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ASSESSMENT OF MOLECULAR DIVERSITY AND ESTABLISHING PHENOTYPIC RELATIONSHIPS IN FEMALE AND MALE GENOTYPES OF SPINE GOURD (*Momordica dioica* Roxb.)

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Spine gourd (*Momordica dioica* Roxb.) is a highly nutritious vegetable crop with dioecious reproductive nature. Forty-eight spine gourd genotypes including 32 female and 16 male genotypes were assessed for molecular divergence to establish phenotypic relationships using ISSR markers. Twenty-two out of a total of 25 ISSR primers studied yielded a total of 88 bands of which 80 bands were polymorphic, with three of them being unique in their profile. Each primer thus produced a mean of 4.0 bands per marker, with 3.64 mean polymorphic bands per marker. Fifteen primers showed 100 percent polymorphism**.** In the dendrogram, genotypes were distinguished from each other with a similarity range of 0.465 to 0.959. A wider range of molecular diversity detected by ISSR markers reflected the presence of a high level of genetic variation forming different 5 broad groups of clusters. The clustering pattern based on molecular variation during this investigation revealed five clusters; of which cluster three had twenty-eight (all 16 malealong with 12 female genotypes) genotypes; while cluster 4 and 5 were mono-genotypic.

Key words: cucurbit, dioecious, divergence, ISSR analysis, Spine gourd

INTRODUCTION

Spine gourd also known as Teasle gourd or kartoli, belongs to the family Cucurbitaceae under the genus Momordica. Momordica genus (annual or perennial climbers) contains around 60 species (SCHAEFER and RENNER, 2010). This genus is native to tropical regions of Asia with extensive distribution in China, South East Asia, Japan, Polynesia besides tropical Africa and South America. As many of the species of this genus have been found to grow wildly in South to East Asia (NAGARANI *et al*., 2014) it indicated that this region might be the origin of *Momordica dioica*. The genus Momordica possesses different sex forms (monoecious, dioecious, hermaphrodite) and has basic chromosome number x=14. Spine gourd, *M. dioica* Roxb. (2n = 2x

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= 28) and its wild relative, *M. cochinchinensis* Spreng. (2n = 4x = 56) are dioecious and mainly propagated vegetatively through tuberous roots (SARKAR *et al*., 2017). Both the species are perennial, tuberous rooted but they differ in their stem, leaf, fruit and seed characters. It grows in warm and humid weather and tuberous roots are planted in pits. In India, it grows widely near the hills of Peninsular Plateau and Norh East as well as in the deserts of Rajasthan, and several others pockets of south India and Andaman Islands (NAGARANI *et al*., 2014).

The tender green fruits of the spine gourd are used for culinary purpose. Sometimes fruits are sliced, blanched and dried for further use. In some regions, the young tender leaves are used as vegetable (NAGARANI *et al*., 2014). Besides being a vegetable, the seeds of spine gourd yield oil which can be used as an illuminant and also as an admixture with drying oils in the formulation of paints and varnishes. Its root froths in water and therefore, is used as washing soap.

These plants exhibits various medicinal properties like anti-diabetic, anti-cancer, anti-fertility abortifacient, anti-inflammatory, antioxidant activity and cures jaundice and bleeding piles (BAWARA *et al.,* 2010; TALUKDAR and HOSSAIN, 2014; NAGARANI *et al*., 2014; HASSAN *et al*., 2022). Phytochemical investigations have revealed the presence of traces of alkaloids and ascorbic acid in fruits. Owing to its multifold uses, its systematic cultivation would be a boon both for horticulture as well as the pharmaceutical industry, especially in tropical countries like India. Fruits contain the high amounts of protein, calcium, phosphorous, iron, and highest amount of carotene amongst the cucurbitaceous vegetables (BHARATHI *et al.,* 2007).

