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Genetic relationships among seven specialized forms of *Fusarium oxysporum* determined by DNA sequencing of the ITS region and AFLPs

R. Arroyo-García[1,4], J. L. Cenis[2], J. Tello[3], J. M. Martínez-Zapater[1,4] and D. Cifuentes[5]*

[1] *Centro Nacional de Biotecnología-CSIC. Cantoblanco. 28049 Madrid. Spain*
[2] *IMIDA. C/ Mayor, s/n. 30150 La Alberca (Murcia). Spain*
[3] *ETSIA. Universidad de Almería. Cañada de San Urbano, s/n. 04120 Almería. Spain*
[4] *INIA. Ctra. Coruña, km 7.5. 28040 Madrid. Spain*
[5] *ETSIA. Universidad Politécnica de Cartagena. P.° Alfonso XIII, s/n. 30203 Cartagena (Murcia). Spain*

Abstract

The fungus *Fusarium oxysporum* Sch.:Fr. presents a high biological and genetic variability, manifested by the existence of many specialized forms and races. With the goal of evaluating the genetic relationship among different specialized forms, an experiment was carried out involving the application of two types of molecular markers. One consisted of sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA of 17 isolates belonging to seven forms: *F.o. melonis* (4 isolates), *F.o. dianthi* (2), *F.o. niveum* (4), *F.o. lycopersici* (2), *F.o. radicis-lycopersici* (3), *F.o. lagenaria* (1) and *F.o. luffae* (1). Analysis of these sequences revealed that isolates from different forms present an identical sequence while isolates of the same form appear distributed in different groups of the dendrogram. This observation was confirmed by an AFLP analysis. In addition to the previously cited forms, ten more were studied in this experiment, belonging to *F.o. ciceris*, *F.o. cucumerinum*, *F. proliferatum* and *F.o. asparagi*, as well as two non-pathogenic isolates. The dendrogram calculated with the AFLP markers did not reveal any genetic structuration of the 10 specialized forms. These data, in line with those obtained by other authors, seem to suggest that in general, the specialized forms of *F. oxysporum* do not constitute monophyletic lineages because they evolve in a divergent way. It rather seems that widely different genotypes could share similar genetic factors conferring pathogenic specificity.

Key words: phylogeny, PCR, fungi, molecular markers.

Resumen

Relaciones genéticas entre siete formas especializadas de *Fusarium oxysporum* Sch.:Fr. determinadas mediante la secuenciación de la región ITS y AFLPs

El hongo *Fusarium oxysporum* Sch.:Fr. presenta una elevada variabilidad biológica y genética que se manifiesta por la existencia de numerosas formas especializadas y razas. Con el fin de evaluar la relación genética entre diferentes formas especializadas se aplicaron dos tipos de marcadores genéticos. En primer lugar se secuenció la región ITS (*internal transcribed spacer*) del ADN ribosomal de 17 aislados pertenecientes a siete de dichas formas: *F.o. melonis* (4 aislados), *F.o. dianthi* (2), *F.o. niveum* (4), *F.o. lycopersici* (2), *F.o. radicis-lycopersici* (3), *F.o. lagenaria* (1) y *F.o. luffae* (1). El análisis de estas secuencias reveló que aislados de diferentes formas presentan una secuencia idéntica, mientras que aislados de la misma forma aparecen en grupos diferentes del correspondiente dendrograma. Esta observación se confirmó mediante marcadores AFLP de las mismas formas especializadas y 10 más pertenecientes a *F.o. ciceris*, *F.o. cucumerinum*, *F. proliferatum* y *F.o. asparagi*, así como dos aislados no patogénicos. El dendrograma obtenido no reveló ninguna estructuración genética de las 10 formas especializadas. Estos datos, que están en la línea de los obtenidos por otros autores, parecen indicar que algunas formas especializadas de *F. oxysporum* no constituyen linajes monofiléticos, ya que evolucionan de forma divergente. Parece más bien que genotipos muy diferentes podrían compartir factores similares de especifidad y especialización patogénica.

Palabras clave: filogenia, PCR, hongos, marcadores moleculares.

