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ORIGINAL PAPER

Mycorrhizal diversity and specificity in *Lecanorchis* (Orchidaceae)

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Abstract *Lecanorchis* is a nonphotosynthetic plant genus in Vanilloideae, Orchidaceae. Because of the distribution of many *Lecanorchis* taxa in various climate conditions, we hypothesized that mycorrhizal diversity and specificity are different among the different taxa of *Lecanorchis*. In the present study, identities of mycorrhizal fungi were examined for 90 individuals of 10 *Lecanorchis* taxa at 26 sites from Niigata to Okinawa Prefectures in Japan. Phylogenetic analyses of *Lecanorchis* taxa based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (rDNA) divided the examined *Lecanorchis* taxa into three groups, groups A, B, and C. ITS rDNA sequences suggested that fungi associating with *Lecanorchis* were ectomycorrhiza-forming fungi in *Lactarius*, *Russula*, Atheliaceae, and *Sebacina*, with *Lactarius* and *Russula* dominant. Our results suggested some degree of mycorrhizal specialization among *Lecanorchis* taxa. Interestingly, the *Lecanorchis* group C had some specific relationships with *Lactarius*, whereas less specificity was found in the relationships with *Russula*. However, observed specificity results may be biased by geographic opportunity, and we suggest further research to assess whether *Lecanorchis* species are limited to the associations we observed.

Keywords Atheliaceae · *Lactarius* · Mycoheterotrophic plants · Russulaceae · *Sebacina*

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Introduction

Mycorrhizal symbioses are essential for the growth and reproduction of orchids in natural habitats. Orchid seeds are usually quite small and lack endosperm. Accordingly, seed germination and subsequent growth at the protocorm stage requires colonization by mycorrhizal fungi to acquire carbohydrates and other nutrients. This nutritional mode is known as mycoheterotrophy (Leake 1994). Most orchid species are photosynthetic after forming leaves above ground, whereas some orchids are achlorophyllous even at the adult stage. These mycoheterotrophic orchids depend entirely on colonized fungi for carbon throughout their lives (Leake 1994). Most mycoheterotrophic orchids associate with ectomycorrhiza-forming fungi (Smith and Read 2008). The orchids, their mycorrhizal fungi, and ectomycorrhizal trees associate with one another in such tripartite relationships (Taylor and Bruns 1997), and tree photosynthate is supplied to the orchids through the fungal mycelia (Bidartondo et al. 2004; Julou et al. 2005).

Some previous studies showed that orchid phylogenies are related to the identities of associated fungi in mycoheterotrophic orchids (Taylor et al. 2004; Kennedy et al. 2011) as well as in some chlorophyllous orchids (Shefferson et al.

2007, 2010). Furthermore, associations with confined clades of fungi are often found in mycoheterotrophic orchids. The high specificities can be found at the plant species level as shown in *Hexalectris* Raf. (Kennedy et al. 2011) or even at the intraspecific plant genotype level as shown in *Corallorhiza maculata* (Taylor et al. 2004). These results indicate that mycorrhizal specialization can evolve rapidly in mycoheterotrophic orchids.

Lecanorchis Blume is an achlorophyllous orchid genus in Vanilloideae that is characterized by a calyculus, a cup-like structure between the base of the perianth and the apex of the ovary (Szlachetko and Mytnik 2000). Approximately 20 taxa in this genus are widely distributed from southwest part of Japan to Indonesia and Bangladesh (Pridgeon et al. 2003). No other mycoheterotrophic orchid genera have such a large number of taxa distributed in various climatic conditions. We hypothesized that mycorrhizal diversity and specificity are different among the taxa of *Lecanorchis* because of their distributions in various climate conditions, to which the diversification of *Lecanorchis* may have some relationships. In our preliminary observation, we found that the habitats of *Lecanorchis* species were invariably located in understories of ectomycorrhizal trees, which strongly suggested their association with ectomycorrhiza-forming fungi. In the present study, we have identified mycorrhizal fungi in ten *Lecanorchis* taxa in 26 sites from Niigata to Okinawa Prefectures in Japan to examine whether *Lecanorchis* associates with ectomycorrhiza-forming fungi, and whether the phylogeny of *Lecanorchis* is related to the identities of the mycorrhizal fungi.

