Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil

Olinto Liparini Pereira, Maria Catarina Megumi Kasuya, Arnaldo Chaer Borges, Elza Fernandes de Araújo

Abstract: To initiate a conservation program of the Orchidaceae from the Brazilian Atlantic rain forest with the purpose of ex situ conservation or reintroduction in the State of Minas Gerais, seven mycorrhizal *Rhizoctonia*-like fungal strains were isolated from roots of seven neotropical orchid species from three different Atlantic rain forest fragments. Taxonomic studies revealed that the isolates belong to the genera *Ceratorhiza* and *Epulorhiza*. The *Epulorhiza* isolates were identified as *Epulorhiza repens* (N. Bernard) R.T. Moore and *Epulorhiza epiphytica* Pereira, Rollemberg et Kasuya. RAPD analysis indicated higher polymorphism between *Epulorhiza epiphytica* and *Epulorhiza repens* than found in the PCR–RFLP analysis. RAPD and morphological analyses indicated a degree of relatedness among the *Ceratorhiza* isolates obtained from the roots of different *Oncidium* species. A combination of morphological and molecular characterizations permitted integration of fungal strain identification with genetic relatedness among the isolates, thus allowing some inferences to be made on specificity of these endosymbionts under field conditions.

Key words: biodiversity, *Ceratorhiza*, *Epulorhiza*, orchid mycorrhiza, *Rhizoctonia*-like, symbiosis, specificity.

Résumé : Les auteurs travaillent à mettre sur pied un programme de conservation pour les Orchidaceae de la forêt ombrophile atlantique du Brésil, avec l'objectif d'effectuer de la conservation ex situ et de la réintroduction, dans l'état de Minas Gerais. À cette fin, ils ont isolé sept souches de champignons de type *Rhizoctonia,* à partir de racines de sept espèces d'orchidées néotropicales, provenant de trois fragments différents de la forêt ombrophile de l'Atlantique. Les études taxonomiques révèlent que les isolats appartiennent aux genres *Ceratorhiza* et *Epulorhiza*. Ils ont identifié les isolats d'*Epulorhiza* comme *Epulorhiza repens* (N. Bernard) R.T. Moore et *Epulorhiza epiphytica* Pereira, Rollemberg et Kasuya. L'analyse RAPD montre un polymorphisme plus grand entre l'*Epulorhiza epiphytica* et l'*Epulorhiza repens,* que celui qu'on retrouve avec les analyses PCR–RFLP. Les analyses RAPD et morphologiques indiquent un certain degré de relation entre les isolats provenant des *Ceratorhiza* obtenus des racines de différentes espèces d'*Oncidium.* La combinaison des caractéristiques morphologiques et moléculaires permet d'intégrer l'identification des souches fongiques avec la relation génétique entre les isolats, permettant ainsi de déduire des informations sur la spécificité de ces endosymbiontes, sous des conditions de terrain.

Mots clés : biodiversité, *Ceratorhiza, Epulorhiza*, mycorhizes des orchidées, type *Rhizotonia*, symbiose, spécificité.

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Introduction

In nature, orchids utilize mycorrhizal fungi to initiate seed germination and for seedling establishment, at least in the early stages of their development (Andersen and Rasmussen 1996; Harvais and Hadley 1967; Currah et al. 1997; Peterson et al. 1998; Rasmussen 2002). The fungi provide exogenous carbon sources, resulting in the orchids dependence on endomycorrhizal fungi as an absolute requirement for completing their life cycle, in one of the most pervasive mutualistic relationships found in terrestrial ecosystems (Leake 1994; Smith and Read 1997). The Brazilian Atlantic rain forest is one of the most threatened tropical ecosystems in the world (Mori 1989). This ecosystem is recognized for its extreme biodiversity, with a very diverse array of terrestrial and, especially, epiphytic orchids (Miller et al. 1996). However, disturbances caused by land-clearing for agriculture, forestry, urbanization, pastureland, natural resource and extraction, as well as frequent fires, have resulted in an increased threat of extinction for many orchid species in Minas Gerais (Mendonça and Lins 2000) and other Brazilian states (Ruschi 1986; Miller et al. 1996).

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Mycorrhizal fungi associated with the roots of orchids have thus been isolated, characterized, and stored as important resources for the conservation of orchid species (Arditti et al. 1990; Zettler 1997*a*, 1997*b*; Batty et al. 2001*a*, 2001*b*). Studies have employed mycorrhizal fungi for symbiotic seed germination of some endangered orchid species (Zettler and McInnis 1993; Zettler and Hofer 1998; Zettler et al. 2000; Zettler et al. 2001; Takahashi et al. 2001; Stewart and Zettler 2002) with potential to be used to establish orchid populations in restored habitats. However, most work on orchid mycorrhizal fungi has dealt with terrestrial, temperate orchids, and very few reports on associations between endomycorrhizal fungi and epiphytic or tropical species exist in the literature (Bayman et al. 1997; Richardson and Currah 1995; Richardson et al. 1993; Zettler et al. 1998, 1999; Pereira et al. 2003*b*).

Identifying *Rhizoctonia* and *Rhizoctonia*-like endophytes from mycorrhizal orchid roots is very problematic because of a paucity of sufficiently distinctive and stable morphological features in culture (Andersen 1990). However, a variety of approaches have been developed to identify them: morphological characters (Currah and Zelmer 1992; Currah and Sherburne 1992; Andersen 1996; Currah et al. 1997), anastomosis groups (Carling et al. 1999; Sen et al. 1999), and the use of molecular techniques (Sen et al. 1999; Kristiansen et al. 2001; Shan et al. 2002; Otero et al. 2002; Ma et al. 2003).

The objective of our study was to characterize *Rhizoctonia*-like mycorrhizal fungi associated with seven neotropical orchid species native to Minas Gerais State, Brazil, by employing morphological and molecular techniques.

Materials and methods

Collection of orchid root samples

Intact young and healthy roots of seven neotropical orchid species from Atlantic rain forest fragments in Minas Gerais State, Brazil, were selected for isolation of orchidaceous endosymbiotic fungi (Table 1). Root fragment samples from about five plants of each species were collected in their native habitat between 2000 and 2001, transported within 1 h to the laboratory, and washed under running tap water. Samples were collected only when the plants were flowering to facilitate identification of the orchid species. Each sample consisted of about 15–20 roots fragments of 5–10 cm each. Part of the root sample was selected for anatomical studies and the other part for endosymbiont isolation.

