Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(6): 1576-1580
Received: 12-09-2018
Accepted: 15-10-2018

BABU AG
Directorate of Research, University of Horticultural Sciences, Bagalkot, Karnataka, India

Prabhuling G
Directorate of Research, University of Horticultural Sciences, Bagalkot, Karnataka, India

Rashmi S Karani
Senior Research Fellow, Center For Biotechnology Research, University of Horticultural Sciences, Bagalkot, Karnataka India

Satish D
College of KRCCH, Arabhavi, University of Horticultural Sciences, Bagalkot, Karnataka, India

Rohini K Patil
Senior Research Fellow, Center For Biotechnology Research, University of Horticultural Sciences, Bagalkot, Karnataka, India

Mulla SR
College of Horticulture, University of Horticultural Sciences, Bagalkot, Karnataka, India

Raghavendra G
College of Horticulture, University of Horticultural Sciences, Bagalkot, Karnataka, India

Jagadeesha RC
College of Horticulture, University of Horticultural Sciences, Bagalkot, Karnataka, India

Correspondence
BABU AG
Directorate of Research, University of Horticultural Sciences, Bagalkot, Karnataka, India

Genetic diversity analysis among banana cultivars through ISSR markers

Babu AG, Prabhuling G, Rashmi S Karani, SATISH D, Rohini K Patil, Mulla SR, Raghavendra G, and Jagadeesha RC

Abstract
In Karnataka, several landraces of banana are cultivated in different parts, known by different local names. Eight banana cultivars (Sugandhi Bale, Rajapuri Bale, Nanjanagud Rasbale, Sakkare bale, Basrai dwarf bale, Hanuman bale, Kalyani bale and Kari Bale) from Karnataka state were selected for genetic diversity study. Genetic variations and relationships among ten important banana cultivars were evaluated using 20 Inter Simple Sequence Repeat (ISSR) primers. The ISSR-PCR technique was used in the present study for the evaluation of genetic diversity within the Banana cultivars. Among 20 ISSR primers representing tri-, tetra, Penta and other repeats 12 primers revealed polymorphic patterns. Using those 12 primers, 72 bands in total were obtained, out of which 42 were polymorphic and scored giving an average of 3.5 bands amplified per primer. The used primers produced specific patterns for each banana cultivar. This study reveals there is a huge genetic dissimilarity among banana cultivars reveals their different genomic constitution and different evolutionary pattern of the cultivars. Hence need for cultivar wise cultivation, conservation, propagation and commercial production.

Keywords: Banana, ISSR, polymorphic, diversity, genetic dissimilarity

Introduction
Bananas and plantains are the fourth most important crop in developing countries after rice, wheat and maize. Banana is cultivated in 102 million ha in humid tropics and subtropics in Americas, Africa, and Asia extending up to Europe and Australia with an annual production of 102 million tons approximately. India is the largest producer of banana contributing 19.71% to the global output; 19.19 million tons from 0.565 million ha (FAO, 2012) [6].

The vast majority of cultivated bananas and plantains are sterile and originate from inter and intraspecific crosses between two diploid (2n=2x=22) wild species, *Musa acuminate* (AA genome) and *Musa balbisiana* (BB genome). The wild diploids have seed bearing fruits with little starch and are of no economic value. Domesticated bananas and plantains are mainly triploids (AAA, AAB, ABB genomes) and fruit develop by vegetative parthenocarpy. There are also seedless cultivated diploid and tetraploids. Mainly the sweet dessert bananas have AAA genome and most plantains have AAB or ABB genome. There are over 1000 cultivars or land races of banana known worldwide. The advent of DNA finger printing opened up new vistas in banana germplasm characterization. Molecular phylogeny using RFLP, RAPD, AFLP microsatellite, ISSR primers and in situ hybridization generated ample information to precisely establish the genetic relations between banana cultivars (Lu *et al.*, 2011) [7].

