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ORIGINAL PAPER

Taxonomic and functional characterisation of fungi from the *Sebacina vermifera* complex from common and rare orchids in the genus *Caladenia*

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Abstract The terrestrial orchid genus *Caladenia* contains many species which are threatened with extinction. They have highly specific associations with *Sebacina vermifera* and closely related fungi, and conservation of these terrestrial orchids, in part, relies on symbiotic propagation to produce plants for reintroduction and ex situ conservation collections. However, little is known of the diversity of mycorrhizal fungi associating with natural populations. Here, restriction fragment polymorphism analysis, internal transcribed spacer and nuclear large subunit sequencing and symbiotic seed germination were used to investigate the taxonomic and functional diversity of fungal isolates from single populations of six endangered *Caladenia* species and one common species across the same biogeographic range. Fifty-nine fungal isolates were collected for investigation including ten isolates from the six endangered species *Caladenia audasii*, *Caladenia amoena*, *Caladenia* sp. aff. *fragrantissima* (Central Victoria), *Caladenia* sp. aff. *patersonii*, *Caladenia rosella* and *Caladenia orientalis* and 49 isolates from six populations of the common species *Caladenia tentaculata*. While the common species associated with three distinct *S. vermifera*-like taxa, the six endangered species were restricted to one of these fungal taxa. No direct relationship between the taxonomic identity of the fungi and their ability to stimulate seed germination was observed; however, the majority of the fungi isolated from the *Caladenia* species were capable of germinating seed in vitro, indicating their mycorrhizal status and potential for symbiotic propagation in conservation programmes.

Keywords Orchid mycorrhiza · Mycorrhizal diversity · *Sebacina vermifera* complex · ITS and nLSU sequencing · Symbiotic seed germination · Threatened species reintroduction

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Introduction

Orchids are extremely vulnerable to extinction. Habitat clearance, fragmentation and degradation have impacted on the population size and distribution of many species (Duncan et al. 2005; Swarts and Dixon 2009). Orchid species have highly specialised pollination mechanisms that rely on specific insect pollinators for effective seed set (Schiestl et al. 1999; Gaskett et al. 2008; Phillips et al. 2009); they also depend on colonisation by mycorrhizal fungi for seed germination (Rasmussen 1995; Zettler 1997). Mycorrhizal associations continue through the life of the orchid, with increasing evidence that they play an important role in adult nutrition (Cameron et al. 2006, 2007; Girlanda et al. 2006). The distribution of compatible mycorrhizal fungi is likely to be a limiting factor in seedling recruitment. Further, the role of these fungi in adult nutrition means that fungal persistence is required for orchids to maximise growth and reproduction. Thus, mycorrhizal fungi are routinely used in propagation of threatened orchids (Zettler 1997; Batty et al. 2006; Swarts and Dixon 2009; Wright et al. 2009) to increase plant

numbers in natural and augmented populations in conjunction with ex situ collections, significantly reducing extinction risks. In reintroductions, the provenance and genetic variation of plant material is routinely taken into account (Fay and Krauss 2003), but such considerations are overlooked when sourcing fungal inoculum.

For most threatened orchid species in need of recovery, little is known of the natural diversity of the associated fungi. The majority of fungi isolated from orchids belong to the anamorphic form genus *Rhizoctonia* (Filipello-Marchisio and Berta 1985); however, an increasing number of non-photosynthetic orchids have been shown to associate with non-*Rhizoctonia* fungi (Dearnaley 2007). The identification of *Rhizoctonia* teleomorphs by classical means (morphology) is difficult, as they rarely sporulate in culture. With the application of molecular techniques, orchids have been found to associate with an array of different teleomorphic genera including *Ceratobasidium*, *Thanatephorus*, *Tulasnella* and *Sebacina* (Rasmussen 2002) from a number of distantly related basidiomycete lineages (Taylor et al. 2003).

Both morphological and molecular studies of orchid mycorrhiza reveal that many terrestrial orchids have extremely specific associations, often with fungi from a single fungal (teleomorphic) genus (Warcup 1971, 1981; Taylor et al. 2003, 2004; Shefferson et al. 2007). This high mycorrhizal specificity suggests that appropriate fungi need to be supplied during plant reintroductions.

Australia has extremely high terrestrial orchid diversity, especially the southern region, with over 1,200 named species (Backhouse 2007). Many species are threatened with extinction, and active conservation efforts are required to save them (Duncan et al. 2005; Swarts and Dixon 2009). The genus *Caladenia* contains at least 243 species (Hopper and Brown 2004), with the vast majority endemic to Australia. It has the largest number of endangered species of any Australian orchid genera, which in turn comprise of 5% of Australia's entire threatened flora species (Dixon and Hopper 2009). Because many endangered *Caladenia* species occur in small fragmented populations, they are increasingly vulnerable to extinction. *Caladenia rosella*, once widespread across the Victorian Goldfields (a ca. 3,500-km^2 region), is now only known from four locations (Jeanes and Backhouse 2006). *Caladenia audasii* is known from five individuals in three widely separated locations (Duncan et al. 2003) and is no longer found at the type location, and *Caladenia* sp. aff. *fragrantissima* is known from a single population of two plants (Jeanes and Backhouse 2006). These and many more critically endangered *Caladenia* species are being symbiotically propagated for conservation purposes (Wright et al. 2009); however, there is limited information on the diversity of fungi associated with these plants.

Warcup and Talbot (1967) and Warcup (1971, 1981), using morphological studies of the teleomorph, identified the fungus *S. vermifera* in association with several *Caladenia* species. Sequencing of the nuclear ribosomal DNA of Warcup's *S. vermifera* isolates has shown that these do not belong to a single species but are part of a species complex which also contains fungal endophytes from a number of vascular and non-vascular plants from four continents (Weiss et al. 2004). The sequence divergence and host range of these and related fungi were used as evidence to suggest that the Sebacinaceae constitutes a new order, the Sebacinales, of which Warcup's *S. vermifera* isolates belong to subgroup B (Weiss et al. 2004). Subsequent molecular studies of mycorrhizal fungi from *Caladenia* have confirmed that these orchids associate with *S. vermifera*-like fungi (Bougoure et al. 2005; Bonnardeaux et al. 2007; Swarts 2007; Huynh et al. 2009); however, it is still unclear whether individual *Caladenia* species can utilise a diverse range of *S. vermifera*-like fungi or associate with a single fungus.

Understanding the diversity of mycorrhizal fungi associating with threatened orchids is essential for effective conservation of existing populations and development of successful reintroduction programmes. It would inform decision making relating to the source and range of mycorrhizal inoculum used in orchid reintroductions and may aid understanding why some species are in decline where other species are still relatively common.

The objectives of this study are to (1) examine the identity, diversity and distribution of mycorrhizal fungi within and between different populations of the common *Caladenia*, *Caladenia tentaculata*, (2) establish whether fungal identity affects the ability of mycorrhizal fungi to induce seed germination in vitro and thus affects their potential use in symbiotic propagation, and (3) compare the identity, diversity and distribution of mycorrhizal fungi associating with common and endangered *Caladenia* species across the same geographic range.

Materials and methods

Caladenia study species

All species in this study belonged to *Caladenia* subgenus *Calonema*, which is one of the six currently accepted subgenera in the genus (Hopper and Brown 2004; Dixon and Hopper 2009). *C. tentaculata* is a relatively common summer-dormant terrestrial orchid with a wide distribution throughout southeastern Australia (Jeanes and Backhouse 2006). The six study populations were all located in Victoria near Maldon (144.06722 E, −36.99750 S), Chewton (144.27696 E, −37.07590 S), Eltham (145.1458 E, −37.7222 S), Inverleigh (144.05056 E, −38.03028 S), Anglesea (144.18361 E, −38.4111S) and Wonthaggi

(145.58944 E, −38.68900 S; see Fig. 1). Six critically endangered *Caladenia* species (International Union for Conservation of Nature Red List criteria; Backhouse and Cameron 2005) are endemic to Victoria, with *C.* sp. aff. *fragrantissima* (Central Victoria) and *Caladenia* sp. aff. *patersonii* known from single populations. Populations of *C. audasii*, *Caladenia amoena*, *C. rosella* and *Caladenia orientalis* are highly fragmented across wide distributions, and many have extremely small plant numbers. Selected study populations of these critically endangered species were all the same biogeographic region as one of the *C. tentaculata* study populations, i.e. *C. audasii*, *C.* sp. aff. *fragrantissima* (ca. 40 and 50 km from the Maldon population, respectively), *C. amoena* (ca. 15 km from the Eltham population), *C. rosella* (ca. 20 km from the Eltham population), *C.* sp. aff. *patersonii* (ca. 30 m from the Inverleigh population) and *C. orientalis* (ca. 4 km from the Wonthaggi population; Fig. 1; Table 1).

