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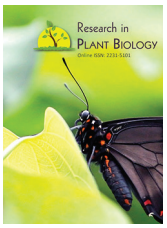

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Intra Specific Analysis of *Gloriosa superba* (L.) through ISSR finger printing and DNA sequencing of ecotypes collected from different accessions of Tamil Nadu State, India

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ABSTRACT

Diversity within and among the population of *Gloriosa superba* collected from five different location of Tamil Nadu State, India, were explored by using Inter Simple Sequence Repeat (ISSR) DNA sequencing method. A total of 86.27% of polymorphic bands were seen in 94 reproducible bands generated from 12 number of ISSR primer ranging between 200bp to1000bp of band width. ISSR primer produces the bands with an average polymorphism of more than ninety nine percent among all the ecotypes. The dendrograms drawn for the analysis of genetic similarity in all the ecotypes. Our studies reveals that, genetic variations among different accessions of *Gloriosa superba(L)* was very well identified with the help of ISSR finger printings technic.

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INTRODUCTION

Gloriosa superba (L) is an emerging medicinal plant belongs to the family Colchicaceae found widely throughout tropics and extends its occurrence in Africa, India and Southeastern Asia. The plant initially grown for its showy, broad and beautiful flower, locally named as "Senganthal malar" which is the state flower of Tamil Nadu State, India. Later the crop was evidenced to have high medicinal value both in formal and informal medicine. Its utility is pronounced both traditional and in modern medicine because of the high alkaloid accumulation in various parts of the plant.

The tuber portion of the plant contain two important alkaloids namely Colchicine ($C_{22}H_{25}$ O_6N) and Colchicoside ($C_{27}H_{33}NO_{11}$) [1] which is used for curing cold, ulcers, hemorrhoids [2] but in the recent study which is used for the treatment of bruises and sprains, colic, chronic ulcer, haemorrhoids, cancer, impotence, nocturnal emission, skin diseases, snake bites, intermittent fever, leprosy and also for inducing labour pains and for abortions [3]. In modern medicine, thiocolchicoside a semi – synthetic derivative of cholchicoside is used as a muscle relaxant, analgetic and for anti inflammatory actions. Seeds and tubers of *Gloriosa superba* are used to treat gouts and rheumatism [4].

The genus *Gloriosa* comprises of about 10 to 15 known species apart from *Gloriosa superba*, *viz. G. lulea*, *G.plantii*, *G. latifolia*, *G.longifolia*, *G.magnifica*, *G.rothschildiana*, *G.abyssinica*, *G. congijolia and G.simplex*. In all these the important species found in India are *G.superbaa* and *G. rothschidiana* [5]. Its distribution spreads to Nasik, Ratnagiri, Savanthwadi (Maharastra), Uttar karnataka, Hasson, Chikmangalur, Coorg, Mysore (Karnataka), Cannanore Palakkad, Trivandrum (Kerala), Tamil Nadu and Goa. In Tamil Nadu the largest area are under hilly region and is up to 6000 acres and spread over seven district viz Karur, Tirupur, Dindigul, Salem, Ariyalur, Perembalur and Nagapattnam [6]. The planting of the crop is seasonal and it falls on the kharif month (June – July).

There is no specific released variety is available in this crop, only ecotypes with variable proportion of chemical constitutions in accumulation are available. This chemical constitution especially the alkaloid content were quantified and seems to be varying along its distribution and found very much influenced by the micro climatic factors of that particular accession. For further breeding and hybridization between natural populations, it is mandatory to known the genetic relationship that exists between the ecotypes of different accession, the identification

of similarities and difference between the ecotypes taps the novel genes within the ecotypes for future research purpose.

ISSR- PCR has been widely used DNA-based molecular marker technique used to analyze the relationship between the species and ecotypes [7]. The characterization of accessions through finger printing can be very well done using ISSR-PCR technique [8] among many types of DNA markers, the Inter simple sequence repeat (ISSR-PCR) can be very well utilized, as the ISSR distribution throughout the genome can be very well addressed by this technique. The marker based on microsatellites simple sequence repeats (SSRS) provides a co-dominate highly reproducible and genetically informative marker system [9].

