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Study on the taxonomy status of alfalfa with cream flowers

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ABSTRACT

Alfalfa with cream flowers is differentiable and genetically stable; however, describing it in scientific research is difficult and ambiguous because of its taxonomical status. This research attempts to delimit the taxonomical status of 14 accessions, including *Medicago sativa* ssp. *sativa*, alfalfa with cream flowers, *Medicago sativa* ssp. *falcata* and *Medicago ruthinica* L., using morphology, chromosome karyotypes and inter simple sequence repeat (ISSR) markers. From morphology and the ISSR-PCR analysis, three accessions of *M. ruthenica* L. were clustered into an independent group, and the remaining 11 accessions were clustered into three groups apparently based on flower color. Alfalfa with cream flowers had its own remarkable chromosome karyotype. The genetic distance between alfalfa with cream flowers and *M. sativa* ssp. *sativa* was almost equal to that between *M. sativa* ssp. *falcata* and *M. sativa* ssp. *sativa*. In conclusion, alfalfa with cream flowers may be an independent population just like *M. sativa* ssp. *falcate*, representing a subspecies of *M. sativa* ssp. according to certain taxonomic principles.

Key words: Alfalfa with cream flower, Chromosome karyotype, ISSR marker, Morphology, Taxonomical status.

INTRODUCTION

Alfalfa is the most important and widely grown forage legume worldwide. One of the most desirable traits of alfalfa is its high nutritive quality as animal feed (Tanja *et al*., 2011). Alfalfa cultivars are synthetic populations formed from eight to 200 parents and thus, have a broad genetic base (Bernadette *et al.*, 2000; Flajoulot, 2005 ;). The subspecies composing the *M. sativa* complex, including subspecies *sativa* and *falcata* (2n = 4x = 32). *M. sativa* ssp. *sativa* are characterized by purple flowers and coiled pods (Quiros and Bauchan, 1988). Plants of the subspecies *falcata* are characterized by yellow flowers with straight to sickle-shaped pods. However, a large population with cream flowers observed in experimental and wild fields was commonly reported in early literature (Westgate, 1909; Lesins, 1956 ;). Charles *et al*. (1978) reported that cream-colored flowers had no taxonomic significance at the subspecies level, and they would best be handled as a form as if these phenotypes required a formal rank and name. However, Gakgroglu (2009) deemed that flower color should be used as a taxonomical classification index. Much research on alfalfa having cream flower was conducted (Yun, *et al*., 2004; Fengping Wu, 2008), but owing to the lack of rank and name, the descriptions of these populations were problematic. In my field experiments, alfalfa with cream flowers has its own significant differences. Alfalfa with cream flowers has always been cream flower from flowering to withering. Alfalfa with cream flowers was tentatively classifying according to the flower (Barnes, 1972). There was not still enough clear scientific evidence indicating how alfalfa with cream flowers was produced or where its origins.

The current taxonomic status of species and subspecies were addressed using morphological, karyotype and molecular data (Villalobos *et al.*, 2004). Taxonomy relies greatly on morphology to discriminate groups (Vincenzo *et al*., 2011). Morphological traits, such as flower color, pod shape and ploidy, have traditionally being used for taxonomic classifications (Touil, 2009; Gakgroglu, 2009). Modern experimental techniques in higher plant taxonomy are more accurate than orthodox taxonomy in determining classifications. The data used are not only based on morphological characteristics, but other additional evidence, such as chromosomal features, have also been taken into consideration, owing to the great contributions from the works of taxonomists (Suranto, 2002). As a rule, systematic and cytological approaches have led to conclusions that were in general agreement (Villalobos, 2004; Gömürgen, 2011; Feng Yu *et al.*, 2013). Molecular genetic markers represent one of the most powerful tools for the analysis of genomes and the association of heritable traits with underlying genetic variation (Jacqueline and Edwards, 2009). Molecular genetic markers like inter simple sequence repeat (ISSR)-PCR markers have been used extensively in determining relationships, delimitations and the biodiversity of alfalfa and other species (DeSalle *et al*., 2005; Maryam *et al*., 2013). In considering breeding purpose, it is very important to

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broaden the genetic background of the initial materials; therefore, it is favorable to involve wild or semi-wild accessions in this process. To design successful controlled crosses between these cultivated and semi-wild accessions, it is necessary to know their ploidy level (Falconer, 1983; Lapina *et al.*, 2011).

