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Assessment of genetic relation of Indian isolates of four predatory fungi through RAPD markers

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ABSTRACT

The polymerase chain reaction (PCR) method was used to differentiate 4 commonly occurring deuteromycetes (fungi imperfecti), viz. *Paecilomyces lilacinus, Verticillium chlamydosporium, Duddingtonia flagrans* and *Arthrobotrys oligospora* using short sequence oligonucleotide primers. A total of 146 amplicons were identified in the genomic DNA of 4 fungi. Maximum genetic similarity was found in between *Paecilomyces lilacinus* and *Verticilium chlamydosporium* (39.96%) while *Duddingtonia flagrans* and *Verticilium chlamydosporium* showed the least (22.5%). Though the fungi are placed in the same group (fungi imperfecti) the results indicate the status of their distinct genotype at molecular level.

Key words: Genetic relation, Predatory fungi

In recent years, nematophagus fungi of the class Deuteromycetes have shown a great potential for the control of helminths of livestock origin (Larsen 2000). In India, out of the 2 wild isolates of nematode-trapping fungi, viz. *Arthrobotrys oligospora* and *Duddingtonia flagrans*, the later has shown great promise in the control of gastrointestinal nematodiasis in livestock (Sanyal 2000). Besides these, recently 2 wild isolates of egg-parasitic fungi, viz. *Paecilomyces lilacinus* and *Verticillium chlamydosporium*, were also isolated (Sanyal *et al*. 2008), which are having capability to attack and destroy the long lasting egg stages of animal parasites. *Verticillium chlamydosporium* and other *Verticillium* spp. enzymatically degrade the egg shells of *Ascaris lumbricoides* of pigs (Lysek and Krajci 1987, Lysek and Sterba 1991, Kunert 1992), *Toxocara canis* of dogs (Araujo *et al*. 1995), *Parascaris equorum* of horses and *Nematodirus filicollis* of cattle (Larsen 2000). Indian isolates of the fungi *P. lilacinus* and *V. chlamydosporium* were reported to degrade eggs of *Gigantocotyle explanatum* and amphistomes in *in-vitro* condition (De *et al*. 2008).

The use of these fungi as biocontrol agent in livestock requires their inundative application along with feed so that they appear in the faeces and predate on the immature stages of parasites in the faeces/soil and thus prevent infection to the susceptible hosts (definitive/intermediate) (Larsen 2000, Sanyal 2000). These 4 fungi, along with others, are grouped as 'fungi imperfecti' as the mechanism of sexual reproduction is not known and the taxonomic relation of these fungi is not clear. The identification of this group of fungi using taxonomic keys does not allow differentiation between fungi which may exhibit substantial biological differences (Sanyal 2000). In contrast, modern molecular techniques offer the potential to monitor the release of a particular isolate provided an appropriate molecular signature is found (Tigano-Milani *et al*. 1995b, Faedo *et al*. 2001, Sanyal and Mukhopadhyaya 2002).

RAPD polymorphism is carried out using randomly synthesized primers in a PCR reaction. DNA fragments in a genomic DNA complimentary to the primer strand will be amplified and product will be detected in a gel (Bhusan *et al*. 2008). Hence, similarity in banding pattern indirectly indicates the genetic relation between 2 genomic DNA sample (between individuals or isolates) APD markers has been used successfully to estimate genetic similarity and variation in different livestock (Bhusan *et al*. 2008) different quantitative traits (Li-Xiang Long *et al*. 2004) and for estimating inbreeding (Bhattacharya *et al*. 2003). To address

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the immediate need for a reliable method of differentiation of the recently obtained Indian isolates of egg parasitic fungi and nematode-trapping at molecular level, this study was taken up to generate consistent randomly amplified polymorphic DNA (RAPD) markers for the measurement of genetic relation between these fungi.

