Molecular phylogeny and RNA secondary structure of *Fusarium* species with different lifestyles

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The definition of endophytes ranges from symbiotic to balanced antagonism and/or latent plant pathogens. This indicates a close affiliation between endophytes and pathogens but molecular evidence confirming these relationships are still to be elucidated. We investigated the relationship of endophytic, saprobic and pathogenic *Fusarium* species as a model genus based on ITS sequences and ITS2 secondary structure analysis. Altogether 212 *Fusarium* species mostly named in GenBank with ranging lifestyles were used in this study. Species deposited in GenBank were found to be often wrongly named based on sequence comparison as species with the same names were polyphyletic, while differently named species often had the same sequences. Phylogenetic analysis by three different methods (UPGMA, NJ and MP) revealed that similar named species clustered together, but did not form well distinct clades reflective of their lifestyles. Several species were polyphyletic, while several other unrelated species appear to share the same ITS sequences. Since ITS rDNA sequence based phylogeny did not showed distinct relationships, ITS2 consensus secondary structures were used. Endophytes showed close molecular structural affinity with pathogens but structural features of saprobes was very different. Although care must be taken when using sequences from named *Fusarium* species in GenBank this study provides molecular evidence that endophytes share a close relationship with pathogens and agrees with the assumption that endophytes are latent pathogens.

Key words – *Fusarium* spp. – Lifestyle – Phylogeny – Secondary structures analysis

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Introduction

There have been numerous reports on endophytic fungi colonizing various plant hosts and speculation on their functional roles in the environment (Hyde & Soyong 2008, Albrectsen et al 2010, Saikkonen et al 2010). Most definitions regard endophytes as internal plant tissue colonizers that do not cause any disease symptoms. Such a definition implies a symbio-

tic or mutualistic relationship between the host plant and the endophytic microbe. There have however, been differences in opinion concerning the relationships that exist between the endophyte and their respective host. Strobel (2002) suggested that the relationship can range from borderline pathogenic, to commensalism and to a symbiotic relationship. There are also studies that have explored relationships

between endophytes and their roles as saprobes (Hyde et al. 2007, Promputtha et al. 2007, 2010, Purahong & Hyde 2011). There are also many examples showing endophytes to be latent pathogens (Hyde & Soyong 2008). In fact, several fungal taxa reported as endophytes closely resemble plant pathogenic fungi. Such findings suggest an inherent genomic modulation and adaptability in endophytic fungi. Even though there are hypotheses providing evidence for these transitional forms; molecular level investigations with regard to their different lifestyles are few (Promputtha et al. 2007). We have attempted to study the relationships between endophytic, saprobic and pathogenic *Fusarium* species as a model organism based on nuclear Internal Transcribed Spacer (ITS) sequences and RNA secondary structures study.

Fusarium is a highly diverse genus with species exhibiting many different lifestyles (Summerell et al 2010, 2011). They are generally regarded as plant pathogens causing serious diseases (Amata et al 2010). Some are also reported to cause diseases in humans especially in immuno-compromised patients, while others live as saprobes in the soil and play a role in nutrient recycling. *Fusarium* species have commonly been isolated as endophytes and reported from various plant hosts (Strobel and Daisy 2003, Xu et al. 2008, Deng et al. 2009). The existence of *Fusarium* species occupying different lifestyles is confusing because morphologically they cannot be well-differentiated. Molecular techniques have often been used to identify *Fusarium* species (Amata 2010, Summerell et al 2010, 2011), but there is little information on their phylogeny and variations at molecular level considering isolates existing in different forms.

The Internal Transcribed Spacer (ITS) regions of rDNA have commonly been used in species differentiation. They form specific secondary structures which are needed for correct recognition of cleavage sites and provide the binding sites for nucleolar proteins and RNAs during ribosome maturation (Hausner and Xang 2005). ITS regions that comprised two transcribed intergenic spacers (ITS1 and ITS2) have not only been used for phylogenetic and taxonomic studies of fungi but also for species delimitation and ecological analysis (Pinto

et al. 2004; Anderson and Perkin 2007). The ITS2 region has been found to vary in both primary sequences and secondary structures, so it could be used as possible marker in molecular systematics and phylogenetic reconstruction (Schultz & Wolf 2009). Several researchers have already demonstrated the potential applications of ITS2 for taxonomic classification and phylogenetic reconstruction at both the genus and species levels for eukaryotes, including animals, plants and fungi (Coleman 2007, Miao et al. 2008, Keller et al. 2009). Secondary structural data analysis of this region can also improve the phylogenetic resolution to a considerable extent (Keller et al. 2008). Since structures are more conserved than sequences, the aim of this study was to infer phylogenetic relationships and to address variations among *Fusarium* spp. having different lifestyles based on ITS sequence and structure analysis.

Materials and Methods

Isolation of the source organisms

Fusarium strains occurring in different habitats were isolated from various sources (Table 1). Endophytic strains were obtained from their respective hosts by surface sterilization procedure. The technique used for isolation was as follows: Barks samples were immersed sequentially in 70% ethanol for 3 min and 0.5% NaOCl for 1 min and rinsed thoroughly with sterile distilled water. The excess water was dried under laminar airflow chamber. Then, with sterile scalpel, outer tissues were removed and the inner tissues were carefully dissected and placed on Petri-dishes containing Potato Dextrose Agar (PDA) medium and incubated at 25°C. Saprobian strains were obtained by dilution plate method. Soil samples were serially diluted and each dilution was plated on Potato Dextrose Agar medium and incubated at 30°C for week. Pathogenic strains comprised of one plant pathogen and one human pathogen. The plant pathogenic strain was obtained from the experimental farm of Orissa University of Agriculture and Technology, Odisha, India and were isolated from an infected immature coconut. The human pathogenic strain was obtained from the Microbiology Division of Kasturba Medical

Table 1 *Fusarium* spp. isolated from various sources used in present study.

