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Genetic relatedness among weevil resistant *Medicago* species and Indian susceptible cultivars of lucerne (*Medicago sativa* L.) using SSR and RAPD markers

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Abstract

Fifty-two species of genus *Medicago* were screened against lucerne weevil (*Hypera postica* Gyll.) and accessions belonging to five species *viz,. M. laciniata*, *M. muricoleptis*, *M. rugosa*, *M. tenoreana* and *M. scutellata* were identified as potential resistance source. Genetic relatedness using SSR and RAPD markers of these species were estimated along with five Indian weevil susceptible cultivars namely Anand 2, LLC 3, RL 88, T 9 and CO 1. Both SSR and RAPD based dendrograms revealed grouping of all five Indian cultivars into one cluster. Moreover, RAPD based dendrogram indicated sub-grouping of *M. scutellata* accession with Indian cultivars. Both data sets showed more closeness of *M. scutellata* and *M. muricoleptis* with Indian cultivars as genetic distance between these species were comparable over other suggesting use of such species for improvement of Indian cultivars of lucerne for weevil resistance.

Key words: Genetic relatedness, *Medicago* species, PCR, RAPD, SSR, weevil resistance

Introduction

Lucerne or alfalfa (*Medicago sativa* L.) is one of the important forage crops of India and occupies more than one million hectare area and yield 60 to 130 tones per hectare green forage [1]. The genus *Medicago* includes highly cross-pollinated perennial and annual species [2]. Perennial species are largely tetraploids (2n = 4x = 32), however diploid (2n = 2x = 16) and hexaploid (2n = 6x = 48) were also reported. The majority of the annual *Medicago* species are diploid and have 2n = 2x =16 chromosomes with the exception of *M. constricta* Dur., *M. murex* Willd., *M. polymorpha* L., *M. praecox* DC. and *M. rigidula* Desr. as they contain 2n = 2x = 14. *M. scutellata* Mill. and *M. rugosa* Desr. are the only annual species with 2n = 30 [3].

The lucerne weevil, *Hypera postica* Gyll. (Curculionidae: Coleoptera) is a major limiting factor in lucerne forage production not only in India but also in other lucerne growing areas of the world. In India, the pest remains active in colder months (December through March) with highest incidence in February [4]. Majority of the non-dormant Indian cultivars are susceptible to this pest [4]. Insect damage causes 10 to 15 % annual green biomass loss where forage quality is not taken into account. Shade *et al.* [5, 6] reported that glandular-haired annual species were highly resistant to weevil larvae and potato leafhopper nymphs, two highly destructive alfalfa insects. Our results also indicated the tolerance of *M. scutellata*, *M. rugosa*, *M. laciniata, M. tenoreana* and *M. muricoleptis* to lucerne weevil when they were evaluated under natural hot-spot conditions [7]. However, the most challenging task in this genus has been the successful crossing. Moreover in most of these crosses, attention was more towards generation of hybrid than those of improvement of agronomical traits especially weevil resistance. Sandeun *et al.* [8] reported successful crossing between *M. sativa* and glandular haired *M. scutellata*, however progenies were either sterile or generated unhealthy seeds. Recently cell fusions have resulted into S_1 offspring from *M. sativa* and *M. rugosa,* while no seeds were obtained with self-pollinating plants of the crosses of *M. sativa* and *M. scutellata* [9]. Thus, these studies indicated identification of accessions or species genetically closer to *M. sativa* which could be able to yield hybrids possessing weevil resistant trait. Studies have been conducted using molecular markers to assess the level of variations between and within perennial *Medicago* [10-11] as well as annual species [12]. Diwan *et al.* [13] reported the presence and frequency of simple sequence repeats (SSR) DNA sequences in the alfalfa genome. Eujayl *et al.* [14] demonstrated the utilization of *M. truncatula* EST

based SSRs markers in other species of *Medicago*. However, these studies were not targeted to understand the genetic relatedness among weevil resistant/ susceptible species. As boundaries between species in many crops are extremely difficult to define because of wide range of diversity, the study of an intra-species variations and its inter-specific similarity becomes important to understand the probable evolution of the genus. This will also help in designing the breeding strategies for incorporation of desirable traits like weevil tolerance. Muller *et al*. [15] reported that the domesticated species pool of alfalfa (*M. sativa* L.) contains an average 31 % less diversity than the wild pool, but with a high heterogeneity among loci. Present study was undertaken to assess the genetic relatedness among Indian cultivars and their closeness with the annual species showing low levels of weevil infestation.

