

A study of variability in the Sicilian faba bean landrace 'Larga di Leonforte'

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Abstract In this paper molecular analysis, physical, technological, and chemical traits were used to estimate the level of variation on five accessions of a locally adapted Sicilian faba bean (*Vicia faba* L.) landrace named 'Larga di Leonforte'. DNA analysis was performed using the Amplified Fragment Length Polymorphism (AFLP) molecular marker class and two other faba beans (cv. 'Aguadulce Samba'—Spain—and landrace 'Locale di Modica'—Sicily) were used as controls. Although the accessions of 'Larga di Leonforte' varied significantly for most of the agronomical and physical traits, this landrace generally had a heavy seed weight, short but large pods, and no more than two seeds per pod. This last characteristic allowed for erect pod angle attitude at maturity. Idratation data showed difference among accessions in seed weight at full hydration and in

absorption rate at the very beginning of the hydration process, while any difference among accessions emerged in terms of cooking properties. The six AFLP Eco+3/Mse+3 different primer combinations applied in this research revealed different levels of polymorphism among the faba bean accessions and a total number of 346 amplicons were generated. Around 60% of amplicons displayed a polymorphic pattern among different accessions. Cluster analysis on morphological, technological, chemical and molecular data placed the all five 'Larga di Leonforte' accessions into a separated group, and the Sicilian material shares a fairly large amount of similarity with respect to the cultivar 'Aguadulce Samba' selected in Spain.

Keywords AFLP · Genetic diversity · Landrace · Physical, technological and chemical traits · *Vicia faba* L.

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Introduction

For decades, the faba bean (*Vicia faba* L.) has represented the only grain legume widely grown in Europe, but nowadays its cultivation is continuously declining, due to low yield and yield instability. Stresses of both biotic and abiotic origin are the major causes in limiting the realization of full yield potential. In this view, the locally adapted landraces could represent an economically valuable opportunity

for farmers in marginal areas (Hawtin et al. 1997), and become the basis for plant breeders to develop new varieties (Nadal et al. 2003; Terzopoulos et al. 2008). Autochthonous landraces, in fact, evolved from ancient types through conscious and unconscious phenotypic selection by farmers, contain adaptive genes to different agroecological conditions (Chahal and Gosal 2002). Among Sicilian locally adapted landraces, the 'Larga di Leonforte', belonging to *V. faba* var. *faba* shows characteristics with great appeal for the consumer, such as large seed dimension (1,000 seeds weight >2,000 g). There are also peculiar traits appreciated by both farmers and breeders such as the erect pod angle at maturity which allows mechanized harvest and drought resistance-tolerance mechanisms. Various investigations have been conducted to acquire information concerning the diversity among and within faba bean landraces in Sicily based on morphological and agronomic traits (Abbate et al. 2001, 2006) or physiological aspects (Avola et al. 2008). Moreover, the study of model of the kinetic of seed hydration and cooking, applied to compare genotypes (Abu-Ghannam and McKenna 1997; Ibarz et al. 2004; Avola et al. 2009), are not amenable to statistical analysis in particular when large numbers of accessions are involved. The above mentioned models of identification are also unstable because of environmental effects on the characteristics, and it is debatable if a classification scheme using only morphological/agronomic traits is adequate for the development of gene pools since these traits are either influenced by environmental factors and stage of plant development.

For this purpose, DNA marker technology, such as RFLP, AFLP and SSR (Weising et al. 2005) has been widely used for investigating genetic variability in *Vicia* species and *V. faba* accessions and in generating genetic linkage maps (Link et al. 1995; Pozárková et al. 2002; Zeid et al. 2003; Román et al. 2004; Terzopoulos and Bebeli 2008). In particular AFLP proved to be very useful for *V. faba* studies (Zeid et al. 2003; Román et al. 2004) since they combine the reliability of RFLPs with the power of PCR and the possibility of testing several loci in each experiment. AFLP has been used in intra-accession diversity in barley germplasm with the aim of avoiding selection and genetic drift in the gene bank collection (van Treuren et al. 2001).

