Geographical range and host breadth of *Sebacina* orchid mycorrhizal fungi associating with *Caladenia* in south-western Australia

RYAN D. PHILLIPS\(^1,2,3,\ast\), MATTHEW D. BARRETT\(^2,3\), EMMA L. DALZIEL\(^2,3\), KINGSLEY W. DIXON\(^2,3,4\) and NIGEL D. SWARTS\(^5,6\)

\(^1\)Evolution, Ecology and Genetics, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia
\(^2\)Botanic Gardens and Parks Authority, Fraser Ave, West Perth, WA 6005, Australia
\(^3\)School of Plant Biology, The University of Western Australia, Crawley, WA 6009, Australia
\(^4\)Department of Agriculture and Environment, Curtin University, Kent Street, Bentley, WA 6012, Australia
\(^5\)Royal Tasmanian Botanical Gardens, Queens Domain, Hobart, TAS 7000, Australia
\(^6\)Tasmanian Institute of Agriculture, University of Tasmania, Private Bag 98, Hobart, TAS 7000, Australia

Received 3 February 2016; revised 13 May 2016; accepted for publication 31 May 2016

Specialized mycorrhizal interactions have the potential to limit the geographical range of plant species and contribute to reproductive isolation. We investigated these predictions in *Caladenia* (Orchidaceae) from south-western Australia, a group known to have specialized mycorrhizal associations with the genus *Sebacina* s.l. Sequencing of fungal isolates from 47 of the 136 species of Western Australian *Caladenia* was undertaken to resolve the geographical range and habitat preferences of mycorrhizal fungal operational taxonomic units (OTUs) and their host breadth in *Caladenia*. Eight different fungal OTUs were used by *Caladenia*, with the more frequently detected OTUs occurring in a wide range of habitats and geographical regions. Given the comparatively narrow geographical ranges of most Western Australian *Caladenia* taxa, this suggests that the geographical ranges of fungal OTUs are unlikely to limit the geographical range of *Caladenia* spp. Extensive sharing of fungal OTUs between closely related orchid species was detected, suggesting that in the main there is little potential for mycorrhizal fungi to contribute to reproductive isolation between *Caladenia* spp. Our data mostly support previous work suggesting high mycorrhizal specificity in *Caladenia*, but this may not be the case in all subgenera, highlighting that *Caladenia* may offer powerful opportunities for investigating the evolution of specialized mycorrhizal associations. © 2016 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2016, **182**, 140–151

**ADDITIONAL KEYWORDS:** biogeography – endophytes – mycorrhiza – operational taxonomic units – orchid – rarity – reproductive isolation – specialization.

**INTRODUCTION**

Most plants have generalist mycorrhizal associations (Brundrett, 2009; but see Bidartondo & Bruns, 2005), but highly specialized relationships have evolved repeatedly in Orchidaceae, sometimes involving just a single fungal species (Ogura-Tsujita & Yukawa, 2008; Bougoure et al., 2009; Swarts et al., 2010; Phillips et al., 2011a; Linde et al., 2014). As orchids have minute, dust-like seeds that typically lack an endosperm (Arditti & Ghani, 2000), terrestrial orchids are dependent on mycorrhizal fungi for germination and annual growth, with the fungi providing organic carbon and mineral nutrients (Cameron, Leake & Read, 2006). As seen in other forms of ecological interactions (Pauw & Bond, 2011; Phillips et al., 2014), this high level of specialization, combined with a physiological dependence, creates
the possibility that the geographical range and habitat of the plant could be constrained by the distribution and ecology of its interacting partner.

Despite the relative prevalence of specialized interactions in some groups of orchids, investigations across multiple spatial scales have found limited evidence for mycorrhizal associations limiting their geographical range. Seed burial experiments have demonstrated that although there are numerous microsites with fungi that are capable of supporting germination within populations, most remain unoccupied (Rasmussen & Whigham, 1993; Baty et al., 2001a; Phillips et al., 2011a; Tešitelová et al., 2012; McCormick & Jacquemyn, 2014; but see McCormick et al., 2012). Further, at larger geographical scales (e.g. biogeographic regions), evidence suggests that the fungal species associating with orchids have wide distributions and broad habitat tolerances (Waterman et al., 2011; McCormick & Jacquemyn, 2014; Davis et al., 2015). However, data on most of these fungal species remain limited due to the reliance on molecular methods to detect them. Consequently, the geographical range and habitat preferences of most mycorrhizal fungi and their role in constraining orchid distribution at present remain poorly resolved.

