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RESEARCH ARTICLE

Characterization of the lentil landrace Santo Stefano di Sessanio from Abruzzo, Italy

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Abstract In the world lentil is grown on more than 3 million hectares and is one of the most important, low-cost, food source of protein. In Italy lentil has been cultivated since ancient times, but in the last decades its cultivation has been confined to marginal areas, small islands and hilly, mountainous areas of central and southern Italy. Local varieties are still common and are often greatly appreciated for their taste and cooking qualities. Several accessions from the Santo Stefano di Sessanio area, Abruzzo Region, were collected and phenotypically and genotypically characterized in order to look for the existing variability within and between populations. Image analysis of seeds was also used. Populations grown in Santo Stefano di Sessanio and in the neighbouring area basically share most of their characteristics. However, some of the accessions anonymously gathered from the local market were shown to be different from those collected from farmers. The paper reports and discusses how this local product needs be characterized and promoted in order to avoid fraud that could negatively affect the local economy and put valuable, adapted, genetic resources at risk of erosion.

Keywords Cluster, discriminant and principal components analysis · Image analysis · *Lens culinaris* Medik. · Phenotypic and genotypic characterization

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Introduction

Lentil (*Lens culinaris* Medikus) is one of the oldest and most appreciated grain legumes of the Old World. It is an annual, herbaceous, diploid ($2n = 2x = 14$), self-pollinating species (Sharma et al. 1995). Its centre of origin is the Near East, where it was domesticated earlier than the 7000 BC reported by Zohary (1972) (Sonnante et al. 2009) and, by the end of the Bronze Age, it was common in the Nile valley, the whole Mediterranean Basin, and extended to Central Europe and the Indian Subcontinent (Cubero 1981; 1984). According to seed size cultivated lentil comprises the small seeded *L. culinaris* Medik. ssp. *microsperma* (Baumg.) Barul. and the large seeded *L. culinaris* Medik. ssp. *macrosperma* (Baumg.) Barul. (Barulina 1930). Its importance in the human diet is basically due to the high seed protein content (about 25%), constituting an important protein source in many rural communities (Zohary 1995). Worldwide, lentil is

grown on a total area of 3.3 million hectares (FAOSTAT 2008). Presently it is widely cultivated throughout the Indian Subcontinent, the Middle East (Turkey in particular), North Africa, South Europe, North and South America, as well as in Australia and West Asia (Ford and Taylor 2003). According to recent FAO statistics, the world production increased from 850 thousand tonnes in 1961 to 2.8 million tonnes in 2008. In Europe lentil is one of the three most important pulse crops, following pea (*Pisum sativum* L.) and bean (*Phaseolus* spp.; Horneburg and Becker 2008).

In Italy lentil has been cultivated since ancient times and until few decades ago it was one of the cheapest sources of dietary protein in rural and urban communities. In recent times the cultivated area has shrunk to include only marginal and mountainous areas of Central and Southern Italy and some small islands (Piergiovanni 2000). Local landraces, well adapted to harsh, local conditions, are almost the only source of seeds. Italian lentils are greatly appreciated by consumers for their taste and cooking qualities. Some of them are considered a typical example of a "niche product", marketed at higher prices than those imported that make their cultivation profitable in marginal areas. In Central Italy, Castelluccio di Norcia, in Umbria Region, and Santo Stefano di Sessanio, in Abruzzo Region, are two typical examples of local landraces; both are grown at altitudes well above 1.000 m a.s.l., the former on over 600 ha, while the latter on about 50 ha. Castelluccio di Norcia was the first lentil landrace to receive the PGI (Protected Geographic Identification) from the EU and from this recognition the number of hectares steadily increased, so that local communities of farmers consider it as an essential step towards the safeguarding of its survival.

Most of these landraces are populations hosting significant variability based on a multitude of pure lines, as confirmed by morpho-physiological characters and by modern genetic characterization techniques. Genetic characterization of local varieties/landraces in the genus *Lens* has been assessed through several methods. Erskine et al. (1989) characterized accessions from different countries through quantitative traits such as time to maturity, lowest pod height and 100-seed weight. Ahmad et al. (1997) employed morphological characters to assess phylogenetic relationships in *Lens* species and their hybrids. Allozyme polymorphism was used by Ferguson and Robertson (1996) to assess genetic diversity. Molecular markers such as RFLP (Havey and Muehlbauer 1989), RAPD (Sharma et al. 1995; Ford et al. 1997), AFLP (Sharma et al. 1996) and ISSR (Sonnante and Pignone 2007) have been widely used to study the diversity and phylogeny within and among *Lens* taxa.

More recently image analysis has been placed alongside phenotypic and genotypic characterization, as a complementary tool to distinguish landraces/local varieties belonging to the same species. Electronic tools have provided the possibility of replacing human visual assessment, and this has spurred a wealth of research in this area (Bacchetta et al. 2008). Image analysis is being used for the inspection of various agricultural products such as fruit (Schatzki et al. 1997), vegetables (Zhang et al. 2003) and cereals (Liao et al. 1994; Sainis et al. 2006). Measurements of the morphological and textural characteristics of seeds of many legume species, including lentil, have also been reported (Shahin and Symons 2003a; Venora et al. 2007). This is particularly important in species where seeds are the primary product and, hence, could be the object of frauds when the market price is high.

The objective of the present research was to characterize the Santo Stefano di Sessanio lentil landrace using three methods: morpho-agronomic traits, AFLP markers and image analysis of seeds.

Materials and methods

Plant material

In 2004 twenty-four seed samples of lentils were collected from local farmers in Santo Stefano di Sessanio and neighbouring villages in the region Abruzzo, central Italy. Four commercial seed samples were purchased on the market and used as controls: Eston, a Canadian variety, Kislik, a Turkish variety, Castelluccio di Norcia and Altipiano del Rascino, two landraces collected from other areas of central Italy, the former from Umbria, the latter from Latium regions (Table 1).

