Mycorrhizal specificity in endemic Western Australian terrestrial orchids (tribe Diurideae): Implications for conservation

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BSc (Hons)

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University
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I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

The specificity of fungal isolates from endemic Western Australian orchid species and hybrids in the tribe Diurideae was investigated using symbiotic seed germination and analysis of the fungal DNA by amplified fragment length polymorphism (AFLP). The distribution of the fungal isolates in the field was also assessed using two different seed baiting techniques. The information from these investigations is essential for developing protocols for reintroduction and translocation of orchid species.

Two groups of orchids in the tribe Diurideae were studied. Firstly, a number of Caladenia species, their natural hybrids and close relatives from the southwest of Western Australia were selected because orchid species from the genus Caladenia are considered to have among the most specific mycorrhizal relationships known in the orchid family – an ideal situation for the investigation of mycorrhizal specificity. Secondly, species of Drakaea and close relatives, from the southwest of Western Australia and elsewhere in Australia, which are never common in nature and occur in highly specialised habitats, were selected to investigate the influence of habitat on specificity.

Seed from the common species Caladenia arenicola germinated on fungal isolates from adult plants of both C. arenicola and its rare and endangered relative C. huegelii, while seed from C. huegelii only germinated on its own fungal isolates. The AFLP analysis grouped the fungal isolates into three categories: nonefficacious fungi, C. huegelii type fungi, and C. arenicola type fungi. The group of C. huegelii type fungi included some fungal isolates from C. arenicola. An analysis of the AFLP fingerprints of C. arenicola fungal isolates from different collection locations showed that some, but not all, populations were genetically distinct, and that one population in particular was very variable.

Despite being thought to have very specific mycorrhizal relationships, Caladenia species hybridise frequently and prolifically in nature, often forming self-perpetuating hybrid lineages. Five natural hybrids within Caladenia and its closest relatives were investigated. Symbiotic cross-germination studies of parental and hybrid seed on fungi from the species and the naturally occurring hybrids were compared with AFLP
analyses of the fungal isolates to answer the question of which fungi the hybrids use. The germination study found that, while hybrid seeds can utilise the fungi from either parental species under laboratory conditions, it is likely that the natural hybrids in situ utilise the fungus of only one parental species. Supporting these observations, the AFLP analyses indicated that while the parental species always possessed genetically distinct fungal strains, the hybrids may share the mycorrhizal fungus of one parental species or possess a genetically distinct fungal strain which is more closely related to the fungus of one parental species than the other.

The work on *Caladenia* hybrids revealed that *C. falcata* has a broadly compatible fungus that germinated seeds of *C. falcata*, the hybrid *C. falcata x longicauda*, and species with different degrees of taxonomic affinity to *C. falcata*. In general, germination was greater from species that were more closely related to *C. falcata*: seeds from *Caladenia* species generally germinated well on most *C. falcata* isolates; species from same subtribe (Caladeniinae) germinated well to the stage of trichome development on only some of the fungal isolates and rarely developed further; and seeds from species from different subtribes (Diuridinae, Prasophyllinae, Thelymitrinae) or tribes (Orchideae, Cranichideae) either germinated well to the stage of trichome development but did not develop further, or did not germinate at all. The AFLP analysis of the fungal isolates revealed that the fungi from each location were genetically distinct.

*In situ* seed baiting was used to study the introduction, growth and persistence of orchid mycorrhizal fungi. A mycorrhizal fungus from *Caladenia arenicola* was introduced to sites within an area from which the orchid and fungus were absent, adjacent to a natural population of *C. arenicola*. In the first growing season, the fungus grew up to 50 cm from its introduction point, usually persisted over the summer drought into the second season and even into the third season, stimulating germination and growth to tuber formation of the seeds in the baits. Watering the inoculated areas significantly increased seed germination.

Mycorrhizal relationships in Drakaeinae were less specific than in Caladeniinae. A study of the species *Spiculaea ciliata* revealed that this species, when germinated symbiotically, develops very rapidly and has photosynthetic protocorms, unlike all
other members of the Drakaeinae. An AFLP analysis of the fungal isolates of this species grouped the isolates according to whether they had been isolated from adult plants or reisolated from protocorms produced in vitro. Isolates were genetically distinct when compared before germination and after reisolation. A cross-species symbiotic germination study of seeds of three Drakaea species and one Paracaleana species against fungal isolates from the same species and several other Drakaeinae species revealed lower specificity in this group than previously thought. A number of fungal isolates from Drakaea and Paracaleana species germinated two or more seed types, while all seed types germinated on fungal isolates from other species and the seed of Drakaea thynniphila germinated to some extent on every fungal isolate tested. An AFLP analysis of the Drakaeinae fungal isolates supported this information, revealing little genetic differentiation between the fungi of different orchid species.

An ex situ seed baiting technique was used to examine the role of mycorrhizal fungi in microniche specialisation in the narrow endemic Drakaea. Soil samples from within and outside two Drakaea populations were tested for germination of the relevant seed types. In both cases, germination was significantly higher on soil samples from within than outside the populations, suggesting that the relevant mycorrhizal fungi may be restricted to the same microniches as the Drakaea species. The presence of similar fungi at distant, disjunct locations may be related to the extreme age and geological stability of the Western Australian landscape.

The information from these investigations is essential for developing protocols for reintroduction and translocation of orchid species. It appears that the mycorrhizal relationships in these groups of orchids are not as specific as was previously thought. For reintroduction work, a broad sampling strategy is necessary, as it cannot be assumed that the same orchid species has the same fungus at different locations. A broadly compatible fungus may be of considerable utility in conservation work, such as in situations where a specific fungus appears to have poor saprophytic competence or where soil conditions have been altered. Seed baiting studies provide additional data on fungal distribution in situ. In general, molecular data do not provide information about efficacy or fungal distribution, so research programs that combine symbiotic germination studies with seed baiting investigations and genetic analyses of
the fungi will provide the maximum benefit for designing more effective conservation programs.
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Chapter 1 - Introduction

1.1 – The family Orchidaceae

1.1.1 – Conservation status of Orchidaceae

The family Orchidaceae is the most species-rich plant family in the world, with an estimated 17500 to 35000 species (Batty et al., 2002; Cribb et al., 2003). Orchids can be found from the tropics to the arctic regions, as epiphytes (typically tropical), terrestrials (typically temperate), and even include subterranean forms (Batty et al., 2002; Cribb et al., 2003). A large proportion of orchid species are rare and endangered as a direct or indirect result of human activities, including collection, habitat destruction and degradation, and loss of pollinators and fungal partners (Batty et al., 2002; Cribb et al., 2003; Zettler et al., 2003). The greatest potential for loss of orchid species is in the tropics, where species diversity is highest, and this is the area in which orchids have been studied least (Batty et al., 2002; Cribb et al., 2003). However, the Western Australian terrestrial orchids also exhibit considerable diversity and many species are threatened. Although there has been extensive research on these species (Batty et al., 2002; Cribb et al., 2003), much more is needed before they can be effectively conserved.

1.1.2 - Western Australian orchids

The southwest of Western Australia has a mediterranean type climate, is considered to be a world biodiversity hotspot, and possesses a highly diverse and unique terrestrial orchid flora (Dixon, 1990). Long-term geographical isolation and highly infertile soils have led to great biodiversity (about 10000 species) and endemism (over 80 %) in the flora of the southwest (Batty et al., 2002). There are over 340 orchid species in this region, more than 85 % of which are endemic (Hoffman & Brown, 1998). This diverse orchid flora is also highly endangered, with 34 taxa classified as critically endangered and a further 34 known from few locations and requiring further study.
(Briggs & Leigh, 1996; Batty et al., 2002). Even the relatively common species are declining in range and number due to habitat destruction and degradation (Dixon, 1990).

In a Mediterranean type climate, orchids follow a strict growth cycle according to the seasons. Growth commences in late autumn when the rains of the cool, moist winter begin; this is also the season for seed germination (Dixon, 1991). Flowering usually occurs in late winter or spring, prior to the hot, dry summer, during which period the orchids survive as aestivating tubers (Dixon, 1991). A seed that germinates in autumn must form a tuber before summer if it is to survive to adulthood; seeds that do not germinate during their first winter after dispersal do not survive to another season (Dixon, 1991; Batty et al., 2001a). The growing season is only five to eight months long, decreasing from south to north (Dixon, 1991).

The terrestrial orchids of the southwest are found in a wide range of habitats, with open woodland, heath and ephemeral wet sites particularly favoured, and many are highly specialised and restricted (Dixon, 1991). Unusual and specialised pollination mechanisms are common, such as sexual deception of male thynnid wasps (Dixon, 1991). Reproduction occurs from seed, with seedling survival greatest near the parent plant where the appropriate fungus is present, and clonally by the production of daughter tubers (Dixon, 1991). According to Batty et al. (2002), the low recruitment levels of Western Australian orchids are suspected to be due to the patchy distribution of appropriate mycorrhizal fungi in the soils and the scarcity of suitable habitats. Mycorrhizal specificity is still poorly understood, and as a fungal partner is vital to an orchid’s existence, this is a very important area for current and future research (Cribb et al., 2003; Zettler et al., 2003).
1.2 - Fungal ecology and specificity

1.2.1 - Orchid mycorrhizal associations

Orchid mycorrhizas are different to most other types of mycorrhizas in a number of ways (Brundrett, 2002). They can occur in stems as well as roots, and the fungi often recolonise older cells after the previous generation of pelotons has been digested (Brundrett, 2002; Batty et al., 2002). Other differences occur in the morphology of the mycorrhizas, and a phylogenetically distinct group of fungi is involved (Brundrett, 2002; Batty et al., 2002; Zettler et al., 2003). These rhizoctonia-type fungi are more closely related to groups of pathogenic and saprophytic fungi than to other mycorrhizal fungi (Pope & Carter, 2001; Brundrett, 2002). This means that knowledge of other mycorrhizal associations cannot safely be applied to orchid mycorrhizas (Brundrett, 2002; Batty et al., 2002).

Mycorrhizas are present in all terrestrial orchids, and in many epiphytes (Batty et al., 2002; Bayman et al., 2003). Different genera of terrestrial orchids have different patterns of fungal colonisation within their roots or stems, which may be associated with particular types of fungi (Ramsay et al., 1986; Batty et al., 2002; Zettler et al., 2003). The main area of infection is the cortex, where the fungus forms a peloton (a coil of undifferentiated fungal hyphae) inside each cell (Brundrett, 2002; Batty et al., 2002; Zettler et al., 2003). The peloton is separated from the orchid’s cytoplasm by the cell membrane, through which nutrient transfer occurs (Brundrett, 2002; Zettler et al., 2003). There is evidence from isotope studies of transfer of carbon compounds and of nutrients (including P and N) from the fungus to the orchid but not vice versa (Smith et al., 1994; Brundrett, 2002; Zettler et al., 2003).

All orchids are mycotrophic, at least to some extent during some life stages, and it is sometimes argued that they are parasites on their fungi (Hadley, 1982; Zettler, 1997; Brundrett, 2002; Zettler et al., 2003). The achlorophyllous orchids are the most extreme case of mycotrophy, and are totally dependent on their fungal partners (Brundrett, 2002; Zettler et al., 2003). Achlorophylly has arisen independently in about 20 separate orchid lineages (Batty et al., 2002). These orchids have been found
to participate in a three-way relationship with photosynthetic plants which subsidise the orchids, transferring carbon compounds and nutrients to the orchid via the fungal partner (Hadley, 1982; Warcup, 1988; McKendrick et al., 2000; Batty et al., 2002).

1.2.2 – Fungal specificity

Mycorrhizal specificity in orchids varies considerably. Some orchids form mycorrhizal relationships with more than one fungus, and some fungi with more than one orchid species (Hadley, 1970, 1982; Zettler et al., 2003; Fay & Krauss, 2003). It has also been found that orchids often utilise different fungi at different life stages, in particular associating with different fungi during the seedling and adult stages (Zelmer & Currah, 1997; Zettler et al., 2003). In southwestern Western Australia, most orchid species are highly specific, often possessing a one-to-one relationship with a particular fungal strain (Dixon, 1991). Such highly specific relationships are not as common in other terrestrial orchids, particularly those of the northern temperate zone which have been intensively studied (Zettler et al., 2003). Specificity varies even within the orchids of southwestern Western Australia, with different genera of orchids often possessing different patterns of specificity (Warcup, 1973, 1975; Dixon, 1991). The highly specific mycorrhizal relationships of Western Australian orchids have considerable implications for conservation (Dixon, 1991; Ramsay & Dixon, 2003). The appropriate fungal partner is likely to be only obtainable from adult plants of the same species, which can be a risky and therefore undesirable procedure with a highly endangered species (Ramsay & Dixon, 2003). Also, in any reintroduction program, it is possible that the appropriate fungus must also be reintroduced (Ramsay & Dixon, 2003).

1.2.3 – The saprophytic stage

An important question in orchid ecology concerns what happens to orchid mycorrhizal fungi when they are not within the plants. There is evidence that some orchid fungi (such as the fungi of achlorophyllous orchids) can form mycorrhizal relationships with plants from other families, but this is uncommon (Warcup, 1988; McKendrick et al., 2000; Batty et al., 2002). Most orchid fungi are saprophytes, and the rhizoctonia-type
fungi also include soil saprophytes which are not known to form mycorhizal relationships (Batty et al., 2002; Zettler et al., 2003). The fungi have been found (by seed baiting studies) a considerable distance from the nearest adult plant (McKendrick et al., 2000; Batty et al., 2002). Orchid fungi can digest cellulose (Hadley, 1969; Warcup, 1975; Rasmussen, 1992a), and can presumably survive in the soil in the absence of the orchid indefinitely. The distribution of orchid fungi in soil may be similar to that of other mycorrhizal fungi, and is likely to be correlated with certain soil properties (Batty et al., 2002). Fungal distribution in soils is likely to vary considerably over time as well as space (Batty et al., 2002; Rasmussen & Whigham, 2002).

1.3 - Phenology of infection

1.3.1 - Orchid seed germination

Orchid seeds can be germinated both symbiotically (in the presence of an appropriate fungal isolate) and asymbiotically (Johansen & Rasmussen, 1992; Ramsay & Dixon, 2003). It has been found that seeds tend to germinate more rapidly, grow faster, and produce larger, stronger and more robust seedlings in a shorter time in the presence of an appropriate fungus than when grown asymbiotically (Hadley & Williamson, 1971; Arditti et al., 1990; Peterson et al., 1998; Ramsay & Dixon, 2003). The lack of stored nutrient reserves in the tiny seeds of orchids means that it is likely that mycorrhizal fungi are normally essential for germination (Peterson et al., 1998; Batty et al., 2002; Zettler et al., 2003; Ramsay & Dixon, 2003).

Symbiotic seed germination

It is probable that most orchid seed germination in situ occurs symbiotically, certainly in terrestrial orchids (Arditti et al., 1990; Zettler et al., 2003; Ramsay & Dixon, 2003). The most common method of in vitro symbiotic germination involves soaking the seeds in a sterilising solution (commonly calcium hypochlorite) before sowing, generally onto oatmeal agar, and a fungus (usually an isolate from an adult orchid of
the same species) is inoculated onto the agar plate before or after seed sowing (Arditti et al., 1990; Johansen & Rasmussen, 1992; Ramsay & Dixon, 2003). Once the fungus enters the seed, germination commences (Peterson et al., 1998; Zettler et al., 2003; Ramsay & Dixon, 2003). Pelotons form in the cortical cells of the lower part of the protocorm or seedling (Peterson et al., 1998; Zettler et al., 2003). In terrestrial orchids, it is generally some weeks before a shoot forms and photosynthesis begins (Peterson et al., 1998; Zettler et al., 2003; Ramsay & Dixon, 2003). Prior to shoot formation, the seedling is fully mycotrophic and dependent on the fungus (Peterson et al., 1998; Zettler et al., 2003). It has been found that symbiotic seedlings can form dormant tubers without ever becoming photosynthetic (Batty et al., 2002). Seedling germination and survival in situ is generally highest close to adult plants, possibly due to the presence of an appropriate fungus (Dixon, 1991; Batty et al., 2002; McKendrick et al., 2002).

Asymbiotic seed germination

Although it is probable that symbiotic germination is the usual condition in situ, orchid seeds can be germinated asymbiotically in vitro (Arditti et al., 1990; Johansen & Rasmussen, 1992; Ramsay & Dixon, 2003). This requires a complex medium containing sugars and hormones to substitute for the supply of essential compounds from the fungus (Arditti et al., 1990; Johansen & Rasmussen, 1992; Peterson et al., 1998; Ramsay & Dixon, 2003). Asymbiotic germination is more successful with epiphytic orchids, which can form chloroplasts in the protocorms and become fully photosynthetic within days of germination, than with terrestrial orchids, which take much longer to become photosynthetic (Arditti et al., 1990; Johansen & Rasmussen, 1992; Peterson et al., 1998; Ramsay & Dixon, 2003). It has been found that orchid seedlings germinate and grow more slowly under asymbiotic conditions than symbiotically (Arditti, 1990; Peterson et al., 1998; Markovina & McGee, 2000; Ramsay & Dixon, 2003).

Ecological and potential specificity

Specificity has been discussed above with regards to adult orchids in situ. This type of specificity, defined as the range of fungi with which adult orchids are found to associate in situ, is known as ecological specificity (Masuhara & Katsuya, 1994; Fay
& Krauss, 2003). However, it has been shown that a wider range of fungi can support germination of orchid seeds in vitro than the species is found to associate with in situ (Masuhara & Katsuya, 1994; Perkins et al., 1995; Batty et al., 2002; Fay & Krauss, 2003). This type of specificity, defined as the range of fungi which can support germination of an orchid species in vitro, is known as potential specificity (Masuhara & Katsuya, 1994; Fay & Krauss, 2003). Potential specificity has been found to be much wider than ecological specificity (Masuhara & Katsuya, 1994; Fay & Krauss, 2003). The additional fungal types involved in potential specificity may not support growth to adulthood despite their ability to germinate the seeds, or may induce a much slower growth rate of the seedling, in either of which cases the fungi may not be sufficiently effective for growth and survival in situ (Masuhara & Katsuya, 1994; Batty et al., 2002). It is essential to distinguish between ecological and potential specificity (Masuhara & Katsuya, 1994; Batty et al., 2002; Fay & Krauss, 2003). True germination, and hence true specificity, should be taken as the establishment of healthy seedlings with leaves or a tuber and hence a good chance of survival to adulthood (Masuhara & Katsuya, 1994; Zettler & Hofer, 1998; Batty et al., 2002).

1.3.2 - Adult orchids

Fungal hyphae enter and exit the adult orchid via the root hairs or trichomes (Hadley, 1982; Zettler et al., 2003). The infected organ may be the roots, the collar (stem infection just below ground level) or a rhizome (Ramsay et al., 1986). The fungus forms pelotons within the cortical cells of the infected organ and it is here that nutrient transfer occurs (Hadley, 1982; Zettler et al., 2003). Most orchids of mediterranean type climates survive the dry summers as rhizomes or dormant tubers; the resting tuber is not infected by the mycorrhizal fungus (Dixon, 1991). It is likely that an orchid’s roots or stem follow the same or similar routes through the soil each year, as each replacement tuber is formed very close to the previous one (Dixon, 1991; Batty et al., 2002). The remnants of mycorrhizal tissue from the previous season probably form an important fungal source for reinfection, and a fibrous sheath formed over many years often surrounds the collar of stem infected orchids (Dixon, 1991; Batty et al., 2002).
1.4 - Identity of orchid mycorrhizal fungi

1.4.1 - Conventional taxonomy

Most orchid mycorrhizal fungi are members of the form-genus *Rhizoctonia* (Currah et al., 1997; Zettler et al., 2003). Orchid rhizoctonias are distinguished by their appearance in culture, but are rarely identified beyond this level by conventional methods, which rely heavily on spore morphology, as it is extremely difficult to induce sexual sporulation (production of basidiospores) of orchid rhizoctonias in culture (Currah et al., 1997; Zettler et al., 2003). Most orchid rhizoctonias occur as anamorphs (the asexual state, with no production of basidiospores), and more rarely as the corresponding teleomorphs (the sexual state, which produces basidiospores) (Currah et al., 1997; Zettler et al., 2003).

Genera/species which occur as orchid mycorrhizal fungi

Orchid rhizoctonias which have been identified in culture belong to the teleomorph genera *Ceratobasidium*, *Sebacina*, *Thanatephorus*, *Tulasnella*, and *Waitea*, and the corresponding anamorphs *Ceratorhiza* (*Ceratobasidium*), *Epulorhiza* (*Sebacina* and *Tulasnella*), and *Moniliopsis* (*Thanatephorus* and *Waitea*) (Batty et al., 2002; Currah et al., 1997; Zettler et al., 2003). Many Australian orchid species are associated with these genera (Currah et al., 1997), including a number of the species studied here (Table 1.1).
Table 1.1 - Named fungi from mycorrhizas of Australian orchid genera (from Currah et al., 1997). ‘A’ indicates anamorphs (asexual states) and ‘T’ teleomorphs (sexual states).

<table>
<thead>
<tr>
<th>Orchid taxon</th>
<th>Fungal taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caladenia</em></td>
<td>Sebacina vermifera (T)</td>
</tr>
<tr>
<td></td>
<td>Tulasnella calospora (T)</td>
</tr>
<tr>
<td><em>Chiloglottis reflexa</em></td>
<td>Tulasnella asymmetrica (T)</td>
</tr>
<tr>
<td><em>Corybas dilatatus</em></td>
<td>Tulasnella allantospora (T)</td>
</tr>
<tr>
<td><em>Cyanicula sericea</em></td>
<td>Sebacina vermifera (T)</td>
</tr>
<tr>
<td><em>Diuris</em></td>
<td>Tulasnella calospora (T)</td>
</tr>
<tr>
<td><em>Elythranthera brunonis</em></td>
<td>Sebacina vermifera (T)</td>
</tr>
<tr>
<td><em>Microtis unifolia</em></td>
<td>Sebacina vermifera (T)</td>
</tr>
<tr>
<td></td>
<td>Tulasnella calospora (T)</td>
</tr>
<tr>
<td><em>Prasophyllum</em></td>
<td>Ceratorhiza goodyerae-repentis (A)</td>
</tr>
<tr>
<td></td>
<td>Ypsilonidium anomalum (T)</td>
</tr>
<tr>
<td></td>
<td>Thanatephorus cucumeris (T)</td>
</tr>
<tr>
<td><em>Pterostylis</em></td>
<td>Ceratorhiza goodyerae-repentis (A)</td>
</tr>
<tr>
<td></td>
<td>Thanatephorus cucumeris (T)</td>
</tr>
<tr>
<td></td>
<td>Ceratobasidium angustisporum (T)</td>
</tr>
<tr>
<td><em>Pyrorchis nigricans</em></td>
<td>Tulasnella calospora (T)</td>
</tr>
<tr>
<td><em>Rhizanthella gardneri</em></td>
<td>Thanatephorus gardneri (T)</td>
</tr>
<tr>
<td><em>Thelymitra</em></td>
<td>Thanatephorus sterigmaicus (T)</td>
</tr>
<tr>
<td></td>
<td>Tulasnella calospora (T)</td>
</tr>
<tr>
<td></td>
<td>Tulasnella asymmetrica (T)</td>
</tr>
<tr>
<td></td>
<td>Tulasnella violea (T)</td>
</tr>
<tr>
<td></td>
<td>Tulasnella cruciata (T)</td>
</tr>
</tbody>
</table>

Identification of orchid mycorrhizal fungi

Methods which have been used for the identification and phylogenetic grouping of orchid mycorrhizal fungi include colonial morphology, anastomosis grouping, pectic zymograms, and molecular methods (see below) (Currah et al., 1997; Zettler et al., 2003). Fungi which are only present as anamorphs can usually be identified to genus level on the basis of colonial morphology without the necessity of sexual sporulation (Currah et al., 1997; Zettler et al., 2003). A good morphological key is given by Currah et al. (1997).

Anastomosis grouping involves classifying fungal isolates according to the reactions between their hyphae when two isolates encounter one another. These reactions range from anastomosis (fusing) of the hyphal cells between (presumably) closely related isolates to antagonistic reactions between more distantly related fungi. Anastomosis grouping has been used to group fungi from the *Rhizoctonia* complex. However, orchid mycorrhizal fungi belong to only a few anastomosis groups and some isolates cannot be assigned to a specific anastomosis group, so this method is of limited utility.
for identification of orchid mycorrhizal fungi. Anastomosis grouping has been used to group orchid rhizoctonias with pathogenic and saprophytic rhizoctonias to create a world-wide phylogeny of *Rhizoctonia* (Ramsay et al., 1987; Cubeta et al., 1991; Perkins & McGee, 1995; Yang et al., 1995; Sen et al., 1999; Pope & Carter, 2001).

Pectic zymograms are created by the analysis of enzymes produced by fungi when growing on a pectin substrate. This can classify fungi into much smaller groups of (presumably) related isolates than can be achieved by morphology or anastomosis grouping, and has been used to characterise phytopathogenic rhizoctonias (Yang et al., 1995).

1.4.2 - Molecular techniques for taxonomy

Molecular techniques are ideal for the identification of orchid mycorrhizal fungi, as identity can be established in the absence of spores, identical or closely related fungal isolates can be identified, and fungal phylogenies can be created (Zettler et al., 2003). Some of these techniques are useful for determining the relatedness of a group of fungi or for provenance analysis, while others are more useful for providing a specific name for a fungus or for the creation of a phylogeny. The use of molecular methods for studying orchid fungi has so far been limited (Zettler et al., 2003).

**Amplified fragment length polymorphism (AFLP)**

Amplified fragment length polymorphism involves fragmenting the fungal DNA with restriction enzymes, amplifying selected fragments by the polymerase chain reaction (PCR), and running the fragments on a polyacrylamide gel to produce a characteristic banding pattern (Mueller & Wolfenbarger, 1999). AFLP has been widely used with plants, where it has been found to be useful for provenance analysis, identifying clones, paternity analysis, and population genetics (Mueller & Wolfenbarger, 1999). With fungi, AFLP is useful for grouping fungi according to how closely related they are, and for provenance analysis and the creation of phylogenetic trees.

The use of AFLP for genetic analysis of orchid mycorrhizal fungi has not been reported. Most published genetic studies of orchid mycorrhizal fungi have used site
specific sequencing (for example Pope and Carter, 2001; Sen et al., 1999; Taylor and Bruns, 1999). AFLP has been used, however, for genetic analysis of other types of fungi, such as arbuscular mycorrhizal fungi (Rosendahl & Taylor, 1997), shiitake mushrooms (Terashima et al., 2002a,b), phytopathogenic fungi (Majer et al., 1996; Abeln et al., 2002; Bock et al., 2002; Hurtado & Ramstedt, 2002; Keiper et al., 2003; Kothera et al., 2003), and mycoparasites (Grendene et al., 2002). The advantages of AFLP over other genetic techniques include minimal development time, high stringency and reproducibility, and potentially a large number of polymorphic markers (Mueller & Wolfenbarger, 1999). It is thus a useful technique for rapid analysis of genetic variation in orchid mycorrhizal fungi.

**Sequencing**

Sequencing involves determining the order of bases in a particular section of the DNA, most commonly the internal transcribed spacer (ITS) region in fungi. Sequencing has been used, sometimes in conjunction with RFLP (see below) to identify fungi to species level by comparison with registered sequences from fungi of known identity (Taylor & Bruns, 1999; Kristiansen et al., 2001; McKendrick et al., 2002; Taylor et al., 2003), and additionally to determine how closely related they are or to create a phylogenetic tree (Taylor & Bruns, 1997; Kristiansen et al., 2001; Pope and Carter, 2001; Taylor et al., 2003).

**Other methods**

Other molecular methods which have been used for taxonomy of orchid mycorrhizal fungi include restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). These methods generally provide less sensitivity to genetic variation. RFLPs have been used, sometimes in conjunction with anastomosis groupings or sequencing, to characterise rhizoctonias from pine trees, orchids and phytopathogens (Vilgalys & Gonzalez, 1990; Taylor & Bruns, 1997; Taylor and Bruns, 1999; Sen et al., 1999; McKendrick et al., 2002; Taylor et al., 2003). RAPDs have been used, also sometimes in conjunction with anastomosis and pectic zymogram groupings, to characterise rhizoctonias from orchids (Shan et al., 2002) and phytopathogens (Yang et al., 1995).
1.5 - Thesis objectives

In this thesis a variety of methods will be used to analyse the fungal specificity of some highly endemic Western Australian terrestrial orchids from tribe Diurideae. In particular it will investigate:

- Fungal diversity and specificity within and between populations and within three species of the *Caladenia arenicola* complex
  
  Hypothesis:
  - Fungal genetic variation mirrors the genetic variation of orchid populations in *Caladenia*

- Mycorrhizal specificity in selected *Caladenia* hybrids
  
  Hypothesis:
  - Hybrid terrestrial orchids utilise the same mycorrhizal fungus as only one parental species

- The breadth of the efficacy and specificity of the mycorrhizal fungi of *Caladenia falcata*
  
  Hypotheses:
  - Mycorrhizal fungi from *C. falcata* populations germinate seeds of species from all *Caladenia* subgenera
  - Mycorrhizal fungi of *C. falcata* are genus-specific in germination efficacy
  - *C. falcata* possesses a similar mycorrhizal fungus in geographically disjunct populations

- Fungal specificity and genetic variation in the subtribe Drakaeinae
  
  Hypothesis:
  - Fungal specificity and genetic variation mirrors species phylogeny in a highly specialised, narrowly endemic orchid group (the subtribe Drakaeinae)

- The distribution of mycorrhizal fungi in and around *Drakaea* populations and whether *Drakaea* and *Paracaleana* species can utilise the same mycorrhizal fungi
  
  Hypotheses:
- Fungal distribution mirrors plant distribution
- *Drakaea* and *Paracaleana* species can interchange mycorrhizal fungi

The persistence, colonisation, and growth of naturally occurring and introduced mycorrhizal fungi of *Caladenia arenicola* over a period of three years

**Hypotheses:**
- Fungal distribution mirrors orchid distribution
- Fungi introduced into areas lacking the mycorrhizal fungi successfully colonise soil and persist over time
- Watering improves fungal growth and persistence and seed germination
Chapter 2 – General methods

2.1 – Fungal isolation and culture

2.1.1 - Collection of plant material
Adult orchid plants were sampled, generally while in flower, by removing the above ground parts and digging up the infected organs (collar or roots, shown in Ramsay et al., 1986). The underground tuber, usually fully formed by the time of collection, was undisturbed to enable growth of the plant to continue unaffected in the following season. The infected organs were stored in moist paper towelling at 4 °C for up to one week before fungal isolation. The flower was pressed as a herbarium specimen and used to confirm identification. Herbarium specimens were lodged in the Kings Park and Botanic Gardens herbarium.

2.1.2 - Fungal isolation
The mycorrhizal fungi were isolated by the single peloton isolation method (Batty et al., 2001; Clements & Ellyard, 1979; Ramsay & Dixon, 2003). The collar or roots were cleaned by hand in running water, followed by surface washing by vigorous shaking in two to five rinses of sterile distilled water. Under sterile conditions, the collar or roots were teased apart in water to release the pelotons from the plant tissue (Figure 2.1A). The pelotons were collected in a sterile glass capillary tube (made by heating and stretching a pasteur pipette) and rinsed two to six times in sterile distilled water (Figure 2.1B,C). The pelotons were then plated out on either Fungal Isolation Medium (FIM) or modified Soil Solution Equivalent (SSE) agar (Appendix 1; Angle et al., 1991; Dellar and Lambert, 1992) with 0.01 M streptomycin sulfate. Once the pelotons had started to grow (Figure 2.1D), hyphal tips of the fungi were subcultured to new plates of FIM, SSE or oatmeal agar (Appendix 1). The rhizoctonia-type fungal isolates were all found to be anamorphic (asexual – see section 1.4.1) and did not
produce basidiospores in culture. Two or three non-sporulating isolates per plant were selected for further culture, testing for germination efficacy and experimentation.

![Figure 2.1](image)

**Figure 2.1 – Fungal isolation by the single peloton method.** A: The mycorrhizal tissue is teased apart to release the pelotons. B and C: The pelotons are collected in a sterile glass capillary tube and rinsed. D: A peloton starting to grow on agar. Scale bar = 1 mm approx.

### 2.1.3 - Fungal culture

Fungal cultures were maintained on 90 mm petri dishes of food grade agar (Davis Gelatin, Australia) (Appendix 2); oatmeal agar was used for *Drakaea* and *Paracaleana* fungi, and FIM or SSE for the remaining fungi. In addition, backup cultures were maintained on slopes of oatmeal agar or 1/5 Potato Dextrose Agar (PDA) (Appendix 1) in 10 mL plastic screw-topped tubes (TechnoPlas, Australia). After experimentation, some cultures were placed into cryostorage at -196 °C in liquid nitrogen (Batty et al., 2001b).
### 2.2 – Symbiotic seed germination in vitro

#### 2.2.1 - Seed collection and storage

Orchid seeds were collected from the same locations as the adult plants used for fungal isolation. The plants were hand pollinated to ensure seed set. Pollinated plants were tagged and the seed capsules collected prior to dehiscence 3 to 4 weeks later, according to climatic conditions and the consequent degree of capsule ripeness. The green seed capsules were stored with their stalks in water until the capsules ripened and turned brown, at which time they were air dried in paper bags to collect the seeds. The seeds were removed from the capsules, sieved, and placed in 1.5 mL cryostorage vials (Nunc), then dried for 24 hours over silica gel (Batty et al., 2001b). The seeds were stored at 4 °C (short term) or at −196 °C in liquid nitrogen (long term) (Batty et al., 2001b).

#### 2.2.2 - Seed germination in vitro

Symbiotic seed germination in vitro was carried out according to the method of Ramsay & Dixon (2003). The seeds were wrapped in packets of filter paper (Whatman’s No. 1), then sterilized in 1 % calcium hypochlorite for 30 minutes and rinsed twice in sterile distilled water (ten minutes per rinse). The sterile seeds were either spread directly onto plates of oatmeal agar (Appendix 1), or the filter papers containing the seeds were cut into several pieces and the seeds, on the filter paper, placed onto the agar (Ramsay & Dixon, 2003). For symbiotic germination, oatmeal agar previously inoculated with a fungus, which had grown to cover the plates by the time of seed sowing, was used. Food grade agar (Davis Gelatin, Australia) was used for germination of orchid species from subtribe Caladeniinae, and purified agar (Sigma) for species from Drakaeinae (Appendix 2). Three replicates were used in all cases, and asymbiotic controls were set up on uninoculated plates of oatmeal agar. The germination plates were incubated at 22 °C in the dark until germination was evident (usually at 4 weeks after seed sowing) and then the plates were moved into the light (Figure 2.2). Germination was scored at 4, 8, 12 and 16 weeks after sowing with reference to the germination stages of Ramsay et al. (1986) as shown in Figure 2.3.
2.3 – Fungal DNA extraction

The fungi were grown for DNA extraction in 10 mL of liquid modified Soil Solution Equivalent (SSE) medium (Appendix 1) in a 250 mL glass jar at room temperature. The growth rates of the fungi in liquid SSE were so slow that insufficient biomass had been produced even after several months. When an equal volume of vegetable juice (Campbell’s V8 vegetable juice diluted to 1/5 and autoclaved) was added to the medium (Pope & Carter, 2001), sufficient biomass for DNA extraction was produced after three to four weeks. The fungal mycelium was removed from the jar, blotted dry with filter papers (Whatman’s), and placed in a 1.5 mL eppendorf tube. Experimentation showed that efficient extraction of high quality DNA could only be achieved by freezing the mycelium with liquid nitrogen (Appendix 4). No grinding was required as this resulted in sheared DNA (Appendix 4). This was followed by use of the Qiagen DNeasy® Plant Mini Kit according to the manufacturer’s instructions. The DNA was quantified on a Pharmacia Biotech GeneQuant II spectrophotometer, and visualised on a 1 % agarose gel with ethidium bromide.
Figure 2.3 – Orchid germination stages. Two developmental routes seen in different species. Of the orchids studied here, species from the genera *Pterostylis*, *Drakaea*, and *Paracaleana* followed Route A (labelled *Pterosylis sanguinea*), while species from all other genera followed Route B (labelled *Diuris laxiflora*, *Microtis media*). Not to scale. From Hollick (1999), adapted from Ramsay *et al.* (1986). Picture: P Hollick.
2.4 – Amplified fragment length polymorphism (AFLP)

DNA fingerprints were produced from high quality DNA using AFLP, adapted from
the four step process described in Krauss & Hopper (2001).

1. **Restriction digest of the DNA.** Approximately 200 – 250 ng of DNA was
digested with 1 µL EcoRI/MseI restriction enzyme in a reaction volume of 12.5
µL per sample, incubated at 37 °C for two hours, then at 70 °C for 15 minutes
before cooling on ice.

2. **Ligation of adapters.** 0.5 µL of T4 DNA ligase and 12 µL of adapter/ligation
solution were added to each digested sample, which were then incubated at 20
°C overnight.

3. **Preselective amplification by PCR.** 1.25 µL of the ligation mix was added to
10 µL of pre-amplification primer solution (pre-amp primer mixes I and II were
both tested), 1.25 µL of 10x PCR buffer for AFLP, 0.1 µL of *Taq* DNA
polymerase, 0.375 µL of MgCl2, and 0.375 µL of water in a PCR plate. PCR
was performed for 20 cycles of 94 °C for 30 seconds, 56 °C for 2 minutes, 72
°C for 2 minutes. The pre-amplification product was diluted 1:30 in 0.1 M TE
buffer.

4. **Selective amplification by PCR.** 1.25 µL of each diluted, pre-selective DNA
sample was added to 1 µL of 10x PCR buffer, 0.05 µL of *Taq* DNA
polymerase, 0.3 µL of MgCl2, 3.4 µL of water, 0.2 mM of each of four dNTPs,
and primers (fluorescently labelled *EcoRI* primers e-act, e-agg, and e-acc; the 2
and 3 base *MseI* primers m-ca, m-ct, m-cg, m-cc, m-ctg, m-ctc, and m-cac were
tested). PCR was performed for 3 cycles of 94 °C for 10 minutes, 70 °C for 2
minutes, 72 °C for 2 minutes, then 8 touchdown cycles of 94 °C for 30 seconds,
69 °C for 2 minutes, 72 °C for 2 minutes, then 23 cycles of 94 °C for 30
seconds, 61 °C for 2 minutes, 72 °C for 2 minutes, followed by a 60 °C hold
for 30 minutes.

All AFLP reagents were purchased in kit form from Invitrogen (Carlsbad, CA, USA).
PCR was performed on an Applied Biosystem GeneAmp® 9700 Thermocycler. The
fluorescently labelled amplified fragments were analysed by gel electrophoresis (6%
polyacrylamide gels) by an ABI Prism 377 Automated DNA Sequencer. Fragments
were sized through the inclusion of GeneScan Rox 500 internal size standard (Applied
Biosystems, Warrington, UK). Multi-locus profiles were visualised by ABI GeneScan software (Applied Biosystems, Foster City, USA). AFLP profiles were scored for the presence/absence of fragments between 50 and 500 base pairs in size, as well as for fragment intensity as determined by peak height.

### 2.5 – Sequencing

DNA sequences of the ITS region of the genomic DNA were produced and analysed by the following four step process.

1. **ITS amplification by PCR.** 20-50 ng of template DNA was added to 5 µL of 10x DNA polymerase buffer, 3 µL of 50 mM MgCl₂, 2 µL of each of four 100 µM dNTPs, 2.5 units of Taq DNA polymerase, 1 µL at 100 pmol/µL of each of the oligonucleotide primers ITS1 and ITS4, and ddH₂O (total volume of H₂O and DNA was 37.5 µL). PCR was performed for 35 cycles of 1 minute at 96 °C, 30 seconds at 50 °C, 2 minutes at 72 °C.

