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Clarifying the ploidy of some accessions in the USDA alfalfa germplasm collection

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Abstract: Cultivated alfalfa was selected from a complex taxonomic group called the *Medicago sativa-falcata* complex. The complex includes a number of diploid (2n = 2x = 16) and tetraploid (2n = 4x = 32) taxa. Prior information of ploidy is vital for the effective utilisation of genetic resources in alfalfa breeding programs. From the US National Plant Germplasm System, we obtained the seeds of 256 wild accessions thought to be from the 3 diploid subspecies included in the *M. sativa-falcata* complex, in order to determine their ploidy level using flow cytometry: *M. sativa* subsp. *caerulea*, *M. sativa* subsp. *falcata*, and *M. sativa* subsp. *hemicycla*. A majority of the accessions classified as subsp. *hemicycla* were found to be either tetraploid (27 out of 32) or a mixture of diploid and tetraploid (2 out of 32), suggesting that they ought to be reclassified as subsp. *varia*. Only 3 accessions consisted of purely diploid genotypes. Out of 71 accessions classified as subsp. *caerulea*, 7 were found to be tetraploid and 8 accessions were admixed. Of the 153 accessions of subsp. *falcata*, 67 were diploid, 82 were tetraploid, and 4 accessions showed within-accession ploidy-level variation. We recommend reclassifying the tetraploid accessions to reflect the ploidy levels detected in this study.

Key words: Alfalfa germplasm, ploidy, *M. Sativa-falcata* complex

ABD Tarım Bakanlığı Bitki Genetik Kaynaklar Sistemindeki bazı yonca aksesyonlarının ploidi düzeyinin tespiti

Özet: Kültürü yapılan yonca, karmaşık bir taksonomik gurup olan *Medicago sativa* tür kompleksi ya da *Medicago sativa-falcata* kompleksi olarak bilinen bir birimden ıslah yolu ile geliştirilmiştir. Kompleks aralarında tozlaşma engeli bulunmayan diploid (2n = 2x = 16) ve tetraploid (2n = 4x = 32) alttürlerden oluşmaktadır. Ploidi düzeyinin bilinmesi genetik kaynakların etkin kullanımı için hayati öneme haizdir. Bu çalışmada tohumları Amerika Birleşik Devletleri Ulusal Bitki Genetik Kaynaklar Sistemi kapsamında depo edilen ve *M. sativa-falcata* kompleksinin diploid alttürleri *M. sativa* subsp. *caerulea*, *M. sativa* subsp. *falcata* ve *M. sativa* subsp. *hemicycla* olarak geçici olarak sınıflandırılan 256 aksesyonun ploidi düzeyleri flov sitometri metodu kullanılarak tesbit edilmiştir. Subsp. *hemicycla* alttürü olarak sınıflandırılan 32 aksesyonun büyük çoğunluğunun ya tetraploid olduğu (27 aksesyon) ya da diploid-tetraploid karışımı (2 aksesyon) olduğu ve bunların subsp. *varia* olarak yeniden sınıflandırılmaları gerektiği tespit edildi. Subsp. *caerulea* olarak sınıflandırılan aksesyonlardan 71 aksesyonun ploidi düzeyi tespit edildi. Bunlardan 7 aksesyonun tetraploid olduğu ve 8 aksesyonunda tetraploid-diploid karışımı olduğu gözlemlendi. Subsp. *falcata* olarak sınıflandırılan ve ploidi düzeyini ölçtüğümüz 153 aksesyondan 67 tanesinin diploid, 82 tanesinin tetraploid ve 4 tanesinin aksesyon içi ploidi düzeyi çeşitliliğine sahip olduğu tespit edildi. Bu bulgular dikkate alındığında tetraploid aksesyonların yeniden sınıflandırılması gerekmektedir.

