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Diversity studies on the endophytic fungi of *Vitex negundo* L.

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Abstract

A total of 143 endophytic fungal isolates were recovered from 1350 bark, twig and leaf segments of *Vitex negundo* L. which has important traditional medicinal value in the Indian system of medicine. The isolates belonged to ascomycetes (24%), coelomycetes (21%), hyphomycetes (51%) and mycelia sterilia (4%) groups. *Lasiodiplodia* sp. was the dominant endophyte in all the plant parts. The endophytic taxa were identified on the basis of morphology and DNA sequence data. Twig samples harboured higher number of endophytic taxa than the bark and leaf tissues of *V. negundo*. Phylogenetic relationships of the endophytic fungi were estimated from the sequences of the ITS region. Our studies indicate that diverse endophytic genera are grouped into six clades with respective fungal endophytes grouped in separate clades.

Keywords – colonization frequency – diversity indices – endophytes – ITS sequencing – phylogenetic analysis

Introduction

The endophytic microorganisms colonize the intracellular spaces between cells of higher plants, which provide a unique and specialized biological niche. Endophytes establish in the living internal tissues of their hosts without producing symptoms of disease (Sakalidis et al. 2011). Endophytic fungi have been reported from various plant species, which contribute to the diversity of microorganisms in natural environments (Nalini et al. 2014) and produce various bioactive compounds that play a major role in inherent surroundings (Samaga et al. 2013, Qadri et al. 2014, Tiwari et al. 2014).

The composition of the fungal community usually differs between host species (Saikkonen 2007), among the geographically separated individuals of the same host species (Collado et al. 1999), and also within the tissue or organs of a host plant (Kumar & Hyde 2004). Variation in the diversity of fungi may also be associated with location, climate and plant age (Petrini 1991, Asai et al. 1998). Endophytic fungi have been recognised as sources for new secondary metabolites with useful biological activity. Interest in fungal endophytes is largely due to their chemical diversity. These represent a virtually untapped source of chemical reservoir that finds use in agriculture and therapeutics. Sampling and characterization of fungal endophyte diversity is an emerging challenge, which leads to the discovery of new species producing novel compounds and a better understanding of their role in ecosystems (Saikkonen 2007, Rodriguez et al. 2009).

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Vitex negundo L. belongs to the family Verbenaceae, and is commonly known as 'Nirgundi' in Sanskrit, and 'Five leaved chaste tree' in English. It is a large aromatic shrub with typical five foliate leaf patterns. The species occurs in tropical to temperate regions of the world. India, one of the 11 mega biodiversity countries, has 13 species of *Vitex*. *V. negundo* is a shrub or small tree (4–8 m). The leaves have five leaflets in a palmate arrangement and pointed at both ends. The bluish purple flowers are numerous. The fruit is succulent, black and rounded (Gautam et al. 2008).

Vitex negundo L. is one of the common plants used in traditional medicine in India and has a variety of pharmacological activities (Basal & Kurmi 2006). Although, all parts of *V. negundo* are used as medicine in the indigenous system of medicine, the leaves are most potent for the treatment of inflammation, eye disease, etc. The plant contains many polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids (Alam & Gomes 2003, Dharmasiri et al. 2003).

The mycota from medicinal plants generally produces the bioactive and chemically novel metabolites with medicinal and agricultural potentials (Strobel & Daisy 2003). The present study was taken up to investigate the composition and diversity of fungal endophytes associated with *V. negundo* and to generate the ITS sequence data to validate the diversity of selected endophytic fungi.

Materials & Methods

Collection and sampling site

Bark, twig and leaf samples of *V. negundo* were collected from the Western Ghats region (10° 10′ N 77° 04′ E) stretching across Shimoga and Chikmagalur districts and Mysore district (12.30° N 76.65° E) of Karnataka state during the monsoon season of 2013. A herbarium specimen was prepared and submitted to the herbarium collection of the Department of Studies in Biotechnology. Bark pieces (5.0 × 5.0 cm) from the trunk were cut 1.5−2.0 m above the ground level with the help of alcohol (70%) swabbed machete. The samples were placed in polythene bags, labelled, transferred in ice box to the laboratory and placed in a refrigerator at 4°C. The samples were processed within 24h of collection.

