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Interactions of Newly Isolated Orchid Mycorrhizal Fungi with Korean *Cymbidium kanran* Hybrid 'Chungsu'

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Two fungal isolates obtained from roots of *Cymbidium goeriingii* in Jeju island were confirmed to be symbiotic with orchid plantlets, and were compared with other orchid mycorrhizal (OM) fungi previously isolated. The two isolates differed in their peloton structures formed in the roots of *Cymbidium kanran* hybrid 'Chungsu' and in responses of orchid plant. These two isolates differed from the additionally tested OM fungi in some features, and from root damaging species of *Rhizoctonia* and *Fusarium* as based on cluster analysis after PCR-RAPD with the primers, Bioneer-28 and OPO-2. With this simple and fast technique, it was possible to distinguish OM fungi from the plant root pathogenic fungi based on calculation of their polymorphic bands. This technique can therefore be helpful to distinguish the OM fungi from the root pathogens. Particularly, the new isolates are considered as new resource of symbiotic fungi for horticultural industries.

KEYWORDS: Mycorrhizae, Orchid, PCR-RAPD, Peloton, *Rhizoctonia*

Mycorrhizae are reported as symbiotic associations between plant roots and fungi. The ectomycorrhizal (EM) and arbuscular mycorrhizal (AM) fungi provide nutrients to plants and stimulate plant growth under certain environmental stress or unfavorable conditions (Harley and Smith, 1983). However, orchid mycorrhizal (OM) symbiosis is little known in this respect, even though their fungus, *Rhizoctonia*, was reported to be symbiotic with orchid roots and possibly play an important role in growth and survival of orchids in nature (Arditti, 1992). Techniques for inoculation of EM fungi onto plant roots have been intensively investigated during the last two decades, e.g. for *Tuber* (Hall *et al.*, 1994) or *Tricholoma matsutake* production (Lee and Hong, 1998). But inoculation of orchids with their symbiotic fungi is not frequently applied (Lee and You, 2000).

Several species of *Cymbidium* occurring in Eastern North Asia, e.g., *Cymbidium goeringii* and *C. kanran*, are important in oriental floricultural industries. Much attention has been paid to tissue cultures of orchids regarding cultivation and seed germination for breeding. However, these could not properly performed without any OM fungi (Lee *et al.*, 2001; Warcup, 1981; Peterson and Currah, 1990). The mortality of orchid plantlets was 60~90% a year under greenhouse conditions in Korea after they were transplanted to soil substrate. OM fungi are known to stimulate growth of orchid plantlets under such conditions (Harley and Smith, 1983; Arditti, 1992), providing the plants with nitrogen and stimulating growth of roots (Lee *et al.*, 1998b). Further, OM fungi were found to protect the tissue-originated plantlets from root pathogenic fungi (soft rots), and resulted in growth stimulation of *Cymbidium* hybrids (Lee *et al.*, 2001). Recently, the inoculation of OM fungal isolates to *Cymbidium* hybrids was reported to increase survival rates, and they protected plants from root rots or soil pathogens (Lee *et al.*, 2000, 2001). However, different responses of orchid plants were observed after inoculation with various OM isolates collected from different areas in Korea. Therefore studies on OM fungi of Korean native orchids are considered to be important for their cultivation and breeding in commercial industries.

Tulasnella repens was isolated from the roots of *C. goeringii* collected from SeoSan Island in Korea and revealed to be symbiotic with several commercial orchid species (Lee *et al.*, 1997a). A second OM fungus isolated from roots of *C. goeriingii* collected from Southern area, was an unknown species of *Tulasnella* and showed different responses with various Korean orchid plantlets (Lee and You, 2000; Lee *et al.*, 1997a, 1998b). This isolate was found to be symbiotic with the roots of *Cymbidium* hybrids and to induce pelotons in the cortical cell causing subtle responses of the orchid, which were different from those of OM fungi previously isolated. One strain of OM fungi isolated from Jeju Island, differed from those of inland orchid plantlets. The objectives of this study were to screen and select of orchid mycorrhizal isolates that contribute to be better plant growth and survival and to be

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establish new and reliable RAPD analysis method to identify and distinguish known orchid mycorrhizal isolates.