2. **Sequencing reaction.** The PCR product was purified using a QIAQuick® purification kit according to the manufacturer’s instructions. 20-40 ng of the purified PCR product was added to 2 µL of Terminator Ready Reaction PreMix, 1 µL of 5x BD buffer, 1.6 pmol of primer, and ddH₂O (total volume of H₂O and DNA was 5.4 µL). Sequencing reactions were carried out for 1 minute at 96 °C, followed by 25 cycles of 10 seconds at 96 °C, 5 seconds at 50 °C, 4 minutes at 60 °C, terminated at 4 °C. The DNA was sequenced in both the forward and reverse directions for samples which contained a lot of ambiguity in the sequence.

3. **Sequence analysis.** Primers, fluorescents and dNTPs were removed using a Qiagen DyeEx™ 2.0 Spin Kit according to the manufacturer’s instructions. The DNA was eluted in 1.5 µL of loading buffer (5:1 deionised formamide: 25 mmol/L EDTA (pH 8), 0.05 % mass/volume blue dextran), placed in a water bath for 2 minutes at 95 °C followed by 5 minutes on ice. The sequences were loaded onto 6 % thermopage polyacrylamide gels and run on an ABI Prism™ 377 XL sequencer (Applied Biosystems) for 4 hours.
4. **Sequence alignment and identification.** The strands of the sequences were edited and aligned using Sequencher™ 3.0 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). Consensus sequences were constructed for DNA strands sequenced in both directions. Sequences of similar taxa published in GenBank (http://www.ncbi.nlm.nih.gov/) were obtained by performing a BLAST search on sequences of each isolate.

The oligonucleotide primers ITS1 (5’-TCC-GTA-GGT-GAA-CCT-GCG-G) and ITS4 (5’-TCC-TCC-GCT-TAT-TGA-TAT-GC), used by White et al. (1990) and obtained from Gene Work (Perth, Australia), were used to amplify the ITS region of the genomic DNA. All PCR reagents were purchased in kit form from Invitrogen (Carlsbad, CA, USA). PCR was performed on an Applied Biosystem GeneAmp® 9700 Thermocycler. The PCR amplification products were quantified on a Pharmacia Biotech GeneQuant II spectrophotometer, and visualised on a 1% agarose gel with ethidium bromide. The reagents used in the sequencing reaction were supplied in kit form with the ABI Prism™ Dye Terminator.
Chapter 3 – Mycorrhizal diversity and specificity in the related species *Caladenia arenicola*, *C. huegelii* and *C. georgei*

3.1 – *Introduction*

3.1.1 – The relationship between genetic variation in orchids and fungi

Many studies have been devoted to investigating the genetic variation and/or phylogeny of orchid species (Kores et al., 2001; Chase et al., 2003; Fay & Krauss, 2003). It has also become common to use genetic tools to investigate the diversity and specificity of mycorrhizal fungi associated with a particular group of orchids (usually only one or a few related species), and to construct a phylogeny of these fungi (Section 1.4.2). Comparisons between orchid and fungal genetic variation on a wider scale are much rarer, and the question of whether or not the patterns of fungal variation mirror those of the orchid is only now being investigated. Have orchids and their mycorrhizal fungi evolved in parallel? This chapter will consider the genetic variation among mycorrhizal fungi from three species in the *Caladenia arenicola* complex (*C. arenicola*, *C. huegelii* and *C. georgei*), and compare it to the published information on genetic variation between the orchid species and provenances within the species *C. arenicola* (Fay & Krauss, 2003; S Krauss, unpublished data).

3.1.2 – The *Caladenia arenicola* complex

The *Caladenia arenicola* complex is well suited for the study of the relationship between genetic variation in orchids and fungi, as the genetic variation of some members of the species complex has been investigated (Fay & Krauss, 2003; S Krauss, unpublished data) and some initial studies have been undertaken on the mycorrhizal fungi (Batty et al., 2000, 2001a). The complex has a somewhat confused taxonomic
history, discussed by Hopper & Brown (2001), and is currently thought to include more than twenty species, most of which do not occur in the Perth area (Hopper & Brown, 2001). The three species studied here, Caladenia arenicola Hopper & AP Br., C. georgei Hopper & AP Br., and C. huegelii HG Reichb. (Figure 3.1) occur on the Swan Coastal Plain from the Perth area southwards. Caladenia arenicola is the most common and C. huegelii (a declared rare plant) the rarest (Hopper & Brown, 2001). The taxonomy of the group is still debated, and the species C. arenicola and C. georgei are closely allied and hybridise in some areas (Hopper & Brown, 2001). An investigation of genetic variation and provenance delineation by amplified fragment length polymorphism (AFLP) failed to differentiate between C. arenicola, C. georgei and C. huegelii (S Krauss, unpublished data). Despite the apparent lack of genetic differentiation between the three species, they can be distinguished morphologically by the relative length of the petals and sepals, presence or absence and relative size of osmophores, the shape and length of the labellum marginal calli, floral colouration, and habitat distribution (Hopper & Brown, 2001; Figure 3.1).

Figure 3.1 – A: Caladenia arenicola (Photograph: EP Tay). B: C. georgei (Photograph: M Brundrett). C: C. huegelii (Photograph: A Batty).
Fay and Krauss (2003) investigated the genetic variation and provenance delineation of *Caladenia arenicola* (Figure 3.2). They sampled seven populations (locations shown in Figure 3.3), of which four were located in Kings Park and Botanic Garden (populations KP1-4), one in Bold Park (BP), one at Wireless Hill (WH), and one at Armadale Road (AR) (Figure 3.2; Fay & Krauss, 2003). Three of the four Kings Park populations (KP2-4) were grouped together in the genetic analysis (Figure 3.2; Fay & Krauss, 2003). However, the fourth Kings Park population (KP1) was markedly differentiated from these other populations, but was grouped together with the Bold Park (BP) and Armadale Road (AR) populations (Figure 3.2; Fay & Krauss, 2003). The Wireless Hill population (WH) was moderately genetically differentiated from all of the other populations (Figure 3.2; Fay & Krauss, 2003).

![Figure 3.2 – Ordination of AFLP data revealing provenance delineation for seven populations of *Caladenia arenicola* (adapted from Fay & Krauss, 2003).](image)

The soil seed-bank dynamics and fungal ecology of *Caladenia arenicola* have been examined (Batty et al., 2000, 2001a). The soil seed bank of *C. arenicola* is transient, lasting for less than one growing season (Batty et al., 2000). Such short-lived seeds will only produce a seedling and, ultimately, an adult orchid plant if an appropriate
mycorrhizal fungus is present (Batty et al., 2000, 2001a). Fungal presence is correlated with the presence of adult C. arenicola plants, but vacant recruitment sites (with an appropriate fungus present) are available (Batty et al., 2001a). As C. arenicola occurs in discrete populations (Hopper & Brown, 2001), it is possible that its fungi may be differentiated according to population as well.

The Caladenia species C. arenicola, C. georgei, and C. huegelii are ideal for a study of fungal diversity and specificity. The genetic variation and provenance delineation of C. arenicola has been studied (Fay & Krauss, 2003), as have its soil seed-bank dynamics and fungal ecology (Batty et al., 2000, 2001a). The three species are separated morphologically but not genetically. Fungal isolates can be obtained from all three species. The genetic variation of the fungi can thus be compared to the established orchid variation, and may assist in clarifying the issue of species separation.

### 3.1.3 - Objectives

The objective of this chapter was to investigate fungal diversity and specificity within and between populations and within three species of the Caladenia arenicola complex. Hypothesis: Fungal genetic variation mirrors the genetic variation of orchid populations in Caladenia

### 3.2 – Materials and Methods

#### 3.2.1 – Seed and inoculum sources, with site descriptions

The collection locations for seed and inoculum of Caladenia arenicola, C. huegelii and C. georgei used in this study are shown in Figure 3.3, and a summary of seed and inoculum sources in Table 3.1. All of the study sites originally supported Banksia-Allocasuarina-Eucalyptus woodland on sandy soil, and all sites except for the AR site were highly disturbed and substantially altered.
Figure 3.3 – Collection locations for *Caladenia arenicola*, *C. huegelii* and *C. georgei*. A: Perth and surrounding area (after Andrew Batty) – see Table 3.1 for explanation of location codes. B: Kings Park and Botanic Garden.
### Table 3.1 – Seed and inoculum sources for Caladenia arenicola, C. huegelii and C. georgei.

<table>
<thead>
<tr>
<th>Species</th>
<th>Year of collection</th>
<th>Collection location</th>
<th>No of adult plants from which fungi isolated</th>
<th>Seed collection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caladenia arenicola</em></td>
<td>1999, spring</td>
<td>Kings Park and Botanic Garden site 1 (KP1)</td>
<td>1 +</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2000, spring</td>
<td>Kings Park and Botanic Garden site 1 (KP1)</td>
<td>1 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Armadale Road (AR)</td>
<td>3 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2001, autumn</td>
<td>Kings Park and Botanic Garden site 1 (KP1)</td>
<td>3 −</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2001, spring</td>
<td>Kings Park and Botanic Garden site 1 (KP1)</td>
<td>3 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kings Park and Botanic Garden site 2 (KP2)</td>
<td>3 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kings Park and Botanic Garden site 3 (KP3)</td>
<td>2 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kings Park and Botanic Garden site 4 (KP4)</td>
<td>3 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kings Park and Botanic Garden site 5 (KP5)</td>
<td>3 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bold Park (BP)</td>
<td>5 −</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wireless Hill (WH)</td>
<td>4 −</td>
<td>+</td>
</tr>
<tr>
<td><em>Caladenia georgei</em></td>
<td>2000, spring</td>
<td>Armadale Road (AR)</td>
<td>3 −</td>
<td>−</td>
</tr>
<tr>
<td><em>Caladenia huegelii</em></td>
<td>1999, spring</td>
<td>Bannister Road (BR)</td>
<td>1 −</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2000, spring</td>
<td>Armadale Road (AR)</td>
<td>2 +</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>2001, spring</td>
<td>Armadale Road (AR)</td>
<td>2 −</td>
<td>+</td>
</tr>
</tbody>
</table>

**KP1-5 – Kings Park and Botanic Garden sites**

This 400 hectare area of native bushland and recreation areas is located in the centre of Perth (Figure 3.3A). KP1 was near the depot compound (Figure 3.3B). This is one of the most intensively studied terrestrial orchid populations in Australia (Batty et al., 2000, 2001a). A number of other orchid species are common in the area, including *Caladenia flava*, *Pterostylis sanguinea*, and *Microtis media*. The population is typical of *Caladenia arenicola*, being discrete and with clear boundaries. Adult plants were collected from this population in September 1999, 2000 and 2001 (spring), and in May 2001 (autumn) and their mycorrhizal fungi were isolated (Chapter 2). Seed was collected in 1999 (Chapter 2).

KP2 was located in bushland, in a frequently used area of the park (Figure 3.3B). KP3 was next to a walking trail in the centre of the park, and was thus less disturbed than KP1 and KP2 (Figure 3.3B). KP4 was near the lake and picnic area in the centre of the park, but relatively undisturbed (Figure 3.3B). KP5 was near the Thomas Street corner
of Kings Park and Botanic Garden, in an area of heavy use and disturbance (Figure 3.3B). Adult plants were collected from these four populations in September 2001 and their mycorrhizal fungi were isolated (Chapter 2).

**AR – Armadale Road**

The AR population of *Caladenia huegelii* was located within relatively undisturbed habitat approximately 25 km south of Kings Park and Botanic Garden (Figure 3.3A). A nearby site, substantially altered by sand mining, had a population of *C. huegelii* as well as a possibly hybridising population of *C. arenicola* and *C. georgei*. Adult plants of all three species were collected from both sites in September 2000 and their mycorrhizal fungi were isolated (Chapter 2). Seeds of *C. huegelii* were collected (Chapter 2). Additional adult plants of *C. huegelii* were collected in September 2001 and their mycorrhizal fungi isolated (Chapter 2).

**BP – Bold Park**

The BP population of *Caladenia arenicola* was located near Zamia Trail in the centre of Bold Park, which is approximately 5 km west of Kings Park and Botanic Garden, near the coast (Figure 3.3A). The area is substantially disturbed, but work has recently begun on weed control and revegetation. Adult plants were collected from this population in September 2001 and their mycorrhizal fungi were isolated (Chapter 2).

**WH – Wireless Hill**

The WH population of *Caladenia arenicola* was located at Wireless Hill park, which is on the south side of the Swan River, approximately 15 km south of Kings Park and Botanic Garden (Figure 3.3A). The area is substantially disturbed and heavily used for recreation, but a number of orchid species are common. Adult plants were collected from this population in September 2001 and their mycorrhizal fungi were isolated (Chapter 2).

**BR – Bannister Road**

The BR population of *Caladenia huegelii* was located approximately 25 km southeast of Kings Park and Botanic Garden (Figure 3.3A). A number of orchid species are common in the area. Adult plants were collected from this population in September 2001 and their mycorrhizal fungi were isolated (Chapter 2).
A list of the fungal isolates used in this study, and the experiments for which the isolates were used, is given in Table 3.2.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Year of collection</th>
<th>Collection location</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>Symbiotic germination experiment no</th>
<th>AFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caladenia arenicola</td>
<td>1999, spring</td>
<td>KP1</td>
<td>1</td>
<td>a</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2000, spring</td>
<td>KP1</td>
<td>1</td>
<td>a</td>
<td></td>
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<td></td>
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<td>b</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>2001, autumn</td>
<td>KP1</td>
<td>1</td>
<td>b</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>b</td>
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<td></td>
<td></td>
<td>3</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2001, spring</td>
<td>KP1</td>
<td>1</td>
<td>a</td>
<td>1,2</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>a</td>
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<td>3</td>
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<td></td>
<td>b</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>KP2</td>
<td>1</td>
<td>a</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>2</td>
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<td>b</td>
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<tr>
<td></td>
<td></td>
<td>KP3</td>
<td>1</td>
<td>a</td>
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<td>+</td>
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<td>KP4</td>
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<td>f</td>
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<td>a</td>
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<td></td>
<td></td>
<td>f</td>
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<td></td>
<td></td>
<td>KP5</td>
<td>1</td>
<td>a</td>
<td></td>
<td></td>
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<td>b</td>
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<td>a</td>
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<td></td>
<td></td>
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<td>a</td>
<td></td>
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<td></td>
<td></td>
<td>b</td>
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<td>BP</td>
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<td>c</td>
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<td></td>
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<tr>
<td></td>
<td>2</td>
<td></td>
<td>a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>f</td>
<td>1,2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 – Fungal isolates used in this study.
3.2.2 – Symbiotic germination

Two symbiotic seed germination experiments were carried out according to the methods described in Chapter 2. In the first experiment, seed of *Caladenia arenicola* was tested against a number of fungal isolates from *C. arenicola* and one isolate each from *C. huegelii* and *C. georgei*. In the second experiment, seed of *C. huegelii* was tested against three fungal isolates from *C. arenicola* and three from *C. huegelii*. The second experiment was carried out in collaboration as described in Swarz (2003). The collection locations for the seed batches are listed in Table 3.1, and the fungal isolates used in each experiment in Table 3.2. Germination was scored as development to stage 3 and above, and to stage 5 and above, according to the germination stages of Ramsay et al. (1986) shown in Figure 2.3. Germination was scored at 12 weeks from sowing for Experiment 1 and 6 weeks for Experiment 2. Germination was scored as a percentage of viable seed (i.e. stage 0 (Figure 2.3) was not included). The percentage of nonviable seed differed between but not within each seed type. An asymbiotic control was used in each experiment.
3.2.3 – Statistics
Seed germination was statistically analysed by ANOVA. Fisher’s PLSD with a 95% confidence interval was then applied to arcsine-transformed data to make comparisons between means. Analysis was performed using the statistical program Statview® (Statview, SAS Institute Inc.).

3.2.4 – DNA extraction and amplified fragment length polymorphism (AFLP)
DNA extraction and AFLP were carried out according to the methods described in Chapter 2 on the fungal isolates indicated in Table 3.2. A Principal Coordinates Analysis (PCA) based on genetic distance (Euclidean distance) was performed on each set of AFLP fingerprints to illustrate the pattern of genetic variation within and between fungal source species, using the genetic analysis program GenAlEx (Peakall & Smouse, 2001).

3.3 – Results

3.3.1 – Asymbiotic germination
No germination of seed of either species tested (Caladenia arenicola and C. huegelii) occurred under asymbiotic conditions, in the absence of a fungal isolate (Tables 3.3 – 3.4).

3.3.2 – Symbiotic germination
Experiment 1 – Caladenia arenicola
The C. arenicola seeds germinated to or beyond stage 3 on every fungal isolate except that from location WH (Table 3.3). This germination was, however, very variable on some isolates, resulting in high standard errors, and was statistically significantly higher than the zero germination of the asymbiotic controls only on four of the C. arenicola isolates and the C. georgei isolate (Table 3.3). There was no significant
difference in germination to or beyond stage 3 between the fungal source species (Table 3.3). The pattern was similar for germination to or beyond stage 5, which was statistically significantly higher than on the asymbiotic controls on two of the C. arenicola isolates and the C. georgei isolate (Table 3.3). Again, there was no significant difference in germination to or beyond stage 5 between the fungal source species (Table 3.3).

### Table 3.3 – Symbiotic germination of *Caladenia arenicola* seeds on fungi from *C. arenicola* and its relatives, *C. georgei* and *C. huegelii*, at 12 weeks after sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Year isolated</th>
<th>Location</th>
<th>Plant no.</th>
<th>Isolate no.</th>
<th>% to and beyond stage 3 (SE)</th>
<th>% to and beyond stage 5 (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymbiotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. arenicola</em></td>
<td>1999</td>
<td>KP1</td>
<td>1</td>
<td>a</td>
<td>55.3 (6.93) *</td>
<td>49.6 (5.50) *</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>KP1</td>
<td>1</td>
<td>b</td>
<td>34.4 (17.26)</td>
<td>32.0 (16.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AR</td>
<td>3</td>
<td>a</td>
<td>68.9 (3.51) *</td>
<td>53.3 (4.70) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KP1</td>
<td>1</td>
<td>a</td>
<td>48.8 (3.54) *</td>
<td>27.3 (3.55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KP2</td>
<td>2</td>
<td>a</td>
<td>34.6 (17.29)</td>
<td>27.7 (13.89)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KP3</td>
<td>1</td>
<td>b</td>
<td>64.8 (6.29) *</td>
<td>32.0 (9.36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KP4</td>
<td>3</td>
<td>a</td>
<td>28.7 (28.67)</td>
<td>24.0 (24.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BP</td>
<td>2</td>
<td>f</td>
<td>36.1 (18.09)</td>
<td>8.6 (4.70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WH</td>
<td>1</td>
<td>a</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. georgei</em></td>
<td>2000</td>
<td>AR</td>
<td>2</td>
<td>b</td>
<td>76.8 (1.75) *</td>
<td>60.9 (5.08) *</td>
</tr>
<tr>
<td><em>C. huegelii</em></td>
<td>2000</td>
<td>AR</td>
<td>2</td>
<td>b</td>
<td>50.0 (40.82)</td>
<td>18.2 (14.90)</td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)

### Experiment 2 – *Caladenia huegelii*

The *C. huegelii* seeds germinated to or beyond stage 3 on every fungal isolate except *C. arenicola* isolate KP1 1a (Table 3.4). The germination was, however, very low on *C. arenicola* isolate BP 2f and very variable on isolates *C. arenicola* KP3 1b and *C. huegelii* BR 1a, resulting in high standard errors, and was statistically significantly higher than the zero germination of the asymbiotic controls only on two of the *C. huegelii* isolates (Table 3.4). Germination to or beyond stage 3 was statistically significantly higher on isolates from *C. huegelii* than *C. arenicola* (P < 0.05) (Table 3.4). The only development to or beyond stage 5 was not statistically significantly higher than zero, and occurred on the two *C. huegelii* isolates which supported significant germination to or beyond stage 3 (Table 3.4). There was no significant difference in germination to or beyond stage 5 between the fungal source species (Table 3.4). Germination was scored at six weeks from seed sowing, rather than at twelve weeks as for Experiment 1.
Table 3.4 – Symbiotic germination of *Caladenia huegelii* seeds on fungi from *C. huegelii* and its relative *C. arenicola* at 6 weeks after sowing (from Swarz, 2003).

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Year isolated</th>
<th>Location</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>% to or beyond stage 3 (SE)</th>
<th>% to or beyond stage 5 (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Asymbiotic 0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. arenicola</em></td>
<td>2001</td>
<td>KP1</td>
<td>1</td>
<td>a</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KP3</td>
<td>1</td>
<td>b</td>
<td>25.4 (25.41)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BP</td>
<td>2</td>
<td>f</td>
<td>0.2 (0.22)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2001</td>
<td>AR</td>
<td>2</td>
<td>b</td>
<td>47.8 (6.47) *</td>
<td>3.3 (3.33)</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>AR</td>
<td>1</td>
<td>e</td>
<td>69.4 (8.97) *</td>
<td>4.7 (3.03)</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td>BR</td>
<td>1</td>
<td>a</td>
<td>25.8 (25.79)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)

3.3.3 – Amplified fragment length polymorphism (AFLP)

A total of 248 markers were scored, all of which were polymorphic. Of the total variation, 82 % was partitioned within populations and 18 % among populations. The Principal Coordinates Analysis (PCA) of the AFLP fingerprints produced three non-overlapping groups of fungal isolates based on source species and compatibility (Figure 3.4). The largest group, of *Caladenia arenicola* type fungal isolates, contained 46 of the 72 isolates analysed, from *C. arenicola* from every location and *C. georgei* from location AR, and showed less genetic diversity than either of the other groups (Figure 3.4). The second group, of *C. huegelii* type fungal isolates, contained all of the fungal isolates from *C. huegelii* (from location AR), as well as isolates from *C. arenicola* from locations BP, KP1, WH and AR, and isolates from *C. georgei* from location AR (Figure 3.4). The third group contained fungal isolates from *C. arenicola* from locations BP, KP3, KP4 and AR, which may all be nonefficacious for germination as the only isolate from this group tested against seed was nonefficacious (Figure 3.4).

The genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between these fungal isolates was greater (approximately double) between isolates from *Caladenia huegelii* and those of the other species than between isolates from *C. arenicola* and *C. georgei*, or between isolates of *C. arenicola* from different populations (Appendix 3). The genetic distance between isolates from *C. arenicola*
and *C. georgei* was similar to that between isolates from different populations of *C. arenicola* (Appendix 3).

The group of *Caladenia arenicola* type fungal isolates which had not been separated by the first PCA was considered separately. Of the 89 markers scored, 96% were polymorphic. Of the total variation, 69% was partitioned within populations and 31% among populations. This second PCA grouped the fungal isolates according to collection location (Figure 3.5). The fungal isolates from locations AR, WH and KP3 were genetically distinct, while the fungal isolates from all of the other locations overlapped to some extent (Figure 3.5). The fungal isolates from location KP1 contained more genetic diversity than any of the other groups of isolates (Figure 3.5).
3.4 – Discussion

The orchid species studied here appear to show different degrees of mycorrhizal specificity: *Caladenia arenicola* seeds germinated readily on fungal isolates from all three orchid species, while *C. huegelii* seeds generally germinated better on their own fungi (Tables 3.3 and 3.4). *Caladenia huegelii* thus appears to be more specific in its fungal requirements than its close relative *C. arenicola*, and may therefore be a more recently evolved offshoot of the widespread and common *C. arenicola* (or vice versa). *Caladenia huegelii* may have evolved from *C. arenicola* to utilise a more specialised fungus, or *C. arenicola* from *C. huegelii* to utilise a less specialised one. If *C. arenicola* can utilise both fungi, while *C. huegelii* can only use one, this may, at least partially, explain the comparative rarity of *C. huegelii*. It is also tempting to speculate that the seed of *C. arenicola* may colonise and occupy *C. huegelii* fungal sites, and may in time displace *C. huegelii* seedlings and increase the rarity of the species.

The apparently greater specificity of *Caladenia huegelii* than *C. arenicola* in its fungal requirements was supported by the AFLP results. Some fungal isolates from *C. arenicola* were grouped with those from *C. huegelii* (Figure 3.4). This infers that *C. arenicola* can use the fungus of *C. huegelii* but not vice versa. These results are
supported by the genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between the fungal isolates. The genetic distance was greater (approximately double) between isolates from *Caladenia huegelii* and those of the other species, than between isolates from *C. arenicola* and *C. georgei* or between isolates of *C. arenicola* from different populations (Appendix 3). It is, however, possible that the smaller sample size from *C. huegelii* than from *C. arenicola* led to an artificial separation between the fungi of the two orchid species, as the full range of genetic variation of the *C. huegelii* fungi may not have been sampled. The inclusion of some fungal isolates from *C. arenicola* in the *C. huegelii* type group supports the suggestion arising from the seed germination data that *C. huegelii* diverged from *C. arenicola* as it was able to take advantage of a more specialised fungus which was already available, or that *C. arenicola* diverged from *C. huegelii* to utilise a new fungus but retained its ability to use the old fungal type.

There was no separation of the *C. georgei* fungi from those of *C. arenicola*, but this may be due to the limited sampling of *C. georgei*, which was only collected from one location in which there may be some hybridisation with *C. arenicola*. The lack of separation between the *C. georgei* and *C. arenicola* fungi is supported by the genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between the fungal isolates, which was similar between isolates from the two species as between isolates from different populations of *C. arenicola* (Appendix 3). This is in accordance with the investigation of genetic variation and provenance delineation of the orchid species themselves, which failed to differentiate between *C. arenicola*, *C. georgei* and *C. huegelii* (S Krauss, unpublished data).

Overall, the genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between the isolates from *Caladenia huegelii* and *C. arenicola* was considerably lower (less than quarter) than between isolates from other *Caladenia* species (Chapter 4, Appendix 3), while the genetic distance between the fungal isolates from different populations of *C. arenicola* was considerably lower (about half) than between different populations of *Caladenia falcata* (Chapter 5, Appendix 3). This indicates that the overall genetic variation within the fungi from *C. arenicola* was lower than within *C. falcata*, and that the fungi from *C. huegelii* were more similar to those of *C. arenicola* than was recorded between fungi of different species of *Caladenia* in Chapter 4. The
overall low genetic variation within the *C. arenicola* fungi may be due to the small geographic separation of the populations, as the different populations of *C. falcata* were considerably further apart (Chapter 5), while the similarity of the *C. huegelii* fungi to those of *C. arenicola* supports the idea of *C. huegelii* being closely related to, but taxonomically distinct from, *C. arenicola*. It is also possible that the smaller sample size from *C. huegelii* than from *C. arenicola* resulted in less genetic variation being sampled from the *C. huegelii* fungi than the *C. arenicola* fungi, and hence an artificially close relationship between the fungi of the two orchid species.

There were two main areas in which the germination experiments might be refocussed. Firstly, it was difficult to make direct comparisons between the two germination experiments. As the *Caladenia huegelii* germination was scored at six weeks from seed sowing, rather than at twelve as for *C. arenicola*, it might be expected that the levels of germination and development of *C. huegelii* would be less than those of *C. arenicola*. This might, therefore, explain the lack of development to or beyond stage 5 of the *C. huegelii* seedlings (Table 3.4). Secondly, as only one seed batch of each species was used, this would hide any population specificity in germination within each species. It would therefore be useful to conduct a fully reciprocal trial of all three species in which seed batches from each separate population were germinated against fungal isolates from each population, using greater numbers of fungi, to investigate the issue of population specificity.

One fungal isolate used in each experiment was nonefficacious (did not support germination – Tables 3.3 and 3.4). It has been found for orchids from a variety of taxa and habitats that different fungal associates may be present during germination and adult life (Harvais & Hadley, 1967; Rasmussen, 1995; Zelmer & Currah, 1997; Zettler, 1997; Markovina & McGee, 2000; Takahashi et al., 2000, 2001). The presence of nonefficacious fungi within adult orchids implies that a broad sampling strategy is necessary, in order to cover the full range of available fungi. In addition, seed baiting to test for fungal diversity which is not utilised by adult plants may be advisable. It would be useful if methods could be developed so that fungi that are nonefficacious for germination could be tested for symbiotic ability with adult plants, and perhaps in analyses of genetic variation the nonefficacious fungi should be treated separately.
There did not appear to be any direct link between geographic distance and genetic distinctiveness between the populations of fungal isolates from *Caladenia arenicola*. The geographical scale was, of course, small, with a maximum distance between populations of approximately 30 km. Slight genetic differentiation could be detected over the broader geographic area, as both of the populations from south of the Swan River (populations WH and AR) were genetically distinct from the populations from north of the river (Figure 3.5). These two populations, however, were relatively genetically dissimilar, both being more genetically similar to some northern fungal isolates than to each other (Figure 3.5). The genetic variation of the fungi may be related to microsite requirements, or be simply a result of serendipity of fungal distribution.

There were both similarities and differences between the patterns of genetic variation of the fungal and orchid populations (Figures 3.2, 3.4 and 3.5). The fungi of *Caladenia huegelii* could be distinguished from those of *C. arenicola*, while those of *C. georgei* could not, while no differentiation was seen between the orchid species (S Krauss, unpublished data). In *C. arenicola*, for both the orchids and the fungi, population WH was more or less genetically distinct from the other populations, while population AR was close to (fungi) or overlapping with (orchids) population KP1, which in turn overlapped with population BP (Figures 3.2 and 3.5). In contrast, fungal population KP3 was genetically distinct from all other populations, while overlapping with populations KP2 and KP4 for the orchids, and orchid population KP1 was genetically distinct from the other KP populations, while overlapping with populations KP2, KP4 and KP5 for the fungi (Figures 3.2 and 3.5). However, these details must be qualified by the small sample sizes, which mean that the error associated with a ‘cluster’ is weakly known, and the consequences of larger sample sizes on relationships and overlap also unknown.

It is interesting that molecular tools applied to the fungi support the separation of the three species which was clear on morphological grounds, but molecular genetic analysis of the orchid species showed no differentiation between species. This situation should be clarified by a more extensive genetic analysis of the orchid species using different molecular tools, such as sequencing or microsatellites. A more extensive molecular analysis of the fungi of the three species might be useful in
determining whether the fungal differentiation between the three orchid species is consistently greater than that between populations, in which case the genetic variation of the fungi would clearly support the separation of the three orchid species.

The contrast of two closely related species, one common and one rare, such as Caladenia arenicola and C. huegelii, may help us to understand some of the driving forces of rarity in orchids. Caladenia arenicola certainly possesses a number of more ‘weedy’ characteristics when compared to C. huegelii, including the narrow specificity of C. huegelii and the more general specificity of C. arenicola. Caladenia huegelii tends to have a more restricted occurrence in the landscape, being restricted to topographically linked sites (K Dixon, pers. comm.), while C. arenicola can occur throughout the Spearwood and Bassendean dune systems (Hopper & Brown, 2001).

Caladenia arenicola has become more common in Kings Park since the 1970s, increasing steadily in both abundance and locations (K Dixon, pers. comm.). However, changes in bushland management have occurred over this period (e.g. controlled burning to no-burn policy, active weed control and suppression) which may have contributed to the increase in the orchid’s abundance. The genetic variation of the Kings Park populations, both of orchids and of fungi, may be related to the geology of the area. Kings Park possesses a unique limestone bluff, and in some ways acts as a confluence of several geological systems (the Quindalup, Spearwood and Bassendean dune systems). It is, therefore, a frontier environment, which may explain the greater genetic variation of the fungi from Kings Park than in all of the other locations.
Chapter 4 – Mycorrhizal specificity in *Caladenia* hybrids

4.1 – *Introduction*

4.1.1 – *Caladenia* hybrids

The genus *Caladenia* (spider orchids) is one of the most diverse in southwestern Western Australia, with more than 110 species occurring in this region of mediterranean type climate (Hoffman & Brown, 1998; Hopper & Brown, 2001). *Caladenia* species have among the most specific mycorrhizal relationships recorded in the orchid family (Warcup, 1981, 1988; Ramsay et al., 1986). However, *Caladenia* species also hybridise frequently and prolifically in nature, often forming self-perpetuating hybrid lineages (Adams & Lawson, 1993; Hoffman & Brown, 1998; Hopper & Brown, 2001). All but one of the more than 110 species of *Caladenia* found in the southwest have been observed to form natural hybrids, many of which are fertile, and some of which are quite common (Hopper & Brown, 2001).

*Caladenia* hybrids are ideal for studying mycorrhizal specificity, as they can provide an insight into the inheritance of specificity. If *Caladenia* species have such specific mycorrhizal relationships, how do the natural hybrids survive? Do the hybrids use one parental fungus, both parental fungi, or a completely different fungus? There has even been speculation that hybridisation (Adams & Lawson, 1993) and the utilisation of fungi may be a possible path to speciation (K Dixon, pers. comm.).

*Caladenia* hybrids vary in frequency, often depending on the closeness of the relationship between the parental species (Hopper & Brown, 2001). As might be expected, it has been observed that more closely related species hybridise more often than those that are more distantly related (Hopper & Brown, 2001). According to the taxonomic system of Hopper & Brown (2001), *Caladenia* is divided into five
subgenera: *Caladenia* (one species only in southwest Western Australia), *Calonema* (55 endemic species in the southwest), *Drakonorchis* (four species), *Elevatae* (five species in the southwest, four of which are endemic), and *Phlebochilus* (45 species in the southwest, all but three endemic). Some of the most common hybrids occur within a subgenus, and hybrids between different subgenera are less common (Hopper & Brown, 2001). Of the five hybrids selected for study (Table 4.1), two combinations were relatively common and occurred within a subgenus, two were rare and occurred between different subgenera, and one was an extremely rare intergeneric hybrid, occurring between genera which were once included in *Caladenia* (Hopper & Brown, 2001). The five hybrids are listed in Table 4.1, and illustrated, along with their parental species, in Figure 4.1.

<table>
<thead>
<tr>
<th><em>Caladenia</em> hybrid (parental species)</th>
<th>Hybrid name</th>
<th><em>Caladenia</em> subgenus</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Elythranthera brunonis</em> (Endl.) A.S. George <em>x Cyanicula sericea</em> (Lindl.) Hopper &amp; A.P. Br.</td>
<td><em>x Cyanthera glossodioides</em></td>
<td>Intergeneric (<em>Caladenia</em> allies, which were once included in <em>Caladenia</em>)</td>
</tr>
</tbody>
</table>

4.1.2 - Objectives

The objective of this chapter was to investigate mycorrhizal specificity in selected *Caladenia* hybrids to test the hypothesis:

Hybrid terrestrial orchids utilise the same mycorrhizal fungus as only one parental species.

4.2 – Materials and Methods

4.2.1 – Seed and inoculum sources, with site descriptions

The locations of the sites from which the hybrids and their parental species were collected are shown in Figure 4.2, and a summary of seed and inoculum sources in Table 4.2. A list of the fungal isolates produced, the codes used for each isolate, and the experiments for which they were used, is given in Table 4.3.

![Map of Collection locations for Caladenia hybrids in southwest Western Australia](image)

**Figure 4.2 – Collection locations for Caladenia hybrids in southwest Western Australia:** KP = Kings Park and Botanic Garden (*C. flava x latifolia*), B = Brookton Highway (*C. falcata x longicauda*), M = Medina (*C. flava x latifolia*), D = Darkan (*C. flava x longicauda, C. chapmanii x longicauda*), MR = Margaret River (*C. flava x latifolia, Elythranthera brunonis x Cyanicula sericea*).
**Table 4.2 – Seed and inoculum sources for the studied *Caladenia* hybrids**

<table>
<thead>
<tr>
<th>Caladenia hybrid</th>
<th>Seed source</th>
<th>Inoculum source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. falcata x longicauda</em></td>
<td>Brookton Highway (B)</td>
<td>Brookton Highway (B)</td>
</tr>
<tr>
<td><em>C. flava x latifolia</em></td>
<td>Medina (M)</td>
<td>Medina (M) (parental species only)</td>
</tr>
<tr>
<td></td>
<td>Kings Park and Botanic Garden</td>
<td>Margaret River (MR)</td>
</tr>
<tr>
<td></td>
<td><em>(C. flava only)</em></td>
<td>Kings Park and Botanic Garden (KP)</td>
</tr>
<tr>
<td><em>C. flava x longicauda</em></td>
<td>Darkan (D)</td>
<td>Darkan (D)</td>
</tr>
<tr>
<td></td>
<td>Kings Park and Botanic Garden</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(C. flava only)</em></td>
<td></td>
</tr>
<tr>
<td><em>C. chapmanii x longicauda</em></td>
<td>Darkan (D)</td>
<td>Darkan (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Elythranthera brunonis x Cyanicula sericea</em></td>
<td>Roleystone <em>(E. brunonis only)</em></td>
<td>Margaret River (MR)</td>
</tr>
</tbody>
</table>

**Kings Park and Botanic Garden (KP)**

*C. flava x latifolia*

Kings Park and Botanic Garden is located in the centre of Perth, overlooking the Swan River estuary. Most of the park consists of substantially modified *Eucalyptus-Banksia-Allocasuarina* woodland. The limestone scarp overlooking the river has recently undergone substantial revegetation and ecological restoration work, although some parts, further from the city centre, remained in relatively good condition. Despite the urban nature of the park, fifty orchid species have been recorded in the area. In September 2001, two hybrid plants of *C. flava x latifolia* were discovered flowering on an unrestored area of the scarp. These two plants, and two individuals from each parental species from the same area, were collected and their fungi isolated (Chapter 2).

**Brookton Highway (B)**

*C. falcata x longicauda*

The Brookton Highway site is 100 km east of Perth, just beyond the eastern edge of the moist jarrah (*Eucalyptus marginata*) forest. The site consists of substantially modified york gum-jam (*Eucalyptus loxophleba-Acacia acuminata*) woodland on a clay-loam (eroded laterite) soil in a road reserve, with the understorey almost entirely replaced by weeds. Nevertheless, the area is rich in orchids, particularly *Caladenia* species. In September 2000, three individuals of the hybrid *C. falcata x longicauda* and three
individuals of each parental species were collected and their fungi isolated (Chapter 2). Hand pollinations were carried out to produce capsules of parental seed, and of hybrid seed of both maternal types. The pollinated plants were tagged and the capsules collected just prior to seed dehiscence (Chapter 2).

**Medina (M)**

*Caladenia flava x latifolia*

Medina is 30 km south of Perth. The site consists of relatively pristine *Eucalyptus-Banksia-Allocauswarina* woodland on deep white sands (the Bassendean dune system). The site is known for its orchids, including the rare and beautiful *Thelymitra variagata*. In October 2000, the hybrid *C. flava x latifolia* was reported to have been found at the site. Subsequent searches failed to locate the hybrids, but three individuals of each parental species were collected from an area where both were abundant and their fungi isolated (Chapter 2). Hand pollinations were carried out to produce capsules of parental seed, and of hybrid seed of both maternal types. The pollinated plants were tagged and the capsules collected just prior to seed dehiscence (Chapter 2).