<i>Fusarium</i> spp.	Isolation source	Lifestyle	GenBank Accession No.
<i>F. solani</i>	<i>Taxus baccata</i> bark	Endophyte	FJ719812.1
<i>F. proliferatum</i>	<i>Ipomoea carnea</i> bark	Endophyte	HM145946.1
<i>F. proliferatum</i>	Immature coconut	Pathogen	GU363955.1
<i>F. solani</i>	Corneal scrape	Pathogen	HM145945.1
<i>F. oxysporum</i>	Grassland soil	Saprobe	GU363956.1
<i>F. incarnatum</i>	River belt soil	Saprobe	HQ221741.1

College, Manipal University, Manipal, India. The strain was isolated on Sabouraud Dextrose Agar (SDA) medium from corneal scrape of 38 years old male patient.

Isolation of genomic DNA, PCR amplification and sequencing

Total genomic DNA was extracted from mycelia of the fungi using a CTAB method (Cai et al. 2005). DNA amplification was performed by PCR. The PCR was set up using the following components: 5µL Buffer (10×), 3µL MgCl₂ (25mM), 1µL dNTPs (10mM), 1.5µL Taq Polymerase (5U), 1.5µL Forward primer (10µM), 1.5µL Reverse Primer (10µM), 3µL DNA template and 34.7µL distilled water. Initial denaturation was at 95°C for 5 min denaturation, annealing and elongation were done at 95°C for 1min, 52°C for 30 sec and 72°C for 1 min, respectively in 45 cycles. Final extension was at 72°C for 10 min and hold at 4°C. For amplification of ITS-rDNA region ITS4 and ITS5 primers were used according to the method described by White et al. (1990). The PCR product, spanning approximately 500–600bp was checked on 1% agarose electrophoresis gel. It was then purified using a quick spin column and buffers (washing buffer and elution buffer) according to the manufacturer's protocol (QIA quick gel extraction kit Cat No. 28706). DNA sequencing was performed using the above mentioned primers in an Applied Biosystem 3130xl analyzer.

Taxon sampling

Six *Fusarium* strains were obtained as isolates. The sequences were annotated and submitted to GenBank. ITS rDNA sequence data from 2437 *Fusarium* species were also retrieved from GenBank (on 26-12-2010). The sequences were also trimmed for ITS2 using annotation tools based on Hidden Markov Mo-

del (Keller et al. 2009). The sequences were filter searched and 206 sequences were selected based on their mode of occurrence as endophytes, saprobes and pathogens. Altogether 212 ITS rDNA sequences were considered for phylogenetic analysis and same numbers of ITS2 sequences were used for secondary structure study.

Sequence alignment and phylogenetic analyses

Multiple sequence alignments were performed using CLUSTALW software utilizing default settings. ITS rDNA sequences were used for phylogenetic relationship inferences. Phylogenetic trees were generated by three different methods (UPGMA, NJ and MP) by MEGA 4.0 (Tamura et al. 2007). All characters were equally weighted and unordered. Alignment gaps were treated as missing data. The optimal trees with the sum of branch length were 0.47152190 for UPGMA and 0.48761537 for NJ. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap was 500 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). In the MP method, the tree was generated from 344 most parsimonious trees. The consistency index was 0.582822, the retention index was 0.979115 and the composite index was 0.570650. The MP tree was obtained using the Close-Neighbor-Interchange algorithm. There were a total of 238 positions in the final dataset, out of which 65 were parsimony informative.

Consensus RNA secondary structures prediction

The ITS2 sequences of *Fusarium* occurring as endophytes, saprobes and pathogens (plant pathogenic and human pathogenic) were used to generate consensus RNA secondary structures using LocARNA version 1.5.2 (Smith et al. 2010). It performed multiple alignments and generated consensus secondary structures of each category using realistic energy model for RNAs. The structures were observed and variations were analyzed amongst different groups.

Results and Discussion

Isolation, identification and retrieval of *Fusarium* spp.

Six *Fusarium* strains were isolated from various sources; two strains were endophytic, two were saprobic and one each was a phytopathogen and human pathogen (Table 1). All strains were morphologically and microscopically identified as *Fusarium* species. Species identification of *Fusarium* using morphological traits has always been a difficult task for mycologists because characteristics like mycelial pigmentation, formation, shape and size of conidia are unstable and highly dependent on composition of media and environmental conditions. Phenotypic variation is abundant and considerable expertise is required to distinguish between closely related species (Nelson 1983). Therefore molecular characterization was carried out based on ITS rDNA data. The strains were sequenced and species level confirmations were done by BLAST search analysis. Among several molecular techniques, ITS rDNA sequence analysis has been a method of choice for species confirmation because they are conserved regions of the genome and genetically variable and have proven to be very useful for investigating relationships between fungi at all taxonomic levels including genus and species (Bruns et al. 1991). All the sequences were deposited in GenBank and GenBank index numbers were obtained. In addition, 206 species of *Fusarium* with ITS2 sequences were retrieved from ITS2 database based on their habitat and locations (Table 2).

