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RESEARCH ARTICLE

Evaluation of Genetic Diversity of *Piper betle* cultivars using ISSR markers

Shalini Goyat[1], Anita Grewal*[1], Devendra Singh[2], R.S. Katiyar[2], S.K. Tewari[2], R.C Nainwal[2], K. Hima Bindu[3]

1 Department of Biotechnology Engineering, University Institute of Engineering &Technology, Kurukshetra University, Kurukshetra- 136119
2 CSIR National Botanical Research Institute, Lucknow- 226001
3 Section of Medicinal Crops, Indian Institute of Horticultural Research, Bangalore- 560089

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Corresponding Author

Anita Grewal

Abstract

Betel vine (*Piper betle*) is an important cash crop of India. ISSR markers were used to assess the genetic diversity and relationships of 15 cultivars of betelvine. Out of 28 ISSR primers tested, 18 were selected for their reproducibility, reliability and high polymorphism. Eighteen ISSR primers amplified 138 bands with 118 (85.51%) polymorphic ones. Average polymorphic information content (PIC) was 0.32 over all primers used. The genetic diversity and relatedness among 15 betelvine cultivars was computed using Jaccard's similarity coefficient. The dendrogram grouped cultivars in 3 main clusters. PCA (principal component analysis) results were comparable to cluster analysis. The results suggested that ISSR markers can successfully reveal variability among the cultivars. ISSR can be useful for genetic diversity studies, to provide practical information for parental selection and to assist breeding programs.

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Introduction

Betel vine (*Piper betle* L.) belongs to the family Piperaceae and is popularly known as "paan" in India. It is a dioecious (male and female plants are different), shade loving perennial root climber. Betel vine is very popular in India with social, cultural and religious values. Fresh leaves are traditionally used for chewing in their natural raw condition along with many other ingredients like sliced areca nut, slaked lime, coriander, aniseed, clove, cardamom, sweetener etc. The most probable place of origin of betel vine is Malaysia (Chattopadhyay and Maity, 1967). In India it is widely cultivated in the states of Uttar Pradesh, Bihar, Madhya Pradesh, Northeastern India, Maharashtra, Karnataka, Orissa, West Bengal, Andhra Pradesh, Tamil Nadu, Kerala and Andamans. Propagation of betel vine as a crop is through vegetative means only. The leaves of betelvine possess antimicrobial, carminative, stimulant and antiseptic activities (Agarwal et al., 2012). Studies also revealed that the leaf improves immune system and inhibits cancer growth (Amonkar et al., 1989). There is a high demand of this plant in food and pharmaceutical industries so, it is required to further increase the productivity of betel vine. However, to achieve a further increase in productivity, information regarding the crop's genetic diversity is essential for breeding programs. Also, the knowledge of genetic diversity of this crop is very important for rational planning of conventional, modern breeding, for the purpose of improving the yield and quality of its produce. The recognition of intellectual property rights on plant varieties in the country's Plant Varieties Protection and Farmers Rights Act 2001 necessitates their efficient characterization.

More than 100 local cultivars (landraces) of betel vine are being cultivated in India and are often named after the locality or village where they are grown. Screening of the landrace names and their etymology, suggests that a given landrace may be named differently in different regions and more than one landrace may have the same name. Thus landraces with prefix Desi in their names invariably refer to the landrace 'Bangla' in West Bengal, landrace

'Kapoori' in Maharashtra and landrace 'Desavari' in Madhya Pradesh (Balasubrahmanyam et al., 1995). There is a need to rationalize the different landraces and to identify duplicates among them. On the basis of chemical constituent analysis of leaf essential oils, five prominent groups of betelvine landraces, namely, Bangla, Kapoori, Meetha, Sanchii and Desawari have been recognized (Rawat et al., 1989).