Field evaluation as spaced plants

The evaluation was carried out in 2005 in Corfinio (42° 12′ N, 13° 84′ E, 330 m a.s.l.). Experimental

Table 1 Lentil accessions and geographic information of the collecting sites

No.	Code	Farm/Landrace/Variety	Collection site/origin	Geographic information		
				Latitude (E)	Longitude (N)	Altitude (m a.s.l.)
1	Eston	ESTON	CANADA			
2	Kislik	KISLIK	TURKEY			
3	Castel	Barcaroli Livio	Castelluccio di N.	42.8292°	13.2082°	1430
4	Rascino	De Michelis A. Maria	Altip. del Rascino	42.2648°	13.1255°	980
5	SS CVE	Ciarrocca Ventidio	S. Stefano di Sessanio	42.3442°	13.6431°	1240
6	SS CRO	Ciarrocca Rosa				
7	SS DPI	D'Alessandro Pio				
8	SS UDO	Ursini Domenico				
9	SS RLA	Rusciolelli Laura				
10	SS CRM	Ciarrocca Remo				
11	SS CLU	Capoverde Luca				
12	SS LAN	Leone Annina				
13	SS DAN	D'Aloisio Antonio				
14	SS CDO	Cardelli Domenico				
15	SS CGI	Chiarelli Giovanni				
16	SS SMA	Setacci Marcella				
17	SS CGA	Costantini Gabriella				
19	SS Market	Local market				
20	SS FCA	Fulgenzi Carmelo				
18	CC Market	Local market	Castelvecchio Calvisio	42.3106°	13.6885°	1040
21	CC MPI	Marsilia Pina				
22	BA MGI	Matergia Giovanni	Barisciano	42.3255°	13.5935°	960
23	CA CDO	Ciccone Domenico	Calascio	42.3266°	13.6981°	1240
24	CA GAL	Gentile Alessandra				
25	CA AMA	Antonacci Mario				
26	CM GMA	Giuliani Massimo	Castel del Monte	42.3645°	13.7258°	1320
27	CM PG1	Petronio Giulio				
28	RM ZAN	Zannetti Giambattista	Rocca di Mezzo	42.2058°	13.5203°	1280

units of 20 plants (two rows of 10 plants) were arranged in a randomized block design with four replications, in order to have a total of 80 plants per entry. On 4 April 2005, 2–3 seeds per planting post were sown at a distance of 60 × 30 cm, thinned to one seedling after emergence. Hand weeding was carried out twice during the growing season. The recorded characters are listed in Table 2.

Field evaluation in dense stands

The evaluation was carried out in 2005 in Calascio (42° 32′ N, 13° 70′ E, 1,230 m a.s.l.), a site representative of the lentil growing area. Experimental units of 2 m^2 were arranged in a randomized block design with four replications and were sown on 9 May 2005 at 13 g m^{-2}, a typical seeding rate in the area. The characters recorded in dense stand are listed in Table 2.

AFLP analysis

Sixty plants per accession were grown in a glasshouse. DNA was extracted by bulking the leaflets of 30 plants, thus having at disposal two samples (A and B) per accession. DNA extraction was carried out using the Plant miniprep kit (Sigma) and quantified by the DU650 Spectrophotometer (Beckman). Total DNA (500 ng) was restricted-ligated and pre-amplified according to the protocol of Vos et al. (1995). AFLP

Table 2 Morpho-agronomic traits of lentil accessions studied

	Morphological traits
1	Time to flowering (days from sowing)
2	Growth habit (1 = erect; 3 = semi erect; 5 = horizontal)[a]
3	Leaf shape (1 = elliptic; 2 = ovate; 3 = rectangular)[a]
4	Plant height (cm)
5	Plant pigmentation at the base of the stem (1 = absent; 9 = present)[a]
6	Time to maturity (days from sowing)
7	Seed yield (g/plant)
8	Pod shape (1 = truncate; 2 = truncate to pointed; 3 = pointed)[a]
9	Number of seed per pod
	Agronomic traits
10	Ground cover index (1 = min; 9 = max)
11	Average plant height (cm) (ten random measurements per plot)
12	Time to flowering (no. of days from sowing)
13	Flower colour (violet stripes of standard) (1 = absent; 9 = present)
14	Score on diseases (1 = no symptoms, 5 all plants attacked)
15	Plant pigmentation at the base of the stem (1 = absent; 9 = present)[a]
16	Maturity index 1 (1 = green pods; 9 = brown, mature pods) in mid of June
17	Maturity index 2 (1 = green pods; 9 = brown, mature pods) at the end of June
18	Pod dehiscence (1 = indehiscent; 4 = completely dehiscent)
19	Seed yield (g/plot)

[a] UPOV 2003

amplifications were performed in a 20-µl reaction mix containing 1/100 of the pre-amplified DNA, 50 ng fluorescent-labelled *Eco*RI + 3 oligonucleotide primer, 50 ng of unlabeled *Mse*I primer (Table 3), 2 µl PCR buffer (Invitrogen), 0.2 mM dNTPs, 0.4 U *Taq* polymerase (Invitrogen). After PCR, 8 µl of loading buffer (98% formamide, 2% dextran blue, 0.25 mM EDTA) were added to each tube. Samples were denatured at 95°C for 10 min and then immediately placed in ice water and run on an ABI377 sequencer (Applied biosystems). DNA analysis was based on a total of 8 primer combinations.

Image analysis

The images of a random sample of seeds per accession were acquired using a flatbed scanner (ScanMaker 9800 XL, Microtek Denver, CO) and randomly assigned to a training set and a test set. Size, shape and colour components of seed images were analyzed by the image analysis software KS-400 V3.0 (Carl Zeiss, Vision, Oberkochem, Germany). Before image acquisition, the scanner was calibrated with a Q60 Kodak colour chart (scanned at 200 dpi) for colour matching, following the protocol of Shahin and Symons (2003b). For each seed set only intact seeds were used, singularly placed on the flatbed scanner in order to obtain a 512 × 512 pixel digital image at a resolution of 200 dpi. A 10 cm high cardboard box was used as the scanner cover, with non-reflecting black paper to eliminate any spurious or reflecting light.

The analysis is based on segmentation, a process that reduces images to information by dividing them into regions corresponding to structural units undergoing a foreground/background separation (Venora et al. 2007). Seed images were analyzed for size (diameter, area, perimeter, shape factor, roundness) and colour (RGB—Red, Green and Blue channels, and HLS—Hue, Light and Saturation channels, models, measured as mean grey levels). The shape factor (SF) was calculated for each object, according to the formula:

$$SF = 4\pi\frac{A}{P^2}$$

where A is the area and P the perimeter of the measured object.

Table 3 AFLP primer combination used

Name	Sequence		
Adaptor			
Eco RI	5′-CTCGTAGACTGCGTACC-3′		
	3′-CTGACGCATGGTTAA-5′		
Mse I	5′-GACGATGAGTCCTGAG-3′		
	3′-TACTCAGGACTCAT-5′		
AFLP primer combinations and selective bases			
E-CCA/M-ACT	E-CCA/M-ACG	E-CCA/M-ATC	E-CAG/M-ACT
E-CAG/M-AGG	E-CAG/M-ATC	E-CAC/M-ACT	E-CAC/M-AGG

Statistical analysis

Univariate analysis of variance was performed for morphological and agronomic data. Mean data of 19 variables from both field experiments were standardized and used into multivariate procedure, UPGMA clustering based on a Euclidean distance matrix, and in a Principal Component Analysis (PCA).

