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Restriction Fragment Length Polymorphism Analysis of the Mitochondrial DNA of *Fusarium oxysporum* f. sp. *ciceris*

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With 3 figures

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Abstract

Seven isolates of *Fusarium oxysporum* f. sp. *ciceris*, representing pathogenic races 1, 2, 3, and 4 from India and 0, 5, and 6 from Spain, were assayed for restriction fragment length polymorphisms (RFLPs) in the mitochondrial DNA (mt DNA). The mt DNA fraction of total fungal DNA was purified and digested with the restriction endonucleases *Bam* HI, *BgI* II, *Eco* RI, *Kpn* I, *Sac* I, *Sal* I, *Sma* I, and *Xho* I. The mt DNA is a circular molecule of 40.5 kb. No RFLP in the mt DNA was detected among the seven races of *F. o. ciceris*. The identical restriction patterns of mt DNA indicates an extensive conservation in the gene composition of mt DNA without sequence variation, and suggests that mt DNA of *F. o. ciceris* may not be responsible for pathogenic diversity. The restriction map of mt DNA from the race 6 isolate Fo 8272 was constructed by digestion of the mt DNA with five restriction enzymes: *Eco* RI, *Kpn* I, *Sac* I, *Sal* I, and *Xho* I, either singly or in selected pairs.

Zusammenfassung

Analyse von Restriktionsfragmentlängenpolymorphismen der Mitochondrien-DNA von *Fusarium oxysporum* f. sp. *ciceris*

Sieben Isolate von *Fusarium oxysporum* f. sp. *ciceris*, die die physiologischen Rassen 1, 2, 3 und 4 (aus Indien) und 0, 5 und 6 (aus Spanien) repräsentierten, wurden auf Restriktionsfragmentlängenpolymorphismen (RFLPs) in der Mitochondrien-DNA (mt-DNA) untersucht. Die mt-DNA-Fraktion der gesamten DNA wurde gereinigt und mit den Restriktionsendonukleasen *Bam* HI, *BgI* II, *Eco* RI, *Kpn* I, *Sac* I, *Sal* I, *Sma* I und *Xho* I geschnitten. Die mt-DNA ist ein ringförmiges Molekül von 40,5 kb. Bei den sieben *F. o. ciceris*-Rassen wurden keine RFLPs in der mt-DNA festgestellt. Die identischen Restriktionsmuster der mt-DNA weisen darauf hin, daß die genetische Zusammensetzung der mt-DNA hochgradig konserviert ist und die Sequenzen nicht variieren. Sie legen außerdem nahe, daß die mt-DNA von *F. o. ciceris* nicht für die physiologische Diversität verantwortlich ist. Durch Verdau der mt-DNA mit fünf Restriktionsenzymen (*Eco* RI, *Kpn* I, *Sac* I, *Sal* I und *Xho* I, entweder einzeln oder in ausgewählten Paaren) wurde die Restriktionskarte der mt-DNA des Isolats Fo 8272 (Rasse 6) erstellt.

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, is a major constraint to chickpea (*Cicer arietinum* L.) production in many countries (Haware, 1990; Nene and Reddy, 1987). Seven races of *F.o. ciceris* (races 0–6) have been identified, which occur in India (races 1–4) (Haware and Nene, 1982), California and Spain (races 0, 1, 5 and 6), Morocco (races 1 and 6) and Tunisia (race O) (Jiménez-Díaz et al., 1989, 1993). The identification of races of *F.o. ciceris* is based on the differential disease reactions of 10 chickpea lines to isolates of the pathogen (Haware and Nene, 1982; Jimenez-Diaz et al., 1989), which is expensive, time-consuming, and may be influenced by variability inherent in the experimental system (Alcalá-Jiménez et al., 1992, Bhatti and Kraft, 1992a, b). Furthermore, the differential disease reactions do not provide information about the genetic relationship and variability among, and within, the pathogenic races.