The purpose of this research was to 1) delimitate the taxonomical status of alfalfa with cream flowers; and 2) broaden the genetic background of initial breeding materials.

MATERIALS AND METHODS

Morphologic observations, chromosome karyotyping and ISSR-PCR marker analyses were carried out in 14 alfalfa accessions, including three alfalfa with cream flowers, denoted as No. 1, 2 and 3; three sickle alfalfa with yellow flowers, denoted as No. 4, 5 and 6; five alfalfa with purple flowers, denoted as No. 7, 8, 9, 10 and 11, and three *Ruthenia medic*, denoted as No. 12, 13 and 14.

Morphological evaluation

Ten plants of each accession were characterized at the morphological level according to the 'Descriptors and Data Standards for Medic (Medicago Linn.) (Lingqing Yu and Anling Gu, 2010)' and 'Descriptors and Data Standard for Medic (*Medicago ruthenica* (Linn) Trautv.)' (Hongyan Li and Zongli Wang, 2007). The 20 closest indices to taxonomical classification, including stem (form), leaf, flower color, flag petal, ala petal, keel petal, sepal (length) and pod (shape), were characterized. The morphological characteristics were analyzed using the SPSS 19.0 software. Phonological dendrograms were constructed based on Euclidean distance coefficients. Photomicrographs of flowers, including flag, ala and keel petals, were taken using the software ARTCAM-900MI-C from the Olympus microscope SZ.

Chromosome karyotype evaluation

Seeds were germinated on filter paper in Petri dishes at 25°C. Root tips were obtained 60 h after germination was initiated, then pretreated in an ice bath for 24 h, and fixed in Carnoy's Fluid (3:1 v/v, 95% ethanol:glacial acetic acid) for 24 h. The dissociation of root tips was carried out in 1 mol/L HCl at 60°C for 8 min after fixing in Carnoy's Fluid and then, they were stained in carbolic acid and magenta staining solution for at least 30 min. A single fixed root tip was placed in a drop of 45% acetic acid for 2–3 min on a microscope slide. The dissection of dividing root tip cells was accomplished using sharpened 0.5 cm needles. Cells were gently warmed and squashed under a cover slip. The slides were dried on a hot plate at 55–60°C for 10–12 min. Twenty cells containing well-spread C-banded chromosomes were observed clearly from the Olympus microscope BX41. Photomicrographs were taken using the software ARTCAM-900MI-C with measurements. The ideograms of the chromosomes in five selected photomicrographs were based on lengths, centromere positions and banding patterns.

ISSR-PCR marker evaluation

DNA extraction: Total genomic DNA was extracted from the young leaves of 14 plant materials using a modified Cetrimonium Bromide (CTAB) method (Doyle and Doyle, 1989). The quality and quantity of extracted DNA were examined using 1% agarose gel. The PCR was carried out according to Table 1.

Primers and ISSR-PCR assays: Fourteen primers were screened for their ability to detect the genetic relationships among the 14 plant materials. Template DNA was initially denatured at 94°C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 30 seconds at 94°C, primer annealing for 30 sec at a temperature based on the primer composition (50°C or 52°C) and primer extension for 30 sec at 72°C. The sequences of the primers used in ISSR-PCR are listed in Table 2A final incubation for 10 min at 72°C was performed to ensure that the primer extension reaction preceded to completion. The ISSR products were separated by electrophoresis on a 1% agarose gels using 1× TBE (5×Tris Boracic acid) buffer for 45 min. Photo documentation was performed under UV light using a photo imaging system.

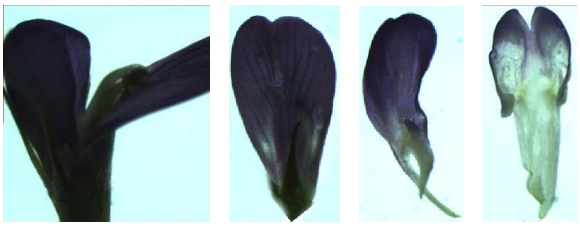
Data scoring and statistical analysis: The number of bands generated by different primers was scored and the frequency of the polymorphisms was calculated. ISSR bands were treated as binary characters and coded accordingly (presence = 1, absence = 0). Pop gene software (version 1.31) was used for the correlation analysis. A cluster analysis based on the unweighted pair group method with arithmetic average (UPGMA) using a sequential agglomerative hierarchical nested (SAHN) cluster analysis was performed with NTSYSpc software.

