified using a Jetsorb kit (Genomed, Oeynhausen, Germany) following the manufacturer's instructions.

### PCR primers

The following oligonucleotide primers were used in this analysis: the nucleotide sequence of NL1 (5'-GCTATCCTGAGGGGAACTTC-3'), designed by Mori et al. (2000); the nucleotide sequence of Ctb6 (5'-GCATATCAATAAGCGGAGG-3') and TW14 (5'-GCTATCCTGAGGGGAACTTC-3'), which were kindly provided by Dr. G.S. Saenz, University of New Mexico, USA; and BN1 (5'-GATCCTTGACGTGATAAG-3'), which was designed based on the nucleotide sequence of the ITS2 region of rDNA of *Russula* spp. (Shimono et al. 2000).

For amplifications of the 5'-end of the large subunit rDNA that included the D1 and D2 regions, primer set BN1/TW14 was used for the first amplification. Partial nested primer set Ctb6/TW14 was then used for the second amplification. If the second amplification was not achieved, the further partial nested primer set NL1/TW14 was used for a third amplification.

### DNA sequencing

Nucleotide sequences of the PCR products were obtained for both strands using direct sequencing in an Applied Biosystems 373A sequencer (the alignment is available upon request from the corresponding author). The sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The primers NL1, NL2, NL3, and TW14 were used for the sequencing of the LSU rDNA in both directions.

Table 1. Specimens sequenced

Taxon	Collection no.	Extract no.	Origin	Accession no.	Classification (sect., subsect.)
<i>Russula</i>					
<i>Russula eburneareolata</i> Hongo	OSA-MY-1708	297	Japan, Kyoto city	AB154696	<i>Pelliculariae, Guayarenses</i>
<i>R. japonica</i> Hongo	OSA-MY-1709	153	Japan, Uji city	AB154697	<i>Delicoarchoaeae</i>
<i>R. chloroides</i> (Krombh.) Bres.	OSA-MY-1710	358	Japan, Minoo city	AB154698	<i>Plorantes</i>
<i>R. chloroides</i> (Krombh.) Bres.	OSA-MY-1711	343	Japan, Fuchuu city	AB154699	<i>Plorantes</i>
<i>R. delica</i> Fr.	OSA-MY-1712	156	Japan, Neyagawa city	AB154700	<i>Plorantes</i>
<i>R. compacta</i> Frost & Peck apud Peck	OSA-MY-1713	206	Japan, Kyoto city	AB154701	<i>Crassotunicatae</i>
<i>R. adusta</i> (Pers.) Fr.	OSA-MY-1714	351	Japan, Yamanashi Pref.	AB154702	<i>Compactae</i>
<i>R. densifolia</i> Gill.	OSA-MY-1715	294	Japan, Kashihara city	AB154703	<i>Compactae</i>
<i>R. densifolia</i> Gill.	OSA-MY-1716	352	Japan, Yamanashi Pref.	AB154704	<i>Compactae</i>
<i>R. densifolia</i> Gill.	OSA-MY-1717	3510	Japan, Minoo city	AB154705	<i>Compactae</i>
<i>R. nigricans</i> Fr.	OSA-MY-1718	195	Japan, Katano city	AB154706	<i>Compactae</i>
<i>R. nigricans</i> Fr.	OSA-MY-1719	356	Japan, Uji city	AB154707	<i>Compactae</i>
<i>R. nigricans</i> Fr.	OSA-MY-1720	398	Japan, Sendai city	AB154708	<i>Compactae</i>
<i>R. nigricans</i> Fr.	OSA-MY-1721	4010	Japan, Katano city	AB154709	<i>Compactae</i>
<i>R. subnigricans</i> Hongo	OSA-MY-1722	251	Japan, Uji city	AB154710	<i>Compactae</i>
<i>R. subnigricans</i> Hongo	OSA-MY-1723	252	Japan, Uji city	AB154711	<i>Compactae</i>
<i>R. subnigricans</i> Hongo	OSA-MY-1724	253	Japan, Uji city	AB154712	<i>Compactae</i>
<i>R. subnigricans</i> Hongo	OSA-MY-1725	261	Japan, Shimonoseki city	AB154713	<i>Compactae</i>
<i>R. subnigricans</i> Hongo	TNS-F-237524	391	Japan, Kyoto city	AB154714	<i>Compactae</i>
<i>R. subnigricans</i> Hongo	TNS-F-237524	392	Japan, Kyoto city	AB154715	<i>Compactae</i>
<i>R. subnigricans</i> Hongo	OSA-MY-1726	407	Japan, Mie Pref.	AB154716	<i>Compactae</i>
<i>Russula</i> sp.	OSA-MY-1729	342	Japan, Fukushima Pref.	AB154719	<i>Compactae</i>
<i>R. earlei</i> Peck	OSA-MY-1727	354	Japan, Saitama Pref.	AB154717	<i>Compactae</i>
<i>R. dissimulans</i> Shaff.	OSA-MY-1728	399	Japan, Musashimurayama city	AB154718	<i>Compactae</i>
<i>R. dissimulans</i> Shaff.?	OSA-MY-1730	137	Japan, Kashihara city	AB154720	<i>Decolorantes, Decolorantinae</i>
<i>R. rubescens</i> Bwardslee	OSA-MY-1731	3115	Japan, Kyoto city	AB154721	<i>Decolorantes, Decolorantinae</i>
<i>Russula</i> sp.	OSA-MY-1732	347	Japan, Okayama Pref.	AB154722	<i>Ingratae, Fistulosinae</i>
<i>R. earlei</i> Peck	OSA-MY-1733	291	Japan, Nara city	AB154723	<i>Ingratae, Foeteninae</i>
<i>R. foetens</i> Pers.: Fr.	OSA-MY-1734	292	Japan, Hirakata city	AB154724	<i>Ingratae, Foeteninae</i>
<i>R. laurocerasi</i> Melzer	OSA-MY-1735	293	Japan, Minoo city	AB154725	<i>Ingratae, Foeteninae</i>
<i>R. laurocerasi</i> Melzer	OSA-MY-1736	289	Japan, Nara city	AB154726	<i>Ingratae, Foeteninae</i>
<i>R. pectinatooides</i> Peck	OSA-MY-1737	288	Japan, Kyoto city	AB154727	<i>Ingratae, Foeteninae</i>
<i>R. sororia</i> (Fr.): Romell	OSA-MY-1738	12cyG1	Japan, Kyoto city	AB154728	<i>Rigidae, Cyanoxanthinae</i>
<i>R. cyanoxantha</i> (Schaeff.) Fr.	OSA-MY-1739	296	Malaysia, Kota Kinabalu city	AB154729	<i>Rigidae, Cyanoxanthinae</i>
<i>Russula</i> sp.	OSA-MY-1740	374	Japan, Minoo city	AB154730	<i>Rigidae, Cyanoxanthinae</i>
<i>R. cutefracta</i> Cke.	OSA-MY-1741	373	Japan, Minoo city	AB154731	<i>Rigidae, Cyanoxanthinae</i>