Materials and methods

Genomic DNA from five Indian cultivars of lucerne *viz.*, Anand 2, LLC 3, RL 88, T 9 and CO 1 and accession of five *Medicago* species *viz*., *M. laciniata* EC 541616, *M. muricoleptis* EC 541637, *M. rugosa* EC 547732, *M. tenoreana* EC 547746 and *M. scutellata* EC 541686 were isolated from fresh, young and disease free leaves by 2X CTAB buffer following Iqbal *et al*. [16]. In the present study genomic DNA of *M. falcata* was also included considering that most of the *Medicago* lines are derived from this. Ten individual plants of each cultivar of lucerne and six *Medicago* species were used for the isolation of DNA. All five accessions of *Medicago* species namely *M. laciniata*, *M. muricoleptis*, *M. rugosa*, *M. tenoreana* and *M. scutellata* were identified as the resistance source where weevil infestation was 5 to 10 % [7]. Each RAPD PCR amplification was performed in a final volume of 20 ml of reaction mixture containing 67 mM Tris-HCl (pH 8.0), 16.6 mM $(NH_4)_2SO_4$, 0.45 % (v/v) Triton X-100, 4 mg BSA, 3.5 mM $MgCl_2$, 150 mM of each of dATP, dCTP, dGTP and dTTP, 7.5 pmol (15 ng) primer, 25 ng genomic DNA template and 0.5 unit *Taq* polymerase (Bangalore Genei, India), and finally it was overlaid by 5-10 ml light mineral oil. Amplifications were performed on a DNA thermal cycler PTC-200 (MJ Research, USA) with the cycling program at 94°C for 1 min, 37°C for 1 min and 72°C for 2 min for 40 cycles followed by 41st cycle at 37°C for 1 min and finally at 72°C for 10 min extension. The amplified products were kept at 4°C until loaded on to the gel. Amplification products were separated by electrophoresis on 1.6 % agarose gel. In total 25 RAPD primers namely OPG 8, OPN 4, 6, OPP 1, 9, OPR 4, 7, OPO 6, OPT 6, OPU 1, OPJ 13, OPQ 9, OPX 1, OPAB 1, 3, 5, 10, 16, OPAE 2, 3, 12, OPAF 13, OPAK 18, OPAL 11 and OPAH 3 and were purchased from Operon Inc. USA.

Ten SSR primer pairs (Table 1) developed based on genomic sequences [17] were custom synthesized by Sigma Genosys, Bangalore, India. The PCR reactions for SSR markers was carried using the touch-down PCR profile: an initial denaturation step of 3 min at 94°C was followed by 45 cycles with denaturation at 94°C for 30 s and extension of 72°C for 30 s, respectively. The annealing temperature was decreased in 0.5°C increments from 55°C in the first cycle to 50°C after the 10th cycle and was kept constant for the remaining 35 cycles (always 30 s). After 45 cycles a final extension step was performed at 72°C for 5 min. The SSR PCR was carried out in 10 µl reactions consisting of 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.5 µM of each primers, 100 µM of each nucleotide, 1 X PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % triton X-100, and 1 unit *Taq* DNA polymerase. PCR amplifications were carried out using a MJ Research Thermocycler (PTC 200). PCR products were separated on 7 % acrylamide gel using Tris-glycine buffer at 100 V for 5 hrs. Gels were stained with ethidium bromide and visualized under UV transilluminator.

For both SSR and RAPD markers input binary data matrix of 11 accessions of *Medicago* was developed by entering the data by assigning 1 to presence or 0 to absence of bands. Only reproducible and unambiguous RAPD and SSR fragments obtained after three runs were used for scoring. Degree of polymorphism between accessions was calculated using index of genetic distance (1-F). F values (degree of similarity) were obtained by using the method of Nei and Li [18].

