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ESTIMATING GENOME SIZE AND CONFIRMING PLOIDY LEVELS OF WILD TETRAPLOID ALFALFA ACCESSIONS (*Medicago sativa* subsp. × *varia)* USING FLOW CYTOMETRY

*Muhammet SAKIROGLU [*1], Muge Mavioglu KAYA[2],*

[1]Kafkas University, Department of Bioengineering, Kars, TURKEY
[2]Kafkas University, Department of Biology, Kars, TURKEY,
** Corresponding author: msakiroglu@kafkas.edu.tr*

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ABSTRACT

The taxonomic group *Medicago sativa-falcata* continuum includes the important cultivated forage legume, alfalfa, along with a number of other perennial, outcrossing, and morphologically differentiated subspecies at diploid and tetraploid levels. Prior information of morphology, ploidy, and genome size is vital for accurate classification of the taxa included in the complex and thus for effective usage of genetic resources in alfalfa breeding programs. The United States Department of Agriculture-National Plant Germplasm System (USDA-GRIN) has an extensive collection of the members of *Medicago sativa-falcata* continuum gathered from all the centres of diversity. However, accessions classified in the complex are occasionally misidentified. Furthermore, the accessions identified as *M. sativa* subsp. ×*varia* (T. Martyn) Arcang. in the USDA GRIN collections have not been evaluated based on morphological traits, ploidy level or genome size. In this study, we evaluated morphological traits and determined ploidy levels and genome size of plants from 25 wild accessions classified as *Medicago sativa* L. subsp. ×*varia* using flow cytometry. All of the accessions classified as subsp. ×*varia* were found to be tetraploid; however, deviations from flower colour, pod shape expectations were observed. This will be a major step toward effective utilization of germplasm resources classified as *M. sativa* subsp. ×*varia.*

Keywords: Alfalfa; flow cytometry; genome size; *M. sativa-falcata* complex*; M. sativa* subsp. ×*varia;* ploidy

INTRODUCTION

The nuclear DNA content of an unreplicated diploid cell (in G1 phase) is considered to have a 2C DNA amount (Swift, 1950) and this value is used to determine genome size of plant species. The knowledge of C-value is vital for many scientific disciplines, including molecular biology, systematic, and ecology (Dolezel and Bartos, 2005). Despite the wide range of usage, C-value of only less than 2% of all angiosperms are known (Hanson et al., 2003). Ploidy is another significant aspect of plant genome and plays an important role in evolution and diversity (Grant, 1971; Wang et al., 2006; Gaeta et al., 2007). Allopolyploidy arises from interspecific hybridity and stabilizes the hybrid genome whereas autopolyploidy occurs within species in which there are more than two sets of homologous chromosomes (Grant, 1971; De Laat et al., 1987). In addition to the evolutionary implications, polyploidy has a tremendous significance for agriculture. A number of economically important crops such as wheat, sugar cane, potato, coffee, alfalfa, and cotton are polyploids (Leitch and Bennett, 1997).

The *M. sativa* species complex (or sometimes the *M. sativa-falcata* continuum) includes the important cultivated forage legume, alfalfa, and a number of other perennial, outcrossing, and morphologically differentiated but often interfertile taxa at diploid and tetraploid levels. The taxonomic status of the taxa included in the complex has been unsettled until recently (Sakiroglu et al., 2010). Some authors have considered the taxa as species (Sinskaya, 1961; Lesins and Lesins, 1982; Ivanov, 1988) and others considered them as subspecies (Quiros and Bauchan, 1988). Majority of the taxa has been demoted to the subspecies level recently and the nomenclature has been widely adopted (Sakiroglu et al., 2010). Diploid (2n=2x =16) subspecies include *Medicago sativa* L. subsp. *falcata* (L.) Arcang. (yellow flowers, sickle shaped pods), *M. sativa* subsp. *caerulea* (purple flowers, coiled pods), and their natural hybrid, *Medicago sativa* L. subsp. ×*hemicycla* (Grossh.) C.R. Gunn. The analogous set of tetraploid (2n=4x=32) subspecies have similar distinguishing phenotypes to the diploids and include *M. sativa* subsp. *falcata*, *M. sativa* subsp. *sativa* L., and the tetraploid hybrid *Medicago sativa* L. subsp. ×*varia* (T. Martyn) Arcang. (Small, 1985; Quiros and Bauchan, 1988). The key characters distinguishing among the member of the complex are morphology (flower colour, pod shape, and pod pubescence) and ploidy.

