available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/mycres

Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions

Yumiko BONNARDEAUX[a,*], Mark BRUNDRETT[b], Andrew BATTY[a,c], Kingsley DIXON[c], John KOCH[d], K. SIVASITHAMPARAM[a]

[a]School of Earth and Geographical Sciences (Soil Science Discipline), Faculty of Natural & Agricultural Sciences, University of Western Australia, Crawley, WA 6009, Australia
[b]School of Plant Biology, Faculty of Natural & Agricultural Sciences, University of Western Australia, Crawley, WA 6009, Australia
[c]Science Directorate, Botanic Gardens and Parks Authority, Kings Park and Botanic Garden, West Perth, WA 6005, Australia
[d]Alcoa World Alumina Australia, PO Box 252, Applecross, WA 6953, Australia

ARTICLE INFO

Article history:
Received 3 August 2006
Received in revised form
9 October 2006
Accepted 3 November 2006
Published online 6 February 2007
Corresponding Editor:
Derek Thomas Mitchell

Keywords:
Disa bracteata
Fungal specificity
Mycorrhizas
Orchids
Pyrorchis nigricans
Rhizoctonia
Seed germination
Western Australia

ABSTRACT

The diversity of mycorrhizal fungi associated with an introduced weed-like South African orchid (*Disa bracteata*) and a disturbance-intolerant, widespread, native West Australian orchid (*Pyrorchis nigricans*) were compared by molecular identification of the fungi isolated from single pelotons. Molecular identification revealed both orchids were associated with fungi from diverse groups in the *Rhizoctonia* complex with worldwide distribution. Symbiotic germination assays confirmed the majority of fungi isolated from pelotons were mycorrhizal and a factorial experiment uncovered complex webs of compatibility between six terrestrial orchids and 12 fungi from Australia and South Africa. Two weed-like (disturbance-tolerant rapidly spreading) orchids — *D. bracteata* and the indigenous Australian *Microtis media*, had the broadest webs of mycorrhizal fungi. In contrast, other native orchids had relatively small webs of fungi (*Diuris magnifica* and *Thelymitra crinita*), or germinated exclusively with their own fungus (*Caladenia falcata* and *Pterostylis sanguinea*). Orchids, such as *D. bracteata* and *M. media*, which form relationships with diverse webs of fungi, had apparent specificity that decreased with time, as some fungi had brief encounters with orchids that supported protocorm formation but not subsequent seedling growth. The interactions between orchid mycorrhizal fungi and their hosts are discussed.

Introduction

Terrestrial orchids require the presence of a compatible fungal partner for seed germination and continued growth (Rasmussen 1995; Peterson *et al.* 1998). In this study, fungal specificity is defined as the diversity of mycorrhizal fungi compatible with an orchid and the shape of compatibility links between orchids and fungi referred to as webs. The

* Corresponding author.
E-mail address: yumiko@cbbc.murdoch.edu.au
0953-7562/$ – see front matter © 2006 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2006.11.006

diversity of compatible fungi is expected to influence the competition, survival and distribution of orchids (Rasmussen 1995; Currah et al. 1997; Batty et al. 2002).

Most orchid mycorrhizal fungi belong to the form genus *Rhizoctonia*, a diverse polyphyletic group of pathogens, endophytes, saprophytes and mycorrhizal fungi (Warcup 1981; Sivasithamparam 1993; Rasmussen 1995; Currah et al. 1997; Roberts 1999). These fungi are difficult to classify due to the scarcity of sexual sporulation (necessary to define teleomorphic genera) and the morphological similarity of anamorphic genera in this artificial group (Roberts 1999; Currah et al. 1997). Consequently, molecular methods have become the standard means of assigning orchid fungi to groups within the *Rhizoctonia* alliance (Taylor et al. 2003; McCormick et al. 2004; Weiss et al. 2004; Shefferson et al. 2005). Most studies found terrestrial orchids to have mycorrhizal associations with a narrow diversity of fungi in the *Rhizoctonia* alliance, but fungal diversity varies across habitats, or between seedlings and adult plants in other cases (Hadley 1970; Rasmussen 1995; Currah et al. 1997; Brundrett 2006).

The Southwest Floristic Region of Western Australia (WA) is known as a centre of worldwide significance in terms of plant biodiversity (Myers et al. 2000; Hopper & Gioia 2004). Over 400 orchid species occur in this isolated region of Mediterranean-type climate, most of which are endemic (http://florabase.calm.wa.gov.au). These are most diverse in higher rainfall zones where land clearing, weeds and salinity have resulted in substantial loss of suitable habitats. Although a few of these orchids can be described as 'weed-like' due to their ability to rapidly spread in a wide range of habitats, the majority are restricted to particular habitats and there are 76 rare or poorly known orchids in this region (http://florabase.calm.wa.gov.au).

This study aimed to investigate the ecological consequences of orchid–fungus specificity by contrasting the diversity of mycorrhizal fungi compatible with a weed-like South African orchid *Disa bracteata* with a widespread, native Australian orchid *Pyrorchis nigricans* and other WA orchids. Orchid–fungus specificity was investigated using molecular fungus identification in conjunction with symbiotic orchid seed germination assays using seven orchid species. Another objective of this study was to establish whether the mycorrhizal associations of orchids influenced their capacity to grow in disturbed habitats, by contrasting weed-like and non weed-like orchids. The diversity of fungi compatible with different orchids is compared in an attempt to gain a better understanding of the mycorrhizal compatibility and distribution of different fungal groups within the *Rhizoctonia* complex.

Materials and methods

Fungal isolations were primarily from *Disa bracteata* and *Pyrorchis nigricans*. *D. bracteata* has spread rapidly across southern Australia since its introduction in 1994 to Albany, WA (Erickson 1965). The orchid has the capacity to inhabit a wide range of habitats but is particularly common in disturbed areas such as roadside verges and rehabilitated minesites (Hoffman & Brown 1992; Grant & Koch 2003). The common native Western Australian orchid, *P. nigricans*, also has an extensive distribution in Australia but is not present in disturbed sites (Hoffman & Brown 1992; Collins et al. 2005). Seed and fungi from five relatively common and widespread native orchids, *Microtis media*, *Thelymitra crinita*, *Caladenia falcata*, *Diuris magnifica* and *Pterostylis sanguinea*, each belonging to a different subtribe in the *Orchidoideae* subfamily of *Orchidaceae* (Freudenstein et al. 2004), were also used in germination assays. Orchid names follow Hoffman & Brown (1992), except *Disa (Monadenia) bracteata*. The taxonomic groups to which orchids belong are shown in Fig 1, except for *P. nigricans*, which is in the *Drakaeinea* subtribe of the *Diuridaea*.

Orchids were collected during the growing season from the locations in Fig 2 and Table 1. The Jarrahdale sites, from which the majority of *D. bracteata* and *P. nigricans* were sampled, included natural bushland, as well as mine rehabilitation sites with or without recent disturbance by fire.

