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RESEARCH ARTICLE

EVALUATION OF GENETIC DIVERSITY OF THE IMPORTANT MEDICINAL PLANT CRAB'S EYE (*ABRUS PRECATORIUS* L.) USING RAPD AND ISSR MARKERS

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ABSTRACT

Genetic relationships between five genotypes (Abrus Precatorius. L) with different seed coatcolours (Red with black eyespot, black, white brown and maroon) were collected from different places of Andhra Pradesh were analyzed using RAPD and ISSR markers. Ten RAPD (decamers) and five (ISSR) primers were prescreened for their amplication potential. Of which 3 RAPD and 3 ISSR primers produced clear and reproducible amplified products. For RAPD analysis, 3 random primers (OPA – 02, OPA – 16 and OPC – 09) were used which amplified 25bandsof which 15 were polymorphic with and ever age polymorphism of 60.00%. the amplified products varied size from 500 – 3000 bp. For ISSR analysis, 3 primers were used which produced 32 bands, 23 of which were polymorphic (71.87%). The size of amplified bands ranged from 500 – 3000 bp. The efficiency of primers in generating sufficient information for genetic diversity analysis was computed using discriminatory power (Di), which ranged from 0.00 – 0.24 for RAPDs and 0.00 – 0.28 for ISSRs. The date analysis based on Jacquard's similarity coefficient and UPGMA Clusters analysis revealed that genotypes with Red and brown on seed coats were more diverse as compared to other three genotypes (RAPD Clusters) while as in ISSR cluster analysis. Maroon and red seed coats were more diverse as compared to black. Because maroon and coated genotype was collected from Seshachalam Forest, which is located inChittor District, while the rest with four genotypes were collected from east Godavari district, Andhra Pradesh.

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INTRODUCTION

RAPD (Random Amplified polymorphic DNA) is a fast and sensitive method and in able to provide reproducible and characteristic fingerprints of complex genomes without prior sequence information. The use of short primers of arbitrary sequence during PCR results in amplification of different segments of genomic DNA, which after gel electrophoresis gives characteristic band patterns. Most informative DNA bands on RAPD are usually of the 300 – 3000 bp range. RADP provides a cost effective method for the precise and routine evaluation of variability. It may also be used to Identity areas of maximum diversity. Thus molecular technique provides a powerful tool for the study of plant population genetics. RAPD has been used for genetic finger printing (Wilde *et al.,* 1992), locating disease resistance genes (Martin *et al.,* 1991, Michel more *et al.,* 1991), identifying chromosome-specific markers (Quiros *et al.,*. 1991). In the past few years the RAPD technique has been widely accepted in labs throughout the world. Many medicinal important species have been analyzed by this techniques.

This techniques helps to identify a large number of markers whichcan be used for estimating genetic variation. Abrus precatorius L. Commonly known as crab's eye in English, Rate in HindiGunja in Sanskrit and Gurivinda in Telugu is a medicine plant belonging to family Fabaceae. It is a climbing perennial herb which grows wild in the dry regions at low elevations. The unique characteristic of this plant is that it has toxic red seeds with black mark at the base Crensah, 2011). Plant parts such as leaf extracts were used for lucoderma, the seed having abrin is used as a purgative and abortive and the root extract used against coughs in the ayurvedic system of medicine. The sends of this plant exhibit potent HIV – 1 PR inhibitory activity (Marshall, 1998). Medicinally A. Precatorius is well reputed for its antitumor properties in ayurvedic medicine. Two toxic antitumor proteins Abrin A and B were isolated from seeds. (Prakash and Nainwal, 2013). Seeds are containing several chemical constituents and promise in treatment to several diseases (R Jaram *et al.,* 1992 and Rohan and Jenardhan, 1995). Indian gold smiths used its seeds ass weights in ancient times (Nadkavni, 1976). The one ameagrereports were available on Genetic diversity and Genetic characterization in AburnprecatoriusMathur *et al.,* 2013, john De Brilto *et al.,* 2011 and chittora and purohit

2012). The present study has undertaken the genetic diversity potential diversity potential of RAPD and ISSR markers among 5 genotypes of Abrusprecatorius (L)

MATERIALS AND METHODS

Fresh leaves of five genotypes of AbrusPrecatorius (L) with different seed coat colours such as Red, with black spot, Black, White, Brown and Maroon were collected from the field grown plants at different geographical regions of Andhra Pradesh, India viz. Rampachodavaram (Red and Black spots) Addathegala (white), Maredumilli (Black) in East Godavari District and in Seshachalam Forest in Chittoor District (Brown and Maroon seeds genotypes) for the investigation of genetic diversity of the AbrusPrecatorius (L).

