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Inter simple sequence repeat (ISSR) markers as reproducible and specific tools for genetic diversity analysis of betelvine germplasm and *Piper* species

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ABSTRACT

Investigations on the levels and distribution of genetic variability are essential for conservation and genetic improvement programme. Genetic diversity analysis was carried out in 37 accessions of betelvine and two accessions of *P. hamiltoni* and one of *P. colubrinum* using inter simple sequence repeats (ISSR) markers. Out of 60 ISSR primers tested, 15 were selected based on high and consistent polymorphism. They generated a total 82 bands of which 72 were polymorphic. The different band statistics and efficiency parameters showed that the primers, *viz.*, UBC-822, 825, 826, 863 and ISSR-1, ISSR-15 were more efficient to study the genetic diversity. The UPGMA dendrogram and PCA plot revealed *P. colubrinum* to be the most distant of the three species. The accessions from Andaman clustered based on geographical origin and shared 70% similarity. A distinct gender-based clustering was observed among cultivated clones of betelvine.

Key words: Gender, ISSR markers, *Piper betle* accessions.

INTRODUCTION

Betelvine (*Piper betle* L.) is a perennial, evergreen creeper belonging to *Piperaceae* family. The *Piperaceae* family includes 10 genera and over 1,000 species of herbs, shrubs, and climbers (Parthasarthy *et al*., 6). Some of the commercially important species of *Piper* are *P. betle*, *P. nigrum*, *P. chaba*, *P. longum etc*. The green heart shape leaves of betelvine are popularly known as *Paan* in India. The most probable place of origin of betelvine is Eastern Malaysia (Chattopadhyay and Maity, 1). In Asiatic region its ranks second to coffee and tea in terms of daily consumption (Kumar *et al*., 4). The leaves are widely used in Indian system of medicine and health. It has a positive stimulatory influence on intestinal digestive enzymes, especially lipase, amylase and disaccharides (Sandhya *et al*., 13). The anti-inflammatory properties of the ecotypes of this plant have been investigated and three purified chemicals namely chavibetol, chavibetol acetate and chavicol were identified (Khozirah *et al*., 3). It is commercially propagated through stem cutting. The cultivated betelvine cultivars or landraces are named with local or vernacular names. These landraces have been named differently in different regions and more than one landrace may have the same name. In the absence of any systematic attempts to resolve this nomenclature problem, a few isolated efforts have been made to rationalize the different landraces and to identify similar and dissimilar types among them.

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Many studies have classified the cultivars into different categories based on leaf traits, morphological, anatomical and phytochemical properties. Molecular markers are widely used in germplasm characterization and fingerprinting. DNA markers have the potential to enable individual clones to be differentiated reliably and unambiguously. inter simple sequence repeat (ISSR) markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al*., 9). The ISSR markers were used in studies for assessing genetic diversity of *Vanilla* species by Verma *et al*. (15); fingerprinting analysis of mango cultivars by Pandit *et al*. (5) and they proved to be ideal for phylogenetic studies. Betelvine is a dioecious plant, the identification of male and female flowering in the germplasm collection and profuse fruit setting in certain geographical regions like Bengaluru and North East conditions of India opened up the possibilities of orthodox breeding of betelvine (Raghvendra and Maiti, 8). Assessing the genetic diversity of the crop is an essential step for plant breeding programmes. In the present study systematic molecular characterization of betelvine germplasm using ISSR markers was attempted to assess the variability and identify the diverse genotypes for further breeding programs.

MATERIALS AND METHODS

The research work was carried out during 2010-11 utilizing 38 *Piper betle* accessions and 2 *Piper* species.

The material was sampled from the betelvine garden maintained at Central Horticultural Experimental Station (CHES), Hirehalli, a substation of IIHR, Bengaluru. Young leaves were collected and stored at -80ºC until the total genomic DNA was extracted using modified CTAB protocol (Doyle and Doyle, 2). Frozen tissue (2-3 g) was grounded with mortar and pestle in liquid nitrogen and homogenized in 12 ml of pre-heated (65ºC) DNA extraction buffer containing 100 mM Tris-Hcl (pH 8.0); 1.5 M NaCl; 20 mM EDTA (pH 8.0); CTAB (2% w/v) and β-mercaptoethanol (0.2% v/v) with the addition of pinch of polyvinyl pyrollidone (PVP). The DNA was re-suspended in 200 µl Tris-EDTA buffer and quantification was carried out by using Hoefers Dyna quant (Pharmacia Biotech, USA) and diluted in autoclaved double-distilled water to 25 ng/µl for further PCR reaction.

