Applied Biological Research 19 (3): 290-298; (2017)
DOI: 10.5958/0974-4517.2017.00042.8

GENETIC DIVERSITY ANALYSIS AND DNA FINGERPRINTING OF ELITE STOCK OF GLADIOLUS (*Gladiolus hybridus* Hort.) USING ISSR MARKERS

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(Received 11 February, 2017; accepted 24 September, 2017)

ABSTRACT

Molecular characterization of 48 genotypes of gladiolus was carried out using PCR based ISSR technique. A total number of 239 loci were generated out of which 216 loci ranging in size of 200 to 1000 bps were found polymorphic (90.38% polymorphic bands). The PIC value of primers ranged from 0 to 0.22 with mean value of 0.15 primer^{-1} and most informative marker was related to UBC 853 followed by UBC 859. Based on banding pattern, the cluster analysis was done using UPGMA and dendrogram was constructed. The similarity coefficient ranged from 0.34 to 0.74 based on dissimilarity matrix. The dendrogram grouped the genotypes into 3 main groups with group I and II having maximum number of genotypes. The pattern of genotype grouping into clusters was independent of their eco-geographical regions and gave a useful insight into their phylogenetic relationships. Analysis of molecular variance (AMOVA) indicated that maximum genetic diversity was partitioned within groups (84%) and only 16% of total variation occurred between the groups. The phylogenetic relationship was determined by principal co-ordinate analysis (PCoA) that explained 23.41% cumulative variance. PCoA revealed similar clustering pattern of genotypes along the axis, thereby corroborating the clustering of genotypes in UPGMA dendrogram.

Key words: Gladiolus, genetic diversity, ISSR markers

INTRODUCTION

Gladiolus (*Gladiolus hybridus* Hort.) is one of the most widely cultivated economically important flowering plant of *Iridaceae*. Due to unique colourful spikes, it is one of the four famous cut-flowers in the world trade market (Shufang *et al.,* 2010). The plant is very popular in beautification and has good prospects in gardening, owing to its bright coloured florets and long bloom period. Gladiolus is very rich in variable varietal wealth and has been classified into different groups based on biological characteristics like growth, flowering time, flower shape, diameter and colour (Chan, 2001). However, this method of classification is often laborious and occasionally misleading in some specific cultivars which make it necessary to have a revisit into the classification system and genetic relationship among cultivars of *G. hybridus* (Jingang *et al*., 2006). However, assessment of genetic diversity with DNA-based molecular markers has been more convincing because unlike morphological traits, DNA fingerprinting reflects plant variability directly at genetic level. Characterization of genotypes or cultivars using molecular markers not only helps in establishing the relationship and affinities among various taxa, but shall also pave the way for selection of elite

genotypes for hybridization programme and broadening of genetic base. Inter simple sequence repeat (ISSR) markers have successfully been used in identification and to determine the relationships at species, population and cultivar levels in many plant species, including several aromatic and medicinal plants (Sangwan *et al*., 1999; Nan *et al*., 2003). The method involving the use of ISSR primers is widely applicable because these primers are rapid, inexpensive, require small amounts of template DNA and, unlike SSR markers, do not require prior knowledge of DNA sequences (Godwin *et al*., 1997). Therefore, the present study was aimed to assess the magnitude of genetic diversity present in elite germplasm and to compare the information content of the ISSR markers system which may act as an aid in formulating the breeding and conservation programmes of gladiolus.

MATERIALS AND METHODS

Experimental material

The experimental material for molecular characterization comprised of 48 genotypes of gladiolus, given as under, representing the germplasm which include prominent exotic genotypes and some indigenous hybrid genotypes developed under Indian conditions.

