GENETIC DIVERSITY AMONG SOME *Vicia narbonensis* L. VARIETIES AS REVEALED BY KARYOTYPE AND PROTEIN ANALYSIS

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The genus *Vicia* belongs to the Legumes, family leguminosae which is considered one of the largest families of flowering Plants and represents tremendous morphological, ecological and genetic diversity. It includes approximately 140 species (Weber and Schifino-Wittman, 1999) in which *Vicia narbonensis* L. (narbon bean) is one of an annual leguminous species with the potential to become an important grain and straw crop for animal feed. The understanding of the interpretation of evolutionary pathway depends on karyotype studies are of a particular importance. *V. narbonensis* represents a species with relatively large genome (7.3 Pg/1C) (Navrátilová *et al*., 2003) and the previous studies revealed that all accessions of *V. narbonensis* have one karyotype (Perino and Pignome, 1981; Singh and Lelley, 1982; Cremonini *et al.*, 1998a; Ashour *et al.*, 2005). Others found different karyotype formulae for some accessions (Svesschnikova, 1927; Yamamoto, 1973; Cremonini *et al.*, 1998b; Koul *et al.*, 1999; Navrátilová *et al.*, 2003).

The use of gel electrophoresis of seed protein in phylogeny is supported by the fact that mature seeds posses the same protein components unchanged with age or environmental stress, and thus provide valid evidence for genetic relatedness (Crawford, 1990). Sammour (1987) carried out a comparative study on seed proteins of a number of wild *Vicia* species and its varieties. The study showed variation among *V. narbonensis* var. *Aegyptiaca, which* originated from *V. narbonensis* var. *narbonensis* and *V. narbonensis* var *jordanica*. El-Badan (2003) found close similarity in the seed protein profiles of the cultivated *Vicia* species and *Vicia narbonensis* var *narbonensis* indicated that this species can be considered as the immediate progenitor of *V. faba*. The previous studies confirmed that *V. narbonensis* is the closest genome of the wild species to *V. faba* and may share some common gene loci from a common ancestor and evolved in different pathways (Ashour *et al.*, 2005). Consequently, for breeding and seed production, reliable genotype identification is necessary.

In this study seeds of *V. narbonensis* from 9 locations in the Middle East were used for karyological and protein analysis. This study aims to clarify the genetic diversity among *V. narbonensies* varieties collected from different geographical regions and to join three unknown *V. narbonensis* varieties to their similar group (variety) based on karyotype and protein analysis.

MATERIALS AND METHODS

Collection of samples

Seeds from 27 accessions representing three varieties of *V. narbonensis* L. in addition to three unknown varieties (collected from different geographical regions) was used in this karyological and protein studies. The seeds were obtained from ICARDA (International Center for Agricultural Research in the Dry Area) seed bank (Table 1).

Karyotype analysis

Seeds were scarified with sandpaper before sterilization in 30% sodium hypochlorite for five minutes, then washed under running water for 15 minutes, and then germinated at 25±1°C. Root tips of one to two cm long were treated by 0.002 M 8-hydroxyquinoline for four hours at 4ºC before fixation in ethanol- glacial acetic acid (3:1 V/V) for 24 hrs. Roots were stained by modified carbol fuchsin (Millerd *et al.*, 1971). Semi-permanent slides were examined by light microscopy. Five well spread metaphase chromosomes per root were measured using "Micro Measure" computer program (Reeves, 2001). Chromosomal nomenclature was carried out according to Levan *et al.* (1965). The obtained karyotype data were used to calculate relative length (RL), arm ratio (AR), symmetry (S%) and total formula (TF%) (Ramesh and Salimuddin, 1992). Interchromosomal asymmetry (A2) was estimated according to Zarco (1986). Total chromosome volume (TCV) was estimated by using the formula of Naranjo *et al.* (1998) (TCV= $2(\Pi \times r^2 \times TCL)$; where r is the mean chromatid radius, and TCL is the total chromosome length).

