Hindawi Publishing Corporation
Journal of Mycology
Volume 2013, Article ID 172056, 10 pages
http://dx.doi.org/10.1155/2013/172056

Research Article

Biodiversity, Antimicrobial Potential, and Phylogenetic Placement of an Endophytic *Fusarium oxysporum* NFX 06 Isolated from *Nothapodytes foetida*

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Received 30 September 2013; Revised 31 October 2013; Accepted 5 November 2013

Academic Editor: Simona Nardoni

Biodiversity of endophytic fungi associated with the medicinal plant *Nothapodytes foetida* of Agumbe forest was determined and evaluated for its microbial activity. A total of 170 endophytic isolates were obtained from leaf, stem, seed, and fruit tissues of *Nothapodytes foetida*. The dominant endophytic fungi belong to genera *Fusarium*, *Penicillium*, *Aspergillus*, and *Colletotrichum*. Maximum endophytic isolates were obtained from leaves segments followed by fruit, stem, and seed tissues. Hyphomycetes were the dominant group found with 75.29% over other fungal groups. Shannon-Weiner and Simpson indexes showed rich diversity of endophytic fungi suggesting even and uniform occurrence of various species. 88.57%, 74.28%, 62.85%, and 65.71% of isolates have shown activity against *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 69548), respectively. One of the isolate NFX 06 isolated from leaf has showed considerable antimicrobial activity against all the test pathogens. It was identified as *Fusarium oxysporum* by ITS sequence analysis; the nucleotide sequence was submitted in the GenBank with an accession number KC914432. Phylogenetic relationship confirmed that the strain *F. oxysporum* NFX 06 has evolved from an endophytic ancestor.

1. Introduction

Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effect [1]. Endophytic fungi represent an important and quantifiable component of fungal diversity, with an estimate of at least 1 million species [2–4]. They are found in nearly all plant families including bryophytes [5], pteridophytes [6], gymnosperms [7], and both monocotyledonous [8] and dicotyledonous angiosperms [9, 10]. Endophytic fungal community has been found in plants living in a unique ecosystem [11], which can be a source for a variety of bioactive metabolites such as antibiotics, antimycotics, immunosuppressants, and anticancer compounds [12].

The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens [13–15]. The antimicrobial compounds can be used as drugs and also as food preservatives in the control of food spoilage and foodborne diseases [16]. Thus there is a huge interest across the world to determine the biodiversity of endophytic mycoflora within the host for its novel and undescribed species [17, 18]. In this study an attempt has been made to isolate the endophytic mycoflora of *N. foetida*, to screen for their antimicrobial activity against selected human pathogens and to determine the phylogenetic relationship of *Fusarium* sp. 2 NFX06 with other endophytic and pathogenic isolates of different plant species.

2. Materials and Method

2.1. Study Area and Collection of Plant Material. *N. foetida*, commonly known as "Amrutha" is a medicinal plant that belongs to the family Icacinaceae and it was selected for the study because of its well known pharmacological properties.

Figure 1: *Nothapodytes foetida*.

The plant samples were collected in the month of August from Agumbe forest located at 13°30″N and 75°2″E in the Western Ghats, Dakshina Karnataka, India (Figure 1). The plant material was identified and authenticated by an experienced botanist. Fresh and healthy parts of the plant like leaves, stem, seed, and fruits were cut with a sterile scalpel and stored at 4°C in a sterile polythene bag prior to use.

2.2. Isolation and Identification of Endophytic Fungi. Isolation was carried out as described by Wang et al. [19], with little modifications. Plant samples were surface sterilized with 75% ethanol (1 minute) and 2.5% sodium hypochlorite (3 minutes) followed by washing with sterile distilled water (2 minutes). Each plant sample was cut aseptically into 0.5 cm long segment and placed on petri dishes containing potato dextrose agar (PDA) supplemented with chloramphenicol (50 μg/mL, Sigma) and streptomycin sulphate (250 μg/mL Sigma). The plates were sealed using Parafilm and incubated at 25°C ± 1°C in a light chamber with 12 hours of light followed by 12 hours of dark cycles [20]. The petri dishes were monitored every day to check the growth of endophytic fungal colonies from the plant segments. As and when the hyphal tips emerged out from plant segments they were isolated and subcultured and brought to pure culture by serial subculturing. Identification of fungal endophytes was carried out based on their microscopic and macroscopic characteristics. Lactophenol cotton blue staining method was used for staining the fungal cultures [21] and visualized under microscope (MOTIC BA 400).

2.3. Determination of Species Diversity. Colonization rate (CR) was calculated as the total number of plant tissue segments infected by fungi divided by the total number of segments incubated [22]. Isolation rate (IR) was determined as the number of isolates obtained from plant segments divided by the total number of segments incubated. Colonization frequency (CF %) was calculated as the number of plant segments colonized by a single endophyte divided by the total number of segments observed × 100. Isolation frequency (IF %) was calculated as the total number of isolates of one species divided by the total number of isolates in that sample × 100. Simpson dominance index and Shannon-Wiener's diversity index were calculated for fungal diversity [23, 24].

Simpson's index of diversity was calculated using formula 1–D as given in the following equation:

$$D = \frac{\sum n(n-1)}{N(N-1)}, \tag{1}$$

where n is the total number of organisms of a particular species and N is the total number of organisms of all species.

Shannon-Wiener diversity index was calculated using the following equation:

$$H_s = -\sum_{i=1}^{S} (P_i)(\ln P_i), \tag{2}$$

where H_s is the symbol for the diversity in a sample of S species or kinds, S is the number of species in the sample, P_i is the relative abundance of ith species or kinds measures = n/N, N is the total number of individuals of all kinds, n_i is the number of individuals of ith species, and ln is the log to base 2.

