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Diversity in Lucerne (*Medicago sativa* L.) germplasm for morphology, yield and molecular markers and their correlations

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Abstract

Lucerne is an important legume forage grown worldwide due to its high nutritive value, yield potential, quality and survival under highly contrasting environments. Estimation of genetic diversity in germplasm is an important criterion in breeding programme and is done based on phenotypic characters, biochemical and molecular markers. Thirty one accessions of Lucerne collected from Maharashtra and Gujarat were evaluated for morphological, yield characters, molecular markers and were compared with national check RL-88. The diversity analysis of morphological and yield characters indicated wide range of variation within the accessions. The accession RLG 08-1 recorded significant differences for plant height, number of tillers per plant, internodal length, green fodder, dry matter and crude protein yield (P<0.05) over RL-88. Green fodder yield was significantly correlated with plant height (0.72), no. of tillers per plant (0.71**) and internodal length (0.71**). Dry matter yield was found significantly correlated with plant height, no. of tillers per plant, internodal length and crude protein yield (0.70**, 0.69**, 0.70** and 0.99**). Leaf stem ratio was not linearly correlated with green fodder, dry matter and crude protein yield. Principal component analysis (PCA) demonstrated that the first two PCs contributed to 93.7% of total variance among the accessions. The accession RLG 08-1 was found superior among all the accessions. Thirteen Inter Simple Sequence Repeats (ISSRs) markers showed 82.8% polymorphism. The dendrogram revealed slight geographical structuring and RLG 08-1 was found genetically distinct from other accessions.Mantel correlation was not observed between morphological and yield characters as well as molecular markers.**

Key words: Forage, correlation, genetic diversity, ISSRs, morphology

Introduction

Lucerne or alfalfa (*Medicago sativa* L.) is often described as the queen of forages because of its high protein content and adaptation to different soil and environmental conditions. Perennial types of Lucerne can supply green fodder for three to four years continuously from the same field. Lucerne is distributed worldwide and grown in highly contrasting environments. In India, it is mostly cultivated in the states of Punjab, Haryana, Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamilnadu with assured irrigation (Ahlawat 2008). This extensive geographical adaptation promotes genetic variation providing an opportunity to the plant breeders for using highly diverse gene pools. Local populations as a starting source in alfalfa breeding program aimed at creating cultivars of a broad genetic base (Popovic et al. 2006). Evaluation and determination of genetic diversity in breeding program is one of the most important criteria for selection of the parental lines. It is usually done based on morphological and yield characters, biochemical and molecular studies. Morphological characterization is the first step towards the classification of crop germplasm and is based on environmental conditions (Cholastova and Knotova 2012). DNA-based molecular markers allow a more précised and environment independent way of studying genetic diversity. Several studies have been done on alfalfa using DNA-based markers like Random Amplified Polymorphic DNA (RAPD) (Musial et al. 2002), Amplified Fragment Length Polymorphism (AFLP) (Segovia-Lerma et al. 2003), Restriction Fragment Length Polymorphism (RFLP) (Maureira et al. 2004), Simple Sequence Repeat (SSR) (Flajoulot et al. 2005) and Sequence Related Amplified Polymorphisms (SRAP) (Vandemark et al. 2005). Use of Inter Simple Sequence Repeats (ISSRs) markers is simple and quick method in DNA fingerprinting,

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conservation biology (Li et al. 2005), phylogenetic studies (Kochieva et al. 2006) and population studies (Zhang and Dai 2010). ISSRs were used specifically for characterization of *Medicago* sp. and equal level of variance was observed within and among populations (Xavier et al. 2011). Genetic diversity in *Medicago sativa* L. populations were estimated using ISSRs and recorded 60% polymorphism with four main groups (Touil et al. 2008).

The present study was conducted to analyze and compare the genetic diversity among the collected and accessions of Lucerne by morphological yield attributes. Diversity analysis was also done at molecular level using ISSR markers.

