Species confirmation of fungal isolates by molecular analysis

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Received: 22 February 2008 / Accepted: 15 October 2008
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Abstract Traditional taxonomy of hyphomycetes has been based on conidial morphology and development. In order to confirm species level for the detection and identification of the entomopathogenic fungus, we analysed the species-specific fingerprints to investigate molecular characteristics within isolates of six species and to resolve morphologically atypical isolates. The extent of fingerprint profile observed by RAPD was sufficient to confirm the species level of all the isolates. The genetic similarity among morphologically identified isolates of each species was considerably higher, allowing us to conclude that all the isolates are of same species. These results establish a molecular framework for further taxonomic, phylogenetic and comparative biological investigations.

Keywords *Beauveria bassiana* · Entomopathogenic fungi · *Isaria felina* · *Metarhizium anisopliae* · *Nomuraea rileyi*

Introduction

Entomopathogenic fungi, which are mostly facultative parasites, are subjected to many environmental factors. Environmental habitat selection determines the population structure [1]. The population structure and gene diversity of isolates can arise either due to diversification of a single genotype into several forms through accumulations of changes in the genome or due to recombination between genetically different individuals either by sexual or parasexual means [2]. Individual isolates of a particular entomopathogenic fungus may display considerable specialisation in host range, and it is becoming increasingly apparent that identification up to the species level is necessary to place the fungal isolate within a taxonomic rank.

Classification of these fungi is based on morphological characteristics, which are often highly subjective, with unambiguous identification. Additionally, the morphological differences observed may be the product of simple mutations or media/cultivation [3]. Phenotypic characters of a fungal species often displayed a range of variation, which overlaps, with those of another species [4]. Obviously, taxonomic procedures are becoming more and more complex and it is generally accepted that molecular identification techniques are needed in addition to the traditional morphological characteristics formally used to classify fungal species [5].

Molecular analysis can potentially place an isolate within a genus or even to a confirmed species level without having to observe the latter [6]. Different molecular techniques were used for various applications and on different fungi [7–10], including identification using RAPD [11–13]. Since its development, the RAPD protocol has acquired a diversity of uses, such as: the establishment of the genetic

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similarity degree between individuals within a population [14], the construction of genetic maps as well as the localization of economically interesting genes [15], the production of a genomic fingerprint [16], the evaluation of recombination processes [17] and for the study of genetic diversity along with the identification of species and isolates including entomopathogenic fungi [18, 19].

Using the RAPD analysis, we attempted to confirm the species level of some indigenous entomopathogenic fungal isolates collected from the diverse habitats of Central India. A second objective was to assess whether molecular analysis support the species level confirmation for fast identification process necessary for specific and sensitive systems for identification of fungi prior to deposition in long-term storage and third objective was to compare genetic diversities, genetic similarities and genetic distances among the isolates of an individual species.

Material and methods

Monosporic fungal cultures were prepared from stored lyophilised stocks. All the isolates were easily identified to the genus level on the basis of morphological criteria. For our analysis we selected 15 isolates of *Paecilomyces fumosoroseus*, 12 isolates of *Beauveria bassiana*, 11 isolates of *Nomuraea rileyi*, 8 isolates of *Paecilomyces farinosus*, 5 isolates of *Metarhizium anisopliae* and 4 isolates of *Isaria felina*, which were assigned to a known species using microscopic morphological characters (Table 1).

DNA extraction, concentration estimation was carried out according to Thakur and Sandhu [20]. Primer survey was carried out using random decamer primers from kits A, F, G, H, K, J and X (Operon Technologies, Alameda, USA). A total of 32 random primers were screened using DNA from one isolate of each species. Those primers that gave