The initial screening of 60 primers of ISSR previously in *Piper longum* by Saji (12) and UBC primers obtained from Bioserve Pvt. Ltd., India were employed in 38 accessions of betelvine and 2 *Piper* sp. (Table 1). Based on the ability to generate polymorphic and reproducible profiles, 15 primers were selected (Table 2) for the subsequent analysis. The polymerase chain reaction (PCR) amplification for the ISSR was performed in 25 µl reaction volume, containing of 10X PCR buffer (10 mM tris-HCl pH 8.0), 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 1U of *Taq* polymerase, 12.5 pmol primers and 50 ng of genomic DNA template. PCR amplification was carried out using thermal cycler (Eppendorf, Germany), which was programmed for an initial step of 4 min. at 94ºC followed by 35 cycles of 1 min. 94ºC, annealing temperature standardized according to the primer (Table 3) for the 45 sec. and 1 min. for 72ºC for 10 min. final extension and product was stored at 4ºC until loading. PCR products were separated on 1.5% agarose gel, later stained with ethidium bromide (1X), products were visualiszed and photographed with gel documentation system (UVPRO, UK).

The band profiles of each primer were scored visually. The consensus profiles were recorded on the basis of presence (1) or absence (0) of bands and binary qualitative data matrices was constructed. Among the various similarity indices those of Jaccard's coefficient were chosen as the most appropriate for dominant markers. Data were used to obtain cluster analysis of the similarity matrices following Unweighted Pair Group with Arithmetic Averages (UPGMA) and Principal component analysis (PCA) using SAHN function of NTSYS-pc version 2.1 program (Rohlf, 10). The group analysis of similarity index was calculated from the similarity matrics given in Table 3, by using the relation $SI = \sum(1 - S)$, where, *S* is the similarity between landrace pairs. Betelvine landraces were considered in four groups named as 'Kapoori', 'Bangla', 'Andaman' and 'Others',while the two outgroup *Piper* species *P. hamiltonii* and *P. colubrinum* were included as a fifth group.

Polymorphic information content (PIC) or average heterozygosity was calculated as per Rolden-Riuz *et al*. (11). Effective multiplex ratio (EMR) was calculated. Observed heterozygosity (H_o) was calculated by average of the sum of bands (S) present for each allele divided by number of genotypes (NC) under study, ($H_o=\sum S/NC$). Marker index (MI) is the product of PIC and EMR Powell *et al.* (7). Resolving power was calculated as, $R_p = \sum Ib$ Where, Band informativeness (Ib) = 1 – [2(0.5 – p)] Where, 'p' is the proportion of accession containing the band. Diversity index is the expected heterozygosity and was calculated as DI= $1\text{-}1/L \sum_l \sum_l P_i^2$ Where, p_i is the frequency of i^{th} allele at the 1 locus and L is the number of loci.

RESULTS AND DISCUSSION

Genetic variability and genetic relationships of *P. betle* and Piper species were studied using ISSR markers. All 15 primers recorded clear sharp reproducible bands and high polymorphism and which are efficient in diversity study even low level of genetic variations. The polymorphic banding pattern of IT-1 and UBC-827 is shown in Fig. A and B. Majority of the primers (10 of 15 = 66.66%) exhibited 100% polymorphism, while least polymorphism (50%) was recorded in UBC-828. The primers yielded total 82 scorable bands (Table 2) and their size varied from 250 to 1200 bp. Out of 82 bands 77 (91.82%) were polymorphic and only 5 bands (8.18%) were monomorphic, indicating that the selected primers have shown across the accessions (91.82%). The number of scorable loci resolved per amplification was primer dependent and varied from 2 (UBC-828) to 8 (UBC-822) with average 5.4 loci per primer.

The polymorphism recorded (91.82%) in betelvine is greater than what was reported in vanilla (Verma *et al*., 15) and similar to mango (Pandit *et al*., 5). The present study shows that ISSR primers efficient tools for studying genetic diversity of betelvine. The resolving power (Rp) of 15 ISSR primers ranged from 0.3 for primer ISSR-2 to 4.65 for primer UBC-822 with an average of 2.48. Among the primers used UBC-825 and UBC-826 recorded higher polymorphism (85.71 & 100%) and also possess the high Rp values (3.5 and 3.4,respectively) suggesting that these primers are highly useful in molecular studies on betelvine. Polymorphic information content (PIC) ranged from 0.2 for primer UBC-863 to 0.48 for primer UBC-828 with an average of 0.35. The range obtained for other efficiency parameters like the marker index (MI), diversity index (DI), and effective

Table 1. List of betelvine germplasm and *Piper* sp. with their geographical origin and sex form.

