

FULL PAPER

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## Molecular phylogeny of *Lactarius volemus* and its allies inferred from the nucleotide sequences of nuclear large subunit rDNA

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**Abstract** The phylogenetic relationships of *Lactarius volemus* and its relatives were investigated using the nucleotide sequences of the nuclear-encoded large subunit ribosomal DNA (LSU rDNA). Thirty-six sequences from *L. volemus*, *L. corrugis*, and *L. hygrophoroides*, including three sequences obtained from the GenBank database, were used in this study. Samples studied were divided into four major subclades (A–D) in both neighbor-joining (NJ) and maximum-parsimony (MP) trees. *Lactarius volemus* and *L. corrugis* formed one large clade in both NJ and MP trees (bootstrap value, 100%), which was divided into three subclades (A–C). Subclade A included three clusters of *L. volemus* strains, i.e., velvet, red, and Chinese types. Subclade B included the common and red types of *L. corrugis*. Subclade C included the common and yellow types of *L. volemus*. Subclade D included only one type of *L. hygrophoroides*. An analysis of the fatty acid composition supported the divisions found in the molecular analysis. Analyses of nucleotide sequence, fatty acid composition, morphological characteristics, and the taste of the fruiting bodies all led us to conclude that the common, velvet, red, and Chinese types of *L. volemus*, and the common and red types of *L. corrugis*, may each belong to different species, subspecies, or varieties. Further studies with more material from a wide range of regions are required to conduct taxonomic revision of these types. The LSU rDNA region may be a useful tool to investigate phylogenetic relationships within the section *Dulces* of the genus *Lactarius*.

**Key words** Fatty acid composition · *Lactarius corrugis* · *Lactarius hygrophoroides* · *Lactarius volemus* · Section *Dulces*

### Introduction

The genus *Lactarius*, consisting of 400 species (Kirk et al. 2001), is distributed from tropical regions to extremely cold regions and mostly form obligately ectotrophic mycorrhizae with many kinds of forest trees. Some *Lactarius* species form mycorrhizae with conifers, whereas other species form mycorrhizae with hardwood trees, mainly of the orders Salicales and Fagales (Singer 1986). Eleven species are known in the section *Dulces* (Singer 1986), and 4 of these species have been identified in Japan, i.e., *L. volemus* (Fr.) Fr., *L. corrugis* Peck, *L. hygrophoroides* Berk. & M.A. Curt., and *L. luteolus* Peck. Of these 4 species, *L. volemus* and *L. corrugis* are similar in macro-morphological characteristics, e.g., color and surface structure of the pileus and stipe. *Lactarius corrugis* has a darker and distinctly wrinkled pileus surface comparing with *L. volemus*. However, a type that has a pileus intermediate between *L. corrugis* and *L. volemus* in color and surface structure is often seen (Hesler and Smith 1979). Therefore, it is difficult to distinguish between these 2 species based on macro-morphological characteristics. *Lactarius volemus* is a major species of the genus *Lactarius* that is widely distributed in the warm temperate or northern districts of the Northern Hemisphere, and it is known as an edible mushroom. In Europe, the pileus color of *L. volemus* is variable and not taxonomically important (Heilmann-Clausen et al. 1998). Similarly, *L. volemus* and *L. corrugis* are known to vary in the color and texture of the pileus and stipe in North America (Hesler and Smith 1979). In Japan, there are also some variations in the color and surface texture of the pileus of *L. volemus*, i.e., a red pileus, a yellow pileus with a long stipe, or a velvety surface texture. In Japanese *L. corrugis*, two types are recognized based on the color of the pileus, i.e., a common type typically with dark ferruginous color in the pileus and a red type

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with reddish color. Our preliminary examination of their fatty acid composition suggested that there are also differences among these types of *L. volemus* and *L. corrugis*.

Recent advances in molecular techniques (Drehmel et al. 1999; Hopple and Vilgalys 1999; 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. The present study aims to identify species of the section *Dulces* and to provide information on their phylogenetic relationships based on the nucleotide sequence of their LSU rDNA and on their fatty acid composition.