2.4. Fungal Cultivation and Determination of Antimicrobial Activity

2.4.1. Test Microorganisms. The test microorganisms used in this study included three bacterial strains (*S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853)) and one yeast *C. albicans* (ATCC 69548). Inoculum of the test microorganisms was adjusted to a McFarland 0.5 standard in optical density corresponding to 1 to 2 × 10^8 cfu/mL and 3 × 10^8 cfu/mL for bacteria yeasts were used. The concentrations were confirmed via spectrophotometer readings at 580 and 626 nm, respectively. Yeast samples were inoculated on Sabouraud agar (1% peptone, 4% dextrose, and 2% agar), and the bacteria were plated on Brain Heart Infusion agar (Difco) using a swab.

2.4.2. Fermentation. The fungal fermentation was carried out using 100 mL of potato dextrose broth (PDB) in 250 mL of Erlenmeyer flask inoculated with (1 cm × 1 cm) mycelial agar plug taken from an actively growing colony on potato dextrose agar plate. It was then incubated at a temperature of 25° ± 1°C and at 120 rpm under light and dark conditions of 12 hours. After 14 days of fermentation the biomass was separated from the broth by filtration using sterilized preweighed filter cloth.

2.4.3. Antimicrobial Screening of Fungal Endophytes. The antimicrobial activity of the fermentation broth was carried out for the 35 endophytic fungal isolates. The fermented broth was sterilized by filtration with 0.22 μm Millipore syringe filters and 50 μL of the broth were tested by modified agar well diffusion assay as described by NCCLS [25]. The antimicrobial activity was assessed by the diameter (mm) of inhibition zones relative to those of positive and negative

Table 1: Colonization and isolation rate of endophytic fungi from different plant tissues of *N. foetida* collected from Agumbe forest.

	Leaf (NFX)	Stem (NFS)	Seeds (NFB)	Fruit (NFT)	Total
No. of samples	100	50	50	50	250
No. of samples yielding fungi	88	18	12	36	154
No. of isolates	110	24	23	46	170
Colonization rate (CR %)	88	36	24	72	61.6
Isolation rate (IR)	1.1	0.48	0.46	0.92	0.68

controls. Streptomycin and Fluconazole were coassayed as positive antimicrobial references with DMSO as negative control.

2.5. PCR Amplification, DNA Sequencing, and Phylogenetic Analysis of Representative Endophytic Fungi. Polymerase chain reaction was performed to amplify specifically the ITS region of the fungal genome. The fungal ITS regions, including the intervening 5.8 S gene and the flanking ITS1 and ITS2, were amplified with universal primers ITS 4 (5′TCCTCCGCTTATTGATATGC3′) and ITS 5 (5′GGAAGTAAAAGTAACAAGG3′) designed by White et al. [26]. Amplification was performed in a 50 μL reaction mixture containing 28 μL MilliQ Water, 5 μL of 10X Mg free PCR buffer, 2 μL of 25 mM $MgCl_2$, 5 μL of 2.5 mM deoxyribonucleotide triphosphates (dNTPs), 2 μL each of 10 μM primers (ITS4 and ITS5), 2 μL of DNA template, 2 μL of 2.5 units of Taq DNA polymerase, and 2 μL of DMSO. The thermal cycle consisted of 3-minute initial denaturation at 94°C, followed by 35 cycles of 30-second denaturation at 94°C, 30-second primer annealing at 54°C, 1-minute extension at 72°C, and a final 10-minute extension at 72°C. The PCR products were examined by electrophoresis in 1% (w/v) agarose gel with ethidium bromide (10 mg/mL) with a 100-base-pair ladder and checked for size and purity. The purified PCR product was sequenced using the forward and reverse primer in Applied Biosystems 3130 Genetic Analyser.

The consensus sequence obtained was subjected to BLAST search to assign putative identity, designation of operational taxonomic units based on sequence similarity measures, and phylogenetic inference. It was then aligned with other sequences of endophytic and pathogenic *F. oxysporum* downloaded from GenBank to determine their possible evolutionary relationship using MEGA program [27].

3. Results and Discussion

Endophytic fungi are known to be ubiquitous in nature and every plant species examined to date has been found colonized with fungal endophytes. It has been found that a single plant species may harbour hundreds of endophytes and may inhabit all available tissues, including leaves, petioles, stems, twigs, bark, xylem, roots, fruit, flowers, and seeds [28–30].

3.1. Species Diversity. In the present study a total of 170 isolates of endophytic fungi were recovered from 250 samples of leaf, stem, seed, and fruit of ten *N. foetida* trees. The population of Zygomycetes and basidiomycetes were totally absent in this study and usually they are isolated in very low numbers in plant endophyte research as reported by Suryanarayanan et al. [31]. Colonization rates (CR %) of leaf, stem, seed, and fruit were found to be (88.0%, 36.0%, 24.0%, and 72.0%) and isolation rates (IR) of leaf, stem, seed, and fruit were found to be (1.1, 0.48, 0.46, and 0.92), respectively (Table 1). It indicates a wide variation between the segments of plants selected. Endophytic fungi were more prevalent on leaf tissue (39.45%) than on stem (16.32%), seed (14.96%), and fruit tissues (29.25%) which comprises 6.47% ascomycetes, 34.70% coelomycetes, 75.29% hyphomycetes, and 1.17% sterile fungi, respectively. Colonization frequency (Table 2) indicates that *Colletotrichum* sp. 1, *Pestalotiopsis* sp., *Aspergillus flavus*, *Alternaria* sp., *Fusarium* sp. 1, *Fusarium* sp. 2, *Fusarium* sp. 3, and *Penicillium* sp. 2 were the common isolates found in leaf, stem, seed, and fruit whereas *Chaetomium* sp., *Drechslera* sp., and Mycelia sterilia were restricted to the leaf tissues.

