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Molecular variance and population structure of lentil (*Lens culinaris* Medik.) landraces from Mediterranean countries as revealed by simple sequence repeat DNA markers: implications for conservation and use

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Abstract

The Mediterranean region has a rich history of domestication and cultivation of lentil (*Lens culinaris* Medik.). Landraces have been grown and repeatedly selected by local farmers under different agro-environments. Characterization of molecular variation and genetic differentiation helps to ensure enhanced valorization, conservation and use of these genetic resources. Nineteen Simple Sequence Repeat DNA markers were used for molecular variance analysis (AMOVA) and population structure assessment underlying 74 lentil landraces from four Mediterranean countries: Morocco, Italy, Greece and Turkey. Based on AMOVA, presence of population structure and genetic differentiation at different levels were evidenced. Genetic diversity among Turkish landraces was higher than that of other countries. These landraces were more homogeneous as shown by low genetic differentiation among individuals within each landrace. Whereas Moroccan landraces followed by Italian and Greek provenances showed higher diversity and differentiation among individuals within landraces. The wide genetic variability of these landraces could help to better adaptation to biotic and abiotic stresses. Moreover, they could provide useful alleles related to adaptive traits for breeding purposes. Based on structure analysis, we obtained indications of possible presence of two major gene pools: a northern gene pool composed of Turkish, Italian and Greek landraces, and a southern gene pool composed of Moroccan landraces. Our results could be of interest when designing future diversity studies, collection missions, conservation and core collection construction strategies on Mediterranean lentil landraces.

Keywords: AMOVA, genetic differentiation, Mediterranean lentil landraces, SSR markers

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Introduction

Lentil (*Lens culinaris* Medik.; Fabaceae) is one of the most important food legumes worldwide. It contributes to reduce hunger and malnutrition especially with low-income people. Its grains are largely consumed as staple food especially in developing countries and as vegetarian meal and in salads in many parts of the word, providing a cheap source of proteins, vitamins and some important micronutrients like iron and zinc (Grusak, 2009; Grusak and Coyne, 2009; Carbonaro *et al.*, 2015). Increased production and consumption of this mineral-rich food could reduce mineral malnutrition affecting more than half of the world's population (Mayer *et al.*, 2008; White and Broadley, 2009; Shahzad *et al.*, 2014). Furthermore, the crop provides a number of additional agronomic, environmental and economic benefits. Lentils are widely cultivated in the Middle East, North Africa, Ethiopia, the Indian subcontinent, North America and Australia (Bhatty, 1988; Erskine *et al.*, 1990; Muehlbauer and Tullu, 1997; Ferguson and Erskine, 2001; Sarker *et al.*, 2002; Yadav *et al.*, 2007; Coyne and McGee, 2013).

The Mediterranean basin is known for its species richness with 'diversity hot spots' for various food legumes (Davis *et al.*, 1994; Akeroyd, 1999; Maxted and Bennett, 2001). It is the center of diversity of a number of important crop species, such as some cereals, legumes and olives, among others. It has also one of the richest floras of the world containing some 25,000 plant species (Maxted and Bennett, 2001). The Mediterranean region has a rich history of domestication and cultivation of lentil; local farmers have repeatedly selected landraces and local cultivars for adaptation to biotic and abiotic stress conditions over a long period of time. A wide diversity of agro-environments (highlands, drylands, more favourable areas) is also known to occur, thanks to the diversity of climatic and edaphic conditions. Lentils collected from these different regions most probably have high molecular diversity and different responses to abiotic and biotic stresses as a result of reproductive isolation and evolutionary differences among populations (Heywood, 1995; Akeroyd, 1999; Idrissi *et al.*, 2015, 2016).

Molecular characterization of the genetic diversity and population structure of genetic resources such as landraces is an important element in defining appropriate strategies for their collection, management, efficient conservation and utilization in breeding programmes. An effective lentil breeding programme requires utilizing lentil genetic resources effectively and efficiently to develop valuable genotypic and phenotypic variations in promising new lentil varieties (Gepts, 2006; Singh *et al.*, 2014). Several studies on genetic diversity and relationships between lentil landraces have been performed in a number of Mediterranean countries using different molecular markers (Ferguson *et al.*, 1998; Sonnante and Pignone, 2001; Sonnante *et al.*, 2003; Duran and Perez De La Vega, 2004; Toklu *et al.*, 2009; Bacchi *et al.*, 2010; Zaccardelli *et al.*, 2011; Lombardi *et al.*, 2014; Idrissi *et al.*, 2015, 2016; Khazaei *et al.*, 2016). These authors reported high genetic diversity but population structure still not well clarified in the Mediterranean region. We therefore focused on analysing molecular variance and determining the population structure using Simple Sequence Repeat (SSR) markers of lentil landraces from this region.

