

Ploidy level, genome size and genetic variability among a collection of *Medicago sativa* L. Gabsi as revealed by flow cytometry

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Abstract - The flow cytometry technique has been applied in order to check the ploidy level of seven provenances of local alfalfa (*Medicago sativa* L.) Gabsi, to estimate the genome (pg DNA) size of these alfalfa lines and to verify whether any genetic differences existed between these provenances belonging to the same population. Flow cytometry technique enabled us to show that all sources are tetraploid but also showed genetic variability that can be explained by the effect of microclimate, even if it is obvious that all these sources belong to a same unique population.

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1. Introduction

Alfalfa is the oldest plant that has been exclusively grown for forage and the most important forage legume in the world (Quiros and Bauchan 1988). Cultivated alfalfa, *Medicago sativa* L., is an autotetraploid that in reality has originated from the *Medicago sativa-falcata* complex (Quiros and Bauchan 1988). In reality *Medicago sativa* is a polyploidy complex comprising two ploidy levels, diploid ($2n = 2x = 16$) and tetraploid ($2n = 4x = 32$) as described by Lesins and Lesins (1979), with only weak hybridization barriers within the same and across ploidy levels (the latter through unreduced $2n$ gametes being produced from a diploid parent (Quiros and Bauchan 1988). The taxa included in the complex are distinguished based on morphology (mainly flower color, pod shape, and pollen morphology) and ploidy.

The diploid subspecies is recognized as *M. sativa* subsp. *caerulea* and the tetraploid as *M. sativa* subsp. *sativa*. (Şakiroğlu and Brummer 2011). In this respect, flow cytometry is an alternative tool for plant breeders to determine ploidy level and has been characterized as a robust method for determining the ploidy level of *M. sativa* accessions (Brummer et al. 1999). Perennial species are mainly tetraploids ($2n = 4x = 32$) and out crossing (Quiros and Bauchan 1988).

Characterization of genetic variation in alfalfa, *Medicago sativa* L., using morphological traits is sometimes insufficient, especially when closely related populations or those with a narrow genetic base are used. The development and application of molecular cytogenetics to *Medicago sativa* will give an idea about genome size and base pair composition that, according to Ohri & Khosoo (1986), can in turn provide useful data about the affinity of species.

In order to evaluate the nuclear DNA content and to assess genetic diversity among seven provenances of local populations of *Medicago sativa* L. Gabsi, we used flow cytometry as it is generally used for ploidy analysis and considered as the most accurate tool for determining the ploidy level (Doležal et al. 1998; Ochatt 2008). Besides, it is also a rapid and robust technique that allows accurate determination of DNA content in a large number of nuclei (Brown 1993).



This technique quantifies the intensity of light emitted by isolated nuclei stained with DNA specific fluorochromes after excitation. The fluorescent signal of each individual nucleus is proportional to the amount of fluorochrome stoichiometrically bound to the DNA (Gamiette et al. 1999).

Use of flow cytometry in plant biology have increased rapidly because it has several advantages when compared with other methods: rapid sample preparation, non-destructive sampling, rapid detection of mixed samples or endopolyploidy and relatively low operating costs (Ochatt 2008; Suda et al. 2007), thus a large population can be measured in a short time and the presence of subpopulations may be detected (Shapiro 2003).

The aim of this study was to determine the ploidy level, to estimate genome size and to assess genetic diversity within different provenances of local *Medicago sativa* L. Gabsi.

2. Materials and methods

2.1. Plant collection and in vitro culture

Analyses were carried out on seven different provenances of local *Medicago sativa* L. Gabsi plants that were collected in different oases on south of Tunisia (Table1).

For seed scarification we used a 96% sulfuric acid for 8 minutes followed by several washes with sterile water (120°C for 20 min). For sterilization, they were immersed in commercial bleach (50%, v/v 12Cl) for 8 minutes and washed 5 times. Finally we put them in 75% ethanol for 5 minutes and then we washed them 5 times.

Seven to eight seeds were planted per Petri dish with MSO medium (Murashige and Skoog 1962). Petri dishes were sealed with Parafilm and incubated in dark at 25°C until germination. They were then transferred to light for plant growing for 2 to 3 weeks.