Accession	Local Name/Acc. No.	Geographical origin	Sex of plant
IIHRBV-01	Tellaku, Ponnur	Andhra Pradesh	Male
IIHRBV-04	Tellaku, Chinthalapudi	Andhra Pradesh	Male
IIHRBV-06	Kuljedu Cuddapah	Andhra Pradesh	Male
IIHRBV-08	Vasani Kapoori	Maharashtra	Male
IIHRBV-09	Shirpurkata	Maharashtra	Male
IIHRBV-10	Kapoori	Tamil Nadu	Male
IIHRBV-11	Kapoori Chinacheppalli	Andhra Pradesh	Male
IIHRBV-18	Yellow Leaf	Andhra Pradesh	Male
IIHRBV-20	Patchaikodi	Tamil Nadu	Male
IIHRBV-47	Swarna Kapoori	Andhra Pradesh	Male
IIHRBV-30	Ramtek Bangla	Maharashtra	Female
IIHRBV-31	Kalipathi	Maharashtra	Female
IIHRBV-32	Bangla Nagaram	Uttar Pradesh	Female
IIHRBV-33	Godi Bangla	Odisha	Female
IIHRBV-35	Bangla	Uttar Pradesh	Female
IIHRBV-42	Simarali Babna	West Bengal	Female
IIHRBV-43	Simarali Babna local	West Bengal	Female
IIHRBV-45	Khasi Pan	Assam	Female
IIHRBV-37	SGM-1	Tamil Nadu	Female
IIHRBV-105	Vellaikodi-2	Tamil Nadu	Female
IIHRBV-58	CARI-6	Andaman Islands	Male
IIHRBV-59	CARI-2	Andaman Islands	Female
IIHRBV-67	IIHRBV-67	Andaman Islands	Female
IIHRBV-68	IIHRBV-68	Andaman Islands	Female
IIHRBV-74	IIHRBV-74	Andaman Islands	Female
IIHRBV-75	IIHRBV-75	Andaman Islands	Female
IIHRBV-76	IIHRBV-76	Andaman Islands	Unknown
IIHRBV-107	IIHRBV-107	Gujarat	Female
IIHRBV-109	IIHRBV-109	Karanataka	Female
IIHRBV-110	IIHRBV-110	Andaman	Unknown
IIHRBV-111	IIHRBV-111	Andaman	Unknown
IIHRBV-112	IIHRBV-112	Andaman	Unknown
IIHRBV-113	IIHRBV-113	Andaman	Unknown
IIHRBV-114	IIHRBV-114	Andaman	Unknown
IIHRBV-115	Dogapan Sada	West Bengal	Unknown
IIHRBV-116	Awanipan-2 (*P. hamiltoni*)	West Bengal	Unknown
IIHRBV-117	Ambadi koppa	Karanataka	Male
IIHRBV-118	Kariyele	Karanataka	Unknown
IIHRBV-PC-1	*Piper colubrinum*	Kerala	Hermaphrodite
IIHRBV-38	Awanipan-1 (*P. hamiltoni*)	West Bengal	Male

Table 2. List of ISSR primers, banding pattern and its statistics in betelvine germplasm and *Piper* sp.

Primer	Sequence (5'-3')	Total No. of bands	Polymorphic bands (No.)	Monomorphic bands (No.)	Percent of polymorphism	Polymorphic information content (PIC)	Resolving power (Rp)	Effective Multiplex Ratio (EMR)	Diversity Index (DI)	Marker index (MI)	Observed heterozygosity (H_o)
ISSR-1	CTCTCTCTCTCTCTCTTG	7	7	0	100	0.29	2.3	7	0.46	3.25	0.54
ISSR-2	CTCTCTCTCTCTCTCTAC	4	3	1	75	0.13	0.3	3	0.63	1.9	0.37
ISSR-5	CACACACACACACGT	5	5	0	100	0.37	2.75	5	0.43	2.13	0.58
ISSR-7	CACACACACACACGG	4	4	0	100	0.46	2.95	4	0.37	1.48	0.63
ISSR-9	GTGTGTGTGTGTGG	4	4	0	100	0.4	1.6	4	0.36	1.45	0.64
ISSR-15	GTGGTGGTGG	7	7	0	100	0.33	2.1	7	0.41	2.9	0.59
ISSR-18	GATAGATAGATAGG	5	5	0	100	0.43	2.85	5	0.29	1.43	0.72
IT-1	GGTAACAAGGTTTCC	6	5	1	83.33	0.29	2.4	5	0.62	3.1	0.38
IT-2	AGTTTCTTCTCCTCC	5	5	0	100	0.44	2.75	5	0.33	1.63	0.68
UBC-822	TCTCTCTCTCTCTCTCA	8	8	0	100	0.31	4.65	8	0.58	4.63	0.42
UBC-825	ACACACACACACACACT	7	6	1	85.71	0.29	3.5	6	0.64	3.85	0.36
UBC-826	ACACACACACACACACC	6	6	0	100	0.46	3.4	6	0.32	1.9	0.68
UBC-827	ACACACACACACACACC	6	6	0	100	0.46	2.75	6	0.23	1.38	0.77
UBC-828	TGTGTGTGTGTGTGTGA	2	1	1	50	0.48	0.7	1	0.35	0.35	0.65
UBC-863	AGTAGTAGTAGTAGTAGT	6	5	1	83.33	0.2	2.2	5	0.78	3.9	0.22
	Total	82	77	5	-	-	-	-	-	-	-
	Mean	-	5.1	0.33	91.82%	-	-	-	-	-	-

