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Molecular identification of endophytic fungi associated with orchids from Mount Cameroon region

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Abstract

The orchidaceae (orchid family) is the second largest family of flowering plants after the Asteraceae. Orchids are important in herbal medicine, the food industry, perfumery industry and as ornamentals. They rely on mycorrhizal fungi to provide the carbon sources for seed germination and seedling establishment. The Mount Cameroon Region has a rich diversity of orchids which is under threats from land use patterns. This study was aimed at (i) identifying fungi associated with orchid mycorrhiza; and (ii) identifying non-mycorrhizal endophytic fungi. Nine species of orchids were selected for this study, three each from the different life forms. Selection criteria were based on vulnerability, scarcity and abundance. Mycorrhizal and non-myccorrhizal endophytic fungi were isolated from dissected single pelotons and from non-peloton root tissues respectively. Identification of fungi was based on morphological and sequence-based molecular methods. A total of 18 fungi species belonging to 12 genera were identified with *Penicillium* being the most abundant. The inferred phylogenetic tree grouped all endophytes into 9 major clusters belonging to 2 phyla. Clustering was independent of whether endophytes were mycorrhizal or non-mycorrhizal. The results of this study could contribute to orchid conservation and for the discovery of bioactive compounds.

Keywords: *Orchid, Mycorrhiza, Non-mycorrhizal, Endophytic, Fungi, Molecular*

1. Introduction

The Orchidaceae (the orchids' family) is the second largest family of flowering plants after Asteraceae. The importance of orchids in herbal medicine, the food industry, perfumery industry and as ornamentals worldwide cannot be overstated. In Japan, legend has it that a sterile Emperor's wife inhaled the inebriating perfume of *Cymbidium ensifolium* and went on to have 13 children (*Berliocchi*, 2004). The enthusiasm for orchids has

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grown to an extent that it is associated with a lot of myths and folklores. The medicinal use of orchids continues in Chinese herbal medicine to this day. In Europe, the Greeks referred to testicles as *orchis*, and Theophrastus (372–286 BC) named the orchids from that word, as the underground tubers of many European terrestrial orchids resemble a pair of testicles (Theophrastus, 1664). In his *Enquiry into Plants*, he reported that the orchids had medicinal properties (Reinikka, 1995). In the first century AD, Dioscorides, who was a Greek working as a Roman military physician, wrote his *De Materia Medica*, including two terrestrial orchids (*Berliocchi*, 2004). He adopted and promoted the 'Doctrine of Signatures' whereby plants were used for medicinal purposes according to their resemblance to parts of the human anatomy, for example by shape or color. Naturally this led to orchid tubers being used to heal diseases of the testicles, and to stimulate lust. Moreover, if given to men as whole fat new tubers this was supposed to produce male progeny, and if the shriveled old tubers were given to women, this should produce female children. Orchids are collected or grown because of the beauty of their flowers. The admiration of orchid flowers has driven many persons into orchid cultivation for the exploitation of their flowers and has also led to the production of many hybrids. Orchids are collected or grown because of the beauty of their flowers. The admiration of orchid flowers has driven many persons into orchid cultivation for the exploitation of their flowers and has also led to the production of many hybrids. Orchid flowers usually have good cents and are often exploited in the perfume industries for the production of perfumes using gas-liquid chromatography or mass spectrometry (Gross *et al*., 2016). Underground tuber of the purple orchids called *orchis mascula* is used for cooking the hot Turkish beverage salep which is an aphrodisiac. *Vanill planifolia* is a commercially important orchid used as flavoring in baking worldwide.

