Studies in Fungi 2 (1): 26–37 (2017) www.studiesinfungi.org ISSN 2465-4973
Article

Doi 10.5943/stif/ 2/1/4

The molecular phylogeny and taxonomy of endophytic fungal species from the leaves of *Vitex negundo* L.

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Ramesh V, Arivudainambi USE, Rajendran A 2017 – The molecular phylogeny and taxonomy of endophytic fungal species from the leaves of *Vitex negundo* L. Studies in Fungi 2(1), 26–38, Doi 10.5943/sif/2/1/4

Abstract

Enormous fungal species live within the healthy plant tissues, some of which presumably occur in a symbiotic association with host. Some fungal endophytes are widespread and can be found in many different plant species, whereas others are highly specific to single hosts. In this study, we isolated three endophytic fungi from the medicinal plant *Vitex negundo*. They were identified based on morphological characteristics such as size, shape, and colour of the spore and it was reinforced by 18s rRNA gene sequence analysis. The phylogenetic tree showed that C. *gloeosporioides* VN1 and *Pestalotiopsis virgatula* VN2 were closely relationship between. But they were not closely relationship between the other endophytic fungal species that were obtained from geographically different part of the world. This aspect can be further explored to understand the relationships between plant hosts and their fungal endophyte.

Key words – Endophytic fungi – MEGA 6.0 – phylogenetic relationship – rRNA

Introduction

Endophytes are to be found in virtually every plant on earth. They reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic (Strobel et al. 2004). Endophytes receive nutrition and protection from the host plant, while the host plant may benefit from enhanced competitive abilities and increased resistance to herbivores, pathogens, and various abiotic stresses by attaining the metabolic substances of endophytes (Saikkonen et al. 1998, Tan & Zou 2001, Zhang et al. 2006). Endophytic fungi have been found in all plant families so far investigated, which represent many species in different climatic regions of the world (Spurr & Welty 1975, Petrini & carroll 1981, Petrini et al. 1992). Endophytes have been reported from all major groups of plants including algae (Zuccaro et al. 2008, Suryanarayanan et al. 2010), lichens (Suryanarayanan et al. 2005), mosses (Schulz et al. 1993), ferns (Petrini et al. 1992), conifers (Giordano et al. 2009) and angiosperms (Saikkonen 2007), and may persist even in aseptically cultured plants (Lucero et al. 2008). Endophytic fungi are reported from plants that grow in various environments including tropic (Mohali et al. 2005), temperate (Ganley et

Submitted 16 August 2016, Accepted 20 February 2017, Published online 27 March 2017
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al. 2004), xerophytic (Suryanarayanan et al. 2005) coastal mangroves (Kumaresan & Suryanarayanan 2001, Okane et al. 1998) and aquatic environment (Sati & Belwal 2005). Environment plays an important role on endophyte biodiversity, while the species diversity is dependent upon the nature of the host plant and their ecological location.

Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Strobel et al. 2004, Wiyakrutta et al. 2004, Kumar et al. 2005, Tejesvi et al. 2007). Plant with pharmaceutical importance is being exploited because of their healing properties. However, large scale harvesting of medicinal plants has already become a major threat to biodiversity. As an alternative, microbe which lives inside the plants (endophytes) may often become a tremendous potential source of therapeutic compounds.

Traditional classification and identification of endophytic fungi depends upon microscopic features, colony characteristics on artificial media and biochemical reactions (Sutton & Cundell 2004). This kind of methods have served in the past but they have major drawbacks as they cannot be applied to non cultivatable organisms and occasionally biochemical characteristic of some organisms do not fit into the patterns of any known genus and species. Amplification and sequencing of target regions within the ribosomal DNA gene complex has emerged as a useful adjunctive tool for the identification of endophytic fungi and does not depend on fungus sporulation for identification (Buzina et al. 2001, Iwen et al. 2002, Rakeman et al. 2005, Schwarz et al. 2006).

