doi: 10.17221/10/2015-CJGPB

Evaluation of Genetic Variability in Algerian Clover (*Trifolium* L.) Based on Morphological and Isozyme markers

Imane MEDOUKALI, Inès BELLIL and Douadi KHELIFI

Laboratoire de Génétique Biochimie et Biotechnologies Végétales, Faculté des Sciences de la Nature et de la Vie, Université Constantine, Constantine, Algérie

Abstract

Medoukali I., Bellil I., Khelifi D. (2015): Evaluation of genetic variability in Algerian clover (*Trifolium* L.) based on morphological and isozyme markers. Czech J. Genet. Plant Breed., 51: 50–61.

Genetic variation within and among fifteen *Trifolium* species represented by 157 accessions was assessed using morphological and isozyme markers. Most of morphometric characters contributed to the discrimination of the species. No significant relationship between the environment of the collection site and morphological features was detected. The two isozyme systems analysed, esterase (EST) and glutamate oxaloacetate transaminase (GOT), proved polymorphic. Phenotype diversity of isozyme markers ranged from 0.07 to 0.61 with an average of 0.31 based on the polymorphic information content. The pairwise Jaccard's similarity coefficient ranged between 0.10 and 0.60, indicating that the collection represents genetically diverse species. A considerable number of species-specific zymograms were detected which can be used for the species identification. The clustering pattern of isozyme markers was incongruent with the groupings based on quantitative traits. The rich isozyme variability present among the Algerian clover species indicates that they can provide good gene resources for breeding.

Keywords: clover; isozymes; morphology; PAGE; polymorphism

The genus *Trifolium* L. (Leguminosae) or clover includes 250–300 annual and perennial species, distributed throughout the temperate and subtropical regions (Allen & Allen 1981; Zohary & Heller 1984; Ellison *et al.* 2006). About 10% (20–30) of them are used as forage plants in commercial agriculture, and a greater number is used locally for fodder in their native zones (Williams & Nichols 2011). In particular, *Trifolium repens* (white clover), *Trifolium pratense* (red clover) and *Trifolium alexandrinum* (berseem clover) are widely used in the temperate livestock agriculture (Abberton 2007).

The genus has been divided into eight sections: *Lotoidea*, *Paramesus*, *Mystillus*, *Vesicaria*, *Chronosemium*, *Trifolium*, *Tricocephalum* and *Involucrarium*. *Lotoidea* is the largest and has served as a source taxon for the evolution of other sections (Zohary & Heller 1984).

Trifolium species occur in a wide range of habitats, including meadows and prairies, open woodlands, semi-deserts, mountains, and alpine peaks. A common feature of these diverse habitats is high solar radiation, hence, few clover species tolerate shade. The greatest species diversity is found in three geographic regions: the Mediterranean basin, western North America, and the highlands of eastern Africa (Ellison *et al.* 2006).

Population genetic diversity and variability play a vital role for a successful breeding program. The conservation and sustainable use of plant genetic resources require accurate identification of their accessions (Asci 2011). Morphological characterization is the first step in description and classification of genetic resources (Smith & Smith 1989). When various sets of clover populations were analysed, large genetic diversity was determined among and within populations using morphological traits (Kongkiatngam *et al.* 1995; Greene *et al.* 2004; Dias *et al.* 2008a; Asci 2011).

Isozyme markers when used in combination with other approaches are of considerable value in un-

doi: 10.17221/10/2015-CJGPB

derstanding the crop plant evolution (Malaviya *et al*. 2008). Despite the use of DNA markers, isozyme polymorphism is also used effectively in species delimitation and conservation, genetic relationship between individual populations and closely related species. Sporadic reports within the genus *Trifolium* based on isozymic studies are available (Kongkiatngam *et al*. 1995; Lange & Schifino-Wittmann 2000; Malaviya *et al*. 2005, 2008; Dias *et al*. 2008b).

Algeria is one of the Mediterranean countries rich in genetic diversity; it is represented by 37 clover species in its natural flora (Quezel & Santa 1962). However, there are few reports regarding the interrelationships of Algerian *Trifolium* species based on works using morphological and agronomic traits (Ghoubay & Abdelguerfi 1991; Issolah & Abdelguerfi 2000, 2003). Therefore, the present investigations were carried out to determine the similarity and differences between populations of the genus *Trifolium* L., which naturally grow in Northern Algeria by using both morphological and isozyme markers.

MATERIAL AND METHODS

Sample collection. The study was conducted on 15 species of the genus *Trifolium* L. (*T. angustifolium*, *T. lappaceum*, *T. resupinatum*, *T. tomentosum*, *T. scabrum*, *T. campestre*, *T. fragiferum*, *T. pallidum*, *T. pallescens*, *T. squarrosum*, *T. glomeratum*, *T. cherleri*, *T. stellatum*, *T. repens and T. spumosum*) represented by 157 accessions. These species were collected from 32 sites covering a wide range of bioclimatic stages in Northern Algeria from May to July 2013. The collecting sites extended from the coastal region to the Tellian Atlas Mountains, including the coastal plains and the steppe highlands, along a gradient of increasing aridity from North to South (Figure 1). For every collection seven main ecological parameters were determined (Table 1): altitude (Alt), latitude (Lat), longitude (Lon), average annual rainfall (*Pm*), the average of the minimum temperature of the coldest month (*m*), the average of the maximum temperature of the hottest month (*M*) and classical Emberger's coefficient (*Q*2), which was calculated according to Stewart (1974):

$$Q2 = 3.43\ P/(M - m) \quad (1)$$

where:

P – annual rainfall

M – average of the maximum temperature of the hottest month

m – average of the minimum temperature of the coldest month

Morphological markers. For each accession, 10 fruiting heads were taken from different plants randomly and 10 quantitative traits related to fruiting head, pod and seed were scored: size of fruiting heads: length (LF), width (WDF); number of pods per fruiting head (PF); number of seeds per pod (SP); number of seeds per fruiting head (SF); seed size: length (LS), width (WS); weight of 30 fruiting heads (WTF); weight of 1000 seeds (WTS) and the ratio weight of seeds/weight of fruiting heads (RW).

Isozyme markers. Polyacrylamide gel electrophoresis (PAGE) as described by Singh *et al*. (1991) was used to analyse the variation in two enzymatic systems: EST (E.C. 3.1.1.) and GOT (E.C. 2.6.1.1). Extraction of enzymes was performed from six-days-old seedlings according to Zoro Bi *et al*. (1999).