Materials and Methods

Fungal isolates. Roots of *Cymbidium goeriingii*, native in the south western coast of Korea, were collected, washed with tap water, sealed in polyethylene bags, and stored at 4 until usage. Pure cultures of fungi were made, and accompanied by microscopic observations with the methods as outlined by Currah *et al.* (1989) and Lee (1991). The roots, sliced and sterilized with methyl alcohol or hypochlorite, were placed on water agar or GS agar (for details, Lee *et al.*, 1997a, 1998a, b) and incubated at room temperature. Mycelia were appeared around the root pieces one to two weeks after inoculation. They were transferred to Potato Dextrose agar (PDA, Difco), and sub-cultured to new PDA every month, and were controlled by light microscopy (Lee *et al.*, 1997a, 1998a). Two of the obtained fungal isolates, P-22 to P-23 in Table 1 were compared with previously established OM isolates P-01 to P-12 (Lee and You, 2000), and confirmed as symbiotic endophytes of orchid roots. Some strains of an anastomosis group (AG) of *Rhizoctonia solani* (Sneh *et al.*, 1991; Singleton *et al.*, 1992) were kindly provided by Kim (1993; RDA, Suwon) and *Fusarium oxysporum* was provided by Choi *et al.* (1997; Dr. Lee, Y. S. Kangwon National University). Their genomic DNA was compared to that of the other isolates of the present study.

Synthesis of mycorrhizae. Plantlets of Chungsu, a *Cymbidium kanran* hybrid (Korean 'Jeju' × Japanese 'Paek Myo') having two or three bulbs per plant were uniformly transplanted into glass bottles as used for tissue cultures (diameter 10 × height 15 cm) and were sealed with plastic caps. The mycelia grown on PDA were transferred into bottles containing 2% Oatmeal agar (OA) and were incubated for a week until they covered the surface (usually a gray-white layer on OA; Lee *et al.*, 1998b, 1997b). Five to six plantlets were raised from the rhizome grown in Hyponex (Kano, 1965) and planted on the OA agar previously inoculated with the OM fungus. Hence the plantlets became inoculated in the bottles under sterile conditions. They were cultivated to measure eight weeks under light of 15 μmole $s^{-1}m^{-2}$. The plantlets were harvested for growth parameters (shoots and roots; Lee *et al.*, 1997a, b, 1998a, b), and were afterwards cleaned with tap water for planting into soil. In addition, cleaned roots were stained with trypan blue as for arbuscular mycorrhizal fungi (Eom *et al.*, 1994; Koske and Gemma, 1989) for observations of pelotons, which is the mycelial mass only found in the cortical cells of roots during symbiotic relationship (Currah *et al.*, 1989).

PCR. Total genomic DNA was extracted from the mycelia according to the procedure described by Gosselin *et al.* (1995). Nucleic acids were precipitated in isopropanol, re-suspended in STE (1.5 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5) and re-precipitated with 2 volumes of ethanol. The genomic DNA was repeatedly purified with CTAB extracting buffer until a single band was obtained on the gel. The total genomic DNA extracted was amplified by PCR using the random ten-mer primers Bioneer-28 (5' GGGC CCG TTG '3; http://www.bioneer.com) and OPO-2 (5' CCC GCC GTTG '3) and conditions listed by Lee and You (2000). The reaction mixture was kept at 94°C for 5 min, then subjected to 40 cycles each of 94°C for 1 min, 37°C for 1 min, and 72°C for 1 min, and then stored at 4°C. A sample in each amplification was electrophoresed in 1.4% agarose gel (TAE buffer) at 100 volts (Lee and You, 2000).

Dendrograms. The gels with the PCR-RAPD bands were photographed by Kodak digital Camera (DC 120 Zoom series) and transferred into matrix data by Imager III™. Bands in the agarose plates were recorded as present (1) or absent (0). The polymorphic DNA fragments obtained with the two primers were scored and a data matrix was constructed. Cluster analysis was carried out by SPSS version 9.0 and a dendrogram was constructed

Table 1. The isolates from orchid mycorrhizas collected from roots of *Cymbidium* species in Korea

Isolates	Fungal species	Characteristics of hyphae[a]			Collection site of the orchid (species name)
		Colony type	GR	HT	
P-22	Isolated in this work	Submerged, brown	5~6	2.0~3.0	Mt. Halla, Jeju (*C. goeringii*)
P-23	Isolated in this work	Submerged, white cream	4	2.5~2.8	Mt. Halla, Jeju (*C. goeringii*)
P-01	Tulasnella repens	Submerged, white cream	4	2.5~4.0	Seosan, Chungnam (*C. goeringii*)
P-02	Ceratobasidum cornigerum	Aerial hyphae, brown	20~25	6~7	Muju, Junbuk (*C. sinense*)
P-07	*Tulasnella* sp.	Submerged, white cream	4	3.0	Haenam, Junnam (*C. goeringii*)
P-08	*Tulasnella* sp.	Submerged, white cream	4	3.0	Kumsansa, Junpuk (*C. goeriingii*)
P-10	Unknown	Aerial hyphae, white	12	7.0	Namwon, Junpuk (*C. goeriingii*)
P-12	Tulasnella repens	Submerged, white cream	4	2.5~3.0	Jeongup, Junpuk (*C. goeringii*)

