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Genetic diversity study in *Piper* spp. using inter simple sequence repeat (ISSR) markers

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Abstract

Genetic diversity analysis of 27 *Piper* species using ISSR (Inter Simple Sequence Repeat) markers indicated that the analysis placed them in six clusters in the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram. The molecular marker based clustering of the species gave supporting evidence to the earlier groupings proposed by the taxonomists using traditional tools. We have identified 35 species specific bands from different species. *P. galeatum* had a maximum number of four unique bands.

Keywords: dendrogram, molecular marker, Principal Coordinate Analysis (PCA), polymorphism, UPGMA

Introduction

The genus *Piper* includes about 3000 diverse species of herbs, shrubs and climbers, a few of which are economically important as spice or medicinal plant. There is ample diversity which makes it a potential candidate for ecological, evolutionary and geographic distribution studies (Jaramillo & Manos 2001). *Piper* species show relatively little morphological variation and high species richness (Dyer & Palmer 2004). Though a few morphological phylogenetic studies are attempted in the genus (Ravindran & Babu 1996; Mathew *et al.* 2001; Mathew & Mathew 2002; Saji 2006) comprehensive genomic studies involving *Piper nigrum* and its secondary gene pool is still at large.

Molecular markers are powerful tools in aiding genetic characterization, conservation and improvement in crops. RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Polymorphism) are the most commonly used marker strategies and ISSR is found to be more robust and reliable even in case of closely related individuals (Martins-Lopes *et al.* 2007; Christopoulos *et al.* 2010). ISSRs are highly variable, require less investment and generate high percentages of polymorphic loci (Jabbarzadeh *et al.* 2010; Li *et al.* 2010). The utility of ISSR markers for discriminating the accessions of *P. nigrum* from other *Piper* species was demonstrated by George *et al.* (2005).

Other attempts have also been made in the past to analyze genetic variation and differentiation of species or populations of *Piper* (Morell *et al.* 1995; Sen *et al.* 2010; Jiang & Liu 2011).

Molecular markers like RAPD (Nisha *et al.* 2007; Sreedevi *et al.* 2005; Chikkaswamy *et al.* 2007; Nazeem *et al.* 2005) and AFLP (Amplified Fragment Length Polymorphism) by Shi *et al.* (2009) have been employed in analysis of genetic relationships and discrimination of black pepper. However, studies involving ISSR markers is lacking in *Piper* sp.

This is the first attempt to screen a large population of 27 species involving 17 ISSR primers in a single study. Indian Institute of Spices Research (IISR) possesses the world's largest repository of *Piper* species and realising the responsibility of conservation, management and effective bioprospecting of these species, the present study was undertaken.

Materials and methods

Plant material

The work was carried out during 2010–11 at IISR, Kozhikode. DNA was extracted from fresh leaves of twenty seven species of *Piper* (Table 1) conserved at the *Piper* Germplasm Repository at the Experimental Farm, Peruvannamuzhi (N. 11° 36' 25', E 75° 49' 22) using a modified CTAB (Cetyl trimethyl ammonium bromide) method (Ausubel *et al.* 1995). Three samples were collected per accession and pooled for DNA extraction. DNA quality was checked on (0.8%) agarose gel and stored at-20°C.

PCR amplification

Polymerase chain reaction was performed in a final volume of 25 mL consisting of 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM Primer, 1U Taq polymerase and 20-30 ng DNA. PCR was carried out in a master cycler gradient with the following profile - initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C-55°C for 1 min and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The annealing temperature was calculated for each primer. A total of 28 anchored primers were screened on DNA samples from 27 morphologically distinct *Piper* species. Eighteen primers were selected based on number of polymorphic bands produced, band size, amplification intensity and reproducibility. The amplification products were separated by gel electrophoresis in 2% agarose gel with 1X TAE buffer stained by ethidium bromide (10 mg mL^{-1}) and were photographed using gel documentation system (Alpha Imager 2200).

