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ISSR BASED GENOTYPIC DIFFERENTIATION OF GRAPE (*VITIS VINIFERA* L.)

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ABSTRACT
In order to study the genetic variability of four grape cultivars (Nanasaheb purple, Sonaka, Thompson seedless and Ganesh), 10 ISSR primers were screened of which seven were found polymorphic. These polymorphic primers produced a sum total of 86 bands of which 56 were polymorphic. The grape cultivars grouped into two major clusters at 51 per cent similarity. The first cluster had only Nanasaheb purple whereas; the second cluster contained Sonaka, Thompson seedless and Ganesh cultivars. Thompson seedless and Ganesh in the second cluster showed a similarity coefficient of 0.63. Clustering was strongly supported by high bootstrap values. Resolving power of the ISSR primers ranged between 3 (UBC 850) and 10 (UBC 810), PIC value from 0.78 (UBC 850) to 0.88 (UBC 811, UBC 815 and UBC 834), and Marker indices (MI) from 3.89 (UBC 850) to 8.80 (UBC 815 and UBC 834) with a mean value of 6.14, 0.85, and 6.88, respectively. The results revealed that ISSR could be a better tool for evaluation of genetic diversity among the grape cultivars.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important perennial fruit crops botanically belonging to the family Vitaceae and is divided into 12 genera. *Vitis vinifera* is the most widely cultivated species of the genus *Vitis* and is grown throughout the temperate and tropical regions. *Vitis vinifera* is known for its wide morphological and genetic diversity and there exist a large number of cultivars (Teixeira *et al.,* 2013). In India, Grapes are cultivated over an area of 111.4 thousand hectares with a production of 1,234 thousand tonnes and productivity 11.1 tonnes per hectare (NHB, 2011). Besides being eaten as fresh fruit, grapes are also used for grape wine, grape juice, raisins and canned products. It contains high levels of easily absorbable glucose, protein, vitamins, amino acids, lecithin and minerals; and contain flavonoids which are a powerful antioxidant, eliminate free radicals, and prevent aging (Pandey and Rizvi, 2009).

Although, most of the commercially cultivated grapes in India are introduced from major grape growing countries, and hence, interrelationships between them are not very clear (Jogaiah *et al.,* 2013). Despite botanical homogeneity, grape varieties possess wide and unique phenotypic variability regarding berries size, shape and colour, and quality traits including berry composition, content of sugars, acidity and organic acids (Coombe, 1992; Shiraishi *et al.,* 2010). Accurate identification of grape cultivars is difficult due to the vegetative propagation and reliance upon ampelography (Dhanorkar *et al.,* 2005), and often, same variety is known by different names which may lead to confusion in nomenclature (Soyolt *et al.,* 2013). Therefore, knowledge on genetic relationships and correct identification of varieties is essential for evolutionary studies, germplasm collection, and *in situ* conservation.

However, use of molecular markers for grapevine identification is regarded as an alternative or supplementary to ampelography (Herrera *et al.,* 2002; Bahurupe *et al.,* 2013). Molecular markers provide powerful tools to reveal polymorphism at the DNA sequence level and are robust to detect genetic variability and are not influenced by the environment or the developmental stage of a plant, making them ideal for genetic relationships studies (Akhare *et al.,* 2008). ISSR (Inter Simple Sequence Repeats) is a PCR-based technique and provide a reliable marker system for many organisms, especially plants (Modgil *et al.,* 2005; Kandasamy *et al.*, 2013) because of its simple, fast, high stability, no prior requirement of sequence information, cost effectiveness and versatility of markers. It involves amplification of the DNA segment present at an amplifiable distance in-between two identical microsatellite repeat regions oriented in opposite directions (Zietkiewicz *et al.,* 1994). ISSR has been widely used for varietal fingerprinting or genetic diversity analysis, characterization of genetic relatedness among populations, detection of clonal variation, cultivar identification, phylogenetic analysis, detection of genomic instability, and assessment of hybridization (Bornet and Branchard, 2004;

Tamhankar *et al.,* 2001; Herrera *et al.,* 2002; Hassan *et al.,* 2011; Joshi *et al.,* 2013). Therefore, the present study was aimed at using ISSR markers to assess the levels of genetic diversity among the selected grape cultivars.