Isolation, identification and preservation of endophytes

The samples were washed thoroughly in running water before processing. Bark, twig and leaf tissues were surface sterilized by immersing in 70% ethanol (v/v) for one min and 3.5% NaOCl (v/v) for three min, then rinsed with sterile water thrice and allowed to surface dry under sterile conditions. Bits of 1.0 × 1.0 cm size were excised with the help of a sterile blade. Four hundred and fifty segments each of bark, twig and leaves were placed on water agar (15 g/L) (WA) medium supplemented with Streptomycin (100 mg/l) contained in 9 cm diameter Petri dishes. Ten to fifteen segments were placed on solidified 20 ml WA medium in each Petri dish. The Petri dishes were incubated at 22°C with 12h light and dark cycles upto 3−4 weeks (Suryanarayanan 1992). After incubation, individual fungal colonies were picked from the edge with a sterile fine tipped needle and transferred onto potato dextrose agar (PDA) medium for further identification. The fungal endophyte that did not sporulate was inoculated onto sterilized banana leaf bit (1 cm^2) impregnated on agar to ensure sporulation (Matsushima 1975). The endophytic identification was done based on the conidial morphology and conidial characters. All endophytic isolates have been maintained in cryovials on PDA layered with 15% glycerol (v/v) at -80°C in an Ultra freezer (Cryoscientific Pvt. Ltd., Chennai, India) at the Department of Studies in Biotechnology, University of Mysore, Mysore, India.

Molecular identification

The endophytic fungal isolates were cultured in Potato Dextrose Broth (PDB, Himedia™, India) at 27°C. The fungal mat were separated and subjected to freeze-drying process. The mycelial mat was quenched in liquid nitrogen before the extraction of genomic DNA. The genomic DNA was extracted with slight modification following the method of Guo et al. (2000). The DNA was

quantitatively determined by a Nanodrop method spectrophotometrically (BioRad, California, USA). DNA was amplified with the universal ITS primers ITS1 (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 (5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') in a Thermo cycler (Eppendorff, Germany) (White et al. 1990). Amplification was performed in a 25 µl reaction volume which contained PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 200 µM of each deoxyribonucleotide triphosphate, 10 pmols of each primer, 50 ng template DNA and 2.5 U of Taq DNA polymerase (20 mM Tris-HCl, 0.1 mM EDTA, 0.1 M KCl, 0.5% Tween 20 (v/v) and 50% glycerol (v/v)).

The amplification was achieved by setting the following PCR conditions: 3 min initial denaturation at 95°C, followed by 35 cycles of 1 min denaturation at 92°C, 1 min primer annealing at 52°C, 2 min extension at 72°C and a final 10 min extension at 72°C. Fifteen microliters of PCR products from each PCR reaction were examined by electrophoresis at 100V for 1−2 h in an 1% (w/v) agarose gel in 1X TBE buffer (pH 8.4) stained with ethidium bromide and visualized under a gel documentation system (Gel Doc 2000, BioRad, California, USA).

Sequencing of ITS region and phylogenetic analysis

PCR products were sequenced with primer pairs as mentioned above at Eurofins Genomics India Pvt Ltd., Bengaluru. The sequence obtained from each isolate was further analyzed using BLAST from the National Centre for Biotechnology Information (NCBI) website (www.blast.ncbi.nlm.nih.gov). Sequences obtained were subjected to Clustal W analysis using MEGA software version 6.05 (online) (Tamura et al. 2013). The analyzed sequences were uploaded to NCBI.

Data analysis

The colonisation density, colonisation rates and isolation rates of fungal diversity were calculated as the percentage of segments colonized by one or more isolate(s) from the total number of segments of each tissue plated following the method of Petrini & Fisher (1988).

Total no. of segments in a sample yielding ≥ 1 isolate.

$$\text{Colonization rate (CR)} = \frac{\text{Total no. of isolates in a given sample}}{\text{Total no. of segments in a sample}} \times 100$$

$$\text{Isolation rate (IR)} = \frac{\text{Total no. of isolates yielded in a sample}}{\text{Total no. of segments in sample}}$$

The relative frequency of colonization (%CF) was calculated as the number of isolates of a taxon from each segments, divided by the total number of segments plated × 100 (Fisher & Petrini 1987) and dominant endophytes were calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes × 100 (Kumaresan & Suryanarayanan 2001).

Species diversity indices were measured with Shannon diversity index (H'), Shannon evenness index (J') and Simpson diversity index (D) and calculated for the evaluation of fungal species richness of endophytes isolated from *V. negundo* using the Shannon calculator (Zar 2004). It was based on the data provided in Table 2. The endophyte species present in the particular sample type was taken as 1 and the same endophyte absent in the other sample type was taken as 0.

Results

Colonization and isolation rates of endophytic fungi

A total of 143 isolates of endophytic fungi were recovered from 1350 tissue segments of *V. negundo*. CR of bark, twig and leaf tissue (22.22%, 22.66% and 21.33%) as well as IR of bark, twig and leaf tissue (0.12, 0.12 and 0.08), and the colonization and isolation rates of endophytic

fungi in twigs was higher followed by bark and leaf tissues. Much variation was not observed between the twig and bark assemblage, while small variation was observed in the leaf tissue (Table 1).

Table 1 Colonization and isolation rates of endophytic fungi in *Vitex negundo*.

