Endophyte fungal diversity in *Nothapodytes nimmoniana* along its distributional gradient in the Western Ghats, India: are camptothecine (anticancer alkaloid) producing endophytes restricted to specific clades?

Singh Shweta[1,2,5,6], B. R. Gurumurthy[3], M. M. Vasanthakumari[1], G. Ravikanth[4], S. Dayanandan[5,6], R. Storms[5], M. B. Shivanna[2] and R. Uma Shaanker[1,4,*]

[1]School of Ecology and Conservation and Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bengaluru 560 065, India
[2]Department of Applied Botany, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shivamogga 577 451, India
[3]Department of Crop Physiology, University of Agricultural and Horticultural Sciences, Shivamogga 577 201, India
[4]Ashoka Trust for Research in Ecology and the Environment, Royal Enclave, Srirampura, Jakkur PO, Bengaluru 560 064, India
[5]Centre for Structural and Functional Genomics and Biology Department, Concordia University, 7141 Sherbrooke West, Montreal, Quebec, H4B 1R6, Canada
[6]Quebec Centre for Biodiversity Sciences, 1205, Dr Penfield Avenue, Montreal, Quebec, Canada

***Nothapodytes nimmoniana* Graham (Icacinaceae) is a small tree distributed along a 1600 km mountain range in the Western Ghats biodiversity hotspot in southern India. The stem wood of *N. nimmoniana* accumulates high concentration (about 0.3% by dry weight) of the anti-cancer alkaloid, camptothecine (CPT). Several endophytic fungi isolated from this plant have been shown to produce camptothecine *in vitro*. In this study, we examined the diversity and distribution of fungal endophytes of *N. nimmoniana* along its entire distributional range in the Western Ghats and investigated if the CPT-producing endophytes are restricted to any specific clade. The leaf and stem of *N. nimmoniana* were sampled from 18 sites along the Western Ghats spanning 8–18°N lat. Endophytes were recovered from all sites with the colonization frequency ranging from 0% to 52% across the sites. One hundred and four endophytic fungal isolates were recovered from 118 plants and characterized both morphologically and by sequencing the internally transcribed spacer region of the nuclear rDNA gene. The fungal isolates belonged to 45 species (44 Ascomycetes and one Basidiomycetes). *Fusarium* and *Hypoxylon* were the most predominant genera comprising over half of the total isolates. Interestingly, CPT-producing endophytes were not restricted to any specific clade. We discuss these results in the context of the growing interest in endophytic fungi as possible alternative sources of plant secondary metabolites.**

K**eywords:** Camptothecine, endophytic fungi, fungal diversity and distribution, *Nothapodytes nimmoniana*.

Introduction

NOTHAPODYTES NIMMONIANA Graham, formerly known as *Nothapodytes foetida* Sleumer and *Mappia foetida* Meirs, is a small tree of the family Icacinaceae and is commonly referred to as 'stinking tree'. It is distributed throughout the Western Ghats in southern India[1] and in Sri Lanka, Myanmar, Indonesia and Thailand. In recent years, *N. nimmoniana* has gained considerable interest as it is reported to contain a high concentration (about 0.3% on a dry wt basis) of the quinolone alkaloid, camptothecine (CPT)[2]. CPT is a potent inhibitor of eukaryotic topoisomerase I, and is the third largest anti-cancer drug. Several semi-synthetic derivatives of CPT are in clinical use against ovarian, small lung and refractory ovarian cancers[3,4]. In India, extensive extraction of *N. nimmoniana* from naturally occurring populations in the Western Ghats has resulted in considerable decline of the trees[1,5]. In order to reduce the pressure on the natural populations of *N. nimmoniana*, efforts have been made to identify alternative plant and endophytic sources of camptothecine[6–9].

Puri *et al.*[10] isolated an endophytic fungus, *Entrophospora infrequens* from *N. foetida* (synonym of *N. nimmoniana*) and demonstrated the production of CPT by the fungus *in vitro*. Later, Gurudatt *et al.*[11] reported 26 endophytic fungi from the inner bark tissue of 15 individuals of *N. nimmoniana* that produced camptothecine in

*For correspondence. (e-mail: umashaanker@gmail.com)

culture, independent of the host tissue. Seventeen of these were identified using nucleotide sequences of the internally transcribed spacer (ITS) region of the nuclear rDNA gene. Twelve fungal species belonged to the genus *Fusarium*, while the remaining five belonged to the genera *Diaporthe*, *Irpex*, *Botryosphaeria* and *Galactomyces*. These results suggest that the ability to produce CPT, a plant-specific secondary metabolite, is shared by a wide diversity of endophytes. This feature is mirrored by another well-studied plant secondary metabolite, taxol, also produced by endophytes. To date, a number of endophytic fungi from a variety of plant species have been shown to produce taxol in culture, independent of the plant host[12,13]. These studies have raised interesting yet challenging questions both at the proximate level, about the possible mechanisms through which the endophytes are able to synthesize the host specific compounds, and at the ultimate level, about their possible evolutionary significance[14].

In the present study, we examine the diversity and distribution of endophytic fungi associated with *N. nimmoniana* covering its entire geographical distributional range in the Western Ghats, India. Specifically, we address the following questions: (a) What is the endophytic fungal diversity in *N. nimmoniana* across its distribution range and how does this compare with intra-specific endophytic fungal diversity reported for other species in the same region? (b) Is there a significant turnover in endophytic fungal diversity in relation to the geographical region or latitudinal gradient of collections? (c) Are there significant differences in the endophytic fungal isolates from leaves and stems of *N. nimmoniana*? (d) Are endophytic fungal isolates producing camptothecine restricted to any specific clade or are they widespread? We discuss the results in the light of the growing worldwide interest in endophytes as possible alternative sources of plant secondary metabolites. We specifically discuss, how, apparently diverse fungal endophytes could produce CPT in culture, independent of the host.