RESULTS AND DISCUSSION

Morphological evaluation: Fig 1 and Fig 2 present hierarchical clustering based on the morphological characteristics of the 14 accessions. At a rescaled distance of 14, the accessions separated clearly into four groups. The three accessions of *M. ruthenica* L. were clustered in an independent group. The remaining 11 accessions clustered into three groups based on flower color. In fact, alfalfa of different colors (purple, cream and yellow) each had their own linked internal and external characteristics. Alfalfa with cream flowers and alfalfa with purple flowers also had a

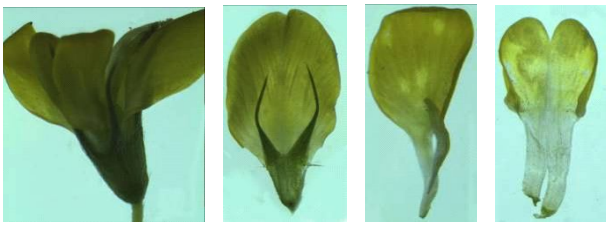
Table 1: Optimized ISSR-PCR reaction

To provide (µl)	Sample content
6.5	Double distilled water
10	Mix[0.1 U Taq Polymerase/µl, 500 µM dNTP, 20 mM Tris-HCl (pH 8.3), 100 Mm KCl,3 mM $MgCl_2$]
1.5	DNA (10 ng ng/µl)
2	Primer (10 µmol)
20	total

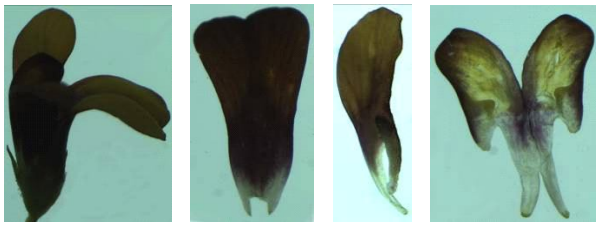


a. *Medicago sativa* ssp. *sativa*

b. Alfalfa with cream flower



c. *Medicago sativa* ssp. *falcata*



d. *Medicago ruthenica* L.

Fig 1: Photomicrographs of flowers, including flag, ala and keel petals

closer relationship compared with alfalfa having yellow flowers. Meanwhile, alfalfa with yellow flowers had a much closer relationship with *M. ruthenica* L. than the others (Table 3).

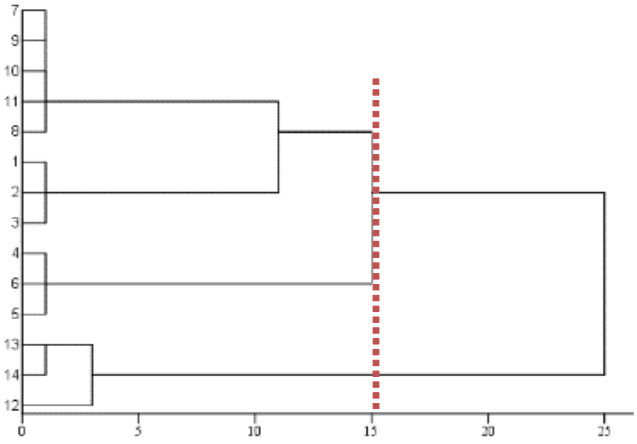


Fig 2: Hierarchical clustering of the 14 *Medicago sativa* genotypes based on morphological characteristics.

Chromosome karyotype evaluation: *M. sativa* ssp. *sativa*, which is 2n = 4x = 32, is auto-tetraploid and the chromosomes lengthened gradually. The karyotype was the 2A type due to the ratio of the longest to shortest chromosome (1.647) and the asymmetric coefficient (59.16%) with the formula 2n = 4x = 24m + 8sm. There were six median region chromosomes, Nos. 1, 2, 3, 4, 5 and 6, and the others were submedian region chromosomes, including Nos. 7 and 8 (Table 4, Fig. 4).