In this paper, we have investigated at molecular, morphological, technological, and chemical levels, samples of 'Larga di Leonforte' accessions of a locally adapted faba bean landrace. This was done to assess the genetic variation present in the studied landrace. This information may be useful for the certification of typical products of faba bean, for the recognition of protected geographical indication (IGP), and for to assess the consistency of farmers' naming of the faba bean landrace they grow.

Materials and methods

Seeds from five accessions (named A, B, C, D and E) of the 'Larga di Leonforte' landrace were collected in spring 2006 in a restricted area where this landrace has been cultivated from long time. During December 2006, all the material was sown in experimental plots of 10 × 10 m and harvested during the first 10 days of June 2007. For the measurements, seeds were collected on the middle two rows of each plot.

Pods dimension and physical and technological properties of seeds

Number of seeds per pod, pod dimension (mm), seed weight (g) and seed size (length, width and thickness mm) were measured at harvest. Thickness was determined at the hilum end and at the centre of the seed, and the results were expressed as the mean of the two determinations.

The incidence of seed coat on entire seed weight (%) was determined gravimetrically. Hydration tests were performed by soaking 10 seeds in 400 ml deionized water (pH 7) at 25°C temperature. To determine the kinetic hydration, the seeds were removed from the soaking water at timed intervals starting from 30 min up to 24 h. Weight gain was taken as the amount of water absorbed and expressed as the hydration capacity: $HC = [(weight\ after\ soaking)/(weight\ before\ soaking)] \times 100$. Each test was repeated three times to determine mean values.

To model the water uptake kinetics, we chose a two parameter asymptotic regression model

$$y_t = y_0(1 - \exp(-kx)) \quad (1)$$

where y_t is hydration (g seed⁻¹) after soaking for time t (hours), y_0 is the asymptote of the curve estimating

the seed weight at full hydration (H_{\max}), k is a curve parameter that is related to the absorption rate at the very beginning (H_{rate}).

To determine the cooking properties, pre-hydrated seeds (after 24 h of hydration) were placed in 1 l deionized boiling water and removed at timed intervals (10, 20, 30 and 40 min) for seed textural determination. Seed textural changes after the soaking process (at 24 h) and during the cooking process were tested using a texturemeter (Bertuzzi, Italy) equipped with an 60 mm diameter conical plunger. The peak compression force (expressed by $N\text{ cm}^{-2}$) was recorded as the measure of the force necessary to compress a seed to 50% of its original thickness at hilum. For each measurement, results were expressed as the mean of ten determinations.

The texture property as a function of cooking time was calculated following the exponential decay model:

$$F_t = F_{\text{mr}} + (F_{\text{mr}} - F_0) \exp(-kt) \quad (2)$$

where F_t is the firmness of seed at time t , F_{mr} is the maximum retainable firmness at $t = \infty$, F_0 is the initial firmness of seed, at time $t = 0$ and k is the rate constant.

Chemical analysis

The samples were dried at 70°C in preparation for chemical analysis and were ground through a 1-mm screen with a micro-fine mill (model MF10, IKA® Group, Germany). Initial moisture, starch, ash, iron, magnesium and calcium contents were determined according to AOAC methods (AOAC 2000). Crude protein content was determined by the Kjeldahl method ($N \times 5.7$), using the coefficient reported by Mossé (1990) for the calculation of protein in grain legumes. Tannin content was determined using a Hach D800 meter and Hach tannin test kit. Triplicate samples were used to determine chemical composition. All determinations were expressed on dry matter basis.