Total seed protein electrophoresis

SDS- polyacrylamide gel procedure was carried out according to Laemmli (1970). 50 mg of crushed seeds (without their testa) were used for each variety of *Vicia narbonansis*. Samples were defatted with acetone (10ml/gm) and vortexed for 3min. The supernatant was centrifuged for 15 minutes at 12,000 rpm. Defating was repeated three times and the final residue was left over night to dry. For soluble protein extraction, 0.5ml of buffer (20ml of 1M Tris-HCL pH 6.8, 10 g sucrose, 2 g SDS) was added to 0.05 g of seeds meal and stored overnight at 4°C. Plyacrylamide gel electrophoresis was performed in 2.5% stacking gel solution poured onto the main gel of 15% acrylamide. The gel was then left to settle, electrophoresis was carried out using Tris/glycine pH 8.3 electrode buffer. Electrophoresis was started at 20mA/gel until the marker dye reached the end of

the stacking gel. Then electrophoresis was continued at 25 ma/gel until the marker dye reached the end of the main gel. The gels were stained and de-stained according to Laemmli (1970) and photographed and analyzed by Total Lab Program (www.Totallab.com).

Statistical analysis

Analysis of variance and the dissimilarity genetic distance were carried out for karyotype parameters. The presence and absence of each protein band was treated as a binary character in a data matrix (coded 1 and 0). Using a genetic distance, a dendrogram was constructed using the unweighted pair-group method analysis (UPGMA) with SAHN module of NTSYS-pc (Rolf, 2002) to compare the studied accessions.

RESULTS AND DISCUSSION

The diploid chromosome number of *Vicia narbonensis* was found to be 14 in all studied accessions, and this result agrees with the previous counts (Yamamoto, 1973; Bisht *et al*., 1998; Kamel, 1999; Raina *et al*., 2001; Ashour *et al*., 2005). The presence and length of satellite in the studied *V. narbonensis* varieties showed considerable variation. It varied from complete disappearance in the unknown varieties number 2 and 3 up to 3.5 µm in variety number 4 (Table 2). Satellite was observed on long arm of the shortest chromosome and also in all known studied varieties of *V. narbonensis*. These results agree with those obtained by Yammamoto (1973), Cremonini *et al*. (1998 a&b), Raina *et al.* (2001) and Navrátilová *et al.* (2003). It seems that the absence of satellite in the unknown varieties, accessions 2, 3 and the presence of a longest satellite in the unknown variety, accession 4, play an important role in the evolution of *V. narbonensis* varieties. Sinha and Acharia (1972) suggested that the complete disappearance of satellite is due to translocation, and hybridization might have given rise to the varieties without satellite chromosome.

Karyotype formula was found to be "2st+ 5sm" for var. *salmonea* and "1 st +1m +5sm" for variety 2, while the formula for variety 3, 4 and 6 was found to be "1 st +6sm" (Table 2 and Fig. 1). The karyotype formula "7 sm" was found for all accessions of var. *affinis* except accession number 9 was "1 st + 6 sm". Karyotype formulas varied among studied varieties and this was in agreement with the previous findings (Cremonini *et al.* 1998b; Koul *et al*., 1999). However, others found one karyoptye formula for all accession of *V. narbonensis* (Singh and lelley, 1982; Cremonini *et al.*, 1998a; Ashour *et al*., 2005). This might be due to that the studied accessions were obtained from the similar geographic regions and or the same altitude, which is different in the present study. The longest DCL and MCL were found in the unknown variety 4, while the lowest values were found in varieties 2 and 3. Var. *affinis* had the highest total chromosome volume (190.06) due to the highest value of its chromatid radius (0.65 µm).

The results of the Analysis of Variance (ANOVA) showed that the total diploid chromosome lengths (TDL) were significantly different among all *V. narbonensis* varieties studied ($P < 0.005$) (Fig. 3). The chromosomes from the two unknown varieties 2 and 3 were separated from all varieties and had the lowest total diploid chromosome length. The unknown variety 4 had the longest total diploid chromosome length and was also separated from all investigated varieties. The total length of diploid chromosomes in var. *salmonea* (accesstion 1) occupied the same range of var. *narbonensis* (accession 5) and that of accession 9 of var. *affinis*. The chromosomes of var. *affinis* accessions 6, 7 and 8 were nearly equivalent (Fig. 1). This difference probably due to chromosome deletion or different levels of condensation as was suggested by El-Nahas (2000).

