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Fungal endophytes from two orchid species – pointer towards organ specificity

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Fungal endophytes may influence plant communities by altering the host's fitness either positively or negatively. Little is known, however, about their host/organ specificity, life style and role in plant-fungus symbiosis under varying environmental conditions. We compared the leaf and root endophyte assemblages of two orchids (*Bulbophyllum neilgherrense* and *Pholidota pallida*) from natural forests and greenhouse conditions. *Xylariaceae* species were consistently associated with leaf and root tissues, while *Guignardia* and *Pestalotiopsis* were found predominantly in the leaf tissues of both orchids. Correspondence analysis of the endophyte assemblages showed that the endophytes exhibited distinct organ but little host specificity. More endophytes were shared by the two different orchids growing in the same location when compared to endophyte assemblages of a single orchid from different locations. Considering the influence of endophytes in shaping the host's community, diverse habitats must be screened vigorously to address questions regarding the role of endophytes in host-endophyte interactions.

Key words: fungal endophytes, environment, orchids, *Bulbophyllum neilgherrense*, *Pholidota pallida*.

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Rostlinná společenstva mohou být ovlivněna působením houbových endofytů, které se odráží ve změnách fitness jejich hostitelů, ať už pozitivních či negativních. Avšak málo je známo o hostitelské či orgánové specificitě endofytů, jejich životním stylu a roli v symbiotickém vztahu s rostlinou při různých podmínkách prostředí. Autoři srovnávali společenstva listových a kořenových endofytů dvou orchidejí (*Bulbophyllum neilgherrense* a *Pholidota pallida*), rostoucích v přirozených lesích a skleníkových podmínkách. Druhy z čeledi *Xylariaceae* byly pevně spjaty s listovými i kořenovými pletivy, zatímco druhy rodů *Guignardia* a *Pestalotiopsis* jsou nacházeny převážně v listových pletivech obou orchidejí. Korespondenční analýza společenstev endofytů ukázala, že tyto houby vykazují zřetelnou orgánovou specificitu, ale nízkou specificitu hostitelskou. Více společných endofytů měly dvě různé orchideje rostoucí na společné lokalitě ve srovnání se společenstvy endofytů jednotlivých rostlin z různých lokalit. Vzhledem k vlivu endofytů na utváření společenstev svých hostitelů bude ještě potřebný důsledný průzkum různých substrátů pro ozřejmení role endofytů v interakcích s jejich hostiteli.

INTRODUCTION

Endophytes are symptomless colonisers of internal plant tissues and cause no significant harm to their host, with few reports suggesting that they can even improve their host's fitness (Rodriguez et al. 2009). Endophytes have been isolated from all major plant groups screened to date (Suryanarayanan et al. 2011) and constitute a major part of plant microbiomes (Porras-Alfaro & Bayman 2011). However, the focus on fungal endophyte research remains concentrated on their purportedly high diversity (Hyde & Soytong 2008), their ability to elicit bioactive metabolites (Aly et al. 2010), and their potential as biocontrol agents (Arnold et al. 2003). Evidences indicate that biotic and abiotic factors may significantly influence the endophyte diversity in a given host (Arnold & Herre 2003). Questions on host/organ specificity of fungal endophytes have been addressed by several researchers (see Petrini 1991, Stone & Petrini 1997). *Rhabdocline parkeri* has been reported to be strictly host-specific, found associated with Douglas-fir and not on any other sympatric conifers (Carroll 1988), while very little host specificity was reported from conspecific hosts growing in dry tropical forests from India (Suryanarayanan et al. 2011). Kumaresan et al. (2002) observed distinct endophyte species colonising different organs of a mangrove tree; similarly Rivera-Orduña et al. (2011) recorded distinct organ specificity in fungal endophytes colonising *Taxus globosa*. However, the role of the environment in influencing the occurrence and distribution of endophytic fungi in host lineages growing across geographical localities in tropical communities is not yet clear.

