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ORIGINAL PAPER

Functional Genetic Diversity Analysis and Identification of Associated Simple Sequence Repeats and Amplified Fragment Length Polymorphism Markers to Drought Tolerance in Lentil (*Lens culinaris* ssp. *culinaris* Medicus) Landraces

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Abstract Genetic diversity of 70 Mediterranean lentil (*Lens culinaris* ssp. *culinaris* Medicus) landraces was assessed using simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs). These landraces were also assessed for variation in root and shoot traits and drought tolerance as estimated by relative water content (RWC), water losing rate (WLR) and wilting score (WS). Genetic diversity and clear differentiation of Moroccan landraces from those from northern Mediterranean regions (Italy, Turkey and Greece) were found. High genetic variation in root and shoot traits and traits related to drought tolerance was also observed. No relationship was found between drought tolerance of landraces and their geographic origin. Landraces with higher dry root biomass, chlorophyll content and root–shoot ratio were drought tolerant as evidenced by higher RWC and lower WLR and wilting severity. Kruskal–Wallis non-parametric test (K-W) was used to find SSRs and AFLPs associated with RWC, WLR and WS. Regression analysis showed six SSR and AFLP alleles explaining the highest phenotypic variation of RWC, WLR and WS (ranging from 21 to 50 % for SSRs and from 14 to 33 % for AFLPs). Functional genetic diversity analysis showed relationships between drought response of landraces and linked SSR and AFLP alleles to RWC, WLR and WS according to K-W test using canonical discriminant analysis. Our results confirm the feasibility of using association mapping to find DNA markers associated with drought tolerance in larger numbers of lentil landraces.

Keywords Lentil landraces · SSR · AFLP · Genetic diversity · Drought · Stress ecophysiology · Marker–trait association

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Introduction

Lentil (*Lens culinaris* ssp. *culinaris* Medicus) is an annual grain legume widely cultivated in the Middle East, North Africa, Ethiopia, the Indian subcontinent, North America and Australia for its protein-, mineral- (Fe, Zn,…) and vitamin-rich seeds. It is also a valued straw for animal feed (Bhatty 1988; Erskine et al. 1990; Ferguson and Erskine 2001; Grusak 2009). Lentil has yet a number of other agronomic benefits thanks to its ability to fix atmospheric nitrogen in symbiosis with Rhizobium species. It is also an important rotation component in cereal-based cropping systems, enhancing soil fertility and sustainability. Average annual global production of lentil is 4.55 million tons (Mt) from 4.2 million hectares (FAOSTAT 2012).

Lentil domestication probably occurred around 7000 BC in the foothills of the mountains between Turkey and Syria in the Eastern Mediterranean (Ladizinsky 1979 and 1987). After domestication, lentil spread to Greece, Central Europe, Egypt, Central Asia and India. Lentil probably reached North Africa, Spain and the Italian islands of Sardinia and Sicily eventually either from Central Europe or the Levant (Sonnante and Pignone 2001; Faratini et al. 2011). After the discovery of the New World, lentil was introduced into North and South America and more recently to Australia (Ferguson and Erskine 2001).

Globally, drought is one of the most challenging abiotic stresses causing yield losses limiting benefits to farmers. With increasing global warming in the context of climate change becoming more and more important, drought episodes are expected to worsen and become more frequent. Thus, improving plant tolerance and adaptation to water-limited conditions to maintain growth and yield is an important strategic research focus for breeders. Breeding for drought tolerance is a major objective in arid and semi-arid areas. Landraces selected over centuries are valuable genetic resources for developing genotypes adapted to different abiotic stresses, particularly drought.

Screening methods that use parameters reflecting water status in plants, such as relative water content, water losing rate and wilting score, have been reported as suitable and effective for genetic studies (Levitt 1980; Verslues et al. 2006; Shrestha et al. 2006; Razavi et al. 2011; Jain and Chattopadhyay 2010; Mullan and Pietragalla 2012; Singh et al. 2013; Khazaei 2013; Ammar et al. 2015; Idrissi et al. 2015b; Iglesias-García et al. 2015; Esmaeilpour et al. 2015). Well-developed roots, vigorous shoots at early seedling stage, high root–shoot ratio and chlorophyll content (Soil Plant Analysis Development (SPAD) value) have all been reported to be important indicators in promoting drought avoidance in lentil and other food legumes (Sarker et al. 2005; Kashiwagi et al. 2005; Vadez et al. 2008; Gaur et al. 2008; Aswaf and Blair 2012; Idrissi et al. 2015b).

Association of molecular markers with such traits of interest as those linked to drought tolerance is being studied using mapping populations to identify quantitative trait loci; in addition, unrelated genetic resources such as landraces are being used in association mapping to take advantage of the historic linkage between phenotypic and genetic variations during the process of selection and adaptation. Based on genetic diversity analysis, Singh et al. (2013) reported Simple Sequence Repeats (SSR) markers associated with Fusarium wilt (*Fusarium udum*) resistance in cultivated pigeon pea (*Cajanus cajan*), Razavi et al. (2011) identified Amplified Fragment Length Polymorphism (AFLP) and Expressed Sequence Tag (EST) candidate gene markers associated to water-deficit response in *Fragaria*, whereas Mondal et al. (2010) reported association of SSR markers with genes for rust and late leaf spot resistance in cultivated groundnut (*Arachis hypogaea* L.).

The Mediterranean region is expected to harbor high genetic diversity in lentil thanks to the rich history of domestication and cultivation as well as because of the frequency of biotic and abiotic stresses as selection pressure. In Mediterranean environments, lentil, as well as other crops, experiences intermittent drought during vegetative growth and end-cycle drought associated with increasing temperatures during reproduction stage (Silim et al. 1993; Materne and Siddique 2009). This offers opportunities for the identification of biotic and abiotic stress-resistant landraces. Although genetic diversity and relationships between lentil landraces have been reported from 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. 2015a), to our knowledge, no studies have reported on lentil genetic diversity in association with drought tolerance.

Thus, the objectives of our study were to (1) analyze genetic diversity of 70 landraces from different Mediterranean countries (Morocco, Italy, Turkey and Greece) using SSR and AFLP DNA markers, (2) characterize their root and shoot traits and to evaluate their drought tolerance using physiological parameters and (3) analyze their functional genetic diversity in association with drought tolerance as a first and preliminary step of testing association mapping studies in lentil.

Materials and Methods

Plant Materials

Seventy landraces collected in four different Mediterranean countries (Morocco, Italy, Turkey and Greece; Table 1) were evaluated for their genetic diversity using SSR and AFLP DNA markers and for their drought tolerance under greenhouse conditions using relative water content (Barr and Weatherley 1962; Verslues et al. 2006), water losing rate (Suprunova et al. 2004) and wilting score (Singh et al. 2013) as drought characterization parameters. The genetic diversity of Moroccan landraces according to their respective agro-environments was evidenced in Idrissi et al. (2015a).

DNA Extraction

All landraces were planted in the ILVO greenhouse (Melle, Belgium) in 2014. Young leaves were collected from 2- to 3-week-old plantlets and lyophilized. For each landrace, genomic DNA was isolated from five single plants according to the NucleoSpin® Plant (MACHEREY-NAGEL, MN; Duren, Germany) kit protocol. Concentration and quality of DNA

Table 1 List of the 70 lentil landraces analyzed and their respective origins

Name	Code	Origin[a]	Name	Code	Origin[a]
ALTAMURA	I1	Italy	MGB1032	M15	Morocco
TIPO ASSTELLUCCIO	I2	Italy	MGB1034	M16	Morocco
MOUNTAIN LENTIL	I3	Italy	MGB1035	M17	Morocco
TIPO TURCHE NO2	I4	Italy	MGB1036	M18	Morocco
MG110288	I5	Italy	MGB1045	M19	Morocco
MG110438	I6	Italy	MGB1049	M20	Morocco
MG106892	I7	Italy	MGB1050	M21	Morocco
MG110287	I8	Italy	MGB1051	M22	Morocco
MG111854	I9	Italy	MGB1052	M23	Morocco
MG111863	I10	Italy	MGB1053	M24	Morocco
MG106899	I11	Italy	MGB1054	M25	Morocco
MG111849	I12	Italy	MGB1055	M26	Morocco
AKCA MUCIMEGI	T1	Turkey	MGB1056	M27	Morocco
YERLI1	T2	Turkey	MGB1058	M28	Morocco
ADI	T3	Turkey	MGB1008	M29	Morocco
YERLI2	T4	Turkey	MGB1010	M30	Morocco
ILL183	T5	Turkey	MGB1043	M31	Morocco
ILL171	T6	Turkey	MGB1044	M32	Morocco
ILL306	G1	Greece	MGB996	M33	Morocco
ILL312	G2	Greece	MGB997	M34	Morocco
ILL298	G3	Greece	MGB999	M35	Morocco
MGB1000	M1	Morocco	MGB1026	M36	Morocco
MGB1013	M2	Morocco	MGB1027	M37	Morocco
MGB1015	M3	Morocco	MGB1037	M38	Morocco
MGB1016	M4	Morocco	MGB1038	M39	Morocco
MGB1017	M5	Morocco	MGB1039	M40	Morocco
MGB1019	M6	Morocco	MGB1040	M41	Morocco
MGB1020	M7	Morocco	MGB1041	M42	Morocco
MGB1022	M8	Morocco	MGB1042	M43	Morocco
MGB1023	M9	Morocco	MGB1047	M44	Morocco
MGB1024	M10	Morocco	MGB1060	M45	Morocco
MGB1025	M11	Morocco	MGB1061	M46	Morocco
MGB1029	M12	Morocco	MGB1062	M47	Morocco
MGB1030	M13	Morocco	L24 (local cultivar)	M48	Morocco
MGB1031	M14	Morocco	L56 (local cultivar)	M49	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, US Department of Agriculture, USA

were verified using a NanoDrop® Spectrophotometer ND-1000 (Isogen; De Meern, The Netherlands). Isolated DNA was diluted to 15 ng/μl and subsequently stored at −20 °C.

SSR Analysis

SSR analysis was carried out as described in Idrissi et al. (2015a). Nineteen microsatellite markers developed by Hamwieh et al. (2005) and evaluated in Idrissi et al. (2015a) were used in this study (Table 2). Polymerase Chain Reaction (PCR) analysis was performed as described in De Keyser et al. (2010) according to the Qiagen Multiplex PCR kit protocol (Multiplex PCR Kit; Qiagen; Manchester, UK).