Materials and methods

Sample collection

Root and shoot samples from 90 individuals of ten *Lecanorchis* taxa, i.e., *Lecanorchis flavicans* var. *acutiloba* Hashimoto, *L. flavicans* var. *flavicans* Fukuyama, *Lecanorchis japonica* var. *hokurikuensis* (Masam) T. Hashim., *L. japonica* var. *japonica* Blume, *L. japonica* var. *kiiensis* (Murata) T. Hashim., *Lecanorchis kiusiana* var. *kiusiana* Tuyama, *L. kiusiana* var. *suginoana* (Tuyama) T. Hashim., *L. nigricans* Honda, *L. trachycaula* Ohwi, and *Lecanorchis virella* Hashimoto, were collected at 26 sites from Kaetsu in the Niigata Pref. to Iriomote in Okinawa Pref. in Japan (Fig. 1, Table S1). Almost all *Lecanorchis* taxa in Japan except some extremely rare taxa were collected, which was about half of the taxa in the world. *Quercus* spp. and *Castanopsis* spp. in Fagaceae were the dominant trees in

Fig. 1 Sampling sites of *Lecanorchis* species in Japan

many sampling sites. Samples were collected during the flowering stage to allow species identification. The samples kept cool in plastic bags were processed within several days.

Molecular investigations

The roots and stems of flowering shoots were cut into 1-cm fragments, and DNA was extracted from each fragment using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Root cross sections were observed under a compound microscope to select root fragments with fungal colonization. One fragment each of root and stem was arbitrarily selected for each plant individual.

The internal transcribed spacer (ITS) region of the fungal nuclear ribosomal RNA gene (rDNA) was amplified using the primers ITS1F and ITS4 (Gardes and Bruns 1993), with the TaKaRa Ex Taq Hot Start Version (Takara Bio, Otsu, Japan). The PCR reaction mixture contained 2 μl of the extracted DNA solution, 0.75 U of Taq polymerase, 0.25 μM of each primer, 200 μM of each deoxynucleotide triphosphate, and 3 μl of the supplied PCR buffer in 30 μl of the total volume. The PCR program performed on the Program Temp Control System PC-818S (Astec, Fukuoka, Japan) was as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 25 s, at 55°C for 30 s, at 72°C for 1 min, and final elongation at 72°C for 5 min. The plant ITS rDNA was amplified using primers ny43 and ny47 (Cameron 2005) with the aforementioned PCR reaction mixture and PCR program. Amplified PCR products were purified using the PCR Purification Kit (Qiagen), and direct sequencing was performed for the purified PCR product with the PCR primers by the dideoxy sequencing method using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) with the Genetic Analyser 3130 (Applied Biosystems). When direct sequencing was unsuccessful, PCR products were cloned using the pGEM-T Easy Vector System I (Promega, Tokyo, Japan) and Competent high DH5α (Toyobo, Osaka, Japan). The DNA insert was then sequenced using T7 and SP6 promoter primers. The DNA sequences obtained were deposited in the DDBJ database under accession numbers AB597720–AB597768 for plant DNA and AB597630–AB597719 for fungal DNA. The DNA sequences obtained were subjected to a BLAST search (Altschul et al. 1997), and similar data were downloaded from the GenBank database. Multiple sequence alignment was performed using ClustalX (Larkin et al. 2007), and further manual editing was conducted using SeaView (Galtier et al. 1996), in which gaps were treated as missing data.

For phylogenetic analysis of *Lecanorchis* taxa, Bayesian analyses were conducted with the MrBayes 3.1.2 program (Ronquist and Huelsenbeck 2003). The general time-reversible model under the assumption of a discrete gamma-shaped rate variation without a proportion of invariable sites (GTR+G) was estimated as the best-fit likelihood model using MrModeltest 2.2 (Nylander 2004) and PAUP* 4.0b10 (Swofford 2002). Posterior probabilities (PP) were approximated by a Metropolis-coupled MCMC method. Two parallel runs were conducted with one cold and seven heated chains each for 1,000,000 generations, starting with a random tree. The seven chains were heated at 0.2 for the both datasets. Trees were saved to a file every 100th generation. We judged that the two runs reached convergence when the average SD of split frequencies dropped below 0.01. Trees obtained before reaching convergence were discarded using the "burn-in" command, and the remaining trees were used to calculate a 50% majority consensus topology and to determine PP for individual branches. The alignment datasets were further analyzed by the neighbor-joining (NJ) (Saitou and Nei 1987) and the maximum parsimony (MP) using MEGA 5 (Tamura et al. 2011). Evolutionary distances in NJ analysis were estimated using Gamma-distributed rates. The trees obtained in these analyses were drawn with the Treeview software (Page 1996).