Analysis of DNA restriction fragment length polymorphisms (RFLPs) is one of the several molecular techniques that are used to determine genetic variation in phytopathogenic fungi (Michelmore and Hulbert, 1987). Recently, variation of mitochondrial DNA (mt DNA) has been used extensively to assess inter- and intra-species relationships in various fungi, including *F. oxysporum* (Jacobson and Gordon, 1990; Kim et al., 1992; Kistler and Benny, 1989; Kistler et al., 1987) and other plant pathogenic fungi (Correll et al., 1992; Förster et al., 1988, 1989; Gray and Hepburn, 1992; Ko et al., 1993; Kohn et al., 1988; Martin and Kistler, 1990). In fungi, the mt DNA is small and present in high copy number and shows abundant polymorphisms resulting from length mutations (Bruns et al., 1991; Taylor, 1986), which makes it suitable for restriction enzyme analysis. Because of the rapid rate of evolution of mt DNA, which is estimated in animals to be 10 to 100 times faster than that of nuclear DNA (Brown et al., 1982; Wilson et al., 1985), analysis of mt DNA may provide a more sensitive measurement of genetic divergence in populations than other methods. The purpose of this study was to analyse RFLPs in the mt DNA from isolates of *F. o. ciceris* differing in pathogenicity to chickpea lines and geographic origin in order to identify genetic variation among them. A preliminary report of this work has been published (Pérez-Artés et al., 1992).

Materials and Methods

Fungal strains and growth conditions

Seven isolates of *F.o. ciceris*, representative of races 0–6, were used in this study (Table 1). Isolates of races 1–4 from India were kindly

Table 1
Isolates of *Fusarium oxysporum* f. sp. *ciceris* used to determine mt DNA restriction fragment length polymorphisms

Designation	Race[a]	Year of isolation	Location of isolation[b]
Fo 7952	0	1979	Andalucía, southern Spain
Fo 7989	1	1979	Hyderabad, southern India
Fo 8605	2	1980	Kanpur, northern India
Fo 8606	3	1980	Gurdaspur, northern India
Fo 8607	4	1980	Jabalpur, central India
Fo 8012	5	1980	Andalucía, southern Spain
Fo 8272	6	1982	Andalucía, southern Spain

[a] Determined in previous studies (Haware and Nene, 1982; Jiménez-Díaz et al., 1989).
[b] Isolates from India were provided by M. P. Haware, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

provided by M. P. Haware, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. Isolates had been previously characterized for race (Table 2) (Haware and Nene, 1982; Jimenez-Diaz et al., 1989) and vegetative compatibility (Nogales-Moncada et al., 1993). They were single-spored, increased, and stored in sterile soil tubes. An active culture of each isolate was obtained by plating a small aliquot of soil culture on potato-dextrose agar (PDA) and incubating at 25°C with a 12 h photoperiod of fluorescent light and near UV light at 36 $\mu E.m^{-2}.s^{-1}$. Stock cultures of the isolates were maintained on PDA during the course of the studies. Mycelia for DNA preparation were obtained from flask cultures in potato-dextrose broth gyrated at 110–120 rpm for 3 days at same temperature and light conditions as above. Aproximately 25 g of fresh mycelium were harvested from each *F. o. ciceris* isolate by filtration through Miracloth (Calbiochem, San Diego, CA, USA). The mycelial mat was frozen in liquid nitrogen and lyophilized.

DNA isolation

The lyophilized mycelium was ground up to a very fine powder with a mortar and pestle. Total nucleic acids were extracted by the method of Raeder and Broda (1985) with slight modifications. Five grams of powdered mycelium were resuspended in 10 volumes of extraction buffer (200 mM Tris-HCl, pH 8,5; 100 mM NaCl; 10 mM EDTA; 0,5% SDS). The slurry was mixed homogeneously with seven volumes of equilibrated phenol and extracted with three volumes of chloroform: isoamylalcohol (24:1). The suspension was centrifuged at 13 000 × *g* (20°C) for 1 h.

The upper aqueous phase was immediately transferred to a new tube and DNA was precipitated by addition of 0.54 volumes of cold isopropanol. DNA precipitated visibly into a lump, was removed with a glass rod, washed twice in 100% ethanol, dried and resuspended in 30 ml of 10 mM Tris:1 mM EDTA buffer. DNAs were separated by isopycnic centrifugations in CsCI gradients (density = 1.68), using the fluorescent DNA-binding dye bisbenzimide (Sigma Chemical Co., Dorset, UK) at a concentration of 120 μg/ml. Mt DNA fractions were purified by two rounds of centrifugations at 242 000 g for 24 h and 328 000 *g* for 20 h in the verticial rotors Beckman vTi 50 and vTi 80 respectively.

Table 2
Reactions of differential chickpea lines to races of *Fusarium oxysporum* f. sp. *ciceris*[a]

Differential line	0	1	2	3	4	5	6
12-071/10054	S	M	R	R	R	R	R
JG 62	R	S	S	S	S	S	S
C 104	M	M	S	S	S	S	M
JG 74	R	R	S	R	R	M	R
CPS 1	R	R	S	M	M	M	R
BG 212	R	R	S	M	M	R	R
WR 315	R	R	R	S	R	R	R
ICCV 2	R	R	M	M	M	S	M
ICCV 4	R	R	S	S	S	S	M
P 2245	S	S	S	S	S	S	S

[a] Assessed on 0–4 scale according to percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0%, 1 = 1 – 33%, 2 = 34 – 66%, 3 = 67 – 100%, 4 = dead plant) 40 days after inoculation. Scores ⩽1 and ⩾3 were considered as resistant (R) and susceptible (S) reactions, respectively. Scores in between were considered as moderately susceptible reaction (M).