Materials and methods

Study sites and sample collection

Samples of leaves and bark from the stem of *N. nimmoniana* trees ($N = 118$ individuals) were collected from 18 sites/populations located in 6 regions – southern Kerala (SKE), northern Kerala (NKE), southern Karnataka (SKA), Central Karnataka (CKA), northern Karnataka (NKA) and Maharashtra (MAH) along the Western Ghats region distributed across a latitudinal range 8–18°N (Figure 1, Table 1). The Western Ghats is a 1600 km long mountain chain running parallel to the west coast of India and is one of the 34 biodiversity hotspots of the world[15]. *N. nimmoniana* trees are distributed all along the Western Ghats (from 8–18°N). The sampling was designed to cover the entire distributional range of the species. Each sampled tree was assigned a unique identification number and details of the sample: tissue collected, name of the site, and latitude and longitude of the collection site were recorded in a registry maintained at the School of Ecology and Conservation, University of Agricultural Sciences, GKVK, Bengaluru, India. For the collection of stem bark samples, the outer bark at breast height was scrapped using a knife and a section of 2 cm thick inner bark (5 cm × 5 cm) was collected into a sterile plastic bag and sealed. Leaf samples were also collected and sealed in plastic bags and transported to the laboratory within 48 h and stored at 4°C.

Isolation of endophytic fungi

The endophytic fungi were isolated from stem bark and leaf samples following Shweta *et al.*[16]. In the case of leaf, 10–15 segments (including mid vein of the lamina) of approximately 0.5 cm^2 were excised at random from 4 or 5 leaves using a pair of sterile scissors. Similar procedure was followed for obtaining sections from the stem bark. The segments were thoroughly washed with distilled water, surface disinfected by immersion in ethanol (70%, v/v) for 1 min followed by immersion in NaOCl (5%) for 5 min, and then rinsed three times for 60 sec each with sterile demineralized water. The surface disinfected tissue segments were incubated on petri plates with agar (1.5% agar, HiMedia, Mumbai) amended with streptomycin (200 μg/ ml) at 28 ± 2°C, until fungal growth emerged at the cut ends. When the hyphae grew to a length of about 2 cm, single hyphal tips were isolated and subcultured on potato dextrose agar (PDA, HiMedia, Bengaluru) and incubated at 28 ± 2°C. A serial subculturing was performed to obtain a pure culture. Fungal isolates were stored in their vegetative form as slants and in 50% (v/v) glycerol at –8°C. Based on the emergence of fungal growth, the per cent colonization frequency was computed as the ratio of the number of tissue segments showing emergence to the total number of tissue segments incubated[17]. To check for the effectiveness of sterilization, surface impressions of the surface disinfected leaf/stem segments were made on the PDA plates and incubated at 28°C for 96 h. The plates were monitored for fungal growth.

Fungal identification using spore morphology and DNA sequences of rDNA

The fungal mycelia emerging from tissue segments were picked from the growing margin with a fine-tipped sterile needle, transferred to fresh PDA plates and incubated for 5 or 6 days. Isolates of sporulating fungi were identified to the genus and species levels where possible, based on

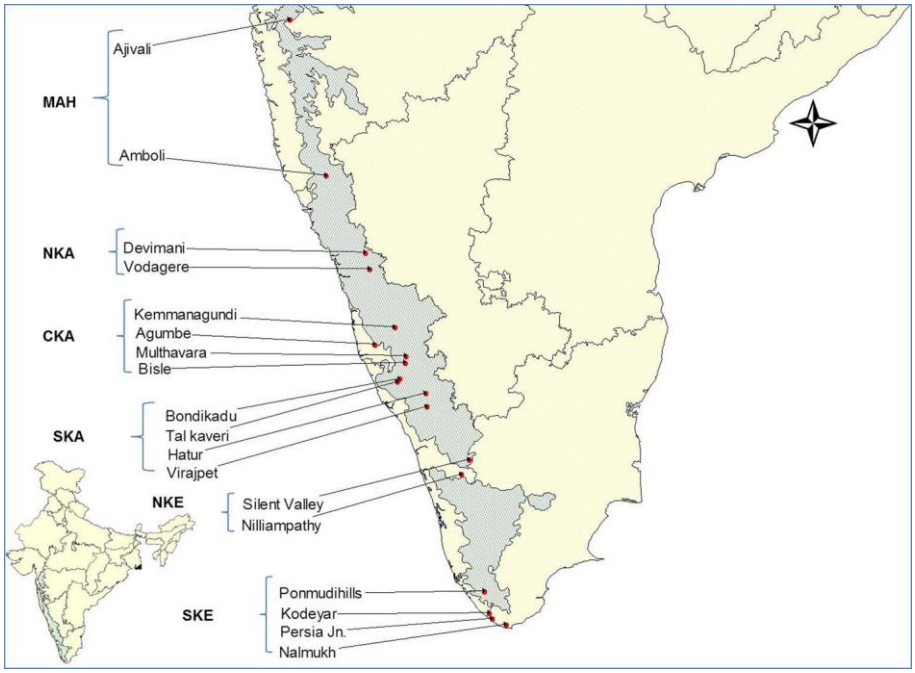

Figure 1. Sampling sites (dots) of *Nothapodytes nimmoniana* in the Western Ghats (shaded region), India. SKE, Southern Kerala, NKE, Northern Kerala, SKA, Southern Karnataka, CKA, Central Karnataka, NKA, Northern Karnataka, and MAH, Maharashtra.