For the mycobionts of *Lecanorchis*, phylogenetic analyses were conducted by the maximum likelihood (ML) method with additional NJ using MEGA 5 in each fungal group, *Lactarius*, *Russula*, Atheliaceae, and *Sebacina*. The best-fit ML trees were inferred under GTR+GAMMA model which was estimated with MrModeltest 2.2 (Nylander 2004) in PAUP* 4.0b10 (Swofford 2002). To check statistical support for the tree topology obtained, the bootstrap option was used under the automatically assigned, GTA+CAT model, setting the number of replicates to 1,000.

Statistical analysis

The specificity levels in the plant–fungi relationships may be different depending on the fungal groups, *Lactarius*, *Russula*, Atheliaceae, and *Sebacina*. In order to quantify levels of specificity for each fungal group, mean pairwise distances were computed among the fungal ITS rDNA sequences in each *Lecanorchis* taxon by the maximum composite likelihood method in MEGA 5. Overall mean was also computed for each fungal group. All positions containing gaps and missing data were eliminated from the dataset with complete deletion option.

Results

Phylogenetic analysis of *Lecanorchis*

In total, 49 sequences of the ITS rDNA region were obtained from ten *Lecanorchis* taxa (Table S1). From the

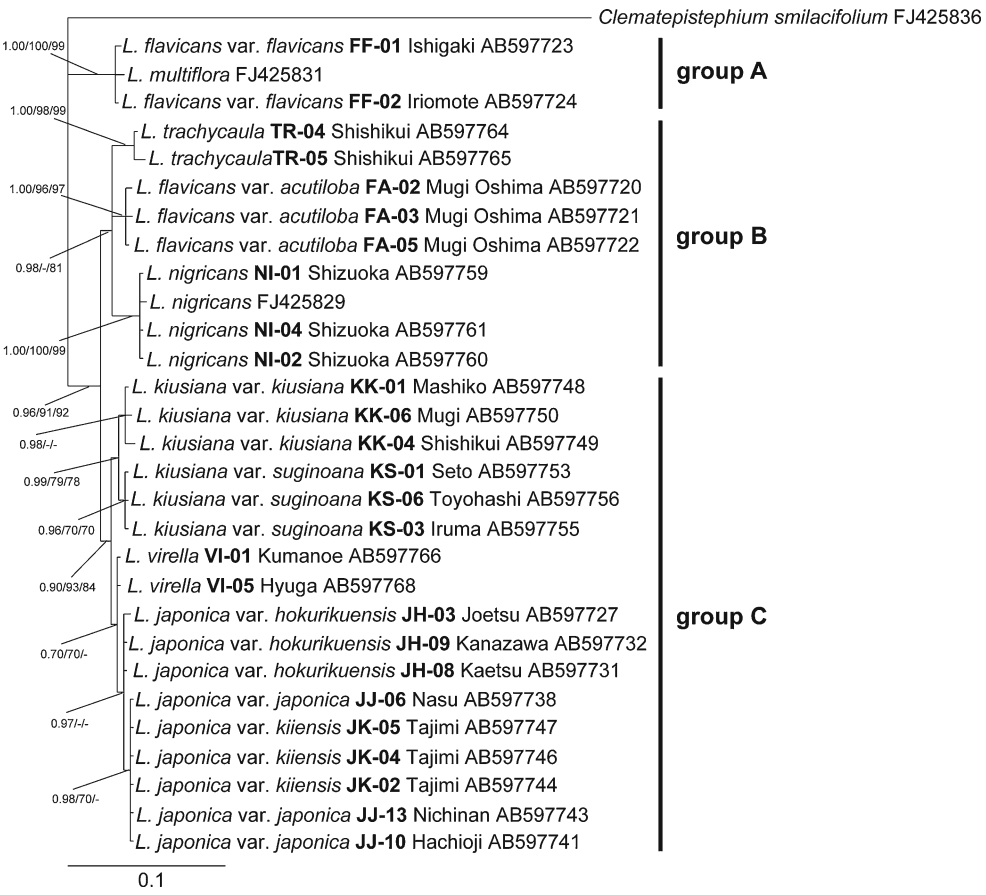

Fig. 2 Bayesian 50% majority rule consensus topology obtained from 5,050 trees based on the partial ITS sequences of rDNA of *Lecanorchis* spp. *Clematepistephium smilacifolium* in Vanilloideae is used as an outgroup. Bayesian posterior probabilities (PP), Bootstrap values with 1,000 replications in neighbor-joining (NJ) analysis (NJBS) and maximum parsimony (MP) analysis (MPBS) are indicated as PP/NJBS/MPBS at branches of nodes. For BS values, those over 70% are only shown. The DNA sequences obtained are shown with the plant number, plant species, and sampling region. Plant number identifiers are shown in Table S1. Accession numbers are provided for all sequences