Data analysis

The electrophoretic pattern was visually analyzed and DNA bands were scored as present (1) or absent (0). The matrix obtained was entered into NTSYS-pc program (Rohlf 1993) and Jaccard's Similarity Index (JSI) was calculated for each pair of samples. A UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was constructed based on JSI. The robustness of each phenogram was evaluated by a boot strap analysis of each data using the computer program WINBOOT (Yap & Nelson 1996). Each phenogram was reconstructed 1000 times by repeated sampling with replacement. The strength of the clustering was determined based on frequency with which a particular grouping repeated. Principal Coordinate Analysis (PCA) of the original binary data matrix was also performed using NTSYS-pc to demonstrate multiple dimension distribution of the *Piper* accessions.

Results and discussion

PCR amplification of genomic DNA of 27 *Piper* species produced clear, reproducible and polymorphic banding pattern that allowed discrimination of the species used in the study. A representative gel is shown in Fig. 1. The primers, their sequence, annealing temperature, mean number of amplified bands and percent polymorphism are given in Table 2. Table 3 gives the discriminatory primers and number of unique bands. Dendrogram constructed based on UPGMA analysis with the ISSR data is shown in Fig. 2. Jaccard's similarity coefficient ranged from 0.12 to 0.60. At 36% variation, the dendrogram separated the species into six distinct clusters. Cluster I and IV were the largest clusters and consisted of 8 species, each. Five species were accommodated in the cluster II and three in cluster III. Cluster V and VI consisted of one species each. Majority rule

Table 1. *Piper* species used in the study and their salient features

Serial No.	Coll No.	Place of collection	Species name	Long. and Lat.	Remark
1	Acc. 5526	Totopara/ New Jalpaiguri West Bengal, India	*P. peepuloides*	26° 59′ 92" N 88° 29′13" E	A climber with tender shoots pink in colour. Erect cylindricalspike from the axil of each leaves from the fruiting branches. Bract peltate orbicular. Fruits fused.
2	—	Jurong Bird Sanctuary, Singapore	*P. sarmentosum* (*)	1° 19′15" N 103° 42′ 43" E	Resembles *P. longum* but fruiting branches are erect. Cylindrical spike with peltate orbicular bract. Fruits fused.
3	Acc. 5540	Port Blair, Andaman and Nicobar Islands, India	*P. longum* (*)	11° 37′ 19" N 92° 44′18" E	A slender shy climber. Leaves adnate or shortly petiolate. Spike erect and cylindrical. Bracts peltate orbicular. Fruits fused.
4	—	Kuala Lumpur, Malaysia	*P. sarmentosum* (*)	3° 8′ 88" N 101° 41′ 08"E	Resembles *P. longum* but fruiting branches are erect. Cylindrical spike with peltate orbicular bract. Fruits fused.
5	Acc. 5301	Silent Valley/ Palaghat, Kerala, India	*P. mullesua*	11° 11′80" N 76° 26′20" E	A glabrous slender climber found medium to high elevation. Leaves on the runner shoots are ivy like. Spike globose, bracts peltate.
6	Acc. 5528	Totopara/ New Jalpaiguri, West Bengal, India	*P. thomsoni*	26° 50′92" N 88° 29′13" E	A bushy plant with erect fruiting branch. Spike globose and erect. Bracts peltate.
7	Acc. 6096	Parambikulam WLS/ Palaghat, Kerala, India	*P. longum* (*)	10° 23′20" N 76° 41′20" E	A slender shy climber. Leaves adnate or shortly petiolate. Spike erect, cylindrical. Bracts peltate orbicular. Fruits fused.
8	Acc. 3119	Palaruvi/ Kollam, Kerala, India	*P. hapnium*	9°00′00" N 71° 11′ 70" E	Resembles *P. longum* but leaves unequally cordate with incurved auricles. Spike erect, cylindrical and thick. Fruits fused.
9	—	Kuala Lumpur, Malaysia	*Piper* species (**) (Malaysia)	3° 8′88" N 101°41′ 08"E	A climber, cordate leaves with incurved auricles. Pendent spike with peltate bract. Petiole base purple coloured.
10	Acc. 3177	Shillong, Meghalaya, India	*P. sylvaticum*	25°34′71"N 91° 53′58" E	A slender climber with thin leaves. Spike fragrant, erect filiform. Bracts sessile, oblong and adnate to the rachis.
11	Acc. 3089	Shillong, Meghalaya, India	*Piper* species (**) (Northeast India)	25 ° 34′71"N 91° 53′58" E	Spike pendent. Leathery leaves.
12	Acc. 4607	ICIMOD, Kathmandu, Nepal	*P. nepalense*	27° 38′84" N 85° 19′83" E	Berries deep red while ripening, colour persisting, leaves hairy. Tender runner shoots puple in colour. Spike pendent.
13	Acc. 5336	South Andamans, A & N Islands, India	*P. hamiltonii*	11° 33′91" N 92° 41′33" E	Leaves leathery, Spike pendent, bract sessile, oblong and adnate to the rachis.