## Materials and methods

### Sample sources

Thirty-six samples of *L. volemus*, *L. corrugis*, and *L. hygrophoroides*, and three other species from the section *Dulces*, i.e., *L. piperatus* (Scop.) S.F. Gray (AF218556), *L. subumbonatus* Lindgren (AF506412), and *L. lignyotus*

Fr. (AY631898) were studied. Their collection locations, specimen numbers, and accession numbers in the nucleotide sequence databases (DDBJ) are provided in Table 1. Of 36 samples used in this study, 23 were from the species *L. volemus*, 10 were from *L. corrugis*, and 3 were from *L. hygrophoroides*.

The specimens were dried and deposited in the Natural History Museum of Osaka City. The fungal species were identified based on Imazeki and Hongo (1989).

### Macro-morphological characteristics

The main macro-morphological characteristics of the respective types of *L. volemus* and *L. corrugis* used in this study are as follows.

#### Types of *L. volemus*

Common type (Fig. 1): This type is distributed in various kinds of broadleaf forests and is the most widespread type

**Table 1.** List of the species of *Lactarius volemus* and allies used in this study

Species	Collection no.	Extract no.	Origin	Type	Accession no.
<i>L. volemus</i> (Fr.) Fr.	OSA-My-3993	5313	Japan, Koriyama city	Velvet	AB238645
<i>L. volemus</i> (Fr.) Fr.	OSA-My-3994	539	Japan, Ibaraki city	Velvet	AB238646
<i>L. volemus</i> (Fr.) Fr.	OSA-My-3995	429	Japan, Koriyama city	Velvet	AB238647
<i>L. volemus</i> (Fr.) Fr.	OSA-My-3996	611	Japan, Iwaki city	Velvet	AB238648
<i>L. volemus</i> (Fr.) Fr.	OSA-My-3997	5312	Japan, Koriyama city	Red	AB238649
<i>L. volemus</i> (Fr.) Fr.	OSA-My-3998	562	Japan, Koriyama city	Red	AB238650
<i>L. volemus</i> (Fr.) Fr.	OSA-My-3999	L12	Japan, Fukushima Pref.	Red	AB238651
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4000	612	Japan, Koriyama city	Red	AB238652
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4001	616	Japan, Minoo city	Red	AB238653
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4002	543	China	Chinese	AB238654
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4003	563	China	Chinese	AB238655
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4004	5311	Japan, Koriyama city	Common	AB238656
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4005	581	Japan, Abiko city	Common	AB238657
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4006	582	Japan, Abiko city	Common	AB238658
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4007	L10	Japan, Fukushima Pref.	Common	AB238659
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4008	624	Japan, Koriyama city	Common	AB238660
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4009	542	Japan, Fukushima Pref.	Yellow	AB238661
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4010	L9	Japan, Fukushima Pref.	Yellow	AB238662
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4011	4210	Japan, Koriyama city	Common	AB238663
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4012	L4	Japan, Koriyama city	Common	AB238664
<i>L. volemus</i> (Fr.) Fr.	OSA-My-4013	L5	Japan, Koriyama city	Common	AB238665
<i>L. volemus</i> (Fr.) Fr.		Database			AF042574
<i>L. volemus</i> (Fr.) Fr.		Database			AF506413
<i>L. corrugis</i> Peck	OSA-My-4014	L1	Japan, Fukushima Pref.	Red	AB238666
<i>L. corrugis</i> Peck	OSA-My-4015	541	Japan, Fukushima Pref.	Red	AB238667
<i>L. corrugis</i> Peck	OSA-My-4016	L8	Japan, Fukushima Pref.	Red	AB238668
<i>L. corrugis</i> Peck	OSA-My-4017	L2	Japan, Fukushima Pref.	Red	AB238669
<i>L. corrugis</i> Peck	OSA-My-4018	L3	Japan, Fukushima Pref.	Red	AB238670
<i>L. corrugis</i> Peck	OSA-My-4019	5310	Japan, Ibaraki city	Common	AB238671
<i>L. corrugis</i> Peck	–	ch23	Japan, Kanuma city	Common	AB238672
<i>L. corrugis</i> Peck	OSA-My-4020	613	Japan, Iwaki city	Common	AB238673
<i>L. corrugis</i> Peck	OSA-My-4021	614	Japan, Fukushima Pref.	Common	AB238674
<i>L. corrugis</i> Peck		Database			U11919
<i>L. hygrophoroides</i> Berk. & Curt.	OSA-My-4022	L6	Japan, Koriyama city		AB238675
<i>L. hygrophoroides</i> Berk. & Curt.	OSA-My-4023	L7	Japan, Koriyama city		AB238676
<i>L. hygrophoroides</i> Berk. & Curt.	OSA-My-4024	561	Japan, Koriyama city		AB238677
<i>L. piperatus</i> (Scop.) S.F. Gray		Database			AF218556
<i>L. subumbonatus</i> Lindgr.		Database			AF506412
<i>L. lignyotus</i> Fr.		Database			AY631898