obtained sequences, two or three sequences were arbitrarily selected for the phylogenetic analysis (Fig. 2). Some DNA sequences of *Lecanorchis* spp. including the same region were downloaded from the GenBank database. A common region of 485–501 bp was used in Bayesian, NJ, and MP analyses, and sequence identity among the obtained *Lecanorchis* sequences in that region computed by ClustalX varied from 94% to 100%. *L. flavicans* var. *flavicans* from the neotropical islands Ishigaki and Iriomote formed clade A with *L. multiflora* JJ Smith. The other variety of this species,

Table 1 Relationship between *Lecanorchis* phylogeny and affiliations of the *Lecanorchis* mycobionts based on the phylogenetic analyses on ITS rDNA

Lecanorchis taxon and phylogeny	*Lecanorchis* ITS group	Number of the *Lecanorchis* mycobionts								
		Lactarius				*Russula*	Atheliaceae		*Sebacina*	Others
		Lac 1	Lac 2	Lac 3	Other		Ath 1	Ath 2		
Lecanorchis flavicans var. *flavicans*	A								2	
Lecanorchis trachycaula	B		1			5	2		1	
Lecanorchis flavicans var. *acutiloba*	B					1		6		
Lecanorchis nigricans	B	3	2	1		5				
Lecanorchis kiusiana var. *kiusiana*	C		6			3				
Lecanorchis kiusiana var. *suginoana*	C	9	1							
Lecanorchis virella	C		5			1				
Lecanorchis japonica var. *hokurikuens*	C	1		8		4			1	1
Lecanorchis japonica var. *japonica*	C	1		5	1	7				2
Lecanorchis japonica var. *kiiensis*	C			4		1				

L. flavicans var. *acutiloba* was unexpectedly separated into clade B. The other *Lecanorchis* taxa were divided into two clades, clade B with *L. trachycaula*, *L. nigricans* and *L. flavicans* var. *acutiloba*, and clade C with *L. kiusiana*, *L. virella* and *L. japonica*. In clade B, the three taxa separated properly into their own clades with high Bayesian posterior probabilities and bootstrap support. *L. nigricans* (FJ425829) from voucher Yukawa s. n. (WIS) by Cameron (2009) was also included in the *L. nigricans clade*. In clade C, the two varieties of *L. kiusiana* formed their own clades.