Table 1 List of fungal cultures used in the present investigation

FGCC No.	Name of fungus	Insect Host	District	State
E 13	<i>Metarhizium anisopliae</i>	Grass hopper	Jabalpur	MP
E 14	<i>Metarhizium anisopliae</i>	<i>Eutectona machaeralis</i>	Jabalpur	MP
E 15	<i>Metarhizium anisopliae</i>	<i>Eutectona machaeralis</i>	Mandla	MP
E 16	<i>Metarhizium anisopliae</i>	<i>Eutectona machaeralis</i>	Mandla	MP
E 17	<i>Metarhizium anisopliae</i>	<i>Eutectona machaeralis</i>	Mandla	MP
E 18	<i>Paecilomyces farinosus</i>	Unidentified insect	Dantewara	CG
E 19	<i>Paecilomyces farinosus</i>	<i>Eutectona machaeralis</i>	Dantewara	CG
E 20	<i>Paecilomyces farinosus</i>	Adult <i>Hyblaea</i>	Dantewara	CG
E 21	<i>Paecilomyces farinosus</i>	Unidentified beetle	Bastar	CG
E 23	<i>Paecilomyces fumosoroseus</i>	Stick insect	Bastar	CG
E 24	<i>Paecilomyces fumosoroseus</i>	Unidentified grub	Jabalpur	MP
E 26	<i>Nomuraea rileyi</i>	<i>Spodoptera litura</i>	Narsimhapur	MP
E 27	<i>Nomuraea rileyi</i>	<i>Diachrysia orichalcea</i>	Narsimhapur	MP
E 33	<i>Nomuraea rileyi</i>	<i>Diachrysia orichalcea</i>	Jabalpur	MP
E 69	<i>Beauveria bassiana</i>	<i>Eutectona machaeralis</i>	Jabalpur	MP
E 70	<i>Beauveria bassiana</i>	<i>Eutectona machaeralis</i>	Jabalpur	MP
E 71	<i>Beauveria bassiana</i>	<i>Hyblaea puera</i>	Jabalpur	MP
E 72	<i>Beauveria bassiana</i>	<i>Hyblaea puera</i>	Jabalpur	MP
E 73	<i>Beauveria bassiana</i>	<i>Eutectona machaeralis</i>	Jabalpur	MP
E 74	<i>Beauveria bassiana</i>	<i>Eutectona machaeralis</i>	Jabalpur	MP
E 75	<i>Beauveria bassiana</i>	<i>Hyblaea puera</i>	Jabalpur	MP
E 76	<i>Beauveria bassiana</i>	<i>Hyblaea puera</i>	Jabalpur	MP
E 77	<i>Beauveria bassiana</i>	<i>Hyblaea puera</i>	Jabalpur	MP
E 78	<i>Beauveria bassiana</i>	<i>Eutectona machaeralis</i>	Mandla	MP
E 81	<i>Beauveria bassiana</i>	<i>Eutectona machaeralis</i>	Mandla	MP
E 82	<i>Beauveria bassiana</i>	<i>Eutectona machaeralis</i>	Mandla	MP
E 145	<i>Nomuraea rileyi</i>	<i>Diachrysia orichalcea</i>	Jabalpur	MP
E 146	<i>Nomuraea rileyi</i>	<i>Diachrysia orichalcea</i>	Jabalpur	MP
E 148	<i>Nomuraea rileyi</i>	<i>Diachrysia orichalcea</i>	Katni	MP

