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Molecular characterization through IGS sequencing of formae speciales of *Fusarium oxysporum* pathogenic on lamb's lettuce

KRISHNAMOORTHY SRINIVASAN, GIOVANNA GILARDI, DAVIDE SPADARO, MARIA LODOVICA GULLINO and ANGELO GARIBALDI

Agroinnova – Centro di Competenza per l'Innovazione in Campo Agro-ambientale, Università di Torino, Via Leonardo da Vinci 44, 10095 Grugliasco, Torino, Italy

Summary. Twenty-nine strains of *Fusarium oxysporum* isolated from wilted lamb's lettuce plants (*Valerianella olitoria*) along with eight ATCC reference strains were examined for differences in the nucleotide sequences of the ribosomal DNA (rDNA) intergenic spacer (IGS) region, which is about 2.5 kb long in these strains. A phylogenetic (neighbour-joining) analysis was performed on the strains and identified four phylogenetic groups, I, II, III, and IV. Most *F. oxysporum* isolates recovered from wilted lamb's lettuce plants cultivated in northern Italy clustered in group I and were very similar to *F. oxysporum* f. sp. *raphani*. All the isolates, including the 8 control strains, were tested for pathogenicity on lamb's lettuce cv. Trophy cultivated in the glasshouse. Most isolates from northern Italy were pathogenic on lamb's lettuce, displaying varying degrees of virulence, and their IGS sequence were similar to the forma specialis *raphani*. However, four isolates were not pathogenic on lamb's lettuce. Among the control strains, six showed moderate virulence; these belonged to the formae speciales *raphani* and *conglutinans*; and two, belonging to the forma specialis *matthioli* (ATCC16602 and ATCC16603) were not pathogenic on lamb's lettuce. In conclusion, analysis of the IGS sequences indicated that the isolates studied had different origins, and that phylogeny and pathogenicity were related; non-pathogenic isolates differed genetically from isolates that were poorly, moderately or highly virulent. To the best of our knowledge, this is the first report on the differences between formae speciales of *F. oxysporum* on lamb's lettuce plants as determined by IGS sequence analysis.

Key words: Fusarium wilt, phylogeny, ribosomal intergenic spacer, *Valerianella olitoria*.

Introduction

Lamb's lettuce (*Valerianella olitoria* L.), also known as corn salad, is increasingly grown in Italy, and is used in the preparation of mixed fresh cut salads. In summer 2003, plants of lamb's lettuce (cv. Trophy and Palmares) exhibiting wilt symptoms were seen in several commercial greenhouses close to Bergamo in northern Italy. Wilting appeared in 30-day-old plants at the time of thinning when temperatures ranged from 28 to 35°C. *Fusarium oxysporum* von Schlechtendal was consistently isolated from affected tissue and the first report of Fusarium wilt on lamb's lettuce worldwide was published in 2004 by Garibaldi and co-workers. The distribution of the disease was generally uniform in the greenhouses, from 30 to 50% of plants being affected. Besides being wilted, the vascular tissue of affected plantlets appeared red or brown, later turning brown or black. Affected plants were stunted and had yellowed leaves. From 2003 to 2009, wilted lamb's lettuce plants were frequently found in many plastic greenhouses in some of the major lamb's lettuce growing areas in Italy. The newly discovered wilt occurred in the same areas where similar diseases had previously been seen:

Corresponding author: D. Spadaro
Fax: +39 011 6709307
E-mail: davide.spadaro@unito.it

wilt of lettuce, caused by *F. oxysporum* f. sp. *lactucae* (Garibaldi *et al.*, 2002) and wilt of wild rocket (*Diplotaxis tenuifolia*) and cultivated rocket (*Eruca sativa*), caused by both *F. oxysporum* f. sp. *raphani* and *F. oxysporum* f. sp. *conglutinans* (Garibaldi *et al.*, 2003; Catti *et al.*, 2007). Among these isolates, *F. oxysporum* ff. spp. *raphani* and *conglutinans* were the major pathogens, causing Fusarium wilt of rocket grown in the same areas (Garibaldi *et al.*, 2006; Gilardi *et al.*, 2008).

With more than 80 putative host-specific formae speciales described (Katan, 1999), members of the *Fusarium oxysporum species complex* (FOSC) represent the most commonly encountered and economically important species complex within the *Fusarium* genus. The formae speciales of *F. oxysporum* can be differentiated using pathogenicity tests with the appropriate host, and by these tests they are assigned to vegetative compatibility groups (VCG) determined by heterokaryon formation between anastomosing nitrate non-utilizing (*nit*) mutants (Gilardi *et al.*, 2008). The pathogenicity tests suggest that there is genetic diversity among the *F. oxysporum* isolates from wilted lamb's lettuce plants in Italy. However, these tests are time consuming, labour intensive and subject to changes in the environmental or culture growth conditions while they are being carried out (Woo *et al.*, 1996; Mbofung *et al.*, 2007). Molecular techniques such as restriction fragment analysis of PCR-amplified ribosomal intergenic spacers (IGS) can rapidly characterise large *F. oxysporum* populations (Edel *et al.*, 1995). Of the relatively small number of genes that have been sampled to date within the *Fusarium* genus (IGS rDNA, EF-1α, polygalacturonases, mitochondrial small subunit ribosomal RNA, phosphate permease, β-tubulin, nitrate reductase, MAT 1 and MAT2), only EF-1α (Amatulli *et al.,* 2010) and IGS rDNA genes (Mbofung *et al.,* 2007) appear to have much of a phylogenetic signal. The IGS region, which separates the rDNA repeat units, is particularly suitable for studying intraspecific relationships (Appel and Gordon, 1996; Mbofung *et al.*, 2007). Recent studies have shown that phylogenetic analysis of the IGS region sequence is very useful to study the composition of genetic populations of *F. oxysporum* (Kawabe *et al.*, 2005; 2007; Enya *et al.*, 2008; Dissanayake *et al.*, 2009). No study of *F. oxysporum* genetic diversity in lamb's lettuce has been reported.

