Electrophoretic phenotypes of different enzymes in some entries of *Lathyrus sativus* L.

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Introduction

In Italy, grasspea (*Lathyrus sativus* L.) is one of the least common grain legumes. Today, it is not included in the official statistics of Italy and its survival is due to the praiseworthy action of a small number of farmers in very limited areas of peninsular and insular central-southern Italy. Since the Second World War the sharp reduction in cultivation, similarly for other grain legumes, has certainly contributed to relegate grasspea among neglected crops in the Italian and European agricultural research programs.

The development of sustainable and environmentally sound agricultural systems has stimulated a rising interest in the role of grain legumes and of this crop, in particular**,** in the world scientific community [2,4,8]. As for other neglected grain legumes**,** the lack of varieties has favoured breeding programs using local material generally represented by old landraces [15]. The renewed interest of both farmers and plant breeders for grasspea has induced a concomitant and necessary action on the primary gene pool of this endangered species. In particular, research programs have been started for safeguarding, collecting and establishing several collections at different scientific institutions, complemented by diversity evaluation programs on them [11].

Among molecular methods, the electrophoretic analysis of isoenzyme variation has proved to be particularly useful in defining more precisely the size and structure of genetic diversity in the gene pools of different grain legumes [6,7,10]. In grasspea, the literature on both genetic diversity and intra and interspecific-relationships among collections is quite poor [1,16].

The aims of this study were:

a) to add information on the genetic structure of *Lathyrus sativus* L.
b) to verify the possibility of classifying grasspea entries based on isoenzyme traits.

Materials and Methods

Twelve grasspea populations, selected for morpho-agronomic traits from the Southern Italy collection maintained at the Centro Inter-universitario del Germoplasma Mediterraneo (CIGM), University of Basilicata, Potenza, Italy were used. The accessions are described for 1000-seed weight, seed protein content and seed coat colour (Table 1). Ten plants per population were grown in a controlled environment chamber and analysed for 9 enzyme systems: acid phosphatase (APH), phospho-gluconate dehydrogenase (PGD), aspartate aminotransferase (AAT=GOT), shikimate dehydrogenase (SKD), endopeptidase (END), glucose-6-phosphate dehydrogenase (G-6-PDH), glutamate dehydrogenase (GDH), diaphorase (DIAP) and leucine aminopeptidase (LAP).

Table 1. List of *Lathyrus sativus* L. entries tested.

Entry*	1000 Seed weight (g)	Seed protein content (%)	Seed coat colour
ACL 2	372	24.8	White
ACL 29	327	24.9	“
ACL 53	332	26.5	“
ACL 77	325	25.2	“
ACL 80	294	25.2	“
ACL 94	369	25.2	“
ACL 95	356	26.0	Grey
ACL 104	316	25.1	White
ACL 109	317	25.2	“
ACL 130	339	25.7	“
ACL 137	351	25.4	“
ACL 146	303	25.4	“

*Entry number of Alba’s collection of *Lathyrus* spp. maintained at CIGM, University of Basilicata, Potenza, Italy.

Five hundred mg of younger leaves were picked from plants at the 4 to 5 true-leaf stage, and homogenised with 3 ml of Tris-HCl 75 mM pH 7.5 added with 5% sucrose, 5% PVP w/v, 14 mM β-mercaptoethanol 0.1% v/v, 50 mM ascorbic acid, 10 mM diethyldithiocarbamate, albumin bovine serum 0.1% w/v [14]. After centrifuging at 10 000 rpm for 15

minutes, the supernatant was removed and stored at –80°C until it was used. The extracts were electrophoresed on 12% starch gel at 4°C and 180 mA. As regards the SKD enzyme system, a buffer of histidine-citrate 0.2 M pH 6 was used, while a solution obtained from the running buffer with water (1:6 ratio) was used for the gel. For all other systems a buffer composed of Tris-citrate 0.2M pH 8.3 and lithium-borate 0.5M pH 8.3 (9:1 ratio) was applied. At the end of the run, starch gel slices about 1.5 mm thick were stained [(14)].