Table 1. Continued

Taxon	Collection no.	Extract no.	Origin	Accession no.	Classification (sect., subsect.)
<i>R. variata</i> Ban. apud Pk.	OSA-MY-1742	273	Japan, Kyoto city	AB154732	<i>Rigidae, Cyanoxanthinae</i>
<i>R. heterophylla</i> (Fr.) Fr.	OSA-MY-1743	339	Japan, Okayama Pref.	AB154733	<i>Rigidae, Lividinae</i>
<i>R. vesca</i> Fr.	OSA-MY-1744	338	Japan, Okayama Pref.	AB154734	<i>Rigidae, Lividinae</i>
<i>R. aeruginea</i> Lindbl. apud Fr.	OSA-MY-1745	283	Japan, Nara city	AB154735	<i>Rigidae, Subcompactinae</i>
<i>R. grisea</i> Fr.	OSA-MY-1746	282	Japan, Kashihara city	AB154736	<i>Rigidae, Subcompactinae</i>
<i>R. amoena</i> Quél.	OSA-MY-1747	12AG2	Japan, Kyoto city	AB154737	<i>Rigidae, Amoeniniae</i>
<i>R. flavida</i> Frost & Peck apud Peck	OSA-MY-1748	2612	Japan, Nagano Pref.	AB154738	<i>Rigidae, Amoeniniae</i>
<i>R. flavida</i> Frost & Peck apud Peck	OSA-MY-1749	333	Japan, Tatsuno city	AB154739	<i>Rigidae, Amoeniniae</i>
<i>R. flavida</i> Frost & Peck apud Peck	OSA-MY-1750	349	Japan, Koube city	AB154740	<i>Rigidae, Amoeniniae</i>
<i>Russula</i> sp.	OSA-MY-1751	4011	Japan, Katano city	AB154741	<i>Rigidae, Amoeniniae</i>
<i>R. bella</i> Hongo	OSA-MY-1752	1712	Japan, Takarazuka city	AB154742	<i>Rigidae, Amoeniniae</i>
<i>R. violipes</i> Quél.	OSA-MY-1753	223	Japan, Neyagawa city	AB154743	<i>Rigidae, Amoeniniae</i>
<i>R. alboareolata</i> Hongo	OSA-MY-1754	124	Japan, Kyoto city	AB154744	<i>Rigidae, Virescentinae</i>
<i>R. virescens</i> (Schaeff.) Fr.	OSA-MY-1755	144	Japan, Kutsuki mura	AB154745	<i>Rigidae, Virescentinae</i>
<i>R. virescens</i> (Schaeff.) Fr.	OSA-MY-1756	363	Thailand, Chiang Mai city	AB154746	<i>Rigidae, Virescentinae</i>
<i>R. viridibrulimbata</i> Ying	OSA-MY-1757	2510	Japan, Uji city	AB154747	<i>Rigidae, Virescentinae</i>
<i>R. viridibrulimbata</i> Ying	OSA-MY-1758	281	Japan, Nara city	AB154748	<i>Rigidae, Virescentinae</i>
<i>R. lilacea</i> Quél.	OSA-MY-1759	348	Japan, Uji city	AB154749	<i>Rigidae, Lilaceinae</i>
<i>R. rosea</i> Quél.?	OSA-MY-1760	3116	Japan, Kyoto city	AB154750	<i>Rigidae, Roseinae</i>
<i>R. rosacea</i> (Pers. ex) S.F. Gray	OSA-MY-1761	284	Japan, Nara city	AB154751	<i>Rigidae, Lepidinae</i>
<i>R. xerampelina</i> (Schaeff.) Fr.	OSA-MY-1762	361	Thailand, Chiang Mai city	AB154752	<i>Rigidae, Xerampelinae</i>
<i>R. omiensis</i> Hongo	OSA-MY-1763	7N1	Japan, Kyoto city	AB154753	<i>Rigidae</i>
<i>R. kansaiensis</i> Hongo	OSA-MY-1764	285	Japan, Kyoto city	AB154754	<i>Russula, Puellarinae</i>
<i>R. emetica</i> (Schaeff.) Fr.) S.F. Gray	OSA-MY-1765	213	Japan, Kutsuki mura	AB154755	<i>Russula, Russula</i>
<i>R. sanguinea</i> (Bull.) Fr.	OSA-MY-1766	344	Japan, Yamanashi Pref.	AB154756	<i>Russula, Sanguineae</i>
<i>R. aurea</i> Pers.	OSA-MY-1767	298	Japan, Nagano Pref.	AB154757	<i>Russula, Firmiores</i>
<i>Lactarius</i>					
<i>Lactarius vellereus</i> (Fr.) Fr.	OSA-MY-1768	375	Japan, Minoo city	AB154758	<i>Albati</i>
<i>Lactarius</i> sp.	OSA-MY-1769	254	Japan, Uji city	AB154759	<i>Plinthogali</i>

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

**Table 2.** Sequences from database used in this study

Species	Accession no.	Classification (sect., subsect.)
<i>Russula</i>		
<i>Russula radicans</i> Heim	AF218547	<i>Pelliculariae, Radicantes</i>
<i>Russula discopus</i> Heim	AF218543	<i>Pelliculariae</i>
<i>Russula adusta</i> (Pers.) Fr.	AF218544	<i>Compactae</i>
<i>Russula earlei</i> Peck	AF042571	<i>Ingratae, Fistulosinae</i>
<i>Russula foetentula</i> Peck	AF218546	<i>Ingratae, Foetentinae</i>
<i>Russula virescens</i> (Schaeff.) Fr.	AF041548	<i>Rigidae, Virescentinae</i>
<i>Russula xerampelina</i> (Schaeff.) Fr.	AF218542	<i>Rigidae, Xerampelinae</i>
<i>Russula brunneola</i> Burl.	AF218548	<i>Rigidae, Modestinae</i>
<i>Russula mairei</i> Singer	U11926	<i>Russula, Russula</i>
<i>Russula romagnesii</i> Singer	AF042572	<i>Russula, Urentes?</i>
<i>Russula campinensis</i> Henkel, Aime et Miller	AF218560	NA
<i>Russula campinensis</i> Henkel, Aime et Miller	AF218564	NA
<i>Russula campinensis</i> (ectomycorrhizae)	AF218565	NA
<i>Lactarius</i>		
<i>Lactarius corrugis</i> Peck	U11919	<i>Dulces</i>
<i>Lactarius volemus</i> (Fr.) Fr.	AF042574	<i>Dulces</i>
<i>Lactarius fumosus</i> var. <i>fumosus</i> Peck	AF218551	<i>Plinthogali</i>
<i>Lactarius lignyotellus</i> Sm. & Hesl.	AF218557	<i>Plinthogali</i>
<i>Lactarius deceptivus</i> Peck	AF218550	<i>Albati</i>
<i>Lactarius piperatus</i> (Scop.) S.F. Gray	AF042573	<i>Albati</i>
<i>Lactarius piperatus</i> (Scop.) S.F. Gray	AF218556	<i>Albati</i>
<i>Lactarius speciosus</i> Burlingham	AF218555	<i>Lactarius</i>
<i>Lactarius subpurpureus</i> Peck	AF218553	<i>Lactarius</i>
<i>Lactarius subdulcis</i> (Fr.) S.F. Gray	AF218552	<i>Russulares</i>
<i>Lactarius peckii</i> var. <i>peckii</i> Burlingham	AF218554	<i>Russulares</i>
<i>Lactarius panuoides</i> Singer	AF281561	<i>Panuoidei</i>
<i>Lactarius uyedae</i> Singer	AF281562	<i>Panuoidei</i>
Other		
<i>Macowanites americanus</i> Sing. & Sm.	AF265540	NA
Outgroups		
<i>Bondarzewia berkeleyi</i> (Fr.) Singer	AF218563	NA
<i>Bondarzewia berkeleyi</i> (Fr.) Fr.	AF287849	NA
<i>Boletus edulis</i> Bull.	AF071457	NA

NA, not applicable

## Data analysis

The obtained sequences were initially aligned using the Clustal V package (Higgins et al. 1992). The alignment was then refined visually using a word processing program with color-coded nucleotides. (The data matrix is also available upon request from the corresponding author.) Phylogenetic trees were obtained from the data by distance and parsimony methods. For the parsimony analysis, we used the maximum-parsimony method with a heuristic search using PAUP version 4.0b 8a (Swofford 2001). 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 for branch-swapping algorithm.

