Stepwise Evolution of Races in *Fusarium oxysporum* f. sp. *ciceris* Inferred from Fingerprinting with Repetitive DNA Sequences

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ABSTRACT

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Plant pathogens often exhibit variation in virulence, the ability to cause disease on host plants with specific resistance, evident from the diversity of races observed within pathogen species. The evolution of races in asexual fungal pathogens has been hypothesized to occur in a stepwise fashion, in which mutations to virulence accumulate sequentially in clonal lineages, resulting in races capable of overcoming multiple host plant resistance genes or multiple resistant cultivars. In this study, we demonstrate a simple stepwise pattern of race evolution in *Fusarium oxysporum* f. sp. *ciceris*, the fungus that causes Fusarium wilt of chickpeas. The inferred intraspecific phylogeny of races in this fungus, based on DNA fingerprinting with repetitive sequences, shows that each of the eight races forms a monophyletic lineage. By mapping virulence to each differential cultivar (used for defining races) onto the inferred phylogeny, we show that virulence has been acquired in a simple stepwise pattern, with few parallel gains or losses. Such a clear pattern of stepwise evolution of races, to our knowledge, has not been demonstrated previously for other pathogens based on analyses of field populations. We speculate that in other systems the stepwise pattern is obscured by parallel gains or losses of virulence caused by higher mutation rates and selection by widespread deployment of resistant cultivars. Although chickpea cultivars resistant to Fusarium wilt are available, their deployment has not been extensive and the stepwise acquisition of virulence is still clearly evident.

Additional keywords: *Cicer arietinum*, pathotype evolution, transposable elements.

The origin of pathogenic variation has intrigued plant pathologists since the discovery of races in the early 20th century. Most recent work in this area has focused on the molecular mechanisms that allow pathogens to avoid recognition by plant defenses (9,14,43). From an evolutionary perspective, however, mutations and recombination among avirulence (*avr*) genes in sexually reproducing pathogens (3,37) have been postulated as the mechanisms responsible for variation in races. For asexual species lacking regular mechanisms of recombination, races capable of overcoming multiple resistance genes are thought to evolve by the successive accumulation of mutations. This type of stepwise mutation model for the evolution of races has been proposed for some pathogens (44), but has not been demonstrated adequately. The rice blast pathogen, *Magnaporthe grisea*, provides one of the few examples showing correlations between virulence (defined as the ability to cause disease on plants with race-specific resistance) and clonal lineages (29,30,48), but without clear evidence for stepwise evolution. For most fungal plant pathogens, including more diverse populations of *M. grisea*, this type of association is weak or not detectable (10,11,17,49), probably because of relatively high mutation rates for virulence and strong selection to overcome resistance genes deployed in host populations, resulting in parallel gains and losses that obscure any stepwise process. Therefore, the stepwise mutation model for the evolution of pathogenic races may be simpler to test in asexual species in which virulence evolves more slowly and has fewer parallel gains or losses.

Pathogenic variation of the putatively asexual plant-pathogenic fungus *Fusarium oxysporum* Schlechtend.:Fr. complex has been studied at two different levels. First, this species complex comprises plant-pathogenic taxa that are specialized to different host species (referred to as formae speciales or f. sp.). Some formae speciales have evolved once and are therefore monophyletic (1, 25), whereas others have evolved multiple and independent times and therefore are polyphyletic (1,33). At the second level of variation, the evolution of races within formae speciales has been studied in less detail. For example, multiple gene genealogies of *F. oxysporum* f. sp. *vasinfectum*, which mainly infects cotton, revealed that this forma specialis and at least one of the races were polyphyletic (41). Other formae speciales, however, have been reported to be monophyletic and the close genetic relatedness among races has led to the hypothesis that races were derived in a stepwise process rather than evolving independently (12). Although this is a reasonable hypothesis, the lack of phylogenetic

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains supplemental material not included in the print edition. The online supplement presents the raw binary data matrix from DNA fingerprints of *Fusarium oxysporum* f. sp. *ciceris* isolates generated with repetitive probes FocB10, FocO2, and FocP18 combined.

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resolution within monophyletic formae speciales makes it difficult to analyze the evolutionary pathways of pathogenic variation, and to our knowledge, has not yet been tested.

The forma specialis that causes Fusarium wilt of chickpeas (*Cicer arietinum* L.), *F. oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato, exhibits considerable pathogenic variation even though it is monophyletic (22). This disease, one of the most important limiting factors of chickpea production worldwide, has been the target of breeding for resistance (19). Resistant cultivars are one of the few means for managing Fusarium wilt of chickpeas, but their deployment has not been extensive because of undesirable agronomic characteristics. However, pathogenic variability in *F. oxysporum* f. sp. *ciceris* may limit the effectiveness of resistance (15,20). Two pathotypes have been distinguished based on the distinct yellowing or wilting symptoms they cause in chickpeas (47). In addition to major symptom types, eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6) can be identified by disease reactions on a set of differential chickpea cultivars (15,20). Races 0 and 1B/C induce yellowing symptoms (yellowing pathotype), whereas the other races cause wilting (wilting pathotype) (20,24). The eight races also have distinct geographic distributions. Races 2, 3, and 4 have only been reported in India (15), whereas races 0, 1B/C, 5, and 6 are found mainly in the Mediterranean region and the United States (California) (13,20). Unlike the other races, race 1A is more widespread and has been reported in India, California, and the Mediterranean region (20, 23). Despite this high degree of pathogenic variation, all isolates of *F. oxysporum* f. sp. *ciceris* studied to date—regardless of pathotype, race, and geographic origin—are members of the same vegetative compatibility group (VCG) (32) and are genetically identical based on mitochondrial DNA restriction fragment length polymorphisms (RFLPs) (35) and multilocus sequence genotypes (22). Race diversity in *F. oxysporum* f. sp. *ciceris* is consistent with Correll's model III, with different races within a forma specialis originating within the same VCG (6).

