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Genetic diversity and structure of *Fusarium oxysporum* f.sp. *lentis* isolates from Iran, Syria and Algeria

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Abstract Lentil is an important cool season food legume. Vascular wilt incited by *Fusarium oxysporum* f.sp. *lentis* (FOL) is the most important disease of lentil worldwide. Knowledge of pathogen genetic structure is crucial to develop effective control strategies. In this study, the genetic structure of a collection of FOL isolates from Iran, Syria and Algeria was analysed using SSR markers. Eight markers were developed in this study and constitute a valuable resource for future molecular studies in FOL. Our results showed that there is a high molecular variation within regions, with isolates from North West Iran showing the highest gene diversity. Variation among regions also exists, with Iranian populations differing significantly from non-Iranian ones, having some private alleles. The clustering of isolates was well in agreement in both distance-based and model-based approaches suggesting the presence of seven ancestral FOL lineages, being three present exclusively in Iran while the others were distributed across all the regions. These results suggest that Iran could be a regional center of origin for FOL.

Keywords Lentil (*Lens culinaris*) · *Fusarium oxysporum* f.sp. *lentis* · Population structure · Pathotype · SSR

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Introduction

Lentil (*Lens culinaris* Medikus subsp. *culinaris*) is one of the most important cool season food legumes in India, northern Africa, western Asia, southern Europe, North and South America, and Australia (Erskine and Bayaa 1996). World production of lentil is estimated at 4.95 million tons from an estimated 4.34 million ha with an average yield of 1140 Kg/ha (FAOSTAT 2013 http://faostat.fao.org/faostat/collections). Their seeds are a rich source of proteins, minerals and vitamins for human nutrition, and the straw is used for animal feeding. As for most crops, a number of biotic and abiotic stresses decrease lentil yield (Chen et al. 2009). Of them, vascular wilt, caused by *Fusarium oxysporum* f.sp. *lentis* Gordon, from now on referred as FOL, is the most important disease of lentil worldwide (Bayaa et al. 1998; Khare 1981). The pathogen is able to attack the plant in almost every stage of the growth and can

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survive for several seasons in the soil without the host (Erskine and Bayaa 1996).

The most effective, economical and environmentally friendly method to control the disease is the use of resistant cultivars (Bayaa et al. 1995; Kraft et al. 2000). Health or disease is the result of a battle between plants and their pathogens, the final outcome depending on the interaction between the genetic factors of both parts of the interaction. Therefore, knowledge of the genetic structure of pathogens populations is relevant for planning managing strategies to control the diseases and enhance resistance durability. Thus, the level of genetic diversity and the capacity of a pathogen to evolve and create more aggressive isolates determine the potential of pathogens to overcome plant resistance genes.

Molecular markers offer a powerful method for revealing and identifying population structure of a pathogen and have been extensively used to study genetic diversity in different pathogens including different *formae speciales* and races of *F. oxysporum* (Grajal-Martin et al. 1993; Bentley et al. 1994; Manulis et al. 1994; O'Donnell et al. 1998; Baysal et al. 2009; Lievens et al. 2009).

Genetic structure analysis and variability of FOL populations have been tackled using different methods such as vegetative compatibility groups, IGS (intergenic spacer), ISSRs (inter-simple sequence repeat), SSRs (simple sequence repeat), RAPDs (random amplified polymorphic DNA), rDNA (ribosomal DNA) and AFLPs (amplified fragment length polymorphism) (Belabid and Fortas 2002; Belabid et al. 2004; Taheri et al. 2010; Datta et al. 2011; Mohammadi et al. 2012; Al-Husien et al. 2017; Nourollahi and Madahjalali 2017). Among molecular markers, SSRs have a number of advantages such as high level of polymorphisms, locus specificity, co-dominance, reproducibility, and random distribution throughout the genome (Saghai Maroof et al. 1995). However, only a few SSR are available for FOL. Thus, the only SSRs used so far for this species are SSRs developed by Bogale et al. (2005) for the *F. oxysporum* complex.

Previous studies on FOL population genetics have studied isolates from a specific geographic region such as India, Iran, Syria or Algeria (Belabid et al. 2004; Taheri et al. 2010; Datta et al. 2011; Mohammadi et al. 2011; Al-Husien et al. 2017; Nourollahi and Madahjalali 2017). In addition, some of these studies analysed the relationship between genetic structure and isolates aggressiveness. However, as FOL pathoypes have only recently been identified (Pouralibaba et al. 2016), nothing is known about the possible relationship between genetic structure and pathoypes.