Isolation and morphological characterization

Samples of healthy root fragments from epiphytic orchid species were surface-sterilized by immersion for 1 min in a 70% ethanol solution, followed by immersion for 5 min in 2% sodium hypochloride solution and finally by rinsing five times with sterilized distilled water. The roots were then decorticated with a sterile scalpel, macerated with a pestle in a porcelain mortar, spread on Petri dishes containing 25 mL of modified Melin-Norkrans medium (Marx 1969), and incubated at 26 °C in the dark. Pelotons that were visible on the isolation medium surface under the light microscope were monitored for the presence of active hyphal growth. Hyphal tips were selected, removed aseptically with a scalpel, and transferred into Petri dishes containing potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan), and incubated for 7 d at 26 °C for the isolation of only peloton-forming fungi (Pereira et al. 2003*b*). Agar plugs (9 mm in diameter) from the border of resulting colonies were transferred into Petri dishes containing either PDA, corn meal agar (CMA, Difco), coconut milk agar, malt extract agar, or oat meal agar (Zelmer and Currah 1995) for morphological characterization. Colony diameter, colour, border, and aerial mycelial aspect were recorded for five replicate plates after 72 h of incubation in the dark at 26 °C. Monilioid cells were examined in colonies grown on CMA, and runner hyphae were measured in colonies grown on PDA. Nuclei numbers were counted in young hyphal cells after staining with HCl-Giemsa (Saksena 1961). Cultures were kept on PDA or in sterilized distilled water at 4 °C for long-term storage (Sneh and Adams 1996).

Enzymatic assays

Isolates were grown on minimal medium with carboxymethyl cellulose as the sole carbon source for cellulase (Teather and Wood 1982). After 7–10 d of incubation at 26 °C, the agar medium was flooded with an aqueous solution of Congo red (1 $mg \cdot mL^{-1}$) for 15 min. The Congo red solution was then poured off, and the medium was treated further by flooding with 1 $mol \cdot L^{-1}$ NaCl for 15 min. The cellulose hydrolysis zone was readily observable on the medium surface. Polyphenol oxidase activity was detected using the method of Davidson et al. (1938).

Identification of fungal species

The isolated fungi were identified using a dichotomous key for genera of mycorrhizal fungi associated with orchids (Currah and Zelmer 1992), and a key to *Epulorhiza* species (Currah et al. 1997) that includes *Epulorhiza repens* (N. Bernard) R.T. Moore (Moore 1987, 1996), *Epulorhiza anaticula* (Currah) Currah (Currah et al. 1990), *Epulorhiza albertaensis* Currah and Zelmer (Currah and Zelmer 1992), *Epulorhiza calendulina* Zelmer and Currah (Zelmer and Currah 1995), and *Epulorhiza inquilina* Currah, Zettler, and McInnis (Currah et al. 1997). Specimen subcultures are being maintained in the Departamento de Microbiologia / BIOAGRO (see Table 2 for isolate codes) and voucher specimens were deposited at VIC Herbarium. Random amplified polymorphic DNA (RAPD) and polymerase chain reaction – restriction fragment length polymorphism (PCR–RFLP) analyses of the rDNA internal transcribed spacer (ITS) region were performed to supplement the limited morphological characteristics available for *Rhizoctonia*-like fungi identification.

Teleomorph induction experiments

Induction of basidiome production in the seven isolates was attempted by the soil-over-culture (Tu et al. 1969) and plant infection (Flentje 1956) techniques. *Eucalyptus grandis* W. Hill ex Maiden and *Lycopersicum esculentum* Mill. seedlings were used for infection. The induction was also carried out in Petri dishes containing 25 mL of each media: PDA, CMA, coconut milk agar, malt extract agar, oat meal agar (Zelmer and Currah 1995), V-8 agar juice

Table 1. Orchid species, habitat, and location from which *Rhizoctonia*-like endophytes were isolated and used in our study.

Orchid species	Habitat	Location*	Isolate code
Epidendrum rigidum Jacq.	Epiphytical	Pedra do Anta	M1
Oncidium flexuosum (Kunth) Lindl.	Epiphytical	Viçosa	M2
Isochilus lineares (Jacq.) R. Br.	Epiphytical	Carangola	M3
Maxillaria marginata Fenzl	Epiphytical	Carangola	M4
Oeceoclades maculata (Lindl.) Lindl.	Terrestrial	Viçosa	M5
Polystachya concreta (Jacq.) Garay and Sweet	Epiphytical	Pedra do Anta	M6
Oncidium varicosum Lindl. and Paxton	Epiphytical	Viçosa	M7

*Cities from Minas Gerais State, Brazil.

(Dhingra and Sinclair 1995), and vegetable–broth–agar medium (Pereira et al. 2003*a*). Cultures were incubated in the dark for 48 h and later submitted to photoperiods of 12 h near-ultraviolet irradiation : 12 h darkness (Leach 1962) for 25 d at 25 °C. The experiments were routinely observed for fruit body formation. Each treatment was replicated three times and the experiment was carried out twice.

Microscopic observations

Roots collected from wild orchids were transversally cut using a freezing-stage microtome in 30-µm-thick slices that were mounted on glass slides and examined using an light microscope for peloton presence. Pelotons were also visualized on the Melin-Norkrans agar surface using an inverted microscope during isolation.

DNA extraction

Prior to DNA extraction, the isolates were grown on cellophane membranes overlaid on PDA for 2 weeks in the dark at 26 °C. The outer borders of young (7 d old) colonies were excised and the underlying cellophane was removed. Total DNA was extracted from 0.8 to 1.0 g of the fresh mycelium, according to the procedures of Speacht et al. (1982). The DNA pellet was dissolved in 30 µL of double-distilled water and the DNA concentration was estimated by comparison with known standards in 0.8% agarose gels stained with ethidium bromide. DNA samples were stored in Eppendorf tubes at –20 °C.

