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Genotyping of Low β-ODAP Grass Pea (*Lathyrus sativus* L.) Germplasm with EST-SSR Markers

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HIGHLIGHTS

- 22 low β-ODAP grass pea accessions present a wide variation.
- The mean polymorphic information content (PIC) value is 0.49.
- Genetic distances are higher between GP6/GP11, GP4/GP11 and GP5/GP8 accessions.

Abstract: Grass pea (*Lathyrus sativus* L.) is an important protein source in arid regions as both human and animal food. Despite its significance, the use of grass pea is limited by the presence of β-N-oxalyl-L-a,b-diaminopropionic acid (β-ODAP) which can cause neurological disorders. Breeding studies in grass pea have therefore focused on developing high-yielding varieties with low β-ODAP content. However, the narrow range of genetic diversity and the restricted genomic tools in grass pea have slowed progress in such breeding. The present investigation was conducted to explore the genetic diversity of low β-ODAP germplasm consisting of 22 accessions with 31 EST-SSR markers. The molecular analyses revealed a total of 133 alleles ranging from 142 to 330 bp with a mean number of alleles per locus of 4.29. The mean polymorphic information content (PIC) value was calculated as 0.49, and the EST-SSRs in loci S5, S6 and S116 were of the most informative PICs. A dendrogram based on Nei's genetic distance matrix revealed that breeding lines were grouped in two main clusters. Genetic distances were higher between GP6/GP11, GP4/GP11 and GP5/GP8 accessions which could be further used in crop improvement studies for developing wider genetic diversity.

Keywords: genetic diversity; microsatellite; molecular selection.

INTRODUCTION

The genus *Lathyrus* belongs to the Leguminosae family, and consists of about 160 annual and perennial species [1]. Grass pea (*Lathyrus sativus* L.) is a member of the genus *Lathyrus*, and has high protein and carbohydrate content of up to 31% and 65% of the seeds, respectively [2,3]. This legume crop has many agricultural advantages such as tolerance to drought and water-logging, nitrogen fixation and high grain-yielding capacity. Grass pea plays a vital role as human food in rural areas because its seeds are used as a major protein source by the poor and as survival food during drought and famine [4].

Despite its desirable traits, the use of grass pea is limited by the presence of a neurotoxic, β-N-oxalyl-L-a,b-diaminopropionic acid (β-ODAP), which can cause the neurological disorder, lathyrism, through excessive consumption of seeds [5,6,7,8]. β-ODAP accumulates in all tissues of grass pea during all stages of development, although the highest concentrations have been reported in young seedlings [9,10] and seed embryos [5]. Therefore, the main objectives of worldwide grass pea breeding programs tend toward develop high-yielding varieties with <0.2% β-ODAP content [11,12]. Some promising lines with low β-ODAP and high yield have been evaluated in different countries [4,13]. However, the polygenic nature of β-ODAP content [14,15], the narrow range of current genetic variation of grass pea [13] and environmental effects [16] make breeding studies difficult. So far, no toxin-free varieties have been developed [4].

Wider genetic diversity is significant for assessment of plant genetic resources, and for identifying the differences of genotypes [17]. The measurement of genetic diversity using morphological traits is direct, inexpensive and easy. However, these traits are highly affected by the environment or by cultural practices [18,19]. Molecular markers provide huge information and they are highly effective and non-sensitive to environmental fluctuations changes compared to agronomic traits [20]. However, most genetic diversity studies have been conducted using morphological traits in grass pea and little information is available at the molecular level, as with many other orphan legume crops [21]. Molecular markers such as RAPD [22], RFLP [23] and AFLP [24] have been used to determine genetic relationships and diversity in grass pea. Among the molecular markers, SSRs and EST-SSRs have specifically been selected by breeders because of their polymorphic character and co-dominant inheritance, as well as the large number of alleles per locus and abundant in distribution throughout the genome [25,26]. They are also useful for genetic diversity studies [27] in spite of the fact that there are limited studies on genetic diversity specifically in grass pea using SSR and EST-SSR markers [21,25,28,29,30].

The demand for resilient food crops are increasing therefore improvement of grass pea is still considered a priority by international breeding studies [31]. Information about the genetic variation of low β-ODAP population is a great breeding value to exploit the genetic resource and identify promising lines. From this perspective, the current study evaluated the genetic diversity of a low β-ODAP population consisting of 22 grass pea lines using EST-SSR markers. The data generated in our study will be useful in the utilization of this specific grass pea population, and in selecting diverse parental lines for the future improvement of low β-ODAP and high-yield grass pea genotypes.