Degree of similarity, $F = (2.N_{ab})/(N_a+N_b)$

Degree of polymorphism = (1-F)

where

N_{ab} = number of shared bands between species 'A' and 'B'

N_a = number of bands in species 'A'

N_b = number of bands in species 'B'

This method has the advantage over other general similarity indices because of the increased weighting of band matches versus that of non-matches. The NTSYS program version 2.0 was used to produce the similarity matrix (Simqual function) for both SSR and RAPD data. Dice similarity coefficient was used to

estimate the genetic similarity. The resulting data were further processed with SAHN cluster analysis using the un-weighted pair group average method (UPGMA) and tree display was followed for generation of dendrogram (NTSYS tree phenogram).

Results and discussion

After screening 52 species of genus *Medicago*, accessions of five species namely *M. laciniata*, *M. muricoleptis*, *M. rugosa*, *M. tenoreana* and *M. scutellata* were identified as potential resistance materials against lucerne weevil (*Hypera postica* Gyll.) [7]. A total of 498 RAPD bands were scored with 25 RAPD primers when 11 accessions representing seven species of *Medicago* including five cultivars of *M. sativa* was used for RAPD analysis (Fig. 1a). RAPD analysis revealed genetic distance indices among these accessions ranging from 0.29 to 0.90 (Table 2). When cluster analysis using RAPD data was performed, *M. falcata* made a distinct node whereas all five Indian cultivars made one separate cluster which joined most closely to *M. scutellata* (Fig. 2a). Group of these five accessions joined with the third cluster made by four different species namely *M. laciniata, M. muricoleptis, M. rugosa* and *M. tenoreana* (Fig. 2a). Among the Indian cultivars four cultivars made one small sub-cluster which finally joined with cv CO1 indicating distinctness of cv CO1 to those of others. The separate node as made by *M. falcata* joined to other species of *Medicago* at different similarity levels indicating closeness of *M. falcata* as this belongs to

Table 1. SSR primers used in the study

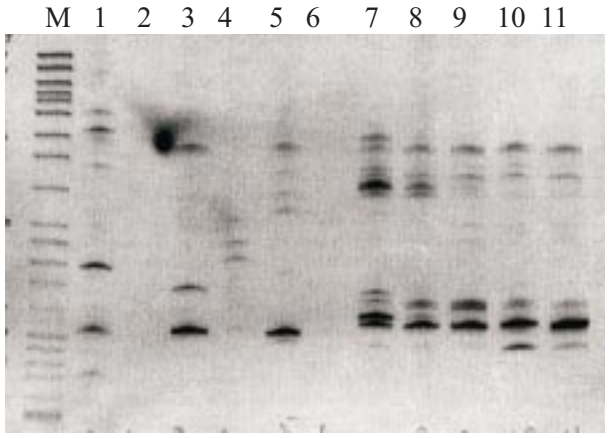
Primers	5' end primer sequence (Forward)	3' end primer sequence (Reverse)	Core motif	Ref.
AFct60	cctccctaactttccaaca	tggatcaacgtgtctttca	$(CT)_{21}$	27
AFct11	ggacagagcaaaagaacaat	ttgtgtggaaagaataggaa	$(CT)_{12}$	27
MTLEC2A	cggaaagattcttgaatagatg	tggttcgctgttctcatg	$(AT)_{19}$	27
AFca16	ggtcgaaccaagcatgt	taaaaaacattacatgacctcaaa	$(CA)_{12}$	27
AFca11	cttgagggaactattgttgagt	aacgtttcccaaaacatactt	$(CA)_{11}$	27
AFct45	taaaaaacggaaagagttggttag	gccatcttttcttttgcttc	$(CT)_8AT(CT)_3$	27
AFct32	tttttgtcccacctcattag	ttggttagattcaaagggttac	$(CT)_{14}$	27
AFctt1	cccatcatcaacattttca	ttgtggattggaacgagt	$(CTT)_9(CAA)_3$	27
AFat15	ttacgggtctagattagagagtatag	caaaatgagtatagggagtgg	$(AT)_{23}$	27
AFca1	cgtatcaatatcgggcag	tgttatcagagagagaaagcg	$(CT)_4(CA)_{10}$	27

