The functional significance for the orchid *Caladenia tentaculata* of genetic and geographic variation in the mycorrhizal fungus *Sebacina vermifera* s. lat. complex

Magali Wright[1,2]*, Rob Cross[2], Roger D. Cousens[1], Tom W. May[2] and Cassandra B. McLean[1]

[1]   Melbourne School of Land and Environment, The University of Melbourne, Burnley Campus, 500 Yarra Boulevard, Richmond, Victoria 3121, Australia

[2]   Royal Botanic Gardens Melbourne, Birdwood Avenue, South Yarra, Victoria 3141, Australia

* Corresponding author. e-mail: mmwright@unimelb.edu.au

Abstract

To investigate the influence of provenance of mycorrhizal fungi on functional diversity in orchids, six isolates of two taxa in the *Sebacina vermifera* complex, were sourced from Victorian *Caladenia tentaculata* populations. Germination of orchid seeds with isolates from their own and other populations in vitro and utilisation of a range of carbon and nitrogen substrates for growth in culture were tested. There was considerable inter- and intra-taxon variation in seed germination abilities but no evidence of a 'home site' advantage for fungal isolates. All isolates utilised the carbon compounds carboxymethyl cellulose and pectin, and inorganic nitrogen in the form of NH_4+ but not NO_3-, as their sole nitrogen source. However, the two fungal taxa differed in ability to utilise sucrose as a sole carbon source. Implications for sourcing mycorrhizal fungi to propagate orchid plants for conservation purposes are discussed.

Keywords: orchid mycorrhiza; seed germination; nutrient utilisation

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Introduction

Orchid seeds require colonisation by mycorrhizal fungi for germination (Zettler 1997) and depend on this interaction for nutrition into adulthood (Cameron *et al.* 2006, 2007; Girlanda *et al.* 2006). The dependence of orchids on fungi often results in highly dispersed populations and, as a result, these plants face heightened extinction risks (Rasmussen & Rasmussen 2009). Species of the summer dormant terrestrial genus *Caladenia* represent an astounding 5% of Australia's threatened flora (Dixon & Hopper 2009), thus presenting a significant conservation challenge. The ability of orchid mycorrhizal fungi to induce orchid seed germination has been harnessed to propagate plants (symbiotic propagation) for *ex situ* collections and reintroduction into natural habitats (Scade *et al.* 2006; Brundrett 2007; Wright *et al.* 2009). Many critically endangered Victorian orchid species have been reduced to small fragmented populations (Backhouse & Cameron 2005). Thus fungal provenance is a potentially important factor when sourcing mycorrhizal isolates for symbiotic propagation; but one that is currently overlooked.

The majority of mycorrhizal fungi isolated from photosynthetic orchids belong to the anamorphic form genus *Rhizoctonia* (Rasmussen 2002). The *Rhizoctonia* fungi associated with orchids represent an assemblage of taxonomically diverse fungal groups whose teleomorphic (sexual) stages belong to three distantly related basidiomyceteous lineages in the Sebacinales (Sebacinaceae) and Cantharellales (Tulasnellaceae and Ceratobasidiaceae) (Taylor *et al.* 2003; Weiβ *et al.* 2004; Taylor & McCormick 2008). *Caladenia* have highly specific mycorrhizal interactions with fungi from the *Sebacina vermifera* complex (Weiβ *et al.* 2004; Dearnaley *et al.* 2009; Wright e*t al.* 2010; Swarts *et al.* 2010). Members of the Sebacinales are not exclusive in their interactions with orchid plants and are found

to undertake a variety of other ecosystem roles, such as saprotrophs (Roberts 1999), bryophyte endophytes (Newsham & Bridge 2009) and ectomycorrhizas (McKendrick *et al.* 2000; Selosse *et al.* 2002). Despite the ecological importance of these fungi and the varied interactions they form with plants, there are no baseline studies on their nutritional requirements.

In a mycorrhizal symbiosis the fungus transfers soil-derived nutrients to the plant host (Smith & Read 1997). Many Australian soils are nutrient-poor (Attiwill & Leper 1987; Schmit & Steward 1997) and most of the macronutrients essential for plant growth are associated with the organic fraction (King & Buckney 2002), thus ability to access nutrients in organic forms makes mycorrhizal fungi in these soils of significant benefit to their hosts. Orchid mycorrhizal interactions differ from those found in the majority of plant families, as the fungi provide carbon (Cameron *et al.* 2006; Girlanda *et al.* 2006), as well as nitrogen (and phosphorus) to their plant partners (Cameron *et al.* 2007, 2008). While the ability to germinate orchid seed is an essential functional attribute when selecting mycorrhizal fungi for conservation purposes, it is also important to consider their ability to harness different sources of nutrients that can be made available for orchid growth.