Table 1. Colonization frequency (CF) of endophytic fungi in leaf and stem segments of *Nothapodytes nimmoniana* from different locations in the Western Ghats, India

Site	Location	Latitude (°N)	Longitude (°E)	Number of plants	Total segments	Leaf isolates	Stem isolates	CF in leaf (%)	CF in stem (%)
Nalmukh	SKE	8.15	77.45	3	12	0	3	0	37.5
Persia Junction	SKE	8.25	77.20	1	5	0	1	0	33.333
Kodeyar	SKE	8.35	77.15	5	26	0	2	0	11.765
Ponmudihills	SKE	8.73	77.06	8	80	4	6	14.286	11.538
Nilliampathy	NKE	10.783	76.65	6	36	2	2	16.667	8.333
Silent Valley	NKE	11.04	76.79	7	38	1	3	7.692	12
Virajpet	SKA	11.968	76.03	9	72	9	6	36	12.765
Hatur	SKA	12.201	76.00	7	28	3	5	30	27.777
Tal kaveri	SKA	12.201	76.00	2	15	0	1	0	10
Bondikaudu	SKA	12.4	75.50	1	8	0	1	0	20
Bisle	CKA	12.45	75.54	8	30	0	4	0	20
Multhavara	CKA	12.732	75.64	4	15	2	0	40	0
Agumbe	CKA	12.85	75.65	5	30	3	1	30	5
Kemmanagundi	CKA	13.05	75.09	12	65	3	9	13.043	23.809
Vadagere	NKA	13.35	75.45	10	45	2	3	12.5	10.345
Devimani	NKA	14.37	75.00	6	25	0	3	0	18.750
Ajivali	MH	14.66	74.92	19	50	9	12	52.941	36.343
Amboli	MH	16.004	74.22	5	20	2	1	28.571	7.692
Total				118	600	40	64	19.230	16.326

SKE, Southern Kerala; NKE, Northern Kerala; SKA, Southern Karnataka; CKA, Central Karnataka; NKA, Northern Karnataka and MAH, Maharashtra.

the culture characteristics, morphology of the fruiting bodies and characteristics of the conidia/spores following standard mycological manuals[18–22]. Isolates of non-sporulating fungi were classified based on their cultural and hyphal characteristics. The identities of the fungi were further confirmed by sequencing the *ITS* of the nuclear rDNA region of the genome.

Each endophytic fungal isolate was cultured in potato dextrose broth (PDB) at 25°C in flasks at 250 rpm for 7 days in a rotary shaker. The fungal mycelia were

freeze-dried and the genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method[23]. About 500 mg of fungal mycelia were crushed in liquid nitrogen to obtain a fine powder. The powdered mycelial mass was added to 10 ml extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.7 M NaCl, 2% cetrimide, 1% SDS and 50 µl β-mercaptoethanol), mixed gently and incubated at 65°C for half an hour with continuous shaking. The lysate was extracted and added with an equal volume of chloroform/isoamylalcohol (24 : 1) and centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was transferred to a new tube and the genomic DNA was precipitated with 2 volumes of chilled isopropanol and centrifuged at 4°C for 10 min at 10,000 rpm. The resulting pellet was washed twice with 70% ethanol, air-dried and dissolved in 20 µl of sterile Millipore water. The *ITS* region of each DNA sample was amplified with *ITS*1 and *ITS*4 primers following protocols described previously[24]. The PCR conditions used were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 15 sec, 50°C for 1 min, 72°C for 45 sec, and a final extension at 72°C for 7 min. The 50 µl reaction mixture contained 1× PCR buffer, each dNTP (200 µM), 1.5 mM $MgCl_2$, 10 pmol of each primer (Sigma life sciences, USA), 1–5 ng DNA and 2.0 units *Taq* DNA polymerase. The PCR fragments were sequenced at the McGill University and Génome Québec Innovation Centre, Montreal, Canada.

All sequences were analysed using the Basic Local Alignment Search Tool (BLAST) at the NCBI GenBank. The BLAST analysis was performed with full-length ITS1 and ITS2 sequences as queries to identify similar nucleotide sequences in the fungal nucleotide sequence database at the NCBI[25,26]. The sequences were matched with type strain sequences and/or the reference sequences obtained from NCBI, ITS2, UNITE, ENA, Staininfo, Mycobank and Cbs-Knaw (NCBI, http://ncbi.nlm.nih.gov/ (last accessed on 1 February 2014); ITS2 database, http://its2-old.bioapps.biozentrum.uni-wuerzburg.de (last accessed on 1 February 2014); unite, http://unite.ut.ee/ (last accessed on 1 February 2014); ENA, http://www.ebi.ac.uk/ena/ (last accessed on 3 February 2014); staininfo, http://www.staininfo.net (last accessed on 1 February 2014); Mycobank, http://www.Mycobank (last accessed on 3 February 2014); cbs-knaw, http://www.cbs.knaw.nl/ (last accessed on 3 February 2014)). The sequence with the highest homology, maximum query coverage and maximum score was used as a reference to assign the identity of the endophytic fungus[27,28]. The genus and species of the database match were accepted whenever the identity of the sequence of the study and that of the database *ITS* was greater than or equal to 97%; only the genus was accepted when the identity of the database match was greater than or equal to 95–96%, and when the similarity was less than 95%, such isolates were considered unidentified. All *ITS* sequences of fungal species were deposited in the GenBank (see Table S1, Supplementary material online).

Phylogenetic analysis

The nucleotide sequences were edited using BioEdit v7.1.11 (ref. 29; http://www.mbio.ncsu.edu/bioedit/bioedit.html) and then aligned using the MUSCLE software[30]. The 50% majority rule consensus phylogenetic tree was reconstructed using Bayesian approach as implemented in MrBayes[31]. The most plausible model of sequence evolution was determined using JModelTest[32], which yielded the HKY + G model. Thus, phylogenetic analyses were performed using the HKY + G model. One million iterations of Markov Chain Monte Carlo (MCMC) analysis were performed and initial 25% of trees were discarded. The software program FigTree v1.3.1 (tree.bio.ed.ac.uk/software/figtree) was used for visualization of the resulting phylogenetic trees.

Endophytic fungal diversity analysis

The data based on the endophytic fungal species identified through morphological and ITS-rDNA sequencing were used for fungal diversity analysis. Species richness and species diversity were calculated for all sampling sites; Shannon diversity and beta diversity indices were calculated using PAST version 2.14 (ref. 33). The endophytic fungal diversity was compared across sites and locations, between parts and across other studies[34,35] using Jaccard's similarity index. A principal component analysis (PCA) was carried out to determine the presence of any distinct clustering of the sites of collection with respect to their endophytic fungal assemblages using the PAST version 2.14 software[33]. The rarefaction analysis of endophytic fungal species richness in the stem and leaf was also carried out using the above software[33]. The data were first analysed together for the leaf and stem segments, and then analyses were performed for stem and leaf segments separately. Similarly, the data were analysed separately for sites (N = 18) and locations (N = 6).