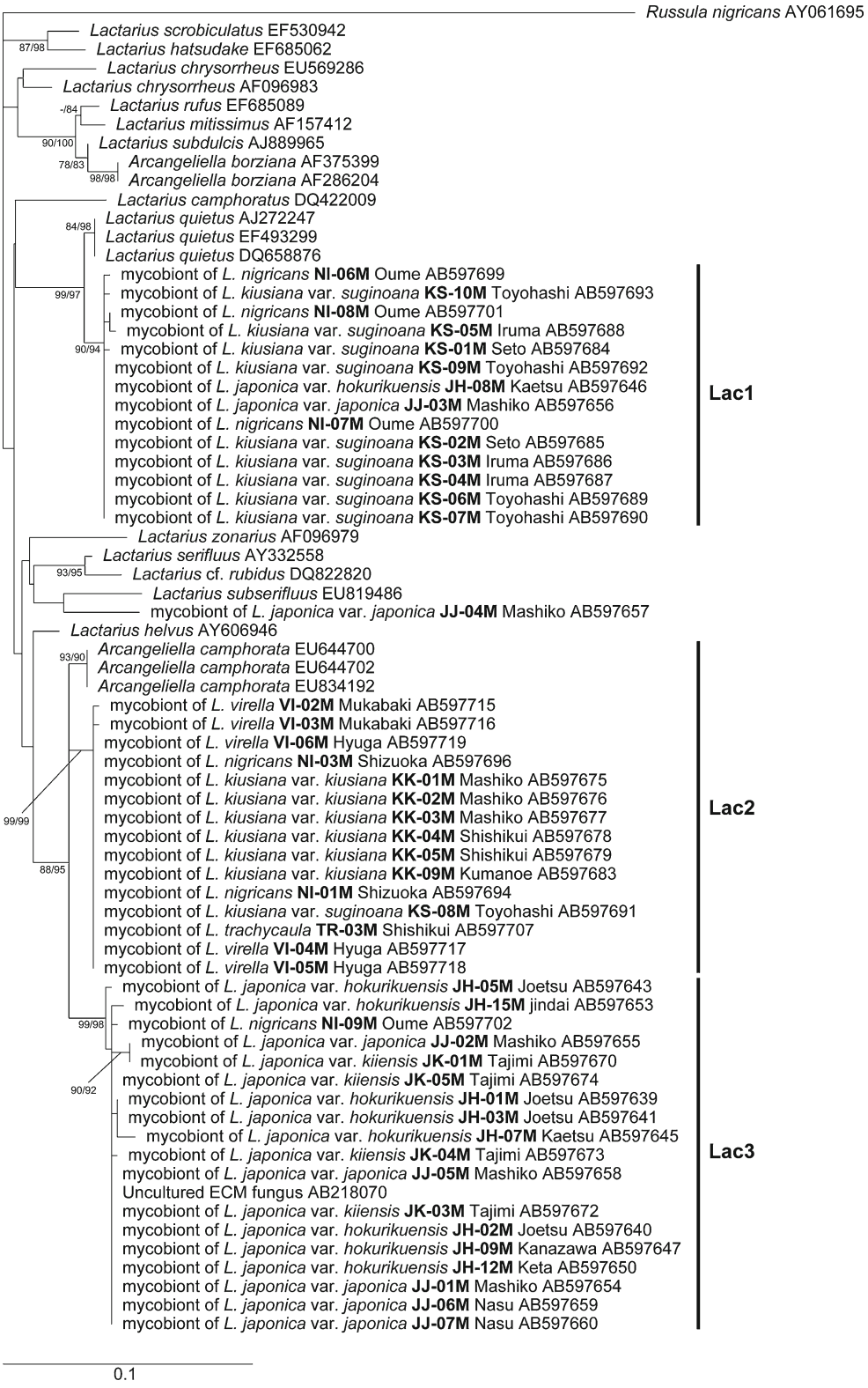

Fig. 3 The ML tree (In L=−1,974.65) based on the partial ITS sequence of rDNA of *Lactarius* spp. in Russulaceae obtained from the roots of *Lecanorchis* spp. in this study and from the Genbank database. The tree is rooted to *Russula nigricans* (Russulaceae). The fungal DNA sequences obtained are shown with the fungal number and sampling region in *bold* characters. Fungal number identifiers are shown in Table 1. Bootstrap values with 1,000 replications in ML analysis (MLBS) and NJ analysis (NJBS) are indicated as MLBS/NJBS at branches of nodes. For BS values, those over 70% are only shown. A scale is shown to infer evolutionary distances. Accession numbers are provided for all sequences

Phylogenetic analysis of mycorrhizal fungi

One fungal sequence of ITS rDNA was obtained from each *Lecanorchis* individual. In total, 90 fungal sequences were obtained. Almost all sequences were obtained by direct sequencing, but one sequence, KS-01M, was obtained after cloning. After determination of the fungal affiliations by BLAST searches (Table 1), phylogenetic analysis was performed for each of the fungal taxa: *Lactarius* (Fig. 3), *Russula* (Fig. 4), Atheliaceae (Fig. S1), and *Sebacina* (Fig. S2).

More than half of the identified fungi were *Lactarius*, and most of them were divided into three clades in the phylogenetic tree: *Lactarius* clade 1 (Lac1), clade 2 (Lac2), and clade 3 (Lac3), in which Lac2 and Lac3 were closely related (Fig. 3). In addition, *Arcangeliella camphorata* (Russulaceae) was closely related to Lac2 and Lac3. *Lecanorchis* group C had some specific relationships with the groups of

