Full paper

Strong primer bias for *Tulasnellaceae* fungi in metabarcoding: Specific primers improve the characterization of the mycorrhizal communities of epiphytic orchids

Kento Rammitsu^{a,b}, Tadashi Kajita^{b,c}, Ryosuke Imai^c, and Yuki Ogura-Tsujita^{a,b,*}

^a Faculty of Agriculture, Saga University, 1 Honjyo-machi, Saga 840-8502, Japan

^b The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-8580, Japan

^c Tropical Biosphere Research Center, University of the Ryukyus, Uehara, Yaeyama, Okinawa, 907-1541, Japan

ABSTRACT

Primer bias toward *Tulasnellaceae* fungi during PCR is a known issue with metabarcoding analyses for the assessment of orchid mycorrhizal communities. However, this bias had not been evaluated for the fungal communities of epiphytic orchids, which account for 69% of all orchid species diversity. We compared the mycorrhizal communities detected using two primer pairs, a fungal universal primer pair (ITS86F/ITS4) and *Tulasnella*-specific primer pair (5.8STulngs/ITS4-Tul2), using a mock community of fungal isolates from epiphytic orchids and also environmental samples, including orchid roots and a tree bark tip from the host tree of an epiphytic orchid collected. The detected mycorrhizal communities differed widely depending on the primer pairs used. The fungal universal primer pair successfully identified *Ceratobasidiaceae* and *Serendipitaceae* fungi but did not reflect *Tulasnellaceae* diversity. *Tulasnellaceae* fungi were mainly detected using the *Tulasnella*-specific primer pair. These tendencies were observed in both the mock community and environmental samples. These results strongly suggest that the use of a *Tulasnella*-specific primer in combination with a fungal universal primer is essential for assessing the mycorrhizal communities of orchids through metabarcoding analysis, especially in epiphytic orchids. Our study contributes to further understanding of the diversity of mycorrhizal fungi in orchids.

Keywords: epiphytes, mycorrhizal fungi, next generation sequencing, *Orchidaceae*, primer choice

Article history: Received 1 February 2021, Revised 18 June 2021, Accepted 20 June 2021, Available online 13 July 2021.

1. Introduction

Environmental DNA metabarcoding using next generation sequencing is a growing approach for the assessment of orchid mycorrhizal communities (Kaur, Phillips, & Sharma, 2020; Xing et al., 2020). This approach allows for the easy detection of a larger number of fungal species from a single mycorrhizal community than does the traditional Sanger sequencing-based DNA barcoding approach (Tedersoo et al., 2010). Due to the vast amounts of data available, the metabarcoding approach has rapidly replaced the Sanger sequencing-based approach. In terrestrial orchids, large numbers of studies have investigated mycorrhizal communities using the metabarcoding approach, and the findings of such studies continue to enhance our understanding of orchid mycorrhizal associations (Oja, Kohout, Tedersoo, Kull, & Kõljalg, 2015; Waud, Brys, Van Landuyt, Lievens, & Jacquemyn, 2017; Duffy, Waud, Schatz, Petanidou, & Jacquemyn, 2019; Kaur et al., 2020). However, few studies have used the metabarcoding approach to examine ep-

iphytic orchids (Herrera et al., 2019; Xing et al., 2020), which account for 69% of all orchid species (Zotz, 2013), and thus the optimal experimental methods for accurately assessing the mycorrhizal communities of epiphytic orchids remain poorly understood.

The utility of DNA metabarcoding for orchid mycorrhizal communities using universal primers for fungal detection is limited due to primer bias, which prevents the detection of particular fungi present in a sample by primer mismatches during PCR (Waud, Busschaert, Ruyters, Jacquemyn, & Lievens, 2014). The mycorrhizal fungi of orchids mainly belong to three *Basidiomycota* families, the *Tulasnellaceae*, *Ceratobasidiaceae*, and *Serendipitaceae*, collectively described as 'rhizoctonia-like' (Yukawa, Ogura-Tsujita, Shefferson, & Yokoyama, 2009). Although the *Tulasnellaceae* are the most common partner of orchids (Dearnaley, Martos, & Selosse, 2012), its molecular detection is made difficult by mismatches with fungal universal primers (Suárez et al., 2006; Taylor & McCormick, 2008). This is because *Tulasnellaceae* fungi underwent accelerated evolution of the nuclear ribosomal operon (Taylor & McCormick, 2008), which increased the rate of base substitutions at the primer site compared with other *Basidiomycota* families. Therefore, *Tulasnella*-specific primers were used to prevent the underrepresentation of *Tulasnellaceae* fungi with the Sanger sequencing-based ap-

* Corresponding author.

Faculty of Agriculture, Saga University, 1 Honjyo-machi, Saga 840-8502, Japan.
E-mail address: ytsujita@cc.saga-u.ac.jp (Y. Ogura-Tsujita).

This is an open-access paper distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivative 4.0 international license (CC BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>).

proach (Ogura-Tsujita, Yokoyama, Miyoshi, & Yukawa, 2012; Kartzinel, Trapnell, & Shefferson, 2013).