MATERIAL AND METHODS

Plant Material

The genetic material used in the study was comprised of 22 grass pea accessions originating from Bangladesh (Table 1). The seeds were collected from each accession for β-ODAP content calculation and it was determined by spectrophotometrically [32]. This low β-ODAP content germplasm is maintained by the ICARDA genebank. Detailed information about quality and quantity traits for this germplasm has been reached in ICARDA international nurseries data management system (http://indms.icarda.org). The seeds of the grass pea accessions were germinated in the experimental fields of Akdeniz University, Antalya, Turkey (36°53´N, 38°30´E and altitude 33 m) during the 2018 growing season. Grass pea leaves were collected from plants and stored at -80 °C for DNA extraction.

Table 1. The list of low β-ODAP grass pea germplasm used in present study

Genotypes	Accession number	ICARDA Gen-bank Number	β-ODAP con-centration (% weight)
GP2	Bang-190	117250	0.386
GP3	Bang-234	117378	0.408
GP4	Bang-186	117241	0.566
GP5	Bang-237	117388	0.316
GP6	Bang-187	117243	0.244
GP7	Bang-202	117283	0.415
GP8	Bang-235	117381	0.267
GP10	Bang-182	117209	0.428
GP11	Bang-255	117424	0.319
GP12	Bang-290	117524	0.368
GP13	Bang-309	117586	0.564
GP14	Bang-261	117440	0.566
GP15	Bang-313	117601	0.352
GP16	Bang-241	117400	0.497
GP17	Bang-265	117454	0.321
GP18	Bang-310	117594	0.573
GP19	Bang-185	117229	0.587
GP20	Bang-188	117248	0.583
GP21	Bang-191	117251	0.504
GP22	Bang-233	117375	0.388
GP23	Bang-242	117401	0.565
GP24	Bang-249	117408	0.448

Molecular Study

The leaves from three plants of each accession were mixed for genomic DNA extraction. The CTAB procedure [33] was used for DNA isolation with minor modifications. Integrity and quality of DNA were evaluated by electrophoresis on 1% w/v agarose gels by comparison with a DNA standard.

The set of 31 EST-SSR primers [28] used in this study from *Lathyrus* genus ESTs and was sourced from the NCBI database. The sequences of primers, repeat motifs and putative functions of each locus are given in Table 2.

Table 2. The information of 31 EST-SSR markers used in this study.

Locus	Primer sequences (5'-3')	Repeat motif	GenBank acc. no	Putative function[1]
S5	F: TGTGGGGCTTGTTACACTGA R: AGCTACCATAACAGACAAAACC	$(GT)_8$	DY396423	gonadotropin beta chain
S6	F: CTGCAACAAGAAGCCATTCA R: TATGGGTCCGTCGGAATAAC	$(CT)_9$	GO317614	conserved hypothetical protein
S7	F: CAATAGGGGCCCCACTAAAT R: CGACGGTTAGGAAACAGAGC	$(AAC)_5$	GO317636	unknown
S19	F: CGATGCCAAATATGGAGGTT R: GAAGCACCACCATGTCCAC	$(TGG)_5$	GO316451	dormancy-associated protein
S33	F: TGGTTTTGTTGTGGAAAGTGAG R: ACTGCAAAAGCCTCAAAGGA	$(TTTTG)_3$	GO315340	conserved hypothetical protein
S43	F: GCTCTCAGCCTCACCGTAAC R: CTCAATTAGCGTCCGGGTAA	$(AGTTCT)_3$	GO317439	predicted protein
S52	F: CGCCCCTCATCTTATCATTC R: GTTGTTGGGTGAAGGAATCG	$(CTCGCT)_3$	GO316642	inositol phosphate kinase
S63	F: ACAGCGAAACCCTCATTCAG R: AGAGTGCCGTTAGGGGATTT	$(TCT)_6$	GO317080	Conserved hypothetical protein
S70	F: GTGCAACCTTTCATCAATCA R:CGGTGAAGCTAAAGAAGAAGAA	$(GGTTT)_3$	GO315117	bZIP transcription factor bZIP11
S83	F: TCTTCCAACCCAAACCCTAA R: GACGACGGTTTGAACCTGAT	$(CTC)_5$	GO314361	predicted protein
S97	F: GGTTTCTTCTCACGGGTGTT R: GAAATACTTGAACCACCACTTCC	$(GAA)_5$	GO316810	predicted protein
S98	F: GTCCCCACGTTAACTGAAGC R: GACGTAGGGTTGTTGCGATT	$(GATCA)_4$	GO316847	conserved oligomeric Golgi complex
S102	F: GTTGGGAATCCGTCTTCAAA R: GTCGAGAGAGGTGAGGTTGG	$(CGACGG)_3$	GO319283	leucine-richrepeat
S103	F: CCTCCGACATGTCCATCATT R: TCAGCATTGTGTTGGTCGAT	$(CAA)_5$	GO318479	cysteineprotease, putative
S114	F: CTAGCATCCCGCCATTTATC R: AACATTTCAGCCCTGACCAC	$(CCT)_5$	GO318925	GTP-binding signal Rec. particle SRP54
S116	F: TCGAGAGACTTCTCTTCTCTCCTC R: CGACATCTGACCATCCATTG	$(CTT)_6$	GO318959	unknown
S117	F: CATTATCTTCCCCAGCTCCA R: AAGGGATGAGCCACAGAATG	$(CCTCCA)_3$	GO318966	glutathione peroxidase, putative
S118	F: TTCCCTCCCTTTCCTTCACT R: TGTTTGGTTTGGTTTGGTGA	$(TTGTC)_3$	GO319719	class II KNOX homeobox transcription factor
S119	F: CCCTCCCCTTCTTCATCTTC R: TTGTGAAAGATGCTGGTTGTG	$(ACCAA)_3$	GO319719	class II KNOX homeobox transcription factor
S135	F: CACATAGCAAAAACGCTAGGC R: CTCCGACGAGAGATGATTCG	$(ACA)_5$	GO320656	PREDICTED: hypothetical protein
S137	F: GAGATCATCTTTGTCGGTGGA R: TTCAGTAGGACCCAGCAACC	$(ATTTCC)_3$	GO319063	Glycoside transferase, six-hairpin, subgroup
S142	F: TTGAATCCAACGGAAGATCC R: CAGTTCCCGGTCAGAACAAT	$(GATTCT)_3$	GO318214	conserved hypothetical protein
S147	F: CCAGGGAGGACTATCACGAA R: AAACAGAAACGCGATGAACC	$(CTT)_8$	GO319933	PREDICTED: hypothetical protein
S155	F: CGACGATCTACAACCACCAA R: TGGAAGGAGGGAAGAGAGGT	$(CAC)_6$	GO317900	Serine/threonine protein kinase, active site