Extraction and estimation of camptothecine using HPLC-DAD

Out of the total 104 endophytic fungal isolates collected, 45 isolates belonging to 28 species were randomly chosen and cultured in liquid media[7] for assessment of CPT production. Each culture was prepared by inoculating a single hyphal tip into 50 ml of pre-sterilized PDB medium in a 250 ml conical flask. Flasks were incubated in a rotary shaker at 250 rpm and 28°C for 4 days. After 4 days, the mycelia mass was separated from the broth, washed in sterile water and dried (5% moisture content) in a

hot-air oven at 60°C for 4 days. The dried mycelial mass was crushed to a fine powder using a sterile pestle and mortar. The powdered mycelial mass was then transferred to 15 ml vials and extracted in 10 ml of 61% ethanol at 60°C for 3 h in a shaking water bath. After cooling to room temperature, the extract was centrifuged at 10,000 rpm (Eppendorf, Germany) for 10 min at 10°C.

The supernatant was filtered through a 0.2 µm membrane filter (Tarsons, India) and analysed for the presence of CPT using RP-HPLC separation performed on a (Shimadzu, Japan) liquid chromatography system equipped with a LC-20AD solvent delivery system (Shimadzu, Japan), a SPD-M 20A photo diode array detector (Shimadzu, Japan), a SIL-20ACHT injector with 50 µl loop volume and a Luna C18, 250 mm × 4.6 mm ID, 5 µm pore size column (Phenomenex, USA).

Chromatographic separations were performed using an injection volume of 20 µl, a mixture of 25% acetonitrile (pump A) and 75% water + 0.1% trifluro-acetic acid (pump B) as the mobile phase, a flow rate of 1.5 ml min^{-1}, with detection at 240 nm and a run time of 40 min. The presence of CPT in the experimental samples was detected by comparing the retention time with that of the standard (see Figure S1, Supplementary material online).

Liquid chromatography-mass spectrometry analysis

The presence of CPT in the samples was further confirmed by LC-ESI-MS analysis (LCMS-2020, Shimadzu, Japan). The LC analysis was coupled to the ion trap mass analyser. The mass analyser was equipped with atmospheric pressure ionization source electrospray ionization (ESI). High-purity nitrogen from a nitrogen generator was used as a carrier gas. LC conditions for the analysis were as follows: RPC-C18, 250 mm × 4.6 mm, 5 µm size (Phenomenex), detector: UV-visible, wavelength: 254 nm, flow rate: 0.2 ml/min, injection volume: 5 µl, mobile phase: pump A: 25% acetonitrile and pump B: 75% water in an isocratic mode. The total analysis run time was 40 min (see Figure S2, Supplementary material online). The conditions for mass spectrum during the analysis were set as follows: dry gas flow rate 10 l/min, nebulizer pressure 35 psi, nebulising gas flow 1.5 l/min, DL temperature of 250°C and mass range was from 100 to 700 *m/z*. The presence of CPT in the samples was also confirmed by ESI-MS/MS (Thermo Scientific, San Jose, CA), operating in the positive ion mode. Instrumental parameters for the mass analyser were as follows: 110 psi nebulizer gas (N_2) pressure, and 5 kV spray voltage on an ion trap LTQ XL mass spectrometer, flow rate 5.0 µl/min, damping gas: helium at 1.0 ml/min approx. Applying these conditions, the data acquisition was accomplished using Xcalibur software for CPT and its derivatives.

Results and Discussion

Endophytic fungal diversity in N. nimmoniana across its distribution range

The colonization frequency ranged from 0% to 52.94% (for leaf segments) and 0% to 37.5% (for stem segments; Table 1). The overall mean colonization frequency from leaf was 19.23% compared to 16.32% from stem segments (Table 1). Ajivali in the northern region of the Western Ghats had the highest colonization frequency for leaf segments (52.94%) and Nalmukh in southern Kerala had the highest colonization frequency for stem segments (37.5%; Table 1 and Figure 2). Among the different regions, the colonization frequency was highest in Maharashtra (30%) followed by southern Karnataka (18.6%) and Central Karnataka (17.1%). The colonization frequency recorded in this study appears to be far less than that reported in several other species[36,37]. Gamboa and Bayman[38] reported up to 95–98% of leaf fragments of *Guarea guidonia* in Puerto Rico to be colonized by endophytes but only 21–30% of samples of *Euterpe oleracea* in Brazil yielded culturable endophytes[39]. Cannon and

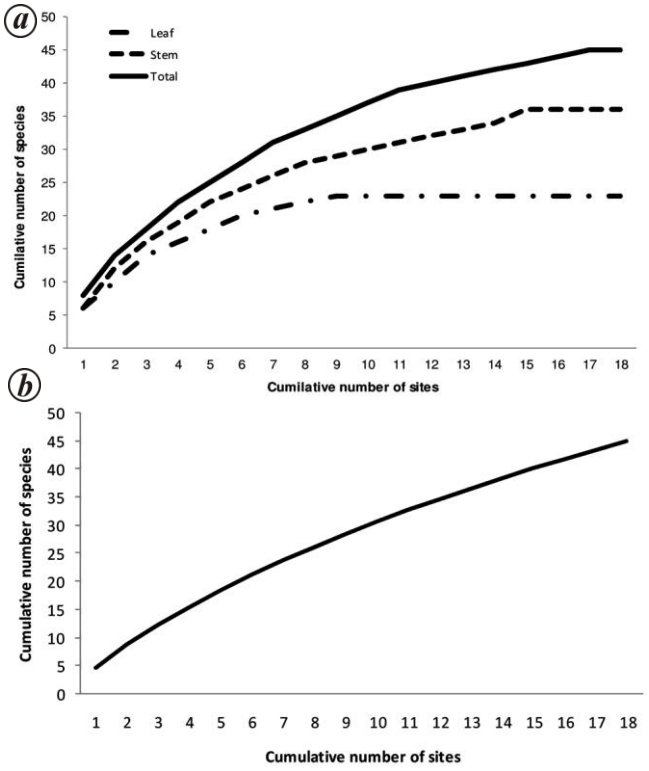

Figure 2. ***a***, Cumulative number of fungal species as a function of the number of collections. Solid line indicates the species accumulation curve for both the stem and the leaf community; continuous dashed line indicates the species accumulation curve for stem community, and the dashed and dotted line indicates species accumulation curve for the leaf community. ***b***, Rarefaction curve of the number of endophytic species obtained from *N. nimmoniana* across 18 sites in the Western Ghats, India.