	Bark*	Twig*	Leaf*	Total
No. of samples yielding fungi	100	102	96	298
No. of isolates	52	53	38	143
Colonization rate (%)	22.22	22.66	21.33	22.07
Isolation rate	0.12	0.12	0.08	0.11

*Based on 450 bits plated per sample (n=10)

Of the 143 fungal isolates recovered from the sample, 139 sporulated and were assigned to 23 spp. representing 16 genera based on the morphological characteristics. The identity of the fungal endophytes were further confirmed by sequencing the ITS region of the large subunit of the rRNA gene (Table 2). The fungal endophytic composition included 14 species in bark, 16 species in twig and 12 species in leaf tissue and consisted of 24% ascomycetes, 21% coelomycetes, 51% hyphomycetes and 4% mycelia sterilia. Various endophytic fungi were obtained from *V. negundo* collected from nine locations (Fig. 1; Table 2), of these, seven were from Shimoga, six from Kodachadri, five from Hebri, four from Udupi, three from Sringeri, eight from Kudremukh, four from Hornadu, five from Chikmagalur and five from Mysore.

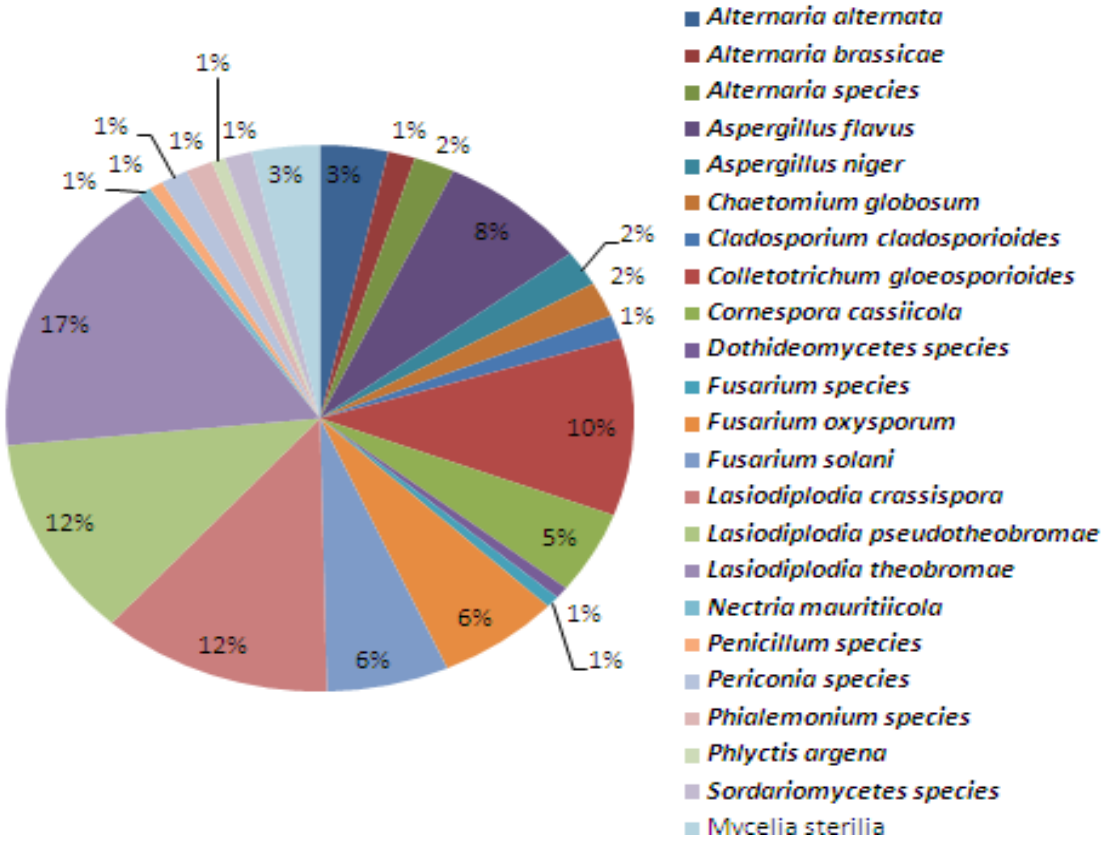


Fig. 1 − Relative frequencies of isolation of endophytic fungi from *V. negundo*.