Table 1 Continued

E 152	<i>Nomuraea rileyi</i>	<i>Diachrysia orichalcea</i>	Katni	MP
E 158	<i>Nomuraea rileyi</i>	<i>Diachrysia orichalcea</i>	Katni	MP
E 159	<i>Paecilomyces farinosus</i>	<i>Hyblaea puera</i>	Bastar	CG
E 165	<i>Paecilomyces fumosoroseus</i>	<i>Eutectona machaeralis</i>	Dantewara	CG
E 168	<i>Paecilomyces farinosus</i>	<i>Eutectona machaeralis</i>	Dantewara	CG
E 185	<i>Paecilomyces fumosoroseus</i>	<i>Hyblaea puera</i>	Bastar	CG
E 187	<i>Paecilomyces farinosus</i>	<i>Hyblaea puera</i>	Bastar	CG
E 200	<i>Paecilomyces fumosoroseus</i>	<i>Eutectona machaeralis</i>	Mandla	MP
E 218	<i>Paecilomyces fumosoroseus</i>	<i>Hyblaea puera</i>	Seoni	MP
E 222	<i>Paecilomyces farinosus</i>	Unidentified spider	Balaghat	MP
E 223	<i>Nomuraea rileyi</i>	<i>Spodoptera litura</i>	Bhopal	MP
E 224	<i>Nomuraea rileyi</i>	<i>Spodoptera litura</i>	Bhopal	MP
E 227	<i>Nomuraea rileyi</i>	<i>Spodoptera litura</i>	Bhopal	MP
E 229	<i>Isaria felina</i>	Unidentified beetle	Hoshangabad	MP
E 238	<i>Paecilomyces fumosoroseus</i>	<i>Hyblaea puera</i>	Hoshangabad	MP
E 239	<i>Paecilomyces fumosoroseus</i>	<i>Hyblaea puera</i>	Hoshangabad	MP
E 240	<i>Paecilomyces fumosoroseus</i>	Unidentified insect	Chhindwara	MP
E 241	<i>Paecilomyces fumosoroseus</i>	Unidentified insect	Chhindwara	MP
E 242	<i>Paecilomyces fumosoroseus</i>	Unidentified insect	Jabalpur	MP
E 243	<i>Paecilomyces fumosoroseus</i>	<i>Eutectona machaeralis</i>	Mandla	CG
E 244	<i>Paecilomyces fumosoroseus</i>	<i>Eutectona machaeralis</i>	Mandla	CG
E 245	<i>Paecilomyces fumosoroseus</i>	<i>Hyblaea puera</i>	Jabalpur	MP
E 246	<i>Paecilomyces fumosoroseus</i>	<i>Hyblaea puera</i>	Chhindwara	MP
E 251	<i>Isaria felina</i>	Unidentified beetle	Jabalpur	MP
E 252	<i>Isaria felina</i>	Unidentified beetle	Jabalpur	MP
E 253	<i>Isaria felina</i>	Unidentified beetle	Jabalpur	MP

FGCC = Fungal Germplasm Collection Centre, Jabalpur, MP, India.

MP = Madhya Pradesh State.

CG = Chhattisgarh State.

reproducible results were selected and used in the RAPD analysis of selected isolates separately for each species. The set of primers was selected for each species to generate specific patterns of bands and construct a species-specific fingerprint. The sizes of RAPD products were estimated using 100 bp ladder + Lambda/*Hind* III digest marker (Bangalore Genei Pvt. Ltd., Bangalore, India).

The standard RAPD program for amplifications and the electrophoresis of amplified product were carried out according to Thakur et al. [21]. The presence and absence of RAPD band patterns was scored visually. The recorded data were statistically analysed. Jaccard similarity (S_j) coefficient values for each pair wise comparison between accessions were calculated and dendrogram based on similarity coefficient matrix using average linkage procedure was constructed separately for each species on the basis of reproducible amplification fragments from the scored data of all the six species by SAHN (Sequential agglomerative

hierarchical nested cluster analysis) clustering method-using UPGMA (unweighted pair-group method for arithmetic average analysis) to generate and visualize the relationship by NTSYS (Numerical Taxonomy System, Applied Biostatistics, Setauket, New York) computer programme [22].

Results and discussion

The selected set of primers were capable of amplifying multiple polymorphic DNA fragments of all the six species of entomopathogenic fungi, which resulted in either very distinct amplification, sub-optimal, indistinct amplification products or complete lack of amplifications (Table 2). However, among these primers, the amplifications obtained using OPA-09, OPG-16, OPJ-06, OPK-12, OPK-19, and OPX-06 was persistent in 15 isolates of *Paecilomyces fumosoroseus* and also in the amplifications obtained using

Table 2 Amount of RAPD-PCR amplified fragments per primer for comparative isolates of *Paecilomyces fumosoroseus*, *Beauveria bassiana*, *Nomuraea rileyi*, *Paecilomyces farinosus*, *Metarhizium anisopliae* and *Isaria felina*