The key elements for the evolution of the endophytes are quite complex, involving various types of interactions between the host plant, numerous levels of happenstance, and multidirectional flows, they are also influenced by random events, such as living and non living factors, which guide the process of co-evolution between endophytic fungi and their hosts (Saikkonen et al. 2004).

Eventhough knowledge regarding the ecology, life cycle and phylogeny of endophytic fungi has quickly increased and accumulated over the last three decades, questions concerning their evolutionary origin, species and ecological role are not yet completely understood (Saikkonen et al. 2004). There is good reason to believe that partnership co-evolution was essential for the survival of both, and in this case, the symbiosis was mutualistic (Read et al. 2000).

The ribosomal DNA (rDNA) is present in all organisms and its evolution is rapid, so it is used to discriminate related species or even varieties of the same species. The ITS regions are flanked by preserved segments (18S, 5.8S and 28S genes). These preserved regions provide the information about the phylogeny and the taxonomic level, since their evolution is slow and they are highly similar within different taxa

Considering the importance of the *Vitex negundo* L. as a medicinal plant, the aim of the present work was to determine the phylogenetic relationship of three endophytic fungi comparison with other endophytes from different geographic region that were deposited in NCBI database.

Materials & Methods

Plant material and study area

Healthy leaves of medicinal plant *Vitex negundo* L. were collected from various seasons in Botanical garden, Department of Botany, VHNSN College, Virudhunagar, Tamil Nadu, India (Fig.1) Leaves were cut from the plants and placed in plastic bags after removal of excess moisture. The leaf samples were stored at 4°C.

Isolation of endophytic fungi

The leaf samples were washed thoroughly under running tap water and air dried before they were processed. An endophytic fungus was isolated according to the reported protocol (Petrini 1986), which was modified slightly based on preliminary testing. All the leaf samples were washed twice in distilled water and then surface sterilized by immersion for 1 min in 70% v/v ethanol, 4 min in sodium hypochlorite (3% v/v available chlorine) and 30 s in 70% v/v ethanol, and further washed three times in sterilized distilled water for 1 min each time. After surface sterilization, the samples were cut into 5-7 mm pieces and aseptically transferred to Petri plates containing potato dextrose agar (PDA) with 50 µg/mL of streptomycin to suppress bacterial growth. The Petri plates were incubated at 30°C with normal daily light and dark periods. The plates were examined daily for up to 1 month for the development of fungal colonies growing on the leaf segments. The fungi growing on the leaf tissue were subsequently transferred onto fresh PDA plates without antibiotics.

Microscopic analysis

The endophytic fungi were grown on PDA at 30 ^{o}C for 7 - 9 d, and the formation of conidia was examined under a microscope. Moreover, slide culture technique was also used to observe the morphology of the fungi. For spore dimensions determinations we were used 50 spores. Lacto phenol cotton blue and distilled water were used as mounting media for microscopic analysis. Photography was carried out with the assistance of light microscope and binocular microscope (COSLAP) with computer attached. The isolated endophytic fungi were identified at Centre for Advanced Studies in Botany, University of Madras, Tamil Nadu, India.

DNA extraction, amplification and sequencing

Fungal isolates were incubated a week at 30 ˚C on PDA. The mycelia were harvested and transferred into 2 ml plastic tubes using a sterile spatula and lyophilized for DNA isolation. Genomic DNA was isolated by using the method of Doyle & Doyle **(**1987**).** Further, the ribosomal DNA amplification, ITS1-5.8S-ITS2 region, was carried out and primers ITS1 and ITS4 were used as described by White et al. (1990). Isolates of 18s rRNA fungal sequences obtained were submitted to GenBank (NCBI, USA) (accession numbers: HQ191217, JF795287 and JF795288). All the studies of DNA isolation and sequencing were done by Synergy Scientific Services, Chennai.

