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Characterization and taxonomic placement of *Rhizoctonia*-like endophytes from orchid roots

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Abstract: Twenty-one *Rhizoctonia*-like fungal strains were isolated from the roots of four terrestrial orchid species from various locations in Hong Kong. The cultural morphology, nuclear number of the hyphal cell, pore ultrastructure, and RAPD and CAPS analyses of rDNA fragments revealed that most of these isolates were associated with the genera *Ceratorhiza* and *Epulorhiza*. RAPD analysis showed the presence of genetic diversity between the isolates from different hosts and locations. The compatibility between a selection of these *Ceratorhiza* and *Epulorhiza* isolates and 14 orchid species was determined using a symbiotic germination method. The germination and development of three orchid species, *Arundina chinensis*, *Spathoglottis pubescens*, and *Spiranthes hongkongensis*, were strongly stimulated by the *Epulorhiza* isolates. *Habenaria dentata* was found to form symbionts successfully with a *Ceratorhiza* isolate.

Key Words: *Ceratorhiza*, *Epulorhiza*, mycorrhiza, orchid, pore ultrastructure, *Rhizoctonia*, ribosomal DNA, symbiont

INTRODUCTION

It has long been established that under natural and horticultural conditions, there exists an association between orchid roots and certain species of fungi (Hadley 1982). Such an orchid-fungus relationship is formed at the onset of seed germination. Orchid seeds are relatively minute and lack sufficient food reserves, so their germination depends on the external supply of nutrients from compatible fungi. The isolation and identification of orchid mycorrhizal fungi are thus of importance to the understanding of the orchid-fungus relationship.

Most orchid mycorrhizal fungi have been assigned

to the form genus *Rhizoctonia* (Sneh et al 1991). The genus represents an assemblage of taxonomically diverse groups that differ in many features, including anamorph and teleomorph stages (Curtis 1939, Warcup and Talbot 1966, 1967, 1980, Currah et al 1987). Most species in this genus are mycelia sterilia and a variety of approaches have been developed to identify them. The conventional method of identifying these *Rhizoctonia* fungi has been based on their cultural morphology, which includes colony color, monilioid cells, sclerotia, and other mycelium characteristics (Curtis 1939, Currah et al 1987). Anastomosis group (AG) based on anastomosis behavior of vegetative hyphal fusion and intraspecific group (ISG) within AGs based on combined evidence from anastomosis behavior, pathogenicity, morphology, and other criteria have been the most frequently adopted methods to identify *R. solani* complex and study their genetic diversity (Vilgalys and Cubeta 1994). Ramsay et al (1987) assigned some Australian orchid *Rhizoctonia*-like endophytes to five anastomosis groups. However, DNA data showed that while most AGs represent monophyletic evolutionary units, hyphal anastomosis behavior may not be the best indication of evolutionary relationship between different ISGs (Vilgalys and Cubeta 1994).

Moore (1987) assigned doliporous *Rhizoctonia* and *Rhizoctonia*-like fungi into the groups *Ceratorhiza*, *Epulorhiza* and *Monilioiopsis* according to the cell nuclear number and septum ultrastructural features. This framework has been adopted by several other researchers recently when identifying orchid mycorrhizal fungi because it offers a taxonomically correct and justified approach to define taxa. Species concepts within these genera can then be erected on the basis of finer culture characteristics such as mycelia morphology on specific media (Currah et al 1990, Currah and Zelmer 1992, Zelmer and Currah 1995, Andersen 1996).

The use of DNA fingerprinting techniques has proved to be a useful, fast and accurate approach to the study of variations among inter- and intraspecific strains in many fungi complexes (Cubeta and Vilgalys 1997, Sen et al 1999). Random amplified polymorphic DNA (RAPD) analysis surveys multiple loci of the whole genome simultaneously with each single primer and detects nucleotide differences between

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TABLE I. Sources of *Rhizoctonia*-like isolates studied in this investigation

Orchid species	Habitat	Location	No. of root specimens	Fungal accession no.	Collection time
<i>Eulophia flava</i>	Exposed grassy hillside	Lamma Island, Hong Kong	10	Eu3	July 1996 (after flowering)
<i>Goodyera procera</i>	Rocky banks of streams	Ma On Shan, Kowloon peninsula	10	G23, G38, G44, G45, G46, G47, G48	April 1996, April 1997
		Shing Mun, Kowloon peninsula	10	G13, G15, G16, G17, G19, G20, G29	March–April 1996
		Tai Po Kau, Kowloon peninsula	5	G55	March 1996
<i>Habenaria dentata</i>	Exposed grassy hillside	Sai Kung, Kowloon peninsula	10	H29, H30, H31, H34	April 1997
<i>Spiranthes hongkongensis</i>	Damp but sunny hillside	Ma On Shan, Kowloon peninsula	10	Spir36	May 1996

isolates. The analysis of restriction fragment length polymorphisms (RFLP) and cleaved amplified polymorphic sequences (CAPS) of DNA (Drenkard et al 1997) in general can provide information on a few specific loci in the genome, thus reflecting the relationships between isolates in a relatively more conservative way than RAPD. These techniques have been widely employed to distinguish subspecific groups of *Rhizoctonia solani* and showed similar patterns of variations within and among ISGs (Vilgalys and Gonzalez 1990, Duncan et al 1993, Vilgalys and Cubeta 1994, Matsumoto et al 1996, Cubeta and Vilgalys 1997), but very few studies have been carried out to determine the genetic relationships among orchid *Rhizoctonia*-like endophytes and identify them on the basis of molecular characteristics, pore ultrastructure, and number of nuclei in combination (Andersen 1996, Sen et al 1999).

