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Identification of Symbiotic Rhizoctonias from Naturally Occurring Protocorms and Roots of *Dactylorhiza aristata* (Orchidaceae)

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Introduction

Orchids have extremely small seeds and have an intercalated stage, the protocorm, between the seed and plantlet. Chlorophyllous orchid species become autotrophic after the seedling stage. Orchids maintain symbiotic relations with fungi throughout their life cycle. Protocorms depend on symbiotic fungi to provide nutrients for their growth and adult plants have mycorrhiza¹⁹⁾. The relationship between orchids and their symbiotic fungi in nature has been studied^{3, 4, 14, 24, 29-31)}, most of these studies, however, involved fungi associating with roots of mature plants. Our knowledge of the fungi associated with the protocorm stage is restricted because of the difficulty of collecting the minute protocorms from soil in nature. The isolates from roots are often assumed to be symbiotic based on their ability to induce orchid seed germination *in vitro*, but it is still unclear whether the symbiotic relationship is the same in heterotrophic protocorms as it is in roots of autotrophic adult plants.

Accurate identification of fungal isolates is necessary to investigate the relationship between fungi and orchids. Orchid mycorrhizal fungi are mostly Basidiomycetes, and rhizoctonias are well known as orchid mycorrhizal fungi. Recently, non-rhizoctonias with clamp connections have also been reported from achlorophyllous orchids^{23, 35, 37)}. Moore¹³⁾ split anamorphs of the form-genus *Rhizoctonia* De Candeille into 5 genera based on the number of nuclei per cell, septal ultrastructure and known or suspected teleomorphs, and 3 of which, *Moniliopsis* Ruhland, *Ceratorhiza* Moore, and *Epulorhiza* Moore, are associated with orchids. Although these anamorphic genera are well defined, species concepts within each of the genera are not well developed. There are few morphological characteristics in these fungi, so identification to species level is difficult. The anastomosis grouping technique for identification of rhizoctonias has been used extensively

for pathogenic isolates¹⁵⁾ and each anastomosis group (AG) is known to have a moderately specialized host range^{1,22)}. The AGs are genetically distinct⁹⁾, and the concept of AGs has aided species and subspecies identification. We considered that anastomosis testing could determine the teleomorph of an isolate that belonged to one of the AGs for which its teleomorph had been determined. Identification of orchid mycorrhizal fungi to AG is expected to give a better understanding of the relationship between symbiotic fungi and orchids. However, application of this technique to rhizoctonias isolated from orchids has been reported only from Australia^{16,17)} and Japan²⁷⁾.

The objectives of this study were to determine: (1) whether there was any taxonomic difference between symbiotic rhizoctonias isolated from protocorms and from adult plants of the same species, *Dactylorhiza aristata* (Fisch.) Soó, using identifications based on AG technique, (2) whether symbiotic fungi from both protocorms and adult plants of *D. aristata* could induce the germination of seeds of this species *in vitro*.

Materials and Methods

Plant collections: *Dactylorhiza aristata* formed large colonies on a mountain roadside of the Nakayama Pass (831 m above sea level) in Sapporo, Hokkaido. From 2 sites (location 1 and 2) which are 3.4 km apart, whole plants were collected with rhizosphere soil (20×20×15 cm) in July, August and October, 1993. The sampling sites were exposed to full sun, but the soil was always wet and was composed of mainly live moss and plant debris. Protocorms and seedlings that had shoots, resulting from naturally germinated seeds, were collected from the soil surrounding mature plants. The number of plant samples and their collection dates are shown in Table 1.

Fungal isolation: Symbionts were isolated from the plants at different growth stages (Table 1). The subterranean samples of *D. aristata* were divided into roots and palmate root-stem tuberoids⁶⁾ (Figure 1), which were used for isolation of symbionts. Two methods were used for isolation of mycorrhizal fungi from adult plants and seedlings. In one, root segments, obtained by ran-

Table 1. The number of samples of *Dactylorhiza aristata* and their collection date (1993)

