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Research Article

Genetic Diversity of *Fusarium oxysporum* f. sp. *dianthi* in Southern Spain

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The diversity of races and prevalence of pathogenic populations of *Fusarium oxysporum* f. sp. *dianthi* (Fod) were surveyed in an area in southern Spain. From 54 farms, 132 isolates were collected from wilted carnation plants. Isolates were characterized by RAPD-PCR, DNA sequence analysis of the *TEF1-α* gene, and race-specific molecular markers. Selected isolates from RAPD groups were phenotypically evaluated by pathogenicity tests. Data analysis showed that Fod race 2 was the most frequent and prevalent race in the study area, followed by race 1/8. Moreover, phylogenetic analyses showed similar results, which were different to those of the race-specific PCR assays. It was concluded that (i) seven isolates were not classified in groups where Fod testers were clustered; even they showed different results when race-specific markers were used, (ii) ten isolates with retarded race 1 or race 8 specific band were characterized as *F. proliferatum* by *TEF1-α* gene sequencing and clustered into an outgroup, and (iii) six isolates failed to generate an amplification signal using race-specific markers. Furthermore, three of them were grouped close to race 2 tester according to the phylogenetic analyses, showing the same differential pathogenicity as race 2. This may indicate a Fod race 2 subgroup in this region.

1. Introduction

Fusarium oxysporum Schlechtend. Emend. W. C. Snyder & H. N. Hans is a species complex of morphologically similar fungi with multiple phylogenetic origins that fall into three well-supported clades [1]. Pathogenic isolates of *F. oxysporum* often display a high degree of host specificity and can be subdivided into *formae speciales* on the basis of the plant species affected. The *forma specialis dianthi* (Fod) causes Fusarium wilt of carnation (*Dianthus caryophyllus* L.), a disease that is responsible for severe losses in areas where this flower is grown. The pathogen is widespread in soils worldwide [2–5].

In 2009, Andalusia accounted for 55.4% of the total carnations produced in Spain, and 62.1% of this was from the province of Cádiz [6], which is the greatest producer in Europe [7]. In this area, Fusarium wilt disease appeared five years after the introduction of the first carnation plants in 1975. Since then, it has been reported continuously [8]. Despite this, exhaustive studies on race diversity and pattern of Fod distribution in this area are lacking [5].

Fod can be distinguished into physiological races on the basis of the capacity of the pathogen to attack differential carnation cultivars. To date, ten races have been described worldwide [5]. Races 1 and 8 apparently originated in the Italian Riviera, where they are associated with Mediterranean carnation ecotypes found in Italy, France, and Spain. Race 2 is widespread in all areas of carnation cultivation in the world. Race 4 is found in carnation cultivars in the United States, Italy, Israel, Spain, and Colombia. Race 3 was initially classified as a Fod race, but DNA-based methods recently reclassified it as *F. redolens*, revealing that *F. redolens* and *F. oxysporum* not only are different species but also lack a sister group relationship [1]. The other races described have not been found in carnation crops in the Spanish areas studied so far [9].

Race classification of Fod can be established by testing its virulence using differential carnation cultivars [4, 5] or PCR

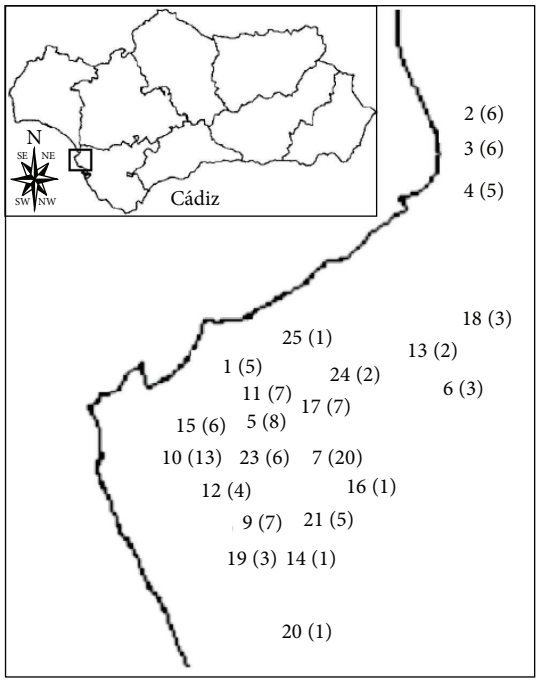

Figure 1: Wilted carnation plants collected in the north-west of the province of Cadiz. Top left: map of Andalusia showing the north-west area of the province of Cadiz. Bottom: the locations of some of the areas studied are represented in bold with code numbers and referenced in Table 1. In parentheses: numbers of isolates recovered in each zone.

with specific molecular markers to determine Fod races 2, 4, and, jointly, 1 and 8 [10]. Regarding race-specific primers, they were designed on the basis of transposable elements *Fot1* and *impala*, whose restriction profiles show a race-associated polymorphic pattern using Southern hybridization [10]. Fingerprinting techniques (such as random amplification of polymorphic DNA polymerase chain reaction, RAPD-PCR) have been applied to evaluate the level of genetic diversity in various *F. oxysporum formae speciales,* including *dianthi* [3, 4, 11]. This technique can also separate Fod races [4, 11]. Moreover, previous studies to establish a reliable molecular marker for phylogenetic studies on different Fusarium species have been conducted. The *TEF1-α* gene is considered highly informative for differentiating *Fusarium* spp. and many *formae speciales* within the *F. oxysporum* species complex [12–15]. These phylogenetic techniques could provide further information and reinforce the separation of Fod races using specific markers.

The determination of race and genetic diversity in pathogen populations in a given region provides relevant information of practical significance [16], which is useful for developing resistance breeding programs aimed at selecting cultivars that can reduce the devastating effects of *Fusarium* wilt in a given area [17, 18]. The aim of this study was to survey the racial diversity and prevalence of a Fod population in the north-west of the Cádiz province, a region with a long tradition of carnation cultivation, using phenotypic and molecular assays.

2. Materials and Methods

2.1. Sampling Process. Wilted carnation plants were sampled from 54 randomly selected farms in three municipal areas of Andalucia, Spain (Chipiona, Sanlucar de Barrameda, and Rota) from 2004 to 2006. One isolate was obtained from each disease focus in different greenhouses per farm. The sampling locations are shown in Table 1 and Figure 1.

2.2. Pathogen Isolation. Plants were cut at the stem base and the obtained pieces were surface-sterilized using 10% (v/v) sodium hypochlorite for 2 min. After two wash steps in sterile distilled water (SDW), the stem fragments were air-dried and transferred to Komada's *Fusarium* semiselective medium [19]. Plates were incubated at 25°C and fungi were monitored daily. Later, fungi were transferred to potato dextrose agar (PDA, Merck) plates. A total of 132 isolates displaying *Fusarium* morphology were recovered. Two monosporic cultures from each isolate were stored in silica gel at 5 to 10°C [20].

2.3. Total DNA Isolation. Fungi stored on silica gel were grown on sterile cellophane disk plates on PDA and incubated at 25°C for 7 to 8 days. Mycelium was recovered and transferred to a sterile Eppendorf tube. Tubes were frozen and then lyophilized (Telstar Cryodos, Telstar, UK) for 24 h. Total genomic DNA was extracted from the lyophilized isolates as described by Cassago et al. [21], with minor modifications. Approximately 0.2 g of lyophilized mycelium was used. DNA was resuspended in 40 μL of TE-Rnase (10 μg/mL) at 37°C for 30 min. Concentration and purity of total DNA were determined by spectrophotometric measurements at 260 nm and 280 nm.