RAPD analysis

PCR amplification of DNA sequences was performed with each of the arbitrarily selected primers: OPH 06, OPD 04, OPG 11, OPF 14, OPH 03, OPP 01, OPG 05, OPE 02, OPC 02, OPC 05, OPC 08, OPC 13, OPJ 02, OPW 07, OPH 14, OPW 02, and OPE 19 (Operon Technologies Inc., Alameda, California). Each 20-µL PCR reaction contained 10 mmol·L^{-1} Tris-HCl (pH 8.3), 50 mmol·L^{-1} KCl, 2 mmol·L^{-1} $MgCl_2$, 100 µmol·L^{-1} each of dATP, dCTP, dGTP, and dTTP, 0.4 µmol·L^{-1} of one random primer, 10 ng of genomic DNA, and 1 U of *Taq* DNA polymerase. Negative controls without DNA were run in all experiments. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, Massachusetts), programmed for 40 cycles, with each cycle consisting of one denaturating step at 94 °C for 60 s, one annealing step at 50 °C for 60 s, and one extension step at 72 °C for 90 s. After the 40th cycle, a final extension step was performed at 72 °C for 7 min. Amplification products were electrophoresed in 1.5% agarose gels immersed in TEB running buffer (90 mM Tris-borate, 1 mmol·L^{-1} EDTA, pH 8.0) and run for 4 h at 100 V. The gels were stained with ethidium bromide (0.5 µg mL^{-1}) and photographed under UV light with a Polaroid camera. The images were also captured and stored using a photodocumentation system (Eagle Eye II, Stratagene, La Jolla, California).

PCR amplification and RFLP analysis

Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), constructed for molecular phylogenetic studies (White et al. 1990), were used for amplification of the fungal rDNA ITS region of all isolates. The components for 25-µL PCR reactions were 10 ng of total DNA, 40 pmol of each primer, 10 mmol·L^{-1} Tris-HCl (pH 8.3), 50 mmol·L^{-1} KCl, 2 mmol·L^{-1} $MgCl_2$, 0.1 mmol·L^{-1} of each dNTP (dATP, dCTP, dGTP, and dTTP), and 1 U of *Taq* DNA polymerase. The cycling parameters were 40 cycles, each cycle consisting of a denaturation step at 94 °C for 60 s, an annealing step at 52 °C for 60 s, and an extension step at 72 °C for 90 s. After the 40th cycle, a final extension step at 72 °C for 7 min was performed. The amplification reaction was performed in a PTC-100 thermocycler (MJ Research, Inc.). After amplification, the DNA products were analysed by eletrophoresis in a 1.5% (*w*/*v*) agarose gel immersed in TBE buffer (90 mmol·L^{-1} Tris-borate, 2 mmol·L^{-1} EDTA, pH 8.0) or precipitated for RFLP analysis. DNA was precipitated by adding ammonium acetate to a final concentration of 2.5 mol·L^{-1} followed by 2.5 volumes of absolute ethanol. The samples were kept at –20 °C for 5 h and centrifuged at 14 000*g* for 30 min. Pellets were washed with 70% (*v*/*v*) ethanol, resuspended in 10 µL H_2O, and digested with five arbitrarily selected endonucleases (*Alu*I, *Hae*III, *Hin*fI, *Eco*RI, and *Cla*I) in a 20-µL final volume. DNA digestion was performed following the manufacturer's instructions. DNA fragments were size-fractionated in 2% (*w*/*v*) agarose gel. The gels were stained with ethidium bromide (0.5 µg·mL^{-1}), and photographed under UV light with a Polaroid camera. The images were also captured and stored using the photodocumentation system Eagle Eye II.

Numerical analysis

Qualitative morphological features were used to define morphological groups. The selected morphological characteristics were based on cultural features known to be of important taxonomical value for mycorrhizal *Rhizoctonia*-like fungi (Currah and Zelmer 1992). The scoring was done con-

sidering the presence (1) or absence (0) of such qualitative morphological features.

Scoring of PCR–RFLP and RAPD analyses was done considering presence (1) or absence (0) of determined DNA fragments for different samples. The presence of a determined band (similar size) in all genotypes compared indicated similarity, while presence in one and absence in the other indicated dissimilarity. The data were analyzed by the statistical program GENES (Cruz 1997). The genetic distances among the isolates were calculated using Nei–Li's coefficient (Nei and Li 1979), according to the formula:

$$S_{ii'} = 2a/(a + b + c)$$

where $S_{ii'}$ is the Nei–Li's coefficient for genotypes i and i', a is the band or the same allelic form present in genotypes i and i', b is the band or the same allelic form present in genotype i, and c is the band or the same allelic form present in genotype i'.

Cluster analyses of the morphological and molecular data were performed by the unweighted pair-groups method algorithm (UPGMA), using the STATISTICA program, version 4.2 (Statsoft 1995). To aid the characterization of the groups, the Tocher cluster method and rPearson method, as described by Cruz and Regazzi (1994) were also performed. Agreement between values in the three matrices was estimated from the coincidence coefficient, which represents the correlation between the highest and lowest genetic distance values, starting from the average value of each matrix (Cruz and Regazzi 1994).

Results

Peloton observation

The majority of root sections showed intense colonization. Intact (Figs 1, 2) or degraded (Fig. 3) pelotons were observed in about 75%–80% of the root cortical cells of *Isochilus lineares*, *Maxillaria marginata*, *Oncidium flexuosum*, *Oncidium varicosum*, and *Oeceoclades maculata*, whereas *Epidendrum rigidum* and *Polystachya concreta* showed very low colonization frequency, with only about 5%–10% of the root cortical cells colonized. In intensely colonized species, the large cortical cells in the middle regions of the root sections contained clumps of degenerating hyphae, whereas cells at the edge contained intact hyphae. Only one distinct peloton-forming fungus was found in all root segment samples of a given species. Intact pelotons of *Epidendrum rigidum*, *P. concreta*, and *Oeceoclades maculata* were formed by narrower hyphae, whereas intact pelotons of the remaining species were formed by broader hyphae. Fungal hyphae were observed on the velamen surface of root fragments of all epiphytic species examined in our experiment.

Isolation

It was much more difficult to isolate endosymbiotic fungi from epiphytic orchid species than from the terrestrial orchid, *Oeceoclades maculata.* Dark septate endophytes (Jumpponen and Trappe 1998), were consistently isolated from the culture media of both types of orchid species. All isolates from a given orchid species appeared to be the same fungal species, based on morphological and morphometric data (not shown). Therefore, one isolate was selected for each plant species and codified, in order of isolation, as M1 to M7, corresponding to the host species *Epidendrum rigidum*, *Oncidium flexuosum*, *Isochilus lineares*, *M. marginata*, *Oeceoclades maculata*, *P. concreta*, and *Oncidium varicosum*, respectively (Table 1).

