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Allele Frequency and Genetic Diversity of Some Species of Genus *Vicia* L. Using SDS-PAGE Technique

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GENETIC diversity refers to genetic variability within and among species. Each individual species possesses alleles which are the source of its own unique adaptation features to the future biotic and abiotic stresses. The greater the genetic diversity within a species, the greater that this species have chances of long-term survival. Studying seed storage proteins patterns of different species of *Vicia* germplasm was aimed to characterize and assess the genetic diversity and phylogenetic relationship among defined 51 accessions collected by ICARDA (International Center for Agricultural Research in Dry Area) from different countries and habitats. These represent nine *Vicia* species and subspecies; *V. ervilia, V. monantha, V. villosa* subsp. *villosa*, *V. villosa* subsp. *dasycarpa*, *V. villosa* subsp. *eriocarpa, V. sativa* subsp. *nigra*, *V. sativa* subsp. *amphicarpa, V. sativa* subsp. *macrocacrpa* and *V.sativa* subsp. *sativa*. Dry seeds were used to extract total storage proteins since each seed is regarded as a fixed physiological state. The pattern of variation in molecular size of total storage proteins in those accessions were studied by the numerical analysis of data obtained by using SDS-PAGE technique. The descriptive population statistics calculations were based on band presence versus absence and allele frequencies. Consequently, these data generate pertinent genetic information for the complement of passport data of ICARDA germplasm collections.

Keywords: Allele frequency, Electrophoresis, Seed storage proteins, UPGMA dendrogram.

Introduction

Genus *Vicia* L. is widely distributed along several regions of Europe, North and South America (Weber & Wittmann, 1999) and the Mediterranean area which is its principal center of distribution and diversification (Naranjo et al., 1998) where polymorphism has been associated with geographical origin of germplasm (ICARDA, 2013).

Vicia species growing in wide agro-ecological conditions, were considered as model crops for genetic and molecular studies (Singh et al., 2007). Inceer & Ayaz (2005) found that genus *Vicia*L. is represented in Turkey by 64 species and 22 subspecies. In Egypt Täckholm (1974) recognized twelve species, while Boulos (1999) enumerated fourteen species.

Genus *Vicia* L. contains the extensively cultivated faba bean, *Vicia faba*, the minor forages *V. narbonensis*, *V. sativa* subsp. *sativa* (common vetch) and the taxa that have a high potential for use as forages of the future: *V. hyaeniscyamus*, *V. noeana*, and *V. sativa* subsp. *amphicarpa* (Maxted et al., 1991; Maxted, 1995). The three species, *V. sativa* L., *V. villosa* Roth and *V. narbonensis* L., are cultivated (in Spain, Turkey, Jordan, Syria and Iraq) for their high-quality fodder and protein-rich seeds (28 to 32%) (Siddique et al., 1996; Caballero et al., 2001). *Vicia sativa* subsp. *sativa*, which is known as the common vetch, is one of the most commonly grown winter cover crop, or

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green mature, and is also used as pasture, silage, and hay as mentioned by Hueze et al. (2011) and Sullivan (2003). Due to its economic and ecological advantages, common vetch is now widespread through many parts of the world, including the Mediterranean basin, West and Central Asia, China, Eastern Asia, India, and the USA (Sullivan, 2003; Tate & Ennenking, 2006; Hueze et al., 2011).

Previously, morphological or cytological assay procedures were used to estimate existing genetic variability in crops of commercial importance (Islam & Shepherd, 1991). These assay procedures though were successful in many cases, were not considered suitable for large scale screening mainly because of limited number of such markers and time consuming. Later on, protein markers (especially seed storage proteins) are being used for better and more reliable estimation of genetic distances among species/lines/populations (Weber et al., 2005). The technique of Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) is a commonly used technique for separating different molecules of proteins on the basis of their size. This technique is commonly used for the estimation of genetic diversity (Deif et al., 2006; Yasmin, 2010).

The fact that proteins are direct products of gene transcription and translation makes them ideally suited for plant variety identification. Analysis of protein pattern can be considered to be an analysis of gene expression and a comparison of the patterns of a particular set of proteins becomes a comparison of the genetic differences between individuals. Since varieties are different from one another, and the differences must be at least in some part genetically based, protein pattern forms an ideal means of variety discrimination and identification.

Seed storage protein patterns are considered to be particularly reliable, as they are largely independent of environmental factors. They are synthesized only in seeds, in protein bodies, during mid-maturation stage of seed development and used up during germination and lack any functional activity (Mirali, 1987). Seed protein patterns obtained by electrophoresis have been successfully used to resolve the taxonomic and evolutionary problems of several crop plants (Ladizinsky & Hymowitz, 1979; Sammour, 1989; Khan, 1992; Das & Mukherjee, 1995). They have also provided a tool to distinguish allele frequency of cultivars of particular crop species, genetic diversity and evaluation of taxonomic and genetic associations in the *Vicia* and numerous other species at generic, specific and intraspecific levels (Ladizinsky & Hymowitz, 1979; Moller & Spoor, 1993; Jha & Ohri, 1996; El- Badan, 2004; Mirali et al., 2007; Çelebi et al., 2009; Hameed et al., 2009; Emre et al., 2010; Emre, 2011). Profiling seed storage proteins using SDS-PAGE technology, is particularly considered as a simple, valid and consistent tool for economic characterization of germplasm of plant groups (Javid et al., 2004; Iqbal et al., 2005; Sher et al., 2010) and to formulate hypotheses on their phylogeny (Mirali, 2007).

Materials and Methods

Mature dry seeds of 51 wild accessions of four*Vicia*species from three sections were studied, section Vicia (*V. sativa* L.), section Cracca (*V. monantha* Retz, *V. villosa* Roth) and section Ervum (*V. ervilia* L.). All accessions were obtained from the International Center of Agricultural Research in Dry Area (ICARDA), Aleppo (Table 1).

Bulked mature dry seeds from each accession were crushed and milled manually after removing their testa. Samples of 50mg of seeds powder from each accession and location were analyzed separately. Total storage proteins were extracted and electrophoresed as described by Laemmli (1970) and Hames & Rickwood (1990) respectively. A kit of medium range of standard protein marker formed from 97, 66, 45, 30, 20.1 and 14.4kDa (Sigma Aldrich-chemie, Germany) was used. Electrophoresis was performed in Hoefer Scientific Instrument; vertical slab gel unit-Model SE 400. The gels were stained with Coomassie Blue R250 (Sigma Aldrich-chemie, Germany). Five microliter protein extract of each sample and 6µl of marker protein were loaded onto the 15% SDS-PAGE. Electrophoresis was performed using Tris/glycine pH 8.3 as electrode buffer at 20 mA, then continued at 25mA.

Phenotypic data scoring

Digital images of protein gels were analyzed and band sizes were determined using the TotalLab image analysis software (version 1.1.4301, 26877). Only Well-resolved bands were scored and recorded as absent (0) or present (1). A locus was considered polymorphic if a consistent band

was present in one or more, but not all accessions otherwise it was considered as monomorphic band. According to Liengsiri et al., 1990, the percentage of the polymorphic bands (Pb %) is calculated as percentage of total bands for each accession – common bands for all accessions/ total bands for all accessions.

Inter-accessional variation is expressed on the basis of dissemilarity indices. Principal Coordinate Analysis was carried out using the first two most informative eigenvalues that showed the maximum variation by PAST software (Davis, 1986) and the results were expressed as plot.

Genotypic diversity

The first step in any investigation of population genetics of a species is an assessment of its total genetic diversity, which is estimated through several descriptive measures of diversity (De Vicente et al, 2003). To start, locus means band observable on a gel and an allele is an estimated entity based on dominant data.

i. Percent of polymorphic loci: a locus is considered polymorphic if two alleles were detected, regardless of their frequencies. According to Liengsiri et al. (1990), the percentage of the polymorphic loci (Pb= Pl %). is calculated as: The percent of total loci for each sample (species) minus total common bands for all samples divided by total bands for all samples.

ii. Allele frequency: Many molecular as well as proteins data generate dominant markers, thus heterozygotes cannot be distinguished (Miller, 1997). Accordingly, each band was assumed to correspond to a single locus and the similarity of band size (M.wt.) is an indicator of homology (O'Hanlon & Peakall, 2000). To calculate allele frequencies, Hardy-Weinberg equilibrium is assumed. Only two alleles are considered to exist for a dominant marker locus, the dominant allele"p"(presence of band) and the nullallele"q"(absence of band). The presence of a band indicates either homozygote or heterozygote dominant allele. And thus, allele frequencies could be estimated (De Vicente et al., 2003; Deif et al., 2006).

iii. Average interlocus gene diversity (H_i): It is the mean of the sum of the interlocus gene diversity for accessions of the same species:

$$H_{i=} \sum h_i / N$$

where, $h_i = 1- p^2 – q^2$; where p is the mean frequency of dominant allele, q is the mean frequency of recessive "null" allele and N is the total number of loci (polymorphic and monomorphic).

