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Analysis of the chromosome variation within some natural populations of subterranean clover (*Trifolium subterraneum* L., *Fabaceae*) in Algeria

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Abstract. Nine natural populations of subterranean clover (*Trifolium subterraneum* L.) coming from different eco-geographical sites of the North-East Algeria, have been studied for their chromosome number and karyotype features. The study is part of the evaluation and valorization of plant genetic resources of fodder and pastoral interest in Algeria. The results of mitosis detect two groups of populations, and reveal diversity in the number among and within populations. The Algerian populations of *T. subterraneum* are characterized by two chromosomic formulas. The first formula (2n=2x=16m) (median), more common in most of the studied populations, is in conformity with previous reports in this species. The karyotype of these populations is symmetrical for size and form. The second (2n=2x= 18m), is detected for the first time and described as a new chromosomal formula in *T. subterraneum*. The latter is relatively more frequent than the first one and characterizes the populations coming from high altitude areas. The karyotype (2n=2x=18m) is relatively symmetrical. At the level of the two established Karyotypes, satellites are highlighted at the first pair. A variation in the size and frequency of these satellites is observed. The species exhibits regular meiotic behaviour, confirming the presence of two basic chromosome numbers (x=8 and 9). The study also highlights the role of ecological factors (Altitude and Rainfall) of the originating environment of Algerian populations in the variation and evolution of chromosome numbers in *T. subterraneum.* The new cytogenetic data can be exploited in the taxonomy of the species in Algeria in order to select and develop this plant genetic resource in the agricultural field.

Keywords. Chromosomes, Intraspecific variability, Karyotype, Subterranean clover, *Trifolium subterraneum* L.

INTRODUCTION

The genus *Trifolium* is one of the largest genera of the *Fabaceae* family (sub-family, *Papilionoideae*). It has more than 255 annual and perennial spe-

cies (Zohary and Heller 1984; Gillet and Taylor 2001). Most of them are of great agricultural importance and widely grown as fodder and green manure (Ellison et al. 2006). The genus *Trifolium* is originating from Mediterranean, because of the greatest diversity of numbers and chromosome forms have been found in this region (Taylor 1985). It has been subdivised into eight sections: *Lotoidea*, *Paramesus*, *Mystillus*, *Vesicaria*, *Chronosemium*, *Trifolium*, *Trichocephalum* and *Involucrarium* (Zohary and Heller 1984). The principal geographical centers of diversity of *Trifolium* are the Mediterranean basin, the West of North America, and the highlands of Eastern Africa (Ellison et al. 2006). The cytotaxonomic studies carried out on *Trifolium* have shown that it presents a surprising variety of chromosome numbers, and the changes in the number of chromosomes have played a large part in its evolution (Falistocco et al. 2013). Britten (1963) and Pritchard (1969) have shown that an aneuploid series of basic numbers x=5, 6, 7 and 8 are found in this genus. The presence of x=8 in about 80% of the species suggests that x=8 is the ancestral number of the genus (Senn 1938; Pritchard 1969; Zohary and Heller 1984; Ellison et al. 2006), from which the numbers x = 7, 6 and 5 are derived. Polyploidy is more common in perennial species (Kiran et al. 2010; Falistocco et al. 2013).

Subterranean clover (*Trifolium subterraneum* L., sect *Trichocephalum*), commonly known as the burrowing clover or sower, is a winter annual species, native to the Mediterranean Basin, West Asia and the Atlantic coast of Western Europe (Gladstones and Collins 1983 ; Zohary and Heller 1984). The plant of subterranean clover is autogamous, characterized by mechanisms of burial of reproductive structures, ensuring thus, its own self-regenerating (Masson 1997). The species constitutes an heterogenous complexesis, divided into three subspecies: *subterraneum*, *brachycalycinum* and *yanninicum* (Katznelson 1984), identifiable enough by their morpho-physiology, karyotypes, isozymes and polymorphisms for molecular markers (Piluzza et al. 2005). In Algeria, the subterranean clover is very common in the Tell and the mountain meadows (Quezel and Santa 1962), adapted to different ecological conditions (Issolah et al. 2015). This species is represented by three varieties belonging to the *subterraneum* subspecies (Subsp. *subterraneum* Var. *subterraneum*, Var. *brachycladum*, Var. *flagelliforme*) on the eight varieties described in Algeria (Zohary and Heller 1984). Despite the agronomic importance of the species in the world, as cattle feed and soil improvement, its cytological characterization remains very restricted.

This is because of the small size of chromosomes like all the other species of *Trifolium* (Zohary and Heller 1984).

The first investigations on *T. subterraneum* focused only on the determination of the chromosome number (2n=16), but without establishing the karyotype (Weselxen 1928; Yates and Brittan 1952; Brock 1953; Hutton and Peak 1954; Zohary and Katznelson 1958; Kliphuis 1962; Britten 1963; Katznelson and Morley 1965a). Later, some karyotype studies were performed in Spain (Angelo et al. 1975, 1977, 1983), Iran (Hezamzadeh Hijazi and Ziaeinasab 2006) and Italy (Falistocco et al. 1987; Falistocco et al. 2013).

The present study is interested in the evaluation and the valorization of the phytogenetic resources of fodder and ¦pastoral¦ interest in Algeria.

Its aim is the analysis of the chromosomal diversity presents in the natural populations of *Trifolium subterraneum* L., and the establishment of its karyotype.