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

–, a specimen of *L. corrugis* ch23 is missing

in Japan. The pileus is 5–12 cm across and is initially convex, becoming flat to slightly concave with age. The surface of the pileus has a fine velvety texture when young and is ochre to cinnamon, reddish-orange, orange-brown, or dark brick red in color. The lamellae are slightly decurrent, and they are pale cream to light yellow, often brown-spotted when bruised. The flesh is very firm and pale cream, turning pale brownish with age. It has a mild and delicious taste and a strong herring-like smell when dry. Latex is abundant, white and staining brown.

**Yellow type (Fig. 2):** This type occurs earlier in the season than the common type. The color of the pileus is pale yellow, the stipe is slender, and the general appearance is similar to a yellowish *L. piperatus*. The occurrence of fruiting bodies is infrequent.

**Velvet type (Fig. 3):** This type occurs from early July, earlier than either the common or the red types of *L. volemus*. The surface of the pileus is velvety, particularly when young. The pileus is darker in color in the inside than the outside.

**Red type (Fig. 4):** This type occurs from early August to early October in the Tohoku district. The pileus is a darker red than the common type (Fig. 1), particularly when young. The latex is darker (dark brown when dry) than the latex of the common type.

**Chinese type:** The color of the pileus is similar to the common type. Fruiting bodies, imported from China, were obtained from the market in Tochigi Prefecture.

#### Types of *L. corrugis*

**Common type (Fig. 5):** This type is widely distributed in broadleaf forests in Japan. The surface of the stipe has a whitish bloom when mature. The pileus is 5–12 cm across, initially convex, becoming concave with age. Its surface is conspicuously velvety and minutely wrinkled, and its color is orange-cinnamon to ferruginous, the margin sometimes being paler than the center. The stipe is 5–7 cm long, 1.5–2.5 cm thick, velvety, minutely wrinkled, grayish-brown, and paler than the pileus.

**Red type (Fig. 6):** This type has been found only on higher land in the town of Aizu Tajima in Fukushima Prefecture. The stipe surface appears obscurely pruinose at first but soon becomes glabrous. The pileus is red-colored as in the red type of *L. volemus*; hence it is difficult to distinguish this type from the red and common types of *L. volemus* on the basis of the pileus color.

#### DNA extraction and PCR amplification

DNA was isolated from the lamellae of the fruiting bodies using the method reported by Suyama et al. (1996) and Shimono et al. (2004). The LSU rDNA region was amplified two or three times by polymerase chain reaction (PCR) using nested primer sets, and the PCR reactions were conducted in 50- $\mu$ l volumes as previously described (Hirata and Takamatsu 1996). A negative control that lacked template DNA was included in each set of reactions. The PCR prod-

uct was subjected to electrophoresis in 1.5% agarose gel in a TAE buffer. The DNA product from each amplification was excised from the ethidium bromide (0.01  $\mu$ g/ml)-stained gel and purified using a JETSORB kit (GENOMED, Oeynhausen, Germany) following the manufacturer's instructions.

#### PCR primers

The oligonucleotide primers were the same as those used in previous studies (Shimono et al. 2004). For amplifications of the 5'-end of the large subunit (LSU) rDNA including the D1 and D2 regions, the primer set BN1/TW14 was used for the first amplification. The nested primer set Ctb6/TW14 was used for the second amplification. If the second amplification was not successful, the primer set NL1/TW14 was used for the 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 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 large subunit rDNA in both directions.

#### 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 available upon request from the corresponding author. Phylogenetic trees were obtained by distance and parsimony methods. For the parsimony analysis, we used 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 the branch-swapping algorithm.

