**IN VITRO SYMBIOTIC SEED GERMINATION AND MOLECULAR CHARACTERIZATION OF ASSOCIATED ENDOPHYTIC FUNGI IN A COMMERCIALLy IMPORTANT AND ENDANGERED INDIAN ORCHID VANDA COERULEA GRIFF. EX LINDL.**

SIMMI AGGARWAL1,*, C. NIRMALA1, SHANUJA BERI2, SACHIN RASTOGI2, and ALOK ADHOLEYA2

1 Department of Botany, Panjab University, Chandigarh – 160 014, India. Tel.: 91-09815231311; Fax: 91172-2696587
2 Biotechnology and Bioresources Division, The Energy and Resources Institute, IHC, Lodhi Road, New Delhi – 110003, India
*Corresponding author: simmi.aggarwal@rediffmail.com

**ABSTRACT**

The technique of symbiotic seed germination-using fungi to cultivate orchid seedlings _in vitro_ leading to their reintroduction _in situ_ has considerable potential for conservation as evidenced by studies mostly in Australia and North America. However, its use has yet to be fully realized throughout the world. On the Indian subcontinent, which harbors a considerable number of orchid species, symbiotic germination has been virtually unexplored. In the present studies, we provide a protocol for the symbiotic seed germination and eco-restoration of an endangered orchids _Vanda coerulea_ Griff. ex Lindl., which is a floriculturally significant epiphyte used to progenate a vast variety of hybrids. Seeds were obtained from the mature un-dehisced capsules and sown on oat meal agar medium with the fungus isolated from the roots of mature _V. coerulea_ plants _in situ_. Using molecular characterization techniques, cultures were assignable to _Rhizoctonia zeae_ with teleomorph _Thanatephorus cucumeris_. All the seeds germinated within 5 wks of culture and very healthy, dark green protocorms were obtained in 5 month old cultures. Seedlings with 1–2 roots and 2–3 leaves were obtained in 8 months. These were acclimatized in the greenhouse for a year and introduced to their natural habitat at Manipur in North East India. Seedling growth and development was continuously monitored, demonstrating active growth during monsoon season (April–July). Out of 29 plants reintroduced, 23 survived and are growing well with the formation of new roots and leaves, observed after twelve months of reintroduction.

*Keywords*: _Vanda coerulea_, symbiotic seed germination, _Rhizoctonia zeae_, endangered orchid, reintroduction, molecular identification, _Thanatephorus cucumeris_

**Introduction**

Within the plant kingdom, orchids are particularly vulnerable to extinction because they must consume free-living, mycorrhizal fungi as a carbon source (= mycotrophy) to prompt seed germination and seedling development in nature (Arditti 1966; Clements 1988; Rasmussen 1995). Mycorrhizal associations continue throughout the life of the orchid and play an important role in nutrition of adult plants (Cameron et al. 2006, 2007; Girlanda et al. 2006). Consequently, any loss in mycorflora from habitat destruction poses a threat to orchid conservation because such plants would be unable to complete their life cycle. Moreover, habitat clearance, land fragmentation and degradation has reduced the population size and distribution of many species (Duncan et al. 2005; Swartz and Dixon 2009). Therefore, the long-term survival of orchids in managed or restored habitats requires the presence of appropriate fungal mycobiont for seedling recruitment and plant nutritional support (Zettler 1997a). The applications of the mycorrhizal association for horticultural and conservation purposes have recently gained considerable attention (Rasmussen 2002; Rasmussen and Rasmussen 2007; Zettler et al. 2007; Swartz and Dixon 2009). The technique of symbiotic seed germination is most frequently applied for seedling recruitment (Clements et al. 1986; Dixon 1987; Zettler 1997b; Betty et al. 2006).