It follows the different studies carried out on natural fodder legumes (Issolah and Abdelguerfi 1999a; Issolah and Khalfallah 2007; Issolah et al. 2006, 2012, 2015, 2016).

MATERIAL AND METHODS

Plant materials

The *Trifolium subterraneum* specimens were collected by INRAA (National Institute of Agronomic Research of Algeria), in July 2010. Nine natural populations sampled from North-East Algeria (Issolah et al. 2015), were the subject of a karyological study (Table 1).

Chromosome counting

The seeds belonging to the nine studied populations, were scarified to remove in tegumentary hardness, and then germinated on wet filter paper in Petri dishes at room temperature. The root tips meristems (1 to 1.5 cm in length) were excised in the morning between 8 am- 8.30 am and pretreated with α-bromonaphthalene (1%) at room temperature for 2h45mn. The use of this pretreatment increases the number of metaphase mitotic cells, allows the chromosomes to be well spread in the cell, straightens the chromatids, and contracts the chromosomes, which makes primary and secondary constrictions very noticeable (Singh 2018). For chromosomes analysis, root tips were hydrolyzed in 1N HCl and stained in lactopropionic orcein (Dyer 1963). The chromosomic observations were repeated several times. For each population, five plates of chromosomes were selected from at least 30 individuals (seeds). Then, they were observed and photographed using a Primo Star Zeiss

microscope. Chromosome counts were performed on metaphase plates with well individualized chromosomes.

Karyotype analysis

The karyomorphological analysis was carried out according to the following parameters: the lenght of long arm (L), short arm (S), the total length of the chromosome (LT = L + S), the difference between arms (d = L-S), and the relative length (LR (‰) = 1000 x TL/ ΣTL). Centromere position and chromosome types were determined from the two parameters: arm ratio (r= L/S), and centromeric index (CI % = S/LT x100) according to the nomenclature of Levan et al. (1964). For determining the asymmetry of the karyotype, three parameters were estimed: [(Ias. K% = (Σ L/Σ LT) x100 (Aran and Saito 1980)], the ratio between the longest and the shortest chromosome pairs (R), and the inter-chromosomal asymmetry coefficient (A2) (standard deviation of chromosome length / mean chromosome length) (Romeo Zarco 1986). Chromosome measurements, based on five plates per population, were performed using the Axiovision software (1999-2009). The different karyotype calculations were made thanks to Excel (2007).

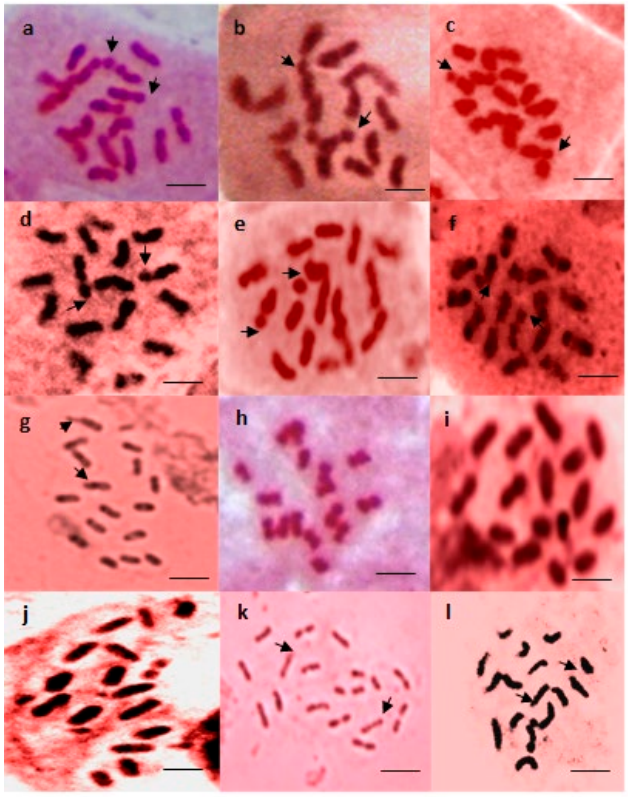

Figure 1. Mitotic metaphases of Algerian natural populations of *Trifolium subterraneum* L. with two chromosomes numbers 2n=16 and 2n=18 respectively: (a) population 12/10; (b) population 13/10; (c) population 19/10; (d) population 20/10; (e) population 22/10; (f) population 23/10; (g) population 25/10; (h) population 26/10; (i) population 33/10; (j) population 22/10 (2n=18); (k) population 25/10 (2n=18); (l) population 26/10(2n=18) . Arrows: satellites. Bar: 2.5µm.

Meiosis

To confirm the results corresponding to the numbers found by mitosis (presence of supernumerary chromosome pair for certain populations), the meiotic behaviour of the nine populations was also analysed. For this purpose, a trial has been conducted at the experimental station of INRAA (November 2014). Each population was represented by twenty individuals (seeds) and sowed in total randomization (field) for identifying the different phases of meiosis (laboratory). The flower buds collect period was spread over a month before flowering (recovering flower buds of variable size). For each plant, at least five flower buds were collected (April 2015) in the early morning (from 8h), then fixed in Carnoy solution (Ethanol-acetic acid 3 : 1, v/v) for at least 48 h at 4C°. After dissection of the anthers, the pollen mother cells (PMC) were crushed in an acetic carmine drop 1% (Jahier et al. 1992). Observations and photographs at different phases were performed using a Primo Star Zeiss microscope.

