

## New Records of *Liparis purpureovittata* (Orchidaceae) and Identification of its Mycorrhizal Fungi

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(Received 12 May 2016; accepted 22 June 2016)

**Abstract** We revise the distribution area of *Liparis purpureovittata* with new records from western Japan (Ehime Pref. and Kochi Pref.) and central to northern Japan. In molecular analyses of mycorrhizal fungi of *L. purpureovittata*, *Tulasnella* (Tulasnellaceae), which is among predominant mycobiont groups of the Orchidaceae including the other *Liparis* species, was detected from the plants, suggesting its status of the fungal partner.

**Key words:** Japan, *Liparis*, mycorrhiza, orchid, *Tulasnella*.

### Introduction

*Liparis* Rich. is a cosmopolitan orchid genus consisting of about 320 species, including epiphytes and terrestrial plants (Pridgeon *et al.*, 2005). In Japan, 17 species of *Liparis* are recognized (Yukawa, 2015). *Liparis purpureovittata* Tsutsumi, T.Yukawa & M.Kato was recently described as a new species (Tsutsumi *et al.*, 2008). The species is morphologically and phylogenetically close to *L. fujisanensis* F.Maek. ex Konta & S.Matsumoto, *L. kumokiri* F.Maek. and *L. koreojaponica* Tsutsumi, T.Yukawa, N.S.Lee, C.S.Lee & M.Kato. It can be distinguished from the congeners by the shapes of labellum and column and the flower color (Tsutsumi *et al.*, 2008).

*Liparis purpureovittata* was recorded in several areas in Hokkaido, Niigata, Nagano, and Gunma Prefectures in central to northern Japan (Tsutsumi *et al.*, 2008). Subsequently, the species

was also reported from Toyama Pref., central Japan (Toyama Prefecture, 2012). Recently, we found the species from Ehime Pref. and Kochi Pref. in Shikoku, western Japan. Furthermore, the species was also observed in several localities from central to northern Japan. In this report, we revise the distribution area of *L. purpureovittata*.

Mycorrhizal symbiosis is a key issue to determine habitats or geographical distributions of orchid plants (Rasmussen, 1995; Batty *et al.*, 2001; Ogura-Tsujita and Yukawa, 2008; Barrett *et al.*, 2010; Roche *et al.*, 2010). Most mycorrhizal fungi in the Orchidaceae belong to narrow ranges of taxa in Basidiomycota, such as Ceratobasidiaceae, Tulasnellaceae, and Sebacinaceae, whereas Glomeromycota, forming arbuscular mycorrhizae that are common in most land plants, have not been recorded from the Orchidaceae (Yukawa *et al.*, 2009). In several *Liparis*

species such as *L. japonica* (Miq.) Maxim., *L. kumokiri*, *L. liliifolia* (L.) A. Rich. ex Lindl., and *L. loeselii* (L.) Rich., which are closely related to *L. purpureovittata* (Tsutsumi *et al.*, 2007), *Tulasnella* (Tulasnellaceae) has been found with molecular techniques (McCormick *et al.*, 2004; Illyés *et al.*, 2005; Shimura *et al.*, 2009; Ding *et al.*, 2014). To clarify mycorrhizal partners of *L. purpureovittata*, molecular identification of the fungi was performed.

### Materials and Methods

Leaves of *Liparis purpureovittata* collected from Saijo-shi, Ehime Pref. (*Y. Hagino et al. FOS-009187, FOS-009189*) were used for molecular identification of plants. DNA from the leaf samples was extracted using a QIAGEN DNeasy Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instruction. The internal transcribed spacer (ITS) regions with the 5.8S region of nuclear ribosomal DNA were examined as described by Tsutsumi *et al.* (2007). The obtained sequencing patterns were compared to those of the sample of *L. purpureovittata* examined by Tsutsumi *et al.* (2007).