The aim of this study was to use SSR markers to study the genetic structure of FOL populations covering a wide geographical region (Iran, Algeria and Syria), and to study whether there is any relation between this genetic structure and pathotypes. In addition, in order to perform a more accurate analysis, covering a higher genomic area, some new SSRs were developed for FOL.

Materials and methods

Fungal material

Forty seven FOL isolates and one *F. oxysporum* f. sp. *pisi* (FOP) isolate were used in this study (Table 1). FOL isolates originated from Iran (isolates from North East of Iran kindly provided by Dr. N. Mohammadi, Tarbyat Modarres University,Tehran), Syria (kindly provided by Dr. S. Ahmed, ICARDA, Syria) and Algeria (kindly provided by Dr. L. Belabid, University of Mascara, Algeria). The FOP isolate originated from Pullman, USA and was kindly provided by Dr. W. Chen. Isolates were first obtained from naturally yellowed/wilted lentil plants and subsequently purified to obtain mono-conidial isolates, which were stored at −80 °C as micro conidial suspensions in 150 ml vials containing 70 ml spore suspension: 30 ml sterile 98% glycerol. Isolates were characterized as *F. oxysporum* on Carnation Leaf Agar (CLA), using the key proposed by Nelson et al. (1983) and kindly confirmed by Prof. A. Trapero-Casas, University of Córdoba, Spain. Pathogenicity and characterization of isolates as FOL was tested by inoculating the lentil susceptible cultivar "Ardebil Local" with the isolates. To avoid any possible loss of virulence due to the effect of long storage or consecutive growing *in medium*, all isolates were inoculated on and re-isolated from the susceptible lentil cultivar "Ardebil Local" before performing the studies.

DNA extraction and SSR analysis

DNA was extracted from mycelium obtained from the fungus growing in PDB (Potato Dextrose Broth) liquid medium. Fungal isolates were grown in a 250-ml flasks containing 100 ml PDB (200 g potato: 20 g glucose: 1 l

Table 1 Code, origin and pathotype of the *F. oxysporum* f. sp. *lentis* (FOL) and the *F. oxysporum* f. sp. *pisi* (FOP) isolates used in the studies

Isolate code	Isolate number in Pouralibaba et al. 2016	Region	Province, location/village	Pathotype[b]
S01	52	Algeria	Saida,ITGC2	3
S02	54	Algeria	Mascara,Site 1	4
S03	56	Algeria	Sidi Bel-Abbas, Site 1	7
S04	57	Algeria	Sidi Bel-Abbas, Site 2	7
S05	40	Syria	Alhaskeh,Eyn Divar	2
S06	41	Syria	Aleppo,ICARDA	3
S07	42	Syria	Aleppo,ICARDA	1
S08	43	Syria	Aleppo,Eshraf	6
S09	44	Syria	Hama, Al-Eskandaryeh	3
S10	45	Syria	Idlib,Maharez	7
S11	47	Syria	Idlib,Termantin	1
S12	48	Syria	Dar'a, Mohatat Al- Bohouth	7
S13	3	NW[a] Iran	Varzeghan, km 10th Varzeghan - Ahar	2
S14	4	NW Iran	Bukan, Km 10th Bukan - Saqez	6
S15	5	NW Iran	Bukan	2
S16	6	NW Iran	Naghadeh, Km 5th Naqadeh - Piranshahr	3
S17	7	NW Iran	Oshnavyeh, Km 8th Oshnavyeh - Orumiyeh	1
S18	8	NW Iran	Miyandoab, Km 20th Miyandoab - Mahabad	7
S19	10	NW Iran	Varzeghan, km 10th Varzeghan - Kharvana	7
S20	12	NW Iran	Moghan, Ghare-Ghasemlu	7
S21	13	NW Iran	Moghan, Zargaran	7
S22	14	NW Iran	Moghan,Gog-Tapeh	5
S23	15	NW Iran	Moghan, Ruh-Kandi	2
S24	17	NW Iran	Varzeghan	7
S25	19	NW Iran	Moghan,Damirchi	2
S26	20	NW Iran	Moghan, Bilehsavar	7
S27	22	NW Iran	Varzeghan	2
S28	24	NW Iran	Moghan,Gog-Tapeh	5
S29	27	NW Iran	Ahar, km 10th Ahar - Tabriz	2
S30	28	NW Iran	Moghan, Gog-Tapeh	2
S31	32	NW Iran	Ahar, km 15th Ahar - Kaleybar	5
S32	34	NW Iran	Moghan, Gog-Tapeh	2
S33	35	NW Iran	Moghan,Gog-Tapeh	7
S34	36	NW Iran	Moghan,Gog-Tapeh	1
S35	2	NE Iran	Mash'had, Pardis	3
S36	9	NE Iran	Mash'had, Pardis	5
S37	11	NE Iran	Bojnourd	7
S38	16	NE Iran	Maneh-Samlaghan	3
S39	18	NE Iran	Maneh-Samlaghan	7
S40	21	NE Iran	Mash'had, Pardis	7
S41	23	NE Iran	Bardaskan	3
S42	29	NE Iran	Bardaskan	7
S43	30	NE Iran	Bojnourd	2
S44	31	NE Iran	Maneh-Samlaghan	3

