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Studies on morphological and genetical similarities of *Medicago murex* and *M. doliata* to *M. scutellata*

Amaresh Chandra

Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi - 284 003, India

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Abstract: *Lucerne (M. sativa L., 2n = 4x = 32) is susceptible to weevil (Hypera postica Gyll) insect, hence incorporation of desirable gene (s) from M. scutellata (2n = 30) is an important researchable issue. Incompatibility due to incongruous chromosomal arrangements in these two species necessitated the identification of closer species to M. scutellata (possibly progenitors). After screening 197 accessions comprising 50 Medicago species, M. murex (2n = 2x = 14) and M. doliata (2n = 2x = 16) have been identified as morphologically similar having compatible ploidy and genetically closer to M. scutellata as observed with 17 simple sequence repeats (SSR) and 8 enzymes based isozyme markers. The identified accessions namely IL-04-223 and IL-04-151 of M. doliata and M. murex respectively showing low levels (< 5%) of weevil infestation can be contemplated with diploid M. sativa (2n = 2x = 16) to generate weevil resistant lines.*

Key words: *Hypera postica, Isozyme, Medicago species, SSR, Weevil resistance*
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Introduction

Lucerne (2n = 4x = 32) (alfalfa) is one of the important perennial forage legumes of many temperate and tropical countries including India. However, major problem associated with this legume is the infestation caused by lucerne weevil (*Hypera postica* Gyll.) which amounts to >20% green fodder loss (quality is not taken into account). *M. scutellata* has been reported as potential weevil resistant species possessing glandular hairs on leaves and stems (Barnes and Ratcliffe, 1969; Shade *et al.,*1975, 1979) and thus hinders laying of eggs by insect. Barring few reports and that to with the application of gibberallic acid on peduncle and pedicels (Sangduen *et al.,* 1982), no major success has been reported regarding transfer of weevil resistant trait in perennial lucerne (Mizukami *et al*., 2006). Chromosome elimination in the hybrid is largely contributed by factors like nature of donor species, genetic distance, culture condition (*in vitro* cell fusion) and incompatible ploidy levels of fusing partners (Oberwalder *et al*., 1998). As such very few reports are available where inter-specific crosses in genus *Medicago* have been successful (McCoy and Smith, 1986; McCoy and Echt, 1991; Nenz *et al.,* 1996; Mizukami *et al.,* 2006). The major barrier in recovery of perennial *Medicago* interspecific hybrids is post-fertilization (Oldemeyer, 1956; Sangduen *et al.,* 1983; McCoy, 1985) and incompatible chromosomal arrangements as the case with annual *M. scutellata* (2n = 30) and perennial *M. sativa* (2n = 4x = 32). Partly, the presence of two pairs of SAT chromosomes in *M. scutellata* also contributed in unsuccessful production of hybrids with this species. One pair of this is separated from the main body of the chromosome by relatively long distance (Bauchan and Elgin Jr., 1984). Further, several theories have been proposed by which a somatic number of 2n = 30 can be derived. One possibility is the hybridization of a 2n = 14 with 2n = 16 species followed by the polyploidization of the hybrid. Another possibility would be the loss of a pair of chromosomes from 2n = 32 polyploid. The gain of a pair of chromosomes is unlikely because 2n = 28 species has not been found in the genus *Medicago* (Bauchan and Elgin Jr., 1984). Phenolic-taxometric studies have indicated the closeness of *M. rigidula* (L.) All., *M. murex* Willd., *M. doliata* Carmian, *M. muricoleptis* Willd. and *M. rotata* Boiss to *M. scutellata* (Classen *et al.,* 1982). However, among these, two possible (2n = 14 and 2n = 16) species reasonably more closer to *M. scutellata* have not been identified so far despite that isozyme markers have been reported in identification of alfalfa plants (Quiros, 1980) and progenitor-derivative relationships study (Small, 1992). This is important because 2n = 30 species (*M. scutellata*) may have arisen through hybridization followed by polyploidization (Bauchan and Elgin Jr., 1984).

The objectives of the present work are to delineate the closest species to *M. scutellata* based on sharing of SSR and isozyme bands which can be directly utilized in breeding programmes.