Mycorrhizal specialization could also contribute to reproductive isolation between orchid species, potentially leading to speciation (Taylor et al., 2003). For example, in Corallorhiza striata Lindl., each lineage of orchids is associated with a specific clade of Tomentella mycorrhizal fungi (Barrett et al., 2010). Should divergent lineages of orchids evolve an incompatibility with the reciprocal clade of mycorrhizal fungi, this could generate reproductive isolation. The contribution of mycorrhizal fungi to reproductive isolation will be strongly influenced by the range of orchid species with which each fungal species associates. Evaluation of this possibility requires the sampling of multiple co-occurring orchid species. Some studies have shown that closely related orchid species tend to share genetically defined fungal operational taxonomic units (OTUs), but use them in different relative frequencies (Wright et al., 2010; Jacquemyn et al., 2011a, b, 2012; Tešitelová et al., 2013). In an extreme case of fungal sharing, several co-occurring species of Drauchaea Lindl. all share a single species of Tulasnella, as defined by multiple sequence loci (Phillips et al., 2011a; Linde et al., 2014). Due to frequent fungal sharing, in some of these instances mycorrhizas are unlikely to contribute strongly to reproductive isolation. However, it often remains unconfirmed whether orchids are forming a symbiotic interaction with the fungi detected through sequencing, raising the possibility that closely related orchids may require different specific fungi for growth, but have other species present as non-beneficial (and non-pathogenic) endophytes.

The recent proliferation of molecular studies of orchid mycorrhizal relationships has revealed that southern Australia is characterized by an unusually high incidence of specialized mycorrhizal associations relative to other orchid floras (Phillips et al., 2011a). There have been multiple evolutionary origins of mycorrhizal specialization in the Australian orchid flora, with highly specialized species already known from approximately seven orchid genera (Bougoure et al., 2009; Roche et al., 2010; Swarts et al., 2010; Wright et al., 2010; Otero et al., 2011; Phillips et al., 2011a; Linde et al., 2014; Davis et al., 2015). Southern Australia has a diverse, but highly threatened terrestrial orchid flora, with > 1000 species (Jones, 2006), over 170 of which are currently federally listed as rare or threatened (Department of Environment, 2015). Although there is a trend for groups with specialized pollination systems to exhibit a high level of intrinsic rarity, the strongest correlate with rarity is the occupation of unique or naturally fragmented edaphic environments (Phillips et al., 2011b). The diversity of the Australian orchid flora, and the association between rarity and edaphic environment, raises the question of whether mycorrhizal specialization or narrow geographical ranges of fungi have contributed to intrinsic rarity (i.e. natural rather than human induced rarity) and speciation of orchids.

Of the orchid genera with specialized mycorrhizal associations, Caladenia R.Br. is one of the most diverse, with > 370 recognised species and subspecies (Phillips et al., 2009a) in six subgenera (Hopper & Brown, 2001). The centre of diversity of the genus is south-western and south-eastern Australia, but a small number of species are found in New Zealand, with one species occurring as far as New Caledonia and Indonesia (Phillips et al., 2009a). Although Caladenia taxa occur in a wide range of habitats, ranging from subalpine woodlands to the margins of the arid zone, the areas of highest diversity are in temperate woodlands and forests, where numerous species often co-occur (Jones, 2006; Hoffman & Brown, 2011). Caladenia is of particular interest due to the high number of intrinsically rare species, typically associated with local soil types or unusual geological features (Phillips et al., 2011b). Furthermore, Caladenia contains > 70 taxa currently federally listed as rare or threatened (Department of Environment, 2015), many being naturally (intrinsically) narrow range endemics. The species investigated so far have all associated with either one or few putative species (OTUs) of Sebacina s.l. (Swarts et al., 2010; Wright et al., 2010) in ‘Group B’ of Sebacinales (Oberwinkler et al., 2014).

© 2016 The Linnean Society of London, Botanical Journal of the Linnean Society, 2016, 182, 140–151
Here we focus on the ecology of mycorrhizal fungi associated with *Caladenia* in south-western Australia. We sequenced mycorrhizal fungi isolated from 47 *Caladenia* spp. to test: 1. How many fungal OTUs are associated with Western Australian *Caladenia*? 2. What is the distribution and habitat breadth of mycorrhizal fungi associated with Western Australian *Caladenia*? 3. What is the phylogenetic breadth of *Caladenia* with which mycorrhizal fungi will associate? Resolution of these questions will aid in understanding if mycorrhizal fungi are likely to either constrain geographical range or contribute to reproductive isolation in *Caladenia*.