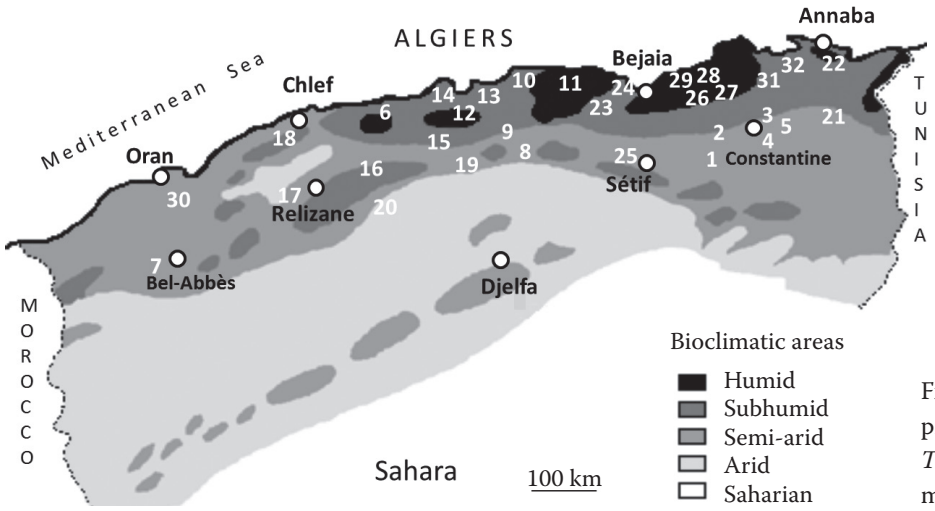

Figure 1. Locations of the sampled populations of the genus *Trifolium* L. in relation to bioclimate in northern Algeria

doi: 10.17221/10/2015-CJGPB

Isozyme staining protocols followed methods as described by Bendiab *et al.* (1993).

Statistical analysis. Morphometric traits were analysed using the principal component analysis (PCA). Mean, standard deviation (SD) and coefficient of variation (CV) of the morphological traits for the 15 studied species were computed. The correlation between morphological variability and bioclimate was examined by another PCA including seven ecological parameters (Alt, Lat, Lon, *Pm*, *m*, *M* and *Q*2). UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) dendrogram based on Euclidean distances was constructed to evaluate phenetic relationships between species. All analyses based on morphological markers were performed using the STATISTICA version 6.0 software (Statsoft, Inc. 1995). For each enzyme system, zones of activity and bands were numbered sequentially from the origin. All isozymes that appeared in a zone were considered in identifying isozyme phenotypes that were lettered

Table 1. Sampling sites and the main bioclimatic parameters used in this study

No.	Localities	Sample size	Alt	Lat	Lon	*Pm*	*m*	*M*	*Q*2
1	Oued Athmenia	4	757	36°16'17.87''N	6°16'36.31''E	568	2.5	31.3	67.65
2	Mila east	9	831	36°21'48.77''N	6°19'56.83''E	742	4.4	31.1	95.32
3	Constantine North	7	429	36°31'04.89''N	6°33'52.68''E	704	3.2	31.4	85.63
4	Constantine west	6	850	36°18'08.13''N	6°27'50.96''E	558	3.0	32.2	65.55
5	Constantine center	5	584	36°20'20.08''N	6°37'27.04''E	624	3.3	32.0	74.58
6	Tipaza	4	203	36°34'48.16''N	2°34'06.74''E	626	8.4	30.1	98.95
7	Sidi Belabbes	5	465	35°14'20.82''N	0°37'03.24''E	450	4.8	29.7	61.99
8	Bouira	6	537	36°22'09.18''N	3°51'33.64''E	506	0.2	30.9	56.53
9	Bouira west	4	144	36°34'38.26''N	3°33'16.25''E	711	5.9	31.9	93.80
10	Boumerdès	5	20	36°44'52.57''N	3°41'42.29''E	739	6.8	30.8	105.62
11	Tizi Ouzou	8	129	36°43'54.04''N	4°17'54.66''E	896	6.2	32	119.12
12	algeirs center	4	120	36°44'43.41''N	3°04'10.22''E	707	8.1	28.5	118.87
13	Mohammadia	7	3	36°44'21.62''N	3°08'50.41''E	670	7.6	29.8	103.52
14	algeirs west	7	172	36°44'00.09''N	3°01'33.28''E	762	6.9	28.4	121.57
15	Blida	8	70	36°32'48.32''N	2°48'23.72''E	791	7.0	30.9	113.52
16	Ain Defla	5	257	36°09'57.25''N	1°43'25.04''E	593	6.0	33.5	73.96
17	Relizane	2	49	35°55'35.18''N	0°47'33.24''E	348	6.8	31.1	49.12
18	Chlef	4	135	36°14'45.30''N	1°14'17.86''E	405	6.6	32.6	53.43
19	Medea	9	385	36°20'33.96''N	2°46'04.83''E	736	2.5	30.6	89.84
20	Tissemsilt	6	575	36°00'36.95''N	2°09'11.39''E	609	1.1	30.1	72.03
21	Guelma	4	798	36°22'08.93''N	7°13'59.93''E	564	1.9	32.1	64.06
22	Annaba	10	132	37°02'56.98''N	7°23'19.39''E	712	8.2	28.1	122.72
23	Akbou	2	207	36°30'03.58''N	4°34'28.30''E	659	6.2	31.3	90.05
24	Tichy	5	2	36°40'21.52''N	5°08'59.99''E	856	8.7	28.9	145.35
25	Sétif	1	1089	36°11'41.65''N	5°24'25.79''E	473	0.3	31.7	51.67
26	Jijel South	4	166	36°47'29.52''N	5°41'26.87''E	818	8.2	29.3	132.97
27	Jijel east	3	132	36°47'56.92''N	5°42'56.70''E	818	8.2	29.3	132.97
28	Jijel North	1	9	36°48'56.16''N	5°42'04.92''E	818	8.2	29.3	132.97
29	Jijel west	3	21	36°47'26.38''N	5°39'46.16''E	818	8.2	29.3	132.97
30	Oran	2	87	35°37'40.78''N	0°36'01.72''O	383	8.2	26.6	71.40
31	oued Righa	4	32	36°53'46.84''N	7°03'56.20''E	767	7.1	28.5	122.94
32	filfila	3	159	36°53'11.44''N	7°04'56.50''E	794	5.7	29.6	113.95

Alt – Altitude; Lat – Latitude; Lon – Longitude; *Pm* – the average annual rainfall; *m* – the average of the minimum temperature of the coldest month; *M* – the average of the maximum temperature of the hottest month; *Q*2 – bioclimatic Emberger's coefficient, calculated according to Stewart (1974), see Eq. (1)

doi: 10.17221/10/2015-CJGPB

arbitrarily. The polymorphism information content (PIC) for each zone:

$$PICi = 1 - P_{ij}^2 \quad (2)$$

where:

P_{ij} – frequency of the j^{th} phenotype for the i^{th} zone

To estimate the isoenzymatic variation among the species UPGMA dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard 1908) by the aid of XLSTAT version 2014.1.09 program (Addinsoft 2007). The correlation between the morphological and isozyme distances was assessed by the Mantel test (Mantel 1967).

RESULTS

Morphological characters and their relationship with ecological parameters. Mean, range, SD and CV values of the morphological characteristics studied are shown in Table 2. The number of pods per fruiting head (PF) was found to be the most variable characteristic with CV 45.03% ranging from 8.00 mm to 91.00 mm followed by the number of seeds per fruiting head (SF) with CV 33.04% ranging between 3.33 mm and 86.00 mm. The length of fruiting heads (LF) presented an average variability coefficient with 17.25% followed by WDF and WTF with CV 2.60% and 1.85%, respectively. The remaining characters displayed a margin variation across all the species.