2.4. RAPD-PCR Analysis. Population profiles were created by RAPD-PCR fingerprinting. Five 10-mer oligonucleotides were chosen on the basis of (i) previous genetic diversity studies with Fod and other *F. oxysporum* pathogens (OPB-01 [22, 23] and OPE-11 and OPE-16 [4]) and (ii) polymorphic band patterns (OPH-19 and OPH-20 (Operon Technologies, Alameda, CA, USA)).

The amplifications were performed in a total volume of 20 μL containing 7.5 ng μL^{-1} of genomic DNA, 1x Biotools standard reaction buffer, 2.5 mM of $MgCl_2$, 100 μM of dNTP (Biotools, Madrid, Spain), 1 μM of each primer, and 1 U of Biotools DNA polymerase (Biotools, Madrid, Spain). The amplification conditions were as follows: a denaturation step for 5 min at 94°C, followed by 45 amplification cycles of 30 s at 94°C, 1 min at 36°C, and 2 min at 72°C. A final extension step was performed for 5 min at 72°C. Reactions were performed with a thermal cycler (Gene Amp PCR System 2700, Applied Biosystems). Amplified DNA products were run on 1.5% agarose gels and separated by electrophoresis and DNA was visualized and photographed under UV light with a Transilluminator (BioDoc-it System, UVP).

Each band in the RAPD profiles was considered an independent locus with two alleles and transformed into binary codes on the basis of presence versus absence of amplification products (1 for presence and 0 for absence). The amplification products of different primers recorded on photos were scored

Table 1: *Fusarium oxysporum* populations from diseased carnation cultivars in the north-west of the province of Cadiz, ordered by farm.

Isolate[a]	Municipal areas[b]	Zones[c]	Code zone[d]	Farm[e]	Cultivar[e]	Race[f]
C4-06	Chipiona	Espantamonos	1	1a	Ashely	R1-8
C8-13	Chipiona	Espantamonos	1	1a	Ashely	R1-8
C8-15	Chipiona	Espantamonos	1	1b	Bellami	R2
C8-23	Chipiona	Espantamonos	1	1c	Pasodoble	R1-8
C9-06	Chipiona	Espantamonos	1	1c	Pasodoble	R1-8
C4-10	Sanlúcar de Barrameda	La Algaida	2	2a	Rossini	R2
C9-21	Sanlúcar de Barrameda	La Algaida	2	2a	Rossini	R2
C10-06	Sanlúcar de Barrameda	La Algaida	2	2a	Rossini	R2
C7-04	Sanlúcar de Barrameda	La Algaida	2	2b	Báltico	R2
C7-05	Sanlúcar de Barrameda	La Algaida	2	2b	Delphi	R2
C9-24	Sanlúcar de Barrameda	La Algaida	2	2b	Liberty	R2
C4-13	Sanlúcar de Barrameda	La Algaida Punta del Muro	3	3a	Liberty	R1-8
C9-02	Sanlúcar de Barrameda	La Algaida/Punta del Muro	3	3b	Liberty	R2
C9-10	Sanlúcar de Barrameda	La Algaida/Punta del Muro	3	3b	Liberty	R2
C9-03	Sanlúcar de Barrameda	La Algaida/Punta del Muro	3	3c	Ivana Orange	R2
C9-04	Sanlúcar de Barrameda	La Algaida/Punta del Muro	3	3c	Ivana Orange	R2
C9-11	Sanlúcar de Barrameda	La Algaida/Punta del Muro	3	3c	Ivana Orange	R2
C4-17	Sanlúcar de Barrameda	La Algaida C/C	4	4a	Gram Slam	R1-8
C4-18	Sanlúcar de Barrameda	La Algaida C/C	4	4a	Gram Slam	R1-8
C10-08	Sanlúcar de Barrameda	La Algaida C/C	4	4a	Gram Slam	R1-8
C4-19	Sanlúcar de Barrameda	La Algaida C/C	4	4b	Orange Prestige	R2
C4-20	Sanlúcar de Barrameda	La Algaida C/C	4	4b	Orange Prestige	**R1-8**
C4-21	Chipiona	Los Rizos	5	5a	Gaudina	R1-8
C4-22	Chipiona	Los Rizos	5	5a	Gaudina	R1-8
C4-23	Chipiona	Los Rizos	5	5a	Gaudina	R1-8
C5-01	Chipiona	Los Rizos	5	5b	Liberty	R2
C5-16	Chipiona	Los Rizos	5	5b	Liberty	R2
C6-21	Chipiona	Los Rizos	5	5b	Liberty	R2
C5-05	Chipiona	Los Rizos	5	5c	While Ashley	R1-8
C5-06	Chipiona	Los Rizos	5	5c	While Ashley	R1-8
C5-03	Sanlúcar de Barrameda	Hato de la Carne	6	6a	Barbara	R1-8
C5-04	Sanlúcar de Barrameda	Hato de la Carne	6	6a	Barbara	R1-8
C6-23	Sanlúcar de Barrameda	Hato de la Carne	6	6a	Barbara	R1-8
C5-07	Chipiona	Las Machuelas	7	7a	Gaudina	R1-8
C5-08	Chipiona	Las Machuelas	7	7a	Gaudina	R1-8
C5-09	Chipiona	Las Machuelas	7	7a	Gaudina	R1-8
C5-19	Chipiona	Las Machuelas	7	7b	Pilar	R2
C5-20	Chipiona	Las Machuelas	7	7b	Pilar	R2
C5-24	Chipiona	Las Machuelas	7	7b	Pilar	R2
C6-14	Chipiona	Las Machuelas	7	7c	Pilar	NB
C6-15	Chipiona	Las Machuelas	7	7c	Pilar	NB
C7-02	Chipiona	Las Machuelas	7	7c	Pilar	NB
C7-08	Chipiona	Las Machuelas	7	7d	Capineira	R1-8
C10-11	Chipiona	Las Machuelas	7	7d	Capineira	R1-8
C10-24	Chipiona	Las Machuelas	7	7d	Capineira	R1-8
C7-09	Chipiona	Las Machuelas	7	7e	Natila	R1-8
C9-07	Chipiona	Las Machuelas	7	7e	Natila	R1-8
C10-20	Chipiona	Las Machuelas	7	7e	Natila	R1-8
C8-03	Chipiona	Las Machuelas	7	7f	Ticotico	R2
C8-04	Chipiona	Las Machuelas	7	7f	Ticotico	R2
C8-05	Chipiona	Las Machuelas	7	7f	Ticotico	R2

Table 1: Continued.