Band frequencies were calculated for each entry and for each isozyme system. The intra-population variation was estimated by the Shannon-Weaver (H') diversity index [(12)]:

$$H' = -\sum_{i=1}^{n} p_i \log_2 p_i$$

where p_i is the frequency of the ith band and n is the number of bands observed for each enzyme. This index applied by different authors (Hutchenson, 1970; Polignano et al., 1998) provides a quantitative estimate of the intra-population diversity.

Based on the matrix of band frequencies and standardised data, the inter-population variation was examined by cluster analysis, and Ward's method (PROC CLUSTER) was utilized to calculate Euclidean distances [(1)].

Results and Discussion

Polymorphism was present for 19 out of the 43 isozymes resolved in 9 enzyme systems (Table 2). the results obtained for each enzyme system are explained below:

Acid phosphatase (APH). Eighteen patterns and three zones of activity were detected: a cathodal (APH-1), an intermediate (APH-2) and an anodal zone (APH-3). The zone APH-3 with two bands (g, h) was monomorphic. The zone APH-2 was polymorphic with three bands (d, e, f). Similarly, the zone APH-1 was polymorphic with a slow (a), an intermediate (b) and a fast band (c).

Phospho-gluconate dehydrogenase (PGD), g*lucose-6-phosphate dehydrogenase* (G6PDH) and g*lutamate dehydrogenase* (GDH). Of the two zones of activity detected for these enzyme systems, some electrophoretic variation was found only for the cathodal zone, PGD-1, G6PDH-1 and GDH-1, which displayed both homozygous and heterozygous genetic structures. The fastest band was always present, on the contrary, the slowest one was highly discriminating.

Glutamate oxalacetate transferase (GOT=AAT). This enzyme system exhibited two isozymes (GOT-1 and GOT-2) corresponding to three phenotypes. GOT-1 was polymorphic, GOT-2 monomorphic (band a). Heterozygous individuals exhibited a three-banded pattern.

Diaphorase (DIAP). Three isozymes were detected in gel slices stained for DIAP. Of the three zones of activity resolved for this enzyme system, some electrophoretic variation was found only for the intermediate one, characterized by five bands (c, d, e, f, g).

Endopeptidase (ENP). One zone of endopeptidase activity was present. Heterozygous individuals exhibited a three-banded pattern.

Leucine amino peptidase (LAP). Two clear regions of LAP activity were resolved, some electrophoretic variation was found only for the cathodal one, LAP-1, which exhibited both homozygous and heterozygous phenotypes

Shikimate dehydrogenase (SKD). Assays showed two regions of activity. The five band variants observed (a, b, c, d, e) suggest the presence of two loci SKD-1 and SKD-2. The latter was made visible in one monomorphic band in all surveyed individuals. Three bands of isozyme activity were observed in the SKD-1 zone among the analysed individuals.

A total of 43 bands with frequency values ranging from 0 to 1 were observed. Average frequencies below 5% indicate a rare allelic presence; instead, if the frequency of the most frequent allele to a fixed locus is less than 95% the population is considered to be polymorphic (Brown and Weir, 1983). Values lower than 5% were estimated for the following bands: APH-a (entries 137 and 146), APH-b (entries 104 and 137), SKD-a (entries 146 and 53), END-a (entry 137) and DIAP-g (entries 53, 80 and 137). The bands APH-g-h, PGD-b-c-d, AAT-a-d, SKD-b-d-e, END-c, G6PDH-a-c-d, GDH-a-c-d, DIAP-a b-c-e-h and LAP-b-c were found in all entries. The average values of the bands for each enzyme system ranged from 4.3 to 4.7.

Table 2. Band frequencies for 9 enzyme systems in 12 *Lathyrus sativus* L. entries (ACL number, see Table 1). Some bands are grouped signifying that frequencies were all of value 1.