Hence, our main objective was to differentiate the isolates of *F. oxysporum* from wilted lamb's lettuce in Italy according to sequence variation within the IGS region and to correlate any difference with the pathogenicity.

Materials and methods

Isolates of *F. oxysporum*

Table 1 indicates the names of the isolates, the year of isolation, the geographical origin and the original host. All isolates were maintained on potato dextrose agar (PDA, Merck, Darmstadt, Germany) slants at 4°C.

Inoculum production and pathogenicity tests

The *F. oxysporum* isolates (Table 1) were grown in 100 ml of casein hydrolysate at 25°C with shaking at 0.1 *g*. After 10 days, the cultures were aseptically filtered to collect the conidia, which were brought to a final density of 10^6 conidia ml^{-1} in sterile water. Surface-sterilized seeds of lamb's lettuce cv. Trophy were sown in a steamed substrate (peat:perlite, 70:30 v:v) in plug trays (Oktpac 160, Arca, Bergamo, Italy) kept at 21 to 25°C, with 12 hours of fluorescent light per day. Roots of 15-day-old plants were washed, trimmed to 5 cm and dipped in a 200 ml spore suspension of the isolate for 10 min. Inoculated plants were then transplanted to 10 litre containers filled with a steamed substrate (peat:clay:perlite, 70:20:10 v:v:v). Control plants were prepared in the same way and soaked in sterile deionised water. Fifteen plants were used per isolate, arranged in a randomized block design. Three replications were used during the study. Uninoculated plants maintained in the same glasshouse served as the healthy control. Plants in the glasshouse at 24 to 30°C were watered and fertilized in accordance with the practice of local growers. Three trials were carried out, with all isolates being tested at least twice under glasshouse conditions. Each trial lasted 33 to 53 days. Starting 10 days after inoculation, plants were checked for symptoms at 7 day intervals and wilted plants were counted. At the end of the experiments, re-isolation was carried out from inoculated and control plants. A disease index was adopted to rank the plants every week; where 0 indicated healthy plants; 25, vascular discolouration, slight

Table 1. Strains of *Fusarium oxysporum* used in this study. The disease index obtained after pathogenicity tests and their reaction on lamb's lettuce is also reported.