Table 1 (continued)

Isolate code	Isolate number in Pouralibaba et al. 2016	Region	Province, location/village	Pathotype[b]
S45	33	NE Iran	Maneh-Samlaghan	2
S46	38	NE Iran	Maneh-Samlaghan	2
S47	39	NE Iran	Mash'had, Pardis	2
FOP		USA	Pullman	

[a] *NE* and *NW* North East and North West, respectively

[b] According to Pouralibaba et al. 2016

distilled water) at 28 to 30 °C for 3–4 days on a shaker in darkness. To obtain mycelia samples, the culture was filtered through four layers of sterile cheesecloth, washed in distilled sterile water, dried with filter paper and stored at −80 °C. DNA was isolated using CTAB method (Lassner et al. 1989), with the modifications described by Torres et al. (1993). Nine SSR developed by Bogale et al. (2005) for the *F. oxysporum* complex (MB2, MB5, MB9, MB10, MB11, MB13, MB14, MB17, MB18) and 11 developed by Vogelgsang et al. (2011) for *Fusarium poae* (FP01, FP03, FP05, FP06, FP06, FP07, FP08, FP09, FP10, FP11, FP12, FP14) were surveyed in the FOL populations. In addition, we designed new SSRs primers. In the absence of available sequences for FOL, we made use of the *F. oxysporum* f. sp. *pisi* HDV247 whole genome shotgun sequencing project (GenBank accession AGBI00000000.1). Using these *F. oxysporum* f. sp. *pisi* sequences as templates, 23 SSR were identified and primers designed using BatchPrimer3 software (You et al. 2008). Primer design parameters were: length range = 18–23 nucleotides, with 21 as optimum, optimum annealing temperature 55 °C and GC content 40–60%.

For SSRs earlier described by Bogale et al. (2005) and Vogelgsang et al. (2011) PCR reaction mix and amplification conditions initially used were as reported by the authors. In the case of absence of amplification, different annealing temperatures and $MgCl_2$ concentrations were tested. For the SSRs newly developed in this study, amplification conditions were as reported by Bogale et al. (2005) and annealing temperatures and $MgCl_2$ concentration were optimized (Table 2). Amplification products were first subjected to electrophoresis performed in a 2% agarose with Tris–borate EDTA Buffer gels for 3 h at 90 V. Gel green-stained gels were visualized on an ultraviolet light trans-illuminator and photographed. Only SSRs showing a clear unique band were selected for fragment analysis. For fragment analysis, SSRs were amplified in PCR reactions in a total volume of 15 μL, containing: 50 ng of genomic DNA; 10x NH_4 reaction buffer (670 mM Tris-HCl (pH 8.8 at 25 °C), 160 mM $(NH_4)_2SO_4$, 100 mM KCl, 10, 0.1% stabilizer); 160 μM of dNTPs; 1.5 or 2.5 mM of $MgCl_2$ depending on the primer (Table 2); 0.2 μM of primers and 0.6 U BIOTAQ DNA polymerase (Bioline). Forward primers were labeled with fluorophores 6FAM or HEX (Sigma-Genosys Ltd.) at the 5′ ends. The thermal profile consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing temperature for 30 s (Table 2) and 72 °C for 30 s, concluding with a final extension step at 72 °C for 10 min. Amplification products were separated in an automated capillary sequencer (ABI 3130 Genetic Analyzer, Applied Biosystems /HITACHI) at the Genomics Unit of the Central Service of Research Support at the University of Córdoba (Spain). The size of the amplified bands were calculated based on an internal standard DNA (400HD-ROX, Applied Biosystems) with GeneScan software (v 3.x Applied Biosystems) and the results analysed using the Genotyper software (v 3.7, Applied Biosystems).