Materials and methods

Plant materials

Seventy-four macro- and microsperma lentil landraces collected in four different Mediterranean countries (Morocco, Italy, Turkey and Greece; Table 1) were genotyped using SSR markers. Landraces were provided by the Moroccan National Gene Bank, INRA-Settat, Morocco; the Italian National Council of Research, Institute of Biosciences and Bioresources, Bari, Italy; and by the National Plant Germplasm System, United States Department of Agriculture, USA (for landraces from Turkey and Greece).

DNA extraction

All seeds were planted in the greenhouse. Young leaves were collected from 2- to 3-week-old plantlets and lyophilized. As landraces could be composed of a mixture of different genotypes, DNA was isolated from five single plants from each landrace representing different collection sites.

Genomic DNA was isolated according to the NucleoSpin Plant (MACHEREY-NAGEL, MN; Duren, Germany) kit protocol. Tissue Lyser (Qiagen; Manchester, UK) was used to homogenize 20 mg of dry weight (lyophilized) plant material. Then, 450 µl of PL2 lysis buffer was added to the resulting powder allowing to solubilize the cell membrane and therefore release DNA. Tubes were then mixed thoroughly and 15 µl of RNase A was added to remove RNA before incubating the mixture for 30 min at 65°C. After adding 112.5 µl of PL3 buffer and mixing, tubes were incubated for 5 min on ice in order to precipitate SDS completely, followed by a 5 min of 14,000 rpm centrifugation step. The obtained crude lysate was loaded onto a NucleoSpin® Filter column and centrifuged for 2 min at 11,000 rpm to collect the clear flow-through. For adjusting DNA binding conditions, 675 µl of PC buffer was added and tubes contents mixed thoroughly. After that, a maximum of 700 µl of each sample was loaded to a new collection of tubes using the NucleoSpin® Plant II Column followed by a centrifugation step for 1 min at 11,000 rpm. Wash buffers PW1

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Table 1. List of the 74 lentil landraces analysed and their respective origins

Name	Origin[a]	Name	Origin[a]
ALTAMURA	Italy	MGB1035	Morocco
TIPO ASSTELLUCCIO	Italy	MGB1036	Morocco
MOUNTAIN LENTIL	Italy	MGB1045	Morocco
TIPO TURCHE NO2	Italy	MGB1049	Morocco
MG110288	Italy	MGB1050	Morocco
MG110438	Italy	MGB1051	Morocco
MG106892	Italy	MGB1052	Morocco
MG110287	Italy	MGB1053	Morocco
MG111854	Italy	MGB1054	Morocco
MG111863	Italy	MGB1055	Morocco
MG106899	Italy	MGB1056	Morocco
MG111849	Italy	MGB1058	Morocco
AKCA MERCIMEGI	Turkey	MGB1008	Morocco
YERLI1	Turkey	MGB1010	Morocco
ADI	Turkey	MGB1043	Morocco
YERLI2	Turkey	MGB1044	Morocco
ILL183	Turkey	MGB996	Morocco
ILL171	Turkey	MGB997	Morocco
ILL306	Greece	MGB999	Morocco
ILL312	Greece	MGB1026	Morocco
ILL298	Greece	MGB1027	Morocco
MGB1000	Morocco	MGB1037	Morocco
MGB1013	Morocco	MGB1038	Morocco
MGB1015	Morocco	MGB1039	Morocco
MGB1016	Morocco	MGB1040	Morocco
MGB1017	Morocco	MGB1041	Morocco
MGB1019	Morocco	MGB1042	Morocco
MGB1020	Morocco	MGB1047	Morocco
MGB1022	Morocco	MGB1060	Morocco
MGB1023	Morocco	MGB1061	Morocco
MGB1024	Morocco	MGB1062	Morocco
MGB1025	Morocco	MGB7377	Morocco
MGB1029	Morocco	MGB7386	Morocco
MGB1030	Morocco	MGB7389	Morocco
MGB1031	Morocco	MGB7457	Morocco
MGB1032	Morocco	L24 (local cultivar)	Morocco
MGB1034	Morocco	L56 (local cultivar)	Morocco

[a]Landraces from Morocco were provided by Moroccan National Gene Bank, INRA-Settat, Morocco. Landraces from Italy were provided by Italian National Council of Research, Institute of Biosciences and Bioresources, Italy. Landraces from Turkey and Greece were provided by National Plant Germplasm System, United States Department of Agriculture, USA.