Molecular data were arranged in a data matrix. AFLP fragments were scored as 1 or 0 for presence or absence of the band, respectively, using the Genescan software (Applied biosystem). Genetic similarity (GS) was estimated using the similarity coefficient of Jaccard (1908): $GS_{(ij)} = a/(a + b + c)$, where $GS_{(ij)}$ is the genetic similarity between individual i and j; a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. Genetic similarities among samples were clustered by the unweighted pair-group method of arithmetic average (UPGMA; Sneath and Sokal 1973). The goodness-of-fit of cluster analysis was validated through bootstrap analysis (Felsenstein 1985), cophenetic procedure (Rohlf and Sokal 1981), and Principal Coordinate Analysis (Gower 1966). The correlation between agronomic traits and genetic data was tested by the test of Mantel (1967) after a transformation of the genetic similarity matrix into a genetic distance matrix ($GD = 1 - GS$).

The variability among populations, measured in terms of seed colour components (in RGB and HLS models) and seed morphology (shape and size), was analysed in a training set and in a test set; the former set was used to develop the classifiers using Stepwise Linear Discriminant Analysis, the latter to validate the model and obtain the percentage of correct re-classification.

The ANOVA procedures were computed with the SAS statistical package (SAS 2008), linear discriminant analysis with the SPSS statistical package release 15 (SPSS 2006), Euclidean distances, similarity estimates, cluster analysis, Mantel test and Principal Coordinate Analysis were carried out by NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) software package version 2 (Rohlf 1993), bootstrap analysis was performed by WinBoot software (Yap and Nelson 1996).

Results

Morphological and agronomic characterisation

In the spaced plant trial, differences in leaf shape, plant height, maturity time, interval of days between flowering and maturity time, seed yield and pod shape were not statistically significant. Significant differences among entries were instead found for growth habit, anthocyanin pigmentation at the base of the plant in the early stage of plant growth, flowering time and number of seeds per pod (Table 4).

Eston variety showed a basically erect growth habit while, at the other extreme, two landraces from Calascio (CAGAL and CACDO) were semi-erect (3.0 and 2.9, respectively; $P < 0.05$); all other entries had intermediate values. As for anthocyanin pigmentation at the base of the plant, the two control varieties, Eston and Kislik, ranked at both extremes: the former with a pigmentation significantly higher than the latter (6.6 vs. 1.5, $P < 0.05$). Almost all entries (mean scores ranging from 2.7 to 1.3) ranked

Table 4 Mean and *P* level of significance of several morphological and agronomic traits recorded in 28 lentil entries

Morphological traits (spaced plants)	Mean	*P*	Agronomic traits (dense stand)	Mean	*P*
Time to flowering	21.61	***	Ground cover index	4.35	**
Growth habit	2.63	**	Crop height	16.36	***
Leaf shape	1.44	NS	Time to flowering	17.76	***
Plant height	20.82	NS	Flower colour	5.58	NS
Anthocyanin pigmentation	2.81	***	Anthocyanin pigmentation	4.58	**
Time to maturity	78.06	NS	Maturity index (1)	6.18	**
Seed yield/plant	1.27	NS	Maturity index (2)	6.69	**
Pod shape	1.01	NS	Pod dehiscence	2.09	**
Number of seed per pod	1.37	*	Seed yield/plot	44.94	***

NS not significant

*, **, *** Significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively

between these two extremes and did not significantly differ from Kislik), with the exception of SSMarket (6.1, not differing from Eston) and of CCMarket (7.6, significantly higher than Eston, $P < 0.05$). CCMarket and SSMarket showed the same behaviour also in terms of flowering time, being among the latest flowering populations in the experiment, similar to Eston but significantly higher than Kislik, the earliest entry ($P < 0.05$). Interestingly, they showed the lowest number of seeds per pod, ranking together at the bottom of the list of all the lentil entries.

Seedling establishment in dense stand was normal for all entries but at 3 weeks from sowing many local populations showed a significantly higher rate of soil cover than the two varieties and the two landraces used as a control ($P < 0.05$). Mean crop height at full flowering time was generally low (16.3 cm) but in line with the average crops of the growing area, ranging from 14.7 cm for Kislik and CCMarket to 18.0 cm for SSLAN. Plant height of most landraces from Santo Stefano di Sessanio and surrounding villages were generally higher than the controls ($P < 0.05$) although local landraces were confirmed to be early flowering types. In dense stands the two control varieties were the latest to flower, Rascino performed in a similar way, while Castelluccio was amongst the earliest entries.

The anthocyanin pigmentation at the base of plants grown in dense stands confirmed the results found for spaced plants: Eston and Kislik were at both extremes, the former with a pigmentation score significantly higher than the latter (9 vs. 1, $P < 0.05$). SSMarket and CCMarket, the two populations coming from the local market had a mean scores of 9, which was similar to Eston but higher than all the other entries. The score of Rascino was similar to that of Kislik ($P < 0.05$).

Seed yields were very different among entries (from 15.7 to 75.3 g of seeds m^{-2}). For this trait adaptation to the harsh environmental conditions of the cultivation area was confirmed to be a key factor: the yield of Castelluccio (43.1) was within the average of the experiment, while the yields of the other three controls, Kislik (15.7), Eston (27.2) and Rascino (23.7), were the lowest. As many as 11 local landraces were able to yield over 50 g of seeds m^{-2}. Interestingly, CCMarket and SSMarket were characterized by very low yields (25.8 and 15.8, respectively) suggesting that these two samples might not be local. In addition, it is striking that the yields of the only two entries from Castelvecchio Calvisio village, namely CCMPI from a farmer and SSMarket, ranked at the extremes of the list (75.3 and 15.8, respectively).

The multivariate PCA and UPGMA clustering analysis, as performed on morphological and agronomic data, generated the same results. Eigenvalues of the first three principal components were greater than 1 and accounted for 65.8% of the total variation (Table 5). The first component, accounting for 33% of the total variation, was correlated with growth habit, flowering time, plant height, seed yield and maturity index at the end of June. The second component, accounting for 22% of the total variation, was correlated with flowering time (from the trial as spaced plants), pod shape and maturity index

Table 5 Principal Component Analysis based on morphological and agronomic traits of 28 lentil entries, and significant loadings (in bold) of the first three principal components with the original variables

	PC1	PC2	PC3
Eigenvalue	6.51379	4.36673	2.28692
Percent of total variation	32.6	21.8	11.4
Cumulative	32.6	54.4	65.8
Seed yield (dense stand)	**0.7530**	−0.3104	−0.3955
Plant height	**0.7445**	−0.0611	0.3706
Maturity index (2)	**0.7353**	−0.2173	0.0972
Seed yield (spaced plants)	**0.6933**	0.1031	0.2003
Growth habit	**0.6480**	0.2470	0.1511
Flow. time in dense stand	**−0.7172**	0.5212	−0.1240
Flow. time at spaced plant	0.0489	**−0.8002**	0.2091
Maturity index (1)	0.3714	**−0.7887**	0.1975
Pod shape	−0.1939	**−0.7029**	0.0728
Anthocyanin pigmentation	0.5232	−0.2461	**0.7333**

recorded in mid June, while the third component, accounting for 11%, was correlated with anthocyanin pigmentation. Figure 1 reports the centroids of the 28 entries according to the first two components. With the exception of the two samples collected from the market, the accessions from Santo Stefano di Sessanio area were grouped all together and comprised Castelluccio di Norcia, one of the control populations. Eston, Rascino and Kislik were progressively plotted further one another and from the Santo Stefano di Sessanio group. The results of UGMA cluster analysis, as based on the Euclidean distance matrix, showed close agreement with those reported for the PCA (Fig. 2).