Data analysis

Total number of alleles and expected heterozygosity (H_E) of each of the 12 microsatellite markers in a set of 47 *Fusarium oxysporum* f. sp. *lentis* isolates as well as the average number of markers (N_{av}), the number of private markers (N_{pr}), and unbiased expected heterozygosity (H_E) within each of four regions [P1 Algeria, P2 Syria, P3 North West (NW) Iran and P4 North East (NE) Iran], were calculated using ARLEQUIN ver. 3.0 (Excoffier et al. 2005). Allelic richness (N_{ar}) and private allelic richness (N_{par}) within each population were estimated after controlling for differences in sample size

Table 2 Primer sequences, SSR motif, amplification conditions, amplicon size range, diversity traits and putative chromosome location of SSRs analysed in the collection of 47 *F. oxysporum* f.sp. *lentis* isolates

No.	Marker[a]	Primers sequences (3′-5′)	SSR motif	Annealing Temp. (°C)	$MgCl_2$ (mM)	Amplicon size range (bp)	No of alleles	H_E[b]	Chromosome position (Chr.: bp)[c]
1	MB14*	Fw:CGTCTCTGAACCACCTTCATC Rv: TTCCTCCGTCCATCCTGAC	$(CCA)_5$	57	2.5	201–213	2	0.322	Chr. 1: 3,925,915
2	MB11*	Fw: GTGGACGAACACCTGCATC Rv: AGATCCTCCACCTCCACCTC	$(GGC)_7$	68	2.5	190–200	4	0.707	Chr. 4: 1,635,005
3	MB18	Fw: GTAGGAAATGACGAAGCTGAC Rv: TGAGCACTCTAGCACTCCAAAC	$(CAACA)_6$	57	2.5	286–310	5	0.784	Chr. 4: 3,657,593
4	SSR17	Fw: AGGATTTGGTTTCTTGTTCTC Rv: ACCTCATGATCTATCCTGTCC	$(AGT)_4$	50	2.5	169–179	4	0.603	Chr. 5: 3,446,927
5	SSR18	Fw: GAAGGTAAGGTTTGGTGAAGT Rv: GTAGGCCGTACCTACCTAGC	$(CCTGG)_3$	50	1.5	142–194	6	0.748	Chr. 5: 3,453,750
6	SSR8*	Fw: TCATATTATTGGGCTGAGAGA Rv: AGGCTCTGAAGCTGTTTTATT	$(AC)_6$	50	2.5	144–157	7	0.714	Chr. 5: 4,413,845
7	SSR6	Fw: CAGGCTTCAGTCCCTAATATC RV: CATGATGAAAATATACGAGAA	$(TCACT)_3$	50	2.5	158–165	4	0.679	Chr. 5: 4,446,929
8	SSR14	Fw: GATCGTTAGGAGAGCTAAAGG Rv: CAATCTAGGCATTCTCTTTCA	$(CA)_6$	50	1.5	166–168	2	0.313	Chr. 7: 2,310,847
9	SSR15*	Fw: GCAGGTGTCTGTCACTTGTAT Rv: CGTTGATGTAGAGAACAAAGG	$(TCG)_6$	50	2.5	161–187	7	0.818	Chr. 7: 2,318,036
10	SSR21	Fw: TCTCCAATAACATCATCCTCA Rv: GAGGATAGCGAAGAGAAGAAG	$(TCC)_6$	55	2.5	149–161	4	0.638	Chr. 8: 1,806,301
11	SSR23*	Fw: GATGATGATGTTTGGGAGAC Rv: CGCGACACTTCATATAAAAAC	$(CGTGAC)_3$	55	2.5	152–173	5	0.688	Chr. 8: 1,810,577
12	MB2*	Fw: TGCTGTGTATGGATGGATGG Rv: CATGGTCGATAGCTTGTCTCAG	$(GT)_{11}(GA)_6$	57	2.5	236–285	10	0.867	Chr. 9: 2,166,733

[a] SSR markers are newly developed in the present study and MB markers are those reported by Bogale et al. 2015

[b] Expected heterozygosity

[c] Obtained by blasting (BLASTN) the sequence amplified by the primers against the genome sequence of *Fusarium oxysporum f. sp. lycopersici* 4287 (Sequence ID: NC_030994.1)

*SSR selected for analyses concerning genetic relationships and population structure

using the rarefaction method (Kalinowski 2004) implemented in the program HP-Rare (Kalinowski 2005).

For each SSR, the sequences located between the forward and reverse primers in GenBank accession AGBI00000000.1 were blasted (discontiguous megablast) against the whole genome sequence of *Fusarium oxysporum* f. sp. *lycopersici* 4287 (Sequence ID: NC_030994.1). Linkage disequilibrium (LD) between all pairs of loci was calculated within each population using GENEPOP ver. 4.7.0 (Rousset 2008). The sequential Bonferroni adjustments were applied to correct for the effect of multiple tests using SAS release 8.02 (SAS Institute 2004).