RESULTS

Chromosome counting

All mitotic metaphase plates of investigated populations of the species *Trifolium subterraneum* L. showed a diploid number of chromosomes (2n = 16) (Figure 1). This number is frequently observed in individuals of the populations 12/10; 13/10; 19/10; 20/10 and 33/10. However, the somatic metaphases of the four populations 22/10; 23/10; 25/10; 26/10, have presented along with the characteristic number of the species (2n = 16), a second and new number of chromosomes (2n = 18), often encountered during this study in these later populations (Figure 1). The two chromosome numbers (2n = 16 and 18) are observed within the cells of the same individual, and also in different individuals of the same population. This indicates a chromosomical variation within and between the populations of *Trifolium subterraneum*.

The analysis of 15 individuals per population, indicated that the variation of the chromosome numbers (2n = 16 and 18) was not in the same frequency in these

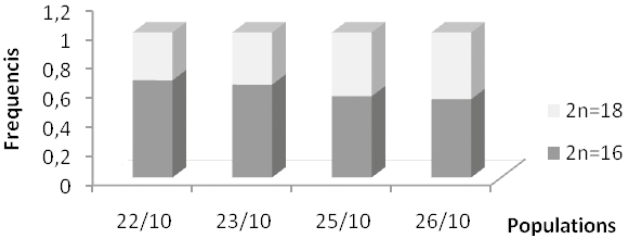

Figure 2. Frequencies of the two chromosomes numbers (2n=16 and 18) in the four populations of *Trifolium subterraneum* L. (15 individuals / population).

latter populations (Figure 2). Indeed, within the populations (22/10 and 23/10), the frequency of the number (2n = 16) represents twice the frequency of the number (2n=18) (0.67 and 0.33; 0.64 and 0.36, respectively). However, very similar frequency values are shown in the other two populations (25/10 and 26/10) (0.56 and 0.44; 0.5 and 0.46 respectively) (Figure 2).

Karyotype analysis

In all investigated populations, the morphology and chromosome structure are almost identical (Table 2-5). Our results showed that the chromosomes of the Algerian population of the species *Trifolium subterraneum* L. are small. The size of the chromosomes varies from 1.02 µm (Table 3) to 3.01 µm (Table 2). The total lengths of diploid chromosome set are comprised between 12.82 µm (Table 4) and 18.87 µm (Table 2). The mean value of the total length (TLG) of all studied populations is 1.92µm. The results of this study indicate also that the population 22/10 (2n =16) is characterized by the highest values for the selected parameters, like the mean value of chromosome length, which gives an estimated size of the genome (18.87 µm) and the largest first pair and eighth pair (3.01µm-1.72µm) (Table 2). Thus, we note that the two additional chromosomes present in the populations (2n = 18), have the same form, with a mean size of 1.09 µm (Figure 1, Table 3 and 5). The results of this study indicated also that satellites are located at the first chromosome pair within all investigated populations. A variation of the size and an abundance of these satellites are noticed. Thus, the metaphase plates of the populations characterized by (2n= 16), present a considerable size of these satellites compared to that noted on the plates of the populations characterized by (2n=18) with 0.25 µm ± 0.022; 0.18 µm ± 0.025, respectively.

These satellites are more abundant in the metaphases of populations with 2n = 18 compared to those with 2n = 16. Their frequencies are 0.70 and 0.44, respectively (Figure 1). Otherwise, the results of the centromeric index (Ic) and the ratio between the long arm and the short arm (r) allowed us to determine the homologous chromosomes and to classify the different chromosomal types. Therefore, all the studied populations are characterized by the karyograms, presenting median chromosomes (Figure 3b).

Table 1. Geographical origin and ecological characteristics of the sampling sites of nine populations of *Trifolium subterraneum* L. in Algeria

N° of populations	Origin	Altitude (m)	Rainfall (mm)
12/10	Guelma	170	600
13/10	Guelma	200	558
19/10	Tarf	665	661
20/10	Tarf	555	661
22/10	Souk Ahras	950	800
23/10	Souk Ahras	1040	700
25/10	Souk Ahras	800	900
26/10	Souk Ahras	1110	700
33/10	Skikda	110	562

Source (Issolah et al. 2015)

Table 2. Morphometric data within the population 22/10 (2n=16) of *Trifolium subterraneum* L. in Algeria.

Ch p	L (µm) (±SD)	S (µm) (±SD)	TL (µm)	RL ‰	d	r	Ci %	Ct
1	1.69 (0.41)	1.32 (0.26)	3.01	159.36	0.37	1.28	43.90	m-sat
2	1.42 (0.31)	1.26 (0.43)	2.68	141.96	0.17	1.13	46.86	M
3	1.53 (0.38)	1.08 (0.35)	2.61	138.52	0.45	1.42	41.33	M
4	1.26 (0.50)	1.09 (0.40)	2.35	124.38	0.17	1.15	46.45	M
5	1.23 (0.50)	1.05 (0.43)	2.28	120.85	0.18	1.17	45.98	M
6	1.18 (0.40)	1.05 (0.31)	2.23	118.11	0.13	1.12	47.12	M
7	1.05 (0.29)	0.94 (0.32)	1.99	105.65	0.11	1.12	47.16	M
8	0.92 (0.31)	0.80 (0.19)	1.72	91. 17	0.12	1.15	46.61	M
	I1as% =54.48	ΣTL=18.87	TLG=2.36	R1=1.75	$A_{2(1)}$=0.14			