**METHODS**

**Sampling design and methodology**

Our sampling strategy was designed to gain an estimate of the total number of OTUs associating with *Caladenia* in south-western Australia and to maximize the chance of detecting fungal OTUs associating with multiple orchid species. As such, we opted to sample fungi from a small number of individuals for a large number of orchid species. To maximize the phylogenetic breadth of orchid species sampled, we sampled fungi from all four subgenera of *Caladenia* present in south-western Australia (subgeneric classification follows Hopper & Brown, 2001). Seventy-eight fungal isolations across 47 *Caladenia* spp. were performed, sampling one to five orchid individuals per species (Supporting Information, Tables S1 and S2; due to issues relating to sampling permits, representative vouchers of some but not all species were submitted to the Western Australian Herbarium). These data were supplemented with accessions on GenBank, giving fungal isolate sequences for 49 Western Australian *Caladenia* spp. across 62 sites, representing 36.0% of the 136 Western Australian *Caladenia* spp. (Brown & Brockman, 2015). In addition, for the OTUs detected in the present study, we searched GenBank for sequences from orchids outside Western Australia and from studies outside orchids (e.g. environmental DNA or other types of mycorrhiza).

To resolve the breadth of habitats in the South-west Australian Floristic Region that each OTU occurs, sampling was conducted from *Caladenia* spp. in each of the biogeographic regions in south-western Australia (Hopper & Gioia, 2004; Phillips et al., 2011b), encompassing the full range of habitats occupied by Western Australian *Caladenia*. At each sampling location the soil type and vegetation community was recorded. Given the wide variety of soil types encountered in terms of colour, origin and particle size, broad categories of soil types were sufficient to distinguish between habitat types without quantifying traits such as pH and soil nutrients. To quantify the climatic conditions encountered by each OTU, temperature and rainfall records were collated from the nearest weather station (using Bureau of Meteorology data as of December 2013) to the sampling location of the corresponding orchid mycorrhizal fungus (OMF) DNA sequence (average distance to station = 19.6 ± 15.3 (SD) km; range = 0.9–54.4 km). The variables quantified are regularly used for bioclimatic niche models (Xu & Hutchinson, 2011): annual rainfall, rainfall in the wettest quarter, rainfall in the driest quarter, rainfall in the wettest month, mean maximum temperature of the warmest month, mean maximum temperature of the coldest month and mean minimum temperature for the coldest month. As annual rainfall is highly correlated with biogeographic regions in south-western Australia (Hopper & Gioia, 2004; Phillips et al., 2011b), we focused on this variable for quantifying climatic conditions.

In *Caladenia*, OMF colonize a ‘collar’ region of the underground stem subtending the leaf (Ramsay, Dixon & Sivasithamparam, 1986), where they reside as pelotons of undifferentiated hyphae within the cells of the cortex (Dixon & Tremblay, 2009). During collection, the collar region of the orchid was either sectioned with a scalpel allowing the plant to remain intact or the whole stem was removed. Plant material was placed in moist paper towel and stored at 4 °C for a maximum of 3 days until processing (usually 1 day). In the laboratory, plant material was rinsed with water to remove soil particles and surface contaminants. Under sterile conditions, pelotons were excised from the cortical cells, rinsed thoroughly through three successive changes of autoclaved deionized water, and one randomly selected peloton was individually plated onto nutrient agar medium.

**Figure 1.** Phylogenetic analysis of mycorrhizal fungi isolated from Western Australia *Caladenia* (Orchidaceae), using a maximum likelihood analysis of the ITS sequence locus. Numbers above the line are bootstrap values from a maximum likelihood analysis, numbers below the line are posterior probabilities from a Bayesian analysis. Squares denote fungal isolates from orchids. Note that *Sebacina vermifera* was the only named fungus in any of the clades of orchid fungi, suggesting that most of these OTUs belong to undescribed entities. Black = *Caladenia* subgenus *Calonema*, White = *Caladenia* subgenus *Phlebochilus*, Red = *Caladenia* subgenus *Drakonorchis*, Blue = *Caladenia* subgenus *Elevatæ*, Yellow = other genera in Caladeniinae. # denotes samples from south-eastern Australia.
containing 1% streptomycin as described in Bonnardeaux et al. (2007). In all cases hyphal growth was observed. Single hyphal tips chosen arbitrarily were subcultured onto new plates to ensure that only a single fungal entity would be cultured on each plate. Clean cultures were further subcultured onto Petri plates containing potato dextrose agar (PDA) for DNA sequencing. Only one culture per plant was sequenced.