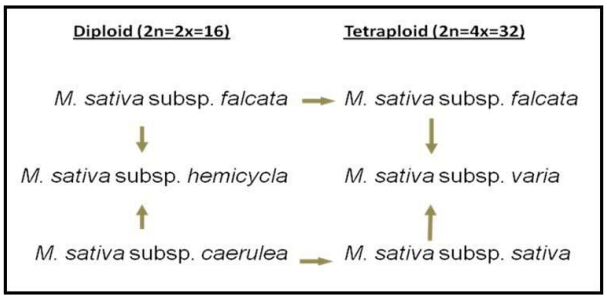

Figure 1. Taxonomic relationship among the members of *Medicago sativa* species complex

Chromosome counting of stained root tips under microscope was long regarded as the convention method for ploidy determination in plants (Sakiroglu and Brummer, 2011); however, the method has been noted as laborious and occasionally misleading (Brummer et al., 1999; Tuna et al., 2001). The two early approaches that were employed to determine the 2C DNA content of a given organism were the analysis of DNA extracted from a large number of cells and the measurements taken from the individual nuclei (Dolizel and Bartos, 2005). The estimate obtained from the first approach does not represent the 2C DNA amount, since source tissue could contain cells at different phases of the cell cycle and thus different DNA amounts. Although the estimation based on single nucleus approach yields higher precision, the technique is noted to be very demanding (Dolizel and Bartos, 2005). Flow cytometry serves as an accurate and fast alternative tool for plant scientists to determine ploidy level and to estimate the genome size of *M. sativa* accessions (Blondon et al., 1994; Brummer et al., 1999; Sakiroglu and Brummer, 2011). United States Department of Agriculture, Germplasm Resources Information Network (USDA-GRIN) maintains one of the largest alfalfa germplasm collections with more than 4000 accessions collected from the entire distribution range of all the members of the complex. However, the accessions are occasionally misidentified in the USDA-GRIN system (Sakiroglu and Brummer, 2011). The earlier attempts to determine ploidy level of *Medicago sativa* accessions either focused on subspecies *falcata* (Brummer et al., 1999) or diploid subspecies (Sakiroglu and Brummer, 2011). The evaluated accessions in the two above mentioned studies collectively correspond to less than 3% of the entire collection. Hence, majority of accessions still lack the information of ploidy level. As one of the subspecies considered in the complex, *Medicago sativa* L. subsp. ×*varia* has been incorporated into breeding material and has significant economic value. Accessions collected in the USDA-GRIN system and denoted as subsp. ×*varia* have not been evaluated based morphology and ploidy. The only genome size estimate of the subsp. ×*varia* is estimated from a single plant and extrapolation of that single plant estimate to the entire subspecies could be misleading. Therefore, there is need to evaluate subsp. ×*varia* accessions based on morphology, ploidy, and genome size.

In this study, we evaluate morphological traits and ploidy levels of 25 accessions of *Medicago sativa* L. subsp. ×*varia* gathered from one of the centre of diversity and estimated genome size of accessions using flow cytometry.

MATERIALS AND METHODS

We used 25 accessions from the USDA National Plant Germplasm System identified in GRIN that were originally collected from Turkey and denoted as *M. sativa*, subsp. ×*varia.* All the accessions are also listed as local landraces. A tomato plant (*Solanum lycopersicum* L.) from cultivar SC2121 (Arumuganathan and Earle, 1991), a diploid (CADL), and a tetraploid (Buldog 505) plant of known ploidy (Brummer et al., 1999, Sakiroglu and Brummer, 2011) were selected as diploid and tetraploid standards and genome sizes of all the samples were compared to the two standards.

Alfalfa seedlings were grown in Kafkas University greenhouse for about six weeks. Young leaves were harvested from each plant for flow cytometry analyses. Each individual sample consisted of 10 mg of leaf tissue of tomato plant and 15 mg of leaf tissue of individual genotypes from each accession. Each of the individual genotype from each accession was run independently.