In the present study, morphological characteristics and the number of nuclei of all strains, and pore ultrastructure of some strains of orchid endophytes were investigated to segregate them into groups. RAPD and CAPS analyses were undertaken in order to understand the genetic relationships among the orchid-associated *Rhizoctonia*-like isolates from different geographic locations and hosts in Hong Kong. An attempt was made to establish the relationship between our isolates and reference strains of *Rhizoctonia* at the genetic level in order to identify the isolates from orchids. The compatibility between seeds of several orchid species and a selection of the fungal strains was tested for any correlation between symbiont-forming ability and specific groupings of these strains.

MATERIALS AND METHODS

Sources of isolates.—Endophytic fungi were isolated from roots of four terrestrial orchid species *Eulophia flava*, *Goodyera procera*, *Habenaria dentata*, and *Spiranthes hongkongensis*. Only *Rhizoctonia*-like fungi (21 isolates) were selected for culturing based on the characteristics described by Sneh et al (1991). Their sources are listed in detail in TABLE I.

Five fungal strains identified to species level were used as references. They were *Ceratorrhiza cornigerum* (Bourdot) Rogers (ATCC 34054, USA), *C. globosporum* Warcup & Talbot (CBS 569.83, The Netherlands), *Rhizoctonia repens* Bernard (CBS 298.32), *R. solani* Kühn (CBS 174.83), and *Thanatephorus cucumeris* (Frank) Donk (CBS 233.93).

Sampling procedure and isolation of endophytes.—Soil was carefully removed from the orchid plants, keeping the roots intact. Healthy roots were rinsed under running tap water and surface sterilized in 70% ethanol for 5 s, then transferred to 0.1% HgCl₂ for 1–5 min and rinsed in five changes of distilled water. The roots were then aseptically cut into approximately 2 mm portions and placed singly in 5 cm Petri dishes containing Potato Dextrose Agar (PDA, Difco) with 3 mg/L rose bengal and 50 mg/L streptomycin (Sigma). The plates were incubated in the dark at 25 C until hyphae were visibly growing from root specimens onto the medium. Pure cultures were then obtained by transferring hyphal tips onto PDA. All cultures were incubated at 25 C in the dark.

Culture of isolates and induction of teleomorph.—Besides PDA, isolates were also grown on Cornmeal agar (CMA, Difco) and Oatmeal agar (2.5% oatmeal flakes, OMA) at 25 C in the dark to observe the cultural characteristics. Some cultures were left for at least 6 wk to allow the development of monilioid cells and the formation of sclerotia. For the observation of nuclei number and hyphal morphology, isolates were grown on dialysis membranes placed

on CMA. Cultures were kept on PDA at 4 C or in sterilized distilled water at room temperature for long term storage.

To induce teleomorph formation, the media employed were PDA, CMA, CMA with orchid leaf-discs, OMA, water agar (1% Bacto-agar, Difco) and V-8 agar (Johnston and Booth 1983). Isolates on these media were incubated under near UV-light for 12 h and darkness for 12 h. All cultures were examined weekly.

The soil-agar method of Stretton et al (1964) was also used to induce sporulation. Isolates were grown on PDA in 9 cm Petri dishes for 7–10 d. The lids were then removed, and each culture was covered to a depth of approximately 1 cm with sterilized soil which was watered 1–3 times a day to maintain constant moisture. The cultures were observed over a three-month period.

Cultural characteristics.—Colony surface and reverse colors were determined according to the descriptions in Methuen Handbook of Colour (Kornerup and Wanscher 1978) and coded with the prefix MHC.

The measurements of hyphal diameter and dimensions of monilioid cells were made by examination of the mycelium mounted in lactophenol on microscope slides.

Growth rates were determined according to the technique of Currah et al (1987). A small fragment of mycelium (approximately 1 mm²) was inoculated onto the middle of a 9 cm PDA plate. Radial increments in colony diameter were measured in two directions every 48 h over a two-week period. Growth rates were represented by averages based on three replications.

Nuclei number of hyphal cells.—Determination of the nuclei number in vegetative cells was carried out by modifying the procedure of Sneh et al (1991) as follows: hyphae growing on the dialysis membrane were fixed in 2% formaldehyde for 2 min and rinsed in distilled water for 1 min. Hyphae were then stained with 5 µg/ml of 4'-6'-diamidino-2-phenylindole (DAPI) for 10 min, and destained with water for 2 min. The material was finally placed in a drop of 50% glycerine on a microscope slide. A cover slip was used without pressing down onto the hyphae.