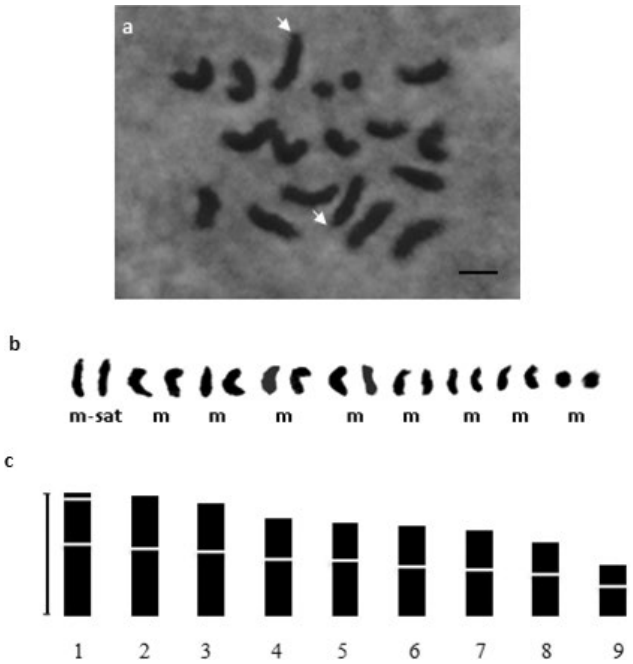

Figure 3. Karyotype of *Trifolium subterraneum* L. in Algeria. (a) Somatic metaphasis (2n=18, population 23/10); (b) Karyogram; (c) Idiogram; arrow (satellites). Bar: 2µm.

The values of the asymmetry index Ias% (Arano and Saito, 1980), the ratio between the largest and the smallest chromosome pairs (R), and the interchromosomal index A2 (Romero Zarko 2006) gives indications on the evolution of chromosomes in plants. The results of the three parameters [(R1: 1.75, R3 =1.78), (I1as% = 54.48, I3as% = 55.81), and (A2 (1) = 0.14, A2(3) = 0.19)] (Table 2 and 4) are weak and indicate that the karyotype (2n=16m) is very symmetrical for the size and the form. It is therefore primitive. Nevertheless, although the asymmetry indices are low (I2as%: 56.69, I4as% 55.07) in the populations (2n = 18), they showed a karyotype with more or less uniform sizes except for the ninth pair. This is reflected by relatively high values of the ratio (R) and interchromosomal asymmetry A2, compared to those found for the karyotype (2n = 16) (Table 3 and 5).

Meiosis analysis

The study of meiotic behaviour showed that the nine natural populations of the species *Trifolium subterraneum* exhibit normal and regular meiosis, with dominance of bivalents at the diakinesis, metaphases I and

Table 3. Morphometric data within the population 23/10 (2n=18) of *Trifolium subterraneum* L. in Algeria.

Ch p	L (µm) (±SD)	S (µm) (±SD)	TL (µm)	RL ‰	d	r	Ci %	Ct
1	1.45 (0.17)	1.05 (0.14)	2.50	146.23	0.40	1,38	42.06	m-sat
2	1.39 (0.13)	1.02 (0.16)	2.41	140.86	0.37	1,37	42.27	M
3	1.33 (0.11)	0.93 (0.08)	2.26	132.46	0.39	1,42	41.31	M
4	1.09 (0.32)	0.89 (0.08)	1.98	116.14	0.20	1,23	44.84	M
5	1.07 (0.25)	0.80 (0.22)	1.87	109.50	0.27	1,33	42.97	M
6	1,01 (0.24)	0.81 (0.19)	1.82	106.51	0.21	1,26	44.30	M
7	0.92 (0.25)	0.81 (0.19)	1.73	101.41	0.11	1,13	46.86	M
8	0.85 (0.30)	0.63 (0.04)	1.48	86.93	0,21	1,34	42.77	M
9	0.57 (0.24)	0.45 (0.15)	1.02	59.96	0.11	1,25	44.42	M
	I2as%=56.69	ΣTL=17.08	TLG=1.90	R2=2.45	$A_{2(2)}$=0.25			

Table 4. Morphometric data within the population 25/10 (2n=16) of *Trifolium subterraneum* L. in Algeria.

Ch p	L (µm) (±SD)	S (µm) (±SD)	TL (µm)	RL ‰	d	r	Ci %	Ct
1	1.14 (0.23)	0.91 (0.02)	2.05	159.96	0.22	1.24	44.57	m-sat
2	1.12 (0.10)	0.82 (0.01)	1.94	151.56	0.31	1.37	42.14	M
3	0.96 (0.09)	0.80 (0.01)	1.76	137.50	0.16	1.20	45.60	M
4	0.99 (0.01)	0.73 (0.02)	1.72	134.38	0.26	1.35	42.30	M
5	0.83 (0.06)	0.67 (0.02)	1.50	117.00	0.16	1.24	44.50	M
6	0.80 (0.02)	0.61 (0.15)	1.41	110.16	0.19	1.31	43.09	M
7	0.73 (0.06)	0.56 (0.20)	1.29	100.78	0.17	1.30	43.41	M
8	0.60 (0.17)	0.56 (0.18)	1.15	90.04	0.04	1.08	48.16	M
	I3as%=55.81	ΣTL=12.82	TLG=1.6	R3=1.78	$A_{2(3)}$=0.19			

Table 5. Morphometric data within the population 26/10 (2n=18) of *Trifolium subterraneum* L. in Algeria.

