Evaluating multilocus Bayesian species delimitation for discovery of cryptic mycorrhizal diversity

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Abstract

The increasing availability of DNA sequence data enables exciting new opportunities for fungal ecology. However, it amplifies the challenge of how to objectively classify the diversity of fungal sequences into meaningful units, often in the absence of morphological characters. Here, we test the utility of modern multilocus Bayesian coalescent-based methods for delimiting cryptic fungal diversity in the orchid mycorrhiza morphospecies Serendipita vermifera. We obtained 147 fungal isolates from Caladenia, a speciose clade of Australian orchids known to associate with Serendipita fungi. DNA sequence data for 7 nuclear and mtDNA loci were used to erect competing species hypotheses by clustering isolates based on: (a) ITS sequence divergence, (b) Bayesian admixture analysis, and (c) mtDNA variation. We implemented two coalescent-based Bayesian methods to determine which species hypothesis best fitted our data. Both methods found strong support for eight species of Serendipita among our isolates, supporting species boundaries reflected in ITS divergence. Patterns of host plant association showed evidence for both generalist and specialist associations within the host genus Caladenia. Our findings demonstrate the utility of Bayesian species delimitation methods and suggest that wider application of these techniques will readily uncover new species in other cryptic fungal lineages.

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1. Introduction

The study of evolution and ecology relies on an ability to partition biodiversity into species—the basic taxonomic unit. While traditionally based on morphological characters, biologists have become increasingly reliant on DNA sequences as a source of biological variation upon which to base phylogenetic hypotheses of species boundaries. Previously, phylogenetic species delimitation sought to satisfy the criterion of reciprocal monophyly across independent gene trees (Knowles and Carstens, 2007). However, meeting this criterion is frequently hindered by discordant gene tree topologies resulting from gene flow or incomplete lineage sorting—a common finding, especially in groups with recent or rapid divergence (Degnan and Rosenberg, 2009; Edwards, 2009; Fujita et al., 2012).

Coalescent theory now offers a treatment of multilocus phylogenies to explicitly incorporate gene tree conflicts into a model of phylogenetic history for the populations or species concerned (Carstens and Knowles, 2007; Degnan and Rosenberg, 2009; Fujita et al., 2012; Yang, 2015). These techniques aim to integrate sequence data from multiple species across multiple loci in order to estimate a single species tree accommodating the demographic history of the ancestral populations (Rannala and Yang, 2003; Fujita et al., 2012; Aydin et al., 2014). By combining multispecies...
As a group that confounds traditional taxonomic techniques, sequence-based phylogenetic species delimitation offers much promise for describing the cryptic diversity of these ubiquitous Serendipitaceae (and more broadly, order Sebacinales) (Weiß et al., 2011). Furthermore, given their potential role in beneficial plant-fungal mutualisms (Barazani et al., 2005; Weiß et al., 2016), resolving the species diversity of the group is crucial for enhancing our understanding of plants and soils in both natural and managed landscapes (Ray and Craven, 2016). In particular, Serendipitaceae play a crucial role as obligate symbionts in the germination of orchid seed. The extent to which individual orchid species specialize on specific fungal taxa varies widely (Szweczyk et al., 2012; Linde et al., 2014), but in some taxa there is evidence that specificity in the orchid-fungus partnership may facilitate orchid diversity through niche partitioning (Tesitelová et al., 2013). The objective of this study was to apply multilocus coalescent approaches to characterize the diversity of Serendipita symbionts associated with the diverse Australian orchid genus Caladenia, and to evaluate the extent of fungal symbiote sharing between orchid species. We used two cutting edge Bayesian coalescent techniques to choose among four species delimitation hypotheses, the one that best fitted our data. We then used coalescent gene tree reconstruction methods to elucidate phylogenetic relationships among our newly delimited species.

Specifically, we address the following three questions:

1. Using multilocus coalescent methods, how many fungal taxa associate with 18 species of Caladenia orchids sampled at a continental scale in Australia?
2. Do the two primary methods of multilocus Bayesian coalescent species delimitation (Bayes factor delimitation and Bayesian Phylogenetics and Phylogeography (BP&P)) agree in their delineation of the fungal taxa?
3. Do Caladenia orchid species show specialized partnerships with fungal taxa?

