

Molecular Identification of Mycorrhizae of *Cymbidium kanran* (Orchidaceae) on Jeju Island, Korea

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Abstract A fungal internal transcribed spacer region was used to identify the mycorrhizae of *Cymbidium kanran*. The family Russulaceae was found to be the most frequently occurring group in both root and soil samples. In phylogenetic analyses, the majority of the Russulaceae clones were clustered with *Russula brevipes* and *R. cyanoxantha*. Therefore, *C. kanran* may form symbiotic relationships with the genus *Russula*.

Keywords *Cymbidium kanran*, ITS sequencing, Orchid mycorrhizae, *Russula*

Orchidaceae is comprised of five subfamilies and ~870 genera with approximately 20,000 to 35,000 species [1]. Orchids are found in almost every conceivable environment, including arctic tundra, deserts, and rainforests [2, 3]. The orchid family represents the most evolved group among flowering plants and is known to be niche-specific and to require optimal conditions in its natural habitats [4]. Therefore, Orchidaceae may be locally abundant but can occur only in a limited number of locations. Recently, certain wild orchid species became critically endangered due to forest destruction, global climate change, and overcollection for food, traditional medicine, and horticultural trade.

Cymbidium kanran is among these endangered species and is facing extinction. This species is exclusively distributed in a few neighboring countries in Northeast Asia (China, Japan, Korea, and Taiwan). Jeju Island is the only place in

Korea where a small number of wild populations still exist. *C. kanran* has long been known for its attractive color, shape, and the scent of its flowers. Accordingly, illegal poaching has been a constant problem on Jeju Island and is the main reason for the dwindling numbers of these plants in the wild. Habitat destruction for the creation of tangerine farms and pasture lands is also responsible for the decline in the orchid population [5]. For these reasons, a natural habitat of *C. kanran* in Sanghyo-dong, Jeju was designated as a Korean National Monument (No. 432) in 2002 as a conservation strategy.

Outreach seems to be the most successful way to conserve the wild-orchid habitat. Nevertheless, conservation through reserves alone is unlikely to provide adequate protection to endangered orchid species [6]. Reintroduction can be defined as an attempt to restore a species population in areas that it formerly inhabited. Reintroduction is considered an effective but challenging *ex situ* conservation method. It usually involves seed collection, germination, the establishment of seedlings and sustainable populations, and reintroduction of the new plants into the wild. Because most terrestrial orchid seeds have an obligate relationship with mycorrhizal symbionts, seed recruitment is highly dependent on mycorrhizae [7-9]. Orchid mycorrhizae perform pivotal functions during germination because orchid seeds contain almost no energy reserves and even some photosynthetic adult orchids continue to obtain carbon from their mycorrhizal fungi [10, 11]. Hence, assessment of fungal populations that may contribute to the symbiosis in the orchid root system is important for successful orchid seedling recruitment.

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Recently developed molecular taxonomic techniques have been successfully utilized for studies on the identity and diversity of the fungal partners of epiphytic orchids [12–14]. PCR-based approaches allow for direct identification of the fungi most frequently associated with orchid roots; thus, the severe biases of traditional culture-dependent methods can be avoided. On the other hand, the specificity of orchids for their mycorrhizal partners and the effects of the fungi on orchid growth are still unclear.

The aim of this study was to identify the mycorrhizal fungi most frequently occurring in the roots and surrounding soil of *C. kanran*. The information gained from this study may shed light on the factors affecting germination, growth, and distribution of *C. kanran* on Jeju Island.

Sample collection and DNA extraction. Root and soil samples were collected at three sites in the natural habitat of *C. kanran* (33°17' N, 126°35' E; Sanghyo-dong, Seogwipo, Jeju-do, Korea) in the spring of 2013. The root and rhizosphere samples were carefully separated and pulverized in liquid nitrogen with a sterile mortar and pestle. Total community DNA was extracted with a Tris-HCl DNA extraction buffer consisting of 0.1 M Tris-HCl (pH 9.0–9.5), 10 mM EDTA (pH 8.0), and 1 M KCl. Sterile glass beads (Glass Bead 3; Glastechnique Mfg., Gräfenroda, Germany) were added and the mixture was vortexed to facilitate the extraction. Crude DNA was further purified with a Wizard DNA Clean-Up System (Promega, Madison, WI, USA) to remove PCR inhibitors such as humic acids.

