Non-mycorrhizal endophytic fungi from orchids

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Orchidaceae is one of the largest flowering plant families of the plant kingdom. The habitats of orchids are highly diverse, ranging from tree bark and damp forest floors to rock crevices, sandy dunes and semi-arid deserts. The diversity of endophytes (internal symbiotic fungi) associated with orchids is enormous. Most studies of endophytic fungi from orchids in the past have focused on mycorrhizal endophytes (internal symbiotic fungi associated with plant roots). There has, however, been an increasing trend to study non-mycorrhizal endophytes from orchids because of their physiological roles and their potential as sources of novel bioactive compounds. This review discusses the methods used in the isolation and identification of endophytic fungi from orchids, their diversity and host-specificity, their significance in orchid conservation and cultivation, and their potential application in the discovery of bioactive compounds.

Keywords: Bioactive compounds, diversity, non-mycorrhizal endophytic fungi, orchids.

Introduction

ORCHIDACEAE is one of the largest flowering plant families of the plant kingdom, which comprises more than 899 genera and 27,801 species (The Plant List 2013). Of these, over 200 genera have been studied for their endophytic fungal diversity (see Appendix 1), which is less than 30% of the total orchid genera. Orchids with horticultural, ornamental, medical and commercial importance have been studied for the presence of endophytes[1,2]. Rare or endangered orchids, including species in *Cypripedium*, *Holcoglossum* and *Paphiopedilum* have also received attention[3–5]. Terrestrial orchids which make up nearly one-third of all orchid species occupy approximately half of the endangered orchid list (The World Conservation Union 1999)[6]. Many of them have also been subjected to endophyte research (Table 1). Orchid species in the genera *Aa*, *Hadrolaelia*, *Gavilea* and *Satyrium* have been poorly studied and can be regarded as new topics for research[7–10].

Research on endophytic fungi in orchids has been carried out in all trophic groups (i.e. photosynthetic, mixotrophic and mycoheterotrophic) of all growth habits (i.e. terrestrial, epiphytic and lithophytic), from highly diverse habitats (e.g. rainforests, evergreen forests, coniferous forests, bamboo forests, ectomycorrhizal forests, wetlands, swamps, calcareous coastal plains, botanical gardens and greenhouses) in all continents except Antarctica (Table 1). Some orchids occur in a wide range of habitats, while others are endemic to certain regions. For example, *Platanthera minor* grows in forests on slopes and alpine meadows at elevations 90–3000 m in China, Japan and Korea[11,12]. *Satyrium nepalense* was reported to be distributed from grassy hill slopes at varying altitudes (600–4600 m) in India[10]. *Ophrys benacensis* occurs only in northern Italy[13] and *Piperia yadonii* only in North America[14]. The epiphytic orchid *Sarcochilus parviflorus* survives only with its main host *Backhousia myrtifolia*[15].

The purpose of this article is to review the studies on non-mycorrhizal endophytic fungi of orchids and present the main conclusions from the research.

Isolation and identification of fungal endophytes from orchids

Isolation

Orchid mycorrhizal fungi are known to be associated with roots of orchids[16,17]. Therefore, most endophyte studies on orchids have investigated orchid roots for mycorrhizal and endophytic diversity[18]. Other orchid parts, including leaves, rhizomes, mature bulbs, tubers, stems and stem-collars have also been studied for endophytes[19,20]. Since endophytes are commonly defined as 'all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host'[21,22], only healthy organs were used in these studies[23].

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Table 1. Orchid non-mycorrhizal endophytes

Orchid	Organs	Orchid growth habit	Orchid habitat	Isolation method	Identification method	No. of taxa or OTUs	Fungal trophic group	Reference
Aa achalensis	Root	Terrestrial (photosynthetic)	Natural habitat, west Argentina	From surface-sterilized tissues	Morphology, ITS	1	Endophytes	7
Acampe praemorsa	Root, leaf	Epiphytic (photosynthetic)	Similipal Biosphere Reserve, Odisha, India	From surface-sterilized tissues	ITS	6	Endophytes	112
Acianthus pusillus	Root	Terrestrial (photosynthetic)	Open forests, Australia	Crush peloton from surface-sterilized tissues	ITS-RFLP, ITS	2	Endophytes	30
Aphyllorchis caudata	Root	Terrestrial (mycoheterotrophic)	Evergreen forest, Thailand	No isolation	ITS, 28S, or mt-LSU-rDNA	11	ECM fungi, endophyte	18
Aphyllorchis montana	Root	Terrestrial (mycoheterotrophic)	Forests in Thailand	No isolation	ITS, 28S, or mt-LSU-rDNA	4	ECM fungi, endophytes and saprobes	18
Bletilla ochracea	Leaf	Terrestrial (photosynthetic)	Mountains (1–1.6 km high), China	From surface-sterilized tissues	Morphology, ITS, TUB2, ACT, GAPDH	17	Endophytes	33
Bletilla ochracea	Root, leaf	Terrestrial (photosynthetic)	Mountain (1310 m high), China	No isolation	ITS, cloning	17	Endophytes	64
Bulbophyllum kaitiense	Root	Epiphytic or lithophytic (photosynthetic)	Kolli hills (80–869 m high), India	From surface-sterilized tissues	Morphology, ITS	2	Endophyte	71
Bulbophyllum neilgherense	Mature bulb and aerial root	Epiphytic (photosynthetic)	River Kali, Kaiga forest, India	From surface-sterilized tissues	Morphology	17	Endophytes	20
Bulbophyllum neilgherrense	Root, leaf	Epiphytic (photosynthetic)	Natural forest and greenhouse, India	From surface-sterilized tissues	ITS	14	Endophytes	28
Caladenia (eight species)	Stem-collar	Terrestrial (photosynthetic)	Roadside, open forests, woodland, and heath, swamp, SWAFR	No isolation	ITS	1	Endophyte	63
Caladenia carnea	Root	Terrestrial (photosynthetic)	Helidon Hills, Australia	Crush peloton from surface-sterilized tissues	ITS-RFLP, ITS	1	Endophyte	30
Cattlteya skinneri	Root	Terrestrial (mixotrophic)	Botanical Gardern, southern Mexico and orchidarium, Santo Domingo	From surface-sterilized tissues	Morphology	10	Endophytes	2
Cephalanthera (2 species)	Root	Terrestrial (photosynthetic)	ECM forests, northeast Bavaria, Germany	No isolation	ITS, 28S, mt-LSU-rDNA	6	ECM fungi	53
Cephalanthera exigua	Root	Terrestrial (mycoheterotrophic)	Evergreen forest, Thailand	No isolation	ITS, 28S, or mt-LSU-rDNA	6	ECM fungi, saprobes (11%)	18
Cephalanthera longifolia	Root	Terrestrial (mixotrophic)	Calcareous coastal plain at Pussa, west of Estonia	No isolation	ITS, ITS-RFLP	12	ECM fungi, endophytes, saprobes	52
Chamaegastrodia sikokiana	Rhizome	Terrestrial (non-photosynthetic)	Coniferous (350 m high) and evergreen broadleaved forest (780 m high), Japan	Crush peloton from surface-sterilized tissues	ITS	1	ECM fungi	78

