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MORPHO-AGRONOMIC AND GENETIC DIVERSITY AMONG TWELVE SICILIAN AGRO-ECOTYPES OF LENTIL (*LENS CULINARIS*)

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ABSTRACT

Although Sicily is relatively small (about 25000 km^2), it accounts for several agro-ecotypes of lentil (*Lens culinaris*Medik.), for many of which no data on morphological, agronomic, and organoleptic characteristics are available to date. Thus, experiments were performed to characterize 12 lentil agro-ecotypes from different areas of Sicily, including some small islands surrounding the main island, and to assess the extent of genetic diversity (by means of six inter-simple sequence repeat [ISSR] primers). A famous agro-ecotype from central Italy (CastelluccioNorcia) and two commercial varieties from Canada (Eston and Laird) were also included in the study. The results showed a large degree of genetic diversity (based on ISSR markers) and variability in pheno-morphological and agronomic traits. In contrast, the intra-accession variability was on average rather low. The agronomic (productivity, nitrogen fixation capacity) and qualitative (lipid content, hydration coefficient) attributes of several Sicilian agro-ecotypes were more pronounced than those of the controls. This fact certainly represents a prerequisite for their future economic valorization. Moreover, the observed variability may be of interest from the point of view of breeding.

Keywords: Phenotypic diversity; Molecular markers; ISSR; Hydration coefficient; Nitrogen fixation.

INTRODUCTION

Lentil (*Lens culinaris*Medik.) is a self-pollinating diploid (2n = 2x = 14) species (Havey and Muehlbauer 1989). It is one of the most ancient cultivated legumes and it is a staple food appreciated by consumers around the world because of its sensorial properties, shorter cooking time than most other pulses, and high nutrient value. Indeed, lentil seeds are high in proteins, micronutrients, and vitamins content (Jood *et al.* 1998; Iqbal *et al.* 2006).

Over the past 50 years, the harvested area of lentil worldwide has gradually increased, rising from just over 1.5 million hectares to over 4.2 million (FAOSTAT 2013). Asia is the continent historically most interested in the cultivation of lentil but today this cultivation is widespread in Canada, Australia, and USA, where the amount of surface invested in lentil is rapidly increasing. In Europe, about 60000 ha are sown with lentil, with Spain as the leading producer. Italy has a long history of cultivating lentil and was in the past a major European producer, but today the crop is grown on only about 2000 ha (FAOSTAT 2013). Lentil production still takes place in restricted areas of central and southern Italy and on some small islands, generally on farms linked to traditional farming systems (Piergiovanni 2000). As in many countries in which lentil is traditionally grown, Italian lentil production is mostly based on the cultivation of agro-ecotypes, or local populations progressively selected by farmers over time and well adapted to the agro-environment in which they are grown (Zaccardelli *et al.* 2012). Some Italian lentil agro-ecotypes, such as the Castelluccio di Norcia (from Umbria), have been characterized in previous studies for both morpho-agronomic and genetic traits(Sonnante and Pignone 2001, 2007;Piergiovanni and Taranto2005; Amato *et al.* 2006; Scippa *et al.* 2008, 2010; Torricelli *et al.* 2012; Zaccardelli *et al.* 2012).

Although Sicily is relatively small (about 25000 km^2), it is home to several lentil agro-ecotypes, probably because of both the great pedo-climatic variability and the long history of cultivation of the species across the island, factors that may have driven the intraspecific differentiation of lentil. Most Sicilian lentil agro-ecotypes are currently at risk for severe genetic erosion or extinction, as they are grown on only few hectares and their survival is often in the hands of older farmers. To this day, no molecular studies have been performed to assess the extent of genetic variability within the Sicilian lentil germplasm, and, moreover, no information is available on the morphological, agronomic, or organoleptic characteristics of most Sicilian lentil agro-ecotypes. Thus, we performed an experiment to characterize the morphological, agronomic, nutritional, and technological traits and to assess the extent of genetic diversity (by means of six inter-simple sequence repeat [ISSR] primers) of 12 lentil agro-ecotypes from different areas of Sicily, including some small islands surrounding the main island. This knowledge is essential for identifying the agro-ecotypes better able to increase their market value; furthermore, this knowledge will be useful

in planning effective breeding programs and in defining priorities for conservation programs.

MATERIALS AND METHODS

Plant material and experimental site: Twelve lentil agro-ecotypes collected throughout Sicily were studied (Fig. 1). Three out of these agro-ecotypes were from three small Sicilian islands (Linosa, Pantelleria, and Ustica). Also, one well-known Italian agro-ecotype from the central Apennines (CastellucciodiNorcia) and two Canadian commercial varieties (Eston and Laird) were included in this study as outgroups. Some traits relative to the seeds of the accessions under study are reported in Table 1.

Two field trials were conducted in a hilly area of Sicily (37°30' N, 13°31' E, 178 m asl) during two growing seasons (2005–2006 and 2007–2008) on a VerticHaploxerept soil. The topsoil (0–0.40 m) had the following characteristics: 380 g/kg clay, 250 g/kg silt, and 370 g/kg sand; pH 8.4 (1:2.5 H_2O); 12.7 g organic matter/kg soil and 8.5 g total Nitrogen (N)/kg soil. The study site has a semiarid Mediterranean climate with a long-term mean annual rainfall of 550 mm, mostly concentrated during the autumn–winter period (74%) and with a lesser amount during spring (18%). The average minimum and maximum temperatures are 10.0 and 23.3°C, respectively. The total rainfall during the first growing season was 558 mm, which was very close to the long-term average, whereas the mean monthly temperature was slightly lower than average. During the second growing season, the total rainfall was 479 mm, 10% less than the long-term average, and the mean monthly temperature was similar to the average.

Agronomic evaluation in dense stands: The 15 accessions were sown on 22 December 2005 at 300 viable seeds/m^2 in plots (eight rows 6 m long and 0.18 m apart). A randomized block design with four replications was used. Plots were not fertilized, and the trial was performed under rainfed conditions. Weeds were manually removed.

For each accession, at maturity plant height, aboveground biomass production, grain yield, and seed weight were recorded. Nitrogen and lipid contents were determined in the grain flour using Kjeldahl and Soxhlet methods, respectively.

Nitrogen fixation was estimated using the ^{15}N isotope dilution technique (McAuliffe *et al.* 1958). Isotope-labelled (^{15}N) fertilizer was applied in the amount of 8 kg N/ha ($[NH_4]_2SO_4$ with an isotopic composition of 10 atom% ^{15}N) to a 1.5-m^2microplot (five rows 1.6 m long) in the middle of each plot. The ^{15}N fertilizer was equally divided and uniformly applied in liquid form at the emergence of the crop and again 60 d after plant emergence. Durum wheat (cv Simeto) was used as a reference crop to calculate the percentage of N derived from the atmosphere (%Ndfa) by the legumes. Wheat was planted at a rate of 350 viable seeds/m^2 in rows 0.18 m apart and was treated as the legume.