M. sativa ssp. (with cream flowers), which is 2n = 4x = 32, is auto-tetraploid, and the chromosomal lengths were gradually-varied. The karyotype was the 1A type due to the ratio of the longest to the shortest chromosome (1.778) and the asymmetric coefficient (59.4%) with the formula 2n = 4x = 24m + 8sm (SAT). There were six median region chromosomes, Nos. 1, 2, 4, 5 and 6, and the others were submedian region chromosomes, including Nos. 7, with a satellite, and 8 (Table 5, Fig. 5).

M. sativa ssp. *falcata*, which is 2n = 4x = 32, is auto-tetraploid, and the chromosomal lengths were gradually-varied. The karyotype was the 2A type due to the ratio of the longest to the shortest chromosome (1.44) and asymmetric coefficient (60.09%) with the formula 2n = 4x = 24m + 6sm + 2T (SAT). There were six median region chromosomes, Nos. 1, 2, 3, 4, 5 and 6. The No. 7 chromosome was a submedian region chromosome. Half of the No. 8

Table 2: ISSR primer sequences and their melting temperatures

Primer	Sequence 5’ - 3’	Melting temp (°C)	Primer	Sequence 5’-3’	Melting temp (°C)
3	ACACACACACACACACTC	52	UBC825	ACACACACACACACACT	52
13	AGAGAGAGAGAGAGAGGC	52	UBC823	TCTCTCTCTCTCTCTCC	50
15	AGAGAGAGAGAGAGAGCA	52	UBC827	ACACACACACACACACG	52
UBC835	AGAGAGAGAGAGAGAGYC	52	UBC844	CTCTCTCTCTCTCTCTRC	52
UBC860	TGTGTGTGTGTG GTGRA	52	UBC846	CTCTCTCTCTCTCTCTRC	52
UBC810	GAGAGAGAGAGAGAGAT	52	UBC848	CACACACACACACACARG	52
UBC812	GAGAGAGAGAGAGAGAA	52	UBC857	ACACACACACACACACYG	52

Table 3: Phenotypic characterization of alfalfa with purple, cream and yellow flowers (mm)

Indices and Traits			*Medicago sativa* ssp. *sativa*	Cream	*Medicago sativa* ssp. *falcata*	*Medicago ruthenica* L.
Stem		Form	Erect	Erect	oblique	Prostrate
Leaf		Length	29.070	18.868	13.883	9.469
		Width	10.449	4.919	5.951	4.237
		Length / Width	2.825	3.863	2.370	2.252
		Color	Yellow green	Grey green	Dark green	Dark green
Flower	Flag petal	Length	10.280	9.533	8.273	6.830
		Width	5.357	4.342	4.317	3.160
		Length / Width	1.926	2.207	1.924	2.161
		Shape	Wide eggshape	Long elliptic	Oblong	Spoonform of oblong
		Tip shape	Truncate	Convex	Retuse	Sinus
	Ala petal	Length	8.100	7.650	6.280	5.370
		Width	2.690	2.680	2.590	1.880
		Length / Width	3.011	2.852	2.425	2.856
		Shape	Oblong	Long drepaniform	Long drepaniform	Oblong
	keel petal	Length	7.080	7.450	6.290	5.330
		Width	3.050	2.363	1.780	3.400
		Length / Width	2.321	3.191	3.534	1.568
	Color		Purple	Cream	Yellow	Yellow with purple stripes
Sepal		Length	6.697	4.978	6.397	2.82
Pod		Form	2–4coils	1–1.5 coils	Drepaniform	Ovate

Table 4: Chromosomal parameters of alfalfa (purple flowers)

Length of chromosome (LA+SA=Total)/μm	Relative length (%)	Arm ratio	Centromere Index	Type
1.03+0.93=1.95	2.928	1.109	47.416	m
1.05+0.85=1.90	2.853	1.238	44.697	m
1.30+1.00=2.30	3.453	1.302	43.453	m
1.18+0.88=2.05	3.078	1.344	42.669	m
1.23+0.85=2.08	3.078	1.344	42.669	m
1.33+0.88=2.20	3.303	1.511	39.843	m
1.28+0.70=1.98	2.965	1.826	35.450	sm
1.48+0.73=2.20	3.303	2.028	33.036	sm