Continuation of table 2

Locus	Primer sequences (5'-3')	Repeat motif	GenBank acc. no	Putative function[1]
S156	F: GCCGAACCAGAAAACACAAT R: TCGGTGCTCATAATTCCAGA	$(AAG)_5$	GO320751	PREDICTED: hypothetical protein
S159	F: CGATCTCCCACTTTAGAAAACA R: GGTGGCTATAACCAATGATGG	$(AAAC)_4$	GO321140	putative plasma membrane intrinsic protein
S163	F: ACACAGAAACACCACCACCA R: GATTTTCCGTAACGGCTTGA	$(CAT)_5$	GO318661	AT2G42690
S165	F:CGCTCTAGAGATAGGAGAGAGAGG R: GCTTCACAAGAACCCATTTCA	$(GA)_8$	GO318696	Nucleic acid binding protein, putative
S167	F: TGACTTCTTGCTCTCCCATGT R: GAGAGTGTAGGCGGAACCTG	$(TCC)_5$	GO319158	PREDICTED: hypothetical protein
S168	F: CCAAAACGACACCCCCTTAT R: TGTGACATACAAACGGAAGC	$(ATCAAA)_3$	GO319159	unknown
S177	F: GGGAGTGAATCAAAACCAACA R: GAAGTTCTCCGTAGCGGTGT	$(AAAG)_4$	GO320015	gamma-glutamylcysteine synthetase precursor

[1]Ref. [28]

Polymerase chain reactions (PCR) were performed in 10 µL reaction volumes consisting of 1µL of 10X PCR buffer, 0.3 mM of dNTPs mix, 2.5 mM of $MgCl_2$, 0.3 µM of each primer, 1 unit of Taq DNA polymerase (DreamTaq DNA Polymerase, Thermo Fischer Scientific Biosciences, Germany), 1 µL genomic DNA template and Milli-Q water to a final volume of 10 µL.

The PCR cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, annealing at the optimized temperature for each primer pair for 45 s and 72 °C for 45 s; the last cycle ending with a five-minute at 72 °C. PCR amplified products were electrophoretically separated on 2% agarose gel and then visualized under UV light to validate band amplification. The expected bands were also monitored in the Fragment Analyzer™ (Advanced Analytical Technologies GmbH, Heidelberg, Germany) for accurate sizing and relative quantification of fragments. The DNF-905-K1000 reagent kit was used for qualitative analysis. The gel, inlet buffer, capillary conditioning solution and marker were prepared according to manufacturers' instructions. The data were normalized to 1 bp (lowest) and 500 bp (highest) and calibrated to the 1–500 bp range DNA ladder. The virtual gel image was analyzed and scored using the software PROSize 2.0 (Advanced Analytical Technologies, AMES, IA, USA).