* Corresponding author: dina.cifuentes@upct.es
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Introduction

The fungus *Fusarium oxysporum* Schlechtend.:Fr. is present in almost all cultivated soils worldwide. It is predominantly an abundant and active saprophytic fungus of soil and organic matter. However, a very small proportion of the total soil population has pathogenic activity and causes serious crop diseases especially as a result of vascular wilting (Tello and Lacasa, 1990). The economic importance of diseases caused by *F. oxysporum* is difficult to estimate precisely but is known to be considerable. Vascular wilting appears in almost all horticultural crops in regions of the world where intensive agriculture is practiced. In intensive horticultural crops, either grown outdoors or in greenhouses in the southeast of the Iberian Peninsula or in the Canary Islands, the incidence of this disease is an important factor in crop management. Treatments of this fungus have included routine soil disinfection, in some cases using very aggressive products. This has resulted in high economic inputs for the crop and microbiological degradation of soils that have given rise to other associated problems. In some cases, acceptable levels of control have been achieved using resistant varieties. Development of these varieties has made it necessary to study in detail an important aspect of the biology of *F. oxysporum*, namely its pathogenic and genetic variability. This variability is manifested by the existence of specialized forms and races. At present, more than 150 specialized forms have been described (Baayen *et al*., 2000), which, in turn, present a variable number of races. As described by Kistler (1997), it was originally thought that isolates of a specialized form that share the same host, share more genetic similarities than others with a different parasite specialization. The evolutionary interpretation of this concept is that specialized forms are monophyletic and that the isolates that share a host are all derived from a single pathogenic genotype. The races would be derived from point mutations in this genotype, selected by selective pressure imposed by the cultivation of varieties with resistance genes. This predominant point of view has been corroborated by certain experiments. Hence, Tantaoui *et al*. (1996) demonstrated via a vegetative compatibility group study (VCGs), using random amplified polymorphic DNA (RAPD-PCR) and restriction fragment length polymorphisms (RFLPs), that isolates of *F. oxysporum* f. sp. *albedinis* that affect Moroccan and Algerian palms form a monophyletic lineage. In turn, Plyler *et al*. (2000) provided data suggesting that *F. oxysporum* f. sp. *canariensis* is a monophyletic lineage with a moderate level of genetic diversity. On the other hand, Jiménez-Gasco *et al*. (2002) demonstrated, by the sequenciation of introns of five genes (EF-1α, β-tubulin, histone 3, actin and calmodulin) of *F. oxysporum* f.sp. *ciceris*, that the isolates of this specialized form are grouped into a single clonal lineage.

However, opposing this predominant idea, there is evidence to support an alternative view. O'Donnell *et al*. (1998) studied the phylogeny of a wide collection of isolates of *F. oxysporum* f. sp. *cubense* by comparing the sequences of a small subunit of mitochondrial (mtSSU) rDNA and of the elongation factor (EF-1α). The phylogeny inferred from the combination of the two sets of data show that the isolates of *F.o. cubense* are distributed in five significantly different lineages. Two of these five clonal lineages differ, in turn, in their chromosome number. From these results they deduced that the specialized form that causes «Panama disease» in the banana is caused by fungi of very different evolutionary origins. A second important work in this line was that of Baayen *et al*. (2000), in which a total of 89 isolates representative of eight specialized forms were studied by AFLP (amplified fragment length polymorphisms) and by sequentiation of mtSSU and of the elongation factor EF-1α. The results indicated that in contrast to clearly monophyletic forms such as *F.o. lilii* and *F.o. tulipae*, there are others such as *F.o. asparagi*, *dianthi*, *gladioli*, *lini*, *opuntiarum*, and *spinaciae* which reveal multiple evolutionary origins.