PCR-based techniques have been used extensively in genetic analysis and identification of molecular markers in plants. Simple sequence repeats (SSRs) are considered as a marker of choice for genetic mapping and genetic estimations of germplasm resources (Kantartzi *et al.*, 2010) [11]. ISSR (Inter-Simple Sequence Repeat) analysis, developed by Zietkiewicz and co-worker (Zietkiewicz *et al.*, 1994) which uses the SSR motif per se as the single primer in PCR amplifications, does not require the knowledge of flanking sequences and has wide applications for all organisms, regardless of the availability of information about their genome sequence. ISSR has been proven to be a simple and reliable marker system for many organisms, especially plants, with highly reproducible results and abundant polymorphisms (Modgil *et al.*, 2005) [5]. ISSR analysis has been successfully applied in gene tagging (Ammiraju *et al.*, 2001) [3], variety fingerprinting or genetic diversity analysis (Bornet *et al.*, 2005) [4] and the evaluation of microsatellite motif frequencies in the rice genome (Blair *et al.*, 1999) [2].

ISSR technique is considered as a very simple, fast, cost-effective, highly discriminative and reliable technique for genetic diversity analysis (Pradeep *et al.*, 2002) [10]. For successful ISSR

analysis, pairs of SSRs (inversely oriented) must occur within a short distance on the same chromosome, which is amplifiable by a PCR reaction to give a band that is resolvable on agarose or polyacrylamide gels. The primers used in ISSR analysis may be developed from any SSR motifs (di-, tri-, tetra-nucleotides). The potential applications of ISSR analysis for diverse aims depend on the variety and frequencies of microsatellites within the specific genomes. The diversity within the *Musa* cultivars were analyzed using 16 ISSR primers out of which 9 primers revealed polymorphic patterns (Pillai PR *et al.*, 2011) [3]. Hence in the present study, ISSR molecular marker technique was used to investigate genetic relativity/variability among selected banana cultivars of around Karnataka.

Materials and Methods

Plant Materials: In Karnataka eight banana cultivars *viz*., Sugandhi Bale, Rajapuri Bale, Nanjanagud Rasbale, Sakkare bale, Basrai dwarf bale, Hanuman bale, Kalyani bale and Kari Bale were selected for the genetic variability study using ISSR markers and their location and genome is indicated in Table1. Plant samples were collected randomly from their respective locations within Karnataka state.

Table 1: Details of banana cultivars and its location used for ISSR studies

S. No	Banana Cultivars	Location (within Karnataka state, India)	Genomic group
1	Sugandhi Bale	Kamalapur (Ballary Dist)	AAB
2	Rajapuri Bale	Arabhavi (Belagavi Dist)	AAB
3	Nanjanagudu Rasbale	Rangasamudra (Mysore)	AAB
4	Sakkare bale	Kamalapur (Ballary Dist)	AB
5	Basrai dwarf bale	Sector 70, MHREC (Bagalkot)	AAA
6	Hanuman bale	Sector 70, MHREC (Bagalkot)	AAA
7	Kalyani bale	Kamalapur (Ballary Dist)	AAA
8	Kari Bale	Sector 70, MHREC (Bagalkot)	AAB

Table 2: List of ISSR primers used for diversity analysis with their sequence.

S. No.	Primer Name	Sequence (5'-3')	S. No.	Primer Name	Sequence (5'-3')
1	UBC811	GAG AGA GAG AGA GAG AC	11	SPS7	$(GTG)_5$
2	UBC841	GAG AGA GAG AGA GAG AYC	12	SPS1	$(GAC)_5$
3	UBC826	ACA CAC ACA CAC ACA CC	13	I3	$(GA)_9A$
4	UBC818	CAC ACA CAC ACA CAC AG	14	ISSR1	$(CT)_8$ TG
5	UBC834	AGA GAG AGA GAG AGA GYT	15	ISSR2	$(CT)_8$ AC
6	UBC835	AGA GAG AGA GAG AGA GYC	16	ISSR4	$(AGC)_4$ GT
7	UBC850	GTG TGT GTG TGT GTG TYC	17	ISSR5	$(CAC)_3GC$
8	I2	$(GA)_9T$	18	ISSR6	$(CTC)_3$ GC
9	SPS8	$(GGA)_4$	19	ISSR7	$(GACA)_3$
10	SPS4	$(AGG)_6$	20	ISSR8	$(GACA)_3$ GC

2.1 Genomic DNA Extraction

Fresh Leaf samples were harvested and homogenized to fine powder in liquid nitrogen (-80°C) using mortar and pestle. Total DNA was extracted from fresh leaves of individual eight cultivars using cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) [5]. After ethanol precipitation DNA was resuspended in 1X TE buffer (pH 8.0). The DNA was quantified spectrophotometrically by taking the absorbance at 260 nm by using Nano Drop™ 2000/2000c spectrophotometer.

