High Genetic Diversity Among Strains of *Fusarium oxysporum* f. sp. *vasinfectum* from Cotton in Ivory Coast

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ABSTRACT

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Seventeen isolates of *Fusarium oxysporum* f. sp. *vasinfectum* from the Ivory Coast were characterized using vegetative compatibility group (VCG), restriction fragment length polymorphism of the ribosomal intergenic spacer region (IGS), and mating type (MAT) idiomorph, and compared with a worldwide collection of the pathogen containing all available reference strains. Some of the isolates were identical to known reference strains for all three traits, whereas others had previously unknown varieties of IGS and (possibly) VCG. One or the other MAT idiomorph was present in each of the new isolates and the reference strains. The new isolates and reference strains were grouped based upon the three traits. Strains from the Ivory Coast were found in 7 of 11 groups detected, suggesting multiple sources for Fusarium wilt in the country. Despite the presence of both MAT idiomorphs among isolates, no evidence for recombination was found.

The cultivation of cotton has a long and venerable history in Africa traceable to the earliest written records in Egypt. In the millennia since, cultivation of cotton has expanded to many parts of the continent including Ivory Coast, where it has become one of the most important crops grown for export. Tracheomycosis caused by *Fusarium oxysporum*, well known in many cotton growing areas of Africa, Asia, and North America, is relatively recent in Ivory Coast. The disease was first noted in much of Africa in 1933, but was not reported in the Ivory Coast until 1983 or 1984 (16,22). It is now known throughout the country, and is one of the major pests of the crop.

The morphospecies *F. oxysporum* is a species complex with an unknown sexual stage; however, it is nested phylogenetically within the *Gibberella* clade (25,27,32). Pathogenic forms of *F. oxysporum* have been further divided into formae speciales. These are taxa of convenience for the organism based on their putative specificity to the host plant. The cotton vascular wilt pathogen is referred to as forma specialis *vasinfectum.* This forma specialis has been further subdivided into pathotypes or races, defined by differential pathogenicity to various cultivars of cotton. Within the forma specialis, the races have been shown to be monophyletic, but the forma specialis is polyphyletic as are some other formae speciales of *F. oxysporum* (2,6,19,20,28,31). At least 12 vegetative compatibility groups (VCGs) have been identified within *F. oxysporum* f. sp. *vasinfectum* (15,18). Earlier studies with eight isolates from Ivory Coast found three VCGs in the country and a low level of genetic variability among them, and identified all isolates as belonging to VCGs and restriction fragment length polymorphism (RFLP) types known from outside the country (15). Our objective here was to characterize new isolates of *F. oxysporum* f. sp. *vasinfectum* taken from regions of the Ivory Coast that had not previously been sampled. We were interested in determining whether they represented a limited number of genotypes or whether they represented new genotypes defining emergent strains. This leads us to two hypotheses. The first is that the new strains are clonally related to other known strains of *F. oxysporum* f. sp. *vasinfectum* and will be identical with these known strains for any arbitrary combination of molecular and/or genetic markers. The second hypothesis is that there are newly emergent strains that have arisen through recombination and/or mutation within a population (not necessarily the local population) and that they will not be identical for these markers and may show signs of recombination among genetic loci. These hypotheses are not mutually exclusive, as a mixture of "old" clonally derived strains and "new" strains derived from recombination and/or mutation is also possible. In any case, the use of molecular techniques to identify these strains coupled with more classical phenotypic methods such as vegetative compatibility analysis should give one the ability to sample a combination of loci sufficient for drawing of comparisons.

While no sexual stage of *F. oxysporum* has yet been described, molecular techniques have been used to show the presence of the two mating type (MAT) idiomorphs in *F. oxysporum* f. sp. *lycopersici* and other formae speciales (4). We used the MAT idiomorphs as a molecular marker for identification of these isolates, with the expectation that the strains would vary for this character, having either one of the MAT idiomorphs, or both (putatively homothallic), or neither. As a means of further identification of these isolates, we analyzed the ribosomal DNA intergenic spacer region (IGS) of these strains. This region is well known to be polymorphic within species, and even more so when viewed between species (2,3,13,17,23,29,30). Thus, strains that are highly similar or identical in sequence in this region are presumed to have close affinities. As an additional examination of the hypothesis of sexual origin, we tried to determine if strains with the same variants of this highly polymorphic locus also had

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both MAT idiomorphs by employing the above mentioned molecular techniques to detect MAT genes.