The majority of fungi isolated from orchids belong to the anamorphic genera _Rhizoctonia_ (Filippello-Marchisio and Berta 1985). _Rhizoctonia_ like fungi include the anamorphic genera e.g. _Ceratobasidium, Ceratorhiza, Epulorhiza, Moniliopsis, and Rhizoctonia_ and a variety of teleomorphs e.g. _Ceratobasidium, Thanatephorus, Tulasnella_, and _Sebacina_ (Rasmussen 2002; Dearnaley 2007). The approaches that have been used to identify the _Rhizoctonia_ like fungi include 1) cytomorphological characterization of anamorph and telemorph and anastomosis group (Sneh et al. 1991), 2) characterization of ultrastructure of cell wall and septal pore (Currah and Sherburne 1992; Wells and Bandoni 2001; Suarez et al. 2006), and molecular characterization (Otero et al. 2002; Shan et al. 2002; Suarez et al. 2006; Taylor and McCormick 2008). From the above mentioned methods, molecular method is one of the most efficient because a finer distinction between strains is possible (Rasmussen 2002).

In India, about 1300 orchid species in 180 genera are reported (Rao 2008) and nearly 300 species in 75 genera are endemic (Rao 1991). Majority of the Indian orchids are epiphytes (nearly 800 species), mainly distributed in North East Himalaya followed by nearly 300 species in Western Ghats and about 200 species in North West Himalaya. Most of the commercially important orchids have been collected from the wild to the point of rarity resulting in the extinction of many. In addition, habitat destruction associated with urbanization and natural disasters has also added to the reduction in the size
and frequency of native orchids. Several Indian species including the focus of this study (Vanda coerulea Griff. Ex. Lindl.), are threatened while other species, e.g., Phaius tankeri (Lindl.) are thought to be extinct. To augment in situ conservation, a blend of various approaches (= integrated conservation; Swartz 2007; Stewart 2007) will be needed, including the recovery, use and long term storage of mycorrhizal fungi for propagation (i.e., symbiotic seed germination, and growth of protocorms and seedlings). Symbiotic seed germination has practical merit for both conservation and horticulture, but its widespread use has been limited mostly to temperate climates such as Australia (e.g. Batt et al. 2006) and North America (Stewart et al. 2003).

Vanda coerulea Griff. Ex. Lindl., commonly known as ‘blue vanda’, (Fig. 1a) is a floriculturally important and endangered species of the epiphytic and monopodial orchids. It is found at elevations of 1000–1500 m and is endemic to the state of Meghalaya and Manipur in India and northern range of Thailand and Burma. Flowers are known to have a long shelf life (2–3 months). This beautiful orchid has been extensively used to propagate a vast variety of remarkable hybrids (Bose et al. 1999). The species is also known for its ethnobotanical importance; its flowers are used as pectoral, and the juice from its leaves is used to cure diarrhoea, dysentery and dermal disorders (Nadkarni 1954). However, this attractive orchid is faced with extensive collection and habitat destruction processes which has resulted in the decline of populations in some remote areas (Pradhan 1985). It has been listed as an endangered species of Red Data Book on Indian Orchidaceae-1 (Pradhan 1985) and as threatened by the International Union for Conservation of Nature and Natural Resource (Seeni and Latha 2000). As a result, the Committee of International Trade in Endangered species of World Fauna and Flora has imposed a ban on its trade (Seeni and Latha 2000).

As only limited information is available for mycorrhizal fungi in epiphytic orchids, which constitute a majority of orchids, it is important to isolate and identify orchid mycorrhiza and understand orchid-fungus relationship in epiphytic orchids. Keeping this in view, the present studies were planned with the objective to reestablish the in vitro symbiotically raised seedlings back to their natural habitat so that Vanda coerulea can be restored to protected habitats. Fungus from V. coerulea has been isolated and morphologically identified (Saha and Rao 2006) and symbiotic technique has been successfully used to grow Cymbidium elegans, C. giganteum, and Thunia alba (Raghuvanshi et al. 1991). However, molecular work is still lacking and for accurate identification, these techniques are considered more authentic (Shan et al. 2002; Dearnaley 2007; Yagame et al. 2008). The present work involves isolation and identification of mycorrhiza of V. coerulea both by conventional and molecular methods and to reestablish the in vitro symbiotically raised seedlings back to their natural habitat.