A significant correlation between Total formula (TF) and the altitude was found ($P< 0.005$) (Fig. 4). Completely stable karyotype in sexually reproducing species is unlikely to occur. Slight significant variation has been found on a geographic scale and reported in *Scilla vindobonensis*, *S. bifolia* and *S. mischtschenkoana* (Greilhuber and Speta, 1977 & 1978), *Adoxa* (Greilhuber, 1979), several *Allium* taxa and *Brimeura fastigiata* (Vosa, 1976 a&b; Vosa, 1979), or several *Hordeum* taxa (Linde-Laursen *et al.*, 1980).

Cluster analysis based on chromosome characters indicated the discrimination into two clusters at 0.67 similarity coefficient (Fig. 5). The first cluster comprised one of the unknown varieties (accession 4) with *V. narbonensis* var. *narbonensis* (5) and one of the var. *affinis* (accession 8).The second cluster further classified into two sub clusters, one comprised the unknown variety 3 with var. *salmonea* and accession 7 of var. *affinis* (accession 7), while the second sub cluster included the unknown variety 2 with accessions 6 and 9 of var. *affinis*.

Total seed protein analysis for the studied accessions indicated the presence of 45 bands. There was only one common band in the varieties studied at M.wt 30 KDa, which could be used as fingerprint for *V. narbonensis* (Table 3 and Fig. 6). The differences in the protein profile of individual seeds of each *V. narbonensis* variety was expected since Mudzana *et al*. (1995) and Goodrich *et al*. (1985) found that there was variability in the total seed storage protein profiles of individual seeds within a variety. This was probably due the cross fertilization nature of the genus.

The number of accession specific bands ranged from one band in var. *salmonea* to nine bands in var. *affinis* accession 6. There were no specific bands in two of the unknown varieties accessions 2, 3 and the three accessions of var. *affinis* (accessions 7, 8 and 9). The percentage of polymorphic bands ranged from 13.04% in var. *salmonea* to 39.97% in the unknown variety accession 3. The percentage of polymorphic bands of the

unknown varieties 2 and 3 was 17.39% equaled that of accessions 7 and 8 of var. *affinis*.

The cluster analysis indicated the discrimination into two clusters at 1.53 similarity coefficient (Fig. 7). The first cluster grouped one of the unknown varieties (4) with var. *narbonensis* (5) indicating their relationship. The second cluster comprised the two other unknown varieties (2 and 3) in which the unknown variety (2) was closely related to var. *salmonea* and the other unknown variety (3) was more related to var. *affinis.* At the same cluster var. *affinis* accession number 6 was separated at 1.16 coefficient, probably because it had the highest number of specific bands.

Both clusters indicated the distribution of var. *affinis* accessions throughout the dendrogram, which can be attributed to the absence of specific bands (Table 3). Both clusters also confirmed that the unknown variety 4 is closely related to *V. narbonensis* var. *narbonensis*.

The present study of karyotype and protein pattern of the *V. narbonensis* varieties revealed the presence of high polymorphisms among the studied varieties and the accessions of var. *affinis.* Studying the species from different geographic regions and altitudes, indicates that the species may be still evolving in different pathways, contrary to a previous study which considered *V. narbonensis* accessions donated by the Agriculture Research Center of Egypt (probably from the same geographic region) to be the most stable and primitive type among the genus *Vicia* (Ashour *et al*., 2005).The wide geographic ranges may explain the high degree of variation among varieties and should be taken into account both in conservation programs and in the genetic improvement of this economically important wild *Vicia* species.

SUMMARY

Mitotic chromosomes of nine accessions of three *Vicia narbonensis* varieties (*V. narbonensis* variety *salmonea*, *V. narbonensis* variety *affinis* and *V. narbonensis* variety *narbonensis*) and three unknown varieties were studied. Satellite chromosomes were considered to be the most variable part among the karyotypes of the studied varieties. The total loss of the satellite region was detected in two of the unknown varieties 2 and 3 and was suggested to be due to either translocations and/ or hybridization. The total diploid chromosome lengths were significantly different among all studied *V. narbonensis* varieties ($P < 0.005$). Karyotype formula was also variable between studied varieties. The present study revealed a significant correlation between total formula (TF) as a measure of symmetry and the altitude ($P<0.005$) consequently, the wide geographic distribution of the species might be or is one of the factors which may explain the high levels of genetic