Isolation of genomic DNA: Fresh, young and disease free leaves of five genotypes of Abrusprecatorius (L) were collected and immediately kept in ice to reduce the nuclease activity. It was bought to the laboratory weighed (2 grams each) and frozen in liquid nitrogen and stored 70^{o}C till further use. Genomic DNA was isolated by following the protocol (Doyle and Doyle, 1990) with little modifications. Two grams of fresh and young leaf samples was grinded with 2% insoluble PVPP to make a fine powder in a cold mortar and pestle with repeated addition of liquid nitrogen. Thawing was avoided to a 50 ml centrifuge tube containing 10 ml of pre-warmed (60^{o}C) 2% (TAB-DNA extraction buffer (10% CTAB; 4M Nacl ; 0.5M EDTA, PH8; 1M Tris-Hcl, PH 8; 2% β-mercipto-ethanol) and was mixed vigorously. The mixture was incubated in a water bath for one hour at 65^{o}C with intermittent gentle shaking. After incubation, the mixture was cooled to room temperature and emulsified with an equal volume of phenol:chloroform: Iso amylalcohol (25:24:1) and was gently mixed. Then it was centrifuged at 10,000 rpm for 20 min in a cooling centrifuge (C-24B1, Remi) at room temperature. The upper aqueous phase was pipette out with the help of a micropipette into another 50 ml centrifuge tube and mixed with 2.5 volume of pre-chilled dehydrated ethanol. After quick inversion, DNA, like a mass of certain threads was precipitated. The precipitated nucleic acid was spooled out with a bend glass Pasteur pipette, washed twice with 70% ethanol, stored in a 1.5 ml microcentrifuge tube and dried. The dried DNA was dissolved in an excess amount of T_{10} E_1 buffer (Tris.cl 10 mmol, EDTA 1 mmol with PH 8).

Purification of genomic DNA

The dissolved DNA was impure with proteins, RNA and phenolic in some cases so the crude DNA was purified and RNA was removed. The RNA was removed by giving RNase treatment. For 1 ml of crude DNA solution, 60 mg of RNase A was added, and the solution was incubated with continuous shakingin a water bath and an equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) wasadded and gently mixed thoroughly. The solution was then centrifuged in cooling centrifuge at 10,000 rpm for 20 min at 20^{o}C and the upper aqueous phase was pipette out. It was again washed with chloroform: Isoamyl alcohol (24:1) twice and centrifuged at 10,000 rpm for 20 min at room temperature. The upper aqueous phase was separated after centrifugation and mixed with 1/10thvolume of 3m sodium acetate (PH 4.8).DNA was precipitated by adding 2.5 volume of chilled absolute ethanol and pelleted by spinning. The pellet was washed twice with 70% ethanol, carefully and dried under vacum. The dried DNA was dissolved in minimum amount of T_{10} E_1 buffer (PH.8)

Test for quality and quantity of the purified DNA: The quality and quantity of DNA were measured by UV-VIS spectrophotometer (Model evolution 220, Thermo Fisher Scientific). The total DNA quantity was measured by taking the absorbance at 260 nm wavelength and the quality of the DNA was confirmed from the absorbance ratio at 260 nm and 280 nm. It was reported that if the ratio is about 1.8 to 2.0 then the quality as well as the quantity of DNA, the DNA was loaded in 0.8% agarose gelalongside diluted uncutlambda DNA as standard and electrophoresed. It was observed that the DNA from all the samples was very good in quality. After quantification, the DNA was diluted with $T_{10}E_1$ buffer to a working concentration of 25 mg/ml for RAPD and ISSR analysis.

RAPD and ISSR amplification: Two types of polymerase chain reaction (PCR) based molecular techniques namely RAPD and ISSR were utilized for the present study.