Knowledge of the phylogeny and phylogeography of *F. oxysporum* is important to understand how the fungus is introduced and spreads and to design detection probes based on its DNA polymorphisms. The goal of this work was to study the phylogeny and genetic associations of seven specialized forms, some of considerable economic importance in Spain, that have been little studied in these aspects: *F.o. melonis*, *F.o. dianthi*, *F.o. niveum*, *F.o. lycopersici*, *F.o. radicis-lycopersici*, *F.o. lagenaria* and *F.o. luffae*. Two types of markers were used for the genetic analysis. One has been widely used in evolutionary genetic studies, such as sequencing of the ITS region (internal transcribed spacer) of ribosomal DNA (White *et al*., 1990). Another type of markers, such as AFLPs (Vos *et al*., 1995; Majer *et al*., 1996; Baayen *et al*., 2000), are less used but they are very useful for detecting variability in different types of organisms.

Material and methods

Isolates used

In the present work, a total of 36 isolates of *Fusarium oxysporum* and one of *F. proliferatum* were studied (Table 1). Two of the *F. oxysporum* isolates were not pathogenic, and the rest belonged to 10 specialized forms. Of all the isolates used in the present work, 27 were studied by the AFLP technique and 17 by sequencing the ITS region.

The isolates mainly came from three collections. One of these was the one kept by J. Tello (Almería University) with isolates from the southeast, Levante and

Table 1. Isolates of *Fusarium oxysporum* used in this work and their origins. Isolates used in the AFLP experiment and in ITS sequencing are specified, including in this case the accession number in the Gen Bank

Code	Origin	Race	AFLP	ITS
Fusarium oxysporum f. sp. *lycopersici*				
Fol 39	Aguilas (Murcia)	1	+	AY354400
Fol 59	Aguilas (Murcia)	1		AY354385
Fusarium oxysporum f. sp. *radicis-lycopersici*				
Forl 1	Mazarrón (Murcia)	1	+	AY354401
Forl 12	Mazarrón (Murcia)	1		AY354386
Forl 15	Mazarrón (Murcia)	1	+	
Forl 19	Aguilas (Murcia)	1	+	AY354387
Fusarium oxysporum f. sp. *dianthi*				
Fod Gri1	Ribadavia (Ourense)	4	+	
Fod Gsa1	Salnés (Pontevedra)	2	+	
Fod Gsa2	Salnés (Pontevedra)	4		AY354389
Fod F276	Colección de A. Garibaldi (Turin)	8	+	
Fod Sur2	Crevillente (Alicante)	2	+	
Fod Sur10	San Pedro (Alicante)	2		AY354388
Fusarium oxysporum f. sp. *niveum*				
Fon 1	Cullera (Valencia)			AY354396
Fon 7	Cullera (Valencia)			AY354397
Fon NM11	CHN (cl) PCR-F5 (89) (China)			AY354395
Fon NM14	FL-7III-1 (Florida)		+	
Fon NM22	TX-J-NAV (88) (Texas)		+	
Fon NM30	RS-2-LRGV (86) (Taiwan)			AY354394
Fusarium oxysporum f. sp. *melonis*				
Fom MM1	ATCC 16413 (Florida)		+	
Fom MM2	ATCC 28858 (France)	1,2w	+	AY354390
Fom MM3	ATCC 28862 (Israel)	1	+	
Fom MM6	K-RM2 (Israel)	2	+	AY354391
Fom MM7	KNH (Israel)	1,2		AY354392
Fom 28	San Miguel (Alicante)	1		AY354393
Fusarium oxysporum f. sp. *cucumerinum*				
Foc CM1	NETH 10782 (89) (Holland)		+	
Foc CM2	NETH 20286 (Holland)		+	
Foc CM3	NETH 11179 (Holland)		+	
Foc CM4	PSU 1266 (Canada)		+	
Fusarium oxysporum f. sp. *lagenaria*				
Fola LM1	ATCC 18143 (Japan)		+	AY354398
Fola LM2	ATCC 38363 (Japan)		+	

Table 1 (cont.)

