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ORCHID MYCORRHIZAL SYMBIOSIS: EVOLUTION, MOLECULAR MECHANISM AND ROLE IN ORCHID DISTRIBUTION

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Abstract

Mycorrhizal fungi are intricately linked to orchid life cycle as the orchids are dependent on these fungi for the supply of nutrients especially during the initial stages of their development. The association of orchid mycorrhizal (OM) fungi is also believed to have been a crucial event in the evolution of orchidaceae. The additional supply of carbon by OM fungi to the orchid as a direct result of the association influence the physiological and morphological changes of the orchids which subsequently directed their evolutionary pathway. Besides factors such as pollinators, seed dispersal agents *etc*, mycorrhizal fungi are also known to play an important role in distribution of orchids. Nonetheless, these factors that influence the distribution of orchids are closely connected and untwining a single factor is often difficult. Degree of specificity of orchids with their mycorrhizal partners and spatial distributional patterns of OM fungi has been proposed to propel distribution and rarity of orchids. However, such assumption may be too simplistic as several studies have revealed that OM fungi are widespread and present even without orchids. Interestingly, environmental factors affecting OM fungi distribution are also known to indirectly influence orchid distribution. The present paper summarizes recent findings concerning roles of OM fungi in influencing orchid distribution, evolution of OM symbiosis and comparative study of molecular mechanisms participating in OM symbiosis.

Introduction

ORCHIDACEAE, THE most species-rich plant family amongst angiosperms is known for its spectacular diversity in floral characters. Orchids exhibit both epiphytic and terrestrial growth forms and are ubiquitous in nature. Orchid distribution and abundance vary between continents and within regions (Myers *et al.*, 2000). Richest concentration of epiphytic orchids is found in the Northern Andes of South America, Madagascar, Sumatra and Borneo. SouthWestern Australia is a centre of terrestrial orchid richness and IndoChina harbours both epiphytic and terrestrial species (Cribb *et al.*, 2003). Despite their wide occurrence, the major impediment to orchid distribution is perhaps their predisposition to a patchy distribution because of their highly specialized and restricted habitats. It is well known that flowering plants are inextricably linked to pollinators, herbivores, seed dispersal agents, pathogens and mutualistic soil organisms. These interactions have likely participated in their evolution and diversification. The breadth of the plant niche and adaptive capability to a variety of environmental pressures and evolutionary processes are defined by ecological specialization between plants and their interacting partners (Wardle *et al.*, 2004). These abiotic and biotic interactions have allowed continuation of symbiosis from generalists to highly specialized, plants being at the specialized end of the scale, often rendering plants rare or vulnerable to extinction (Swarts and Dixon, 2009). Among these, an interesting and complex interaction involving orchids with soil micro-organisms particularly fungi may be cited. Fungi associated with orchid roots, also known as orchid mycorrhizal (OM) fungi play an important role in their life cycle and evolutionary history (Rasmussen, 2002; Rasmussen and Rasmussen, 2009). This paper focuses on the role of mycorrhizal fungi in the distribution of orchids with evidences from latest researches including molecular studies in orchid mycorrhizal association.

Evolution of the OM Symbiosis (Differences/ Similarities with other Symbiosis)

Mycorrhizal fungal association for mineral nutrition is a pervasive phenomenon across the plant kingdom. Owing to their minute seeds which lack endosperm, orchids in particular, are dependent upon mycorrhizal fungi for the uptake of nutrients and water to the developing plant (Rasmussen, 1995). In both terrestrial and epiphytic green orchids, the association is not restricted to germination/seedling stage but mycorrhizae are retained even during the adult stage. Such orchids are known to have mixotrophic mode of nutrition and augment their carbon (C) requirements via photosynthesis and mycorrhizal fungi (Bidartondo *et al.*, 2004; Gebauer and Meyer, 2003; Julou *et al.*, 2005; Selosse *et al.*, 2004). Achlorophyllous orchids, on the other hand, are dependent on their mycorrhizal fungal partner for their C requirement throughout their

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lifecycle, and hence called fully mycoheterotrophic plants.

The Evolution of Orchids is Presumed to Have Been Driven by Mycorrhiza

Extant species in Apostasioideae (Dressler, 1993) with plicate leaves and faintly zygomorphic epigynous flowers, poorly developed column with partly adnate (united) filaments and styles, and loosely aggregated pollen (Dressler, 1993; Dressler and Dodson, 1960; Freudenstein and Rasmussen, 1999) are believed to be representative of the ancestral orchids. Mycorrhizal associations endow orchids with additional carbon source which ultimately set the physiological and morphological evolutionary path for the orchids (Rasmussen and Rasmussen, 2014). Physiologically, these changes include delayed development of photo-assimilating structures or the complete exclusion of photo assimilation (Rasmussen and Rasmussen, 2014). The morphological changes include reduction of seed size, multi-seed production, aggregated pollen masses, zygomorphy in the flower, and column specializations (Rasmussen, 1995; Rasmussen and Rasmussen, 2014).

