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Seeking the needle in the haystack: Undetectability of mycorrhizal fungi outside of the plant rhizosphere associated with an endangered Australian orchid

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ABSTRACT

Co-occurrence and abundance of suitable mycorrhizal fungi are expected to be important drivers for orchid seedling establishment and development, as well as mature plant distribution. However, limited information is available on the occurrence and spatial patterns of orchid mycorrhizal fungi in soil independent of the orchid host. In this study, we investigated the *in situ* distribution of *Tulasnella* spp. associated with the critically endangered Australian orchid *Diuris fragrantissima*. We tested and implemented a meta-barcoding approach (fungal ITS1 region) using three soil sources: orchid rhizosphere, orchid-associated bulk soil and bulk soil from the orchid native site. The quality-filtered data set revealed that the occurrence of *Tulasnella* spp. *in situ* is restricted to the orchid rhizosphere, suggesting that a limited number of potential recruitment micro-sites with suitable mycorrhizal taxa exists in the *D. fragrantissima* natural habitat. The meta-barcoding approach also revealed a distinctive fungal community associated with the orchid rhizosphere. Overall, Next Generation Sequencing technology has proven to be a suitable method for large-scale screening of mycorrhizal fungi in orchid-associated soil.

1. Introduction

Seed germination, transition to seedling and early development of terrestrial orchids in natural conditions are reliant on colonization by mycorrhizal fungal symbionts (Harley and Smith, 1983; Leake, 1994). Lack of an extensive root system makes nutrient acquisition by fungal symbionts critical throughout the life of the orchid (Harley and Smith, 1983). Despite the diversity of fungi associated with terrestrial orchids varying across habitats, as well as between seedlings and adult plants (Brundrett, 2006; Dearnaley et al., 2012) many such orchids form mycorrhizal associations with a relatively narrow diversity of fungi in the 'rhizoctonia' group. 'Rhizoctonia' fungi are a polyphyletic assemblage encompassing pathogens, endophytes, saprotrophs and mycorrhizal fungi (Warcup, 1981; Sivasithamparam, 1993; Currah et al., 1997; Roberts, 1999; Rasmussen et al., 2015), comprising important orchid mycorrhizal genera such as *Ceratobasidium, Tulasnella*, *Thanatephorus*, *Serendipita* and *Sebacina* (Weiß et al., 2004, 2016; Dearnaley, 2007; Veldre et al., 2013; Linde et al., 2017).

Co-occurrence and abundance of suitable mycorrhizal fungi is expected to be an important driver not only for orchid seedling establishment and development, but also mature plant occurrence, and the significance of distribution/dispersal linkages between orchids and fungi has been documented in several studies (reviewed in McCormick and Jacquemyn, 2014). While on a local scale limited presence of compatible fungi may represent a major constraint (McCormick et al., 2012, 2016), especially for those orchids displaying narrow fungal specificity (Brundrett et al., 2003; Bonnardeaux et al., 2007; Dearnaley, 2007; Ogura-Tsujita et al., 2009), our understanding of dispersal dynamics and ecology of mycorrhizal fungi independent from orchids is still limited. Indeed, in many cases orchid mycorrhizal fungi occur outside orchid patches (McKendrick et al., 2002; Tešitelová et al., 2012; Oja et al., 2015), indicating that suitable fungi can also be distributed in soil independently from their hosts. However, in several instances germination has been found to decline with increasing distance from adult plants, suggesting that the occurrence of orchid mycorrhizal fungi may be ephemeral without existing orchids (McKendrick et al., 2000, 2002; Batty et al., 2001; Diez, 2007; Jacquemyn et al., 2012). Given the importance of fungi as drivers

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of orchid dispersal (Batty et al., 2002; Jacquemyn et al., 2014; McCormick et al., 2016; Rock-Blake et al., 2017), being able to resolve the *in situ* distribution of mycorrhizal fungi in both occupied and unoccupied orchid habitats is predicted to be a crucial requirement to identify appropriate micro-sites for reintroduction activities (McCormick et al., 2016).

'Baiting' techniques using orchid seed packets are commonly implemented to investigate the distribution of orchid mycorrhizal fungi (e.g. Perkins and McGee, 1995; McKendrick et al., 2000; Batty et al., 2001; Brundrett et al., 2003; Otero et al., 2007; McCormick et al., 2016). However, this method can only detect locations where both fungi and environmental conditions are appropriate for seed germination, while actual fungal occurrence may be more widespread (McCormick et al., 2016). Recently, DNA meta-barcoding using Next Generation Sequencing (NGS) has emerged as an essential complementary molecular tool for the effective profiling of microbial communities from a wide range of environments, representing an increasingly popular alternative for monitoring biodiversity (Taberlet et al., 2012; Thomsen and Willerslev, 2015), including occurrence of rare fungi (e.g. Geml et al., 2014). Nevertheless, recent studies using this method reported relatively low rates of detection for orchid fungi in soil (Jacquemyn et al., 2014, 2017; Oja et al., 2015; Han et al., 2016). These limitations could be due to biases inherent to PCR amplification (Suzuki and Giovannoni, 1996), as well as DNA extraction method, limited affinity of conventional NGS primers for orchid mycorrhizal fungi (Taylor and McCormick, 2008; Tedersoo et al., 2014) and sequencing platform employed (Nguyen et al., 2015; Fouhy et al., 2016). In particular, fungal primers typically used for amplicon-based NGS are unsuitable for many species within the family Tulasnellaceae (Tedersoo et al., 2014; Oja et al., 2015). Therefore, the ability of meta-barcoding to reliably and consistently detect orchid mycorrhizal fungi in soil is still debated (see for example McCormick et al., 2016).