DNA SEQUENCING AND PHYLOGENETIC ANALYSIS

Approximately 100 mg of hyphae was scraped from the plate surface of each fungal isolate and ground in 1 mL lysis buffer (2% CTAB, 0.1 M Tris–HCl, 1.4 M NaCl, 0.02 M EDTA) with 5 μl RNase using a plastic pestle. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Melbourne, Australia) following the manufacturer’s instructions. Genomic DNA was amplified by polymerase chain reaction (PCR) using the universal fungal primers ITS1 (5'-TCCGTAAGT GAACTTGCCG-3') and ITS4 (5'-TCCCTCCGTTATT GATATGC-3') (White, Bruns & Taylor, 1990), amplifying ca. 550 base pairs of ITS1, 5.8S and ITS2 of nuclear rDNA. PCR conditions and preparation for sequencing followed Swarts et al. (2010). All fungal cultures yielded PCR products, demonstrating that these primers were appropriate for amplifying the study taxa. Bi-directional sequencing was undertaken by Macrogen Inc. (Seoul, Korea). Returned sequences were edited using Geneious Pro v7.1.4. (Kearse et al., 2012). BLAST searches (Altschul et al., 1997) of the NCBI sequence database (http://www.ncbi.nlm.nih.gov) were performed to assess the identity of our sequences and identify suitable outgroups. Included in the alignment were several sequences derived from other Western Australian Caladenia spp. (Swarts et al., 2010; Sommer et al., 2012), Sebacina isolated from other Western Australian orchid genera and several outgroups from Sebacinaeae s.s. (= Sebacinales Group A) (see Supporting Information, Table S1). A MUSCLE sequence alignment using default parameters was undertaken in Geneious v7.1.4. (Kearse et al., 2012), followed by manual editing of gap boundaries.

Phylogenetic analyses were undertaken using both maximum likelihood and Bayesian approaches. A maximum likelihood phylogenetic analysis with 1000 replicate bootstraps was performed using PHYML (Guindon & Gascuel, 2003) via a plug-in in Geneious v7.1.4 (Kearse et al., 2012). A Bayesian analysis was performed using MrBayes v3.2.3 through the CIPRES portal (Miller, Pfeiffer & Schwartz, 2010). The analysis involved two parallel runs with four chains, running for three million generations with sampling every 1000 generations. The first 60 000 generations were discarded as burn-in. The analysis ceased when the average standard deviation of the split frequencies reached < 0.01 after c. 1.6 million generations. Both sets of analyses were performed using the GTR + G model of nucleotide substitution, as all other models are nested within this model. Results of the phylogenetic analyses were viewed in Figtree v1.4.2 (Rambaut, 2014).

Fungal OTUs were defined using a 3% sequence divergence cut-off value, which has been used in previous ecological research (Tedersoo et al., 2003, 2008; Kartzinel, Trapnell & Shefferson, 2013). Although lower values (e.g. 1%) have been used in some community studies focused on a single site, we consider a higher level of divergence to be appropriate for the 800-km spatial scale of the present study, given that fungal species are expected to show phylogeographic structure. Further, the 3% cut-off was found to correlate well with OTUs identified from multilocus species delimitation methods in Tulasnellia (Linde et al., 2014). For each clade with fungi isolated from Western Australian Caladenia, the maximum level of sequence divergence within the OUT and the minimum genetic divergence between OTUs were measured in Geneious Pro v7.1.4. (Kearse et al., 2012), calculated from the percentage of differing base pairs between all pairwise comparisons of fungal sequences.

GERMINATION TESTING OF OMF ISOLATES

To confirm that mycorrhizal fungi isolated from the orchids were indeed functional in the lifecycle of the orchid, a subset of cultures was chosen for in vitro seed germination (Supporting Information, Table S1). These fungi were isolated from ten species of Caladenia subgenus Calonema (Lindl.) Hopper & A.P.Br, three species of subgenus Phlebochilus (Benth.) Hopper & A.P.Br and one species of subgenus Drakonorchis Hopper & A.P.Br. In all cases fungi were tested for germination efficacy with seeds of the same species from which they had been isolated. This trial supplemented existing data for C. huegelii Rehb.f., C. arenicola Hopper & A.P.Br, C. longicauda Lindl. (subgenus Calonema) and C. flav R.Br. (subgenus Elevatae Hopper & A.P.Br) already presented in Swarts et al. (2010). Orchid seed sourced from the same orchid population as the selected OMF isolate was placed onto 25% oatmeal agar plates as described in Swarts et al. (2010) and a 0.5 cm² block of nutrient agar containing the OMF isolate was subcultured onto the plate. Germination plates were incubated in the dark for 4 weeks at 21 °C. After an additional 4 weeks under artificial light, germination was scored following Ramsay et al. (1986). The OMF association was considered functional when seedlings reached the green leaf.
stage (stage 3 as per Batty et al., 2001b; modified from Ramsay et al., 1986). Although there are examples of orchids germinating with a broader range of fungi in vitro than in situ (Masuhara & Katsuy, 1994), it should be noted that in C. huegelii the fungal OTU from adult plants also supports germination in the field (Swarts et al., 2010).

