Morphological and genetic differentiation among four pigment producing Indian species of *Phoma* (Saccardo, 1899)

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Abstract A PCR-based technique, involving the random amplification of polymorphic DNA (RAPD), was used for assessing genetic relatedness among isolates of the genus *Phoma*. Randomly Amplified Polymorphic DNA (RAPD) revealed the presence of interspecific genetic variation among the pigment producing isolates of *Phoma* and has shown distinct phylogenetic cluster. The major objective of the study was to study the genetic variation, if any. Study was aimed to differentiate four pigment producing species of *Phoma* based on morphological studies and molecular markers in general and RAPD in particular. We found that the test species of *Phoma* can be very well differentiated using molecular markers. *Phoma sorghina* was differentiated from *P. exigua*, *P. fimeti* and *P. herbarum*. RAPD profiles of *P. herbarum* and *P. fimeti* has shown the maximum similarity, which indicates the genetic relatedness among these two species which were considered earlier as distinct species based on morphological observation.

Keywords RAPD · *Phoma* · Genetic variation

Introduction

Phoma is a taxonomically difficult genus and is not fully understood. It belongs to order Sphaeropsidales of Deuteromycotina. It is a unique form of pycnidiales, which occurs ubiquitously and have been reported from a wide variety of hosts particularly from plant and soil. It has also been recovered from aquatic and aerial environment [1], marine environment [2], entomopathogenic [3] and have been found to cause disease in human beings [4, 5].

The existing Indian species of *Phoma* have been erected on the basis of host alone, and thus the importance of host specificity for the taxonomy of *Phoma* has been much emphasized and overestimated. The assumption that each host genus or species was colonized by a specialized *Phoma* species prompted many mycologists to ignore morphological characters when erecting new *Phoma* species. Usually, a morphological species may attack various host plants. For example, *P. exigua* has been reported by investigators on different hosts [6]. The criterion of identification should be in such a way so that it should be possible to identify a *Phoma* species in case host identification is difficult particularly when floral parts are lacking, or when the fungus is grown on artificial media.

Molecular techniques have revolutionized analyses of the diversity of fungi and studies of the interaction with their hosts. Particularly, the polymerase chain reaction (PCR) assay has provided a framework to understand taxonomy and population structure. The assessment of genetic diversity is required for the correct species identification and recognition of physiological strains (pathotypes). The random-amplified polymorphic DNA (RAPD) method has been successfully used to identify strains [7–9], to characterize races [10] and to analyze virulence variability related to

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genetic polymorphisms [11–15] in phytopathogenic fungi. It has also been used in the study of inter- and intraspecific variability among populations from different [16–18] and from the same geographic regions [19–21].

Since its development, the random-amplified polymorphic DNA (RAPD) protocol has acquired a diversity of uses, such as: the establishment of the genetic similarity degree between individuals within a population [22], the construction of genetic maps as well as the localization of economically interesting genes [23], the production of a genomic fingerprint [24], and the study of genetic diversity along with the identification of fungi [25–30].

We used a RAPD assay to determine the genetic variability among four pigment producing species of *Phoma*.

Materials and methods

Fungal strains isolation and growth conditions

During the course of the present study four different pigment producing *Phoma* species were selected. These included *P. sorghina*, *P. exigua*, *P. herbarum* (MTCC 2319) and *P. fimeti* (MTCC 2323), out of these *P. exigua* was isolated from soil and leaf litter, while *P. sorghina* from petiole of *Carica papaya*.

Isolations were made by cutting the infected portions from the junctions of healthy and diseased region of the leaves and by surface sterilizing with 70% ethanol and putting it onto petriplate containing sterilized PDA (Potato Dextrose Agar) and malt agar. Isolations from soil were performed by serial dilution.

All the isolates were grown on PDA and malt agar. The cultures of *Phoma* were maintained on PDA and malt agar (Hi-Media, Mumbai) slants at 4°C.

Cultural studies

The cultural studies were carried on potato dextrose agar and malt agar media. The culture plates were inoculated by *P. sorghina*, *P. exigua*, *P. herbarum* and *P. fimeti* and incubated at 22°C for 7 days.

