Diversity and seasonal variation of endophytic fungal communities associated with some medicinal trees of the Western Ghats, Southern India

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A total of 17 561 fungal isolates were isolated from a total of 19 800 leaf segments incubated from 33 medicinal trees. These fungal isolates belonged to teleomorphic Ascomycota (11.7%), anamorphic Ascomycota producing conidiomata (35.2%). anamorphic Ascomycota without conidiomata (34.4%), Zygomycota (1.3%), and sterile forms (5.8%). *Colletotrichum gleosporioides*, *Phyllosticta* spp., *Cladosporium cladosporioides, Phomopsis* spp., *Pestalotiopsis* spp., *Chaetomium globosum,* and *Aspergillus niger* were frequently isolated from the host plants studied. The number of isolates was higher in the winter season than during monsoon and summer season. The colonization of endophytic fungi varied and differed significantly between monsoon, winter and summer seasons.

Key words: endophytes, abundance, seasonality, Malnad region.

Endophytic fungi are ubiquitous plant symbionts that live asymptomatically within plant tissues (Carroll 1988). The biology of endophytes varies greatly from true mutualistic associations (Saikkonen *et al.* 1998) to latent infections by potential pathogens (Photita *et al.* 2004). In mutualistic associations, these microbes can have profound effect on plant health, growth, development, and yield making host plants ecologically fit also in adverse conditions like water deficit, stress, high temperatures, soil acidity, and nutrient deficiency (Malinowski *et al.* 2000, Redman *et al.* 2002).

The infected host plants get benefited by defense against microbial pathogens with the production of various alkaloids (Clay & Schardl 2002, Kim *et al.* 2007, Shankar *et al.* 2009). In addition, these fungal endophytes have been recognized as a repository of novel secondary metabolites some of which exhibit beneficial biological activities (Strobel & Daisy 2003). A study has indicated that 51 % of bioactive substances isolated from endophytic fungi were previously unknown (Schutz 2001). Due to growing interest from ecologists, bioprospectors and mycologists, an expansion of research infrastructure in the tropics and development of new methods, the study of tropical endophytes is more accessible now than ever before.

Medicinal plants have been used to cure the diseases since time immemorial. It has been reported that the micro organisms resides in these plants can mimic the biological properties of their hosts. Western Ghats of India (one of the hot spots of global biodiversity) is reported to have a diverse population of endophytic fungi (Raviraja 2005, Krishnamurthy *et al.* 2008, Shankar *et al.* 2008). Few studies on the endophytic fungi of these plants have been conducted. The present study was undertaken in order to investigate the diversity of endophytic fungi and their seasonal colonization pattern in medicinal trees used by the local people for curing various diseases in the Malnad region, Western Ghats of Karnataka, Southern India.

Materials and Methods

Sample collection and isolation of endophytes

Apparently healthy looking leaf samples of 33 medicinal trees (Tab.1) growing in different sites of the Malnad region were collected, brought in sterile polythene bags to the laboratory and processed within 24 h of collection. From each host 200 segments were randomly selected from the leaves of two individuals (1 km apart) per season. Surface sterilization of samples was done by cleaning leaves under running tap water and cutting them into 1 cm segments followed by stepwise washing with 70% ethanol for 2 min, sodium hypochlorite solution for 5 min and 70% ethanol for 30 s. The leaf segments were then allowed to surface-dry under sterile conditions. This method of surface sterilization has been shown to effectively eliminate contaminants from endophyte cultures (Arnold *et al.* 2000). Leaf segments were placed on 9 cm Petri plates containing potato dextrose agar (PDA, Hi Media Laboratories, Mumbai, India) medium amended with streptomycin 250 (mgL^{-1}) to suppress bacterial growth. The efficacy of surface sterilization was confirmed by pressing the sterilized leaf segments onto the surface of PDA medium. The absence of growth of any fungi on the medium confirmed that the surface sterilization procedure was effective (Schulz *et al.* 1993). Petri plates were incubated at 28 ºC ±1 ºC with a 12 h photoperiod, and sporulation was induced by incubation in a UV light chamber for one to twelve days. Fungi growing out from the leaf segments were subsequently transferred onto fresh PDA plates. Pure cultures were spread on fresh PDA slants. Endophytic fungal species were identified on the basis of cultural characteristics and morphology of fruiting bodies and spores by using standard keys (Subramanian 1971, Sutton 1980, Barnett & Hunter 1998). Cultures that failed to sporulate were recorded as sterile forms. All the isolates were numbered and are maintained in the Culture Collection Centre of Department of Applied Botany, Kuvempu University, Shankaraghatta, India.