MATERIALS AND METHODS

Fungi

Wild isolates of *V. chlamydosporium*, *P. lilacinus*, *A. oligospora* and *D. flagrans* maintained as mother cultures in 2.5% corn meal agar (CMA) medium were cultured in sterilized potato dextrose broth (2.5%) and incubated at 26°C. Following incubation for a fortnight, the fungal broth was centrifuged and pellet of fungal material was washed 3 times in phosphate buffer saline (PBS; pH 7.2) and was preserved in 70% alcohol at –20°C till further use.

Isolation of genomic DNA

Genomic DNA from the fungi was obtained by phenol-chloroform extraction (Sambrook and Russel 2001) and quantified using Gelquant software after comparing with standard molecular weight marker (Generuler, Fermentas, USA). Working solutions of quantified DNA (5 ng/ml) were prepared by diluting the stock solution in nuclease free water and stored at –20°C.

RAPD-PCR

Ten arbitrary primers were used for this RAPD-PCR, (Table 1). The amplification reactions were carried out in 25 ml volume containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100, 4 mM $MgCl_2$, 100 mM dNTPs each, 15–20 ng of primer (Bio Basic Inc., Canada), 25 ng of DNA and 1.5 units of *Taq* DNA polymerase. To check the repeatability of RAPD finger printing, same sample was screened in duplicate. After initial denaturation at 95°C for 5 min, PCR reaction was carried out in 35 cycles, 95°C for 45 sec, 36°C for 45 sec and 72°C for 45 sec followed by a step of final extension at 72°C of 10 min. PCR products were analyzed after electrophoresis in 1.5% (w/v) agarose gel and visualized in ethidium bromide.

RAPD amplicon profiling: Amplified products (5 ml) were loaded in 1.2% agarose gel containing ethidium bromide along with DNA marker (100 bp and 1 kb). Electrophoresis was done at 50V. After sufficient migration photographs were taken in a Gel documentation system.

Analysis of RAPD fingerprints: RAPD bands were assessed by observing the presence or absence of the amplified fragments in atleast 2 reactions. Positional homology of amplified fragments were assumed and only polymorphic bands were considered in analysis. Similarity coefficients (S) between fungi were calculated using the following formula:

$$S = 2N_{xy}/(N_x + N_y)$$

where N_x and N_y are the number of fragments amplified in fungus X and Y, respectively. N_{xy} is the number of bands shared by 2 fungi (Nei and Li 1979).

Table 1. Sequence details of 10 arbitrary primers used in the study

Code	5'–3'	M. W.
OP-01	ACCTGGACAC	2997
OP-02	GGAGGAGAGG	3182
OP-03	CAGAAGCCCA	3006
OP-04	CCGCCTAGTC	2964
OP-05	TGTTCCACGG	3019
OP-06	AAGGCGGCAG	3102
OP-07	CAGCGACAAG	3046
OP-08	TTTGCCCGGT	3010
OP-09	TGGAGAGCAG	3117
OP-10	ACAACGCGAG	3046

RESULTS AND DISCUSSION

Ten oligonucleotide primers (10-mer) of arbitrary sequence revealed the presence of 146 scorable amplicons in the genomic DNA of the 4 fungi. Distinct fingerprints between the fungi were obtained by most of the primers (Figs

Table 2. Similarity coefficient among various fungal species to a specific primer