All micrographs were made using an Olympus BX60 microscope equipped with an Olympus fluorescence accessory with mercury lamp.

Transmission electron microscopy (TEM).—Isolates examined were grown on PDA for 7 d and then TEM work was carried out following the methods of Wong et al (1998).

Preparation of genomic DNA.—Each isolate was grown on PDA for 15–30 d at 25 C. A 0.25 cm² piece of mycelium was then scraped from the surface of the plate for DNA extraction. DNA was extracted according to the protocols described by Doyle and Doyle (1987), and Bult et al (1992) with the following modifications:

Mycelium was ground in 2 × CTAB buffer and incubated at 60 C for 25–30 min. After extraction with 2/3 volume phenol: chloroform: isoamyl alcohol (25:24:1), the sample was extracted 2–3 times using chloroform: isoamyl alcohol (24:1) until the aqueous layer was clear. The aqueous layer was then precipitated in 2 volumes cold absolute ethanol (–20 C) for 2 h, centrifuged and the supernatant discarded.

TABLE II. Primers used in RAPD analysis

Primer	Sequence (5'-3')
UBC-016	GGT GGC GGG A
UBC-031	CCG GCC TTC C
UBC-034	CCG GCC CCA A
UBC-065	AGG GGC GGG A
UBC-066	GAG GGC GTG A
UBC-070	GGG CAC GCG A
UBC-071	GAG GGC GAG G
UBC-073	GGG CAC GGG A
UBC-089	GGG GGC TTG G
UBC-091	GGG TGG TTG C

The DNA pellet was dissolved in TE buffer with 0.8 µg/mL RNaseA stock (10 mg/mL, Promega) and stored at 4 C.

DNA amplification with random primers (RAPD).—The RAPD protocol described by Liew and Irwin (1994) was followed with slight modification. Each polymerase chain reaction (PCR) was performed in a total volume of 25 µL. The reaction mixture consisted of approximately 30 ng template DNA, 2.5 µL 10 × Taq DNA polymerase buffer (Promega), 4 mM MgCl₂ (Promega), 200 µM of each dNTP, 1 unit of Taq polymerase (Promega), 0.5 µM primer (Set #1, Nucleic Acid-Protein Service Unit, University of British Columbia, Canada) and MilliQ water. Mineral oil (30 µL, Promega) was overlaid on the reaction mixture. A PTC-100[®] Programmable Thermal Controller (MJ Reearch Inc.) was programmed for 5 min at 94 C, followed by 40 cycles of 1 min at 94 C, 1 min at 37 C, and 2 min at 72 C. The final cycle was followed by an extension step for 8 min at 72 C.

Template DNA was amplified using 10-base primers. Ten primers were used and their sequences are listed in TABLE II. A control sample (*C. cornigerum* × primer UBC-065) was included in each PCR run. The amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels (G_{IB} BRL) in Tris Borate (TBE) buffer containing 0.3 µg/mL ethidium bromide (EtBr), at 2.5 V/cm. The gels were visualized and photographed on a uv-transilluminator.

PCR amplification of the ribosomal DNA.—The internal transcribed spacers (ITS1 and ITS2), the 5.8S subunit and partial 18S subunit of the ribosomal DNA defined by the primers NS5 and ITS4 were amplified by PCR according to White et al (1990). Primer NS5 is located in the 18S rDNA region and ITS4 is located in the 28S rDNA region.

PCR amplification was performed in a reaction mixture with a final volume of 50 µL. The mixture contained 50–100 ng of genomic DNA, 300 pM each of both primers NS5 and ITS4, 5 µL 10 × Taq DNA polymerase buffer (Promega), 1.5 mM MgCl₂ (Promega), 200 µM of each dNTP, 1.5 unit of Taq polymerase (Promega). Mineral oil (30 µL, Promega) was overlaid onto the reaction mixture. The reaction was performed in a PTC-100[®] Programmable Thermal Controller (MJ Reearch Inc.) for 31 cycles of: denaturation at 95 C for 1 min, annealing at 52 C for 50 s, and extension at 72 C for 1 min 20 s, with an initial denaturation of 3 min at 95 C before cycling and a final extension of 15

min at 72 C after cycling. A fraction (5 μ L) of the PCR products was size fractionated in a 0.8% agarose gel (G_{IB} BRL, Spain) with 0.3 μ g/mL EtBr. The presence of a single bright band (ca 1.3 kb) for each sample indicated successful amplification. The remaining PCR product was purified and concentrated using Wizard[®] PCR Preps Purification System (Promega) for restriction enzyme digestion.

Restriction of PCR-amplified fragments.—PCR-amplified products were digested using restriction endonucleases, DpnII, EcoRI, HaeIII, HpaII and RsaI (New England Biolabs, Inc.), as recommended by the manufacturer. The digestion products were separated by electrophoresis in a 2% agarose gel (G_{IB} BRL) containing 0.3 μ g/mL EtBr in TBE, at 2.5V/cm. The DNA size marker used in this experiment was a 100 base-pair ladder (Pharmacia Biotech).

