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Molecular analysis of *Ascochyta rabiei* (Pass.) Labr., the pathogen of ascochyta blight in chickpea

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Abstract Genetic diversity in *Ascochyta rabiei* (Pass.) Labr., the causative agent of ascochyta blight of chickpea, was determined using 37 Indian, five American (USA), three Syrian, and two Pakistani isolates. A total of 48 polymorphic RAPD markers were scored for each isolate and the data used for cluster analysis. Most of the isolates clustered in the dendrogram essentially according to geographic origin. Based on the two major clusters A and B, Indian isolates were grouped into two categories, type-A and type-B. Isolates of *A. rabiei* within the Punjab state were more diverse than isolates from other states in northwestern India. A DNA marker ($ubc756_{1.6\ kb}$), specific to Indian isolates was identified. This is the first report of a molecular diversity analysis of Indian isolates of *A. rabiei*. The information may assist Indian chickpea breeders in the proper deployment of blight-resistant cultivars and in disease management.

Keywords RAPD markers · Genetic diversity · Phytopathogen

Introduction

Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labr., is a devastating disease of chickpea (*Cicer arietinum*. L) in most of the chickpea-producing countries (Saxena and Singh, 1987). The pathogen grows asexually on the host, and its short-distance spread within a field is primarily dependent on rainsplash dispersal of conidiospores. The disease initially appears in small areas within affected fields and spreads rapidly when cool and wet conditions prevail (Kaiser 1973, 1992). The perfect stage of *A. rabiei*, *Didymella rabiei* (Kovachevski) Arx., has been identified on over-wintering chickpea debris in several countries (Kaiser 1995). While its influence on disease epidemiology and pathogen diversity is still unclear, a few reports suggest that the teleomorph is more widespread than previously thought and that ascospores may serve as a primary inoculum (Kaiser and Hannan 1987; Trapero-Casas et al., 1996).

Genetic diversity analysis in plant pathogen populations is necessary to understand co-evolution in plant pathosystems (McDonald et al., 1989). However, evaluating genetic diversity in the field requires a set of highly discriminating, selectively neutral and reliable criteria for genotype analysis. *A. rabiei* is known for variation in its morphology (Kaiser 1973; Grewal 1984), pathogenicity (Gowen et al., 1989; Porta-Puglia 1992) and phytotoxin production (Alam et al., 1989; Hohl et al., 1990). Therefore, any identification based on these characters is difficult and suffers from several disadvantages. Biological pathotyping is time-consuming and labor-intensive and its reproducibility is often poor. In recent years, DNA polymorphisms have increasingly been used to complement traditional markers in the analysis of genetic identity, variability and relatedness in fungi. Two methods have been employed to assess genetic variation in fungi: (1) DNA fingerprinting with multilocus probes detecting numerous restriction fragment length polymorphisms simultaneously (Pena et al., 1993, Sastry et al., 1995), and (2) RAPD, a PCR-based approach that amplifies anonymous regions of a genome (Welsh and McClelland 1990; Williams et al., 1990). Both approaches have yielded valuable insights into the population genetics, pathotype diversity, mating systems and phylogeny of a wide variety of fungal species (Hamer et al., 1989; McDonald and Martinez 1990, 1991; Crowhurst et al.,

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Table 1 Isolates of *A. rabiei* used in random amplified polymorphic DNA studies