The objective of this study was to investigate the influence of fungal provenance on the functional diversity of orchid mycorrhizal fungi from the *Sebacina vermifera* complex. This was achieved by 1) confirming the identity of isolates associating with six geographically distinct *Caladenia tentaculata* study populations growing in a range of vegetation types, edaphic and climatic conditions using ribosomal DNA (rDNA) sequencing, 2) assessing the ability of these isolates to induce *in vitro* germination of orchid seeds from each of the study populations and, 3) comparing
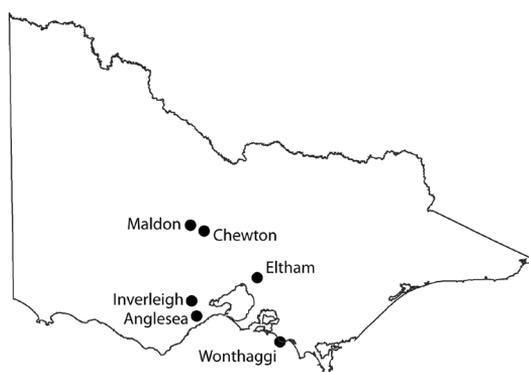

Figure 1. A map showing the relative locations of the six Victorian *Caladenia* populations selected for this study.

their abilities to utilise a range of carbon and nitrogen sources for growth in axenic culture.

Materials and Methods

Study populations

Six geographically distinct *Caladenia tentaculata* populations in Victoria, Australia were selected to represent a range of vegetation types and climatic conditions (Table 1). The six study populations (Fig. 1) were located near Maldon (144.06°E, -36.99°S), Chewton (144.27°E, -37.07°S), Eltham (145.14°E, -37.72°S), Inverleigh (144.05°E, -38.03°S), Anglesea (144.18°E, -38.41°S) and Wonthaggi (145.58°E, -38.68°S).

To investigate edaphic conditions present at each site, twenty 10 cm deep soil core samples were taken at 1 m intervals along 20 m transects at each site adjacent to the largest concentration of *C. tentaculata* plants. The soil samples were dried at 40 °C and sent to the State Chemistry Laboratory (Department of Primary Industry, Werribee) for testing. The samples were mixed to make

Table 1. Site characteristics for each *C. tentaculata* population, including Ecological Vegetation Class (EVC), maximum air temperature, mean annual rainfall and mean soil nutrient content of the composite sample from twenty 10 cm soil cores.

Population	EVC	Max air temp. Jan/Jul (°C)	Rainfall (mm/yr)	Total C (%w/w)	Total N (%w/w)	N as NH_4+ (mg/kg)	N as NO_3- (mg/kg)	pH
Anglesea	Heathy forest	23.0/12.6	814	3.8	0.12	4.4	0.9	5.2
Chewton	Box ironbark forest	27.9/11.7	597	2.9	0.12	2.9	3.7	4.8
Eltham	Grassy dry forest	25.1/11.4	748	6.2	0.32	7.2	1.0	4.8
Inverleigh	Grassy woodland	24.4/10.9	562	1.4	0.06	2.3	0.5	5.2
Maldon	Grassy dry forest	28.2/11.3	621	2.8	0.15	8.1	7.4	5.7
Wonthaggi	Coastal heathland	28.2/13.5	945	7.9	0.38	5.6	2.0	5.4

Table 2. The isolate name, accession number (RBGM living collection), GenBank number for ITS sequence and information on the closest Genbank match for the six isolates.

Population	Isolate name	RBGM accession	GenBank accession	Identity (percentage match) of the closest match against GenBank ITS sequences. Numbers in parentheses are the base pairs matching/base pairs compared
Anglesea	A2	080313	EF470248	98% (506/512) DQ983815 *Sebacina vermifera*
Chewton	C3	080314	EF470249	98% (506/512) DQ983815 *S. vermifera*
Eltham	E2	080315	EU526279	97% (407/419) DQ983815 *S. vermifera*
Inverleigh	I4	061048	EU526280	97% (433/446) DQ983815 *S. vermifera*
Maldon	M4	080316	EU526281	96% (410/424) DQ983816 *S. vermifera*
Wonthaggi	W2	080313	EU526282	98% (512/518) DQ983815 *S. vermifera*

a single composite soil sample (w/w) and were tested for pH, total carbon (% w/w), total nitrogen (% w/w), amount of nitrogen as NH_4^+ (mg/kg) and amount of nitrogen as NO_3^- (mg/kg).

Mycorrhizal isolates

Six fungal isolates (Table 2) were obtained, one from each of the six study *C. tentaculata* populations (Fig. 1). Isolates were cultured from stem-collars after removal from the plants using the peloton rinsing method of Rasmussen *et al.* (1990). Isolates were cultured, stored under sterile water and lodged in the Royal Botanic Gardens Melbourne (RBGM) Living Collection as described by Wright *et al.* (2010). Isolate accession numbers are shown in Table 2.