MATERIALS AND METHODS

Fungal isolates. Seventeen new pathogenic isolates (Fov1 to Fov17; Table 1) were collected from four different regions in Ivory Coast in 1994 to 1996: Tiéningboué (seven isolates), Béoumi (four isolates), Bouaké (four isolates), and Kounahiri (two isolates). Two isolates (Fov6 and Fov7) were taken directly from soil collected in cotton fields that had contained diseased plants using the procedure described by Edel et al. (12). Fifteen isolates were collected from diseased plants that were diagnosed by the presence of symptoms of wilt and brown discolored vessels in their stems. The plants were cut into small pieces, and the pieces were surface-sterilized by alternate washing in a 10 to 15% solution of sodium hypochlorite and sterile distilled water. The pieces were placed on malt agar supplemented with 250 mg of citric acid per liter and incubated at 25°C for 3 or 4 days in the dark. Colonies growing out from the fragments were identified as *F. oxysporum* by cultural characteristics and microscopic morphology following the practice of Nelson et al. (26) and Burgess et al. (7). Strains were isolated from single spores. Pathogenicity of the 17 new isolates was verified by inoculation onto roots of susceptible cultivars of cotton (5,16). Seedlings of cotton (*Gossypium hirsutum*) cvs. GL7 and ISA205K were grown in loose soil for 2 weeks. *Fusarium* isolates were grown on potato dextrose agar (PDA) (Sigma Chemical, Saint Quentin Fallavier, France), and the conidia were harvested by scraping the surface of a colony with a bent glass rod using distilled water and aspirating the resultant spore suspension. The suspensions were diluted to 10^6 conidia per ml. Seedlings were gently removed from their containers and rinsed in sterile water to free the roots. The roots of eight plants were dipped into each conidial suspension for 15 min and then transplanted to 500-ml pots in sterile soil. Plants dipped in sterile water were employed as negative controls. Two

TABLE 1. Isolates of *Fusarium oxysporum* f. sp. *vasinfectum* analyzed in this study

Group	Isolate	Race	IGS type[a]	MAT[b]	VCG[c]	Origin	Source (reference)[d]
1	Fov1		14	1	0112	Ivory Coast (Bouaké, 1994)	This study, CBS 116611
	Fov6		14	1	0112	Ivory Coast (Bouaké, 1995)	This study, CBS 116616
	Fov21	2	14	1	0112	USA	IMI 141148 (W. O'Neill)
	Fov23	1	14	1	0112	Tanzania	IMI 292248 (W. O'Neill)
	Fov26	a	14	1	0112	Tanzania	11 (15)
	Fov45	2	14	1	0112	USA	NRRL 25424, BBA 65635 (31)
	Fov46	2	14	1	0112	USA	NRRL 25425, BBA 65636 (31)
	Fov36	2	14	1	0112	USA	ATCC 16611, BBA 66844 (15,19,31)
2	Fov20	1	14	1	0111	USA	IMI 141146 (W. O'Neill)
	Fov35	1	14	1	0111	USA	ATCC 16421, BBA 66843 (15,19,31)
1 or 2	Fov2		14	1	011-HSI	Ivory Coast (Bouaké, 1995)	This study, CBS 116612
3	Fov11		14	2	1	Ivory Coast (Tiéningboué, 1996)	This study, CBS 116867
	Fov13		14	2	1	Ivory Coast (Tiéningboué, 1996)	This study, CBS 116622
	Fov17		14	2	1	Ivory Coast (Tiéningboué, 1996)	This study, CBS 116626
	Fov44	2	14	2	1	Egypt	NRRL 25421, BBA 64495 (31)
	Fov41	1	14	2	1	USA	NRRL 25422, BBA 65634 (31)
	Fov12		14	2	011-HSI	Ivory Coast (Tiéningboué, 1996)	This study, CBS 116621
	Fov22		14	2	011-HSI	Tanzania	IMI 195176 (W. O'Neill)
	Fov7		14	2	011-HSI	Ivory Coast (Tiéningboué, 1995)	This study, CBS 116617
4	Fov19	4	3	1	0114	USA	IMI 141112 (W. O'Neill)
	Fov29	4	3	1	0114	India	ATCC 16613, BBA 66846 (15,19)
	Fov30	4	3	1	0114	China	CH1 (15)
	Fov34	4	3	1	0114	India	NRRL 25434, BBA 69519 (31)
	Fov47	4	3	1	0114	India	NRRL 25436, BBA 69521 (31)
	Fov48	4	3	1	0114	India	NRRL 25435, BBA 69520 (31)
5	Fov14		65	2	01110	Ivory Coast (Béoumi, 1996)	This study, CBS 116623
	Fov32	a	65	2	01110	Ivory Coast	CIAN (15)
6	Fov3		65	2	0115	Ivory Coast (Bouaké, 1995)	This study, CBS 116613
	Fov31	a	65	2	0115	Ivory Coast	CYSA (15)
5 or 6	Fov4		65	2	011-HSI	Ivory Coast (Tiéningboué, 1994)	This study, CBS 116614
7	Fov15		66	1	0116	Ivory Coast (Béoumi, 1996)	This study, CBS 116624
	Fov39	6	66	1	0116	Brazil	ATCC 36198, BBA 66847 (19,31)
	Fov8		66	1	0116	Ivory Coast (Kounahiri, 1996)	This study, CBS 116618
8	Fov10		66	1	2	Ivory Coast (Béoumi, 1996)	This study, CBS 116620
	Fov16		66	1	2	Ivory Coast (Kounahiri, 1996)	This study, CBS 116625
7 or 8	Fov25	6	66	1	011-HSI	Brazil	IMI 338126 (W. O'Neill)
7 or 8	Fov5		66	1	011-HSI	Ivory Coast (Tiéningboué, 1995)	This study, CBS 116615
9	Fov24	5	67	1	0113	Sudan	IMI 325576 (W. O'Neill)
	Fov28	3	67	1	0113	Israel	Mh3 (15)
	Fov33	3	67	1	0113	Egypt	NRRL 31667, BBA 62375 (31)
	Fov37	3	67	1	0113	Egypt	ATCC 16612, BBA 66845 (19,31)
	Fov40	5	67	1	0113	Sudan	NRRL 25432, BBA 65654 (31)
	Fov42	5	67	1	0113	Sudan	NRRL 25431, BBA 65650 (31)
	Fov43	3	67	1	011-HSI	Egypt	NRRL 25429, BBA 67521 (31)
10	Fov9		2	1	3	Ivory Coast (Béoumi, 1996)	This study, CBS 116619
11	Fov18	a	21	2	0117	Benin	W. O'Neill