PCA revealed that the first three axes accounted for 86.60% of the total variation with 52.41%, 24.21% and 9.97% for PC1, PC2 and PC3, respectively. Two-dimensional (2-D) plot was prepared using the first two PCs (Figure 2a). All quantitative traits showed a discriminant power and the most important characters in accession differentiation were PF and SF, hence, they had high loadings on the first two principal components. The remaining characters displayed a significant correlation between them and contributed highly to the formation of PC1, excluding RW and SP, which contributed mainly to PC2 and PC3, respectively. According to PCA performed to evaluate the correlation between morphology and bioclimate (Fig-

Table 2. Mean, range, standard deviation (SD) and coefficient of variation (CV) of the analyzed traits for 15 *Trifolium* species

Traits	Min	Max	Mean	SD	CV (%)
LF (mm)	4.82	53.20	15.75	11.68	17.257
WDF (mm)	4.19	28.18	10.90	4.53	2.600
PF (score)	8.00	91.00	36.58	18.86	45.030
WTF (g)	0.36	16.20	3.51	3.83	1.853
SP (score)	0.00	6.00	1.18	0.16	0.003
SF (score)	3.33	86.00	26.87	16.16	33.047
LS (mm)	0.89	2.51	1.59	0.41	0.021
WS (mm)	0.60	1.89	1.14	0.31	0.012
WTS (g)	0.16	5.50	1.40	1.13	0.163
RW (g/g)	0.14	1.68	0.56	0.33	0.014

LF – length of fruiting head; WDF – width of fruiting head; PF – No. of pods per fruiting head; WTF – weight of 30 fruiting heads; SP – No. of seeds per pod; SF – No. of seeds per fruiting head; LS – length of the seed; WS – width of the seed; WTS – weight of 1000 seeds; RW – ratio weight of seeds per weight of fruiting heads

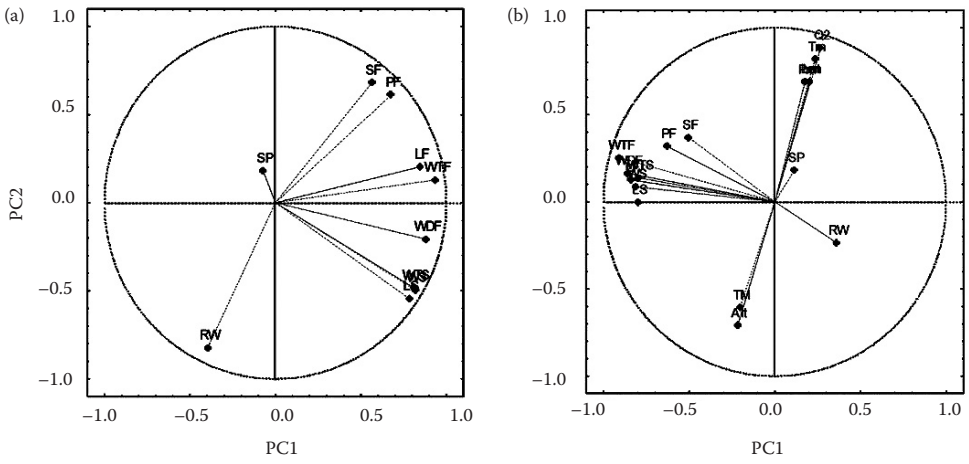

Figure 2. Principal component analysis depiction of ten morphological characters (a) and their relationship with bioclimate (b)
LF – length of fruiting head; WDF – width of fruiting head; PF – No. of pods per fruiting head; WTF – weight of 30 fruiting heads; SP – No. of seeds per pod; SF – No. of seeds per fruiting head; LS – length of the seed; WS – width of the seed; WTS – weight of 1000 seeds; RW – ratio weight of seeds per weight of fruiting heads

doi: 10.17221/10/2015-CJGPB

Table 3. Phenotype frequencies at seven zones of enzymatic activity in fifteen *Trifolium* species

Zones	Phenoptypes	Total	Species														
			TA 22	*Tlap* 18	*Tsc* 20	*Tcp* 23	*Ttm* 14	*TR* 10	*Tst* 9	*Tsq* 8	*Tps* 8	*Tpd* 7	*Tch* 6	*Tg* 5	*Tf* 4	*Trp* 2	*Tsp* 1
	A: 1+2	0.04	–	–	–	–	–	0.60	–	–	–	–	–	–	–	–	–
	B: 2	0.20	0.18	–	–	–	1.00	0.40	0.89	–	–	–	–	–	–	–	1.00
	C: 2+4	0.06	0.41	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D: 2+5	0.01	–	–	–	–	–	–	0.11	–	–	–	–	–	–	–	–
	E: 2+4+5	0.06	0.41	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	F: 3	0.01	–	0.11	–	–	–	–	–	–	–	–	–	–	–	–	–
	G: 3+4	0.10	–	0.89	–	–	–	–	–	–	–	–	–	–	–	–	–
	H: 5	0.03	–	–	–	0.22	–	–	–	–	–	–	–	–	–	–	–
	I: 5+6	0.05	–	–	–	–	–	–	–	1.00	–	–	–	–	–	–	–
Got-1	J: 5+7	0.03	–	–	–	–	–	–	–	–	–	–	–	–	1.00	–	–
	K: 5+9	0.08	–	–	–	0.52	–	–	–	–	–	–	–	–	–	–	–
	L: 5+9+11	0.04	–	–	–	0.26	–	–	–	–	–	–	–	–	–	–	–
	M: 6	0.01	–	–	–	–	–	–	–	–	–	–	0.17	–	–	–	–
	N: 6+7	0.03	–	–	–	–	–	–	–	–	–	–	0.83	–	–	–	–
	O: 6+9	0.06	–	–	–	–	–	–	–	–	1.00	–	–	–	–	1.00	–
	P: 7	0.03	–	–	–	–	–	–	–	–	–	0.71	–	–	–	–	–
	Q: 7+8	0.01	–	–	–	–	–	–	–	–	–	0.29	–	–	–	–	–
	R: 10+12	0.03	–	–	–	–	–	–	–	–	–	–	–	1.00	–	–	–
	S: 13	0.13	–	–	1.00	–	–	–	–	–	–	–	–	–	–	–	–
	A: 14	0.04	–	–	–	–	–	–	–	–	–	–	1.00	–	–	–	–
	B: 14+16	0.01	–	–	–	–	–	–	–	–	–	–	–	–	0.50	–	–
	C: 14+16+17	0.01	–	–	–	–	–	–	–	–	–	–	–	–	0.25	–	–
	D: 15+17	0.03	–	–	–	0.17	–	–	–	–	–	–	–	–	–	–	–
	E: 16	0.32	–	0.94	–	–	1.00	0.10	1.00	0.13	–	1.00	–	–	0.25	–	1.00
Got-2	F: 16+17	0.06	–	–	–	–	–	0.90	–	0.13	–	–	–	–	–	–	–
	G: 17	0.38	1.00	–	–	0.78	–	–	–	0.75	1.00	–	–	1.00	–	0.50	–
	H: 17+19	0.01	–	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–
	I: 18	0.07	–	–	0.55	–	–	–	–	–	–	–	–	–	–	–	–
	J: 19	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–	0.50	–
	K: Null	0.06	–	0.06	0.45	–	–	–	–	–	–	–	–	–	–	–	–
	A: 1+2	0.08	–	–	–	–	0.86	–	–	–	–	–	–	–	–	–	–
	B: 2	0.01	–	–	–	–	0.14	–	–	–	–	–	–	–	–	–	–
	C: 2+4	0.03	–	–	0.05	–	–	–	–	–	0.50	–	–	–	–	–	–
	D: 2+5	0.03	–	–	0.10	–	–	–	–	–	0.13	–	0.17	–	–	–	–
	E: 2+4+5	0.01	–	–	–	–	–	–	–	–	0.25	–	–	–	–	–	–
	F: 2+3+6	0.01	–	–	–	–	–	–	–	–	–	–	0.17	–	–	–	–
	G: 2+5+6	0.01	–	–	–	–	–	–	–	–	–	–	0.17	–	–	–	–
Est-1	H: 2+6	0.01	–	–	–	–	–	–	–	–	–	–	0.17	–	–	–	–
	I: 3+6	0.01	–	–	–	–	–	–	–	–	–	–	0.33	–	–	–	–
	J: 4	0.05	–	–	0.30	–	–	–	–	–	0.13	–	–	–	0.25	–	–
	K: 4+5	0.06	–	–	0.35	–	–	–	–	–	–	–	–	–	0.75	–	–
	L: 5	0.03	–	–	0.20	–	–	–	–	–	–	–	–	–	–	–	–
	M: 6	0.23	–	1.00	–	–	–	0.50	–	1.00	–	–	–	1.00	–	–	–
	N: Null	0.44	1.00	–	–	1.00	–	0.50	1.00	–	–	1.00	–	–	–	1.00	1.00