For fungal identification, fungal hyphae were isolated from five corms of *Liparis purpureovittata* collected from Saijo-shi, Ehime Pref. The corms were thoroughly washed in running water. After the outer tissues of the corms were trimmed, the remaining inner tissues were sliced into pieces using clean razor blades. Each piece was washed three times in sterilized distilled water and crushed using a sterilized glass rod in a Petri dish, into which about 15 mL of corn meal agar (CMA; Nissui Pharmaceutical Co., Tokyo) containing 150 ppm streptomycin and 50 ppm tetracycline were added. A few days after incubation at approximately 25°C in the dark, fungal hyphae growing from pelotons (hyphal coils) were transferred to new CMA plates for purification. The isolates were transferred and cultured on potato dextrose agar slants (Nissui Pharmaceutical Co.) at approximately 25°C in the dark.

The hyphae were directly used for polymerase

chain reaction (PCR) amplification without DNA extraction. The internal transcribed spacer (ITS) regions with the 5.8S region of nuclear ribosomal DNA were used for fungal identification. A pair of primers ITS1-F and ITS4 (White *et al.*, 1990; Gardens and Bruns, 1993) was used. PCR was performed using a Perkin-Elmer 9700DNA thermal cycler (Applied Biosystems, Foster, CA) with Ex Taq DNA polymerase (TaKaRa Bio, Tokyo) and Ampdirect Plus (Shimadzu, Kyoto); the reaction conditions were as follows: 30 denaturation, annealing, and elongation cycles for 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C, respectively, with a final elongation step for 7 min at 72°C. The PCR products were purified using illustra ExoProStar (GE Healthcare, Buckinghamshire) following the manufacturer's instructions. Sequences were analyzed using ABI3130xl or ABI 3500xl (Applied Biosystems) and assembled using Seqman II (DNASTar Lasergene, WI). The sequences analyzed in this study were registered in Genbank (LC158688–LC158689 for *Tulasnella*, LC158690 for *Exophiala*).

The sequences were submitted to BLAST searches (Altschul *et al.*, 1997) against the NCBI sequence database (Genbank) to detect closely matched sequences. We assigned the genus or family names to our samples based on the registered sequences with >96% ITS similarity. The BLAST searches revealed that *Tulasnella* are candidates of mycorrhizal partner of *L. purpureovittata* (see Results and Discussion). Therefore we performed molecular phylogenetic analyses using our samples identified as *Tulasnella*, 30 registered sequences with high Max scores by the BLAST searches, and ITS sequences analyzed by Girlanda *et al.* (2011). Girlanda *et al.* (2011) comprehensively analyzed a close relative group of *Tulasnella* to our samples. All assembled sequences were aligned using the Clustal X program (Thompson *et al.*, 1997) and then aligned manually. The alignment was easily performed, whereas the nuclear ribosomal operon of the Tulasnellaceae is known to evolve exceedingly rapidly (Taylor *et al.*, 2002; Binder *et al.*, 2005; Moncalvo *et al.*, 2006). For outgroups,

mycorrhizal fungi from *Dendrobium crumenatum* (AJ313438) and *Vanda* 'Miss Joaquim' (AJ313443) were used in the ITS analysis based on the result of phylogenetic analysis in Girlanda *et al.* (2011).

Phylogenetic analyses were performed using Bayesian analysis. Ambiguous bases and gaps were treated as unknown (N) and missing data, respectively. MrModeltest 2.0 was used to determine nucleotide substitution models (Nylander, 2004). GTR + I + G models were selected. Bayesian searches were conducted using Markov Chain Monte Carlo with two independent sets of four chains; each was run for 10 million generations with sampling every 100 generations by using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The program Tracer (Rambaut and Drummond, 2009) was used to check the runs that had reached stationarity; the effective sample size of all the parameters was high ( $>200$ ). The first 2.5 million generations were discarded as burn-in periods and the remaining trees were used to calculate posterior probabilities.