Table 2. Index of genetic distance (1-F) obtained from 498 RAPD bands (bold number) and 191 SSR bands between 11 accessions representing seven species of *Medicago*

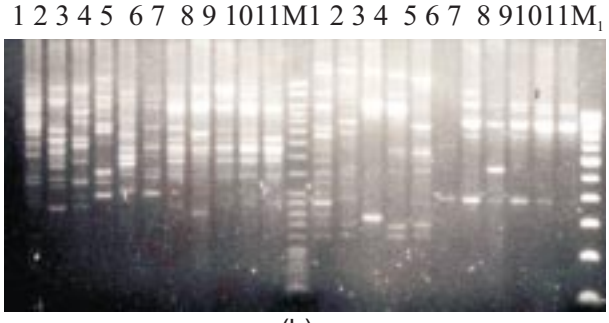
	M. laciniata	*M. muricoleptis*	*M. rugosa*	*M. tenoreana*	*M. scutellata*	*M. falcata*	*M. sativa* cv. Anand 2	*M. sativa* cv. LLC 3	*M. sativa* cv. RL 88	*M. sativa* cv. T9	*M. sativa* cv. CO 1
M. laciniata	**0.00**	0.80	0.77	0.87	0.80	0.89	0.78	0.70	0.75	0.75	0.74
M. muricoleptis	**0.68**	**0.00**	0.74	0.77	0.77	0.79	0.75	0.74	0.71	0.72	0.76
M. rugosa	**0.76**	**0.70**	**0.00**	0.73	0.61	0.92	0.78	0.77	0.80	0.73	0.74
M. tenoreana	**0.74**	**0.74**	**0.70**	**0.00**	0.88	0.92	0.94	0.90	0.88	0.89	0.
M. scutellata	**0.75**	**0.81**	**0.78**	**0.72**	**0.00**	0.80	0.64	0.70	0.73	0.67	0.68
M. falcata	**0.89**	**0.89**	**0.87**	**0.90**	**0.86**	**0.00**	0.87	0.88	0.87	0.83	0.88
M. sativa cv Anand 2	**0.67**	**0.72**	**0.74**	**0.72**	**0.74**	**0.83**	**0.00**	0.30	0.30	0.30	0.35
M. sativa cv LLC 3	**0.75**	**0.80**	**0.76**	**0.72**	**0.74**	**0.86**	**0.56**	**0.00**	0.12	0.30	0.30
M. sativa cv RL 88	**0.75**	**0.71**	**0.74**	**0.77**	**0.72**	**0.76**	**0.38**	**0.58**	**0.00**	0.30	0.34
M. sativa cv T 9	**0.72**	**0.72**	**0.71**	**0.75**	**0.72**	**0.77**	**0.33**	**0.61**	**0.29**	**0.00**	0.26

the primary gene pool of genus *Medicago* from where most of the species have been derived. In comparison to other species, *M. scutellata* showed better closeness with Indian cultivars as this has formed an immediate cluster with all five Indian lucerne cultivars (Fig. 2a) suggesting utilization of this species over other namely *M. laciniata*, *M. muricoleptis*, *M. rugosa* and *M. tenoreana* species for improvement of Indian lucerne cultivars for weevil resistance.