It being an underutilized vegetable, extensive research program has not been given towards the improvement of this crop in India. Collection, evaluation of the existing germplasm and selection of better parents are the prerequisite for commencing a breeding program in any crop like spine gourd. Besides, knowledge of genetic diversity and relationship among the sets of germplasm is critical to accelerated plant improvement (YADAV *et al*., 2020). For quick reference, variability based on morphological and physiological features are handy to use in preliminary screening. In spite of its many advantages, only a few studies have been conducted to analyze the genetic relatedness, diversity, and cultivar identification among spine gourd cultivars using molecular markers.

Various DNA-based molecular markers have been used for cultivar or species identification in a variety of plant species. ISSR markers have been employed in genetic diversity analysis of different cucurbits (DJE *et al.,* 2006; SARKAR and SINGH, 2018; BORGES and DE MELO, 2019; VASAVA *et al.,* 2021; PANDEY *et al.,* 2021). The phylogenetic relationship among the different monoecious and dioecious Momordica species has also been studied using plastid and mitochondrial DNA based markers (SCHAEFER and RENNER, 2010; RAMESH *et al*., 2022). No systematic efforts have yet been made to understand the existing diversity pattern and phonetic relationships among the cultivated varieties included under Indian Momordica spp., using molecular markers. Molecular marker technique is fast and independent of environmental variations and provides a simple methodology for analysis of plant material at any stage of development. ISSR marker has proven quite useful in the genetic study of many plant species.ISSRis a universal dominant marker, which does not require target sequence information for the designing of primers. ISSR marker system has the ability to amplify DNA from dispersed polymorphic loci in between SSR repeats that are distributed throughout the genome and hence have the power to resolve low genetic divergence (MOKATE *et al*., 2017; BHARATHI *et al*., 2012).

Hence, this research work was aimed to study the diversity among male and female genotypes of spine gourd by using molecular markers.

MATERIALS AND METHODS

Materials

The molecular diversity among female and male genotypes of spine gourd was conducted during *Kharif* 2014-2015 at State Level Biotechnology Centre, Mahatma Phule Krishi Vidyapeeth (M.P.K.V.), Rahuri. Thirty-two female and sixteen male genotypes of spine gourd (*Momordica dioica* Roxb.) were obtained from the Plant Breeder, All India Coordinated Research Networkon Potential Crops, M.P.K.V., Rahuri for this study (Table 1).

Table 1. List of genotypes used for study

Sr.No.	Code	Genotype	Sr. No.	Code	Genotype
Female genotypes					
1.	F_1	RKFG-10-1-12	17.	F_{17}	RKFG-11-5-4
2.	F_2	RKFG-10-1-14	18.	F_{18}	RMF-37 (NC)
3.	F_3	RKFG-10-2-2	19.	F_{19}	RMFG-09-6
4.	F_4	RKFG-10-2-6	20.	F_{20}	RMFG-09-3
5.	F_5	RKFG-10-2-3	21.	F_{21}	RMFG-09-4
6.	F_6	RKFG-10-2-10	22.	F_{22}	RMFG-09-23
7.	F_7	RKFG-10-2-12	23.	F_{23}	RMFG-09-34
8.	F_8	RKFG-10-3-2	24.	F_{24}	RMFG-09-19
9.	F_9	RKFG-10-3-4	25.	F_{25}	RMFG-09-22
10.	F_{10}	RKFG-11-4-3	26.	F_{26}	RMFG-09-33
11.	F_{11}	RKFG-11-4-4	27.	F_{27}	RMFG-09-21
12.	F_{12}	RKFG-11-4-5	28.	F_{28}	RMFG-09-1
13.	F_{13}	RKFG-11-4-10	29.	F_{29}	RMFG-09-26
14.	F_{14}	RKFG-11-4-14	30.	F_{30}	RMFG-09-29
15.	F_{15}	RKFG-11-5-2	31.	F_{31}	RKFG-10-3-10
16.	F_{16}	RKFG-11-5-3	32	F_{32}	RKFG-10-3-3
Male genotypes					
1.	M_1	RKMG-10-2	9.	M_9	RKMG-09-17
2.	M_2	RKMG-10-3	10.	M_{10}	RKMG-09-30
3.	M_3	RKMG-10-4	11.	M_{11}	RKMG-09-31
4.	M_4	RKMG-10-5	12.	M_{12}	RKMG-09-29
5.	M_5	RKMG-09-2	13.	M_{13}	RKMG-09-34
6.	M_6	RKMG-09-5	14.	M_{14}	RKMG-09-21
7.	M_7	RKMG-09-8	15.	M_{15}	RKMG-09-18
8.	M_8	RKMG-09-11	16.	M_{16}	RKMG-09-14

