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Gene genealogies support *Fusarium oxysporum* f. sp. *ciceris* as a monophyletic group

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Fusarium oxysporum f. sp. *ciceris* (Foc), the causal agent of fusarium wilt of chickpea, consists of two pathotypes (yellowing and wilting) and eight races (races 0, 1B/C, 1A and 2–6) of diverse geographical distribution. Six Foc isolates, one each of races 0, 1B/C, 1A, 4, 5 and 6, representing the two pathotypes and the geographical range of the pathogen, showed identical sequences in introns of the genes for translation elongation factor 1α (*EF1*α), β-tubulin, histone 3, actin and calmodulin. Eleven additional Foc isolates representative of all races, pathotypes and geographical range, and three isolates of *F. oxysporum* (Fo) nonpathogenic to chickpea were further analysed for sequence variation in the *EF1*α gene. All isolates pathogenic to chickpeas shared an identical *EF1*α gene sequence, which differed from that shared by the three Fo isolates nonpathogenic to chickpea. *EF1*α gene sequences from the 17 Foc isolates and the three Fo isolates were compared with 24 *EF1*α gene sequences in GenBank from isolates of 11 *formae speciales* of *F. oxysporum* by parsimony analysis. Foc isolates formed a grouping distinct from other *formae speciales* and nonpathogenic isolates. These results indicate that *F. oxysporum* f. sp. *ciceris* is monophyletic.

Keywords: chickpea, *Cicer arietinum*, evolutionary origin, fusarium wilt

Introduction

Asexually reproducing fungi do not undergo regular recombination, and genetic variation results mainly from the accumulation of mutations. As a consequence, in these fungi the whole genome is linked, transmitted as a unit from one generation to the next, and different regions in the genome should share the same evolutionary history (Taylor *et al.*, 1999b). Therefore, the evolution of phenotypic traits in asexual plant pathogenic fungi, such as host specificity or relatedness among pathogenic races, can be studied by analysing genealogies of genes that do not have a direct functional relationship to the phenotypes of interest (e.g. O'Donnell *et al.*, 1998; Taylor *et al.*, 1999a; Steenkamp *et al.*, 2000).

Fusarium oxysporum is an ubiquitous, asexual species complex. Isolates of *F. oxysporum* can cause vascular wilt or cortical rot diseases in many agricultural crops and have been classified into *formae speciales* based on their host specificity (Nelson *et al.*, 1981). Isolates within a *forma specialis* are generally more similar genetically than isolates with different host specificities and have been assumed to have a monophyletic origin (Tantaoui *et al.*, 1996; Kistler, 1997). Also, clonality in *F. oxysporum* has been associated with vegetative compatibility (Gordon & Martyn, 1997; Kistler, 1997), with isolates belonging to a vegetative compatibility group (VCG) showing high genetic similarity, as determined by mitochondrial (mt) DNA or intergenic spacer region (IGS) haplotyping (Gordon & Martyn, 1997). However, a few gene genealogy studies have shown that some *formae speciales* of *F. oxysporum* can have multiple independent origins (i.e. polyphyletic), with pathogenicity and virulence evolving more than once (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000). Similarly, notable exceptions have been found in the correlation between VCG and mtDNA or IGS haplotypes. For example, a *F. oxysporum* f. sp. *melonis* isolate was identified in VCG 0131 that shared mtDNA and IGS haplotypes with pathogenic isolates from VCG 0134, instead of with other isolates representative of VCG 0131 (Appel & Gordon, 1995). Also, nonpathogenic *F. oxysporum* isolates vegetatively compatible with *F. oxysporum* f. sp. *melonis* in VCGs 0131 and 0134 had nucleotide sequences in the IGS region of rDNA that were distinct from the pathogenic isolates (Appel & Gordon, 1996). This latter example demonstrates that in some cases vegetative compatibility may be coincidental, possibly arising by convergence rather than common descent.