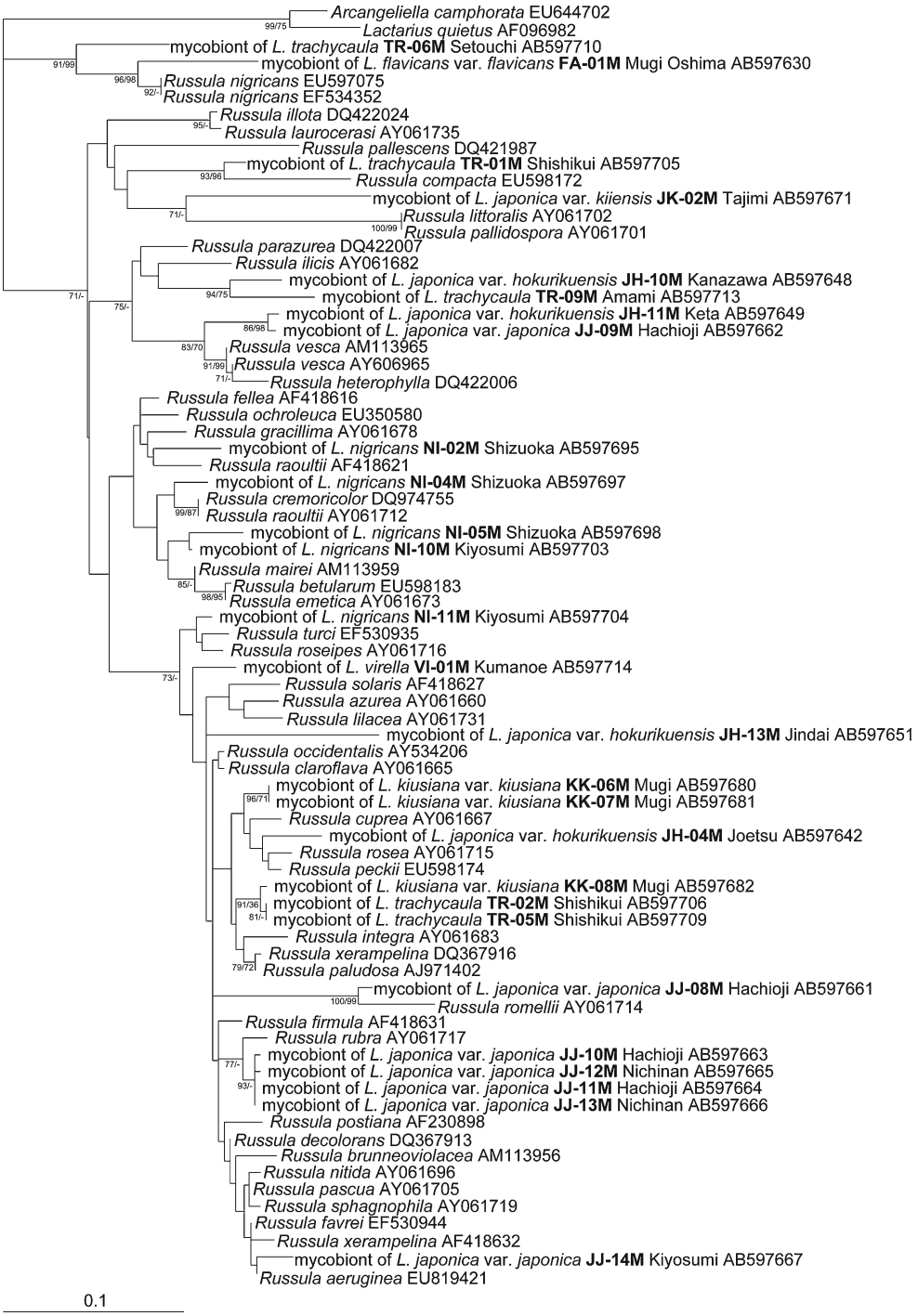

Fig. 4 The ML tree (In *L*= −1,405.95) based on the partial ITS sequence of rDNA of *Russula* spp. in Russulaceae obtained from the roots of *Lecanorchis* spp. in this study and from the Genbank database. The tree is rooted to *Arcangeliella camphorate* and *Lactarius quietus* (Russulaceae). Fungal DNA sequences obtained are shown with the fungal number and sampling region in *bold* characters. Fungal number identifiers are shown in Table 1. Bootstrap values with 1,000 replications in ML analysis (MLBS) and NJ analysis (NJBS) are indicated as MLBS/NJBS at branches of nodes. For BS values, those over 70% are only shown. A scale is shown to infer the evolutionary distances. Accession numbers are provided for all sequences

