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ISSR studies on small and large seed varieties of *Glycine max*

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Abstract
Plant molecular identity (ID) is required to describe molecular characteristics of plants, which is should contain all of the required information. Molecular markers are popularly used to characterize plant genetic diversity. In the present study, DNA was isolated from the two different *Glycine max* varieties, for ISSR based marker analysis. For molecular characterization total 30 primers of ISSR were tested for the both varieties. The results obtained using ISSR primers for two *Glycine max* varieties showed recordable monomorphic and polymorphic band patterns. Overall, ISSR has shown 39.2 % polymorphism. In this, primers UBC888, UBC889 and UBC814 showed 100% polymorphism, followed by 73.33% in UBC881 and 77.78% in UBC817. Thus primers UBC888, UBC889 and UBC814, have given high polymorphism and proved the best to distinguish both varieties from each other.

Keywords: *Glycine max*, DNA, ISSR, Variety

Introduction
Molecular marker techniques play an important role in identification of plant at variety levels (Singh and Kumar, 2009) [25]. Molecular characterizations in plant breeding have proven by DNA markers, and are widely used in fingerprinting, diversity analyses, and gene mapping. Many types of molecular markers i.e. Hybridization-based molecular markers (e.g., RFLP) PCR-based molecular markers (e.g., AP-PCR, CAPS, STS, RAPD, SCAR, AFLP, SSAP, SSR, ISSR, EST) and sequencing-based molecular markers (e.g., SNP) are becoming increasingly important for cultivar identification and diversity analysis (Mandaliya *et al.*,2010, 2011) [12,13]. These markers play an important role in, studies of genetic diversity and variability, tracking individuals or lines carrying particular genes and the structure of linkage maps (Kalendar *et al.*, 2011) [8].
Earlier many authors reported that inter-simple sequence repeat (ISSR) markers are successfully applied for genetic analysis of plants (Nagaoka and Oginara, 1997; Kojima *et al.*, 1998; Prevost and Wilkinson, 1999; Joshi *et al.*, 2000; Arcade *et al.*, 2000) [17, 9, 20, 7, 1]. ISSR is a PCR-based method, which involves amplification of DNA segment present at a distance that amplify in between two identical microsatellite repeat regions oriented in opposite direction.
ISSR is a straight forward, rapid, and competent technique with high reproducibility due to the use of longer primers (16 - 25 mers). ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology (Shahnawaz *et al.*, 2017) [24]. ISSR analysis is faster and it amplifies and detects a greater number of bands per primer (Godwin *et al.*, 1997) [5]. ISSR marker system accesses variation in the numerous microsatellite regions dispersed throughout the genome (Semagn *et al.*, 2006) [23] and circumvents the challenge of characterizing individual loci that other molecular approaches require. ISSR is extremely useful marker due to several properties of microsatellite such as high variability among taxa, high copy number in eukaryotic genomes and ubiquitous occurrence (Morgante *et al.*, 1996) [15].
Diversity in several crops such as soybean has been recognized based on morphological and agronomical traits (Lee and Kaltsikes, 1973; Ford *et al.*, 1991; Lu *et al.*, 2002) [10, 4, 11]. Such characters are strongly influenced by the developmental stage of the plant and environmental factors. Since the early, molecular markers such as RAPDs, SSRs and ISSRs have been developed as extremely effective in population genetics studies and their alternative methods (Prasad *et al.*, 2000) [19]. Considering to this, ISSR primers were used in the present study to determine the genetic diversity between two varying variety of *Glycine max.*

Materials and Methods
Plant material
Certified seeds of *Glycine max* (Small (S) and large (L) seed size) were selected for the study.

