The cost of production of fungus on standard media ranges from about Rs 70 to 120 l^{-1}, while the cost for the medium prepared by procuring every constituent from the market is Rs 2.43 l^{-1}, which is much cheaper and from a single standard petri plate, one can prepare 40 flasks of each fungi.

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Rapid and molecular discrimination of host-specific fungal plant pathogens in pulse crops using genome profiling

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A rapid and accurate identification of potential plant pathogens below the species level is highly desirable to understand the genetic basis of host–pathogen interactions and thus to effectively manage plant diseases. In this study, a genome profiling (GP) technique was applied to identify 14 common seed-borne fungal pathogens from five different legume plant hosts in Rajasthan, India. Six species belonging to different taxonomic orders were successfully identified and classified topologically to the same position with their phenotypic traits. Next, we demonstrated that GP could be used to discriminate fungal pathogenic strains below the species level by classifying 10 different strains of *Aspergillus niger* and *Aspergillus flavus* based on plant host specificity. These results suggest that accurate identification of plant pathogenic subspecies is likely to become an easier task, and the resulting GP-based database can be an ideal platform for timely and unambiguous identification of fungal species, with pathogenic or beneficial relation to plant host.

Keywords: Fungal plant pathogens, genome profiling, host–pathogen interaction, plant diseases, species identification.

THE high mortality associated with the increasing number of fungal and fungal-like diseases in recent times, has highlighted the need for rapid and accurate identification of fungal pathogenic species[1]. Classical identification of pathogenic isolates based on culture-based morphological, anatomical and biochemical analyses is not only inadequate in some instances but also time-consuming and laborious. Another important limitation of classical methods is the inability to differentiate pathogenic from nonpathogenic strains that belong to the same microbial species. In addition, most species of fungal plant pathogens are known to possess a broad host range, such as, two morphologically identical strains of a fungal species may have quite different infection capacity on two different hosts[2]. Pathogenic strains have therefore been assigned to

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so-called *formae speciales* (f. spp.) based on specificity to host species; while, a particular *forma specialis* which is pathogenic to a certain plant species, strains belonging to other *formae speciales* may be nonpathogenic or even beneficial to the same plant species[3]. Therefore, a rapid and accurate method to discriminate pathogenic and nonpathogenic strains towards a specific crop is required and essential for virtually all aspects of plant pathology including predicting disease outbreaks.

Molecular approaches are increasingly being employed as a superior alternative which not only resolves evolutionary relationships, but can also contribute to revising the classification of a particular genera or species[4,5]. Although many technologies to genotyping already exist, they are often not applicable to the comparison of more distantly related species. At present, sequence-based approaches, such as ribosomal RNA (rRNA) genes sequence analysis, are the most definitive solution for species identification. A DNA array approach containing genus-, species- and *formae speciales*-specific detector oligonucleotides has recently been developed for the identification of *Fusarium oxysporum*[6]. Nevertheless, array approaches require specialized robotics and imaging equipment that are not affordable in general. Another concern is that these and other similar molecular approaches are based on the detection of polymorphisms in ubiquitously conserved genes, and housekeeping genes do not generally reflect sufficient sequence variation to place a species at the appropriate position on the phylogenetic tree[7]. This is particularly important in the analysis of fungal species that exhibit a high level of sequence homology in rRNA genes but less than 50% genomic DNA similarity. Naturally, the gene similarity between strains in a species is much higher and far more difficult to discriminate. Therefore, a method which can sample a sufficient amount of sequence information from original genomic DNA is required to solve these strain delineation problems. The current genome sequence-based technologies, such as, random amplified polymorphic DNA (RAPD) and others, are best suited for discrimination at the strain level, but are poor at resolving the higher levels of taxonomy, mainly due to insufficient informational content. In addition, there have been criticisms mainly based on its lack of repeatability and reproducibility, because of the low stringency conditions employed[8]. Secondly, a cohesive computational environment based on existing cyber infrastructure is clearly required to vastly accelerate the global application of such genome-based taxonomic approaches. As a solution to these problems, genome profiling (GP) technique has been developed and demonstrated as a reliable, rapid and cost-effective molecular method which can exploit integrated sequence information obtained from several functionally important genes derived by analysing random PCR products by the use of microtemperature gradient gel electrophoresis (μTGGE), and thus represents a powerful tool for species identification[9–11]. Importantly, the inclusion of the internal reference method[12] and computer-aided data processing[13] in GP technique eliminate experimental fluctuations and generate highly reliable and reproducible data.