Serial No.	Coll No.	Place of collection	Species name	Long. and Lat.	Remark
14	Acc. 5525	Jaigon/ New Jalpaiguri, West Bengal, India	*P. ribesiodes*	26 ° 55'18" N 88 ° 56'69" E	Very profuse vine, large leaves, light green shoot tip, leaf base oblique.
15	Acc. 6061	Nelliampathy/ Palaghat, Kerala, India	*P. sugandhi*	10° 32'60" N 76° 41'90" E	A vigorous vine. Cordate leaves, spike purple in colour, cupular bracts. Fruis bold and slightly stipitate.
16	Acc. 6426	Siruvani/ Palaghat, Kerala, India	*P. nigrum*	10° 59'00" N 76° 35'30" E	Closest relative of cultivated black pepper but unisexual. Cordate leaves, spike pendent and filiform, bracts copular and fruits sessile.
17	Acc. 6437	Siruvani/ Palaghat, Kerala, India	*P. trichostachyon*	10 ° 57'90" N 76 ° 89'10" E	Large woody climber, ovate leathery leaves, spike filiform and minutely hairy, pendent, bracts fleshy connate, transformed in to cup, berries bold.
18	Acc. 6026	Suganthagiri/ Wayanad, Kerala, India	*P. galeatum*	11 ° 32'50" N 76 ° 00'70" E	Large woody climber, ovate leathery leaves, spike filiform, pendent, bracts fleshy connate, transformed in to cup, berries bold.
19	Acc. 6004	Pakshipathalam/ Wayanad, Kerala, India	*P. bababudani*	11 ° 56'28" N 75 ° 57'71" E	Woody climber, Cordate leaves. Spikes pendent filiform, fleshy and bracts are copular, bold berries.
20	Acc. 7111	Charmadi hills/ Chikamangalore, Karnataka, India	*Piper* species (**)	13 ° 07'14" N 75 ° 30'74" E	Spike filiform, pendent, spike white in colour and fleshy
21	Acc. 5369	Palaruvi/ Kollam, Kerala, India	*P. argyrophyllum*	8 ° 57'86" N 77 ° 8'59" E	Slender climber, leaves five nerved. Shoot tip slightly hairy. Spike thin, filiform pendent.
22	Acc. 5345	Sholayar/ Palaghat, Kerala, India	*P. hymenophyllum*	10 ° 33'10" N 76 ° 58'20" E	Slender climber, resembles *P. argyrophyllum* but leaves are pubescent.
23	Acc. 4634	Kuttikanam/ Kottayam, Kerala, India	*P. attenuatum*	9 ° 35'06" N 76 ° 58'20"E	A glabrous climber, leaves thin, seven nerved. Very long spikes with conical or round berries. Bract sessile, oblong and adnate to the rachis.
24	Acc. 613	Brymore/ Nagarcoil, Tamil Nadu, India	*P. barberi*	8 ° 38'1" N 77 ° 15'60"E	An endangered species, leaves with reticulate venation. Spikes cylindrical with long pedicel. Bracts peltate, orbicular.
25	Acc. 565	Kumta/ Sirsi Karnataka, India	*P. betle*	14 ° 25'59" N 74 ° 25'06" E	Cordate leaves, Spike pendent and cylindrical. Bract peltate, orbicular.
26	Acc. 3362	Secondary source	*P. ornatum*		Ornamental climber with variegated cordate leaves. Spike pendent.
27	Acc. 5816	Secondary source	*P. magnificum*		Ornamental shrub. Leaves purple coloured and eucamptodromous venation. Spike cylindrical, bracts peltate, orbicular.