In contrast to the monomorphism for most genetic markers, random amplified polymorphic DNAs (RAPDs) are variable and correlate to pathotypes and races (23,24). Therefore, we hypothesize that *F. oxysporum* f. sp. *ciceris* may be an example in which races evolved from one another, possibly in a stepwise fashion. Although the association between RAPDs and races made it possible to develop diagnostic markers for races, they lack the resolution necessary for inferring the evolution of races (23,24). Our other attempt—using multiple gene genealogies—to address the evolution of races in this fungus failed because of a complete lack of polymorphism in the genes sequenced (22). Discerning the evolutionary pathways for pathogenic variation within this forma specialis, however, may be possible by using genetic markers with the appropriate level of genetic variation. Therefore, the aim of this study was to test whether pathotypes and races of *F. oxysporum* f. sp. *ciceris* evolved in a stepwise fashion. To achieve this aim, we inferred the evolution of lineages using DNA fingerprinting and mapped virulence onto the inferred phylogeny to determine if races acquired virulence in a stepwise process. We show in this report that virulence in *F. oxysporum* f. sp. *ciceris* has evolved in a relatively simple pattern, following a stepwise process with few parallel gains or losses.

MATERIALS AND METHODS

Fungal isolates. Thirty-six *F. oxysporum* f. sp. *ciceris* isolates, representative of the eight described races, and three *F. oxysporum* isolates from roots of healthy plants but nonpathogenic to chickpea were used in this study (Table 1). These isolates were used in previous studies of genetic variation (23,24) and were selected for this study to maximize geographic variation. Races of

TABLE 1. Isolates of *Fusarium oxysporum* f. sp. *ciceris* and *F. oxysporum* nonpathogenic to chickpea used in the DNA fingerprinting analysis with the repetitive DNA probes FocB10, FocO2, and FocP18, including origin and fingerprint similarity

Isolate reference[a]	*N*[b]	Geographic origin[c]	Mean similarity (SD)	Range of similarities
F. oxysporum f. sp. *ciceris*				
Race 0	12		0.636 (0.0958)	(0.475, 0.897)
Foc-7802, -7952, -8207, -82108, -82113, -9018 PV1, -9018 JG62, -9032, -91108, -91114		Spain		
Foc-9605, -T3		Tunisia		
Race 1B/C	3		0.749 (0.0460)	(0.696. 0.778)
Foc-USA 3-1JG62, -1987-W17		United States		
Foc-9602		Tunisia		
Race 1A	3		0.741 (0.0874)	(0.654, 0.829)
Foc-7989		India		
Foc-9168		Morocco		
Foc-8272		Spain		
Race 2	2		0.778	Not applicable[d]
Foc-8605, -1992 R2N		India		
Race 3	2		0.917	Not applicable[d]
Foc-8606, -1992 R3N		India		
Race 4	2		0.781	Not applicable[d]
Foc-8607, -1992 R4N		India		
Race 5	5		0.929 (0.0458)	(0.852, 1.0)
Foc-8012, -9035, -9094 JG62		Spain		
Foc-USA1-1 JG62, -USA W6-1		United States		
Race 6	7		0.879 (0.0639)	(0.774, 1.0)
Foc-9023, -9027 PV1		Spain		
Foc-9164, -9165, -9166, -9170		Morocco		
Foc-Tonini		United States		
F. oxysporum				
Nonpathogenic to chickpea	3		Not done	
Fo-8250, -9009, -90105		Spain		

[a] Race was determined by pathogenicity tests on chickpea differential lines (20,23,24).
[b] Sample size for each race.
[c] Isolates from Morocco, Spain, and the United States (California) were obtained from the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain; isolates from India were provided by M. P. Haware, ICRISAT, Hyderabad, India; and isolates from Tunisia were obtained from M. H. Halila, Institute Nationale de la Recherche Agronomique, Ariana, Tunisia.
[d] Only one similarity value can be estimated.

F. oxysporum f. sp. *ciceris* isolates were determined in previous studies by assessing virulence on differential host cultivars (20, 23,24) as described in Table 2. Isolates are stored in the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain as monoconidial cultures in sterile soil in glass tubes at 4°C. Methods for culturing and obtaining mycelia for DNA extraction were as described previously (21). Total genomic DNA was purified from lyophilized, ground mycelium using a small-scale method (36) with slight modifications (21).

Southern blots and hybridizations. For each isolate, 3 to 5 µg of total genomic DNA was digested with 20 to 30 units of *Eco*RI restriction endonuclease (Pharmacia LKB, Uppsala, Sweden) for 12 to 16 h at 37°C. Restriction fragments were separated by electrophoresis in 0.7% agarose gels in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 1 $V \cdot cm^{-1}$ overnight. The size-fractionated DNA fragments were then transferred to nylon membranes by a vacuum transfer system (VacuGene XL, VacuPump; Pharmacia LKB). Three repetitive DNA sequences from the genome of *F. oxysporum* f. sp. *ciceris*, namely FocB10, FocO2, and FocP18, were used as hybridization probes for generating DNA fingerprints. Sequences of FocO2 and FocP18 were previously reported to share similarities with fungal transposons (21); the sequence of FocB10 was obtained according to methods described previously (21) and is reported in this work. For probe preparation, inserts were removed from cloning vectors and labeled using DIG-11-dUTP (digoxigenin-3-O-methylcarbonyl-amino-caproyl-5-(3-aminoallyl)-uridine-5′-triphosphate) by random-primed labeling according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Hybridization conditions were as described by Sambrook et al. (39) at a high stringency temperature (68°C). Hybridizations were visualized by a chemiluminescent method using the DIG Luminescent Detection Kit according to manufacturer's instructions (Roche Diagnostics).

Data analyses. A binary data matrix was constructed in which DNA fingerprint fragments were scored as present ('1') or absent ('0'). Restriction fragments of the same electrophoretic mobility were considered identical in state. We calculated similarities among haplotypes using Jaccard's coefficient (42) in NTSYSpc2.0 (Exeter Software, Setauket, NY) and manually calculated mean similarities within races. The phylogeny of *F. oxysporum* f. sp. *ciceris* isolates was inferred from fingerprint haplotypes in PAUP* 4.0b4a (Sinauer Associates, Sunderland, MA) by neighbor-joining (38) and parsimony analyses. Parsimony analysis was performed using the heuristic search option with simple addition of the tree-bisection-reconnection, branch swapping algorithm, and the MULPARS option on. Support for each branch in the inferred trees was evaluated by 1,000 bootstrap replicates.