The procedure explained in Galbraith et al. (1983) was used for the flow cytometry. The leaf material was chopped with a one-sided sharp razor blade in a Petri dish containing chopping buffer prepared according to Galbraith et al. (1983) to isolate nuclei. After chopping, the buffer, containing cell constituents and large tissue remnants, was passed sequentially through nylon filters of 50 µm and 20 µm mesh size to separate nuclei from the cell debris. The buffer with nuclei was then centrifuged at high speed (800 rpm for 5 minutes), the supernatant was discarded, and the pellet was resuspended in 100 µl propidium iodide (PI) staining solution of 100 µg/ml. All the sample preparation procedures were performed in darkness on ice until the analyses were completed.

The samples were analyzed on a Cytomics FC 500 (Beckman-Coulter, Fullerton, CA**)** flow cytometer at the Flow Cytometry Facility of Trakya University Hematology Lab with a wavelength of 488 nm. Mean DNA content was based on analysis of 10000 nuclei. Ploidy levels of accessions were determined based on diploid and tetraploid standards (CADL and Buldog 505) and the DNA content was determined based on tomato plant.

Since the morphological traits such as flower colour and the pod shape are employed to assign accessions into subspecies, we recorded flower colour and pod shape from each genotype under greenhouse conditions. The flower colour of each accession was visually classified following Barnes (1972). For tetraploid subspecies, yellow flowered accessions were considered as subsp. *falcata*, purple flowered accessions as subsp. *sativa*, and the variegated flowers as subsp. ×*varia*. The pod shape data was

gathered following Sakiroglu et al. (2010). Briefly, pod shape among the members of *Medicago sativa-falcata* complex ranges from straight or falcate pods to highly coiled pod shape. We recorded the coil number for ten individual pods from each genotype and computed an average coiling value of the accession across all genotypes. Pods were scored in 1/4 coil intervals.

Table 1. Wild diploid alfalfa accessions along with geographic coordinates and the subspecies information.

No	Population	Taxonomic status given by USDA	Location of Collection	Number of plants assayed
1	PI 171719	Medicago sativa subsp. ×varia	Tokat/Turkey	3
2	PI 172429	Medicago sativa subsp. ×varia	Ardahan/Turkey	3
3	PI 206281	Medicago sativa subsp. ×varia	Sivas/Turkey	3
4	PI 206282	Medicago sativa subsp. ×varia	Sivas/Turkey	3
5	PI 238145	Medicago sativa subsp. ×varia	Tunceli/Turkey	3
6	PI 238150	Medicago sativa subsp. ×varia	Sivas/Turkey	3
7	PI 238151	Medicago sativa subsp. ×varia	Sivas/Turkey	3
8	PI 383693	Medicago sativa subsp. ×varia	Pertek/Turkey	3
9	PI 464800	Medicago sativa subsp. ×varia	Sivas/Turkey	3
10	PI 464801	Medicago sativa subsp. ×varia	Elazığ/Turkey	3
11	PI 464802	Medicago sativa subsp. ×varia	Bingöl/Turkey	3
12	PI 464803	Medicago sativa subsp. ×varia	Kars/Turkey	3
13	PI 464804	Medicago sativa subsp. ×varia	Kars/Turkey	3
14	PI 464805	Medicago sativa subsp. ×varia	Kars/Turkey	3
15	PI 464806	Medicago sativa subsp. ×varia	Kars/Turkey	3
16	PI 464807	Medicago sativa subsp. ×varia	Kars/Turkey	3
17	PI 464808	Medicago sativa subsp. ×varia	Kars/Turkey	3
18	PI 464809	Medicago sativa subsp. ×varia	Erzincan/Turkey	3
19	PI 464810	Medicago sativa subsp. ×varia	Sivas/Turkey	3
20	PI 464811	Medicago sativa subsp. ×varia	Sivas/Turkey	3
21	PI 464812	Medicago sativa subsp. ×varia	Yozgat/Turkey	3
22	PI 464813	Medicago sativa subsp. ×varia	Yozgat/Turkey	3
23	PI 464814	Medicago sativa subsp. ×varia	Ankara/Turkey	3
24	PI 577511	Medicago sativa subsp. ×varia	Erpeler/Turkey	3
25	PI 577512	Medicago sativa subsp. ×varia	Erzincan/Turkey	3
26	Buldog 505	Cultivar	Tetraploid Standard	2
27	CADL	Cultivar	Diploid Standard	1

RESULTS AND DISCUSSION

Ploidy level of the accessions:

In order to assess the ploidy level of the accession, we compared the fluorescence density of samples to the previously known diploid and tetraploid standards. Fluorescence densities of diploid and tetraploid standards were first detected. The peak from diploid standard was occurred at 183 nm whereas the peak of the tetraploid standard was occurred at 361 nm (Figure 2a and 2b). The peaks obtained from the samples were compared to the ones from standard plants (Figure 2a and 2b). All the accessions were found to have fluorescent density equal to the tetraploid standard confirming tetraploid status of the accessions. We did not observe any within accession ploidy level variations (Table 2).