Phylogenetic analyses of *Fusarium* spp.

A total of 212 ITS rDNA sequences of *Fusarium* from different lifestyles were ob-

tained as final dataset for phylogenetic study. Sequence analysis of the ITS region of nuclear ribosomal DNA has been widely used for taxonomic placement (Guo et al. 2001, Wang et al. 2005) and phylogenetic diversity of fungi (Tejesvi et al. 2009). This region has also been used in taxonomy and phylogeny of *Fusarium* species (Schroers et al. 2009). Phylogenetic trees were generated by three different methods (UPGMA, NJ and MP) using MEGA 4.0.2 (Tamura et al. 2007). All the trees showed that more or less similar species of *Fusarium* clustered together but did not form distinct clades as per their lifestyles. One of the most parsimonious trees showing evolutionary relationships among *Fusarium* spp. is shown in Figure 1. The result also indicated that several species appear to be polyphyletic and several other unrelated species appear to share the same ITS sequences. This may be due to wrongly named species being submitted to GenBank as in other genera (Cai et al. 2009). Endophytic, saprobic and pathogenic taxa did not cluster into separate groups even in the case of similarly named species. This was particularly obvious in common species of *Fusarium* such as *F. oxysporum* and *F. solani*. Several workers have however clearly differentiated endophytic and pathogenic fungi by analyzing the ITS regions (Photita et al. 2005, Wei et al. 2007b). Such findings do not agree with our result, where consideration of ITS rDNA sequence phylogeny did not elucidate variations among closely related species of *Fusarium* based on their lifestyles. The ambiguity may be due to consideration of many sequences of *Fusarium* species which is genetically diverse with different lifestyles or the fact that they are wrongly named in GenBank. There are also significant drawbacks in using the ITS region such as (i) insufficient hypervariability to distinguish the various species in the *Fusarium* species complexes; (ii) its failure to distinguish between closely related species (sibling species) and (iii) problems with the reliability of the ITS sequences deposited in the reference databases (e.g., GenBank/EMBL/DDBJ) (Nilsson et al. 2006, Cai et al 2009). Moreover, primary sequences of ITS regions often contain insertions and deletions (indels) making alignment difficult beyond infraspecific levels (Kruger & Gargas, 2008). Since consideration

Table 2 *Fusarium* spp. used in the present study with their isolation sources, lifestyles and GenBank numbers.

Species	Source	Lifestyle	GenBank Index
<i>F. solani</i> *	<i>Taxus baccata</i>	Endophyte	224551656
<i>F. proliferatum</i> *	<i>Ipomea carnea</i>	Endophyte	296410105
<i>F. solani</i>	<i>Camptotheca accuminta</i>	Endophyte	117650700
<i>F. solani</i>	<i>Nothapodytes nimmoniana</i>	Endophyte	205319823
<i>F. solani</i>	<i>Rhizophoraceae</i> sp.	Endophyte	291293624
<i>F. solani</i>	Agarwood	Endophyte	296042316
<i>F. solani</i>	<i>Eleuterococcus seaticosus</i>	Endophyte	299790610
<i>F. solani</i>	<i>Triplaris cumingiana</i>	Endophyte	304333705
<i>F. solani</i>	Mulberry Roots	Endophyte	186694316
<i>F. equiseti</i>	<i>Curcuma longa</i>	Endophyte	144905075
<i>F. equiseti</i>	<i>Vitis vinifera</i>	Endophyte	154721227
<i>F. equiseti</i>	Mulberry Roots	Endophyte	186694315
<i>F. equiseti</i>	-	Endophyte	222477058
<i>F. equiseti</i>	<i>Holcus lanatus</i>	Endophyte	240936106
<i>F. equiseti</i>	Agarwood	Endophyte	296042314
<i>F. equiseti</i>	Agarwood	Endophyte	296042333
<i>F. equiseti</i>	<i>Piper reticulatum</i>	Endophyte	304333726
<i>F. oxysporum</i>	<i>Paris polyphylla</i>	Endophyte	146217051
<i>F. oxysporum</i>	Mangrove	Endophyte	159024208
<i>F. oxysporum</i>	<i>Dioscorea zingiberensis</i>	Endophyte	170785449
<i>F. oxysporum</i>	Mulberry Roots	Endophyte	186694314
<i>F. oxysporum</i>	Mexican Yew Leaf	Endophyte	203285112
<i>F. oxysporum</i>	<i>Nothapodytes nimmoniana</i>	Endophyte	205319842
<i>F. oxysporum</i>	<i>Palicourea tetraphylla</i>	Endophyte	217331371
<i>F. redolens</i>	<i>Paris polyphylla</i>	Endophyte	146217055
<i>F. redolens</i>	<i>Rehmannia glutinosa</i>	Endophyte	299851415
<i>F. redolens</i>	<i>Rehmannia glutinosa</i>	Endophyte	299851446
<i>F. tricinctum</i>	<i>Centaurea stoebe</i>	Endophyte	148357869
<i>F. tricinctum</i>	<i>Dendrobium</i>	Endophyte	295855229
<i>F. tricinctum</i>	<i>Dendrobium</i>	Endophyte	295855230
<i>F. tricinctum</i>	<i>Dendrobium</i>	Endophyte	295855231
<i>F. tricinctum</i>	<i>Dendrobium</i>	Endophyte	295855242
<i>F. pulverosum</i>	<i>Centaurea stoebe</i>	Endophyte	152060774
<i>F. poae</i>	<i>Centaurea stoebe</i>	Endophyte	152060775
<i>F. sporotrichioides</i>	<i>Centaurea stoebe</i>	Endophyte	152060776
<i>F. incarnatum</i>	<i>Vitis vinifera</i>	Endophyte	154721229
<i>F. culmorum</i>	Coffee Plant	Endophyte	157986165
<i>F. culmorum</i>	<i>Holcus lanatus</i>	Endophyte	240936141
<i>F. lateritium</i>	Mangrove	Endophyte	199599783
<i>F. lateritium</i>	Mexican Yew Leaf	Endophyte	203285125
<i>F. lateritium</i>	Mexican Yew Leaf	Endophyte	203285140
<i>F. chlamydosporum</i>	Mexican Yew Leaf	Endophyte	203285050
<i>F. chlamydosporum</i>	Mexican Yew Leaf	Endophyte	203285051
<i>F. chlamydosporum</i>	Mexican Yew Leaf	Endophyte	203285052
<i>F. chlamydosporum</i>	Mexican Yew Leaf	Endophyte	203285068
<i>F. chlamydosporum</i>	<i>Dracaena</i> sp.	Endophyte	295814524
<i>F. oxysporum</i>	<i>Nothapodytes nimmoniana</i>	Endophyte	205319826
<i>F. aqueductum</i>	Mangrove	Endophyte	223016912
<i>F. polyphialidicum</i>	<i>Hevea brasiliensis</i>	Endophyte	229560273
<i>F. polyphialidicum</i>	<i>Pharus virescens</i>	Endophyte	304333709
<i>F. polyphialidicum</i>	<i>Ananas comosus</i>	Endophyte	304333925
<i>F. larvarum</i>	<i>Pinus halpensis</i>	Endophyte	298356922
<i>F. arthrosporioides</i>	<i>Taxus cuspidata</i>	Endophyte	46318076
<i>F. oxysporum</i> *	Soil	Saprobe	283854593
<i>F. incarnatum</i> *	Soil	Saprobe	312064792
<i>F. oxysporum</i>	Soil	Saprobe	11692812
<i>F. oxysporum</i>	Soil	Saprobe	148733622