Genetic distances between pairs of samples (47 FOL and 1 FOP isolate) were calculated based on six microsatellite loci using the proportion-of-shared-alleles distances (D_{psa}; Bowcock et al. 1994) as implemented in MICROSAT (Minch et al. 1997). Cluster analysis was performed using the Fitch-Margoliash method in the PHYLIP ver. 3.6b software (Felsenstein 2004). The reliability of the tree topology was assessed via bootstrapping (Felsenstein 1985) over 1000 replicates generated by MICROSAT and subsequently used in FITCH and CONSENSE programs in PHYLIP. The tree was rooted using FOP isolate as an outgroup.

The analysis of molecular variance (AMOVA; Excoffier et al. 1992) was carried out using ARLEQUIN. AMOVA was used to partition the total microsatellite diversity among and within regions. The variance components were tested statistically by non-parametric randomisation tests using 10,000 permutations. Pairwise population comparisons examined with AMOVA resulted in values of ϕ_{ST} that are equivalent to the proportion of the total variance that is partitioned between two regions.

A model-based clustering method was applied on multilocus microsatellite data to define the number of clusters in the dataset using the software STRUCTURE ver. 2.3.4 (Pritchard et al. 2000). Thirty runs per each K were done by setting the number of clusters (K) from 1 to 11 on the Isabella computer cluster at the University of Zagreb, University Computing Centre (SRCE). Each run consisted of a burn-in period of 200,000 steps followed by 10^6 Monte Carlo Markov Chain replicates assuming admixture model. The choice of the most likely number of clusters (K) was carried out by calculating an ad hoc statistic ΔK based on the rate of change in the *log* probability of data between successive K values, as described by Evanno et al. (2005). The program STRUCTURE HARVESTER v0.6.92 was used to process the STRUCTURE results files (Earl Dent and von Holdt 2012). Runs were clustered and averaged using CLUMPAK (Kopelman et al. 2015).

The likelihood-ratio chi-square test in SAS (SAS Institute 2004) was used to test for dependence between cluster membership and pathotype of the isolates. The strength of association was assessed by calculating Cramér's V, the measure that reaches the maximum value of 1 when the two variables (i.e. classification criteria) are equal to each other. The pathotypes represented by less than five isolates (4 and 6) were excluded from the analysis.

Results

SSR analysis

Twelve SSRs, four from Bogale et al. (2005) and eight new SSRs developed in this study, were successfully amplified and yielded clear reproducible bands in the FOL populations analysed (Table 2). In some isolates, for some SSRs, fragment analysis identified more than one band. In this case only those clear bands, with a reproducible pattern in the population and of the expected size were selected. None of the SSRs developed by Vogelgsang et al. (2011) amplified in our samples.

The average number of alleles per SSR was five, being MB2 the most polymorphic, showing 10 different alleles, and SSR14 and SSR17 the least polymorphic, both showing only two alleles. Expected heterozygosity ranged from 0.313 (SSR14) to 0.867 (MB2) being 0.657 as average (Table 2).

Blasting of the expected sequence amplified by the SSRs against *Fusarium oxysporum* f. sp. *lycopersici* genome identified sequences highly similar (from 96 to 99% identity) in *F. oxysporum* f. sp. l*ycopersici* chromosomes 1, 4, 5, 7, 8 and 9 (Table 2). Several of the markers analysed were located in the same chromosome. Considering the isolates from the largest population (NW Iran), the significant linkage disequilibrium ($P < 0.05$) was observed between loci MB11 and MB18 ($P = 0.004$) both located in the chromosome 4. The same was true for the loci SSR14 and SSR15 ($P = 0.017$) in the chromosome 7 and between loci SSR21 and SSR23 ($P = 0.000$) in the chromosome 8. Three out of six pairwise tests for

linkage disequilibrium between four loci located in the chromosome 5 (SSR6, SSR8, SSR17, SSR18) were significant including the test between the most distant loci (SSR17 and SSR6; $P = 0.002$) (Table 3). Therefore, in order to avoid linkage disequilibrium effect, only one SSR per chromosome was selected (Table 2) for further analyses concerning genetic relationships and population structure.

FOL population genetic characterization

Regarding FOL populations, four regions were initially considered, Algeria, Syria, North West Iran (NW Iran) and North East Iran (NE Iran). NW Iran showed the highest allelic richness (N_{ar}) as well as the highest unbiased expected heterozygosity (H_E) (Table 4.). In addition, the private alleles were found exclusively in Iranian populations, although it could be the effect of the sample size as shown by the values of private allelic richness.