Enzyme	Band	Entry												Mean
		104	109	130	137	146	2	29	53	77	80	94	95	
APH	**a**	0.10	0.30	0.60	0	0	0.10	0.20	0.30	0.40	0.60	0.50	0.70	0.32
	b	0.07	1	0.90	0	0.70	0.60	0.50	1	0.70	0.70	0.80	1	0.41
	c	1	0.70	0.70	1	1	1	1	0.60	0.80	0.90	0.70	0.50	0.41
	d	0.50	0.80	1	0.90	0.60	0.60	0.90	0.70	0.90	0.50	0.40	0.80	0.63
	e	0.80	1	1	0.70	0.90	1	0.90	1	1	1	0.90	1	0.35
	f	1	0.60	0.70	1	0.90	1	0.70	0.80	0.80	0.80	1	0.50	0.48
	g&h	1	1	1	1	1	1	1	1	1	1	1	1	1
PGD	**a**	0.50	0.50	0.40	0.50	0.50	0.50	0.50	0.50	0.60	0.30	0.50	0.60	0.49
	b,c&d	1	1	1	1	1	1	1	1	1	1	1	1	1
AAT	**a&d**	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	0.60	0.50	0.60	0.40	0.60	0.50	0.70	0.70	0.60	0.60	0.60	0.70	0.59
	c	0.30	0.20	0.30	0.20	0.20	0.10	0.30	0.40	0.30	0.40	0.10	0.30	0.26
SKD	**a**	0.80	0.90	0.30	0.60	0	0.90	0.20	0	0.80	0.30	0.60	0.70	0.51
	b,d&e	1	1	1	1	1	1	1	1	1	1	1	1	1
	c	0.40	0.30	0.20	0.50	0.40	0.20	0.20	0.60	0.50	0.70	0.10	0.60	0.39
END	**a**	0.10	0.30	0.20	0	0.10	0.10	0.30	0.20	0.30	0.10	0.40	0.10	0.18
	b	0.70	0.80	0.50	0.50	0.90	0.90	0.70	0.80	0.70	0.40	0.70	0.50	0.68
	c	1	1	1	1	1	1	1	1	1	1	1	1	1
G6PDH	**a,c&d**	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	0.60	0.20	0.60	0.30	0.50	0.40	0.40	0.60	0.40	0.40	0.50	0.60	0.46
GDH	**a,c&d**	1	1	1	1	1	1	1	1	1	1	1	1	1
	b	0.60	0.40	0.40	0.40	0.60	0.40	0.60	0.70	0.50	0.40	0.50	0.60	0.51
DIAP	**a,b,c,e&h**	1	1	1	1	1	1	1	1	1	1	1	1	1
	d	0.40	0.50	0.50	0.50	0.40	0.40	0.40	0.40	0.30	0.30	0.60	0.40	0.43
	f	0.50	0.50	0.40	0.40	0.40	0.40	0.60	0.50	0.60	0.30	0.50	0.40	0.46
	g	0.20	0.10	0.20	0	0.10	0.20	0.20	0	0.20	0	0.20	0.20	0.13
LAP	**a**	0.50	0.60	0.60	0.60	0.80	0.80	0.60	0.30	0.40	0.60	0.50	0.30	0.55
	b&c	1	1	1	1	1	1	1	1	1	1	1	1	1
Mean alleles per locus		**4.7**	**4.7**	**4.7**	**4.3**	**4.5**	**4.7**	**4.7**	**4.7**	**4.7**	**4.7**	**4.6**	**4.7**	

The Shannon-Weaver diversity index H' was calculated to compare the diversity expressed by the enzyme systems and the entries (Table 3). For a single enzyme system a low H' value indicates unbalanced frequency classes and lower diversity levels. On the contrary, higher H' values indicate a greater balance among frequency classes and greater diversity levels. The average value of H', estimated for each entry, ranged from 0.56 (entry 2) to 0.89 (entry 137). Similarly, the average H' value for each enzyme system ranged from 0.28 (END) to 0.94 (G6PDH). Lower H' values were estimated for APH system except for entry 146 (0.61); for the latter enzyme system, the entry 137 was monomorphic (1.00).