RESULTS

We generated DNA fingerprints by hybridizing Southern blots with labeled clones of FocB10, FocO2, and FocP18 as probes. A BLAST search of GenBank revealed that the translated amino acid sequence from FocB10 (GenBank Accession No. AY255836) has 86% similarity with the *Fot*1 transposable element in *F. oxysporum* f. sp. *melonis* (8). DNA fingerprints generated by all three probes were complex, with hybridization patterns showing clear differences between *F. oxysporum* f. sp. *ciceris* and *F. oxysporum* isolates nonpathogenic to chickpea, as well as among pathogenic races. Probe FocO2 hybridized to 20 to 30 restriction fragments of approximately 1.5 to 12 kb in the *F. oxysporum* f. sp. *ciceris* isolates, but to only 3 and 10 fragments in nonpathogenic isolates Fo-8250 and Fo-90105, respectively (Fig. 1). Similarly, probe FocB10 hybridized to 20 to 30 *Eco*RI fragments in pathogenic isolates compared with 9 and 15 fragments from nonpathogenic isolates Fo-8250 and Fo-90105, respectively. Probe FocP18 produced very intense hybridization patterns; up to 16 clearly resolved bands were easily scored in pathogenic isolates, although bands of 6 to 12 kb could not be resolved reliably (data not shown). Probe FocP18 hybridized to three and six fragments in nonpathogenic isolates Fo-8250 and Fo-90105, respectively. No hybridization was detectable from the nonpathogenic isolate Fo-9009 to any of the three fingerprinting probes used in this study (Fig. 1).

DNA fingerprint similarity within and between races. We scored a total of 88 fragments in the hybridization patterns of 36 *F. oxysporum* f. sp. *ciceris* and three nonpathogenic *F. oxysporum* isolates with the three combined probes. Fingerprint similarity varied considerably within each race (Table 1). Average fingerprint similarities within races are difficult to interpret because samples vary in size and extent of geographic distribution for each race. Nonetheless, with samples roughly comparable in size and geographic variation, the most extreme difference was between the average similarities within races 0 and 5, which were approximately 64 and 93%, respectively.

Inferred phylogeny of races based on DNA fingerprints. Neighbor-joining and parsimony analyses performed on fingerprint data from the combined data set from all probes in general gave similar results (Fig. 2). Pathogenic isolates grouped together in a clade with high bootstrap support and were clearly delineated from isolates nonpathogenic to chickpea. Within the pathogenic isolates, two well-supported clades clearly correlated with the yellowing and wilting pathotypes, and fingerprint lineages generally correlated to races (Fig. 2). The inferred neighbor-joining tree showed that each race corresponds to a unique lineage with moderate to high bootstrap support for most races (Fig. 2A). We obtained two most parsimonious trees (Fig. 2B), each with a rela-

TABLE 2. Disease reaction of differential chickpea lines to the inoculation with pathogenic races of *Fusarium oxysporum* f. sp. *ciceris*[a]

Differential chickpea line	Code[b]	Pathogenic race							
		0	1A	1B/C	2	3	4	5	6
12-071/10054	A	S	M	S	R	R	R	R	M
JG-62	B	R	S	S	S	S	S	S	S
C-104	C	M	M	R/M	S	S	S	S	M
JG-74	D	R	R	R	S	R	R	M	R
CPS-1	E	R	R	R	S	M	M	M	R
BG-212	F	R	R	R	S	M	M	R	R
WR-315	G	R	R	R	R	S	R	R	R
ICCV-2	H	R	R	R	S	S	S	S	M
ICCV-4	I	R	R	R	S	S	S	S	M
P-2245	J	S	S	S	S	S	S	S	S

[a] Disease evaluated on a 0 to 4 severity scale depending on the percentage of affected foliar tissue (0 = 0%, 1 = 1 to 33%, 2 = 24 to 66%, 3 = 67 to 100%, and 4 = dead plant) at 40 days after sowing in infested soil (15,20). Average disease reactions of <1 and >3 were considered resistant (R) and susceptible (S), respectively. Intermediate disease reactions were considered moderate susceptible (M) (15,20).

[b] Code was used to map each differential cultivar onto the neighbor-joining tree inferred from DNA fingerprints (Fig. 3).

tively low consistency index, CI = 0.416, indicating a considerable amount of homoplasy as found for fingerprinting markers in other fungi (4). The topologies of the parsimony trees, however, were generally similar to the neighbor-joining tree (Fig. 2) except that one isolate of race 1B/C (Foc-9602) was placed in the race 0 clade, races 1A and 6 did not form a distinct clade, and the resolution of races 3, 4, and 5 was not as distinct as in the neighbor-joining tree. Neither tree showed any association between fingerprint lineage and geographic origin, except for consistently grouping races 2, 3, and 4, which were reported only from India.

Stepwise evolution of races. Virulence to each differential cultivar (denoted as cultivars A through J), which collectively defines races (Table 2), was mapped onto the neighbor-joining tree (Fig. 3). Mapping of virulence was done manually to minimize the number of evolutionary events, especially parallel gains and losses of virulence. Although many pathways may be possible, we present two that we consider the simplest. First, considering only the gain, but not loss, of virulence, we inferred the pathway shown in Figure 3A. In this scenario, the common ancestor of all pathogenic races ("unknown 1" in Figure 3A) is only virulent to chickpea differential cultivar J and partially virulent to cultivar C. The first major branching within pathogenic races shows acquisition of virulence (either full or partial virulence, as indicated by the completely susceptible [S] or moderately susceptible [M] reactions of differential cultivars, respectively, described in Table 2) to cultivar A giving rise to the yellowing races, while virulence to cultivar B is associated with all wilting races. Thereafter, yellowing race 1B/C evolved from race 0 by gain of virulence to cultivar B. The common ancestor to the wilting races (unknown 2) is a race that has not been found in nature (virulence only to cultivars B and J and partial virulence to cultivar C). However, wilting races evolved in a stepwise manner. For example, race 1A evolved from unknown 2 by addition of partial virulence to cultivar A, and race 6 evolved from race 1A by addition of partial virulence to cultivars H and I. Similarly, races 2, 3, 4, and 5 evolved in a stepwise fashion. Races 4 and 5 evolved from the common ancestor of this clade (unknown 3), although this particular race is not currently known, and races 2 and 3 evolved from race 4. Note, however, that in addition to inferring the existence of three ancestral races that are not known to be extant, this evolutionary scenario requires a parallel gain of virulence to cultivar B, which evolved twice (in the root of the wilting isolates and again in race 1B/C). In two instances, we infer that full and partial virulence to the same cultivars evolved independently. Full virulence to cultivar A evolved in the yellowing isolates (races 0 and 1B/C), but partial virulence to cultivar A evolved independently in races 1A and 6. Similarly, full virulence to cultivars H and I evolved in the ancestor to races 2, 3, 4, and 5, while partial virulence to cultivars H and I evolved independently in race 6.