AFLP Analysis

The standard AFLP protocol (Vos et al. 1995) was used following De Riek et al. (2001), with minor modifications as described in Idrissi et al. (2015a). Seven primer combinations were used: *EcoRI*-ACA + *MseI*-CAG; *EcoRI*-ACA + *MseI*-CTG; *EcoRI*-

Table 2 Primer sequences and PCR conditions used for the amplification of the microsatellites in the 70 lentil landraces tested

Locus name	Primer sequences (5′-3-)		Ta (°C)	Alleles size range (bp)	No. of cycles	PCR multiplex set	Fluorescent label
	Forward	Reverse					
SSR113	CCGTAAGAATTAGGTGTC	GGAAAATAGGGTGGAAAG	53	209–245	25	1	NED
SSR154	GGAATTTATCACACTATCTC	GACTCCCAACTTGTATG	53	261–381	25	1	FAM
SSR199	GTGTGCATGGTGTGTG	CCATCCCCCTCTATC	53	180–213	25	2	FAM
SSR124	GTATGTGACTGTATGCTTC	GCATTGCATTTCACAAACC	56	174–177	25	3	NED
SSR233	CTTGGAGCTGTTGGTC	GCCGCCTACATTATGG	56	126–161	25	3	HEX
SSR80	CCATGCATACGTGACTGC	GTTGACTGTTGGTGTAAGTG	60	129–157	25	4	FAM
SSR184	GTGTGTACCTAAAGCCTTG	GTAAGTTGATCAAACGCCC	60	190–271	25	5	FAM
SSR48	CATGGTGGAATAGTGATGGC	CTCCATACACCACTCATTCAC	60	152–195	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	177–246	30	9	HEX
SSR336	GTGTAACCCAACTGTTCC	GGCCGAGGTTGTAACAC	56	233–282	30	9	FAM
SSR119	GAACTCAGTTTCTCATTG	GAACATATCCAATTATCATC	50	243–297	30	10	HEX
SSR212-1	GACTCATTGTTGTACCC	GCGAGAAGAATGGTTG	50	159–248	30	10	NED
SSR215	CATTAATATTTCTTTGGTGC	CTTTTCTTCTCTTCCCC	50	251–447	30	10	FAM
SSR130	CCACGTATGTGACTGTATG	GAAAGAGAGGCTGAAACTTG	56	195–198	30	11	NED
SSR33	CAAGCATGACGCCTATGAAG	CTTTCACTCACTCAACTCTC	56	239–321	30	11	HEX

ACA + *MseI*-CTT; *EcoRI*-ACG + *MseI*-CAA; *EcoRI*-AGC + *MseI*-CAA; *EcoRI*-AGC + *MseI*-CAG; *EcoRI*-AGC + *MseI*-CTG. Fragments were separated, sized and visualized as described for SSRs.

Root and Shoot Characterization and Drought Tolerance Evaluation

Landraces were evaluated for drought tolerance in a plastic pot experiment in a greenhouse arranged in a completely randomized block design with three replications. Four uniformly germinated seeds were planted in plastic pots (H 35 cm×D 24 cm) filled with fine perlite in order to be able to extract intact roots without damage. Standard nutrition solution EEG MESTSTOF 19-8-16 (4) [NO3 11 %, NH4 8 %, P 2O5 8 %, K2O 16 %, MgO 4 %, B 0.02 %, Cu EDTA 0.03 %, Fe EDTA 0.038 %, Mn EDTA 0.05 %, Mo EDTA 0.02 %, Zn EDTA 0.01 %] was supplied only during the first week after plant emergence. Water supply was then stopped in order to expose plants to progressive drought stress. Initial moisture in all pots was 70 % of field capacity and decreased to about 20 % at the eighth week after sowing. In the greenhouse, temperature ranged from 8 to 15 °C with 20 to 35 % relative humidity. The photoperiod was 11/13-h light/dark with light intensity of 240 W m^{-2}. The experiment was carried out at Ghent University greenhouse, Melle, Belgium, during November and December 2014.

Response of landraces to drought stress was assessed based on three fast and resource-effective phenotyping methods widely used in plant breeding programs: wilting score (WS), leaf relative water content (RWC) and leaf water losing rate (WLR). WS estimates visual symptoms of tissue damages under drought stress as the degree of wilting severity using the following 0–4 score scale as described by Singh et al. (2013): 0 = healthy plants with no visible symptoms of drought stress, 1 = green plants with slight wilting, 2 = leaves turning yellowish green with moderate wilting, 3 = leaves yellow–brown with severe wilting and 4 = completely dried leaves and/or stems. RWC measures the plant water status in plant tissues estimating dehydration avoidance under drought stress. Fresh weight (FW) was recorded on fully expanded excised leaves after 4-h drying on filter paper (at room temperature under a constant light); then, leaves were soaked for 4 h in distilled water at room temperature under constant light to determine turgid weight (TW). Total leaf dry weight (DW) was recorded after oven-drying at 72 °C for 48 h. RWC was calculated according to Barr and Weatherley (1962): RWC (%)=[(FW−DW)/(TW−DW)]×100.

WLR estimates rate of water loss of leaves exposed to dehydration and was determined on a separate set of young fully expanded leaves. Weight after 4-h drying on filter paper

Table 3 Simple sequence repeats (SSR) polymorphism parameters in the 70 studied lentil landraces

Locus name	Number of observed alleles (*no*)	Number of expected alleles (*ne*)	Shannon information index (*I*)	Observed heterozygosity (*Ho*)	Expected heterozygosity (*He*)	Probability of identity (*PI*)
SSR113	19	10.11	2.52	0.0403	0.9024	0.0088
SSR154	12	2.50	1.47	0.7708	0.6018	0.0224
SSR199	5	2.20	1.06	0.3311	0.5480	0.1069
SSR124	2	1.12	0.24	0.0095	0.1115	0.7283
SSR233	13	2.98	1.59	0.5545	0.6661	0.0698
SSR80	14	7.95	2.28	0.0476	0.8757	0.0118
SSR184	22	4.34	2.11	0.1516	0.7713	0.0572
SSR48	17	6.87	2.22	0.0526	0.8557	0.0217
SSR19	10	5.43	1.84	0.0466	0.8174	0.0519
SSR99	2	1.07	0.15	0.0000	0.0694	0.5161
SSR302	16	3.29	1.75	0.2322	0.6974	0.0873
SSR309_2	8	3.88	1.57	0.8899	0.7439	0.0591
SSR204	7	3.46	1.40	0.0521	0.7127	0.0642
SSR336	15	7.09	2.11	0.4509	0.8604	0.0255
SSR119	24	10.13	2.60	0.0000	0.9027	0.0095
SSR212_1	22	13.14	2.77	0.0947	0.9253	0.0080
SSR215	26	10.32	2.80	0.7273	0.9046	0.0272
SSR130	2	1.13	0.26	0.0116	0.1207	0.7671
SSR33	25	4.61	2.09	0.3567	0.7845	0.0217
Total	261					4.89×10^{-24}
Average	13.73	5.35	1.73	0.2537	0.6775	
Standard deviation	7.72	3.59	0.82	0.2923	0.2776	

(W4) (at room temperature under constant light) was recorded, and total leaf DW was recorded after oven-drying at 72 °C for 48 h. Leaf WLR was calculated according to Suprunova et al. (2004): WLR (g h^{-1} g^{-1} DW)=[(FW−W4)]/[DW×4].

RWC and WLR were measured twice for each landrace and each replication at week 6 after sowing using separate sets of leaves. WS was estimated 1 day before harvest. At 60 days after sowing, plants were carefully extracted, the roots were washed without damage, and then, shoots and roots were put into separate plastic bags.

Chlorophyll content was estimated via SPAD values measured at 48 days after sowing using a SPAD-502 Plus chlorophyll meter (Konica Minolta, Japan) and four measures were performed on fully expanded leaves per plant. Shoot length was measured as stem length (cm) at 12 and 22 days after sowing. Dry root and shoot biomass (DRW, DSW; mg

Table 4 Amplified fragment length polymorphism (AFLP) primer combinations polymorphism parameters in the 70 lentil landraces

Primer combinations	Number of fragments	Polymorphic fragments			Fragment size range (bp)	PIC
		Number	Standard deviation	Percentage		
EcoRI-ACA + *MseI*-CAG (PC1)	148	101.2	25.4	68.33	52-480	0.4497
EcoRI-ACA + *MseI*-CTG (PC2)	127	68.75	21.97	54.13	50-499	0.3387
EcoRI-ACA + *MseI*-CTT (PC3)	162	91.42	16.25	56.43	50-469	0.3588
EcoRI-ACG + *MseI*-CAA (PC4)	96	43.87	19.83	45.70	50-486	0.3195
EcoRI-AGC + *MseI*-CAA (PC5)	104	53.28	17.39	51.23	51-493	0.3259
EcoRI-AGC + *MseI*-CAG (PC6)	92	48.39	18.16	52.60	52-491	0.3393
EcoRI-AGC + *MseI*-CTG (PC7)	83	42.77	9.25	51.54	50-499	0.3249
Total	812	449		54.28		0.3509
Average	116	64.24				

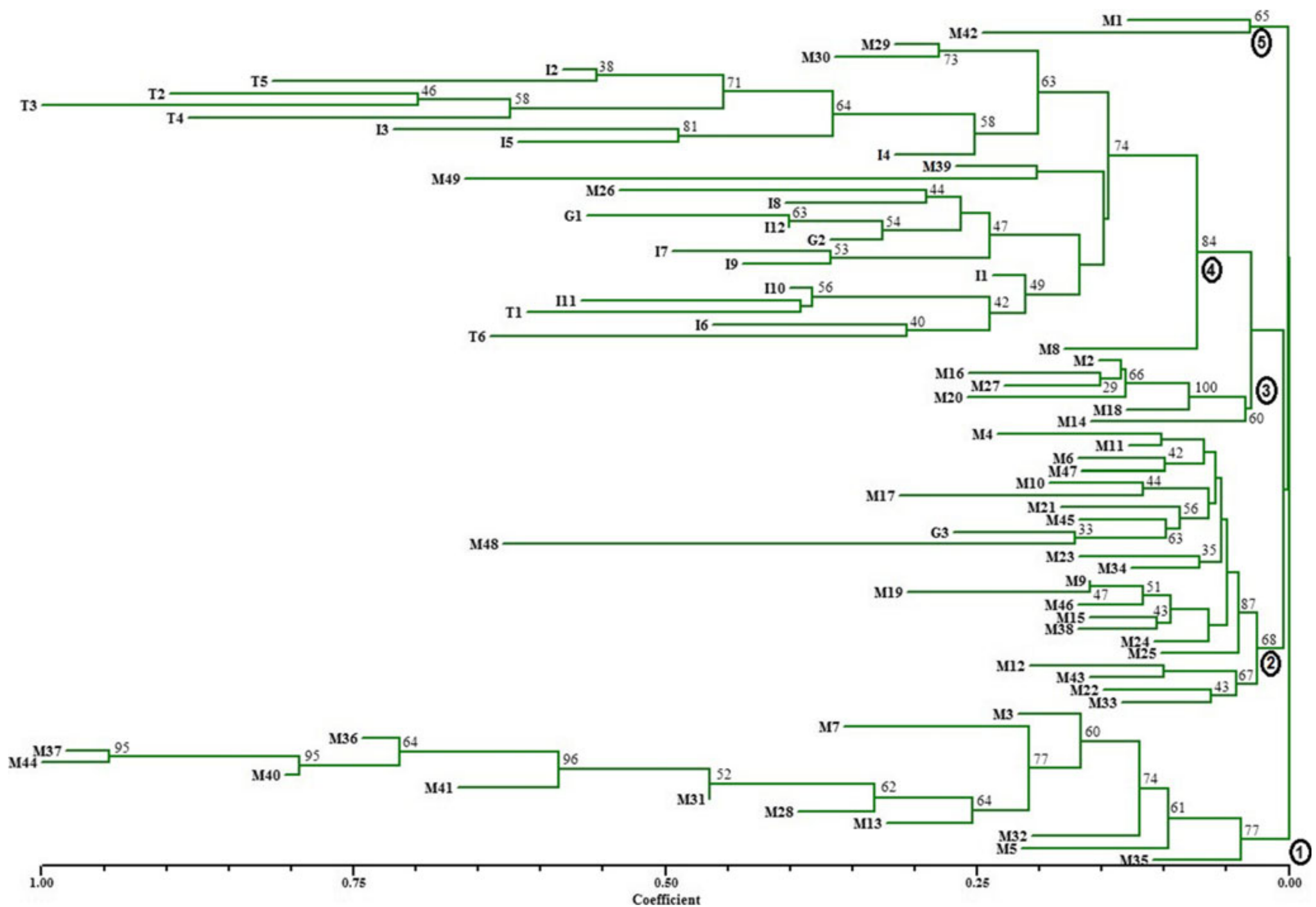


Fig. 1 Neighbor-joining (NJ) dendrogram of the 70 lentil landraces obtained via Nei genetic distance from SSR markers. Bootstrap values are given at the nodes