Isolate[a]	Municipal areas[b]	Zones[c]	Code zone[d]	Farm[e]	Cultivar[e]	Race[f]
C10-04	Chipiona	Las Machuelas	7	7f	Ticotico	**R1-8**
C10-13	Chipiona	Las Machuelas	7	7g	Star	**R1-8**
C5-21	Chipiona	Huerta San Juan	8	8a	Eveling	R2
C6-24	Chipiona	Huerta San Juan	8	8a	Eveling	R2
C9-20	Chipiona	Huerta San Juan	8	8a	Eveling	R2
C5-22	Chipiona	Majada de las vacas	9	9a	Máster	NB
C5-23	Chipiona	Majada de las vacas	9	9a	Master	NB
C5-25	Chipiona	Majada de las vacas	9	9a	Master	R1-8
C6-01	Chipiona	Majada de las vacas	9	9a	Master	NB
C6-03	Chipiona	Majada de las vacas	9	9b	Pilar	R2
C6-04	Chipiona	Majada de las vacas	9	9b	Pilar	R2
C6-07	Chipiona	Majada de las vacas	9	9b	Pilar	R2
C6-06	Chipiona	Escalereta	10	10a	Báltico	R2
C6-08	Chipiona	Escalereta	10	10a	Báltico	R2
C10-10	Chipiona	Escalereta	10	10a	Báltico	R2
C6-09	Chipiona	Escalereta	10	10b	Maestro	R2
C6-10	Chipiona	Escalereta	10	10b	Maestro	R2
C6-25	Chipiona	Escalereta	10	10b	Maestro	R2
C7-12	Chipiona	Escalereta	10	10c	Imagine	**R1-8**
C7-13	Chipiona	Escalereta	10	10c	Imagine	**R1-8**
C7-14	Chipiona	Escalereta	10	10c	Imagine	R2
C7-17	Chipiona	Escalereta	10	10c	Imagine	R2
C7-20	Chipiona	Escalereta	10	10c	Imagine	**R1-8**
C9-18	Chipiona	Escalereta	10	10c	Imagine	R2
C10-03	Chipiona	Escalereta	10	10c	Imagine	R2
C6-11	Chipiona	Pinar Martín	11	11a	Berry	R1-8
C7-01	Chipiona	Pinar Martín	11	11a	Berry	R1-8
C6-12	Chipiona	Pinar Martín	11	11b	Medea	R1-8
C6-13	Chipiona	Pinar Martín	11	11b	Medea	R2
C8-10	Chipiona	Pinar Martín	11	11c	Rayo de Sole	R2
C8-11	Chipiona	Pinar Martín	11	11c	Rayo de Sole	R2
C10-05	Chipiona	Pinar Martín	11	11c	Rayo de Sole	**R1-8**
C6-16	Chipiona	El Olivar	12	12a	Dover	R2
C6-17	Chipiona	El Olivar	12	12a	Dover	R2
C7-03	Chipiona	El Olivar	12	12a	Dover	R2
C11-03	Chipiona	El Olivar	12	12b	Arena	R2
C7-06	Sanlúcar de Barrameda	Madre del Agua	13	13a	Firato	R2
C10-17	Sanlúcar de Barrameda	Madre del Agua	13	13a	Firato	R2
C7-11	Chipiona	Majadales	14	14a	Tempo	R2
C7-16	Chipiona	Los Llanos	15	15a	Lolita	R2
C7-18	Chipiona	Los Llanos	15	15a	Lolita	R2
C11-04	Chipiona	Los Llanos	15	15a	Lolita	R2
C10-14	Chipiona	Los Llanos/Envidio	15	15b	Claudia	R1-8
C10-15	Chipiona	Los Llanos/Envidio	15	15b	Claudia	R1-8
C10-16	Chipiona	Los Llanos/Envidio	15	15b	Claudia	R1-8
C8-02	Chipiona	Cerro Colón	16	16a	Delfi	R2
C8-06	Chipiona	Loma Baja	17	17a	Splendido	R2
C8-07	Chipiona	Loma Baja	17	17a	Splendido	R2
C8-08	Chipiona	Loma Baja	17	17a	Splendido	**R1-8**
C8-09	Chipiona	Loma Baja	17	17a	Splendido	**R1-8**
C8-19	Chipiona	Loma Baja	17	17b	Eilat	R2

Table 1: Continued.

Isolate[a]	Municipal areas[b]	Zones[c]	Code zone[d]	Farm[e]	Cultivar[e]	Race[f]
C8-20	Chipiona	Loma Baja	17	17b	Eilat	R2
C8-24	Chipiona	Loma Baja	17	17b	Eilat	R2
C8-22	Sanlúcar de Barrameda	Santa Tecla	18	18a	Montezuma	R2
C8-14	Sanlúcar de Barrameda	Santa Tecla	18	18b	Tauroc	R1-8
C9-05	Sanlúcar de Barrameda	Santa Tecla	18	18b	Tauroc	R1-8
C8-21	Chipiona	Majadales alto	19	19a	Solar Oro	R1-8
C9-17	Chipiona	Majadales alto	19	19b	White Fleurette	R1-8
C11-02	Chipiona	Majadales alto	19	19c	Millenium	R2
C9-08	Rota	Peña del Águila	20	20a	Rebeca	R1-8
C4-01	Chipiona	Aulagar	21	21a	Pink Ashley	R2
C4-02	Chipiona	Aulagar	21	21a	Pink Ashley	R2
C4-03	Chipiona	Aulagar	21	21a	Pink Ashley	R2
C4-04	Chipiona	Aulagar	21	21b	Pink Ashley	R2
C7-10	Chipiona	Aulagar	21	21b	Pink Ashley	R2
C4-14	Chipiona	Monteruco	22	22a	Cerise R. Barb.	**R1-8**
C4-15	Chipiona	Monteruco	22	22a	Cerise R. Barb.	**R1-8**
C4-16	Chipiona	Monteruco	22	22a	Cerise R. Barb.	**R1-8**
C10-07	Chipiona	Monteruco	22	22a	Cerise R. Barb.	R1-8
C7-07	Chipiona	Monteruco	22	22b	Orbit	R1-8
C9-14	Chipiona	Monteruco	22	22b	Orbit	R1-8
C9-25	Chipiona	Monteruco	22	22b	Orbit	R1-8
C7-21	Chipiona	Lopina	23	23a	Nogalte	R2
C7-22	Chipiona	Lopina	23	23a	Nogalte	R2
C7-23	Chipiona	Lopina	23	23a	Nogalte	R2
C7-24	Chipiona	Lopina	23	23a	Nogalte	R2
C7-25	Chipiona	Lopina	23	23a	Nogalte	R2
C8-01	Chipiona	Lopina	23	23a	Nogalte	R2
C9-15	Chipiona	La Loma	24	24a	Spectro	**R1-8**
C9-19	Chipiona	La Loma	24	24b	Spectro	R1-8
C10-02	Chipiona	Copina	25	25a	Delphi	R2

[a]Names of population isolates

[b]Municipal areas (Chipiona, Sanlúcar de Barrameda, and Rota)

[c]Zones in which farms were studied

[d]A random code assigned to each zone

[e]Carnation cultivar finding in the corresponding farm, which isolates were obtained. Code zone with the same number but different letters means same zone but different farms

[f]*Fusarium oxysporum* f. sp. *dianthi* (Fod) determination using specific primers [10]. R1-8: Fod race 1 or race 8; R2: Fod race 2; R1-8 in bold: retarded electrophoretic mobility band; NB: nonamplified band.

manually and a binary matrix was thus generated [24]. Forty-one markers were analyzed using five operon primers. From the binary data matrix, the distances were estimated using the distance estimation method described by Link et al. [25], using TREECON [26] of the Windows software package. Tree topology was inferred with the UPGMA (unweighted paired group method with arithmetic averages) clustering method to construct an unrooted tree [24]. Amplifications were repeated once with each monosporic culture from each isolate.

2.5. Isolate Determination by DNA Sequencing. A portion of the translation elongation factor 1-α gene (*TEF1-α*) was amplified using EF1 and EF2 primers. Each 50 μL of PCR reaction contained 7.5 ng μL^{-1} of genomic DNA, 1x Biotools standard reaction buffer, 3.75 mM of $MgCl_2$, 100 μM of dNTP (Biotools, Madrid, Spain), 0.5 μM of each primer, and 1 U of Biotools Pfu DNA polymerase (Biotools, Madrid, Spain). The PCR for both primer pairs was run at 95°C for 2 min, followed by 10 cycles of 94°C for 1 min, 54°C for 2 min, and an elongation phase at 72°C for 2 min, and 20 cycles of 94°C for 1 min, 54°C for 2 min, and an elongation phase at 72°C for 2 min with an increase of 4 s per cycle, concluded by a final elongation phase at 72°C for 5 min. Reactions were performed with a thermal cycler (Gene Amp PCR System 2700, Applied Biosystems). Amplified DNA products were run on 1.5% agarose gels and DNA was photographed under UV light, as described previously. The expected amplification product size was 700 bp. PCR products were purified using Speedtools PCR Clean-Up Kit (Biotools, Madrid, Spain). Sequencing was performed by Secugen (Secugen, Madrid, Spain).