Materials and methods

Explorations were done in traditional Lucerne growing areas in Ahmednagar and Aurangabad districts of Maharashtra and Kutch and Surendranagar districts of Gujarat state (Table 1). A total of thirty one accessions were collected from the farmer's fields.

Morphology and yield evaluation

Field studies were conducted in augmented block design during 2011-14 at BAIF Development Research Foundation, Central Research Station, Urulikanchan, Pune (M.S.). Thirty-one accessions of Lucerne were evaluated in comparison with national check variety RL-88 for seven morphological and yield traits *viz.,* plant height (cm), number of tillers, internodal length (cm), leaf stem ratio, green fodder yield (q/ha), dry matter yield (q/ha) and crude protein yield (q/ha). The data were collected for twenty-seven cuts and subjected to analysis of variance (ANOVA) to detect significance. The correlation coefficient studies of morphological and yield traits were done using MS Excel. Principal Component Analysis (PCA) was performed for all traits to understand superiority within the accessions using PAST program (Hammer et al. 2001).

Molecular evaluation

Genomic DNA was extracted from leaves of all accessions using a modified CTAB method (Murray and Thompson 1980). Thirteen ISSR (Inter Simple Sequence Repeats) primers were used in this study (Table 2). PCR was performed for a 20 µl final volume containing 10 µl (2x) Go Taq green master mix (Promega, India), 0.6 (10 mM) µl ISSR primer, 8.4 µl PCR water and 20ng genomic DNA. PCR cycling were carried out in a thermo-cycler (MJ research PTC-200) with an initial denaturation 94^{o}C for 5 min followed by 45 cycles at 94^{o}C for 30 sec, annealing at 50^{o}C for 45 sec, extension at 72^{o}C for 60 sec and then final extension step at 72^{o}C for 5 min (Bahulikar et al. 2004). The amplified products were separated on 2% Agarose gel in 1X TAE Buffer. Bands were detected by Ethidium Bromide staining. Gels were photographed using gel documentation system (Bio Rad, India). All polymorphic and reproducible bands were scored as presence (1) or absence (0). The resultant data were used for Unweighted Pair Group Method with Arithmetic Means (UPGMA) cluster analysis and dendrogram was constructed using PAST 3.12 software (Hammer et al. 2001).

Results and discussion

Morphology and yield analysis

Performance of Lucerne accessions for morphological and yield characters revealed significant ($P< 0.05$) difference between accessions for various traits (Table 1). Range of variation for green fodder yield was 626.00 (ALS-10) to 2066.28 (RLG 08-1) q ha^{-1} with an average of 1489.08 q ha^{-1}, as compared to check (RL-88) (1238.07 q ha^{-1}). The dry matter yield ranged from 178.71 (ALS-10) to 635.17 (RLG 08-1) q ha^{-1} with an average of 416.85 q ha^{-1} compared to 340.78 q ha^{-1} in control. The crude protein yield ranged from 31.45 (ALS-10) to 107.79 (RLG 08-1) q ha^{-1} with an average of 71.86 q ha^{-1} compared to 61.95 q ha^{-1} in check. The accession RLG 08-1 had significantly higher yields for green fodder, dry matter and crude protein over the check RL-88. The range of variation for yield traits was observed higher than the results obtained by Tucak et al. (2014).

Plant height ranged from 22.40 (ALS 10) to 75. 91 cm (RLG 08-3, ALS-3) with an average of 57.06 cm as compared to check (45.26 cm). Tucak et al. (2014) studied alfalfa population for agro-morphological traits, forage quality and yield and reported that the plant height ranged from 66.77 cm to 79.39 cm. The plant height is an important yield component and is often used as criteria for choosing a superior genotype in an early stage of selection (Tucak et al. 2008b). Number of tillers per plant ranged from 2.82 (ALS-10) to 10.70 (ALS-3) with an average of 7.92 as compared to check variety (6.0), which was higher than reported by (Benabderrahim et al. 2009). Internodal length was in the range of 2.00 cm (ALS-10) to 6.24 cm (ALS-3) with an average of 4.81 cm in comparison to check, 3.91 cm. Sengul (2002) studied alfalfa landraces and indicated that internodal length ranged from 0.29 to

Table 1. Mean performance of Lucerne accessions for morphological and yield characters.