Isolate number[a]	Place of collection	Pathogenicity[b]
AR54	Ludhiana, Punjab, India	
AR55	Gurdaspur, Punjab, India	
AR56	Delhi, India	Race 4
AR57	Hissar, Haryana, India	
AR58	Srinagar, Jammu and Kashmir, India	
AR59	Pantnagar, Uttar Pradesh, India	
AR60	Berthin, Himachal Pradesh, India	
AR135	SriGanganagar, Rajasthan, India	
AR208	Gurdaspur, Panjab, India	Race 1
AR209	Ludhiana, Punjab, India	Race 2
AR210	Gurdaspur, Punjab, India	
AR211	Unknown	
AR212	Gurdaspur, Punjab, India	Race 5
AR213	Abohar, Punjab, India	
AR214	Unknown	
AR215	Unknown	Race 6
AR216	Ludhiana, Punjab, India	Race 7
AR217	Gurdaspur, Punjab, India	Race 3
AR218	SriGanganagar, Rajasthan, India	Race 6
AR219	Ludhiana, Punjab, India	
AR220	Ludhiana, Punjab, India	
AR221	Delhi, India	
AR222	Hissar, Haryana, India	Race 8
AR223	SriGanganagar, Rajasthan, India	
AR224	Berthin, Himachal Pradesh, India	Race 9
AR225	Sundar Nagar, Himachal Pradesh, India	Race 10
AR226	Palampur, Himachal Pradesh, India	
AR658	Berthin, Himachal Pradesh, India	
AR659	Pantnagar, Uttar Pradesh, India	
AR673	Hissar, Haryana, India	
AR815	Gurdaspur, Punjab, India	
AR816	Jammu, Jammu and Kashmir, India	
AR817	Rajasthan, India	
AR818	Hissar, Haryana, India	
AR819	Haryana, India	
AR820	Haryana, India	
AR821	Palampur, Himachal Pradesh, India	
AR628	Syria	Highly virulent
AR652	Syria	
AR655	Syria	
AR89	Pakistan	
AR82-I	Pakistan	
4b (ATCC76501)	USA	
6 (ATCC76502)	USA	
AR275	Palouse, WA, USA (pea)	
AR630	Palouse, WA, USA (lentil)	
AR281	Palouse, WA, USA (wheat)	

[a] Isolate numbers as maintained at USDA-ARS Western Regional Plant Introduction Station, Washington State University, Pullman, Wash., USA

[b] Race classification of Indian isolates after Singh (1990)

1991; Goodwin and Annis 1991; Levy et al., 1991). RAPD analysis is advantageous over RFLPs because it requires a small amount of DNA, is technically easy, and a large number of samples can be analyzed in a short period of time.

In general, a high level of genetic variation in a *A. rabiei* population has been noted when utilising both DNA markers and morphological characters. For example, extensive genetic diversity within *A. rabiei* from Tunisia based on oligonucleotide fingerprinting was reported by Morjane et al. (1994). Similar results were found in Dutch (Klein-Bolting 1992) and Italian isolates (Fischer et al., 1995) using RAPD markers. Significant genetic variation within *A. rabiei* isolates of Indian origin based on morphological and cultural variation has also been observed (Singh 1990; Ambardar and Singh 1996). However, there is no report on the molecular analysis of Indian isolates of *A. rabiei* based on DNA markers. The objective of the present study was to elucidate the extent of genetic variability present in 37 isolates of *A. rabiei* from India and to determine the similarity/diversity of isolates from USA, Pakistan and Syria with these Indian isolates by using DNA markers.

Materials and methods

Isolates of *A. rabiei*

Forty seven isolates of *A. rabiei* were from a collection established and maintained by W.J. Kaiser, USDA-ARS, Washington State University, Pullman, Wash., USA (details presented in Table 1).

Fungal material

Single-spore cultures of all isolates were used to inoculate chickpea stem pieces, which were then stored in a dry state for future use. Isolates from these chickpea stem pieces were revived as follows: a small section of chickpea stem was scraped from the surface with a sterile knife and cultured on 2% water agar (WA) media in 9-cm diameter Petri dishes. These dishes were incubated at 21–23°C under fluorescent light for 12 h. A 1-cm^2 piece of WA containing the fungal culture was cut from the plate of each isolate with a sterile knife and used to inoculate 50-ml of liquid broth of potato-dextrose (Difco Laboratories, Detroit, Mich.) in 250-ml conical flasks which were then kept in an incubator shaker at 21–23°C with a 150 rpm speed for 7 days. Mycelia were harvested by filtration through four layers of muslin cloth, lyophilised in liquid nitrogen, stored at –70°C and later used for DNA extraction.