Data Analysis

The EST-SSRs generated unambiguous bands of different molecular weights that were handled for data analysis. The POPGENE package (version 3.2) [34] was used to calculate the number of alleles, effective number of alleles, expected, observed heterozygosity and Wright's fixation index (F) for each locus. Shannon's information index value was also calculated according to Shannon and Weaver [35]. The genetic distance among genotypes were calculated based on Nei's parameters [36]. To determine the genetic relationships among the breeding lines studied, a phylogenetic dendrogram was constructed from the molecular phylogenic tree using the unweighted pair group method with arithmetic mean. MEGA software [37] was used to modify the constructed phylogenetic tree. The polymorphic information content (PIC) value of each EST-SSR was identified using the Excel MS Toolkit (version 3.1) [38]. A principal coordinate analysis (PCoA) was performed with the PAST (version 3.23).

RESULTS

A total of 22 grass pea accessions were fingerprinted using 31 EST-SSR markers. PCR products were obtained for all the studied loci (Figure 1) and a considerable level of variability was observed among the breeding lines. Table 3 presents the genetic parameters of size range, number of alleles (no), effective number of alleles (ne), observed (Ho) and expected (He) heterozygosity, polymorphic information content (PIC) and Shannon's index value. All the studied EST-SSR loci were polymorphic, yielding 133 amplification fragments ranging from 142 to 330 bp. The mean number of alleles per locus was 4.29. The highest number of alleles (10) was detected in locus S6, which was followed by 8 alleles in both locus S5 and locus S116. The

lowest (2) was observed in loci S117, S142, S163, S167, S168 and S177. The effective number of alleles varied from 1.09 for locus S177 to 8.00 for locus S6, with an average of 2.76.