[a]The mycelia of each isolate grown on PD agar (all isolates having two nuclei per cell). GR: Growth rates (mm/day), HT: thickness of hyphae (μm) measured on PD agar grown at 25°C.

using squared Euclidean distance and Between-groups linkage methods.

Results

Isolates. The mycelial growth on PDA or OA agar was observed and measured (Table 1). For the *Rhizoctonia* strains, typical features were observed; monilioid hyphae, two nuclei per cell and simple septa. Isolate P-22 was similar to P-02 in its brown colony, but differed in its hyphal thickness and growth rate. Isolate of P-23 was similar to P-01 and P-12 in all studied features. Isolate P-10, collected from NamWon (Southern West area in Korea), was similar to P-02, in hyphal thickness, but differed in their colony type and growth rate. Monilioid cells were frequently observed in slide preparations of P-01 and P-23, but not in P-22. Isolate of P-23 showed many monilioid 10~12 μm wide hyphae in PDA and OA agar, similar in length to or longer than those of P-01. Therefore, the isolate pairs P-22 and P-02 as well as P23 and P10, are not regarded as related, although they posses the same colony color on PD agar. In conclusion, isolate P-23 is very similar to P-01 and P-12. Therefore it is possibly a *Tulasnella repens* isolate. Strain P-22, however, differs from strain P-23 and from all previously isolated cultures in many respects. Therefore, this strain is a different fungus.

Synthesis of mycorrhizae. Orchid plantlets of *C. kanran* 'Chungsu' hybrid and *Phalaenopsis aphrodite* inoculated with isolates P-22 or P-23 grew well during eight weeks, as compared to those inoculated with the remaining isolates. Hybrid 'Chungsu' of *C. kanran* inoculated with P-22 and P-23 produced higher fresh weight than those inoculated with isolates P-07, P-08, P-10 and were similar in weight to those inoculated with P-01 and P-12 (Table 2). Isolates P-22 and P-23 increased not only fresh weight but also the amount of new roots and root length. This effect is again similar to the results obtained with P-01 and P-12. Isolates P-07 and P-10 did not stimulate growth in comparison to the control. The orchid plantlets inoculated with the isolate P-02 grew worse and half of them were finally dead within eight weeks. Therefore, these data were from the statistic evaluation. Fresh weight of the plants was correlated with plant height and with the values of leaf length multiplied by width and number of leaves (Table 1). These parameters were also correlated with these values of the root parameters. Isolates P-01/P-22/P-23 stimulated root production and inhibited degeneration of roots (dead roots). Isolates P-22 and P23 collected from the sub-tropic area of Halla Mountains, differed in agar culture, but were similar to another with respect to plant performance.

Peloton. The pelotons formed in the cortical cells of *C. kanran* hybrid Jeju were not earlier observed than after eight weeks in this hybrid of cultivation. All symbiotic isolates formed many pelotons within eight weeks, but pelotons were not formed in root cells of *C. goeringii* within this time (data not shown). Pelotons were stained deeply in blue within *C. kanran* roots inoculated with P-01, P-12 and P-23 (A, B, E, F, J-L in Fig. 1, respectively). Loosely coiled hyphae were found in roots inoculated with isolate P-01 and P-23 (Fig. 1B and L). The digested pelotons of P-07 and P-10 were stained in pale blue (Fig. 1C and D). The pelotons formed with P-22 were different from those of the other isolates and consisted of thick hyphae (Fig. 1G-I). They were composed of several hyphal aggregations and stained in pale blue (Fig. 1I). The hyphal thickness in the pelotons was similar to that of on agar cultures.