2. Materials and methods

2.1. Sampling and fungal isolations

We sampled fungi from 16 orchid species early in the Australian spring (Sep–Oct) by cutting flowers at the stem, below the specialized collar region where fungal association takes place (Ramsay et al., 1986). Our sampling strategy concentrated on the southwest of Australia, one of the hotspots for Caladenia diversity (Phillips et al., 2009). For species listed as “Declared Rare Flora”, rather than taking a whole stem, we exposed the collar in situ and shaved off a section of tissue with a fresh scalpel blade before replacing the topsoil. For detailed fungal isolation methods, see supporting information.

We collected and grew a total of 138 fungal isolates from field collections for DNA analysis. Nine isolates in our culture collection that were originally collected from Caladenia orchids were also included in the study, bringing the number of host species sampled to 18 (Table S6). For clarity, we use host names along with a sample code to denote each isolate in this paper.

2.2. DNA extraction and sequencing

Lyophilized fungal tissue was extracted using a Qiagen (Valencia, CA, USA) Plant Mini Kit following the manufacturer’s instructions. Seven loci were sequenced: ITS (including ITS1, 5.8s, and ITS2), the nuclear large subunit (nLSU), ATP6 and four loci specifically developed for use in Serendipita (Table S1) (Ruibal et al., 2013).
Amplification and sequencing followed the protocol of Ruibal et al. (2014). Because ITS is a multigene copy region and some multicoeys were observed, PCR products were cloned using Invitrogen’s TOPO® TA Cloning® Kit for Subcloning, with One Shot® TOP10 Chemically Competent E. coli cells according to the manufacturer’s instructions. Two white colonies were picked and placed in 20 μl of Milli-Q water. Colony PCR was carried out with 2 μl of the cell suspension and M13 F and R primers in 30 μl reactions.

2.3. Bayesian phylogenetic analyses

The workflow for our Bayesian species delimitation is shown in Fig. 1. Before running phylogenetic analyses, we aligned our sequences, tested assumptions and assigned best-fit partitioning schemes and models of substitution by the methods described in supporting information. Bayesian phylogenetic analyses were run using *BEAST* (Bouckaert et al., 2014). See supporting information for more detailed description of priors, models of molecular evolution employed and the analysis strategy.

We also built a phylogeny for the host genus *Caladenia* using publicly available sequences for four genes (Swarts et al., 2014; Clements et al., 2015) (Table S2). Resolution of traditional plant phylogenetic markers for *Caladenia* is poor and in many cases we lack DNA sequence data for the host plant species sampled in this study. Where we lacked data for a host, we substituted sequence data for the closest allied orchid species which did have sequence availability. In cases where we did not have sequence data for the host plant species sampled in this study, we concatenated the nuclear genes into a single alignment treating each variable site as an allele, and coded the linked mitochondrial loci as a single locus where each mtDNA haplotype represented a different allele. We ran the STRUCTURE (Pritchard et al., 2000) analysis under the conditions described in supporting information and determined optimal K using the Evanno method (Evanno et al., 2005) (Table S3) implemented in STRUCTURE (Earl, 2012).

The K = 2 species hypothesis was based on two conserved loci (C11488 (mtDNA) and nLSU) to obtain a conservative delimitation of species. Sequence data from these loci were treated with a PCoA analysis in GenAlEx v6.501 (Peakall and Smouse, 2006, 2012) to cluster sequences based on haploid genetic distance. This indicated the existence of three clusters (Fig. S1). Lastly, the K = 8 species delimitation hypothesis was based on percentage pairwise ITS sequence divergence among all isolates, calculated in Geneious v6.1.8. The frequency distribution of pairwise sequence distances at ITS was used to search for a ‘barcode gap’: a discontinuity in the frequency distribution of sequence divergence indicating a transition between inter- and intraspecific divergence (Meyer and Paulay, 2005). The discontinuity closest to 100% sequence similarity was designated as the barcode gap threshold value. We then clustered samples into groups of sequences separated by a divergence greater than the observed barcode gap. The results of our ITS divergence species model included one group with only a single isolate (CLM0204 from host *Caladenia cairnsiana*). Because *BEAST* requires multiple samples per putative taxon in order to properly model the coalescent process, we also lumped this singleton with its closest relative thereby generating an additional K = 7 species delimitation hypothesis.