2.2 PCR Conditions

Twenty ISSR primers produced amplicons were selected by primary screening and were selected for genetic diversity analysis by taking into consideration the repeatability, sharpness and intensity of bands (Table 2).

The test solutions were made up to a final volume of 20µl containing 50 ng of template DNA, 10 pM decamer primer, 1x reaction buffer, 0.1 mM dNTP mix and 1.0 U Taq DNA Polymerase. The amplification was performed using Eppendorf Thermocycler with a hot start for 2 minutes at 94ºC; followed by 35 cycles of denaturing at 94°C for 30 seconds; annealing at 54°C for 30 seconds; and product extension for 5 minutes at 72°C. The PCR products after ISSR amplification were analyzed in 1.5% agarose gel containing Ethydium bromide electrophoresis system to resolve the different molecular configuration of a DNA molecule as well as to separate DNA fragments of different weights. DNA was stained by soaking the gel in a 0.5-mg/mL ethidium bromide solution and visualized under Gel documentation system.

2.3 Data Analysis

Similarity matrices and dendrogram using NTSY Spc programme

NTSYS pc is a computer programme for distance/similarity estimation. It is used to discover pattern and structure in multi Variate data. Similarity matrices are matrices whose elements are the relative distance between points located on a certain manifold. Reproducible amplicons were scored against the presence or absence of a fragment and denoted as '1' or '0' respectively. Dendrogram is a 'treelike' diagram that summaries the process of clustering. Equal interest is the discovery that the variations in some subsets of variables are highly inter-correlated (clustered).

A Dendrogram was constructed using NTSYS pc software programme. On the basis of this analysis, the populations were clustered into groups. Similarity matrices and Dendrogram were constructed based on diversity coefficients generated for individual primers and also for pooled data by using UPGMA methods using NTSYS pc.

Results and Discussion

3.1 Genetic diversity analysis

In the present study, the total of twenty ISSR primers was used to study genetic relativity among the selected eight

Banana cultivars (Sugandhi Bale, Rajapuri Bale, Rasbale, Sakkare bale, Basrai bale, Hanuman bale, Kalyani bale and Kari Bale).

The utility of individual marker systems and their combination was also discussed by Smolik (Smolik *et al.*, 2004) [12]. Genetic variation in plants has proven valuable in plant conservation and management, for identification of populations, species and sub-species, and for estimating contributions to stock mixtures. The present work results demonstrated a high level of intercultivar ISSR polymorphism in the banana cultivars which were analyzed. Therefore, for assessing genetic relationships between banana cultivars, ISSR-PCR could be effectively used in banana breeding programs. Rational use of genetic resources in breeding programmes is essential to widen the existing gene pool of *Musa* and to develop new varieties while conserving the existing elite or superior cultivars (Yasodha *et al.*, 2004) [13].

ISSR are present to detect difference between SSRs (Ziekiewicz *et al.*, 1994) [15]. Compared with other molecular markers, ISSR can reveal high polymorphism, which is helpful in distinguishing individual at inter-and/or intra-species level. Moreover, ISSR has its specific advantage over other markers as follows: no prior sequence information, simple operation, high stability and low cost. Therefore ISSR has been proposed as a more economical and reliable DNA marker system (Bornet *et al.*, 2001,) [3].

In the present study out of twenty primers, twelve primers *viz.*UBC826, ISSR1, ISSR2, ISSR4, ISSR5, ISSR6, ISSR7, ISSR8, UBC841, UBC811, p-261, SPS8 showed polymorphism for eight Banana cultivars. In total, 72 scorable bands were produced in eight cultivars with 12 ISSR primers. This data was utilized for further computations. The mean number of bands per primer was 6.0. Out of 72 bands, 42 bands were polymorphic (61.3%).

The mean number of polymorphic ISSR bands was 3.5 per primer. Out of total 20 ISSR primers eight primers (UBC818, UBC834, UBC835, UBC850, I2, SPS4, SPS7 and I3) showed monomorphic. Out of 12 primers two (ISSR1 and ISSR 6) showed 100 per cent polymorphism with all the identifiable ISSR bands being polymorphic for eight banana cultivars (Table 3).