The *Orchidaceae* are one of the largest plant families, consisting of almost 10 % of all flowering plant species (Pillon & Chase 2007). Epiphytic orchids are highly valued as ornamental plants and play a vital role in rainforest ecosystems; they are extremely sensitive to disturbances and are threatened by habitat loss (Pillon & Chase 2007). Though extremely diverse, the plants of this family have mostly been screened for their mycorrhizal symbiosis (Otero et al. 2002) and studies on fungal endophyte communities are relatively few (Bayman et al. 1997, Tao et al. 2008, Chen et al. 2011). The aim of this study was to study the biodiversity of endophytes in *Orchidaceae* and to understand the host/organ specificity of endophytes associated with them. We selected two orchid species growing in the Western Ghats region and screened their root and leaf tissues for endophytes. The same two orchids grown under greenhouse conditions were investigated simultaneously for comparison.

MATERIAL AND METHODS

Isolation methods. *Bulbophyllum neilgherrense* and *Pholidota pallida* were collected from their natural habitat in the villages of Hiriadka and Agumbe, near the Western Ghats, Karnataka, India (13.35–13.50° N, 74.85–75.10° E). These two orchids were also grown in a greenhouse in the Manipal Life Sciences Centre and simultaneously investigated for fungal endophytes. The plant samples were brought to the laboratory in sterile polythene bags and processed within 6 hours of collection. Leaf and root samples (10–20 orchid samples/location) were thoroughly washed in tap water, cut into 0.5 cm^2 segments and then surface sterilised by treating in 70% alcohol for 30 seconds, a sodium hypochlorite solution (4%) for 90 seconds and sterile distilled water for one minute. After removing the excess water, the segments were placed on Petri plates (9 cm diam.) containing Potato Dextrose Agar (PDA) amended with chloramphenicol (150 mg/L). A total of 100 segments were screened for every set of sample. The plates were incubated at 26 °C and fungal colonies were monitored regularly. The efficacy of the sterilisation protocol was tested by making an imprint of a surface sterilised segment onto a PDA medium. No fungal colonies grew from these, suggesting that the sterilisation protocol was effective (Hyde & Soytong 2008). Upon appropriate growth of fungal endophytes, the fungi were sub-cultured onto separate PDA plates. The cultured fungi were classified into different morphotypes based on culture morphology and growth characteristics.

Fungal DNA extraction. Total genomic DNA was extracted from all fungal morphotypes using the phenol-chloroform method. Fresh mycelia were scraped from the surface of the Petri plate and transferred to a sterile microfuge tube containing 500 µL of DNA extraction buffer (0.1 M NaCl, 50 mM Tris, 10 mM Na_2EDTA, 2% SDS, pH 8.0). The samples were ground using a sterile toothpick and mixed with 500 µl of chilled phenol, followed by centrifugation at 12,000 rpm for 15 minutes at 4 °C. The upper aqueous phase (200–300 µL) was transferred to a fresh tube to which an equal volume of chilled chloroform : isoamylalcohol (24 : 1) was added and mixed to obtain a white suspension. The tubes were then centrifuged at 12,000 rpm for 15 minutes at 4 °C. The aqueous phase was transferred into a fresh microfuge tube to which 0.6 volume of chilled isopropanol and $1/10^{th}$ volume of 3 M sodium acetate was added and gently mixed. Microfuge tubes were kept at –80 °C for 2 hours and centrifuged at 12,000 rpm for 15 minutes at 4 °C whereby the supernatant was discarded. The pellet was washed with 200 µL of 70% ethanol and centrifuged at 12,000 rpm for 10 minutes at 4 °C. The supernatant was discarded and the pellet air-dried, resuspended in 50 µL of sterile distilled water and stored at –20 °C.

PCR amplification and sequencing of fungal ITS region. The fungal 5.8S rDNA with its flanking ITS regions (ca. 600 bp) was amplified using the

ITS4 and ITS5 primers (White et al. 1990). The 25 µL reaction mix consisted of 10X PCR buffer, forward and reverse primers (10 µM each), 4 mM dNTPs, 1 Unit of Taq DNA Polymerase, 1% DMSO, 25 mM $MgCl_2$ and approx. 50 ng of fungal genomic DNA as template. The reactions were performed in a Master Cycler Thermocycler (Eppendorf, USA) at the following conditions: 94 °C for 3 minutes, followed by 34 cycles of 94 °C for 30 seconds, 54 °C for 30 seconds, 72 °C for 1 minute, and finally 72 °C for 10 minutes. The amplicons were visualised on 1% agarose gel to determine product size and purity. The purified PCR products were sequenced using the ITS4 primer in an automated sequencer (ABI 3130 Genetic Analyzer) following the manufacturer's protocols. The sequences were manually edited and used as a query sequence to search for the closest match in the NCBI database using the Blastn algorithm, and also in the CBS database. The sequences were submitted to GenBank, where accession numbers were obtained (JX157850–JX157874).