Results

Total seed soluble storage protein analysis of the different accessions of the studied species indicated the presence of twenty-four to forty-five different bands. Polymorphism was observed in all molecular weight regions. High intensity common bands appeared in all phenograms, however some accessions showed some specific bands with different molecular weights. All studied accessions showed a common band of M.wt.~ 40kDa.

Vicia ervillia L. had 5 biotypes and expressed 24 bands, 9 of them were common (M.wt.: 74.5, 60.2, 40.1, 36.3,33.1, 26.5, 21.3, 20.1, 18.7kDa). Unique bands occurred in two accessions only, one in e6 (M.wt.: 24.6kDa) and five in e7 (M.wt.: 87.3, 54.3, 45.3, 25.1, 22.7kDa) (Table 2). The UPGMA cluster analysis separated *V. ervilia* L. accessions e5 from other Syrian accessions at 71% similarity distance (Fig 1). The other 6 accessetions formed the second group which was divided into two subgroups one included e6 and e7with the highest number of specific bands, the other included the four accssetions in two clusters of which cluster containing e1 and e2 expressed with the highest similarity distance (93%).

Vicia monantha Retz. was represented by 10 accessions with different altitudes, latitudes and longitudes (Table 1). Table 3 indicated that *Vicia monantha* accessions expressed 28 bands three of them were common (54, 46, 13.2kDa). Unique bands characterized several accessions. Band of M.wt. 70.5kDa in accession m2; bands of M.wt. 23.6 and in accession m9, and six unique bands were found in accession m10 (M.wt.: 88.1, 78.2, 63.6, 24.2, 19.3, 16.1kDa). Polymorphism among accessions ranged from 25 to 50% (Table 3). The cluster analysis for *V. monantha* Retz. accessions (Fig. 2) showed that accession m2 formed a separate group at 64% similarity distance. Accession m9 and m10 formed separate clusters from the second group at 66% and 67% similarity distances respectively However, accessions m5, m7 and m8 showed 100% similarity.

TABLE 1. Passport information of the 51 *Vicia* investigated accessions from ICARDA. m.asl: meter above sea level, IG: ICARDA catalogue number, IFVI: Crop_nr.

Section	*Vicia* species	Accessions	Country	Province	(IFVI)	IG	Longitude°	Latitude (N)	Alt m.asl
Ervum	*V. ervilia* L.	e 1	MOR	Meknes	5361	112083	W05° 21′ 58′′	N35° 01′ 55′′	310
		e 2	MOR	Taza	3620	63569	W04° 15′	N34° 35′	1500
		e 3	DZA	Tiaret	4207	64156	E01° 58′	N34° 15′	950
		e 4	LBN	Baalbek	2698	62647	E36° 04′	N34° 02′	100
		e 5	SYR	Idlib	2542	62491	E36° 41′ 02′′	N36° 12′ 05′′	350
		e 6	SYR	Sweida	3399	63348	E36° 35′ 40′′	N32° 42′ 00′′	1150
		e 7	JOR	Tafila	4244	64193	E35° 35′	N30° 54′	900
Cracca	*V. monantha* Retz	m1	SYR	Homs	2960	62909	E36° 21′ 40′′	N34° 43′ 35′′	290
		m 2	SYR	Sweida	5129	108430	E36° 34′ 30′′	N32° 49′ 50′′	920
		m 3	JOR	Zarqa	4222	64171	E36° 12′	N32° 05′	650
		m 4	EGY	Marsa Matrouh	4262	64211	E28° 20′	N31° 03′	30
		m 5	TUN	Kairovan	5117	108288	E10° 13′	N35° 38′	50
		m 6	TUN	Kasserine	5103	168274	E08° 31′	N35° 02′	720
		m 7	DZA	Oasis	2507	62456	E02° 58′	N33° 49′	820
		m 8	DZA	Tiaret	2506	62455	E02° 23′	N34° 12′	1300
		m 9	MOR	Marrakech	4883	107589	W07° 05′	N31° 47′	765
		m 10	TUR	Gaziantep	3730	63679	E36° 55′	N36° 50′	475
Sativa	*V. sativa* L. subsp. *Amphicarpa*	s.am1	SYR	Hama	2650	62599	E37° 02′ 05′′	N35° 16′ 57′′	450
		s.am2	SYR	Sweida	5132	108433	E36° 43′ 00′′	N32° 52′ 00′′	1106
		s.am 3	JOR	Karak	4236	64185	E35° 48′	N31° 26′	360
	V. sativa L. subsp. *Macrocarpa*	s.mac 1	TUR	Aydin	4144	64093	E27° 21′	N37° 45′	200
		s.mac 2	TUN	Ariana	5235	111315	E09° 11′ 30′′	N36° 53′ 36′′	200
		s.mac 3	DZA	Batna	2505	62454	E06° 20′	N35° 42′	850
		s.mac 4	MOR	Tetouan	4595	64544	W05° 32′	N35° 33′	230

TABLE 1. Cont. Passport information of the 51 *Vicia* investigated accessions from ICARDA. m.asl: meter above sea level, IG: ICARDA catalogue number, IFVI: Crop_nr.

Section	*Vicia* species	Accessions	Country	PROVINCE	IFVI	IG	Longitude °	LAT°(N)	Alt m.asl
Sativa	*V. sativa* L.	s.n 1	TUR	IZMIR	1416	61365	E27° 39′	N38° 24′	210
		s.n 2	PAK	Punjab	5678	117736	E74° 41′ 47′′	N32° 05′ 43′′	265
		s.n 3	EGY	Al Buhayrah	4215	64164	E30° 02′	N31° 09′	~6
		s.n 4	EGY	Marsa Matrouh	4212	64161	E27° 17′	N31° 15′	~1
		s.n 5	EGY	Alexandria	4268	64217	E29° 57′	N31° 10′	10
		s.n 6	TUN	Le Kef	5107	108278	E08° 27′	N35° 41′	650
		s.n 7	DZA	Alger	4402	64351	E03° 09′	N36° 33′	50
		s.s 1	LBN	Baalbek	2695	62644	E36° 04′	N34° 02′	1000
		s.s 2	TUR	Yozgat	3610	63559	E34° 56′	N39° 51′	1300
		s.s 3	TUR	Manisa	1154	61103	E28° 00′	N38° 35′	120
		s.s 4	IRN	Sharekod	455	60404	E50° 52′	N32° 20′	2070
		s.s 5	SYR	Idlib	2541	62490	E36° 41′ 02′′	N36° 12′ 05′′	350
		s.s 6	JOR	Balqa	4317	64266	E35° 43′	N32° 06′	760
		s.s 7	JOR	Amman	4216	64165	E35° 49′	N31° 56′	880
		s.s 8	CYP	Nicosia	708	60657	E33° 21′	N35° 09′	~134
		s.s 9	DZA	Medea	4414	64363	E03° 04′	N36° 12′	850
		s.s 10	DZA	Tiaret	4428	64377	E01° 43′	N35° 24′	1100
		s.s 11	DZA	Mostaganem	2509	62458	E00 °25′	N35° 43′	90
		s.s 12	MOR	Tetouan	4363	64312	W05° 03′	N35° 04′	900
		s.s 13	MOR	Meknes	4775	107458	W06° 04′	N33° 28′	1060
		s.s 14	MOR	Marrakech	4362	64311	W07° 45′	N31° 20′	880
Triphaca	*V. villosa* Roth	v.d 1	MOR	Khenifra	4828	107522	W05° 20′	N33° 18′	1015
		v.d 2	MOR	Meknes	4787	107470	W06° 00′	N33° 31′	880
		v.d 3	TUR	Antalya	4059	64008	E31° 11′	N36° 56′	90
		v.v 1	SYR	Hama	3372	63321	E36° 19′ 20′′	N35° 54′ 30′′	720
		v.v 2	DZA	Tiaret	4454	64403	E00° 51′	N35° 04′	1050
		v.e	SYR	Al Hasakah	683	66320	E41° 14′ 26′′	N37° 02′ 57′′	464

