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RESEARCH ARTICLE

Microsatellite marker based cross species amplification and genetic diversity analysis in the genus *Piper*

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Abstract

Microsatellite markers have been used for cross-species amplification and genetic diversity analysis in twenty three *Piper* species, including Western Ghats, North East and Exotic regions. Sixteen microsatellite primers developed from black pepper were used for genotyping out of which, six polymorphic markers could generate 48 alleles. The number of alleles per locus obtained with each marker varied from 4 to 11 alleles with an average of 8 alleles per locus. The Polymorphism information content value of each marker varied for all the tested SSR loci ranged from 0.15 to 0.32. The highest PIC value 0.32 was recorded for PNB5 and the lowest PIC value 0.15 was recorded for PND10. The phylogram generated with SSR data discriminated 23 *Piper* species into eight clusters, three of which were further divided in to sub clusters. The microsatellite markers developed in *P. nigrum* L. have been found to clearly demarcated diversity among Indian and exotic species. Hence these markers can be effectively used to study genetic diversity of the genus *Piper* across species. This is the first report on molecular characterization of major Indian and some exotic *Piper* species using microsatellite markers.

INTRODUCTION

The genus *Piper* is considered to be one of the largest genera of angiosperms (Kubitzki *et al*., 1993) and the main centers of distribution of the genus are Central and South America and South Asia (Trelease and Yuncker, 1950. The family Piperaceae was classified under the sub class Monoclamidae in the series Microembryeae (Bentham and Hooker, 1880). The genus *Piper* comprises about 3000 species of which 115 are of Indian origin (Saji, 2006). The *Piper* species are mainly herbs, shrubs, creepers and climbers. *Piper* species occurring in India are unisexual, but the Central and South American species are generally bisexual types. However the cultivated black peppers are bisexual in nature.

Both economically and medicinally important species are coming under the genus *Piper* of which black pepper (*Piper nigrum* L.) is the oldest, most widely used and the most valuable economically important spice crop and the best known agricultural product in the genus *Piper*. Long pepper (*Piper longum* L.) and betel vine (*Piper betle* L.) are the other two economically important *Piper* species grown in India.

Taxonomically *Piper* is a confusing genus; it's a diverse assemblage of dicots named paleoherbs *i.e*., plants resembles monocots in some vegetative characters (Donoghue and Doyle, 1989; Loconte and Stevenson 1991). The

genus also shows extreme reduction of floral characters which are the key factors for phylogenetic analysis. Under such circumstances molecular markers give better results for phylogenetic analysis. The studies on molecular diversity of genus *Piper* will help in better understanding of species interrelationships and help in crop improvement programmes.

Earlier studies on molecular characterization of *Piper* were mostly using RAPD (Nirmal Babu *et al.*, 2003; Sen *et al*., 2010), AFLP (Babu *et al*., 2003: Joy *et al*., 2007; Shi *et al*., 2009; Chowdhury *et al*., 2014), and ISSR markers (Jiang and Liu, 2013; Sheeja *et al*., 2013.). Recently, Microsatellite markers (SSRs) play a significant role in estimation of genetic variation in germplasm collections (Powell *et al*., 1996; Ma *et al*., 2011). Microsatellites, being their high mutation rate, multiallelic, co dominant, rich polymorphism and high genome coverage are the most frequently used molecular markers for genetic diversity studies. The transfer of potential microsatellite markers developed in one species to other (Cross species amplification/ transferability) offers an alternative to the *de novo* development of microsatellite markers in specific crops (Peakall *et al*., 1998). This is the first attempt of genotyping 23 *Piper* species using polymorphic microsatellite markers.

Materials and Methods

Plant materials

The study was carried out with 23 *Piper* species obtained from the ICAR- IISR experimental farm, Peruvannamuzhi, Kozhikode, Kerala which includes Western Ghats, North East and Exotic species (Table 1).

