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Molecular characterization and Phylogenetic analysis of *Lecanicillium spp.*

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Abstract
The entomopathogenic fungus *Lecaniicillium spp.* is a naturally available biological control and it is considered to be one of the best mycoinsecticide agents against the destructive sucking pests of cotton like whitefly and mealy bugs. In this study molecular detection by polymerase chain reaction with specific primers ITS1 and ITS4 was performed on 3 isolates of *Lecaniicillium spp.* The pattern showed by PCR analysis was identical for all the isolates tested confirming their identity as *Lecaniicillium spp*. The ITS1/ITS4 rDNA sequences were compared with those available in the NCBI GenBank database for *Lecaniicillium spp.* (accession number: MW131533).The amplification of the ITS region resulted in a single product size of 600 bp for the isolate VL-2 (*Lecaniicillium sp.)*. The Clustal W alignments and phylogenetic analysis confirmed the taxonomic identity of the potential entomopathogenic isolate, evaluated in the present study as *Lecaniicillium sp.* with accession numbers MN889410.1, KT446000.1, MF-770736.1 and MW131533.1 (VL-2) isolates grouped in the same cluster with more than 93% sequence similarity of NCBI GenBank database. The other isolates were also matched > 93% sequence similarity, however clustered into three major clustered. Thus, indicating the variation among the different isolates as per geographical variations, hosts and genetic relatedness among *Lecaniicillium sp.* isolates.

Keywords: Entomopathogen, 18S rRNA, Isolation, PCR, Phylogenetic analysis

1. Introduction
Lecaniicillium sp. are important entomopathogenic fungi ubiquitously distributed in soils, although these fungi are mainly isolated from insects. *Lecaniicillium* species have a broad host range including insects, phytopathogenic fungi and plant-parasitic nematodes (Hall, 1981) [2]. Earlier polymorphism of *Verticillium* isolates was studied using traditional (morphological and cultural) methods such as colour, consistency and growth rate. On examination of 46 isolates previously accepted as *V. Lecanii* from various hosts and geographical locations, Sugimoto *et al.* (2003) suggested that phialide morphology were inappropriate to support differentiation of species of *Verticillium / Lecaniicillium*. Classical taxonomic and morphological characters are often not sufficiently reliable for revealing differences among the different species of *Verticillium*. Fungal systematics is an essential part of biological research especially in the context of ecological and economic implications. *V. Lecanii* was considered to be a complex species, including several distinct taxa defined by their molecular profiles (Steenberg and Humber, 1999). Hence, researchers started using molecular phylogeny as a tool to classify the complex *Verticillium lecanii* group. Zare and Gams (2001) [6, 8] transferred a major part of the species formerly classified in Verticillium sect. Prostrata, especially *V. lecanii* and *V. psalliotae*, to the genus *Lecaniicillium*. It was in agreement with PCR-RFLPs of ITS, mt-DNA types and α-tubulin gene profile results. Entomopathogenic isolates of V. Lecanii have now been grouped under the genus *Lecanicillum* with four distinct species based on ITS sequences and on molecular phylogeny (Zare *et al.*, 2000, 2001 [8]; Gams and Zare, 2001 [6, 8]; Sung *et al.*, 2001) [6]. In India, no reports are available on the molecular phylogeny of *L. lecanii* and Indian isolates are still referred to as *L. lecanii*.
In the present study, attempts were made to classify the isolates of *L. lecanii* based on the DNA sequence data obtained using internal transcribed spacers, ITS region sequence and suggesting the taxonomic grouping based on the sequence similarity of 18S rRNA and also their evolutionary relationship.

2. Material and Methods

2.1 Fungal DNA extraction

DNA extraction was done using the established CTAB method with some modification**.** Liquid nitrogen, extraction buffer, chloroform/isoamyl mixture, isopropanol, 70% ethanol, TE buffer was executed as reagents.

2.2 Sample preparation

The DNA was extracted from five to seven days old fungal cultures grow either in 20 ml PD broth in 100 ml flask or culture plate at 25 ºC temperature. The fungal mass from the culture plate was scraped out with the help of a fine spatula and fungal mass from the culture broth was harvested on a Wattman's filter paper and washed three times with sterile distilled water that allow broth to pass through while retaining the fungal mass. Dry the mycelia mat and store at -20 ºC until use.