Table 2: Response of five carnation cultivars tested to races 1, 2, 4, 6, and 8 of *Fusarium oxysporum* f. sp. *dianthi*.

	Race 1	Race 2	Race 4	Race 6	Race 8
Eilat	R	S	R	R	R
New Elsy	S	R	R	R	R
Suprema	R	S	NT	NT	R
Reina	R	S	R	NT	NT
Mayor	S	S	NT	S	NT

R: resistant; S: susceptible; NT: not tested.

Table 3: Selected isolates from the RAPD study.

Isolate[a]	Municipal area[b]	Species name[c]	Race[d]	RAPD Group	TEF Group	GenBank accession[e]
C5-20	Chipiona	*F. oxysporum*	2	I	I	GU199330
C6-15	Chipiona	*F. oxysporum*	NB	I	I	GU199333
C6-25	Chipiona	*F. oxysporum*	2	I	I	GU226824
C9-10	Sanlúcar de B.	*F. oxysporum*	2	II	I	GU226828
C5-23	Chipiona	*F. oxysporum*	NB	IV	IV	GU199331
C6-01	Chipiona	*F. oxysporum*	NB	IV	IV	GU199332
C10-07	Chipiona	*F. oxysporum*	1-8	V	IV	GU226826
C8-08	Chipiona	*F. oxysporum*	RB	VI	IV	GU226825
C4-17	Sanlúcar de B.	*F. oxysporum*	1-8	VII	III	GU199328
C4-22	Chipiona	*F. oxysporum*	1-8	VIII	III	GU199329
C10-14	Chipiona	*F. oxysporum*	1-8	VIII	III	GU226827
C4-15	Chipiona	*F. proliferatum*	RB	IX	V	GU191842
R1	Marismas de Lebrija	*F. oxysporum*	1-8	VIII	III	
R2	Chipiona	*F. oxysporum*	2	I	I	
R4	Italy	*F. oxysporum*	4	III	II	
R8	Italy	*F. oxysporum*	1-8	VIII	III	
Fr	South África	*F. redolens*	—	—	—	

[a]R1 (race 1), R2 (race 2), R4 (race 4), R8 (Race 8), and Fr (*Fusarium redolens*)
[b]Municipal area as shown in Table 1
[c]determined by query and comparison with sequence databases using *TEF1-α* gene sequences
[d]Race assignments using specific primers; 2: Race 2; NB: no band amplification; 1-8: race 1 or race 8; RB: retarded race 1 or race 8 band; 4: race 4
[e]Accession numbers of *TEF1-α* gene sequences.

Sequences were edited and then blasted against the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To match the *TEF1-α* gene in conjunction with GenBank, the *Fusarium*-ID database (http://fusarium.cbio.psu.edu) was used. This database is publicly available and contains vouchered and well-characterized sequences of *Gibberella fujikuroi*, *F. oxysporum,* and *F. solani* species [15]. Accession numbers of *TEF1-α* regions from selected isolates are shown in Table 3.

2.6. Phylogenetic Distance Tree Using TEF1-α Gene Sequences. Sequences were edited using Sequence Scanner v1.0 software (Applied Biosystems) and aligned to construct a phylogenetic tree using the MEGA5 software [27]. Evolutionary history was inferred from the maximum likelihood method based on the Kimura 2-parameter model [28]. The tree with the highest log-likelihood (−1413.6427) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search (Close-Neighbor-Interchange algorithm) were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise, the BIONJ method with a maximum composite likelihood (MCL) distance matrix was used. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 137 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 640 positions in the final dataset.

2.7. Bacterial, F. redolens, and Fod Race-Specific PCR Assays. Bacterial 16S rDNA was used to control the presence and accessibility of eubacterial DNA, which could amplify false RAPD markers. For this purpose, 704f and 1495r primers [29] were used. Specific *Fusarium redolens* (Fr) primers, *redolens*-F, and *redolens*-R [1], were used to detect the presence of this pathogen in the population. Fod races were determined using

a multiplex PCR with race-specific primers (Ft3f, IMP2f, R8.1r, R2.1r, and R4.2r) designed by Chiocchetti et al. [10]. Primers were obtained from cVIRAL (Alcobendas, Madrid, Spain). Negative and positive controls were included in all experiments. The bacterial isolate 911 (Collection of Dr. M. Aviles, University of Seville, Spain), *F. redolens* (provided by Dr. E.T. Steenkamp, University of Pretoria, Pretoria, South Africa), and the Fod race testers (provide by Dr. J.M. Melero, CSIC, Córdoba, Spain) were used as positive controls for each PCR assay.

Twenty microliters of reaction volume containing 7.5 ng μL^{-1} of genomic DNA, 1x Biotools standard reaction buffer, 2.5 mM of $MgCl_2$, 100 μM of dNTP (Biotools, Madrid, Spain), and 1 U of Biotools DNA polymerase (Biotools, Madrid, Spain) were used. Primers were added as follows: 0.5 μM each for eubacterial DNA detection [29], 0.5 μM each for Fr detection [1], and 2.5 μM each for race determination [10]. Reactions were performed with a thermal cycler (Gene Amp PCR System 2700, Applied Biosystems) following the respective authors' protocols. After amplification, the reaction mixture was loaded onto a 1.5% agarose gel and photographed under UV light as described previously. The expected amplification product sizes were 800 bp for eubacteria, 386 bp for Fr, and 295, 564, and 1315 bp for Fod races 1/8, race 2, and race 4, respectively. Analyses were repeated one with each monosporic culture from each isolate.

2.8. Fusarium Wilt Pathogenicity Test. Determination of race is based on the response of the different cultivars to a particular isolate. For this phenotypic assay, at least one isolate of each RAPD group was selected on the basis of the similarity index after the fingerprinting study [9, 16, 23]. Pathogenicity test was performed with five different carnation (*D. caryophyllus*) cultivars (Reina, New Elsy, Mayor, Suprema, and Eilat; summarized response in Table 2) in a greenhouse in Seville (Seville, Spain) from June to August 2009. Plants were provided by Barberet & Blanc (Murcia, Spain) as bare-root cuttings (8 to 12 true-leaf stages). Fungi were grown for 7 to 10 days on AMAP (10 g L^{-1} agar, 10 g L^{-1} malt extract (Difco, Le Pont de Claix, France), and 2 g L^{-1} asparagine (Difco, Le Pont de Claix, France)) plates at 25°C. Five mL of SDW were added to the plates and the surface was scraped with a sterile spatula. Inocula were filtered through two layers of sterile gauze. The concentration of conidia was determined with a hemocytometer and then diluted in SDW to give suspensions of 10^6 conidia mL^{-1}. These suspensions were used to inoculate carnation cuttings by root dipping for 30 min using 50 to 80 mL per plant. Controls were carried out by dipping plant roots in sterile water. Inoculated cuttings were individually planted in pots containing peat fertilized with 0.5 g L^{-1} of Peter's Foliar Feed (27 + 15 + 12; N + P_2O_5 + K_2O, and micronutrients; Scotts, Heerlen, the Netherlands) and 0.5 g L^{-1} of Fertigreen (Burés, Barcelona, Spain). The experimental design involved five plants for each isolate and cultivar combination, randomized within each cultivar.

