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Transferability of SSR markers across twelve species of forage legumes for germplasm characterization and evaluation

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Abstract

Simple sequence repeat (SSR) or microsatellite marker is the most preferred and reliable molecular marker system. However, development and use of such markers for evolution and diversity assessment is limited to few tropical forage legumes. In the present investigation, 43 SSR markers of *Medicago truncatula* and *Trifolium repens* from existing data bases were used to assess their transferability in 24 accessions of 12 tropical legume species of forage importance. Cross-genus amplification of *M. truncatula* based SSR was 32.08% whereas only 23.97% transferability of *T. repens* markers was observed. Seventy six polymorphic amplicons were identified could be used as DNA markers. Identified markers were demonstrated in characterization of 24 accessions of *Desmanthus,* thus indicated their suitability. The transfer of SSR markers across species or genus can be a very efficient approach for DNA markers development, especially for less explored legumes of tropical forage importance.

Key words: Forage legume, genetic resources, SSR, transferability

Introduction

In general tropical forage legumes are neglected crop commodity in terms of development and use of SSR markers particularly for evolution and diversity assessment. Though *Trifolium alexandrinum* (Berseem) and *Medicago sativa* (Lucerne) occupy more than 3 million ha land in India [1], other forage legumes like *Lablab purpureus, Macroptelium atropurpureum, Clitoria ternatea, Cyamopsis tetragonolobu, Desmanthus virgatus, Vicia narbonensis, Lathyrus sativus, Stylosanthes* species, *Vigna unguiculata* and *Melilotus parviflora* are also agronomically important and grown discretely across the country and other parts of the world. Except *Medicago,* forage legumes of other genus are less molecularly characterized mainly due to the lack of adequate simple sequence repeat (SSR) markers and expressed-sequence-tagged (EST) sequence information in the databases. Being one of the major forage legumes of the country, *T. alexandrinum* (also referred as King of Fodder) has not been characterized with SSR markers and no molecular breeding work has been taken up due to non-availability of such markers. Along with this, a significant numbers of germplasm of other forage 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. Because of non-availability of molecular data, germplasm of these forage legumes are described solely by morphological and agronomic characters. This may lead to slow utilization of germplasm in future. A large set of markers, 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 [2]. As such isolation of microsatellites and development of markers for each species is expensive and time consuming [3]. Transferability of SSR markers from related or distant species has been considered as an alternate strategy to characterize those germplasm where SSR are not available. Depending on sequence conservation in the primer binding sites flanking the microsatellite loci and the stability of those sequences during evolution, largely decide the level of transferability of SSR markers across species or genera [4]. The transfer of SSR loci across species or genera has been reported in many crop species [5-8]. Many SSRs have been developed within the legume family [5-7] and have been successfully used in major crops like soybean [8] and *Medicago* [5]. In the present investigation, 43 SSR markers of *Medicago truncatula* and *Trifolium repens* from existing data bases were used to assess their transferability in 24 accessions of 12 tropical legumes

species of forage importance and also to validate the markers in a subset of germplasm accessions of *Desmanthus.*

Material and methods

In total 24 accessions of tropical fodder legumes representing 12 genus were selected (Table 1). Seeds of most of the legumes were obtained from Indian Grassland and Fodder Research Institute, Jhansi mid term storage module. The accession or species chosen were based on their importance as forage legumes being grown under different agro climatic zones of the country and evaluated for different agronomical traits. To test the polymorphic markers, 24 accessions of *Desmanthus* were chosen for characterization.

Genomic DNA was isolated from seedlings as well as leaves (especially of *Desmanthus* accessions) using liquid nitrogen. DNA was extracted using 1 X CTAB and exclusively buffer 'S' (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 50 mM EOT A pH 8.0, and 2 % SDS) for *Stylosanthes* [9]. A set of 9 genomic based SSR markers of *Medicago* [9] and 28 EST based SSR primers of *T. repens* [10] were used in the present study. Besides, six new SSRs were designed from the ESTs of *M. truncatula* (Table 2). The PCR was performed in PTC 200 thermocycler (MJ Research, USA). The genomic DNA (25 ng) was used in 15 µl total volume with a final concentration of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.5 µM of each primers, 100 µM of each nucleotide and 0.5 unit/µl *Taq* DNA polymerase (Bangalore Genei, India). The PCR reactions 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 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. Although the annealing temperature for primers ranges from 50 to 60°C, touch down programme with 55 to 50°C was used for all amplifications. 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 V. For calculation of cross species amplification percentage, the amplifications from the primer-designed species were excluded.