But the loci of ISSR in the *Gloriosa* is unknown, this may be replaced by the advanced technique called ISSR- PCR technique, which has been found to be an efficient and reliable technique [10] as this technique need -no information on the DNA sequence prior to amplification, low cost, done with micro samples, perform with simple operations gives stability and produces abundance of genomic information.

Several factors are important including the geographic range which is strongly associated in determining the genetic diversity within the species it is also noted in this study that the general endemic species have lower genetic diversity than the wide spread species [11]. So, it is of great need to find a genetic marker for all the five ecotypes collected from different accessions of Tamil Nadu for authentication and standardization of chemical constituents for further clinical, pharmacological and research purpose through highly reliable ISSR-PCR marker Systems. The objective of the present study is to identify specific, effective and reproducible markers for five ecotype of *Gloriosa superba* (L) collected from different commercially cultivable accessions.

MATERIALS AND METHODS

Planting Materials

Ecotypes of *Gloriosa superba* (L) were taken from five different accessions from different regions such as Sirumalai (GA1), Mulanoor (GA2), Thuraiyur (GA3), Konganapuram (GA4) and Vedaranyam (GA5) for the investigation. The Randomized Block Design (RBD) with three replication was done. The experimental area mixed with red earth, sand, vermicompost and farmyard manure in the ratio of 1:1:1:1 was applied in the agriculture field in order to enhance the micro and macro nutrient of the soil for better plant growth. The plots were irrigated at seven to ten days interval regularly, the recommended agronomic and plant protection methodology were implemented during the cultivation.

Genetic Variability Studies And Separation Of DNA

Genetic variability studies for five ecotypes from 5 different accessions. Samples taken from five different accessions were used for the present studies (The reagents used to amplify the genomic DNA for ISSR analysis is given in the Table -1) and the DNA was extracted from bulk, fresh leaf tissues from each population of *Gloriosa superba* (L) as per the procedure [12] and the DNA was assessed by UV-vis spectrophotometer (Techomp 8500) in order to find out the purity and the quantity of DNA [13] and the DNA were separated by electrophoresis in agarose gel (1.0%) and was visualized by UV-vis trans illuminator and photographed. DNA pooling was done by pooling equal amount of purified economic DNA from the individuals of each five species and aliquot from those combined sample was used for PCR.

Inter Simple Sequence (ISSR Analysis)

The genetic variability studies were conducted by ISSR analysis using modified method [10].Earlier twenty primers were tested and twelve primers producing reproducible bands were selected for five accessions. Primers representing different random knock out DNA sequences were issued for ISSR-assay. The experiment were repeated three times and conformed for the reproducibility of bands the amplified products were checked by running through 1.5% agarose gel stained with ethidium bromide, the gel was viewed using UV-trans-illuminator and gel was documented using photo documentation system. The banding patterns were analyzed.

Inter Simple Sequence (ISSR Analysis)

The genetic variability studies were conducted by ISSR analysis by using modified method [10]. Earlier twenty primers were tested and in which twelve primers producing reproducible bands were selected for the five accessions. Primers representing different random knock out nucleotide sequences were repeated three times for confirmation of the reproducibility of bands. An initial denaturing period of 2min at 94degree was followed by 35 cycles of 30sec at 94degree and then 35 cycles for 45 sec at 42degree followed by 35 cycles of 1min-30 sec at 72 degree for final extension was done (Table 2).The reaction was done with25 μl containing about 15.9 μl of sterile water, 4 μl of dNTPS,1.5μl of Mgcl2.,1 μl of Taq polymerized and 0.5 μl of plant DNA. The amplified products were checked by running through 1.5%agrosegel stained with ethidium bromide. The gel was viewed using UV-Tran-illuminator and the gel was documented using photo documentation system. The banding patterns were analyzed for knowing the similarities and difference between the ecotypes. Out of 20 primers, 12 reproduced primers were chosen for running ISSR-PCR analysis (Table 3).

