

Mycorrhizal fungi isolated from Japanese endangered plant *Liparis truncata* (Orchidaceae)

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Abstract *Liparis truncata* is endemic to Japan and ranked as critically endangered (CR) in the Red List of Japanese vascular plants. The information of the mycorrhizal fungi is useful for plant conservation because a mycorrhizal association is necessary for seed germination in orchids. We performed a molecular analysis and a preliminary symbiotic germination test using the isolated mycorrhizal fungi. The result suggests that *Tulasnella* (Tulasnellaceae) is a predominant mycorrhizal partner of *L. truncata*.

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). Among them, 17 species of *Liparis* occur in Japan (Yukawa, 2015). *Liparis truncata* F.Maek. ex T.Hashim. was described by Hashimoto (1987) based on cultivated specimens. It is morphologically similar to *L. krameri* Franch. & Sav. distributed in Japan, Korea, Russia and China (Xinqi *et al.*, 2009). It differs from *L. krameri* by the height of flowering plant (3–10 cm in *L. truncata* vs. 10–25 cm in *L. krameri*), habitat (epiphytic vs. terrestrial), and the shape of lip apex (truncate with apiculate tip vs. acutely caudate) (Hashimoto, 1987). It is also close to, but distinct by the floral characters from *L. nanlingensis* H.Z.Tian & F.W.Xing in China, *L. nikkoensis* Nakai in Japan, *L. reckoniana* T.C.Hsu in Taiwan, *L. sasakii* Hayata in Taiwan, and *L. tsii* H.Z.Tian et A.Q.Hu in China (Tian *et al.*, 2012; Hsu, 2013; Tian *et al.*, 2015; Yukawa, 2015). Molecular phylogenetic studies using *L. truncata*

and relatives suggested that *L. truncata*, *L. krameri*, *L. nanlingensis*, *L. sasakii*, and *L. tsii* are closely related to each other and form a monophyletic clade (Tsutsumi *et al.*, 2007; Tian *et al.*, 2012; Tian *et al.*, 2015). They belong to the terrestrial *Liparis* subclade with conduplicate leaves sensu Cameron (2005) (Tsutsumi *et al.*, 2007).

Liparis truncata is treated as Critically Endangered (CR) in the Red List of Japanese vascular plants (Ministry of the Environment of Japan, 2015). It is endemic to Japan, distributed from Tohoku to Kyushu districts (Yukawa, 2015) and lives on old tree trunks of *Fagus* and *Quercus* in cool-temperate forest. The conservation of the species must be preferentially undertaken, but information of its detailed distribution, mycorrhizal partner, pollinators, and preferable habitat is deficient.

Mycorrhizal symbiosis is a key issue to determine the 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) because all orchid species are myco-heterotrophic, at least in

the early stage of life history (Warcup, 1981; Ardittii, 1992). The germination of small, dust-sized seeds depends on mycorrhizal associations, which provide fixed carbon and mineral nutrients (e.g., Alexander *et al.*, 1984; Hadley, 1984; Alexander and Hadley, 1985; Eriksson and Kainulainen, 2011). 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 terrestrial *Liparis* species such as *L. japonica* (Miq.) Maxim., *L. kumokiri* F.Maek., *L. liliifolia* (L.) A.Rich ex Lindl., *L. loeselii* (L.) Rich., and *L. purpureovittata* Tsutsumi, T.Yukawa & M.Kato, *Tulasnella* fungi (Tulasnellaceae) have been found with molecular techniques (McCormick *et al.*, 2004; Illyés *et al.*, 2005; Shimura *et al.*, 2009; Ding *et al.*, 2014; Tsutsumi *et al.*, 2016).

To clarify the mycorrhizal partners of *L. truncata*, this study performed molecular identification and preliminary symbiotic germination test using the fungi isolated from *L. truncata*.

Materials and Methods

Fungal Isolation

For molecular identification, fungal hyphae were isolated from corms of *Liparis truncata* collected from three localities; Minamiuonuma-gun, Niigata Prefecture; Nantan-shi, Kyoto Prefecture; and Yufu-shi, Oita Prefecture (Table 1).