Molecular characterisation

The scoring of AFLP gels showed the presence of 698 fragments, 404 of these (57%) were polymorphic (Table 6), with an average of polymorphic bands per primer combinations ranging from 32 (CAG-ACT) to 85 (CAG-ATC) and an average fragment length ranging from 60 (CAG-ACT) to 489 bp (CCA-ACT).

The UPGMA procedure found a total of 8 ties giving rise to 8 dendrograms whose differences were

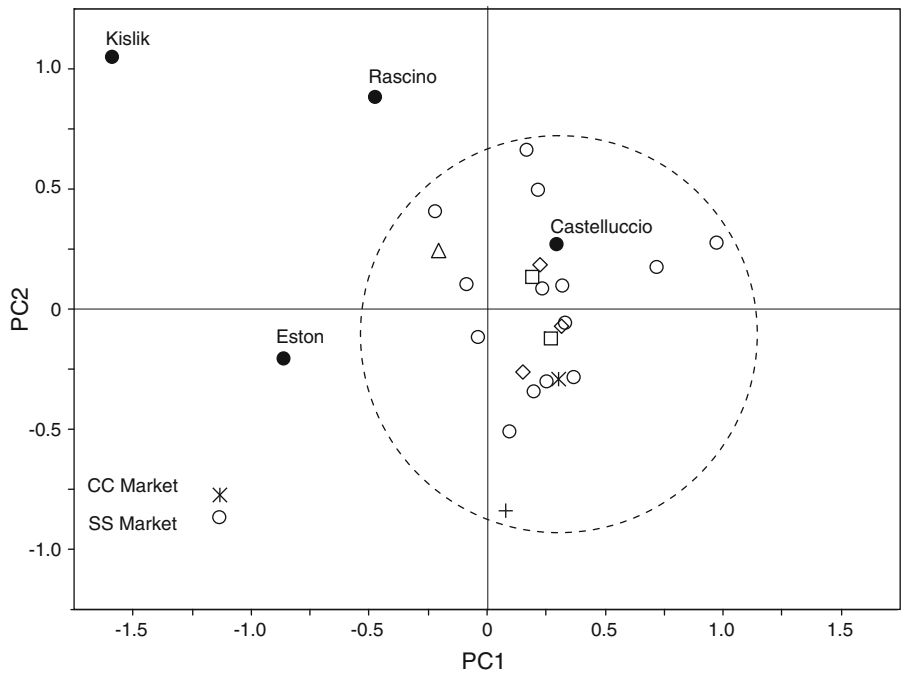

Fig. 1 Centroids of 24 lentil landraces from Abruzzo (*open symbols*), and 4 controls (*closed circles*): Kislik, Eston (*commercial varieties*), Castelluccio and Rascino (landraces from Central Italy), plotted according to the first two principal components obtained from morphological and agronomic traits. The symbols of the landraces differ according to the village of collection: (*open circle* Santo Stefano di Sessanio; *open diamond* Calascio; *open triangle* Rocca di Mezzo; *open square* Castel del Monte; *plus* Barisciano; *star* Castelvecchio Calvisio)

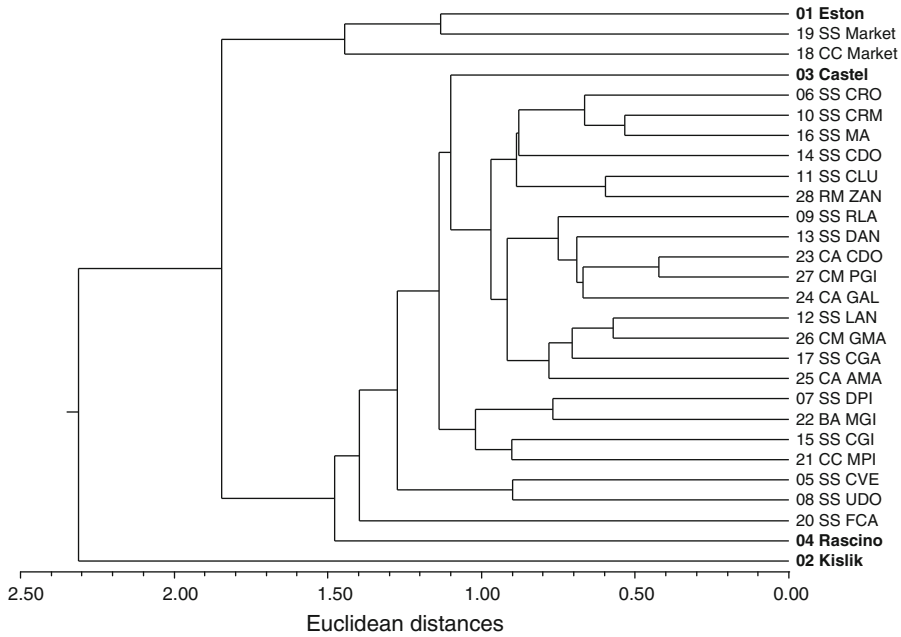

Fig. 2 UPGMA clustering of 28 entries of lentils according to Euclidean distances estimated from morphological and agronomic traits

Table 6 Primer combinations and level of AFLP polymorphisms

Primer combinations	Number of fragments	Polymorphic fragments		Fragment size (bp)
		No.	%	
E-CCA/*M*-ACT	99	61	61.61	60.4–489.2
E-CCA/*M*-AGG	83	50	60.24	71.6–444.7
E-CCA/*M*-ATC	104	64	61.53	65.8–468.0
E-CAG/*M*-ACT	67	32	47.76	60.0–430.8
E-CAG/*M*-AGG	55	34	61.81	61.7–464.0
E-CAG/*M*-ATC	137	85	62.04	71.2–436.5
E-CAC/*M*-ACT	82	43	52.44	66.1–461.9
E-CAC/*M*-AGG	71	35	49.29	66.8–394.1
Total	698	404	–	–
Mean	87.25	50	57.09	–

