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Genotyping and Assessment of Genetic Diversity in *Pisum* Accessions, using AFLP Markers

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□ ABSTRACT □

In order to identify efficient strategies for the sustainable management of the genetic resources of the landraces, we have investigated in this study the level of genetic diversity in 81 accessions of *Pisum* belonging to the two species *P. sativum* and *P. fulvum*. The accessions had 6 different origins. DNA was extracted and analyzed by amplified fragment length polymorphism (AFLP) technique. Seven primer combinations were used to produce DNA fingerprints for the 81 accessions. 289 polymorphic fragments were identified. The number of polymorphic fragments in the different accessions varied from 46 to 225, and the percentage of polymorphic fragments ranged between 18.93% and 92.59%. A specific DNA pattern for each accession was identified. Based on the data of AFLP, we estimated the genetic distance between the different accessions. The level of genetic diversity detected within species was higher than the one detected between species. The utility of the molecular markers in the development of collection strategies is also discussed in this paper.

Keywords: *Pisum*, Genetic diversity, Molecular markers, AFLP, Germplasm resources.

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البصمة الوراثية وتقدير التباينات الوراثية في مدخلات البازلاء باستخدام مؤشرات الـ AFLP

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□ الملخّص □

تم في هذه الدراسة تقدير مستوى التنوع الوراثي لأحد وثمانين مدخلاً من الجنس *Pisum* ينتمون للنوعين *P. sativum* و *P. fulvum* باستخدام مؤشرات AFLP وذلك بهدف تحديد الية مناسبة للادارة المستدامة للأصول الوراثية. تم جمع العينات من ستة أماكن مختلفة. استخدمت سبعة أزواج من بادئات الـ AFLP في تحليل DNAالمدخلات.

أظهرت النتائج وجود 289 قطعة مختلفة من الـ DNA، وقد تباين عدد هذه القطع ما بين المدخلات المختلفة حيث تراوح عددها ما بين 46 و 225 قطعة، وهذا يقابل نسبة تباينات تتراوح ما بين 39و18% و59و92%. تم تحديد بصمة وراثية خاصة بكل مدخل ومن ثم تم حساب البعد الوراثي بين المدخلات اعتماداً على معطيات تقنية الـ AFLP، و أنشئت المخططات التي تعكس الاختلافات بين أنواع *Pisum* المختلفة. أظهرت النتائج بان مستوى التباين الوراثي ضمن النوع الواحد هو أكبر من ذلك الموجود بين النوعين.

تمت، في نهاية البحث، مناقشة إمكانية الاستفادة من المؤشرات الجزيئية في تطوير طرق والية لجمع العينات النباتية لحفظ الأصول الوراثية للبازلاء.

تم تنفيذ البحث في المركز الدولي للبحوث الزراعية في المناطق الجافة(ايكاردا)، حلب، سوريا عام 2005.

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الكلمات المفتاحية: الجنس *Pisum* ، التباين الوراثي، المؤشرات الجزيئية، تقنية الـ AFLP ، الأصول الوراثية.

1. Introduction:

Peas were reported to be originally cultivated as a winter annual crop in the Mediterranean region (Smart, 1990). It belongs to the genus *Pisum* which contains two species, *P. sativum* and *P. fulvum*, both with 2*n* = 14 chromosomes (Muehlbauer 1991). Cultivated peas are classified within *P. sativum* ssp. *sativum* which contains var. *sativum*, the horticultural types, and var. *arvense*, which are the fodder and winter types.

The biodiversity of a species has built up during millions of years of naturally arising mutations and natural selection. The genetic variation inherent to a species, its biodiversity, is the sum of the different sequences present in all the members of that species. It represents the genetic 'wealth' of the species, allowing adaptation to new environmental stimuli such as habitat change or disease. One of the big problems facing biodiversity research is the measurement of genetic variation across thousands or millions of organisms. Molecular markers are used to assess biodiversity of a variety of crop plant species and their wild relatives. They are employed to detect polymorphism among individuals at the DNA level from which associations pertaining to entire genomes may be inferred (Saiki *et al.* 1988). For this, a number of fast and economical techniques may be used that may or may not require preliminary sequence knowledge. PCR techniques commonly used for diversity analyses are the arbitrary amplification methods of random amplified polymorphic DNA (RAPD) analysis (Welsh and McClelland, 1990; Williams *et al.* 1990), amplified fragment length polymorphism (AFLP) analysis (Vos *et al.* 1995), and other derivatives (Virk *et al.* 1995).

Amplified fragment length polymorphism (AFLP) marker is a molecular fingerprinting technique that can be applied to DNAs of any source or complexity. Total genomic DNA is digested using two restriction enzymes. Double-stranded nucleotide adapters are ligated to the DNA fragments to serve as primer binding sites for PCR amplification. Primers complementary to the adapter and restriction site sequence, with additional nucleotides at the 3'-end, are used as selective agents to amplify a subset of ligated fragments. Polymorphisms are identified by the presence or absence of DNA fragments following analysis on polyacrylamide gels. This technique has been extensively used with plant DNA for the development of high-resolution genetic maps, for the positional cloning of genes of interest and for the estimation of genetic diversity , (He and Prakash 1997; Hongtrakul *et al.*. 1997; Eujayl *et al.*. 1998; Choumane *et al.*. 1998; Gracia-Mas *et al.*. 2000; Teulat *et al.*. 2000; Hayashi *et al.*. 2001, Yin and Boyle 2003; Choumane *et al.* 2004, Hamwieh *et al*. 2005; Madini *et al*. 2005; Barkley *et al.* 2006).

2. Objectives:

The aims of this research were to fingerprinting and to investigating the level of genetic diversity in 81 accessions of *Pisum* belonging to the two species *P. sativum* and *P. fulvum*. This study will contribute to the identification of efficient strategies for the sustainable management of the genetic resources of the landraces.

3. Material and Methods:

3.1. Plant Material

Eighty one accessions belonging to the two species *P. sativum* (69 accessions representing 12 groups) and *P. fulvum* (12 accessions) are used in this study. The species and sub species names, the number of accessions per species and sub species and the collection sites are presented in Table (1). This study was carried on in the biotechnology laboratory at the International Center for agriculture Research in the Dry Areas (ICARDA), in Aleppo, in the year 2005.

3.2. DNA isolation

Seedlings were grown in the glass house for three weeks. Leaves of each accession were harvested separately and ground into a fine powder in liquid nitrogen. Genomic DNA was isolated using a modified CTAB procedure according to Rogers & Bendich (1985).

3.3. Amplified Fragment Length Polymorphism (AFLP) reaction:

The protocol for the AFLP assay was carried out as described by Vos *et al.*. (1995) with minor modifications. 0.5 µg of DNA was digested with the restriction enzymes *Pst*I and *Mse*I. Pre-amplification and selective amplifications were performed as described in the original protocol. Polymorphic bands were detected by silver staining (Bassam et al. 1991). Twenty five primer combinations were tested to screen for polymorphism between the DNA samples. The best seven primer combinations were selected to be used in the analysis of the all collection (Table 2).