Restriction endonuclease analyses

Restriction enzymes were obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden, or from Boehringer Mannheim, Germany. Mt DNA aliquots from the seven *F. o. ciceris* isolates were digested for 2 h at 37°C with a variety of restriction endonucleases, (*Bam* HI, *Bgl* II, *Eco* RI, *Kpn* I, *Sac* I, *Sal* I, *Sma* I and *Xho* I), according to the manufacturers' specifications. Restriction fragments were separated by electrophoresis in 0.7% agarose gels in Tris:acetate:EDTA buffers containing ethidium bromide. Restriction endonuclease fragments of phage lambda DNA digested with *Hind* III were used as size markers.

Results

Identification of mt DNA

Isopycnic centrifugation of *F. o. ciceris* total DNA resulted in three distinct bands. The lowermost band corresponded to nuclear DNA, and the uppermost one was a band of non-identified DNA. The intermediate band contained high molecular weight DNA that was identified as mt DNA since it gave rise to a discrete number of well-defined bands after digestion with a variety of restriction endonucleases and separation in agarose gels. Spectrophotometric quantification and gel electrophoresis analyses indicated that about 1% of the total DNA extracted from the *F. o. ciceris* isolates was mt DNA. We routinely recovered 10 μg of mt DNA and 1000 μg of nuclear DNA from the crude extract of 5 g of lyophilized mycelium.

Restriction fragment pattern of mt DNA within *F. o. ciceris*

Mt DNA purified from the seven *F. o. ciceris* isolates was digested to completion with each of eight restriction endonucleases. All isolates produced identical restriction fragment patterns when digested with each of the enzymes used (Fig. 1). Therefore, no RFLPs in mt DNA were detected among these isolates.

Size and restriction map of mt DNA

The mt DNA from race 6 isolate Fo 8272 was treated with restriction endonucleases *Bgl* II, *Eco* RI, *Kpn* I, *Sac* I, *Sal* I, and *Xho* I, by single digests as well as all selected pairwise combinations of enzymes. The size of mt DNA is about 40.5 kilobase pairs (kb). It was determined by averaging the sum of all digests containing DNA fragments of less than 15 kb (Fig. 2). A mt DNA restriction map constructed with the enzymes *Eco* RI, *Kpn* I, *Sac* I, *Sal* I, and *Xho* I is shown in Fig. 3. The mt DNA of *F. o. ciceris* is a circular DNA molecule. Location of target site for *Bgl* II was not included in the restriction map because of the extremely high number of bands obtained in all double digestions with this enzyme. Digestion with enzymes *Bgl* II plus *Sac* I lacks two of the expected bands; digestion with enzymes *Bgl* II plus *Xho* I lacks four of the expected bands; and digestion with enzymes *Eco* RI plus *Xho* I lacks one of the expected bands. The absence of these bands is probably due to the small fragment size produced after the double cutting with restriction endonucleases.