Fungal isolations

Fungi were isolated from single pelotons dissected out of mycorrhizal organs of mature plants and rinsed in five changes of sterile distilled water, before dispersal on the agar surface. This method was used for the majority of isolations, as it has been established to be a highly reliable method for isolating mycorrhizal fungi from orchids (Rasmussen 1995; Batty et al. 2002). Several fungi were also isolated from 1–2 mm root segments surface sterilised in 3 % (w/w) hydrogen peroxide solution for 20 s before being plated onto nutrient agar. Culture media used for fungal isolations contained (in grams per litre): NH_4NO_3, 0.4; KH_2PO_4, 0.0136; $MgCl.6H_2O$, 0.61; NaCl, 0.058; $CaSO_4.2H_2O$, 0.861; FeEDTA(Na), 0.073. This media has been modified to replicate the composition of Australian soil nutrients in order to optimise growing conditions for Australian soil fungi (Mursidawati 2004).

Fungus colonies of consistent appearance that grew from a peloton were transferred onto fresh media. Hyphal tip cultures from each isolate were placed on three Petri plates using fungus isolation media and backup cultures were kept in cryostorage. The majority of fungi growing from pelotons were members of the *Rhizoctonia* complex, as established by hyphal morphology in culture (Currah et al. 1997).

Symbiotic orchid seed germination

Seed from naturally pollinated plants of the selected orchids were collected within 15 km of the centre of Perth. A factorial symbiotic seed germination assay using seed of six orchids and 12 fungus isolates plus uninoculated control plates was conducted. A standard protocol for symbiotic germination of Australian orchids was used (Warcup 1981; Ramsay et al. 1986; Batty et al. 2006; Hollick et al. 2005). Dried seed stored at 5 °C was placed in packets folded from filter paper (Whatman no. 1, 7 cm diam) and sterilised in 1 % calcium hypochlorite for 30 min before rinsing in sterile deionised water (Hollick et al. 2005). The filter paper was cut into pieces containing approximately equal amounts of seed as shown in Fig 3. A 3×3 mm^2 block of fungus culture was inoculated in the centre of Petri plates of oat agar (2.5 g l^{-1} ground rolled oats, 8 g l^{-1} agar) surrounded by seed of the six orchids on filter paper

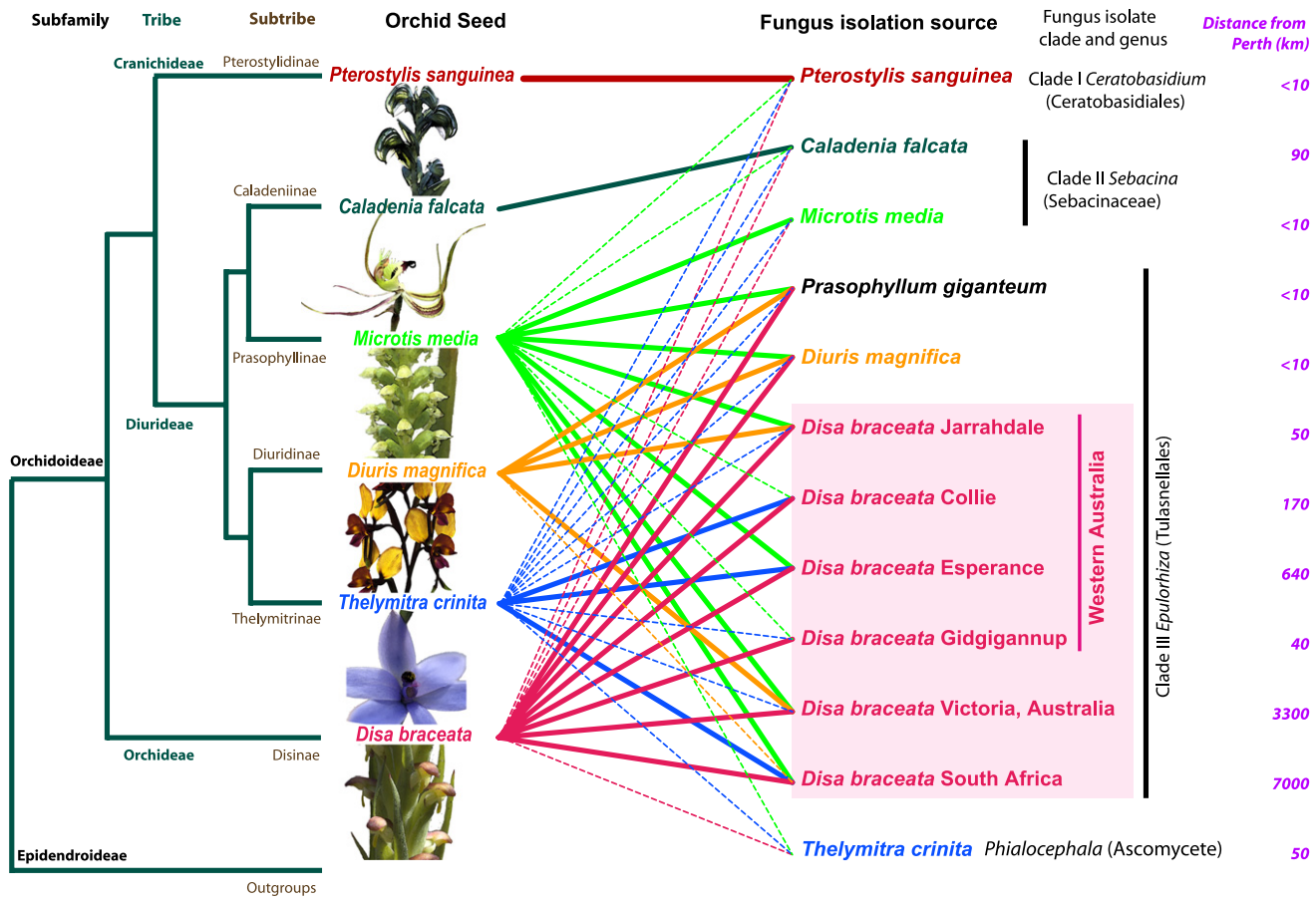

Fig 1 – Webs of mycorrhizal compatibility between orchids and fungi. Thick solid lines are fully compatible symbioses. Dashed narrow lines are partially compatible mycorrhizal associations where seedlings later aborted. Orchids are organised in taxonomic hierarchy (Chase *et al.* 2003) using a recent phylogenetic tree (Freudenstein *et al.* 2004). Mycorrhizal fungi are labelled according to their host plant of origin within major taxonomic groups of the *Rhizoctonia* alliance. The distance of the origin of fungal isolates from Perth is shown.

triangles (Fig 3). There were three replicates for each of the 12 fungal isolates tested and control plates containing uninoculated orchid seeds.

Seed germination was recorded eight and 16 weeks after sowing and assigned to standardised germination stages (Ramsay *et al.* 1986). Randomly sampled protocorms were stained and examined microscopically to confirm the presence of mycorrhizal fungi. *Pyrorchis nigricans* seed was also included in germination assays, but repeated attempts using different media and dormant breaking treatments failed to produce consistent germination results for this species (<5 %), presumably due to low seed vitality. Low seed viability due to inbreeding depression has been found in other orchids that primarily reproduce clonally (Peakall & Beattie 1996).