MATERIALS AND METHODS

Planting Material

Four grape cultivars (Nanasaheb Purple, Sonaka, Thompson seedless and Ganesh) were collected from the Grapes and Onion Research Centre, Pimpalgaon (Maharashtra, India). The morphological characterization of these four varieties used in the present study has been described in Table 1.

Isolation and quantification of DNA

The young immature leaves (200-300mg) were ground using liquid nitrogen to a fine powder with mortar and pestle (frozen rapidly at -20ºC) and were immediately transferred to a 1.5mL microcentrifuge tube containing 700μL of prewarmed CTAB buffer for isolation of DNA (Piccolo *et al.,* 2012). The DNA preparation was treated with RNase-A (Bangalore GeNei, India) for 1 hour at 37ºC to remove RNA contamination and the samples were diluted to a concentration of 50 ng/μL.

Selection of primers

Ten ISSR primers with good resolving power were procured from UBC primer set (University of British Columbia, Vancouver, Canada) and were screened for polymorphism against the grape varieties. Of these, 7 ISSR primers (Table 2) produced distinct banding pattern with good quality of amplification and reproducibility, however, no band was detected in any negative control.

Inter simple sequence repeat-PCR

PCR reactions were performed in a 20 μL reaction volume [2.5 μL of 10X Taq buffer (with $MgCl_2$), 5.0 μL of 100 mM dNTPs, 2.0 μL (50 ng) of genomic DNA, 2.0 μL 10 pM primer, 0.3 μL of Taq DNA polymerase (1 U), 8.2 μL of sterile water] using Eppendorf Master Cycler (Eppendorf, USA). PCR was performed by using following thermal profile: 94ºC for 5 minutes (1 cycle); 94ºC for 1 minute, 40ºC for 1 minute, 72ºC for 2 minutes (35 cycles); final extension at 72ºC for 7 minutes (1 cycle) and cooling of samples at 4ºC.

Agarose gel electrophoresis

The amplified PCR products were run on 1.4% agarose gel using 1X TAE buffer stained with ethidium bromide along with 1 kb marker (ëDNA). The profile was visualized under UV transilluminator and documented using gel documentation system (UVItec, Cambridge, UK).

Data collection and analysis

The documented ISSR profiles were carefully examined for banding pattern, polymorphism and number of bands. A locus was considered to be polymorphic if the band was present in one variety and not in the other (Khalekar *et al.,* 2014). Resolving power (Rp) (Prevost and Wilkinson, 1999), Polymorphic information content (PIC) (Smith *et al.,* 1997) and Marker index (MI) (Manimekalai and Nagarajan, 2006) were calculated for the primers for better understanding relation among the grapes cultivars. The data was analysed using numerical taxonomy system of multivariate statistical programme (NTSYSpc 2.02i) software package (Rohlf, 2000). It was further used for construction of dendrogram using unweighted pair group method of arithmetic averages (UPGMA) and two-dimensional (2D) and three-dimensional (3D) principal component analyses (PCA) using Jaccard's coefficient, EIGEN and PROJ modules of NTSYSpc. Bootstrapping was done using WINBOOT software (Yap and Nelson, 1996) with 1,000 replications.

RESULTS AND DISCUSSION

ISSR analysis

The preliminary screening of ten ISSR primers showed that only seven primers (UBC 810, UBC 811, UBC 815, UBC 834, UBC 835, UBC 850 and UBC 888) produced polymorphic amplification pattern (Fig. 1), which were finally selected for molecular profiling of the grape varieties. A total number of bands produced by the polymorphic primers varied from 5 (UBC 850) to 10 (UBC 815). The total number of bands, number of polymorphic bands, percent polymorphism (%P), polymorphic information content (PIC), resolving power (Rp) and marker indices (MI) are summarized in Table 2. The resolving power (Rp) of the ISSR primers ranged between 3 (UBC 850) and 10 (UBC 810) with an average of 6.14. The polymorphic information content (PIC) value for 7 primers varied between 0.78 (UBC 850) and 0.88 (UBC 811, UBC 815 and UBC 834) with average of 0.85. The marker indices (MI) of ISSR primers ranged from 3.89 (UBC 850) to 8.80 (UBC 815 and UBC 834) with a mean of 6.88.