DNA isolation

DNA was isolated from lyophilised mycelia using the miniprep method of Doyle and Doyle (1987) with slight modifications. Two to three grams of mycelium from each isolate were submerged in liquid nitrogen and ground to a fine powder. The powder was quickly transferred to a tube containing 7.5 ml of ice-cold extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA, pH 7.5). The tube was briefly shaken and 7.5-ml of nuclei lysis buffer (2 M NaCl, 0.2 M Tris, 50 mM EDTA, 2% CTAB, pH 7.5) was then quickly added, followed by 3 ml of 5% sarkosyl solution. Sample sets were incubated in a 65°C water bath for 20 min, allowed to cool for a few min, and 18 ml of chloroform/isoamyl alcohol (24:1) was added to each tube. The tubes were centrifuged at 5000 g for 15 min; the aqueous layer was removed and extracted again with a 15-ml chloroform/isoamyl alcohol mixture. The aqueous layer was transferred to a new tube and DNA was precipitated with a double volume of chilled ethanol. The dry DNA pellet was suspended in 500 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and DNA was quantified with the mini-gel method (Sambrook et al., 1989) by comparing band intensities with that of a standard lambda/*Hin*dIII DNA marker (Gibco BRL, Bethesda, Md.) in ethidium bromide-stained gels. DNA was then diluted to a 5 ng/µl concentration and used in the polymerase chain reaction (PCR).

Oligonucleotide primers

Primers used in PCR were obtained from three different sources. The CS primers and ARP1, ARP2 and ARP3 (P3, P5 and P6, respectively, of Fischer et al., 1995) were synthesized by Genosys Biotechnology Inc., USA. The UBC primers were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, and five primers were from Operon Technologies Inc. (Alameda, Calif.).

Polymerase chain reaction

For RAPD analysis, PCR-amplification was performed in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton ×100, 2.5 mM $MgCl_2$, 100 µM of dNTP, 0.24 µM of primer, 20–25 ng of DNA and 1 unit of *Taq* polymerase per 25-µl reaction volume with the following cycle repeated 40-times: denaturing at 94°C for 20 s, annealing at 36°C for 1 min, a 3-min ramp to 72°C and elongation at 72°C for 1 min. A final elongation was performed for 8 min. The polymerase chain reaction was carried out in a Perkin Elmer Cetus Gene Amp PCR system 9600. The PCR products were separated electrophoretically in 2% agarose gels using 1×TBE buffer (Sambrook et al., 1989). The gels were stained with ethidium bromide at a concentration of 0.5 µg/ml and banding patterns were visualised by a UV-transilluminator. RAPD gels were photographed on thermal photographic paper (Mitsubishi) using the FOTO/Analyst Mini Visionary Benchtop Digital Documentation System (Fotodyne). Since RAPD markers are dominant, a marker locus was considered to be polymorphic if the band was present in a few isolates but absent in other isolates. Only clear and reproducible DNA bands were scored. The RAPD marker loci were designated according to the primer name.

Molecular-data analysis

Polymorphic RAPD markers were scored as phenotypic data with 1 and 0 character states for the presence and absence of a polymorphic RAPD band, respectively. Using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) version 1.80 (Rohlf 1993), a data matrix was constructed using the binary characters 1 or 0. A similarity matrix using Dice's coefficient was made by employing the SIMQUAL program. Genetic cluster analysis was performed to analyse genetic differences between isolates and a dendrogram was generated by an unweighted pair-group method with arithmetic average (UPGMA) in the SAHN program and constructed using the program TREE. To determine the robustness of the dendrogram, the data were bootstrapped with 1000 replications using the computer program WINBOOT (Nelson et al., 1994).

