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Survey and molecular characterization of *Nomuraea rileyi* isolates

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ABSTRACT

After conducting a roving survey in Chittoor district for the natural occurrence of *Nomuraea rileyi,* a few fungal infected and died (mummified) cadavers of *Spodoptera litura* and *Bombyx mori* were found and collected. With microscopic studies, the fungus was identified as *Nomuraea rileyi* and *Beauveria bassiana*. The molecular characterization of 7 isolates of *N.rileyi* was done by RAPD-PCR for studying the genetic variability/similarity. RAPD banding profile with 12 different random primers *viz.,* 3 primers from OPD, 3 primers from OPY, 2 primers from OPM, 2 primers from OPA, 1 primer from OPC and 1 primer from OPW (Operon technology) showed 88.6 per cent polymorphism as all the bands obtained were polymorphic with size ranging from 100 bp to 3000 bp. Jaccards similarity co-efficients between the *N.rileyi* isolates showed 97.50 per cent genetic variation between isolates of Tirupati and V.Kota-1. While the isolates V.Kota-2 and Madanapalli were found to be genetically similar as 70.3 per cent similarity was observed between the isolates. In the resulted dendrogram V. Kota-2 and Madanapalli isolates formed one group and V. Kota-2 and Kanumakupalli another group and remaining isolates did not form any group.

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INTRODUCTION

Variability between strains facilitates posterior ecological studies of introduced isolates, the precise identification of which is a prerequisite for patent registration and the production of commercial products (Vargas *et al.,* 2003). Phenotypically, *N.rileyi* isolates display less variability than other entomopathogenic fungi such as *B.bassiana* and *Metarhizium anisopliae*. To date, characterization by biological and morphological parameters failed to produce information that can readily differentiate strains. For example plating of *N.rileyi* at different times on the same medium incubated under identical conditions produced variable sporulation patterns (Boucias *et al.,* 2000). The implementation of PCR based tools for characterization of organisms has greatly advanced on understanding of the phylogenetic and species boundaries. The widespread method of PCR for variability study was RAPD (Randomly Amplified Polymorphic DNA) (Vargas *et al.,* 2003). The RAPD primers have so far provided differentiation at the intraspecific level, allowing differentiation of closely related isolates with in single species (Bridge *et al.,* 1998).

MATERIAL AND METHODS

The molecular characterization of 7 isolates of *N.rileyi* was conducted in the S.V. Agricultural College and Frontier Technology Lab, Tirupati during 2011- 2012.

Table A : The details of Entomopathogenic fungi collected during survey

Sr. No.	Entomopathogen	Host insect	Place/location	Agricultural Division
1.	*Beauveria bassiana*	*Bombyx mori*	Kosuvaripalli	Madanapalli Division
2.	*Beauveria bassiana*	*Bombyx mori*	Kosuvaripalli	Madanapalli Division
3.	*Beauveria bassiana*	*Bombyx mori*	Kosuvaripalli	Madanapalli Division
4.	*Beauveria bassiana*	*Bombyx mori*	Taruguvaripalli	Madanapalli Division
5.*	*Nomuraea rileyi*	*Bombyx mori*	Taruguvaripalli	Madanapalli Division
6.	*Beauveria bassiana*	*Bombyx mori*	Taruguvaripalli	Madanapalli Division
7.	*Beauveria bassiana*	*Bombyx mori*	Truguvaripalli	Madanapalli Division
8.	*Beauveria bassiana*	*Bombyx mori*	Nayanavaripalli	Madanapalli Division
9.*	*Nomuraea rileyi*	*Bombyx mori*	kanumakupalli	Palamaneru division
10.	*Beauveria bassiana*	*Spodoptera litura*	Ramachandrapuram	Puttur Division

Survey on the occurrence of entomopathogenic fungi in Chittoor district :

A roving survey was carried out in major crops and major silkworm rearing villages of four Agricultural divisions *viz*., Puttur, Srikalahasti, Palamaneru and Madanapalli of Chittoor district for the occurrence of *Nomuraea rileyi*, on lepidopteran caterpillars from December 2010 to Febrauary 2011 at fortnight intervals (Table A). In the groundnut fields of Ramachandrapuram village and field bean fields in dry land farm, RARS, Tirupati, a few fungal infected caterpillars of *S.litura* were collected. In some of the silk warm rearing units in Kanumakupalli (Palamaneru division), Kosugavaripalli and Taruguvaripalli (Madanapalli division), a few fungal infected and died (mummified) cadavers of *Bombyx mori* were found and were carried separately to the laboratory in sterilized vials.

The diseased cadavers were sterilized with 0.5 per cent sodium hypochlorite and larvae were blotted dry and placed in moist chamber for mycelial growth and sporulation. With microscopic observation, *N.rileyi* was identiied. The conidia were isolated on SMAY slants and sub-culturing was done repeatedly to obtain pure *N.rileyi* culture. For DNA extraction, *N.rileyi* was grown on liquid SMY medium.

