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ORIGINAL ARTICLE

ITS2 RNA secondary structure analysis reveals close affinity between endophytic and pathogenic fungi: A case study in *Fusarium* species

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Abstract Endophytes are microbes which colonize inner plant tissues without causing any disease symptoms. Many of the fungi isolated as endophytes show a close morphological resemblance to plant pathogenic fungi. It is commonly believed that pathogenic/non-pathogenic fungi become endophytic/pathogenic due to virulence loss/acquisition, respectively, but the molecular basis of such transformations and the shared characteristics are still to be elucidated. We have investigated the relationships between endophytes and pathogens based on internal transcribed spacer 2 (ITS2) sequence and secondary structure analyses in *Fusarium* as a model organism. We found that the ITS2 sequence-based phylogeny indicated close genetic proximity among endophytic and pathogenic strains of *Fusarium* species, suggesting that strains of this fungus can easily change between an endophytic and a necrotrophic lifestyle. We also observed a considerable discrepancy in the positions of bases in the ITS2 nucleotide sequences. RNA secondary structures of both endophytic and pathogenic forms of *Fusarium* were generated to distinguish between conserved and variable regions within the ITS2 sequence. The generated structures showed some structural similarities between the endophytic and pathogenic forms, with coincident variations in their respective junctions, hairpin loops, terminal loops and internal loops. Such findings suggest that *Fusarium* lifestyles are not stable but rather are dynamic and likely influenced by the genetic makeup of the fungal species, host factors and changing environment. Our research highlights the importance of the ITS2 sequence and its secondary structure as possible molecular markers to establish relationships and variations between the endophyte and pathogen lifestyle.

Keywords Endophyte · *Fusarium* spp. · ITS2 secondary structure · Pathogen

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Introduction

Fungi are highly diverse and found in divergent environments, with lifestyles that range from saprobic to symbiotic to biotrophic or necrotrophic. Fungi that colonize inner plant tissues without causing any disease symptoms are known as 'endophytic fungi'. These fungi are believed to have complex lifestyles that extend from borderline pathogenic to commensalism and ultimately to a symbiotic relationship. The relationships between endophytes and their roles as saprobes have been explored (Hyde et al. 2007, Promputtha et al. 2007, 2010; Purahong and Hyde 2011). There are also many examples of endophytes which are latent pathogens (Hyde and Soytong 2008) and of endophytes which have colonized a host asymptomatically but which behave as pathogens under changed environmental conditions.

Colonization by endophytes benefits the plant host in various ways. Endophyte colonization promotes plant growth through the production of hormones and confers enhanced resistance to various pathogens (Clay and Schardl 2002; Arnold et al. 2003) by producing antibiotics (Ezra et al. 2004). Endophytes also produce unusual secondary metabolites that are important to the plant (Taechowisan et al. 2005). Nevertheless, little is known about why some fungi live asymptomatic as endophytes whereas others cause disease symptoms after colonization and behave as pathogens. In fact, several fungal taxa

reported as endophytes closely resemble plant pathogenic fungi. There are also reports that endophytes can become parasites under certain conditions and vice versa (Müller and Kraus 2005; Schulz and Boyle 2005). Recent work has demonstrated that the switch from mutualism to parasitism is governed by a mutation in a single microbial gene (Tanaka et al. 2006). This finding indicates that endophytic and pathogenic fungi have a close affinity and suggests an inherent genomic propinquity and adaptability.

During our ongoing study on the diversity of endophytic fungi from various plant species, we have isolated several *Fusarium* species as endophytes in our laboratory (Mohanta et al. 2008; Tayung et al. 2011; Tayung et al. 2012). This fungus is commonly known to be a pathogen which can cause diseases in both plants and animals. The occurrence of *Fusarium* with endophytic and pathogenic lifestyles is quite confusing and speculative because morphologically these species are quite indistinguishable despite their different lifestyle. Although hypotheses have been proposed to explain these transitional forms (Promputtha et al. 2007), investigations at the molecular level aimed at demonstrating a shared relationship are essentially lacking.