Gladiolus genotypes analyzed for molecular characteristics along with their parents wherever available

S. No.	Genotype	Parentage	S. No.	Genotype	Parentage
1	True Yellow	Unknown	25	Pusa Kiran	Unknown
2	Dark Jamini	Unknown	26	Aldebaran	Unknown
3	Shagun	White Oak x Oscar	27	Priscilla	Diamond x Leona
4	Yellow Stone	Unknown	28	Shobha	Wild Rose induced mutant
5	Legend	Unknown	29	Bis Bis	Unknown
6	Sylvia	Unknown	30	Punjab Dawn	Suchitra x Melody
7	Red Beauty	Unknown	31	Arka Anmol	Unknown
8	Nova Lux	Unknown	32	PG-23-55	Jacksonville Gold x Hunting Song
9	Hunting Song	Unknown	33	Anglia	Eloise x Rosemarie Pfitzer
10	Red Advance	Unknown	34	Dhanavantri	Jr. Prom x Lucky Star
11	Punjab Flame	Sylvia x White Prosperity	35	PG-6-16	Happy End x True Yellow
12	Peter Pears	Saiome x Maolete	36	Jacksonville Gold	Unknown
13	Rose Supreme	Unknown	37	Arka Kesar	Unknown
14	Punjab Pink Elegance	Suchitra x White prosperity	38	Kum kum	Watermelon Pink x Lady John
15	White Prosperity	Unknown	39	Punjab Glance	Happy End x Yellow Stone
16	PG-9-2	Happy End x Yellow Stone	40	Fidelio	Unknown
17	Wind Song	Unknown	41	Jessica	Unknown
18	Sunset	Unknown	42	Punjab Lemon Delight	Jacksonville Gold x White prosperity
19	Suchitra	Sylvia x Jo Wagenaar	43	Happy End	Unknown
20	Chandni	Green Woodpecker x White Butterfly	44	Punjab Glad1	Happy End x True Yellow
21	Alexander Great	Unknown	45	PG-12-19	CPG x White Prosperity
22	Blue Sky	Unknown	46	PG-20-64	Nova Lux x Hunting Song
23	Praha	Unknown	47	PG-19-15	Nova Lux x Priscilla
24	CPG	Unknown	48	Sancerre	Unknown

The introduced exotic genotypes were first subjected to clonal selection for several years and then desirable clones having greater adaptability and desirable morphological characteristics were selected for diversity analysis. The samples for molecular characterization were procured from the research field trials, laid out in the year 2013-2014 in a randomized block design with three replications at the Department of Floriculture and Landscaping, PAU, Ludhiana (India). The molecular study was conducted in the Central Laboratory of Department of Plant Breeding and Genetics, PAU Ludhiana, India.

Experimental methodology

DNA extraction: DNA was extracted from 150 to 300 mg of gladioli leaf materials using modified Doyle and Doyle (1987) method. Samples (fresh young leaves) were collected from healthy plants and stored at -80°C until use. The leaves were ground in liquid nitrogen in a sterile pre-chilled mortar and pestle and were put in test tubes. About 15 mL extraction buffer (100 m mol tris-HCl (pH 8.0), 20 m mol EDTA (pH 8.0); 2% β-mercaptoethanol, 2% PVP, 1.4 mol NaCl and 1.5% CTAB) in test tubes was incubated at 60°C in water bath for 30 min with occasional inversion. After the tubes were cooled to room temperature, 20 mL chloroform-isoamyl alcohol (24:1 v/v) was added to each tube and shaken vigorously to form a dark green emulsion. Tubes were placed on a rotary shaker for 30 min and then centrifuged at 8000 rpm at room temperature for 20 min. After centrifugation, 30 mL aqueous phase was transferred to a new sterile tube and 20 mL cold isopropanol added to each tube and then inverted gently several times till white cotton threads of precipitated DNA were formed. The floating DNA was hooked out using a sterile hooked Pasteur pipette and transferred into a clean sterile 2.0 mL microfuge tubes. Resulting pellets were washed 3 times with equal volume of 70% ethanol and pellets dried and resuspended in 2 mL TE (tris + ethylene diamine tetra-acetic acid) buffer with 10 µL RNAase and incubated at 37°C for 1 h. The quantification of purified DNA was done by using NanoDrop ND-100 spectrophotometer (Nano-Drop Technologies, Wilmington). The dilution of DNA samples was done to a uniform concentration of 20 ng μL^{-1} by adding double distilled deionized water and stored at 4°C till further use.

Primers: Forty eight anchored primers (designed by University of British Columbia, Canada) were screened for polymorphism using a few DNA samples to select primers suitable for study. After preliminary screening, 40 primers were selected for further study on the basis of number of polymorphic bands produced, band size, amplification intensity and reproducibility (Table 2). Primers were excluded if banding patterns were difficult to score accurately on 1.8% agarose gels.