For each lentil accession, seed diameter was measured on eight samples (two subsamples per replicate) of 100 seeds each using the image analysis software Image Tool 3.0 (UTHSCSA 2002).

Seed thickness was also measured using a digital calliper. The seed coat was removed after moistening and drying and its weight determined. Seed diameter and seed thickness measures were used to calculate the total surface area of the seed, considering it as a double spherical cap.

For each lentil accession, seed hydration coefficients were calculated on a subsample of each replicate by measuring the water uptake by 5 g seeds soaked in 25 mL distilled water at room temperature (Hulse *et al.* 1977). After 10, 20, 40, 80, 160, 320, 640, and 1280 min, each subsample was removed from the water and blotted dry and its weight recorded. Subsamples were then returned to the water.

At maturity, a sample of total aboveground biomass was taken from each microplot that had received the isotope-labelled (^{15}N) fertilizer; the biomass was dried at 60°C to a constant weight, ground to a fine powder (sieved using a 0.1-mm mesh) in a fast-running mill, and analysed for total N and ^{15}N enrichment. Data on the ^{15}N enrichment of the biomass were used to calculate the percentage of N derived from symbiotic N_2 fixation (%Ndfa) and the total amount of N fixed on a basis area according to Giambalvo *et al.* (2012).

Pheno-morphological characterization under spaced-plant conditions: Seeds from the 15 lentil accessions were sown into Jiffy pots on 15 December 2007. After 6 weeks, 20 randomly chosen seedlings of each accession were transplanted to the field with 0.5 m between each plant. A completely randomized design was used. The soil was not fertilized, and weeds were manually removed throughout the growing season. The trial was conducted under rainfed conditions, although each plot was initially irrigated immediately following transplantation (10 mm). Deltamethrin (at a rate of 6.25 g a.i./ha) was applied on 11 and 21 April 2008 to control aphid attacks. Ten plants per accession were randomly selected, and the following pheno-morphological characters were recorded during the crop cycle: days to first flower from 1 April (hereafter, flowering time); plant height; growth habit (1 = prostrate, 9 = erect); leaflet area, number of leaflets per leaf, leaf petiole length, leaf tendril length (all measured on three fully developed leaves per plant); number of flowers per raceme; number of seeds per pod; and seed diameter. The leaf and seed measurements were obtained on digital photographs

using the image analysis software Image Tool 3.0 (UTHSCSA 2002).

Characterization by ISSR molecular markers: Total genomic DNA was extracted from 0.3 g young, healthy, and fresh leaflets according to the procedure described by Lodhi *et al.*(1994). For each lentil agro-ecotype, DNA was extracted from 10 individual plants (with the exception of Castelluccio di Norcia and Maniace, for which 9 and 8 individual plants, respectively, were used). For each of the two commercial varieties (Eston and Laird), DNA was extracted from a bulk of young leaflets from 10 individuals. The DNA concentration of each sample was quantified by measuring absorbance at 260 nm as described by Sambrook *et al.* (1989) and adjusted to a concentration of 25 ng/µl. Genetic analysis was performed using six ISSR primers (Table 2) as provided by Dr. S. Lucretti of the Biotechnology and Agriculture Division, ENEA C.R. Casaccia, Italy. These ISSR primers were already used in other studies (Ruisi *et al*. 2011; Gristina *et al*. 2014). Polymerase chain reaction (PCR) amplification was performed according to the following conditions: a 25-µl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2mM $MgCl_2$, 0.5 µM each primer, 800 µMd NTP, 1 unit Taq polymerase, and 25 ng genomic DNA. PCR reactions were performed in a 96-well thermocycler GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) under the following cycle program: initial denaturation at 94°C for 4 min, followed by 36 cycles at 94°C for 30 s (denaturation), 53–56°C (depending on primer) for 45 s (annealing), and 72°C for 120 s (extension), followed by a final extension step at 72°C for 7 min. PCR-amplified DNA fragments were separated on a 1.5% agarose gel containing 1 × TBE (45 mMTris-borate, 1 mM EDTA) and a 0.5 µg/ml aqueous solution of ethidium bromide. About 25 µl reaction product (with an adequate amount of loading buffer) was loaded and the gel was run for 7 h at 100 V. The gel was then visualized under ultraviolet light.

Statistical analysis: In both field experiments, an analysis of variance (ANOVA; SAS Institute, 2008) was performed for each trait according to the experimental design to test the significance of the variation among accessions. The means of the accessions were compared using Tukey's test at the 5% probability.

For the molecular analysis, amplified bands from each primer were scored as present (1) or absent (0) for all of the genotypes analysed. Only bands showing consistent amplification were considered; smeared and weak bands were excluded from the analysis. Nei's (1972) genetic distance between each accession pair was determined. To show a graphical representation of the genetic relationships among the studied genotypes, a principal coordinate analysis (PCoA) was performed using molecular data, and a scatter plot of the first two principal coordinates made. A cluster analysis based on Nei's genetic distances was performed according to the unweighted paired group method with arithmetic mean (UPGMA) and the relative dendrogram was constructed. The following additional statistics were computed to estimate the degree of polymorphism among the lentil agro-ecotypes studied: number of amplified loci, percentage of polymorphic loci, average gene diversity among and within population, and coefficient of gene differentiation. Intra-population variability was expressed as the average number of polymorphic loci identified for the individual, percentage of polymorphic loci of the total number of loci analysed, actual average number of observed alleles, Shannon index (Lewontin 1972), and gene diversity within each population. Finally, Analysis of Molecular Variance (AMOVA) was used to separate the total genetic variance into within and among populations. All calculations and analyses were conducted using POPGENE, version 1.31 (Yeh *et al.* 1999) and GenAlex 6.5 (Peakall and Smouse 2006).