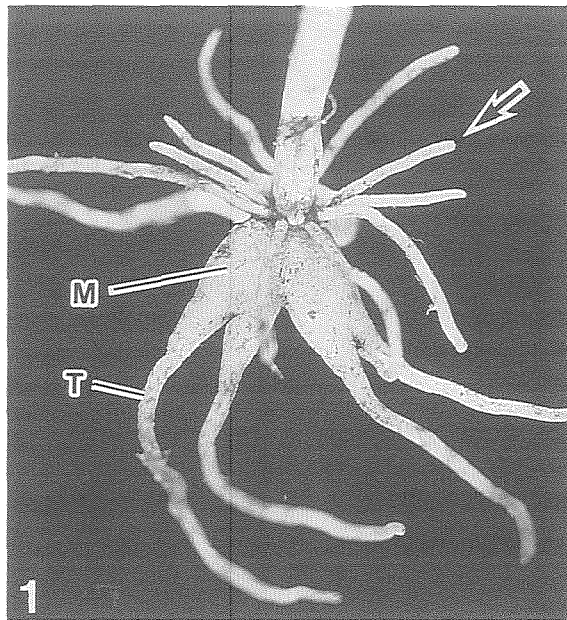
Growth stage	26 July		25 August		5 October		Total
	site		site		site		
	I	II	I	II	I	II	
Adult plants	8	7	6	3	5	2	31
Seedlings	3	4	24	44	22	12	109
Protocorms	54	25	101	145	94	22	441

domly selecting 2 or 3 roots, were surface-sterilized in 0.5% sodium hypochlorite solution, rinsed three times in sterile distilled water, and sectioned into 1 mm thick slices with a sterile scalpel. They were blot-dried with absorbent paper and placed on either water agar or Czapek-Dox medium supplemented with yeast extract (100 $\mu\text{g/ml}$) and streptomycin (30 $\mu\text{g/ml}$)¹⁴. In the second method, 5 mm long root segments were transferred to a drop of sterile water in a sterile Petri dish. Pelotons, observed as intracellular fungal coils, were teased out from cortical cells into the water. After large pieces of torn root segments were removed, the cooled but still liquid medium was poured into the Petri dishes containing the isolated pelotons and solidified³².

The protocorms (Figure 2) were surface-sterilized and used to isolate fungi using the same media as described for the roots. Then, they were cut in half longitudinally and each half was used for one of the two isolation methods described for roots. These plates for both methods were incubated in darkness at 20–25 °C until hyphal growth was visible. Hyphal tips were transferred to potato dextrose agar (PDA) with streptomycin to free them of contaminating bacteria, and then to PDA slants for storage at 15 °C.

Identification of rhizoctonia isolates: The isolates were identified by the anastomosis grouping technique³⁴. First, they were divided into multinucleate or binucleate isolates by staining a piece of agar medium with hyphae from fungal culture on PDA with aniline blue and observing it with a light microscope²⁶. Within these two groups, among the isolates having similar morphological characteristics, hyphal fusions were observed on water agar with a light microscope. Each temporary anastomosis group was then tested by hyphal fusion with tester isolates and assigned to one of the authentic AGs. Tester isolates of AG-1 to AG-10 (multinucleate) and AG-A to AG-S (binucleate) belonged to the Laboratory of Plant Pathology, Hokkaido University. *Thanatephorus orchidicola* Warcup & Talbot (ATCC 24752) was also used as a tester isolate to identify a multinucleate isolate.

Symbiotic germination *in vitro*: Five isolates (2 of R.r.1²²), 1 of AG-G, and 2 of AG-C) from roots and 7 isolates (4 of R.r.1, 2 of AG-C, and 1 of AG-H) from protocorms of *D. aristata* were used. Symbiotic germination was tested using the methods of Tomita and Tsutsui²⁵. Seeds of *D. aristata* were surface-sterilized in sodium hypochlorite solution (0.5 % available chloride) for 1 min and rinsed three times in sterilized distilled water. They were placed on the surface of oatmeal agar (OMA) (3.0 g oatmeal powder + 15 g agar + 1000 ml distilled water) slants. Inoculum from a 7-day-old fungal culture was transferred onto the upper part of OMA slants. The culture tubes were incubated in darkness at 25 °C for two weeks and then transferred a growth chamber with a 16 h daylight length at 25 °C. Five replicates were prepared for each isolate. Seven weeks after inoculation, three slants were randomly selected and the growth of the protocorms was assessed. The formation of protocorm hairs indicated germination. Growth of



Figures 1 and 2. Materials of *Dactylorhiza aristata* used for isolations. 1: The underground organs of an adult plant at anthesis. T, tapering extension of a palmate root-stem tuberoid; M, main part of a palmate root-stem tuberoid; Arrow = root. 2: Protocorms resulting from naturally germinated seeds collected from a soil which were sampled with an adult plant. In a 9 cm Petri dish.

seeds was classified into four categories: no germination -; protocorm hair formation +; protocorm formation ++; and shoot formation +++ . Then percentage of each growth stage was combined using the following formula : (+) % $\times 1$ ++ % $\times 2$ +++ % $\times 3$. For each percentage of seeds germinated and combined growth of protocorms, the statistical significance of differences in mean was tested using an one-way analysis of variance (ANOVA) followed by Fisher's PLSD test, for each isolate (n=3).