Phenotypic Plasticity Enabled Orchids to Jump Partners

Based on studies of close monocot relatives, arbuscular mycorrhizal (AM) fungi were believed to have been the progenitors of OM symbiosis. The orchids then switched from AM to non-ectomycorrhizal (ECM) basidiomycete fungi as evident by the retention of AM-like internal mycorrhizal structure (Taylor and Bruns, 1997). Interestingly, some tropical mycoheterotrophic orchids are known to form associations with non-*Rhizoctonia* saprophytic fungi in the absence of ECM fungi (Dearnaley *et al.*, 2013; Smith and Read, 2008). Taylor and Bruns (1997) also suggested that the ultimate shift from non-ECM *Rhizoctonia* fungi to various ECM fungi is believed to have been driven by the need to have access to a more continuous supply of carbon. Additionally, the change from one mycobiont to another would also enable the orchid to inhabit new niches. Prevalence of primitive non-ECM associations in members of the photosynthetic but diverse species of *Cephalanthera* and *Corallorhiza,* however, suggested that the combined phenomenon of a jump to association with ECM fungi and a loss of photosynthesis occurred independently in these two orchids (Taylor and Bruns, 1997). Rasmussen and Rasmussen (2014) argues phenotypic plasticity (McCormick *et al.*, 2006), enables the orchids to successfully associate with different fungi.

Recently, Rasmussen and Rasmussen (2014) proposed two probable hypotheses for the evolutionary origin of orchid mycorrhiza (Fig. 1). In the first type, orchid mycorrhiza is believed to have evolved via infection of the ancestral orchid by pathogenic *Rhizoctonia,* resulting in a successful mutualistic association made possible by the fine tuning of the host defense reaction to prevent necrosis of plant cells and complete elimination of the invader. Such hypothesis is evidently supported by the close relationship of some extant orchid mycobionts such as *Ceratobasidium* sp. to pathogenic genotypes (Veldre, 2011). The second type involved association of the ancestral orchid with *Paris*-type AM. The *Paris*-AM is characterized by the formation of intracellular hyphal coils which are believed to function as transporter of phosphorus (P) to the plant (Feddermann *et al.*, 2010) and also mutualistically to fungi whenever the plant partner is photosynthetic (Hodge *et al.*, 2010). Such characteristic would in all probability support the transfer of C from the fungi to the plant. In fact, the similarities in histological pattern and the tendency towards mycoheterotrophy between *Paris*-AM and orchid mycorrhiza have been documented (Rasmussen and Rasmussen, 2014).

Orchid Mycorrhiza Evolved via Endophytism

Mycoheterotrophic orchids are now known to be associated with several independent saprotrophic lineages in the Hymenochaetales, Psathyrellaceae, Mycenaceae and Marasmiaceae. Green (photosynthetic) orchids on the other hand are still mostly associated with a polyphyletic assemblage of saprophytic *Rhizoctonia* taxa (*i.e.*, members of Sebacinales, Ceratobasidiales and Tulasnellales) (Dearnaley *et al.*, 2013). Other saprotrophic mycorrhizal fungi reported in green orchids include *Mycena* species in *Anoectochilus roxburghii* (Guo *et al.*, 1997) and *Cymbidium sinense* (Fan *et al.*, 1996). However, presence of these saprotrophic fungi in green orchids does not necessarily indicate true mycorrhizal association as they could be simple contaminants, or form mycorrhizal structures on small portions of the root or colonize tissues as endophytes (Selosse *et al.*, 2010). Nonetheless, mycorrhizal association in orchids is believed to have evolved via saprotrophism to endophytism and then to mycorrhizal association especially with respect to mycoheterotrophic orchids (Selosse *et al.*, 2009). Shefferson *et al.* (2007) reported similar evolutionary pattern of association in certain *Rhizoctonia*- associated species of *Cypripedium*.