[a] The intergenic spacer (IGS) types are defined in Table 3.
[b] Mating type idiomorph.
[c] VCG indicates vegetative compatibility group and HSI indicates heterokaryon self-incompatibility.
[d] ATCC = American Type Culture Collection, Manassas, VA; BBA = Biologische Bundesanstalt für Land-und Forstwirtschaft, Berlin, Germany; CBS = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; IMI = International Mycological Institute, Surrey, UK; NRRL = National Center for Agricultural Utilization Research, Peoria, IL.

weeks after inoculation, plants were observed for symptoms of wilt and browning of the vessels. Presence of *Fusarium* sp. was confirmed by transfer of browned stem sections onto PDA and malt agar and the recovery of fungal colonies identified as *F. oxysporum*.

Finally, the collection of 46 strains of *F. oxysporum* f. sp. *vasinfectum* analyzed included the 17 new isolates (Fov1 to Fov17) from Ivory Coast collected in this study and 29 reference strains: 2 strains from Ivory Coast, 11 strains from other countries in Africa, and 16 strains from other continents (Table 1). The reference strains are representative of all known VCGs within the forma specialis *vasinfectum*, with the exception of VCG 0118, VCG 0119, VCG 01111, and VCG 01112, for which the corresponding strains were not found in any collection.

Molecular characterization. DNA was extracted from the isolates of *F. oxysporum* by a rapid minipreparation procedure (13). All the isolates were characterized by RFLP analysis of the ribosomal IGS as previously described (11,12). A fragment of the IGS was amplified by polymerase chain reaction (PCR) with oligonucleotide primers PNFo (5′-CCCGCCTGGCTGCGTCCG-ACTC-3′) and PN22 (5′-CAAGCATATGACTACTGGC-3′) and digested with seven restriction enzymes: *Alu*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Scr*FI, and *Xho*I. Each isolate was assigned to an IGS type defined by the combination of the restriction patterns obtained with the seven enzymes (12,13,23,30).