Table 2 Mean pairwise distances among fungal ITS rDNA sequences in the fungal groups, *Lactarius*, *Russula*, Atheliaceae, and *Sebacina*, for each *Lecanorchis* taxon

Lecanorchis taxon	*Lecanorchis* ITS group	Fungal group			
		Lactarius	*Russula*	Atheliaceae	*Sebacina*
Lecanorchis flavicans var. *flavicans*	A	–	–	–	0.00557
Lecanorchis flavicans var. *acutiloba*	B	–	–	0.00220	–
Lecanorchis nigricans	B	0.04810	0.08116	–	–
Lecanorchis trachycaula	B	–	0.17568	0.00162	–
Lecanorchis japonica var. *hokurikuens*	C	0.01045	0.15917	–	–
Lecanorchis japonica var. *japonica*	C	0.03543	0.05496	–	–
Lecanorchis japonica var. *kiiensis*	C	0.00463	–	–	–
Lecanorchis kiusiana var. *kiusiana*	C	0.00000	0.03903	–	–
Lecanorchis kiusiana var. *suginoana*	C	0.01648	–	–	–
Lecanorchis virella	C	0.00184	–	–	–
Overall average		0.02943	0.10551	0.07404	0.10697

Lactarius fungi (Table 1). All three varieties of *L. japonica* associated with Lac3 fungi. They were collected from six sampling sites between Kaetsu and Tajimi located in the northeastern area; these sites were at most separated by 307 km, whereas *L. virella*, which is closely related to *L. japonica*, associated with Lac2 fungi. The two varieties of *L. kiusiana* were distinguished in their *Lactarius* mycobionts, that is, var. *suginoana* had a preference to associate with Lac1 fungi, while var. *kiusiana* associated with Lac2 fungi. The mean pairwise distances among the obtained sequences of the *Lactarius* fungi showed that those of the species in *Lecanorchis* group C are generally lower values than the overall average, which suggested that these species have evolved to specialize on slightly different suites of fungi (Table 2). *Lactarius* fungi are also detected in *Lecanorchis* group B, in which less specificity was suggested by the higher mean pairwise distance of *L. nigricans* compared to the overall average.

In eight *Lecanorchis* taxa, 27 fungi were identified as *Russula* spp. (Table 1, Fig. 3). In contrast to those in *Lactarius*, less specificity was found in the relationships by the mean pairwise distances. However, four fungi from *L. japonica* var. *japonica* formed a clade in the phylogenetic analysis. These four fungi were collected in the samples from Hachioji and Nichinan, which are separated by 868 km, indicating some preference of *L. japonica* var. *japonica* to the group of fungi.

L. flavicans var. *acutiloba* and *L. trachycaula* exhibited high mycorrizal specificity. In Atheliaceae, two fungi from *L. trachycaula* and six fungi from *L. flavicans* var. *acutiloba* each formed clades Ath1 and Ath2, respectively (Table 1, Fig. S1). Both of these *Lecanorchis* taxa are in group B. The samples with Ath1 were collected from Setouchi and Amami separated about 781 km. Furthermore, the two fungi of *L. trachycaula* in Ath2 collected from Setouchi and Mugi-oshima with 22.1 km of separation had identical sequences. The mean pairwise distances among the sequences of Atheliaceae fungi in each *Lecanorchis* taxa are lower than the overall average (Table 2).

In *L. flavicans* var. *flavicans*, *L. japonica*, and *L. trachycaula*, four fungi were identified as *Sebacina* (Table 1. Fig. 2S). The two fungi detected in two samples of *L. flavicans* var. *flavicans* in *Lecanorchis* group A isolated from two different islands, Ishigaki and Iriomote, were closely related (Fig. 2S).

Moreover, fungi belonging to *Phialocephala* (Helotiales), *Tuber* (Tuberaceae), and Typhulaceae were detected in some plants (Table 1). *Tuber* is a well-known ectomycorrhizal genus of Ascomycota (Berndt et al. 1990). *Phialocephala* is a dark septate fungal genus (Jumpponen and Trappe 1998), and Typhulaceae includes some snow mold fungi (Hsiang et al. 1999).