Anahtar sözcükler: Yonca genetik kaynakları, *M. sativa-falcata* kompleksi, ploidi

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Introduction

Ploidy is an important aspect of plant genomes and a majority of plant species are proposed to have undergone polyploidisation in their history (Grant, 1971). About 80% of all angiosperms are polyploids (Masterson, 1994), including the majority of economically important crops such as alfalfa, wheat, maize, sugar cane, potato, coffee, and cotton (Leitch & Bennett, 1997). Allopolyploidy is 1 of the 2 main types of polyploidy, resulting from interspecies hybridisation followed by chromosome doubling via unreduced gametes to stabilise the hybrid genome. In contrast, autopolyploidy refers to the doubling (or higher order increase) of the chromosome complement within a species (Grant, 1971; De Laat et al., 1987). Polyploidy is important for certain crop species because desirable agronomic and horticultural traits such as size and vigour are expressed in polyploids more often than in their diploid counterparts (Elliot, 1958).

Cultivated alfalfa, *Medicago sativa* L., is an autotetraploid (Stanford, 1951) derived from the *Medicago sativa-falcata* complex, which includes a number of species and subspecies that share the same karyotype (Quiros & Bauchan, 1988). The taxa included in the complex are differentiated based on morphology (mainly flower colour, pod shape, and pollen morphology) and ploidy. Only 2 ploidy levels are detected in the complex, diploid (2n = 2x = 16) and tetraploid (2n = 4x = 32) (Lesins & Lesins, 1979; McCoy & Bingham, 1988). The species or subspecies status of the taxa included in the complex was once considered to be contentious (Sinskaya, 1950; Lesins & Lesins, 1979; Ivanov & Brezhnev, 1988), but recently all of the taxa have been given subspecific status within the *M. sativa-falcata* complex (Quiros & Bauchan, 1988), a nomenclature that has been widely adopted (Şakiroğlu et al., 2010).

Among the subspecies included in the complex, *M. sativa* subsp. *falcata* has yellow flowers and sickle-shaped pods. Its distribution ranges from Germany to eastern Siberia and from the Black Sea to northern Russia. It is very well adapted to cold regions and thought to contribute winter hardiness to many modern cultivars (Quiros & Bauchan, 1988; Muller et al., 2006). Subsp. *falcata* also has the potential to increase yield in alfalfa breeding efforts (Riday et al., 2002; Riday & Brummer, 2002a, 2002b, 2004). In nature, subsp. *falcata* exists both in diploid and tetraploid levels.

The 2 purple-flowered subspecies in the complex that have coiled pods are different from each other based solely on ploidy level. The diploid subspecies is known as *M. sativa* subsp. *caerulea* and the tetraploid as *M. sativa* subsp. *sativa*. The purple-flowered species are adapted to temperate regions that include the Near and Middle East, the Mediterranean region, and Central and South Asia. The natural diploid hybrid between diploid *M. sativa* subsp. *falcata* and *M. sativa* subsp. *caerulea* has variegated flower colour along with intermediate pod coiling and is named *M. sativa* subsp. *hemicycla*. The tetraploid natural hybrid of *M. sativa* subsp. *falcata* and *M. sativa* subsp. *sativa* is *M. sativa* subsp. *varia*, which possesses a morphology similar to that of subsp. *hemicycla*. Hybridisation among taxa is common and gene flow between different ploidy levels is possible as a result of unreduced gametes (McCoy & Bingham, 1988).

Germplasm collections are vital to plant breeding programs since they can be used to enhance breeding material and aid in the selection of appropriate germplasm for genetic studies and conservation efforts (Lincy & Sasikumar, 2010; Sahai et al., 2010). The US Department of Agriculture's Germplasm Resources Information Network (USDA-GRIN) maintains 1 of the largest alfalfa germplasm collections in the world, including more than 4000 accessions collected throughout the natural distribution range of alfalfa and its close relatives. Although the majority of alfalfa cultivars are released at the tetraploid level, diploid cultivars have been developed, particularly for subsp. *falcata*. The gene flow across ploidy levels has been documented (Bingham & Sounders, 1974) and is more common from diploid to tetraploid. Thus, diploid genetic variation should be able to be transferred to tetraploid germplasm (McCoy, 1982; Veronesi et al., 1986). Moreover, in order to avoid complexities arising from tetrasomic inheritance, diploids are preferred in genetic studies, particularly since diploid and tetraploid genetic maps are highly syntenic (Kaló et al., 2000).