DNA extraction :

The DNA was extracted using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) protocol (Murray and Thompson,1980).

Nomuraea rileyi isolates were lyophilized in liquid nitrogen, and transferred to steriled eppendorf tubes and suspended in 900 µl of extraction buffer. The samples were centrifuged with equal volumes of phenol:chloroform followed by chloroform : isoamyl alcohol (24:1) at 10,000 rpm for 10 minutes. To the supernatant 0.6 volume of ice cold isopropanol and 0.1 volume of sodium acetate were added and incubated at -20°C for overnight for precipitation of DNA. The pellet obtained was washed with 70 per cent ethanol (100 µl) and to the pellet RNase (1µl/100 µl) was added and stored at 20°C for further use. The concentration and quality of DNA was estimated using nano drop spectrophotometer at 260 nm and running sample on 1.0 per cent agarose along with 1 kb marker.

RAPD profiles through polymerized chain reaction (PCR) :

Twelve different random primers using 3 primers from OPD, 3 primers from OPY, 2 primers from OPM, 2 primers from OPA, 1 primer from OPC and 1 primer from OPW were screened. The experiment was repeated thrice and results were reproducible. The PCR conditions were optimized in terms of concentration of template DNA from 25 ng to 100ng in reaction volume of 25 µl. A reaction volume of 25 µl and 100ng of DNA gave maximum number of reproducible bands and thus was considered ideal and used subsequently in the analysis. The final amplification assay contained 100ng genomic DNA, 1.5 Units *Taq* DNA polymerase, 0.2 mM of dNTPs mix, 2.5 mM $MgCl_2$, 10 pmole primer and 1X *Taq* buffer in a PCR reaction volume of 25 µl. The amplification profile followed was initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 seconds, annelation at 35°C for 30 seconds and extension at 72°C for 1 min repeated 45 cycles with final extension at 72°C for 7 min.

Agarose gel electrophoresis:

Amplified products were separated on 1 per cent agarose gel and stained with Ethidium bromide (0.5µg/ml of gel).

Statistical analysis:

The data matrix generated was used to calculate Jaccard's similarity co-efficient for each pair-wise comparison. The similarity co-efficients were subjected to unweighted Pair-Group Method of Arithmetical averages (UPGMA) cluster analysis to group the genotypes based on their overall similarities. Statistical Package for Social Sciences (SPSS) package was used for the cluster analysis and subsequent dendrogram preparation.

RESULTS AND DISCUSSION

The findings of the present study as well as relevant

discussion have been presented under the following heads :

RAPD profiles through PCR and Agarose gel electrophoresis :

The PCR conditions were optimized as given in materials and methods. The final amplification assay contained 100 ng genomic DNA, 1.5 Units *Taq* DNA polymerase, 0.2 mM of dNTPs mix, 2.5 mM $MgCl_2$, 10 pmole primer and 1X *Taq* buffer in a PCR reaction volume of 25 µl. Amplified products were separated on agarose gel electrophoresis.

Primer selection and survey:

Primer survey was carried out with the following 12 primers and all the primers gave reproducible and scorable bands with high percentage of polymorphism.

Numbers of amplification products obtained were specific to each primer and it ranged from 1 to 12 (Table 1). These primers showed 88.6 per cent polymorphism as all the bands obtained were polymorphic with size ranging from 100 bp to 3000 bp. A maximum number of 12 bands were obtained with the seven isolates with OPD-18 out of which 10 were polymorphic. These results are similar to the findings of Boucias *et al.* (2000) who determined that the 24 *Nomuraea* isolates produced maximum scorable bands in RAPD with 24 operan primers *viz.*, 2 OPB primes, 1 OPC primer, 5 OPD primers, 2 OPE, 2 OPI, 2 OPN, 2 OPW, 2 OPX, 4 OPY primers. The size of amplification product ranged from 3800bp to less than 493bp. Vargas *et al*. (2003) characterized five *N.rileyi* isolates/strains using RAPD analysis, virulence studies and assessment of chitinolytic and proteolytic activity. RAPD analysis divided the strains into two groups with a similarity co-efficient of 0.76 per cent. Out of the 10 primers tested OPW-13 showed maximum polymorphism. Suwannkut *et al.* (2005) evaluated genetic diversity of 79 *N.rileyi* isolates using six different primers. The total scorable bands were 111 with 105 polymorphic bands evenly distributed from 700-3000bp. Uma *et al*. (2007) undertaken a study to investigate the possibility of genotypic diversity in isolates of *N.rileyi* sampled from an epizootic population from south India through AFLP. The size of fragment ranged from 46-404 bp. Out of the 143 AFLP bands, 113 were polymorphic bands resulting in a polymorphism level of 79 per cent.