PCR conditions and gel electrophoresis: Polymerase chain reaction (PCR) was performed in final volume of 20 µL comprising of PCR buffer (1 x buffer, 100 n mol L^{-1} tris HCl {pH 8.3}, 500 m mol L^{-1} KCl and 1.5 m mol L^{-1} $MgCl_2$), 200 µ mol dNTPs, 1 U Taq DNA polymerase, 0.5 µ mol primer, and 60 ng genomic DNA. Double distilled deionized water was supplemented for the rest. PCR conditions were optimized for each primer pair by adapting the melting temperature (Tm). The PCR cycle was as follows: 5 min initial denaturation at 95ºC, 40 cycles of amplification [45sec at 94 ºC, 45 sec at optimized annealing temperature, 1.30 min extension at 72 ºC], followed by final extension for 7 min at 72ºC. All PCRs for ISSR primers were performed on Eppendorf Master Cycler gradient (Eppendorf Scientific, Germany). PCR products were separated on 1.8 % agarose gel in 0.5 Tris–borate–ethylenediamine tetraacetic acid (TBE) buffer at 120 V, stained with ethidium bromide, and visualized under ultraviolet (UV) light and photographed in a gel documentation system (Alphaimager HP, Alpha Innotech). A 100 bp Plus DNA ladder (Fermentas International Inc., Sankt Leon-Rot, Germany) was used as molecular size standard.

Data analysis

DNA polymorphism was evaluated and scored on the basis of presence or absence of bands (1 or 0) and each ISSR fragments was treated as a unit character. For each primer, the numbers of total alleles and polymorphic ones as well as the percentage of polymorphic bands (PPB) were calculated. The PPB was determined as the percentage of polymorphic bands over total number of bands. Polymorphism information content (PIC*i*) of a band was calculated as per Anderson *et al*. (1993) as follow:

$$PIC_i = 1 - \sum fij^2$$

where *fij* is the frequency of *jth* pattern of *ith* band (note that dominant markers have two patterns for a band as being present and absent). Then, the PIC of each primer was calculated as:

$$PIC = 1/n \sum PIC_i$$

Where n is the number of polymorphic bands.

The dissimilarity coefficients between cultivars were analyzed and clustering carried out using Unweighted Pair Group Method and Arithmetic Average (UPGMA) and Neighbour Joining (NJ) method through DARwin software (Dissimilarity Analysis and Representation for Windows version 5.0.148) (*http://darwin.cirad.fr/darwin*, Perrier and Jacquemoud-Collet, 2006). The principal coordinate analysis (PCoA) was used for the analysis of phylogenetic relationship among gladioli genotypes. PCoA and analysis of molecular variance (AMOVA) were performed using software GenAlEx V6.5 (Peakall and Smouse, 2012).

RESULTS AND DISCUSSION

ISSR polymorphism

Forty-eight ISSR primers were employed to differentiate the studied gladiolus populations. Of these, 40 primers generated 216 sharp and polymorphic bands of 200 to 1000 bp size (Table 1). In total, 239 alleles were produced among different gladiolus genotypes using these markers. The selected primers produced an average of 90.38% polymorphic bands. These results are in line with the ISSR based studies in chrysanthemum (Baliyan *et al*., 2014) and lilium (Zahao *et al*., 2014). About 93.6% polymorphism was also produced by ISSR markers in gladiolus (Jingang, 2008). Polymorphism information content values were calculated as marker index to show the informative-ness of utilized markers with mean value of 0.15 by the range of variation from 0.00 to 0.22. The most informative marker was related to genotype UBC 853 followed by UBC 859 and UBC 812