Internal transcribed spacer (ITS) regions that comprise two transcribed intergenic spacers (ITS1 and ITS2) have been used for phylogenetic and taxonomic studies of fungi as well as for species delimitation and ecological analysis (Pinto et al. 2004; Anderson and Parkin 2007). The ITS2 region has been found to vary in terms of its primary sequence and secondary structure, suggesting its usefulness as a possible marker in molecular systematics and phylogenetic reconstruction (Schultz and 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; Samaga et al. 2014). Secondary structural data analysis of this region can also improve the phylogenetic resolution to a considerable extent (Keller et al. 2008; Rampersad 2014; Poczai et al. 2015). Therefore, the aim of this study was to investigate the relationships between endophytic and pathogenic strains within the same species based on the phylogenetic approach and ITS2 RNA secondary structure analysis, using *Fusarium* as the model organism.

Materials and methods

Isolation of the source organisms

Fusarium species were isolated from various sources (Table 1). Endophytic strains were obtained from their respective hosts using a surface sterilization procedure. Briefly, samples of healthy plant tissues were collected, thoroughly washed in distilled water, then immersed sequentially in 70 % ethanol for 3 min and 0.5 % NaOCl for 1 min and finally rinsed thoroughly with sterile distilled water. The excess water was removed by drying in laminar airflow chamber. The outer tissues were removed from the plant sample using a sterile scalpel, and the inner tissues were carefully dissected and placed on Petri dishes containing Potato Dextrose Agar (PDA) medium and incubated at 25 °C. Pathogenic *Fusarium* sp. was isolated from diseased tissue of coconut palm.

Isolation of genomic DNA, PCR amplification and sequencing

The fungi were first cultured on PDA medium, and a small amount of the mycelia was subsequently suspended in 40 μl Milli Q water. Genomic DNA was isolated by the cetyltrimethyl ammonium bromide (CTAB) method (Clarke 2009). A portion of the genomic DNA was diluted to 50 ng/μl for use in the PCR analyses. The nuclear ribosomal DNA and ITS region of the isolate were amplified using the universal primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR reaction mix contained 2.5 μl buffer (10×), 1.5 μl $MgCl_2$

Table 1 Isolation source, lifestyle and accession number of fungi used for the internal transcribed spacer RNA secondary structure study

Fungal species	Lifestyle	Isolation source	Country	GenBank accession
Fusarium solani	Endophytic	*Taxus baccata*	India	FJ719812.1[a]
F. solani	Endophytic	*Rhizoparacea* sp.	China	GU595038.1
F. solani	Pathogenic	*Theobroma cacao*	Mexico	KF010166.1
F. solani	Pathogenic	*Theobroma cacao*	Mexico	KF010168.1
F. proliferatum	Endophytic	*Ipomoea carnea*	India	HM145946.1[a]
F. proliferatum	Endophytic	*Morinda citrifola*	Malaysia	GU066627.1
F. proliferatum	Pathogenic	Immature coconut	India	GU363955.1[a]
F. proliferatum	Pathogenic	Mango	China	GU074004.1