Disease severity was scored using the symptom severity scale described by Baayen and van der Plas [30] as follows: 0 = asymptomatic plant (0% disease); 1 = weakly affected plant (5%); 2 = local base-stem symptoms (20%); 3 = unilateral and well-developed symptoms (50%); 4 = strong disease symptoms throughout the plant (80%); 5 = dead plant (100%). Disease severity was monitored twice a week. At each assessment, the mean disease severity per pot was calculated. The standardized area under the disease progress curve (AUDPC) was calculated by disease severity integrated between symptom onset and the final time of the assay and divided by the total duration (days) of the epidemic in each bioassay. Therefore, AUDPC data can range from 0 to 1. High severity was considered when AUDPC was higher than 0.45. Moderate severity was considered when AUDPC data range between 0.45 and 0.30 and low severity was considered when AUDPC data range between 0.30 and 0.20. Pathogenicity test was repeated twice. Each test lasted three months.

2.9. Statistical Analyses. Data collected from the pathogenicity bioassay were analyzed with the Statgraphics Plus program (version 5.1; Statistical Graphics Corp., Rockville, MD). Data were analyzed using one-way ANOVA. The means were compared using Tukey's test ($P < 0.05$).

3. Results

3.1. Classification of Isolates Using RAPD Analysis. All isolates were independently subjected to RAPD-PCR analysis employing five random decamer primers. In the preliminary experiments, all the tested primers produced distinct and reproducible band profiles (Figure 2). Reproducible markers were used in cluster analysis. On the basis of a similarity matrix, a dendrogram using the UPGMA was constructed to illustrate the level of similarity among isolates (Figure 3(a)). According to the patterns of all five primers, isolates were classified into nine RAPD groups "haplogroups," corresponding to a similarity of more than 90%. Each RAPD group showed high genetic homogeneity.

Distance-based analyses of the RAPD groups are shown in Figure 3(a). Cluster analysis separated the Fod race testers with an approximately 60% similarity index, with a bootstrap support of 93%. Seventy-three isolates (plus the Fod race 2 tester) were placed in RAPD group I. The RAPD group II showed one isolate (C9-10), while Fod race 4 was clustered in RAPD group III (Figure 3(a)). Between RAPD groups III and VII, seven isolates were clustered in three distinct groups (RAPD groups IV, V, and VI), The RAPD group VII showed only one isolate (C7-14). Forty isolates were clustered in RAPD group VIII, together with the Fod races 1 and 8 testers. Finally, ten isolates were outgroups (Figure 3(a)) and clustered in RAPD group IX; *F. redolens* was also clustered as an outgroup, closer to RAPD group IX.

3.2. Phylogeny Using the TEF1-α Sequence. Phylogenetic analysis of the partial *TEF1-α* gene from all isolates (Figure 3(b)) revealed five groups and *F. redolens* as an outgroup. The Fod race 2 tester was found in TEF group I, along with 74 isolates (the same as RAPD groups I and II). Race 4 tester, as in RAPD analysis, was the only one to cluster in TEF group II. Races 1 and 8 testers and 41 isolates (the same as RAPD groups VII

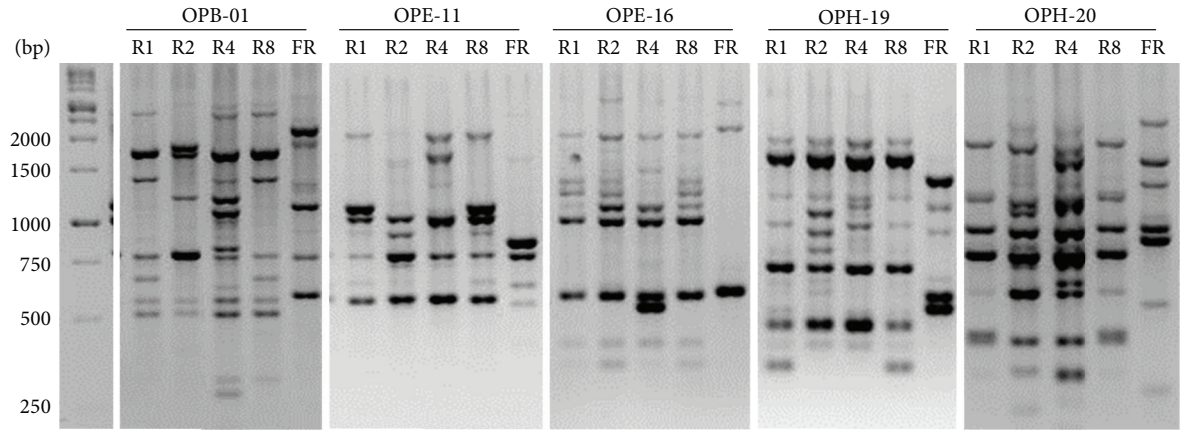

Figure 2: Random amplified polymorphisms DNA (RAPD) patterns of the different Fod race testers and one *F. redolens* obtained with five primers. Left: molecular weight marker (1 kb DNA ladder). Races belonging to R1: Fod race 1, R2: Fod race 2, R4: Fod race 4, R8: Fod race 8, and FR: *Fusarium redolens*.

and VIII) were clustered in TEF group III. Isolates clustered in the RAPD groups IV, V, and VI (Figure 3(a)) were in TEF group IV (Figure 3(b)), while TEF group V comprised the same ten isolates as RAPD group IX. The *TEF1-α* gene region from C4-15 (a representative isolate from RAPD group IX) was subjected to a BlastN enquiry in GenBank, which showed that this isolate had high sequence similarity to *F. proliferatum* (a single mismatch) and had 100% similarity with isolate 165PG/F (Accession no. GU066714).

3.3. Bacterial, F. redolens, and Fod Race-Specific PCR Assays. Race-correlated amplifications from 95.45% of the isolates were obtained using race-specific primers, as described by Chiocchetti et al. [10]. For the 132 isolates, Forty-three and seventy of the isolates produced a 295 bp (diagnostic for race 1 or race 8) or a 564 bp (specific for race 2) amplification product, respectively. The 1,315 bp amplification product, corresponding to race 4, was never detected in the sampled isolates. Thirteen isolates produced race 1 or race 8 band with retarded electrophoretic mobility, while six isolates did not show any amplification signal/band. All isolates are classified in Table 1 by race using race-specific primers. Neither *F. redolens* nor eubacteria were detected among the tested isolates. Race characterization and presence of specific amplification bands for *F. redolens* (Figure 4(b)), eubacteria (Figure 4(c)), and certain isolates (Figure 4(a)) are shown in Figure 4.

Isolates C6-14, C6-15, and C7-02 (from RAPD group I and TEF group I, where Fod race 2 tester is included) did not show a race-specific band. Furthermore, seven isolates were distributed in three RAPD groups (RAPD groups IV, V, and VI) that were clustered in the same TEF group (TEF group IV). RAPD group IV (three isolates) did not produce any race-specific band. RAPD group V (only one isolate) showed a typical race 1- or race 8-specific band. RAPD group VI (three isolates) showed an unusual race 1 or race 8 specific band with retarded electrophoretic mobility. Similarly, isolates from RAPD group IX (all included in the TEF group V) showed race 1 or race 8 band with retarded electrophoretic mobility (see isolate C4-15). Certain isolates from those RAPD groups are shown in Figure 4(a).

Isolates C10-07 (RAPD group V), C8-08 (RAPD group VI), and C4-15 (RAPD group IX) and the Fod race 8 tester showed an amplification product (race 1 or race 8 band with retarded electrophoretic mobility, ~295 bp) only with the race-specific primers Ft3/R8.1. As expected, sequencing of this amplicon (Figure 5) showed that it included the 3′ end of *Fot1* transposon and *Fot1*-flanking genomic region (Fot1-37, GenBank accession X64799), similar to that observed in the Fod race 8 tester. Moreover, the difference in mobility of the amplified bands from isolates C4-15 and C8-08 could be explained by an additional sequence of 17 nucleotides (CAGGGGGGGTCGGTTAC) located downstream to the 3′ inverted terminal repeat of the *Fot1* transposon, which was not observed in the Fod race 8 tester.