Strain	Year of isolation	Geographical origin	Original host	Genbank accession	Forma specialis	Disease index 0 – 100 [a]	Reaction on lamb's lettuce [b]
F. oxysporum FV-1/03	2003	Lombardy (I)	Lamb's lettuce	GQ914734	*raphani*	89.6 f	H
F. oxysporum FV-2/03A	2003	Lombardy (I)	Lamb's lettuce	GQ914735	*raphani*	60.4 cdef	H
F. oxysporum FV-2/03B	2003	Lombardy (I)	Lamb's lettuce	GQ914736	*raphani*	58.3 cdef	M
F. oxysporum FV-3/03	2003	Lombardy (I)	Lamb's lettuce	GQ914737	*raphani*	52.1 bcdef	M
F. oxysporum FV-4/03	2003	Lombardy (I)	Lamb's lettuce	GQ914738	*raphani*	83.3 ef	H
F. oxysporum FV-5/03	2003	Lombardy (I)	Lamb's lettuce	GQ914739	*raphani*	83.3 ef	H
F. oxysporum FV-6/03	2003	Lombardy (I)	Lamb's lettuce	GQ914740	*raphani*	64.6 cdef	H
F. oxysporum FV-1/04	2004	Lombardy (I)	Lamb's lettuce	GQ914741	*raphani*	35.4 abc	M
F. oxysporum FV-2/04	2004	Lombardy (I)	Lamb's lettuce	GQ914742	*raphani*	75.0 def	H
F. oxysporum FV-3/04	2004	Lombardy (I)	Lamb's lettuce	GQ914743	*fabae*	00.0 a	NP
F. oxysporum FV-4/04	2004	Lombardy (I)	Lamb's lettuce	GQ914744	*raphani*	70.8 cdef	H
F. oxysporum FV-5/04-MYA3072	2004	Lombardy (I)	Lamb's lettuce	GQ914745	*raphani*	72.9 cdef	H
F. oxysporum FV-6/04	2004	Lombardy (I)	Lamb's lettuce	GQ914746	*medicaginis*	00.0 a	NP
F. oxysporum FV-7/04	2004	Lombardy (I)	Lamb's lettuce	GQ914747	*raphani*	52.1 bcdef	M
F. oxysporum FV-8/04	2004	Lombardy (I)	Lamb's lettuce	GQ914748	*raphani*	83.3 ef	H
F. oxysporum FV-9/04	2004	Lombardy (I)	Lamb's lettuce	GQ914749	*raphani*	50.0 bcdef	M
F. oxysporum FV-10/04	2004	Lombardy (I)	Lamb's lettuce	GQ914750	*raphani*	54.2 bcdef	M
F. oxysporum FV-1/05A	2005	Lombardy (I)	Lamb's lettuce	GQ914751	*raphani*	81.3 def	H
F. oxysporum FV-1/05B	2005	Lombardy (I)	Lamb's lettuce	GQ914752	*raphani*	43.8 bcde	M
F. oxysporum FV-2/05	2005	Lombardy (I)	Lamb's lettuce	GQ914753	*raphani*	58.3 cdef	M
F. oxysporum FV-1/06	2006	Lombardy (I)	Lamb's lettuce	GQ914754	*fabae*	00.0 a	NP
F. oxysporum FV-SD/04	2006	Lombardy (I)	Lamb's lettuce	GQ914755	*raphani*	47.9 bcdef	M
F. oxysporum FV-2/06	2006	Lombardy (I)	Lamb's lettuce	GQ914756	*raphani*	60.4 cdef	H
F. oxysporum FV-11/04	2004	Lombardy (I)	Lamb's lettuce	GQ914757	*medicaginis*	00.0 a	NP
F. oxysporum FV-1/09A	2009	Lombardy (I)	Lamb's lettuce	GQ914758	*raphani*	70.8 cdef	H
F. oxysporum FV-1/09B	2009	Lombardy (I)	Lamb's lettuce	GQ914759	*raphani*	64.6 cdef	H
F. oxysporum FV-2/09	2009	Lombardy (I)	Lamb's lettuce	GQ914760	*raphani*	56.3 cdef	M
F. oxysporum FV-3/09	2009	Lombardy (I)	Lamb's lettuce	GQ914761	*raphani*	58.3 cdef	M
F. oxysporum FV-7/09	2009	Lombardy (I)	Lamb's lettuce	GQ914762	*conglutinans*	12.5 cdef	L
Control strains used in the pathogenicity tests and in the phylogenetic study:							
F. oxysporum f. sp. *raphani* ATCC58110 (a) [c]	n.d.	Unknown	*Raphanus sativus*	GQ914763	*raphani*	56.3 cdef	M
F. oxysporum f. sp. *raphani* ATCC16601 (a)	n.d.	USA	*R. sativus*	GQ914765	*raphani*	40.0 abcd	M
F. oxysporum f.sp. *raphani* 6-MYA3041(b)	2002	Lombardy (I)	*Eruca vesicaria*	GQ914768	*raphani*	41.7 abcde	M
F. oxysporum f. sp. *conglutinans* race 1 ATCC52557 (a)	n.d.	Wisconsin (USA)	*Brassica oleracea*	GQ914767	*conglutinans*	47.9 bcdef	M

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Table 1 continued

Strain	Year of isolation	Geographical origin	Original host	Genbank accession	Forma specialis	Disease index 0 – 100 [a]	Reaction on lamb's lettuce [b]
F. oxysporum f. sp. *conglutinans* race 2 ATCC58385 (a)	n.d.	USA	*B. oleracea*	GQ914770	*conglutinans*	39.6 abcd	M
F. oxysporum f. sp. *conglutinans* ATCC16600 (a)	n.d.	North Carolina (USA)	*B. oleracea*	GQ914766	*conglutinans*	45.8 bcde	M
F. oxysporum f. sp. *matthioli* race 1 ATCC16602 (a)	n.d.	USA	*Matthiola incana*	GQ914764	*matthioli*	00.0 a	NP
F. oxysporum f. sp. *matthioli* race 2 ATCC16603 (a)	n.d.	USA	-	GQ914769	*matthioli*	00.0 a	NP
Control strain sequence used in the phylogenetic study:							
F. oxysporum f. sp. *raphani* NRRL22553	2009	USA	-	FJ985463	*raphani*	-	
F. oxysporum f. sp. *raphani* NRRL53154	2009	USA	-	FJ985676	*raphani*	-	
F. oxysporum f. sp. *conglutinans* NRRL53158	2009	USA	-	FJ985678	*conglutinans*	-	
F. oxysporum f. sp. *conglutinans* NRRL36364	2009	USA	-	FJ985583	*conglutinans*	-	
F. oxysporum f. sp. *fabae* NRRL26411	2006	USA	-	DQ831902	*fabae*	-	
F. oxysporum f. sp. *medicaginis* Fom004	2007	USA	-	EU313446	*medicaginis*	-	
F. oxysporum f. sp. *matthiolae* NRRL22545	2009	USA	-	FJ985457	*matthioli*	-	

[a] Means in the same column followed by the same letter do not differ according to Tukey's test ($P<0.05$).

[b] Reaction: NP, non-pathogenic strain; L, low virulence (10–30%); M, moderate virulence (31–60%); H, high virulence (61–100%).