Fungal isolates

In July 2005, longitudinal tissue slices, 2 to 3 mm in thickness, were removed from the stem collars of six randomly selected *C. tentaculata* plants from each of the six study populations (Fig. 1). On return to the laboratory, the tissue slices were surface-sterilised with 0.5% NaOCl for 30 s, and pelotons were plated on to fungal isolating medium (FIM; Clements et al. 1986) with 0.05% streptomycin using the peloton rinsing method described by Rasmussen et al. (1990). Hyphae growing from the pelotons were subcultured on to fresh FIM. Multiple fungal isolates were cultured from each of the 36 plants harvested. Isolates were labelled with a unique identifier indicating which plant they were cultured from, e.g. isolate 17.1 was the first isolate cultured from plant 17. After the first subculture, five to ten cubes of agar were excised from the growing edge of the culture and transferred aseptically into McCartney bottles containing sterile deionised water. For each fungal isolate, two such McCartney bottles were lodged in the Royal Botanic Gardens Melbourne (RBGM) Living Collection (see Table 2 for accession numbers). Fungal isolates were also cultured in Petri dishes containing potato dextrose agar and dried in a domestic food dehydrator (Sunbeam®) for 12 h at 35°C, and the resulting dried agar and fungal mycelium was lodged at the National Herbarium of Victoria (MEL; Table 2).

Isolates from the six critically endangered *Caladenia* species were collected from wild populations and stored under sterile water (as described above) in the RBGM Living Collection for symbiotic propagation of the orchid species they were isolated from. The isolates from *C. audasii*, *C.* sp. aff. *fragrantissima* and *C.* sp. aff. *patersonii* were recent isolations (2005); however, those from *C. amoena* (2000), *C. rosella* (1994) and *C. orientalis* (2001) had been isolated prior to this study. The isolates from *C. amoena*, *C. rosella*, *C. orientalis* and *C. audasii* were collected from whole stem collars (as described by Huynh et al. 2004), whereas the isolates from *C.* sp. aff. *fragrantissima* and *C.* sp. aff. *patersonii* were cultured from stem-collar tissue slices as described for the *C. tentaculata* plants. All isolates were cultured from single pelotons and subcultured, stored and maintained as described for the *C. tentaculata* isolates.

DNA isolation and amplification

Fungal mycelia from each of the isolates (Table 2) were grown for 4 weeks in low carbon and nitrogen modified

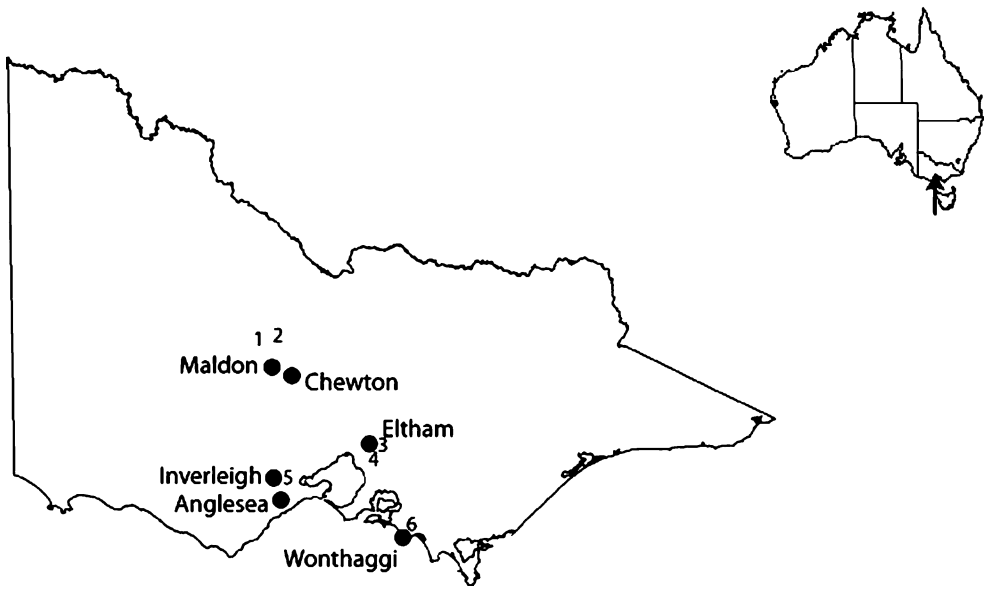


Fig. 1 A map showing the relative locations of the *Caladenia* populations selected for this study. The *black circles* indicate the six *C. tentaculata* populations with the name of each population next to the *circle*. The study population of the endangered *Caladenia* populations are indicated by the *numbers 1–6*: *1 C. audasii*, *2 C.* sp. aff. *fragrantissima* (Central Victoria), *3 C. amoena*, *4 C. rosella*, *5 C.* sp. aff. *patersonii* and *6 C. orientalis*

Table 1 The EVC and presence of human disturbance (type of disturbance) at the *Caladenia* populations sampled in this study

Species	Location	EVC	Disturbed/remnant
C. tentaculata	Anglesea	Heathy forest	Disturbed (vehicular tracks)
C. tentaculata	Chewton	Box ironbark forest	Disturbed (prior gold mining)
C. tentaculata	Eltham	Grassy dry forest	Remnant
C. tentaculata	Inverleigh	Grassy woodland	Remnant
C. tentaculata	Maldon	Grassy dry forest	Disturbed (herbivory and erosion)
C. tentaculata	Wonthaggi	Coastal heathland	Disturbed (vehicular tracks)
C. amoena	Wattle Glen	Box ironbark forest	Disturbed (herbivory and erosion)
C. rosella	Cottles Bridge	Grassy dry forest	Disturbed (herbivory and erosion)
C. orientalis	Wonthaggi	Coastal heathland	Remnant
C. sp. aff. *patersonii*	Inverleigh	Grassy woodland	Remnant
C. sp. aff. *fragrantissima* (Central Victoria)	Mandurang	Valley grassy forest	Disturbed (prior timber harvesting, vehicular tracks)
C. audasii	Bendigo	Grassy dry forest	Disturbed (prior timber harvesting, vehicular tracks)

EVC ecological vegetation type

Melin–Norkrans liquid medium (low CN MMN; Marx and Bryan 1975; 0.3 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.14 g $MgSO_4.7H_20$, 66.23 mg $CaCl_2.2H_2O$, 25 mg NaCl, 3 mg $ZnSO_4$, 12.5 mg ferric EDTA, 0.13 mg thiamine and 5.0 g glucose in 1 L of deionised water) with the pH adjusted to 5.0–6.0 before the addition of the ferric EDTA on a shaker at 2.5 rpm. The mycelium was removed from the liquid medium, 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 internal transcribed spacer (ITS) region of the DNA was amplified using the primers ITS 1 and 4 (White et al. 1990) and the nuclear large subunit (nLSU) region using the primers Ctb6 and TW14 (Taylor et al. 2003). The 25-μL reactions contained 1 ng DNA, 0.5 mM deoxynucleotide triphosphates mix, 1.5 mM $MgCl_2$, 2.5 μL 10× reaction buffer, 1 μM of each primer and 1 U Taq polymerase made to volume with sterile MilliQ® water. A Corbett thermal cycler was used to amplify the ITS region (94°C for 8 min, 29 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, and one cycle of 72°C for 5 min). A negative control reaction was electrophoresed with each PCR by replacing the DNA with MilliQ® water. Amplification of the nLSU differed only with an annealing temperature of 51°C rather than 56°C.