Genetic distance and cluster analysis

The cluster analysis was carried out based on Jaccard′s similarity coefficients generated from ISSR bands ranged from 0.34 to 0.74. The maximum dissimilarity coefficient was found between genotypes Punjab Flame and True Yellow (0.66), which can be utilized in hybridization for attaining more heterosis. However, the minimum dissimilarity coefficient (0.26) was recorded between genotypes Punjab Pink Elegance and Chandni thus indicating that these two genotypes are genetically similar although quite different in morphology and have light pink and yellow coloured florets, respectively. This appears due to highly heterozygous nature of this crop (Kumar *et al*., 2016). The UPGMA dendrogram constructed using Jaccard's dissimilarity matrix of ISSR data discriminated all the genotypes into 3 groups (Fig 1). Group I was divided into two sub-groups and comprised of 19 genotypes. In this group both exotic and indigenous developed hybrid genotypes were found together. The maximum genotypes of this group had light reddish or pinkish florets, except Chandni, Praha and Yellow Stone which had light yellowish florets. Genotypes Punjab Glance and PG-6-16 were grouped with their common parent Happy End. Punjab Glance had orange coloured florets with yellow centre and PG-6-16 had lemon coloured florets unlike their common parent having red coloured florets. Thus, it can be inferred that the genotypes which look different in flower colour may genetically be related to each other. So, classification on the basis of morphological traits may not be similar to ISSR diversity (Kiani and Memariani, 2012). Punjab Flame showed close resemblance to one of its parents Sylvia. Sylvia is brick red to maroon in colour and produces carmine pink coloured florets when crossed with White Prosperity. Thus, it can be assessed that the hybrid genotypes showing close similarity with each other and with one of their parents may be closely related to that parent as compared to the other parent. The ISSR analysis of the genetic resource of *G. hybridus* showed that if the kinship among varieties is close, species with different colours from different places can assemble together within the same cluster (Jingang *et al.*, 2008). Raycheva *et al.* (2011) who examined genetic diversity and relations among seven Bulgarian species from the Iridaceae family by ISSR markers observed that grouping of studied species did not coincide with the classification schemes based on morphological features, but was in agreement with the phylogenic studies.

Table 1: The selected ISSR primers, annealing temperature, number of polymorphic bands and PIC value used in the study

S. No.	Primers	Repeat motif (5' – 3')	Annealing temperature (ºC)	Total bands	Polymorphic bands	%age polymorphism	PIC value
1	UBC 806	(AG)9T	52	6	6	100	0.14
2	UBC 808	(AG)8C	46	6	6	100	0.14
3	UBC 809	(GA)9T	49	8	8	100	0.11
4	UBC 810	(GA)8T	48	5	4	80	0.19
5	UBC 811	(GA)8C	43	8	8	100	0.11
6	UBC 812	(CT)8T	43	4	3	75	0.21
7	UBC 814	(CT)8A	48	5	4	80	0.17
8	UBC 816	(CA)8A	46	9	9	100	0.10
9	UBC 818	(CA)8G	50	6	6	100	0.14
10	UBC 819	(GT)8C	51	4	2	50	0.19
11	UBC 820	(GT)8T	48	6	6	100	0.14
12	UBC 821	(TC)8A	45	6	4	66.67	0.18
13	UBC 824	(TC)8G	46	9	7	77.78	0.11
14	UBC 825	(AC)8RT	48	9	7	77.78	0.12
15	UBC 826	(AC)8C	51	6	6	100	0.13
16	UBC 827	(AC)8G	48	9	9	100	0.10
17	UBC 828	(TG)8A	49	7	7	100	0.12
18	UBC 829	(TG)8C	51	4	3	75	0.20
19	UBC 830	(TG)8G	51	4	3	75	0.20
20	UBC 834	(AG)8CTT	52	5	5	100	0.15
21	UBC 836	(AG)8CTA	52	5	5	100	0.16
22	UBC 840	(GA)8CTT	52	5	5	100	0.15
23	UBC 846	(CA)8RT	48	3	1	33.33	0.00
24	UBC 847	(CA)8RC	48	6	6	100	0.14
25	UBC 848	(CA)8RG	48	9	9	100	0.10
26	UBC 851	(GT)8CTG	54	3	3	100	0.15
27	UBC 853	(TC)8RT	49	4	3	75	0.22
28	UBC 854	(TC)8RG	51	4	4	100	0.19
29	UBC 855	(AC)8CTT	52	6	6	100	0.14
30	UBC 856	(AC)8CTA	52	5	5	100	0.16
31	UBC 858	(TG)8RT	48	7	7	100	0.12
32	UBC 859	(TC)8RC	51	4	3	75	0.21
33	UBC 861	(ACC)6	58	5	4	80	0.19
34	UBC 862	(AGC)6	60	5	5	100	0.16
35	UBC 864	(ATG)6	45	5	4	80	0.18
36	UBC 865	(CCG)6	72	7	7	100	0.12
37	UBC 866	(CTC)6	48	6	6	100	0.14
38	UBC 867	(GGC)6	72	6	5	83.33	0.16
39	UBC 868	(GAA)6	45	9	6	66.67	0.10
40	UBC 873	(GACA)4	47	9	9	100	0.09
	Total			239	216	90.38	