DNA extraction and AFLP analysis

The DNA extraction and AFLP analysis were carried out in all the above-mentioned accessions. Two controls (the Sicilian landrace 'Locale di Modica' and the cultivar 'Aguadulce Samba', both belonging

to *V. faba* var. *faba*) were also added. Leaf tissues from 9 plants per accession were collected *in situ* and immediately transferred into liquid nitrogen to prevent degradation. Genomic DNA was extracted from 0.5 to 1 g of fresh tissues using the Genelute Plant genomic DNA miniprep kit (Sigma). DNA concentration was assessed through optical density reading (DU650 spectrophotometer, Beckman) and confirmed by agarose gel electrophoresis. AFLP analysis was carried out according to Vos et al. (1995) with modifications as reported in Porceddu et al. (2002). After PCR 10 µl of Hi-Di Formamide (Applied Biosystems) were added to 1 µl of each reaction, denaturated at 94°C for 4 min and then immediately placed in ice. Samples were separated by an ABI 3130xl capillary sequencer (Applied Biosystems). An AFLP locus was considered to be polymorphic if the amplified band was present in some accessions and absent in others, and monomorphic if the band was present in all the evaluated accessions. To avoid underestimation of the genetic similarities, all loci, polymorphic or not, were considered. AFLP fragments were scored as 1 (presence of the band) and 0 (absence of the band), using the Genemapper 4.0 software (Applied biosystems); all data collected were entered into a data matrix.

Statistical data analysis, genetic diversity and divergence analysis

The pod dimension and the physical, technological and chemical characters (quantitative traits) were analysed for their means, ranges and standard errors. Data was also analyzed for variance (ANOVA), and to identify pairs of means that are significantly different. The LSD value (0.05 significance) was calculated using the SNK test.

The parameters of the mathematical models used in this study (water uptake kinetics and cooking kinetics) were estimated using a non-linear regression procedure performed using the SigmaPlot software (SigmaPlot 6.0 scientific graphing software from SPSS Inc., Chicago). The suitability of the models was evaluated and compared using the coefficient of determination (R^2), and the Root Mean Square Error (RMSE). Each parameter and the relative standard error reported by the nonlinear regressions were used to compare the different accessions by means of

pooled *t* statistics according to the approach suggested by Glantz and Slinker (1990).

Different measures of genetic variability were used to estimate the levels of polymorphism within and between variety/accessions. The informative content of the markers scored was evaluated by the MR index (Powell et al. 1996) number of polymorphic loci per primer combination—and the polymorphic index content (PIC; Botstein et al. 1980).

P-distances (pairwise distances) were calculated on AFLP data to construct a neighbour-joining tree using the MEGA 3.1 software (Kumar et al. 2004). For each single accession (genetic material from 2 or 4 plants), ordination analysis was performed according to the neighbour-joining (NJ) method clustering algorithm (Saitou and Nei 1987). Dendrograms were constructed from the symmetrical genetic similarity matrix.

Dissimilarity analysis of the data obtained from quantitative traits were constructed using a dissimilarity measure calculated using the method proposed by Laghetti et al. (2008).

Cluster analysis of all quantitative and genetics data was constructed based on Euclidean distances, nearest neighbour method and performed with data analysis package StatistiXL 1.5. (StatistiXL Ltd).

Results and discussion

Morphological, physical and technological properties of seeds

The main morphological traits of 'Larga di Leonforte' seeds are summarized in Table 1. Although these

accessions varied significantly for most of the traits, this landrace is characterized by a heavy seed weight (2,705 mg for 1,000 seeds), short but large pods, and no more than two seeds per pod. This last characteristic allows erect pod angle attitude at maturity. The analysis of variance showed significant difference in length, width and weight of the seed. No differences emerged on thickness.

Seed coat incidence was slightly but significantly affected by genotypes and 'A' was characterized by the highest value, 'B', D and 'E' by the lowest (Table 2). In contrast, both the hydration capacity as well as the seed textural changes after 24 h of soaking process did not vary in relation to genotypes. The amount of water absorbed was about 1.5-fold of seed weight before soaking, and the force needed to compress a seed was $97.5 N cm^{-2}$.