polymorphisms among varieties. Cluster analysis based on chromosome parameters and protein bands indicated the distribution of variety *affinis* accessions through all clusters, which can be attributed to the absence of variety specific bands. Both clusters also confirmed that the unknown variety 4 is closely related to *V. narbonensis* var. *narbonensis*. Cluster analysis based on chromosome parameters alone grouped the unknown variety (3) with variety *salmonea* and variety *affinis* (7) besides the unknown variety (2). The unknown variety 4 is separated alone probably due to the longest DCL and MCL. On the other hand cluster analysis of protein profile showed that the unknown variety 2 is more related to variety *salmonea* and the unknown variety 3 more allied to variety *affinis*.

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Table (1): Accession number, origin and altitude of *V. narbonensis* varieties used in the study. (ICARDA information).

Varieties (accession no.)	Accession number	Origin	Altitude
Var. salmonea (1)	IG 63676	Turkey	600
?var. (2)	IG 60698	Turkey	900
? var.(3)	IG 63775	Turkey	500
?var. (4)	IG 64282	Jorden	650
var. narbonensis (5)	IG 64179	Jorden	950
var. affinis (6)	IG 63701	Turkey	800
var. affinis (7)	IG 60697	Turkey	1100
var. affinis (8)	IG 63034	Syria	105
var. affinis (9)	IG 63871	Syria	580

Table (2): Karyotype parameters of *V. narbonensis* varieties.

Varieties	Accession number	DCL (µm)	TCV (µm)	TF%	S%	MCL	A2	Sa
Var. *salmonea*	1	86.94^{a}	136.50^{a}	13.36^{a}	69.04^{ab}	6.21^{a}	0.135^{a}	1
unknown	2	10.14^{b}	0.40^{b}	14.00^{a}	48.42^{bd}	0.72^{b}	0.228^{ab}	0
	3	11.71^{b}	0.18^{c}	14.86^{ac}	58.33^{ab}	0.84^{b}	0.175^{a}	0
	4	132.49^{c}	133.13^{a}	14.25^{a}	64.30^{ab}	9.46^{c}	0.145^{a}	1
Var. *narbonensis*	5	92.73^{ae}	93.18^{d}	14.07^{ac}	63.40^{abd}	6.62^{a}	0.162^{a}	1
Var. *affinis*	6	65.07^{d}	200.23^{e}	14.77^{ac}	69.82^{ab}	4.65^{d}	0.126^{a}	1
	7	63.57^{ad}	143.72^{af}	13.56^{ac}	66.30^{abd}	4.54^{d}	0.124^{a}	1
	8	66.32^{d}	149.94^{f}	15.24^{bc}	73.06^{abc}	4.74^{d}	0.103^{a}	1
	9	86.56^{ad}	266.36^{e}	14.50^{ac}	70.46^{abc}	6.18^{ad}	0.120^{ac}	1
F ($p<0.001$)		1.00	113.63	1.06	3.90	134.88	0.78	
StDev		60.12	13.95	1.91	6.43	0.41	0.16	

DCL = Diploid Chromosome Length, TCV = Total Chromosome Volume, TF% = Total Form, S% = Symmetry index, MCL = Mean Chromosome Length, A2 = interchromosom°al asymmetry index and Sa = presence of satellite chromosome 1 and absence 0. Means with similar letters in a column are not significantly different at 0.05.

Table (3): Relative concentration (band %) and molecular weight (M.wt) of protein bands of *Vicia narbonensis* varieties.