Tab. 1. – Medicinal tree species studied for isolation of endophytic fungi from Malnad region of Western Ghats, Southern India.

Host plant	Family	Collection site	Medicinal uses
Aegle marmelos (L.) Corr. Serr.	Rutaceae	Kumsi	Diarrhoea, dysentery, constipation
Anacardium occidentale L.	Anacardiaceae	Sagar	Skin diseases
Annona squamosa L.	Annonaceae	Koppa	Diarrhoea, dysentery, insecticide
Anthocephalus cadamba Roxb.	Rubiaceae	Koppa	Diabetes, dental care
Azadirachta indica Juss.	Meliaceae	Shankaraghatta	Anthelminthic, diabetes, antifungal
Bixa orellana L.	Bixaceae	Shankaraghatta	Fever, dysentery, insect repellant
Cinnamomum zeylanicum Blume	Lauraceae	Thirthahalli	Anti oxidant, antimicrobial
Embelia ribes Burm. f.	Myrsinaceae	Lakkavalli	Fever, antidote, intestinal disorders
Eucalyptus globulus Labill.	Myrtaceae	Shankaraghatta	Analgesic, antibacterial, inflammation, antiseptic
Ficus glomerata Rox b.	Moraceae	Sagar	Piles, diarrhea
Ficus religiosa L.	Moraceae	Sagar	Diarrhea, dysentery, asthma
Garcinia indica Choisy	Clusiaceae	Thirthahalli	Antioxidant, antifungal
Holarrhena antidysenterica Roxb. Ex Fleming	Apocyanaceae	Shankaraghatta	Dysentery, antibacterial
Limonia acidissima L.	Rutaceae	Lakkavalli	Dysentery, diarrhoea
Michelia champaca L.	Magnoliaceae	Sagar	Diuretic, skin diseases
Mimusops elengi L.	Sapotaceae	Chikmagalur	Gastro intestinal disorders
Myristica fragrans Houtt.	Myristicaceae	Chikmagalur	Emetic, antiemetic, purgative
Nyctanthes arbor-tristis L.	Oleaceae	Koppa	Gastro intestinal disorders
Persea macrantha (Nees) Kosterm	Lauraceae	Lakkavalli	Asthma, inflammation
Phyllanthus emblica L.	Euphorbiaceae	Shankaraghatta	Hemorrhages, diarrhea
Plumeria rubra L.	Apocyanaceae	Thirthahalli	Eczema, arthritis, constipation
Pongamia pinnata (L.) Pierre	Fabaceae	Lakkavalli	Antibacterial
Pterocarpus marsupium Roxb.	Fabaceae	Chikmagalur	Fever, skin diseases
Putranjiva roxburghii Wall.	Euphorbiaceae	Koppa	Antifungal
Santalum album L.	Santalaceae	Shankaraghatta	Skin diseases
Strychnos nux vomica L.	Strychnaceae	Sagar	Nervous disorders, psoriasis
Syzygium cumini L.	Myrtaceae	Sagar	Dysentery, diuretic, diabetics
Terminalia bellerica Roxb.	Combretaceae	Shankaraghatta	Laxative, anthelminthic, bronchitis
Terminalia chebula Retz.	Combretaceae	Koppa	Emetic, antiemetic, purgative
Terminalia paniculata Roth.	Combretaceae	Shankaraghatta	Antifungal
Terminalia tomentosa W. & A.	Combretaceae	Koppa	Antifungal
Thevetia peruviana (Pers.) K. Schum.	Apocyanaceae	Shimoga	Gastrointestinal disorders
Wrightia tinctoria R. Br.	Apocyanaceae	Shankaraghatta	Anthelminthic diarrhoea