Table 1: Reagents used to amplify the genomic DNA for ISSR analysis

S.No.	Constituents	Quantity
1.	Sterile water	15.9μl
2.	dNTPs	4.0 μl
3.	MgCl2	1.5 μl
4.	10X PCR Buffer	2.5 μl
5.	Primers	0.5 μl
6.	Taq polymerase	0.1 μl
7.	Plant DNA	0.5 μl
Total		25.0 μl

Data Analysis

The genetic distance matrix was obtained and analyzed through cluster method inorder to construct the UPGMA (Unweighted Pair Group method with Arithmetic mean Dendrogram using MEGA 4.0 [14].

RESULTS AND DISCUSSION

ISSR Polymorphism

Earlier twenty primers were taken and screened for the studies. Primer producing clear and reproducible bands of 12 numbers were selected for further amplification with genome of five ecotypes of *Gloriosa Superba* (L).The oligonucleotide sequence of the primer with the percent content along with the amplification temperature were taken and their appropriate sequences were used for the analysis of ISSR. The twelve primers on amplification produces 7.8 bands per primer. The size range varies between 200bp to 1000 bp and a total of 94 polymorphic fragments were produced for all the five ecotypes of *Gloriosa superba* (L) which were summarized.

Genetic Diversity

The 5' anchored primers produces total of 94 polymorphic fragments in which the total diversity (Hg) varies between 0.2479 and 0.4880, and diversity within the population (Hs) varies between 0.0924 and 0.1984. The genetic differentiation (gst) of the produced fragment population range within 0.4418 to 0.8436 with a range of 0.2179-0.6387 of gene flow (Nm) (Hr,Hs,Gst and Nm) With the mean average of 1.9647 numbers of alleles,a mean average of 1.6471 numbers of alleles were found to be effective. The mean Nei's gene diversity between GA1-GA5 were 0.0931, 0.1477,0.1285,0.1193 and 0.1013 respectively, with an average mean value of 0.4041 as shown (Table 4). Nei's Unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) were shown (Table 5). Genetic distance of all loci between the population obtained by assessing the ISSR-PCR banding pattern shown in table 6. The Similarity matrix shows a maximum similarity of (0.9276) that occurs between GA1 and GA5and a minimum similarity of (0.6307) that occurred between GA2 and GA3 which is given in the similarity matrix (Table 7).The number of polymorphic loci in all the five ecotypes varies between 26 (GA1) to 45(GA3), and the percentage of polymorphism varies from 26.36% (GA1) to 40.64 (GA3) which were represented (Table 8).The value of total number of observed alleles(na), Effective number of alleles(ne), Nei's gene diversity (h), Shannan's information index (I) were given as 1.9647, 1.6471, 0.4041, 0.5607 respectively and the over all polymorphic loci and its percentage were given as 92 and 86.27 respectively were given in Table 9.

The genetic relationship between five ecotypes was arrived by closing it into groups by UPGMA analysis based on Nei's genetic diversity within the population. A dendrogram was generated based on the cluster analysis through which the genetic relationship between the ecotypes can very well be studied. The genetically, ecotypes of GA4 and GA5 were closely related and ecotypes of GA3 and GA2 are widely placed, which were shown in dendrogram (Figure 1).

Table 2: Conditions followed for ISSR – PCR analysis and the reaction steps

S.No.	Activity	Temperature	Time	Cycles
1.	Initial denaturation	94°C	2 min	One
2.	Denaturation	94°C	30 sec	35
3.	Annealing	42-46°C	45 sec	
4.	Extension	72°C	1 min. 30 sec	
5.	Final Extension	72°C	20 min	One
6.	Storage	4°C	For ever	

Table 3: ISSR-primers used for ISSR-PCR analysis of five accessions of *Gloriosa superba* (L)

S.No.	Primers	Sequence 5'-3'	GC content (%)	Temperature (C⁰)
1.	HBI0809	(AG) 8G	52.9	52.8
2.	HBI0810	(GA) 8T	47.1	50.4
3.	HBI0812	(GA) 8A	47.1	50.4
4.	HBI0816	(CA) 8T	47.1	50.4
5.	HBI0834	(AG) 8T	47.2	52.6
6.	HBI0835	(AG) 8C	52.9	52.8
7.	HBI0836	(AG) 8A	47.1	50.4
8.	HBI0840	(GA) 8T	47.1	50.4
9.	HBI0842	(GA) 8G	52.9	52.8
10.	HBI0855P	(8TG) 6	51.6	46.9
11.	HBI0864	(CTC) 6	33.3	48.7
12.	HBI0873P	(GACA) 4	49.2	50.2

DISCUSSION

It is of urgent need to standardize and authenticate the chemical constituents of *Gloriosa superba*(L) ecotypes obtained from various accessions of Tamil Nadu as it varies with varying geological and agroclimatic conditions. Earlier work on *Gloriosa superba* (L) was done with ISSR-PCR system to evaluate genetic fidelity of micro propogated *Gloriosa superba*(L) using ISSR marker [15] was seems to be single work regarding genetic studies of the plant.