Table 3. The distribution of pair-wise similarity index within and between betelvine landrace groups and the outgroup plants.

Parameter Similarity range	Within *Kapoori*	Within *Bangla*	Within *Andaman*	Between *Kapoori* and rest of the landraces groups	Between *Bangla* and rest of the landraces groups	Between *Andaman* and rest of the landraces groups	Between *P. colubrinum* and rest of the landraces groups	Between *P. hamiltonii* and rest of the landraces groups
	Proportion (%) of genotypes pairs in the similarity range							
0.00-0.10	-	-	-	-	-	-	-	-
0.11-0.20	-	-	-	-	-	-	-	-
0.21-0.30	-	-	-	-	-	-	-	-
0.31-0.40	-	3.03	-	1.67	1.08	1.60	55.26	
0.41-0.50	10.90	7.57	1.51	20.87	16.30	16.66	47.73	56.41
0.51-0.60	30.90	31.81	22.72	64.30	53.98	51.28	-	41.02
0.61-0.70	32.72	40.90	45.45	12.79	25.72	25.32	-	2.56
0.71-0.80	21.81	16.66	19.69	1.34	2.89	5.12	-	-
0.81-0.90	3.63	-	10.60	-	-	-	-	-
No. of pairs	55	66	66	297	276	312	38	37
Av. similarity	0.63	0.61	0.66	0.55	0.57	0.57	0.39	0.43

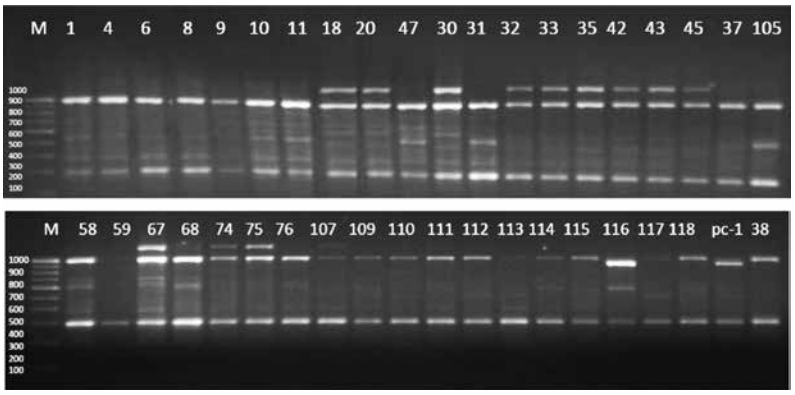

Fig. A. Profiles of IT-1 primer with *Piper betle* accessions and *Piper* sp. same order as listed in Table 1.

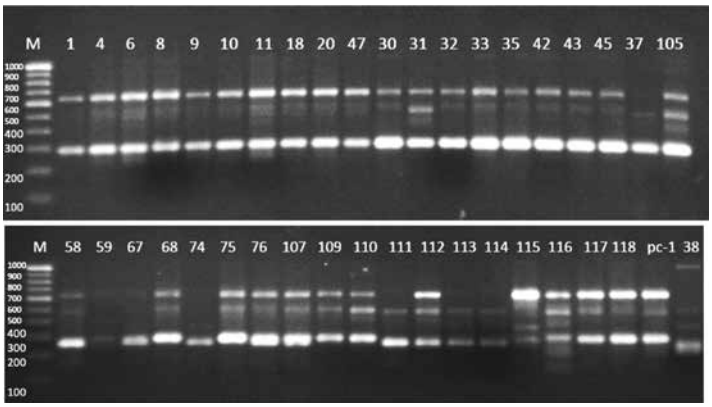

Fig. B. Profiles of UBC-872 primer with *Piper betle* accessions and *Piper* sp. same order as listed in Table 1.

multiplex ratio (EMR) and Observed heterozygosity (H_o) is presented in Table 3. It is evident from the study the efficiency parameters (Table 3) that UBC-822, 825, 826, 863 and ISSR-1, ISSR-15 are the most efficient primers and the UBC-828 is the least efficient primer.