An alternative scenario for the evolution of virulence is shown in Figure 3B. In this scenario, we allow virulence to be gained or lost. The inferred common ancestor of all pathogenic isolates is race 1A. The yellowing race 1B/C evolved from wilting race 1A by a change from partial to complete virulence to cultivar A; subsequently, race 0 evolved from race 1B/C by the loss of virulence to cultivar B. Race 6 evolved from race 1A (as described previously) by the gain of partial virulence to cultivars H and I. The remaining wilting races appear to have their origin in a series of mutational events in one clade resulting in virulence to cultivars C, H, and I, partial virulence to cultivar E, and loss of virulence to cultivar A; as in the previous scenario, this hypothetical race (unknown 3, Fig. 3B) has not been found in nature. The only parallel evolution in this scenario is for full and partial virulence: full virulence evolved to cultivar D in race 2 and partial virulence to cultivar D in race 5, and as described previously, full and partial virulence evolved independently to cultivars H and I in unknown 3 (and subsequently in races 2, 3, 4, and 5) and race 6, respectively. The mapping of virulence onto the parsimony tree generally gave similar patterns but yielded a pattern a little more complicated than with the neighbor-joining tree (data not shown). Nonetheless, the overall pattern for both trees in this scenario, like the one described previously, is of a stepwise process in which one race evolves from another, usually by the gain of virulence, but also sometimes from loss.

DISCUSSION

In putatively asexual fungi such as *F. oxysporum* f. sp. *ciceris*, mutations, including to virulence, should accumulate sequentially over time (7,45). In this work, we confirmed findings of previous studies that *F. oxysporum* f. sp. *ciceris* has a clonal genetic structure (22–24), and we further demonstrate that pathogenic phenotypes—both pathotypes, as determined by an isolate's ability to cause a particular symptom type in the host plant, and races, as determined by the disease reaction on differential cultivars—correlate to inferred DNA fingerprint lineages. A similar correlation was suggested earlier from RAPD data, although with consider-

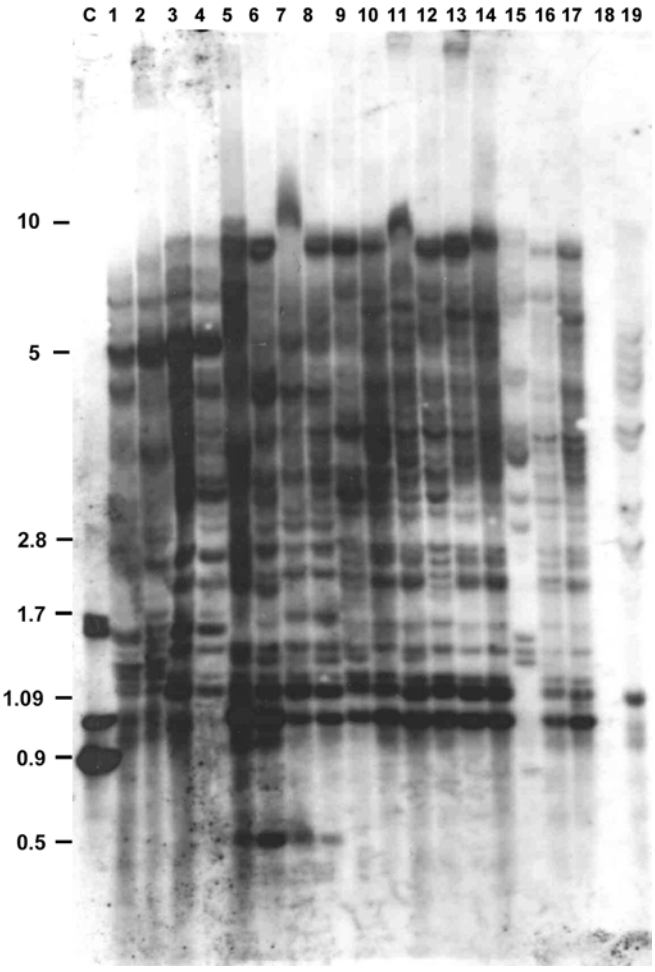

Fig. 1. Hybridization patterns of *Eco*RI-restricted genomic DNA of *Fusarium oxysporum* f. sp. *ciceris* and *F. oxysporum* isolates nonpathogenic to chickpea probed with the repetitive DNA sequence FocO2. Numbers on the left are approximate molecular size (kilobases). Lanes correspond to the following isolates: lanes 1 and 2, race 0 Foc-9018 JG62 and -7802; lanes 3 and 4, race 1B/C Foc-USA 3-1 JG62 and -1987 W17; lane 5, race 2 Foc-8605; lane 6, race 4 Foc-1992R4N; lanes 7 and 8, race 5 Foc-8012 and -9035; lanes 9, 12, and 16, race 1A Foc-7989, -9168, and -8272; lanes 10, 11, 13, 14, 16, and 17, race 6 Foc-9166, -9165, -9027 PV1, -Tonini, and -9170; and lanes 15, 18, and 19, *F. oxysporum* Fo-8250, -9009, and -90105, respectively. Lane C is a positive hybridization control containing DNA used to construct the probe.

ably less resolution (23,24). More importantly, we demonstrated that virulence to differential cultivars that define races could be mapped onto the inferred phylogeny of clonal lineages with minimal parallel gains or losses. This analysis suggests that races of *F. oxysporum* f. sp. *ciceris* evolved by the stepwise accumulation, and possibly the occasional loss, of virulence to differential cultivars. To our knowledge, this type of stepwise process has not been demonstrated previously for plant-pathogenic fungi.