DNA extraction and sequencing

Fungal isolates were grown in a 20 % solution of commercial V8 juice (Campbell's®), pH 5.5, adapted from Pope & Carter (2001) for DNA extraction. Genomic DNA was extracted from mycelia according to standard protocols adapted from Cenis (1992). Oligonucleotide primers ITS1 (5′-TCC-GTA-GGT-GAACCT-GCG-G) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC) used by White *et al.* (1990) were used to amplify the ITS region of genomic DNA. The reactions were performed in an Applied Biosystem GeneAmp PCR system 9700 thermal cycler and consisted of initial denaturing at 96 °C for 1 min, followed by 35 cycles of denaturation at 96 °C for 1 min, annealing at 50 °C for 30 s and extension at 72 °C for 2 min. The PCR product was purified using a QIAQuick® purification kit prior to sequencing which was carried out in an Applied Biosystem GeneAmp® PCR system 9700 thermal cycler. Strands of sequences were manually edited and aligned with Sequencher™ 3.0 (Gene Codes, Ann Arbor, MI) and consensus sequences were constructed for DNA strands sequenced in both directions.

Sequence and phylogenetic analyses

A Blast search (www.ncbi.nlm.nih.gov/BLAST) was conducted on all the sequences to determine their closest known relatives and to confirm the ITS region of the nuclear rDNA was sequenced. Before tree generation, pre-alignment was undertaken and sequences were trimmed. Sequences of closely related taxa representing well-characterised isolates from different clades of *Rhizoctonia*, obtained from GenBank, were

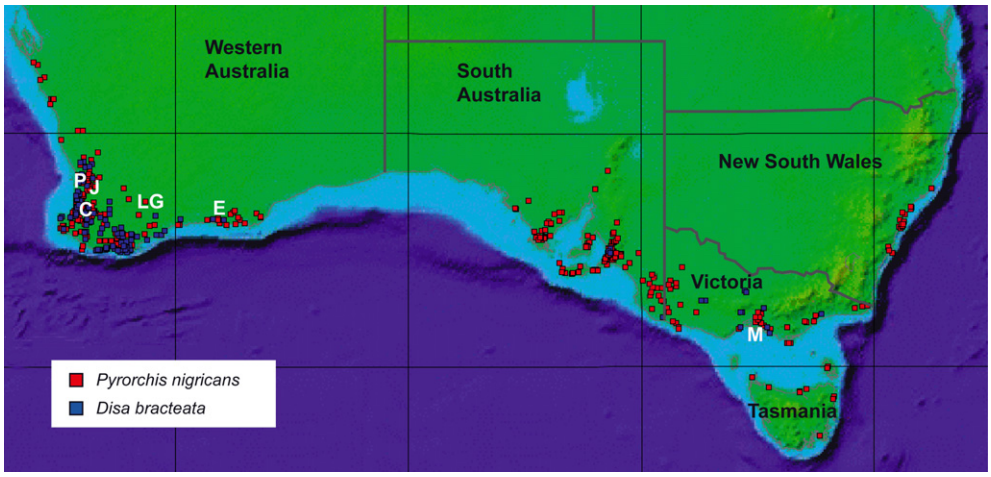

Fig 2 – Distribution of *Disa bracteata* and *Pterostylis nigricans* orchids in Australia and sampling locations (yellow stars): P, Perth and includes the sampling sites of Kings Park and Botanic Garden, Warwick and Bold Park E, Helms Arboretum site; J, Jarrahdale; C, Collie; LG, Lake Grace; M, Melbourne. (Distribution map provided by Australian Virtual Herbarium: http://www.anbg.gov.au/avh/.)

included in the alignment and analysis, in addition to some from other Australian native orchids (Table 1). The multiple sequence alignment was carried out using the T-coffee multiple sequence alignment program Version 1.37 (Notredame *et al.* 2000). The sequence alignment was manually edited using MacClade 4.05. The matrix was analysed with PAUP (Swofford, 2000) using parsimony methods with ericoid mycorrhizal fungi obtained from *P. nigricans* (isolates P27 and P28, Table 1) defined as the outgroup. A heuristic search was performed using 100 random sequence addition with tree bisection–reconnection (TBR) branch swapping, saving 100 trees per replicate. Branch reliability was based on BS analysis with 100 simple sequence replicates and TBR branch swapping.

Results and discussion

Germination trial

In this study, the symbiotic germination trials used 12 fungal isolates from *Disa bracteata* and the West Australian native orchids, collected in WA, Victoria and South Africa (Fig 1). These fungi were later assigned to three major clades in the *Rhizoctonia* alliance (two separate experiments were conducted in parallel). With the exception of an endophytic ascomycete isolated from *Thelymitra crinita*, fungi sustained growth of the orchid from which they were isolated and most were also compatible with other orchids. Results of the germination assay revealed complex 'webs' of interaction linking fungi to compatible orchids. The shape of these fungal diversity webs varied considerably (Fig 1), from broad webs where orchids were compatible with diverse fungal groups, to narrow webs where orchids were compatible with one fungus or a small group of fungi.

D. bracteata germinated with isolates obtained throughout its 10,000 km geographic range confirming its compatibility with geographically disjunct fungi (Fig 1). Many of these same fungi were also compatible with the Australian orchids *Microtis media*, *Diuris magnifica* and *Thelymitra crinita*. Indeed, *M. media* and *T. crinita* were compatible with a South African fungus isolated from *Disa bracteata*. Pope & Carter (2001) also found that an Australian orchid (*Pterostylis acuminata*) had mycorrhizal fungi with close relatives in South Africa.

The largest webs of fungal associates were recorded for *D. bracteata* and *M. media*, but these did not overlap completely. *M. media* was compatible with members of a second fungus clade (*Sebacina*), while *D. bracteata* only germinated with fungi from the *Epulorhiza* clade. *Diuris magnifica* and *T. crinita* had intermediate fungal diversity with subsets of *Epulorhiza* isolates. *Disa bracteata* also germinated with *Epulorhiza* isolates sourced from *Diuris* and *Prasophyllum*.

In contrast to the orchids compatible with diverse webs of fungi, *Caladenia falcata* and *Pterostylis sanguinea* germinated exclusively with their own fungus isolates from the *Sebacina* and *Ceratobasidium* clades, respectively (Fig 1). This study only included single fungi that were compatible with *Caladenia* or *Pterostylis* species, but the narrow fungus specificity of orchids in these genera is well known from other studies (Warcup 1981; Ramsay *et al.* 1986; Pope & Carter 2001; Huynh *et al.* 2004; Hollick *et al.* 2005).