all of minor importance and due to slightly different order within the same cluster. The goodness-of-fit of the analysis was validated by the high and significant correlation coefficient between the similarity and cophenetic matrices ($r = 0.994$, Mantel $t = 8.859$, $P < 0.001$). Data were clustered into two main groups (100% of the bootstraps) at a similarity value of 0.67, grouping Rascino and Kislik from the rest of the populations (Fig. 3). Rascino and Kislik, two apparently distinct populations, shared a similarity score of 0.90. The largest cluster was, in turn, split into two groups at a similarity value of 0.77; the first group included Eston and the two populations purchased in the market of Santo Stefano di Sessanio and Calvecchio Calvisio, while the second, rather large group, comprised Castelluccio di Norcia and all landraces collected in Santo Stefano di Sessanio and surrounding areas. In the latter cluster Castelluccio was divided from all the others at a similarity index of 0.92. The iteration of the bootstrap procedure confirmed the goodness-of-fit of the above clusters. Interestingly, with the exception of the two entries

purchased on the market and sold as local landraces, all populations from Santo Stefano di Sessanio and surrounding areas clustered together. It is also remarkable that at these levels of similarities sometimes the two replicates of the same population (A and B) did not cluster together, but clustered with samples of other populations from the Santo Stefano di Sessanio area, indicating that accessions from the area of study are largely similar (Fig. 3).

A further statistical validation of these results is supported by Principal Coordinate Analysis, where the projections of the 56 samples plotted against the axes and representing the most significant eigenvectors (Fig. 4), show a similar trend as reported in the dendrogram of Fig. 3. The first three eigenvalues were able to explain as much as 69% of the total variation (39, 21 and 9%, respectively), each of them able to significantly explain more variation than expected under the broken stick model (8, 6 and 5%, respectively; Joliffe 1986).

The correlation between the matrices based on morpho-agronomic field data and AFLP markers was highly significant ($r = 0.79$, $P < 0.01$). By examining and comparing the dendrograms the only minor difference was in Rascino, which clustered much closer to Kislik according to the genetic data.

In conclusion, the two sets of data (1) are in good agreement and (2) with the exception of the two samples purchased in the market that clustered apart in both cases, the landraces from Santo Stefano di Sessanio area were always grouped together.

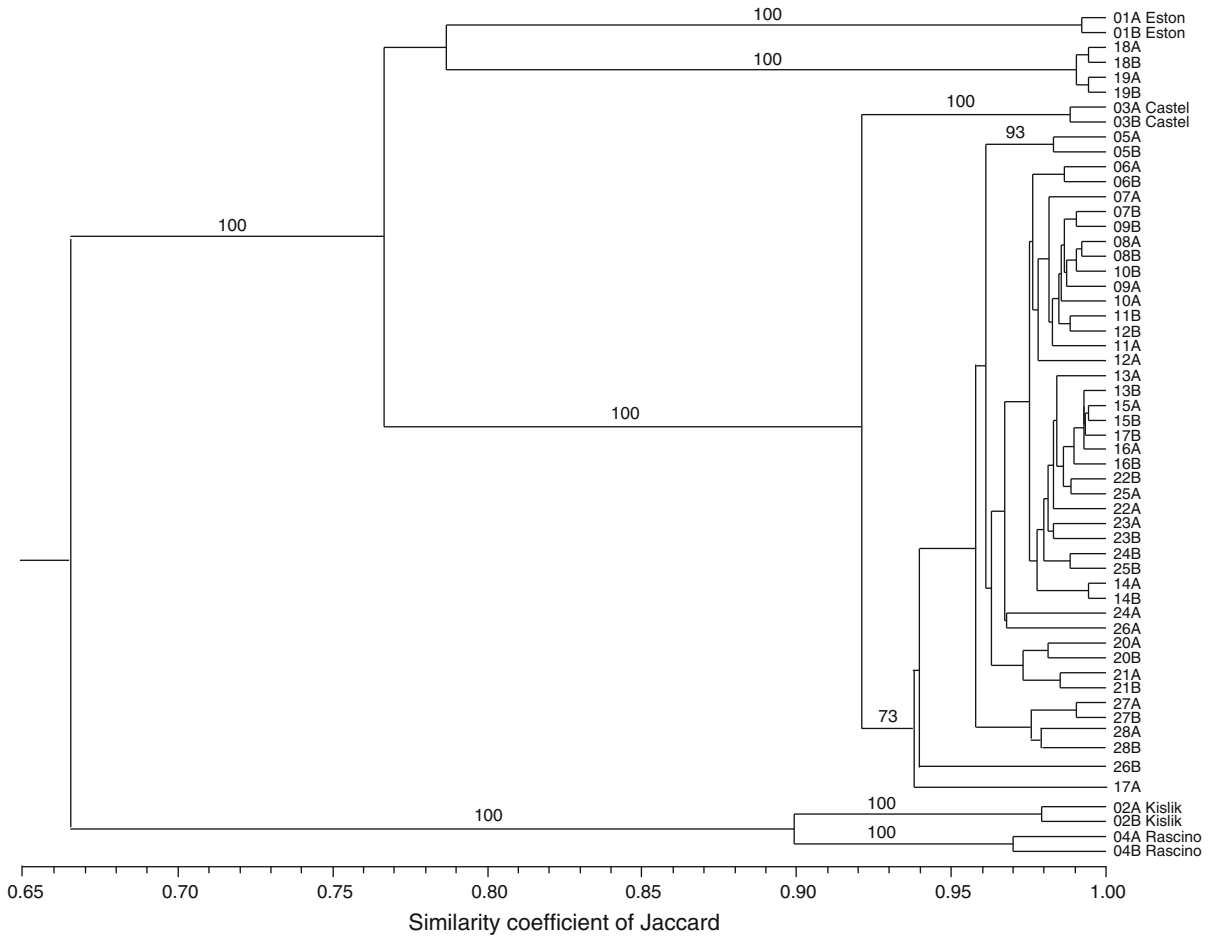

Fig. 3 UPGMA clustering of Jaccard genetic similarity coefficients of 28 lentil entries, as based on AFLP data. *A* and *B* are two samples from the same entry, each based on a bulk of 30 individuals

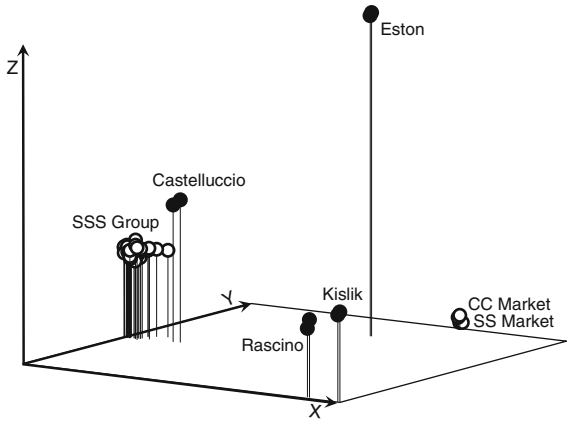

Fig. 4 Centroids of 24 lentil landraces from Abruzzo (*open symbols*), and 4 controls (*closed circles*): Kislik, Eston (*commercial varieties*), Castelluccio and Rascino (landraces from Central Italy), plotted according to the first three principal coordinates analysis to test the goodness-of-fit of cluster analysis as reported in Fig. 3

Image analysis

The data generated with image analysis were basically used in a discriminant procedure, therefore the statistical, biological significance and interpretation of differences among accessions in terms of RGB spectrum and other morphometric characters are not reported (see Tables in the Appendix). Discriminant analyses were carried out firstly on data from individual accessions (on all 28 entries) and later on accessions grouped according to the origin of collection (10 entries), but excluding SSMarket and CCMarket, the two accessions collected from the market and of doubtful origin according to the molecular and morphological results.