(two washes of 400 and 700 µl followed by a centrifugation of 1 min at 11,000 rpm) and PW2 (200 µl, 2 min at 11,000 rpm centrifugation) were used to wash away contaminants and dry the silica membrane. Finally, genomic DNA was eluted with low salt elution buffer PE (65°C) with a twice repeated step of adding 50 µl, incubation at 65°C for 5 min and a centrifugation of 1 min at 11,000 rpm. Concentration and quality of DNA were verified using a NanoDrop Spectrophotometer ND-1000 (Isogen; De Meern, The Netherlands). Isolated DNA was then diluted to 15 ng/µl and stored at −20°C.

SSR analysis

Thirty microsatellite markers developed by Hamwieh *et al.* (2005) were evaluated in this study. All SSRs were first tested for amplification and polymorphism on a subset of 16 DNA samples. Based on the published polymerase chain reaction (PCR) conditions (Hamwieh *et al.*, 2005), annealing temperature (T_a) and number of PCR cycles were optimized for each marker to produce clear and reproducible microsatellite profiles. Of the 30 tested SSRs, 19 were polymorphic and as such selected for further use in this study (Table 2). PCR analysis was performed according to the Qiagen Multiplex PCR kit protocol with a final volume of 10 µl per reaction. Each reaction mix contained 5 µl of 2× Qiagen MultiPlex Mastermix (Multiplex PCR Kit; Qiagen; Manchester, UK), 0.2 µl of each primer (10 µM), RNase-free water and 1 µl of DNA (15 ng/µl). Different multiplex sets, with similar reaction conditions, were composed containing two or three microsatellites. Forward primers were labelled fluorescently (FAM, HEX and NED, Table 2).

PCR was conducted in a GeneAmp 9700 Dual thermocycler. The Hot StarTaq DNA polymerase enzyme was activated with a heating step of 15 min at 95°C, followed by 25 or 30 cycles (Table 2) of 30 s at 94°C (denaturation), 90 s at T_a (annealing, Table 2) and 60 s at 72°C (extension) with a final extension step of 30 min at 60°C. Samples were then stored at −20°C and protected against light. Of the final PCR product, 1 µl was mixed with 13.5 µl Hi-DiTM Formamide (Applied Biosystems; Carlsbad, California, USA) and 0.5 µl of GeneScanTM-500 Rox Size Standard (Applied Biosystems; Carlsbad, California, USA). Products were denatured by heating for 3 min at 90°C. Capillary electrophoresis and fragment detection were performed on an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems). GENEMAPPER 4.0 software (Applied Biosystems) was used for scoring the alleles.

Data analysis

Analysis of molecular variance (AMOVA) and population differentiation as estimated by fixation indices (Wright, 1978) with 1023 permutations were assessed using

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Table 2. Primer sequences and PCR conditions used for the amplification of the microsatellites in the landraces (Idrissi *et al.*, 2015)