(*Contd*)

Table 1. (*Contd*)

Orchid	Organs	Orchid growth habit	Orchid habitat	Isolation method	Identification method	No. of taxa or OTUs	Fungal trophic group	Reference
Changnienia amoena	Rhizome	Terrestrial (non-photosynthetic)	Indian cedar forest and broadleaved forests in ZJ, HB, AH, China	From surface-sterilized tissues	Morphology, ITS	17	Endophytes	42
Chiloglottis (six species)	Tuber, rhizome	Terrestrial (photosynthetic)	Moist, sheltered places: five in NSW, and one in ACT, Australia	From surface-sterilized tissues	ITS, mt-LSU	3	Endophytes	56
Corallorhiza maculata	Root or rhizome	Terrestrial (mycoheterotrophic)	CA, WA, OR, OH, WI in USA	Single peloton	ITS-RFLP; ITS	23	ECM fungi	29
Corallorhiza mertensiana	Root or rhizome	Terrestrial (mycoheterotrophic)	Sonoma, WA, OR, UT, Tehama in USA	Single peloton	ITS-RFLP; ITS	3	ECM fungi	29
Corybas recurvus	Root, stem	Terrestrial (photosynthetic)	Open heath, SWAFR	No isolation	ITS	1	Endophyte	63
Cryptostylis ovata	Root	Terrestrial (photosynthetic)	Granite outcrop, SWAFR	No isolation	ITS	1	Endophyte	63
Cymbidium aloifolium	Root, leaf	Epiphytic or lithophytic (photosynthetic)	Similipal Biosphere Reserve, Odisha, India	From surface-sterilized tissues	ITS	3	Endophytes	112
Cymbidium spp.	Root	Terrestrial (mixotrophic)	Southwest of China	From surface-sterilized tissues	ITS	3	Endophytes	70
Cypripedium (seven species)	Root	Terrestrial (photosynthetic)	Baltic coast in Estonia, forests in USA	No isolation	ITS, 28S, mt-LSU, RFLP	6	Parasites	3
Dendrobium (ten species)	Root, stem, leaf	Epiphytic (photosynthetic)	580–1200 m amsl in Guizhou, Yunnan, China	From surface-sterilized tissues	Morphology, ITS	80	Endophytes	1
Dendrobium (seven species)	Root	Epiphytic (photosynthetic)	Tropical rainforest in Xishuangbanna, China	From surface-sterilized tissues	Morphology, ITS, 28S, TUB2	18	Endophytes	73
Dendrobium nanum	Root	Epiphytic (photosynthetic)	Kolli hills (80–869 m high), India	From surface-sterilized tissues	Morphology, ITS	2	Endophyte	71
Dendrobium nobile	Root, stem, leaf	Epiphytic (photosynthetic)	Trunks or branches of standing trees, Yunnan, China	From surface-sterilized tissues	Morphology, ITS	33	Endophytes, saprobes	19
Dirus (two species)	Root	Terrestrial (photosynthetic)	Open and closed forests, SWAFR	No isolation	ITS	1	Endophyte	63
Epipactis (four species)	Root	Terrestrial (photosynthetic)	ECM forest and wetland, Germany	From surface-sterilized tissues, and no isolation	ITS, 28S, mt-LSU-rDNA	6	ECM fungi	53
Epipactis atrorubens	Root	Terrestrial (photosynthetic)	Meadow, ash hill, coast and forest, Estonia	No isolation	ITS, 5.8S, mt-LSU, ITS-RFLP	4	ECM fungi, parasite	67
Epipactis microphylla	Root	Terrestrial (mycoheterotrophic)	Alps (930 m), forest (90 m), in Mont Maurice (910 m), France	No isolation	ITS, 28S, cloning-RFLP	7	ECM fungi	44
Epipactis thunbergii	Root	Terrestrial (photosynthetic)	Banks of bogs and drainage ponds, Japan	No isolation	ITS, mt-LSU	1	Endophytes	57