Materials and Methods

After initial screening of 197 accessions belonging to 50 species of *Medicago*, 14 species having 2n = 2x =16 and 2n = 2x = 14 ploidy levels namely *M. arabica* (L.) Hudson (2n = 2x = 16), *M. blancheana* Boiss (2n = 2x = 16), *M. coronata* (L.) Bartal. (2n = 2x = 16), *M. doliata* Carmign. (2n = 2x = 16), *M. intertexta* (L.) Miller (2n = 2x = 16), *M. muricoleptis* Tineo (2n = 2x = 16), *M.*

* **Corresponding author:** amaresh62@yahoo.com

rotata Boiss. (2n = 2x = 16), *M. truncatula* Gaertner (2n = 2x = 16), *M. hybrida* (Pourret) Trautv. (2n = 2x = 16), *M. polymorpha* L. (2n = 2x = 14), *M. constricta* Durieu (2n = 2x = 14), *M. murex* Willd. (2n = 2x = 14), *M. praecox* DC (2n = 2x = 14), *M. rigidula* (L.) All. (2n = 2x = 14) were further established in consecutive years along with *M. scutellata* (2n = 30) in the nursery of Indian Grassland and Fodder Research Institute (IGFRI) (78°33'18"E/ 25°31'36"N), Jhansi, India. These accessions were previously selected based on the similar morphological features like presence of hairs on the stem, leaf morphology, serration and the level of weevil infestation under natural hot spot condition (Chandra *et al.,* 2006). Of these species, six accessions each of *M. murex* (2n = 2x = 14) and *M. doliata* (2n = 2x = 16), 4 of *M. rigidula* (2n = 2x = 14), 5 of *M. scutellata* (2n = 30) and 3 accessions of *M. muricoleptis* (2n = 2x = 16) were selected based on significant morphological similarities to *M. scutellata* as well as earlier reports (Classen *et al.,* 1982) indicating similarities based on phenolic-taxometric studies for further work. All these species were annual and self pollinating in nature (Heyn, 1963; Lesins and Lesins, 1979). The National Bureau of Plant Genetic Resources (NBPGR), New Delhi, the nodal agency through which seed materials were obtained from *Medicago* Germplasm conservation and exchange centre USDA-ARS, USA. In case of isozyme analysis equal amounts of leaf tissues from all accessions of a species was ground together in isozyme extraction buffer and in case of DNA leaves from each accession was ground separately. Growth habit, leaf shape and level of weevil infestation were recorded after 60 days of germination.

Genomic DNA was isolated using liquid nitrogen. Fine ground powder was mixed in 1: 2 ratio with extraction buffer (made by mixing 10 ml of Tris-HCl pH 7.5, 2.5 ml of 5 M NaCl, 2.5 ml of 0.5 M EDTA pH 8.0, 2.5 ml of 10% SDS and 32.5 ml of double distilled water) in autoclaved Eppendorf tube. Before adding the buffer into powder, 0.1% 2-mercaptoethanol was freshly added into the buffer. The slurry was incubated at 65°C for 1 hr with occasional mixing. The homogenate was centrifuged at 10000 rpm for 10 min and supernatant was extracted with chloroform. After extraction and centrifugation, liquid phase was collected in fresh tube and DNA was precipitated by adding 0.6 volume of isopropanol. RNase treatment was applied to remove the RNA, and the resultant DNA was checked on gel and diluted to the final concentration of 5 ng μl^{-1} for PCR analysis. For enzyme extract, leaves were ground in one ml grinding buffer (50 mM Tris-HCl pH 7.5, 1mM EDTA, 5% (w/v) sucrose, 0.2% (v/v) 2-mercaptoethanol, 7% (w/v) PVP). The homogenates were centrifuged at 10000 rpm at 4°C and supernatant was used as enzyme source. The pooled samples extract representing five species were compared for eight enzymes namely peroxidase (PRX), esterase (EST), superoxide dismutase (SOD), acid phosphatase (ACP), polyphenol oxidase (PPO), malate dehydrogenase (MDH), amylase (AMY) and glutamate oxalo acetate transaminase (GOT). Vertical polyacrylamide gel (10 %) electrophoresis method of Laemmli(1970) with discontinuous buffer system was used for studying the isozyme profiles. Gels were stained for PRX (Veech, 1969) and for EST, SOD, PPO, GOT, MDH, AMY and ACP (Wendel and Weeden, 1989). About 150 µg of protein sample mixed with 5 µl of bromophenol blue (tracking dye) was loaded onto the well from cathodal end. The gel was run at 100 V till dye crossed the stacking gel and then at 200 V till the dye was 0.5-1.0 cm away from bottom. One common control was put in all the plates to equalize the movement of major band of control across the plates. The bands were given numbers from the origin.