Genetic variability

A total of 10 ISSR primers were screened for detection of polymorphism among the four grape varieties, of these 7 primers gave polymorphic bands. The sequences of the primers giving polymorphic bands indicate that the microsatellites more frequently contain repeated dinucleotides (AG)n, (GA)n, (GT)n and (CA)n. Overall, the number of amplicons produced with the ISSR primers ranged from 6 (for UBC 850) to 20(for UBC 810). However, the average number of bands and average number polymorphic bands/primer for 7 ISSR primers were 12.8 and 8 respectively. Earlier findings in grapevine cultivars also suggest wide variation in allele produced per primer (Seyedimoradi *et al.,* 2012). Such allelic variation may be due to difference among the cultivars or varieties used and concentration of the agarose gel (Powell *et al.,* 1996).

Polymorphism involves the existence of different forms (alleles) of the same gene in plants or a population of plants. These differences are tracked as molecular markers to identify desired genes and the resulting trait. It has been reported that the ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker system (Sivaprakash *et al.,* 2004). The term polymorphic information content (PIC), originally introduced by Botstein *et al.* (1980), refers to the value of a marker detecting polymorphism within a population depending on the number of detectable alleles and their frequency. In the present investigation, 7 ISSR primers produced PIC value ranging between 0.78 (UBC 850) to 0.88 (UBC 811, UBC 815 and UBC 834) with an average of 0.85, which was comparable

Table 1: Description/morpho-taxonomic characterization of the four grape varieties

Sl. No.	Variety	Berries shape	Colour	*T.S.S. (brix)	*Acidity	*Size (mm)
1.	Thompson Seedless	Oval to oblong	Golden yellow	18-22ºC	0.5-0.7%	16-18
2.	Sonaka	Elongated, Cylindrical	Amber	20-22ºC	0.4-0.7%	16-19
3.	Nanasaheb Purple	Elliptical	Purple	18-21ºC	0.5-0.7%	18-20
4.	Ganesh	Ovoid	Green to Amber	20-22ºC	0.5-0.65%	15-20