For metabarcoding analysis, primer bias was assessed against broad fungal taxa associated with terrestrial orchids, including *Tulasnellaceae* fungi in Waud et al. (2014). This study evaluated the performance of a variety of fungal universal primers and specific primers for orchid mycorrhizal fungi to characterize orchid mycorrhizal communities, and suggested several suitable primer pairs. The fungal universal primer pair ITS86F/ITS4 outperformed other primer combinations. Many studies have investigated mycorrhizal communities using ITS86F/ITS4 in both terrestrial and epiphytic orchids (Cevallos, Sánchez-Rodríguez, Decock, Declerck, & Suárez, 2017; Johnson, González-Chávez, Carrillo-González, Porras-Alfaro, & Mueller, 2020). However, Waud et al. (2014) pointed out that this primer pair excludes the *Tulasnellaceae* fungi including *Tulasnella* subgroup A defined by Girlanda et al. (2011). Furthermore, previous large-scale surveys using Sanger sequencing-based approaches have shown that the mycorrhizal communities of epiphytic orchids differ from those of terrestrial orchids (Martos et al., 2012; Xing et al., 2019). Despite these issues, no assessments of primer bias have been performed in epiphytic orchids. Appropriate primer selection is required to accurately assess the fungal communities of epiphytic orchids.

In this study, we tested whether primer choice in metabarcoding affects the diversity of the fungal communities, especially *Tulasnellaceae* communities, detected from epiphytic orchid roots. Two primer pairs, a fungal universal primer pair (ITS86F/ITS4) and a *Tulasnella*-specific primer pair, were compared by evaluating their abilities to characterize the mycorrhizal communities of bulk DNA samples, which are mock communities of fungi isolated from orchids, and environmental samples including orchid roots or a tree bark tip. Furthermore, to examine whether primer biases were related to particular *Tulasnellaceae* taxa, a phylogenetic analysis of *Tulasnellaceae* fungi was performed with additional information on the number of mismatches against used primer sequences.

2. Materials and methods

2.1. Creation of mock fungal communities

Mycorrhizal fungi were isolated from nine epiphytic and two terrestrial orchid species distributed in Japan based on the methods of Rammitsu et al. (2021). Then, we identified the fungal taxa of these isolates by sequencing their internal transcribed spacer regions (ITS; spanning the ITS-1, 5.8S, and ITS-2 regions). These sequences were assigned to operational taxonomic units (OTUs) based on 97% sequence similarity across the whole ITS region (Martos et al., 2012).

We choose 20 rhizoctonia isolates (18 OTUs) including 8 *Tulasnellaceae* (8 OTUs), 11 *Ceratobasidiaceae* (9 OTUs), and 1 *Serendipitaceae* (1 OTU) isolates. The other *Basidiomycota* family, *Brachybasidiaceae*, isolated from epiphytic orchids, were also mixed as a non-rhizoctonia sample. To test whether we were able to identify different fungi within the same OTU, three *Ceratobasidiaceae* isolates (CE18A, CE18B, and CE18C) within OTU CE18 were added to the mock community. CE18B and CE18C showed 99.9% (593/597 bp) sequence similarity and had 12 or 13 base substitutions compared with CE18A, respectively. All of the isolate sequences were registered in the DNA Data Bank of Japan (LC597338–LC597358). Eighteen fungal isolates were deposited in the NITE Biological Resource Center in Japan (Table 1; NBRC114085–NBRC114087, NBRC114098, NBRC114194, NBRC114196, NBRC114198, NBRC114909–NBRC114918, NBRC114326).

Fungal DNA was extracted from the isolates using the methods of Izumitsu et al. (2012). Mycelia (0.1–1 µg) were obtained from a colony on culture medium using a sterilized toothpick and suspended in 50 µL of Tris–EDTA (TE) buffer in a 1.5-mL tube. The tubes were microwaved (600 W) for 1 min. After 30 s at room temperature, the tubes were microwaved for another minute. The tubes were cooled at –20 °C for 10 min and then centrifuged at 10,000 rpm for 5 min. The supernatant was used as the template for PCR. The DNA was quantified with a Qubit dsDNA HS Assay Kit (Invit-

Table 1 List of fungal isolates in the mock community.

Fungal isolates	Isolation source		Information of fungal isolates			
	Life form	Orchid species	Isolate ID	Fungal family	Accession No.	NBRC Accession No.
CE4	E	<i>Thrixspermum japonicum</i>	F416	<i>Ceratobasidiaceae</i>	LC597338	NBRC 114914
CE5	E	<i>Thrixspermum japonicum</i>	F417	<i>Ceratobasidiaceae</i>	LC597339	NBRC 114915
CE6 ^a	E	<i>Vanda falcata</i>	F313	<i>Ceratobasidiaceae</i>	LC597340	NBRC 114194
CE9 ^a	E	<i>Thrixspermum japonicum</i>	F414	<i>Ceratobasidiaceae</i>	LC597341	NBRC 114913
CE15 ^a	E	<i>Vanda falcata</i>	F210	<i>Ceratobasidiaceae</i>	LC597342	NBRC 114086
CE16	E	<i>Vanda falcata</i>	F214	<i>Ceratobasidiaceae</i>	LC597343	NBRC 114087
CE18A	T	<i>Gastrodia pubilabiata</i>	F86	<i>Ceratobasidiaceae</i>	LC597344	–
CE18B ^a	E	<i>Gastrochilus japonicus</i>	F324	<i>Ceratobasidiaceae</i>	LC597345	NBRC 114198
CE18C	E	<i>Dendrobium officinale</i>	F356	<i>Ceratobasidiaceae</i>	LC597346	NBRC 114326
CE21	E	<i>Gastrochilus japonicus</i>	F468	<i>Ceratobasidiaceae</i>	LC597347	NBRC 114916
CE22	E	<i>Phalaenopsis japonica</i>	F499	<i>Ceratobasidiaceae</i>	LC597348	NBRC 114917
TU3 ^a	E	<i>Thrixspermum japonicum</i>	F267	<i>Tulasnellaceae</i>	LC597349	NBRC 114098
TU10 ^a	E	<i>Dendrobium officinale</i>	F205	<i>Tulasnellaceae</i>	LC597350	NBRC 114085
TU11 ^a	E	<i>Vanda falcata</i>	F320	<i>Tulasnellaceae</i>	LC597351	NBRC 114196
TU12	T	<i>Geodorum densiflorum</i>	F227	<i>Tulasnellaceae</i>	LC597352	NBRC 114909
TU18 ^a	E	<i>Staurochilus lutchuensis</i>	F330	<i>Tulasnellaceae</i>	LC597353	NBRC 114910
TU22 ^a	E	<i>Liparis bootanensis</i>	F372	<i>Tulasnellaceae</i>	LC597354	NBRC 114912
TU23 ^a	E	<i>Luisia teres</i>	F348	<i>Tulasnellaceae</i>	LC597355	NBRC 114911
TU36	T	<i>Gastrodia pubilabiata</i>	F98	<i>Tulasnellaceae</i>	LC597356	–
SE1 ^a	E	<i>Dendrobium officinale</i>	F345	<i>Serendipitaceae</i>	LC597357	–
BR1 ^a	E	<i>Dendrobium moniliforme</i>	F446	<i>Brachybasidiaceae</i>	LC597358	NBRC 114918