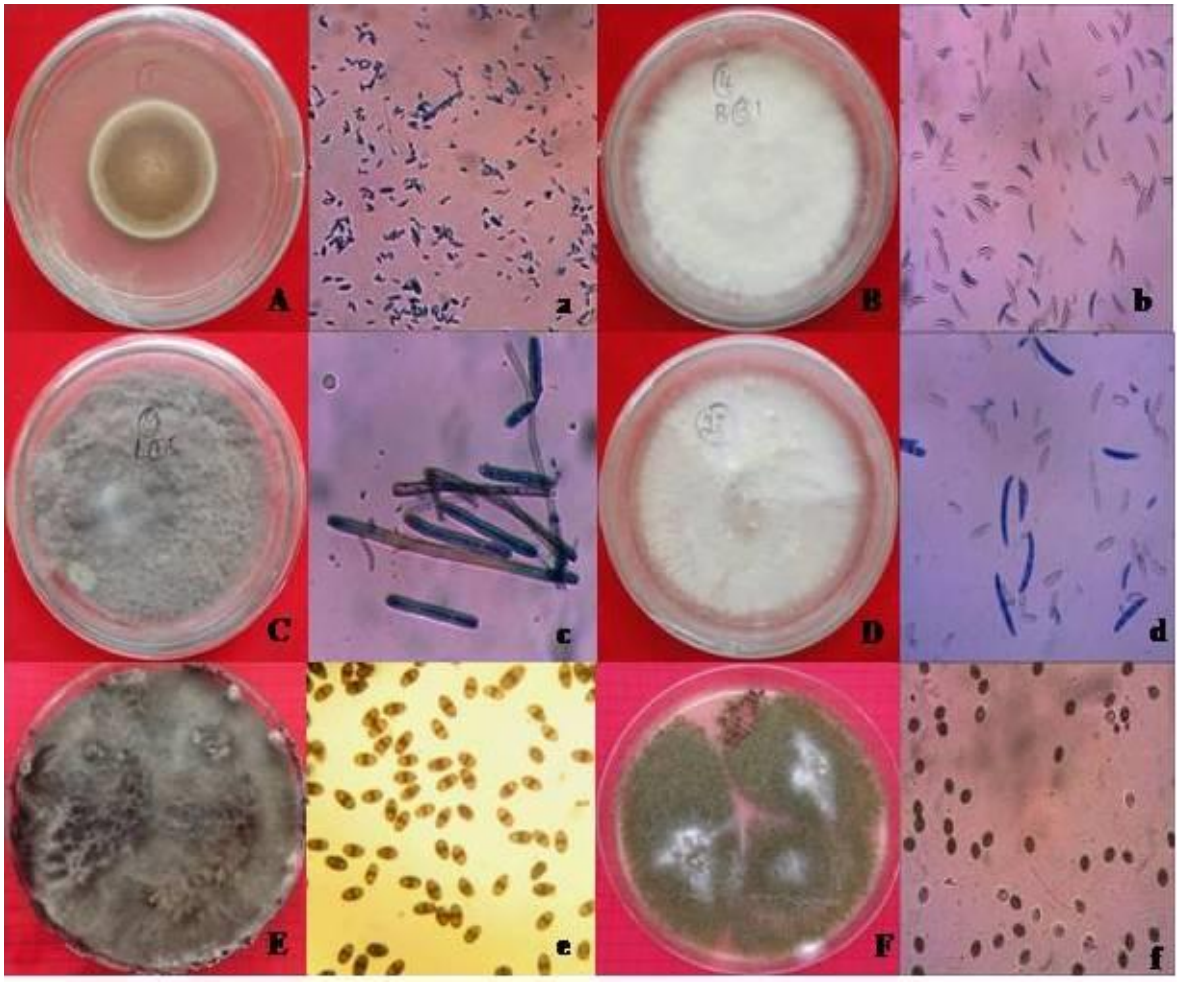
Endophytic fungi were isolated from *V. negundo* by surface sterilization and plating techniques. The total endophytic fungal isolates obtained were considered as hundred. The relative frequencies were calculated for each endophytic genera and represented as percentage.

Table 2 Frequency of endophytic fungi isolated from bark, twig and leaf samples of *Vitex negundo*.

	Shimoga			***Kodachadri***			***Hebri***			***Udupi***			***Sringeri***			***Kudremukh***			***Hornadu***			***Chikmagalur***			***Mysore***				
ENDOPHYTES	***B***	***T***	***L***	***B***	***T***	***L***	***B***	***T***	***L***	***B***	***T***	***L***	***B***	***T***	***L***	***B***	***T***	***L***	***B***	***T***	***L***	***B***	***T***	***L***	***B***	***T***	***L***	***Total***	***Frequency of dominant endophytes***
Alternaria alternata	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*6.0*	*0*	*0*	*0*	*0*	*5*	*3.49*
Alternaria brassicae	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*1.39*
Alternaria species	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*4.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*2.09*
Aspergillus flavus	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*4.0*	*12.0*	*0*	*0*	*0*	*11*	*7.69*
Aspergillus niger	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*2.09*
Chaetomium globosum	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*6.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*3*	*2.09*
Cladosporium cladosporioides	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*1.39*
Colletotrichum gloeosporioides	*6.0*	*0*	*6.0*	*0*	*2.0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*6.0*	*4.0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*12*	*8.39*
Cornespora cassiicola	*0*	*0*	*2*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*4.0*	*0*	*0*	*0*	*0*	*0*	*0*	*4.0*	*7*	*4.89*
Dothideomycetes species	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0.69*
Fusarium species	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0.69*
Fusarium oxysporum	*0*	*0*	*2.0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*8.0*	*0*	*0*	*4.0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*9*	*6.29*
Fusarium solani	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*16.0*	*0*	*0*	*9*	*6.29*
Lasiodiplodia crassispora	*0*	*4.0*	*4.0*	*2.0*	*0*	*2.0*	*8.0*	*4.0*	*4.0*	*0*	*0*	*0*	*0*	*2.0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*17*	*11.88*
Lasiodiplodia pseudotheobromae	*0*	*0*	*2.0*	*6.0*	*0*	*0*	*2.0*	*6.0*	*2.0*	*0*	*2.0*	*0*	*2.0*	*4.0*	*8.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*17*	*11.88*