Results and discussion

Cross genus amplification was considered only when the amplicons were generated from other genera but not from its own genus from which primers were designed [2]. Only clear and reproducible bands were considered as the amplified products across species to genera. In total 266 reactions generated clear bands, representing 28.9% (266/918) of the total tested reactions. In case of *M. trancatula* based primer maximum amplicons obtained were 4 whereas in case of *T. repens* it was 6. In comparison to *T. repens* (23.97%), primers of *M. trancatula* showed more cross species amplification (32.08 %). Wang *et al*. [2] reported 30.8 % cross genus amplification of *Medicago,* soybean, cowpea, and peanut SSR markers across the legume family. In comparison with the size of original amplicons, the cross genus amplifications varied greatly (80 to 700 bp), probably due to the presence of introns. Thiel *et al*. [11] reported the product of the expected size only with 50% of EST-SSRs developed and used among *Hordeum* species. The primer pairs prs256 from *Trifolium,* generated an amplicon of approximate 259 bp in *T. alexandrinum* but generate an amplicon of approximate 400 bp from stylo and an amplicon of approximately 750 bp from *Vigna* (Fig. 1). The primer pair Fct 45 from *Medicago* generated five amplicons of 123 to 156 bp in *Medicago* but generated amplicons of approximately 140 bp in *Melilotus* and 160 bp from *Desmanthus.* Primers AW698894, AFct32, AFcal, AFct45 from *Medicago* and primers prs461 and prs256 from *Trifolium* have amplified PCR products across all the genera tested (Fig. 1). Primer like prs256 of *Trifolium* gave polymorphism in most of the accessions of different genera but not in the same genus e.g., *T. alexandrinum* (Fig. 1). Out of six EST based SSR primers designed from *M. trancatula* based genome, three have amplified and generated more than one fragment in almost all genera, however in *M. scutellala* it has produced single band. These primers also showed polymorphism among accessions of few species (*Stylosanthes* and *Cyamopsis*) suggested easy transfer of *Medicago* EST derived SSR into other genera in comparison to *T. repens* EST derived SSRs. The degree of conservation for SSR transfer analysis across the legume of forage importance was very variable (Table 3). For example, *Medicago* genome as well as EST derived SSR transferred maximum in *Stylosanthes* (53.3%) and minimum in *Lablab* and *Vicia* (20% in each) whereas *T. repens* EST derived SSR transferred maximum in *T. alexandrinum* (46.4%) and minimum in *Vicia* and *Medicago* (10.7% in each). These

Table 1. Details of the plant species used in molecular analysis

S. No.	Ace. no./ identification no.	Species	Common name	Source	Origin
1	CTIL07-3	*Clitoria ternatea*	Butterfly pea	Indigenous collection	India
2	CITL01530-1	*Clitoria ternatea*	Butterfly pea	Indigenous collection	India
3	BG-1	*Cyamopsis tetragonoloba*	Guar/ cluster bean	Indigenous collection	India
4	BG-2	*C 'yamopsis tetragonoloba*	Guar/ cluster bean	Indigenous collection	India
5	BG-3	*Cvamopsis tetragonoloba*	Guar/ cluster bean	Indigenous collection	India
6	BL-1	*La blab purpureus*	Lablab	Indigenous collection	India
7	IL04-125	*Vlcia narbonensis*	Vetch	Indigenous collection	India
8	IL-145	*Macroptelium atropurpureum*	Siratro	Indigenous collection	India
9	IL-140	*Macroplelium atropurpureum*	Siratro	Indigenous collection	India
10	EC539028	*Lathyrus sativus*	Lathyrus	NBPGR, New Delhi	Syria
11	IC-791-2004	*Melilotus parviflora*	Sanji	Indigenous collection	India
12	IC-792-2004	*Melilotus parviflora*	Sanji	Indigenous collection	India
13	EC-401035	*Desmanthus virgatus*	Hedge lucerne	ILCA, Addis Ababa	-
14	EC-401045	*Desmanthus virgatus*	Hedge lucerne	ILCA, Addis Ababa	-
15	cv. Wardan	*Trifolium alexandrinum*	Berseem	IGFRI, India	India
16	JHB-146	*Trifolium alexandrinum*	Berseem	IGFRI, India	India
17	JHB-BB-3	*Trifolium alexandrinum*	Berseem	IGFRI, India	India
18	IG-411	*Stylosanthes hamata*	Caribbean stylo	ILRI, Ethiopia	Colombia
19	EC408404	*Stylosanthes seabrana*	Caatinga stylo	CSIRO, Australia	Brazil
20	CPI 41117A	*Stylosanthes fruticosa*	African stylo	CS1RO, Australia	-
21	CPI 40292	*Stylosanthes scabra* cv. seca	Shrubby stylo	CSIRO, Australia	Brazil
22	IGFRI-95-1	*Vigna unguiculata*	Cowpea	IGFRI, India	India
23	EC541686	*Medicago scutellata*	Snail medick	USDA, ARS	-
24	cv. LLC-3	*Medicago sativa*	Lucerne	IGFRI, India	India