**Darkan (D)**

*Caladenia flava x longicauda*  
*Caladenia chapmanii x longicauda*

The Darkan site is located 200 km southeast of Perth, and consists of relatively unmodified *Eucalyptus* woodland on a clay-loam soil. The site is known for its abundant and varied orchids, especially *Caladenia* and *Elythranthera* species. Despite an extremely dry season inland, two *Caladenia* hybrids were found there in September 2000. Two individuals of *C. flava x longicauda*, three of *C. chapmanii x longicauda*, and three individuals of each parental species (five of *C. longicauda*) were collected from the site and their fungi isolated (Chapter 2). Hand pollinations were carried out to produce capsules of parental seed, and of hybrid seed of both maternal types. The pollinated plants were tagged and the capsules collected just prior to seed dehiscence (Chapter 2).
Margaret River (MR)

*Caladenia flava x latifolia*

*Elythranthera brunonis x Cyanicula sericea*

Margaret River is located 300 km south of Perth. The two hybrids from this area were collected from separate sites, both of which consisted of substantially modified karri (*Eucalyptus diversicolor*) forest. The first site is about 50 km southeast of Margaret River, on private land used for cattle grazing. Despite the impact of the livestock, the area is rich in orchids, and in particular supports literally hundreds of *Caladenia flava x latifolia* in a self-perpetuating hybrid lineage. In October 2000, three individuals of the hybrid and three individuals of each of the parental species were collected from the site and their fungi isolated (Chapter 2). The second site is about 5 km west of Margaret River, on a road reserve. The extremely uncommon *Elythranthera brunonis x Cyanicula sericea* was found there in October 2000. The single hybrid plant and three individuals of each parental species were collected and the fungi isolated (Chapter 2).
Table 4.3 – Fungi isolated from five *Caladenia* hybrids and their parental species, and the experiments for which they were used.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Location of collection</th>
<th>Plant number</th>
<th>Isolate number</th>
<th>Symbiotic germination</th>
<th>AFLP</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caladenia chapmanii</em> x <em>longicauda</em></td>
<td>D</td>
<td>1</td>
<td>b</td>
<td>+</td>
<td>+</td>
<td></td>
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<td></td>
<td></td>
<td>2</td>
<td>c</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>a</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Caladenia falcata</em> x <em>longicauda</em></td>
<td>B</td>
<td>1</td>
<td>a</td>
<td>+</td>
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<td></td>
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<td>2</td>
<td>b</td>
<td>+</td>
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<tr>
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<td></td>
<td>3</td>
<td>a</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Caladenia flava</em> x <em>latifolia</em></td>
<td>MR</td>
<td>1</td>
<td>a</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
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<td>b</td>
<td>+</td>
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<td>3</td>
<td>a</td>
<td>+</td>
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<tr>
<td><em>Caladenia flava</em> x <em>longicauda</em></td>
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<td>b</td>
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<td>b</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Elythranthera brunonis</em> x <em>Cyanicula sericea</em></td>
<td>MR</td>
<td>1</td>
<td>c</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Caladenia chapmanii</em></td>
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<td>3</td>
<td>a</td>
<td>+</td>
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<td><em>Caladenia falcata</em></td>
<td>B</td>
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<td>b</td>
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<td>c</td>
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<td><em>Elythranthera brunonis</em></td>
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<td>+</td>
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</tr>
<tr>
<td><em>Elythranthera brunonis</em></td>
<td>MR</td>
<td>1</td>
<td>a</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>c</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
4.2.2 – Symbiotic germination

Symbiotic seed germination was carried out according to the methods described in Chapter 2. For each hybrid, each of the available seed stocks (hybrid and parental species) was tested against each of the available fungal isolates (from the hybrid and the parental species) in a symbiotic germination matrix. Where available, seed stocks from the same location as the fungal isolates were used. The fungal isolates used are listed in Table 4.3, and the seed stocks in Table 4.2. Where more than one seed type was tested against a fungal isolate, they were tested in the same petri dish, on separate pieces of filter paper. Germination was scored according to the germination stages of Ramsay et al. (1986) as shown in Figure 2.3. Germination was scored as the number of seedlings at stage 3 and above, and at stage 5 and above, as a percentage of viable seed (i.e. stage 0 (Figure 2.3) was not included). The percentage of nonviable seed differed between but not within each seed type. An asymbiotic control was used for each experiment.

4.2.3 - Statistics

Seed germination data were statistically analysed by ANOVA. Fisher’s PLSD with a 95% confidence interval was then applied to arcsine-transformed data to make comparisons between means. Analysis was performed using the statistical program Statview® (Statview, SAS Institute Inc.).

4.2.4 – DNA extraction and amplified fragment length polymorphism (AFLP)

DNA extraction and AFLP were carried out according to the methods described in Chapter 2, on all of the fungal isolates listed in Table 4.3 as being used for AFLP. A principal coordinates analysis (PCA) based on genetic distance (Euclidean distance) was performed on each set of AFLP fingerprints to illustrate the pattern of genetic variation within and between fungal source species, using the genetic analysis program GenAlEx (Peakall & Smouse, 2001).
4.2.5 – Sequencing

Sequencing was carried out according to the methods described in Chapter 2, on all of the fungal isolates listed in Table 4.3 as being used for sequencing. Four fungal isolates from different *Caladenia* species (*C. chapmanii*, *C. falcata*, *C. flava* and *C. longicauda*) from three locations were sequenced, and a BLAST search performed on the sequences. The BLAST search compared the sequence of the ITS region of the fungal isolates to sequences of fungi submitted to GenBank by other authors, and identified the closest relatives to the fungal isolates.

4.3 – Results

4.3.1 – Asymbiotic germination

No seeds of any parental species or hybrids germinated under asymbiotic conditions (Tables 4.4 – 4.12).

4.3.2 - *Caladenia falcata x longicauda* (*C. x cala*)

Symbiotic germination

The *C. longicauda* and the hybrid seed had very similar patterns of germination across all fungal isolates (Table 4.4). Both seed types germinated well on all fungal isolates except isolate *C. falcata* 3b, with the highest levels of germination for both seed types produced by isolates *C. falcata* 1b and 2c (Table 4.4). The *C. falcata* seed only germinated on isolates *C. falcata* 1b and 2c (Table 4.4). All three seed types produced their highest levels of germination on these two fungal isolates. The levels of germination were all statistically significantly higher than zero, except for those of *C. longicauda* and the hybrid on isolate *C. longicauda* 2a, and of the hybrid on isolate hybrid 1a (Table 4.4).

All fungal isolates except *C. longicauda* 2a and *C. falcata* 3b supported statistically significantly higher levels of germination than the asymbiotic treatment across all seed types (Table 4.4). Isolate hybrid 3a supported a statistically significantly higher level
of germination than the other *C. longicauda* and hybrid isolates across all seed types, and isolates *C. falcata* 1b and 2c supported statistically significantly higher levels of germination than any other isolates (Table 4.4). Isolate *C. falcata* 2c had the highest level of germination across all seed types (Table 4.4). Across all fungal isolates, the *C. longicauda* seed displayed the highest germination, and *C. falcata* the lowest (Table 4.4).

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Plant no.</th>
<th>Isolate no.</th>
<th>Seed type $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. longicauda</em></td>
</tr>
<tr>
<td>Asymbiotic $^2$</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. longicauda</em></td>
<td>1 a</td>
<td>25.2 (9.39) *</td>
<td>11.8 (4.53) *</td>
</tr>
<tr>
<td></td>
<td>2 a</td>
<td>4.9 (4.94)</td>
<td>1.4 (1.42)</td>
</tr>
<tr>
<td></td>
<td>3 b-c</td>
<td>11.2 (4.41) *</td>
<td>5.4 (2.97) *</td>
</tr>
<tr>
<td>Hybrid</td>
<td>1 a</td>
<td>13.7 (3.11) *</td>
<td>1.1 (1.08)</td>
</tr>
<tr>
<td></td>
<td>2 b-c</td>
<td>18.5 (6.98) *</td>
<td>9.6 (2.41) *</td>
</tr>
<tr>
<td></td>
<td>3 a</td>
<td>30.1 (10.32) *</td>
<td>8.7 (2.54) *</td>
</tr>
<tr>
<td><em>C. falcata</em></td>
<td>1 b</td>
<td>36.4 (8.21) *</td>
<td>61.0 (8.90) *</td>
</tr>
<tr>
<td></td>
<td>2 c</td>
<td>42.5 (3.17) *</td>
<td>71.1 (2.11) *</td>
</tr>
<tr>
<td></td>
<td>3 b</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Germination statistically significantly higher than zero (P < 0.05)

$^1$ Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types (P < 0.05)

$^2$ Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates (P < 0.05)

A similar pattern emerged when germination to more advanced stages (stages 5 and beyond, Figure 2.3) was considered (Table 4.5). Development to stage 5 and beyond occurred in all cases where germination to stage 3 and beyond was statistically significantly higher than zero (Tables 4.4 and 4.5). Once again, all three seed types germinated best on isolates *C. falcata* 1b and 2c, except for the *C. longicauda* seed, which germinated better on isolate hybrid 3a (Table 4.5). All germination levels were statistically significantly higher than zero, except for those of the hybrid seed on isolate *C. longicauda* 3b and of the *C. longicauda* seed on isolate hybrid 1a (Table 4.5).

Fungal isolates *C. longicauda* 1a, hybrid 2b and 3a, and *C. falcata* 1b and 2c displayed statistically significantly higher levels of germination to more advanced stages than the
asymbiotic treatment across all seed types (Table 4.5). Isolates \textit{C. falcata} 1b and 2c had the highest levels of germination to more advanced stages across all seed types (Table 4.5). There was no significant difference between the seed types in levels of germination to more advanced stages across all fungal isolates (Table 4.5).

**Table 4.5 – Mean percentage germination to or beyond stage 5 (standard error) of \textit{Caladenia falcata x longicauda} and parental species’ seeds on fungal isolates from the hybrid and both parental species at 12 weeks from sowing.**

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Plant (n^a)</th>
<th>Isolate (n^b)</th>
<th>(C. longicauda)</th>
<th>Hybrid</th>
<th>(C. falcata)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic (a)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
<td>0 (0)</td>
</tr>
<tr>
<td>(C. longicauda)</td>
<td>1 (\alpha^bc)</td>
<td>11.0 (6.68) *</td>
<td>7.3 (6.38) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>2 (\alpha^a)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>3 (\beta^b)</td>
<td>5.0 (2.52) *</td>
<td>0.5 (0.51)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Hybrid</td>
<td>1 (\alpha^a)</td>
<td>2.5 (2.47)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>2 (\beta^b)</td>
<td>10.6 (3.85) *</td>
<td>5.8 (2.14) *</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>3 (\alpha^c)</td>
<td>30.1 (10.32) *</td>
<td>4.6 (2.67) *</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>(C. falcata)</td>
<td>1 (\beta^d)</td>
<td>25.3 (6.93) *</td>
<td>29.1 (7.00) *</td>
<td>51.9 (8.07) *</td>
<td>45.3 (8.33) *</td>
</tr>
<tr>
<td>2 (\epsilon^d)</td>
<td>15.5 (3.58) *</td>
<td>34.0 (4.49) *</td>
<td>45.3 (8.33) *</td>
<td>45.3 (8.33) *</td>
<td></td>
</tr>
<tr>
<td>3 (\beta^d)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

1 Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types (P < 0.05)

2 Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates (P < 0.05)

**Amplified fragment length polymorphism (AFLP)**

All of the 106 markers scored were polymorphic. Of the total variation, 78 % was partitioned within populations and 22 % among populations. The Principal Coordinates Analysis (PCA) of the AFLP fingerprints grouped the fungal isolates according to source species, with the isolates from the hybrid lying between those from the two parental species (Figure 4.3). None of the groups of isolates overlapped (Figure 4.3). The two isolates from \textit{C. longicauda} were closely grouped together, as were the three isolates from the hybrid (Figure 4.3). The two isolates from \textit{C. falcata} were not closely grouped, however. Isolate 1b, which supported high levels of germination of all three seed types, was positioned close to the isolates from the other source species, while isolate 3b, which supported no germination of any seed type, was positioned at some distance from the other isolates (Figure 4.3).
4.3.3 - *Caladenia flava x latifolia* (C. x spectabilis)

**Symbiotic germination**

The *C. flava x latifolia* seed germinated very well (statistically significantly higher than zero) on most fungal isolates. Exceptions were isolates *C. latifolia* MR3b and *C. flava* KP3a and KP3b, which showed no germination, and isolates hybrid MR1a, KP1a and KP2b, and *C. latifolia* KP2a, which resulted in very low germination (statistically indistinguishable from zero) (Table 4.6). It is noteworthy that of the seven exceptions listed above, three produced no significant germination of any seed type, while the remaining four, which only germinated *C. flava* seed, were all from the KP location (Table 4.6). The hybrid seed only germinated well on one (of six) KP fungal isolates, and that one was from *C. latifolia* (Table 4.6).

The *C. flava* seed also displayed a relatively wide specificity, but a preference for its own or the hybrid’s fungi (Table 4.6). The *C. flava* seed germinated well (statistically significantly higher than zero) on five (of eight) *C. flava* isolates, four (of five) hybrid isolates, and three (of eight) *C. latifolia* isolates, poorly (statistically indistinguishable from zero) on the remaining three *C. flava* isolates (M2c, M3b and MR3b) and one *C. latifolia* isolate (KP2a), and not at all on the remaining hybrid isolate (MR1a) and four *C. latifolia* isolates (all three M isolates and MR3b) (Table 4.6). There was also a location component to the *C. flava* germination, with greater specificity evident at the M location than elsewhere (Table 4.6). Of the isolates from the M location, the *C.
flava seed germinated well or poorly on its own isolates, and not at all on the C. latifolia isolates (Table 4.6). The specificity was lower on the fungal isolates from the MR and KP locations, as the C. flava seed germinated well on two (of three) isolates from each source species from the MR location, and five (of six) isolates from the KP location (Table 4.6).

In comparison, the C. latifolia seed displayed a much narrower specificity, germinating well (statistically significantly higher than zero) on five (of eight) of its own fungal isolates and one (of eight) C. flava isolates, and poorly (statistically indistinguishable from zero) on three (of eight) C. flava isolates and one (of five) hybrid isolate (Table 4.6). Again, specificity was greatest at the M location, with the C. latifolia seed germinating well on all three of its own fungal isolates and poorly or not at all on the C. flava isolates (Table 4.6). The C. latifolia seed did not germinate at all on any of the hybrid isolates from the MR location, and poorly or not at all on five (of six) isolates from the KP location (Table 4.6).

It is noteworthy that there were three fungal isolates which germinated all three types of seed well (statistically significantly higher than zero), which were isolates C. latifolia KP1a and MR1a, and isolate C. flava MR1b (Table 4.6). In some cases, the seed of one parental species germinated better on an isolate than the seed of the source species (this occurred for isolates C. latifolia KP2a and MR2a, and isolates hybrid KP1a and KP2b) (Table 4.6).

All of the fungal isolates displayed a statistically significantly higher germination rate than the asymbiotic plates (on which no germination of any seed type occurred) across all seed types, except for isolates hybrid MR1a, C. latifolia MR3b and KP2a, and C. flava KP3a and KP3b (Table 4.6). The fungal isolates C. latifolia KP1a and MR1a, and isolate C. flava MR1b, which germinated all three seed types, supported statistically significantly higher germination than the other isolates across all seed types (Table 4.6). The hybrid seed displayed statistically significantly higher germination than either parental species across all fungal isolates (Table 4.6).
A similar pattern emerged when germination to more advanced stages was considered (Table 4.7). The main difference was an increased specificity for the *C. latifolia* seed, with the only germination to more advanced stages occurring on its own fungi (Table 4.7). The only statistically significant germination to more advanced stages of the *C. flava* seed was on isolates hybrid KP1a and KP2b and *C. latifolia* KP1a, with poor germination (statistically indistinguishable from zero) on six (of eight) *C. flava* isolates, two (of the remaining three) hybrid isolates, and two (of the remaining seven) *C. latifolia* isolates (Table 4.7). The statistically significant hybrid germination to more advanced stages was once again found with all types of fungal isolates, occurring on isolates *C. flava* MR1b and MR2a, hybrid MR1b, and *C. latifolia* MR1a, MR2a and KP1a, and poor germination (statistically indistinguishable from zero) occurred on four (of the remaining six) *C. flava* isolates, one (of the remaining four) hybrid isolates, and two (of the remaining five) *C. latifolia* isolates (Table 4.7).

Table 4.6 – Mean percentage germination to or beyond stage 3 (standard error) of *Caladenia flava x latifolia* and parental species’ seeds on fungal isolates from the hybrid and both parental species at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Loc.</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>Asymbiotic*</th>
<th>Seed type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C. flava¹</td>
<td>Hybrid²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. flava</em></td>
<td>M</td>
<td>a⁵</td>
<td>30.2 (4.37) *</td>
<td>21.8 (8.15) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c⁶</td>
<td>11.1 (1.12)</td>
<td>38.2 (5.46) *</td>
<td>2.3 (2.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b³</td>
<td>5.0 (1.72)</td>
<td>22.4 (21.50)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. latifolia</em></td>
<td>M</td>
<td>a⁷</td>
<td>0 (0)</td>
<td>17.4 (3.40) *</td>
<td>33.3 (2.55) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a⁸</td>
<td>0 (0)</td>
<td>30.8 (17.92) *</td>
<td>33.7 (12.68) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a⁹</td>
<td>0 (0)</td>
<td>15.0 (3.32) *</td>
<td>50.4 (1.88) *</td>
</tr>
<tr>
<td><em>C. flava</em></td>
<td>MR</td>
<td>b⁴</td>
<td>19.9 (5.04) *</td>
<td>62.9 (6.96) *</td>
<td>17.5 (4.25) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a⁵</td>
<td>19.0 (15.61) *</td>
<td>52.5 (3.85) *</td>
<td>1.1 (0.57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b⁶</td>
<td>8.9 (4.49)</td>
<td>52.0 (5.67) *</td>
<td>1.0 (0.98)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>MR</td>
<td>a⁶</td>
<td>0 (0)</td>
<td>6.8 (5.33)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b⁷</td>
<td>20.3 (5.19) *</td>
<td>39.5 (4.37) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a¹</td>
<td>16.1 (10.46) *</td>
<td>53.8 (8.59) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. latifolia</em></td>
<td>MR</td>
<td>a⁸</td>
<td>10.0 (3.55) *</td>
<td>71.5 (12.02) *</td>
<td>8.3 (2.12) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a⁹</td>
<td>12.1 (6.69) *</td>
<td>48.8 (13.73) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b⁰</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. flava</em></td>
<td>KP</td>
<td>a⁵</td>
<td>9.0 (8.01) *</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b⁶</td>
<td>10.9 (3.37) *</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>KP</td>
<td>b⁷</td>
<td>18.3 (5.40) *</td>
<td>1.8 (1.75)</td>
<td>0.4 (0.42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a⁸</td>
<td>17.4 (2.20) *</td>
<td>4.3 (4.35)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. latifolia</em></td>
<td>KP</td>
<td>a⁹</td>
<td>1.7 (1.67)</td>
<td>1.0 (0.98)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a¹</td>
<td>22.9 (3.85) *</td>
<td>39.9 (1.57) *</td>
<td>54.4 (4.54) *</td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

¹ Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types (P < 0.05). Fungi from KP analysed separately.

² Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates (P < 0.05)
Fungal isolates *C. flava* M1a, MR1b, MR2a and KP3b, *C. latifolia* M1a, M3a, MR1a, MR2a and KP1a, and hybrid MR1b, KP1a and KP2b supported levels of germination to more advanced stages that were statistically significantly higher than that of the asymbiotic plates across all seed types (Table 4.7). As occurred with germination to stage 3 and beyond, the hybrid seed germinated to more advanced stages at a statistically significantly higher level than either of the parental species (Table 4.7).

Table 4.7 – Mean percentage germination to or beyond stage 5 (standard error) of *Caladenia flava x latifolia* and parental species’ seeds on fungal isolates from the hybrid and both parental species at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Loc.</th>
<th>Plant n*</th>
<th>Isolate n*</th>
<th>Seed type 1</th>
<th>Seed type 2</th>
<th>Seed type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C. flava 1</td>
<td>Hybrid 1</td>
<td>C. latifolia 1</td>
</tr>
<tr>
<td>Asymbiotic 0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. flava</td>
<td>M</td>
<td>1</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.2 (4.11)</td>
<td>12.3 (6.88)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3 (0.28)</td>
<td>13.3 (6.67)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>b&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7 (0.68)</td>
<td>19.8 (18.94)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. latifolia</td>
<td>M</td>
<td>1</td>
<td>a&lt;sup&gt;bol&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>5.8 (3.06)</td>
<td>13.6 (0.92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>15.2 (6.53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>a&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>7.5 (3.95)</td>
<td>26.3 (5.39)</td>
</tr>
<tr>
<td>C. flava</td>
<td>MR</td>
<td>1</td>
<td>b&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.6 (4.16)</td>
<td>46.4 (4.83)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>28.7 (5.77)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>b&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>1.8 (1.75)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>MR</td>
<td>1</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.9 (0.93)</td>
<td>17.1 (6.96)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>a&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>4.8 (4.76)</td>
<td>5.0 (3.83)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. latifolia</td>
<td>MR</td>
<td>1</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.4 (0.18)</td>
<td>61.3 (10.33)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>2.2 (2.22)</td>
<td>32.5 (13.26)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. flava</td>
<td>KP</td>
<td>3</td>
<td>a&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>6.2 (6.25)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>9.1 (3.80)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>KP</td>
<td>2</td>
<td>b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.8 (5.90)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.4 (2.20)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. latifolia</td>
<td>KP</td>
<td>2</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.6 (5.15)</td>
<td>21.7 (1.02)</td>
<td>2.6 (1.36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.6 (5.15)</td>
<td>21.7 (1.02)</td>
<td>2.6 (1.36)</td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

1 Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types (P < 0.05). Fungi from KP analysed separately.

2 Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates (P < 0.05)

Amplified fragment length polymorphism (AFLP)

All of the 199 markers scored were polymorphic. Of the total variation, 90 % was partitioned within populations and 10 % among populations. The PCA of the AFLP fingerprints grouped the fungal isolates according to source species and collection
location (Figure 4.4). The isolates from the KP location were grouped separately to those from the other locations (Figure 4.4). Within the KP isolates, grouping was according to source species, with none of the groups of isolates overlapping (Figure 4.4). Of the two *C. flava* KP isolates, one grouped closely with the other KP isolates, while the other grouped more closely to the *C. flava* isolates from the other locations (Figure 4.4). The *C. latifolia* isolates from M and MR formed distinct groups, relatively close to one another (Figure 4.4). In contrast, the *C. flava* isolates from M and MR, and the hybrid isolates from MR, all overlapped one another in a single large group (Figure 4.4).

![Figure 4.4 - Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from *Caladenia flava* x *latifolia* and both parental species. Each point represents one fungal isolate.]

### 4.3.4 - *Caladenia flava* x *longicauda* (*C. x triangularis*)

**Symbiotic germination**

The seed of the hybrid *C. flava* x *longicauda* germinated well (statistically significantly higher than zero) on all but one (*C. flava* 2a – poor germination, statistically indistinguishable from zero) fungal isolate (Table 4.8). In contrast, the *C. longicauda* seed germinated on all of the hybrid and *C. longicauda* isolates, although only well (statistically significantly higher than zero) on isolate hybrid 2a (Table 4.8). The *C. flava* seed was highly specific, only germinating on its own fungi (statistically
significantly well on isolates 1b and 1c, poorly (statistically indistinguishable from zero) on isolate 2a) (Table 4.8).

All of the fungal isolates supported germination levels statistically significantly higher than the zero of the asymbiotic plates across all seed types (Table 4.8). The hybrid seed displayed higher germination across all fungal isolates than either of the parental species (Table 4.8).

Table 4.8 – Mean percentage germination to or beyond stage 3 (standard error) of *Caladenia flava x longicauda* and parental species’ seeds on fungal isolates from the hybrid and both parental species at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>C. flava</th>
<th>Hybrid</th>
<th>C. longicauda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic*</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. flava</em></td>
<td>1</td>
<td>b&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>16.4 (11.07) *</td>
<td>45.6 (16.52) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.8 (0.97) *</td>
<td>20.9 (4.93) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.3 (3.22)</td>
<td>2.5 (2.47)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Hybrid</em></td>
<td>1</td>
<td>b&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>11.0 (3.77) *</td>
<td>7.7 (6.21)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>50.1 (6.30) *</td>
<td>18.9 (6.11) *</td>
</tr>
<tr>
<td><em>C. longicauda</em></td>
<td>4</td>
<td>a&lt;sub&gt;cde&lt;/sub&gt;</td>
<td>0 (0)</td>
<td>43.2 (7.73) *</td>
<td>6.2 (3.61)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>b&lt;sub&gt;de&lt;/sub&gt;</td>
<td>0 (0)</td>
<td>59.8 (12.47) *</td>
<td>8.8 (5.91)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c&lt;sub&gt;d&lt;/sub&gt;</td>
<td>0 (0)</td>
<td>36.2 (11.10) *</td>
<td>2.8 (2.78)</td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

1 Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types (P < 0.05)

2 Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates (P < 0.05)

The pattern was similar for germination to more advanced stages (Table 4.9). The hybrid seed germinated to more advanced stages well (statistically significantly higher than zero) on all three *C. longicauda* isolates and on isolates hybrid 2a and *C. flava* 1b, and poorly (statistically indistinguishable from zero) on isolates hybrid 1b and *C. flava* 1c (Table 4.9). There was no significant germination to more advanced stages of the *C. longicauda* seed, but poor germination (statistically indistinguishable from zero) occurred on isolates hybrid 1b and 2a and *C. longicauda* 4a and 5b (Table 4.9). The only germination to more advanced stages of the *C. flava* seed was on isolate *C. flava* 1b, and was not statistically significant (Table 4.9).
All three *C. longicauda* isolates and isolates *C. flava* 1b and hybrid 2a supported a level of germination to more advanced stages that was statistically significantly higher than that on the asymbiotic plates (Table 4.9). The hybrid seed displayed a statistically significantly higher level of germination to more advanced stages across all fungal isolates than either of the parental species (Table 4.9).

### Table 4.9 – Mean percentage germination to or beyond stage 5 (standard error) of *Caladenia flava x longicauda* and parental species’ seeds on fungal isolates from the hybrid and both parental species at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Plant n°</th>
<th>Isolate n°¹</th>
<th>C. flava²</th>
<th>Hybrid²</th>
<th>C. longicauda³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic⁴</td>
<td></td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. flava</em></td>
<td>1</td>
<td>b⁵</td>
<td>6.1 (3.61)</td>
<td>37.0 (21.02) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c⁶</td>
<td>4.4 (4.44)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>2</td>
<td>a⁷</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. longicauda</em></td>
<td>4</td>
<td>a⁸</td>
<td>0 (0)</td>
<td>22.2 (0.83) *</td>
<td>4.2 (4.17)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>b⁹</td>
<td>0 (0)</td>
<td>37.9 (4.92) *</td>
<td>6.7 (6.67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c¹⁰</td>
<td>24.2 (8.90) *</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)
¹ Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types (P < 0.05)
² Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates (P < 0.05)

### Amplified fragment length polymorphism (AFLP)

All of the 145 markers scored were polymorphic. Of the total variation, 85 % was partitioned within populations and 15 % among populations. The PCA of the AFLP fingerprints grouped the fungal isolates according to source species, with the isolates from *C. longicauda* lying between those from the hybrid and *C. flava* (Figure 4.5). None of the groups of isolates overlapped (Figure 4.5). The closest grouping of isolates consisted of two from *C. longicauda*, two from *C. flava*, and one from the hybrid, with the remaining isolates further from this central grouping (Figure 4.5). The two closely grouped isolates from *C. longicauda* originated from different plants, while the outlier (isolate 5c) was from the same plant as one of the other isolates (Figure 4.5). In contrast, the two closely grouped isolates from *C. flava* originated from the same plant, while the outlier (isolate 2a) was from a different plant (Figure 4.5).
4.3.5 - *Caladenia chapmanii x longicauda* (*C. x eludens*)

**Symbiotic germination**

The seed of the hybrid *C. chapmanii x longicauda* only germinated well on isolate *C. longicauda* 5c, and poorly on isolate *C. longicauda* 5b (Table 4.10). In contrast, the *C. chapmanii* seed displayed a specificity for its own fungi, germinating well (statistically significantly higher than zero) on isolates *C. chapmanii* 2b and 3a (Table 4.10). The *C. longicauda* seed had a wider specificity, germinating well on all of the hybrid and *C. longicauda* isolates (Table 4.10). There was no germination of any seed type on isolate *C. chapmanii* 1a (Table 4.10).

All of the fungal isolates, apart from *C. chapmanii* 1a, supported statistically significantly higher germination than the zero of the asymbiotic plates across all seed types (Table 4.10). The *C. longicauda* seed displayed the highest germination levels across all fungal isolates, and the hybrid seed the lowest (Table 4.10).
The pattern was similar for germination to more advanced stages (Table 4.11). The hybrid seed displayed no statistically significant germination to more advanced stages, but did germinate poorly (statistically indistinguishable from zero) on isolates *C. longicauda* 5b and 5c (Table 4.11). The *C. chapmani* seed germinated statistically significantly well to more advanced stages on isolate *C. chapmani* 3a, but displayed no development to more advanced stages on isolate *C. chapmani* 2b, despite good total germination (Table 4.11). The *C. longicauda* seed germinated to more advanced stages well (statistically significantly higher than zero) on isolates hybrid 2c and *C. longicauda* 4a and 5b, and poorly (statistically indistinguishable from zero) on isolates hybrid 1b and *C. longicauda* 5c, but displayed no development to more advanced stages on isolate hybrid 3a, despite good total germination (Table 4.11). It is noteworthy that a higher proportion of the germinated hybrid seeds had developed to more advanced stages by week 12 than of either parental species (Tables 4.10 and 4.11).

Only isolates *C. chapmani* 3a and hybrid 2c supported statistically significantly higher levels of germination to more advanced stages across all seed types than the asymbiotic plates (Table 4.11). The *C. longicauda* seed displayed a statistically significantly higher level of germination across all fungal isolates than either of the other seed types (Table 4.11).
Table 4.11 – Mean percentage germination to or beyond stage 5 (standard error) of Caladenia chapmanii x longicauda and parental species’ seeds on fungal isolates from the hybrid and both parental species at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>Seed type</th>
<th>C. chapmanii</th>
<th>Hybrid</th>
<th>C. longicauda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic</td>
<td></td>
<td></td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. chapmanii</td>
<td>1</td>
<td>a</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>b</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>14.4 (10.34) *</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>1</td>
<td>b&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
<td>0 (0)</td>
<td>-</td>
<td>1.5 (0.85)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>c</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>33.4 (6.72) *</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. longicauda</td>
<td>4</td>
<td>a&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>9.4 (4.72) *</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>b&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
<td>0 (0)</td>
<td>2.4 (2.38)</td>
<td>7.7 (7.69) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0 (0)</td>
<td>3.9 (1.96)</td>
<td>0.7 (0.67)</td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)
1 Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types (P < 0.05)
2 Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates (P < 0.05)

Amplified fragment length polymorphism (AFLP)

All of the 130 markers scored were polymorphic. Of the total variation, 95 % was partitioned within populations and 5 % among populations. The PCA of the AFLP fingerprints grouped the fungal isolates according to source species, with the isolates from the hybrid lying between those from the two parental species (Figure 4.6). The groups of isolates from the parental species did not overlap, while those from the hybrid overlapped with those from C. longicauda (Figure 4.6). One isolate from each parental species did not group closely with the other isolates (Figure 4.6). The outlying isolate from C. longicauda (isolate 5c) was from the same plant as one of the centrally grouped isolates (Figure 4.6). In contrast, the outlying isolate from C. chapmanii (isolate 1a) was from a different plant to either of the centrally grouped isolates, and was the only isolate to support no germination of any seed type (Figure 4.6).
Figure 4.6 – Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from *Caladenia chapmanii x longicauda* and both parental species. Each point represents one fungal isolate.

4.3.6 - *Elythranthera brunonis x Cyanicula sericea (x Cyanthera glossodiioides)*

Symbiotic germination

*Elythranthera brunonis* seed was the only type available for this experiment. The *E. brunonis* seed germinated well (statistically significantly higher than zero) on the hybrid isolate and *E. brunonis* 1a, and poorly (statistically indistinguishable from zero) on isolate *E. brunonis* 2c (Table 4.12). Development to more advanced stages occurred in both cases of significant germination to stage 3 and beyond, but was only statistically significantly higher than zero on isolate *E. brunonis* 1a (Table 4.12).

Table 4.12 – Mean percentage germination (standard error) of *Elythranthera brunonis* seed on fungal isolates from *Elythranthera brunonis x Cyanicula sericea* and both parental species at 16 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Plant n°</th>
<th>Isolate n°</th>
<th>% to and beyond stage 3 (SE)</th>
<th>% to and beyond stage 5 (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. sericea</em></td>
<td>1</td>
<td>b</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>c</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>1</td>
<td>c</td>
<td>4.9 (3.83) *</td>
<td>0.8 (0.83)</td>
</tr>
<tr>
<td><em>E. brunonis</em></td>
<td>1</td>
<td>a</td>
<td>10.8 (0.76) *</td>
<td>1.7 (1.18) *</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>c</td>
<td>2.3 (1.26)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)
Amplified fragment length polymorphism (AFLP)

Of the 214 markers scored, 98% were polymorphic. Of the total variation, 57% was partitioned within populations and 43% among populations. The PCA of the AFLP fingerprints grouped the fungal isolates according to source species, with the isolates from the hybrid lying between those from the two parental species (Figure 4.7). None of the groups of isolates overlapped (Figure 4.7). The two isolates from *Cyanicula sericea* were closely grouped together, while the two isolates from *Elythranthera brunonis* were not (Figure 4.3). Overall, the genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between these isolates from different orchid genera was considerably greater (approximately twice as much) than between isolates from different orchid species in the same genus (*Caladenia*) (Appendix 3).

![Figure 4.7](image_url)

**Figure 4.7** – Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from *Elythranthera brunonis* x *Cyanicula sericea* and both parental species. Each point represents one fungal isolate.

### 4.3.7 - Sequencing

Four fungal isolates from different *Caladenia* species (*C. chapmanii*, *C. falcata*, *C. flava* and *C. longicauda*) from three locations were sequenced, and a BLAST search performed on the sequences. The results indicated that all four isolates were *Sebacina vermifera* (Table 4.13). *Sebacina vermifera* is, however, a teleomorph (sexual state) and the four isolates sequenced here were anamorphs (asexual), and therefore belong to the corresponding anamorph genus *Epulorhiza* (Currah et al., 1997).
Table 4.13 – Identity of fungal isolates from four *Caladenia* species as indicated by GenBank.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Location</th>
<th>Plant n°</th>
<th>Isolate n°</th>
<th>Close relatives from BLAST search</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caladenia chapmanii</em></td>
<td>D</td>
<td>1</td>
<td>a</td>
<td><em>Sebacina vermifera</em> 18s (orchid mycorrhizal fungus)</td>
</tr>
<tr>
<td><em>Caladenia falcata</em></td>
<td>B</td>
<td>1</td>
<td>b</td>
<td>BLAST reference number: AF202728</td>
</tr>
<tr>
<td><em>Caladenia flava</em></td>
<td>KP</td>
<td>3</td>
<td>a</td>
<td>Country of origin: Australia</td>
</tr>
<tr>
<td><em>Caladenia longicauda</em></td>
<td>D</td>
<td>4</td>
<td>a</td>
<td>Reference: Taylor, DL, Bruns, TD, &amp; Hodges, SA (unpublished)</td>
</tr>
</tbody>
</table>

### 4.4 – Discussion

Germination of all of the seed types used in this study was dependent on the presence of a mycorrhizal fungus, as no germination occurred on the asymbiotic controls (Tables 4.4 – 4.12). This confirms previous reports of fungal-dependent germination for a number of *Caladenia* species, including some of the species studied here (Warcup, 1981; Ramsay et al., 1986). The symbiotic germination of hybrid seed has not previously been reported, but this study indicated that hybrid seeds are as dependent on a mycorrhizal fungus for germination as their parental species.

There were both similarities and differences between the conclusions which could be drawn from the results of the germination experiments and the AFLP analyses. Both techniques indicated that the parental species always use different fungi to each other. However, the germination experiments indicated that the hybrids probably share the fungi of one parental species (Tables 4.4 – 4.12), while the AFLP analyses indicated that the fungi of the hybrids may be the same as those of one parental species or may be different to both but more closely related to one than the other (Figures 4.3 – 4.7). It may be that the orchids are less stringent fungal taxonomists than are AFLP analyses.

The germination studies reported here support the hypothesis that hybrid orchids will utilise the same mycorrhizal fungus as one parental species. *Caladenia falcata x longicauda* appears to share the fungus of *C. longicauda*, as both seed types germinated on both fungal types (Tables 4.4 and 4.5). The issue here is complicated by the broad specificity of the mycorrhizal fungi of *C. falcata*, which germinated all
three seed types (discussed further in Chapter 5). *Caladenia flava x latifolia* probably shares the fungus of *C. flava*, as both seed types (*C. flava* and the hybrid) germinated on both fungal types (from *C. flava* and the hybrid), and this appears to be the case in all three collection locations (Tables 4.6 and 4.7). *Caladenia flava x longicauda* seems to share the fungus of *C. longicauda*, as both seed types (*C. longicauda* and the hybrid) germinated on both fungal types (from *C. longicauda* and the hybrid) (Tables 4.8 and 4.9). The situation with *C. chapmanii x longicauda* is less clear, as the hybrid seeds only germinated on *C. longicauda* fungi (Tables 4.10 and 4.11). However, the hybrid would appear to share the same fungus as *C. longicauda*, as *C. longicauda* seed germinated both on its own and the hybrid’s fungi. It is more difficult to come to a conclusion about *Elythranthera brunonis x Cyanicula sericea* due to the lack of seed of the hybrid and *C. sericea*, but it would seem that the hybrid shares the fungus of at least the *E. brunonis* parent, as the *E. brunonis* seed germinated on the hybrid’s fungus as well as its own (Table 4.12).

The hybrid seeds appeared to have a broader specificity than the seeds of their parental species. With some exceptions, the seeds of three out of the four available hybrids germinated on the fungi of both of their parental species as well as on their own (Tables 4.4 – 4.9). The fourth hybrid, *Caladenia chapmanii x longicauda*, only germinated on *C. longicauda* fungi, but had a higher rate of development to more advanced stages than either of its parental species (Tables 4.10 and 4.11). It is unusual for an orchid to not germinate on its own fungal isolates (Harvais & Hadley, 1967; Markovina & McGee, 2000; Takahashi et al., 2000, 2001). In contrast, it was very rare for any parental seed types to germinate on fungal isolates other than their own or those of the hybrid (Tables 4.4 – 4.12).

A number of fungal isolates which could germinate both parental species, as well as the hybrid, were found. The *C. falcata* fungi germinated any type of seed (Tables 4.4 and 4.5, investigated further in Chapter 5). In some cases, all three types of seed germinated well on the same fungus, including the isolates *C. latifolia* KP1a, *C. latifolia* MR1a, and *C. flava* MR1b (Tables 4.6 and 4.7). In other cases, a different seed type had improved germination over that of the source species, including the isolates *C. latifolia* KP2a, *C. flava x latifolia* KP1a, *C. flava x latifolia* KP2b, and *C.
latifolia MR2a (Tables 4.6 and 4.7). These results indicate that specificity in Caladenia may be broader than has been previously thought.

The concept of species-specific mycorrhizal relationships has been supported by the AFLP genetic fingerprinting of the fungal isolates carried out in this study. Each parental orchid species possessed fungal isolates which were genetically distinct from the fungal isolates of the other parental species (Figures 4.3 – 4.7). There was no overlap between the fungal isolates of any pair of parental species.

Most of the hybrids examined in this study also possessed fungal isolates which were genetically distinct from those of both parental species (Figures 4.3 – 4.7). There was no overlap between the fungal isolates of the hybrids and those of either parental species for Caladenia falcata x longicauda, C. flava x latifolia from location KP, C. flava x longicauda, or Elythranthera brunonis x Cyanicula sericea, indicating that hybrids may select for unique fungal genotypes. However, in two cases the fungal isolates of the hybrids overlapped with those of one parental species (Caladenia flava x latifolia from location MR and C. chapmanii x longicauda), indicating that the fungal isolates of these hybrids were not genetically distinct from those of their parental species.

In all of the cases where the fungal isolates of the hybrids were genetically distinct from those of both parental species, the fungal isolates of the hybrids were more closely related to those of one parental species than the other. The fungal isolates of C. falcata x longicauda were more closely related to those of C. longicauda than those of C. falcata (Figure 4.3), the fungal isolates of C. flava x longicauda were more closely related to those of C. longicauda than those of C. flava (Figure 4.5), and the fungal isolate of Elythranthera brunonis x Cyanicula sericea was more closely related to those of E. brunonis than those of C. sericea (Figure 4.7). This is supported by the Nei Genetic Distance between the hybrids and their parental species, which was always lower for the distance between the hybrid and one parental species than between the hybrid and the other parental species (Appendix 3). It thus appears that the hybrids’ mycorrhizal fungi may or may not be genetically distinct from those of their parental species, but if distinct, are more closely related to the fungi of one parental species than the other.
The patterns of germination specificity and fungal genetic variation differed between locations in the one hybrid collected from more than one site (C. flava x latifolia – Tables 4.6 and 4.7, Figure 4.4). At location M, the fungal isolates were quite specific in their germination effect, while the specificity was lower at location MR and varied between seed types at location KP. The fungal isolates of the hybrid from location MR overlapped with those of C. flava from both MR and M, while at location KP, the fungal isolates of the hybrid were genetically distinct from those of both parental species. These results indicate that there are geographic as well as taxonomic influences on fungal usage and specificity. This has been suggested by Zettler et al. (2003) and clearly demonstrated in the mycoheterotrophic orchids Corallorhiza maculata and C. mertensiana by Taylor & Bruns (1999), who found that mycorrhizal fungal strain was influenced by geography and/or habitat as well as source species.

