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Use of EST database markers from *M. truncatula* in the transferability to other forage legumes

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Abstract

In general tropical forage legumes lack microsatellites or simple sequence repeat (SSR) markers. Development of genic SSR markers from expressed sequence tagged (EST) database is an alternate and efficient approach to generate the standard DNA markers for genome analysis of such crop species. In the present paper a total of 816 EST-SSRs containing perfect repeats of mono (33.5%), di (14.7%), tri (39.3%), tetra (2.7%), penta (0.7%) and hexa (0.4%) nucleotides were identified from 1,87,763 ESTs of *Medicago truncatula*. Along with, 70 (8.5%) SSRs of a compound type were also observed. Seven primer pairs of tri repeats were tested for cross transferability in 19 accessions of forage legumes comprising 11 genera. At two different annealing temperatures (55 and 60°C) all primer pairs except AJ410087 reacted with many accessions of forage legumes. A total of 51 alleles were detected with six *M. truncatula* EST-SSRs primer-pairs against DNA from 19 accessions representing 11 genera where number of alleles ranged from 2 to 13. The cross-transferability of these EST-SSRs was 40.6% at 55°C and 32.3% at 60°C annealing temperature. 24 alleles of the total 50 (48%) at 55°C and 27 of 51 (53%) at 60°C were polymorphic among the accessions. These 27 polymorphic amplicons identified could be used as DNA markers. This study demonstrates the developed SSR markers from *M. truncatula* ESTs as a valuable genetic markers and also proposes the possibility of transferring these markers between species of different genera of the legumes of forage importance. It was evident from the results obtained with a set of *Desmanthus virgatus* accessions where Sequential Agglomerative Hierarchical and Nested (SAHN) cluster analysis based on Dice similarity and Unweighted Pair Group Method with Arithmetic mean Algorithm (UPGMA) revealed significant variability (24 to 74%) among the accessions. High bootstrap values (>30) supported the nodes generated by dendrogram analysis of accessions.

Key words

Forage legume, EST-SSRs, Genetic resources, *Medicago truncatula*, Transferability

Introduction

Both rainfed and cultivated forage legumes are important components of livestock feeding systems in tropical countries. However, limited production due to biotic and abiotic constraints the supplies have been badly affected world wide. The genetic improvement of such neglected crop commodities are largely hampered due to the non availability of the useful molecular markers which can be utilized in molecular mapping and hastening the breeding program. A part from *Trifolium alexandrinum* L. (Egyptian clover or 'berseem') and *Medicago sativa* L. (Alfalfa or 'lucerne'), the two major fodder legumes occupying more than 3 million ha land in India (Hazra, 1995), *Lablab purpureus*, *Macroptelium atropurpureum*, *Clitoria ternatea*, *Cyamopsis tetragonoloba*, *Desmanthus virgatus*, *Vicia narbonensis*, *Lathyrus sativus*, *Stylosanthes* species, *Vigna unguiculata* and *Melilotus parviflora* are also important tropical forage legumes grown discretely across the tropical and sub-tropical countries. Except *Medicago*, forage legumes of other genus are less molecularly characterized mainly due to the lack of adequate microsatellites based molecular markers and sequence information in public databases. Being one of the major winter annual forage legumes of tropical and subtropical countries including Egypt, India, Pakistan, Turkey and others in the Mediterranean region, *T. alexandrinum* (also referred as King of Fodder) has also not been characterized with SSR markers due to non-availability of such markers. Along with this, a significant number of germplasm of other species are maintained at National Bureau of Plant Genetic Resources (NBPGR), New Delhi and in mid-term module at Indian Grassland and Fodder Research Institute (IGFRI),