Results

Identification of symbiotic fungi : Binucleate and multinucleate rhizoctonias were isolated from samples of *D. aristata* shown in Table 1. Each of the fungal groups in this study was assignable to the known AGs and was placed into the corresponding teleomorphic group for the AGs. There were the binucleate isolates, which belonged to R.r.1, (*Tulasnella deliquescens* (Juel) Juel), AG-C (*Ceratobasidium cornigerum* (Bourdot) Roger), AG-G (*Ceratobasidium* sp.) and AG-H (*Ceratobasidium* sp.). All multinucleate isolates were anastomosed with ATCC24752 which is a tester isolate of *T. orchidicola*, and were assigned to the new anastomosis group T.o.1 of *T. orchidicola* (Table 2).

All symbionts of adult plants of *D. aristata* were isolated exclusively from roots or the tip of tapering extensions of palmate root-stem tuberoids, but main parts of palmate root-stem tuberoids were free of colonization. *Tulasnella deliquescens*, AG-C, AG-H and *Thanatephorus orchidicola* were isolated from both protocorms and adult plants of *D. aristata*, whereas AG-G was isolated only once from a single adult plant. The isolation number of anastomosis groups was summarized for their origin (roots or protocorms) and for each sampling soil (Table 2). In a sampling soil, R.r.1 and AG-C were isolated from both roots and protocorms, or only from protocorms. On the other hand, T.o.1 was isolated from both roots and protocorms, or only from roots. T.o.1 was always isolated along with R.r.1. R.r.1 was isolated more frequently than other AGs.

Symbiotic germination : There were no correlations between seed germination and the origin of fungal isolates nor between seed germination and AG of fungal isolates. Statistical analyses performed for seed germination showed that there were two groups among isolates tested (Table 3). In one group, isolates significantly enhanced seed germination ($p < 0.01$), and in another group they showed no difference with controls without fungal inoculation. Analyses for combined growth rate of protocorms for the isolates showed that all isolates significantly promoted protocorm growth, compared with it in the control ($p < 0.05$) (Table 3). There was significant positive correlation between combined growth rate of protocorms and rate of seed germination ($r = 0.86$, $p < 0.01$) (Figure 3). *Tulasnella deliquescens* (8213) and *Ceratobasidium* (163) showed the highest rate of seed germination and the growth rate of protocorms (Table 3). All

germinated seeds were confirmed to be colonized by the isolate which formed pelotons intracellularly.

Germination of controls in which seeds were sown and incubated without fungus was 4.2 %, and no protocorms reached growth stage (++). These protocorms did not contain pelotons.

Table 2. The occurrence of symbiotic rhizoctonias in rhizosphere soil of each adult plant.

Collection month-site -sample number	Number of plant samples ^{b)}						
	Plant samples examined ^{a)}	Anastomosis group					T.o.I
		R.r.1	C	G	H	T.o.I	
Jul-I-1	A	11	2	-	1	1	2
	P	54	6	-	-	1	-
Jul-II-1	A	11	1	-	-	-	-
	P	29	22	-	-	-	-
Aug-I-1	A	13	6	-	-	-	8
	P	132	22	-	-	-	15
Aug-I-2	A	9	7	-	-	-	1
	P	4	4	-	-	-	-
Aug-I-3	A	8	2	-	-	-	-
	P	91	55	-	-	-	-
Aug-II-1	A	16	9	6	-	-	-
	P	8	4	1	-	-	-
Aug-II-2	A	9	9	-	-	-	-
	P	1	1	-	-	-	-
Aug-II-3	A	22	1	-	-	-	-
	P	45	32	-	-	-	-
Oct-I-1	A	3	2	-	-	-	-
	P	0	NT	NT	NT	NT	NT
Oct-I-2	A	1	-	-	-	-	-
	P	8	3	-	-	-	-
Oct-I-3	A	5	5	-	-	-	-
	P	20	11	-	-	-	-
Oct-I-4	A	7	3	-	-	-	-
	P	15	8	1	-	-	-
Oct-II-1	A	11	3	4	-	-	-
	P	22	7	5	-	-	-
Oct-II-2	A	1	1	-	-	-	-
	P	34	7	-	-	-	-
Oct-II-3	A	2	1	-	-	-	-
	P	8	3	-	-	-	-

a) A : Adult plant, P : protocorm

b) -: No isolates were obtained, NT : Not tested.