Previously, extensive deviations at the ploidy level among the accession classified as diploid hybrid subspecies *Medicago sativa* L. subsp. ×*hemicycla* were detected (Sakiroglu and Brummer 2011). Majority of the accessions were proposed to be reclassified as *Medicago sativa* L. subsp. ×*varia.* A similar pattern was observed among other subspecies (Sakiroglu et al., 2010; Sakiroglu and Brummer, 2011; Havananda et al., 2010). Here, we did not observe any deviations from the expected ploidy level.

DNA content of the accessions

In order to estimate the DNA content of accessions, the samples including the tomato as the standard plant were run in flow cytometry. DNA content of the subsp. ×*varia* accessions were estimated based on relative florescent density of each sample compared to tomato plant. We initially targeted to estimate DNA content of three plants per accession; however, due to ambiguities of peaks from tomato plant, we could not estimate total nuclear DNA of some individual genotypes. Therefore, DNA content of seven accessions was estimated using two individual genotypes, and that of six accessions was estimated using a single genotype. Estimated DNA content was ranged from 2.85 pg to 4.99 pg with a mean of 3.25 pg (Table 3). These values show a large within subspecies genome size variation. The large intraspecific genome size variations were reported previously both among members of the complex (Blondon 1994) and in other species (Dolizel and Bartos, 2005). The only available genome size estimate for the subsp. ×*varia* is the early estimate obtained by Blondon et al. (1994) and it is based on one single plant. The 2C value of subsp. ×*varia* was 3.47pg. The estimate of genome in our study is 3.25 is based on average of 56 plants and is probably more robust. Given the sampling nature of two studies and extent of the variation within subsp. ×*varia*, the difference is not unexpected. Therefore, we conclude that

Table 2. Information on the ploidy level, flower colour, and pod coiling of the Medicago sativa accessions evaluated.

No	Accession	Subspecies	Ploidy level	Flower Colour	Average pod coiling
1	PI 171719	*Medicago sativa* subsp. ×*varia*	32	Purple	1.24
2	PI 172429	*Medicago sativa* subsp. ×*varia*	32	Purple	NA
3	PI 206281	*Medicago sativa* subsp. ×*varia*	32	Purple	1.46
4	PI 206282	*Medicago sativa* subsp. ×*varia*	32	Purple	1.85
5	PI 238145	*Medicago sativa* subsp. ×*varia*	32	Purple	0.98
6	PI 238150	*Medicago sativa* subsp. ×*varia*	32	Purple	1.03
7	PI 238151	*Medicago sativa* subsp. ×*varia*	32	Purple	1.63
8	PI 383693	*Medicago sativa* subsp. ×*varia*	32	Purple	1.15
9	PI 464800	*Medicago sativa* subsp. ×*varia*	32	Purple	1.28
10	PI 464801	*Medicago sativa* subsp. ×*varia*	32	Purple	1.56
11	PI 464802	*Medicago sativa* subsp. ×*varia*	32	Purple	1.40
12	PI 464803	*Medicago sativa* subsp. ×*varia*	32	Purple	1.32
13	PI 464804	*Medicago sativa* subsp. ×*varia*	32	Purple	1.58
14	PI 464805	*Medicago sativa* subsp. ×*varia*	32	Purple	1.13
15	PI 464806	*Medicago sativa* subsp. ×*varia*	32	Yellow/Purple	NA
16	PI 464807	*Medicago sativa* subsp. ×*varia*	32	Purple	1.65
17	PI 464808	*Medicago sativa* subsp. ×*varia*	32	Purple	1.18
18	PI 464809	*Medicago sativa* subsp. ×*varia*	32	Purple	1.28
19	PI 464810	*Medicago sativa* subsp. ×*varia*	32	Purple	NA
20	PI 464811	*Medicago sativa* subsp. ×*varia*	32	Purple	0.68
21	PI 464812	*Medicago sativa* subsp. ×*varia*	32	Purple	NA
22	PI 464813	*Medicago sativa* subsp. ×*varia*	32	Purple	0.87
23	PI 464814	*Medicago sativa* subsp. ×*varia*	32	Purple	NA
24	PI 577511	*Medicago sativa* subsp. ×*varia*	32	Purple	1.24
25	PI 577512	*Medicago sativa* subsp. ×*varia*	32	Purple	1.18
26	Buldog 505	Cultivar	32		
27	CADL	Cultivar	16		

NA: Not available

genome size estimations be conducted on large number of plants before the estimate is accurately established.