Isolation of genomic DNA

The DNA was isolated from each female and male genotype by following the modified

CTAB DNA extraction method (SAGHAI–MAROOF *et al.,* 1984). The genomic DNA isolated from the individual plant was quantified spectrophotometrically both at 260 ηm and 280 ηm (Nanodrop, ND-1000). The genomic DNA samples with an OD 260/280 ratio of 1.6-2.0 were considered pure and were diluted to working concentrations for further work.

PCR reaction mixture for ISSR markers

The purified genomic DNA extracts of all the genotypes were used as template DNA. PCR amplification was performed with 25 Inter simple Sequence Repeats (ISSR) primers custom synthesized from M/s Bangalore Genei Pvt. Ltd (Table 1). The amplification reaction mixture of 20 µl volume was prepared in 0.2 ml thin-walled flat cap PCR tubes, with a reaction mixture consisting of 1 X *Taq* DNA polymerase Buffer A (with $MgCl_2$), 0.5 mM dNTP mix (0.125 mM each), 1unit *Taq* DNA Polymerase, 20 picomoles of each ISSR primer, 20 ng template DNA.

For PCR amplification of ISSR markers, the thermal profile comprised of initial denaturation for 5 minutes at 94ºC, followed by a PCR regime comprising 40 cycles of denaturation (30 Sec. at 94ºC), annealing (30 Sec. at 43-53ºC) and extension (1 minute at 72ºC), followed by final extension for 10 minutes at 72ºC and finally held at 4ºC till samples were removed.

Gel Electrophoresis

The PCR amplified DNA from 32 female and 16 male genotypes were subjected to1.2% agarose gel electrophoresis in1X Tris Acetate EDTA buffer. A voltage of1.5 V/cm was given for a period of three hours for the separation of PCR fragments. After the run, the gel was viewed under UV light and the DNA banding pattern was imaged directly in the gel documentation unit (gel doc). The banding pattern itself was noted from the digital image of the gels and analyzed further for identifying polymorphic bands.

Data analysis

The clearly resolved PCR products of spine gourd with 25 different ISSR primers were scored manually for their presence (1) and absence (0) in a datasheet. The analyses were carried out using the computer package NTSYSpc 2.02i (ROHLF, 1998). Binary Data matrix was analyzed using SIMQUAL module with Dice similarity coefficients calculated as per model. SAHN module based on Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) method was employed for cluster analysis to generate a tree (dendrogram).

RESULTS AND DISCUSSION

Quality and quantity of genomic DNA of spine gourd genotypes

In the present study average ratio of absorbance 260/280 recorded was 1.81 with the average DNA extracted being 567.58 µg/gm of fresh leaves tissue in spine gourd genotypes (32 female and 16 male genotypes). Similarly, SIMON *et al.* (2009) isolated DNA from fresh leaves tissue by using the CTAB method and reported an amount of DNA ranging from 945-1465 µg/gm with an average 260/280 ratio of absorbance for the sample was 1.77, which was optimal for PCR amplification.

Molecular marker analysis of spine gourd

During the present study, a total of 25 primers were used out of which 22 yielded PCR amplification products (Figure 1), while three primers viz., IS8932816, UBC890 and IS8080 did not amplify. A total of 88 amplicons were generated of which 80 amplicons were polymorphic (including 3 unique bands) with an average of 90.90 percent polymorphism (Table 2). Three amplicons were unique and 8 amplicons were monomorphic in their profile. Each primer thus produced an average of 4.0 amplicons per primer and 3.64 polymorphic amplicons. Fifteen primers showed 100 percent polymorphism. Primer IS-8336 produced less polymorphism (50%) with two monomorphic amplicons.