Group II is the biggest one having 26 genotypes with two subgroups. The first subgroup comprized of only indigenous bred genotypes having reddish coloured florets whereas second subgroup was dominated with both indigenous bred and exotic genotypes most of which were soft coloured. Punjab Lemon Delight showed close similarity with its parent White Prosperity although both are morphologically different having yellow and white coloured florets, respectively. Thus, grouping of genotypes did not match their phenotypes (Sirohi *et al*., 2017). PG-23-55 and PG-20-64 showed close similarity with their common parent Hunting Song. PG-19-15 confirmed its association

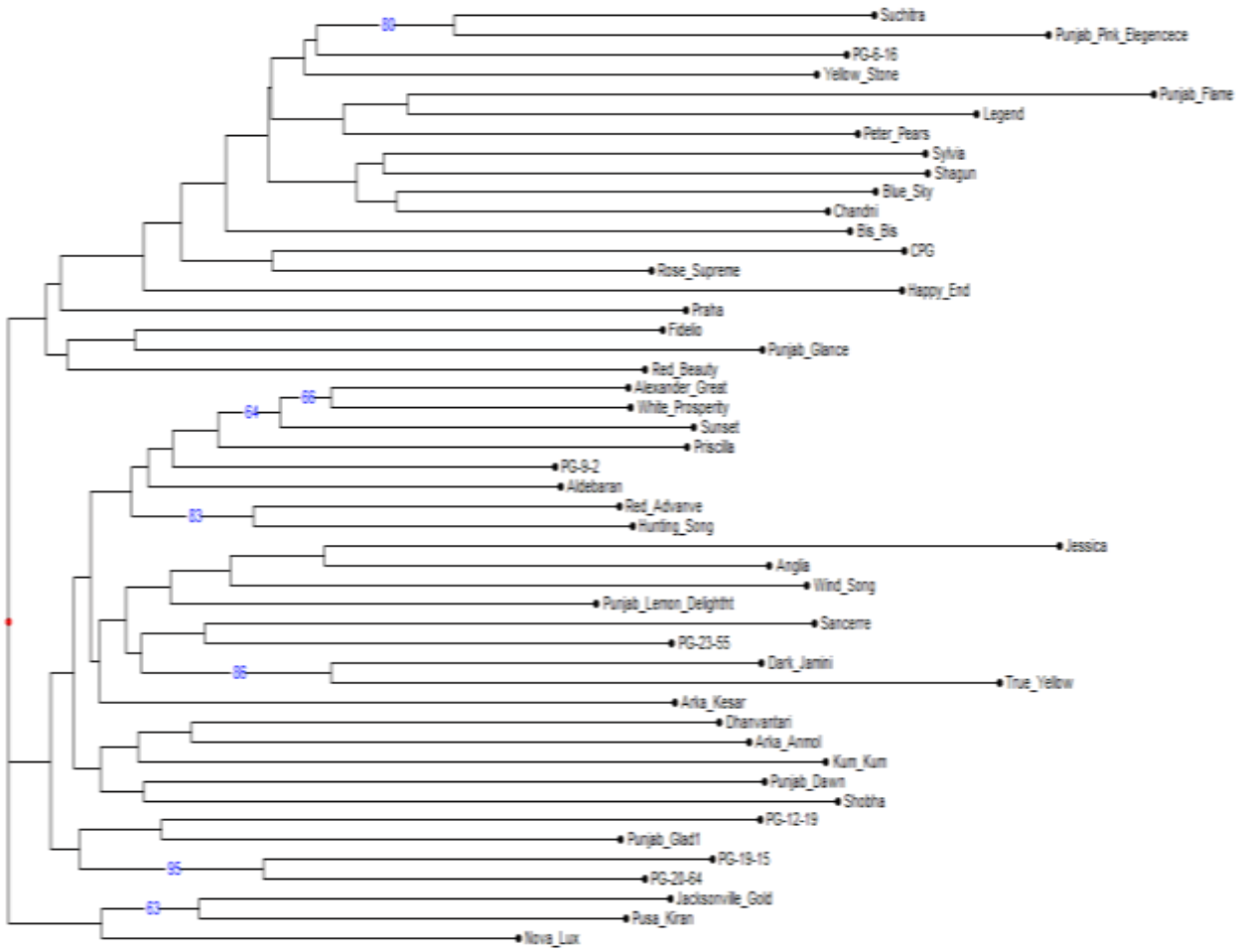

Fig. 1: Phylogenetic tree generated using unweighted Neighbour joining method from similarity matrix with computer software DARwin5