There were outliers in four of the fungal groups (Figures 4.3 – 4.6). In two cases, the outlying fungal isolate was nonefficacious (Caladenia falcata and C. chapmanii – Figures 4.3 and 4.6), and these were the only two nonefficacious isolates observed in this study (Tables 4.4 – 4.12). Of the two remaining outliers (both efficacious), the outlier of C. flava from location D was from a different plant to the other isolates, while the outlier of C. longicauda from location D was from the same plant as one of the other isolates (Table 4.3). This indicates that a single orchid plant can contain two or more genetically distinct mycorrhizal fungi.

The mycorrhizal fungi used by members of different genera and their hybrid (Elythranthera brunonis x Cyanicula sericea) were more distantly related to each other than were the mycorrhizal fungi used by members of the same genus (Caladenia). This is illustrated by the Nei Genetic Difference between the fungi of these groups (Appendix 3), which was approximately twice as great between isolates from different genera as between isolates from the same genus. This is genetic confirmation of morphological observations (Ramsay et al., 1986), which indicated that isolates from members of the same genus tended to be similar, while isolates from different genera tended to be more dissimilar in cultural properties.

As no previous studies have examined the use of AFLP to analyse the patterns of genetic variation in orchid mycorrhizal fungi (Chapter 1), it is difficult to specify the
ideal sample size for studies of this sort. The sample sizes used in this study (isolation
and use of fungi from three adult plants of each species or hybrid in each location)
were chosen for reasons of practicality. The results of this study indicate that more
samples from more locations would have been useful. It is difficult to speculate on the
effect of larger sample sizes on the observed patterns of genetic variation, as there are
no comparable studies in the literature. However, it is possible that a larger sample
would include more genetic variation, and may alter the observed patterns of genetic
variation to some extent. It is not possible to say whether this would affect the
conclusions drawn in this study about the fungi used by hybrid orchids.

All four of the fungi sequenced in this study, which were isolated from different
*Caladenia* species from three subgenera, were identified through GenBank as *Sebacina
vermifera*. *Sebacina vermifera* is, however, a teleomorph (sexual state) and the four
isolates sequenced here were anamorphs (asexual), and therefore belong to the
corresponding anamorph genus *Epulorhiza* (Currah et al., 1997). This included the
isolate from *Caladenia chapmanii* which was nonefficacious in germination. It is thus
likely that all of the *Caladenia* fungi used in this study are of the same species,
although the same conclusion cannot necessarily be drawn about the more distantly
related fungi from the other orchid genera. Other studies have identified the
mycorrhizal fungi of *Caladenia* species as *Sebacina vermifera* (Warcup, 1981, 1988).
In particular, Warcup (1981) used morphological techniques to identify the
mycorrhizal fungi of a number of Australian orchid species. *Caladenia* (at that time
including *Cyanicula*) and *Elythranthera* were found to be associated with *Sebacina

The differences between the conclusions which can be drawn from the results of the
germination experiments and AFLP analyses may be related to the concepts of
ecological and potential specificity (Masuhara & Katsuya, 1994; Rasmussen, 2002;
Fay & Krauss, 2003). Ecological specificity, which is the range of fungi associated
with an orchid species *in situ*, is indicated by the AFLP analyses, wherein it appears
that the hybrids use fungi which are usually different to those of both parental species
but are more closely related to the fungi of one parent than the other. Potential
specificity, on the other hand, which is the range of fungi with which an orchid species
can form viable associations *in vitro*, is indicated by the germination experiments,
wherein it appears that the hybrids can use the fungi of both parental species as well as their own, which are likely to be the same as those of one parental species. It is generally considered that ecological specificity is much narrower than potential specificity (Masuhara & Katsuya, 1994; Rasmussen, 2002; Fay & Krauss, 2003), and that is certainly the case for the hybrids studied here, which appear to be able to use a wider range of fungi in vitro than in situ. It also appears that the hybrids have a wider potential specificity than the parental species.

Some of the results of the germination experiments presented in this study can be interpreted as further evidence for the likelihood of some orchids using different fungi at different life stages (Harvais & Hadley, 1967; Rasmussen, 1995; Zelmer & Currah, 1997; Zettler, 1997; Markovina & McGee, 2000; Takahashi et al., 2000, 2001). In particular, the fact that some of the orchids did not germinate on their own fungi (Caladenia latifolia from locations MR and KP, and C. chapmanii x longicauda) would seem to suggest that these orchids may have used a different fungus to germinate in situ than they possessed as adult plants. The seeds were viable, as shown by their germination on other fungal isolates, and the fungal isolates in question were efficacious with other seed types. The seeds which produced the adult orchids from which the fungi were isolated must have used a fungus to germinate originally. If the fungus the adult orchid possessed did not germinate its own seeds, then it is a reasonable assumption that either the efficacy of the fungus has changed since the orchid germinated, or that the orchid used a different fungus to germinate than it possessed as an adult.

It is possible that hybridisation and the utilisation of fungi may be a path to speciation. The genus Caladenia is considered to be actively speciating (Adams & Lawson, 1993), and one possible path for speciation may be hybridisation and the utilisation of different fungi by the hybrids. The occurrence of fertile hybrids and self-perpetuating hybrid lineages has been observed in the field and is supported by the data. While the avoidance of cross-pollination with either parental species would be necessary for the sustaining of a hybrid lineage into a self-perpetuating new species (Adams & Lawson, 1993), perhaps hybrids only survive (and, in particular, only form self-perpetuating hybrid lineages) where a suitable un- or under-utilised fungus occurs.
Chapter 5 – Investigation of broadly compatible mycorrhizal fungi from *Caladenia falcata*

5.1 – *Introduction*

5.1.1 – *Caladenia falcata*

During the investigation of the mycorrhizal fungi of *Caladenia* hybrids presented in Chapter 4, it was discovered that the fungal isolates from *C. falcata* (Figure 5.1) appeared to have some highly unusual and potentially important ecological features (Section 4.3.2). The seeds of the hybrid *C. falcata x longicauda* germinated on the *C. falcata* fungi, as was usual for hybrid seeds on a parental fungus, but the seeds of *C. longicauda* germinated also, often at a higher percent than on its own fungi (Tables 4.4 and 4.5). Some fungi of *C. flava* and *C. latifolia* were also observed to support germination of each others’ seeds (Section 4.3.3), but never as consistently, or giving such a high percentage germination, as for the *C. falcata* fungi.

![Figure 5.1 – *Caladenia falcata* (Photograph: Gary Brockman)](image)
The mycorrhizal fungi of terrestrial orchids, particularly those of Australian orchids, are generally highly specific (Rasmussen, 1995). High specificity in *Caladenia* was shown by Ramsay et al. (1986), who symbiotically germinated over 50 *Caladenia* species, and Clements (1988), who reported species-specific fungal relationships and germination in *Caladenia*. Indeed, *Caladenia* species are considered to possess among the most specific mycorrhizal relationships of any orchids (K Dixon, pers. comm.).

Other studies have indicated that *Caladenia* seeds tend to be more specific in their fungal requirements than those of other Australian terrestrial genera, while fungal isolates from *Caladenia* may support the germination of unrelated orchid species. Warcup (1981) tested the seeds of four *Caladenia* species and three other orchid species on fungi from eight *Caladenia* species and five other orchids. Many of the *Caladenia* seeds germinated on more than one (sometimes all) of the *Caladenia* isolates but not on fungi from other orchid genera, while the other orchid seeds germinated on a wider range of fungal isolates, often including those from *Caladenia* (Warcup, 1981). However, from the data it is unclear whether germination in this study proceeded fully to the green leaf stage, and if it did not, then it is doubtful whether it can truly be classed as germination (Warcup, 1981). Warcup (1988) later found that four *Caladenia* species germinated on one fungus of *Caladenia* origin, as did four *Microtis* species which also germinated on a number of other isolates of different origin. Clements (1982) reported that a fungus from *Caladenia reticulata* had been found to germinate eleven *Diuris* species and eight *Thelymitra* species. The extent of seedling development is also unclear in these studies, and it is possible that the reported germination may have occurred without fungal infection.

If these other reports of *Caladenia* fungi with broad compatibility are, in fact, reporting true germination to more advanced stages, then this feature of the *C. falcata* fungi studied here can be added to an established list. It is surprising that these fungi with broad compatibility have not been investigated further. Is this a feature of a number of *Caladenia* fungi? Broadly compatible fungi did not appear in any of the other *Caladenia* species investigated in Chapter 4. Why would this one species, out of the five investigated here and many others (K Dixon, pers. comm.), use a fungus of such broad compatibility? Why don’t other co-occurring orchids, such as *Caladenia*
C. longicauda, C. hirta, and C. denticulata, use the same fungus? It has been shown that C. longicauda uses a different fungus, as does the hybrid C. falcata x longicauda (Chapter 4). Does C. falcata possess broadly compatible fungi in more than one population, or only in the one location already investigated? If broadly compatible fungi occur in more than one population, is there one widely distributed, broadly specific fungus, or different fungi in each location? The existence of a fungus with unusually broad compatibility has conservation implications, such as presenting options for investigating rare taxa with saprophytic incompetence problems.

5.1.2 - Objectives

The objective of this chapter was to investigate the breadth of the efficacy and specificity of the mycorrhizal fungi of Caladenia falcata to test the hypotheses:

- Mycorrhizal fungi from C. falcata populations germinate seeds of species from all Caladenia subgenera.
- Mycorrhizal fungi of C. falcata are genus-specific in germination efficacy.
- Caladenia falcata possesses a similar mycorrhizal fungus in geographically disjunct populations.

5.2 – Materials and Methods

5.2.1 – Seed and inoculum sources, with site descriptions

The collection locations for seed and inoculum of Caladenia falcata used in this study are shown in Figure 5.2, and a summary of seed and inoculum sources in Table 5.1.
Figure 5.2 – Collection locations for *Caladenia falcata* in Australia: B is Brookton Highway, C is Corrigin, and Y is York. The shaded area shows the approximate range of *C. falcata* (Hoffman & Brown, 1998; Hopper & Brown, 2001).

Table 5.1 – Seed and inoculum sources for *Caladenia falcata*.

<table>
<thead>
<tr>
<th>Collection location</th>
<th>N° of adult plants from which fungi isolated</th>
<th>Seed collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brookton Highway (B)</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>York (Y)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Corrigin (C)</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

**Brookton Highway (B)**

The Brookton Highway site is located approximately 100 km east of Perth, just beyond the eastern edge of the jarrah (*Eucalyptus marginata*) forest. The site consists of substantially modified york gum-jam (*Eucalyptus loxophleba-Acacia acuminata*) woodland on a clay-loam (eroded laterite) soil in a road reserve, with the understorey almost entirely replaced by weeds. Nevertheless, the area is rich in orchids, particularly *Caladenia* species. In September 2000, three individuals of *C. falcata* were collected and their fungi isolated (Chapter 2). Hand pollinations were carried out to ensure seed set, and the pollinated plants were tagged and the capsules collected just prior to seed dehiscence (Chapter 2).
York (Y)
The York site is located 100 km east of Perth, about 10 km west of York, beyond the eastern edge of the moist jarrah (*Eucalyptus marginata*) forest. The site consists of relatively undisturbed york gum-jam (*Eucalyptus loxophleba-Acacia acuminata*) woodland on a clay-loam (eroded laterite) soil in a nature reserve. The area is rich in orchids. In September 2001, five individuals of *Caladenia falcata* were collected and their fungi isolated (Chapter 2).

Corrigin (C)
The Corrigin site is located approximately 250 km southeast of Perth, about 10 km south of Corrigin in the eastern wheatbelt. The site consists of relatively undisturbed *Eucalyptus*-Proteaceae mallee and low scrub on a loamy sand soil in a nature reserve. The area is populated by a number of orchid species, with *Caladenia falcata* the only large *Caladenia* species present. In August 2001, two individuals of *C. falcata* were collected and their fungi isolated (Chapter 2).

Other orchid species
Seeds of a number of other orchid species were used in this study. The species used, the locations of seed collection, and the experiments for which they were used, are shown in Table 5.2.

A list of the fungal isolates used in this study, and the experiments for which the isolates were used, is given in Table 5.3.
Table 5.2 – Sources of orchid seeds used in this study (classifications from Chase et al., 2003; authorities from Hopper & Brown, 2001 and Hoffman & Brown, 1998).

<table>
<thead>
<tr>
<th>Species</th>
<th>Subgenus (of Caladenia)</th>
<th>Genus</th>
<th>Subtribe (of Diurideae)</th>
<th>Tribe</th>
<th>Collection location</th>
<th>No *</th>
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</thead>
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<td>Caladenia falcata</td>
<td>Calonema</td>
<td>Caladenia</td>
<td>Caladeniinae</td>
<td>Diurideae</td>
<td>Brookton Highway (B)</td>
<td>1,2,3,4</td>
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<tr>
<td>(Nicholls) MA Clem. &amp; Hopper</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Caladenia longicauda</td>
<td>Calonema</td>
<td>Caladenia</td>
<td>Caladeniinae</td>
<td>Diurideae</td>
<td>Brookton Highway (B)</td>
<td>1</td>
</tr>
<tr>
<td>Lindl.</td>
<td></td>
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</tr>
<tr>
<td>Caladenia falcata x</td>
<td>Calonema</td>
<td>Caladenia</td>
<td>Caladeniinae</td>
<td>Diurideae</td>
<td>Brookton Highway (B)</td>
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<tr>
<td>longicauda Hopper &amp; AP Br.</td>
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<td>Caladenia</td>
<td>Caladeniinae</td>
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<tr>
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</tr>
<tr>
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<td>Caladenia</td>
<td>Caladeniinae</td>
<td>Diurideae</td>
<td>Medina</td>
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<td>Hopper &amp; AP Br.</td>
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<td></td>
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<td>Caladenia</td>
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<td>Caladeniinae</td>
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<td>Diurideae</td>
<td>Dalwallinu</td>
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<td>Hopper &amp; AP Br.</td>
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<td>Cyanicula gemmata</td>
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<td>Cyanicula</td>
<td>Caladeniinae</td>
<td>Diurideae</td>
<td>John Forrest National Park</td>
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<tr>
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<td>Diuris</td>
<td>Diuridinae</td>
<td>Diurideae</td>
<td>Warrick</td>
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<td>Microtis media R. Br.</td>
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<td>Microtis</td>
<td>Prasophyllinae</td>
<td>Diurideae</td>
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<tr>
<td>Thelymitra crinita</td>
<td>-</td>
<td>Thelymitra</td>
<td>Thelymitrinae</td>
<td>Diurideae</td>
<td>Gidgegannup</td>
<td>4</td>
</tr>
<tr>
<td>Lindley</td>
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<td>Monadenia bracteata (Sw.)</td>
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<td>Monadenia</td>
<td>-</td>
<td>Orchideae</td>
<td>Gidgegannup</td>
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<td>Pterostylis</td>
<td>-</td>
<td>Cranichideae</td>
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<tr>
<td>DL Jones &amp; MA Clem.</td>
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</table>

* Symbiotic germination experiment number
### Table 5.3 – Fungal isolates used in this study.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Collection location</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>Symbiotic germination (experiment no)</th>
<th>AFLP</th>
</tr>
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<tbody>
<tr>
<td>Caladenia falcata</td>
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<td>1</td>
<td>b</td>
<td>1,2,3,4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>c</td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>b</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>a</td>
<td>2,3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a</td>
<td>2,3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>2,3</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>1</td>
<td>a</td>
<td>2,3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>2,3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a</td>
<td>2,3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>a</td>
<td>2,3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

### 5.2.2 – Symbiotic germination

Four symbiotic germination experiments were carried out according to the methods described in Chapter 2, in which several types of seed were tested against one or more fungal isolates. The seed types used in each experiment are listed in Table 5.2, and the fungal isolates in Table 5.3. Experiment 1 used seeds of species from the same subgenus as *C. falcata*; Experiment 2 used species from the same and different subgenera to *C. falcata*; Experiment 3 used species from different subgenera to *C. falcata*, and from different genera from the same subtribe as *Caladenia*; and Experiment 4 used species from different subtribes and tribes to *Caladenia* (Chase et al., 2003). Experiment 4 was a collaborative study and is reported in part in Bonnardeaux (2003). *Caladenia falcata* seed from location B was used in all four experiments.

In each experiment, all seed types tested against each fungal isolate were placed in the same petri dish, on separate pieces of filter paper. Germination was scored as development to and beyond stage 3, and to and beyond stage 5, according to the germination stages of Ramsay et al. (1986) shown in Figure 2.3. Germination was scored as a percentage of viable seed (i.e. stage 0 (Figure 2.3) was not included). The percentage of nonviable seed differed between but not within each seed type. Asymbiotic controls were used in experiments 1-3.
5.2.3 – Statistics
Seed germination was statistically analysed by ANOVA. Fisher’s PLSD with a 95% confidence interval was then applied to arcsine-transformed data to make comparisons between means. Analysis was performed using the statistical program Statview® (Statview, SAS Institute Inc.).

5.2.4 – DNA extraction and amplified fragment length polymorphism (AFLP)
DNA extraction and AFLP were carried out according to the methods described in Chapter 2, on all of the fungal isolates listed in Table 5.3 as being used for AFLP. A Principal Coordinates Analysis (PCA) based on genetic distance (Euclidean distance) was performed on each set of AFLP fingerprints to illustrate the pattern of genetic variation within and between fungal source species, using the genetic analysis program GenAlEx (Peakall & Smouse, 2001).

5.3 – Results

5.3.1 – Asymbiotic germination
No germination of any orchid species tested occurred under asymbiotic conditions (Tables 5.4 – 5.10).

5.3.2 – Symbiotic germination
Experiment 1 – Germination of species from Caladenia subgenus Calonema on fungal isolates from Caladenia falcata (subgenus Calonema)
All three seed types (C. falcata, C. longicauda, and C. falcata x longicauda) germinated well to or beyond stages 3 and 5 (Figure 2.3) on two of the three fungal isolates from location B (Tables 5.4 and 5.5). No germination occurred on the third fungal isolate (Table 5.4).
Table 5.4 – Mean percentage germination to or beyond stage 3 (standard error), using fungal isolates from *Caladenia falcata* (subgenus *Calonema*), of seeds from species and hybrids from the same subgenus (*Caladenia falcata, C. longicauda*, and the hybrid *C. falcata x longicauda*) at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species (location)</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>Seed type</th>
<th>C. falcata</th>
<th>C. falcata x longicauda</th>
<th>C. longicauda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Asymbiotic</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. falcata</em> (B)</td>
<td>1</td>
<td>b</td>
<td>53.7 (9.80) *</td>
<td>61.0 (8.90) *</td>
<td>36.4 (8.21) *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>c</td>
<td>61.4 (7.52) *</td>
<td>71.1 (2.11) *</td>
<td>42.5 (3.17) *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>b</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

Table 5.5 - Mean percentage germination to or beyond stage 5 (standard error) using fungal isolates from *Caladenia falcata* (subgenus *Calonema*), of seeds from species and hybrids from the same subgenus (*Caladenia falcata, C. longicauda*, and the hybrid *C. falcata x longicauda*) at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species (location)</th>
<th>Plant no</th>
<th>Isolate no</th>
<th>Seed type</th>
<th>C. longicauda</th>
<th>C. falcata x longicauda</th>
<th>C. falcata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Asymbiotic</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. falcata</em> (B)</td>
<td>1</td>
<td>b</td>
<td>25.3 (6.93) *</td>
<td>29.1 (7.00) *</td>
<td>51.9 (8.07) *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>c</td>
<td>15.5 (3.58) *</td>
<td>34.0 (4.49) *</td>
<td>45.3 (8.33) *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>b</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

Experiment 2 – Germination of four species and hybrids from *Caladenia* subgenera *Calonema, Elevatae* and *Phlebochilus* on fungal isolates from *Caladenia falcata* (subgenus *Calonema*)

All four seed types (*C. falcata, C. arenicola, C. flava x latifolia* and *C. chapmanii*) germinated well (statistically significantly better than under asymbiotic conditions) to or beyond stage 3 (Figure 2.3) on nearly all of the fungal isolates used (Table 5.6). Two of the isolates from location Y (1a and 2a) supported levels of germination which were not statistically significantly different from the zero germination in the asymbiotic control (Table 5.6). The most effective fungal isolates across all seed types were isolates Y2b, Y5a, Y5b, Y3a, C1a, B1b, and C1b (in that order) (Table 5.6). The *C. chapmanii* seeds (subgenus *Phlebochilus*) displayed statistically significantly lower germination across all fungal isolates than the other seed types, while the *C. arenicola* seeds (subgenus *Calonema*) displayed statistically significantly higher germination (Table 5.6).
Table 5.6 - Mean percentage germination to or beyond stage 3 (standard error), using fungal isolates from *Caladenia falcata* (subgenus *Calonema*), of seeds from species and hybrids from the same or different subgenera (*Calonema* - *Caladenia falcata* and *C. arenicola*; *Elevatae* - *C. flava* x *latifolia*; and *Phlebochilus* - *C. chapmanii*) at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>L</th>
<th>Pl. n°</th>
<th>Is. n°</th>
<th>Caladenia falcata&lt;sup&gt;a&lt;/sup&gt; (subgenus <em>Calonema</em>)</th>
<th>Caladenia arenicola&lt;sup&gt;c&lt;/sup&gt; (subgenus <em>Calonema</em>)</th>
<th>Caladenia flava x latifolia&lt;sup&gt;b&lt;/sup&gt; (subgenus <em>Elevatae</em>)</th>
<th>Caladenia chapmanii&lt;sup&gt;1&lt;/sup&gt; (subgenus <em>Phlebochilus</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Caladenia falcata</td>
<td>B</td>
<td>1</td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>37.1 (11.04)*</td>
<td>36.0 (2.50)*</td>
<td>77.9 (16.48)*</td>
<td>24.1 (2.03)*</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td>a&lt;sup&gt;f&lt;/sup&gt;</td>
<td>62.6 (6.28)*</td>
<td>37.1 (4.03)*</td>
<td>52.6 (5.29)*</td>
<td>5.4 (1.83)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;i&lt;/sup&gt;</td>
<td>22.6 (3.82)*</td>
<td>71.7 (9.53)*</td>
<td>100 (0)*</td>
<td>26.0 (4.56)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>4.3 (2.29)</td>
<td>0.9 (0.90)</td>
<td>7.4 (5.96)</td>
<td>0.8 (0.78)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>1</td>
<td>a&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0.2 (0.16)</td>
<td>0.5 (0.46)</td>
<td>3.1 (3.08)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;def&lt;/sup&gt;</td>
<td>4.0 (2.00)</td>
<td>2.1 (1.09)</td>
<td>0 (0)</td>
<td>5.2 (2.70)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.7 (2.69)</td>
<td>0.6 (0.62)</td>
<td>0 (0)</td>
<td>2.6 (2.56)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>22.5 (14.17)*</td>
<td>10.4 (3.20)*</td>
<td>3.9 (3.10)</td>
<td>11.7 (10.81)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a&lt;sup&gt;f&lt;/sup&gt;</td>
<td>16.4 (4.31)*</td>
<td>13.3 (3.33)*</td>
<td>15.3 (4.05)*</td>
<td>18.6 (2.78)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.3 (2.30)</td>
<td>1.0 (0.95)</td>
<td>21.9 (10.01)*</td>
<td>0.7 (0.74)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>50.6 (7.84)*</td>
<td>7.5 (1.37)*</td>
<td>7.6 (2.92)*</td>
<td>5.9 (0.73)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>38.5 (9.74)*</td>
<td>9.1 (1.04)*</td>
<td>12.2 (2.13)*</td>
<td>1.3 (1.26)*</td>
<td></td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)

<sup>1</sup> Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types

<sup>2</sup> Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates

L = collection location

When germination to or beyond stage 5 (Figure 2.3) was considered, it was found that seeds from all three subgenera displayed statistically significantly better germination than the asymbiotic controls on at least one fungal isolate (Table 5.7). The most effective fungal isolates across all seed types were isolates Y5a, Y5b, Y3a, C1a, B1b and C1b (in that order) (Table 5.7). The *C. chapmanii* seeds (subgenus *Phlebochilus*) displayed a statistically significantly lower level of germination across all fungal isolates than the other seed types, while the *C. falcata* seeds displayed a statistically significantly higher level of germination (Table 5.7). Detailed examination of the protocorms showed that all species were infected by the mycorrhizal fungus, even when development did not progress beyond stage 3 (results not shown).
Table 5.7 - Mean percentage germination to or beyond stage 5 (standard error), using fungal isolates from *Caladenia falcata* (subgenus *Calonema*), of seeds from species and hybrids from the same or different subgenera (*Calonema* - *Caladenia falcata* and *C. arenicola*; *Elevatae* - *C. flava* *x* *latifolia*; and *Phlebochilus* - *C. chapmanii*) at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>L</th>
<th>Pl. n*</th>
<th>Is. n*</th>
<th>Seed type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Caladenia falcata</em>&lt;sup&gt;c&lt;/sup&gt; (subgenus <em>Calonema</em>)</td>
</tr>
<tr>
<td>Asymbiotic*&lt;sup&gt;j&lt;/sup&gt;</td>
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<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Caladenia falcata</em></td>
<td>B</td>
<td>1</td>
<td>b&lt;sup&gt;ex&lt;/sup&gt;</td>
<td>29.6 (10.59)*</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td>a&lt;sup&gt;k&lt;/sup&gt;</td>
<td>23.5 (8.40)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;l&lt;/sup&gt;</td>
<td>12.4 (2.84)*</td>
<td>60.0 (5.44)*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a&lt;sup&gt;x&lt;/sup&gt;</td>
<td>1.2 (0.61)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>1</td>
<td>a&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;z&lt;/sup&gt;</td>
<td>1.6 (0.92)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a&lt;sup&gt;x&lt;/sup&gt;</td>
<td>12.1 (4.89)*</td>
<td>6.5 (2.84)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;oo&lt;/sup&gt;</td>
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<td>0.5 (0.48)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>a&lt;sup&gt;x&lt;/sup&gt;</td>
<td>30.1 (7.93)*</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b&lt;sup&gt;x&lt;/sup&gt;</td>
<td>21.7 (1.33)*</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)
1 Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types
2 Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates
L = collection location

Experiment 3 – Germination of species from *Caladenia* subgenera *Elevatae* and *Drakonorchis* and subtribe Caladeniinae on fungal isolates from *Caladenia falcata* (subgenus *Calonema*, subtribe Caladeniinae)

Fungal isolates from *Caladenia falcata* from two or more locations supported germination to or beyond stage 3 (Figure 2.3) of seeds from different subgenera of *Caladenia* (*Elevatae* and *Drakonorchis*), but although there was some germination of seeds from the different genera (*Cyanicula* and *Elythranthera*) the levels were not statistically significant (Table 5.8). Three of the fungal isolates supported no germination of any seed type (isolates B1b, Y3a and Y5a), despite the fact that isolate B1b had germinated seeds in Experiments 1 and 2 (Table 5.8, and Tables 5.4 – 5.7). All of the other isolates supported statistically significantly higher germination than the asymbiotic controls across all seed types (Table 5.8). The seeds of *Caladenia falcata* and *C. drakeoides* displayed statistically significantly higher germination across all fungal isolates than the other seed types (Table 5.8).
Table 5.8 - Mean percentage germination to or beyond stage 3 (standard error), using fungal isolates from *Caladenia falcata* (subgenus *Calonema*), of seeds from species from the same or different subgenera (*Calonema - Caladenia falcata; Elevatae - C. latifolia; and Drakonorchis - C. drakeoides*) or different genera (*Elythranthera brunonis* and *Cyanicula gemmata*) at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>L</th>
<th>Pl. n°</th>
<th>Is. n°</th>
<th>Seed type ²</th>
<th>Caladenia falcata ³ (subtribe Caladeniinae, genus Caladenia, subgenus Calonema)</th>
<th>Caladenia latifolia ⁴ (subtribe Caladeniinae, genus Caladenia, subgenus Elevatae)</th>
<th>Caladenia drakeoides ⁵ (subtribe Caladeniinae, genus Caladenia, subgenus Drakonorchis)</th>
<th>Elythranthera brunonis ⁶ (subtribe Caladeniinae, genus Elythranthera)</th>
<th>Cyanicula gemmata ⁷ (subtribe Caladeniinae, genus Cyanicula)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic a ⁸</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cal. falcata</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B1 b ⁹ 0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a²</td>
<td></td>
<td></td>
<td>32.1 (10.79)*</td>
<td>59.9 (11.58)*</td>
<td>38.8 (5.77)*</td>
<td>1.2 (1.25)</td>
<td>3.3 (0.42)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>a¹</td>
<td></td>
<td></td>
<td>18.1 (18.13)</td>
<td>2.2 (2.22)</td>
<td>19.9 (18.20)</td>
<td>0 (0)</td>
<td>0.8 (0.81)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a²</td>
<td></td>
<td></td>
<td>77.8 (0)*</td>
<td>56.9 (0)*</td>
<td>65.1 (0)*</td>
<td>13.6 (0)</td>
<td>9.5 (0)</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>a ¹</td>
<td></td>
<td></td>
<td>55.0 (0)*</td>
<td>72.7 (0)*</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>21.4 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>b²</td>
<td></td>
<td></td>
<td>39.7 (10.29)*</td>
<td>57.1 (1.77)*</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4.2 (4.17)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a²</td>
<td></td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>a²</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)

1 Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types

2 Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates

L = collection location

The effectiveness of the fungal isolates was confirmed when germination to or beyond stage 5 (Figure 2.3) was considered (Table 5.9). Four of the five seed types germinated on at least one of the fungal isolates, but the germination was statistically significantly higher than the asymbiotic controls only for the three *Caladenia* species (Table 5.9). Fungal isolates B3a, C1a, C2a, Y1a, and Y2a supported statistically significantly higher germination than the asymbiotic controls across all seed types (Table 5.9). The seeds of *C. falcata* and *C. drakeoides* displayed statistically significantly higher germination across all fungal isolates than the other seed types (Table 5.9). Detailed examination of the protocorms showed that all species, including *Elythranthera brunonis* and *Cyanicula gemmata*, were infected by the mycorrhizal fungus, even when development did not progress beyond stage 3 (results not shown).
Table 5.9 - Mean percentage germination to or beyond stage 5 (standard error), using fungal isolates from *Caladenia falcata* (subgenus *Calonema*), of seeds from species from the same or different subgenera (*Calonema - Caladenia falcata; Elevatae - *C. latifolia*; and *Drakonorchis - C. drakeoides*) or different genera (*Elythranthera brunonis* and *Cyanicula gemmata*) at 12 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>L</th>
<th>Pl. n°</th>
<th>Is. n° 1</th>
<th>Seed type 2</th>
<th>Asymbiotica*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Caladenia falcata</em></td>
<td>B</td>
<td>b°</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>a²</td>
<td>23.9 (4.71)*</td>
<td>12.4 (8.99)*</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Caladenia latifolia</em></td>
<td>C</td>
<td>a°</td>
<td>15.8 (15.79)*</td>
<td>0 (0)</td>
<td>17.8 (16.12)*</td>
</tr>
<tr>
<td>(subtribe Caladeniinae, genus <em>Caladenia</em>, subgenus <em>Calonema</em>)</td>
<td>2</td>
<td>a²</td>
<td>77.8 (0)*</td>
<td>10.3 (0)*</td>
<td>65.1 (0)*</td>
</tr>
<tr>
<td><em>Caladenia drakeoides</em></td>
<td>Y</td>
<td>a²d</td>
<td>35.0 (0)*</td>
<td>0 (0)</td>
<td>72.7 (0)*</td>
</tr>
<tr>
<td>(subtribe Caladeniinae, genus <em>Caladenia</em>, subgenus <em>Drakonorchis</em>)</td>
<td>2</td>
<td>b°</td>
<td>22.3 (4.64)*</td>
<td>0 (0)</td>
<td>31.0 (15.44)*</td>
</tr>
<tr>
<td><em>Elythranthera brunonis</em></td>
<td>3</td>
<td>a°</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>(subtribe Caladeniinae, genus <em>Elythranthera</em>)</td>
<td>5</td>
<td>a°</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Cyanicula gemmata</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(subtribe Caladeniinae, genus <em>Cyanicula</em>)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)
1 Fungal isolates with different lower case letters as superscripts support statistically significantly different levels of germination across all seed types
2 Seed types with different capital letters as superscripts displayed statistically significantly different levels of germination across all fungal isolates

L = collection location

Experiment 4 – Germination of species from subtribes Diuridinae, Prasophyllinae, and Thelymitrinae, and tribes Orchideae and Cranichideae on fungal isolates from *Caladenia falcata* (subtribe Caladeniinae, tribe Diuridaceae)

*Caladenia falcata* and three of the other orchid species from different subtribes to *Caladenia falcata* (*Monadenia bracteata, Thelymitra crinita, and Microtis media*) germinated to or beyond stage 3 (Figure 2.3) to a level that was statistically significantly higher than zero (Table 5.10). The highest germination was observed for the *Caladenia falcata* seeds, and only these seedlings developed to or beyond stage 5 (Table 5.10). An asymbiotic control was not used, and the seedlings were not examined to determine whether infection had occurred. Germination was scored at 9 weeks from seed sowing, rather than at 12 weeks as for Experiments 1-3.
Table 5.10 - Mean percentage germination, using fungal isolates from *Caladenia falcata* (subtribe Caladeniinae, tribe Diurideae), of seeds from species from the same or different subtribes (Caladeniinae – *Caladenia falcata*; Diuridinae – *Diuris corymbosa*; Prasophyllinae – *Microtis media*; and Thelymitrinae – *Thelymitra crinita*) or different tribes (Cranichideae – *Pterostylis sanguinea*; and Orchideae – *Monadenia bracteata*) at 9 weeks from sowing (from Bonnardeaux, 2003).

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Seed type ¹</th>
<th>Tribe/subtribe</th>
<th>% to or beyond stage 3 (SE)</th>
<th>% to or beyond stage 5 (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caladenia falcata B1b</td>
<td><em>Caladenia falcata</em></td>
<td>Tribe Diurideae, subtribe Caladeniinae</td>
<td>99.5 (0.47) *</td>
<td>78.0 (7.03) *</td>
</tr>
<tr>
<td><em>Diuris corymbosa</em></td>
<td>Tribe Diurideae, subtribe Diuridinae</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Microtis media</em></td>
<td>Tribe Diurideae, subtribe Prasophyllinae</td>
<td>82.5 (4.58) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Thelymitra crinita</em></td>
<td>Tribe Diurideae, subtribe Thelymitrinae</td>
<td>77.7 (1.02) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Pterostylis sanguinea</em></td>
<td>Tribe Cranichideae</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Monadenia bracteata</em></td>
<td>Tribe Orchideae</td>
<td>92.6 (1.33) *</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

¹ Seed types with different lower case letters as superscripts displayed statistically significantly different levels of germination to & beyond stage 3

* Germination is statistically significantly higher than zero (P < 0.0001)

5.3.3 – Amplified fragment length polymorphism (AFLP)

Of the 120 markers scored, 97 % were polymorphic. Of the total variation, 72 % was partitioned within populations and 28 % among populations. The Principal Coordinates Analysis (PCA) of the AFLP fingerprints grouped the fungal isolates according to collection location first, and then according to plant number within location (Figure 5.3). None of the groups of isolates overlapped (Figure 5.3). Most of the isolates were closely grouped together, with one outlier, isolate Y5b, which was efficacious in the germination tests (Figure 5.3). The genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between isolates from populations C and Y was considerably lower (about half) than between isolates from populations C and B or Y and B (Appendix 3).
5.4 – Discussion

All three populations of Caladenia falcata investigated in this study possessed broadly compatible fungi which could germinate seeds from species in the same subgenus as C. falcata, and from each of the other three subgenera investigated (Tables 5.4 – 5.9). This occurred despite the fact that some of the species investigated, such as C. drakeoides, are found well outside the natural range of C. falcata (Hoffman & Brown, 1998).

The general trend of the germination trials on the fungi of Caladenia falcata was that the orchid species tended to germinate better the more closely they were related to C. falcata. While the broadly compatible fungi of C. falcata would support germination of species from every subgenus of Caladenia tested, they would not germinate seeds of closely related genera within the same subtribe, or of species from other subtribes and tribes (Tables 5.4 – 5.10). Although germination to stage 3 occurred in many of these species from outside Caladenia (Cyanicula gemmata, Elytrverthera brunonis, Microtis media, Thelymitra crinita, and Pterostylis sanguinea), there was no further development.
The existence of these broadly compatible fungi suggests that mycorrhizal specificity in *Caladenia* may have been overestimated. The reputation that *Caladenia* has for being highly specific appears to be based on unpublished information (K Dixon, pers. comm.), as the published data indicates that *Caladenia* seeds tend to be more specific in their fungal requirements than those of other genera, while fungal isolates from *Caladenia* may support the germination of unrelated orchid species (Warcup 1981, 1988; Clements 1982). Broadly compatible fungi may, in fact, be fairly common in *Caladenia*. A thorough investigation of the patterns of specificity in *Caladenia* is likely to be a rewarding endeavour.

Some of the fungal isolates used in this study were nonefficacious (did not support germination of any seed types). It is considered that in these cases the orchid species use different fungi for germination and for association with the adult plants (Rasmussen, 1995; Zelmer & Currah, 1997; Zettler, 1997). Orchids from a variety of taxa and habitats have often been found to be associated with fungi which do not germinate their seeds (Harvais & Hadley, 1967; Rasmussen, 1995; Markovina & McGee, 2000; Takahashi et al., 2000, 2001). There may be a habitat benefit to the orchid to be associated with different fungi at different life stages, or different fungi may be more useful to the orchid at different times. If *Caladenia falcata* uses different fungi with, perhaps, a lower competency, during recruitment events, that would explain why *C. falcata* seedlings are always scattered and never occur near adult plants (K Dixon, pers. comm.). On the other hand, orchids often possess a variety of fungal endophytes which are not involved in symbiotic relationships (Rasmussen, 1995), so the presence of a fungus within an orchid should not be taken as sole evidence of its mycorrhizal nature or ecological competency for sustaining the orchid.

The effectiveness of some fungal isolates changed between consecutive experiments (Tables 5.4 – 5.10). This occurred, most notably, with isolates B1b, Y3a and Y5a, which did not support germination (even of the seeds of *Caladenia falcata*) in Experiment 3, when they had all previously supported high levels of germination (Tables 5.4 – 5.9). A number of explanations are possible, from inadvertent contamination of the fungal cultures (unlikely, as no change in visible cultural properties occurred) to a loss of efficacy. It is known that fungal isolates can lose their
efficacy over time in culture (Alexander & Hadley, 1983), so this may provide an explanation in this case.

The genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between the fungal isolates from *Caladenia falcata* (from different populations of the same orchid species) was considerably lower (less than half) than between isolates from different orchid species in the same genus (*Caladenia* – Chapter 4, Appendix 3), while being considerably higher (about twice as much) than between different populations of *Caladenia arenicola* (Chapter 3, Appendix 3). The genetic diversity of these broadly compatible fungi appears, on the basis of this limited sample, to be relatively wide for fungi from several populations of the same orchid species (Appendix 3). This infers that broadly compatible fungi may not be a limited phenomenon.

This is the first intensive study of a highly endemic orchid group with a broadly compatible fungus. It is possible that this situation is not unique (Warcup, 1981, 1988; Clements, 1982), and that other endemic *Caladenia* species may also possess broadly compatible fungi. However, more work needs to be done to clarify this situation. *Caladenia falcata* belongs to a group of orchids which occurs over a wide range of climatic conditions and is not divided into subspecies (Hoffman & Brown, 1998). Perhaps a broadly compatible fungus is advantageous in this situation. It would be useful to search for the occurrence of broadly compatible fungi among other orchid species with wide ranges and no subspecies. Alternatively, *C. falcata* may be in an ecological or geographical expansion phase, for which association with a broadly compatible fungus achieves the goal of niche penetration in a founder seedling.