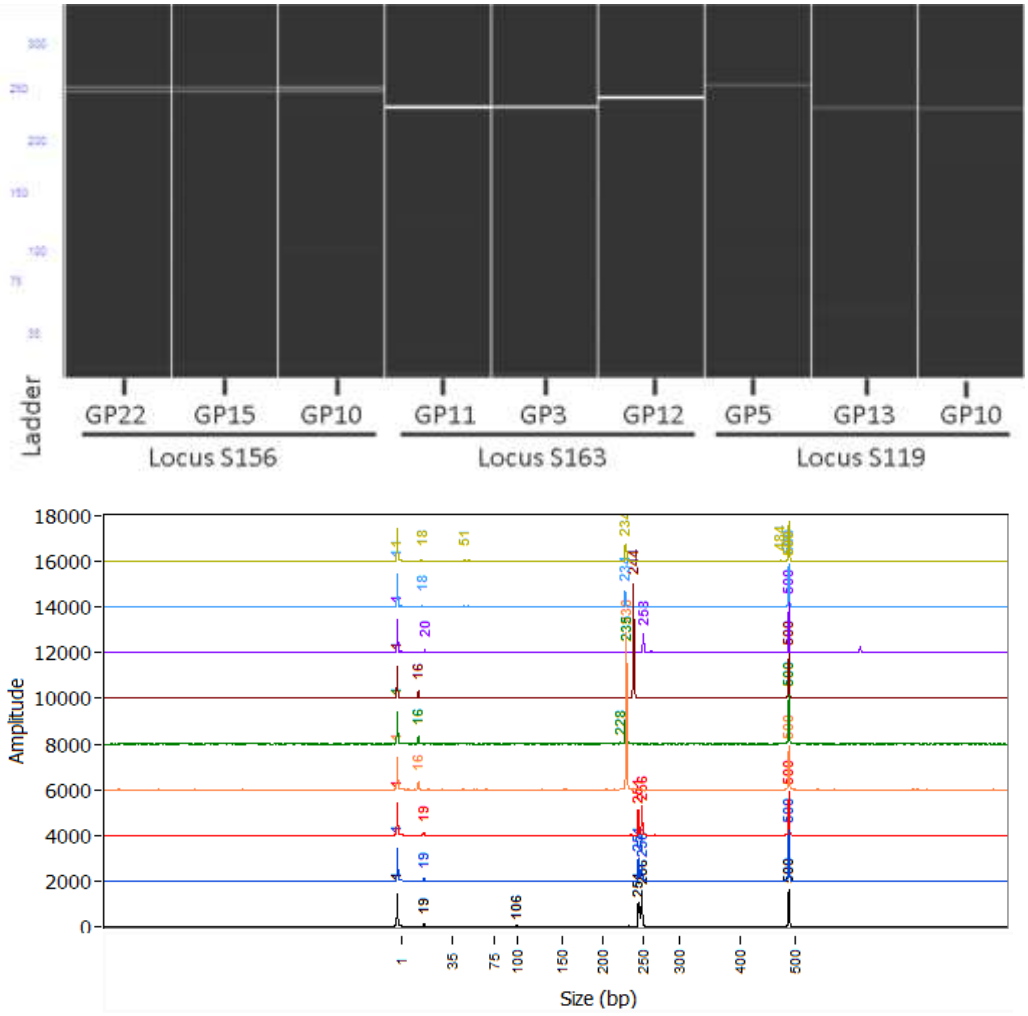

Figure 1. Fragment Analyzer™ shows the gel picture and peak analysis of EST-SRR profiles for selected grass pea breeding lines. Amplifications performed with the markers in loci S156, S163 and S119 with a 1-500 bp ladder.

Table 3. The genetic parameters for 31 EST-SSR loci studied in 22 grass pea accessions

EST-SSR locus	Size range	Na[1]	Ne	Ho	He	PIC	*I*	F
S5	169-205	8	5.63	0.40	0.84	0.80	1.85	0.51
S6	191-211	10	8.00	0.00	0.90	0.86	2.18	1.00
S7	213-231	7	4.84	0.90	0.81	0.77	1.74	-0.1
S19	225-243	5	2.09	0.47	0.54	0.49	1.03	0.09
S33	223-243	5	3.22	0.29	0.71	0.65	1.35	0.57
S43	155-173	4	2.07	0.47	0.54	0.48	0.99	0.09
S52	228-246	4	3.11	0.25	0.70	0.63	1.25	0.63
S63	234-240	3	1.58	0.00	0.38	0.34	0.68	1.00
S70	243-263	3	1.21	0.00	0.18	0.17	0.38	1.00
S83	299-308	4	3.25	0.89	0.71	0.64	1.26	-0.2
S97	183-198	7	3.67	0.76	0.75	0.69	1.50	-0.1
S98	162-172	3	1.70	0.14	0.42	0.37	0.72	0.65
S102	198-210	3	2.17	0.00	0.55	0.48	0.91	1.00
S103	201-222	6	3.28	1.00	0.71	0.65	1.37	-0.4
S114	270-288	7	3.88	1.00	0.76	0.70	1.54	-0.3
S116	309-330	8	5.56	1.00	0.85	0.80	1.84	-0.2
S117	288-294	2	1.15	0.14	0.14	0.12	0.25	-0.1
S118	155-170	4	2.76	1.00	0.66	0.57	1.13	-0.5
S119	233-258	5	3.83	0.28	0.76	0.70	1.44	0.62
S135	188-194	3	2.11	0.00	0.54	0.43	0.83	1.00
S137	227-245	4	2.29	0.36	0.58	0.52	1.05	0.35
S142	142-148	2	1.84	0.05	0.47	0.35	0.65	0.89
S147	147-159	4	1.69	0.17	0.42	0.38	0.80	0.59
S155	208-220	4	1.61	0.18	0.39	0.36	0.75	0.52
S156	250-256	3	2.57	0.95	0.63	0.53	1.00	-0.5
S159	251-263	4	3.25	0.36	0.71	0.64	1.27	0.47
S163	235-244	2	1.89	0.29	0.48	0.36	0.66	0.39
S165	221-235	3	1.20	0.00	0.17	0.16	0.36	1.00
S167	227-236	2	1.56	0.00	0.37	0.30	0.54	1.00
S168	256-258	2	1.47	0.00	0.33	0.27	0.50	1.00
S177	233-237	2	1.09	0.00	0.09	0.08	0.18	1.00
Mean		4.29	2.76	0.37	0.55	0.49	1.03	0.41

[1]Na number of alleles, Ne effective number of alleles, He expected heterozygosity, Ho observed heterozygosity, PIC polymorphism information content, I Shannon's Information index, F Wright's fixation index.