Results and discussion

Strategies for primer selection

Four randomly selected isolates (AR57, AR59, AR211 and AR216) were used to test the potential of RAPD primers for their ability to amplify DNA fragments from *A. rabiei* genomic DNA. Among 76 primers tested, 22 RAPD primers (CS19, CS23, CS27, CS29, CS34, CS39, CS49, CS57, CS60, CS68, UBC78, UBC151, UBC403, UBC727, UBC756, UBC765, OPJ01, OPT20, OPU11, OPV6, ARP1 and ARP3) amplified reproducible and polymorphic bands in the four isolates. However, eight primers produced no DNA amplification, 19 primers generated inconsistent banding patterns and 27 primers generated monomorphic patterns. These 22 primers were then used for all the 47 isolates and only easily detectable and well-resolved bands, which were reproducible in three replications, were considered. The number of bands generated by each primer that produced a polymorphic banding pattern varied from four in the case of UBC151, to 13 in the case of CS34 and CS39. The sizes of the fragments ranged from 300 bp to 2 kb. Quantitative differences in band intensity were not considered in the present study since the reproducibility of quantitative differences was not consistent. Controls, where no template DNA was used, were always included in the PCR reactions, and no bands were produced. One representative profile of amplified products using the RAPD primer UBC756 is shown in Fig. 1. This is the most-polymorphic primer, where five fragments out of six amplified were polymorphic among the isolates. One of these five polymorphic bands of molecular weight 1.6 kb was absent in all non-Indian isolates except for one Syrian isolate, AR628.

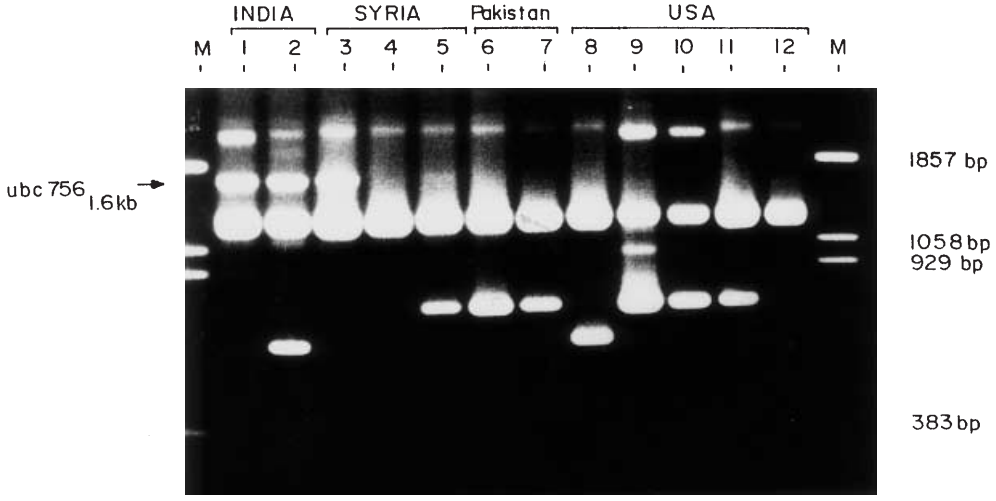

Fig. 1 Random amplified DNA polymorphisms of *A. rabiei* isolates with random primer UBC756. *Lanes 1–12* represent AR820, AR821, AR628, AR652, AR655, AR89, AR82-I, 4b (ATCC76501), 6 (ATCC76502), AR275, AR630, and AR281. *M* indicates molecular-weight-marker *Bst*NI digest of pBR322. The DNA marker specific to Indian isolates is marked with an arrow