Phylogenetic analysis

Phylogenetic analysis was conducted in MEGA 6 software (Tamura et al. 2007). Sequenced ITS1-5.8S-ITS2 regions were aligned initially using the alignment algorithm Clustal W (Thompson et al. 1997) with the gap open penalty 7.0 and gap extension penalty 4.0. Due to some variation in areas of ITS1 and ITS2 regions, an alignment was then improved manually. The evolutionary history was inferred using the neighbor joining method (Saitou & Nei 1987). All positions containing gaps with missing data were eliminated from the dataset. Strengths of internal branches of resulting trees were statistically tested by the bootstrap analysis of 1000 replications **(**Felsenstein 1985). Additional sequences were retrieved from GenBank (Table 1).

Results

Taxonomy

Fungal isolate VN1

The morphological characteristics of the endophytic fungal isolate VN1 was observed

on PDA after 7 days of growth at 30 ^{o}C. Colonies on PDA was circular, raised, at first orange-white, sometimes grey and becoming pale orange with age, aerial mycelia white dense, cottony without visible conidial masses, reverse bright orange but sometimes yellowish-brown to olive-brown and very slow-growing. Acervuli and Setae were absent in culture. Conidia were hyaline, unicellular and cylindrical with obtuse apices and tapering bases. Average conidial size was 14.7 × 3.8 µm. (Fig. 2).

Traditionally, identification of *Colletotrichum* sp. have been based on size and shape of conidia and culture characteristics such as colony colour, growth rate and texture (Smith et al. 1990). Morphological characteristics allowed the identification of the endophytic fungal isolate VN1 as *Colletotrichum gloeosporioides*, which was reinforced by the sequence of its 18S rRNA that gave a 91% sequence similarity to those accessible at the BLAST of *Colletotrichum gloeosporioides* (Fig. 3). The endophytic fungal sequence was deposited at GenBank with Accession No. HQ191217.

Fungal isolate VN2

The fungus growing on PCA was pale buff with sparse aerial mycelium and acervuli containing black, slimy spore masses (Fig.4). All isolates had 5-celled conidia, apical and basal cells were hyaline, while the three median cells were olivaceous; the upper two were slightly darker than the lower one. Conidia were 20.3 × 6.8 µm. They were typically three apical appendages averaging 16.8 µm long. The average basal appendage was 3.8 µm long (Fig. 4). The fungal isolate was initially identified by comparing morphological and cultural characteristics (Size of the conidia, color and length of median cells, length and number of apical appendages and length of basal appendage) to those described in Guba's monograph of *Monochaetia* and *Pestalotia* (Guba 1961).

Morphological characteristics allowed the identification of the endophytic fungal isolate VN2 as *Pestalotiopsis virgatula* which was reinforced by the sequence of its 18S rRNA gene that gave a 98% sequence similarity to those accessible at the BLAST of *Pestalotiopsis virgatula* (Fig. 5). The endophytic fungal sequence was deposited at GenBank Accession No. JF795287.

Based on the above morphological and molecular characteristics the endophytic fungus was identified and designated as *Pestalotiopsis virgatula* VN2. The endophytic fungus *P. virgatula* VN2 belongs to the class Ascomycota.

Fungal Isolate VN3

The fungal isolate VN3 grows rapidly on PDA medium and matures within 5 d. The colony is flat, downy to cottony and may eventually be covered by greyish, short, aerial hyphae. The reverse side is typically brown to black due to pigment production (Fig. 6). They have septate, dark hyphae. They bear simple or branched large conidia (8-16 × 23-50 µm) which have both transverse and longitudinal septations. These conidia may be observed singly or in acropetal chains and may produce germ tube. They are ovoid to obclavate, darkly pigmented muriform, smooth or roughened. The end of the conidium nearest the conidiophere is round while it tapers towards the apex (Fig. 6).

Morphological characteristics of fungus allowed the identification of the endophytic fungal isolate VN3 as *Alternaria alternata* which was reinforced by the sequence of its 18S rRNA gene that gave a 95% sequence similarity to those accessible at the BLAST of *Alternaria alternata*. The endophytic fungal sequence was deposited at GenBank with Accession No. JF795288 (Fig. 7). This endophytic fungus *A. alternaria* VN3 belongs to the class Ascomycota.