Table 2. Colonization frequency, species richness and Shannon diversity of endophytic fungi in *N. nimmoniana* from different locations in the Western Ghats, India

		Species richness		Shannon diversity	
Site	Location	Site	Location	Site	Location
Nalmukh	SKE	2	14	0.6931	2.639
Persia junction		1		0	
Kodeyar		3		1.099	
Ponmudihills		10		2.303	
Nilliampathy	NKE	4	7	1.386	1.946
Silent Valley		4		1.386	
Virajpet	SKA	11	15	2.398	2.485
Hatur		5		1.609	
Talkaveri		1		0	
Bondikaudu		1		0	
Bisle	CKA	5	17	1.609	2.833
Multhavara		2		0.6931	
Agumbe		4		1.386	
Kemmanagundi		11		2.398	
Vadagere	NKA	5	7	1.609	1.792
Devimani		3		1.099	
Ajivali	MH	10	10	2.303	2.485
Amboli		3		1.099	

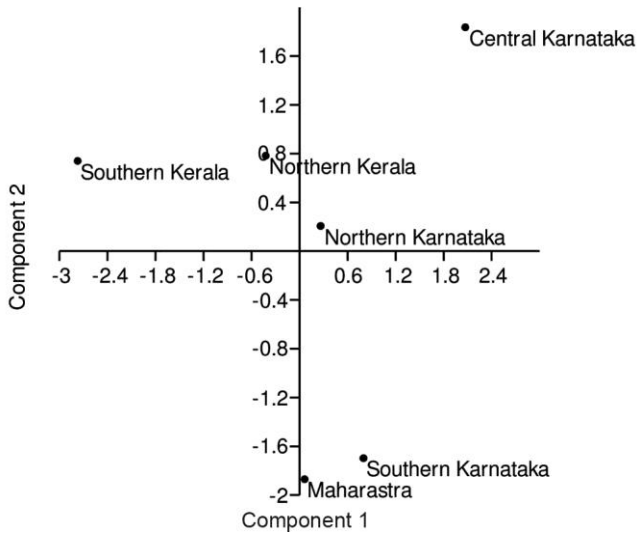

Figure 3. Principal component analysis (PCA) of fungal endophytes isolated from different tissues of *N. nimmoniana* across different locations in the Western Ghats. The first two components explain 58% of the total variation.

Simmons[40] suggested that the comparison between studies might not always be appropriate considering the differences in protocols used across studies. The stringent surface-sterilization method (mainly to avoid epiphytic fungi) employed in the study might have reduced the fungal load. However, significant under-sampling is highly unlikely since the obtained colonization frequency ranged from 0% to 52%, which is comparable to those reported elsewhere. Yet another reason for the apparently poor colonization frequency could be due to the fact that both stems and leaves of the tree produce CPT, which has been shown to be anti-fungal[9,41].

Using both morphological keys and nucleotide sequences of the *ITS*-rDNA, the 104 isolates obtained were assigned to 45 species belongs to 24 genera and 10 orders (see Table S1, Supplementary material online). The cumulative number of endophytes as a function of the number of collections from both the leaf and the stem segments reached an asymptote (Figure 2 *a*). The curve representing the accumulation of species in the stem was steeper compared to that from the leaf (Figure 2 *a*). The species accumulation curve revealed that while 64 endophyte isolates from stem segments were grouped into 36 species, the 40 isolates from leaf segments were grouped into 23 species (Figure 2 *a*). However, rarefaction analysis of the fungal species over 18 sites did not reach an asymptote, indicating that there is a possibility of recovering more fungal species from *N. nimmoniana* should more sampling be done (Figure 2 *b*). Highest number of species occurred in Virajpet and Kemmanagundi (11 species) followed by Ajivali and Ponmudi Hills (10 species each; Table 2). The Shannon diversity index was highest for tissue segments from Virajpet and Kemmanagundi sites (Table 2). Among regions, Central Karnataka had the highest species richness (17) followed by southern Karnataka (15) and southern Kerala (14). A considerably fewer number of species occurred in the northern Karnataka and northern Kerala regions (7). Principal Component Analysis (PCA) of the endophytic fungal species composition indicated a separation based on the location. Only the endophytic fungal species from southern Karnataka and Maharashtra clustered together. The first three axes of the PCA explained 73% of the total variation (Figure 3).

Interestingly, Suryanarayanan *et al.*[42], from a 10-year study on 75 dicotyledonous tree species belonging to 33