As shown in Table 3, this difference in prevalence may be tissue specific which may be due to their anatomical structure [32, 33]. In the comparative study of twig and leaf-associated endophytes in *Quercus ilex* [34], the commonest endophytes were found only in leaves, whereas the remainder were found in both leaves and twigs, but no twig-specific taxa were recovered. Similarly no bark specific taxa were found in *Kigelia pinnata* by Maheswari and Rajagopal [18]. This interestingly coincides with the present study, as there were no stem, seed, or fruit-specific taxa in the overall isolates.

3.2. Effect of Secondary Metabolites of Endophytic Fungi on Selected Pathogens. The endophytic fungi were evaluated for their antimicrobial activity against some clinically significant human pathogens. The fermentation broths of 35 endophytic fungi were screened for antimicrobial activity against three pathogenic bacterial strains, *S. aureus*, *E. coli*, and *P. aeruginosa* and one yeast *C. albicans*. 88.57%, 74.28%, 62.85%, and 65.71% of isolates have shown activity against *S. aureus*, *P. aeruginosa, E. coli*, and *C. albicans*, respectively.

NFX 06-*Fusarium* sp. 2, NFX 23-*Fusarium* sp. 5, and NFB 04-*Penicillium* sp. 3 isolated from leaves and seeds, respectively, were found to exhibit very good activity against all the pathogenic strains with a zone of inhibition of more than 15 mm. NFX 13- *Penicillium* sp. 1 and NFX 20-*Phomopsis* sp. 2 isolated from leaf, NFS 02-*Xylaria* sp. 4 isolated from stem, and NFB 02-*Emericella* isolated from seeds have shown a zone of inhibition of about 10 to 15 mm against the bacterial pathogens and more than 15 mm against *C. albicans*.

Table 2: Endophytic fungi isolated from different parts of *N. foetida*.

Endophytes	Leaf	CF (%)	Stem	CF (%)	Seed	CF (%)	Fruit	CF (%)
Ascomycetes								
Chaetomium sp.	2	2	—	—	—	—	—	—
Xylaria sp.	6	12	1	2	—	—	2	4
Coelomycetes								
Colletotrichum sp. 1	14	14	1	2	1	2	2	4
Colletotrichum sp. 2	3	3	—	—	—	—	1	2
Pestalotiopsis sp.	8	8	2	4	1	2	3	6
Phomopsis sp. 1	12	12	—	—	2	4	5	10
Phomopsis sp. 2	5	5	3	6	—	—	—	—
Hyphomycetes								
Aspergillus flavus	7	7	3	6	4	8	1	2
Aspergillus niger	2	2	—	—	2	4	4	8
Aspergillus terreus	—	—	—	—	1	2	1	2
Curvularia sp.	6	6	1	2	1	2	—	—
Alternaria sp.	6	6	2	4	1	2	2	4
Drechslera sp.	1	1	—	—	—	—	—	—
Fusarium sp. 1	8	8	2	4	1	2	4	8
Fusarium sp. 2	6	6	1	2	2	4	6	12
Fusarium sp. 3	4	4	1	2	2	4	9	18
Trichoderma sp.	4	4	—	—	—	—	2	4
Penicillium sp. 1	5	5	3	6	—	—	1	2
Penicillium sp. 2	9	9	4	8	4	8	3	6
Mycelia sterilia								
Morphotype 1	1	1	—	—	—	—	—	—
Morphotype 2	1	1	—	—	—	—	—	—

Table 3: Species diversity in terms of dominance, richness and evenness of endophytic fungal assemblages in different tissues of *N. foetida*.

Tissue	Total number of taxa	Total number of isolates	Simpson index (I-D)	Shannon-Wiener index (Hs)	Evenness index
Leaf	20	110	0.9302	2.794	0.8175
Stem	12	24	0.8958	2.362	0.8841
Seed	12	22	0.8884	2.335	0.8607
Fruit	15	46	0.8998	2.485	0.8002

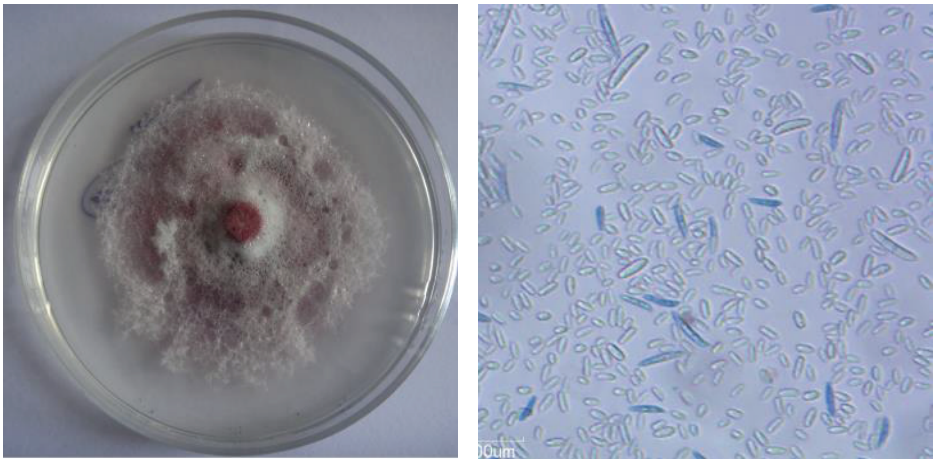

Figure 2: Colony morphology and conidia of endophytic fungus *Fusarium oxysporum* NFX06.

Table 4: Antimicrobial activity of the fermentation broth of endophytic fungi isolated from different parts of *N. foetida*.