RESULTS

Agronomic evaluation: The ANOVA results showed highly significant differences among the 15 lentil accessions for most of the recorded traits (Table 3). On average, plant height ranged from 42 cm (Linosa) to 75 cm (cv Laird). Average grain yield was 1.31 t/ha, with Castelluccio di Norcia (i.e., the agro-ecotype from central Italy) being the most productive accession (2.45 t/ha); among the Sicilian accessions, grain yield ranged from 0.62 t/ha (Nera di Enna) to 2.14 t/ha (Ustica). Several Sicilian agro-ecotypes were more productive than the two Canadian varieties. Great differences among the accessions were observed for total N content (at harvest), which ranged from 112 kg N/ha (cvEston) to 173 kg N/ha (Ustica); biomass production and its N concentration contributed equally to the differences among the accessions in total N content ($r = 0.63$ and 0.69, respectively; $P < 0.05$). Average Ndfa was 39.5%, with no significant differences among the accessions. The amount of N fixed ranged from 35 kg/ha (Villalba) to 74 kg/ha (Pantelleria), with appreciable differences among the accessions. The amount of N fixed appeared to be positively correlated with both total N content ($r = 0.72$; $P < 0.01$) and grain yield ($r = 0.73$; $P < 0.01$). No differences were observed among the accessions for grain protein content (range: 260–274 g/kg), whereas significant differences were found for grain lipid content, which ranged from 5.3 g/kg (Nera di Enna) to 11.7 g/kg (Castelluccio di Norcia).

Large differences were found among the accessions in the morpho-dimensional traits of the seeds (area, diameter, and thickness; Table 4). Accessions from the small islands (Linosa, Pantelleria, and Ustica) surrounding Sicily and Pachino had the smallest seeds,

whereas Villalba and cv Laird had the biggest seeds. Significant differences were recorded for the weight of the seed coat per unit area, which ranged from 0.061 mg/mm (Linosa, Pachino, Ustica, and cvEston) to 0.084 mg/mm (Castelluccio di Norcia), whereas no differences among the accessions were observed for the proportion of the seed coat weight to the total seed weight.

Regarding hydration coefficients, after 10 min, seeds absorbed, on average, water equal to 88 g/kg of the dry weight of the seed, with significant differences among the accessions (range: 37–144 g/kg for Castelluccio di Norcia and Villalba, respectively; Fig. 2). These differences increased until 40 min (range: 125–486 g/kg for Castelluccio di Norcia and Villalba, respectively); after 80 min the amount of water absorbed by seeds was 555 g/kg of the dry weight of the seed, and differences among accessions remained large and significant (range: 354–708 g/kg for Castelluccio di Norcia and cv Laird, respectively). After 640 min, the hydration coefficient was on average 1035 g/kg and ranged from 917 to 1127 g/kg (Ustica and cv Laird, respectively). At the last step (1240 min; i.e., more than 10 h from the previous step) the hydration coefficient showed very little increase, reaching on average 1074 g/kg. The differences among the accessions in hydration coefficients, particularly after 40 min of soaking, were closely and positively related to seed dimension traits (seed area and diameter) and seed weight; Fig. 3 reports the relation between seed weight and the amount of water absorbed by seeds after 40 min.

Pheno-morphological variability: The ANOVA showed significant differences among the lentil accessions for all traits observed for plants grown under spaced conditions (Table 5). Regarding flowering time, Linosa was the first population to flower (4 d from 1 April) and Nera di Enna the last (28 d from 1 April). Leaflet area ranged from 42 mm^2 (Nera di Enna) to 88 mm^2 (Mussomeli). The Sicilian lentil accessions varied widely in the length of their leaf tendrils, which was between 18 mm (Nera di Enna) and 39 mm (Palazzolo), and the number of flowers per raceme, which ranged from 1.4 (Linosa) to 2.8 (Bisacquino). However, low inter-accession variation was observed in the number of leaflets per leaf.

For most of the traits, intra-accession variability was generally low, being for most of the Sicilian agro-ecotypes similar to that of the two Canadian varieties included in the study. On the whole, Nera di Enna and Villalba showed the greatest intra-accession variability (Table 5).

Molecular diversity: Six ISSR primers were used to evaluate all 15 accessions included in the study. On the whole, 113 well-resolved bands were observed (Table 6). The number of ISSR bands obtained with each primer varied from 10 for primer $AG(CA)_8$ to 24 for primer $(ACC)_6CC$, with an average of 18.5 bands per primer. Out of the 113 amplified bands, 72 were polymorphic (63.7% of the total amplified bands). The number of polymorphic markers detected with each primer ranged from 6 for primer $CCAT(GT)_7$ to 21 for primer $(ACC)_6CC$.

The total gene diversity (H_T) in this study averaged 0.151 ± 0.035. The greatest diversity was found among lentil accessions (coefficient of gene differentiation [G_{ST}] = 0.743); in contrast, within-accession diversity (H_S) was very low (0.039 ± 0.002, on average). Also AMOVA indicated that most of the genetic diversity occurred among accessions (75%) while the variability within accessions accounted for only 25% of the observed genetic diversity.

The indices of intra-accession variability calculated for each accession are shown in Table 7. The percentage of polymorphic loci was 11.1% on average and ranged from 0 to 20.4%; gene diversity within the population was between 0 and 0.071. Shannon's index was between 0 and 0.106, with an average of 0.058. Modica showed an absence of internal variability (all individuals were genetically identical), whereas Villalba showed the highest degree of diversity.ThePCoA performed on the molecular data clearly discriminated the lentil accessions (Fig. 4). Moreover, individuals belonging to the same accession were generally close to one another, which confirms the low degree of genetic diversity within accessions. The dendrogram constructed on the basis of Nei's genetic distances using the UPGMA method is shown in Fig. 5. Five main groups were distinguishable. Cluster 1 included the accessions from the small islands surrounding Sicily (Ustica, Pantelleria, and Linosa); these accessions were characterized by early flowering, small leaves and seeds, and a low number of flowers per raceme. Cluster 2 included only two accessions (Nera di Enna and Bisacquino) that showed late phenology, seeds with intermediate dimensions, a low lipid content in the grain, and low grain productivity. Cluster 3 consisted of three accessions (Nissoria, Maniace, and Villalba) from central and north-eastern Sicily that differed in many pheno-morphological traits (Villalba in particular differed from the others). Cluster 4 grouped all of the accessions from south-eastern Sicily (Pachino, Palazzolo, and Modica) plus an accession from central Sicily (Mussomeli) and cvEston (selected from a Turkish accession); also in this case the clustered accessions showed a certain degree of phenotypic variability, particularly Mussomeli from the others. Clusters 5 and 6 each consisted of only one accession: Castelluccio di Norcia (the agro-ecotype from central Italy), the most productive accession included in the study, and cv Laird (selected from a Russian accession), respectively.

Table 1.Prevalent colours and patterns of seed coats and colours of cotyledons of the accessions studied.