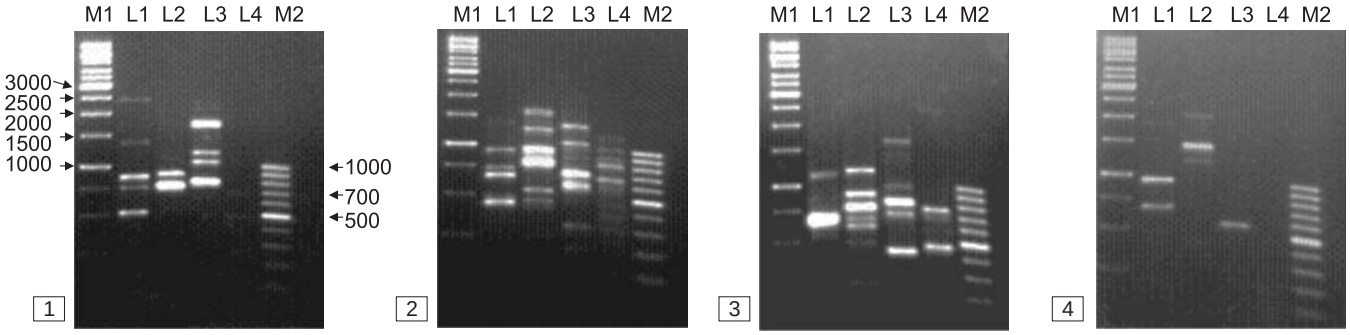
Primer	PL-VC	PL-DF	PL-AO	VC-DF	VC-AO	DF-AO
OP1	0.57	0.22	**	0.33	**	**
OP2	0.40	0.22	0.33	0.00	0.25	0.00
OP3	0.57	0.28	0.40	0.40	0.25	0.25
OP4	0.00	0.00	**	0.00	**	**
OP5	0.00	0.44	**	0.33	**	**
OP6	0.22	0.33	0.54	0.46	0.50	0.76
OP7	0.35	0.43	0.40	0.46	0.42	0.54
OP8	0.40	0.00	**	0.25	**	**
OP9	0.50	0.00	0.00	0.20	0.25	0.00
OP10	0.22	0.28	**	0.33	**	**

PL, *Paecilomyces lilacinus*; VC, *Verticillium chlamydosporium*; DF, *Duddingtonia flagrans*; AO, *Arthrobotrys oligospora*.
** No amplification in *Arthrobotrys oligospora*.

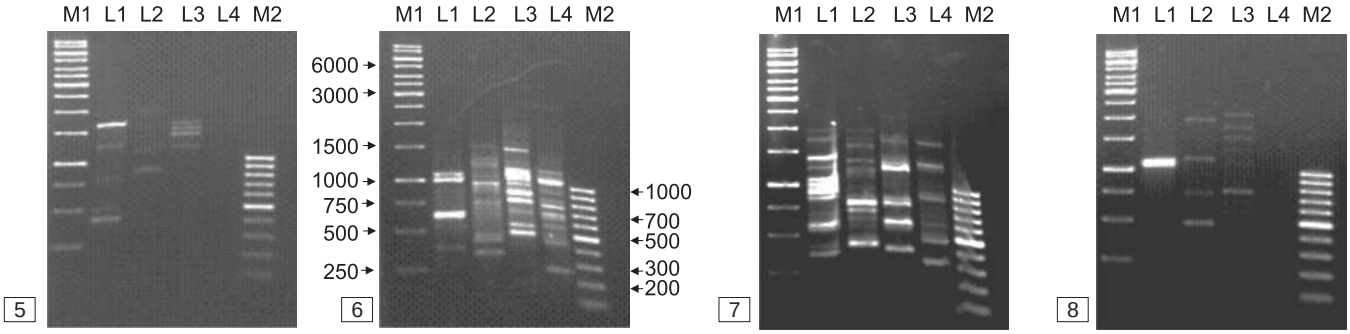
Table 3. Similarity coefficient between the fungi obtained using the combined data generated by the arbitrary primers (OP1- OP10), expressed as percentages

	PL	VC	DF	AO
PL	–	39.96	32.10	28.13
VC	39.96	–	22.50	23.88
DF	32.10	22.50	–	26.87
AO	28.13	23.88	26.87	–

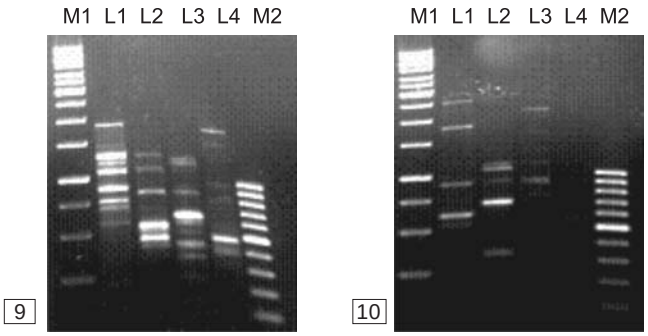
PL, *Paecilomyces lilacinus*; VC, *Verticillium chlamydosporium*; DF, *Duddingtonia flagrans*; AO, *Arthrobotrys oligospora*.