The PCR profile for the pre-amplification program was: 30 sec. at 94 ^{o}C, 30 sec. at 60 ^{o}C, 1 min. at 72 ^{o}C, for 30 cycles. The program for selective amplification was the following: 30 sec. at 94 ^{o}C, 30 sec. at 65 ^{o}C, 1 min. at 72 ^{o}C, for one cycle. This was followed by 11 cycles over which the annealing temperature was decreased by 0.7 ^{o}C per cycle followed by 30 sec. at 94 ^{o}C, 30 sec. at 56 ^{o}C, 1 min. at 72 ^{o}C, for 23 cycles. The amplified fragments were electrophoresed on 6% poly-acrylamide gels and stained with silver nitrate (Bassam *et al.*. 1991). The presence and the absence of the bands were visually recorded.

Table 1. Species and sub species of *Pisum* used in this study and their sites of collection.

Provinces	countries	Scientific names	Number	Provinces	countries	Scientific names	Local accession	Number
Aleppo	SYR	*P.sativum*	42	Homs	SYR	*P.sativum sativum*	local 11102	1
Lattakia	SYR	*P.sativum*	43	aleppo	SYR	*P.sativum sativum*		2
Daraa	SYR	*P.sativum*	44	Tartous	SYR	*P.sativum sativum*		3
aleppo	SYR	*P.sativum*	45	Tartous	SYR	*P.sativum sativum*		4
Idlib	SYR	*P.sativum*	46	Damascus	SYR	*P.sativum sativum*		5
Idlib	SYR	*P.sativum*	47	Al Qunaytirah	SYR	*P.sativum sativum*		6
Homs	SYR	*P.sativum*	48	Damascus	SYR	*P.sativum sativum*	local 11014	7
Sweida	SYR	*P.sativum*	49	Al Hasakah	SYR	*P.sativum sativum*		8
Dahuk	IRQ	*P.sativum*	50	Adana	TUR	*P.sativum sativum*		9
Idlib	SYR	*P.sat arvence*	51	Antakya	TUR	*P.sativum sativum*		10
Al Hasakah	SYR	*P.sat arvence*	52	Antalya	TUR	*P.sativum sativum*		11
Tartous	SYR	*P.sat arvence*	53	Tekirdag	TUR	*P.sativum sativum*		12
Antalya	TUR	*P.sat arvence*	54	Homs	SYR	*P.sativum sativum*	local 11091	13
Antalya	TUR	*P.sat arvence*	55	Lattakia	SYR	*P.sativum sativum*		14
Sweida	SYR	*P.sat arvence*	56	Homs	SYR	*P.sativum sativum*		15
Icarda	SYR	*P.sat var pumilio*	57	Aleppo	SYR	*P.sativum sativum*		16
Icarda	SYR	*p.sat var asiaticum*	58	Idlib	SYR	*P.sativum sativum*		17

Irbid	Jor	*p.sat elatius*	59	Dar'a	SYR	*P.sativum sativum*		18
	Ger	*p.fulvum*	60	Homs	SYR	*P.sativum sativum*	local 10896	19
	Ger	*p.sat sat sat*	61	Homs	SYR	*P.fulvum*		20
	Ger	*p. sat sat exiphilum*	62	Tartous	SYR	*P.fulvum*		21
	Ger	*p.sat abyssicum*	63	Tartous	SYR	*P.fulvum*		22
	Ger	*p.sat abyssicum*	64	Lattakia	SYR	*P.fulvum*		23
	Ger	*p.sat sat speciosum*	65	Homs	SYR	*P.sat sat*	local 10980	24
	Ger	*p.sat sat medulloso*	66	Aleppo	SYR	*P.fulvum*		25
	Ger	*p.sat sat medulloso*	67	Sweida	SYR	*P.fulvum*		26
Sweida	SYR	*p.fulvum*	68	Idlib	SYR	*P.fulvum*		27
Zahle	LBN	*p.sativum*	69	Aleppo	SYR	*P.fulvum*		28
Tafila	JOR	*p.sativum*	70	Damascus	SYR	*P.fulvum*		29
Tartous	SYR	*p.sativum*	71	Idlib	SYR	*P.fulvum*		30
Irbid	JOR	*p.sativum*	72	Lattakia	SYR	*P.sat sub elatius*		31
	Ger	*p.sat sat sat*	73	Homs	SYR	*P.sat sub elatius*		32
	Ger	*p.sat sat sat*	74	Homs	SYR	*P.sat sub elatius*		33
	Ger	*p.sat sat ponderorum*	75	aleppo	SYR	*P.sat sub elatius*		34
	Ger	*p.sat sat speciosum*	76	Lattakia	SYR	*P.sat sub elatius*		35
	Ger	*p. sat sat exiphilum*	77	Tartous	SYR	*P.sat sub elatius*		36
	Ger	*p.sat sat sat*	78	Lattakia	SYR	*P.sat sub elatius*		37
	Ger	*p.sat sat speciosum*	79	Lattakia	SYR	*P.sat sub elatius*		38
	Ger	*p.sat sat speciosum*	80	Homs	SYR	*P.sativum*		39
	Ger	*p.sat sat speciosum*	81	Tartous	SYR	*P.sativum*		40
				Idlib	SYR	*P.sativum*		41

Table 2. Names and sequences of AFLP primers used in the analysis of *Pisum* accessions.

Serial no.	Primer combination	Forward Primer / PstI 5' → 3'	Reverse Primers / MseI 5' → 3'
1	P81/M237	GACTGCGTACATGCAGTAG	GATGAGTCCTGAGTAAGATA
2	P20/M213	GACTGCGTACATGCAGGC	GATGAGTCCTGAGTAATGC
3	P20/M237	GACTGCGTACATGCAGGC	GATGAGTCCTGAGTAAGATA
4	P74/M213	GACTGCGTACATGCAGGGT	GATGAGTCCTGAGTAACTGA
5	P20/M88	GACTGCGTACATGCAGGC	GATGAGTCCTGAGTAATGC
6	P237/M34	GACTGCGTACATGCAGGATA	GATGAGTCCTGAGTAAAAT
7	P81/M82	GACTGCGTACATGCAGTAG	GATGAGTCCTGAGTAATAT

3.4. Data analysis

A matrix of all fragments (bands) scored in the 81 accessions with the 7 primer combinations was generated using "1" when the band was present and "0" when the band was absent. Fragments of the same molecular weight were scored as identical. Each fragment was presumed to represent a single genetic locus. Genes diversity at each AFLP locus and genic variation statistics for all loci were estimated by the software package POPGENE, version 1.32 (Yeh and Boyle, 1997). Analysis of genetic diversity (*GD*) was calculated following the formula of Nei (1987):

$$GD = n(1- \Box p^2)/(n-1)$$

where *n* is the number of samples and *p* is the frequency of one allele.

Gene diversity was calculated as follows:

$H = (1 - \sum p_{ij}^2)$ (Weir 1990)

where p_{ij} is the frequency of jth allele generated with the primer i.