For the analysis of distances methods, 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\*.





**Fig. 1-6.** Fruit bodies of four types belonging in *Lactarius volemus* and of two types belonging in *L. corrugis*. 1-4 *Lactarius volemus*: 1 common type; 2 yellow type; 3 velvet type; 4 red type. 5, 6 *L. corrugis*: 5 common type; 6 red type. Bars 20mm

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

#### Analysis of fatty acid composition

Total lipids were extracted from fresh fruiting bodies according to the Floch's method (Floch et al. 1957) using a

mixture of chloroform and methanol (2:1 vol/vol). The lipids were converted to methyl esters by transesterification with 5% HCl in methanol. The fatty acid methyl esters were analyzed by a Shimadzu GC-8A gas-liquid chromatograph equipped with a flame ionization detector. The analysis conditions were as follows. The packing agent was Diasolid ZF (2m × 3.2mm glass column), the carrier gas was N<sub>2</sub>, the injector and detector temperatures were both 240°C, the column temperature was 205°C, and the integrator was a

Shimadzu CR-1B. Identification of fatty acid methyl esters was performed by comparing their retention times to that of various standards.

## Results

### Molecular phylogenetic analysis

The length of nuclear LSU rDNA amplification products ranged from 778 to 881 base pairs, including the divergent domains D1 and D2 (Hopple and Vilgalys 1999). Based on these sequences, we constructed an alignment data set of 889 sites consisting of 39 ingroup and two outgroup sequences and used it for the phylogenetic analysis. Two sequences of *Bondarzewia berkeleyi* (Fr.) Bond & Sing. (AF218563, AF287849) were used as outgroup sequences. Of the 889 sites, 29 were variable but phylogenetically uninformative, and 114 were phylogenetically informative in parsimony. Using Modeltest (Posada and Crandall 1998), we concluded that the Tamura–Nei model (Tamura and Nei 1993), using equal base frequencies, a gamma-distributed rate heterogeneity model (four rate categories,  $G = 0.6074$ ), and an estimated proportion of invariant sites ( $TrN + I + G$ ), was the most appropriate model of evolution for this data set. A NJ tree produced using this data set is shown in Fig. 7. A set of 72 most parsimonious trees of 236 steps, which differ only in the minor branching order of terminal taxa, was found in a maximum-parsimony (MP) analysis [consistency index (CI) = 0.6822; retention index (RI) = 0.8689]. The MP tree is not shown, as it was almost identical to the NJ tree.

All the members of section *Dulces* studied formed a single large clade, except for a sequence of *L. corrugis* obtained from GenBank. The bootstrap value of the large clade was 55% in the NJ analysis (see Fig. 7) and 51% in the MP analysis. The clade consisted of three subclades, A, B, and C, each containing specimens of *L. volemus* and *L. corrugis*, but not *L. hygrophoroides*. Each of these subclades had a bootstrap value of 100% in both NJ and MP trees. Subclade A contained some isolates of *L. volemus*, and subclade B contained *L. corrugis* isolates that formed a separate subclade with a low bootstrap value. Subclade A in both NJ and MP analyses was divided into three clusters, each containing one of three types of *L. volemus* [velvet type (A-1), red type (A-2), and Chinese type (A-3)], which were identified on the basis of the color of the pileus. Sub-

clade B was divided into two clusters, each containing one of two types of *L. corrugis* [red type (B-1) and common type (B-2)]. The common type of *L. volemus*, having the typical morphological characteristics of *L. volemus*, formed a single group (subclade C) in both NJ and MP trees. In this subclade, type C-1 (common type in Japan) was closely related to C-2 (yellow type). A fourth subclade, subclade D, contained three sequences of *L. hygrophoroides*. These sequences formed a single cluster within the subclade, having a bootstrap value of 100% in both NJ and MP trees. Two sequences of *L. volemus* obtained from GenBank were placed at the base of subclade C in the NJ and MP trees.