The broadly compatible fungi were found in all three populations of *Caladenia falcata* which were sampled, but no other species were investigated (other than the species used in the investigation of *Caladenia* hybrids in Chapter 4). It would be useful to sample a number of other populations of *C. falcata* for the presence of broadly compatible fungi, particularly those from the margins of its range, and also to test for fungi from seedlings or seed baits (Brundrett et al., 2003). It would also be useful to investigate other *Caladenia* species, and a possible starting point would be with related species, co-occurring species, species mentioned in previous studies (Warcup, 1981,
1988; Clements, 1982), and, as mentioned above, species with broad ranges over climatic extremes and no division into subspecies.

A more speculative area for future work would be on the implications of broadly compatible fungi for orchid speciation. The speciation of mycorrhizal plants such as orchids tends to be a ‘chicken or egg’ situation: does a different fungus or a different orchid arise first? Perhaps orchids might diverge by progressively exploiting more and more specialised fungi. In this case, perhaps a broadly compatible fungus is the more ancestral form, and the gradual use of more specific fungi has aided orchids in progressive niche specialisation. It may be that broadly compatible fungi are only still used by orchids that have diverged by other means, such as adaptation to specialised pollinators or extreme environments.

The existence of a fungus with unusually broad compatibility has conservation implications, such as presenting options for investigating rare taxa with saprophytic incompetence problems or where populations of an orchid have decreased to such low numbers that efficacious or ecologically significant fungi are not present (i.e. the more plants the greater the opportunity for highly efficacious fungi to be partnered). It may be possible to use a broadly compatible fungus rather than an orchid’s specific fungus for propagation and restoration if certain features of the orchid’s specific fungus appear to have contributed to the orchid’s decline. A broadly compatible fungus may also be useful for restoration work, particularly in cases where soil conditions have been altered, as a broadly compatible fungus may be more tolerant of extreme conditions than a more narrowly specialised fungus. A range of ethical and conservation issues must then be considered in relation to the use of a fungus for reintroduction work that may not be native to the area.
Chapter 6 – Mycorrhizal specificity in subtribe Drakaeinae

6.1 – Introduction

6.1.1 – The relationship between genetic variation in orchids and fungi
As discussed in Chapter 3, there have been a number of genetic studies on the genetic variation and phylogeny of orchid species (Kores et al., 2001; Chase et al., 2003; Fay & Krauss, 2003) and their mycorrhizal fungi (Section 1.4.2). However, questions still remain about the relationship between orchid genetic variation and phylogeny and that of the mycorrhizal fungi. This chapter will consider the genetic variation among mycorrhizal fungi from a number of genera and species from the orchid subtribe Drakaeinae, which has an established phylogeny (Kores et al., 2001), and compare the two.

6.1.2 – The subtribe Drakaeinae
The subtribe Drakaeinae is a small group of closely related genera, and there is general agreement amongst experts on how the genera are related to one another (Figure 6.1; Kores et al., 2001; Chase et al., 2003). The genera (and species) are endemic (to Australia, the southeast, or the southwest), are very specialised, have high levels of intrinsic rarity, inhabit highly individualised habitats, and possess extremely specialised fungi (Ramsay et al., 1986; Hoffman & Brown, 1998). All of these are key points to consider in using the subtribe Drakaeinae. Each genus is quite small, consisting of only one to a few species (Chase et al., 2003; Hoffman & Brown, 1998), so the subtribe provides a good model for studying relationships within and between genera.
Figure 6.1 – Tree showing relationships of genera within Drakaeinae, and of Drakaeinae to nearest related subtribes, based on combined matK and trnL-F sequences (adapted from Kores et al., 2001).

Some members of the subtribe Drakaeinae (Drakaea, Paracaleana and Spiculaea) are known to have very unusual and culturally distinctive mycorrhizal fungi which are multinucleate (rather than the binucleate fungi common in Australian terrestrial orchids) and have intense pink pigmentation (Ramsay et al., 1986). These genera are wasp pollinated and occupy specialised niches which they share with few other orchids (Ramsay et al., 1986). Seedlings of Drakaea species can be found germinating under adults of different species or under Paracaleana species, and vice versa (K Dixon, pers. comm.). It is therefore likely that the mycorrhizal fungi of this group are related, as it is probable that a seedling found under an adult orchid is utilising the same fungus as the adult plant; the fungi are furthermore easily recognisable and easy to study. It is also possible that orchids from these three genera share similar or related fungi which are interchangeable, or use the same fungus, or can use each others’ fungi. Thus this group is ideal for studying mycorrhizal specificity.
Drakaea (the focus of this study) has only nine currently recognised species (Hoffman & Brown, 1998), all of which are strict endemics to southwest Western Australia. All Drakaea species are highly specialised in their habitat (particularly soil type) requirements, and some have an extremely narrow range (Hoffman & Brown, 1998). It has been suggested that the orchids’ distribution and rarity may be due to fungal habitat preferences (K Dixon, pers. comm.). Drakaea species are all single flowered and have small seed capsules, and the plants are sparsely distributed (Hoffman & Brown, 1998). Pollination is by sexual deception of male thynnid wasps (Adams & Lawson, 1993; Hoffman & Brown, 1998), which only occurs under specific weather conditions (K Dixon, pers. comm.).

Paracaleana species have extremely small capsules, and are wasp pollinated and widespread but low in abundance (Adams & Lawson, 1993; Hoffman & Brown, 1998). It is common to find populations containing two or more Drakaea species, and Paracaleana species are often present as well (Hoffman & Brown, 1998). Very few other orchid groups are found in the specialised niches of Drakaea and Paracaleana species, and even fewer in the moss swards of granite outcrops which are inhabited by Spiculaea ciliata (Ramsay et al., 1986; Hoffman & Brown, 1998).

Four of the most common and widespread Drakaea species are D. livida, D. glyptodon, D. gracilis and D. thynniphila, while the most common and widespread Paracaleana species is P. nigrita. These five species are illustrated in Figure 6.2, and the habitats of Drakaea livida and Spiculaea ciliata are shown in Figure 6.3.

Other genera included in the subtribe Drakaeinae include Caleana, Chiloglottis, Spiculaea, Lyperanthus, Pyrorchis, and Leporella. Of these genera, Caleana and Chiloglottis are the closest relatives of Paracaleana and Drakaea respectively (Figure 6.1), but are confined to southeastern Australia, and are therefore useful in this study as non-sympatric relatives of the focus genera which are believed to possibly use similar fungi (Ramsay et al., 1986; Hoffman & Brown, 1998). Spiculaea ciliata is, perhaps, the most specialised of the Drakaeinae, being confined to the moss swards of granite outcrops, is endemic to southwestern Australia, and shares the distinctive fungal group possessed by Drakaea and Paracaleana (Ramsay et al., 1986; Hoffman & Brown, 1998).
Lyperanthus, Pyrorchis and Leporella are more abundant and widespread than Drakaea; Lyperanthus serratus often occurs in the woodland surrounding the sand lenses occupied by Drakaea species, as do Pyrorchis nigricans and Leporella fimbirata, which are often the only orchid species to occur in any abundance within Drakaea habitat (Ramsay et al., 1986; Hoffman & Brown, 1998). These three species, while possessing a different type of mycorrhizal fungus to the focus genera, are useful as related species displaying varying degrees of sympatry. The remaining species studied here, Arthrochilus byrnesii, is a member of the closely related subtribe Thelymitrinae, which is found in northern Australia.

Figure 6.2 – A: Drakaea livida (L), Drakaea glyptodon (R). B: Drakaea gracilis. C: Drakaea thynniphila. D: Paracaleana nigrita. (Photographs: R Heberle)
Figure 6.3 – Habitats of *Drakaea livida* and *Spiculaea ciliata*. A: *Eucalyptus marginata* woodland. B: Lateritic soil of *E. marginata* woodland with relatively dense vegetation. C: Sand lens within lateritic *E. marginata* woodland, displaying sparse vegetation. D: Sandy soil of sand lens with *Drakaea livida* in flower (red arrows) and *Leporella fimbriata* leaves (blue arrows). E: Moss sward of seasonally moist granite outcrop. F: *Spiculaea ciliata* in bud (red arrow) in moss sward. (Photographs: P Hollick).
6.1.3 - Objectives

The objective of this chapter was to investigate fungal specificity and genetic variation in the subtribe Drakaeinae.

Hypothesis: Fungal specificity and genetic variation mirrors species phylogeny in a highly specialised, narrowly endemic orchid group (the subtribe Drakaeinae).

6.2 – Materials and Methods

6.2.1 – Seed and inoculum sources, with site descriptions

The locations of the sites from which seed and inoculum of southwestern Drakaeinae species were collected are shown in Figure 6.4. Inoculum of other species was sourced from the Kimberley region of northern Western Australia, and from Victoria in southeastern Australia. A summary of the seed and inoculum sources for each species is given in Table 6.1.

Figure 6.4 – Collection locations for seed and inoculum of southwest Western Australian species of Drakaeinae. For explanation of location codes, see text (site descriptions) and Table 6.1.
BH – Brookton Highway

*Drakaea glyptodon, Leporella fimbriata*

The Brookton Highway site is about 50 km east of Perth, in the Darling Range, and consists of a revegetated sand extraction pit within *Eucalyptus marginata* woodland. The borders of the sand pit possess sandy soil, while the surrounding woodland is generally lateritic. The sandy borders of the pit are populated by *Drakaea glyptodon*, while a rotted, half-buried log in the woodland strip between the highway and the pit is populated by about 50 plants of *Drakaea glyptodon* and *Paracaleana nigrita*, which is an unusually dense population for these species. Adult plants of *Drakaea glyptodon* and *Paracaleana nigrita* were collected in September of 2000 and 2002, and their mycorrhizal fungi isolated (Chapter 2). Flowering plants of both species were hand pollinated and tagged, and the seed capsules collected three or four weeks later (Chapter 2) in 2000 and 2002. Two adult plants of *Leporella fimbriata* were collected in September 2002 and their mycorrhizal fungi isolated.

BJR – Baker’s Junction Reserve

*Drakaea glyptodon, Paracaleana nigrita*

Baker’s Junction Reserve is near the south coast of Western Australia, east of Albany, and consists of relatively unmodified jarrah-marri (*Eucalyptus marginata-Corymbia calophylla*) forest on a sandy soil. Adult plants of *Drakaea glyptodon* and *Paracaleana nigrita* were collected in October 2000 and their mycorrhizal fungi isolated (Chapter 2).

C – Collie

*Pyrorchis nigricans*

The Collie site is located approximately 200 km south of Perth, near the Alcoa minesite, and consists of undisturbed jarrah (*Eucalyptus marginata*) forest on a lateritic soil. Three adult plants of *Pyrorchis nigricans* were collected in August 2002 and their mycorrhizal fungi isolated (Chapter 2).
CMR – Canning Mills Road

*Drakaea livida, Lyperanthus serratus, Leporella fimbriata*

The Canning Mills Road site is about 30 km east of Perth, in the Darling Range, in *Eucalyptus marginata* woodland. The soil is generally lateritic, but the study site is in a slight hollow and has a top layer of sand. The sand lens is populated by *Drakaea livida*, while *Leporella fimbriata* occurs both within and outside the sand lens. *Lyperanthus serratus* can be found in the surrounding area. Adult plants of *Drakaea livida* were collected in August or September of 2000 and 2002, and their mycorrhizal fungi isolated (Chapter 2). Flowering plants were hand pollinated and tagged, and the seed capsules collected three or four weeks later (Chapter 2) in 2000 and 2002. Two adult plants each of *Leporella fimbriata* and *Lyperanthus serratus* were collected in September 2002 and their mycorrhizal fungi isolated.

CR – Caves Road

*Drakaea thynniphila*

The Caves Road site is about 300 km south of Perth, to the west of Margaret River, and consists of substantially modified karri (*Eucalyptus diversicolor*) forest on a road reserve. An adult plant of *Drakaea thynniphila* was collected in October 2001 and its mycorrhizal fungi isolated (Chapter 2).

D – Darkan

*Paracaleana nigrita*

The Darkan site is located approximately 200 km southeast of Perth, and consists of substantially modified *Melaleuca uncinata* woodland on a clay-loam soil. The site is on private property and has been used for grazing. The rare orchid *Drakaea confluens* is found in the area, as is *Paracaleana nigrita*. An adult plant of *Paracaleana nigrita* were collected in September 2001 and its mycorrhizal fungi isolated (Chapter 2).

FR – Frosty Road

*Drakaea livida, Paracaleana nigrita*

The Frosty Road site is about 300 km southeast of Perth, off Muir Highway between Manjimup and Mount Barker, and consists of relatively unmodified karri (*Eucalyptus diversicolor*) forest. Adult plants of *Drakaea livida* and *Paracaleana nigrita* were collected in October 2000, and their mycorrhizal fungi isolated (Chapter 2).
KP – Kings Park and Botanic Garden

*Pyrorchis nigricans*

Kings Park and Botanic Garden is a 400 hectare urban bushland remnant in the centre of Perth, overlooking the Swan River estuary. Most of the park consists of substantially modified *Eucalyptus-Banksia-Allocaasuarina* woodland. The limestone scarp overlooking the river has recently undergone substantial revegetation and ecological restoration work, although some parts, further from the city centre, remain relatively undisturbed. Despite the urban nature of the park, fifty orchid species have been recorded in the area. *Pyrorchis nigricans* is one of the most common of these, occurring in large clonal colonies throughout much of the park. Two adult plants were collected in August 2002 and their mycorrhizal fungi isolated (Chapter 2).

RLR – Red Lake Road

*Drakaea glyptodon*

The Red Lake Road site is about 300 km southeast of Perth, off Muir Highway between Manjimup and Mount Barker, and consists of relatively unmodified jarrah-marri (*Eucalyptus marginata-Corymbia calophylla*) forest on a sandy soil. A number of different *Drakaea* species are present in the area, and hybridisation is common. An adult plant which could be confidently identified in the field as *Drakaea glyptodon* was collected in October 2000 and its mycorrhizal fungi isolated (Chapter 2).

W – Warrick

*Pyrorchis nigricans*

Warrick is in the northern suburbs of Perth, approximately 20 km north of Kings Park and Botanic Garden. The site consists of substantially modified *Eucalyptus-Banksia-Allocaasuarina* woodland, which is heavily used for recreational purposes, but a number of orchid species are found in the area. Three adult plants of *Pyrorchis nigricans* were collected in August 2002 and their mycorrhizal fungi isolated (Chapter 2).

WB – William Bay

*Drakaea glyptodon, Drakaea thynniphila, Paracaleana nigrita*

The William Bay site is near the south coast of Western Australia, on the corner of the South Coast Highway and William Bay Road west of Denmark, and consists of
relatively undisturbed karri (*Eucalyptus diversicolor*) forest on private property. The firebreaks of the property are densely populated by *Drakaea glyptodon*, *Drakaea thynniphila* and *Paracaleana nigrita*. Adult plants of all three species were collected in October 2000 and their mycorrhizal fungi isolated (Chapter 2). Flowering plants of all three species were hand pollinated and tagged, and the seed capsules collected three or four weeks later (Chapter 2).

**WCH – West Cape Howe**

*Drakaea thynniphila*

The West Cape Howe site is located on the south coast of Western Australia, between Albany and Denmark, and consists of coastal kwongan heath on a sandy soil. A large population of *Drakaea thynniphila* is present beside a walking trail. Two adult plants were collected in October 2000 and their mycorrhizal fungi isolated (Chapter 2). Flowering plants which had already set seed were also collected for seed (Chapter 2).

**WR – Warradale Road**

*Drakaea glyptodon, Drakaea gracilis*

The Warradale Road site is about 100 km east of Perth close to Brookton Highway, in relatively undisturbed *Eucalyptus wandoo* woodland on a clay-loam soil. The area is known for its abundance and diversity of orchid species. There is a mixed population of *Drakaea glyptodon* and *Drakaea gracilis*, with *Drakaea gracilis* the more abundant species. Two adult plants of each species were collected in September 2000 and their mycorrhizal fungi isolated (Chapter 2).

**Y – York**

*Spiculaea ciliata*

The York site is approximately 100 km east of Perth, about 10 km west of York, beyond the eastern edge of the jarrah (*Eucalyptus marginata*) forest. The site consists of relatively undisturbed york gum-jam (*Eucalyptus loxophleba-Acacia acuminata*) woodland on a clay-loam (eroded laterite) soil in a nature reserve. The area is rich in orchids. A granite outcrop possesses a large population of *Spiculaea ciliata*. Three adult plants were collected in October 2001 and their mycorrhizal fungi isolated (Chapter 2). Flowering plants were hand pollinated and tagged, and the seed capsules collected three or four weeks later (Chapter 2).
Kimberleys – northern Western Australia

*Arthrochilus byrnesii*

The Kimberley region of northern Western Australia is a subtropical area with alternating dry (winter) and wet (summer monsoon/cyclonic) seasons. A number of orchids are found in this area (12 terrestrial and one epiphytic species), including the terrestrial species *Arthrochilus byrnesii*. A fungal isolate from *Arthrochilus byrnesii* which had been collected from Mount Bomford, approximately 400 km north-north-east of Derby, was supplied by Nura Abdul Karim.

Victoria – southeastern Australia

*Caleana major, Chiloglottis trapeziformis, Chiloglottis valida*

Victoria, in southeastern Australia, has a cooler, wetter climate than Western Australia. In the winter and spring of 2002, adult plants of *Caleana major* (one plant from Dereel, called V1), *Chiloglottis trapeziformis* (one plant from each of Enfield State Forest and Inverleigh, called V2 and V3 respectively) and *Chiloglottis valida* (one plant from each of Big River and Sylvan, called V4 and V5 respectively) were supplied from Victoria and their mycorrhizal fungi isolated (Chapter 2).
Table 6.1 – Sources and collection locations for seed and inoculum of orchid species from subtribe Drakaeinae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection location</th>
<th>No of adult plants from which fungi isolated</th>
<th>Seed collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drakaea glyptodon Fitzg.</td>
<td>Brookton Highway (BH)</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Warradale Road (WR)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Red Lake Road (RLR)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>William Bay (WB)</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Baker’s Junction Reserve (BJR)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Drakaea gracilis Hopper &amp; AP Brown</td>
<td>Warradale Road (WR)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Drakaea livida J Drummond</td>
<td>Canning Mills Road (CMR)</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Frosty Road (FR)</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Drakaea thynniphila AS George</td>
<td>Caves Road (CR)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>William Bay (WB)</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>West Cape Howe (WCH)</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Leporella fimbriata (Lindley) AS George</td>
<td>Canning Mills Road (CMR)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Brookton Highway (BH)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lyperanthus serratus Lindley</td>
<td>Canning Mills Road (CMR)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Paracaleana nigrita (J Drummond ex Lindley) Blaxell</td>
<td>Darkan (D)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Frosty Road (FR)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>William Bay (WB)</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Baker’s Junction Reserve (BJR)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Pyrorchis nigricans (R. Br.) DL Jones &amp; MA Clem.</td>
<td>Warrick (W)</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kings Park and Botanic Gardens (KP)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Collie (C)</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Spiculaea ciliata Lindley</td>
<td>York (Y)</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Arthrochilus byrnesii Blaxell</td>
<td>Kimberleys (K)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Caleana major R. Br.</td>
<td>Victoria – one location (V1)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Chiloglottis trapeziformis Fitzg.</td>
<td>Victoria – two locations (V2-3)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Chiloglottis valida D. L. Jones</td>
<td>Victoria – two locations (V4-5)</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

A list of the fungal isolates used in this study, with their source species and locations and the experiments for which they were used, is given in Table 6.2.
Table 6.2 – Fungal isolates from orchids of subtribe Drakaeinae, and the experiments for which they were used.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Collection location</th>
<th>Plant number</th>
<th>Isolate number</th>
<th>Symbiotic germination experiment number *</th>
<th>AFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drakaea glyptodon</td>
<td>BH</td>
<td>1</td>
<td>b +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>b +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>d +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WR</td>
<td>1</td>
<td>a +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a +</td>
<td>+</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>c +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RLR</td>
<td>1</td>
<td>a +</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>c +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WB</td>
<td>2</td>
<td>a +</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td>b +</td>
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<tr>
<td></td>
<td>BJR</td>
<td>1</td>
<td>a +</td>
<td>+</td>
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<td></td>
<td></td>
<td>b +</td>
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<td></td>
<td></td>
<td></td>
<td>c +</td>
<td></td>
<td></td>
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<tr>
<td>Drakaea gracilis</td>
<td>WR</td>
<td>1</td>
<td>a +</td>
<td>+</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>b +</td>
<td></td>
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<td>c +</td>
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<td>b +</td>
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<td></td>
<td></td>
<td></td>
<td>c +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drakaea livida</td>
<td>CMR</td>
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<td>a +</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td>b +</td>
<td></td>
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<td></td>
<td>4</td>
<td>d +</td>
<td>+</td>
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<td>b +</td>
<td>+</td>
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<td></td>
<td>c +</td>
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<td>+</td>
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<td></td>
<td></td>
<td></td>
<td>b +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drakaea thynniphila</td>
<td>CR</td>
<td>1</td>
<td>a +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WB</td>
<td>1</td>
<td>a +</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>a +</td>
<td>+</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>b +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WCH</td>
<td>1</td>
<td>c +</td>
<td>+</td>
<td></td>
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<td></td>
<td></td>
<td>2</td>
<td>b +</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>c +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leporella fimbriata</td>
<td>CMR</td>
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<td>b +</td>
<td>+</td>
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</tr>
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<td>BH</td>
<td>1</td>
<td>a +</td>
<td></td>
<td></td>
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<td>Lyperanthus serratus</td>
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<td>c +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paracaleana nigrita</td>
<td>D</td>
<td>1</td>
<td>a +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b +</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>c +</td>
<td></td>
<td></td>
</tr>
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<td></td>
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<td>b +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WB</td>
<td>1</td>
<td>a +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>a +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>b +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BJR</td>
<td>1</td>
<td>b +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrorchis nigricans</td>
<td>W</td>
<td>2</td>
<td>a +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>b +</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### 6.2.2 – Symbiotic germination

Symbiotic seed germination was carried out according to the methods described in Chapter 2. Two categories of experiments were performed: firstly, species-specific symbiotic germination experiments involving one species of seed and one fungal source species (Experiment No 1 in Table 6.2); and secondly, symbiotic cross-species germination experiments involving more than one species of seed and more than one fungal source species (Experiment No 2 in Table 6.2). The fungal isolates involved in each experiment are listed in Table 6.2. Germination was scored to and beyond stage 3 and to and beyond stage 5 (Figure 2.3), as a percentage of viable seed (i.e. stage 0 (Figure 2.3) was not counted) at 16 weeks after sowing. The percentage of nonviable seed differed between but not within the seed types.

Two species-specific symbiotic germination experiments were carried out with *Spiculaea ciliata*. The first experiment involved two fungal isolates from each of three plants; in the second experiment, some of these isolates were reused and some new
isolates from the same plants were also used. The seeds were sterilised for 30 minutes and spread directly on the pre-inoculated agar. Three replicates were used, and an asymbiotic control. Following the second experiment, some of the symbiotic seedlings from each fungal isolate were surface-sterilised in 3 % H2O2 and plated out onto sterile oatmeal agar. The resulting fungal cultures were used for AFLP to compare pre- and post-germination isolates. Some seedlings were hand sectioned and examined under natural light and UV fluorescence to determine which areas of the protocorms were photosynthetic and which contained pelotons.

A symbiotic cross-germination experiment was performed using seeds of *Drakaea glyptodon*, *D. livida*, *D. thynniphila* and *Paracaleana nigrita* on fungal isolates from a number of different source species (Table 6.2). The seeds were sterilised for 5 minutes and all seed types tested against each fungal isolate were placed in the same petri dish, on separate pieces of filter paper. Three replicates were used, and an asymbiotic control. Purified agar (Sigma) (Appendix 2) with oatmeal was used.

**6.2.3 – Statistics**

Fungal growth rates and seed germination were statistically analysed by ANOVA. Fisher’s PLSD with a 95% confidence interval was then applied to arcsine-transformed data to make comparisons between means. Analysis was performed using the statistical program Statview® (Statview, SAS Institute Inc.).

**6.2.4 – DNA extraction and amplified fragment length polymorphism (AFLP)**

DNA extraction and AFLP were carried out according to the methods described in Chapter 2 on the fungal isolates listed as being used for AFLP in Table 6.2. A principal coordinates analysis (PCA) based on genetic distance (Euclidean distance) was performed on each set of AFLP fingerprints to illustrate the pattern of genetic variation within and between fungal source species, using the genetic analysis program GenAlEx (Peakall & Smouse, 2001).
6.3 – Results

6.3.1 – Symbiotic germination

**Experiment 1 - *Spiculaea ciliata***

The *Spiculaea ciliata* seeds germinated to or beyond stage 3 on all fungal isolates in both experiments (Table 6.5). Of the four cases where a fungal isolate was used in both experiments, two produced almost identical germination rates (isolates 1a and 1b) while the other two produced statistically significantly higher germination in the second experiment (isolates 3b and 3f; $P < 0.0001$) (Table 6.5). There was no germination under asymbiotic conditions (Table 6.5). Although the germination results shown here were scored at 12 weeks after seed sowing, it was noted that the maximum levels of germination were reached very early, and percent germination (to or beyond stage 3 and stage 5) altered very little after four weeks (results not shown).

Development to or beyond stage 5 occurred to a level statistically significantly higher than the zero germination of the asymbiotic controls on all isolates except 1a, 1b, 2a, and 2f, all in the first experiment (Table 6.5). Two of these isolates (1a and 1b) were also used in the second experiment, where they produced significant germination to or beyond stage 5 (Table 6.5). All four of the repeated fungal isolates produced statistically significantly higher germination to or beyond stage 5 in the second experiment than in the first ($P < 0.05$) (Table 6.5). Over all fungal isolates, germination was statistically significantly higher in the second experiment than in the first ($P < 0.05$) (Table 6.5).
Table 6.5 – Symbiotic germination of *Spiculaea ciliata* seeds at 12 weeks after sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Plant n°</th>
<th>Isolate n°</th>
<th>Experiment n°</th>
<th>% to or beyond stage 3 (SE)</th>
<th>% to or beyond stage 5 (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic</td>
<td>1</td>
<td>a</td>
<td>1</td>
<td>71.9 (3.99) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>b</td>
<td>2</td>
<td>71.9 (2.05) *</td>
<td>35.5 (1.59) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c</td>
<td>2</td>
<td>64.2 (3.53) *</td>
<td>0.2 (0.22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d</td>
<td>2</td>
<td>65.2 (6.78) *</td>
<td>7.4 (1.29) *</td>
</tr>
<tr>
<td><em>Spiculaea ciliata</em></td>
<td>1</td>
<td>e</td>
<td>2</td>
<td>55.6 (6.81) *</td>
<td>9.1 (1.14) *</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>f</td>
<td>2</td>
<td>45.3 (1.37) *</td>
<td>3.5 (0.49) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>1</td>
<td>82.5 (3.26) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e</td>
<td>2</td>
<td>64.0 (7.29) *</td>
<td>19.9 (3.89) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>f</td>
<td>1</td>
<td>75.7 (15.64) *</td>
<td>2.3 (2.06)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
<td>1</td>
<td>37.5 (1.62) *</td>
<td>29.0 (0.66) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d</td>
<td>2</td>
<td>77.5 (1.44) *</td>
<td>46.7 (4.66) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>f</td>
<td>1</td>
<td>90.6 (3.05) *</td>
<td>53.4 (7.59) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>38.0 (0.96) *</td>
<td>34.3 (0.49) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>83.8 (4.43) *</td>
<td>47.5 (1.81) *</td>
</tr>
</tbody>
</table>

*Germination is statistically significantly higher than zero (P < 0.0001)*

It was observed that the *Spiculaea ciliata* seedlings at or beyond stage 5 had green (photosynthetic) protocorms as well as green shoots (Figure 6.5 A). Some seedlings were therefore hand sectioned and examined under natural light and UV fluorescence to determine which areas of the protocorms were photosynthetic and which contained pelotons (Figure 6.5 B-F). The upper part of the protocorm (closest to the shoot) was entirely photosynthetic (Figure 6.5 B, F), a transitional zone had photosynthetic tissue under the epidermis and around the vascular tissue (Figure 6.5 C, E, F), and the lower part of the protocorm was nonphotosynthetic (Figure 6.5 B, D, F). The nonphotosynthetic parts of the cortex were infected by the mycorrhizal fungus and contained many pelotons (Figure 6.5 B, D-F).
Figure 6.5 – Symbiotic seedlings of *Spiculaea ciliata*. A: Entire seedlings showing photosynthetic protocorms (white arrow) and pink colouration from mycorrhizal infection (yellow arrow). B-F: hand sections. B: Protocorm showing photosynthetic areas (green areas – white arrows) and pelotons (dark or pink areas within cortical cells – yellow arrows). C: Protocorm showing trichome (red arrow) and photosynthetic layers (green areas – white arrows) under epidermis and around vascular tissue. D: Protocorm showing trichome (red arrow), photosynthetic layers (green areas – white arrows), pelotons (dark or pink areas within cortical cells – yellow arrows) and dropper (tuber stalk) forming internally (blue arrow). E-F: Protocorms under UV fluorescence showing photosynthetic layers (red areas – white arrows) under epidermis and around vascular tissue, and pelotons (pale areas – yellow arrows) in cortex. Scale bars = 1 mm (approx.). (Photographs: P Hollick).
Experiment 2 - Drakaea and Paracaleana

All four seed types germinated to or beyond stage 3 on at least some fungal isolates (Table 6.6). The Drakaea thynniphila seeds germinated on all of the fungal isolates and on the asymbiotic plates as well (Table 6.6). The only germination that was statistically significantly higher than on the asymbiotic plates was of the D. livida and D. thynniphila seeds and was produced by the fungal isolate from D. thynniphila (Table 6.6). In contrast to the Spiculaea ciliata seedlings produced in Experiment 1, germination and development of the Drakaea and Paracaleana seedlings was relatively slow, and it was noted that the percent germination (to and beyond stage 3 and stage 5) was still increasing at 16 weeks from seed sowing (results not shown).

All of the fungal isolates germinated seeds of species other than their source species, and some did so even when their source species did not germinate (isolates D. glyptodon WB2b and P. nigrita BJR1b and FR2b) (Table 6.6). Four isolates germinated all four seed types (isolates D. livida FR1b, D. glyptodon WR1a and BJR1a, and D. thynniphila WCH2c) and two others germinated three seed types (isolates D. livida CMR4d and P. nigrita BJR1b) (Table 6.6). Four fungal isolates supported levels of germination statistically significantly higher than the asymbiotic plates across all seed types (isolates D. livida FR1b, D. glyptodon BJR1a, D. thynniphila WCH2c and P. nigrita BJR1b) (Table 6.6).

The D. thynniphila seeds displayed the highest germination across all fungal isolates, and the P. nigrita seeds the lowest (Table 6.6). The Victorian fungal isolates from Chiloglottis valida, Chiloglottis trapeziformis and Caleana major only germinated D. thynniphila, and never to a level significantly higher than the asymbiotic plates (Table 6.6).
Table 6.6 – Symbiotic germination of *Drakaea* and *Paracaleana* seeds to or beyond stage 3 (SE) on fungal isolates from subtribe Drakaeinae at 16 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Location &amp; isolate n° ¹</th>
<th>Seed type ²</th>
<th>D. glyptodon ³</th>
<th>D. livida ³</th>
<th>D. thynniphila ³</th>
<th>P. nigrita ³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic ⁴</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.8 (0.26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Drakaea gracilis</em></td>
<td>WR2b⁴</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.0 (0.22)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CMR4d⁵</td>
<td>0 (0)</td>
<td>0.5 (0.49)</td>
<td>1.1 (0.54)</td>
<td>1.6 (1.32)</td>
<td></td>
</tr>
<tr>
<td><em>Drakaea glyptodon</em></td>
<td>WR1a⁴</td>
<td>1.0 (0.72)</td>
<td>0.9 (0.09)</td>
<td>1.4 (0.57)</td>
<td>0.2 (0.22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BJR1a⁶</td>
<td>4.3 (1.09)</td>
<td>1.5 (0.69)</td>
<td>2.6 (0.69)</td>
<td>5.5 (5.14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WB2b⁴</td>
<td>0 (0)</td>
<td>0.5 (0)</td>
<td>0.4 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Drakaea thynniphila</em></td>
<td>WCH2c⁷</td>
<td>1.4 (0.71)</td>
<td>12.9 (6.92)*</td>
<td>15.7 (7.56)*</td>
<td>0.5 (0.53)</td>
<td></td>
</tr>
<tr>
<td><em>Paracaleana nigrita</em></td>
<td>BJR1b⁸</td>
<td>3.9 (3.13)</td>
<td>9.7 (5.73)</td>
<td>7.8 (4.35)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FR2b⁹</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.4 (0.23)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Chiloglottis valida</em></td>
<td>V5-1c⁴</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2.3 (0.74)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Chiloglottis trapeziformis</em></td>
<td>V2-2b⁵</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.1 (0.02)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V3-2c⁶</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.3 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><em>Caleana major</em></td>
<td>V1-1c⁷</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.0 (0.50)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V1-1d⁸</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4.1 (2.07)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)
  ¹ Fungal isolates with different lower case letters as superscripts support significantly different levels of germination across all seed types (P < 0.05).
  ² Seed types with different capital letters as superscripts displayed different levels of germination across all fungal isolates (P < 0.05).

There was little development to or beyond stage 5 and none on the asymbiotic plates, but all four seed types developed to or beyond stage 5 on at least one fungal isolate (Table 6.7). The only development to or beyond stage 5 that was statistically significantly higher than on the asymbiotic plates was of *D. glyptodon* seeds on isolate *D. livida* FR1b and of *D. thynniphila* seeds on isolate *D. thynniphila* WCH2c (Table 6.7).

Isolate *D. livida* FR1b produced development to or beyond stage 5 of all four seed types, and isolates *D. glyptodon* BJR1a and *D. thynniphila* WCH2c of two seed types (Table 6.7). Isolates *D. livida* FR1b and *D. thynniphila* WCH2c supported statistically significantly higher levels of development to or beyond stage 5 than the asymbiotic plates across all seed types (Table 6.7). There was no significant difference in development to or beyond stage 5 between the seed types across all fungal isolates (Table 6.7). There was no development to or beyond stage 5 of any seed type on the asymbiotic plates or on any of the Victorian fungal isolates from *Chiloglottis valida*, *Chiloglottis trapeziformis* and *Caleana major* (Table 6.7).
Table 6.7 – Symbiotic germination of *Drakaea* and *Paracaleana* seeds to or beyond stage 5 (SE) on fungal isolates from subtribe Drakeniinae at 16 weeks from sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Location &amp; isolate no</th>
<th>D. glyptodon*</th>
<th>D. livida*</th>
<th>D. thynniphila*</th>
<th>P. nigrita*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymbiotic*</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Drakaea gracilis</em></td>
<td>WR2ba</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>CMR4da</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Drakaea livida</em></td>
<td>FR1bc</td>
<td>2.1 (1.81) *</td>
<td>0.2 (0.22)</td>
<td>0.7 (0.42)</td>
<td>0.6 (0.56)</td>
</tr>
<tr>
<td></td>
<td>WR1aa</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>BJR1ab</td>
<td>0.4 (0.43)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.4 (0.44)</td>
</tr>
<tr>
<td><em>Drakaea glyptodon</em></td>
<td>WB2ha</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.4 (0.40)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Drakaea thynniphila</em></td>
<td>WCH2c</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2.0 (1.40) *</td>
<td>0.5 (0.53)</td>
</tr>
<tr>
<td><em>Paracaleana nigrita</em></td>
<td>BJR1b</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>FR2b</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Chiloglottis valida</em></td>
<td>V5-1c</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Chiloglottis</em></td>
<td>V2-2b</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>trapeziformis</em></td>
<td>V3-2c</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>Caleana major</em></td>
<td>V1-1c</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>V1-1d</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.05)

1 Fungal isolates with different lower case letters as superscripts support significantly different levels of germination across all seed types (P < 0.05).

2 Seed types with different capital letters as superscripts displayed different levels of germination across all fungal isolates (P < 0.05).

### 6.3.2 – Amplified fragment length polymorphism (AFLP)

**Spiculaea ciliata**

Of the 218 markers scored, 99 % were polymorphic. Of the total variation 75 % was partitioned within populations and 25 % among populations. The Principal Coordinates Analysis (PCA) of the AFLP fingerprints of the fungal isolates from *Spiculaea ciliata* grouped the fungal isolates according to isolation from adult plants or laboratory-germinated protocorms (Figure 6.6). The two groups of isolates did not overlap (Figure 6.6). For two isolates, two analyses were done, one before use for germination and the second after reisolation from protocorms. In each case, the PCA distinguished between the adult and protocorm isolates (Figure 6.6). Within each of the two groups (adult or protocorm origin), the fungal isolates were grouped according to plant number (Figure 6.6).
The subtribe Drakaeinae

All of the 245 markers scored were polymorphic. Of the total variation of the fungal isolates from *Drakaea, Paracaleana* and *Spiculaea*, 92% was partitioned within fungal source species and 8% among. The Principal Coordinates Analysis (PCA) of the AFLP fingerprints of the fungal isolates from *Drakaea, Paracaleana* and *Spiculaea* revealed very little genetic differentiation between fungal source species (Figure 6.7). All of the groups of isolates overlapped to some extent (Figure 6.7). The fungal isolates from *Drakaea glyptodon* contained more genetic variation than all of the other *Drakaea* and *Paracaleana* fungal isolates put together, which were mostly subsets of the variation of *D. glyptodon* (Figure 6.7). The fungal isolates from *Paracaleana nigrita* were a subset of those of *D. thynniphila*; the isolates from *D. gracilis* were genetically distinct from those of *P. nigrita* and *D. glyptodon*, while those from *D. livida* overlapped with all three (Figure 6.7). The fungal isolates of *Spiculaea ciliata* were moderately differentiated from those of the other species (Figure 6.7).
When the fungal isolates from the other Drakaeinae species (excluding *Drakaea*, *Paracaleana* and *Spiculaea*) were considered, 74 % of the total variation was partitioned within fungal source species, and 26 % among. There was slightly more genetic differentiation between the fungal isolates from the other Drakaeinae species (excluding *Drakaea*, *Paracaleana* and *Spiculaea*), as demonstrated by the PCA of the AFLP fingerprints (Figure 6.8). All of the groups of isolates except that from *Arthrochilus byrnesii* (which originated from the Kimberleys) overlapped to some extent (Figure 6.8). The three species from Victoria (*Caleana major*, *Chiloglottis valida* and *Chiloglottis trapeziformis*) possessed fungal isolates which were genetically distinct from each other but all overlapped with isolates from at least one Western Australian species (*Pyrorchis nigricans*, *Lyperanthus serratus* and *Leporella fimbriata*), and vice versa (Figure 6.8).
Over all of the fungal isolates from Drakaeinae species, 84% of the total variation was partitioned within fungal source species, and 16% among. The overall PCA of the AFLP fingerprints of all of the fungal isolates from Drakaeinae species demonstrated a lack of genetic differentiation between source species, with all of the groups of isolates overlapping with at least one other group (Figure 6.9). All four of the species which were not from southwest Western Australia (Arthrochilus byrnesii, Caleana major, Chiloglottis valida and Chiloglottis trapeziformis) possessed fungal isolates whose genetic variation was a subset of that of the fungal isolates of Drakaea glyptodon (Figure 6.9). The only species to possess fungal isolates which were substantially different to those of D. glyptodon were Spiculaea ciliata and Pyrorchis nigricans, both of which were southwestern (Figure 6.9).

The genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between these fungal isolates from Drakaeinae species was lowest between isolates from Drakaea, Paracaleana and Spiculaea species, slightly higher between isolates from these species and isolates from Lyperanthus, Leporella and Chiloglottis species, and highest between all of the preceding isolates and those from Caleana major, Pyrorchis nigricans and Arthrochilus byrnesii (Appendix 3).
An analysis of the Drakaeinae fungal isolates according to location revealed a similar lack of genetic differentiation between locations as there was between species (results not shown).