Table 2 (continued) *Fusarium* spp. used in the present study with their isolation sources, lifestyles and GenBank numbers.

Species	Source	Lifestyle	GenBank Index
<i>F. oxysporum</i>	Soil	Saprobe	148733623
<i>F. oxysporum</i>	Soil	Saprobe	148733625
<i>F. oxysporum</i>	Soil	Saprobe	162285917
<i>F. oxysporum</i>	Soil	Saprobe	204463578
<i>F. oxysporum</i>	Soil	Saprobe	237769607
<i>F. oxysporum</i>	Soil	Saprobe	261277540
<i>F. oxysporum</i>	Soil	Saprobe	28208780
<i>F. oxysporum</i>	Soil	Saprobe	28208780
<i>F. oxysporum</i>	Soil	Saprobe	289119520
<i>F. oxysporum</i>	Soil	Saprobe	289718953
<i>F. oxysporum</i>	Soil	Saprobe	289780330
<i>F. oxysporum</i>	Soil	Saprobe	304651424
<i>F. oxysporum</i>	Soil	Saprobe	304651425
<i>F. oxysporum</i>	Soil	Saprobe	304651428
<i>F. oxysporum</i>	Soil	Saprobe	304651429
<i>F. oxysporum</i>	Soil	Saprobe	60593117
<i>F. incarnatum</i>	Soil	Saprobe	134290455
<i>F. incarnatum</i>	Soil	Saprobe	262069425
<i>F. equiseti</i>	Soil	Saprobe	148733624
<i>F. equiseti</i>	Soil	Saprobe	162285925
<i>F. equiseti</i>	Soil	Saprobe	219523076
<i>F. equiseti</i>	Soil	Saprobe	289718950
<i>F. tricinctum</i>	Soil	Saprobe	148733626
<i>F. tricinctum</i>	Soil	Saprobe	148733629
<i>F. tricinctum</i>	Soil	Saprobe	148733631
<i>F. tricinctum</i>	Soil	Saprobe	148733632
<i>F. tricinctum</i>	Soil	Saprobe	237769552
<i>F. tricinctum</i>	Soil	Saprobe	261277537
<i>F. solani</i>	Soil	Saprobe	149132031
<i>F. solani</i>	Soil	Saprobe	197717741
<i>F. solani</i>	Soil	Saprobe	204463676
<i>F. solani</i>	Soil	Saprobe	215513486
<i>F. solani</i>	Soil	Saprobe	215513487
<i>F. solani</i>	Soil	Saprobe	215513488
<i>F. solani</i>	Soil	Saprobe	215513490
<i>F. solani</i>	Soil	Saprobe	215513491
<i>F. solani</i>	Soil	Saprobe	223366136
<i>F. solani</i>	Soil	Saprobe	237769596
<i>F. solani</i>	Soil	Saprobe	237769597
<i>F. solani</i>	Soil	Saprobe	256856198
<i>F. solani</i>	Soil	Saprobe	289780325
<i>F. solani</i>	Soil	Saprobe	289780326
<i>F. solani</i>	Soil	Saprobe	289780327
<i>F. solani</i>	Soil	Saprobe	289780328
<i>F. solani</i>	Soil	Saprobe	289780329
<i>F. solani</i>	Soil	Saprobe	304651421
<i>F. sporotrichioides</i>	Soil	Saprobe	187235530
<i>F. delphinoides</i>	Soil	Saprobe	294653635
<i>F. oxysporum</i>	Soil	Saprobe	297185611
<i>F. brachygibbosum</i>	Soil	Saprobe	298684054
<i>F. proliferatum*</i>	Immature Coconut	Phytopathogen	283854592
<i>F. cerealis</i>	Plant with Head Blight	Phytopathogen	91719251
<i>F. lunulosporum</i>	Plant with Head Blight	Phytopathogen	91719250
<i>F. brasiliense</i>	Plant with Head Blight	Phytopathogen	91719243
<i>F. mesoamericanum</i>	Plant with Head Blight	Phytopathogen	91719226
<i>F. oxysporum</i>	<i>Gladiolus</i>	Phytopathogen	82622243