TABLE 2. Comparative analysis of seed storage proteins of different accessions of *Vicia ervillia* L. and *Vicia villosa* Roth. Pb%: Percent of polymorphic bands

Species	*Vicia ervillia* L							*Vicia villosa* Roth					
Accession	e1	e2	e3	e4	e5	e6	e7	v.d1	v.d2	v.d3	v.v1	v.v2	v.e
Common bands				9							6		
Specific bands	0	0	0	0	0	**1**	**5**	2	2	0	1	0	1
Total bands	14	14	14	14	14	17	18	14	11	11	15	13	13
Pb%	20.83	20.83	20.8	20.83	20.83	33.33	37.50	36.36	22.73	22.73	40.9	31.82	31.82

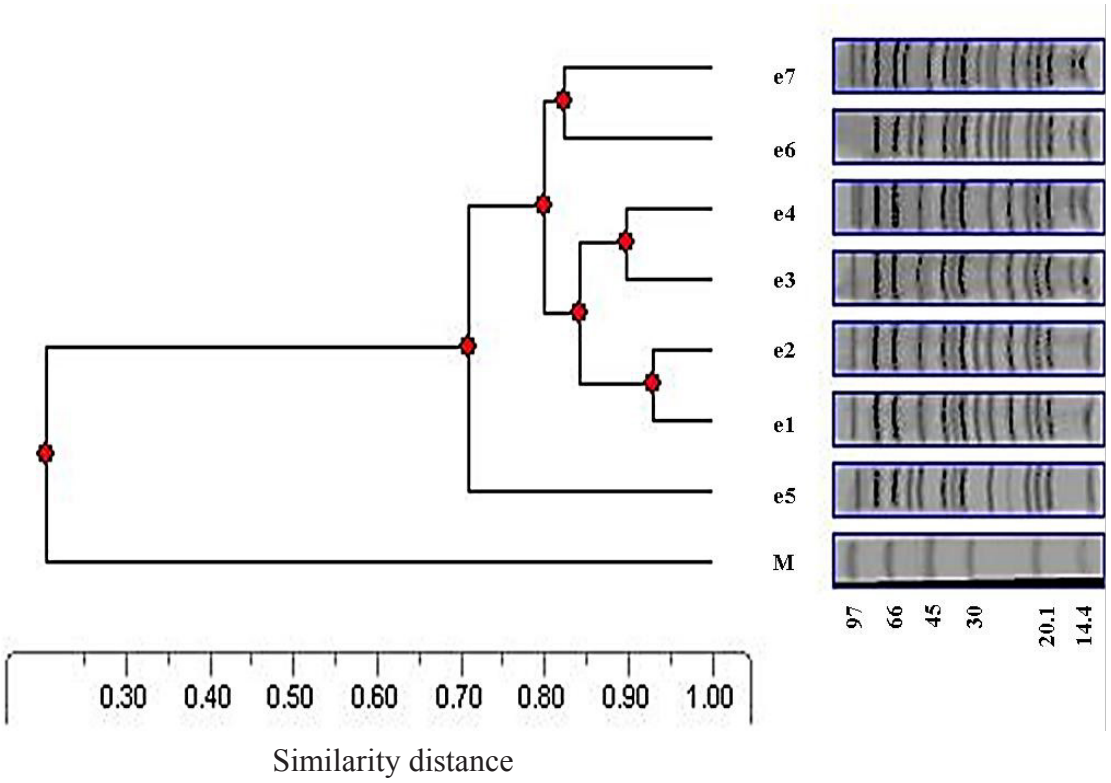

Fig.1. UPGMA cluster analysis of 7 accessions of *Vicia ervillia* L based on similarity distance matrix of SDS-PAGE protein banding pattern (constructed by Totallab v.1.1 software) [Accessions details denoted in table 1 and 2. M: protein marker]

TABLE 3. Comparative analysis of seed storage protein bands of different accessions of *Vicia monantha* Retz. Pb%: Percent of polymorphic bands

Accession	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10
Common bands						3				
Specific bands	0	1	0	0	0	0	0	0	2	6
Total bands	12	10	12	11	12	12	12	12	13	17
Pb%	32.14	25.00	28.57	28.57	32.14	32.14	32.14	32.14	35.71	50.00

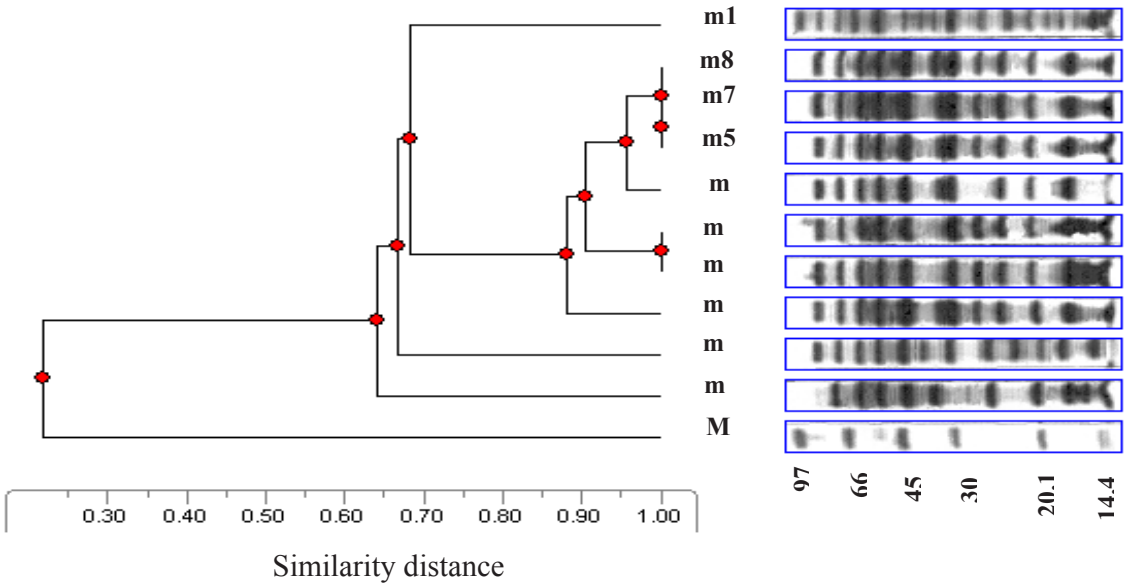

Fig.2. UPGMA cluster analysis of *Vicia monantha* Retz based on similarity distance matrix of SDS-PAGE protein banding pattern (constructed by Totallab v.1.1 software) [Accessions details denoted in table 4. M: protein marker]

Vicia sativa L. was represented by 28 accessions belonging to 4 subspecies (*amphicarpa*, am; *macrocarpa*, mac; *nigra,* nand *sativa,* s) according to ICARDA identification (Table 1). Phenotypes of *V. sativa* complex showed the utmost polymorphisms (ranged from 13 to 46.9%). Four common bands characterize accessions of *Vicia sativa* complex (40, 34, 31, 21kDa) whichshowed their unequivocal identification (Fig. 3).

The UPGMA cluster analysis of ***V. sativa*** species separated the studied accessions into two major groups with accession s.s7 forming the first group at similarity distance of 36% (Fig. 3). All the other 27 accessions formed mixed subspecies clusters at different similarity distances. It was noticed that all representatives of the different subspecies rarely grouped together except in two cases, s.s13 and s.s14 (MOR). Generally, accessions were not grouped according to location, altitude or latitude.

Vicia sativa subsp. *amphicarpa* was represented by 3 accessions.Table 4 indicated that all accessions expressed 25 bands, 7 of them were common (four common bands characterize accessions of *Vicia sativa* complex and 3 bands, 23,18.7, 17.7kDa, characterize subsp.amphicarpa). Eight unique bands were found in s.am3 (87, 78.4, 70.7, 63.3, 58.4, 50, 46.3,28kDa.) and only four in s.am 2 were found (47.6,44.2,30,25.2kDa.). Percent of polymorphic band ranged from 24 to 36%.

