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Original Research Article

Utilization Of SSR Markers For Identification Of Horse Gram (*Macrotyloma uniflorum* (Lam.) Genotypes

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Abstract: Horse gram is the poor man's pulse crop in southern India where the seeds are parched and then eaten after boiling or frying, either whole or as a meal. Horse gram (*Macrotyloma uniflorum*) varietal characterization and identification of distinguishing characters is most important in agricultural crops to assess genetic purity of seed in seed production programme. Characterization of twenty two horse gram genotypes *viz.,* AK-38, AK-42,AK-42C, BGM-1, BJPL-2, CRHG-4, CRHG-17, CRHG-18, HG-07, HG-34, PHG-9C, VLG-22, VLG-21, TCR-81, TCR-73, TCR-117, TCR-223, TCR-244, TCR-268, TCR-614, TCR-74 and PHG-9 was carried out with different SSR markers of the runner bean or scarlet runner bean (*Phaseolus coccineus),* which will be useful in broad classification of genotypes. A set of 10 SSR primers amplified 26 alleles from 22 horse gram genotypes. On an average of 2.6 alleles per marker were amplified. All most all the primers amplified minimum of 2 alleles. Some of the primers *viz.,* CA-910489, CA-911990, CA-911600 and CA-912170 amplified maximum of 3 alleles. Highly distinct genotypes among the selected germplasm were BGM-1 with TCR-74 and AK-38 followed by TCR-74 with TCR-614 and VLG-22 with TCR-223 which could be successfully utilized in the breeding programme and genetic purity testing of horse gram crop improvement.

Key words: Germplasms, NBPGR, SSR

Introduction

Horse gram (*Macrotyloma uniflorum (Lam.)* verdc. belongs to genera Macrotyloma, family Fabaceae (alt. *Leguminosae*), subfamily *Faboideae,* tribe *Phaseoleae,* subtribe *Phaseolinae*. It as synonyms of *Dolichos uniflorus* and *Dolichos biflorus* with chromosome number of 2n=20, 22 and 24. Macrotyloma comprises about 25 species; most of them are restricted to Africa. Within *Macrotyloma uniflorum* 4 varieties have been distinguished *viz.*, *Var. Uniflorum, Var. Stenocarpum, Var. Verrucosum and Var. Benadirianum.*

The origin of horse gram is not known but the region of maximum genetic diversity is considered to be in the Old World Tropics, especially in India and Himalayas (Zeven and de Wet, 1982). It is protein rich (20-24%) cattle feed largely used in south India. Although, horse gram is an important crop of south India and predominantly grown in about 2 million hectares, the latest survey indicates that its cultivation has been drastically reduced to nearly 0.6 million hectares, indicates its replacement by other crops. Horse gram is the poor man's pulse crop in Southern India where the seeds are parched and then eaten after boiling or frying, either whole or as a meal. The seeds are important food for cattle and horses and are usually given after boiling. The stems, leaves and husks are used as fodder. In Burma, the dry seeds are boiled, pounded with salt and fermented to produce a sauce similar to soy sauce from *Glycine max*. Horse gram is also grown as a green manure crop. It is also said to be good for patients suffering from urinary and kidney problem.

Until recently, the crop improvement programme in horse gram was not given the required attention and very few selections *viz.,* CO.1 (Tamil Nadu), Hebbal Huralli-1 and Hebbal Hurali-2 (Karnataka), Sina and man (Maharastra), HPK (Himachal Pradesh), BK-1 (Bihar) have been released for cultivation. The productivity level is still below the profitable line. Hence, there is immense scope to improve upon. Though, traditionally, it is being grown in South India, recently in North India substantial area is under horse gram cultivation. In this background, NBPGR regional station, Akola has assembled the