Endophytic fungal taxa	Strain no.	Microorganism tested			
		S. aureus	*P. aeruginosa*	*E. coli*	*C. albicans*
Alternaria sp.	NFX 01	+	−	−	+
Aspergillus niger	NFX 02	+	+	−	−
Aspergillus flavus	NFX 03	+	−	−	−
Xylaria sp.	NFX 04	++	+	−	−
Fusarium sp. 1	NFX 05	+	+	+	++
Fusarium sp. 2	NFX 06	+++	+++	+++	+++
Colletotrichum sp. 1	NFX 07	++	+	+	+
Colletotrichum sp. 2	NFX 08	+	+	+	++
Phomopsis sp. 1	NFX 09	+++	++	++	++
Pestalotiopsis sp. 1	NFX 10	+	+	+	+
Pestalotiopsis sp. 1	NFX 11	++	+	−	−
Fusarium sp. 2	NFX 12	++	+	+	++
Penicillium sp. 1	NFX 13	++	++	++	+++
Penicillium sp. 2	NFX 14	++	+	+	+
Xylaria sp.	NFX 15	−	−	−	−
Pestalotiopsis sp. 2	NFX 16	+	−	−	−
Curvularia sp.	NFX 17	−	−	−	−
Drechslera sp.	NFX 18	+	+	−	−
Pestalotiopsis sp. 2	NFX 19	+	−	−	−
Phomopsis sp. 2	NFX 20	++	++	++	+++
Phomopsis sp. 2	NFX 21	+	−	−	−
Phomopsis sp. 1	NFX 22	++	+	+	+
Fusarium sp. 1	NFX 23	+++	+++	+++	+++
Xylaria sp.	NFS 01	−	−	−	−
Xylaria sp.	NFS 02	++	++	++	+++
Phomopsis sp. 2	NFS 03	+	+	+	+
Aspergillus terreus	NFB 01	+	+	+	+
Aspergillus niger	NFB 02	++	++	++	+++
Colletotrichum sp. 2	NFB 03	+	+	+	++
Penicillium sp. 2	NFB 04	+++	+++	+++	+++
Pestalotiopsis sp. 2	NFT 01	++	++	++	++
Fusarium sp. 2	NFT 02	++	++	+	++
Colletotrichum sp. 1	NFT 03	++	+	++	+++
Colletotrichum sp. 2	NFT 04	+	+	+	+
Xylaria sp.	NFT 05	−	−	−	−
	Streptomycin	+++	+++	+++	−
	Fluconozole	−	−	−	+++

NFT 01-*Pestalotiopsis* sp. 3 isolated from fruit have shown zone of inhibition of about 10 to 15 mm against all the tested microorganisms. NFX 09-*Phomopsis* sp. 1 has shown a zone of inhibition more than 15 mm against *S. aureus* while showing 10 to 15 mm against all the other tested microorganisms.

NFX 01-*Trichoderma* sp. 1, NFX 02 -*A. niger*, NFX 03-*A. flavus*, NFX 04-*Xylaria* sp. 1, NFX 05-*Fusarium* sp. 1, NFX 16-*Pestalotiopsis* sp. 2, NFX 18-*Fusarium* sp. 4, NFX 19-*Pestalotiopsis* sp. 2, and NFX 21-*Phomopsis* sp. 3 were found to exhibit low activity of less than 10 mm zone of inhibition. According to Fabry et al. [35], the endophytic fungi that showed low antimicrobial activity might possess the active compound in a lesser concentration. And the concentration of these compounds could be enhanced by considering some factors like mode of fermentation, concentration of inoculum, period of incubation, solvents used for extraction, and most importantly purification process [36].

NFX 15-*Xylaria* sp. 2, NFX 17-*Drechslera* sp. 1, NFS 01-*Xylaria* sp. 3, and NFT 05-*Lasiodiplodia* sp. 1 isolated from leaf, stem, and fruit, respectively, were found to exhibit no activity against the selected human pathogens. Among all the isolates, NFX 06-*Fusarium* sp. 2 (Figure 2) has shown maximum zone of inhibition, as shown in Table 4, of about 17.7 mm hence further studies were carried out with this isolate.

3.3. Phylogenetic Placement of F. oxysporum. Molecular methods to determine the genetic diversity within the species

Table 5: List of *F. oxysporum* strains used in this study and their GenBank accession numbers.