Phylogenetic analysis. The ITS1-5.8S-ITS2 sequence fragments of the four species of *Xylariaceae* obtained in this study were aligned with 160 sequences available from the GenBank database. The sequences were aligned using ClustalW (Thomson et al. 1994) with default settings. The aligned sequences were then manually adjusted and an initial neighbor-joining analysis was performed to select 54 closely related sequences which were used for the final analysis. Evolutionary tree of the datasets were inferred using the neighbor-joining method (Saitou & Nei 1987) by using MEGA version 5.05 (Tamura et al. 2011). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980). Bootstrapping with 1,000 replicates was used to assess branch support (Felsenstein 1985). All ambiguous positions were removed for each sequence pair and a total of 611 positions were included in the final dataset.

Data analysis. The similarity between assemblages was calculated using the Morisita-Horn (MHI) similarity coefficient (Magurran 2004). The cumulative species richness and the correspondence analysis were performed using the BiodiversityPro software, version 2 (McAleece et al. 1997).

RESULTS AND DISCUSSION

Endophytes identified in host tissues

In our study, 725 fungal isolates were isolated from root and leaf tissues of two orchids growing at two different locations. Based on morphological characters, the isolated fungi were identified up to species level wherever possible. Further, all the endophytic fungi were grouped into distinct genotypes based on ITS rDNA sequencing. A threshold of 98% sequence similarity was set as a minimum for accepting species names and those below 98 % were grouped at the genus or family

Tab. 1. Fungal endophytes obtained in the present study with their GenBank accession number and the nearest match obtained from GenBank and CBS databases. Values in brackets indicate the percentage of similarity.

GenBank No.	Identified as	Nearest match in GenBank database	Nearest match in CBS database
JX157850	*Chaetomium* sp. 1	*Chaetomium globosum* (100 %)	*Chaetomium globosum* (100 %)
JX157851	*Chaetomium* sp. 2	*Chaetomium cupreum* (98 %)	*Chaetomium fusiforme* (98 %)
JX157852	*Colletotrichum gloeosporioides* complex	*Colletotrichum gloeosporioides* (100 %)	*Colletotrichum cliviae* (99 %)
JX157853	*Colletotrichum* sp. 1	*Colletotrichum capsici* (100 %)	*Colletotrichum capsici* (100 %)
JX157854	*Cylindrocarpon* sp. 1	*Cylindrocarpon* sp. (99 %)	*Cylindrocarpon olidum* (93 %)
JX157855	*Phomopsis* sp. 1	*Diaporthe phaseolorum* (99 %)	*Phomopsis* sp. (100 %)
JX157856	*Fusarium* sp. 1	*Fusarium equiseti* (100 %)	*Fusarium equiseti* (100 %)
JX157857	*Fusarium* sp. 2	*Fusarium solani* (100 %)	*Fusarium solani* species complex (100 %)
JX157858	*Fusarium* sp. 3	*Fusarium oxysporum* (100 %)	*Fusarium oxysporum* (100 %)
JX157859	*Lasiodiplodia* sp. 1	*Lasiodiplodia pseudotheobromae* (100 %)	*Lasiodiplodia pseudotheobromae* (100 %)
JX157860	*Lasiodiplodia* sp. 2	*Lasiodiplodia theobromae* (99 %)	*Lasiodiplodia theobromae* (100 %)
JX157861	*Penicillium* sp. 1	*Penicillium purpurogenum* (100 %)	*Penicillium purpurogenum* (100 %)
JX157862	*Penicillium* sp. 2	*Penicillium funiculosum* (100 %)	*Penicillium funiculosum* (100 %)
JX157863	*Pestalotiopsis* sp. 1	*Pestalotiopsis microspora* (100 %)	*Pestalotiopsis microspora* (100 %)
JX157864	*Phoma* sp. 1	*Phoma tropica* (99 %)	*Phoma minor* (100 %)
JX157865	*Phyllosticta* sp. 1	*Guignardia bidwellii* (97 %)	*Guignardia bidwellii* (97 %)
JX157866	*Phyllosticta* sp. 2	*Guignardia mangiferae* (100 %)	*Guignardia mangiferae* (100 %)
JX157867	*Sordaria* sp. 1	*Asordaria prolifica* (100 %)	*Asordaria prolifica* (100 %)
JX157868	Sterile form 1	Ascomycota sp. (99 %)	Ascomycota sp. (100 %)
JX157869	Sterile form 2	*Prosthecium pyriforme* (94 %)	*Prosthecium pyriforme* (95 %)
JX157870	*Trichoderma* sp. 1	*Trichoderma* sp. (100 %)	*Trichoderma virens* (100 %)
JX157871	*Xylariaceae* sp. 1	*Daldinia eschscholzii* (100 %)	*Daldinia eschscholzii* (100 %)
JX157872	*Xylariaceae* sp. 2	*Entonaema liquescens* (99 %)	*Entonaema liquescens* (99 %)
JX157873	*Xylariaceae* sp. 3	*Hypoxylon fragiforme* (99 %)	*Nodulisporium indicum* (99 %)
JX157874	*Xylariaceae* sp. 4	*Nemania* sp. (99 %)	*Nemania* sp. (100 %)