## Results and Discussion

### New localities

*Liparis purpureovittata* was found in cool-temperate mixed conifer and deciduous forests at the border between Saijo-shi, Ehime Pref. and Agawa-gun, Kochi Pref. (Y. Hagino *et al.* FOS-009187, FOS-009189, ca. 1670m alt., 2 Jul. 2015 [MBK0272829, MBK0272831]; Figs. 1, 2. T. Ishijo and M. Hyodo 17922, 17923, ca. 1700m alt., 29 Jun. 2015. Saijo-shi, Ehime Pref. [TNS]). Identification was confirmed by molecular analyses; the ITS sequences of the three individuals collected by Hagino *et al.* were identical to those previously examined by Tsutsumi *et al.* (2007), except one ambiguous nucleotide site (data not shown). It is the first record of the species from western Japan. The habitats were characterized by leaf litter and mosses along roadsides or at the sides of open spaces. There were more than 30 individuals, about five of which were flowering

at 2 July 2015. The large individuals with about nine flowers were observed in partially shaded places under the shrubs, and the small individuals with 3–6 flowers and juveniles were in light places. Some individuals had no purple tints in the green flowers. In this area, several individuals of the orchid *Malaxis monophyllos* (L.) Sw. occurred together.

We also recorded *L. purpureovittata* from other localities in central and northern Japan (Fig. 2); Shari-gun, Hokkaido, 280m alt., 21 Jul. 2013 (A. Uchida *s.n.* [TNS]), Rebun Island, Hokkaido (S. Miyamoto pers. comm., determined by photographs), Yuza-machi, Yamagata Pref., 1120m alt. (K. Sawa and S. Kawakami, pers. comm., determined by photographs), Yama-gun, Fukushima Pref., 1385m alt., 9 Jul. 2013 (Y. Yamashita 264 [FKSE 69074]), and Ohno-gun, Gifu Pref. (H. Nakayama pers. comm., determined by photographs). In the Shari-gun plants, green flowers without purple tints were also observed. Those flowers are morphologically similar except in the flower color.

In Shari-gun, Hokkaido, three individuals were observed in the semi-open and partially shaded places. In Yuza-machi, Yamagata Pref., almost 10 individuals were found on the damp ground with *Nephrophyllidium crista-galli* (Menzies ex Hook.) Gilg. In Yama-gun, Fukushima Pref., 30 individuals occurred along a trail in the mountain grassland. Based on those results, we found out that *L. purpureovittata* is widely distributed in light and partially shaded places in mountain grasslands, in damp grounds or along roadsides in open places of the cool temperate zones from western to northern Japan.

### Mycorrhizal partner

We analyzed the ITS regions of 13 isolates randomly selected from 30 fungal isolates obtained from *L. purpureovittata*. BLAST searches for the ITS regions of the samples showed that four of the 13 isolates had high affinities with *Tulasnella* (Tulasnellaceae, Basidiomycota) and a single isolate was closely related to *Exophiala* (Herpotrichiellaceae, Ascomycota).