Code	Origin	Race	AFLP	ITS
Fusarium oxysporum f. sp. *luffae*				
Folu LUM1	ATCC 28860 (Japan)		+	AY354399
Fusarium oxysporum f. sp. *ciceris*				
Foci-1	(Córdoba University) 8207 Yellow		+	
Foci-2	(Córdoba University) 8012 Wilt		+	
Fusarium oxysporum f. sp. *asparagi*				
Foe 10	Campillos (Málaga)		+	
Fusarium oxysporum no pagogénico				
FONP-1	(Córdoba University) 9081		+	
FONP-2	(Córdoba University) 90101		+	
Fusarium proliferatum				
Fpro	L1-11		+	
Sequences used from the GenBank				
F. proliferatum X94171				
F. solani AF130141				
F. o. vasinfectum AF322076				

Galicia. A second collection was that of R. Martyn (Texas University A&M, College Station, USA), with isolates collected from all over the world Cucurbitaceae. Finally, isolates of *F.o. ciceris* and non pathogenic *F. oxysporum* isolates from the collection of Córdoba University were used.

The molecular variability of these isolates was previously studied by RAPD-PCR (Cifuentes, 2001) and is representative of the genetic variability of a much larger group of 217 isolates of *F. oxysporum* from the three collections cited above.

AFLP experiment

AFLP analysis was done following the protocol described by Vos *et al.* (1995). The DNA of the different isolates was digested by the enzymes *Mse*I (New England Biolabs) and *Eco*RI (Amersham Pharmacia). Digestion of 150 ng DNA was done in a final volume of 35 µl in 10 mM Tris acetate, 10 mM magnesium acetate, 50 mM DTT, pH 7.5, 10u *Eco*RI, 8u *Mse*I, for 3 h at 37ºC. Next, the two adaptors of *Eco*RI and *Mse*I were ligated, adding to the digestion 5 µl of a mixture containing 5 pmol of *Eco*RI adaptor, 50 pmol of adaptor *Mse*I, 8 mM ATP, 10 mM Tris acetate, 10 mM magnesium acetate, 50 mM DTT, pH 7.5 and 1.4u DNA ligase T4 (Roche). The ligation was incubated for 3 h at 37ºC and at 4ºC for 6 h. The sequences of the *Eco*RI and *Mse*I adaptors are shown in Table 2.

The digested and ligated fragments were diluted 1:7 to be used as templates in a first preamplification reaction. This preamplification consisted in a PCR reaction using primers complementary to the *Eco*RI and *Mse*I adaptors, with an additional selective 3' nucleotide to reduce the complexity of the collection of DNA fragments, since only 1/16 of the total of digested fragments were amplified. PCR reactions were carried out in a 20 µl volume containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 30 ng of each primer E11 and M20 (Genset) (Table 2), 0.4 u of *Taq* DNA-polymerase (Roche) and 3 µl of diluted DNA fragments. The PCR amplifications were carried out in a Perkin Elmer 9600 thermocycler with 28 cycles, each consisting of 30 s at 94ºC, 1 min at 60ºC and 1 min at 72ºC.

Ten microlitres of preamplification products were diluted by adding 90 µl of distilled water. This was used as starting material for the selective radioactive amplification. For this reaction, only *Eco*RI primers were radioactively labeled. Both the primers *Eco*RI and *Mse*I used in this reaction had the same sequence as those used in the preamplification reaction but with 3 additional selective nucleotides at the 3' end. The

Table 2. Adaptor and primer sequences used in AFLP analysis

Code	Type	Sequence
	Adaptor *Eco*RI	5´-CTCGTAGACTGCGTACC-3´ 3´-CTGACGCATGGTTAA-5´
	Adaptor *Mse*I	5´-GACGATGAGTCCTGAG-3´ 3´-TACTCAGGACTCAT-5´
E11	Primer *Eco*RI + 1	5´-GACTGCGTACCAATTCA-3´
M20	Primer *Mse*I + 1	5´-GATGAGTCCTGAGTAAC-3´
E36	Primer *Eco*RI + 2	5´-GACTGCGTACCAATTCAC-3´
M31	Primer *Mse*I + 2	5´-GATGAGTCCTGAGTAACT-3´
M39	Primer *Mse*I + 2	5´-GATGAGTCCTGAGTAATC-3´

PCR reaction was carried out in a volume of 20 µl of 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.08 mM of each dNTP, 4 ng of primer [^{33}P]-*Eco*RI, 24 ng of primer *Mse*I, 0.4 u of *Taq* DNA-polymerase and 5 µl of diluted preamplified DNA. Selective amplification was done using the following parameters: 1 cycle of 30 s at 94ºC, 30 s at 65ºC, 1 min at 72ºC. Next, 12 cycles were programmed in which the annealing temperature decreased by 0.7ºC per cycle, followed by 23 cycles of 1 min at 94ºC, 30 s at 56ºC and 1 min at 72ºC. In the present analysis, two primer combinations were used for the selective amplification: 1-E36/M31 and 2-E36/M39 (Table 2).