DNA extraction

For isolation of DNA from *Phoma sorghina*, *P. exigua*, *P. herbarum* and *P. fimeti* the test isolates were grown in 40 ml malt broth in 150 ml conical flask in dark and maintained in a growth chamber adjusted at 22°C for 7-days. Mycelial mat was then separated and washed with sterile distilled water twice to remove the traces of media. Mycelium was then dried on a sterile filter paper. Fungal genomic DNA

was extracted from dried mycelium using modification of method given by Vandemark et al. [31]. Two gram of mycelium was macerated in liquid nitrogen using mortar and pestle. The ground mycelium was hydrated for 10 minutes at room temperature in 3 ml of extraction buffer [10 mM Tris-Cl, 250 mM NaCl, 10 mM EDTA, pH 8.5 (Qualigens Fine Chemicals, Mumbai), and 0.5% w/v Sodium Dodecyl Sulphate (Sigma Aldrich Chemie, Germany). DNA was extracted with two volume of phenol: Chloroform: isoamyl alcohol [25:24:1] (Sigma Chemie, Germany). The nucleic acid was precipitated from upper aqueous layer with twice the volume of ice-cold ethyl alcohol (–20°C). After centrifugation for 5 minutes at 4000 rpm, the pellet was washed with 70% ethyl alcohol twice, air dried in laminar flow and resuspended in 1X Tris-EDTA buffer [1.0 M Tris-HCl and 0.1 M EDTA] (Sigma Chemie, Germany)]. Dissolved nucleic acid was stored at –20°C for long term storage.

Screening for random amplified polymorphic DNAs (RAPD)

DNA from each fungal isolate was screened for RAPD markers generated by 22 random decamer primers (Operon Technologies, Inc. Alameda, CA). Five primers were selected finally for the RAPD analysis (Table 1). Each reaction mixture (25 µl) for PCR amplification consisted of 10X assay buffer for Taq DNA polymerase with 15mM MgCl₂ (MBI Fermentas), 1U of Taq DNA polymerase (MBI Fermentas), 200 µM dNTP mix (MBI Fermentas), 0.4 µM decamer primer (Operon Technologies, USA), and approximately 50 ng genomic DNA template. PCR amplification conditions were as follows: Initial extended step of denaturation at 94°C for 5min, followed by each 40 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min, and elongation at 72°C for 2 min. The 40th cycle was followed by final extension step at 72°C for 7 min and then being held at 4°C until electrophoresis was done. PCR was carried out in Whatman Biometra 2000 (Germany) thermocycler. PCR products were mixed with 5.0 µl of 6X gel loading dye (MBI Fermentas) and the amplification products were electrophoresed on 1.5% w/v M.B. grade agarose gel at 50 volts in 1X TAE buffer (MBI Fermentas). Gene ruler 500 bp and Lambda EcoRI/Hind III digest (MBI Fermentas) were used as molecular size standards. The gels were stained

Table 1 Primers and their sequences used for RAPD analysis

S. No.	Primer	Sequence
1.	OPA-02	TGCCGAGCTG
2.	OPA-04	AATCGGGCTG
3.	OPA-08	GTGACGTAGG
4.	OPA-10	GTGATCGCAG
5.	OPF-01	ACGGATCCTG

with 0.5 µg/ml of ethidium bromide (Sigma), visualized under ultra violet light, and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). For all isolates, bands on RAPD gels were scored as present (1) or absent zero (0). The results were repeated three times to check the consistency of the RAPD profile.

Data scoring and statistical analysis

Amplicons were scored for the presence (1) or absence of bands zero (0) across all the lanes. The binary RAPD data were analyzed to produce a matrix of similarity values based on Jaccard's coefficient of similarity (J_{ij}) [32]. Clustering of lines was done using unweighed pair-group method based on arithmetic averages (UPGMA) analysis using the programme Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) software version 2.1 [33].

Results

Cultural characteristics of *Phoma* species

All the four species of *Phoma* were selected for the study in order to assess their inter-specific variations and relatedness using Random Amplified Polymorphic DNAs.

P. exigua was isolated from soil (Leaf litter) by serial dilution method and *P. sorghina* was isolated from leaf petiole of *Carica papaya*. Both the species were grown on potato dextrose agar (PDA) and malt agar (Hi-Media, Mumbai) for their morphological and cultural studies and were maintained in slants at 4°C.

The cultures of *Phoma* were identified by using identification key [34, 35]. Two species were sourced from MTCC (Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh, India).