The genetic distance between pairs of FOL isolates based on the proportion-of-shared-alleles distance measure ranged from $D_{psa} = 0.000$ (76 genetically identical pairs of isolates) to $D_{psa} = 1.000$ (256 pairs of isolates having no alleles in common) with the average of D_{psa} a = 0.709.

AMOVA showed that the observed genotypic variation in the FOL isolate collection was mainly due to the variation present within regions (accounting for 86.55% of the observed variation), but that variation among regions also exist and these differences were statistically

Table 3 Significance level of linkage disequilibrium (LD) between all pairs of loci located on same chromosome in the *F. oxysporum* f.sp. *lentis* isolates from North-West Iran

Chromosome	Locus1	Locus2	P^a
Chr. 4	MB11	MB18	0.004
Chr. 5	SSR6	SSR8	0.051
Chr. 5	SSR6	SSR17	0.002
Chr. 5	SSR6	SSR18	0.000
Chr. 5	SSR8	SSR17	0.314
Chr. 5	SSR8	SSR18	0.066
Chr. 5	SSR17	SSR18	0.000
Chr. 7	SSR14	SSR15	0.017
Chr. 8	SSR21	SSR23	0.000

[a] Significance levels after sequential Bonferroni adjustments

Table 4 Sample size (n), average number of alleles (N_a), allelic richness (N_{ar}), number of private alleles (N_{pr}), private allelic richness (N_{par}) and unbiased expected heterozygosity (H_E) of 47 *Fusarium oxysporum* f. sp. *lentis* isolates for different regions based on 12 microsatellite loci

Region	n	N_a	N_{ar}	N_{pr}	N_{par}	H_E
Algeria	4	1.833	1.361	0	0.321	0.431
Syria	8	2.833	1.567	0	0.597	0.618
NW[a] Iran	22	4.750	1.662	8	0.404	0.679
NE Iran	13	3.000	1.427	3	0.356	0.445

[a] *NE* and *NW* North East and North West, respectively

significant (Table 5). Pairwise ϕ_{ST} values showed that Algeria and Syria isolates differed significantly from Iranian isolates (Table 6).

To further verify these results, an additional AMOVA was carried out to check whether the observed genotypic variation was caused by the variation present among Iranian vs non Iranian regions. This AMOVA showed that considering Iranian regions as one group and non-Iranian regions as another group, differences within groups were not significant, but the groups differed significantly from each other (Table 5).

Population structure analysis performed by using STRUCTURE software also reinforced these results. For choosing the most likely number of clusters (K) we used ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values, as described by Evanno et al. (2005). The best results were obtained at K = 7 ($\Delta K = 40.68$) followed by those at K = 2 ($\Delta K = 23.35$) (Fig. 1). At K = 2 the cluster A included the FOP isolate along with 21 isolates originating exclusively from Iran (NW and NE), while 26 isolates from all the regions were assigned to the cluster B (Fig. 2). At K = 7, cluster A was divided in three clusters (A1, A2 and A3), being the FOP isolate located in cluster A1. Clusters B was divided in four clusters. Clusters B1 and B2 included isolates only from Syria and North West Iran, while clusters B3 and B4 included isolates from all regions (Fig. 2). The clusters identified by STRUCTURE were in agreement with the distance-based analysis (i.e. Fitch-Margoliash tree) results (Fig. 2).

The association between cluster membership and the pathotype of the isolates was not significant neither at K = 2 ($\chi^2 = 0.55$; df = 4; $P = 0.986$; Cramér's V = 0.11) nor at K = 7 ($\chi^2 = 23.37$; df = 24; $P = 0.498$; Cramér's V = 0.32).

Table 5 Analysis of molecular variance (AMOVA) for the partitioning of genetic diversity of *Fusarium oxysporum* f. sp. *lentis* (FOL) isolates based on six microsatellite loci (A) among and within regions (Algeria, Syria, NW Iran, NE Iran, and (B) between groups of regions (Iranian vs. non-Iranian), between regions within groups and within regions

Analysis	Source of variation	df	Variancecomponents	% Total variance	ϕ-statistics	$P(\phi)^a$
(A)	Among regions	3	0.21	13.45	0.134	0.0002
	Within regions	43	1.32	86.55		
	Between groups	1	0.16	10.17	0.102	<0.0001
(B)	Between regions within groups	2	0.11	7.07	0.079	0.0268
	Within regions	43	1.32	82.76	0.172	0.0002

[a] ϕ-statistic probability level after 10,000 permutations

Discussion

Understanding the genetic structure of plant pathogen populations is a strategy that estimates the pathogen evolutionary potential and allows management strategies to be chosen, with the aim of enhancing the durability of resistant varieties and determines cultivar deployment strategies (McDonald and Linde 2002). Simple sequence repeats (SSRs) provide a powerful tool for taxonomic and population genetic studies (Bogale et al. 2005). They are locus specific, highly reproducible and can be scored in a high-throughput way. However, up to now only a few SSRs were available for FOL. In fact, the only SSRs used so far for this pathogen were the nine SSRs markers reported by Bogale et al. (2005) for the *Fusarium oxysporum* complex. In the present study we have developed eight new SSR markers that were successfully amplified in FOL, increasing markedly the molecular markers available for this specie.