The PCR reactions using genomic (Diwan *et al.,* 1997, 2000) and EST based SSR primers generated from *M. truncatula* ESTs (Table 1) was carried out using the following 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 30s, respectively. The annealing temperature was decreased in 0.5°C increments from 60°C in the first cycle to 55°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. Both sets of PCR were 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 model thermocycler (*PTC 200,* USA). PCR products were separated on 3% high quality Sigma agarose gels in 0.5 X TBE buffer (90mM Tris-borate and 2 mM EDTA, pH 8.0) at 70 V for 3 hrs. Gels were stained with ethidium bromide and visualized under gel documentation system (Alpha Imager, 2200, Alpha Innotech Corp., USA). Each band in case of isozyme and SSR was treated as distinct alleles. Band present is scored as 1 and absence as 0 in both isozyme and SSR markers. The percentage of sharing of bands among accessions and species was calculated accordingly.

Results and Discussion

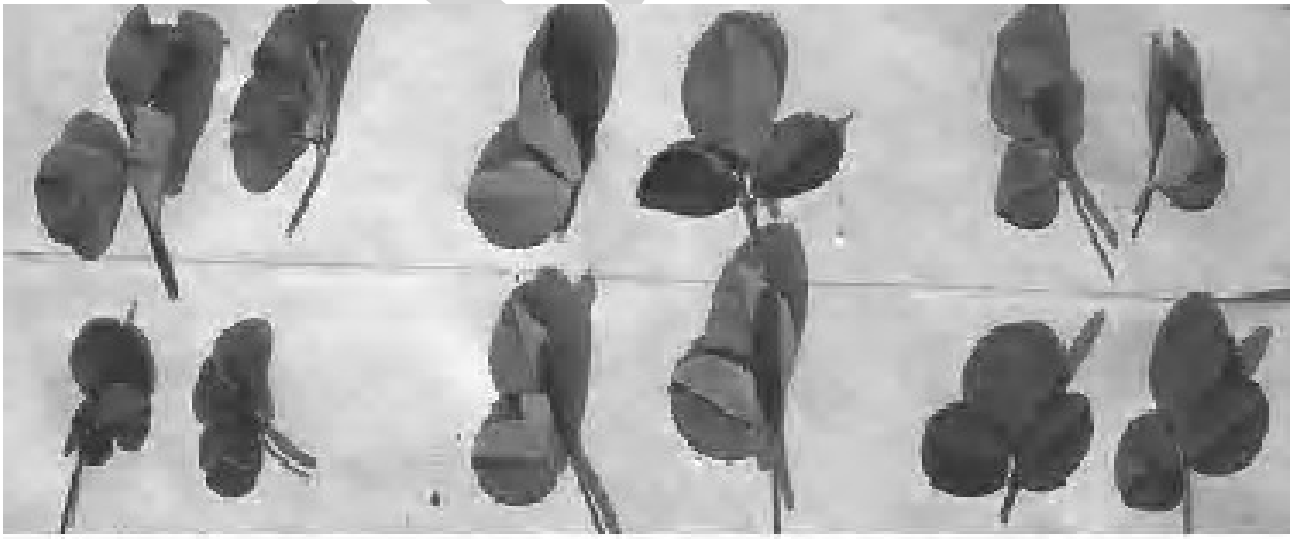
Of the 14 *Medicago* species possessing compatible levels to that of *M. scutellata*, *M. doliata* Carmign. and *M. muricoleptis* Tineo (both 2n = 2x = 16), and *M. murex* Willd. and *M. rigidula* (L.) All. (both 2n =2x =14) were morphologically identified as most similar species to the accessions of *M. scutellata* species. Leaf shape (Fig. 1), serration, presence of hairs on leaf and stem and growth habit of these species were close to each other, hence isozyme and SSR banding patterns were only analyzed of these species along with *M. scutellata*. In total, eight enzyme systems namely PRX, EST, SOD, PPO, GOT, MDH, AMY and ACP yielded 19 isozyme bands (Fig. 2). Glutamate oxaloacetate transaminase enzyme generated two bands of which one was exclusively present in *M. scutellata* (2n = 30) whereas other was observed only in other four morphologically similar species of *Medicago.* In case of superoxide dismutase, 4 isozyme bands which were present in *M. scutellata* were shared by four species namely *M. murex, M. rigidula,*

Table - 1: Original sequence accession codes of *M. trancatula* EST database, motifs of SSR, predicted length in base pairs (bp), annealing temperature (Tm), number of alleles and amplification range in bp obtained with the *Medicago* species