The estimation of genetic diversity and the genetic distance was realized using the software pakage Numerical Taxonomy and Multivariate Analysis System, version 2.01 (NTSYS-pc, Rohlf 1997). The construction of dendrograms were performed using the Unweighted Pair Group Mean Average Method (UPGMA) (Sneath and Sokal 1973).

4. Results:

4.1. DNA-Analysis by AFLP

The analysis of DNA samples from 81 accessions of *Pisum* with seven primer combinations has produced two hundred and ninety four distinct and polymorphic DNA fragments (Figure 1). All primers were able to detect polymorphism between the accessions analyzed. The number of total fragments detected varied between the primer combinations used in the analysis. It ranges from 21 fragments with the combination P81/M237 to 66 fragments with P20/M237 (Table 3).

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40M

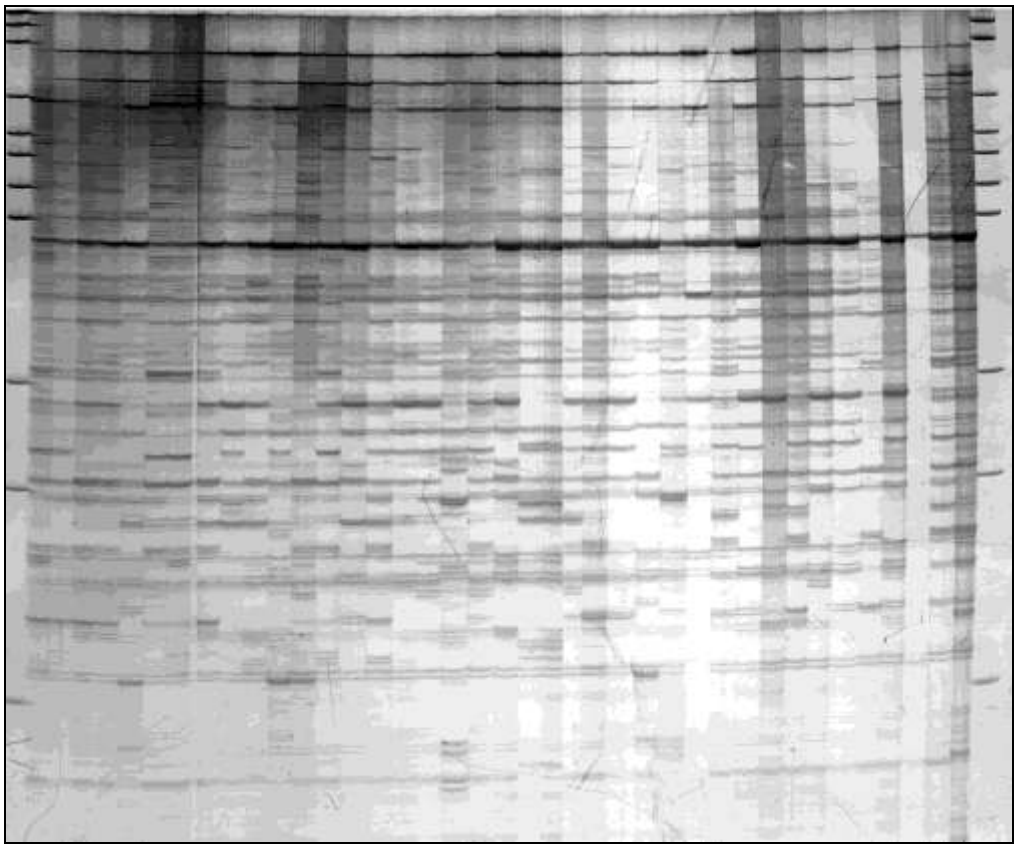

Figure 1. Polymorphism between *Pisum* accessions detected with the AFLP primer combination (P74/M213). PCR products were electrophorized on 6% polyacrylamide gel and polymorphic fragments were revealed by silver nitrate staining.

Table 3. Number of polymorphic DNA fragments detected in each species by using seven primer combinations.

Primer combinations	Number of polymorphic DNA fragments detected in each species		Total number of polymorphic DNA fragments detected in *Pisum*
	Pisum sativum	*Pisum fulvum*	
1- P20/M88	48	42	49
2- P74/M213	54	46	54
3- P81/M82	29	26	29
4- P81/M237	20	21	21
5- P20/M213	35	34	35
6- P20/M237	66	66	66
7- P273/M34	38	37	38
Total of detected fragments	292	274	294

Some primers combinations (e.g. P20/M88, P74/M213 and P81/M82) revealed some fragments in *P. sativum* which were absent in *P. fulvum* (Table 3). The number of polymorphic fragments detected in *P. sativum* (292) was higher than that revealed in *P. fulvum* (274). The comparison of AFLP patterns between accessions of the same sub species demonstrated the presence of high level of variability between accessions. No identical accessions were detected in any sub species and no species specific bands (DNA fragments) were revealed. Although some fragments were amplified only in *P. sativum* but they couldn't be specific in the species *P. sativum* because they are not present in all its accessions (fig.1). The polymorphism detected between the two species (*P. Sativum* and *P. fulvum*) was less important than those detected within species.

The number of polymorphic fragments varied between the different sub species. It ranges from 46 fragments in *P. sativum* sp. *medullosa* to 223 in *P. sativum* which correspond to 18.93% and 91.77% of polymorphic fragments, respectively (Table 4).

The presence and the absence of fragments were recorded in each accession and used to prepare a matrix data. This matrix was used to calculate the level of similarity and the genetic distance between the 81 accessions and the data was used to establish the dendrogram presented in Figure 2. The dendrogram shows that the majority of accessions of *P. sativum sativum* (12 accessions) were regrouped in one cluster with a 0.2 value of genetic distance but the others accessions (7 accessions) were dispersed between the others samples. The accessions collected from the same site were not clustered closer to each other than the ones collected from different sites. The second cluster contains many accessions of *P. sativum* (9 accessions) and they are closer to *P. sativum sativum* than the others groups. For the species *P. fulvum* , its accessions were regrouped in small groups dispersed between the other accessions of *P. sativum*.

For each accession and each group, the observed number of alleles (na), the gene diversity (h), the number and the percentage of polymorphic AFLP loci were calculated and the results for each group were accumulated and presented in Table 4.

4.2. Genetic variability

The highest mean of polymorphic DNA fragments was detected in *P. fulvum* where 12 plants were analyzed, while the smallest mean value was in *P. s. medullosa* where only 2 plants were analyzed (Table 4). The number of observed alleles (na) was evaluated for each locus (That means it was evaluated for the 294 loci but the table of results is not presented). The means were calculated and presented in Table 4. Usually, this value ranges from 1 (minimum level of gene diversity) to 2 (maximum level of gene diversity). This value will be compared only for groups where more than one accession was analyzed. The smallest na was in *P.* s. *medullosa* (1.1893) and the highest one was in *P. sativum* (1.9177). The calculated values of gene diversity varied between groups, some groups possessed high level of gene diversity as (0.3483) in *P. fulvum* and (0.3455) in *P. s. elatius*, while this value was only (0.0947) in *P.* s. *medullosa.*

Table 4. Number of accessions per group (Size of population), observed number of alleles (na), the gene diversity (h) , polymorphic fragments and percentage of polymorphic fragments detected in *Pisum*. The means and the standard deviation (St. D) were calculated for each group.

