RESEARCH ARTICLE

SSR GENOTYPING OF GERMPLASM COLLECTIONS OF *SACCHARUM EDULE* HASSK

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Abstract

Saccharum edule Hassk. is cultivated for its aborted edible inflorescence in pacific Islands. The most accepted classification of *Saccharum* comprises of six species including *S.edule,* but the status and origin of *S.edule* was in debate due to its similarities with *S.robustum*.Though molecular studies have been widely utilized in sugarcane systematic; *S.edule* was not included in any of these studies. In this study, 17 accessions of *S.edule* along with five other species of *Saccharum* were characterized using eight SSR markers. Multiple bands were observed for all the primers used and the highest PIC value/ highest number of bands were observed for the primer SMC334BS and NKS5 was revealing the least polymorphism. *S.spontaneum* and *S.robustum* showed lesser number of bands across the primers. In cluster analysis three main clusters were formed the other species clones except *S.robustum* found to cluster in one group along with six *S.edule* accessions and the *S.robustum* with another clone of *S.edule*. Probably the less number of accession used in other species might have resulted in such grouping. However, the grouping of accession in three distinct clusters indicates the wide genetic variability within the collection.

Key words: Genotyping, *Saccharum edule*, SSR primers, polymorphism

Introduction

Saccharum edule Hassk. is being cultivated from New Guinea to Fiji for its edible aborted inflorescence. It has many common names, like *Coastal pitpit, duruka, dule* in Fiji and Fiji *asparagus, naviso, pit-pit* in Melanesia. The unopened flower heads of *Saccharum edule* are gathered and after removing the leaf sheath the 'flower head' is used as a vegetable (Fig. 1), eaten either raw or cooked. The genus *Saccharum* consists of six species out of which two are wild viz., *S. spontaneum* L. and *S. robustum* Brandes and Jesweit ex Grassl and four are cultivated viz., *S. officinarum* L., *S. barberi* Jesw., *S. sinense* Roxb. and *S. edule* Hassk. The status and origin of *S. edule* was in debate due to its close resemblance with *S. robustum* for most of the morphological characters except for its aborted inflorescence. Due to the similarity in gross morphology, Lennoux (1939) and Brandes et al. (1939) suggested that it

might have originated from *S. robustum* as a result of spontaneous mutation. Grassl (1967) studied the variability of *S. edule* and suggested that *it* consisted of two groups viz., the New Guinea type originated from *S.robustum* x *Miscanthus* introgression and the Fijian type evolved from *S.officinarum* x *Miscanthus* introgression. Daniel and Roach (1987) considered *S. edule* as a product of introgression of *S. officinarum* or *S.robustum* with other genera. Molecular markers have been evolved as a powerful tool for genetic diversity analysis within and between the members of *Saccharum* complex. Among the molecular markers SSR based diversity analysis is more accurate owing to its codominant inheritance, multiallelism, reproducibility, amenability to high throughput and ability to detect polymorphism even between closely related genotypes. The SSR markers are effectively used for studying relatedness among sugarcane cultivars from

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Fig. 1. The edible inflorescence of S. edule
(A. un opened inflorescence, B- The 'flower head' after removing the leaf sheath, C- the loosened 'flower head' without axis)

breeding programme in China (Chen et al. 2009; Zhi-Nian Deng et al. 2015), studying diversity among sugarcane cultivars (Neil et al. 2009), genotyping of Florida sugarcane clones (Pan et al. 2003), studying the inter and intra specific relationship among *Saccharum* species (Brown et al. 2007; Selvi et al. 2006) and screening for sugar related traits (Mishra et al. 2015). However, the molecular studies on *Saccharum* species (Selvi et al. 2006; Brown et al. 2007), *S. edule* has not been included for diversity analysis or for finding any inter-specific relationship.

The latest studies on *S. edule* by Saraswathi et al. (2013) and Chandran et al. (2014) showed that considerable variation was available within the population for agro-morphological and quality traits. Though the species remain under exploited, it's potential as a edible crop plant has been highlighted by Mudaliar (2007) and Saraswathi et al. (2013) and suggested that there is a definite need to identify superior varieties for commercial exploitation. The conventional hybridization programme for crop improvement is out of question in this species due to its aborted inflorescence and the only plausible method to identify genetically diverse clones with yield potential for commercial exploitation is by selection from the natural population. Hence, the present study was undertaken to genotype the *S. edule* collection available in the world collection of sugarcane germplasm along with representative genotypes from other species of *Saccharum* to find the relationship between different genotypes within the species and with other species using SSR markers.

