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Molecular characterisation of vegetative compatibility groups in *Fusarium oxysporum* f. sp. *radicis-lycopersici* and f. sp. *lycopersici* by random amplification of polymorphic DNA and microsatellite-primed PCR

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Abstract

Random amplification of polymorphic DNA (RAPD-PCR) analysis was conducted on 48 isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) from different geographic regions, representing all known vegetative compatibility groups (VCGs) except VCG 0097 and VCG 0099 and on eight isolates of *F. oxysporum* f. sp. *lycopersici* (*F.o.l.*), representing VCGs 0030, 0031, 0032 and 0033. Upon UPGMA (unweighted pair-group method with arithmetic averages) analysis of 86 RAPD-PCR markers generated by 16 informative primers and 44 markers obtained with eight microsatellite primers, a close relatedness was evident for *F.o.r.l.* isolates in VCGs 0090, 0092, 0096, and, to a lesser extent, for those in VCG 0093. Representatives of VCG 0091 formed a distinct group, while *F.o.r.l.* isolates in VCGs 0094 and 0098 were not distinguishable by the tested markers, most of which were also shared by *F.o.l.* isolates belonging to VCGs 0031 and 0033. *F.o.l.* isolates in VCGs 0030 and 0032 shared most of the molecular markers. The correlation between RAPD-PCR and microsatellite genetic distance was highly significant ($R^2 = 0.77$; P by Mantel test < 0.001). The molecular variability observed in both *formae speciales* is discussed in relation to the development of *F.o.r.l.*- and *F.o.l.*-specific diagnostic tools.

Fusarium oxysporum f. sp. *radicis-lycopersici* (*F.o.r.l.*) is the causal agent of crown and root rot on tomato. First described in Japan as a new race of *F. oxysporum* f. sp. *lycopersici* (*F.o.l.*), it was later reported in a number of tomato growing areas worldwide (Jarvis, 1988). Both pathogens may occur simultaneously on the same tomato plant, causing overlapping symptoms (Katan et al., 1997; Rosewich et al., 1999; Gale et al., 2003). However, the two fungi present considerable differences in both host susceptibility (Jarvis and Thorpe, 1976; Menzies et al., 1990; Brayford, 1996) and epidemiological behaviour (Rowe et al., 1977; Jarvis, 1988; Rekah et al., 2001). Consequently, accurate distinction between the two *formae speciales* has essential diagnostic and control implications.

Physiological races of *F.o.r.l.* have not been reported, but the distribution of vegetative compatibility groups (VCG; Katan et al., 1991; Katan and Katan, 1999; Di Primo et al., 2001) and of nuclear restriction fragment length polymorphism

(RFLP) markers (Rosewich et al., 1999; Kalman et al., 2001) are indicators of a high level of genetic variation within this pathogen. Nine VCGs (0090, 0091, 0092, 0093, 0094, 0096, 0097, 0098 and 0099) were identified among isolates obtained from the Mediterranean region, Western Europe and North America (Katan et al., 1991; Katan and Katan, 1999; Rosewich et al., 1999; Di Primo et al., 2001). Based on the intensity of hyphal complementation, the VCGs 0090, 0091 and 0094 could be subdivided into at least two subgroups each. Among 191 isolates collected in five geographic regions in Italy, 65 isolates were assigned to VCG 0090; 99 to VCG 0091; 23 to VCG 0092; 2 to VCG 0093; and 2 to VCG 0096 (Di Primo et al., 2001). A similar distribution was reported for a population of the pathogen in Israel (Katan et al., 1991). Other VCGs, such as VCG 0094, which is widespread in North-Western Europe and in Florida and VCGs 0098 and 0099, found in Florida (Katan and Katan, 1999; Rosewich et al., 1999), have not been identified to date in Italy. Finally, VCG 0097 has only been reported for Belgian samples and is probably very rare (Katan and Katan, 1999). The purpose of this study was to determine the level of genetic diversity within and among VCGs of *F.o.r.l.* based on random amplification of polymorphic DNA (RAPD-PCR; Welsh and McClelland, 1990; Williams et al., 1990) and microsatellite-primed PCR (MP-PCR; Weising et al., 1995), as a first step aimed to find diagnostic markers and to explore their suitability to distinguish isolates of different VCGs of this *forma specialis*.