2.4. Generating species delimitation hypotheses

We generated four competing hypotheses for species delimitation by clustering samples by different methods and data sources. We denote each of these schemes by a name “K = x”, where x is equal to the number of clusters that resulted from that method. We obviously did not know the outcome of each clustering analysis in advance, and only use this notation here for clarity.

A K = 2 species hypothesis was generated by the Bayesian clustering package STRUCTURE (Pritchard et al., 2000). Here we concatenated the nuclear genes into a single alignment treating each variable site as an allele, and coded the linked mitochondrial loci as a single locus where each mtDNA haplotype represented a different allele. We ran the STRUCTURE (Pritchard et al., 2000) analysis under the conditions described in supporting information and determined optimal K using the Evanno method (Evanno et al., 2005) (Table S3) implemented in STRUCTURE (Earl, 2012).

The K = 3 hypothesis was based on two conserved loci (C11488 (mtDNA) and nLSU) to obtain a conservative delimitation of species. Sequence data from these loci were treated with a PCoA analysis in GenAlEx v6.501 (Peakall and Smouse, 2006, 2012) to cluster sequences based on haploid genetic distance. This indicated the existence of three clusters (Fig. S1).

Lastly, the K = 8 species delimitation hypothesis was based on percentage pairwise ITS sequence divergence among all isolates, calculated in Geneious v6.1.8. The frequency distribution of pairwise sequence distances at ITS was used to search for a ‘barcode gap’: a discontinuity in the frequency distribution of sequence divergence indicating a transition between inter- and intraspecific divergence (Meyer and Paulay, 2005). The discontinuity closest to 100% sequence similarity was designated as the barcode gap threshold value. We then clustered samples into groups of sequences separated by a divergence greater than the observed barcode gap. The results of our ITS divergence species model included one group with only a single isolate (CLM0204 from host *Caladenia cairnsiana*). Because *BEAST* requires multiple samples per putative taxon in order to properly model the coalescent process, we also lumped this singleton with its closest relative thereby generating an additional K = 7 species delimitation hypothesis.

2.5. Bayesian species delimitation: BFD and BP& P

We applied and compared the outcomes of two different complementary approaches for Bayesian species delimitation, *BEAST* for estimation of species trees coupled with *Bayes factor delimitation* (BFD) of competing species hypotheses, and ‘Bayesian Phylogenetics and Phylogeography’ (BP&P); a method that assigns probabilities to speciation events incorporating uncertain and conflicting gene tree topologies (Yang, 2015). For detailed description of priors and settings for both of these methods, see supporting information.

Our method for Bayes factor species delimitation broadly followed the four steps outlined in Grummer et al. (2014), i.e. we constructed competing topological hypotheses and used Bayes factor comparisons to test them. For example, to contrast a hypothesis that our sequences form K = 3 monophyletic groups (H1), versus clustering our samples into K = 2 monophyletic groups (H2), we enforced these two constraints and via marginal-likelihoods, computed a Bayes factor describing which of the two models is favoured. Marginal likelihoods were calculated via the stepping stone routine in the ‘Path Sampler’ add-on package for *BEAST* 2 (Xie et al., 2011; Bouckaert et al., 2014), which was run once for each of our four species hypotheses with a chain length of 10^6 for 200 steps and 50% burn in. Results from test runs were not dissimilar in 100 versus 200 step runs leading us to conclude that we obtained stable and replicable marginal likelihoods. Marginal likelihoods were then
compared among pairwise competing species hypotheses by calculation of Bayes factors (BF) (Kass and Raftery, 1995) which estimate the posterior odds in favour of H₂. We followed the guidelines of Kass and Raftery (1995) who use 2lnBF > 10 as indicative of “decisive” support of one model over another, 2lnBF = 6–10 indicating “strong” support, 2lnBF = 2–6 indicating “positive” support, and 2lnBF = 0–2 means “not worth more than a bare mention”.