doi: 10.17221/10/2015-CJGPB

Table 3 to be continued

Zones	Phenoptypes	Total	Species														
			TA 22	*Tlap* 18	*Tsc* 20	*Tcp* 23	*Ttm* 14	*TR* 10	*Tst* 9	*Tsq* 8	*Tps* 8	*Tpd* 7	*Tch* 6	*Tg* 5	*Tf* 4	*Trp* 2	*Tsp* 1
	A: 7	0.02	–	–	–	–	0.21	–	–	–	–	–	–	–	–	–	–
	B: 7+8	0.06	–	–	–	–	0.71	–	–	–	–	–	–	–	–	–	–
	C: 7+8+9	0.12	0.36	0.06	–	–	–	–	–	–	1.00	–	–	–	–	1.00	–
	D: 7+8+9+11	0.09	0.64	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E: 7+9	0.21	–	–	1.00	–	–	0.90	–	–	–	–	–	–	1.00	–	–
	F: 7+8+10	0.01	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–	–
	G: 8	0.06	–	0.06	–	–	–	–	0.78	–	–	–	–	–	–	–	1.00
	H: 8+9	0.06	–	0.11	–	–	–	–	–	0.13	–	–	0.50	0.60	–	–	–
	I: 8+9+10	0.03	–	0.22	–	–	–	–	–	–	–	0.14	–	–	–	–	–
Est-2	J: 8+9+10+11	0.01	–	0.06	–	–	–	–	–	–	–	–	–	–	–	–	–
	K: 8+9+11	0.01	–	0.06	–	–	–	–	–	–	–	–	–	–	–	–	–
	L: 8+10	0.02	–	0.17	–	–	–	–	–	–	–	–	–	–	–	–	–
	M: 8+10+11	0.01	–	0.06	–	–	–	–	–	–	–	–	–	–	–	–	–
	N: 8+11	0.03	–	0.22	–	–	–	–	–	–	–	–	–	–	–	–	–
	O: 9	0.05	–	–	–	–	–	0.10	–	0.13	–	0.14	0.50	0.40	–	–	–
	P: 9+10	0.01	–	–	–	–	–	–	–	–	–	0.14	–	–	–	–	–
	Q: 9+11	0.06	–	–	–	–	–	–	–	0.63	–	0.57	–	–	–	–	–
	R: 11	0.01	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–
	S: Null	0.16	–	–	–	1.00	–	–	0.22	–	–	–	–	–	–	–	–
	A: 12	0.34	1.00	0.44	0.20	0.09	–	0.60	0.11	–	0.13	0.29	0.17	1.00	0.25	–	–
	B: 12+13	0.10	–	–	0.70	0.09	–	–	–	–	–	–	–	–	–	–	–
	C: 12+13+15	0.05	–	–	–	0.35	–	–	–	–	–	–	–	–	–	–	–
	D: 12+13+15+16	0.03	–	–	–	0.17	–	–	–	–	–	–	–	–	–	–	–
	E: 12+13+16	0.01	–	–	–	0.09	–	–	–	–	–	–	–	–	–	–	–
Est-3	F: 12+14	0.03	–	0.22	–	–	–	–	–	–	0.13	–	–	–	–	–	–
	G: 12+15	0.03	–	–	–	0.17	–	–	–	–	–	–	–	–	–	–	–
	H: 12+16	0.06	–	–	–	0.04	–	–	0.78	–	–	–	–	–	–	–	1.00
	I: 13	0.01	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–	–
	J: 14	0.15	–	–	–	–	1.00	–	–	0.38	0.13	–	0.83	–	–	–	–
	K: Null	0.20	–	0.33	0.05	–	–	0.40	0.11	0.63	0.63	0.71	–	–	0.75	1.00	–
	A: 17	0.32	0.73	0.17	–	0.39	0.21	0.10	0.89	–	0.50	–	–	1.00	–	1.00	–
	B: 17+18	0.04	–	–	–	–	0.50	–	–	–	–	–	–	–	–	–	–
	C: 17+19	0.23	–	–	1.00	–	–	0.90	–	–	0.38	–	0.67	–	–	–	–
	D: 17+20	0.08	–	–	–	0.57	–	–	–	–	–	–	–	–	–	–	–
	E: 17+20+21	0.01	–	–	–	–	–	–	–	–	–	0.29	–	–	–	–	–
	F: 17+20+21+22	0.02	–	–	–	–	–	–	–	–	–	0.43	–	–	–	–	–
	G: 17+21	0.04	0.27	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Est-4	H: 18	0.03	–	–	–	–	0.29	–	–	–	–	–	–	–	–	–	–
	I: 19	0.04	–	–	–	–	–	–	–	0.13	0.13	–	–	–	1.00	–	–
	J: 20	0.02	–	0.11	–	0.04	–	–	–	–	–	–	–	–	–	–	–
	K: 20+21	0.01	–	0.11	–	–	–	–	–	–	–	–	–	–	–	–	–
	L: 21	0.06	–	0.50	–	–	–	–	–	–	–	0.14	–	–	–	–	–
	M: 21+22	0.01	–	0.06	–	–	–	–	–	–	–	–	–	–	–	–	–
	N: 20+21+22	0.01	–	–	–	–	–	–	–	–	–	0.14	–	–	–	–	–
	O: Null	0.08	–	0.06	–	–	–	–	0.11	0.88	–	–	0.33	–	–	–	1.00