[a] Isolates from our own laboratory

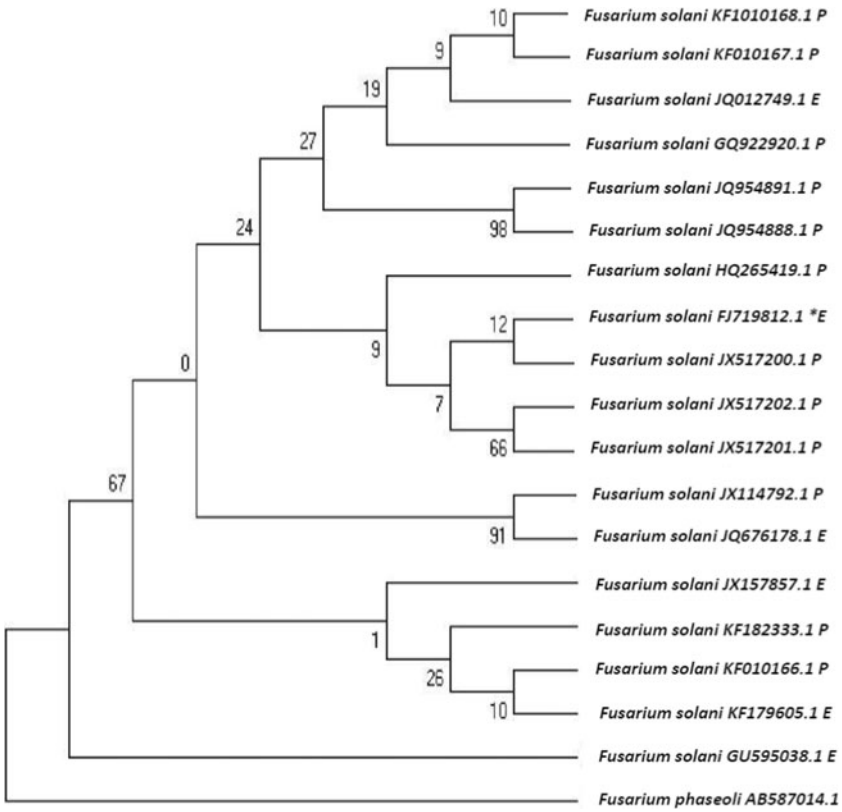


Fig. 1 Phylogenetic tree showing evolutionary relationships of 19 taxa of *Fusarium solani* which occur as an endophyte (*E*) or pathogen (*P*) isolated from various sources. *Asterisk* Isolate of *F. solani* from own laboratory

(25 mM), 2.5 μl dNTPs (2 mM), 0.2 μl Promega Taq (5 U/μl; Promega Corp., Madison, WI), 1.0 μl each of forward and reverse primers (5 pm/μl) and 6.0 μl DNA from the diluted extract. The amplification conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 45 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min and holding at 4 °C. After PCR cycling, a 2-μl sample of the product was run on a 1 % agarose gel as control. DNA sequencing was performed using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA).

Taxon sampling

An intensive search for sequences of *Fusarium* with endophytic and pathogenic lifestyles was also carried out using the GenBank database. Altogether 967 sequences of *F. solani* and 262 sequences of *F. proliferatum* were retrieved from GenBank (on 26-10-2013). The sequences were filter-searched, and those sequences having 18S partial, ITS1, 5.8S, ITS2 and 28S partial rRNA genes (ITS rDNA) were selected for phylogenetic analysis. Similarly, the sequences were also trimmed for ITS2 using annotation tools based on the Hidden Markov Model (Keller et al. 2008). *F. solani* and *F. proliferatum* with ITS2 sequences were used to generate RNA secondary structures.

Phylogeny and RNA secondary structure analysis

For phylogenetic analysis, multiple sequence alignments were performed using CLUSTALW software utilizing default settings, and phylogenetic trees were generated by the character-based maximum parsimony method

Table 2 Nucleotide variations in internal transcribed spacer 2 of the studied pathogenic and endophytic isolates of *Fusarium solani*

Position of base	Pathogens		Endophytes	
	KF010168	KF010166	GU595038	FJ719812[a]
22	G	**C**	G	G
25	T	**C**	T	T
43	A	A	**G**	G
44	G	G	**A**	**A**
45	G	G	G	**A**
46	C	C	C	**G**
52	T	T	**C**	T
54	C	**T**	**T**	C
61	A	**G**	A	A
106	T	T	**C**	T
128	A	A	**G**	A

Nucleotide variations are highlighted in bold font

[a] Isolate from our own laboratory

using MEGA 4.0 (Tamura et al. 2007) . RNA secondary structures were generated using the mfold web server (Zuker 2003).