2.3 PCR Conditions

The PCR amplification of fungal DNA was performed with primers ITS-1(5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5' - TCCTCCGCTTATTGGTAT-3'). The reaction mixture for each sample was prepared by dissolving 10X PCR buffer with $MgCl_2$ 1.5 µL, dNTPs 0.4 µL, primer 0.5 µL each, taq polymerase 0.3 µL, DNA 1.5 µL and final volume adjusted to 25 µL by adding 15.4 µL nuclease free water. The PCR was performed in applied bio system thermal cycler. An initial denaturation at 94 °C for 4 min, 35 cycles, each cycle consisting of a denaturation at 94 °C for 45 sec, annealing at 57 °C for 1min and extension at 72 °C for 2 min; a final extension step at 72 °C for 7 min. The PCR products were analyzed on 1.5 per cent agarose gel.

2.4 Molecular identification of potential isolate

After cultivation of potential entomopathogenic fungus, genomic DNA was extracted from the isolate grown on PDA media for 7 days at 26±2 °C according to the CTAB method (Doyle and Doyle, 1987) [1]. The polymerase chain reaction (PCR) for amplification of Internal Transcribed spacers (ITS) region was performed by universal primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990) [7] to obtain estimated amplicon product size of 500-600 bp. The amplification reaction conditions consisted of 2 min at 94°C followed by 40 cycles of 15 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C with a final extension of 4 min at 72 °C. The PCR amp icon size of product was visualized under UV light in 1.2% agarose gels stained by ethidium bromide in gel electrophoresis unit. The PCR product was purified by ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Bio systems™). The nucleotide sequencing was performed by AgriGenome Labs Pvt. Ltd, Kochi, Kerala-682 042 by Sanger method with sequencer (Applied Bio systems, ABI3730XL). After sequencing, the sequences were assemble for consensus sequence with CAP3 sequence assembly program (Huang and Madan, 1999) [3].

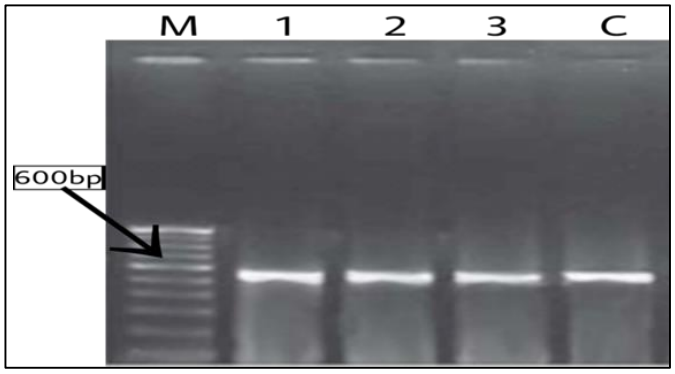
2.5 Molecular variability

The molecular evolutionary genetic analysis (MEGA X) was used to detect the genetic variations and phylogenetic relatedness among the different potential isolates of *Lecaniicillium spp*. Phylogenetic tree with similar sequences of the ITS1/ITS4 regions was completed by retrieving sequences of after BLASTn with NCBI GenBank database. For phylogenetic analysis, maximum likelihood method (ML) was applied to evaluate the strength of the topology, the tree was assessed by Tamura-Nei model with 1000 bootstrap replicates and evolutionary analyses were conducted in MEGA X.

3. Result and discussion

3.1 Molecular characterization

The ITS1/ITS4 rDNA sequences were compared with those available in the NCBI GenBank database for *Lecaniicillium sp.* (Accession number: MW131533).The amplification of the ITS region resulted in a single product size of 600 bp for the isolate VL-2 (*Lecanicillium sp.)*, (Plate 1). The Clustal W alignments and phylogenetic analysis confirmed the taxonomic identity of the potential entomopathogenic isolate, evaluated in the present study as *Lecaniicillium sp.* with accession numbers MN889410.1, KT446000.1, MF-770736.1 and MW131533.1 (VL-2) isolates grouped in the same cluster with more than 93% sequence similarity of NCBI GenBank database. The other isolates were also matched > 93% sequence similarity, however clustered into three major clusters (Plate 2, Table 1). Thus, indicating the variation among the different isolates as per geographical variations, hosts and genetic relatedness among *Lecaniicillium sp.* isolates.