ITS-RFLP analysis

ITS-restriction fragment polymorphism (RFLP) analysis was used to guide the selection of isolates for DNA sequencing. The ITS PCR products were purified using a Qiagen® PCR purification kit according to the manufacturer's instructions. The ITS PCR product was digested with the restriction enzymes Hinf I, Mbo I, Hha I and Alu I (Promega®; Taylor and Bruns 1999; Taylor et al. 2003). Each 20-μL reaction contained 1-μg/μL PCR product, 2 μL 10× reaction buffer, 0.2 μL acetylated bovine serum albumin (10 μg/μL), 5 U enzyme and was made up to the final volume with sterile MilliQ® water. The reactions were incubated at 37°C for 4 h. The entire digested product was mixed with 10× gel loading dye (GibcoBRL®Blue Juice, Australia) and loaded on a 2% agarose gel containing 50 ng/L of ethidium bromide alongside 3 μL of undigested ITS PCR product. Electrophoresis of samples was conducted for 2 h at 110 V and visualised with UV light. Restriction sizes were determined using a Gene Ruler® 100 bp ladder as a reference (MBI Fermentas, Germany) with Kodak Digital Imaging® software. All samples judged to have the same fragment pattern with a certain enzyme from different gels were checked by re-amplification, digestion and electrophoresis of the products on the same gel.

ITS and nLSU sequencing

At least one isolate from each ITS-RFLP group which did not have an additive fragment length twice the original PCR product was selected for ITS sequencing (Table 2). At least two isolates were selected from each ITS clade for nLSU sequencing. In preparation for sequencing, PCR products were purified using a Qiagen® PCR purification kit according to the manufacturer's instructions. The sequencing reactions had a final volume of 20 μL and contained 3 μL PCR product, 1 μL Big Dye v. 3.1, 5 μL of 5× reaction buffer and 3pM forward or reverse primer, made up to the final volume with sterile MilliQ® water. The sequencing reactions were cleaned up using sodium acetate/

Table 2 Isolate, associated orchid species, locality and voucher data for the 59 fungal isolates from *Caladenia* investigated in this study

Orchid species	Isolate identifier	Population	RBGM accession number	MEL number	ITS sequence	nLSU sequence	ITS-RFLP group	ITS clade	Closest GenBank match	Germination (+/−)
C. tentaculata	17.5	Maldon	080553	2334380	GQ996338	–	1	A	DQ983816 *S. vermifera*	−
C. tentaculata	18.5	Maldon	080554	2334381	GQ996339	–	1	A	DQ983816 *S. vermifera*	−
C. tentaculata	28.3	Maldon	080549	2334382	GQ996340	GQ996363	1	A	DQ983816 *S. vermifera*	+
C. tentaculata	29.3	Maldon	080550	2334383	GQ996341	–	1	A	DQ983816 *S. vermifera*	+
C. tentaculata	30.1	Maldon	080551	2334384	–	–	1	–		+
C. tentaculata	30.2	Maldon	080551	2334385	GQ996343	–	1	A	DQ983816 *S. vermifera*	+
C. tentaculata	30.6	Maldon	080551	2334386	GQ996344	–	2	C	DQ983815 *S. vermifera*	+
C. tentaculata	31.1	Maldon	080552	2334387	GQ996345	–	1	A	DQ983816 *S. vermifera*	+
C. tentaculata	32.2	Chewton	080555	2334388	–	–	5	–		+
C. tentaculata	32.4	Chewton	080555	2334389	–	–	5	–		+
C. tentaculata	32.6	Chewton	080555	2334390	–	–	5	–		+
C. tentaculata	33.4	Chewton	080556	2334391	–	–	3	–		+
C. tentaculata	34.4	Chewton	080557	2334392	GQ996346	GQ996364	4	B	AY634116 uncultured fungus	−
C. tentaculata	35.4	Chewton	080558	2334393	GQ996347	GQ996365	6	C	DQ983815 *S. vermifera*	+
C. tentaculata	36.2	Chewton	080559	2334394	–	–	2	–		+
C. tentaculata	37.2	Chewton	080560	2334395	–	–	6	–		+
C. tentaculata	38.4	Eltham	080561	2334396	GQ996348	GQ996366	2	C	DQ983815 *S. vermifera*	+
C. tentaculata	39.1	Eltham	080562	2334397	–	–	2	–		+
C. tentaculata	39.2	Eltham	080562	2334398	–	–	2	–		+
C. tentaculata	39.5	Eltham	080562	2334399	–	–	2	–		+
C. tentaculata	40.3	Eltham	080563	2334400	–	–	2	–		+
C. tentaculata	41.1	Eltham	080564	2334401	GQ996349	GQ996367	7	C	DQ983815 *S. vermifera*	+
C. tentaculata	42.5	Eltham	080565	2334402	–	–	2	–		+
C. tentaculata	43.3	Eltham	080566	2334403	–	–	2	–		+
C. tentaculata	44.3	Anglesea	080567	2334404	–	–	8	–		+
C. tentaculata	44.4	Anglesea	080567	2334405	–	–	8	–		+
C. tentaculata	44.6	Anglesea	080567	2334406	GQ996350	–	8	C	DQ983815 *S. vermifera*	+
C. tentaculata	45.2	Anglesea	080568	2334407	–	–	1	–		+
C. tentaculata	46.4	Anglesea	080569	2334408	–	–	9	–		+
C. tentaculata	48.5	Anglesea	080570	2334409	–	–	9	–		−
C. tentaculata	49.4	Anglesea	080571	2334410	GQ996351	–	2	C	DQ983815 *S. vermifera*	+
C. tentaculata	50.1	Inverleigh	090856	2334411	–	–	2	–		+
C. tentaculata	51.6	Inverleigh	090857	2334412	–	–	2	–		+
C. tentaculata	53.3	Inverleigh	090858	2334413	–	–	2	–		+
C. tentaculata	54.3	Inverleigh	090859	2334414	–	–	10	–		+

Table 2 (continued)

Orchid species	Isolate identifier	Population	RBGM accession number	MEL number	ITS sequence	nLSU sequence	ITS-RFLP group	ITS clade	Closest GenBank match	Germination (+/−)
C. tentaculata	57.2	Inverleigh	090860	2334415	–	–	10	–		+
C. tentaculata	57.4	Inverleigh	090860	2334416	–	–	10	–		+
C. tentaculata	57.6	Inverleigh	090860	2334417	–	–	2	–		+
C. tentaculata	58.3	Inverleigh	090861	2334418	–	–	10	–		+
C. tentaculata	59.2	Wonthaggi	090863	2334419	–	–	5	–		+
C. tentaculata	59.3	Wonthaggi	090863	2334420	–	–	5	–		+
C. tentaculata	59.5	Wonthaggi	090863	2334421	–	–	5	–		+
C. tentaculata	59.6	Wonthaggi	090863	2334422	–	–	5	–		+
C. tentaculata	60.2	Wonthaggi	090864	2334423	–	–	5	–		+
C. tentaculata	61.1	Wonthaggi	090865	2334424	GQ996352	GQ996368	11	B	DQ983814 *S. vermifera*	−
C. tentaculata	62.6	Wonthaggi	090862	2334425	–	–	5	–		+
C. tentaculata	63.3	Wonthaggi	090866	2334426	–	–	5	–		+
C. tentaculata	64.2	Wonthaggi	090867	2334427	–	–	5	–		+
C. tentaculata	64.6	Wonthaggi	090867	2334428	–	–	5	–		+
C. amoena	CAM	Wattle Glen	060848	2334429	GQ996353	–	1	A	DQ983816 *S. vermifera*	+
C. rosella	CRS	Cottles Bridge	070389	2334430	GQ996358	–	1	A	DQ983816 *S. vermifera*	+
C . orientalis	CFO	Wonthaggi	061409	2334431	GQ996357		1	A	DQ983816 *S. vermifera*	+
C. audasii	CAU1	Bendigo	061348	2334432	GQ996354	–	1	A	DQ983816 *S. vermifera*	+
C. audasii	CAU2	Bendigo	061348	2334433	GQ996355	GQ996369	1	A	DQ983816 *S. vermifera*	+
C. audasii	CAU3	Bendigo	061348	2334434	GQ996356	–	1	A	DQ983816 *S. vermifera*	+
C. sp. aff. *fragrantissima* (Central Victoria)	CAFA1	Mandurang	061353	2334435	GQ996359	–	1	A	DQ983816 *S. vermifera*	+
C. sp. aff. *fragrantissima* (Central Victoria)	CAFA2	Mandurang	061353	2334436	GQ996360	–	1	A	DQ983816 *S. vermifera*	+
C. sp. aff. *patersonii*	ISO1	Inverleigh	070364	2334437	GQ666361	–	1	A	DQ983816 *S. vermifera*	+
C. sp. aff. *patersonii*	ISO9	Inverleigh	070364	2334438	GQ996362	–	1	A	DQ983816 *S. vermifera*	+