To create the mock community, DNA of each isolate was adjusted to 0.1 ng/µL and pooled with 5 µL.

^a DNA samples from the isolates with DNA < 0.1 ng/µL were also pooled with 5 µL. E, epiphytic species; T, terrestrial species; –, isolates without accession numbers.

rogen, Carlsbad, CA, USA). We diluted each DNA sample to the minimum concentration of detection (0.1 ng/ μ L) and mixed 5 μ L of each to make a mock community of fungal isolates. The DNA concentrations in 12 samples were below the detection limit of the assay kit and failed to quantify the exact value; thus, the extracted DNA was less than 0.1 ng/ μ L and pooled with the 5 μ L. The mixture was used as template for PCR amplification.

2.2. Creation of bulk root samples and preparation of the tree bark sample

Roots from three epiphytic orchid species (*Dendrobium okinawense* Hatus. et Ida, *Luisia teres* (Thunb.) Blume, and *Thrixspermum japonicum* (Miq.) Rchb.f.) and a tree bark tip were used as environmental samples (Supplementary Table S1). These samples were collected at two locations in Japan over 1,000 km apart. We collected 1–3 roots from each individual and observed hand-sliced sections of them under a microscope to assess the presence of fungal colonization. Then, the mycorrhizal roots were cut into 0.5–2 cm root fragment samples. We produced six bulk root samples, each of which mixed 2–4 fragment samples from 1–3 orchid individuals (Supplementary Table S1). We also sampled the bark surface (1 cm²) of *Rhododendron indicum* (L.) Sweet from near (< 1 cm) the roots of *T. japonicum*.

Fungal DNA was obtained from hyphal coils colonizing the root fragments using the methods of Rammitsu, Yukawa, Yamashita, Isshiki, & Ogura-Tsujita (2020) because the velamen of epiphytic orchids harbors a highly diverse group of ascomycetes (Herrera, Suárez, & Kottke, 2010), which are non-mycorrhizal endophytic fungi. The fragments were washed with TE buffer under a stereomicroscope to remove debris and crushed using tweezers to disperse the hyphal coils into a fresh volume of the same buffer. For each fragment, 100–400 hyphal coils were collected using a micro-pipette, rinsed three times in TE buffer, and triturated in 20 μ L of TE buffer within a 1.5-mL tube using a BioMasher II homogenizer (Nippi, Tokyo, Japan). DNA was extracted from the suspension following the above-described method. The tree bark was homogenized with 3-mm zirconia beads at 3,000 rpm for 2 min twice and then DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The DNA was quantified, diluted to 0.1 ng/ μ L, and mixed according to the protocols used for the mock community.

2.3. PCR amplification and next-generation sequencing

Fungal nuclear ribosomal DNA ITS sequences were amplified using the standard fungal universal primer pair ITS86F/ITS4 (White, Bruns, Lee, & Taylor, 1990; Turenne, Sanche, Hoban, Karlowsky, & Kabani, 1999). Furthermore, we employed a *Tulasnella*-specific primer pair of 5.8S-Tulngs (5'-CATTCGATGAAGACCGTTGC-3') that was slightly modified from 5.8S-Tul (Suárez et al., 2006), and ITS4-Tul2 (5'-TTCTTTTCTCCGCTGAWTA-3'; Oja et al., 2015). To design primers to detect diverse *Tulasnellaceae* taxa associated with epiphytic orchids, we aligned 39 *Tulasnellaceae* sequences obtained from epiphytic orchids in recent studies (Martos et al., 2012; Kartzinel et al., 2013; Riofrio et al., 2013; Xing et al., 2019) or in our fungal collections. A new primer was designed for the conserved 5.8S region using Primer3 software (Koressaar & Remm, 2007; Untergasser et al., 2012) and named 5.8S-Tulngs; it has a one-base deletion from 5.8S-Tul designed by Suárez et al. (2006). Sequencing libraries were produced using a two-step protocol, as suggested by Berry, Mahfoudh, Wagner, and Loy (2011). A first PCR amplification was conducted in a total volume of 25 μ L

