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Genotyping with RAPD and microsatellite markers resolves pathotype diversity in the ascochyta blight pathogen of chickpea

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Abstract The poor definition of variation in the ascochyta blight fungus (*Ascochyta rabiei*) has historically hindered breeding for resistance to the chickpea (*Cicer arietinum* L.) blight disease in West Asia and North Africa. We have employed 14 RAPD markers and an oligonucleotide probe complementary to the microsatellite sequence $(GATA)_4$ to construct a genotype-specific DNA fragment profile from periodically sampled Syrian field isolates of this fungus. By using conventional pathogenicity tests and genome analysis with RAPD and microsatellite markers, we demonstrated that the DNA markers distinguish variability within and among the major pathotypes of *A. rabiei* and resolved each pathotypes into several genotypes. The genetic diversity estimate based on DNA marker analysis within pathotypes was highest for the least-aggressive pathotype (pathotype I), followed by the aggressive (pathotype II) and the most-aggressive pathotype (pathotype III). The pair-wise genetic distance estimated for all the isolates varied from 0.00 to 0.39, indicating a range from a clonal to a diverse relationship. On the basis of genome analysis, and information on the spatial and temporal distribution of the pathogen, a general picture of *A. rabiei* evolution in Syria is proposed.

Key words Ascochyta blight of chickpea · Resistance breeding · DNA fingerprinting · Pathogen variability · Phylogeny

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Introduction

The ascochyta blight fungus *Ascochyta rabiei* (Pass) Lab. is considered the most damaging pathogen of the chickpea (*Cicer arietinum* L.). This disease occurs worldwide in chronic epidemic cycles despite the frequent introduction of new resistant chickpea cultivars (Singh and Reddy 1993). The absence of durable blight resistance in the field has been attributed to the appearance of new pathotypes (Singh et al. 1992) and high levels of polymorphism in aggressiveness in pathogen populations (Vir and Grewal 1974; Reddy and Kabbabeh 1985; Malik and Rahman 1992; Porta-Puglia 1992). However, the nature of this polymorphism is subject to much debate (Malik and Rahman 1992; Porta-Puglia 1992), marked by strongly contrasting views on the diversity and stability of pathotypes.

The pathotype of an *A. rabiei* isolate is determined by assaying its pathogenicity on a set of differential chickpea cultivars. Using such an assay, Vir and Grewal (1974) reported finding more than ten pathotypes among field isolates from India. In similar studies Reddy and Kabbabeh (1985) identified six pathogenic groups among 50 isolates from Syria. Nene and Reddy (1987) found five pathogenic groups and several strains, respectively, among isolates from Pakistan and Turkey. However, these workers used different assay procedures and different cultivars for the pathogenicity tests. These assays were conducted by artificial inoculation either in the field or under plastic-house conditions. In these assay procedures there was very little control on the environment, therefore the assay results necessarily fluctuate. Furthermore, Porta-Puglia (1992) judged that pathogenicity or aggressiveness within the same isolate/line combination could vary according to the environmental conditions. For example, Reddy and Kabbabeh (1985) reported the aggressiveness of the six isolates to be in the order $6 > 5 > 4 > 3 > 2 > 1$ under plastic-house conditions. For the same isolates

Weising et al. (1991) reported a different aggressiveness ($6 > 4 > 2 > 1 > 5 > 3$) under growth-chamber conditions. In addition, several workers have suggested that difficulty in standardizing the assay procedures (Reddy et al. 1990; Porta-Puglia 1992) and the disease rating scales (Harrabi and Halila 1992; Porta-Puglia 1992) leads to inflated estimates of pathotypic diversity and variability. The key to resolving these issues is to provide a genetic definition for the phenotypic variation observed in pathogenicity assays.

Although a variety of genetic markers can be used to study fungal phytopathogen populations (McDermott and McDonald 1993), few have been definitive for the ascochyta blight fungus. For example, Bouznad et al. (1997) found that the isozyme profile of *A. rabiei* isolates from Algeria, Tunisia and Syria are nearly uniform, and differ little from other species of the genus *Ascochyta*. However, Weising et al. (1991) showed that oligonucleotide sequences complementary to microsatellites could be efficiently used as probes in RFLP analysis to detect genetic variation among six isolates of *A. rabiei* from Syria.

The combined use of pathotype- and genome-analysis techniques can provide insight into population processes affecting the genetic structure of pathogen populations in response to a changing agro-ecological environment (Burdon 1993). Understanding population structure and the processes affecting its change is important for designing suitable strategies to reduce disease and to deploy resistant cultivars in the field. However, this information is lacking in *A. rabiei*.

In this report, our main objective is to resolve the issue of pathotype diversity in the ascochyta blight pathogen of chickpea by providing a genetic definition of the phenotypic variation observed in the pathogenicity assays. For this purpose, we employed a set of RAPD and microsatellite markers to detect genetic diversity within and among the periodically sampled isolates belonging to the major pathotypes of *A. rabiei*. We also attempted to determine whether the different isolates of a pathotype of the pathogen are clonal lineages or if isolates of a pathotype can be further divided into genotypes. Based on the information of spatial and temporal distribution of the genetic variation, we speculate on the population processes acting on this pathogen.