Another key observation was that the four orchid species with relatively diverse webs of compatible fungi (*Disa bracteata*, *Diuris magnifica*, *M. media* and *T. crinita*) formed short-term relationships (brief encounters) whereby the orchid seeds developed into protocorms (swollen embryos with trichomes) with many isolates tested, but only a subset of these fungi supported substantial orchid seedlings with a leaf (Fig 1). In all cases protocorms contained mycorrhizal fungi (microscopy results not presented) and germination was much faster than in uninoculated controls. Only fully compatible fungi resulted in orchid seedlings with a leaf (lasting relationships). The same orchids also germinated to the protocorm stage on plates without fungi and had short-term relationships with the endophyte from *T. crinita* (*Phialophora*

Table 1 – Fungal isolates obtained from *Pyrorchis nigricans* (A), *Disa bracteata* (B) and Australian native orchids (C)

A. *Pyrorchis nigricans* isolates

Code	Site	Extraction	BLAST e value, percent identity	Close relative from GenBank
P02	Eucalypt and banksia woodland, Warwick, WA	Peloton	3e-105, 100%	• *Rhizoctonia* sp. Eab-S4 (Pathogen) GenBank code: AJ242884 Country of origin: Spain Source: Salazar *et al.* (unpublished)
P19	Eucalypt forest, Jarrahdale, WA	Peloton	9e-96, 98%	
P09	Eucalypt forest, Jarrahdale, WA	Tissue block	2e-100, 99%	
P17			6e-103, 99%	
P11	Eucalypt and banksia woodland, Warwick, WA	Peloton	5e-107, 97%	• *Thanatephorus cucumeris* (anamorph: Rhizoctonia solani) GenBank code: AY154318 Country of origin: Brazil Source: Kuramae *et al.* (unpublished)
P12			5e-107, 97%	
P24	Eucalypt and banksia woodland, Kings Park,WA	Peloton	2e-85, 97%	• *Tulasnella danica* specimen voucher KC 388 (orchid fungus) GenBank code: AY373297 Country of origin: USA Source: McCormick *et al.* 2004
P25			2e-85, 97%	
P20	Eucalypt forest, Jarrahdale, WA	Peloton	7e-72, 99%	
P35			3e-90, 97%	
P31			1e-89, 100%	
P30	Eucalypt forest, Jarrahdale, WA	Tissue block	1e-89, 100%	
P33	Eucalypt and banksia woodland, Warwick, WA	Peloton	1e-89, 100%	
P27	Eucalypt forest, Jarrahdale, WA	Tissue block	7e-125, 95%	• Ericoid mycorrhizal sp. Sd9 GenBank code: AF269067 Country of origin: Italy Source: Bergero *et al.* 2000
P28			7e-125, 95%	

B. *Disa bracteata* isolates

Code	Origin	Extraction	BLAST e value, percent identity	Close relative from GenBank
*SA1	Sand heathland (Proteaceous/Ericaceous heath), Betty's Bay, South Africa	Peloton	3e-130, 99%	• *Tulasnella* sp. 224 GenBank code: AY373272 Country of origin: USA Source: McCormick *et al.* 2004
SA2			3e-130, 99%	
*D44	Urban bushland, Warwick, WA	Peloton	1e-157, 96%	
*D10	Urban bushland, Melbourne, Victoria	Peloton	2e-124, 99%	• *Tulasnella* sp. JT0307 GenBank code: DQ061111 Country of origin: Australia Source: Otero (unpublished)
D51			2e-124, 99%	
D60	Mined Jarrah forest, Jarrahdale, WA	Peloton	3e-158, 98%	
*D37			1e-160, 99%	
*D32			1e-160, 99%	
*D20			e=0, 99%	
*D29	Mined Jarrah forest, Jarrahdale, WA	Tissue block	1e-160, 99%	
*D30			e=0, 99%	
*D33			e=0, 99%	
*D03	Pine plantation, Helms Arboretum, WA.	Peloton	e=0, 99%	
*D34	Mined Jarrah forest, Jarrahdale, WA	Peloton	2e-67, 95%	• *Epulorhiza* sp. Am8 (orchid fungus) GenBank code: AJ313448 Country of origin: Singapore Source: Ma *et al.* 2003
*D35			2e-67, 95%	
*D38			2e-67, 95%	

(*continued on next page*)

Table 1 (*continued*)

B. *Disa bracteata* isolates

*D46 D47	Rural bushland, Collie, WA.	Peloton	3e-37, 89% 3e-37, 89%	• *Tulasnella pruinosa* specimen voucher DAOM 17641 (orchid fungus) GenBank code: AY373295 Country of origin: USA Source:McCormick *et al.* 2004
*D31	Mined Jarrah forest, Jarrahdale, WA.	Tissue block	3e-127, 96%	• Ericoid mycorrhizal sp. Sd9 GenBank code: AF269067 Country of origin: Italy Source: Bergero *et al.* 2000
D52	Rural bushland, Lake Grace, WA.	Peloton	e=0, 99%	• *Nectria mauritiicola* (pathogen) GenBank code: AJ557830 Country of origin: Russia Source: Vasilenko *et al.* (unpublished)
D41	Rural bushland, Gidgegannup, WA.	Pelotone	2e-59, 98%	• *Leptodontidium orchidicola* GenBank code: AF214578 Country of origin: Canada Source: Addy *et al.* 2000

C. Isolates from other West Australian orchids

Origin	Host plant	BLAST e value, percent identity	Close relative from GenBank
Eucalypt and banksia woodland, Bold Park, WA.	*Thelymitra crinita*	3e-175, 91%	• *Phialophora* sp. GS6N4b GenBank code: AY465455 Country of origin: USA Source: Ganley & Newcombe 2006
Eucalypt and banksia woodland, Bold Park, WA.	*Prasophyllum giganteum* *Diuris magnifica (corymbosa)*	1e-176, 98% 1e-176, 98%	• *Tulasnella calospora* (orchid fungus) GenBank code: AY373298 Country of origin: USA Source: McCormick *et al.* 2004
Eucalypt and banksia woodland, Kings Park, WA.	*Caladenia falcate* *Microtis media*	7e-178, 99% 1e-178, 99%	• *Sebacina vermifera* (orchid fungus) GenBank code: AF202728 Country of origin: USA Source: Taylor *et al.* (unpublished).
Eucalypt & banksia woodland, Bold Park, WA.	*Pterostylis sanguinea* *Pterostylis recurva*	2e-150, 97% 1e-117, 94%	• *Vouchered mycorrhizae* (Ceratobasidium) GenBank code: DQ028808 Country of origin: Australia Source: Otero (unpublished).

Jarrah = *Eucalyptus marginata*

Isolates from the current study with very similar sequences are grouped together with a selected close relative (BLAST e value and percentage identity given) from GenBank that has been well studied. GenBank accession numbers are pending for the new isolates. Mycorrhizal formation by isolates preceded by an asterisk has been confirmed by germination assays.

sp.). Orchids with broad specificity also germinated slowly on the asymbiotic plates, but orchids with narrow specificity did not, providing further evidence that germination requirements differ between these two groups of orchids. Earlier studies have achieved asymbiotic germination of orchid seed when using complex media (Arditti *et al.* 1990).