* The quantitative data shows average of the values obtained from 50 plants

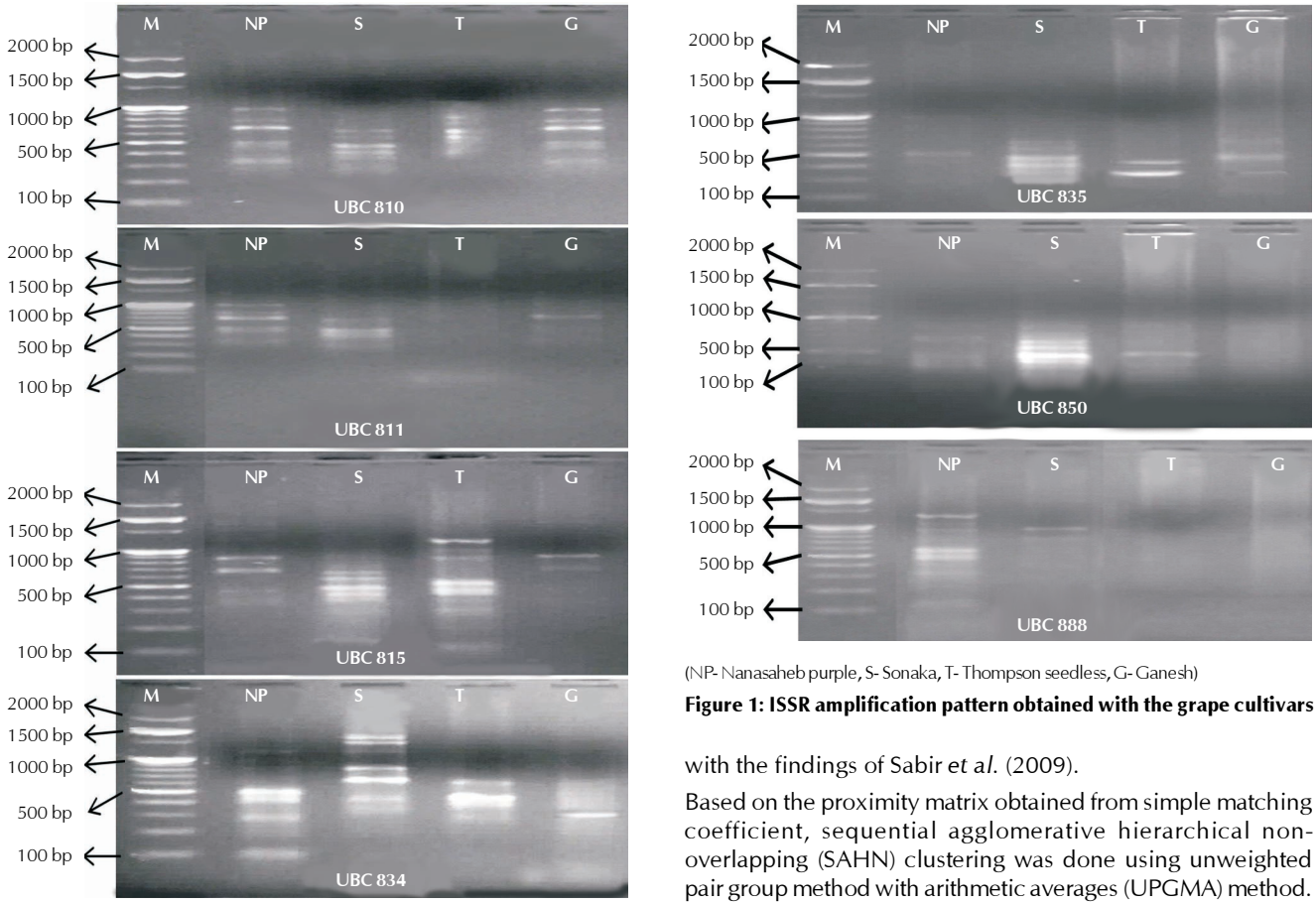
Table 2: ISSR primers used for fingerprinting of the grape cultivars

Sl. No.	Primer name	Primer sequence (5′ to 3′)	Total no. of bands	No. of Polymorphic bands	%P	PIC	Rp	MI
1.	UBC 810	GAG AGA GAG AGA GAG AT	20	7	35.0	0.87	10	6.06
2.	UBC 811	GAG AGA GAG AGA GAG AC	10	9	90.0	0.88	5	7.92
3	UBC 815	CTC TCT CTC TCT CTC TG	15	10	66.7	0.88	7.5	8.80
4.	UBC 834	AGA GAG AGA GAG AGA GYT	15	10	66.7	0.88	7.5	8.80
5.	UBC 835	AGA GAG AGA GAG AGA GYC	12	8	66.7	0.85	6	6.78
6.	UBC 850	GTG TGT GTG TGT GTG TYC	6	5	83.3	0.78	3	3.89
7.	UBC 888	BDB CAC ACA CAC ACACA	8	7	87.5	0.84	4	5.91
Total			86	56	65.1	5.97	43	48.15
Average			12.28	8	-	0.85	6.14	6.88

%P = percentage polymorphism; PIC = polymorphic information content; Rp = resolving power; MI = marker indices

Table 3: Similarity matrix obtained with ISSR primers in selected grape cultivars using Jaccard's similarity coefficient

	Nanasaheb purple	Sonaka	Thompson seedless	Ganesh
Nanasaheb purple	1.0000000			
Sonaka	0.2456140	1.0000000		
Thompson seedless	0.3859649	0.4736842	1.0000000	
Ganesh	0.5438596	0.4912281	0.6315789	1.0000000



(NP- Nanasaheb purple, S- Sonaka, T- Thompson seedless, G- Ganesh)

Figure 1: ISSR amplification pattern obtained with the grape cultivars

with the findings of Sabir *et al.* (2009).