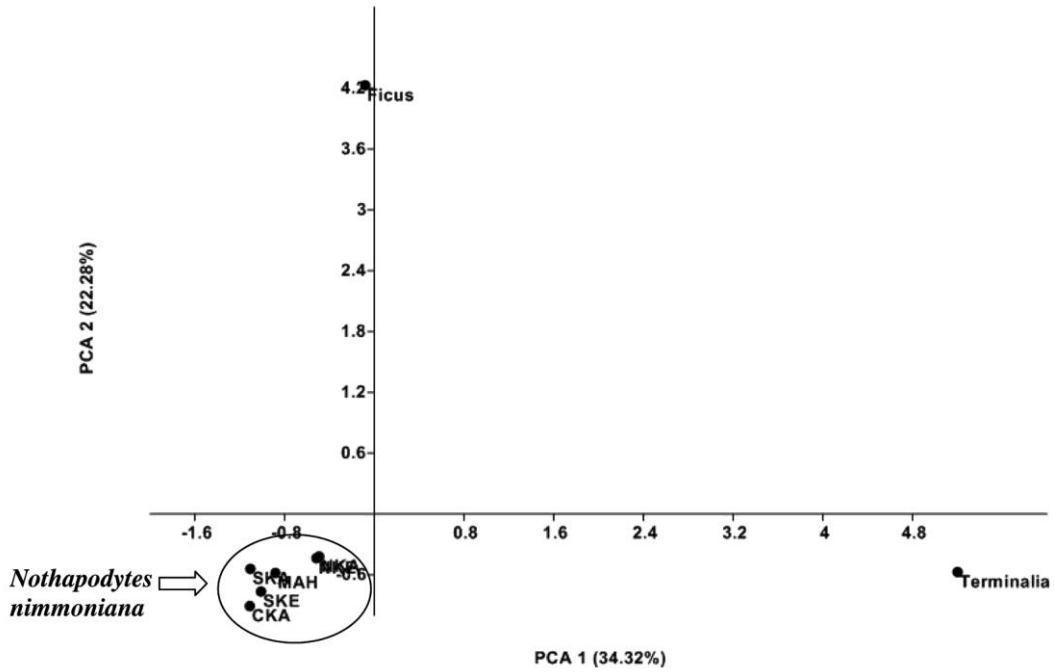

Figure 4. PCA of fungal endophytes isolated from different tissues of *N. nimmoniana* (present study), *Terminala arjuna* (Tejesvi *et al.*[35]) and *Ficus benghalensis* (Suryanarayan and Vijaykrishna[44]) across different locations in Southern India.

families in 3 different forest types of the Western Ghats, concluded that endophyte diversity in the region was not high due to a non-specific host affiliation of several endophyte species. In a previous study involving dry thorn and dry-deciduous forests of South India, Murali *et al.*[43] reported that sampling 15 tree species could capture 95% of the endophyte species diversity in these plant communities. However, compared to these studies and notwithstanding the fact that the asymptote was not reached, the absolute endophyte count in the present study was substantially higher. This could be attributed to the fact that the present study covers a nearly complete natural distribution range of *N. nimmoniana*, whereas most other studies were restricted to a narrow geographic area.

The intra-specific endophytic species assemblages of *N. nimmoniana* were compared with those of *Ficus benghalensis* and *Terminalia arjuna*, the only other species that have been investigated in southern India[31,44]. The PCA indicated that the endophytic fungal species assemblages were completely different across these species (Figure 4). This analysis supports the view that tropical plant species harbour a rich diversity of endophytes and that the endophyte species assemblages associated with different species are different. This is intriguing since sampling of a large number of tree species for their foliar endophytes in the Western Ghats by Suryanarayanan *et al.*[42] indicated a low species diversity. Clearly more studies are needed to determine the influence of host plant distribution on endophyte diversity.

Among the 45 species identified through molecular analysis, 44 belonged to Ascomycetes and their anamorphs, and only one to Basidiomycetes, a feature earlier reported by Rungjindamai *et al.*[45] (Figure 5). Most species belonged to the order Hypocreales (33.3%) followed by Xylariales (13.3%) and Botryosphaeriales (11.1%, Figure 3). About 60% of isolates belonged to *Fusarium* and *Hypoxylon* genera (Figure 3). Many of the genera found in this study (for example *Alternaria*, *Botryosphaeria*, *Colletotrichum*, *Fusarium*, *Trichoderma*, *Penicillium* and *Xylaria*; see Table S1, Supplementary material online) are similar to the genera reported in other studies[40,46-49]. However, several other genera that are not commonly isolated from other plants such as *Phialemonium*, *Phanerochaete*, *Irpex* and *Galactomyces* were also detected.

Endophytic fungal diversity in leaves and stems

Previous studies have shown a distinct association of endophytic fungal species based on the tissue of origin[50,51]. In the present study, differences in the endophytic fungal assemblage between leaf and stem bark tissues were detected. Out of the 45 endophytic fungal species recovered, 14 were from both stem and leaf samples, 9 were only from leaf samples and 22 were from stem segments (see Table S1, Supplementary material online). The species isolated from both stems and leaves belonged to genera *Botryosphaeria*, *Corynespora*, *Fusarium*, *Hypoxylon*, *Phomopsis* and *Lasiodiplodia* (see Table S1, Supplementary material online). The two different parts of the tree were dominated by different endophytic species. In the case of stems, *Fusarium* was the dominant genus