DNA Isolation

High quality genomic DNA was isolated from the fresh leaves of 23 *Piper* species following the protocol described by Doyle and Doyle (1990) with little modifications. Leaf tissues were powdered with liquid N_2, homogenized with 2% CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer. After 1 hours of incubation with intermittent shaking equal volume of chloroform: isoamyl alcohol mixture (24:1) was added and mixed thoroughly. DNA was precipitated by the addition of 2 v/v of ice cold isopropanol and dissolved in 0.5 ml 1 × TE buffer. The DNA was further purified. The quality and concentration of the DNA was assessed by spectrophotometer and agarose gel electrophoresis.

SSR markers and PCR amplification

Sixteen previously developed black pepper microsatellite primer pairs were used for the cross species amplification and genetic diversity study (Nine microsatellite primers developed by Menezes *et al*. (2009) and 7 microsatellite primers by Joy *et al*. (2011). Initially, all the 16 SSR primer pairs were used to standardize the PCR-amplification conditions and then the working primer pairs were checked for their utility as potential genetic markers as for cross species transferability.

Amplification of microsatellite primers were carried out in a 25 µl reaction mixture containing 1X Taq buffer (Genei, Bangalore), 1.5 mM $MgCl_2$ (Genei, Bangalore), 0.25mM dNTP's (Fermentas, Canada), 10pmols of forward and reverse primers (Sigma, USA), 20-40 ng genomic DNA and 1U *Taq* DNA polymerase (Genei, Bangalore). The reaction was performed in thermal cycler (Eppendorf, master cycler gradient S) with the following program: an initial denaturation at 94^0C (3 min) followed by a 35 cycle reaction profile programmed at 94^0C (30 s), Ta^0C (45 s, Ta- varied for individual primers), 72^0C (1 min), and a final extension of 72^0C (10 min).

Electrophoresis separation and visualization of amplified products

After PCR amplification PCR products were resolved on 3% agarose gels to check for amplification and to reveal polymorphism. To determine the size variation 10-15% denatured polyacrylamide gels were used. 5 µl of the PCR product was mixed with an equal volume of loading buffer (98% formamide, 10mM EDTA (pH 8), 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95^0 and were then snap cooled using ice. The samples were then resolved on 10-15% denatured polyacrylamide gel electrophoresis (7M Urea, IX TBE buffer) at a constant power of 200V for 6-8 h using Mini Protean System (Biorad, USA). Gels were stained with silver nitrate as described by Bassam *et al*. (2007).

Microsatellite data analysis

The size of the most intensely amplified band for each microsatellite markers were checked against its migration distance relative to molecular weight marker 100bp and Ultra Low Range DNA ladder (Fermentas,

Canada). The gel image was scanned using Image Scanner (Wipro). The reproducibility of amplification products was confirmed twice for each primer.

For assessing genetic diversity among the genotypes, only clear and prominent bands were scored. The variability at each locus was determined in terms of number of alleles per locus. For the characterization of *Piper* species only those markers showing 100% transferability were considered. The presence (1) and absence (0) of bands were recorded for all the genotypes and the data were entered into binary matrix.

This binary matrix was used to estimate the genetic similarity as Dice coeffecient using SIMQUAL subroutine in the similarity routine of NTSYS- pc version 2.02i software package (Rohlf, 1993). The resultant similarity matrix was applied to construct phylogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighed Pair-Group Method with Arithmetic Average (UPGMA) to test the goodness of fit between the UPGMA clustering and the original similarity matrix, the cophenetic correlation coefficient was computed using NTSYS- pc version 2.02i. The robustness of the phylogram was evaluated with a bootstrap analysis performed on the binary data set using 1000 permutations in Past 3 software using Neighbour joining method (Hammer *et al*., 2001). Principal Coordinate Analysis (PCA) was also performed based on the binary data matrix obtained from the obtained from the molecular data using NTSYS-pc.