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Orchid	Organs	Orchid growth habit	Orchid habitat	Isolation method	Identification method	No. of taxa or OTUs	Fungal trophic group	Reference
Epiplema grandiflorum	Root	Terrestrial (photosynthetic)	Open shrub, SWAFR	No isolation	ITS	1	Endophyte	63
Epipogium aphyllum	Rhizome	Terrestrial (mycoheterotrophic)	France (45°N, 02°–04°E), Russia (51°N, 82°–102°) and Japan (35°N, 138°E)	Crush peloton from surface-sterilized tissues	Morphology, ITS, 28S, ITS-RFLP	25	ECM fungi, parasites, endophytes, saprobes	69
Eulophia alta	Root	Terrestrial (mycoheterotrophic)	National Wildlife Refuge, Avon Park, Florida, USA	From surface-sterilized tissues	Morphology	3	Endophyte	113
Eulophia zollingeri	Root	Terrestrial (mycoheterotrophic)	Japan, Myanmar, Peninsula, Taiwan	No isolation	ITS	1	Endophyte	65
Gastrochilus acaulis	Root	Terrestrial (photosynthetic)	Kolli hills (80–869 m high), India	From surface-sterilized tissues	Morphology, ITS	1	Endophyte	71
Gastrodia confusa	Root	Terrestrial (non-photosynthetic)	Dense bamboo forests (5–1000 km apart), Japan	No isolation	ITS, 28S	5	Saprobes	85
Gastrodia similis	Root	Terrestrial (mycoheterotrophic)	Rainforests and second-growth forest, France	Single peloton	ITS, 28S	7	Saprobes and endophytes	66
Geodorum densiflorum	Root	Terrestrial (photosynthetic)	Kolli hills (80–869 m high), India	From surface-sterilized tissues	Morphology, ITS	1	Endophyte	71
Grammatophyllum (three species)	Root	Terrestrial (photosynthetic)	Mount Kinabalu, Malaysia	From surface-sterilized tissues	Morphology	28	Endophytes, parasitism fungi	72
Habenaria radiata	Root	Terrestrial (photosynthetic)	Banks of bogs and drainage ponds, Japan	No isolation	ITS, mt-LSU	1	Endophytes	57
Himantoglossum adriaticum	Root	Terrestrial (photosynthetic)	Dry calcareous grassland (969–1047 m), Italy	No isolation	ITS, cloning	4	Endophytes	62
Holcoglossum (nine species)	Root	Epiphytic (photosynthetic)	Yunnan, Guangxi and Hainan, China	From surface-sterilized tissues	ITS	15	Endophytes	5
Lepanthes (seven species)	Root and leaf	Epiphytic and lithophytic (mixotrophic)	Rainforests, Puerto Rico	From surface-sterilized tissues	Morphology	10	Saprobes, endophytes	47
Limodorum abortivum	Root	Epiphytic (mixotrophic)	Woodlands (1420 m) in France and Italy	From surface-sterilized tissues	Morphology, cloning, ITS-RFLP	8	ECM fungi, mycobionts, endophytes	49
Microtis (five species)	Root	Terrestrial (photosynthetic)	Granite outcrop, swamp, open forests, SWAFR	No isolation	ITS	3	Endophytes	63
Neottia nidus-avis	Root	Terrestrial (mycoheterotrophic)	Eight regions (90–1400 m high) in France	No isolation	ITS, ITS-RFLP	12	ECM fungi, endophytes	79
Orchis militaris	Root	Terrestrial (photosynthetic)	Flowering stage, hills, Italy	No isolation	ITS	3	ECM fungi, endophytes	68
Orchis tridentata	Root, tuber	Terrestrial (photosynthetic)	Mountain, poor grasslands on calcareous soil, Italy	From surface-sterilized tissues	Morphology, ITS-cloning	9	Endophytes	60
Paracaleana nigrita	Stem-collar	Terrestrial (photosynthetic)	Open shrub, SWAFR	No isolation	ITS	2	Endophytes	63
Pecteilis susannae	Root	Terrestrial (photosynthetic)	Three field sites in Chiang Mai, Thailand	From surface-sterilized tissues	Morphology, ITS	1	Endophytes	43

(*Contd*)

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Orchid	Organs	Orchid growth habit	Orchid habitat	Isolation method	Identification method	No. of taxa or OTUs	Fungal trophic group	Reference
Pholidota pallida	Root, leaf	Terrestrial (photosynthetic)	Natural forest and greenhouse, India	From surface-sterilized tissues	ITS	10	Endophytes	28
Platanthera chlorantha	Root	Terrestrial (photosynthetic)	Ectomycorrhizal forest, northeast Bavaria, Germany	No isolation	ITS, 28S, mt-LSU-rDNA	2	ECM fungi	53
Platanthera minor	Root	Terrestrial (mixotrophic)	Nine regions, altitude from 90~810 m, Japan	Crush coils from surface-sterilized tissues	ITS, 28S	6	Endophytes, ECM fungi	12
Prasophyllum (three species)	Root	Terrestrial (photosynthetic)	Open forests, SWAFR	No isolation	ITS	3	Endophytes	63
Pseudorchis albida	Root	Terrestrial (photosynthetic)	Mountain meadows, park, Czech	From surface-sterilized tissues	ITS	66	Endophytes, pathogenic fungi, saprobes	51
Satyrium nepalense	Root, tuber	Terrestrial (autotrophic)	Grass hilly slopes (600–4600 m), India	From surface-sterilized tissues	Morphology, ITS	1	Saprobes, endophytes	10
Spiranthes spiralis	Root	Terrestrial (photosynthetic)	The Euganean Hills, Italy	From surface-sterilized tissues	ITS	8	Endophytes	23
Thelymitra (three species)	Root	Terrestrial (photosynthetic)	Open forests, granite outcrop, roadside, SWAFR	No isolation	ITS	3	Endophytes	63
Vanda testacea	Root, leaf	Epiphytic (photosynthetic)	River Kali, Kaiga forest, India	From surface-sterilized tissues	Morphology	20	Endophytes, entomopathogenic fungi	20
Vanda testacea	Root, leaf	Epiphytic (photosynthetic)	Similipal Biosphere Reserve, Odisha, India	From surface-sterilized tissues	ITS	5	Endophytes	112
Wullschlaegelia aphylla	Root, rhizome	Terrestrial (mycoheterotrophic)	Rainforests, Guadeloupe, France	Single peloton	ITS, 28S	11	Saprobes, endophytes	66

*nrDNA, Nuclear ribosomal DNA; ITS, Internal transcribed spacer; 5.8S rDNA, 5.8S ribosomal DNA; 28S rDNA, 28S ribosomal DNA; mt-LSU-rDNA, Mitochondrial large subunit ribosomal DNA; nr-LSU, Nuclear ribosomal large subunit; TUB2, β-tubulin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ACT, Actin; RFLP, Restriction fragment length polymorphism; NSW, New South Wales; ACT, Australian Capital Territory; ZJ, Zhe Jiang Province; HB, Hu Bei Province; AH, An Hui Province; CA, California; WA, Washington; OR, Ore, OH, Ohio; WI, Wisconsin; UT, Utah; SWAFR, Southwest Australian Floristic Region. Most number of taxa or OTUs was counted by fungal species and less by genera according to references.