RAPD Amplification: PCR amplification (Williams *et al.,* 1990) was performed with arbitrary decamer primers obtained from operon technologists, Alamed a USA (Table1). Amplification were performed in 25 ml reaction volumes containing 2.5 ml of 10 x ass=4 buffer (100 mr Tris-Cl; PH 8.3, 500 mrKcl, 15 mre $rgcl_2$), 02 mr of each dNTPs (dATP, dCTP, dGTP and dTTP), 5 pg of primer, 1.0 unit of Taq DNA polymerase and 30 mg template DNA. The amplification reaction was carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 43 cycles using the following amplification profile: initial denaturation of template DNA at 94^{o}C for 5 minutes followed by 43 amplification cycles of denaturation at 92^{o}C for 1 minute primer annealing at 37^{o}C for 1 minute and elongation at 72^{o}C for 2 minutes followed by a final extension step at 72^{o} C for 7 minutes. After completion of amplification, PCR product was resolved on 1.5% agarosegel in 1x TAE buffer by electrophoresis at 120V for 1h and 20 minutes then visualized with under the UV light and photographed in a gel documentation system (Syngene, UK). The sizes of the amplicons were determined by comparing them with the 100 bp ladders (Ferments, USA). The entire process was repeated atleast twice to confirm the reproducibility.

ISSR amplification: A total of five ISSR primers were used for the analysis (Table1). PCR amplification was performed with minor modifications (Zeitkiewiez *et al.,* 1994). ISSR amplification reactions were carried out in 25 ml reaction volumes containing 2.5 ml of 10 x assay buffer (100 mr Tris.cl; PH 8.3, 500 mr KCP, 15 mr rgcl2), 0.2 mr of each dNTPs (dATP, dCTP, dGTP, and dTTP), 5 pg of primer, 1.0 unit of Taq DNA polymerase and 25 mg template DNA. The amplification reaction was carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 43 cycles using the following amplification profile : initial denaturation of template DNA at 94^{o}C for 5 minutes followed by 43 amplification cycles of denaturation at 92^{o}C for 1 minute primer annealing at 50-56^{o}C (ISSR) for 1 minute and elongation at 72^{o}C for 2 minutes followed by a final extension step at 72^{o}C for 7 minutes. The amplified products were electrophoresed at 120V on a 1.5% agarosegel in 1 x TAE buffer using 100 bp ladder (Fermentas, USA) as the molecular weight standard.

Data Scoring: The data were scored as '1' band presence and '0' for absence for each primer genotype combination for RAPD and ISSSR analysis. All bands (monomorphic and polymorphic) were considered to avoid under/over estimation of the genetic diversity (Gneradi et al 1998). The data were used as discrete variables in a binary matrix. The data was analyzed using NTSYS software package (Rohlf, 1997). The SIMQUALprogramme was used to calculate Jaccord's coefficient (Jaccord, 1908). The clustering was done using Sequential Agglomerative Heirachial Nested Clustering (SAHN) routine and a dendrogram constructed using UPGMA (Sneath and S.OKal 1973) with NTSYS package.

RESULTS

RAPD analysis: From 10 random decamere oligonucleotide primers three primers were selected OPA-02, OPA-16, and OPC-09 were amplified. All the five genotypes of AbrusPrecatorius L. each in triplicates was used which produced distinct reproducible amplicons. The DNA profiles as obtained by RAPO markers were represented in Table 2 (Fig.1).

ISSR analysis: Out of 5 primers 3 primers resulted in the amplification of 32 fragments. The ISSR banding pattern in five genotypes is represented in table-2. The primer (AG)8C produced maximum number of bounds (13) while primer (AC)8C (10) and (AC)8G-9 produced minimum number of bands (9). The bands were amplified the range of 500-3000 bp and no unique bands were formed. From 32 bands amplified 23 were polymorphic on 9 were monomorphic in nature. Among these ISSR primers the highest percentage of polymorphism (80%) was recorded with primer ISSR-3 (AC) 8C and minimum percentage of polymorphism was encountered (55.56%) with primer ISSR-1 (AC) 8G. Details of ISSR banding. The two types of markers (RAPD and ISSR) were employed for essessment of genetic similarity among 5-genotypes of Abrusprecatorius L. A total of 57 bands were amplified with the entire marker out of which 38 were polymorphic and rest 19 were monomorphic (table-2) All the samples were correlated with each other with an average similarity of a 24 which ranged between 0.24 to 0.00.