Forty-eight pathogenic strains, representing the most frequently observed VCGs of *F.o.r.l.*, were analysed and compared to eight reference isolates of *F.o.l.* belonging to VCGs 0030, 0031, 0032 and 0033 (Table 1). Genomic DNA was purified from lyophilised mycelium (Migheli et al., 1996). RAPD-PCR was carried out in 25 μl of reaction mix containing: 10 mM Tris–HCl, pH 9.0; 1.5 mM $MgCl_2$; 50 mM KCl; 0.2 μM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI, USA); 0.2 μM of 10-mer primer; about 5 ng of template DNA for each isolate; and 1.0 U of Red*Taq* DNA polymerase (Sigma). Thirty 10-mer oligonucleotides were randomly chosen among the series OPA, OPB, OPE and OPL (Operon Technologies, Alameda, CA, USA) and tested as primer sequences. Amplification was run in a Gene Amp PCR System 9600 (Applera, Norwalk, CT, USA) programmed for one cycle of 1 min at 94 °C; 40 cycles of 1 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C; one final cycle of 10 min at 72 °C. MP-PCR was carried out essentially as described for RAPD-PCR by using the primers ($5' \rightarrow 3'$): GAGGGTGGCGGTTCT (matching the phage M13 core sequence); $(AAC)_8$; $(AAG)_8$; $(AC)_{10}$; $(AGT)_5$; $(ATC)_5$; $(CAT)_5$; $(CT)_8$; $(GACA)_4$; $(GATA)_4$; $(GT)_8$; $(GTG)_5$; $(TATG)_4$; $(TGTC)_4$; $(TTC)_8$; choice of primers was based on previous studies on fungal characterisation by MP-PCR or restriction enzyme length polymorphism (RFLP) by using microsatellite probes (Meyer et al., 1993; DeScenzo and Harrington, 1994; Hantula et al., 1996; Geistlinger et al., 1997; Barve et al., 2001). The following amplification programme was adopted: one cycle of 5 min at 94 °C; 40 cycles of 1 min at 94 °C, 90 s at 50 °C [for primers GAGGGTGGCGGTTCT; $(AAC)_8$; $(AAG)_8$; $(AC)_{10}$; $(GT)_8$; $(GTG)_5$; $(TGTC)_4$ and $(TTC)_8$] or at 37 °C [for primers $(AGT)_5$; $(ATC)_5$; $(CAT)_5$ and $(CT)_8$; $(GACA)_4$; $(GATA)_4$ and $(TATG)_4$], 2 min at 72 °C; one final cycle of 7 min at 72 °C. Half of the amplification product was loaded in a 1.5% electrophoresis grade agarose (Gibco BRL, Carlsbad, CA, USA) gel containing 0.5 μg of ethidium bromide ml^{-1}. Electrophoresis was performed for 1 h 30 min at 3.5 V cm^{-1} in 1 × TAE (Maniatis et al., 1982) running buffer, and amplicons were observed over a UV light source. Comparison of each profile for each of the 16 most informative RAPD-PCR primers and of eight microsatellite primers was based on the presence [1] vs. absence [0] of amplicons that migrated to the same position in the gel. Bands of the same size obtained by the same primer were scored as identical and only bands repeatable in at least two experiments with the same primer at different times were evaluated. For RAPD-PCR, microsatellite markers and the combined dataset, a UPGMA topology of individual isolates using the Nei and Li (1979) distance estimator was generated using Treecon software ver 1.3b (Van de Peer and De Wachter, 1994; http://bioc-www.uia.ac.be/u/yvdp/treeconw.html). The strength of each node of the dendrogram was calculated by bootstrapping over presumed loci with 1000 replicates. Correlation between the RAPD-PCR and microsatellite pairwise genetic distance matrix was tested using the Mantel test (Mantel, 1967) implemented in

Table 1. Isolates tested in this work, listed according to vegetative compatibility groups and subgroups, geographic origin, and source