The isolate identifier for *C. tentaculata* indicates the plant sampled and the number of the isolate from that plant (plant.isolate). Isolates selected for ITS and nLSU sequencing and their corresponding GenBank accession numbers are also shown, alongside the ITS-RFLP groups, ITS clades (where defined) and seed germination ability (+/−) of each isolate

ethanol precipitation (3 M sodium acetate, pH 5.2). Sequencing was performed using an ABI capillary automated sequencer at the Australian Genome Research Facility, Brisbane, Australia.

Sequence analysis

Both the ITS and nLSU sequences were BLAST searched to find the closest matching sequences on GenBank (all sequence references are shown in figure legends). Relationships among the *Caladenia* mycorrhizal fungi were investigated by analysing all ITS sequences together (416 bp) with closely matched sequences downloaded from GenBank, including those from Australian terrestrial orchids (Table 3), using *Geastrum saccatum* as the outgroup. Another analysis was conducted with 361 bp of ITS sequences and included an ITS sequence of *Caladenia falcata* (EF160069; Bonnardeaux et al. 2007). To establish the position of the *Caladenia* mycorrhizal fungi in relation to members of the Sebacinales, 490 bp of selected nLSU sequences was aligned with 21 sebacinoid sequences downloaded from GenBank with *G. saccatum*, *Filobasidiella floriforme* and *Trichosporon cutaneum* as an outgroup (Weiss et al. 2004). Alignments were constructed using Clustal X and manually edited in MEGA version 4 (Tamura et al. 2007). The evolutionary history was inferred using the maximum parsimony (MP) method (Eck and Dayhoff 1996). A bootstrap consensus tree was inferred from 10,000 replicates. The MP tree was obtained using the close-neighbour interchange algorithm (Nei and Kumar 2000) with search level 3 (Nei and Kumar 2000) in which the initial trees were obtained with the random addition of sequences (ten replicates). All positions containing gaps and missing data were eliminated from the datasets. Phylogenetic analyses were conducted in MEGA version 4 (Tamura et al. 2007).

Estimates of mean evolutionary divergence over ITS sequence pairs between and within the clades observed in the maximum parsimony analysis of the ITS sequences were obtained using the maximum composite likelihood

Table 3 Isolate, associated orchid species, GenBank accession numbers, voucher data and reference for the fungal isolates from Australian terrestrial orchid species downloaded from GenBank and used in phylogenetic analysis

Orchid species	Culture collection and accession number	Isolate identifier	ITS sequence	nLSU sequence	Reference
Caladenia tentaculata	RBGM 080313	A2	EF470248	EU526283	Wright (2007)
C. tentaculata	RBGM 080314	C3	EF470249	–	Wright (2007)
C. tentaculata	RBGM 080315	E2	EU526279	–	Wright (2007)
C. tentaculata	RBGM 061048	I4	EU526280	EU526286	Wright (2007)
C. tentaculata	RBGM 080316	M4	EU526281	EU522687	Wright (2007)
C. tentaculata	RBGM 080313	W2	EU526282	EU526288	Wright (2007)
C. tessellata	MAFF 305838	Warcup 907	DQ983816[b]	DQ983816[b]	Deshmukh et al. (2006)
C. catenata	MAFF 305835	Warcup 770	DQ983814[b]	DQ983814[b]	Deshmukh et al. (2006)
C. dilatata[a] (*C. catenata*)	Not reported	Warcup 750	–	AY505550	Weiss et al. (2004)
C. dilatata	CBS 572.83	Warcup 750	–	AF202729	Taylor et al. (2004)
C. dilatata	MAFF 305837	Warcup 846	DQ983815[b]	DQ983815[b]	Deshmukh et al. (2006)
C. formosa	RBGM	F1Cs	AY463171	–	Huynh et al. (2009)
C. carnea	BRIP 43825	CC2-1	AY643802	AY643801	Bougoure et al. (2005)
C. falcata	KPBG	Cf01	EF160069	–	Bonnardeaux et al. (2007)
Glossodia major	Not reported	Warcup 768	–	AY505551	Weiss et al. (2004)
Microtis uniflora	Not reported	Warcup 963	–	AY505554	Weiss et al. (2004)
M. rara[a] (*M. uniflora*)	Not reported	Warcup 977	–	AY505555	Weiss et al. (2004)
Eriochilus cucullatus[a] (*E. scaber*)	Not reported	Warcup 140	–	AY505548	Weiss et al. (2004)

RBGM National Herbarium of Victoria Living Collection (Melbourne, Australia), *CBS* Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (The Netherlands), *MAFF* Ministry of Agriculture, Forestry and Fisheries Genebank, Microorganisms Section (Japan), *KPGB* Kings Park and Botanic Gardens Herbarium (Perth, Australia), *BRIP* Queensland Department of Primary Industries Herbarium (Brisbane, Australia)

[a] Indicates sequences from Warcup's isolates for which Weiss et al. (2004) reported orchid host species contrary to the original publication by Warcup (1981); the orchid species reported by Warcup (1981) are listed here with those reported by Weiss et al. (2004) in brackets

[b] Indicates those sequences that included the ITS and 28S regions of nuclear ribosomal DNA

method in MEGA version 4 (Tamura et al. 2007). A total of 390 positions were analysed for both the within groups (clades) and between groups (clades) analysis, and all positions containing gaps were eliminated from the dataset.

Seed germination

To confirm the mycorrhizal status of the isolates, *Caladenia* seed batches from multiple plants from each of the study species and population (Table 2) were separately surface sterilised in 0.5% NaOHCl (with a drop of Tween 80 per 100 mL) for 3 min and then rinsed six times in sterile deionised water. Suspended sterilised seed was pipetted onto filter paper segments and placed on 2.5% oatmeal agar (Clements and Elyard 1979). The Petri dishes containing these seed batches were inoculated with isolates from the corresponding orchid species (or population in the case of *C. tentaculata*) by placing a 3-mm^3 cube of agar containing a single fungal isolate with three to six replicate plates for each isolate listed in Table 2. The Petri dishes were incubated in a Conviron® growth cabinet with a daily cycle of 17 h at 20°C, 1 h at 15°C and a 6-h dark period at 10°C. The presence of protocorms at stages 3 and 4 (Clements et al. 1986) was observed 8 weeks (critically endangered species) or 12 weeks (*C. tentaculata*) after inoculation and used to indicate positive seed germination.