levels (Tab. 1). Based on these values, we managed to isolate 27 different endophyte species representing 18 different fungal genera from the two orchids (Tab. 2). The sterile forms isolated were identified as belonging to *Mycoleptodiscus* (Ascomycota) and *Prosthecium* using the ITS sequence similarity; however, for both these sterile morphotypes, the sequence similarity values were lower when compared to other morphotypes.

The colonisation frequency of endophytes ranged from 85 % to 96 % (Tab. 2). The root assemblage of *Bulbophyllum neilgherrense* from greenhouse comprised

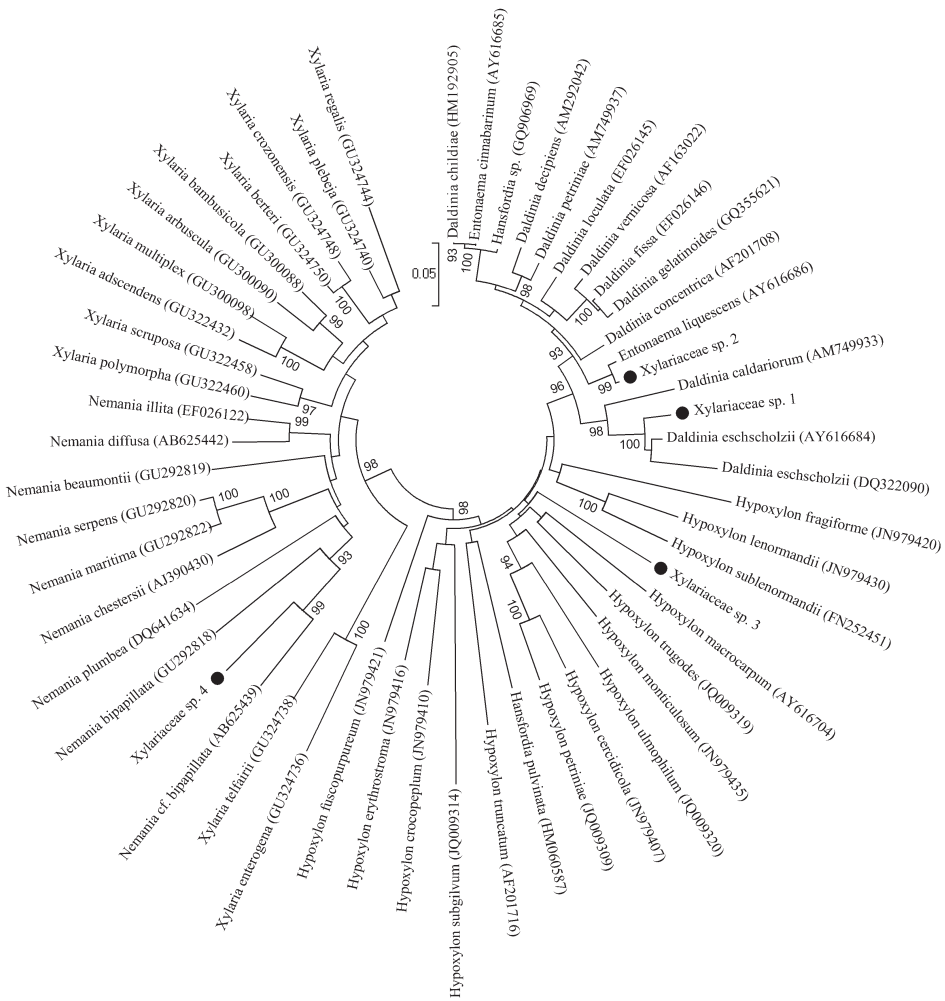

Fig. 1. Neighbor-joining analysis based on aligned ITS rDNA sequences of species of *Xylariaceae* and other related species. GenBank accession numbers of sequences are given in parentheses. Bootstrap values >90 % from 1000 replications are shown at the branches.

9 different species while the root assemblage from the forest hosted 5 different species. The leaf assemblage of *B. neilgherrense* from forest also included 5 different species as endophytes. Species of *Xylariaceae* were dominant in the root tissues while *Pestalotiopsis* was the dominant endophyte associated with leaf tissues. *Trichoderma* and *Fusarium* were associated with the root tissue only, while *Guignardia* sp. and *Colletotrichum gloeosporioides* complex were restricted to leaf endophyte assemblages. Earlier studies have also shown that *Guignardia*

spp. (anamorph *Phyllosticta* spp.) and *Diaporthe* spp. (anamorph *Phomopsis* spp.) are frequently isolated as foliar endophytes from tropical plant hosts (Murali et al. 2006). Although *Trichoderma* and *Fusarium* are commonly encountered as pathogens of plant tissues, other studies have shown that these two fungi can also occur as endophytes (Fisher & Petrini 1990, Bayman et al. 1997, Leslie et al. 2001, Suryanarayanan et al. 2011).

For the four *Xylariaceae* species isolated in the present study, we performed the neighbor-joining analysis to infer their evolutionary history. The bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were found to be collapsed (Fig. 1). The results showed that the four *Xylariaceae* species belonged to four different genera, namely *Daldinia*, *Entonaema*, *Hypoxylon* and *Nemania*. We observed different species of *Xylariaceae* co-dominating the root endophyte assemblage of both orchids. Earlier studies have shown species of *Xylariaceae* to be associated with all plant