Note: m, Median region; sm, Submedian region

Table 5: Chromosomal parameters of “alfalfa” with cream flowers

Length of chromosome (LA+SA=Total)/μm	Relative length (%)	Arm ratio	Centromere Index	Type
1.38+1.35=2.73	3.225	1.021	49.500	m
1.55+1.28=2.83	3.343	1.213	45.199	m
1.53+1.15=2.68	3.166	1.328	42.958	m
1.68+1.18=2.85	3.373	1.425	41.245	m
1.65+1.05=2.70	3.195	1.573	38.882	m
1.58+0.95=2.53	2.988	1.659	37.608	m
1.48+0.80=2.28	2.692	1.846	35.141	sm*
1.65+0.85=2.50	2.959	1.944	33.983	sm

chromosome with a satellite was a submedian region chromosome, and the other was a terminal point chromosome, named No. 9 for convenience (Table 6, Fig. 6).

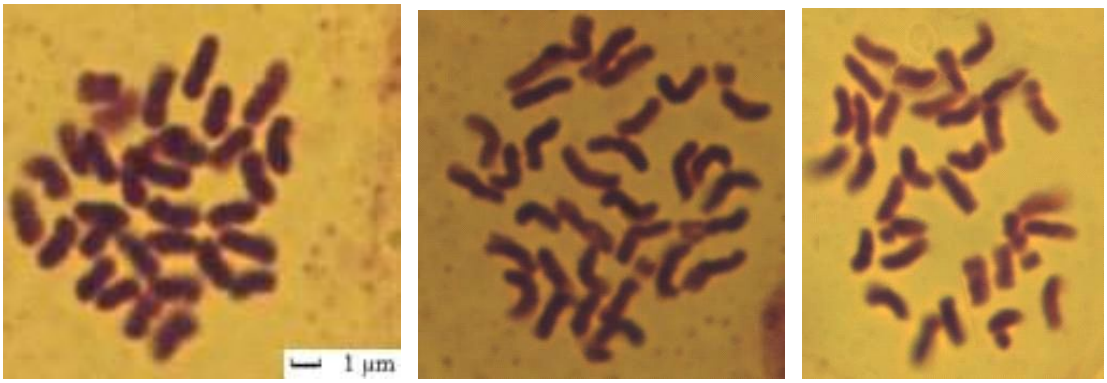
ISSR-PCR marker evaluation

A cluster analysis (UPGMA NTSYS) based on classifications of genetic distances from 0.84 was divided into four groups (Fig. 3). The first group of alfalfa with the cream flowers consisted of No. 1, 2 and 3 genotypes, and the average similarity was 0.88. The second group of *M. sativa* (purple flower) consisted of No. 7, 8, 9, 10 and 11 genotypes, and the average similarity coefficient was 0.87. The third group of *M. sativa* ssp. *falcata* (yellow flower) consisted of No. 4, 5 and 6 genotypes, and the average similarity coefficient was 0.85. The last group consisted of genotypes No. 4, 5 and 6 (*Medicago ruthenica* L.) as the control, and the average similarity coefficient was 0.90 (Fig. 7).

The similarity coefficient between the first group of alfalfa with cream flowers (0.9085) and the second group of *M. sativa* ssp. *sativa* (purple flower) was almost equal to that of the third group of *M. sativa* ssp. *falcata* (yellow

Table 6: Chromosomal parameters of sickle alfalfa (Yellow Flower)

Length of Chromosome (LA+SA=Total)/μm	Relative Length (%)	Arm Ratio	Centromere Index	Type
1.18+1.10=2.28	3.232	1.069	48.361	m
1.15+1.00=2.15	3.054	1.148	46.569	m
1.40+1.05=2.45	3.480	1.332	42.893	m
1.23+0.85=2.08	2.947	1.441	40.981	m
1.38+0.90=2.28	3.232	1.528	39.565	m
1.33+0.83=2.15	3.054	1.605	38.404	m
1.40+0.68=2.08	2.947	2.071	32.647	sm
1.70+0=1.70	2.415	“	-	T*
1.30+0.50=1.80	2.557	2.600	27.778	sm*



Medicago sativa ssp. *Sativa* Alfalfa with cream flowers *Medicago sativa* ssp. *falcata*

Note: Chromosome karyotype evaluations did not involve *Medicago ruthenica* L. due to it being an explicit diploid. (Arrows indicate the SAT chromosomes.)