DNA isolation from soybean seed

DNA was isolated according to "Dellaporta" method (1983) [3]. One g seed was crushed in a mortar pastel with liquid nitrogen and make fine powder was made, added 30 ml extraction buffer and 2.1 ml 20% SDS, mixed well by inversion and Incubate at 65°C for 30 min. After incubation mixture was centrifuged for 20 min at 3,000 x g. Supernatant was transferred and added 6 ml 5M KAc vigorously shacked for 1 min, kept it for 30 min on ice and centrifuge for 20 min at 3,000 x g. supernatant was transferred. Equal volume of isopropanol was added and mixed gently for 1 min. Mixture was incubated on ice for 5 min and centrifuged for 20 minutes at 3,000 x g. Supernatant was removed and pellet was air-dried at 37°C until all isopropanol residues is evaporated. The pellet was then dissolved 10 ml 10 mM Tris-EDTA buffer (pH 8.0). The samples were shacked for 1 h at 100 rpm 37 ºC. DNA was purified by phenol: chloroform: isoamy lalcohol (25:24:1) was added and mixed well and centrifuged for 20 min. Upper aqueous phase was carefully transferred to a new tube. Then 900 µl 3M NaoAc and 6 ml isopropanol was added and mixed gently by inversion, Incubate on ice for 5 min and centrifuged for 20 min at 3,000 g to pellet the DNA. Supernatant was removed and pellet was air-dried at 37°C until all isopropanol residues is evaporated.

Pellet was dissolved in 1 ml TE buffer and shacked for 1 hour. DNA solution was transferred to a new 2.0 ml micro centrifuge tube, 10 µl RNase A (mg/ml) was added, gently mixed and incubated for 20 min at 37°C. Again DNA was extracted by 800 µl phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well for 1 minute and centrifuged for 10 minutes. Upper aqueous phase (about 900 µl) was transferred, then 800 µl chloroform was added, mixed well for 1 min and centrifuged for 10 min at maximum speed. Upper aqueous phase (about 800 µl) was transferred and added 90 µl 3M NaoAc and 600 µl isopropanol and mixed gently by inversion for 1 min, kept it for 5 min on ice and centrifuged for 1 min. All supernatant was removed and 1 ml 70% ethanol was added gently to wash the DNA pellet. The samples were kept for shaking for 1 h and centrifuged for 5 min. Supernatant was removed and air-dried the pellet at 37°C until all ethanol residues was evaporated. 200 µl 10 mM Tris-EDTA buffer (pH 8.0) was added to the DNA pellet and allow dissolving for about 10 h at 4°C. The quality and concentration of the DNA was confirmed by measuring optical density 260/280 nm ratio.

DNA purity and quantification

The DNA concentration was determined at 260 nm, and purity was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (Sambrook and Russel, 2001) [22] using the micro plate reader (µ Quant, Bio-Tek instrument, USA).

Agarose Gel Electrophoresis

Electrophoresis was carried out on 2 % agarose gel at constant voltage of 60 V in 1x TAE buffer pH 8.0. 100 bp DNA ladder and Supermix marker, both from Bangalore Genei, India, were used as molecular size standards.

Amplification of ISSR marker

A set of 30 ISSR primers were used for this study (Table 1). DNA was amplified in a total volume of 25 µl. The reaction mixture contained 2.5 µl 10X buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Trion X-100), 2.5 µl 25mM MgCl2, 1µl 10mM deoxynucleoside triphosphate, 10 µM primer and 1 µl of Taq DNA polymerase, 2 µl DNA template. PCR reaction was performed using the Verity ™ Thermal Cycler with 40 cycles of denaturation at 92 ºC temperature for 2 min., annealing (36-60 ºC) for 1 min., extension was done at 72 ºC for 2 min and final extension 72 ºC for 7 min. Amplified DNA fragments were analyzed though 2 % agarose gel electrophoresis.

Table 1: ISSR primer set

Sr. No.	Primer Name	Sequence	Ta (°C)
I-1	UBC803	(AT)8C	36
I-2	UBC808	(AG)8C	48
I-3	UBC814	(CT)8A	48
I-4	UBC817	(CA)8A	44.2
I-5	UBC822	(TC)8A	48
I-6	UBC826	(AC)8C	48
I-7	UBC834	(AG)8TT	44.2
I-8	UBC840	(GA)8TT	44.2
I-9	UBC862	(AGC)6	48
I-10	UBC864	(ATG)6	48
I-11	UBC867	(GGC)6	48
I-12	UBC868	(GAA)6	48
I-13	UBC872	(GATA)4	48
I-14	UBC873	(GACA)6	48
I-15	UBC876	(GATA)2(GACA)2	48
I-16	UBC880	(GGAGA)3	48
I-17	UBC881	(GGGGT)3G	48
I-18	UBC884	HBH(AG)7	48
I-19	UBC885	BHB(GA)7	48
I-20	UBC886	VDV(CT)7	48
I-21	UBC888	BDB(CA)7	48
I-22	UBC889	DBD(AC)7	48
I-23	UBC891	HVH(TG)7	48
I-24	ISSR1	(AGC)5GC	55
I-25	ISSR2	(CA)7AC	44.2
I-26	ISSR3	(GT)7AC	44.2
I-27	ISSR4	GCA(GA)7	47.2
I-28	ISSR5	(GA)9C	51.7
I-29	ISSR6	(GA)9A	49.5
I-30	ISSR7	(CG)8C	60