Species	No. of base pairs	Source/host	Country	Genbank accession no.
		Endophytic isolates		
Fusarium oxysporum NFX06	510 bp	*Nothapodytes foetida*	India	KC914432*
F. oxysporum AC30	594 bp	*Acorus calamus*	India	GU056168
F. oxysporum f. sp. gladioli UAS007	528 bp	*Nothapodytes nimmoniana*	India	FJ158124.1
F. oxysporum JRE 1	497 bp	*Juniperus recurva*	India	EF591767.1
F. oxysporum D 16	517 bp	*Annona squamosa*	China	EF488410.1
F. oxysporum D 17	510 bp	*Annona squamosa*	China	EF488411.1
F. oxysporum	543 bp	*Scuticaria irwiniana*	Brazil	FJ605247.1
F. oxysporum UFMGCB 1376	543 bp	*Acianthera teres*	Brazil	FJ605244.1
F. oxysporum F35	515 bp	*Taxus cuspidata*	South Korea	AY555719.1
F. oxysporum DC-1-67	493 bp	*Dracaena cambodiana*	China	FJ449900.1
F. oxysporum EYR11	549 bp	*Pinus massoniana*	China	EU888922
F. oxysporum FuO139	561 bp	*Schlumbergera truncata*	Italy	KC196121
F. oxysporum CMT6	527 bp	*Phaseolus vulgaris*	Brazil	JQ754006
F. oxysporum	545 bp	*Paris polyphylla* var. *chinensis*	China	JF776163
F. oxysporum DB0612101	515 bp	—	—	HQ682196
F. oxysporum SFCF20120912-05	548 bp	*Pinus thunbergii*	Korea	KF313101.1
F. oxysporum REF213	555 bp	*Ephedra distachya*	Hungary	JN859433
F. oxysporum DH-42	581 bp	*Rehmannia glutinosa*	China	HM346538.1
F. oxysporum JJ002	541 bp	*Ginkgo biloba*	China	DQ166550
F. oxysporum FOFB62	564 bp	*Grevillea robusta*	Kenya	HQ651161
F. oxysporum Aug021	543 bp	*Pecteilis susannae* (*L.*) *Rafin*	Thailand	GQ862347
		Pathogenic Isolates		
F. oxysporum 18P	487 bp	Bean	Mexico	FJ619940
F. oxysporum f. cubense 115HT	569 bp	Banana	India	DQ889176.1
F. oxysporum GXF-6	544 bp	Hybrid *bamboo*	China	EU285554.1
F. oxysporum 544	544 bp	*Bupleurum chinensis*	China	EU862240
F. oxysporum 6*e* − 35	515 bp	*Capsicum annum*	Mexico	AY728210
F. oxysporum nazar 1	545 bp	*Agave tequilana*	Mexico	EU161243
F. oxysporum f. sp. niveum 1ZF1	545 bp	Watermelon	China	EU588397
F. oxysporum f. sp. lilli FF-2	543 bp	Lily	Taiwan	AY684919
F. oxysporum f.sp. *vasinfectum* F1TP0001	542 bp	Tulip	Mexico	DQ979010
F. oxysporum f. sp. vasinfectum	545 bp	Cotton	China	EU849584
F. oxysporum R1	543 bp	*Atractylodes*	China	JX885462
F. oxysporum 857	564 bp	*Macrocephala*	China	JN232190
F. oxysporum GLB3	567 bp	—	China	GU136492
F. oxysporum 08YSJG	567 bp	—	China	GU136493
F. oxysporum	527 bp	*Pinus sylvestris*	Sweden	HM036595
F. oxysporum	564 bp	*Grevillea robusta*	—	HQ651161
F. oxysporum	552 bp	—	UK	AY147369
F. oxysporum XSD-73	570 bp	*Dioscorea collettii* var. *hypoglauca*	China	EU326215
F. oxysporum XSD-78	571 bp	*Polygonatum sibirilum red*	China	EU326216
F. oxysporum OT0330	546 bp	Roots of willows	—	GU934524

*Endophytic fungus used in the present study.

of *F. oxysporum* [37] and genetic diversity within pathogenic and nonpathogenic isolates with endophytic isolates from various plants [38, 39] and also with other isolates that exist as pathogens, saprophytes, and endophytes [40] have been reported. In this study, an attempt was made to place an endophytic *F. oxysporum* isolated from *N. foetida* leaves with other endophytic and pathogenic *F. oxysporum* of different plant species (Table 5). Phylogenetic tree was generated using maximum parsimony method. The generated tree showed twelve clades and one independent lineage which have descended from a pathogenic *F. oxysporum* ancestor (Figure 3).

The evolutionary relationship of 40 taxa revealed consistency index (0.229865), retention index (0.534473), and composite index (0.122857), respectively, with a tree length of 6295. Clade I showed two clusters with an endophytic *F. oxysporum* and in Clade II all the isolates were pathogenic *F. oxysporum* whereas both the clades shared common