A prerequisite for using germplasm in taxonomic, genetic, or breeding experiments is the determination of its ploidy level. Ploidy determination in plants has conventionally been conducted by chromosome counting of stained root tips using microscopy, but this method is laborious and occasionally misleading (Brummer et al., 1999; Tuna et al., 2001). Flow cytometry has been offered as an alternative tool for plant breeders to determine ploidy level and has been shown to be a robust method for determining the ploidy level of *M. sativa* accessions (Brummer et al. 1999). As the first comprehensive attempt to determine the ploidy level of a wide range of alfalfa accession, Brummer et al. (1999) used both the chromosome counting method and flow cytometry to evaluate the ploidy level of 283 accessions, mostly from subsp. *falcata*, that are maintained by the USDA-GRIN. This figure corresponds to less than 1% of the entire collection, however, and a majority of accessions still lack information on ploidy level. For this reason, the classification in the Germplasm Resources Information Network (GRIN) may be incorrect. In this study, we used flow cytometry to investigate ploidy levels of accessions putatively classified as 1 of 3 subspecies, subsp. *falcata*, subsp. *caerulea*, and subsp. *hemicycla*.

Materials and methods

Plant materials

We used 256 accessions from the USDA National Plant Germplasm System identified in GRIN that were originally collected from across the natural distribution of the 3 diploid subspecies of *M. sativa*: subsp. *falcata*, subsp. *caerulea*, and subsp. *hemicycla*. We sampled subsp. *falcata* extensively, since it includes both diploid and tetraploid accessions (total of 153 accessions). We tested 71 and 32 accessions from subsp. *caerulea* and subsp. *hemicycla*, respectively. Because flower colour is the main character used to distinguish among the current taxa, we recorded this information from each of the genotypes under greenhouse conditions in order to further verify their classification.

Sample preparation

Alfalfa seedlings were grown in greenhouses at Iowa State University and the University of Georgia for about 4 weeks. Young leaves were harvested from the freshly picked true leaves of each plant. An equal amount of leaf tissue from 4 individual genotypes from each accession was combined into 1 sample of 50 mg. If variation in ploidy level was detected in the pooled sample, a new sample of 50 mg of leaf tissue was prepared from each of the individual genotypes and run independently.

We followed the procedure explained by Galbraith et al. (1983) for the flow cytometry. In order to recover nuclei, the leaf material was chopped with a one-sided sharp razor blade in a petri dish containing chopping buffer prepared according to the method of Galbraith et al. (1983). 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 min), the supernatant was discarded, and the pellet was resuspended in 100 µL of a propidium iodide (PI) staining solution at a concentration of 100 µg/mL. All of the sample preparation procedures were performed in darkness on ice and the analyses were performed within a couple of hours of preparation.

The samples were analysed on a Cytomics FC 500 (Beckman-Coulter, Fullerton, CA) flow cytometer at the University of Georgia's Flow Cytometry Facility and on an EPICS ALTRA (Beckman-Coulter, Fullerton, CA) flow cytometer at the Iowa State University Flow Cytometry Facility with a wavelength of 488 nm. Mean DNA content was based on the analysis of 10,000 nuclei. Diploid (PI440531) and tetraploid (PI499550) plants of known ploidy (Brummer et al., 1999) were selected as diploid and tetraploid standards and genome sizes of all samples were compared to the 2 standards.

Results and discussion

The USDA-GRIN system has adopted nomenclature from Quiros and Bauchan (1988) for taxa included in the *M. sativa-falcata* complex, and we used USDA-GRIN nomenclature to assign accessions into subspecies. Out of 256 accessions analysed, 71 accessions were classified as subsp. *caerulea* in the USDA-GRIN system, 153 accessions as subsp. *falcata*, and 32 as subsp. *hemicycla*.