plant^{-1}) were measured after oven-drying at 72 °C for 48 h. Root–shoot ratio (RS ratio) was calculated by dividing dry root weight by dry shoot weight. Seedling vigor (SV) was recorded following the 1–5 IBPGR and ICARDA (1985) scale: 1 = very poor, 2 = poor, 3 = average, 4 = good, and 5 = excellent. All variables were analyzed as mean values based on four plants per pot and per genotype.

Data Analysis

For both SSR and AFLP analyses, allele pattern profiles corresponding to amplification products were visualized, sized and automatically scored using GENEMAPPER 4.0 software (Applied Biosystems). Unique SSR pattern profiles corresponded with homozygous individuals, while two different profiles corresponded with heterozygous ones. Binary matrices were constructed based on scoring presence of amplification products of all SSR loci and AFLP fragments of all primer combinations as (1) and absence as (0) using MS Access and MS Excel. Considering all genotypes (five single plants represent each landrace), genetic diversity parameters were estimated for SSRs taking into consideration whether the individual is homozygous or heterozygous at each given locus (observed number of alleles, *na*; expected number of alleles, *ne*; Shannon's information index, *I*; Nei's genetic distances (Nei 1973); observed heterozygosity, *Ho*; and expected heterozygosity, *He*) and, for AFLP (number of fragments, percentage of polymorphic fragments), using POPGENE 1.31 (Yeh et al. 1999). The probability of identity (*PI*) between all genotypes for SSR markers was calculated using the IDENTITY 1.0 program (Wagner and Sefc 1999). Polymorphic information content (PIC) was calculated for AFLP using $PIC=1-\sum P_i^2$, where P_i is the fragment frequency of the ith allele (Smith et al. 1997).

Genetic distance matrices between all pairwise genotypes based on Nei's genetic distance (Nei 1973) using binary matrices for SSR and AFLP as well as Mantel test (Mantel 1967) were computed and performed on NTSYS-PC 2.1 (Rohlf 2004) program to construct neighbor-joining clusters to show the associations between the studied landraces. Bootstrap analysis of neighbor-joining dendrograms was performed using TREECON software (Van de Peer and De Wachter 1993) to test confidence and faithfulness of the obtained groupings.

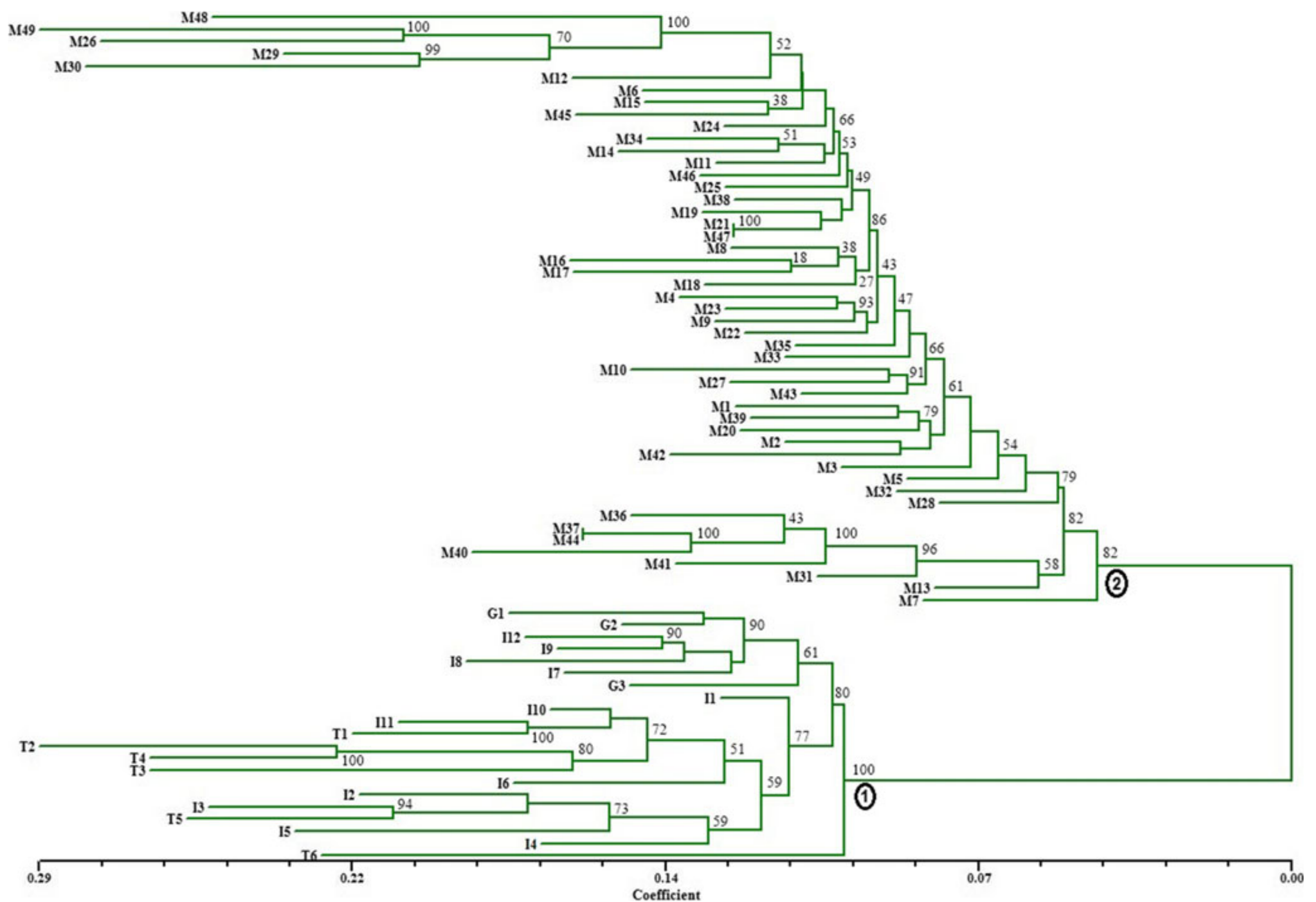


Fig. 2 Neighbor-joining (NJ) dendrogram of the 70 lentil landraces obtained via Nei genetic distance from AFLP markers. Bootstrap values are given at the nodes

SPSS Statistics 22 was used for normality test, variance, correlation, and principal component analyses of root and shoots traits, drought parameters, and genetic data from SSR and AFLP markers. It was also used to perform the non-parametric Kruskal–Wallis analysis to test the associations between individual SSR and AFLP markers and drought parameters as measured by WS, RWC and WLR. In order to test functional groupings according to drought responses of landraces, canonical discriminant analyses based on genetic distance between landraces from SSR (chi-square) and AFLP (Jaccard similarity index) markers linked to the three drought parameters were performed using prior information on landraces' response to drought as follows. The five classes obtained according to WSs (Singh et al. 2013) were used as grouping variable. Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate ($52.5 \leq \text{RWC} < 60$) and tolerant (RWC ≥ 60). Similarly, three classes were defined for WLR: sensitive (WLR ≥ 0.56), intermediate ($0.56 < \text{WLR} \leq 0.50$) and tolerant (WLR <0.50). Regression analysis based on SSR and AFLP markers linked to the three drought measures was performed to confirm association revealed by the K-W test and to identify the markers explaining the highest phenotypic variation. Canonical discriminant and regression analyses were performed using SPSS Statistics 22.

Results

Genetic Diversity

For all landraces' genotypes, 19 SSRs produced a total of 261 alleles with an average of 13.73 alleles per locus whereby the number of alleles per locus ranged from 2 to 26. SSR215 locus produced the largest number of observed alleles (*no*) while SSR124, SSR99 and SSR130 loci produced the lowest number of alleles. Average Shannon information index was 1.73, ranging from 0.15 for SSR99 to 2.80 for SSR215. The level of genetic diversity as estimated by expected heterozygosity (*He*), expressing the probability at a given locus of two alleles taken at random from the population to be different of each other, ranged from 0.0694 (SSR99) to 0.9253 (SSR212-1) with an average over all loci for all landraces

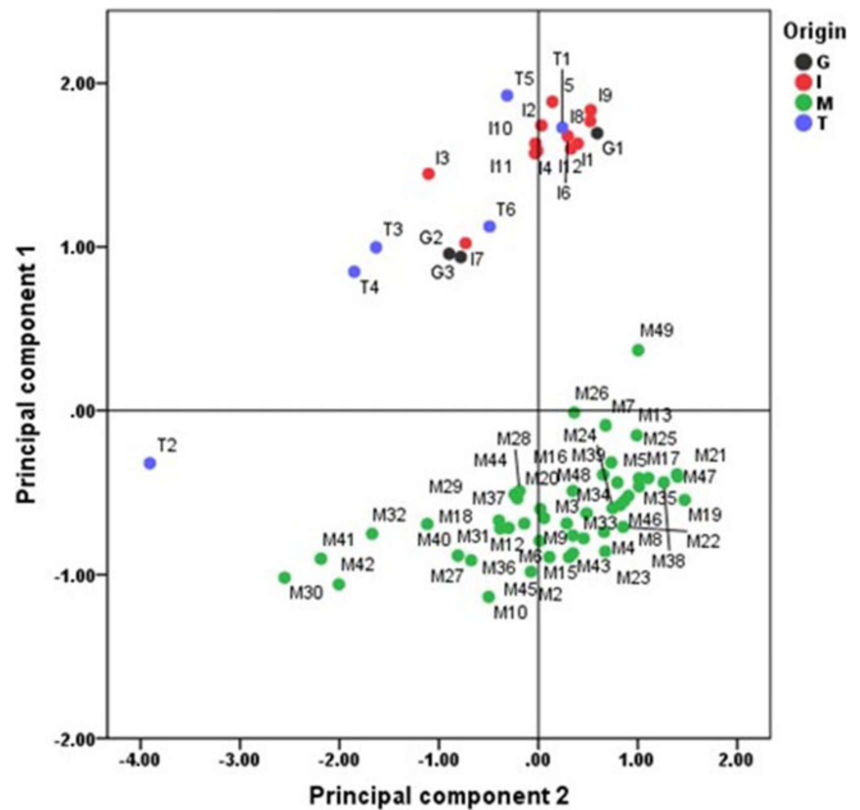


Fig. 3 Principle component analysis (PCA) scatter plot based on combined SSR and AFLP data sets of the 70 landraces sorted by country of origin: *G* Greece, *I* Italy, *M* Morocco, *T* Turkey

of 0.6775. Total probability of identity (*PI*) between two randomly chosen genotypes of the landraces over all loci was as low as 4.89 10^{-24} (Table 3).