![Principal Coordinates Analysis of AFLP fingerprints of fungal isolates from Drakaeinae species.](image)

There was insufficient differentiation between the fungal isolates of the different orchid species to construct a phylogenetic tree showing the relationships of the fungal isolates of Drakaeinae species.

### 6.4 – Discussion

The germination of the *Spiculaea ciliata* seeds was very interesting for a number of reasons. Firstly, this species demonstrated very rapid germination and development, reaching maximum germination as early as four weeks from seed sowing. This is in marked contrast to the *Caladenia* species studied in Chapters 3-5, which achieved maximum germination at 8-12 weeks, and in particular to the other Drakaeinae species studied in this chapter, which achieved maximum germination later than 16 weeks.

Secondly, the protocorms, as well as the shoots, were photosynthetic, which is rarely recorded for herbaceous terrestrial orchids (K Dixon, pers. comm.), with the
photosynthetic zones occurring under the epidermis and around the vascular tissue in the upper part of the protocorm (Figure 6.5). It is well known that epiphytic orchids can form chloroplasts in their protocorms and become fully photosynthetic within days of germination (Johansen & Rasmussen, 1992; Ramsay & Dixon, 2003), but terrestrial orchids are considered to take much longer to become photosynthetic as they usually can only form chloroplasts in their shoots.

Both of these features may be a result of the highly specialised niche occupied by *Spiculaea ciliata*, which is confined to the moss swards of seasonally moist granite outcrops (Ramsay et al., 1986; Hoffman & Brown, 1998) (Figure 6.3 E-F). This environment is extremely poor in nutrients and organic matter, is subject to rapid and frequent fluctuations in moisture content during a very short growing season, and dries out very early in spring. Under such harsh conditions, it would obviously be advantageous to an orchid species to be able to germinate and develop rapidly in order to form a dormant tuber before the early onset of the summer drought, and to maximise the photosynthetic ability of its seedlings in order to minimise fungal dependence.

A third unique feature of the *Spiculaea ciliata* germination was the dramatic increase in efficacy between the first and second experiments of the four fungal isolates which were used in both experiments (Table 6.5). All four of the repeated fungal isolates produced significantly higher germination in the second experiment than in the first, and over all fungal isolates, germination was significantly higher in the second experiment than in the first (Table 6.5). This is the first reported instance of fungal efficacy increasing in culture, although fungi have been known to lose their efficacy over time in culture (Chapter 5; Alexander & Hadley, 1983).

The increase in efficacy of the *Spiculaea ciliata* fungi following use for germination was supported by the genetic analysis, which divided the fungi into those which were isolated from adult plants and those which were reisolated from laboratory germinated protocorms (Figure 6.6). This means that the increase in efficacy following use for germination was accompanied by a measurable genetic change in the fungi. It thus appears that the orchid may be able to influence the fungus’ pliable genetic system to suit its particular needs. With the particular and peculiar habitat requirements of *S.*
ciliata, a highly efficacious fungal partner would be an advantage allowing exploitation of the particularly harsh and specialised niche it occupies.

The existence of, apparently, two distinct forms of the fungus, which may be called the seedling and parental forms, opens up a wide field of questions for speculation and further investigation. The parental form, once isolated from an adult orchid, appears to be converted to the seedling form during use for germination. Can the fungus revert to the parental form after conversion to the seedling form? This would seem to be likely, as other fungi have been observed to lose their efficacy over time in culture (Chapter 5; Alexander & Hadley, 1983). Is the loss of efficacy in fungi accompanied by a similar genetic change? Can a similar increase in efficacy occur in other types of fungi, and does this involve a genetic alteration? It may be possible to revitalise fungi which have lost some efficacy in culture by cycling them through seedlings, rather than making new isolations from adult plants. In the case of rare species this may enhance both the survival of the adult plants and the prospects for reintroduction.

The Drakaea and Paracaleana species demonstrated a remarkable lack of specificity in their germination, with D. thynniphila germinating on all fungi and (at a low percent) on the asymbiotic controls (Table 6.6). The highest germination of this species, however, was produced in combination with its own fungus. Of the other species, D. glyptodon also germinated best on one of its own fungi, while D. livida and P. nigrita germinated best on fungi from other species (Table 6.6). Three of the nine Western Australian fungal isolates supported germination of other seed types even when their source species did not germinate, and four isolates germinated all four seed types (and two isolates germinated three of the four). The germination rates were, however, generally very low, which meant a lack of statistical significance for the results. This may be due to the apparently stringent requirements of Drakaea and Paracaleana species with regards to germination conditions (Appendix 2). The germination protocol for these genera is likely to need further refinement.

The lack of specificity demonstrated by the Drakaea and Paracaleana species suggests that these species may be using the same fungi. The mycorrhizal fungi from this group of orchids (including Spiculaea) are known to be very unusual and culturally distinctive, and it has been suggested that cohabiting taxa from this group may share
fungi (Ramsay et al., 1986). It is interesting to note that a majority of the least specific and most efficacious fungal isolates came from locations at which more than one *Drakaea* and/or *Paracaleana* species occurred, which suggests that multiple-species populations may be more likely to possess fungi which are non-specific and highly efficacious. Although it has been noted that some non-Western Australian genera, such as *Caleana* and *Chiloglottis*, possess similar fungi to the *Drakaea* group (Warcup, 1981; Ramsay et al., 1986), in this study the fungal isolates from *Caleana* and *Chiloglottis* species failed to germinate any of the Western Australian species at a higher rate than the asymbiotic controls.

The results of the genetic study supported the idea that *Drakaea* and *Paracaleana* species may utilise the same fungi, as there was very little genetic differentiation between the source species (Figures 6.7 – 6.9). The genetic variation of the fungal isolates from most of the other Drakaeinae species were subsets of the variation of the *Drakaea glyptodon* fungi, with only the fungi of *Spiculaea ciliata* and *Pyrorchis nigricans* moderately genetically differentiated (Figure 6.9). The link between cohabiting taxa hypothesised by Ramsay et al. (1986) is supported by the lack of genetic differentiation between the fungi, and it appears likely that *Drakaea* and *Paracaleana* species may indeed use closely related fungi. It is interesting that the fungal isolates from the Victorian orchids (*Caleana* and *Chiloglottis*), which did not germinate the Western Australian orchid seeds, were genetically undifferentiated from the efficacious Western Australian fungal isolates. It is therefore clear that molecular data does not inform about efficacy and that effective research programs should address both areas.

An analysis of the Drakaeinae fungal isolates according to location revealed a similar lack of genetic differentiation between locations as there was between species (results not shown). There was some evidence of geographic partitioning in *Drakaea glyptodon* and *Paracaleana nigrita*, but this was not related to geographic distance. Thus, similar fungi occurred in the same species at distant locations. This may be related to the ancient landscape of Western Australia, as it would clearly take time for similar fungi to spread to suitable habitats which are widely separated. It is interesting that, considering the disjunct nature of the distributions of the orchids (for example, *Drakaea, Paracaleana* and *Spiculaea* species are rarely contiguous), they should use
genetically undifferentiated fungi within their disjunct, discrete and distant populations. It is possible that there are some habitats where niche occupancy by orchid species depends on the availability of a broad genetic palette of fungi, while other sites possess a narrow genetic palette of fungi (and perhaps therefore also possess a narrow range of orchid species).

While the lack of genetic differentiation between fungi from different locations infers that similar fungi are found at distant locations, it cannot be assumed that an orchid species possesses the same fungus at different sites. This has implications for conservation, restoration, and research, as any reintroduction program must first consider whether a fungus is suitable for a particular location. It is therefore necessary to understand whether habitats can tolerate broader or narrower ranges of fungi, and analyses of genetic variation in fungi from different populations of orchid species are a good start on this issue.

The genetic distance (Nei Genetic Distance, GenAlEx, Peakall & Smouse, 2001) between the fungal isolates from Drakaeinae species was lowest between isolates from Drakaea, Paracaleana and Spiculaea species, slightly higher between isolates from these species and isolates from Lyperanthus, Leporella and Chiloglottis species, and highest between all of the preceding isolates and those from Calanea major, Pyrorchis nigricans and Arthrochilus byrnesii (Appendix 3). The small genetic distance between isolates from Drakaea, Paracaleana and Spiculaea species confirms the previously hypothesised link between the fungi of these taxa (Ramsay et al., 1986), while the different genetic distances between the fungi of these and other taxa did not appear to be related to the genetic or geographic distance between the taxa (Appendix 3).

The genetic distance between isolates from different Drakaea species was lower than that found between isolates from different Caladenia species (more similar to that found between isolates from different populations of the same Caladenia species – Chapters 3 and 5, Appendix 3), and the genetic distance between isolates from orchids belonging to different Drakaeinae genera was also lower than that found elsewhere between isolates from different Caladeniinae genera (more similar to that found between isolates from different Caladenia species – Chapter 4, Appendix 3). This
infers that the overall genetic variation within Drakaeinae fungi is lower than within Caladeniinae fungi.

The patterns of genetic variation among the fungal isolates from Drakaeinae species did not appear to be linked to the interrelationships of the Drakaeinae species themselves (Figure 6.1). The most closely related fungi were those from Drakaea, Paracaleana and Spiculaea, while each of these orchid genera was more closely related to another orchid genus (Drakaea to Chiloglottis and Paracaleana to Caleana – Figure 6.1) which possessed more distantly related fungi. There does not appear to be a geographic element to the fungal genetic variation, either, as some non-Western Australian fungal isolates were relatively closely related to those from the core group of Drakaea, Paracaleana and Spiculaea, while some Western Australian isolates were more distantly related.

The possible reasons for the lack of specificity and genetic differentiation of fungi in Drakaeinae are many, but some conclusions can be drawn from the known facts about these orchids. The fungal isolates from Drakaeinae species, particularly the core group of Drakaea, Paracaleana and Spiculaea, are particularly slow growing (Appendix 2) and may be the least robust of all Australian orchid fungi (K Dixon, pers. comm.). The fungi may, therefore, be restricted to microniches in order to avoid competition, as they might have lower saprophytic competence than other orchid fungi. These possibly habitat-restricted fungi may, however, have a wide distribution due to the extreme age of the Australian landscape. Investigation of the distribution of Drakaeinae fungi within and beyond the habitats of the orchid species (Chapter 7), and of fungal saprophytic competence, would be fruitful areas for future research in attempting to elucidate these issues.

The orchids themselves (Drakaea, Paracaleana and Spiculaea) are highly specific in their habitat requirements (Figure 6.3), and occupy specialised niches which they share with few other orchids (Ramsay et al., 1986). In general, the only other orchid species commonly found in the same areas as Drakaea and Paracaleana species are Pyrorchis nigricans and Leporella fimbriata, both of which also belong to Drakaeinae. In a classic ‘chicken or egg’ question, it is unclear whether it is the fungal distribution that restricts the distribution of the orchids, or vice versa (or neither). The exploitation of
both a unique group of fungi and particular microniche habitats may have given
*Drakaea* and *Paracaleana* species a competitive advantage over other orchid species.

The consistent and considerable localisation of *Drakaea* and *Paracaleana* species
inference that they may be comparatively recent speciants, and they do indeed occupy a
relatively derived position in the phylogeny of Drakaeinae (Figure 6.1). Recent
speciation of *Drakaea* and *Paracaleana* species, and indeed of Drakaeinae as a whole,
may explain the lack of fungal specificity and genetic differentiation. Drakaeinae
species mostly possess highly specialised pollination mechanisms, so adaptation to
different pollinators rather than to fungi may have driven speciation in this group
(Adams & Lawson, 1993).
Chapter 7 – The role of mycorrhiza in microniche specialisation in the narrow endemic *Drakaea*

7.1 – Introduction

7.1.1 – The study of orchid mycorrhizal fungi *ex situ*

Many aspects of the orchid mycorrhizal symbiosis can be studied *ex situ* in the laboratory or glasshouse, but there are limitations in applying the results to the field (Brundrett et al., 2003). The types of *ex situ* work that are vital include fungal isolation, symbiotic seed germination (to confirm efficacy of fungi), and the production of plants for reintroduction. For reintroduction work, knowledge of the distribution of appropriate mycorrhizal fungi is essential. Techniques to elucidate this include *in situ* and *ex situ* seed baiting, each of which has its own advantages and disadvantages (Brundrett et al., 2003). *In situ* seed baiting will be discussed in Chapter 8.

*Ex situ* seed baiting involves taking a soil sample, consolidating the organic matter from the sample (the primary source of fungal material), and baiting with orchid seeds under laboratory conditions (Brundrett et al., 2003). This innovative new method was developed by Brundrett et al. (2003), who found that the coarse organic matter (>2mm) from the litter layer and topsoil was the most important reservoir of fungal inoculum. *Ex situ* seed baiting can efficiently survey a wider area than *in situ* baiting, as many samples can be taken from over a wide area and each tested under a micro-sampling array system (Brundrett et al., 2003). It also uses less orchid seed than *in situ* seed baiting, the developing seeds can be observed more easily and without disturbance of the seeds or fungus, and several types of seed can be tested at once on the same soil sample (Brundrett et al., 2003). *Ex situ* seed baiting also avoids the seasonal constraints of *in situ* seed baiting, as the soil samples can be collected at any
time of year (Brundrett et al., 2003). On the downside, *ex situ* seed baiting necessarily takes place under more artificial conditions than does *in situ* seed baiting.

### 7.1.2 – Microniche specialization in *Drakaea* species

The study of mycorrhizal fungal distribution in *Drakaea* species is particularly important, as *Drakaea* species are all microniche specialists, many are narrow endemics, and most species are highly endangered (Hoffman & Brown, 1998). The genus *Drakaea* is confined to the southwest of Western Australia; some of the species can be found through much of this area, while others have an extremely narrow range (Hoffman & Brown, 1998). All *Drakaea* species are highly specialised in their habitat requirements, being found only in highly nutrient-poor sandy soils (Figure 6.2). They often occur in sand lenses that are only a few metres in diameter in an otherwise lateritic or clay-loam landscape (Hoffman & Brown, 1998). The narrow soil type requirements of *Drakaea* species leads to the question of whether their limited distribution is due to the absence of appropriate mycorrhizal fungi in the surrounding areas. In other words, the issue is whether it is the *Drakaea* species or their mycorrhizal fungi that require the siliceous sandy substrate.

*Ex situ* seed baiting is an ideal method to investigate this question. While the spatial scale of *Drakaea* populations is relatively small, and therefore possible to investigate by *in situ* seed baiting, there are a number of other features of *Drakaea* species that make *in situ* seed baiting impractical. Foremost amongst these is the problem of obtaining large numbers of seed. *Drakaea* species are all single flowered and have small seed capsules containing relatively few seeds for an orchid, and the plants are sparsely distributed (Hoffman & Brown, 1998). Pollination is by sexual deception of male thynnid wasps (Adams & Lawson, 1993; Hoffman & Brown, 1998), which only occurs under specific weather conditions (K Dixon, pers. comm.). If the necessary conditions do not occur during the flowering period of the orchids, pollination does not take place. The combination of these features means that it is difficult to obtain large quantities of seed of any species, and therefore *ex situ* seed baiting is far more suitable for investigation of the fungal relationships of this genus.
A genus which is closely related to *Drakaea* is *Paracaleana*. *Paracaleana* species can be multi-flowered, have extremely small capsules containing very few seeds for an orchid, and are also wasp pollinated and sparsely distributed (Adams & Lawson, 1993; Hoffman & Brown, 1998). It is common to find populations containing two or more *Drakaea* species, as well as one or more *Paracaleana* species (Hoffman & Brown, 1998). Seedlings of one species (or genus) can be found under adults of another (K. Dixon, pers. comm.). These observations lead to the question of whether *Drakaea* species (and even *Paracaleana* species) may be able to utilise each others’ mycorrhizal fungi. *Ex situ* seed baiting is, again, an ideal method to investigate this issue, as more than one type of seed can be tested for germination on the same soil sample.

### 7.1.3 – Objectives

The objective of this study was to investigate the distribution of mycorrhizal fungi in and around *Drakaea* populations and to test whether *Drakaea* and *Paracaleana* species can utilise the same mycorrhizal fungi.

**Hypotheses:**

- Fungal distribution mirrors plant distribution
- *Drakaea* and *Paracaleana* species can interchange mycorrhizal fungi

### 7.2 – Materials and methods

#### 7.2.1 – Site descriptions

**Brookton Highway**

The Brookton Highway site is located 50 km east of Perth, in the Darling Range, and consists of a revegetated sand extraction pit within *Eucalyptus marginata* woodland (Figure 7.1). The borders of the sand pit possess sandy soil, while the surrounding woodland is generally lateritic. The sand pit is adjacent to Brookton Highway, separated by a ditch and a narrow strip of woodland. The sandy borders of the pit are
populated by *Drakaea glyptodon*, while a rotted, half-buried log in the woodland strip between the highway and the pit is populated by about 50 plants of *Drakaea glyptodon* and *Paracaleana nigrita*, which is an unusually dense population for these species. No *D. glyptodon* or *P. nigrita* plants occur in the revegetated sand pit or the surrounding lateritic woodland. The locations of the soil samples are shown in Figure 7.1 and are as follows:

- **B1** – between log and highway, outside populations of *D. glyptodon* and *P. nigrita*
- **B2** – left-hand end of log, within populations of *D. glyptodon* and *P. nigrita*
- **B3** – middle of log, within populations of *D. glyptodon* and *P. nigrita*
- **B4** – right-hand end of log, within populations of *D. glyptodon* and *P. nigrita*
- **B5** – far bank of ditch beyond log, within *D. glyptodon* population
- **B6** – within *D. glyptodon* population
- **B7** – within *D. glyptodon* population, further around sand pit
- **B8** – within *D. glyptodon* population, at back corner of sand pit

![Diagram](image.png)

*Figure 7.1 – Drakaea glyptodon (B2 – B8) and Paracaleana nigrita (B2 – B4) populations at the Brookton Highway site, showing the locations of the soil samples. Shaded area is laterite, unshaded area is sandy soil.*
Canning Mills Road
The Canning Mills Road site is located 30 km east of Perth, in the Darling Range, in *Eucalyptus marginata* woodland. The surrounding soil is generally lateritic, while the study site is in a slight hollow and has a top layer of sand. The sand lens is populated by *Drakaea livida*, the related orchids *Leporella fimbriata* and *Pyrorchis nigricans* (Drakaeinae) and the unrelated orchid *Caladenia flava* (Caladeniinae) occur both within and outside the sand lens, and the surrounding lateritic area is densely populated by the unrelated orchid *Elythranthera brunonis* (Caladeniinae). The transect was placed through the centre of the population and is shown in Figure 7.2. The soil samples were taken every 2.5 m along the transect, from 0 m to 17.5 m, and are as follows:
C1 (0m) – outside *D. livida* population
C2 (2.5m) – outside *D. livida* population
C3 (5m) – 0.9 m outside *D. livida* population (first adults at 5.9m)
C4 (7.5m) – within *D. livida* population, adults present
C5 (10m) – within *D. livida* population (closest adults at 10.6m)
C6 (12.5m) – within *D. livida* population, adults present
C7 (15m) – 0.7 m inside boundary of *D. livida* population (last adults at 15.7m)
C8 (17.5m) – outside *D. livida* population
Figure 7.2 – Transect through Drakaea livida population at the Canning Mills Road site. The D. livida population extends for over 20 m on each side of the transect. In addition to the orchids shown, the entire transect was densely populated by Leporella fimbriata in numbers too great to count. The soil samples were taken every 2.5 m along the transect in positions C1-8, from 0 m to 17.5 m.

7.2.2 – Seed sources

Seeds were collected from wild plants of the study species from a variety of locations. The seed types used and the locations from which they were collected are shown in Table 7.1. Hand pollination was carried out to maximise seed set and the seed capsules were collected just prior to seed release. The contents of the seed capsules were combined and thoroughly mixed. The seeds were dried over silica gel for 24 hours at room temperature and stored in airtight containers at 4 °C prior to use.
Table 7.1 – Orchid species used for seed baiting, locations of seed collection, study sites for which seed batches used, and reasons for selection of species

<table>
<thead>
<tr>
<th>Orchid species</th>
<th>Location of seed collection</th>
<th>Study sites for which seed used</th>
<th>Reason for selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drakaea glyptodon</td>
<td>Denmark (south coast of Western Australia)</td>
<td>Brookton Highway</td>
<td>Native to study site</td>
</tr>
<tr>
<td>Paracaleana nigrita</td>
<td>Denmark (south coast of Western Australia)</td>
<td>Brookton Highway, Canning Mills Road</td>
<td>Native to Brookton Highway site</td>
</tr>
<tr>
<td>Drakaea livida</td>
<td>Canning Mills Road</td>
<td>Canning Mills Road</td>
<td>Native to and obtained from study site</td>
</tr>
<tr>
<td>Elythranthera brunonis</td>
<td>Canning Mills Road</td>
<td>Canning Mills Road</td>
<td>Native to and obtained from area surrounding study site</td>
</tr>
</tbody>
</table>

7.2.3 – *Ex situ* seed baiting

Soil samples were collected from the sites described above (section 7.2.1). Each soil sample consisted of topsoil and litter layer, and was taken from an area of 20 cm by 20 cm to a depth of approximately 2-3 cm. The fractionation and use of the soil samples is shown in Figure 7.3.
The soil samples were divided into two parts. Sample N° 2 was dried for 5 days at 35 °C, then stored at room temperature for six months before being sieved. Sample N° 1 was sieved, the fine fraction (less than 1.4 mm) was dried (as above) and the organic matter floated off. The fine organic matter was also stored at room temperature for six months (Figure 7.3).

The medium-sized fraction of Sample N° 1 was placed in 100 mm square petri dishes (Sarstedt) and covered with a layer of sterile white quartz sand (to make a smooth surface) and a layer of 100 μm nylon mesh (Australian Filter Specialists) (Figure 7.4). The soil and sand was wetted to field capacity. Seeds were scattered on 9 mm squares of 0.45 μm membrane filter (Millipore), which were then placed at random in a grid pattern on the nylon mesh (Figure 7.4). Two or three species were replicated four times on each plate. The plates were sealed and stored at 20 °C in the dark. Germination was scored at 4, 8, 12, 16, 20 and 24 weeks from sowing, and assessed according to the germination stages of Ramsay et al. (1986) as shown in Figure 2.3. Germination was scored as the percent of viable seeds which had reached stage 3 or greater (i.e. seeds at stage 0 (Figure 2.3) were not counted). The experiment was repeated after 6 months using the medium fraction of Sample N° 2 and the organic matter of the fine fraction of Sample N° 1, both of which had been stored at room temperature for the intervening period (Figure 7.3).
7.2.4 – Statistics

Seed germination was statistically analysed by ANOVA. Fisher’s PLSD with a 95% confidence interval was then applied to arcsine-transformed data to make comparisons between means. Analysis was performed using the statistical program Statview® (Statview, SAS Institute Inc.).

7.3 – Results

7.3.1 – Brookton Highway

Germination of Drakaea glyptodon seeds on soil samples from within the population (B2 – B8) was significantly higher than on those from outside (P = 0.0022) when the data from all three experiments were pooled (data not shown). The overall picture was less clear when each experiment was considered separately, but additional information
was evident. The germinants never developed beyond stage 3 (Figure 2.3), and after the maximum germination was achieved the seedlings began to senesce and the level of germination decreased. Detailed examination of the stage 3 seedlings produced indicated that they were infected, with distinctive peloton-like structures visible (results not shown).

Germination of *Drakaea glyptodon* seeds occurred on the fresh medium fraction of soil samples B2 – B8 (Table 7.2). Of these, the germination levels on samples B2, B3, B5, B6, and B8 were statistically significantly higher than zero (Table 7.2). Very little germination of *Paracaleana nigrita* seeds occurred, and was not statistically significantly higher than zero (Table 7.2). Detailed examination of the *P. nigrita* seedlings indicated that were infected, with distinctive peloton-like structures visible (results not shown).

**Table 7.2 – Germination of orchid seeds on fresh medium fraction of soil samples from Brookton Highway at 16 weeks after sowing. Results shown as percent germination (standard error). Shading shows species which occur naturally at the location of each soil sample.**

<table>
<thead>
<tr>
<th>Location</th>
<th>Drakaea glyptodon</th>
<th>Paracaleana nigrita</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B2</td>
<td>3.3 (0.42) *</td>
<td>0.5 (0.49)</td>
</tr>
<tr>
<td>B3</td>
<td>3.8 (1.80) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B4</td>
<td>0.6 (0.56)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B5</td>
<td>10.1 (1.06) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B6</td>
<td>5.8 (2.89) *</td>
<td>0.6 (0.58)</td>
</tr>
<tr>
<td>B7</td>
<td>0.5 (0.51)</td>
<td>0.1 (0.13)</td>
</tr>
<tr>
<td>B8</td>
<td>8.2 (2.14) *</td>
<td>0.3 (0.32)</td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

Germination of *D. glyptodon* seeds occurred on the stored medium fraction of all eight soil samples (Table 7.3). The germination levels on samples B2, B3, B4, B6, B7, and B8 were statistically significantly higher than zero (Table 7.3). No germination of *P. nigrita* seeds occurred (Table 7.3).
Germination reached its maximum level sooner on the fine fraction of soil that had been stored for 6 months (Table 7.4). Consequently, results are given for germination at 12 weeks for seeds on the fine fraction and 16 weeks for seeds on the medium fractions (both fresh and stored), as these were the times of maximum germination.

Germination of *D. glyptodon* seeds occurred on samples B2 – B8 of the stored fine fraction of the soil samples, and was statistically significantly higher than zero on samples B2, B3, B4, B6, and B8 (Table 7.4). No germination of *P. nigrita* seeds occurred (Table 7.4).

<table>
<thead>
<tr>
<th>Location</th>
<th>Seed type</th>
<th>Location</th>
<th>Seed type</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.6 (0.35)</td>
<td>B1</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B2</td>
<td>1.5 (0.65) *</td>
<td>B2</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B3</td>
<td>2.3 (0.69) *</td>
<td>B3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B4</td>
<td>1.8 (1.20) *</td>
<td>B4</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B5</td>
<td>0.3 (0.29)</td>
<td>B5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B6</td>
<td>2.3 (1.19) *</td>
<td>B6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B7</td>
<td>2.4 (2.23) *</td>
<td>B7</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B8</td>
<td>2.7 (1.22) *</td>
<td>B8</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)
7.3.2 – Canning Mills Road

Germination of *Drakaea livida* seeds on soil samples from within the population (C4 – C7) was significantly higher than on those from outside (P = 0.0206) when the data from all three experiments were pooled (data not shown). The germinants never developed beyond stage 3 (Figure 2.3), and after the maximum germination was achieved the seedlings began to senesce and the level of germination decreased. Detailed examination of the stage 3 seedlings produced indicated that they were infected with distinctive peloton-like structures (results not shown).

Seeds of *Drakaea livida* germinated on soil samples C3 – C7 of the fresh medium soil, but none of the germination levels was statistically significantly higher than zero (Table 7.5). The *Elythranthera brunonis* seeds germinated very well on all soil samples, and the *Paracaleana nigrita* seeds showed low (not significant) levels of germination on samples C2, C3, C5, and C7 (Table 7.5). Detailed examination of the *P. nigrita* seedlings indicated that were infected, with distinctive peloton-like structures visible (results not shown).

<table>
<thead>
<tr>
<th>Location</th>
<th>Seed type</th>
<th>Location</th>
<th>Seed type</th>
<th>Location</th>
<th>Seed type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Drakaea livida</em></td>
<td></td>
<td><em>Elythranthera brunonis</em></td>
<td></td>
<td><em>Paracaleana nigrita</em></td>
</tr>
<tr>
<td>C1</td>
<td>0 (0)</td>
<td>C2</td>
<td>0 (0)</td>
<td>C3</td>
<td>0.3 (0.31)</td>
</tr>
<tr>
<td>C4</td>
<td>2.3 (1.32)</td>
<td>C5</td>
<td>1.1 (1.11)</td>
<td>C6</td>
<td>2.9 (2.94)</td>
</tr>
<tr>
<td>C7</td>
<td>1.9 (1.85)</td>
<td>C8</td>
<td>0 (0)</td>
<td></td>
<td>81.8 (0.40) *</td>
</tr>
<tr>
<td></td>
<td>77.9 (4.91) *</td>
<td></td>
<td>67.7 (7.36) *</td>
<td></td>
<td>70.1 (8.18) *</td>
</tr>
<tr>
<td></td>
<td>66.6 (6.54) *</td>
<td></td>
<td>65.9 (15.89) *</td>
<td></td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>82.3 (6.50) *</td>
<td></td>
<td>5 (0)</td>
<td></td>
<td>0.2 (0.16)</td>
</tr>
<tr>
<td></td>
<td>81.8 (0.40) *</td>
<td></td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)
The levels of germination of *D. livida* seeds were higher on the medium fraction that had been stored for six months than when it was fresh (Table 7.6). Germination occurred on samples C1 and C3 – C8, and of these, the levels of germination on samples C3 – C5 was statistically significantly higher than zero (Table 7.6). The *E. brunonis* seeds germinated well on all soil samples, but less well than on the fresh soil (Table 7.6). No germination of *P. nigrita* seeds occurred (Table 7.6).

Table 7.6 – Germination of orchid seeds on stored medium fraction of soil samples from Canning Mills Road at 16 weeks after sowing. Results shown as percent germination (standard error). Shading shows species which occur naturally at the location of each soil sample.

<table>
<thead>
<tr>
<th>Location</th>
<th>Seed type</th>
<th>Drakaea livida</th>
<th>Elythranthera brunonis</th>
<th>Paracaleana nigrita</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0.3 (0.32)</td>
<td>40.9 (3.74) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>0 (0)</td>
<td>55.5 (5.37) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>1.6 (0.62) *</td>
<td>63.4 (7.17) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>3.2 (0.58) *</td>
<td>64.2 (5.83) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>2.9 (1.43) *</td>
<td>50.2 (7.05) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>0.5 (0.47)</td>
<td>82.1 (5.10) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td>0.3 (0.31)</td>
<td>58.3 (3.41) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>2.0 (1.96)</td>
<td>50.8 (2.46) *</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

Germination reached its maximum levels sooner on the fine fraction of soil that had been stored for 6 months than on either of the medium fractions (Table 7.7). Therefore, results are given for germination at 12 weeks for seeds on the fine fraction and 16 weeks for seeds on the medium fractions, as these were the times of maximum germination.

Germination of the *D. livida* seeds occurred on soil samples C2 – C4, C6, and C8 of the fine fraction, but of these, only samples C2 and C4 produced germination levels statistically significantly higher than zero (Table 7.7). The *E. brunonis* seeds germinated well, but at a lower rate than on either of the medium fractions (Table 7.7). Once again, no germination of *P. nigrita* seeds occurred.
Table 7.7 – Germination of orchid seeds on stored fine fraction of soil samples from Canning Mills Road at 12 weeks after sowing. Results shown as percent germination (standard error). Shading shows species which occur naturally at the location of each soil sample.

<table>
<thead>
<tr>
<th>Location</th>
<th>Seed type</th>
<th>Drakaea livida</th>
<th>Elythranthera brunonis</th>
<th>Paracaleana nigrita</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0 (0)</td>
<td>30.1 (4.51) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>1.4 (0.81) *</td>
<td>27.5 (6.51) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>1.0 (0.76)</td>
<td>43.6 (5.46) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>1.5 (0.77) *</td>
<td>40.5 (3.64) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>0 (0)</td>
<td>45.5 (3.13) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>1.2 (1.18)</td>
<td>47.1 (2.78) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td>0 (0)</td>
<td>41.2 (4.01) *</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>0.9 (0.53)</td>
<td>35.0 (5.08) *</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Germination statistically significantly higher than zero (P < 0.05)

7.4 – Discussion

A key finding of this study was the statistically significant correlation of the germination of Drakaea seeds with the natural distribution of the adult plants. This was the case for both D. glyptodon at the Brookton Highway site and D. livida at the Canning Mills Road site (Tables 7.2 – 7.7). The correlation was very close for D. glyptodon, as there was only one instance of germination on a soil sample from outside the population (statistically insignificant) (Table 7.3), and germination on soil samples from within the population was statistically significantly higher than that on soil samples from outside the population. The correlation was less clear cut for D. livida, as there was germination on soil samples from outside the population for all three soil fractions, and the stored medium and fine soil fractions could induce significant germination outside the orchid’s natural area (Tables 7.5 – 7.7). However, germination of D. livida seeds was statistically significantly higher on soil samples from within the population than from outside the population. This provides good support for the hypothesis that fungal distribution mirrors orchid distribution (Section 7.1.3). It would appear, from the admittedly small number of samples studied in this experiment, that the mycorrhizal fungi of Drakaea species are indeed absent (or at
least present at a lower inoculum potential) in the areas surrounding their small populations.

The picture was somewhat different for the other seed types used in this experiment. *Paracaleana nigrita* occurred naturally throughout the Brookton Highway site, co-occurring with *D. glyptodon*, and was absent from the Canning Mills Road site. Germination of *P. nigrita* seeds occurred on soil samples from both sites, but was not statistically significant (Tables 7.2 and 7.5). The seed batch was viable, as germination (and development to stage 5) occurred in vitro (Chapter 6) and the seedlings were infected with mycorrhizal fungi, but the levels of germination were always low. Thus, although an appropriate fungus was present at both sites, it is not clear why the levels of germination of the *P. nigrita* seeds were so low.

Germination of the *Elythranthera brunonis* seeds was high on all soil samples. No adult plants of this species were observed to occur within the sand lens of the study site, but they were very common on the surrounding laterite. A possible explanation is that the mycorrhizal fungus of *E. brunonis* occurred within the sand lens even in the absence of the orchid. Alternatively, the *E. brunonis* seeds may have been utilising a different fungus within the sand lens (which did not support development beyond stage 3) as its own fungus was not present. Further tests, such as isolating the fungus from germinated seedlings of *E. brunonis* on soil samples from both within and outside the sand lens, would be required to resolve this issue.

The levels of germination of all seed types used in this experiment altered, or appeared to do so, with storage of the soil samples. The germination of both *Drakaea* species appeared to increase overall with storage of the soil samples, although this apparent effect was not statistically significant. This picture changed with division of the soil samples into those from within and outside the *Drakaea* populations. Germination appeared to decrease with soil storage for those soil samples from within the populations, but to increase for those from outside (again, not statistically significant). The only case in which *D. glyptodon* seeds germinated on a soil sample from outside the population occurred on stored medium fraction soil, and both stored medium and fine soil fractions could induce significant germination of *D. livida* seeds on soil.
samples from outside the population, which did not occur on fresh soil samples. Further tests with higher replication are necessary to determine whether the apparent trends, which are not statistically significant in these experiments, are real.

In contrast to the *Drakaea* species, germination of the other seed types significantly decreased with storage of the soil samples. The levels of germination of the *E. brunonis* seeds remained high throughout, but decreased from fresh medium fraction to stored medium fraction to stored fine fraction, and the only germination of the *P. nigrita* seeds occurred on the fresh soil samples.

One possible explanation for this alteration in germination levels with storage of the soil samples is that the inoculum potential of the mycorrhizal fungi in the soil samples was altered by a period of storage, and this then affected the levels of seed germination (Garrett, 1963). It is well documented that after a fallow period, beneficial micro-organisms dominate in soils (Garrett, 1963); this could explain the apparent increase in germination of *Drakaea* seeds on soil samples from outside the populations after a period of storage, but not the decrease in germination of the other species and of the *Drakaea* seeds on soil samples from within the populations.

A number of other interesting features emerged from the results of this experiment. Firstly, while both the medium and fine fractions of the soil samples supported germination to similar levels, the germination reached its maximum level sooner on the fine fractions. A possible explanation for this phenomenon is that the inoculum potential of the mycorrhizal fungi was higher in the fine fraction of the soil. One problem with this explanation is that a higher inoculum potential might be expected to produce not only more rapid germination, but also a higher percentage of germination, which did not occur. Alternatively, the fungal spores in the fine fraction of the soil might have been less dormant than those in the medium fraction, and thus able to germinate and grow more rapidly when the soil was taken out of storage. This explanation also has its problems, including the fact that it does not explain the earlier peaking of seed germination on the stored fine fraction than on fresh soil, which would, by this theory, support more rapid germination, as the fungus would be more active than in stored soil. An alternative explanation is that the seedlings began to
decompose earlier on the fine fractions of the soil samples due to a greater pathogenic presence, and did not achieve as high a maximum level of germination as on the medium fractions of the soil samples. It is also a possibility that a lesser quantity of sterile white sand was required to produce a flat surface in the micro-array sampling plates on the fine fraction, and so the fungus was able to reach the seeds sooner. It is impossible to distinguish statistically between these theories, as all soil samples supported germination to statistically similar levels.

It is also interesting that the germinated seedlings never developed beyond stage 3 (Figure 2.3). Previous experiments have found *ex situ* seed baiting to support germination of other species to stage 5 (Brundrett et al., 2003). This can, perhaps, be explained by the fact that the mycorrhizal fungi of *Drakaea* species are the slowest growing and least robust of all Australian orchid fungi (Ramsay et al., 1986). Even under the most conducive of conditions, these fungi do not support high levels of germination (Chapter 6; Ramsay et al., 1986). The seedlings were, however, observed to be infected, so some development to later stages might be expected. It is, of course, possible that the amount of organic matter available to a fungus during *ex situ* seed baiting was insufficient for the fungus to support extensive germination or development of the seeds (Brundrett et al., 2003). The greater development of seedlings of other species during *ex situ* seed baiting (Brundrett et al., 2003) may be due to the more weedy nature of these orchids, and hence a lower reliance on fungal nutrition. Some form of supplementary nutrition, such as the addition of sucrose, may be of benefit during *ex situ* seed baiting. If, however, seedlings of some orchid groups never develop beyond stage 3 during *ex situ* seed baiting, the validity of the method to relate to the situation *in situ* is open to question.

As an experimental technique, *ex situ* seed baiting is still undergoing evaluation (Brundrett et al., 2003). No doubt further refinement of the technique will occur as additional research adds to the body of knowledge on which it is based. However, a number of conclusions concerning the general utility of the technique can be drawn from the results currently available. Firstly, a great advantage of this technique is its ability to determine the presence or absence of mycorrhizal fungi from an area while only using a small quantity of seed (Brundrett et al., 2003). Secondly, a number of
different seed types can be conveniently tested against one soil sample, and the space required for the experiment is minimal (Brundrett et al., 2003). Thirdly, the time course of germination can be easily observed without disturbing the germinating seeds or their hyphal connections to the soil (Brundrett et al., 2003). Fourthly, ex situ seed baiting can be carried out at any time of year, and even after storage of the soil samples (Brundrett et al., 2003). All of these points indicate the distinct advantages of ex situ seed baiting over the older in situ method.

On the other hand, there are also a number of disadvantages and potential problems involved in ex situ seed baiting. The conditions under which the seed baiting occurs are, of necessity and by definition, somewhat artificial. Many orchid species are known to react poorly to soil disturbance, and so may not germinate naturally on a sieved and homogenised soil sample. The seeds are separated from the soil and exposed on the surface, which is hardly a natural state. It can be difficult to maintain suitable moisture content, while simultaneously allowing for oxygen penetration. The impracticality of sterilising soil sieves between usages, especially when a large number of samples are being processed, means that cross-contamination of samples is a possibility, however remote. The failure of the seedlings produced in this experiment to develop beyond stage 3, despite mycorrhizal infection, leads to questions about the ability of the technique to indicate the real potential for germination. The potential of supplementary nutrition to enable further seedling development should be investigated, while keeping in mind that this will increase the artificiality of the experimental situation. All of these issues are, to at least some extent, resolved by the use of in situ seed baiting instead.

To summarise, ex situ seed baiting offers both advantages and disadvantages as an experimental method. There is a definite place for this technique, but until some of the problems can be further resolved, it should be used in conjunction with in situ seed baiting, which takes place under far more natural conditions. The issue of greatest concern is the lack of seedling development beyond stage 3 despite mycorrhizal infection, as this may mean that ex situ seed baiting indicates a level of germination and development that differs significantly from the real percentage of germination and
degree of seedling development that would occur in the field and lead to the establishment of new adult plants.