Materials and methods

Fungal material

The 53 isolates used in this study were sampled during the years 1982, 1991, 1992 and 1993 from different chickpea growing regions of the Syria and Lebanon (Fig. 1). Isolate numbers 1–6 were described previously (Reddy and Kabbabeh 1985). These isolates are representative of six pathogenic groups observed in the collection of 50 isolates from Syria and Lebanon sampled during 1982. Pathogenic grouping of these six isolates was based on a pathogenicity assay conducted in a partially controlled environment during 1982 (Reddy and Kabbabeh 1985). Since chickpea is cultivated extensively in the Northeast part of Syria, and ascochyta blight is a major constraint for production, more samples were collected from this area (Fig. 1). In Syria Ghab 2 (ILC 3279) is the most popular variety. Therefore most of the isolates were collected from farmers fields involving the cultivar ILC 3279. Our previous results (unpublished results; Anonymous 1995) showed that there is a very little or no variation within and among the isolates collected from a single lesion, a single plant and a single field. Over 80% of the isolates from a field belong to a single genotype. Therefore, only one representative sample was randomly collected from every field. The fields sampled are located at least 1 km away from each other. Stem lesions were obtained from individual diseased plants and temporarily preserved in silica gels. The individual stem lesions were cut into small pieces of 1 cm, leaving a small healthy portion on either side. Then the lesion was washed thoroughly in sterile distilled water and surface-sterilized in 0.5% sodium hypochlorite solution for 10 min. After re-washing in sterile distilled water (once), the lesion was placed in 70% alcohol for 30 s, then washed three times in sterile distilled water and placed on water agar (2% agar) plates. Subsequently (after 2–4 days) a small sector of growing mycelium from a single lesion was transferred onto chickpea-seed meal agar plates (CSMA; extract from 4 g of ground chickpea seeds, 3 g dextrose, 1.8 g agar and 100 ml of distilled water). Single spores of the isolates were subsequently obtained from each single-lesion as follows.

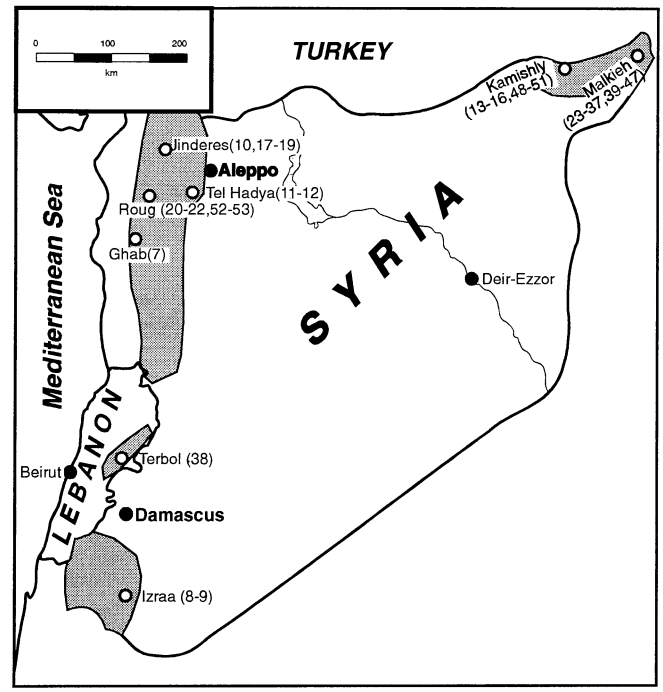

Fig. 1 Map with sampling sites (*open circles*) and isolate numbers of *A. rabiei* collected (*in parenthesis*) in different chickpea growing regions (*marked area*) of Syria and Lebanon

Stock cultures of the fungi were immersed in sterile distilled water to release spores from the pycnidia. The resulting spore suspension was diluted to a concentration of 3.5×10^5 spores/ml, dispersed on water agar (2%) plates, and incubated at 20°C to induce germination. After 24 h, a 20×20 mm agar block was transferred onto a microscopic slide and dissected into smaller squares (1.5×1.5 mm) aseptically. Squares with single germinating spores were identified microscopically and transferred to fresh CSMA medium for culture. All cultures were maintained on CSMA, incubated at 18°C and illuminated for 14 h per day.