Orchids reproduce sexually through seeds that once released depend on mycorrhizal fungi to germinate and sustain early embryo growth (Murguía and Lee, 2007; Pereira *et al*., 2005; Smith and Read, 2008). This dependency is due to the lack of developed endosperm in orchid seeds. The fungi supply relevant nutrients to the embryo such as carbon, nitrogen, and phosphorus (Arditti and Ghani 2000; Smith and Read, 2008). Many orchid species establish a close association with these fungi, sometimes considered parasitic, but the energetic cost of the interaction may be relatively low for the fungal partner (McKendrick *et al*., 2000). At adult stages, many species maintain symbiotic associations with fungi that colonize the cortical cells of their roots (Pereira *et al*., 2014; Atala *et al*., 2015; Herrera *et al*., 2017).

The identification and isolation of fungi associated with orchids could help in the conservation and ecological restoration of orchid species, many of which have known conservation problems (Novoa *et al*., 2015; Atala *et al*., 2015 and 2017). Historically, much knowledge about orchid mycorrhizas has been acquired from in vitro isolation of fungi. This has allowed basic fungal identification and simple in vitro seed germination experiments conducted with some root-isolated fungi (Warcup, 1971; Clements, 1988). A hurdle in these types of investigations has been an inability to accurately identify the isolated fungal partners and this has been especially critical to orchid conservation procedures involving restorative work; moreover, isolation often provided mostly contaminants or endophytes (i.e., fungi that for all or part of their life cycle inhabit living plant tissues but do not form pelotons nor cause any obvious disease symptoms; (Wilson, 1995). Molecular taxonomy approaches have enhanced fungal taxonomy, especially by isolating fungal DNA and sequencing the nuclear ribosomal DNA (Seiffert, 2009). The fungal partners of orchid mycorrhizas can be more accurately and routinely identified from cultured fungi or directly from orchid protocorms, roots, tubers and rhizomes (Bougoure *et al*., 2005; Martos *et al*., 2009; Swarts *et al*., 2010). For mycobionts recalcitrant to axenic growth, PCR amplification of colonized orchid tissues using fungus-specific primers is commonly used (Dearnaley and Le Brocque, 2006; Dearnaley and Bougoure, 2010). Sequencing of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations (White *et al*., 1990; Gardes and Bruns, 1993) has been the method of choice for identifying orchid mycobionts over the past decade.

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 nonmycorrhizal endophytes from orchids because of their physiological roles and their potential as sources of novel bioactive compounds (Xiaoya *et al*., 2015).

This study aims at identifying fungi associated with orchid mycorrhizal endophytes and identifying the much-neglected non-mycorrhizal endophytic fungi in orchids from mount Cameroon, following the increasing interest in their physiological roles and their potential as sources of novel bioactive compounds.

2. Materials and methods

2.1. Selection of species of orchids

Nine species of orchids were selected for this study, three each from the different life forms. Orchids were selected based on the different life forms; the criteria of selections were based on some striking characteristics of the respective species. *Ansellia africana* is classified under International Union for Conservation of Nature (IUCN) as a vulnerable species, likewise in the study area only one stand of it was recorded on the 1959 lava which is currently undergoing excavation. *Habenaria procera* is the most abundant orchid species in the Mount Cameroon Region (MCR) with the highest success rate registered in this study. *Bulbophyllum lupulinum* is the second most abundant species yet had low success rate. *Angraecum birrimense* was the only orchid species that was found in all the vegetational zones both on the windward and leeward sides of the mountain. *Polystachya laxiflora* was peculiar in that, different individuals flowered at a given point in time the whole year round without a clear cut defined flowering period.

2.2. Preparation of media

Potato dextrose agar (SIGMA) was used for the isolation of fungi. Media preparation was based on manufacturer's instructions in which the powder (21 g) was weighed and dissolved in distilled water (500 ml) in an autoclavable conical flask. The medium was then autoclaved at 121 °C for 15 min, allowed to cool to about 50 °C. Streptomycin (antibiotic) was added at a concentration of 0.1 g / L and mixed properly by gently swirling the conical flask. The medium was poured into 9 mm diameter sterile petri dishes and allowed to cool and solidify.