Vicia sativa subsp. *macrocarpa* was represented by 4 accessions (s.mac1 to s.mac4). Table 4 indicated that all accessions gave 30 different bands, 5 of them were common (four common bands characterize accessions of *Vicia sativa* complex and one band, 59kDa, characterize subsp.macrocarpa). Four unique bands were found in s.mac1 and s.mac4 accessions (91.7, 65.9,37.1 kDa. and 107.5, 76, 25.8,.19kDa. respectively) and only one specific band was found in s.mac2 accession (106.4kDa). Percent of polymorphic band ranged from 30 to 40%.

Vicia sativa subsp. *nigra* was represented by 7 accessions expressed 32 bands, 5 of them were common bands (40.1,34,31,26.6,21kDa). Unique bands were found in 2 accessions only; s.n1 (99.8kDa) and s.n2 (80.9,69.3,60.2,37.8kDa), Percent of polymorphic band ranged from 18.8 to 46.9%, (Table 4).

Vicia sativa subsp. *sativa* were represented by the largest number of accessions. Table 5 indicated that all accessions expressed 46 bands, 4 of them were common (40.1,34,31,26.6,21kDa). Unique bands were found in 6 accessions. s.s3 (100,29.23kDa.), s.s5 (44.2kDa.), s.s6 (88.8,27.2kDa.), s.s7 (54,41.8, 32.6 kDa.), s.s8 (16.7kDa.), and *s.s*13 (24.6kDa.). Percent of polymorphic band ranged from 13 to 28.3%.

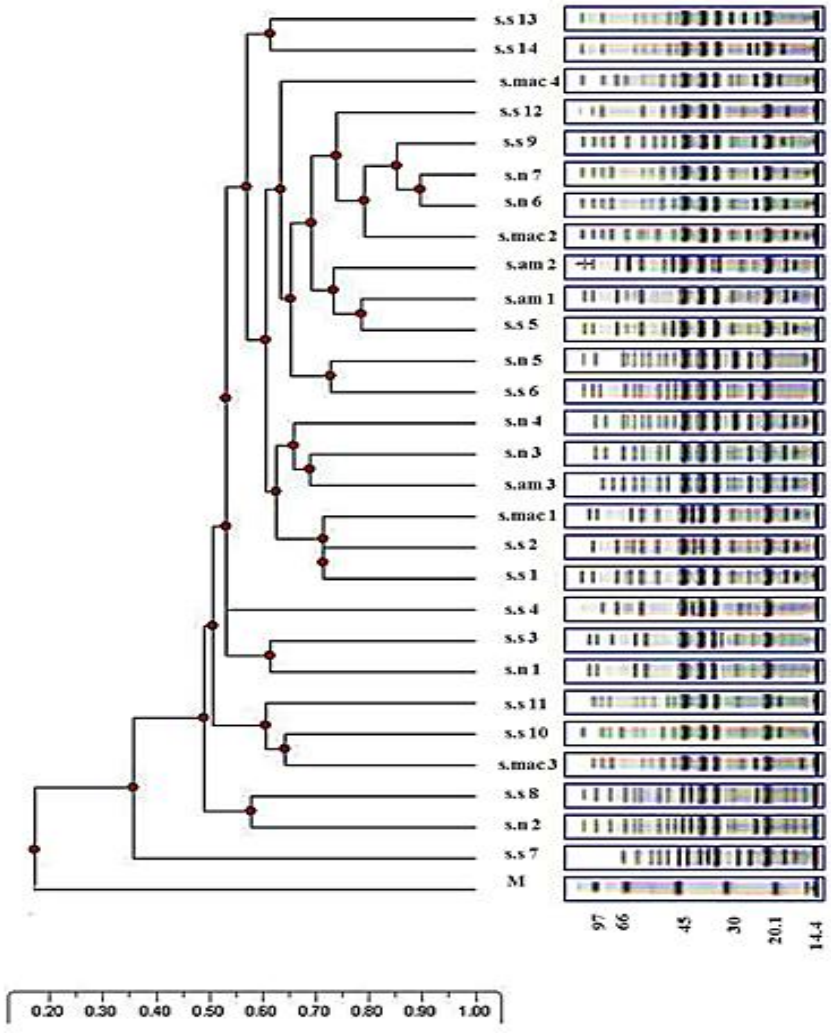

Fig.3. Dendragram using UPGMA cluster analysis of 28 accessions of *Vicia sativa* L. subspecies based on similarity distance matrix of SDS-PAGE protein banding pattern (constructed by Totallab v.1.1 software) [Accessions details denoted in tables 1. M: protein marker]

TABLE 4. Comparative analysis of seed storage protein bands of different accessions of *Vicia sativa L. subsp. Amphicarpa , subsp. Macrocarpa and subsp.nigra. Pb%: Percent of polymorphic bands*

***Vicia sativa* L.**	**subsp. *amphicarpa***			**subsp. *macrocarpa***					**subsp. *nigra***					
Accession	**s.am1**	**s.am2**	**s.am3**	**s.mac1**	**s.mac2**	**s.mac3**	**s.mac4**	**s.n1**	**s.n2**	**s.n3**	**s.n4**	**s.n5**	**s.n6**	**s.n7**
Common bands		7				5					5			
Specific bands	0	4	8	4	1	3	4	1	4	0	0	0	0	0
Total bands	13	16	16	14	17	14	16	11	20	16	19	17	16	16
Pb%	24.00	36.00	36.00	30.00	40.00	30.00	36.67	18.75	46.88	34.38	43.75	37.5	34.38	34.38

TABLE 5. Comparative analysis of seed storage protein bands of different accessions of *Vicia sativa* L. subsp. *sativa*. Pb%: Percent of polymorphic bands

Accession	s.s1	s.s2	s.s3	s.s4	s.s5	s.s6	s.s7	s.s8	s.s9	s.s10	s.s11	s.s12	s.s13	s.s14
Common bands							4							
Specific bands	0	0	2	0	1	2	3	1	0	0	0	0	1	0
Total bands	13	13	12	10	13	16	14	17	16	14	14	10	13	13
% Pb	19.57	19.57	17.39	13.04	19.57	26.09	21.74	28.26	26.09	21.74	21.74	13.04	19.57	19.57

Vicia villosa Roth was represented by 6 accessions, belonged to 3 subsp. *V. villosa* subsp. *villosa* (v.v1 andv.v2), *V. villosa* subsp. *dasycarpa* (v.d1, v.d2, v.d3) and *V. villosa* subsp. *eriocarpa* (v.e). They were collected from different altitudes, latitudes and longitudes (Table 1). Table 3 indicated that all accessions expressed 22 bands, 6 of them were common bands (47.6,43,35.7,29.6,17,15 kDa.). Unique bands were found in 4 accessions, two in subspecies *dasycarpa* (vd1; 66.3, 21.6kDa. and vd2; 79, 50.4kDa.), one in each of v.v1 (54.2kDa.) and v.e (19kDa.). The UPGMA cluster analysis of *V. villosa* Roth showed high similarity level (72%). They were separated into two main groups at 70% similarity (Fig. 4). Percent of polymorphic band ranged from 22.7, 41%.

Data analysis

Generally ANOVA test was used to analyze the present results at the species level, it showed that there was no significance among altitudes with respect to common bands, specific bands, total bands and percent of polymorphic bands (P= 0.688, 0.111, 0.862 and 0.614, respectively). However, number of specific bands and total bands were significantly correlated with country location (P= 0.001 and 0.01, respectively). Also, common bands, total bands and percent of polymorphic bands were significantly affected by kind of species (P= 0.000, 0.01 and 0.002, respectively).

Inter-accessional variation is expressed on the basis of dissemilarity indices (Appendix I). The first two components accounted for a maximum of 44.68% of the total variace among the accessions which is attributed to Eigen values of 0.1 and 0.3. Principal Coordinate Analysis (PCo) was carried out using the first two most informative eigenvalues and the results were expressed as plot (Fig. 5). It showed four main patches reflecting the four studied *Vicia* species. Within each species' patch, some accessions from same geographic location lie very close to each other with few exceptions. The accessions of *Vicia sativa* complex were grouped in one patch with the separation of accessions of subsp. *macrocarpa* and *amphicarpa* from each other and from subsp. *sativa* and subsp. *nigra*, which were intermingled. PCo was used to choose accessions for DNA finger printing.