Fungal community analysis. The internal transcribed spacer (ITS) region containing two ITSs and the 5.8S rRNA gene (ITS1–5.8S–ITS2) was amplified with the primers ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by Gleason *et al.* [15]. The PCR products were purified with the LaboPass PCR Purification Kit (Cosmo Genetech, Seoul, Korea). The purified amplicons were ligated into the

pGEM Easy vector (Promega, Madison, WI, USA) and used to transform *Escherichia coli* DH5 α cells. Thirty eligible colonies from each sample were randomly selected, and the recombinant plasmids were extracted with the Dokdo Plasmid Mini-Prep Kit (Elpis-Biotech, Daejeon, Korea). The identity of the plasmids containing the correct inserts was verified by agarose gel electrophoresis and sequencing at Macrogen (Seoul, Korea).

Phylogenetic analysis of the ITS inserts. The sequences obtained from the clone library were compared to ITS gene sequences in the National Center for Biotechnology Information (NCBI) GenBank database using the BLASTN algorithm [16], and closely related sequences were downloaded. The clone sequences and closely related sequences were manually imported into and aligned in the Molecular Evolutionary Genetics Analysis (MEGA) software, ver. 6.0 [17], with the ClustalW tool. The best-fit nucleotide substitution model (K2 + G) was selected by means of MEGA ver. 6.0 on the basis of the Bayesian information criterion. This model was used to build a maximum likelihood (ML) phylogenetic tree with 1,000 bootstrap replicates [18]. *Albatrellus ellisii* JLF1838 (JX415333) was used as an outgroup. The DNA sequences obtained in this study were submitted to the NCBI database (accession Nos. KU141146–KU141325) (Supplementary Tables 1 and 2).

The ITS inserts were amplified from the DNA of three root and three soil samples with the fungi-specific primer pair, ITS1f and ITS4. Then, a clone library was constructed, and 180 clones were sequenced. Taxonomic assignment was performed by means of top-scoring matches from BLAST searches covering at least 90% of the query sequence length and matching the target sequence with a minimal identity of 90%, 85%, and 75% for a species, genus, and family, respectively [19]. ITS amplicons with insufficient coverage or identity were designated as unclassified fungal clones. The occurrence of mycorrhizal fungi in the root and soil samples on the basis of family is summarized in

Table 1. The frequency (%) of the fungal taxa identified in each sample type

Root			Rhizosphere		
Family	Occurrences	%	Family	Occurrences	%
Aspergillaceae	2	2.2	Aspergillaceae	2	2.2
Clavicipitaceae	2	2.2	Clavicipitaceae	2	2.2
Cystofilobasidiaceae	1	1.1	Hypocreaceae	1	1.1
Hyaloscyphaceae	3	3.3	Inocybaceae	2	2.2
Inocybaceae	1	1.1	Lasiosphaeriaceae	1	1.1
Lyophyllaceae	1	1.1	Mortierellaceae	2	2.2
Mortierellaceae	14	15.6	Myxotrichaceae	1	1.1
Ophiostomataceae	1	1.1	Pseudeurotiaceae	1	1.1
Russulaceae	52	57.8	Russulaceae	51	56.7
Sebacinaceae	1	1.1	Sebacinaceae	8	8.9
Thelephoraceae	2	2.2	Tremellaceae	1	1.1
Unclassified	10	11.1	Unclassified	18	20.0
Total	90	100.0	Total	90	100.0