Phylogenetic analysis

Phylogenetic relationships inferred from ITS1-5.8S-ITS2 region sequences of three species are shown in Figure 8. The tree is divided into three main clusters (A, B and C) and further each one divided into two sub-clusters like A1, A2, B1, B2, C1 & C2. Based on the evolution, among the present three fungal endophytes *Colletotrichum gloesporoides* VN1 and *Pestalotiopsis virgatula* VN2 were grouped into the single sub cluster B2. Another present endophytic fungal species of *Alternaria alternata* VN3 was located in the subclaade A1. In sub-cluster A1 *Colletotrichum gloeosporoides, Pestalotiopsis funerea* strain SYJM13, *Pestalotiopsis* sp. Strain F4875 and *Alternaria alterna* VN3 were grouped together. *Pestalotiopsis* sp. MA165, *Pestalotiopsis* sp. MA129 and *A. compacta* strain IR13 were grouped together in sub-cluster A2. In sub-cluster B1 *C. gloeosporiodes* strain FL1-ML2, *Alternaria* sp. Abs and *Pestalotiopsis* sp. Z4-08 were grouped together. The phylogenetic tree results showed *C. gloeosporioides* VN1 and *Pestalotiopsis virgatula* VN2 were closely relationship between. But they were not closely relationship between the other endophytic fungal species that were obtained from geographically different parts of the world.

Discussion

Vitex negundo L. (*Verbenaceae*) is a woody, aromatic shrub growing to a small tree. It commonly bears tri or penta foliate leaves on quadrangular branches, which give rise to bluish purple colored flowers in branched tomentose cymes. It thrives in humid places or along water courses in wastelands and mixed open forests and has been reported in many countries. It is grown commercially as a crop in parts of Asia, Europe, North America and the West Indies (de Padua et al. 1999). It is an important medicinal plant used in the traditional medicine and has a variety of pharmacological activities. Hence, in the present study we were used this plant as a host for endophytic fungal isolation. Three endophytic fungal species were isolated from leaves of *V. negundo*.

Table 1 Species and GenBank accession number used in the study

Name of the Species	**Geographic origin**	**GenBank No**
Colletotrichum gloeosporioides VN1*	India	HQ191217
Colletotrichum gloeosporioides Strain JS1-SAS12	China	KP900300
Colletotrichum gloeosporioides Strain FL1-ML2	China	KP900236
Colletotrichum gloeosporioides Strain W-2	China	HQ845101
Colletotrichum gloeosporioides Strain JL5	China	KM513573
Colletotrichum gloeosporioides Strain FL1-CJL1	China	KP900235
Pestalotiopsis virgatula VN2*	India	JF795287
Pestalotiopsis funerea strain SYJM13	India	JF923833
Pestalotiopsis sp. Z4-08	China	HQ262524
Pestalotiopsis sp. 1 AE-2013 Strain F4875	Panama	KF746126
Pestalotiopsis sp. MA129	Thailand	GQ254681
Pestalotiopsis sp. MA165	Thailand	GU592005
Alternaria alternata VN3*	India	JF795288
Alternaria compacta Strain IR13	Iran	KU323573
Alternaria sp. HT-M18-LS	China	KJ527010
Alternaria sp. HT-M18-L	China	KJ527009
Alternaria sp. Abs	Serbia	JF742668
Alternaria sp. B5A	USA	EF432299

Asterisks indicate the sequences obtained from the present study

Endophytic fungal species are complex anamorphic genus. For example *Pestalotiopsis* was established by Steyaert (1949). It can be lived as saprobes, plant pathogens or endophytes (Suto & Kobayashi 1993, Rivera & Wright 2000, Karakaya 2001, Gonthier et al. 2006, Sousa et al. 2004). The identification of endophytic fungal species based on morphology is however, complicated because there are few morphological characters available to distinguish taxa at the species level. Hence, nuclear small subunit ribosomal RNA gene regions are usually used as a molecular tool to analyze fungal taxa at a family or order level and ITS regions are commonly used to examine phylogenetic positions or relationship at a species or intra species level. Morphological characters are important in identifying *Pestalotiopsis* species (Steyaert 1949, Guba 1961, Sutton 1980, Nag Raj 1993). Characters used however are few and often overlap. This results in identification problems and difficulties in differentiating species. In the case of *Colletotrichum* sp. molecular phylogeny has been helpful in establishing species concepts (Photita et al. 2005).