Data analysis

The colonization rate of endophytic fungi was determined as the total number of segments yielding ≥ 1 isolate in a host sample divided by the total number of segments incubated in that sample x 100. Frequency of colonization by individual taxa was calculated as total number of isolates/colonies of that species divided by the total number of segments incubated in that sample x 100. Significance of differences in the frequency of colonization among the host plants was determined by the Kruskal-Wallis method (Gibbons 1976). Differences between winter, monsoon, and summer seasons were tested by ANOVA. Shannon diversity index (H'), Shannon evenness index (J') and Simpson diversity index (1/D) were used for the evaluation of the fungal species richness (Zar 2004). Jaccard's Similarity Coefficient (JI) was used to describe the taxonomic affinity of the endophytic mycobiota among sampling sites (Arnold *et al.* 2000).

Results

A total of 17 561 fungal isolates were isolated from a total of 19 800 leaf segments incubated from 33 medicinal trees *Colletotrichum gleosporioides* (8.32%), *Phyllosticta* spp. (6.16%), *Cladosporium cladosporioides* (Fresen.) de Vries (5.22%), *Phomopsis* spp. (5.08%), *Pestalotiopsis* spp. (4.08%), *Chaetomium globosum* Kunze & Schm. (3.76%), and *Aspergillus niger* Tiegh. (3.15%) were frequently isolated from the host plants studied (Tab. 2). The total colonization rate (Fig. 1) and frequency of endophytic fungi varied and differed significantly between monsoon, winter, and summer seasons (F = 12.73). The colonization frequency (CF %) was high in *Nyctanthes arbor-tristis* L. (137.0%) and low in *T. bellerica* (35.5%) during monsoon season. It was highest in *Thevetia peruviana* (Pers.) K. (176.0%) and lowest in *T. bellerica* (35.5 %) during the winter season. Similarly CF (%) was highest in *E. globulus* Labill. (94.0%) and lowest in *T. bellerica* (15.0%) during the summer season (Tab. 3). Percentages of colonization did not differ significantly among the tree species, when tested by the Kruskal-Wallis method. The Shannon diversity index (H') indicated that *Myristica fragrans* Houtt. had a high endophytic diversity (H' value = 1.58), and a low diversity was observed in *T. bellerica* (H' value = 0.78). The Shannon Evenness index (J') showed that the relative diversity or evenness was high in *Aegle marmelos* Carr., *Pterocarpus marsupium* Roxb., and *Terminalia chebula* Retz. with a J' value of 0.92 each, and it was lowest in *Holarrhena antidysenterica* Roxb. ex Flem. with a J' value 0.73. Similarly, according to Simpson's diversity index, species abundance was high in *A. marmelos* and *Limonia acidissima* L. with a 1/D value of 14.2 each. Low species abundance was observed in *Persea macrantha* (Nees) Kosterm. (1/D = 4.16) (Tab. 4). Isolates belonging to *Alter-*

naria, Aspergillus, Botryosphaeria, Chaetomium, Cladosporium, Colletotrichum, Pestalotiopsis, Phomopsis, and *Phyllosticta* were frequently isolated during monsoon and winter seasons. *Cladosporium, Penicillium, Bipolaris*, and *Septocylindrium* spp. were frequently isolated during the summer season. No significant difference was observed in the endophytic assemblages among the study sites: the colonization frequency was high in Shimoga (118.5%) followed by Kumsi (105.3%), Koppa (93.3%), Sagar (92.3%), Chikmagalur (89.7%), Shankaraghatta (83.7%), and Lakkavalli (78.6%), whereas it was lowest in Thirthahalli (75.9%). The dendrogram based on clustering of the sites shows that Kumsi, Sagar, Shankaraghatta, and Thirthahalli have similar endophytic assemblages whereas Shimoga is well separated from the other sites (Fig. 2).