Jhansi. In lack of molecular data, germplasm of these legumes are described solely by morphological and agronomic characters. This may lead to slow utilization of germplasm in future. A large set of markers and that to be highly variable among accessions, easy to use and potentially high through put is required to assess the genetic diversity in many accessions of fodder legumes. Microsatellites or simple sequence repeats generally meet these requirements for assessing the genetic diversity (Yang *et al*., 1996; Brown-Guedira *et al*., 2000; Wang *et al*., 2004). As such isolation of microsatellites and development of SSR markers from genomic libraries for each species is expensive and time consuming (Powell *et al.,* 1996). Transferability of SSR markers from related or distant species has been postulated as an alternate strategy to characterize those germplasm where SSR are not available. Generally DNA sequence conservation in the primer sites flanking the microsatellite loci and the stability of these sequences during evolution largely decides the level of transferability of SSR markers across species or genera (Choumane *et al*., 2000; Decroocq *et al*., 2003; Zucchi *et al*., 2003). With the availability of the large numbers of expressed sequence tags (ESTs) and other sequence data, development of SSR markers through data mining has become a fast, efficient, and low-cost option for legume like *M. truncatula* (Danesh *et al.,* 2002; Mahalakshmi *et al*., 2002; Eujayl *et al*., 2004). This is due to the fact that the time consuming and expensive processes of generating genomic libraries and sequencing of large numbers of clones for finding the SSR containing DNA regions are not needed in this approach. A modest 1 to 5% of the ESTs in various plant species have been found to have SSRs of suitable length (20 nucleotides or more) for marker development (Kantety *et al.,* 2002). The large number of such SSRs is possible where great numbers of ESTs have been generated like *M. truncatula,* which is an annual forage legume and have been identified as model plant species for molecular biology work.

The transfer of SSR loci across species or genera has been reported in legumes (Peakall *et al.,* 1998; Eujayl *et al*., 2004; Wang *et al.,* 2004), tall fescue (Saha *et al.,* 2004, 2006), barley (Thiel *et al*., 2003; Varshney *et al*., 2005), paspalum (Wang *et al*., 2006), rice (Zhao and Kochert, 1993), wheat (Roder *et al*., 1995) and *Eucalyptus* (Louise dos Santos *et al.,* 2007). Many SSRs have been developed within the legume family (Diwan *et al.,* 2000; Barrett *et al*., 2004; Eujayl *et al*., 2004; Sethy *et al*., 2006) and have been successfully used in major crop like soybean (Cregan *et al*., 1999) and model plant *Medicago.* It has been shown that closely related species are more likely to share microsatellites priming sites than more distantly related ones, but it is possible to transfer functional microsatellite primers even from more distantly related species (Lorieux *et al.,* 2000). In general ESTs based SSR markers are more transferable (>50%) across species within a genus (Gattan-Solis *et al*., 2002) than those of genomic SSR across genera and beyond (White and Powell, 1997; Peakall *et al.,* 1998). This is largely because of the fact that EST-SSR are derived from transcribed regions of the DNA and thus more conserved (Scott *et al*., 2000), however the nature of EST-SSRs may limit their polymorphism. Another advantage of EST-SSR markers is that they may get associated with gene expression and gene function. Baquerizo-Audio *et al*. (2001), Danesh *et al.* (2002), Mahalakshmi *et al*. (2002) and Eujayl *et al*. (2004) have reported the development of EST-SSR markers from the *M. truncatula* EST database. However, they have either tested the developed markers within the *Medicago* genus or only limited to *Pisum sativum*, *Phaseolus vulgaris* and *Glycine max*.

The objectives of the study are to develop the SSR markers from the *M. truncatula* EST database and testing them in fodder legumes like *T. alexandrinum*, *Clitoria ternatea*, *Stylosanthes* species, *Macroptelium atropurpureum*, *Desmanthus virgatus*, *Cyamopsis tetragonoloba*, *Lablab purpureus*, *Lathyrus sativus*, *Vicia narbonensis* and *Melilotus parviflora*, to estimate the extant of transferability. The utility of transferred markers were tested in a set of *Desmanthus virgatus* accessions by estimating the genetic relatedness/ variability among them.

Materials and Methods

EST database and development of markers: The EST database of *M. trancatula* was extracted from the NCBI in FASTA format. A flow chart summarizing the individual steps of the data mining procedure is given in Fig. 1. EST sequences of larger than 700 bp were clipped at their 3' side to avoid the inclusion of low quality sequences in the further analysis (Thiel *et al.,* 2003). Once SSR–containing ESTs were identified, flanking primers were designed using Primer 3 software (Rozen and Skaletsky, 1998).