Isolate pathotyping

Our previous results on pathotyping (Anonymous 1989) with a set of nine differentials showed that all the differentials could be grouped into three classes (susceptible, moderately resistant and highly resistant). Instead of nine differentials, only three (one differential from each class) are sufficient for obtaining the same pathotyping results. Therefore, in the present study, only three differentials, namely ILC 1929 (susceptible), ILC 482 (moderately resistant) and ILC 3279 (highly resistant), were used for pathotyping. Healthy seeds of the differential with uniform size were hand picked and germinated in a germination box (17.2 cm × 14.2 cm × 11.5 cm) (watering with regular intervals) for 36 h at room temperature. The seeds with uniform germination were selected and transferred to Jiffy-7 pots. Seedlings were grown in a growth chamber. Temperature and light settings during seedling growth are presented in Table 1. After 7 days, four seedlings of each of the differentials (in total 12 plants per box) were arranged in a random fashion in a germination box having a compatible transparent cover and a trough at the bottom. Wicks were placed in the trough to supply water through capillary movement.

A spore suspension was prepared using a 14-day old culture grown on CSMA lacking agar (CSM) at the temperature and light regimes described above. The resulting suspension was adjusted to 1.6×10^5 spores/ml with sterile water and sprayed onto plants. Each box (with four plants of each of the three differentials) was considered as a replication and each isolate was sprayed on two boxes. Two hundred millilitres of spore suspension were used for each treatment. After spraying, the plants were covered with transparent plastic covers to maintain leaf wetness during incubation. Light intensity was reduced and the temperature was set at 18°C (Table 1). The plants were incubated at this condition for 40 h after which the plastic covers were removed and temperature and light conditions were adjusted as before (Table 1). Readings for disease severity on a single-plant basis were taken from the 3rd to 14th day after inoculation using the following scale:

1 = no symptoms
2 = small round tissue depression or spot
3 = elongated spots
4 = coalescent spot
5 = stem girdling
6 = stem breaking
7 = lesion growths downward from breaking points
8 = whole plant nearly dead
9 = plant dead.

Table 1 Temperature and light setting of the growth chamber[a]

Time	Temperature (°C)	Light setting
Before and after inoculation		
4	16	11
6	16	22
7	18	33
8	20	44
10	22	44
16	22	33
17	22	22
18	21	11
19	20	11
20	20	00
21	18	00
22	16	00
During inoculation		
4	18	10
20	18	00

[a] The growth chamber used was Conviron, model No. E15, manufactured by Control Environment Ltd., Manitoba, Canada

Extraction of DNA

DNA was extracted according to a modified CTAB method (Weising et al. 1991). The lyophilized fungal mycelia (0.2 g) were ground to a fine paste in 12 ml of hot (60°C) 2× CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris-HCl pH 8, 20 mM EDTA, 0.2% β-mercaptoethanol). After 30 min of incubation at 60°C with gentle swirling, the resulting cell lysate was extraction with an equal volume of chloroform/isoamylalcohol (24 : 1, v/v). Then, the cell lysate was centrifuged (5000 g, 20°C, 25 min). The aqueous phase was transferred into another tube, followed by precipitation with a 0.6 vol of isopropanol and collected by centrifugation (5000 g, 15 min, 20°C). Pellets were washed with 70% ethanol, dried and then dissolved in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) overnight at 4°C. Un-dissolved DNA was removed by an additional centrifugation (5000 g for 5 min) step. DNA was quantified by absorbance at 260 nm.

RAPD analysis

Eighty different primers (kits H–M), obtained from Operon Technologies (Alameda, California), were tested on a small sub-set of isolates to identify polymorphic primers. Among the primers tested, a total of 14 (OPI-01, OPI-16, OPJ-01, OPJ-10, OPJ-15, OPJ-18, OPK-09, OPK-12, OPK-15, OPK-19, OPL-08, OPL-05, OPL-16 and OPM-02) revealed polymorphism. These primers were further tested for polymorphism on 53 isolates of *A. rabiei* and one isolate of *A. lentis*. DNA amplifications were performed in a 25-µl reaction, containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ and 0.001% gelatin), 100 µM each of dATP, dTTP, dGTP and dCTP, 5 pmol of a single 10-base primer, 200 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase (Boehringer Mannheim), and topped with a drop of mineral oil. Amplifications were performed in a thermocycler (Perkin Elmer, 9600) programmed for 2 min at 94°C; for 40 cycles of 1 min each at 92°C, 1 min at 36°C and 2 min at 72°C; with a final extension of 5 min at 72°C. The reaction mix without template DNA served as a control and was electrophoresed along with the samples. Amplified DNA products were loaded onto 1.2% agarose gels and electrophoresed in 1× TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA). Gels were stained with ethidium bromide and photographed under UV light. RAPD analysis with each of the primers was repeated twice and only consistent and reproducible banding patters were scored.

Microsatellite analysis

Genomic DNA was digested either with *Taq*I or *Bam*H1 and Southern hybridization was performed using digoxigenated $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$ and $(GTG)_5$ as probes. Southern hybridization and colorigenic detection were performed according to Bierwerth et al. (1992).

