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In vitro Seed Propagation and Conservation of the Rediscovered Rare *Liparis hostifolia* (Orchidaceae)

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Abstract *Liparis hostifolia* is a rare orchid species that was recently rediscovered, after an approximately 80-year gap in the record, on Minami-iwo-to Island in the Bonin (Ogasawara) Archipelago, Japan. We conducted *in vitro* propagation of *L. hostifolia* with asymbiotic and symbiotic seed germination techniques. Two strains of *Tulasnella* (Tulasnellaceae) were isolated from *L. hostifolia* and used as fungal partners for symbiotic germination. While the germination rate was low and the protocorm growth was slow in an asymbiotic culture, symbiotic cultures produced a high germination rate and rapid protocorm growth. Symbiotic *in vitro* culture will be useful for propagation and conservation of this rare, endangered orchid.

Key words: Asymbiotic germination, conservation, *Liparis*, mycorrhiza, orchid, propagation, symbiotic germination, *Tulasnella*.

Introduction

Liparis Rich. is a cosmopolitan orchid genus consisting of about 320 species (Pridgeon *et al.*, 2005). In Japan, there are 17 recognized species of *Liparis* (Yukawa, 2015). *Liparis hostifolia* (Koidz.) Koidz. ex Nakai was recorded on Chichi-jima Island and Minami-iwo-to Island of the Bonin (Ogasawara) Archipelago, Japan (Koidzumi, 1916; Tuyama, 1937). However, there had been no records from Minami-iwo-to Island since 1936 or from Chichi-jima Island since 1938. Recently, Takayama *et al.* (2019) rediscovered the species in a 2017 field survey of Minami-iwo-to Island and used the living collection to examine its biological properties. Molecular phylogenetic analyses showed that *L. hostifolia* is likely a sister to the clade comprising *L. longiracemosa* Tsutsumi, T.Yukawa, & M.Kato; *L. makinoana* Schltr.; *L. suzumushi* Tsutsumi, T.Yukawa, & M.Kato; and *L. yongnoana* N.S. Lee, C.S.Lee & K.S.Lee. Analyses also suggested that *L. hostifolia* had probably immigrated from East Asia and had long been isolated in the Bonin Archipelago. *Liparis hostifolia* may currently survive in the natural environment of Minami-iwo-to Island, which has been well preserved because the island is unpopulated. In contrast, the species may be extinct from Chichi-jima Island, the vegetation of which is excessively exploited. This species is ranked as Critically Endangered at the national level (Ministry of the Environment, Japan, 2020). The only *ex-situ* collection of *L. hostifolia* consists of three individuals cultivated at Tsukuba Botanical Garden, National Museum of Nature and Science in Tsukuba, Japan. Consequently, development of artificial propagation methods should be prioritized for conservation of this species.

Under natural conditions, orchid seed germination requires an association with compatible mycorrhizal fungi (Rasmussen, 1995). Germina-

tion of the dust-sized seeds depends on these 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 associated with Orchidaceae belong to a small number of taxa within the Basidiomycota, such as Ceratobasidiaceae, Tulasnellaceae, and Sebacinaceae, whereas Glomeromycota, which are common as arbuscular mycorrhizae in most land plants, have not been recorded with the Orchidaceae (Yukawa *et al.*, 2009; Leake and Cameron, 2012; Martos *et al.*, 2012). *Tulasnella* (Tulasnellaceae) is associated with several *Liparis* species that are closely related to *Liparis hostifolia*, including '*L. japonica* (Miq.) Maxim.' [the correct name should be *L. makinoana* or a relative (Tsutsumi *et al.*, 2019)]; *L. kumokiri* F.Maek.; *L. liliifolia* (L.) Rich. ex Lindl.; *L. loeselii* (L.) Rich.; and *L. purpureovittata* Tsutsumi, T.Yukawa & M.Kato. (McCormick *et al.*, 2004; Illyés *et al.* 2005; Shimura *et al.*, 2009; Ding *et al.*, 2014; Tsutsumi *et al.*, 2016).