Figs 1–4. **1.** RAPD fingerprints with primer OP-1 on agarose gel. **2.**RAPD fingerprints with primer OP-2 on agarose gel. **3.** RAPD fingerprints with primer OP-3 on agarose gel. **4.** RAPD fingerprints with primer OP-4 on agarose gel.



Figs 5–8. **5.** RAPD fingerprints with primer OP-5 on agarose gel. **6.** RAPD fingerprints with primer OP-6 on agarose gel. **7.** RAPD fingerprints with primer OP-7 on agarose gel. **8.** RAPD fingerprints with primer OP-8 on agarose gel.



Figs 9–10. **9.** RAPD fingerprints with primer OP-9 on agarose gel. **10.** RAPD fingerprints with primer OP-10 on agarose gel.

1–10). Similarity coefficient analysis of polymorphic pattern showed 100% different banding pattern in amplification of genomic DNA of 4 fungi using OP4 oligonucleotide primer (Fig.4). The same type of banding pattern (100% dissimilarity) was also obtained by amplification of genomic DNA of *Paecilomyces lilacinus* and *Verticillium chlamydosporium* using OP5 oligonucleotide primer (Fig.5), *Paecilomyces lilacinus* and *Duddingtonia flagrans* and between *Duddingtonia flagrans* and *Arthrobotrys oligospora* using OP9 (Fig.9) oligonucleotide primer (Table 2). Similarity coefficients between the fungi using the combined data generated by the arbitrary primers (OP1 to OP10) revealed microhomogeneity among the fungi in a range of 22.5%–39.96% (Table 3). The results revealed maximum genetic similarity between *Paecilomyces lilacinus* and *Verticillium chlamydosporium* (39.96%) while *Duddingtonia flagrans* and *Verticillium chlamydosporium* showed the least (22.5%).

The main emphasis of the present study was to distinguish between the four predacious fungi (*P. lilacinus*, *V. chlamydosporium*, *D. flagrans* and *A. ologospora*) by studying their polymorphic amplification pattern. Most of the earlier molecular works on *Fungus imperfecti* of the class Deuteromycetes, were carried out to distinguish different strains within a genus of fungus (Tigano-Milani *et al*. 1995a, Tigano-Milani *et al*. 1995b, Mukhopadhyaya *et al*. 2004, Faedo, 1998, Sanyal and Mukhopadhyaya 2002).

In the present study, arbitrarily designed oligonucleotide primers revealing detectable polymorphs among the fungi were identified. On the basis of the similarity coefficients (Table 2) and combined similarity coefficients (Table 3), these 4 fungi appear to have much microheterogeneity (60.04–74.5%), which is reflected in their fingerprints. This has reconfirmed the status of their distinct genotype at molecular level. The most pronounced feature of DNA fingerprints was the variation in the intensity of the bands in the same reaction possibly because of priming within the repeated region and indicative of presence of unique genotype of the corresponding fungus. This has been well exemplified in case of *Echinococcus granulosus* of bubaline origin (Reddy *et al*. 1998) and *Trypanosoma evansi* (Basagoudanavar *et al*. 1999).

Absence of RAPD bands in *A. oligospora* genomic DNA by the primers OP1, OP4, OP5, OP8 and OP10 (Figs1, 4, 5, 8, 10) is indicative of the absence of the priming sites of these primers in the fungal DNA.

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