Results and discussion

Fusarium species are ubiquitous and found in diverse environments. They can exist in different lifestyles, such as saprophytes (Fracchia et al. 2000), plant pathogens (Chandra et al. 2008) and endophytes (Bacon and Hinton 1996). Morphologically they may be identical and therefore difficult to study. Thus, there is a need to utilize molecular-based methods to differentiate *Fusarium* taxa of different lifestyles. To this end, we have attempted to differentiate pathogenic and endophytic forms of *Fusarium* and to reveal their close genomic proximity. Of the *Fusarium* species studied, three isolates originated from our own laboratory, with one isolated as a pathogen from infected coconut palm and the other two obtained as endophytes from surface-sterilized healthy tissues of *Taxus baccata* and *Ipomoea carnea*. Species confirmation of the isolates was carried out by molecular characterization of the ITS region of the rDNA sequence since morphological characterization of *Fusarium* species has been always tedious

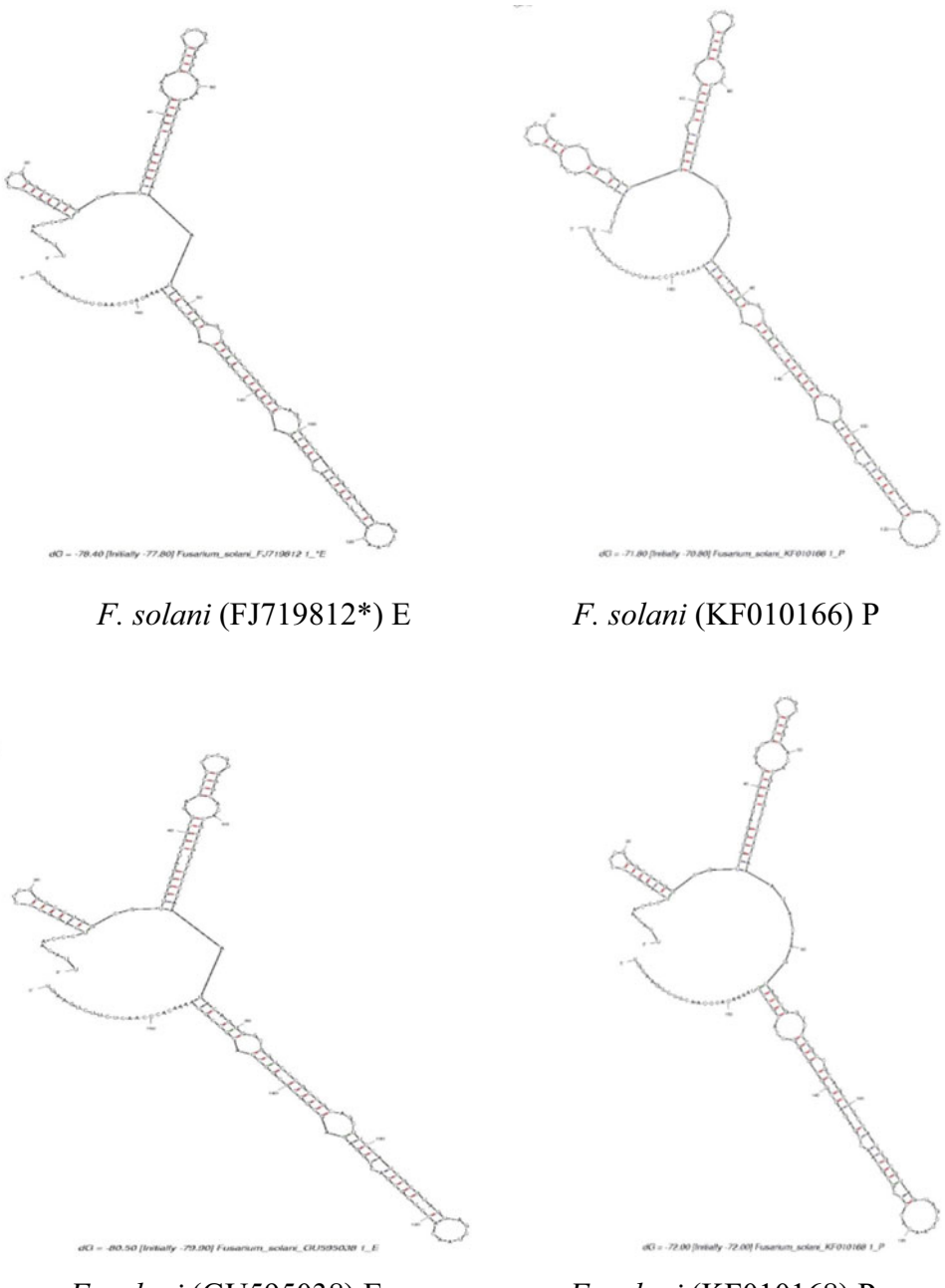


Fig. 2 Internal transcribed spacer 2 (ITS2) RNA secondary structures of pathogenic (*P*) and endophytic (*E*) isolates of *F. solani*. Accession number is given in parenthesis

