Study of Phylogenetic Relationships Among *Fusarium oxysporum* f. sp. *dianthi* Isolates: Confirmation of Intrarace Diversity and Development of a Practical Tool for Simple Population Analyses

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Abstract

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Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *dianthi*, is the most important disease of carnation worldwide. Knowing the diversity of the *F. oxysporum* f. sp. *dianthi* population present in a carnation growing area is a key component of preventing dramatic losses in production. Sequence analyses of partial β -tubulin, translation elongation factor 1 α genes, and the full-length ribosomal DNA intergenic spacer (IGS) were conducted to resolve phylogenetic relationships in a wide collection of Spanish *F. oxysporum* f. sp. *dianthi* isolates, along with some representatives from Italy. We found that, among the three different gene regions, the IGS sequence was the best choice to resolve phylogenetic relationships among *F. oxysporum* f. sp. *dianthi* isolates. The phylogenetic tree generated with the complete IGS region was the only one showing a clear clustering of isolates according to the molecular group (virulence grouping) and the vegetative compatibility group. In order to

develop a more practical tool based on a shorter DNA sequence to quickly analyze diversity in *F. oxysporum* f. sp. *dianthi* populations, we examined IGS nucleotide alignments and identified a region of approximately 300 bp that accumulates enough “informative” changes to resolve intraspecific relationships and determine pathogenic variants in *F. oxysporum* f. sp. *dianthi*. Moreover, the “condensed” alignment of this short IGS region showing only the informative positions revealed the existence of virulence group-discriminating positions. In addition to clarifying the phylogenetic relationships among *F. oxysporum* f. sp. *dianthi* isolates of the recently described race groups by using multigene genealogies, we have developed simple tools for the phylogenetic analyses of *F. oxysporum* f. sp. *dianthi* populations and the determination of the molecular group of uncharacterized *F. oxysporum* f. sp. *dianthi* isolates.

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *dianthi*, is considered the most serious and severe disease worldwide affecting carnation (*Dianthus caryophyllus* L.) (Garibaldi and Gullino 1987). Since the use of methyl bromide for disease management was banned, use of carnation cultivars resistant or tolerant to the pathogen has become a key measure to control this disease. Because cultivar resistance is usually effective against only one or a few races of *F. oxysporum* f. sp. *dianthi*, the efficient use of resistant cultivars requires a prior knowledge of the genetic diversity of the pathogen in the growing area.

Pathogenic and genetic diversity in *F. oxysporum* f. sp. *dianthi* populations has been extensively studied. The approaches used for these analyses include race determination by pathogenicity tests using differential carnation cultivars (Garibaldi 1975, 1977; Prados-Ligero et al. 2007), vegetative compatibility group (VCG) determination (Aloi and Baayen 1993; Gómez-Lama and Pérez-Artés 2014; Katan et al. 1989), restriction fragment length polymorphism (RFLP) (Manicom and Baayen 1993), a combination of VCG and RFLP analysis (Baayen et al. 1997; Lori et al. 2004; Manicom et al. 1990), esterase profiles (Baayen et al. 1997), amplified fragment length polymorphism (AFLP) (Baayen et al. 2000), and random amplified polymorphic DNA (RAPD) (Gómez-Lama et al. 2012; Manulis

et al. 1994; Migueli et al. 1998). Initially, the global model of diversity observed in *F. oxysporum* f. sp. *dianthi* included 10 physiological races that fell into six VCGs (Baayen et al. 1997; Wright et al. 1996). Each VCG was considered to have a clonal origin (Baayen et al. 1997; Lori et al. 2004); this consideration included two VCGs that comprise more than one race, because races within the same VCG shared molecular characteristics and a similar virulence spectrum (Baayen et al. 1997). Nevertheless, the homogeneous concept of *F. oxysporum* f. sp. *dianthi* races has been recently questioned (Gómez-Lama et al. 2012). Molecular analysis of a collection of 107 *F. oxysporum* f. sp. *dianthi* isolates, obtained during the period 1998 to 2008 from both soils and carnation plants in the most important growing areas in Spain, showed that genetic diversity (molecular groups) existed in race 1 (R1) and race 2 (R2) of *F. oxysporum* f. sp. *dianthi*, and that this diversity correlated with differences in virulence (virulence groups) (Gómez-Lama et al. 2012). Thus, isolates of R1 and R2 in molecular group I (R1I and R2I isolates) and isolates of R1 “type” (R1t isolates) were all highly virulent, whereas isolates of R1 and R2 in molecular group II (R1II and R2II isolates) all exhibited a low virulence profile. VCG analysis of this collection of *F. oxysporum* f. sp. *dianthi* isolates also found that, contrary to all previous results which assigned isolates of R1 and R2 to different VCGs (i.e., VCG 0022 and VCG 0021, respectively) (Gómez-Lama and Pérez-Artés 2014), isolates of R1 and R2 can share the same VCG whenever they belong to the same molecular group (virulence group). Thus, isolates of races R1I and R2I grouped together in VCG 0021, isolates of race R1t were placed in VCG 0022, and isolates of races R1II and R2II constituted the new VCG 002- (Gómez-Lama and Pérez-Artés 2014).