Table 2. EST-SSRs markers, their sequences and core motif developed in the present study from *M. trancatula* EST database

Primers (Forward)	5' end primer sequence (Reverse)	3' end primer sequence	Core motif	Tm	Product size
AW698723	gaaattgaagttggctggga	ccaaaattcatttcctccaaa	$(AAG)_5(A)_{10}$	60°C	112-161
AW698672	agctcattttcaccaccgtc	cctcaccatttttccatttcaa	$(TGC)_5$	60°C	141-147
CB858137	atgactgcgtaccaattcgc	tcctgagtaagcatttcgcc	$(GAA)_5$	61°C	113-176
AW698894	acattcagcaggaggagcat	ctgcaacccagacactttca	$(GAT)_8$	59°C	172-209
AJ248338	gtggcaaaggtgaacgactt	tgctacctacgccacctctt	$(GGA)_5$	60°C	115-119
AJ410087	tcttggcaaacttgcagatg	cagcagcgaagcgtagttag	$(GAA)_7$	60°C	169-189

results indicate relatedness between genera. *Medicago,* for example, is more closely related to *Stylosanthes* than to *Lablab* and *Vicia.*

Some of the transferred polymorphic SSR markers were demonstrated in germplasm characterization of 24 accessions of *Desmanthus.* For example, marker prs055 and prs256 from *T. repens* was transferred to

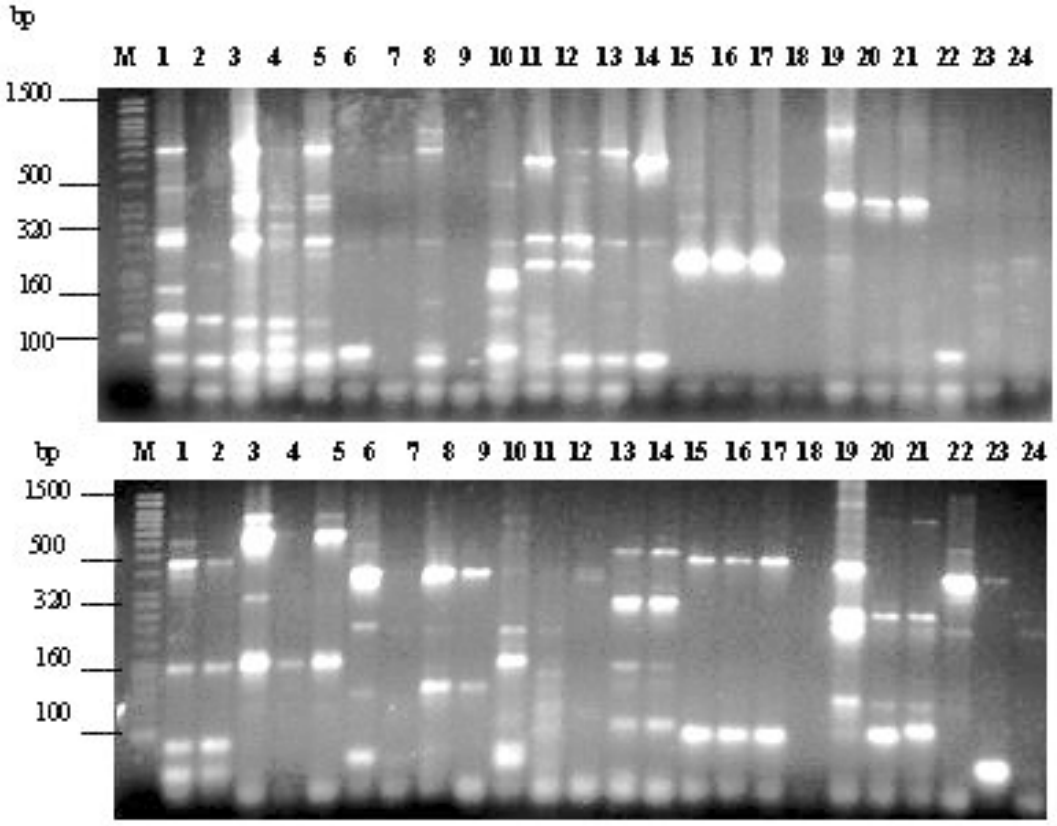

Fig. 1. PCR products generated with 24 different forage legumes with primer pairs prs256 (*T. repens*) (top) and AW698894 (*M. trancatula*) separated by electrophoresis on a 3% agarose gel. M = molecular marker of the 20bp ladder (Bangalore Genei, India), 1-24 plant materials as given in Table 1

