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Morphology and RAPD analysis of certain potentially entomopathogenic isolates of *Metarhizium anisopliae* Metsch. (Deuteromycotina: Hypocreales)

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ABSTRACT

Diversity within the entomopathogenic fungi have been traditionally analyzed using morphological features but morphology alone can lead to ambiguity pertaining to identification at species level. Therefore utilization of molecular methods to detect the level of polymorphism among species helps minimize the problem. Nine isolates of Metarhizium were morphologically characterized by assessing their colour, media pigmentation, size and shape of conidia. Molecular characterization was carried out by random amplified polymorphic DNA analysis. The RAPD - PCR assay for nine isolates were performed by amplifying random sequences using three RAPD primers. The amplification products for the different isolates were compared with each other and were screened for the presence or absence of specific bands. The scored band data was subjected to cluster analysis. A genetic similarity matrix was constructed using Jaccard's coefficient method. Colony colour varied from pale green to blackish green. Pigment production was observed for four isolates. Average width of spores ranged from 2.10 - 4.10µm and length 3.20 - 7.69 µm. The spores were grouped as either oval, round or elongated. The three primers generated a total of 166 reproducible distinct bands among the 9 isolates and the similarity was estimated on the basis of number of shared bands. The Jaccard's similarity coefficient between isolate pairs ranged from 0.00 to 0.70 indicating a high genetic diversity. The maximum similarity was noticed between isolates MIS13 and MIS18. A dendrogram was generated from RAPD patterns of the Metarhizium isolates. Grouping of isolates into clusters correlated with similarities in their RAPD DNA patterns.

Key words: Entomopathogens, *Metarhizium anisopliae*, Characterization, Morphology, Molecular, RAPD

INTRODUCTION

Constraints on the use of chemical pesticides have lead to the development of several biological control options and their implementation in integrated pest management (IPM) programs today. Need to reduce or eliminate the use of conventional chemical pesticides, both in agriculture and forestry, has fostered a search for alternative products and strategies that have a much lesser impact on human health and the environment. Biological control involves the use of one organism to moderate or control the behavior of another organism and typically involves an active human role. In biological control, attempts are made to locate a natural enemy of a pest and augment its population to control unacceptable population levels of the pest. It employs natural enemies such as predators, parasitoids, pathogens, or competitors of a pest to help keep its number in check. The entomopathogenic fungus, *M. anisopliae*, the agent of green muscardine disease of insects is one among the most exploited hypocrelean fungi for insect pest

management. This fungus has gained significant attention as biocontrol agent due to its vast spectrum of pathogenicity and infectivity to wide range of insect pests.

Diversity within the entomopathogenic fungal species has been traditionally analyzed using morphological features by assessing the phenotypic characteristics. However, assessing the species diversity by analyzing morphological features alone could lead to considerable ambiguity with regard to identification at species level, due to extensive divergence of morphological characters produced by a high level of genetic variability. Therefore utilization of molecular methods to detect the level of polymorphism among species help minimize the problem. Different molecular techniques have been employed to study the genetic diversity of entomopathogenic fungi. Some of the most commonly employed and relatively less complex techniques include RFLP, RAPD-PCR, AFLP, SSR analysis, ITS sequence analysis etc. With the employment of these techniques, many ecological and taxonomic complexities in the species level classification of Metarhizium could be resolved. RAPD analysis has been a very effective and easy to adopt tool in detecting genetic diversity in several species. The objective of this study was to determine the level of diversity among some of the isolates of *Metarhizium anisopliae,* collected from different sources and regions by phenotypic and genotypic characterization methods. The results of this study demonstrated considerable morphological and genetic diversity existed among the isolates studied.