Specificity has predominantly been determined through isolation of fungi from orchid roots and/or *in vitro* germination trials (Hadley 1970; Masuhara & Katsuya 1994; Perkins & McGee 1995; Perkins *et al.* 1995). The implication of using these techniques exclusively to describe orchid–fungi relationships has not yet been verified, and associations that are examined under *in vitro* conditions may not always represent the symbiotic germination that occurs in natural situations (Masuhara & Katsuya 1994; Rasmussen 1995; Peterson *et al.* 1998). However, our observations on orchid fungus compatibility are in agreement with earlier studies that also found orchid seeds germinate into protocorms in the presence of certain fungi that did not sustain larger seedlings, or were not obtained from adult plants of the same orchid species (Warcup 1981; Ramsay *et al.* 1986; Masuhara & Katsuya 1994; Esitken *et al.* 2005). We do not believe that that these observations can be explained by *in vitro* orchid–fungi specificity generally being broader than *in situ* specificity due to the impact of artificial growing conditions, because the fungi forming lasting relationships *in vitro* belonged to the same particular

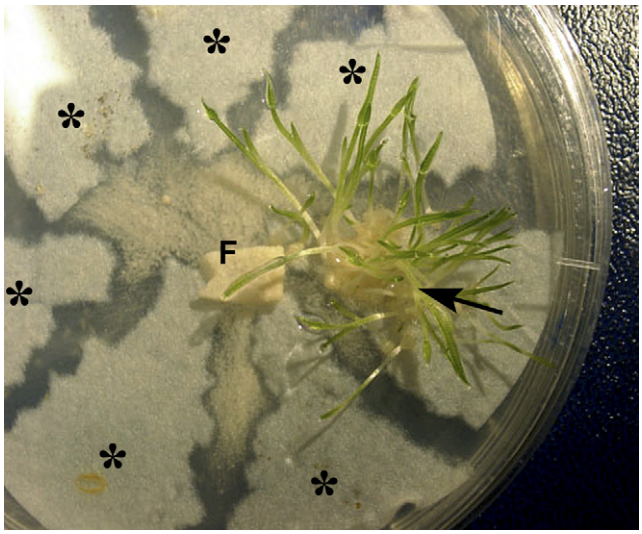

Fig 3 – Testing the specificity of orchid–fungus associations by sterile culture assays using orchid seed and a mycorrhizal fungus. Only one orchid species is compatible with this fungus (F) resulting in seedlings with a leaf (arrow) and seed of other orchids did not germinate (asterisks).

groups within the *Rhizoctonia* alliance that were isolated from orchids *in situ*. It is not known if orchids with broad specificity also form short-term relationships with a wide diversity of fungi *in situ* but perhaps this could be resolved by further research. Unfortunately, it is very difficult to measure the diversity and inoculum levels of orchid fungi in soils, so *in vitro* experiments remain the most effective means of evaluating compatibility between orchids and fungi.

We recommend that compatible fungi be designated as those that support the orchid host to an advanced seedling stage where the leaf exceeds the protocorm in length, to avoid confusion between lasting relationships and brief encounters which may not sustain seedlings in the long-term. We have shown that seedlings of this size are capable of survival after transplanting. Previous studies that defined orchid–fungus compatibility by germination to an early protocorm stage should be re-evaluated.

There was some evidence that mycorrhizal associations of orchids with narrow fungal diversity were more efficient, as a significantly higher proportion of germinated seeds for *C. falcata* and *P. sanguinea* (90–98 % ± 4 % - 95 % CI) was observed, relative to *Diuris magnifica* and *T. crinita* (56–64% ± 14%). However, germination of the weedy orchids *Disa bracteata* and *M. media* was also relatively efficient (77–84% ± 8%).

Fungal isolation and identification

Molecular identification established that almost all of the fungi isolated from pelotons were members of the *Rhizoctonia* complex, further confirming that there was a high overall success rate for isolating mycorrhizal fungi using this method (Table 1). Huynh *et al.* (2004) established that most fungi isolated from pelotons from an Australian orchid when they were growing or flowering were mycorrhizal fungi. *Rhizoctonia* isolates were also obtained from surface-sterilised tissue blocks, but this method was found to be less reliable for isolating mycorrhizal fungi. Fungi isolated from tissue blocks were discarded if they demonstrated rapid growth and sporulation, characteristic of conidial fungi. In this study, a three-stage confirmation process was used to identify orchid mycorrhizal fungi: (1) direct isolation from mycorrhizal structures (pelotons); (2) symbiotic germination assays using a well-established culture protocol (oat agar media); and (3) molecular confirmation that fungi belonged to groups within the *Rhizoctonia* alliance known to contain orchid mycorrhizal fungi. Consequently, there can be little doubt that the majority of these fungi were mycorrhizal. It is recommended that orchid mycorrhizal fungi be designated with caution when less evidence is available.

Accurate identification of orchid mycorrhizal fungi using molecular methods is necessary to reveal the diversity of fungi compatible with orchids. However, symbiotic germination remains the only practical method to confirm compatibility between orchids and fungi. We have found that symbiotic germination tests in combination with accurate fungal identification provided more information about the specificity of orchid fungi relationships than either method alone would provide.

Non-*Rhizoctonia* fungi that were sequenced and identified using GenBank (Table 1), included *Verticillium*, *Leptodontium*, *Dactylella* (a nematode-destroying fungus) and *Phialophora dimporphospora* (probably an ericoid mycorrhizal fungus). Endophytic growth of mycorrhizal fungi in non-host plants is common and seems to be especially common in orchids (Bayman & Otero 2006; Brundrett 2006).

The wide divergence of rDNA ITS sequences of *Rhizoctonia* alliance fungi made it very difficult to align their DNA, it was still possible to use these sequences for phylogenetic analysis (Fig 4). This analysis classified isolates into distinct groups, most of which had high bootstrap support (Fig 4). These groups of fungi include representatives of the three major clades in the *Rhizoctonia* alliance found in other studies of orchid fungi; *Epulorhiza*, *Sebacina* and *Ceratobasidium* (e.g. Shan *et al.* 2002; Taylor *et al.* 2003; Kristiansen *et al.* 2004; Weiss *et al.* 2004; Shefferson *et al.* 2005). Many isolates were closely related to well-studied pathogenic and orchid fungi in the *Rhizoctonia* alliance, (Fig 4, Table 1). Earlier studies of Australian orchids in the genera *Caladenia*, *Pterostylis*, *Microtis* and *Thelymitra* assigned mycorrhizal fungi to the same broad fungal genera by morphological classification (Warcup 1981; Ramsay *et al.* 1986; Milligan & Williams 1988).

The largest and most diverse group (Fig 4) consisted of members of the anamorph genus *Epulorhiza* in the Tulasnellales. All the *D. bracteata* isolates belonged to the *Epulorhiza* group with one exception, which was a non-orchid fungus. Isolates from the Australian orchids *Prasophyllum giganteum* and *Diuris magnifica* also belonged to this group. Members of the two other major groups within *Rhizoctonia*, *Sebacina* and *Ceratobasidium*, were isolated from *Caladenia falcata*, *Pterostylis* spp. and *M. media*. These fungi were closely related to pathogenic *Ceratobasidium* isolates and putative ectomycorrhizal *Sebacina* isolates from the Northern Hemisphere (Table 1).