For distance analyses, the most appropriate evolutionary model was determined for a given data set using PAUP\* and Modeltest 3.06 (Posada and Crandall 1998). A starting tree was obtained using the neighbor-joining (NJ) method. With this tree, likelihood scores were calculated for 56 alternative models of evolution by PAUP\*. The output file was then imported to Modeltest to compare the models using Akaike's (1974) information criterion (AIC). Once a model

of evolution was chosen, it was used to construct phylogenetic trees with NJ methods using PAUP\*.

The strength of internal branches from the resulting trees was tested by bootstrap analysis using 1000 replications (Felsenstein 1985) with TBR branch-swapping algorithm.

## Results

The length of nuclear LSU rDNA amplification products ranged from 853 to 889 base pairs of the divergent domains D1 and D2 (Hopple and Vilgalys 1999). Based on these sequences, we constructed an alignment data set of 945 sites. We excluded 72 ambiguously aligned sites and 45-bp insertions of *B. edulis* from the data set. The remaining 873 sites were used for the phylogenetic analysis. Of the 873 sites, 304 were variable and 204 were phylogenetically informative. Using Modeltest (Posada and Crandall 1998) under the likelihood ratio test criterion, we concluded that the Tamura-Nei model (Tamura and Nei 1993), with equal base frequencies, a gamma-distributed rate heterogeneity model (four rate categories,  $G = 0.5351$ ) and an estimated proportion of invariant sites (0.4918) was the most appro-

appropriate model of evolution for this data set. A NJ tree produced using this data set is shown in Fig. 1. An island of 816 most parsimonious trees of 1102 steps, which differ in only the minor branching order of terminal taxa, was found in a maximum-parsimony (MP) analysis [consistency index (CI) = 0.3575; retention index (RI) = 0.6871]. A strict consensus tree of the MP trees is shown in Fig. 2.

Six lineages (groups A–F) were found in the Russulaceae based on the NJ analysis (Fig. 1), and four lineages (groups A, D, E, and F) were found based on the MP analysis (Fig. 2). Group A includes all *Lactarius* species. The genus *Russula* was divided into five large clades in the NJ tree: groups B, C, D, E, and F. In the MP tree, other than for groups B and C, the genus *Russula* was divided into three clades. Groups B and C were split into two separate clades in the MP tree. Groups D and F were supported with low bootstrap values (64% and 54%, respectively) in the NJ analysis and 67% and 58%, respectively, in the MP analysis. The bootstrap supports of groups A and E were lower than 50%. Taxa included in groups E, F-1, F-2, F-3, F-5, and D-1 of the MP tree completely formed the taxa of the groups of the NJ tree.

#### Group A

Singer (1986) divided the genus *Lactarius* into nine sections. In group A, three small clades, groups A-1, A-2, and A-3, were found. Group A-1 consists of *L. fumosus*, *L. lignyotellus*, and *Lactarius* sp. (section *Plinthogali*). Group A-2 consists of *L. vellereus*, *L. deceptivus*, and *L. piperatus* (section *Albati*), *L. volemus* (section *Volemi*), and *L. panuoides* and *L. uyedae* (section *Panuoidei*). In section *Albati*, *L. vellereus* and *L. deceptivus* formed small clades with moderate bootstrap support (79%), but *L. piperatus* was not included in this clade. The pleurotoid *L. panuoides* and *L. uyedae* were not included in this clade either. Group A-3 consisted of *L. peckii* and *L. subdulcis* (section *Russulares*), *L. scrobiculatus* and *L. speciosus* (section *Lactarius*), and *L. subpurpureus* (section *Dapetes*). Although groups A-1 and A-2 were grouped into a clade in the NJ tree, groups A-1 and A-3 formed a clade in the MP tree.

#### Group B (sections *Plorantes* and *Archaeinae*)

Singer (1986) divided the genus *Russula* into ten sections and one supplemental section, *Archaeinae* sensu Heim. Clade B was divided into two subclades. In the NJ tree, *R. delica*, *R. chloroides*, and *R. brevipes* formed group B-1 with high bootstrap support (96%). *Russula earlei* (section *Ingratae*; Singer 1986), which was collected in Japan and America, was included in group B-2 with a high bootstrap value (100%). Groups B-1 and B-2 grouped into a large clade (group B) with a low bootstrap value.

#### Group C (sections *Russula* and *Delicoarchaeae*)

In the NJ tree, group C-1 consisted of two subclades. One subclade was composed of *R. japonica* and *R. romagnesii*

and the other subclade was composed of *R. campinensis*. Although the former subclade was strongly supported by the bootstrap analysis in both the NJ and MP trees, the character of the fruiting body is clearly different between the two species. *Russula japonica*, which belongs to section *Delicoarchaeae*, has a large stout fruiting body, whereas *R. romagnesii* (subsection *Urentes* of section *Russula*) has a small fragile yellow pileus (Romagnesi 1996; Sarnari 1998). Groups C-1 and C-2 did not group into one clade in the MP tree.

#### Group D (sections *Russula*, *Decolorantes*, and *Rigidae*, and Gasteroid *Russula*)

Group D is a large clade that consists of four small clades: group D-1 (section *Decolorantes*, subsection *Puellarinae* of section *Russula*, and subsection *Xerampelinae* of section *Rigidae*), group D-2 (section *Rigidae* and subsection *Firmiores* of section *Russula*), group D-3 (section *Rigidae* and subsections *Sanguineae* and *Russula* of section *Russula*), and group D-4 (sections *Rigidae* and *Russula*). This clade is mostly composed of colored spore print taxa. Excluding *R. rosacea*, the color of the spore print in group D-4 is yellow, whereas in groups D-1 and D-2 it is slightly yellow. Other than *R. sanguinea*, group D-3 has a white spore print and is acrid in taste. The suprahilar spot on the spore of the species belonging to section *Russula*, *R. emetica*, *R. rosea*, and *R. xerampelina* shows an amyloid reaction with Melzer's reagent.