[c] (a), Source of information on the ATCC strains is the website http://www.lgcstandards-atcc.org/; (b), strain deposited in the ATCC collection and previously evaluated (Garibaldi *et al.,* 2002; Garibaldi *et al.,* 2006; Catti *et al.,* 2007).

n.d., Not determined (year of isolation unknown).

leaf chlorosis and growth reduction of around 25% compared with the healthy control; 50, vascular discoloration, chlorosis and strong growth reduction; 100, dead plants. Data of the replications of repeated experiments were pooled and analysed together. The mean disease index values for each treatment were calculated. Data were analysed using the SPSS software (SPSS Inc., version 17.0, Chicago, IL, USA). Statistical significance was judged at the level of $P<0.05$. When the analysis of

Table 2. Primers used in this study.

Primer	Sequence (5' → 3')	Reference
M13 forward (-40)	GTTTCCCAGTCAGAC	-
M13 reverse	AACAGCTATGACCATG	-
SP6 promoter	CATTTAGGTGACACTATAG	-
T7 promoter	GTAATACGACTCACTATAG	-
Intergenic spacer region		-
CN34[b]	CCAACACATGGGTGGTACCG	Mbofung *et al.,* 2007
CN61[a]	GGTTCAATTTGATGTCGGCT	Mbofung *et al.,* 2007
CNL12[a]	CTGAACGCCTCTAAGTCAG	Abderson and Stasovsky, 1992
CNS1[b]	GAGACAAGCATATGACTACTG	White *et al.,* 1990
CNS12[b]	GCACGCCAGGACTGCCTCGT	Mbofung *et al.,* 2007
IGSF4[a]	CCAGACTTCCACTGCGTGTC	Mbofung *et al.,* 2007
RCN61[b]	AGCCGACATCAAATTGACC	Mbofung *et al.,* 2007
RU3[a]	GTGTGAAATTGGAAAGTCGG	Mbofung *et al.,* 2007
RU46.67[a]	GTGTCGGCGTGCTTGTATT	Mbofung *et al.,* 2007
U46.67[b]	AATACAAGCACGCCGACAC	Appel and Gordon, 1996

[a] Forward primer.
[b] Reverse primer.

variance was statistically significant, Tukey's test was used to compare the means.

DNA extraction

Total DNA was extracted from about 100 mg of mycelium scraped from Petri dishes containing PDA using the NucleoSpin kit (Macherey Nagel GmbH and Co., Duren, DE, USA), according to manufacturer's instructions, adding 10 μl of a solution Proteinase K (10 mg ml^{-1}) and 10 μl of RNAse A (12 mg ml^{-1}) to the lysis buffer in each tube used for the extraction. The extracted DNA was loaded on 1% agarose gel containing 1 μl 100^{-1} of SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, USA). DNA concentrations were measured with a spectrophotometer (Eppendorf, Hamburg, Germany). Purified DNA was stored at 4°C.

IGS sequence analysis

The complete IGS region of 29 *F. oxysporum* isolates was amplified using the primers CNL12 and CNS1 (see Table 2 for primer sequences and references) in a PCR performed in 50 μl containing 10 ng of gDNA; 1 μM of each primer, 5 Units of Fast Start Taq DNA Polymerase (Roche, Basel, Switzerland) and 10 μl of colourless 10´ Fast Start Taq DNA Polymerase buffer containing dNTPs (Roche). We used a T-Gradient thermal cycler (Biometra, Göttingen, Germany) programmed to 95°C for 5 min, followed by 38 cycles with denaturation at 94°C for 1 min; annealing at 60°C for 90 sec, extension at 72°C for 3 min, and final extension at 72°C for 10 min. A negative control (no template DNA) was included. About 7 μl of PCR products were electrophoresed on 0.8% agarose gel (Agarose D-1 LOW EEO, Eppendorf) containing 1 μl 100 ml^{-1} of SYBR safe DNA gel stain (Invitrogen) for 30 min at 3.3 V cm^{-1} in 1´ TAE running buffer (Maniatis *et al.*, 1982), and amplimers were viewed under UV lighting. Gel images were acquired with a Gel Doc EC (Bio-Rad, Hercules, CA, USA). Band size was estimated by comparing them with a Gel Pilot Wide Range Ladder (Qiagen, Chatsworth, CA, USA).

The PCR products were purified using a QIAquick PCR purification kit (Qiagen) and measured with a spectrophotometer. They were ligated with a pDrive cloning vector using the Qiagen cloning kit according to manufacturer's instruc-

tions, in a 10 μl volume of ligation mixture, incubated at 4–12°C for 2 h. After incubation, 1–2 μl of the ligation mixture was inoculated in a tube containing Qiagen EZ competent cells, mixed gently and incubated on ice for 5 min. The tubes were then heated at 42°C for 30 s without shaking and finally incubated on ice for 2 min; 250 μl of super optimal broth with catabolite repression (SOC, Hanahan, 1983) was added to the tubes and directly plated with each transformation mixture on plates containing Luria-Bertani medium (LB) as well as the selection markers of 100 μg ml^{-1} of ampicillin (Merck), 30 μg ml^{-1} of kanamycin (Merck), isopropyl β-D-thiogalactopyranoside (IPTG; 50 μg ml^{-1}) and 80 μg ml^{-1} of 5-bromo-4-choloro-3-indolyl β-D-galactopyranoside (X-Gal) at 37°C for 15 to 18 h for the blue/white screening of recombinant colonies. The white colonies were screened using M13 forward and reverse primers (see Table 2) to check for inserts. PCR was performed in a total volume of 20 μl containing: 60 μM of each dATP, dCTP, dGTP and dTTP; 1 μM of each primer, 1 Unit of Taq DNA polymerase (Qiagen); and 2 μl of 10´ colourless buffer using whole cells as template. The PCRs were run as described above but decreasing the annealing temperature to 50°C.

