Indian J. Agric. Res., 52(4) 2018: 434-438
Print ISSN:0367-8245 / Online ISSN:0976-058X

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
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ISSR marker based diversity assessment of *Piper spp*. in Bay Islands, India

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Received: 26-10-2017 Accepted: 31-05-2018 DOI: 10.18805/IJARe.A-4941

ABSTRACT

Inter-simple sequence repeat (ISSR) analysis was used to study the genetic diversity of *Piper spp.* with its relative genus *Peperomia pellucida*. Among the 38 ISSR primers used, 10 primers generated 1083 bands, of which seven (80.8%) were polymorphic. The maximum polymorphism were obtained from the primers UBC 881, UBC 889, UBC 848 and UBC 830. Cluster analysis grouped the species studied into two major clusters. One cluster comprised the different accessions of *Piper nigrum* and is distinct from the other species of Piperaceae. The other cluster grouped the four species of Piperaceae namely *Piper longum*, *Piper sarmentosum*, *Piper betle* and *Peperomia pellucida*. The variability studies also revealed that the ISSR marker could group the accessions within the same species based on their similar geographical origin. Assessment of genetic diversity among the different species of the family Piperaceae through ISSR marker would help in rapid identification of polymorphism, assist in future germplasm collection, conservation and domestication programmes.

Key words: Genetic diversity, ISSR, *Piper sp*, Piperaceae.

INTRODUCTION

Piper is one of the well represented genera in the family *Piperaceae*, which have wide distribution in tropical and subtropical regions of the world. The genus *Piper* comprises more than 3000 species reported from the tropical and subtropical regions around the world. About 115 sp of *Piper* are of Indian origin (Saji, 2006). However, the genus is rather poorly represented among the tropical island of Andaman and Nicobar in the Bay of Bengal with only seven species *viz., Piper longum* L., *P. betle* L., *P. miniatum Bl., P. ribesoides* Wall, *P. sarmentosum Roxb., P. pedicellosum* Wall ex Dc and *P. nigrum* L. (Mathew *et al*., 2004). However, there is an endemic species of Piper distributed in the Nicobar group of Islands namely *P. clypeatum Wall.ex Hook .f.* (Pandey and Diwakar, 2008). Among the different *Piper sp* black pepper is the important commercial species in the Bay Islands which is grown on coconut, arecanut or Glyricidia standards. *P. betle*, *P. sarmentosum, P longum* and *P. pedicellosum* are the important species commonly used in the indigenous medicinal practices in the Island. It is taxonomically a very difficult genus because of the greater range of variability and minute nature of flowers (Sharma and Bhattacharya, 1959). The Island ecosystem is highly fragile as prone to various natural calamities. In this regard knowledge of genetic diversity of different populations is important to form a basis for conservation, genetic improvement and promotion of domestication of populations with desirable traits (Ahmad *et al*., 2010). The extent of genetic diversity among the different *Piper sp* is very much essential for maintenance and utilization in further breeding programmes. Diversity analysis of *Piper sp*. based on morphological, cytological studies were carried out by Sharma and Bhattacharya (1959) and based on chemical constituent data by Sebastian and Sujatha (1996). But differentiation of species through morphological features is inefficient and inaccurate. Most morphological traits are highly influenced by environmental conditions or vary with the development of the plant and isozymes are limiting due to low levels of polymorphisms (Asha *et al*., 2006). Consequently, DNA based techniques such as RAPD, ISSR and micro-satellites or simple sequence repeats (SSRs) are effective in assessing genetic diversity of plant species because they provide unlimited potential markers to reveal differences at molecular level (Ghafoor *et al*., 2007). When compared to other classes of markers, DNA based markers, often carry high numbers of alleles at very low frequencies (Vinod *et al*., 2007). Inter-simple sequence repeat (ISSR) is the molecular marker which was first employed by ZIetkiewicz *et al*, (1994) and Gupta *et al*, (1994) 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. A few genetic studies using molecular markers such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) have been conducted on the analysis of genetic diversity within Indian cultivars of black pepper (*P. nigrum* L.) (Pradeep kumar *et al*., 2003 and Joy *et al*., 2007). The genetic diversity analysis of different *Piper sp* was studied using ISSR marker (Jiang and Liu, 2001) in China. The present study has been undertaken with the objective to assess the level of genetic diversity among different species of piper

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namely P. *longum* L., *P. sarmentosum* Roxb., *P. betle* L., *P. nigrum* L.,with a relative genus *Peperomia pellucida* (L.) from different parts of South Andaman using ISSR marker.