The FOL collection studied included isolates from Iran, Syria and Algeria, being, so far, the study analysing the genetic diversity of FOL covering the widest geographical region. Our results showed that there is a high molecular variation within regions, being NW Iran the region that showed the highest genetic diversity. Previous studies have also concluded that most of the variance of genetic variation occurred within populations (Al-Husien et al. 2017; Nourollahi and Madahjalali 2017). This high diversity within populations can be due to a high rate of mutation or presence or transposable elements in the genome as reported by Ma et al. (2010), and could explain the existence of different pathotypes in FOL.

Despite this high molecular variation within isolates from the same region, our studies also show that there are significant differences between regions. Thus, Iranian populations differed significantly from non-Iranian populations and private alleles were identified exclusively in populations collected in Iran. In addition, cluster A, identified by STRUCTURE software at K = 2 was only present in Iran.

No sexual stage is yet known for *F. oxysporum* and populations are expected to be formed by a mixture of

Table 6 AMOVA's pairwise ϕ_{ST} values and their significance between regions

Region	Syria	NW Iran	NE Iran
Argelia	0.211^{ns}	0.166^{*}	0.426^{**}
Syria		0.086^{*}	0.277^{***}
NW Iran			0.048^{ns}

P value significance levels: *** $P < 0.001$; ** $0.001 < P < 0.01$, * $0.01 < P < 0.05$, ^{ns} $P > 0.05$

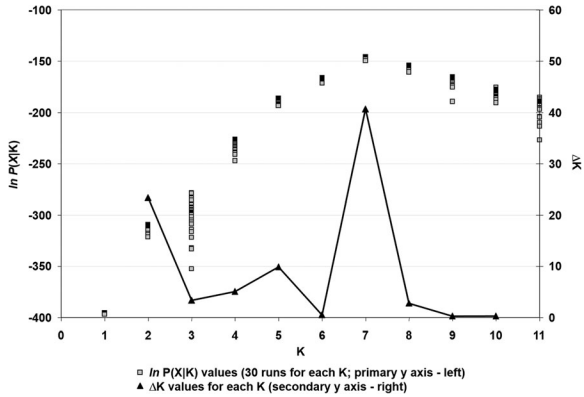

Fig. 1 The choice of the most likely number of clusters (K) inferred from six microsatellite markers of 47 *Fusarium oxysporum* f. sp. *lentis* (FOL) and a *F. oxysporum* f. sp. *pisi* (FOP) isolates using a model-based clustering method of Pritchard et al. (2000): *ln P(X|K)* values for each of the 30 independent runs for each K and ΔK values for each K based on the second order rate of change of the likelihood function with respect to K described by Evanno et al. (2005)