Orchid Potentially Indulges in Cheating with their Fungal Partner

Mutualism in the orchid mycorrhizal symbiosis has been

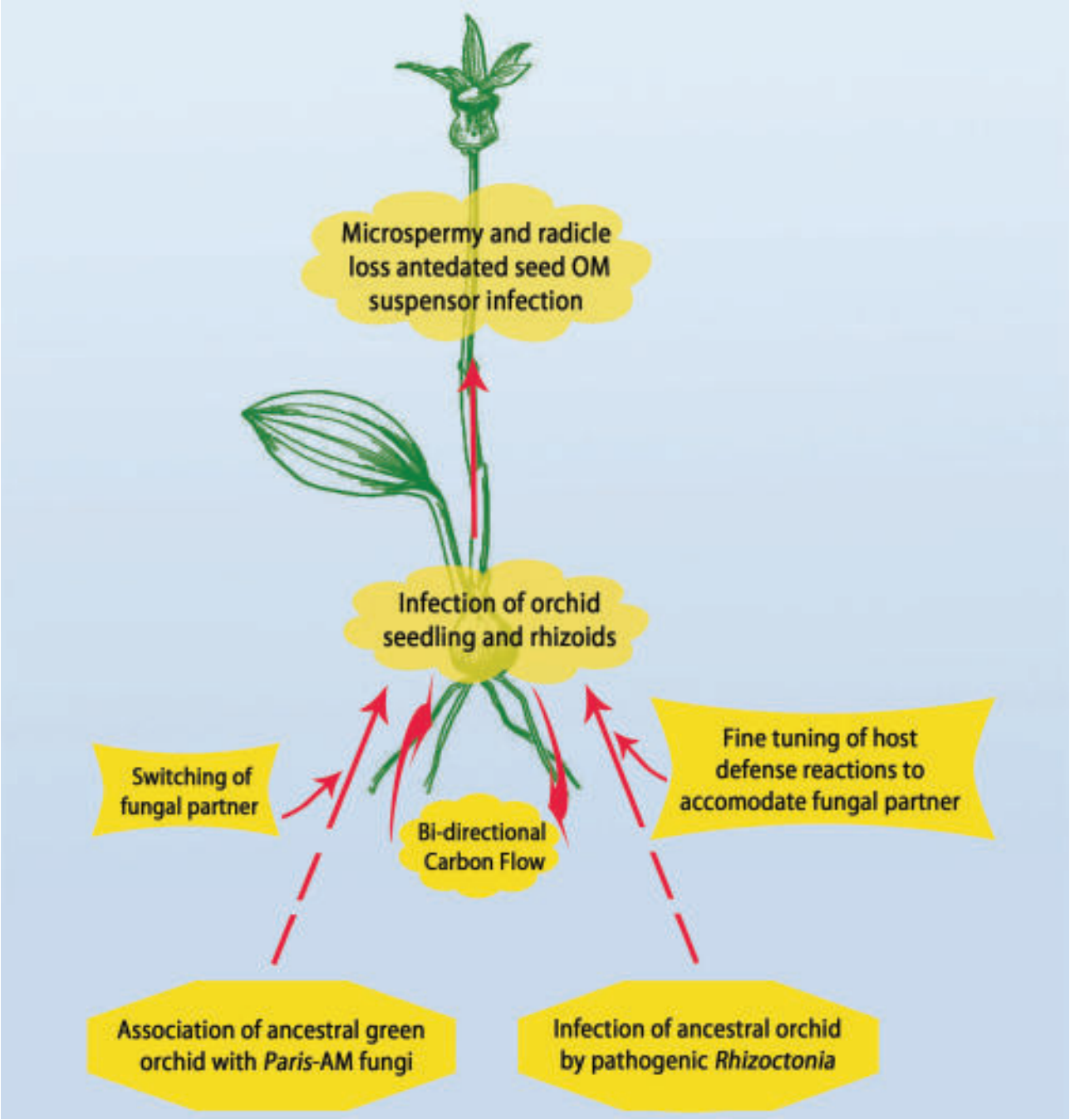

Fig. 1. Hypothesis for evolutionary origin of orchid mycorrhiza (Adapted from Rasmussen and Rasmussen, 2014).

debated by many scientists as the fungi supply both nutrients and C to the germinating seeds which produces achlorophyllous, heterotrophic seedlings called protocorms (Eriksson and Kainulainen, 2011). In some associations, however, adult plants have been reported to supply C to the fungus (Cameron *et al.*, 2008), but still in others adult photosynthetic and non-photosynthetic plants continue to acquire C from mycorrhizal fungi (Stockel *et al.*, 2014; Yagame *et al.*, 2012). The presence of full heterotrophy in 30 independent orchid lineages (Merckx, 2013), as well as the existence of partial heterotrophy which is considered as an evolutionary intermediate to full heterotrophy (Roy *et al.*, 2013) does point towards cheating of their fungal partner by orchids. Selosse (2014) however, argued that the OM symbiosis could also involve other possible benefits to the fungus such as transfer of vitamins, and/or the protection of hyphae within the roots apart from transfer of carbon (C) and other nutrients such as phosphorus (P) and nitrogen

(N) (Cameron *et al.*, 2006, 2007).

Molecular Mechanism of OM Symbiosis

The molecular mechanism underlying the OM symbiosis (Fig. 2) is the least understood among mycorrhizal symbiosis (Zhao *et al.*, 2014). However, in recent years, application of transcriptome, expressed sequence tags (ESTs) and proteome analysis are beginning to help unravel the key molecular events during the plant-fungus interactions (Li *et al.*, 2012; Perotto *et al.*, 2014; Valadares *et al.*, 2014; Zhao *et al.*, 2013, 2014).