Primer	Sequence 5' – 3'	Total amount of RAPD amplified fragments					
		<i>P. fumosoroseus</i>	<i>B. bassiana</i>	<i>N. rileyi</i>	<i>P. farinosus</i>	<i>M. anisopliae</i>	<i>I. felina</i>
OPA-09	GGGTAACGCC	14	–	–	–	26	18
OPA-13	CAGCACCCAC	–	17	23	–	21	10
OPA-16	AGCCAGCGAA	–	13	14	–	–	06
OPF-01	ACGGATCCTG	–	–	–	08	11	10
OPF-02	GAGGATCCCT	–	–	–	09	05	–
OPF-06	GGGAATTCGG	–	–	07	08	13	–
OPF-08	GGGATATCGG	05	–	08	08	–	–
OPF-10	GGAAGCTTGG	10	–	11	07	–	–
OPG-08	TCACGTCCAC	06	–	08	11	–	–
OPG-15	ACTGGGACTC	–	–	11	–	–	–
OPG-16	AGCGTCCTCC	14	–	08	12	–	–
OPH-02	TCGGACGTGA	–	13	10	–	24	16
OPH-12	ACGCGCATGT	05	14	16	08	08	10
OPH-16	TCTCAGCTGG	07	–	–	15	17	23
OPJ-01	CCCGGCATAA	–	–	–	09	–	–
OPJ-06	TCGTTCCGCA	20	13	–	16	04	–
OPJ-09	TGAGCTCAC	03	–	–	11	–	–
OPJ-16	CTGCTTAGGG	–	–	–	09	06	05
OPK-09	CCCTACCGAC	–	–	–	11	–	–
OPK-12	TGGCCCTCAC	22	15	17	10	–	14
OPK-19	CACAGGCGGA	27	15	09	07	–	10
OPX-06	ACGCCAGAGG	19	13	17	25	–	–
OPX-08	CAGGGGTGGA	06	11	18	04	–	–
OPX-09	GGTCTGTTG	–	18	09	–	–	–
OPX-15	CAGACAAGCC	02	09	20	–	–	–
Total amount of fragments		160	151	206	188	135	122

OPA-13, OPH-02 OPH-12, OPJ-06, OPX-09 and OPX-15 in all 12 isolates of *Beauveria bassiana* and by using OPA-13, OPF-10, OPG-15, OPK-12, OPX-09 and OPX-15 in all 11 isolates of *Nomuraea rileyi*. The amplifications obtained using OPF-01, OPG-08, OPH-16, OPJ-06, OPK-09 and OPX-06 was persistent in 8 isolates of *Paecilomyces farinosus* and also in the amplifications obtained using OPA-13, OPF-01, OPF-06, OPH-02, OPH-12 and OPJ-06 in all 5 isolates of *Metarhizium anisopliae* and by using OPA-09, OPA-13, OPF-01, OPH-16, OPK-14, and OPK-19 in all 4 isolates of *Isaria felina*, however the extent of polymorphism varied with each primer. Therefore the results of amplification patterns from these primers were used in the statistical analysis.

The RAPD analysis of *P. fumosoroseus* comprises of 15 indigenous isolates from various sites of 7 different districts of Central India which were recovered from 5 different types of insect hosts (Fig. 1). A total of 160 bands were obtained ranging between 300–4500 bp and 124 bands were scorable and showed polymorphism. This resulted in 77.50% polymorphism. The dendrogram (Fig. 2) obtained for *P. fumosoroseus* consists of 3 clusters (50% similarity). The results indicated the presence of high level of genetic

diversity. A fairly wide range in the value (0.33 to 0.89) of Jaccard similarity (S_j) coefficient was observed among all the isolates. These data indicated that 11–50% differences have evolved between isolates. The average S_j among the 15 isolates of *P. fumosoroseus* species was found to be 0.61.

The RAPD analysis of *B. bassiana* comprises of 12 indigenous isolates from various sites of 2 different districts of Central India which were recovered from 2 types of lepidopteran insect hosts (Fig. 3). A total of 151 bands were obtained ranging between 290–2300 bp and 119 bands were scorable and showed polymorphism. This resulted in 78.81% polymorphism. The dendrogram (Fig. 4) obtained for *B. bassiana* consists of 2 clusters (higher than 50% similarity). The results indicated the presence of high level of genetic diversity. Sets of bands showed identical patterns to others were grouped at 100% similarity. A fairly wide range in the value (0.29 to 1.00) of Jaccard similarity (S_j) coefficient was observed among all the isolates. These data indicated that 0–45% differences have evolved between isolates. The average S_j among the 12 isolates of *B. bassiana* species was found to be 0.65.