Data analysis.—The data from RAPD and restriction of PCR-amplified fragments were scored for the presence or absence of bands at each size location, designated 1 or 0 respectively. The data matrices were analyzed by the SIMQUAL program of NTSYS-PC, version 1.8 (Rohlf 1994). The similarity index (F) between two individuals x and y was calculated based on the theory of Nei and Li (1979) using the formula $F = 2m_{xy}/(m_x + m_y)$, where m_{xy} was the number of bands shared by the two individuals x and y, and m_x and m_y were the numbers presented by the individuals x and y, respectively. A phenogram based on the data matrix was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis to visualize the relation between the samples.

In this study, differences in band intensity were not taken into account. For a given primer, we assumed fragments amplified from different individuals but which had the same molecular weight to be identical.

Orchid seed collection and sowing.—Seeds of 14 orchid species in dehiscing capsules were obtained from their natural habitats in Hong Kong, with the exception of those of *Phaius tankervilleae* which were collected from cultivated plants in the garden of The University of Hong Kong. The 13 wild species were the terrestrial species *Arundina chinensis*, *Cymbidium ensifolium*, *Eulophia flava*, *Goodyera procera*, *Habenaria dentata*, *H. rhodocheila*, *Malaxis* sp., *Paphiopedilum* sp., *Peristylus tentaculatus*, *Spathoglottis pubescens*, *Spiranthes hongkongensis*, *Zeuxine gracilis*, and the epiphytic species *Liparis longipes*.

All seeds were sown within 24 h after harvest. Seeds were surface sterilized by suspending them in 0.1% HgCl₂ with 0.1% of Tween 80 (surfactant) and shaken for 5 min. The seeds were then rinsed in five changes of sterilized water.

Approximately 300 seeds were distributed over the surface of a sterile filter paper strip (1 \times 3 cm, Whatman No. 1) resting on the surface of an OMA plate (Rasmussen and Rasmussen 1991). An 8 mm³ block of Potato Dextrose Agar (Difco) with or without mycorrhizal fungus was placed at the edge of each filter paper strip in symbiotic and asymbiotic cultures respectively. The plates were then sealed with parafilm and incubated in continuous light of approximately 500 Lux provided by Gro-Lux fluorescent light tubes at a temperature of 25 C (Yam and Weatherhead 1988, An-

derson 1991). Three replicates of each treatment were prepared. For seeds that failed to germinate, a dark germination condition was also tried.

One or more fungal strains from different morphological groups were selected to test their ability to form symbionts with orchids. They were Eu3, G16, G19, G38, G55, H30, H34 and Spir36.

Assessment of germination and developmental stages.—Germination (rupture of the seed coat by enlarged embryo) and developmental stages were assessed using a scale of 1–4 which is adopted from Zettler and McInnis (1993) but modified by combining the stage 3 and 4 as one. Stage 0: No germination; Stage 1: Enlarged embryo and seed coat rupture, occasionally a rhizoid present; Stage 2: Embryo 2–3 times enlarged and rhizoids present; Stage 3: Leaf primordium present and appearance of first true leaves; Stage 4: Elongation of initial leaves and root differentiation.

RESULTS

Twenty-one *Rhizoctonia*-like isolates were obtained, *Eulophia flava* (1), *Goodyera procera* (15), *Habenaria dentata* (4), and *Spiranthes hongkongensis* (1) (TABLE I).

Fungal morphology.—All isolates in the present study can be segregated into four morphological groups (MG) on the basis of their morphological characteristics. TABLE III summarizes the significant features observed in the isolates.

RAPD analysis.—Twenty two fungal isolates (21 orchid endophytes and one a reference and outgroup strain *Ceratobasidium cornigerum*) were examined. A total of 207 bands were scored for polymorphisms. A phenogram constructed using UPGMA cluster analysis is shown in FIG. 1a. The similarity index between the isolates examined ranges from approximately 18% to 100%. Six main groups with a similarity index lower than 23% between each other can be observed. The MG II isolates, which were isolated from *Goodyera procera* and *Eulophia flava*, are grouped into two main clusters. The MG IV isolates, which were isolated from *Habenaria dentata*, form one of the main clusters and show a low level of heterogeneity (similarity index >95%). The MG I isolate H34, MG III isolate Spir36, and the reference strain *C. cornigerum*, are assigned to three different main clusters. Among the isolates, H34 is most distantly related to the others.