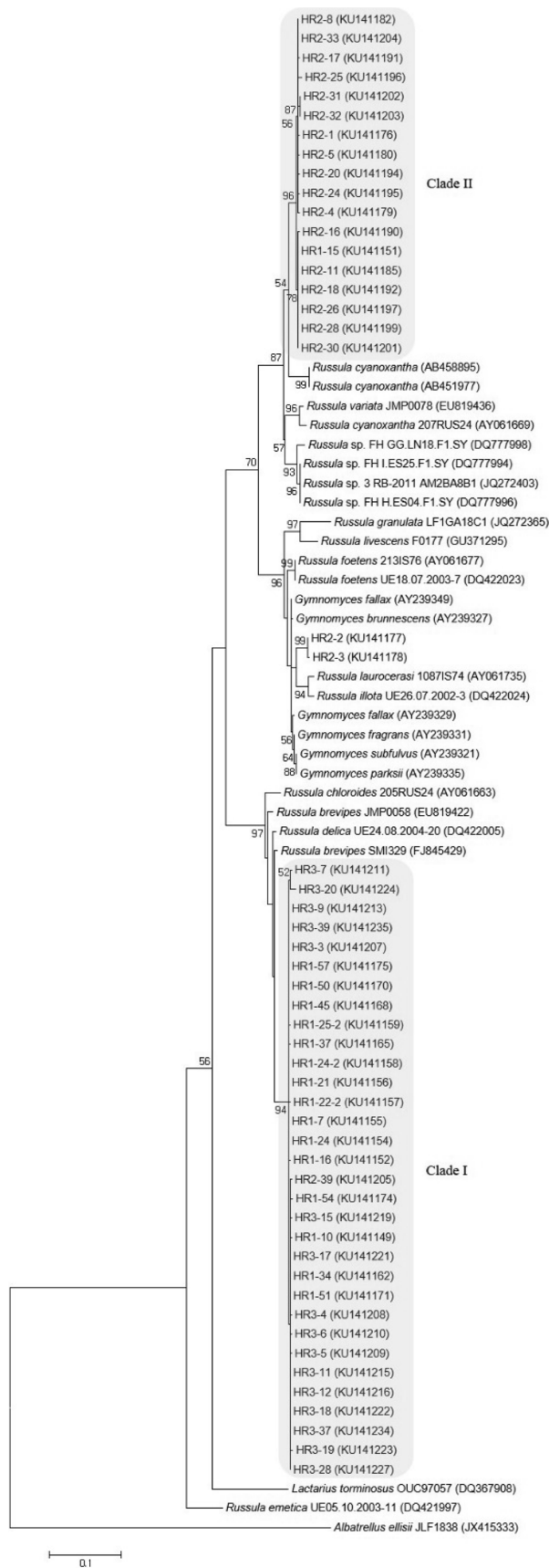


Fig. 1. Phylogenetic relationship of the root clones of Russulaceae and *Russula brevipes* (Clade I) and *R. cyanoxantha* (Clade II) inferred from the internal transcribed spacer sequence data. The scale bar represents a 1% difference in nucleotide sequences.