doi: 10.17221/10/2015-CJGPB

Table 3 to be continued

Zones	Phenoptypes	Total	Species														
			TA 22	*Tlap* 18	*Tsc* 20	*Tcp* 23	*Ttm* 14	*TR* 10	*Tst* 9	*Tsq* 8	*Tps* 8	*Tpd* 7	*Tch* 6	*Tg* 5	*Tf* 4	*Trp* 2	*Tsp* 1
Est-5	A: 23	0.17	0.05	–	–	0.35	–	1.00	–	–	0.13	0.29	–	–	1.00	–	–
	B: 23+24	0.08	–	–	–	0.13	–	–	1.00	–	–	–	–	–	–	–	–
	C: 23+25	0.09	–	–	–	–	1.00	–	–	–	–	–	–	–	–	–	–
	D: 23+25+26	0.03	–	–	–	–	–	–	–	–	–	–	–	1.00	–	–	–
	E: 23+25+27	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–	0.50	–
	F: 23+25+28	0.01	–	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–
	G: 23+27	0.05	0.32	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–
	H: 23+27+29	0.06	0.23	–	–	–	–	–	–	–	0.50	–	–	–	–	–	–
	I: 23+29	0.11	0.27	0.06	–	–	–	–	–	–	–	0.71	1.00	–	–	–	–
	J: 23+27+30+32	0.01	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–
	K: 23+28	0.01	–	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–
	L: 24	0.04	–	–	0.10	0.17	–	–	–	–	–	–	–	–	–	–	–
	M: 24+27	0.01	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–	1.00
	N: 25+26	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–	0.50	–
	O: 27	0.03	0.09	–	0.10	–	–	–	–	–	–	–	–	–	–	–	–
	P: 27+29	0.01	–	–	–	–	–	–	–	–	0.25	–	–	–	–	–	–
	Q: 27+30	0.03	–	–	–	–	–	–	–	0.63	–	–	–	–	–	–	–
	R: 27+30+32	0.01	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–
	S: 27+32	0.01	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–
	T: 28	0.01	–	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–
	U: 29	0.03	0.05	0.11	0.05	–	–	–	–	–	–	–	–	–	–	–	–
	V: 28+29	0.09	–	0.78	–	–	–	–	–	–	–	–	–	–	–	–	–
	W: 28+29+31	0.01	–	0.06	–	–	–	–	–	–	–	–	–	–	–	–	–
	X: Null	0.12	–	–	0.70	0.22	–	–	–	–	–	–	–	–	–	–	–

TA – T. angustifolium; *Tlap – T. lappaceum*; *Tsc – T. scabrum*; *Tcp – T. campestre*; *Ttm – T. tomentosum*; *TR – T. resupinatum*; *Tst – T. stellatum*; *Tsq – T. squarrosum*; *Tps – T. pallescens*; *Tpd – T. pallidum*; *Tch – T. cherleri*; *Tg – T. glomeratum*; *Tf – T. fragiferum*; *Trp – T. repens*; *Tsp – T. spumosum*

ure 2b), none of the morphological characters showed a close relationship with the climatic variables taken into account. This analysis explained 77.36% of the total variation. Although most of the morphometric traits contributed to the formation of PC1, PC2 was influenced mainly by ecological parameters.

Cluster analysis based on quantitative traits. The phenogram based on Euclidean distances (Figure 3) revealed that all the species were grouped into three major clusters. The first cluster (I) consisted of eight species and could be further divided into three sub-clusters: *T. angustifolium* formed the first one (IA) at a distance of 31.23, sub-cluster IB was composed of six species (*T. pallidum*, *T. lappaceum*, *T. campestre*, *T. fragiferum*, *T. pallescens*, *T. repens*) and sub-cluster IC was formed by *T. squarrosum* and linked to IB at a distance of 17.46. The second cluster (II) was composed of six species that were subdivided into two sub-clusters at a level distance of 26.51: IIA (*T. stellatum*, *T. cherleri*, *T. scabrum* and *T. tomentosum*) and IIB (*T. resupinatum* and *T. glomeratum*). The longest branch separated *T. spumosum* from the rest of the species at a taxonomic distance of 65.18; this species was the lone member of cluster III.

Isozyme phenotype frequencies. Phenotype frequencies observed for each isozyme are presented in Table 3, with a total of 51 bands attributed to 113 phenotypes that were detected for the EST and GOT isozymes. Examples of zymograms and their diagrams are shown in Figure 4.

Two zones of activity (*Got-1* and *Got*-2), with 19 and 11 phenotypes for *Got-1* and *Got-2*, respectively,

doi: 10.17221/10/2015-CJGPB

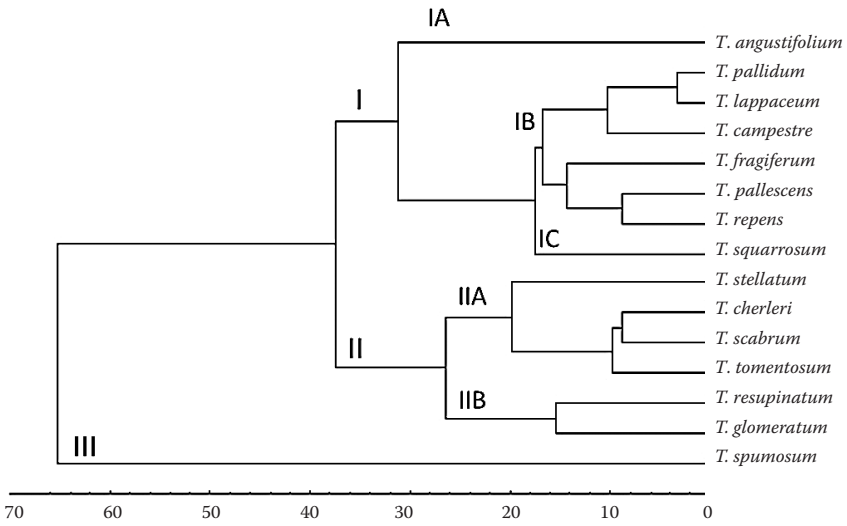

Figure 3. UPGMA phenogram based on morphological characters showing relatedness among *Trifolium* species

were revealed. The phenotypes *Got-1*B (band 2), *Got*-2G (band 17) and *Got*-2E (band 16) were the most frequent in their zones with frequencies 0.20, 0.38 and 0.32, respectively. All other phenotypes were species-specific at different levels of frequency, except *Got*-1 phenotype O, which was shared by *T. pallescens* and *T. repens,* and *Got*-2 phenotype F, which was shared by *T. resupinatum* and *T. squarrosum.* The null form *Got*-2K was observed only in two species *T. lappaceum* and *T. scabrum* with frequency0.06.