In this study, we aimed to assess the distribution of mycorrhizal fungi associated with *Diuris fragrantissima*, a critically endangered Australian native orchid (EPBC Act, 1998). *D. fragrantissima* grows in association with a narrow taxonomic range of mycorrhizal fungi (*Tulasnella* spp.) within the cosmopolitan family Tulasnellaceae (Smith et al., 2010). The limited survival of cultivated plants of *D. fragrantissima* after reintroduction has been correlated with the loss of associated fungi during the transition from *ex situ* to *in situ* (Smith, 2006). Nevertheless, monitoring the presence of the fungus using traditional 'baiting' techniques has proven to be unreliable in this situation, resulting in the inability to locate compatible mycorrhizal fungi in the orchid natural habitat (Smith, 2006). We therefore tested the suitability of the meta-barcoding technique, using primers targeting the whole fungal ITS (internal transcribed spacer) region, to identify *Tulasnella* sp. in soil. This molecular approach was then implemented to investigate the distribution of *D. fragrantissima*-associated mycorrhizal fungi in the orchid rhizosphere and adjacent soil, as well as to characterise fungal diversity across the orchid natural habitat.

2. Material and methods

2.1. Site description and soil sampling

D. fragrantissima, Sunshine *Diuris*, is a perennial, terrestrial orchid endemic to the basalt plains immediately to the west of Melbourne (Australia) in the Victorian Volcanic Plain IBRA Bioregion (*sensu* DEH, 2000). Following habitat degradation, weed invasion, predation by introduced herbivores, altered burning regimes and illicit collection, range and abundance of this native Australian orchid underwent a dramatic decline (Smith, 2006; Murphy et al., 2008). To date, only a single wild population, comprising approximately 30 plants, remains in a grassland reserve within a fenced area of approximately 1200 m^2 situated in Sunshine (Melbourne, Victoria, Australia) (Murphy et al., 2008). Orchids are located in the north-eastern side of this fenced portion of the reserve, in a patch measuring approximately 15×10 m (Supplementary Fig. 1), although the area is dominated by several Australian native plant species, including *Themeda triandra*, *Austrodanthonia* sp., *Dianella longifolia*, *Dianella revoluta, Tricoryne elatior*, *Dichanthium sericeum* and *Pimelea humilis*. The soil is shallow heavy clay, with several exposed basalt boulders also present (Murphy et al., 2008).

We collected soil samples from three distinct sources: orchid rhizosphere soil (in direct contact with the orchid root), orchid bulk soil (adjacent to the orchid plants), and site bulk soil (Fig. 1). Firstly, to explore the composition of the fungal community directly influenced by the orchid roots, two samples from the rhizosphere soil surrounding the roots of four native plants *in situ* ('orchid rhizosphere soil') were collected by scraping the first cm of soil in direct contact with the root surface, for a total of eight samples. Secondly, to investigate the orchid 'zone of influence' — i.e. how far from the plant we can still find traces of its mycorrhizal symbiont — five plants at the external boundaries of the orchid patch were further examined by collecting adjacent to the plant (i.e. soil in contact with the stem, but not in contact with the root, from herein referred to as '0 cm' samples) along with three points at incremental distance from each plant (10, 20, 40 cm). Samples were taken along between one to four transects in the directions north, south, east and west, depending on the orchid location (some transects were not possible due to presence of rocks), for a total of 35 additional samples ('orchid bulk soil'). Sampling was performed using a soil corer (2 cm diameter) to a depth of 10 cm, following Prober et al. (2015). Thirdly, to investigate the distribution of *Tulasnella* spp. and the occurrence of possible micro-sites suitable for orchid translocation, soil samples were collected from the site at which the orchid was present ('site bulk soil'). The sample pool comprised soils from areas where orchids were and were not present. To achieve systematic sampling, 5 m^2 grids were designated within the fenced area (Supplementary Fig. 1). For each plot, the middle point was identified and four soil cores (2 cm diameter $\times$ 10 cm length) were collected within a small radius (25 cm) of

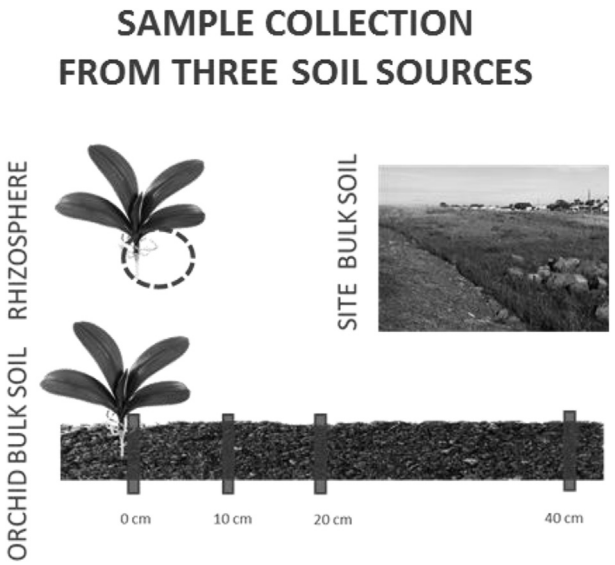

Fig. 1. Experimental workflow.