The mean PIC value was calculated as 0.49, and the EST-SSRs in loci S5 (0.80), S6 (0.86) and S116 (0.80) were determined to be the most informative with regard to PIC values. In addition, 13 markers were observed as more informative by showing PIC values above 0.5. The EST-SSR in locus S177 had the lowest PIC value of 0.08. The highest and lowest expected heterozygosity were obtained in loci S6 and S177 with values of 0.86 and 0.09, respectively, with an average of 0.55. The highest observed heterozygosity value was obtained in loci S103, S114, S116 and S118, and the lowest was seen in loci S6, S63, S70, S102, S135, S165, S167, S168 and S177, with a mean value of 0.37. The highest Shannon's index (I) value was determined in locus S6 (2.18), while locus S177 had the lowest value (0.18), with a mean of 1.03 across the EST-SSR loci (Table 3). The fixation index (F) ranged from −0.5 for S118 and S156 to 1.00 for S6, S63, S70, S102, S135, S165, S167, S168, S177 with an average of 0.41. The genetic distances between accessions were minimal and maximal for GP4/GP18 and GP6/GP11, respectively (Table 4). Accessions with a genetic distance higher than 0.9 were GP4/GP11, GP5/GP8, GP8/GP11, GP10/GP11, GP11/GP12, GP11/GP18 and GP21/GP24.

The dendrogram based on Nei's genetic distance matrix revealed that the 22 accessions were grouped in two main clusters (I and II) including 20 and 2 breeding lines, respectively (Figure 2). Cluster I was further divided into four sub-clusters; IA consisting of 6 breeding lines, IB of 8 and IC of 5 lines. Sub-cluster ID included one breeding line, GP13, which was not grouped. Cluster II consisted of the accessions GP11 and GP14. Principal coordinate analysis (PCoA) indicated the existence of four subgroups among 22 accessions of our study (Figure 3). The first second coordinated explained 11.0% and 9.45% of variation, respectively. The grass pea panel was also divided into three subgroups.

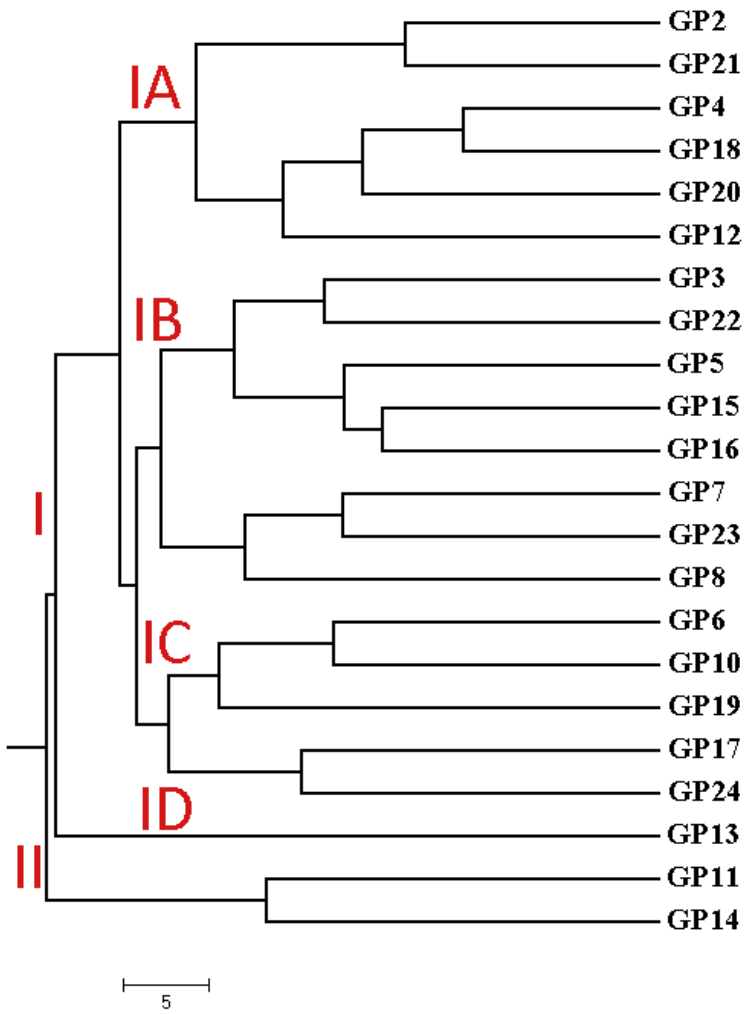

Figure 2. UPGMA dendrogram showing genetic relationships among 22 accessions of grass pea accessions using 31 EST-SSR markers