In total 191 SSR bands were scored with 10 genomic based [17] (Table 1) SSR primer pairs (Fig. 1b). The genetic distance among 11 accessions of *Medicago* species ranged from 0.12 to 0.94, thus indicated more diversity among the accessions in comparison to RAPD based diversity (Table 2). Dendrogram developed by using 191 SSR bands revealed two distinct clusters where all *Medicago sativa* Indian cultivars grouped into one cluster whereas other six species including *M. falcata* formed separate cluster. Among Indian cultivars there were two small sub-cluster. More closeness of *M. sativa* cv RL 88 with *M. sativa* cv T9 and *M. sativa* cv LLC3 with *M. sativa* cv CO1 was observed. *M. scutellata* showed 48 to 55 % similarity with five cultivars of lucerne. Of the six different *Medicago* species, *M. laciniata* was observed as most distinct accessions than those of others (Fig. 2b). *M. falcata* clustered with *M. tenoreana* and *M. muricoleptis* whereas in case of RAPD patterns *M. falcata* was observed as a distinct node and joined with rest of the 10 accessions. Contrary to the RAPD based dendrogram, SSR based dendrogram revealed more closeness of accession to each other. The SSR analysis of *Medicago* species using both genomic and EST-based SSR have been reported indicated the usefulness of markers in selecting the potential species for improvement of annual and perennial *Medicago* for different agronomical traits [13, 14, 17]. Based on phenolic-taxometric studies, *M. rigidula* (L.) All., *M. murex* Willd., *M. doliata* Carmian, *M. muricoleptis* Willd. and *M. rotata* Boiss species showed a close relationship to *M. scutellata* [19]. Two of the species are 2n = 14 (*M. murex* and *M. rigidula*) and the others are 2n = 16. The presence of these species and their close relationships with *M. scutellata* suggest that



(a)



(b)

Fig 1. (a) SSR profiles of 11 accessions representing 7 species of *Medicago* with primer AFct11. (b) RAPD profile with primers OPAB03 and OPAB01. Lanes 1 to 11 are *M. laciniata*, *M. muricoleptis*, *M. rugosa*, *M. tenoreana, M. scutellata, M. falcata, M. sativa* cv Anand 2, LLC 3, RL 88, T 9 and CO 1. M = 20 bp DNA ladder; M_1 = 100bp DNA ladder as molecular weight marker

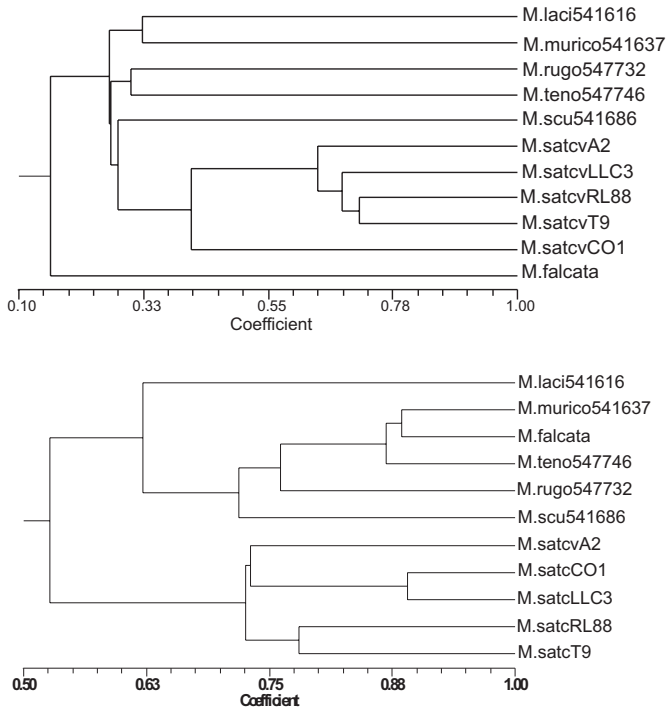


Fig. 2. Dendrogram based on (a) RAPD and (b) SSR similarity coefficients using UPGMA module

2n = 30 chromosome bearing species like *M. scutellata* may have arisen through hybridization followed by polyploidization [4], however genomic in situ hybridization (GISH) technique could provide insight about the evolution of 2n = 14 species and origin of polyploids like *M. rugosa* and *M. scutellata* (2n = 30) [20]. Thus, being *M. muricoleptis* as weevil resistant and one of the putative progenitors of *M. scutellata,* this particular species showing more closeness with *M. sativa* can also be used for improvements of Indian lucerne applying gene introgression strategy [19]. The identified species like *M. scutellata* showing comparable closeness in terms of genetic distance with Indian lucerne cultivars, thus, may ultimately be useful in transferring the characters into *M. sativa* cultivars.

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