The RAPD analysis of *N. rileyi* comprises of 11 isolates collected from various soybean field sites of 4 different

Fig. 1 Example of RAPD banding pattern obtained for 15 *Paecilomyces fumosoroseus* isolates with primer OPK-19. The lanes labelled M correspond to the standard molecular weight markers. Separation conditions were 70 V (5.38 V cm^{-1}) for 180 min in 1.4% agarose.

Fig. 2 Dendrogram of 15 isolates of *Paecilomyces fumosoroseus* based on RAPD data.

districts of Central India (Fig. 5). The dendrogram showing the genetic relationship between *N. rileyi* isolates based on the total number of amplified fragments is presented in Fig. 6. A total of 206 bands were obtained ranging between 350–3000 bp and 172 bands were scorable and showed polymorphism. This resulted in 83.50% polymorphism. The dendrogram obtained for *N. rileyi* consists of 2 closely knit clusters (higher than 65% similarity). Sets of bands showed identical patterns to others were grouped at 100%

similarity. A moderate range in the value (0.63 to 1.00) of Jaccard similarity (S_j) coefficient was observed among all the accessions. These data indicated that 0–34% differences have evolved between isolates. The average S_j among the 11 isolates of *N. rileyi* species was found to be 0.82.

Different isolates of indigenous *P. farinosus* of Central India were analysed using RAPD technique comprises of 8 isolates from various sites of 3 different districts of Central India and recovered from 5 different insect hosts (Fig. 7).

Fig. 3 Example of RAPD banding pattern obtained for 12 *Beauveria bassiana* isolates with primer OPH-12. The lanes labelled M correspond to the standard molecular weight markers. Separation conditions were 130 V (10 V cm^{-1}) for 70 min in 1.2% agarose.

Fig. 4 Dendrogram of 12 isolates of *Beauveria bassiana* based on RAPD data.

A specific banding pattern was observed for each isolate of *P. farinosus* when selected random primers were used. A total of 188 bands were obtained ranging between 200–4500 bp and 162 bands were scorable and showed polymorphism. This resulted in 86.17% polymorphism. The dendrogram (Fig. 8) for *P. farinosus* consists of 2 closely knit clusters (higher than 50% similarity). A moderate range in the value (0.45 to 0.74) of Jaccard similarity (S_j) coefficient was observed among all the isolates. These data indicated that 26–47% differences have evolved between isolates. As none of the isolate was found to be at 100% similarity level,

thus each isolate is considered to be of individual genotype. The average S_j among the 8 isolates of *P. farinosus* species was found to be 0.60.

The RAPD analysis of *M. anisopliae* comprises of 5 isolates collected from various sites of two different districts of Central India (Fig. 9). Four isolates were recovered from *Eutectona machaeralis* and one from a grasshopper host. The dendrogram showing the genetic relationship between *M. anisopliae* isolates based on the total number of amplified fragments is presented in Fig. 10. A total of 135 bands were obtained ranging between 300–3000 bp and 118 bands

Fig. 5 Example of RAPD banding pattern obtained for all 11 *Nomuraea rileyi* isolates with primer OPX-09. The lanes labelled M correspond to the standard molecular weight markers. Separation conditions were 130 V (10 V cm^{-1}) for 80 min in 1.5% agarose.

Fig. 6 Dendrogram of 11 isolates of *Nomuraea rileyi* based on RAPD data.

were scorable and showed polymorphism. This resulted in 87.41% polymorphism. The dendrogram obtained for *M. anisopliae* separates the grasshopper isolates from lepidopteran host clusters (higher than 50% similarity). A fairly wide range in the value (0.42 to 0.96) of Jaccard similarity (S_j) coefficient was observed among all the accessions. These data indicated that 4–50% differences have evolved between isolates. The average S_j among the 5 isolates of *M. anisopliae* species was found to be 0.69.

Four isolates of indigenous *I. felina* of Central India were analysed using RAPD technique were collected from various sites of two different districts of Central India and recovered from unidentified insect hosts (Fig. 11). A total of 122 bands were obtained ranging between 400–2000 bp and 96 bands were scorable and showed polymorphism. This resulted in 78.69% polymorphism. The dendrogram for *I. felina* is shown in Fig. 12 (higher than 60% similarity). A moderate range in the value (0.57 to 0.78) of Jaccard

Fig. 7 Example of RAPD banding pattern obtained for 8 *Paecilomyces farinosus* isolates with primer OPX-06. The lanes labelled M correspond to the standard molecular weight markers. Separation conditions were 50 V (3.85 V cm^{-1}) for 200 min in 1.5% agarose.