Ch p	L (µm) (±SD)	S (µm) (±SD)	TL (µm)	RL‰	d	r	Ci %	Ct
1	1.36 (0.49)	1.25 (0.36)	2.61	160.10	0.11	1.09	47.94	m-sat
2	1.22 (0.43)	1.04 (0.41)	2.26	138.20	0.18	1.17	46.01	M
3	1.25 (0.58)	0.90 (0.33)	2.15	131.45	0.35	1.38	41.96	M
4	1.14 (0.50)	0.85 (0.39)	1.99	121.50	0.29	1.34	42.75	M
5	1.00 (0.52)	0.75 (0.43)	1.75	107.40	0.25	1.33	42.94	M
6	0.91 (0.38)	0.70 (0.28)	1.61	98.97	0.21	1.30	43.50	M
7	0.82 (0.16)	0.68 (0.23)	1.50	92.08	0.14	1.20	45.42	M
8	0.77 (0.30)	0.71 (0.25)	1.48	90.55	0.06	1.09	47.88	M
9	0.56 (0.05)	0.47 (0.02)	1.03	62.48	0.09	1.20	45.15	M
	I4as%=55.07	ΣTL=16.38	TLG=1.82	R4=2.54	$A_{2\ (4)}$=0.26			

Ch p: chromosome pair, L: long arm, S: short arm, LT: total length of chromosome, LR (‰) : relative length, d : long arm - short arm ; r : long arm / short arm, Ic : centromeric index, Ct : chromosome type, Ias% : asymmetry index, R : longest / shortest pair, **Σ**TL : total lenght of diploid set, TLG : average of total length, A2: interchromosomal asymmetry index, (SD) : standard deviation, sat: satellites.

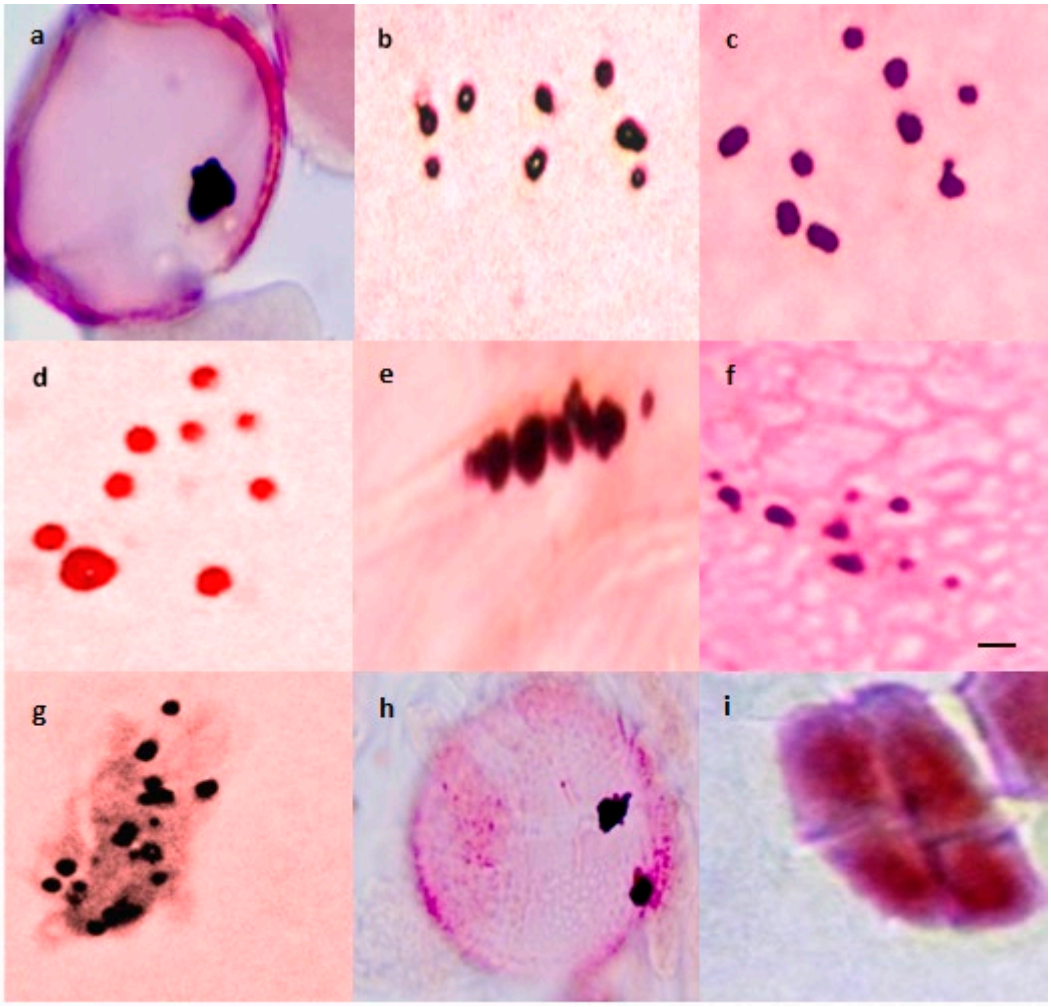

Figure 4. Pollen meiosis in some natural populations of *Trifolium subterraneum* L. in Algeria L. (a) pollen cell ; (b) Diakinesis (population 22/10, n=x=8) ; (c) Diakinesis (22/10, n=x=9) ; (d) Diakinesis (population 23/10, n=x=8) ; (e) Metaphase I (population 23/10, n=x=8) ; (f) Metaphase I (population 23/10 n=x=9) ; (g) Anaphase I ; (h) Telophase I ; (i) Tetrade. Bar: 2 µm.