Lasiodiplodia theobromae	*0*	*4.0*	*2.0*	*2.0*	*4.0*	*0*	*12.0*	*4.0*	*2.0*	*2.0*	*0*	*2.0*	*0*	*0*	*2.0*	*4.0*	*4.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4.0*	*24*	*16.78*
Nectria mauritiicola	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0.69*
Penicillum species	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0.69*
Periconia species	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*1.39*
Phialemonium species	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*1.39*
Phlyctis argena	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0.69*
Sordariomycetes species	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*4.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2*	*1.39*
Mycelia sterilia	*4.0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*2.0*	*0*	*0*	*0*	*0*	*2.0*	*2.0*	*0*	*5*	*3.49*
Total No. of isolates recovered	*6*	*5*	*11*	*6*	*3*	*2*	*13*	*9*	*6*	*9*	*2*	*1*	*6*	*5*	*9*	*4*	*7*	*2*	*0*	*8*	*2*	*0*	*5*	*6*	*10*	*1*	*5*	*143*	
Total CF%	*12*	*10*	*22*	*12*	*06*	*04*	*26*	*18*	*12*	*18*	*04*	*02*	*12*	*10*	*18*	*08*	*14*	*04*	*0*	*16*	*04*	*0*	*10*	*12*	*20*	*2*	*10*		

Note: B − Bark; T − Twig; L − Leaf



Figs. 2 − Colony morphology and conidial characters of important fungal endophytes isolated from *Vitex negundo*. (A) *Colletotrichum gloeosporioides* colony (a) and conidial characteristics (B) *Fusarium oxysporum* colony (b) and conidial characteristics (C) *Corynespora cassiicola* colony (c) and conidial characteristics (D) *Fusarium solani* colony (a) and conidial characteristics (E) *Lasiodiplodia theobromae* colony (e) and conidial characteristics (F) *Chaetomium globosum* colony (f) and conidial characteristics.

The most frequently isolated endophytic fungus was *Lasiodiplodia* sp. *viz. L. crassispora, L. pseudotheobromae* and *L. theobromae*, identified on the basis of sequence analysis of ITS region. The other common endophytes of *V. negundo* included *Aspergillus flavus, A. niger, Colletotrichum gloeosporioides, Fusarium oxysporum* (Fig. 2). Twig yielded the highest number of endophytic fungi (12%), followed by bark (11%) and leaf (8%).

Diversity of fungal endophytes

The Shannon-Wiener diversity indices of endophytic fungi was high in twig (2.48) > bark (2.36) > leaf (2.28) (Table 3). Simpson's diversity index value ranged between 0.10 and 0.12, the highest value indicates an increase in the species diversity. The endophytes of leaf exhibited greater diversity compared to the endophytes of other plant parts examined (Table 3). Species richness was found to be higher in twig samples with 17 species, followed by bark with 15 species and leaf with 13 species

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Table 3 Diversity of fungal endophytes from *V. negundo*.

	Shimoga			Kodachadri			Hebri			Udupi			Sringeri			Kudremukh			Hornadu			Chikmagalur			Mysore		
	B	**T**	**L**	**B**	**T**	**L**	**B**	**T**	**L**	**B**	**T**	**L**	**B**	**T**	**L**	**B**	**T**	**L**	**B**	**T**	**L**	**B**	**T**	**L**	**B**	**T**	**L**
Shannon-Wiener Diversity Index	1.33	1.05	1.85	1.24	0.64	0.69	1.31	1.52	1.56	1.43	0.69	0	1.56	1.33	1.43	0.69	1.35	0.69	0	1.49	0.69	0	0.67	0	0.64	0	1.05
Species Richness (S)	4.0	3.0	7.0	4.0	2.0	2.0	5.0	5.0	5.0	5.0	2.0	1.0	5.0	4.0	5.0	2.0	4.0	2.0	0	5.0	2.0	0	2.0	1.0	3.0	1.0	3.0
Total Abundance	6	5	11	6	3	2	13	9	6	9	2	1	6	5	9	4	7	2	0	8	2	0	5	6	10	1	5
Simpson Diversity Index (D)	0.28	0.36	0.17	0.33	0.56	0.5	0.33	0.23	0.22	0.28	0.5	1.0	0.22	0.28	0.28	0.5	0.27	0.5	0	0.25	0.5	0	0.52	1.0	0.66	1.0	0.36
Evenness	0.96	0.96	0.94	0.89	0.92	1.0	0.81	0.95	0.97	0.89	1.0	0	0.97	0.96	0.87	1.0	0.98	1.0	0	0.93	1.0	0	0.97	0	0.58	0	0.96
Shannon Entropy	1.92	1.52	2.66	1.79	0.92	1.0	1.89	2.19	2.25	2.06	1.0	0	2.25	1.92	2.06	1.0	1.95	1.0	0	2.16	1.0	0	0.97	0	0.92	0	1.52