Species and sub species	Mean and St. deviation	Population Size	na*	h*	No. of polymorphic DNA fragments	% of polymorphic DNA fragments
P. sativum sativum (PSS)	Mean	19	1.9136	0.2997	222	91.36%
	St. Dev		0.2816	0.1598		
P. sativum (local) (PSS)	Mean	5	1.6543	0.2653	159	65.43%
	St. Dev		0.4766	0.2038		
P. fulvum (Pful)	Mean	12	1.9259	0.3483	225	92.59%
	St. Dev		0.2624	0.1496		
P. sativum (PS)	Mean	16	1.9177	0.298	223	91.77%
	St. Dev		0.2754	0.1513		
P. s. abyssicum (PSAbsy)	Mean	2	1.1934	0.0967	47	19.34%
	St. Dev		0.3958	0.1979		
P. s. arvense (PSA)	Mean	6	1.535	0.2046	130	53.5%
	St. Dev		0.4998	0.2026		
P. s. asiaticum (PSA)	Mean	1	1	0	179	0
	St. Dev		0	0		
P. s. elatius (PSE)	Mean	9	1.8642	0.3455	210	86.42%
	St. Dev		0.3433	0.1709		
P. s. exiphilum (PSExi)	Mean	1	1	0	119	0
	St. Dev		0	0		
P. s. medullosa (P*SMed*)	Mean	2	1.1893	0.0947	46	18.93%
	St. Dev		0.3926	0.1963		
P. s. ponderorum	Mean	1	1	0	142	0

(PSPon)	St. Dev		0	0		
P.s. pumilio (PSpum)	Mean	1	1	0	183	0
	St. Dev		0	0		
P.s. speciosuum (PSSpec)	Mean	5	1.7819	0.2974	190	78.19%
	St. Dev		0.4138	0.1783		
Total	Mean	81	2	0.3647	294	100%

4.3. Genetic distance

The values of genetic distance between accessions of the same sub species was calculated (Tables 5,6,7, 8 and 9) and their dendrograms were constructed (figures 3, 4, 5, and 6). The level of genetic diversity within groups was very high, which is reflected by the high value of genetic distance detected between accessions. The highest level of genetic distance was detected in *P. sativum*. ssp *eliatus* (Coefficient of Nei = 0.82, Figure 3) while the smallest value was detected in *P. sativum* ssp. *arvense* (Coefficient of Nei = 0.32, Figure 4).

The comparison between dendrograms showed that the level of genetic diversity (represented by the genetic distance) was much higher between accessions belonging to the same group (Intra-group) than that detected between groups (Inter-groups).

Table 5: Coefficient of genetic distance between accessions belonging to *P. s. eliatus*

	31PSE	32PSE	33PSE	34PSE	35PSE	36PSE	37PSE	38PSE	59PSE
31PSE	0.000								
32PSE	0.213	0.000							
33PSE	0.317	0.205	0.000						
34PSE	0.501	0.576	0.556	0.000					
35PSE	0.331	0.449	0.429	0.619	0.000				
36PSE	0.687	0.599	0.702	0.791	0.712	0.000			
37PSE	0.803	0.730	0.967	1.146	0.893	0.444	0.000		
38PSE	0.346	0.449	0.475	0.648	0.430	0.777	0.800	0.000	
59PSE	0.399	0.521	0.558	0.593	0.392	0.805	0.995	0.439	0.000

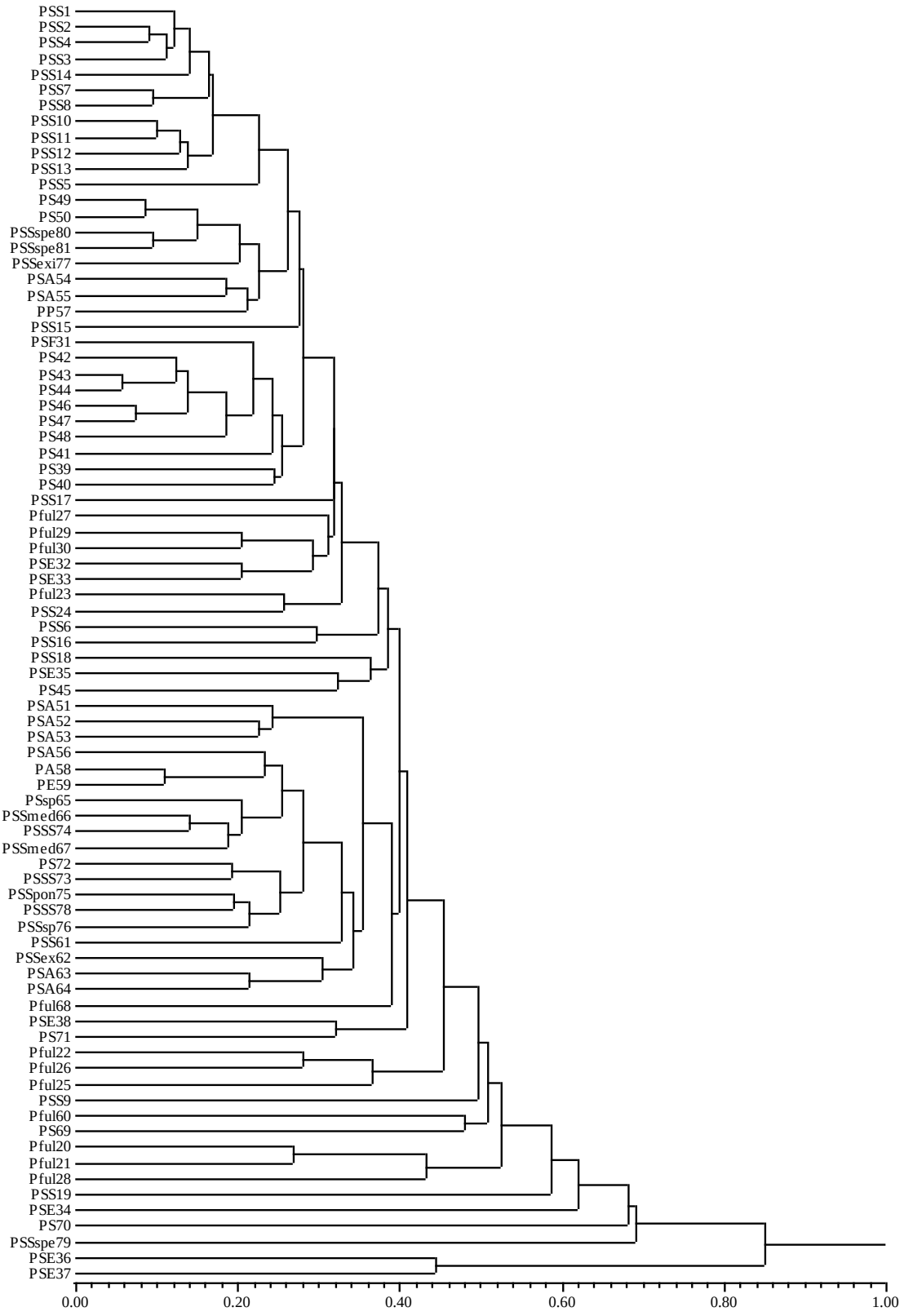

Figure 2. Dendrogram representing the genetic distance between 81 accessions based on AFLP data. To refer to Table 4 for abbreviation.