2.3. Treatment of roots prior to isolation of endophytic fungi

Orchid plants were carefully collected with a scalpel such that the roots were not destroyed and parked in zip-lock bags. The collected samples were taken to the Life Science Laboratory of the University of Buea. The roots were washed thoroughly with running tap water to remove soil particles. Isolation was done following standard procedure described by Hallmann *et al*. (2007), whereby collected plant roots were washed with running tap water, chopped (1 × 1 mm) with a sharp knife, then sterilized in 70% ethyl alcohol for a minute, and then surface-sterilized to remove microorganisms by immersion in 4% $NaHPO_3$ for 3 min, rinsed again in 70% ethyl acohol for a minute, then in deionized sterile distilled water to remove sterilants and blotted dry on sterile tissue paper. The sterilized root fragments were chopped roots were cultured on PDA. Two fragments of each orchid species were placed in a petri dish and each inoculated petri dish was duplicated.

Treatment of roots prior isolation of mycorrhizal fungi from peloton was such that the root segments were surface sterilized in a 20% solution of household bleach for 1 min, rinsed twice in sterile distilled water, and decorticated with a sterile scalpel. Clumps of cells were removed from the inner cortex, macerated in a drop of sterile water, plated on PDA, incubated in the dark at 18 °C until hyphae grew from the cortical cells into the media (Currah *et al*., 1987). Hyphal tips were transferred to fresh PDA and sub-cultured until pure cultures were obtained. Table 1 indicates the different species that were selected for the study of orchidaceous mycorrhizas.

Table 1: Selected Orchid species and codes of fungal isolates

Life forms	Selected species	Codes for fungi from root segments	Codes of fungi from cortex (peloton)
Lithophytes	*Ansellia Africana*	AA	MAA
	Habenaria procera	HP	MHP
	Bulbophyllum lupulinum	BL	MBL
Epiphytes	*Angraecum birrimense*	AB	MAB
	Polystachya laxiflora	PL	MPL
	Liparis nervosa	LE	MLE
Terrestrial	*Vanda* Miss Joaquim	VM	MVM
	Arachnis Maggie Oei	VS	MVS
	Bletillia striata	BS	MBS

2.4. Molecular characterization of endophytic fungi community in the MCR

Genomic DNA Extraction: DNA of 18 non-mycorrhizal fungi from root segments and 13 mycorrhizal fungi from peloton, were extracted from pure isolates in the Biotechnology Unit of the University of Buea. The protocol for extraction was that described by Liu *et al*. (2000) in which a sterile tooth pick was used to collect a small lump of mycelia from the pure culture and transferred into a 1.5 ml eppendorf tube containing a lysis buffer (400 mM Tris-HCL (pH 8), 60 mM EDTA- pH 8.0, 150 mM NaCl and 1% sodium dodecyl sulphate). The contents were briefly vortexed to disrupt mycelia after which it was maintained at room temperature for 10 min. 150 μl of Potassium acetate was added to the eppendorf tube, it was briefly vortexed and finally centrifuged at >13,000 x g for 1min. The supernatant was transferred to another 1.5 ml eppendorf tube and centrifuged again as described above. After transferring the supernatant to a new 1.5 ml eppendorf tube, an equal volume of isopropyl alcohol was added. The tube was thoroughly mixed by inversion and spun at >13,000 x g for 2 min and the supernatant was discarded. The resultant DNA pellet was washed in 300 μl of 70% ethanol after which it was spun at 10,000 rpm for 1 min and then the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 μl of 1X Tris- EDTA and 1 μl of purified DNA was used in 24 μl of PCR mixture.

PCR amplification of fungi DNA: PCR amplification was done on the 31 extracted fungi genomic DNA (18 fungi DNA from root segments and 13 fungi DNA from peloton) using the Internal Transcribed Spacers (ITS) (Araújo *et al*., 2010). The forward (ITS-1F) and reverse (ITS-4R) primers used in the PCR reactions, designed by White *et al*. (1990) was used to amplify the ITS region of the rRNA operon (Michaelsen *et al*., 2006). Table 2 shows the sequence of both the forward and reverse primers of ITS.