Comparative morphological and cultural studies of four species of *Phoma*, viz., *P. sorghina*, *P. exigua*, *P. herbarum* and *P. fimeti* are provided in Table 2.

P. herbarum was verified on cultural characteristics. Colonies were ashy to green with compact aerial mycelium; pycnidia black, globose to sub-globose; conidia hyaline 1-celled, ovoid, yellow discolouration of the medium was observed on malt agar. Red pigments were produced which on application of NaOH turned blue.

P. sorghina was verified on the basis of colour of the colony (Pinkish-Red), Yellow discolouration of the medium was observed. The mycelium was profuse, erect and pinkish-red. A typical character of this species is production of red pigment and chlamydospores.

P. exigua was identified on the basis of morphological characters and spot-test. Discolouration of agar medium was found on application of NaOH. Colonies were gray to black with irregularly scalloped margins attained a diameter of 6–7 cm in 7 days.

P. fimeti by its very slow rate of growth. Colonies were ashy to green with compact aerial mycelium, attaining the diameter of 1.0–1.5 cm on malt agar; pycnidia black, globose to sub-globose; pycnidiospores hyaline, 1-celled, ovoid; dull-yellow discolouration of the medium on malt agar.

DNA isolation and characterization by RAPD-PCR

Fungal genomic DNA was extracted from juvenile mycelium using modification of method given by Vandemark et al. [31]. A significant yield was noted using modification of the protocol. Fig. 1 depicts the DNA isolated from four species.

Twenty-two primers were used for the RAPD analysis of which only five primers detected polymorphism. A total of 129 bands were obtained by using five primers ranging between 200–3000 bp out of which 82 bands were scorable

Table 2 Colony characteristics of different pigment producing species of *Phoma* (one week old culture)

Name of species	Pycnidia colour, shape and size	Colony diameter	Colour	Pigmentation	Herbarium/Host
<i>P. sorghina</i>	Black, Flask shaped, globose to sub-globose, 75–155 µm	5–7 cm	Pink to red	Yellow discolouration of the medium in acidic condition below the colony. Yellow colour changes to red with NaOH.	<i>Carica papaya</i> Linn.
<i>P. exigua</i>	Black, Globose to sub-globose coalesce to form irregular fructification, 79–296 µm	6–7 cm	Gray to black	Greenish blue discolouration changes to reddish on application of NaOH	Soil
<i>P. herbarum</i>	Black, Flask-shaped, 74–150 µm	4–5 cm	Gray to green	Red pigment turns blue on application of NaOH	MTCC 2319
<i>P. fimeti</i>	Black, Globose to sub-globose, pseudoparenchymatous, 74–160 µm	1–1.5 cm	Ashy-green	Dull-yellow discolouration	MTCC 2323

Fig. 1 Photograph (Alpha Imager 2000) showing DNA isolated from *Phoma* species on 0.7% agarose gel. M, Lambda Eco RI/Hind III digest (MBI Fermentas); Lane 1, *P. sorghina*; Lane 2, *P. exigua*; Lane 3–5, *P. fimeti*; Lane 4–6, *P. herbarum*

Fig. 2 Photograph (Alpha Imager 2000) showing RAPD profile of four pigment producing *Phoma* species obtained by OPA-02 decamer primer (Operon Technologies, USA) on 1.5% agarose gel stained with ethidium bromide. M, Gene Ruler 500 bp (MBI Fermentas); Lane 1, *P. sorghina*; Lane 2, *P. exigua*; Lane 3, *P. herbarum*; Lane 4, *P. fimeti*

and showed polymorphism. This resulted in 63.56% polymorphism. The numbers of amplified products were ranged from 5 to 11. All the primers produced amplification products; however the extent of polymorphism varied with each primer. Percent genetic similarity using RAPD markers was 22 using OPA-02. Figs. 2 and 3 depicts a section of the RAPD profile obtained with primer OPA-02 and OPF-01 respectively. No banding pattern was observed in negative control

Genetic similarity among isolates

Jaccards pair-wise similarity estimates between species within the genus *Phoma* were calculated (Fig. 4). The average similarity coefficient among four *Phoma* species revealed by five RAPD markers found to be 0.22. The species namely *P. herbarum* and *P. fimeti* showed 0.2564 similarity coefficient which was found to be the maximum as compared to all other isolates (Table 3). The least similarity was found between *P. sorghina* and *P. herbarum* (0.1892) using OPA-02 primer.