DNA isolation, amplification and sequencing

Fungal mycelia from each of the isolates (Table 2) were grown for 4-6 weeks in low carbon and nitrogen Melin Norkrans liquid media (low CN MMN; Marx & Bryan 1975). The mycelium was then removed, blotted dry, weighed and then crushed to a powder in a mortar and pestle using liquid nitrogen. Qiagen® DNeasy mini kits were used to isolate DNA from each sample according to the manufacturer's instructions. The ITS region of the DNA was amplified using the primer set ITS1- ITS4 (White *et al.* 1990). The PCR reactions and thermal cycling conditions followed those outlined in Wright *et al.* (2010). PCR products were then purified using a Qiagen® PCR purification kit according to the manufacturer's instructions to prepare for ITS sequencing. Purified PCR products were sequenced following the methods outlined in Wright *et al.* (2010).

Sequence analysis

The ITS sequences were edited manually by checking nucleotides against the corresponding chromatographs for sequencer reading errors. The edited ITS sequences were then BLAST searched on GenBank to find the closest matches. Relationships among the *Caladenia* mycorrhizal fungi were investigated by aligning ITS sequences (416 bp) with closely matched sequences downloaded from GenBank, using *Geastrum saccatum* as the out-group. Alignments were constructed using Clustal X and edited manually in MEGA version 4 (Tamura *et al.* 2007). The evolutionary history was inferred using the Maximum Parsimony (MP) method (Eck & Dayhoff 1996) in MEGA. A bootstrap consensus tree was inferred from 10,000 replicates. The MP tree was obtained using the Close-Neighbour-Interchange algorithm with search level 3 (Nei & Kumar 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the datasets.

In vitro symbiotic seed germination

Seeds from multiple *Caladenia tentaculata* plants from each of the six populations were surface sterilised (3 minutes in 0.5% NaOCl and rinsed six times in deionised water). They were then placed on individual filter paper wedges in each replicate Petri dish (Bonnardeaux *et al.* 2007), which contained 2.5% oatmeal agar (containing 2.5g crushed rolled oats, 0.1g yeast extract and 10g of agar in 1L of deionised water, pH 5-6, modified from Clements & Elyard 1979). Each Petri dish was inoculated with a single isolate from one of the six populations (Table 2), with a treatment left

uninoculated as a negative control. This process was replicated ten times. The Petri dishes were arranged in a randomised complete block design and exposed to continuous light with a photosynthetic photon flux of 8.3 μmol m^{-2}s^{-1} at 21°C.

Statistical analysis: *in vitro* seed germination

Germination data were recorded as the number of stage 3 and stage 4 protocorms (Ramsey & Dixon 2003) on each piece of filter paper as a proportion of the initial seed number (the number of seeds without embryos subtracted from the total number of seeds on each piece of filter paper). The mean initial seed number (± standard error of the mean) was 51.3 ± 2.9. The data were analysed using a generalised linear model with binomial errors in GenStat®. A separate statistical analysis was performed on the seed germination data for each individual seed batch.

Carbon and nitrogen utilisation

The six fungal isolates (Table 2) were grown with four different carbon and three different nitrogen sources (described below) as well as in the absence of both carbon and nitrogen in liquid low CN MMN (Marx & Bryan 1975). Thirty mL of each medium was aliquoted into 120mL tissue culture tubes, which were inoculated with a single 5mm^3 cube of agar excised from the growing edge of colonies of the fungal isolates on agar plates. There were six replicates of each isolate for each medium. A control without fungal inoculation was included for all media (also replicated six times). The tubes were incubated, unagitated, at 21°C. For both the carbon and nitrogen experiments, the positions of the culture tubes from each treatment (media × isolate) were randomly assigned in six blocks. After six weeks the fungal mycelium was separated from the liquid medium by filtration through pre-weighed pieces of filter paper (Sartorium®, Grade 603N) and dried at 65°C for one week. The dry weight of the mycelium from each tube was measured. The mean dry weight for the control was subtracted from the dry weight data for the corresponding medium to correct for any interference due to the weight of residual media on the filter paper (especially particulate matter present in the media containing chitin).

Media modifications

The pH of the low CN MMN was adjusted to 5.0-6.0 before the addition of the ferric EDTA and autoclaved at 121°C for 20 minutes. The four carbon sources used were glucose (Sigma®), sucrose (Sigma®), carboxymethyl cellulose (sodium salt, Sigma®) and pectin (from apple, Sigma®), each to a final concentration of 2gL^{-1} (as in Midgley *et al.* 2006). The carbon source was excluded for the negative control.

The three nitrogen sources were nitrate (NaNO$_3$), ammonium ((NH$_4$)$_2$ HPO$_4$), and chitin (powdered from crab shells, Sigma®), each to a final concentration of 53mgL^{-1}. The nitrogen source was excluded for the negative control. In the nitrogen comparison, the amount of glucose was adjusted so that each medium had a C:N ratio of 39:1 (as in Midgley *et al.* 2004). Two chitin media were used; Chitin 1 included the carbon content of the chitin in the C:N ratio and Chitin 2 did not.