In this study, we have attempted to apply GP to investigate the genetic relationship among 14 common seed-borne fungal pathotypes, isolated from 5 different legume plant hosts (chickpea, *Cicer arietinum*; lentil, *Lens culinaris*; urad bean, *Vigna mungo*; mung bean, *Vigna radiata*; moth bean, *Vigna aconitifolia*) in India. The objective of this study is first, to establish if identification of fungal plant pathotypes using morphological character can be supported by a rapid and molecular GP approach; and secondly, to define the genetic relationship and correlate any difference with respect to the pathogenicity between morphologically similar species on different plant hosts.

Seed samples from five common pulses were collected from different agricultural fields of Rajasthan (Table 1). All the experiments were conducted in the laboratory carefully, and in well-sterilized conditions. Fifty seed samples of each pulse were randomly collected and tested, by employing standard blotter method in three replications. Three layers of sterilized blotters were jointly soaked in sterilized distilled water and placed in sterilized disposable plastic petri plates of 9 cm diameter. All the infected seeds were surface sterilized with sodium hypochlorite (0.1%) for 2 min and subsequently rinsed thrice in distilled water. Both unsterilized- and surface-sterilized seeds were equidistantly placed on a blotter in separate petri plates, in the ratio of 10 seeds per plate. Three plates were incubated in a biochemical oxygen demand (BOD) incubator at 25 ± 2°C for 8 days, under 12 h alternating cycles of near-ultraviolet (nUV) light and darkness. After incubation of 8 days, the exposed seeds were examined by naked eye and under stereoscopic microscope for the associated seed-borne pathogens, and the fungi were identified based on the habit and colony

Table 1. Fungal plant pathogens species and their hosts used in this study

Species	Host (cultivar)
Aspergillus niger	Lentil (*Lens culinaris*)
Aspergillus niger	Moth bean (*Vigna aconitifolia*)
Aspergillus niger	Mung bean (*Vigna radiata*)
Aspergillus niger	Urad bean (*Vigna mungo*)
Aspergillus niger	Chickpea (*Cicer arietinum*)
Aspergillus flavus	Lentil (*Lens culinaris*)
Aspergillus flavus	Moth bean (*Vigna aconitifolia*)
Aspergillus flavus	Mung bean (*Vigna radiata*)
Aspergillus flavus	Urad bean (*Vigna mungo*)
Aspergillus flavus	Chickpea (*Cicer arietinum*)
Rhizopus nigricans	Chickpea (*Cicer arietinum*)
Fusarium oxysporum	Chickpea (*Cicer arietinum*)
Alternaria alternata	Chickpea (*Cicer arietinum*)
Chaetomium globosum	Chickpea (*Cicer arietinum*)

characters. Following purification, all the isolated samples were preserved in potato dextrose agar (PDA) medium and detailed fungal characters were observed in detail. Their identification was confirmed with the help of standard literature[14–16].