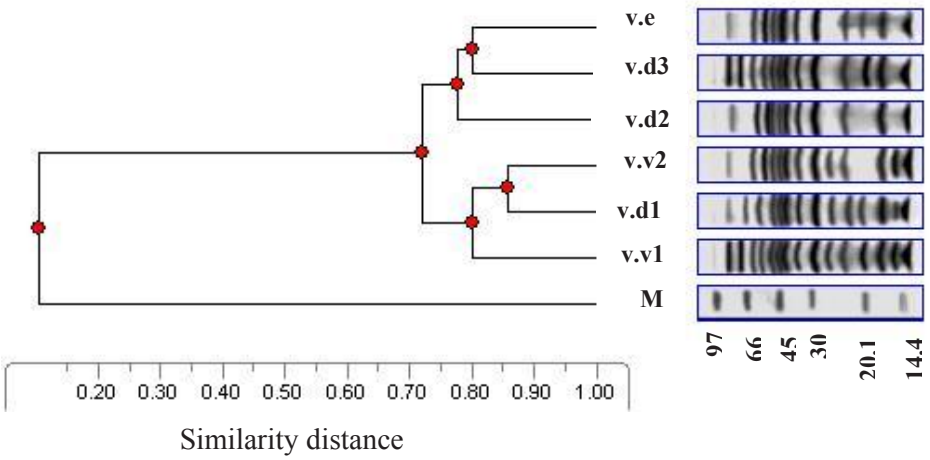

Fig.4. Dendrogram using UPGMA cluster analysis of 6 accessions of *Vicia villosa* Roth based on similarity distance matrix of SDS-PAGE protein banding pattern (constructed by Totallab v.1.1 soft ware) [Accessions details denoted in table 1.M: protein marker]

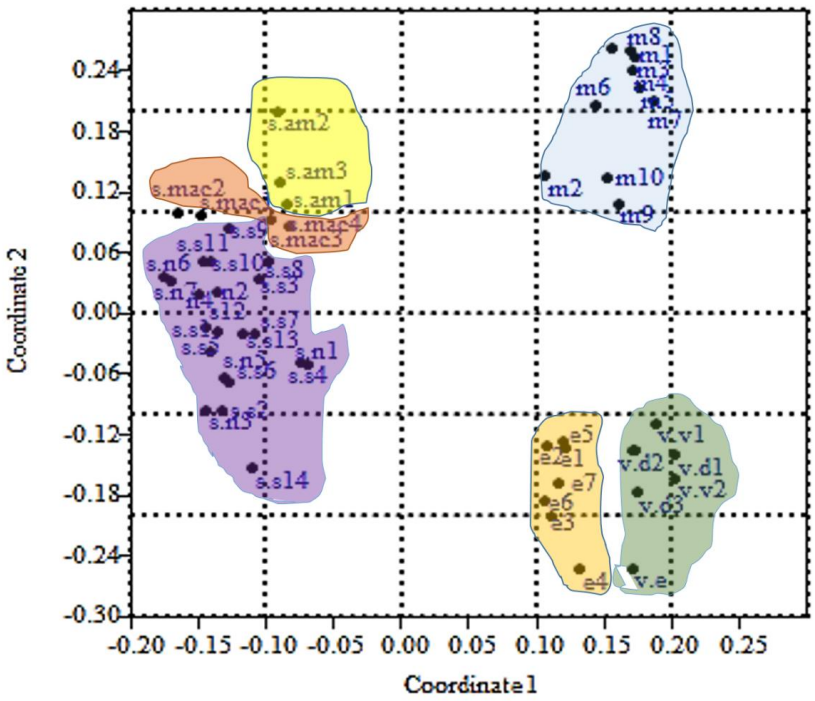

Fig. 5. PCo based on Gower similarity of SDS-PAGE for all *Vicia* species. (PAST software)

Allele frequency and genetic diversity

Genetic diversity was calculated for all studied species according to the presence and absence of protein bands (loci) from which allele frequencies were calculated. Genetic diversity of accessions growing at two main geographic regions; North Africa (Egypt, Tunis, Algeria Morocco) and West Asia (Lebanon, Syria, Jordon, Turkey, Pakistan, Iran) were compared as well as those found at different altitude ranges.

V. ervilia L., data presented in Table 6 and Fig. 6 revealed that all loci of North Africa accessions were monomorphic except 4 loci giving lower percent of polymorphism (16.7%) than those of West Asia (62.5%), although the average homozygous recessive allele was the same in both regions (0.6). In addition, it was found that the average gene diversity/locus was higher in West Asia (0.2) than that of North Africa (0.07). With respect to altitudes, the average homozygous recessive allele (q) ranged from 0.4 to 0.5 in all altitude ranges. The medium altitude range (900-950m) showed the lowest average allele frequency of the null allele (q), the highest percent of polymorphic loci (33%) and the highest average gene diversity/locus (0.14).

TABLE 6. *V. ervilia* L. Accessions: Average allele frequency, percent of polymorphic loci and average gene diversity/locusbetween two geographic regionsand three altitudes ranges

Location		Average allele frequency		Percent of polymorphic loci (%)	Gene diversity/Locus (Average, H_i)
		q	p		
Geographic region	North Africa	0.45	0.55	16.67	0.07
	West Asia	0.45	0.55	62.50	0.22
Altitude range	310-350	0.45	0.55	16.67	0.07
	900-950	0.40	0.60	33.33	0.14
	1000-1500	0.42	0.58	29.17	0.11

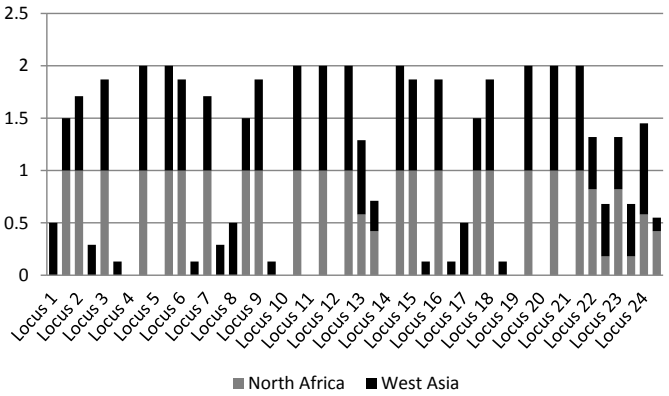

Fig. 6. Allele frequency for each locus in North Africa and West Asia populations of *V.ervilia* L.

V. monantha Retz allele frequencies were presented in Table 7 and Fig 7. There were 13 and 6 monomorphic loci found in North Africa and West Asia accessions respectively. However, the percent of polymorphic loci were found to be high in West Asia accessions. While, the average allele frequencies were the same in both localities (q= 0.65, p= 0.35). The average gene diversity/locus, showed the same trend as that of percent of polymorphic loci, it was lower in North Africa than in West Asia (1.1 and 1.3, respectively). In spite of having three altitude ranges, the low and medium ranges gave analogues average allele frequencies (0.6 for q allele and 0.4 for p allele). Higher altitudes gave high value for q allele (0.7) and subsequently low frequency for p allele (0.3). The percent of polymorphic loci was the highest (75%) in the medium altitude range while, it was 11% and 46% for the low and high altitude ranges respectively. The average gene diversity/locus was very low in low altitudes (0.05) and it was more than 1 in medium and high altitudes (1.2 and 1.1, respectively).

TABLE 7. *V. monantha* Retz. Accessions: Comparison between average allele frequencies, percent of polymorphic loci and average gene diversity/locus between two geographic regions and three altitudes ranges

Location		Average allele frequency		Percent of polymorphic loci (%)	Gene diversity/ Locus (Average, H_i)
		q	p		
Geographic region	North Africa	0.65	0.35	53.57	1.12
	West Asia	0.65	0.35	78.57	1.27
Altitude range	<300	0.61	0.39	10.71	0.05
	400-800	0.61	0.39	75.00	1.24
	>800	0.68	0.32	46.43	1.14

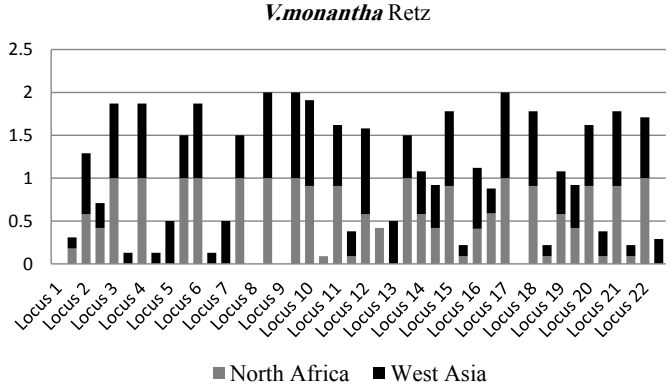

Fig.7. Allele frequency for each locus in North Africa and West Asia populations of *V. monanth* Retz.