In the training set of the analysis carried out on the 28 accessions, a stepwise procedure included 16 out of 20 variables, with an overall correct classification of 88.5% (Table 7). The classification coefficients for each variable and accession (not reported) were used on the test set data and were able to achieve an overall correct re-classification of seeds equal to 87.6% (Table 7). The percentage of correctly classified seeds of the training and of the test set were highly and significantly correlated ($r = 0.951$, $df = 26$, $P < 0.001$).

The stepwise discriminant analysis on accessions grouped by the origin of collection included 16 out of 20 variables. The excluded variables were all those related to seed size (circumference equivalent, minimum and maximum diameter and their ratio). The nine discriminant functions were highly significant ($P < 0.001$), with the first three able to explain as much as 89.2% of the total variation. Figure 5 reports the centroids of the discriminant scores of the first two functions. Function 1, explaining as much as 62.4% of the total variation, was related to the mean and standard saturation, mean and standard green, while Function 2, explaining 16.7% of the total

Table 7 Number of seeds used in the training set and in the test set and percentage of correct classification according to discriminant analysis

Acc.	Code	Training set		Test set	
		No	%	No	%
1	Eston	523	99.4	271	98.9
2	Kislik	436	99.8	226	99.6
3	Castel	242	91.3	111	90.1
4	Rascino	339	87.6	173	90.8
5	SS CVE	352	85.2	169	82.8
6	SS CRO	169	79.3	87	82.8
7	SS DPI	175	77.7	96	84.4
8	SS UDO	204	88.7	101	92.1
9	SS RLA	409	94.4	226	90.7
10	SS CRM	420	75.5	207	75.9
11	SS CLU	1104	96.6	560	95.4
12	SS LAN	348	87.9	176	86.4
13	SS DAN	156	76.9	81	69.1
14	SS CDO	116	66.4	62	67.7
15	SS CGI	154	64.3	87	63.2
16	SS SMA	64	71.9	47	72.3
17	SS CGA	159	89.3	83	88.0
19	SS Market	549	82.9	276	80.8
20	SS FCA	37	97.3	22	100.0
18	CC Market	291	91.4	138	87.7
21	CC MPI	147	80.3	75	78.7
22	BA MGI	325	83.4	169	80.5
23	CA CDO	204	77.5	108	77.8
24	CA GAL	235	80.0	126	78.6
25	CA AMA	164	68.9	84	67.9
26	CM GMA	1037	94.0	531	93.6
27	CM PG1	906	85.7	457	84.2
28	RM ZAN	503	84.9	257	83.7
	Overall	306.0	88.5	155.1	87.6

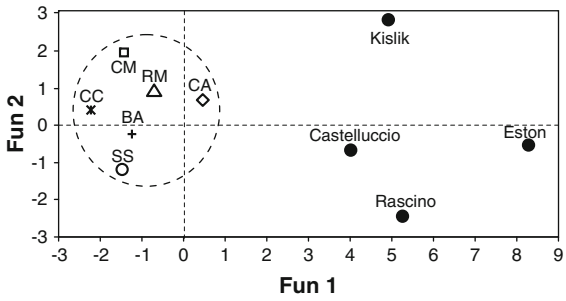

Fig. 5 Centroids of lentil landraces from Abruzzo (*open symbols*), and 4 controls (closed circles): Kislik, Eston (commercial varieties), Castelluccio and Rascino (landraces from Central Italy), plotted according to the two discriminant functions obtained from the seed image analysis data. The symbols of the landraces differ according to the village of collection: (*open circle* Santo Stefano di Sessanio; *open diamond* Calascio; *open triangle* Rocca di Mezzo; *open square* Castel del Monte; *plus* Barisciano; *star* Castelvecchio Calvisio)

variation, was basically related to the standard blue. The correct re-classification of seeds in the training set and in the test set (Table 8) were highly correlated ($r = 0.979$, $df = 8$, $P < 0.01$). Interestingly, after grouping accessions by locations, the seeds correctly re-classified were rather high for some entry, and low for others, as shown on the diagonal of Table 8. The four control accessions were correctly re-classified with an average level of 92.6%, particularly high for Kislik and Eston. At a first sight misclassification was rather high for Castelvecchio Calvisio and Barisciano, 46.9 and 49.5%, respectively. In fact, many seeds of Castelvecchio Calvisio were classified as Santo Stefano di Sessanio (30%), Castel del Monte (19%) and a few others as Calascio and Barisciano. Similarly, 47% of seeds of Barisciano were classified as Santo Stefano di Sessanio and a few others as Calascio. Moreover, as many as 89.4% of seeds from Santo Stefano di Sessanio were correctly re-classified, while 10.4% were misclassified, but erroneously classified in accessions from the same area, and only 7 seeds out of 3867 were misclassified in accessions used as controls. In other words, the results of discriminant analysis indicate that all accessions from Santo Stefano di Sessanio and neighbourhood locations were actually very similar one another, confirming the results of morphological characters and genetic markers, and indicating the existence of a pool of accessions sharing a high amount of similarities, and thus constituting a sort of metapopulation.

Discussion

Traditional crop varieties, generally known as landraces, are resources still grown by farmers in areas where agriculture is carried out in marginal conditions. In Italy the landraces of lentils are still common, particularly along the Apennines and in many small islands (Piergiovanni 2000), where they are part of the local productive systems, often based on practices and traditions strictly linked to food (Porfiri et al. 2009). Local varieties are the result of a dual selection pressure: a kind of indirect phenotypic selection by farmers, who often re-sow seeds harvested from their best plants, and by the agro-environmental conditions in which they have been grown for decades. Often of low productivity, landraces are well known to farmer as materials highly adapted to harsh, marginal agricultural conditions. This is particularly true as conditions become more and more marginal, where a thin line divides crop harvesting from crop failure. These are the conditions in Santo Stefano di Sessanio and in the Apennines areas in general. At the moment the existing lentil landraces from this region, from Castelluccio di Norcia and from other areas are an example of spontaneous on-farm conservation as a result of better prices they are sold compared to imported seeds from Canada, the USA and Turkey.