Locus name	Primer sequences (5′−3−)		T_a (°C)	Alleles size range (bp)	No of cycles	PCR multiplex set	Fluorescent label
	Forward	Reverse					
SSR113	CCGTAAGAATTAGGTGTC	GGAAAATAGGGTGGAAAG	53	211–245	25	1	NED
SSR154	GGAATTTATCACACTATCTC	GACTCCCAACTTGTATG	53	261–381	25	1	FAM
SSR199	GTGTGCATGGTGTGTG	CCATCCCCCTCTATC	53	180–211	25	2	FAM
SSR124	GTATGTGACTGTATGCTTC	GCATTGCATTTCACAAACC	56	174–177	25	3	NED
SSR233	CTTGGAGCTGTTGGTC	GCCGCCTACATTATGG	56	126–159	25	3	HEX
SSR80	CCATGCATACGTGACTGC	GTTGACTGTTGGTGTAAGTG	60	129–157	25	4	FAM
SSR184	GTGTGTACCTAAAGCCTTG	GTAAGTTGATCAAACGCCC	60	216–271	25	5	FAM
SSR48	CATGGTGGAATAGTGATGGC	CTCCATACACCACTCATTCAC	60	163–195	25	5	HEX
SSR19	GACTCATACTTTGTTCTTAGCAG	GAACGGAGCGGTCACATTAG	60	255–276	25	6	HEX
SSR99	GGGAATTTGTGGAGGGAAG	CCTCAGAATGTCCCTGTC	60	153–164	25	6	FAM
SSR302	CAAGCCACCCATACACC	GGGCATTAAGTGTGCTGG	60	231–276	30	7	FAM
SSR309-2	GTATGTCGTTAACTGTCGTG	GAGGAAGGAAGTATTCGTC	50	171–193	25	8	FAM
SSR204	CACGACTATCCCACTTG	CTTACTTTCTTAGTGCTATTAC	56	177–195	30	9	HEX
SSR336	GTGTAACCCAACTGTTCC	GGCCGAGGTTGTAACAC	56	235–270	30	9	FAM
SSR119	GAACTCAGTTTCTCATTG	GAACATATCCAATTATCATC	50	263–297	30	10	HEX
SSR212-1	GACTCATTGTTGTACCC	GCGAGAAGAATGGTTG	50	159–207	30	10	NED
SSR215	CATTAATATTTCTTTGGTGC	CTTTTCTTCTCTTCCCC	50	361–441	30	10	FAM
SSR130	CCACGTATGTGACTGTATG	GAAAGAGAGGCTGAAACTTG	56	195–198	30	11	NED
SSR33	CAAGCATGACGCCTATGAAG	CTTTCACTCACTCAACTCTC	56	250–321	30	11	HEX

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Arlequin ver 3.11 programme (Excoffier *et al.*, 2005). For the total Mediterranean population, genetic variation was tested among countries, among landraces within countries, and among individuals (accessions) within landraces and within individuals. For each country, three levels of structure were considered: individuals among landraces, landraces among countries and countries among the total Mediterranean population.

Structure 2.3.4 software (Pritchard *et al.*, 2000, 2010) was used to investigate the population structure of the lentil Mediterranean germplasm studied including all genotypes (five for each landrace) using SSR multilocus genotype data. The admixture model was assumed to perform 10 runs for $K = 1$ to $K = 10$ with 'length of burning period' and 'number of Markov chain Monte Carlo' repeats of 100,000 both. In the admixture model, both possibilities of correlated allele frequencies and independent allele frequencies were tested. The output of the 10 runs were used to estimate the most likely number of gene pools (k) according to the method described by Evanno *et al.* (2005) using the *ad hoc Delta k* (ΔK) statistic. This method allows to identify genetically homogeneous groups of individuals using a Bayesian algorithm. It is based on the rate of change in log probability (computed from posterior likelihoods) of data generated by successive K values, whereby the highest ΔK corresponds to the true number of gene pools (K). The ΔK was estimated following the formula of Evanno *et al.* (2005):

$$\text{Delta}\, K = [L''(K)]/S,$$

where S is the standard deviation of the estimated probability values $[L(K)]$ from the 10 runs, and $L''(K)$ is the absolute value of the second-order rate of change of the likelihood distribution.

Results

Molecular variance and population differentiation analysis

Molecular variance in Mediterranean lentil landrace populations was found to be higher within accessions (individual landraces) with up to 41.6% of total variance. Variations among individuals within landraces and among landraces within countries were also high with 29.9 and 21.3% of total variance, respectively (Table 3).

When performing AMOVA for each country, variation within individuals of landraces was higher for Moroccan and Italian landraces with 46.2 and 37.8% of total variation, respectively; while it was higher among individuals within landraces for Greek germplasm and among landraces for Turkish genetic material with 45.0 and 77.5% of total variation, respectively (Table 4). Variation among accessions of Turkish landraces was the lowest compared to other origins. The obtained negative value of −0.4 should be considered as close to zero because individuals of these landraces are more related to each other than landraces from the same population (Excoffier *et al.*, 2005). Genetic variation among landraces was highest for Turkish landraces, followed by Italian, Greek and Moroccan provenances with 77.5, 33.7, 25.1 and 17.0%, respectively.