Genetic diversity within the *Musa* cultivars using 16 ISSR primers representing di-, tri-, tetra-, and penta- repeats, of which 9 primers revealed polymorphic patterns. Using those 9 primers, 63 bands in total were obtained, out of which 49 were polymorphic and scored giving an average of 5 bands amplified per primer. These primers produced cultivar specific banding patterns for each selected banana cultivars (Pillai PR, Savarimuthu *et al.*, 2011) [3].

Ying Lu *et al*., (2011) [3] used the ISSR markers to survey of the genomes of 30 *Musa* cultivars obtained from Hainan province of South China. He found 85.1% of the bands generated using ISSR were polymorphic, which provided evidence of a large genetic diversity among the tested cultivars.

Table 3: Polymorphic banding pattern of 12 ISSR primers for 12 banana cultivars.

S. No.	Primer Name	Sequence (5'-3')	Total bands produced	Number of polymorphic bands	Per cent polymorphism
1	ISSR2	$(CT)_8$ AC	6	5	83.3
2	ISSR4	$(AGC)_4$ GT	4	3	75.0
3	ISSR1	$(CT)_8$ TG	4	4	100.0
4	UBC841	GAG AGA GAG AGA GAG AYC	4	3	75.0
5	UBC826	ACA CAC ACA CAC ACA CC	6	2	33.3
6	ISSR5	$(CAC)_3$GC	9	8	88.8
7	ISSR6	$(CTC)_3$ GC	4	4	100.0
8	ISSR7	$(GACA)_3$	7	2	28.3
9	ISSR8	$(GACA)_3$ GC	9	6	66.6
10	UBC811	GAG AGA GAG AGA GAG AC	10	2	20.0
11	SPS1	$(GAC)_5$	5	2	40.0
12	SPS8	$(GGA)_4$	4	1	25.0
Total			72	42	735.3
Mean			6.0	3.5	61.3

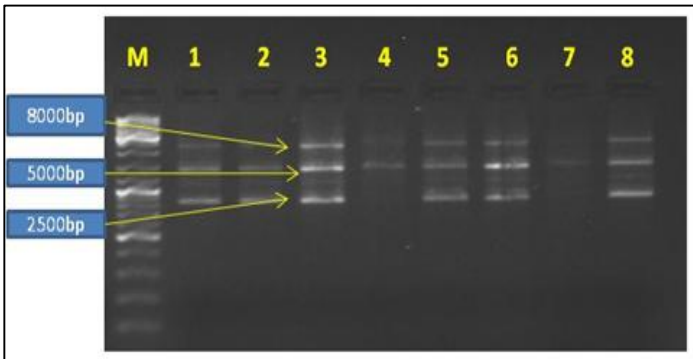

Fig 3. Lane M-Marker 1kb ladder (GenedireX), Lane 1-8 banana cultivars (1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale, 5-Basrai bale, 6-Hanuman bale, 7-Kalyani bale, 8- Kari bale) amplified using ISSR6 primer.

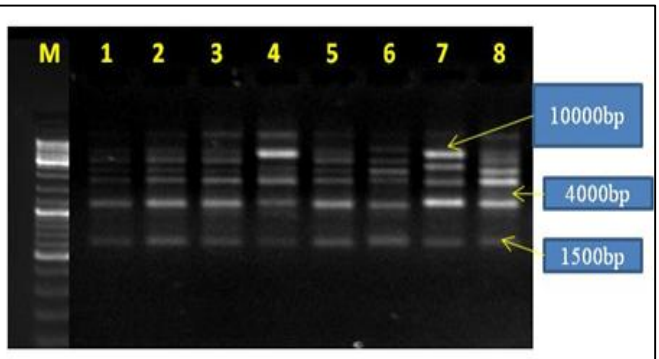

Fig 4. Lane M-Marker 1kb ladder (GenedireX), Lane 1-8 banana cultivars(1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale, 5-Basrai bale, 6-Hanuman bale, 7-Kalyani bale, 8- Kari bale) amplified using ISSR7 primer.