MATERIALS AND METHODS

Isolation and maintenance of cultures of *M. anisopliae*

Among the nine fungal isolates (MIS1 to MIS25) used in this study, 6 were isolated either from soil or from infected insects and 3 procured from different institutions. The infected larvae were surface-sterilized by dipping sequentially in 70% ethyl alcohol, 1% sodium hypochlorite, and sterile distilled water; each for 3 minutes. The larvae were dissected and placed on PDA/Veen's medium and incubated at 28±1°C and 90% RH to facilitate growth and sporulation of the fungus.

Soil samples were collected upto a depth of 30cm from different study areas. *Galleria* bait method was used to isolate the fungus from soil samples. After removing roots and gravel, soil samples were sifted through a 5 mm sieve. Thereafter, plastic boxes (10 cm high, 8 cm diameter) were filled with 100 g of soil and ten *G. mellonella* late instar larvae were introduced. The lids were punched for making air holes. Boxes were incubated at 20°C in dark conditions. During the first five days, the boxes were turned once daily to make bait insects penetrate as much soil as possible. After 7-10 days, boxes were examined every other day and dead larvae were collected. Cadavers were surface-sterilized by dipping sequentially in 70% ethyl alcohol, 1% sodium hypochlorite, and sterile distilled water; each for 3 minutes. The larvae were dissected and placed on PDA/Veen's medium and incubated at 28±1°C and 90% RH to facilitate growth and sporulation of the fungus. Slant cultures were prepared from a single colony and stored at -20°C. The viability and virulence of the cultures were maintained by sub culturing and passaging through the host at regular intervals [1] [2].

Morphological characterization of *Metarhizium* isolates

Morphological features like the colour, media pigmentation, size and shape of conidia were recorded. The length and width of spores were measured at 1000 X magnification using an optical micrometer under phase contrast objective.

Molecular Characterization of *Metarhizium* isolates

DNA extraction

Whole-cell DNA was isolated from 7-day-old mycelia collected from cultures grown on PDA media supplemented with 1% (w/v) of yeast extract. For each fungal isolate, the freeze dried mycelium sample (50mg) was ground with a motar and pestle in liquid nitrogen, suspended in 500µl DNA extraction buffer (100mM Tris, pH8; 10mM EDTA; 2% SDS; 100µg/ml proteinase K; 1% β-mercaptoethanol; 1/10 vol. of 10% CTAB and 1.4M NaCl) and incubated for 10min at 65°C. After incubation, one volume of 24:1 chloroform: isoamyl alcohol was added, gently emulsified, incubated at 0°C for 30 min and centrifuged at 3500 rpm for 10 min at 4°C. The top phase was transferred to fresh 1.5 ml microfuge tube, ½ vol. of 5M NH_4OAc was added, mixed gently, incubated in ice for 60 min and centrifuged at 3500 rpm for 10 min at 4°C. The supernatant was discarded and 10mg/ml stock RNase was added to the tubes. DNA was precipitated with 0.55 vol. isopropanol and spun immediately at 3500 rpm for 50-10 min. The supernatant was aspirated and the DNA pellet was washed twice with 70% ETOH, air dried for 20 min at room temperature and re-suspended in 50µl TE buffer pH 8.0 and incubated at 4°C overnight.

RAPD – PCR

The nine isolates were analyzed by amplifying random sequences using three RAPD primers - OPD-07 (5'-TTGGCACGGG-3'), OPD-08 (5'-GTGTGCCCCA-3') and M13 F (5'- GTAAAACGACGGCCAGT-3'). The RAPD -PCR assay was performed with a 25µl reaction mixture containing 0.5 µl 10mM dNTPs, 2µl primer (25pmol), 2.5µl 10x PCR buffer, 1.5 µl $MgCl_2$ (1.5mM), 0.5 µl Taq DNA Polymerase (0.5U), 1 µl DNA template and 17 µl nuclease free water. For each isolate, PCR-RAPD of the extracted DNA was performed in an Applied Biosystem thermal cycler set at the following profile: an initial denaturation at 94°C for 5 min; 42 cycles, each cycle consisting of a denaturation at 94°C for 1min, annealing at 37°C for 1min and extension at 72°C for 2 min; a final extension step at 72°C for 7 min. Amplified products were resolved in 1.55 (w/v) agarose gels in 1 x TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.0) at 100V for 3.5h. A 1kb and 100bp DNA ladders were used as molecular weight markers. Gels were stained with ethidium bromide and imaged with the gel documentation system [3].