Data analysis

The fragment pattern of each isolate derived from microsatellite and RAPD analysis was coded in a binary form, i.e. as 1s and 0s, representing the presence and absence of each fragment, respectively. Nei's genetic distance (Nei 1972; a dissimilarity index) was computed

from the binary data for all pair-wise combinations of isolates. The unweighted pair-group method for the arithmetic average (UPGMA; Sneath and Soakal 1973) and neighbor joining (both midpoint and out-group) method (NJM; Saitou and Nei 1987) were used to estimate the phenogram and phylogram, respectively, on the basis of the distance matrix data. Since NJM yields a more accurate tree (Rohlf 1993) than UPGMA and parsimony, only the result of NJM is presented. Clusters that separated at 90% similarity levels were given capital-letter designations. The strict-rule consensus tree (Sokal and Rohlf 1981) for the set of NJM alternative trees was worked out. All the data analysis was performed using the software package NTSYS-pc (Rohlf 1993). Genetic diversity (H) was calculated according to the following equation (Nei 1987):

$$H = [n/(n-1)] \cdot \left[1 - \sum_{i=1} x_i^2\right],$$

where x is the frequency of the ith genotype in the population, and n the number of isolates examined.

Results

Isolate pathotyping

Based on the pathogenicity tests, all isolates were classified into three pathotypes, namely pathotype I (least aggressive, kills ILC 1929, but not ILC 482 and ILC 3279), pathotype II (aggressive, kills both ILC 1929 and ILC 482, but not ILC 3279) and pathotype III (most aggressive, kills all three cultivars). Typical disease development curves for the three pathotypes are presented in Fig. 2. Out of 53 isolates, 13 (24.5%) grouped as pathotype I, 5 (9.5%) as pathotype II and 35 (66.0%) as pathotype III. Furthermore, there was no association between pathotype and the host cultivar from which the isolate was obtained. All three pathotypes were sampled in fields grown with the popular Syrian variety, Ghab 2.

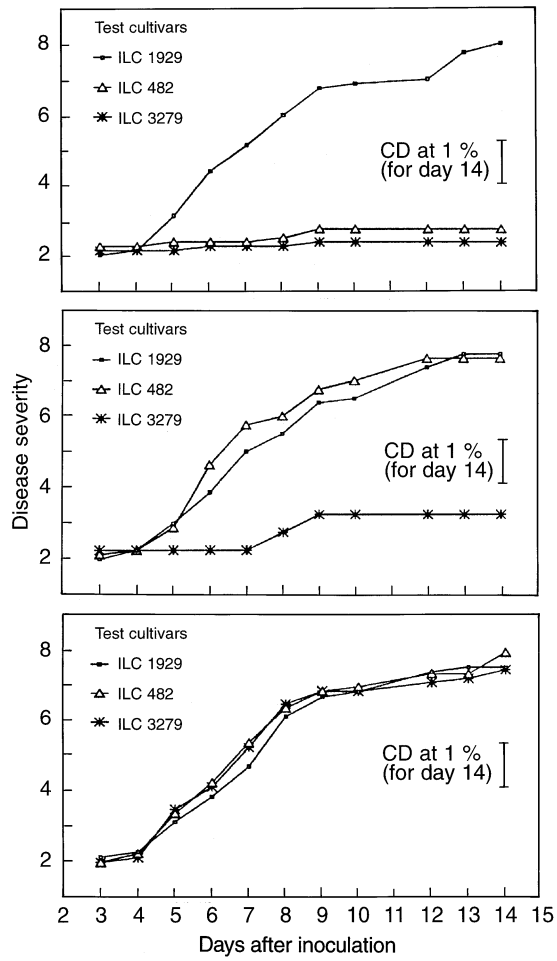

Fig. 2 Typical disease development curves of three pathotypes of *A. rabiei*: pathotype I (upper), pathotype II (middle) and pathotype III (lower). The disease severity was measured on a 1–9 scale (1 = no symptoms and 9 = plant dead) and on three differential cultivars (ILC 1929, ILC 482 and ILC 3279). The critical difference (*CD*) at 1% was calculated for the disease severity level on day 14 after inoculation

RAPD analysis

Depending on the primer, 1–8 DNA fragments were amplified from a given template DNA. The number of genotypes defined, the average number of amplified fragments per genotype, and the range and numbers of loci for the *A. rabiei* collection, are shown in Table 2.

The discriminating power of the various primers varied greatly. Out of 80 primers initially screened, only 14 revealed polymorphism and amplified a total of 78 loci. Figure 3 shows the RAPD patterns of one such a polymorphic primer. All 14 primers, along with the primers which did not detect polymorphism among the isolates of *A. rabiei*, showed polymorphism between *A. rabiei* and *A. lentis* (ascochyta blight pathogen of lentil), and the latter formed a distinct group (unpublished data). Among the informative primers OPJ-01 and OPJ-15 had a higher discriminating power and could define 8 and 13 genotypes, respectively. With some of the primers the discriminating power was minimal, i.e. they could define only two genotypes (Table 2). The genetic diversity detected by the individual primers also varied considerably and estimates ranged from 0.07 (primer OPK-15) to 0.63 (primer OPJ-01). The estimates for each primer for all isolates examined did not correlate with the average number of amplified fragments per genotype ($r = 0.07$), indicating that the number of amplified fragments is not an indicator of the discriminating power of a primer. From the composite data of all the primers we were able to classify the isolates into 22 genotypes.