The pure DNA from fungal mycelium was extracted by a rapid CTAB (cetyltrimethylammonium bromide) buffer-based extraction method[17]. A fungal mycelium (near 2 g) was ground in liquid N_2 with the help of a mortar and pestle. The homogenized material was then transferred to 10 ml pre-warmed (60°C) DNA isolation buffer and incubated for 30 min at 60°C in a water bath, with occasional mixing. This was followed by the addition of a 10 ml mixture of chloroform and isoamyl alcohol (24 : 1), and the solution was mixed through inversion, for 15 min, to ensure emulsification of the phases. The mixture was centrifuged at 15,000 rpm for 15 min at 4°C. The aqueous phase was taken out and transferred to another propylene tube and mixed with two volumes of ice-cold absolute alcohol or 0.6 volume of isopropanol. The precipitated DNA–CTAB complex was collected by centrifugation at 15,000 rpm for 15 min at 20°C. The pellet was washed using 20 ml of 70% alcohol for 20 min with mild agitation. The pellet was collected by centrifugation at 10,000 rpm for 5 min at 4°C and dissolved in 1 ml of *Tris* buffer (pH 8.0) followed by leaving it overnight at room temperature, without any agitation.

Purification of DNA was carried out through the following steps: a 2.5 µl of RNase was added to 0.5 ml of isolated crude DNA solution, mixed gently and incubated at 37°C for 1 h. A 0.4 ml solution of chloroform : isoamyl alcohol (24 : 1) was added and mixed thoroughly for 15 min till an emulsion was formed. The emulsion was centrifuged at 15,000 rpm for 15 min at 4°C and the supernatant was recovered by avoiding the whitish layer at the interface of emulsion. The DNA was reprecipitated by adding double the quantity of absolute alcohol. The tube was centrifuged for 5 min at 10,000 rpm and the pellet was washed with 70% alcohol and dried overnight. The DNA was dissolved in 0.5 ml of *Tris* buffer (pH 8.0) and stored for further use.

Oligonucleotide primers for species-specific PCR were used for the selective amplification of the internal transcribed spacer 1 (ITS1) and its flanking regions of the rDNA[18]. For specific detection of the genus *Aspergillus*, a set of forward primer (UD_{Asp}: 5′-CAGCGAGTACATCACCTTGG-3′) and reverse primer (DR_{Asp}: 5′-CCATTGTTGAAAGTTTTAACTGATT-3′) was used. For specific detection of the species *Aspergillus niger* and *Aspergillus flavus*, two primer sets were used which consist of a common forward primer ($UD_{A.nig/fla}$: 5′-ACTACCGATTGAATGCGTCG-3′) and different reverse primers ($DR_{A.nig}$: 5′-ACGCTTTCAGACAGTGTTCG-3′ for *A. niger* and $DR_{A.fla}$: 5′-TTCACTAGATCAGACAGACT-3′ for *A. flavus*). All these oligonucleotides were commercially obtained (JBioS Oligo Service Corp., Japan). Each genus or species-specific DNA was amplified in a total volume of 10 µl of a PCR mixture consisting of 1 × *ExTaq* PCR buffer (Takara Bio Inc., Japan), 0.2 mM of each dNTP, 2 µM each of the forward and reverse primers, and 0.5 U of *ExTaq* DNA polymerase. PCR was performed at 95°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 60 sec. A final PCR product extension was performed at 72°C for 10 min. PCR products of 1 µl aliquots were analysed and further confirmed by polyacrylamide gel electrophoresis (6% T, 90 mM TBE, and 8 M urea) and scanned using a lab-made LED transilluminator (μ Transilluminator, patent pending) and by silver staining.