Materials and methods

The world collection of sugarcane germplasm is maintained at ICAR - Sugarcane Breeding Institute-Research centre, located at Kannur, Kerala, India (11°52 N, 75°25 E, 11m MSL, mean annual rainfall 3350 mm). List of accession studied and passport information is given in table 1. All the 22 accessions were planted in 2 m x 1 row during 2014-15 and the recommended packages and practices followed for sugarcane is adopted.

Total genomic DNA was extracted from individual plants using CTAB method as described by (Murray and Thomson 1980) and quantified by ethidium bromide staining after agarose gel electrophoresis using known concentration of DNA.

Optimum polymerase chain reaction (PCR) conditions were standardized by varying the concentration of $MgCl_2$, dNTPs, Taq DNA polymerase and genomic DNA and other parameters, *e.g.* melting temperature (Tm, °C), annealing time etc. Eight SSR primers (Table 2) available on public domain were used for genotyping. The PCR amplification was carried out in a 10 µL final volume containing 30ng of genomic DNA as template, 0.2 mM of each dNTP (Thermo Scientific) 0.25 µM of each primer, 1.0 unit of Taq DNA polymerase (Thermo Scientific) in 1 x PCR reaction buffer. The PCR conditions

Table 1. The passport information of the 22 *Saccharum* genotypes

S. No	Identity	Species	Country of origin	Collection place
1	28NG 82	*S. edule*	New Guinea	NA
2	28NG 201	*S. edule*	New Guinea	NA
3	28NG 272	S. edule	New Guinea	NA
4	NG77-1	*S. edule*	New Guinea	Sovam
5	57NG 27	*S. edule*	New Guinea	NA
6	57NG234	*S. edule*	New Guinea	NA
7	IJ76-312	S. edule	Indonesia	NA
8	IJ76-329	*S. edule*	Indonesia	NA
9	IJ76-336	*S. edule*	Indonesia	Digul R
10	IJ76-337	*S. edule*	Indonesia	Digul R
11	IJ76-338	S. edule	Indonesia	Digul R
12	IJ76-360	*S. edule*	Indonesia	Butiptiri
13	IJ76-375	*S. edule*	Indonesia	Butiptiri
14	IJ76-552	*S. edule*	Indonesia	Manokwari
15	IS76-119	S. edule	Indonesia	ParigiKampung
16	NG77-10	*S. edule*	New Guinea	Wewak
17	NG77-235	*S. edule*	New Guinea	Koabu,fly R
18	21NG 3	*S. officinarum*	New Guinea	NA
19	NG77-94	S. robustum	New Guinea	Marpik area
20	UBA Seedling	*S. sinense*	China	NA
21	Dhaulu	*S. barberi*	India	Punjab
22	IND 82-330	*S. spontaneum*	India	NA

consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 56-60 °C for 45 seconds and extension at 72 for 45 seconds, with a final 72 °C extension for 10 min. The amplified products were resolved in 7.5% polyacraylamide gel and visualised through silver staining

The SSR banding patterns on the gel were scored for absence or presence, using a gel documentation system (Alpha Imager EC).The polymorphism information content (*PIC*) was calculated as follows:

$$PIC = 1 - \sum_{j=1}^{n} Pij^2$$

Where, P_{ij} is the frequency of the jth allele for the ith locus with summation extended to n alleles.

Cluster analysis was done based on jaccard's coefficient of dissimilarity following nearest neighbour joining (unweighted) method using Darwin 6.0.10 version. To assess the reliability of the dendrogram a bootstrap analysis with 10000 random sampling was applied. The morphological data from the earlier studies by the same author (Chandran et al. 2014) is used for discussing the cluster analysis data for better clarity. Principal component analysis (PCA) was carried out using SAS, JMP 9.0 version to identify the principal