Cultural characteristics, nuclear condition, enzymatic assays, and isolate identification

The morphological characteristics analyzed and the results of the enzymatic tests are listed in Table 2. All isolates were binucleate (Fig. 4) and formed monilioid cells in culture (Figs. 5–8). Although all isolates were binucleate, two distinct groups could be recognized, based on morphology, growth on culture media, and enzymatic tests (Fig. 9). The first group, including isolates M1, M5, and M6, consists of fungi with scant and submerged mycelia, with very slow growth in culture, thin vegetative hyphae, mostly spherical monilioid cells, and absence of polyphenol oxidase activiy. The second group, containing isolates M2, M3, M4, and M7, consists of fungi with abundant, cottony aerial mycelia, high growth rates in culture, broad vegetative hyphae, mostly cylindrical to barrel-shaped monilioid cells, and presence of polyphenol oxidase activity. No clamped hyphae were observed and pelotons formed by hyphae with clamps were not noted.

All peloton-forming isolates were *Rhizoctonia*-like fungi based on morphological criteria. Isolates M1, M5, and M6 belong to the genus *Epulorhiza* and isolates M2, M3, M4, and M7 belong to *Ceratorhiza*. *Epulorhiza* isolates were identified at the species level, *Epulorhiza repens* (N. Bernard) R.T. Moore and *Epulorhiza epiphytica* Pereira, Rollemberg et Kasuya (Pereira et al. 2003*b*) (Table 2), while species identification was not possible for the *Ceratorhiza* isolates because the morphological features of these organisms were not sufficiently distinctive and stable in culture. No *Ceratorhiza* isolate was assignable to *C. pernacatena* Zelmer & Currah (Zelmer and Currah 1995).

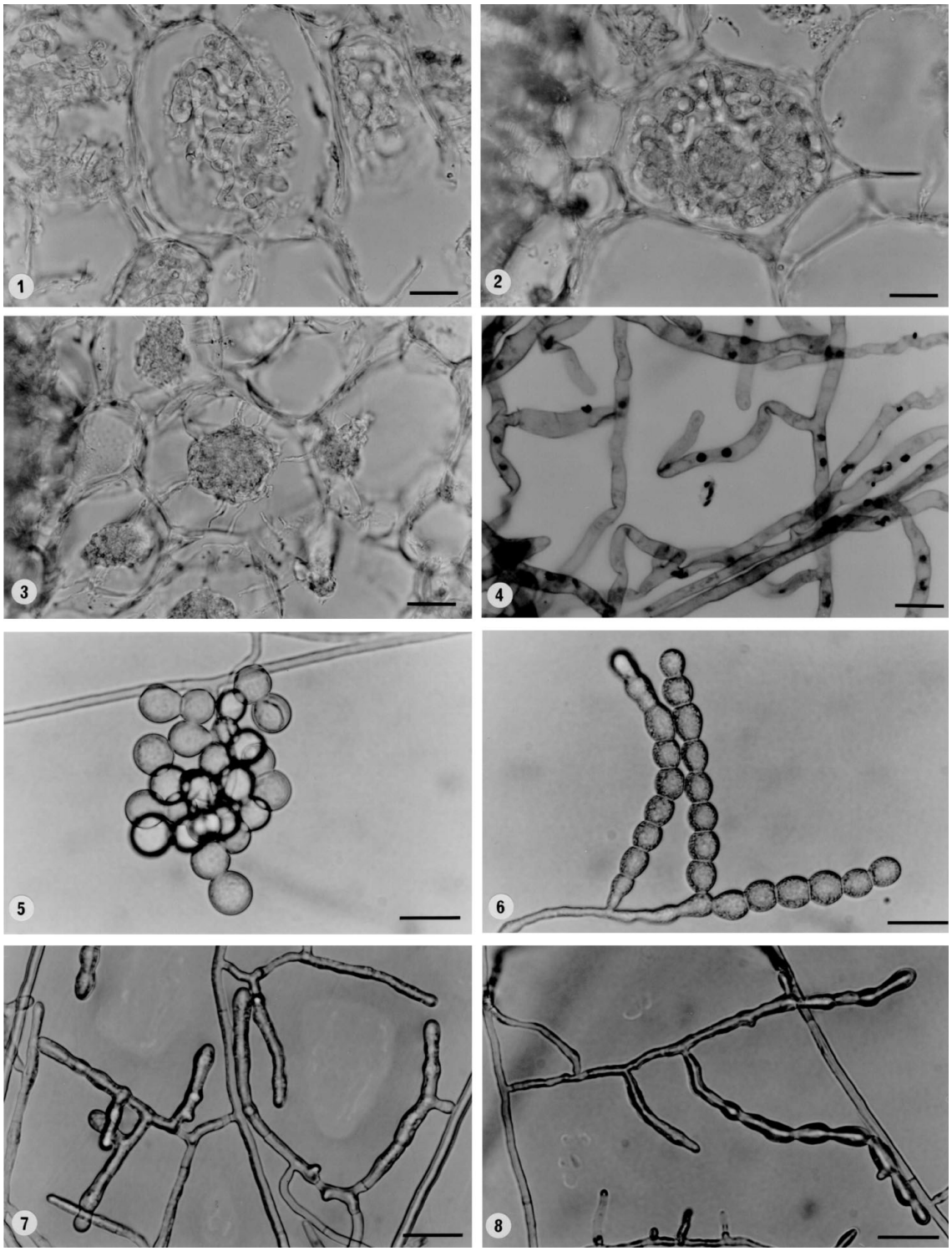
Teleomorph induction

Induction of basidiome production was unsuccessful in all seven isolates.

PCR–RFLP analysis

The ITS region, targeted by the universal primers ITS1 and ITS4 was amplified in all seven isolates (Fig. 10). PCR products varied in size from 640 to 730 bp. Amplification of ITS sequences from *Epulorhiza* isolates M1, M5, and M6 generated a common fragment of approximately 640 bp. Fragments from *Ceratorhiza* isolates M2, M3, M4, and M7 ranged from 660 to 730 bp (Table 3). The longest fragment (730 bp) was from the sole sclerotia-forming *Ceratorhiza* isolate (M7). The smaller polymorphic fragment products, produced by digestion of the ITS regions with the five endonucleases, *Alu*I, *Hae*III, *Eco*RI, *Hin*fI, and *Cla*I, are shown in Table 3. Endonucleases *Alu*I, *Eco*RI, *Hin*fI, and *Cla*I generated the same restriction pattern for all *Epulorhiza* isolates while *Hae*III generated different restriction patterns, separating *Epulorhiza repens* (M5) from both *Epulorhiza epiphytica* isolates (M1 and M6). The pair of *Epulorhiza epiphytica* isolates M1 and M6 produced the same restric-