MATERIALS AND METHODS

Plant materials and DNA extraction: Five accessions from each species of *Piper longum*, *Piper sarmentosam*, *Peperomia pellucida*, *P. betle* and *P. nigrum* were collected from different areas of South Andaman (Table 1) and propagated in our polyhouse at the experimental field of Garacharma, ICAR-CIARI. Genomic DNA from leaves of ten plants per accession studied were extracted according to the CTAB method (Saghai Maroof *et al*, 1984; Doyle and Doyle, 1990) with minor modifications. Leaf tissue of 5 g was ground to fine powder in liquid nitrogen, followed by incubation in 15 ml of pre-heated extraction buffer (2% w/v CTAB; 1.4 M NaCl; 100 mMTris-HCl,pH 8; 20 mM EDTA; 2% PVP, and 0.1%v/v *b*-mercaptoethanol) for 2 h at 55°C.The homogenate was extracted once with chloroform:iso-amyl alcohol (24:1) and centrifuged at 10000 rpm at 20°C for 10 minutes. Nucleic acids were precipitated in equal volumes of ice-cold iso-propanol and kept at -20°C for overnight. Nucleic acids were precipitated by centrifugation at 10000 rpm, 15 min, at 20°C.The precipitate was washed with 70% ethanol, air-dried and dissolved in TE buffer (10 mM TrisHCl, pH 8; 1 mM EDTA). DNA was treated with bovine pancreatic RNase and extracted with phenol: chloroform (1:1) and chloroform: iso-amyl alcohol (24: 1) in succession. The purified DNA was quantified in a fluorometer and dissolved to appropriate dilution in TE buffer. After that, DNA was quantified and adjusted to 25-50 ng/µl for PCR amplification and DNA quality and purity were evaluated by electrophoresis on 0.8% agarose gel and the ratio of A260/A280.

ISSR assay: For primer selection, 38 ISSR primers were employed for amplification with the template DNA of *Piper longum*, *Piper sarmentosam*, and *P. nigrum*. Out of the 38 ISSR primers tested, 10 ISSR primers that gave satisfactory results were used for the genetic diversity analysis of the plant material. The components of the amplification reaction were optimized and a typical 20µl PCR mixture comprised 50ng genomic DNA- 2.0µl, 2X PCR Taq Mixture (HIMEDIA; Cat.No.MBT061)-10µl and 1mM primer (Clontech) - 2 µl. PCR reactions were carried out on eppendorf-Mastercycler-nexus gradient DNA thermal cycler. After a predenaturation step of 4 minute at 94°C, amplification reactions were cycled 40times at 94°C for 1 minute, 52°C for 1 minute and 72°C for 2 minutes followed by 5 minutes at 72°C. Amplification products were separated by electrophoresis on 1.4% agarose gels in 1X TAE buffer (pH 8.3) and detected by ethidium bromide staining (Yang *et al*., 1996)100 bp DNA ladder (amresco, code No. N550-300 µl) was used to determine the size of the ISSR fragments. The gel pictures were taken under UV light using gel documentation system (UVP-MultiDoc-It-Digital image system).

Data analysis: ISSR analysis were repeated twice and only clear bands produced twice were recorded for all samples. Amplified products were scored across the lane with respect to their molecular size. All the genotypes were scored for presence and absence of the ISSR bands. The 0/1 matrix was used to calculate similarity as Jaccard's coefficient using SIMQUAL subroutine in similarity routine. The resultant similarity matrix was employed to construct dendrogram by UPGMA method with NTSYS 2.02 software (Rohlf, 1998).

Table 1: Different species of Piperaceae collected from different places of South Andaman.

Species name	Accession No.	Code	Collection area
Piper longum	1.	Pl-1	Glass house, ICAR-CIARI
	2.	Pl-2	Gene garden, ICAR-CIARI
	3.	Pl-3	Polyhouse, ICAR-CIARI
	4.	Pl-4	Gene garden, ICAR-CIARI
Piper sarmentosam	1.	Ps-1	Gene garden, ICAR-CIARI
	2.	Ps-2	Jirkatang
	3.	Ps-3	Gene garden, ICAR-CIARI
	4.	Ps-4	Polyhouse, ICAR-CIARI
Peperomiapellucida	1.	Pp-1	Dairy farm
	2.	Pp-2	Lambaline
	3.	Pp-3	Garacharam farm, ICAR-CIARI
	4.	Pp-4	Garacharam farm, ICAR-CIARI
Piper betle	1.	Bl-1	Near Field crops division
	2.	Bl-2	Polyhouse, ICAR-CIARI
	3.	Bl-3	Polyhouse, ICAR-CIARI
	4.	Bl-4	Jirgatang
Piper nigrum	1.	Bp-1	Garacharam farm, ICAR-CIARI
	2.	Bp-2	Garacharam farm, ICAR-CIARI
	3.	Bp-3	Sippighatfarm
	4.	Bp-4	Jirkatang

