Journal of Biological Control, 25 (4): 337–341, 2011

Research Note

ITS sequencing of Indian isolates of *Lecanicillium* species

B. RAMANUJAM*, M. BALACHANDER, G. ROOPA, R. RANGESHWARAN and PRITAM KARMAKAR
National Bureau of Agriculturally Important Insects, HA Farm Post, Bellary Road, Bangalore 560 024, Karnataka, India
**Corresponding author E-mail:* bonamramanujam58@gmail.com

ABSTRACT: The genetic diversity of thirty one isolates of *Lecanicillium* species isolated from insect hosts from various geographical regions of India were studied. Their phylogenetic relationships were determined using internal transcribed spacer, ITS1, ITS2 and 5.8S gene of rRNA sequence. Based on the sequence similarity of ITS region and construction of subsequent phylogenetic analysis (neighbour-joining method), 15 isolates were grouped as *Lecanicillium lecanii*, 11 isolates as *L. attenuatum*, 3 isolates as *L. longisporum* and 2 isolates as *L. muscarium*.

KEY WORDS: ITS region, Phylogenetic analysis, *Lecanicillium* sp.

(Article chronicle: Received: 5-8-2011 Sent for Revision: 16-9-2011 Accepted: 21-10-2011)

Lecanicillium species are important entomopathogenic fungi ubiquitously distributed in soils, although these fungi are mainly isolated from insects. *Lecanicillium* species have a broad host range including insects, phytopathogenic fungi and plant-parasitic nematodes (Hall, 1981). 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/ Lecanicillium.* 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) transferred a major part of the species formerly classified in *Verticillium* sect. *Prostrata*, especially *V. lecanii* and *V. psalliotae*, to the genus *Lecanicillium*. 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; Gams and Zare, 2001; Sung *et al*., 2001). 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 Indian 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.

Fungal Isolates

Thirty-one Indian isolates of *V. lecanii* obtained from different geographical locations (Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra and Orissa) were used. Among the 31 isolates, 29 were from insect hosts, one was from fallen coffee berries and one from coffee rust fungus, *Hemileia vastatrix*. All the isolation were done from 2001 to 2009 (Table 1, 2 and 3; Fig. 1). These isolates were maintained on Sabouraud dextrose agar supplemented with yeast extract (SDAY). A single spore of each isolate was cultured on SDAY agar and used in subsequent investigations.

DNA extraction

For DNA extraction mycelia were grown in Sabouraud dextrose yeast extract broth (SDYB) for 7 days at 26±

Table 1. Fungal isolates identified as *Lecanicillium lecanii* based on ITS sequence analysis and GenBank accessions

Sl. No.	Isolate Number	Insect host	Place of Collection	Accession number	Reference Sequence
1.	NBAII-Vl-3e	*Coccus viridis*	Chettalli, Karnataka	JF718693	*Lecanicillium lecanii* 1. Isolate ARSEF 4065 (EF512995.1) 2. Isolate ARSEF 5126 (EF 512996.1) 3. Isolate ARSEF 4025 (EF 512994.1) 4. Isolate ARSEF 5491 (EF 512997.1)
2.	NBAII-Vl-5	*Meconellicoccus hirsutus*	Pune, Maharashtra	JF718694	
3.	NBAII- Vl-6	*Rhophalosiphum maidis*	Bangalore, Karnataka	JF718695	
4.	NBAII-Vl-7	*Bemisia tabaci*	Bangalore, Karnataka	JF718696	
5	NBAII-Vl-10	*Aphids* (Unidentified)	Bangalore, Karnataka	JF718697	
6	NBAII-Vl-15	*Lepidosaphes* spp.	Appangala, Karnataka	JF718698	
7	NBAII-Vl-17	*Planococcus lilacinus*	Chettalli, Karnataka	JF718699	
8	NBAII-Vl-19	Unidentified insect larvae	Bapatla, Andhra Pradesh	JF718700	
9	NBAII-Vl-20	*Singhiella cardamomi*	Parathode, Kerala	JF718701	
10	NBAII-Vl-24a	*Singhiella cardamomi*	Moolathara, Kerala	JF718702	
11	NBAII-Vl-25a	*Coccus viridis*	Noriampara, Kerala	JF718703	
12	NBAII-Vl-26	Unidentified insect from guava	Kanchiar, Kerala	JF718704	
13	NBAII-Vl-27	Unidentified insect from cocoa	Swaraj, Kerala	JF718705	
14	NBAII-Vl-28	Unidentified scale insect from guava	Vellilamkandam, Kerala	JF718706	
15	NBAII-Vl-29	Unidentified insect from Tea	Chappath, Kerala	JF718707	