Isolate	*Forma specialis*	VCG	Geographic origin	Source[a]
DP 1	*radicis-lycopersici*	0090 I	Sicily, Italy	P.D.P
DP 51	*radicis-lycopersici*	0090 I	Calabria, Italy	P.D.P
DP 83	*radicis-lycopersici*	0090 I	Sardinia, Italy	G.I.
S1 = PVS 318	*radicis-lycopersici*	0090 I	Sardinia, Italy	A.F.
DP 251 = FRC-0-1090/1	*radicis-lycopersici*	0090 I	Canada	T.K.
S6	*radicis-lycopersici*	0090 I	Sardinia, Italy	G.I.
S13 = PVS 233	*radicis-lycopersici*	0090 I	Sardinia, Italy	A.F.
S16 = PVS 234	*radicis-lycopersici*	0090 I	Sardinia, Italy	A.F.
DP 253 = FORL II D	*radicis-lycopersici*	0090 II	Israel	T.K.
DP 254 = FORL II E	*radicis-lycopersici*	0090 II	Israel	T.K.
DP 95	*radicis-lycopersici*	0090 III	Sardinia, Italy	A.F.
S2 = PVS 319	*radicis-lycopersici*	0090 III	Sardinia, Italy	A.F.
S3 = PVS 320	*radicis-lycopersici*	0090 III	Sardinia, Italy	A.F.
S4 = PVS 321	*radicis-lycopersici*	0090 III	Sardinia, Italy	A.F.
S5 = PVS 221	*radicis-lycopersici*	0090 III	Sardinia, Italy	A.F.
S7	*radicis-lycopersici*	0090 III	Sardinia, Italy	G.I.
S8	*radicis-lycopersici*	0090 III	Sardinia, Italy	G.I.
S9	*radicis-lycopersici*	0090 III	Sardinia, Italy	G.I.
S10	*radicis-lycopersici*	0090 III	Sardinia, Italy	G.I.
S11	*radicis-lycopersici*	0090 III	Sardinia, Italy	G.I.
S12 = PVS 322	*radicis-lycopersici*	0090 III	Sardinia, Italy	A.F.
S14	*radicis-lycopersici*	0090 III	Sardinia, Italy	A.F.
DP 61	*radicis-lycopersici*	0091 I	Calabria, Italy	P.D.P
DP 63	*radicis-lycopersici*	0091 I	Calabria, Italy	P.D.P
DP 236	*radicis-lycopersici*	0091 I	Sicily, Italy	P.D.P
DP 237	*radicis-lycopersici*	0091 I	Sicily, Italy	P.D.P
DP 238	*radicis-lycopersici*	0091 I	Sicily, Italy	P.D.P
DP 25/A	*radicis-lycopersici*	0091 II	Sicily, Italy	P.D.P
DP 28	*radicis-lycopersici*	0091 II	Sicily, Italy	P.D.P
DP 31	*radicis-lycopersici*	0091 II	Sicily, Italy	P.D.P
DP 93	*radicis-lycopersici*	0092	Sardinia, Italy	G.I.
DP 155	*radicis-lycopersici*	0092	Sicily, Italy	P.D.P
DP 231	*radicis-lycopersici*	0092	Sicily, Italy	P.D.P
DP 37	*radicis-lycopersici*	0093	Sicily, Italy	P.D.P
DP 44	*radicis-lycopersici*	0093	Sicily, Italy	P.D.P
DP 264 = FORL C204/6	*radicis-lycopersici*	0093	Israel	T.K.
PB 1	*radicis-lycopersici*	0094	Florida, USA	H.C.K.
PB 11	*radicis-lycopersici*	0094	Florida, USA	H.C.K.
PB 20	*radicis-lycopersici*	0094	Florida, USA	H.C.K.
PB 84	*radicis-lycopersici*	0094	Florida, USA	H.C.K.
DP 265 = FORL 01150/6	*radicis-lycopersici*	0094	Belgium	T.K.
DP 266 = FORL 01152/31	*radicis-lycopersici*	0094	Belgium	T.K.
DP 267 = FORL C 624A/5	*radicis-lycopersici*	0096	Israel	T.K.
DP 268 = FORL C 624A/3	*radicis-lycopersici*	0096	Israel	T.K.
DP 282	*radicis-lycopersici*	0096	Sicily, Italy	P.D.P.
PB 9	*radicis-lycopersici*	0098	Florida, USA	H.C.K.
PB 19	*radicis-lycopersici*	0098	Florida, USA	H.C.K.
PB 51	*radicis-lycopersici*	0098	Florida, USA	H.C.K.
FOL M	*lycopersici*	0030	Israel	T.K.
FOL R	*lycopersici*	0030	Israel	T.K.
B-FOL 51	*lycopersici*	0031	Louisiana, USA	T.K.
OSU 451 B/25	*lycopersici*	0031	Ohio, USA	T.K.
FOL MM 59/5	*lycopersici*	0032	Arkansas, USA	T.K.
FOL MM 66	*lycopersici*	0032	Arkansas, USA	T.K.