Table 2 (continued) *Fusarium* spp. used in the present study with their isolation sources, lifestyles and GenBank numbers.

Species	Source	Lifestyle	GenBank Index
<i>F. oxysporum</i>	<i>Confier norsum</i>	Phytopathogen	68508671
<i>F. oxysporum</i>	<i>Confier norsum</i>	Phytopathogen	68508670
<i>F. oxysporum</i>	Asparagus root	Phytopathogen	68267976
<i>F. oxysporum</i>	Lotus	Phytopathogen	62948004
<i>F. pseudograminearum</i>	Wheat	Phytopathogen	32135112
<i>F. oxysporum</i>	Bean	Phytopathogen	302311065
<i>F. poae</i>	Wheat	Phytopathogen	288965987
<i>F. poae</i>	Zea mays	Phytopathogen	83272581
<i>F. lateritium</i>	Hezel nut	Phytopathogen	293321577
<i>F. lateritium</i>	Wheat	Phytopathogen	288965977
<i>F. solani</i>	<i>Daucus carota</i>	Phytopathogen	284507210
<i>F. incarnatum</i>	Banana	Phytopathogen	83595204
<i>F. oxysporum</i>	Chickpea wilt	Phytopathogen	260101097
<i>F. polyphialidicum</i>	Bean	Phytopathogen	224472853
<i>F. tricinctum</i>	Kiwi fruit	Phytopathogen	209360972
<i>F. oxysporum</i>	Cotton	Phytopathogen	194580114
<i>F. oxysporum</i>	Watermelon	Phytopathogen	183673382
<i>F. chlamydosporum</i>	Guava	Phytopathogen	295814506
<i>F. chlamydosporum</i>	Ginger	Phytopathogen	239819638
<i>F. oxysporum</i>	Lentil	Phytopathogen	161876806
<i>F. oxysporum</i>	<i>Citrus sinensis</i>	Phytopathogen	157073411
<i>F. sinensis</i>	Wheat	Phytopathogen	156254128
<i>F. langsethiae</i>	Wheat	Phytopathogen	151335753
<i>F. asiaticum</i>	Wheat	Phytopathogen	148596890
<i>F. asiaticum</i>	Wheat	Phytopathogen	148596892
<i>F. virguliforme</i>	soybean	Phytopathogen	148535005
<i>F. virguliforme</i>	<i>Glycine max</i>	Phytopathogen	57117619
<i>F. tucumaniae</i>	Soybean	Phytopathogen	148534981
<i>F. tucumaniae</i>	Soybean	Phytopathogen	62528633
<i>F. phaseoli</i>	Soybean	Phytopathogen	148534979
<i>F. phaseoli</i>	Soybean	Phytopathogen	37788533
<i>F. cuneirostrum</i>	Soybean	Phytopathogen	148534976
<i>F. cuneirostrum</i>	Soybean	Phytopathogen	148534977
<i>F. brasiliense</i>	Soybean	Phytopathogen	148534971
<i>F. redolens</i>	<i>Allium fistulosum</i>	Phytopathogen	148277513
<i>F. redolens</i>	Ginger	Phytopathogen	239819656
<i>F. sporotrichioides</i>	Solanaceous plant	Phytopathogen	134103851
<i>F. equiseti</i>	Wheat	Phytopathogen	126567419
<i>F. equiseti</i>	Bamboo	Phytopathogen	157704073
<i>F. oxysporum</i>	<i>Solanum tuberosum</i>	Phytopathogen	116710911
<i>F. oxysporum</i>	<i>Agave tequilana</i>	Phytopathogen	157922341
<i>F. solani</i>	Sorghum	Phytopathogen	139538584
<i>F. solani</i>	<i>Lotus japonicus</i>	Phytopathogen	146219020
<i>F. oxysporum</i>	Banana	Phytopathogen	114329283
<i>F. culmorum</i>	Wheat	Phytopathogen	126567411
<i>F. culmorum</i>	Wheat	Phytopathogen	126567412
<i>F. oxysporum</i>	<i>Confier norsum</i>	Phytopathogen	68508669
<i>F. solani</i> *	Cornea	Human Pathogen	296410104
<i>F. culmorum</i>	Cystric fibrousis patient	Human Pathogen	85375905
<i>F. solani</i>	Human	Human Pathogen	134269487
<i>F. solani</i>	Human Nail	Human Pathogen	134269492
<i>F. solani</i>	Human Skin	Human Pathogen	134269503
<i>F. solani</i>	Cornea	Human Pathogen	145909935
<i>F. solani</i>	Contact lens	Human Pathogen	198401846
<i>F. solani</i>	Contact lens	Human Pathogen	198401859
<i>F. solani</i>	Diabetic foot	Human Pathogen	45862178
<i>F. solani</i>	Nails	Human Pathogen	256575429