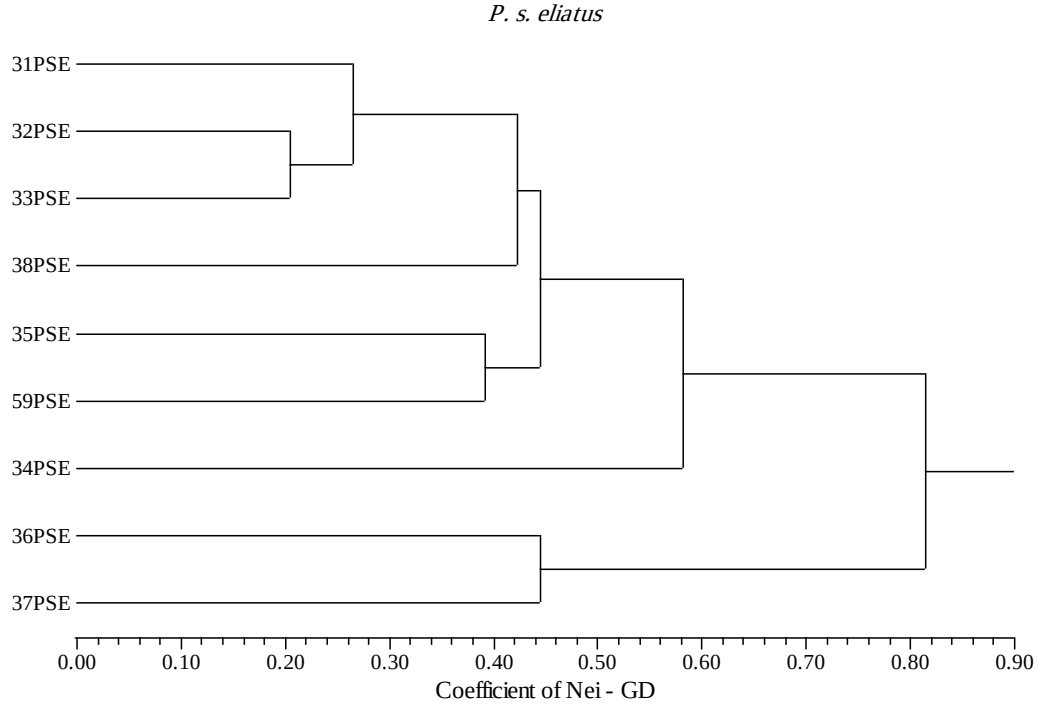

Figure 3. Dendrogram of genetic distance values based on the AFLP data calculated according to Nei coefficient for *P. s. eliatus* accessions.

Table 6: Values of genetic distance between accessions belonging to *P. s.arvense*

	51PSA	52PSA	53PSA	54PSA	55PSA	56PSA
51PSA	0.000					
52PSA	0.234	0.000				
53PSA	0.252	0.227	0.000			
54PSA	0.308	0.308	0.325	0.000		
55PSA	0.340	0.242	0.269	0.184	0.000	
56PSA	0.331	0.343	0.341	0.235	0.270	0.000

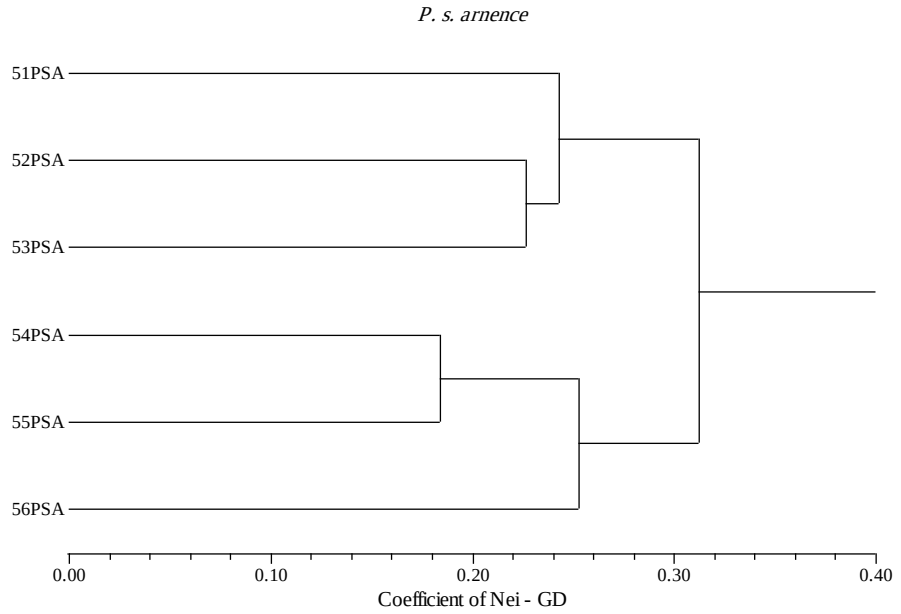

Figure 4. Dendrogram of genetic distance values based on the AFLP data calculated according to Nei coefficient for *P. s. arvence* accessions.

Table 7: Values of genetic distance between accessions of *P. fulvum*

	20Pful	21Pful	22Pful	23Pful	25Pful	26Pful	27Pful	28Pful	29Pful	30Pful	60Pful	68Pful
20Pful |	0.000											
21Pful |	0.269	0.000										
22Pful |	0.482	0.421	0.000									
23Pful |	0.573	0.487	0.264	0.000								
25Pful |	0.586	0.551	0.425	0.379	0.000							
26Pful |	0.528	0.464	0.281	0.309	0.305	0.000						
27Pful |	0.642	0.605	0.370	0.328	0.424	0.328	0.000					
28Pful |	0.468	0.394	0.475	0.479	0.537	0.401	0.459	0.000				
29Pful |	0.562	0.532	0.396	0.333	0.431	0.281	0.352	0.322	0.000			
30Pful |	0.580	0.574	0.343	0.328	0.396	0.318	0.304	0.362	0.203	0.000		
60Pful |	0.573	0.590	0.615	0.583	0.596	0.560	0.576	0.575	0.579	0.546	0.000	
68Pful |	0.685	0.636	0.660	0.603	0.654	0.569	0.476	0.511	0.455	0.427	0.557	0.00