Table 2. Protocols used for surface sterilization in orchid endophyte studies

Tissue	Protocol	Reference
Root	5% solution of 'Domestos' (20–30 min) – sterilized water	114
Root	0.1% $HgCl_2$ in 20% ethanol – sterilized distilled water (4~5 changes)	115
Root	Several changes in sterile water	116
Root	20% 'Milton' (15–20 min)	117
Root	20% solution of household bleach (1 min) – sterile distilled water	39
Root	70% ethanol (30 s) – 5.25% household bleach (10 min)	118
Root	75% ethanol (35 s) – 3% $NaClO_2$ (1 min) – 75% ethanol (30 s)	73
Root	75% ethanol (30 s) – 0.5% $NaClO_2$ (3–5 min)	42
Root	70% ethanol (30 s) – 95% ethanol, 5.25% $NaClO_2$, sterile H_2O_2 (1 : 1 : 1) (1 min)	43
Root	75% ethanol (1 min) – 3.4% NaClO (10 min) – 75% ethanol (30 s)	47
Root	5.25% NaClO (1 min) – sterile water (2 times)	119
Root	70% ethanol, 2.5% NaClO (1 min) – 70% ethanol (1 min)	36
Root	70% ethanol (30s) – 2.6% NaClO (3 min)	120
Root	70% ethanol (1 min) – 2% NaClO (5 min)	121
Stem	75% ethanol (40 s) – 4% NaClO (10 min)	19
Root, leaf	70% ethanol (30 s) – 4% NaClO (90 s)	28
Root	95% ethanol (20 s) – 5% NaClO (3 min)	23
Root	70% ethanol (2 min) – 10% NaClO (3 min)	7
Root	70% ethanol (1 min) – NaClO with 1% available chlorine (1 min)	78
Rhizome	70% ethanol (30 s) – NaClO with 1% available chlorine (30 s)	69
Root	30% H_2O_2 (1 min) – sterile water	49
Root	3% H_2O_2 (10 min) – sterile distilled water (three times)	4
Root	75% ethanol (30 s) – 0.1% HgCl2 (5 min)	122
Root	70% ethanol (1–2 min) – 0.1% $HgCl_2$ (7–8 min)	70
Root	Detergent solution (5–6 min) – 10% $Ca(ClO)_2$ (7–8 min)	123

The study of endophytic fungi starts with a collection of orchid samples, followed by isolation in the laboratory. Epiphytic microorganisms are removed via surface sterilization prior to isolation[24]. All surface sterilization procedures in orchid endophytic research have used sterilizing reagents, including ethanol, chlorine (Cl_2), sodium chlorite ($NaClO_2$), sodium hypochlorite (NaClO), mercury (II) chloride ($HgCl_2$), hydrogen peroxide (H_2O_2) and calcium hypochlorite ($Ca(ClO)_2$) to disinfect tissues via sequentially immersing tissues in reagents (see Table 2 for details). The concentration and time for surface sterilization vary depending on the sterilizing reagents and the type of orchid tissues studied. The concentration of reagents is important. Sterilization with 0.1% or 0.2% $HgCl_2$ for 3 min did not kill *Bacillus* species, but using 0.3% $HgCl_2$ for 10 min successfully killed the bacteria[25]. NaClO has been reported[26] to be more damaging to tissues than $Ca(ClO)_2$. The degree of surface sterilization greatly affects the fungal endophytes recovered[22]. Therefore, Schulz *et al.*[27] suggested leaf imprinting to test the effectiveness of the protocol. However, in most orchid endophyte studies to date, leaf imprinting was not carried out. The work of Sawmya *et al.*[28] was the only orchid endophyte study that tested the effectiveness of their surface sterilization protocol. No microorganisms grew on media after imprinting the surface-sterilized tissues on agar, which indicated that their surface sterilization protocol was successful.

The isolation of non-mycorrhizal endophytes has involved teasing apart or crushing surface-sterilized root pieces or rhizomes aseptically to liberate hyphae on media or sterilized water[29,30]. Cultivation of surface-sterilized segments on media has also been widely used for all orchid tissues to isolate endophytic fungi (Table 1).

Antibiotics were used in culture-dependent isolation to prevent bacterial contamination. Streptomycin sulphate and potassium penicillin G restrained the growth of G^- bacteria and G^+ bacteria respectively[31]. Sometimes researchers added several kinds of antibiotics to prevent contamination. Otero *et al.*[32] applied streptomycin, tetracycline and penicillin together to prevent contamination. A study in which *Colletotrichum* species were isolated from *Bletilla ochracea* used streptomycin and chloramphenicol to prevent contamination[33].

Different protocols may be tried for isolating endophytic fungi. *Epulorhiza* fungi could be isolated using either single peloton or root section protocol and they grew more quickly when bacteria were present than if excluded[31,34]. Some mycorrhizal fungi, however, were isolated using root section because they did not form massive hyphal colonization[4,32,35,36]. Moreover, not all studies on orchid endophytes used isolated fungi as materials for fungal identification. Direct sequencing of DNA extracted from orchid tissues containing fungi has also revealed diversity of fungal endophytes. However, it is necessary to emphasize that although some fungal-specific primers are available, they do not necessarily amplify only fungal DNA. For example, primer ITS1F (ref. 37) is intended to be specific to fungi and it can also amplify DNA of many species of eu-dicots and some

orchids[38]. Therefore, analysis and interpretation of the results from such protocols must be treated with caution. Comparison of the sequences of fungal endophytes with those of well-characterized fungi in GenBank is necessary to name the species.

Identification of fungal endophytes

Fungi can be identified using morphology, molecular analysis or a combination of both approaches. In the past, fungal identification relied on morphological characteristics such as colony, mycelium and spore characters[39]. However, molecular approaches have more recently been applied to identify orchid endophytes[40]. The use of combined morphological–molecular data is probably a better approach[41], but most studies on orchid endophytes have used either morphology or molecular analysis (Table 1). Only a few studies performed both[33,42,43].

Morphological identification

Even though endophytes can be directly visualized inside the tissues by staining[22], most studies of orchid endophytes did not use this method. Majority of fungal endophyte studies have adopted surface-sterilized tissues which can be problematic because not all endophytic fungi grow in culture, or epiphytic fungi are not completely killed[29]. Orchid mycorrhizal fungi can be distinguished by hyphal coils (pelotons); however, many non-mycorrhizal fungal endophytes from orchids do not possess any specific characters or have some overlapping traits in culture[32,44].

Problems with identification of orchid endophytic fungi using morphology: Morphological identification of orchid fungal endophytes to species or sometimes even genus level is not always possible[41]. Many endophytic fungi will not sporulate, even if sporulation-inducing methods are applied[7,45]. These include ectomycorrhizal fungi (ECM fungi) such as *Russula*[46]. Apart from the nature of the fungi, morphological identification requires researchers to have a good understanding of basic fungal taxonomy and good skills in handling fungal cultures. Morphological identification may take more time than molecular identification, as endophytic fungi may need at least three to four weeks to sporulate[47,48]. However, employing morphological characterization to identify endophytes is less expensive.