*Indicates the different accessions of the same species; **Species not identified

Fig. 1. Representative figure showing amplification of 27 *Piper* species using ISSR-28 primer. Lane (1) *P. peepuloides*; (2) *P. sarmentosum* (*); (3) *P. longum* (*); (4) *P. sarmentosum* (*); (5) *P. mullesua*; (6) *P. thomsoni*; (7) *P. longum* (*); (8) *P. hapnium*; (9) *Piper* species (**); (10) *P. sylvaticum*; (11) *Piper* species (**); (12) *P. nepalense*; (13) *P. hamiltonii*; (14) *P. ribesiodes*; (15) *P. sugandhi*; (16) *P. nigrum*; (17) *P. trichostachyon*; (18) *P. galeatum*; (19) *P. bababudani*; (20) *Piper* species (**); (21) *P. argyrophyllum*; (22) *P. hymenophyllum*; (23) *P. attenatum*; (24) *P. barberi*; (25) *P. betle*; (26) *P. ornatum*; (27) *P. magnificum*; (M) 1 Kb DNA ladder.
*The different accessions of the same species
**Species not identified

Fig. 2. UPGMA dendrogram representing genetic relationship among 27 *Piper* species (Number at branches represents bootstrap values generated by 1000 replicates using Winboot program)

consensus tree constructed using the bootstrap of the binary data showed a high degree of correspondence. PCA by and large agreed with the UPGMA clustering (Fig. 3).

The genus *Piper*, the largest of the *Piperaceae* family, with several forms of growth, from herbs, small trees, and vines, forming about 3,000 species, is pantropical in distribution. About 113 of them are found in India (Saji 2006) and a good number in Brazil (about 400) (Jaramillo & Manos 2001; Quijano-Abril *et al.* 2006). Though there is morphological variation

Table 2. Primer sequence, mean number of amplified products, their size range and % polymorphism generated by different ISSR primers in *Piper* species.

ISSR Primers	Primer sequence 5' to 3'	Annealing temperature (°C)	Mean number of amplified bands	% polymorphism
ISSR-2	AGAGAGAGAGAGAGAGG	54°C	4.6	100
ISSR-3	CACACACACACACAG	54°C	4.6	100
ISSR-6	GACAGACAGACAGACA	54°C	4.1	100
ISSR-7	AGAGAGAGAGAGAGAGT	52°C	6.2	100
ISSR-8	CACACACACACACACAA	54°C	4.6	100
ISSR-9	ACACACACACACACACC	52°C	5.7	100
ISSR-11	GAGAGAGAGAGAGAGAC	54°C	4.1	100
ISSR-14	AGAGAGAGAGAGAGAGC	55.4°C	7.7	100
ISSR-15	GAGAGAGAGAGAGAGAG	54°C	3.9	100
ISSR-16	GAGAGAGAGAGAGAGAT	52°C	5.5	100
ISSR-19	ACACACACACACACACG	54°C	6.2	100
ISSR-22	GACAGACAGACAGC	50°C	6.0	100
ISSR-23	ACACACACACACACT	50°C	7.3	100
ISSR-24	ACACACACACACACC	50°C	4.9	100
ISSR-25	ACACACACACACACG	50°C	6.8	100
ISSR-26	CTCCTCCTCGC	50°C	4.4	100
ISSR-27	GACAGACAGACA	50°C	6.5	100
ISSR-28	AGTGAGTGAGTGGG	50°C	6.0	100