Tab. 2. – Colonization frequency (%) of different fungal groups isolated from medicinal trees.

Host plants	Fungal groups					Total*
	Teleomorphic Ascomycota	Anamorphic Ascomycota		Zygomycota	Sterile isolates	
		With conidiomata	Without conidiomata			
Aegle marmelos (L.) Corr. Serr.	16.6	39.8	44.0	0.0	2.1	105.3
Anacardium occidentale L.	10.3	49.0	15.1	0.0	16.5	91.0
Annona squamosa L.	0.0	40.6	49.1	2.2	0.0	92.0
Anthocephalus cadamba Roxb.	11.6	13.0	26.0	1.0	2.8	54.6
Azadirachta indica Juss.	3.5	4.0	12.6	1.5	25.1	49.6
Bixa orellana L.	0.0	0.0	64.0	0.0	6.1	70.1
Cinnamomum zeylanicum Blume	19.1	15.1	26.8	0.0	10.8	72.0
Embelia ribes Burm. f.	3.6	62.3	35.1	2.8	5.8	109.8
Eucalyptus globulus Labill.	15.8	36.8	41.0	11.8	0.0	105.5
Ficus glomerata Roxb.	2.8	60.5	46.5	0.0	0.0	109.8
Ficus religiosa L.	4.5	37.6	17.5	1.5	2.6	63.8
Garcinia indica Choisy	16.3	5.3	26.5	0.0	5.8	54.0
Holarrhena antidysenterica Roxb. ex Fleming	16.8	49.8	20.1	0.6	0.0	87.5
Limonia acidissima L.	32.8	20.8	23.3	0.0	0.0	77.0
Michelia champaca L.	34.1	16.3	34.6	0.0	10.3	95.5
Mimusops elengi L.	43.5	21.1	18.0	2.8	0.0	85.5
Myristica fragrans Houtt.	4.1	38.5	33.0	0.0	10.3	86.0
Nyctanthes arbor-tristis L.	0.0	45.5	52.0	0.0	6.6	104.1

Table 2. – continued

Host plants	Fungal groups					Total*
	Teleomorphic Ascomycota	Anamorphic Ascomycota		Zygomycota	Sterile isolates	
		With conidiomata	Without conidiomata			
Persea macrantha (Nees) Kosterm.	0.0	62.8	8.1	3.1	7.0	80.8
Phyllanthus emblica L.	34.5	22.5	8.6	1.1	11.0	77.8
Plumeria rubra L.	9.5	54.3	35.6	2.3	0.0	101.8
Pongamia pinnata (L.) Pierre	12.6	39.0	20.16	0.0	6.6	78.5
Pterocarpus marsupium Roxb.	13.3	36.6	44.5	3.0	0.0	97.5
Putranjiva roxburghii Wall.	0.0	41.5	77.8	0.0	4.0	123.3
Santalum album L.	9.3	20.1	81.6	0.0	7.3	118.0
Strychnos nux vomica L.	21.6	32.1	21.5	0.0	12.8	87.6
Syzygium cumini L.	6.6	44.3	47.5	0.0	7.8	106.3
Terminalia bellerica Roxb.	0.0	14.8	17.1	1.3	0.0	33.3
Terminalia chebula Retz.	0.0	37.1	34.5	3.1	11.1	86.0
Terminalia paniculata Roth.	12.5	56.6	38.8	0.0	3.1	111.1
Terminalia tomentosa W. & A.	26.0	38.5	38.6	2.1	0.0	100.5
Thevetia Peruviana (Pers.) K. Schum.	0.0	42.8	57.8	3.1	14.6	118.5
Wrightia tinctoria R. Br.	7.6	63.0	20.8	0.6	2.1	94.3

* Percentages higher than 100 % result from multiple infections of single host segments. (See calculation of the colonization rate in the methods part.)