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germplasm under exploited or orphan multiutility economic crop. Several exploration trips have been under taken by the scientists of the NBPGR headquarters and its regional stations to capture the variability of this crop. However, the Akola regional station has been actively engaged in characterization, evaluation, documentation and conservation activities of this crop. Further, scope of crop improvement depends on the conserved line of genetic variability and diversity in the plant breeding programmes and use of new biotechnological tools. Despite its economical importance, horse gram molecular genetics and genomics have not been explored yet. So, as a first step, there is need to put effort for the isolation and characterization of genotypes. Characterization based on phenological and morphological characters usually varies with environments and evaluation of these traits requires growing the plant to full material prior to identification. Now, the rapid development of biotechnology allows easy analysis of a large number of loci distributed throughout the genome of plants. Recent trend of identification of genotypes is through simple sequence repeats (SSR).

SSR's are short sequence elements arranged in simple internal repeat structure (Tautz 1989) that are densely and randomly distributed throughout eukaryotic genomics. The number of microsatellite/ SSR has been shown to be highly variable within and between species and subspecies. Because of their high mutation rate, they constitute the molecular markers with the highest polymorphic information content. This characteristic has promoted the application of SSR's as molecular markers in fingerprinting (Weising *et al.,* 1995; Diwan and Cregan 1997; Shikawa *et al.,* 1999), genome mapping (Marion *et al.,* 1995; Broun and Tanksley 1996; McCouch *et al.,* 1997; Rader *et al.,* 1998; Winter *et al.,* 1999; Scotti *et al.,* 2000), marker assisted breeding and population genetics (Goldstein *et al.,* 1999).

Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationship within and among species (Cooke, 1999). In the present investigation the main objective was to assess / identify the efficiency of SSR markers in characterization of twenty two horse gram genotypes.

Material and methods

Plant material:

Authentic seeds of twenty two horse gram genotypes were procured from All India Coordinated Research Project (AICRP) on legumes ZARS, UAS, Bangalore and were listed in **Table 1**. The genotypes were raised in seed trays at National Seed Project, UAS, Bangalore.

Table 1: Elite genotypes of horse gram used for the study

1	AK-38	12	PHG-9C
2	AK-42	13	TCR-73
3	AK-42C	14	TCR-74
4	BGM-1	15	TCR-81
5	BJPL-2	16	TCR-117
6	CRHG-4	17	TCR-223
7	CRHG-17	18	TCR-244
8	CRHG-18	19	TCR-268
9	HG-07	20	TCR-614
10	HG-34	21	VLG-21
11	PHG-9	22	VLG-22

Sample preparation:

The leaf samples from 21 days old seedlings were collected and frozen in liquid nitrogen and isolated in at -80^{0}c freezers.

Isolation of DNA:

DNA was extracted as per the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Cao and Oard, 1997) with slight modifications. 1.5-2g frozen leaves were taken in 2 ml centrifuge tube with addition of 6 ml ice cold extraction buffer (0.1 M Tris, 0.5 M NaCl, 0.05 M EDTA and 0.01 M β-mercaptoethonol). The sample was grinded in to fine mixture by using fine grinder along with beads and the ground powder was incubated at 65^{0}C for 30 minutes with intermittent shaking. The mixture was centrifuged at 4000 rpm for 10 minutes at 4^{0}C, then supernatant was taken into separate tube and equal amount of wet chloroform was added and again centrifuged at 4000 rpm for 10 minutes. This step was repeated twice. For the final supernatant equal volume of ice cold isopropanol was added and kept in -20^0 C for overnight. The solution was centrifuged at 4000 rpm for 10 minutes and supernatant was discarded and the DNA pellet was washed with 70% ethanol. The dried DNA pellet was resuspended in 1 ml TE (Tris-EDTA) buffer and transferred to 2 ml eppendorf tubes. The final pellet was dissolved in 0.5 ml TE. The DNA concentration was determined by using "Nanophotometer" (procedure as given by manufacturer) and the

quality verified by electrophoresis on a 0.8% agarose gel.