Table 2. Fungal isolates identified as *Lecanicillium attenuatum* based on ITS sequence analysis and GenBank accessions

Sl. No.	Isolate Number	Insect host	Place of Collection	Accession number	Reference Sequence
1.	NBAII-Vl-1	Unidentified insect	Bangalore, Karnataka	JF718708	*L. attenuatum* Isolate CBS 170.76 (EF679164.1)
2.	NBAII-Vl-2d	*Coccus viridis*	Mattikad, Kerala	JF718709	
3.	NBAII-Vl-3	*Coccus viridis*	Madikere, Karnataka	JF718710	
4	NBAII-Vl-4	*Hemileia vastatrix* Coffee Rust fungus	Chettalli, Karnataka	JF718711	
5.	NBAII-Vl-14	*Toxoptera aurantii*	Valparai,Tamil Nadu	JF718712	
6	NBAII-Vl-16	*Aleurodicus* spp.	Chettalli, Karnataka	JF718713	
7	NBAII-Vl-18	Fallen Coffee berries	Ooty,Tamil Nadu	JF718714	
8	NBAII-Vl-21a	*Singhiella cardamomi*	Thalayankanu, Kerala	JF718715	
9	NBAII-Vl-22	*Coccus viridis*	Karakunne, Kerala	JF718716	
10	NBAII-Vl-23	Unidentified Coleopteran beetle	Balagram, Kerala	JF718717	
11	NBAII-Vl-30	*Pyrilla perpusilla*	Odanga, Orissa	JF718718	

Table 3. Fungal isolates identified as *Lecanicillium longisporum and L. muscarium* based on ITS sequence analysis and GenBank accessions

Sl. No.	Isolate Number	Insect host	Place of Collection	Accession number	Reference Sequence
	Lecanicillium longisporum isolates				*L. longisporum* Isolate ARSEF 974 (EF512987.1) *L. muscarium* Isolate ARSEF 2323 (EF513017.1)
1.	NBAII-Vl-12	Unidentified insect	Trichi, Tamil Nadu	JF718719	
2.	NBAII-Vl-21	*Singhiella cardamomi*	Thalayankanu, Kerala	JF718720	
3.	NBAII-Vl-24	*Singhiella cardamomi*	Moolathara, Kerala	JF718721	
	Lecanicillium muscarium isolates		*L. muscarium*		
1	NBAII-Vl-8	Unidentified insect	Chennai, Tamil Nadu	JF718722	
2	NBAII-Vl-22a	*Coccus viridis*	Balagram, Kerala	JF718723	

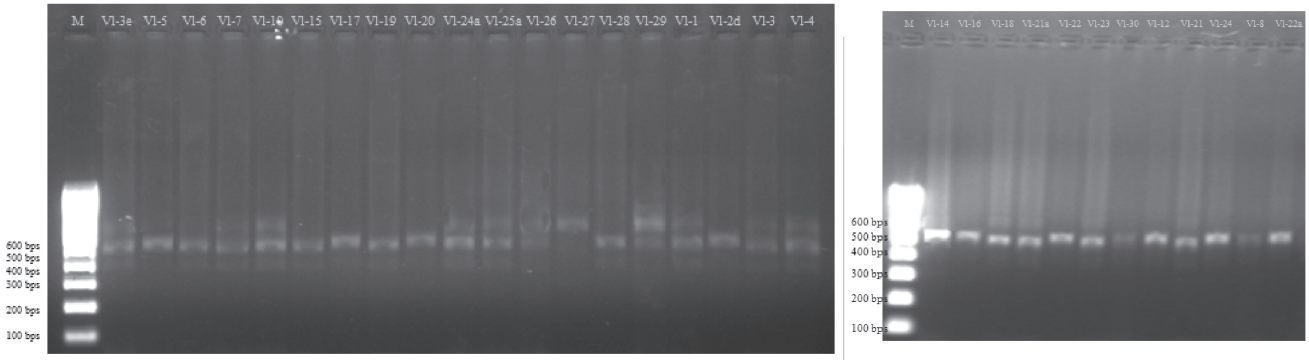

Fig. 1. PCR amplification of ITS-1 and ITS-2 (566 bps) regions of the 31 Indian isolates of *Lecanicillium* spp.