Band no.	M.wt. (KDa)	Band %								
		V. narbonensis								
		Var. *salmonea*	unknown varieties			Var. *narbonensis*	Var. *affinis*			
		1	2	3	4	5	6	7	8	9
1	150.0	10.17*								
2	145.0							2.51	3.31	16.7
3	140.0		9.57	4.90						
4	118.0				1.04	1.78				
5	110.0				0.64	2.76				
6	100.0				1.37	1.63	3.32			
7	94.0						3.33*			
8	93.5						1.72*			
9	93.0						1.41*			
10	90.0				1.44*					
11	70.0						3.61*			
12	65.0				9.11	1.67				
13	60.0	44.60	32.81	23.94			3.38	35.21	25.5	25.46
14	58.0				4.99*					
15	50.0				7.65*					
16	48.0					1.94*				
17	46.0					7.42*				
18	45.0				1.48*					
19	42.0	18.85	7.12	9.08			4.50	3.69	13.04	13.53
20	41.0						8.38*			
21	40.0				10.29		4.52			
22	38.0					6.48*				
23	35.0				1.38		5.96			
24	33.0						10.53*			
25	32.0							7.37	8.28	4.52
26	30.0	3.00	4.34	12.38	5.80	12.87	9.39	12.01	4.76	8.35
27	28.0	5.95	10.20	11.20		12.17				
28	27.0				2.70	4.42				
29	26.0				7.39			4.12	4.69	5.90
30	25.0		3.80	4.56	9.61	8.15	3.84			
31	23.0						4.96*			
32	20.0					8.70*				
33	18.5	7.31	17.53	5.57				5.58	5.72	5.96
34	18.0					6.45*				
35	16.5						10.35*			
36	16.0						3.88*			
37	15.8				7.96*					
38	15.0					1.64		7.35		
39	14.5		14.49	15.86		1.98	6.89			
40	14.0				3.75		2.29			5.75
41	13.5					8.30*				
42	13.0	10.13	0.13	12.51		11.67	3.20	21.79	17.82	6.51
43	12.0				8.14*					
44	11.0								16.83	7.33
45	10.0				8.99*					
Number of variety specific bands		1	0	0	7	6	9	0	0	0
Percentage of polymorphic bands		13.04	17.39	17.39	39.97	34.78	39.13	17.39	17.39	19.57

*= accession specific band

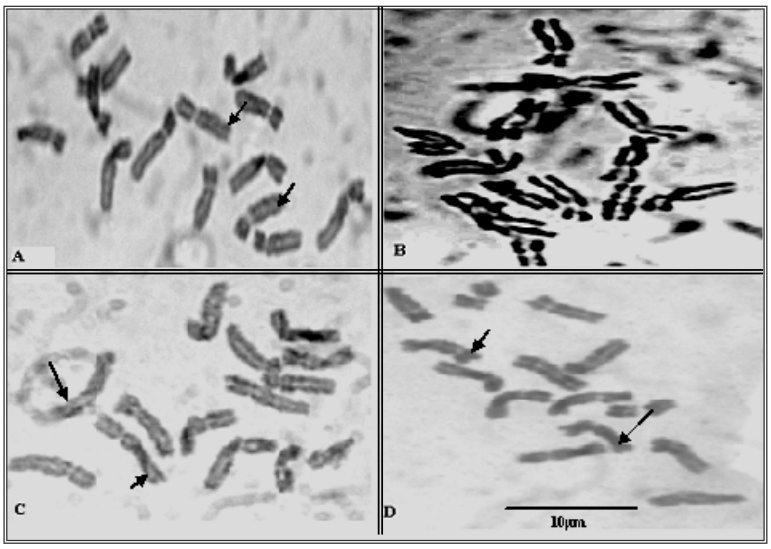


Fig. (1): Somatic metaphase chromosomes of the *V. narbonensis.* (A) var. *salmonea*, (B) unknown variety 2, (C) unknown variety 4 and (D) var. *affinis.*