Seven AFLP primer combinations yielded a total of 812 fragments ranging from 50.08 to 499.54 bp over all landraces, with an average of about 116 fragments per primer combination. The highest number of fragments was produced by primer combination *EcoRI*-ACA + *MseI*-CTT (PC3) with 162 fragments, while the lowest number was produced by primer combination *EcoRI*-AGC + *MseI*-CTG (PC7) with 83 fragments. Of all fragments obtained, 449 (64.24 %) were polymorphic. Polymorphic band percentages ranged from 45.70 (*EcoRI*-ACG + *MseI*-CAA (PC4)) to 68.33 % (*EcoRI*-ACA + *MseI*-CAG (PC1)). Polymorphic information content (PIC) ranged from 0.3195 (*EcoRI*-ACG + *MseI*-CAA (PC4)) to 0.4497 (*EcoRI*-ACA + *MseI*-CAG (PC1)), with an average over the seven primer combinations of 0.3509 (Table 4).

Table 5 Variation among root and shoot traits and drought parameters in the 70 lentil landraces

Traits	Mean±SD	Minimum	Maximum	Coefficient of variation (%)
Shoot length at 12 days after sowing (SL12DAS)	6.82±1.42	3.53	10.13	20.82
Shoot length at 22 days after sowing (SL22DAS)	17.17±3.46	10.53	21.15	20.15
Seedling vigor (SV)	3.38±0.93	1.66	4.66	27.51
Dry shoot weight (DSW)	0.8490±0.19	0.4763	1.2220	22.37
Chlorophyll content (SPAD)	38.23±3.18	31.10	46.6	8.31
100-seed weight (SeedW)	4.13±1.38	2.02	5.16	33.41
Dry root weight (DRW)	0.6578±0.1912	0.3177	1.1823	29.06
Root–shoot ratio (RSRatio)	0.7906±0.2188	0.3125	1.5501	27.67
Leaf relative water content (RWC)	56.03±9.98	40.12	75.13	17.81
Leaf water losing rate (WLR)	0.5158±0.1221	0.3717	0.7027	23.67
Wilting score (WS)	1.92±0.8128	0.33	3.66	42.33

Table 6 Correlations between root and shoot traits and drought parameters in the 70 lentil landraces

	SL12DAS	SL22DAS	SV	DSW	SPAD	SeedW	DRW	RSRatio	RWC	WLR	WS
SL12DAS	1	0.577**	0.578**	0.320**	0.059	0.050	−0.015	−0.222	−0.062	0.013	0.167
SL22DAS	0.577**	1	0.761**	0.533**	−0.050	0.524**	0.040	−0.372**	−0.098	0.214	0.267*
SV	0.578**	0.761**	1	0.571**	0.005	−0.177	0.127	−0.259*	−0.077	0.095	0.252*
DSW	0.320**	0.533**	0.571**	1	0.105	0.235	0.460**	−0.453**	0.052	0.072	0.126
SPAD	0.059	−0.050	0.005	0.105	1	−0.177	0.573**	0.298*	0.335**	−0.325**	−0.538**
SeedW	0.050	0.524**	−0.177	0.235	−0.177	1	−0.153	−0.313**	−0.232	0.310*	0.319*
DRW	−0.015	0.040	0.127	0.460**	0.573**	−0.153	1	0.737**	0.482**	−0.288*	−0.411**
RSRatio	−0.222	−0.372**	−0.259*	−0.453**	0.298*	−0.313**	0.737**	1	0.362**	−0.256*	−0.374*
RWC	−0.062	−0.098	−0.077	0.052	0.335**	−0.232	0.482**	0.362**	1	−0.577**	−0.610**
WLR	0.013	0.214	0.095	0.072	−0.325**	0.310*	−0.288*	−0.256*	−0.577**	1	0.571**
WS	0.167	0.267*	0.252*	0.126	−0.538**	0.319*	−0.411**	−0.374**	−0.610**	0.571**	1

**Significant at 0.01 level; *significant at 0.05 level

Genetic Relationship Between Landraces as Revealed by SSR and AFLP DNA Markers

Genetic relationship among landraces was assessed for both microsatellite and AFLP markers taken separately using neighbor-joining (NJ) method and the combined data sets using principle component analysis (PCA).

Based on SSR markers, the NJ dendrogram generated five groups. Landraces from the northern Mediterranean (Italy, Turkey and Greece) were grouped together in group 4 separately from those of Morocco, except for six landraces (M29, M30, M39, M49, M26 and M8). The four other groups were from Morocco (Fig. 1).

NJ grouping based on AFLP markers (Fig. 2) discriminated between landraces from Morocco and those from northern Mediterranean. Landraces from Italy, Turkey and Greece were clustered in group 1. Landraces from Morocco could be separated into four groups, one large group containing 36 landraces, two groups containing 7 and 5 landraces, respectively, and one single landrace (M7) separated from the rest.

Genetic similarity matrices between lentil landraces from the two data sets (SSRs and AFLPs) were compared using the Mantel test. A significant correlation between the two matrices was found with $r=0.6485$ and Mantel $t=5.7477$ ($P<0.001$). Same grouping patterns as shown in Figs. 2 and 3 were obtained based on all 350 genotypes (five genotypes per landrace) analyzed for both DNA markers (data not shown). Combined data sets from SSR and AFLP analyses were used to construct a consensus grouping of landraces by performing PCA. The first and second axes of PCA explained 37.69 and 25.40 % of total variance and separated lentil landraces into two main groups discriminating Moroccan landraces from those of Italy, Turkey and Greece. Landraces from both the northern Mediterranean region as well as from Morocco enclose high genetic diversity (Fig. 3).

Root and Shoot Characterization and Drought Tolerance Evaluation

All variables were normally distributed. A slight deviation from normal distribution was observed for WS, RS ratio, and shoot lengths at 12 and 22 days after sowing. Analysis of variance showed a significantly high variation for all traits measured (Table 5): shoot lengths at 12 and 22 days after

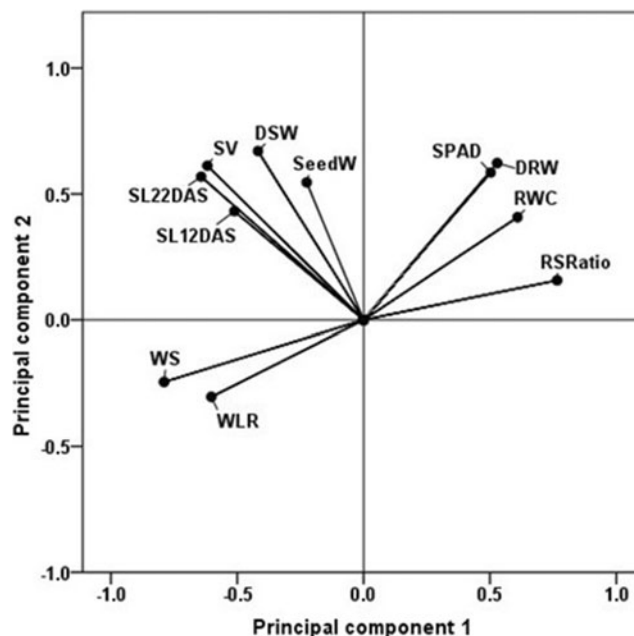


Fig. 4 Principal component analysis (PCA) scatter plot based on all traits measured on the 70 landraces tested (shoot length at 12 days after sowing (*SL12DAS*), shoot length at 22 days after sowing (*SL22DAS*), seedling vigor (*SV*), dry shoot weight (*DSW*), chlorophyll content (*SPAD*), 100-seed weight (*SeedW*), dry root weight (*DRW*), root–shoot ratio (*RSRatio*), leaf relative water content (*RWC*), leaf water losing rate (*WLR*) and wilting score (*WS*))