S.No.	Name of accessions	Source of collection	Plant height (cm)	No. of tillers/ plant	Internodal length (cm)	Leaf stem ratio	Green fodder (q/ha)	Dry matter yield yield(q/ha)	Crude protein yield (q/ha)
1	BAL08-1	Aurangabad	56.94	8.44	4.78	1.13	1761.68	505.43	88.96
2	BAL08-2	Aurangabad	53.55	9.20	4.66	1.20	1621.63	482.84	82.01
3	BAL08-3	Aurangabad	65.46	9.38	5.68	1.05	1747.50	476.24	84.28
4	BAL08-4	Aurangabad	64.58	9.36	5.44	1.24	1454.86	418.05	72.35
5	BAL08-5	Aurangabad	62.79	8.66	5.25	1.27	1679.51	492.43	86.89
6	BAL08-6	Aurangabad	58.05	8.61	4.94	1.13	1499.89	421.54	77.26
7	BAL08-7	Aurangabad	66.08	9.19	5.63	1.21	1676.23	466.66	80.34
8	BAL08-8	Aurangabad	71.82	9.66	5.84	1.23	1598.59	445.57	79.19
9	RLG 08-1	Ahmednagar	70.78	10.26	5.94	0.85	2066.28	635.17	107.79
10	RLG 08-2	Ahmednagar	63.71	10.32	5.09	0.90	1758.97	484.82	82.99
11	RLG 08-3	Ahmednagar	75.91	9.27	5.83	1.17	1841.06	546.75	88.08
12	RLG 08-4	Ahmednagar	55.73	7.93	4.59	1.27	1702.40	509.95	87.43
13	RLG 08-5	Ahmednagar	68.81	9.14	6.13	1.02	1553.60	456.80	80.22
14	RLG 08-6	Ahmednagar	63.40	8.46	5.38	1.05	1412.19	374.19	59.26
15	RLG 08-8	Ahmednagar	65.09	9.42	5.62	1.06	1301.31	368.99	63.47
16	RLG 08-9	Ahmednagar	61.26	7.77	4.83	0.95	1323.02	362.31	60.04
17	RLG 08-10	Ahmednagar	63.00	8.60	5.37	1.26	1312.34	368.21	64.08
18	RLG 08-11	Ahmednagar	57.39	7.61	5.33	0.96	1855.60	528.24	88.85
19	RLG 08-12	Ahmednagar	63.96	8.59	5.28	1.24	1583.81	447.23	80.30
20	ALS-1	Kutch	30.33	3.15	2.71	0.97	826.62	207.23	35.87
21	ALS-2	Surendranagar	30.93	3.95	2.70	1.14	1209.25	332.27	59.98
22	ALS-3	Surendranagar	75.91	10.70	6.24	1.23	1543.06	444.87	77.97
23	ALS-4	Surendranagar	58.09	7.36	4.65	1.11	1534.21	403.34	67.01
24	ALS-5	Surendranagar	42.28	5.19	3.66	1.18	1168.90	318.23	55.44
25	ALS-6	Surendranagar	49.35	8.44	4.33	1.24	1236.57	288.37	47.84
26	ALS-7	Kutch	53.27	7.81	4.49	1.21	1267.99	318.49	55.85
27	ALS-8	Kutch	44.36	5.69	3.65	1.18	1479.64	371.21	64.83
28	ALS-9	Kutch	56.86	7.12	4.54	1.17	1373.98	377.91	66.92
29	ALS-10	Kutch	22.40	2.82	2.00	1.18	626.10	178.71	31.45
30	ALS-11	Kutch	54.16	7.12	4.48	1.18	1643.98	448.40	76.36
31	ALS-12	Kutch	42.55	5.42	3.66	1.18	1500.68	441.80	74.22
32	RL-88	Released variety	50.75	6.69	4.24	1.17	1555.67	438.19	80.51
Mean			56.86	7.85	4.78	1.14	1491.16	417.51	72.13
SE (m)+			6.97	1.07	0.56	0.02	11.56	3.78	0.64
CD at 5 %			19.53	3.02	1.57	0.07	33.50	10.96	1.87