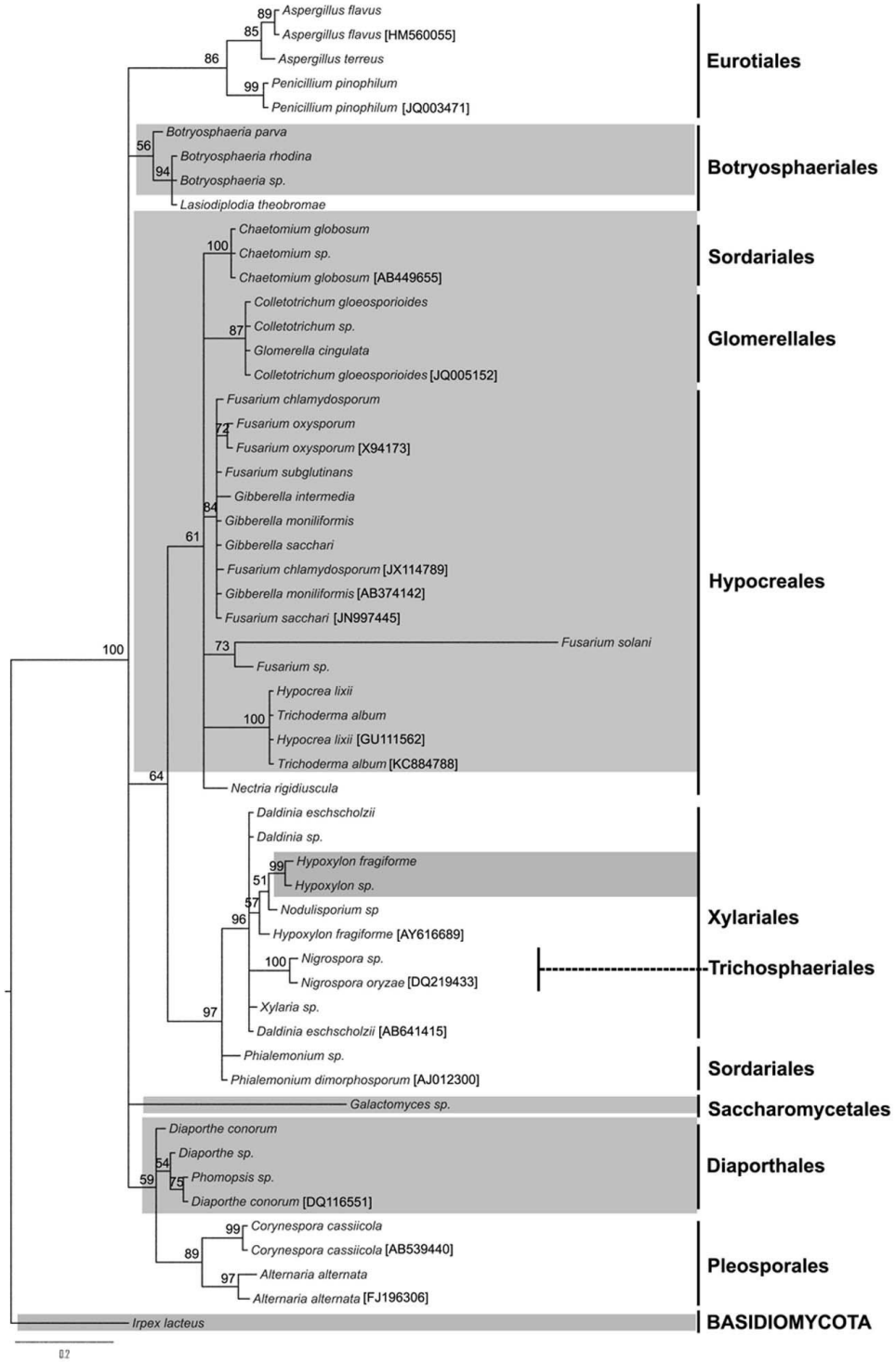

Figure 5. The 50% majority rule consensus tree obtained based on Bayesian analyses of the nucleotide sequences of *ITS*2 region of the nuclear rDNA of endophytic fungal isolates obtained from *N. nimmoniana*. Species (n = 25) within the grey shaded area with the exception of the reference sequences (indicated by the GenBank numbers) were screened and found positive for camptothecine (CPT) in liquid culture; three species which were positive for CPT are not shown here due to sequencing errors. For the rest of the species outside the grey shaded area, CPT was not determined. The accession numbers assigned to the species of the present study are given in Table S1 (see Supplementary material online).

followed by *Botryosphaeria*, whereas in the leaf samples, *Hypoxylon* was the dominant genus followed by *Fusarium*. The Jaccard's similarity index between endophytic fungal species of leaf and stem segments was 0.325. The species composition and frequency of endophytes are known to vary with different tissues of host plants[39,52]. Plant organs may represent distinct microhabitats with reference to fungal endophyte infections and therefore the

presence of distinct endophytes in different parts is expected[53]. Studies have suggested that leaf samples harbour a greater number of species since leaves appear to have less infection barriers compared to the sapwood[54]; however, in the present study higher species richness was found from the inner bark tissue. This could be due to the fact that stems are persistent over a long period of time, whereas deciduous leaves of *N. nimmoniana* are short-lived providing a relatively short period for fungal colonization. Li *et al.*[55] also reported a higher colonization frequency in the stem compared to the leaf in the case of five dominant but deciduous species in the Baima Snow Mountains in Southwest China.

Turnover of endophytic fungal species across locations

Since most studies on intra-specific endophytic diversity are limited to narrow geographical regions[35,44], endophytic fungal diversity turnover patterns across populations are not clearly known. In the present study, distinct turnover patterns of the endophytic fungal species composition across populations were noticeable. The beta diversity was high, indicating a low similarity of endophytic species composition between locations (Table 3). Such variation in the geographical distribution of the endophytic fungal species in two sympatric coastal grasses has also been reported[56]. Although not statistically significant, an inverse relationship between the similarity of the endophytic species assemblages and the distance between locations was found in the present study. There have been many reports of an inverse relationship with respect to the similarity of endophytic assemblages and distances between locations[48,57]. The location-specific assemblages of endophyte species documented in the present study suggest that specific microclimate in a location could also influence the species composition of the endophytes.

Are CPT producing endophytes restricted to specific clades?

The phylogenetic tree based on the Bayesian analyses of the *ITS* nucleotide sequences revealed five major clades, and *Alternaria* and *Corynespora* occupied a basal position (Figure 5). One of the clades comprised a single species, *Irpex lacteus* of Basidiomycota. Among the remaining four clades, the largest clade comprised species of the genera *Chaetomium*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Galactomyces*, *Gibberella*, *Glomerella*, *Hypocrea*, *Nectria*, *Phomopsis* and *Trichoderma*. One of the remaining three clades comprised of species of *Botrosphaeria* and *Lasiodiplodia*, while the other clade contained species of *Aspergillus* and *Penicillium*. The fifth clade consisted of *Daldinia*, *Hypoxylon*, *Nigospora*, *Nodulisoposium*, *Phialemonium* and *Xylaria*. All 45 isolates belonging to 28 species produced CPT in liquid culture (see Figures S1 and S2, Supplementary material online). These fungi represented all orders, except Pleosporales and Eurotiales. Thus the CPT-producing ability of the endophytic fungal species was not restricted to any specific clade (Figure 5). Eighty five percentage of all the endophytic fungal species that are known to produce CPT[7,10,11,16,58,59], including endophytes isolated from *N. nimmoniana* and related plant species in families Icacinaceae and Nyssaceae, were recovered in the present study. These results, apart from indicating that diverse endophytic fungi are able to produce CPT, raise interesting questions on how the fungi are able to do so and what, if any, could be its evolutionary significance.