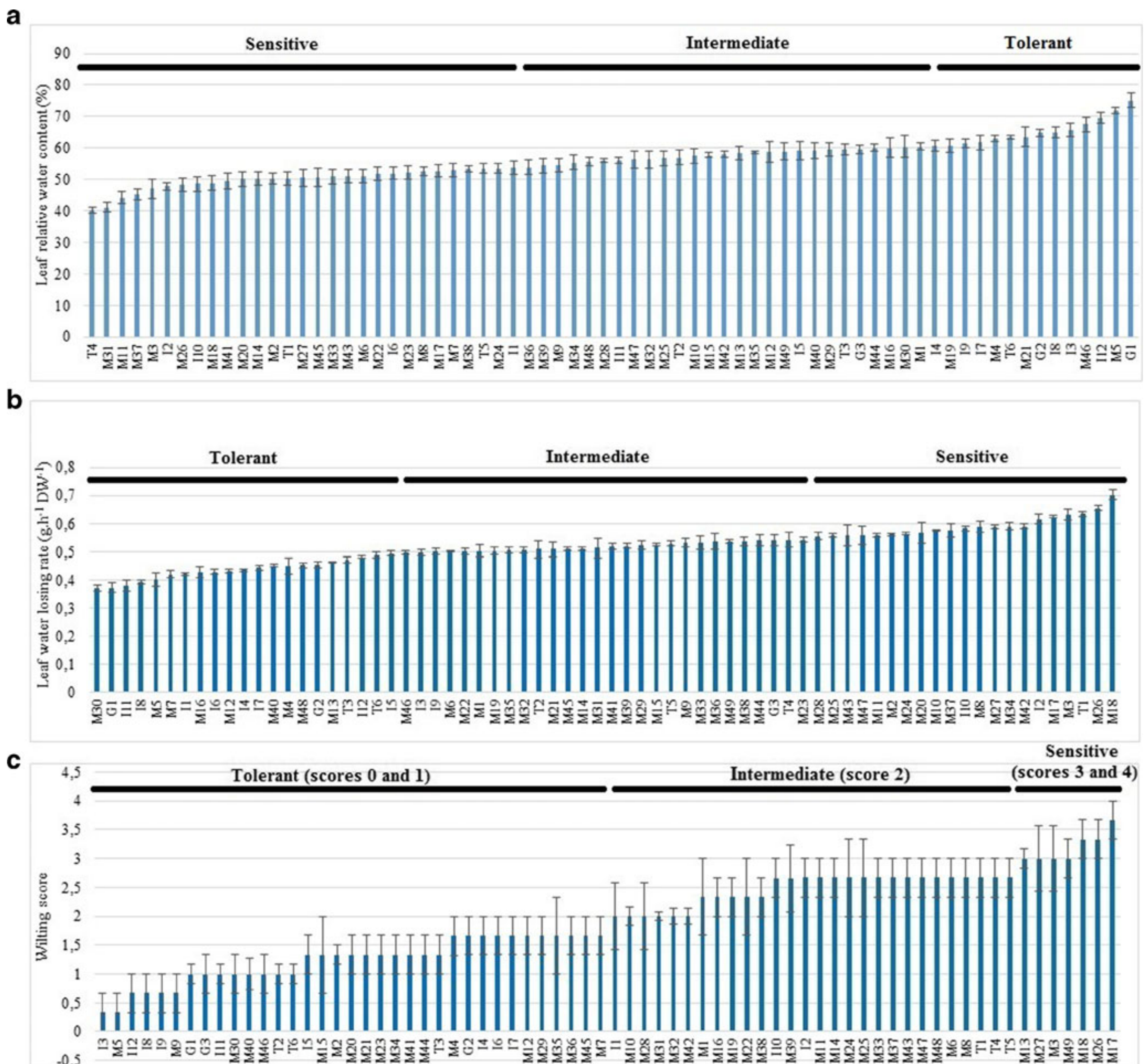


Fig. 5 Variation of leaf relative water content (**a**), leaf water losing rate (**b**) and wilting score (**c**) among the 70 lentil landraces tested. Wilting score: 0 to 4 corresponds to the following 0–4 score scale as described by Singh et al. (2013): 0 = healthy plants with no visible symptoms of drought stress, 1 = green plants with slight wilting, 2 = leaves turning yellowish-green with moderate wilting, 3 = leaves yellow–brown with severe wilting and 4 = completely dried leaves and/or stems. Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate (52.5≤RWC<60) and tolerant (RWC ≥60). Similarly, three classes were defined for WLR: sensitive (WLR ≥0.56), intermediate (0.56<WLR≤0.50) and tolerant (WLR <0.50)

sowing, SV, dry shoot weight, chlorophyll content as estimated by the SPAD values, 100-seed weight, dry root weight, RS ratio, RWC, WLR and WS (Table 5). Also, variations were significant within each geographic origin.

Significant correlations were shown between the following: SV and WS (0.252); SPAD and leaf RWC (0.335), WLR (−0.325), and WS (−0.538); dry root weight and dry shoot weight (0.460), SPAD (0.573), RWC (0.482), WLR (−0.288), and WS (−0.411); and RS ratio and RWC (0.362), WLR (−0.256) and WS (−0.374) (Table 6). The three drought parameters were also significantly correlated to each other. WLR and WS were positively correlated (0.571), while RWC was negatively correlated to both parameters with values of −0.577 and −0.610, respectively.

We also performed PCA based on all variables among landraces. The first and second axes explained 34.16 and 24.59 % of total variation, respectively (Fig. 4). Principal component 1 was positively correlated with RS ratio

(0.766), leaf RWC (0.609), dry root weight (0.529) and chlorophyll content (0.503), and negatively correlated with WS (−0.789), WLR (−0.603), shoot lengths at 12 and 22 DAS (−0.511; −0.643), SV (−0.618) and dry shoot weight (−0.418). Principal component 2 was positively correlated with dry shoot weight (0.670), dry root weight (0.623), SV (0.612), chlorophyll content (0.585), shoot lengths at 12 and 22 days after sowing (0.431; 0.569) and leaf RWC (0.408). Weak but still significant negative correlations of principal component 2 were observed with WLR (−0.303) and WS (−0.244). Weak but significant differentiation (low eigenvalues of discriminant analysis) according to geographic origin was observed based on phenotypic data, and landraces from Morocco and Greece had slightly higher shoot length, biomass, and seedling early vigor compared to those from Italy and Turkey. Turkish landraces had the lowest biomass (Supplementary materials: Figs. S1 and S2).

Drought tolerance level as evaluated by RWC, WLR and WS showed high genotypic variations among landraces. RWC ranged from 40.12 % in T4 to 75.13 % in G1; WLR ranged from 0.3717 in M30 to 0.7027 in M18; WS ranged from 0.33 in I3 to 3.66 in M17 (Fig. 5). No correlation between landrace origin and drought response was observed.

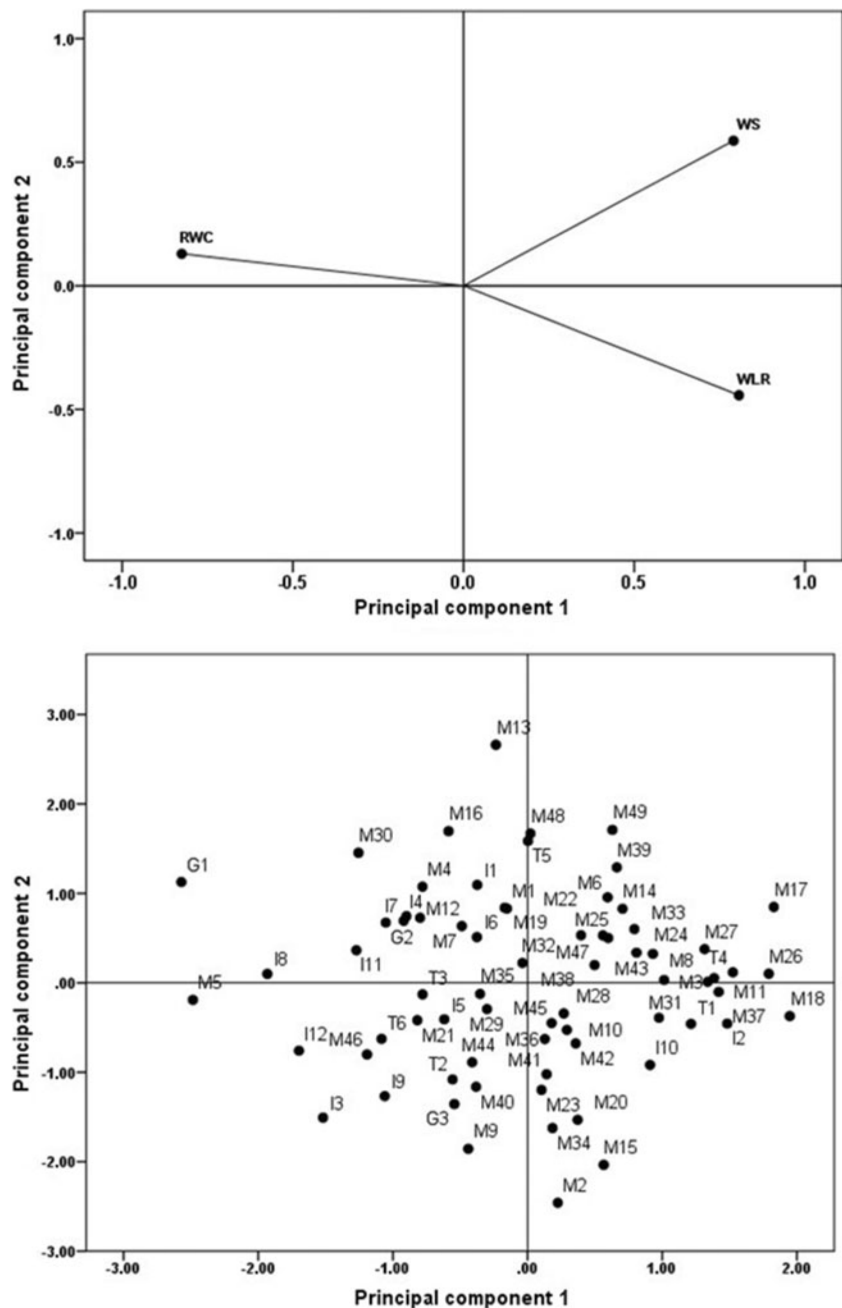


Fig. 6 PCA of the 70 lentil landraces based on leaf relative water content (RWC), leaf water losing rate (WLR) and wilting score (WS). The *first upper figure* sorts the three variables as associated to the two principal components (PC) while the *lower part* shows landraces according to the two PCs

PCA was performed with the three parameters used to estimate drought tolerance (leaf RWC, leaf WLR and WS) in order to sort the landraces according to a consensus classification in response to drought stress (Fig. 6). Principal components 1 and 2 explained 65.25 and 18.57 % of total variation, respectively. The first axis was highly correlated with the three parameters: −0.826 with leaf RWC, 0.807 with WLR and 0.791 with WS. Higher values of this axis indicated sensitive landraces, while lower values indicated tolerant landraces.

SSR and AFLP Markers Associated to Drought Tolerance

In order to determine SSR and AFLP markers that are linked to the individually measured physiological traits, a Kruskal–Wallis analysis was applied. The test was based on the ranking of landraces according to leaf RWC, WLR, and WS separately and testing the association to the markers one by one as grouping variable. Six, four and five SSRs were identified to be associated with leaf RWC, leaf WLR and WS, respectively (Table 7). On the other hand, 91, 105 and 51 AFLP markers were found to be associated with leaf RWC, WLR and WS, respectively (Tables 8, 9 and 10).

In order to test the genetic differentiation of landraces according to their drought reaction as measured by the three parameters, we tested prior information related to their grouping based on RWC, WLR, and WS, and canonical discriminant analyses were performed using pairwise genetic distances between landraces generated from SSR and AFLP markers linked to the respective parameters. The analyses highly discriminated landraces according to their drought reaction into the predefined groups based on RWC, WLR, and WS for both SSRs and AFLPs linked to these parameters (Figs. 7 and 8). First discriminant functions explained 96.9, 84.5, and 93.7 % of total variation with canonical correlations of 0.883, 0.683, and 0.975 and eigenvalues of 3.53, 0.876 and 19.57 for SSRs linked to RWC, WLR and WS, respectively. Although significant, second functions explained only a small amount of variation for SSRs linked to the three parameters. Some overlapping was observed for SSRs linked to WLR (eigenvalues <1), but the three groups still could be well-differentiated.

For AFLPs linked to RWC, WLR and WS, first discriminant functions explained 62.3, 58 and 73.5 % of total variation with canonical correlations of 0.987, 0.991 and 0.995 and eigenvalues of 37.49, 53.14 and 91.97, respectively. Second discriminant functions explained 37.7, 42 and 13.3 % of total variation with canonical correlations of 0.979, 0.987 and 0.971 and eigenvalues of 22.66, 38.44 and 16.63, respectively, for AFLPs linked to RWC, WLR and WS.