Table 1. (Continued)

Isolate	*Forma specialis*	VCG	Geographic origin	Source[a]
FOL MM 10	*lycopersici*	0033	Arkansas, USA	T.K.
DA 1/7	*lycopersici*	0033	Florida, USA	H.C.K.

[a] *Sources*: P.D.P. – P. Di Primo, University of Reggio Calabria, Italy; A.F. – A. Franceschini, University of Sassari, Italy; G.I. – G. Ionta, Cagliari, Italy; T.K. – T. Katan, ARO-Volcani Center, Bet Dagan, Israel; H.C.K. – H. C. Kistler, University of Minnesota, USA.

PopTools ver 2.4 software (www.cse.csiro.au/CDG/poptools) with 1000 permutations.

In a first screening, RAPD-PCR and microsatellite-primed patterns were obtained with 30 RAPD-PCR primers and with 15 microsatellite primers from DNA of all the *F. oxysporum* isolates listed in Table 1. Representative results are given in Figure 1. Sixteen RAPD-PCR primers and eight microsatellite primers were selected as the most informative, generating 130 markers (Table 2).

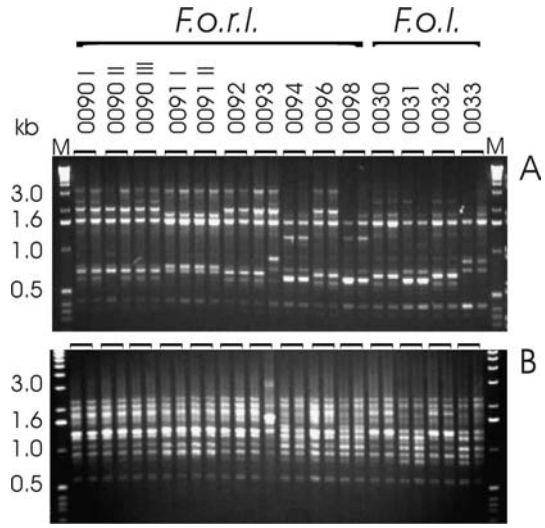

Figure 1. Comparison of amplification patterns obtained (A) by random amplified polymorphic DNA (RAPD) with primer OPL-07 and (B) by microsatellite-primed PCR with primer $(GTG)_5$ from genomic DNAs of (from left to right): isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*): DP 83, DP 251 (VCG 0090 I); DP 253, DP 254 (VCG 0090 II); S8, DP 95 (VCG 0090 III); DP 237, DP 238 (VCG 0091 I); DP 25/A, DP 28 (VCG 0091 II); DP 155, DP 231 (VCG 0092); DP 44, DP 264 (VCG 0093); PB 20, DP 266 (VCG 0094); DP 268, DP 282 (VCG 0096); PB 9, PB 19 (VCG 0098); *F. oxysporum* f. sp. *lycopersici* (*F.o.l.*): FOL M, FOL R (VCG 0030); B-FOL 51, OSU 451 B/25 (VCG 0031); FOL MM 59/5, FOL MM 66 (VCG 0032); FOL MM 10, DA 1/7 (VCG 0033); M: molecular weight marker (1 kb DNA ladder; Life Technologies, Gaithersburg, MD, USA). Molecular weight in kb of some marker bands is specified on the left margin.