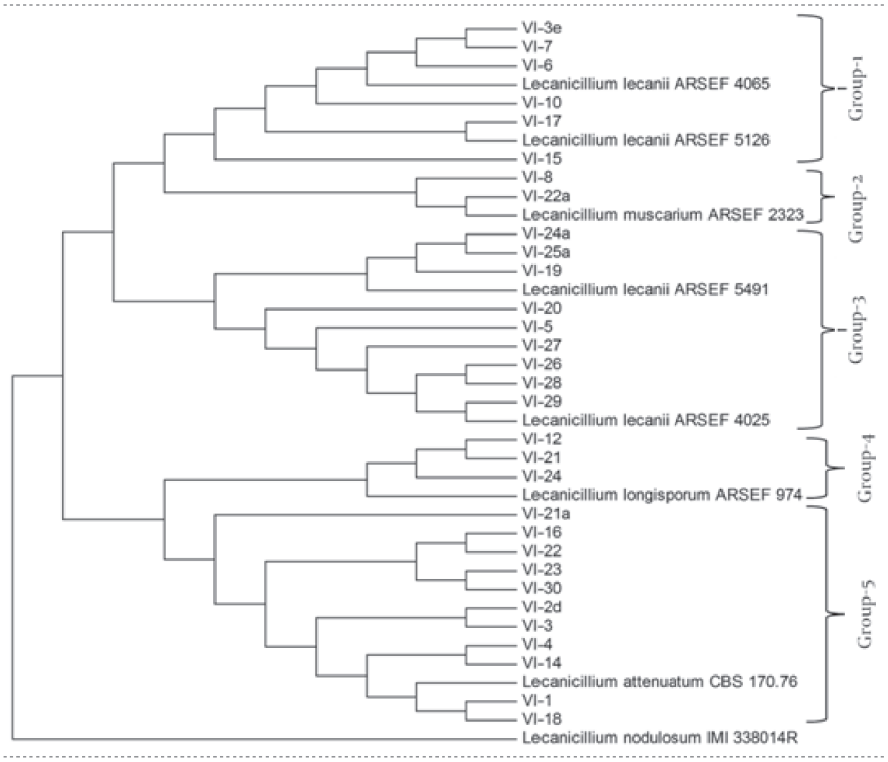

Fig. 2. Phylogenetic tree analysis of *Lecanicillium* isolates based on the nucleotide sequence of ITS. The Phylogenetic tree was constructed by neighbor-joining (NJ) method

0.2°C without shaking. After excess liquid was removed, mycelia were ground with 9 ml of pre-warmed CTAB extraction buffer and incubated for 60-90 minutes, with occasional inversion at 65 ºC in water bath. The samples were cooled by immersion in a trough of water at room temperature (25-30°C) for 5 minutes. To that 5ml of mixture of chloroform: isoamyl alcohol (in the ratio of 24:1) were added and rotated on the tube roller for 5 minutes and the samples were centrifuged for 15 mins at 7000 rpm at 20ºC. The top aqueous layer was transferred into a fresh tube containing 25µl of RNase A (20mg/ml) and incubated for 30 minutes at room temperature. To this 6ml of ice-cold isopropanal was added to each tube and mixed gently by inversion to get a white fluffy DNA precipitate and centrifuged at 7000rpm for 10 mins at 4ºC. The precipitated DNA was washed separately with 9ml of cold CTAB wash buffer and 70% ethanol at 7000rpm for 10 mins at 4ºC. DNA pellets were dissolved in 100 µl of TE (10mM Tris–HCl+0.1mM EDTA at pH 8.0).