Statistical analysis: carbon and nitrogen utilisation

Corrected dry weight data were analysed using a general analysis of variance in GenStat®, with media and isolates as factors, and the block fitted as a random factor. The plot of residuals versus fitted values was checked visually for homogeneity of variance and no transformation of the data was required. The significance of the difference between pairs of means was assessed using the least significant difference (l.s.d.). As the ability of the isolates to utilise each of the carbon and nitrogen sources individually was also of interest, the analysis described above was also conducted for each isolate's biomass on the range of carbon and nitrogen media separately (with medium as a single factor).

Results

Edaphic conditions

All the soils were acidic (Table 1) and were well below the optimal horticultural level for nitrate (41-60mg/kg, Pivot Ltd 2007). All soils, except that from Chewton, had more nitrogen as ammonium (NH$_4$$^+$) than as nitrate (NO$_3$$^-$). The soil from Inverleigh was below the optimal total carbon (>1.85%) recommended for horticultural crops (Pivot Ltd 2007).

GQ996353 cultured mycorrhiza from *Caladenia amoena*
GQ996343 cultured mycorrhiza from *C. tentaculata*
GQ996356 cultured mycorrhiza from *C. audasii*
GQ996338 cultured mycorrhiza from *C. tentaculata*
GQ996358 cultured mycorrhiza from *C. rosella*
GQ996343 cultured mycorrhiza from *C. tentaculata*
GQ996355 cultured mycorrhiza from *C. audasii*
GQ996339 cultured mycorrhiza from *C. tentaculata*
GQ996354 cultured mycorrhiza from *C. audasii*
GQ996361 cultured mycorrhiza from *C.* sp. aff. *patersonii*
GQ996362 cultured mycorrhiza from *C.* sp. aff. *patersonii*
GQ996340 cultured mycorrhiza from *C. tentaculata*
GQ996341 cultured mycorrhiza from *C. tentaculata*
87
EU526281 M4 isolate from *C. tentaculata*
GQ996345 cultured mycorrhiza from *C. tentaculata*
DQ983816 *Sebacina vermifera* from *C. tessellata*
77
GQ996357 cultured mycorrhiza from *C. orientalis*
98
GQ996359 cultured mycorrhiza from *C.* sp. aff. *fragrantissima*
97
GQ99360 cultured mycorrhiza from *C.* sp. aff. *fragrantissima*
91
AY463171 cultured mycorrhiza from *C. formosa*
GQ996367 cultured mycorrhiza from *C. tentaculata*

Clade I

98

GQ9963352 cultured mycorrhiza from *C. tentaculata*
99
DQ983814 *S. vermifera* from *C. catenata*
GQ996346 cultured mycorrhiza from *C. tentaculata*
75
AY634116 uncultured mycorrhiza from *Epipactis gigantea*

Clade II

AY643802 cultured mycorrhiza from *C. carnea*
GQ996344 cultured mycorrhiza from *C. tentaculata*
GQ996348 cultured mycorrhiza from *C. tentaculata*
EU526279 E2 isolate from *C. tentaculata*
98
GQ996349 cultured mycorrhiza from *C. tentaculata*)
88
GQ996350 cultured mycorrhiza from *C. tentaculata*
EF470248 A2 isolate from *C. tentaculata*
GQ996351 cultured mycorrhiza from *C. tentaculata*
DQ983815 *S. vermifera* from *C. dilatata*
GQ996347 cultured mycorrhiza from *C. tentaculata*
EF470249 C3 isolate from *C. tentaculata*
EU526280 I4 isolate from *C. tentaculata*
EU526282 W2 isolate from *C. tentaculata*