The biosynthetic pathway of CPT in plants is only partially characterized[60,61]. Strictosidine is considered as the precursor of CPT[62]. Strictosidine is formed by the coupling of secologanin (from the mevalonate pathway) to tryptamine (from the shikimate pathway) in a reaction catalysed by the enzyme strictosidine synthase (*STR*). Orthologs of *STR* and other downstream genes have not been located in any of the 50-odd non-endophytic fungal genomes sequenced thus far. In the only study so far, Kusari *et al.*[63] showed the presence of *TDC* (shikimate pathway) and *G10H* and *SLS* (mevalonate pathway) genes, but not *STR* in the CPT producing endophytic fungus, *Fusarium solani* isolated from *Camptotheca acuminata*[63]. In summary, therefore, the molecular events leading to the synthesis of CPT by the endophytes in culture remain a puzzle. Currently, efforts are being made by several labs to solve this puzzle by whole genome sequencing of endophytes producing CPT. Recently, Sachin *et al.*[14] proposed an interesting and plausible hypothesis that might explain the production of CPT by diverse endophytes.

Table 3. Jaccard's similarity index (lower diagonal) and beta diversity (upper diagonal) of endophytic fungal isolates from *N. nimmoniana* for different locations

Location	Southern Kerala	Northern Kerala	Southern Karnataka	Central Karnataka	Northern Karnataka	Maharastra
Southern Kerala	1	0.80952	0.84615	0.80645	0.9	0.76923
Northern Kerala	0.10526	1	0.89474	0.75	0.84615	0.89474
Southern Karnataka	0.083333	0.055556	1	0.65517	0.88889	0.58333
Central Karnataka	0.10714	0.14286	0.20833	1	0.65217	0.72414
Northern Karnataka	0.052632	0.083333	0.058824	0.21053	1	0.66667
Maharastra	0.13043	0.055556	0.26316	0.16	0.2	1

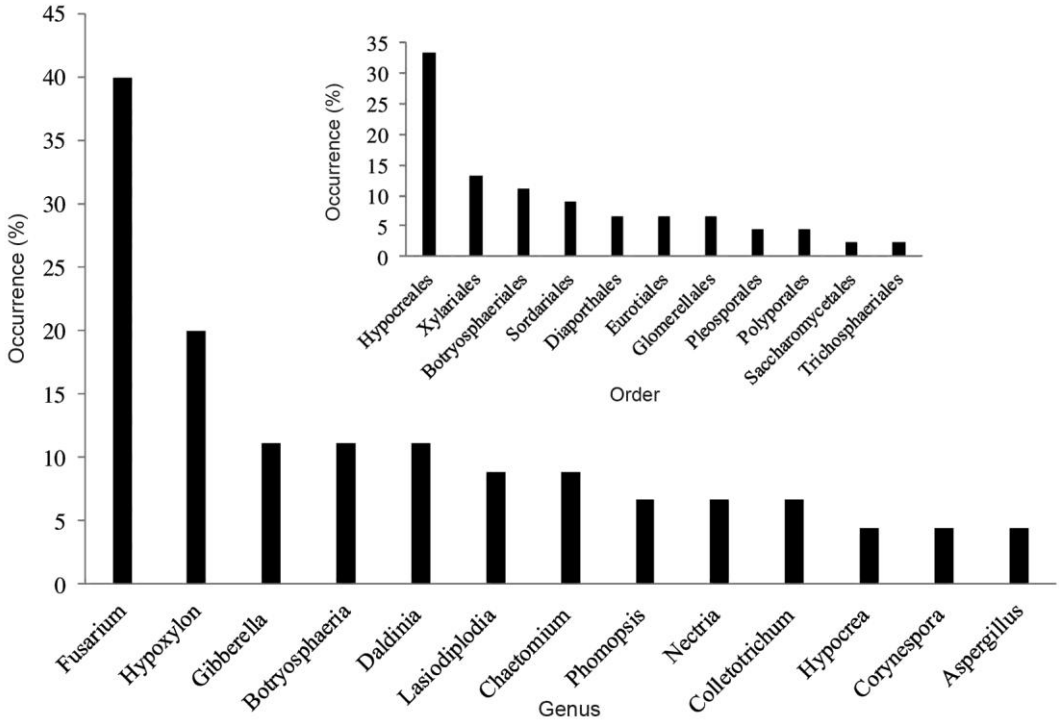

Figure 6. Percentage occurrence of endophytic fungal genera of *N. nimmoniana*. Only genera having three or more species are included. Unidentified fungal species have not been included. (Inset) Percentage occurrence of endophytic fungal species placed under order.

They hypothesized that endophytic fungi may carry critical gene clusters for secondary metabolite synthesis in extra-chromosomal elements (ECEs) or plasmids borne on endophytic bacteria. The genes themselves may have evolved independently or acquired from the host tissue through horizontal gene transfer. Accordingly, any endophyte resident in the host tissue could carry the gene clusters and based on the basic scaffold of shikimate and mevalonate pathways that the fungi already possess to produce CPT. Efforts to examine this hypothesis are underway in our laboratory. Viewed in this context, the production of CPT by the fungi, may be regarded as a metabolic spin-off, so long as the fungi are in the metabolic ecosystem of the host plant. Once outside of the host, the endophytes would be able to continue to produce the plant secondary metabolite, here CPT, so long as the ECEs or plasmids are sustained in the fungi. In fact, it is now well documented that endophytes generally tend to attenuate the production of CPT after a few subcultures[11,16]. In the light of the hypothesis by Sachin *et al.*[14], it is likely that over subculture, endophytes tend to lose the ECEs or plasmids bearing the critical genes responsible for CPT biosynthesis.

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