Table 1. Sequence information of RAPD and ISSR oligonucleotide primers used for amplications and polymorphism study in 5 genotypes of Abrusprecatorius L

S.No.	RAPD & ISSR Primer, Code	Primer sequence 5^1 to 3^1
	RAPD	
1	OPA – 02	TGCCGAGCTG
2	OPA – 10	GTGATCGCAG
3	OPA – 14	TCTGTGCTGG
4	OPA – 16	AGCCAGCGAA
5	OPA – 20	GTTGCGATCC
6	OPC – 05	GATGACCGCC
7	OPC – 09	CTCACCGTCC
8	OPC – 11	AAAGCTGCGG
9	OPC – 14	TGCGTGCTTG
10	OPC – 20	ACTTCGCCAC
	ISSR	
11	ISSR – 1	5^1 (AC) 8 G – 3^1
12	ISSR – 2	5^1 (AC) 8 T – 3^1
13	ISSR – 3	5^1 (AC) 8 C – 3^1
14	ISSR – 4	5^1 (AC) 8 C– 3^1
15	ISSR – 5	5^1 (AC) 8 T – 3^1

Table 2. Details of primers and banding pattern of RAPD and ISSR analysis in 5 genotypes of Abrusprecatorius L.

Primer	Nucleotide sequence	Range of amplificous (in dp)	Total No. of Bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphism. E
RAPD						
OPA–02	TGCCGAGCTG	100-1500	11	6	5	54.54
OPA–16	AGCCAGCGAA	100-1000	8	5	3	62.50
OPC–09	CTCACCGTCC	400-2000	6	6	2	66.67
Total			25	15	10	60.00
ISSR						
ISSR – 1	5^1 (AC) 8 G – 3^1	100-2500	9	5	4	55.56
ISSR -3	5^1 (AC) 8 G – 3^1	100-2000	10	8	2	80.0
ISSR–4	5^1 (AG) 8 G – 3^1	300-1000	13	10	3	76.92
Total			32	23	9	71.87

A total of 25 bands were amplified, 15 of which were found to be polymorphic and 10 were monomorphic nature. The highest number of bands (11) were amplified with primer OPA-02 (500-3000 bp) and lowest number of band (6) was amplified with primed OPC-09 (500-3000 bp). No unique bands were found will all the primers. Average number of bands per primer was found to be 8.33. The highest percentage of polymorphim (66.67%) were recorded with primer OPC-09 and the lowest percentage of polmorphysm (54.54%) was observed with primer OPA-02. The details of RAPD banding pattern and amplification with different primers and genotype of A. precotorius on presented in Table – 2.

The dendrogram constructed using jaccard's similarity coefficient, separated the five genotypes into two major custers (RAPD) cluster I with four genotypes and cluster II with one genotype at similarity coefficient of 0.24 (Fig 8). Similarly, the didrogram constructed with Jaccord's similarity coefficient separated the five genotypes into three major clusters (ISSR) cluster I with three genotypes and rest of cluster II and III. One each genotype at similarity coefficient of 0.26 (Fig 8). The similarity ranged from 0.00 – 0.26 respectively. Among the five genotypes maroon coloured seed type is more distinct