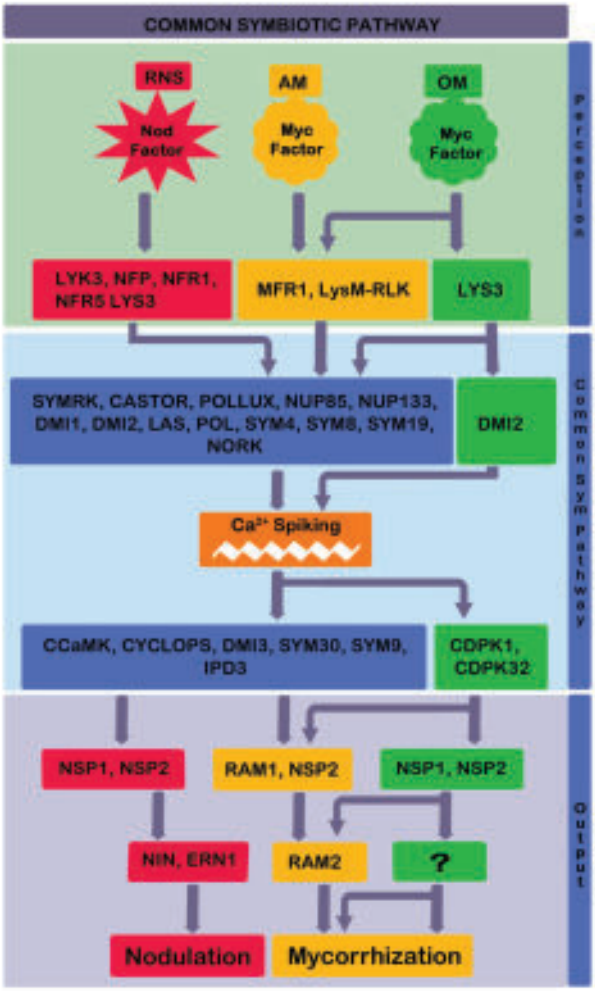

Fig. 2. Common symbiosis pathway underlying the orchid mycorrhizal (OM) symbiosis: Nod/Myc Factor produced by *Rhizobia*/Mycorrhizal fungi are perceived by Nod/Myc factor receptors (NFR1, LYK3, MFR1) which in turn form a complex with symbiosis receptor-like kinase (SYMRK/DMI2). The symbiosis signal is then transduced through the common symbiosis pathway. Calcium spiking induced by nuclear ion channel DMI1 are further perceived and decoded by calcium/calmodulin-dependent protein kinase (DMI3/CCaMK), which consequently activates downstream transcription regulators (NSP1, NSP2, RAM1, NIN, ERN1) which govern the expression of nodulation/mycorrhization genes. Names inside green boxes represent genes that have so far been identified in the orchid mycorrhizal symbiosis.

Genes Involved in OM Symbiotic Signal Transduction Pathway

Calcium-dependent protein kinases (CDPKs) are calcium sensors that play an important role in the symbiotic signaling transduction pathways including mycorrhizal symbiosis. Zhao *et al.* (2013) were the first to report CDPKs genes in the OM symbiosis. Using suppression subtractive hybridization (SSH) cDNA library of symbiotically germinated *Dendrobium officinale* seeds, they identified and characterized two CDPKs genes- CDPK1 and CDPK32. These genes were presumed to play an important regulatory role in the *D. officinale* - *Sebacina* sp. symbiosis as they showed a tissue specific expression pattern and were also both up-regulated in the symbiotically germinated seeds. Two homologues of CDPKs genes were also observed to be up-regulated in roots of *Cymbidium hybridum* co-cultivated with different mycorrhizal fungi (Zhao *et al.*, 2014). They also detected up-regulation of two genes presumably encoding lysin motif-receptor-like kinases LysM similar to LYS3 from *Lotus japonicus*, one ion channel protein and one leucine-rich-repeat receptor-like-kinase DMI2 gene homologue from *Medicago truncatula*. In the nitrogen-fixing root nodule symbiosis (RNS), DMI2 and the nuclear membrane bound channel proteins DMI1, POLLUX and CASTOR, responsible for generation of symbiotic calcium oscillations are activated downstream of the LysM perceptions leading to nodulation and infection. Expressions of mycorrhization or nodulation genes are regulated by GRAS family of transcription factors. These factors constitute NSP1 and NSP2 in RNS, and NSP2 and Arbuscular Mycorrhization 1 (RAM1) in AM symbiosis (Oldroyd, 2013). However, in roots of *C. hybridum* co-cultivated with mycorrhizal fungi, two homologue of NSP1 from *L. japonicus* were also detected and found to be up-regulated, interestingly, the homologue of NSP2 from *M. truncatula* was down regulated. Calcium signals or calcium spiking is a key trigger in the symbiosis signaling pathway in AM and RNS interactions (Roberts *et al.*, 2013). Calcium signaling is also believed to be at the heart of OM regulation as suggested by differential accumulation of proteins such as calmodulin, a core component of the calcium signal transduction pathway (Yang and Poovaiah, 2003), and inositol-5-phophatase, presumed to be involved in IP3 hydrolysis (Chen *et al.*, 2008), in symbiotically germinated green protocorms of *Oncidium sphacelatum* (Valadares *et al.*, 2014).