As complementary data, to determine whether the *Fot-1* transposon was moved or the primer target sequences modified, Southern blot analyses using a *Fot1*-specific probe were performed with two isolates (C6-14 and C6-15), without specific amplification but clustered near the Fod race 2 tester by both phylogenetic analyses. The restriction pattern confirmed that these two isolates had the same restriction pattern as the Fod race 2 tester, therefore confirming that they indeed belonged to Fod race 2 (data not shown).

3.4. Pathogenicity Assay. Given the high number of isolates, a pathogenicity test was conducted with a subset of the population (Table 3) using RAPD analysis as the reference. For this purpose, at least one representative isolate from each RAPD group was selected. Cultivars "Suprema," "Reina," and "Eilat" were susceptible to isolates C5-20, C6-15, C6-25, and C9-10 and race 2 tester but resistant to others (Table 4). But only "Eilat" showed moderate severity when inoculated with race 4 tester. Moreover, the cultivar "Reina" showed lower severity values than cultivars "Suprema" and "Eilat". The opposite was found with the cultivar "New Elsy," which was susceptible to isolates C4-17, C4-22, and C10-14 and to race 1 tester but not race 8 tester. This cultivar could be

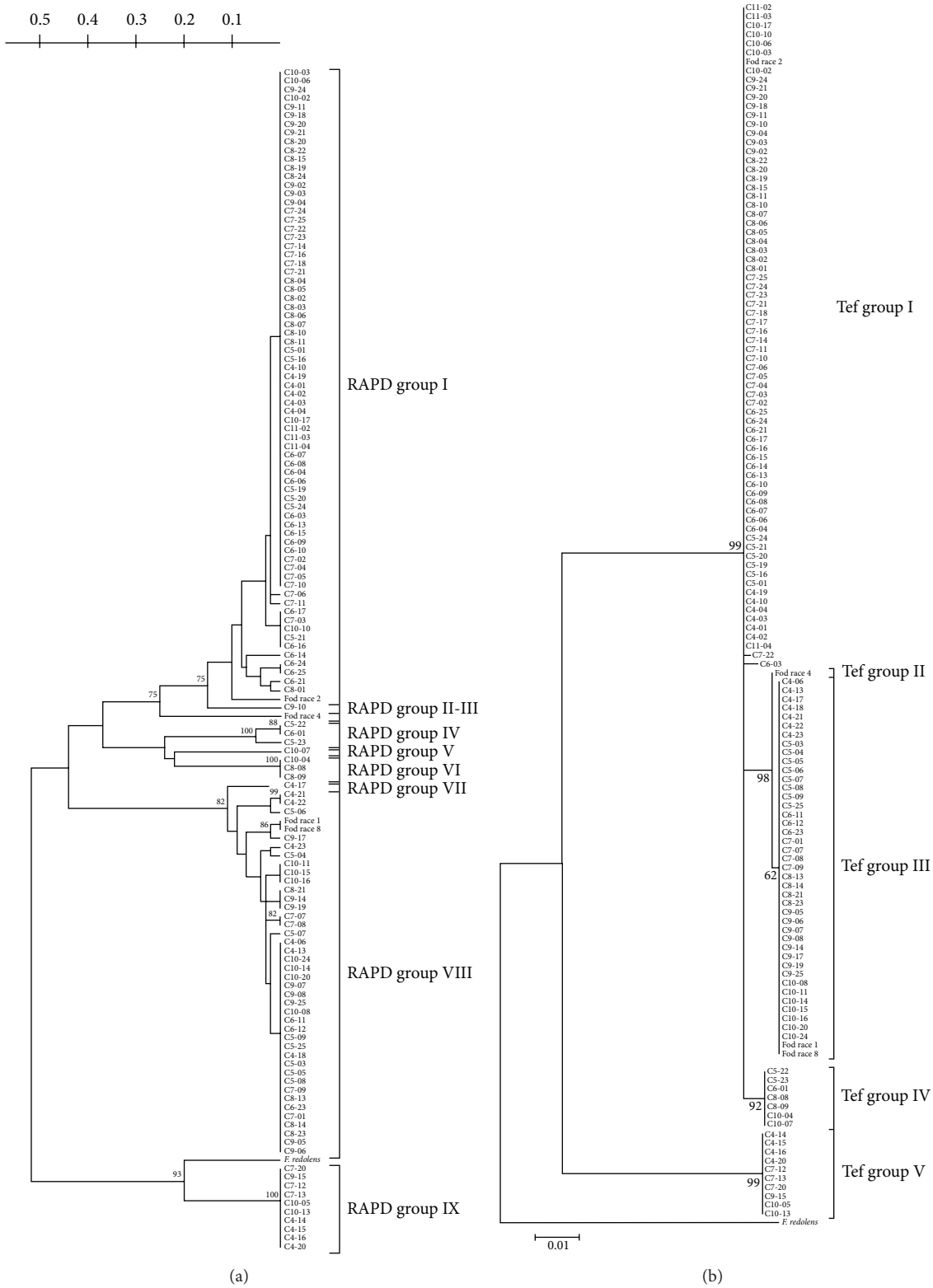

Figure 3: Phylogenetic analyses using molecular tools: (a) cluster analysis of the RAPD data was performed by the UPGMA method. RAPD groups are shown on the right of the tree. (b) Most likelihood trees were generated by *TEF1-α* gene sequencing. Evolutionary history was inferred from the maximum likelihood method based on the Kimura 2-parameter model. The tree with the highest log-likelihood (−1413.6427) is shown. TEF groups are shown on the right of the tree. The numbers on the branches are values from bootstrap analysis, in percentages, with 100 replications for both types of phylogenetic analyses.