Microsatellite analysis

The resolving power of the various microsatellites varied greatly. Out of four oligonucleotides complementary to the microsatellite sequences, namely

Table 2 RAPD analysis of *A. rabiei*: number of genotypes defined, range of fragments amplified, and genetic diversity (*H*) estimated by the primers

Primers	No. of genotypes defined	Range of fragments amplified	Average No. of fragments/genotype	No. of loci amplified	Genetic diversity/(*H*)
OPI-01	2	5–6	5.50	6	0.21
OPI-16	6	1–6	4.33	7	0.57
OPJ-01	8	2–4	2.62	6	0.63
OPJ-10	2	7–8	7.50	8	0.26
OPJ-15	13	5–8	6.15	12	0.60
OPJ-18	2	2–3	2.50	3	0.11
OPK-09	3	5–8	6.33	9	0.34
OPK-12	3	1–3	2.00	3	0.18
OPK-15	2	1–3	2.00	4	0.07
OPK-19	3	3–4	3.33	5	0.14
OPL-05	7	1–4	2.42	5	0.59
OPL-08	3	1–3	2.00	3	0.42
OPL-16	4	3–5	4.00	5	0.49
OPM-02	2	1–2	1.50	2	0.44
Composite RAPD data	22	1–8	3.71	78	0.70

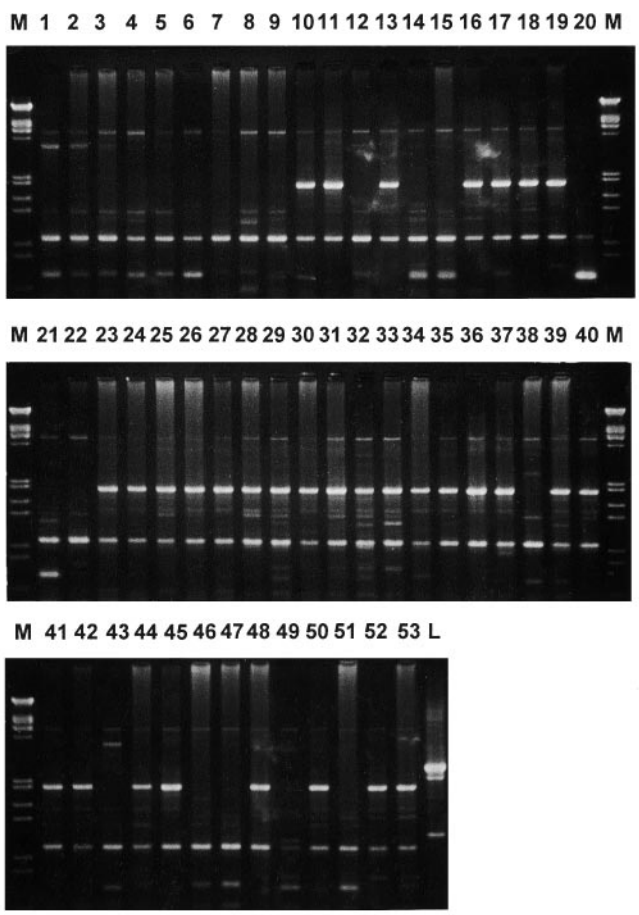

Fig. 3 RAPD patterns of *A. rabiei* isolates as revealed by primer OPJ-01. The *lane numbers* indicate the isolate numbers of *A. rabiei*; *lane L* represents the RAPD pattern of *A. lentis*, and *lane M* is a lambda *Eco*RI-*Hin*dIII digest (molecular-weight standard)

$(GATA)_4$, $(GACA)_4$, $(GGAT)_4$ and $(GTG)_5$, screened for polymorphism on a sub-sample of isolates, only the first three revealed polymorphism. Among the informative microsatellites, $(GATA)_4$, had the highest discriminating power, followed by $(GACA)_4$ and $(GGAT)_4$. $(GTG)_5$ did not reveal any polymorphism (data not shown). Among the enzymes tested for microsatellite analysis, *Taq*I gave more polymorphic profiles of restriction fragments with $(GATA)_4$. Hence the probe-enzyme combination $(GATA)_4$-*Taq*I was used for genotyping of the rest of the isolates (Fig. 4). This probe-enzyme defined 18 genotypes.

Genetic distance and diversity estimation

The pair-wise genetic distance (Nei 1972) was calculated for all 115 fragments (loci) of the isolates, derived from the composite data of microsatellites and RAPDs, and the genetic diversity estimates for year-wise collections are presented in Table 3. The genetic distance for the 53 isolates varied from 0.00 to 0.39, indicating a range from clonal to diverse relationships. Overall genetic diversity (*H*) as detected by all the primers among all the isolates was 0.70. The maximum genetic diversity (0.93) was observed for isolates sampled during 1982 and 1991, followed by 1993 (0.72) and 1992 (0.47).