Ahmednagar and Aurangabad : Districts of Maharashtra; Kutch and Surendranagar: Districts of Gujrat

0.54 cm. Leaf stem ratio ranged from 0.85 (RLG 08-1) to 1.29 (RLG 08-10) with an average of 1.14 compared to 1.0 of check, Popovic et al. (2006) concluded that leaf stem ratio can be used as an indirect selection criterion for quality improvement.

Table 2. Phenotypic correlation coefficients between the analysed traits for thirty one accessions of Alfalfa

	Plant height	No. of tillers/plant	Internodal length	Leaf stem ratio	Green fodder yield	Dry matter yield	Crude protein yield
Plant height	1						
Number of tillers/plant	0.94**	1					
Internodal length	0.98**	0.94**	1				
Leaf stem ratio	–0.067NS	–0.051NS	–0.095NS	1			
Green fodder yield	0.72**	0.71**	0.71**	–0.17NS	1		
Dry matter yield	0.70**	0.69**	0.70**	–0.16NS	0.98**	1	
Crude protein yield	0.68**	0.68**	0.69**	–0.12NS	0.96**	0.99**	1

*,** = NS significant differences between the levels of a factor at P ≤ 0.01, P ≤ 0.05 and Non Significant respectively

Correlation coefficients between the analysed traits showed positive correlation between green fodder yield and plant height (0.72**); no. of tillers per plant (0.71**), internodal length (0.71**) and dry matter yield (0.70**, 0.69**, 0.70**), respectively (Table 2). These correlations are in acquaintance with previous reports (Bernadette Julier et al. 2000; Julier et al. 2000; Tucak et al. 2008a). Moreover, dry matter yield found significantly correlated with plant height, no. of tillers per plant, internodal length and crude protein yield (0.70**, 0.69**, 0.70** and 0.99**). Recognition of correlation provides the opportunity for improvement of a larger number of traits at the same time. The PCA was performed to identify the variability and genetic diversity within the accessions. PCA is a data reduction method that allowed a better understanding of yield and morphological characters of the entire collection and to identify the most suitable variables among the studied accessions (Zimisuhara et al. 2015). In present study, PCA (Fig. 1) revealed 93.7 % of total variance among the accessions contributed by first two PCs. Tucak et al. (2009) studied twenty seven populations and cultivars of alfalfa using five phenotypical traits by PCA analysis and reported that first four PCs contributed 89.02% of the variability among the cultivars and populations for all the traits.

In PCA, the accessions fall in three groups.