Table 2. SSR Primer details and polymorphism in 22 genotypes of *Saccharum*

S. No	Primer	Sequence Forward	Reverse	Annealing temperature (°C)	No of Bands	BP	PIC	Type
1	mSSCIR4	TTC CAG CAGCAG CAT CAA T	CCC ACT AGG AGA AGC AAT AAC T	56	21	210-500	0.9436	Gen
2	NKS5	ATAGCTCCCAC ACCAAATGC	TTGGCAAAAT TGACCCAAAT	56	13	110-450	0.8342	Gen
3	SCB10	GGCTCCACCGCA CCAACTCC	CGCCTCGCTC GTCTTGGTCTC	60	18	150-500	0.7562	EST
4	mSSCIR56	ATT TGA CGC TAC GAT GGT G	ATC CGT TTT T CA GCA GAG C	56	19	150-500	0.9064	Gen
5	SGM16	ATTTACTTACACA TCCGCCAC	GCTACTACAGC AACTGGAACT	57	27	120-500	0.8770	Gen
6	NKS23	TAA ACC CCC GA A AAA GAA CC	TCCGGAGGTA GATCCATTTG	59	23	160-480	0.9418	Gen
7	UGSM417	GCTAGCAACAGA TCGGAGTGTC	GTGTACCGTGT GTATGTCTGTC	56	14	150-485	0.8644	EST
8	SMC334BS	CAATTCTGACC GTGCAAAGAT	CGATGAGCTTG ATTGCGAATG	56	28	150-350	0.9540	EST

Table 3. Dissimilarity matrix of 22 accessions based on Jaccard's Coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
2	0.699																				
3	0.722	0.119																			
4	0.746	0.586	0.606																		
5	0.708	0.676	0.682	0.661																	
6	0.750	0.654	0.676	0.567	0.650																
7	0.766	0.672	0.677	0.679	0.673	0.593															
8	0.779	0.690	0.677	0.746	0.673	0.596	0.222														
9	0.806	0.765	0.738	0.750	0.723	0.754	0.721	0.745													
10	0.729	0.697	0.704	0.596	0.636	0.464	0.627	0.630	0.646												
11	0.722	0.622	0.643	0.617	0.678	0.631	0.673	0.695	0.764	0.617											
12	0.722	0.692	0.699	0.639	0.678	0.541	0.696	0.695	0.741	0.306	0.656										
13	0.806	0.723	0.733	0.680	0.745	0.667	0.721	0.745	0.756	0.578	0.667	0.500									
14	0.769	0.780	0.780	0.800	0.795	0.840	0.722	0.692	0.711	0.707	0.796	0.705	0.643								
15	0.757	0.756	0.732	0.781	0.741	0.727	0.741	0.737	0.660	0.700	0.826	0.694	0.708	0.711							
16	0.768	0.750	0.743	0.831	0.754	0.758	0.755	0.727	0.780	0.774	0.785	0.746	0.755	0.783	0.702						
17	0.797	0.671	0.658	0.789	0.773	0.789	0.848	0.857	0.797	0.789	0.691	0.764	0.807	0.807	0.765	0.719					
18	0.786	0.766	0.761	0.813	0.776	0.776	0.755	0.793	0.702	0.774	0.821	0.821	0.837	0.837	0.724	0.737	0.677				
19	0.794	0.686	0.692	0.719	0.786	0.746	0.740	0.759	0.766	0.696	0.754	0.754	0.698	0.658	0.797	0.850	0.766	0.768			
20	0.810	0.791	0.817	0.786	0.730	0.718	0.770	0.766	0.814	0.731	0.811	0.725	0.759	0.771	0.779	0.754	0.803	0.714	0.781		
21	0.813	0.765	0.759	0.838	0.627	0.805	0.848	0.788	0.817	0.789	0.746	0.781	0.847	0.789	0.817	0.738	0.722	0.776	0.785	0.681	
22	0.821	0.813	0.812	0.814	0.750	0.831	0.846	0.857	0.854	0.793	0.841	0.783	0.848	0.791	0.828	0.842	0.813	0.731	0.714	0.707	0.754

Note: Name of the accessions 1-22 are given in the table 1.

components involved in maximum variation and to display the inter-relationship between the various *Saccharum* species.