Fixation indices were calculated to assess the degree of population structure at different levels (Table 5). This resulted in significant tests at $P < 0.001$, and population structure according to subpopulations was found to be present at individual, landrace and country levels except among individuals within Turkish landraces. When considering all Mediterranean germplasm, moderate genetic differentiation among countries was obtained with a value of $F_{CT} = 0.102$. Higher genetic differentiation was at individual level with a value of $F_{IT} = 0.564$ corresponding to very important differentiation according to Wright (1978). F_{ST} expressing the amount of genetic variance that can be explained by population structure within subpopulations of each country was high to very high showing considerable genetic differentiation of landraces.

Population structure analysis

Results obtained from the STRUCTURE programme using SSR markers for all genotypes are reported in Fig. 1. According to the method suggested by Evanno *et al.* (2005) for the estimation of the most likely number of gene pools (k) in a population based on the *ad hoc* ΔK

Table 3. AMOVA of all tested Mediterranean landraces

Source of variation	df	Sum of squares	Variance components	Percentage of variation (%)
Among countries	4	138.977	0.27399 V_a	7.22
Among landraces within countries	69	793.450	0.80897 V_b	21.32
Among individuals within landraces	276	1061.492	1.13371 V_c	29.87
Within individuals	350	552.500	1.57857 V_d	41.59
Total	699	2546.419	3.79524	

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Table 4. AMOVA by Country

Source of variation	df				Sum of squares				Variance components				Percentage of variation (%)			
	M	I	G	T	M	I	G	T	M	I	G	T	M	I	G	T
Among landraces	52	11	2	5	628.9	199.4	23.4	118.1	0.73	1.43	0.87	2.7	16.93	33.7	25.1	77.5
Among individuals within landraces	199	47	10	20	1027.9	189.6	41.8	6.4	1.58	1.21	1.57	−0.4[a]	36.9	28.5	45	−13[a]
Within individuals	252	59	13	26	501	95	13.5	32.5	1.99	1.61	1.03	1.25	46.17	37.8	29.8	35.8
Total	503	117	25	51	2157.9	484	78.7	157	4.30	4.25	3.5	3.49				

M, Morocco; I, Italy; G, Greece; T, Turkey.
[a]Negative variance components can sometimes occur, because they are rather covariances (Excoffier *et al.*, 2005).

statistic, the Mediterranean lentil germplasm used in this study could be divided into two or three gene pools (Fig. 1).

The best population genetic structure model is likely to be at $K = 2$, which displays the clear highest value of ΔK (317.61). For $K = 3$, a fairly higher value of ΔK (68.25) compared to other values of ΔK suggests also the possibility of three gene pools (Fig. 1).

For $K = 2$, landraces from the northern Mediterranean countries were clustered together in one gene pool (green cluster, Fig. 1(a)) with high membership proportions of assignation of genotypes of 73.7, 97.8 and 99.9% for landraces from Greece, Italy and Turkey, respectively. The second gene pool (red cluster, Fig. 1(a)) contained predominantly landraces from Morocco with high proportions of membership of each sub-population sorted by geographic origin of 99.0, 82.3, 80.6, 96.7, 98.9, 49.5 and 99.2% for Chaouia (I), Zaer (II), middle Atlas mountains (III), Abda (IV), Sais Meknès, local cultivars (VI) and unknown origin (VII), respectively. The two gene pools shared only small proportions of landraces from the northern and southern Mediterranean region, except for the two local Moroccan cultivars that were shown to be assigned to the two different gene pools. Genetic diversity in the same cluster estimated by expected heterozygosity was as high as 0.59 and 0.72 for the gene pool containing Moroccan landraces and for the ones containing landraces from the northern Mediterranean, respectively.

For $K = 3$, landraces from the northern Mediterranean countries were shown to belong to the same gene pool (green cluster, Fig. 1(b)) as found for $K = 2$ with closely similar proportions of membership (74.2, 96.5 and 99.6%, respectively, for landraces from Greece, Italy and Turkey). Moroccan landraces were assigned to the three different gene pools with different proportions. Moreover, they were mainly clustered together in the same gene pool as shown for $K = 2$ (red cluster, Fig. 1(b)). Proportions of membership for the latter gene pool (87.2, 69.7, 55.3, 99.2, 50.0 and 71%, respectively, for Chaouia, Zaer, middle Atlas mountains, Sais Meknès, local cultivars and unknown origin) were the highest compared to the two other gene pools except for landraces from Abda region. The third gene pool (blue cluster, Fig. 1(b)) contained 56.7% of landraces from Abda region, which is the highest proportion of membership for this gene pool. Expected heterozygosity in the same cluster was 0.58, 0.69 and 0.56 for the red, the green and the blue cluster, respectively.