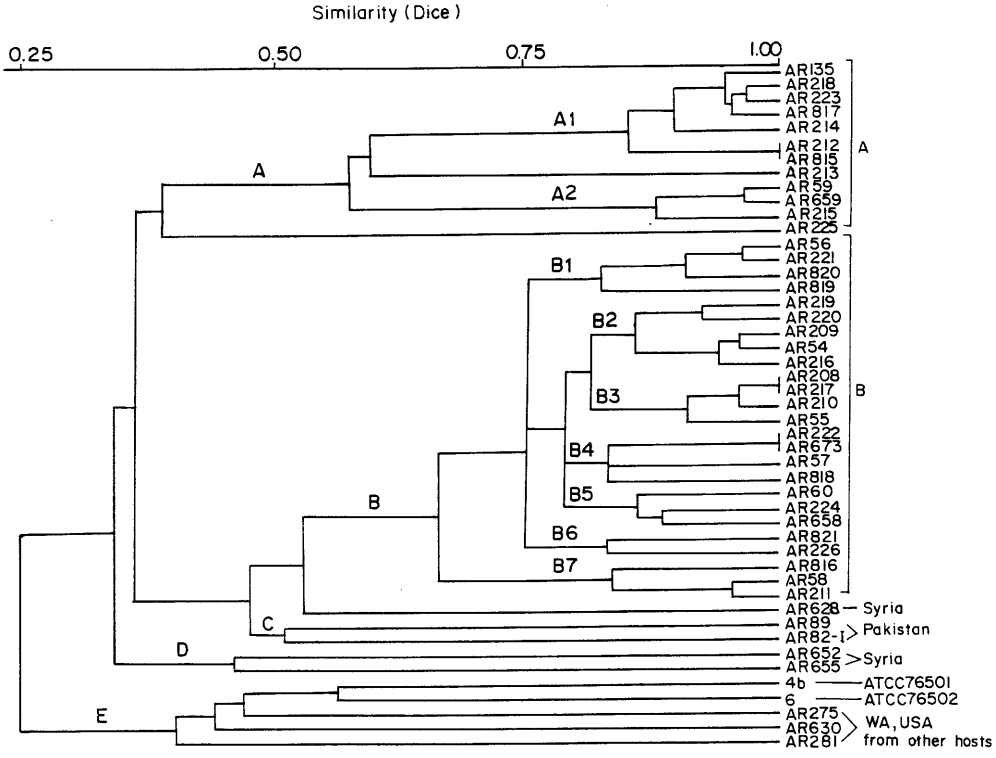

Fig. 2 Dendrogram showing relationship among *A. rabiei* isolates based on RAPD markers

RAPD analysis clusters *A. rabiei* isolates according to their geographic origin

An attempt was made to assess genetic diversity among *A. rabiei* isolates and to establish possible genetic relationships. Thirty seven Indian isolates formed two major clusters, A and B, each consisting of two and seven subgroups, respectively (Fig. 2). Subgroup A1 included four isolates from Rajasthan (AR135, AR218, AR223 and AR817), two isolates from Gurdaspur, Punjab (AR212 and AR815), and AR214 of unknown origin. The presence of AR214, together with isolates from Rajasthan in subgroup A1, suggested that AR214 was probably collected from Rajasthan. Isolates AR59 and AR659 from Pantnagar, Uttar Pradesh, and AR215 from unknown place of collection, comprised subgroup A2. It was noted that within group A, the isolates AR213 and AR225 were loosely grouped with A1 and A2. Two isolates collected from Delhi (AR56 and AR221), and AR820 and AR819 from Haryana, clustered into subgroup B1, whereas five isolates from Ludhiana (AR219, AR220, AR209, AR54 and AR216) and four isolates from Gurdaspur (AR208, AR217, AR210 and AR55) in the state of Punjab formed subgroups B2 and B3, respectively. Isolates AR222, AR673, AR57 and AR818 collected from Hissar, Haryana, generated a subgroup B4. Isolates present in subgroups B5 and B6 were collected at Berthin (AR60, AR224 and AR658) and Palampur (AR821 and AR226) of Himachal Pradesh, respectively, whereas B7 consisted of