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Fusarium oxysporum f. sp. *ciceris*, the causal agent of fusarium wilt of chickpeas (*Cicer arietinum*), is pathogenic to *Cicer* spp. (Kaiser *et al.*, 1994) and occurs worldwide (Jalali & Chand, 1992). Variation in symptom types (pathotypes) and pathogenic races have been reported to correlate to different geographical regions and to polymorphisms in molecular markers. Two pathotypes, designated yellowing and wilting, have been differentiated by pathogenicity tests (Trapero-Casas & Jiménez-Díaz, 1985). The yellowing pathotype induces progressive foliar yellowing with vascular discoloration, followed by plant death within 40 days of inoculation. The wilting pathotype induces severe chlorosis and flaccidity combined with vascular discoloration, followed by plant death within 20 days of inoculation. These two pathotypes can be distinguished unambiguously by random amplified polymorphic DNA (RAPD) markers; they formed two distinct groups in cluster analyses (Kelly *et al.*, 1994), suggesting that they may not be closely related. In addition to variation in symptom type, there are eight races of *F. oxysporum* f. sp. *ciceris* (races 0, 1A, 1B/C, 2, 3, 4, 5 and 6) which are identified by differential disease reactions on a set of chickpea cultivars (Haware & Nene, 1982; Jiménez-Díaz *et al.*, 1993). As with pathotypes, some of these races can be distinguished by RAPD markers (Jiménez-Gasco *et al.*, 2001). In susceptible chickpea cultivars, races 0 and 1B/C induce the yellowing syndrome (yellowing pathotype), whereas races 1A, 2, 3, 4, 5 and 6 induce the wilting syndrome (wilting pathotype) (Trapero-Casas & Jiménez-Díaz, 1985; Jiménez-Díaz *et al.*, 1993). These races also have distinct geographical distributions. Races 2, 3 and 4 have only been described from India (Haware & Nene, 1982), whereas races 0, 1B/C, 5 and 6 are found mainly in the Mediterranean region, as well as in the USA (California) (Jiménez-Díaz *et al.*, 1993; Halila & Strange, 1996). Race 1A has been reported in India (Haware & Nene, 1982), California and the Mediterranean region (Jiménez-Díaz *et al.*, 1993). However, despite variation in symptom type, race and geographical distribution, all *F. oxysporum* f. sp. *ciceris* isolates tested so far are in a single VCG (Nogales-Moncada, 1997).

The phenotypic and geographic diversity observed within *F. oxysporum* f. sp. *ciceris* raises the possibility that this taxon is polyphyletic. Therefore, the aim of the present study was to test the null hypothesis that *F. oxysporum* f. sp. *ciceris* is monophyletic. To achieve this objective, the extent and nature of sequence variation in introns of conserved genes were studied. These sequences were compared with those of other *formae speciales* of *F. oxysporum* to test whether f. sp. *ciceris* is monophyletic within this species complex. The secondary objective was to compare sequence variation in relation to variation in pathotype, race and geographical distribution.

Materials and methods

Fungal isolates

Seventeen isolates of *F. oxysporum* f. sp. *ciceris* representative of the eight described races and from a wide geographical range were used in this study (Table 1). In addition, three isolates of *F. oxysporum* obtained from roots of healthy chickpeas and nonpathogenic to chickpea were analysed (Table 1). All of these isolates were characterized for pathotype and race in previous studies (Jiménez-Díaz *et al.*, 1993; Kelly *et al.*, 1994; Jiménez-Gasco *et al.*, 2001). At least two isolates of each race, chosen to maximize geographic variation within race whenever possible (some races have limited geographical distribution), were analysed. Isolates from California, Morocco and Spain were collected by R. M. Jiménez-Díaz. Isolates from India were a gift from Dr M. P. Haware (International Crops Research Institute for Semiarid Tropics, Hyderabad, India). Isolates from Tunisia were provided by Dr M. H. Halila (Institut Nationale de la Recherche Agronomique, Ariana, Tunisia). All isolates were stored as monoconidial cultures in sterile soil at 4°C in the dark in the culture collection of the Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. Active cultures of isolates were obtained by subculturing stored cultures on potato dextrose agar at 25°C with a 12-h

Table 1 Geographical origin and race information for isolates of *Fusarium oxysporum* f. sp. *ciceris* (Foc) and *Fusarium oxysporum* (Fo) nonpathogenic to chickpea used in this study

Isolates[a]	Origin[b]	Pathogenic race[c]
Group A		
Foc-7802	Spain	0
Foc-1987-W17	USA (California)	1 B/C
Foc-7989	India	1 A
Foc-1992 R4N	India	4
Foc-9035	Spain	5
Foc-1987T	USA (California)	6
Group B		
Foc-9601	Tunisia	0
Foc-USA 3-1 JG62	USA (California)	1 B/C
Foc-9602	Tunisia	1 B/C
Foc-9168	Morocco	1 A
Foc-8605	India	2
Foc-1992 R2N	India	2
Foc-8606	India	3
Foc-1992 R3N	India	3
Foc-8607	India	4
Foc-9094 JG62	Spain	5
Foc-9093 PV1	Spain	6
Fo-90101	Spain	NP
Fo-90105	Spain	NP
Fo-9169	Morocco	NP