We inferred two different scenarios for the evolution of races in *F. oxysporum* f. sp. *ciceris*. One is based entirely on the gain of virulence (Fig. 3A). However, this scenario requires at least three parallel gains of virulence and postulates the existence of three ancestral races that have never been observed. In contrast, allowing two losses of virulence (or partial virulence) in the other scenario made it possible to map virulence onto the phylogenetic tree with only one parallel event and only one hypothetical ancestral race (Fig. 3B).

Both of these scenarios merit further discussion in the context of our current knowledge of pathogenic variation in this fungus. In the first scenario, the yellowing races 0 and 1B/C (yellowing pathotype) and the wilting races 1A, 2, 3, 4, 5, and 6 evolved first from an ancestor virulent only to cultivar J and partially virulent to cultivar C (unknown 1). We cannot, however, infer from this scenario whether unknown 1 was a yellowing or wilting pathotype. However, from previous studies using RAPD markers, we hypothesized that the wilting pathotype was derived from the yellowing races (23,24). This hypothesis is supported by the following information. First, race 0 is virulent on the fewest differential cultivars of all pathogen races (20) and is the most widespread race in the Mediterranean region, although it has not been reported from the Indian subcontinent (13,15,19,20). Second, several lines of evidence indicate that *F. oxysporum* f. sp. *ciceris* has a monophyletic origin (22,32,35). Monophyletic formae speciales of *F. oxysporum* have been assumed to be derived from parasitic, nonpathogenic populations or single individuals that acquired pathogenicity on a particular host plant (6,28). Therefore, we would expect an ancestral race to be virulent on fewer resistant cultivars than the more recently derived races. Moreover, mutations would be expected to accumulate over time, resulting in greater diversity in older lineages. The relatively low average similarity of fingerprint haplotypes (higher diversity) within race 0 is consistent with the hypothesis of it being an old race. Although this diversity estimate is based on relatively few isolates in this study, a more extensive sampling of races with RAPD data confirms that race 0 is the most diverse race (23).

The second scenario inferred in this study for the evolution of races of *F. oxysporum* f. sp. *ciceris* (Fig. 3B) seems to be simpler than the first. It proposes that race 1A, a wilt-inducing race, is the common ancestor of all races, instead of a race that is not known to be extant, as proposed in the first scenario. As expected for an ancient lineage, race 1A is the most geographically widespread race of *F. oxysporum* f. sp. *ciceris*; it was first described in India (15) and was later found in the Mediterranean region and California (20). This makes race 1A the only race found in India that is also found in other chickpea production areas. Without more extensive sampling and analysis of race 1A isolates, however, it is not possible to make any further conclusions as to which scenario is more likely to reflect the actual evolution of races.

The inferred phylogeny of *F. oxysporum* f. sp. *ciceris* races provides some insight into long-distance migrations that have occurred for this pathogen. Previous findings that *F. oxysporum* f. sp. *ciceris* is monophyletic (22), with generally little genetic variation for neutral genetic markers (23,24), argues for pathogenicity on chickpeas having evolved only one time, and relatively recently. Although we cannot determine where it first arose, we can conclude that at least two migration events have occurred between the Mediterranean region and India, most likely as a consequence of transporting seeds (16). If *F. oxysporum* f. sp. *ciceris* evolved in the Mediterranean region, then race 1A and the common ancestor of races 2, 3, and 4 (either unknown 3 or race 4)