containing 1 μ L of sample DNA, 12.5 μ L of 2 $\times$ KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA), and 0.75 μ L of each primer (0.3 μ M). The PCR protocol was 95 °C for 3 min; 35 cycles of denaturing at 98 °C for 20 s, annealing at 57 °C for 15 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 1 min. For annealing, we tested the optimum temperature using both primer pairs in the range 55–60 °C in preliminary experiments, because previous metabarcoding studies annealed fungal DNA from epiphytic orchids at 55 °C (Cevallos et al., 2017) or 60 °C (Cevallos, Declerck, & Suárez, 2018; Izuddin, Srivathsan, Lee, Yam, & Webb, 2019). PCR amplifications succeeded at 55–58.4 °C, although 55 °C produced non-specific amplicons visible as a smear after gel electrophoresis. Therefore, we choose 57 °C, the lowest temperature without smears. Each PCR was run with an appropriate negative control (no template). The PCR products were extracted from 1.5% agarose gels and cleaned using a FastGene Gel/RCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The purified PCR products were used as template for a second PCR to attach index and flow cell sequences to the amplicons. The PCR protocol was 98 °C for 3 min; 10 cycles of 98 °C for 20 s and 72 °C for 15 s; and a final extension at 72 °C for 5 min. After purification, the second PCR products were quantified using a Qubit fluorometer and pooled in equimolar quantities to prepare sequencing libraries. The libraries were paired-end sequenced (150 bp $\times$ 2) using the Illumina iSeq 100 sequencing platform (San Diego, CA, USA).

2.4. Data processing

Due to the PCR product size ranging from 250 to 350 bp, a large number of the pair-end 150 bp reads did not overlap. Furthermore, most of the region included in the forward read was covered by the 5.8S ribosomal region, which is a highly conserved sequence. Therefore, we analyzed only the reverse read, which covered most of the ITS2 region with high sequence divergence.

The extraction of high-quality sequences was performed with the QIIME2 package (Bolyen et al., 2019). All 151 bp reads were trimmed to 81 bp by removing 64 bp and 6 bp from the 3' and 5' ends of the reads, respectively, as the inclusion of these low-quality regions caused the mean quality score to drop below Q30. The DADA2 was used for denoising and the removal of chimeras. The sequences were assigned to OTUs at 97% sequence similarity. The total number of reads for each OTU obtained with ITS86F/ITS4 and 5.8S-Tulngs/ITS4-Tul2 was calculated, and we excluded OTUs with fewer than 100 total reads to remove sequence errors and contaminants. For the remaining OTUs, the taxonomic assignment for each OTU was performed using the UNITE database ver. 8.2 (Abarenkov et al., 2020) with reference sequences of isolates included in a mock community. To ensure the identity of mycorrhizal OTUs, we also compared OTUs against the NCBI nucleotide database using BLAST. In the mock community, the sequences of assigned OTUs had 0–3 base substitutions versus the reference sequences of the isolates, probably due to sequence errors. To attribute these sequences, we performed phylogenetic analyses of the *Ceratobasidiaceae* and *Tulasnellaceae* sequences (Supplementary Fig. S1). OTUs that formed monophyletic clades with the reference sequences with high bootstrap values (81%) were regarded as the same isolates.

2.5. Phylogenetic analysis

We conducted a phylogenetic analysis of the *Tulasnellaceae* using the 5.8S-ITS2 regions of 76 sequences (Supplementary Table S2). Among these, 8 were from isolates within a mock community

and 25 were from the main fungi detected from multiple epiphytic orchid species in previous large-scale studies (Martos et al., 2012; Herrera, Kottke, Molina, Méndez, & Suárez, 2018; Xing et al., 2019). To reveal their phylogenetic placement, sequences of representative *Tulasnellaceae* fungi in Girlanda et al. (2011) and Freitas et al. (2020) were added to the analysis. Two sequences of *Multi-clavula* species were used to represent outgroup taxa, following Cruz, Suárez, Kottke, Piepenbring, and Oberwinkler (2011) and Shefferson, Kull, and Tali (2008).

The sequences were aligned using the MAFFT online server (Madeira et al., 2019). All positions with less than 90% site coverage were eliminated (i.e., < 10% alignment gaps, missing data, and ambiguous bases were allowed at any position). The final dataset comprised 273 bp positions. Phylogenetic analyses were performed using MEGA7 (Kumar, Stecher, & Tamura, 2016). The phylogenetic tree was constructed using the maximum likelihood method based on the Kimura two-parameter model (Kimura, 1980), which was the best substitution model identified by MEGA7. We estimated the relative robustness of branches using the bootstrap method (Felsenstein, Carney, Iacoviello, & Hirsch, 1985) with 1,000 replicates. Furthermore, we counted the number of mismatches against the four primers used for each sequence. The sequences lacking the primer sites were excluded from the mismatch calculation. This alignment was deposited in TreeBASE (<http://www.treebase.org/>) under the accession number S28425.

3. Results

3.1. Mock community

The number of *Tulasnellaceae* isolates included in the mock community was assessed more accurately using the *Tulasnella*-specific primer pair than using the fungal universal primer pair (Fig. 1). All eight *Tulasnellaceae* isolates were detected by 5.8S-Tulngs/ITS4-Tul2, while seven of them were detected by ITS86F/ITS4 (Supplementary Table S3). *Tulasnellaceae* isolates were relatively more abundant with 5.8S-Tulngs/ITS4-Tul2 (99.5%; 159,306 reads) than with ITS86F/ITS4 (0.05%; 124 reads). Both primer pairs detected all *Ceratobasidiaceae* isolates, but the relative abundances were much greater with ITS86F/ITS4 (83.9%; 207,814 reads) than with 5.8S-Tulngs/ITS4-Tul2 (0.37%; 592 reads). The *Serendipitaceae* isolate, SE1, was detected only with ITS86F/ITS4 amplification with 0.14% (334 reads) of all reads. Two OTUs that were not included in the mock community, one each for *Tulasnellaceae* and *Serendipitaceae*, were detected with a total of 278 reads. The sequences of CE18B and CE18C belonging to a single *Ceratobasidiaceae* OTU, CE18, could not be distinguished using either primer pair (Supplementary Table S3), while that of CE18A was identified by its unique substitutions located downstream of the ITS2 sequence.