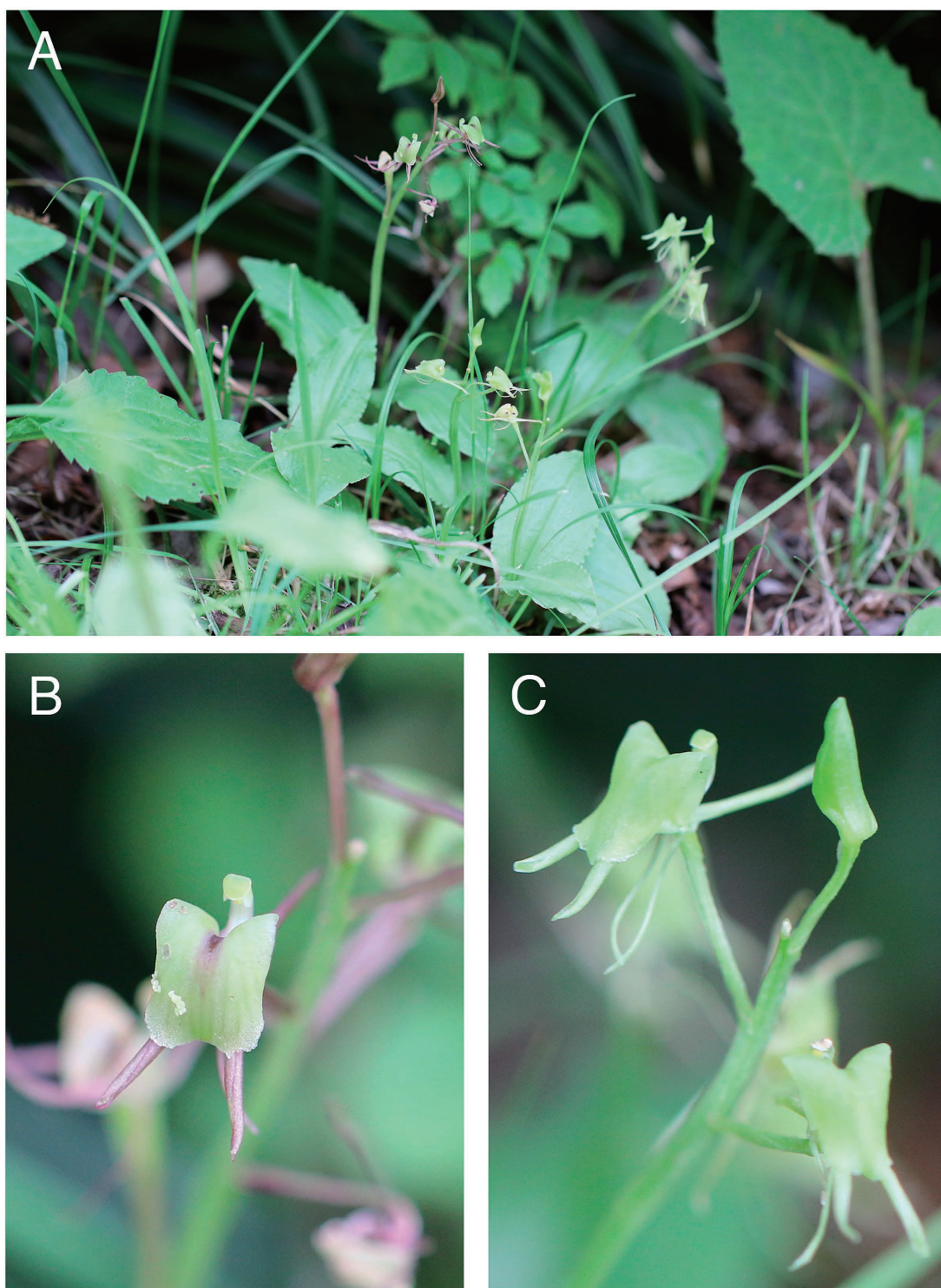


Fig. 1. *Liparis purpureovittata* from new locality, at border of Saijo-shi, Ehime Pref. and Agawa-gun, Kochi Pref. A, habitat. B, flower with a purple dot at center of lip. C, green flowers with no purple dot.

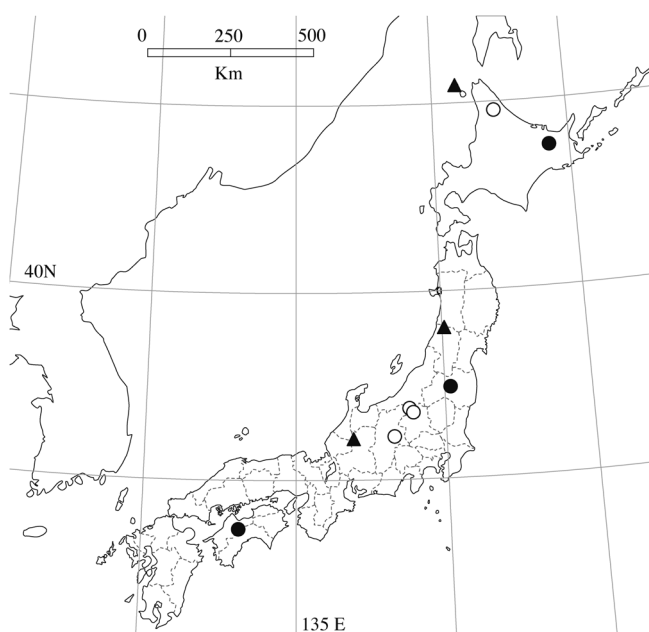


Fig. 2. Distribution map of *L. purpureovittata*. Solid circles, new localities determined by voucher specimens; solid triangles, new localities determined by photographs; open circles, localities recorded in Tsutsumi *et al.* (2008).