Knowing the phylogeny of a diverse population can contribute to an understanding of its origin and help to predict its possible evolution. In the case of *F. oxysporum* f. sp. *dianthi* populations, this knowledge could be of special relevance because they are

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highly diverse and population structure in the soil appears to have changed over time (Gómez-Lama et al. 2012). Techniques based on DNA sequence analysis are increasingly used to assess relationships among individuals. Nevertheless, their applications to the analysis of *F. oxysporum* f. sp. *dianthi* populations have been limited to inclusion of some *F. oxysporum* f. sp. *dianthi* isolates in two

broad studies of the evolutionary relationships among formae speciales of *F. oxysporum* (Baayen et al. 2000; O'Donnell et al. 2009). Therefore, the objective of the present study was to clarify the phylogenetic relationship among *F. oxysporum* f. sp. *dianthi* isolates by using multigene genealogies. For phylogenetic analyses, the genomic region chosen has to be sufficiently variable to reveal

Table 1. *Fusarium oxysporum* f. sp. *dianthi* isolates used for the phylogenetic analysis

Isolate	Geographic origin ^a	Source ^b	Year	Race ^c	VCG ^d	GenBank accession numbers		
						β-Tub	TEF	IGS
86	Lebrija (Se) Spain	Soil	2002	R1t	0022 (002-)	KJ433894	KJ433859	JQ423252.1
87	Lebrija (Se) Spain	Soil	2002	R1t	0022 (002-)	KJ433895	KJ433860	JQ423253.1
108	Chipiona (Ca) Spain	Icardina	2008	R1t	0022 (0021) (002-)	KJ433949	KJ433847	KJ433932
110	Chipiona (Ca) Spain	Megu	2008	R1t	–	KJ433950	KJ433848	KJ433933
120	Chipiona (Ca) Spain	Grand Slam	2008	R1t/R8	0022	KJ433951	KJ433849	KJ433934
128	Chipiona (Ca) Spain	Grand Slam	2009	R1t/R8	0022	KJ433952	KJ433850	KJ433935
200	Chipiona (Ca) Spain	Master	2011	R1t/R8	0022	KJ433953	KJ433851	KJ433936
103	Italy	Soil	–	R1I	0021	KJ433896	KJ433861	JQ423246.1
104	Italy	Soil	–	R1I	0021	KJ433897	KJ433862	JQ423247.1
9	Chipiona (Ca) Spain	Soil	1998	R2I	0021	KJ433898	KJ433863	JQ585759.1
14	Chipiona (Ca) Spain	Soil	1998	R2I	0021	KJ433899	KJ433864	JQ585760.1
29	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433900	KJ433865	JQ585761.1
37	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433901	KJ433866	JQ585762.1
38	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433902	KJ433867	JQ585763.1
46	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433903	KJ433868	JQ585764.1
47	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433904	KJ433869	JQ585765.1
48	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433905	KJ433870	JQ585766.1
55	Chipiona (Ca) Spain	Soil	2000	R2I	0021 (002-)	KJ433906	KJ433871	JQ585767.1
58	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433907	KJ433872	JQ585768.1
62	Chipiona (Ca) Spain	Soil	2000	R2I	0021	KJ433908	KJ433873	JQ585769.1