Table 3: Summary AMOVA for ISSR markers

Source	df	SS	MS	Estimated variance	Variance (%)
Among groups	2	566.029	283.015	15.529	16
Within groups	45	3578.638	79.525	79.525	84
Total	47	4144.667		95.054	100

with one of its parents Priscilla. Punjab Glad 1 also showed close resemblance to its parent True Yellow. Punjab Glad 1 and True Yellow are morphologically different having light orange and yellow coloured florets, respectively. However, molecular analysis revealed that these are similar to each other. The group III is smallest one comprised of only three genotypes. From the diversity analysis it appeared that the genotypes were grouped irrespective of their geographical origin which confirmed earlier results of Kumar *et al*. (2016) in gladiolus; Agarwal and Srivastava (2010) in chickpea and Khar *et al.* (2008) in garlic. The results also inferred that there is no clear- cut demarcation between the exotic and locally developed hybrid genotypes. This is might be due to the reason that the indigenous bred hybrids have been developed by hybridizing the exotic genotypes which might have resulted in the random distribution of genotypes among various groups. Thus, the origin of the contemporary *Gladiolus hybridus* Hort is very complicated due to years of interspecific or inter-varietal hybridizations and most of these species planted all over world are not pure original species (Jingang *et al.*, 2008)

Factorial analysis for variability

Analysis of molecular variance (AMOVA) (P< 0.05) for 999 permutations for group comparisons was conducted to partition the genetic variation among and within groups. The results demonstrated that the greatest variation can be attributed to intra-group variation (84%) (Table 3 & Fig. 3). The lower level of genetic variation among the groups was observed in intra-group (16%) as compared to the genetic variation within groups which presumably appears due to small population size. The principal coordinate analysis (PCoA) was used to evaluate phylogenetic relationship among the genotypes. The biplot analysis provided a better picture of genotype relationships. The major advantage of ordination methods over cluster analysis is that these methods facilitate detection of individuals or populations that show some intermediary between two groups (Lessa, 1990). The PCoA showed significant grouping of germplasm that were plotted into 3 plots, each group representing with distinctive features (Fig. 4). The representation of genetic relatedness by two-dimensional PCoA revealed diversity among different gladiolus genotypes. The first three coordinates

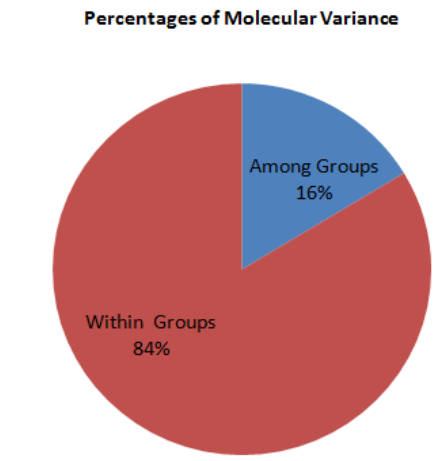

Fig. 2: Analysis of molecular variance (AMOVA) of 48 gladiolus genotypes based on ISSR data