Table 2: The ITS primer pair used in this study

Primer	Sequence 5' → 3'
ITS1 (forward)	TCCGTAGGTGAACCTGCGG
ITS4 (reverse)	TCCTCCGCTTATTGATATGC

Each PCR mixture contained 10 μl of Red taq ready mix, 0.5 μl of each primer pair, 8 μl of analytical grade sterile water (Sigma-Aldrich) and 5 μl of genomic DNA giving a total volume of 24 μl. The thermo cycling program used was composed four stages: (i) an initial denaturation stage (94 °C for 5 min) and 30 cycles of subsequent denaturation (94 °C for 1 min), (ii) annealing stage (60 °C for 1 min), (iii) elongation stage (72 °C for 1 min) and (iv) a stabilization stage (72 °C for 5 min) (Michealsen *et al*., 2006). The genetic materials were electrophoresed on 2% agarose gel in Tris acetate- EDTA buffer and the gel was stained with ethidium bromide and observed in UV detector (Sambrook *et al*., 1989). Approximated molecular sizes of the amplicons were determined using molecular weight marker 1kb Plus DNA ladder (Invitrogen Carlsbad, California, USA).

2.5. DNA sequencing of amplicons of fungi

Amplicons were sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria – South Africa for sequencing. The sequences were then blasted in the Biotechnology Unit of the University of Buea, against known sequences in the GenBank using BLAST (Basic Alignment Search Tool) to find regions of local similarity between sequences so as to provide species identification. A phylogenetic tree was constructed based on the available DNA data obtained during this study using the neighbor-joining method.

3. Results

3.1. Isolation of endophytic fungi

A total of 60 endophytic fungi isolates were obtained in pure culture. Of this number, 40 were non-mycorrhizal endophytes isolated from non-peloton-forming root tissues while 20 were mycorrhizal fungi isolated from the pelotons (Table 3). There were nine non-mycorrhizal isolates and seven mycorrhizal isolates from the lithophytes, giving a total of 15 endophytes; 15 non-mycorrhizal isolates and seven mycorrhizal isolates from the epiphytes, making a total of 22 endophytes while there were 16 non-mycorrhizal isolates and six mycorrhizal isolates from the terrestrial orchids, summing up to 22 endophytes. However, some of the fungal isolates occurred in two or more orchid species, bringing down the number to 31 distinct isolates.

Table 3: Abundance of fungal endophytes in the different orchid life forms in the MCR

Life forms	Species	Non-mycorrhizal fungi	Mycorrhizal fungi	Total	
Lithophytes	*Ansellia africana*	03	02	05	16
	Habenaria procera	03	03	06	
	Bulbophyllum lupulinum	03	02	05	
Epiphytes	*Angraecum birrimense*	06	02	08	22
	Polystachya laxiflora	03	02	05	
	Liparis nervosa	06	03	09	
Terrestrial	*Vanda Miss Joaquim*	06	02	08	22
	Arachnis Maggie Oei	05	02	07	
	Bletilla striata	05	02	**07**	

3.2. Molecular characterization

DNA Extraction and Amplification: DNA extraction was successful for all 31 isolates. However, amplification was only successful for 21 isolates; 11 non-mycorrhizal fungi and 10 mycorrhizal fungi. The 500 bp region corresponding to the ITS genes of the fungi isolates were successfully amplified by PCR.

Sequencing of PCR amplicon and identification of isolates: Sequencing was successful for all 21 amplicons. Blasting and subsequent identification was equally successful for all with percentage identity ranging from 94% to 100%. However, isolates MVS2 and MVS turned out to be the same species while isolate VS4 had no significant match with sequences in the gene bank for the ITS gene.