82	Chipiona (Ca) Spain	Soil	1999	R2I	0021	KJ433909	KJ433874	JQ585770.1
90	Chipiona (Ca) Spain	Soil	2003	R2I	0021	KJ433910	KJ433875	JQ585771.1
593	Italy	Soil	1983	R2I	0021	KJ433911	KJ433876	JQ585796.1
21	Italy	–	–	R2I	0021	KJ933912	KJ433877	JQ423254.1
751	Italy	–	1972	R2I	0021	KJ933913	KJ433878	JQ423255.1
1121I	Italy	Soil	1987	R2I	0021	–	–	JQ423256.1
MSAI	Italy	Soil	–	R2I	0021	KJ433914	KJ433879	JQ423257.1
113	Chipiona (Ca) Spain	Olimpia	2008	R2I	0021	KJ433946	KJ433844	KJ433929
116	Chipiona (Ca) Spain	Pilar	2008	R2I	0021	KJ433947	KJ433845	KJ433930
185	Chipiona (Ca) Spain	Candela	2010	R2I	0021	KJ433948	KJ433846	KJ433931
158	Chipiona (Ca) Spain	Kabul	2010	R2I	0021	KJ433957	KJ433855	KJ433940
213	Lebrija (Se) Spain	Bizzet	2011	R2I/R1I	0021	KJ433958	KJ433856	KJ433941
1	Chipiona (Ca) Spain	Soil	1998	R1II	–	KJ433915	KJ433880	JQ423248.1
6	Chipiona (Ca) Spain	Soil	1998	R1II	002-	KJ433916	KJ433881	JQ423249.1
79	Chipiona (Ca) Spain	Soil	1999	R1II	002-	KJ433917	KJ433882	JQ423250.1
102	Spain	Soil	–	R1II	002- (0021)	KJ433918	KJ433883	JQ423251.1
3	Chipiona (Ca) Spain	Soil	1998	R2II	002- (0021)	KJ433919	KJ433884	JQ585772.1
10	Chipiona (Ca) Spain	Soil	1998	R2II	002-	KJ433920	KJ433885	JQ585773.1
15	Chipiona (Ca) Spain	Soil	1998	R2II	002-	KJ433921	KJ443886	JQ585774.1
17	Chipiona (Ca) Spain	Soil	1998	R2II	002-	KJ433922	KJ443887	JQ585775.1
20	Chipiona (Ca) Spain	Soil	1998	R2II	002- (0021)	KJ433923	KJ443888	JQ585776.1
21	Chipiona (Ca) Spain	Soil	1998	R2II	002-	KJ433924	KJ443889	JQ585777.1
22	Chipiona (Ca) Spain	Soil	1999	R2II	002-	KJ433925	KJ443890	JQ585778.1
31	Chipiona (Ca) Spain	Soil	2000	R2II	–	KJ433926	KJ443891	JQ585779.1
65	Chipiona (Ca) Spain	Soil	2000	R2II	002- (0021)	KJ433927	KJ443892	JQ585780.1
67	Chipiona (Ca) Spain	Soil	2000	R2II	002-	KJ433928	KJ443893	JQ585781.1
78	Chipiona (Ca) Spain	Soil	1999	R2II	002-	KJ433944	KJ433842	JQ585782.1
84	Chipiona (Ca) Spain	Soil	1999	R2II	002- (0021)	KJ433945	KJ433843	JQ585783.1
179	Chipiona (Ca) Spain	Soil	2010	R2II/R1II	–	KJ433954	KJ433852	KJ433937
260	Chipiona (Ca) Spain	Soil	2011	R2II/R1II	–	KJ433955	KJ433853	KJ433938
275	Chipiona (Ca) Spain	Soil	2011	R2II/R1II	–	KJ433956	KJ433854	KJ433939
156	Marismas (Ca) Spain	Brunelo	2010	R1II	002- (0021)	KJ433959	KJ433857	KJ433942
256	Chipiona (Ca) Spain	Soil	2011	R1II	–	KJ433960	KJ433858	KJ433943

^a Ca = Cádiz province and Se = Sevilla province.

^b Soil or cultivar.

^c Race group assignment obtained by pathogenicity test on differential carnation cultivars and amplification of molecular markers (Gómez-Lama et al. 2012).

When race group assignment was determined only by molecular markers, the two possible assignments are separated by a bar. R1t = race 1 molecular group type, R1I = race 1 molecular group I, R1II = race 1 molecular group II, R2I = race 2 molecular group I, R2II = race 2 molecular group II, and R8 = race 8.