The development of molecular (DNA) marker provides new dimension, accuracy and perfection in the screening of germplasm (Tar'an et al., 2005). Efficient and quick screening of such genotypes speedup the process of varietal evaluation, thus molecular marker plays pivotal role in this regard. Inter-simple sequence repeat (ISSR) was first employed by Zietkiewicz et al. (1994) and Gupta et al. (1994) and it has been proved to be a highly useful tool for estimating genetic diversity and assessing genetic relationships because it is simple, fast, cost-effective, reliable and highly discriminating (Ci et al., 2008; Crespe et al., 2009; Zhang and Dai, 2010; Uysal et al., 2010; Petros et al., 2008). ISSR are ideal markers for population studies because of their abundance and high degree of polymorphism between individuals within a population of closely related genotypes (Hokanson, 1998).An additional advantage is that knowledge of the DNA sequences is not necessary to apply these techniques. The properties of ISSR make them useful for assessing population genetic parameters such as within-population and between-population genetic diversity. The ISSR method has been reported to be more reproducible (Goulao et al., 2001) and produces more marker patterns than the RAPD approach (Chowdhury et al., 2002), which is advantageous when differentiating closely related cultivars. ISSR has also been used for cultivar identification in numerous plant species, including sorghum (Medraoui et al., 2007), apple (Goulao et al., 2001; Dhyani et al., 2015) and strawberry (Arnauet al., 2003). PCR-based ISSR markers have been widely used in assessing genetic variation within a species by measuring genetic diversity in *Piper betle* (Patra et al., 2011) and many species, including medicinal plants like *Withania somnifera* (Tripathi et al., 2012a), *Bacopa monnieri* (Tripathi et al., 2012b), *Ocimum* sp. (Patel et al., 2015). The aim of the present investigation was to study the genetic relatedness and diversity among 15 cultivars of *P. betle* with ISSR-PCR fingerprints.

Plant material

Plants of *Piper betle* germplasm lines (Table 1) from different locations of country including Indian Institute of Horticultural Research (IIHR), Bangalore and National Botanical Research Institute (NBRI), Lucknow were collected. A total of 15 cultivars were used as experimental material.

DNA extraction

Genomic DNA was extracted from leaf samples of 15 cultivars of *P. betle*. Young leaves were washed free off dust and dirt, de-ribbed and powdered in liquid nitrogen. Powdered tissue was stored at -70°C for further use. Total genomic DNA was extracted using modified CTAB extraction method of Doyle (1990) and dissolved in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) and stored at -20°C until further use. DNA quantification was performed by calculating the ratio of absorbance at 260 nm to that of 280 nm (OD_{260}/OD_{280}). DNA concentration and purity were also determined by electrophoresis on 0.8 % agarose gel containing 5µg/ml of ethidium bromide using alpha imager software. The re-suspended DNA was then diluted in sterile distilled water to the concentration of 1µg/µl for further use.

ISSR analysis

Of 28 primers (Bangalore Genei, Bangalore, India) tested, 18 primers (Table 2) that revealed polymorphism and exhibited good resolution were selected for further analysis. Each sample was amplified in a reaction mixture containing 50 ng genomic DNA, *Taq* polymerase 1.5 units (Bangalore Genei, India), 1x *Taq* buffer with 1.5 µm $MgCl_2$, and 0.25 mM of each dNTP mixture (Bangalore Genei, India), 1.0 µm of ISSR primers. Amplification was programmed with the following time and temperature profiles: initial denaturation of 5 min at 94°C, 30 cycles each of 1 min at 94°C for denaturation, 2 min for annealing at 45°C-52°C, 1 min for 72°C for extension, and a final extension at 72°C for 5 min. PCR mixture was cooled to 4°C and stored at -20°C until electrophoresis. Following PCR the samples were separated on 1.5% agarose gels buffered with 1X TBE and stained with ethidium bromide (5µg/ml). The electrophoretic patterns of the PCR products were visualized under UV and recorded using an Alpha Imager^{TM} EC (Alpha Innotech) Gel documentation system.

Data analysis
PCR products from ISSR analyses were scored qualitatively for the presence or absence of bands. Each ISSR band was considered an independent locus, and only distinct, reproducible, and well resolved fragments were scored visually, as absent (0) or present (1), for each of the 15 cultivars. A locus was considered polymorphic if a consistent band was present in one or more, but not all, individuals of the population. Only clear and apparently unambiguous bands were taken into account for analysis. Genetic similarities between the cultivars were measured by the Jaccard's (1908) similarity coefficient based on the proportion of shared alleles using NTSYS-PC version 2.0e (Exeter Software, Setauket, NY, U.S.A.) software package. The resultant similarity matrix data was used to construct dendrogram by using the unweighted pair-group method with an arithmetic average (UPGMA) subprogram of NTSYS-PC (Rohlf 1998). The polymorphism information content (PIC) of each marker that provides an estimate of the discriminatory power of a locus or loci was calculated.