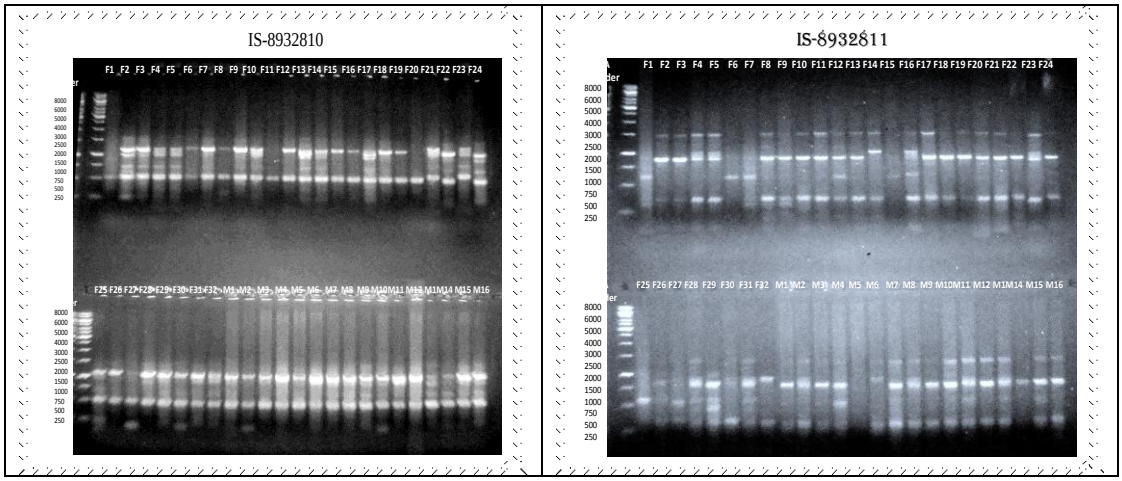

Figure1. ISSR Profile representing genetic variability present among the female and male genotypes of Spine gourd

DEY *et al.* (2006) screened 38 genotypes of *M. charantia* with 116 random decamer primers of which 29 were polymorphic and informative enough to analyze these genotypes. Similarly, RASUL *et al.* (2007) reported genetic relationship and variation of 29 accessions of teasle gourd (*Momordica dioica* Roxb.) and 1 accession of *Momordica cochinchinensis* Spreng. (Wild relatives of teasle gourd) by RAPD analysis using 44 decamer primers. The results of our findings are also in conformity with these studies. RAI *et al.* (2012) assessed the genetic stability of the regenerated plants using RAPD markers, the amplification products were monomorphic. SIKDAR *et al.* (2010) used biochemical and molecular markers on eleven species of Cucurbitaceae among 40 primers examined, 14 RAPD and 10 ISSR primers selected for the analysis generated RAPD (100) and ISSR (100) fragments showing high variations among the species. BHARATHI *et al.* (2012) studied genetic diversity for establishing phenetic relationships among 35 Momordica and five Luffa genotypes by using twenty-one RAPD and twelve ISSR primers. A total of 436 RAPD

and 230 ISSR fragments were produced of which 99.8% fragments showed polymorphism. The varieties belonging to dioecious Momordica species (75.6%) showed a higher level of polymorphism.