^d VCG = vegetative compatibility group (Gómez-Lama and Pérez-Artés 2014). VCG in parenthesis = group to which the isolate has cross-compatibility.

informative differences among individuals; we have analyzed polymorphisms at three different nuclear loci that included rapidly evolving noncoding DNA sequences derived from partial β -tubulin (β -tub) and translation elongation factor 1 α (EF-1 α) genes and the full-length intergenic spacer (IGS), which separates repeated units of the rDNA genes. In addition, analysis of our results has led to the development of practical molecular tools to quickly analyze *F. oxysporum* f. sp. *dianthi* populations, which are also described.

Materials and Methods

Fungal isolates. In total, 53 *Fusarium oxysporum* f. sp. *dianthi* isolates were used for the phylogenetic analysis (Table 1) (Gómez-Lama et al. 2012). Forty-six of these isolates were obtained in the major carnation-producing areas of Spain (provinces of Seville and Cádiz) in sampling studies conducted between 1998 and 2011. The other seven *F. oxysporum* f. sp. *dianthi* isolates came from Italy. In all, 39 of the Spanish isolates had been characterized to race group both by performing pathogenicity tests on differential carnation cultivars and by using molecular markers, while 7 of them had been assigned to a race group only by using molecular markers (Table 1) (Gómez-Lama et al. 2012). The seven isolates from Italy had been characterized by both pathogenicity tests on carnation and molecular markers. Isolates were usually recovered from soil, although some came from diseased carnation plants, using methodology described by Leslie and Summerell (2006). Single-conidia

cultures were prepared for each isolate as previously described (Gómez-Lama et al. 2012). Isolates were stored as conidial suspensions in glycerol at -80°C .

DNA isolation, polymerase chain reaction amplification, and sequencing. *F. oxysporum* f. sp. *dianthi* isolates were grown on potato dextrose agar plates for 6 to 7 days and DNA was isolated as described previously (Gómez-Lama et al. 2012). To amplify regions of the β -tub and the EF-1 α genes and the full-length IGS, we used the primer sets T1 and T2 (O'Donnell and Cigelnik 1997), EF1 and EF2 (O'Donnell et al. 1998), and CNL12 and CNS1 (Anderson and Stasovski 1992; White et al. 1990), respectively. Each amplification reaction mixture contained approximately 10 ng of DNA, 0.5 μM each primer, 200 μM deoxynucleoside triphosphates (dNTPs), and 0.2 U of Expand High Fidelity (Roche, Indianapolis, IN, USA) in the reaction buffer. Cycling conditions consisted of 35 cycles at 95°C for 35 s, 50°C for 40 s, and 72°C for 1 min (β -tub and EF-1 α) or 3 min (IGS). Each polymerase chain reaction (PCR) was preceded by an initial denaturation step at 95°C for 5 min and concluded with a final extension step at 72°C for 7 min. PCR products were purified from agarose gels using the NucleoSpin Gel and PCR clean-up kit (Mackerey-Nagel, Düren, Germany). For sequencing of the entire IGS fragment, the internal primers U46.67 (Appel and Gordon 1996) and RU46.67, CN34, and RCN61 (Mbofung et al. 2007) were used.

PCR-RFLP analysis of the short IGS region. PCR amplification of the short IGS region for diagnostic purposes was performed in 50- μl

Fig. 1. Phylogenetic trees based on neighbor-joining analysis of partial sequences of *Fusarium oxysporum* f. sp. *dianthi* isolates. A, β -Tubulin (β -tub) and B, translation elongation factor 1 α (EF-1 α). Bootstrap values are given when above 70% (1,000 replicates). *F. proliferatum* was used as an outgroup. Vertical bars on the right indicate molecular group clustering of the isolates.

Phylogenetic analysis. DNA sequences were edited and assembled using the BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Sequence alignments were performed with MUSCLE (Edgar 2004), followed by visual inspection and manual adjustment. Trees

Results

Phylogenetic analyses based on partial β -tub and EF-1 α sequences and the full-length IGS region. Partial β -tub and EF-1 α sequences were generated for 52 *F. oxysporum* f. sp. *dianthi* isolates and the full-length IGS sequence for 53 isolates (Table 1). The lengths of the sequence alignments, including gaps, were 516, 663, and 2,623 sites for the β -tub, EF-1 α , and IGS datasets, respectively. Phylogenetic trees generated from the sequence data of each region separately showed different resolutions. Thus, the phylogenetic analysis based on the NJ method of the β -tub sequence showed the lower resolution.