The analysis of the slopes and the intercepts calculated from the non-linear regression showed differences between accessions (Table 3). All the accessions but 'C', showed H_{max} higher than 1.50 g

Table 2 Some technological characteristics of 'Larga di Leonforte' seed

Accession	Seed coat Incidence (%)	Hydration capacity (%)	Seed textural ($N cm^{-2}$)
A	16.4a	147	95.5
B	14.5c	145	94.9
C	15.4b	141	140.9
D	14.8bc	146	100.4
E	14.5c	147	91.9
Average	15.1	144	97.5

Different letters indicate significant differences for $P < 0.05$

Table 1 Some morphological seed characteristics of 'Larga di Leonforte' accessions

Accession	Pod length (cm)	Pod width (cm)	Seed pod ⁻¹ (n)	1,000 Seed weight (g)	Seed dimension		
					Length (mm)	Width (mm)	Thickness (mm)
A	9.2	3.1b	1.9	2,812b	30.2b	21.4ab	7.25
B	9.6	2.9c	1.9	2,695c	28.1c	20.8bc	7.54
C	8.3	2.8d	1.9	2,522d	27.2d	20.1bc	7.46
D	9.0	3.2a	2.0	2,570d	30.5ab	21.2ab	7.55
E	8.7	3.2a	1.8	2,926a	31.1a	21.8a	7.55
Average	9.0	3.1	1.9	2,705	29.4	21.1	7.47

Different letters indicate significant differences for $P < 0.05$

Table 3 The parameters of the non-linear regression analysis of hydration curves of ‘Larga di Leonforte’

Accession	y_0 (%)	$k\% \text{ h}^{-1}$	R^2
A	155a	0.205b	0.981
B	154a	0.164b	0.989
C	139b	0.455a	0.995
D	154a	0.209b	0.986
E	154a	0.235b	0.982
All data combined	150	0.233	0.994

Different letters indicate significant differences for $P < 0.05$

Table 4 The parameters of the non-linear regression analysis of cooking curves of ‘Larga di Leonforte’

Accession	y_0	k	b	R^2
A	13.6	94.3	0.074	0.987
B	12.0	94.8	0.075	0.988
C	21.5	104.5	0.103	0.991
D	17.0	99.4	0.098	0.988
E	13.1	91.7	0.078	0.994
All data combined	15.8	96.9	0.233	0.992

$\text{H}_2\text{O g dry seed}^{-1}$, while ‘C’ hydrated faster than the other faba bean accessions (0.455 g min^{-1}) and also contained the least amount of water ($1.36 \text{ g H}_2\text{O g dry seed}^{-1}$).

From the analysis of the cooking kinetic parameters, no one genotype showed significant difference in terms of cooking characteristics (Table 4).

Chemical characteristics

The parameters under investigation varied significantly except for ash, among all the accessions (Table 5). Starch ranged between 43.7% of ‘A’ and 48.1% of ‘C’. The data revealed that, except for the genotype ‘C’, there were no significant differences in protein content with the mean value being 24.9%. Content of crude fibre (7.7%) and ash (4.1%) were nearly similar in all the accessions. Genotypic differences were noted for tannin, once again due to the very high value recorded in accession ‘C’. Mg and Fe content did not show appreciable variations among accessions, while accessions ‘A’ and ‘B’ presented a calcium content significantly higher than the others.

Table 5 Some chemical characteristics of ‘Larga di Leonforte’ seeds

Accession	Starch (g 100 g ⁻¹ dw)	Crude protein (g 100 g ⁻¹ dw)	Crude fibre (g 100 g ⁻¹ dw)	Ash (g 100 g ⁻¹ dw)	Tannin content (g 100 g ⁻¹ dw)	Fe (mg 100 g ⁻¹ dw)	Ca (mg 100 g ⁻¹ dw)	Mg (mg 100 g ⁻¹ dw)
A	43.7c	25.1a	7.9a	4.1	2.5b	2.9a	69.0a	71.1a
B	45.7b	25.3a	7.7ab	4.2	2.1c	1.9c	60.5a	65.9a
C	48.1a	24.1b	7.4b	4.2	3.3a	2.7ab	35.0b	47.0b
D	46.1b	25.1a	8.4a	4.1	2.3bc	2.2bc	40.5b	63.1a
E	47.2ab	25.1a	7.4b	4.0	2.2bc	2.2bc	44.4b	71.3a
Average	46.2	24.9	7.7	4.1	2.5	2.4	49.9	63.7