Results
Out of the tested 28 ISSR primers, only 18 ISSR primers produced DNA fragments which were scorable. All the 18 scorable ISSR primers were polymorphic and reproducible. A total of 138 fragments were produced, out of which, 118 were polymorphic (Fig. 1). The size of the amplified products ranged from 150 bp to 2250 bp. The range of polymorphism was between (43%) for 1737-028primer up to (100%) for 1737-023, 9974-057, 9974-058, 9974-061 and 9974-053primers. The polymorphic information content (PIC) varied from 0.17 (1737-020 and 9973-008) to 0.45 (1737-021) with an average PIC of 0.32.The number of polymorphic loci varied from 2 (1737-025) to 13 (9974-053) with an average of 6.5 per primer (Table 2).

Genetic similarity matrices were constructed using Jaccard's similarity coefficient (Fig. 2). According to the results, genetic similarity ranged from 0.20 (low similarity) between U4, F5 and U2 up to 0.74 (high similarity) between M2 and M3 followed by 0.72 between U4 and U5. Cluster analysis using UPGMA revealed three main groups with six, three and four betelvine cultivars in each group and two cultivars namely M4 and U2 lying separately (Fig. 3). Cluster I consisted of F1, F4, F5, M1, F3, F2. Cluster II consisted of U3, U4 and U5 and Cluster III was comprised of M2, M3, U1, M5 in which M2 and M3 were more closely related to each other (Fig. 3). The result of principal component analysis (PCA) was comparable to the cluster analysis (Fig. 4).

Discussion
ISSR markers can be used in population genetic studies of plant species as they effectively detect very low levels of genetic variation (Zietkiewicz et. al., 1994). This method also may have potential for analyzing biogeographic patterns among populations of a single plant species (Li et al., 2008). High reproducibility of ISSR may be due to the use of longer primers and higher annealing temperatures (Morenoet al., 1998) compared with those used normally for other DNA amplification based techniques, such as the RAPD. Assessment of genetic variability within a cultivated crop has important consequences in plant breeding and the conservation of genetic resources. It is particularly useful in the characterization of individual accessions and cultivars for detecting duplications of genetic material in germplasm collections, and for selection of parents for breeding hybrids (Davilla et al., 1998). Studies have also revealed that knowledge on genetic diversity and of genetic relationships among breeding materials has a great impact on crop improvement (Ganesh and Thangavelu, 1995).

18 ISSR primers were tested on 15 betelvine cultivars with the aim to assess the genetic diversity of these 15 cultivars at molecular level for identification of superior parents for the purpose of recombination breeding. In the present investigation, 18 ISSR primers produced 118 polymorphic and 20 monomorphic bands (85.5% polymorphism) that unambiguously discriminated 15 *P. betle* cultivars into three major clusters. Results indicated the presence of wide genetic variability despite being a vegetatively grown crop, which reflects a high level of polymorphism at the DNA level. Variations in DNA sequences lead to polymorphism and greater polymorphism are indicative of greater genetic diversity. Ranade et al. (2002), Verma et al. (2004) and Patra et al. (2011) also reported high polymorphism level in betelvine. Betelvine exhibits less variability in morphological characters as compared to other vegetatively propagated plants. The ISSR profiles could reveal variability among the cultivars. Nomenclature ambiguity can easily be demonstrated in this study. U4 and U5 both the cultivars were having local name 'Bangla' but were collected from different geographical regions. Genetic similarity study showed that both shared a similarity index of 0.72. This clearly states that even though both the cultivars are named similar and also share some genetic similarity but they are not the same.