Note: B − Bark; T − Twig; L − Leaf

Molecular identification of endophytic fungi based on ITS rRNA sequence

The quantity of DNA obtained ranged from 27.5 ng/µl to 480 ng/µl. Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because of its high copy number of rRNA genes, which allows easy amplification even from small quantities of DNA, and it possesses a high degree of variation even between closely related species. The amplified ITS sequences of the isolated endophytes were subjected to BLAST search, which yielded high homology with the respective species in the GenBank database from NCBI (Table 4).

Molecular identification, on the basis of sequence analysis, revealed that the isolated endophytic fungi were greatly attributed to seven genera, *Alternaria, Periconia, Lasiodiplodia, Corynespora, Colletotrichum, Nectria* and *Fusarium.* The sequences of some nearest neighbours of the endophytic fungal isolates retrieved from GenBank (NCBI) were used to construct the phylogenetic tree. Based on the phylogenetic tree, two clusters were formed. Cluster 1 contained *Lasiodiplodia* sp., *Corynespora cassiicola, Periconia byssoides* and *Alternaria* sp. Cluster 2 contained *Fusarium* sp., *Nectria mauritiicola* and *Colletotrichum gloeosporioides.* Six clades were identified, and the respective endophytic fungi were grouped in one clade, showing the appropriate identification. In cluster 2, *Periconia* sp. was nested within *Nectria* sp., which also possessed identical sequence alignment from the retrieved neighbour sequence (Fig. 3).

Table 4 Molecular identification of endophytes isolated from *V. negundo* through ITS sequences based on BLAST queries in NCBI.

Sl. No.	Endophyte Taxa	GenBank Accession No.	Closest blast match (GenBank accession No.)	Query/reference ITS length (similarity %)
1	*Alternaria alternata*	KF907249	GU325663	516/516 (100%)
2	*Alternaria alternata*	KF923869	FJ809940	516/516 (100%)
3	*Alternaria brassicae*	KF923864	JX857165	536/538 (99%)
4	*Alternaria* sp.	KF923865	KC895541	531/535 (99%)
5	*Cladosporium cladosporioides*	KF907248	KC880082	506/507 (99%)
6	*Colletotrichum gloeosporioides*	KF923853	KF541090	519/519 (100%)
7	*Colletotrichum gloeosporioides*	KF923867	JX902437	527/529 (99%))
8	*Colletotrichum gloeosporioides*	KF923871	KF053200	526/529 (99%)
9	*Colletotrichum gloeosporioides*	KF923863	JF710559	528/528 (100%)
10	*Colletotrichum gloeosporioides*	KF923860	HQ264180	523/525 (99%)
11	*Corynespora cassiicola*	KF846520	KC428403	425/433 (98%)
12	*Corynespora cassiicola*	KF907250	JN541214	515/520 (99%)
13	*Dothideomycetes* sp.	KF907245	KC341979	507/508 (99%)
14	*Fusarium oxysporum*	KF907239	HQ384394	499/503 (99%)
15	*Fusarium oxysporum*	KF907241	JF807394	493/495 (99%)
16	*Fusarium oxysporum*	KF907243	HQ671184	491/492 (99%)
17	*Fusarium oxysporum*	KF923858	GQ121297	491/492 (99%)
18	*Fusarium oxysporum*	KF923862	HQ451888	490/491 (99%)
19	*Fusarium solani*	KF923870	JN786598	524/525 (99%)
20	*Fusarium solani*	KF923859	KF030978	533/538 (99%)
21	*Fusarium* sp.	KF923861	JF819150	494/495 (99%)
22	*Lasiodiplodia crassispora*	AB872222	JF923841	727/785 (93%)
23	*Lasiodiplodia pseudotheobromae*	KF923854	EU860391	560/572 (98%)
24	*Lasiodiplodia theobromae*	KF923857	KF814724	935/993 (94%)
25	*Nectria mauritiicola*	KF923866	AJ558115	538/539 (99%)
26	*Periconia byssoides*	KF923856	KC954160	512/515(99%)
27	*Periconia byssoides*	KF907244	KC954157	512/515 (99%0)
28	*Phialimonium* sp.	KF907246	JQ425380	521/538 (97%)
29	*Phialimonium* sp.	KF907242	GU219470	527/537 (98%)
30	*Sordariomycetes* sp.	KF907240	KF160002	536/538 (99%)