and difficult. The BLAST search analysis of the sequences revealed the pathogenic isolate to be *F. proliferatum* and the two endophytic isolates *as F. solani* and *F. proliferatum* (Table 1). We also retrieved sequences of *Fusarium* with endophytic and pathogenic lifestyles from GenBank, selecting only those sequences having 18S partial, ITS1, 5.8S, ITS2 and 28S partial rRNA genes (ITS rDNA) for phylogenetic analysis. Separate phylogenetic tree was constructed for each species, taking into account both endophytic and pathogenic lifestyles. ITS rDNA-based phylogeny of *F. solani* revealed that the species did not cluster together according to lifestyle. The phylogenetic tree indicated that pathogenic and endophytic forms of *F. solani* shared similar clades and did not form distinctive clades according to individual lifestyle. The tree also showed several pathogenic and endophytic forms of *F. solani* clustered together within the clade. The phylogenetic tree was also reconstructed using ITS2 sequences to enhance phylogenetic resolution. This approach revealed that although most of the species did not cluster together according to lifestyle, there was some degree of uniformity present (Fig. 1), indicating that there is close genetic proximity between the endophytic and pathogenic strains. These results also suggest that *Fusarium* species could have both endophytic and pathogenic stages in their life cycle. This possibility is supported by the results of several phylogenetic studies which also indicate the transformation of an endophytic fungus to a pathogenic lifestyle and vice-versa, thus suggesting that fungi can easily change between an endophytic and a necrotrophic lifestyle (Carroll 1988; Freeman and Rodriguez 1993; Hyde and Soytong 2008; Eaton et al. 2011).

Studies have indicated that fungi may express different lifestyles in response to host genotype or environmental factors. This phenomenon has been well demonstrated in *Colletotrichum magna* which under certain conditions endophytes may become pathogens that causes symptomatic infection and vice-versa (Freeman and Rodriguez 1993). Such findings suggest that a gene mutation or nucleotide change may control the switch in lifestyle, either to mutualism through the loss of pathogenicity or, alternatively, to pathogenicity that enables a wide range of hosts to be invaded. Therefore, we attempted to determine whether any variations do exist in the ITS2 nucleotide sequence of pathogenic and endophytic *F. solani*. We chose this nucleotide sequence for study since sections of ITS2 transcripts are consistently predicted to form conserved stem-loop structures. In addition, complementary base changes and the recovery of secondary structure motifs