The pooled binary data from 15 ISSR primers was utilized for cluster (Fig. C) and principle component analysis (Fig. 4) using the NTSys pc vers. 2.02 (Rohlf, 10). Dendrogram was generated using the genetic similarity matrices (Table 4) by UPGMA method. The genetic similarity value in the matrices ranged from 0.32 to 0.88, suggesting a moderate to very high diversity among the betelvine germplasm and *Piper* species. The least genetic similarity values (0.32) are recorded between *P. betle* (CARI-2) and *P. colubrinum*. Dendrogram showed two (I, II) major clusters at 41% similarity, *P. colubrinum* clustered separately from *P. betle* accessions, where depicting species level differentiation. It is also evident from the dendrogram that *P. colubrinum* and *P. hamiltonii* are divergent from *P. betle.* Two accessions of *P. hamiltoni* clustered separately showing the possible variability present within the species, it can be concluded that *P. hamiltonii* is relatively closer to *P. betel* than *P. colubrinum.* Among the 15 accessions, 10 accessions grouped together at 60% similarity, except CARI-2 and IIHRBV67 which are distinct and clustered separately. Different ancestry from others or natural variations over the period of time might be the reasons for their uniqueness. The highest genetic similarity of 0.88 was observed between IIHRBV-110 and IIHRBV-111, followed by 0.87 between IIHRBV-112 and IIHRBV-114 as these collections belong to the same geographical area may have originated from same ancestor. In *P. betle* accessions, clustering based on geographical origin was observed only among collections from Andaman Islands and they clustered separately from the material from mainland. Grouping based on the sex of the plants is not observed among Andaman Islands collection. Isolation of Andaman area from mainland and limited diffusion of genetic material may be the reasons for their uniqueness from the cultivated material and similarities among them.

Next level of clustering showed two major groups at 50% similarity; one major cluster with 37 accessions and Kalipathi grouped seperately. In the sub-clusters CII cluster contained two sub-clusters, CII-a consisting 10 male accessions and CII-b 12 female accessions showing gender based grouping irrespective their place of origin. Results from PCA analysis shows similar trend as that of cluster analysis. Very distinct gender based grouping in betelvine was also reported earlier by Verma *et al.* (14) in their study of 53 clones using 11 RAPD markers. The clear distinct grouping of male and female is not observed in collections from Andaman Islands otherwise evident in cultivated clones. Landraces which are cultivated are generally selected for their suitability for specific region consumer preferences. In betelvine, male and female clones are grown in different regions and under different systems of cultivation. Being cultivated in isolation, in absence of sexual reproduction, clones could have acquired specific adaptation features which are reflected in their grouping. Whereas such gender based grouping is absent in the collection from the natural forest of Andaman area where both male and female co-exist.

Data given in Table 3 indicate the trend for the distribution of pairwise similarities within and between landrace groups of betelvine and *Piper* species. The average similarity between the betelvine landraces and *P. colubrinum* (0.39), *P. hamiltoni* (0.43) was very low which was following the pattern observed in dendrogram and PCA analysis. The average similarity values for the ISSR profiles varied from 0.61 to 0.68 within the betelvine landrace groups (Bangla, Kapoori and Andaman), while between the groups the plants had similarity value in the range 0.39 to 0.57. The genetic similarity present within the kapoori, bangla and Andaman groups is almost similar, showing all the groups hold considerable diversity.

Present investigations revealed that ISSR markers are powerful tool for delineating the diversity in the betelvine. From the result of various efficiency parameters UBC-822, 825, 826, 863 and ISSR-1, ISSR-15 are the most efficient primers and can be used in further studies. It is seen that *P. colubrinum* and *P. hamiltoni* are distinctly different from the *P. betle*. Among the cultivated clones clear gender based grouping is seen irrespective their geographical place of origin. But collections from Andaman Islands showed clustering based on geographical origin. The study shows that Andaman area harbour valuable germplasm of betelvine and needs to be conserved. The ISSR markers proved to be highly efficient in identifying diverse *P. betle* genotypes. The information on the diverse male (Ambadikoppa and CARI-6) and female (CARI-2 and Khasipan) clones is useful in further breeding programmes of betelvine. Though, betelvine vegetatively is propagated countable divergence still present among the cultivated clones, which can be exploited through hybridization.

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Table 4. Genetic similarity matrix (Jaccard's) of *Piper betle* germplasm and *Piper* sp.