Fig. 8 Dendrogram of 8 isolates of *Paecilomyces farinosus* based on RAPD data.

similarity (S_j) coefficient was observed among all the isolates. These data indicated that 22–36% differences have evolved between isolates. As non of the isolate was found to be at 100% similarity level, thus each isolate is considered to be of individual genotype. The average S_j among the 4 isolates of *I. felina* species was found to be 0.68.

In the present study, the considerable variability among isolates of each species was obtained by RAPD technique, which also reflected a high level of polymorphism at DNA

level in each species. Clustering analysis by UPGMA demonstrated that all isolates of a one species were identical. Cluster analysis has been used widely to consider banding patterns from gels. This technique in general has been developed for independent characters and bands in RAPD analysis are only arguably independent. However, cluster analysis is a hierarchical method that will place isolates in groups with some level of implicit relatedness. The dendrogram from RAPD data clearly gave unique result and showed that there were distinct homogenous populations within the very diverse species *P. farinosus*. It also showed some clustering in relation to host specificity. Isolates recovered from one host *E. machaeralis* order Lepidoptera clustered irrespective of geographical origins and so did isolates from the other lepidopteran host *Hyblaea puera*.

Although the isolates of *P. fumosoroseus* used in this study showed morphological as well as host-species similarities, they showed extreme diversity in PCR-amplified fragments. No differences were found in the RAPD banding pattern for all the isolates of the *B. bassiana*. Identical

banding patterns were also found among all the isolates of other species. The electrophoretic profile of the chosen isolates supports use of RAPD analysis for species level confirmation. Many of the isolates showed identical PCR fragments for one or two of the primers but no two isolates were identical for all the primers. We found some evidence of host clustering in *M. anisopliae*. This study provided no evidence of host clustering in *N. rileyi* isolates which were

Fig. 9 Example of RAPD banding pattern obtained for all 5 *Metarhizium anisopliae* isolates with primer OPH-02. The lanes labelled M correspond to the standard molecular weight markers. Separation conditions were 80 V (6.15 V cm^{-1}) for 90 min in 1.2% agarose.

Fig. 11 Example of RAPD banding pattern obtained for all 4 *Isaria felina* isolates with primer OPH-16. The lane labelled M correspond to the standard molecular weight markers. Separation conditions were 80 V (6.15 V cm^{-1}) for 90 min in 1.2% agarose.

Fig. 10 Dendrogram of 5 isolates of *Metarhizium anisopliae* based on RAPD data.

isolated in MP from the soybean looper hosts showed all bands in common. But in other cases also no correlation between host species and PCR-amplified fragments was found. Nevertheless, the extent of genetic similarity was higher in indigenous isolates of *N. rileyi* than other species.

The lack of host clustering among isolates of may stem from the wide host range of these entomopathogenic fungi, or because the insect hosts from which these isolates were recovered also represent an evolutionary diverse group within the Insecta. There were no apparent correlations

Fig. 12 Dendrogram of 4 isolates of *Isaria felina* based on RAPD data.

between subgroups and particular geographical locations or host insect origin. Differences can, however be found between most of the individuals so far recovered and consequently RAPD can be used for species characterization and authentication. Available molecular data support the status of two species of anamorphic genera, *Paecilomyces*, *P. farinosus* and *P. fumosoroseus*. Both form distinct, easily identifiable conidia, which, however, are often difficult to identify to species. Further taxonomic work on the status of the various species will require inclusion of additional, less conservative genes (e.g. ITS [23]).

Assessment of species confirmation by RAPD analysis involves the assumption that bands of similar size are homologous. Obviously, data could be misinterpreted and faulty conclusions drawn if different DNA fragments have similar sizes. To minimize this effect we used four different primers for one species which collectively allowed comparisons between a large numbers of bands. Similar approach was applied by Bidochka et al. [24] for the differentiation of species and strains of entomopathogenic fungi by RAPD. The level of resolution provided by the present study could be combined with the classical morphological criteria and species-specific assays for fine accurate diagnosis and identification of many if not most of the indigenous entomopathogenic fungal species could be designed. The RAPD analyses provided important information as to the degree of genetic similarity and the relationship between the isolates for each species investigated, revealing polymorphism and establishing electrophoretic profiles useful to characterize the entomopathogen. PCR-fragment-pattern

polymorphisms and the construction of probes from one or more of these fragments may provide a useful and rapid tool for identifying species of entomopathogenic fungi. The correct fungal species was successfully detected showing that molecular technique allows rapid and secure detection and identification of fungal isolates.