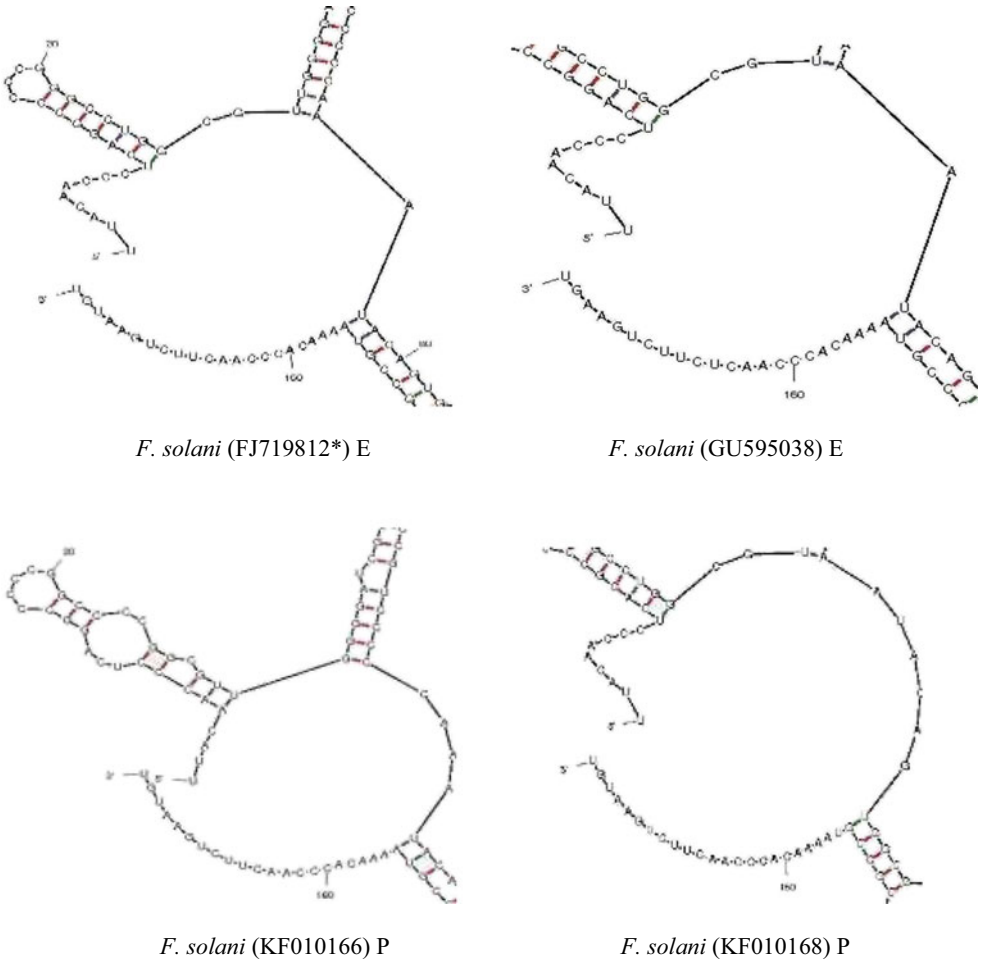


Fig. 3 Variation in RNA secondary structure in endophytic (*E*) and pathogenic (*P*) isolates of *F. solani*. Accession number is given in parenthesis

F. solani (FJ719812*) E

F. solani (GU595038) E

F. solani (KF010166) P

F. solani (KF010168) P

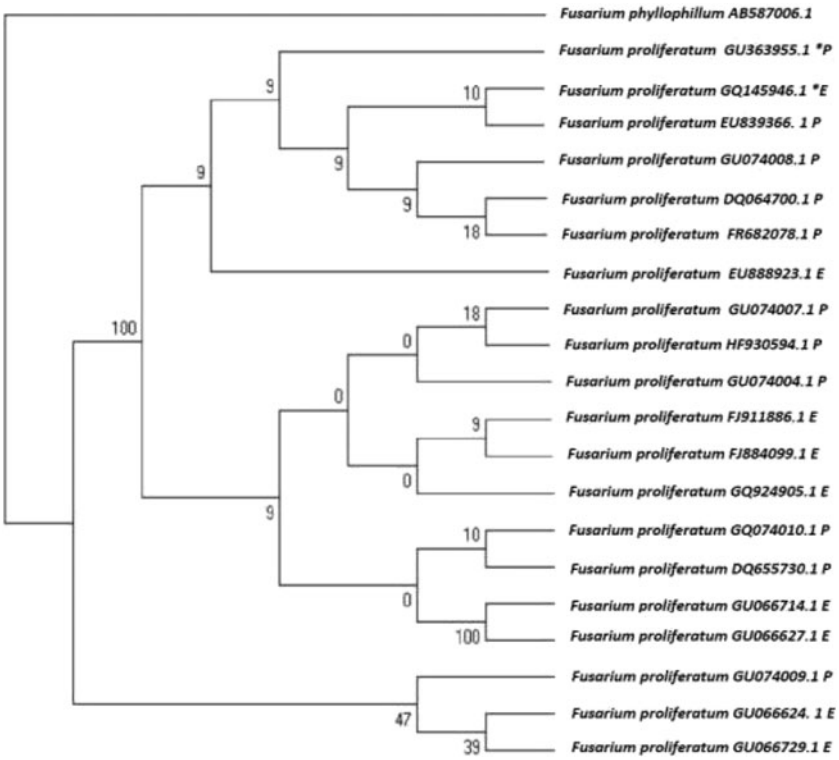


Fig. 4 Phylogenetic tree showing evolutionary relationships of 20 taxa of *F. proliferatum* occurring as endophyte (*E*) and pathogens (*P*) isolated from various sources. *Asterisk* Isolate of *F. proliferatum* from own laboratory