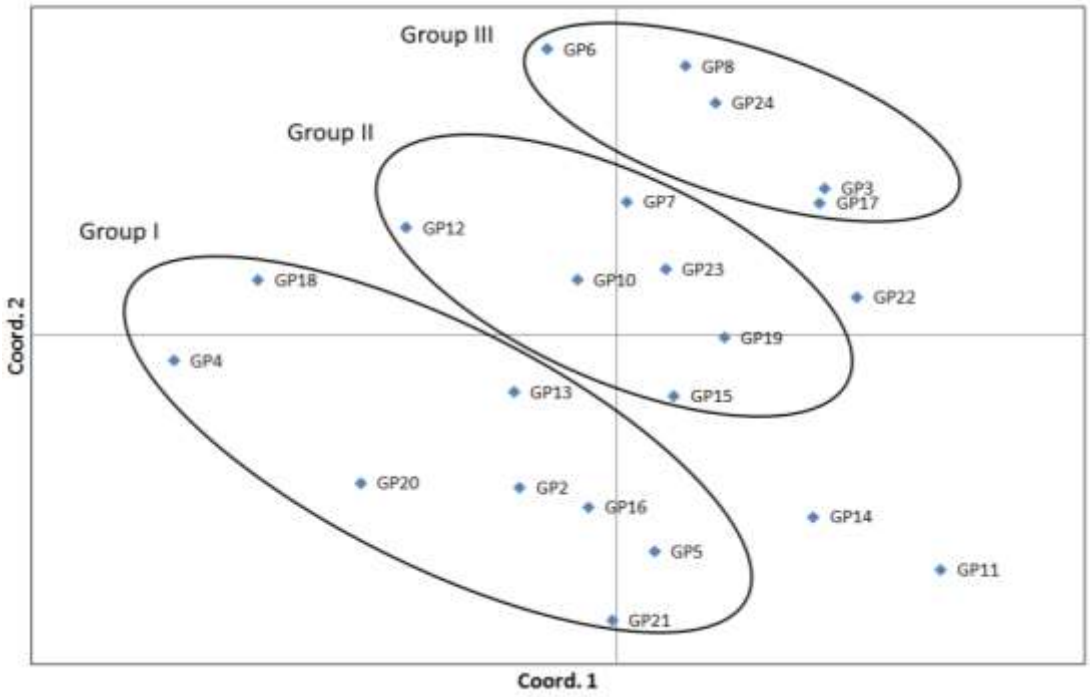

Figure 3. Principal coordinate analysis (PCoA) of 22 sesame accessions based on 31 EST-SSR markers.

DISCUSSION

Wider genetic diversity is highly important for sustainable plant breeding programs because it allows identification and improvement of desirable traits, especially relating to yield, human diet and resistance to biotic and abiotic stresses. Grass pea is a neglected crop, and enhanced genetic diversity would contribute to the utilization of this crop for breeding studies [30]. Limited genetic and genomic resources are available in the public domain for *Lathyrus*, and the application of established molecular markers in the estimation of genetic variation is a promising way of demonstrating the genetic potential of this legume crop [29].

The present investigation showed that EST-SSRs are useful tools for the diagnosis of genetic diversity in grass pea. The average number of alleles obtained in our germplasm (4.29) was higher than studies reported by Shiferaw and coauthors [21] and Gupta and coauthors [25] who detected 4.0 and 3.72 alleles per locus using 11 and 18 EST-SSRs, respectively. Similarly, lower value was observed in grass pea panel which was screened with 288 microsatellites [39]. An average number of alleles per locus of 8.6 has been reported in a highly diverse grass pea collection consisting of 283 genotypes and screened with 30 SSR loci [30]. This larger number of alleles per locus might result from differences in the number of genotypes with diverse geographical origins [21,40].

A low average PIC value of EST-SSR markers was observed in the present study (0.49), and similar low percentages have been reported in grass pea [29,41] and other related grain legumes such as pea [42] and faba bean [43]. This might be caused by the genetic structure of EST-SSR primers which tend to be less polymorphic in crops because of greater DNA sequence conservation in transcribed regions [26,44,45]. The average observed heterozygosity value was 0.37 in our collection, and this was higher than Italian grass pea germplasm [46] and comparable to reported values of 0.39 and 0.40 in Ethiopian grass pea germplasms studied by Ponnaiah and coauthors [47] and Shiferaw and coauthors [21], respectively.