About 5 ml of LB medium containing the same amounts of selective antibiotics as did the plates was inoculated with a single transforming colony and incubated for 16 h at 37°C. Plasmids were purified from bacterial cells following manufacturer's instruction (Qiagen) and were sequenced by the BMR Genomics Centre (Padova, Italy) using the ABI PRISM 3730Xl DNA Sequencer. The complete sequence of the IGS region due to its large size, required additional internal primers (see Table 2) and sequencing was performed in both directions. The IGS sequences obtained were deposited in GenBank (accession numbers are shown in Table 1) and aligned using the DNA Baser v2.71.0 program (Heracle Software, Lilienthal, Germany). Sequence alignments were manually adjusted to remove mismatches. The sequences were submitted to the National Center for Biotechnology Information (NCBI), USA (Table 1). The IGS sequences of strains of *Fusarium oxysporum* isolated from other plant species obtained from GenBank were used as reference sequences and out-group (Table 1).

Phylogenetic analysis

Phylogenetic analysis was conducted using MEGA version 4 software (Tamura *et al.*, 2007). IGS sequence data were analysed to determine the relationships using the distance and parsimony methods. A distance matrix for the aligned sequences was calculated using Kimura's two-parameter model (Kimura, 1980), and was analysed with the neighbour-joining method (Saitou and Nei, 1987) using the ClustalW v.1.6 program (Thompson *et al.*, 1994), excluding positions with gaps and missing data (complete deletion option). Bootstrap analysis was based on 10,000 re-samplings.

Results

Pathogenicity test

The results of the pathogenicity trials carried out on lamb's lettuce plants with 29 isolates of *F. oxysporum* along with 8 ATCC strains are shown in Table 1. The typical symptoms of Fusarium wilt were first seen 12 to 14 days after inoculation. Symptoms included stunting and chlorosis of plants, with brown or black streaks in the vascular system and were similar to those described by Garibaldi *et al*. (2004). Lamb's lettuce was infected by twenty-five isolates recovered from northern Italian lamb's lettuce (FV-1/03, FV-2/03A, FV-2/03B, FV-3/03, FV-4/03, FV-5/03, FV-6/03, FV-1/04, FV-2/04, FV-4/04, FV-5/04-MYA3072, FV-7/04, FV-8/04, FV-9/04, FV-10/04, FV-1/05A, FV-1/05B, FV-2/05, FV-SD/04, FV-2/06, FV-1/09A, FV-1/09B, FV-2/09, FV-3/09 and FV-7/09) and by three strains of *F. oxysporum* f. sp. *raphani* (ATCC58110, ATCC16601, and 6-MYA3041). The disease index was high, reaching a maximum of 89.6% in glasshouse conditions. For the reference strains ATCC58110, ATCC16601, and *F. oxysporum* 6-MYA3041, the disease indices were 56.3, 40.0, and 41.7%, respectively. Similarly, for the reference strains of *F. oxysporum* f. sp. *conglutinans* ATCC16600, race 1 ATCC52557, and race 2 ATCC58385, the disease indices were 45.8%, 47.9%, and 39.6%. Six strains (FV-3/04, FV-1/06, FV-6/04, FV-11/04, race 1 ATCC16602 and race 2 ATCC16603) were not pathogenic on lamb's lettuce. The first two strains were later identified, by pathogenicity tests on faba bean and alfalfa, VCG and IGS sequencing, as *F. oxysporum* f. sp.

fabae, while the following two strains were identified as *F. oxysporum* f. sp. *medicaginis* (Table 1). *F. oxysporum* was consistently re-isolated from inoculated plants at the end of the experiments. Re-isolation was performed using the semi-selective media for Fusaria (Komada, 1975) from both symptomless and affected plants (Garibaldi *et al*., 2004). Similar results were obtained in all the trials performed.

IGS sequencing and phylogenetic analysis

The PCR reaction produced a clear single product varying from 2,493 to 2,592 bp in the isolates tested. The internal primers generated smaller fragments (ranging from 800 to 1,000 bp) with the exception of CNL12, used only to amplify the IGS sequence in each isolate.

The assembly of the IGS contigs and their alignment yielded a sequence of 1,971 bp in the final dataset, of which 81 were parsimony-informative. Twenty-four isolates collected in northern Italy from 2003 to 2009 had highly similar IGS sequences, differing only by single nucleotide polymorphisms (SNPs), distributed in different positions. Differences in the SNPs were the most common differences between the pathogenic isolates.