Experimental plant materials and DNA extraction: In the present investigation in total 22 genotypes of 12 tropical fodder legumes were used (Table 1) as plant materials. Additionally, 16 diverse genotypes of *D. virgatus* were used to assess the utility of the developed markers. Genomic DNA was isolated from seedlings as well as leaves (especially of *Desmanthus* accessions) using liquid nitrogen. Fine ground powder was mixed in 1: 2 ratio with CTAB extraction buffer 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. Instead of CTAB, buffer 'S' (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 50 mM EDTA pH 8.0, and 2% SDS) was used for isolation of DNA from stylo following the procedure of Liu and Musial (1995). The primer pairs were custom synthesized by Sigma Genosys, Bangalore, India.

Polymerase chain reactions: The PCR reactions of EST based generated primers were 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 (30 s) and extension of

72°C (30 s), 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 (5 min). Similarly the second set of PCR was carried out where 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). 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 primer, 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). In total seven EST-SSRs of *M. truncatula* designed primers were used in the present study. Although the annealing temperature for primers ranges from 50 to 60°C, touch down programme with 60 to 55°C and 55 to 50°C was used for all PCRs. To ensure that the results were reproducible each of the amplification was repeated at least twice. All PCR products were separated on a 3% agarose gel (High resolution Sigma agarose) in 0.5 X TBE buffer by electrophoresis for 4 hr at 70 volts. The PCR products were stained by ethidium bromide, visualized by UV light and sized by representing with a 20 bp DNA ladder on the same gel. For calculation of cross species amplification percentage, the amplifications from the primer-designed species were excluded.

Scoring of SSR bands and data analysis: SSR bands were scored for presence (1) and absence (0). Only bands present in two replicated PCRs were considered. Amplification failure of a sample or missing data was coded as 9. The NTSYS program version 2.0 was used to produce the similarity matrix (Simqual function). Dice similarity coefficient and unweighted pair-group method with arithmetic average (*UPGMA*) clustering algorithm were applied to the three marker data sets and SAHN clustering and tree display was followed for generation of dendrogram (NTSYS tree phenogram). The reliability of the generated dendrogram was also tested by bootstrap analysis using WinBoot software (Yap and Nelson, 1996) with 1,000 iterations.

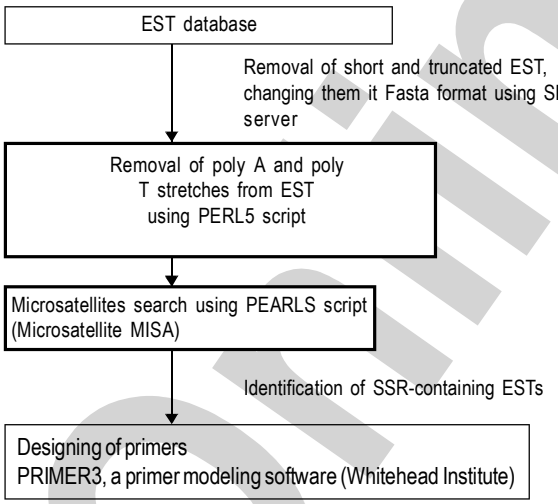

Fig. 1: Steps involve in development of EST-derived microsatellite markers. Removal of poly A and poly T and microsatellites search are achieved through Perl module (thick border) whereas operation of EST database like changing them in Fasta format and designing of primers were achieved through additional programs (lighter box)

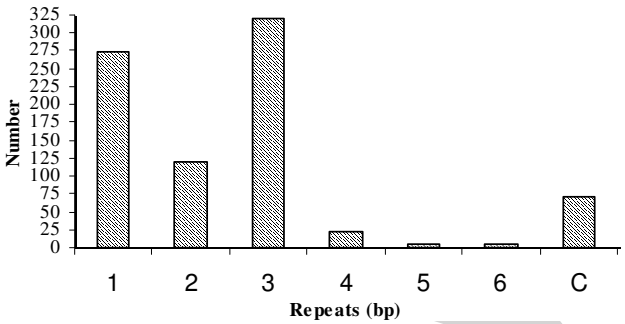

Fig. 2: Distribution of EST-SSRs of *Medicago truncatula* developed by *in silico* data mining. (C = compound microsatellites)