### Fatty acid composition of fruiting bodies

The fatty acid compositions of the stipe of the mature fruiting bodies of *L. volemus*, *L. corrugis*, and *L. hygrophoroides* are shown in Table 2. The percentage of 18:1 and 18:2 fatty acids in the common type of *L. volemus* was similar to that in the yellow type. The percentage of the fatty acid 18:3 in the common, yellow, and velvet types of *L. volemus* was higher (0.9%–2.6%) than in the other types (0%–0.2%). The percentage of 18:2 was higher than that of 18:1 in the velvet type of *L. volemus*, but not in the other types of *L. volemus* and *L. corrugis*. The percentage of fatty acid 18:1 was higher than that of 18:2 in the red types of *L. volemus* and *L. corrugis* and in the common type of *L. corrugis*. The percentage of 16:1 was higher in two *L. corrugis* types than in the other types, but the percentage of 16:1 differed between these two types of *L. corrugis*. That is, the *L. corrugis* red type contained twice as much 16:1 fatty acid as the common type. *Lactarius hygrophoroides* contained a high percentage of the fatty acids 18:2 (77.4%) and 16:0 (9.4%).

## Discussion

In this molecular study, a sequence of *L. corrugis* obtained from the GenBank DNA database (U11919) did not cluster with the other sequences of the section *Dulces* of the genus *Lactarius*. This sequence clustered with members of the species *L. subumbonatus* and *L. lignyotus* Fr. Shimono et al. (2004) showed that *L. corrugis* (U11919) did not form a clade with *L. volemus* sequences obtained from GenBank (AF506413, AF042574) in either NJ or MP trees. Eberhardt (2002) also reported that this same *L. corrugis* sequence

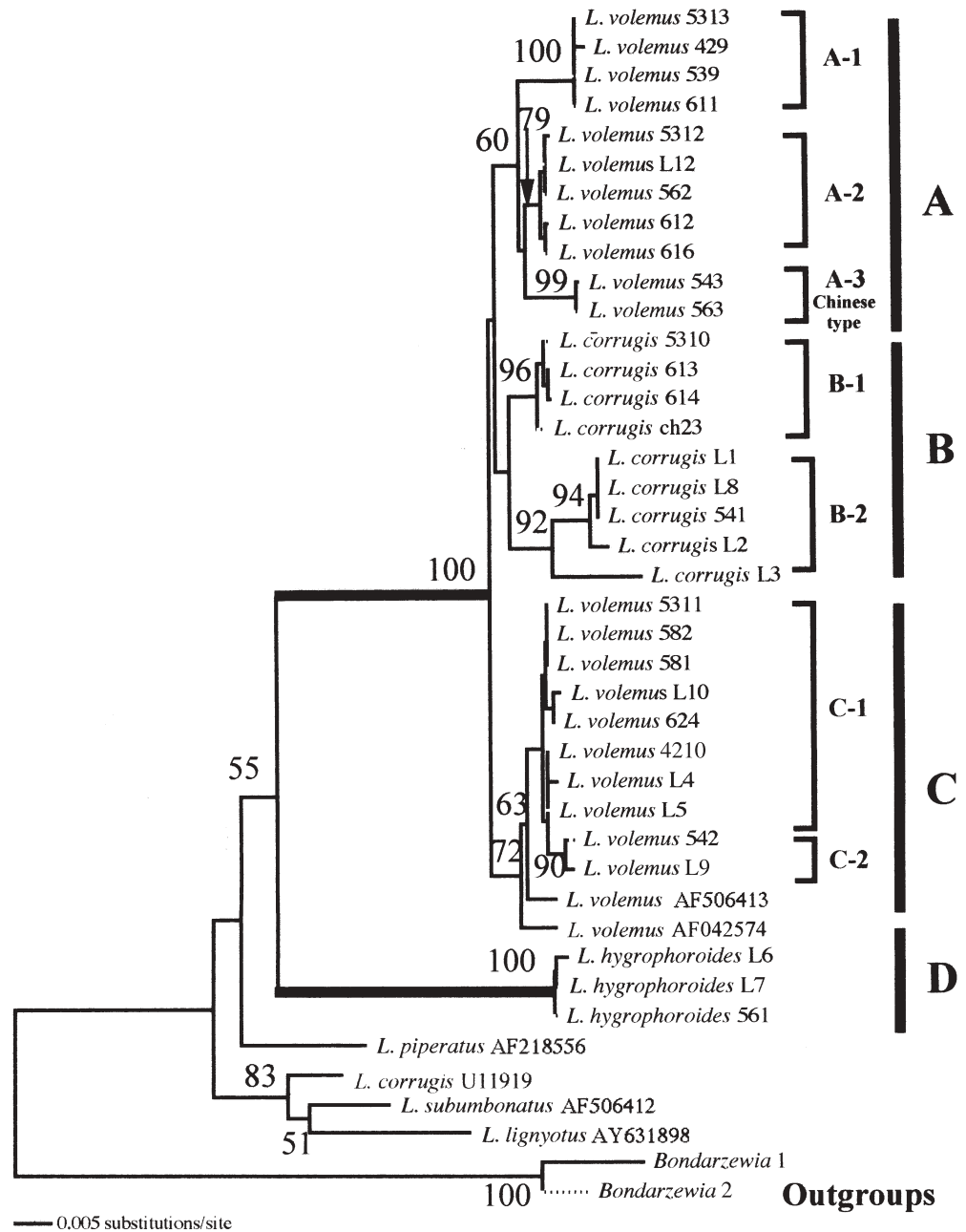
**Table 2.** Fatty acid composition in the stipe of mature *Lactarius volemus* and allies