A high H' value was estimated for the PGD system in all entries except for entries 130 and 80, which were monomorphic. On the contrary, the AAT system was polymorphic in entries 2, 29, 53, 94, 95 and 146.

For both SKD and END systems lower H' values were estimated in all entries. In particular, the entries 146 and 53 resulted monomorphic for the SKD system, and the entry 137 for the END system. The highest H' values were estimated for both enzyme systems G6PDH and GDH but not in all entries. The latter systems showed the highest number of monomorphic entries with an index value of 1.

Table 3. Shannon-Weaver (H') diversity indices for 9 enzyme systems observed in 12 *Lathyrus sativus* L. entries (see Table 1).

Entry	Enzyme									Mean
	APH	PGD	AAT	SKD	END	G6PDH	GDH	DIAP	LAP	
ACL 104	0.19	0.93	0.96	0.21	0.20	*0.81*	0.81	0.34	0.94	0.60
ACL 109	0.22	0.93	0.96	0.17	0.23	0.88	*1.00*	*0.31*	0.81	0.61
ACL 130	0.18	*1.00*	0.96	0.26	0.28	*0.81*	*1.00*	0.34	0.81	0.63
ACL 137	*1.00*	0.93	*1.00*	0.24	*1.00*	*1.00*	*1.00*	*1.00*	0.81	*0.89*
ACL 146	0.61	0.93	0.90	*1.00*	*0.13*	0.94	0.81	0.32	*0.50*	0.68
ACL 2	*0.17*	0.93	*0.83*	*0.15*	*0.13*	*1.00*	*1.00*	0.35	*0.50*	*0.56*
ACL 29	0.22	0.93	0.90	0.24	0.26	*1.00*	0.81	0.33	0.81	0.61
ACL 53	0.22	0.93	0.90	0.97	0.21	*0.81*	*0.69*	*1.00*	*1.00*	0.74
ACL 77	0.22	*0.81*	0.96	0.19	0.26	*1.00*	0.94	0.33	*1.00*	0.63
ACL 80	0.24	*1.00*	0.96	0.23	0.16	*1.00*	*1.00*	*1.00*	0.81	0.71
ACL 94	0.25	0.93	*0.83*	0.20	0.26	0.94	0.94	0.32	0.94	0.62
ACL 95	0.23	*0.81*	0.90	0.21	0.25	*0.81*	0.81	0.35	*1.00*	0.60
Mean	**0.31**	**0.93**	**0.93**	**0.34**	**0.28**	**0.94**	**0.88**	**0.50**	**0.81**	**0.66**

The DIAP system showed generally medium to low H' value and three entries (137, 53 and 80) resulted monomorphic. Similarly, the last system, LAP, showed medium to high H' values except three entries (523, 77 and 95) which resulted monomorphic.

Figure 1 is a phenogram constructed using Ward's minimum variance method. The most appropriate number of clusters was found by looking for a consensus among the four statistics R^2 (RSQ), cubic clustering criterion (CCC), pseudo-F (PSF) and pseudo-t^2 (PST^2) [9]. Consequently, four main clusters may be identified, which explain a moderate variance (49%).

Five of the twelve entries are included in cluster III (29, 77, 94, 109 and 130). Cluster I is represented by one entry which is very distant from the other clusters. Entries 53 and 95 are included in cluster II. The cluster IV includes four entries with entry 137 being more distant from the other entries (2, 104 and 146).

Conclusions

Despite the limited number of tested entries and isozyme loci examined in this study, some useful conclusions may be drawn. *Lathyrus sativus* entries showed a wide polymorphism for all the enzyme systems being tested. Other similar results have been reported for *L. sativus* [1,16]. A total of 43 bands have been observed, including 19 with different frequency percentage, and 24 which were always present. No variation was observed in the average number of bands/enzyme.