Other Genes Involved in OM Symbiosis

Apart from genes involved in the OM signaling

pathway, Zhao *et al.* (2014) also identified several other up-regulated genes related to cellular organization, protein processing, nutrient transport, defense-related and phytohormone. Among these, a homologue of cellulose synthase A (CesA) catalytic subunit of *Vitis vinifera* was found to be up-regulated 32-fold. In *L. japonicus,* these CesA genes have been reported to be involved in cell expansion during arbuscule development (Guether *et al.*, 2009) and are therefore presumed to play similar function in OM symbiosis. Substantial number of genes believed to be homologues of AM and ectomycorrhizal symbiosis (Guether *et al.*, 2009), which are associated with protein metabolism and turnover, membrane dynamics and cell wall synthesis were also specifically induced by OM. Syntaxin SYP 132, a member of membrane fusion related proteins is presumed to be vital for compatibility in the *Lotus*-mycorrhizal fungi interactions. Two homologues of *Arabidopsis thaliana* syntaxin SYP 132 were found to be induced 16- and 6- fold by mycorrhizal symbiosis in *C. hybridum*, indicating their role in OM symbiosis. Interestingly, protein metabolism and processing regulated genes were found to be drastically up-regulated in mycorrhizal symbiosis in *C. hybridum.* This underpins the importance of protein metabolism and processing in mycorrhizal symbiosis (Fiorilli *et al.*, 2009; Gande *et al.*, 2012; Guether *et al.*, 2009).

Transfer of nutrients such as P from mycorrhizal fungi to an orchid is a well documented process in the OM symbiosis. Transcriptional profiling of co-cultured *C. hybridum* roots with mycorrhizal fungi also revealed co-induction of 7 nutrient transport related genes, including two inorganic phosphate transporters (Zhao *et al.*, 2014). These inorganic phosphate transporters were further found to be homologues of inorganic phosphate transporters 1-4 of *A. thaliana,* LjPT3, LjPT4 of *L. japonicus* and MtPT6 of *M. truncatula.* Besides inorganic phosphate transporters, genes encoding putative plant plasma membrane proton ATPases, nitrogen transport, amino acid transporters, ATP binding cassette (ABC) transporter were found to be up-regulated in symbiotic roots of *C. hybridum* (Zhao *et al.*, 2014).

Specificity of OM Symbiosis Versus Orchid Distribution

Studies on specificity of orchids for fungi during mycorrhizal association are important to - understand orchid biology, plan conservation strategies, population restoration (Dearnaley, 2007), and also to gain insight into the distribution of orchid populations. Specificity between orchids and mycorrhizal fungi has been a subject of debate among scientists for decades with the general consensus that specificity varies noticeably amongst orchid taxa (Swarts *et al.*, 2010). Based on his studies of australian terrestrial orchids and *Rhizoctonia* sp., Warcup (1973, 1975, 1981), was the first to suggest the probable existence of specificity between orchids and fungi at least at the genus level. Specificity is broadly distinguishable as - high/narrow specificity which results in limited distribution, and low/wide specificity which could be attributed for the wide-spread distribution of a species. Wide specificity in an OM symbiosis can be defined as several mycorrhizal fungi associating with the same orchid (Timms and Read, 1999), *e.g.*, two australian orchids *Disa bracteata* and *Microtis media* could germinate readily to green leaf stage with a range of phylogenetically diverse fungal species, indicating their wide specificity range (Bonnardeaux *et al.*, 2007). Consistent with the earlier report, *M. media* was shown to associate with a broad taxonomic spectrum of mycobionts *i.e.*, *Ceratobasidium* sp., *Piriformospora indica*, *Sebacina vermifera*, and *Tulasnella calospora*, which enabled them to be wide-spread and common (De Long *et al.*, 2013). These traits may prove advantageous to the associating orchid by allowing them to be tolerant to a range of habitats. On the other hand, there are certain groups of orchids that are known to prefer particular fungal species over others. For instance, all mycorrhizal isolates from six *Chiloglottis* taxa across Eastern Australia were restricted to only a phylogenetically narrow group of *Tulasnella* fungi (Roche *et al.*, 2010). The existence of specificity is perhaps, best indicated by the regular yield of the same fungus or group of fungi from a given orchid over different geographical ranges. Based on extensive sampling over its distribution range, *Caladenia huegelii* was shown to associate exclusively with a specific mycorrhizal fungus (Swarts *et al.*, 2010). It was suggested that distribution of *C. huegelli* was limited to locations only where its mycorrhizal fungus occurred, thus providing evidence for the cause of rarity of this species as a consequence of narrow mycorrhizal specificity. Smith *et al.* (2010) reported *Diuris* to associate with narrow monophyletic groups of *Tulasnella* mycorrhizal symbionts. Further mycorrhizal fungi isolated from seven different populations of *Liparis japonica* belonged to a single clade of *Tulasnella* indicating a very specific relationship between the orchid and its associated fungi (Ding *et al.*, 2014). Many studies have found narrow mycorrhizal specificity to be a predominant feature of fully mycoheterotrophic orchids. These orchids were very often found to associate with fungi that form ectomycorrhizal relationships with trees (McKendrick