Lane M, Molecular marker GeneRuler™ 100 bp DNA ladder.
Lane 1-3, *Lecanicillium spp*. isolates.
Lane C, positive control of *Lecanicillium spp.* obtained from ICAR-CICR, Nagpur

Fig 1: PCR amplification of genomic DNA from *Lecaniicillium spp.* by using primers ITS1/ITS4

The development of PCR amplification method from different rDNA regions from fungi has greatly revolutionized the study of fungi taxonomy. The multiple sequence alignments and phylogenetic analyses confirmed the *Lecaniicillium sp. as* taxonomic identity of potential isolate. Analyses of ITS-rDNA sequences have been applied to determine the genetic diversity of *Lecaniicillium sp.* (Ramanujan *et al.* 2011, LianMing Lu *et al.* 2015) [4]. Similarly, Sugimoto *et al.* 2003, made the genetic characterization of *Lecaniicillium sp*. strains who obtained PCR amplicon product of 600-800 bp size from the ITS1/ITS4 rDNA fragment regions. The size of the DNA fragments from *Lecaniicillium spp.* isolate in the present study (620 bp) was similar to those ones reported for the *Lecaniicillium sp.* (MW131533.1) reference strain with 620 bp. Comparison of ITS-rRNA obtained from fungal isolates in regarding to the GenBank database (NCBI) indicated that the MN889410.1, KT446000.1, MF-770736.1, KY495194.1,

LC375372.1, KF766521 isolates corresponding to *Lecaniicillium fusisporum* 1ITS-rDNA region was amplified by the ITS1/ITS4 primers, wherein amplicon was observed with an average size of 600 bp was sequenced. The results showed that three strains indicated themselves as monophyletic group. However, other clustered group also represented more than 92% identity with *Lecaniicillium sp.* The MW131533.1 though showed a genetic identity with *Lecaniicillium sp.* > 93% but clustered at genetically distantly related. Thus, it might be concluded that *Lecaniicillium sp.* fungi present different capacity cause mortality of the insects, with the *Lecaniicillium sp.* MW131533.1 (VL-2) isolate of the present study with the highest virulence.