RESULTS

PHYLOGENETIC ANALYSIS

By combining phylogenetic analysis and 3% cut-off levels of sequence divergence of the ITS region, we inferred the presence of eight fungal OTUs associated with Western Australian Caladenia, all of which belonged to the genus Sebacina (Fig. 1, identical sequences removed for analysis in Supporting Information, Table S3). In the maximum likelihood analysis, bootstrap support for each OTU was generally high (> 90%). Maximum levels of sequence divergence within OTUs ranged from 0.2% to 2.7%, whereas the minimum level of sequence divergence between OTUs ranged from 3.5% to 13.4%. The delineation of sequences into OTUs was largely congruent regardless of whether a 3% or 5% cut-off was used. However, if a cut-off value of 5% is used then samples from south-eastern Australian would also be considered part of OTU4. OTUs 2 and 3 also exhibited < 5% sequence divergence from each other, but we have not used this classification, as these OTUs are sympatric over several 100 km without intermediate genotypes and OTU3 is more closely related to other OTUs in the phylogenetic analysis than OTU2. When seed was sown on fungi isolated from the same orchid species, all of the tested isolates yielded germination through to at least the origin of the leaf (Supporting Information, Table S1).

Of the eight OTUs detected, three of them accounted for 85% (80 out of 94) of the isolates sequenced from Western Australian Caladenia. Of the three fungal OTUs that were regularly detected (observed more than ten times), all occurred in a broad range of vegetation communities, soil types, climatic conditions and biogeographic regions (Table 1; Fig. 2; Supporting Information, Table S4). Among the three fungal OTUs that were infrequently detected (N = 3–4), they were typically detected from more than one broad habitat type and biogeographic region. OTU 4 and OTU 6 were detected too infrequently to speculate on their habitat range. Using GenBank accessions, OTUs 1, 2, 5 and 7 were also recorded in south-eastern Australia. However, there were no cases of OTUs associated with Caladenia being recorded outside Australia or from plants other than orchids.

The number of Caladenia spp. that each OTU associated with ranged from one to 21 (Table 2). Among the three OTUs that were regularly detected, all associated with a wide range of orchid species (> 10). Although the two most commonly recorded OTUs were found to associate with three different subgenera of Caladenia, there was a strong bias in the subgenus with which each most frequently associated. Out of the 45 times that OTU 1 was detected from Caladenia, on 38 occasions it was associated with subgenus Calonema, and out of the 21 times that OTU 2 was detected, on 19 occasions it was associated with subgenus Phlebochilus. OTU 3, which was detected on 14 occasions, was only found to associate with subgenus Calonema.

Each of the orchid subgenera showed a different pattern of association with OTUs, with subgenus Calonema associating primarily with OTUs 1 and 3, subgenus Phlebochilus associating with only OTUs 1 and 2, and subgenus Elevatae associating with six different OTUs, despite much more limited sampling (Table 2). This study was not designed to test for specificity of the orchids, but when multiple isolates were included from the same orchid species they always used the same OTU, except for C. flava (three OTUs from five samples) and C. longicauda (three OTUs from six samples).

DISCUSSION

MYCORRHIZAL FUNGI OF SOUTH-WEST AUSTRALIAN CALADENIA

Sequencing of mycorrhizal fungi isolated from 47 south-western Australian Caladenia spp. confirmed the initial findings of Swarts et al. (2010), Wright et al. (2010) and Sommer et al. (2012) that the genus associates exclusively with members of the genus Sebacina s.l. Using a phylogenetic analysis of the ITS region, we detected eight Sebacina OTUs. Based on a cut-off of 3% sequence divergence and the broad sympathy exhibited by these clades we believe it is likely that these OTUs represent distinct biological species. Indeed, an analysis using multiple newly developed sequence loci has suggested that ITS alone and a cut-off of 3% provides a close approximation of species boundaries in this group of Sebacina (Ruibal et al., 2014). Three main clades of Sebacina were regularly recorded associating with Caladenia, with an additional five OTUs being occasionally detected. By relying on orchids to detect fungi, we cannot resolve whether these five infrequently detected OTUs are less common, have a narrower host range or associate with orchids less frequently.
Habitat types and biogeography of *Sebacina* orchid mycorrhizal fungi