PCR amplification:

The basic protocol reported by Williams *et al.,* (1990) for PCR was followed with slight modifications. The runner bean or scarlet runner bean (*Phaseolus coccineus)* is a plant in the Fabaceae family: these species primers were used for the study, because horse gram specific SSR markers are not yet developed. The PCR reactions were carried out in 20 µl reaction mixture containing template DNA (15 ng/µl), reaction buffer (10X) 1µl, dNTPs (2 mM) 1µl, 5 pmol of primer, Taq DNA polymerase (3 U/µl) 0.3 µl and 2 mM $MgCl_2$. The mixture was overlaid with one drop of mineral oil to prevent evaporation of the reaction mixture. Amplification was performed in a thermal cycler Bio rad for 35 cycles after an initial denaturation temperature 94^0C for 3 minutes, touch down 10 cycles (-0.5c/ cycle), denaturation 94^0C for 30 seconds, primer annealing 58^0C for 30 seconds, primer extension 72^0C for 1 minute, complete final extension 72^0C for 10 minute and hold temperature 4^0C until PCR plate was removed. SSR primers used for the study are listed in Table 2.

Table 2: SSR primers used for the study

Sl. No.	Primer Name	Forward sequence	Reverse sequence
1	CA906441	CTTCCCCACTGATTCATTTCTGTT	CACTCCCCTTTGTTCTTGATTATG
2	CA909226	GATCTCTTTCACGCTGGACAT	CACATAGCGACATGCTAGAACACT
3	CA910489	AGAAAAAGGAGGGAAATGCTGCTA	TTGCCTCCGCTTTCTTTATCTTTC
4	CA910598	CCTCCAATTCGGTTCCAGCACTTC	GCCCTTGATCTTCCTCTTTTTACG
5	CA911600	AACTGGTTCATCGCATTACTCTTC	TGGCATCACCTTGTTTGGCATTAG
6	CA911990	TTTCATCCCCAGCATCAGCAGTTT	TGGGGGTGGGGCAGAATCA
7	CA912170	TGGAGAAAGCATGGCAGGATACT	CCCCGGAGGCAAGATTTCAA
8	CA914165	GATAATTTTGACGAGGGAGCAGA	GGTGGAGAACAGGAGCAAAGTATT
9	CA912542	TTATTGGAGTTGCCCTGTTCTTCA	GCCCCGTCTGCATTCCTTTGT
10	CA906286	CGGGCGGGGGAGATTGT	CCCAGCCCACGTAAGGTAAGAAC
11	CA910928	CGGAATCTGGTCGGCCTACATCTA	TGATACCCCTCCGGCAACAAG
12	CA907856	ACACTTGAGGGAAAGCCATCA	TTCCAAGCCAAAGCAATAAC
13	CA906298	TAGTTGACCGGTTATCTGTTGTTT	GCGCCTACGAGCATCACTTA
14	CA912634	GACGGAATGAAGTTGAAGGTGGAT	AGATGGGCCAAGATATTTTAGACT
15	CA913150	CGGAGATGGTGGCGTGTTATT	CTTGTGCAGCCTTGTTTTCAGC
16	CA911895	CATCTGCTTTTGCCCATTACG	CACCAAGCCGCTCTAGCAAGTTAT
17	CA911460	GAGCTGCAACAAGGAATGGATG	ATGAGGGCTAACAGGAAAAGAAGG
18	CA902380	GGCCCCCTCAGACAGAT	AGATAAATTTGGGCTTGAA
19	CA907467	AGCCTGTCAATTCCAAACTCCACT	ATCCAACTTCAGCACCCAGACAAA
20	CA907742	TCGGTTGAAAAAGAAAAGCAGAAG	ATAGGCACTCAGGAAGGGAATGTC
21	CA900698	TTGTTCTCAGCGCAATGTGG	CTGAGCACCAAAGCCCCTGACA
22	CA900229	TGCCGAGTCATCCCGAACAT	CTCCAAAGCTGAAGCGAACCA
23	CA914163	ATGGTGAATGCCTGAAACAAATAC	CATGACAAATGCCTGAATAAGACT
24	CA912177	CTTGGTCCGCCATACTCTGAT	TTTTCTTTCCTCCTCTGGTATGG

