

ASSESSMENT OF VARIETAL DISTINCTNESS OF MONGOLIAN ALFALFA VARIETY ‘BURGALTAI’: SIMPLE-SEQUENCE REPEAT MARKERS AND MORPHOLOGICAL CHARACTERISTICS

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ABSTRACT. Alfalfa (*Medicago sativa* L.) is a perennial herbaceous plant in the family Fabaceae and the world's most widely cultivated nutritious forage. The ‘Burgaltai’ variety of alfalfa was created by selecting and hybridizing *Medicago sativa* L. and *Medicago falcata* L. in Mongolia's forest-steppe. Ensuring the identity of this variety is imperative since this variety is cold-tolerant and well-adapted in Mongolia. In this study, we observed morphological characteristics and analyzed varietal genetic similarity using simple-sequence repeat (SSR) markers to distinguish the ‘Burgaltai’ variety from other varieties. ‘Burgaltai’ variety had deep purple and yellow variegated flowers, which was a reliable and “descriptor” morphological characteristic. In this study, we applied eleven simple-sequence repeat (SSR) markers; 10 markers (90.9%) gave amplified products and exhibited polymorphism. Three SSRs (MTIC64, MTIC451, and MTIC471) were variety-specific and applicable to identify the ‘Burgaltai’ variety from others. PIC values of markers ranged from 0.32 to 0.86, with an average of 0.52, proving that these markers were appropriate for varietal identification and can be used in further genetic diversity analysis. Moreover, the study results showed that two Turkish varieties belonged to one cluster in the dendrogram, whereas the ‘Burgaltai’ variety was closer to two Russian varieties in another cluster for the genetic profile. This paper first reports on the molecular characterization of the Mongolian alfalfa plant by using SSR markers.

Keywords: SSR markers, genetic similarity, alfalfa, varietal identification

INTRODUCTION

Mongolia has 126.0 million hectares of pasture land, which makes up about 80% of the country's total area. With mean annual temperatures ranging from -6.2°C in the North to roughly +4°C in the Gobi Desert, Mongolia's climate is often colder than other nations at the same latitude due to its high altitude. Plant communities in Mongolia have evolved and adapted to sustained grazing pressure from wild and domesticated animals. [1]. Alfalfa is highly nutritious, especially for cattle, horses, and poultry. It contains protein, vitamins (A, C, E, and K), minerals (calcium, magnesium, potassium, and phosphorus), and fiber. Also, alfalfa's symbiotic relationship with soil nitrogen-fixing bacteria converts atmospheric nitrogen into a form that plants can use, enriching the soil with nitrogen. [2]. Additionally, the root of alfalfa can absorb water about 5 m deep, allowing plants to survive in arid land for long-term drought [3]. Researchers of the Research Institute of Animal Husbandry (RIAH) have created the ‘Burgaltai’ variety by selection and hybridization of native *Medicago falcata* L. and Russian *Medicago sativa* L. in Mongolia's forest-steppe zone and registered it as a variety due to its high forage production in 1988 [2]. This alfalfa variety was highly efficient in improving vegetation and producing hay [4]. Alfalfa seeds are imported from countries such as France, Germany, and Canada. Due to environmental conditions, the imported seeds produce a crop

once, and the yield deteriorates significantly in Mongolia the following year [5]. As the 'Burgaltai' variety has comparatively stronger cold tolerance [4], it became crucial to ensure its identification and distinguish it from other varieties. This study aims to determine the 'Burgaltai' variety using commonly applied methodologies, including morphological observation and SSR markers.

The conventional classification of alfalfa is based on morphological characteristics, including leaves, cotyledons, ploidy level, organ hairiness, and flowers. Flower colors and pod and leaf shapes are standard morphological features that are mainly employed for the identification of alfalfa varieties [6]. Several molecular markers have recently been applied for varietal identification, genetic diversity evaluation, and genetic mapping analysis. Molecular identification techniques can accurately differentiate between closely related plant varieties that may look similar morphologically. Using molecular markers enhances the possibility to study, conserve, and utilize plant genetic resources effectively [7]. Simultaneously, choosing a marker system that is appropriate for the identification of plant varieties is important. Several molecular markers have previously been applied to distinguish alfalfa varieties. For example, restriction fragment-length polymorphism (RFLP) markers [8] and amplified fragment-length polymorphism (AFLP) markers [9]. We were able to distinguish various alfalfa varieties.