We sampled all of the *hemicycla* accessions from the USDA collections available at the time. Out of the 32 accessions evaluated, only 3 accessions were found to be completely diploid (PI634111, PI641615, and PI641619). The flower colour of accessions PI641615 and PI641619 were purple; however, genomic composition based on molecular markers confirmed that they indeed belonged to subsp. *hemicycla* (Şakiroğlu et al., 2010). Two of the accessions classified as subsp. *hemicycla* (PI634128, PI641593) were found to consist of diploid and tetraploid individuals. Variation in the ploidy level of alfalfa has been previously reported within accessions (Brummer et al., 1999). The rest of the 27 accessions were found to be tetraploid. The entire set of USDA-GRIN subsp. *hemicycla* accessions was gathered from Kazakhstan, and ploidy levels were determined using chromosome counting from the stained root tips (S. Greene, personal communication). Due to the extensive deviation in the ploidy level of subsp. *hemicycla* accessions from the GRIN report, we measured the ploidy levels of these accessions twice at each of the 2 different institutions involved in this study; our results were consistent between the 2 locations. Chromosome counting from the stained root tips was noted to be occasionally erroneous for determining the ploidy level of alfalfa accessions (Brummer et al., 1999). Therefore, provided that the morphological traits agree with hybrid expectations, we propose that these 27 accessions should be reclassified as subsp. *varia*.

In general, flower colour alone is not sufficient enough to accurately assign accessions to subspecies at a given ploidy level. We noted, based on genome composition analyses, that the hybrid subspecies *hemicycla* could also express yellow and purple flowers in addition to the expected variegated flower colour (Şakiroğlu et al., 2010). When flower colour is considered along with pod shape data, however, it is possible to accurately assign populations into subspecies (Şakiroğlu et al., 2010). Yellow-flowered accessions with pods having fewer than one coil could be denoted as subsp. *falcata*, whereas those featuring yellow flowers and pods with one coil or more are in fact subsp. *hemicycla*. Similarly, subsp. *caerulea* accessions have pods with more than 1.5 coils. If subsp. *hemicycla* accessions have purple flowers, they tend to have pods with fewer than 1.5 coils (Şakiroğlu et al., 2010).

Since subsp. *caerulea* is known as the diploid version of subsp. *sativa*, all of the accessions are expected to be diploid; any tetraploid accession in this group is considered as a misidentification and ought to be reclassified as subsp. *sativa*, provided that it has purple flowers and coiled pods. We evaluated the ploidy levels of 72 out of 88 available subsp. *caerulea* accessions, a figure that corresponds to more than 80% of the entire USDA subsp. *caerulea* collection. We found that 7 subsp. *caerulea* accessions (PI312458, PI440512, PI464711, PI631954, PI634122, PI634126, and PI634178) were tetraploid; they should be reclassified as subsp. *sativa*. Within-accession variation in ploidy level was detected in 8 accessions (PI206453, PI315465, PI325318, PI502435, PI502436, PI502437, PI577539, and PI577541). Among the diploid accessions, some had deviations in flower colour. This implies that in addition to ploidy level, flower colour should also be evaluated in order to ensure a more accurate assignment of accessions to subspecies. The Afghani accession PI222198 was classified as subsp. *caerulea* in the USDA-GRIN system; however, it was found to have yellow flowers and should thus be reclassified as subsp. *falcata*. The Russian accession PI315460 and Kazakh accession PI641603 had within-accession variation of flower colour. Each accession had individuals with variegated flower colour in addition to individuals with purple flowers. These 2 accessions were proposed to be reclassified as subsp. *hemicycla* based on genome composition deduced from SSR marker profiles (Şakiroğlu et al., 2010).

Subsp. *falcata* contains both diploid and tetraploid accessions and the ploidy level of the majority of accessions is not known. The USDA collections include over 400 accessions denoted as subsp. *falcata*; we screened 153 of those accessions. Our results found 67 accessions (approximately 44%) to be diploid, 82 (approximately 54%) to be tetraploid, and 4 to demonstrate within-accession ploidy-level variation (PI115365, PI440527, PI631571, and PI631708) (Table). It should be noted that these proportions do not reflect the true proportions of subsp. *falcata* germplasm at each ploidy level in the USDA collections, because in some cases, we selected questionable diploid accessions in order to confirm their status. The real proportion of diploid accessions denoted as subsp. *falcata* was reported to be around

Table. Information on the ploidy level, flower colour, and country of origin of the Medicago sativa accessions evaluated. Accessions are categorised based on their current GRIN assignments. Proposed reclassifications, based on ploidy or flower colour, are also identified.