Results and discussion

SSR polymorphism

More than two amplified products assumed to be alleles were observed for all the primers used in the study which was accrued to the high ploidy level as reported by Brown et al. (2007) in species of *Saccharum*. The eight primers produced a total of 163 alleles across the clones studied. The highest numbers of bands were produced by the primer SMC334BS and the NKS5 the least (Table 3). All the primers except mSSCIR56 produced bands specific to a particular clone. The primer NKS 23 produced seven clone specific bands followed by mSSCIR4 (Fig. 2), NKS 5, SGM 16, UGSM 417 and SMC 334BS with 4 clone specific bands. The

primer SCB 10 produced only one clone specific bands.

Only the clearly visible bands between 100 to 500bp were scored for the analysis. High PIC values were observed for most of the primers used in this study. The PIC values ranged from 0.75 to 0.95.The highest PIC value was recorded by the primer SMC334BS (0.954) which is a genomic based SSR and the least in EST based sequence SCB10 (0.756).This primer also produced only one clone specific bands. The lower PIC value of the primer SCB 10 may be due to the higher allelic frequency indicating that the alleles are present in almost all the clones studied. The lower PIC value for EST primers is in accordance with Cordeiro et al. (2000), where they observed the average PIC values of EST based SSR (0.66) were inferior to PIC values of genomic SSR primers (0.72) screened over five *Saccharum* genotypes.
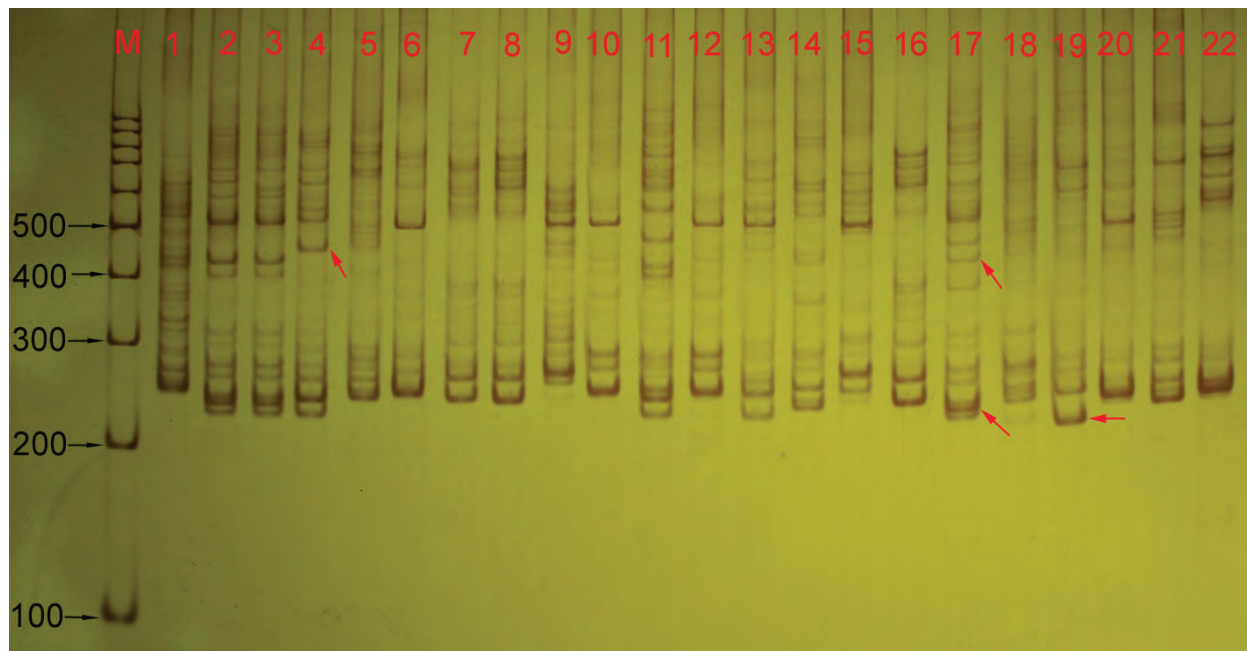