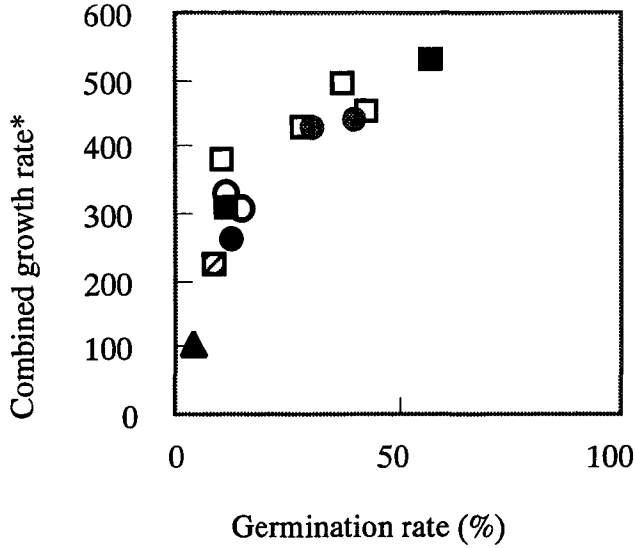


Figure 3. Relationship between germination rate and combined growth rate of an *in vitro* symbiotic germination test of *Dactylorhiza aristata*. There is a positive correlation between them ($r=0.86$, $p<0.01$). * = The percentage of each growth stage (Table 3) was summarized using the following formula: $(+)\% \times 1 + (++)\% \times 2 + (+++)\% \times 3$.

- | | | |
|--------------------|----------------------|-----------|
| ■ AG-C, protocorm | ● AG-G, adult plant | ▲ Control |
| □ R.r.1, protocorm | ○ AG-C, adult plant | |
| ▨ AG-H, protocorm | ● R.r.1, adult plant | |

Discussion

In this study, the mycorrhizal isolates from *D. aristata* were identified as *Tulasnella deliquescens* (R.r.1), *Ceratobasidium cornigerum* (AG-C), *Ceratobasidium* sp. (AG-G, AG-H) and *Thanatephorus orchidicola* (T.o.1). Previously, Uetake *et al.*²⁷⁾ reported *Thanatephorus cucumeris* (AG-6), *Ceratobasidium* sp. (AG-E, AG-I) and *Tulasnella deliquescens* (R.r.1) from *D. aristata*. From *Dactylorhiza purpurella* (T. & T.A. Steph.) Soó, *D. sambucina* (L.) Soó and *D. maculata* (L.) Soó, *Ceratobasidium* sp., *Thanatephorus orchidicola* and *Tulasnella calospora* (Bourdot) Talbot (= *T. deliquescens*) have been reported^{7,8,33)}. In these reports, three kinds of rhizoctonia teleomorphs were associated with the genus *Dactylorhiza*.

Tulasnella deliquescens (*Epulorhiza repens*) has been reported world wide^{5,7,8,11,29-33)}, and was always isolated from this orchid in the present study. *Thanatephorus orchidicola*, which is reported here for the first time from Japan,

Table 3. Germination and growth rate of seeds of *Dactylorhiza aristata* in the presence of symbiotic rhizoctonias *in vitro*