MAT idiomorph for each strain was determined by adapting the PCR methods of Arie et al. (4). Primers were designed using Primer 3 (Center for Genome Research, Cambridge, MA) from the published sequences of the MAT genes from *F. oxysporum* (Accession nos. AB011378 and AB011379). The primers for the MAT 1 idiomorph were generated using the highly conserved alpha-box sequence, and those for the MAT 2 were designed from the similarly conserved HMG box of the MAT 2 gene. The primers used were as follows: for the MAT 1 idiomorph, MAT 1L (5′-TGGCAGCTAGTTTCACA-3′) and MAT 1R (5′-GGCAAA-AAGGAAGTTGC-3′); and for the MAT 2 idiomorph, MAT 2L (5′-ACCGTAAGGAACGTCACCAG-3′) and MAT 2R (5′-CTT-TTTCTGATCCGCCATCT-3′). A 205-bp fragment is predicted from the use of primers MAT 1L and MAT 1R and a 190-bp fragment is predicted from the use of primers MAT 2L and MAT 2R. The specificity of the primers for the respective MATs was confirmed by the use of reference strains SUF 959 (MAT 2) and 88061a-1 (MAT 1) (4). PCR amplifications were performed in a final volume of 25 µl by mixing 1 µl of template DNA with 0.1 µM of each primer, 250 µM each of dATP, dCTP, dGTP, and dTTP, 2 units of *Taq* DNA polymerase (Q-BIOgene, Evry, France), and PCR reaction buffer containing 1.5 mM $MgCl_2$. PCR conditions were as follows: an initial denaturation at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, and a final elongation of 5 min at 72°C. PCR products were visualized by electrophoresis in 1% agarose gels stained with ethidium bromide, using molecular weight marker 8 (MBI Fermentas, Burlington, Canada) as a size standard. Strains were scored as either MAT 1 or MAT 2 if amplified DNA from the strain produced either a 205-bp fragment using the MAT 1 primers or a 190-bp fragment using the MAT 2 primers. In order to confirm the specificity of the primers designed to amplify the MAT genes, the sequences of the PCR products obtained with primers MAT 1L and MAT 1R for strains Fov6 and Fov9 and the PCR products obtained with primers MAT 2L and MAT 2R for strains Fov3 and Fov13 were determined. The four PCR products were cloned with the pGEM-T Easy Vector System (Promega, Madison, WI) and sequenced using primers SP6 and T7 (MWG, Ebersberg, Germany). For each PCR product, sequences from both strands were assembled using ChromasPro version 1.3 (Technelysium Pty. Ltd., Tewantin, Australia). Sequence identities were determined using BLAST from the National Center for Biotechnology Information (NCBI) (BLAST is available online from NCBI).

VCG testing. Vegetative compatibility was determined for each strain following the method of Correll et al. (8) by the generation of *nit* mutants using chlorate resistance as the selective character. Mutants of each strain were characterized phenotypically for their ability to grow on nitrate-, nitrite-, or hypoxanthine-containing medium. Mutants of different types were paired on nitrate medium and scored compatible if vigorous growth occurred at the point of contact between the two paired mutants. As a control, different mutants of the same isolate were paired on nitrate medium. Those that failed to produce vigorous growth at the point of contact were scored as heterokaryon self-incompatible (HSI).

RESULTS

Molecular characterization. Oligonucleotide primers PNFo and PN22 permitted the amplification of a single DNA fragment of about 1.7 kb for each of the 46 isolates. The PCR products were digested with each of the seven restriction enzymes. Depending on the enzyme, two to five different restriction patterns were obtained for each enzyme among the 46 isolates of *F. oxysporum* (Table 2). Seven different combinations of patterns representing seven IGS types were identified among the 46 isolates (Table 3). IGS type 14 was the most abundant, found in 19 of the 46 isolates of *F. oxysporum* f. sp. *vasinfectum* analyzed.

TABLE 2. Restriction patterns of polymerase chain reaction-amplified intergenic spacer (IGS) fragments of *Fusarium oxysporum* isolates