3.2. Mycorrhizal communities of root and tree bark samples

The *Tulasnella*-specific primer pair allowed for the detection of more *Tulasnellaceae* OTUs than did the fungal universal primer pair (Fig. 2). Eight *Tulasnellaceae* OTUs were detected from seven environmental samples with 5.8S-Tulngs/ITS4-Tul2, while five of these were identified by ITS86F/ITS4 (Supplementary Table S4). *Tulasnellaceae* OTUs were much more abundant with 5.8S-Tulngs/ITS4-Tul2 (94.3%; 896,375 reads) than with ITS86F/ITS4 (0.09%; 1,501 reads; Supplementary Table S4). Four *Ceratobasidiaceae* OTUs were detected using both primer pairs, but the relative abundance of obtained reads was much larger with ITS86F/ITS4 (6.6%; 114,653 reads) than with 5.8S-Tulngs/ITS4-Tul2 (0.04%; 369 reads).

Four *Serendipitaceae* OTUs were detected with ITS86F/ITS4 (34.0% of all reads; 585,188 reads), and two of these were also identified with 5.8S-Tulngs/ITS4-Tul2 (4.7% of all reads; 44,611 reads). For other *Basidiomycota* fungi, the relative abundances with ITS86F/ITS4 or 5.8S-Tulngs/ITS4 were 22.6% (14 OTUs; 388,366 reads) and 0.28% (8 OTUs; 2,616 reads), respectively. *Ascomycota* fungi were detected more frequently with ITS86F/ITS4 (72 OTUs; 26.1% of all reads) than with 5.8S-Tulngs/ITS4-Tul2 (24 OTUs; 0.092% of all reads).

3.3. Phylogenetic analysis and assessment of primer mismatch numbers

Based on the number of mismatches against the primers used, *Tulasnellaceae* sequences including the phylogenetic analysis were assigned to four groups (Fig. 3). Sequences within Group I had 3–4 and 2–3 mismatches with ITS86F and ITS4, respectively, while they perfectly matched with both 5.8S-Tulngs and ITS4-Tul2, except for one sequence (JQ240476). The sequences within Group II had 1–3 and 2–4 mismatches with ITS86F and ITS4, respectively, 0–3 mismatches with 5.8S-Tulngs, and were perfectly matched with ITS4-Tul2, except for one sequence (DQ925497). Group III sequences included 3–5 mismatches against ITS86F, ITS4, and 5.8S-Tulngs, but no mismatches for ITS4-Tul2. Group IV sequences matched completely with ITS86F, except for one sequence (DQ925537), but had 3–4 mismatches with 5.8S-Tulngs. Groups I and III corresponded to monophyletic clades supported by bootstrap values of 99% and 98%, respectively (Fig. 3). Group IV included two monophyletic clades with bootstrap values of 85% and 97%, respectively. Of the eight sequences from isolates within the mock community, seven and one fell into Groups I and II, respectively. The representative *Tulasnellaceae* fungi of epiphytic orchids in previous studies (Martos et al., 2012; Herrera et al., 2018; Xing et al., 2019) were positioned in Groups I (12 sequences), II (10 sequences), and III (3 sequences).

4. Discussion

Our results reveal that the diversity of *Tulasnellaceae* detected using a metabarcoding analysis of the mycorrhizal communities of epiphytic orchids differed widely depending on the primer pairs used. A *Tulasnella*-specific primer pair, 5.8S-Tulngs/ITS4-Tul2, successfully identified all *Tulasnellaceae* isolates within a mock community with high relative abundances, while the fungal universal primer pair ITS86F/ITS4 did not detect one *Tulasnellaceae* isolates, and the abundance was much lower than that for the specific primer pair (Fig. 1; Supplementary Table S3). Conversely, all *Ceratobasidiaceae* isolates were detected with both primer pairs and showed high abundance with ITS86F/ITS4 in the mock community assessment. A *Serendipitaceae* isolate was detected only by ITS86F/ITS4 at low frequency. The identification of mycorrhizal communities from environmental samples showed similar results with that of the mock community (Fig. 2). The primer pair 5.8S-Tulngs/ITS4-Tul2 detected mainly *Tulasnellaceae*, while ITS86F/ITS4 detected *Ceratobasidiaceae* and *Serendipitaceae* well. These results show that using a *Tulasnella*-specific primer pair in combination with a fungal universal primer is essential to accurately assess the diversity of mycorrhizal fungi associated with epiphytic orchids.