Clade III

90
AF490395 *S. incrustans*
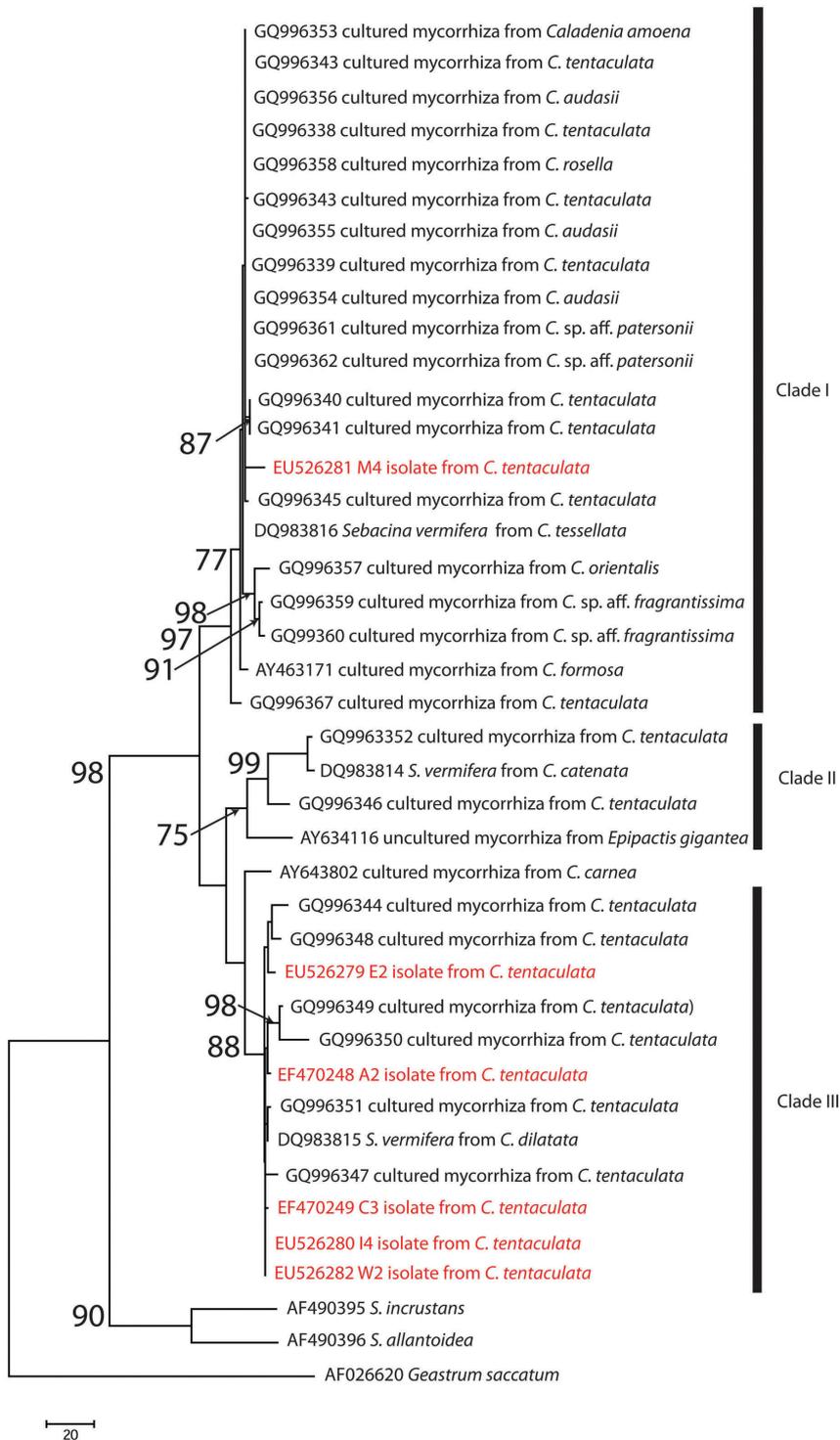AF490396 *S. allantoidea*
AF026620 *Geastrum saccatum*

20

Figure 2. Maximum parsimony analysis of ITS sequences of a range of mycorrhizal fungi from *Caladenia* and other terrestrial orchids (red font = isolates from current study). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The bootstrap values represent percentages of 10,000 replicates. Only bootstrap values of 70 or over are displayed.

Isolate identification

The ITS sequences from isolates A2, C3, E2, I4 and W2 were 97-99% similar to DQ983815, a *S. vermifera* isolate from *Caladenia dilatata* R.Br originally obtained by J.H. Warcup but M4 was a 96% match to DQ983816, a *S. vermifera* isolate from *C. tessellata*, also originally isolated by J.H. Warcup (Deshmukh *et al.*, 2006; Table 2). The ITS alignments contained a total of 359 positions in the final dataset, of which 125 were parsimony informative. The six isolates from this study fell within two well-supported clades previously described by Wright *et al.* (2010). MP analysis of these sequences revealed that the majority fell within clade III (88% bootstrap support), along with DQ983815, whereas M4 belonged to clade I (97% bootstrap support) as did DQ983816 (Fig. 2).

In vitro symbiotic seed germination

The mycorrhizal status of the six isolates was confirmed with positive seed germination recorded for each. All germination percentages were relatively low, with none exceeding 50%. Differences were observed in the specificity of the six isolates. M4 was the only isolate to germinate *Caladenia* seeds from all six populations. Four isolates (A2, C3, E2 and I4) failed to germinate seed from the Maldon population and another isolate (W2) produced only 2.4% germination of seed from this population (Table 3). There were also significant differences among germination percentages induced by the six isolates in relation to seeds from the Anglesea, Chewton and Eltham populations (*P*<0.05, Table 3). The general trend was that inoculation with the A2, E2 and I4 isolates gave significantly higher germination than the C3, M4 and W2 isolates. None of the isolates gave significantly higher germination of seeds from their own population when compared to isolates from other populations. C3 gave significantly lower germination percentages from Chewton seeds and W2 failed to germinate seeds from its own Wonthaggi population.