Discussion

The phylogeny of *Lecanorchis* based on ITS rDNA sequences generally reinforced natural species. The two varieties of *L. kiusiana*, var. *kiusiana* and var. *suginoana*, were separated into different but closely related clades (Fig. 4). The three varieties of *L. japonica,* var. *japonica*, var. *hokurikuensis*, and var. *kiiensis* belonged to the same clade, in which no sequence difference was found between var. *japonica* and var. *kiiensius* (Fig. 4). In addition, *L. virella* was close to *L. japonica*. However, the sequences of *L. flavicans* var. *flavicans* obtained from the different subtropical islands, Ishigaki and Iriomote, were not closely related to those of *L. flavicans* var. *acutiloba* but formed a clade with *L. multiflora* (Cameron 2009).

Phylogeny of Vanilloideae based on the combined sequences of nuclear 26S, 5.8S and 18S rDNA gene,

mitochondrial *atpA* gene and *nad1b-c* intron placed *Lecanorchis* sister to *Clematepistephium* and *Eriaxis* distributed in New Caledonia (Cameron 2009). Furthermore, the *Lecanorchis* taxa in group A, which was shown to be first diversified in the phylogeny, are distributed in tropical or subtropical regions (Seidenfaden and Wood 1992; Pridgeon et al. 2003). *Lecanorchis* probably diversified from ancestors in the tropics and subsequently migrated to temperate regions, including Japan.

We examined relationships between the *Lecanorchis* taxa and mycorrhizal fungi as follows, but the number of collected plant samples was limited because of the rarity of the examined *Lecanorchis* taxa. Furthermore, only one fungal sequence was obtained from each individual. Therefore, it is probable that mycorrhizal associations may actually be broader than those described in this study. Even though, some plant–fungi specificities were suggested as follows. In the *Lecanorchis* group A, two *Sebacina* fungi in *L. flavicans* var. *flavicans* were detected in two different subtropical islands, Iriomote and Ishigaki. The two *Sebacina* fungi were close in the phylogeny suggesting the high specificity in the relationship. The three *Lecanorchis* taxa in goup B, *L. flavicans* var. *acutiloba*, *L. nigricans*, and *L. trachycaula* are distributed from the temperate to the subtropic regions (Hashimoto 1990). Among the seven identified fungi of *L. flavicans* var. *acutiloba*, six were identified as belonging to the Atheliaceae clade Ath2. The Atheliaceae fungi were exclusively detected in *Lecanorchis* group B. To the best of our knowledge, no studies have identified Atheliaceae as orchid mycorrhizal fungi, although Atheliaceae have previously been found as arbutoid and ectomycorrhizal fungi (Rosling et al. 2003; Vincenot et al. 2008; Obase et al. 2009; Kjøller and Clemmensen 2009). The *Lecanorchis* group C consisted of *L. japonica*, *L. kiusiana*, and *L. virella*. These *Lecanorchis* taxa are known to distribute in the temperate region (Hashimoto 1990). The *Lecanorchis* taxa in this group developed specificities in the associations with *Lactarius* fungi that were divided into Lac1, Lac2, and Lac3 in the phylogenetic analysis. The two varieties of *L. kiusiana*, var. *kiusiana* and var. *suginoana*, differed in associations with mycorrhizal fungi, that is var. *kiusiana* associated with Lac2 fungi and var. *suginoana* mainly associated with Lac1 fungi. Meanwhile, all three varieties of *L. japonica* were strongly associated with Lac3 fungi. The difference in mycorrhizal association among closely related *Lecanorchis* taxa in group C suggests a potential relationship between plant diversification and mycorrhizal specialization that should be further investigated. In contrast, less specificity was found in the relationship with *Russula*. An interesting next step would be to examine the physiological and functional differences between more and less specific associations in the mycorrhizal symbioses of *Lecanorchis*. Cross-colonization studies might be also interesting to test whether these suites of fungi are genuine reflections of the specialization or caused by low opportunities to associate with other potential symbionts.

The majority of the mycorrhizal fungi found in *Lecanorchis* belonged to *Lactarius* and *Russula* in the Russulaceae (Table 1). Russulaceae have been found as orchid mycorrhizal fungi in some orchids (Taylor and Bruns 1997; Selosse et al. 2004; Girlanda et al. 2006; Bougoure and Dearnaley 2005; Yamato and Iwase 2008), and most of them were in *Russula*. *Lactarius* has only been detected in *C. maculata* Raf. (Taylor and Bruns 1999) and *Epipogium aphyllum* (Roy et al. 2009) as minorities. Therefore, this is the first study to show the predominance of *Lactarius* species as mycorrhizal fungi in orchids.

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