Discussion

The importance of morphological characters and conidial ontogeny for the taxonomy of fungi in general and *Phoma*

Fig. 3 Photograph (Alpha Imager 2000) showing RAPD profile of four pigment producing *Phoma* species obtained by OPF-01 decamer primer (Operon Technologies, USA) on 1.5% agarose gel stained with ethidium bromide. M, Lambda Eco RI/Hind III digest (MBI Fermentas); Lane 1, *P. sorghina*; Lane 2, *P. exigua*; Lane 3, *P. herbarum*; Lane 4, *P. fimeti*; Lane 5, Negative control

Fig. 4 Dendrogram based on Jaccard's Similarity Coefficient generated by UPGMA analysis of four *Phoma* species using five RAPD markers

Table 3 Jaccard's Similarity Coefficient among four *Phoma* species revealed by five RAPD primers

	<i>P. sorghina</i>	<i>P. exigua</i>	<i>P. herbarum</i>	<i>P. fimeti</i>
<i>P. sorghina</i>	1.0000			
<i>P. exigua</i>	0.2286	1.0000		
<i>P. herbarum</i>	0.1892	0.2143	1.0000	
<i>P. fimeti</i>	0.2059	0.2308	0.2564	1.0000

in particular has been strongly overestimated and many species have been erected from India. If morphology alone is taken to separate the taxa, it may result in considerable confusion because sometimes the identification based on morphology itself proves to be wrong or where a morphological species might show different characters with changed environmental conditions such as pigment production on agar medium.

For this reason, it is essential to study genetic diversity or relatedness using molecular markers in order to create a more realistic and usable classification of *Phoma* and *Phoma* related fungi in general and pigment producing species of *Phoma* in particular.

RAPD analysis is extremely powerful tool and can separate individuals having intra- and interspecific variability among the pathogen population as it is based on the entire genome of an organism.

Using RAPD markers, diversity within the four pigment producing isolates was studied. Prevalence of genetic diversity among four isolates revealed that they can be very well differentiated from each other using PCR based RAPD markers. Cultural characteristics of *P. fimeti* and *P. herbarum* revealed that these species show variety of

differentiating morphology-based characters. The diameter of the colony when considered as a differentiating character, it was found that diameter of *P. fimeti* was 1.0–1.5 cm while that of *P. herbarum* was 4.0–4.7 cm and in case of *P. sorghina* it was 6.0–7.0 cm after 7 days of incubation under same growth conditions and on the same growth medium (PDA). Slow growth of *P. fimeti* clearly differentiates it from other pigment producing species within the genus *Phoma*. Secondly, the conidia of *P. fimeti* are ovoid and 1-celled but, in case of *P. herbarum* conidia are sometimes bi-celled. Only colour of the colony in this case is some what similar. Irrespective of morphological characters that differentiates these two species, the RAPD profiles of these two species have shown maximum similar bands that has been given by screening with different decamer primers. Genetically these species showed maximum similarity than with other species such as *P. sorghina* and *P. exigua*.

UPGMA cluster analysis using Jaccards similarity coefficient showed two main clusters. The four pigment producing species were grouped into three distinct sub-clades. We could differentiate the species using RAPD markers. The genetic diversity of these isolates studied by RAPD markers is prerequisite for developing a diagnostic tool for the identification and differentiation of *Phoma* and other allied genera.

The purpose of this study was to elucidate the genetic relationship between related pigment producing *Phoma* isolates because they secrete commercially useful anthraquinones of antimicrobial nature, that are often isolated from plant material, soil samples and sometimes as opportunistic pathogens in immunocompromised hosts [4, 34]. An earlier study showed the usefulness of RAPD analysis in detecting

significant polymorphisms between *Didymella bryoniae* and *Phoma* species [36–38]. The present study expands the previous study by using RAPD analysis on a pigment producing collection of four isolates to determine their phylogenetic relationships. RAPD analysis of four pigment producing *Phoma* species used in this study sub-divided the isolates into three unique sets of genetic fingerprints. The present sampling of a collection of isolates confirms the previous observation regarding *P. fimetii* and *P. herbarum*. The species that show genetic similarity can be included as a new variety, but further biochemical marker based studies are required for validation of the data for operational taxonomic units.

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