Both *D. bracteata* and *P. nigricans* associated with a relatively broad diversity of fungi belonging to the *Epulorhiza* clade which clustered into four discrete groups in Fig 4. Indeed,

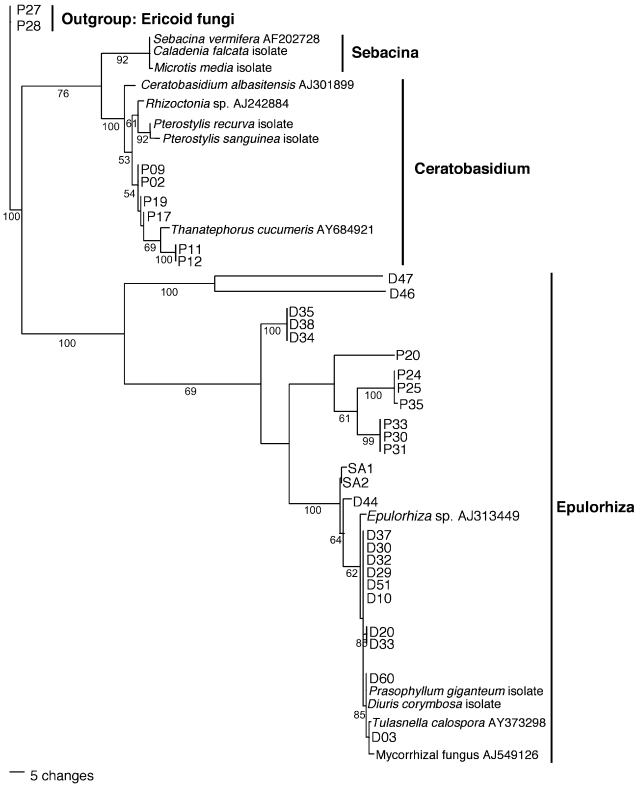

Fig 4 – Phylogenetic tree of the ITS sequences of fungi isolated from *Disa bracteata* (D), *Pyrorchis nigricans* (P) and other Australian native orchids, with sequences of related fungi from GenBank. Numbers under branches correspond to values. See Table 1 for more information about fungi and bootstrap host plants.

P. nigricans appeared to be symbiotic with the widest diversity of fungi, including isolates of *Epulorhiza* and *Ceratobasidium*. Different fungi were isolated from *P. nigricans* plants collected from locations only meters apart, which further confirms this orchid's ability to utilise a wide diversity of fungi. We were not able to confirm that all of the fungi isolated from *P. nigricans* were mycorrhizal due to very low rates of seed germination. However, we are confident the majority of these fungi were mycorrhizal as they were isolated from single pelotons with sufficient replication to confirm that the dominant fungi were obtained. *Pyrorchis nigricans* is one of Australia's most widespread native orchid species and it occupies an unusually diverse range of habitats, including dense forests and open heathlands (Jones 1988; Hoffman & Brown 1992), but it rarely colonises disturbed habitats (Collins *et al.* 2005). The capability of *P. nigricans* to utilise a diverse range of fungi that are widespread may be linked to its capacity to grow in more diverse habitats than most other orchids. This orchid typically reproduces vegetatively under natural conditions, giving rise to clonal populations, and tends to only flower after a hot summer fire (Jones 1988; Hoffman & Brown 1992). These restrictions on seed production would also be an important factor limiting its recruitment into disturbed habitats.

Even though *D. bracteata* and *P. nigricans* were chosen to represent opposite ends of an ecological continuum (disturbance tolerant or intolerant), both had a wide diversity of fungal partners. Differences in the fungal associates used by the orchids in the Jarrahdale mine site sampling region may also be attributed to variations in soil conditions and disturbance, as *P. nigricans* plants only occurred in undisturbed bushland areas while most *D. bracteata* plants were from adjacent rehabilitated mined areas.

The widespread distribution of fungi from terrestrial orchids was made evident by the closely related, or in some cases identical fungi (in relation to the ITS region sequenced) sampled from widely separated geographic locations (Table 1, Figs 2, 4). In particular, there were closely related fungi associated with *D. bracteata* from locations up to 10,000 km apart, extending from eastern Australia to South Africa. These *Epulorhiza* isolates belonged to a clade that appears to have a worldwide distribution, with close relatives known from Northern Hemisphere orchids (Table 1). The cosmopolitan distribution of these fungi probably explains why *D. bracteata* spread rapidly after its arrival in Australia 60 y ago. However, the rapid spread of this orchid is also facilitated by efficient seed set due to self-pollination (Linder & Kurzweil 1999). Consequently, broad webs of compatible mycorrhizal fungi may be as important as other ecological attributes such as high fecundity for weed-like orchids such as *D. bracteata* and *M. media*. *D. bracteata* responds favourably to disturbance in South Africa, but seems to be restricted to a narrower range of habitats than in Australia (Linder & Kurzweil 1999).

Orchids with broad webs of fungi should encounter a compatible fungus after dispersal more often than other orchids, and thus be more capable of distribution or migration to new habitats, as was the case with *D. bracteata* — a South African orchid rapidly invading Australia. Thus, we should expect other weed-like orchids such as *M. media* to have broad webs of compatible fungi and to also have the capacity to spread rapidly if introduced to other continents. Indeed, the closely related species *M. unifolia* and *M. parvifolia* also associate with *Epulorhiza* (Milligan & Williams 1988; Perkins *et al.* 1995). Both these species are widespread in eastern Australia especially in frequent disturbed habitats and *M. unifolia* occurs throughout Asia (Jones 1988).

It would be expected that disturbance-tolerant orchids require disturbance-tolerant fungi. Most fungi compatible with *D. bracteata* and *M. media* belonged to *Epulorhiza*, suggesting that this group of fungi may be more likely to occur in disturbed habitats (Table 1). However, Australian orchids that primarily occur in undisturbed habitats, such as *Prasophyllum* and *Diuris*, also associated with *Epulorhiza* isolates overlapping with those compatible with *D. bracteata* and *M. media*. These results suggest that *Epulorhiza* isolates are common in both undisturbed and disturbed habitats. The results of this study suggest that *D. bracteata* may compete for niche space with Australian orchids such as species of *Diuris* and *Prasophyllum* that have overlapping webs of fungi. However, this alien invader is unlikely to compete with orchids that utilise fungi other than *Epulorhiza* (e.g. species of *Caladenia* and *Pterostylis*). There is currently great concern surrounding the aggressive spread of *D. bracteata* (Backhouse 2000; Fleming 2002), but it is not yet clear whether the orchid will have any major impacts on the ecology of native Australian orchids.

Recognition processes are an essential part of most symbiotic associations (Radutoiu *et al.* 2003), but the occurrence of short-term relationships suggests that orchids with broad webs of fungi lack these recognition processes. Thus, seed of these orchids seem to germinate in the presence of any fungus with sufficient endophytic competence, but many of the resulting protocorms abort because of physiological imbalances with these fungi. Recognition processes seem to be more complex in orchids with narrow fungus diversity, which only germinate in the presence of a suitable fungus. Orchid cells containing coils of hyphae are considered to maintain a delicate metabolic balance to control 'untamed' fungi differing from other mycorrhizal fungi as these fungi have not evolved to take part in mutualistic associations with plants (Brundrett 2002, 2004). This risky fungus recruitment process may explain the seed strategy of orchids where millions of dust-like seed are efficiently dispersed but few survive (Benzing & Atwood 1984; Brundrett 2002). Orchids have switched to myco-heterotrophy (where fungi replace photosynthesis) far more often than other plants (Molvray *et al.* 2000; Brundrett 2002; Taylor *et al.* 2003), perhaps because of the flexible nature of their fungal recruitment strategies which allow them to exploit new lineages of fungi.