et al., 2002; Selosse *et al.*, 2002; Taylor *et al.*, 2004) and *Lecanorchi*s sp. associated with ectomycorrhiza forming fungi *i.e., Atheliaceae, Lactariu*s, *Russula*, and *Sebacina* (Okayama *et al.*, 2012). On the basis of these studies, it can be assumed that orchids which maintain a generalist association with numerous mycorrhizal partners may exhibit better adaptive ability in a changing or highly fragmented environment. On the contrary, narrowing mycorrhizal partners which are most likely highly ecologically proficient and niche-specialised may guarantee survival in specific habitats or may be highly adaptive under a narrow range of environmental conditions but limits the potential for survival if the environmental changes are not favourable to the plant or fungus (Swarts and Dixon, 2009). The transition from generalist (Jacquemyn *et al.*, 2010, 2011; Otero *et al.*, 2002; Stark *et al.*, 2009) to specialist (Ogura-Tsujita and Yukawa, 2008; Otero *et al.*, 2004; Roche *et al.*, 2010; Swarts *et al.*, 2010) mode of association is a continuous process. Most studies on orchid-fungus specificity have focussed either on terrestrial or fully mycoheterotrophic orchids; however, epiphytes forming the major group of orchids (Jones, 2006) are comparatively little studied. In order to have a clear picture of orchid-fungus specificity, the underlying mechanisms of orchid-fungus interactions in each of the above systems have to be well understood. Stable carbon and nitrogen isotope signatures have greatly helped our understanding of mycoheterotrophic mycorrhizae (Bidartondo *et al.*, 2004; Gebauer and Meyer, 2003) and these techniques could also be used to understand the basis for the existence of different levels of specificity in other orchids.

Specificity Versus Rarity

Pate and Hopper (1993) suggested that a particular species may be recognized as rare if it has low numerical strength compared to others, as organisms are known to vary enormously in their distribution and abundance. Rare species can be categorized into three groups on the basis of space, time or group relatedness (Harper, 1981). Species falling under the first category are those which may be abundant locally, but restricted to only a limited number of sites due to high niche specificity or barriers lowering dispersal potential. Very often these species are local endemics and vulnerable to threatening processes. Fluctuations in population number of species following adverse erratic or cyclical events, such as drought or fire results in time-dependent rare species (Koopowitz *et al.*, 2003). Group-dependent rarity comprises populations of a rare species which occupy a specialized niche with a limited distribution. Orchids belong to all these categories. Degree of specificity of orchids with their mycorrhizal partners, purportedly, is the major factor driving and determining rarity in orchids but the intensity of its effect on rarity is not much known or studied. Orchids are believed to have intrinsic (natural biotic processes limiting the abundance and distribution of species) rarity and constitute one of the angiosperm families with the greatest share of endangered species (Swarts and Dixon, 2009). In order to check orchid rarity, it is important to examine and document the array of mycorrhizal fungi that exists across the natural distribution range of orchids. Determining functional significance as to which fungal species participate during orchid seed germination and throughout its growth and maturity, and assessing how this interaction works to limit orchid abundance and distribution leading to rarity in nature is essential. In this context, use of *in situ* and *ex situ* seed baiting germination techniques have been successfully established (Rasmussen and Whigham, 1993). *In situ* experiments were conducted by placing small packets of seeds (enclosed in muslin cloth/fine nylon netting) in the habitat of the adult orchid and ultimately recovering them for evaluating the presence of mycorrhizal fungi (Brundrett *et al.*, 2003). The *in situ* and *in vitro* seed germination studies of *Caladenia huegelii* provided further evidence for the association of orchid with specific mycorrhizal fungi as the most important factor influencing rarity of orchids (Swarts *et al.*, 2010). Although, it may be mentioned here that germination under *in vitro* conditions is generally less specific and is known to associate with a broad range of fungi than under natural conditions (Masuhara and Katsuya, 1994; Perkins and McGee, 1995; Rasmussen, 2002). However, even under *in vitro* conditions, *C. huegelii* germinated only with its own fungal isolate, thus, demonstrating a highly specialized nature of mycorrhizal association. *Sarcochilus weinthalii*, a rare australian epiphytic orchid was also reported to have narrow fungal specificity by associating with a single species of *Ceratobasidium* at three structurally different sampling sites in south-east Queensland, Australia (Graham and Dearnaley, 2012). Several other scientists also linked orchid rarity with narrow specificity of orchids for their mycorrhizal fungi (Okayama *et al.*, 2012; Shefferson *et al.*, 2005; 2007; Stewart and Kane, 2007).