Data analysis

The ISSR products were scored according to Vafaie-Tabar *et al.,* (2004) [27] for each primer and % polymorphism was calculated.

Results and Discussion

Molecular markers are essential tools in cultivar identification (DNA typing), assessment of genetic variability and relationships, management of genetic resources and biodiversity, studies of phylogenetic relationships and in genome mapping (Semagn *et al*., 2006) [23]. The isolation of good quality DNA is the prerequisite for molecular research. Successful application of PCR-based downstream applications requires efficient recovery of good quality and quantity of DNA. In this work, two different varieties of soybean were selected for ISSR marker analysis. The soybean seed was containing a high amount of protein that interferes with total DNA isolation procedures and PCR-based downstream applications. The plants also produce huge amounts of secondary metabolites as well as other substances of medicinal and industrial importance. These secondary compounds impede the extraction of DNA and the downstream application of the DNA preparation like nucleotide sequencing, polymerase chain reaction, and molecular marker analysis (Sirakov, 2016) [26].

The total genomic DNA of soybean seed was isolated from

the liquid nitrogen pulverized samples using "Dellaporta" method. In this study, DNA quality was assayed by gel electrophoresis and intense bands showed on 1.0 % agarose (Fig. 1). The yield and purity of DNA is presented in Table 2. The A260/A280 ratio was in the range of 1.35% to 1.68% which indicated the purity of the nucleic acid obtained using our method. The small seed DNA concentration was 16.65 µg/ml, while concentration of large seed DNA was 4.5µg/ml.

The quantity and the quality of the DNA extracted by this method were high enough to perform PCR reactions. Thus, this protocol gave suitable result for downstream application and bulk of PCR reactions.

In this work SDS was used for the lysis of cells because of its high affinity to bind to proteins and denature them quickly. The addition of KAc participates in precipitation of these complexes by replacing sodium ions with potassium ions (Dellaporta *et al*., 1983) [3]. RNase treatment is the generally accepted method to remove RNA. The contaminated RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikbart and Villeponteau, 1993) [18]. The phenol/chloroform extraction was used to remove major protein from the cell debris contamination (Murray and Thompson, 1980) [16]. Thus, residual proteins and other cellular contaminants were removed or separated from the DNA using phenol/chloroform.

Assessment of genetic diversity in a crop species is a helps to generate genetically diversified breeding populations and prerequisite to its improvement. Considerable variation has been recorded for physiological, morphological, and agronomic traits in *G. max* crop (Moe and Girdthai, 2013) [14]. In the present study, two soybean verities were used for ISSR analysis. It is interesting to know that these verities showed relationship with each other or not. In the present study total 30 ISSR primers were used, out of 26 ISSR produced the bands. The reaction products were electrophoresis on a 2% agarose gel.

In this work ISSR amplification of soybean DNA polymorphic and monomorphic band pattern observed in electrophoresis (Fig- 2-6). Clearly detectable amplified ISSR bands were obtained ranging in size from 100 to 3000 bp for the two Soybean cultivars. Among the 30 primers, 6 were more suitable and give high polymorphism. The total bands were scored as 0 and 1 for absence and presence of bands. Amongst the primers studied 26 primers generated sharp, clear and reproduciable 320 bands (Table 3). The polymorphic 108 and 212 monomorphic bands were observed thus percentage of polymorphic bands observed 39.2 and of monomorphic 60.8. The average 8.2 amplicon per primer was observed. The maximum number of amplified product was 20 (I-10) and minimum 7 (I-13, I-26), the total number of amplified products was 214 (an average of 8.23 bands per primer) ranging from 200 to 1,500 bp, being monomorphic amplicon 106 (an average of 4.07,) polymorphic amplicon 108 (an average 4.15) of them, total band was 320. Being three primers UBC888, UBC889 and UBC814, 100% polymorphism observed, followed by 73.33% (UBC881) and 77.78% (ISSR4). So, in this work primers UBC888, UBC889 and UBC814, given high polymorphism and prove best to distinguish soybean both varieties from each other. UBC822 was not given any polymorphism.