Upon UPGMA analysis of RAPD-PCR data, the tested haplotypes could be clustered within two major groups: one included representatives of *F.o.r.l.* VCGs 0090, 0091, 0092, 0093 and 0096; a second cluster included isolates belonging to *F.o.r.l.* VCGs 0094 and 0098 and all the *F.o.l.* isolates (not shown).

MP-PCR experiments proved that microsatellite primers such as $(GTG)_5$, GAGGGTGGCGGTTCT, $(AC)_{10}$, $(ATC)_5$, $(GACA)_4$, $(TGTC)_4$, $(AAC)_8$ and $(AAC)_8$ allow to recognise isolates from both *formae speciales* belonging to the same VCG. It is interesting to note that the sequence $(ATC)_5$ was also identified as one of the best fingerprinting probes for races of *F. oxysporum* f. sp. *ciceris* (Barve et al., 2001). RFLP analysis of this pathogen and of *A. rabiei* (Geistlinger et al., 1997) revealed that oligonucleotide probes with a low GC content were more informative than those with a higher GC content. Our analysis, which is based essentially on MP-PCR, supports this evidence also in the case of *F.o.r.l.* and *F.o.l.*, as most of the primers generating polymorphic amplification profiles had ≥50% AT content (Table 2).

The correlation between RAPD-PCR and microsatellite genetic distance was highly significant ($R^2 = 0.77$; P by Mantel test < 0.001). To represent with a higher resolution the relationship between haplotypes, as shown for other organisms (Sun et al., 1999; Simioniuc et al., 2002), the two datasets were combined and analysed simultaneously by UPGMA. The resulting dendrogram (Figure 2) confirms the close relatedness of *F.o.r.l.* VCGs 0090, 0092, 0096 and, to a lesser extent, of VCG 0093; VCG 0091 formed a distinct group, while VCGs 0094 and 0098 were not distinguishable by the tested markers, most of which were also shared by *F.o.l.* representatives of VCGs 0031

Table 2. Primers tested in this work, sequence, total number of markers scored for each primer, and percentage of polymorphic markers between and among isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) and f. sp. *lycopersici* (*F.o.l.*)

Primer code	Sequence (5′ → 3′)	*F.o.r.l.* + *F.o.l*		*F.o.r.l.*		*F.o.l.*	
		No.[a]	% Poly.	No.	% Poly.	No.	% Poly.
RAPD analysis							
OPA-02	TGCCGAGCTG	2	100	1	100	2	50
OPB-15	GGAGGGTGTT	2	100	2	50	1	100
OPE-01	CCCAAGGTCC	7	100	6	83.3	4	100
OPE-18	GGACTGCAGA	4	100	4	100	3	100
OPL-01	GGCATGACCT	4	75	5	80	3	0
OPL-02	TGGGCGTCAA	2	50	2	50	1	100
OPL-04	GACTGCACAC	7	85.7	5	80	5	66.7
OPL-05	ACGCAGGCAC	7	100	6	100	3	100
OPL-07	AGGCGGGAAC	10	100	9	100	3	100
OPL-08	AGCAGGTGGA	11	100	9	100	7	100
OPL-10	TGGGAGATGG	2	50	2	50	2	50
OPL-11	ACGATGAGCC	7	71.4	5	60	4	50
OPL-12	GGGCGGTACT	6	100	5	100	3	66.7
OPL-13	ACCGCCTGCT	3	66.7	3	67.2	3	67.2
OPL-16	AGGTTGCAGG	6	100	5	100	4	50
OPL-17	AGCCTGAGCC	6	100	6	100	2	50
Microsatellite-primed analysis							
M02	$(GTG)_5$	8	87.5	8	50	7	85.7
M03	$(AC)_{10}$	2	50	2	50	2	50
M05	$(ATC)_5$	5	60	5	40	4	50
M07	$(GACA)_4$	8	87.5	7	85.7	6	83.3
M09	$(TGTC)_4$	7	100	7	100	2	100
M10	$(AAC)_8$	2	100	2	100	1	0
M12	$(AAG)_8$	7	42.9	6	33.3	6	0
M13	GAGGGTGGCGGTTCT	5	60	5	60	3	33.3

[a] Total number of markers (No.) and percentage of polymorphism (% Poly.).

and 0033; *F.o.l.* isolates in VCGs 0030 and 0032 shared most of the molecular markers, supporting the hypothesis that both originated from a common ancestor (Cai et al., 2003; Gale et al., 2003).