Fig 3: Microscopic views of chromosomes from *Medicago L.* having different flower colors

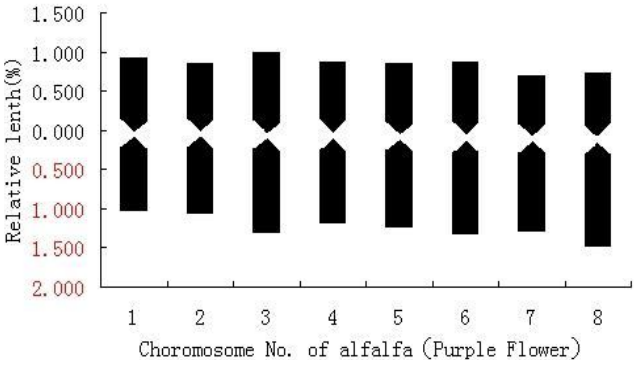


Fig 4: Chromosome mode of alfalfa (Purple Flowers)

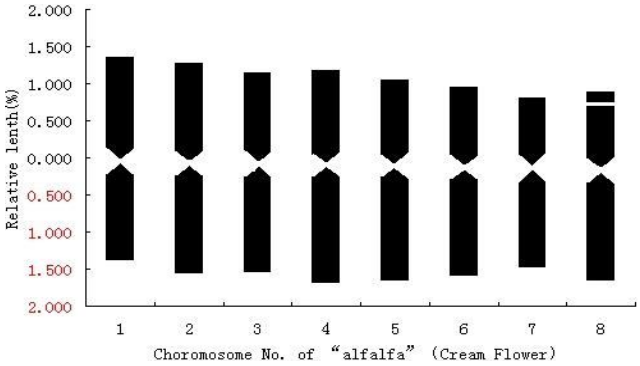


Fig 5: Chromosome mode of “alfalfa” with Cream flowers

flower) and the second group of alfalfa with purple flowers (0.9006). This indicated that they had almost the same relationships. For *M. ruthenica* L., the similarity coefficients of the first group of alfalfa with cream flowers, 0.5263, and the second group of alfalfa with purple flowers, 0.5731, also indicated a close relationship ; meanwhile, the third group of alfalfa with yellow flowers had a much closer relationship (0.6065) (Table 7).

The classification of taxa in the *M. sativa* complex as species or subspecies has been controversial (Sinskaya, 1961; Quiros and Bauchan, 1988). According to Charles *et al*., (1978), Sinskaya was one of the first taxonomists who integrated legume characteristics and flower colors to recognize their taxonomic importance in the *M. sativa* complex. Gakgroglu (2009) also argued that flower color and pod shape should be primary characteristics used in taxonomic classification. Thus, alfalfa with the distinct trait of cream flowers ought to be a subspecies of *M. sativa* according to certain criteria of Chinese taxonomists (Flora of China, 1998). Many natural and well-defined genera and families were established by Linnaeus mainly using morphology (Wafaa, 2005), which plays an indisputable role in species taxonomy. Later, chromosome karyotype and molecular marker analyses were effectively used, independently or cooperatively, to delineate species in taxonomy for further accuracy (Sulieman *et al.*, 2013; Mehme *et al.*, 2014; AYSENIHAL *et al*., 2011). In the present study, using morphological evidence, alfalfa with cream flowers appeared to have a close relationship with *M. sativa* ssp. *sativa*. *M. sativa* ssp. *falcata* has a close relationship with *M. ruthenica* L., which our research confirmed. Our conclusions regarding the taxonomical status of alfalfa with cream flowers were consistent with those of Charles *et al.*, (1978) and the "Flora of China" (1998). However, there was no established criterion to differentiate species or subspecies even though the groups could be separated.