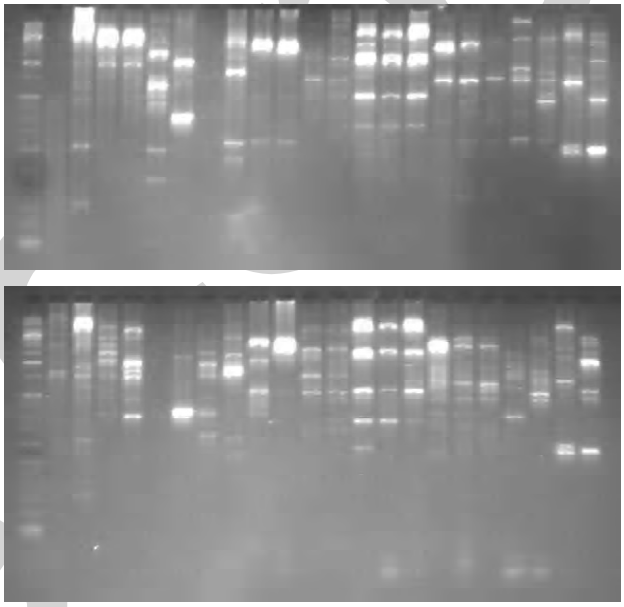

Fig. 3: Amplification of 22 accessions representing 12 genera of forage legumes with primer AW698894 at 55°C (top) and 60°C (bottom) annealing temperature (touch down). Only intense bands were considered actual SSR alleles. The faint bands were mostly non-specific and disappeared when PCR was done at fixed (60°C) annealing temperature. Loading of samples is as given in Table 1. M. = 20 bp DNA ladder as molecular weight marker

Results and Discussion

In total 816 EST-SSRs containing perfect repeats were observed from 187,763 ESTs of *Medicago truncatula.* In comparison to the earlier reports (Mahalakshmi *et al.,* 2002; Eujayl *et al*., 2004), EST-SSRs markers identified in the present study were less (0.4%) which might be due to the removal of many truncated and smaller

Table - 1: Details of the plant species used

No.	Accessions identification no.	Species with ploidy status	Common name	Source	Origin
1	CTIL07-3	*Clitoria ternatea* (D)	Butterfly pea	Indigenous collection	India
2	CITL01530-1	*Clitoria ternatea* (D)	Butterfly pea	Indigenous collection	India
3	BG-1	*Cyamopsis tetragonoloba* (D)	Guar/ cluster bean	Indigenous collection	India
4	BG-3	*Cyamopsis tetragonoloba* (D)	Guar/ cluster bean	Indigenous collection	India
5	BL-1	*Lablab purpureus* (D)	Lablab	Indigenous collection	India
6	IL04-125	*Vicia narbonensis* (D)	Vetch	Indigenous collection	India
7	IL-145	*Macroptelium atropurpureum* (T)	Siratro	Indigenous collection	India
8	EC539028	*Lathyrus sativus* (D)	Lathyrus	NBPGR, New Delhi	Syria
9	IC-791-2004	*Melilotus parviflora* (D)	Sanji	Indigenous collection	India
10	IC-792-2004	*Melilotus parviflora* (D)	Sanji	Indigenous collection	India
11	EC-401035	*Desmanthus virgatus* (D)	Hedge lucerne	ILCA, Addis Ababa	-
12	EC-401045	*Desmanthus virgatus* (D)	Hedge lucerne	ILCA, Addis Ababa	-
13	cv. Wardan	*Trifolium alexandrinum* (D)	Berseem	IGFRI, India	India
14	JHB-146	*Trifolium alexandrinum* (D)	Berseem	IGFRI, India	India
15	JHB-BB-3	*Trifolium alexandrinum* (T)	Berseem	IGFRI, India	India
16	EC408404	*Stylosanthes seabrana* (D)	Caatinga stylo	CSIRO, Australia	Brazil
17	CPI 41117A	*Stylosanthes fruticosa* (T)	African stylo	CSIRO, Australia	-
18	CPI 40292	*S. scabra* cv.Seca (T)	Shrubby stylo	CSIRO, Australia	Brazil
19	IGFRI-95-1	*Vigna unguiculata* (D)	Cowpea	IGFRI, India	India
20	EC541686	*Medicago scutellata* (D)	Snail medick	USDA, ARS	-
21	cv. LLC-3	*Medicago sativa* (T)	Lucerne	IGFRI, India	India
22	EC547747	*Medicago truncatula* (D)	Barrel medic	USDA, ARS USA	Cyprus