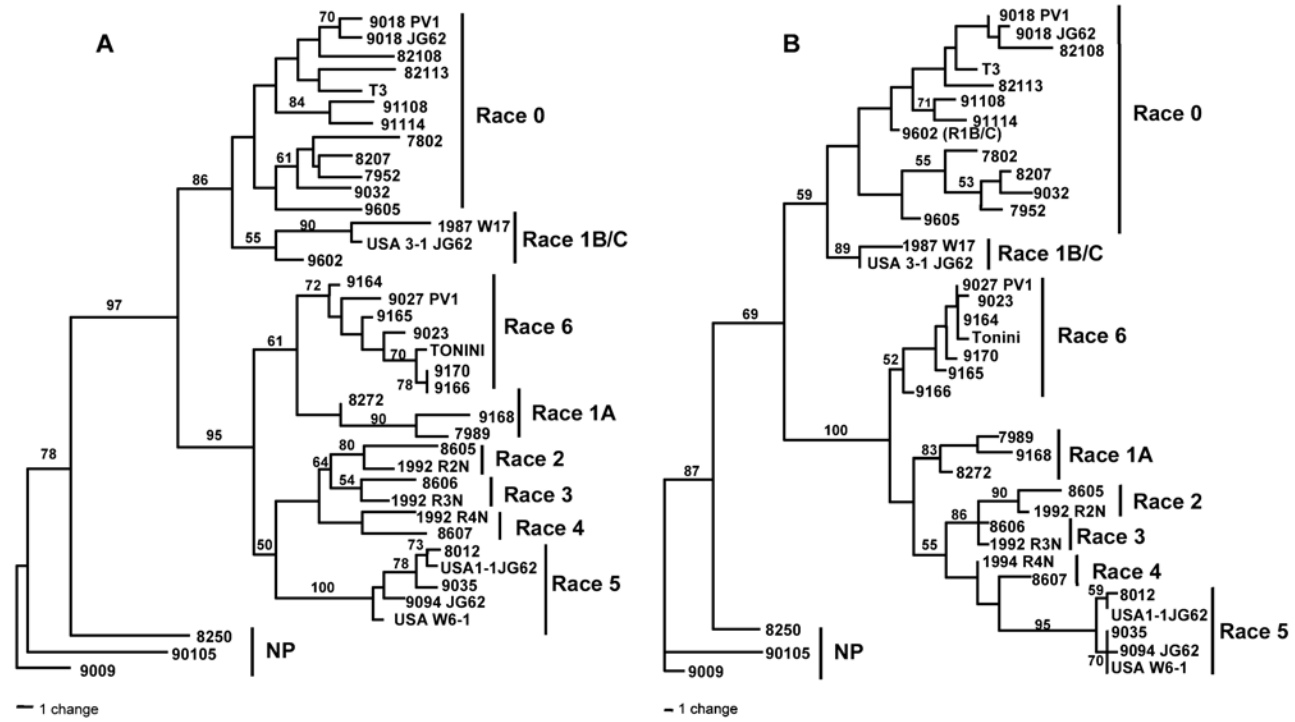

Fig. 2. Inferred phylogeny of races within *Fusarium oxysporum* f. sp. *ciceris* based on **A,** neighbor-joining and **B,** parsimony analyses of DNA fingerprint haplotypes. Fingerprints were generated by hybridization of genomic DNA to three repetitive probes: FocB10, FocO2, and FocP18. Isolates of *F. oxysporum* f. sp. *ciceris* represent all known pathogenic races from a wide geographic range (Table 1); three *F. oxysporum* isolates nonpathogenic (NP) to chickpea were included for comparison. Numbers on branches represent bootstrap values of >50% based on 1,000 replicates. **B,** The parsimony tree is one of two most parsimonious trees (202 steps; consistency index, CI = 0.416; retention index, RI = 0.786; and rescaled consistency index, RC = 0.327).

must have been introduced into India. Alternatively, if it evolved in India, then race 1A, which may be the progenitor of all other races (Fig. 3B), migrated in the opposite direction. In addition, a common ancestor of races 2, 3, 4, and 5 (unknown 3) also would have migrated from India to the Mediterranean region. In either case at least two different races have migrated in or out of India. We speculate that the Mediterranean region, or the Fertile Crescent, which is the center of diversity for *Cicer* sp. (29), is the most likely origin of this pathogen, sometime since domestication of chickpeas 8,000 to 9,000 years ago (29,40). Regardless of the origin, the similarities of race composition between the Mediterranean region and California indicate that repeated migration has occurred between these latter areas rather than the independent evolution of races in different regions. It may be possible to estimate the rate and direction of migration from genealogical data (18,46), although our earlier attempt to do this was unsuccessful because of genetic homogeneity (22).

The combined analysis of virulence and DNA fingerprinting has given us insight into some discrepancies in recent studies of *F. oxysporum* f. sp. *ciceris* (2,5). For example, a close relationship between races 1A and 4 was suggested from analyses of microsatellites (2) and RFLPs of the intergenic spacer region of ribosomal RNA genes (5). However, in those studies only one isolate of each of races 1A, 2, 3, and 4 from India were genotyped, and no races from other geographic areas were analyzed (2,5). Although only a few isolates of the races from India were available to us for this study, the combination of fingerprint and virulence data indicates that races 1A and 4 have distinct evolutionary histories.

Mapping of virulence onto a phylogeny of *F. oxysporum* f. sp. *ciceris* was possible because we used genetic markers that could resolve the lineage structure within this taxon (1). The fingerprint probes used in this study are repetitive sequences with similarity to known transposable elements in other fungi (8,27,34). Resolving the patterns of descent within *F. oxysporum* f. sp. *ciceris* could only be done with a fingerprinting marker because other markers did not adequately resolve differences among races (23, 24,35), including multiple gene sequences that revealed no variation among pathogenic isolates (22). Fingerprinting with transposable elements has its limitations, however, and needs to be interpreted with caution. Analyses that rely entirely on fingerprint data may incorrectly infer hierarchical patterns of descent because identical fingerprints can be derived by different patterns of descent, e.g., parallel gains or losses (4). In our analyses, low to moderate bootstrap support for some lineages and a low consistency index in parsimony analysis are indications of the uncertainty associated with inferences from fingerprint data on the patterns of descent. Nonetheless, the high correlation between races and fingerprint lineages gives us some degree of confidence in the inferred phylogeny even though the detailed pathways may not be completely accurate.

The stepwise accumulation of virulence inferred in *F. oxysporum* f. sp. *ciceris* included one relatively large evolutionary jump giving rise to races 2, 3, 4, and 5 (especially in the scenario in Figure 3B) in the simultaneous acquisition of virulence to cultivars C, H, and I, partial virulence to cultivar E, and loss of partial virulence to cultivar A. Although this may seem like a massive mutational event, the underlying genetics of host resistance or virulence are not known in this system, and correlations among these virulence phenotypes could conceivably be caused by relatively few genetic changes with pleiotropic effects. For example, the coincident evolution of full and partial virulence to cultivars H and I implies that cultivars H and I (cvs. ICCV2 and ICCV4, respectively, in Table 2) may have the same resistance factors. Future genetic studies of resistance in chickpeas might be directed toward testing this hypothesis.

It is unlikely that the stepwise evolution of races in *F. oxysporum* f. sp. *ciceris* has resulted from selection by specific resistances in host populations. Some races are widely distributed geographically, even where resistant cultivars have not been deployed. For example, resistant cultivars generally have not been used in the Mediterranean region, but this region has a high diversity of races (13,20,21,23,24). Conversely, widespread use of race 1A-resistant cultivars H and I (cvs. ICCV2 and ICCV4 in Table 2) in India has not yet lead to reports on development of race 6, which specifically overcomes that resistance and derives from race 1A (Table 2) (20); rather, races 2, 3, and 4, which are virulent to cultivars H and I, were reported in India long before (15) release of these cultivars (26). Thus, unlike other pathosystems (e.g., rice blast), there may have been little or no selection for resistance-breaking races of *F. oxysporum* f. sp. *ciceris*, minimizing the probabilities of obtaining parallel changes in virulence, which