Isolate	Origins ^{a)}	AG ^{b)}	Germination rate(%) ^{c)}	Growth rate of germinated seeds ^{d)}			
				+	++	+++	Combined growth rate
Control ^{e)}	-	-	4.2 ^f	4.2	0.0	0.0	100.0 ^k
82A1	A	R.r.1	38.9 ^l	6.5	32.4	0.0	183.1 ^{mn}
138	A	R.r.1	29.6 ^l	5.3	24.4	0.0	182.6 ^{mn}
122H1	A	G	12.7 ^f	7.5	5.2	0.0	139.7 ^{lm}
212	A	C	15.1 ^g	7.4	7.7	0.0	152.8 ^{lm}
234	A	C	11.9 ^{fg}	5.2	9.4	0.0	156.3 ^{lm}
816	P	R.r.1	43.3 ^l	5.1	40.6	0.0	186.6 ^{mn}
213	P	R.r.1	28.6 ^h	5.3	23.4	0.0	155.5 ^{lm}
8213	P	R.r.1	38.0 ^{hl}	2.2	34.6	1.2	197.2 ^{mn}
11	P	R.r.1	10.7 ^{fg}	3.3	7.3	0.0	169.0 ^m
163	P	C	57.4 ^l	5.1	45.1	7.2	202.7 ⁿ
2520	P	C	12.1 ^{fg}	6.0	6.2	0.0	152.5 ^{lm}
152	P	H	9.0 ^{fg}	6.2	2.8	0.0	136.6 ^l

a) Growth stage of orchids from which the fungus was isolated ;

A, adult plant (root) ; P, protocorm

b) AG : anastomosis group

c) Mean germination percentage (total percentage of all seeds germinated) of 3 replicates ; there is no significant difference between numbers followed by the same letter ($p < 0.01$)

d) Growth assessment of germinated seeds : +, epidermal hairs formation ; ++, protocorm formation ; +++, shoot formation. Combined growth rate were summarized using the following formula : $(+)\% \times 1 + (++)\% \times 2 + (+++)\% \times 3$

e) Control, without fungus

was previously isolated from *Coeloglossum viride* (L.) Hartm. in Scotland, and from *Dactylorhiza mascula* (L.) L. in England³²⁾.

So far, the groups of R.r.1 and R.r.2 (*T. deliquescens*), AG-A, AG-C, AG-E, AG-I (*Ceratobasidium*), AG-5 and AG-6 (*Thanatephorus cucumeris*) have been reported from 21 genera including 30 species of orchids in Japan²⁷⁾, and here AG-G, AG-H and T.o.1 (*T. orchidicola*) are newly reported as orchid mycorrhizal fungi. Among these, R.r.1, R.r.2 and T.o.1 have been reported only from orchids, and AG-H and AG-6 have been isolated from soil and orchids. On the other hand, each of the other groups has several host plants, and isolates of AG-A, AG-E and AG-I, AG-5 and AG-6 are known to be pathogens or weak-pathogens on non-orchid species and AG-C has been considered as non-pathogens^{12,21)}. The range of AGs associating with orchids, based on present information, appears to be restricted. Excluding R.r.1 and R.r.2 in *Tulasnella* and T.o.1 in *Thanatephorus orchidicola*, only 6 of 17 binucleate AGs and 2 of 11 multinucleate AGs were reported from orchids.

In addition to AGs isolated in this study, isolation of AG-E, AG-I and AG-6 from *D. aristata* have been reported²⁷⁾. This suggests that several other AGs can colonize *D. aristata* depending on locations. Further work is needed to reveal the distribution of these symbionts in other habitats. There is a strong possibility that the new AGs will be found with additional sampling, since the known groups are based on the isolates from plant pathological studies. A study by Ramsay *et al.*¹⁷⁾ supports this prediction. In their study, isolates of 10 AGs were isolated, but no group corresponded to previously reported AGs. In Japan, several undetermined AGs have been found in both bi- and multinucleate rhizoctonias isolated from orchids^{22,27)}. The presence of subgroups in *T. deliquescens* has also been suggested (Ogoshi, unpublished data). More studies on anastomosis groups of rhizoctonias associated with orchids are necessary for a better understanding of the relationship between orchids and their mycorrhizal fungi.

Although the symbiotic association between orchids and fungi has been studied by many authors^{3~5,17,21,29,31)}, most isolates of symbiotic fungi have been derived from roots of adult plants. It is necessary to determine the fungi associating with germinating seeds or protocorms of orchids, because for chlorophyllous orchids these are heterotrophic stages, and it is not clear whether the symbiotic relationship is the same as that in autotrophic adult plants. Recently, efforts have been made to trap the fungi associating with the early stages of orchids by enclosing seeds in packets buried in the soil of their natural habitats^{10,16,19,20,36,37)}. Using cotton gauze packets, Masuhara and Katsuya¹⁰⁾ reported *E. repens* and an undescribed multinucleate *Rhizoctonia* from the seeds of *S. sinensis*; and using polyester filter fabric packets, Zelmer and Currah³⁶⁾ isolated *Ceratorhiza* sp. from *S. lacera* (Rafinesque) Rafinesque and an *Epulorhiza* sp. was isolated from *S. magnicamporum* Sheviak³⁷⁾. Although the materials of the packets may influence the results, the seed packet method promises to lead to a better understanding of the fungi associating with the early stage of orchids.