Table 3. Discriminatory primers and unique bands specific to different species

*Species	Unique bands (no.)	Discriminatory primer $_{\text{unique band(bp)}}$
P. peepuloides Roxb.	2	ISSR-25$_{1100}$, ISSR-26$_{375}$
P. sarmentosum Roxb.(*)	1	ISSR-24$_{250}$
P. longum L. (*)	1	ISSR-25$_{1750}$
P. sarmentosum Roxb (*)	1	ISSR-23$_{3000}$
P. mullesua Buch. -Ham.	1	ISSR-8$_{1250}$
P. thomsoni Hook. F.	3	ISSR-8$_{1800}$, ISSR-25$_{1350}$, ISSR-27$_{900}$
P. hapnium Buch. -Ham.	1	ISSR-9$_{250}$
Piper sp. (**)	3	ISSR-16$_{250}$, ISSR-19$_{2000}$, ISSR-28$_{275}$
P. sylvaticum Roxb.	2	ISSR-27$_{1750}$, ISSR-27$_{1500}$
Piper sp. (**)	1	ISSR-9$_{1450}$
P. nepalense Miq.	1	ISSR-28$_{1450}$
P. ribesiodes Wall.	2	ISSR-14$_{1750}$, ISSR-28$_{1200}$
P. sugandhi Babu et Naik	2	ISSR-6$_{900,550}$
P. trichostachyon C. DC.	3	ISSR-8$_{2250,300}$, IISR-9$_{1650}$
P. galeatum C. DC	4	ISSR-8$_{280}$, ISSR-23$_{250}$, ISSR-24$_{1300}$, ISSR-25$_{325}$
P. bababudani Rahiman.	1	ISSR-22$_{1500}$
P. argyrophyllum Miq.	1	ISSR-7$_{500}$
P. betle L.	3	ISSR-19$_{875}$, ISSR-27$_{2000}$, ISSR-28$_{1500}$
P. ornatum N.E.Br.	2	ISSR-22$_{250}$, ISSR-25$_{1500}$

One accession of each *P. longum* and *Piper* sp.; *P. hamiltonii, P. nigrum* L, *P. hymenophyllum. P. attenatum, P. barberi* and *P. magnificum* could not be discriminated by unique bands
*The different accessions of the same species
**Species not identified

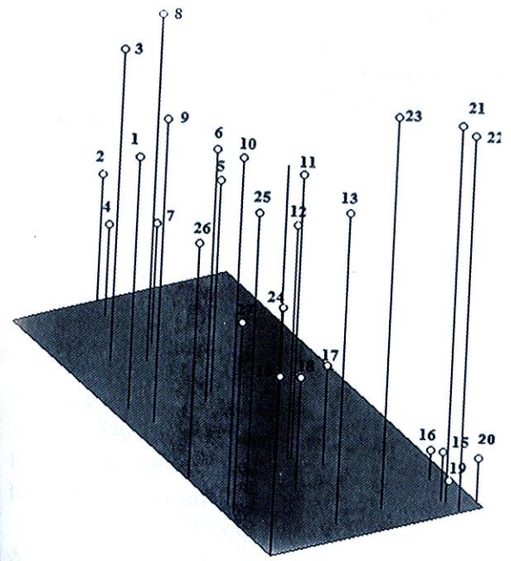

Fig. 3. Three D plot of 27 *Piper* species by Principal Coordinate Analysis. (1) *P. peepuloides;* (2) *P. sarmentosum* (*); (3) *P. longum* (*); (4) *P. sarmentosum* (*); (5) *P. mullesua;* (6) *P. thomsoni;* (7) *P. longum* (*); (8) *P. hapnium;* (9) *Piper* species (**); (10) *P. sylvaticum;* (11) *Piper* species (**); (12) *P. nepalense;* (13) *P. hamiltonii;* (14) *P. ribesiodes;* (15) *P. sugandhi;* (16) *P. nigrum;* (17) *P. trichostachyon;* (18) *P. galeatum;* (19) *P. bababudani;* (20) *Piper* species (**); (21) *P. argyrophyllum;* (22) *P. hymenophyllum;* (23) *P. attenatum;* (24) *P. barberi;* (25) *P. betle;* (26) *P. ornatum;* (27) *P. magnificum.*

*The different accessions of the same species
**Species not identified

among the gene pool of *Piper*, informative and neutral molecular markers, with a high degree of polymorphism, are important for evaluation of the variability existing in germplasm collections as they supplement the morphological variability, providing a better focus for conservation efforts, and generating guidelines for the development of cultivar improvement programs (Souza *et al.* 2004).