Different letters indicate significant differences for $P < 0.05$

Table 6 Dissimilarity matrix based on the 19 physical, technological and chemical descriptors studied

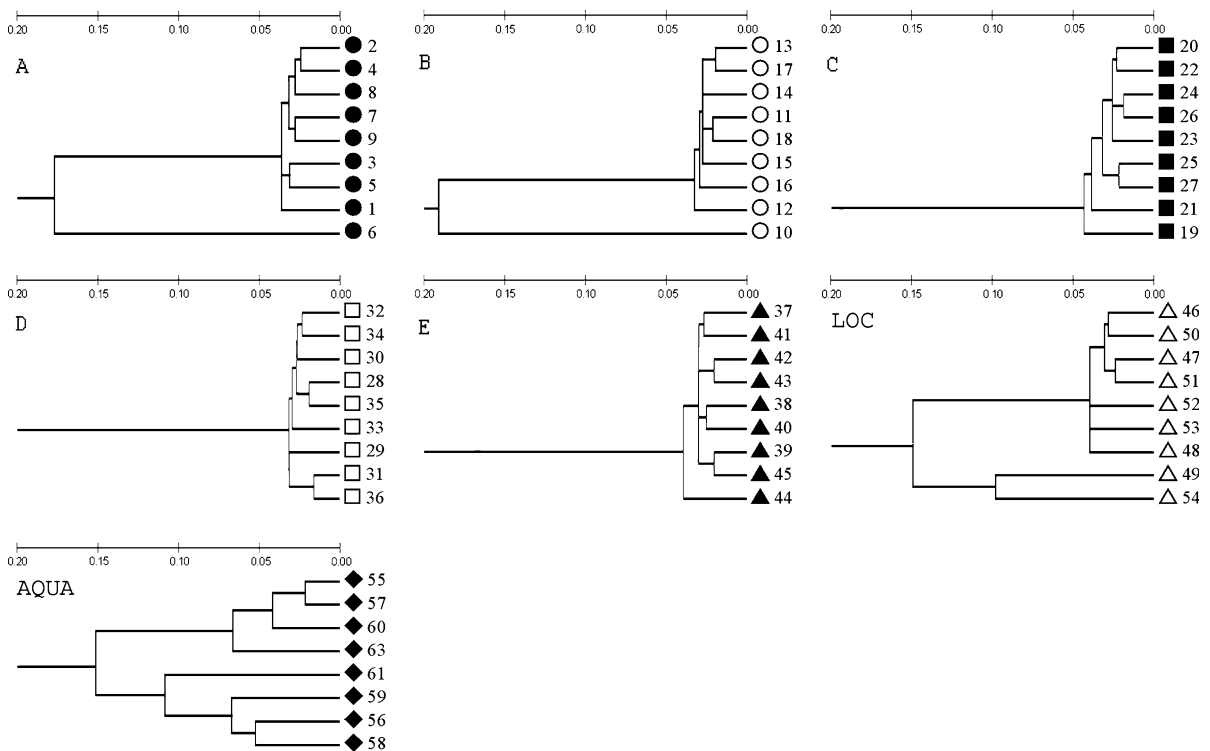
Accession	B	C	D	E
A	3.94	10.82	3.68	4.98
B	–	9.64	2.42	4.70
C		–	8.74	10.16
D			–	4.26

Dissimilarity analysis based on physical, technological and chemical data

Table 6 reports the dissimilarity matrix based on the 19 physical, technological and chemical descriptors. The lowest dissimilarity between pair-wise comparisons was found in 'B' and 'D' (2.42), whilst the the highest value was detected for 'A' and 'C' (10.82).