A considerable amount of future work is required to further investigate the issue of microniche specialisation in *Drakaea* species. This experiment has provided some confirmation for the idea that the orchids are restricted in their distribution by fungal presence. Further work, involving both *ex situ* and *in situ* seed baiting at a number of different *Drakaea* populations, would be necessary to reinforce this concept. If it is indeed the case that the fungi are a limiting factor in *Drakaea* distributions, then the question arises of why *Drakaea* fungi are so specific in their habitat requirements. A possible avenue to explore this issue would involve work on the saprophytic competence of the *Drakaea* fungi. These slow growing and least robust of all Australian orchid fungi may be restricted to microniches to avoid competition, as they might have lower saprophytic competence than other ‘common’ orchid fungi. Experimental work to explore this issue would be very valuable in developing conservation prescriptions for these highly specialised and narrowly endemic orchids.
Chapter 8 – Introduction, growth and persistence in situ of orchid mycorrhizal fungi

8.1 – Introduction

8.1.1 - Orchid mycorrhizal fungi in situ

The study of mycorrhizal fungi of terrestrial orchids in their natural environment, in situ, provides more realistic data than ex situ studies, but there are many problems associated with such investigations (Brundrett et al., 2003). In situ work can investigate issues which cannot be studied ex situ, such as soil seed bank dynamics (Batty et al., 2000; Rasmussen & Whigham, 1993 & 1998b), mycorrhizal fungal distribution (Batty et al., 2001a; Masuhara & Katsuya, 1994; McKendrick et al., 2000; Perkins & McGee, 1995), and fungal specificity in situ (Masuhara & Katsuya, 1994; McKendrick et al., 2000; Perkins & McGee, 1995; Rasmussen & Whigham, 1993 & 1998b; Zelmer & Currah, 1997).

The problems associated with in situ investigation of orchid mycorrhizal fungi include the fact that there are many fungi present in soil, and aggressive, fast growing fungi can mask the presence of other, more benign fungi. As orchid mycorrhizal fungi are usually saprophytes (Brundrett et al., 2003), the fungi specific to each orchid species are present in the soil (presumably in the organic matter and litter layer) as well as in the host plants. An orchid mycorrhizal fungus is active during the growing season, and may be found in germinating seedlings and adult orchids as well as in the surrounding soil. It survives the summer drought as dormant spores in the soil and the dead remnants of infected orchid tissue, but not usually in the orchid’s resting tubers (Dixon, 1991; Brundrett et al., 2003). Orchid mycorrhizal fungi are thus easiest to locate in the infected tissues of adult plants, but locating fungi in the soil can be difficult (Rasmussen & Whigham, 1993 & 1998b; Brundrett et al., 2003). In the absence of a precise molecular tool, one of the most efficient and effective ways to
discover whether a particular fungus is present is to use seed baiting (Rasmussen & Whigham, 1993 & 1998b; Brundrett et al., 2003).

8.1.2 – In situ seed baiting

In situ seed baiting involves burying a packet of orchid seeds for a period of time to see whether any seeds germinate and/or become infected by a mycorrhizal fungus (Rasmussen & Whigham, 1993 & 1998b; Batty et al., 2001a). As orchid seeds are extremely small (Rasmussen, 1995), it is necessary to find a way of containing the buried seeds so that the packet can be retrieved (Rasmussen & Whigham, 1993). This problem has been overcome by the use of fine nylon mesh held together by photographic slide frames, with an attached cord to aid retrieval (Rasmussen & Whigham, 1993; Batty et al., 2001a). The first published use of this method was by Rasmussen & Whigham (1993); it has been modified and used by Batty et al. (2000, 2001a) and others, such as Masuhara & Katsuya (1994), Perkins & McGee (1995), Zelmer & Currah (1997), Rasmussen & Whigham (1998b), McKendrick et al. (2000), and Brundrett et al. (2003).

Each packet of seed can be assumed to only test a small area, as the area over which a fungus can reasonably be expected to find seeds is not clear (Brundrett et al., 2003). This means that it is difficult to know how far apart the seed baits should be placed. Fungal distribution is generally quite patchy but often correlated with proximity of adult plants (Perkins & McGee, 1995; Batty et al., 2001a; Brundrett et al., 2003). However, fungi can be found a long way from adult plants, presumably representing unoccupied potential orchid habitats (McKendrick et al., 2000; Batty et al., 2001a; Brundrett et al., 2003). The handicaps of in situ seed baiting are that it is labour intensive and uses a lot of seed, and is temporally limited to the growing season (Brundrett et al., 2003). Some of these handicaps can be overcome by using ex situ seed baiting (Chapter 7), which has its own set of limitations (Brundrett et al., 2003).
8.1.3 – Objectives
The objective of this chapter was to investigate the persistence, colonisation, and growth of naturally occurring and introduced mycorrhizal fungi of Caladenia arenicola over a period of three years.
Hypotheses:
- Fungal distribution mirrors orchid distribution
- Fungi introduced into areas lacking the mycorrhizal fungi successfully colonise soil and persist over time
- Watering improves fungal growth and persistence and seed germination

8.2 – Materials and Methods

8.2.1 – Site description
The study site consisted of substantially altered Banksia-Allocasuarina-Eucalyptus woodland habitat within Kings Park, Perth, Western Australia. The climate is mediterranean type, with cool wet winters and warm dry summers. Caladenia arenicola is typical of many orchids of the southwest of Western Australia. Growth and flowering occur during the wet season (winter) and plants die back to a dormant tuber during the summer drought (Dixon, 1991).

The population of Caladenia arenicola selected for this study had been mapped previously, so the occurrence of the orchids and the mycorrhizal fungus was known (Batty et al., 2001a). Using the seed baiting method described below, Batty et al. (2001a) found that the mycorrhizal fungus was localised in its occurrence and closely associated with the adult plants. Over the study site, therefore, locations could be selected where adult plants and/or the mycorrhizal fungus were known to be present or absent.

The populations selected for this study occurred along transects 3 and 4 of Batty et al. (2001a), being most dense where the transects intersect. Batty et al. (2001a) inserted
seed baits every 0.5 m along each 50 m transect and scored for germination. In the survey, adult plants and seed germination occurred within the first 26 m of transect 3 (Batty et al., 2001a), and within the first 30 m of transect 4. No adult plants were found and no germination occurred beyond these points (Batty et al., 2001a). Most germination occurred in the area where the transects intersected (from 20-26 m on transect 3 and 13-18 m on transect 4) (Batty et al., 2001a). This was selected as the ideal location for control sites with the mycorrhizal fungus already present, and all other sites were selected outside the population. A sketch of the two transects, the extent of the *C. arenicola* population and previous germination, and the locations of the sites used in this study, is shown in Figure 8.1.

**Figure 8.1 – Map of the study site, showing the transects used by Batty et al. (2001a), the extent of the *C. arenicola* population, and the locations of the seed baiting sites (indicated by A – R) utilized in the current work. For an explanation of the treatments used, see Table 8.1.**
8.2.2 – Seed and inoculum sources

Seeds were collected from wild plants of *C. arenicola* in the population to be studied. Hand pollination was carried out to ensure seed set, and the seed capsules were collected prior to dehiscence 3 to 4 weeks later, according to climatic conditions and the consequent degree of capsule ripeness. The green seed capsules were stored with their stalks in water until the capsules ripened and turned brown, at which time they were air dried in paper bags to collect the seeds. The contents of the seed capsules were combined and thoroughly mixed. The seeds were dried over silica gel for 24 hours at room temperature and stored in airtight containers at 4 °C prior to use. Fresh seed was collected each year for use in the following season. The inoculum consisted of a fungus isolated in 1999 (prior to the first year of experimentation) from an adult *C. arenicola* from the same population. The fungus was tested for germination efficacy against each seed batch used, to provide a baseline *in vitro* germination percentage. This did not differ significantly from the germination percentages achieved *in situ*.

8.2.3 – *In situ* seed baiting

Seed baits

*In situ* seed baiting was carried out according to the method of Batty et al. (2001a). Seed baits were made by placing approximately 100 – 200 seeds between two layers of 100 µm nylon mesh (Australian Filter Specialists) held together by a photographic slide frame and bulked out with 0.5 g of sterile white quartz sand (Figure 8.2). A length of nylon cord was attached to each slide frame to enable retrieval. The seed baits were placed under the mulch layer at an approximate depth of 4 cm (vertically or at 45° to vertical). At each location that was inoculated, the seed baits were placed within the inoculation zone, and at distances of 5, 10 and 50 cm from the edge of the inoculation zone (replicated four times at each distance) (Figure 8.3).
Inoculation

Inoculation was carried out by removing a soil core (8 cm diameter and approximately 5 cm deep) from the centre of each location. The soil was thoroughly mixed with an
approximately equal fresh weight of inoculum before being replaced in its original location. The inoculum consisted of oat agar colonised by a fungus previously tested for germination efficacy of *C. arenicola* (Section 8.2.2). The centre of each inoculation zone was marked with a wire stake. Locations which were not inoculated had sterile oat agar mixed with the soil. The seed baits were buried in mid-May of each study year, after the autumn rains had begun, and removed in September, before the soil dried out at the beginning of the summer drought, providing an experimental period of 4-5 months, depending on the year.

**Treatments**

In the first year of the study (2000), nine seed baiting locations were set up (locations A to I – Figure 8.1 and Table 8.1). Three of these (A-C) were located within the *C. arenicola* population, at points where Batty et al. (2001a) had found the fungus to be present in the soil (Figure 8.1, Table 8.1). The remaining sites (D-I) were located outside the *C. arenicola* population. Of these, three (D-F) were not inoculated and three (G-I) were inoculated with the mycorrhizal fungus (Figure 8.1, Table 8.1). All nine sites (A-I) were baited for three years (2000-2002) (Figure 8.1, Table 8.1). In the second year of the study (2001), three additional sites (J-L) were inoculated with the mycorrhizal fungus and baited for two years (2001-2002) (Figure 8.1, Table 8.1). In the third year of the study (2002), six more sites (M-R) were inoculated and baited (Figure 8.1, Table 8.1). Another treatment, watering, was introduced; three of the new sites were watered once a week during the period from seed burial to removal (M, O, and Q), while three were not (N, P, and R) (Figure 8.1, Table 8.1).
Table 8.1 – Treatments used at each of the seed baiting locations (Figure 8.1).

<table>
<thead>
<tr>
<th>Location</th>
<th>C. arenicola present?</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Within C. arenicola population</td>
<td>Fungus present in soil (Batty et al., 2001) No inoculation Baited 2000-2002</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>No inoculation Baited 2000-2002</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Outside C. arenicola population</td>
<td>No fungus present in soil (Batty et al., 2001)</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>No inoculation Baited 2000-2002</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Outside C. arenicola population</td>
<td>No fungus present in soil (Batty et al., 2001) Inoculated 2000 Baited 2000-2002</td>
</tr>
<tr>
<td>H</td>
<td></td>
<td>Inoculated 2001 Baited 2001-2002</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>Outside C. arenicola population</td>
<td>No fungus present in soil (Batty et al., 2001)</td>
</tr>
<tr>
<td>K</td>
<td></td>
<td>Inoculated 2001 Baited 2001-2002</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>Outside C. arenicola population</td>
<td>No fungus present in soil (Batty et al., 2001)</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>Inoculated 2002 Baited 2002</td>
</tr>
<tr>
<td>O</td>
<td></td>
<td>M, O, Q watered N, P, R not watered</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Germination

Scoring of seed germination stages was carried out by opening the seed packets and examining the seed/sand mix under a dissecting microscope. Seeds and seedlings were scored for germination according to the germination stages of Ramsay et al. (1986) as shown in Figure 2.3. Germination was scored as a percentage of viable seed (i.e. stage 0 (Figure 2.3) was not included). The percentage of nonviable seed did not differ significantly between the treatments or from the figures recorded in vitro.

Germination data are given for seedlings that reached stage 3 and above, and also for those that reached stage 5 and above (Figure 2.3).

8.2.4 – Statistics

Seed germination was statistically analysed by ANOVA. Fisher’s PLSD with a 95% confidence interval was then applied to arcsine-transformed data to make comparisons between means. Analysis was performed using the statistical program Statview® (Statview, SAS Institute Inc.).
8.3 – Results

8.3.1 – Climatic conditions during the study period

During the study period (2000 – 2002), Perth experienced three years of considerably below-average rainfall (Figure 8.4). Not only was the total rainfall low between seed burial and retrieval, but there were also periods of no or very little rain (not shown). This means that the inoculated fungus and the buried seeds had to cope with dry winters which included ‘drought’ periods when the soil became much drier than is usual during the growing season.
Figure 8.4 – Monthly rainfall for the Perth region during the study period 2000 – 2002 (Source: Bureau of Meteorology).
8.3.2 – Germination and development to stages 3 and above

Germination at locations where the fungus was previously recorded to be present in the soil (A-C) was extremely variable both within and between the three sites and the three years of the study (Table 8.2). In 2000, germination never exceeded 0.5 %, while in 2001 and 2002 germination was sometimes over 30 % (Table 8.2). It can also be seen that the number of locations within sites A-C where no germination occurred decreased over the three years of the study (Table 8.2).

More anomalous is the occurrence of low levels of germination from sites that the earlier survey had shown to be free of the fungus (controls D-F) in the year 2000 (Table 8.2). No germination occurred in later years. Detailed examination of the stage 3 protocorms from these areas indicated that they were not infected by a mycorrhizal fungus (results not shown).

At sites that were inoculated in 2000, good germination occurred in the first year after inoculation (2000) at all three locations (G-I), decreasing with distance from the inoculation zone (Table 8.2). Germination occurred 10 cm from the inoculation zone in all three locations, and 50 cm away in one, indicating wide growth and spread of the fungus. In the second year of the study (2001) the fungus persisted at two of the three locations (Table 8.2), and at one of these locations (G) the fungus was still flourishing in the third year (2002), at high percentages of germination up to at least 10 cm from the original inoculation zone (Table 8.2).

Of the three sites inoculated in 2001 (J-L), seed germination was observed in the first year in all three, but only within the inoculation zone (Table 8.2). The fungus persisted at all three locations into the second growing season (2002), producing higher levels of germination at one of the three locations (Table 8.2). In one location (J), the fungus had spread at least 5 cm from the inoculation zone by the second season (Table 8.2).
Table 8.2 – Mean percentage germination at 0 – 50 cm from an inoculation point of *Caladenia arenicola* seeds to or beyond stage 3 at 4-5 months from seed burial. Standard errors are shown in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Location</th>
<th>Distance (cm)</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within population, fungus present</td>
<td>A</td>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.3 (0.29)</td>
<td>12.6 (12.60)</td>
<td>6.1 (5.25)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0</td>
<td>0 (0)</td>
<td>2.9 (2.94)</td>
<td>5.9 (2.53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.4 (0.35)</td>
<td>3.4 (3.37)</td>
<td>23.1 (4.43) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.2 (0.20)</td>
<td>6.0 (3.96)</td>
<td>25.2 (7.56) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.2 (0.16)</td>
<td>31.2 (7.25) *</td>
<td>37.0 (22.55) *</td>
</tr>
<tr>
<td>Control (no fungus)</td>
<td>C</td>
<td>0</td>
<td>0.5 (0.18)</td>
<td>0 (0)</td>
<td>3.2 (3.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.1 (0.12)</td>
<td>12.0 (12.04)</td>
<td>4.1 (2.98)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4.7 (2.78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0 (0)</td>
<td>20.8 (10.45) *</td>
<td>13.0 (9.19)</td>
</tr>
<tr>
<td>Inoculated 2000</td>
<td>D</td>
<td>0</td>
<td>0.8 (0.35)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.2 (0.11)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.1 (0.12)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.1 (0.09)</td>
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* Germination is statistically significantly higher than zero (P < 0.013)
Table 8.3 – Mean percentage germination at 0 – 50 cm from an inoculation point of *Caladenia arenicola* seeds to or beyond stage 5 at 4-5 months from seed burial. Standard errors are shown in parentheses.

<table>
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<th>Treatment</th>
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<th>Year</th>
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<th>2002</th>
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<tr>
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</tr>
<tr>
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</table>

* Germination is statistically significantly higher than zero (P < 0.02)
8.3.3 - Germination and development to more advanced stages (stages 5 and above)

The pattern of germination and development to stages 5 and above (Table 8.3) was very similar to that of germination to stages 3 and above (Table 8.2), but the levels were generally much lower. No seedlings developed to stage 5 in the control areas where there was no natural fungus and no inoculation (D-F) (Table 8.3). There was considerable germination in the locations with natural fungus (A-C) in the years 2001 and 2002 (Table 8.3).

Of the three locations inoculated in 2000 (G-I), germination to advanced stages occurred in all (Table 8.3). This occurred within the inoculation zone at one location, and 5 and 10 cm from the inoculation zone at the other two locations (Table 8.3). Germination to advanced stages occurred in two of these locations in 2001, and one in 2002. It was, however, the locations with the least initial fungal spread in 2000 in which the fungus persisted for the longest time (Table 8.3).

Of the three locations inoculated in 2001 (J-L), germination to advanced stages occurred in all three in the first year, but only one in the second (Table 8.3). In all cases, the germination to advanced stages only occurred within the inoculation zone (Table 8.3).

8.3.4 - Effect of watering on germination

Germination occurred in all six locations within the inoculation zone, 5 cm from the inoculation zone at three locations (N, O, and R) and 10 cm at one location (M) (Table 8.4). Germination to advanced stages (to or beyond stage 5) occurred in only five locations, and was always within the inoculation zone (Table 8.4). Watering the sites once a week from seed burial to retrieval increased the level of germination (Table 8.4). Within the inoculation zone, the watered sites M, O, and Q had significantly higher germination than the unwatered sites N, P and R (P < 0.0001), while overall there was significantly higher germination within the inoculation zones than beyond (P < 0.03).
Table 8.4 - Effect of watering on mean percentage germination at 0 – 50 cm from an inoculation point of *Caladenia arenicola* seeds at 4-5 months from seed burial. All sites were inoculated in 2002.

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<th>Location</th>
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<th>Mean percentage germination to or beyond stage 5 (SE)</th>
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<td>10.2 (1.64) *</td>
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<tr>
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<td>5</td>
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<td>+</td>
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<td>22.7 (12.32) *</td>
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<td>10</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than zero (P < 0.013)

8.4 – Discussion

The very patchy germination within the *Caladenia arenicola* population (locations A-C, Tables 8.2 and 8.3) supports earlier conclusions that, even within the *C. arenicola* population, the distribution of the mycorrhizal fungus is very patchy (Masuhara & Katsuya, 1994; Perkins & McGee, 1995; McKendrick et al., 2000; Batty et al., 2001a). Also in accordance with previous work (McKendrick et al., 2000; Batty et al., 2001a), germination (and presumably fungal presence) was demonstrated outside the population, perhaps representing unoccupied sites. The patchy nature of mycorrhizal fungus distribution has been indicated by other studies. Masuhara & Katsuya (1994) found that seed germination was not affected by proximity of adult plants in the orchid.
species *Spiranthes sinensis*. In contrast, Perkins & McGee (1995) found a correlation between the distribution of fungus and the host orchid *Pterostylis acuminata*.

It is interesting that germination within the *Caladenia arenicola* population was so poor in the year 2000 (Tables 8.2 and 8.3), never exceeding 0.5%. This low germination was most likely a result of poor climatic conditions, the winter of 2000 being very short and dry (Figure 8.4). The effect of unusually dry weather after inoculation and seed baiting would be adverse to both the inoculated fungus and the seeds. An inoculated fungus might be unable to flourish during or even survive a dry spell, if it was unable to from resistant spores, while a similar adverse effect might be expected for a naturally occurring fungus. It has been observed that if orchid seeds dry out after imbibition has occurred, the seeds usually become nonviable and fail to germinate even if conditions become favourable again (Batty et al., 2000). Given the hydrophobic nature of Western Australian soils, and the time taken for a hydrophobic soil to become moist again after drying out, the effect of dry weather extends well beyond the end of the dry spell itself.

It can also be seen that the number of locations where no germination (A-C) was recorded decreased over the three years of the study (Table 8.2), which perhaps represents the recovery and spread of the fungus after the poor season of 2000. However, the following seasons were also poor, so an alternative explanation may be that the fungus was better able to cope with successive dry seasons.

An anomalous result was the occurrence of low levels of germination in the year 2000 from sites that, in the earlier survey (Batty et al., 2001a), were shown to be free of the fungus (controls D-F) (Table 8.2). No germination occurred in later years, and the occurrence of germination in an especially poor year was an odd result. These sites were several metres from the nearest locations where germination had been found to occur previously (Figure 8.1, Batty et al., 2001a), and the maximum rate of fungal spread (and subsequent persistence) found in this study was 10 cm in one season (Table 8.2), so it is unlikely that the naturally occurring fungus had reached these sites. The lack of infection by a mycorrhizal fungus of these seedlings indicates that this low
level of apparent ‘germination’ may be due to imbibition and may not represent true germination.

The widespread growth of the fungus in locations G-I in the year 2000, and the persistence of the fungus for two years in location H and three years in location G (Tables 8.2 and 8.3), indicates that reintroduced fungi can grow and persist over time in situ. This result is supported by the growth of the fungus in locations J-L in the year 2001 and the persistence of the fungus for two years in location J (Tables 8.2 and 8.3), as well as the growth of the fungus in locations M-R in the year 2002 (Table 8.4). It seems clear that a reintroduced fungus will grow throughout its first season after introduction, and has perhaps a 50% chance of persisting into a second season (as occurred in three out of six locations in this study). The chance of a reintroduced fungus persisting into a third season is somewhat lower (one out of two locations in this study), but the one fungal colony that persisted for three years in this study (G) was flourishing. This is the first study to investigate the growth and persistence of a reintroduced fungus in situ, and offers encouragement for future reintroduction programs for rare and endangered orchids (and, perhaps, other mycorrhizal plants).

Germination to stage 3 or 4 is insufficient to indicate likely survival of seedlings over summer and into the next season as an adult plant (Batty et al., 2001a). Seedlings may reach stage 3 without forming a true symbiosis. For these reasons, germination to more advanced stages (stage 5 and greater) was considered more biologically significant in this study. Levels of germination to more advanced stages were generally much lower than recorded for stages 3 and greater, and some of the anomalous results were removed (Tables 8.2 and 8.3). No seedlings developed to stage 5 in the control areas where there was no natural fungus and no inoculation (D-F) (Table 8.3). The levels of advanced stage germination were similar for locations with natural fungus (A-C) and with introduced fungus (G-R), indicating that an introduced fungus is capable of supporting similar levels of germination to a naturally occurring fungus. The presence of advanced stage germination into the third year of fungal persistence (location G) provides additional support for the hypothesis that an introduced fungus can persist despite poor seasonal conditions, and indicates that an
introduced fungus can support germination to a high enough level for the establishment of adult plants.

The use of watering is recommended to aid plant reintroduction programs in Australia (Atkins et al., 1997). The results of this study indicate that watering can also aid the reintroduction of a mycorrhizal fungus. This has implications for reintroduction programs for orchids and other mycorrhizal plants. It may even be of use in the conservation of existing populations, where watering may assist with natural recruitment, particularly in dry years.

In any consideration of the use of watering to aid reintroduction efforts, consideration should be made of whether the benefit is sufficient to warrant the considerable expense of a watering program. In this case, the increase in germination due to watering, while significant, was small and the expense of a watering program would be better spent on increasing the number of seeds or seedlings introduced than on improving the survival rate.

However, there is evidence from this study that the fungi may be severely limited in poor years. It may therefore be of benefit to supplement the water supply to natural or reintroduced populations in dry years. The fungus may be more sensitive than adult plants or seeds to seasonal variability. One of the drivers for orchid abundance may therefore be fungal vitality in the soil. A promising area for future research would be on the effect of stresses, such as dry seasons, on the inoculum potential of the mycorrhizal fungi. The growth rates of orchid mycorrhizal fungi are very low compared to other saprophytes, which implies that their saprophytic competence and ecological competitiveness may not be great. This means that, even at the best of times, the inoculum potential of these fungi may be low, and any additional stresses may have a significant impact.

This study of fungal distribution, reintroduction, growth, and persistence over time in situ resulted in a number of implications for orchid reintroduction programs. Firstly, fungal distribution generally mirrors orchid distribution. Thus, a reintroduction program to an area where the orchid does not currently occur should first use a seed
baiting method to determine whether a compatible fungus occurs in the area. Secondly, reintroduced fungi successfully colonise soil, grow out from an inoculation point, and persist over time. Thus, orchid reintroduction programs should include a fungal reintroduction program, which can occur at the same time as the orchid reintroduction. Thirdly, watering improves fungal growth and seed germination. Thus, where cost effective, a watering program to extend the growing season and negate any dry spells could be used to improve the success of a reintroduction program.
Chapter 9 – General discussion

The results presented here have implications in the areas of speciation, habitat specialisation, and conservation. Taylor et al. (2003) hypothesised that mycorrhizal interactions have contributed to orchid diversification. Here, it is suggested that orchid speciation may occur by hybridisation and the utilisation of novel fungi (Chapter 4) or by evolution of new species that utilize new fungi resulting in the ability to grow in more specialised habitats (Chapter 3). These possibilities will be discussed below.

The *Caladenia* hybrids investigated in Chapter 4 appear to use either the same fungus as one parental species, or a novel fungus which is more closely related to the fungus of one parental species than the other. It is possible that the self-perpetuating hybrid lineages which occur so frequently in the genus *Caladenia* can only survive and thrive if they are able to exploit a novel un- or under-utilised fungus. This novelty in terms of orchid associates may provide for habitat specialisation as a possible prerequisite for speciation. The utilisation by *Caladenia* hybrids of fungi different to those of either parental species may be interpreted as evidence for the concept of ‘spare’ fungal capacity which is available to be exploited. The only other evidence for the presence of unutilised fungi in the environment is the South African species *Disa (Monadenia) bracteata*, which has invaded the southwest of Western Australia and utilises fungi which do not appear to used by any native species (Bonnardeaux, 2003).

The evidence presented in Chapter 6 (concerning the species *Spiculaea ciliata*), that orchids may be able to genetically modify their fungal partners, introduces the intriguing possibility that hybrids may initially use the same fungus as one parental species, but over time it becomes modified and adapted to the hybrid. This may be the mechanism by which a new and distinctive fungal type becomes associated with the hybrid lineage which in time may evolve into a new species able to colonise new habitats (Figure 9.1).
In contrast to this situation is the case of the ‘frontier’ species *Caladenia falcata* (Chapter 5), which has a broadly compatible fungus which may be able to tolerate an unusually wide range of habitats as well as orchid partners. Perhaps a broadly compatible fungus is advantageous to a frontier orchid species, or *C. falcata* may be in an ecological or geographical expansion phase and association with a broadly compatible fungus allows niche penetration in founder seedlings.

One question raised by the existence of a broadly compatible fungus is why other orchid species, sympatric with *C. falcata*, which might be expected from the germination studies (Chapter 5) to be able to utilise this broadly compatible fungus, do not do so. Perhaps more specific fungi are advantageous to these species for the converse of any of the reasons given for the benefits of associating with a broadly compatible fungus, or perhaps the seeds of *C. falcata* outcompete those of all other species in colonising and exploiting fungal sites.
The existence of a broadly compatible fungus may have implications for orchid speciation as well as niche penetration. Orchids may diverge by exploiting progressively more and more specialised fungi, but only if the widespread ancestral species cannot utilise the more specialised habitats and fungi, or if the specialised orchid in combination with the specialised fungus has a competitive advantage. Perhaps association with a broadly compatible fungus is the ancestral condition, and the gradual use (or production) of more specific fungi is associated with progressive niche specialisation. However, broadly compatible fungi may still be used only by orchids that have diverged by other means, such as adaptation to specialised pollinators or extreme environments. If association with a broadly compatible fungus is the ancestral condition and more derived orchids use more specialised fungi, then a possible implication is that the more derived and specialised an orchid is, and the more specialised its fungus, the more susceptible the orchid may be to change (such as human impact) and hence the more likely it is to be endangered or rare. This hypothesis might be investigated by comparing the fungi from related common and rare orchid species to see if more common species possess more broadly compatible fungi, or alternatively if more common species can utilise a broader range of fungi.

The common species *Caladenia arenicola*, and its rare relative *C. huegelii*, display different degrees of habitat specialisation and fungal specificity (Chapter 3), and would appear to provide an example that supports the above hypothesis. The common *C. arenicola* is less specific in both habitat requirements and fungal associations than *C. huegelii*. These two species may have diverged relatively recently, perhaps because *C. huegelii* was able to take advantage of a more specialised fungus, or, conversely, because *C. arenicola* could utilise a more generalistic partner. *Caladenia arenicola* shows more ‘weedy’ characteristics and can utilise the fungus of *C. huegelii* as well as its own, and occurs in the more specialised subhabitat occupied by *C. huegelii* as well as the surrounding areas. The contrast in specialisation and specificity of these two closely related species, one common and one rare, may assist in reaching an understanding of some of the driving forces of rarity in orchids. Theoretically, the seedlings of *C. arenicola* may possess a competitive advantage over those of *C. huegelii*, as *C. arenicola* seed may colonise and occupy *C. huegelii* fungal sites, thus pre-empting and displacing *C. huegelii* seedlings. For *C. huegelii* to persist, it is likely
that its seedlings together with its specialised fungi have some competitive advantage over *C. arenicola* in the more specialised habitat. Otherwise, *C. huegelii* might be expected to become extinct.

There is some evidence that *Caladenia arenicola* does not occupy all available habitat, and that there may be unexploited ecological capacity to support this species. Vacant recruitment sites (with an appropriate fungus present) are available (Batty et al., 2001), and *C. arenicola* has become more common in Kings Park since the 1970s (K Dixon, pers. comm.), so the species may be gradually exploiting the vacant recruitment sites. The increase in the orchid’s abundance may, however, also be linked to changes in bushland management which have occurred over this period. The fungus itself may also be spreading. It has been demonstrated here that the fungus of *C. arenicola* can be successfully introduced to suitable habitat in the absence of the orchid (Chapter 8). This provides further confirmation for the hypothesis that there is unexploited ecological capacity to support orchids. The evidence mentioned here, together with the work on *Disa bracteata* mentioned above, and the utilisation of novel fungi by *Caladenia* hybrids, indicates that there may be a good chance of fungi existing in environments that are not currently being used by orchids. Molecular tools would be essential to confirm this speculation.

As has been suggested above, the association with a broadly compatible fungus may be the ancestral condition for orchids, and more derived orchids may use more specialised fungi. Data from the other group of orchids studied here, the Drakaeinae, do not support this hypothesis. It is possible to consider the genus *Drakaea* and related species, with their complicated pollination mechanisms by sexual deception, specialised pollinators, and microniche habitats (Nilsson, 1992), to be among the most highly evolved and specialised orchids in existence, and may be more vulnerable to environmental change than more generalised species. The genus *Drakaea*, in fact, has a very high level of rarity, with five of the nine currently recognised species considered to be endangered (Briggs & Leigh, 1996). Such highly specialised orchid species might be expected to associate with equally specialised fungi, but this does not appear to be the case. The fungal associates of *Drakaea* do appear to be habitat limited (Chapter 7), but fungi from the different species are genetically undifferentiated, have
a very broad geographical spread, and a broad intergeneric specificity with Drakaeinae species (Chapter 6).

It may appear to be somewhat contradictory that a highly evolved orchid, which is itself highly habitat specialised, should possess a fungal associate which, although perhaps equally habitat specialised, is very unspecific in its association with orchid species. A consideration of the habitats occupied by many Drakaeinae species may, however, resolve the apparently contradictory nature of Drakaeinae fungal specificity and habitat specialisation. The species of *Drakaea* occupy precision habitats which can be best described as impoverished white sand lenses isolated within a surrounding environment with a different soil type and vegetation (Hoffman & Brown, 1998). These sand lenses are typified by an extremely low organic content, very low nutrient levels, low residual moisture capacity, and much sparser vegetation than the surrounding areas (Figure 6.3 A – D). The fungi of *Drakaea* species were absent outside the orchid populations (Chapter 7). It is possible that only a few fungi can cope with such a hostile environment. The situation may in fact be a tradeoff for the orchids; perhaps species that occupy such highly specialised, extreme habitats lose the opportunity for association with unique fungal types. If the hostile environment excludes most fungal types, and the fungi which we know to occur there are broadly compatible (at least within Drakaeinae), then perhaps any orchid which can utilise the habitat can utilise the fungi, or vice versa.

Of the remaining genera of Drakaeinae, *Paracaleana* species tend to occupy similar habitats to *Drakaea* species, and *Spiculaea ciliata* is found in still more extreme conditions, growing as it does only in the moss swards of seasonally moist granite outcrops (Figure 6.3 E – F) (Ramsay et al., 1986; Hoffman & Brown, 1998). *Leporella fimbriata* and *Pyrorchis nigricans* are among the only other orchid species to consistently co-occur in any abundance within *Drakaea* habitat (another indication that perhaps any orchid which can utilise the habitat can utilise the fungi, or vice versa), but also occur in the surrounding woodland, which is often populated by *Lyperanthus* species (Hoffman & Brown, 1998). The occurrence of these genera, which seem to use the apparently genetically undifferentiated Drakaeinae fungi, outside the precision habitats of *Drakaea*, contradicts the finding that *Drakaea* fungi may be habitat limited. More work is required to establish whether the fungi are in
fact genetically undifferentiated, or are distinctive and the *Drakaea* type is habitat limited.

The genera of *Caleana* and *Chiloglottis* from southeastern Australia tend to occupy woodland and moist woodland habitats respectively. The Drakaeinae genera examined here occupy an environmental continuum from the xeric habitats of *Spiculaea* to the more mesic habitats of *Chiloglottis*. The broad geographic spread of the undifferentiated Drakaeinae fungal types thus also includes a broad environmental spread, as there appears to be no fungal partitioning between the orchid species. This is a further contradiction between the findings that, on the one hand, Drakaeinae fungi are undifferentiated, and, on the other hand, that *Drakaea* fungi are confined to their precision habitats. This paradox must be resolved by further research.

The exploitation by orchids of such extreme habitats, such as the sand lenses of *Drakaea* and the granite outcrops of *Spiculaea*, may in fact only have been possible in a landscape as stable as that of southwestern Australia. The last glaciation event occurred more than 250 million years ago, the area is tectonically stable, with no volcanism, and significant human impact has only occurred over the last 200 years. Extreme habitats in such an ancient landscape may be relatively stable environments, thus allowing exploitation by groups which might have been excluded by environmental variability in a less stable landscape.

One of the most fascinating results produced by this study was the evidence (concerning the species *Spiculaea ciliata* in Chapter 6) that orchids may be able to genetically modify their fungal associates. The evidence, however, is preliminary; the experiment should be repeated and expanded to determine the nature of the genetic changes before firm conclusions can be drawn. This possibility, however tenuous the evidence may be, has immense implications in all areas of orchid research and conservation. If orchids can induce genetic changes in their fungi, then this would presumably lead to a high degree of specialisation of the fungus to the orchid, and vice versa, and perhaps provide a driving force for speciation. If the genetic modification of the fungi is directional, then it would be theoretically possible for orchids to induce new fungal types which might then be instrumental in colonisation of new habitats and even orchid speciation (Figure 9.1). It has frequently been found that orchids associate
with different fungal types at different life stages (Zelmer & Currah, 1997; Zettler, 1997); perhaps the orchid itself may have played a role in modifying its fungus to suit particular life stages.

The association of adult orchids with fungi which do not germinate their seeds is a common phenomenon (Harvais & Hadley, 1967; Rasmussen, 1995; Markovina & McGee, 2000; Takahashi et al., 2000, 2001), and may hypothetically be accounted for by the genetic modification of fungi by their orchid hosts. It is possible that fungi which are efficacious seed germinators are less effective as adult plant associates, and vice versa (Rasmussen, 1995; Takahashi et al., 2000, 2001). One issue with the association of orchids with different fungi at different life stages is the problem of how seeds find (or are found by) the appropriate seed germination efficacious fungus, if the fungus associated with the adult orchid is more abundant in the soil around the adult plants than the one efficacious for seed germination. If, as seems possible from the evidence in Chapter 6 (Spiculaea ciliata), a fungus can be altered genetically by passing through a seedling, it is possible that seeds that encounter an adult associate fungus may be unable to germinate or do so at low frequency. It is also theoretically possible that seeds which do germinate with such a mycorrhizal association are able to alter the fungus genetically so it becomes efficacious for germination. However, this is not to suggest that most or all fungi found in adult plants that are not efficacious for germination might be genetically modified from such a condition. Orchids often possess a variety of fungal endophytes which are not involved in symbiotic relationships (Rasmussen, 1995), so the presence of a fungus within an orchid should not be taken as sole evidence of its mycorrhizal nature or ecological competency for sustaining the orchid.

The hypothetical ability of orchids to genetically modify their fungi may be linked to the phenomenon of collar infection. Most orchids from the subtribes Caladeniinae and Drakaeinae possess this form of fungal infection, in which fungal presence is concentrated in a swollen section of stem just below the surface of the soil, rather than in the roots (Dixon, 1991). One of the benefits of collar infection is considered to be the localisation of the fungal inoculum in last season’s collar, present as a fibrous sheath formed over many years surrounding the collar, through which the orchid’s shoot passes each year when sprouting from its aestivating tuber (Dixon, 1991). In this
manner, it is almost certain that the orchid will be infected by the appropriate fungus early in each growing season. If the orchid has genetically modified its fungal associate, it becomes even more important for the orchid to pick up the same fungal type each year. Collar infection may, in fact, be a particularly efficient mechanism by which an orchid can ensure constancy of fungal type from year to year, and therefore provide stability, habitat (and fungal) specialisation, and the most appropriate fungal genetic composition. It may even be possible that fungal specificity may be higher, and other forms of fungal specialisation may occur more often, in collar infected orchids due to this efficient mechanism for picking up the same fungus each year. A rewarding area for future research would be to examine the degree to which fungal characters change over time in adult orchids, and between individuals of the same orchid species in different habitats.

The two focal genera of this study, *Caladenia* and *Drakaea*, possess a number of contrasting characteristics (Table 9.1). These are important because the correlation of particular features may help us to understand aspects of orchid biology and ecology. Firstly, fungal specificity appears to be higher in *Caladenia* than in *Drakaea*, and a possibly linked feature is that broadly compatibly fungi appear to be more common in (unspecific) *Drakaea* than in (specific) *Caladenia*. A number of possible reasons for these characteristics have been discussed earlier. *Drakaea* species also appear to be more habitat specialised than *Caladenia* species, as do their fungal associates. *Caladenia* species tend to occur in populations which may or may not be discrete within a relatively homogeneous habitat, and there are vacant recruitment sites where the fungus occurs but the orchid does not. This infers that a generalised habitat may possess unexploited fungi. *Drakaea* species, on the other hand, tend to occur in disjunct, discrete, distant populations which occupy specialised niches which are often separated by considerable distances of unsuitable habitat, and their specialised niches appear to be foci for their highly habitat specific fungi. This infers that specialised niches may possess a limited range of fungi.
Table 9.1 – A comparison of Caladenia and Drakaea.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Caladenia</th>
<th>Drakaea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungal specificity</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Broadly compatible fungi</td>
<td>Unusual</td>
<td>Common</td>
</tr>
<tr>
<td>Habitat specialisation of orchid</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Habitat specialisation of fungus</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Orchid populations</td>
<td>Disjunct to continuous, discrete, distant to close</td>
<td>Disjunct, discrete, distant</td>
</tr>
<tr>
<td>Habitat and environment</td>
<td>Relatively homogeneous</td>
<td>Niches often separated by considerable distances of unsuitable habitat</td>
</tr>
<tr>
<td>Fungal distribution in situ</td>
<td>Vacant recruitment sites (fungus present, orchid absent)</td>
<td>Fungus rarely found outside specialised niches</td>
</tr>
<tr>
<td>Rarity</td>
<td>Majority of species common</td>
<td>Majority of species rare</td>
</tr>
</tbody>
</table>

The genetic diversity generated during the evolution of the orchid species in subtribe Drakaeinae does not appear to have been paralleled by the genetic diversity of their mycorrhizal fungi. The mycorrhizal fungi of Drakaeinae appeared to be genetically undifferentiated (Chapter 6), despite the contradictory finding of habitat limitation of Drakaea fungi (Chapter 7), and the clear genetic differentiation of the orchid species (Kores et al., 2001). In contrast, genetic diversity in Caladeniinae does appear to be paralleled by the genetic diversity of their fungi (Chapters 3 – 5). Not only do some Caladenia hybrids appear to utilise novel fungal types (Chapter 4), but the three species studied in the Caladenia arenicola complex were genetically indistinguishable, morphologically distinct, and possessed genetically distinct fungal types (Chapter 3). It is therefore clear that fungal diversity can vary considerably between different orchid groups, and this has implications for fungal specificity. Pollinator specificity, however, is extremely high in Drakaeinae (Nilsson, 1992; Adams & Lawson, 1993); in Drakaea, for example, each species of orchid is pollinated by a different species of thynnid wasp (Nilsson, 1992; Adams & Lawson, 1993). Highly species-specific pollination may overcome the apparent importance of fungal differentiation.