Based on the proximity matrix obtained from simple matching coefficient, sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group method with arithmetic averages (UPGMA) method.

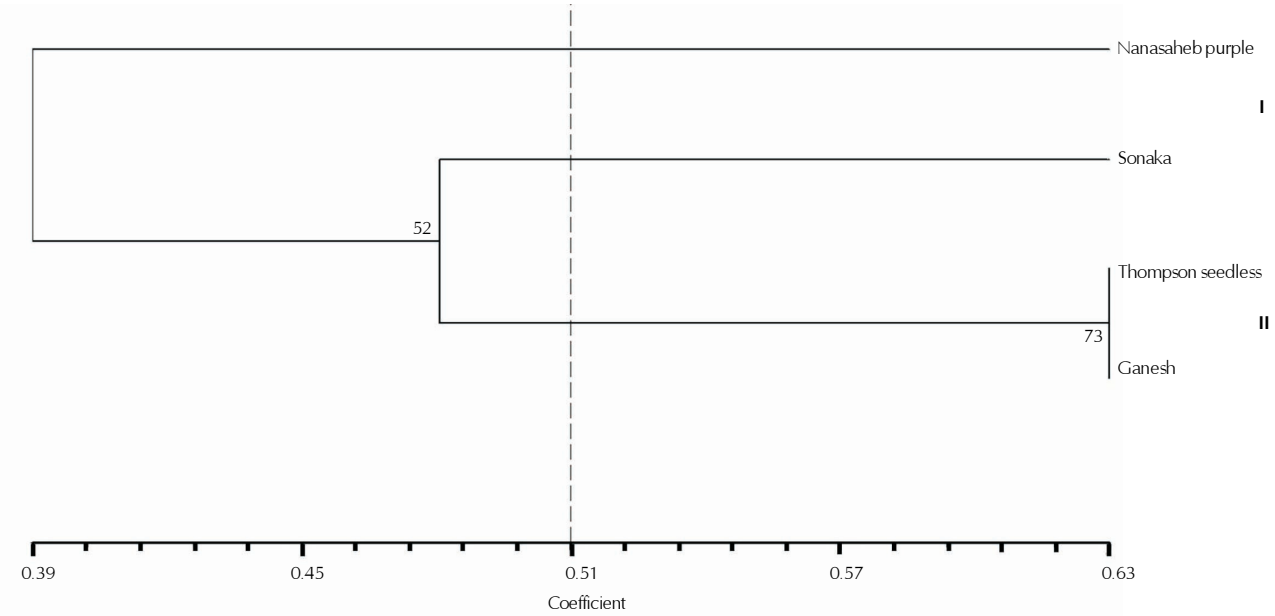
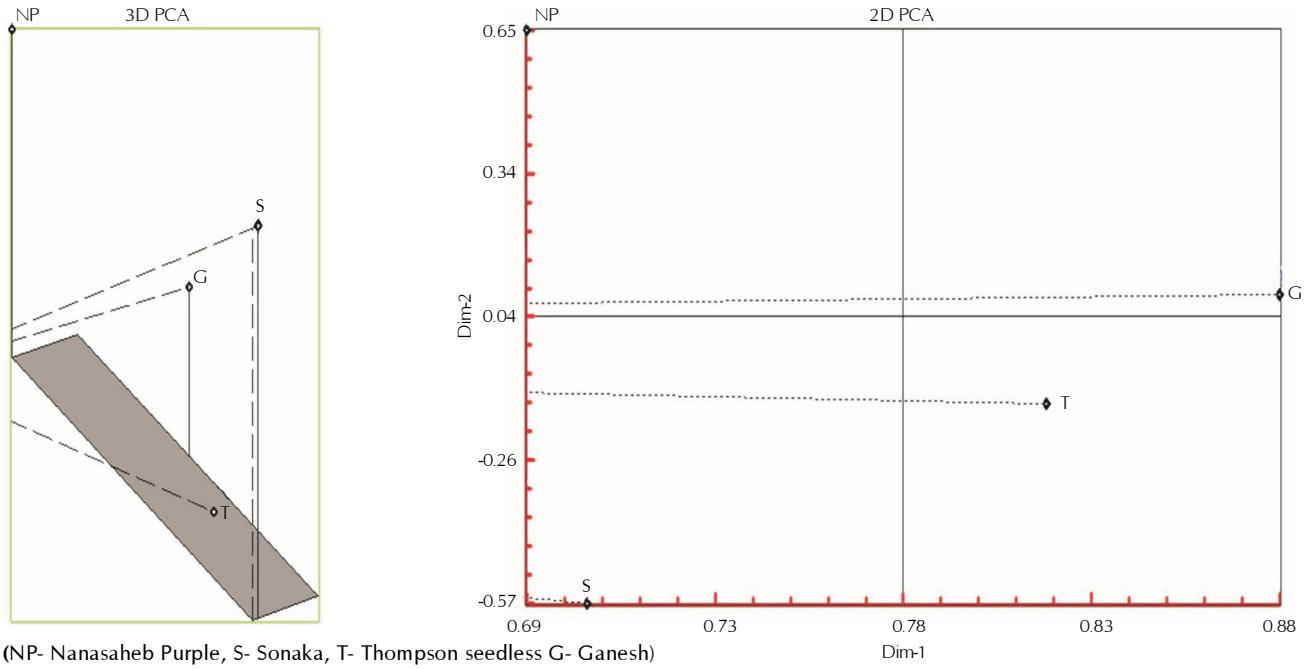