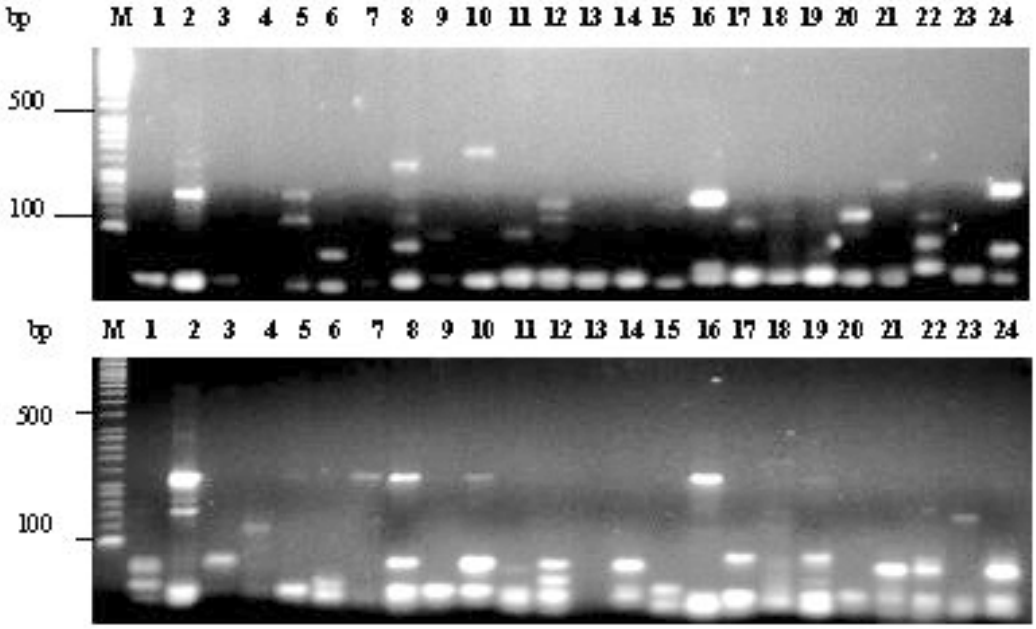

Fig. 2. Transferability of SSR markers and their use in characterization of 24 lines of *Desmanthus* by transferred SSR markers. Amplicons generated from markers prs055 (top) and prs256 (bottom) from *T. repens* were separated on a 3% agarose gel. M = molecular marker of the 20bp ladder (Bangalore Genei, India)

Table 3. Cross-genera amplification with transferred SSR markers

Primer source	Cross-genera amplification %												
	Primer set	*Medicago*	*Clitoria*	Guar	*Lablab*	*Vicia*	Siratro	Sanji	*Lathyrus*	Hedge	*Trifolium*	Stylo	*Vigna*
Medicago	15	46.6	46.6	26.6	20.0	20.0	26.6	33.3	33.3	33.3	33.3	53.3	26.6
T. repens	28	24.9	32.1	35.7	14.2	10.7	17.8	28.5	14.2	14.2	46.4	39.2	10.7

Desmanthus, it was polymorphic among the accessions of *Desmanthus* (Fig. 2). Since the polymorphism was so clear, these accessions were categorized into seven clusters by these markers on 3 % agarose gel. These accessions will be further characterized and evaluated by more polymorphic markers.

With increasing genome sequence information, the traditional classification can be more refined. In the current context the transferable SSR markers in the forage legumes 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 species to another [12]. However, less information is available about the easy transfer of plants SSRs. Wang *et al*. [2] 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 [13-16].

The difference in rate of transferability of SSR markers can be attributed to sequence conservation 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 [17]. 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*. [2] have also reported the mis-priming as one of the important factors in cross species and cross genera amplifications. Based on the available literature on transferability, lower annealing temperature (50°C) was used in the present study. 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 legumes (32%) was still lower than the rate (40 and 43%) from peach SSRs to apple and strawberry [18] but it is higher than the rate (26%) from barley SSRs to oat [19]. However, the transferred markers could be used to characterize and evaluate the fodder legume germplasm especially of *T. alexandrinum, C. ternatea, Stylosanthes* species, *M. atropurpureum, D. virgatus, C. tetragonoloba, L. purpureus, L. sativus, V. narbonensis* and *M. parviflora.* 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. Even the EST-SSRs 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.

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