Isolates that are highly similar were from the same host species and location. For example, G13, G15, G16, G17, G19, and G29 from Shing Mun show similar RAPD patterns and have similarity indices of above 95%. However, not all of the isolates from the same host species and location show a high level of similarity. G20 from Shing Mun is distinct from the

TABLE III. Summary of cultural, morphometric characteristics of *Rhizoctonia*-like isolates (all characteristics were observed on PDA except where otherwise indicated)

	Group I	Group II	Group III	Group IV
Color of young colony (surface)	Yellowish to white (MHC 4A2-4A3)	White (MHC 1A1)	Grey to greyish yellow (MHC 2B1)	Yellowish white (MHC 2A1)
Color of young colony (reversed)	Butter yellow (MHC 4A4-4A5)	Milk to pale yellow (MHC 1A1-1A2)	Yellowish grey (MHC 4B2)	Yellow grey (MHC 4B2)
Change in colony color at 4C	No	No	No	Yes
Colony appearance	Cottony	Glabrous or fluffy	Flat or felted	Glabrous
Color of vegetative hyphae	Hyaline	Hyaline	Hyaline	Hyaline
Diameter of vegetative hyphae (μm)	3.8-7.5	1.8-5.0	1.8-5.0	1.8-5.0
Shape of monilioid cells (if present)	Ellipsoidal or elongate barrel shape	Irregularly ellipsoidal to nearly spherical	Absent	Nearly spherical
Dimension of monilioid cells (μm)	(7.5-15.0) \times (10.0-25.0)	(7.5-20.0) \times (7.5-12.5)	Absent	(3.8-6.3) \times (3.8-7.5)
Color of sclerotial masses on OMA	White to yellowish (MHC 2A1)	White (MHC 1A1)	White (MHC 1A1)	White (MHC 1A1)
Colony growth rate (mm/hr)	0.42-0.52	0.14-0.36	0.14-0.16	0.08-0.12
Septal structure (only some strains were examined)	Not determined	Dolipore septa, imperforate parentheses (Eu3, G16, and G19)	Dolipore septa, imperforate parentheses (Spir36)	Not determined
Nuclear condition	Binucleate cells	Binucleate cells	Binucleate cells	Binucleate cells
Teleomorph	Absent	Absent	Absent	Absent
Material examined	H34, ATCC 34054	Eu3, G13, G15, G16, G17, G19, G20, G23, G29, G38, G44, G45, G46, G47, G48, G55, CBS 298.32	Spir36	H29, H30, H31

other Shing Mun isolates, and is assigned to another main group. Isolates from the same host species but different locations show a high level of heterogeneity and are separated into distinct clusters. For example, although G16, G38, and G55 are all from *Goodyera procera*, the similarity index between G16 (from Shing Mun) and G38 (from Ma On Shan) is approximately 48% while G16 (from Tai Po Kau) clustered within a separate main group from that of G16 and G38.

CAPS analysis of ITS1, ITS2, 5.8S, and part of the 18S rDNA.—Eleven orchid endophytes were chosen for the CAPS study on the basis of RAPD results. Most of the selected isolates are heterogeneous according to the RAPD data, except G16 and G29 which are similar (FIG. 1a). Five strains, *Ceratobasidium cornigerum*,

C. globisporum, *Rhizoctonia repens*, *R. solani*, and *Thanatephorus cucumeris* were included in the study as references.

A total of 71 bands were scored and a phenogram (FIG. 1b) was derived on the basis of UPGMA cluster analysis. The MG II isolates, which were isolated from *Eulophia flava* and *Goodyera procera*, show a high level of similarity and were assigned to a distinct cluster with a similarity index of >60%. Among the reference strains, *Rhizoctonia repens* shows the closest relationship with the MG II isolates. The MG I isolate H34, *Ceratobasidium cornigerum*, *C. globisporum*, and *Thanatephorus cucumeris* were assigned to one cluster at a 60% similarity level. In this cluster, H34 is most closely related to *C. cornigerum*, with a similarity index of approximately 88%. H30 is most dissimilar to all the other strains tested.

Screen tests for host-fungal compatibility.—Germination and development of *Arundina chinensis*, *Spathoglottis pubescens*, and *Spiranthes hongkongensis* was strongly stimulated by the selected fungal strains in MG II (Eu3, G16, G19, G38, G55) except for G19 which was not tested with *A. chinensis* and which failed to form successful symbionts with *S. hongkongensis* (TABLE IV). Strains in other morphological groups could not enhance germination and development of these three orchid species.

A few seeds of *L. longipes* and *P. tankervilleae* developed to seedling stage (stage 4) with some MG II strains such as Eu3. Germination and development of seeds of *H. dentata* was strongly stimulated by H34 in MG I only (TABLE IV).

No fungal strain selected in the tests formed successful symbionts with the other eight orchid species studied (TABLE IV). G16, G19, G38, G55 from *G. procerata* and Spir36 from *S. hongkongensis* all failed to stimulate germination of the seeds of the orchid species from which they had been obtained.

DISCUSSION

Moore (1987) assigned anamorphic binucleate *Rhizoctonia* species with dolipore septa and perforate parentheses to the genus *Ceratorhiza*, with the teleomorph *Ceratobasidium*. In our study, although septal ultrastructure of H34 in morphological group I (MGI) was not determined, the high similarity between H34 and *Ceratobasidium cornigerum* (ATCC 34054) indicated by CAPS analysis suggests that H34 could be assigned to the group of the anamorphic genus *Ceratorhiza*. Besides the close genetic relationship, the morphology of this strain is also similar to that of *C. cornigerum* in this study and that of *Ceratorhiza goodyerae-repentis* described by Richardson, Currah and Hambleton (1993).