Bayesian species delimitation was also conducted using the program BP&BP v3.1 (Yang, 2015). The method applies multispecies coalescent theory to compare different models of species delimitation in a Bayesian framework, accounting for incomplete lineage sorting due to ancestral polymorphism and gene tree–species tree conflicts (Rannala and Yang, 2003; Yang and Rannala, 2010, 2014). Each analysis was run at least twice to confirm consistency between runs. As BP&BP evaluates models by collapsing nodes, we used the "Beast K = 8 species tree as a guide tree in order to begin with a hypothesis of more, rather than fewer, species.

2.6. Comparison with GenBank accessions

To assess the monophyly of our delineated taxa and to place our samples in context with previously published molecular diversity, we gathered ITS sequences from GenBank. We used representative sequences in context with previously published molecular diversity, 85 sequences generated in this study. We built a phylogeny for the eight fungal taxa de

assumptions of stationarity, reversibility and homogeneity (Table S4) and, therefore, were included in downstream analysis. The gene trees inferred for the five different partitions (combined mtDNA, nDNA C16699, nDNA C28586, nDNA LSU, and ITS) revealed eight major lineages supported by strong Bayesian posterior probabilities (BPP > 0.90, Figs. S2–S5, Fig. 3). However, the inferred relationships among the eight major lineages differed somewhat among the data sets. For example, in the ITS gene tree, taxon C (the taxon incorporating fungal isolates sampled from Caladenia longicauda) formed a clade with taxa C, D, E, F (BPP = 0.94). However in the mtDNA gene tree, taxon C was placed in a clade with taxa A and B (BPP = 1). The gene trees for C16699 and C28586 (Figs. S3 and S4) were not well supported at the deepest nodes, and disagreed in their placement of species C. Similarly conflicting topologies were found for taxon B (Figs. 2, Figs. S2–S5). Despite these disagreements in the deeper relationships among lineages, all phylogenetic analyses were consistent in their support for the existence of eight clades defined by > 4.1% ITS sequence divergence.

3.3. Species delimitation

Bayes factor support for the four competing species hypotheses revealed K = 7 and K = 8 to be decisively supported (2lnBF > 10) or near decisively supported over both the K = 2, or K = 3 species hypothesis (Table 1). When compared to one another, the K = 8 hypothesis garnered positive but not strong support (2lnBF = 2–6) over the K = 7 model (Table 1). The BP&BP analysis was congruent with the BFD analysis, finding decisive support for the K = 8 hypothesis. BP&BP speciation probabilities strongly supported all eight taxa assuming population size parameters of 1 or 10 differences per kb. At higher differences per kb (30 and 50), only 7 taxa, with taxon H lumped with G, were strongly supported (Table S5). The species tree generated from the K = 8 species hypothesis also showed strong Bayesian posterior support (>0.99) for three out of six nodes (Fig. 4) and BP&BP speciation probabilities were uniformly strong (1.0) among all internal nodes. Based on the deepest divergence in the species tree, the eight fungal taxa defined here fall into two broader clades. Overall, the species tree (Fig. 4) most closely reflected the mtDNA gene tree (Fig. 5).
Fig. 3. Bayesian gene tree for *Caladenia*-associated *Serendipita* isolates based on ITS variation. Species hypotheses are colour coded. Bayesian posterior probabilities are reported for selected nodes.
multi-species monophyletic clades were identified: one including taxa D, E and F; the other including taxa G and H. Using divergence depth between taxa in these monophyletic clades as a guide, and incorporating node support, we were able to show that our other three taxa (A, B and C) were distinct, separated from other clades by Serendipita diversity not sampled here. Taxon F was not represented by ITS sequence in the GenBank database, however all other taxa had at least one previously published representative sequence.