RESULTS AND DISCUSSION

Analysis of genetic diversity: The ten selected primers amplified a total of 1042 alleles, of which seven (80.8%) were polymorphic. The number of alleles produced by each marker varied from 5 to 11, with an average of 104.2 bands per primer (Table 2). Markers UBC 862 and UBC 864 produced the largest number of clear bands 136, 144 and 135 respectively, while marker UBC 848 produced the smallest number of bands 31 only. The PCR profile of the different species of Piperacae using ISSR markers are represented in Fig 1. Four markers (UBC 881, UBC 889, UBC 848 and UBC 830) were found to be polymorphic. The ten markers which produced discrete polymorphic banding pattern were taken up for cross species transferability and genetic diversity study. Information regarding the original source, repeat motifs, primer sequences, Number of alleles and PIC of each SSR markers which were completely transferable to all the five *Piper* species were given (Table 2). The number of alleles and polymorphic nature of these ISSR markers indicated their robustness in fingerprinting of species (Joy *et al*., 2007). The resulting genotyping of all five (four accessions from each species)species were aligned. From these data, a homology tree was constructed by the Multiple Sequence Alignment Program that showed 63 to 97% similarities among the 20 accessions of five species of *Pipeaceae* (Fig. 2). The maximum homology of 100% was observed between *P.longum* accessions and in the third and fourth accessions of *Peperomia pellucida*. The 20 accessions from five species of the family piperaceae clustered into two major groups. Group I consisted of 16 accessions from four species and could be further divided into three subgroups, group IA, group IB and group IC. Group IA comprised eight accessions from two species *P.longum and P. sarmentosam* and all these

Table 2: ISSR pimers used and polymorphism given by them.

Prime Name	Sequence	% of expression	Av. No. of bands	Maximum no of alleles per primer	Total no of scorable bands	% of polymorp hism	Marker range (bp)	PIC value
UBC 856	ACACACACACAC ACACYA	94.7	11. 40	10	114	60	150-800	0.4 97
UBC 862	AGCAGCAGCAGC AGCAGC	94.7	12. 36	11	136	90	200-900	0.4 99
UBC 873	GACAGACAGACA GAC	94.7	14. 40	10	144	40	200-1000	0.4 89
UBC 864	ATGATGATGATG ATGATG	84.2	13. 50	10	135	50	250-1000	0.5 00
UBC 881	GGGTGGGGTGGG GTRG	78.9	11. 00	7	177	100	300-1000	0.4 68
UBC 889	ACACACACACAC AC	78.9	12. 60	10	126	100	200-1000	0.4 98
UBC 848	CACACACACACA CACARG	31.5	3.8 7	8	131	100	200-900	0.2 64
UBC 844	CTCTCTCTCTCTC TCTRC	89.4	12. 55	9	113	78	200-800	0.4 99
UBC 859	TGTGTGTGTGTGT GTGRC	73.6	8.0 0	11	88	90	250-900	0.4 65
UBC 830	TGTGTGTGTGTGT GTGG	84.2	15. 60	5	78	100	200-650	0.4 80