The polymorphism information content (PIC) for each individual SSR allele was calculated following the formula described by Weir (1990) *i.e*. PIC= 1- $\sum P_i^2$, where P_i is the frequency of the i^{th} allele in the genotypes examined, which can be simplified to PIC= 2PiQi, where P_i is the frequency of presence and Q_i is the frequency of absence of a particular band (Tehrani *et al*., 2008).

Results and Discussion

Polymorphism and Cross species amplification (Transferability) of SSR markers

The SSR primer pairs developed in one species could be used to detect the diversity in other species or genera. The potentiality to successful transfer of microsatellite markers across species is called cross species amplification or transferability. When the evolutionary distance between species increases the transferability success rate decreases (Steinkellner *et al*., 1997; Barbará *et al*., 2007) *i e*., the transfer rate is agree with the phylogentic distance and sequence conservation among the species (Kalia *et al*., 2011).

Sixteen microsatellite markers developed in Black pepper were used to analyze the cross transferability and genetic variability among twenty three *Piper* species. All the sixteen SSR primers tested were amplified for more than four species, in which seven primers (43%) were successfully amplified in all the twenty three *Piper* species, and six markers were found to give good polymorphic scorable banding pattern. Out of the seven amplified primers five were developed by Menezes *et al*. (2009) and two primers were referred from Joy *et al*. (2011). Menezes *et al* (2009) also reported the amplification of same five primers when tested on four *Piper* species *P. attenuatum* Buch.-Ham., *P. hispidinervium* C. DC., *P. tuberculatum* Jacq. and *P. colubrinum* Link.

Four markers were found to be polymorphic but they could not amplified in majority of the species. The primer pairs which didn't amplify in all the twenty three species tested and those which couldn't produce distinct banding pattern were excluded from genotyping. The six 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 23 *Piper* species were given (Table 2).

Table 1: List of Twenty three *Piper* species used for the study

SI. No.	Name of *Piper* species	Acc. No.
1	*P. betle* L.	5473
2	*P. nigrum* L.	6426
3	*P. sugandhi* Babu et Naik.	6021
4	*P. trichostachyon* C.D.C.	639
5	*P. galeatum* C.D.C.	4577
6	*P. barberi* Gamble.	613
7	*P. attenuatum* Buch.Han.ex Wall.	4613
8	*P. argyrophyllum* Miq.	5369
9	*P. hymenophyllum* Miq.	644

10	*P. bababudani* Rahiman.	5396
11	*P. longum* L.	5565
12	*P. hapnium* Buch.-Ham.	5501
13	*P. peepuloides* Roxb.	5526
14	*P. sarmentosum* Roxb.	5466
15	*P. ribesioides* Wall.	5525
16	*P. thomsoni* Hook.	5528
17	*P. hamiltonii* C.D.C.	5536
18	*Piper sp*	3177
19	*P. chaba* Hunter.	692
20	*P. colubrinum* Link ex Kunth Link ex C.D.C	392
21	*P. arboreum* Aubl.	3363
22	*P. ornatum* N.E.Br.	3362
23	*P. magnificum* Hort.ex.Gentil.	5816

Table 2: Microsatellite marker repeat motifs, sequence information, No. of alleles, PIC of six polymorphic markers used in the present study