Our fungal strains formed a segregated claade with *A. alternata*, *C. gloeosporioides* and *P. virgatula* supported by low bootstrap values of 38, 18 and 46 %, respectively. Similar results were obtained in the phylogenetic analysis of *Xylaria* species from Western Gahts of Curtallum Hills (Ramesh et al. 2012). Phylogenetic analysis, based on rDNA sequencing, enabled us to show that there is genetic variability among the isolates of the three endophytic fungi. Moreover, they were not closely related between the other endophytic fungal species that were obtained from geographically different parts of the world.

Fig. 1 - Medicinal plant *Vitex negundo* L.

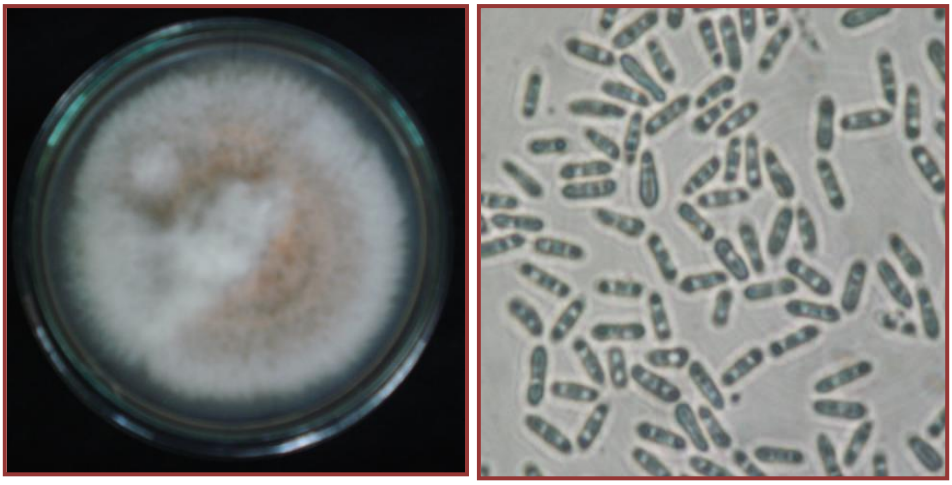

Fig. 2 - Morphology characteristics of *Colletotrichum gloeosporioides*

TCTACACCCTTTGTGACATACCTATAACTGTTGCTTCCGCGGGTAAGGTCCCCGT
GACCCTCCCGGGCTCCCGCCCCCCGGGCGGGTCGGCGCCCGCCCGAAGAAAACC
CAACTCTGATTTAACGACCTTTCTTCTGAATGGTACAAGCAAATAATCCAAACTT
TTAACAACGGATCTCTTGGTTCTGGCATCCATGAAAAACGCAGCGAAATGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCGCCCACATTCTGGCGGGCATGGCTGTTCCAACGTCCTTTTCAACCCTCAAG
CTCTGCTTGGTGGTGGGGGCCCTACACTGATGTTAGGCCCTCAAGGTAATGGCGG
AACCCTCCCCGAACCCCCTTTGCGTTATAACTTTTACGTCTCGCACTGGGGATCC
GGAAGGGACTCCTTGCCCGAAAACCCCCAATTTTTCCAAAGGTTGACCTCGGATC
AGGTAAGAAATACCCCGCTGAACTTTAACATATCAATAACCGGAAGA

Fig. 3 - 18S rRNA sequence of *Colletotrichum gloeosporioides*

Colony morphology Conidial spore (200 X)