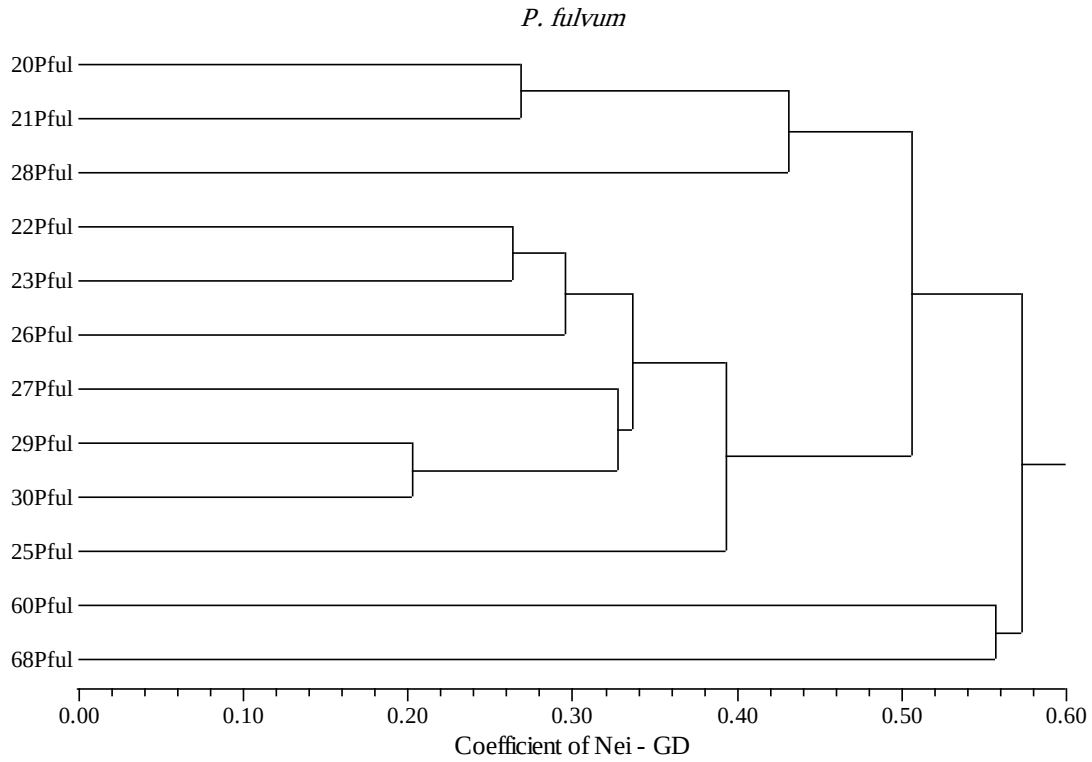

Figure 5. Dendrogram of genetic distance values based on the AFLP data calculated according to Nei coefficient for P. s. eliatus accessions.

Table 8: Values of genetic distance between accessions belonging to *P. sativum*

	39PS	40PS	41PS	42PS	43PS	44PS	45PS	46PS	47PS	48PS	49PS	50PS	69PS	70PS	71PS	72PS
39PS	0.000															
40PS	0.245	0.000														
41PS	0.314	0.290	0.000													
42PS	0.196	0.239	0.152	0.000												
43PS	0.219	0.189	0.204	0.103	0.000											
44PS	0.252	0.207	0.253	0.142	0.056	0.000										
45PS	0.389	0.309	0.415	0.288	0.227	0.209	0.000									
46PS	0.234	0.241	0.250	0.150	0.129	0.162	0.275	0.000								
47PS	0.226	0.240	0.219	0.140	0.108	0.140	0.250	0.074	0.000							
48PS	0.342	0.267	0.329	0.229	0.167	0.163	0.197	0.194	0.174	0.000						
49PS	0.273	0.280	0.282	0.255	0.203	0.226	0.366	0.222	0.196	0.317	0.000					
50PS	0.294	0.271	0.280	0.216	0.162	0.176	0.304	0.204	0.174	0.261	0.085	0.000				
69PS	0.547	0.558	0.499	0.458	0.420	0.441	0.505	0.475	0.441	0.422	0.436	0.433	0.000			
70PS	0.787	0.676	0.583	0.596	0.582	0.571	0.719	0.672	0.613	0.689	0.639	0.636	0.520	0.000		
71PS	0.420	0.445	0.504	0.407	0.396	0.376	0.487	0.411	0.378	0.469	0.328	0.291	0.650	0.741	0.000	
72PS	0.443	0.371	0.430	0.348	0.325	0.330	0.386	0.402	0.365	0.384	0.351	0.280	0.418	0.540	0.348	0.000

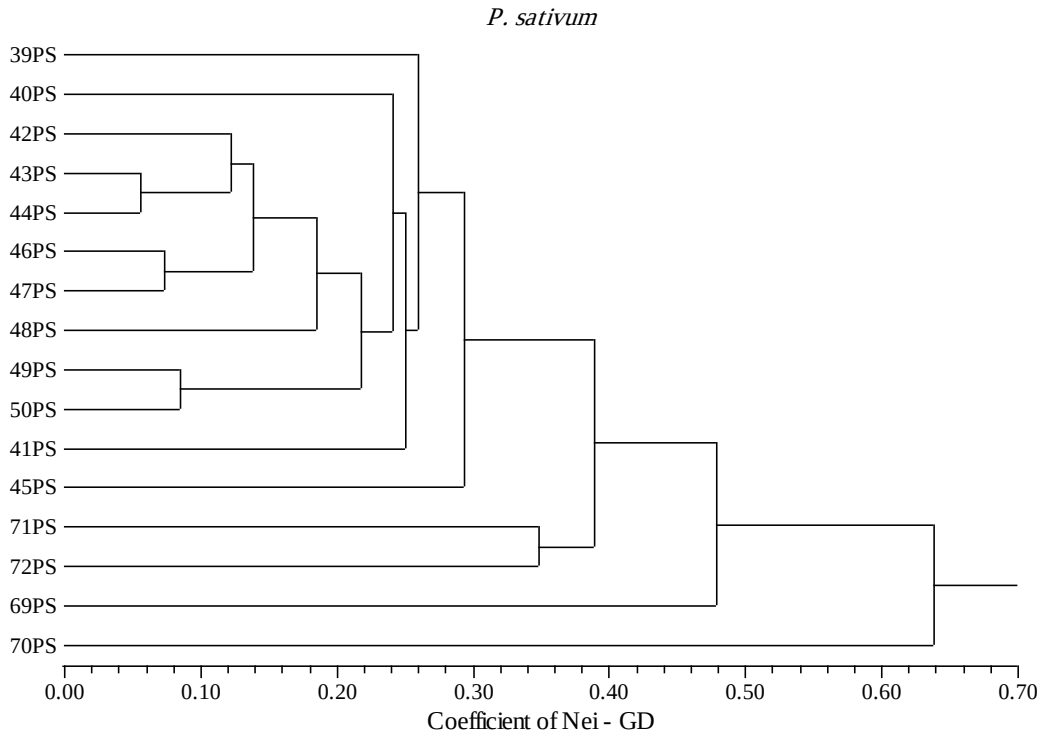

Figure 6. Dendrogram of genetic distance values based on the AFLP data calculated according to Nei coefficient for *P. sativum sativum* accessions.