Sequence accession number	Primer sequences 5' 3'	Core motif	Tm (°C)	Predicted product length (bp)	Number of alleles and amplification range (bps)
AW698723	Forward GAAATTGAAGTTGGCTGGGA Reverse CCAAAATTCATTTCCTCCAAA	$(AAG)_5(A)_{10}$	60	112-161	(5) 80-240
AW698672	Forward AGCTCATTTTCACCACCGTC Reverse CCTCACCATTTTCCATTTCAA	$(TGC)_5$	60	141-147	(7) 50-290
CB858137	Forward ATGACTGCGTACCAATTCGC Reverse TCCTGAGTAAGCATTTCGCC	$(GAA)_5$	61	113-176	(2) 60-70
AW698894	Forward ACATTCAGCAGGAGGAGCAT Reverse CTGCAACCCAGACACTTTCA	$(GAT)_8$	59	172-209	(6) 60-240
AJ248338	Forward GTGGCAAAGGTGAACGACTT Reverse TGCTACCTACGCCACCTCTT	$(GGA)_5$	60	115-119	(2) 100-120
AJ410087	Forward TCTTGGCAAACTTGCAGATG Reverse CAGCAGCGAAGCGTAGTTAG	$(GAA)_7$	60	169-189	(4) 50-80

Table - 2: Comparative sharing of SSR and isozyme bands as amplified by *M. murex* and *M. rigidula (*2n = 2x = 14) and *M. muricoleptis* and *M. doliata* (2n = 2x = 16) with that of *M. scutellata* (2n =30). Percentage of sharing is given in parentheses

Species	Number of DNA fragments/ isozyme bands (%)
SSR markers	
SSR markers in *M. scutellata* shared by other species	25
SSR markers shared by *M. muricoleptis* (2n = 2x = 16) with *M. scutellata*	14 (56)
SSR markers shared by *M. murex* (2n = 2x = 14) with *M. scutellata*	17 (68)
SSR markers shared by *M. doliata* (2n = 2x = 16) with *M. scutellata*	17 (68)
SSR markers shared by *M. rigidula* (2n = 2x = 14) with *M. scutellata*	12 (48)
Isozyme bands	
Isozyme bands in *M. scutellata* shared by other species	19
Isozyme bands shared by *M. muricoleptis* (2n = 2x = 16) with *M. scutellata*	15 (79)
Isozyme bands shared by *M. murex* (2n = 2x = 14) with *M. scutellata*	14 (74)
Isozyme bands shared by *M. doliata* (2n = 2x = 16) with *M. scutellata*	16 (64)
Isozyme bands shared by *M. rigidula* (2n = 2x = 14) with *M. scutellata*	12 (63)



M. murex (2n=14) *M. scutellata* (2n=30) *M. doliata* (2n=16)

Fig. 1: Similarity of the trifoliate leaves between *M. murex* (2n =14), *M. scutellata* (2n = 30) and *M. doliata* (2n=16)