V. sativa L. was the largest studied species (28 accessions) containing four important subspecies; *amphicarpa*, *macrocarpa*, *nigra* and *sativa*. Each one was characterized by different number of loci (25, 30, 32 and 46, respectively). The average allele frequencies ranged from 0.44 for q and 0.56 for p of subsp. *sativa*, to 0.65 for q and 0.35 for p for subsp. macrocarpa. Percent of polymorphic loci ranged from 91.3% in subsp. *sativa* to 72% in subsp. *amphicarpa*. This was expressed as the highest average gene diversity/ locus for subsp. *sativa* and the lowest for subsp. *amphicarpa* (Table 8).

V. sativa subsp. *nigra* expressed similar average allele frequencies for both North Africa and West Asia (q= 0.6 and p= 0.4) with higher percent of polymorphic loci found for North Africa accessions (63%) than those of West Asia (41%). Also, the average gene diversity/locus was higher in North Africa than those of West Asia (0.24 and 0.17, respectively) (Table 9 and Fig. 8). All accessions of this subspecies were found at generally low altitude ranges (1-50m.asl and 210-650 m.asl) with similar allele frequencies but the polymorphism was higher in the accessions of the respectively higher altitude range (210-650m. asl) than those of the lower ones.

TTABLE 8. Average allele frequency, percent of polymorphic loci and average gene diversity/locus among *V. sativa* L. subspecies

***Vicia sativa* subspecies**	**Average allele frequency**		**Percent of polymorphic loci (%)**	**Gene diversity/ Locus (Average, H_i)**
	q	**p**		
amphicarpa	0.47	0.53	72.00	0.26
macrocarpa	0.65	0.35	86.67	0.30
nigra	0.62	0.38	84.38	0.27
sativa	0.44	0.56	91.30	0.45

TABLE 9. *V. Sativa* subsp.*nigra* accessions:Average allele frequency, percent of polymorphic loci and average gene diversity/locus between two geographic regions and two altitudes ranges

Location		**Average allele frequency**		**Percent of polymorphic loci (%)**	**Gene diversity/Locus (Average, H_i)**
		q	**p**		
Geographic region	North Africa	0.59	0.41	62.50	0.24
	West Asia	0.60	0.40	40.63	0.17
Altitude range	1-50	0.59	0.41	62.50	0.23
	>210-650	0.63	0.37	65.63	1.21

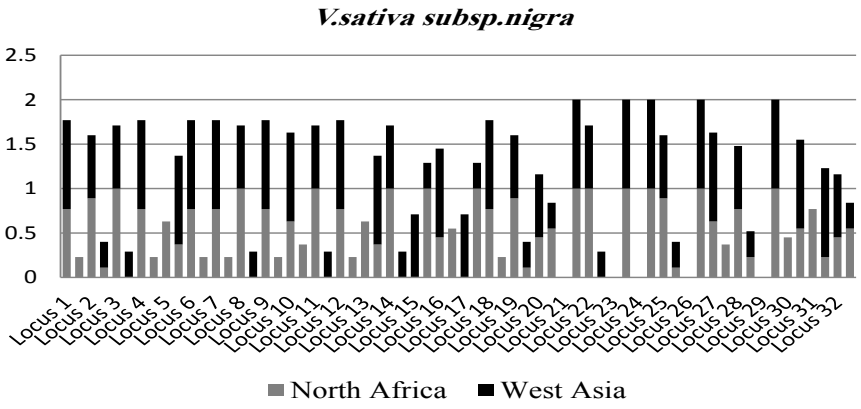

Fig. 8. Allele frequency for each locus in North Africa and West Asia populations of *V. sativa* susp. *nigra*.

V. sativa subsp. *sativa* showed similar average allele frequencies but the percent of polymorphic loci were higher in West Asia accessions (70%) than those of North Africa (43%) (Table 10 and Fig. 9). The average gene diversity/locus was also slightly higher in West Asia accessions (0.2) than in those of North Africa (0.1). However, the lower altitude range gave higher polymorphism (87%) than those of the higher altitude range (54%) in spite of having similar averages allele frequencies and gene diversity/locus.

V. villosa Roth allele frequency was presented in Table 11 and Fig. 10, genetic diversity was calculated for the two subspecies, *dasycarpa* and *villosa* only for the two geographic locations and two altitude ranges (90-720m.asl 800-1050 m.asl). *V. villosa* subsp. *dasycarpa* had allele frequencies of q= 0.6 and p= 0.4 with 55% polymorphism. While subsp. *villosa* had the reverse allele frequencies (q= 0.4 and p= o.6) with 18% polymorphism. The average gene diversity/locus within *V. villosa* subsp. *dasycarpa* accessions was higher than that of *V. villosa* subsp. *villosa* (0.2 and 0.1, respectively). The accessions of North Africa were found at altitudes ranging from 800 m to 1050m, while those accessions of West Asia were found to be at altitudes ranging from 90 to 720m. Average allele frequencies in accessions of both localities and both altitude ranges were 0.5 for both, q and p alleles with higher polymorphism (55%) in North Africa than West Asia (36%). Average gene diversity was high (1.2) in West Asia and low (0.14) in North Africa.

TABLE 10. *V. Sativa* subsp.*sativa* accessions:Average allele frequency, percent of polymorphic loci and average gene diversity/locusbetween two geographic regions andthree altitudes ranges

Location		Average allele frequency		Percent of polymorphic loci (%)	Gene diversity/ Locus (Average, H_i)
		q	p		
Geographic region	North Africa	0.78	0.22	43.48	0.13
	West Asia	0.79	0.21	69.57	0.18
Altitude range	90-900	0.79	0.21	86.96	0.19
	1000-2070	0.81	0.19	54.35	0.16

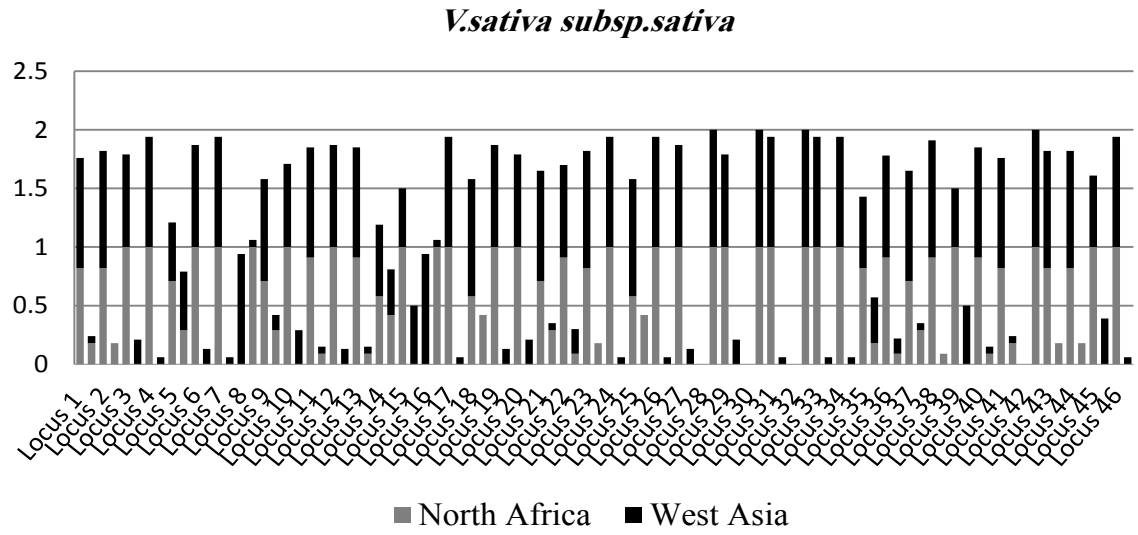

Fig. 9. Allele frequency for each locus in North Africa and West Asia populations of *V. sativa* subsp. *sativa.*

TABLE 11. *V.villosa* Rothaccessions:Average allele frequency, percent of polymorphic loci andaverage gene diversity/locus between two geographical regions and two altitudes ranges

Location		Average allele frequency		Percent of polymorphic loci (%)	Gene diversity/Locus (Average, H_i)
		q	p		
Geographic region	North Africa	0.48	0.52	36.36	0.14
	West Asia	0.54	0.46	54.55	1.15
Altitude range	800-1050	0.48	0.52	36.36	0.14
	90-720	0.54	0.46	54.55	1.15