Results

BLAST searches of the ITS sequences revealed (Table 2) that each of the *Caladenia* isolates was most closely related to one of the three Warcup *S. vermifera* isolates from Australian *Caladenia* sequenced by Deshmukh et al. (2006; GenBank accession numbers: DQ983814, *Caladenia catenata*; DQ983815, *Caladenia dilatata* and DQ983816, *Caladenia tessellata*) with the exception of isolate 34.4 which most closely matched an uncultured mycorrhizal fungus from *Epipactis gigantea* (AY634116). The ITS alignments contained a total of 359 positions in the final dataset, of which 125 were parsimony informative. Phylogenetic analysis of these sequences (Fig. 2) revealed that the *C. tentaculata* isolates fell into three well-supported clades (I, 97% bootstrap support; II, 75% bootstrap support and III, 88% bootstrap support) each containing one of the three Warcup *S. vermifera* isolates that were the closest matches in the BLAST searches. Isolates from the endangered *Caladenia* species were restricted to clade I, which also contained an isolate from another endangered Australian *Caladenia* species, *Caladenia formosa* (Huynh et al. 2009). Most isolates in clade I had identical ITS sequences (Fig. 2). Nine of the *C. tentaculata* isolates fell within clade I, two were in clade II and 11 were in clade III. A separate maximum parsimony analysis of 361 bp of ITS sequence revealed that an isolate of *C. falcata* (EF160069; Bonnardeaux et al. 2007) fell within clade III. The estimates of average evolutionary divergence within the ITS sequences (390 positions) from clade I were 1.7%, clade II 8.3% and clade III 3.8%. Those between clades I and II were 16.8%, clade I and III 14.2% and clade II and III 14.3%.

The nLSU alignments contained a total of 457 positions in the final dataset, of which 128 were parsimony informative. Analysis of nLSU sequences was consistent with the ITS analysis, identifying the same three well-supported clades (Fig. 3), each containing one of the same three Warcup *S. vermifera* isolates as in the ITS analysis. Isolates of Sebacinales subgroup B as described by Weiss et al. (2004) grouped separately from other fungal taxa of the Sebacinales, with strong bootstrap support (87%).

Eleven ITS-RFLP groups were identified among the 59 *Caladenia* isolates investigated. The *C. tentaculata* isolates were found to belong to all 11 groups, whereas the isolates from the six endangered species all belonged to a single ITS-RFLP group (group 1). Each of the ITS-RFLP restriction patterns generated for a given ITS-RFLP group was unique, with no combinations of restriction patterns of more than one ITS-RFLP group observed (Table 4).

The *C. tentaculata* isolates from ITS-RFLP group 1 clustered within clade I. All the other isolates in clade I (from the endangered species) also belonged to ITS-RFLP group 1. Isolates of *C. tentaculata* from ITS-RFLP groups 4 and 11 fell within clade II and those from groups 2, 6, 7 and 8 fell within clade III (Fig. 2). The sequences within clade III from groups 6, 7 and 8 were nested with sequences from group 2, and the restriction fragment patterns of these groups differed by a single enzymatic digest, e.g. group 7 differed from group 2 when digested with Hinf I whereas group 6 differed from group 2 when digested with Mbo I (Table 4). There was some slight variation among the sequences from both of the RFLP groups (1 and 2) for which multiple sequences were aligned.

The undigested ITS PCR products were between 650 and 670 bp long, with differences due to internal deletions and insertions. The additive fragment length of ITS-RFLP group 3 when digested with the enzymes Mbo I, Hinf I and Alu I was approximately twice the undigested PCR product (Table 3). ITS-RFLP groups 5, 9 and 10 all had banding patterns with additive fragment length twice the length of the undigested ITS PCR product when digested by one of the four enzymes used (Table 4).

There were isolates from two ITS clades present at three out of the six *C. tentaculata* populations studied. Isolates from a single plant (plant 30) from Maldon belonged to different clades: two from clade I and one from clade III. Isolates from clade III were the most widespread, occurring in five of the six *C. tentaculata* populations (Table 5). Of

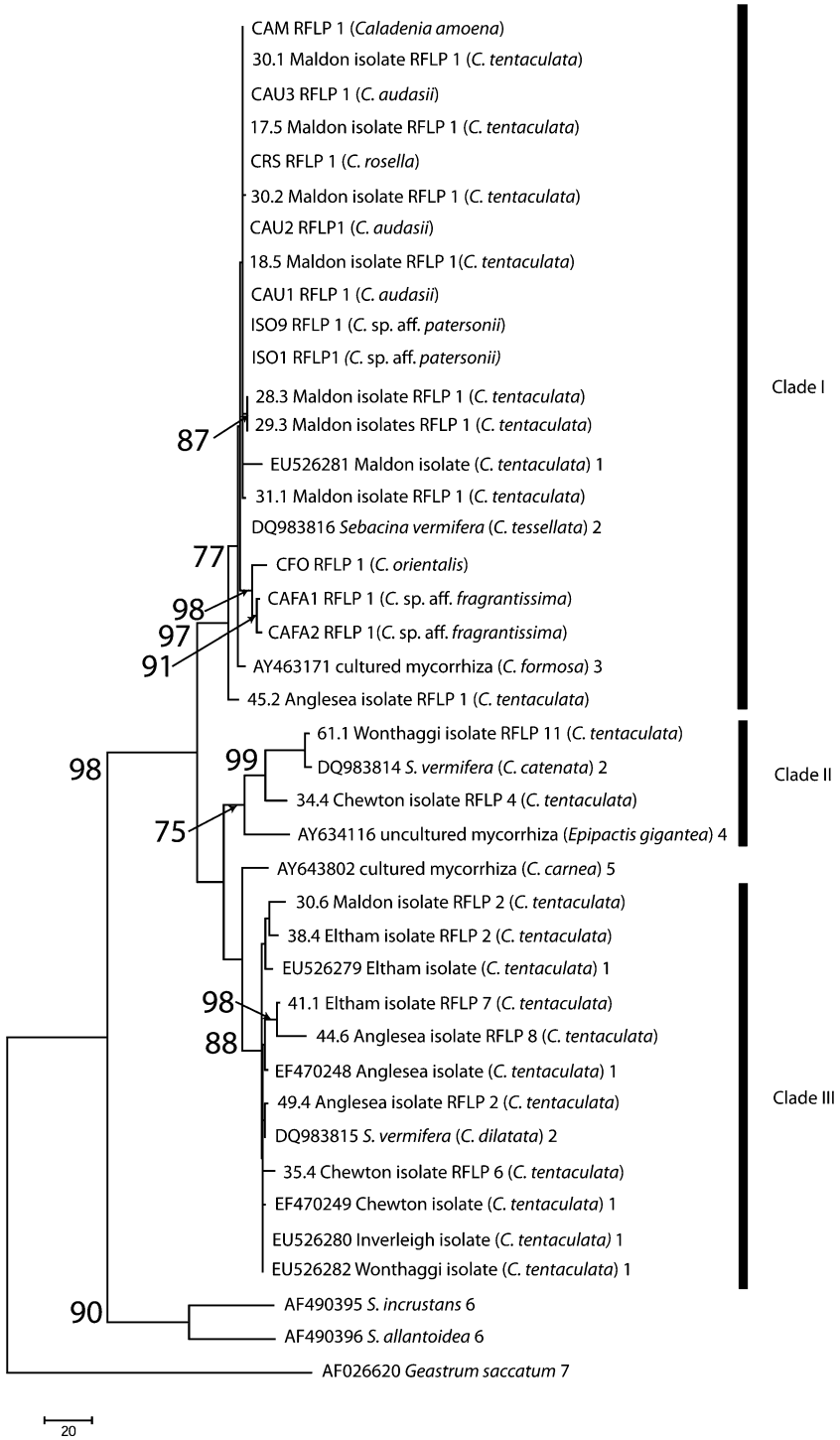


Fig. 2 Maximum parsimony analysis of 416 bp of ITS sequences of the *Caladenia* mycorrhizal isolates sampled with those downloaded from GenBank. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The bootstrap values represent percentages of 10,000 replicates. Only bootstrap values of 70 or over are displayed. References for the sequences downloaded from GenBank are as follows: *1* Wright (2007), *2* Deshmukh et al. (2006), *3* Huynh et al. (2009), *4* Bidartondo et al. (2003), *5* Bougoure et al. (2005), *6* Selosse et al. (2002), *7* Hibbett et al. (1997)

those isolates assigned to a clade, clade III isolates were the most common in four of the populations (Table 5). Isolates from clade I were found at two populations (Maldon and Anglesea); however, they were only the most common at the Maldon population (Table 5). Clade II was only found at Wonthaggi and Chewton (Table 5).

More than one ITS-RFLP group was identified from each population of *C. tentaculata* (Table 2). The most common ITS-RFLP group was different in all populations except Chewton and Wonthaggi, which shared group 5 as the most common group. ITS-RFLP group 2 was widespread, present in all populations except Wonthaggi, while the majority of ITS-RFLP groups were unique to a single population.