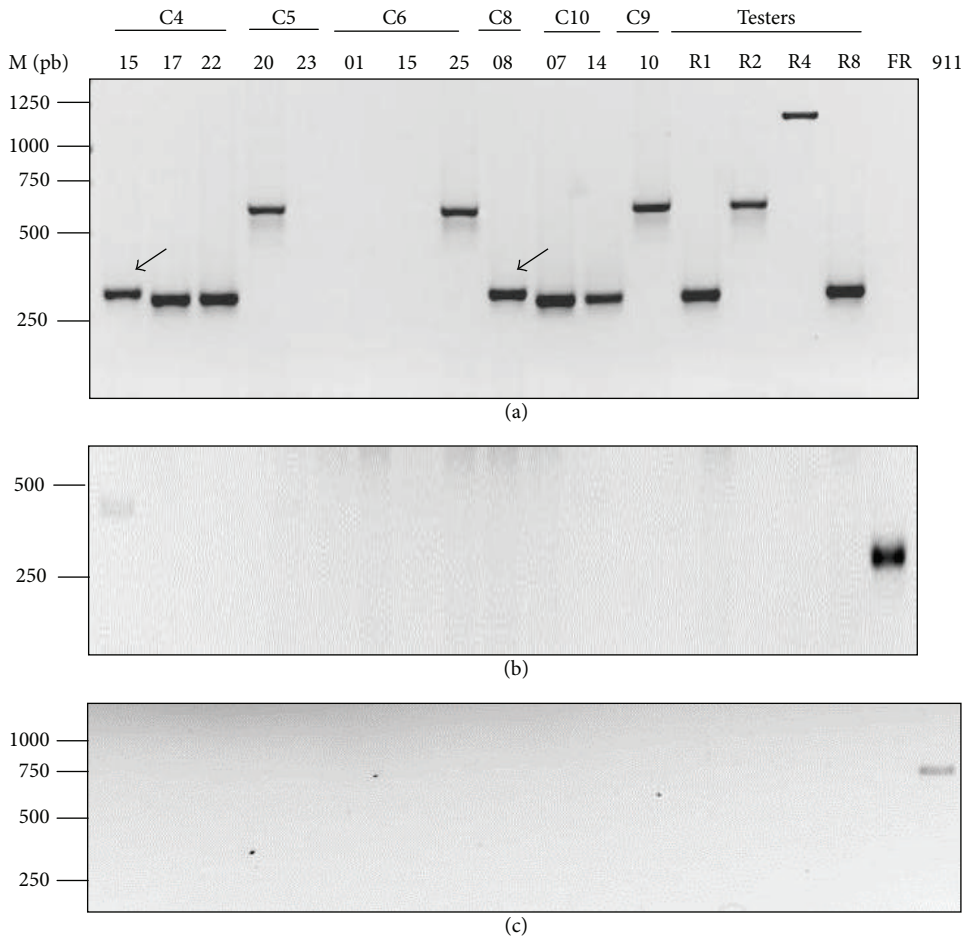

Figure 4: Specific Fod-race primers showing different amplification products. Agarose gel electrophoresis (1.5%) of PCR products from the genomic DNA of 12 representative RAPD group isolates, four Fod testers, and one *Fusarium redolens* isolate. (a) Multiplex PCR in which five primers were mixed in the same reaction tube, where combinations of Ft3/R8.1, Ft3/R2.1, and Ft3/R4.2 resulted in bands of 295 (race 1/8), 564 (race 2), and 1315 bp (race 4), respectively; bands with retarded electrophoretic mobility are shown with an arrow. (b) Multiplex PCR products obtained with the LSU (not shown) and *Fusarium redolens*-specific primers, with a resulting band of 386 bp. (c) PCR of the eubacterial primers 704f and 1495r where a 900 bp product was not amplified. Fod testers R1 (race 1), R2 (race 2), R4 (race 4), and R8 (race 8); Fr: *Fusarium redolens* and 911 (bacterial tester). M: molecular size marker (1 kb ladder, Biotools); sizes (base pairs) are indicated on the left.

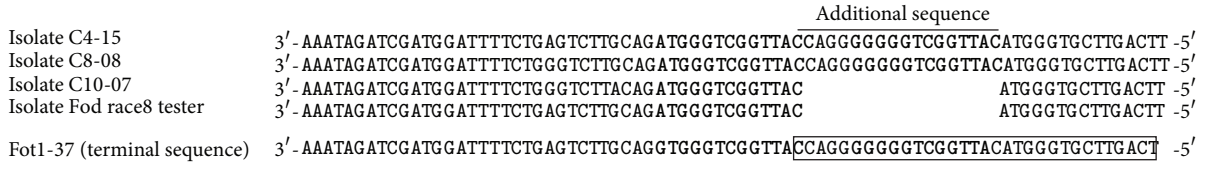

Figure 5: Aligned DNA sequences of the *Fot1* final portion and the inverted terminal repeat (ITR) from *F. melonis*; Fod race 8 (tester) and three isolates from our population, using primers Ft3/R8.1. A sequence complementary to the *Fot1* primer in the ITR is displayed in the box. The perfect direct repeats in the ITR are given in bold.

used to differentiate between races 1 and 8. Cultivar "New Elsy," considered resistant to race 2 in our assay conditions showed a moderate severity for C6-15 and C6-25 isolates and a low severity for C5-20 and C9-10. Isolate C6-15 was confirmed to belong to race 2, as expected from the results of the phylogenetic analyses (Figure 3). The isolates C5-23, C6-01, C8-08, and C10-07 were not pathogenic to any cultivar, although C10-07 isolate showed 0.19 AUDPC value in "New Elsy." In the cultivar "Mayor," these four isolates did not show severity as opposed to the other isolates and Fod races 1 and 2 testers. This last result agrees with data supported in Table 2. *F. proliferatum* (isolate C4-15) produced low disease severity in cultivars "Eilat" and "New Elsy," although severity was significantly higher than in the control (data not shown).

3.5. Fod Race Frequency and Prevalence. Taking into account all the data, the frequency and prevalence of each race in the tested area were calculated (Table 5). Regarding frequency,

Table 4: Standardized AUDPC for carnation cultivars.

RAPD Group	Isolates/Testers	Carnation cultivars				
		Suprema	Reina	New Elsy	Eilat	Mayor
I	C5-20	0.56 (0.02) a	0.34 (0.06) ab	0.23 (0.04) bc	0.67 (0.00) a	0.64 (0.02) a
I	C6-15	0.61 (0.06) a	0.43 (0.05) a	0.31 (0.02) b	0.75 (0.02) a	0.64 (0.01) a
I	C6-25	0.64 (0.03) a	0.45 (0.04) a	0.31 (0.07) b	0.74 (0.01) a	0.62 (0.01) a
II	C9-10	0.54 (0.03) a	0.47 (0.03) a	0.21 (0.03) bc	0.65 (0.02) a	0.61 (0.03) a
IV	C6-01	0.07 (0.02) bcd	0.04 (0.02) c	0.10 (0.01) cd	0.05 (0.02) c	0.03 (0.01) b
V	C5-23	0.05 (0.01) cd	0.04 (0.01) c	0.13 (0.02) cd	0.05 (0.05) c	0.02 (0.01) b
VI	C10-07	0.18 (0.06) b	0.07 (0.01) c	0.19 (0.04) bc	0.10 (0.01) c	0.01 (0.00) b
VII	C8-08	0.09 (0.02) bc	0.12 (0.04) bc	0.14 (0.02) cd	0.05 (0.01) c	0.08 (0.03) b
VIII	C4-17	0.00 (0.00) d	0.00 (0.00) c	0.56 (0.04) a	0.02 (0.01) c	0.63 (0.02) a
VIII	C4-22	0.01 (0.01) cd	0.02 (0.01) c	0.61 (0.02) a	0.02 (0.01) c	0.66 (0.02) a
IX	C10-14	0.00 (0.00) d	0.02 (0.01) c	0.58 (0.00) a	0.02 (0.01) c	0.55 (0.08) a
VIII	R1	0.01 (0.01) d	0.03 (0.01) c	0.57 (0.02) a	0.04 (0.01) c	0.54 (0.04) a
I	R2	0.50 (0.06) a	0.37 (0.04) a	0.13 (0.04) cd	0.63 (0.02) a	0.59 (0.03) a
III	R4	0.00 (0.00) d	0.13 (0.08) c	0.06 (0.02) d	0.41 (0.11) b	0.06 (0.03) b
VIII	R8	0.06 (0.01) bcd	0.05 (0.04) c	0.17 (0.01) bc	0.01 (0.01) c	0.07 (0.05) b

Each cultivar was infested using a root-dipping technique in a solution with 10^6 conidia mL^{-1} from each isolate. Testers isolates: R1, Fod race 1; R2, Fod race 2; R4, Fod race 4; and R8, Fod race 8. For each cultivar (columns), numbers with the same letter were not significantly different according to Tukey's test at $P < 0.05$. In parentheses, SE of the mean ($n = 5$).

Table 5: Frequency and prevalence of each race by PCR using specific primers and *TEF1-α* gene.