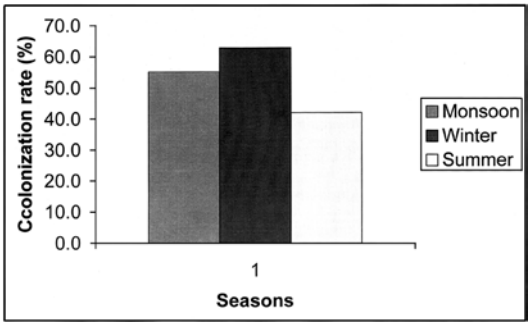

Fig. 1. – Total Colonization rate (%) of endophytic fungi during different seasons in medicinal tree species.

Tab. 3. – Colonization frequency of endophytic fungi (%) during seasons, dominant genus and number of species isolated from medicinal trees.

Host Plants	Dominant Genus	Colonization frequency (%)			No. of species
		Monsoon	Winter	Summer	
Aegle marmelos (L.) Corr. Serr.	Chaetomium	116.5	126.0	73.5	20
Anacardium occidentale L.	Phyllosticta	89.0	137.5	46.5	14
Annona squamosa L.	Colletotrichum	84.5	129.5	62.0	16
Anthocephalus cadamba Roxb.	Phyllosticta	48.0	82.0	34.0	15
Azadirachta indica Juss.	Sterile form	54.5	63.5	31.0	12
Bixa orellana L.	Aspergillus	76.5	99.0	35.0	14
Cinnamomum zeylanicum Blume	Chaetomium	74.0	101.5	40.5	13
Embelia ribes Burm. f.	Phomopsis	103.5	158.0	68.0	22
Eucalyptus globulus Labill.	Colletotrichum	108.0	114.5	94.0	18
Ficus glomerata Rox b.	Colletotrichum	121.5	144.0	64.0	21
Ficus religiosa L.	Colletotrichum	73.5	94.5	23.5	18
Garcinia indica Choisy	Xylaria	68.0	67.0	27.0	11
Holarrhena antidysenterica Roxb. ex Fleming	Colletotrichum	99.0	91.5	72.0	14
Limonia acidissima L.	Chaetomium	76.0	101.5	53.0	18
Michelia champaca L.	Chaetomium	84.0	148.0	54.5	19
Mimusops elengi L.	Botryosphaeria	88.0	102.5	66.0	12
Myristica fragrans Houtt.	Botryodiplodia	95.0	113.0	50.0	11
Nyctanthes arbor-tristis L.	Colletotrichum	137.0	92.0	83.5	15
Persea macrantha (Nees) Kosterm.	Botryodiplodia	76.0	101.5	53.0	18
Phyllanthus emblica L.	Sordaria	80.5	95.5	57.5	14
Plumeria rubra L.	Phyllosticta	122.0	120.5	63.0	14
Pongamia pinnata (L.) Pierre	Colletotrichum	82.0	105.5	48.0	14
Pterocarpus marsupium Roxb.	Phomopsis	109.0	112.0	71.5	13
Putranjiva roxburghii Wall.	Phyllosticta	119.0	158.0	93.0	17
Santalum album L.	Aspergillus	111.5	165.0	77.5	15
Strychnos nux vomica L.	Xylaria	76.5	134.0	52.5	13
Syzygium cumini L.	Phomopsis	135.5	119.0	64.5	16
Terminalia bellerica Roxb.	Phyllosticta	35.5	49.5	15.0	08
Terminalia chebula Retz.	Phyllosticta	84.0	114.5	59.5	14
Terminalia paniculata Roth.	Phyllosticta	113.5	164.0	56.5	19
Terminalia tomentosa W. & A.	Phomopsis	98.5	131.0	72.0	16
Thevetia peruviana (Pers.) K. Schum.	Colletotrichum	134.0	176.0	45.5	21
Wrightia tinctoria R. Br.	Phomopsis	87.5	125.0	70.5	14

Tab. 4. – Diversity indices of endophytic fungi in some medicinal trees of Malnad region, Karnataka.