For the EST isozyme system, five zones and 32 bands that attributed to 14, 19, 11, 15 and 24 phenotypes for *Est-1*, *Est-2*, *Est-3*, *Est-4* and *Est-5*, respectively, were recorded. Null phenotypes of *Est-1*N, *Est-2*S, *Est-3*K, *Est-4*O and *Est-5*X were present at frequencies 0.44, 0.16, 0.20, 0.08 and 0.12, respectively. *Est-1* phenotype M was the most frequent following the null form *Est-1*N at frequency 0.23 in the *Est-1* zone. Phenotype E of *Est-2* and phenotype A of *Est-3*, *Est-4* and *Est-5* were the most frequent at the 0.21, 0.34, 0.32 and 0.17 proportions, respectively, followed by *Est-2*C, *Est-3*G, *Est-4*C and *Est-5*I with frequencies 0.12, 0.15, 0.23 and 0.11, respectively. The remaining phenotypes were considered to be rare or species-specific with relatively low frequencies.

Phenotypic polymorphism. Table 4 represents the polymorphism information content (PIC) for each zone in each species. The average PIC value was estimated to be 0.30. *T. spumosum* species was excluded from intraspecific variation because it was represented uniquely by one accession. All isozyme zones were monomorphic in some species and polymorphic in others. EST isozymes were considered to be the most informative with PIC values varying from 0.32 to 0.37. *Got-1* and *Got-2* zones had the lowest

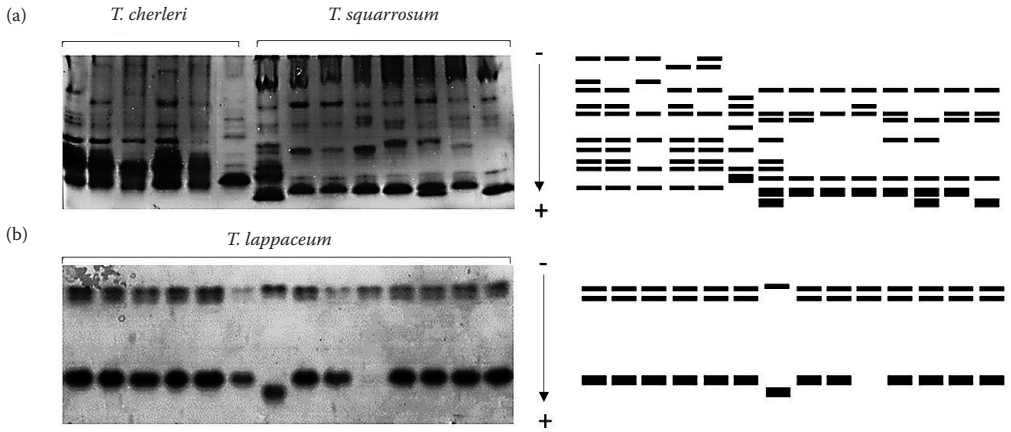

Figure 4. Zymograms of EST in *Trifolium cherleri* and *T. squarrosum* accessions (a) and GOT in some *T. lappaceum* accessions (b)

doi: 10.17221/10/2015-CJGPB

Table 4. Polymorphism information content (PIC) for each zone in each studied species

Species	Zones							Mean
	Got-1	*Got-2*	*Est-1*	*Est-2*	*Est-3*	*Est-4*	*Est-5*	
T. angustifolium	0.63	0.00	–	0.46	0.00	0.40	0.76	0.38
T. lappaceum	0.20	0.10	0.00	0.85	0.64	0.69	0.38	0.41
T. scabrum	0.00	0.50	0.74	0.00	0.47	0.00	0.49	0.31
T. campestre	0.61	0.36	–	–	0.79	0.53	0.78	0.61
T. tomentosum	0.00	0.00	0.24	0.44	0.00	0.62	0.00	0.19
T. resupinatum	0.48	0.18	0.50	0.18	0.48	0.18	0.00	0.29
T. stellatum	0.20	0.00	–	0.35	0.37	0.20	0.00	0.19
T. squarrosum	0.00	0.41	0.00	0.56	0.47	0.22	0.56	0.32
T. pallescens	0.00	0.00	0.66	0.00	0.56	0.59	0.66	0.35
T. pallidum	0.41	0.00	–	0.61	0.41	0.69	0.41	0.42
T. cherleri	0.28	0.00	0.78	0.50	0.28	0.44	0.00	0.33
T. glomeratum	0.00	0.00	0.00	0.48	0.00	0.00	0.00	0.07
T. fragiferum	0.00	0.63	0.38	0.00	0.38	0.00	0.00	0.20
T. repens	0.00	0.50	–	0.00	–	0.00	0.50	0.20
T. spumosum	–	–	–	–	–	–	–	–
Mean	0.20	0.19	0.37	0.34	0.37	0.33	0.32	0.30

PIC values of 0.20 and 0.19, respectively. Whence, *T. tomentosum*, *T. pallescens* and *T. glomeratum* were found monomorphic for the GOT isozymes. *T. lappaceum*, *T. campestre* and *T. resupinatum* displayed variation for the two GOT zones. All other species were monomorphic for one zone and polymorphic for the other. On the other hand, *Trifolium* species disclosed more or less important enzymatic variability based on PIC values for each zone. *T. campestre* showed the highest intraspecific variation across all zones with PIC value of 0.61, followed by *T. pallidum* and *T. lappaceum* with PIC values of 0.41 and 0.42, respectively. Nevertheless, the least intraspecific variation was represented by *T. glomeratum* (PIC = 0.07), which was polymorphic solely for the *Est-2* zone.

Cluster analysis based on isozyme markers. Similarities between species were evaluated by Jaccard's coefficient (J), and distance values were represented in a UPGMA dendrogram (Figure 5). According to the isoenzyme variability, clover species were classified into four clusters. Cluster I was composed of the two least distant species *T. spumosum* and *T. stellatum* at a distance 0.40. *T. tomentosum* formed cluster II that was linked to the first one at a distance 0.75. Clus-

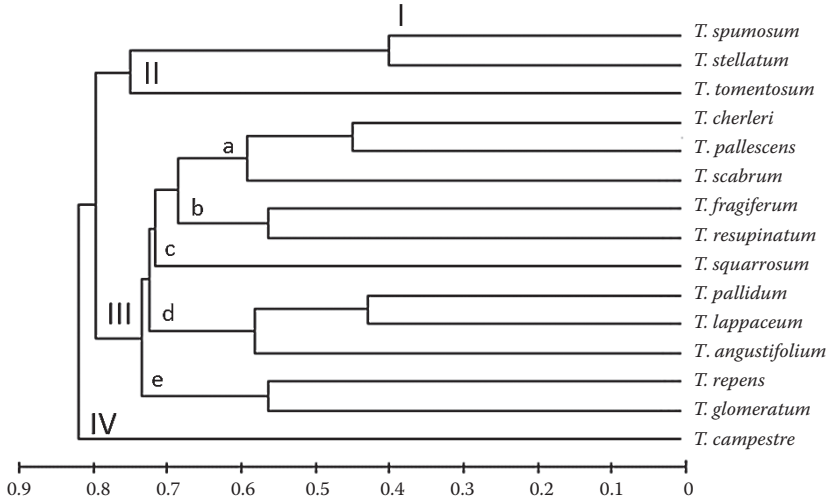

Figure 5. UPGMA dendrogram based on isozyme markers showing relatedness among *Trifolium* species