The mycorrhizal fungi associating with *Caladenia* all had broad geographical ranges in south-western Australia (including OTUs with three or more samples), being recorded from between two and eight biogeographic provinces. By comparison, approximately half of all south-western Australian *Caladenia* spp. occur in just a single botanical province (Phillips et al., 2009a). The most commonly used fungal OTUs all occurred in a diverse range of plant communities and soil types, typically encompassing sands, clay loams and granitic soils. Similarly, the well sampled fungal OTUs occurred across a rainfall gradient of > 500 mm. This is a broader range of edaphic and climatic conditions than is seen in the majority of Western Australian *Caladenia* (Hopper & Brown, 2001; Hoffman & Brown, 2011). Further, Davis et al. (2015) demonstrated that some of the fungi found associating with south-western Australian orchids are also present in south-eastern Australia, despite being separated by the arid Nullarbor Plain. As such, there is no evidence that the absence of suitable mycorrhizal fungi constrains the overall geographical range of orchid species associating with *Sebacina* in south-western Australia. However, at the microsite scale, mycorrhizal fungi could still play a role in determining the distribution of orchids through either a patchy distribution (McCormick et al., 2012), low inoculum potential of the fungus or interactions between the environment and the ability of the orchid to form an effective symbiosis with the

### Table 1. Summary of habitat and biogeographic information for *Sebacina* fungi isolated from Western Australian *Caladenia*

<table>
<thead>
<tr>
<th>OTU</th>
<th>N</th>
<th>Vegetation community</th>
<th>Rainfall range (mm)</th>
<th>Soil types</th>
<th>Biogeographic region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td><em>Agonis</em> woodland, <em>Banksia</em> woodland, eucalypt woodland, jarrah forest, karri forest, mallee shrubland</td>
<td>384–1301</td>
<td>Clay loam, grey sand, granitic sand, laterite</td>
<td>EM, LN, NS, S, SF, SoW, SwW</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td><em>Acacia</em> shrubland, <em>Allocasuarina</em> woodland, eucalypt woodland, granite in jarrah forest, jarrah forest, mallee shrubland, <em>Melaleuca</em> shrubland, sandplain heath, salt-lake shrubland</td>
<td>306–1077</td>
<td>Clay loam, sandy loam, granitic sand, gravelly sand, moist grey sand, pale-brown sand, red sand</td>
<td>DS, F, K, LN, NS, NW, S, SoW, SwW</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td><em>Agonis</em> woodland, <em>Allocasuarina</em> woodland, <em>Banksia</em> woodland, eucalypt woodland, jarrah forest, low heathland, <em>Melaleuca</em> swamp, shrubland on granite, swampy flats</td>
<td>425–967</td>
<td>Clay loam, moist loam, sandy loam, granitic sand, grey sand,</td>
<td>LN, NS, SF</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Unknown</td>
<td>635–707</td>
<td>Unknown</td>
<td>B, S</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td><em>Banksia</em> woodland</td>
<td>537–829</td>
<td>Grey sand</td>
<td>B, S</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Granite heathland</td>
<td>596</td>
<td>Granite</td>
<td>E</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td><em>Agonis</em> woodland, <em>Allocasuarina</em> woodland, granite in jarrah forest</td>
<td>385–1077</td>
<td>Grey sand, sandy loam, loam</td>
<td>LN, S, SwW</td>
</tr>
</tbody>
</table>

Biogeographic regions based on those specifically identified for the orchid flora in Phillips et al. (2011b).

Biogeographic regions: B, Brookton; DS, Darkan-Stirlings; E, Esperance; EM, Esperance Mallee; F, Fitzgerald; K, Kalbarri; LN, Leeuwin-Naturaliste; NS, Northern Sandplain; NW, Northern Wheatbelt; SF, Southern Forests; S, Swan Coastal Plain; SoW, Southern Wheatbelt; SwW, South-west Wheatbelt.
fungus. These possibilities could be evaluated by combining manipulations of environmental variables that are likely to affect the formation of symbioses with independent PCR assays of fungal availability in the soil using specific primers (McCormick & Jacquemyn, 2014).