	1	4	6	8	9	10	11	18	20	47	30	31	32	33	35	42	43	45	37	105	58	59	67	68	74	75	76	107	109	110	111	112	113	114	115	116	117	118	PC	38
1	1.00																																							
4	0.82	1.00																																						
6	0.60	0.67	1.00																																					
8	0.75	0.77	0.62	1.00																																				
9	0.62	0.65	0.53	0.75	1.00																																			
10	0.70	0.75	0.63	0.85	0.73	1.00																																		
11	0.62	0.74	0.67	0.74	0.68	0.75	1.00																																	
18	0.49	0.59	0.72	0.53	0.47	0.55	0.66	1.00																																
20	0.64	0.69	0.56	0.72	0.57	0.70	0.63	0.60	1.00																															
47	0.76	0.69	0.54	0.72	0.66	0.70	0.59	0.51	0.67	1.00																														
30	0.50	0.60	0.52	0.60	0.53	0.58	0.59	0.61	0.63	0.55	1.00																													
31	0.49	0.51	0.44	0.60	0.56	0.55	0.48	0.45	0.55	0.54	0.60	1.00																												
32	0.48	0.55	0.50	0.61	0.60	0.57	0.60	0.63	0.60	0.56	0.76	0.61	1.00																											
33	0.51	0.59	0.51	0.59	0.58	0.58	0.58	0.58	0.55	0.51	0.80	0.56	0.83	1.00																										
35	0.54	0.62	0.54	0.62	0.53	0.60	0.59	0.61	0.63	0.60	0.76	0.59	0.72	0.73	1.00																									
42	0.49	0.55	0.56	0.59	0.55	0.60	0.55	0.58	0.53	0.51	0.64	0.45	0.66	0.70	0.67	1.00																								
43	0.47	0.55	0.52	0.50	0.50	0.54	0.51	0.54	0.56	0.58	0.60	0.38	0.62	0.62	0.63	0.72	1.00																							
45	0.49	0.56	0.56	0.49	0.42	0.50	0.56	0.58	0.53	0.49	0.59	0.38	0.58	0.61	0.54	0.58	0.66	1.00																						
37	0.48	0.52	0.50	0.50	0.44	0.52	0.50	0.57	0.57	0.50	0.69	0.50	0.68	0.75	0.66	0.72	0.71	0.63	1.00																					
105	0.52	0.52	0.52	0.55	0.54	0.54	0.51	0.55	0.56	0.58	0.52	0.54	0.62	0.54	0.55	0.54	0.58	0.54	0.65	1.00																				
58	0.48	0.58	0.58	0.61	0.54	0.60	0.57	0.55	0.57	0.53	0.50	0.44	0.51	0.47	0.55	0.60	0.59	0.58	0.54	0.56	1.00																			
59	0.50	0.52	0.40	0.50	0.45	0.49	0.46	0.40	0.51	0.52	0.42	0.36	0.40	0.37	0.47	0.49	0.50	0.46	0.45	0.50	0.59	1.00																		
67	0.45	0.50	0.55	0.53	0.54	0.50	0.50	0.52	0.52	0.53	0.53	0.42	0.57	0.55	0.58	0.58	0.66	0.55	0.54	0.55	0.63	0.53	1.00																	
68	0.47	0.54	0.59	0.57	0.53	0.58	0.56	0.53	0.58	0.54	0.59	0.42	0.55	0.59	0.59	0.70	0.69	0.62	0.63	0.55	0.66	0.63	0.67	1.00																
74	0.62	0.64	0.58	0.59	0.55	0.57	0.53	0.55	0.57	0.56	0.51	0.45	0.50	0.58	0.61	0.66	0.65	0.58	0.57	0.51	0.60	0.54	0.66	0.66	1.00															