D= Diploid, T = Tetraploid

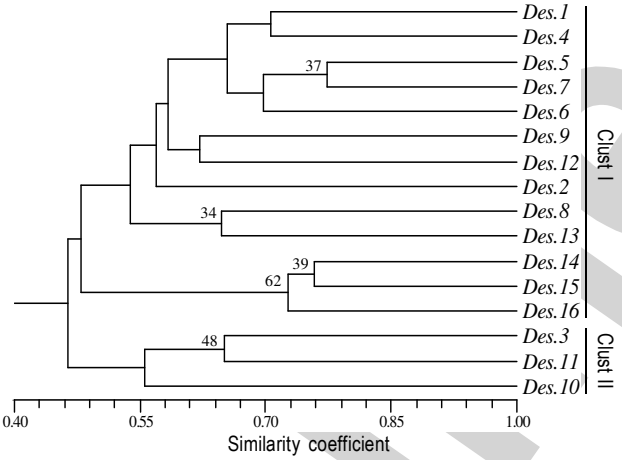

Fig. 4: UPGMA dendrogram for the *Desmanthus virgatus* accessions in study. Bootstrap values (>30) indicated on various nodes

ESTs as well as allowing computer software to use longer length of tandem repeats (>20 bp) in course of designing the primer pairs. The proportion of SSR unit sizes were not evenly distributed in the genome: 273 (33.5%) were mononucleotide, 120 (14.7%) dinucleotide, 321 (39.3%) trinucleotide, 22 (2.7%) tetranucleotide, 6 penta- and 4 hexanucleotide microsatellites (Fig. 2). Apart from this, 70 (8.5%) SSRs were compound type. Regarding dimeric SSRs, the motif AG (60%) was the most common, whereas no SSRs with CG were obtained. Low number of SSRs bearing GC motif have been also reported by Eujayl *et al.* (2004) in *Medicago*. Similar to genomic SSRs in soybean (Brown-Guedira *et al.,* 2000) and other legumes, the AT-rich SSRs were also found in the present study (30.8%) (Table 2). Among trimeric microsatellites, AAG (30.5%), AAC (13.4%) and ATA (7.2%) were the most-common motifs. The most-frequent tetrameric microsatellites motifs were AATT and GACA. As reported earlier (Thiel *et al.,* 2003) the dominance of trimeric SSRs in the present study can be explained by the suppression of non-trimeric SSRs in coding regions due to the risk of frame-shift mutations that may occur when those microsatellites alternate in size of one unit (Mertzgar *et al.,* 2000).

Cross genus amplification was considered only when the amplicons was generated from other genera but not from its own genus from which primers were designed (Wang *et al.,* 2004). Only clear and reproducible bands were considered as the amplified products across species or genera. Of the total seven primer pairs employed, only one primer AJ 410087 did not show reactions with any accessions at both annealing temperature. In total 63 at 55°C and 50 at 60°C reactions generated clear bands, representing 41 and 32.4% (63/134 and 50/134) of the total tested reactions (Table 3). The cross-transferability of these EST-SSRs in 11 tropical legumes was 40.6% at 55°C and 32.3% at 60°C annealing temperature. Twenty four alleles of the total 50 (48%) at 55°C and 27 of 51 (53%) at 60°C were polymorphic among the accessions. These 27 polymorphic amplicons identified could be used as DNA markers. Wang *et al*. (2004) reported 30.8% cross genus amplification of *Medicago*, soybean, cowpea, and pea nut SSR markers across the legume family. In comparison with the size of original amplicons (with *M. truncatula*) the cross genus amplifications varied greatly (110 to 1500 bp) (Table 4). Also with a primer pair,