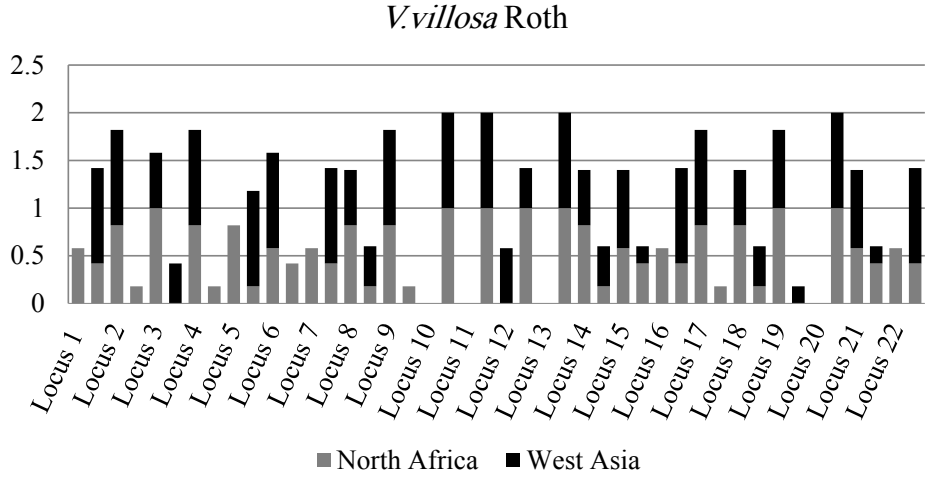

Fig. 10. Allele frequency for each locus in North Africa and West Asia populations of *V.villosa* Roth

Discussion

The fact that proteins are direct products of gene transcription and translation made them ideally suited for plant variety identification purposes. Analysis of protein composition as one for analysis of gene expression and the comparison of the composition of a particular set of proteins for genetic differences between individuals. Protein markers (especially seed storage proteins) are being used for identification and characterization of crop and herbage cultivars (Karihaloo et al., 2002; Yüzbaşıoğlu et al., 2008) and also for more reliable estimation of genetic distances among species/lines and populations. The technique of Sodium Dodecyl Sulphate Poly Acryl amide Gel Electrophoresis (SDS-PAGE) is commonly used for separating different molecules of proteins on the basis of their molecular weight.

The present study showed some variation in banding profile among accessions of the same species in the same geographic region. The difference in band profile could be attributed to polymorphism in the coding regions involved in their synthesis. These polymorphisms may result from difference in the DNA sequences that code for the peptides in different alleles. However, Berber & Yaşar (2011) suggested that the genetic relatedness among genotypes of wild species could be the result of close proximity of collection sites (having similar environmental and climatic conditions) and due to intensive breeding in case of economic species. Also, Crawford (1990) suggested that seed protein profiles reflect genetic affinities within a taxon and even between different biological entities based on the assumption that closely related species show more similar electrophoretic protein patterns than those that are phylogenetically less related.

Accordingly, accessions with similar banding pattern were classified as biotypes of the same species, therefore, different numbers of biotypes had been recorded for most *Vicia* species which were also recorded by El- Badan (2004). Herein, common protein bands, representing common loci, among various accessions indicated that the genes coding these proteins are conserved. Most accessions of each species were distinguishable even they did show some identical number of bands with similar mobility. Meanwhile, there was no distinct molecular weight area of protein diversity in the studied accessions of genus *Vicia*, but the variation ranged from below 14 kDa to over 100kDa This finding contradicted that of Bertozo & Valls (2001) studying genus *Arachis* who detected polymorphism only in the 14 to 24kDa range.

*V. villosa***,** in the present study, showed that each of its accessions belonged to different subspecies, and separate biotypes having the same six common bands specifying their relationship. This finding confirmed that of Sammour (1989) who found not much similarity in protein profile among subspecies (except that of common bands). Herein, it is recommended that an increase in the number of accessions of each subspecies is a must to elucidate the marked recorded differences.

Contrarly, accessions of *V. sativa* aggregate were clearly delineated by their electrophoretic patterns. The results indicated that the four subspecies shared four common bands which indicated the high similarity in the genetic construction of these accessions. However, each subspecies has its own 'protein portrait' except some accessions of subspecies *nigra* and subspecies *sativa*. Since certain amount of cross pollination could occur in *V. sativa* aggregates (Hanelt & Mettin, 1970), the observed genetic variation in protein banding pattern among accessions of the same subspecies were based on the difference of both intra- and inter-geographical range with different latitudes, longitudes and altitudes. This finding is in agreement with El- Shanshoury (2007), who confirmed that this variation resulted in the diversification of gene products (seed storage proteins). In the meantime, Potokina (1997) stated that although the wild populations of the *V. sativa* aggregate consisted of well defined taxa, some morphological intermediate forms had been recorded. Herein, protein level analyses recorded intermediate forms as shown as different banding patterns (biotypes) among accessions. All the plants having intermediate protein patterns were found in the populations where different taxa grow sympatrically (i.e having the same geographical area). This could support the view that these intermediate forms could be the result of gene flow (Potokina et al., 2000).

It was established, by the present study, that seed proteins of all studied *Vicia* species were heterogeneous in total number of bands which was reflected as different polymorphic band percents in the different accessions. It revealed the extensive polymorphism with high intra-specific variation even from the same geographical region that formed different biotypes (5, 8, 3, 4, 6 and 14 biotypes in the following respective species; *V. ervilia*, *V. monantha, V. sativa* subsp. *amphicarpa*, *V. sativa* subsp. *macrocarpa*, *V. sativa* subsp. *nigra* and *V. sativa* subsp. *sativa*). The occurrence of different biotypes for each species had also been reported by Thakare et al. (1987) in *chickpea* accessions, Mehrani (2002) in *Pisum*, Emre (2007) in *Lathyrus,* EL- bakatoushi & Ashour (2009) in *Vicia narbonensis*. This finding pointed out that these variations among accessions from different geographic regions and altitudes indicated that those species may be still evolving in different pathways. Herein, the wide geographic ranges may explain the high degree of variation among accessions. Also, intra-specific differences among accessions varied due to the abundance of common bands. This is obvious since *V. ervillia* and *V. villosa* accessions showed the lowest intraspecific variation and the highest number of common bands (9 and 6, respectively), while, *V. monantha* and *V. sativa* species showed the highest intraspecific variation and the lowest number of common bands (3 and 4, respectively).

The current results implied that there is a common band (approx. 20-21kDa) in *V. ervilia* and all *V. sativa* complex which was considered to be one of the most specific banding regions of the vicilin zone as suggested by Potokina et al. (2000). Also, these species shared the major band at 40kDa that was denoted by Sammour (1989) as globulin. However, all studied subspecies of *V. sativa* and *V. villosa* were characterized by the presence of a band at ~30kDa which is suspected to be lectin protein that is found in most species of family Fabaeceae as proposed by Luzia et al. (2004). On the basis of these findings, it is clear that crosses among different gene pools of

species could create more genetic variability than crosses among similar accessions. The protein pattern of low molecular weight polypeptides, (around 20 and <14kDa) was almost present in all examined accessions. All *V. sativa* accessions were characterized by the presence of a band at ~34kDa. However, Ayaz et al. (1999) found that the reducing protein profile of all studied taxa of *Vicia* (*V. sativa* subsp. *nigra* one of them) had a common band with an estimated molecular weight of 34.7kDa, in addition, most storage proteins were mol. Wt. ranges between 30-40 and 20kDa. Commonly, this protein is legumine like globulins (Debyshire et al., 1976) was reported to be the predominant storage protein in leguminous seed plants (Fischer & Schopfer, 1988).