There is a significant ongoing discussion on whether the observed interspecies genome fluctuations are real or due to experimental artifacts and the discussions are comprehensively reviewed by Greilhuber (2005). Predominantly phenolic endogenous staining inhibitors in the plant material have been proposed to affect the genome size measurements (Greilhuber, 1986, 1988). Nonetheless, since we are reporting an average estimate of genome size for subsp. ×*varia*, the overall genome size should provide a robust estimate for the subspecies despite the evident variation.

Morphological traits

Flower colour of all the accessions evaluated was purple with the exception of PI464806. Within accession flower colour variation was detected from PI464806 one of the individual genotype found to have yellow flowers (Table 2). The within accession flower colour variations are not unusual given the outcrossing nature of the *M. sativa*, particularly when the location of collection is considered. The Eastern Anatolia is considered as one the centre of diversity for the members of *M. sativa* species complex (Sinskaya 1961; Lesins and Lesins, 1982; Quiros and Bauchan, 1988; Small et al., 1990; Sakiroglu and Brummer, 2012). All the subspecies are grown sympatricaly and the mixtures during the seed collection is very likely. The mixture at the seed collection sides during the seed increase could also be the cause of the within accession flower colour variations. The other important morphological trait that is used for classification of the accessions is pod shape and it is measured quantitatively. The average pod coiling of the putative subsp. ×*varia* accessions are measured from 20 accessions and ranged from 0.68 to 1,85 coils with an average of 1,28 (Table 3). Accessions of *M. sativa* subsp. ×*varia* are ought to have variegated flower colour which is distinct morphology of hybrid subspecies. The purple flowers however are distinctive characteristic of subsp. *sativa*. The classification of hybrid subspecies solely based on flower colour or pod coiling data could be misleading because these two morphological traits are governed by a small number of genes. In a previous study evaluating diploid subspecies using genome-wide marker data, it was