The complete IGS sequence of each isolate was analysed to infer the phylogenetic relationship by using the neighbour-joining method (Figure 1). Four different clusters (Groups I, II, III and IV) were identified. The maximum parsimony (MP) analysis found the tree length to be 157; the consistency index was 0.936 (0.895), the retention index was 0.976 (0.976), and the composite index was 0.914 (0.873) for all sites (figures for parsimony-informative sites are shown in parentheses). The IGS sequence analysis supported the four groups indicated by the neighbour-joining method.

Twenty-four isolates from lamb's lettuce (FV-1/03, FV-2/03A, FV-2/03B, FV-3/03, FV-4/03, FV-5/03, FV-6/03, FV-1/04, FV-2/04, FV-4/04, FV-5/04-MYA3072, FV-7/04, FV-8/04, FV-9/04, FV-10/04, FV-1/05A, FV-1/05B, FV-2/05, FV-SD, FV-2/06, FV-1/09A, FV-1/09B, FV-2/09 and FV-3/09) as well as *F. oxysporum* f. sp. *raphani* ATCC58110 and ATCC16601, *F. oxysporum* 6-MYA3041 and the NCBI *raphani* strains NRRL53134 and NRRL22553, belonged to the "*raphani*" group, (Group I), with a bootstrap value of 100%, while the remaining isolates were separated from this group by not having the consensus sequence TCCATGGTAG (position 562–571). Isolates from the same area did not necessarily cluster in a single group. Of the 24 isolates, 5 isolates (FV-1/03, FV-2/03A, FV-1/04, FV-1/05B and FV-1/09B) formed a sub-group within Group I, supported by a boot strap value of 86%.

Interestingly, isolate FV-7/09 had a sequence very similar to that of *F. oxysporum* f. sp. *conglutinans* ATCC16600, ATCC52557 and ATCC58385: these clustered together in Group II with a bootstrap value of 96%. Two of the non-pathogenic isolates of lamb's lettuce (FV-6/04 and FV-11/04) and FOM004 isolated from *Medicago sativa* formed a distinct group (Group III, with a bootstrap value of 97%). Group IV consisted of two non-pathogenic isolates (FV-3/04 and FV-1/06). Isolate NRRL 36364 of *Fusarium oxysporum* f. sp. *conglutinans* was separated from the other isolates, including the ATCC strains, by not having the consensus sequences CGTCGATAGGA (position 390–400 bp), TGTGTGTTGG (position 413–422 bp), GCAGGGTAGGCTGCTTGGA (position 428–446 bp), CGAGGATCGATTCGAGGGCCGGCCTGTCGATGAT (position 458-492 bp), and CAGAGTCGGGTCTAGGGTAGGC (position 1702–1723 bp) that were found in the other isolates tested, with a bootstrap value of 100%. The ATCC strains 16602 and 16603 and strain NRRL 22545 were not similar to the other isolates tested and they were grouped in another cluster, including the forma specialis *matthioli* isolates, with a bootstrap value of 99%.

The similarity index of the 29 isolates and the ATCC strains ranged from 86 to 100%. The cluster analysis separated the 24 isolates into two major groups at a genetic similarity of more than 86%. One group included isolates FV-6/04 and FV-11/04 with 100% genetic similarity. Varying levels of genetic relatedness were found among the IGS types resolved through the formae speciales.

Discussion

The *F. oxysporum* isolates examined differed in their disease severity index, which ranged from 0.0 to 89.6%. These findings are consisting with previous studies, which showed variations in the virulence of *F. oxysporum* isolates on lamb's let-