Table 9: Values of genetic distance between accessions belonging to *P. sativum sativum*

	1PSS	2PSS	3PSS	4PSS	5PSS	6PSS	7PSS	8PSS	9PSS	10PSS	11PSS	12PSS	13PSS	14PSS	15PSS	16PSS	17PSS	18PSS	19PSS	24PSS	61PSS
1PSS	0.000																				
2PSS	0.090	0.000																			
3PSS	0.136	0.126	0.000																		
4PSS	0.138	0.089	0.098	0.000																	
5PSS	0.190	0.217	0.168	0.174	0.000																
6PSS	0.294	0.247	0.297	0.282	0.358	0.000															
7PSS	0.184	0.134	0.203	0.169	0.256	0.213	0.000														
8PSS	0.165	0.109	0.175	0.133	0.247	0.227	0.094	0.000													
9PSS	0.341	0.319	0.327	0.314	0.356	0.441	0.309	0.268	0.000												
10PSS	0.149	0.140	0.178	0.154	0.231	0.274	0.168	0.137	0.252	0.000											
11PSS	0.165	0.143	0.202	0.177	0.274	0.263	0.172	0.141	0.311	0.100	0.000										
12PSS	0.189	0.158	0.194	0.161	0.244	0.300	0.183	0.175	0.289	0.121	0.137	0.000									
13PSS	0.163	0.160	0.213	0.181	0.262	0.274	0.210	0.164	0.282	0.154	0.127	0.135	0.000								
14PSS	0.154	0.139	0.132	0.135	0.229	0.305	0.180	0.180	0.311	0.177	0.174	0.152	0.172	0.000							
15PSS	0.260	0.236	0.237	0.236	0.313	0.428	0.289	0.258	0.471	0.294	0.276	0.269	0.265	0.191	0.000						
16PSS	0.381	0.311	0.395	0.368	0.471	0.297	0.305	0.367	0.586	0.360	0.296	0.353	0.386	0.365	0.428	0.000					
17PSS	0.253	0.212	0.271	0.267	0.353	0.331	0.287	0.259	0.376	0.258	0.225	0.282	0.267	0.264	0.432	0.341	0.000				
18PSS	0.321	0.346	0.290	0.326	0.285	0.486	0.408	0.378	0.573	0.370	0.413	0.319	0.387	0.299	0.316	0.498	0.444	0.000			
19PSS	0.516	0.487	0.494	0.471	0.509	0.610	0.517	0.569	0.978	0.567	0.564	0.528	0.631	0.493	0.508	0.487	0.651	0.483	0.000		
24PSS	0.303	0.280	0.306	0.284	0.356	0.354	0.289	0.300	0.592	0.308	0.306	0.326	0.342	0.314	0.350	0.416	0.388	0.316	0.394	0.000	
61PSS	0.381	0.322	0.342	0.318	0.386	0.587	0.397	0.369	0.676	0.415	0.431	0.418	0.470	0.384	0.397	0.539	0.505	0.405	0.544	0.392	0.000

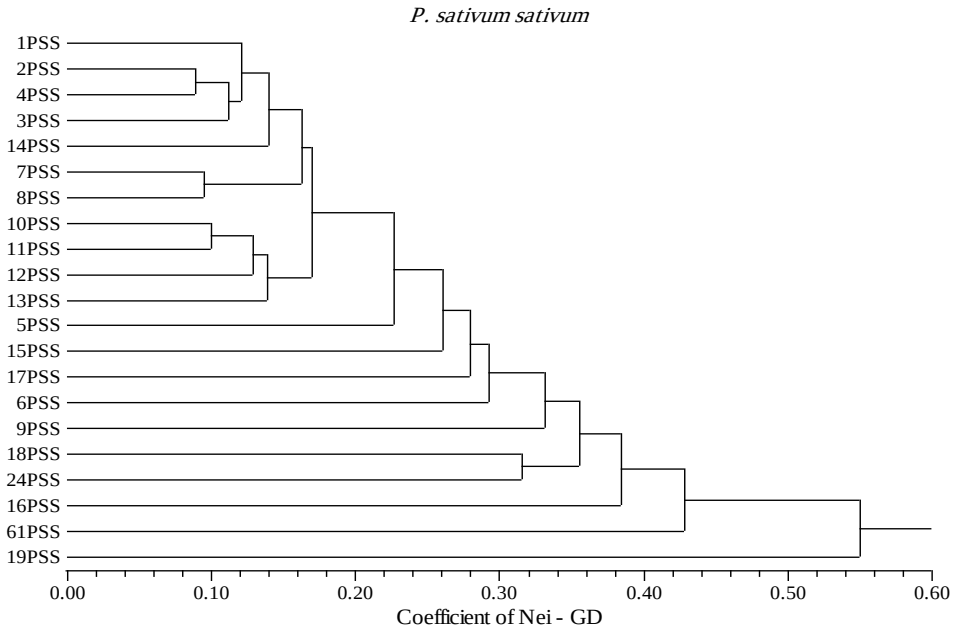

Figure 7. Dendrogram of genetic distance values based on the AFLP data calculated according to Nei coefficient for *P. sativum sativum* accessions.

4.5. Genetic relationship within *Pisum*

In order to estimate the genetic relationship between the 13 different groups of the two *Pisum* species, the values of their genetic distance were calculated (Table 10) and they were used to establish the dendrogram in Figure 7. The cluster was divided into 2 main branches. The first branch contains *P. s. exiphium*, which was the most distant sub species of *Pisum* and the second one contained the 12 other groups. The second branch was divided into 3 branches. *P. s. ponderoru* and *P.s. pumlio*, each one belongs to a branch separated from the other by a value of genetic distance equal to 0.27.

The third branch was clearly separated from the two previous ones and includes 2 distinguish clusters separated by a coefficient of genetic distance equal to 0.26. One cluster contains 9 groups and the other contains only one group, *P.s. asiaticum*. The 9 groups are regrouped in two clusters, one of them contains *P.s. abyss* and *P.s. medullosa* and the other contains the last 7 groups. These 7 groups form 4 branches, the first contains *P. sativum sativum* whish is the most close group to the groups of local plants used as controls with a coefficient of genetic distance of about 0.04. *P. sativum* and *P. s. speciosum* are close to each other and the coefficient of genetic distance between them is about 0.08. *P. fulvum* and *P. s. elatius* are the closet to each other and they are separated by a value of genetic distance equal to 0.14. We can notice that the 6 groups in the three branches were very close to each other where the value of the coefficient of genetic distance didn't exceed 0.1. *P. s. arvense* is the most distant sub species in the cluster. It's separated from the 6 sub species by a value of genetic distance equal to 0.18.