Fig. 2. Phylogenetic relationships inferred from neighbor-joining analysis of intergenic spacer (IGS) sequences of 53 *Fusarium oxysporum* f. sp. *dianthi* isolates. Bootstrap values are given when above 70% (1,000 replicates). *F. proliferatum* was used as an outgroup. Vertical bars on the right indicate molecular group clustering of the isolates.

followed by the tree of EF-1 α (Fig. 1). In both trees, an incongruent clustering of one of the isolates (isolate 2I) was observed. Interestingly, in the EF-1 α tree, the only branches that were consistently supported included the R It isolates and one small group of isolates of molecular group II (GII). In an attempt to obtain a higher phylogenetic signal, we also performed MP and ML analysis. However, no increase in resolution was obtained when using MP or ML with β -tub and EF-1 α datasets. The trees displayed essentially the same topology as with NJ (data not shown). The phylogenetic tree from the NJ analysis of the complete IGS sequence showed, on the other hand, a clear grouping of the isolates in clusters with bootstrap values that consistently supported the branches (Fig. 2). From the clades identified, the one that included the isolates from molecular group I (GI) and the one that included the isolates from the molecular group R It showed 100% bootstrap support, whereas the clade that included the isolates from GII showed 70% bootstrap support. Isolates in this latter group were

[illegible]

Fig. 3. Parsimony informative positions identified in the multiple sequence alignment of a short intergenic spacer (IGS) region (about 300 nucleotides) of 53 *Fusarium oxysporum* f. sp. *dianthi* isolates. Isolates from different molecular groups are separated by bold horizontal lines. When the isolates from the heterogeneous GI1 molecular group show three or more changes in these informative positions, a thin horizontal line separates them. Exclusive nucleotide changes leading to differences in restriction patterns in molecular groups GI1 and R1t are labeled.

12, and 72 for β -tub, EF-1 α , and IGS, respectively. Interestingly, from the 72 singletons in the IGS sequence, 36 belonged to the isolate *F. oxysporum* f. sp. *dianthi* 179, which indicated that the IGS sequence of this isolate was clearly different from the rest. From the sequence alignment comparisons, we appreciated that the IGS dataset contains proportionally more informative sites than the other datasets.

Identification of a short IGS region useful for phylogenetic analysis that allows molecular group discrimination. In order to develop a practical approach for routine analysis, we explored the long IGS region to try to identify a short sequence that would allow us to resolve intraspecific relationships in *F. oxysporum* f. sp. *dianthi* with a high resolution. Detailed analysis of the nucleotide alignment of the IGS region from the 53 *F. oxysporum* f. sp. *dianthi* isolates included in this study showed that a fragment of approximately 300 bp located between positions 1,303 and 1,624 of the alignment accumulated a high number ($n = 19$) of parsimony informative positions. This fragment was flanked by two conserved regions, where the specific primers SHORT IGS-F (5'-GGAGGACGATCTTGGCTGG-3') and SHORT IGS-R (5'-TATCCCGCCCTGGACGGC-3') can be used for amplification. Furthermore, the condensed alignment showing only these 19 informative positions revealed the existence of group-discriminating features in this region (Fig. 3). Thus, it was observed that the GI isolates were very homogeneous, all showing identical nucleotide composition in the 19 positions. Interestingly, the C residue found at position number 19 exclusively in this group of isolates created a unique restriction target for the enzyme *HpaII*. Therefore, amplification of this short variable region and digestion with *HpaII* can be very useful to discriminate GI isolates without the need for sequencing. On the other hand, it was also observed that the R1t isolates were very homogenous, sharing identical nucleotide composition in these 19 sites. In this group of isolates only, the T at position number 18 was substituted by a C, and this substitution resulted in the loss of a restriction target for the enzyme *XcmI*. Thus, lack of digestion with this restriction enzyme can be used as a diagnostic tool to discriminate R1t isolates. In the case of the GII isolates, we observed that the nucleotide composition of these positions was more variable but that two main subgroups could be distinguished. Furthermore, restriction analysis showed that all GII isolates lacked the *HpaII* target but contained the *XcmI* one (Supplementary Fig. S2). To validate this proposed diagnostic tool, nine *F. oxysporum* f. sp. *dianthi* isolates of known race group but not included in the phylogenetic analyses presented in this work were selected for short IGS PCR amplification and digestion (Fig. 4). As expected, PCR products from GI isolates presented restriction sites for both enzymes *HpaII* and *XcmI* and those from R1t isolates for neither; finally, PCR products from GII isolates were digested with *HpaII* but not with *XcmI* (Fig. 4B).