Carbon source utilisation

The effects of the different carbon sources on the mycelium dry weights were significantly different (*P*<0.001, Table 4). The dry weight of the isolates was significantly greater when grown in the glucose, pectin and carboxymethyl cellulose media in comparison to the sucrose medium and the medium lacking a carbon source. The effect of isolate was not significant (*P*>0.05) and there was no significant interaction between carbon source and isolate (*P*>0.05). Only isolate M4 produced significantly greater mycelium (dry weight) in the sucrose medium than the zero carbon control (l.s.d=17.4, *P*>0.05).

Nitrogen source utilisation

The different nitrogen sources had a significant effect on the mycelium dry weights (*P*<0.001, Table

Table 3. Mean percentage seed germination (stage 3 plus stage 4) ± standard error of the mean for seed from all six populations inoculated with fungi from all six populations at 16 weeks. The same letters indicate transformed means that are not significantly different within a single column (p<0.05).

Seed \ Isolate	Anglesea	Chewton	Eltham	Inverleigh	Wonthaggi	Maldon
Anglesea (A2, Clade III)	45.7 ± 18.9[b]	52.3 ± 14.1[b]	41.8 ± 12.6[b]	42.2 ± 12.6[a]	12.5 ± 12.5[a]	0
Chewton (C3, Clade III)	17.3 ± 7.0[a]	5.7 ± 2.6[a]	20.5 ± 10.2[ab]	29.7 ± 13.4[a]	6.3 ± 4.8[a]	0
Eltham (E2, Clade III)	46.3 ± 6.5[b]	2 8.8 ± 5.1[b]	27.4 ± 9.1[ab]	33.4 ± 7.5[a]	11.5 ± 6.6[a]	0
Inverleigh (I4, Clade III)	36.7 ± 7.0[b]	33.4 ± 7.6[b]	34.4 ± 8.2[b]	44.2 ± 9.5[a]	5.6 ± 5.6[a]	0
Wonthaggi (W2, Clade III)	2.4 ± 12.6[ab]	24.1 ± 12.5[ab]	7.3 ± 6.3[ab]	7.7 ± 3.8[a]	0	2.4 ± 1.2[a]
Maldon (M4, Clade I)	5.4 ± 3.2[a]	8.6 ± 4.5[ab]	4.9 ± 2.8[a]	15.8 ± 5.6[a]	29.8 ± 20.6[a]	15.6 ± 6.6[a]
No fungi	0	0	0	0	0	0

Table 4. Mean dry weight of mycelium (mg) ± the standard error of the mean of the six fungal isolates growing in low CN MMN liquid media with a variety of carbon sources.

Isolate / Carbon source	A2 Clade III	C3 Clade III	E2 Clade III	I4 Clade III	W2 Clade III	M4 Clade I	Mean for carbon source l.s.d = 6.3
Glucose	18.9 ± 5.0	18.3 ± 4.7	18.6 ± 4.2	17.6 ± 4.9	20.6 ± 3.0	20.6 ± 4.1	19.1
Sucrose	8.3 ± 2.9	10.3 ± 1.3	3.0 ± 1.4	5.1 ± 3.6	3.9 ± 2.6	20.3 ± 5.5	8.4
Cellulose	19.0 ± 3.6	27.3 ± 8.7	18.8 ± 8.1	19.5 ± 8.0	11.2 ± 5.2	26.2 ± 4.1	20.3
Pectin	18.8 ± 6.0	13.7 ± 2.3	21.7 ± 3.6	11.5 ± 6.0	18.3 ± 5.0	18.0 ± 5.1	17.0
No Carbon	0.0 ± 0.0	3.0 ± 3.4	0.0 ± 0.0	1.1 ± 1.1	4.2 ± 2.7	0.0 ± 0.0	1.4
Mean for isolate l.s.d = 6.9	13.0	14.5	12.4	11.0	11.7	17.0	

5). The mean for ammonium was the highest and was significantly greater than all other nitrogen sources and the medium lacking nitrogen. There was no significant difference between the mean for nitrate and that for the no nitrogen control. The mean for the Chitin 2 medium was also significantly greater than the no nitrogen control, nitrate and the Chitin 1 medium. There was no significant effect of isolate or a significant interaction between nitrogen source and isolate were significant (P>0.05).

Discussion

There was both genetic and functional diversity among the six *Caladenia tentaculata* isolates, revealing that fungal provenance is an important consideration when selecting isolates for symbiotic propagation for conservation. The isolates belonged to two different taxa within the *Sebacina vermifera* complex, and differed both in their effectiveness to germinate orchid seeds and their specificity for seeds from different *Caladenia*

populations. While there was little variation in ability to utilise different carbon and nitrogen sources, there was strong evidence that these fungi can act as saprotrophs and utilise forms of inorganic nitrogen available to them in Australian orchid habitats. To our knowledge, the results of this study provide the first baseline data on the nutrient requirements of fungi from the Sebacinales.