An overall description of GP-based identification method of host-specific pathogens is given in Figure 1. This method consists of three major steps: first, generation of melting profiles of isolated genomic DNA using random PCR and temperature gradient gel electrophoresis (TGGE). In contrast to specific-PCR, random PCR is a process in which DNA fragments are sampled randomly from genomic DNA through a mismatch-containing hybridization of a primer to a template DNA[19]. Thus, random PCR enables the extraction of specified partial information from the whole genome DNA, through random sampling, using a single primer of an arbitrary nucleotide sequence at lower annealing temperatures. This generates a set of DNA fragments, mostly originating from incomplete hybridization (mismatch and/or bulge-containing) of primers to template DNAs. A single primer of dodeca-nucleotides (pfM12, dAGAACGCGCCTG) which has been recommended for general use, including the application to animal cells, was used for random PCR[20]. A 10 µl of PCR mixture consisting of 200 mM dNTPs (N = G, A, T, C), 0.5 mM primer, 10 mM *Tris*-15 HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.02 unit/ml *Taq* DNA polymerase (Takara Bio, Shiga, Japan) and a particular amount of template DNA. Random PCR was carried out with 30 cycles of denaturation (94°C, 30 sec), annealing (26°C, 2 min) and extension (47°C, 2 min) using a thermal cycler (PTC-100TM PCR machine, MJ Research, Inc., USA). The DNA samples were subjected to μ-TGGE[21], which adopts a tiny slab gel of $24 \times 16 \times 1\ mm^3$ for electrophoresis using a temperature-gradient generator (Lifetech Co. Ltd, Japan). For normalization procedure, two internal reference DNAs (200 and 600 bp) were co-migrated in each run of electrophoresis. The gel used was composed of 6% (w/v) acrylamide and bis-acrylamide (19 : 1) containing 90 mM TBE buffer (0.1 M *Tris*, 0.09 M boric acid, 0.001 M EDTA (pH 8.4)) and 6.5 M urea. A 8 µl of a random PCR solution containing both the internal reference DNAs and triple dyes (Nippon Gene, Japan) was electrophoresed under the condition of 100 V for 10 min duration at a linear temperature gradient of 15–55°C in 450 mM TBE buffer. After electrophoresis, DNA bands were detected using a lab-made LED transilluminator and by silver straining.