We further analyzed the potential of this short IGS region not only as a diagnostic tool to discriminate isolates but also to study phylogenetic relationships among *F. oxysporum* f. sp. *dianthi* isolates. The NJ phylogenetic tree generated using only the short variable IGS region showed an identical clustering and well-supported branches that the one produced with the full-length IGS sequence (Fig. 5). Thus, the clades that included isolates from GI and R1t showed a 100 and 99% bootstrap support, respectively, and the GII isolates, that formed the most heterogeneous group, showed a subgrouping identical to the one observed with the complete IGS sequences, with a bootstrap support higher than 70%. These results led us to conclude that this short region in the IGS sequence accumulates enough variation to be used for the analyses of phylogenetic relationships among *F. oxysporum* f. sp. *dianthi* isolates.

Discussion

Phylogenetic approaches are tools not only to study the origin of pathogenic diversity in phytopathogenic fungi but also to predict the potential of evolution of this diversity in the population. These studies are crucial when resistant cultivars, one of the best alternatives to control fungal diseases, are to be used. To resolve phylogenetic relationships in closely related individuals such as *F. oxysporum* f. sp. *dianthi* individuals, it is necessary to have a genomic region that accumulates enough variation, and this variation must be informative. In the present

study, rapidly evolving noncoding β -tub, EF-1 α , and IGS rDNA sequences were evaluated separately and in combination for their ability to resolve phylogenetic relationships of a collection of *F. oxysporum* f. sp. *dianthi* isolates. Isolates in this collection had been previously characterized to race by pathogenicity tests and to molecular group by multiplex-PCR amplifications, and also assigned to their correspondent VCG (Gómez-Lama and Pérez-Artés 2014; Gómez-Lama et al. 2012).

From the three genomic regions chosen for this study (β -tub, EF-1 α , and IGS), only the latter seems to contain enough variation to clearly establish relationships among *F. oxysporum* f. sp. *dianthi* isolates. Although the use of the complete IGS sequence for *F. oxysporum* formae speciales phylogenetic studies has led to some discrepancies (Mbofung et al. 2007; O'Donnell et al. 2009), it seems to work well for phylogenetic resolution within the forma specialis *dianthi*. Thus, the IGS sequence grouped *F. oxysporum* f. sp. *dianthi* isolates very efficiently, with high values of bootstrap support. Phylogenetic analyses based on IGS sequences presented here confirm previous results (Gómez-Lama and Pérez-Artés 2014; Gómez-Lama et al. 2012), in which *F. oxysporum* f. sp. *dianthi* isolates in R1 and R2 were separated into different molecular groups (I and II) that correlated with differences in virulence and different VCGs. Consistent with this, the IGS tree shows that R1 and R2 isolates in GI, all highly virulent and belonging to VCG 0021, and isolates of the same races in GII, all of low virulence and belonging to the recently described VCG 002-, constitute two distinct lineages, supported by bootstrapping of 100 and 70%, respectively. On the other hand, *F. oxysporum* f. sp. *dianthi* isolates of R1t, all highly virulent and belonging to VCG 0022, fell into a third clade (bootstrap support 100%). The two clades constituted by highly virulent isolates (GI and R1t) are very homogeneous, whereas the clade that contains the less virulent isolates (GII) is much more heterogeneous. The maximum exponent of this heterogeneity is represented by the *F. oxysporum* f. sp. *dianthi* 179 isolate, which does not cluster with any of the groups formed, in concordance with the high number of singletons found in its sequence compared with the rest. Our results also show, for the first time, a clear subgrouping of the isolates in GII. These low-virulence isolates (VCG 002-) are distributed between two subclades that exhibit different degrees of heterogeneity among them. Both subclades contain R1 and R2 isolates but only one of them (the most heterogeneous one) includes a high proportion of isolates showing cross-compatibility with one or a few isolates in VCG 0021 (GI isolates). At this point, it is interesting to note that although most of the branches in the EF-1 α tree were not well supported, the one that included one of these subgroups of GII isolates (the most homogeneous one) showed a bootstrap value of 98%. The strong support for this