Molecular identification

Molecular identification of orchid endophytes can be done using polymerase chain reaction (PCR) to amplify a specific DNA region and subsequently cleave the PCR product using specific restriction endonucleases (i.e. PCR-RFLP)[30,45,48,49]. However, the more commonly used molecular identification is sequence-based approach by which a selected DNA region is sequenced. Then the DNA sequence can be blasted in the public database (e. g. GenBank) and/or used to construct a phylogenetic tree (Table 1). Selection of genes/regions for molecular identification is particularly important[48]. The ITS region (i.e. internal transcribed spacers of the rDNA gene or ITS1-5.8S rDNA-ITS2) is the region of choice because of its high degree of variation and the fact that it is the most common sequence generated[29,50]. Therefore, applying ITS sequence approaches to identify fungi increases the possibility to find similar or homologous sequences. For example, 66 distinct operational taxonomic units (OTUs) were isolated from *Pseudorchis albida* and identified through only ITS sequencing and phylogenetic analysis[51].

However, using ITS region alone for identification of some groups of fungi is not adequate. As a result, multiple gene loci are usually sequenced[52,53]. Besides ITS, regions of DNA that have been used in sequence-based identification of orchid fungal endophytes include the nuclear coding regions, i.e. 28S rDNA, β-tubulin (TUB2), glycerdalehyde-3-phosphate dehydrogenase (GAPDH), actin (ACT), and the mitochondrial large subunit rDNA (mt-LSU-rDNA) (Table 1). For example, the ITS region alone compared with a combination of ITS, TUB2 and *tef1* gave relatively poor species resolution in identification of *Pestalotiopsis* species[54] isolated from *Dendrobium nobile*[1] and *Pholidota pallida*[28], as well as *Fusarium* from *Pecteilis susannae* and *Cattlteya skinneri*[2,43,55]. For identification of fungal endophytes from *P. pallida* to generic level, the ITS region was used; however, the ITS region combined with TEF, GPDH and ACT was used for interspecific distinction[28,55]. Similarly, ITS in combination with mt-LSU-rDNA were used to identify endophytes from *Habenaria radiata*, *Epipactis thunbergii* and six species of *Chiloglottis*[56,57]. Huang *et al.*[58] reported that sequencing multiple barcodes of fungi from *Phalaenopsis microbiome* using next-generation sequencing gave much higher fungal diversity than that sequencing nuclear-ITS alone.

Problems with identification of orchid endophytes using molecular methods: There are several disadvantages in relying on molecular methods for identifying endophytes, including low quality and misidentification of a large number of ITS sequences in GenBank[41]. These problems are now being addressed[41]. For example, Cai *et al.*[59] compared ITS sequences of ex-type specimens of *Colletotrichum* with the sequences in GenBank and reported that the majority of *Colletotrichum* ITS sequences in GenBank are wrongly named. Recently, some researchers advocated applying sequences of fungal ex-type for constructing phylogenetic backbone which may avoid improper identification[41,55]. Furthermore, some fungal-specific

primers may fail to amplify DNA of some fungi. The fungal primers ITS1F and ITS4 cannot efficiently amplify DNA of fungal species in the family Tulasnellaceae[60]. Therefore, although in most of the cases morphological identification or molecular identification alone is adequate, it is best, where possible, to use both morphological and molecular methods. We recommend a combination of two methods for endophytic identification in orchids. This is because endophytes may contain some fungal taxa that grow vigorously on media[61], but others are only revealed when molecular methods are applied.

Diversity of orchid non-mycorrhizal endophytes

The orchid non-mycorrhizal endophytic fungi contain over 110 genera, which are more diverse than mycorrhizal endophytes (Table 3). At least 39 genera of Sordariomycetes (i.e. *Cylindrocarpum*, *Hypocrea*, *Nigrospora*, *Pestalotiopsis*)[1,62,63], 25 genera of Dothideomycetes (i.e. *Alternaria*, *Cercospora*, *Lasiodiplodia*, *Phyllosticta*)[28,35,64], 12 genera of Leotiomycetes (i.e. *Chaetomella, Sclerotinia*)[1,63] in Ascomycota and 32 genera of Agaricomycetes in Basidiomycota (i.e. *Conocybe*, *Gymnopus*, *Hydropus*, *Psathyrella*, *Resinicium*)[65,66] have been reported as orchid non-mycorrhizal endophytic fungi. They also involve a few species of Pezizomycetes (i.e. *Geopora*)[67], Eurotiomycetes (i.e. *Talaromyces*)[44], Chaetothyriomycetes (i.e. *Exophiala*)[67], Helotiales and Xylariales of ascomycetes (i.e. Nemania)[19] in Ascomycota and Tremellomycetes (i.e. *Cryptococcus*)[64] as well as Pucciniomycetes (i.e. *Tuberculina*)[68] in Basidiomycota. Orchid non-mycorrhizal fungi related to Chytridiomycota (i.e. *Olpidium*)[69], Glomeromycota[57] and Zygomycota (i.e. *Umbelopsis*)[70] have also been reported. Among all genera observed in orchid non-mycorrhizal fungi, *Colletotrichum* and *Fusarium* frequently appeared in different orchids such as *S. nepalense* and *D. nobile*[10,19]. *Aspergillus*, *Trichoderma* and *Verticillium* have also been repeatedly found in orchids[47,71] (Table 3).

Since the traditional protocol of surface sterilization has a significant influence on the fungal endophytes obtained, it is possible that some surface contaminants could be mistakenly identified as orchid non-mycorrhizal endophytes. This may be particularly true for species of *Aspergillus*, *Penicillium* and *Cladosporium* which are common surface contaminants[22], as well as *Trichoderma hamatum* and *Verticillium* sp., which are soil-dwelling fungi but reported as fungal endophytes from orchids[63,71,72].

Specificity and factors affecting fungal diversity

Host-specificity between orchids and their non-mycorrhizal endophytic fungi has been less well studied compared to their biodiversity. Endophytes of certain non-photosynthetic orchids appeared to be more specific than in green photosynthetic orchids[3]. *Psathyrella candolleana* is specific to the mycoheterotrophic orchid *Eulophia zollingeri*[66]. However, fungal specificity could be observed in some photosynthetic orchids. The photosynthetic orchids like *Dendrobium* spp. have frequent associations with fungi in Xylariaceae[1,19,73]. *Grammatophyllum speciosum* was reported to be colonized by *Fusarium* and *Trichoderma*[72]. Endophytic fungi isolated from another photosynthetic orchid – *Orchis militaris* were found to be host-specific[68]. Specificity was also observed in mycoheterotrophic orchids. Thirteen different taxa occurred on a single sample in the study of endophytes from the mycoheterotrophic orchid, *Aphyllorchis montana*[18].