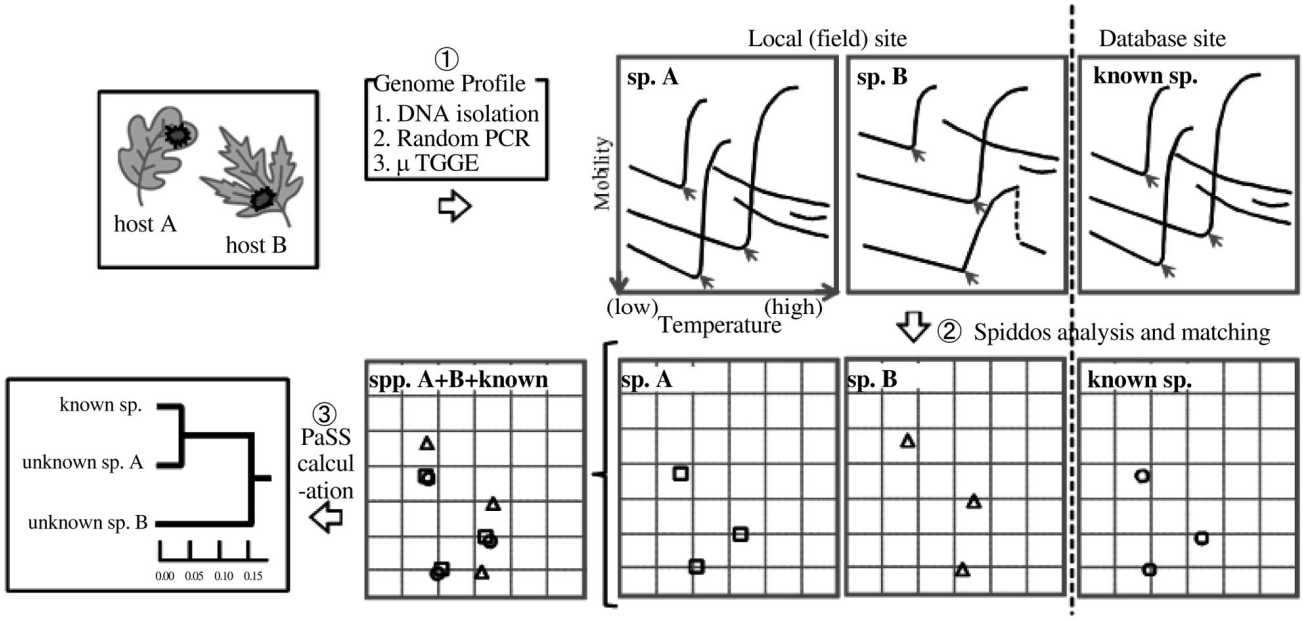

Figure 1. Overall procedure used to discriminate host-specific plant pathogens using GP technique. GP are generated by temperature-gradient gel electrophoresis of random PCR products obtained from the genomic DNA of infected plant source followed by computer-aided data processing (spiddos and *PaSS* calculation). Resultant values of GP from unknown isolate are then matched with the nearest GP (known species) registered in the database and the unknown species is identified and placed at the appropriate position on the phylogenetic tree based on the genome distance with reference to the matched known species in database.

Every electrophoretic experiment was conducted twice or thrice, to confirm the reproducibility of data.

Second, computer-aided normalization and data processing were performed by assigning spiddos (species identification dots) derived from featuring points[12]. Featuring points correspond to those points where structural transitions of DNA occur, such as double-stranded to single-stranded DNA, and are shown by arrow in Figure 1 (ref. 22). A set of featuring points can be assigned for each genome profile displayed on the computer and converted to spiddos.

Third, calculating pattern similarity scores (*PaSS*) value which is a good measure to quantify the closeness or the distance between two genomes was done as described elsewhere[10,12]. A set of spiddos can be used to provide a sufficient amount of information for identifying species. Using spiddos, we can define the *PaSS* between two genomes as follows

$$PaSS = 1 - \frac{1}{n}\sum_{i=1}^{n}\frac{|\vec{P}_i^{(1)} - \vec{P}_i^{(2)}|}{|\vec{P}_i^{(1)}| + |\vec{P}_i^{(2)}|}, \quad (0 < PaSS \le 1), \qquad (1)$$

where $\vec{P}$ of each spiddo is its corresponding position vector and a function of temperature and mobility (i.e. $\vec{P} = P(t, m)$). The parenthesized superscripts (1) and (2) represent genomes 1 and 2 respectively, and i denotes the serial number of spiddos (supplementary comment: If the two species are sufficiently close, the assignment of the corresponding feature points is self-evident). However, as they become exceedingly distant, it gets more and more probabilistic to assign the corresponding feature points. Therefore, we have introduced a general definition for the *PaSS* value. The *PaSS* value between two species is assumed to be the maximum value obtained after the computer-aided exhaustive combinations of a set of spiddos between two organisms. In brief, the *PaSS* value provides a measure of how two sets of spiddos can be closely superposed, generating a higher value (maximum: 1) when they are more closely related mutually (see Figure 1). The effectiveness of this approach has been experimentally supported[11] and theoretically considered[19]. A database site has also been constructed[23] in order to make a provisional identification of unknown species by subjecting to a search of the closest species in the database[13]. Finally, phylogenetic trees were generated using clustering software, *Free Lighter*[11], for cluster analysis.

A variety of five common pulses were selected to study seed-borne fungal pathogens (Table 1). Seeds were obtained from fungal-infected plants and the testing of seeds was performed through visual examination. Identification of fungi was done on the basis of spore morphology and colony character such as pattern of formation of conidia and conidiophores. Two major species of fungus, *A. niger* and *A. flavus*, were particularly noticed and identified. Fungus colony of *A. niger* grows slowly, consisting of a compact to fairly loose white to faint-yellow basal mycelium, with conidia. Conidia are typically spherical and very dark in colour. Conidial heads are

typically large and spherical or split into two or more loose to reasonably well-defined columns. Fungus colony of *A. flavus* is yellow to deep yellow-green in colour. Conidia are typically spherical to sub-spherical, conspicuously spiny and variable in diameter. Although the identification of seed-borne fungi on seeds by morphological examination has been used for many years, this system usually displays low detection sensitivity, and that make these methods less ideal for identification purpose.