organs of the epiphytic orchid *Dendrobium nobile* (Yuan et al. 2009), while Bayman et al. (1997) found widespread occurrence of species of *Xylariaceae* in different species of the orchid genus *Lepanthes* growing in Puerto Rico. Thus, our study adds further evidence to the fact that diverse species of *Xylariaceae* are frequently associated as fungal endophytes with plant hosts (Petrini & Petrini 1985, Rodrigues & Petrini 1997, Osono et al. 2013) especially in tropical communities, since they play an important role in litter decomposition.

Similarity of endophyte assemblages

The Morisita-Horn similarity index showed that the foliar endophyte assemblages of the two orchids from the forest shared several taxa (MHI = 0.94). Similarly, the assemblages from the two orchids grown under greenhouse conditions shared more taxa (MHI = 0.65) when compared to assemblages from the same orchid growing at different locations (MHI = 0.5 and 0.62) (Fig. 2). This suggests that the microenvironment may play a larger role in determining the endophyte community associated with a given host than supposed. However, such a clear trend was not noticeable in root assemblages. Hoffman & Arnold (2008) studied endophyte communities associated with cupressaceous trees from different sites and found that each site harboured distinct endophyte communities. In contrast, Gange et al. (2007), while studying endophytes associated with *Cirsium arvense* and *Leucanthemum vulgare* from five different sites, found that *L. vulgare* did not show any correlation between endophyte assemblage similarity and distance between the sites; in *C. arvense*, similarity between assemblages decreased with increasing distance between sites. Interestingly, in our study, the dominant endophyte species in the two orchids were common in assemblages from both locations. Our results further corroborate the view of Rodriguez et al. (2009) that

Tab. 2. Endophytes isolated from the orchids *Bulbophyllum neilgherrense* and *Pholidota pallida*. Host codes: BNFL – *Bulbophyllum neilgherrense* leaf assemblage from forest; BNGL – *B. neilgherrense* leaf assemblage from greenhouse; PPFL – *Pholidota pallida* leaf assemblage from forest; PPGL – *P. pallida* leaf assemblage from greenhouse; BNFR – *B. neilgherrense* root assemblage from forest; BNGR – *B. neilgherrense* root assemblage from greenhouse; PPFR – *P. pallida* root assemblage from forest; PPGR – *P. pallida* root assemblage from greenhouse.