Table 2 (continued) *Fusarium* spp. used in the present study with their isolation sources, lifestyles and GenBank numbers.

Species	Source	Lifestyle	GenBank Index
<i>F. oxysporum</i>	Onychomycosis	Human Pathogen	194339137
<i>F. oxysporum</i>	Contact lens	Human Pathogen	198401858
<i>F. oxysporum</i>	Patient with keratitis	Human Pathogen	256575427
<i>F. oxysporum</i>	Bronchial secretions	Human Pathogen	256575431
<i>F. oxysporum</i>	Blood infection	Human Pathogen	256575437
<i>F. dimerum</i>	Cornea	Human Pathogen	198401865
<i>F. dimerum</i>	Keratitis	Human Pathogen	256575433
<i>F. dimerum</i>	Clinical source	Human Pathogen	148535529
<i>F. incarnatum</i>	Cornea	Human Pathogen	164654195
<i>F. solani</i>	Cornea	Human Pathogen	164654201
<i>F. mangiferae</i>	Bronchial secretions	Human Pathogen	256575432
<i>F. nelsonii</i>	Human	Human Pathogen	262476204
<i>F. nelsonii</i>	Human	Human Pathogen	262476206
<i>F. brachygybbsum</i>	Human	Human Pathogen	262476220
<i>F. armeniacum</i>	Human	Human Pathogen	262476232
<i>F. flocciferum</i>	Human	Human Pathogen	262476235
<i>F. equiseti</i>	Human	Human Pathogen	262476540
<i>F. equiseti</i>	Human	Human Pathogen	262476542
<i>F. equiseti</i>	Human	Human Pathogen	262476547
<i>F. equiseti</i>	Human	Human Pathogen	262476549
<i>F. equiseti</i>	Human	Human Pathogen	262476553
<i>F. equiseti</i>	Human	Human Pathogen	262476592
<i>F. equiseti</i>	Human	Human Pathogen	262476595
<i>F. equiseti</i>	Human	Human Pathogen	262476601
<i>F. equiseti</i>	Human	Human Pathogen	262476611
<i>F. lacertarum</i>	Human	Human Pathogen	262476541
<i>F. concolor</i>	Human	Human Pathogen	262476622
<i>F. virguliforme</i>	Human	Human Pathogen	270155615
<i>F. tucumaniae</i>	Human	Human Pathogen	270155616
<i>F. polyphialidicum</i>	Keratitis	Human Pathogen	39725403
<i>F. solani</i>	Trachea	Human Pathogen	56578581
<i>F. solani</i>	Ocular sources	Human Pathogen	198401863
<i>F. oxysporum</i>	Onychomycosis	Human Pathogen	194339138
<i>F. oxysporum</i>	Onychomycosis	Human Pathogen	194339139
<i>F. oxysporum</i>	Onychomycosis	Human Pathogen	194339140
<i>F. oxysporum</i>	Onychomycosis	Human Pathogen	194339144
<i>F. oxysporum</i>	Onychomycosis	Human Pathogen	194339145
<i>F. solani</i>	Ocular sources	Human Pathogen	198401847
<i>F. solani</i>	Ocular sources	Human Pathogen	198401848
<i>F. solani</i>	Ocular sources	Human Pathogen	198401867
<i>F. solani</i>	Ocular sources	Human Pathogen	198401868
<i>F. solani</i>	Corneal Infections	Human Pathogen	164654196
<i>F. solani</i>	Corneal Infections	Human Pathogen	164654197

- No data available, * Own isolates used in the present study.

of ITS sequence phylogeny did not yield evidence on variation of *Fusarium* species occupying different lifestyles, ITS2 consensus secondary structures were investigated for observation of discrepancy because structures are more conserved than sequences.

ITS2 secondary structure prediction

Consensus secondary structures of four different forms of *Fusarium* i.e. endophytes, saprobes, phytopathogens and human patho-

gens were generated and comparison of structures among the diverse forms were studied. Endophytic forms showed close structural similarities with pathogenic forms but structural features of saprobic forms were totally different. The structural similarities of endophytic and pathogenic forms were observed in their longest helices which are arrowed in Figure 2. However, variations amongst them were seen in their respective junctions, hairpin loops, terminal and internal loops. The ITS2

consensus secondary structure analysis has pro-

vided information to conclude that endophytic

Fig. 1 – Phylogenetic tree showing evolutionary relationships of 212 *Fusarium* spp. of different lifestyles. The evolutionary history was inferred using the Maximum Parsimony method. The numbers indicate GenBank indices.