NP, nonpathogenic to chickpea.
[a]Isolates in group A were analysed for sequence variation in the genes for translation elongation factor 1α (*EF1*α), β-tubulin, histone 3, actin and calmodulin. Isolates in group B were analysed for sequence variation only in the *EF1*α gene.
[b]Isolates from California, Morocco and Spain were collected by R. M. Jiménez-Díaz; isolates from India were provided by Dr M. P. Haware, International Crops Research Institute for the Semiarid Tropics, Hyderabad, India; isolates from Tunisia were a gift from Dr M. H. Halila, Institut Nationale de la Recherche Agronomique, Ariana, Tunisia.
[c]Determined by pathogenicity tests on differential lines of chickpea (Alcalá-Jiménez, 1995; Jiménez-Gasco *et al.*, 2001).

Table 2 Primer sets and corresponding amplified targets

Target gene	Primers	Primer sequence (5′ → 3′)
EF1α[a]	EF1	ATGGGTAAGGA(A/G)GACAAGAC
	EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT
β-*tub*[b]	Bt1a	TTCCCCCGTCTCCACTTCTTCATG
	Bt1b	GACGAGATCGTTCATGTTGAACTC
H3[b]	H3-1a	ACTAAGCAGACCGCCCGCAGG
	H3-1b	GCGGGCGAGCTGGATGTCCTT
Act[c]	ACT-512F	ATGTGCAAGGCCGGTTTCGC
	ACT-783R	TACGAGTCCTTCTGGCCCAT
Cal[c]	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC
	CAL-737R	CATCTTTCTGGCCATCATGG

Primers described in [a]O'Donnell *et al.* (1998), [b]Glass & Donaldson (1995) and [c]Carbone & Kohn (1999).

photoperiod of fluorescent and near-UV light at 36 μE m^{-2} s^{-1}. Methods for culturing the isolates and obtaining mycelia for DNA extraction were as described previously (Jiménez-Gasco *et al.*, 2001). Genomic DNA was purified from lyophilized ground mycelium using a small-scale method (Raeder & Broda, 1985).

DNA amplification and sequencing

Genes were selected that contained conserved sequences among ascomycetes and that included at least one intron. The genes chosen were those for: translation elongation factor 1α (*EF1α*), β-tubulin (β-*tub*), histone 3 (*H3*), actin (*Act*) and calmodulin (*Cal*). Polymerase chain reaction (PCR) primers and amplification of these target genes were as described previously (Glass & Donaldson, 1995; O'Donnell *et al.*, 1998; Carbone & Kohn, 1999) (Table 2). PCR products were purified using the QIAquick PCR purification and QIAquick gel extraction kits (Qiagen Inc., Chatsworth, CA, USA). All primers were synthesized by the Cornell University Oligonucleotide Synthesis Facility. Sequencing of PCR products was done by the DNA Sequencing Facility at Cornell University, Ithaca, NY, USA.

Phylogenetic analysis

Sequences were edited and aligned with homologous sequences from the *F. oxysporum* species complex obtained from GenBank using Sequencher 3.1.1 (Gene Codes, Ann Arbor, MI, USA). Phylogenetic analysis was performed with PAUP* 4·0b4a (Swofford, 2000). Indels were coded as single events. Parsimony analysis was performed using the heuristic search option with 1000 random addition sequences of the tree-bisection-reconnection, branch-swapping algorithm, and the MULPARS option on. Bootstrap analysis was based on 1000 replications.

Results

The genes *EF1α*, β-*tub*, *H3*, *Act* and *Cal* were first sequenced and analysed from one isolate of each of races 0, 1B/C, 1A, 4, 5 and 6 of *F. oxysporum* f. sp. *ciceris* (Table 1, group A) to search for sequences associated with pathotypes, races and geographical distribution in *F. oxysporum* f. sp. *ciceris*. However, sequences were identical among all six isolates for all five genes (GenBank accession numbers AF346503, AF346505 to AF346508, respectively).