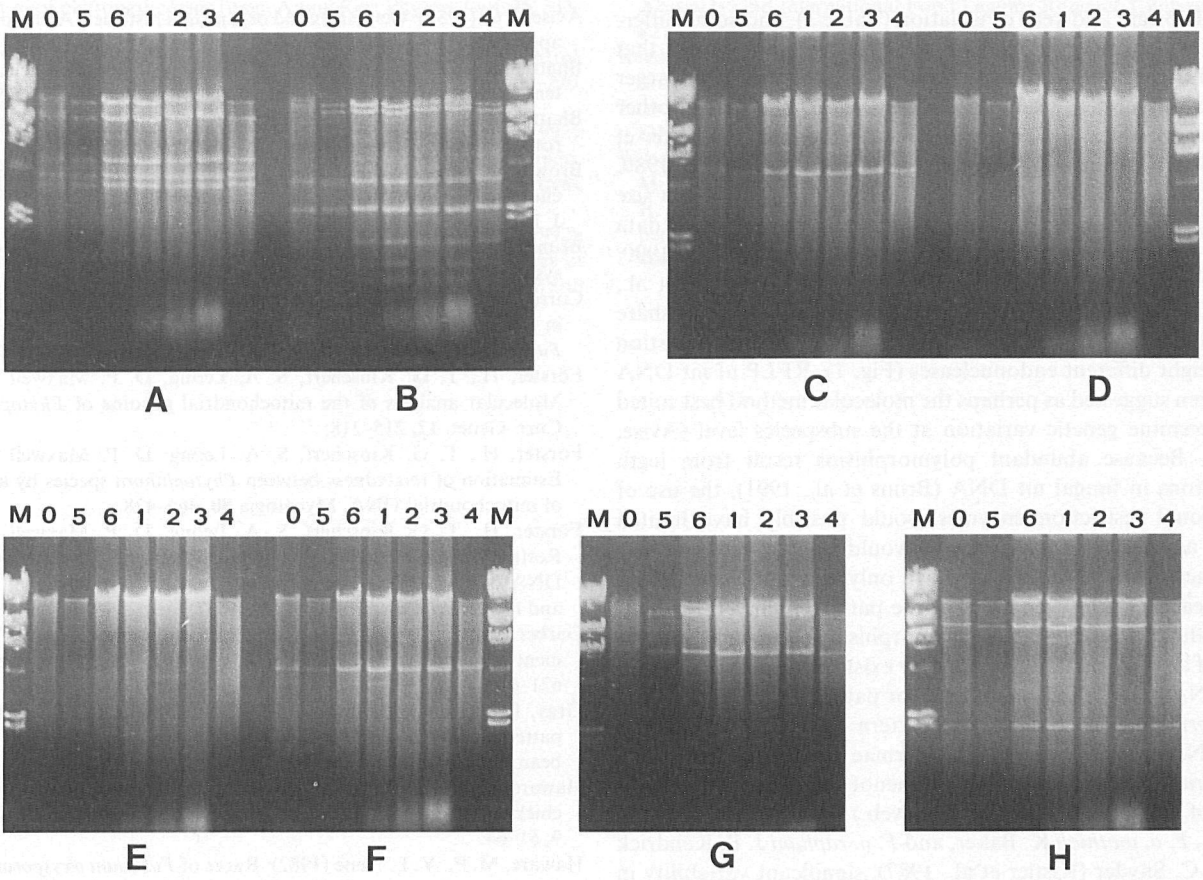

Fig. 1 Agarose gel electrophoresis of mt DNA from races 0,5 and 6 (Spain) and races 1, 2, 3 and 4 (India) of *Fusarium oxysporum* f. sp. *ciceris* digested with *Eco* RI (A) *BgI* II (B), *SaI* I (C), *Bam* HI (D), *Sma* I (E), *Sac* I (F), *Kpn* I (G) and *Xho* I (H). In each digest, lane M corresponds to *Hind* III fragments of lambda DNA

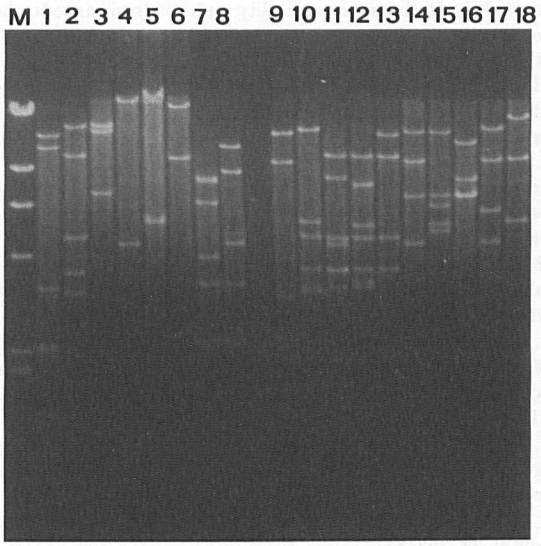

Fig. 2 Agarose gel electrophoresis of mt DNA from *Fusarium oxysporum* f. sp. *ciceris* race 6 isolate Fo 8272. Lanes: 1) *BgI* II, 2) *Eco* RI, 3) *Kpn* I, 4) *Sac* I, 5) *Sal* I, 6) *Xho* I, 7) *BgI* II plus *Kpn* I, 8) *BgI* II plus *Sac* I, 9) *BgI* II plus *Xho* I, 10) *Eco* RI plus *Kpn* I, 11) *Eco* RI plus *Sac* I, 12) *Eco* RI plus *SaI* I, 13) *Eco* RI plus *Xho* I, 14) *Kpn* I plus *Sac* I, 15) *Kpn* I plus *SaI* I, 16) *Kpn* I plus *Xho* I, 17) *Sac* I plus *Xho* I, and 18) *SaI* I plus *Xho* I. Lane M corresponds to *Hind* III fragments of lambda DNA