Enzymes[a]	Fragments (bp)
*Alu*I	
1	850, 500, 270, 85
20	1115, 500, 85
21	690, 500, 270, 145, 85
*Hae*III	
1	460, 145, 130, 115*, 100, 95*, 85, 80, 60, 55*
4	500, 175, 145, 130, 100, 95*, 85, 80, 70, 60, 55
8	460, 145, 130, 115, 100, 95*, 85, 80, 70, 60, 55*
22	460, 175, 145, 130, 115, 100, 95*, 85, 60, 55
30	460, 270, 145, 130, 100, 95*, 85, 60, 55*
*Hin*fI	
2	700, 550, 210, 120, 80
5	700, 580, 210, 120, 80
20	700, 550, 210, 130, 80
26	700, 550, 210, 150, 80
*Msp*I	
1	560, 275, 200, 105, 95, 85, 75, 60*, 50
6	560, 275, 200, 105, 95, 75, 60*, 50
25	440, 275, 200, 160, 105, 95, 85, 75, 60*, 50
26	560, 200, 185, 105, 95*, 85, 75, 60*, 50
27	625, 275, 200, 120, 105, 75, 60*, 50
*Rsa*I	
1	610, 560, 400, 90
3	1200, 400, 90
9	650, 540, 400, 90
20	560, 400, 365, 245, 90
*Scr*FI	
1	460, 215, 180, 170, 135, 110, 90, 85, 60
21	460, 215, 180, 170, 135, 120, 85*, 60
*Xho*I	
1	1300, 370
2	1670

[a] For each restriction enzyme, the various patterns are indicated by numbers in the first column and the sizes in base pairs of the corresponding restriction fragments are indicated in the second column. Estimates of fragment sizes were determined by electrophoresis in 4 to 6% Nusieve 3:1 agarose (FMC, Rockland, ME) and by comparison with the molecular weight marker VIII (Roche Diagnostic, Meylan, France) with measurements rounded to the nearest 5 bp. This was done for purposes of comparison among isolates; the values do not reflect absolute base pair fragment sizes. Restriction fragments less than 50 bp were not taken into consideration because they were not clearly resolved by electrophoresis. Asterisks indicate two restriction fragments of the same size (doublet). Numbers corresponding to the different restriction patterns follow those previously described (12,13,23,30).

The 19 strains of *F. oxysporum* f. sp. *vasinfectum* from Ivory Coast were distributed in four IGS types. Two additional IGS types were identified among African strains. The 16 strains from other continents were distributed in four IGS types, of which three IGS types were common to African strains. Within *F. oxysporum* f. sp. *vasinfectum*, IGS types 2 and 65 were only detected among strains from the Ivory Coast.

Specificity of the primers designed in this study for characterization of MATs was confirmed by determining the nucleotide sequences in both directions of two PCR products obtained with the MAT 1 and MAT 2 primers. Sequences of fragments obtained with MAT 2 primers from strains Fov3 and Fov13 (Accession nos. DQ132899 and DQ132900, respectively) were identical over the 190 nucleotides sequenced. BLAST analysis revealed 100% identity between these sequences and other sequences of MAT 2-1 genes of *F. oxysporum* (Accession nos. AB106001 to AB106008). Sequences of fragments obtained with MAT 1 primers from strains Fov6 and Fov9 (Accession nos. DQ132901 and DQ132902, respectively) showed 99% identity over the 250-bp region sequenced. BLAST analysis revealed 98% identity between these sequences and other sequences of MAT 1-1 genes of *F. oxysporum* (Accession nos. AY527415 to AY527422).

The MAT idiomorph screen revealed that 32 isolates were MAT1 and 14 were MAT 2 (Table 1). One IGS type (IGS type 14) had some strains that possessed either a MAT 1 or a MAT 2 idiomorph. All other IGS types possess only a single MAT idiomorph.

VCGs. Strains from Ivory Coast belonged to seven VCGs, four of these groups (0112, 0115, 0116, and 01110) were identified to previously described groups (15) and three appear to represent new VCGs arbitrarily assigned to provisional VCGs 1, 2, and 3 (Table 1). In addition, VCG 1 was also found among the strains we acquired from reference collections. There were eight strains, five from Ivory Coast and three reference strains, that were scored as HSI and could not be classified to a VCG.

All strains that belong to the same VCG also have the same IGS type and the same MAT idiomorph. Three IGS types contain multiple VCGs, i.e., VCGs 0111, 0112, and (provisional) VCG 1 within IGS type 14, VCG 0116 and (provisional) VCG 2 within IGS type 66, and VCGs 0115 and 01110 within IGS type 65. The same is true of pathological race in which the race testing has been reported in other publications (31), with all strains of the same race sharing an IGS type, although there may be a number of races within any given IGS type. Among the strains identified as IGS type 14, there are strains of races 1 and 2. Race 1 strains within this IGS type include strains with VCGs 0111 and 0112 as well as provisional VCG 1, and also include strains with MAT 1 and MAT 2 idiomorphs. Similarly, race 2 strains within IGS type 14 found in VCG 0112 and provisional VCG 1 also possess both MAT idiomorphs. Strains with IGS type 67 are found with either race 3 or 5, but unlike the strains in IGS type 14, they all belong to the same VCG (0113) and share the MAT 1 idiomorph.