In the present study, a large number of naturally occurring protocorms was found in the rhizosphere of the adult plants of *D. aristata*. From protocorms of *D. aristata*, the groups of R.r.1, T.o.1, AG-C and AG-H were isolated. However, in addition to these fungi, AG-G was isolated from only roots of adult plants. From roots and protocorms collected from soil samples (about 20 cm x 20 cm x 15 cm), R.r.1 and AG-C were isolated from both, or only from protocorms, whereas T.o.1 was isolated from roots and protocorms, or only from roots. In addition, T.o.1 was always isolated along with R.r.1, but R.r.1 and AG-C were isolated alone respectively. These results suggest that *D. aristata* is mainly associated with R.r.1, and also AG-C in the early growth stage in the sampling sites. It is important to compare isolates from mycorrhizal roots with isolates from protocorms, especially if these were collected from the same sampling soil, and to determine the fungi associated with protocorms and roots of orchids.

So far, in *in vitro* test of symbiotic germination, two types of penetration of

hyphae have been reported. In one, hyphae penetrate into the suspensor region before hair formation (*Pterostylis* spp.²⁾, *Spiranthes sinensis*²⁸⁾, *Platanthera* spp.²¹⁾). In the other, penetration of hyphae occurs only through epidermal hairs as reported in *Dactylorhiza purpurella* (T. & T.A. Steph.) Sođ³³⁾ and *D. majalis* (Rchb. f.) Hunt & Summerhayes¹⁸⁾. Colonization to seeds of *D. aristata* also occurs only through epidermal hairs (data not shown), hence, only germinated seeds can be colonized by fungus. Therefore, the high germination rate compared to controls suggests that hair formation was enhanced by fungal isolates. In addition, because all protocorms treated with isolates were colonized by the isolates, growth enhancement of protocorms was caused by fungus. The most effective ones were one isolate of AG-C from protocorms and isolates of R.r.1 from protocorms and roots. It is suggested that mycorrhizae of adult plants have a role to supply effective fungi for symbiotic seed germination.

Because seed germination of *D. aristata* was affected by external hyphae before fungal colonization and growth enhancement of protocorm was affected by internal colonization of hyphae, for assessment of the ability to be symbionts for the isolates, seed germination rate and growth rate of protocorms must be considered separately. From this point of view, isolates fell into 2 groups by the ability of enhancement of germination. This suggests that the effects of fungal isolates on seed germination and on growth of protocorms are different. In addition, the positive correlation between enhancements of seed germination and of protocorm growth suggests some relationship between both functions of fungus. Further work is necessary to determine these functions of the fungi to symbiotic germination of orchids.

Summary

Symbiotic fungi were isolated from naturally occurring protocorms, roots of seedlings and roots of adult plants *Dactylorhiza aristata* (Orchidaceae). The fungi were all assigned to the form genus *Rhizoctonia* (rhizoctonias). Using the hyphal anastomosis technique, they were identified as *Ceratobasidium cornigerum* (anastomosis group; AG-C), *Ceratobasidium* sp. (AG-G and AG-H), *Tulasnella deliquescens* (R.r.1), and *Thanatephorus orchidicola* (T.o.1). Protocorms of *D. aristata* yielded four AGs (R.r.1, AG-C, AG-H and T.o.1), and in addition to these groups, AG-G was found in the roots. From roots and protocorms that were collected from the same soil samples, R.r.1 and AG-C were isolated from both or only from protocorms, whereas T.o.1 was isolated from both or only from roots. In *in vitro* test of symbiotic germination of *D. aristata* seeds, origin of isolates did not affect the results. Isolates used in this test fell into two groups whether these enhanced formation of protocorm hairs, which are entrance points of fungus, or not. In the former, isolates belonged to R.r.1 and AG-C and in the latter, all AGs were found. From these results, it is suggested that *D. aristata* in this study has close associa-

tion with R.r.1, and in protocorm of this plant also has association with AG-C.

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