Orchid tissues used for the fungal endophyte study also affect the diversity of non-mycorrhizal endophytes. The diversity of non-mycorrhizal endophytic fungi in orchids is higher in leaves than roots[20,73]. Tao *et al.*[64] found that there was overlap in the case of few endophytic fungi in roots and leaves of *Bletilla ochracea*. They pointed out that orchid leaves and roots had different endophyte associations and speculated that this was probably because the organ texture provided different ecological habitats (air or below ground) with varying physiology and chemistry for the taxa[64,74].

The diversity of orchid non-mycorrhizal endophytic fungi probably also depends on the localities from where the orchids were collected. Sudheep and Sridhar[20] reported that relatively similar endophytic fungal assemblages were isolated from distantly related orchids *Vanda testace* and *Bulbophyllum neilgherrense* sampled in the same habitat, i.e. the Kaiga forest of the Western Ghats, India. There was no overlap in taxa of non-mycorrhizal endophytic fungi isolated from individuals of *Epipactis atrorubens* sampled respectively, at a meadow in a coastal farm and at Ash Hill[67]. Bunch *et al.*[75] found that fungal endophytes in *Cypripedium acaule* were significantly influenced by geography and soil. Therefore, when studying orchid non-mycorrhizal endophytic fungi, sampling at different niches will help understand their fungal ecology. Furthermore, as climate change occurs, this may alter orchid niches by impacting their surroundings such as soil moisture and rainfall[74]. Endophyte diversity in plants may also be affected by insect-induced galls, which can change fungal colonization and diffusion[76].

Groups of non-mycorrhizal endophytes from orchids

Orchid non-mycorrhizal endophytes can be classified into several groups according to their lifestyles, i.e. ECM fungi, saprobes, parasites and latent pathogens (Table 1). However, fungal lifestyles are not always stable traits. Some endophytic fungi can switch to a necrotrophic lifestyle at an ecological timescale[77]. Further studies on the

Table 3. Mycorrhizal and non-mycorrhizal endophyte genera from orchids

Mycorrhizal	Non-mycorrhizal						
Ceratobasidium (anamorph: *Ceratorhiza*)[53]	*Acephala*[51]	*Cladophialophora*[63]	*Entonaema*[28]	*Hebelom*[69]	*Marasmiellus*[85]	*Paraconiothyrium*[1]	*Schizophyllum*[1]
Coprinus[53]	*Acremonium*[1]	*Cladosporium*[5]	*Exidia*[57]	*Helicomyces*[72]	*Meliniomyces*[51]	*Penicillium*[20]	*Sclerotinia*[113]
Lactarius[124]	*Alternaria*[1]	*Clonostachys*[19]	*Exophiala*[63]	*Humicola*[51]	*Menispora*[51]	*Periconiella*[1]	*Scytalidium*[70]
Leptodontidium[53]	*Ampelomyces*[1]	*Cochliobolus*[123]	*Fusarium*[112]	*Hyalodendron*[1]	*Merismodes*[125]	*Pestalotia*[47]	*Steccherinum*[30]
Moniliopsis[119]	*Amphinema*[52]	*Colletotrichum*[33]	*Fusicoccum*[1]	*Hydropus*[85]	*Metarhizium*[69]	*Pestalotiopsis*[28]	*Stephanonectria*[30]
Sebacina[3]	*Annulohypoxylon*[73]	*Conocybe*[85]	*Galactomyces*[51]	*Hymenogaster*[44]	*Mortierella*[70]	*Pezicula*[1]	*Strumella*[44]
Thanatephorus[57] (anamorph: *Rhizoctonia*)	*Armillaria*[113]	*Cortinarius*[44]	*Geomyces*[42]	*Hypocrea*[66]	*Mycosphaerella*[51]	*Phaeosphaeria*[30]	*Talaromyces*[44]
Trichosporiella[126]	*Arthrinium*[1]	*Cosmospora*[5]	*Geopora*[67]	*Hypoxylon*[28]	*Myrmecridium*[5]	*Phialophora*[63]	*Terfezia*[79]
Tuber[10]	*Ascobolus*[42]	*Cryptococcus*[64]	*Geotrichum*[72]	*Laccaria*[79]	*Nectria*[69]	*Phoma*[47]	*Thelephora*[69]
Tulasnella[112] (anamorph: *Epulorhiza*)	*Aspergillus*[2]	*Cryptosporiopsis*[5]	*Gibberella*[64]	*Lachnum*[51]	*Nemania*[42]	*Phomopsis*[73]	*Tomentella*[44]
	Aureobasidium[1]	*Curvularia*[72]	*Gliocladium*[20]	*Lasiodiplodia*[28]	*Neonectria*[63]	*Phyllosticta*[28]	*Trechispora*[66]
	Bionectria[42]	*Cylindrocarpon*[5]	*Gloeophyllum*[30]	*Lasiosphaeria*[57]	*Nigrospora*[1]	*Pleospora*[1]	*Trichoderma*[28]
	Botrytis[2]	*Daldinia*[73]	*Glomerularia*[1]	*Leohumicola*[51]	*Nodulisporium*[73]	*Podospora*[42]	*Umbelopsis*[70]
	Candida[60]	*Davidiella*[23]	*Guignardia*[19]	*Lepiota*[85]	*Oidiodendron*[51]	*Protoventuria*[69]	*Varicosporium*[51]
	Cercophora[1]	*Didymella*[69]	*Gymnomyces*[49]	*Leptosphaeria*[23]	*Olpidium*[69]	*Psathyrella*[66]	*Verticillium*[1]
	Chaetomella[1]	*Dioszegia*[64]	*Gymnopus*[85]	*Leptosphaerulina*[64]	*Paecilomyces*[69]	*Pseudogymnoascus*[42]	*Wilcoxina*[52]
	Chaetomium[72]		*Halocyphina*[30]	*Macowanites*[49]	*Paneolus*[85]	*Resinicium*[66]	*Xylaria*[19]

evolution of endophytic fungi at the gene and ecological levels need to be carried out to explore their roles in orchids[78].