Species/fatty acid	No. of sample	16:0	16:1	18:0	18:1	18:2	18:3	Others
<i>L. volemus</i> (common type)	7	3.4	2.2	1.1	61.7	28.1	2.0	1.5
<i>L. volemus</i> (yellow type)	2	5.2	1.2	1.7	60.9	26.9	2.6	1.5
<i>L. volemus</i> (velvet type)	4	5.3	2.0	1.1	42.7	44.9	0.9	3.1
<i>L. volemus</i> (red type)	5	4.7	0.7	1.4	49.9	41.1	0.2	2.0
<i>L. corrugis</i> (red type)	2	2.3	7.5	0.5	55.9	32.6	0.1	1.4
<i>L. corrugis</i> (common type)	3	3.7	4.2	1.1	50.1	38.1	0.0	2.8
<i>L. hygrophoroides</i>	2	9.4	0.5	1.7	8.8	77.4	0.1	2.1

Values are % in each type



**Fig. 7.** A neighbor-joining tree based on large-subunit rDNA data for 36 samples of *Lactarius volemus* and allies: *L. piperatus* AF218556, *L. subumbonatus* AF506412, *L. lignyotus* AY631898, and two outgroup species. Modeltest parameters: equal base frequencies with rate heterogeneity; gamma-shape parameter = 0.6074; proportion of invariable sites = 0.6163; six rate categories; Tamura–Nei model (Tamura and Nei 1993) with transformation parameters [A–C] = 1.00, [A–G] = 5.92, [A–T] = 1.00, [C–G] = 1.00, [C–T] = 12.30, and [G–T] = 1.00. Percent bootstrap support (1000 replications) is shown above nodes



clustered with *L. camphoratus* (Bull.: Fr.) Fr. In this study, the sequence similarities between four types of *L. volemus*, two types of *L. corrugis*, and one type of *L. hygrophoroides* studied ranged from 94.5% to 98%. However, the sequence similarity between these taxa and the *L. corrugis* sequence was only 94%. Therefore, the *L. corrugis* sequence (U11919) seems to have been obtained from an incorrectly identified specimen, as suggested by Eberhardt (2002).

Hesler and Smith (1979) reported that *L. corrugis* differs from *L. volemus* in a number of characteristics, i.e., larger spores, larger pleurocystidia, and strikingly darker colors, although some morphological characteristics are shared between *L. volemus* and *L. corrugis*. Nara (2004a,b) reported that *L. volemus* differs from *L. corrugis* in its shorter pileicystidia (*L. volemus*: 40–60 × 4–6 μm; *L. corrugis*: 3–4 μm)

and caulocystidia (*L. volemus*: 36–43 × 4 μm; *L. corrugis*: 70–150 × 4–5 μm). In this phylogenetic analysis, *L. corrugis* formed a single clade (subclade B), although the bootstrap value of the clade was less than 50% in both NJ (Fig. 7) and MP trees. *Lactarius corrugis* also differed from *L. volemus* in the composition of its fatty acids. All these results suggest that *L. corrugis* forms a separate species different from *L. volemus*.