anaphases I (Figure 4). This allowed us to authenticate the basic haploid number (x = 8) for the populations (12/10; 13/10; 19/10; 20/10; 33/10). Likewise, it confirms the presence of the two chromosome numbers (2n = 16 and 18) detected in mitosis, within the four populations (22/10; 23/10; 25/10; 26/10), through the appearance of two basic haploid numbers (x = 8) and (x = 9).

DISCUSSION

In this study, the chromosome numbers, karyogram, idiogram and karyotype asymmetry of naturel populations of *Trifolium subterraneum*, were determined. Mitotic metaphases showed both the same chromosome number (2n=16) in all studied populations. This number was previously reported by several authors within different ecotypes and varieties from several areas (Senn 1938; Angelo 1975, 1977, 1983; Zohary and Heller 1984; Hezamzadeh Hijazi and Ziaeinasab 2006; Vizintin et al. 2006; Falistocco et al. 1987; Falistocco et al. 2013), considering x=8, as being the ancestral basic chromosome number of the species. Meanwhile, four populations presented two numbers of chromosome (2n=16 and 18) within the cells of the same individual, and also in different individuals of the same population.

The number of chromosomes, as one of the genetic variations, is extremely variable ranging from low numbers to relatively high numbers (Eroğlu and Per 2016). A change in the basic chromosome number of a species represents dysploidy (Yakovlev 1996). According to the same author, this change can occur either in the direction of an increase (ascending dysploidy) or a decrease (downward dysploidy). In plants, this last case seems to be the most frequent, it results from the simultaneous or successive action of several cytogenetic mechanisms (robertsonian translocation, deletion …) (Yakovlev 1996).

Contandriopoulos (1978) reports 2n = 30, 32 and 34 for *Sideritis libanotica* Labill. This author notes that dysploidy still seems anarchic and has not succeeded to form populations with stable karyotypes having their own geographical distribution and a particular morphological differentiation. In such case, according to the same author, it would seem more judicious to speak about hyper and hypoaneuploidy. Aneuploidy may present the beginning of the mechanism leading to dysploidy, provided that the individuals carrying the aneuploid number are able to multiply then impose itself in the population (Contandriopoulos 1978).

Yakovlev (1996) considers that a variable chromosome number within the same population is both an aneuploidy and dysploidy phenomenon, witch is difficult to draw the line between these two phenomena, especially when it is polyploid taxa. An Intra-specific dysploidy represents a transitional step towards a definitive change in the basic chromosome number (Yakovlev 1996). The populations in which such change has occurred and fixed represent, well probably, the direct ancestors of future dysploïde species (Yakovlev 1996).

In the genus *Trifolium*, many variations of the nombre de chromosomes (2n = 16, 14, 12, and 10) characterize different diploid species, and in some instances cytological variants occur within the same species (Falistocco et al. 2013).

Brock (1953) counted two different chromosome numbers (2n = 12 and 16) in the species *Trifolium subterraneum* growing in various regions. This author suggested that the difference could be the result of a chromosomal rearrangement without loss of genetic material.

In the same genus, two basic numbers (X = 8 and 9) were highlighted within the populations of two species of *Trifolium: T. ornithopodiodes* from the British Isles (Rutland 1941; Muñoz-Rodríguez 1995), and also in *T. montanum* var. *montanum.* of Iberian Peninsula (Bleier 1925a; Muñoz-Rodríguez 1995). Issolah and Abdelguerfi (1999b), evenly showed the presence of two basic chromosomes numbers (x = 5 and 6) in the Algerian populations of *Trifolium scabrum*.

According to Pritchard (1969) and Zohary and Heller (1984), the dysploidy is consistently linked to the annual species, and are most common within sections that are at a more advanced stage of evolution, such as *Trifolium* and *Tricocephalum*, in which all the four basic numbers (x =8,7,6 and 5) may be found. Uslu (2012) has shown that taxa in the *Trifolium* section, growing in Turkey, have three numbers (x = 6, 7 and 8).

Within the tribe *Trifolieae*, Darlington and Jamaki (1945) and Darlington and Wylie (1945) reported three basic numbers (x = 7, 8, and 9). The last basic number (x = 9) was detected in Europe in *Trigonella ornithopodiodes* L. (DC) (Darlington and Wylie 1945). This species was reclassified later, for taxonomic reasons, in the *Trifolium* genus (Allen and Allen 1981).

Within the *Fabaceae* family, several cases, observing more than one basic chromosome number, have been reported in different genera including *Onobrychis*, with x = 7 and x= 8 (Hejazi et al. 2010, Arslan et al. 2012) and *Genista* where the most common number of chromosomes is 2n = 48, with the exception of the aneuploid number (2n = 44) revealed in *Genista ovina* (Bacchetta et al. 2012). The same process was detected in species of the genus *Hedysarum*, among which, *H. pallidum* (2n = 16 and 18) (Benhizia et al. 2003); *H. coronarium* (2n =

16 and 2n = 18) (Issolah et al. 2006) and *H. perrauderianum* (2n = 32 and 18) (Benhizia et al. 2013).