3.3. Inventory of fungi of different orchid life forms

A total of 18 fungi species belonging to 12 genera were identified based on molecular evidence. Five of these genera (*Acrmonium, Ceriporia, Trichoderma, Penicilium* and *Talaromyces*) were non-mycorrhizal endophytes, five (*Phellinus, Xylaria, Aspergillus, Cladosporium and Curvularia*) were mycorrhizal endophytes while two (*Lasiodiplodia and Fusarium*) occurred in both groups. The most represented genera were *Penicillium* with five species, *Fusarium* and *Cladosporium* with two species each, while all the other genera had just one species. The species *Trichoderma virens* was present in all epiphytic orchids and also in two of the three species of lithophytic orchids. It was completely absent in the terrestrial life form (Table 4). Meanwhile, *Lasiodiplodia theobromae* occurred in all three terrestrial species sampled and in one lithophyte and equally occurred as a mycorrhizal endophyte in *Liparis nervosa* (an epiphyte). *Trichoderma virens* was present in both lithophytic and epiphytic forms but absent in terrestrial forms. It is worth noting that no fungal species occurred as both mycorrhizal and none-mycorrhizal for the same species of orchid. Table 4 shows the names of the respective species of fungi encountered and their assession numbers.

Table 4: Inventory of endophytic fungi in orchids from the MCR

Orchid life forms	Orchid species	Non-peloton-forming fungi			Peloton-forming fungi		
		Isolates code	Species name	Accession number	Isolates code	Species name	Accession number
		AA1	*Fusarium nematophilum* Nirenberg & G. Hagedorn	KF77902.1			

Table 4 (Cont.)

Orchid life forms	Orchid species	Non-peloton-forming fungi			Peloton-forming fungi		
		Isolates code	Species name	Accession number	Isolates code	Species name	Accession number
Lithophytes	*Ansellia africana*	AA2	*Ceriporia lacerate* N. Maek., Suhara & R. Kondo	KJ780757.1	MAA	*Fusarium oxysporum* SchItdl(Snyder & Itansen)	KX421432.1
		AA3	*Acremonium macroclavatum* Ts. Watanabe	HQ897806.1			
	Habenaria procera	HP1	*Trichodema virens* (J.H. Mill., Giddens & A.A. Foster) Arx	KU990874.1	MHP	*Phellinus noxius* (Corner) Cunningham	KJ654485.1
		HP2	PINK FUNGI				
	Bulbophyllum lupulinum	BL1	*Penicillium charlesii* G. Smith	FJ430768.1	MBL	*Xylaria multiplex*(Mont. et P.Joly) T. Shumacher	GU300099.1
		BL2	*Trichodema virens* J.H. Mill., Giddens & A.A. Foster) Arx	KU990874.1			
		BL3	*Acremonium macroclavatum* Ts. Watanabe	HQ897806.1			
		BL4	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.	KU990874.1			
Epiphytes	*Angraecum birrimense*	AB1	*Penicillum aculeatum* Raper & Fennell	HQ392496.1	MAB	*Aspergillus sclerotiorum*G.A. Huber	KP329622.1
		AB5	*Trichodema virens* J.H. Mill., Giddens & A.A. Foster) Arx	KU990874.1			
	Polystachya laxiflora	PL1	*Trichodema virens* J.H. Mill., Giddens & A.A. Foster) Arx	KU990874.1			
		PL2	PINK FUNGI				

Table 4 (Cont.)