Hesler and Smith (1979) reported that the pileus color is variable within North American strains of *L. volemus*. In Japan, we also found three macro-morphological variants of *L. volemus* (common, red, and velvet types) that differed in the color and surface characteristics of the pileus, as described in Materials and methods. Nara (2004b) reported that the velvet type of *L. volemus* was similar to the com-

mon type in the cuticle structure of the pileus, the cheilocystidia, the pleurocystidia, and its spore size, but differed in the characteristics of the pileocystidia and the caulocystidia (common type, dispersed; velvet type, fasciculate). In this phylogenetic analysis, the velvet type (A-1), red type (A-2), and Chinese type (A-3) (the last one from China) formed one clade (subclade A) with a low bootstrap value in the NJ tree (60%), but they did not cluster with the common and yellow types of *L. volemus* (subclade C) (see Fig. 7). The yellow type of *L. volemus* (C-2) has a yellow pileus with a slender stipe, thin cystidia in the stipe, and small spores (Nara 2004a,b). The fatty acid composition of the yellow type is similar to that of the common type of *L. volemus*, especially in the percentages of the fatty acids 18:1, 18:2, and 18:3 (see Table 2). This study shows that the yellow type is included in the same subclade as the common type of *L. volemus* (C-1), which has moderate bootstrap values in the NJ and MP trees (72% and 73%, respectively) (see Fig. 7). Hesler and Smith (1979) proposed a new variety of *L. volemus*, *L. volemus* var. *flavus* Hesler and A.H. Smith, for a *L. volemus* variant having a yellow pileus with a slender stipe. In this molecular study, the yellow type formed a small cluster with a high bootstrap value in the NJ tree (90%). The yellow type of *L. volemus* analyzed here may be identical with *L. volemus* var. *flavus*, as the morphological characteristics of it studied here are in good agreement with the variety described by Hesler and Smith (1979).

Two foreign *L. volemus* from database (AF506413 and AF042574) were grouped into the subclade C (see Fig. 7) along with the Japanese common type and yellow type. This result suggests that they are closely related to the common (C-1) and yellow (C-2) types of the Japanese *L. volemus*.

In the phylogenetic analysis reported here, three sequences of *L. hygrophoroides* formed a single cluster (subclade D), which was supported by a bootstrap value of 100% in both NJ and MP trees (100%). In the fatty acid analysis, the percentages of 18:2 and 16:0 in *L. hygrophoroides* were higher than those in *L. volemus* and *L. corrugis* (see Table 2). Based on the study of morphological characteristics, *L. hygrophoroides* is also distinct from *L. volemus* and *L. corrugis*, i.e., the lamellae are more widely spaced, the ornamentation of the spores is not clearly reticulate, there are no pleurocystidia, and the length of cystidia in the stipe is more than 200  $\mu\text{m}$  (Nara 2004b). Therefore, we conclude that the Japanese *L. hygrophoroides* corresponds to *L. hygrophoroides* var. *hygrophoroides* Berk. & M.A. Curtis.

From the molecular analyses reported here, we found that there are four groups in the species *L. volemus*, two groups in *L. corrugis*, and one group in *L. hygrophoroides*, in Japan. We also found some differences in the composition of fatty acid and macro- and micro-morphological characteristics between the groups. Hiroi and Shimono (unpublished data) conducted a test of the taste of several types of *L. volemus* and *L. corrugis* using high school students and members of the Tochigi Mycological Club as tasters. The red and velvet types of *L. volemus* and the common type of *L. corrugis* were found to have a good taste, but not the common type of *L. volemus*. Based on the molecular

analysis, fatty acid composition, morphological characteristics, and taste of the fruiting body, the common, velvet, red, and Chinese types of *L. volemus* can each be divided into different species, subspecies, or varieties. Further phylogenetic studies using materials collected from a wide range of regions and sequence data from other DNA regions, as well as morphological studies, are required to conduct taxonomic revision of these types.

The present study revealed that the DNA sequence of the LSU of rDNA and the fatty acid composition of the fruiting bodies are useful tools for phylogenetic analysis of the section *Dulces* of the genus *Lactarius*. To clarify the phylogenetic relationships within the section *Dulces*, further studies of the genus *Lactarius* should be made using molecular markers capable of distinguishing between closely related species.

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