Figs. 1 and 2. Intact pelotons observed in root cortical cells of two neotropical orchid species. Fig. 1. *Isochillus lineares*. Scale bar = 10 μm. Fig. 2. *Oncidium varicosum*. Scale bar = 10 μm. **Fig. 3.** Digested peloton observed in the root cortical cell of *Polystachia concreta*. Scale bar = 10 μm. **Fig. 4.** Binucleate nuclear condition of young hyphae cell of *Epulorhiza repens*, stained with HCl-Giemsa. Scale bar = 10 μm. **Figs. 5–8.** Monilioid cells formed in corn meal agar by four *Rhizoctonia*-like endophytes. Fig. 5. *Epulorhiza repens* isolate M5. Scale bar = 20 μm. Fig. 6. *Epulorhiza epiphytica* isolate M6. Scale bar = 20 μm. Fig. 7. *Ceratorhiza* sp. isolate M7. Scale bar = 30 μm. Fig. 8. *Ceratorhiza* sp. isolate M3. Scale bar = 30 μm.



tion patterns for all five endonucleases utilized in the PCR–RFLP analysis, as did the pair of *Ceratorhiza* isolates M3 and M4. All endonucleases generated different restriction patterns for the M2 and M7 and the M3 and M4 pairs of *Ceratorhiza* isolates (Table 3). A genetic distance matrix was constructed (not shown) based on restriction products

Table 2. Summary of culture and morphometric characteristics, and enzimatic assay results for the seven *Rhizoctonia*-like isolates.

	Isolate code						
Characteristics	M1	M2	M3	M4	M5	M6	M7
Colony on PDA							
Color	Cream	Pale brown	White	White	Cream	Cream	Pale brown
Appearance	Sebaceous	Cottony	Cottony	Cottony	Sebaceous	Sebaceous	Cottony
Margin	Submerged	Aerial	Aerial	Aerial	Submerged	Submerged	Aerial
Aerial mycelia on PDA							
Appearance	Scant	Abundant	Abundant	Abundant	Scant	Scant	Abundant
Diameter of hyphae (µm)	2	5.0–6.0	4.0–5.0	4.0–5.0	2.0–3.0	2	5.5–6.5
Nuclear condition	Binucleate	Binucleate	Binucleate	Binucleate	Binucleate	Binucleate	Binucleate
Monilioid cells on CMA							
Dimensions (µm)	(7.0–10) × (8.0–9.5)	(20–26) × (8.0–10)	(17–25) × (9.5–14)	(17–27) × (9.5–14)	(14–16) × (10–15)	(7.0–10) × (8.0–9.5)	(18–24) × (7.5–10)
Shape	Globose	Barrel	Barrel	Barrel	Globose to ellipsoidal	Globose	Barrel
Surface	Foveate	Smooth	Smooth	Smooth	Smooth	Foveate	Smooth
Sclerotia**	Absent	Absent	Absent	Absent	Absent	Absent	Present
Teleomorph	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Enzimatic assay							
For cellulase	Present	Present	Present	Present	Present	Present	Present
For polifenol-oxidase	Not detectable	Present	Present	Present	Not detectable	Not detectable	Present
Identification	*Epulorhiza epiphytica*	*Ceratorhiza* sp.	*Ceratorhiza* sp.	*Ceratorhiza* sp.	*Epulorhiza repens*	*Epulorhiza epiphytica*	*Ceratorhiza* sp.
Voucher	VIC 27802	VIC 27803	VIC 27804	VIC 27805	VIC 27806	VIC 27807	VIC 27808

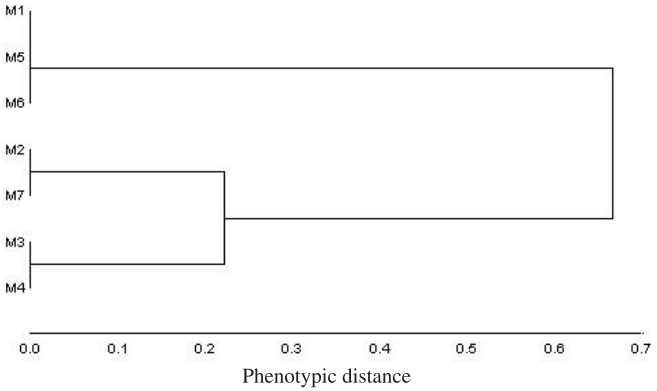
Note: PDA, potato dextrose agar; CMA, corn meal agar.

Table 3. Fragment lengths produced by each isolate after PCR–RFLP of the rDNA ITS region, followed by digestion with the restriction enzymes *Alu*I, *Hae*III, *Eco*RI, *Hin*fI, and *Cla*I.

Isolate	Total length of PCR products (bp)	Length of digestion fragments (bp) generated by restriction enzymes				
		*Alu*I	*Hae*III	*Eco*RI	*Hin*fI	*Cla*I
M1	640	600	600	640	180, 150, 130, 90*	610
M2	660	420, 110, 80*	370, 110*, 80*	360, 300	350, 300	380, 240
M3	660	450, 190	350, 110,* 100*	370, 290	350, 300	410, 230
M4	660	450, 190	350 110,* 100*	370, 290	350, 300	410, 230
M5	640	600	490, 100*	640	180, 150, 130, 90*	610
M6	640	600	600	640	180, 150, 130, 90*	610
M7	730	280, 190, 120	540, 120, 110	380, 330	360, 350	400, 280

Note: Identification of the isolates is according to Table 2.
*Possible doublet.

Fig 9. Cluster analysis of the seven *Rhizoctonia*-like isolates based on qualitative morphological data. The dendrogram was generated from phenotypic similarity coefficients based on the unweighted pair-group method using the arithmetic average. Identification of the isolates was according to Table 2.



from the seven rDNA regions analysed. Cluster analysis based on these genetic distances separated the isolates into three groups: group I, containing *Epulorhiza* isolates; group II, containing the *Ceratorhiza* isolate M2; and group III, containing *Ceratorhiza* isolates M3, M4, and M7 (Fig. 11).