Because the *EF1α* gene has been extensively studied in several *formae speciales* of *F. oxysporum* (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000), its sequences were obtained from 11 additional isolates of *F. oxysporum* f. sp. *ciceris* and three isolates of *F. oxysporum* nonpathogenic to chickpea (Table 1, group B). All *EF1α* gene sequences from *F. oxysporum* f. sp. *ciceris* were identical among themselves. Similarly, *EF1α* gene sequences from the three nonpathogenic *F. oxysporum* isolates (GenBank accession number AF346504) were identical among themselves, but different from the pathogenic isolates. Sequence differences were found only in the three introns of the *EF1α* gene, as described by O'Donnell *et al.* (1998) for other taxa within the *F. oxysporum* complex.

To test whether the *EF1α* gene genealogy supports *F. oxysporum* f. sp. *ciceris* as a monophyletic group, the *EF1α* gene sequences from the 17 *F. oxysporum* f. sp. *ciceris* isolates and the three nonpathogenic *F. oxysporum* isolates were compared with 24 *EF1α* gene sequences obtained from GenBank. These sequences were from isolates of 11 different *formae speciales* (*asparagi*, *canariensis*, *cubense*, *dianthi*, *gladioli*, *lini*, *lycopersici*, *melonis*, *radicis-lycopersici*, *spinaciea* and *tulipae*), as well as from isolates of putatively nonpathogenic *F. oxysporum*. Sequences were chosen to represent the main clades of *F. oxysporum* (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000). Parsimony analysis was done on 701 characters, of which 30 were informative. Three equally parsimonious trees, rooted to an outgroup species, *Fusarium* spp. NRRL 22903 (O'Donnell *et al.*, 1998), were obtained. *F. oxysporum* f. sp. *ciceris* isolates formed a distinct grouping when compared with other *formae speciales* (Fig. 1). This distinct group, with 62% bootstrap support, was resolved within clade 1 *sensu* O'Donnell *et al.* (1998). The three isolates of *F. oxysporum* nonpathogenic to chickpea, Fo-90101, -90105 and -9169, showed identical *EF1α* gene sequences regardless of different geographical origins (Fo-9169 from Morocco and Fo-90101 and -90105 from Spain); these sequences were also identical to another putatively nonpathogenic *F. oxysporum* isolate (NRRL 25356) and to one isolate of f. sp. *melonis* (NRRL 26178). Sequences of these isolates were resolved within clade 2 (O'Donnell *et al.*, 1998) together with f. sp. *lini* and one sequence from f. sp. *cubense* (NRRL 25609).

Discussion

The primary objective of this study was to determine whether *F. oxysporum* f. sp. *ciceris* has a monophyletic evolutionary origin. Despite the diversity in geographical origin, symptom type and cultivar-specific pathogenicity, isolates representing six different races had identical sequences in introns of five genes. Sequences from an