Orchid life forms	Orchid species	Non-peloton-forming fungi			Peloton-forming fungi		
		Isolates code	Species name	Accession number	Isolates code	Species name	Accession number
		LE1	*Trichodema virens* J.H. Mill., Giddens & A.A. Foster) Arx	KU990874.1	MLN	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.	LC074359.1
	Liparis nervosa	LE2	*Talaromyces verruculosus* (Peyronel) Samson, Yilmaz, Frisvad & Seifert	HQ607919.1			
		LE6	*Penicillium singorense* Visagie, Seifert & Samson	LT558940.1			
Terrestrial	*Vanda Miss Joaquim*	VM4	*Talaromyces verruculosus* (Peyronel) Samson, Yilmaz, Frisvad & Seifert	HQ607919.1	MVM1	*Cladosporium cladosporioides* (Fresenius)GA de Vries	AB763554.1
		VM5	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.	KU990874.1	MVM2	*Cladosporium tenuissimum* Cooke	KX349488.1
	Vanda sp	VS1	*Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.	KU990874.1	MVS	*Curvularia lunata* (Wakker) Boedijin	KX685659.1
		VS3	*Fusarium nematophilum* Nirenberg & G. Hagedorn	KF77902.1			
	Bletillia striata	BS2	*Ceriporia lacerate* N. Maek., Suhara & R. Kondo	KJ780757.1	MBS1	*Penicillium steckii* K.M. Zalessky	KX674639.1
		BS3	*Fusarium nematophilum* Nirenberg &G. Hagedorn	KF77902.1	MBS2	*Penicillium brocae* S.W. Peterson, Jeann.Pérez, F.E.Vega & Infante	KX674623.1
		BS5	*Acremonium macroclavatum* Ts. Watanabe	HQ897806.1			
		BS4	*Lasioplodia theobromae* (Pat.) Griffon & Maubl.	KU990874.1			