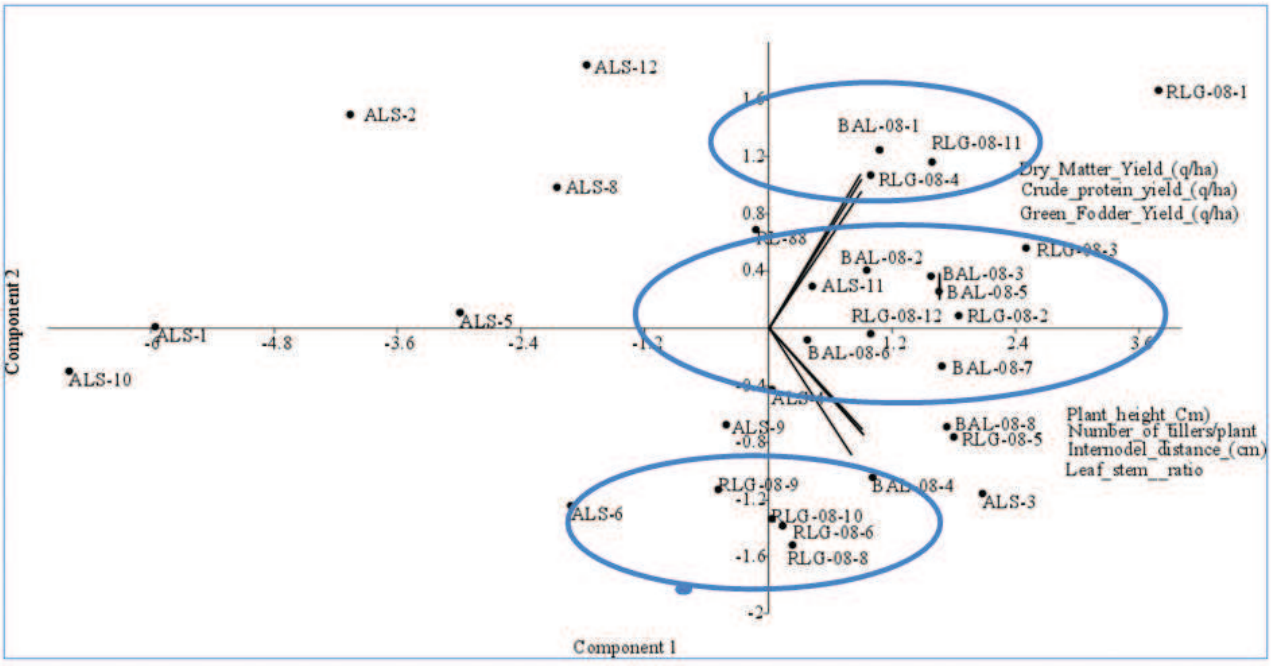

Fig. 1. Principle component analysis showing grouping of lucerne accessions with reference to morphological and yield traits

Group-1 contained three accessions from Aurangabad and Ahmednagar which were associated with vectors of traits such as high green fodder yield, dry matter yield and crude protein yield. Group 2 was the largest than other groups and was composed of nine accessions, positioned in between Group 1 and Group 3 due to mixed or average range of traits. Group 3 includes five accessions from Aurangabad and Ahmednagar which were associated with vectors *viz*., higher plant height, more number of tillers, more internodal distance and higher leaf stem ratio. The accession RLG08-1 from Ahmednagar was found distinct among other accessions and was characterized by the highest green fodder, dry matter and crude protein yield, highest plant height, maximum number of tillers and more internodal length.

Marker analysis

Out of 32 ISSR primers studied, 13 were found informative, which produced 93 bands; of them 77 were polymorphic. Polymorphic bands ranged from 2 to 11 for different accessions. Habibi et al. (2012) found 63 bands from 8 ISSR primers for 18 alfalfa genotypes of which 51 bands were polymorphic. Rashidi et al. (2013) reported 44 bands from 8 ISSR primers for 17 genotypes of which 40 bands were polymorphic ranging from 3-7. Polymorphic primers comprised from AG, CA and GA repeats. Highest number of bands (13) were recorded for $(AG)_8YC$ primer and lowest number of bands (4) for primer $(AG)_8T$. The primers $(AG)_8C$, $(AG)_8YC$ and $(GA)_8C$ gave 100% polymorphic bands (Table 3).

In the present study, the percentage of polymorphism for the lucerne accessions ranged from 40 to 100 with an average of 80.25, whereas 60% of average polymorphism was recorded using 19 ISSR primers (Touil et al. 2008). Similarly, 73.39% of polymorphism from 8 ISSR primers has been reported previously (Habibi et al. 2012).