A cluster analysis revealed four distinct groups in which entries revealed a greater similarity. In other words, the entries belonging to a cluster showed more similar patterns for the enzyme systems compared to those located in different clusters. None of the nine enzyme systems observed was exclusive to define one or more groups. In fact, all the enzyme systems showed a similar trend of variation in the groups obtained.

The wide intra-population variation observed suggests the opportunity to extend the survey to wider segments of the collection. More detailed studies including more differentiated materials could be useful to investigate the associations among morpho-agronomic and isozyme traits. Moreover, the inclusion of materials from different geographical regions could be useful to ascertain the available variation and its distribution. Finally, the lack of information on the genetic control of single enzymes in *Lathyrus* has not allowed the examination of allelic frequencies. Future research should be directed to more accurate investigations to elucidate the genetics of different enzyme systems.

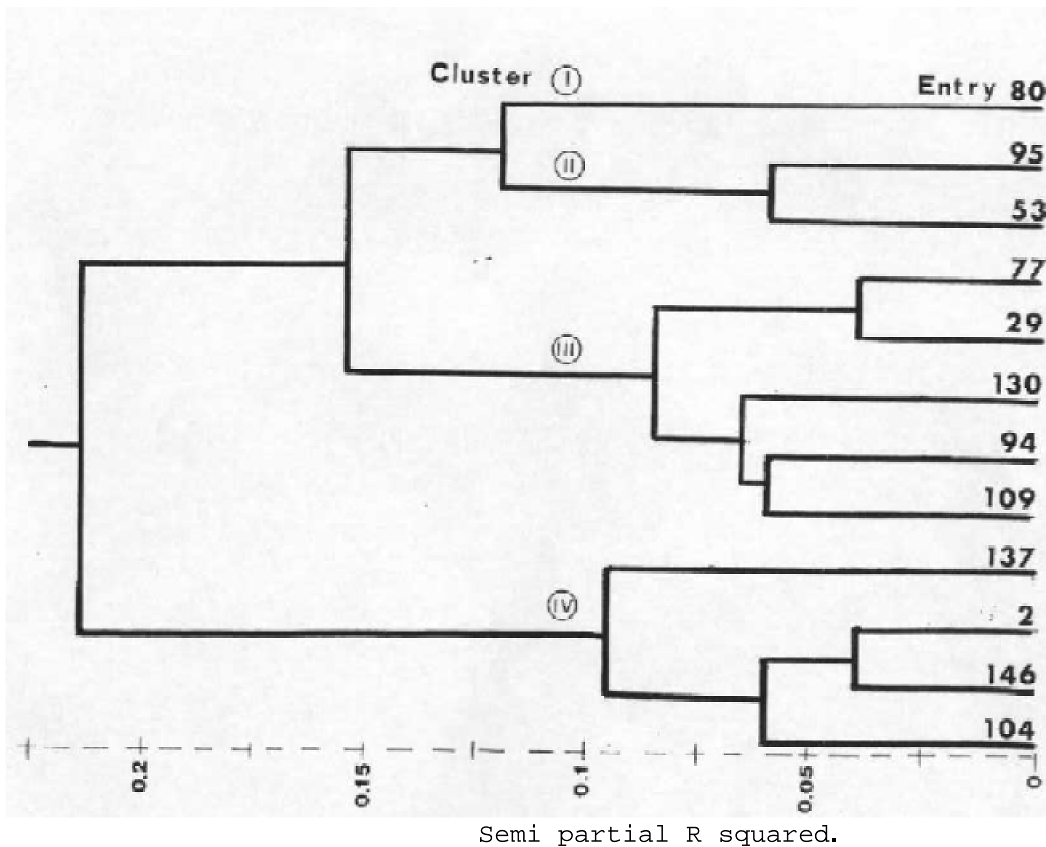

Fig. 1. Phenogram from cluster analysis of twelve *Lathyrus sativus* L. entries (ACL number, see Table 1) based on a distance matrix of allele frequencies at 19 polymorphic loci.

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