PCR-RAPD. More than ten primers were employed during this work, but only the data from two primers were used for cluster analysis. The bands obtained with primer Bioneer-28, with lengths of 2100 to 107 base pairs (bps), and those with primer OPO-2 (2016 to 257 bp bands) were selected for the analysis of the seven OM isolates (Fig. 2). Four groups were obtained from cluster analysis (Fig. 3). Group 1 comprised the *Fusarium* species (F-80,

Table 2. Average growth of *Cymbidium kanran* hybrid 'Chungsu' cultivated with symbiotic fungi for eight weeks

Fungal isolates	Fresh weight (g)	Plant height (cm)	Leaves				Roots		
			Length (cm)	Width (mm)	Numbers	Total numbers	New	Dead	Length (cm)
Control[b]	1.23a	18.14a	14.3a	5.4a	4.2b	5.4ab	1.5c	1.5b	3.6a
P-22	2.06d	22.66d	17.1b	7.9c	3.8ab	6.2abc	2.3de	0.1a	5.8b
P-23	1.81cd	21.92c	16.8b	7.4bc	3.2a	6.1abc	2.8ef	0a	5.3b
P-01	1.99d	22.30d	17.2b	7.2b	4.1b	6.4abc	2.8ef	0a	5.1b
P-07	1.17a	21.30bcd	17.5b	7.4bc	3.3a	4.6a	0a	1.8bc	3.6a
P-08	1.59bc	19.88ab	17.7b	7.1b	3.8ab	5.9abc	1.7cd	0.3a	3.6a
P-10	1.36ab	20.31bcd	16.9b	6.8b	4.1b	5.4ab	0.8b	0.3a	3.1a
P-12	1.97d	18.17a	13.5a	5.3a	6.1c	7.2c	3.1f	2.3c	5.0b

[a]The different letters behind the average values indicate significance level of P<0.05 (Duncans multiple range test; ten replicates).
[b]Orchid plantlets not inoculated with OM fungi.