Groups of strains were defined by shared IGS, VCGs, and MATs (Table 1). This grouping method allowed us to place some of the HSI strains in groups solely on the basis of MAT and IGS type. Grouping of some of the HSI strains is ambiguous since they could be placed into two possible groups (Table 1). The 46 strains of *F. oxysporum* f. sp. *vasinfectum* analyzed were placed into 11 such groups, seven of which were detected among strains from the Ivory Coast.

DISCUSSION

Evidence that some of the pathogens are new groups within *F. oxysporum*. Several of the isolates we examined belonged to previously undescribed groups. These included IGS types, and may include VCGs as well. We were able to obtain only 8 of the 12 previously described VCGs of forma specialis *vasinfectum* from culture collections, and we found three VCGs in our sample of new strains that were not in any of the eight previously described VCGs. Thus, there may be up to three new VCGs for this forma specialis among our newly acquired isolates. Given the variability displayed by the IGS region in other isolates of *F. oxysporum*, the finding of an additional three IGS types to the 48 already described (11–13,23,30) was not unexpected. Although some of the strains examined had IGS profiles identical to those previously described for strains isolated in soils in France and Argentina where cotton had never been cultivated (13,23), it is unknown whether the soil isolates are members of the forma specialis *vasinfectum* because they were not tested for cotton pathogenicity.

Comparison of the new isolates with previously described *F. oxysporum* strains and their affinities. We used the IGS region of the rDNA array as a target to analyze the genetic diversity among the strains studied. This was done because of the extremely high variability displayed by this region. IGS is known to vary within biological species (2,3,13,17,23,29,30), and strains that share IGS sequence, or in the case reported here, a significant fraction of the sequence determined by placement and sequence of restriction sites (92 total sites, 57 polymorphic), are thus likely to be closely related.

Diversity of the pathogens observed in Ivory Coast is relatively high compared with other populations within this species complex found elsewhere, but it is similar to studies of other populations of forma specialis *vasinfectum* (19). Other outbreaks of *F. oxysporum* f. sp. *vasinfectum* have usually contained fewer VCGs, e.g., two in Australia (9). We found seven VCGs among the Ivory Coast sample, which is similar to that reported by Fernandez et al. (15) who found three VCGs among eight strains (two of which are included in this study). Other studies of *F. oxysporum* have shown high levels of VCG diversity. For example, five VCGs were found in a sample of 160 *F. oxysporum* f. sp. *radicis-lycopersici* isolates in Sicily (10) and four VCGs were

TABLE 3. Intergenic spacer (IGS) types and restriction patterns of *Fusarium oxysporum* isolates revealed by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified IGS sequences

IGS type[a]	Representative isolate[b]	Restriction patterns of amplified IGS fragments digested with enzymes[c]							Geographic distribution
		*Alu*I	*Hae*III	*Hin*fI	*Msp*I	*Rsa*I	*Scr*FI	*Xho*I	
2	Fov9	1	1	2	1	1	1	1	Ivory Coast
3	Fov19	1	1	2	1	1	1	2	India, China, USA
14	Fov1	1	4	5	1	3	1	1	Ivory Coast, Benin, Tanzania, Egypt, USA
21	Fov18	1	8	2	6	1	1	1	Benin
65	Fov3	1	1	2	25	1	1	1	Ivory Coast
66	Fov5	20	30	26	26	20	1	2	Ivory Coast, Brazil
67	Fov24	21	22	20	27	9	21	2	Sudan, Egypt, Israel

[a] IGS types represent the combination of patterns obtained with seven restriction enzymes. The numbers assigned to IGS types 2 to 21 follow those previously described (12,13). The restriction patterns are described in Table 2.
[b] Isolates are described in Table 1.
[c] Numbers designate the various patterns obtained for each restriction enzyme and follow those previously described (12,13,23,30).

found in a sample of *F. oxysporum* f. sp. *cubense* in a single country (21). In forma specialis *phaseoli* in Greece, eight VCGs were detected among 23 isolates, with most of the isolates belonging to a single VCG (14), whereas in Spain, only three VCGs were detected (2).