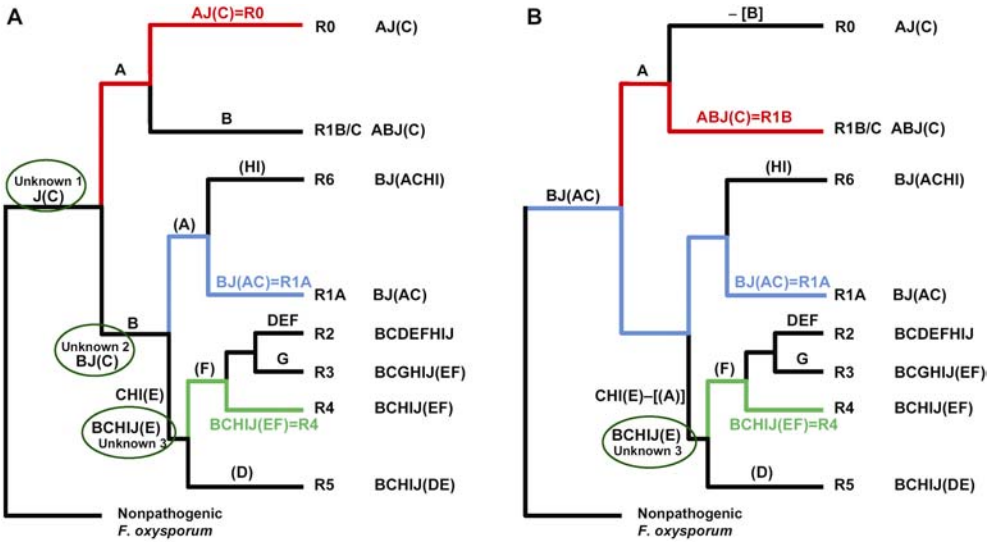

Fig. 3. Mapping of virulence acquisition by races of *Fusarium oxysporum* f. sp. *ciceris* onto the inferred neighbor-joining tree in Figure 2A. Each race is defined by virulence to differential cultivars (Table 2). Letters indicating the cultivars to which each race is virulent (coded A through J) are shown next to each race label (R0, R1B/C, etc.); letters in parentheses indicate partial virulence. Acquisition of virulence to each cultivar was mapped onto the tree manually to minimize the number of parallel events. Colored branches indicate the races inferred to be ancestral during the evolution of other races. Races that are inferred but have not been found in nature are labeled unknown. **A,** Scenario that allows only the gain, not loss of virulence. **B,** Scenario that allows both gains and losses of virulence. Loss of partial virulence to cultivar A is indicated by –[(A)]; loss of virulence to cultivar B is indicated by –[B].

are frequently observed in other systems. Limited selection by resistance deployment might help to explain why we can still detect a relatively simple relationship between clonal lineages and virulence acquisition, with few parallel events.