DNA electrophoresis:

Amplified DNA fragments were separated out on 2.5% agarose gel in 1X TBE buffer stained with ethidium bromide. Running buffer containing Tris-base, boric acid and EDTA (p^H 8.3) was used for electrophoresis. Wells were loaded with 10 µl of reaction volume and 4µl of loading dye (sucrose and bromophenol blue dye) together. Electrophoresis was conducted at 40-75V for 5-8 hr and the gel photographed by using Vilber lourmat– Doc gel documentation unit.

DNA analysis:

The bands generated by SSR primers were scored by binary coding treating 11 for first level of band and 22 for second level of band and 33 for third level of band for a given primer. Analysis of banding pattern was carried on NTSYS ver 2.0. The program used was joining cluster analysis (tree clustering) with raw input data. The main parameters which guided the joining cluster process linkage rule was Unweighed Pair Grouping Average (UPGMA) and the distance was computed from raw data using squared Euclidean distances and Dendrogram was obtained.

Results

The quality of DNA isolated was obtained by electrophoresis and it was also confirmed by Nanophotometer reading. In both the cases quality of DNA observed was 50-500 ng/µl for all the genotypes.

All the twenty two genotypes were tested for DNA profiling to know the extent of genetic relatedness and for fingerprinting using microsatellite (SSR) markers. A set of twenty four SSR markers were used for molecular profiling. The products were run on 2.5 per cent agarose gel. Here only 10 SSR primers showed polymorphic bands out of twenty four primers for horse gram genotypes.

Genetic analysis:

A set of 10 SSR primers amplified 26 alleles from 22 horse gram genotypes. On an average of 2.6 alleles per marker were amplified. All most all the primers amplified minimum of 2 alleles. Some of the primers *viz.,* CA-910489, CA-911990, CA-911600 and CA-912170 amplified maximum of 3 alleles (Fig. 1 and Table 3).

Table 3: Number of alleles in different genotypes of horse gram

Locus	No. of alleles
CA906441	2
CA909226	2
CA910489	3
CA910598	3
CA911600	3
CA911990	3
CA912170	3
CA914165	2
CA912542	3
CA906286	2
Mean	2.6

Cluster analysis was based on similarity matrices obtained with unweighted pair group method analysis (UPGMA). Based on the shared allele's genetic similarity co-efficient was estimated for each pair of the 22 horse gram genotypes which ranged from 0.1 to 0.9. Except for AK-42, AK-42C, BJPL-2, CRHG-44, CRHG-17, CRHG-18, HG-34 and PGH-9C, the dendrogram clearly discriminated all the remaining horse gram genotypes. Genotypes AK-42, AK-42C, BJPL-2 and CRHG-4, CRHG-17 with CRHG-18 and HG-34 with PHG-9C were remained together with similarity of 90 per cent.