Fig. 4 - Morphology characteristics of *Pestalotiopsis virgatula*

TGTGAACTTACCTTTTGTTGCCTCGGCAGAAGTTATAGGTCTTCTTATAACTGCTG
CCGGTGGACCATTAAACTCTTGTTATTTTATGTAATCTGAACGTCTTATTTTAATA
AGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
CGCACATTGCGCCCATTAATATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCA
ACCCTTAAGCCTAACTTAGTGTTGGGGAATCTACTTCTTTATAGTTGTAGTTCCTG
AAATACAACGGCGGATTTGTAGTATCCTCTGAGCGTAGTAATTTTTTTCTCGCTTT
TGTTAAGTGCTATAACTCCCAGCCGCTAAACCCCCAATTTTTTGTGGTTGACCTCG
GATCACGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAATAA
CCTTTTTTAGTTTTCTAATCTCCATCCATGTGACTTACCTTTAGTTGACTCGCAAA
GTTATATGTCTTCTT

Fig. 5 - 18S rRNA sequence of *Pestalotiopsis virgatula*

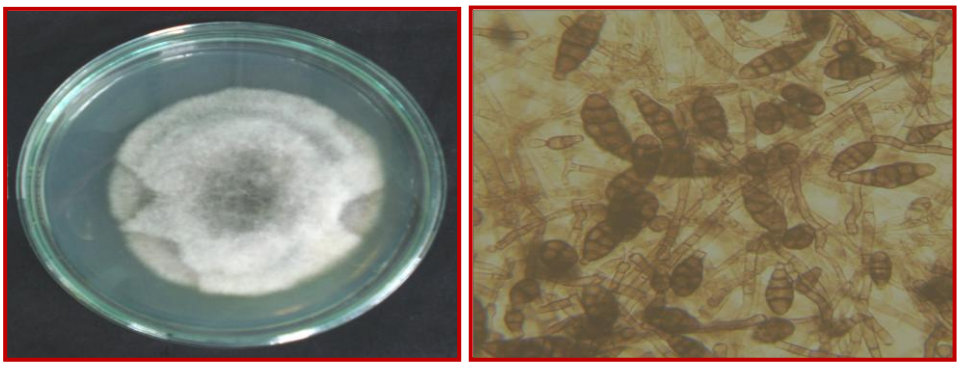

Fig. 6 - Morphology characteristics *Alternaria alternata*

TCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTT
TCCTTGGTGGGTTCCCCCCCCACTAAGACAAACATAAACCTTTTGTAATTGCAAT
CCGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCT
GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTC
AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCA
TGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGT
CTCTAGCTTTGCTGGAGACTCCCTTAAAGTAATTGGGCAGCCGGCCTACTGGTTT
TCGGAGCGCAGCACAAGTCCCCACTCTCTATCAGCAAAGGTCTAACCATCCCATT
AAGCCTTTTTTTTTCAACTTTTTGACCCTCGGGATCCAGGTAGGGAATACCCCGCT
GAAACTTAAACCATAATCAATAAGCGGAAGAAAAAAATCATTACACAAATAATG
AAAGGGCGGGCTGGAATC

Fig. 7 - 18S rRNA sequence of *Alternaria alternata*

Endophytic fungi are everywhere and occur within all plant parts in various ecosystems, but the geographic differences in endophyte diversity, community composition and host preference have not been well documented. To understand the ecology of fungal endophytes, data regarding fundamental parameters of endophyte symbiosis are require from regional to continental scales and encompassing entire ecosystems (Peay et al. 2010). It is hoped that powerful, high throughput molecular techniques like sequencing technology will make the global assessment of endophyte diversity a reality and open up the ‘black box’ of fungal ecology.