The type species of *Ceratorhiza*, *Ceratorhiza goodyerae-repentis* (Constantin and Dufour) Moore was initially described by Constantin and Dufour (1920) based on an isolate from *Goodyera repens* (L.) R. Br. Later, Downie (1941) gave another description of this species in detail (Andersen and Staplers 1994). Warcup and Talbot (1966) determined that *Rhizoctonia* (*Ceratorhiza*) *goodyerae-repentis* was an anamorph of *Ceratobasidium cornigerum* by inducing fruiting bodies from a *R. goodyerae-repentis* isolate identified according to the concept of Downie (Hadley 1969, Andersen and Staplers 1994). This species has been commonly obtained from either terrestrial or epiphytic orchid mycorrhizas in Australia and Britain (Warcup and Talbot 1967, Warcup 1981, Alexander and Hadley 1983), Canada (Currah et al 1990, Currah and

Zelmer 1992), Costa Rica (Richardson et al 1993) and Malaya (Hadley and Williamson 1972).

Moore (1987) segregated binucleate *Rhizoctonia* species with dolipore structure and imperforate parentheses to the genus *Epulorhiza*, with teleomorphs *Tulasnella* and *Waitae* (Sneh et al 1991). According to Moore's classification (1987), isolates Eu3, G16, and G19 in MG II and isolate Spir36 in MG III can be identified as *Epulorhiza* species. The RFLP analysis of rDNA fragments showed a relatively high level of similarity between isolates of Eu3, G16 and the other isolates in MG II. This result suggests that all the isolates in MG II may belong to the form genus *Epulorhiza*. The similarity of morphological characteristics between isolates of MG II and those of *Rhizoctonia* (*Epulorhiza*) *repens* and *Tulasnella* sp. (described by Warcup and Talbot 1980, Currah et al 1987), and the genetic closeness of these isolates to *Rhizoctonia* (*Epulorhiza*) *repens* shown by rDNA CAPS analysis further support the above classification.

The type species of *Epulorhiza*, *Epulorhiza repens* (Bernard) Moore, was first described by Bernard (1909) based on isolates from *Laelio-Cattleya Canhamiana*. Curtis (1939) named a group of isolates from seven orchid species as *R. repens* Bernard according to the cultural morphology, especially the morphology of monilioid cells that were similar to those Bernard had illustrated. Warcup and Talbot (1967) induced teleomorph from *R. repens* isolates and identified it as *Tulasnella calospora*.

Fungi in the genus *Epulorhiza* are distinct and have been continuously isolated from orchids (Hadley 1982, Currah et al 1990, 1997, Currah and Zelmer 1992, Zelmer et al 1996). For example, Marchisio et al (1985) described some orchid endophytes in Italy, which can be placed in *Epulorhiza* according to the reassignment of Moore (1987). Currah et al (1987) isolated *E. repens* from some terrestrial orchids in Alberta, Canada and described the morphology in detail. Some researchers suggest that *Epulorhiza* endophytes may be common to all species of *Spiranthes* (Masuhara and Katsuya 1994, Zelmer and Currah 1997). The identification of Spir36 here also shows there is a relationship between *Spiranthes hongkongensis* and the genus *Epulorhiza*.

Both the RAPD and rDNA CAPS analyses show that MG IV isolates are highly dissimilar to all the other strains examined. Since septal ultrastructures of the isolates in this group was not determined, the MG IV isolates are tentatively assigned to the genus *Rhizoctonia*.

Andersen (1996) reported that RFLP patterns, pore ultrastructure, and sclerotial morphology of *Ceratorhiza* (*Ceratobasidium* anamorph) strains were not distinct from *Moniliopsis* (*Thanatephorus* anamorph)