The majority of the *C. tentaculata* isolates from clades I and III were capable of germinating seeds from the

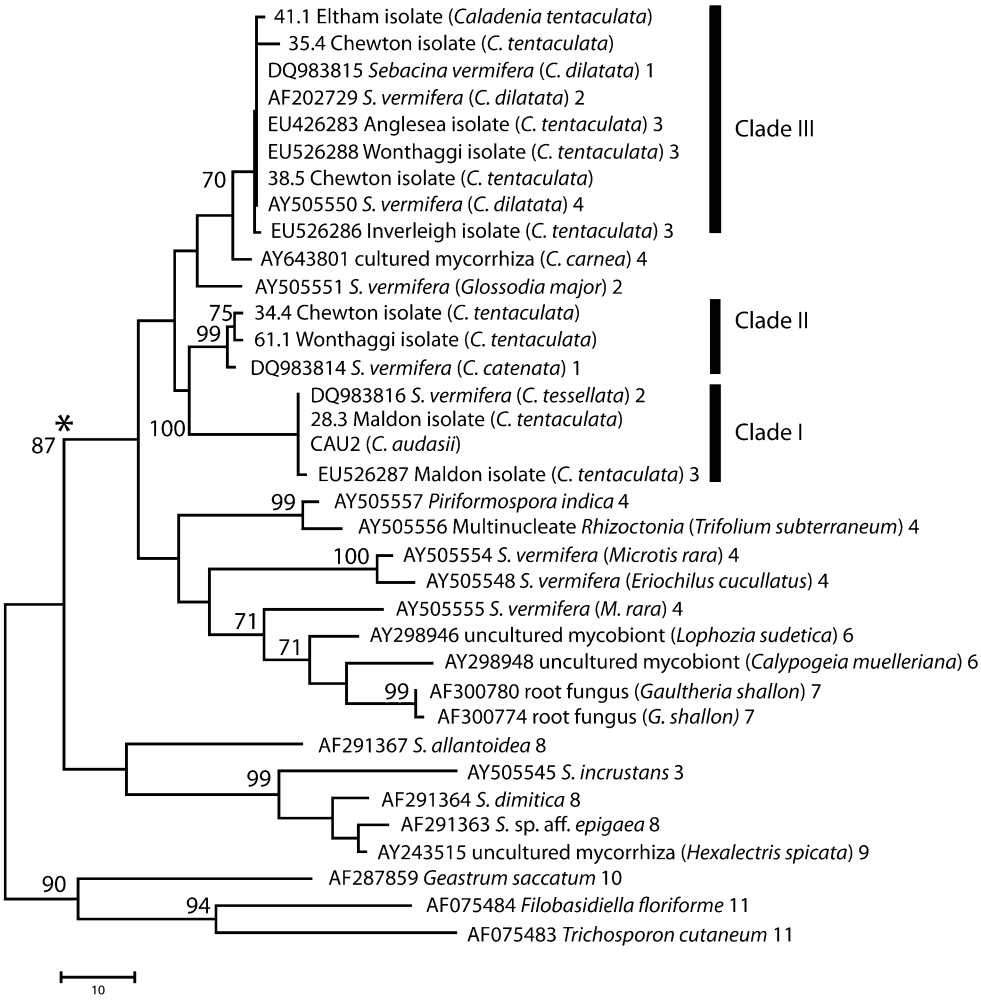


Fig. 3 Maximum parsimony analysis of 490 bp of nLSU sequences of the mycorrhizal *Caladenia* isolates sampled with those downloaded from GenBank. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The bootstrap values represent percentages of 10,000 replicates. Only bootstrap values of 70 or over are displayed. References for the sequences downloaded from GenBank are as follows: *1* Deshmukh et al. (2006), *2* Taylor et al. (2004), *3* Wright (2007), *4* Weiss et al. (2004), *5* Bougoure et al. (2005), *6* Kottke et al. (2003), *7* Allen et al. (2003), *8* Weiss and Oberwinkler (2001), *9* Taylor et al. (2003), *10* Hibbett et al. (2000), *11* Fell et al. (1999). Sebacinales subgroup B is indicated by asterisk

populations they were isolated from, whereas the two isolates from clade II were not (Table 2). The isolates from clades I and III that failed to germinate seeds came from the same populations and shared an ITS-RFLP group with isolates that germinated seeds. All ten isolates from the endangered species were capable of germinating the seeds from the species they were isolated from.

Discussion

Plants of the common orchid species, *C. tentaculata* associated with three closely related fungal taxa from the *S. vermifera* complex. In contrast, the endangered orchid species associated with a single fungal taxon across the same geographic range. There was no direct relationship between the taxonomic identity of the fungi and their ability to induce seed germination. The majority of the fungi isolated from the *Caladenia* species were capable of germinating seed in vitro indicating their potential use for symbiotic propagation in conservation programmes.

Identification and classification of fungi associated with *C. tentaculata* populations

All of the ITS sequences from the *C. tentaculata* isolates closely matched sebacinoid sequences from previous studies (Bidartondo et al. 2003; Deshmukh et al. 2006). Phylogenetic analysis of nLSU sequences (Fig. 3) revealed that these fungi belonged to the order Sebacinales within subgroup B as distinguished by Weiss et al. (2004). The molecular divergence found by Weiss et al. (2004) between subgroup B (containing a number of isolates identified as *S. vermifera*) and subgroup A (containing other *Sebacina* species including the type, *Sebacina incrustans*) indicates that the two subgroups should be recognised at least at generic rank. The generic name *Serendipita* is based on *S. vermifera* (Roberts 1999). However, Weiss and Oberwinkler (2001) express doubt as to the identity of the type of *S. vermifera* with isolates placed under that name by Warcup and Talbot (1967) and subsequent authors. Pending resolution of the taxonomy and nomenclature of the group, we refer to *S. vermifera* in the sense of Warcup and Talbot (1967), but note

Table 4 Fragment lengths of the 11 ITS RFLP groups when digested with enzymes—Mbo I, Hinf I, Alu I and Hha I—and their corresponding ITS clades

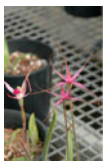
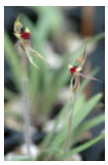
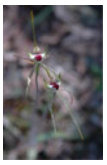
RFLP group	Hinf I	Mbo I	Alu I	Hha I	Corresponding ITS clade
1	350, 300 (650)	370, 200 (570)	650	540, 130 (670)	I
2	270, 170, 120, 100 (660)	320, 120, 100, 70 (610)	530, 150 (680)	340, 210, 130 (680)	III
3	350, 270, 230, 180, 150, 100 (1,280)	410, 320, 160, 120, 70 (1,080)	650, 530, 150 (1,330)	340, 210, 130 (680)	NA
4	300, 210, 120 (630)	250, 200, 180 (630)	530, 150 (680)	340, 210, 130 (680)	II
5	270, 170, 120, 100 (660)	410, 320, 120, 100, 70 (1,020)	530, 150 (680)	340, 210, 130 (680)	NA
6	270, 170, 120, 100 (660)	320, 160, 120, 100, 70 (770)	530, 150 (680)	340, 210, 130 (680)	III
7	270, 170, 140, 120, 100 (800)	320, 120, 100, 70 (610)	530, 150 (680)	340, 210, 130 (680)	III
8	270, 170, 120, 100 (660)	320, 160, 120, 70 (670)	530, 150 (680)	340, 210, 130 (680)	III
9	300, 270, 250, 170, 150, 100 (1,240)	320, 120, 100, 70 (610)	530, 150 (680)	340, 210, 130 (680)	NA
10	330, 270, 170, 120, 100 (990)	320, 120, 100, 70 (610)	530, 150 (680)	340, 210, 130 (680)	NA
11	180, 150, 120, 100 (550)	250, 200, 180 (630)	650	340, 210, 130 (680)	II

The number in brackets indicates the additive length in base pairs of the fragments

that some reports and GenBank accessions refer to *Serendipita vermifera*.