Accession	Seed coat colour	Seed coat pattern	Seed coat pattern colour	Cotyledon colour
Bisacquino	Brown	Marbled	Grey	Yellow
Linosa	Brown	Marbled	Grey	Orange
Maniace	Green/brown	Marbled	Grey	Yellow
Modica	Green/brown	Marbled	Green/grey	Yellow
Mussomeli	Light brown	Absent	−	Yellow
Nera di Enna	Black	Absent	−	Yellow/orange
Nissoria	Light brown	Marbled	Grey	Yellow
Pachino	Brown	Complex	Grey	Yellow/orange
Palazzolo	Light brown	Marbled	Grey	Yellow
Pantelleria	Brown	Absent	−	Orange
Ustica	Brown	Absent	−	Orange
Villalba	Light brown	Absent	−	Yellow
Castelluccio di Norcia	Light brown	Complex	Green/brown	Orange
Eston	Light brown	Absent	−	Yellow
Laird	Light brown	Absent	−	Yellow

Table 2. Inter simple sequence repeat (ISSR) used for assessment of genetic diversity.

ISSR Name	Sequence 5'-3'	Core sequence	Annealing Temperature (°C)
P1 - ENEA34	$(ACC)_6CC$	ACCACCACCACCACCACC	56
P2 - ENEA36	$CC(ATG)_6$	ATGATGATGATGATGATG	56
P3 - ENEA12	$CCAT(GT)_7$	GTGTGTGTGTGTGT	52
P4 - ENEA13	$GCA(AC)_7$	ACACACACACACAC	52
P5 - ENEA14	$GGG(AC)_7$	ACACACACACACAC	54
P6 - ENEA47	$AG(CA)_8$	CACACACACACACACA	52

Table 3. Main traits observed in the agronomic evaluation in dense stand for the 15 lentil accessions.

	Plant height	Grain yield	Biomass production	Total N content	Ndfa	N fixed	Grain protein content	Grain lipid content
	(mm)	(t/ha)	(t/ha)	(kg/ha)	(%)	(kg/ha)	(g/kg)	(g/kg)
Bisacquino	590	0.76	6.97	134.3	33.1	44.8	274	7.2
Linosa	420	1.64	5.33	120.1	40.7	49.4	267	9.0
Maniace	620	1.86	6.64	147.6	46.5	69.0	269	9.7
Modica	630	1.11	7.22	138.9	29.0	39.4	270	8.8
Mussomeli	690	1.00	6.81	120.2	37.9	45.9	260	9.4
Nera di Enna	600	0.62	6.33	121.8	36.8	43.9	274	5.3
Nissoria	550	1.69	6.56	156.2	40.6	63.7	271	8.6
Pachino	480	1.06	5.67	123.4	49.4	60.7	264	10.2
Palazzolo	640	0.87	6.56	136.2	38.4	51.3	262	7.8
Pantelleria	470	1.96	7.06	161.0	46.2	73.8	270	10.5
Ustica	450	2.14	7.36	172.5	38.5	67.2	268	9.9
Villalba	600	0.89	6.31	122.1	29.4	35.4	267	7.7
Castelluccio di Norcia	450	2.45	6.39	135.6	44.0	58.2	262	11.7
Eston	570	0.70	5.58	112.2	45.0	50.6	274	9.3
Laird	750	0.82	6.89	119.1	37.1	43.6	267	11.5
Mean	570	1.31	6.51	134.8	39.5	53.1	268	9.1
P value	<0.0001	<0.0001	0.0009	0.0002	0.3477	0.0425	0.5900	0.0079
HSD ($P \leq 0.05$)	56.0	0.40	0.92	25.1	−	23.5	−	2.9

Table 4. Seed traits observed in the agronomic evaluation in dense stand for the 15 lentil accessions.

	Seed area	Seed thickness	Seed diameter	Seed weight	Weight of seed coat	Seed coat to total seed weight
	(mm^2)	(mm)	(mm)	(mg)	(mg mm^{-2})	(g/kg)
Bisacquino	59.7	2.5	5.6	53.6	0.082	90.9
Linosa	37.2	2.6	4.1	27.0	0.061	83.9
Maniace	51.9	2.7	5.1	40.6	0.066	83.7
Modica	40.5	2.3	4.5	29.7	0.065	87.5
Mussomeli	66.1	2.4	6.0	54.8	0.070	83.9
Nera di Enna	53.5	2.6	5.2	40.4	0.066	87.2
Nissoria	53.9	2.5	5.3	44.5	0.071	85.6
Pachino	34.0	2.5	4.0	23.7	0.061	88.0
Palazzolo	38.4	2.3	4.4	27.7	0.074	101.2
Pantelleria	36.1	2.4	4.1	27.4	0.068	87.6
Ustica	35.0	2.2	4.2	24.9	0.061	85.7
Villalba	74.3	2.5	6.4	62.1	0.070	83.8
Castelluccio di Norcia	37.5	2.3	4.3	30.1	0.084	101.9
Eston	39.2	2.2	4.5	27.4	0.061	87.9
Laird	76.2	2.5	6.5	58.3	0.067	88.0
Mean	48.9	2.4	4.9	38.1	0.068	88.5
P value	<0.0001	<0.0001	<0.0001	<0.0001	0.0383	0.1327
HSD ($P \leq 0.05$)	5.4	0.1	0.4	2.84	0.014	−

Table 5.Pheno-morphological traits (mean ± SD) observed for the 15 lentil accessions grown under spaced-plant conditions.