There were just four accessions representing Australian Serendipita diversity not found in our sample, and each of these likely represents a different taxon nested among the diversity sampled in our study.

3.5. Host association

Our phylogeny for host genus Caladenia (Fig. 6) was unable to resolve the topology within the subgenus Calonema, in which most of our sampled host orchids are placed. This is in line with published phylogenies utilizing the same loci (Swarts et al., 2014; Clements et al., 2015). The subgenus Calonema was associated with four different Serendipita lineages, with the most frequently recovered fungal isolates being Serendipita A and G. Taxon G was only seen in subgenus Calonema, while taxon A was also found in the common and widespread Caladenia latifolia (subgenus Elevatae). All of the isolates of eastern Australian origin included in this study (one from Caladenia pusilla and two from Caladenia tentaculata) were also shared with western Australian orchids, demonstrating the widespread distributions of these fungal taxa over a continental scale exceeding 2500 km.

4. Discussion

4.1. How many species of Serendipita?

The orchid mycorrhizal fungal genus Serendipita exemplifies the problem of how to delimit species with a paucity of taxonomic characters (Weiß et al., 2011). With sequence data from seven loci from both mtDNA and nuclear genomes, treated under multiple approaches to objectively clustering our samples, we tested four different hypotheses for species delimitation for culturable Serendipita fungi found in association with Caladenia orchids. Using statistical species delimitation the best-supported model divided our sampled Serendipita diversity into eight taxa, which we equate to the level of species. One of these taxa (H) was, however, represented by only a single isolate, and therefore our K = 7 hypothesis (lumping H with G) was not decisively better than the K = 8 hypothesis. The discovery of multiple representatives of the H taxon in published ITS GenBank sequences, however, leads us to conclude that this entity is indeed distinct from taxon G.

Our integrated approach to molecular species delimitation has, therefore, revealed the existence of eight species of Serendipita. Although species description is ideally and traditionally done with a combination of sequence and non-sequence characters, there is an increasing need for pragmatic sequence-based methods of species description to tackle cryptic fungal diversity (Lumbsch and Leavitt, 2011; Hibbett and Taylor, 2013). Here, the integration of multiple independent nuclear and mtDNA loci, objective clustering of sequence variation coupled with Bayes factor model-testing, and generation of multilocus species trees under a coalescent framework provides a sure footing on which to base subsequent DNA sequence-based species description.

We predict that applying these methods in other morphospheres of Sebacinales will uncover widespread cryptic species diversity. The high species diversity of Caladenia-associated Serendipita observed here is consistent with the implied outcomes from the data in Weiss et al. (2004), which included only three Serendipita vermicula isolates from Caladenia sensu lato and found all to belong to distinct and deep-branching lineages in Serendipitaceae (Weiss et al., 2004). Other work on two distinct Sebacina morphospheres indicated each lineage was composed of at least three cryptic lineages (Riess et al., 2013). A global study of Sebacinaeae (sister family to Serendipitaceae) also found evidence for the existence of 11 clades in that sample, each containing a...
Fig. 5. Maximum clade credibility tree for Serendipita ITS sequences from this study and GenBank accessions. Branch labels provide maximum-likelihood bootstrap support before Bayesian posterior probabilities. Shaded boxes denote the taxa delimited in this study.
diversity of less well supported subclades that in many cases likely reflect real species (Tedersoo et al., 2014). The reality of a broad and unrecognized diversity of *Serendipita* is further underscored by the evidence for at least four other Australian lineages indicated by singleton GenBank accessions that were not uncovered in our field sampling (Fig. 5).

### 4.2. Phylogenetic insights from Bayesian methodology

The two Bayesian species delimitation methods were in agreement in their support of the $K=8$ species hypothesis. Although BFD found positive but not strong support for $K=8$ over $K=7$, BP&P analysis was unequivocally in favour of a $K=8$ model over any possible $K=7$ scenario. The species tree resulting from our *BEAST analysis matched the topology of the mtDNA tree, however, it showed conflicts with trees derived from our nuclear gene partitions. This conflicting topology could be due to incomplete lineage sorting or introgression in emerging lineages. Regardless of its cause, this result demonstrates that although ITS proves useful as a means for delineating *Serendipita* clades by sequence divergence, its use for elucidating species relationships is weak without additional genetic evidence.