Molecular methods are among the most precise tools for differentiation between species and identification of new strains. A PCR-based approach targeting on conserved sequences of ITS1 ribosomal DNA and its flanking region was used for sequence-based identification of the *Aspergillus* at genus and species levels, using a single pair of primers and a specific set of nested primers respectively[18]. A PCR product of 0.5 kbp in length, including the whole ITS1 region, and 0.3 kbp in length, including a specific region within the ITS1 was used as indicator to analyse the genus and species of *Aspergillus* respectively. All the 10 strains of *A. niger* and *A. flavus* isolated from the infected seeds of each of the five pulses were analysed and the specific amplification of genus-specific (Figure 2 *a*) and species-specific (Figure 2 *b*) products were successfully observed. Only those PCR reactions which are carried out with correct primer-sets generated the species-specific products. Although the results are clear, the specific-PCR based detection of fungal pathogens is not a realistic approach since the genus *Aspergillus* comprises a few hundred species[24]. Therefore, the specific-PCR based approach is rather labour-intensive and expensive, and one has to be careful to optimize the conditions so that all the different amplicons can be generated efficiently.

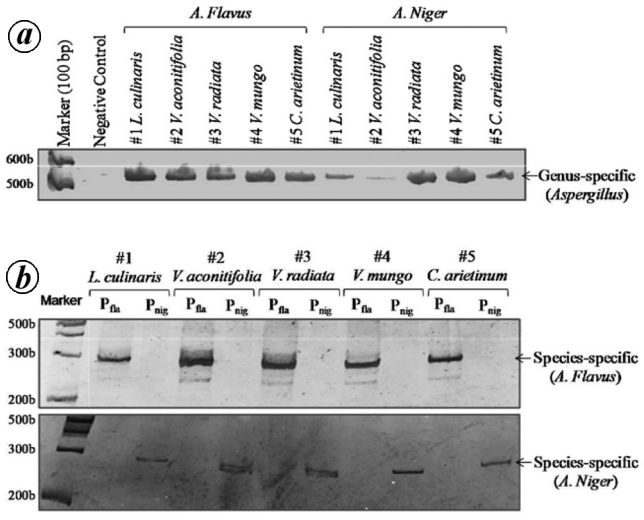

Figure 2. Electrophoretic detection of specific PCR-amplified *Aspergillus* fungal pathogens from five different legume plants. Genus-specific (***a***) and species-specific (***b***) PCR products are marked with arrows. A common primer is used to confirm genus *Aspergillus* and two specific primers, Pfla for *A. flavus* and Pnig for *A. niger*, are used to identify strains at species level. The left most electrophoretic lane is the molecular size marker (100 bp DNA ladder) and the negative control lane shows PCR products with no DNA template.

In order to develop a simple and universal method, GP has been introduced and demonstrated as a potential tool to discriminate species[11]. As shown in Figure 1, GP is based on the statistical concept of random sampling and the rapid acquisition of sequence information from the whole genome. In order to validate the utility of GP in this study, a total of six species of fungi belonging to five different taxonomic orders were selected and analysed (Table 2). The results of GP and spiddos representations are shown in Figure 3 *a*. To represent the mutual relationships of fungal species, a phylogenetic tree was drawn based on the genome distance, which is equivalent to the value of 1-*PaSS*, obtained from the GP. For confirmation, the classical phylogenetic tree was also constructed based on the taxonomical knowledge of their phenotypic traits. Interestingly, all the species examined were classified topologically to the same position in both the phenotype-based and GP-driven genotype-based trees (Figure 3 *b*). These data indicate that GP can classify species simply and robustly, and conserve congruence with phylogenetic trees constructed through the classical (phenotype-based) approach.

Plant pathogenic strains of a fungal species can be grouped into special forms called *formae speciales* (f. spp.), according to the plant host species on which they cause disease, and can be grouped further into races according to crop cultivar specificity. Therefore, plant species and cultivars should also be used for correct strain identification of pathogens. While a particular *forma specialis* may cause disease in a certain plant species, strains belonging to other *formae speciales* may be harmless[3]. This is because the ability of a fungus to infect a particular plant species may depend on specific genes encoding host-determining 'virulence factors', that distinguish virulent from non-virulent strains. Therefore discrimination of *formae speciales* of a fungal species is essential for effective disease control. In this study, we have also used GP for reliable identification of host-specific fungal pathogens and to analyse the taxonomic position of 10 different strains of *A. niger* and *A. flavus*, isolated from five different legume plant hosts.