	Plant height	Flowering time	Leaf petiole length	Leaf tendril length	Number of leaflets per leaf	Leaflet area	Number of flowers per raceme	Seed diameter	Number of seeds per pod	Plant habit
	(mm)	(days from 1 April)	(mm)	(mm)	(no.)	(mm^2)	(no.)	(mm)	(no.)	(1–9)
Bisacquino	340 ± 23	25 ± 4.4	40 ± 4.7	28 ± 4.7	13.9 ± 1.31	61 ± 5.2	2.8 ± 0.27	5.6 ± 0.42	1.3 ± 0.18	5.0 ± 1.50
Linosa	320 ± 33	4 ± 4.7	32 ± 3.1	32 ± 3.1	11.6 ± 0.69	58 ± 9.6	1.4 ± 0.18	4.3 ± 0.20	1.9 ± 0.19	8.3 ± 1.06
Maniace	340 ± 62	12 ± 4.8	40 ± 5.5	36 ± 5.5	11.6 ± 0.90	61 ± 14.5	2.2 ± 0.23	4.6 ± 0.31	1.5 ± 0.19	7.0 ± 1.00
Modica	320 ± 43	15 ± 5.2	39 ± 6.1	35 ± 6.1	12.3 ± 0.54	59 ± 8.9	2.7 ± 0.20	4.3 ± 0.13	1.6 ± 0.25	7.2 ± 0.92
Mussomeli	350 ± 30	23 ± 3.6	46 ± 5.4	32 ± 5.4	12.2 ± 0.35	88 ± 11.0	2.6 ± 0.24	5.7 ± 0.25	1.4 ± 0.20	6.8 ± 0.83
Nera di Enna	300 ± 67	28 ± 9.3	37 ± 4.9	18 ± 4.9	12.2 ± 1.18	42 ± 10.9	2.7 ± 0.29	5.2 ± 0.31	1.2 ± 0.20	6.9 ± 1.36
Nissoria	360 ± 49	7 ± 5.3	36 ± 3.1	31 ± 3.1	12.2 ± 1.25	56 ± 13.8	1.8 ± 0.23	4.7 ± 0.45	1.7 ± 0.33	5.8 ± 0.44
Pachino	280 ± 42	18 ± 4.2	37 ± 5.4	33 ± 5.4	12.1 ± 0.57	54 ± 14.5	2.6 ± 0.29	4.2 ± 0.27	1.7 ± 0.23	5.7 ± 1.16
Palazzolo	340 ± 33	10 ± 4.2	41 ± 4.7	39 ± 4.7	11.8 ± 0.70	67 ± 19.1	2.7 ± 0.36	4.5 ± 0.28	1.7 ± 0.20	6.5 ± 1.20
Pantelleria	300 ± 39	11 ± 3.7	37 ± 4.2	37 ± 4.2	12.1 ± 1.02	64 ± 16.3	1.7 ± 0.16	4.3 ± 0.31	1.8 ± 0.25	6.3 ± 0.67
Ustica	320 ± 31	12 ± 2.7	36 ± 2.4	28 ± 2.4	12.3 ± 0.86	51 ± 10.0	2.0 ± 0.26	4.4 ± 0.13	1.6 ± 0.25	5.7 ± 1.11
Villalba	350 ± 43	23 ± 6.3	42 ± 6.0	23 ± 6.0	12.1 ± 0.77	83 ± 20.2	2.5 ± 0.47	6.4 ± 0.42	1.3 ± 0.26	5.9 ± 0.78
Castelluccio di Norcia	310 ± 51	22 ± 4.7	46 ± 3.8	40 ± 3.8	12.7 ± 0.91	64 ± 8.0	2.9 ± 0.22	4.1 ± 0.29	1.6 ± 0.22	5.0 ± 1.29
Eston	320 ± 31	15 ± 3.5	41 ± 4.6	32 ± 3.6	12.8 ± 0.60	66 ± 13.2	2.1 ± 0.23	4.6 ± 0.22	1.6 ± 0.22	6.7 ± 0.58
Laird	370 ± 39	19 ± 4.9	41 ± 5.8	24 ± 5.8	11.4 ± 0.53	79 ± 10.2	2.3 ± 0.31	5.9 ± 0.30	1.5 ± 0.32	6.4 ± 0.47
Mean	330	16	39	31	12.2	64	2.3	4.8	1.6	6.3
P value	0.0005	<0.0001	<0.0001	0.0014	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
HSD ($P \leq 0.05$)	64	7.4	7.3	15.1	1.23	19.1	0.41	0.44	0.41	1.44

Table 6.Genetic parameters of the 15 lentil accessions according to each ISSR primer.

Primer	Number of amplified loci	Number of polymorphic loci	Percentage of polymorphic loci
P1	24	21	87.5
P2	20	15	75.0
P3	17	6	35.3
P4	23	10	43.5
P5	17	10	58.8
P6	10	8	80.0
Total	113	72	63.7

Table 7.Intra-population variability for each lentil agro-ecotype.

Population	Observed number of alleles	Shannon's information index	Gene diversity within population	Percentage of polymorphic loci
Bisacquino	1.062	0.033	0.022	6.2
Linosa	1.044	0.017	0.011	4.4
Maniace	1.195	0.096	0.064	19.5
Modica	1.000	0.000	0.000	0.0
Mussomeli	1.053	0.026	0.018	5.3
Nera di Enna	1.097	0.053	0.036	9.7
Nissoria	1.159	0.088	0.059	15.9
Pachino	1.186	0.097	0.065	18.6
Palazzolo	1.053	0.020	0.012	5.3
Pantelleria	1.150	0.086	0.059	15.0
Ustica	1.159	0.076	0.050	15.9
Villalba	1.204	0.106	0.071	20.4
Castelluccio di Norcia	1.080	0.050	0.035	8.0

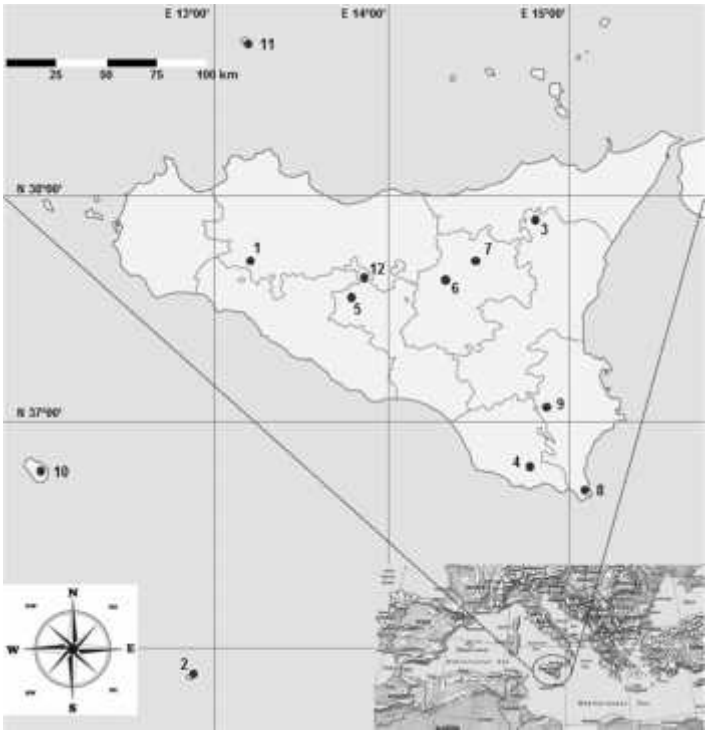

Fig. 1.Geographic locations of the 12 agro-ecotypes collected in Sicily, Italy. 1 = Bisacquino; 2 = Linosa; 3 = Maniace; 4 = Modica; 5 = Mussomeli; 6 = Nera di Enna; 7 = Nissoria; 8 = Pachino; 9 = Palazzolo; 10 = Pantelleria; 11 = Ustica; 12 = Villalba.