75	0.51	0.53	0.51	0.49	0.47	0.50	0.53	0.60	0.55	0.49	0.53	0.41	0.55	0.55	0.58	0.66	0.65	0.61	0.65	0.57	0.57	0.59	0.63	0.69	0.75	1.00														
76	0.46	0.53	0.53	0.50	0.51	0.54	0.55	0.65	0.57	0.54	0.63	0.50	0.65	0.60	0.68	0.66	0.64	0.66	0.67	0.62	0.65	0.53	0.62	0.71	0.65	0.77	1.00													
107	0.50	0.58	0.55	0.53	0.57	0.54	0.52	0.60	0.62	0.53	0.55	0.50	0.56	0.60	0.66	0.63	0.64	0.47	0.59	0.56	0.50	0.53	0.72	0.63	0.74	0.68	0.64	1.00												
109	0.53	0.61	0.58	0.61	0.57	0.62	0.57	0.54	0.70	0.58	0.61	0.50	0.65	0.66	0.66	0.69	0.67	0.55	0.64	0.62	0.59	0.50	0.66	0.68	0.62	0.65	0.64	0.80	1.00										
110	0.48	0.58	0.58	0.55	0.57	0.57	0.52	0.60	0.62	0.53	0.55	0.52	0.56	0.55	0.63	0.66	0.61	0.52	0.59	0.59	0.68	0.64	0.66	0.71	0.68	0.71	0.70	0.77	0.74	1.00									
111	0.47	0.57	0.59	0.54	0.58	0.60	0.59	0.61	0.63	0.52	0.57	0.54	0.58	0.53	0.61	0.64	0.60	0.54	0.58	0.60	0.69	0.63	0.64	0.72	0.66	0.69	0.71	0.78	0.71	0.88	1.00								
112	0.46	0.53	0.56	0.53	0.55	0.55	0.53	0.57	0.62	0.49	0.63	0.50	0.60	0.60	0.63	0.68	0.59	0.55	0.67	0.62	0.62	0.59	0.66	0.77	0.62	0.71	0.70	0.70	0.70	0.83	0.83	1.00							
113	0.46	0.53	0.55	0.55	0.57	0.57	0.60	0.57	0.57	0.46	0.61	0.55	0.59	0.60	0.63	0.63	0.53	0.50	0.62	0.56	0.62	0.50	0.54	0.63	0.54	0.60	0.62	0.62	0.62	0.74	0.75	0.83	1.00						
114	0.44	0.55	0.60	0.53	0.51	0.59	0.59	0.61	0.59	0.46	0.62	0.50	0.59	0.59	0.67	0.67	0.55	0.54	0.63	0.55	0.67	0.55	0.56	0.70	0.59	0.64	0.69	0.66	0.66	0.78	0.85	0.87	0.85	1.00					
115	0.53	0.63	0.58	0.58	0.57	0.57	0.58	0.60	0.67	0.56	0.58	0.50	0.54	0.58	0.58	0.63	0.63	0.51	0.50	0.59	0.56	0.65	0.54	0.55	0.63	0.60	0.62	0.65	0.67	0.67	0.73	0.79	0.73	0.77	1.00				
116	0.45	0.52	0.45	0.50	0.50	0.49	0.49	0.49	0.53	0.47	0.47	0.46	0.53	0.51	0.54	0.46	0.40	0.39	0.50	0.50	0.48	0.50	0.48	0.52	0.49	0.54	0.58	0.58	0.55	0.64	0.62	0.61	0.58	0.63	0.67	1.00			
117	0.50	0.50	0.50	0.57	0.59	0.56	0.51	0.46	0.59	0.47	0.57	0.49	0.61	0.62	0.57	0.65	0.55	0.41	0.58	0.55	0.56	0.50	0.53	0.62	0.56	0.62	0.58	0.70	0.73	0.70	0.68	0.76	0.70	0.68	0.70	0.60	1.00		
118	0.56	0.61	0.56	0.61	0.55	0.57	0.55	0.55	0.68	0.54	0.53	0.48	0.57	0.55	0.58	0.58	0.51	0.46	0.57	0.54	0.57	0.59	0.60	0.61	0.60	0.60	0.54	0.71	0.38	0.38	0.44	0.44	0.55	0.55	0.43	0.39	0.39	1.00	
PC	0.41	0.39	0.44	0.39	0.39	0.43	0.40	0.41	0.41	0.39	0.39	0.40	0.44	0.43	0.42	0.38	0.36	0.34	0.44	0.38	0.33	0.32	0.37	0.36	0.41	0.43	0.38	0.40	0.38	0.38	0.44	0.45	0.44	0.44	0.43	0.39	0.39	0.41	1.00
38	0.47	0.45	0.45	0.52	0.53	0.53	0.46	0.49	0.51	0.50	0.50	0.57	0.50	0.49	0.47	0.46	0.42	0.44	0.50	0.50	0.48	0.50	0.50	0.48	0.57	0.54	0.51	0.53	0.55	0.48	0.55	0.62	0.59	0.53	0.55	0.49	0.55	0.50	0.46	1.00