Ecto-mycorrhizal fungi

Roots of many mycoheterotrophic orchids with internal hyphal coils of saprotrophic fungi, were found to be associated with ECM Ascomycota (e.g. Terfeziaceae, Saroscyphaceae)[79] and/or ECM Basidiomycota (e.g. Russulaceae, Thelephoraceae, Clavulinaceae, and Sebacinaceae)[18,29,44,79] of trees and shrubs. ECM symbiosis has long been understood as the way orchids derive carbon from the surrounding ectomycorrhizal trees. This hypothesis was later verified by the McKendrick *et al.*[80], who used $^{14}CO_2$ to track the transfer of carbon from ectomycorrhizal tree seedlings via hyphal connections to the mycoheterotrophic orchid *Corallorhiza trifida* in the field that was later confirmed by other studies[69,81]. Interestingly, photosynthetic orchids were also found to be associated with ECM fungi in the roots[12,53,68,67] and were partial exploiters of fungal carbon[12,53,69]. The degree of specificity between orchids and their ectomycorrhizal partners, therefore, largely but not entirely depends on the degree of dependency of orchids on the fungal carbon. Achlorophyllous orchids and species with inefficient photosynthesis were reported to be specifically associated with narrow groups of ECM fungi, including *Russula*[49,79], whereas chlorophyllous orchids were associated with a wide range of ECM fungi[82]. However, Roy *et al.*[18] studied orchid–fungal associations in tropical regions and revealed the absence of specificity in two and the presence of specificity in one mycoheterotrophic species.

The role of ECM fungi in orchids, however, is probably not limited to carbon transport. It has been speculated that mycorrhizal networks increase the bioactive zones of infochemicals by serving as the direct connecting superhighways for plants to communicate underground[83]. Even though arbuscular mycorrhizal fungi are presently the only group of fungi that have been proven to transport compounds between multiple plant species through common hyphal networks[84], it will be interesting to investigate if ECM fungi play this role in orchids.

Saprobic fungi

Many saprobic species of Agaricomycetes (i.e. *Hydropus*, *Gymnopus*, *Marasmiellus*)[85] and Sordariomycetes (i.e. *Clonostachys*, *Resinicium*)[19] have been identified as orchid non-mycorrhizal endophytic fungi. Endophytes are important saprobic decomposers[22]. Gymnopoids and mycenoids saprobes isolated from mycoheterotrophic orchids *Gastrodia similis* have been reported to secrete laccases and peroxidases[44,66,86,87]. *Resinicium* spp. living in *G. similis* are also wood-decaying fungi[66]. *Lasiosphaeria* spp. found in the photosynthetic orchid *Habenaria radiataare* are important ligninolytic saprotrophs[57,88].

Latent pathogen

Some of the non-mycorrhizal endophytes are plant pathogens. For example, *Fusarium oxysporum* can cause plant wilt and rot diseases[89]. *Alternaria, Aspergillus, Chaetophoma* and *Trichoderma* have relationships with cotton plant disease[90]. *Xylaria* is a well-known pathogen from decaying plant organs[91]. *Paeciliomyces* sp. isolated from *Vanda testacea* is also reported as an entomopathogen[20].

Latent pathogens in plants have been noticed from the 1950s (ref. 92). They may exist as endophytes and probably become pathogens during a later period of life, especially when plants are stressed[67]. Some *Colletotrichum* species are pathogens of orchids such as *Oncidium flexuosum*, *Bulbophylum cylindrum* and *Coelogyne cristata*[33,93], while they have also been isolated as endophytes from healthy orchids, such as species in *Lepanthes* and *Dendrobium*[1,47]. In fact, endophytes in plant stems and leaves can switch from latent pathogens to mutualistic symbionts[94]. Freeman and Rodriguez[95] found that non-pathogenic and pathogenic strains in plants can restrict the growth of each other, and mutualists may also be pathogens. Orchids at different life stages perhaps carry latent pathogens to different extents because all plants have been found potentially infected by endophytes and when competition for energy occurs between plants and fungi, plants may tend to be more susceptible to the pathogens[96]. Furthermore, some well-known virulent taxa such as *Fusarium* species, which are often isolated from orchids, tend to be asymptomatic endophytes rather than pathogens under optimal growth conditions[62,97]. Therefore, although we speculate that latent pathogens exist in orchids, only further investigations can identify their roles in host tissues.

Role of non-mycorrhizal endophytes in orchids

The role of orchid non-mycorrhizal endophytes has rarely been addressed. In general, plant endophytes are thought to be the resources for bioactive compounds. For example, a *Trichoderma* species from Cupressaceae was shown to have antimicrobial properties[98]. Screening bioactive compounds for disease treatment from higher plants has increased[99]. Potential pharmaceutically important substances are abundant in orchids and this to some extent may be a result of extreme diversity of non-mycorrhizal fungal metabolites. *Alternaria* sp. and *F. oxysporum* isolated from orchids in Brazil showed strong inhibition to *Escherichia coli*[100]. From the orchid *Anoectochilus setaceus*, an antibacterial nortriterpenoid helvolic acid was extracted from the endophytic taxon *Xylaria* sp.[101]. These orchid non-mycorrhizal endophytes may