Alfalfa is an outcrossing autotetraploid, and adequate co-dominant genetic markers are required for molecular genetic research. Simple sequence repeat (SSR) markers are highly polymorphic, abundant, co-dominant, and inheritable; the results are reproducible [10]. Therefore, these markers are feasible for constructing diploid and tetraploid alfalfa linkage maps. Diwan *et al.* [11] were the first to develop SSR markers in alfalfa and confirmed that SSRs can be a powerful tool for characterizing genetic relationships and diversity within alfalfa populations. Furthermore, a set of 107 SSRs identified in the EST database of *Medicago truncatula* was mapped in *M. sativa* [12]. Cholastova and Knotova confirmed that only three SSR markers were enough to characterize genetic variation and analyze the genetic relationships among *Medicago* genotypes [13]. The study results of Flajoulot *et al.* showed that seven SSRs were able to differentiate eight alfalfa cultivars [14]. In this study, eleven SSR markers present on the genomes of alfalfa were applied for varietal identification. The study findings may contribute to assessing the differences between populations, for variety distinction, or the management of genetic resources.

MATERIALS AND METHODS

Plant Material and Morphological Observation

Plant seeds were provided by RIAH (Table 1), and all experiments were carried out from Sept. of 2023 to November 2024 in RIAH. Forty plants from every variety were grown in approximately 200 g of soil in a pot, watered 3 times a week, and grown at 2500 lux light intensity for 16 hours and 8 hours of darkness at $21\pm2^{\circ}\text{C}$ under controlled conditions until flowers developed correctly in the growth room. The number of leaves and branching distance were measured at the flowering stage, and flower color was observed for each variety.

Table 1. Description of Alfalfa Varieties

№	Variety name	Country	Variety creator	Date
1	Tuyana	Russian Federation	Buryat State Agricultural Academy [16]	1995
2	Gozlu 1	The Republic of Turkey	General Directorate of Agricultural Industry of Turkey [17]	2012
3	Burgaltai	Mongolia	Research Institute of Animal Husbandry [5]	1988
4	Vega 87	Russian Federation	V.R. Williams All-Russian Fodder Research Institute [18]	1988 2025.12.27 12:33:00
5	Bilensoy 80	The Republic of Turkey	Field Crops Central Research Institute [19]	1984

SSR Markers

Eleven transferrable SSR primers [15] were employed to amplify the DNA of five alfalfa varieties. Each primer was amplified twice to ensure stability and reproducibility (Table 2).

Table 2. Nucleotide Sequence of Markers

Marker name	Sequence	
	5' - 3'	3' - 5'
MTIC48	TTTTTGTTAGTTTGATTTAGGTG	GCTACAAAGTCTTCTTCCACA
MTIC64	CCCGTTCTTTTATGTTGTGG	AACAAACACAATGGCATGGA
MTIC77	TCTTCATCGCTTTCTTCTATTTC	GCCGTATGGTGTTGTTGATG
MTIC93	AGCAGGATTTGGGACAGTTG	TACCGTAGCTCCCTTTTCCA
MTIC230	GTAAGCGCCTGCTTGGACT	GAGATTCTGCCAAAATGCAA
MTIC232	TAAGAAAGCAGGTCAGGATG	TCCACAAATGTCTAAAACCA
MTIC238	TTCTTCTTCTAGGAATTTGGAG	CCTTAGCCAAGCAAGTAAAA
MTIC248	TATCTCCCTTCTCCTTCTCC	GGATTGTGATGAAGAAATGG
MTIC441	CTTCCTTATCATCGCTTCC	CAGAGATTGAGAATCGAGAAG
MTIC451	GGACAAAATTGGAAGAAAAA	AATTACGTTTGTGTTGGATGC
MTIC471	ATCAGGTGATGATTGGTTTT	CCAACCATCTTTGTTTCCTA