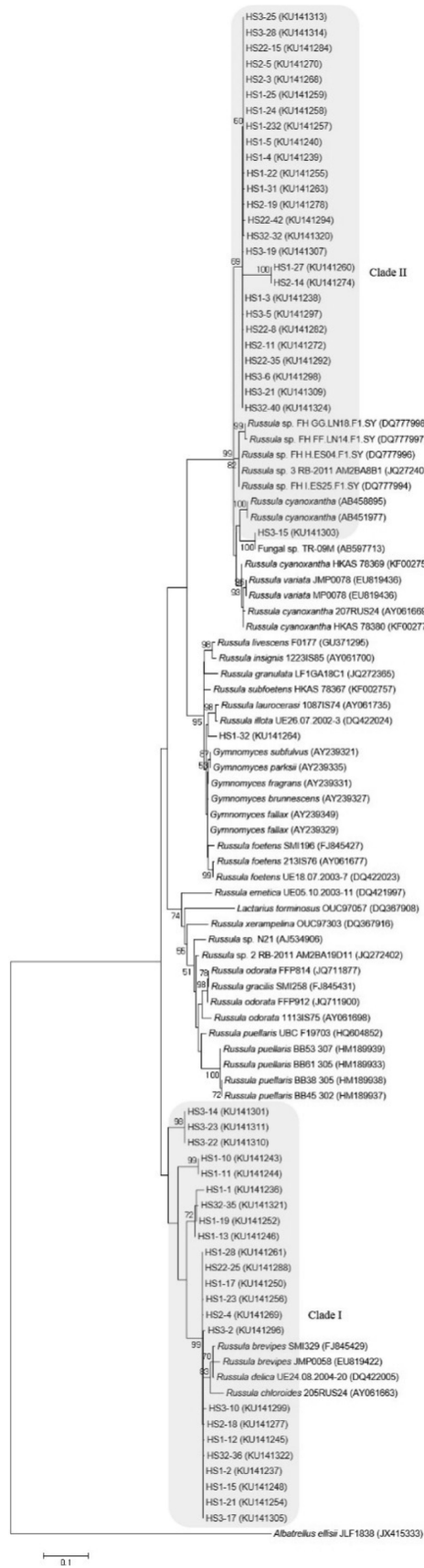


Fig. 2. Phylogenetic relationship of the soil clones of Russulaceae and *Russula brevipes* (Clade I) and *R. cyanoxantha* (Clade II) inferred from the internal transcribed spacer sequence data. The scale bar represents a 1% difference in nucleotide sequences.

Table 1. The family Russulaceae was the predominant group in both the root samples (57.8%) and soil samples (56.7%). The second largest group in the roots and soil was shared by Mortierellaceae (15.6%) and Sebacinaceae (8.9%). Twenty-eight ITS sequences (15.6%) from our samples corresponded to unidentified fungal clones.

A number of studies have shown that members of Russulaceae are among the most frequent symbionts for a wide range of achlorophyllous orchid species [20–24]. In contrast, Ogura-Tsujita *et al.* [25] stated that mycoheterotrophic *Cymbidium* species (*C. macrorhizon* and *C. aberrans*) are highly dependent on Sebacinaceae, whereas Russulaceae species are more abundantly found near the root system of chlorophyllous mixotrophic *Cymbidium* species (*C. goeringii* and *C. lancifolium*). In addition, they reported that a chlorophyllous autotrophic *Cymbidium* species (*C. dayanum*) is predominantly dependent on Tulasnellaceae. Therefore, Russulaceae can be considered the most frequent fungal partner of both mixotrophic and mycoheterotrophic *Cymbidium* species.

Our ML analyses revealed that the majority of the Russulaceae clones were clustered with either *Russula brevipes* or *R. cyanoxantha*, indicating that they are the most commonly occurring species in the root and rhizosphere of *C. kanran* (Figs. 1 and 2). Even though the limited sample size does not allow us to draw definitive conclusions, we can hypothesize that the genus *Russula* establishes symbiotic relationships or specific associations with *C. kanran*.

The genus *Russula* represents approximately 750 species of mycorrhizal gilled mushrooms worldwide. Previous studies have reported that some of the *Rhizoctonia* isolates, isolated from the roots of Korean indigenous *C. goeringii* and inoculated into a *C. kanran* hybrid, successfully promoted the growth of the orchid hybrids under nursery conditions [26, 27]. To date, no attempt has been made to test *Russula* spp. for their symbiotic relationships with *C. kanran* probably because axenic cultivation of Russulaceae spp. is difficult and at times impossible [20, 22, 28]. Isolation and characterization of Russulaceae and the other frequently occurring families, Mortierellaceae and Sebacinaceae, from the natural habitat of *C. kanran* using enhanced culture methods would be warranted in future studies to elucidate the nature and specificity of *C. kanran*-fungus associations. We hope that our results will contribute to the conservation efforts toward the endangered orchid species.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including two tables can be found with this article online <http://www.mycobiology.or.kr/src/sm/mb-43-475-s001.pdf>.

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