On the contrary, Phillips *et al.* (2011) found no connection between mycorrhizal specificity and high intrinsic rarity in the genus *Drakaea*. The authors suggested that due to wide distribution and abundance of fungal partners, the expected frequency of orchid seeds encountering their fungal partners would be high.

Also, efficient exchanges between the partners leading to better growth and development of the plant would be possible when plant species have specialized interactions with one or more fungi (Otero *et al.*, 2005). Recently, Fracchia *et al.* (2014) reported that restricted distribution of *Gavilea australis* was not linked to its mycorrhizal specificity as the species was found to establish association with numerous fungal partners. *Dactylorhiza fuchsia*, an abundant and widespread orchid was found to associate with a narrow range of fungal partners, both in terms of phylogenetic breadth and number of fungal species than the rapidly declining or rare orchid *Anacamptis morio* (Bailarote *et al.*, 2012). In fact, *A. morio* exhibited wide specificity by associating with a greater range of fungi. Phillips *et al.* (2011) attempted to explain the phenomenon of rare orchids being widely specific by citing an alternate pattern of mycorrhizal specificity which is not based on associations with narrow phylogenetic range of fungi. They suggested that orchids could associate locally with a narrow range of fungi, but along the species distribution range with many different fungal partners. This pattern permits a locally specialized species to remain an ecological generalist and has been observed for a number of orchid species (Martos *et al.*, 2009; McKendrick *et al.*, 2002). In such a pattern, the association of a rare orchid with rare fungi would cause rapid decline of the orchid species. However, *A. morio's* association with possibly ubiquitous mycorrhizal fungi distributed in different ecological conditions and environments, does not qualify this species as a specialist candidate. Jacquemyn *et al.* (2011) also failed to relate orchid rarity and decline to mycorrhizal specificity. Perhaps, other factors such as human-induced disturbances leading to rarity could have greater impact than originally thought.

Distribution of Orchids in Relation to Distribution of OM Fungi

It is a general hypothesis that OM fungi distribution and specialization affects orchid distribution and consequently rarity. To test this hypothesis, comprehensive information of the associated OM fungi precisely the distribution of OM endophytes across the orchid's distribution range and life cycle, the functional and ecological importance of the symbiosis and the natural distribution range of the associating OM fungi are required. However, little is known about the distribution of OM fungi in the wild. Conventionally, fungal diversity was examined by isolation of mycorrhizal fungal cultures from colonized tissues of mature plants and their morphological comparisons. However, these methods were not very effective as these fungi rarely produced teleomorphs *in situ* or in axenic cultures. Additionally, different cultural and morphological standards for identification have resulted in an unresolved taxonomy where orchid mycorrhizae are usually allocated to the basidiomycete form genus (Moore, 1987). Recently combination of molecular systematics (DNA sequencing and phylogenetic analysis) and *in situ* and *ex situ* seed baiting techniques to perceive OM endophytes in field sites (Batty *et al.*, 2001; Bidartondo and Read, 2008; Brundrett *et al.*, 2003) have been useful in dealing with problems related to distribution and ecological requirements of OM endophytes. In a recent review, McCormick and Jacquemyn (2014) concluded that many orchid species showed greatly scattered distribution patterns at the landscape scale in spite of producing numerous seeds with the potential to disperse across long distances (Arditti and Ghani, 2000) and some species had the potential to live for many years in the soil (Whigham *et al.*, 2006). This implied that many sites were unsuitable for germination and seedling establishment (Munzbergova and Herben, 2005). Possible reasons for this could be the restricted/uneven distribution of OM fungi within suitable habitats. So, if high specificity exists between the two associating partners, orchids would be able to survive only in those sites where the fungus occurs. For example, vast distribution and abundance of *Ionopsis utricularioides*, a common tropical epiphyte, might have been due to this orchid's preference for a specific but widespread mycorrhiza (Otero *et al.*, 2007). On the contrary, if OM fungi actually had limited distribution, specific association with highly restricted OM partner would confine the orchid resulting in its rarity. Nevertheless, assessment of mycorrhizal associations in a broad range of orchid species has shown that many OM fungi occur in a variety of habitats, widespread and lived even in the absence of orchid host (Batty *et al.*, 2001). For instance, OM fungi of the genus *Orchis* was found to be widespread from Mediterranean up to Northern Belgium and was recovered from a variety of habitats *i.e.*, dry calcareous grasslands, mesic grasslands, and both pine and temperate deciduous forests (Jacquemyn *et al.*, 2011). Wide distribution of OM fungi symbiotic with orchid species such as *Caladenia* and *Drakaea* has been reported (Phillips *et al.*, 2011; Swarts *et al.*, 2010). Presence of OM fungi irrespective of the presence of orchid host were also seen in *Arachnorchis behrii* (Feuerherdt *et al.*, 2005), and whereby, it's closely related fungal lineages even occurred at different continents (Shefferson *et al.*, 2007).