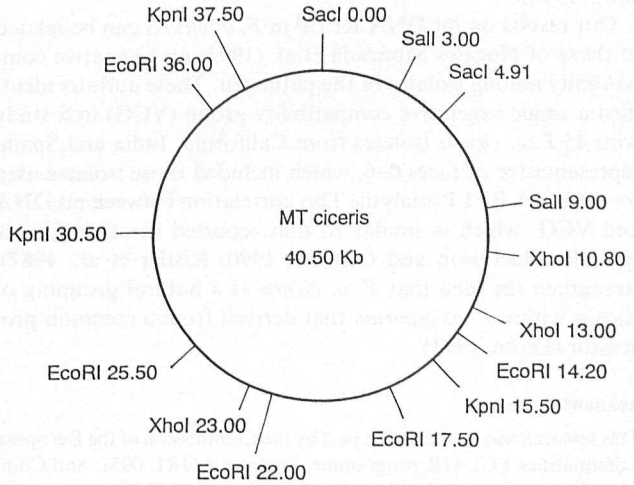

Fig. 3 Restriction map of *Fusarium oxysporum* f. sp. *ciceris* race 6 isolate Fo 8272

Discussion

The purpose of this study was to explore the occurrence of genetic variation in a sample of isolates of *F. o. ciceris* by RFLP analysis of mt DNA. Although the sample size was small, the isolates used differ widely in pathogenicity (Table 2) (Haware and Nene, 1982; Jiménez-Díaz et al., 1989), as well as in geo-

graphic origin and year of isolation (Table 1), and some differences in their genetic make up might occur among them that would encourage a further search of genetic diversity in a larger sample of isolates. Mt DNA of *F. o. ciceris*, like that of other formae speciales of *F. oxysporum* and other fungi (Förster et al., 1987; Garber and Yoder, 1984; Kistler and Benny; 1989, Marriot et al., 1984), is a circular molecule (Fig. 3) with a size of 40.5 kb, which is consistent with previously published data of mt DNA from *F. oxysporum* (Jacobson and Gordon, 1990; Kistler and Benny, 1989; Kistler et al., 1987, Marriot et al., 1984). Isolates of *F. o. ciceris* in this study (Tables 1,2) share identical mt DNA restriction fragment patterns, after digestion with eight different endonucleases (Fig. 1). RFLP of mt DNA has been suggested as perhaps the molecular method best suited to determine genetic variation at the subspecies level (Avise, 1989). Because abundant polymorphisms result from legth mutations in fungal mt DNA (Bruns et al., 1991), the use of additional restriction enzymes would possibly have limited value as most length differences would be detected with few enzymes. Thus, although we have only examined one isolate from each of the seven races of the pathogen currently identified, the lack of mt DNA polymorphism found suggests that little if any internal divergence may exist within races, and that mt DNA may not be responsible for pathogenic diversity in *F. o. ciceris*. In *F. oxysporum*, the pattern of genetic variation in mt DNA seems to vary among formae speciales. Thus, while no variation in mt DNA RFLP phenotypes was found within each of *F. o. conglutinans* (Wollemweb.) W. C. Snyder & H. N. Hans., *F. o. mathioli* K. Baker, and *F. o. raphani* J. B. Kendrick & W. C. Snyder (Kistler et al., 1987), significant variability in mt DNA restriction fragment patterns was detected among isolates of *F. o. melonis* W. C. Snyder & H. N. Hans. (Jacobson and Gordon, 1990) and *F. o. niveum* (E. F. Sm.) W. C. Snyder & H. N. Hans (Kim et al., 1992). However, in neither case was there a correlation between pathogenic races and mt DNA restriction fragment patterns (Jacobson and Gordon, 1990; Kim et al., 1992).

Our results on mt DNA RFLP in *F. o. ciceris* can be related to those of Nogales Moncada et al. (1993) on vegetative compatibility among isolates of the pathogen. These authors identified a single vegetative compatibility group (VCG) in a study with 45 *F. o. ciceris* isolates from California, India and Spain, representative of races 0–6, which included those isolates used for mt DNA RFLP analysis. This correlation between mt DNA and VCG, which is similar to that reported in other formae speciales (Jacobson and Gordon, 1990; Kistler et al., 1987), strengthen the idea that *F. o. ciceris* is a natural grouping of strains within *F. oxysporum* that derived from a common progenitor (Leslie, 1993).

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