### 4.3. ITS divergence and barcode gap

The best-supported species hypothesis clustered samples based
on a minimum 4.1% ITS sequence divergence between taxa (Table 1, Fig. 3). This was determined by our sequence similarity distribution, which identified a gap in pairwise sequence similarity between 94 and 95.9, corresponding to 4.1%–6% divergence (Fig. 2). This barcode gap is slightly larger than the benchmark 3% ITS divergence that has previously been used in fungal delimitation, but consistent with recognized variation in barcode gap among lineages of Basidiomycota (Nilsson et al., 2008). Tedersoo et al.'s (2014) global analysis of the ectomycorrhizal Sebacinaeae found a more or less continuous distribution of ITS divergence and as a result did not determine a definitive ITS barcode gap for the group. The conflict between that result and the findings here may be due to a number of factors: (1) the very wide diversity and geographic area sampled in Tedersoo et al. (2014) is more likely to capture between-lineage variation in the substitution rate of ITS, and (2) the large sample of sequences from publicly accessible databases is more likely to capture variation in sequence quality which could blur the distinction between intra- and interspecific sequence divergence. Therefore, while ITS performs well to distinguish species in our data and other studies (e.g. Linde et al., 2014), it is important to point out that it will not necessarily behave in the same fashion when applied in other systems (e.g. Gazis et al., 2011).

4.4. In context with wider ITS diversity

When previously published ITS sequences were included, four other ITS lineages were found nested among the sampled Serendipita phylogenetic diversity (Fig. 5). Nevertheless, our taxa were still well supported as distinct entities within the ITS phylogeny and frequently included additional published accessions. Interestingly, we found 25 GenBank accessions allied to our singleton H sample. All but one of these accessions were Serendipita isolates from the orchid Pheladenia deformis, published in a study of continent-wide fidelity in mycorrhizal specificity (Davis et al., 2015). The ITS phylogeny crown depth and node support for this clade are consistent with our conclusion that taxon H warrants species status.

Most Australian ITS accessions within 86% ITS sequence divergence of our sample set represented taxa defined here in our analysis. Of the four exceptions, three were from orchid hosts, the other was a soil sample (DQ388872) that necessarily lacked host information. We found no related sequences on GenBank for our taxon F (associated with 2 species of orchid), and thus it represents the discovery of a previously unsampled Serendipita taxon.

4.5. Biogeography and host association

While our sampling focused on Australia’s biodiversity hotspot in the southwest, three isolates from eastern Australia provide some continent-wide context. Each of these eastern isolates matched a taxon also present over 2500 km away on the western side of the continent. When considering the related GenBank accessions (Fig. 5), we find five of the taxa in both east and west Australia, while three taxa (C, G, F) are represented only by samples in western Australia. The extraordinary distribution of single Serendipita taxa with low sequence divergence among distant regions, as well as the close sympatric occurrence of divergent Serendipita taxa (within meters of one another), adds biogeographical evidence to the molecular evidence supporting their species status. The distribution of the same Serendipita species across the major biogeographical divide of the Australian Nullarbor Plain is proving to be a common pattern known also from Serendipita MOTUs associated with P. deformis (Davis et al., 2015). Tulasnellia orchid mycorrhizal fungi (Linde et al., 2014), and basidiomycete macrofungi (May, 2002). Further sampling will be required to determine if taxa C, G and F are endemic to south-western Australia, or simply yet to be detected in eastern Australia.