Fig. 2 – Predicted global consensus ITS2 RNA secondary structures of (a) endophytes, (b) saprobes, (c) Human Pathogens and (d) Phytopathogens. Compatible base pairs are colored, where the hue shows the number of different types C-G, G-C, A-U, U-A, G-U or U-G of compatible base pairs in the corresponding columns. In this way the hue shows sequence conservation of the base pair. The saturation decreases with the number of incompatible base pairs. Thus, it indicates the structural conservation of the base pair. The similar regions are arrow marked.

forms of *Fusarium* have close affinity with pathogenic forms while sequence based phylogeny did not show this result. Such findings agree with the assumption that endophytes are latent pathogens and close relationships exist between them (Sieber et al. 2007). There are numerous examples that endophytes become pathogens, however to our knowledge there is no molecular evidences to support their affinity.

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References

Ahvenniemi P, Wolf M, Lehtonen MJ, Wilson P, German-Kinnari M, Valkonen JP. 2009 – Evolutionary diversification indicated by compensatory base changes in

ITS2 secondary structures in a complex fungal species, *Rhizoctonia solani*. Journal of Molecular Evolution 69, 150–163.

Amata RL, Burgess LW, Summerell BA, Bullock S, Liew ECY, Smith-White JL. 2010 – An emended description of *Fusarium brevicatenulatum* and *F. pseudoanthophilum* based on isolates recovered from millet in Kenya. Fungal Diversity 43, 11–25.

Anderson IC, Parkin PI. 2007 – Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules. Journal of Microbiological Methods 68, 248–253.

Albrechtsen BR, Bjorken L, Varad A, Hagner A, Wedin M, Karlsson J, Jansson S. 2010 – Endophytic fungi in European aspen (*Populus tremula*) leaves-diversity, detection, and a suggested correlation with herbivory resistance. Fungal Diversity 41, 17–28.

- Bruns TD, White TJ, Taylor JW. 1991 – Fungal molecular systematics. Annual Review of Ecology and Systematics 22, 525–564.
- Cai L, Jeewon R, Hyde KD. 2005 – Phylogenetic evaluation and taxonomic revision of *Schizothecium* based on ribosomal DNA and protein coding genes. Fungal Diversity 19, 1–21.
- Cai L, Hyde KD, Taylor PWJ, Weir B, Waller J, Abang MM, Zhang JZ, Yang YL, Phoulivong S, Liu ZY, Prihastuti H, Shivas RG, McKenzie EHC, Johnston PR. 2009 – A polyphasic approach for studying *Colletotrichum*. Fungal Diversity 39, 183–204.
- Coleman AW. 2003 – ITS2 is a double-edged tool for eukaryote evolutionary comparisons. Trends in Genetics 19, 370–375.
- Coleman AW. 2007 – Pan-eukaryote ITS2 homologies revealed by RNA secondary structure. Nucleic Acids Research 35, 3322–3329.
- Deng BV, Liu KH, Chen WQ, Ding XW, Xie XC. 2009 – *Fusarium solani*, Tax-3, a new endophytic taxol-producing fungus from *Taxus chinensis*. World Journal of Microbiology and Biotechnology 25, 139–143.
- Freeman S, Rodrigues RJ. 1993 – Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science 260, 75–78.
- Guo LD, Hyde KD, Liew ECY. 2001 – Detection and Taxonomic Placement of Endophytic Fungi within Frond Tissues of *Livistona chinensis* Based on rDNA Sequences. Molecular Phylogenetics and Evolution 20, 1–13.
- Hausner G, Wang X. 2005 – Unusual compact rDNA gene arrangements within some members of the Ascomycota: evidence for molecular co-evolution between ITS1 and ITS2. Genome 48, 648–660.
- Hyde KD, Soyong K. 2008 – The fungal endophyte dilemma. Fungal Diversity 33, 163–173.
- Hyde KD, Bussaban B, Paulus B, Crous PW, Lee S, McKenzie EHC, Photita W, Lumyong S. 2007 – Diversity of saprobic microfungi. Biodiversity and Conservation 16, 7–35.
- Keller A, Schleicher T, Forster F, Ruderisch B, Dandekar T, Müller T, Wolf M. 2008 – ITS2 data corroborate a monophyletic chlorophycean DO-group (Sphaeropleales). BMC Evolutionary Biology 8, 218.
- Keller A, Schleicher T, Schultz J, Müller T, Dandekar T. 2009 – 5.8S-28S rRNA interaction and HMM-based ITS2 annotation. Gene 430, 50–57.
- Kruger D, Gargas A. 2008 – Secondary structure of ITS2 rRNA provides taxonomic characters for systematic studies and a case in Lycoperdaceae (Basidiomycota). Mycological Research 112, 316–330.
- Landis FC, Gargas A. 2007 – Using ITS2 secondary structure to create species-specific oligonucleotide probes for fungi. Mycologia 99, 681–692.
- Miao M, Warrenb A, Songa W, Wangc S, Shanga H, Chena Z. 2008 – Analysis of the Internal Transcribed Spacer 2 (ITS2) Region of Scuticociliates and Related Taxa (Ciliophora, Oligohymenophorea) to Infer their Evolution and Phylogeny. Protist 159, 519–533.
- Muller T, Philippi N, Dandekar T, Schultz J, Wolf M. 2007 – Distinguishing species. RNA 13, 1469–1472.
- Nelson PE, Toussoun TA, Marasas WFO. 1983 – *Fusarium* species: An Illustrated Manual for Identification. Pennsylvania State University Press, University Park.
- Nilsson RH, Ryberg M, Kristiansson E, Abarenkov K, Larsson KH, Koljalg U. 2006 – Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS One 1, 59.
- Photita W, Taylor PWJ, Ford R, Hyde KD, Lumyong S. 2005 – Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. Fungal Diversity 18, 117–133.
- Pinto PM, Resende MA, Koga-Ito CY, Ferreira JA, Tendler M. 2004 – rDNA- RFLP identification of *Candida* species in immunocompromised and seriously diseased patients. Canadian Journal of Microbiology 7, 514–520.
- Promptuttha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon R. 2007 – A phylogenetic evaluation of whether endophytes become saprotrophs