RAPD analysis

PCR reactions with 17 primers produced a total of 432 reproducible bands. No bands were present in negative controls, and most fragments considered for analysis were polymorphic. Figure 12 shows the amplification pattern obtained with primers OPH 03, OPH 06, and OPP 01. The number of fragments per primer varied between 18 and 35, with an average of 25. As in the PCR–RFLP analysis, RAPD analysis resulted in the same patterns for *Epulorhiza* isolates M1 and M6 and *Ceratorhiza* isolates M3 and M4. Cluster analysis based on pairwise genetic distances among isolates grouped them into three groups: group I, containing the *Epulorhiza* isolates; group II, containing *Ceratorhiza* isolates M3 and M4; and group III, containing *Ceratorhiza* isolates M2 and M7 (Fig. 13).

Correlation and coincidence analyses between morphological and molecular characterizations

A simple coincidence analysis was performed using the genetic distance values obtained between isolate pairs that revealed high superior and inferior coincidence percentages (Table 4). Thus, a high degree of similarity is observed among the groups formed by the different analyses (morphological, PCR–RFLP of the rDNA ITS region, and RAPD) (Table 5).

Discussion

Root colonization

All mycorrhizas of epiphytic orchids were very similar, possessing intact pelotons in the outermost layer of cortical cells, as described by Zelmer et al. (1996) for *Corallorhiza* species. These pelotons probably serve as a source of inoculum for the recolonization of cells deeper in the root cortex (Zelmer et al. 1996), and they may also serve as an important inoculum source for colonization of seeds on or near the roots of the mother plant. We have observed that protocorms, as well as seedlings, are commonly present on the root surface of mature epiphytic orchid species in the field, as reported by Miller and Warren (1995) and Miller et al. (1996). Thus, the presence of young pelotons in the border may be an important reproductive strategy used by epiphytic orchids in their natural environments. Patterns consisting of synchronously formed and degraded patches of pelotons (Zelmer et al. 1996) were not observed in our experiment, probably because the root fragments of each plant species used in our study were colonized by only one fungal species. The isolation of morphologically identical fungi from each orchid species also supports this hypothesis.

Isolation, identification, and morphological characterization

Epulorhiza epiphytica from the roots of *Epidendrum rigidum* and *P. concreta* was the first mycorrhizal fungi isolated from a Brazilian neotropical species (Pereira et al. 2003*b*). The same plant population, from the same geographical region was selected for the present study. A seasonal search for a *Tulasnella* species was conducted at this site; however, no hymenia were observed. Failure to induce basidiome production in *Epulorhiza epiphytica* and the other isolates was expected. Hymenia of teleomorphs connected to *Rhizoctonia*-like fungi are very rarely observed in culture media, and basidiome induction in *Ceratorhiza* and *Epulorhiza* is often unsuccessful (Zelmer et al. 1996; Shan

Fig 10. Fragments produced by PCR–RFLP of the rDNA ITS region (A) and the fragments produced after restriction with the enzyme *Eco*RI (B). Lane Y, molecular fragment size marker ØX 174 digested with *Hae*III. Identification of the isolates was according to Table 2.

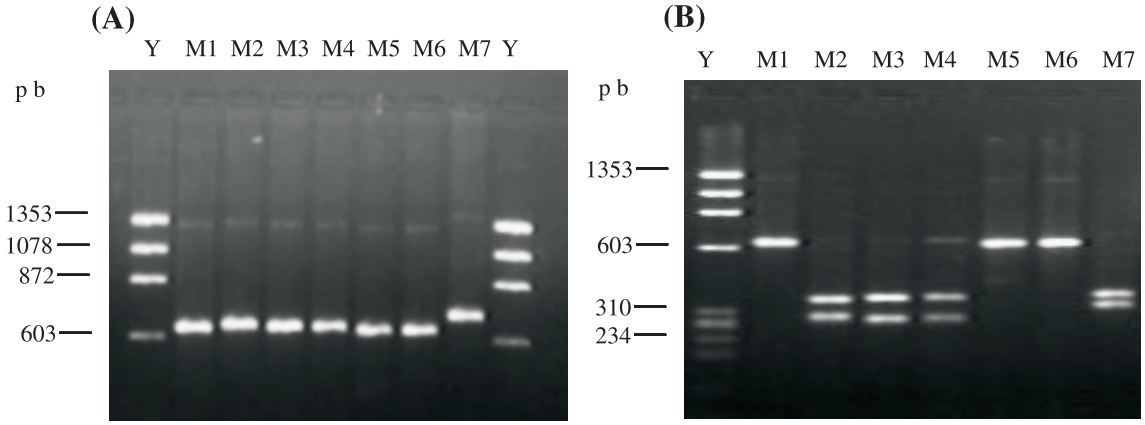
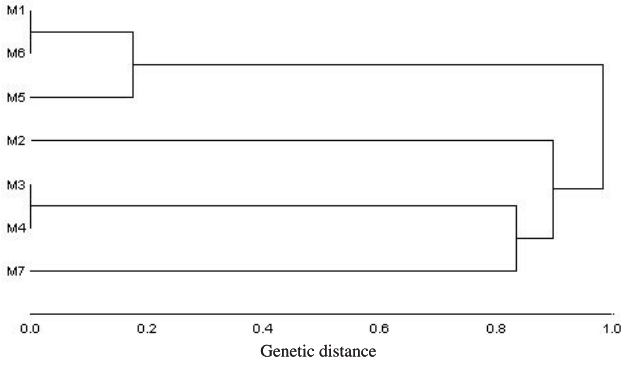


Fig 11. Cluster analysis of the seven *Rhizoctonia*-like isolates based on results of PCR–RFLP of the rDNA ITS region. The dendrogram was generated from genetic similarity coefficients based on the unweighted pair-group method using the arithmetic average. Identification of the isolates was according to Table 2.



et al. 2002; Ma et al. 2003). It appears to be easier to induce the sexual stage in mycorrhizal multinucleate *Rhizoctonia* in culture (Currah 1986; Zelmer et al. 1996). *Epulorhiza epiphytica* and *Epulorhiza repens* were identified in our study since they possess characteristic monilioid cells. However, the *Ceratorhiza* isolates possess extremely variable, growth-medium-dependent morphological characters, which have little taxonomical value for species identification (Andersen 1990).

Epulorhiza repens, anamorph of *Tulasnella deliquescens* (Juel) Juel, isolated from the mycorrhizas of *Oeceoclades maculata*, is reported for the first time in Brazil. Otero et al. (2002) also tried to isolate the mycorrhizal partner of *Oeceoclades maculata*, but the isolates obtained by them were too divergent from the other *Rhizoctonia*-like species to be included in their phylogenetic analysis. *Epulorhiza repens* is a widespread fungal species (Roberts 1999), and the ability of *Oeceoclades maculata* to associate with this fungus may be contributing to the dispersal success of this orchid species, regarded as a invasive weed in some situations.