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LITERATURE CITED

1. Baayen, R. P., O'Donnell, K., Bonants, P. J. M., Cigelnik, E., Kroon, L. P. N. M., Roebroeck, E. J. A., and Waalwijk, C. 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. Phytopathology 90:891-900.
2. Barve, M. P., Haware, M. P., Sainani, M. N., Ranjekar, P. K., and Gupta, V. S. 2001. Potential of microsatellites to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India. Theor. Appl. Genet. 102:138-147.
3. Brown, J. K. M. 1999. The evolution of sex and recombination in fungi. Pages 73-95 in: Structure and Dynamics of Fungal Populations. J. J. Worrall, ed. Kluwer Academic, Dordrecht, The Netherlands.
4. Carbone, I., Anderson, J. B., and Kohn, L. M. 1999. Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. Evolution 53:11-21.
5. Chakrabarti, A., Mukherjee, P. K., Sherkhane, P. D., Bhagwat, A. S., and Murthy, N. B. K. 2001. A simple and rapid molecular method for distinguishing between races of *Fusarium oxysporum* f. sp. *ciceris* from India. Curr. Sci. 80:571-575.
6. Correll, J. C. 1991. The relationship between formae speciales, races, and vegetative compatibility groups in *Fusarium oxysporum*. Phytopathology 81:1061-1064.
7. Crow, J. F. 1988. The importance of recombination. Pages 56-73 in: The Evolution of Sex: An Examination of Current Ideas. R. E. Michod and B. R. Leven, eds. Sinauer Associates, Sunderland, MA.
8. Daboussi, M. J., Langin, T., and Brygoo, Y. 1992. *Fot1*, a new family of fungal transposable elements. Mol. Gen. Genet. 232:12-16.
9. De Witt, P. J. G. M. 1995. Fungal avirulence genes and plant resistance genes: Unraveling the molecular basis of gene-for-gene interactions. Adv. Bot. Res. 21:148-185.
10. Goodwin, S. B., Sujkowski, S. L., and Fry, W. E. 1995. Rapid evolution of pathogenicity within clonal lineages of the potato late blight disease fungus. Phytopathology 85:669-676.
11. Goodwin, S. B., Webster, R. K., and Allard, R. W. 1994. Evidence for mutation and migration as sources of genetic variation in populations of *Rhynchosporium secalis*. Phytopathology 84:1047-1053.
12. Gordon, T. R., and Martyn, R. D. 1997. The evolutionary biology of *Fusarium oxysporum*. Annu. Rev. Phytopathol. 35:111-128.
13. Halila, H. M., and Strange, R. N. 1996. Identification of the causal agent of wilt of chickpea in Tunisia as *Fusarium oxysporum* f. sp. *ciceris* race 0. Phytopathol. Mediterr. 35:67-74.
14. Hammond-Kosack, K., and Jones, J. 2000. Responses to plant pathogens. Pages 1102-1156 in: Biochemistry and Molecular Biology of Plants. B. Buchanan, W. Gruissem, and R. Jones, eds. American Society of Plant Physiologists, Rockville, MD.
15. Haware, M. P., and Nene, Y. L. 1982. Races of *Fusarium oxysporum* f. sp. *ciceris*. Plant Dis. 66:809-810.
16. Haware, M. P., Nene, Y. L., and Rajeshwary, R. 1978. Eradication of *Fusarium oxysporum* f. sp. *ciceri* transmitted in chickpea seed. Phytopathology 68:1364-1367.
17. Hovmøller, M. S., Justesen, A. F., and Brown, J. K. M. 2002. Clonality and long-distance migration of *Puccinia striiformis* f. sp *tritici* in northwest Europe. Plant Pathol. 51:24-32.
18. Hudson, R. R., Slatkin, M., and Maddison, W. P. 1992. Estimation of levels of gene flow from DNA sequence data. Genetics 132:583-589.
19. Jalali, B. L., and Chand, H. 1992. Chickpea wilt. Pages 420-444 in: Plant Diseases of International Importance. Vol. 1. Diseases of Cereals and Pulses. U. S. Singh, A. N. Mukhopadhayay, J. Kumar, and H. S. Chaube, eds. Prentice Hall, Englewood Cliffs, NJ.
20. Jiménez-Díaz, R. M., Alcalá-Jiménez, A. R., Hervás, A., and Trapero-Casas, J. L. 1993. Pathogenic variability and host resistance in the *Fusarium oxysporum* f. sp. *ciceris*/*Cicer arietinum* pathosystem. Pages 87-94 in: Proc. 3rd Eur. Semin. Fusarium Mycotoxins, Taxonomy, Pathogenicity and Host Resistance. Plant Breeding and Acclimatization Institute (Hod. Ròsl. Aklim. Nasienn.), Radzikóv, Poland.
21. Jiménez-Gasco, M. M., and Jiménez-Díaz, R. M. 2003. Development of a specific polymerase chain reaction-based assay for the identification of *Fusarium oxysporum* f. sp. *ciceris* and its pathogenic races 0, 1A, 5, and 6. Phytopathology 93:200-209.
22. Jiménez-Gasco, M. M., Milgroom, M. G., and Jiménez-Díaz, R. M. 2002. Gene genealogies support *Fusarium oxysporum* f. sp. *ciceris* as a monophyletic group. Plant Pathol. 51:72-77.
23. Jiménez-Gasco, M. M., Pérez-Artés, E., and Jiménez-Díaz, R. M. 2001. Identification of pathogenic races 0, 1B/C, 5, and 6 of *Fusarium oxysporum* f. sp. *ciceris* with random amplified polymorphic DNA (RAPD). Eur. J. Plant Pathol. 107:237-248.
24. Kelly, A., Alcalá-Jiménez, A. R., Bainbridge, B. W., Heale, J. B., Pérez-Artés, E., and Jiménez-Díaz, R. M. 1994. Use of genetic fingerprinting and random amplified polymorphic DNA to characterize pathotypes of *Fusarium oxysporum* f. sp. *ciceris* infecting chickpea. Phytopathology 84:1293-1298.
25. Kistler, H. C. 2001. Evolution of host specificity in *Fusarium oxysporum*. Pages 70-82 in: *Fusarium.* Paul E. Nelson Memorial Symposium. B. A. Summerell, J. F. Leslie, D. Backhouse, W. L. Bryden, and L. W. Burgess, eds. The American Phytopathological Society, St. Paul, MN.
26. Kumar, J., Haware, M. P., and Smithson, J. B. 1985. Registration of four short duration Fusarium wilt-resistant kabuli (garbanzo) chickpea germplasm. Crop Sci. 25:576-577.
27. Langin, T., Capi, P., and Daboussi, M. J. 1995. The transposable element *impala*, a fungal member of the *Tc-1/mariner* superfamily. Mol. Gen. Genet. 246:19-28.
28. Leslie, J. F. 1993. Vegetative compatibility in fungi. Annu. Rev. Phytopathol. 31:127-151.
29. Lev-Yadun, S., Gopher, A., and Abbo, S. 2000. The cradle of agriculture. Science 288:1602-1603.
30. Levy, M., Correa-Victoria, F. J., Zeigler, R. S., Xu, S., and Hamer, J. E. 1993. Genetic diversity of the rice blast fungus in a disease nursery in Colombia. Phytopathology 83:1427-1433.
31. Levy, M., Romao, J., Marchetti, M. A., and Hamer, J. E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95-102.
32. Nogales-Moncada, A. M. 1997. Compatibilidad Vegetativa en *Fusarium oxysporum* f. sp. *ciceris* y *Fusarium oxysporum* f. sp. *melonis*, Agentes, Respectivamente de las Fusariosis Vasculares del Garbanzo y del Melón. Ph.D. thesis. University of Córdoba, Spain.
33. O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. USA 95:2044-2049.
34. Okuda, M., Ikeda, K., Namiki, K., and Tsuge, T. 1998. *Tfo1*: An *Ac*-like transposon from the plant pathogenic fungus *Fusarium oxysporum*. Mol. Gen. Genet. 258:599-607.
35. Pérez-Artés, E., Roncero, M. I. G., and Jiménez-Díaz, R. M. 1995. Restriction fragment length polymorphism analysis of the mitochondrial DNA of *Fusarium oxysporum* f. sp. *ciceris*. J. Phytopathol. 143:105-109.
36. Raeder, U., and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1:17-20.
37. Roelfs, A. P., and Groth, J. V. 1980. A comparison of virulence phenotypes in wheat stem rust populations reproducing sexually and asexually. Phytopathology 70:855-862.
38. Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.
39. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
40. Singh, K. B. 1997. Chickpea (*Cicer arietinum* L.). Field Crop Res. 53:161-170.
41. Skovgaard, K., Nirenberg, H. I., O'Donnell, K., and Rosendahl, S. 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. Phytopathology 91:1231-1237.
42. Sneath, P., and Sokal, R. 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification. W. H. Freeman & Co., San Francisco.
43. Staskawicz, B. J., Mudgett, M. B., Dangl, J. L., and Galan, J. E. 2001. Common and contrasting themes of plant and animal diseases. Science 292:2285-2289.
44. Steele, K. A., Humphreys, E., Wellings, C. R., and Dickinson, M. J. 2001. Support for a stepwise mutation model for pathogen evolution in Australasian *Puccinia striiformis* f. sp. *tritici* by use of molecular markers. Plant Pathol. 50:174-180.
45. Taylor, J. W., Jacobson, D. J., and Fisher, M. C. 1999. The evolution of

asexual fungi: Reproduction, speciation, and classification. Annu. Rev. Phytopathol. 37:197-246.
46. Templeton, A. R. 1998. Nested clade analyses of phylogeographic data: Testing hypotheses about gene flow and population history. Mol. Ecol. 7:381-397.
47. Trapero-Casas, A., and Jiménez-Díaz, R. M. 1985. Fungal wilt and root rot diseases of chickpea in southern Spain. Phytopathology 75:1146-1151.
48. Xia, J. Q., Correll, J. C., Lee, F. N., Marchetti, M. A., and Rhoads, D. D. 1993. DNA fingerprinting to examine microgeographic variation in the *Magnaporthe grisea* (*Pyricularia grisea*) population in two rice fields in Arkansas. Phytopathology 83:1029-1035.
49. Zeigler, R. S., Cuoc, L. X., Scott, R. P., Bernardo, M. A., Chen, D. H., Valent, B., and Nelson, R. J. 1995. The relationship between lineage and virulence in *Pyricularia grisea* in the Philippines. Phytopathology 85:443-451.