Discussion

The Western Ghats is one of the hot spots and is endowed with a rich biodiversity of more than 4700 different plant species (Rajeshkumar & Singh 2012, Vineet et al. 2013). A new species

of endophyte, *Muscodor kashayum* was recently isolated from the medicinal species *Aegle marmelos* (Bael tree) from the Western Ghats of Muthanga region of Wayanad Wildlife Sanctuary, Kerala, India (Vineet et al. 2013). *V. negundo* is an important medicinal plant used in the traditional medicine and has a variety of pharmacological activities. Our results indicated that twigs harboured more endophytic species, followed by bark and leaf tissues. The results are on par with our previous study, where twigs showed the highest assemblage of endophytic fungi from the medicinal plant *Boswellia serrata* Roxb. (Sunayana & Prakash 2012). One possible reason is that the isolation from twig samples is that the structure and substrate are different between bark and leaf tissues, which influence the colonization and distribution of endophytic fungi (Carroll & Petrini 1983, Rodrigues 1994).

Sun et al. (2012) cited that high species richness for endophytic fungi was observed in twigs than in leaves from a study of three plant species of *Betula platyphylla, Quercus liaotungensis* and *Ulmus macrocarpa*. Sun et al. (2008), recovered more endophytic taxa from twigs than the leaves of six medicinal plant species from China. Kumar & Hyde (2004) stated that the overall colonization rate of endophytes in the leaves was significantly higher than those in root bark, root xylem, flowers and twig bark. Tejesvi et al. (2005) found that the endophytic colonization frequency was greater in inner bark (18.5%) than twigs (4.6%), in *Terminalia arjuna,* while the bark samples harboured more endophytes than twig samples in *Crataeva magna* (Nalini et al. 2005). Similar results have been reported in many previous endophyte studies (Guo et al. 2008, Thalavaipandian 2011).

A total of 143 isolates of endophytic fungi were recovered from 1350 tissues of *V. negundo.* Colonization of endophytes is affected by their host and the climatic factors (Collado et al. 2000). The colonization (22.66 – 21.33) and isolation rates (0.12 – 0.08) of endophytic fungi was recorded from *V. negundo* in the present study. Sun et al. (2008), reported that 973 isolates of endophytic fungi were recovered from 1144 tissue fragments of six medicinal plant species belonging to four families. There were high colonization rates (47.9% – 63.1%) and isolation rates (0.7 – 0.93) observed for endophytic fungi. Similarly, a total of 343 isolates were recovered from 500 samples of leaves, root bark, root xylem, flowers and twig bark of *Tripterygium wilfordii*. The mean overall colonization and isolation rates of endophytes from *T. wilfordii* were 57.8% and 65.4% respectively, as stated by Kumar & Hyde (2004).

Previous studies have revealed that the species composition and frequency of endophytes vary with different host tissues (Sun et al. 2012). For example, the fungal endophytic species noticeably differed between branches and leaves in six medicinal plant species on the basis of cluster analysis (Sun et al. 2008).

In our study, common endophytic taxa found in all the tissues were *A. flavus, A. niger, C. gloeosporioides, F. oxysporum, L. crassispora, L. pseudotheobromae* and *L. theobromae*. These endophytes were found as common endophytic fungi reported on different hosts and were also supported by many previous studies (Okane et al. 1997, Cannon & Simmons 2002). *Aspergillus* sp. and *Penicillum* sp. were isolated from *V. negundo* as endophytes, and the same have been reported on other hosts (Aline et al. 2012, Maheshwari & Rajagopal 2013).

Endophytes were isolated form *V. negundo* and genomic DNA isolated and amplified using ITS primers. The PCR products were sequenced and the sequence obtained from each isolate was subjected to BLAST search. The numbers at each branch point represent percentage bootstrap support calculated from 1000 replicates using MEGA software version 6.05 (online). GenBank accession numbers underlined represent endophytes obtained from *V. negundo*

The Shannon-Wiener diversity index was high in twigs (Shannon diversity: 2.48, Simpson's diversity: 0.10, Species richness: 17), followed by bark and leaf. Sunayana & Prakash (2012) have also reported similar results from diversity studies on endophytes of the *B. serrata.* Higher Shannon-Wiener diversity indices for endophytic fungi in twigs than in leaves of six medicinal plants have been reported (Kumar & Hyde 2004, Sun et al. 2008).