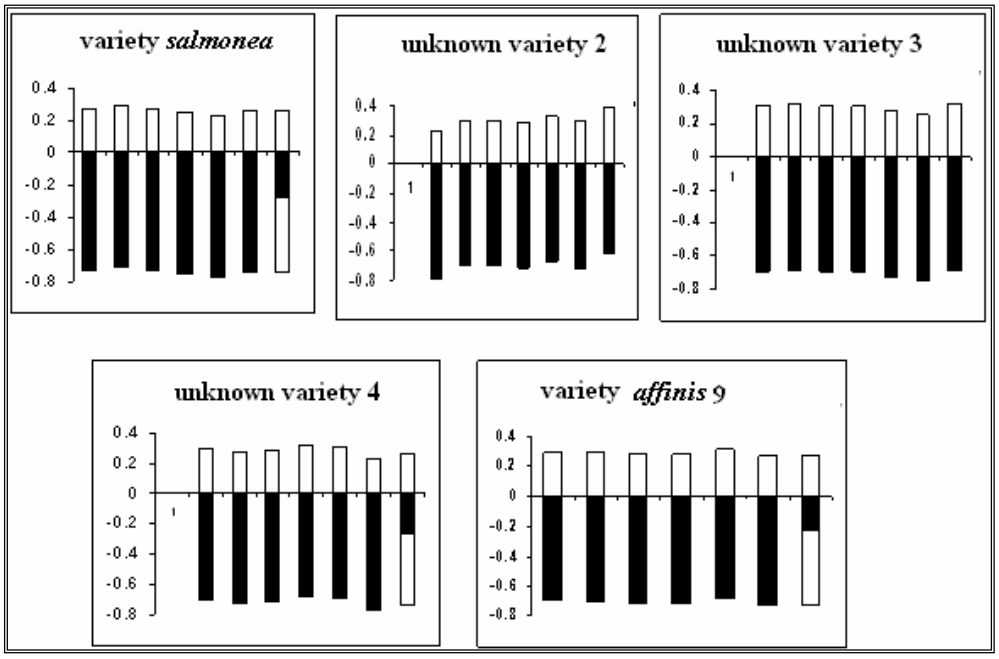


Fig. (2): Idiogram of the *V. narbonensis* varieties.

Source	df	SS	MS	F	P
Factor	8	36098.8	4512.4	135.41	0.000
Error	18	599.8	33.3		
Total	26	36698.7			
S = 5.773		R-Sq = 98.37%		R-Sq(adj) = 97.64%	

Individual 95% CIs for Mean Based on Pooled StDev

Level	N	Mean	StDev	---+---------+---------+---------+
V. narbonensis var. *salmonea*	3	86.94	1.12	(-*-)
V. narbonensis ? 2	3	10.15	0.99	(-*-)
V. narbonensis ? 3	3	11.71	0.91	(-*-)
V. narbonensis ? 4	3	132.49	5.93	(-*-)
V. narbonensis var. *narbonensis*	3	92.73	8.97	(-*-)
V. narbonensis var. *affinis* 6	3	65.07	6.86	(-*-)
V. narbonensis var. *affinis* 7	3	62.32	4.41	(-*-)
V. narbonensis var. *affinis* 8	3	66.32	0.85	(-*-)
V. narbonensis var. *affinis* 9	3	86.58	10.68	(-*-)
Pooled StDev = 5.77				---+---------+---------+---------+
				35 70 105 140

Fig. (3): Analysis of variance of the total diploid chromosome length of the accessions studied.