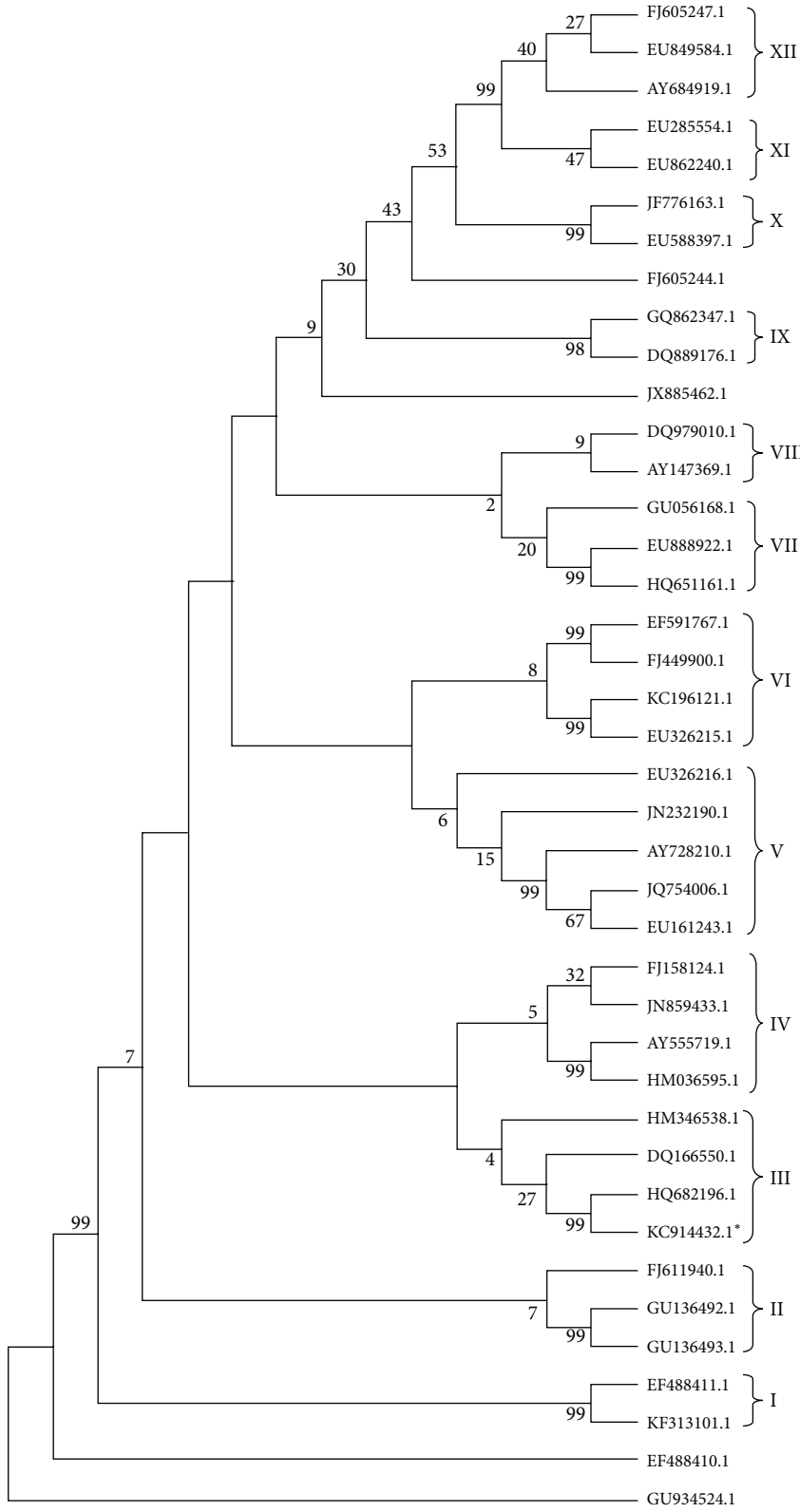

Figure 3: Tree showing phylogenetic relationship of 40 *F. oxysporum* strains. The evolutionary history was inferred using the maximum parsimony method. Tree number 1 out of the 280 most parsimonious trees (length = 6295) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. * denotes the endophytic fungus used in the present study.

endophytic ancestor. Clade III has all endophytic descendants with a common endophytic ancestor. Clades IV and VI have two coevolved endophytic isolates with two major clusters giving rise to one pathogenic descendant. Clades VIII and XI have two clusters with all pathogenic isolates. Clades VII and XII have two endophytic isolates and one pathogenic isolate which depicts the possible evolution of a pathogenic strain from an endophytic isolate. Clades IX and X have two clusters each with an endophytic and pathogenic isolate coexisting together which shares a common pathogenic ancestor.

Based on the phylogenetic analysis we hypothesize that endophytic strains have given rise to pathogenic forms. This hypothesis is supported by the fact that there is a great deal of genetic relatedness between pathogenic and nonpathogenic *F. oxysporum* isolates, which has led some researchers to conclude that some particular pathogenic isolates might have evolved from nonpathogenic strains by mutations involving a few loci [41, 42].

4. Conclusions

However, this is perhaps the first report on endophytic fungal diversity of the endangered medicinal plant *N. foetida* of Agumbe forest. This study contributes to the understanding of species richness of endophytic fungi in different tissues of the plant like leaf, stem, seed, and fruit. More number of endophytic isolates in leaf tissue may be due to the fact that the sampling was done in a wet season and in many instances leaves sampled during the wet season harboured more endophytes than those screened during the dry season [43, 44]. The rich diversity of *Fusarium* species as endophytes in different tissues of both the plants may be due to germination of more number of spores of this fungus due to favourable environmental condition. Most of the species isolated comprise mostly *Fusarium, Penicillium, Colletotrichum, Pestalotiopsis, Aspergillus*, and *Alternaria* which was in agreement with the results of Jeewon et al. [45] from *Antidesma madagascariense*. The result of antimicrobial activity indicated that most of the isolates showed varying degree of activity against the selected human test pathogens. Hence the endophytic fungi existing in the plant are potential sources of antimicrobial substances as reported by several researchers [46–49]. Among the 35 selected endophytes *Fusarium* sp. 2 has shown very good activity which was further identified based on ITS sequencing to be *F. oxysporum* strain NFX06 and submitted in the GenBank with the accession number KC914432. Phylogenetic analysis of the endophytic strain *F. oxysporum* NFX06 with the other endophytic and pathogenic *F. oxysporum* indicated that there is a strong relationship between the pathogenic and nonpathogenic *F. oxysporum* and from the results it was concluded that *F. oxysporum* NFX06 has evolved from an endophytic ancestor.

Acknowledgments

The authors are thankful to Manipal Life Science Centre for providing the facilities for identification of endophytic fungus. And they are grateful to retired Professor G. K. Bhat, Poornaprajna College Mangalore, for helping them in finding and authenticating the plant.

References

[1] C. W. Bacon and J. White, *Microbial Endophytes*, Marcel Dekker, New York, NY, USA, 2000.

[2] M. M. Dreyfuss and I. H. Chapela, "Potential of fungi in the discovery of novel, low molecular weight pharmaceuticals," in *The Discovery of Natural Products With Therapeutic Potential*, V. P. Gullo, Ed., pp. 49–80, Butterworth-Heinemann, Boston, Mass, USA, 1994.

[3] A. E. Arnold, Z. Maynard, and G. S. Gilbert, "Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity," *Mycological Research*, vol. 105, no. 12, pp. 1502–1507, 2001.

[4] I. R. Sanders, "Plant and arbuscular mycorrhizal fungal diversity—are we looking at the relevant levels of diversity and are we using the right techniques?" *New Phytologist*, vol. 164, no. 3, pp. 415–418, 2004.