Genome profiles and spiddos obtained from the GP experiments using a single probe (primer, *pfM12*) for *A. flavus* and *A. niger* species isolated from five different plant hosts are shown in Figure 4 *a*. The calculated *PaSS* values were used to position all the species in a phylogenetic tree (Figure 4 *b*). As expected, both the host-specific species grouped in two clusters and each of the strain was distantly mapped in the phylogenetic tree. These results are interesting since, from a taxonomical point of view, it suggests that the species are equivalently distant from each other at the genome level, irrespective of their phenotypic classification. All the strains belonging to

Table 2. Phenotype-based taxonomic classification of fungal plant pathogens species used in this study

Phylum	Class	Order	Family	Genus	Species
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	*A. niger*
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Aspergillus	*A. flavus*
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*F. oxysporum*
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	*C. globosum*
Ascomycota	Mucormycotina	Mucorales	Mucoraceae	Rhizopus	*R. nigricans*
Ascomycota	Pleosporomycetidae	Pleosporales	Pleosporaceae	Alternaria	*A. alternata*

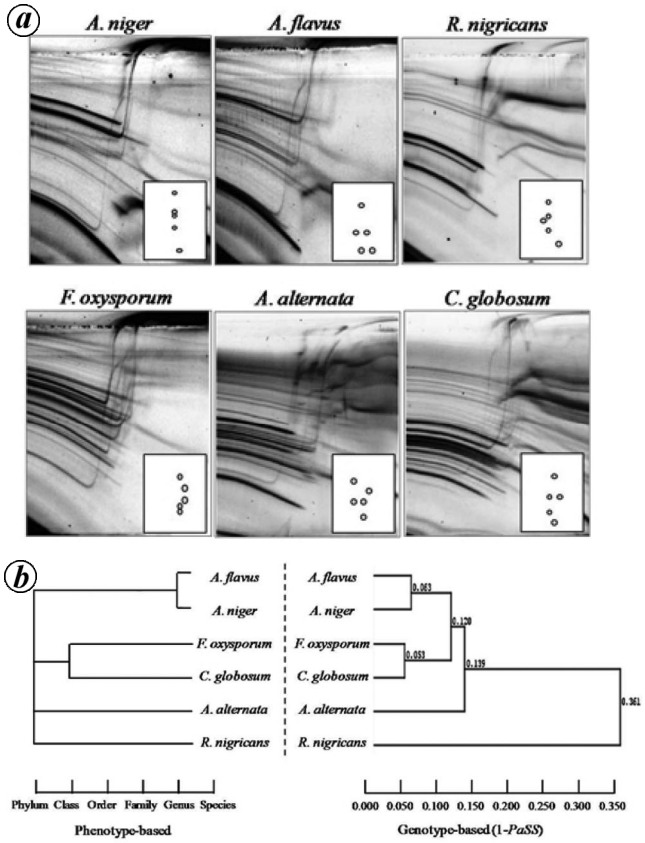

Figure 3. GP-based species identification of fungal plant pathogens. ***a***, Genome profiles of six species from phylum *Ascomycota*, spiddos pattern are shown in inset. ***b***, Phenotypic (left) and GP-based genotypic (right) phylogenetic tree are drawn on the basis of taxonomic hierarchy and *PaSS* value respectively. All the species showed good correspondence between both the trees.