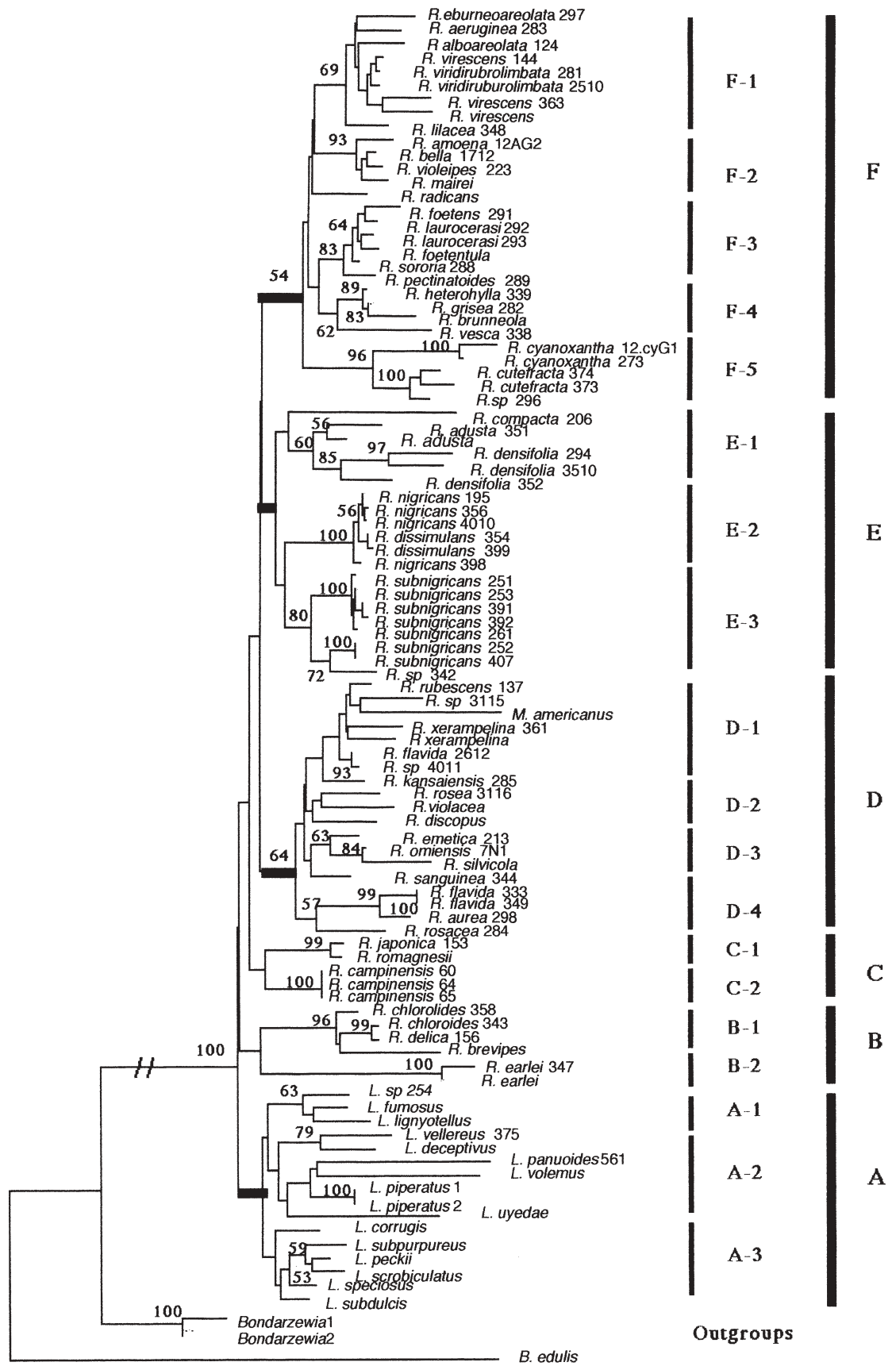
#### Group E (sections *Compactae* and *Crassotunicatae*)

Nineteen taxa belonging to section *Compactae* used in this study were divided into three subclades (E-1, E-2, and E-3) in the NJ and MP trees. E-1 consists of *R. adusta*, *R. densifolia*, and *R. compacta*, E-2 consists of *R. nigricans*, and E-3 consists of *R. subnigricans*. Although the bootstrap support of group E-1 was less than 50%, groups E-2 and E-3 were supported with high bootstrap values in the NJ and MP trees.

*Russula compacta* (section *Crassotunicatae*) grouped with group D in the MP tree with low bootstrap support (<50%). Group E-3 was divided into three smaller groups: one group contained a type specimen of *R. subnigricans* (extract nos. 391 and 392), while another contained extract nos. 407, 252, and 342 of *R. subnigricans*. The LSU rDNA sequence of extract no. 407 was identical to that of no. 252. The two specimens were very similar in morphology, and differed somewhat from no. 342. The complex of *R. subnigricans* from Japan could be divided into at least three different species based on the present morphological and molecular analyses.

#### Group F (sections *Rigidae*, *Ingratae*, and *Pelliculariae*)

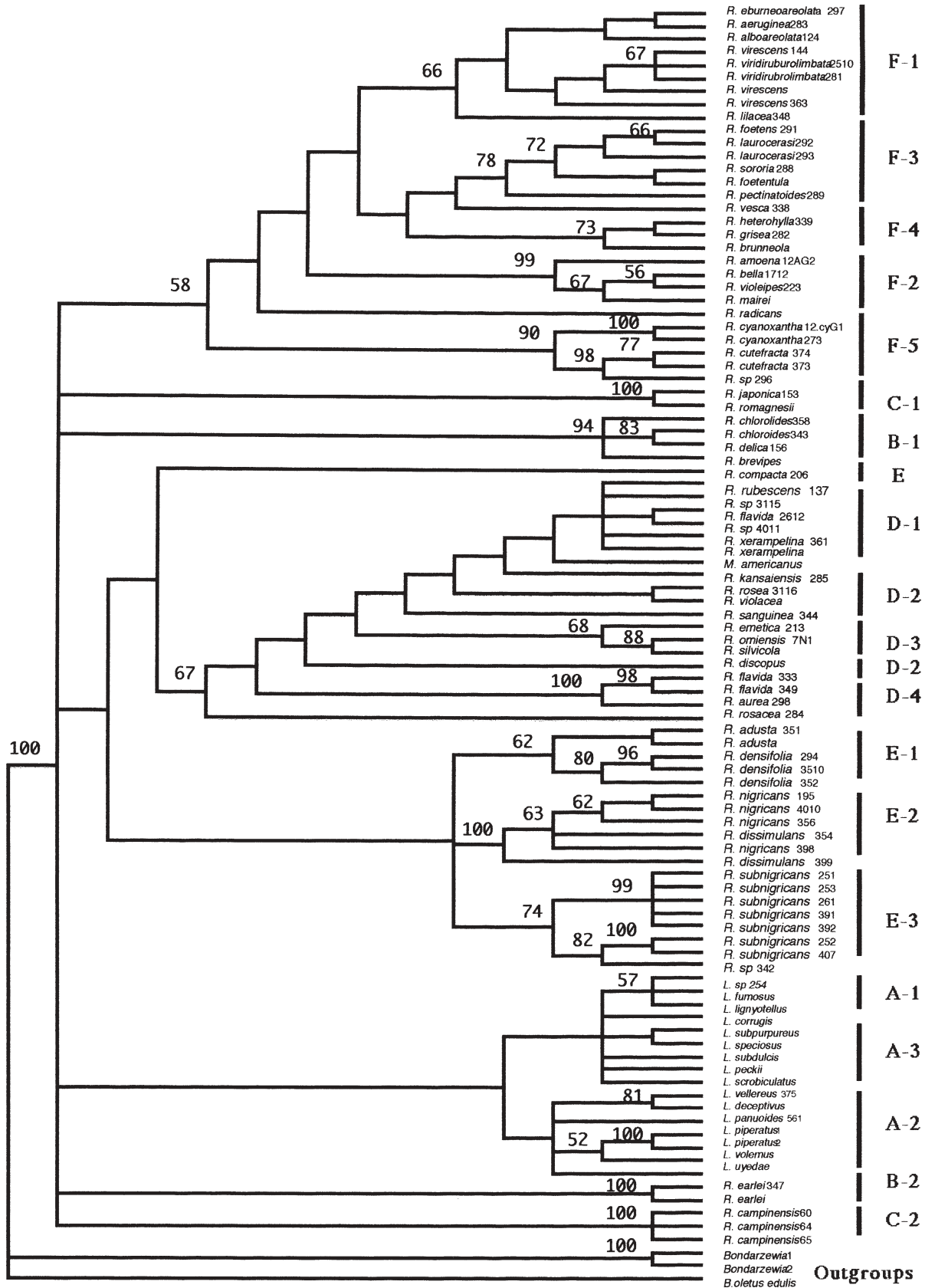
Group F consists of five subgroups and *R. radicans* in the NJ tree: group F-1 (subsections *Virescentinae*, *Subcompactinae*,