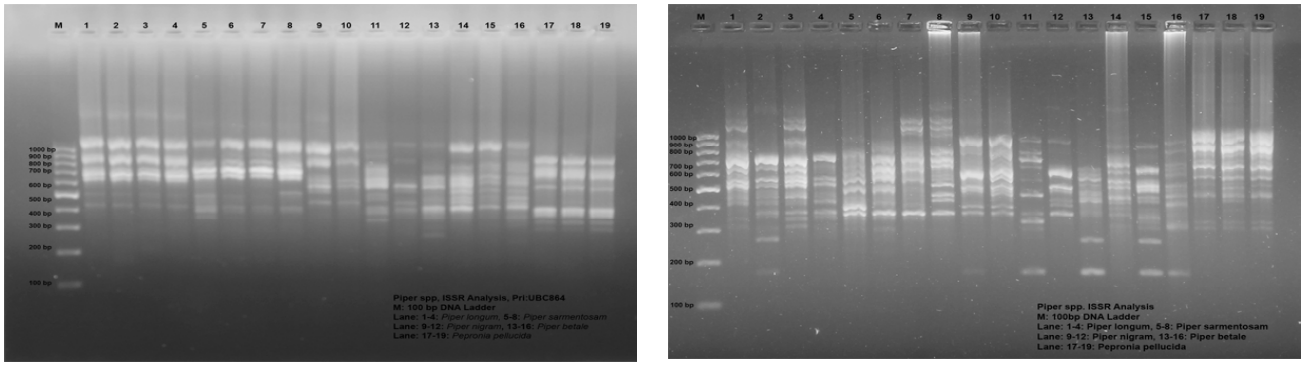
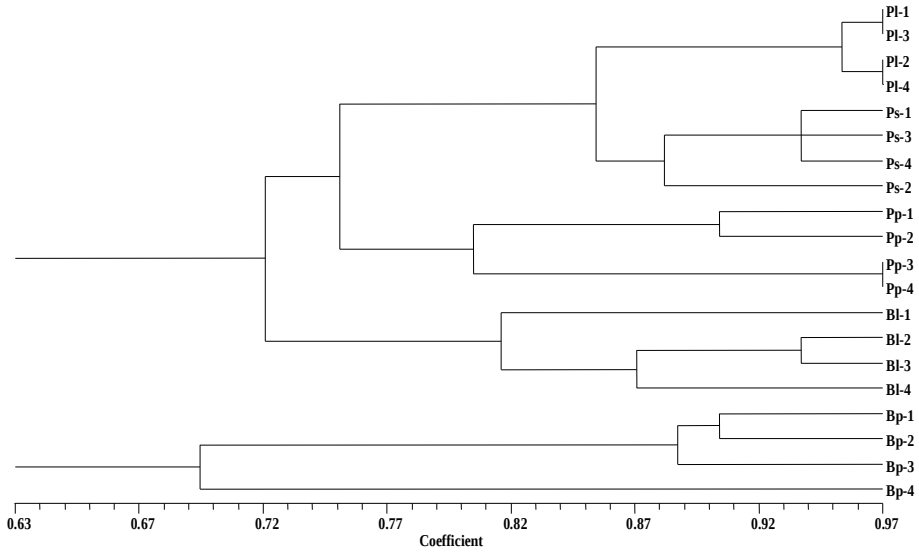


Fig 1: PCR profile of Piper sp using ISSR primers



Key: Bp-*Piper nigrum*,Pp-*Peperomiapellucida*, Ps-*Piper sarmentosum*, Pl –*Piper longum*
Fig 2: Dendrogram of the genetic relationship among *Piper sp* of South Andaman by ISSR markers

accessions shared 85% homology. Group IB consisted of four accessions from *Peperomia pellucida* and these accessions showed 79% homology. The accessions of Group IA showed 79% homology with group IC, which consisted of four accessions of *P.betle* and with group II accessions showing 63% homology. The accessions of *P. nigrum* are grouped into a separate cluster which indicates that, this particular species is different from the other species of Piperaceae. Further the genotypes within the cluster are grouped based on their geographical locations in three species studied. All the accessions of *P. longum*, *P. nigrum* accessions 1 and 2, *Peperomia pellucida* accessions 1 and 2 grouped in same cluster because of their similar geographical origin. Similar studies on use of ISSR markers to study the genetic diversity of *Piper sp* of China (Joy *et al*., 2007). There was less genetic difference between the different accessions of the same species. This may be due to the fact the vegetative propagation of *Piper sp* tend to narrow down the genetic base of the species (Joy *et al*., 2007). Selfing with occasional out crossing is the predominant mode of pollination in bisexual black pepper varieties (Sasikumar *et al*., 2004). Fruit set in some dioecious clones of black pepper is due to apomixes (Gentry, 1955). Few wild species of *Piper* are not self compatible (Prakash *et al*, 1994) which might have led to out crossing and thus the genetic variation between the different species of the family Piperaceae. There are only a very less number of species of *Piper* distributed in the Andaman and Nicobar Islands and hence the interspecific variation may also be considerably less except for the species *Piper nigrum*. The low genetic difference among the different species of Piperaceae like *Piper longum*, *Piper betle*, *Piper sarmentosum* and *Peperomia pellucida* indicates that there was more gene flow within agro-ecological zones (Kingdom *et al*., 2007). The analysis of overall diversity revealed that the inter specific diversity is more that the intra specific diversity. This low intra-specific diversity is due to high degree of gene flow in population through random mating without barrier (Ahmad *et al*., 2010)

In conclusion, the results of this study indicate that ISSR markers can be reliably used for quantification of genetic diversity and relationships, species identification and evaluation of conservation status of wild species of *Piper*.

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