Table - 2: Representation of the occurrence of SSRs in a set of 1,87,763 *M. truncatula* ESTs

SSR Motif	Number of repeats												Total
	5	6	7	8	9	10	11	12	13	14	15	>15	
A/T	-	-	-	-	-	111	50	34	26	11	9	25	266
C/G	-	-	-	-	-	-	-	-	5	1	-	1	7
AC/GT	-	5	1	2	1	1	1	-	-	-	-	-	11
AG/CT	-	20	11	2	3	4	6	4	3	3	1	15	72
AT	-	13	6	3	-	6	1	1	-	-	2	5	37
AAC/GTT	26	8	4	4	-	1	-	-	-	-	-	-	43
AAG/CTT	60	20	9	3	4	2	-	-	-	-	-	-	98
AAT/ATT	7	2	4	-	1	-	-	-	-	-	-	-	14
ACC/GGT	10	3	-	1	1	-	-	-	-	-	-	-	15
ACG/CGT	13	1	-	-	-	-	-	-	-	-	-	-	14
ATA/TAT	17	3	-	1	-	-	-	1	1	-	-	-	23
ACT/TGA	12	2	6	-	-	-	1	-	-	-	-	-	21
AGG/CCT	5	7	-	-	-	-	-	-	-	-	-	-	12
ATG/TAC	6	4	1	-	-	-	-	-	-	-	-	-	11
TCT/AGA	12	4	1	-	-	1	-	-	-	1	-	-	19
ATC/GAT	10	5	-	-	-	-	-	-	-	-	-	-	15
AGC/GCT	8	-	-	1	-	-	-	-	-	-	-	-	9
GTG/CAC	7	1	-	-	-	-	-	-	-	-	-	-	8
ACA	2	3	-	1	1	-	-	-	-	-	-	-	7
GAG/CTC	2	3	-	-	-	-	-	-	-	-	-	-	5
CTG/GAC	3	-	-	1	-	-	-	-	-	-	-	-	4
CGG	2	1	-	-	-	-	-	-	-	-	-	-	3
TCTT/TTAT	-	4	-	-	-	-	-	-	-	-	-	-	4
TTTC/GACA	5	-	-	-	-	-	-	-	-	-	-	-	5
AATT/TTTG	11	-	-	-	-	-	-	-	-	-	-	-	11
ACTC/ATGT	1	1	-	-	-	-	-	-	-	-	-	-	2
CAAAC	1	-	-	-	-	-	-	-	-	-	-	-	1
CAACT	-	-	1	-	-	-	-	-	-	-	-	-	1
AAACA	2	-	-	-	-	-	-	-	-	-	-	-	2
GGTTT	1	-	-	-	-	-	-	-	-	-	-	-	1
TTTAT	-	1	-	-	-	-	-	-	-	-	-	-	1
ACAATA	3	-	-	-	-	-	-	-	-	-	-	-	3
ATGAAC	1	-	-	-	-	-	-	-	-	-	-	-	1
Compound	-	-	-	-	-	-	-	-	-	-	-	-	70
Total													**816**