— 0.005 substitutions/site

**Fig. 1.** Neighbor-joining based on large subunit rDNA data for 95 taxa of *Russula* and *Lactarius* including gasteroid species and three outgroup taxa. Modeltest parameters: equal base frequencies with rate heterogeneity; gamma shape parameter = 0.5351; proportion of invariable sites = 0.4918; six rate categories; Tamura–Nei model (Tamura

and Nei 1993) with transformation parameters [A–C] = 1.00, [A–G] = 6.62, [A–T] = 1.00, [C–G] = 1.00, [C–T] = 17.61, and [G–T] = 1.00. Present bootstrap support (1000 replications) is shown above nodes. Bar 0.005 substitutions/site



**Fig. 2.** Strict consensus equally parsimonious trees based on large subunit rDNA data for 95 taxa of *Russula* and *Lactarius* including gasteroid species and three outgroup taxa. Present bootstrap support (1000 replications) is shown above nodes. Consistency index (CI) is 0.3575, retention index (RI) is 0.6871, and rescaled consistency index (RC) is 0.2437



and *Lilaceinae* of section *Rigidae* and subsection *Guayarenses* of section *Pelliculariae*), F-2 (mainly subsection *Amoeninae* of section *Rigidae* and subsection *Russula* of section *Russula*), F-3 (section *Ingratae*), F-4 (subsections *Virescentinae*, *Compactinae*, and *Lividinae* of section *Rigidae*), and F-5 (subsection *Cyanoxanthinae* of section *Rigidae*). The MP tree supports the groupings of the NJ tree.

#### Group F-1

This clade contained three subsections of sections *Rigidae* and *Pelliculariae* and is supported with a bootstrap value of 69%. *Russula virescens*, *R. viridirubrolimbata*, and *R. alboareolata* (subsection *Virescentinae* of section *Rigidae*) formed a small clade, but two *R. virescens* isolates collected in the United States and Thailand showed a 2.2% genetic distance from the Japanese isolate. Group F-1 includes the morphologically divergent taxa *R. eburneoareolata*, *R. aeruginea*, and *R. lilacea*. If we analyze more isolates of these taxa in the near future, this group will be split into several small clades.

Concerning *R. eburneoareolata*, Buyck (1995) stated that section *Pelliculariae* is a heterogeneous assemblage of small, very thin, frequently annulate species, some of which belong to subsections with a worldwide distribution (e.g., *Amoeninae*). Because *R. eburneoareolata* was placed into group F-1 and *R. radicans*, which had an annular veil, seems to be a distinct branch in both Fig. 1 and Fig. 2, section *Pelliculariae* should be regarded as an artificial section. Section *Pelliculariae* has long been considered as primitive in the genus *Russula*, but our results do not support this hypothesis.

#### Group F-2

This clade contained three species of subsection *Amoeninae* of section *Rigidae* and *R. mairei* (section *Russula*) with a high bootstrap value (93%).

#### Group F-3

This clade contained only section *Ingratae*. Along with two sequences obtained from the DNA database, eight sequences belonging to section *Ingratae* were used in this study. Excluding two *R. earlei* sequences, six sequences of section *Ingratae* grouped into a single clade with high bootstrap values (83% and 78%) in the NJ and MP trees.

#### Group F-4

This clade contained subsections *Lividinae*, *Modestinae*, and *Subcompactinae* of section *Rigidae* with a low bootstrap value (62%). *Russula brunneola* belongs to subsection *Modestinae*.

#### Group F-5

This clade contained five species (subsection *Cyanoxanthinae* of section *Rigidae*), namely, *R. cyanoxantha*, *R. cutefracta*, and *Russula* sp. collected in Malaysia. Subsection *Cyanoxanthinae* formed one lineage with high bootstrap values (96% and 90%) in the NJ and MP trees, respectively. In subsection *Cyanoxanthinae*, stipes and lamellae do not change color with  $\text{FeSO}_4$ .

## Discussion

### Delimitation between the genera *Russula* and *Lactarius*

Historically, delimitation between the genera *Russula* and *Lactarius* is very difficult because of the presence or absence of latex and the presence of spherocysts in the hymenophoral trama. The genus *Russula* has spherocysts in the trama of the lamellae, but not latex, whereas the genus *Lactarius* has latex and no spherocysts. Recently, intermediate species between *Russula* and *Lactarius* have been commonly found in the tropics (Buyck 1995). *Lactarius compiensis*, which was transferred to the genus *Russula* by its morphology and systematic affinities (Henkel et al. 2000), is one of these species. These two genera have a few common characteristics, i.e., a *Virescens* structure and a similar fruiting body nature. The *Virescens* structure is ciliate dermatocystidium, which consists of a few basal cells that are short cylindrical or spherocyst like and end in one or more terminal cells (Largent et al. 1977). This characteristic is shared by subsection *Virescentinae* and section *Pelliculariae* (subsections *Guayarensis* and *Discopodinae*) in *Russula* and section *Plinthogali* in *Lactarius*. In this study, three species belonging to section *Plinthogali* constituted one clade with a low bootstrap value (63%), while subsection *Virescentinae* and section *Pelliculariae* constituted another clade (group F). Singer (1986) reported that there are only three species groups where the two genera seem to “touch” each other, i.e., the *Albati* (*Lactarius*)–*Plorantes* (*Russula*), *Archaeinae* (*Russula*)–*Lactariopsis* (*Lactarius*), and *Archaeinae* (*Russula*)–*Polysphaerophori* (*Lactarius*) complexes. Similarities between sections *Albati* and *Plorantes* are that they have hard, rigid, and unchanging or slightly stained pink lamellae. A study of LSU rDNA (Henkel et al. 2000) supported the report of Maire (1910) that some members of the genus *Russula*, such as *R. brevipes*, which are mostly white with dense tissue, are similar to some species of *Lactarius*.

We used a few taxa belonging to section *Plorantes* and section *Albati* in this study. As shown in Fig. 1, section *Plorantes* constituted group B with *R. earlei* (section *Archaeinae*), but section *Albati* was placed into group A-2, which only included *Lactarius*. Thus, section *Plorantes* is not closely related to section *Albati*. In the NJ tree, taxa of *Lactarius* formed a single clade without the genus *Russula* and placed in the basal position of the phylogenetic tree, while *Russula* consisted of three large clades that excluded

groups B and C (see Fig. 1). In the MP tree, taxa of *Lactarius* often are placed in a large clade that includes group F of *Russula*. Although the genera *Lactarius* and *Russula* have many similar morphological characters, such as the surface of the pileus and flesh condition, the genus *Lactarius* forms a clade that consisted of only *Lactarius* taxa (monophyletic) in the NJ tree and was paraphyletic in the MP tree. On the other hand, the genus *Russula* was largely monophyletic in the NJ tree and polyphyletic in the MP tree.

Redhead and Norveil (1993) stated that the Russulaceae had a lineage that diverged from plesiomorphic saprophytic or parasitic *Lactarius*-like species through possibly several sections of the mycorrhizal genus *Lactarius* to the genus *Russula* by loss of latex production and increased spherocyst production. Buyck (1995), on the basis of studies of variability of many features of the tropical Russulaceae, stated that there was a diminishing distinction between the genera *Lactarius* and *Russula*. Miller et al. (2001) supported Buyck (1995) on the basis of studies of Russulales that included agaricoid, gasteroid, and pleurotoid taxa using a phylogenetic analysis of LSU rDNA. In this study, the taxa of *Lactarius* formed a single clade without *Russula* and were placed in the basal position of the phylogenetic tree. The scenario that the genus *Russula* originated from the genus *Lactarius* was not clear because of the low bootstrap value. However, the lack of latex cells might occur once with the increased spherocyst in trama. In contrast, the gasteroidization may have occurred many times in the genus *Russula*. An analysis of additional taxa would be required to clarify the relationship between *Lactarius* and *Russula*. In particular, additional samples of sections *Plorantes* and *Pallidosporinae* sensu Bon in *Russula* and section *Albati* in *Lactarius* would be necessary.