Materials and Methods

Procurement of roots for isolation of mycorrhiza

Roots were procured from two naturally growing plants of V. coerulea that originated from a natural population in the vicinity of Imphal Manipur. (North East India; latitude of 23°83’N–25°68’N and longitude of 93°03’E–94°78’E) at an altitude of 5500 m. These were collected in May–June during their active vegetative growth, stored in the paper bags/ziplock and transferred to the laboratory in < 12 hrs.

Isolation of fungal endophytes

Within 24 hrs of collection, the root segments were rinsed with cold tap water and scrubbed with a soft brush to remove debris attached to the surface of the roots. Thin transverse sections (approx. 1 mm) of the roots were cut and stained with lactophenol tritylcyro cotton blue and observed under light microscope for the presence of fungal colonization. The roots showing the presence of fungal pelotons were selected for further experimentation. Isolations were carried out by slightly modifying the method of Currah et al. (1987). Under aseptic conditions, root segments were surface-sterilized in a 20% solution of household bleach for one minute, rinsed twice in sterile distilled water and decorticated with sterile scalpel. Clumps of the cells were removed from the inner cortex, macerated in a drop of sterile deionized water and plated on Fungal Isolating Medium (FIM, Clements et al. 1986) in 90 mm sterile disposable petri plates (Hi Media, Mumbai, India). Plates were sealed with the help of parafilm and incubated both dark and light at ±25 °C until the hyphae grow from the cortical cells into the medium. For dark incubation, the plates were wrapped in aluminium foil. Hyphal tips were transferred to Potato Dextrose Agar (PDA, Hi Media, Mumbai, India) medium and serially transferred for 3–4 times until pure cultures were obtained. Regular subculturing was made at 15 days interval to fresh PDA medium for use of the fungal inoculum. Some cultures were left for 6 weeks on PDA plates to allow the development of moniloid cells and formation of sclerotia.

Fungal Identification (Morphological and Molecular)

Morphological identification. The macroscopic features examined were colony colour, growth pattern and sclerotia formation. Growth rates were determined according to the technique of Currah et al. (1987). A small fragment of mycelium (approx. 1 mm²) was inoculated in the middle of a 90 mm PDA plate. Radial increments in colony diameter were measured in two directions every 48 hrs over a period of two weeks. Growth rates were calculated as average of five replicates. For microscopic examination, fungal hyphae were mounted both in 10%
a solution of 5% (w/v) CaOCl₂ + 1% (v/v) tween-80 for 24 hours. After this, seeds were stained with 1% TTC (2,3,5-triphenyltetrazoliumchloride) for 24 hrs in darkness. Seeds were rinsed three times with sterile distilled water to remove excess of TTC. These were examined under light microscope and seeds containing pinkish-brown embryos were considered as viable.

**Symbiotic seed germination**

The capsules were scrubbed with a neutral detergent “Teepol” (Qualigens Fine Chemicals, Mumbai, India) with the help of a soft brush to clean the surface of the capsule. After drying on a filter paper, these were swabbed with absolute alcohol and were placed in a 250 ml flask (Scott Duran, Germany). These were washed in running tap water for 20 mins and transferred to Laminar Air Flow Cabinet. For surface sterilization, a treatment of 0.5% HgCl₂ was given for 5 minutes with occasional stirring followed by washing with sterile distilled water for three times. Finally, the capsules were dipped in absolute alcohol and flamed for two seconds and put in sterile distilled water. The surface sterilized capsules were placed on a filter paper in a glass Petri dish (90 mm) and dissected longitudinally with the help of sterile surgical blade (No. 23) fitted on a sterile scalpel handle (stainless steel No. 4). The seeds were scooped with the help of forceps and some seeds were placed on a glass slide in 10% glycerine solution, covered with a cover slip and examined under light microscope. The seeds were cultured on PDA in the dark at room temperature. DNA from the isolate OM3 was extracted using DNeasy Plant MiniKit (Qiagen). For fungal identification, the SSU-ITS region of nuclear rDNA was amplified using the primer pairs ITS1 F (Gardes and Bruns 1993) as forward primer and TW13 (White et al. 1990) as reverse primer. The PCR product was purified using the PCR Purification kit from Qiagen and was then sequenced using the BigDye Terminator 3.1 (Applied Biosystems). All sequences obtained by the above study were analyzed by NCBI database using BLASTn program.