Appendix 1. Orchid genera in endophytic research

Orchid genera	Reference	Orchid genera	Reference	Orchid genera	Reference
Aa	7	*Dryadella*	135	*Oerstedella*	151
Acampe	112	*Dryandra*	93	*Oncidium*	32
Acianthera	100	*Dryas*	93	*Onychium*	152
Acianthus	30	*Elythranthera*	63	*Ophrys*	145
Aerangis	127	*Encyclia*	143	*Orchis*	142
Aeranthes	66	*Epiblema*	63	*Oreorchis*	139
Aerides	123	*Epidendrum*	32	*Ornithidium*	153
Amerorchis	39	*Epipactis*	44	*Orthoceras*	117
Anacamptis	23	*Epipogium*	69	*Paphiopedilum*	123
Angraecopsis	66	*Eriochilus*	117	*Paracaleana*	154
Angraecum	127	*Erythrodes*	32	*Pecteilis*	43
Anoectochilus	128	*Erythrorchis*	140	*Pelexia*	134
Aphyllorchis	18	*Eulophia*	113	*Peristeranthus*	117
Aplectrum	129	*Galeola*	144	*Phaius*	144
Apostasia	130	*Gastrochilus*	71	*Phajus*	139
Appendiculata	131	*Gastrodia*	85	*Phalaenopsis*	123
Arachnis	114	*Gavilea*	9	*Pholidota*	28
Arachnorchis	132	*Gennaria*	145	*Piperia*	155
Arthrochilus	117	*Geodorum*	71	*Platanthera*	53
Arundina	131	*Glossodia*	117	*Platylepis*	66
Beclardia	66	*Gomesa*	146	*Plectorrhiza*	117
Benthamia	66	*Gongora*	135	*Pleione*	64
Bipinnula	133	*Goodyera*	147	*Pleurothallis*	118
Bletilla	33	*Grammatophyllum*	72	*Plocoglottis*	144
Brassia	129	*Graphorchis*	66	*Pogonia*	129
Bromheadia	131	*Graphorkis*	127	*Polystachya*	127
Buddleja	134	*Gymnadenia*	142	*Pomatocalpa*	117
Bulbophyllum	28	*Habenaria*	57	*Prasophyllum*	63
Caladenia	30	*Hadrolaelia*	8	*Pseudorchis*	51
Calanthe	131	*Hetaeria*	135	*Psychilis*	32
Caleana	53	*Hexalectris*	148	*Pteroceras*	117
Calochilus	117	*Hexisea*	135	*Pterostylis*	30
Calopogon	129	*Himantoglossum*	62	*Pterygodium*	156
Calypso	39	*Hoffmannseggella*	8	*Pyrorchis*	63
Camaridium	119	*Holcoglossum*	5	*Renanthera*	123
Campylocentrum	32	*Holothrix*	66	*Rhinerrhiza*	117
Catasetum	135	*Hymenocallis*	93	*Rhizanthella*	117
Cattlteya	2	*Ionopsis*	32	*Rhynchostylis*	157
Cephalanthera	53	*Isochilus*	45	*Robiguezia*	135
Chamaegastrodia	78	*Jacquinella*	135	*Robiquetia*	117
Changnienia	42	*Jumellea*	66	*Rodriguezia*	135
Chiloglottis	56	*Laeliocattleya*	114	*Rossioglossum*	158
Clivia	93	*Lecanorchis*	124	*Saccolabiopsis*	117
Coeloglossum	119	*Lepanthes*	47	*Sacoila*	134
Coelogyne	64	*Leporella*	117	*Sarcochilus*	116
Coppensia	136	*Leucorchis*	142	*Sarcoglottis*	159
Corallorhiza	80	*Limodorum*	49	*Satyrium*	10
Corybas	63	*Liparis*	147	*Scaphyglottis*	135
Corycium	137	*Lirope*	73	*Serapias*	145
Corymborkis	66	*Listera*	142	*Sobralia*	135
Cranichis	138	*Loroglossum*	149	*Sophronitis*	100
Cremastra	139	*Ludisia*	126	*Spathoglottis*	131
Cryptopus	66	*Luisia*	150	*Spiculaea*	63
Cryptostylis	63	*Lycaste*	129	*Spiranthes*	23
Cymbidium	139	*Lyperanthus*	63	*Stanhopea*	160
Cynorkis	66	*Macodes*	144	*Stelis*	118
Cypripedium	3	*Maxillaria*	138	*Taeniophyllum*	117
Cyrtosia	140	*Microtis*	63	*Thelymitra*	63
Cyrtostylis	141	*Miltonia*	135	*Thrixspermum*	114
Cystopus	135	*Myoxanthus*	135	*Tipularia*	147
Dactylorchis	142	*Myrmechis*	129	*Tolumnia*	32
Dactylorhiza	53	*Neottia*	79	*Trichoglottis*	117

(*Contd*)

Appendix 1. (*Contd*)

Orchid genera	Reference	Orchid genera	Reference	Orchid genera	Reference
Dendrobium	1	*Neottianthe*	162	*Trichopilia*	135
Dendrochilum	131	*Nervilia*	163	*Trichosalpinx*	135
Dichaea	135	*Neuwiedia*	164	*Trigonidium*	135
Dichromanthus	161	*Nidema*	135	*Trizeuxis*	138
Didymoplexis	135	*Nigritella*	165	*Vanda*	114
Dimerandra	135	*Notylia*	138	*Vanilla*	138
Diplocaulobium	34	*Oberonia*	150	*Vrydagzynea*	135
Dipodium	125	*Octomeria*	135	*Wullschlaegelia*	66
Disa	63	*Odontoglossum*	166	*Yoania*	167
Diuris	63	*Oeceoclades*	32	*Zeuxine*	168
Dryadella	135	*Oeonia*	66		

occur in other plants and possibly be involved in the production of bioactive compounds. Gogoi *et al.*[102] screened bioactive metabolites from *Hypocrea* spp. isolated from *Dillenia indica*. *Hypocrea* species have also been isolated from orchids, such as *Wullschlaegelia aphylla* and *Himantoglossum adriaticum*[66,82]. Xu *et al.*[103] found that approximately 160 metabolites isolated from *Pestalotiopsis* species had anti-tumour, anti-fungal or anti-microbial potential. This perhaps provides hope for decreasing pressure for the huge requirement for taxol, as the anti-tumour drug is restricted to yew trees[104].

Besides highly bioactive alternatives, Hou and Guo[105] showed that dark septate endophytes isolated from *Dendrobium* and *Leptodontidium* spp., interacted with the seedlings of *D. nobile* in a manner similar to that of orchid mycorrhizal fungus. The endophyte formed peloton-like structures in cortical cells of the orchid and greatly enhanced the growth and biomass of the orchid seedlings. Non-mycorrhizal *Fusarium* was reported to promote seed germination in *Cypripedium* and *Platanthera* orchids, even though the effect was relatively minor when compared to that of specific orchid *Rhizoctonia* mycorrhiza[106]. Similarly, *Umbelopsis nana* isolated from *Cymbidium* spp. has a vigorous effect on development of *Cymbidium hybridum*, enhancing K, Ca, Cu, Mn contents in symbiotic plantlets[81]. Researchers detected fuel potential in volatile organic compounds isolated from *Phomopsis* sp. from orchid *Odontoglossum* sp.[107]. Applications of endophytes of other plants have been shown to have industrial potential, which may be worth exploring in orchid endophytes. For example, endophytic antioxidant activities have been reported in many plants[108]. *Phoma*, *Alternaria* and *Aspergillus* species are metal-resistant and play roles in phytoremediation[109]. *Phomopsis* isolates can secrete enzymes, including cellulases, lipases, pectinases, pectate, lyases and proteases[110]. *Cladosporium*, *Alternaria* and *Fusarium* species that are major groups of endophytic fungi in grasses have close relationships with allergen exposure, which may help in understanding the evolution of immune reaction to respiratory allergens[111].

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