Analysis of RAPD data

The amplification products for the different isolates were compared with each other and were screened for the presence or absence of specific bands. Each amplified DNA fragment was considered a DNA marker and manually scored '1' for presence and '0' for the absence of bands for each primer. The binary data was used to estimate the levels of polymorphism by dividing the number of polymorphic bands by the total number of scored bands. The results were analyzed based on the principle that a band is considered to be 'polymorphic' if it is present in some individuals and absent in others and 'monomorphic' if present in all the individuals. All amplifications were repeated twice and only reproducible bands were considered for scoring. The RAPD data (binary data) generated with the three primers were used for statistical analysis. The scored band data (presence or absence) was subjected to cluster analysis using STATISTICA. The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm [4]. The data from the three primers were used to estimate the similarity on the basis of the number of shared bands. A genetic similarity matrix was constructed using Jaccard's coefficient method.

RESULTS AND DISCUSSION

Isolation of fungal strains

Details of the isolates characterized and their source and place of collection are furnished in Table 1. Isolation of *M. anisopliae* from dead cadavers on agar medium and from soil samples by *Galleria* bait assay has been carried out by various researchers [5]. Baiting soil samples with larvae of *G. mellonella* is a widely applied tool to screen for species of entomopathogenic fungi and is therefore useful for isolation of the spectrum of entomopathogenic fungi present in soils. Fungus from the insect cadavers was isolated by incubating a part of the cadaver in agar medium [2].

Table 1: List of isolates of *M. anisopliae* collected from different sources/locations

Isolates	Host insect/source	Place Of Collection
MIS1	Unknown larva	Mysore, Karnataka
MIS2	Unknown larva	Madhurai, Tamil Nadu
MIS3	Unknown larva	Sirsi, Karnataka
MIS7	*Eutectona machoeralis* (Lepidoptera)	Tirupathi, Andhra Pradesh
MIS13	soil	Bangalore, Karnataka
MIS18	soil	Salem, Tamil Nadu
MIS19	Unknown larva	NBAII
MIS20	Unknown larva	NBAII
MIS24	*Diatraea saccharalis* (Lepidoptera)	ARSEF

Morphological characterization

Morphological characteristics of the isolates were studied after purifying the cultures. The colour, shape and size of conidia etc are furnished in Table 2. Colony borders of all the isolates were regular. Colonies were initially white or creamy, mycelia bearing clumps of conidiophores, becoming shades of yellow green or olivaceous green to dark green during sporulation with the presence of colorless exudate drops. Colony colour varied from pale green to blackish green. Pigment production was observed at the base of the growth medium for four isolates. Average width of spores ranged from 2.10 - 4.10µm and average length from 3.20 - 7.69 µm. Based on length and width, the spores were grouped as either oval, round or elongated.

This observation akins with those made by many workers on this aspect; studies carried out by Fernandes *et al*. [6] showed that colonies of *Metarhizium* species and varieties in his study were predominantly dark green, light green, yellow, white or brownish. The reverses of most colonies were brownish, orange, yellow or not coloured. He also reported variation in conidia size from 5.62 x 3.98 to 10.57 x 4.74 µm. The findings of Tangthirasunun *et al*. [7] also supports the observations of the present study as they also reported variation in colour of *Metarhizium* isolates from light green to dark green, green to yellow pigmentation and size of the spores in the range of 2.06 to 3.73 µm (width) and 5.12 to 10.77 µm (length). Mythili *et al*. [8] reported dark herbage green or yellowish green or olivaceous colonies for surface culture of *M. anisopliae* after 7 days of incubation and the spore shape was cylindrical to oval measuring 7.5 mm. Colonies of *Metarhizium* were described as light green ovoid spores measuring < 9mm [9]. Caston [10] reported yellowish green colonies of *M. anisopliae* on PDA while Bridge *et al*. [11] recorded dark-green colour spore mass for *Metarhizium* isolates. Kheng Hoe *et al*. [12] reported yellowish green to thick green mycelium with white to orange pigmentation and round shaped spores for *Metarhizium* cultures.