Principal coordinate analysis (PCo) indicated that regardless of the wide range of collection sites, accessions of each species were clustered together revealing that they share a common gene pool. The 28 studied accessions of *Vicia sativa* were also clustered together forming a completely separate patch, this was confirmed by Emre (2011). The most notable issue is that inspite of *V. ervilia* (section Ervum) and *V. villosa* (section Cracca) were from different sections, their accessions formed two closely related groups and clustered together proving that they belonged to the same subgenus, vicilla. This finding contradicted with that of Mirali et al. (2007) and Arslan et al. (2010) where the former found that both *V.sativa* and *V.ervilia* accessions were in the same group and far from *V. villosa*, while the latter found that his studied *Vicia* accessions were grouped in two main clusters. One cluster included all accessions of section Vicia (*V. sativa*) and section Cracca (*V. monantha* and *V. villosa*), while the other cluster contained all accessions of section Ervum (*V. ervilia*). In addition, 28 accessions of *V. sativa* complex formed a separate group which was differentiated into three closely related subgroups amounting to PCo of 44.7% variation (subsp.sativa, macrocarpa and amphicarpa), indicating their separate entities. This finding is in accordance with that of Mirali *et ai.*(2007). However, subsp. *nigra* was found to be intermingled with accessions of subsp. *Sativa.* This is in agreement with Potokina et al. (2000), who used both seed protein and RAPDs parameters. It is also, compatible with that of De la Rosa & Gonzalez (2010) who suggested that the native wild populations are composed of a mixture of genotypes that may offer survival advantages in changeable environmental conditions. Outcrossing could also be a source of variation in *V. sativa* subsp. *amphicarpa* (Mirali et al., 2007) and other types of vetch.

The total amount of variability accounted for the principal coordinate analysis was 44.68% which indicated that the accessions showed good association probably due to parallel evolution and extensive gene flow in the different geographical regions (Sammour et al., 2007).

The current study also revealed that *V. villosa* subsp. *eriocarpa* was delimited from the other subspecies forming an outgroup branch for its accessions. This result agreed with El- Shanshoury & Soliman (1996) who mentioned that *V. villosa* subsp. *eriocarpa* is a single phonetic line and they opposed its inclusion as a subspecies of *V. villosa.* The Possible gene flow in populations of *V. sativa* aggregate especially subspecies *sativa* and *nigra* could explain the intermingling of accessions of those subspecies. They were probably cross compatible as suggested by Potokina et al. (2000).

The present study is concerned with a scientific idea using protein pattern as markers for genetic diversity among different *Vicia* species and its accessions. This technique had been applied by various authors since 2003 (De Vicente et al., 2003; Wallace, 2003) later on, Deif et al. (2006) to calculate allele frequency of date palm species. Abdel Khalic & Al-Gohary (20013) used protein pattern to evaluate different *Vicia species.* In 2020, Debaroti et al. (2020) used protein pattern as well as DNA finger printing in the differentiation and evolutionary history of a certain gene (GALC) in different human population.

In spite of, proteins could be used as dominant markers since they are direct gene, there was no clear correlation among accessions and altitude or longitude. Therefore, accessions were grouped into two major geographical regions; North Africa and West Asia and different altitude ranges. Allele frequencies were estimated according to Hardy-Weinberg equilibrium for accessions of each species belonging to these groups to measure the genetic diversity (allele frequency, percent of polymorphic loci and estimated gene diversity). Accordingly, the genetic diversity measures indicated that accessions of *V. ervilia, V. monantha, V. villosa* and *V. sativa* subsp. *sativa* belonging to West Asia expressed higher genetic

diversity and percent of polymorphic loci than those of North Africa. Contrarily, *V. sativa* subsp. *nigra* of North Africa expressed high average gene diversity/locus and high percent of polymorphic loci than those of West Asia. However, with respect to different altitude ranges each species had its own ranges; for example, *V. ervilia, V. monantha* and *V. sativa* subsp. *sativa* accessions which were characterized by high average gene diversity/locus were found in the higher altitudes ranges. While, accessions of *V. sativa* subsp. *nigra* and *V. villosa* expressed the high average gene diversity/locus and percent of polymorphic loci at the lower altitude ranges. It is evident that there is high genetic diversity found among accessions of species within the two major geographical regions. These wild accessions were collected from characteristic geographical distribution and suitable altitude with high average gene diversity/ locus. Since the heterozygosity of a gene is a measure of the extent of genetic variation (diversity) in a population, so an increase or decrease of genetic diversity within these two geographic regions could be strong evidence that the gradient of biotypes of a species in a region could have an adaptive significance (David et al., 1989). Perry & McIntosh (1991) suggested that differentiation according to geographical regions is useful in confirming the postulated regions of diversity or gene centers.

Finally, on going research using DNA finger printing will support the finding of the present study.

Conclusion

Using SDS page, *Vicia accessions* were grouped into two major geographical regions; North Africa and West Asia and different altitude ranges. Allele frequencies were estimated according to Hardy-Weinberg equilibrium to measure the genetic diversity. Accordingly, accessions of *V. ervilia, V. monantha, V. villosa* and *V. sativa* subsp. *sativa* belonging to West Asia and high altitude ranges expressed higher genetic diversity and percent of polymorphic loci than those of North Africa. Contrarily, accessions of *V. sativa* subsp. *nigra* and *V. villosa* expressed higher values at low altitude ranges. So an increase or decrease of genetic diversity within these two geographic regions and altitude ranges could be strong evidence that the gradient of biotypes of a species in a region could have an adaptive significance.

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Authors contribution: Ghada E. El-Badan; performed the measurements, processed the experimental data using different universal computer programs to validate the data and writing the original draft, Laila M. El-Sadek; suggested the study and supervised it, reviewed the paper and conceived the presented idea, Amal W. Amin; supervised the study, Data curation and reviewed the paper, Fatma M. Ashour; supervised and reviewed the paper, All authors discussed the results and contributed to the final manuscript.

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التنوع الوراثى ومعدل الاالليلات لبعض أنواع جنس الفشيا باستخدام الفصل الكهربى لبروتين البذور

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يشير التنوع الوراثى إلى التباين الجيني داخل الأنواع وفيما بينها. كل نوع على حدة يمتلك جينات (والليلاتها) التي هي مصدر خصائصها الفريدة للتكيف مع الضغوط الحيوية وغير الحيوية في حياتها. و كلما زاد التنوع الجيني داخل الأنواع، زادت فرص بقاء الأنواع على المدى الطويل. وقد أدت الأهمية الاقتصادية العالية لجنس الفشيا إلى مجموعة كبيرة من الدراسات البيوكيميائية والجزيئية لكل نوع والتحقيق في العلاقات التطورية فيما بينها. ينتشر جنس الفشيا على نطاق واسع على امتداد عدة مناطق من أمريكا الشمالية والجنوبية وأوروبا وآسيا ومنطقة البحر المتوسط التي تعد المركز الرئيسي للتنوع حيث ارتبط تعدد السلالات بالمنشأ الجغرافي للمحتوى الجينى.

و قد أمكن تقييم مدى التنوع الجيني في السلالات والأنواع من خلال التوصيف المورفولوجي والواسمات الجينية التى تساعد على تميز الأنواع للمهتمين بتهجين النباتات على اختيار السلالات التي سيتم استخدامها في برامج التهجين. وقد أدى ظهور التقنيات الكيميائية الحيوية والجزيئية إلى إجراء تقييم أكثر دقة للمحتوى الجيني والبيئي للتنوع الوراثى.

هدفت دراسة الفصل الكهربى لبروتين البذور والتى تمت على أنواع مختلفة من جنس الفشيا إلى توصيف وتقييم التنوع الجيني والعلاقة التطورية بين 51 عينة تم جمعها من قبل هيئة الإيكاردا (المركز الدولي للبحوث الزراعية في المناطق الجافة) من أماكن ذات خطوط طولية وعرضية وارتفاعات مختلفة، تمثل تسعة أنواع وتحت نوع من فيسيا ارفيليا، وفيسيا مونانثا، وفيسيا فيللوزا (تحت نوع كل من فيللوزا، ديسيكاربا، اريوكاربا) وفيسيا ساتيفا (تحت نوع كل من ساتيفا وماكروكاربا وانفيكاربا ونيجرا). استخدمت البذور الجافة فى الفصل الكهربى لبروتين البذور حيث أن كل بذرة تعتبر حالة فسيولوجية ثابتة وتمت دراسة نمط التباين في الحجم الجزيئي لبروتينات البذور باستخدام تقنية SDS-PAGE. اعتمدت حسابات التوصيف الإحصائي لوراثة العشائر على بيانات الشكل المظهري (وجود / عدم وجود الشريط البروتينى) والتركيب الجينى (معدل الأالليلات المنتجة لهذه البروتينات) وبالتالي، فإن هذا البحث أعطى معلومات ذات صلة لاستكمال البيانات الوراثية لسلالات الأنواع المتواجدة بهيئة لإيكاردا.