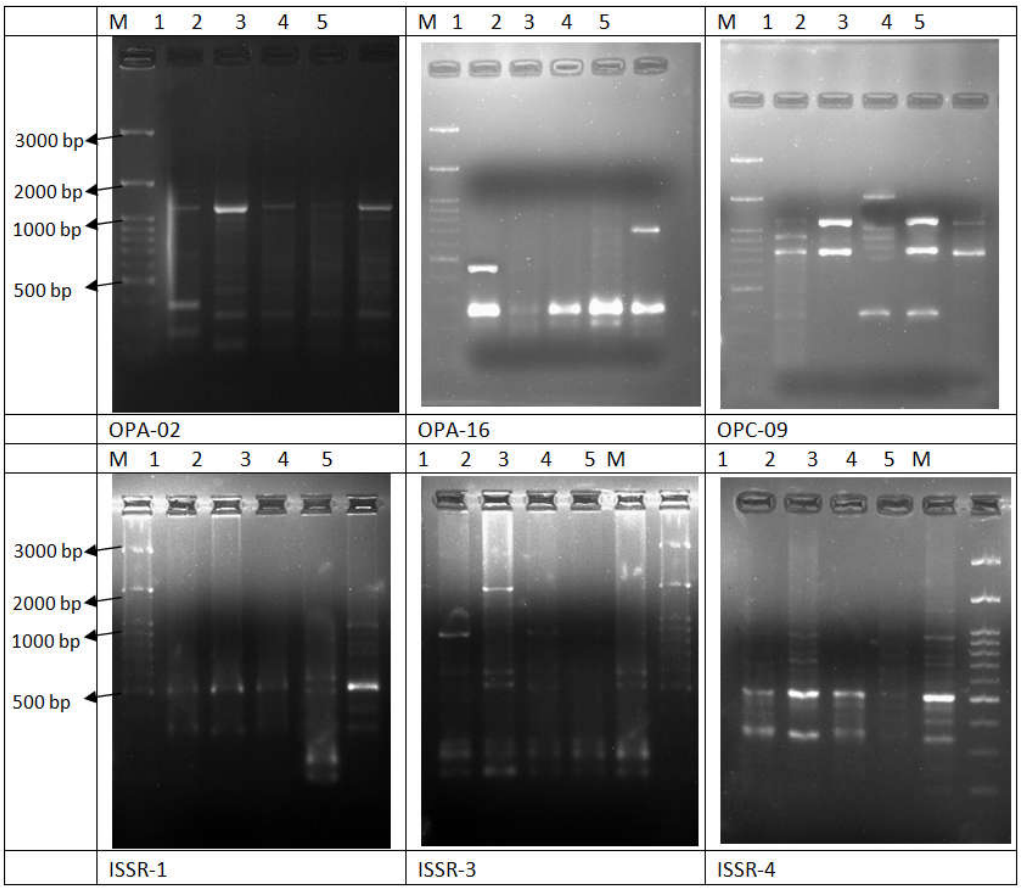

Figure 1. Figure showing agarose gel images of RAPD and ISSR markers. Lane M-1000bp markers; Lane 1-red black;Lane2-black;Lane 3-White;Lane 4-brown; Lane 5-maroon

Figure-1 Amplication products from all the five genotypes of Abrusprecatorius L. with OPA 02 (RAPD primer)

Figure 2 Amplication products from all the five genotypes of Abrusprecatorius L With OPA-16(RAPD primer)

Figure 3 Amplication products from all the five genotypes of Abrusprectorius L With OPC-09(RAPD primer)

Figure 4 Amplication products from all the five genotypes of A.prectoriusL.with ISSR-1primer

Figure -5 Amplication products from all the five genotypes of A. prectorius L with ISSR-3 primer

Figure -6 Amplication products from all the five genotypes of A.prectorius L with ISSR-4 primer

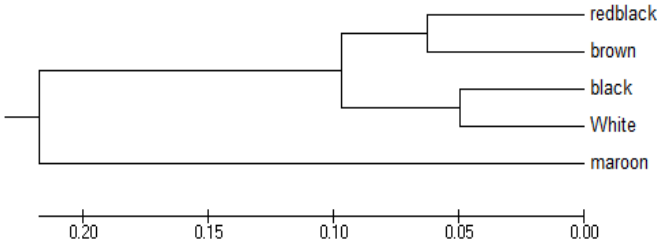

Figure 7. Dendrogram for five genotypes using UPGMAcluster analysis based on RAPD markers

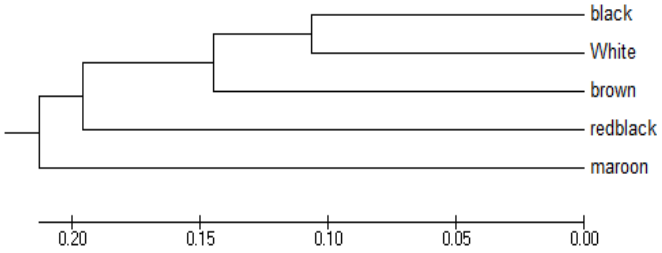

Figure 8. Dendrogram for five genotypes using UPGMA cluster analysis based on ISSR markers

because this was collected from Seshachalam which is distenty geographical region. Dispute the great and similar discriminating power of both markers, some differences between the two could be detected 1) discriminating power volues lower for RAPDs then ISSRs and 2) higher number of total, polymorphic and discriminant fragments ISSRs then RAPDs. Although further investigations need to be made to confirm this observation, comparatively low polymorphism and less reproducibility of RAPD in the present study indicate ISSR as marker of the choice.