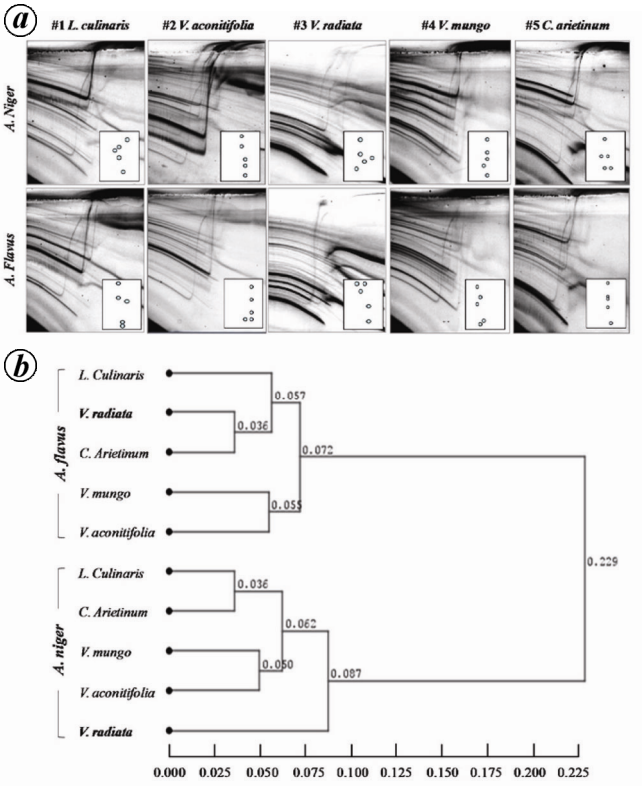

Figure 4. GP-based host-specific strain identification of fungal plant pathogens. ***a***, Genome profiles of *A. niger* and *A. flavus* isolated from five different plant hosts, spiddos pattern are shown in inset. ***b***, GP-based phylogenetic tree is drawn on the basis of *PaSS* value obtained from their genome profiles.

A. niger and *A. flavus* isolated from different host positioned distantly though they look very similar morphologically. It was interesting to note that the disputed positions of *A. niger* f. sp. *radiata* and *A. flavus* f. sp. *radiata* in the phylogenetic tree showed with conviction that GP can be a very robust method for unambiguous identification of the *forma specialis* and races, based on host-specific interactions, and thus can be an important tool for host- and cultivar-specific pathogenicity.

In this study, we have shown the utility of GP for reliable identification of host-specific fungal pathogens. Fourteen common seed-borne fungal pathotypes from five different legume host plants were identified and the genetic relationship within strains was drawn. In the first study, six fungal pathogens belonging to five different taxonomic orders were classified topologically at the same position with their phenotypic traits. In the next study, two different species from five different host plants were identified and distantly mapped in the phylogenetic tree. Interestingly, the ambiguous position of *V. radiata* indicates a differential interaction between host cultivars and pathogenic races. The disputed position of *A. niger* f. sp. *radiata* from the two clusters in the phylogenetic tree suggests that *A. niger* may have a harmless or even a beneficial relation with *V. radiata* which is in concordance with the recent study that reported a significant effect on growth and nutrient uptake in the *V. radiata*

by *A. niger* seed inoculation[25]. However, the appearance of *A. flavus* f. sp. *radiata* within the cluster along with other plant hosts indicates the host-specific pathogenicity of *A. flavus*. The production of B2 aflatoxin in *A. flavus* with higher concentrations (40–185 ppm) as compared with low concentration (19–31 ppm) in *A. niger* supports this notion[26]. Therefore, it clearly indicates that two closely related species which looks morphologically similar may not be genotypically similar and, thus can be re-identified using GP-based molecular approaches. It is therefore, believed that GP technology has the potential to revolutionize concepts and can be employed to evaluate the genetic diversity in order to reveal the genetic relationship among a large population of microbes, especially fungal pathogens which causes serious damage to important crops such as pulses. In addition, GP can be used for early detection of pathogens, which is a crucial step in diagnosis and management programmes for pulse crops. In the near future, an online GP-based portable and affordable system, which is presently under development, will be available for routine identification of plant pathogens directly in the field, to undertake appropriate disease control measures as quickly as possible.

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