At the end of the selective radioactive amplification, samples were denatured by adding an equal volume of formamide buffer (98% formamide, 10 mM EDTA pH 8, 0.05% of bromophenol blue and 0.05% of xylene cyanol) and heating for 3 min at 94ºC. A total of 3 µl of each sample were loaded into a gel of 4.5 % acrylamide/bisacrylamide 19:1, and 7.5 M urea. Electrophoresis was carried out in a BioRad Sequigen GT sequenciation cell, of 38 x 50 cm and 1X TBE buffer for 2 h.

Sequencing of the ITS region

The sequence of the ITS region of ribosomal DNA was obtained by first amplifying this region using the universal primers described by White *et al.* (1990). These primers hybridize in regions of the genes 28S, 5.8S and 18S that are highly conserved in most fungal genera and families. Different combinations of primers were tested finally adopting the combination *Its4* (5'- TCC TCC GCT TAT TGA TAT GC) and *Its5* (5'- GGA AGT AAA AGT CGT AAC AAG G), that amplified a 560 bp fragment including the regions ITS 1, ITS 2 and the gene 5.8S. Amplification of the ITS fragments was done by PCR following the protocol described by White *et al.* (1990) and consisted in an initial denaturization of 2 min at 95ºC, followed by 30 cycles of: denaturization for 30 s at 95ºC; annealing of primers for 30 s at 55ºC and polymerization for 1 min at 72ºC. These 30 cycles were followed by a final extension of 10 min at 72ºC. Afterwards, 10 µl of the amplified product were precipitated by adding an equal volume of isopropanol, centrifuged at 13000 rpm for 30 min and the precipitate was dried, and redissolved at a concentration of 20 ng μl^{-1}. Ten microliters of this solution were sent to the DNA Sequencing Service of the Centro de Investigaciones Biológicas of the CSIC (Madrid).

Genetic data analysis

To determine the genetic similarity between isolates from the data obtained in the AFLP experiment, the Dice coefficient was calculated (Sneath and Sokal, 1973). This coefficient is based on the proportion of bands shared between two isolates, but weighted to some extent, according to the formula: $GS(ij) = 2a / (2a + b + c)$, where $GS(ij)$ is the genetic similarity between the individuals i and j; a is the number of polymorphic bands shared by i and j; b is the number of bands present in i and absent from j; c is the number of bands present in j and absent from i.

The calculation process starts by recording in a rectangular matrix the presence or absence of the amplified bands in each isolate, as 0 for an absence and 1 as presence of a selected band. This matrix was used in the NTSYS program (Numerical Taxonomy System) (Rohlf, 1988), that calculated Dice's coefficient of similarity between all the pairs of isolates studied in each experiment, establishing a triangular similarity matrix.

The similarity between the isolates was visualized by an UPGMA dendrogram (unweighted pair group method using arithmetic averages), which was also calculated by the NTSYS program.

The sequences obtained in the sequentiation experiment, together with another three existing in the GenBank, belonging to *F. oxysporum* f.sp. *vasinfectum*, *F. solani* and *F. proliferatum* (Table 1), were aligned using the CLUSTALW program (http://searchlauncher.bcm.tmc.edu). After their alignment, all the sequences obtained were analyzed by the MEGA program (www.megasoftware.net). By this analysis, a dendrogram was drawn up using the Kimura model with 2 parameters (Kimura, 1980) and the significance test was done by bootstrap analysis with 1000 permutations.