Regression analysis based on SSR alleles linked to RWC, WLR and WS showed moderate associations with $R^2=0.504$, $R^2=0.289$ and $R^2=0.363$, respectively, for the three drought measures as dependent variables. SSR19_7 and SSR80_12 explained the highest phenotypic variation of RWC with 33 and 30 %, respectively. SSR336_22 and SSR184_17

Table 7 SSR markers linked to drought parameters according to Kruskal–Wallis H test

SSRs linked to drought parameters	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*
Leaf relative water content (RWC)					
SSR113_5	221	15.32	6	0.018	−0.24*
SSR184_17	263	8.36	3	0.039	0.42**
SSR19_7	262	7.30	2	0.02	0.32**
SSR233_13	155	11.5	5	0.04	0.26*
SSR48_3	165	3.9	1	0.04	0.25*
SSR80_12	153	18.1	7	0.01	0.27*
Leaf water losing rate (WLR)					
SSR215_9	388	6.07	2	0.04	−0.33**
SSR154_4	361	6.95	2	0.04	0.27*
SSR184_17	263	8.86	3	0.04	−0.28*
SSR336_22	279	10.7	4	0.04	−0.28*
Wilting score (WS)					
SSR119_5	271.50	4.8	1	0.02	0.25*
SSR154_12	379	3.96	1	0.04	0.24*
SSR19_7	270.50	14.45	6	0.02	0.25*
SSR204_1	177	5.64	1	0.01	0.36**
SSR48_3	165.50	4.8	1	0.03	−0.32**

*Significant at $p<0.05$; **significant at $p<0.01$

Table 8 AFLP markers linked to relative water content (RWC) according to Kruskal–Wallis H test

AFLPs linked to RWC	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance	Correlation	AFLPs linked to RWC	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*
PC1_111	111	11.57	3	0.009	0.30*	PC3_59	59	11.94	4	0.036	0.25*
PC1_114	114	11.92	4	0.036	0.33**	PC3_64	64	7.92	3	0.048	0.24*
PC1_127	127	13.71	4	0.018	−0.20*	PC3_69	69	13.49	4	0.009	0.26*
PC1_145	145	10.97	4	0.027	−0.20*	PC3_88	88	14.53	4	0.006	0.23*
PC1_152	152	10.82	4	0.029	0.36**	PC3_91	91	12.55	4	0.028	0.25*
PC1_171	171	12.97	4	0.024	0.27*	PC3_93	93	13.04	4	0.023	0.26*
PC1_217	217	12.47	4	0.029	0.19*	PC3_97	97	18.63	4	0.002	0.27*
PC1_218	218	10.69	4	0.030	0.26*	PC3_333	333	11.85	4	0.037	0.25*
PC1_219	219	12.23	4	0.032	−0.27*	PC3_384	384	17.93	4	0.003	0.26*
PC1_234	234	10.85	4	0.028	0.32**	PC4_152	152	11.28	3	0.01	0.25*
PC1_236	236	15.84	4	0.007	0.33**	PC4_179	179	14.19	4	0.014	0.36**
PC1_238	238	17.30	4	0.016	−0.30*	PC4_196	196	13.49	4	0.019	−0.37**
PC1_240	240	11.64	4	0.020	0.31**	PC4_270	270	12.64	4	0.027	0.28*
PC1_290	290	14.77	4	0.011	0.19*	PC4_300	300	15.74	4	0.008	0.35**
PC1_291	291	13.18	4	0.022	0.35**	PC4_302	302	13.02	4	0.023	0.40**
PC1_299	299	13.79	4	0.017	−0.27*	PC4_303	303	11.73	4	0.039	0.41**
PC1_314	314	14.87	4	0.011	0.42**	PC4_377	377	11.78	4	0.038	0.26*
PC1_319	319	11.78	4	0.038	0.19*	PC4_380	380	13.70	4	0.018	0.24*
PC1_323	323	13.02	4	0.023	0.35**	PC4_444	444	13.16	4	0.011	0.30*
PC1_327	327	15.32	4	0.009	0.49**	PC4_81	81	16.10	4	0.007	0.32**
PC1_329	329	12.63	4	0.027	0.37**	PC4_89	89	11.67	4	0.020	−0.25*
PC1_355	355	17.37	4	0.004	0.44**	PC4_93	93	14.06	4	0.015	0.48**
PC1_400	400	13.63	4	0.018	0.33**	PC5_104	104	15.93	4	0.007	0.29*
PC1_419	419	12.43	4	0.029	0.33**	PC5_134	134	12.49	4	0.029	0.22*
PC1_422	422	14.99	4	0.010	0.41**	PC5_193	193	12.02	4	0.034	0.37**
PC1_447	447	16.13	4	0.006	0.45**	PC5_248	248	13.88	4	0.016	0.24*
PC1_456	456	19.36	4	0.002	0.46**	PC5_283	283	14.66	4	0.012	0.38**
PC1_458	458	13.01	4	0.023	0.21*	PC5_350	350	18.27	4	0.032	0.30*
PC1_53	53	12.38	4	0.030	0.36**	PC5_435	435	12.33	4	0.015	0.40**
PC1_75	75	14.18	4	0.014	0.33**	PC5_436	436	11.92	4	0.036	0.23*
PC1_98	98	13.50	4	0.019	0.41**	PC6_121	121	18.75	4	0.002	0.29*
PC2_108	108	12.47	4	0.029	0.22*	PC6_123	123	11.80	4	0.038	0.36**
PC2_120	120	13.43	4	0.020	0.33**	PC6_150	150	10.26	4	0.036	0.26*
PC2_166	166	14.78	4	0.011	0.36**	PC6_321	321	11.98	4	0.035	−0.27*

Table 8 (continued)

AFLPs linked to RWC	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance	Correlation	AFLPs linked to RWC	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*
PC2_250	250	15.14	4	0.010	0.33**	PC6_478	478	12.54	4	0.028	0.24*
PC2_352	352	15.03	4	0.010	0.32**	PC6_484	484	12.18	4	0.032	0.23*
PC2_64	64	11.27	4	0.046	0.32**	PC6_68	68	11.79	4	0.038	0.45**
PC2_98	98	17.09	4	0.004	0.44**	PC6_74	74	13.06	4	0.023	−0.24*
PC3_113	113	12.28	4	0.031	0.35**	PC7_126	126	17.63	4	0.001	0.31**
PC3_140	140	15.72	4	0.008	−0.27*	PC7_234	234	15.72	4	0.008	−0.24*
PC3_184	184	12.39	4	0.030	0.25*	PC7_253	253	15.14	4	0.032	0.37**
PC3_185	185	13.78	4	0.017	0.29*	PC7_360	360	11.86	4	0.037	0.26*
PC3_261	261	15.85	4	0.008	0.30*	PC7_479	479	12.67	4	0.027	0.24*
PC3_305	305	14.52	4	0.024	−0.26*	PC7_63	63	20.043	4	0.001	−0.37**
PC3_311	311	12.27	4	0.031	0.28*	PC7_92	92	10.06	4	0.039	0.24*
PC3_471	471	7.87	3	0.049	0.30*						

*Significant at $p<0.05$; **significant at $p<0.01$

explained the highest phenotypic variation of WLR with 50 and 41 %, respectively, whereas SSR19_7 and SSR204_1 explained the highest phenotypic variation of WS with 33 and 21 %, respectively. Linked SSR alleles with major effects on the drought parameters are reported in Supplementary material Table S1.

Regression analysis based on AFLP alleles linked to RWC, WLR and WS showed high associations with R^2=0.753, R^2=0.912 and R^2=0.832, respectively, for the three drought measures used as dependent variables. PC1_400 and PC7_92 explained the highest phenotypic variation of RWC with 32 and 14 %, respectively. PC4_484 and PC4_239 explained the highest phenotypic variation of WLR with 28 and 16 %, respectively. PC7_400 and PC1_314 explained the highest phenotypic variation of WS with 33 and 17 %, respectively. Linked AFLP alleles with major effects on the drought parameters are reported in Supplementary material Table S2.

Higher correlations were observed between matrices based on drought parameters (RWC, WLR, WS) and similarity matrices based on the linked SSR and AFLP markers, compared to matrices based on total and randomly selected markers. This confirms the reliability of genetic differentiation according to drought response classes revealed by the markers linked to the traits. The latter clearly discriminated between groups of landraces corresponding to the drought response classes (sensitive, intermediate and tolerant). Also, closely similar patterns of clustering based on total markers as in Figs. 1 and 2 were obtained differentiating the two major groups of landraces (Moroccan versus Northern Mediterranean) when using the linked markers.

Discussion

High genetic variation was shown to exist among Mediterranean landraces originating from Morocco, Italy, Turkey and Greece by using both SSR and AFLP DNA markers. Overall, 261 alleles with an average expected heterozygosity of 0.6775 and number of observed alleles ranging from 2 to 26 were reported at 19 loci, for SSRs. Sonnante et al. (2007) reported 170 alleles and between 2 and 22 alleles at 16 loci for Italian landraces. Idrissi et al. (2015a) obtained 213 alleles at the same 19 loci using Moroccan landraces. For AFLPs, a total of 812 fragments were obtained whereby 64.24 % were polymorphic with an average PIC of 0.3509 over the seven primer combinations. Idrissi et al. (2015a) reported 766 fragments whereby 54.78 % were polymorphic using the same primer combinations in Moroccan landraces, whereas Torricelli et al. (2011) reported 698 fragments where 57.09 % were polymorphic using eight primer combinations on Italian lentil landraces. Toklu et al. (2009) reported 212 fragments whereby 56.1 % were polymorphic

Table 9 AFLP markers linked to water losing rate (WLR) according to Kruskal–Wallis H test