**Molecular Identification.** Plates of OM3 (coded for present fungal cultures) were grown on PDA in the dark at room temperature. DNA from the isolate OM3 was extracted using DNeasy Plant MiniKit (Qiagen). For fungal identification, the SSU-ITS region of nuclear rDNA was amplified using the primer pairs ITS1 F (Gardes and Bruns 1993) as forward primer and TW13 (White et al. 1990) as reverse primer. The PCR product was purified using the PCR Purification kit from Qiagen and was then sequenced using the BigDye Terminator 3.1 (Applied Biosystems). All sequences obtained by the above study were analyzed by NCBI database using BLASTn program. Sequence obtained from above study were analyzed by NCBI database using BLASTn program. Sequence obtained from above study were analyzed by NCBI database using BLASTn program.
gation of initial leaves and root differentiation. Fungal infection/mycotrophy was also confirmed by examining selected symbiotically raised seedlings (stage 4) for the presence of pelotons using a light microscope.

**Transfer of seedlings to clay pots for acclimatization**

The seedlings formed within 36 week old cultures with 2–3 leaves and 1–2 roots were removed from the oat meal agar medium using a forcep, washed gently with lukewarm water to remove any agar sticking to them and planted in small earthen pots containing a mixture of brick pieces, charcoal pieces, coir chucks at 1 : 1 : 1. Fresh moss was applied to pots. The transferred seedlings were watered daily once in winters and twice in summers.

**Reintroduction to the natural habitats**

Well aclimatized plants maintained in the green house for one year were carried to their natural home (Bamonkampu, Imphal East, Manipur) within two days and were reintroduced on mango trees and the direction of the plants with relation to sun was south and slightly shady. The plants were attached to tree bark with the help of some cow dung and fresh moss and tied with a thread to the bark. The distance of the tree trunk from the ground where the plants were reintroduced was 6 feet. The plants were monitored weekly for further growth and development.

**Results**

Peloton formation was observed in the cortical cells of the roots (Fig. 1b) attached to the substratum, demonstrating that this species is mycotrophic at maturity. The extent of root colonization was significantly higher in May–June than November–December. On PDA plate, the colony appeared white, cottony, radial growth 1 cm in diameter after 48 hrs of culture and 4.5 cm after 15 days, hyphal growth aerial thin and dense (Fig. 1c), vegetative hyphae septate right-angle branching pattern of hyphae with constriction at branching prints, moniloid cells thin walled, barrel shaped, nearly spherical branched chains (Fig. 1d,e). The hyphal and moniloid cells were binucleate measuring approximately 5 microns in width and 20 microns in length. The ITS region characterization of the endophyte isolated from *V. coerulea* confirmed its taxonomic affinity i.e. as on BLASTn search, phylogenetic analysis showed that the isolate OM3 has a high homology (97%) to *Rhizoctonia zeae* (Fig. 2). Sequence obtained from the isolate clustered with known Rhizoctonia sequences retrieved from the Genbank with 79% bootstrap support. This is the first report of occurrence of *R. zeae* in orchid *V. coerulea* from India.