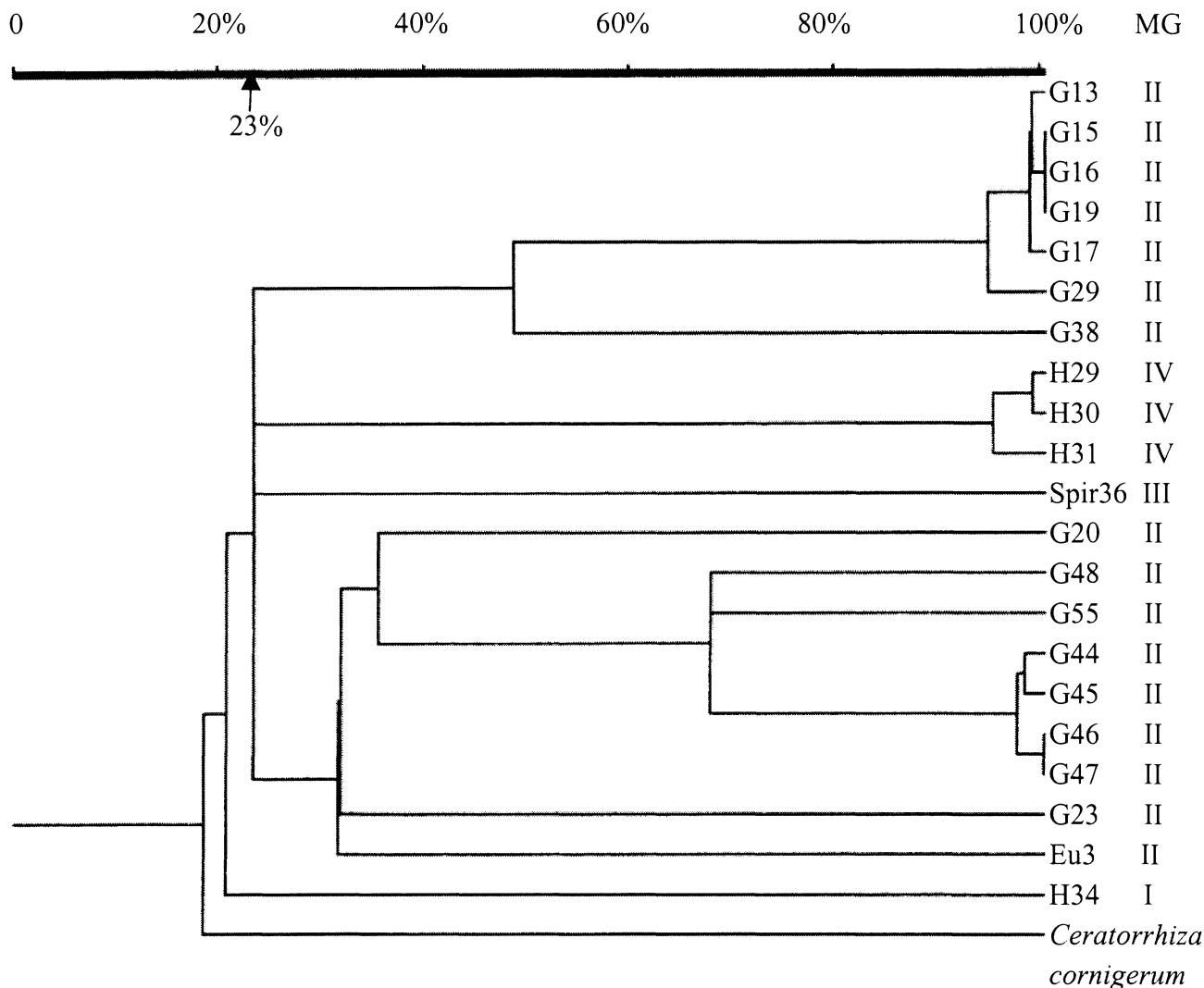


FIG. 1a. Phenogram showing the genetic similarity between *Rhizoctonia*-like isolates based on RAPD analysis.

strains. Earlier studies of sclerotial morphology (Tu and Kimbrough 1975, 1978) also showed a close relationship between *Ceratobasidium* and *Thanatephorus* anamorph. On the basis of rDNA CAPS analysis presented here, *Thanatephorus* is again considered to be close to *Ceratobasidium*.

Among the *Epulorhiza* species, only strains from morphological group II (MG II) showed strong ability to stimulate germination and development of some orchid seeds. CAPS patterns showed that there were close genetic relationships between these *Epulorhiza* species. The morphological group I (MGI) strain could stimulate seeds of only one orchid species to plantlet stage. This indicates that a specific group of fungi may be required by some orchid species during their germination and early development. And CAPS analysis is likely to have the potential to help us to find compatible mycorrhizal fungi for these orchids rapidly.

Some fungal isolates from roots of *Goodyera procera* and *Spiranthes hongkongensis* could not enhance seed germination of their hosts. However, the isolates from *G. procera* could form successful symbionts with seeds of other orchids such as *Spathoglottis pubescens*. One explanation for this phenomenon is that the fungal symbionts required by the adult orchid plants (or any intervening stage) may not necessarily be the same as those required for germination (Warcup 1973, Smericu and Currah 1989, Zelmer and Currah 1997). Another explanation is that the orchid plants were infected by several fungi at the same time and those endophytes we obtained were not the ones forming symbionts with their hosts.

In conclusion, it has been shown that DNA analysis can be used to provide molecular data to delineate *Rhizoctonia* strains at generic and sub-generic levels, especially when morphological characteristics are taken into account. In the current study, RAPD analysis