AFLPs linked to WLR	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*	Linked AFLPs to WLR	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*
PC1_111	111	14.08	3	0.003	−0.24*	PC3_306	306	12.29	4	0.031	0.26*
PC1_114	114	11.26	4	0.046	−0.25*	PC3_308	308	11.82	4	0.037	0.25*
PC1_117	117	17.71	4	0.003	−0.34**	PC3_323	323	11.86	4	0.037	−0.27*
PC1_127	127	12.48	4	0.029	−0.24*	PC3_350	350	12.91	4	0.024	0.28*
PC1_140	140	12.66	4	0.027	−0.24*	PC3_424	424	13.87	4	0.016	0.24*
PC1_143	143	12.73	4	0.026	0.24*	PC3_471	471	11.26	3	0.01	−0.29*
PC1_164	164	10.06	4	0.039	−0.26*	PC3_59	59	15.38	4	0.009	−0.27*
PC1_175	175	14.36	4	0.013	−0.39**	PC3_69	69	17.26	4	0.002	−0.29*
PC1_178	178	12.11	3	0.007	−0.24*	PC3_82	82	13.05	4	0.023	0.26*
PC1_213	213	12.54	4	0.028	−0.27*	PC3_88	88	12.36	4	0.015	−0.29*
PC1_234	234	9.65	4	0.047	−0.27*	PC3_97	97	13.17	4	0.022	−0.30*
PC1_238	238	20.40	4	0.005	0.41**	PC4_136	136	10.77	4	0.029	−0.30*
PC1_254	254	9.32	3	0.025	−0.23*	PC4_181	181	15.81	4	0.007	−0.39**
PC1_255	255	12.31	4	0.015	−0.24*	PC4_184	184	13.13	4	0.022	−0.27*
PC1_258	258	15.77	4	0.008	−0.25*	PC4_190	190	13.94	4	0.016	0.35**
PC1_288	288	14.72	4	0.012	−0.30*	PC4_216	216	14.70	4	0.012	−0.33**
PC1_290	290	14.60	4	0.012	−0.24*	PC4_235	235	14.89	4	0.011	−0.38**
PC1_291	291	12.96	4	0.024	−0.31**	PC4_239	239	13.98	4	0.016	−0.24*
PC1_299	299	18.30	4	0.003	0.30*	PC4_300	300	17.05	4	0.004	−0.33**
PC1_306	306	11.58	4	0.041	0.26*	PC4_380	380	11.34	4	0.045	−0.31**
PC1_329	329	13.48	4	0.019	−0.24*	PC4_484	484	17.036	4	0.004	−0.29*
PC1_333	333	11.58	4	0.041	−0.24*	PC4_84	84	12.68	4	0.025	−0.32**
PC1_343	343	11.76	4	0.038	−0.28*	PC4_90	90	14.79	4	0.011	0.35**
PC1_399	399	12.04	4	0.034	−0.30*	PC5_131	131	12.26	4	0.031	−0.24*
PC1_400	400	11.99	4	0.035	−0.30*	PC5_147	147	11.70	4	0.020	−0.24*
PC1_458	458	13.80	4	0.017	−0.25*	PC5_183	183	12.91	4	0.024	−0.32**
PC1_97	97	12.08	4	0.034	−0.24*	PC5_187	187	14.47	4	0.011	−0.39**
PC1_98	98	12.95	4	0.024	−0.36**	PC5_192	192	12.85	4	0.025	0.27*
PC2_104	104	13.80	4	0.017	−0.35**	PC5_193	193	13.68	4	0.018	−0.26*
PC2_108	108	15.80	4	0.007	−0.24*	PC5_213	213	12.15	4	0.033	−0.24*
PC2_134	134	11.87	4	0.037	0.25*	PC5_350	350	17.41	4	0.043	−0.24*
PC2_143	143	13.65	4	0.018	0.24*	PC5_436	436	11.45	4	0.043	−0.33**
PC2_186	186	15.14	4	0.010	0.32**	PC5_59	59	11.96	4	0.035	−0.26*

Table 9 (continued)

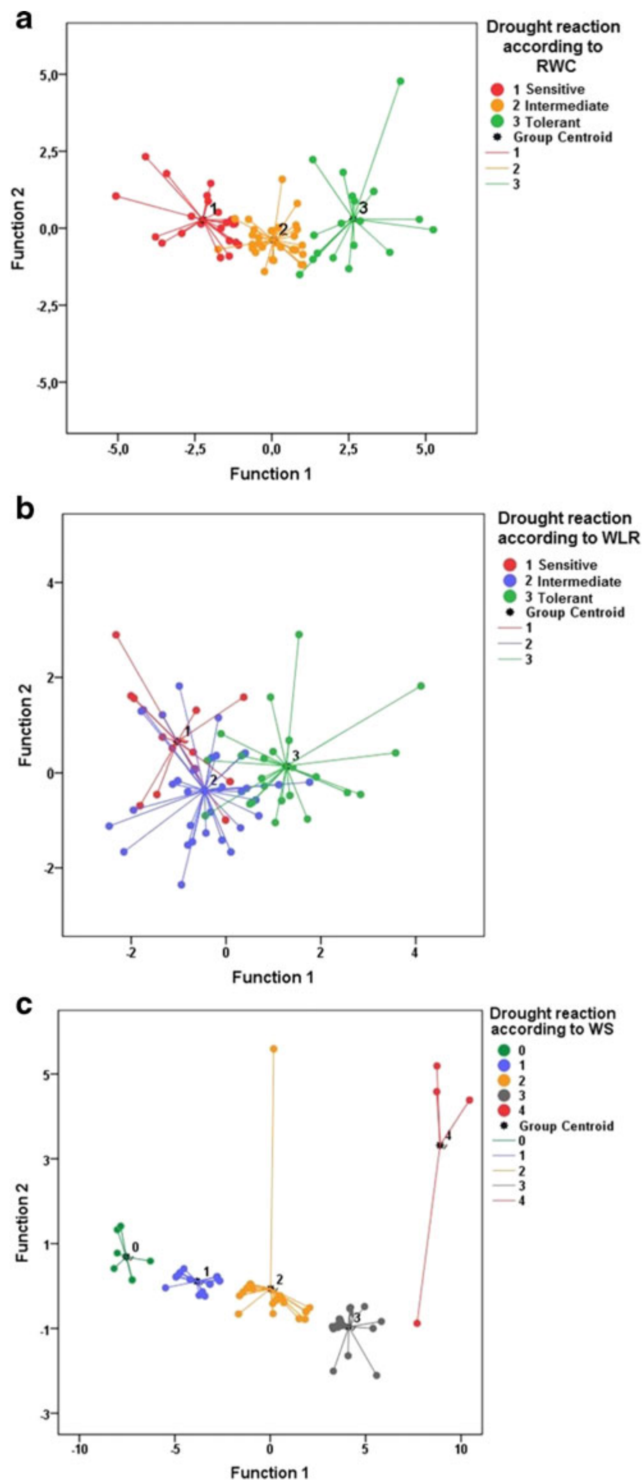
AFLPs linked to WLR	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*	Linked AFLPs to WLR	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*
PC2_192	192	11.58	4	0.041	−0.24*	PC5_70	70	17.12	4	0.004	−0.27*
PC2_220	220	11.78	4	0.038	0.24*	PC6_123	123	11.49	4	0.035	−0.29*
PC2_309	309	13.04	4	0.011	−0.39**	PC6_136	136	11.79	4	0.038	−0.35**
PC2_423	423	12.25	4	0.032	−0.32**	PC6_150	150	10.88	4	0.028	−0.26*
PC2_466	466	12.25	4	0.031	−0.30**	PC6_163	163	13.47	4	0.019	−0.24*
PC2_64	64	12.77	4	0.026	−0.36**	PC6_185	185	15.08	4	0.010	−0.27*
PC2_65	65	11.32	4	0.023	−0.32**	PC6_263	263	12.17	4	0.033	−0.33**
PC3_105	105	12.04	3	0.007	0.25*	PC6_271	271	16.92	4	0.005	0.25*
PC3_111	111	12.32	4	0.031	0.27*	PC6_318	318	16.68	4	0.005	−0.38**
PC3_113	113	11.76	4	0.038	−0.28*	PC6_321	321	12.03	4	0.034	0.24*
PC3_125	125	14.40	4	0.013	0.25*	PC6_323	323	11.98	4	0.035	0.24*
PC3_128	128	15.06	4	0.010	0.26*	PC6_391	391	16.22	4	0.003	−0.44**
PC3_151	151	12.56	4	0.028	0.29*	PC6_475	475	12.87	4	0.025	−0.30*
PC3_172	172	14.03	4	0.015	0.30*	PC6_484	484	12.86	4	0.025	−0.30*
PC3_184	184	22.90	4	0.000	−0.26*	PC7_126	126	11.27	4	0.024	−0.30*
PC3_185	185	13.62	4	0.018	−0.27*	PC7_187	187	10.35	4	0.035	−0.32**
PC3_225	225	11.68	4	0.039	−0.24*	PC7_253	253	12.20	4	0.032	−0.27*
PC3_237	237	15.28	4	0.009	0.24*	PC7_397	397	14.07	4	0.015	0.25*
PC3_245	245	13.77	4	0.017	0.24*	PC7_465	465	11.80	4	0.038	−0.27*
PC3_305	305	15.47	4	0.017	0.29*						

*Significant at $p<0.05$; **significant at $p<0.01$

Table 10 AFLP markers linked to wilting score (WS) according to Kruskal–Wallis H test

AFLPs linked to WS	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance*	Correlation*
PC1_114	114	11.94	4	0.036	−0.27*
PC1_143	143	13.84	4	0.017	0.35**
PC1_217	217	12.97	4	0.024	−0.24*
PC1_314	314	13.47	4	0.019	−0.24*
PC1_333	333	13.57	4	0.019	−0.25*
PC1_355	355	16.86	4	0.005	−0.26*
PC1_399	399	13.94	4	0.016	−0.36**
PC1_468	468	15.99	4	0.007	−0.39**
PC1_73	73	13.29	4	0.021	−0.28*
PC1_75	75	16.35	4	0.006	−0.28*
PC1_92	92	10.82	4	0.029	−0.35**
PC2_104	104	11.21	4	0.047	−0.35**
PC2_166	166	12.95	4	0.024	−0.25*
PC2_250	250	13.40	4	0.020	−0.28*
PC3_113	113	12.48	4	0.029	−0.30*
PC3_131	131	9.71	3	0.021	0.26*
PC3_137	137	16.66	4	0.005	0.27*
PC3_184	184	16.59	4	0.005	−0.29*
PC3_211	211	11.87	4	0.036	0.24*
PC3_213	213	10.08	4	0.039	0.25*
PC3_274	274	14.42	4	0.013	0.26*
PC3_305	305	15.47	4	0.017	0.30*
PC3_360	360	11.92	4	0.036	0.26*
PC3_64	64	10.63	3	0.014	−0.27*
PC3_69	69	9.66	4	0.047	−0.30*
PC3_87	87	14.04	4	0.015	0.26*
PC3_88	88	11.58	4	0.006	−0.31**
PC4_117	117	10.23	4	0.037	−0.35**
PC4_136	136	9.86	4	0.043	−0.34**
PC4_152	152	12.58	3	0.006	−0.24*
PC4_179	179	12.94	4	0.024	−0.33**
PC4_184	184	11.89	4	0.036	−0.37**
PC4_219	219	8.16	3	0.043	−0.25*
PC4_235	235	11.47	4	0.043	−0.39**
PC4_239	239	12.28	4	0.031	0.25*
PC4_300	300	14.93	4	0.011	−0.31**
PC4_380	380	13.41	4	0.020	−0.28*
PC4_66	66	12.59	4	0.027	−0.28*
PC4_75	75	12.55	4	0.028	0.24*
PC5_104	104	16.27	4	0.006	−0.28*
PC5_126	126	15.89	4	0.007	0.34**
PC5_192	192	11.87	4	0.036	0.37**
PC5_248	248	12.37	4	0.030	−0.24*
PC5_88	88	13.56	4	0.019	0.43**
PC6_121	121	12.46	4	0.029	−0.30*
PC6_271	271	12.55	4	0.028	0.42**
PC6_323	323	12.69	4	0.026	0.24*
PC6_391	391	12.014	4	0.017	−0.41*
PC6_97	97	11.42	4	0.044	0.38**
PC7_280	280	17.52	4	0.004	0.51**
PC7_400	400	12.81	4	0.025	−0.24*