Host Plant	Shannon Diversity Index	Shannon evenness (J)	Simpson Diversity Index (1/D)
Aegle marmelos (L.) Corr. Serr.	1.20	0.92	14.28
Anacardium occidentale L.	1.00	0.87	9.09
Annona squamosa L.	0.95	0.79	5.26
Anthocephalus cadamba Roxb.	1.00	0.85	7.69
Azadirachta indica Juss.	0.89	0.83	5.55
Bixa orellana L.	0.92	0.80	5.88
Cinnamomum zeylanicum Blume	0.97	0.87	7.69
Embelia ribes Burm. f.	1.15	0.85	10.00
Eucalyptus globulus Labill.	1.09	0.87	9.09
Ficus glomerata Roxb.	1.18	0.89	12.50
Ficus religiosa L.	1.10	0.88	10.00
Garcinia indica Choisy	0.92	0.88	7.14
Holarrhena antidysenterica Roxb. ex Fleming	0.84	0.73	4.54
Limonia acidissima L.	1.16	0.92	14.28
Michelia champaca L.	1.11	0.87	10.00
Mimusops elengi L	0.90	0.84	6.25
Myristica fragrans Houtt.	1.58	1.51	6.66
Nyctanthes arbor-tristis L.	1.04	0.88	8.33
Persea macrantha (Nees) Kosterm.	0.79	0.75	4.16
Phyllanthus emblica L.	0.99	0.86	8.33
Plumeria rubra L.	1.03	0.90	10.00
Pongamia pinnata (L.) Pierre	0.92	0.80	5.88
Pterocarpus marsupium Roxb.	1.03	0.92	10.00
Putranjiva roxburghii Wall.	1.09	0.88	12.50
Santalum album L.	0.99	0.84	8.33
Strychnos nux vomica L.	0.96	0.86	8.33
Syzygium cumini L.	1.04	0.86	9.09
Terminalia bellerica Roxb.	0.78	0.86	5.00
Terminalia chebula Retz.	1.05	0.92	11.11
Terminalia paniculata Roth.	1.08	0.85	9.09
Terminalia tomentosa W. & A.	1.04	0.86	9.09
Thevetia peruviana (Pers.) K. Schum.	1.18	0.89	12.50
Wrightia tinctoria R. Br.	0.85	0.74	4.34

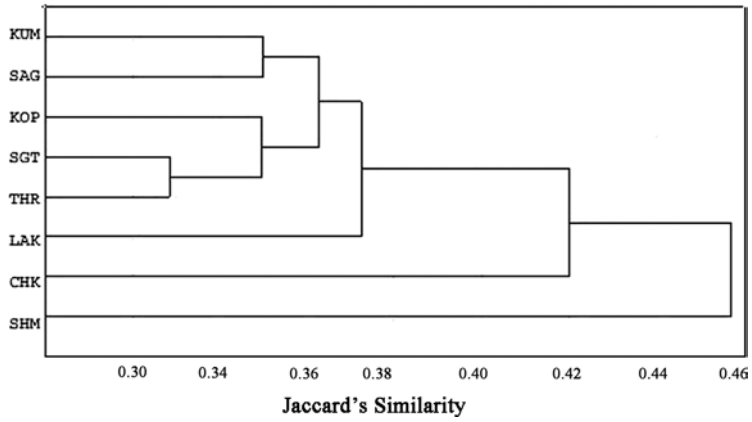

Fig. 2. – Dendrogram showing the Jaccard's similarity of endophytic fungi at the different study sites (KUM = Kumsi, SAG = Sagar, KOP = Koppa, SGT = Shankaraghatta, THR = Thirtahalli, LAK = Lakkavalli, CHK = Chikmagalur, SHM = Shimoga)