For both cases ($K = 2$ and $K = 3$), genomes of some landraces include segments from different gene pools with proportions over all genotypes of 10.28 and 15.42%, respectively.

Discussion

Earlier results related to genetic diversity and population structure of Mediterranean lentil landraces using molecular

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Table 5. Fixation indices of tested Mediterranean landraces when grouped and by country of origin

Fixation indices	Tested Mediterranean landraces	Morocco	Italy	Greece	Turkey
F_{IS}	0.418*	0.444*	0.429*	0.602*	−0.594[a]**
F_{SC}	0.230*				
F_{CT}	0.102*				
F_{ST}		0.169*	0.337*	0.251*	0.775*
F_{IT}	0.564*	0.539*	0.621*	0.702*	0.642*

Genetic differentiation among individuals within landraces (F_{IS}), among landraces within countries (F_{SC}), among countries (F_{CT}), among landraces (F_{ST}) and among individuals (F_{IT}).
[a]F_{IS}: Negative variance components can sometimes occur, because they are rather covariances. Their associated fixation indices can also be seen as correlation coefficients (Excoffier *et al.*, 2005).
*Significant at $P < 0.001$; **not significant $P = 1.1$.

markers were reported by Lombardi *et al.* (2014) and Khazaei *et al.* (2016). Both authors mentioned high level of variation and fair dispersion and deviation of these landraces across the classification of other landraces from different agro-ecological zones. Thus, we aimed to elucidate enclosed molecular variance at different levels and the presence of different gene pools within landraces from four Mediterranean countries: Morocco from the southern, Italy, Greece and Turkey from the northern part.

The higher genetic diversity found among Turkish landraces compared to the three other countries is in agreement with the fact that Turkey is part of the Fertile Crescent, the lentil center of origin where it was initially domesticated (Ladizinsky, 1979; Sandhu and Singh, 2007; Cubero *et al.*, 2009; Hamwieh *et al.*, 2009; Faratini *et al.*, 2011). The site of Göbekli Tepe in southeastern Turkey, which lies northeast of the modern city of Şanlıurfa, was a religious center of the Early Neolithic World of Upper Mesopotamia. This region is considered to be the place where civilizations first came into being (Schmidt, 2011). This region is also very rich in terms of lentil genetic resources. As a matter of fact, Toklu *et al.* (2009) and Hamwieh *et al.* (2009) reported that Turkish lentil landraces exhibit considerable genetic diversity. However, we found Turkish landraces to have higher homogeneity (as evidenced by low genetic variation among individuals within landraces) compared to Greek, Moroccan and Italian provenances that have higher variability between individuals. Still, most farmers grow lentil landraces in the southeastern region of Turkey. Villagers keep several lots of their own seeds to sow the next season crop. Selecting the different type of seeds and keeping the remaining part may over the time increase the purity of landraces. This selection process may be the cause of the higher genetic diversity among landraces but the lowest one among the accessions within landraces. Morocco, Greece and Italy showed lower genetic diversity among landraces. Seed exchange, migration and management by farmers that might have resulted from gene flow between these landraces could be suspected to take place with higher intensity in these countries. Overall, this is more evident in Morocco compared to the northern Mediterranean countries included in this study, probably as a result of different farmer practices after drought episodes (more frequent in Morocco) that could have led to the reduction or loss of functional on farm seed reserve in a given region, thus introducing seeds of other landraces from different regions for the following cropping season. Benbrahim *et al.* (2017) have observed similar fact in Zaer region of Morocco (northwestern). Same result was reported for Italian landraces by Viscosi *et al.* (2010) who has obtained greater genetic variability within (56%) than among (44%) 12 landrace populations. Although a different result was reported by Sonnante and Pignone (2007) for 11 Italian landraces with variation among of 85% and within of 15%, they observed high levels of genetic diversity in some landraces from the Apennine ridge (Colfiorito, S. Stefano and Capracotta) and mainland of Sicily (Villalba). High variability was reported also by Piergiovanni and Taranto (2003) and Torricelli *et al.* (2011). Hence, higher diversity is still enclosed within landraces of these three countries namely Morocco, Italy and Greece. This should be taken in consideration in future diversity studies and collection strategies for genetic resource conservation by sampling from more locations in short distances for these three countries, while sampling from more distant locations in Turkey would be more suitable to yield higher diversity. On the other hand, high genetic heterogeneity of landraces from Morocco, Italy and Greece could be seen as an advantage that may help to better adaptation to biotic and abiotic stresses, such as diseases, pests, drought, low nutrients availability in poor soils, etc. (Maxim, 2010). This germplasm thus could provide valuable alleles of adaptive traits for breeding programmes. Preliminary indications of specific adaptation to contrasted agro-environmental zones were reported for Moroccan landraces by Idrissi *et al.* (2015).