*Significant at $p<0.05$; **significant at $p<0.01$



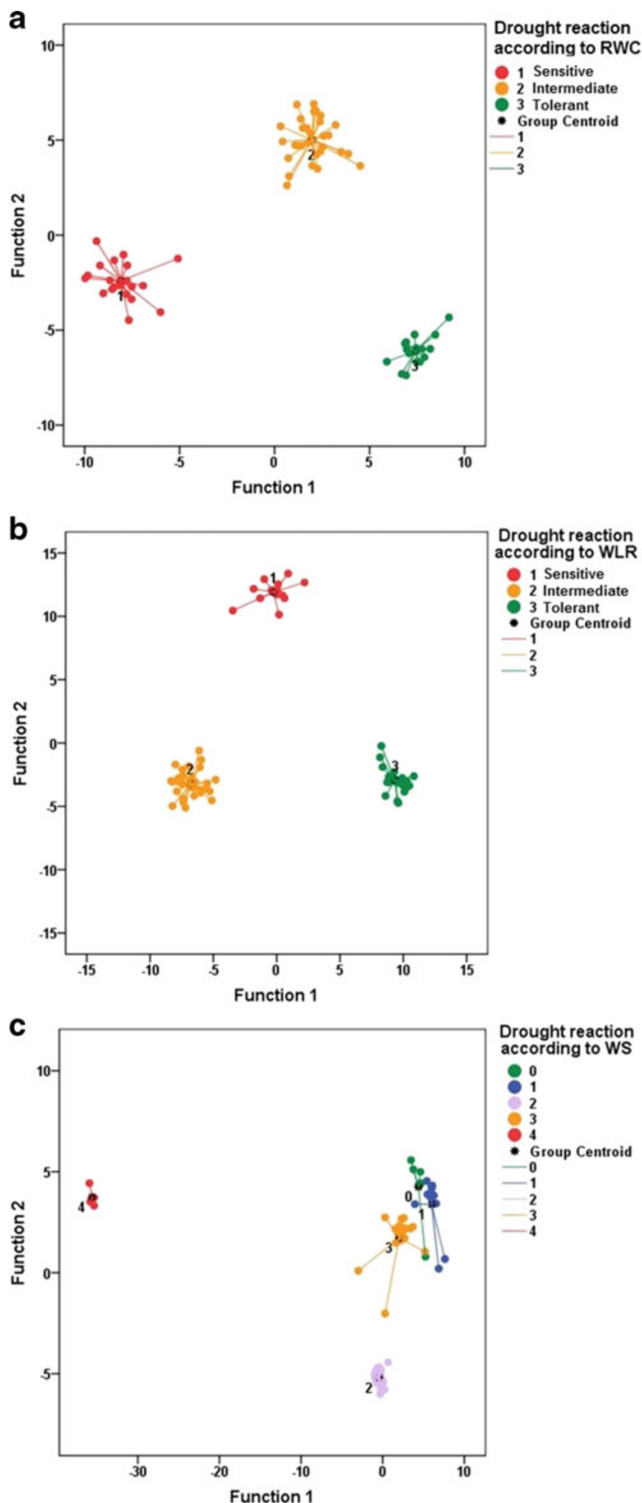
◀ **Fig. 7** Discriminant analysis based on SSRs linked to relative water content (**a**), water losing rate (**b**) and wilting score (**c**): 0 to 4 corresponds to the following 0–4 score scale as described by Singh et al. (2013): 0 = healthy plants with no visible symptoms of drought stress, 1 = green plants with slight wilting, 2 = leaves turning yellowish-green with moderate wilting, 3 = leaves yellow–brown with severe wilting and 4 = completely dried leaves and/or stems. Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate (52.5≤RWC<60) and tolerant (RWC≥60). Similarly, three classes were defined for WLR: sensitive (WLR≥0.56), intermediate (0.56<WLR≤0.50) and tolerant (WLR <0.50)

and with an average PIC of 0.579 using six primer combinations in Turkish landraces.

Based on NJ dendrogram and PCA using SSR and AFLP DNA markers separately, and the combined data sets, landraces from the northern Mediterranean, i.e., from Italy, Turkey and Greece, could clearly be differentiated from those originating from the southern Mediterranean, i.e., from Morocco. Landraces from Italy, Turkey and Greece also differed between them as well. This confirms the presence of high genetic diversity in the Mediterranean region for lentil landraces and the possibility of different gene pools. Our results are in agreement with those of Lombardi et al. (2014) who reported very high levels of genetic diversity among lentil landraces from the Mediterranean region using single-nucleotide polymorphism markers. Similar results of geographic differentiation have been reported for Mediterranean tetraploid wheat landraces by Oliveira et al. (2014) showing four groups: an eastern group (Cyprus, Croatia, Egypt, Iran, Iraq, Israel, Jordan, Lebanon and Turkey), a western group (Algeria, France, Morocco, Portugal, Spain and Tunisia), a second mainly eastern cluster (some accessions not only from Croatia and Turkey, but also from Greece and one Portuguese accession), and a fourth cluster (all Italian accessions and also accessions from Spain and Tunisia).

The rich history of the Mediterranean region regarding lentil domestication and cultivation together with the frequency and diversity of biotic and abiotic stresses makes this region an important source for genotypes that have developed tolerance mechanisms. Laghetti et al. (2008), Toklu et al. (2009) and Idrissi et al. (2015a) reported the importance and genetic differentiation of lentil genetic resources for adaptive traits of some landraces from Italy, Morocco and Turkey.

High genetic variation for root and shoot traits as well as for drought response as estimated by leaf RWC, WLR and WS was observed among the Mediterranean landraces included in our study. The association of these latter traits with drought tolerance in lentil and other crops has been reported often before (Sarker et al. 2005; Kashiwagi et al. 2005; Vadez et al. 2008; Gaur et al. 2008; Aswaf and Blair 2012; Kumar et al. 2012; Idrissi et al. 2015b). Under water-limited conditions, the first plant response is to maintain water content as close as possible to that of the non-stressed situation by stomatal control to limit water loss and by faster root growth and increased RS ratio to improve water uptake. Increased root growth and the capacity to maintain higher water content



◀ **Fig. 8** Discriminant analysis based on AFLPs linked to relative water content (**a**), water losing rate (**b**) and wilting score (**c**): 0 to 4 corresponds to the following 0–4 score scale as described by Singh et al. (2013): 0 = healthy plants with no visible symptoms of drought stress; 1 = green plants with slight wilting; 2 = leaves turning yellowish green with moderate wilting; 3 = leaves yellow–brown with severe wilting; and 4 = completely dried leaves and/or stems. Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate (52.5≤RWC<60) and tolerant (RWC≥60). Similarly, three classes were defined for WLR: sensitive (WLR≥0.56), intermediate (0.56<WLR≤0.50) and tolerant (WLR <0.50)

levels are important in order to maintain plant growth and production under drought stress conditions (Verslues et al. 2006) compared to other mechanisms which have a more negative effect on yield.

Significant positive correlations were obtained between dry root biomass and dry shoot biomass and SPAD. This highlights the possibility of indirect selection for this underground trait using simple measures of chlorophyll content and aboveground biomass weight in breeding programs targeting vigorous root systems. Landraces with higher dry root weight, chlorophyll content and RS ratio were the most drought tolerant as evidenced by their higher leaf RWC and lower WLR and WS. Thus, selection of accessions that score well on these parameters under water-limited conditions would result in developing improved cultivars with drought tolerance. No correlation between drought tolerance and geographic origin of landraces was observed. Thus, selection has to be based on the individual response of each genotype. Significant but rather weak grouping based on shoot and root traits was observed showing landraces from Morocco with slightly higher shoot length, biomass, and seedling early vigor compared to those from northern Mediterranean (low eigenvalues of discriminant analysis, data not shown). Additional phenotypic characterization including morphological and phenological traits is needed to understand the genetic differentiation shown by SSR and AFLP markers.

Significant marker–trait associations of SSR and AFLP DNA markers with leaf RWC, WLR and WS were evidenced based on Kruskal–Wallis test. Six, four and five SSRs and 91, 105 and 51 AFLPs were identified to be linked to the three drought parameters, respectively. SSR- and AFLP- linked allele markers highly discriminated landraces according to their drought reaction highlighting genetic differentiation according to their drought tolerance level (high eigenvalues of discriminant analyses). Landraces with higher RWC and lower WLR and WS could be clearly separated from those with lower RWC and higher WLR and WS. Among these markers, alleles SSR19_7 and SSR80_12, SSR336_22 and SSR184_17, and SSR19_7 and SSR204_1 explained the highest phenotypic variation of RWC, WLR and WS, respectively, as shown by the regression analysis (ranging from 21 to 50 %). These markers can thus be considered as associated markers and potential functional markers to be used in functional genetic diversity analysis related to finding adaptive traits to drought tolerance. The highest phenotypic variation explained by linked AFLPs ranged from 14 to 33 %. This

finding suggests the reliability of association mapping studies for evidencing drought tolerance on a large number of landraces in lentil as an interesting approach for the identification of genes and quantitative trait loci (QTLs) controlling traits of interest for marker-assisted selection (Kumar et al. 2015). Joshi-Saha and Reddy (2015) identified three SSR alleles associated with drought tolerance using K-W test in 60 genotypes of chickpea (*Cicer arietinum*). Using the same method, Razavi et al. (2011) reported five and 13 EST and 47 and 85 AFLP markers linked to leaf RWC and WLR in 23 *Fragaria* cultivars, respectively. Iglesias-García et al. (2015) reported four QTLs associated with drought adaptation as estimated by leaf RWC in pea (*Pisum sativum* L.).

Conclusion

Our study evidenced substantial genetic variation in Mediterranean lentil landraces for traits related to drought tolerance and for molecular diversity at several SSR and AFLP loci. Further phenotypic evaluation is needed to understand the genetic differentiation between landraces from Morocco and those from the northern Mediterranean. Germplasm included in this study has great potential for lentil breeding for developing drought-tolerant lentil varieties. High variability for root and shoot traits and physiological parameters related to drought tolerance observed in this study showed no correlation with geographic origin. Higher dry root biomass, chlorophyll content and RS ratio were associated with higher drought tolerance. Association of certain aboveground traits with root biomass indicates the potential for reliable indirect selection for drought tolerance in lentil.

A number of DNA markers were identified to be associated with drought tolerance, and phenotypic classes according to drought response better corresponded to groupings based on these correlated markers. Although plant response to drought stress is a complex trait involving many aspects, this study showed evidences of genetic differentiation according to drought response. Thus, further studies involving larger numbers of landraces and unrelated genotypes in association mapping and quantitative trait studies based on mapping populations from contrasted parents using more efficient and effective DNA markers like single-nucleotide polymorphism markers would allow better understanding of the genetic basis of their drought tolerance.

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Conflict of Interest The authors declare that they have no conflict of interest.

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