Many of the groups we examined appear to be widely distributed, evidenced by the fact that strains that were identical for all the characters we examined (IGS, VCG, and MAT) were found on different continents. We theorize that the global trade in agricultural products has resulted in the relatively recent geographic dispersal of these pathogens. Evolutionary relationships among strains of forma specialis *vasinfectum* have been described previously (31). In this study, subsequently extended by Kim et al. (19), five lineages were defined. Our results generally support these lineages, with isolates from lineage I (sensu Skovgaard et al. [31]), which includes races 3 and 5, all found in our group 9, and isolates from lineage IV, which includes race 4 strains, all in our group 4. Lineage II isolates, which include races 1, 2, and 6, are found in four different groups, our numbers 1, 2, 3, and 7. In the study cited above, it was lineage II that showed the greatest sequence diversity with some subgrouping possible. We also find high diversity in this lineage, with two different IGS types, four different VCGs, and the presence of both MAT idiomorphs. Kim et al. (19) also reported high diversity in lineage II, finding three IGS types within the lineage, and low diversity in lineage IV, with only a single IGS type in 13 isolates grouped in this lineage. However, they did report greater IGS divergence in lineage I than we found, with three IGS types in their isolates compared with our single type. In general, the results presented here support the interpretation of polyphyly of forma specialis *vasinfectum* (6,19,31).

Evidence for clonality of pathogens. Other studies of pathogenic *F. oxysporum* f. sp. *vasinfectum* have generally been consistent with clonal reproduction (1,15,19,31). These studies have shown that in some lineages, notably lineage IV, a clonal origin is likely for all isolates (19,31). Our results support the clonal origin of all lineage IV isolates that we tested (group 4), as they are identical for IGS, MAT idiomorph, VCG, and pathogenic race. Likewise, we found no differences among strains in lineage I (group 9), although some differentiation of IGS type has been reported elsewhere (19). *F. oxysporum* has no known sexual stage, although phylogenetic analysis has shown that it is nested within the *Gibberella* clade (27,32). This is consistent with what is known about putatively asexual taxa in general, as the unifying theme among them is their close relationships with sexual species (32). The ability to clearly group the strains, both the reference strains and the new isolates, into clusters determined by IGS type, VCG, and MAT idiomorph suggests that sexual reproduction may be absent. There are some strains that might be placed into more than one group, but this is because the vegetative compatibility phenotype of the strains is unknown due to HSI. The proportion of strains that were HSI (4 out of 17 strains from Ivory Coast) was also quite high. This might be due to the relatively low sample size and the chance collection of such strains, or it may reflect a real tendency of the strains toward self-incompatibility. More strains need to be collected to determine which of these hypotheses is correct.

Isolates in lineage II (groups 1, 2, 3, and 7) are clearly not all from the same clone, and the presence of both MAT idiomorphs in the lineage suggests that there may have been recombination in the evolutionarily recent past. Included among strains belonging to this lineage are those that share IGS type 14. We have been able to place these isolates into three groups corresponding to three potential clonal lineages, each potential lineage sharing IGS and VCG type along with MAT idiomorph. Two of them (groups 1 and 2) differ only in their VCG phenotypes and thus may be related by a single mutational event altering a *vic* locus. The third (group 3) is much more problematic since it differs not only for the VCG phenotype but also contains a different idiomorph at the MAT locus. Two possibilities are offered to explain the origin of group 3. Either it arose through recombination of the pathogenic and *vic* genes to create a pathogenic group with a different VCG phenotype, a hypothesis supported in part by the presence of two MATs in the IGS group, or it arose by the acquisition of a mutation that rendered a MAT 2 strain pathogenic. Without further evidence of recombination in this group, these hypotheses are not testable. Taylor et al. (32) showed that for a large sample of fungal taxa, asexual fungi are very closely related phylogenetically to sexual taxa, and that there are no strictly asexual clades above the species level among the fungi. We would expect, then, that ultimately all the strains would have a sexual origin, whether they are clonal (at present) or not, as asexual taxa among eukaryotes are ephemeral (32). As noted by Maynard Smith et al. (24), many organisms follow a middle path of evolution in which sexual recombination produces variants followed by an epidemic increase arising from clonal propagation of opportunistic varieties. The epidemic model of strain origin fits our data well, suggesting that there has been more than one epidemic outbreak from within the *F. oxysporum* complex, giving rise to the many different varieties of the forma specialis.

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