The seeds showed 96% viability by TTC test and all the seeds appeared monoembryonate under light microscope (Fig. 1g). The germination (stage 1 = rupture of testa) commenced within 3 weeks of fungal inoculations in all the seeds (Fig. 1h). Visual contamination rate was 4%. Non-inoculated seeds (control) required more time to germinate (5 wks) and these failed to develop further even after 3 months of inoculations. In contrast, a higher percentage of inoculated seeds (80%) developed to stage 4 (Fig. 1i) within 9 months and the rest (16%) in protocorm stage turned brown and perished on OMA medium even after fresh subcultures. The seedlings remained in vitro for another three months to allow additional leaves and roots development/elongation (Fig. 1j). Of the total seedlings formed, 60 were transferred to clay pots (Fig. 1k) for greenhouse acclimatization for another one year. The survival rate was 96.67% (58 plants) with the onset of new leaf and root growth. Twenty-nine plants were retained in the greenhouse and the remaining 29 plants were reintroduced to their natural habitat (Fig. 1l) leading to a significantly higher survival rate of 79.31% (23 plants). These were monitored for a period of over year starting from April 2010 to March 2011. The plants adapted well with the formation of new roots and leaves.

Results are based on five replicates and each Petri dish contained 80–100 seeds.

**Discussion**

*In vitro* symbiotic seed germination has become a favoured and useful technique for orchid seed propagation and use in plant reintroduction. Fungal colonization was observed in the root cortical cells of *V. coerulea* in the roots attached to the substratum and the extent of pelotonization was more during active growth season. Occurrence of mycorrhiza in epiphytic orchids has been earlier reported in the root portions attached to the tree bark (Arditti 1992; Porras-Alfaro and Bayman 2007). Seasonal variations in fungal infestation with higher colonization during the summer and rainy season of active growth and flowering as compared to slow growth in winter season has been observed earlier (Siddique and Raghuvanshi 1993; Chang 2007), suggesting higher

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<th><em>In vitro</em> germination and development stage of seeds of <em>Vanda coerulea</em> with mycorrhiza.</th>
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<td><strong>Medium</strong></td>
<td><strong>Time in weeks for onset of</strong></td>
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<td><strong>Stage 0</strong></td>
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<td>OMA-fungus</td>
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colonization to meet high nutrient requirement for active growth and flowering.

Mycorrhiza associated with orchids are basically endophytic and the fungi which form mycorrhizal associations belong to the group Armillaria, Ceratobasidium, Erythromyces, Moniliopsis, Mycena, Russulaceae, Serendipita, Thanatephorus, and Tulasnella. Of these, the most common associations are with Ceratobasidium, Thanatephorus, Tulasnella, and Sebacina, which have been authenticated with molecular studies (Rasmussen 2002). In India, studies on mycorrhizal associations are very scanty (Aggarwal and Zettler 2010). Saha and Rao (2006) isolated mycorrhiza from V. coerulea and on the basis of morphological characters, have assigned them to the genera Rhizoctonia and Cochliobolus. However, molecular studies have not been conducted. Radhika and Rodrigues (2007) have reported mycorrhizal colonizations in Rhynchospora retusa, but the fungus has not been identified. In our present work, the fungus Rhizoctonia zeae with teleomorph Thanatephorus cucumeris has been isolated and identified based on morphological as well as molecular studies. Identification on morphological basis was done based on features as described earlier (Currah et al. 1987; Rasmussen 1995; Otero et al. 2002; Shan et al. 2002; Zhu et al. 2008). These fungi are characterized by right-angle branching, a constriction at the branch point and a septum in the branch hypha near its point of origin. The genus Rhizoctonia represents an assemblage of taxonomically diverse groups that differ in cultural and morphological features including anamorph and teleomorph stages (Warcup and Talbot 1980; Currah et al. 1987). Production of chains of small inflated, spherical, globular moniloid cells is an important anamorphic feature among Rhizoctonia fungi (Sneh et al. 1991; Taylor et al. 2002; Shan et al. 2002). These fungi include the asexual (anamorphic) genera Ceratorhiza, Epulorhiza, Moniliopsis, and Rhizoctonia (Moore 1987) and sexual (teleomorphs) such as Ceratobasidium, Thanatephorus, Tulasnella, and Sebacina (Warcup and Talbot 1966, 1971; Anderson and Rasmussen 1996). According to the excellent book on turfgrass diseases (Smiley et al. 2005), the fungal genus Rhizoctonia contains many species and all of these share similar characteristics such as hyphae with right angle branching including a constriction at the branch point as also is evident from the present studies.