The other eight isolates were assigned to members of *Clonostachys rogersoniana*, *Myrothecium gramineum*, *M. roridum*, *Paraphaeosphaeria neglecta*, *Peyronella glomerata*, *Pleosporeales* sp., *Tolypocladium* sp., and *Trichoderma polysporum*. They were excluded from the candidates of mycorrhizal fungi, because they were suggested to be members of Hypocreales and Pleosporales, which have been regarded as either endophytic fungi or contaminants (Dearnaley *et al.*, 2012; Oliveira *et al.*, 2014).

The four isolates were obtained from four of the five forms of *L. purpureovittata*, suggesting that *Tulasnella* are commonly associated with this species. Three of the four isolates of *Tulasnella* had 100% identical sequence and the fourth was 99.2% identical to the other three in the ITS region. Considering that *Tulasnella* has been found in other *Liparis* species (McCormick *et al.*, 2004; Illyés *et al.*, 2005; Shimura *et al.*, 2009; Ding *et al.*, 2014), *Tulasnella* are likely represent of the main fungal partner of *L. purpureovittata*. *Exophiala* was detected only once

from a single sample in this study; it is unlikely that *Exophiala* is the main mycorrhizal partner of *L. purpureovittata*. *Exophiala* has previously been identified as possible orchid endophytes (Stark *et al.*, 2009), but their ecological role in orchids is unclear (Pecoraro *et al.*, 2013).

Preliminary molecular phylogenetic analyses using several sequences of Tulasnellaceae registered in Genbank suggested that the four isolates of *Tulasnella* were included in the “A1” clade in Girlanda *et al.* (2011) (data not shown). The ITS phylogenetic tree using the four *Tulasnella* isolates detected in this study, closest matching sequences of them revealed by BLAST searches, and representative sequences in “A1” clade used by Girlanda *et al.* (2011), revealed that the fungi isolated from *L. purpureovittata* was *Tulasnella calospora* or relatives (Fig. 3). The tree also showed that the fungi of *L. purpureovittata* were close to those of several terrestrial orchids, *Anacamptis laxiflora* (Lam.) R.M.Bateman, Pridgeon & M.W.Chase, *Chloraea blettioides* Lindl., *Dactylorhiza incarnata* (L.) Soó, *Evotella rubiginosa*