2.2. Sample preparation for DNA counting

A small amount of young leaves (4 to 5 leaves) from different plants were put separately in plastic Petri dishes. Leaves were chopped with a razor blade in nuclei extraction and staining buffer DNA 1-Step (Partec GmbH, Germany) with DAPI solution (4,6-diamidino-2-phenylindole), an AT-specific pair binding fluorescent dye, to liberate the nuclei into suspension. Released nuclei were then sieved through a 50µm plastic sieve in plastic hemolysis tubes, and incubated for 5 minutes at room temperature.

2.3. Flow cytometry analyses

Firstly, different samples were analysed alone in order to examine attributed picks. Then, they were all ran again simultaneously with an internal standard reference (Ochatt 2008; Ochatt et al. 2013) which in our case were leaflets of *Medicago truncatula* that were chopped and added in all samples (5.0%). All measurements were performed using a Partec PAS-II (Partec GmbH, Münster, Germany) equipped with an HBO-100 W mercury lamp and a dichroic mirror (TK420), whereby stained nuclei were excited using UV as previously reported elsewhere (Ochatt 2008).

2.4. Genome size estimation

The DNA content of the different provenances studied was calculated with the formula

Sample 2C DNA content = {(sample G1 peak mean) / (standard G1 peak mean)} × standard 2C DNA content (pg DNA).

Conventionally, relative nuclear DNA amounts are presented in pg DNA and Mpb, using the formula 1 pg = 978 Mpb as described by Dolezel et al. (2003).

2.5. Genetic diversity

MVSP3.1 software was used to calculate genetic variability among different provenances. A similarity matrix obtained with the Euclidian distance formula was used to draw dendrograms describing genetic variability among the collection studied.

3. Results and Discussion

3.1. DNA content and genome size

In order to assess the DNA amount in seven local alfalfa (*M. sativa L.*) provenances, and to assess the existence of genetic variability within the Gabsi population with flow cytometry, the diploid standard *Medicago truncatula* (Gaertn.) ($2n=2x=16$; 1C DNA/nucleus = 0.48 pg; genome size = 469.44 Mbp ; Ochatt et al. 2013), plant reference very close to *M. sativa L.* is used.

We chose to work with *Medicago truncatula* because an ideal DNA reference should have a genome size close to the target species in order to avoid the risk of nonlinearity and offset errors (Bagwell et al. 1989). Ochatt (2008) has shown that running an internal standard in the same way, is of great importance, as a control, when analysing the ploidy level of G1-cells, firstly the standard is run alone in order to identify the exact position of peaks, and then it is mixed with the study sample, whereby flow cytometry profiles obtained comprise typically of four peaks, i.e. the 2C and 4C peaks of each sample analysed simultaneously and their position permits to establish the relative nuclear DNA content of the material analysed accordingly to its distance from the G0/G1 peak of the internal standard used. In our case, given the very small genome size of barrel medic (Blondon et al. 1994; Ochatt 2008; Ochatt et al. 2013) its G0/G1 and G2/M peaks are expected in a position very close to the ordinates whereas those of the alfalfa genotypes analysed will be farther away, coherent with the larger genome size of alfalfa of 1.72 pg DNA/nucleus at 2C as reported by Blondon et al. (1994) and Bennett and Leitch (1995).

Flow cytometry analysis applied to nuclear DNA of the seven provenances of *M. sativa* Gabsi thus gave histograms (Figure1) with a dominant G0/G1 population, representing the amount of nuclear DNA in diploid unreplicated cells, according to Swift (1950). Indeed, in a young leaf the majority of cells are not undergoing division, but are in the G0 stage instead. Consequently, their relative nuclear DNA content reflects well the ploidy level of the plant. Cells in division pass from the G0/G1 stage to the G2 stage where they have double the nuclear DNA content (De Laat et al. 1987; Ochatt 2008).