ITS region sequencing is the most common and powerful technique for accurate identification of orchid mycorrhizal fungi (Taylor and Bruns 1997, 1999; Kristiansen et al. 2001; Sharon et al. 2008). Presently, the molecular characterization results showed its teleomorph with
Thanatephorus cucumeris. In the earlier studies, the teleomorph of R. zeae and R. oryzae has been described earlier as Waitia circinata (Warcup and Talbot 1962). The anamorphic species Rhizoctonia zeae, originally described as the causal agent of ear rot of maize, is a synonym of Waitia circinata. Rhizoctonia spp. are well known as widely distributed pathogens, saprophytes and also mycorrhizal fungi of orchids (Masuhara and Katsuya 1994). The species has been isolated from plant roots, leaves and stems and have been found in various habitats including cultivated land and natural forests (Ogashi 1987).

Both morphological and molecular studies of orchid mycorrhiza reveal that many terrestrial orchids have extremely specific associations often with fungi from a single teleomorph (Taylor et al. 2004; Shefferson et al. 2007). However, in epiphytic orchids, compared to the terrestrial orchid taxa, very little work has been done on symbiotic seed germination. Symbiotically propagated orchids have higher survival rate when they are transplanted ex vitro (Ramsay and Dixon 2003). Much research needs to be done to unravel the underlying mechanism of mycorrhizal associations in epiphytic orchids which may help to prevent their decline and extinction in nature.

V. coerulea is a commercially important endangered plant and asymbiotically propagated plants have high mortality rate (about 90%; present study) after transfer to natural habitat. In earlier reports, Anderson (1991) found that 2 out of 40 non-symbiotic seedlings of S. magnicamporum survived soil transfer under aseptic conditions, whereas 40 of 40 symbiotic seedlings survived and flowered under same conditions. The present observation reveals that 80% of the symbiotically propagated plants survived after reintroduction and thus, this symbiotic technique has practical merit for the conservation of this highly important orchid. Further studies are suggested of using fungi from other orchids to evaluate their effect on seed germination and seedling growth in V. coerulea and also on its ex vitro survival in greenhouse/natural habitat.

Conclusions

For orchid reintroduction, the mycobiont-infected seedlings can be very effectively produced through in vitro symbiotic seed germination. The current study represents a successful protocol for reintroduction of a threatened and endangered orchid, V. coerulea by using its own mycorrhiza in vitro. The occurrence of Rhizoctonia zeae as evident from molecular identification is the first report from India in V. coerulea.
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REFERENCES