Fig. 2. PCR amplification profile of 22 genotypes of *Saccharum* sps., using the SSR marker mSSCIR4 (Lane:- M-100 bp ladder,1-28NG 82, 2-28NG 201, 3-28NG 272, 4-NG77-1, 5-57NG 27, 6-57NG234, 7-IJ76-312, 8-IJ76-329, 9-IJ76-336, 10-IJ76-337, 11-IJ76-338, 12-IJ76-360, 13-IJ76-375, 14-IJ76-552, 15-IS76-119, 16-NG77-10, 17-NG77-235, 18-S. *officinarum* (21NG 3), 19-S. *robustum* (NG77-94), 20-S. *sinense* (UBA Seedling), 21-S. *barberi* (Dhaulu), 22-S. *spontaneum* (IND82-330) Red arrow indicates clone specific bands

However, Cordeiro et al. (2000) inferred that PIC value for SSR primers are not constant but may serve as a reference for the relative ability of the primer to detect genetic variability. In a comparative study of SSR Genomic and EST based SSR primers in 59 accessions of sugarcane it was observed that EST based primers are more promising for revealing diversity than genomic based primers (Parthiban et al. 2018). Here such comparison could not be made as the primers used were the highly polymorphic ones obtained from the previous studies by screening 300 primers belonging to Genomic, EST, Enriched genomic and Unigene based SSR in *Saccharum* species (data unpublished). Among the species studied, *S. spontaneum* and *S. robustum* had lesser number of bands across the eight primers. According to the earlier report by Brown et al. (2007) *S. officinarum* and *S. spontaneum* clones revealed lesser number of bands compared to other species. This disparity maybe aroused due to the selection of single accession from *S. officinarum* in the present study.

Cluster analysis

Three main clusters were formed in the cluster analysis based on nearest neighbour joining (un weighted) method with Jaccard's coefficient as distance measure (Fig. 3). In the first cluster there was ten accessions, six of *S. edule* and other four accessions belong to four species viz*., S. officinarum*, *S. barberi*, *S. sinense* and *S. spontaneum*. Earlier molecular markers studies by Lu et al. (1994) also showed the close relationship of these species and concluded that the origin of *S. barberi* and *S. sinense* is from cross of *S. spontaneum* and *S. officinarum*. Among the *S. edule* accessions four of them are New Guinean collection and two Indonesian collections. Four out of the six non flowering accessions of *S. edule* were clustered in this group. This cluster were further sub clustered into three with the *S. officinarum* and four *S. edule* collection in one, *S. spontaneum*, *S. barberi* and *S. sinense* and 57 NG 27 (*S. edule*) in the second. The third sub cluster consists of only one accession 28 NG 82.
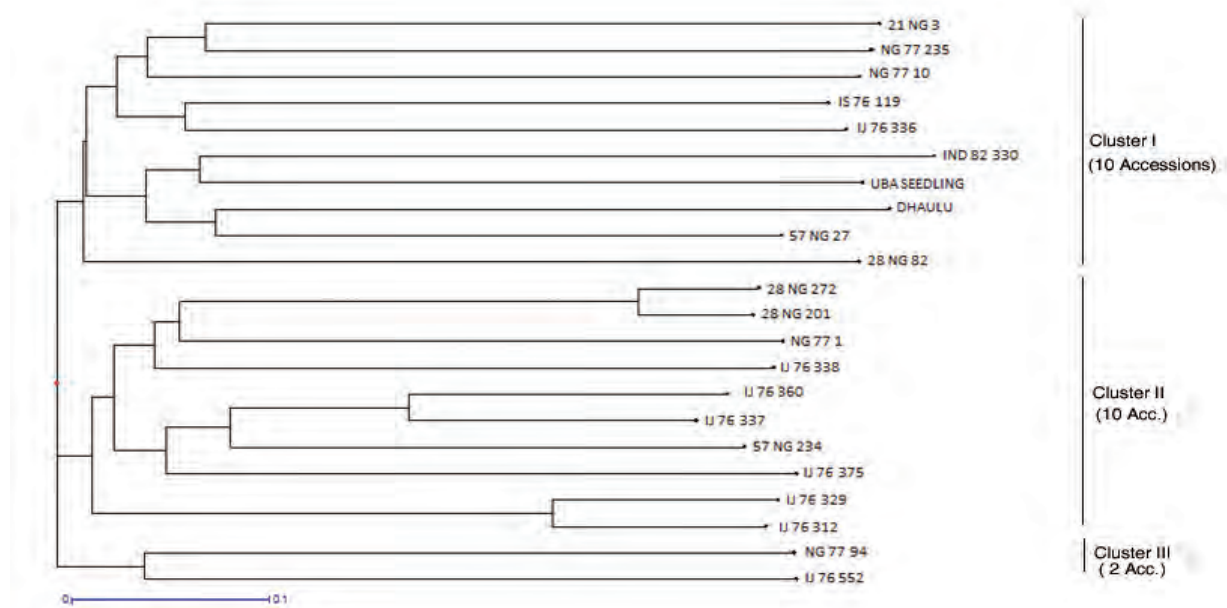