Comparison with the geographical ranges and biogeographic patterns in other lineages of mycorrhizal fungi is challenging, chiefly due to the reliance on detecting them through molecular studies of mycorrhizal or soil fungal communities. The available information supports the notion of broad geographical ranges in some taxa, across a range of fungal genera (McCormick & Jacquemyn, 2014), although methodological limitations make it more challenging to confirm small range size. However, the broad

Figure 2. Geographical range of fungal operational taxonomic units (OTUs) isolated from Western Australian orchids. Each of these fungal OTUs is in the genus Sebacina s.l. Biogeographic regions are based on those identified in Phillips et al. (2011b). When more than one OTU was recorded at a site, colours representing both OTUs have been shown.

Table 2. Summary of fungal OTUs used by different subgenera of Caladenia

<table>
<thead>
<tr>
<th>OTU</th>
<th>Total N</th>
<th>C</th>
<th>P</th>
<th>D</th>
<th>E</th>
<th>Number of Caladenia spp.</th>
<th>Maximum sequence divergence (within OTUs), %</th>
<th>Minimum sequence divergence (between OTUs), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>38</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>21</td>
<td>2.7</td>
<td>13.4</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>1</td>
<td>19</td>
<td>0</td>
<td>1</td>
<td>15</td>
<td>2.4</td>
<td>4.8</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>1.3</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0.2</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1.0</td>
<td>5.1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>5.1</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2.1</td>
<td>7.2</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0.8</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Minimum sequence divergence is with the most similar OTU.

N, the number of fungal isolates; C, subgenus Calonema; P, subgenus Phlebochilus; D, subgenus Drakonorchis; E, subgenus Elevatae.

© 2016 The Linnean Society of London, Botanical Journal of the Linnean Society, 2016, 182, 140–151
geographical ranges of these mycorrhizal fungi OTUs parallel findings with southern Australian macrofungi. Only 10.6% of macrofungi occurring in southwestern Australia are thought to be endemic to this region (May, 2002). Further, several relatively recently described species have subsequently been collected from south-eastern Australia (May, 2002). These findings for both mycorrhizal fungi and macrofungi are in contrast with the flora of south-western Australia, which contains high levels of endemicism at both the species and genus levels (Hopper, 1979; Hopper & Gioia, 2004).

**PHYLOGENETIC PATTERNS OF HOST BREADTH: REPRODUCTIVE ISOLATION**

The association of a diverse range of orchid species with each of the most widespread fungal clades suggests that mycorrhizas are unlikely to contribute to reproductive isolation in many *Caladenia* spp. In particular, in *Caladenia* subgenus *Phlebochilus*, 79% of sampled individuals associated with OTU 2, indicating a high degree of phylogenetic conservatism, and preventing fungus-mediated reproductive isolation. A similar situation exists in *Caladenia* subgenus *Calonema*, in which the vast majority of isolates was either OTU 1 or OTU 3. However, if orchid species were specific to a single OTU, then rare partner shifts could still contribute to reproductive isolation. The potential for this scenario to arise is highlighted in *Caladenia huegelii* (subgenus *Calonema*), which germinates on OTU 1 but not OTU 3 (Swarts et al., 2010). A more detailed test of reproductive isolation would require sampling fungi from multiple individuals per orchid species, *in vitro* germination trials and, ideally, wild reciprocal introductions, to test if the fungus is capable of supporting the adult orchid in the wild. Comparison of the fungal partners of sister species of orchid is needed to determine the frequency with which speciation is associated with a shift in mycorrhizal fungus. Both of these approaches would be most effective if focused on a small group of related orchids, which will be aided by ongoing phylogenetic work in *Caladenia* as, at present, species complexes and the relationship of species within them are poorly resolved.

If a contribution of mycorrhizal fungi to reproductive isolation was confirmed, its importance for overall reproductive isolation would vary between *Caladenia* spp. depending on their pollination strategy. In species pollinated by food deception of nectar foraging insects, visitation is expected to be by a range of insect species (Phillips et al., 2009b), suggesting little pre-zygotic isolation. However, many *Caladenia* spp. are pollinated by sexual deception of thynnine wasps, a highly specialized system in which strong reproductive isolation is achieved by the use of one or few pollinator species, which typically differ between orchid species (Stoutamire, 1983; Phillips et al., 2009b; Whitehead & Peakall, 2014). In sexually deceptive *Caladenia*, any impediment to germination between fungal species is likely to be a secondary isolating barrier, with differences in pollinator attraction being the primary isolating barrier.