Jaccards similarity co-efficients between the *N.rileyi* isolates are presented in Table 2. Similarity matrix produced showed 97.5 per cent genetic variation between isolates of Tirupati and V.Kota-1closely followed by Dharwad and V.Kota-1 (95.6%). While the isolate V.Kota-2 and

Table 1 : RAPD primers, number of bands and polymorphic bands

RAPD-primers		Total no. of bands	No. of polymorphic bands
OPA-10	GTGATCCGAG	06	06
OPA-15	TTCCGAACCC	03	03
OPC-19	GTTGCCAGCC	08	06
OPD-08	GTGTGCCCCA	05	04
OPD-11	AGCGCCATTG	09	08
OPD-18	CACAGCGACA	12	10
OPM-4	GGCGGTTGTC	04	03
OPM-10	TCTGGCGCAC	10	09
OPY-05	GGCTGCGACA	04	04
OPY-06	AAGGCTCACC	08	08
OPY-10	AAGGCTCACC	04	04
OPW-13	CACAGCGACA	06	04
Total		79	69

Table 3 : Jaccard's similarity co-efficients of 7 isolates of *N.rileyi* based on polymorphism obtained with 12 random primers.

Isolates	Tirupati	Bangalore	Dharwad	Kanumakupalli	V.Kota-1	V.Kota-2	Madanapalli
Tirupati	1.000						
Bangalore	.279	1.000					
Dharwad	.527	.355	1.000				
Kanumakupalli	.482	.361	.618	1.000			
V.Kota-1	.025	.051	.044	.045	1.000		
V.Kota-2	.542	.321	.633	.681	.057	1.000	
Madanapalli	.489	.288	.490	.600	.071	.703	1.000

*The similarity co-efficient 1.00 indicates the 100 per cent similarity.

Madanapalli were found to be genetically similar as 70.3 per cent similarity was observed between the isolates. This similarity index indicated more similarity among the four isolates *viz.*, Kanumakupalli, V-Kota-1, V-Kota-2 and Madanapalli isolates. The similarity co-efficients were subjected to SPSS software to produce a dendrogram. In the resulted dendrogram V.Kota-2 and Madanapalli isolates formed one group and V.Kota-2 and Kanumakupalli in another group and remaining isolates did not form any group. Though the Dendrogram (Fig. 1) V.Kota-1 as more variable isolate from all other isolates followed by Bangalore isolate. The Bangalore isolate may be having the more genetic variation as there was no reproducible bands with V.Kota-1 with the all 12 primers.

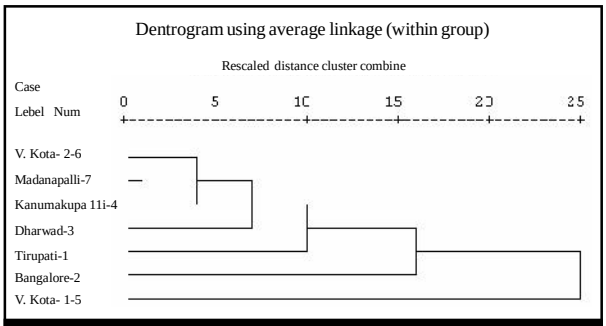

Fig. 1 : Dendrogram illustrating genetic relationships among 7 *N.rileyi* isolates, generated by UPGMA cluster tree analysis

The present findings of considerable genetic variation among the seven isolates of *N.rileyi* in RAPD analysis may be due to difference in host insect from which fungus was extracted rather than location, because Tirupati isolate obtained from *Spodoptera litura* and V.Kota-1 isolated from *Bombyx mori* showed a maximum of 97.5 per cent genetic difference where as V.Kota-2 and Madanapalli though belong to different geographical locations found to be genetically similar as they are isolated from same host insect *i.e. B.mori.* This result is in accordance with works of Suwannkut *et al.* (2005) who evaluated genetic diversity of 79 *N.rileyi* isolates using AFLP analysis. The cluster analysis separated the *N.rileyi* isolates into two major groups and seven sub groups. They demonstrated that AMOVA revealed no significant differences (p=0.3421) among *N.rileyi* isolates from different continents, where as genetic variation among the *N.rileyi* populations from different host insects within each continent was significantly different (p<0.0001). In contrary Vargas (2003) worked with other *N.rileyi* strains and stated that there was high degree of homology between the different strains, irrespective of their geographic origin and the host from which they were isolated. Whereas Leal *et al.* (1997), able to differentiate *M.anisopliae,* isolates from different countries and also between the isolates from different geographic sites within the country. Uma *et al*. (2007) reported a great genotypic diversity in isolates of *N.rileyi* sampled from an epizootic population from south India through AFLP.

All the isolates though showing genetic difference to a considerable extent they formed tight clusters. This may be due to less diversity of isolates with respect to geographical locations as all the isolates belonged to same region and less host diversity *i.e.* all the seven isolates were isolated from two host insects *i.e. B.mori* and *S.litura*.

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