Primer bias is caused by sequence mismatches between primers and their target sequences in the *Tulasnellaceae* taxa. Phylogenetic analysis showed that the *Tulasnellaceae* fungi identified from epiphytic orchids mainly fell into Group I or II, which have more than

Fig. 1 – Relative abundance of fungal isolates within a mock community detected using fungal universal (ITS86F/ITS4) and *Tulasnella*-specific (5.8S-Tulngs/ITS4-Tul2) primer pairs. Two fungal isolates, CE18B and CE18C, sharing over 97% sequence similarity could not be distinguished. In total, 247,576 and 160,083 reads were obtained from the ITS86F/ITS4 and 5.8S-Tulngs/ITS4-Tul2 analyses, respectively. Details of the read numbers obtained for each isolate are in Table S3.

three mismatches with the fungal universal primer set ITS86F/ITS4 (Fig. 3). Primer mismatches toward Groups I and II were also observed by Waud et al. (2014) in their assessment of primer biases using a fungal mock community from terrestrial orchids. The primer pairs used by Waud et al. (2014) showed poor amplification of *Tulasnella* subgroup A, defined by Girlanda et al. (2011), which mainly belonged to Groups I and II in our phylogenetic analysis (Fig. 3). Because the dominant *Tulasnellaceae* fungi of epiphytic orchids from Réunion Island (Martos et al., 2012), Ecuador (Herrera et al., 2018), and China (Xing et al., 2019) mostly fell into Groups I and II, using 5.8S-Tulngs/ITS4-Tul2 will be effective in identifying

the *Tulasnellaceae* fungi associated with orchids, especially in epiphytic species. For fungi within Group IV, ITS86F will be a suitable primer for detection, but the reverse primer needs to be evaluated (Fig. 3). A new forward primer will be required for the detection of fungi within Group III due to many mismatches against both ITS86F and 5.8S-Tulngs, but ITS4-Tul2 seems effective as a reverse primer.

Pair-end sequencing of 151 bp may allow us to assess the mycorrhizal diversity of orchids. In fact, the present analysis, which used only 81 out of the 151 bp, identified all OTUs, including those in a mock community (Supplementary Table S3). However, two of the

Fig. 2 – Frequency distribution of rhizoctonia OTUs detected from orchid roots and a bark tip using fungal universal (ITS86F/ITS4) and *Tulasnella*-specific (5.8S-Tulngs/ITS4-Tul2) primer pairs. The number of reads obtained is represented by the color gradient from white to red. Details of the read numbers obtained are in Table S4. Tree bark sample (Bar) was collected from under the *Thrixspermum japonicum* roots (Thj). Six bulk root samples were produced from the roots of three orchid species, *Thrixspermum japonicum* (Thj), *Luisia teres* (Lut) and *Dendrobium okinawense* (Deo). Thj: a mixing DNA from four root fragments from one individual; Lut1 and Lut2: mixtures of three roots from each of the two individuals; Deo: a mixture of two root fragments from a single root; DeoP1 and DeoP2: mixtures of three roots from three individuals from each of the two populations. Details of the origin of bulk samples are in Supplementary Table S1.

three groups belonging to CE18 were indistinguishable from each other in this study; further evaluation of the sequencing conditions, e.g., the number of sequencing cycles for reverse reads, may allow read lengths longer than 81 bp.

5. Conclusion

Our results strongly suggest that it is essential to use *Tulasnella*-specific primer sets in combination with fungal universal primer sets to assess the mycorrhizal communities of orchids through metabarcoding analysis, especially for epiphytic orchids. A commonly used fungal universal primer pair sufficiently detected *Ceratobasidiaceae* and *Serendipitaceae* fungi but failed to detect *Tulasnellaceae* diversity. Our results also suggest that particular *Tulasnellaceae* taxa have strong primer biases toward the primers used. Some *Tulasnellaceae* fungi might have been overlooked in previous metabarcoding analyses of orchid mycorrhizal fungi due to such biases. Our study contributes to further understanding of the diversity of mycorrhizal fungi in epiphytic orchids.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

Acknowledgments

We thank S. Abe, T. Abe, M. Amano, M. Ichikawa, A. Kinoshita, N. Kotaka, M. Kudaka, N. Kudaka, N. Morita, Y. Ohshima for collecting environmental samples; Y. Nagano for DNA analyses and valuable advices; S. Inaba for depositing fungal isolates. We also thank the Handling Editor and two anonymous reviewers for their helpful comments. The DNA sequencing analyses were made using a Genetic Analyzer spectrometer at Analytical Research Center for Experimental Sciences, Saga University. This study was partially supported by TBRC Joint Usage Project Grant 2019-2020.

References

- Abarenkov, K., Zirk, A., Piirmann, T., Pöhönen, R., Ivanov, F., Nilsson, R. H., & Kõljalg, U. (2020). UNITE QIIME release for Fungi. *UNITE Community*, <https://dx.doi.org/10.15156/BIO/786385>
- Berry, D., Mahfoudh, K. B., Wagner, M., & Loy, A. (2011). Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. *Applied and Environmental Microbiology*, 77(21), 7846–7849. <http://dx.doi.org/10.1128/AEM.05220-11>
- Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., ... Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, 37(8), 852–857. <https://doi.org/10.1038/s41587-019-0209-9>

Fig. 3 – Maximum likelihood tree for 5.8S-ITS region of the *Tulasnellaceae* OTUs included in a mock community. The origin of each sequence is indicated by a symbol; sequences with no symbols had unknown origins. The final dataset comprised 273 bp positions. The number of base mismatches against the primers used is indicated by a color gradient from white to red to black. The bar indicates fungal groups created based on the number of base mismatches against the primer pairs used. The sequences obtained using the reverse primer ITS4 were removed from the mismatch calculation. Only bootstrap values greater than 70% are shown. The tree is drawn to scale, with branch lengths reflecting the number of substitutions per site.