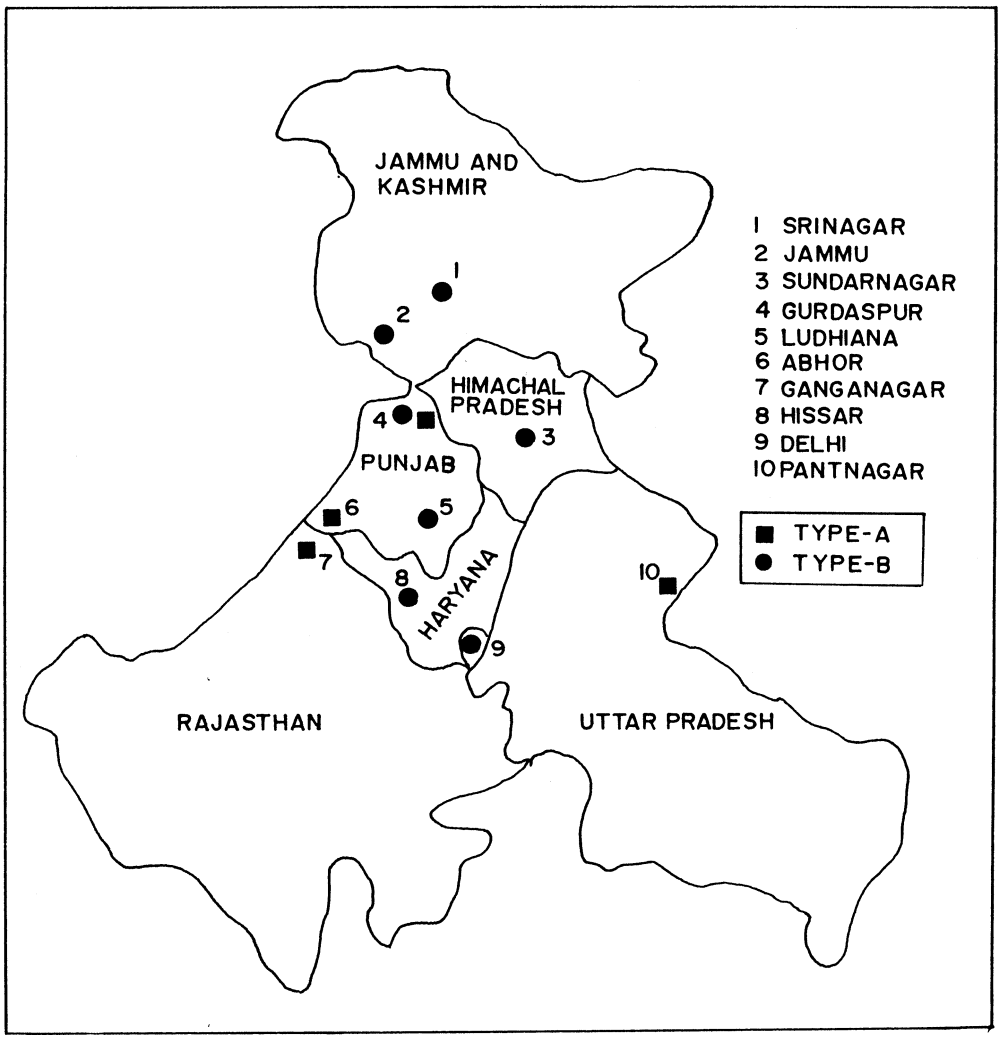

Fig. 3 Outline map of the northwestern states of India showing the relative occurrence of type-A and -B *A. rabiei* isolates

the two isolates collected from Jammu and Kashmir (AR816, AR58) and one isolate (AR211) of unknown origin. The similarity index of Indian isolates within each sub-group varied from 0.83 to 1.0. Gurdaspur isolates AR212-AR815 and AR208-AR217 were 100% similar. Two isolates from Hissar (AR222 and AR673) also showed 100% similarity.

Most of the isolates in the dendrogram (Fig. 2) were clustered essentially according to their geographic origin. Isolates collected from Rajasthan, Ludhiana, Hissar, Berthin, Palampur, and Jammu and Srinagar, formed individual clusters according to their places of collection. However, there were a few exceptions to this geographic clustering. For example, most of the isolates collected from Gurdaspur clustered in subgroup B3, whereas isolates AR212 and AR815 from Gurdaspur clustered with isolates collected from Rajasthan in subgroup A1.