Fig. 3. Dendrogram based on unweighted neighbor joining of Jaccard's coefficient

This accession of *S. edule* had unique combination of rind characteristics such as ivory mark, splits, weather marks and with low wax, zigzag internode alignment, narrow ovate bud with broad lozenges, dark purple growth ring greenish purple dew lap, and compact erect canopy. The clustering based molecular data support the earlier studies based on morphological data (Chandran et al. 2014) where, 28 NG 82 was morphologically very distinct from other clones and remained as an independent node in the dendrogram. However, the dendrogram based on molecular data was not strictly conform to the earlier clustering of *S. edule* accessions based on morphological data in the case of other accessions. Similar observation were recorded by Brown et al. (2007) that agronomic characters based grouping and SSR based grouping had both similarities for some accessions and differences to others in *Saccharum* species.

The second cluster was exclusively with ten *S. edule* accessions with four New Guinean collection and six of Indonesian collection. The lowest distance measure (0.119) was observed between two New Guinean collection 28 NG 272 and 28 NG 201 (Table 3). This cluster also immediately formed two sub clusters the first with 8 accessions (five of New Guinean collection and three Indonesian collection) and the second sub cluster with two Indonesian accessions (IJ 76-329 and IJ 76-312). Between these two Indonesian accessions distance coefficient was less (0.222) showing high similarity (Table 5). Chandran et al. (2014) also observed close similarity between 28 NG 272 and 28 NG 201 as well as IJ 76-329 and IJ 76-312 based on morphological features.

In the third cluster, the *S. robustum* accession was grouped with one *S. edule* accession IJ 76-552 with thick zigzag cane having dorso-ventral hairiness on leaves. Though the present study do not completely corroborate with the studies based on

morphological characters, the molecular diversity profile has the advantage over morphology based diversity, as they are stable and detectable in all tissues regardless of growth, development, age or expression of any physiological adaptation of the cell. Moreover they are not influenced by environmental, pleiotropic and epistatic effects (Linda Mondini et al. 2009).

The clustering of accession revealed the distinct grouping within the collection of *S. edule* which supports the view that the *S. edule* has multiple botanical origins. The clustering of New Guinean collection and Indonesian collection indicate an early movement of this species for its edible inflorescence. The report of Chowing (1963) also highlight that an Indonesian cognate for *S. edule* could not be discovered in her linguistic studies. The grouping of IJ 76-552 (*S. edule*) and NG 77-94 (*S. robustum*) in same cluster indicate that, the former might have originated from *S. robustum* as a result of spontaneous mutation as suggested by Brandes et al. (1939). The other accessions of *S. edule* of two distant clusters might have originated as a product of introgression of *S. officinarum*/*S. robustum* with other genera like Miscanthus (Grassl 1967; Daniel and Roach 1987) and Zea (Janaki Ammal 1941). Though relationship studies between the species might have slightly hampered by selecting only one representative clone from species other than *S. edule,* the diversity studies showed a clear distinct grouping of the accessions within the *S. edule* indicating probable multiple botanical origin of these species which need further confirmation.

Principal component analysis

Principal component Analysis (PCA) is used to identify the primer which is contributing most to the variation among the population and to study the relationship based on principal components. The first component (PC1) describes larger

variation and the following PC successively explains smaller parts of the original variance. The principal component analysis (PCA) and their correlation are shown in score plot (Fig. 4) The PC1 represents 12.1 per cent of variability, while the second component PC2 represents 10.6 per cent of variability among the data. The accessions were occupied on the entire quadrant of biplot but more predominantly in first and fourth quadrant.