Locus	Repeat motif	Primer sequence	No. of alleles	PIC	Bibliographic references
PNB5	$(TG)_{14}$	F 5′ GTTTTGAATGGGTCGGTGAT 3′ R 5′ ATTGTTCTGATTTCTTCGTTATTG 3′	5	0. 32	Menezes *et al*., 2009
PNE3	$(CA)_{13}$	F 5′ TTTGTGTCCTCTCCCTCTCC 3′ R 5′ AAGACTAAATAGGCAAGGCAAA 3′	10	0. 19	Menezes *et al*., 2009
PNG11	$(AC)_5$	F 5′ TTACTAGTGTCCACCCCCACT 3′ R 5′ TCGATGGAAAGTCACCCTCT 3′	9	0. 16	Menezes *et al*., 2009
PND10	$(GT)_{13}$	F 5′ GTGTTACCTTTGGGGCATTCA 3′ R 5′ TGTGTCAGGGCATCAAACC 3′	11	0. 15	Menezes *et al*., 2009
PnAG30	$(CT)_4TT$ $(CT)_{16}$	F 5′ ACTAAGGCTAATGTGATAACCTGAGGA 3′ R 5′ ATCCCTGGATGGAAATTTGAAGGCTTGC 3′	9	0. 26	Joy *et al*., 2011
PnGT2	$(GT)_5$ AT $(GT)_4$ AT $(GT)_{17}$ GG $(GT)_{19}$	F 5′ CTAGAGAGTAACAGTTATCACTTCACAG 3′ F 5′ CTAGCAAATTTGTTCTCTAATTCACATGT 3′	4	0. 28	Joy *et al*., 2011