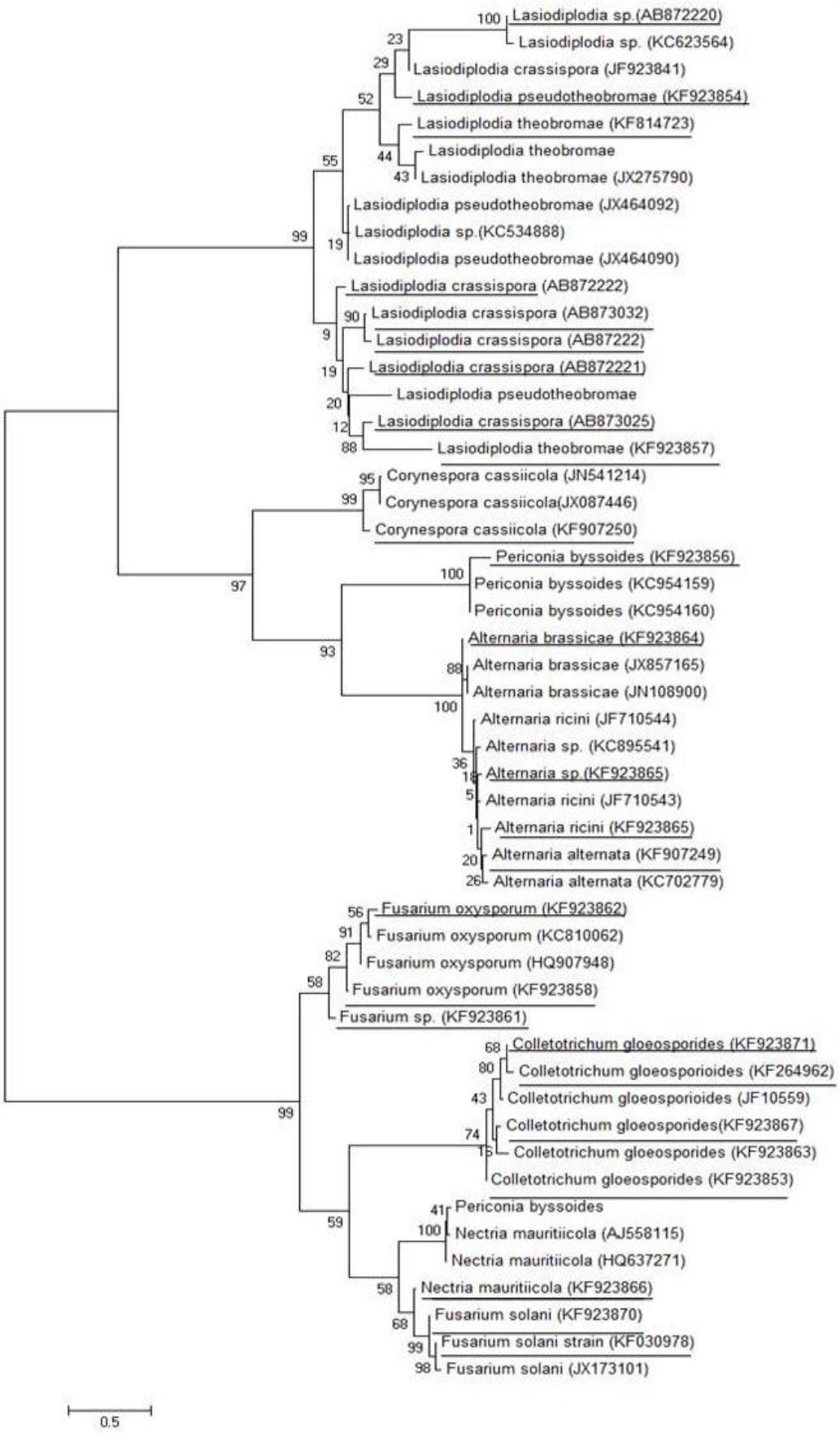

Fig. 3 – Phylogenetic tree inferred from the nearest neighbour of fungal endophytes presented in this study, based on the maximum parsimony method.

The molecular identification, of endophytes based on the sequence analysis showed that most of the endophytic fungi in *V. negundo* belonged to seven genera, such as *Alternaria, Periconia, Lasiodiplodia, Colletotrichum, Corynespora, Nectria* and *Fusarium.* It possessed identical sequence alignments from the retrieved neighbour sequences study. Sim et al. (2010) in their study on the endophytic fungi of *G. mangostana* and *G. parvifolia*, characterized them using ITS, and 10 genera were retrieved into two main clusters of phylogenetic tree.

The present investigation, gives an insight into the fungal endophytes associated with the medicinal plant, *V. negundo* and their diversity. The distribution and composition of endophytic fungi were noticeably affected by hosts and tissues. In recent years interest in fungal endophytes is largely generated due to the diversity of bioactives they are able to produce, and finds use in therapeutics. The isolation and characterization of the bioactive compounds from selected endophytic fungi is under progress.

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