Environmental Factors Influence Orchid Distribution by Affecting OM Fungi Distribution

In spite of the presence of compatible OM fungi at suitable sites, failure of orchids to recruit/germinate and establish might be due to lack of environmental factors conducive for successful symbiotic interactions. The distribution of fungi within habitat patches and the benefits conferred by the association to both partners is known to be influenced by different edaphic factors mainly soil moisture, organic content, pH and nutrient levels (Batty *et al.*, 2001; Diez, 2007). These factors possibly limit the environmental conditions for abundance of mycorrhizal fungi and eventually the orchids that depend upon them. Diez (2007) reported that germination of *Goodyera pubescens* with compatible fungal species increased in sites with favourable conditions *i.e.*, higher soil moisture, organic content and lower pH. McCormick *et al.* (2009) recommended that the population of *Corallorhiza odontorhiza* might have been determined by the distribution of particular drought-resistant fungi. Scattered orchid distribution and purportedly related fungal distribution were well demonstrated in other studies (Batty *et al.*, 2001; Jacquemyn *et al.*, 2007), and higher seed germination in proximity to adult orchids was observed in some cases, and in others, germination was not related to growing sites of adults. Such differences in germination possibly reveal that adult plants either retain their germination-enhancing fungi or switch to another fungi as an adult (Rasmussen and Rasmussen, 2009). Abundance of OM fungi was a significant predictor of presence, number and size of protocorms of *Goodyera pubescens, Liparis liliifolia* and *Tipularia discolor* and their germination seemed to be limited by factors that influenced OM fungi abundance (McCormick *et al.*, 2012). Indirect effect on germination mediated via effects on the abundance of OM fungi was implied. However, the hypothesis that compatible OM fungi limit orchid distribution is contradictory as many scientists have found that OM fungi were not restricted to sites harboring orchid populations. The use of seed-baiting techniques in these studies confirmed presence of both OM fungi and appropriate environmental conditions for the development of symbiosis. Another hypothesis for the patchy occurrence of orchid population and their absence from seemingly favourable habitat patches is the considerable distance from existing orchid populations that minute seeds usually do not reach these habitats (McCormick and Jacquemyn, 2014; Munzbergova and Herben, 2005). Limitation by seed arrival/dispersal rather than the absence of appropriate microsites presumably drives scattered distribution of orchid populations (Batty *et al.*, 2001; Diez, 2007; Jacquemyn *et al.*, 2007; McKendrick *et al.*, 2002).

Conclusions

Application of molecular techniques for direct identification of OM fungi without axenic isolation has overcome many problems related to taxonomy of OM fungi (otherwise based on morphology of rarely forming sexual structures and vegetative characteristics). Molecular techniques will also to a certain extent, help resolve identities of several OM fungi and degrees of specificity for their fungal partners. Specificity, OM fungi distribution, influence of environmental factors on OM fungi distribution, and/or collective effects of all these factors is so far limited to studies on terrestrial orchids. On the other hand, epiphytic orchids having different niche specializations/specialized niche requirements are very little studied in relation to the above mentioned factors. Perhaps this could be attributed in part to the fact that studies of epiphytic orchids in their natural environment are difficult and constrained by many factors. For example, seed packet techniques based specificity studies showed that seed packets slide mounted on the branches were less successful than on those placed on the ground. Nonetheless, the importance of OM fungi distribution associated with, and their effect on epiphytic orchid distribution cannot be overlooked. While studying specificity the functional role of each of the OM fungal partner at each developmental stage of orchid must be considered instead of simply defining specificity in terms of fungal identity as it is done in most cases. Probably, due to specific nutritional requirements of the orchids, specificity of the association is known to vary at different developmental stages. Low specificity during germination or seedling stage but high at adult stage or vice versa is not an uncommon occurrence in some orchids. *In vitro* specificity of *Dendrobium chrysanthum* at different developmental stages has been reported earlier along with the reports of variations in nutrient acquisition ability of different OM fungi associating with numerous species of australian orchids. Other than nutrient supply, orchid endophytes probably have different roles as well such as promotion of growth and development of plants and antagonism against certain plant pathogens. Therefore, in order to better understand factors affecting orchid distribution, it is necessary to understand the biology of orchid-fungus interaction across all types of orchid habitats. It may not justify when general conclusions on factors affecting orchid distribution are drawn based on only a few studies. Further, studies on viability testing of seeds arriving at purportedly suitable habitats and checking suitability of habitat by assisted

introduction of orchid seeds may help us gain valuable insight into the various factors constraining the distribution of orchids.

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