It is becoming increasing apparent that many terrestrial orchids have highly specific mycorrhizal association, which is a fundamental difference from plants in other families where individual plants associate with a wide diversity of fungi (Brundrett 2002, 2004). These associations have profound consequences as the development of orchids will be intrinsically linked to the productivity of a particular fungus. It is reasonable to expect that orchids that are compatible with a limited range of fungi would be more predisposed to being endangered, as there would be lower probability of seeds encountering a compatible fungus after dispersal compared to orchids that have broader webs of fungi. However, these orchids may also benefit from high specificity, perhaps due to more efficient mycorrhizal associations and seed germination. Presumably, the overall advantages of narrow compatibility webs outweigh the disadvantages, or this strategy would not be so common in terrestrial orchids. Orchids with narrow webs of compatible fungi are common in Australia (e.g. the large genera *Caladenia* and *Pterostylis*). However, these genera also include many rare taxa, perhaps due to limitations in finding compatible fungi in highly complex landscapes. Orchids in these genera also tend to have specific pollinators (Stoutamire 1983; Adams & Lawson 1993).

The primary roles of fungi in the *Rhizoctonia* alliance with secondary roles in mycorrhizal or pathogenic associations are still not well understood, as these fungi are more widespread than their hosts due to their endophytic and saprophytic competencies (Roberts 1999; Sen *et al.* 1999; Brundrett 2002; Rasmussen 2002). These polyphyletic fungi are functionally diverse. Some members of the *Sebacina* clade are considered to be ectomycorrhizal (Selosse *et al.* 2004; Weiss *et al.* 2004), the *Ceratobasidiales* clade includes many pathogens (Gonzales *et al.* 2001) and the *Epulorhiza* clade includes the most isolates so far only known from orchids. Knowledge of the functional diversity of fungi within and between these groups is a cornerstone for orchid conservation, as it will help us to explain the habitat requirements and recruitment patterns of orchids (Batty *et al.* 2002). This knowledge is also required to control the activities of *Rhizoctonia* alliance fungi, which are soil-borne plant pathogens.

The complex relationships between the phylogenies of orchids and their mycorrhizal fungi (Figs 1, 4), suggests that both the narrow and broad mycorrhizal specificities of orchids have multiple origins and it is not clear which strategy is more primitive. Accurate DNA-based identification of orchid fungi is beginning to reveal correlations between mycorrhizal fungus specificity and the ecology of orchids (Kristiansen *et al.* 2004; McCormick *et al.* 2004; Selosse *et al.* 2004; Esitken *et al.* 2005; Shefferson *et al.* 2005). Our results demonstrated that fundamentally different categories of terrestrial orchids exist that vary in the size of their webs of compatible fungi — a key biological property regulating their capacity for recruitment. We are just beginning to understand how the nature of mycorrhizal associations contributes to the uniqueness of these amazing plants.

Acknowledgements

Alcoa, World Alumina provided funding and field site access. The Australian Research Council provided support. Special thanks go to Madeleine Parent and Robyn Taylor for laboratory assistance and to William Kenworthy for his help with the sequence alignments.

REFERENCES

Adams PB, Lawson SD, 1993. Pollination of Australian orchids: a critical assessment of the literature 1882–1992. *Australian Journal of Botany* **41**: 553–575.

Addy H, Hambleton S, Currah RS, 2000. Distribution and molecular characterization of the root endophyte *Phialocephala fortinii* along an environmental gradient in the boreal forest of Alberta. *Mycological Research* **104**: 1213–1221.

Arditti J, Ernst R, Wing Yam T, Glabe C, 1990. The contribution of orchid mycorrhizal fungi to seed germination: a speculative review. *Lindleyana* **5**: 249–255.

Backhouse G, 2000. The occurrence of the South African orchid *Disa bracteata* Sw. in Victoria. *Australian Orchid Review* **65**: 22.

Batty AL, Dixon KW, Brundrett MC, Sivasithamparam K, 2002. Orchid conservation and mycorrhizal associations. In: Sivasithamparam K, Dixon KW, Barrett RL (eds), *Microorganisms in Plant Conservation and Biodiversity*. Kluwer Academic Publishers, Dordrecht, pp. 195–226.

Batty AL, Brundrett MC, Dixon KW, Sivasithamparam K, 2006. New methods to improve symbiotic propagation of terrestrial orchid seedlings from axenic culture to soil. *Australian Journal of Botany* **54**: 367–374.

Bayman P, Otero JT, 2006. Microbial endophytes of orchid roots. In: Schulz, Boyle, Sieber (eds), *Mycorrhizal Root Endophytes*. Springer Verlag, pp. 153–177.

Benzing DH, Atwood Jr JT, 1984. *Orchidaceae*: ancestral habitats and current status in forest canopies. *Systematic Botany* **9**: 155–165.

Bergero R, Perotto S, Girlanda M, Vidano G, Luppi AM, 2000. Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (*Quercus ilex*). *Molecular Ecology* **9**: 1639–1649.

Brundrett M, 2002. Coevolution of roots and mycorrhizas of land plants. *New Phytologist* **154**: 275–304.