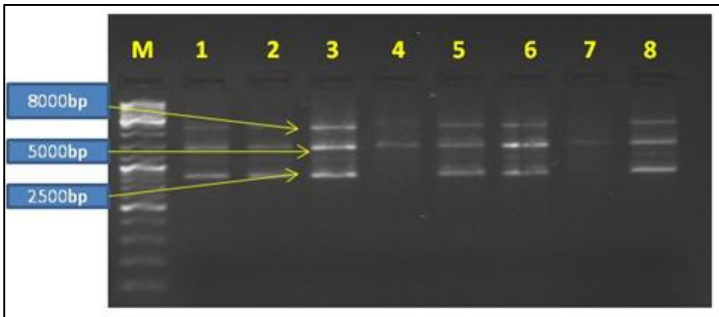

Fig 3. Lane M-Marker 1kb ladder (GenedireX), Lane 1-8 banana cultivars (1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale, 5-Basrai bale, 6-Hanuman bale, 7-Kalyani bale, 8- Kari bale) amplified using ISSR6 primer.

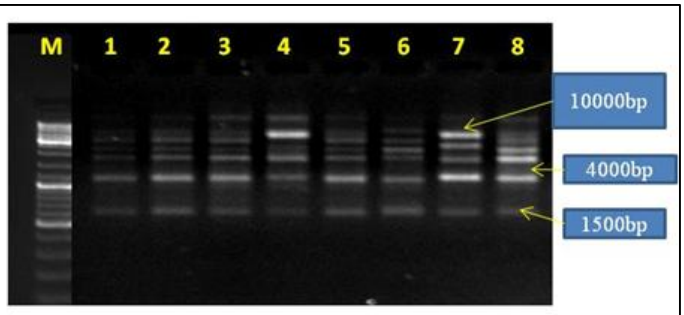

Fig 4. Lane M-Marker 1kb ladder (GenedireX), Lane 1-8 banana cultivars(1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale, 5-Basrai bale, 6-Hanuman bale, 7-Kalyani bale, 8- Kari bale) amplified using ISSR7 primer.

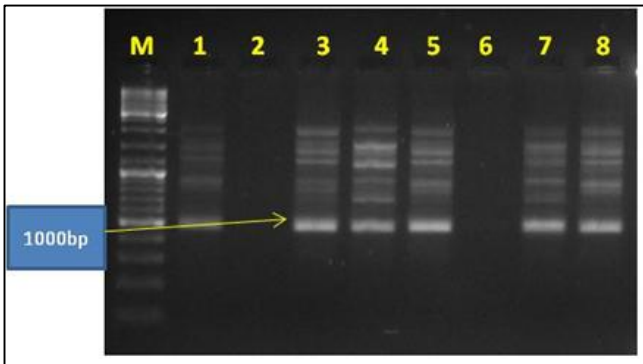

Fig 5. Lane M-Marker 1kb ladder (GenedireX), Lane 1-8 banana cultivars (1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale, 5-Basrai bale, 6-Hanuman bale, 7-Kalyani bale, 8- Kari bale) amplified using ISSR7 primer.

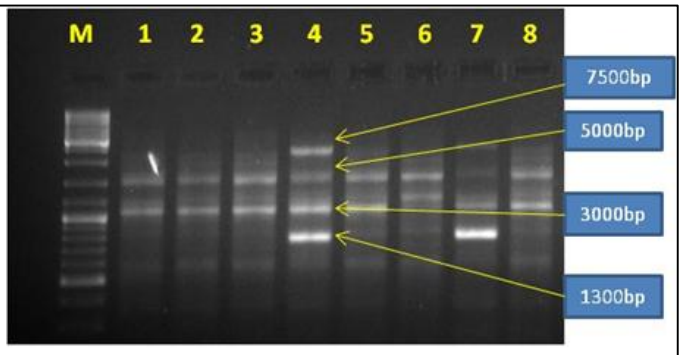

Fig 6. Lane M-Marker 1kb ladder (GenedireX), Lane 1-8 banana cultivars (1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale, 5-Basrai bale, 6-Hanuman bale, 7-Kalyani bale, 8- Kari bale) amplified using ISSR8 primer.

3.2 Genetic similarity for pooled data

The genetic similarity was computed considering all the genotypes from the pooled data and the dendrogram was constructed. These 12 ISSR primers were used for genetic relativity study for eight Banana cultivars. The dendrogram was constructed using NTSY Spc programme. On the basis of this analysis, the populations were grouped into three major clusters (Fig. 7).