ISSR has gradually found widespread application and has become more common. Compared with supplementary molecular markers, ISSR has high polymorphism. Nagaoka and Ogihara (1997)[17] reported that ISSR markers can provide additional genetic information and that currently the accurateness of the ISSR technique is equal to that of random fragment-length polymorphism (RAPD) when investigating genetic relationships in a genus due to its high polymorphism. In the case of ISSR markers, Joshi *et al*., (2001) [6] had found that 25 out of 49 primers showed 51 monomorpic and 239 polymorphic patterns. The maximum number of 17 bands was produced by the primer (GATA) i.e. UBC888, UBC889 (I-21, I-22 numbered in current study) and all bands were polymorphic. They observed that though dinucleotide repeats are more frequent than tetranucleotide repeats in the rice genome. In the present study 26 out of 30 primers showed 108 polymorphic and 212 monomorphic bands. In addition, ISSR markers allow the fast, easy, accurate, reliable, inexpensive, and simultaneous detection of polymorphisms at multiple loci in the genome using low quantities of DNA. Due to these properties markers are very useful for the genetic analysis of different plants (Reddy *et al*, 2002) [21].

Brick and Sivolap, (2001) [2] studied five ISSR primers to study 19 cultivars of soybean. They observed 64 amplification fragments, 45 of which were polymorphic. By these primers the level of polymorphism was 75%. Thus molecular tools are more reliable than phenotypic observations for detection of genetic stability. Thus results show that this DNA isolation method was suitable for molecular analysis. ISSR profile has shown DNA polymorphism among both the varying seed size of soybean. So, this technique could be used in genetic mapping studies of plant varieties as well as to understand their phylogenic relationship.

Table 2: Purity and concentration (µg/ml) of genomic DNA of S seed and L seed varieties of Soybean

Soybean Varieties	Purity	Concentration
Small	1.35 %	16.65 µg/ml
Large	1.68 %	4.5 µg/ml

Table 3: ISSR amplicon profile, % monomorphism and % polymorphism of S seed and L seed varieties of *Glaycine max*

Primer	Total amplicon	Monomorphic amplicon	Polymorphic amplicon	Total band	Monomorphic band	Polymorphic band	% Monomorphism	% Polymorphism
2	10	4	6	14	8	6	57.14	42.86
3	6	2	4	8	4	4	50	50
4	8	2	6	10	4	6	40	60
5	4	4	0	8	8	0	100	0
6	10	7	3	17	14	3	82.35	17.65
7	9	5	4	14	10	4	71.43	28.57
8	7	2	5	9	4	5	44.44	55.56
9	8	3	5	11	6	5	54.55	45.45
10	11	10	1	21	20	1	95.24	4.76
11	7	1	6	8	2	6	25	75
12	6	4	2	10	8	2	80	20

13	4	3	1	7	6	1	85.71	14.29
14	8	7	1	15	14	1	93.33	6.67
15	13	2	11	15	4	11	26.67	73.33
16	10	7	3	17	14	3	82.35	17.65
17	9	6	3	15	12	3	80	20
18	11	6	5	17	12	5	70.59	29.41
19	9	5	4	14	10	4	71.43	28.57
20	11	9	2	20	18	2	90	10
21	9	0	9	9	0	9	0	100
22	8	0	8	8	0	8	0	100
25	9	7	2	16	14	2	87.5	12.5
26	7	0	7	7	0	7	0	100
27	8	1	7	9	2	7	22.22	77.78
28	6	5	1	11	10	1	90.91	9.09
29	6	4	2	10	8	2	80	20
TOTAL	214	106	108	320	212	108		
AVERAGE	8.230769231	4.076923077	4.153846154				60.8	39.2