Plant DNA Isolation and PCR Condition

Fresh leaves (~100 mg) of plants were collected to extract DNA according to the Hexadecyl trimethyl ammonium Bromide (CTAB) procedure [20]. Eleven transferrable SSR markers [12, 13] were employed to amplify DNA of five alfalfa varieties (Table 2). The polymerase chain reaction was performed in a total volume of 25 µL containing 12.5 µL of 2X Hotstart Taq Premix, 1 µL of each primer (forward and reverse), 9.5 µL distilled deionized water, and 1 µL sample DNA. Amplification reactions were performed in compliance with the guidelines suggested by Julier [12]. The cycling consisted of initial denaturation at 94°C for 4 min, denaturation at 94°C for 45 s, annealing at 58°C for 45 s, 1 min extension at 72°C, and a 7 min final extension at 72°C for 30 cycles. PCR products were electrophoresed and separated on 1.5% (w/v) ethidium bromide-stained agarose gel in 1X TAE buffer with 100 bp DNA ladder at 100 W constant power for 30 min. The amplified products were detected as bands on gel visualization using UV illuminator. Each SSR marker's amplified band size (measured in nucleotide base pairs) was established by analyzing its migration in relation to a molecular marker weight size.

RESULTS AND DISCUSSION

Given the large number of plant varieties created for numerous crop species and the fact that seeds are imported from other countries, identifying plant varieties and cultivars has been essential in the agricultural industry. The ‘Burgaltai’ variety is the first and only agronomically improved tetraploid alfalfa variety created by researchers of RIAH. Russian varieties (‘Vega 87’ and ‘Tuyana’) [22] RIAH researchers successfully introduced these varieties to Mongolia from 2014 to 2023. Phenological and agronomic characteristics were assessed extensively in field conditions, and the study results confirmed that these varieties had adapted in Mongolia. [18, 19]. Previously, ‘Gozlu 1’ was compared with ‘Burgaltai’ for salt and drought tolerance under in vitro conditions [3]. The Turkish alfalfa variety ‘Bilensoy 80’ was also applied as a comparative reference.

Morphological Characteristics

Alfalfa flower colors are strongly correlated with agronomic traits such as stress tolerance, yield, and nutritional quality [24]. Having multiple flower color variations of *M. varia* increases the potential resistance to biotic and abiotic stressors [25]. Five varieties of alfalfa were grown in soil; each variety had mixed-colored flowers, and we noted which flower color was prevalent to each variety. ‘Golzu 1’ and ‘Bilensoy 80’ varieties had blue and violet flowers, while ‘Tuyana’ and ‘Vega 87’ had yellow and light blue or violet flowers. The ‘Burgaltai’ variety was distinguished by its deep purple variegation on the top portion and yellow lower portion of the blossom. Additionally, this variety had abundant yellow and a few deep purple flowers (Fig. 1). The plants created a panicle of 2-4 flowers in pot soil [Fig. 1 (a)]. It had an inflorescence of 13-15 flowers in the field condition [Fig. 2. (b) Image by Dr. Alimaa D.].



Fig. 1. Flower colors of ‘Burgaltai’ variety in the pot soil (a) and the field (b)

Although we did not measure differences in leaf size and leaf chlorophyll content, we observed that the leaves of the ‘Burgaltai’ variety were smaller and darker green than the others. Over the past decade, the ‘Burgaltai’ variety has lost its biomass and a low proportion of leaves in the yield (up to 45%) due to cross-pollination. Therefore, the study, conducted to increase the leaf size of this variety through gene transformation, was performed by researchers of RIAH previously [26]. The mean values of leaf number and branching distance characteristics of five varieties were not significantly different ($p > 0.05$) (data not shown).

Polymorphism of SSR Markers

Previously, Yazıcılar B. *et al.* reported that the 11 markers had been used in genomic analysis of genotypes of Turkish alfalfa plants, and all markers produced bands. The PIC values ranged from 0.18 (MTIC238) to 0.37 (MTIC248), and the greatest genetic knowledge was obtained from MTIC441 and MTIC471 [15].

In the present study, we used the same markers; ten markers (90.9%) gave amplified products. However, MTIC44 had no amplification or faint bands; a different DNA fragment detection technique might cause this. Representative gels for MTIC64, MTIC93, MTIC451,

and MTIC471 are shown in Figure 2, respectively. Of these, 34 alleles were detected, of which 8 were unique alleles produced from MTIC48, MTIC64, MTIC77, MTIC451, and MTIC471 primers. All 10 amplified SSR markers in this study exhibited polymorphism (Table 3). The number of detected amplicons ranged from 2 (MTIC48, MTIC77, MTIC238, and MTIC248) to 7 (MTIC451).