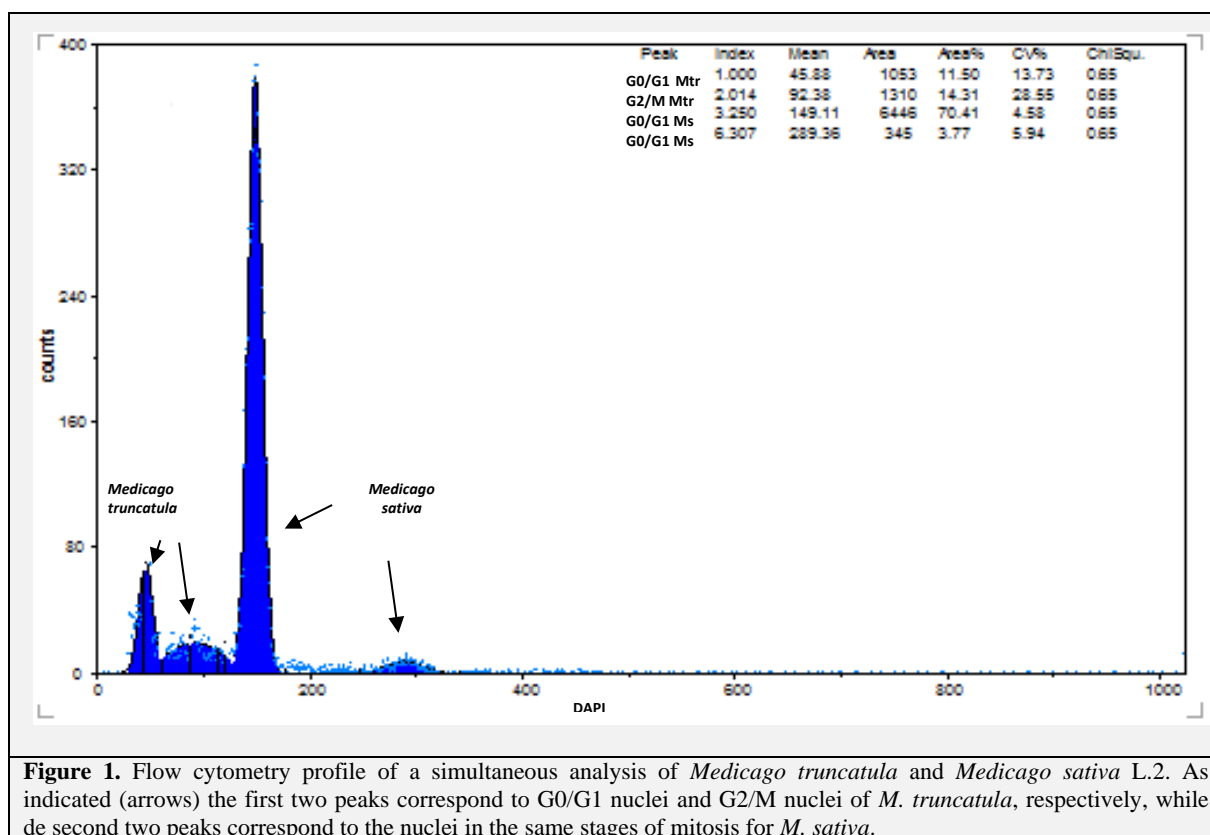


Figure 1. Flow cytometry profile of a simultaneous analysis of *Medicago truncatula* and *Medicago sativa* L.2. As indicated (arrows) the first two peaks correspond to G0/G1 nuclei and G2/M nuclei of *M. truncatula*, respectively, while the second two peaks correspond to the nuclei in the same stages of mitosis for *M. sativa*.