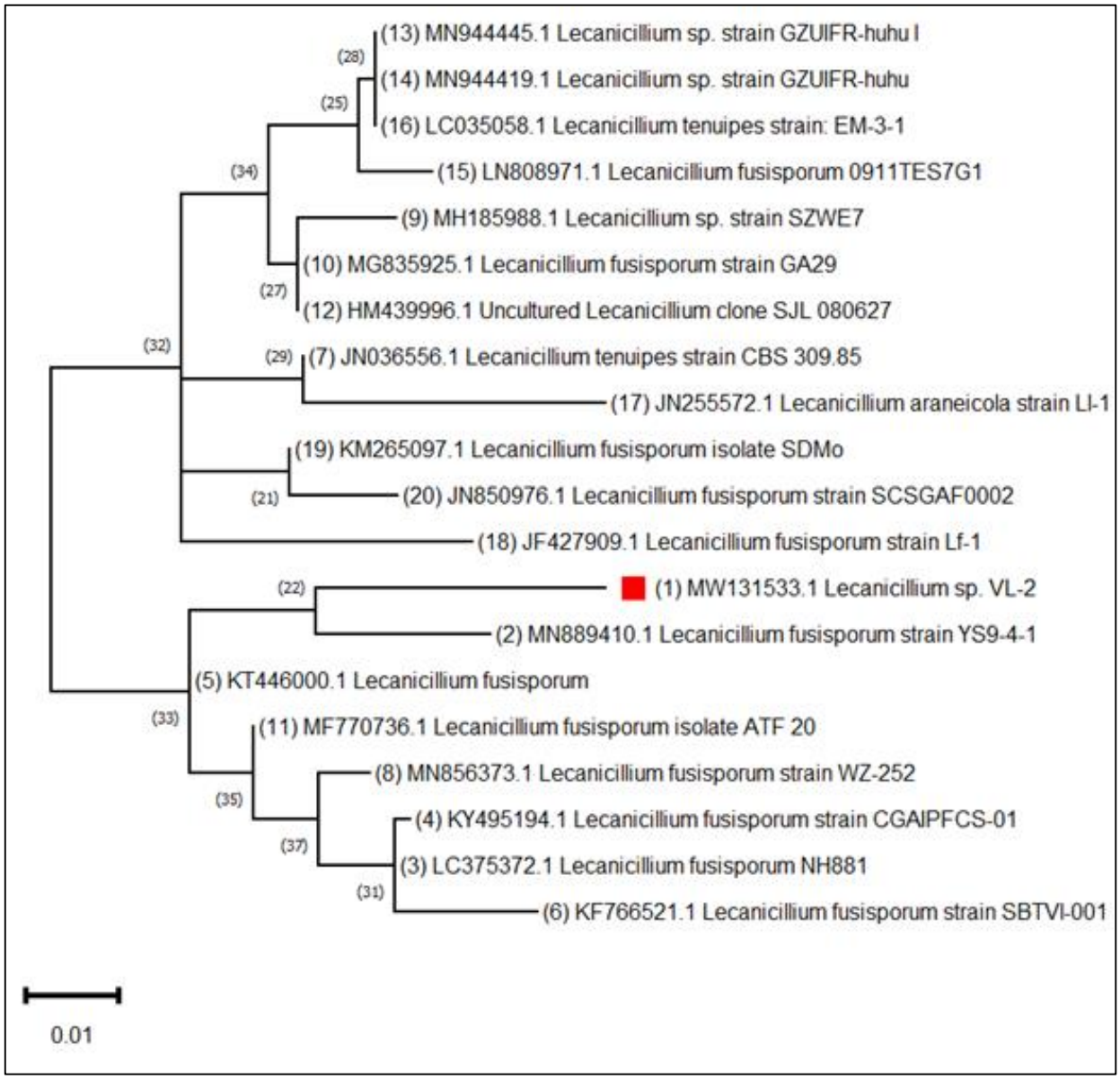


Fig 2: Phylogenetic tree constructed by maximum likelihood method, Tamura-3 parameter model showing the relation between the isolates of *Lecaniicillium* spp. deposited in the GenBank - NCBI database. The sequences used are of the ITS regions of rDNA. The phylogenetic tree built in MEGA X program. The "bootstrap" values for 1,000 replicates.

3.2 Sequence analysis

The result of nucleotide sequences was submitted to NCBI database by using BLASTN. The isolate Vl-2 was amplified using universal primer ITS1 and ITS4 and resulted into 600 bp amp icon product. The PCR product of isolate Vl-2 showed more than 93% similarity with *Lecaniicillium spp*

Table 1: The GenBank accession no. and closest type strains

Sr. No.	Isolate	Accession Number from NCBI	Matching organism in NCBI GenBank database with accession no.	Per cent identity
1	Vl-2	MW131533.1	MN889410.1, KT446000.1, MF-770736.1	93%

4. Conclusion

The molecular identification of all the isolates were done by using ITS1 and ITS4 primers. All the three isolates produced the expected amplicon after successful polymerase chain reaction confirming their identity as *Lecaniicillium spp*. The selective three isolates were sent to the Agro genome,

Bangalore for sequencing. The result of DNA sequencing were searched against other sequences deposited in the NCBI GeneBank database using NCBI-BLAST and Vl-2 isolate showed 93 percent similarity with *Lecaniicillium spp*. Genome.

Lecaniicillium spp. is one of the best biological control agents for successful control of sucking pest viz., aphids, leafhopper, Thrips, white fly and mites without affecting non-target organisms. However, the obtained isolates of *Lecaniicillium spp.* performed better under laboratory conditions against net house reared *A. Gossypii* and need to be evaluated under field conditions for their promotion against sucking insect pests of various Horticulture and Agriculture.

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