The genetic diversity estimates were also evaluated for three pathotypes of the pathogen. Very different estimates of genetic diversity were obtained for the pathotypes with the different primers and the microsatellite marker $(GATA)_4$ (Table 4). For instance, primer OPI-01 could distinguish isolates of pathotypes I and II only, but not those of pathotype III, whereas

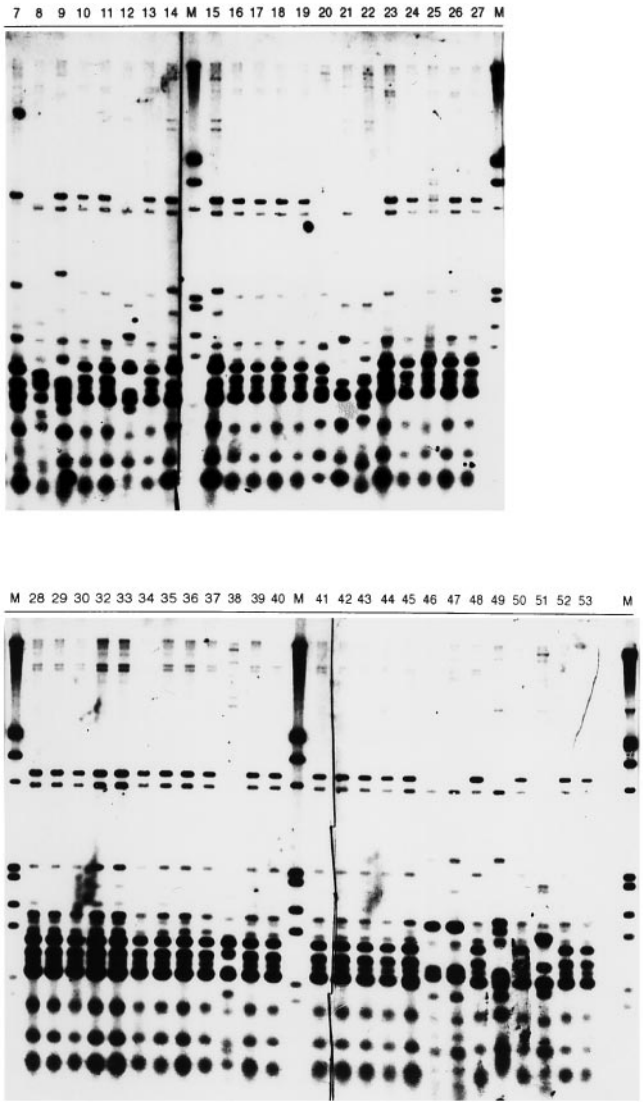

Fig. 4 Microsatellite analysis [probe-enzyme: $(GATA)_4$-*Taq*I] of *A. rabiei* isolates. The *lane numbers* indicate isolate numbers and *lane M* is a lambda *Eco*RI-*Hin*dIII digest (molecular-weight standard)

primer OPJ-18 could discriminate the isolates of pathotype I only, but not those of pathotypes II and III. Similarly, primer OPK-09 differentiated isolates of pathotypes I and III only, but not those of pathotype II, whereas primer OPK-15 differentiated isolates of pathotype II only and not the isolates of pathotypes I and III. Among the primers tested, primer OPJ-01 detected a high degree of genetic diversity in all three pathotypes, followed by OPJ-15. In general, the microsatellite probe detected maximum genetic diversity in pathotypes I and II. On the basis of composite data from all the 14 primers and the microsatellite, pathotype I had maximum genetic diversity (0.97), followed by pathotype II (0.80) and pathotype III (0.32), suggesting a decline in genetic diversity with an increase in aggressiveness (Table 3).

Relationship between phylogeny and pathotype

The relationship between a pathotype and its putative phylogeny was assessed by constructing a dendrogram, based on the composite data (obtained from both RAPD and microsatellite analysis), using the Neighbor-joining (NJM) method (Fig. 5). Isolate no. 1 was placed as the extreme out-group.

At the level of 90% similarity, all the genotypes (A–V) could be grouped into 22 different clusters (Fig. 5). The cluster with genotype H contained 29 isolates and formed a single group belonging to pathotype III. The other clusters of different genotypes contained either one or two isolates. The individual cluster consisted of any one of the three pathotypes, and not multiple pathotypes. The clusters of genotypes H, L, M, N and O consisted of pathotype III, and are very closely related to each other compared to the other two clusters of pathotype III, and even formed a single cluster at a level of 75% similarity. The isolates of pathotype I formed 12 clusters, and the isolates of pathotype II formed three clusters at both levels of similarity. All the clusters of genotypes belonging to

Table 3 RAPD analysis of *A. rabiei*: number of genotypes defined and genetic diversity (*H*) of the 53 isolates, based on the composite data