Table 2: Morphological characteristics of *Metarhizium* isolates

Isolates	Colour	Media pigmentation	Shape of spores	Mean length of spores(µm)	Mean width of spores(µm)
MIS 1	Dark green	Yellow	Oval	5.33± 0.35	3.62±0.21
MIS 2	Dark green	Yellow	Oval	5.17±0.14	3.72±0.06
MIS 3	Pale green	_	Elongated	7.69±0.22	3.23±0.12
MIS 7	Dark green	Golden yellow	Round	4.14±0.15	4.10±0.15
MIS 13	Pale green	Yellow	Elongated	5.70±0.21	3.04±0.04
MIS 18	Blackish green	_	Round	3.20±0.18	2.10±0.18
MIS 19	Dark green	_	Round	4.36±0.11	4.00±0.05
MIS 20	Dark green	_	Elongated	6.52±0.14	2.98±0.13
MIS 24	Green	_	Round	3.78±0.19	3.28±0.12

Molecular Characterization

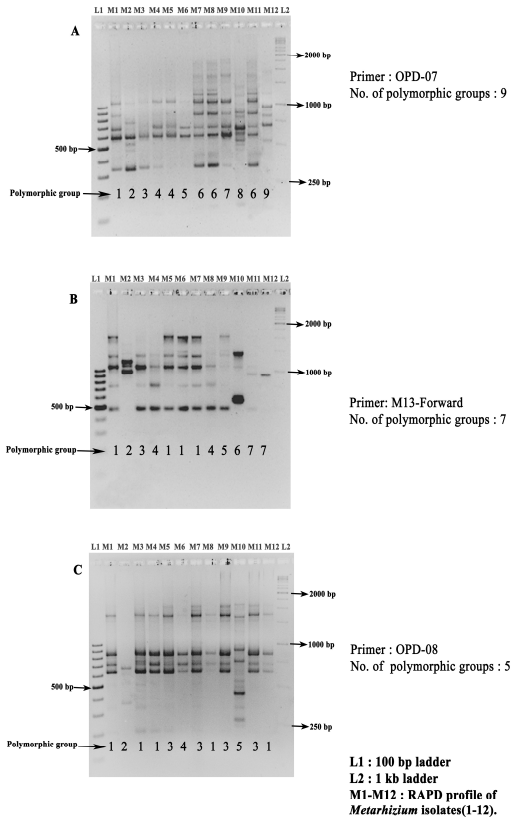
In our preliminary study, various parameters likely to affect the PCR amplification were optimized and data from optimum amplification conditions are presented here. Only well resolved, clear and distinct banding patterns were manually scored from the gel profiles and included for final analysis. The selected RAPD primers, sequence, total number of bands scored, their size and percentage of polymorphic bands for each primer are summarized in Table 3.