Accession	Country of origin	Ploidy level	Flower colour[a]
	Medicago sativa subsp. *caerulea*		
PI179370	Turkey	16	P
PI210367	Iran	16	P
PI212798	Iran	16	P
PI222198[b]	Afghanistan	16	Y
PI243225	Iran	16	P
PI283640	Former Soviet Union	16	P
PI299045	Russian Federation	16	P
PI299046	Russian Federation	16	P
PI307395	Former Soviet Union	16	P
PI314267	Uzbekistan	16	P
PI314275	Uzbekistan	16	P
PI315460[c]	Russian Federation	16	P/V
PI315462	Russian Federation	16	P
PI315466	Russian Federation	16	P
PI325381	Russian Federation	16	ND
PI440500	Kazakhstan	16	P
PI440501	Kazakhstan	16	P
PI440502	Kazakhstan	16	P
PI440505	Kazakhstan	16	P
PI440507	Kazakhstan	16	P
PI440514	Kazakhstan	16	P
PI464712	Turkey	16	P
PI464713	Turkey	16	P
PI464714	Turkey	16	P
PI464715	Turkey	16	P
PI464717	Turkey	16	P
PI464718	Turkey	16	P
PI464719	Turkey	16	P
PI464720	Turkey	16	P
PI464721	Turkey	16	P
PI464722	Turkey	16	P
PI464723	Turkey	16	P
PI464724	Turkey	16	P
PI505871	Former Soviet Union	16	P
PI577542	Russian Federation	16	ND
PI577543	Georgia	16	P
PI577544	Russian Federation	16	ND
PI577545	Russian Federation	16	P
PI577546	Georgia	16	P
PI577547	Georgia	16	P
PI577548	Russian Federation	16	P

Table. (Continued).

Accession	Country of origin	Ploidy level	Flower colour[a]
PI577549	Georgia	16	P
PI577551	Canada	16	P
PI577552	Canada	16	ND
PI631921	Russian Federation	16	P
PI631922	Kazakhstan	16	P
PI631924	Armenia	16	P
PI631925	Kazakhstan	16	P
PI631926	Russian Federation	16	P
PI634119	Kazakhstan	16	ND
PI634136	Kazakhstan	16	P
PI634174	Kazakhstan	16	ND
PI634176	Kazakhstan	16	P
PI641380	Russian Federation	16	P
PI641601	Kazakhstan	16	P
PI641603[c]	Kazakhstan	16	P/V
PI641606	Kazakhstan	16	P
PI312458[d]	Russian Federation	32	ND
PI440512[d]	Kazakhstan	32	ND
PI464711[d]	Turkey	32	ND
PI631954[d]	Pakistan	32	ND
PI634122[d]	Kazakhstan	32	ND
PI634126[d]	Kazakhstan	32	ND
PI634178[d]	Kazakhstan	32	ND
PI206453[d]	Turkey	16/32	ND
PI315465	Russian Federation	16/32	ND
PI325318	Former Soviet Union	16/32	ND
PI502435	Kazakhstan	16/32	ND
PI502436	Kazakhstan	16/32	ND
PI502437	Russian Federation	16/32	P
PI577539	Turkey	16/32	ND
PI577541	Kazakhstan	16/32	P
		Medicago sativa subsp. *falcata*	
PI231731	Former Soviet Union	16	ND
PI234815	Switzerland	16	ND
PI251689	Former Soviet Union	16	ND
PI251690	Former Soviet Union	16	Y
PI251830	Austria	16	Y
PI258752	Russia	16	Y
PI262532	Israel	16	ND
PI263154	Russia	16	ND
PI315471	Russia	16	ND
PI315480	Russia	16	Y
PI325387	Russia	16	Y

Table. (Continued).