In the *Poaceae* family, dysploidy was observed in *Lygeum spartum* L., whose cytogenetic study revealed two basic chromosome numbers, in two Algerian populations of different origins (2n = 16 and 40) (Abddaim-Boughanmi et al. 2009). According to the same authors, the population (2n = 40), also presented a variability of the chromosome number within the same individual.

Yakovlev et al. (2017) have shown that constitutive heterochromatin, DNA GC rich and rRNA are involved in chromosomal rearrangements during the change in basic chromosome numbers in Mediterranean species of the genus *Reichardia* Roth. (*Asteraceae*). These species are characterized by three basic chromosome numbers (x = 9, 8 and 7), which have contributed to the evolution of the genus in the Mediterranean region (Yakovlev et al. 2017).

Concerning chromosome size, our results (1.02-3.1µm) seem to be relatively inferior to those found by Falistocco et al. (2013) on Italian accessions of *Trifolium subterraneum* (2.5-3.5µm). But then, this size appears to be very similar to that recorded in *T. lappaceum* species of Iran (3.03 µm), but smaller than the sizes reported in other *Trifolium* species of Iran (*T. angustifolium:* 14.56 µm, *T. leucanthum:* 12.32 µm, *T. tumens:* 11.09 µm) (Alimardani et al. 2014). Our data are also close to those found within some *Trifolium* species in Turkey, such as *T. echinatum* (1.41-2.74 µm) and *T. phleoides* (1.73-2.78 µm) (Uslu 2012), and appear to be superior to those recorded by kiran et al. (2010) in *T. speciosum* Willd. (0.99-1.64 µm) and *T. campestris* Scherb (1.13-1.73 µm).

Within the same family (*Fabacaea*), the size of *T. subterraneum* chromosomes, found during our study, is relatively close to those reported for some species of the genera *Hedysarum*, *Astragalus* and *Asparagus* studied in Algeria (Benhizia et al. 2003 ; Issolah et al. 2006, Benhizia et al. 2013; Baaziz et al. 2014 and Boubetra et al. 2017).

Our observations highlighted satellites at the first pair of chromosomes. The presence of satellites and their location on the first chromosome pair joins the result found by Falistocco et al. (2013) on Italian accessions. According to Falistocco et al. (1987) and Falistocco et al. (2013), these satellites are present in the three subspecies of *T. subterraneum* (*subterraneum*, *brachycalycinum*, *yanniniccum*), and their size can be used for discriminating the three subspecies. The satellites are more important in *yanninicum* and medium in the other two subspecies (Falistocco et al. 1987).

In all populations, the chromosomes are median. This confirm the results of Falistocco et al. (2013) on Italian accessions, characterized also by median chromosomes, whereas, Angelo et al. (1983) have described two chromosomes types (median and submedian) for Spanish ecotypes. Moreover, two types of karyotypes were identified for the Iranian accessions: the first consists on eight median pairs; the second karyotype is composed by six median pairs and two submedian pairs (Hezamzadeh Hijazi and Ziaeinasab 2006).

Karyotype asymmetry is an important parameter in karyological studies (Eroğlu 2015). In our case, the karyotype (2n=16) of Algerian populations of *Trifolium subterraneum* is very symmetrical. This seems to be a common trait with Italian populations of *T. subterraneum* karyotype (Falistocco et al. 2013), but differs from the Iranian ones. The latter populations of *T. subterraneum* (2n = 16) are characterized by low intrachromosomal symmetry (Hezamzadeh Hijazi and Ziaeinasab 2006).

On the other hand, the karyotype of the population 2n = 18 is considered relatively symmetrical because of the high value of interchomosomal asymmetry. Thus, Muñoz-Rodríguez (1995) does not consider the karyotype of the species *Trifolium ornithopodioides* (2n=18) as asymmetrical, despite the high value of the asymmetry index A2 (0.20). The author noticed this, because of the more or less uniform sizes of the chromosome pairs, except for the first pair, which was larger than the others (Muñoz-Rodríguez 1995).

In the species *Reichardia picroides* (*Asteraceae*), Yakovlev (1986) has suggested that this is a case of secondary symmetry due to chromosomal rearrangements.

The analysis of pollen meiosis confirmed the results obtained in mitosis. At the end of these results we have found that the Algerian populations of *T. subterraneum* are characterized by two chromosomal formulas. The first, (2n = 2x = 16m) (median) usually reported by previous authors, and the second (2n = 2x = 18m) revealed for the first time in this species throughout our present work. It is important to note that the new formla (2n = 2x = 18m) is observed particularly in populations sampled from high altitude sites (800-1110 m), belonging to the same biogeographic area and characterized by a high rainfall (700-900 mm). Consequently, the variation in the chromosome number observed in the populations of this species and the appearance of a new chromosome pair seems to be influenced by these two ecological factors (altitude and rainfall).