#### The suprahilar region of the spore

The suprahilar region of the spore of Russulaceae showed either amyloid or inamyloid dots with Melzer's reagent. Singer (1986) reported that Heim's (1937) description of the presence or absence of amyloid dots in the suprahilar of the spores has not been confirmed by many later researchers but seems to be important for the definition of sections. As a result, species not showing a suprahilar amyloid spot in *Russula* were grouped into the following sections or subsection: *Compactae*, *Crassotunicatae*, *Pelliculariae*, and subsection *Virescentinae* of section *Rigidae*. On the other hand, section *Plorantes* showed an amyloid spot. For *Lactarius*, Heilmann-Clausen et al. (1998) stated that the region of the spores covered by an amyloid substance were in some cases a useful identification tool. To clarify whether this character is useful for the identification of species, amyloid or inamyloid regions are shown in Table 3 based on data from Singer (1986), Imazeki and Hongo (1989), Hesler and Smith (1979), Sarnari (1998), Bon (1987), and Romagnesi (1996).

A large number of taxa are included in groups D and B-1, namely, section *Plorantes* with strongly amyloid regions.

The section and subsections with inamyloid regions were section *Compactae* (group E), subsection *Virescentinae* (group F-1), and subsection *Cyanoxantinae* (group F-5). Concerning spore ornamentation and amyloid or inamyloid regions, Dodd and McCracken (1972) proposed two hypotheses: one is that thin layers of amylose molecules act as oxygen barriers, while the other is that short-chained amylose molecules are soluble in cold water. In the former hypothesis, the amylose layer inhibits the oxygen uptake of amyloid spores to reduce the metabolism. During spore germination, the amylose film would have to be removed. It is not clear why the surface ornamentation of the spore and suprahilar region is amyloid. Based on molecular analysis, the macroscopic and microscopic characteristics that include surface ornamentation are more natural than previously believed (Miller and Buyck 2002). The present analysis supports the report of Miller and Buyck (2002) that amyloidity of suprahilar regions is a useful characteristic for the classification of *Russula*. However, because the degree of amyloid in the surface ornamentation and suprahilar region differs with the age of fruiting bodies and individuals, it is important to use mature fruiting bodies for observations of surface ornamentation and suprahilar regions in *Russula*.

#### Spore color

Four grades of spore color are shown in Table 3, based on Romagnesi's color chart: W for I, CR for II, SY for III, and Y for IV. Many taxa that had colored spore prints were included in group D. In particular, taxa with slightly yellow spores were included in group D-1. In contrast, taxa that belonged to section *Compactae*, subsection *Virescentinae* and subsection *Cyanoxantinae* have white spore prints. The taxa with colored spore prints were strongly amyloid in the suprahilar region. Buyck (1995) reported that in seasonal tropic and cold-temperate alpine climates spore prints of *Russula* and *Lactarius* have a deeper color, whereas Russulaceae in lowland rain forests have mostly white or offwhite spore prints, as in the *R. nigricans* group. There are two reasons why we used many taxa that had a white spore color for the present analysis: one is that there are few taxa with colored spores in Japan, and the other is that many taxa with white spores are distributed in evergreen forests of the western district of Japan. Romagnesi (1967) considered that the white spore print is a plesiomorphic characteristic whereas the colored spore print is an apomorphic characteristic. Although spore color can be changed by environmental factors, it is a useful tool in deciding whether they belong to taxa with primitive or derived characteristics.

Sections *Pelliculariae* and *Compactae*, and two species *Russula earlei* and *R. flavida*

The sequences of two tropical annulate species of *Russula*, *R. radicans* and *R. discopus*, obtained from the database and *R. ebruneoareolata*, which belonged to section *Pelliculariae*, were included in this study. *Russula discopus*