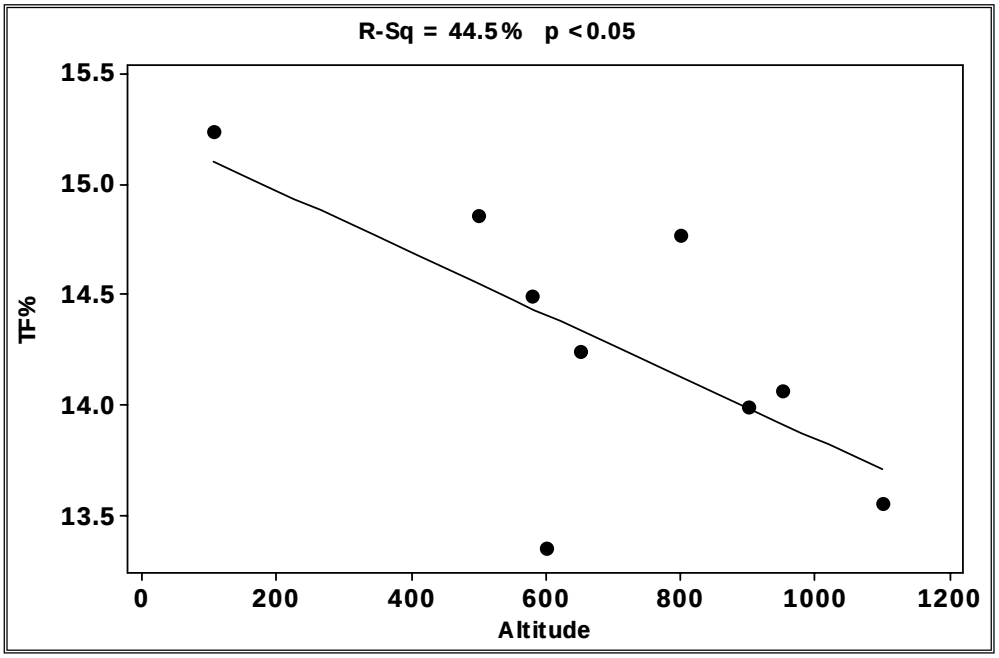


Fig. (4): Correlation between the altitude and TF% of *V. narbonensis*.

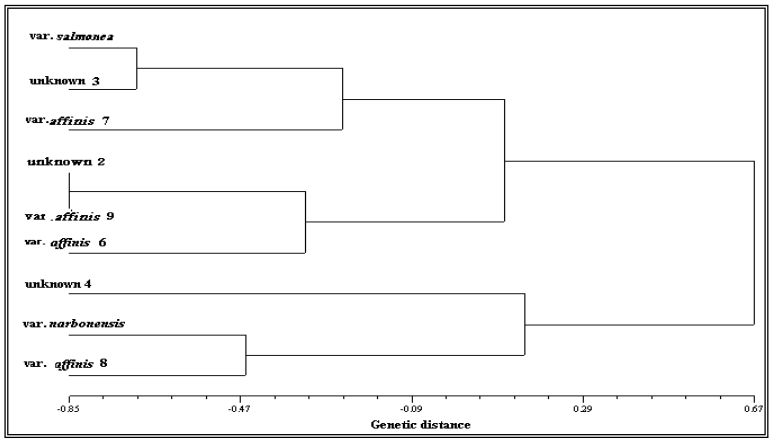
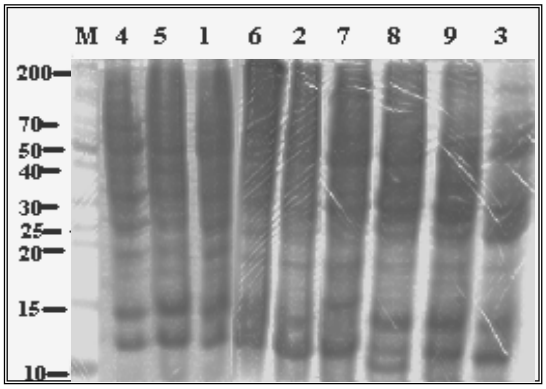


Fig. (5): Dendrogram resulted from UPGMA cluster analysis based on chromosome parameters of the studied *V. narbonensis* accessions.

Fig. (6): Electrophotograph produced by SDS-PAGE analysis of general protein patterns of *V. narbonensis* varieties.



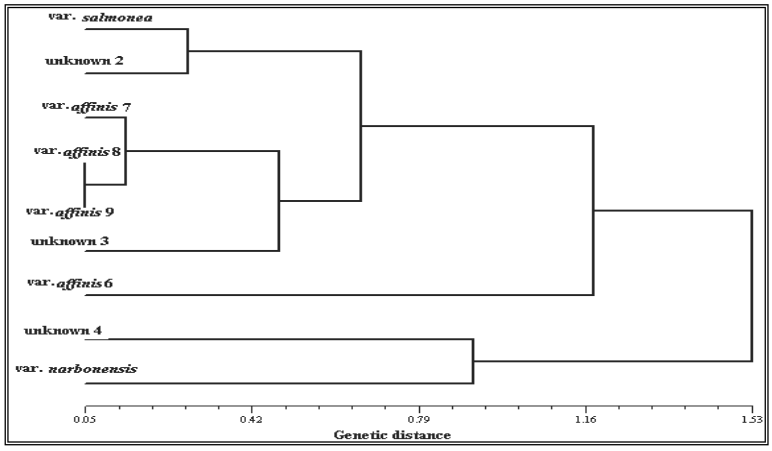


Fig. (7): Dendrogram resulted from UPGMA cluster analysis based on protein electrophoresis of the studied *V. narbonensis* accessions.