Accession	Country of origin	Ploidy level	Flower colour[a]
PI325396	Russia	16	Y
PI325399	Russia	16	Y
PI422560	Former Soviet Union	16	ND
PI464726[e]	Turkey	16	P
PI464727[c]	Turkey	16	P
PI464728	Turkey	16	Y
PI464729	Turkey	16	Y
PI486205	Russia	16	Y
PI486206	Russia	16	Y
PI486207	Russia	16	Y
PI494662	Romania	16	Y
PI494663	Romania	16	ND
PI502438	Russia	16	Y
PI502440	Russia	16	Y
PI502447	Russia	16	Y
PI502448	Russia	16	Y
PI502449	Former Soviet Union	16	Y
PI538987	Russia	16	Y
PI577555	Ukraine	16	Y
PI577556	Bulgaria	16	Y
PI577558	Russia	16	Y
PI631546	Russia	16	Y
PI631549	Russia	16	Y
PI631556	Russia	16	Y
PI631561	Switzerland	16	Y
PI631566	Bulgaria	16	Y
PI631568	Italy	16	Y
PI631577	Italy	16	Y
PI631650	Bulgaria	16	Y
PI631652	Russia	16	Y
PI631654	Russia	16	Y
PI631656	Russia	16	Y
PI631658	Russia	16	Y
PI631660	Russia	16	Y
PI631661	Russia	16	Y
PI631666	Russia	16	Y
PI631667	Russia	16	Y
PI631668	Russia	16	Y
PI631689	Bulgaria	16	Y
PI631691	Bulgaria	16	Y
PI631707	China	16	Y
PI631807	Russia	16	Y
PI631809	Russia	16	Y
PI631812	Russia	16	Y

Table. (Continued).

Accession	Country of origin	Ploidy level	Flower colour[a]
PI631814	Russia	16	P
PI631816	Russia	16	Y
PI631817	Russia	16	Y
PI631818	Russia	16	Y
PI631829	Russia	16	Y
PI631842	Sweden	16	ND
PI634034	Russia	16	Y
PI634106	Ukraine	16	Y
PI641387	Russia	16	ND
PI641394	Germany	16	ND
PI641543	Mongolia	16	Y
PI641544	Mongolia	16	Y
PI325408	Russia	32	ND
PI384506	Russia	32	ND
PI452460	Canada	32	ND
PI440536	Kazakhstan	32	ND
PI502444	Ukraine	32	ND
PI502451	Russia	32	ND
PI542800	Slovenia	32	ND
PI577564	Russia	32	Y
PI631543	Sweden	32	ND
PI631592	Italy	32	ND
PI631594	Greece	32	ND
PI631595	France	32	ND
PI631620	India	32	ND
PI631637	Mongolia	32	ND
PI631640	Mongolia	32	ND
PI631685	Mongolia	32	ND
PI631686	Mongolia	32	ND
PI631690	Bulgaria	32	ND
PI631704	China	32	ND
PI631794	France	32	ND
PI631837	Sweden	32	ND
PI631839	Sweden	32	ND
PI631843	Sweden	32	ND
PI631845	Sweden	32	ND
PI631849	Sweden	32	ND
PI631850	Sweden	32	ND
PI631855	Sweden	32	ND
PI631857	Sweden	32	ND
PI634027	Italy	32	ND
PI634029	Canada	32	ND
PI634031	Canada	32	ND
PI634032	Russia	32	ND
PI634090	Mongolia	32	ND
PI634112	Kazakhstan	32	ND

Table. (Continued).

Accession	Country of origin	Ploidy level	Flower colour[a]
PI634114	Kazakhstan	32	ND
PI634118	Kazakhstan	32	ND
PI634131	Kazakhstan	32	ND
PI634133	Kazakhstan	32	ND
PI634134	Kazakhstan	32	ND
PI634135	Kazakhstan	32	ND
PI634147	Kazakhstan	32	ND
PI634158	Kazakhstan	32	ND
PI634159	Kazakhstan	32	ND
PI634162	Kazakhstan	32	ND
PI634180	Kazakhstan	32	ND
PI634181	Kazakhstan	32	ND
PI634182	Kazakhstan	32	ND
PI634183	Kazakhstan	32	ND
PI641377	Russia	32	ND
PI641381	Russia	32	ND
PI641382	Russia	32	ND
PI641383	Russia	32	ND
PI641384	Russia	32	ND
PI641385	Russia	32	ND
PI641386	Russia	32	ND
PI641388	Russia	32	ND
PI641389	Russia	32	ND
PI641399	Kazakhstan	32	ND
PI641400	Russia	32	ND
PI641401	Russia	32	ND
PI641402	Russia	32	ND
PI641403	Russia	32	ND
PI641416	Russia	32	ND
PI641442	Russia	32	ND
PI641443	Russia	32	ND
PI641463	Russia	32	ND
PI641545	Mongolia	32	ND
PI641546	Mongolia	32	ND
PI641548	Mongolia	32	ND
PI641575	Kazakhstan	32	ND
PI641577	Kazakhstan	32	ND
PI641580	Kazakhstan	32	ND
PI641581	Kazakhstan	32	ND
PI641582	Kazakhstan	32	ND
PI641585	Kazakhstan	32	ND
PI641587	Kazakhstan	32	ND
PI641588	Kazakhstan	32	ND
PI641589	Kazakhstan	32	ND
PI641592	Kazakhstan	32	ND
PI641599	Kazakhstan	32	ND