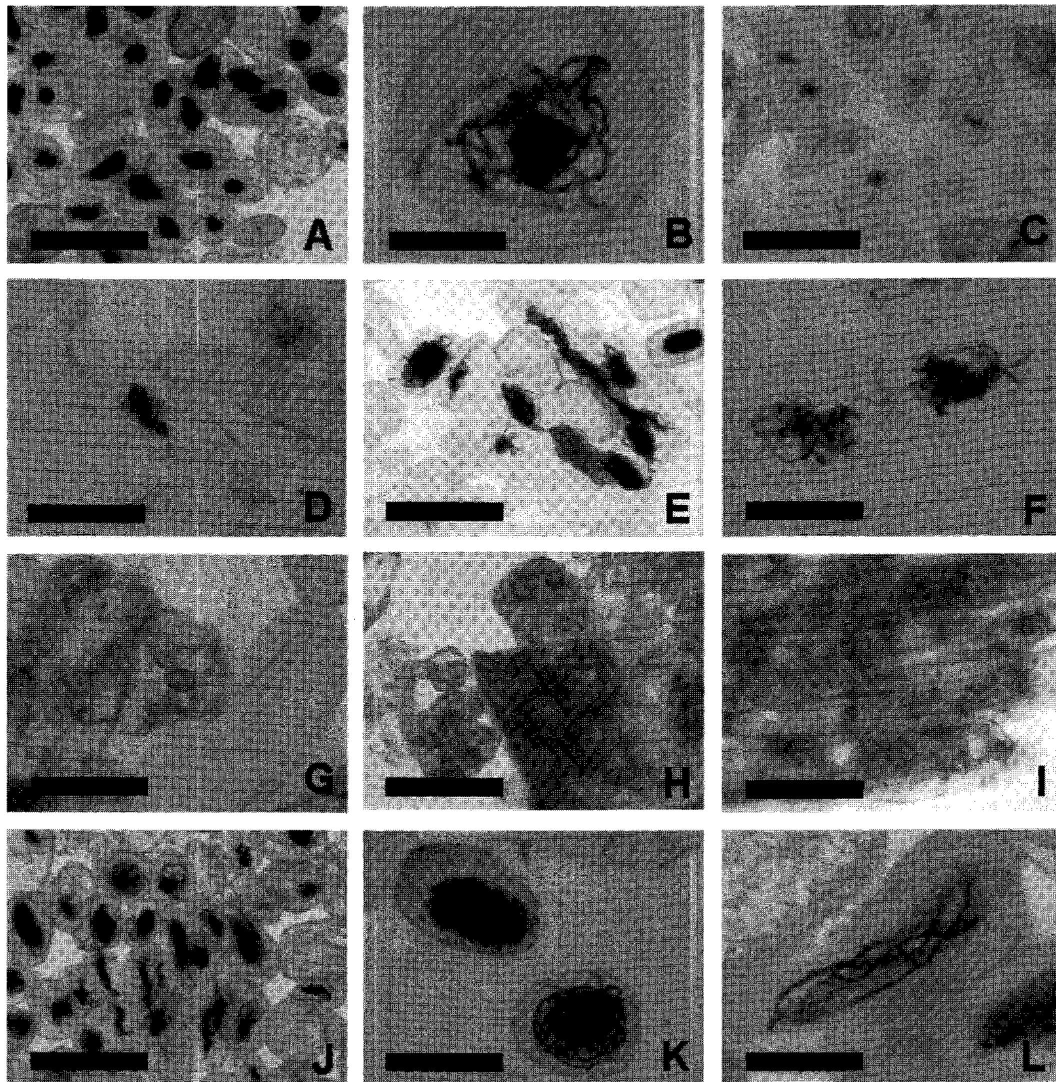

Fig. 1. Various types of pelotons and hyphae in the cortical cells of the roots of *Cymbidium kanran* hybrid 'Chungsu' inoculated with isolate P-01 (A and B, in old roots), isolate P-07 (C in old roots), isolate P-10 (D in old roots), isolate P-12 (E and F in old roots), isolate P-22 (G, H and I in old roots), and isolate P-23 (J, K, and L in old roots). Bar in Fig. A, C, E, and J equals 100 μm; bar in Fig. D, F, and K equals 50 μm; bar in Fig. B, G, H, I, and L equals 25 μm.

F-214, F-354, and F-358), the second group *Rhizoctonia solani* races (Rs-1, Rs-2 and Rs-3) and two OM fungi (P-02 and P-10). The third group formed isolates P-22 and P-23, collected from the islands of Jeju (sub-tropic areas), whereas the OM strains P-07, P-08, P-01, P-02 clustered together. The isolates P-22 and P-23 were closely related to each other, but not closely related to previously identified ones (group 4). The isolates P-22 and P-23 were clearly separated from the groups of the pathogenic fungi (group 1 and 2).

Discussion

Two species of economically important orchids, *C. goeringii* and *C. kanran*, grow in Jeju, whereas only *C. goeringii* is known to occur in areas near the western or south coast of Korean peninsula (Lee, 1985). The OM fungal isolate P22 and P23 collected from Jeju island are, according to the present study, different from those of the Penninsula. The two isolate groups together with *Tulasnella* species and are completely different from the tested plant root pathogens, *Rhizoctonia* and *Fusarium*, at least as far as the RAPD results (Fig. 3). Isolate P-22 differs from isolates of *T. repens* (P-01, P-12) and from P-23 in peloton structure (Fig. 1). The symbiotic relations parallel the results of the dendrogram analysis in Fig. 3. Isolate P-22 is different from the previously isolated OM fungi and can be considered as an important new resource for orchid cultivations. Isolate P-23, however, is similar to already applied strains of *T. repens*.

In crop cultivation, root diseases fungi such as *Fusarium* (Singleton *et al.*, 1992) and *Rhizoctonia* (Sneh *et al.*,