	BNFL	**BNGL**	**PPFL**	**PPGL**	**BNFR**	**BNGR**	**PPFR**	**PPGR**
Chaetomium sp. 1			6					
Colletotrichum sp. 1	9							
Colletotrichum gloeosporioides complex			7	30				
Colletotrichum sp. 2					32			
Cylindrocarpon sp. 1					6		3	
Daldinia eschscholzii (*Xylariaceae* sp. 1)			4	11	32	5	26	15
Entonaema liquescens (*Xylariaceae* sp. 2)		1	5					
Fusarium sp. 1						21		
Fusarium sp. 3					7	3	9	10
Phyllosticta sp. 1	22	42	16	34				
Phyllosticta sp. 2		18						
Hypoxylon sp. (*Xylariaceae* sp. 3)						28	46	26
Lasiodiplodia sp. 1						6		
Nemania sp. (*Xylariaceae* sp. 4)	9							
Penicillium sp. 1		14				5		
Pestalotiopsis sp. 1	50	9	54	19	17			
Phoma sp. 1						12		
Trichoderma sp. 2								27
Isolates	**94**	**89**	**93**	**96**	**94**	**85**	**88**	**86**
Species	**5**	**7**	**7**	**6**	**5**	**9**	**6**	**7**

Fungi with <5 isolates (host codes in brackets): *Chaetomium* sp. 2 (PPFR), *Phomopsis* sp. 1 (BNFL, BNGL, PPGL, PPFR, PPGR), *Fusarium* sp. 2 (BNGR), *Lasiodiplodia* sp. 2 (BNGR), Sterile form 1 (BNGL), *Penicillium* sp. 2 (PPFL), Sterile form 2 (PPGL), *Sordaria* sp. 1 (PPGR), *Trichoderma* sp. 1 (PPGR).

a 'core' group of horizontally transmitted fungi (such as *Colletotrichum*, *Phyllosticta*) may not be influenced by host chemistry and can be isolated as endophytes from various tropical hosts irrespective of the environment in which the host grows; however their exact role in determining the success of the host is not yet clear.

Comparison of endophyte colonisation

A rarefaction curve constructed by combining the endophyte assemblage from both hosts together showed that the assemblage from the greenhouse included more endophyte species when compared to that from the forest (Fig. 3). The

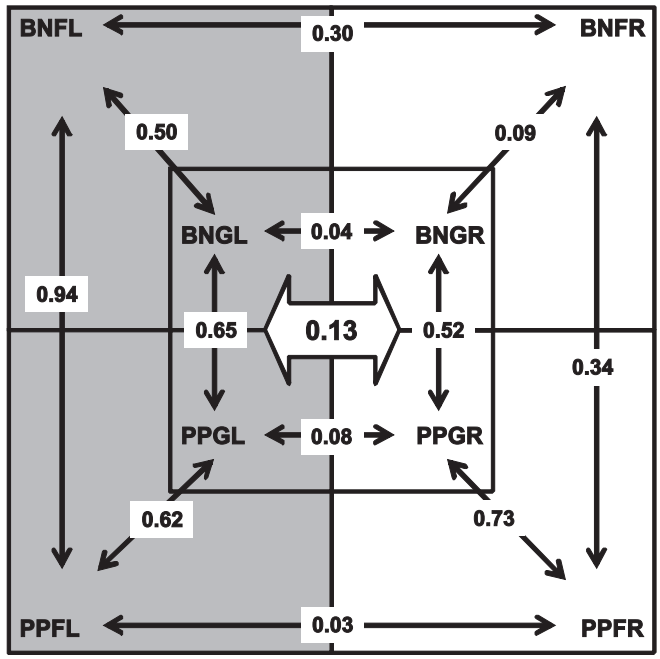

Fig. 2. Similarity coefficient (MHI) between different endophyte assemblages. For codes, see Tab. 2.

higher diversity in greenhouse conditions when compared to natural forests could be due to the reason that opportunistic generalists were able to colonise hosts growing in new environments and survive there as endophytes more successfully than 'specialised' endophytes found associated with hosts inhabiting natural environments. Studies have shown that endophytes can be opportunistic and non-host-specific, especially in plants found growing outside their native communities (see Hoffman & Arnold 2008).