doi: 10.17221/10/2015-CJGPB

ter III was the largest and embodied 11 species that were subdivided into five sub-clusters; sub-cluster IIIa comprised three species, *T. cherleri*, *T. pallescens* and *T. scabrum*, at a distance 0.59. Two species, *T. fragiferum* and *T. resupinatum*, formed sub-cluster IIIb at a distance 0.58. *T. squarrosum* was the lone member of sub-cluster IIIc at a distance 0.71. Sub-clusters IIId and IIIe were composed of three (*T. pallidum*, *T. lappaceum* and *T. angustifolium*) and two (*T. repens* and *T. glomeratum*) species at distances 0.72 and 0.73, respectively. *T. campestre* constituted cluster IV and was the most distant (d = 0.82) from all other species. Pairwise comparison was performed among all species. Jaccard's similarity coefficients calculated from isozyme data varied from 0.10 to 0.60, with a mean value 0.31. The highest similarity coefficient 0.60 was observed between *T. stellatum* and *T. spumosum*, whereas it was lowest between *T. spumosum* and *T. cherleri*.

The Mantel test based on Pearson's correlation showed no significant correlation ($r = 0.051$, $P = 0.620$) between matrices of Euclidean morphological distances and Jaccard's standard distances of isozyme markers.

DISCUSSION

It is the notion among the breeders that the high level of genetic diversity in the breeding material contributes to the significance of selection. To be useful for plant breeders, genetic resources must be characterized by morphological and agronomic traits (Asci 2011).

Results of this study reflect the existence of high variation within and among Algerian clover species based on morphological characters. This is not surprising since several studies have shown that the genome of perennial *Trifolium* spp. is extremely polymorphic due to its strong self-incompatibility (Paplauskienė & Dabkevičienė 2012).

Most of the quantitative traits analysed in this study were found to be important in the discrimination of accessions, especially, number of pods per fruiting head (PF) and number of seeds per fruiting head (SF). A morphological study conducted in seven species of clovers showed the importance of biometrical characteristics (width of fruiting head, weight of 1000 seeds, weight of fruiting heads, number of pods per fruiting head and number of seeds per fruiting head) in comparison with the characteristics of flowering and vegetative development (Issolah 1997). The recorded high morphological variation is consistent with findings of many genetic diversity studies on *Trifolium* ssp. germplasm collections (Kongkiatngam *et al*. 1995; Issolah & Abdelguerfi 1999b; Greene *et al*. 2004; Dias *et al*. 2008a; Asci 2011; Paplauskienė & Dabkevičienė 2012).

Regarding the correlations between morphological characters and the environment of populations, no significant relationship was detected. Nevertheless, a comparison of the same biometric characteristics in *T. scabrum* L. wild populations allowed the selection of populations suitable for cultivation on arid soils of Algeria (Issolach & Abdelguerfi 1999b). Relationships between biometrical characteristics and ecological factors within the populations of several spontaneous clovers in Algeria have also been reported (Issolah & Abdelguerfi 1999a).

Diversity of traits enabled the division of species into eight clusters and sub-clusters. This finding supplements earlier studies that realized different groups of populations based on the contribution of several morpho-agronomic traits (Dias *et al*. 2008a; Drobná 2009; Tucak *et al*. 2009; Asci 2011).

Using isozymes as markers for genetic diversity evaluation would not be as straightforward as in the genus *Trifolium*, because of high variation within and among species (Kongkiatngam *et al*. 1995). A large genetic variability was observed with EST and GOT enzymatic systems, whence, a total of 51 bands and 113 isozyme phenotypes were recorded based on the two enzymes. Such a large number of bands and phenotypes reflects the high level of genetic variation within and among the fifteen *Trifolium* species studied. The genetic variability of phenotypes ranged from 0.07 (*T. glomeratum*) to 0.61 (*T. campestre*) with an average of 0.30 based on the polymorphic information content (PIC). Levels of variability in this study were comparable to those of many enzymatic variation studies on *Trifolium* populations (Kongkiatngam *et al*. 1995; Lange & Schifino-Wittmann 2000; Malaviya *et al*. 2005, 2008; Dias *et al*. 2008b).

EST analysis resulted in more bands and patterns (32 bands and 83 phenotypes) than GOT, which was more conservative (19 bands and 30 phenotypes), since all species were polymorphic for at least one zone of EST, and this is the most suitable enzyme system to assess genetic variability. Several researchers agree with the highest variability for the EST enzymatic system among and within *Trifolium* species (Lange & Schifino-Wittmann 2000; Malaviya *et al*. 2005).

Isozyme zones that showed species-specific phenotypes and allozymes could be used to differenti-

doi: 10.17221/10/2015-CJGPB

ate between species but only if they were present at reasonably high frequencies. Although species-specific forms were found for all zones of each enzyme system (Table 3), they were present at a low frequency across the collection (less than 13%), but some of them were present with high frequency at an intraspecific level (100%). The low frequencies for species-specific phenotypes at the intraspecific level make them less useful for species identification since a large number of plants must be assayed to obtain accurate results (Kongkiatngam *et al*. 1995).

The importance of isozyme variation among the examined *Trifolium* species is demonstrated by the topology of the dendrogram based on Jaccard's similarity coefficient that ranged from 0.10 to 0.60 with a mean of 0.31, this wide range of similarity indices reflects a high degree of polymorphism at the isozyme level. This clustering was inconsistent with that based on morphological markers since the Mantel test ($r = 0.051$, $P = 0.620$) indicated no significant correlation between the matrix based on morphological characters and that derived from the enzymatic analysis. The result suggests that the two marker systems give different estimates of genetic relations among populations. A large number of studies have reported strong differences between the quantitative traits and electrophoretic data suggesting that natural selection acting upon the quantitative traits is responsible for the different patterns of differentiation revealed by neutral and quantitative traits (Kongkiatngam *et al*. 1995; Dias *et al*. 2008a).

The fact that different clusters using isozyme and morphological data were observed can be exploited for breeding purposes. A breeding program can be started within any morphological cluster without risk of inbreeding depression as shown by the large enzymatic variability available within the clusters. Furthermore, enzymatic markers could be used as tools to help in the variety distinction. So, finally morphological and isozyme approaches provide complementary information for breeding.

This study gives an insight into the rich variability present in the Algerian *Trifolium* species core collection at morphological and enzymatic levels, which can prove to be a good source of genes for further improvement programs. Moreover, most of the studied species had specific zymograms that could serve as markers for species identification. Including seed storage proteins and other molecular variation will further help to have a better understanding to select starting material for breeding purposes.

References

Abberton M.T. (2007): Interspecific hybridization in the genus *Trifolium*. Plant Breeding, 126: 337–342.

Addinsoft (2007): XLSTAT, Analyse de données et statistique avec MS Excel. New York, Addinsoft.