Figure 2: Dendrogram on the basis of the ISSR similarity matrix data by unweighted pair group method with arithmetic averages (UPGMA) cluster analysis. Bootstrap values (1000 replicates) are shown next to the branches



(NP- Nanasaheb Purple, S- Sonaka, T- Thompson seedless G- Ganesh)

Figure 3: 2-Dimensional (2D) and 3-Dimensional (3D) principal component analysis (PCA) derived from ISSR analysis of the grape cultivars

The dendrogram of grapes varieties grouped into two major clusters on the basis of the reference line drawn at a similarity coefficient 0.51 (Fig. 2). The highest value for similarity index was obtained for Thompson with Ganesh (0.6315), and least similarity coefficient values obtained for Nanasaheb Purple with Sonaka (0.2456) (Table 3). The first cluster included Nanasaheb purple and second contained Sonaka, Thompson seedless and Ganesh. Thompson seedless and Ganesh variety in second cluster were comparatively more similar with highest similarity coefficient 0.63. Similar results were obtained among cultivars of *V. vinifera* by Alizadeh *et al.* (2009) and Zeinali *et al.* (2012). Clustering based on principal component analysis (PCA) resulted in two clusters which were more or less similar to that of dendrogram clustering (Fig. 3). The first, second and third principal component respectively explain 52.0833%, 27.0833%, and 14.5833% of overall variation; which together accounted for 93.77% of total variation. The Cluster I consist of single ecotypes *i.e.* Nanasaheb purple and Cluster II consist

of three ecotypes *i.e.* Sonaka, Thompson seedless and Ganesh. On comparison of the above two methods, it was observed that Sonaka, which was in cluster II as per the clustering based dendrogram got more separated from Ganesh and Thompson as per PCA. However, Nanasaheb purple represent a distinct cluster in both the ISSR based dendrogram and PCA.

Molecular markers are scattered throughout the genome and their association with various agronomic traits are influenced by the cultivator under selection pressure induced by domestication. Interrelationships between grapes varieties are not very clear and more studies on morphological and agronomic traits and analysis of the clones with more number of reliable DNA markers like SSR, SNP may be helpful in confirming the results. Knowledge of the degree of genetic relationship between these varieties will be important for the germplasm collection, *in situ* conservation and *Vitis* breeding programmes. The results of the present study will be useful in DNA fingerprinting and in determining the genetic diversity among the grapes.

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