Table 2. Details of ISSR primers used for the present study

Sr. No.	Markers	Number of amplicons generated	Number of polymorphic amplicons (Excluding unique)	Number of monomorphic amplicons	Unique amplicons	Annealing temp. (ºC)
1	IS-834	3	3	-	-	51
2	IS-857	4	3	1	-	51
3	IS-827	4	3	-	1	46
4	IS-8336	4	2	2	-	46
5	IS-12	4	4	-	-	47
6	IS-8932805	6	4	1	1	47
7	IS-8932815	4	3	1	-	46
8	IS-8932806	5	5	-	-	44
9	IS-8932811	5	5	-	-	44
10	IS-8932804	7	7	-	-	43
11	IS-8932810	5	4	1	-	43
12	IS-8932807	2	2	-	-	42
13	IS-8932813	3	2	1	-	42
14	IS-8932803	4	4	-	-	44
15	IS-8932809	2	2	-	-	44
16	ISSR-807	5	4	-	1	50
17	ISSR-808	4	3	1	-	50
18	UBC-854	2	2	-	-	53
19	UBC-809	4	4	-	-	46
20	UBC-825	2	2	-	-	46
21	IS-8932799	5	5	-	-	51
22	IS-8932801	4	4	-	-	51
	Total	88	77	8	3	

Recently, PANDEY *et al.* (2021) identified 2171 SSR motifs from seven cucumber chromosomes, to design 70 SSR markers with cross-species transferability among 16 different cucurbits species belonging to six genera. VASAVA *et al.* (2021) designed 15 EST-SSR primers of spine gourd, based on sequence data retrieved comprising of 2,386 (female) and 3,008 (male) SSRs detected. Further, AMEEN *et al.* (2022) could identify two SSR primers, (From MADS-box genes of spine gourd and cucumber) that showed amplified and polymorphism in female genotypes of spine gourd only.

Divergence analysis in spine gourd using ISSR markers

ISSR data-derived clustering analysis was carried out on the basis of banding patterns in 32 female and 16 male genotypes of spine gourd. It was observed that all genotypes studied formed separate clusters, confirming their uniqueness (Figure 2).

In Dice similarity analysis, the similarity coefficient rangedfrom 0.465 to 0.959 with the highest diversity (lowest similarity values range) being observed in RMFG-09-22 (0.465-0.746) and RMFG-09-1 (0.489-0.727). The lowest pair-wise similarity indices were observed between RMFG-09-22 - RKFG-10-1-14 (0.465) followed by RMFG-09-1 -RKFG-11-4-5 (0.489) and RMFG-09-22 – RKFG-10-2-3 (0.511).

Similarly, the highest pair-wise similarity indices were observed between RKMG-09-11-RKMG-09-8 (0.959), RKMG-09-34-RKMG-09-30 (0.924) and RKMG-09-11-RKMG-09-5 (0.923). These results are supported by DEY *et al.* (2006) in *M. charantia*, RASUL *et al.* (2007) reported in 29 accessions of *Momordica dioica* (Roxb.) and 1 accession of *Momordica cochinchinensis* Spreng. SIKDAR *et al.* (2010) studied diversity in eleven species of Cucurbitaceae; while BHARATHI *et al.* (2012) studied 35 genotypes of Indian Momordica species and five genotypes of two Luffa species.

On examination of the dendrogram, all genotypes could be distinguished from each other (Figure 2). They formed 5 different clusters, named 1-5. F25 (RMFG-09-22) and F28 (RMFG-09-1) were found to be the most distinct genotypes, while male genotypes M7 (RKMG-09-8) and M8 (RKMG-09-11) showed the least variability.

Cluster1 which includes four female genotypes is further divided into two sub-clusters 1a and 1b. Sub-cluster 1a included three female genotypes (F1, F6 and F20), while sub-cluster 1b included only a single female genotype (F26).

Cluster 2 included fourteen female genotypes, further divided into five sub-clusters 2a, 2b, 2c, 2d and 2e. Sub-cluster 2a included three female genotypes (F2, F3 and F22). Sub-cluster 2b included four female genotypes (F10, F24, F18 and F19). Sub-cluster 2c included three female genotypes (F4, F5 and F29). Sub-cluster 2d included only one female genotype (F11). Sub-cluster 2e included three female genotypes (F12, F23 and F21).