Table 3: Primers employed, their sequence, the size of the fragments and the percentage of polymorphism for each primer

Gel picture no.	Name of the primer	Primer Sequence (5' - 3')	Amplicon size range (bp)	Total no. of bands	No. of Polymorphic bands	Polymorphism (%)
A	OPD-07	TTGGCACGGG	300-2000	**63**	**16**	**25.4**
B	M13 Frd	GTAAAACGACGGCCAGT	400-1700	**57**	**7**	**12.3**
C	OPD-08	GTGTGCCCCA	200-1700	**46**	**14**	**30.4**

Out of the 8 RAPD primers screened, 3 primers produced unambiguous, consistently reproducible and well amplified uniform banding patterns in two independent experiments and were selected for further analysis. Fig. 1 indicates the banding pattern produced by three primers. Three primers generated a total of 166 reproducible distinct bands ranging from 200 to 2000 bp among the 9 isolates of *Metarhizium*. The number of bands per primer ranged from 63 for primer OPD-07, 57 for primer M13F and 46 for primer OPD-08 with an average of 12.3 polymorphic bands per primer. Out of the 166 bands, 129 (77.7%) were monomorphic and 37 bands (22.3%) were polymorphic revealing low to moderate degree of polymorphism. The polymorphism per primer ranged from 25.4% for OPD-07, 12.3% for M13F and 30.4% for OPD-08.

Fig. 1: RAPD profiles obtained using the primers: OPD-07, OPD-08 AND M13F
L1:100 bp ladder. L2:1 kb ladder. M1-M12: RAPD profile of isolates of *M. anisopliae*



The data from the three primers were used to estimate the similarity on the basis of the number of shared bands. Genetic similarity matrix was calculated on the basis of Jaccards algorithm for RAPD data. The Jaccard's similarity coefficient between isolate pairs was highly variable and ranged from 0.00 to 0.70 indicating a high genetic diversity (Table 4). The maximum similarity was noticed between isolates MIS13 and MIS18 with a high similarity coefficient of 0.70 followed by MIS1 and MIS3, MIS13 and MIS20 and MIS18 and MIS20 with a similarity coefficient of 0.66. Minimum similarity coefficient of 0.01 was recorded for isolates MIS2 and MIS24.

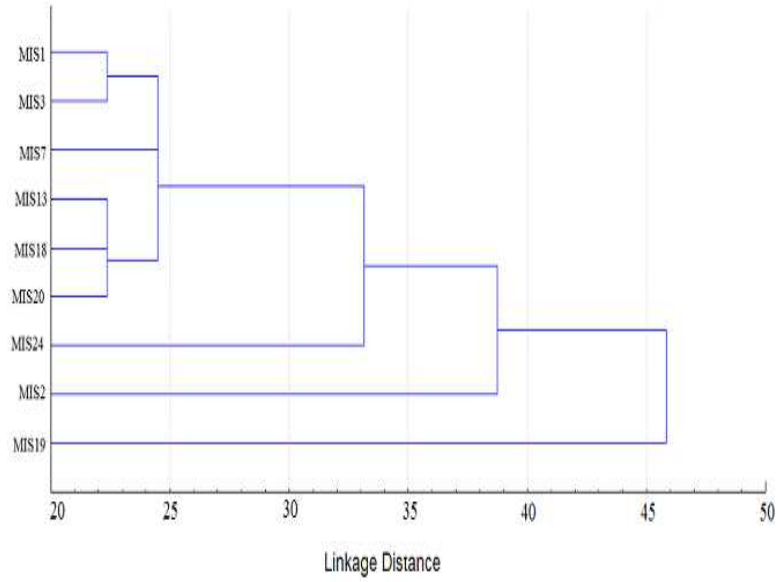
Table 4: Genetic similarity matrix for nine *Metarhizium* isolates generated using Jaccard's Similarity coefficient

Isolates	MIS1	MIS2	MIS3	MIS7	MIS13	MIS18	MIS19	MIS20	MIS24
MIS1	1								
MIS2	0.14	1							
MIS3	0.66	0.16	1						
MIS7	0.60	0.11	0.50	1					
MIS13	0.63	0.12	0.55	0.58	1				
MIS18	0.44	0.04	0.35	0.57	0.70	1			
MIS19	0.15	0.08	0.12	0.08	0.10	0.03	1		
MIS20	0.35	0.09	0.33	0.35	0.66	0.66	0.03	1	
MIS24	0.23	0.01	0.20	0.21	0.14	0.18	0.04	0.25	1