The corms were thoroughly washed in running water. After the outer tissues of the corms were trimmed, the remained 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 containing about 15 mL of corn meal agar (CMA; Nissui Pharmaceutical Co., Tokyo) containing 150 ppm streptomycin and 50 ppm tetracycline. 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.

Molecular Analyses

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, Bucking-

Table 1. Mycorrhizal fungi of *Liparis truncata* analyzed in this study. Asterisks indicate fungi used for symbiotic germination test.

Locality	Plant voucher	Isolation ID of fungi	Fungal genus	Accession No.
Niigata Pref.	Tsutsumi & Hayashi (TNS)	1-1*	<i>Tulasnella</i> (Tulasnellaceae)	LC278368
		2-1*	<i>Tulasnella</i> (Tulasnellaceae)	LC278369
Kyoto Pref.	J. Nagasawa (TNS)	3-1, 3-2, 3-3, 3-4	<i>Tulasnella</i> (Tulasnellaceae)	LC278370
		4-1, 5-1, 5-2, 5-3		
Oita Pref.	K. Tanaka (TNS)	6-1	<i>Ceratobasidium</i> (Ceratobasidiaceae)	LC278371
		6-2	<i>Tulasnella</i> (Tulasnellaceae)	LC278372
		7-1	<i>Tulasnella</i> (Tulasnellaceae)	LC278373
		8-1	<i>Ceratobasidium</i> (Ceratobasidiaceae)	LC278374

hamshire) 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 (Table 1).

The sequences were submitted to BLAST searches (Altshul *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 $\geq 97\%$ ITS similarity. The BLAST searches revealed that *Tulasnella* are candidates of mycorrhizal partner of *L. truncata* (see Results and Discussion). 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). All assembled sequences were aligned using the Clustal X program (Thompson *et al.*, 1997) and then aligned manually. For outgroups, mycorrhizal fungi from *Dendrobium crumenatum* (AJ313438) and *Vanda* 'Miss Joaquim' (AJ313443) were used in the ITS analyses based on the results of phylogenetic analysis in Girlanda *et al.* (2011).

Phylogenetic analyses were performed using Bayesian analyses. 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 1000 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 and that 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.

Symbiotic Germination Test

Seeds were collected from a mature capsule of *L. truncata* cultivated in Tsukuba Botanical Garden (Kyoto Pref. individual: Table 1) just before dehiscence (on 25th November, 2015). The seeds were separated from the capsule tissues, dried at room temperature for a week, and then stored in screw-capped tubes (5 mL) at 4°C.

The seeds were sterilized with 0.25% NaClO solution in the tubes for 30 min and rinsed five times in sterilized distilled water. Approximately 15–50 seeds were sown per 35-mm Petri dish (AGC Techno Glass Co., Chiba). Each dish contained 7 mL of OMA medium (2.5 g oatmeal agar [BD, NJ], 6.5 g agar [Wako Chemical, Kanagawa], and 1 L distilled water, pH 7.0: Yagame *et al.*, 2013). Seeds were inoculated with two fungal isolates collected from Niigata Pref. (Table 1) two or four weeks after the sowing. The mycelium of each isolated fungus was inoculated to two Petri dishes in which the seeds were sown. The dishes were sealed with double layers of Parafilm (Pachiney Plastic Packaging, Menasha, WI) and incubated in the weak light in a room maintained at 25°C. The number of germinated seeds in every dish about 12 weeks after inoculation was counted under a stereomicroscope (Nikon SMZ-10; Nikon Corporation, Tokyo). Germination frequencies were calculated as the ratio of the number of embryos that emerged from the seed coat to the total number of seeds sown.

Results and Discussion

We analyzed the ITS regions of four isolates from two individuals of *Liparis truncata* from Niigata Pref., 14 isolates from three individuals from Kyoto Pref., and six isolates from three individuals from Oita Pref. BLAST searches for the ITS regions showed that 12 of the 24 isolates encompassing all of the sites had high affinities with *Tulasnella* (Tulasnellaceae, Basidiomycota) (Table 1). Two isolates from Oita Pref. were closely related to *Ceratobasidium* (Ceratobasidiaceae, Basidiomycota). The other ten isolates

were assigned to members of *Chloridium* (Chaetosphaeriales) (one strain from Oita), *Diaporthe* (Diaporthales) (one strain from Oita), *Paraconiothyrium* (one strain from Niigata), Pleosporales (two strains from Niigata and Kyoto), *Peyronellaea* (one strain from Kyoto), and *Trichoderma* (four strains from Kyoto) (data not shown). *Diaporthe* is often reported as plant pathogens, non-pathogenic endophytes or saprobes (Gomes *et al.*, 2013), and *Chloridium* is common as endophytic fungi (Rodriguez *et al.*, 2009). The others 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). Therefore the latter ten isolates were excluded from the candidates of mycorrhizal fungi.