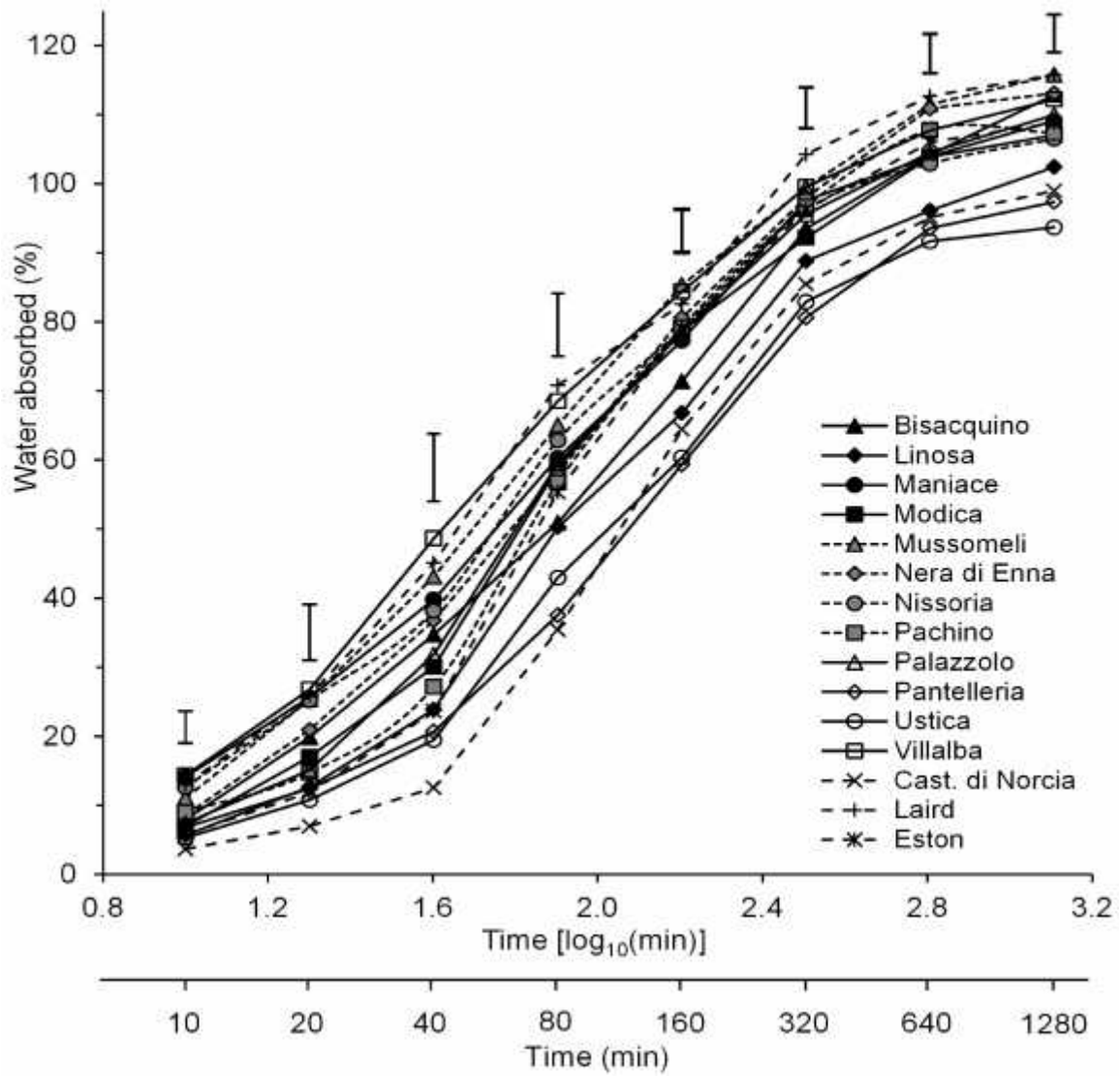

Fig. 2. Water absorbed (as a percentage of the initial seed weight) by seeds of the lentil accessions studied after 10, 20, 40, 80, 160, 320, 640, and 1280 min of soaking. Vertical segments represent HSD (P = 0.05).

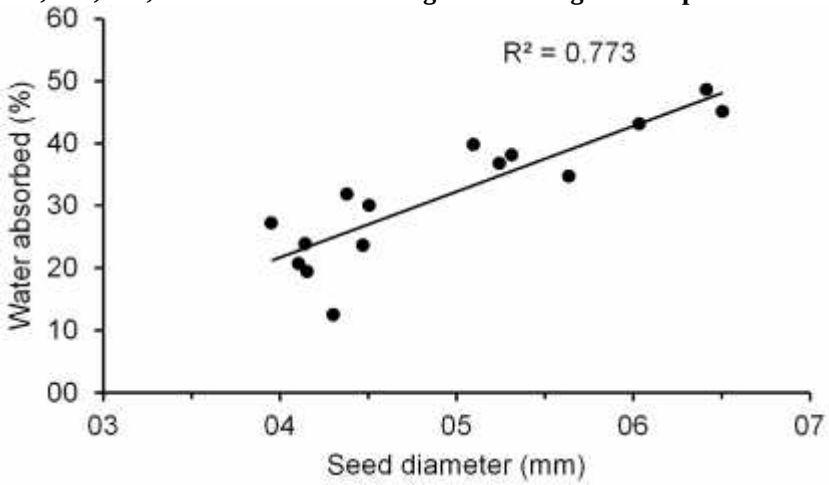

Fig. 3.Relation between mean seed diameter of the lentil accessions and water absorption (as a percentage of the initial seed weight).