Races	Specific primers				Groups	*TEF1-α*			
	Frequency		Prevalence[a]			Frequency		Prevalence	
	Ib	Ib/Ti	*F*	*F*/tF		Ip	Ip/Ti	*F*	*F*/tF
Race 1-8	42	31.8%	21	38.9%	III	41	31.1%	20	37.0%
Race 2	71	53.8%	31	57.4%	I	74	56.1%	32	59.3%
Retarded band	13	9.8%			V (Fp)	10	7.6%	6	11.1%

[a]Prevalence was expressed as the ratio "number of farms in which a specific race was found/total farm numbers," as a percentage.
Ib: Isolates with race-specific amplified band
Ip: Isolates using TEF grouping
Ti: Total isolates ($n = 132$)
F: Numbers of farms in which a specific race *F. proliferatum* or TEF group was found
tF: Total farms ($n = 54$)
Fp: *Fusarium proliferatum*.

using Fod-specific primers, 31.8% (41 isolates plus C10-07) and 53.8% (71 isolates) of the total population belonged to race 1 or race 8 and race 2, respectively. Thirteen of the 132 isolates showed a retarded band, where ten of these could be ascribed to *F. proliferatum*. RAPD-PCR and *TEF1-α* analysis showed similar results. The *TEF1-α* analyses identified 31.1% (41 isolates) and 56.1% (71 isolates plus C6-14, C6-15, and C7-02) of the total population to belong to race 1 or race 8 and race 2, respectively.

In this study, 54 farms in 25 zones (Table 1) were sampled. Analysis with specific primers showed a prevalence of 38.9% and 57.4% for race 1 or race 8 and race 2, respectively. Phylogenetic analysis showed a different prevalence, 37.0% and 59.3% for race 1 or race 8 and race 2, respectively.

Fod race 2 and race 1 or race 8 were located together in 10 zones, but their concurrent presence was found on only one farm (11b, Table 1). Moreover, 11 zones displayed only Fod race 2, while 4 zones had only Fod races 1 or 8, indicating that Fod race 2 was spatially more widespread than Fod race 1 or race 8 in the studied area. The presence of *F. proliferatum* was independent from that of pathogenic Fod races (farms 4b, 10c, 11c, and 22a). Isolates without specific band amplification grouping in RAPD group IV (C5-22, C5-23, and C6-01) were found on the same farm (9a) together with race 1 or race 8 isolate (C5-25). Isolate C10-07, which showed a specific Fod race 1 or race 8 amplification, was found on a farm, coexisting with *F. proliferatum* (22a). RAPD group VI was identified on two farms (7f, 17a) coexisting with Fod race 2 isolates. Isolates without the race 2-specific amplicon (C6-14, C6-15, and C7-02) were found on the same farm (7c).

4. Discussion

4.1. Racial Frequency and Prevalence in the Studied Population. We conclude that Fod race 2 was the most frequent and prevalent in the study area, followed by race 1 or race 8,

which is consistent with another study in the same region [5]. However, the previous report detected a much lower frequency of race 1 than that reported here for 1 or 8. Earlier Fod race diversity reports in other Spanish regions have confirmed frequent and widespread distribution of Fod race 2 in carnation crops [9, 31]. However, race 8 has not been documented in Spain [5, 9, 31]. Genetic variation studies in this region have previously shown a race structure similar to that encountered worldwide [2, 3, 32]. Based on these data, carnations cultivars with resistance to race 2 and races 1 and 8 are the most recommended in the studied area.

4.2. Molecular Tools: RAPD-PCR, Sequencing of the TEF1-α Gene, and Diagnosis of the Fod Race-Specific Primers. Among the diagnostic protocols for *Fusarium* species, PCR assays are considered to be one of the most rapid and reliable methods [33]. RAPD fingerprinting and *TEF1-α* sequence analyses showed strikingly similar and well-resolved clades for Fod races, both approaches complementing each other. Several studies have applied RAPD fingerprinting to identify all major races of Fod present in other countries [4, 11]. Regarding sequence data, the *TEF1-α* gene has been resolved as the most phylogenetically reliable molecular marker of the *Fusarium* genus, relative to the *β*-tubulin gene, ITS regions, and *CYP51C* gene [14]. Our study demonstrated that *TEF1-α* could be used as a molecular marker to group Fod isolates into races, this method taking less time and being more reliable than RAPD-PCR. Indeed, a previous report revealed a higher level of homoplasy shown by RAPD-based phylogeny than the *TEF1-α* and mtSSU rDNA gene sequencing data, using *F. oxysporum* from maize [12]. The high genetic homogeneity within the races found in this population was consistent with that reported in earlier studies [3, 32].

We propose that PCR with specific primers, whose amplification is related with the *Fot1*-transposable element [10], is not sufficient to confirm the presence of Fod in a given area, due to differences in the target genomic sequences. Indeed, these markers showed variable specificity in our population, as described in a previous study [5]. As an example, 13 isolates showed a retarded Fod race 1 or race 8 band when the specific primers were used. Moreover, *Fot1* elements exhibit a very low level of polymorphism in the *Fusarium oxysporum* complex [34]. Our data using race-specific primers showed that Fod race 8 (and probably Fod race 1) has 17 nucleotides less in this region than other *Fusarium* species. A possible horizontal transfer of *Fot1* transposon between *Fusarium* species [34] could create a deletion in this region, differentiating isolates related to Fod from the others (i.e., *Fusarium proliferatum* and *F. oxysporum* isolate C8-08).

Although molecular techniques were reliable, they must be validated with a phenotypic pathogenicity test. Despite the reasonable degree of disease severity found in the pathogenicity test, the assay showed results that were not expected; for example, susceptible responses in New Elsy and Eilat to race 2 and race 4, respectively. Pathogenicity tests conducted in semicontrolled conditions by Ben-Yephet and Shtienberg [35] indicated that the carnation response to Fod is substantially influenced by environmental conditions (mainly solar radiation, temperature, and substrate), which could explain the observed results.

4.3. Pathogenicity Test. In our pathogenicity test, the isolate C6-15 (which did not show a Fod race 2-specific band) behaved like Fod race 2 and was as virulent as other race 2 isolates, such as C6-14 and C7-02. Southern blot analysis (using *Fot1* transposon as the probe) showed that two isolates generated the same restriction pattern as the Fod race 2 tester (data not shown [10]), suggesting that the absence of specific amplification was not due to a movement of *Fot1* but due to a change in the target sequences of these primers. Thus, we propose that the presence of these isolates (C6-14, C6-15, and C7-02) in the population could form a possible Fod race 2 subgroup. To define subgroups within Fod race 2 an extensive virulence study of isolates would have to be carried out.

Isolates selected from the seven clustered in RAPD groups IV, V, and VI and TEF group IV did not show severity in any of the carnation cultivars tested. These isolates, recovered on different farms from wilted carnations, were characterized as *F. oxysporum* by *TEF1-α* sequencing. They may be saprophytic *F. oxysporum*, possibly associated with the carnation rhizosphere, as found in previous studies [4].

Ten isolates (7.6% of the collection) belonging to *F. proliferatum* (RAPD group IX and TEF group V) were found to be nonpathogenic in our phenotypic assay. In a similar study, seven nonpathogenic isolates of *F. proliferatum,* obtained from carnation crops in Italy, Israel, and the Netherlands were clearly recognized on the basis of their RAPD fingerprint [4]. However, Aloi and Baayen [2] obtained 11 isolates of *F. proliferatum* belonging to a distinct vegetative compatibility groups from Fod, which were associated with the basal rot of carnation. This fungus has also been identified as a pathogen in asparagus [36] and as an opportunistic fungus in wheat [37] and palms [13].

5. Conclusions

In conclusion, population characterization showed that Fod race 2 was widely distributed along the north-west of Cádiz, followed by race 1 or race 8. Molecular techniques, such as *TEF1-α* sequencing or RAPD-PCR, allowed (mainly the former) a better identification and clustering of different Fod races than using Fod race-specific primers. Indeed, isolates failing to generate an amplification signal with Fod race-specific primers may indicate the presence of a Fod race 2 subgroup in this area.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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