**PHYLOGENETIC PATTERNS OF HOST BREADTH: SPECIFICITY**

The detection of only three major fungal OTUs, which associated with 85% of the orchid individuals sampled, suggests that many *Caladenia* spp. are likely to be specialized on only one or few *Sebacina* OTUs. In particular, across all species of *Caladenia* subgenus *Phlebochilus* only two fungal OTUs were recorded. This finding provides additional support for earlier studies on the mycorrhizal ecology of *Caladenia*, but also strengthens the trend of highly specialized mycorrhizal interactions in Australian orchids. A potential exception to this trend is *Caladenia flavula* of subgenus *Elevatae*, in which three different OTUs were detected from five individuals. Further, fungi isolated from other members of this subgenus belong to OTUs that are different to those typically recorded from other subgenera, with six different OTUs being recovered from just 11 samples. This finding raises the possibility that not all subgenera of *Caladenia* are characterized by high mycorrhizal specificity. In *Caladenia* a trend is emerging where clades with more specialized mycorrhizal relationships have higher species diversity. The apparently more generalist subgenus (*Elevatae*) is characterized by low species diversity and broader geographical ranges compared with subgenus *Calonema* and *Phlebochilus* (Phillips et al., 2009a). However, we predict that this relationship is likely to be correlational rather than causal. While the sister clades to subgenus *Calonema* and *Phlebochilus* are pollinated primarily by food deception of nectar foraging insects (Phillips et al., 2009b; see Clements, Howard & Miller, 2015, for phylogenetic data), these subgenera contain a large number of species pollinated by sexual deception of thynnine wasps (Phillips et al., 2009b), a strategy that appears to be associated with relatively rapid speciation (Cozzolino & Widmer, 2005; Peakall et al., 2010). Further, sampling of genera allied to *Caladenia* may serve to erode this trend of low specificity in species-poor clades. For example, the closely related monotypic *Pheladenia* D.Jones & M.A.Clem. associates almost entirely with a single OTU of *Sebacina* across its broad geographical range (Davis et al., 2015). When taken together with the extensive sharing of fungal OTUs between *Caladenia*
spp., these lines of evidence suggest that changes in mycorrhizal specificity is unlikely to have been the main driving mechanism in the diversification of some subgenera of Caladenia.

The phylogenetic patterns in fungal use in Caladenia suggest that this would be a suitable genus in which to study the evolution of mycorrhizal specialization. Despite the increasing recognition that mycorrhizal specialization is widespread in terrestrial orchids, the mechanisms underpinning its origin are poorly resolved. Initial predictions that photosynthetic orchids would be consistently more generalist than non-photosynthetic orchids have not been supported by several recent studies (McCormick & Jacquemyn, 2014). Intuitively, specialization would seem to limit germination opportunities, although if mycorrhizal fungi are common and widespread this may not have a strong selective influence. Specialization could arise if there is a trade-off between using a diversity of fungal partners and efficient exploitation of a single partner. Further, Phillips et al. (2011a) predicted that mycorrhizal specificity may be more likely to arise in old landscapes, such as southwestern Australia (Hopper, 2009), through a prolonged opportunity for specialization to occur with the most effective partner, and a trend towards nutrient impoverished soils (Lambers et al., 2010). Given the apparent repeated evolution of high mycorrhizal specificity in Diurideae, the Australian orchid flora may harbour unparalleled opportunities to investigate the evolution of mycorrhizal specialization.

ACKNOWLEDGEMENTS

Funding was provided by Jandakot Airport Holdings, Hermon Slade Foundation, The Australian Orchid Foundation and an ARC Linkage grant to Rod Peakall, Celeste Linde and Kingsley Dixon (LP110100408). RDP was initially supported by an Australian Postgraduate Award. We acknowledge the Western Australian Department of Parks and Wildlife for permission to collect rare flora. Thank you to Celeste Linde for comments that improved the final manuscript and to Keith Smith and Andrew Brown for assistance with study sites and fieldwork. Thanks also to Sacha Ruoss and Matt Hyde for providing technical assistance and Sean Tomlinson for producing Figure 2.

REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Collection details for mycorrhizal fungi isolated from orchids.

Table S2. Vouchers for study populations of rare flora.

Table S3. A list of identical fungal sequences omitted from the figure of the fungal phylogenetic tree.

Table S4. Summary of the range of climatic conditions for the collection locations of each OTU.

© 2016 The Linnean Society of London, Botanical Journal of the Linnean Society, 2016, 182, 140–151