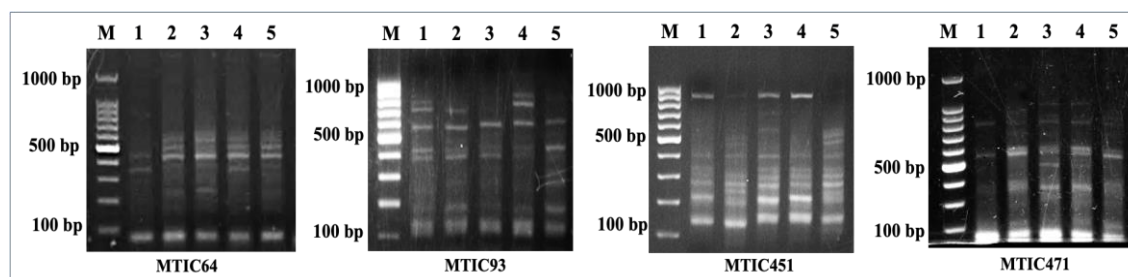


Fig. 2. Amplification products of SSR markers generated from DNA of alfalfa varieties: (M) denoted the size marker (100 bp DNA ladder), (1–5) represented samples from alfalfa

The PIC value of markers ranged from 0.32 to 0.86 with an average of 0.52, and the molecular size of DNA fingerprints ranged between 52 bp (MTIC48) and 895 bp (MTIC451). Alleles of three SSRs, namely MTIC64 (238 bp), MTIC451 (390 bp and 585 bp), and MTIC471 (384 bp), were unique and can be used to identify the ‘Burgaltai’ variety from other varieties.

Table 3. Number of Alleles and PIC Value of SSR Markers

№	Primer name	Number of alleles	PIC value	Band score	Fragment size	
					Smaller size	Larger size
1	MTIC48	2	0.32	3	52	178
2	MTIC64	4	0.44	2	238	447
3	MTIC77	2	0.32	3	145	150
4	MTIC93	5	0.49	3	89	901
5	MTIC230	4	0.57	1	202	515
6	MTIC232	2	0.36	3	142	159
7	MTIC238	3	0.60	2	128	228
8	MTIC248	2	0.48	3	61	360
9	MTIC451	7	0.72	1	128	925
10	MTIC471	4	0.86	2	354	847
Avearage		3.4	0.52			

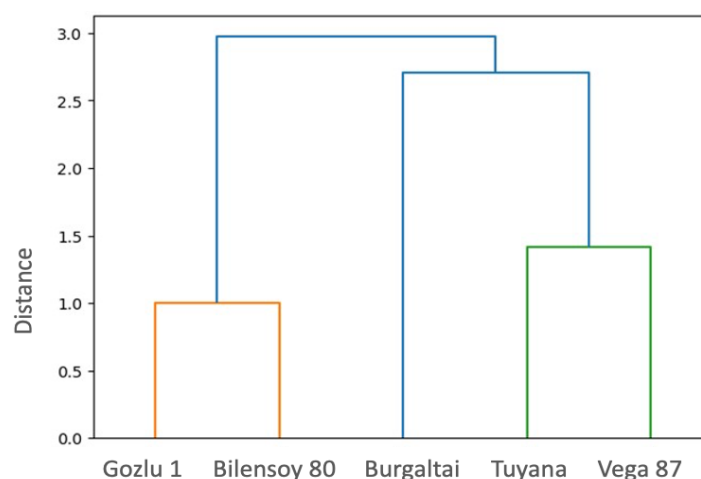


Fig. 3. Dendrogram constructed from SSR marker analysis

The genetic similarity (Euclidean distance) indicated that the highest similarity was observed in 'Gozlu 1' and 'Bilensoy 80' (1.0). At the same time, the most dissimilar varieties were 'Burgaltai' and 'Bilensoy 80' varieties (2.64) (Fig. 3). Assumingly, close genetic isolation caused Mongolian and Russian varieties to be more similar in genetic profile.

CONCLUSION

The 'Burgaltai' variety had a distinctive deep purple and yellow variegated flower color, a unique qualitative characteristic. The set of 10 SSR markers was proven to be applicable and effective in identifying alfalfa varieties, as they were easy to apply, fast, and informative. Three markers (MTIC64, MTIC451, and MTIC471) were variety-specific from all SSR markers regarding their application in identifying the 'Burgaltai' variety. In addition, the results confirmed that the 'Burgaltai' variety had higher genetic similarity with two Russian varieties. The study results can enhance the possibility of effectively classifying, conserving, and utilizing alfalfa genetic resources.

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