The data reported here confirmed previous analyses, which evidenced the considerable genetic diversity of both *F.o.r.l.* (Katan et al., 1991; Katan and Katan, 1999; Rosewich et al., 1999; Di Primo et al., 2001; Kalman et al., 2001) and *F.o.l.* (Elias and Schneider, 1992; Elias et al., 1993; Mes et al., 1994; Cai et al., 2003; Gale et al., 2003). *F.o.r.l.* isolates belonging to VCGs 0090, 0092 and 0096 were indistinguishable by several of the tested primers, except for two Sardinian isolates (coded S2 and S12) assigned to VCG 0090 III, which formed a distinct subgroup by analysing both RAPD-PCR and microsatellite markers. These two isolates were characterised by the presence of additional amplicons which were absent in all other tested isolates. This amplification pattern is constantly associated with the presence of two low-molecular-weight bands (at approximately 2.8–3.2 kb) when total DNA of the two Sardinian isolates is separated by agarose gel electrophoresis (unpublished results). The nature of these extra-chromosomal bands and their role as template regions for both RAPD-PCR and MP-PCR is currently under investigation. The high level of variability observed among *F.o.r.l.* isolates within VCG 0090 (which encompasses the genetic diversity detected between VCGs 0090, 0092 and 0096) suggests that VCG 0090 has the oldest evolutionary history, and that VCGs 0092 and 0096 may have evolved from VCG 0090. Of course this hypothesis may be biased by the sample-size effect.

Diverging amplification profiles were also obtained upon amplification of DNA from three isolates belonging to VCG 0093 with both RAPD-PCR and microsatellite primers. It is noteworthy that VCG 0093 formed a separate heterogeneous