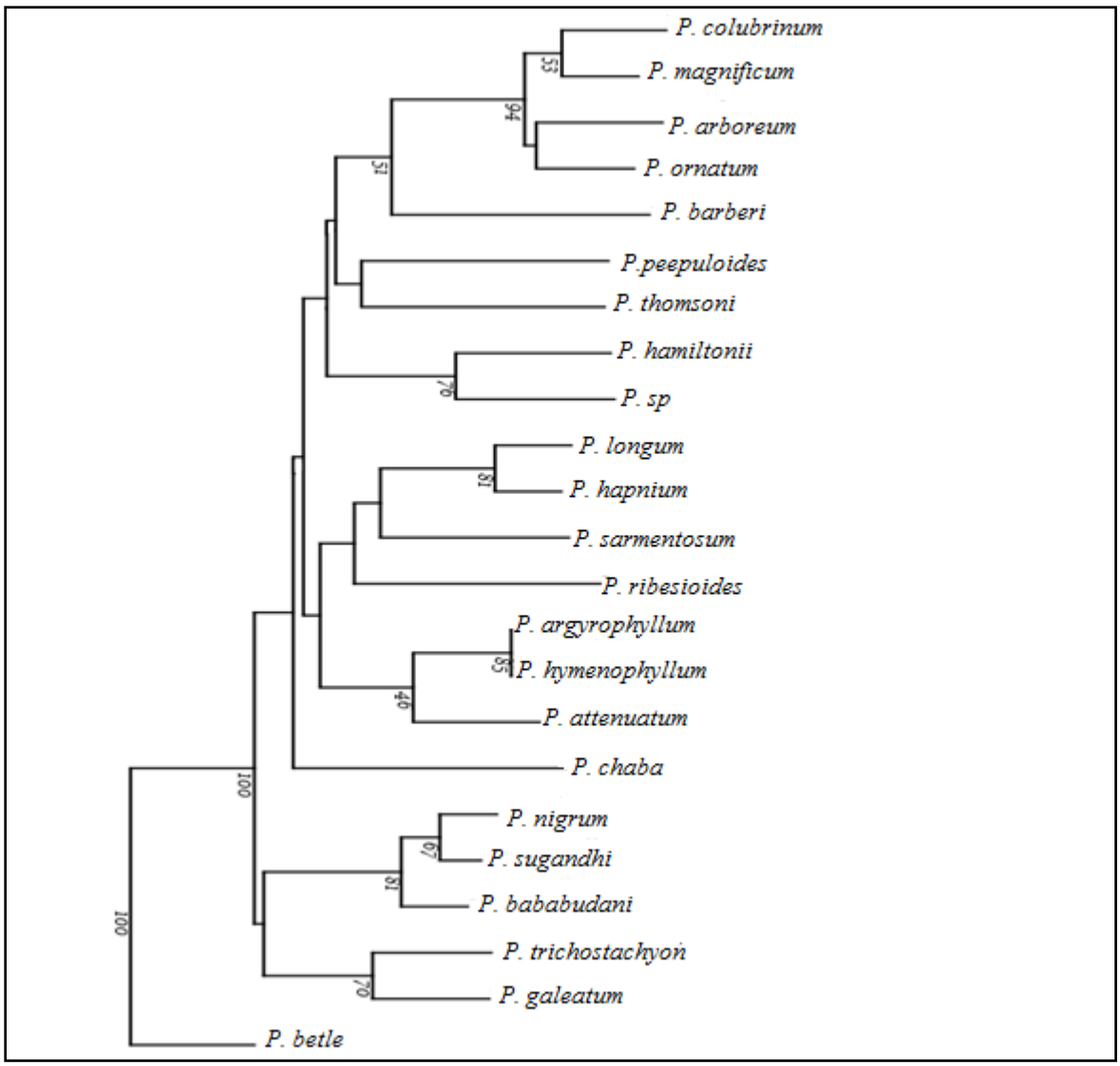

Fig1. Phylogram constructed with Neighbour joining method using 6 polymorphic microsatellite markers to study genetic relationship among 23 *Piper* species based on Dice similarity coefficient. Bootstrap values (based on 1000 permutations) are indicated in each node of the phylogram.

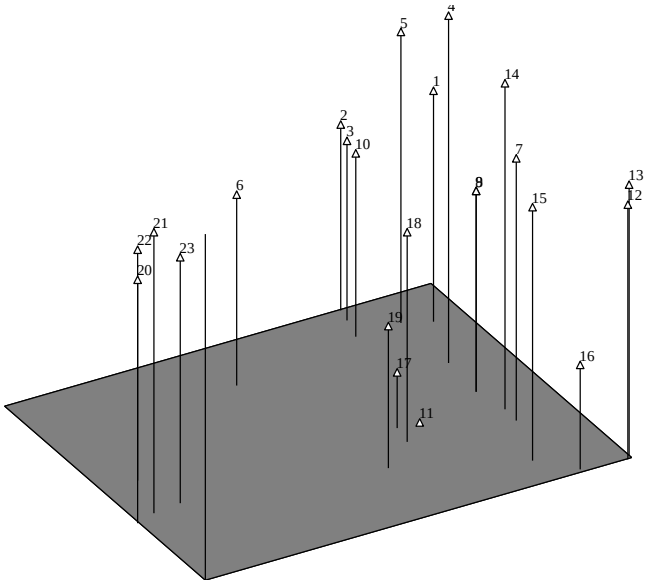

Fig 2. The 3-D plot diagram showing the relationship among 23 *Piper* species based on principal coordinate analysis (PCA) using six microsatellite markers. The number represents the genotypes: (1: *P.betle*; 2: *P.nigrum*; 3: *P.sugandhi*; 4: *P.trichostachyon*; 5: *P.galeatum*; 6: *P.barberi*; 7: *P.attenuatum*; 8: *P.argyrophyllum*; 9: *P.hymenophyllum*; 10: *P.bababudani*; 11: *P.peepuloides*: 12: *P.longum*; 13: *P.hapnium*; 14: *P.chaba*; 15: *P.sarmentosum*; 16: *P.ribesioides*; 17: *P.thomsoni*; 18: *P.hamiltonii*; 19: *Piper* sp; 20: *P.colubrinum*; 21: *P.arboreum*; 22: *P.ornatum*; 23: *P.magnificum*.)

Allelic diversity and PIC value

A total of 48 alleles were observed at the loci of 6 microsatellite markers on genotyping 23 *Piper* species. The number of alleles per locus obtained with each marker varied from 4 to 11 alleles, with an average of 8 alleles per locus. The highest number of alleles (11) was observed for the marker PND10. The PIC value of each polymorphic marker varied for all the tested SSR loci ranged from 0.15 to 0.32. The highest PIC value 0.32 was recorded for PN B5 and the lowest PIC value 0.15 was recorded for PND10.