Epulorhiza epiphytica was consistently isolated from roots of *Epidendrum rigidum*. *Epulorhiza* has rarely been documented in epiphytic orchids, although an *Epulorhiza* sp. isolate was found associated with roots of the epiphytic orchid *Epidendrum conopseum* R. Brown (Zettler et al. 1998). Additionally *Epulorhiza* have been isolated from other *Epidendrum* spp. in Brazil.

Based on the cultural characteristics of *Epulorhiza epiphytica*, this species could be considered congeneric with *Epulorhiza repens*, *Epulorhiza anaticula*, and *Epulorhiza albertensis*, belonging to the group 1 proposed by Ma et al. (2003) in the molecular phylogeny of *Epulorhiza* isolates from tropical orchids in Singapore.

Isolating *Rhizoctonia*-like fungi from the mycorrhizas of the six neotropical epiphytic orchids studied is probably difficult because epiphytic orchid species do not have the massive mycorrhizal colonization observed in terrestrial orchids. Little mycorrhizal infection has also been observed for other tropical epiphytic orchid species (Otero et al. 2002).

Only binucleate *Rhizoctonia*-like fungi were isolated from the six epiphytic orchid species in this study. In contrast, in a study by Bayman et al. (1997) only multinucleate *Rhizoctonia* fungi were isolated from roots of epiphytic orchids, perhaps because of host-specificity, since that study involved only orchids of the genus *Lepanthes* Swartz. Antibiotics were also used in the isolation medium by Bayman et al. (1997), so the mycorrhizal fungal diversity associated to *Lepanthes* spp. may have been underestimated, since some mycorrhizal *Rhizoctonia*-like fungi are sensitive to antibiotics in the isolation medium (Zelmer et al. 1996).

The combination of nuclear condition, mycelial morphological characteristics in culture media, and enzyme activities proved useful for identifying orchid mycorrhizal endosymbionts isolated in our study. Cluster analysis of these combined characteristics identified three groups (Fig. 9). The first group contained the *Epulorhiza* isolates, indicating that the analysis clearly separated the fungi at the genus level. However, only morphometric data and some unique morphological characters, which could not be utilized in the cluster analysis, could separate the species within *Epulorhiza*. The second group contained two *Ceratorhiza* isolates, obtained from different orchid species of the same genus, sampled in the same geographical location, indicating the same or possibly a closely related *Ceratorhiza* species.

Fig 12. Gel electrophoresis of random amplified polymorphic DNA (RAPD) fragments obtained with the primers OPH03 (A), OPH06 (B), and OPP01 (C). Lane Y, molecular fragment size marker λ digested with *Eco*RI, *Bam*HI, and *Hin*dIII. Identification of the isolates was according to Table 2.

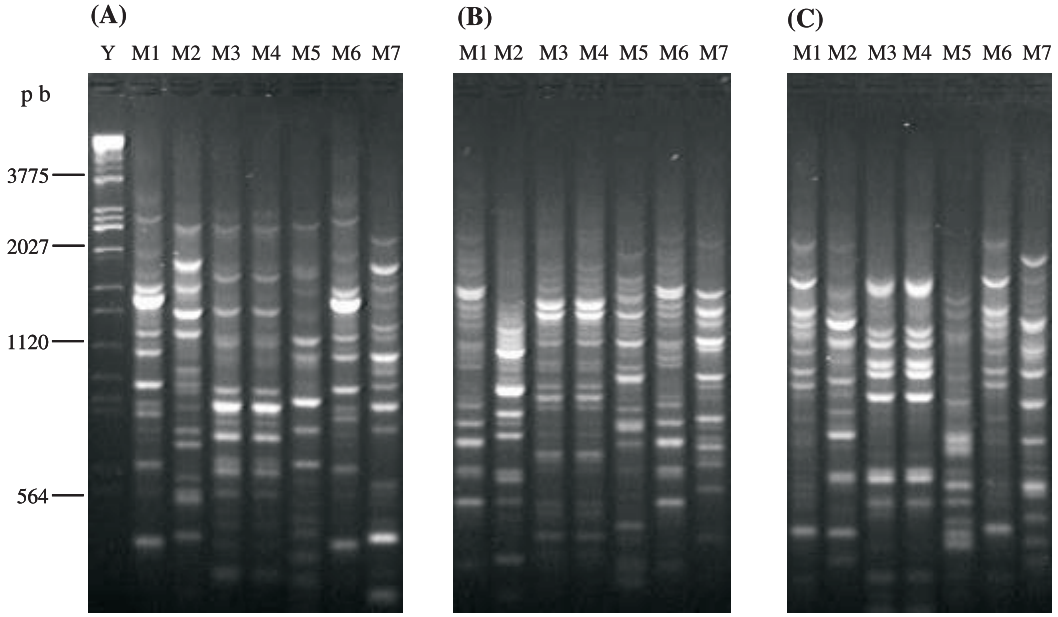
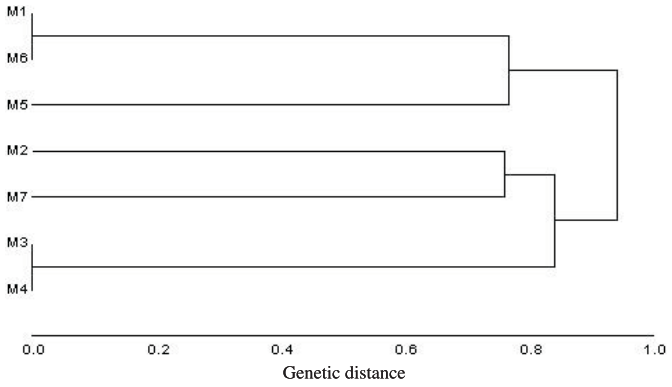


Fig 13. Cluster analysis of the seven *Rhizoctonia*-like isolates based on RAPD–PCR data. The dendrogram was generated from genetic similarity coefficients based on the unweighted pair-group method using the arithmetic average. Identification of the isolates was according to Table 2.



The third group contained two other *Ceratorhiza* isolates obtained from different orchid genera, but from the same geographical region, suggesting the same nonspecific *Ceratorhiza* species.