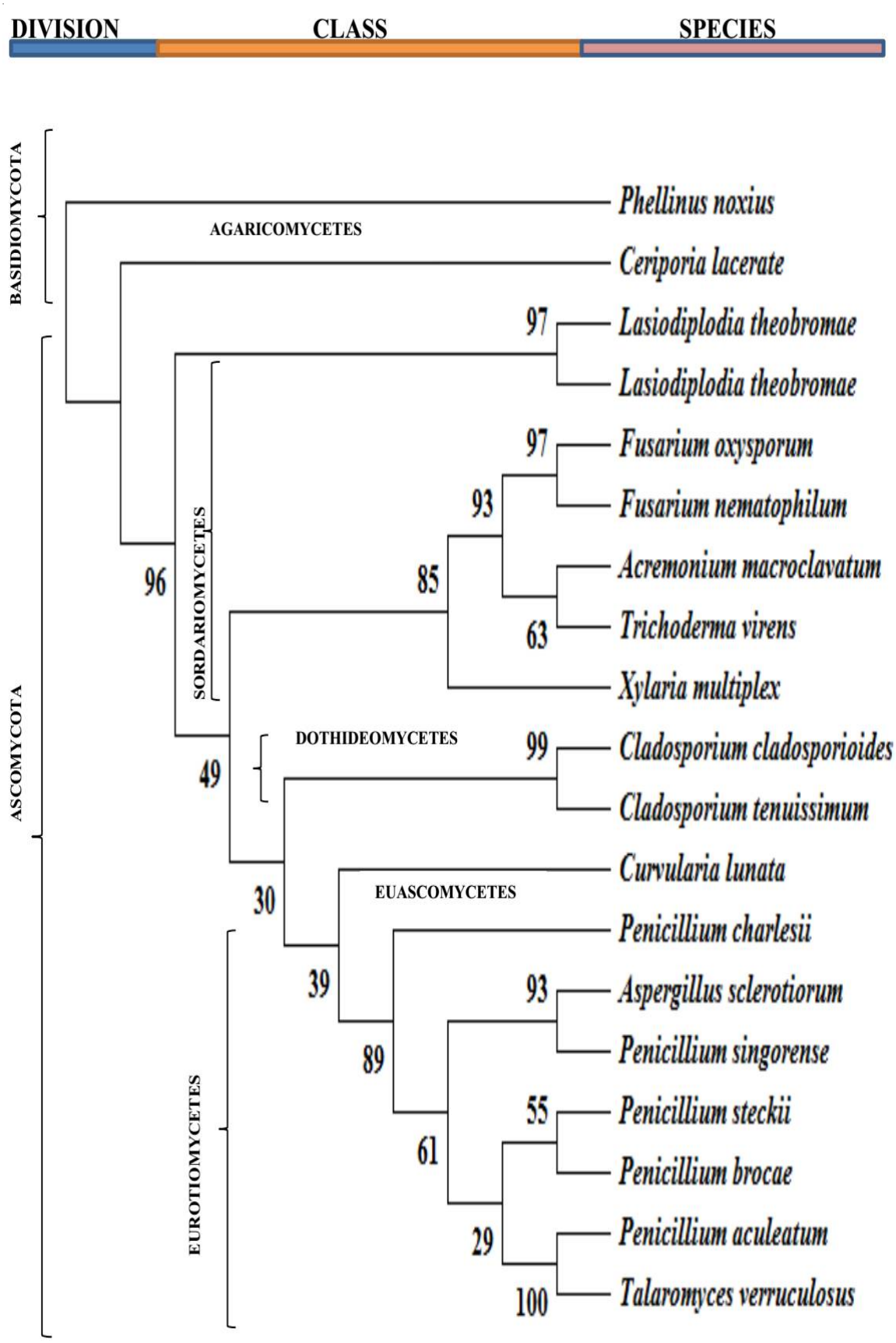

Figure 1: Neighbour-Joining phylogenetic tree showing the phylogenetic relationship of fungi isolated from orchids in the MCR. Bootstrap values are based on percentages of 1000 replicates.

3.4. Phylogeny of fungi found in the different orchid's life forms of the MCR

The phylogenetic relationship of the fungi species isolated from the orchids in the MCR is shown in Figure 1. The fungi belonged to two divisions; Ascomycota and Basidiomycota. Only two of the 18 fungi species, *Phellinius noxius* and *Ceriporia lacerata* belonged to the Basidomycota both in the Agaricomycetes. Euascomycetes had only one fungi species; *Curvularia lunata*. The Sordariomycetes had five species, Dothideomycetes with four species and Eurotiomycetes was the highest with seven species. All species belonging to the Eurotiales formed a distinct cluster.

4. Discussion

Though DNA was successfully extracted for all 31 isolates, amplification was only successful for 21. This observed result may be due to the much reported amplification recalcitrance which has more frequently been associated with members of the Tulasnellaceae. Bidartondo *et al*. (2004) and Selosse *et al*. (2004) reported the problem of amplication recalcitrance of Tulasnellaceae, a frequent orchid mycorrhizal taxon, to the 'universal' fungal ITS primers, because they have highly derived nuclear ribosomal DNA sequences. This entailed the need for additional PCR amplications using Tulasnellaceae-specic PCR primers. To that effect, Suarez *et al*. (2006) introduced the Tulasnellaceae-specic primer 5.8S-Tul to amplify the 5' part of 28S rDNA. This primer works well on a wide range of clades of Tulasnellaceae and is expected to be frequently used in future studies of orchid mycorrhizal fungi because of the high heterogeneity of the ITS alignment (Dearnaley *et al*., 2012).

The blasting results revealed that isolates MVS2 and MVS were the same species of fungi. The differences in the appearance of their cultures that led to them being coded as different isolates may have been due to environmental influences, improper observation of cultural and micromorphological characteristics or they may probably be different forms (anamorph and teleomorph) of the same species of fungi (Chaverri and Samuels 2002; Chaverri et al., 2002; Bechem and Afanga, 2017).