Collection/pathotype	Isolate #	Sample size (n)	No. of genotypes	No. of pathotypes	Genetic diversity (*H*)
All isolates	1–53	53	22	3	0.70
1982	1–6	6	5	2	0.93
1991	7–12	6	5	3	0.93
1992	13–38	26	7	2	0.47
1993	39–53	15	7	3	0.72
Pathotype I	–	13	12	–	0.97
Pathotype II	–	5	3	–	0.80
Pathotype III	–	35	7	–	0.32

Table 4 Genetic diversity (H) within the pathotypic groups of *A. rabiei* as revealed by the RAPD primers and the microsatellite $(GATA)_4$

Marker	Genetic diversity (H)		
	Pathotype I	Pathotype II	Pathotype III
Primer			
OPI-01	0.385	0.600	0.000
OPI-16	0.500	0.800	0.000
OPJ-01	0.736	0.800	0.217
OPJ-10	0.539	0.600	0.111
OPJ-15	0.795	0.800	0.219
OPJ-18	0.385	0.000	0.000
OPK-09	0.500	0.000	0.057
OPK-12	0.295	0.600	0.058
OPK-15	0.000	0.600	0.000
OPK-19	0.154	0.800	0.000
OPL-05	0.154	0.800	0.219
OPL-08	0.385	0.400	0.057
OPL-16	0.590	0.600	0.057
OPM-02	0.282	0.400	0.111
Microsatellite	0.987	0.800	0.113

pathotype I contained only one of the isolates, indicating the genetically diverse nature of the less-aggressive pathotypes as compared to the aggressive and most-aggressive pathotypes.

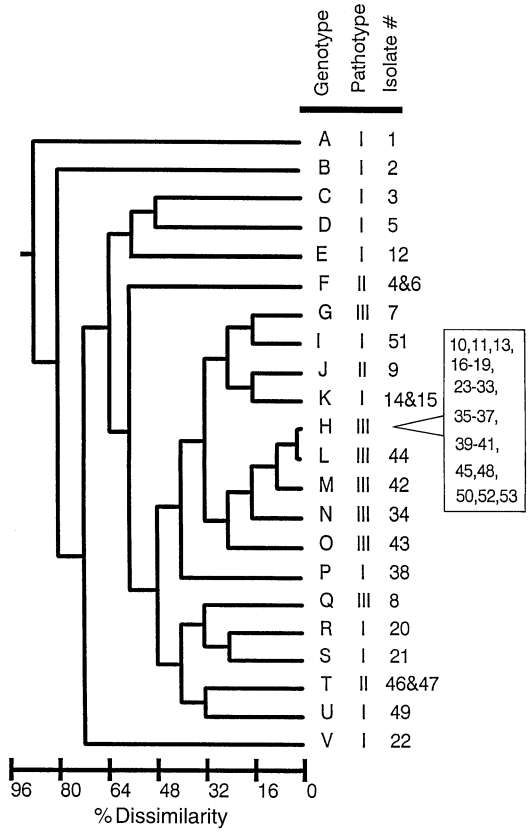

Fig. 5 Phylogenetic tree (neighbor-joining method) of *A. rabiei* isolates constructed based on the genetic distance (Nei 1972) obtained by RAPDs and microsatellite-marker analysis

Discussion

Our experiments demonstrated that both RAPD and microsatellite markers have unprecedented utility for both population genetic and phylogenetic analysis of the ascochyta blight fungus of the chickpea. Since neither requires radioactive isotopes, these methods can be efficiently used by the National Agricultural Research Systems (NARS) of developing countries, where facilities to handle radioactive chemicals are not available.

Our study has also demonstrated that both the microsatellite and RAPD markers are efficient in resolving the pathotype diversity in *A. rabiei*. Although RFLP-based microsatellite analysis is useful, the procedure is time-consuming and expensive. Therefore this analysis is not suitable for handling the large number of samples required for studies in population genetics. On the other hand, RAPD analysis is based on the polymerase chain reaction (PCR), is technically simple and requires only small quantities of DNA. Unlike conventional RFLPs, RAPD analysis does not require cumbersome cloning and membrane hybridization processes and is therefore suitable for the rapid and efficient analysis of a large number of isolates.

Our study showed that the combined use of both RAPDs and microsatellites is more efficient in the genotyping of *A. rabiei*. For instance, isolates 1–6 were classified into four genotypes with microsatellites by Weising et al. (1991), whereas the same isolates were classified into five genotypes in the present study. Our study further classified different pathotypes into genotypes: pathotype I into 12 genotypes, pathotype II into three genotypes and pathotype III into seven genotypes (Table 3), indicating that different isolates of a pathotype of the pathogen are not clonal lineages.

Phenotypic groups, such as pathotypes or vegetative compatibility groups, have been shown to be monophyletic, i.e. to represent evolutionary coherent groups or lineages (Elias et al. 1993; Kelly et al. 1994). On the basis of the phylogram from the present study, however, it appeared that all three pathotypes were polyphyletic. The isolates of pathotype I (least aggressive) formed 12 clusters, pathotype II (aggressive) formed three, and pathotype III (most aggressive) formed three clusters at the 75% similarity level. These groupings will provide a reference base by which to document the emergence of new genotypes as well as the appearance of new lineages. Future investigation of this information with chickpea cropping patterns will provide valuable insight on the influence of host genotypes (resistance genes) to the population biology of the ascochyta blight fungus.