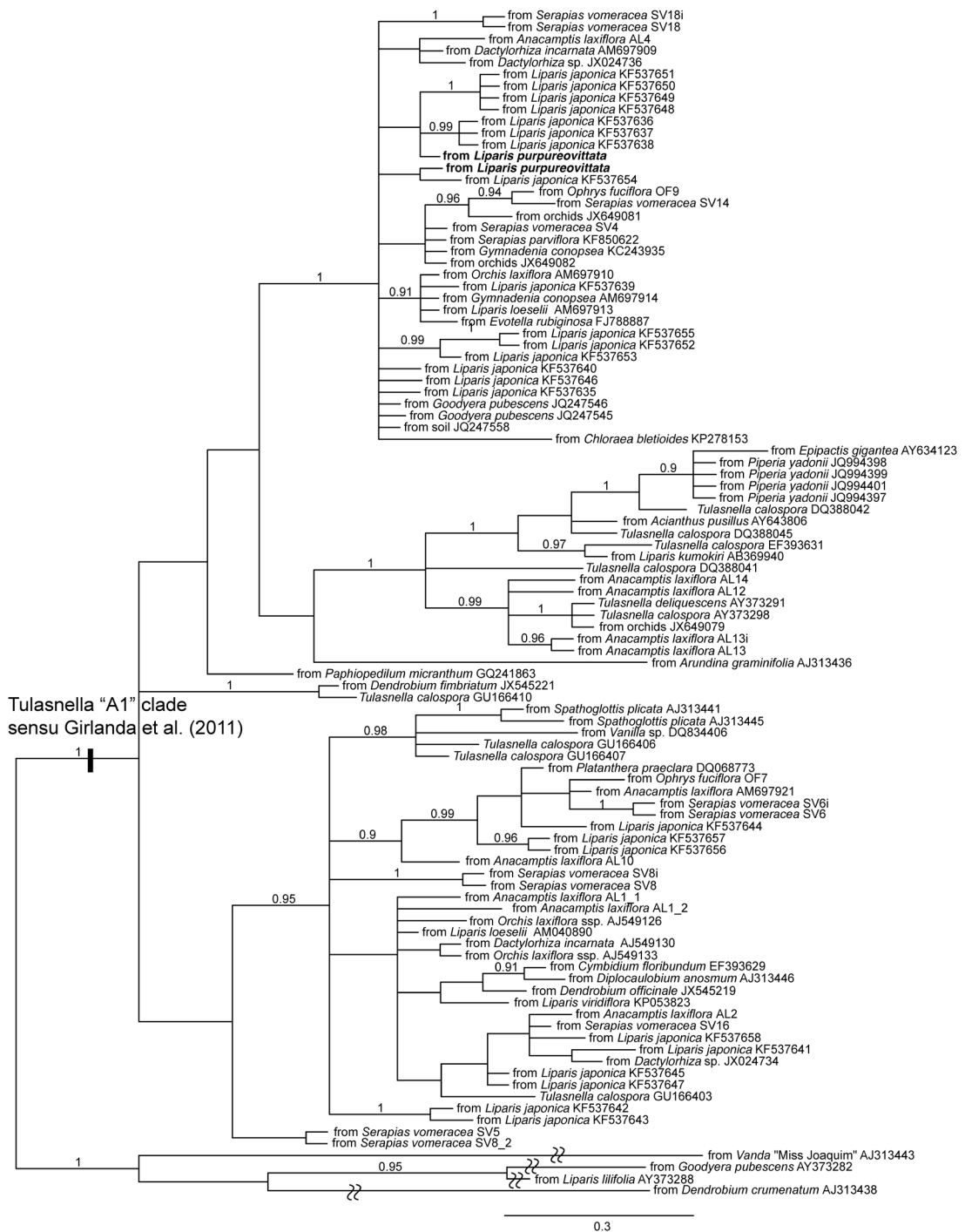


Fig. 3. Consensus tree of *Tulasnella* A1 clade by Bayesian analysis based on ITS sequences (522 bp). Mycorrhizal fungi isolated from *Liparis purpureovittata* are shown by bold font. Other materials than the fungi from *L. purpureovittata* were shown by the names of fungi, or host plants, followed by Genbank accession numbers, or sample ID of Girlanda *et al.* (2011). Figures above branches indicate posterior probabilities ( $p > 0.9$ ) by Bayesian method. Outgroups, fungi from *Dendrobium crumenatum* (AJ313438) and *Vanda* 'Miss Joaquim' (AJ313443), were determined based on the results of Girlanda *et al.* (2011).

(Sond. ex Bolus) Kurzweil & H.P.Linder, *Goodyera pubescens* R.Br., *Gymnadenia conopsea* (L.) R.Br., *Liparis japonica*, *Ophrys fuciflora* (F.W.Schmidt) Moench, *Serapias parviflora* Parl., and *Serapias vomeracea* (Burm.f.) Briq (Fig. 3). These orchids usually live in light and sometimes wet places in temperate grasslands in the northern hemispheres, Chile, and South Africa. Therefore, the mycorrhizal partner of *L. purpureovittata* and its relatives are possibly common in terrestrial orchids distributed in light grasslands in worldwide temperate zones. The four isolates were also close to those from other deciduous terrestrial *Liparis*; *L. japonica*, *L. kumokiri*, and *L. loeselii*, suggesting that these fungi may be predominant mycorrhizal partners of the deciduous terrestrial *Liparis*.

### Acknowledgments

We thank Y. Hagino, S. Hagino, H. Hongo, M. Hyodo, T. Ishijo, S. Kawakami, M. Matsui, S. Mitsuhashi, S. Miyamoto, T. Nagai, H. Nakayama, K. Oikawa, K. Sakamoto, M. Sato, K. Sawa, T. Sekijyo, H. Takeuchi, and K. Watanabe for providing the samples or helping us during our field trips. We are also grateful to K. Suzuki, Y. Yamada, M. Nakajima, and K. Abe for cultivating the living plants and fungi and to P. Abe for teaching us the techniques of fungal isolation.

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