Brundrett M, 2004. Diversity and classification of mycorrhizal associations. *Biological Reviews* **79**: 473–495.
Brundrett MC, 2006. Understanding the roles of multifunctional mycorrhizal and endophytic fungi. In: Schulz, Boyle, Sieber (eds), *Mycorrhizal Root Endophytes*. Springer Verlag, pp. 281–298.
Cenis JL, 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research* **84**: 2380.
Chase MW, Cameron KM, Barrett RL, Freudenstein JV, 2003. DNA data and *Orchidaceae* systematics: a new phylogenetic classification. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (eds), *Orchid Conservation*. Natural History Publications (Borneo), Kota Kinabalu, Sabah, pp. 69–89.
Collins M, Koch J, Brundrett M, Sivasithamparam K, 2005. Recovery of terrestrial orchids in the post-mining landscape. *Selbyana* **25**: 255–264.
Currah RS, Zelmer CD, Hambleton S, Richardson KA, 1997. Fungi from orchid mycorrhizas. In: Arditti J, Pridgeon A (eds), *Orchid Biology: Reviews and Perspectives, VII*. Kluwer Academic Publishers, Dordrecht, pp. 117–170.
Erickson R, 1965. *Orchids of the West*. Paterson Brokensha, Perth.
Esitken A, Ercisli S, Eken C, 2005. Effects of mycorrhiza isolates on symbiotic germination of terrestrial orchids (*Orchis palustris* Jacq. and *Serapias vomeracea* subsp *vomeracea* (Burm.f.) Briq.) in Turkey. *Symbiosis* **38**: 59–68.
Fleming J, 2002. South African weed orchid: identification. In: *Landcare Notes*. Department of Natural Resources and Environment, Victoria, pp. 1–2.
Freudenstein JV, van den Berg C, Goldman DH, Kores PJ, Molvray M, Chase MW, 2004. An expanded plastid DNA phylogeny of *Orchidaceae* and analysis of jackknife branch suport strategy. *American Journal of Botany* **91**: 149–157.
Ganley RJ, Newcombe G, 2006. Fungal endophytes in seeds and needles of *Pinus monticola*. *Mycological Research* **110**: 318–327.
Gonzales D, Carling DE, Kuninaga S, Vilgalys R, Cubeta MA, 2001. Ribosomal DNA systematics of *Ceratobasidium* and *Thanatephorus* with *Rhizoctonia* anamorphs. *Mycologia* **93**: 1138–1150.
Grant CD, Koch J, 2003. Orchid species succession in rehabilitated bauxite mines in Western Australia. *Australian Journal of Botany* **51**: 453–457.
Hadley G, 1970. Non-specificity of symbiotic infection in orchid mycorrhiza. *New Phytologist* **69**: 1015–1023.
Hoffman N, Brown A, 1992. *Orchids of South-West Australia*, 2nd ed. University of Western Australia Press, Perth.
Hollick P, Taylor R, McComb J, Dixon K, 2005. If orchid mycorrhizal fungi are so specific, how do natural hybrids cope? *Selbyana* **26**: 159–170.
Hopper SD, Gioia P, 2004. The Southwest Australian Floristic Region: evolution and conservation of a global hotspot of biodiversity. *Annual Review of Evolution and Systematics* **35**: 623–650.
Huynh TT, McLean CB, Coates F, Lawrie AC, 2004. Effect of developmental stage and peloton morphology on success in isolation of mycorrhizal fungi in *Caladenia formosa (Orchidaceae)*. *Australian Journal of Botany* **52**: 231–241.
Jones DL, 1988. *Native Orchids of Australia*. Reed Books, New South Wales.
Kristiansen KA, Freudenstein JV, Rasmussen FN, Rasmussen HN, 2004. Molecular identification of mycorrhizal fungi in *Newiedia veratrifolia (Orchidaceae)*. *Molecular Phylogenetics and Evolution* **33**: 251–258.
Linder HP, Kurzweil H, 1999. *Orchids of southern Africa*. A.A. Balkema, Rotterdam.
Ma M, Tan TK, Wong SM, 2003. Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. *Mycological Research* **107**: 1041–1049.
Masuhara G, Katsuya K, 1994. *In situ* and *in vitro* specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon.) Ames. var. *amoena* (M. Beiberstein) Hara (Orchidaceae). *New Phytologist* **127**: 711–718.
McCormick MK, Whigham DF, O'Neill J, 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* **163**: 425–438.
Milligan MJ, Williams PG, 1988. The mycorrhizal relationship of multinucleate rhizoctonias from non-orchids with *Microtis* (Orchidaceae). *New Phytologist* **108**: 205–210.
Molvray M, Kores PJ, Chase MW, 2000. Polyphyly of mycoheterotrophous orchids and functional influences on floral and molecular characters. In: Wilson KL, Mossison DA (eds), *Monocots, Systematics and Evolution*. CSIRO, Melbourne, pp. 441–448.
Mursidawati S, 2004. Mycorrhizal association, propagation and conservation of the myco-heterotrophic orchid *Rhizanthella gardneri*. MSc thesis. The University of Western Australia.
Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J, 2000. Biodiversity hotspots for conservation priorities. *Nature* **403**: 853–858.
Notredame C, Higgins DG, Heringa J, 2000. T-coffee, a novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology* **302**: 205–217.
Peakall R, Beattie AJ, 1996. Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentaculata*. *Evolution* **75**: 351–361.
Perkins AJ, Masuhara G, McGee PA, 1995. Specificity of the associations between *Microtis parvifolia (Orchidaceae)* and its mycorrhizal fungi. *Australian Journal of Botany* **43**: 85–91.
Perkins AJ, McGee PA, 1995. Distribution of the orchid mycorrhizal fungus *Rhizoctonia solani*, in relation to it's host, *Pterostylis acuminata*, in the field. *Australian Journal of Botany* **43**: 565–575.
Peterson RL, Uetake Y, Zelmer C, 1998. Fungal symbioses with orchid protocorms. *Symbiosis* **25**: 29–55.
Pope EJ, Carter DA, 2001. Phylogenetic placement and host specificity of mycorrhizal isolates belonging to AG-6 and AG-12 in the *Rhizoctonia solani* species complex. *Mycologia* **93**: 712–719.
Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J, 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425**: 585–592.
Ramsay RR, Dixon KW, Sivasithamparam K, 1986. Patterns of infection and endophytes associated with Western Australian orchids. *Lindleyana* **1**: 203–214.
Rasmussen HN, 1995. *Terrestrial Orchids, From Seed To Mycotrophic Plant*. Cambridge University Press, Cambridge.
Rasmussen HN, 2002. Recent developments in the study of orchid mycorrhiza. *Plant & Soil* **244**: 149–163.
Roberts P, 1999. *Rhizoctonia-forming. Fungi: a taxonomic guide*. Royal Botanic Gardens, Kew.
Selosse M-A, Faccio A, Scappaticci G, Bonfante P, 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (*Neottieae, Orchidaceae*) are associated with ectomycorrhizal septomycetes, including truffles. *Microbial Ecology* **47**: 416–426.
Sen R, Hietala AM, Zelmer CD, 1999. Common anastomosis and internal transcribed spacer RFLP groupings in binucleate *Rhizoctonia* isolates representing root endophytes of *Pinus sylvestris*, *Ceratorhiza* spp. from orchid mycorrhizas and a phytopathogenic anastomosis group. *New Phytologist* **144**: 331–341.
Shan XC, Liew ECY, Weatherhead MA, Hodgkiss IJ, 2002. Characterization and taxonomic placement of *Rhizoctonia*-like endophytes from orchid roots. *Mycologia* **94**: 230–239.
Shefferson RP, Weiss M, Kull T, Taylor DL, 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Molecular Ecology* **14**: 613–626.

Sivasithamparam K, 1993. Ecology of root infecting pathogenic fungi in mediterranean environments. *Advances in Plant Pathology* **10**: 245–279.

Stoutamire WP, 1983. Wasp-pollinated species of *Caladenia (Orchidaceae)* in south-western Australia. *Australian Journal of Botany* **31**: 383–394.

Swofford DL, 2000. *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*, version 4. Sinauer Associates, Sunderland, MA.

Taylor DL, Bruns TD, Szaro TM, Hodges SA, 2003. Divergence in mycorrhizal specialization within *Hexalectris spicata (Orchidaceae)*, a nonphotosynthetic desert orchid. *American Journal of Botany* **90**: 1168–1179.

Warcup JH, 1981. The mycorrhizal relationships of Australian orchids. *The New Phytologist* **87**: 371–381.

Weiss M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F, 2004. *Sebacinales*, a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycological Research* **108**: 1003–1010.

White TJ, Bruns TD, Taylor LS, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: McInnis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols. A guide to method and applications*. Academic Press, San Diego, pp. 315–322.