In phylogenetic analyses of both the ITS and nLSU sequences, the Australian *Caladenia* isolates grouped within three well-supported clades containing members of the *S. vermifera* complex (Figs. 2 and 3). As previously mentioned, species and generic concepts within the Sebacinales are uncertain, particularly around *S. vermifera*.

Table 5 The proportion of associated fungi by ITS clade for populations of common and endangered *Caladenia* species from different locations. ITS clades are assigned from ITS-RFLP groups as presented in Table 4: clade I (white), clade II, (grey), clade III (black) and clade not assigned (dotted). *n* indicates the number of isolates sampled from each population. Letters indicates populations in close proximity (see Fig. 1 for details). *Caladenia audasii* and *C. fragrantissima* photos by Geoff Black and *C. orientalis* photo by Ralph Laby, all other photos by the authors

Location	Anglesea	Bendigo[a]	Chewton[a]	Cottles Bridge[b]	Eltham[b]	Inverleigh	Maldon[a]	Mandurang[a]	Wattle Glen[b]	Wonthaggi
Endangered *Caladenia* populations										
		C. audasii		*C. rosella*		*C.* sp. aff. *patersonii*		*C.* sp. aff. *fragrantissima*	*C. amonea*	*C. orientalis*
										
		n = 3		*n* = 1		*n* = 2		*n* = 2	*n* = 1	*n* = 1
Common *Caladenia* populations										
	C. tentaculata		*C. tentaculata*		*C. tentaculata*	*C. tentaculata*	*C. tentaculata*			*C. tentaculata*
										
	n = 7		*n* = 8		*n* = 8	*n* = 8	*n* = 8			*n* = 10

Warcup (1981) was successful in producing basidia in a number of strains of *S. vermifera*, but on the basis of variable basidiospore morphology and growth parameters of cultures could only conclude that *S. vermifera* is at least a 'variable species' or may be a 'complex of closely allied species'. As these isolates fell in quite divergent lineages based on nLSU sequences, Weiss et al. (2004) suggested that they, whether their morphology was known or not, belong to a broad species complex.

The taxonomic status of the three clades identified in our phylogenetic analysis as species or taxa above or below species level is yet to be established, but the level of ITS divergence strongly suggests that more than one species is involved. The ITS region is routinely used in taxonomic and ecological studies as a 'barcode' for species identity (Peay et al. 2009), although applying an arbitrary percentage sequence similarity to accurately indicate cospecific taxa across different fungal groups has been questioned as the ITS region does not appear to be equally variable between different groups of fungi (Nilsson et al. 2009). While infraspecific variation in the ITS region ranged from 0.2% to 24.4% across different species on the basis of GenBank accessions (Nilsson et al. 2009), it is quite possible that some high values resulted from misidentifications of lodged sequences, with the average within species divergence was 3.33% for Basidiomycota. Hughes et al. (2009) have shown that among 100 basidiomycete sporocarps from a restricted geographic location, the ITS divergence between parental sequences of heterozygotes was no more than 3% in 99% of cases. It is thus reasonable to expect that divergence levels of more than 3% are strongly indicative of species level differences, and studies focusing on ectomycorrhizal diversity across a range of orders, including the Sebacinales, routinely use a threshold for ITS sequence divergence to indicate cospecificity of 3% (Smith et al. 2007; Peay et al. 2009; Tedersoo et al. 2009). Using this threshold, clade I (mean 1.7% sequence divergence) represents a single species, whereas clades III (3.8%) and II (6.8%) are likely to represent more than one species. Much further work is required to provide an integrated view of species limits in the *S. vermifera* group on the basis of molecular, morphological and functional data. For the moment, even a conservative interpretation of the sequence data indicates that more than one species is represented among Australian *Caladenia* isolates of the *S. vermifera* complex.

The RFLP groupings were utilised as an economical means of assigning isolates to clades and revealed only slight sequence variation that is not indicative of further variation of taxonomic significance. ITS sequences for ITS-RFLP groups 6, 7 and 8 differed from group 2 sequences by only a few base pairs more than the difference observed within isolates from RFLP group 2 in the region that was aligned.

The additive fragment lengths of four ITS-RFLP groups (3, 5, 9 and 10; see Table 4) were approximately twice that of the ITS PCR product, and for the majority these of groups, this phenomenon was only observed with a single enzyme digest. This may indicate the presence of more than one closely related fungal organism in these cultures. However, none of the groups comprised of restriction patterns representing combinations of more than one group. If these samples were mixed cultures, at least some of them should contain a combination of two or more of the fungi isolated as single cultures, and their RFLP patterns would reflect this. Significantly, RFLP group 5 was collected from the Chewton and Wonthaggi populations and was the most common group at both (Table 2). It is much more likely that this RFLP pattern represents a single organism with divergent ITS sequences occurring at these two populations than exactly the same combination of two or more different organisms, of which none was isolated as single cultures. As no restriction pattern combinations were observed, it is unlikely that this divergence is due to heterozygosity. It may be due to the presence of pseudogenes (Razafimandimbison et al. 2004) or intragenomic variation on the ITS region (Simon and Weiss 2008) which have both been observed in the ITS region of eukaryotic DNA. To investigate these possibilities, it would be necessary to use cloning to examine a range of single copies of the ITS, which was beyond the scope of the current study.

Diversity and distribution of *C. tentaculata* endophytes

The identification of three different ITS clades (that represent at least three separate species) among fungi isolated from *C. tentaculata* reveals that a range of closely related fungi associate with a single *Caladenia* species. These orchids, while specific in their mycorrhizal associations, do not associate with a single fungus or fungi from a single ITS clade as suggested by earlier studies (Warcup 1971, 1981; Bougoure et al. 2005; Bonnardeaux et al. 2007; Huynh et al. 2009). The utilisation of a group of closely related fungal taxa by a terrestrial orchid species is not unique to *Caladenia*, with observations of this phenomenon in a number of Australian (Irwin et al. 2007), American (Taylor et al. 2003, 2004) and European (McKendrick et al. 2002; Shefferson et al. 2005, 2007) terrestrial orchid species.

Not only can a single *Caladenia* species associate with more than one 'species' of fungus but also this range of fungi can be found in association with plants across geographically distinct populations, within populations and also within individual plants. Three of the *C. tentaculata* populations associated with more than one fungal taxon, as indicated by the presence of multiple ITS clades, and this range of fungal associates was also

observed in an individual plant: One from the Maldon population was colonised by at least two different fungal taxa (Table 2).

The distribution of fungal taxa identified in this study differed considerably, with isolates from ITS clade III found throughout the study range while those from clades I and II were restricted to a few populations (Table 5). Also, different assemblages of fungi were found in association with the plants from each population. The most prevalent isolates from each of the populations differed in their ITS-RFLP groups, with the exception of those from Wonthaggi and Chewton from which fungi from ITS-RFLP group 5 were the most commonly isolated (Table 2). These results indicate that not only does each *Caladenia* population have a unique range of fungal types present; the most common fungal taxon can vary between populations.

The local mycorrhizal diversity described in this study confirms the suggestion made by Batty et al. (2001) that healthy orchid populations associate with a range of fungi. Individual *Caladenia* plants may rely on different fungi for nutrition during different life history stages as shown for the species *Gastrodia elata* (Xu and Guo 2000) and *Tipularia discolor* (McCormick et al. 2004), or under changing environmental conditions as observed in *Erythrorchis cassythoides* (Dearnaley 2006). Each observed fungal taxon may have a different set of optimal environmental conditions for nutrient acquisition and growth, increasing the ability of associated orchid to adapt to environmental changes. It is highly likely that healthy *Caladenia* populations require this suite of fungi to persist.

Ability of *C. tentaculata* endophytes to induce seed germination in vitro

The mycorrhizal status of the majority of the 49 fungal isolates from *C. tentaculata* was confirmed by their ability to germinate seed in vitro. There were representatives of the three ITS clades among the five isolates that failed to germinate seeds (Table 2). In particular, neither of the two isolates from clade II was able to germinate seeds indicating that these isolates, while originating from pelotons growing within the collar of *C. tentaculata* plants, are not functional as mycorrhiza during the germination and seedling stages under in vitro conditions. A fungal isolate from clade II (Weiss et al. 2004) isolated by Warcup (1981) from *C. catenata* was capable of inducing seed germination (Warcup 1981), indicating that a larger sample of this fungal taxon from *C. tentaculata* may reveal isolates capable of germinating seeds in vitro.