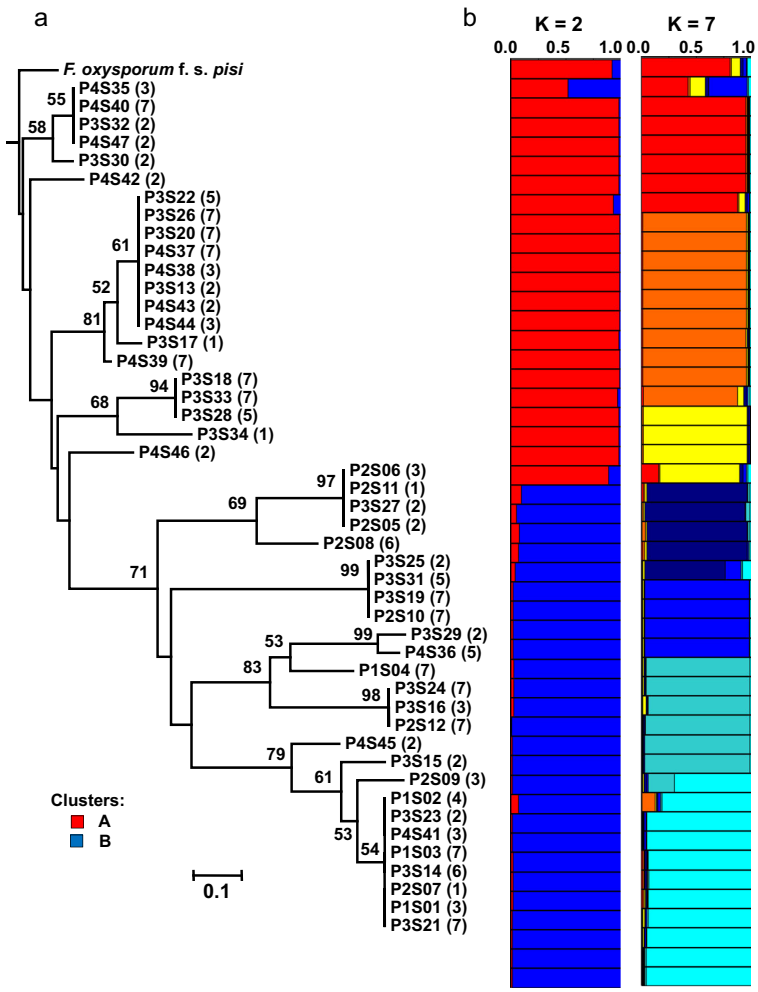

Fig. 2 Fitch-Margoliash tree of 47 *Fusarium oxysporum* f. sp. *lentis* (FOL) isolates and the proportion of membership in each cluster at K = 2 and 7 as defined with a model-based clustering method from Pritchard et al. (2000) based on six microsatellite markers. Region of origin of each sample is indicated as follows: R1 Algeria, R2 Syria, R3 NW Iran, R4 NE Iran. The pathotypes are shown in brackets (P1-P7). The FM tree was rooted using *F. oxysporum* f. sp. *pisi* (FOP) isolate as an outgroup. Bootstrap support values >50% of 1000 replicates are given above branches. Each individual isolate is represented by a single horizontal line divided into colours. Each colour represents one cluster, and the length of the coloured segment shows the individual's estimated proportion of membership in that cluster. Cluster membership of each isolate is indicated on the left (at K = 2) and on the right (at K = 7) of the diagram

clonal lineages. The spatial distribution of these clonal lineages is caused by the dispersion of the asexual propagules. STRUCTURE software predicted that the FOL isolates studied belonged to seven different lineages. Excepting isolate S46, all isolates showed a high value of Q (proportion of membership), suggesting the absence or low level of interbreeding between lineages.

Our results are in agreement with previous studies. Thus, Al-Husien et al. (2017) concluded that the FOL isolates from Syria they studied could be divided in three populations. In our study, for K = 7, the isolates from Syria analysed were also distributed in three different clusters. Similarly, Belabid et al. (2004), reported that FOL isolates from Algeria could be clustered in two main groups, and the isolated from this region we included were also located in two clusters. Nourollahi and Madahjalali (2017) concluded that isolates from Iran could be genetically clustered in three main genetic groups. These groups could correspond to the three main clusters (A1, A2 and A3) identified in our study including only isolates from Iran, although some isolated from Iran were also present in other clusters in our study.

Previous studies concluded the presence from two to three lineages in FOL (Belabid et al. 2004; Datta et al. 2011; Al-Husien et al. 2017; Nourollahi and Madahjalali 2017). While previous studies analysing FOL genetic structure were limited to isolates from a reduced geographical region, our study is the first including isolates from three different countries. This allowed us to identify a higher number of lineages in FOL than previously reported (up to seven) and to obtain information about the evolution and possible spread of FOL across countries. However, the presence

of a peak of *ln* P(X|K) at $K = 2$ suggests that these 7 lineages could derive from two ancestral lineages.

Older fungal populations have more alleles than younger ones as there have been more generations for mutation to occur and then for genetic drift to increase these alleles to a detectable frequency (McDonald and Linde 2002). In our study Iran was the region showing the highest FOL genetic diversity and one of the predicted ancestral clonal lineages A, closely related to FOP was only present in this region. These results suggest that Iran could be a regional center of origin for FOL and that ancestor A could be the oldest clonal lineage. Later on, mutations from this ancestor A could result in the appearance of ancestor B, that was later on dispersed to other geographical regions (Syria and Algeria).

No clear relationship could be established between genetic similarity (i.e. cluster membership) and pathotype of the isolates. Previous studies also concluded that phylogenetic similarity cannot be related to virulence group membership, being different *F. oxysporum formae speciales* spread out across the different lineages (Baayen et al. 2000; Bao et al. 2002; Bogale et al. 2006). This result is not surprising, as differences in virulence could be caused by a single mutation in isolates coming from any ancestral lineage and selected under a certain selection pressure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. All authors have revised the manuscript and approved its submission.

Research involving human participants and / or animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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