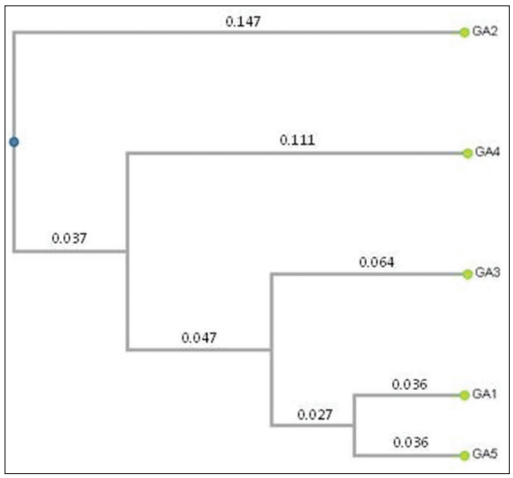

Figure 1: UPGMA dendrogram of *Gloriosa superba* accessions for ISSR analysis

Table 4: Gene diversity within and between the populations of five accessions of ***G.superba***

S.No	Primers	Annealing Temp. (C^0)	No. of polymorphic fragments	Total diversity (H_T)	Genetic diversity within population (H_s)	Genetic differentiation (G_{ST})	Gene flow (N_m)
1	HBI0809	44	8	0.4158	0.1658	0.6587	0.4238
2	HBI0810	43	5	0.3458	0.0924	0.4418	0.3343
3	HBI0812	47	11	0.2510	0.0945	0.5444	0.2179
4	HBI0816	42	6	0.3203	0.1584	0.5389	0.4024
5	HBI0834	46	5	0.2479	0.1065	0.5120	0.2756
6	HBI0835	44	9	0.4789	0.1541	0.6173	0.4891
7	HBI0836	45	9	0.3178	0.1283	0.5410	0.3387
8	HBI0840	42	11	0.4732	0.1074	0.6613	0.4047
9	HBI0842	46	5	0.3496	0.1335	0.6337	0.6387
10	HBI0855P	41	8	0.4880	0.1787	0.8436	0.3001
11	HBI0864	46	9	0.3778	0.0934	0.5454	0.2419
12	HBI0873P	43	8	0.3307	0.1984	0.7896	0.3951

Table 5: Nei's Unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal)

S. No	Accessions	Observed number of alleles (na)		Effective number of alleles (ne)		Nei's gene diversity (h)		Shannon's information index (I)	
		Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
1	GA_1	1.5364	0.5268	1.2632	0.4214	0.0931	0.1643	0.2368	0.3521
2	GA_2	1.4209	0.5871	1.2246	0.4181	0.1477	0.1706	0.2086	0.3679
3	GA_3	1.3545	0.5712	1.3138	0.4255	0.1285	0.1621	0.1932	0.3681
4	GA_4	1.6882	0.5679	1.2030	0.4323	0.1193	0.1731	0.2777	0.3674
5	GA_5	1.4123	0.5426	1.2749	0.4231	0.1013	0.1967	0.2498	0.3570

Table 6: Genetic distance between the populations obtained by assessing the ISSR-PCR banding pattern of *Gloriosa superba* (L) from five accessions.

Accessions	GA_1	GA_2	GA_3	GA_4	GA_5
GA_1	1.000				
Accessions	GA1	GA2	GA3	GA4	GA5
GA_2	0.854	1.000			
GA_3	0.509	0.504	1.000		
GA_4	0.644	0.473	0.518	1.000	
GA_5	0.781	0.618	0.653	0.810	1.000

Table 7: Similarity matrix obtained by assessing the ISSR-PCR banding pattern of five ecotypes of *Gloriosa superba* (L).