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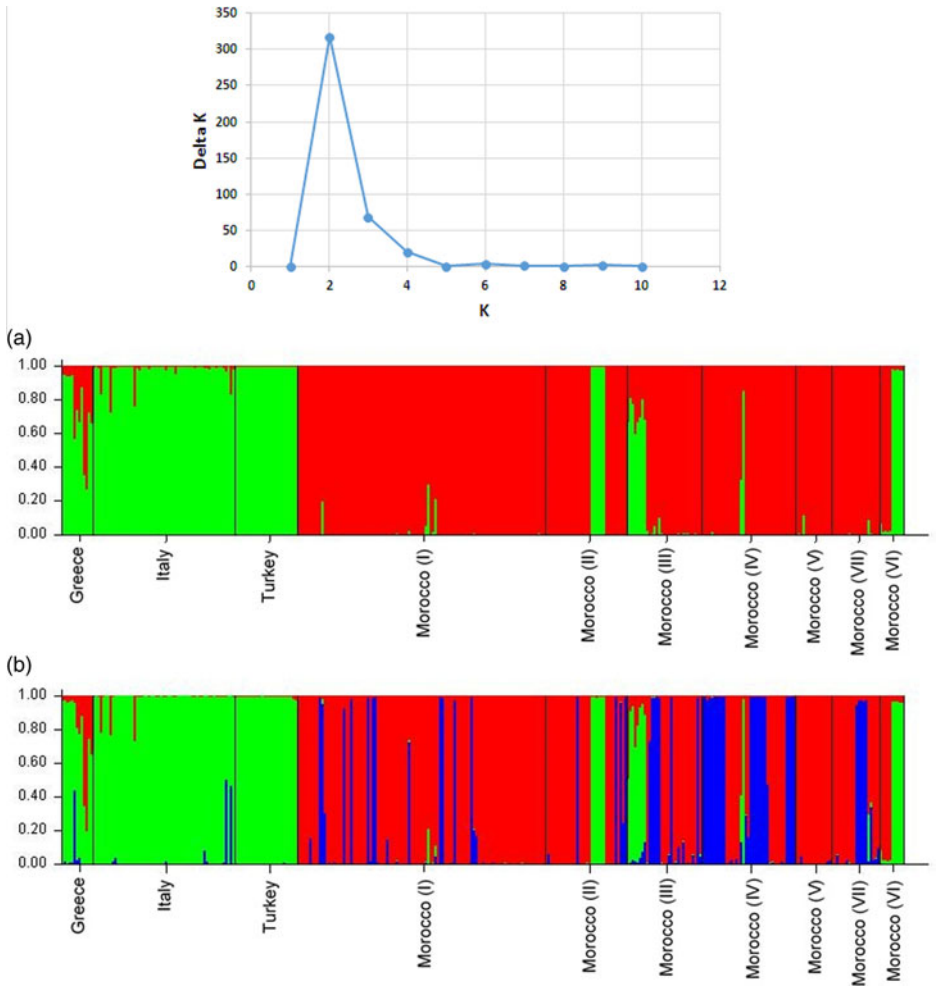

Fig. 1. Variation of Evanno *et al.* (2005) Δ*K* for each *K* calculated for 350 genotypes of the Mediterranean lentil landraces based on SSR markers (upper curve). Inferred population genetic structure for *K* = 2 (a) and *K* = 3 (b) for 350 genotypes of the Mediterranean lentil landraces based on SSR markers. Each individual genotype is presented by a vertical line divided into *K* coloured segments corresponding to the estimated fractions belonging to each gene pool shown in the vertical axis. An admixture model with correlated allele frequencies was assumed. I–VII refer to different regions of origin of Moroccan landraces and local cultivars: *Chaouia, Zaer, middle Atlas Mountains, Abda, Sais-Meknes*, local cultivars and unknown, respectively.