Table 7 Characteristics of amplicons generated by 6 AFLP primer combinations

	EcoRI+3/MseI+3 primer combination	Amplification range	Total fragments	Monomorphic fragments	Polymorphic fragments (Mr)	%Polymorphic fragments	Pic
1	CAG/AGT	79–344	43	17	26	65.4	0.417
2	CAG/ATC	92–377	69	25	44	56.8	0.422
3	CAG/ACG	93–468	40	17	23	73.9	0.606
4	CAC/ACA	60–232	75	30	45	66.7	0.553
5	CAC/ACG	78–194	65	25	40	62.5	0.648
6	CAG/ACA	87–485	54	17	37	45.9	0.482
Total			346	215	131		
Average			57	21		61.9	0.5

**Fig. 1** Dendrograms generated by using AFLP analysis within each of accessions of 'Larga di Leonforte' and the two controls ('Locale di Modica' and 'Aguadulce Samba')

This last accession always accounted the highest dissimilarity values when compared with each of the remaining accessions.

AFLP markers and genetic distances and dendrograms construction

A total of 63 samples were genotyped using 6 AFLP primer combinations and a total of 346 amplification products were scored (Table 7). The percentage of polymorphisms detected ranged from 45.9 (Eco-CAG/Mse-ACA) to 73.9% (Eco-CAG/Mse-ACG) depending on the primer combination, with an average of 61.9% polymorphic bands for the overall data. The molecular weight of the amplification products varied from 60 (E-CAC/M-ACA) to 485 bp (E-CAG/M-ACA) and the average number of bands scored per reaction was 64.3 with a variation from 40 (E-CAG/M-ACG) to 75 (E-CAC/M-ACA). The number of polymorphic products per reaction varied from 23 (E-CAG/M-ACG) to 45 (E-CAC/M-ACA).

The overall number of scoreable fragments per primer was fairly similar to that obtained by Zeid et al. (2003) on faba bean, ranging from 46 to 92 with an average of 66, but differences emerged on the number of polymorphic fragments. These last occurred much less frequently in our analysis compared to that obtained in the above mentioned research, in which the authors accounted 89.5% polymorphic fragments.

Genetic variability was detected among accessions of each landrace or cultivar as well as among landraces of faba bean (Fig. 1). The p-distances within local varieties of *V. faba* ranged from a minimum of 0.034 for accession 'D' to 0.391 for accession 'B' (Table 8). The lowest mean genetic dissimilarity between pair-wise comparisons was found in 'E' and 'D' (0.059 and 0.061, respectively). The highest mean value was estimated for 'Aguadulce Samba' and 'Locale di Modica' characterized by an average p-distance of 0.239 and 0.165, respectively.

Genetic relationships for all studied accessions revealed that the cultivar 'Aguadulce Samba' is well differentiated from all local landraces being that its mean p-distance was as high as 0.228, ranging from 0.222 with accession 'B' to 0.238 with accession 'A' (Table 9). In particular 2 of the 4 selected 'Aguadulce Samba' samples, showed great differentiation not only from other local Sicilian landraces, but also from the other 2 samples of the same cultivar. In fact, they

Table 8 Genetic relationships on over all accessions

	p-Distance min-max	p-Distance average
A	0.043–0.330	0.119
B	0.037–0.391	0.126
C	0.043–0.102	0.068
D	0.034–0.082	0.061
E	0.038–0.087	0.059
Locale di Modica	0.047–0.375	0.165
Aguadulce	0.044–0.364	0.239
Overall average	0.041–0.247	0.120

Within each accession and overall, Min, Max and mean p-distances were calculated using MEGA 3.1 software

Table 9 p-Distance values between accessions

	A	B	C	D	E	LOC	AGUA
A	1.000	–	–	–	–	–	–
B	0.064	1.000	–	–	–	–	–
C	0.060	0.064	1.000	–	–	–	–
D	0.071	0.071	0.080	1.000	–	–	–
E	0.063	0.066	0.066	0.071	1.000	–	–
Locale di Modica	0.082	0.091	0.083	0.088	0.075	1.000	–
Aguadulce	0.238	0.222	0.231	0.222	0.230	0.224	1.000

were located in a well characterized clade (Fig. 2). This is also confirmed by the accession analysis which showed a mean p-distance of 0.239 and two