Chen et al. [25] found a high degree of similarity between *Puccinia striiformis* individuals from the same *formae specialis*, and high degree of polymorphism was observed for individuals of different species. In the same study the RAPD groups formed generally were not associated with the geographic regions either. Similar results were shown by Muller et al. [26] using RAPD assay to investigate the genetic similarity among the isolates of *Bipolaris sorokiniana* collected from different cultivars in wheat producing regions of Brazil. In a work done by Infantino et al. [27] all the Italian isolates of *Pyrenochaeta lycopersici* showed similar RAPD and esterase banding patterns. The overall results indicated a low degree of genetic variability within a collection of 43 Italian isolates. RAPD technique was successfully used to characterize entomopathogenic and mycoparasitic fungi, with special attention to evaluate the genetic stability of the strains that were used as active ingredients in commercial biopesticides [28]. The accuracy of RAPD markers for differentiation of 38 isolates of entomopathogenic fungi isolated from 20 geographic regions of Taiwan and mainland China was shown by Kao et al. [29].

Nucleotide alterations, insertions and deletions at initiation sites may result in polymorphic DNA, which is detectable by the RAPD technique [15]. The variation in intensity

of some fragments in this study was observed, but this might be due to a more efficient amplification at some target sites, or to greater complementarities between the primer used and the fungal DNA [30]. This procedure enables precise characterization and identification of individual isolate and eliminates problems with lower reproducibility of RAPD patterns or problematic interpretation of complex banding patterns as reported by Williams et al. [31]. Optimisation of reaction conditions and selection of suitable primers overcame those disadvantages. RAPD products generated by each particular primer were highly reproducible and no variation was found in reactions from different DNA extractions. Also, precise digital processing of electrophoresis gels is necessary to avoid problems with reproducibility of the obtained data.

Conclusion

Although there are some genetically distinct variations among the morphologically similar isolates; but these RAPD markers were successfully used to confirm the species level of all the morphologically similar isolates of *Paecilomyces fumosoroseus*, *Beauveria bassiana*, *Nomuraea rileyi*, *Paecilomyces farinosus*, *Metarhizium anisopliae* and *Isaria felina* indicating that RAPD markers are suitable technique for intraspecific identification. These results enabled a fast and efficient species confirmation of fungal isolates and also led to the generation of electrophoretic profiles, which discriminated intraspecific polymorphism in the isolates studied. Furthermore, by cloning the genomic DNA regions which have been found to be polymorphic, specific probes may be produced and following their sequence analysis, specific primers could be constructed which might enable rapid detection and identification of certain groups, or even single isolates. The level of neutral nucleotide mutation within nuclear rRNA ITS regions of these indigenous entomopathogenic fungal species may be further investigated. In doing so it is needed to identify unique genetic types, and recognize the evolutionary relationships among isolates infecting various insect species.

This level of identification seems particularly important as it provides a mechanism to validate the purity and identity of a fungal isolate in a fungal germplasm culture collection and that can be used as standard reference to register or protect an individual fungal isolate. The extent of fingerprint similarity was sufficient to confirm the species of all isolates. This feature is very important because the identification of isolates of any fungi has often turned out to be difficult or even impossible when based solely on morphological traits. These results emphasize the ease in using RAPD for taxonomy and the unambiguous identification by classical morphological traits. A major

review of all the genera is required, including the analysis of a more conserved gene region to clarify the phylogenetic relationships.

Acknowledgments The authors are thankful to Council of Scientific and Industrial Research, New Delhi, India for providing Research Associate Fellowship (09/097(0066)2006-EMR-I) to RT and to Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi for providing research project grant (BT/PR1062/PID/22/03/98). The authors are also thankful to Head, Department of Biological Sciences, R.D. University, Jabalpur for providing laboratory facilities.

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