The clustering based on Principal component analysis revealed two main clusters. Cluster I included eleven accessions, while Cluster II included only nine accessions. In cluster I, the genotype of *S. officinarum* (21 NG 3) and *S. edule* (NG 77 235 and NG 77 10) were observed in close proximity. Also the *S. edule* genotypes, IS 76 119 and IJ 76 336 were found together in cluster I which is similar to the cluster analysis result. Cluster I and II formed through PC's mostly confirms
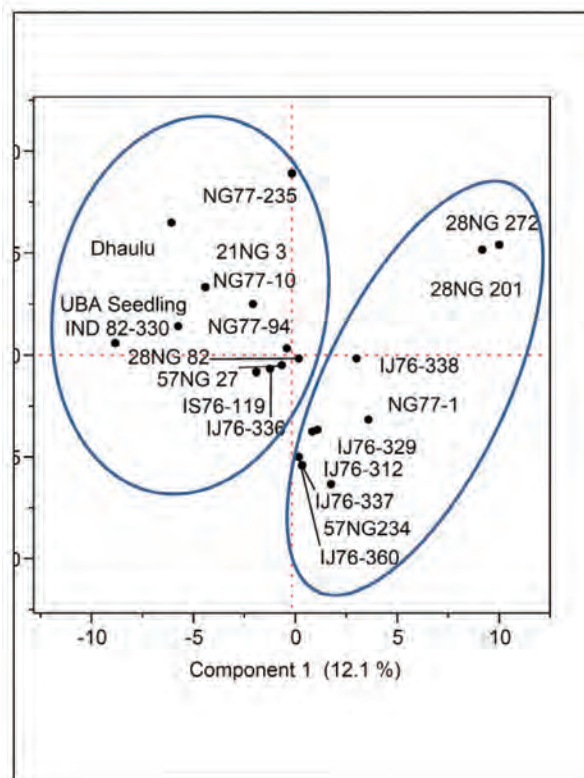

Fig. 4. Score plot of principal component analysis based on primer data

the results of cluster analysis based on complete variables by Jaccard's neighbour joining method. In this analysis due to a few missing data two genotypes viz., IJ76-375, IJ76-552 were excluded from the analysis and the *S.robustum* accession (NG 77-94) was found to occupy the centre of the quadrant showing more affinity towards first cluster instead of forming a separate third cluster as observed in cluster analysis with all variables.

For principal component 1, out of eight primes used, five of them contributed significantly with high load viz., three bands of mSSCIR4 (score 0.571, 0.638 and 0.711), four bands of SCB10 (score 0.546, 0.632, 0.537, 0.729), three bands of SGM16 (score 0.593, 0.624 and 0.600), four bands of NKS23 (Score 0.582, 0.711, 0.729 and 0.530) and four bands of SMC334BS (0.729, 0.579, 0.728 and 0.606). In Principal component 2, all the primers were equally contributed significantly viz., one band for mSSCIR4 (score 0.623), two bands for NKS 5 (score 0.559 and 0.615), one band of SCB10 (0.722), two bands of mSSCIR 56 (score 0.652,0.722), one band of SGM 16 (score 0.562) one band of NKS 23 (score 0.617), one band of UGSM 417(score 0.555) and two bands of SMC334BS (score 0.602, 0.582). Among the primers SCB 10 has contributed significantly for the more loading in both component 1 and component 2 (score 0.729 and 0.722 respectively) and subsequently resulted in more variability among studied genotypes.

S. edule is an under exploited plant species and it has the potential for cultivation as a source of edible inflorescence with high nutritional attributes (Saraswathi et al. 2013). The culms can be supplemented to cogeneration of power in the sugar factories hence a good plant species for crop diversification leading to sustainable agriculture (Chandran et al. 2014). Large scale multiplication of this species can be achieved for producing sufficient planting material

through *in vitro* manipulation of the aborted inflorescence (Chandran et al. 2011). By virtue of its aborted inflorescence its contribution to genetic improvement of sugarcane is distant. However, isolation of the gene or gene complex and incorporation of the gene to sugarcane through biotechnological means is a challenging option (Premachandran et al. 2006) to change the non-edible inflorescence of sugarcane to edible one. Microsatellite markers are found to discriminate even closely related cultivars with very few primer pairs (Jannoo et al. 2011; Olufowote et al.1997) which make them a very potent tool for diversity and species relationship studies. The present studies revealed that the genetically divergent clones of different botanical origin are available in the gene pool of *S. edule* for commercial exploitation.

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