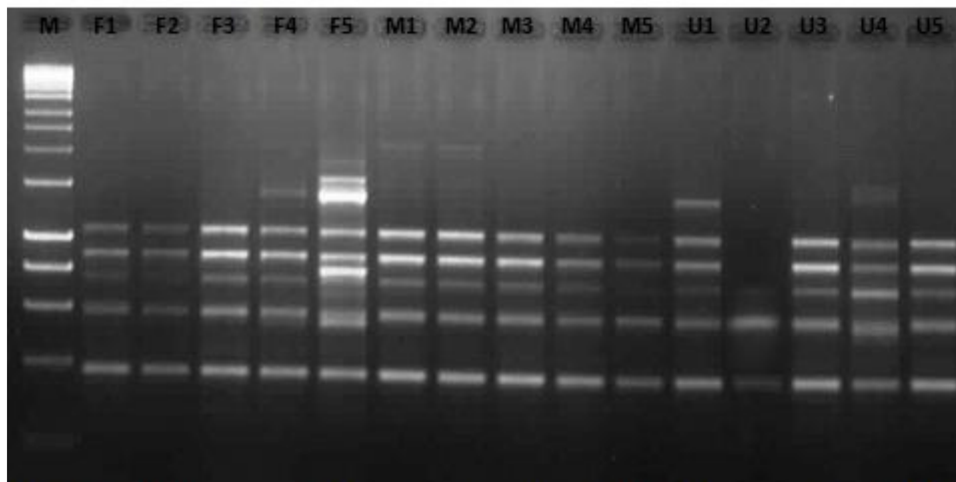

Figure 1: ISSR agarose gel electrophoresis profiles obtained in case of 15 betelvine cultivars using primer 1737-020. Lanes indicated by M contain molecular weight marker. The cultivar numbers are according to table 1 and indicate the template DNA from the appropriate cultivar

	F1	F2	F3	F4	F5	M1	M2	M3	M4	M5	U1	U2	U3	U4	U5
F1	1.00														
F2	0.64	1.00													
F3	0.67	0.63	1.00												
F4	0.69	0.66	0.66	1.00											
F5	0.68	0.56	0.65	0.69	1.00										
M1	0.68	0.56	0.65	0.66	0.71	1.00									
M2	0.60	0.55	0.57	0.50	0.56	0.68	1.00								
M3	0.55	0.49	0.54	0.51	0.52	0.62	0.74	1.00							
M4	0.46	0.44	0.35	0.51	0.39	0.38	0.38	0.45	1.00						
M5	0.42	0.46	0.38	0.42	0.38	0.44	0.49	0.46	0.38	1.00					
U1	0.52	0.54	0.49	0.48	0.45	0.52	0.60	0.67	0.40	0.53	1.00				
U2	0.26	0.32	0.24	0.27	0.20	0.22	0.29	0.29	0.33	0.31	0.27	1.00			
U3	0.67	0.55	0.55	0.53	0.57	0.65	0.71	0.56	0.36	0.43	0.51	0.28	1.00		
U4	0.59	0.44	0.53	0.58	0.57	0.57	0.48	0.46	0.37	0.43	0.46	0.20	0.68	1.00	
U5	0.63	0.48	0.56	0.54	0.53	0.67	0.58	0.55	0.42	0.47	0.57	0.27	0.68	0.72	1.00

Figure 2: A similarity matrix among 15 *Piper betle* cultivars using Jaccard Measure

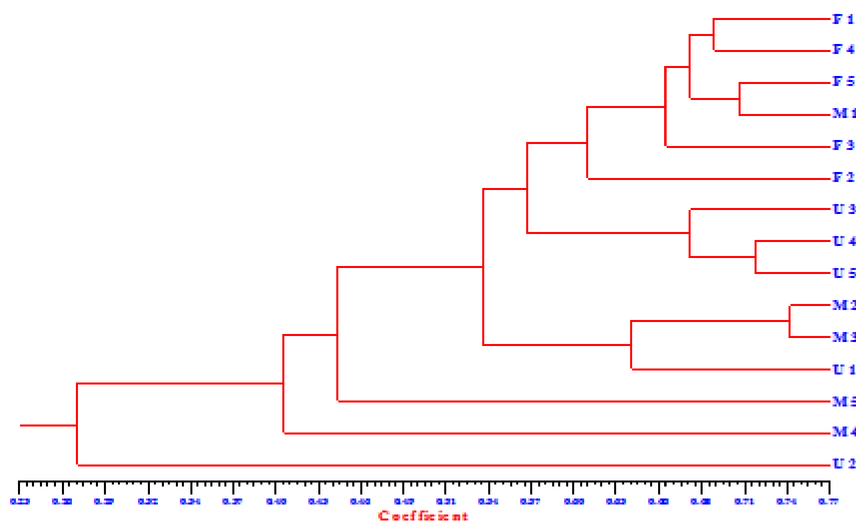

Figure 3: Dendrogram showing the genetic relationship among 15 cultivars of *Piper betle* based on ISSR data, UPGMA clustering method and Jaccard's coefficient