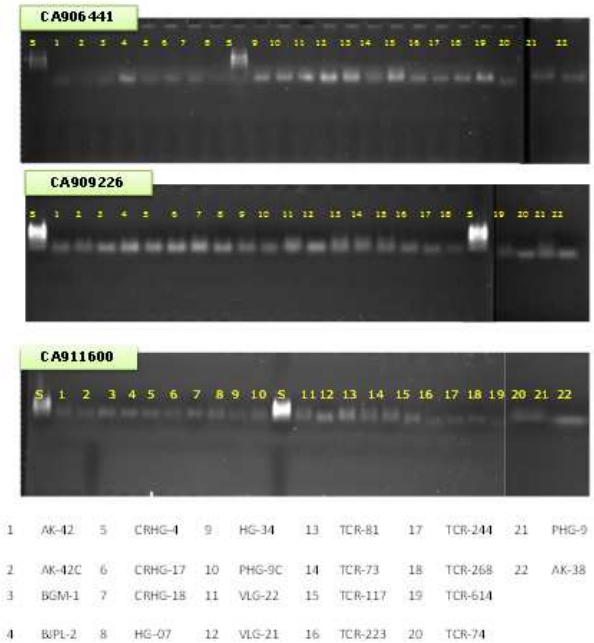

Fig.1: DNA profile of horse gram genotypes amplified by SSR primers

The minimum genetic similarity (10 per cent) in this study was observed between BGM-1 with AK-38 and TCR-74 with TCR-614 and VLG-22 with TCR-223. The cluster analysis grouped 22 horse gram genotypes into 2 main groups. Group A included 15 and

group B included 7 genotypes (Table 4). The major group was again subdivided into sub group I, II and III while 3 subclusters of group B was named as groups IV, V and VI. Genotypes AK-42, AK-42C, BJPL-2 and CRHG-4 showed no difference with each other and were grouped with BGM-1 at a genetic similarity of 88 per cent. The II sub cluster was again sub divided into 3 groups. CRHG-17 and CRHG-18 which are highly similar to each other were grouped with HG-34 and PHG-9C, with a genetic similarity of 88 per cent. Genotype PHG-9 and HG-07 were grouped separately with the above at a genetic similarity of 85 and 83 per cent, respectively.

The III sub cluster was again subdivided into groups with VLG-22 and TCR-81 and TCR-73 and TCR—117 at a genetic similarity of 89 per cent which in turn grouped at a genetic similarity of 85 per cent. The IV sub cluster consisted of VLG-21 and TCR-268 was grouped together at a similarity of 69 per cent. The V sub cluster consisted of TCR-223 and TCR-614, which were grouped at a genetic similarity of 69 per cent while, genotypes AK-38 was grouped separately with TCR-614 and TCR-223. Very low genetic similarity of 50 per cent and inturn was grouped with all the above clusters at genetic similarity of 30 per cent. The last sub cluster consisted of TCR-244 and TCR-74 with a genetic similarity of 69 per cent which inturn was grouped with all the above clusters at a very low genetic similarity of 28 per cent. SSR marker CA-910489 could differentiate maximum three genotypes (TCR-117, TCR-614 and PHG-9) followed by CA-910598 and CA-911600 in which three genotypes *viz.*, TCR-244, TCR-74, AK-38 and VLG-244, PHG-9 respectively. Thus, these five primers CA-910489, CA-910598, CA-91600, CA-911990 and CA-912170 could be used for differentiation of horse gram genotypes with the presence of specific bands.

Discussion

Genetic variation could be caused by genetic recombination and mutation. Although, high cleistogamic nature of horse gram can inhibit extensive gene flow between plants and populations, limited gene-flow would be allowed by insect pollination or wind. If any anonymous mutation occurred in plant, this plant spread these mutations by seed dispersal (Richards, 1997).

The polymorphism detected by these primer pairs did not correlate with the number of repeats in the microsatellite. Although the relationship between the degree of polymorphism and the number of repeats has been reported in some species (Saghai Maroof *et al.,* 1994; Fisher *et al.,* 1998), theoretically the number of repeats is correlated with the mutation rate and not with the degree of polymorphism (Brinkmamm *et al.,* 1998., Xu *et al.,* 2000). Polymorphism may correlate with the product of mutation rate and the generation term of the locus. More recently evolved markers would have fewer polymorphisms because of fewer occasions for mutation, even if they have longer repeats. The twenty two genotypes of horse gram used for characterize analysis were grouped into six clusters (Fig. 2). Among the twenty four primer pair tested, ten gave polymorphism in the tested genotypes. All sample analysis was conducted twice to test for reproducibility and only the reproducible and unambiguous bands were used for the analysis.