Table 4. Genetic distance matrix for 22 accessions based on EST-SSRs

GP3	GP4	GP5	GP6	GP7	GP8	GP10	GP11	GP12	GP13	GP14	GP15	GP16	GP17	GP18	GP19	GP20	GP21	GP22	GP23	GP24	
0.60	0.40	0.50	0.50	0.50	0.80	0.41	0.64	0.53	0.71	0.54	0.44	0.43	0.67	0.53	0.62	0.30	0.30	0.56	0.50	0.67	GP2
	0.70	0.60	0.50	0.50	0.70	0.56	0.68	0.53	0.82	0.51	0.33	0.59	0.55	0.69	0.48	0.72	0.89	0.39	0.47	0.48	GP3
		0.60	0.60	0.50	0.80	0.51	1.03	0.34	0.55	0.70	0.52	0.51	0.89	0.23	0.69	0.25	0.56	0.85	0.64	0.58	GP4
			0.90	0.60	1.00	0.66	0.50	0.64	0.64	0.65	0.40	0.33	0.50	0.75	0.71	0.52	0.53	0.60	0.54	0.73	GP5
				0.50	0.60	0.38	1.23	0.69	0.78	0.79	0.64	0.72	0.64	0.63	0.64	0.71	0.90	0.60	0.70	0.53	GP6
					0.40	0.40	0.66	0.41	0.70	0.55	0.47	0.50	0.68	0.50	0.45	0.59	0.63	0.57	0.37	0.56	GP7
						0.71	0.95	0.73	0.86	0.82	0.66	0.87	0.64	0.78	0.82	0.73	0.88	0.60	0.58	0.52	GP8
							0.93	0.64	0.74	0.56	0.49	0.58	0.50	0.40	0.39	0.51	0.57	0.79	0.51	0.48	GP10
								1.03	0.69	0.46	0.63	0.73	0.72	1.03	0.60	0.74	0.74	0.61	0.55	0.81	GP11
									0.86	0.70	0.53	0.69	0.65	0.52	0.72	0.46	0.77	0.54	0.66	0.6	GP12
										0.72	0.67	0.62	0.56	0.56	0.74	0.76	0.62	0.77	0.78	0.65	GP13
											0.54	0.67	0.80	0.72	0.68	0.68	0.56	0.45	0.72	0.75	GP14
												0.32	0.61	0.60	0.56	0.38	0.67	0.38	0.34	0.50	GP15
													0.68	0.66	0.68	0.39	0.60	0.52	0.61	0.60	GP16
														0.81	0.64	0.84	0.74	0.61	0.68	0.42	GP17
															0.57	0.45	0.66	0.77	0.50	0.55	GP18
																0.71	0.57	0.73	0.68	0.64	GP19
																	0.54	0.61	0.57	0.74	GP20
																		0.78	0.82	0.95	GP21
																			0.44	0.58	GP22
																				0.55	GP23

The expected heterozygosity was greater than the observed heterozygosity which is similar to other results in grass pea [29], pear [48], apple [49] and pea [50]. The highest number of alleles and expected heterozygosity were observed with the EST-SSR marker in locus S6, which was characterized as the most informative fragment because high values for these parameters exhibit the power of an SSR marker for differentiating accessions [51]. Shannon's information index is an important parameter for illustrating the level of polymorphism in a collection [52]. As expected, because of its higher PIC value and heterozygosity, the highest value for Shannon's information index was obtained with the EST-SSR marker in locus S6. Di nucleotide repeat motifs were observed in the EST-SSR markers in loci S5 and S6, which had the highest number of alleles and PIC values as compared to higher order repeat numbers. These results confirm those from Kumar and coauthors [53] and Shiferaw and coauthors [21] in maize and grass pea, respectively.

The genetic distance values were the lowest and the highest between accessions GP4/GP18 and GP6/GP11, respectively and this relationship was monitored in the phylogenetic tree. The dendrogram also showed that accessions GP11, GP13 and GP14 separated from the other breeding lines, indicating high genetic diversity in the studied germplasm. The results obitaned by PCoA analysis conducted with PAST [54] was also supported that these accessions separated from other groups. Therefore, GP11, GP13 and GP14 should be used as parents in crosses creating wider genetic diversity to obtain possible superior recombinations which have low β-ODAP content. This information is extremely important because limited studies have been conducted to identify genetically distant accessions with low β-ODAP content to improve the agronomic performance of this crop [25]. Crosses between grass pea accessions with low and high β-ODAP content might cause an increase of β-ODAP in F1 and further generations. The genetically distant accessions identified in this study therefore provide the advantage of maintaining low neurotoxin content in hybridizations. In addition, the polymorphic markers used in this study should be used in other grass pea germplasm projects, and might also be transferred to cross species that lack of sequence information.

CONCLUSION

The study showed that SSR based markers have powerful tools to characterize genetic detecting variation and could be used genetic analysis of legume crops [21,27,55,56]. Although grass pea cultivation is very suitable for sustainable farming in Mediterranean climate conditions, a limited study has been conducted to identify superior low β-ODAP genotypes [5,16]. This investigation presented genetically distant genotypes and they should be used as parents in breeding studies in that climate conditions. However grass pea is an industrial crop and information on agronomic traits of all genotypes must be handled for optimal commercial usage.

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