Fig. 4. Polymerase chain reaction (PCR) restriction fragment length polymorphism analyses of *Fusarium oxysporum* f. sp. *dianthi* isolates from molecular groups GI, R1t, and GII. **A**, PCR products amplified with the primer pair SHORT IGS-F and SHORT IGS-R using DNA from *F. oxysporum* f. sp. *dianthi* isolates representative of the three different molecular groups GI, R1t, and GII (three of each). **B**, Single digestions of PCR products with *HpaII* and *XcmI* restriction enzymes. Arrows indicate the less stained small DNA fragments generated by the digestion. Lane M refers to molecular size marker (50-bp DNA ladder). The position of the 300-bp band is indicated.

subgroup could indicate that isolates in it were genetically closer, and differentiated from those in the other subgroup of GII isolates. The fact that, in both GII subgroups, there are isolates collected both a long time ago (i.e., 1998) and recently (i.e., 2011) seems to indicate that a diversification of GII isolates occurred in the geographical area considered at one point in the past. In contrast with the GII group, a high genetic stability appears to have been maintained in the isolates of the GI and R1t groups, regardless of their separation in time or even geographically, because all the Italian isolates included in this analysis grouped in one of these homogeneous groups. The genetic similarity observed between Spanish and Italian isolates in group R2I may indicate a common origin. Isolates of R2 are supposed to have originated in Italy (Aloi and Baayen 1993) and they may have been introduced to Spanish carnation-growing areas through propagative material. The origin of GII isolates is less clear and their higher heterogeneity points to a longer evolutionary history. Considering that carnation cultivars have been replaced over the years as more disease-resistant ones have been developed, this group of isolates could represent a native local population,

well adapted to the carnation cultivars grown at the time but weakly virulent on the ones used currently.

The fact that there is a clear association between VCGs and the groups determined by the IGS sequence is in agreement with the concept of clonal lineages previously described in *F. oxysporum* (Klein and Correll 2001; Steinberg et al. 1997). However, contrary to the low divergence observed among *F. oxysporum* f. sp. *dianthi* isolates within the same VCG (Baayen et al. 1997, 2000), our results show a high diversity in the GII group constituting the new VCG 002-. Thus, as mentioned before, two subgroups can be clearly identified in this VCG. This high diversity could reflect a strong evolutionary potential in this particular group of isolates. In fact, the existence of frequent cross-compatibility between these GII isolates and isolates in other clades (GI and R1t isolates) is another indicator of plasticity and could be contributing to the high diversity within this group.

Although the complete IGS sequence was a good choice for robust resolution of *F. oxysporum* f. sp. *dianthi* phylogeny, we studied in detail the alignments of the different IGS sequences and identified

Fig. 5. Phylogenetic tree based on neighbor-joining analysis of short intergenic spacer (IGS) sequences of 53 *Fusarium oxysporum* f. sp. *dianthi* isolates. Bootstrap values are given when above 70% (1,000 replicates). *F. proliferatum* was used as an outgroup. Vertical bars on the right indicate molecular group clustering of the isolates.

a short region (approximately 300 nucleotides [nt]) with considerable variation to be used both as a diagnostic molecular marker to quickly predict the virulence of new isolates and for phylogenetic analyses of *F. oxysporum* f. sp. *dianthi* populations. This approximately 300-nt sequence included 19 informative positions and was flanked by two perfectly conserved regions, which facilitated the design of a pair of primers for its amplification. The detailed analysis of the 19 informative positions showed that some of them allow discrimination of the virulence group by differential restriction analysis. We have applied the proposed restriction analysis to a set of *F. oxysporum* f. sp. *dianthi* isolates, confirming its potential usefulness as a simple diagnostic tool to quickly and accurately discriminate molecular groups in *F. oxysporum* f. sp. *dianthi* populations. The utility of the 19 informative positions identified in the IGS extends to provide useful information about the members of the heterogeneous GII group. Depending on the residue combination of these 19 positions, two clear subgroupings can be distinguished that coincide with the clusters observed in the phylogenetic analysis of *F. oxysporum* f. sp. *dianthi* isolates. However, isolate *F. oxysporum* f. sp. *dianthi* 179 clearly differed from the rest of members of this group. The sequence variation nature of this short IGS region is also enough to resolve *F. oxysporum* f. sp. *dianthi* relatedness, as confirmed by the fact that it generated a high-resolution phylogenetic tree with a topology and branch support similar to one generated with the complete IGS sequence. In summary, this simple tool has a great potential not only to determine phylogenetic relationships among isolates but also to quickly analyze pathogenic or virulence diversity in *F. oxysporum* f. sp. *dianthi* populations in a given area, this information being essential to select resistant carnation cultivars before planting.

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