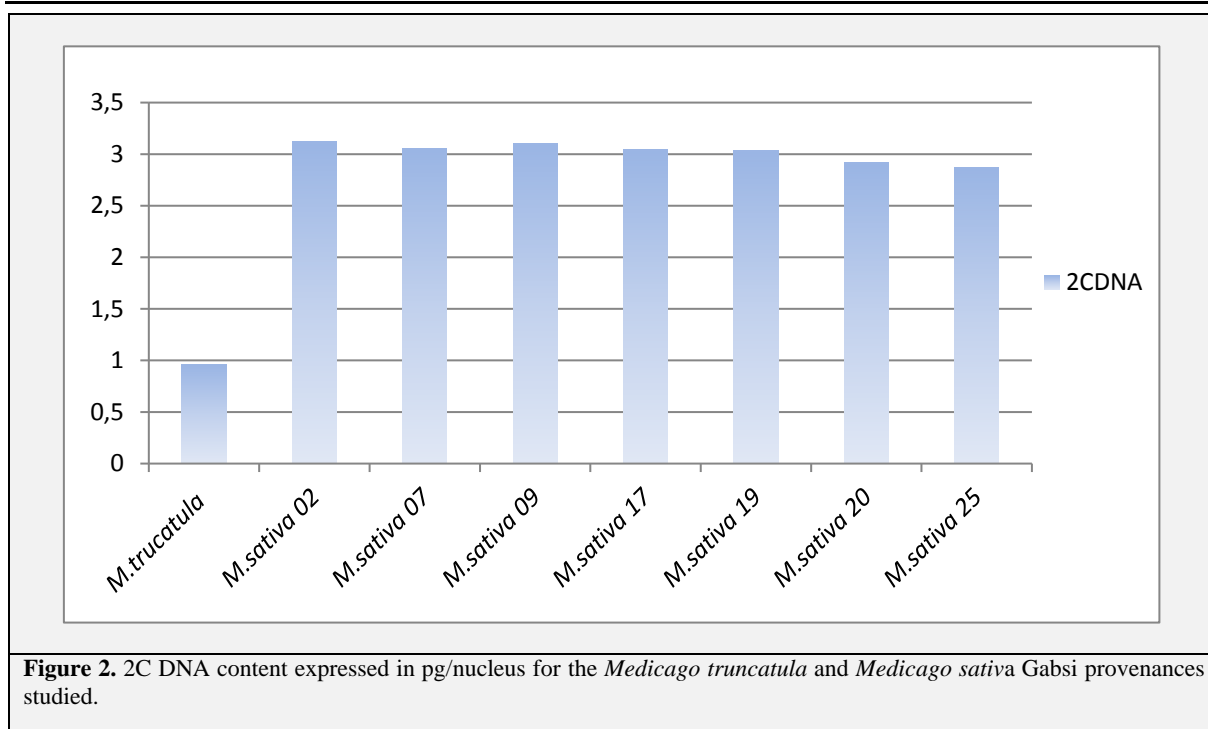
histograms of relative nuclear DNA content. Good histograms should contain minimal amounts of background debris, G1, G2 peaks should be symmetrical and must show low variation, this variation is expressed as the coefficient of variation (CV) = standard deviation/peak mean $\times 100\%$.

According to Dolezel and Göhde (1995), histograms with peak CVs lower than 1 % are very difficult to acquire and possible only under specific conditions. Generally, CVs below 3 % are considered fully acceptable (Galbraith et al. 1998). Such precision may not be attainable in ‘difficult’ species, where CVs below 5% are acceptable. When an internal standard of a smaller genome size is used, as in our studies, this tends to induce a larger CV% for the first two peaks, i.e. those of the internal standard, and thus the flow cytometry parameters used are generally established so that the G0/G1 peaks of the internal standard are as close to the ordinates as possible to try to reduce this CV. The G0/G1 peak of the standard appeared around channel 50 while those of the studied samples appeared between 132.03 and 151.99 (Figure 1). Values obtained on the profiles were used to calculate genome size at 1C DNA in terms of pg and Mpb per nucleus (Table1 and Table2).

Table 1. Genotype code, origins, 2C peaks values and 1C DNA content of studied provenances.							
Species and genotype	Genotype origin	Peak Analysis	Profils of <i>Medicago sativa</i> + <i>Medicago truncatula</i> ^a		Index	1C DNA (pg) ^b	1C DNA (Mpb) ^c
			<i>M. truncatula</i>	<i>M. sativa</i>			
<i>M. truncatula</i> R108	France Dijon	49.73	-	-	1.0	0.48	470
<i>M. sativa</i> 02	Essayah Benguerdane	153.70	45.88	149.11	3.250	1.560	1526
<i>M. sativa</i> 07	Louled Gabes	148.41	47.77	151.99	3.182	1.527	1494
<i>M. sativa</i> 09	Gannouch Gabes	145.30	44.52	144.17	3.239	1.554	1520
<i>M. sativa</i> 17	Mareth Essagy	144.29	44.97	142.60	3.171	1.522	1489
<i>M. sativa</i> 19	Kattana Gabes	143.54	44.51	140.92	3.166	1.520	1486
<i>M. sativa</i> 20	Mareth Essedreya	125.83	43.47	132.03	3.037	1.458	1426
<i>M. sativa</i> 25	Chenini Gabes	138.44	45.01	134.63	2.991	1.436	1404