The UPGMA phenogram constructed based on the similarity index of the ISSR markers placed the 27 *Piper* species into six clusters. The clustering of the species failed to show any pattern of variation that can be related to geographic location of the species. The eight species in the cluster I, originated from diverse regions such as West Bengal in the Eastern part of India and Kerala, Tamil Nadu and Karnataka (South India). All the species in this cluster were robust climbers except *P. barberi* which is a shy climber. However, all the eight species exhibits sexual dimorphism and have long pendant, filiform spikes. One may discern two subclusters in this cluster, with subcluster I comprising of *P. ribesoides, P. sugandhi, P. nigrum* and *P. bababudani*. In fact, *P. sugandhi* is also known as *P. pseudonigrum*, due to its close proximity with *P. nigrum* (Velayudhan & Amalraj 1992; Ravindran 2000) placed the two in one cluster based on the morphotaxonomic traits.

The second subcluster includes three species *viz.*, *P. galeatum, P. trichostachyon* and *P. barberi*. By morphological and biosystematic studies, Ravindran (1990) had suggested *P. galeatum* as one of the putative parents of *P. nigrum*. Based on morphology, palynology and cytology, Mathew & Mathew (2002) included *P. galeatum, P. trichostachyon* and *P. nigrum* to one cluster. Similarly, Gamble (1925) and Ravindran (2000) also grouped *P. galeatum* and *P. trichostachyon* together, based on morphological traits. Cluster II was constituted by 5 species including 3 species collected from Kerala (South India), one from Meghalaya (East India) and the remaining one from Andaman & Nicobar Islands in the Bay of Bengal. Here too, two subclusters were evident. One subcluster accommodated three species *viz.*, *P. argyrophyllum, P. hymenophyllum* and *P. attenuatum*. All 3 slender climbers share many leaf, spike and berry characters (Table 1). Conventional taxonomic studies by earlier workers also placed these 3 species into one cluster (Gamble 1925; Mathew & Mathew 2002; Ravindran 2000).The second subcluster in this cluster consisted of 2 species, *P. hamiltoni* and *P. sylvaticum* with only 24% similarity between the 2, as they differ in spike orientation, fragrance, leaf texture etc. Though three distinct species *viz.*, *P. betle, P. nepalense* and another yet to be identified species formed this cluster the degree of similarity between/among these entities was <24%, attesting their morphological divergence.

Cluster IV comprised of 9 species. Here too, 2

subclusters were discernible. Subcluster I accommodated 4 species *viz.*, *P. mullseua* and *P. thomsonii*, both erect, globose spike producing species as well as *P. hapnium* and *P. longum* (Ravindran 1996) both having erect, cylindrical spikes. All the 4 species are medicinally valued. Earlier workers also placed *P. hapnium* and *P. longum* in one cluster based on conventional taxonomic studies (Mathew *et al.* 2001; Ravindran 1990; Gamble 1925). The second subcluster in this cluster includes 2 accessions of *P. sarmentosum*, *P. longum* (Ravindran & Babu 1996) and a *Piper* species from Malaysia and *P. nepalenses*. The two *P. sarmentosum* entities had maximum similarity of 60% between them. They shared about 36% similarity with *P. longum* (Ravindran & Babu 1996). *P. longum* and *P. sarmentosum* have many common vegetative and reproductive characters (Table 1).The other entities in this subcluster had less than 15% similarity with these species. Cluster V and VI had one species each, *viz.*, *P. magnificum* and *P. ornatum* respectively. Both are exotic ornamental species with entirely distinct morphological features.

The PCA results corresponded well with the UPGMA clustering with minor deviations. The high boot strap values obtained at all major nodes in the phenogram indicated the stability of different clusters.

Thus, the present clustering of the species gave credulous support to the earlier groupings done by the taxonomists. Though the bands may not exactly correspond to a particular morphological feature of the species, it is supportive of the distinct identity of the species.

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