Table - 3: Reactivity of EST-SSRs of *M. truncatula* with other forage legumes

Species (Accessions)	Primers/ annealing temperature / reactivity (yes or no)/ total number of alleles					
	AW 698672 55°C/60°C	AJ 248338 55°C/ 60°C	CB 858137 55°C/ 60°C	AW 698723 55°C/60°C	AW 698869 55°C/60°C	AW 698894 55°C/60°C
Clitoria ternatea (2)	Y/Y (5/2)	Y/N (2/0)	N/N (0/0)	N/Y (0/1)	N/N (0/0)	Y/Y (6/8)
Cyamopsis tetragonoloba (2)	Y/Y (3/2)	N/N (0/0)	N/N (0/0)	N/Y (0/1)	N/N (0/0)	Y/Y (4/10)
Lablab purpureus (1)	Y/N (2/0)	N/N (0/0)	N/N (0/0)	N/Y (0/1)	N/Y (0/1)	Y/N (7/0)
Vicia narbonensis (1)	Y/N (1/0)	N/N (0/0)	N/N (0/0)	N/Y (0/2)	N/N (0/0)	Y/Y (3/1)
Macroptelium atropurpureum (1)	Y/N (1/0)	Y/N (1/0)	N/N (0/0)	N/N (0/0)	N/N (0/0)	Y/Y (1/6)
Lathyrus sativus (1)	Y/N (3/0)	Y/N (1/0)	N/N (0/0)	Y/N (1/0)	N/N (0/0)	Y/Y (2/5)
Melilotus parviflora (2)	Y/Y (3/1)	Y/N (1/0)	N/N (0/0)	Y/Y (1/1)	Y/N (1/0)	Y/Y (3/5)
Desmanthus virgatus (2)	Y/Y (3/2)	N/N (0/0)	Y/Y (1/1)	N/Y (0/1)	N/N (0/0)	Y/Y (3/5)
Trifolium alexandrinum (3)	Y/Y (3/3)	Y/N (2/0)	Y/N (3/0)	N/Y (0/1)	N/N (0/0)	Y/Y (7/9)
Stylo species (3)	Y/N (6/0)	Y/N (1/0)	N/N (0/0)	N/Y (0/1)	N/N (0/0)	Y/Y (4/12)
Vigna unguiculata (1)	Y/N (1/0)	N/N (0/0)	N/N (0/0)	N/Y (0/2)	N/N (0/0)	Y/Y (3/4)

Table - 4: Sequence of seven EST-SSR developed from *M. trancatula* EST database and their core motif, Tm (annealing temperature) and product profiles tested in 19 accessions of forage legumes representing 11 genera

Primers	5' end primer sequence (Forward)	3' end primer sequence (Reverse)	Core motif	Tm (°C)	Expected Product size (bp)	Obtained product size (~bp)
AW698723	gaaattgaagttggctggga	ccaaaattcatttcctccaaa	$(AAG)_5(A)_{10}$	60	112-161	200 and 320
AW698672	agctcattttcaccaccgtc	cctcaccatttttccatttcaa	$(TGC)_5$	60	141-147	200-1400
AW698869	aatgctactttcctagccaacc	cccaaaagcacttcactacctt	$(ACC)_7$	60	144-146	110
AW698894	acattcagcaggaggagcat	ctgcaacccagacactttca	$(GAT)_8$	59	172-209	140-1500
CB858137	atgactgcgtaccaattcgc	tcctgagtaagcatttcgcc	$(GAA)_5$	61	113-176	500-1500
AJ248338	gtggcaaaggtgaacgactt	tgctacctacgccacctctt	$(GGA)_5$	60	115-119	110-1000
AJ410087	tcttggcaaacttgcagatg	cagcagcgaagcgtagttag	$(GAA)_7$	60	169-189	No reaction

size and number of alleles varied in different accessions of forage legumes. The primer pairs AJ 248338 generated fragments of 700 and 1000 bp in *Clitoria*, 280 in *Macroptelium*, 500 in *Lathyrus,* 140 in *Melilotus*, 400 in *Trifolium alexandrinum* and 240 in stylo species whereas in *M. truncatula* the amplicon size was 110 bp. Similarly, four amplicons of sizes of 140, 200, 320 and 400 bps were observed in *M. truncatula* whereas different accessions of other forage legumes showed amplicons of different sizes along with 200 and 400 bps. Thiel *et al.* (2003) reported the product of the expected size only with 50% of EST-SSRs barley. Some of them amplified additional fragments and in 29% cases amplicons were larger in size than expected probably due to the presence of introns. Presumably it is more experience as the case here when SSRs are transferred from one genera to another. The PCR product size indicates variations in number of introns and size in the accessions of forage legumes belonging to different genera. However the amplification of introns may cause problems, since fragments above 500 bp cannot be scored accurately for small differences in fragment size. Additionally, it can be assumed that in several cases the observed polymorphism is caused by a size polymorphism within the intron, which may overshadow a putative polymorphism of the microsatellite (Thiel *et al.,* 2003). Though stringency of the PCR reaction was not much reduced (increased number of bands was observed at both annealing temperature), the non-specific amplification can not be ruled out totally as DNA of different accessions belonging to different genera were used for transferability study. However, number and size of amplicons were not much variable with *M. truncatula* and other two species of *Medicago* used along with DNA of forage legumes of different genera.