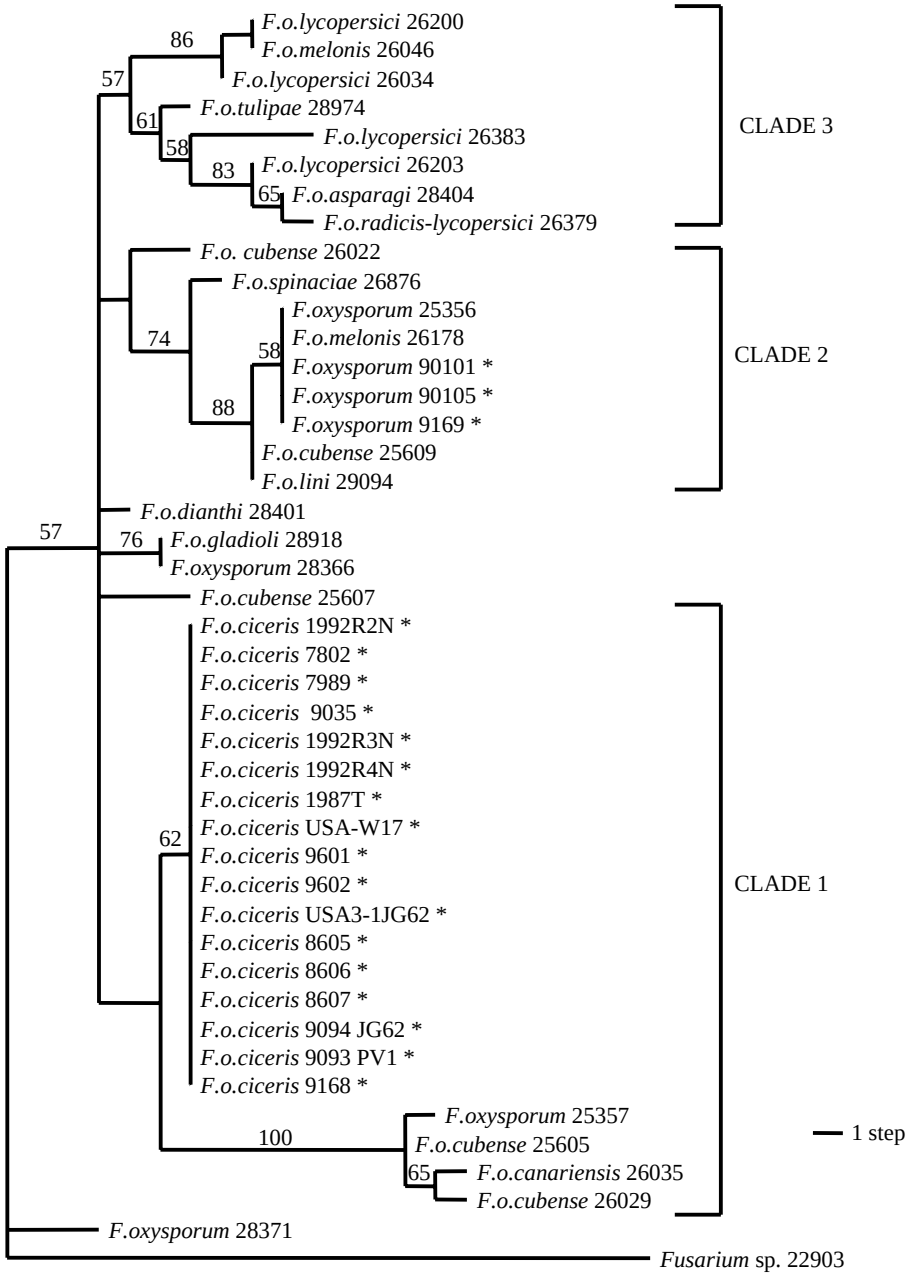

Figure 1 Genealogy of the translation elongation factor 1α (*EF1*α) gene demonstrating monophyly in *Fusarium oxysporum* f. sp. *ciceris*. The tree shown is one of three most-parsimonious trees (73 steps; consistency index, CI = 0·904; retention index, RI = 0·953; rescaled consistency index, RC = 0·862). This tree is based on new sequences from *Fusarium oxysporum* f. sp. *ciceris* and *Fusarium oxysporum* (denoted by * – GenBank accession numbers AF346503 and AF346504, respectively) in comparison to sequences from the *Fusarium oxysporum* complex available in GenBank. Reference sequences from GenBank were chosen to represent the same isolates used by Baayen *et al.* (2000) and O'Donnell *et al.* (1998); these sequences are identified by their NRRL (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA) isolate numbers as previously published. Clades 1, 2 and 3 were defined previously by O'Donnell *et al.* (1998). The tree was rooted with the sequence from *Fusarium* sp. isolate NRRL 22903 (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000). Numbers on branches represent bootstrap values > 50% based on 1000 replicates.

additional set of 11 isolates similarly showed no sequence polymorphism in the *EF1*α gene. When the sequence for this gene from 17 *F. oxysporum* f. sp. *ciceris* isolates was compared with those from three *F. oxysporum* isolates nonpathogenic to chickpeas and from 11 other *formae speciales*, it formed a distinct group (Fig. 1), demonstrating a monophyletic origin. Previous studies showed that isolates in all races of *F. oxysporum* f. sp. *ciceris* have the same mtDNA RFLP pattern (Pérez-Artés *et al.*, 1995), belong to a single VCG (Nogales-Moncada, 1997), carry

the same repetitive element (Kelly, 1996) and are highly similar in cluster analyses of RAPD data (Kelly *et al.*, 1994; Jiménez-Gasco *et al.*, 2001). Although these data would suggest monophyly in *F. oxysporum* f. sp. *ciceris*, a definitive test could only be done by analysis of gene genealogies in relation to related taxa.