In first major cluster consists of the cultivars Sugandhi Bale, Rajapuri Bale and Nanjanagud Rasabale. In second cluster consists of Basarai bale, Hanuman bale and Kari Bale. Whereas, in third cluster consists of the genotypes Sakkare bale and Kalyani. This dendrogram obtained from NTSY Spc software grouped eight Banana cultivars reveals that the banana were grouped according their genome category by twelve ISSR primers.

Amount of genetic similarity observed in molecular studies is dependent on number and type of primers used and the amount of diversity among the genotypes used in the investigation. In the present study the genetic similarity studies among all the eight Banana Cultivars from the pooled data, the genetic similarity obtained was ranged from 60.3% to 94.5% (Fig. 8).

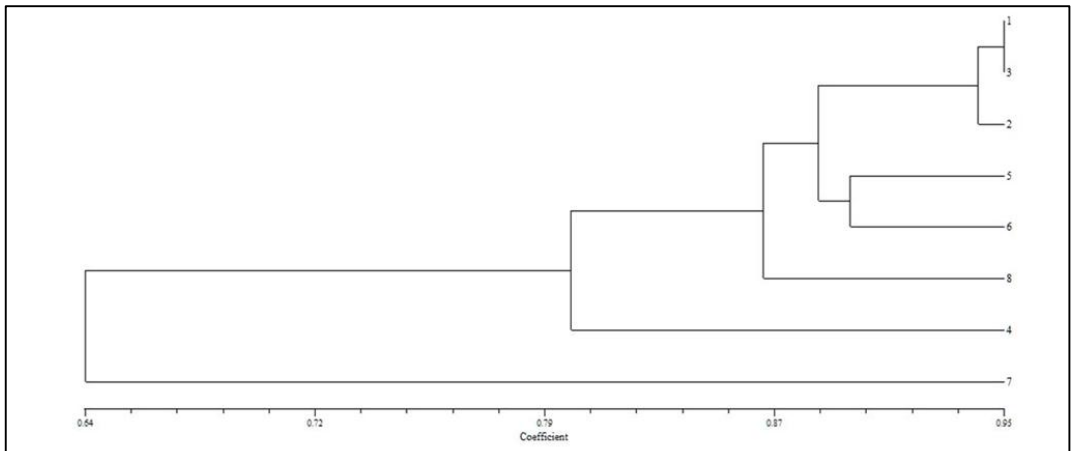

Fig.7: Dendrogram depicting eight Banana cultivars based on the genetic similarity generated by 12 ISSR primers
1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale,
5-Basarai bale, 6-Hanuman bale, 7-Kalyani, bale 8- Kari bale

Rows\Cols	1	2	3	4	5	6	7	8
1	1.0000000							
2	0.9454545	1.0000000						
3	0.9464286	0.9298246	1.0000000					
4	0.8070175	0.7931034	0.7966102	1.0000000				
5	0.8771930	0.8947368	0.8965517	0.7931034	1.0000000			
6	0.8771930	0.8947368	0.8644068	0.7931034	0.8947368	1.0000000		
7	0.6034483	0.6491228	0.6271186	0.6981132	0.6206897	0.6206897	1.0000000	
8	0.8771930	0.8620690	0.8644068	0.8245614	0.8620690	0.8620690	0.6491228	1.0000000

Fig 8: Genetic similarity (%) based on ISSR pooled over the 12 primers in eight Banana cultivars 1-Sugandhi bale, 2-Rajapuri bale, 3-Nanjanagud rasabale, 4-Sakkare bale, 5-Basarai bale, 6-Hanuman bale, 7-Kalyani bale, 8- Kari bale

3.3 Conclusion

The ISSR-PCR technique used in the present study is a suitable method for the evaluation of genetic diversity within the Banana cultivars being analyzed. Among 20 ISSR primers representing tri-, tetra, penta and other repeats 12 primers revealed polymorphic patterns. Using those 12 primers, 72 bands in total were obtained, out of which 42 were polymorphic and scored giving an average of 3.5 bands amplified per primer. The used primers produced specific patterns for each banana cultivar. This study reveals there is a huge genetic dissimilarity among banana cultivars reveals their different genomic constitution and different evolutionary pattern of the cultivars. Hence need for cultivar wise cultivation, conservation and propagation for maintenance and commercial production.

Courtesy

Thanks to Dept. of IT and BT, Government of Karnataka (KBITS), India for financial support.

Consent

It is no applicable.

Ethical Approval

It is no applicable.

Competing Interests

Authors have declared that no competing interests exist.

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