The threatening environmental conditions and the age of farmers, often old people, are exposing these landraces to high risks of genetic erosion. Actions aimed at genetic conservation and safeguarding are therefore imperative. A preliminary step in such conditions is to assess the level of the existing genetic diversity among and within populations in order to acquire insights about the existing, fragmented populations. This information could eventually promote/prevent actions aimed at maintaining/simplifying fragmentation. Monitoring the level of diversity could provide information on the size and direction of natural selection pressures and of the management practices as well.

Morphological characters, highly susceptible to changes due to environmental conditions (Karp et al. 1997) are still required by regulations at national and regional level in the landrace inventory (or regional repertory), in voluntary registers, as well as in production protocols for PGI and/or for Protected Designation of Origin (PDO) marks. Molecular

Table 8 Number and percentage (in bold italics) of correctly classified seeds belonging to the test set, with data grouped by collection areas

To	Eston	Kislik	Castel	Rascino	S.S. Sessanio	Cast. Calvisio	Bari-sciano	Calascio	C. del Monte	Rocca di Mezzo	
From											
Eston	522	1									523
	0.998										
Kislik		436									436
		1.000									
Castel	3	3	203	32	1						242
			0.839								
Rascino	2		46	290	1						339
				0.855							
S.S. Sessanio		1	4	2	3456	15	28	123	190	48	3867
					0.894						
Cast. Calvisio					45	68	2	4	28		147
						0.463					
Barisciano					153		160	12			325
							0.492				
Calascio		2	1		39	12	34	449	66		603
								0.745			
Castel del Monte		1	1		86	1	26	14	1810	4	1943
									0.932		
Rocca di Mezzo					32	2		1	10	458	503
										0.911	

genetics has an important complementary role to play besides the morphological characterization. In the present research a combination of morphological and agronomic traits, AFLP markers and image analysis of seeds were concurrently used in order to characterize 24 lentil accessions collected from Santo Stefano di Sessanio area. In general, the overall results showed high concordance. In terms of the morphological traits analyzed, the within population variability was in some cases very low (as for flowering time, with CV ranging from 3 to 7%), or very high (as for seed yield per plant, with CVs of 127–270%). As plant breeders have never used or manipulated the existing genetic diversity of these inbreeding populations, farmers and the marginal cropping conditions have certainly played a major role in maintaining enough variation for certain traits and limiting its size for certain others, such as those involved in adaptation. As a result, the accessions from Santo Stefano di Sessanio area are likely to be a blend of numerous pure bred lines (Sonnante and Pignone 2007) where the environmental conditions, the farming system and the mating system are the three key factors in determining their genetic structure.

The investigation based on AFLP markers, considered to be independent from selection pressures, indicates that the diversity among the 24 populations from S. S. di Sessanio area is limited, as they shared most of the fragments. The image analysis results further support it. As a result, most of them could be considered as part of a large metapopulation, each storing enough diversity based on slight differences among pure bred lines. It is this richness that ensures low but sustainable yields under marginal cropping conditions.

However, yield sustainability is not translated in economically cost-effective sustainability. Looking at the statistics of the lentil cropping areas in Italy from the 1950s to date, it is clear that lentil quickly disappeared from favourable areas, being restricted to marginal, hilly and mountainous areas, hosting

remnants of a wide existing diversity of the past (Piergiovanni 2000). In these areas, such as those of S. Stefano, not only are yields per unit areas low, but farmers' income is also due to the small farm size (and the average hectarage of grown lentil per farmer is about 1.7 ha). In order to avoid local communities from abandoning their land and migrate to cities and industrial areas, governmental and regional institutions are supporting and supplementing their incomes. The role of the local institutions in supporting local communities and their products could be by funding research seeking to register the PGI or PDO marks, as already traced by Castelluccio di Norcia, another lentil landrace from Central Italy (Piergiovanni 2000). In fact, in 1998, prior to PGI registration, the hectares of lentils in Castelluccio were barely 250, while in 2008 they increased to almost 600, with a total certified seed production rising from 100 to 400 t.

In order to achieve the above objective, the characterization of landraces must be the first step. A second step is the need to define the cultivation area, the cultivation procedures, as well as the marketing strategies, a role covered by the Producer Consortium which must act as a driving force for safeguarding and promoting Santo Stefano di Sessanio lentil. Once PGI or PDO marks are established, the third step is to safeguard the local product from commercial frauds that might occur at two levels: (1) at the trader level (by labelling and marketing as Santo Stefano di Sessanio mixtures of imported, low-cost seeds); (2) at the farmer level (with foreign seeds to be sown in the following season to save on sowing costs).

The results of the present research have clearly shown that in the latter case farmers might incur a substantial decrease of yields because of poorly adapted materials. The very low yields of SSMarket and CCMarket (25.8 and 15.8 g of seeds m^{-2}, respectively) supports this hypothesis and these actions should be discouraged. A further scientific aid to discover this practice has also come from molecular marker analysis, as well as from the image analysis of seeds, a quicker and cheaper tool able to provide an answer with good accuracy.

In image analysis, variables connected to seed size were discarded during the stepwise discriminant procedure, most likely because all entries belonged to the *microsperma* subspecies, while the most useful variables in discriminating the populations of Santo Stefano di Sessanio from the others were those related to the seed coat colours, a trait difficult to assess by visual examination without incurring a bias. The system could be further improved by updating, for example, the database with the seeds produced each year.

The three tools together, as shown in the present research, could be effectively used to overcome the difficulties of characterizing local populations, often hosting high levels of variability.

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Appendix

See Tables 9 and 10.

Table 9 Features of lentil accession seeds obtained from the image analysis in term of RGB colour

Acc.	Code	Red[a]	Std. red	Green[a]	Std. green	Blue[a]	Std. blue	Hue[a]	Std. hue	Light[a]	Std. light	Satur.[a]	Std. satur.
1	Eston	146.9	24.2	128.5	19.3	98.4	11.0	31.2	27.5	122.5	16.8	53.8	15.7
2	Kislik	137.8	27.4	111.9	18.7	95.5	10.2	34.7	54.5	116.1	18.3	49.1	17.8
3	Castel	128.3	23.3	102.3	18.0	85.4	11.2	37.6	59.1	106.1	16.9	52.6	16.4
4	Rascino	132.2	26.7	105.4	21.6	82.5	13.5	37.9	55.2	106.6	19.5	60.9	17.3
5	SS CVE	122.2	19.5	98.3	13.1	87.2	12.1	24.6	44.5	104.3	15.5	42.3	9.4
6	SS CRO	134.9	20.3	109.0	15.8	98.3	12.6	24.2	47.2	116.3	16.0	40.6	10.3
7	SS DPI	125.9	23.3	97.8	15.7	87.0	13.2	27.8	55.5	106.1	18.2	46.4	9.7
8	SS UDO	108.8	18.8	89.3	15.0	82.3	12.1	46.1	75.7	94.9	15.5	36.4	9.6
9	SS RLA	116.9	20.4	97.9	14.7	87.2	12.5	29.5	50.1	101.7	16.4	36.9	9.1
10	SS CRM	124.9	21.5	98.0	15.7	87.5	13.0	30.2	58.5	105.8	17.1	45.0	10.3