PCR amplification

The ITS regions (ITS1 & 2 and the 5.8S gene) were PCR-amplified with the following pair of primers: ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al*., 1990). The PCR reaction mixture consisted of 50 ng DNA, 50 pmol of each primer, a final concentration of 1.25 mM for each dATP, dGTP, dTTP, dCTP, 2.5 units of *Taq* DNA polymerase, 5µl polymerase buffer, 2.5 mM $MgCl_2$, and sterile Millipore water to 50µl. The amplification programme comprised of initial denaturation at 95ºC for 2 min, followed by 36 cycles of denaturation at 94 ºC for 30 seconds, annealing at 60.6ºC for 30 seconds, extension at 72ºC for one minute, and an additional 10 min extension at 72ºC as a final step. The cycles were carried out in a Quantarus Thermal cycler (Model Q-cycler, U.K). PCR products were separated by electrophoresis on a 2% agarose gel and visualized under UV light by ethidium bromide staining.

DNA sequencing

The amplicons were purified, sequenced and processed by Merck Bioscience. For multiple bands the appropriate band near to the expected size of 566 bps was first purified by agarose gel extraction and then sequenced. The sequences were also subjected to BLAST (Basic Local Alignment Search Tool) analysis (Altschul *et al*., 1990) to verify the similarities of the obtained fragments with the sequences of other nucleotides in GenBank. The entire sequences were aligned with Lasergene MegAlign using ClustalW and the Weighted DNA score matrix (Thompson *et al*., 1994; Aiyar 2000). A neighbor-joining dendrogram was generated and the nucleotide sequences were submitted to GenBank. Reference sequences were sought out from NCBI GenBank and sequences of the isolates were submitted to GenBank.

Amplification by PCR using specific primers revealed PCR products of approximately 566 bps (Fig. 2). The output phylogram represent the clustering of the isolates under four species of *Lecanicillium*. Based on the confirmation of BLAST analysis and phylogenetic tree (Fig. 3), *V. lecanii* isolates were reclassified as 15 isolates grouped as *Lecanicillium lecanii* (Table 1), 11 isolates as *L. attenuatum* (Table 2), 3 isolates as *L. longisporum* (Table 3) and 2 isolates as *L. muscarium* (Table 3). Isolates of *L. lecanii* were obtained from different insect species like, *Coccus viridis* (2 isolates), *Meconellicoccus hirsutus* (1 isolate), *Rhophalosiphum maidis* (1 isolate), *Bemisia tabaci* (1 isolate), *Lepidosaphes* spp. (1 isolate), *Planococcus lilacinus* (1 isolate), *Singhiella cardamomi* (2 isolates) and unidentified insects (6 isolates) from Karnataka, Kerala, Tamil Nadu and Maharashtra. *L. attenuatum* isolates were obtained from different insect species like, *Coccus viridis* (3 isolates), *Toxoptera aurantii* (1 isolate), *Aleurodicus* spp. (1 isolate), *Singhiella cardamomi* (1 isolate), *Pyrilla perpusilla* (1 isolate) and unidentified insects (2 isolates), as well as from coffee rust fungus, *Hemileia vastatrix* (1 isolate) and fallen coffee berries (1 isolate) from Karnataka, Kerala, Tamil Nadu and Orissa. Three isolates of *L. longisporum* were obtained from *S. cardamomi* (2 isolates) and unidentified insect (1 isolate). Two isolates of *L. muscarium* were obtained from *C. viridis* (1 isolate) and an unidentified insect (1 isolate) from Tamil Nadu and Kerala. The sequences were submitted to NCBI and accession numbers assigned for *L. lecanii* (JF718693-JF718707), *L. attenuatum* (JF718708-JF718718), *L. longisporum* (JF718719-JF718721) and *L. muscarium* (JF718722-JF718723).

The recent revision of the genus *Verticillium* resulted in the introduction of several new species, some with good support (*L. lecanii, L. muscarium, L. longisporum*, *L. attenuatum* etc.) (Gams and Zare, 2001; Zare and Gams 2001; Zare and Gams, 2004). This revision was based on morphological criteria, such as, the diameter of conidia or the verticillate arrangement of phialides and by molecular analysis of the genomic ribosomal DNA region (Zare *et al*., 2000; Gams and Zare, 2001; Sung *et al*., 2001; Zare and Gams, 2001). The problems arising from using morphological criteria alone or in combination with single gene sequences have been demonstrated in several cases in *Verticillium*. For example, some strains named as *V. albo-atrum* were misclassified and found