independent of primary sequence renders the secondary structure highly appropriate for sequence alignment and taxonomy studies (Krüger and Gargas 2008; Rampersad 2014). Our results revealed a considerable discrepancy in the positions of different bases in the nucleotide sequences (Table 2). Since structures are more conserved than sequences, we also generated RNA secondary structures of both endophytic and pathogenic forms of *F. solani* in order to distinguis between the conserved and variable regions within the ITS2. The generated structures showed some structural similarities between the endophytic and pathogenic forms (Fig. 2), but coincident variations in their respective junctions, hairpin loops, terminal loops and internal loops were also observed (Fig. 3). These results indicate that endophytes possess structural similarities with pathogens and that many of the former might have the same genomic module as the latter, such as virulence factors. There is also evidence suggesting that endophytes have evolved directly from plant pathogenic fungi (Carroll 1988; Isaac 1992). It is therefore possible that the switching off and/or on of virulence factors may determine whether the fungus behaves as an endophyte or a parasite. Our findings also agree with the hypothesis that relationship between endophyte and pathogen can be considered to be plastic and a condition of balanced antagonism with their respective host.

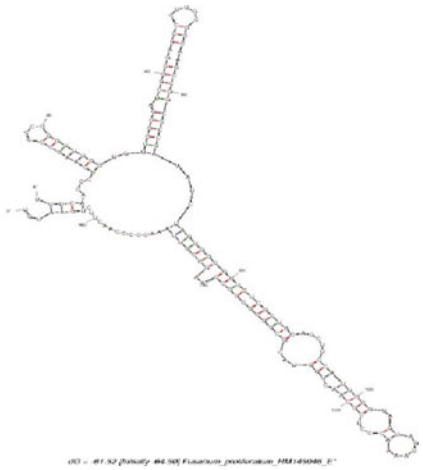
The ITS2-based phylogeny of *F. proliferatum* revealed that the studied species clustered together and formed more or less distinctive clades according to their lifestyle (Fig. 4), indicating few variations in the nucleotide sequences of pathogenic and endophytic forms. This was also observed in the ITS2 nucleotide sequences where only 20 % of the species studied showed variations (Table 3). Similarly, the RNA secondary structures indicated a low degree of discrepancy but the disparity present within the endophytic and pathogenic forms suggested distinct and conserved structural features in the species (Fig. 5). The endophytic and pathogenic forms showed variations only in the internal loops of their secondary structures (Fig. 6). Such findings suggest that *Fusarium* lifestyles are not stable but dynamic and that they are likely to be influenced by the genetic makeup of the fungal species, by host factors and by changing environments. While *Fusarium* species are considered to be destructive plant pathogens, in recent years several *Fusarium* species have been isolated and subsequently

Table 3 Nucleotide variations in internal transcribed spacer 2 of pathogenic and endophytic isolates of *Fusarium proliferatum* used in the present study

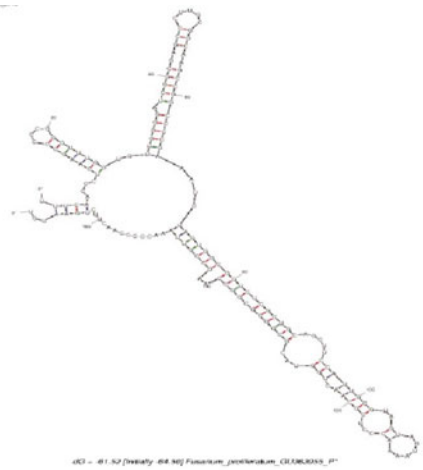
Position of base	Pathogens		Endophytes	
	GU363955[a]	GU074004	HM145946[a]	GU066627
49	T	T	T	**C**
68	A	**G**	A	A
89	C	C	C	**T**
149	A	A	A	**G**