**Table 3.** Color of spore print, amyloid in suprahilar plage, and groups in phylogenetic neighbor-joining (NJ) tree

Taxon <sup>a</sup>	Extract no. <sup>b</sup>	Classification (sect., subsect.)	Color of spore <sup>c</sup>	Suprahilar plage <sup>d</sup>	Group <sup>e</sup>
<i>R. eburneoareolata</i> Hongo	297	<i>Pelliculariae, Guayarenses</i>	W	○	F-1
<i>R. radicans</i> Heim	AF218547	<i>Pelliculariae, Radicantes</i>	NA	NA	F
<i>R. discopus</i> Heim	AF218543	<i>Pelliculariae,</i>	NA	NA	D-2
<i>R. japonica</i> Hongo	153	<i>Delicoarchaeae</i>	SY	●	B-1
<i>R. chloroides</i> (Krombh.) Bres.	358	<i>Plorantes</i>	W	●	B-2
<i>R. chloroides</i> (Krombh.) Bres.	343	<i>Plorantes</i>	W	●	B-2
<i>R. delica</i> Fr.	156	<i>Plorantes</i>	W	●	B-2
<i>R. compacta</i> Frost & Peck apud Peck	206	<i>Crassotunicatae</i>	W	●	E
<i>R. adusta</i> (Pers.) Fr.	351	<i>Compactae</i>	W	○	E-1
<i>R. adusta</i> (Pers.) Fr.	AF218544	<i>Compactae</i>	W	○	E-1
<i>R. densifolia</i> Gill.	294	<i>Compactae</i>	W	○	E-1
<i>R. densifolia</i> Gill.	352	<i>Compactae</i>	W	○	E-1
<i>R. densifolia</i> Gill.	3510	<i>Compactae</i>	W	○	E-1
<i>R. nigricans</i> Fr.	195	<i>Compactae</i>	W	○	E-2
<i>R. nigricans</i> Fr.	356	<i>Compactae</i>	W	○	E-2
<i>R. nigricans</i> Fr.	398	<i>Compactae</i>	W	○	E-2
<i>R. nigricans</i> Fr.	4010	<i>Compactae</i>	W	○	E-2
<i>R. subnigricans</i> Hongo	251	<i>Compactae</i>	W	○	E-3
<i>R. subnigricans</i> Hongo	252	<i>Compactae</i>	W	○	E-3
<i>R. subnigricans</i> Hongo	253	<i>Compactae</i>	W	○	E-3
<i>R. subnigricans</i> Hongo	261	<i>Compactae</i>	W	○	E-3
<i>R. subnigricans</i> Hongo	391	<i>Compactae</i>	W	○	E-3
<i>R. subnigricans</i> Hongo	392	<i>Compactae</i>	W	○	E-3
<i>R. subnigricans</i> Hongo	407	<i>Compactae</i>	W	○	E-3
<i>Russula</i> sp.	342	<i>Compactae</i>	W	○	E-3
<i>R. dissimulans</i> Shaff.	354	<i>Compactae</i>	W	○	E-2
<i>R. dissimulans</i> Shaff.?	399	<i>Compactae</i>	W	○	E-2
<i>R. rubescens</i> Bwardislee	137	<i>Decoloroantes, Decolorantinae</i>	SY	●	D-1
<i>Russula</i> sp.	3115	<i>Decoloroantes, Decolorantinae</i>	SY	●	D-1
<i>R. earlei</i> Peck	347	<i>Ingratae, Fistulosinae</i>	W	○	B-3
<i>R. earlei</i> Peck	AF042571	<i>Ingratae, Fistulosinae</i>	W	○	B-3
<i>R. foetens</i> Pers.: Fr.	291	<i>Ingratae, Foetentinae</i>	CR	⊙	F-3
<i>R. laurocerasi</i> Melzer	292	<i>Ingratae, Foetentinae</i>	CR	⊙	F-3
<i>R. laurocerasi</i> Melzer	293	<i>Ingratae, Foetentinae</i>	CR	⊙	F-3
<i>R. pectinatoides</i> Peck	289	<i>Ingratae, Foetentinae</i>	CR	⊙	F-3
<i>R. sororia</i> (Fr.): Romell	288	<i>Ingratae, Foetentinae</i>	CR	⊙	F-3
<i>R. foetentula</i> Peck	AF218546	<i>Ingratae, Foetentinae</i>	CR	○	F-3
<i>R. cyanoxantha</i> (Schaeff.) Fr.	12cyG1	<i>Rigidae, Cyanoxanthinae</i>	W	○	F-5
<i>Russula</i> sp.	296	<i>Rigidae, Cyanoxanthinae</i>	W	○	F-5
<i>R. cutedracta</i> Cke.	374	<i>Rigidae, Cyanoxanthinae</i>	W	○	F-5
<i>R. cutedracta</i> Cke.	373	<i>Rigidae, Cyanoxanthinae</i>	W	○	F-5
<i>R. variata</i> Ban. apud Pk.	273	<i>Rigidae, Cyanoxanthinae</i>	W	○	F-5
<i>R. heterophylla</i> (Fr.) Fr.	339	<i>Rigidae, Lividinae</i>	W	○	F-4
<i>R. vesca</i> Fr.	338	<i>Rigidae, Lividinae</i>	W	○	F-4
<i>R. aeruginea</i> Lindbl. apud Fr.	283	<i>Rigidae, Subcompactinae</i>	CR	○	F-1
<i>R. grisea</i> Fr.	282	<i>Rigidae, Subcompactinae</i>	CR	○	F-1
<i>R. amoena</i> Quél.	12AG2	<i>Rigidae, Amoeninae</i>	CR	○	F-2
<i>R. flavida</i> Frost & Peck apud Peck	2612	<i>Rigidae, Amoeninae</i>	SY	⊙	D-1
<i>R. flavida</i> Frost & Peck apud Peck	333	<i>Rigidae, Amoeninae</i>	Y	⊙	D-4
<i>R. flavida</i> Frost & Peck apud Peck	349	<i>Rigidae, Amoeninae</i>	Y	⊙	D-4
<i>Russula</i> sp.	4011	<i>Rigidae, Amoeninae</i>	SY	⊙	D-1
<i>R. bella</i> Hongo	1712	<i>Rigidae, Amoeninae</i>	CR	○	F-2
<i>R. violipes</i> Quél.	223	<i>Rigidae, Amoeninae</i>	CR	○	F-2
<i>R. alboareolata</i> Hongo	124	<i>Rigidae, Virescentinae</i>	W	○	F-1
<i>R. virescens</i> (Schaeff.) Fr.	144	<i>Rigidae, Virescentinae</i>	W	○	F-1
<i>R. virescens</i> (Schaeff.) Fr.	363	<i>Rigidae, Virescentinae</i>	W	○	F-1
<i>R. virescens</i> (Schaeff.) Fr.	AF041548	<i>Rigidae, Virescentinae</i>	W	○	F-1
<i>R. viridirubrolimbata</i> Ying	2510	<i>Rigidae, Virescentinae</i>	W	○	F-1
<i>R. viridirubrolimbata</i> Ying	281	<i>Rigidae, Virescentinae</i>	W	○	F-1
<i>R. lilacea</i> Quél.	348	<i>Rigidae, Lilaceinae</i>	W	○	F-1
<i>R. rosea</i> Quél.?	3116	<i>Rigidae, Roseinae</i>	W	●	D-2
<i>R. rosacea</i> (Pers.) S.F. Gray	284	<i>Rigidae, Lepidinae</i>	CR	●	D-4
<i>R. xerampelina</i> (Schaeff.) Fr.	361	<i>Rigidae, Xerampelinae</i>	SY	●	D-1
<i>R. xerampelina</i> (Schaeff.) Fr.	AF218542	<i>Rigidae, Xerampelinae</i>	SY	●	D-1
<i>R. brunneola</i> Burl.	AF218548	<i>Rigidae, Modestinae</i>	W	NA	F-4
<i>R. omiensis</i> Hongo	7N1	<i>Rigidae</i>	W	●	D-3
<i>R. kansaiensis</i> Hongo	285	<i>Russula, Puellarinae</i>	CR	●	D-2
<i>R. emetica</i> (Schaeff.: Fr.) S.F. Gray	213	<i>Russula, Russula</i>	W	●	C-3
<i>R. mairei</i> Singer	U11926	<i>Russula, Russula</i>	W	●	F-2

Table 3. Continued

Taxon <sup>a</sup>	Extract no. <sup>b</sup>	Classification (sect., subsect.)	Color of spore <sup>c</sup>	Suprahilar plage <sup>d</sup>	Group <sup>e</sup>
<i>R. sanguinea</i> (Bull.) Fr.	344	<i>Russula, Sanguineae</i>	SY	●	D-3
<i>R. aurea</i> Pers.	298	<i>Russula, Firmiores</i>	Y	●	D-4
<i>R. romagnesii</i> Singer	AF042572	<i>Russula, Urentes</i>	Y	●	B-1
<i>R. campinensis</i> Henkel, Aime et Miller	AF218560	NA	W	○	B-1
<i>R. campinensis</i> Henkel, Aime et Miller	AF218564	NA	W	○	B-1
<i>R. campinensis</i> (ectomycorrhizae)	AF218565	NA	W	○	B-1
<i>L. corrugis</i> Peck	U11919	<i>Dulces</i>	W	○	A-3
<i>L. volemus</i> (Fr.) Fr.	AF042574	<i>Dulces</i>	W	○	A-2
<i>L. fumosus</i> var. <i>fumosus</i> Peck	AF218551	<i>Plinthogali</i>	W (P)	●	A-1
<i>L. lignyotellus</i> Sm. & Hesl.	AF218557	<i>Plinthogali</i>	CR	○	A-1
<i>Lactarius</i> sp.	254	<i>Plinthogali</i>	W	○	A-1
<i>L. deceptivus</i> Peck	AF218550	<i>Albati</i>	CR	○	A-2
<i>L. vellereus</i> (Fr.) Fr.	375	<i>Albati</i>	W	○	A-2
<i>L. piperatus</i> (Scop.) S.F. Gray	AF042573	<i>Albati</i>	W	○	A-2
<i>L. piperatus</i> (Scop.) S.F. Gray	AF218556	<i>Albati</i>	W	○	A-2
<i>L. speciosus</i> Burlingham	AF218555	<i>Lactarius</i>	W	○	A-3
<i>L. subpurpureus</i> Peck	AF218553	<i>Lactarius</i>	CR	○	A-3
<i>L. subdulcis</i> (Fr.) S.F. Gray	AF218552	<i>Russulares</i>	SY	○	A-3
<i>L. peckii</i> var. <i>peckii</i> Burlingham	AF218554	<i>Russulares</i>	W	●	A-3
<i>L. panuoides</i> Singer	AF281561	<i>Panuoidei</i>	W	○	A-2
<i>L. uyedae</i> Singer	AF281562	<i>Panuoidei</i>	W	○	A-2
<i>M. americanus</i> Sing. & Sm.	AF265540	NA	W	○	D-1
<i>B. berkeleyi</i> (Fr.) Singer	AF218563, AF287849	NA	W	○	–