Table 2. 2C DNA content and ploidy level for studied genotypes.		
Species and genotype	2C DNA	Ploidy (\times)
<i>M. truncatula</i> R108	0,96	2
<i>M. sativa</i> 02	3,12	4
<i>M. sativa</i> 07	3,05443584	4
<i>M. sativa</i> 09	3,10878706	4
<i>M. sativa</i> 17	3,04843229	4
<i>M. sativa</i> 19	3,0393889	4
<i>M. sativa</i> 20	2,9157764	4
<i>M. sativa</i> 25	2,87146856	4

The results obtained (Tables 1 and 2) indicate that the genome size of the seven provenances studied is only slightly variable between the smallest genome size equal to 2.87 pg observed in the provenance Chenini Gabes and the biggest genome size of 3.12pg DNA for the origin Essayah Benguerdane, which results in a mean value of 3.022 pg DNA at 2C DNA per nucleus (Figure2).



The results are close to those of Blondon et al. (1994) who found that the 2C DNA content/nucleus of *M.sativa subsp sativa* ranged from 3.33 pg to 3.46 pg. Our results are also in line with the hypothesis of a relative DNA content value of 0.856 pg DNA corresponding to the basic chromosome number, $x=8$ and thereby confirming the tetraploidy of the studied population.

These values, although, very close, can reveal the existence of a small genetic variability among the studied population. According to Brandizzi and Caiola (1998) a difference in genome size and composition can sometimes be used for the fast discrimination of species as already done among *Medicago* species (Iantcheva et al. 2001) and, likewise, can conceivably permit the distinction between different populations within a single species as previously performed to characterize a large population of *Tnt1* mutants in *Medicago truncatula* (Ochatt 2008). It can also help solve taxonomic problems in some genera as recently done in another legume, *Lathyrus* (Ochatt et al. 2013).

3.2. Genetic variability

For better understanding the observed variability among the studied collection, DNA contents were used to calculate a matrix of genetic distance and the *PHYLIP* software to provide a dendrogram describing the existing interactions.

When observing the dendrogram (Figure 3) describing the genetic relationship, some genetic interactions can be seen between some of the studied provenances of *M.sativa L.* Gabsi, such as those found between lines 02 and 09, 20 and 25 and between 07, 17 and 19.

Importantly, *M.truncatula* R108, having a smaller genome does not show any specific relationship with the studied provenances of *M.sativa L.* Gabsi, but it serves to anchor their flow cytometry peaks and thus confirm their tetraploid nature.

The observed differences may be explained by the following reasons:

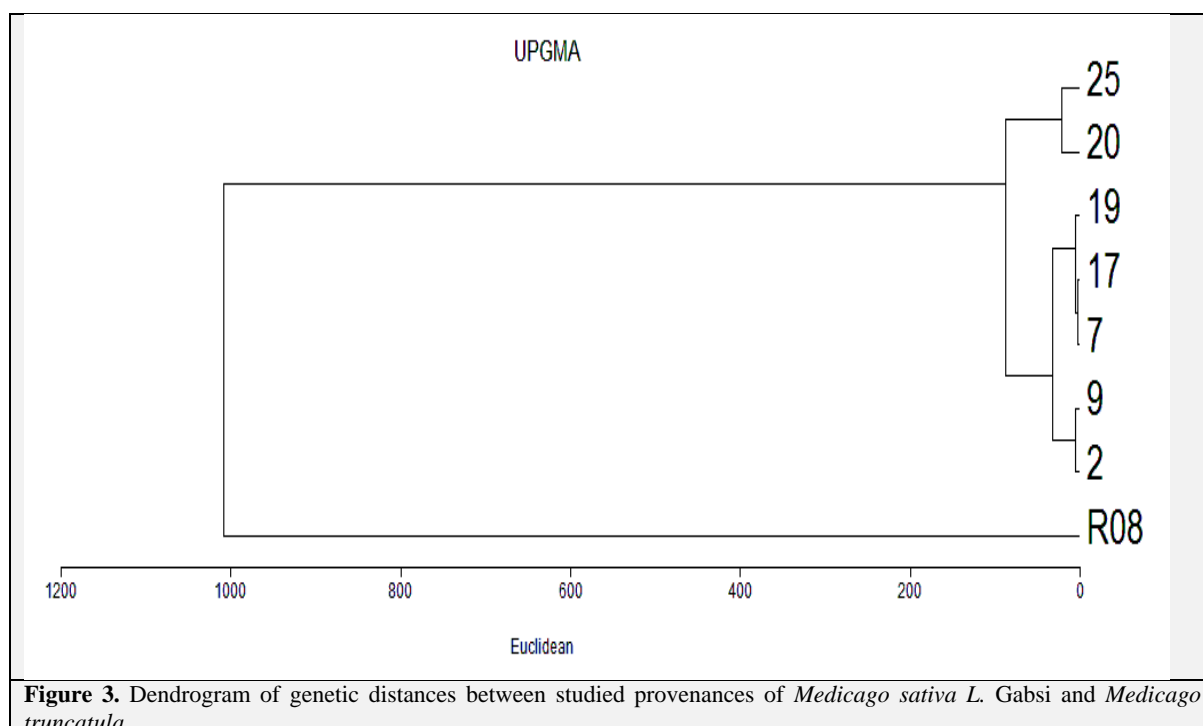
Firstly, the different provenances of the studied populations Gabsi are harvested in different regions of southern Tunisia, where the composition of irrigation water and soil type differ widely. These differences in their microclimates may cause mutations of the genetic information probably correlated with the adaptation of the respective genotypes to such environmental conditions.

Secondly, *Medicago sativa L.* is strictly allogamous, and it can thus receive different characters from other genotypes. According to Bennetzen and Kellogg (1997), the understanding of nuclear genome and its components is very important to provide clues on the mechanisms responsible for genome increase, like activation of Class I retrotransposons, and for genome decrease by deletions (Bennetzen et al. 2005). Variation in BARE-1 retrotransposon copy number was observed in populations of wild

barley in response to differing micro-climates. However, differences between C-values of different populations estimated by flow cytometry were not statistically significant and genome size was only weakly associated with microclimatic gradient (Kalendar et al 2000). Despite this, the study was perceived as supporting previous reports on large intraspecific variations in genome size (Wendel and Wessler 2000).

This intraspecific variation in genome size and its extent is, according to Greilhuber and Obermayer (1997), a difficult and debated field and is now attracting much attention. Intraspecific variation was reported on different species in previous studies, including soybean (Rayburn et al.1997), sunflower (Michaelson et al.1991), pea (Arumuganathan and Earle 1991) and maize (Rayburn et al.1989).

The described variation may in some cases be correlated with environmental gradients or growth conditions. However, geographically isolated populations show great stability of the nuclear genome for *Sesleria albicans* (Lysak et al. 2000), in various species of *Setaria* (Le Thierry d'Ennequin et al. 1998), *Cistus* (Ellul et al. 2002), *Capsicum* (Moscone et al. 2003), and in cultivars of pea and onion (Baranyi and Greilhuber 1995; Bennett et al. 2000b). On the other hand, Dolezel et al. (1998) demonstrated that differences in DNA content estimates observed between different laboratories cannot be interpreted in terms of intraspecific variation, and that small differences may only be identified when using a single instrument. A good practice is therefore to perform replicate measurements on different days, as was done in our studies.



4. Conclusion

Flow cytometric analysis was used in order to verify the ploidy level and evaluate the existence of genetic variability among a collection of seven provenances of local alfalfa (*Medicago sativa* L.).

Such study enabled us to verify that we are handling a tetraploid *Medicago sativa* L. and, precisely, *M.sativa subsp. sativa*.

Secondly, DNA estimates of different provenances showed small genetic differences that may be explained by the response of the plants to the different microclimate conditions where they grow. This result may explain differences in the response of the different provenances to salt stress (result not shown).

Even when they were genetically different, these provenances show a genetic affinity on which we are based to confirm that they all belong to *M.sativa subsp. sativa* Gabsi.

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