Koch et al. (1991) proposed that a direct relationship between phylogenetic and pathogenicity groupings within a species might be expected if pathogenicity is controlled by many genes distributed throughout the fungal genome. An indirect relationship might result from the geographical or mating isolation of individuals having the same pathotype, if pathogenicity is controlled by only few of the genes. Since different pathotypes did not form a single phylogenetic group, we conclude that there is a direct relationship between phylogeny as revealed by the DNA markers and pathotype groupings in *A. rabiei*. Hence, several genes might be controlling the pathogenic-specialization trait in *A. rabiei*. The polygenic nature of the pathogenicity trait could be the reason for not observing a gene-for-gene relationship (de Wit 1992) in the *A. rabiei*/chickpea pathosystem.

Our results strongly support the conclusion that *A. rabiei* pathotypes are stable and reliably distinguished by carefully standardized pathogenicity assays. If pathotypes were subject to sudden, continuous, or wholesale changes, it would have been impossible to consistently identify the most predominant genotype H of pathotype III in the population on the basis of genetic lineage associations maintained over 3 years in nature.

Winter planting of chickpea is a relatively recent practice in Syria, as well as in other countries of West Asia and North Africa (Hawtin and Singh 1984). The cool temperatures and wet weather conditions that prevail during winter, favors the incidence and development of ascochyta blight disease in chickpea fields (Reddy et al. 1990). Over the years, cultivars with improved levels of resistance to the disease have been bred and released (first ILC 482 in 1986 and then ILC 3279 in 1989) for winter planting (Singh and Reddy 1993). Consequently, we speculate that the most-aggressive pathotype evolved through the process of mutation in response to a change in the host resistance in the field and was subsequently selected by the deployed host resistance. Alternatively, it is also possible that pre-existing variability in the pathogen population has been selected by the deployed host resistance. A recent study in a plant pathogenic fungus, *Rhynchosporium secalis*, by Goodwin et al. (1994) also showed that a high rate of mutation could occur for the pathogenicity trait under natural conditions in response to the selection pressure exerted by host resistance. This kind of selection-induced genetic change in fungi is not due solely to random (spontaneous) mutations (McDonald et al. 1989), but also due to the "adaptive mutations" recently described in bacteria and yeast (Hall 1990; Lenski and Mittler 1993; Harris et al. 1994). The evolved aggressive pathotype may have been selected in response to the resistant cultivars deployed in the field over the years, thereby reducing the genetic diversity in the pathogen population in recent times. In addition to periodic strong host selection for specific aggressive genotypes (pathotype), migration may have also played an important role in the evolution of the pathogen. The occurrence of a single genotype over a large area, with increasing frequency, suggests that the most-aggressive genotype migrated, most possibly through infected seeds. This suggestion of migration through infected seeds was further supported by a study conducted by our Socio-economic research group (unpublished results) where it was observed that a majority of farmers in Syria procure seed material of chickpea from another farmer rather than from the Government seed agency. Therefore, there is a little chance that a majority of the seed material used by the farmers has been tested for seed health. This practice thus further facilitated the migration of aggressive genotypes of the pathogen. The wider geographical distribution and the stability of the most predominant genotype over the years, under field conditions, further support the view that the genotypes of this fungus are more stable and that sexual recombination (Kaiser 1992) plays only a minor role in the evolution of this pathogen.

Our study clearly demonstrated that RAPD and microsatellite markers reliably indexed the genetic and pathotypic diversity in Syrian populations of *A. rabiei*. These markers can be used to determine the current geographical organization of pathotype diversity and to monitor its change over time, as well as to identify the dispersal range of specific genotypes. This will improve the processes of breeding, selecting and deploying more durable resistant chickpea cultivars for particular chickpea growing areas. We have already identified the sources of resistance to the predominant genotype (genotype H) of the pathogen from the chickpea gene bank (unpublished results). These sources of resistance are being used in chickpea breeding programs at ICARDA to improve ascochyta blight resistance in agronomically superior cultivars. These markers will also facilitate the study of the rate, direction, and forces of evolution for this important pathogen. A knowledge of the evolutionary forces, such as migration and selection, acting on the pathogen population will help in designing suitable strategies for the integrated management of this disease. For instance, seed treatment with a seed-dressing fungicide (e.g. Tiabendazole), reduced the migration of the predominant genotype of the pathogen, thereby reducing the incidence of the disease (unpublished results) in the released resistant cultivars. Similarly, the selection of an aggressive genotype of the pathogen by host plant resistance could be reduced by crop rotation and also by rotating different sources of resistance in chickpea growing areas.

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the experiments comply with the current laws of Syria and Germany in which the experiments were performed.

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