Results

The results obtained in the AFLPs experiment using two combinations of primers (E36/M31 and E36/M39), were suitable for most of the isolates, with detection of a total of 180 bands. One example of the amplification obtained with the first of the combinations indicated appears in Figure 1. With these bands a dendrogram was drawn up that expresses the genetic similarity of the isolates appearing in Figure 2. The dendrogram reveals the great variability of the collection. The isolate furthest from the rest is *F.o. asparagi* (Foe 10), which branches off at a similarity of 0.34. No clear structuration is observed in the distribution of the isolates in the different branches of the dendrogram, with the exception of the two *F.o. niveum* isolates analyzed (Fon NM14 and Fon NM22), that appear together in a subgroup in the upper part of the dendrogram. A distancing of the Fom MM3 isolate away from the other isolates of *F.o. melonis* is observed. It is also surprising that *F. proliferatum*, a different species to *F. oxysporum*, is included in the *F. oxysporum* group as well as its non pathogenic isolates that usually show a genetic difference to the pathogenic isolates.

A second experiment was designed to develop the phylogeny of the isolates of seven specialized forms. The phylogeny developed using the sequences obtained is represented in the dendrogram from Figure 3. It can be observed that the sequence furthest away from the rest corresponds to a *F. proliferatum* extracted from the GenBank, as expected since this belongs to a different species to *F. oxysporum*. Next, a sequence of the

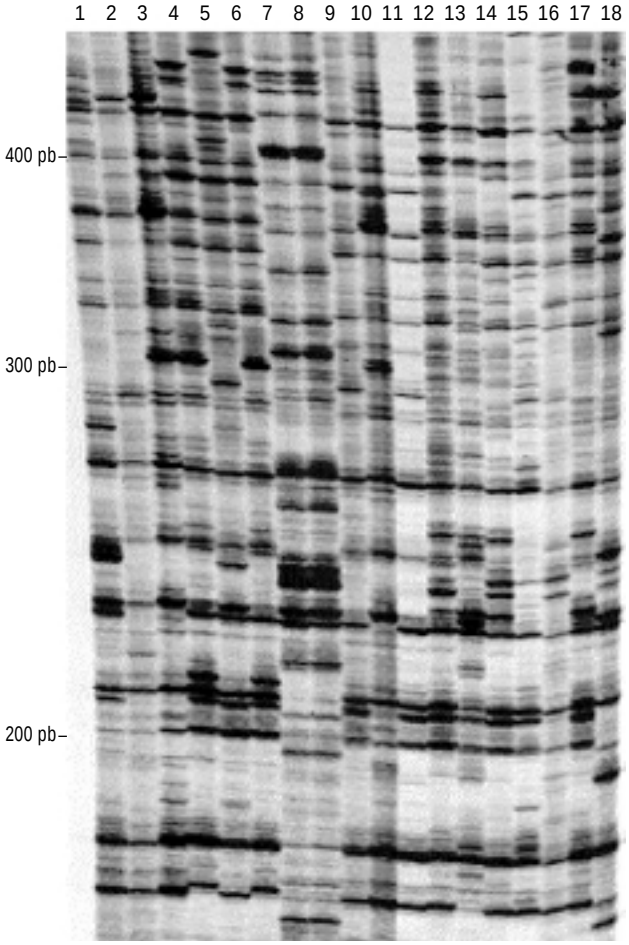

Figure 1. Part of a gel obtained in an AFLP experiment using primers E36/M31. Differences are observed between 18 of the *F. oxysporum* isolates. Lanes: 1, Fon NM14; 2, Fon NM22; 3, Foe 10; 4, Fom MM1; 5, Fom MM6; 6, Fom MM2; 7, Forl 1; 8, Forl 19; 9, Fod Sur 2; 10, Fod Gsa1; 11, Fod Gri1; 12, Fod F276; 13, Fol 39; 14, Fola LM1; 15, Fola LM2; 16, Foci 1; 17, Fpro; 18, Fonp1.