Yuan et al. (2009) reported higher colonisation rates of endophytes in foliar tissues of epiphytic orchids than in other organs. We observed a similar trend with higher colonisation rates of endophytes in leaf tissues, but rarefaction analysis showed that the overall species diversity was higher in root tissues (Fig. 3), with a distinct assemblage of endophytes colonising the root tissues when compared to leaf tissues in both orchids. The Morisita-Horn's index showed that in both orchids screened, the root and leaf tissues from a single site shared very few fungi (MHI = 0.03 to 0.30) while more taxa were shared by the same tissues screened from different sites (Fig. 2). This was further substantiated by the correspondence analysis which clearly separated endophyte assemblages from root and foliar tissues (Fig. 4). Furthermore, a cumulative root assemblage (combining all four root assemblages together) from both orchids shared very few species with the cumu-

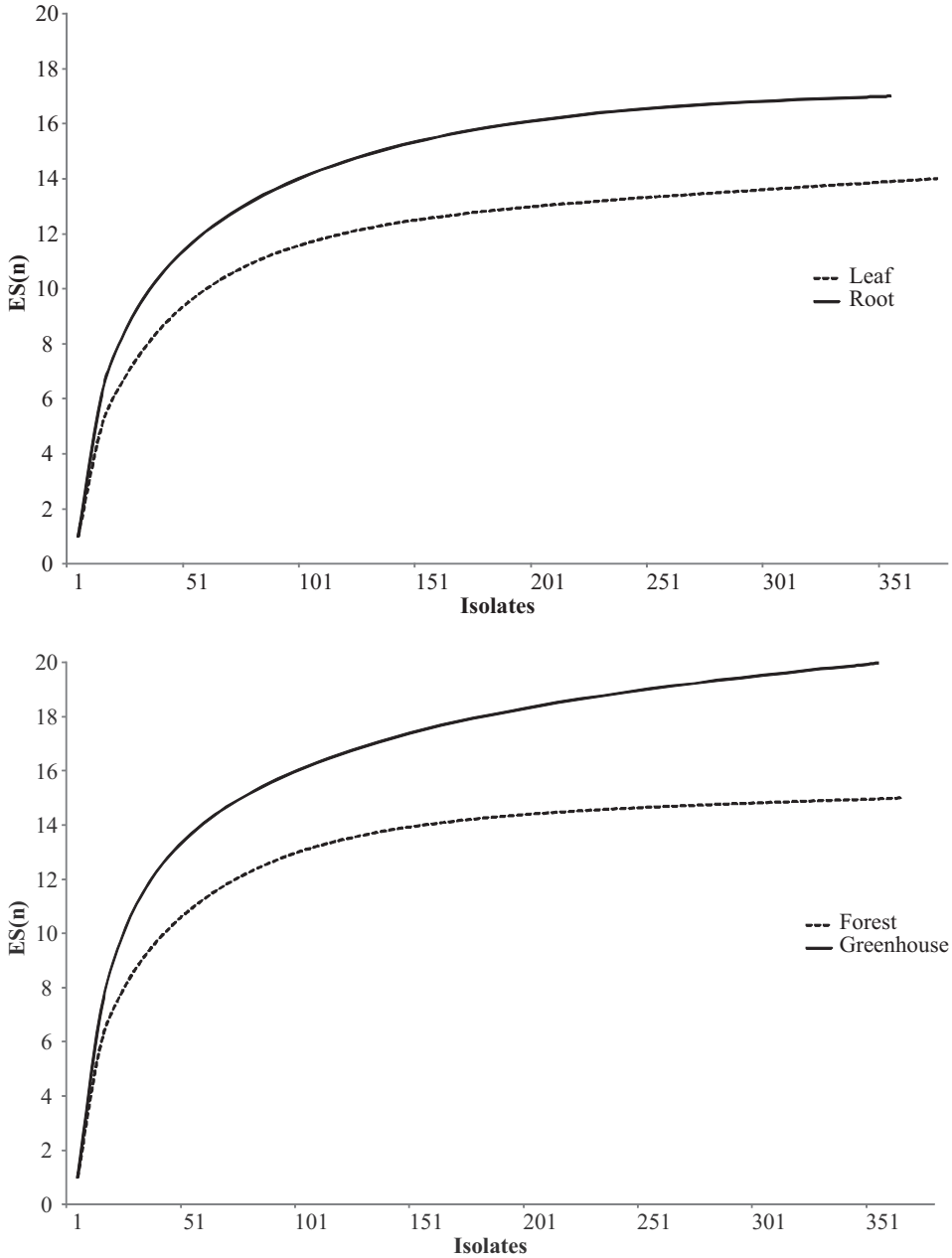

Fig. 3. Cumulative species richness in different endophyte assemblages. ES(n) is the expected number of species for n isolates.

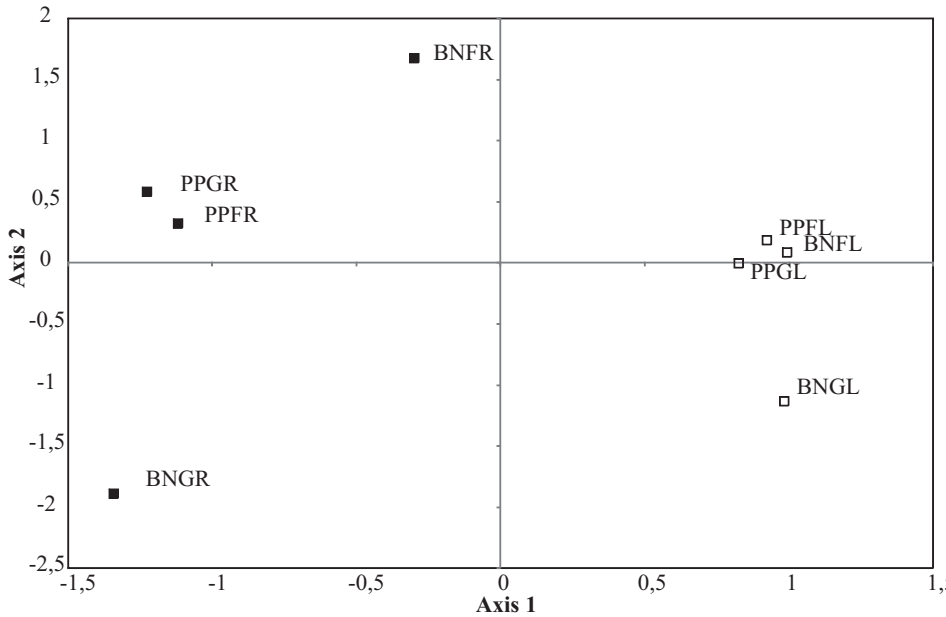

Fig. 4. Correspondence analysis for fungal endophyte assemblages from the two orchids screened. For codes, see Tab. 2. Open squares indicate leaf assemblages, while filled squares indicate root assemblages.

lative foliar assemblage (MHI = 0.13), suggesting that fungal endophytes are organ-specific. Porras-Alfaro & Bayman (2011) opined that such organ specificity can increase the phenotypic plasticity of the host, which may prove advantageous in certain environments.

The traditional method of fungal species identification using morphological characters is slowly replaced by molecular characterisation (Ko et al. 2011). Molecular studies invariably rely on ITS sequencing to differentiate fungal morphotypes; however, due to the inherent shortcomings associated with this approach, a 90% ITS sequence similarity is considered a good match for designating species names for these fungi (Hoffman & Arnold 2008). Even while using the molecular approach, the sterile morphotypes isolated in the present study gave a maximum sequence similarity of 94 % only suggesting that more effort needs to be focused on exploring the true diversity of unculturable and sterile endophytes, especially from tropical hosts. Considering the vast biological and chemical diversity hidden in tropical fungi and the fact that these fungi can act as an alternative source of plant metabolites (Priti et al. 2009), we conclude that further studies of hosts from various natural habitats in tropics are required to explore these fungi.

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