Cluster 3 included twenty-eight (all 16 male along with 12 female genotypes) and they were divided into six sub-clusters 3a, 3b, 3c, 3d, 3e and 3f. Sub-cluster 3a included 1 female (F31) and 1 male genotype (M2). Sub-cluster 3b included a single female genotype (F7). Sub-cluster 3c includes only one female genotype (F9) and six male genotypes (M5, M6, M7, M8, M9 and M12). Sub-cluster 3d included three female genotypes (F15, F27 and F30) and two male genotypes (M4 and M3). Sub-cluster 3e included only six male genotypes (M10, M13, M14, M15, M11 and M16). Sub-cluster 3f included three female genotypes (F8, F13 and F17). Sub-cluster 3g included three

female genotypes (F14, F16 and F32) and a single male genotype (M1).

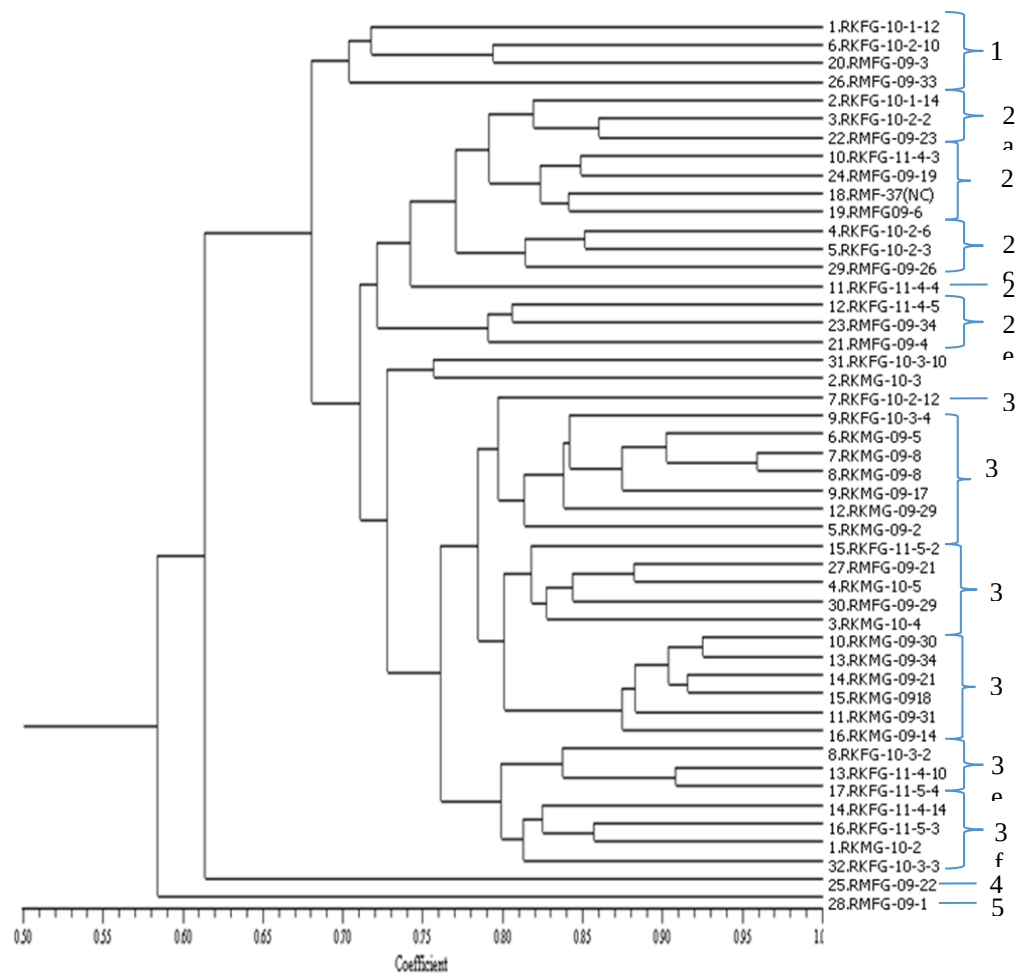

Figure 2. Phylogenetic tree showing clustering of 32 female and 16 male genotypes of spine gourd using ISSR markers.