In vitro asymbiotic germination of seeds is a useful tool for propagation of both ornamental and endangered orchid species. Protocols have been established for many orchid species (Arditti and Ernst, 1984; Rasmussen, 1995; Arditti, 2008). In uncultured species, however, the optimal culture condition should be determined by examining factors such as sterilization, light intensity and duration, temperature, and culture medium. While asymbiotic germination is a popular technique for orchid seed germination, symbiotic seed germination has recently gained popularity in conservation and restoration projects (Kauth *et al.*, 2008). Multiple studies have reported that symbiotic seed germination and seedling development methods hold advantages over asymbiotic methods (see Results and Discussion). However, these advantages appear to be dependent upon selection of a compatible mycobiont. For the *Liparis* species, in particular species allied to *L. hostifolia*, there have been studies on asymbiotic and symbiotic seed germination (*L. fujisanensis*, *L. kumokiri*, *L. koreojaponica* in Tsutsumi *et al.*, 2011; *L. liliifolia* in Rasmussen and Whigham, 1998, Whigham *et al.*, 2002, McCormick *et al.*, 2004; *L. loeselii* in Illyés *et al.*, 2005). Here, we report on propagation of *L. hostifolia* using asymbiotic and symbiotic *in vitro* seed germination techniques.

Materials and Methods

Seed collection

Three *Liparis hostifolia* individuals collected from Minami-iwo-to Island in 2017 (Takayama *et al.*, 2019) are cultivated in the greenhouse of Tsukuba Botanical Garden, National Museum of Nature and Science in Tsukuba, Japan. Artificial self-pollinations were performed in 2017, and artificial cross-pollinations were carried out in 2018. It took 10–16 months for fruits to ripen. After self-pollination in 2017, a mature capsule was harvested and seeds were collected from the capsule on December 26, 2018 (hereafter referred to as 2018-seeds). After cross-pollination in 2018, a mature capsule was harvested and seeds were collected on October 9, 2019 (2019-seeds). The seeds were separated from the pericarp, dried at room temperature, and used for germination analyses within 10 days of collection.

Fungal isolation

Fungal hyphae were isolated from two *Liparis hostifolia* corms. After a corm was thoroughly washed in running water and the outer tissues were trimmed, the remaining inner tissue was sliced into three pieces of ca. 5–10 mm square and ca. 1 mm thick 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 with about 15 mL of corn meal agar (CMA; Nissui Pharmaceutical Co., Tokyo) containing 150 ppm streptomycin and 50 ppm tetracycline. After incubation at approximately 25°C in the dark for 3–5 days, fungal hyphae 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℃ in the dark.

The hyphae were directly used for polymerase chain reaction (PCR) amplification. The internal transcribed spacer (ITS) regions together with the 5.8S region of nuclear ribosomal DNA were used for fungal identification. We used a pair of primers, ITS1F and ITS4 (White *et al.*, 1990; Gardens and Bruns, 1993). The procedures of PCR and purification followed Tsutsumi *et al.* (2016). Sequences were analyzed using ABI 3500xl (Applied Biosystems) and assembled using Seqman II (DNAstar Lasergene, WI).

The sequences were submitted to BLAST searches (Altshul *et al.*, 1997) against the NCBI sequence database (GenBank) to detect closely matched sequences. We assigned genus or family names to our samples based on registered sequences with $>$ 97% ITS similarity. The two isolated *Tulasnella* strains, s1_1_3 and s2_1_3, were used for symbiotic germination (see Results and Discussion). Their sequences were registered at the DNA Data Bank of Japan (DDBJ) (Accession numbers: s1_1_3, LC552073 and s2_1_3, LC552074).