[5] J. M. U'Ren, J. W. Dalling, R. E. Gallery et al., "Diversity and evolutionary origins of fungi associated with seeds of a neotropical pioneer tree: a case study for analysing fungal environmental samples," *Mycological Research*, vol. 113, no. 4, pp. 432–449, 2009.

[6] O. Petrini, T. N. Sieber, L. Toti, and O. Viret, "Ecology, metabolite production, and substrate utilization in endophytic fungi," *Natural toxins*, vol. 1, no. 3, pp. 185–196, 1992.

[7] G. Soca-Chafre, F. N. Rivera-Orduña, M. E. Hidalgo-Lara, C. Hernandez-Rodriguez, R. Marsch, and L. B. Flores-Cotera, "Molecular phylogeny and paclitaxel screening of fungal endophytes from Taxus globosa," *Fungal Biology*, vol. 115, no. 2, pp. 143–156, 2011.

[8] U. Pinruan, N. Rungjindamai, R. Choeyklin, S. Lumyong, K. D. Hyde, and E. B. G. Jones, "Occurrence and diversity of basidiomycetous endophytes from the oil palm, Elaeis guineensis in Thailand," *Fungal Diversity*, vol. 41, pp. 71–88, 2010.

[9] A. B. M. Vaz, R. C. Mota, M. R. Q. Bomfim et al., "Antimicrobial activity of endophytic fungi associated with *Orchidaceae* in Brazil," *Canadian Journal of Microbiology*, vol. 55, no. 12, pp. 1381–1391, 2009.

[10] L. H. Rosa, M. D. L. Almeida Vieira, I. F. Santiago, and C. A. Rosa, "Endophytic fungi community associated with the dicotyledonous plant Colobanthus quitensis (Kunth) Bartl. (Caryophyllaceae) in Antarctica," *FEMS Microbiology Ecology*, vol. 73, no. 1, pp. 178–189, 2010.

[11] G. Strobel, B. Daisy, U. Castillo, and J. Harper, "Natural Products from Endophytic Microorganisms," *Journal of Natural Products*, vol. 67, no. 2, pp. 257–268, 2004.

[12] H. W. Zhang, Y. C. Song, and R. X. Tan, "Biology and chemistry of endophytes," *Natural Product Reports*, vol. 23, no. 5, pp. 753–771, 2006.

[13] J.-H. Song, "What's new on the antimicrobial horizon?" *International Journal of Antimicrobial Agents*, vol. 32, no. 4, pp. S207–S213, 2008.

[14] A. L. Demain and S. Sanchez, "Microbial drug discovery: 80 Years of progress," *Journal of Antibiotics*, vol. 62, no. 1, pp. 5–16, 2009.

[15] H. Yu, L. Zhang, L. Li et al., "Recent developments and future prospects of antimicrobial metabolites produced by endophytes," *Microbiological Research*, vol. 165, no. 6, pp. 437–449, 2010.

[16] X. Liu, M. Dong, X. Chen, M. Jiang, X. Lv, and J. Zhou, "Antimicrobial activity of an endophytic Xylaria sp.YX-28 and identification of its antimicrobial compound 7-amino-4-methylcoumarin," *Applied Microbiology and Biotechnology*, vol. 78, no. 2, pp. 241–247, 2008.

[17] C. P. Narayan, W. K. Kim, S. K. Woo, M. S. Park, and S. H. Yu, "Fungal endophytes in roots of aralia species and their antifungal activity," *Journal of Plant Pathology*, vol. 23, no. 4, pp. 287–294, 2007.

[18] S. Maheswari and K. Rajagopal, "Biodiversity of endophytic fungi in Kigelia pinnata during two different Seasons," *Current Science*, vol. 104, pp. 515–518, 2013.

[19] F. W. Wang, R. H. Jiao, A. B. Cheng, S. H. Tan, and Y. C. Song, "Antimicrobial potentials of endophytic fungi residing in Quercus variabilis and brefeldin a obtained from *Cladosporium sp*," *World Journal of Microbiology and Biotechnology*, vol. 23, no. 1, pp. 79–83, 2007.

[20] V. Gangadevi and J. Muthumary, "Taxol, an anticancer drug produced by an endophytic fungus Bartalinia robillardoides Tassi, isolated from a medicinal plant, Aegle marmelos Correa ex Roxb," *World Journal of Microbiology and Biotechnology*, vol. 24, no. 5, pp. 717–724, 2008.

[21] S. C. Parija, M. R. Shivaprakash, and S. R. Jayakeerthi, "Evaluation of lacto-phenol cotton blue (LPCB) for detection of Cryptosporidium, Cyclospora and Isospora in the wet mount preparation of stool," *Acta Tropica*, vol. 85, no. 3, pp. 349–354, 2003.

[22] O. Petrini, "Fungal endophytes of tree leaves," in *Microbial Ecology of Leaves*, pp. 179–197, Springer, New York, NY, USA, 1991.

[23] R. W. Poole, *An Introduction to Quantitative Ecology*, McGraw-Hill, New York, NY, USA, 1974.

[24] J. V. Groth and A. P. Roelfs, "The concept of measurement of phenotypic diversity in *Puccinia graminis* on wheat," *Phytopathology*, vol. 77, pp. 1394–1399, 1987.

[25] NCCLS, "Methods for determining bactericidal activity of antimicrobial agents," Approved Guideline M26-A, National Committee for Clinical Laboratory Standards, Wayne, Pa, USA, 1996.

[26] T. J. White, T. Bruns, S. Lee, and J. Taylor, "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds., pp. 315–322, Academic Press, New York, NY, USA, 1990.

[27] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, "MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods," *Molecular Biology and Evolution*, vol. 28, no. 10, pp. 2731–2739, 2011.