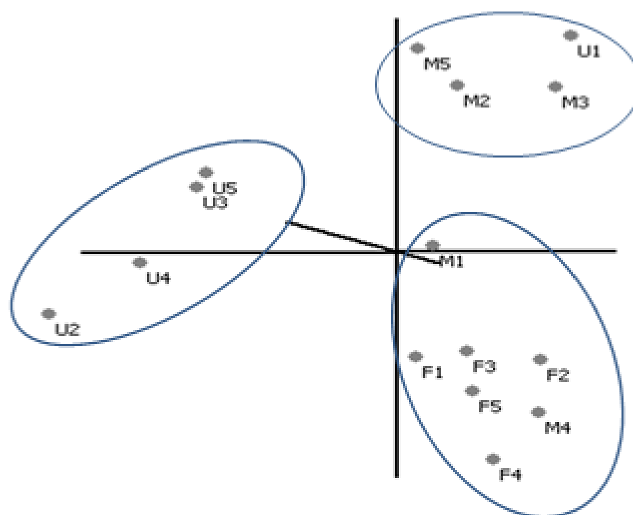

Figure 4: Principal component analysis (PCA) based on combined ISSR data showing genetic relationships among the cultivars of *Piper betle*

Table 1: List of cultivars used in the study

S. No.	Cultivar Name	Place of collection	Code
1	Lakur Local	IIHR, Bangalore	F1
2	Karapaku	Andhra Pradesh	F2
3	Kuljedu	Andhra Pradesh	F3
4	Kalipatti	Andhra Pradesh	F4
5	Mithlai	Bihar	F5
6	Nizamabad	NBRI, Lucknow	M1
7	Bangla Kalkattiya	NBRI, Lucknow	M2
8	Mahoba Bangla	NBRI, Lucknow	M3
9	Noba Cuttack	NBRI, Lucknow	M4
10	Goltapan T.N.	NBRI, Lucknow	M5
11	Desi Patta	Madhya Pradesh	U1
12	Desi Bangla	Madhya Pradesh	U2
13	Cuttak	Madhya Pradesh	U3
14	Bangla	Madhya Pradesh	U4
15	Bangla	Maharashtra	U5

Table 2: ISSR primers and their amplified products used in analyzing 15 *P. betle* cultivars

S. No.	ISSR	Sequence 5'-3'	Number of fragments	Polymorphic fragments	Percentage polymorphism	Allele size (bp)	PIC values
1	1737-026	$(GAA)_6$	8	7	88	1200 − 150	0.21
2	1737-023	$(GA)_8YT$	7	7	100	775 − 210	0.27
3	1737-024	$(GA)_8YC$	8	7	88	1250 − 250	0.32
4	1737-021	$(AG)_8YT$	8	6	75	1900 − 250	0.45
5	1737-025	$(GA)_8YG$	3	2	67	600 − 230	0.31
6	1737-020	$(TG)_8A$	9	7	78	2000 − 240	0.17
7	1737-028	$(AC)_7$	7	3	43	2250 − 400	0.37
8	1737-027	$(GAC)_6A$	6	4	67	2000 − 200	0.40
9	1737-022	$(AG)_8YA$	7	5	71	1400 − 200	0.23
10	9973-011	$(AC)_8YT$	11	10	91	1800 − 150	0.35
11	9974-062	$(CTTCA)_3$	4	3	75	650 − 240	0.42
12	9973-008	$(GA)_8YC$	5	4	80	1180 − 250	0.17
13	9974-057	$ACTT(C)_4ACA$ $GGTTAA(CA)_2$	8	8	100	1400 − 260	0.32
14	9974-054	$(TC)_8G$	10	9	90	1250 − 260	0.37
15	9974-058	TAGATCTGATA TCTGAATTCC	4	4	100	1000 − 440	0.36
16	9974-061	$(CT)_8A$	11	11	100	1100 − 220	0.38
17	9974-053	$(TC)_8A$	13	13	100	1600 − 150	0.36
18	9974-051	$(CT)_8G$	9	8	89	1200 − 150	0.28
Total fragments			138	118			
Average			7.6	6.5	83		0.32

Conclusion

DNA markers have become the markers of choice for the study of the genetic diversity of crop species because they are able to assess the genetic variation more precisely, quickly, and cheaply. In this study, the genetic diversity among Betelvine cultivars from different regions was assessed using ISSR. These allowed an accurate assessment of the genetic diversity among 15 cultivars, which can be combined with the performance of these genotypes in field conditions to direct breeding programs. There is a scope of large scale application of ISSR for genetic differentiation to avoid duplicates and to identify areas of maximum diversity. Using this information, crosses between accessions from different groups can be designed to maximize the chance of getting significant variation in the offspring.

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Conflict of interest

There is no conflict of interest.

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