Similarity index

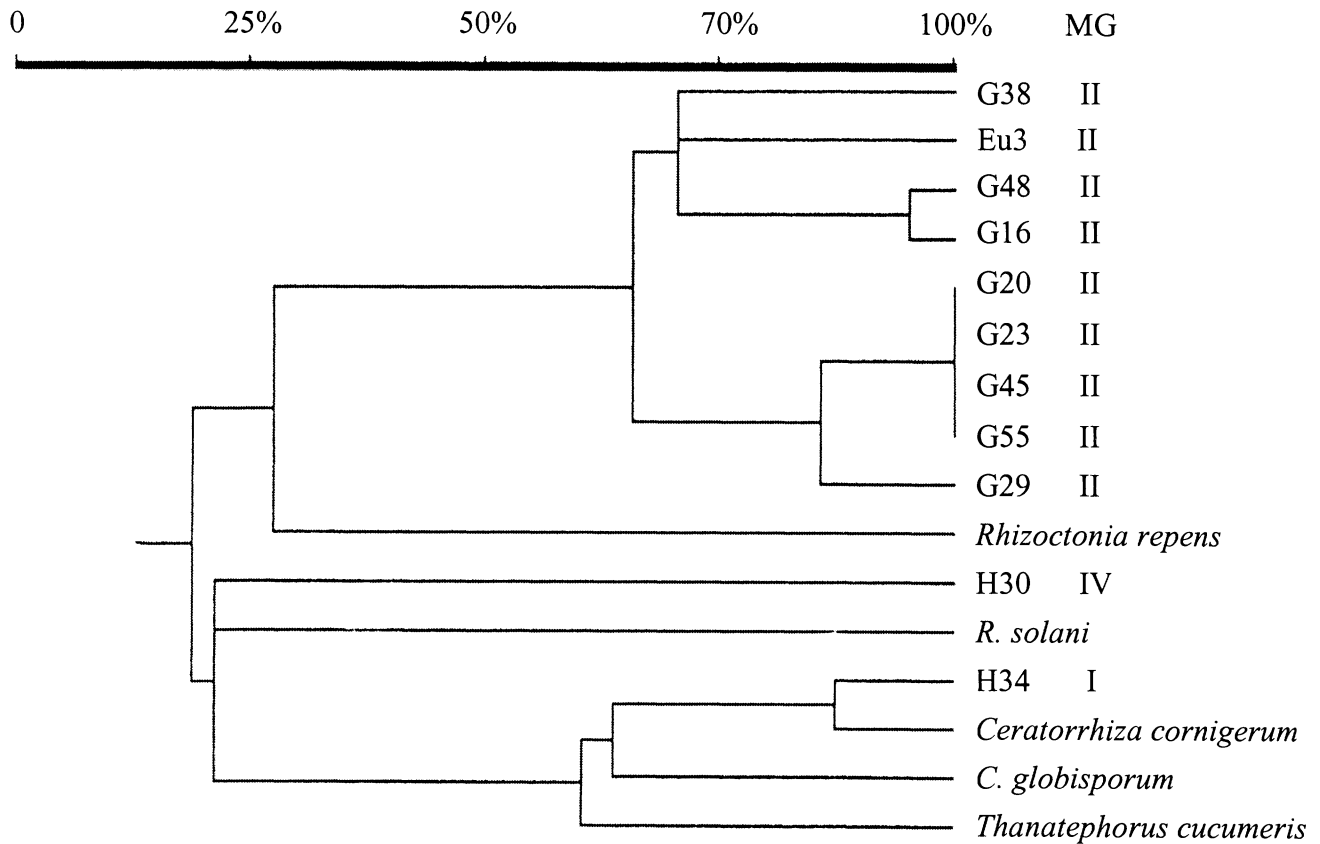


FIG. 1b. Phenogram showing the genetic similarity between *Rhizoctonia*-like isolates and some reference strains based on CAPS analysis.

TABLE IV. Germination and development of various orchid seeds on OMA medium with or without test fungal strain^a

Orchid species	No fungal strain								
	Eu3	G16	G19	G38	G55	H30	H34	Spir36	
<i>Arundina chinensis</i>	2	4	4	— ^b	4	4	2	2	2
<i>Cymbidium ensifolium</i>	0	0	0	0	0	0	0	0	0
<i>Eulophia sinensis</i>	0	0	0	0	0	0	0	0	0
<i>Goodyera procera</i>	0	0	0	0	0	0	0	0	0
<i>Habenaria dentata</i>	1	2	2	2	2	2	4	1	1
<i>H. rhodocheila</i>	0	0	0	0	0	1	0	0	0
<i>Liparis longipes</i>	0	4* ^c	4*	—	—	—	—	—	—
<i>Malaxis</i> sp.	2	2	3	—	—	—	—	—	—
<i>Paphiopedilum</i> sp.	0	0	0	0	0	—	—	—	0
<i>Peristylus tentaculatus</i>	0	0	0	0	0	—	0	—	0
<i>Phaius tankervilleae</i>	1	4*	1	—	1	1	0	0	0
<i>Spathoglottis pubescens</i>	3	4	4	4	4	4	3	0	3
<i>Spiranthes hongkongensis</i>	0	4	4	1	4	4	—	—	0
<i>Zeuxine gracilis</i>	1	2	2	2	2	2	2	0	2

^a Numbers refer to the stage of germination and development.

^b Not tested.

^c* Only one or a few seeds germinated to the stage.

on all isolates grouped the isolates according to their overall genetic similarity. The sample of strains in the CAPS analysis was then reduced to a smaller one by selecting the representative isolates within a high similarity group. By matching CAPS patterns and morphological characteristics, some isolates were then identified to generic level. Although the septal ultrastructure was not determined for all the isolates in this study, the combination of RAPD and CAPS analyses demonstrated the possibility of identifying some of the isolates relatively rapidly, and indicated the genetic variation between the isolates within the same genera. It is suggested that a more thorough and conclusive identification of these orchid endophytes would require DNA sequence analyses and phylogenetic inferences.

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