Asymbiotic in vitro germination

We conducted asymbiotic germination tests using both 2018-seeds and 2019-seeds. The procedures for sowing and culturing seeds followed Miyoshi and Mii (1998). Seeds were sterilized in a solution of NaClO containing 0.25% active chlorine for 30 min, and then rinsed three times with sterilized distilled water. Approximately 150-500 seeds were sown in each of 90 × 20 mm Petri dishes (SH-20S, Terumo Co., Tokyo for the 2018-seeds; SH90-20, AGC Techno Glass Co., Shizuoka for the 2019-seeds). Four replicate plates were prepared. Each plate contained 80 mL of ND medium solidified with 2 g of agar per liter (Tokuhara and Mii, 1993). Immediately after sowing, each plate was sealed with two layers of Parafilm™ (Pechiney Plastic Packaging, Menasha, WI). The plates were incubated in the dark at 25℃. Seed germination and seedling development were monitored for 40 weeks after sowing (WAS) for the 2018-seeds and 15 WAS for the 2019-seeds. After 15 WAS, the plates were moved to incubation with weak light. The protocorms were subcultured after 52 WAS.

The rates of germination and protocorm formation were measured under a stereomicroscope (Olympus SZX-12; Olympus Optical, Tokyo, Japan) at 5, 9, 14, 24, and 40 WAS for the 2018-seeds, and at 5, 10, and 15 WAS for the 2019-seeds. Germination was defined as the stage when an embryo emerged from the ruptured seed coat, and protocorm formation was defined as the stage when both a shoot primordium and rhizoids were visible. The average rates of germination and embryo formation were calculated from those of the four replicates.

Symbiotic in vitro germination

Symbiotic germination tests were performed using the 2019-seeds. The seeds were sterilized as described above. Approximately 150–500 seeds were sown in each of 90 × 20 mm Petri dishes (AGC Techno Glass Co.). Each dish contained 80 mL of OMA medium [2.5 g oatmeal agar (BD, NJ), 6.5 g agar (Wako Chemical, Kanagawa), and 1 L distilled water, pH 5.8 (modified from Yagame *et al.*, 2013)]. Two fungal isolates (s1_1_3 and s2_1_3) were used. At 3WAS, the mycelium of each isolated fungus was inoculated onto three replicate plates on which the seeds were sown. The dishes were sealed with Parafilm and incubated in the dark at 25°C. No germination was observed prior to fungal inoculation. Therefore, monitoring of seed germination and seedling development started after fungal inoculation. The rates of germination and protocorm formation on each plate were calculated at 5 and 10 weeks after fungal inoculation (WAI). The averages of the three replicate cultures were calculated.

Results and Discussion

Asymbiotic germination

Germination rates were quite low ($<$ 1% on average) at 5 weeks after sowing (WAS) for both

2018- and 2019-seeds and gradually increased with incubation time (Figs. 1, 2). Protocorms were observed at 9 WAS, and protocorm rates also gradually increased with time. The rates of seed germination and protocorm formation in the 2018-seeds at 40 WAS were 23.6% and 19.6%, respectively. The rate of protocorm formation was about 25% at 52 WAS (data not shown), before they were subcultured in the new medium, although exact numbers could not be determined because the enlarged protocorms overlapped each other after 40 WAS. We obtained more than 300 protocorms from the 2018-seeds.

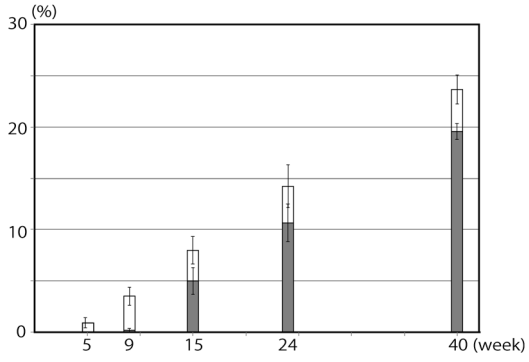

Fig. 1. Average rates of germination (white) and protocorm formation (gray) of 2018-seeds in asymbiotic culture at 4, 9, 15, 24 and 40 WAS (weeks after sowing). Bars represent standard deviations.