Forl 19 isolate of *F.o. radicis-lycopersici* was separated and after a group of two comprised of a sequence of *F.o. melonis* (MM6) and another of *F.o. niveum* (NM11). Finally, one node significantly separates an isolate of *F.o. melonis* (Fom MM7) from the other sequences analyzed, none of which are significantly separated from the others. There are two groups of identical sequences corresponding to isolates of different forms, such as a group of four that includes *F.o. vasinfectum, F.o. luffae, F.o. niveum* and *F.o. melonis*, the group of two that includes *F.o. niveum* and *F.o. dianthi*, or another group of two that includes *F.o. dianthi* and *F.o. radicis-lycopersici*. It is interesting that the sequence of *F. solani* from the GenBank is not clearly

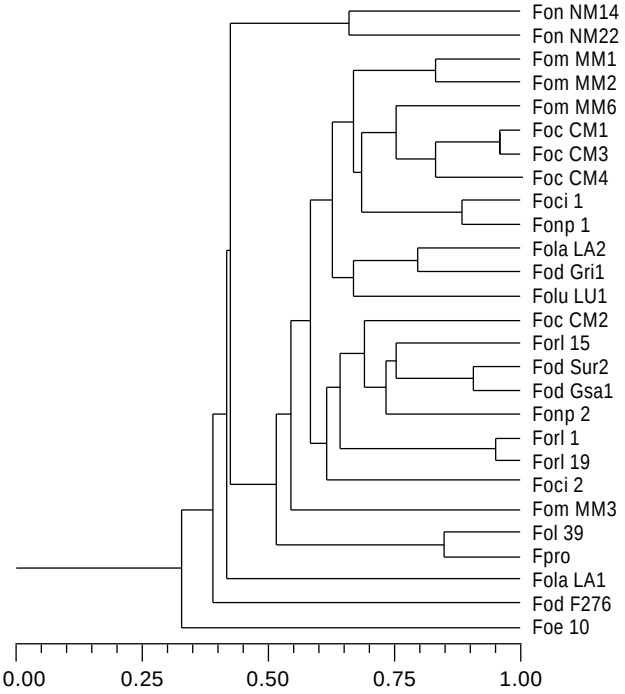

Figure 2. UPGMA dendrogram representing, from AFLP data, the similarity of isolates of 10 specialized forms of *F. oxysporum* and one isolate of *F. proliferatum*.

separated from the *oxysporum* group as expected, but instead seems to be totally integrated with the sequences of this species and does not stand out in any clear way.

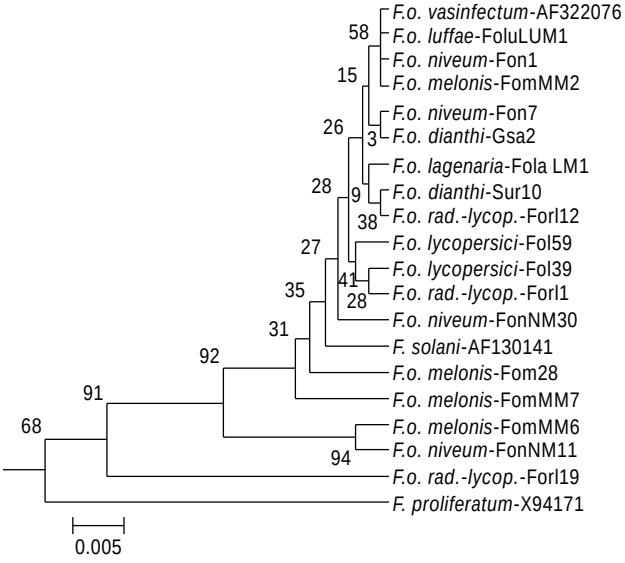

Figure 3. UPGMA dendrogram reflecting the phylogeny of a sequence of *F. proliferatum*, another of *F. solani* and 18 sequences of *F. oxysporum* belonging to eight specialized forms. Bootstrap values were calculated after 1,000 permutations.