Accessions	GA_1	GA_2	GA_3	GA_4	GA_5
GA_1	****	0.7510	0.8840	0.7877	0.9276
GA_2		****	0.6307	0.6900	0.7509
GA_3			****	0.6627	0.8614
GA_4				****	0.8862
GA_5					****

Table 8: Analysis of polymorphism of five accessions of *Gloriosa superba* (L)

S.No.	Number of accessions	Number of polymorphic loci	Percentage of polymorphism
1	GA_1	26	26.36
2	GA_2	29	27.09
3	GA_3	45	40.64
4	GA_4	39	35.82
5	GA_5	38	32.45

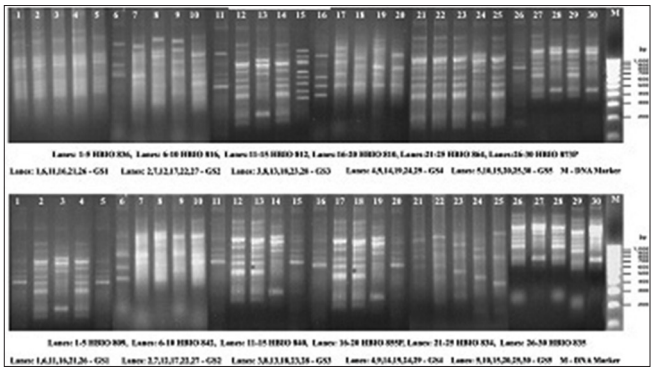

Figure 2: ISSR banding pattern of Gloriosa superba (L).obtained from five different accessions obtained using 12 reproducible primers.

In the present study reveals that the ISSR-PCR system was taken as tool for analyzing the genetic diversity (Figure 2). Twelve number of 5' sequence anchored primer were selected as it produces clear and reproducible fragments, about the 20 primers were taken initially and they were amplified. The ISSR marker technique produces much greater number of total polymorphic and discrimitant fragments then RAPDS [16] and lower cost than AFLPS [17]. Simpler to use than SSR technique [18]. Molecular fingerprints of four different species of *Curcuma amada, C.caesia, C.longa and C.zedoa* [19].Similar results are also reported in three different DNA based technique such as RAPD, ISSR and AFLP were used for fingerprinting *Dactylis glomerata* genotype and for detecting genetic variation between the three subspecies [20]. Various species of genus Saccharina are economically important brown algae cultivated in China.

Table 9: The mean Nei's gene diversity, Shannan's index and polymorphic loci of five accessions

S.No.	Observed number of alleles (na)		Effective number of alleles (ne)		Nei's gene diversity (h)		Shannan's information index (I)		Over all polymorphic loci	Over all percentage of polymorphic loci
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation		
1	1.9647	0.4348	1.6471	0.4990	0.4041	0.2481	0.5607	0.3045	92.0	86.27

DNA based molecular markers such as ISSR to assess the genetic diversity and phylogentic relationships [21].

The ISSR produced fragment shows a 86.27% of polymorphism within 94 reproducible polymorphic fragment, which shows that the inter simple repeat sequences spreads throughout the genome of all the five ecotypes. Similarly high polymorphic fragments were obtained by many workers in so many plants especially in olive, with varying results of 93% [22], 96% [23] when the gene analysis were done with ISSR-PCR system. An average mean of 1.6471 effective number of alleles (ne) were obtained from the average means of 1-9647 number of observed alleles (na). Supporting the identification of novel alleles for further research purposes. Average mean of 0.4041 Nei's gene diversity (h) was arrived using which the dendrogram was constructed using cluster analysis, to find the genetic relationship between the five ecotypes and useful ideal reaction. However, ISSR is a better tool than RAPD for phylogenetic analysis [24, 25]. Methods used to preserve the samples can provides sufficient amount of genomic DNA for ISSR and RAPD analysis [26].

Based on the reproducibility, relatively high polymorphisms were selected generally in optimal condition. Moreover, reliable ISSR-PCR-method for *Gloriosa superba* (L) and established by analyzing amplified bands then successful ideal reaction.

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