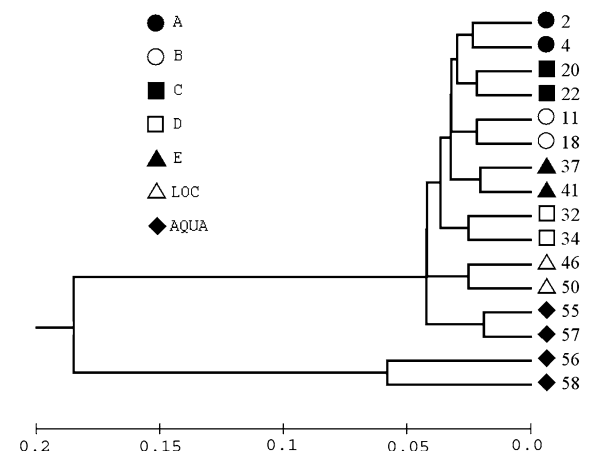


Fig. 2 Dendrogram generated by using AFLP analysis of five accessions of 'Larga di Leonforte' and the two controls ('Locale di Modica' and 'Aguadulce Samba')

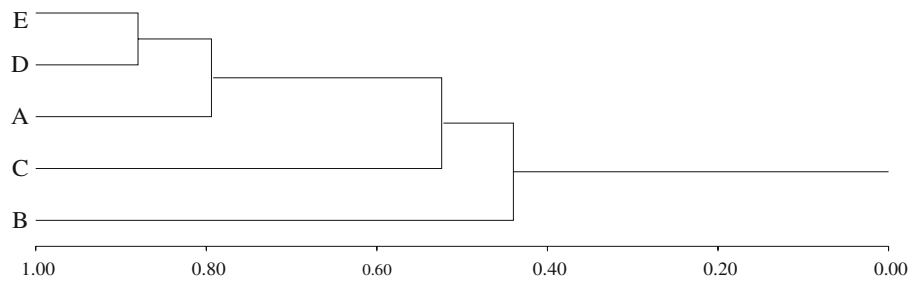


Fig. 3 Cluster analysis based on physical, technological, chemical and genetic data

major clades. The other accessions showed relatively low p-distances values. In fact they ranged from 0.060 between accession ‘A’ and accession ‘C’ to 0.091 between accession ‘B’ and ‘Locale di Modica’ (Table 9).

The results obtained by Terzopoulos and Bebeli (2008) on 20 local Greek faba bean populations using four ISSR primers, suggested that the majority of the observed genetic variability was due to within population variation (75.4%). This was not unexpected for faba bean local populations, since they are partially cross-pollinated and are heterogeneous mixtures of inbreds and hybrids.

Join quantitative and molecular characterization

The dendrogram constructed based on the quantitative and genetic data produced similar results for the similarity/diversity of the accessions (Fig. 3), indicating a high degree of correspondence between the two data sets, and confirmed the above-mentioned genetic relationships for the ‘Larga di Leonforte’ landrace except for ‘C’ which is distantly related to the other accessions for quantitative characteristics, but it is closely related to the D and E accessions for the genetics.

Conclusions

In this research using 6 AFLPs different primer combinations revealed different levels of polymorphism among the faba bean accessions. The five accessions belonging to ‘Larga di Leonforte’ landrace could be placed into one group. In addition, it is interesting to note that the Sicilian material shares a fairly large amount of similarity with respect to the

cultivar ‘Aguadulce’ selected in Spain. ‘Larga di Leonforte’ accessions, in fact, were clustered together with the landrace ‘Locale di Modica’ collected in the South-East of Sicily.

However, a substantial level of genetic variation still exists within accessions of ‘Larga di Leonforte’, as detected by AFLP analysis and confirmed by physical, technological and chemical results. Even when grown in the same location, each accession showed a distinct pattern of polymorphism. This even limited genetic diversity can be used in breeding programs in order to maximize the level of variation present in this landrace by crossing cultivars and populations with greater genetic distance.

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