Discussion

Both in the phylogeny based on ITS sequences and in the genetic similarity dendrogram based on the AFLPs, it can be clearly observed that isolates of different specialized forms are genetically closer than isolates of the same specialized form. This suggests that the specialized forms included in this study are not monophyletic lineages. In contrast to earlier views, specialized forms of *Fusarium oxysporum* are often polyphyletic. The evidence for this was presented for the first time by O'Donnell *et al*. (1998) for *F.o. cubense*. Afterwards, Baayen *et al*. (2000) presented results based on sequence comparisons and AFLPs that indicate that the *F.o. asparagi, dianthi, gladioli* and *lini* forms are polyphyletic. The present work presents data which indicate that *F.o. niveum. F.o. melonis* and *F.o. radicis-lycopersici* could also be polyphyletic. These data have been obtained following the approach of Baayen *et al*. (2000) using AFLP and DNA sequence comparisons. These authors found a good agreement between the phylogenies calculated by both approaches. This is interesting because the two approaches are complementary: AFLP analysis shows random *loci* distributed throughout the genome, while sequence analysis examines the evolution in corresponding genomic regions to defined genes or non coding sequences. In our case, we did not find agreement between the two approaches, since in both cases hardly any structuration was observed in certain groups. This can be partly due to the fact that AFLP markers always show a high level of variability, suitable for interpopulational analysis, but that tend to mask evolutionary relationships. For this reason we considered the data obtained from sequencing of the ITS region to be more relevant. The polyphyletic nature of the three specialized forms mentioned could also be suspected from the structure of their respective vegetative compatibility groups (VCGs). Although, in general there is no correspondence between the phylogenetic clades and the VCGs, the abundance of these indicates a high level of genetic diversification. Hence, 3 VCGs have been described for *F.o. niveum* (Larkin *et al*., 1990), 10 VCGs for *F.o. melonis* (Katan *et al*., 1994) and 10 VCGs for *F.o. radicis-lycopersici* (Katan and Katan, 1999).

In relation to the other specialized forms used in this study, such as *dianthi, lycopersici, lagenaria* and *luffae*, we cannot draw any conclusions owed to insufficient data, although Baayen *et al*. (2000) suggest that *F.o. dianthi* has a polyphyletic character while, according to Mes *et al*. (1999), *F.o. lycopersici* could be mo-

nophyletic. On the other hand, phylogenetic analysis of specialized forms of Cucurbitaceae based on RAPD-PCR (Vakalounakis and Fragkiadakis, 1999), indicate that *F.o. cucumerinum* and *F.o. niveum* could be polyphyletic, while *F.o. radicis-cucumerinum* and *F.o. melonis* would be monophyletic. This partly contradicts our results on *F.o. melonis*, and as a consequence, more research is required to clarify this.

An interesting aspect to consider is the lack of any apparent geographic structuration of the clades. Isolates of *F.o. radicis-lycopersici*, which separate into two different clades, are all from Murcia while those of *F.o. melonis* MM6 and MM7, that also form two clades come from Israel. This could lead us to suspect multiple introduction episodes.

The fact that some specialized forms of the *Fusarium oxysporum* complex are polyphyletic, implies that isolates with a very different genetic background have acquired common pathogenic genes. Apart from the possible existence of multiple mutation and transposition events that could have led to an independent acquisition of pathogenicity to the same hosts, Baayen *et al.* (2000) speculated about the possible mechanisms involved in this process. One of these could be parasexuality, known to occur in *F. oxysporum*, that can have contributed to lateral transfer of pathogenic genes to genetically very distant isolates. Also, horizontal transfer of supernumerary chromosomes that carry pathogenicity genes can take place. A recent review of this area shows that these aspects are beginning to receive more attention (Rosewich and Kistler, 2000). Undoubtedly, the exchange of sequences such as plasmids, introns, transposons and genes should play some role in maintaining a high genetic diversity for an asexual fungus such as *F. oxysporum*. In any case, the previously accepted notion of a specialized form must be revised since the pathogenic genes in different VCGs of a specialized form can have different origins and may not be identical, meaning that the precise nature of this pathogenicity may not be identical either.

An important practical consequence of the results presented here is that it is very difficult to establish a diagnostic method for specialized forms based on AFLP type markers or on ITS sequencing data, that has a global and general validity for all specialized forms of *F. oxysporum*. So far it has been impossible to find a set of amplified bands or DNA sequences specifically and exclusively associated to the set of specialized forms used in this work. It is, therefore, necessary to explore other strategies to try to detect these.

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