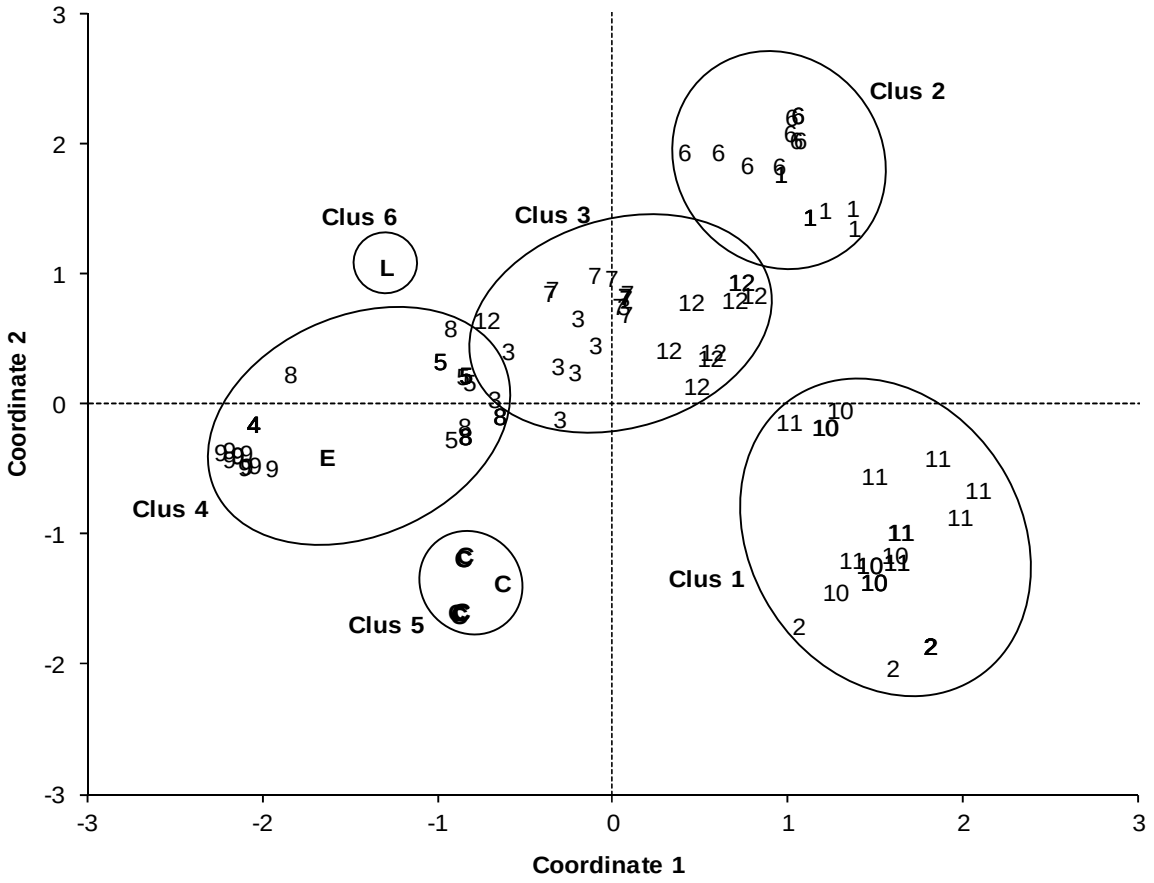

Fig. 4. Principal coordinates biplot of the lentil accessions analysed. Dotted ovals indicate the clusters identified by cluster analysis. 1 = Bisacquino; 2 = Linosa; 3 = Maniace; 4 = Modica; 5 = Mussomeli; 6 = Nera di Enna; 7 = Nissoria; 8 = Pachino; 9 = Palazzolo; 10 = Pantelleria; 11 = Ustica; 12 = Villalba; C= Castelluccio di Norcia.

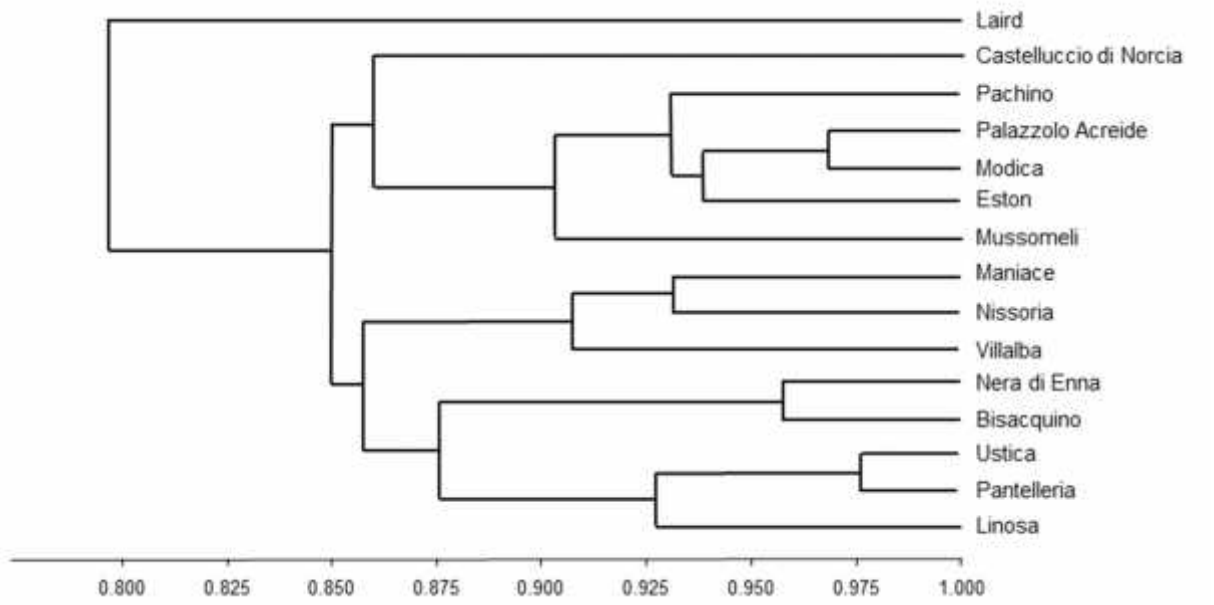

Fig. 5.Dendrogram clustering of 15 lentil accessions based on Nei's genetic distances and calculated using ISSR data.

DISCUSSION

Despite the relatively small size of Sicily, a large genetic and phenotypic variability in the Sicilian germplasm of *L. culinaris* was detected in the present research. The great climatic and pedological variability across the island may have driven the intraspecific differentiation of this species. Moreover, the long tradition of lentil cultivation in Sicily has certainly allowed for the evolution of many agro-ecotypes adapted to restricted areas.

Most of the Sicilian lentil agro-ecotypes had grain yields significantly higher than those of the two Canadian varieties in this study (Eston and Laird). This result agrees with data reported by Avola*et al.* (2001), who found, in a field experiment performed in Sicily, that lentil landraces were more productive than foreign varieties. Also, Zaccardelli *et al.* (2012) found that lentil landraces had better agronomic performance than varieties but only under limiting growing conditions. In the present study, within the Sicilian germplasm the most productive accessions were the earliest ones. In Mediterranean environments, in which drought stress often occurs late in the growing season (as it did in this experiment), early flowering certainly represents an advantage because it reduces the risk of water stress during seed development, thus representing an effective mechanism of escape from drought, as reported by Bacchi *et al.* (2010).

The average percentage of N derived from symbiotic N_2 fixation was 395 g fixed N/kg plant total N in above ground parts, with no significant differences among the accessions. This value is rather low in comparison with those generally reported in the literature for lentil (Kurdali *et al.* 1997; Pilbeam *et al.* 1997; Schmidtke *et al.* 2004; Ruisi *et al.* 2012). It is probable that, in our experiment, the climatic conditions during the study period were favourable to soil organic matter mineralization, thus increasing the availability of inorganic N and, as a consequence, reducing the reliance of the crop on N_2 fixation (Giambalvo *et al.* 2012). The accessions under study differed significantly in the total amount of N fixed per unit area. This trait was positively related to both grain yield and total N content, in accordance with data reported by Salvagiotti *et al.* (2008) on soybean (*Glycine max* L.).