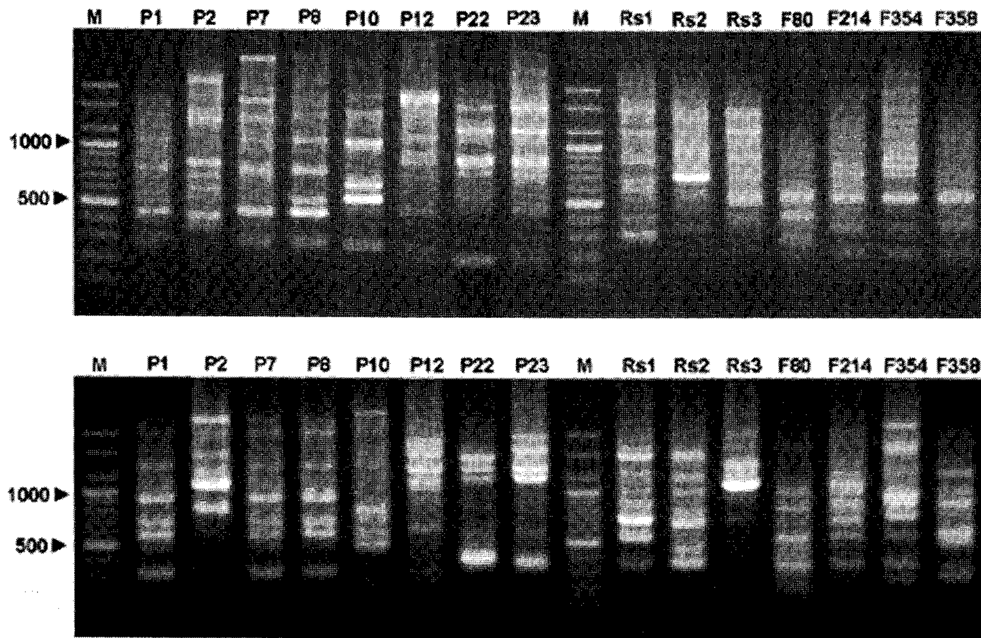

Fig. 2. Random amplified polymorphic DNA analysis of selected isolates of orchid mycorrhizal fungi, P1, P2, P7, P8, P10, P22 and P23, using the primers Bioneer-28 (above) and OPO-2 (below). M = size marker from a 100 bp DNA. Plant pathogenic fungi: *Rhizoctonia solani* Rs-1, Rs-2, and Rs-3 and *forma speciales* of *Fusarium oxysporum*; F-80, F-214, F-354, and F-358 (all obtained from Dr. Y. S. Lee).

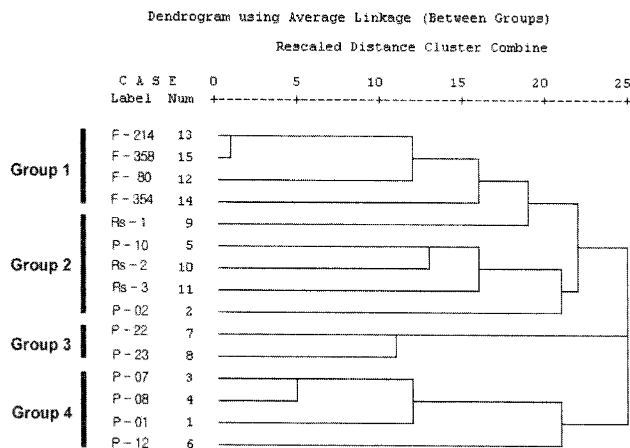

Fig. 3. Dendrogram showing the relationships of the eight isolates of the orchid mycorrhizal and the plant pathogenic fungi (the races of *Rhizoctonia solani* Rs-1, Rs-2, and Rs-3 and *forma speciales* of *Fusarium oxysporum*; F-80, F-214, F-354, and F-358, all obtained from Dr. Y. S. Lee).

1991) cause considerable economical loss. Many horticulture cultivators hesitate to use OM fungi to inoculate orchids, because the anamorph name *Rhizoctonia*, also used for OM fungi, is well known for a dangerous root pathogen. Thus, with respect to biological relations, OM fungi have to be clearly distinguished from plant pathogenic *Rhizoctonia* species, which are well known as anastomosis groups (teleomorph of *Thanatephorus*: Moore, 1987; Sneh *et al.*, 1991; Stalpher and Anderson, 1996). Relationships between the anamorph *Rhizoctonia* and the teleomorph *Tulasnella* were not frequently ascertained although root pathogenic fungi *Rhizoctonia* were intensively studied (Stalpers and Anderson, 1996). The arrangement of the sixteen isolates shown in Fig. 3 is consistent with their systematic relationships (Stalpher and Anderson, 1996; Moore, 1987, 1996). The seven isolates of the fungal pathogens are different from our isolates of OM fungi as based on the polymorphic patterns of PCR-RAPD, but only two of our isolates (P-02 and P-10) were similar to those of pathogens. Consistent with that, orchid growth was apparently not stimulated by the isolates P-02 and P-10. In fact, some strains of *Rhizoctonia solani* although pathogenic to the orchids were already reported to form pelotons in cortical cells of orchids (Currah, 1987; Currah *et al.*, 1989; Harley and Smith, 1983). Therefore, these root pathogens are supposed to inhabit together with OM fungi orchid roots, during commercial cultivations in greenhouses (Lee *et al.*, 2001; Reiw, 1996). The patterns of polymorphic bands (Fig. 2) and cluster analyses (Fig. 3) show that the OM fungi could be distinguished from the root pathogens included in this study.

This PCR-RAPD technique is not very time consuming as for the analysis of pure cultures only two primers have to be applied. This is a prerequisite for horticultural industries. In future, this method could probably still be more simplified when DNA is directly extracted from orchid roots.

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