Genetic diversity analysis of *Piper* species

The neighbor joining phylogram constructed based on the similarity index using SSR markers descriminated the 23 *Piper* species into eight clusters (Figure 1). *P.betle* stood differently in first cluster. The cluster II has sub grouped to two sub clusters. *P. nigrum*, *P. sugandhi*, and *P. bababudani* formed one group, and the other includes *P. galeatum* and *P. trichostachyon*. These data are in accordance with the findings of earlier workers (Ravindran, 2000; Babu *et al*., 2003; Saji 2006). All the species in this cluster are unisexual climbers with pendent filiform spikes. *P. sugandhi* is very close to *P. nigrum* and which is also known as *P. pseudonigrum* (Velayudhan and Amalraj 1992; Ravindran 2000). Both the species share some common characters like pendent filiform spike, cupular bract and pungent fruits. But *P. sugandhi* differs from *P. nigrum* for having stipitate flowers. Mathew and Mathew (2002) reported *P. galeatum*, *P. trichostachyon* and *P. nigrum* in one group because of their similarity in morphological and cytological characters. Based on the morphological characters Ravindran (2000) placed *P. galeatum* and *P. trichostachyon* in one cluster. These two species differ mainly by the presence of minute hairs on spikes of *P. trichostachyon* (Rahiman, 1981; Ravindran, 1991).

P. chaba was individually placed in cluster III. Cluster IV contains 2sub clusters; one comprises *P. attenuatum*, *P. argyrophyllum* and *P. hymenophyllum*. The earlier taxonomists had grouped these three *Piper* species together in one group (Gamble 1925; Mathew and Mathew 2002; Ravindran 2000; Nirmal Babu *et al*., 2003). This grouping is also supported by the molecular characterization using RAPD and ISSR markers (Saji, 2006; Sheeja *et al*. 2013). Though these three scandent unisexual climbers are similar in many morphological characters they differ

from each other by the presence or absence of pubescence in the body. The second sub cluster accommodated *P. longum*, *P. hapnium*, *P. sarmentosum and P. ribesioides*. Both morphological and molecular studies clearly exhibited morphological and genetic similarities of *P. longum*, *P. hapnium* (Saji, 2006). Both the species possess erect and cylindrical spikes (Ravindran, 1996). *P. sarmentosum* is also placed in this cluster which shows morphological similarities with *P. longum* (Mathew *et al*., 2004). In fruit characters *P. sarmentosum* shows resemblances to *P. hapnium* (Mathew *et al*., 2004). Sheeja *et al*. (2013) also reported that *P. longum* and *P. sarmentosum* shares many morphological and reproductive characters and the molecular profiling data with ISSR markers placed *P. sarmentosum* along with *P. longum* and *P. hapnium*.

Cluster V accommodated 2 species *P. hamiltonii* and *Piper* sps (Acc. no. 3177). The same grouping was also reported by Sheeja *et al*. (2013). Cluster VI comprises *P. peepuloides* and *P. thomsoni*. *P. barberi* positioned in cluster VII. *P. barberi* is a distinct South Indian *Piper* species having reticulate venatrion. All the 4 exotic species *viz*., *P. colubrinum*, *P. arborium*, *P. ornatum* and *P. magnificum* were accommodated in cluster VIII. In general the clustering was in tune with the accepted understanding of *Piper* species. The PCA generated with NTSYS-pc software is in agreement with phylogram constructed with NJ method of Past3 software (Figure 2).

The present study fortified the reliability of SSR markers for the estimation of genetic diversity and species interrelationships in the genus *Piper*. The primers which originally developed in *P. nigrum* have been found to clearly demarcated diversity among Indian and exotic species. Hence these markers can be effectively used to study genetic diversity of the genus across the species.

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