[28] K. Saikkonen, S. H. Faeth, M. Helander, and T. J. Sullivan, "Fungal endophytes: a continuum of interactions with host plants," *Annual Review of Ecology and Systematics*, vol. 29, pp. 319–343, 1998.

[29] I. H. Chapela and L. Boddy, "Fungal colonization of attached beech branches. I. Early stages of development of fungal communities," *New Phytologist*, vol. 110, no. 1, pp. 39–45, 1988.

[30] P. J. Fisher, O. Petrini, and B. C. Sutton, "A comparative study of fungal endophytes in leaves, xylem and bark of Eucalyptus in Australia and England," *Sydowia*, vol. 45, pp. 338–345, 1993.

[31] T. S. Suryanarayanan, V. Kumaresan, and J. A. Johnson, "Foliar fungal endophytes from two species of the mangrove *Rhizophora*," *Canadian Journal of Microbiology*, vol. 44, no. 10, pp. 1003–1006, 1998.

[32] K. Rajagopal and T. S. Suryanarayanan, "Isolation of endophytic fungi from leaves of neem (*Azadirachta indica A*. Juss.)," *Current Science*, vol. 78, no. 11, pp. 1375–1378, 2000.

[33] S.-Y. Lee, I. Nakajima, F. Ihara, H. Kinoshita, and T. Nihira, "Cultivation of entomopathogenic fungi for the search of antibacterial compounds," *Mycopathologia*, vol. 160, no. 4, pp. 321–325, 2005.

[34] P. J. Fisher, O. Petrini, L. E. Petrini, and B. C. Sutton, "Fungal endophytes from the leaves and twigs of *Quercus ilex L.* from England, Majorca and Switzerland," *New Phytologist*, vol. 127, no. 1, pp. 133–137, 1994.

[35] W. Fabry, P. O. Okemo, and R. Ansorg, "Antibacterial activity of East African medicinal plants," *Journal of Ethnopharmacology*, vol. 60, no. 1, pp. 79–84, 1998.

[36] P. Powthong, B. Jantrapanukorn, A. Thongmee, and P. Suntornthiticharoen, "Evaluation of endophytic fungi extracts for their antimicrobial activity from *Sesbania grandiflora* (L.) pers," *International Journal of Pharmaceutical Biomedical Research*, vol. 3, pp. 132–136, 2012.

[37] V. Edel, C. Steinberg, I. Avelange, G. Laguerre, and C. Alabouvette, "Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains," *Phytopathology*, vol. 85, no. 5, pp. 579–585, 1995.

[38] K. S. Elais, R. W. Schneider, and M. M. Lear, "Analysis of vegetative compatibility group in nonpathogenic population of *Fusarium oxysporum* isolated from symptomless tomato root," *Canadian Journal of Botany*, vol. 69, pp. 2089–2094, 1991.

[39] S. L. Woo, A. Zoina, G. Del Sorbo et al., "Characterization of *Fusarium oxysporum* f. sp. *phaseoli* by pathogenic races, VCGs, RFLPs, and RAPD," *Phytopathology*, vol. 86, no. 9, pp. 966–973, 1996.

[40] B. P. Barik, K. Tayung, P. J. Narayan, and S. K. Dutta, "Phylogenetic placement of an endophytic fungus *Fusarium oxysporum* isolated from Acorus calamus rhizomes with antimicrobial activity," *EJBS*, vol. 2, no. 1, 2010.

[41] R. P. Baayen, K. O'Donnell, P. J. M. Bonants et al., "Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease," *Phytopathology*, vol. 90, no. 8, pp. 891–900, 2000.

[42] K. Skovgaard, L. Bødker, and S. Rosendahl, "Population structure and pathogenicity of members of the *Fusarium oxysporum* complex isolated from soil and root necrosis of pea (*Pisum sativum L.*)," *FEMS Microbiology Ecology*, vol. 42, no. 3, pp. 367–374, 2002.

[43] K. F. Rodrigues, "The foliar fungal endophytes of the Amazonian palm Euterpe oleracea," *Mycologia*, vol. 86, no. 3, pp. 376–385, 1994.

[44] D. Wilson and G. C. CarrolL, "Infection studies of Discula quercina, an endophyte of Quercus garryana," *Mycologia*, vol. 86, no. 5, pp. 635–647, 1994.

[45] R. Jeewon, J. Ittoo, D. Mahadeb, Y. J. Fakim, H.-K. Wang, and A.-R. Liu, "DNA based identification and phylogenetic characterisation of endophytic and saprobic Fungi from Antidesma madagascariense, a medicinal plant in mauritius," *Journal of Mycology*, vol. 2013, Article ID 781914, 10 pages, 2013.

[46] K. Tayung and D. K. Jha, "Antimicrobial evaluation ofsome-fungal endophytes isolated frombarkofHimalayanyew," *World Journal of AgriculturalSciences*, vol. 2, pp. 489–494, 2006.

[47] J. Mohanta, K. Tayung, and U. B. Mohapatra, "Antimicrobial potentials of endophytic fungi inhabiting three ethnomedicinal plants of similipal biosphere reserve, India," *Internet Journal of Microbiology*, vol. 5, no. 2, 2008.

[48] S. Padhi and K. Tayung, "Antimicrobial activity and molecular characterization of an endophytic fungus, *Quambalaria sp.* isolated from Ipomoea carnea," *Annals of Microbiology*, vol. 63, no. 2, pp. 793–800, 2013.

[49] S. K. Jena and K. Tayung, "Endophytic fungal communities associated with two ethno-medicinal plants of similipal biosphere reserve, India and their antimicrobial prospective," *Journal of Applied Pharmaceutical Science*, vol. 3, p. S7, 2013.