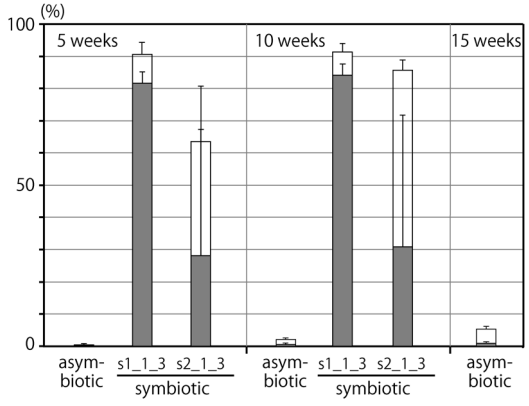

Fig. 2. Average rates of seed germination (white) and protocorm formation (gray) of 2019-seeds in asymbiotic germination at 5, 10, and 15 WAS (weeks after sowing) and in symbiotic germination at 5 and 10 WAI (weeks after inoculation). Two isolated *Tulasnella* stains, s1_1_3 and s2_1_3, were used in symbiotic germination. Bars represent standard deviations.

In the asymbiotic culture, seed germination required more time and the rates of seed germination and protocorm formation were lower than those of allied species, *L. fujisanensis*, *L. koreojaponica*, and *L. kumokiri*, as demonstrated by Tsutsumi *et al.* (2011). Germination rates of the three species in our previous preliminary experiment were more than 80% under the same methods of sterilization, culture medium, temperature, and light conditions as this study (data not shown). The reason for the low rates and slow germination of *L. hostifolia* in the asymbiotic culture remains uncertain. The seeds of *L. hostifolia* have a high germination ability, as indicated by the high rates of germination in the symbiotic culture using seeds from the same capsule as in the asymbiotic culture (Fig. 2). Further analyses are needed to determine appropriate asymbiotic culture conditions.

Fungal identification

We obtained 19 fungal isolates from two *L. hostifolia* corms. BLAST searches of the ITS regions of the 19 isolates showed that 10 had high affinities with *Tulasnella* (Tulasnellaceae, Basidiomycota), which are suggested to be mycorrhizal partners with other allied *Liparis* species (McCormick *et al.*, 2004; Illyés *et al.* 2005; Shimura *et al.*, 2009; Ding *et al.*, 2014; Tsutsumi *et al.*, 2016). Nine of the ten isolates of *Tulasnella* were 100% identical to each other and the remaining one was 99.2% identical (except heteropeaks). One of the nine genetically identical strains (s1_1_3) and the other strain (s2_1_3) were used for symbiotic germination.

Of the other nine isolates, three were assigned to each *Cladosporium*, *Mycoleptodiscus*, and *Neopestalotiopsis*, and the remaining six were members of Pleosporales or Hypocreales. *Cladosporium* is common in many natural environments and considered to be a contaminant. *Mycoleptodiscus* and *Neopestalotiopsis* have been

reported as plant pathogens, non-pathogenic endophytes, or saprobes (Jiang *et al.*, 2018; Hernández-Restrepo *et al.*, 2019). Pelosporales and Hypocreales have been regarded as either endophytic fungi or contaminants (Dearnaley *et al.*, 2012; Oliveira *et al.*, 2014). Therefore, those nine isolates were excluded as candidates of mycorrhizal fungi for *L. hostifolia*.