On the other hand, since isolate VS4 had no significant sequence similarity for the ITS of the nuclear ribosomal DNA with any known fungi species—implying there is a possibility that it could be a new species. However, this conclusion can only be made after all other conditions as spelled out in Matsubara *et al*. (2012) are fulfilled. Matsubara *et al*. (2012) analyzed an isolate of fungus that seemed to be a new type of orchid mycorrhizal fungus, "KMI (refers to Kyoto-Matsubara-Ishii)", obtained from the roots of *Paphiopedilum thailandense*. According to this report, besides other conditions, the strong point was that the ribosomal DNA sequences of 18S and ITS of the said isolate should have no similarity with any known fungal species—which is also the case with isolate VS4 in this study.

Based on molecular evidence, this study identified a total of 18 fungi species belonging to 12 genera. Five of these genera (*Acrmonium, Ceriporia, Trichoderma, Penicilium* and *Talaromyces*) were non-mycorrhizal endophytes, five (*Phellinus, Xylaria, Aspergillus, Cladosporium and Curvularia*) were mycorrhizal endophytes while two (*Lasiodiplodia and Fusarium*) occurred in both groups – implying seven genera in each group with two overlapping. With the exception of *Ceriporia*, six of the non-mycorrhizal endophyte genera identified in this study have previously been reported as recurrent non-mycorrhizal fungal endophytes in orchids (Xiaoya *et al*., 2015). However, *Xylaria*, which has equally been reported as a potential orchid non-mycorrhizal endophyte (Xiaoya *et al*., 2015) was identified in this study as an orchid mycorrhizal endophyte. 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 (Mahdieh and Soltani, 2014). Screening bioactive compounds for disease treatment from higher plants has increased (Aly *et al*., 2010). 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 (Xiaoya *et al*., 2015). *Alternaria sp*. and *F. oxysporum* isolated from orchids in Brazil showed strong inhibition to *Escherichia coli* (Vaz *et al*., 2009). From the orchid *Anoecochilus setaceus*, an antibacterial nortriterpenoid helvolic acid was extracted from the endophytic taxon *Xylaria sp*. (Ratnaweera *et al*., 2014). These orchid non-mycorrhizal endophytes may occur in other plants and possibly be involved in the production of bioactive compounds (Xiaoya *et al*., 2015). 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 (Vujanovic *et al*., 2000). *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 (Aldana *et al*., 2013).

The phylogenetic tree grouped all endophytes into nine major clusters; all of which belonged to two divisions – Ascomycota and Basidiomycota. All the seven genera of orchid mycorrhizal endophytes belonged to the phylum Ascomycota. For many years, orchids were considered to interact largely, if not only, with members of the 'rhizoctonia' complex of the Basidiomycota (Dearnaley *et al*., 2012). Molecular taxonomic identication of orchid mycobionts has now revealed that the diversity of orchid associates is much more complex and that other Basidiomycetes and even Ascomycetes can be involved in orchid mycorrhizas (Dearnaley *et al*., 2012). Although there is general consensus that orchids are more likely to form mycorrhizal associations with species of the division Basidiomycota, it is now established that orchids can equally associate with members of the division Ascomycota. However, the only encountered Ascomycota genera that have been associated with orchid mycorrhiza so far include *Tuber* (Selosse *et al*., 2004), *Tricharina* (Waterman *et al*., 2011) and *Peziza* (Waterman *et al*., 2011).

5. Conclusion

In this study, we identified endophytic fungi associated with orchids from the Mount Cameroon Region. A total of 18 fungi species belonging to 12 genera were identified with Penicillium being the most abundant. The inferred phylogenetic tree grouped all endophytes into nine major clusters belonging to two phyla. Clustering was independent of whether endophytes were mycorrhizal or non-mycorrhizal. The results of this study could contribute to orchid conservation and for the discovery of bioactive compounds. The problem of amplification recalcitrance was encountered, creating the need for additional PCR amplications using Tulasnellaceae-specic PCR primers in future research. In vitro orchid seed germination and seedling establishment trials are recommended to be carried out using mycorrhizal fungal isolates for possible conservation of orchid species from the Mount Cameroon Region.

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