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# 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.



## 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

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coalescent techniques with a model-testing approach, it becomes possible to test competing hypotheses of species delimitation. This framework therefore provides a phylogenetically-informed method for defining species that is not constrained by the requirement for reciprocal monophyly and is also potentially less subject to investigator-driven biases than previous methods (Fujita et al., 2012).

It is estimated that less than 10% of the world's fungal species have been formally described (Bass and Richards, 2011; Hibbett et al., 2011; Hibbett and Taylor, 2013). This is in part because many fungi exist predominantly as inconspicuous sexual stages or only as hyphae, with few phenotypic characters for detection and description (Hughes et al., 2009). The recent rapid increase in the availability of DNA sequence data has increased our rate of detection for new fungal diversity while compounding the scale of the challenge to describe cryptic fungal taxa (Lindahl et al., 2013; Tripp and Lendemer, 2014). DNA sequence-driven species delimitation based on multispecies coalescent theory therefore promises to be a powerful tool in fungal systematics, especially for the many groups that exhibit limited measureable phenotypic variation and/or are unable to be lab cultured (Lekberg et al., 2014; McCormick and Jacquemyn, 2014). While coalescent species delimitation is receiving wide uptake and continued development, it has yet to be widely applied to fungi outside just a few cases (Linde et al., 2014; Sadowska-Deś et al., 2014; Wang et al., 2014; Singh et al., 2015).

To date, most efforts to classify sequence variation divide diversity into 'molecular operational taxonomic units' (MOTU) (Floyd et al., 2002; Peay et al., 2008)—a clustering of similar sequences commonly equated to species level boundaries (Hibbett et al., 2011). For fungi, the most frequently used source of data for detecting MOTUs is variation in the internal transcribed spacers (ITS 1 and 2) region of the nuclear ribosomal repeat unit (Nilsson et al., 2009; Schoch et al., 2012). As well as being one of the first regions to be adopted as a universal fungal barcode, ITS also shows perhaps the strongest evidence for any single marker supporting its suitability for diagnosing species limits (Nilsson et al., 2008; Peay et al., 2008; Hughes et al., 2009; Linde et al., 2014; Tedersoo et al., 2014). For example, in a study on the mycorrhizal genus *Tulasnella*, Linde et al. (2014) found that MOTUs defined by the widely-implemented 3% ITS-divergence threshold agreed with the taxonomic boundaries defined by a multilocus dataset based on seven nuclear and mitochondrial markers.

Notwithstanding the utility of ITS as a barcode for detection and description of fungal diversity, relying on variation in a single gene for taxonomic delineation remains potentially problematic. For example, the rate of evolution in a single gene might vary between lineages or the gene could be present in multiple copies (Nilsson et al., 2008). Ideally then, any molecular based taxonomy should rest on multilocus or genome scale data, defining MOTUs based on concordant evolutionary independence of multiple independent genes (Dupuis et al., 2012; Linde et al., 2014; Tripp and Lendemer, 2014).

The Serendipitaceae (recently reclassified as distinct from the Sebacinaceae (Weiß et al., 2016)) are a globally distributed group of fungi encompassing a wide diversity we are only now beginning to appreciate (Weiss et al., 2004; Weiß et al., 2011; Oberwinkler et al., 2013; Riess et al., 2013; Tedersoo et al., 2014). They form a wide range of plant-fungi partnerships including endophytic, ectomycorrhizal, orchid and ericoid mycorrhizas (Weiss et al., 2004; Weiß et al., 2016). Remarkably, despite their wide distribution and mycorrhizal diversity, only four species of Serendipitaceae have been formally described. This is in large part due to the difficulty of obtaining sexual stages, which when combined with the difficulty of culturing many species, poses serious challenges to traditional taxonomy (Weiß et al., 2016).

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 (Swarts et al., 2010; Jacquemyn 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 (Těšitelová 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 symbiont 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.,