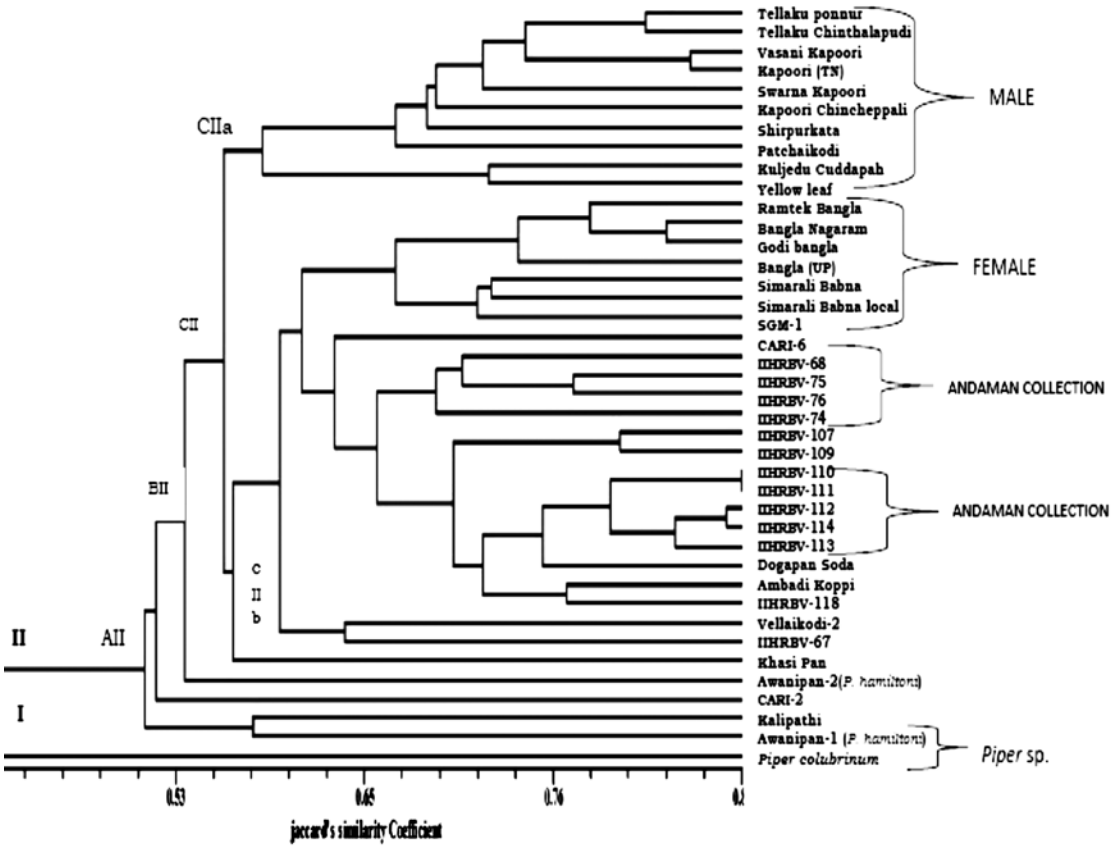

Fig. 3. UPGMA dendrogram of betelvine germplasm and *Piper* sp. based on ISSR primers.

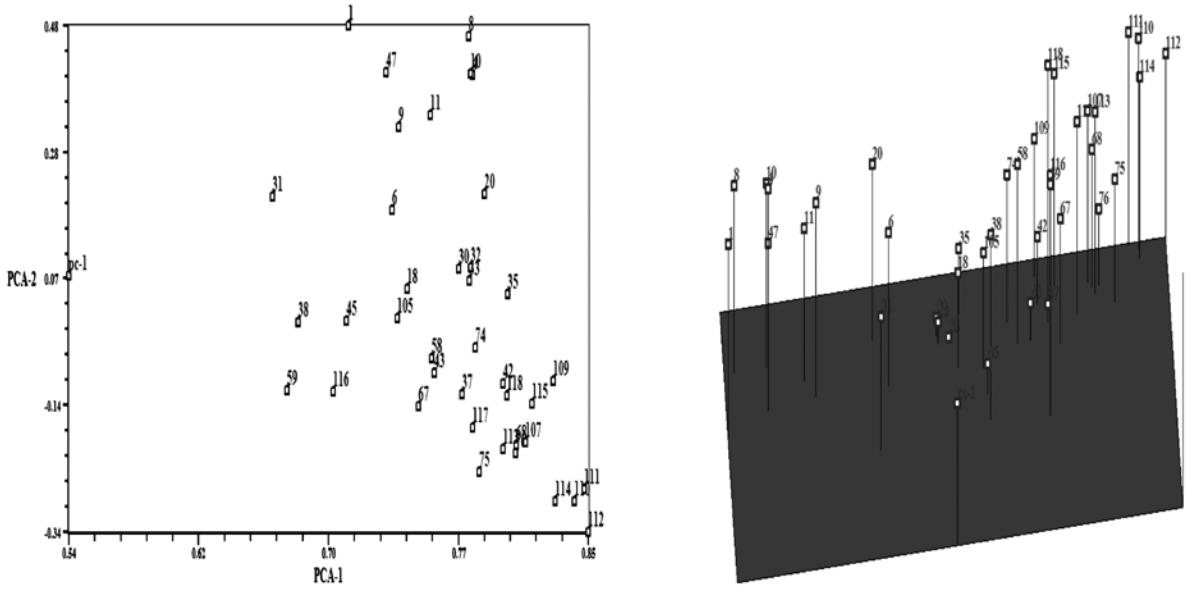

Fig. 4. The 2-D and 3D dimensional plot of principal component analysis based on ISSR primers of betelvine germplasm and *Piper* sp.

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