Dendrogram (Fig. 2) revealed three major clusters. Cluster I represented by accessions BAL-08-3, RLG-08-12 and BAL-08-5. Cluster II have eighteen accessions divided into three sub-groups, the RLG08-10 was out grouped indicating its candidature in the group. Cluster III represented four accessions i.e., BAL-08-4, BAL-08-7. BAL-08-6 and BAL-08-8 which showed geographical structuring. The accessions RLG-08-1 and BAL-08-1 were outgrouped all the three clusters and indicated genetically of distinct nature. The genetic variation of most

Table 3. Comparison of primers, number of fragments scored, number of polymorphic bands and percentage polymorphism

Primer repeat	Total no.of bands	No. of polymorphic bands	% polymorphic bands
AC_8Y*T	7	5	71.4
AG8C	5	5	100.0
AG8T	4	3	75.0
AG8YA	7	5	71.4
AG8YC	11	11	100.0
CA8A	5	2	40.0
CA8R*C	13	12	92.3
CA8RG	8	6	75.0
CT8G	6	5	83.3
GA8A	6	4	66.7
GA8C	9	9	100.0
GA8T	5	4	80.0
TC8G	7	6	85.7
Total	93	77	82.8

Y = pyrimidine and R purine

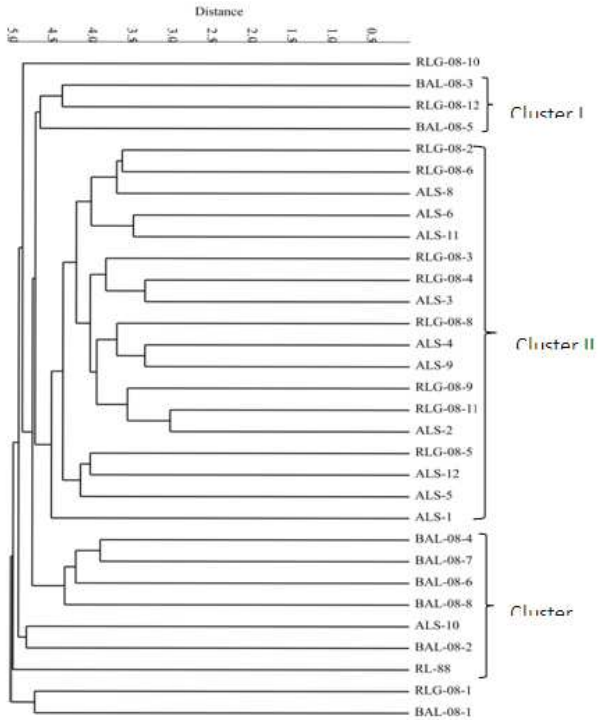

Fig. 2. Dendrogram constructed by UPGMA analysis using Jaccard coefficient of 31 accessions of lucerne using ISSR primers

accessions did not resemble with their geographical distribution. It may be due to long cultivation history of landraces and introduced cultivars in the same area. Similar results have been reported earlier (Touil et al. 2008; Rashidi et al. 2013) in a different set of material. The correlation between the morphological, yield and molecular data were tested by the mantel Z-statistic test (Mantel 1967) and the test did not verify the correlation. Similar results were found by Tucak et al. (2008b). There were significant differences among thirty one accessions studied for seven different morphological and yield traits. The accession RLG-08-1 recorded significant difference for all the traits over check variety RL-88. Green fodder and dry matter yields were significantly correlated with plant height, no. of tillers per plant and internodal length. The accessions RLG-08-1 and BAL-08-1 were genetically distinct than all other accessions. The accession RLG-08-1 has highest values for most of forage yield and morphological traits. It also emerged out as the most distinct cultivar at molecular level over all other accessions. The accessions RLG-08-1 and BAL-08-1 can be utilized for development of new varieties using conventional breeding methods or mutagenesis.

Authors' contribution

Conceptualization of research (PST, JSD, RAB); Designing of the experiments (PST, JSD, RAB); Contribution of experimental materials (PST); Execution of field/lab experiments and data collection (SSJ); Analysis of data and interpretation (PST, RAB, SSJ); Preparation of manuscript (PST, JSD, RAB, SSJ).

Declaration

The authors declare no conflict of interest.

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