Symbiotic germination

The germination rates in symbiotic culture were remarkably high at 5 weeks after fungal inoculation (WAI) (Fig. 2). The rate in cultures with the s1_1_3 strain exceeded 90% at 5 WAI and 10 WAI. The average rate in cultures with the s2_1_3 strain was 63.5% at 5 WAI and 85.6% at 10 WAI. In cultures with the s1_1_3 strain, 84.1% of seeds grew to protocorms at 10 WAI. In cultures with the s2_1_3 strain, the rates of protocorm formation varied depending on the plates and the rates were 0–83.6% at 5 WAI and 0.5–88.5% at 10 WAI. These results suggest that *Tulasnella*, particularly the 1_1_3 strain, is a highly compatible fungal partner of *L. hostifolia.*

Compared to the asymbiotic culture, the seeds germinated well and grew quickly in the symbiotic culture (Figs. 1–3). Thus, symbiotic culture is a useful tool for the propagation and growth promotion of *L. hostifolia.* Several studies have shown that symbiotic seed germination has advantages compared to asymbiotic germination in terrestrial orchid species such as *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh. (Rasmussen *et al.*, 1990); *Epipactis palustris* (L.) Crantz (Rasmussen, 1992); *Pterostylis arenicola* M.A.Clem. & J.L.Stewart (Jusaitis and Sorensen, 1993); *Diuris longifolia* R.Br. and *Elythranthera brunonis* (Endl.) A.S.George (Oddie *et al.*, 1994); *Platanthera integrilabia* (Correll) Luer (Zettler and McInnis, 1994); *Pecteilis radiata* (Thunb.) Raf. (Takahashi *et al.*, 2000); *Eulophia alta* (L.) Fawc. & Rendle (Johnson *et al.*, 2007); and *Caladenia latifolia* R.Br. (Bustam *et al.*, 2014). However, direct comparisons between asymbiotic and symbiotic techniques are questionable because the culture media are very different (Rasmussen *et al.*, 1990). In this study, we were unable to find optimal asymbiotic conditions for *L. hostifolia* because the paucity of the seeds obtained limited the scope of the experiment. Nevertheless, propagation of *L. hostifolia* was achieved under conditions based on previous successful or standard methods used for allied species.

We have also succeeded in *in vitro* flowering using asymbiotic culture in several *Liparis* species closely allied to *L. hostifolia* (C. Tsutsumi, unpubl. observ.). The *Liparis* species are generally difficult to cultivate in pots in the nursery for long periods of time. Therefore, *in vitro* culture is useful and efficient for both propagation and *ex situ* conservation of *Liparis* and other plants that are difficult to maintain in living collections in the nursery.

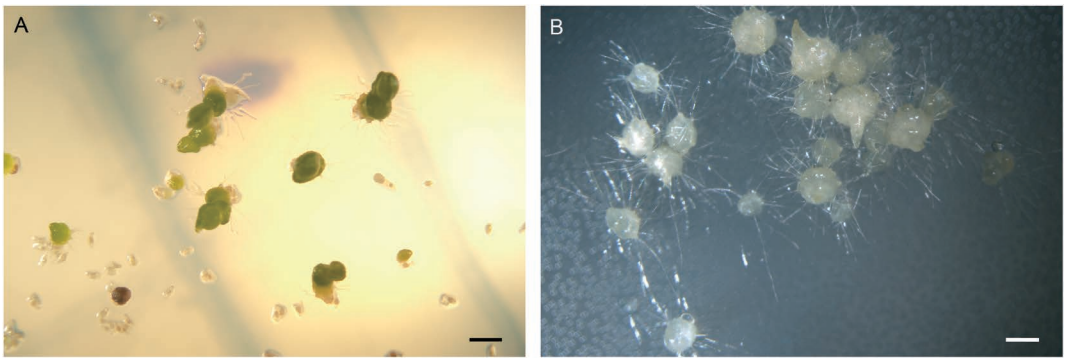

Fig. 3. Seedlings of *Liparis hostifolia.* A, at 40 WAS in asymbiotic culture of the 2018-seeds. B, at 10 WAI in symbiotic culture of the 2019-seeds. Scale bars = 1 mm.

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