On a more local scale, our confined geographic and taxonomic sampling of host plants, as well as the fact that only culturable diversity could be sampled, suggests a potentially wide diversity of these fungi. Our two most intensely sampled sites were Milyeapup and Gracetown (Table S5) where we sampled four and six sympatric species of Caladenia respectively, each from study sites not exceeding 30 m². At both of these sites of high Caladenia species richness we discovered six Serendipita taxa, comprising a diversity that was equal or greater to that of their hosts. While there is evidence that orchid mycorrhizal fungi in different families can differ in their metabolism of soil nutrients (Nurfadilah et al., 2013), we do not know if closely related Serendipita species might differ in their nutrient metabolism. High fungal diversity at a site might be driven by niche partitioning, facilitation, or co-occurring species may simply be competitors. Further studies on the ecology of these species are required to understand the processes that structure fungal community diversity.

Most Australian terrestrial orchids associate with Serendipita, Tulasnellia or Ceratobasidium (Dearlney et al., 2012). However, the emerging patterns of their host association show strong differences among these genera. The patterns of association uncovered in Tulasnellia show a single species of fungus capable of associating with any species within an entire genus of orchids, and even fungal species partnering with species in different orchid genera (Roche et al., 2010; Phillips et al., 2011; Linde et al., 2014). This is in contrast to the varied associations found in Caladenia, where a diversity of fungal species can be shared among different subclades of orchids, confined to particular orchid species, or single orchid species can utilize multiple fungal partners (Swarts et al., 2010; Wright et al., 2010). This diversity of associations formed in Caladenia and Serendipita is also distinct from that observed in the South African orchid tribe Cycniinae, which forms associations with a very broad range of fungal families encompassing six major orchid mycorrhizal lineages (Sebacinaeae, Serendipitaceae, Tulasnellia, Ceratobasidium, Tricharina, and Peziza) rather than just a subclade of a single family of fungi as observed here (Waterman et al., 2011).

It has been proposed that orchid-mycorrhizal-fungi partnerships may facilitate the often observed co-existence of multiple orchid species and genera if germinating orchids can avoid competition for fungal resources through specialization on distinct fungal taxa (Tešitelová et al., 2013). Among the orchids sampled here, we found potential examples of both specialist and generalist relationships. For example, Caladenia flava is a reported mycorrhizal generalist (Swarts et al., 2010) and occupies a geographical range wider than any other orchid sampled here. Accordingly, we found this species to associate with three distinct fungal taxa (Fig. 6), expanding to four when we include the GenBank accessions. Similarly low specificity interactions have been reported in the European genus Orchis (Jacquemyn et al., 2010) where most orchid species associate with several fungal MOTUs.

As well as generalists, many of our sampled hosts within the orchid subgenus Calonema showed narrow taxonomic breadth in their host associations, suggestive of mycorrhizal specialization (Fig. 6). At our two most intensely sampled sites (Gracetown and Milyeapup) we found no two species from subgenus Calonema to share a fungal symbiont. In contrast, at both sites we found fungal sharing between orchids outside the subgenus Calonema (e.g. C. flava and Elythranthera brunonis sharing taxa E, and C. latifolia and Caladenia brownii sharing taxon A). The high species richness of Caladenia subgenus Calonema (Phillips et al., 2009) as well as the growing evidence for a tendency to fungal specificity warrants further study into the role of fungal symbioses in facilitating orchid diversity.
As this study was limited to culturable fungi, a necessary next step would be direct sequencing to determine if apparent specialists are harboring additional undetectable fungal diversity (Jacquemyn et al., 2014). Assigning ecological competence through seed germination experiments would also be invaluable to understanding the significance of these plant-fungal associations.

5. Conclusion

Expanding the incorporation of DNA sequence data is crucial for accelerating species discovery and progress on the systematics of fungi. Our application of Bayesian phylogenetic species delimitation in Serendipita capitalized on three key benefits of this approach for delimiting cryptic fungal diversity: (1) simultaneous estimation of a species tree and species limits, (2) the flexibility to test species hypotheses based on varied methods and data, and (3) the integration of multilocus data. It is also worth noting that the technique is capable of integrating genome-scale data sets (Leaché et al., 2014). As the formal description of fungal species based on DNA sequence data alone becomes increasingly routine (Hibbett and Taylor, 2013), we expect Bayesian phylogenetic species delimitation techniques and their descendants to become increasingly valuable tools in this field.

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Supplementary data

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