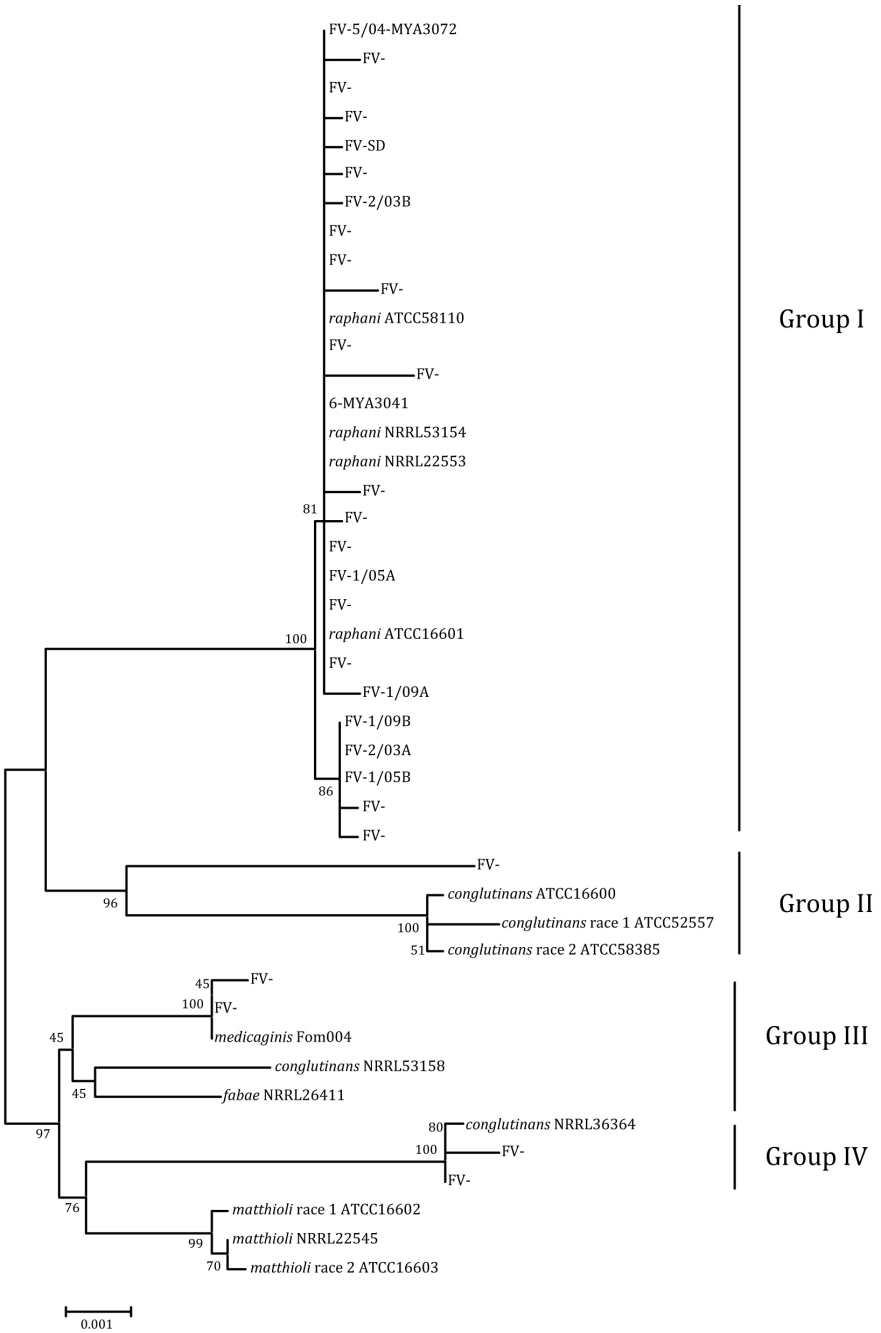

Figure 1. Phylogenetic relationships of 44 isolates (%). The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch lengths = 0.0429 is shown. The percentages of replicate trees in which the associated taxa were clustered together in the bootstrap test (10000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. Evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete delete option). There was a total of 1971 positions in the final dataset. Phylogenetic analyses were conducted using MEGA4.

tuce (Gilardi *et al*., 2008) and other crops (Catti *et al*., 2007; Dissanayake *et al*., 2009). Most *F. oxysporum* isolates were weakly, moderately or highly pathogenic on lamb's lettuce plants. Specifically, some isolates were more virulent than ATCC strains ATCC58110 and ATCC16601 and *F. oxysporum* 6-MYA3041, the first two isolated from *Raphanus sativus*, the last from *Eruca vesicaria*, all belonging to the forma specialis *raphani*. The lowest disease index (12.5%) was recorded in FV-7/09, genetically close to the *conglutinans* isolates. The *conglutinans* strains (ATCC16600; race 1 ATCC52557; and race 2 ATCC58385) were moderately virulent and this species is distinguished from the other isolates of *F. oxysporum* by the susceptibility of the cv. Trophy to it.

Four isolates (FV-3/04, FV-1/06, FV-6/04 and FV-11/04) from lamb's lettuce and two isolates of *Fusarium oxysporum* f. sp. *matthioli*, race 1 ATCC16602 and race 2 ATCC16603, were non-pathogenic on lamb's lettuce. The four isolates from lamb's lettuce were genetically different from the other pathogenic isolates and also from the ATCC strains tested. It cannot however be concluded that these four isolates are saprophytic strains, since they may be pathogenic on other crop species. IGS analysis placed the first two strains in Group III, and the second two strains in Group IV, and their sequences were very close respectively to *F. oxysporum* f. sp. *fabae* and f. sp. *medicaginis*. These isolates may have been pathogenic on crops planted previously in the same field.

Since more than 80 formae speciales have already been described, an enormous number of potential hosts and cultivars would have to be used for conclusive identification (Fravel *et al*. 2003). Although putatively non pathogenic strains have been described, and some have been employed successfully as biocontrol agents to suppress soil borne pathogens (Larkin *et al.,* 1996; Fuchs *et al.,* 1997; Spadaro and Gullino, 2005; Olivain *et al.,* 2006), the null hypothesis that some isolates are non-pathogenic is virtually impossible to test given the huge number of potential vascular plant hosts (O' Donnell *et al.,* 2009). Recently Ma *et al*. (2010) sequenced *F. oxysporum* f. sp. *lycopersici* (FOL) strain 4287. The genome assembly of FOL had 15 chromosomes. The increased genomic territory in FOL compared with other *Fusarium* species was due to additional, unique sequences that mostly resided in extra chromosomes. These unique sequences of FOL are a substantial fraction (40%) of the assembly, they are designated as lineage-specific (LS) regions and they contain 95% of all DNA transposons. Experimentally, Ma *et al*. (2010) demonstrated the transfer of two LS chromosomes between strains of *F. oxysporum*, converting a non-pathogenic strain to a pathogenic strain. The transfer of LS chromosomes between otherwise genetically isolated strains explains why the origin of host specificity is polyphyletic and why new pathogenic lineages in *F. oxysporum* emerge. The non-pathogenic strains can easily be converted to pathogenic strains by transferring lineage-specific genes (van der Does *et al*., 2008) or even chromosomes. Other factors, such as symbiosis with bacteria, can also explain the pathogenicity or non-pathogenicity of some strains of *Fusarium oxysporum* (Minerdi *et al*., 2008). Ectosymbiotic bacteria can silence the expression of genes involved in fungal pathogenesis, thereby changing the characteristics of the hyphae. This may be a direct response to bacterial substances. Consequently from the present study we can only conclude that isolates FV-3/04, FV-1/06, FV-6/04 and FV-11/04 are not pathogenic on lamb's lettuce.