The *Tulasnella* isolates were obtained from all localities analyzed, suggesting that *Tulasnella* are commonly associated with *L. truncata*. Two strains of *Tulasnella* from Oita Pref. had 100% identical sequence. The others were 92.4–97.8% identical to each other in the ITS region. *Ceratobasidium* is also common in orchid mycorrhiza (Yukawa *et al.*, 2009). Further analysis is required to clarify the *Ceratobasidium* is also a

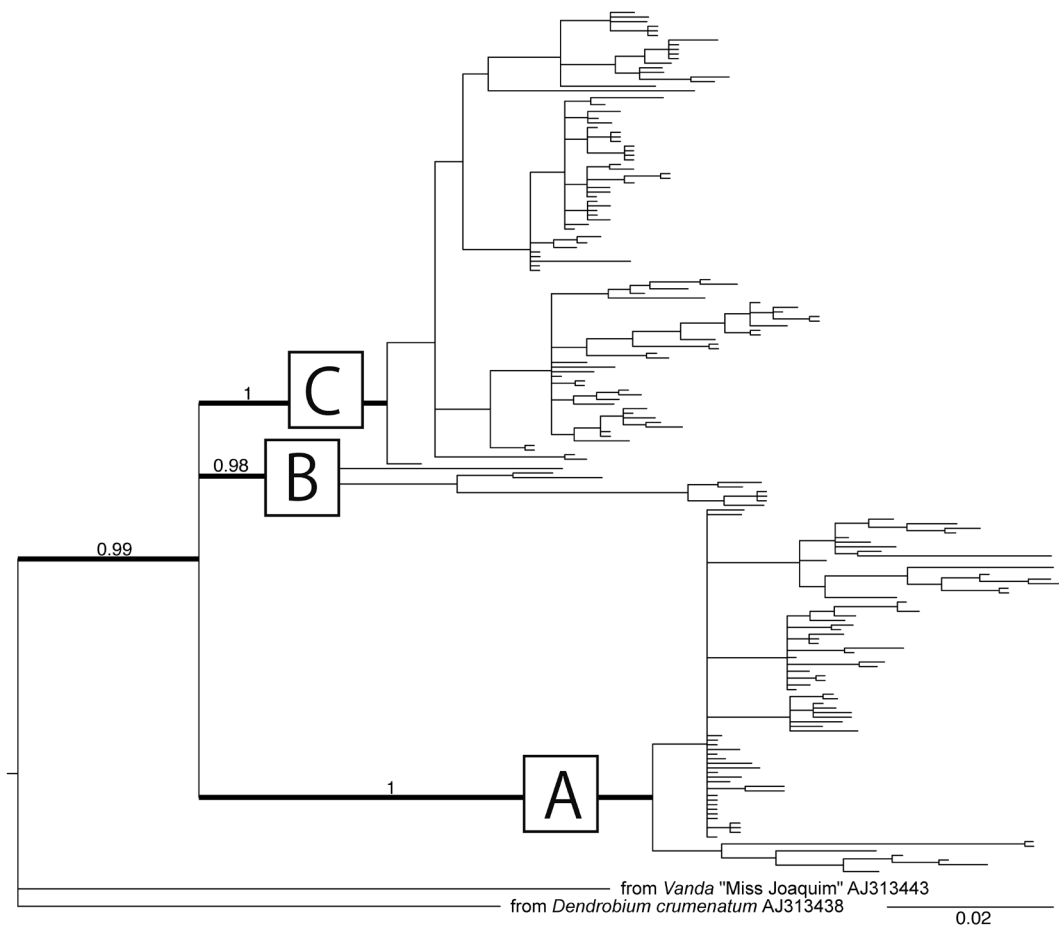


Fig. 1. Diagram of phylogenetic tree of *Tulasnella* by Bayesian analysis based on the nuclear ribosome DNA ITS sequences (526bp.). Numbers at the nodes indicate posterior probabilities ($p > 0.9$) by Bayesian method. Out-groups, viz., mycorrhizal fungi from *Dendrobium crumenatum* (AJ313438) and *Vanda* 'Miss Joaquim' (AJ313443), were determined based on the results of Girlanda *et al.* (2011). The tree consists of three clades (A–C) and all *Tulasnella* isolates from *Liparis truncata* belong to clade A. Details are shown in Figs. 2–3.

Table 2. Number of germinated and non-germinated seeds, and germination frequencies of *Liparis truncata* in each dish by symbiotic germination test.

Fungal strain	Number of germinated seeds	Number of non-germinated seeds	Percentage of germination
Niigata 1-1	0	15	0%
	11	42	20.8%
Niigata 2-1	8	18	30.8%
	3	12	20%

or relatives (Figs. 1–3). The ITS phylogenetic tree comprised three monophyletic clades with high supports (clades A–C in Fig. 1), and all *Tulasnella* isolates from *L. truncata* belonged to the clade A (Fig. 2). The tree also showed that the fungi of *L. truncata* were close to those of several other orchids, i.e., *Chloraea lechleri* Lindl., *Cymbidium goeringii* Rehb.f., *C. floribundum* Lindl., *C. faberi* Rolfe, *Dendrobium nobile* Lindl., *Goodyera maximowicziana* Makino, *G. pubescens* R.Br., *Microtis parviflora* R.Br., *Paphiopedilum armeniacum* S.C.Chen & F.Y.Liu, *P. micranthum* Tang & F.T.Wang, *Platanthera praeclara* Sheviak & M.L.Bowles and *Tipularia discolor* (Pursh) Nutt. (Clade A in Figs. 1–2). These orchids are terrestrial or epiphytic, and live in light, semi-shady, and wet places in temperate to subtropical regions in Asia, Oceania, North