DISCUSSION

The effective conservation and utilization of plant genetic resources requires thorough characterization of genetic structure. Because of its ability to distinguish genotypes at the DNA level, molecular marker analysis is considered the most reliable method for this characterization. Several molecular marker-based methods (RAPD, AFLP, SSR and ISSR etc) On currently in use and allow genotypes to be clustered according to their genetic relationships (Hu *et al.,* 2005; Ojaghi and Akhundova, 2010; Evo3 Poyraz *et al.,* 2012). Combined use of several marker types enables coverage of different parts of the genome and provides more informative data then the use of

individual markers alone (Josich *et al.,* 2008; Kumar *et al.,* 2009). In the present investigation, 2 PCR techniques (RAPD and ISSR) were used to construct genetic profiles for and compare the genetic diversity of 5 genotypes of Abrus Precatorius L. of diverse origin. The RAPD and ISSR primers produced a highly variable number of amplified fragments. The 3 RAPD primers produced a total of 25 reliable fragments and the 3 ISSR primers produced 32 fragments. Neverthless the level of polymorphism reveded by ISSR markers (71.87%) was slightly higher then that with RAPD primers (60.00%). This may be ducts the highly polymorphic and abundant nature of ISSR markers that results from slippage in DNA replication Similar results were also reported by rather *et al.,* (2013) for Abrusprecatorius L. 0.5 by Srivastava *et al* (2007) for Beta Vulgaris. L. were able to obtain a high polymorphism rate (71.87%) using only 3 – ISSR primers in five Abrusprecatorius L. Genotypes. Different markers might reveal different classes of variation and these may correlate with the genome fractions surveyed by each kind of marker and their distribution through out the genome.

The dendrogram separations Abrus accessions into different clusters. In RAPD cluster analysis, all the accessions formed two clusters. I and II. At 24% similarity (Fig.7) cluster I was the largest cluster containing 4 accessions. While the cluster II contained only accession. Genotypes with maroon and red with black eye redcoat were found to be more diverse as compared to genotype with red and coat. Red and coat with block eye genotypes were the most closely related with highest similarity index. This may be the maroon red coat genotype was collected from seshachdemforest, from chiltor district which for distant other genotypes. Which on collected from east Godavari district. The dendrogrend separated Abrus Accessions into different clusters. In ISSR cluster analysis, all the accessions formed three clusters. I, II and III at 0.28% similarity (Fig 8). Cluster I was the largest cluster containing 3 accessions while the cluster II and III contained one each accession. Genotypes with maroon and black red coat. Black red coat colored genotypes were the most closely related with highest similarity index with Black, white and brown and coolidcolours. Cluster II with red and coat with black eye slightly diverse to others 3 types. Similarly cluster III containing maroon red coat colures, closely with highest similarity index. This may be again the maroon redcoat genotype was collected from seshachalam forest. In the present study, RAPD and ISSR profile, indicated very low degree of genetic variability which might occur due to several visions similar results were also repo lid in macohopsis (Sulaimanetal, 1996). Padicluaris furbished (Jayaram and prasad, 2008). Low genetic variation has been reporlid to be due to their reproductive stratifies, such as selling and vegetative propagation. The analysis of polymorphic bonds could prove highly useful in identification of trait linked genes in the genotype determining and coat colures.

Conclusion

Both RAPD and ISSR markers showed genetic variability and on found to be powerful tools for estimating genetic similarities and diversity in the studied Abrus genotypes. The genetic relationships presented among the genotypes on helpful for future breeding programs through selection of genetically diverse parents. This study confirm the suitability of RAPD and ISSR markers on reliable, simple, easy to handle and elephant tool in molecular diagnosis of different accusations available in the germplasm collections Accurate estimates of diversity on a prerequisite for optimizing sampling strategies and for conserving genetic resolvers. Hence the maroon colored seed genotypes conversed as genetically diverse parent for future hybridization program. Currently, it is also proved that the entries that were found to be similar in taxonomical classification based on morphological characters do have divergence at DNA level. The RAPD and ISSR profiles display vast genetic variation indicative of the evolving nature of the taxa.

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