Table 9 continued

Acc.	Code	Red[a]	Std. red	Green[a]	Std. green	Blue[a]	Std. blue	Hue[a]	Std. hue	Light[a]	Std. light	Satur.[a]	Std. satur.
11	SS CLU	108.7	19.4	88.7	15.4	81.8	12.0	54.3	78.9	94.5	15.8	37.4	10.0
12	SS LAN	107.7	19.9	88.4	15.7	77.8	11.9	44.5	68.7	92.2	15.8	41.5	9.8
13	SS DAN	111.3	18.6	96.2	13.6	86.9	11.8	32.6	50.6	98.8	14.9	31.6	9.5
14	SS CDO	115.8	17.9	97.5	12.7	89.5	10.9	30.2	53.6	102.3	14.3	32.5	9.1
15	SS CGI	110.3	19.4	94.6	14.5	87.3	11.8	38.3	62.9	98.4	15.6	30.2	9.3
16	SS SMA	125.0	25.1	101.4	18.3	91.5	13.7	33.6	61.4	107.7	19.5	39.6	10.5
17	SS CGA	121.6	19.7	103.3	12.7	96.4	10.4	29.2	54.0	108.7	14.8	29.3	10.1
19	SS Market	115.8	17.7	98.0	12.3	90.9	10.8	30.5	57.0	103.1	14.1	30.7	9.6
20	SS FCA	119.1	18.5	97.3	12.6	89.2	10.6	41.3	67.9	100.2	17.4	34.0	9.8
18	CC Market	127.6	20.8	100.2	13.6	89.9	11.6	23.8	48.7	108.3	16.1	43.8	9.6
21	CC MPI	128.8	22.2	103.4	15.9	95.5	13.1	26.5	55.7	111.7	17.7	38.0	9.1
22	BA MGI	123.3	22.1	104.6	16.1	94.2	12.6	29.1	43.3	108.5	17.2	34.3	9.5
23	CA CDO	150.0	23.8	127.0	19.5	108.2	14.3	25.0	32.2	128.8	18.6	47.2	12.8
24	CA GAL	137.9	21.5	111.3	16.2	99.0	11.5	22.2	40.6	118.1	16.4	42.4	9.0
25	CA AMA	134.5	24.2	110.0	17.8	98.7	13.0	24.0	43.1	116.3	18.5	39.9	9.8
26	CM GMA	127.5	21.4	104.1	13.6	96.0	10.9	24.5	48.8	111.4	16.0	35.5	10.0
27	CM PG1	103.4	16.4	88.8	13.1	86.9	10.1	79.8	94.8	94.1	13.4	25.3	9.5
28	RM ZAN	110.7	21.0	84.7	15.6	79.2	10.4	68.6	90.3	93.9	16.1	44.4	10.5
	SED	0.63	0.21	0.43	0.13	0.30	0.08	1.31	0.91	0.44	0.14	0.45	0.11

The values are means obtained from a variable number of seeds from each accession, as reported in Table 7 of the text

[a] See "Materials and methods"

Table 10 Morphometric features of lentil accession seeds obtained from the image analysis

Acc	Code	Diameter				Area (mm^2)	Perimeter (mm)	Shape factor	Roundness
		Max (mm)	Min (mm)	Circ. equivalent	Ratio				
1	Eston	5.12	4.71	4.81	0.92	18.24	15.50	0.99	0.88
2	Kislik	4.56	4.17	4.27	0.91	14.37	13.80	0.99	0.88
3	Castel	4.73	4.34	4.44	0.92	15.59	14.34	0.99	0.88
4	Rascino	4.51	4.14	4.23	0.92	14.14	13.68	0.99	0.88
5	SS CVE	4.82	4.40	4.50	0.91	15.97	14.56	0.98	0.87
6	SS CRO	4.28	3.86	3.97	0.90	12.46	12.87	0.99	0.86
7	SS DPI	4.46	4.08	4.18	0.91	13.74	13.50	0.99	0.88
8	SS UDO	4.58	4.17	4.28	0.91	14.43	13.84	0.99	0.87
9	SS RLA	4.16	3.82	3.90	0.92	11.95	12.61	0.99	0.88
10	SS CRM	3.91	3.58	3.66	0.92	10.54	11.86	0.99	0.88
11	SS CLU	3.54	3.16	3.26	0.89	8.35	10.60	0.99	0.85
12	SS LAN	4.31	3.93	4.02	0.91	12.76	13.01	0.99	0.87
13	SS DAN	4.85	4.44	4.55	0.92	16.28	14.67	0.99	0.88
14	SS CDO	5.00	4.54	4.67	0.91	17.18	15.08	0.99	0.88
15	SS CGI	4.35	3.99	4.08	0.92	13.12	13.19	0.99	0.88
16	SS SMA	4.29	3.60	3.83	0.84	11.55	12.46	0.98	0.80

Table 10 continued

Acc	Code	Diameter				Area (mm^2)	Perimeter (mm)	Shape factor	Roundness
		Max (mm)	Min (mm)	Circ. equivalent	Ratio				
17	SS CGA	5.06	4.67	4.76	0.92	17.85	15.35	0.99	0.89
19	SS Market	4.38	4.00	4.10	0.91	13.26	13.24	0.99	0.87
20	SS FCA	4.10	3.68	3.79	0.90	11.36	12.29	0.99	0.86
18	CC Market	4.47	4.11	4.20	0.92	13.91	13.57	1.00	0.88
21	CC MPI	4.47	4.14	4.22	0.93	14.01	13.62	1.00	0.89
22	BA MGI	4.23	3.88	3.97	0.92	12.41	12.82	0.99	0.88
23	CA CDO	4.71	4.32	4.42	0.92	15.43	14.28	0.99	0.88
24	CA GAL	4.52	4.15	4.25	0.92	14.23	13.71	1.00	0.88
25	CA AMA	4.62	4.20	4.31	0.91	14.67	13.94	0.99	0.87
26	CM GMA	4.41	4.04	4.13	0.92	13.46	13.35	0.99	0.88
27	CM PG1	4.32	3.92	4.03	0.91	12.83	13.03	0.99	0.87
28	RM ZAN	4.35	3.98	4.07	0.91	13.08	13.16	0.99	0.87
	SED	0.021	0.021	0.020	0.002	0.134	0.064	0.000	0.002

The values are means obtained from a variable number of seeds from each accession, as reported in Table 7 of the text

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