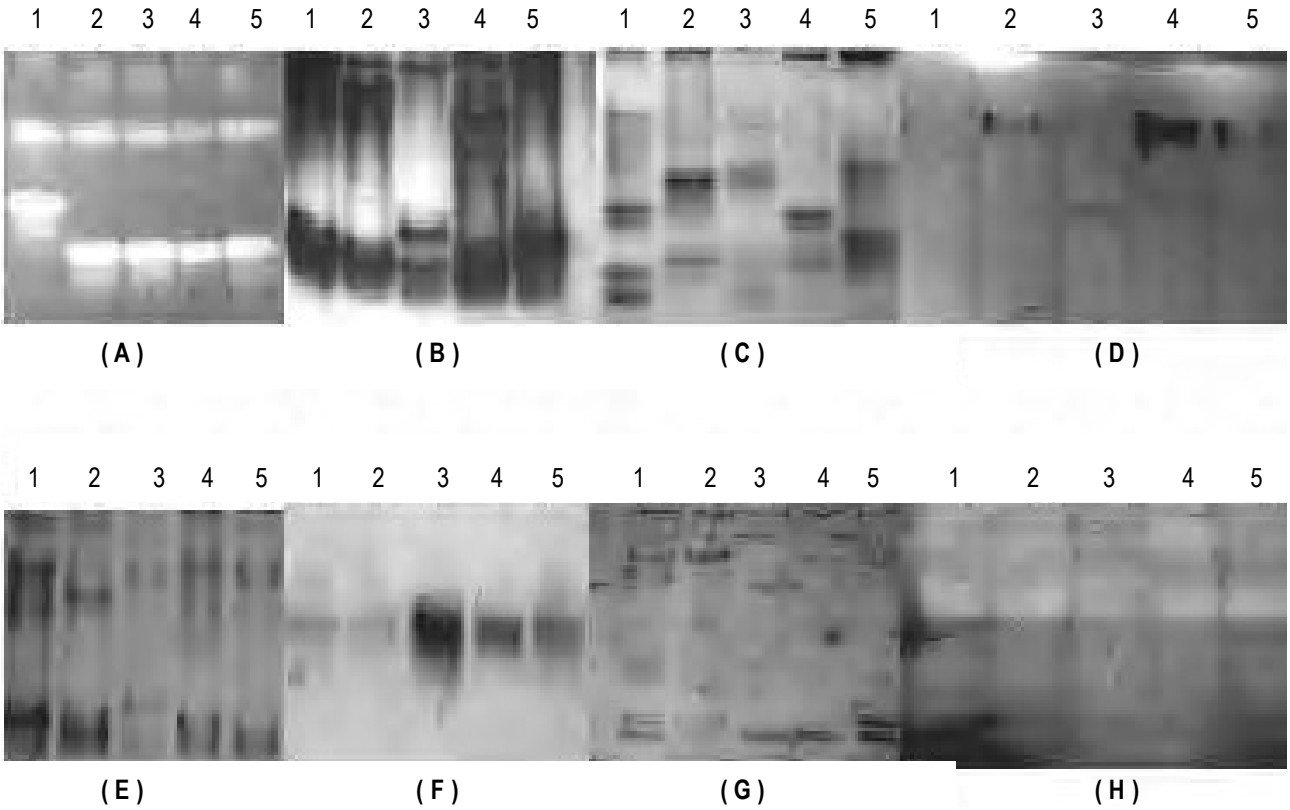


Fig. 2: Isozyme patterns of *M. murex* (lane 1), *M. rigidula* (lane 2), *M. scutellata* (lane 3), *M. muricoleptis* (lane 4) and *M. doliata* (lane 5) showing more similarity of isozyme bands among *M. murex* (2n =14), *M. scutellata* (2n = 30) and *M. doliata* (2n=16). Isozymes analyzed were, A = SOD, B = EST, C = ACP, D = GOT, E = MDH, F = PPO, G = PRX and H = AMY

M. doliata and *M. muricoleptis* representing ploidy levels of both 2n = 14 and 2n = 16 (two each) species. Similarly in case of polyphenol oxidase three bands observed in *M. scutellata* were shared by all four species. In total 19 isozyme bands which was visualized in *M. scutellata*, 14 was present in *M. murex*, 12 in *M. rigidula*, 15 in *M. muricoleptis* and 16 bands in *M. doliata* (Table 2). Of the four putative species representing 2n = 14 and 2n = 16, maximum sharing of isozyme bands was observed with *M. murex* from the category of 2n = 14 whereas *M. doliata* from 2n = 16. Thus, results postulate more closeness of these two species with *M. scutellata* over *M. muricoleptis* and *M. rigidula.*

In total 17 SSR markers derived both from EST and genomic based *Medicago* genome were tested in all 24 accessions of 5 species representing 2n = 14, 2n =16 and 2n = 30 (Fig. 3). In total 25 SSR fragments were shared by *M. scutellata* with other four species (Table 2). In category of 2n =14, maximum 17 bands was shared by *M. murex*, and in case of 2n = 16, maximum 17 bands was shared by *M. doliata*. The other two species namely *M. muricoleptis* showed sharing of 14 bands whereas minimum 12 bands by *M. rigidula* (2n =1 4) with *M. scutellata*. When results of all species were taken together, 23 bands were shared by four species namely *M. murex, M. rigidula*, *M. muricoleptis* and *M. doliata* with 25 bands of *M. scutellata*. Only two bands which was present in *M. scutellata* was not shared by other four species indicated a high level of sharing of bands among these species. Nevertheless, among 2n = 14 category of species maximum sharing of bands was observed with *M. murex* and in case of 2n =16, *M. doliata* indicated more closeness with *M. scutellata*. When same SSR primer sets were tested to visualize the closeness among *S. sativa* cv Anand-2 (Indian variety of lucerne) with *M. scutellata* a similarity of 17% was observed. Similarly *M. sativa* cv Anand-2 revealed 16 and 26% similarity with *M. murex* and *M. doliata* respectively. However, genome-specific markers like sequence-tagged-sites (STS) can be better suited in identifying species or progenitors as reported in other crops (Liu and Musial, 1997; Lem and Lallemand, 2003; Ma *et al.,* 2004).