The data presented here (Chapters 3 – 8) suggest that fungal specificity is much lower than previously thought. The reputation that Caladenia has for being highly specific appears to be based on unpublished information (K Dixon, pers. comm.), as the published data indicates that Caladenia seeds tend to be more specific in their fungal requirements than those of other genera, while fungal isolates from Caladenia often support the germination of unrelated orchid species (Warcup 1981, 1988; Clements
The broadly compatible fungus of *C. falcata* represents an extreme case of nonspecificity in fungal associates; this species may be using a broadly compatible fungus to enable survival and spread in a frontier environment. It also appears, from the data presented here (Chapters 3 – 8), that broadly compatible fungi occur frequently in the subtribe Drakaeinae. If specificity is indeed not as high as previously thought, then there are more opportunities for conservation, for example in the use of broadly compatible fungi in combination with a variety of orchid species, particularly in situations where the environmental conditions have been altered.

Germination was reported in two ways in these studies: germination to and beyond stage 3 and germination to and beyond stage 5 (Figure 2.3). This was due to differences in the biological significance of germination to different stages. Germination to stage 3 indicates some compatibility, but seedlings may reach this stage without forming a true symbiosis (Zettler & Hofer, 1998). Germination to stage 3 or 4 is considered to be insufficient to indicate the likely growth and survival of naturally produced seedlings over summer, including the production of an aestivating tuber, allowing persistence into the next season as an adult plant (Batty et al., 2001a). It is therefore important to report germination to advanced stages (stage 5 and beyond) if results are to be of ecological significance, separately to germination to stage 3 and beyond (which has been frequently reported alone). This conclusion is underlined by the lack of development of some seed/fungus combinations beyond stage 3 in all of the experiments reported here. Examination of some of the stage 3 protocorms showed that the seedlings were infected by the fungi, even when development did not progress any further. These may be examples of semi-compatible relationships, in which infection is possible but development of an adult plant does not occur (Rasmussen, 1995; Zettler & Hofer, 1998).

The differences between the conclusions which can be drawn from the results of the germination experiments and AFLP analyses (Chapters 3 – 6) may be related to the concepts of ecological and potential specificity (Masuhara & Katsuya, 1994; Rasmussen, 2002; Fay & Krauss, 2003). Ecological specificity, the range of fungi associated with an orchid species *in situ*, is indicated by the AFLP analyses, which consider the genetic variation of the fungi isolated from adult plants of the orchid species. Potential specificity, on the other hand, the range of fungi with which an
orchid species can form viable associations in vitro, is indicated by the germination experiments, which consider the production of seedlings from a variety of seed/fungus combinations. It is generally considered that ecological specificity is much narrower than potential specificity (Masuhara & Katsuya, 1994; Rasmussen, 2002; Fay & Krauss, 2003), a conclusion which is supported by the results reported in Chapters 3 – 6, wherein most seed types appear to be able to use a wider range of fungi in vitro than in situ.

It is evident from the data presented here that multiple sampling of orchid plants in the wild is necessary to detect the full range of germination efficacy and genetic variation in the orchids’ fungal associates. For most of the species studied, germination efficacy and genetic variation differed between fungal isolates from different locations. The small sample sizes used in most of the studies (often only three fungal isolates from only one location) were chosen for reasons of practicality. The variation in germination between fungal isolates was not always significant when the considerable variation between replicates was taken into account. In the germination tables presented in Chapters 3 – 8, it can be seen that the standard error is often very high. Greater replication may have reduced the standard error, but the variability in germination makes it clear that more factors influence germination than the fungal isolate used. Orchid germination is evidently a complex process. The small sample sizes were also of concern with regards to the AFLP results, where all of the conclusions were of limited validity as the consequences of larger sample sizes on relationships between and overlap of groups were unknown. It is clear that, in all cases, greater replication and larger sample sizes would have been useful.

In some cases, there appeared to be links between the genetic variation of the fungi and geographic distance (Chapters 3 – 6). Some orchid species appeared to possess genetically distinct fungal types in different populations (Chapters 3 – 5), which in some cases was linked to the distance between the populations, while others showed no genetic differentiation between populations (Chapter 6). It seems a reasonable assumption that fungi are more likely to be more genetically distinct the farther apart they occur, but there is, as yet, no definitive proof to support or refute this assumption. At the same time, it cannot be assumed that the same orchid species will have the same fungus at different sites, as there is evidence of population specificity of fungi.
Genetic data provided by AFLP of fungal isolates does not supply information about germination efficacy and specificity, nor does germination efficacy and specificity provide information about genetic variation. Different information, in turn, is provided by seed baiting. This is supported by the data presented in Chapters 3 – 6. The three techniques, AFLP of fungal isolates, symbiotic germination in vitro, and seed baiting, provide very useful and complementary information, and should be used in conjunction with one another. A research program that combines in vitro symbiotic germination with seed baiting and genetic investigation of fungal associates will provide outcomes that are both of practical use in the areas of conservation and restoration, and add essential details to the body of scientific knowledge about orchid biology and ecology.
Appendix 1 – Culture media

A1.1 – Oatmeal agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oatmeal</td>
<td>2.5</td>
</tr>
<tr>
<td>Agar</td>
<td>8</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
</tbody>
</table>

A1.2 – Fungal Isolation Medium (FIM)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
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</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.5</td>
</tr>
<tr>
<td>Agar</td>
<td>8</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

A1.3 – Modified Soil Solution Equivalent Agar for Western Australian Soils (SSE)

The SSE medium is designed to contain a concentration and balance of nutrients similar to a typical Western Australian soil (Angle et al., 1991; Dellar & Lambert, 1992). Angle et al. (1991) developed a medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution, which was then modified by Brundrett (unpublished data) to correspond to the concentrations found in a typical Western Australian soil (Dellar & Lambert, 1992).
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>0.04</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.00136</td>
</tr>
<tr>
<td>MgCl.6H₂O</td>
<td>0.061</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.0058</td>
</tr>
<tr>
<td>CaSO₄.2H₂O</td>
<td>0.0861</td>
</tr>
<tr>
<td>FeNaEDTA</td>
<td>0.0073</td>
</tr>
<tr>
<td>MES buffer</td>
<td>0.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2</td>
</tr>
<tr>
<td>Agar</td>
<td>8</td>
</tr>
<tr>
<td><strong>pH 5.5</strong></td>
<td></td>
</tr>
</tbody>
</table>

For liquid medium, agar was omitted.

### A1.4 – 1/5 Potato Dextrose Agar (PDA)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA powder (Difco-Bacto)</td>
<td>7.8</td>
</tr>
<tr>
<td>Agar</td>
<td>6</td>
</tr>
<tr>
<td><strong>pH 6</strong></td>
<td></td>
</tr>
</tbody>
</table>

### A1.5 – Streptomycin Sulphate

14.3 g L⁻¹ = 0.01 M (Sigma)

A stock solution of 1 g in 70 mL was filter sterilised (0.22 µm millipore filter) and added to cooled autoclaved agar at a rate of 10 mL L⁻¹.
Appendix 2 – The effect of gelling agents on growth of mycorrhizal fungi and symbiotic germination of Drakaea and Paracaleana species

A2.1 – Introduction

When the first attempts were made to symbiotically germinate seeds of Drakaea and Paracaleana species in vitro (Chapter 6), no germination occurred. This was unusual, as all the species concerned had germinated successfully in the past (Ramsay et al., 1986). The only change in laboratory practice since the successful germination experiments was that food grade agar was now used instead of purified agar. Fungal isolates from the Drakaea and Paracaleana species were extremely slow-growing and displayed considerable sensitivity to media type (Appendix 1), growing fastest on oatmeal agar and slower with higher concentrations of minerals. It was considered possible that the fungi and/or seeds of the Drakaea and Paracaleana species might be sensitive to contaminants in the food grade agar, as food grade agar is known to contain much higher concentrations of many minerals than purified agar (Oddie et al., 1994). Experiments were therefore set up to investigate the effect of different qualities of agar on fungal growth and germination of Drakaea and Paracaleana species.

A2.2 – Materials and Methods

A2.2.1 – Fungal growth rates on media containing different gelling agents

Fungal growth rate experiments were carried out on media containing a number of different gelling agents. The gelling agents used were:
• standard laboratory food grade agar (Gelita, Davis Gelatine Australia, Grade J3) at 8 g L⁻¹ (A)
• carrageenan (Xama, Australia) at 8 g L⁻¹ (C)
• purified agar (Sigma) at 8 g L⁻¹ (PA)
• phytogel (Sigma) at 2 g L⁻¹ with MgCl₂ at 0.5 g L⁻¹ (Pg + Mg)
• purified agar at 8 g L⁻¹ with MgCl₂ at 0.5 g L⁻¹ (PA + Mg)
• DNA grade agarose (Sigma) at 8 g L⁻¹ (Ag)

In each case the carbon source was oatmeal at 2.5 g L⁻¹ (Appendix 1).

The experiment was carried out twice: first with a fungal isolate from Drakaea thynniphila (isolate WCH2c – Chapter 6), and then repeated with the same isolate, plus one each from Drakaea livida (isolate CMR4d – Chapter 6) and Paracaleana nigrita (isolate WB2b – Chapter 6). Each 90 mm plastic petri dish was inoculated by placing a 3 mm by 3 mm cube of agar with fungus (cut from the growing edge of the appropriate culture) in the centre of the dish. Growth of the fungi was quantified by measuring the radius of each fungal culture at four points on each plate three times a week until the cultures had reached the edges of the plates. Six replicates were used in the first experiment, and three in the second.

A2.2.2 – Symbiotic germination on media containing different gelling agents

Symbiotic seed germination was carried out according to the methods described in Chapter 2 on media containing a number of different gelling agents. The gelling agents used are listed above in Section A2.2.1. Two experiments were carried out.

In Experiment 1, two seed types (Drakaea thynniphila and Paracaleana nigrita – Chapter 6) were sown on three fungal isolates (Drakaea thynniphila WCH2c, Paracaleana nigrita BJR1b and Paracaleana nigrita BJR1b pink – Chapter 6) and an asymbiotic control. Two different gelling agents were used, food grade agar (A) and purified agar (PA), with the additional variable of sterilisation time for the seeds (5 or 30 minutes in 1 % calcium hypochlorite). Only one replicate was used. Germination was scored to and beyond stage 3 (Figure 2.3) and to and beyond stage 5, as a
percentage of viable seed (i.e. stage 0 (Figure 2.3) was not counted) at 16 weeks after sowing. The percent of nonviable seed varied between but not within the seed types.

In Experiment 2, *Drakaea thynniphila* seed (Chapter 6) was sown onto plates previously inoculated with *Drakaea thynniphila* fungal isolate WCH2c and used for the first fungal growth rate experiment. The carrageenan plates were not used as the fungus had not grown sufficiently. Of the six plates from the fungal growth rate experiment, the seeds for three replicates were sterilised for 5 minutes in 0.5 % calcium hypochlorite, while the seeds for the other three replicates were sterilised for 5 minutes in the standard 1 % calcium hypochlorite. Germination was scored to and beyond stage 3 (Figure 2.3) and to and beyond stage 5, as a percentage of viable seed (i.e. stage 0 (Figure 2.3) was not counted) at 16 weeks after sowing.

### A2.3 – Results

#### A2.3.1 – Fungal growth rates on media containing different gelling agents

The three fungal isolates all grew at statistically significantly different rates in the presence of the different gelling agents, but the effect varied between the isolates (Figure A2.1). Over all fungal isolates, the statistically significantly fastest growth occurred on purified agar or on phytogel, followed by purified agar with magnesium; DNA grade agarose and food grade agar supported similar but significantly lower fungal growth rates across all isolates, while carrageenan supported the slowest growth rates (P < 0.02). The fastest growing fungus across all gelling agents was that from *Drakaea thynniphila* in the first experiment, while the slowest growing fungus across all gelling agents was that from *Drakaea livida* (P < 0.02).

The effect of the gelling agents on the fungal isolate from *Drakaea thynniphila* varied when the experiment was repeated (Figure A2.1 A and B). The first time the experiment was carried out, the fungus grew significantly faster on purified agar than on any other gelling agent, followed by both purified agar with magnesium and
phytogel, then DNA grade agarose, food grade agar, and carrageenan (in that order) (Figure A2.1A). When the experiment was repeated, however, the fastest fungal growth was produced on both purified agar and food grade agar, followed by DNA grade agarose, purified agar with magnesium, and phytogel, with carrageenan again supporting the slowest growth (Figure A2.1B).

The fungal isolate from Drakaealivida grew fastest on both phytogel and purified agar, followed by food grade agar and purified agar with magnesium (Figure A2.1C). The slowest growth rates were produced on DNA grade agarose and carrageenan (Figure A2.1C).

The fungal isolate from Paracaleana nigrita grew fastest on phytogel, followed by food grade agar, purified agar, and carrageenan (Figure A2.1D). The growth rate of the fungus on DNA grade agarose did not differ significantly from that on food grade agar or purified agar (Figure A2.1D).
Figure A2.1 – Growth of orchid mycorrhizal fungi on media containing different gelling agents. Experiment numbers in parentheses. Error bars represent standard error. Within each fungus, media labelled with different letters support statistically significantly different growth rates over the total period of measurement (P < 0.0012). Note: horizontal scales differ and graph D did not include the medium ‘purified agar + Mg’.
A2.3.2 – Symbiotic germination on media containing different gelling agents

Experiment 1

The *Drakaea thynniphila* and *Paracaleana nigrita* seeds both germinated on food quality and purified agar, but germination of both seed types was statistically significantly higher on purified agar than on food quality agar across all treatments ($P < 0.008$) (Table A2.1). Germination of the *D. thynniphila* seeds was statistically significantly higher than of the *P. nigrita* seeds across all treatments ($P < 0.004$) (Table A2.1). There was no significant difference in germination between the fungal isolates, or between sterilisation times (Table A2.1). There was no germination of either seed type under asymbiotic conditions (Table A2.1).

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Location and isolate no</th>
<th>Gelling agent</th>
<th>Sterilisation time 1</th>
<th>Seed type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Drakaea thynniphila</em></td>
<td>WCH2c</td>
<td>Food grade agar</td>
<td>5 minutes 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purified agar</td>
<td>5 minutes 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 minutes 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td>BJR1b pink 2</td>
<td>Food grade agar</td>
<td>5 minutes 1.1 0</td>
<td>0 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 minutes 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purified agar</td>
<td>5 minutes 7.4 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 minutes 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td><em>Paracaleana nigrita</em></td>
<td>BJR1b</td>
<td>Food grade agar</td>
<td>5 minutes 1.5 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 minutes 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purified agar</td>
<td>5 minutes 6.7 0.9</td>
<td>0.9 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 minutes 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td>BJR1b</td>
<td>Food grade agar</td>
<td>5 minutes 0.4 0</td>
<td>0 0</td>
</tr>
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<td></td>
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<td></td>
<td>30 minutes 2.5 0</td>
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<td></td>
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<td>Purified agar</td>
<td>5 minutes 3.4 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 minutes 0.7 0</td>
<td>0 0</td>
</tr>
</tbody>
</table>

1 Sterilization of seeds was in 1 % calcium hypochlorite (Chapter 2).
2 ‘Pink’ refers to a fungal isolate which had been in the presence of orchid seeds and had changed from nearly colourless to a deep pink.
Experiment 2

The *Drakaea thynniphila* seeds germinated to or beyond stage 3 on all gelling agents, and statistically significantly better on all other agents than on food quality agar (Table A2.2). The highest germination was on phytogel, followed by purified agar with magnesium and DNA grade agarose (Table A2.2). The only development to or beyond stage 5 occurred on purified agar and DNA grade agarose, and was only statistically significantly higher than on food quality agar on DNA grade agarose with 0.5% sterilant (Table A2.2). There was no significant difference in germination between sterilant concentrations (Table A2.2).

Table A2.2 – Symbiotic germination of *Drakaea thynniphila* seeds on media gelled using agar of different qualities and the effect of seed sterilant concentration at 16 weeks after sowing.

<table>
<thead>
<tr>
<th>Fungal source species</th>
<th>Location and isolate n°</th>
<th>Gelling agent ¹</th>
<th>Sterilant conc. ²</th>
<th>% to or beyond stage 3 (SE)</th>
<th>% to or beyond stage 5 (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A ⁵,a</td>
<td>0.5%</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A ⁵,a</td>
<td>1%</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Drakaea thynniphila</td>
<td>WCH2c</td>
<td>A ⁵,a</td>
<td>0.5%</td>
<td>0.2 (0.18)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PA ⁶,b,a</td>
<td>1%</td>
<td>26.9 (12.31) *</td>
<td>0.3 (0.34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PA + Mg ⁸,b,a</td>
<td>0.5%</td>
<td>36.0 (3.08) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PA + Mg ⁸,b,a</td>
<td>1%</td>
<td>35.0 (1.60) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pg + Mg ⁹,b</td>
<td>0.5%</td>
<td>50.7 (5.08) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pg + Mg ⁹,b</td>
<td>1%</td>
<td>36.8 (6.97) *</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag ¹⁰,b</td>
<td>0.5%</td>
<td>37.4 (8.68) *</td>
<td>1.8 (0.91) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag ¹⁰,b</td>
<td>1%</td>
<td>27.3 (6.17) *</td>
<td>0.7 (0.35)</td>
</tr>
</tbody>
</table>

* Germination is statistically significantly higher than for A (P < 0.005)
¹ Gelling agents with different letters as superscripts support statistically significantly different levels of germination (P < 0.05). % to or beyond stage 3 and % to or beyond stage 5 analysed separately; superscript letters separated by commas refer to these.
² Sterilant is calcium hypochlorite (Chapter 2) used for 5 minutes.

A2.4 – Discussion

The overall statistical result indicated that food grade agar was significantly less suitable for fungal growth than purified agar or phytogel. The effect, however, differed considerably between fungal isolates, with the fastest fungal growth usually
occurring on purified agar and/or phytogel (Figure A2.1). There was only one case where food grade agar was as suitable as purified agar or phytogel. The effect of the gelling agents on the growth of the *D. thynniphila* fungal isolate differed significantly between the first and second experiments (Figure A2.1). There are a number of possible explanations for this lack of repeatability, including experimental error (such as media contamination) and a change in fungal quality over time in culture, which has been observed before (Alexander & Hadley, 1983). The lack of repeatability aside, it appears clear that, firstly, food grade agar is less suitable than purified agar for fungal growth, and secondly, that the details of the effect of gelling agents differs between fungal isolates.

A similar picture is presented by the effect of the different gelling agents on germination, where it is clear from both experiments that any of the other gelling agents is preferable to food grade agar (Tables A2.1 and A2.2). In fact, the quality of the gelling agent may be the most important factor, as seedling development to stage 5 only occurred to a significant extent on the DNA grade agarose (Table A2.2). Sterilisation time and sterilant concentration had no significant effect on germination (Tables A2.1 and A2.2).

The overall conclusion on the effect of gelling agents is that purified agar, phytogel and DNA grade agarose are all generally more suitable for fungal growth and germination than food grade agar. The details of the effect differ between fungal isolates, between fungal growth and germination, between seed types and between germination stages, but essentially food grade agar is a poor choice of gelling agent.

The reason for this effect is likely to be the presence of contaminants in food grade agar which are removed during purification. Food grade agar is known to contain much higher concentrations of many minerals than purified agar (Oddie et al., 1994). These contaminants are likely to include those minerals present in the highest concentrations in sea water, such as sodium, chloride, sulfate, and iodide. Further investigation is necessary to determine which minerals are present in the food grade agar which are absent from purified agar, and which of these causes the inhibition of fungal growth and seed germination.
The difference in fungal growth rates between the gelling agents was often considerable, with some fungal isolates growing half as fast on the food grade agar as on the more suitable agents (Figure A2.1). The growth rates of the *Drakaea* and *Paracaleana* fungi, between 0.5 and 1 mm/day even under the most conducive conditions, are very slow compared with 2 mm/day for a fungal isolate from *Microtis media*, 3 mm/day for one from *Diuris laxiflora*, and 5 mm/day for one from *Pterostylis sanguinea* (Hollick et al., 2002). These results suggest that it may be worth while checking preference for gelling agent in slow growing fungi.

The use of a purified gelling agent appears to be even more important in germination. A preference for phytogel or purified agar is practical to check and relatively inexpensive, but DNA grade agar is prohibitively expensive for all but the most important of rare or valuable species. However, as most laboratories use purified agar rather than food grade agar as the standard gelling agent, this issue will often be of only academic interest.
Appendix 3 – Nei Genetic Distance between AFLP fingerprints of fungal isolates

A3.1 – Caladenia arenicola *complex*

Table A3.1 – Pairwise population matrix of Nei Genetic Distance for *Caladenia arenicola*, *C. huegelii* and *C. georgei*

<table>
<thead>
<tr>
<th></th>
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<td>KP1 2001 spring</td>
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<td></td>
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<tr>
<td>KP3 2001</td>
<td>0.054</td>
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<td></td>
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</tr>
<tr>
<td>KP4 2001</td>
<td>0.039</td>
<td>0.033</td>
<td>0.043</td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>KP2 2001</td>
<td>0.038</td>
<td>0.019</td>
<td>0.044</td>
<td>0.045</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>KP5 2001</td>
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<td>0.020</td>
<td>0.036</td>
<td>0.034</td>
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<td></td>
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<tr>
<td>WH 2001</td>
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<td>0.088</td>
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<td>KP1 2000</td>
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<td>0.057</td>
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<td>0.047</td>
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<td>0.052</td>
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<td>0.063</td>
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</tr>
<tr>
<td>KP1 2001 autumn</td>
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<td>0.016</td>
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<td>0.026</td>
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<td>0.068</td>
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<td>0.103</td>
<td>0.108</td>
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<td>AR 2000 C. h.</td>
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<td>0.151</td>
<td>0.132</td>
<td>0.112</td>
<td>0.119</td>
<td>0.121</td>
<td>0.070</td>
<td>0.115</td>
<td>0.122</td>
<td>0.119</td>
<td>0.095</td>
</tr>
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</table>
Table A3.2 – Pairwise population matrix of Nei Genetic Distance for *Caladenia arenicola* (all KP1 collections pooled, and *C. georgei* from AR pooled with *C. arenicola* from AR)

<table>
<thead>
<tr>
<th></th>
<th>BP</th>
<th>KP1</th>
<th>KP3</th>
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<th>KP2</th>
<th>KP5</th>
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A3.2 – Caladenia *hybrids*

Table A3.3 – Pairwise population matrix of Nei Genetic Distance for *Caladenia falcata x longicauda*

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Table A3.4 – Pairwise population matrix of Nei Genetic Distance for *Caladenia flava x latifolia*

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Table A3.5 – Pairwise population matrix of Nei Genetic Distance for *Caladenia flava x longicauda*

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Table A3.6 – Pairwise population matrix of Nei Genetic Distance for *Caladenia chapmanii x longicauda*

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Table A3.7 – Pairwise population matrix of Nei Genetic Distance for *Elythranthera brunonis x Cyanicula sericea*

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A3.3 – *Caladenia falcata*

Table A3.8 – Pairwise population matrix of Nei Genetic Distance for *Caladenia falcata*

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### A3.4 – Drakaeinae

#### Table A3.9 – Pairwise population matrix of Nei Genetic Distance for Drakaeinae

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<th>Drakaea livida</th>
<th>Drakaea thynniphila</th>
<th>Drakaea thynniphila</th>
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<th>Lyperanthus serratus</th>
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Appendix 4 – Optimisation of DNA extraction for AFLP analysis of mycorrhizal fungi of terrestrial orchids (Caladeniinae and Drakaeinae)


Abstract

Published DNA extraction methods presented a number of problems when applied to mycorrhizal fungi of native Australian terrestrial orchids. Grinding with liquid nitrogen sheared the DNA and other pulverisation methods yielded too little DNA. We found that freezing the fungal sample with liquid nitrogen, with no grinding, followed by the Qiagen DNeasy® extraction procedure, produced good yields of high molecular weight DNA. The DNA was then used for AFLP fingerprinting. Good fingerprints were produced by restriction with EcoRI/MseI enzymes, the use of pre-amplification primer mix II (for small genomes), and a 2-base extension MseI primer (m-cc) with 3-base extension EcoRI primers in the selective amplification. This protocol may be of general utility for other fungi with similarly fragile DNA.

Key words: amplified fragment length polymorphism, DNA shearing, fragile DNA, liquid nitrogen, orchid mycorrhizal fungi
A4.1 – Introduction

The Orchidaceae represents one of the most species-rich families of flowering plants with an estimated 25,000 species, many of which are highly endangered and require careful conservation and/or restoration efforts (Batty et al., 2002). Many orchids, especially terrestrial species, are dependant on a mycorrhizal fungus for germination and plant growth (Rasmussen, 1995). It is therefore essential for the conservation, as well as propagation, of orchids that we understand the genetic relationships among the mycorrhizal fungi of populations and species of orchids and particularly the level of genetic specificity of their mycorrhizal relationships (Batty et al., 2002; Rasmussen, 1995).

The purpose of this study was to develop methods for AFLP analysis for the genetic characterisation of the mycorrhizal fungi of some species of terrestrial orchids in the sub-tribes Caladeniinae and Drakaeinae from southwestern Australia (Kores et al., 2001). AFLP (amplified fragment length polymorphism) is a now widely used DNA fingerprinting technique that offers the advantages of minimal development time, high stringency and reproducibility, and potentially a large number of polymorphic markers (Mueller & Wolfenbarger, 1999). However, DNA of very high quality is critical for the success of AFLP.

DNA extraction procedures commonly used for fungi include that of Raeder & Broda (1985) and those discussed by van Burik et al. (1998). Most published methods for DNA extraction of fungi share a number of common elements:

(i) Pulverisation of the fungal sample (usually by grinding with liquid nitrogen, but other methods such as sonication and vortexing with glass beads are discussed by van Burik et al. (1998));

(ii) Addition of an extraction buffer (the composition of which varies between methods) before or after pulverisation;

(iii) Extractions with phenol, chloroform, iso-amyl alcohol, isopropanol, etc., or any combination of the above (procedures vary between methods); and
(iv) Resuspension of the DNA in a buffer, commonly TE (10 mM Tris (pH 8), 1 mM EDTA).
Alternatively, commercially available DNA extraction procedures can be used after the first stage of pulverisation.

We report here that these published methods of DNA extraction do not achieve suitable yields of DNA (in quantity and/or quality) for mycorrhizal fungi of orchids from the sub-tribes Caladeniinae and Drakaeinae (Kores et al., 2001), and describe a DNA extraction protocol which resulted in high quality DNA for AFLP. This protocol may be of general utility for other fungi with similarly fragile DNA.

A4.2 – Materials and Methods

A4.2.1 – Fungal isolation and culture maintenance
The mycorrhizal fungi were obtained by the single peloton isolation method (Batty et al., 2001b; Clements & Ellyard, 1979) from the infected organs of adult orchids of the target species (species of Caladenia, Elythranthera, and Cyanicula (sub-tribe Caladeniinae (Kores et al., 2001)), and Drakaea, Paracaleana, Spiculaea, Pyrorchis, Leporella, Lyperanthus, Chiloglottis, Caleana, and Arthrochilus (sub-tribe Drakaeinae (Kores et al., 2001))). The fungal cultures were then grown and maintained on Modified Soil Solution Equivalent (SSE) medium (0.5 mM NH₄NO₃, 0.01 mM KH₂PO₄, 0.3 mM MgCl₂.6H₂O, 1.0 mM NaCl, 0.5 mM CaSO₄.2H₂O, 0.02 mM FeEDTA(Na), 2 g L⁻¹ sucrose, 8 g L⁻¹ agar, pH 5.5). This medium is designed to contain a concentration and balance of nutrients similar to a typical Western Australian soil (Angle et al., 1991; Dellar & Lambert, 1992). The mycorrhizal nature of each fungus was confirmed by recombination with seeds of the appropriate species in symbiotic germination experiments (Batty et al., 2001b; Clements & Ellyard, 1979).
### A4.2.2 – Fungal growth in liquid culture

The fungi were subcultured into liquid culture for growth for DNA extraction. The growth rates of the fungi in liquid SSE were so slow that insufficient biomass had been produced even after several months. When an equal volume of vegetable juice (Campbell’s V8, diluted 1/5 and autoclaved) was added to the medium (Pope & Carter, 2001), sufficient biomass for DNA extraction was produced after three to four weeks, when the fungus was clearly actively growing.

### A4.2.3 – DNA extraction

The following methods of DNA extraction were compared for fungi of several *Caladenia* species:

1. Grinding in liquid nitrogen to pulverise the fungal sample, followed by one phenol/chloroform extraction, one chloroform extraction, precipitation with isopropanol, and resuspension in TE buffer (Raeder & Broda, 1985);
2. Vortexing with 0.5 g of 0.5 mm glass beads (Sigma) to pulverise the fungal sample, followed by three phenol/chloroform/iso-amyl alcohol extractions, one chloroform/iso-amyl alcohol extraction, precipitation with ammonium acetate, and resuspension in TE buffer (extraction method 1 of van Burik et al. (1998));
3. Vortexing with glass beads (as above), followed by the Qiagen DNeasy® Plant Mini Kit extraction procedure;
4. Vibrating with glass beads using a Spex CertiPrep GenoGrinder 2000, followed by the Qiagen DNeasy® Plant Mini Kit extraction procedure;
5. Grinding in liquid nitrogen with a steel pestle in a 1.5 mL eppendorf tube, followed by the Qiagen DNeasy® Plant Mini Kit extraction procedure;
6. Partial grinding in liquid nitrogen (crushing the frozen fungal sample into several pieces, rather than grinding to a powder), followed by the Qiagen DNeasy® Plant Mini Kit extraction procedure; and
7. Freezing in liquid nitrogen (no grinding), followed by the Qiagen DNeasy® Plant Mini Kit extraction procedure.
A4.2.4 – Quantification and visualisation of the DNA

The DNA was quantified on a Pharmacia Biotech GeneQuant II spectrophotometer, and visualised on a 1 % agarose gel with ethidium bromide to assess shearing.

A4.2.5 – AFLP

DNA fingerprints were produced from high quality DNA (see Results) using AFLP, adapted from the four step process described in Krauss & Hopper (2001).

1. **Restriction digest of the DNA.** Approximately 200 – 250 ng of DNA was digested with 1 µL EcoRI/MseI restriction enzyme in a reaction volume of 12.5 µL per sample, incubated at 37 °C for two hours, then at 70 °C for 15 minutes before cooling on ice.

2. **Ligation of adapters.** 0.5 µL of T4 DNA ligase and 12 µL of adapter/ligation solution were added to each digested sample, which were then incubated at 20 °C overnight.

3. **Preselective amplification by PCR.** 1.25 µL of the ligation mix was added to 10 µL of pre-amplification primer solution (pre-amp primer mixes I and II were both tested), 1.25 µL of 10x PCR buffer for AFLP, 0.1 µL of Taq DNA polymerase, 0.375 µL of MgCl₂, and 0.375 µL of water in a PCR plate. PCR was performed for 20 cycles of 94 °C for 30 seconds, 56 °C for 2 minutes, 72 °C for 2 minutes. The pre-amplification product was diluted 1:30 in 0.1 M TE buffer.

4. **Selective amplification by PCR.** 1.25 µL of each diluted, pre-selective DNA sample was added to 1 µL of 10x PCR buffer, 0.05 µL of Taq DNA polymerase, 0.3 µL of MgCl₂, 3.4 µL of water, 0.2 mM of each of four dNTPs, and primers (fluorescently labelled EcoRI primers e-act, e-agg, and e-acc; the 2 and 3 base MseI primers m-ca, m-ct, m-cg, m-cc, m-ctg, m-ctc, and m-cac were tested). PCR was performed for 3 cycles of 94 °C for 10 minutes, 70 °C for 2 minutes, 72 °C for 2 minutes, then 8 touchdown cycles of 94 °C for 30 seconds, 69 °C for 2 minutes, 72 °C for 2 minutes, then 23 cycles of 94 °C for 30 seconds, 61 °C for 2 minutes, 72 °C for 2 minutes, followed by a 60 °C hold for 30 minutes.

All AFLP reagents were purchased in kit form from Invitrogen (Carlsbad, CA, USA). PCR was performed on an Applied Biosystem GeneAmp 9700 Thermocycler. The
fluorescently labelled amplified fragments were analysed by gel electrophoresis (6% polyacrylamide gels) by an ABI Prism 377 Automated DNA Sequencer. Fragments were sized through the inclusion of GeneScan Rox 500 internal size standard (Applied Biosystems, Warrington, UK). Multi-locus profiles were visualised by ABI GeneScan software (Applied Biosystems, Foster City, USA). AFLP profiles were scored for the presence/absence of fragments between 50 and 500 bp, as well as for fragment intensity as determined by peak height.

**A4.3 – Results and discussion**

**A4.3.1 – DNA extraction, quantification and visualisation**

DNA extraction methods 1 and 5, which involved grinding with liquid nitrogen, sheared the DNA (Table A4.1). Methods 2 and 3, which involved vortexing with glass beads, produced extremely little DNA. The quality of the DNA was higher in method 2 than in 1 and 5, and very good in method 3, but the concentrations were too low to be usable. In method 4, using the GenoGrinder with glass beads, a number of different speed and time settings were tried, but all produced either no DNA or sheared DNA. Method 7, involving freezing with liquid nitrogen (no grinding), produced a large quantity of good quality DNA. More DNA was produced in method 6 when the fungal sample was partially ground, but this sheared the DNA.
Table A4.1 – Quantity and quality of DNA recovered by each of seven extraction methods from mycorrhizal fungi of *Caladenia arenicola*.

<table>
<thead>
<tr>
<th>No</th>
<th>Method</th>
<th>260/280 Ratio</th>
<th>DNA (ng/µL)</th>
<th>DNA sheared?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Raeder &amp; Broda (1985) (grinding with liquid nitrogen)</td>
<td>No reading</td>
<td>No reading</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Van Burik et al. (1998) method 1 (vortexing with glass beads)</td>
<td>No reading</td>
<td>No reading</td>
<td>Nothing visible on agarose gel</td>
</tr>
<tr>
<td>3</td>
<td>Qiagen DNeasy® (vortexing with glass beads)</td>
<td>1.67</td>
<td>0.4</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>Qiagen DNeasy® (GenoGrinder with glass beads)</td>
<td>No reading</td>
<td>No reading</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Qiagen DNeasy® (grinding with liquid nitrogen)</td>
<td>1.71</td>
<td>267.2</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>Qiagen DNeasy® (partial grinding with liquid nitrogen)</td>
<td>1.71</td>
<td>274.2</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>Qiagen DNeasy® (freezing with liquid nitrogen – no grinding)</td>
<td>1.67</td>
<td>93.5</td>
<td>No</td>
</tr>
</tbody>
</table>

A4.3.2 – AFLP

During the AFLP process, it was found that preselective amplification with pre-amp primer mix II (for small genomes) and selective amplification with a 2-base extension *MseI* primer (m-cc) and 3-base extension *EcoRI* primers (e-act, e-agg, and e-acc) produced excellent quality DNA fingerprints with consistent amplification and a good number and spread of peaks. Figure A4.1 shows several restriction digest products of DNA extracted by method 7 (Table A4.1) from mycorrhizal fungi of *Caladenia arenicola* and restricted with *EcoRI/MseI* enzymes.
Figure A4.1 - Restriction digest products (of DNA extracted by method 7 (Table A4.1) from mycorrhizal fungi of *Caladenia arenicola* and restricted with *EcoRI/MseI* enzymes) as visualised on a 1% agarose gel with ethidium bromide (80 V for 60 minutes). Lane 1 shows a low DNA mass ladder (2 µL loaded) with fragment sizes of 2000, 1200, 800, 400, 200, and 100 bp (top to bottom). Lanes 2-15 show restriction digest products of DNA extracted by method 7 from fourteen different isolates of mycorrhizal fungi from *Caladenia arenicola* (10 µL loaded).

The quality of the DNA produced by method 7 was sufficiently high for AFLP fingerprinting. Examples of AFLP electropherograms from DNA extracted by method 7 are shown in Figure A4.2. Many AFLP fingerprints of orchid mycorrhizal fungi have been subsequently generated, all from DNA extracted by method 7. The reproducibility of the technique is excellent, with identical fingerprints generated by different DNA samples from the same fungal isolate. It has also proven possible to detect differences between fungal isolates at a number of different scales (individual, population, species and genus levels) and to discriminate between these scales.
Figure A4.2 – AFLP electropherograms from DNA extracted by method 7 (Table A4.1) of mycorrhizal fungi of Caladenia arenicola. A: Isolate 1 - selective amplification with the fluorescently labelled EcoRI primer e-agg and the 2 base MseI primer m-cc. B: Isolate 2 - selective amplification with the fluorescently labelled EcoRI primer e-agg and the 2 base MseI primer m-cc. C: Isolate 1 - selective amplification with the fluorescently labelled EcoRI primer e-agg and the 3 base MseI primer m-ctg. D: Isolate 2 - selective amplification with the fluorescently labelled EcoRI primer e-agg and the 3 base MseI primer m-ctg.

We have now generated AFLP fingerprints for mycorrhizal fungi from Caladenia (13 species and hybrids), Elythranthera (1 species), Cyanicula (1 species), Drakaea (4 species), Paracaleana (1 species), Spiculaea (1 species), Pyrorchis (1 species),
Leporella (1 species), Lyperanthus (1 species), Chiloglottis (2 species), Caleana (1 species), and Arthrochilus (1 species). The fungi from Caladenia, Elythranthera and Cyanicula (which are closely related genera within the sub-tribe Caladeniinae (Kores et al., 2001)) all had particularly fragile DNA. Fungi from the other genera (which form another group of related genera in the sub-tribe Drakaeinae (Kores et al., 2001)) sometimes produced unsheared DNA when partially ground, but sheared when fully ground with liquid nitrogen. This emphasises the extent of the issue of fragile DNA for orchid mycorrhizal fungi.

The problem of fragile DNA from filamentous fungi has been noted by van Burik et al. (1998), who found that grinding with liquid nitrogen frequently sheared DNA of human pathogenic fungi, and it has also been reported for non-fungal DNA (Nair et al., 1999; Borneman & Triplett, 1997; Carmelo et al., 2000). Alternatives and implications have not, to our knowledge, been discussed. The fragility of our fungal DNA and extent of shearing was surprising, as shearing has not been noted as a problem with other orchid mycorrhizal fungi. Most published genetic studies of orchid mycorrhizal fungi have used site specific sequencing rather than an arbitrary multilocus technique requiring high quality DNA such as AFLP (for example Pope & Carter, 2001; Sen et al., 1999; Taylor & Bruns, 1999), so some shearing may have been acceptable. However, the level of shearing produced by grinding with liquid nitrogen in this study rendered the DNA unusable even for sequencing, so the level of shearing found in this study may have been higher than in other studies. The difference may lie in characteristics of the cell walls or of the DNA itself. It does appear from the literature that DNA shears more readily if the organism lacks a cell wall (Nair et al., 1999; Borneman & Triplett, 1997; Carmelo et al., 2000), and culture conditions can affect hyphal wall thickness.

The simplicity of this DNA extraction procedure, involving freezing in liquid nitrogen (no grinding), followed by use of the Qiagen DNeasy® Plant Mini Kit, makes it attractive for use on other fungal species, particularly where DNA shearing is a problem.
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