Discussion

The colonization frequency (CF %) of fungal endophytes in this study was within the range of many host plants studied in the tropics (Suryanarayanan *et al.* 2003). The difference in colonization by endophytes between the trees might be due to substrate, secondary chemistry, and the physiological state of the host plants (Lodge 1997). Although variation occurred in colonization of endophytes, the assemblages represented not by any unique fungal species but by the commonly occurring in a given host species (Neubert *et al.* 2006). Dominant taxa isolated in this study such as *Colletotrichum, Phyllosticta, Cladosporium, Phomopsis, Pestalotiopsis, Chaetomium* were recovered as endophytes from a wide variety of distantly related host plants indicating that these fungi are well adapted to survive as endophytes (Petrini *et al.* 1992, Kumar & Hyde 2004). *Colletotrichum* spp. have the ability to express different symbiotic life styles based on host genotypes (Redman *et al.* 2001). Many of the strains isolated in this study belonged to genera such as *Aspergillus* and *Penicillium* being saprobic or soil fungi that rarely occur as endophytes in healthy tissues, and may be considered contaminants (Zamora *et al.* 2008). *Cladosporium* spp., which were isolated during all seasons, are common airborne fungi, often found on or in dead plant substrates were reported to live also endophytically in trees, especially in leaves (Petrini 1984). Maximum colonization during the winter season suggests that environmental factors such as humidity and precipitation are positively correlated with endophytic colonization (Bills 1996). No clear host specificity was observed among the host plants studied. Variation in the colonization among the sites might be due to site-specific factors. Differences in

colonization in trees or sites in a region may also be caused by different host genotypes along with prevailing environmental conditions influencing infection by particular fungal endophytes (Elamo *et al*. 1999). Similarly, quantitative surveys of endophyte colonization may be sensitive to leaf size, age, methodology, and growth media (Gamboa *et al*. 2002, Arnold & Herre 2003). One major concern in endophyte studies is, however, that the endophytes are isolated from surface sterilized, living leaves onto artificial media (Ganley & Newcombe 2006, Hyde & Soytong 2007). Because sterile forms, which are often isolated in endophytic studies, cannot be given a taxonomic status (Promputtha *et al*. 2005), and, due to the existence of non-cultivable endophytes, the real number of endophytic species in a sample can be underestimated. Molecular techniques like DNA sequence analyses and DGGE (denaturing gradient gel electrophoresis) have been used effectively for the phylogenetic classification of sterile forms and non-cultivable fungi obtained as endophytes ((Promputtha *et al*. 2005, Jeewon & Hyde 2006, Neubert *et al*. 2006). It has been suggested that both culture and culture-independent methods could be used for the estimation of fungal diversity (Arnold & Lutzoni 2007, Hyde & Soytong 2007).

The screening for endophytic fungi in some medicinal trees described here helps to gain knowledge on their symbiotic fungi. The role of these fungi through mutual interaction in antagonism against phytopathogens or insect herbivores could be speculated. The outcome of microbe/host interactions, however, can be influenced by the genetic diversity of symbionts, the ways in which they are acquired from the environment, their ability to co-colonize individual hosts, their direct and indirect interactions, and their own evolutionary history encapsulated by the genomic architecture associated with pathogenicity or other ecological modes. Recently, steps in understanding the evolutionary origins of symbioses are being taken with large-scale phylogenetic analyses of endophytes (Arnold *et al*. 2007). Even then, much work is needed for understanding the diversity of mycobiota capable of forming endophytic symbioses with plants in any given ecosystem.

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