NA, not applicable

<sup>a</sup>*R.*, *Russula*; *L.*, *Lactarius*; *M.*, *Macowanites*; *B.*, *Bondarzewia*

<sup>b</sup>Accession number for database and extract number of taxon in this study

<sup>c</sup>W, I; CR, II; SY, III; Y, IV, in Romagnesii color chart

<sup>d</sup>●, amyloid; ○, weakly amyloid; ○, nonamyloid in suprahilar plage

<sup>e</sup>Small groups in phylogenetic NJ tree

constituted group D-2 with *R. violacea* in the NJ tree whereas *R. radicans* constituted group F with sections *Ridigae* and *Ingratae* (bootstrap value, 54%). However, these species were not in the same clade as *R. ebruneoaeolata*, which was included with a high bootstrap value (69%) in group F-1 with subsection *Virescentinae* and section *Rigidae*. From their LSU rDNA regions, Miller et al. (2001) considered section *Pelliculariae* as an artificial assemblage that showed affinities with several groups. This conclusion was based on their molecular phylogenetic study of Russulales that included agaricoid, gasteroid, and pleurotoid taxa. In their report, the position of the African annulate species was consistent with the reports of Buyck (1995; Buyck and Horak 1999). Buyck (1989) reported that section *Pelliculariae* was a heterogeneous assemblage of small, very thin, frequently annulate species, some of which belonged to subsection *Amoeninae* with a worldwide distribution. In this study, section *Pelliculariae*, which contained one annulate species, *R. radicans*, and *R. ebruneoaeolata* was related to subsections *Virescentinae* and *Amoeninae*, whereas *R. discopus* was related to group D. Therefore, section *Pelliculariae* should be regarded as an artificial assemblage.

Section *Compactae* sensu Fries includes two species, *R. delica* and *R. nigricans*, based on the presence of regularly intercalated lamellulae and flesh toughness. Singer (1986) restricted section *Compactae* to the *R. nigricans* group and established section *Plorantes* for the species of the *R. delica* group. This concept was supported by morphological–anatomical analyses (Buyck 1989) and by molecular analy-

ses based on LSU rDNA (Miller et al. 2001) and ITS regions (Miller and Buyck 2002). In this study, we found that the two sections were not as closely related as previously believed, which supports the molecular analysis of the LSU rDNA of Miller et al. (2001). We found three different lineages in section *Compactae*: groups E-1 (*R. densifolia*), E-2 (*R. nigricans*), and E-3 (*R. subnigricans*). Group E-2 (*R. nigricans*) has thick lamellae that are very distant, strong reddening before blackening, whereas *R. dissimulans* 399 has slightly narrow lamellae. *Russula adusta* (group E-1) has sturdy and fleshy fruiting bodies, is slightly distant with age in the lamellae, slowly turns very pale pink and then gray, and has finely reticulate spores. *Russula densifolia* shows reddening and then blackening, has narrow lamellae with a cream tinge, and the pileus is mostly dry, dull brown, and sometimes has a slightly olivaceous tinge. *R. subnigricans* (group E-3), a poisonous mushroom, is similar to *R. nigricans* in having thick lamellae and distant reddening, but not blackening. Group E-3 consists of a small clade that has pinkish lamellae, a slightly tuberculate sulcate pileal margin, a characteristic smell, and small warts in the spore ornamentation without a network. The *R. subnigricans* group was divided into at least three species. Buyck (1995) described the impressive diversity of African “*Compactae*” that is currently divided into many subsections. Analyses of more taxa from Africa, the tropics, and Asia are required to clarify the phylogenetic relationship within section *Compactae*. For example, *R. densifolia* is divided into two small clades in this study. Imazeki and Hongo (1989)

reported that the complex around *R. densifolia* contains several species based on macroscopic features. In the future, this group in Japan may be divided into two or three different species. The *R. densifolia* group, which contains *R. albonigra* and *R. adusta*, is divided into five or six small clades in the ITS analysis (data not shown).

The macroscopic features of *R. earlei* are unusual in the genus *Russula*. Singer (1957) placed *R. earlei* into the genus *Hygrophorus* because of its waxy, translucent pileus and widely spaced lamellae. Bills and Miller (1984) stated that the unusual microscopic features of *R. earlei* were the absence of a laticiferous hyphal system and the presence of thick-walled trama hyphal and of small, readily collapsing basidiospores ornamented with isolated, blunt verrucae. Singer (1986) revised *R. earlei* into section *Ingratae*. Bill and Miller (1984) then transferred the species into section *Archaeinae*. From their molecular analysis, Miller et al. (2001) reported that section *Archaeinae* is a sister to section *Compactae*. This study, as well as that of Miller et al. (2001), showed that this species is a sister-group of subsection *Plorantinae* of section *Compactae* (see Fig. 1). In the MP tree, this species was placed in the basal position of the Russulaceae. Therefore, this species could be placed into section *Archaeinae* sensu Heim, not into section *Ingratae*.

*Russula flavida* is characterized by its bright yellow to orange-yellow color, velvety pileus and stipe, and yellow spore print. This species was placed into subsection *Amoeninae* by Singer (1975, 1986). Bill and Miller (1984) reported that *R. flavida* and *R. ochroleuroides* should be placed into another subsection because of the lack of subulate or aciculate dermatocystidia and cheilocystidia and the characteristic odor that is unique in the two species. In this study, four isolates of *R. flavida* were not placed into subsection *Amoeninae* (group F-2). Two were placed into group D-4 (section *Russula* with *R. aurea*) while the other two isolates were placed into group D-1. Specimens from Japan regarded as *R. flavida* represent two different species, which should be placed into group D-4. Because *R. flavida* from Japan possessed the characteristic smell, saffron fruiting body color, and yellow spore print, this species should be placed into section *Russula*, not into subsection *Amoeninae*.

#### The molecular phylogeny and the taxonomic system of the genus *Russula*

Some groups found in the present phylogenetic tree, which included Asian taxa, corresponded to six large basal clades identified by Miller and Buyck (2002). Our group B corresponded to their large clade nos. 1 and 2, group D to nos. 5 and 6, group E to no. 4, and group F to no. 3. Because we had few deep-colored spore taxa, the subgenera of Miller and Buyck (2002) were not found in this study. However, based on molecular data and morphological characteristics, some terminal nodes showed some similar sections and subsections, e.g., sections *Compactae*, *Plorantes*, and subsection *Fotentinae* of section *Ingratae*, and *Virescentinae*, *Cyanoxanthinae*, and *Amoeninae* of section *Rigidae*. The

terminal clades found in the present study might correspond to sections *Ingratae* and *Plotantes* and subsections *Virescentinae*, *Cyanoxanthinae*, and *Amoeninae* of section *Rigidae*.

We used the taxonomic system of Singer (1986), which included Asian taxa, in this study. Miller and Buyck (2002) stated that the classification of Romagnesi (1967) was more suitable than Singer's system for sections *Russula* and *Rigidae* based on morphological and molecular characteristics. In section *Rigidae*, *R. rosacea* (*R. lepida*) does not form a single clade with other subsections of section *Rigidae*, e.g., *Cyanoxanthae*, *Amoeninae*, and *Virescentinae*, as hypothesized by Miller and Buyck (2002). Therefore, section *Rigidae* of Singer must be reorganized as an artificial assembly group that contained many morphologically divergent species. Only a few taxa of section *Rigidae* were included in the present analysis because this group of Russulaceae has not yet been identified in Japan. If species of this group are included in future studies, the phylogeny of the genus *Russula* would become clearer. To clarify the phylogenetic relationships throughout the Russulaceae, many more species of the genus *Lactarius* should also be investigated.

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