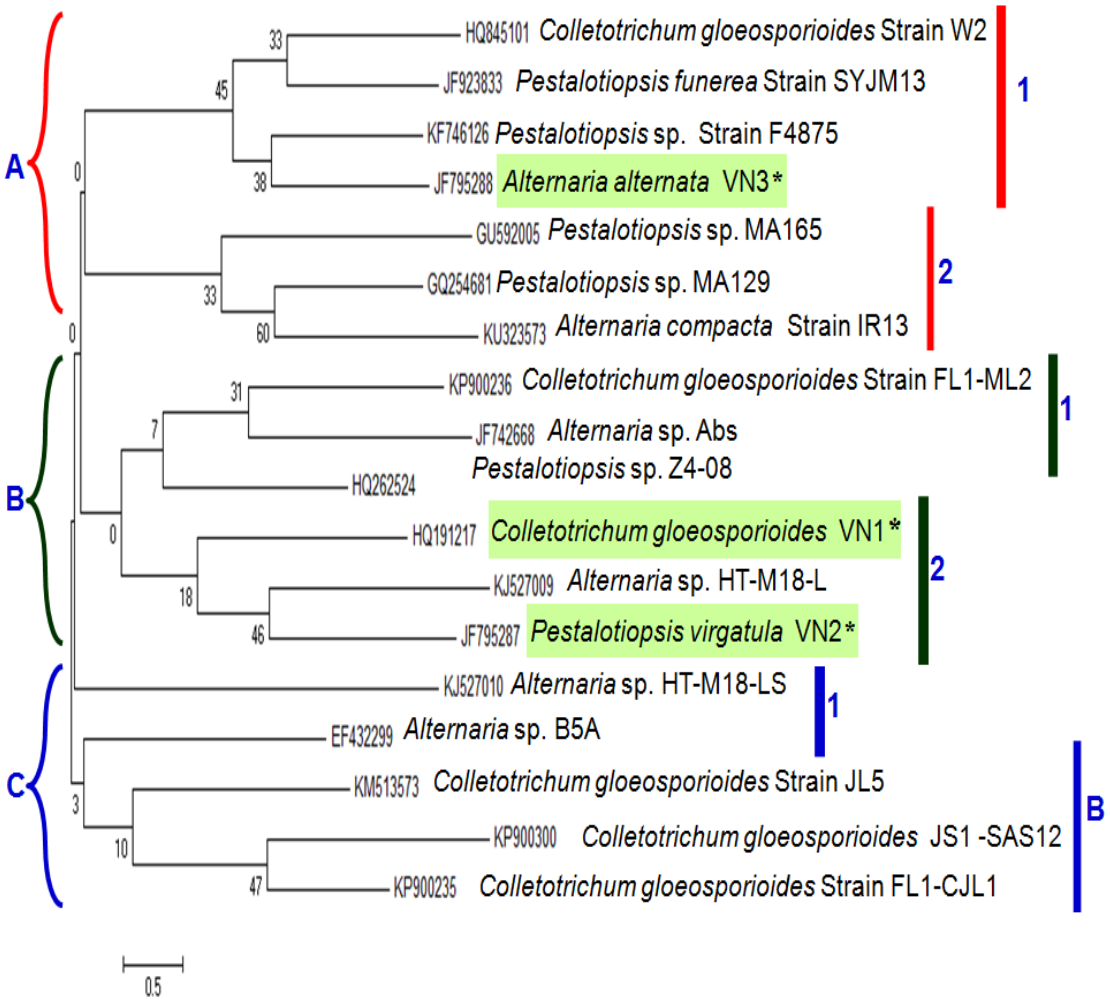

Fig. 8 - Phylogenetic relationship between three endophytic fungal species, inferred from ITS nucleotide sequence data. Bootstrap values are shown for those branches that had >10% support in a bootstrap analysis of 1000 replicates. The numbers of nucleotide changes among taxa are represented by branch length and scale bar equals the number of nucleotide substitutions per site. Asterisks indicate the sequence obtained from the present study. A, B & C indicates major clusters and 1 & 2 indicates sub-clusters referred to in the text.

Acknowledgments

Author thanks the Managing Board of Virudhunagar Hindu Nadar's Senthikumara Nadar College, Virudhunagar-626 001, Tamil Nadu, India for providing research facilities.

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