Nucleotide variations are highlighted in bold font

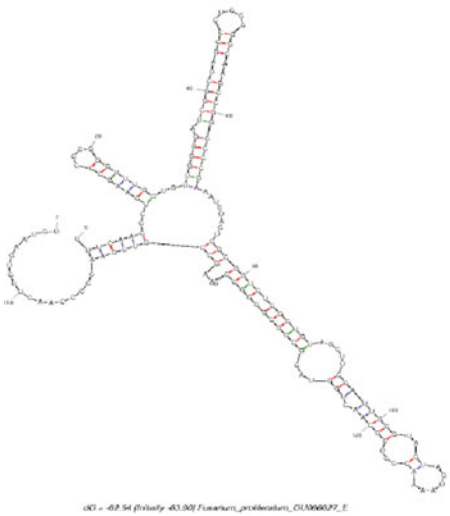
[a] Isolate from our own laboratory



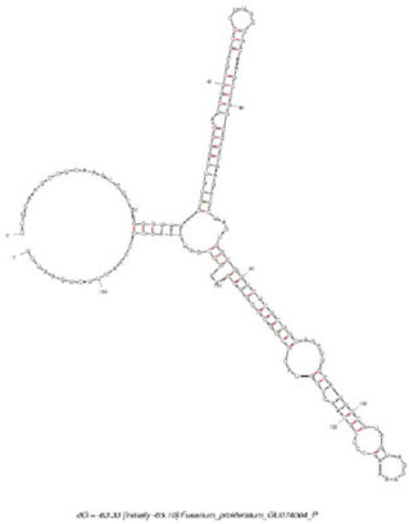
F. proliferatum (HM145946*) E



F. proliferatum (GU074004*) P

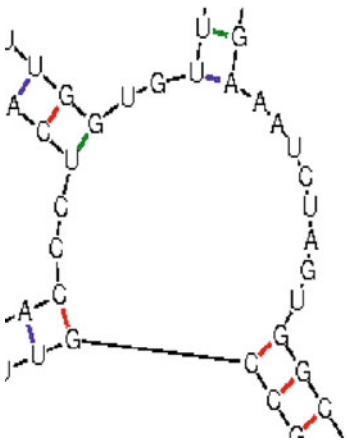


F. proliferatum (GU066627) E



F. proliferatum (GU074004) P

Fig. 5 ITS2 RNA secondary structures of pathogenic (*P*) and endophytic (*E*) *Fusarium proliferatum*. Accession number is given in parenthesis



F. proliferatum (GU066627) E

F. proliferatum (GU074004) P

Fig. 6 Variation in ITS2 secondary structure of endophytic (*E*) and pathogenic (*P*) *F. proliferatum*. Accession number is given *in parenthesis*

identified to be endophytes, including those from many types of plants such as Araceae, Bromeliaceae, Orchidaceae (Petrini and Dreyfuss 1981), Palmae (Rodrigues 1994), Compositae (Fisher et al. 1995), Musaceae and Cactacee (Ratnaweera et al. 2015) and Leguminose (Fernandes et al. 2015). Thus, host–*Fusarium* interactions can range from mutualism through to commensalism and ultimately to parasitism and may depend on various factors. Results from recent studies indicate that fungi may express different lifestyles in response to host genotypes, virulence factors, host defense response and environmental factors which influence the phenotypic plasticity of both partners. The genetic diversity and variability within the *Fusarium* species complex may have facilitated switching between an endophytic and necrotrophic lifestyle at the evolutionary and even ecological timescale. Furthermore, the prevalence of protein disorders is quite high in all forms of *Fusarium* species, implying a complexity and ability to adapt to diverse environments (Barik and Tayung 2012). The results of our study with *Fusarium* species as model organism reveal that mutualistic endophytic fungi have a close genetic proximity with their pathogenic ancestors and are in agreement with the notion that pathogens may either express non-pathogenic lifestyles or infect and remain dormant until plant senescence. Our research highlights the importance of the ITS2 sequence and its secondary structure as a possible molecular marker to establish relationships and variations between endophyte and pathogen.

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