While the majority of isolates from clades I and III induced seed germination, there were also members of these clades that failed to germinate *C. tentaculata* seeds. Thus, not only is there a range of fungi with differing mycorrhizal abilities associated with single *Caladenia* populations but also their functional differences in seedling development cannot be determined by molecular analysis of the ITS region. Huynh et al. (2009) also found this to be the case with the mycorrhizal associates of *C. formosa*. The lack of an obvious correlation between ITS fungal identity and seed germination ability may be unique to isolates from the Sebacinales, as contrary observations have been reported in other fungal groups, i.e. *Tulasnella* (McCormick et al. 2004) and *Ceratobasidium* species (Otero et al. 2005). It is more likely that the large number of isolates and systematic sampling and germination testing performed in this study and that of Huynh et al. (2009) have revealed natural variation of seed germination abilities found within a single fungal taxon. McCormick et al. (2004) and Otero et al. (2005) both tested only two isolates from each ITS clade. It is unlikely that the functional variation seen within a single fungal taxon is restricted to fungi from the Sebacinales, and results of this study suggest that further sampling of isolates is necessary to confirm relationships between fungal identity and seed germination or other functional attributes.

Endangered *Caladenia* species associate with restricted range of fungi

All the isolates from the endangered species induced seed germination indicating their positive mycorrhizal status (Table 2). They belonged to a single ITS clade and were similar to fungi from *C. tentaculata* (in some cases with identical ITS sequences). Interestingly, the fungal diversity observed in association with the common *C. tentaculata* was not observed among the endangered species. *C. tentaculata* plants associated with fungi from clades I, II and III whereas all six endangered *Caladenia* species associated with fungi from clade I (Table 5). ITS sequencing revealed that the fungal associates of another endangered *Caladenia* from southwestern Victoria and South Australia, *C. formosa*, also belonged to clade I (Fig. 2). This restricted range of mycorrhizal fungi is surprising as these endangered orchids species are found in varied habitats across the same geographic range as the *C. tentaculata* populations sampled.

A similar phenomenon has been observed in Western Australian *Caladenia*, with the endangered *Caladenia huegelii* associating with mycorrhizal fungi with identical ITS sequences throughout its entire distribution (Swarts 2007) and the common *Caladenia arenicola* forming associations with a much wider range of fungi (Hollick 2004). It has been suggested that the presence of the appropriate mycorrhizal partner defines a restricted geographic distribution for mycoheterotrophic orchids (Ogura-Tsujita and Yukawa 2008a, b). As common *Caladenia* are able to utilise a wider

range of fungi, their ability to spread to new sites may be enhanced allowing them to retain and expand their geographic range. The ability to take advantage of a broad range of fungi may make orchid species robust to environmental change (Rasmussen and Rasmussen 2007). The restriction of mycorrhizal associates of endangered *Caladenia* species to a single ITS clade may limit their capacity to establish new populations and retain their distribution. It would be necessary to establish whether the full complement of fungal taxa are present in the environment supporting endangered *Caladenia* populations to verify this hypothesis. With this information, it would be possible to confirm whether the restricted mycorrhizal compatibility is an inherent characteristic of these species or if they lack the opportunity to form associations with a wider range of fungi.

The inherent difficultly in culturing fungi from multiple plants of endangered orchid species with extremely small population sizes may have influenced the finding of limited mycorrhizal diversity. Most of the diversity observed among the *C. tentaculata* isolates was revealed by culturing fungi from more than one plant in a population, with only one of the six plants for which multiple isolates were investigated showing variation in ITS sequences, suggesting that the sampling of fungi from the endangered *Caladenia* species may have merely overlooked the true mycorrhizal diversity present in these populations. A survey of the mycorrhizal fungi associated with multiple plants from a single population of the endangered *C. formosa* showed that while there was variation in the ITS region among these isolates, they were restricted to a single ITS clade (Huynh et al. 2009). In this current study, we observed slight ITS sequence variation between isolates of *C.* sp. aff. *fragrantissima* which were cultured from a single plant: It is therefore possible that further sampling from multiple plants of the six endangered *Caladenia* species will reveal some variation in the associated fungi.

Studies of taxa within the *Hexalectris spicata* complex (Taylor et al. 2003) and different genotypes within the species *Corallorhiza maculata* (Taylor et al. 2004) have shown that closely related but genetically distinct orchid plants associate with different lineages of mycorrhizal fungi. Such results have led to the suggestion that mycorrhizal associations may influence orchid evolution (Taylor et al. 2004). There was no evidence of coevolution of mycorrhizal fungi and hosts among the *Caladenia* species investigated in this study. Using the plastid regions matK and trnL-F Hopper and Brown (2004) showed that orchid species from the three subgenera *Caladenia*, *Calonema* and *Phlebochilis* belong to divergent lineages. In contrast, phylogenetic analysis of the ITS and nLSU sequences of *Caladenia* endophytes revealed that orchid species from three subgenera of *Caladenia* (subgen. *Caladenia*: *C. catenata* and *Caladenia carnea*, subgen. *Calonema*: all species investigated in this study and *C. dilatata* and *C. formosa* and subgen. *Phlebochilis*: *C. tessellata*) do not associate with separate lineages of fungi, with isolates from species of subgenus *Calonema* found in all three fungal clades.

Geography and habitat have been found to affect the composition of mycorrhizal fungi found in association with a range of orchid species including the Australian *Microtis parviflora* (Perkins et al. 1995), Japanese populations of *Epipactis helleborine* (Ogura-Tsujita and Yukawa 2008a) and North American *Neottia nidus-avis* (McKendrick et al. 2002) and *C. maculata* (Taylor and Bruns 1999). This phenomenon was also observed among the Western Australian *Caladenia* species studied by Hollick (2004): *Caladenia georgei* and *C. huegelii* plants associate with the same fungi as *C. arenicola* when growing in the same habitat (according to amplified fragment length polymorphism analysis), but the fungi that these species associate with differed between habitats. Although differences were observed in the fungal assemblages associated with *C. tentaculata* populations in different environments (Table 5), differences in habitat did not appear to influence the fungal associations of a wider range of Victorian *Caladenia* species. The endangered *Caladenia* species were sampled from a range of vegetation types (Table 1) and associated with the same group of fungi across this range, not sharing fungal taxa with *C. tentaculata* in the same location (at Wonthaggi and Inverleigh).

While there is difficulty attributing influencing factors to the difference in mycorrhizal diversity observed between *C. tentaculata* and the endangered *Caladenia* in the current study, the remarkable similarity between the fungi associating with the six endangered species is worthy of note. Not only were these fungi from a single ITS clade, those isolated from *C. amoena*, *C. audasii*, *C. rosella* and *C.* sp. aff. *patersonii* had identical ITS sequences (Fig. 2). Interestingly, the fungal taxon associating with these endangered orchids was geographically the most widespread of the three fungal taxa identified in this study, occurring throughout the study range (Table 5). It was also found in association with two of the *C. tentaculata* populations (Maldon and Anglesea). The commonness of this fungal taxon, which associated with both the common and endangered orchids, highlights that orchid mycorrhizal fungi are potentially widely and heterogeneously spaced in the environment.

Conservation and management implications

The present study has shown that for the common *C. tentaculata*, not only do the *S. vermifera*-like mycorrhizal associates differ between populations of the this orchid species but also there can be local mycorrhizal diversity present within individual populations. It is important to

accommodate this diversity during reintroductions by including *Caladenia* plants symbiotically grown with multiple isolates from a range of plants from each population in need of protection.

Endangered *Caladenia* species associate with a restricted range of *S. vermifera*-like fungi in comparison to common species. It appears that there is a reduction in mycorrhizal diversity in threatened *Caladenia* populations, further supporting the need to conserve as much mycorrhizal diversity as possible during plant reintroductions.

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