- at host senescence. *Microbial Ecology* 53, 579–590.
- Promptutha I, Hyde KD, McKenzie EHC, Peberdy JF, Lumyong S. 2010 – Can leaf degrading enzymes provide evidence that endophytic fungi becoming saprobes? *Fungal Diversity* 41, 89–99.
- Purahong W, Hyde KD. 2011 – Effects of fungal endophytes on grass and non-grass litter decomposition rates. *Fungal Diversity* 47, 1–7.
- Redman RS, Ranson JC, Rodriguez RJ. 1999 – Conversion of the pathogenic fungus *Colletotrichum magna* to a nonpathogenic, endophytic mutualist by gene disruption. *Molecular Plant Microbe Interactions* 11, 969–975.
- Ruhl MW, Wolf M, Jenkins TM. 2010 – Compensatory base changes illuminate morphologically difficult taxonomy. *Molecular Phylogenetics and Evolution* 54, 664–669.
- Saikkonen K, Saari S, Helander M. 2010 – Defensive mutualism between plants and endophytic fungi? *Fungal Diversity* 41, 101–113.
- Schroers HJ, O'Donnell K, Lamprecht SC, Kammeyer PL, Johnson S, Sutton DA, Rinaldi MG, Geiser DM, Summerbell RC. 2009 – Taxonomy and phylogeny of the *Fusarium dimerum* species group. *Mycologia* 101, 44–70.
- Schulz B, Boyle C. 2005 – The endophytic continuum. *Mycological Research* 109, 661–686.
- Schultz J, Wolf M. 2009 – ITS2 sequence-structure analysis in phylogenetics: A how to manual for molecular systematics. *Molecular Phylogenetics and Evolution* 52, 520–523.
- Sieber T. 2007 – Endophytic fungi in forest trees: are they mutualists? *Fungal Biology Reviews* 21, 75–89.
- Smith C, Heyne S, Richter AS, Will S, Backofen R. 2010 – Freiburg RNA Tools: a web server integrating IntaRNA, ExaRNA and LocaRNA. *Nucleic Acids Research* 1, 38.
- Strobel GA. 2002 – Microbial gifts from rain forests. *Canadian Journal of Plant Pathology* 24, 14–20.
- Strobel GA, Daisy B. 2003 – Bioprospecting for Microbial Endophytes and Their Natural Products. *Microbiology and Molecular Biology Reviews* 67, 491–502.
- Summerell BA, Laurence MH, Liew ECY, Leslie JF. 2010 – Biogeography and phylogeography of *Fusarium*: a review. *Fungal Diversity* 44, 3–13.
- Summerell BA, Leslie JF, Liew ECY, Laurence MH, Bullock S, Petrovic T, Bentley AR, Howard CG, Peterson SA, Walsh JL, Burgess LW. 2011 – *Fusarium* species associated with plants in Australia. *Fungal Diversity* 46, 1–27.
- Tamura K, Dudley J, Nei M, Kumar S. 2007 – MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.
- Tanaka A, Christensen MJ, Takemoto D, Park P, Scotta B. 2006 – Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *Plant Cell* 18, 1052–1066.
- Tejesvi MV, Tamhankar SA, Kini KR, Rao VS, Prakash HS. 2009 – Phylogenetic analysis of endophytic *Pestalotiopsis* species from ethnopharmacologically important medicinal trees. *Fungal Diversity* 38, 167–183.
- Xu L, Zhou L, Zhao J, Li J, Li X, Wang J. 2008 – Fungal endophytes from *Dioscorea zingiberensis* rhizomes and their antibacterial activity. *Letters in Applied Microbiology* 46, 68–72.
- Young I, Coleman AW. 2004 – The advantages of the ITS2 region of the nuclear rDNA Cistron for analysis of phylogenetic relationships of insects: a *Drosophila* example. *Molecular Phylogenetics and Evolution* 30, 236–242.
- Wang Y, Guo LD, Hyde KD. 2005 – Taxonomic placement of sterile morphotypes of endophytic fungi from *Pinus tabulaeformis* (Pinaceae) in northeast China based on rDNA sequences. *Fungal Diversity* 20, 235–260.
- Wei JG, Xu T, Guo LD, Liu AR, Zhang Y, Pan XH. 2007 – Endophytic *Pestalotiopsis* species associated with plants of Podocarpaceae, Theaceae and Taxaceae in

southern China. *Fungal Diversity* 24, 55–74.

- White TJ, Bruns T, Lee S, Taylor JW. 1990 – Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, a guide to methods and applications* (eds MA Innis, DH Gelfand, JJ Sninsky, TJ White). New York, Academic Press, 315–322.