A dendrogram or a phylogenetic tree was generated from RAPD patterns of the *Metarhizium* isolates. Grouping of isolates into cluster/branch correlated with similarities in their RAPD DNA patterns. For example, the *Metarhizium* isolates that produced the same DNA banding pattern were also recognized as being similar from phylogenetic analysis. These isolates belonged to the same sub-cluster in the phylogenetic tree. The whole dendrogram was distributed between MIS1 to MIS19. The maximum linkage distance between the isolates was 45.9 units. The phenogram for the *Metarhizium* isolates revealed four clusters, A, B, C and D (Fig. 2). The cluster A included 3

isolates distributed between MIS1 and MIS7 and was grouped into two sub-clusters A1 and A2. Sub-cluster A1 included 2 isolates, MIS1 and MIS3 while sub-cluster A2 consisted of only one isolate, MIS7. The cluster B consisted of four isolates and was grouped into two sub-clusters B1 and B2. Sub-cluster B1 consisted of three isolates, MIS13, MIS18 and MIS20. Isolate MIS24 formed the sub-cluster B2. Cluster C and D were solitary clusters consisting of only one isolate each. Cluster C consisted of isolate MIS2 and cluster D MIS19.

Fig. 2: Dendrogram depicting genetic diversity of *Metarhizium* isolates



Several kinds of molecular techniques have been used to study genetic diversity, for example, labeled probes to detect restriction fragment length polymorphism (RFLP) and methods based on the polymerase chain reaction (PCR) such as the use of random amplified polymorphic DNA (RAPD), simple sequences repeat (SSR or microsatellites) analysis and internal transcribed spacer (ITS) sequence analysis [3]. RAPD markers are important tools for evaluation of the variability among different isolates of a species, the degree of genetic relationship among isolates and may also be useful to help distinguish isolates of nonpathogenic and pathogenic fungi [13]. The PCR technique combined with RAPD analysis has been extensively used to determine the genetic diversity of various entomopathogenic fungi. Velasquez *et al*., [3] carried out RAPD analysis of thirty nine Chilean isolates of *Metarhizium anisopliae var anisopliae* which yielded a total of 189 bands with an average of 41% similarity between isolates and the thirty nine isolates were divided into three different clades in the dendrogram. Fegan *et al*. [14] studied the genetic diversity of *Metarhizium* isolates using RAPD and observed a great genetic variability in isolates of *M. anisopliae* var. *anisopliae*. Genetic diversity among 13 isolates of *M. anisopliae* var. *anisopliae* was analysed by Fungaro *et al*. [15] and reported less variability among the isolates collected from insects, suggesting some degree of host specificity. The capability of the RAPD technique to distinguish mutants of *M. anisopliae* var. *anisopliae* was reported by Freire *et al*. [13]. RAPD is a convenient, economical and rapid method as compared to other techniques since it requires no probes and prior sequence information. Further, relatively small number of primers can be used to generate a very large number of fragments from different regions of the genome and hence multiple loci may be examined very quickly. This makes RAPD a powerful technique for screening the germplasm for assessing the genetic diversity [16].

CONCLUSION

Based on the morphological studies, average width of spores ranged from 2.10 - 4.10µm and average length from 3.20 - 7.69 µm. spores were oval, round or elongated and colony pale green to blackish green. Four isolates showed pigmentation. RAPD analysis of the nine isolates revealed that, among the 166 bands produced by the three primers, 129 bands were monomorphic and 37 bands were polymorphic. The Jaccard's similarity coefficients between the isolates ranged from 0.00 to 0.70. Dendrogram showed four clusters, A, B, C and D. Cluster A had three isolates that

formed two sub clusters A1 and A2 while cluster B also consisted of two subclusters B1 and B2 with a total of four isolates. One isolate each formed cluster C and D.

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