The evidence from chromosome karyotyping showed that *M. sativa* ssp. *falcata* had SAT chromosomes, which are a distinguishing trait that differs the subspecies from *M. sativa* ssp. *sativa* (with no SAT chromosomes). The result was consistent with those of Sulieman (2013) and Guixing Yan (2001). Chromosome karyotyping of alfalfa with cream flowers to the best of our knowledge had not been previously performed. The common trait of SAT chromosomes revealed by the karyotype analyses indicated

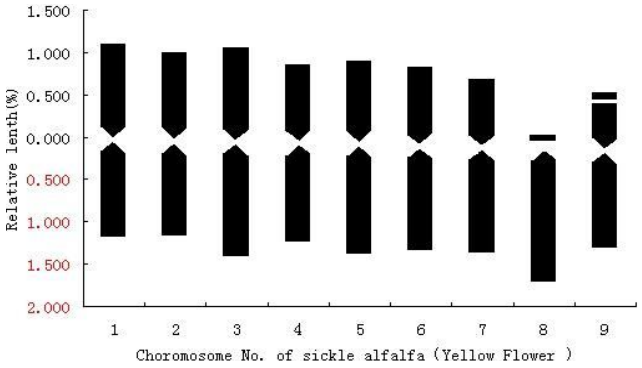


Fig 6: Chromosome mode of sickle alfalfa (Yellow Flower)

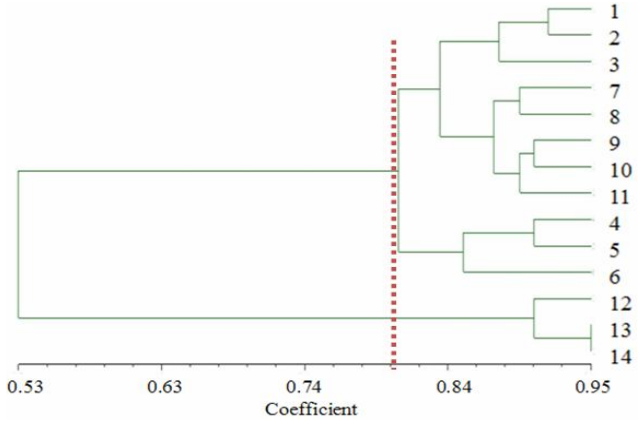


Fig 7: Dendrogram of the cluster analysis based on genetic distances by UPGMA

that alfalfa with cream flowers and *M. sativa* ssp. *falcata* were similar. However, morphologically, alfalfa with cream flowers was more similar to *M. sativa* ssp. *sativa* than *M. sativa* ssp. *falcata*.

The molecular results were similar to those of the morphological analysis in clustering the 14 alfalfa accessions. Many previous studies showed that clustering genotypes based on morphological traits was not consistent with DNA polymorphism analyses (Semagn, 2002; Tahernezhad *et al.*, 2010). Wafaa (2005) concluded that taxonomists should use all of the informative tools available and that morphological taxonomy should not be forgotten. In this study, research material selection relied first on flower colors (a morphological trait) and then, chromosome karyotyping and molecular biology were performed. Thus, morphology is still critical in taxonomy.

Table 7: Nei's original measures of genetic identity and genetic distance

	Cream	***Medicago sativa* ssp. *falcata***	***Medicago sativa* ssp. *sativa***	***Medicago ruthenica* L.**
Cream	***	0.8452	0.9085	0.5263
Medicago sativa ssp. *falcata*	0.1682	***	0.9006	0.6065
Medicago sativa ssp. *sativa*	0.0959	0.1046	***	0.5731
Medicago ruthenica L.	0.6418	0.500	0.5567	***

CONCLUSION

Morphology can explain misunderstandings or paradoxes, and thus plays a leading role in taxonomy. After all, morphology includes the most intuitive characteristics, which are not affected by artificial experimental factors or methods. To clarify morphological relationships, other modern methods can also be used. This analysis suggests, especially considering the significant morphological distinctness, that alfalfa with cream flowers should be a subspecies with a rank and scientific name in *Medicago* L. This would be beneficial to many important plant-associated areas, including germplasm descriptions, variety breeding and gene mining.

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