Most distinct genotypes formed single entity clusters with cluster 4 represented by a single female genotype (F25), while cluster 5 included a single female genotype (F28).

The six most distinct genotypes were all females; with the rest of the clusters sharing genotypes of both sexes. No morphological trait sharing was observed in any of these clusters. The

clustering pattern among female and male genotypes based on morphological (quantitative and quantitative) traits was quite different from that observed during this investigation of molecular variation based on 25 ISSR primers (data not presented). These six most divergent genotypes varied in their morphology. DEY *et al.*, (2006) also didn't observe any correlation between the groupings obtained by RAPD markers with that obtained by morphological traits in 38 genotypes of *Momordica charantia*.

The main cause of mismatch between clustering based on molecular markers and morphological traits may be that most of the quantitative traits are controlled by a large number of genes (polygenes) and these traits are highly influenced by the environment. Besides, molecular markers are randomly distributed throughout the genome and in the majority of cases, most regions of the genome (nearly 90%) are not expressed at the phenotypic level (BEHERA *et al.*, 2012). So, it is very difficult to find out similarities between groupings based on molecular markers and quantitative traits.

For the evaluation of divergence in any crop, the selection of markers is important. The morphological traits selected (including sex of plants) to evaluate the genetic diversity might not explain the genetic variation completely; there could be other traits physiologically and biochemically more important which might explain molecular genetic diversity more precisely. The lack of correlation between molecular and morphological data could be also due to the reduced number of markers used in the cluster analysis.

CONCLUSION

Spine gourd being a dioecious, perennial, tuberous crop shows vegetative growth only under favourable environmental conditions after breaking of tuber dormancy. Further, the morphological markers may reveal little divergence, making the application of molecular markers essential. Further, no clear correlation was observed between ISSR analysis-derived clusters and grouping based on sex or other morphological characters. In the present study, 77 polymorphic markers were generated which were possibly not sufficient to cover the entire spine gourd genome for influencing the expression of quantitative and qualitative traits. Although limited numbers of markers were used in the present study, an effort has been made to study the diversity in the available germplasm of spine gourd. It should also be noted that studies utilizing molecular markers are very limited in this crop. In the future, additional markers showing more polymorphic bands could be utilized for analysis of spine gourd germplasm. The genetically divergent female and male genotypes identified in the present study can be used in future breeding programs for developing spine gourd with improved productivity potential.

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PROCENA MOLEKULARNE RAZNOVRSNOSTI I UTVRĐIVANJE FENOTIPSKIH ODNOSA KOD ŽENSKIH I MUŠKIH GENOTIPOVA *Momordica dioica*Roxb

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Izvod

Momordica dioica Roxb. je visoko hranljiva povrtarska kultura dvodomne reproduktivne prirode. Četrdeset osam genotipova, uključujući 32 ženska i 16 muških genotipova, procenjeno je na molekularnu divergentnost da bi se uspostavile fenotipske veze korišćenjem ISSR markera. Dvadeset dva od ukupno 25 proučavanih ISSR prajmera dala su ukupno 88 traka od kojih je 80 polimorfnih traka , pri čemu su tri od njih jedinstvene u svom profilu. Svaki prajmer je tako proizveo srednju vrednost od 4,0 trake po markeru, sa 3,64 srednjih polimorfnih traka po markeru. Petnaest prajmera je pokazalo 100 % polimorfizam. U dendrogramu, genotipovi su se razlikovali jedan od drugog sa rasponom sličnosti od 0,465 do 0,959. Širi opseg molekularne raznovrsnosti otkriven pomoću ISSR markera odražava prisustvo visokog nivoa genetske varijacije formirajući 5 širokih grupa klastera. Obrazac grupisanja zasnovan na molekularnoj varijaciji tokom ovog istraživanja otkrio je pet klastera; od kojih je klaster tri imao dvadeset osam genotipova (svih 16 muških zajedno sa 12 ženskih); dok su klaster 4 i 5 bili mono-genotipski.

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