From the present study it can be concluded that *M. murex* and *M. doliata* are closer to *M. scutellata* as sharing of both isozyme and SSR bands were higher among these species. Additionally, they were morphologically similar to *M. scutellata*. Accessions IL-04-223 and IL-04-151 of *M. doliata* and *M. murex* respectively

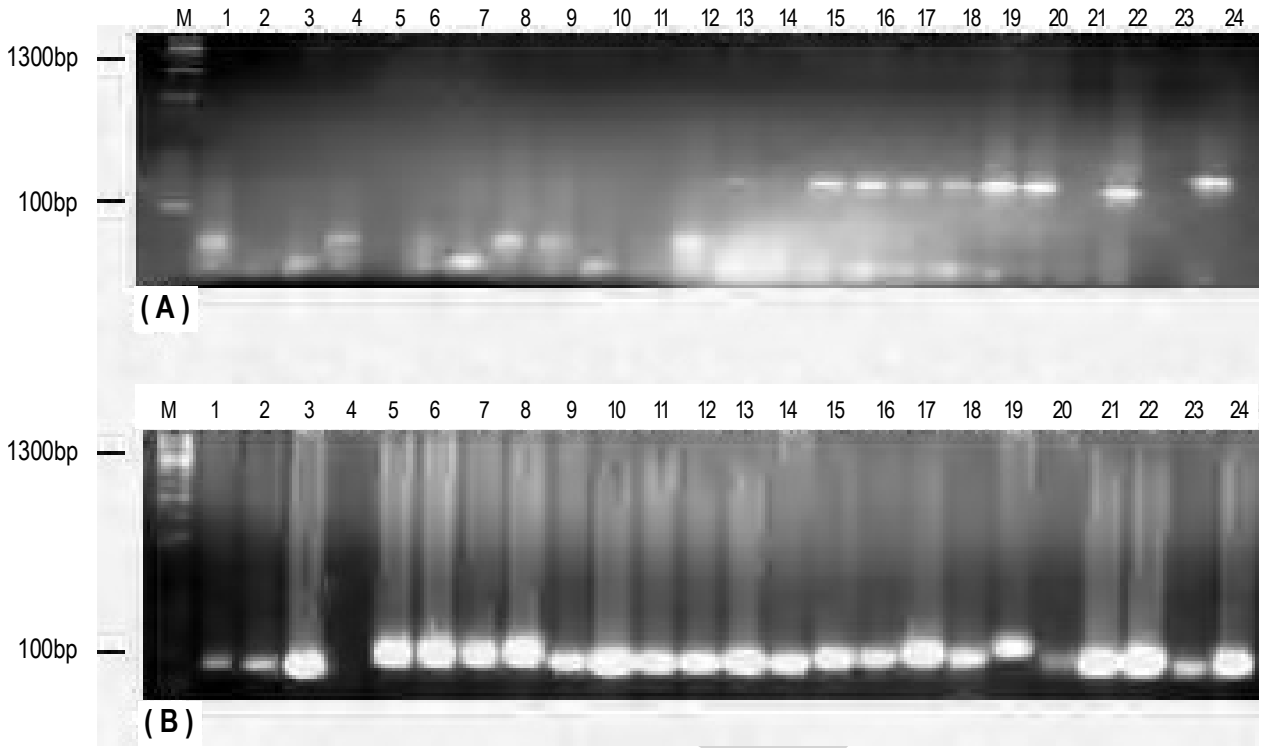


Fig. 3: Simple sequence repeats analysis with primer pairs AFca 1 (A) and AFct 45 (B) of three accessions of *M. muricoleptis* (lanes 1 to 3), five accessions of *M. scutellata* (lanes 4 to 8), six accessions of *M. murex* (lanes 9 to 14), six accessions of *M. doliata* (lanes 15 to 20) and four accessions of *M. rigidula* (lanes 21 to 24). M = 20 bp molecular weight marker

showing low levels of weevil infestation (< 5%) can be utilized as weevil resistant source in developing perennial weevil resistant lucerne through classical breeding or ovule-embryo culture approaches.

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