Genetic diversity of *Caladenia* isolates

The mycorrhizal isolates were closely related, but belonged to two different ITS clades (Fig. 2), with isolate M4 falling in clade I and the other five isolates falling in clade III as defined by Wright *et al.* (2010). The three clades defined by Wright *et al.* (2010) contained members of the *Sebacina vermifera* complex as circumscribed by Weiβ *et al.* (2004). Species and generic concepts within this complex are uncertain (Warcup 1988; Weiβ *et al.* 2004; Wright *et al.* 2010); however, analysis of percentage sequence similarity between the three ITS clades suggested that they are separate at least

Table 5. Mean dry weight of mycelium (mg) ± the standard error of the mean of the six fungal isolates growing in low CN MMN liquid media with a variety of nitrogen sources.

Isolate / Nitrogen source	A2 Clade III	C3 Clade III	E2 Clade III	I4 Clade III	W2 Clade III	M4 Clade I	Mean for nitrogen source l.s.d = 4.4
NO$_3$	5.0 ± 2.1	4.6 ± 1.1	5.8 ± 3.8	7.6 ± 3.0	9.0 ± 2.9	6.1 ±2.5	7.0
NH$_4$	22.5 ± 3.3	13.7 ± 2.1	25.2 ± 3.8	22.5 ± 3.1	24.7 ± 3.9	26.3 ± 3.5	22.5
Chitin 1	5.8 ± 2.0	6.8 ± 12.9	10.4 ± 3.9	5.8 ± 3.7	8.2 ± 3.9	4.9 ± 2.6	6.7
Chitin 2	16.3 ± 8.1	20.3 ± 8.1	10.8 ± 3.1	14.3 ± 3.7	14.2 ± 3.9	10.3 ± 2.6	14.4
No Nitrogen	10.1 ± 3.9	8.3 ± 2.0	10.1 ± 4.0	4.3 ± 2.5	4.9 ± 2.6	4.7 ± 2.1	7.0
Mean for isolate l.s.d = 4.9	11.9	10.7	12.5	10.9	12.2	10.5	

at the species level, with the mean divergence of clade I isolates at 1.7% and that of clade III at 3.8% (Wright *et al.* 2010). Both the well-supported clades identified in the MP analysis contained ITS sequences from a number of other *Caladenia* fungi, corroborating all previous studies, which have found that *Caladenia* species have highly specific mycorrhizal associations with a narrow range of closely related fungi (Warcup 1988; Dearnaley *et al.* 2009; Huynh *et al.* 2009; Swarts *et al.* 2010; Wright *et al.* 2010).

Effect of provenance on *in vitro* symbiotic seed germination

Variation in seed germination effectiveness was observed both within and between the two taxa from the *Sebacina vermifera* complex identified in this study (Table 3). The most striking difference was the lack of germination ability of most clade III isolates with seed from the Maldon population from which the clade I isolate originated. Interestingly, differences were also observed in the specificity of the clade III isolates for seeds from other populations from which the same taxon had been isolated. Similar variation has been observed in the seed germination effectiveness of mycorrhizal isolates from single fungal taxa (as defined by ITS sequence analysis) in *Tulasnella-Chiloglottis* (Roche *et al.* 2010) and other *Sebacina-Caladenia* (Huynh *et al.* 2009) associations, suggesting that either individual orchid fungal taxa contain genetically different strains that differ in mycorrhizal fitness, or ITS sequence divergence is not adequate for defining orchid fungal species. The 3.8% divergence found by Wright *et al.* (2010) among clade III isolates is a little higher than the 3% cut off widely accepted for species level taxa on ITS data (*e.g.* Tedersoo *et al.* 2009) and it is possible that more than one taxon is present within clade III. For some fungal groups it is also becoming apparent that the ITS region may not be adequate for delimiting species (Nilsson *et al.* 2008; Stefani *et al.* 2009) and regions such as actin, β-tubulin, elongation factor 1-α, and ribosomal polymerase B genes give finer resolution and clearer definition of species limits (*e.g.* Bruns & Shefferson 2004; Damm *et al.* 2009). The functional variation observed in this and other orchid mycorrhizal studies suggests that investigation of fungal species limits using a range of gene regions is required.

The results of this study revealed that while *C. tentaculata* fungi differed in their seed germination effectiveness and specificity there was no evidence of "home site" advantage. These results indicate that the use of fungal isolates from the same provenance as seeds in symbiotic propagation of orchids is not necessary when producing large seedling numbers for conservation purposes. Furthermore, genetic studies indicate that there is often a range of fungi present in individual *Caladenia* populations (Wright *et al.* 2010), and therefore it is likely that fungi from a single population will also exhibit a range of seed germination effectiveness and specificity.