Rasmussen HN (1995) Terrestrial orchids: from seeds to mycorro-
Rasmussen HN (2002) Recent developments in the study of orchid
Rasmussen HN, Rasmussen FN (1991) Climatic and seasonal
regulation of seed plant establishment in Dactylorrhiza mahal
inferred from symbiotic experiments in vitro. Lindleyana 6:
221–227.
Rasmussen HN, Rasmussen FN (2007) Trophic relationships in
orchid mycorrhiza – diversity and implications for conserva-
Saha D, Rao AN (2006) Studies on endophytic mycorrhiza of some
selected orchids of Arunachal Pradesh. I. Isolation and identifi-
method for reconstructing phylogenetic trees. Mol Biol Ecol 5:
406–425.
Seeni S, Latha PG (2000) In vitro multiplication and ecorehabi-
tion of the endangered Blue Vanda. Plant Cell Tissue Organ
Cult 64: 1–8.
Sen R, Hietala AM, Zelmer CD (1999) Common anastomosis and
internal transcribed spacer RFLP groupings in binu-
clate Rhizoctonia isolates representing root endophytes of
Pinus sylvestris, Ceratorhiza spp. from orchid mycorrhizas and
a phytopathogenic anastomosis group. New Phytol 144:
331–341.
Shan XCE, Liew CY, Weatherhead MA, Hodkiss IJ (2002) Charac-
terization and taxonomic placement of Rhizoctonia-like endo-
Shimura H, Koda Y (2005) Characterization of mycorrhizal fungi isolated from
the threatened Cypripedium macranthos var. rebunense following inoculation
Characterization of mycorrhizal fungi isolated from the threatened
Cypripedium macranthos in a northern island of Japan: two phylogenetically distinct fungi associated with
Siddique S, Raghuvanshi A (1993) Seasonal changes in Vanda tes-
Pathak P, Seghal RN, Shekhar N, Sharma M, Sood A (eds)
Orchids: Science and Commerce. Bishen Singh Mahendra Pal
Singh, Dehradun, India, pp 35–65.
Smiley RW, Dermoeden PH, Clarke, BB (2005) Compendium of
turfgrass diseases. The American Phytopathological Society,
St. Paul, MN.
Smith SE (1966) Physiology and Ecology of orchid mycorrhizal
fungi with reference to seedling nutrition. New Phytol 65:
488–499.
species St Paul, Minnesota, APS Press.
in the genera Habenaria and Spiranthes: model Orchid conser-
vation system for the Americas, Ph.D. Dissertation. University
of Florida, Gainesville.
Stewart SL, Kane ME (2006) Symbiotic seed germination of Hab-
ernaria macrocrotites (Orchidaceae), a rare Florida terrestrial
semi-aquatic rein orchids (Habenaria repens, H. quinquiesta,
H. macrocrotites) from Florida. Aquat Bot 72: 25–35. doi:
10.1016/S0304-3770(01)00214-5.
Stewart SL, Zettler, LW, Minso J, Brown PM (2003) Symbiotic ger-
mination and reintroduction of Spiranthes brevilabris Lindley,
an endangered orchid native to Florida. Selbyana 24: 64–70.
Diverse tulasnellid fungi from mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycol Res 110:
1257–1270. doi:10.1016/j.myces.2006.08.004.
Swartz ND (2007) Integrated conservation of the rare and endan-
Swartz ND, Dixon KW (2009) Terrestrial orchid conservation in the
Evolutionary Genetics Analysis (MEGA) software version 4.0.
Taylor LD, Bruns TD (1997) Independent, specialized invasion of
telemycorrhizal mutualism by two nonphotosynthetic orchids.
Taylor LD, Bruns TD (1999) Population, habitat and genetic corre-
lates of mycorrhizal specialization in the “cheating” orchids Cor-
primers and sequences for improved characterization of basidi-
Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specif-
cicity and function in myco-heterotrophic plants. In: van der Heijden MGA and Sanders I (eds) Mycorrhizal ecology, Eco-
Van Waes JM, Debergh PC (1986) In vitro germination of some
Warcup JH, Talbot PHB (1962) Ecology and identity of mycella iso-
Warcup JH, Talbot PHB (1980) Perfect states of Rhizoctonias
Part B. Systematics and evolution. Springer Verlag, Heidelberg,
pp 85–120.
White TJ, Bruns TD, Lee S, Taylor Jr, (1990) Amplification and
direct sequencing of fungal ribosomal rna genes for phyloge-
netics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds)
PCR protocols: a guide to methods and applications. Academic,
San Diego, pp 315–322.
mycorrhizal fungi isolated from nonphotosynthetic orchid
Yoder JA, Zettler LW, Stewart SL (2000) Water requirements of
terrestrial and epiphytic orchid seeds and seedlings, and evi-
dence of water uptake by means of mycotrophy. Plant Sci 156:
145–150.
Zettler LW (1997) Orchid fungal symbiosis and its value in con-

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