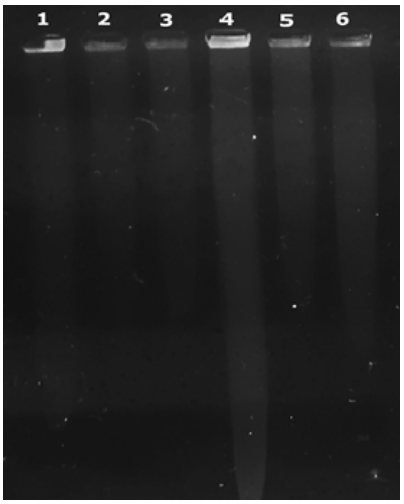

Fig 1: Genomic DNA of S seed (1,2,3) and L Seed (4,5,6)

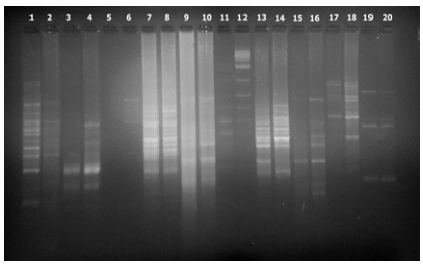

Fig 2: Amplification pattern of selected ISSR primers for S seed and L seed soybean varieties. [1-2:UBC808, 3-4: UBC814, 5-6: UBC822, 7-8: UBC826, 9-10: UBC862, 11-12: Low range ruler- Super mix ruler, 13-14: UBC864, 15-16: UBC867, 17-18: UBC868, 19-20: UBC872]

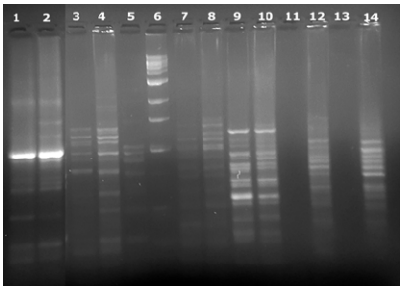

Fig 3: Amplification pattern of selected ISSR primers for S seed and L seed soybean varieties. [1-2: UBC873, 3-4: UBC884, 5-6: Low range ruler- Super mix ruler, 7-8: UBC885, 9-10: UBC886, 11-12: UBC888, 13-14: UBC889]

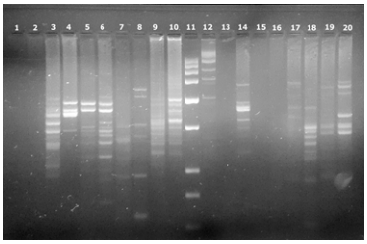

Fig 4: Amplification pattern of selected ISSR primers for S seed and L seed soybean varieties.[1-2: UBC803, 3-4: UBC817, 5-6: UBC834, 7-8: UBC840, 9-10: ISSR2, 11-12: Low range-supermix, 13-14: ISSR3, 15-16: ISSR7, 17-18: ISSR4, 19-20: ISSR6]

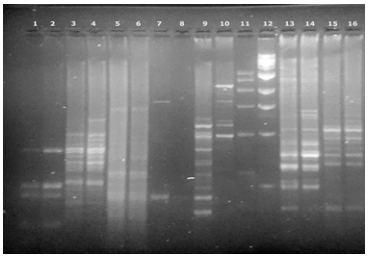

Fig 5: Amplification pattern of selected ISSR primers for S seed and L seed soybean varieties. [1-2: UBC822, 3-4: UBC826, 5-6: UBC862, 7-8: UBC872, 9-10: UBC876, 11-12: Low range ruler- Super mix ruler, 13-14: UBC880, 15-16: UBC881]

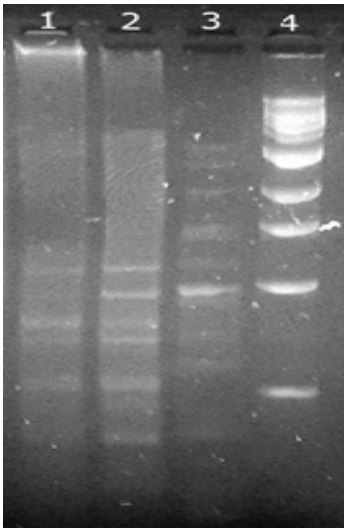

Fig 6: Amplification pattern of selected ISSR primers for S seed and L seed soybean varieties. [1-2: ISSR1, 3-4: Low range ruler- Super mix ruler]

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