America and South America. Therefore, the mycorrhizal partner of *L. truncata* is possibly common worldwide in temperate to subtropical zones. The isolates of *L. truncata* were also close to, but different from a clade of the isolates from other terrestrial *Liparis*; *L. japonica* from China, *L. kumokiri* from Japan, *L. liliifolia* from eastern USA, *L. loeselii* from Hungary, and *L. purpureovittata* from Japan (McCormick *et al.*, 2004; Illyés *et al.* 2005; Shimura *et al.*, 2009; Ding *et al.*, 2014; Tsutsumi *et al.*, 2016) (Clades B and C in Fig. 3).

The symbiotic germination experiment is useful to test symbiotic ability of seeds and juvenile plants with fungi isolated from adult plants. In

American terrestrial *L. liliifolia*, fungi isolated from adults supported its seed germination and protocorm growth (McCormick *et al.*, 2004). Our preliminary symbiotic germination test showed that the two *Tulasnella* strains isolated from Niigata Pref. promoted the seed germination of *L. truncata* (Table 2). Although the frequencies were not high and variable (0–30.8% in Table 2), the germinated seeds grew further and faster in the symbiotic germination than those in asymbiotic cultures (data not shown). The result suggested that the *Tulasnella* isolated from the adult plants are a mycorrhizal partner of *L. truncata* from seeds to adults. The symbiotic cultivation with the *Tulasnella* fungi may be useful for conservation and propagation of endangered *L. truncata*.

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Fig. 3. Part of phylogenetic tree of *Tulasnella* by Bayesian analysis based on ITS sequences (Clades B and C in Fig. 1). Mycorrhizal fungi isolated from *Liparis truncata* are shown in bold. Other sequences are shown by the names of fungi or host plants followed by Genbank accession numbers, or sample ID of Girlanda *et al.* (2011). Numbers at the nodes indicate posterior probabilities ($p > 0.9$) by Bayesian method. Outgroups, viz., mycorrhizal fungi from *Dendrobium crumenatum* (AJ313438) and *Vanda* ‘Miss Joaquim’ (AJ313443), were determined based on the results of Girlanda *et al.* (2011).

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