Sequences of the *EF1*α gene from three *F. oxysporum* isolates from roots of healthy chickpeas, and nonpathogenic to chickpea, were identical to that of an isolate of *F. oxysporum* f. sp. *melonis*. However, another isolate of *F. oxysporum* f. sp. *melonis* had an *EF1*α gene sequence identical to that of an isolate of *F. oxysporum* f. sp. *lycopersici*. The possibility that the three *F. oxysporum* isolates nonpathogenic to chickpea might be pathogenic to other hosts, including melon, was not tested and therefore cannot be ruled out. The *EF1*α gene sequence has proved to be an excellent phylogenetic marker for resolving relationships within *F. oxysporum* (O'Donnell *et al.*, 1998). The current phylogenetic analysis of *EF1*α gene sequences from chickpea isolates and others obtained from GenBank confirms that *F. oxysporum* f. sp. *cubense* and *F. oxysporum* f. sp. *melonis* are polyphyletic (O'Donnell *et al.*, 1998). However, it must be noted that the isolates from which DNA sequences were obtained were not examined and the possibility that they were not correctly identified to *forma specialis* cannot be ruled out.

The simplest interpretation of monophyly and the lack of sequence variation in *F. oxysporum* f. sp. *ciceris* is that it derives from a small founder population – perhaps a single individual – that became pathogenic to *Cicer* spp. As a consequence, variation in symptom types and pathogenicity to different cultivars must have resulted from the accumulation of relatively recent genetic changes (Gordon & Martyn, 1997). These few genetic changes are not reflected in the sequences of the genes studied; however, they can be detected by associations with RAPD markers. Cluster analyses and molecular analyses of variance of RAPD data consistently differentiate yellowing and wilting pathotypes (Kelly *et al.*, 1994) and the various races of *F. oxysporum* f. sp. *ciceris* (Jiménez-Gasco *et al.*, 2001), indicating that RAPD markers, which sample large numbers of loci, are more suitable for such analyses.

Based on the monophyly of *F. oxysporum* f. sp. *ciceris* found in this study, it can be hypothesized that polymorphisms in both pathogenic phenotypes and molecular markers must have arisen after *F. oxysporum* f. sp. *ciceris* diverged from other taxa in this species complex, in particular, that variation in pathotypes and races on different chickpea types arose in geographically isolated subpopulations postdating this divergence. There are two germplasm types, 'kabuli' and 'desi', within *Cicer* spp. (Singh, 1987). 'Desi' chickpeas are grown mainly in the Indian subcontinent, whereas 'kabuli' chickpeas are found mainly in the Mediterranean region and California. Interestingly, the yellowing pathotype (including races 0 and 1B/C) has not been reported from the Indian subcontinent, but it is common in the Mediterranean region and California (Jiménez-Díaz *et al.*, 1993; Halila & Strange, 1996; R. M. Jiménez-Díaz, unpublished data). Race 0 is not pathogenic to chickpea cv. JG 62, the universal suscept of the wilting pathotype (including races 1A and 2–6), and is primarily pathogenic to 'kabuli' chickpeas (Jiménez-Díaz *et al.*, 1993). In contrast, resistance to *F. oxysporum* f. sp. *ciceris* occurs mainly within 'desi' germplasm (Haware *et al.*, 1980) and most 'desi' genotypes are resistant to race 0 (Jiménez-Díaz *et al.*, 1993). Furthermore, races 2, 3 and 4, reported only from India, are the most virulent of the eight reported races of the pathogen (Haware & Nene, 1982; Jiménez-Díaz *et al.*, 1993; Halila & Strange, 1996) and are genetically the most distant from the other races (Kelly *et al.*, 1994; Jiménez-Gasco *et al.*, 2001). This apparent correlation of pathogen pathotypes and races together with chickpea types could be explained if the pathogen consists of geographically isolated populations adapted to local chickpea germplasm, even though they all seem to have evolved originally from the same common ancestor. Clearly, however, monophyly of *F. oxysporum* f. sp. *ciceris* rules out the explanation that different subpopulations evolved from independent origins on the different host types.

The use of gene genealogies has made it possible to reveal the underlying evolution of *F. oxysporum* f. sp. *ciceris* as being derived from a small founder population or a single individual. Although it may be possible for new variants with higher fitness to arise and replace existing populations, i.e. a selective sweep, there is no evidence to suggest this has occurred in *F. oxysporum* f. sp. *ciceris*, unless new genotypes succeeded in replacing existing populations in all major chickpea-growing areas of the world. The uniformity of gene sequences across the entire geographic range is striking evidence for a single origin.

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