Although bioassays are very effective, they are also very time consuming and laborious. Attempts are being made to replace these methods with molecular identification techniques to group the isolates (Lievens *et al*., 2008; Pasquali *et al*., 2008). To understand the genetic relationship among the formae speciales of *F. oxysporum*, it is useful to sequence the IGS region of several isolates of *F. oxysporum*. No study on the IGS analysis of *F. oxysporum* on lamb's lettuce or other crops in Italy has so far been published.

Phylogenetic analysis based on IGS sequences revealed a close relationship between genetic phylogeny and pathogenicity: non pathogenic isolates differed genetically from pathogenic isolates. Isolates from ATCC and those from northern Italy fell into four phylogenetic groups (I to IV). These findings were consistent with Kim *et al*. (1993) and Mbofung *et al*. (2007).

Namiki *et al*. (1994) reported that genetic differences between the two groups of formae speciales *melonis*, which enabled two IGS groups to be identified, could be due to geographic isolation, and to their dispersal throughout the world. Later, Appel

and Gordon (1995) identified 13 IGS haplotypes among a population of 56 *F. oxysporum* isolates collected in Maryland and California. Alves-Santos *et al*. (1999) found 6 IGS haplotypes in 128 isolates of *F. oxysporum*. According to these researchers, the diversity of the IGS haplotype within *F. oxysporum* suggests that sexual reproduction is infrequent or absent in this fungus. More recently, Dissanayake *et al*. (2009) reported 4 clusters in 30 isolates of *F. oxysporum* on onion in Japan.

Our phylogenetic analysis suggests that the strains of *F. oxysporum* f. sp. *raphani* were phylogenetically distinct and had a multiple independent origin. Isolates ATCC58110 and ATCC16601 of *F. oxysporum* f. sp. *raphani* and strain 6-MYA3041 of *F. oxysporum*, clustered as a distinct individual lineage. These findings indicate that the genetic diversity in rDNA IGS sequences is extremely low within the same cluster and that the ATCC strains of the formae speciales *raphani* (ATCC58110 and ATCC16601) and the *F. oxysporum* strain 6-MYA3041, here described for the first time, are genetically different.

Of the 24 pathogenic isolates, 19 formed a distinct group, which was consistently supported with bootstrapping of 81%, whereas the other five pathogenic isolates (FV-1/03, FV-2/03A, FV-1/04, FV-1/05B and FV-1/09B) fell into another subgroup (bootstrap value: 86%). This suggests that the *F. oxysporum* isolates with moderate or high virulence on lamb's lettuce may have a monophyletic origin. Some of the variation in the virulence of these isolates was related to a similar genetic background. It is interesting to note that Groups I and II included weakly, moderately and highly virulent isolates. These isolates may be useful for studying the evolution of this pathogen.

IGS sequence analysis is a precise and reproducible tool to evaluate genetic similarity or identity and it separates formae speciales and physiological races of *F. oxysporum* better than do RAPD and other molecular techniques. Recently, Fujinaga *et al*. (2005) reported that the IGS sequences were useful as an indicator of physiological races of *F. oxysporum*. The polymorphism of this region, which was higher than that of other loci, such as the mitochondrial Small Subunit (mtSSU) or the Elongation Factor 1-α (EF) (Mbofung *et al*., 2007), enabled the genetic diversity detected by VCG to be determined in *Fusarium oxysporum* f. sp. *lactucae* (Fujinaga *et al.,* 2005). The VCG analysis previously conducted on some of the tested isolates (FV-1/04, FV-2/04, FV-4/03, FV-5/03, FV-6/03, and FV-5/04) showed that the pathogenic isolates on lamb's lettuce in northern Italy were uniform (Gilardi *et al.,* 2008).

In any case, recent papers have demonstrated the utility of using multiple gene sequencing inside the FOSC. The widespread genealogical discordance between the IGS rDNA and the EF-1α bipartitions, recently reported by O'Donnell *et al*. (2009), constitutes an argument against using single-locus data for phylogenetic reconstruction and for inferring species limits within the fungi. Although IGS rDNA has become one of the most popular for investigating genetic diversity within the FOSC, and its high levels of nucleotide diversity provide a high degree of discriminatory power useful for isolate identification, yet the evolutionary history of this locus sometimes obscures phylogenetic relationships within this species. Future research should involve the sequencing of more genes in order to better understand the phylogeny of some of the isolates used in this study. The present study provides basic information for breeding lamb's lettuce resistant to Fusarium wilt disease and for establishing disease control strategies.

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