- Cevallos, S., Declerck, S., & Suárez, J. P. (2018). *In situ* orchid seedling-trap experiment shows few keystone and many randomly associated mycorrhizal fungal species during early plant colonization. *Frontiers in plant science*, 9, 1664. <https://doi.org/10.3389/fpls.2018.01664>
- Cevallos, S., Sánchez-Rodríguez, A., Decock, C., Declerck, S., & Suárez, J. P. (2017). Are there keystone mycorrhizal fungi associated to tropical epiphytic orchids? *Mycorrhiza*, 27(3), 225–232. <https://doi.org/10.1007/s00572-016-0746-8>
- Cruz, D., Suárez, J. P., Kottke, I., Piepenbring, M., & Oberwinkler, F. (2011). Defining species in *Tulasnella* by correlating morphology and nrDNA ITS-5.8S sequence data of basidiomata from a tropical Andean forest. *Mycological Progress*, 10(2), 229–238. <https://doi.org/10.1007/s11557-010-0692-3>
- Dearnaley, J. D. W., Martos, F., & Selosse, M. A. (2012). Orchid mycorrhizas: Molecular ecology, physiology, evolution and conservation aspects. In: B. Hock (Eds.), *The mycota: a comprehensive treatise on fungi as experimental systems for basic and applied research* (pp. 207–230). Springer.
- Duffy, K. J., Waud, M., Schatz, B., Petanidou, T., & Jacquemyn, H. (2019). Latitudinal variation in mycorrhizal diversity associated with a European orchid. *Journal of Biogeography*, 46(5), 968–980. <https://doi.org/10.1111/jbi.13548>
- Felsenstein, D., Carney, W. P., Iacoviello, V. R., & Hirsch, M. S. (1985). Phenotypic properties of atypical lymphocytes in cytomegalovirus-induced mononucleosis. *Journal of Infectious Diseases*, 152(1), 198–203. <https://doi.org/10.1093/infdis/152.1.198>
- Freitas, E. F. S., da Silva, M., Cruz, E. da S., Mangaravite, E., Bocayuva, M. F., Veloso, T. G. R., Selosse, M. A., & Kasuya, M. C. M. (2020). Diversity of mycorrhizal *Tulasnella* associated with epiphytic and rupicolous orchids from the Brazilian Atlantic Forest, including four new species. *Scientific Reports*, 10(1), 1–14. <https://doi.org/10.1038/s41598-020-63885-w>
- Girlanda, M., Segreto, R., Cafasso, D., Liebel, H. T., Rodda, M., Ercole, E., Cozzolino, S., Gebauer, G., & Perotto, S. (2011). Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. *American Journal of Botany*, 98(7), 1148–1163. <https://doi.org/10.3732/ajb.1000486>
- Herrera, P., Kottke, I., Molina, M. C., Méndez, M., & Suárez, J. P. (2018). Generalism in the interaction of Tulasnellaceae mycobionts with orchids characterizes a biodiversity hotspot in the tropical Andes of Southern Ecuador. *Mycoscience*, 59(1), 38–48. <https://doi.org/10.1016/j.myc.2017.08.003>
- Herrera, P., Suárez, J. P., & Kottke, I. (2010). Orchids keep the ascomycetes outside: A highly diverse group of ascomycetes colonizing the velamen of epiphytic orchids from a tropical mountain rainforest in Southern Ecuador. *Mycology*, 1(4), 262–268. <https://doi.org/10.1080/21501203.2010.526645>
- Herrera, P., Suárez, J. P., Sánchez-Rodríguez, A., Molina, M. C., Prieto, M., & Méndez, M. (2019). Many broadly-shared mycobionts characterize mycorrhizal interactions of two coexisting epiphytic orchids in a high elevation tropical forest. *Fungal Ecology*, 39, 26–36. <https://doi.org/10.1016/j.funeco.2018.11.003>
- Izuddin, M., Srivathsan, A., Lee, A. L., Yam, T. W., & Webb, E. L. (2019). Availability of orchid mycorrhizal fungi on roadside trees in a tropical urban landscape. *Scientific reports*, 9(1), 19528. <https://doi.org/10.1038/s41598-019-56049-y>
- Izumitsu, K., Hatoh, K., Sumita, T., Kitade, Y., Morita, A., Gafur, A., Ohta, A., Kawai, M., Yamanaka, T., Neda, H., Ota, Y., & Tanaka, C. (2012). Rapid and simple preparation of mushroom DNA directly from colonies and fruiting bodies for PCR. *Mycoscience*, 53(5), 396–401. <https://doi.org/10.1007/s10267-012-0182-3>
- Johnson, L. J., González-Chávez, M. D. C. A., Carrillo-González, R., Porras-Alfaro, A., & Mueller, G. M. (2020). *Vanilla* aerial and terrestrial roots host rich communities of orchid mycorrhizal and ectomycorrhizal fungi. *Plants People Planet*. <https://doi.org/10.1002/ppp3.10171>
- Kartzinel, T. R., Trapnell, D. W., & Shefferson, R. P. (2013). Highly diverse and spatially heterogeneous mycorrhizal symbiosis in a rare epiphyte is unrelated to broad biogeographic or environmental features. *Molecular Ecology*, 22(23), 5949–5961. <https://doi.org/10.1111/mec.12536>
- Kaur, J., Phillips, C., & Sharma, J. (2021). Host population size is linked to orchid mycorrhizal fungal communities in roots and soil, which are shaped by micro-environment. *Mycorrhiza*, 31, 17–30. <https://doi.org/10.1007/s00572-020-00993-5>
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16(2), 111–120. <https://doi.org/10.1007/BF01731581>
- Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33(7), 1870–1874. <https://doi.org/10.1093/molbev/msw054>
- Koressaar, T., & Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23(10), 1289–1291. <https://doi.org/10.1093/bioinformatics/btm091>
- Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A. R. N., Potter, S. C., Finn, R. D., & Lopez, R. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*, 47(W1), W636–W641. <https://doi.org/10.1093/nar/gkz268>
- Martos, F., Munoz, F., Pailler, T., Kottke, I., Gonneau, C., & Selosse, M. A. (2012). The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. *Molecular Ecology*, 21(20), 5098–5109. <https://doi.org/10.1111/j.1365-294X.2012.05692.x>
- Ogura-Tsujita, Y., Yokoyama, J., Miyoshi, K., & Yukawa, T. (2012). Shifts in mycorrhizal fungi during the evolution of autotrophy to mycoheterotrophy in *Cymbidium* (Orchidaceae). *American Journal of Botany*, 99(7), 1158–1176. <https://doi.org/10.3732/ajb.1100464>
- Oja, J., Kohout, P., Tedersoo, L., Kull, T., & Kõljalg, U. (2015). Temporal patterns of orchid mycorrhizal fungi in meadows and forests as revealed by 454 pyrosequencing. *New Phytologist*, 205(4), 1608–1618. <https://doi.org/10.1111/nph.13223>
- Rammitsu, K., Abe, S., Abe, T., Kotaka, N., Kudaka, M., Kudaka, N., Kinoshita, A., & Ogura-Tsujita, Y. (2021). The endangered epiphytic orchid *Dendrobium okinawense* has a highly specific mycorrhizal association with a single *Tulasnellaceae* fungus. *Journal of Forest Research*, 26(3), 215–221. <https://doi.org/10.1080/13416979.2021.1876587>
- Rammitsu, K., Yukawa, T., Yamashita, Y., Isshiki, S., & Ogura-Tsujita, Y. (2020). The mycorrhizal community of the epiphytic orchid *Thrixspermum japonicum* is strongly biased toward a single Ceratobasidiaceae fungus, despite a wide range of fungal partners. *American Journal of Botany* 107(12), 1654–1662. <https://doi.org/10.1002/ajb2.1575>
- Riofrío, M. L., Cruz, D., Torres, E., de la Cruz, M., Iriondo, J. M., & Suárez, J. P. (2013). Mycorrhizal preferences and fine spatial structure of the epiphytic orchid *Epidendrum rhopalostele*. *American Journal of Botany*, 100(12), 2339–2348. <https://doi.org/10.3732/ajb.1300069>
- Shefferson, R. P., Kull, T., & Tali, K. (2008). Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. *American Journal of Botany*, 95(2), 156–164. <https://doi.org/10.3732/ajb.95.2.156>
- Suárez, J. P., Weiß, M., Abele, A., Garnica, S., Oberwinkler, F., & Kottke, I. (2006). Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycological Research*, 110(11), 1257–1270. <https://doi.org/10.1016/j.mycres.2006.08.004>
- Taylor, D. L., & McCormick, M. K. (2008). Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytologist*, 177(4), 1020–1033. <https://doi.org/10.1111/j.1469-8137.2007.02320.x>
- Tedersoo, L., Nilsson, R. H., Abarenkov, K., Jairus, T., Sadam, A., Saar, I., Bahram, M., Bechem, E., Chuyong, G., & Kõljalg, U. (2010). 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. *New Phytologist*, 188(1), 291–301. <https://doi.org/10.1111/j.1469-8137.2010.03373.x>
- Turenne, C. Y., Sanche, S. E., Hoban, D. J., Karlowsky, J. A., & Kabani, A. M. (1999). Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *Journal of Clinical Microbiology*, 37(6), 1846–1851. <https://doi.org/10.1128/JCM.37.6.1846-1851.1999>
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3 – new capabilities and interfaces. *Nucleic Acids Research* 40(15), e115. <https://doi.org/10.1093/nar/gks596>
- Waud, M., Brys, R., Van Landuyt, W., Lievens, B., & Jacquemyn, H. (2017). Mycorrhizal specificity does not limit the distribution of an endangered orchid species. *Molecular Ecology*, 26(6), 1687–1701. <https://doi.org/10.1111/mec.14014>
- Waud, M., Busschaert, P., Ruyters, S., Jacquemyn, H., & Lievens, B. (2014). Impact of primer choice on characterization of orchid mycorrhizal communities using 454 pyrosequencing. *Molecular Ecology Resources*, 14(4), 679–699. <https://doi.org/10.1111/1755-0998.12229>
- White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols: a guide to methods and applications* (pp. 315–322). Academic Press.
- Xing, X., Jacquemyn, H., Gai, X., Gao, Y., Liu, Q., Zhao, Z., & Guo, S. (2019). The impact of life form on the architecture of orchid mycorrhizal networks in tropical forest. *Oikos*, 128(9), 1254–1264. <https://doi.org/10.1111/oik.06363>
- Xing, X., Liu, Q., Gao, Y., Shao, S., Guo, L., Jacquemyn, H., Zhao, Z., & Guo, S. (2020). The Architecture of the network of orchid–fungus interactions in nine co-occurring *Dendrobium* species. *Frontiers in Ecology and Evolution*, 8, 130. <https://doi.org/10.3389/fevo.2020.00130>
- Yukawa, T., Ogura-Tsujita, Y., Shefferson, R. P., & Yokoyama, J. (2009). Mycorrhizal diversity in *Apostasia* (Orchidaceae) indicates the origin and evolution of orchid mycorrhiza. *American Journal of Botany*, 96(11), 1997–2009. <https://doi.org/10.3732/ajb.0900101>
- Zotz, G. (2013). The systematic distribution of vascular epiphytes—a critical update. *Botanical Journal of the Linnean Society*, 171(3), 453–481. <https://doi.org/10.1111/boj.12010>