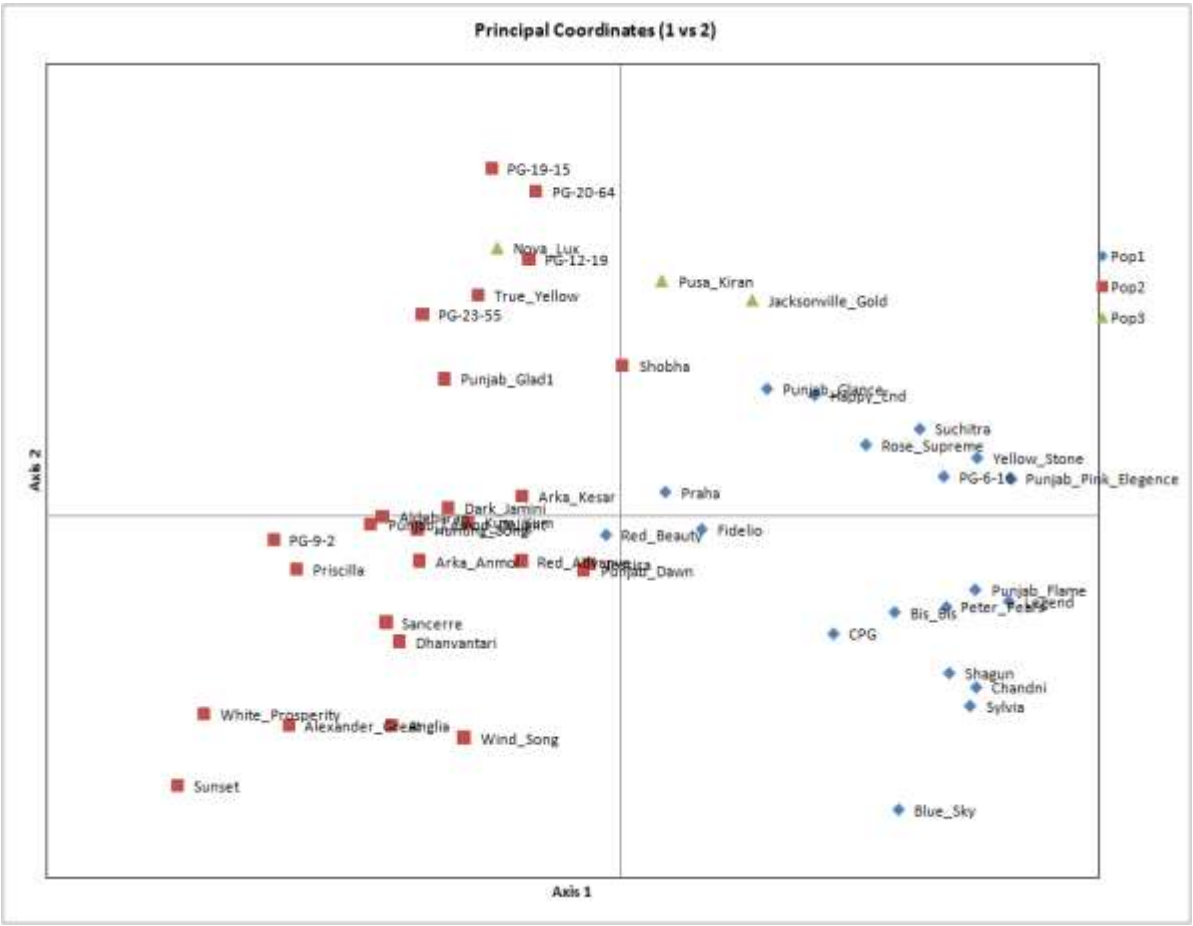

Fig. 3: Principal coordinate analysis (PCoA) of 48 gladiolus genotypes based on ISSR data

explained 23.41% of total molecular variation which accounted for 12.89, 5.85 and 4.66%, respectively, of the observed variations in standardized data set of 48 genotypes. The genotypes were broadly grouped into 3 across the first 2 axes, thereby corroborating the grouping of genotypes in UPGMA dendrogram. Similar results were obtained by Singh *et al*. (2016) in gladiolus. The 3rd group comprising of 3 genotypes (Nova Lux, Pusa Kiran and Jacksonville Gold) showed intermixing across the coordinates. In this coordinate analysis two hybrid genotypes (PG-19-15 and PG-20-64) showed close relationship with one another and also with common mother parent, Nova Lux. PCoA is a good indicator of genetic relationships and both PCoA and cluster analysis grouped the gladiolus genotypes according to their similarity based on their DNA patterns.

Conclusion: The study demonstrated that ISSR technique was very effective in determining the genetic diversity of gladiolus. The ISSR markers reported in present study will facilitate understanding of genetic structure, genetic diversity and evolutionary relationships in gladioli. Application of specific DNA marker systems like SSR or SNPs can be more helpful in marker assisted breeding and in revealing the phylogenetic relationships among gladiolus genotypes.

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