No significant differences were found among the accessions for grain protein content (268 g/kg on average). The values observed for this trait are in line with those reported for the species by many other authors (Erskine *et al.* 1985; Tabera *et al.* 1995; Whitehead *et al.* 2000). As for grain lipid content, a mean value of 9.1 g/kg was recorded, with significant differences among the accessions (range: 5.3 g/kg–11.7 g/kg). These values are in line with those reported by Bhattacharaya *et al.* (2005) and Joshi *et al.* (2010) but are markedly lower than those reported by Jood *et al.* (1998; range 18.3–28.3 g/kg) and by Grela and Gunther (1995) and Iqbal *et al.* (2006; who both reported an average value of 32 g/kg).

Lentil accessions differed markedly on seed hydration capacity. This property is often inversely related to cooking time (Deshpande and Cheryan, 1986; Bishnoi and Khetarpaul, 1993), which is one of the most important properties for determining the quality of pulses used for human consumption (Moscoso *et al.* 1984). In our study, inter-accession differences in seed hydration capacity were significant from the beginning of soaking and were closely and positively related to seed dimensional traits (seed diameter and seed area) and seed weight. Similarly, Williams*et al.* (1983) reported a strong positive correlation between seed size and hydration capacity in chickpea (*Cicerarietinum* L.); also, Wang *et al.* (2003) found that the heaviest seeds in pea (*Pisumsativum* L.) had the highest water hydration capacity. In contrast, Hsu *et al.* (1983) found an inverse relationship between the water absorption rate and seed size in soybean. Inter-accession diversity in the water absorption rate can depend on differences in the cell wall structure of the seed coat (Bhatty, 1995) and/or on the composition of the seed and the compactness of the cells in the seed (Muller, 1967). Moreover, the observed relationship between seed size and hydration capacity can be explained by a higher permeability of the seed coat in large seed types than in small ones as a consequence of the differential presence of microcracks on the seed surface induced by threshing (which is higher in large seed types). Moore (1972) reported that microscopic seed damage increases with an increase in seed sphericity, which, in lentil, is lower in large seed types (as is easily observable from variations in the seed diameter:seed thickness ratio). Obviously, these aspects of cooking quality of the Sicilian lentil germplasm will need further investigation.

Sicilian lentil agro-ecotypes showed significant inter-accession differences for all of the recorded pheno-morphological traits. In contrast, the intra-population variability was rather low and, in most cases, similar to that of the two Canadian varieties. Also analysis on genetic data highlighted that most of the diversity among Sicilian lentil agro-ecotypes occurred among rather than withinaccessions. This result is in agreement with the findings of other authors who have worked on lentil using ISSR markers (Sonnante and Pignone 2007; Scippa *et al.* 2008). The total gene diversity observed in the present study within the Sicilian lentil germplasm was higher than the diversity observed by Ferguson *et al.* (1998) on 100 landrace accessions of cultivated lentil from 10 countries. This difference could be explained by the fact that they used RAPD (randomly amplified polymorphic DNA) markers which are described to have lower sensitivity than other molecular markers, such as AFLPs (amplified fragment length polymorphism) and ISSRs, in

detecting genetic variation at intraspecific level in lentil (Sharma *et al.* 1996; Sonnante and Pignone 2001). Fikiru *et al.* (2007), using four ISSR primers to analyse a collection of seven lentil populations coming from seven different administrative regions (AR) of Ethiopia, observed a degree of total gene diversity similar to that observed in the present research (0.175 *vs* 0.151) but, in their study, the degree of within-population diversity was more than two times higher than the H_S observed in the present research (0.095 *vs* 0.039). It should however be noted that in the work of Fikiru *et al.*, each population was represented by ten accessions from ten sites within each AR and that the authors worked on 15-plant bulk for each accession; thus, they did not really estimate the degree of genetic diversity within the lentil accessions.

In the present study, the highest degree of genetic diversity was observed in some agro-ecotypes from the eastern and central parts of Sicily (Maniace and Pachino, and Villalba, respectively). The relatively high degree of genetic (and phenotypic) variability found in the Villalba accession is in agreement with the results of Sonnante and Pignone (2007) and Zaccardelli *et al.* (2012). We think that it could be a sign of a genetic contamination as a consequence of the introduction in the area in which this agro-ecotype is traditionally grown of allochthonous material; in fact, the great commercial success and problems of harvestability of the local variety could have prompted the farmers to introduce material improved for the mechanization of the harvesting process. Moreover, the noticeable genetic variability of the Villalba accession could be the result of the long history of cultivation of this agro-ecotype and its great diffusion in the origin area (about one third of the lentil produced in Italy during the first half of the last century came from the Villalba area); it is obvious that the longer the time of cultivation and the wider the cultivation, the higher the chances of genetic differentiation. The lowest degree of genetic variability was found in the agro-ecotypes from Modica and the small Sicilian island of Linosa. For the former, the low genetic diversity could be attributed to the occurrence of genetic erosion as a consequence of the very small size of the cultivated area. For the latter, in accordance with Sonnante and Pignone (2007), it is likely that the low genetic diversity observed is a consequence of a bottleneck effect with the (relatively recent) introduction of the species on the island.

The analysis of genetic distance using ISSR markers revealed that, on the whole, accessions from similar areas had a tendency to stay together. For instance, accessions from the south-eastern part of Sicily as well as those from central Sicily appeared to retain a high degree of genetic similarity. Accessions from the small islands surrounding Sicily (Ustica, Pantelleria, and Linosa, all of volcanic origin and characterized by similar agro-climatic environments) were grouped in the same cluster. These three agro-ecotypes, which also showed a good degree of similarity in terms of phenotypic and qualitative traits, probably had a common origin, as reported by Sonnante and Pignone (2001, 2007), because the three small islands have been involved in frequent (and relatively recent) human migration (Maslah 2012). Cultivar Eston showed a certain degree of genetic similarity with some Sicilian accessions mainly coming from south-eastern Sicily. This result could be a sign of a genetic contamination as a consequence of the introduction in this area of cv Eston.

In conclusion, the data presented here provide a precise picture of the degree of diversity present in the Sicilian germplasm of the lentil species, including both the most renowned and many minor agro-ecotypes. Although the germplasm came from a relatively small area, the results of this study document large genetic and phenotypic variability among Sicilian agro-ecotypes of lentil and, moreover, a generally low degree of intra-accession diversity. Many Sicilian agro-ecotypes showed agronomic and/or qualitative characteristics of interest in many cases superior to those of the control varieties, and this fact certainly represents an essential prerequisite for their future economic valorization.

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