Allen O.N., Allen E.K. (1981): The Leguminosae. A Source Book of Characteristics, Uses and Nodulation. Madison, University of Wisconsin Press.

Asci O.O. (2011): Biodiversity in red clover (*Trifolium pratense* L.) collected from Turkey. I: Morpho-agronomic properties. African Journal of Biotechnology, 10: 14073–14079.

Bendiab K., Baaziz M., Brakez Z., Sedra My.H. (1993): Correlation of isoenzyme polymorphism and Bayoud-disease resistance in date palm cultivars and progeny. Euphytica, 65: 23–32.

Dias P.M.B., Julier B., Sampoux J.P., Barre P., Dall'Agnol M. (2008a): Genetic diversity in red clover (*Trifolium pretense* L.) revealed by morphological and microsatellite (SSR) markers. Euphytica, 160: 189–205.

Dias P.M.B., Pretz V.F., Dall'Agnol M., Schifino-Wittmann M.T., Zuanazzi J.A. (2008b): Analysis of genetic diversity in the core collection of red clover (*Trifolium pratense*) with isozyme and RAPD markers. Crop Breeding and Applied Biotechnology, 8: 202–211.

Drobná J. (2009): Yield and forage quality of Romanian red clover (*Trifolium pratense* L.) varieties studied in Slovakia. Notulae Botanicae Horti Agrobotanici, 37: 204–208.

Ellison N.W., Liston A., Steiner J.J., Williams W.M., Taylor N.L. (2006): Molecular phylogenetics of the clover genus (*Trifolium* – Leguminosae). Molecular Phylogenetics and Evolution, 39: 688–705.

Ghoubay A., Abdelguerfi A. (1991): Spontaneous species of the genus *Trifolium* L. in Algeria: Comparison of seeds and inflorescences in thirteen clovers. In: Proc. 4th Int. Rangeland Congr., Montpellier, Apr 22–26, 1991: 375–376.

Greene S.L., Gritsenko M., Vandemark G. (2004): Relating morphologic and RAPD marker variation to collection site environment in wild populations of red clover (*Trifolium pratense* L.). Genetic Resources and Crop Environment, 51: 643–653.

Issolah R. (1997): Comportement, biométrie et caryologie de populations spontanées de treize espèces du genre *Trifolium* L. en Algérie. [MSc. Thesis.] El Harrach, INA.

Issolah R., Abdelguerfi A. (1999a): Chromosome numbers within some spontaneous populations of *Trifolium* species in Algeria. Caryologia, 52: 151–154.

Issolah R., Abdelguerfi A. (1999b): Variability within 31 spontaneous populations of *Trifolium scabrum* L., na-

doi: 10.17221/10/2015-CJGPB

ture of relations with factors of the site of origin. In: 9th Meeting of the FAO-CIHEAM Sub-Network on Mediterranean Pastures and Fodder Crops, Badajoz, Nov 26–29, 1997: 123–127.

Issolah R., Abdelguerfi A. (2000): Study of the variability in 28 spontaneous populations of *Trifolium tomentosum* L.: Relations with factors of the environment of origin. In: Proc. 10th Meeting of the Mediterranean Subnetwork of the FAO-CIHEAM Inter-Regional on Pasture and Fodder Crops, Sassari, Apr 4–9, 2000: 29–32.

Issolah R., Abdelguerfi A. (2003): Morphological variability within four Algerian populations of the species *Trifolium spumosum* L. In: 25th EUCARPIA Fodder Crops and Amenity Grasses Section Meeting, Brno, Sept 1–4, 2003: 205–208.

Jaccard P. (1908): Nouvelles recherches sur la distribution florale. Bulletin de la Société Vaudoise des Sciences Naturelles, 44: 223–270.

Kongkiatngam P., Waterway M.J., Fortin M.G., Coulman B.E. (1995): Genetic variation within and between two cultivars of red clover (*Trifolium pratense* L.): comparisons of morphological, isozyme and RAPD markers. Euphytica, 84: 237–246.

Lange O., Schifino-Wittmann M.T. (2000): Isozyme variation in wild and cultivated species of the genus *Trifolium* L. (Leguminosae). Annals of Botany, 86: 339–345.

Malaviya D.R., Kumar B., Roy A.K., Kaushal P., Tiwari A. (2005): Estimation of variability for isozymes of five enzyme systems among wild and cultivated species of *Trifolium*. Genetic Resources and Crop Evolution, 52: 967–976.

Malaviya D.R., Roy A.K., Kaushal P., Kumar B., Tiwari A. (2008): Genetic similarity among *Trifolium* species based on isozyme banding pattern. Plant Systematics and Evolution, 276: 125–136.

Mantel N. (1967): The detection of disease clustering and a generalized regression approach. Cancer Research, 27: 209–220.

Paplauskienė V., Dabkevičienė G. (2012): A study of genetic diversity in *Trifolium hybridum* varieties using morphological characters and ISSR markers. Žemdirbystė–Agriculture, 99: 313–318.

Quezel P., Santa S. (1962): Nouvelle flore de l'Algérie et des régions désertiques méridionales. CNRS, Paris.

Singh N.K., Shepherd K.W., Cornish G.B. (1991): A simplified SDS-PAGE procedure for separating LMW subunits of glutenin. Journal of Cereal Science, 14: 203–208.

Smith J.S.C., Smith O.S. (1989): The description and assessment of distances between inbred lines of maize: II. The utility of morphological, biochemical and genetic descriptors and a scheme for the testing of distinctiveness between inbred lines. Maydica, 34: 151–161.

Statsoft, Inc. (1995): STATISTICA for Windows. Tulsa, StatSoft, Inc. Available at http://www.statsoftinc.com

Stewart P. (1974): Un nouveau climagramme pour l'Algérie et son application au barrage vert. Bulletin de la Société d'histoire naturelle de l'Afrique du nord, 65: 239–248.

Tucak M., Čupić T., Popović S., Stjepanović M., Gantner R., Meglič V. (2009): Agronomic evaluation and utilization of red clover (*Trifolium pratense* L.) germplasm. Notulae Botanicae Horti Agrobotanici, 37: 206–210.

Williams W.M., Nichols S.N. (2011): *Trifolium.* In: Kole C. (ed.): Wild Crop Relatives: Genomic and Breeding Resources. Clemson, Institute of Nutraceutical Research: 249–272.

Zohary M., Heller D. (1984): The genus *Trifolium*. Jerusalem, Israel Academy of Sciences and Humanities.

Zoro Bi I., Maquet A., Wathelet B., Baudoin J.P. (1999): Genetic control of isozymes in the primary gene pool of *Phaseolus lunatus* L. Biotechnologie Agronomie Société et Environnement, 3: 10–27.

Received for publication January 13, 2015
Accepted after corrections May 14, 2015

Corresponding author:

Prof Douadi Khelifi, Université Constantine, Faculté des Sciences de la Nature et de la Vie, Laboratoire de Génétique Biochimie et Biotechnologies Végétales, Université Constantine 1, 25000 Constantine, Algérie; e-mail: dkhelifi@yahoo.fr