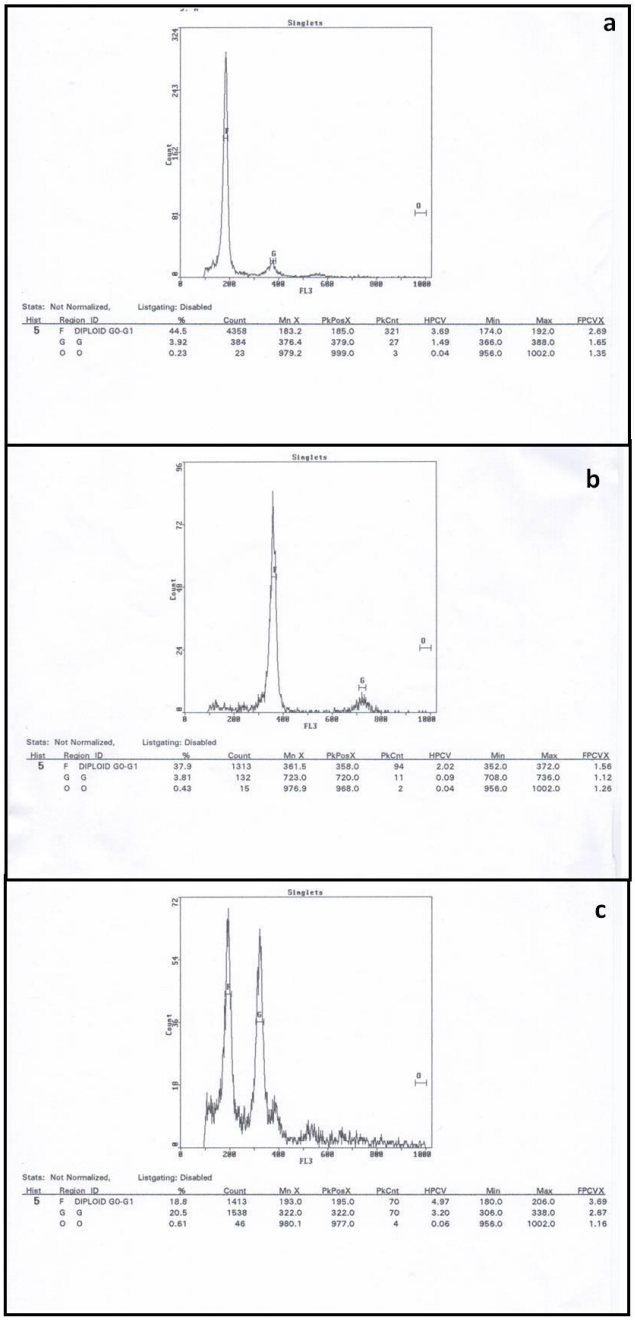

Figure 2. Flow cytometry results as a function of florescence density (nm) of (a) diploid standard CADL, (b) tetraploid standard Bulldog 505, and (c) comparison between tomato (*Solanum lycopersicum*) and *M. sativa* subsp. *varia.*

found that the flower colour could be a distinctive criterion among subspecies only when it is considered with pod coiling data (Sakiroglu et al., 2010). A comparison of genome composition with morphology, the study concluded that in addition to the variegated flower colour, accessions with either yellow or purple flower colour could also be hybrid. Yellow flowers and pods with more than one coil

Table 3. DNA content (pg) of 56 individual genotypes from 25 accessions and the percentage of total genetic variance attributable to each source of variation.

No	Accession	Florescence density of the standard	Florescence density of the sample	DNA content (pg)
1	PI 171719	212	370	3.31
		202	349	3.29
		234	380	3.08
2	PI 172429	219	359	3.12
		237	386	3.10
		228	383	3.20
3	PI 206281	183	315	3.27
		182	312	3.26
4	PI 206282	191	328	3.26
5	PI 238145	212	387	3.47
		220	361	3.12
		204	319	2.97
6	PI 238150	211	364	3.28
		208	360	3.29
7	PI 238151	140	210	2.85
		225	371	3.13
		211	358	3.22
8	PI 383693	203	356	3.33
		201	365	3.45
		190	334	3.35
9	PI 464800	199	351	3.34
		201	366	3.46
10	PI 464801	213	372	3.32
		197	343	3.31
		203	336	3.15
11	PI 464802	207	347	3.18
		178	324	3.46
		153	243	3.02
12	PI 464803	212	416	3.72
13	PI 464804	206	365	3.37
		210	372	3.37
14	PI 464805	188	305	3.08
15	PI 464806	198	339	3.26
16	PI 464807	167	271	3.08
		254	409	3.06
		196	351	3.40
17	PI 464808	226	385	3.23
18	PI 464809	212	358	3.20
		230	373	3.08
		228	366	3.05
19	PI 464810	225	356	3.01
20	PI 464811	191	303	3.01
		248	396	3.04
		235	376	3.05
21	PI 464812	221	366	3.15
		218	353	3.08
		193	297	2.92
22	PI 464813	184	341	3.52
		177	325	3.49
23	PI 464814	195	512	4.99
		192	323	3.20
24	PI 577511	202	351	3.30
		195	322	3.14
25	PI 577512	229	405	3.35
		202	331	3.12
		204	353	3.29
	Mean	212	370	3.25
	Std	21	43	0.29

lead the conclusion that an accession could be classified as hybrid subsp. ×*hemicycla*. The purple flowered accessions could be classified as hybrid subsp. ×*hemicycla* if the pods have fewer than 1.5 coils. Since tetraploid taxa are analogous to the diploid taxa, it is highly likely that a similar morphological relationship is present among tetraploids. Based on this analogy, five out of 20 accessions (PI206282, PI 238151, PI 464801, PI 464804, and PI 464807) are above 1.5 coils and could be reclassified as subsp. *sativa*. However, molecular confirmation of the analogy must be obtained before deriving a definite conclusion and such a confirmation effort with molecular tools could be an interesting research venue.

The current germplasm collections gathered and maintained by USDA provides an excellent source to explore *Medicago sativa* species complex via studying different genomic aspects of the complex. However, the evaluation of accessions based on morphology and genomics is the prerequisite for effective usage in plant breeding. Flow cytometry has been an effective tool employed in estimation of genome size and determination of the ploidy levels in plant populations.

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