Population structure analysis using a Bayesian-based approach confirmed the obtained results by Idrissi *et al.* (2016) from principal component and neighbour-joining analyses, which revealed two distinct gene pools: northern Mediterranean landraces (Greece, Italy and Turkey) versus southern Mediterranean landraces (Morocco). When assuming the possibility of three gene pools as shown following Evanno *et al.* (2005) method, landraces from Abda region (westcentral Morocco), the driest area of origin among those included in this study, were shown to be assigned to a third gene pool. This is in agreement with results reported and discussed by Idrissi *et al.* (2015, 2016) evidencing the genetic differentiation of landraces from this area. Assuming either correlated allele frequencies or independent allele frequencies in the admixture model of Structure software leads to similar results about the detected population genetic structure (Porras-Hurtado *et al.*, 2013).

High genetic diversity obtained by Idrissi *et al.* (2016) was confirmed for each gene pool by high values of expected heterozygosity estimating average genetic distances between landraces obtained from population structure analysis.

The obtained results regarding the two contrasting major gene pools observed suggests that the introduction and/or evolution (once introduced) of lentil to these two areas probably followed different adaptation processes. Furthermore, although lentil has been mainly grown during winter in the Mediterranean zone, different phenological and genetic adaptations occur regarding regions. Late autumn–early winter sowing (decreasing day length followed

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by increasing day length and temperature) is frequent in Morocco, while in northern Mediterranean area there is a shift to late winter–early spring sowing (increasing day length and temperature). This could contribute to explain the genetic differentiation of Mediterranean landraces (Erskine *et al.*, 1989). In fact, significant but low Eigen values from discriminant analysis based on some phenotypic traits linked to drought tolerance (root traits, chlorophyll content, root–shoot ratio, relative water content, water loosing rate and wilting severity) showed distinctiveness of Moroccan landraces from northern Mediterranean ones (Idrissi *et al.*, 2016).

Northern–southern dissemination of lentil to low altitudes from long days to relatively short day regions (Erskine, 1997) may have reduced photoperiodic response and increased earliness. Landraces from Morocco are clearly earliest than Turkish, Greek and Italian ones (Idrissi, observation in field, not yet published). Different level of winter hardiness of landraces because of specific adaptation in northern Mediterranean countries where cold tolerance is an important adaptive trait compared to Morocco may contribute to explain the obtained genetic differentiation. Interestingly, we found some Moroccan landraces originating from highlands (mainly middle Atlas mountains), where cold stress is frequent, to share some genetic backgrounds with northern Mediterranean landraces. Additionally, landraces from Zaer region (northwestern Morocco), favourable areas for lentil growing with more rainfall when compared with other areas where drought and heat stresses are more frequent in Morocco, had also shared proportions of the genome with northern Mediterranean landraces. Erskine *et al.* (1981) reported high cold tolerance of Turkish and Greek lentil landraces as a result of natural and artificial selection in these countries compared to warmer countries such as Egypt.

Similar to our results, Lombardi *et al.* (2014) reported a very high level of genetic diversity for landraces from the Mediterranean region, especially those from Greece and Turkey, using SNP markers. Furthermore, Dikshit *et al.* (2015) concluded that Mediterranean landraces could be used for broadening the genetic base of lentil grown in South Asia. The latter authors attributed the low productivity of lentil in this region to a greater susceptibility to biotic and abiotic stresses due to its narrow genetic base compared to Mediterranean germplasm.

The distinction between the two major gene pools from northern and southern Mediterranean countries as well as the different levels of population differentiation from AMOVA results that we found are important when defining suitable strategies aimed to improve germplasm conservation and utilization. These strategies may be different between these countries. For instance, to prevent the risk of loss of the genetic diversity and genetic erosion in countries with high intra-population (inter-individuals) variation (i.e. Morocco), conservation efforts should focus more on accessions representing each landrace, thus increasing the probability of conserving the available intra-accession genetic diversity. While, conserving more landraces (with less representative accessions for each landrace compared to the previous described case) from different locations should be a priority in countries with higher inter-population variation, such as Turkey. In addition, the evidenced classification could be used prior to the selection of core collections by sampling from both defined groups. Our results from SSR analysis of landraces provide preliminary information about the allelic richness that could be targeted for the construction of such core sets aiming at maximization of genetic variability. Larger number of landraces from as much as possible Mediterranean countries should be considered in future studies to confirm our findings. Further phenotypic characterization and evaluation for biotic and abiotic stresses could help to better understand the obtained genetic differentiation between the two gene pools.

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Conflict of Interest

The authors of this study declare that they have no conflict of interest.

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