Molecular characterization by PCR–RFLP analysis

Molecular characterization of multinucleate *Rhizoctonia* spp. and binucleate *Rhizoctonia*-like fungi is commonly used to infer taxonomic relationships and patterns of genetic diversity among species (Cubeta et al. 1996; Mordue et al. 1996). In our study, cluster analysis based on the results of PCR–RFLP separated the isolates into three groups (Fig. 11). The first group was the same as that found by the morphological analysis containing the *Epulorhiza* isolates. However, this analysis distinguished *Epulorhiza repens* from both *Epulorhiza epiphytica* isolates, with a genetic distance of 18% between the two species. Only the restriction pattern generated by *Hae*III revealed polymorphism among both *Epulorhiza* species.

PCR–RFLP has been used to clarify the structure of multinucleate *Rhizoctonia* populations by detecting genetic variations among species (Cubeta et al. 1996; Cubeta and Vilgalys 1997), although in some cases, the use of few restriction enzymes does not allow detection of polymorphism (Pascual et al. 2000). Binucleate *Rhizoctonia*-like fungi are known to be a diverse group of organisms, composed of many species (Cubeta et al. 1991), and our results emphasize the need to use the largest possible number of endonucleases to generate greater polymorphism in this group of organisms.

Genetic distance between *Epulorhiza epiphytica* isolates M1 and M6, determined by rDNA PCR–RFLP analysis, was null, which is in agreement with the phenotypic distance based on morphological characterization.

At a genetic distance of 90%, all four *Ceratorhiza* isolates were placed in one group. However, at a distance of 88%–89%, isolate M2 was separated from the others. *Ceratorhiza* isolates M2 and M7, closely related by morphological analysis, were distinguished by PCR–RFLP analysis. Based on these results, these isolates appear to be different *Ceratorhiza* species, as suggested by the different sizes of their amplified ITS regions (Table 3). The pair of *Ceratorhiza* isolates M3 and M4, closely related by morphological analysis, showed the same restriction pattern with all five endonucleases used, as well as the same amplified ITS region sizes (Table 3), indicating that both isolates belong to the same *Ceratorhiza* species.

Molecular characterization by RAPD analysis

RAPD-PCR analysis has already been successfully employed in the analysis of genetic variation of *Rhizoctonia solani* (Duncan et al. 1993; Yang et al. 1996) as well as

Table 4. Coincidence analysis based on qualitative and quantitative data from morphological characterization, PCR–RFLP of the rDNA ITS region, and RAPD analyses for the seven orchid mycorrhizal fungal isolates.

Variables	Data No.	Superior coincidence (%)	Inferior coincidence (%)	rPearson
Morphology × PCR–RFLP	19	80	80	0.7700**
Morphology × RAPD	19	90	90	0.6693**
PCR–RFLP × RAPD	19	90	70	0.8303**

**Statistical significance at $p < 0.01$ according to a t test.

Table 5. Comparisons between the variables used for the morphological and molecular characterization of the seven orchid mycorrhizal fungi isolates using the Tocher cluster method.

		Molecular characterization*		
Isolate	Morphological characterization*	PCR–RFLP	RAPD	Identification
M1	a	a	a	*Epulorhiza epiphytica*
M2	b	b	b	*Ceratorhiza* sp.
M3	c	c	c	*Ceratorhiza* sp.
M4	c	c	c	*Ceratorhiza* sp.
M5	a	a	a	*Epulorhiza repens*
M6	a	a	a	*Epulorhiza epiphytica*
M7	b	c	b	*Ceratorhiza* sp.

*The same letter within a column indicates that the means are not significantly different according to the Tocher group analysis.

mycorrhizal binucleate *Rhizoctonia*-like fungi (Shan et al. 2002).

As in the PCR–RFLP analysis, isolates M1 and M6 of *Epulorhiza* and M3 and M4 (Fig. 11) of *Ceratorhiza* showed the same patterns in the RAPD analysis (Fig. 12). For both genera, these results suggest that each pair contains a unique nonspecific isolate, since they were obtained from pairs of orchids belonging to different subtribes. Shan et al. (2002) isolated and characterized 21 *Rhizoctonia*-like fungal strains from four terrestrial orchid species from various Hong Kong locations utilizing morphological and molecular tools and observed that highly similar isolates were isolated from the same host species and locations. In our study, the *Epulorhiza* pair M1 and M6 isolated from *Epidendrum rigidum* and *P. concreta*, respectively, was collected in Pedra do Anta, and the *Ceratorhiza* pair M3 and M4 isolated from *Isochilus lineares* and *M. marginata*, respectively, was collected in Carangola. These results suggest a great geographic influence similar to that observed by Otero et al. (2002) for some *Rhizoctonia*-like fungi associated with tropical epiphytic orchids.

Polymorphism among *Epulorhiza* spp. belonging to group I was more evident in the RAPD analysis than in the PCR–RFLP analysis. RAPD analyzes the entire fungal genome, whereas PCR–RFLP analyzes a specific region of the genome. Since RAPD analyzes multiple loci per each primer, genetic variations were more readily detected.

RAPD analysis also detected broad genetic variation among *Ceratorhiza* isolates M2 and M7; however, at a genetic distance of 75% both isolates were grouped together (Fig. 13). Both isolates seem to have some degree of relatedness that was also detected by morphological analysis, perhaps because they were isolated from the same geographic region and same host genus. In contrast, they appear to be different species and a possible variation in specificity within the single genus *Oncidium* can exist, similar to that observed for the tropical epiphytic orchids *Ionopsis utricularioides* (Sw.) Lindl. and *Ionopsis satyrioides* (Sw.) Rchb. f. (Otero et al. 2002).

Conclusion

The most common fungi associated with Brazilian neotropical orchids belong to two genera: *Epulorhiza* and *Ceratorhiza*. Both morphological and molecular approaches were useful and consistent for grouping the isolates. Considering the low number of plant species and the restricted geographical area investigated, more studies are needed to evaluate the biodiversity and specificity of mycorrhizal fungi associated with Brazilian orchids and to compare their biodiversity and specificity to that of isolates from other countries.

The *Rhizoctonia*-like fungi are taxonomically problematic because they consist of a complex of anamorphic basidiomycetes belonging to different genera and they lack conidiogenesis in their asexual stage (Mycelia Sterilia). These facts complicate mycologists' understanding of their ecology and global diversity. The integration of different methods and techniques should lead to the identification of

markers, which should lead to the standardization of global taxonomical studies of *Rhizoctonia* species.

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