Effect of provenance on utilisation of different carbon and nitrogen sources

There was no variation in the ability of the six isolates to utilise the different carbon and nitrogen sources when all isolates and media combinations were analysed as a factorial (Tables 4 and 5), suggesting that these fungi are very similar with respect to their carbon and nitrogen nutrition. There was, however, considerable evidence that these fungi are able to utilise forms of carbon and nitrogen available to them in orchid habitats.

All six isolates were able to utilise the complex cell-wall-related carbohydrates (carboxymethyl cellulose and pectin, Table 4), indicating the production of some cellulolytic and pectinolytic enzymes. The production of cell wall degrading enzymes suggests that the fungi tested could act as saprotrophs. As cellulose and, to a lesser degree, pectin constitute a large proportion of the organic carbon in leaf litter and soil available to fungi (Kögel-Knabner 2002), these results indicate that *Caladenia* isolates with the ability to germinate seed *in vitro* have the capacity to contribute to the carbon nutrition of orchid plants.

There was very little variation among isolates in their ability to utilise the majority of carbon sources. However, the M4 isolate (the sole representative of *Sebacina vermifera* clade I) was the only isolate capable of growth with sucrose as a sole carbon source, indicating some inter-taxon variation in carbon utilisation. While this result is difficult to interpret on an ecological level, as simple sugars such as sucrose are not considered part of the organic soil fraction (Kögel-Knabner 2002), it does indicate a degree of metabolic variation among members of the *Sebacina vermifera* complex.

It was clear that all six isolates showed a distinct preference for ammonium as an inorganic source of nitrogen and were not able to utilise nitrate (Table 5). The preference for one inorganic nitrogen source over another varies between different forms of mycorrhizal fungi, with studies showing that ammonium is the main inorganic nitrogen source utilised by ectomycorrhizal fungi (France & Reid 1984; Anderson *et al.* 1999; Sawyer *et al.* 2003), whereas ericoid mycorrhizal fungi can utilise both ammonium and nitrate (Chen *et al.* 1999; Midgley *et al.* 2004). The mycorrhizal fungi of some Northern Hemisphere terrestrial orchids have been shown to utilise ammonium and/or nitrate as sources of nitrogen (Stephen & Fung 1971), whereas others are unable to utilise either of these two nitrogen sources (Hadley & Ong 1978). As ammonium is the most abundant form of inorganic nitrogen in most Australian soils (Attiwill & Leeper 1987; Schmit & Steward 1997) including five of the six study sites investigated in this study (Table 1) the capacity of mycorrhizal isolates to utilise this form of inorganic nitrogen would greatly benefit their associated orchids.

All isolates were able to utilise chitin, the organic form of nitrogen used in this study, when the chitinous carbon was not considered in the C:N ratio (Chitin 2 medium). This is the first time that the capacity of orchid mycorrhizal fungi to utilise chitinous nitrogen as its sole nitrogen source has been demonstrated. It remains to be seen if *Sebacina vermifera*-like fungi are able to utilise other organic forms of nitrogen. Organic nitrogen can represent a significant proportion of the nitrogen in Australian soils (Schmidt & Stewart 1997; King & Buckley 2002). Chitin is the main component of fungal cell walls and it has been suggested that senescent fungal hyphae within soil may provide a significant source of nitrogen for mycorrhizal fungi (Kerley & Read 1997).

Mycorrhizal fungi play a significant role in nitrogen acquisition for the orchid species *Goodyera repens* R.Br. (Cameron *et al.* 2006). It is likely that the mycorrhizal fungi of *Caladenia* species also provide nitrogen to their associated orchids. Their capacity to utilise the most prevalent form of inorganic nitrogen in Australian soils, as well as forms of organic nitrogen indicates that they are able to absorb nitrogen present in soils that is not readily available to their orchid associates. It is clear that *Caladenia* mycorrhizal isolates with the ability to germinate seed *in vitro* have the capacity to contribute to the nitrogen nutrition of adult plants.

Conclusions

The genetic identity and functional attributes of the orchid mycorrhizal fungi differed among the six provenances investigated in this study. While there was considerable inter- and intra-taxon variation in seed germination effectiveness and specificity there was no evidence of "home site" advantage, suggesting that fungi from different provenances can be used for successful symbiotic propagation. The similarity in their growth on different carbon and nitrogen sources, suggests that provenance does not influence the ability of these fungi to access these important soil carbon and nitrogen nutrients and therefore their capacity to contribute to the nutrition of their associated orchids. Given that there are at least three species-level taxa in the *Sebacina vermifera* complex that associate with *Caladenia* in Australia, it will be instructive to investigate seed germination effectiveness and specificity in a wider sample of this genetic variation across a number of populations. Testing a wider variety of carbon substrates across more isolates may also reveal more substantial functional variation among the fungal taxa.

Acknowledgments

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