We wanted to determine the similarity/diversity of isolates from USA (4b, 6, AR275, AR630 and AR281), Pakistan (AR89, AR82-I) and Syria (AR628, AR652, AR655) with the Indian isolates. All five isolates from the USA formed a single cluster E which was independent of A and B. As expected, isolates 4b and 6 collected from infested chickpea stems showed more similarity (a similarity index of 0.55) than AR275, AR630 and AR281, which were collected from pea, lentil and wheat plants, respectively, infected by *A. rabiei*, and they remained separate in the major cluster E. The significant amount of genetic diversity among the five isolates of *A. rabiei* collected from the Palouse region of USA may be due to the introduction of this pathogen in to this region from different countries (Kaiser and Muehlbauer 1984, Derie et al., 1985) or else due to sexual recombination (Kaiser and Hannan 1987; Trapero-Casas and Kaiser 1987). Two isolates from Pakistan (AR89 and AR82-I) remained together in cluster C, and were related to the Indian isolates present in cluster B with a similarity index of 0.49. Three isolates from Syria (AR628, AR652 and AR655) also showed similarity with the Indian isolates, among which AR628 showed more similarity to Indian isolates in group B (similarity index 0.52) than AR652 and AR655 in cluster D (similarity index 0.33). These results indicated that RAPD markers were well-suited for the elucidation of the genetic diversity and differentiation present in *A. rabiei* isolates.

Indian isolates of *A. rabiei* could be classified broadly into type-A and type-B corresponding to two major

clusters A and B in the dendrogram. There was a relative predominance of both types of isolates when mapped in the Northwest states of India (Fig. 3), and it was noted that both types of *A. rabiei* (type-A and type-B) were present in the Punjab. A significant amount of diversity within isolates collected from Ludhiana and Gurdaspur was also found. This indicated that the genetic diversity within the isolates in the Punjab was greater than in other states. In a similar observation, Singh (1990) reported that Punjab isolates were more diverse and as many as five different races of *A. rabiei* could be differentiated. The significance of this observation is that ascochyta blight-resistant chickpea varieties developed for this region need to be tested against different isolates prevalent in this state before their release. Thus, this observation may be useful for better deployment of resistant varieties of chickpea in Punjab.

Indian isolate-specific markers

A RAPD marker $UBC756_{1.6\ kb}$ (shown by an arrowhead in Fig. 1) was identified as a common band present in all Indian isolates. Except in one Syrian isolate (AR628) this band is absent in the other two isolates from Syria, in two isolates from Pakistan and also in five isolates from the USA. Recently, isolate AR628 was identified as highly virulent in Syria. The presence of this marker in isolate AR628 can be explained either by the transmission of a virulent *A. rabiei* isolate from India to Syria through international seed exchange (Kaiser 1997) or as the natural occurrence of AR628 similar to the Indian isolate at this marker locus. However, the former possibility seems to be more convincing, since cluster analysis based on polymorphic RAPD marker loci indicates clear similarity between AR628 and the Indian isolates but with less similarity to the other Syrian isolates (Fig. 2). Thus, the specificity of $UBC756_{1.6\ kb}$ can be considered as an Indian isolate-specific DNA marker. Of course, isolates from other countries, isolates from all over Pakistan and more isolates from other regions of India need to be analysed with this RAPD primer. Nevertheless, the occurrence of its absolute specificity to all 37 Indian isolates in the present study is significant.

No correlation between genetic diversity and pathogenicity

Although the pathogenicity of the isolates was not determined in the present study, Singh (1990) reported pathogenicity of 11 of the 37 Indian isolates. No correlation was found, however, between the pathogenicity of these 11 Indian isolates and their respective positions in the dendrogram (Fig. 2). For example, two isolates (AR215 and AR218) were classified as race 6 (Singh 1990) and were grouped in subgroups A1 and A2, respectively. Isolates AR208 and AR217 were classified as race 1 and race 3, respectively, but remained in a single subgroup, B3. Similarly, AR209 and AR216 clustered in B2 and represented race 2 and race 7, respectively. The actual number of primers used in this study is insufficient to detect characteristics that correspond to pathogenicity. Therefore, more primers need to be used for such an analysis. Alternatively, the use of virulence gene specific markers may be a better approach for finding such a correlation. Efforts to correlate the morphological and spore characteristics of *A. rabiei* with its pathogenicity had little success earlier (Singh 1990). To the best of our knowledge, this is the first report of the molecular diversity analysis of *A. rabiei* isolates of Indian origin.

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