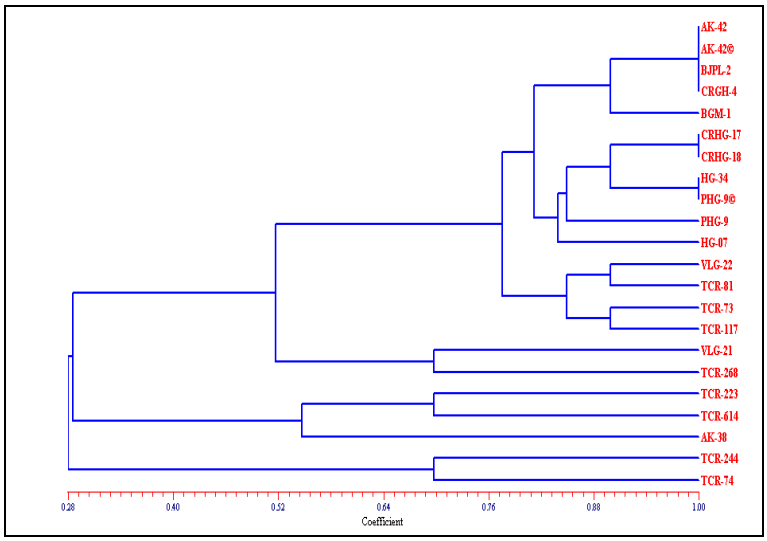

Fig. 2: Dendrogram based on genetic distance calculated from four SSR loci using UPGMA as the cluster method indifferent horse gram genotypes

The minimum genetic similarity (10%) in this study was observed between BGM-1 with AK-38 and TCR-74 with TCR-614 and VLG-22 with TCR-223. The cluster analysis grouped 22 horse gram genotypes into 2 main groups. Group A included 15 and group B included 7 genotypes (Table 4). The major group was again subdivided into sub group I, II and III while 3 subclusters of group B was named as groups IV, V and VI. Genotypes AK-42, AK-42C, BJPL-2 and CRHG-4 showed

no difference with each other and were grouped with BGM-1 at a genetic similarity of 88 per cent. A dendrogram was constructed using the UPGMA method. This approach successfully discriminated most of the horse gram genotypes tested. Cluster analysis with SSR markers resulted in six cluster group. On the basis of polymorphism data, genetic distance values were calculated that ranged from 0.28 to 1.00. TCR-74, AK-42, AK-42C, BJPL-2 and CRHG-4 were found to span the extremes of the dendrogram, with all other germplasm distributed in between at the maximum genetic distance of 0.79.

Table 4: Distribution of horse gram genotypes into different clusters using SSR markers

SI No.	Cluster No.	No. of sub clusters	Sub clusters	No.of the genotypes	Name of the genotypes
1	I	III	I	5	AK-42, AK-42C, BGM-1, BJPL-2, CRHG-4
			II	6	CRHG-17,CRHG-18 HG-07, HG-34, PHG-9 PHG-9C
			III	4	TCR-73, TCR-81, TCR-117, VLG-22
2	II	III	I	2	TCR-268, VLG-21
			II	3	AK-38, TCR-223, TCR-614
			III	2	TCR-74, TCR-244

All TCR and VLG series genotypes except AK-38 were grouped at the lower part of the dendrogram which are all morphologically dwarf type with plant height < 50 cm except TCR-268 and few of the genotypes has heavy test weight and seed shape of kidney in this cluster. Rest of the genotypes like AK, CRHG, HG, PHG, BGM and BJPL were grouped at the upper part of the dendrogram which were morphologically tall genotypes with values ranging from 50 to 70 cm and few of the genotypes had spreading growth habit except AK-42 and AK-42C.

Conclusion

Our study confirmed a high potential of specific microsatellite as excellent molecular markers for horse gram genotypes identification, differentiation and evaluation of their genetic variation. Highly distinct genotypes among the selected germplasm were BGM-1 with TCR-74 and AK-38 followed by TCR-74 with TCR-614 and VLG-22 with TCR-223 which could be successfully utilized in the breeding programme of horse gram crop improvement. The study also shows that there is cross compatibility of markers between horse gram and runner bean.

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