Table 10. Value of genetic similarity between the 13 groups of *Pisum*.

	.s. sativum	P. s. Local	P. fulvum	P. sativum	P. s. abysisum	P. s. arvense	P. s. asiaticum	P. s. elatius	P. s. exiphium	P. s. medullosa	P.s. ponderoru	P. s. pumlio	P. s. speciosum
P. s. sativum	1	0.9525	0.893	0.9228	0.7839	0.8552	0.7695	0.8755	0.7974	0.8223	0.7638	0.8057	0.8961
P. Local		1	0.890	0.8855	0.7219	0.7883	0.7211	0.8601	0.7442	0.7589	0.6962	0.7384	0.846
P. fulvum			1	0.8947	0.7795	0.8204	0.7366	0.9111	0.7307	0.7764	0.7171	0.7562	0.8694
P. sativum				1	0.7943	0.8773	0.7767	0.902	0.7644	0.8317	0.7871	0.8025	0.9082
P. s. abyssium					1	0.81	0.7556	0.7585	0.682	0.8328	0.7512	0.6993	0.8343
P. s. arvense						1	0.8283	0.8282	0.7237	0.8115	0.8021	0.8052	0.8875
P. s. asiaticum							1	0.7336	0.6049	0.8131	0.7449	0.7531	0.824
P. s. elatius								1	0.6986	0.7692	0.746	0.7596	0.8756
P. s. exiphium									1	0.7612	0.679	0.6955	0.8142
P. s. medullosa										1	0.7612	0.7871	0.8866
P.s. ponderoru											1	0.6872	0.8535
P. s. pumlio												1	0.7978
P. s. speciousu													1

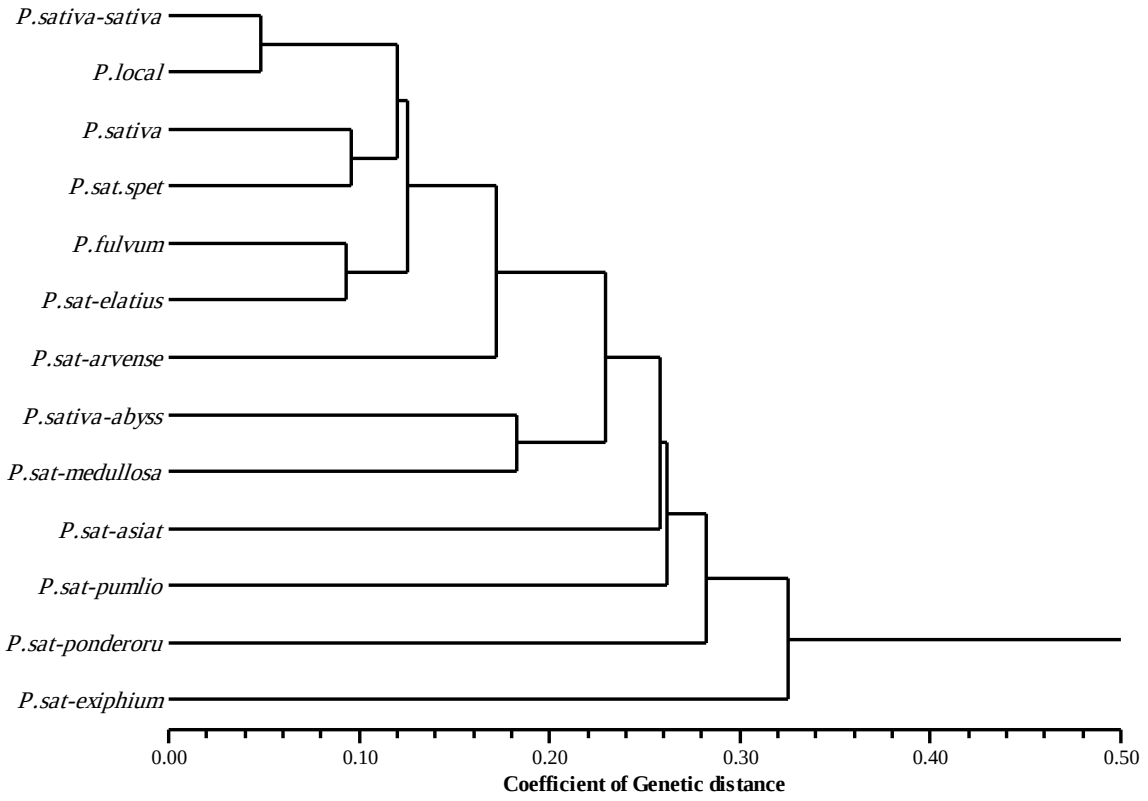

Figure 8: A dendrogram representing the genetic relationships between 13 groups of *Pisum*.

5. General discussion and conclusion:

The use of molecular markers proved to be very useful in the detection and evaluation of genetic diversity in many crops (Gracia-Mas *et al.* 2000; Teulat *et al.* 2000, Choumane *et al*. 2004). In some cases, they were able to regroup the accessions according to their collection sites (Conkle *et al*., 1988). In our study, the analysis of 81 DNA samples belonging to 13 groups (species and sub species) of *Pisum* with 7 AFLP primer combinations allowed to distinguish between the 81 accessions. The number of polymorphic fragments varied from 46 to 225 and the percentage of polymorphic fragments ranges from 18.93% to 92.59%. Each accession had a specific pattern reflecting the presence of high level of genetic diversity within the genus *Pisum*. The comparison between accessions of the same group detected the presence of heterogeneity and impurity within the group. This genetic diversity was affected by the population size and the level of gene diversity. No identical accessions were identified. No species specific DNA fragment was detected. The level of genetic diversity within groups was higher than that detected between groups. Even, the level of diversity within species is higher than that between species. It's clear from the dendrogram (Fig. 7) that *P. fulvum* is closer to *P. sativum* species than other *P. sativum* sub species. These results are in disagreement with those reported by Ford *et al. (*2000) using STMS markes. In that study, *P. fulvum* was well distinguished from *P. sativum*. This disagreement could be explained by 1)- the small number of accessions analyzed 2)- by the use of another germplasm 3)- by the application of another type of molecular markers. Despite the high level of polymorphic fragments identified there was no correlation between any specific fragment and the geographic site of collection. Similar results were detected in *Pinus brutia* where no correlation was revealed between geographic sites and molecular data based on RAPD and AFLP markers (Choumane *et al.* 2004).

These results allowed to conclude that the level of genetic diversity in *Pisum* accessions collected from Syria was very high and represent a very good genetic resources for the breeding programs, but the number of samples analyzed was not sufficient neither representative of the different regions. Therefore, these results are very useful to improve the strategy of collection in the future. New collection should be planed from the regions showed higher level of genetic diversity to avoid any lose of genetic diversity in the genus. Also, more number of accessions from each sub group from each site should be collected to estimate the relationship between the accessions from the different regions and to know how the genetic diversity in the genus *Pisum* is distributed. At the technical level, these results proved that the AFLP markers are very useful in analyzing the genetic diversity in *Pisum.*

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