Meanwhile, the same populations considered through our study have been the subject of previous work on the ecological characterization of the natural habitat of *T. subterraneum* in Algeria (Issolah et al. 2015). Thus, the results of this latest study have shown that the variation of the edaphic, climatic, and topographic characteristics of the origin sites of these popu-

lations influences the distribution of this species in the North-Est Algeria (Issolah et al. 2015). Significant relationships were found between altitude and rainfall and the physico-chemical parameters of the soils of these populations, and the effect of altitude was relatively more pronounced notably on the nitrogen, clay, pH and C / N ratio (Issolah et al. 2015). Abdelguerfi et al. (2006) indicate that *T. subterraneum* is more prevalent in heavily watered and moist regions. Rossiter and Collins (1988a, 1988b) and Cocks (1992) also observed greater variability of subterranean clover populations in high rainfall areas in Australia.

Various studies have shown that differences in the origin's areas of populations and the variation of the environmental factors of the natural habitat may explain the intra-specific differences. Thus, they can affect the variation of chromosome numbers, ploidy level, chromosome structure, and asymmetry of karyotype in certain species belonging to the genera: *Trifolium* (Issolah and Abdelguerfi, 1999b, Issolah 2006); *Hedysarum* (Issolah et al. 2006, Benhezia et al. 2013); *Bellevalia* and *Muscari* (Azizi et al. 2016); *Asparagus* (Boubetra et al. 2017). Environmental factors also, influenced karyotype parameters in *Aegilops* (*Poaceae*) species (Baik et al. 2017). Significant relationships were found between Altitude, total lengths chromosome set and interchromosomal asymmetry on the one hand and, on the other hand, between rainfall and intrachromosomal asymmetry (Baik et al. 2017).

According to Hayward and Breese (1993), natural habitats are rarely, if ever, uniform in space and time and can encompass several distinct micro-niches or go through large seasonal fluctuations. Although *Trifolium subterraneum* is a self-pollinating species, Allard and Adams (1969) and Hayward and Breese (1993), report that fluctuations and variation in edaphic conditions at the site of origin trigger in self-pollinated species, a disruptive selection that produces and maintains high levels of variability in wild populations.

In Italy, a relationship between many morphological characteristics and the ecological factors of the environment of origin (altitude and rainfall) has been determined in several populations of *T. subterraneum* from Sicily (Piano et al.1993, Pecetti and Piano 1998).

In a large collection of subsp. *subterraneum* germplasm of Sardinia, Piano et al. (1996, 2002) found that the level of complexity for various traits varied greatly among populations and was influenced by the climatic characteristics of the collection sites.

Within the genus *Trifolium*, interesting relationships have been found between many morphological characteristics and some ecological factors (altitude and rainfull) of the environment of origin of several spontaneous Algerian populations belonging to various species (*T. campestre*, *T. glomeratum*, *T. tomontosum*, *T. resupinatum*, *T. scrabrum*, *T. lampaceum*, *T. spumosum*) (Issolah and Abdelguerfi 1993, 1995, 2003 ; Issolah 2006). In addition, Medoukali et al. (2015), do not report any significant relationship between the morphological characteristics and the environment of origin of populations belonging to several *Trifolium* species (*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*). Nevertheless, a large genetic variation of isoenzymes has been observed (Medoukali et al. 2015).

Although the species is self-pollinated with cleistogamous flowers (Katznelson and Morley 1965), there is a possibility of occasional cross breeding, and this exceptional rarefaction could be of great importance for the evolution of *T. subterraneum*. Marshall and Broué (1973) estimated the cross-pollination rate of the Australian clover populations at 0.15%. Variation released by occasional hybridization can then be fixed by selfing and made available to natural selective pressures (Cocks 1992b). According to Piano (1984), natural populations of subterranean clover were formed by clusters of several genetically distinct strains. This would probably explain the chromosomal variation observed in this study within and between populations. As a result, the different populations of *T.subterrarenum* would have been crossed.

Meanwhile, four populations from the same region exhibited the same somatic behaviour (2n = 16 and 18) (within the same individual and between different individuals) and meiotic (n = x = 8 and n = x = 9). These populations would probably be evolved in time, since they belong to a species of the "*Trichocephaleum*" section considered**,** according to Zohary and Heller (1984), as the most evolved section compared to other sections of the genus *Trifolium*. This section is therefore composed of species, whose interaction, with the various ecological characteristics of the natural habitat, would affect the chromosomal rearrangements and evolutionary trends of the populations within *T. subterraneum* species.

CONCLUSION

This study permitted to identify and analyse the intraspecific diversity of the chromosome numbers and karyotypes within nine natural populations of *Trifolium subterraneum,* originating from the different areas of the north eastern Algeria. Two chromosome numbers

are distinguished in this species: 2n=16 (x=8) and 2n=18 (x=9). The first number (2n=16), is widely detected by previous authors, while the second one (2n=18) is newly observed in algerian populations of this species. The latter number (2n=18) is frequently met in populations coming from the high altitude areas. The ecological conditions of the origin's environment of the populations would have an effect on the changes in the genetic and karyological structure, particularly the altitude factor. This karyological approach provides new information that will help researchers to elucidate and complete the systematics and the nature of diversity within *Trifolium subterraneum* species. However, thorough investigations of the morphological and molecular aspects of these natural populations would be necessary, to determine the limits of dysploidy. Furthermore, comparative analysis with other populations from different origins would help to understand more about the genome evolution process of *T. subterraneum* populations in their environment of origin. This would permit to valorize and develop this plant genetic resource in the Mediterranean area, especially in Algeria.

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