Primer AW698894 reacted with all accessions except lablab at 60°C annealing temperature (Fig. 3). Primers AW 698872 and AJ 248338 did not reveal polymorphism among three accessions of *Trifolium*, and three species of *Stylosanthes*. Similarly primer AW 698894 which has produced maximum alleles at both 55 and 60°C annealing temperature did not show polymorphism among guar, *Melilotus* and *Desmanthus* accessions. The number of polymorphic alleles (up to 8) as observed with different sets of primer pairs in general indicated more transferability of EST-SSRs in accessions of different tropical forage legumes. Seeing the number of alleles amplified with six sets of primer pairs, *Trifolium* showed maximum reactivity with *M. truncatula* EST-SSRs. Three accessions of *Trifolium* amplified as many as 15 alleles with 6 primer pairs whereas minimum 3 was obtained with *Macroptelium atropurpureum* (Table 3).

When 7 SSR primers (Table 4) were used in amplification of 16 accessions of *Desmanthus virgatus*, a total of 44 SSR bands were scored. Similarity ranged from 26 to 76% among accessions. The *UPGMA* phenogram obtained from the Dice similarity matrix revealed two main clusters (Clust 1 and Clust II) which showed 43% similarity to each other (Fig. 4). The cluster I was further divided into two sub-clusters. Significance among nodes generated by the dendrogram analysis of accessions was supported by high bootstrap values (>30) (Fig. 4). Results indicated divergence among the accessions of *D. virgatus* with the developed markers and thus the utility of markers of one genus to another in diversity analysis.

With increasing genome sequence information, the traditional classification will face new challenges. In the current context the transferable SSR markers in the forage legume will open new vista in characterizing such crops where development of SSR is not economical and time consuming. It is generally accepted that vertebrate SSRs can be easily transferred from one spe@cies to another (Moore *et al.,* 1991; Schlotterer *et al.,* 1991). However, less information is available about the easy transfer of plants SSRs. Wang *et al.* (2004) have reported the transfer and utilization of legume SSR across genera within the legumes. However, many reports are available on the transfer of SSR in closely related genus and that of largely non-leguminous crops (Mertzgar *et al.,* 2000; Saha *et al.,* 2004,2006; Wang *et al.,* 2006; Agarwal *et al.,* 2007).

The difference in rate of transferability of SSR markers can be attributed to sequence conservation of transcribed region of the genome among species, rate and dates of gene diversification which differ for different species as one locus can diverse faster than others, and stability of genome region (Ilic *et al.,* 2003). PCR stringency is also one of the important dependable factors which largely determine the rate of transferability hence this has to be monitored strictly while doing such experiments. Wang *et al.* (2004) have also reported the mis-priming as one of the important

factors in cross species and cross genera amplifications. Though it was expected that a higher percentage of amplification across species and genera would be achieved, however the cross genus amplification rate among *Medicago* and other forage legume (32%) was still lower than the rate (40 and 43%) from peach SSRs to apple and strawberry (Dirlewanger *et al.,* 2002) but it is higher than the rate (26%) from barley SSRs to oat (Li *et al.,* 2000). It would be more appropriate to test more primers with defined PCR conditions for better transferability of the SSR markers. Use of such transferred SSR markers in genetical study of those crops like tropical forage legumes where SSRs are not available opens a new avenue to use them for comparative mapping, functional analysis and assessing genetic diversity. The EST derived SSR with known gene functions can be utilized most appropriately for identification of traits of plant germplasm and thus will lead to the discovery of gene(s) associated with traits. However, caution must be taken that with increasing genetic diversity, less-likely orthologous sequences will be detected.

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