subsequently to be closely related to *V. fungicola* and *V. psalliotae*, when additional molecular data were provided (Carder and Barbara, 1999). The traditionally used ITS1-5.8S-ITS2 region as expected was informative but often not as discriminating as other gene sequences. By studying the entire region here, it was established that the ITS1 was accountable for the variability between species of the genus, in a similar way to that observed for the genus *Penicillium* (Boysen *et al*., 1996), whereas the ITS2 remained less informative for the phytopathogenic species of *Verticillium*. Finally, on examination of 46 isolates previously accepted as *V. lecanii* from various hosts and geographical locations, Sugimoto *et al*. (2003) questioned the validity of phialide morphology as a useful taxonomic character in *Lecanicillium*, as conidial length was found to be a continuous genetic character, and therefore, inappropriate to support differentiation of members as the new species *L. muscarium* and *L. longisporum*. Hence, it is imperative to approach such taxonomic problems with the utmost caution and attempt to combine classical taxonomic data with molecular data from as many genetic traits as possible. Our studies have shown that the collection of 31 isolates of *L. lecanii* which we had previously classified as *V. lecanii* are now placed under four species. The knowledge of genetic diversity of entomopathogenic fungi *Lecanicillium* spp. from different sources and geographical locations will be useful in understanding their identity, their ecological role and their scope for application in biological control programmes. Species that show enhanced activity or the presence of genes such as chitinase, protease and lipase and other enzymes can be further grouped based on the present classification and those species showing consistent activity of such virulent genes can be readily picked up for use in pest management.

ACKNOWLEDGEMENT

The authors are also thankful to Indian Council for Agricultural Research (ICAR) for financial assistance under the AMAAS programme.

REFERENCES

Aiyar, A. 2000. The use of Clustal W and Clustal X for multiple sequence alignment. *Methods in Molecular Biology,* **132**: 221–241.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, **215**: 403–10.

Boysen, M., Skouboe, P., Frisvad, J. and Rossen, L. 1996. Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. *Microbiology*, **142**: 541–549.

Carder, J. H. and Barbara, D. J. 1999. Taxonomic status of putative *Verticillium albo-atrum* isolates. *FEMS Microbiology Letters*, **170**: 211–219.

Gams, W. and Zare, R. 2001. A revision of *Verticillium* sect. Prostrata. III. Generic classification. *Nova Hedwigia,* **72**: 47–55.

Hall, R. A. 1981. Laboratory studies on the effects of fungicides, acaricides and insecticides on the entomopathogenic fungus, *Verticillium lecanii*. *Entomologia Experimentalis et Applicata*, **29**: 39–48.

Steenberg, T. and Humber, R. A. 1999. Entomopathogenic potential of *Verticillium* and *Acremonium* species (Deuteromycotina: Hyphomycetes), *Journal of Invertebrate Patholology,* **73**: 309–314.

Sugimoto, M., Koike, M., Hiyama. N. and Nagao, H. 2003. Genetic, morphological and virulence characterization of the entomopathogenic fungus *Verticillium lecanii*. *Journal of Invertebrate Pathology*, **82:** 176–187.

Sung, G. H., Zare, R., Spatafora, J. and Gams, W. 2001. *Verticillium* section Prostrata. 11. Generic delimitation using ribosomal DNA sequences. *Nova Hedwigia,* **72**: 29–46.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**: 4673–4680.

White, T. J., Bruns, T. D., Lee, S. B. and Taylor, J. W. 1990. Amplication and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315–322. In: Innis M.A., Gelgand, D.H., Suinsky, J.J. and White, J.J., (Eds.), *PCR protocols: A Guide to Methods and Applications*, Academic Press, San Diego.

Zare, R. and Gams, W. 2001. A revision of *Verticillium* section Prostrata. IV. The genera *Lecanicillium* and *Simplicillium* gen. nov. *Nova Hedwigia,* **73**: 1–50.

Zare, R. and Gams, W. 2004. A monograph of *Verticillium* section Prostata. *Rostaniha,* **3**: 1–188.

Zare, R., Gams, W. and Culham, A. 2000. A revision of *Verticillium* sect. Prostrata. 1. Phylogenetic studies using ITS sequences. *Nova Hedwigia,* **71**: 465–480.

Zare, R., Gams. W. and Evans, H. C. 2001. A revision of *Verticillium* section Prostrata. V. The genus *Pochonia*, with notes on *Rotiferophthora*. *Nova Hedwigia*, **73**: 51–86.