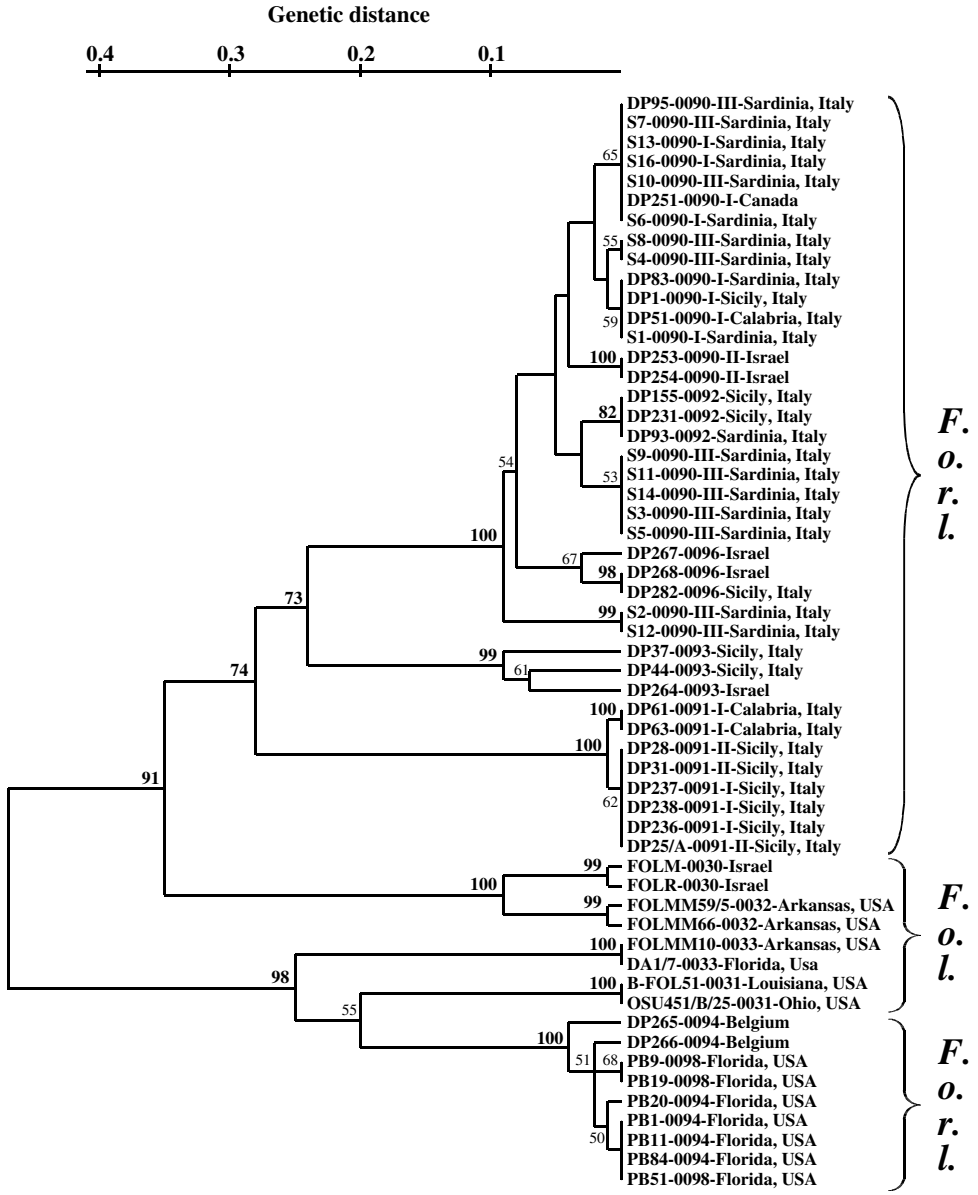

Figure 2. Dendrogram of the genetic distances (Nei and Li, 1979) among 48 isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) and eight isolates of *F. oxysporum* f. sp. *lycopersici* (*F.o.l.*) obtained by combining RAPD and microsatellite-primed PCR molecular data based on the unweighted pair-group method using arithmetic averages (UPGMA). The label of each isolate reports its code, vegetative compatibility group (VCG) and subgroup (where available) and the geographic origin. Bootstrap values (%) higher than 70 were considered significant and are represented in bold.

group upon RFLP analysis, sharing the lowest similarity coefficient with other VCGs (Kalman et al., 2001). Unfortunately, no more isolates of different geographic origin were available for further testing, as this VCG is very rare in nature. Future experiments shall be aimed at the elucidation of the genetic structure of this group of pathogens.

Aiming at developing a diagnostic tool to rapidly distinguish between *F.o.r.l.* and *F.o.l.* isolates, a comparative RAPD-PCR analysis of the two pathogens was previously attempted (Carbonell

et al., 1994). However, the fact that only two haplotypes were observed among isolates of *F.o.l.* (Carbonell et al., 1994) is in contrast to the high frequency of variation at the DNA level found in this pathogen when isolates from different VCGs were compared (Elias et al., 1993; Mes et al., 1994; Cai et al., 2003; Gale et al., 2003). This suggests that the claimed distinction between the two pathogens by analysis of RAPD-PCR markers was biased by the limited number of isolates considered in that study. To our knowledge there is no published data on the distribution of VCGs of *F.o.l.* in Italy, therefore we compared the *F.o.r.l.* isolates from our collection with two representative strains for each of the four known VCGs of *F.o.l.* The newly described VCG 0035 (Cai et al., 2003) was not considered.

The high level of molecular variability observed within and between VCGs in the *forma specialis radicis-lycopersici* hampers development of a *F.o.r.l.*-specific diagnostic tool. Even so, some markers were identified (e.g., upon amplification with RAPD-PCR primers OPL-01 and OPL-11) which allowed us to distinguish unequivocally *F.o.r.l.* isolates in VCGs 0090, 0091, 0092, and 0096 from isolates in VCGs 0094 and 0098, which have not yet been described in Italy, as well as from all *F.o.l.* isolates. Although these findings may provide a useful tool in the identification of *F.o.r.l.* isolates belonging to the prevalent VCGs in Italy, further studies are needed to develop a fast and reliable specific PCR assay based on sequence-characterised amplified regions matching diagnostic RAPD-PCR or microsatellite markers.

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