Table. (Continued).

Accession	Country of origin	Ploidy level	Flower colour[a]
PI641613	Kazakhstan	32	ND
PI641620	Kazakhstan	32	ND
PI115365	Former Soviet Union	16/32	ND
PI440527	Russia	16/32	ND
PI631571	Bulgaria	16/32	Y
PI631708	China	16/32	ND
		Medicago sativa subsp. *hemicycla*	
PI634111	Kazakhstan	16	ND
PI641615	Kazakhstan	16	P
PI641619	Kazakhstan	16	P
PI634121[f]	Kazakhstan	32	ND
PI634123[f]	Kazakhstan	32	ND
PI634129[f]	Kazakhstan	32	ND
PI634130[f]	Kazakhstan	32	ND
PI634139[f]	Kazakhstan	32	ND
PI634146[f]	Kazakhstan	32	ND
PI634160[f]	Kazakhstan	32	ND
PI634163[f]	Kazakhstan	32	ND
PI634166[f]	Kazakhstan	32	ND
PI634170[f]	Kazakhstan	32	ND
PI634172[f]	Kazakhstan	32	ND
PI634175[f]	Kazakhstan	32	ND
PI634179[f]	Kazakhstan	32	ND
PI634184[f]	Kazakhstan	32	ND
PI634185[f]	Kazakhstan	32	ND
PI641571[f]	Kazakhstan	32	ND
PI641574[f]	Kazakhstan	32	ND
PI641576[f]	Kazakhstan	32	ND
PI641578[f]	Kazakhstan	32	ND
PI641579[f]	Kazakhstan	32	ND
PI641586[f]	Kazakhstan	32	ND
PI641590[f]	Kazakhstan	32	ND
PI641597[f]	Kazakhstan	32	ND
PI641602[f]	Kazakhstan	32	ND
PI641609[f]	Kazakhstan	32	ND
PI641612[f]	Kazakhstan	32	ND
PI641616[f]	Kazakhstan	32	ND
PI634128	Kazakhstan	16/32	Y/P/V

[a] P indicates purple flower colour, Y indicates yellow, V indicates variegated colour, and ND indicates that data on flower colour is not available for these accessions.
[b] Should be reclassified as M. sativa subsp. falcata.
[c] Should be reclassified as M. sativa subsp. hemicycla.
[d] Should be reclassified as M. sativa subsp. sativa.
[e] Should be reclassified as M. sativa subsp. caerulea.
[f] Should be reclassified as M. sativa subsp. varia.

32% (Brummer at al., 1999). Three accessions (PI464726, PI464727, and PI631814) initially assigned to subsp. *falcata* had purple flowers and should be reclassified accordingly. It was suggested that accession PI464726 be reclassified as subsp. *caerulea* while the other 2 accessions (PI464727 and PI631814) be reclassified as subsp. *hemicycla* based on genome composition (Şakiroğlu et al., 2010).

The large number of accessions maintained in the USDA-GRIN system provides an excellent base to enhance alfalfa breeding germplasm, since the collection has been gathered from the entire natural distribution range of alfalfa. However, the relationship among the taxa included in the *M. sativa-falcata* complex poses a challenge for the effective usage of genetic resources. The appropriate assignment of accession to subspecies and the characterisation of germplasm is required prior to the incorporation of wild and semiimproved material into the breeding programs. Flow cytometry is a robust method of determining the ploidy level of alfalfa accessions and it offers a fast, reliable, and inexpensive method for determining the ploidy level of all of the accessions maintained by USDA-GRIN.

All findings presented in this paper have been submitted to USDA-GRIN system.

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