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Estimation of Genetic Diversity in *Piper betle* L. based on the analysis of Morphological and Molecular Markers

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Abstract: Paan (*Piper betle* L., family Piperaceae) is an important evergreen cultivated crop of India. We estimated genetic diversity by using morphological and RAPD markers in four cultivars, namely *Piper betle* L. var. Bali, var. Chandrakanta, var. Jhanji and var. Kala Bangla. We had used morphological markers like leaf length, leaf width, and petiole size. By using the morphological markers, hierarchical cluster analysis was carried out, which grouped these four cultivars into two major clusters. In molecular marker analysis, a total of ten RAPD primers used, generating 43 number of amplified bands. Among them, 15 number of polymorphic bands and seven unique bands were found. The genetic diversity and relatedness among the four cultivars were computed using Jaccard's similarity coefficient. The dendrogram grouped all the four cultivars into two main clusters. This RAPD banding patterns can be useful for genetic diversity studies, for cultivar selection, and to marker assist breeding programs.

Keywords: betel vine; genetic diversity; *Piper*; RAPD; similarity index; dendrogram.

1. Introduction

Piper betle L., commonly known as "Paan" is an important crop that belongs to the family Piperaceae. It is cultivated as a perennial evergreen climber, which is distributed in various countries, including India, the Indo-China region, Indonesia, Malaysia, Thailand, Myanmar, Singapore, etc. In India, it is widely cultivated in the state of Uttar Pradesh, Bihar, Madhya Pradesh, Maharashtra, Karnataka, West Bengal, Odisha, Andhra Pradesh, Tamil Nadu, Kerala, and Andamans [1-3]. However, 100 cultivars (landraces) of betel varieties were cultivated in India [4] and are named after the locality or area where they are grown. Four common varieties were cultivated in Odisha. Variety *Bali* and *Chandrakana* cultivated in vineyards of Bahalia revenue area of Balasore district, Odisha; variety *Kala Bangla* cultivated in Torihan Bandha of Puri district, Odisha, and variety *Jhanji* cultivated in Panchama revenue area of Ganjam district, Odisha [5-6]. The landraces with prefix *Bali* due to cultivation in sandy soil, *Chandrakana* due to landrace of the Chandaneswar area, *Kala Bangla* due to landrace West Bengal, and *Jhanji* due to the special flavor of the leaf. The leaves of the betel vine possess antimicrobial, carminative, stimulant, and antiseptic activities [7-9]. Different investigations revealed that leaf improves the immune system and use for oral hygiene [10-11]. This is a demanded crop in the pharma industries—the strong aromatic flavor in betel leaves due to phenolic and terpene compounds [12]. Recent GCMS and LCMS investigations show

leaves contain essential oil composing eugenol, eugenyl acetate, and terpinenol-4 [13-16]. Phytochemical constituents on leaves revealed the presence of amino acids, carbohydrates, steroids, and alkaloids [17]. The estimation of genetic diversity and phylogenetic relationships among the cultivars would endorse the efficient use of genetic diversity in the breeding program [18]. As it is a cash crop of coastal Odisha, many people depend on it for their livelihood. PCR based molecular markers like RAPD (Random amplified Polymorphic DNA), ISSR (Inter simple sequence repeat), AFLP (Amplified fragment length polymorphism), and SSR (Simple sequence repeat) have been demonstrated to be effective indicators for the estimation of genetic diversity related to phenotypic trait [19-21]. RAPD is a widely used technique in the detection of genetic diversity since they have various advantages like technically simple, cost-effective, rapid, and don't involves any radioisotopes, produce an indefinite number of amplicons, ubiquitously spread throughout the genome, and capable of high-level polymorphism [22]. The present study was undertaken to study the polymorphism among the four genotypes of *P. betle* L. using RAPD markers.

2. Materials and Methods

2.1. Source of plant materials.

The study was conducted during the period of 2017-2018. Young leaf tissue was harvested from the field-grown vine, washed properly to remove dust, mopped dry, and quick frozen. Then the frozen leaves were powdered using liquid nitrogen. The powders were either used for isolation of DNA immediately or were stored in a deep freezer (-80ºC) for further uses.

2.2. Methods of DNA profiling using RAPD markers.

2.2.1. Extraction and purification of genomic DNA.

For genomic DNA isolation, about 2 g of leaves powder was taken, and DNA was isolated following the CTAB method [23] with little modifications by adding activated charcoal (0.2% v/v) and PVP (3.5% v/v) to the extraction buffer. The mixture so obtained was suspended in 50 ml Oakridge tube containing 10 ml of extraction buffer. The content of the tube was gently mixed by inversion and incubated in a shaking water bath at 65°C for 1 hour. One-fourth volume (approximately 5 ml) of 5M potassium acetate (pH 7.6) was added to it, followed by uniform mixing, and kept in the ice bucket. After cooling to room temperature, an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to it and mixed gently for 5 minutes by inversion. The suspension was centrifuged at 6000 rpm at 4°C for 20 min using a refrigerated centrifuge (5804R, Eppendorf, Germany), and the supernatant was transferred to another Oakridge tube. To the supernatant equal volume of prechilled isopropanol was added, mixed uniformly, and kept at -20°C for 2 hours. The DNA spool was hooked out by sterile Pasteur capillary pipette and was washed with 70% ethanol at 10000 rpm for 5 min at 4°C. The DNA pellet was dried overnight at room temperature and was dissolved in 2.0 ml Tris-EDTA (TE) buffer (10 mM Tris; 1 mM EDTA, pH 8.0) and stored at 4°C. The solution was treated with 7 µl of RNAse-A (10mg/ml) and incubated at 37°C for 1 hour. Again the solution was treated with 10 µl of proteinase-K (20 mg/ml) and incubated at 37°C for 30 min and 65°C for 10 min, respectively. The solution was added with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), mixed gently, and centrifuged at 5000 rpm for 10 min. The supernatant was added with an equal volume of chloroform: isoamyl alcohol (24:1) mixed

gently and centrifuged at 5000 rpm for 10 min. This step was repeated thrice to remove the traces of phenol. To the supernatant, 1/10th volume of 3M ammonium acetate (pH 5.8) and 2.5 volume of chilled ethanol was added and mixed well. The DNA was hooked out, washed with 70% ethanol, air-dried, and dissolved in 200 µl of $T_{10}E_1$ buffer as explained above, and kept at -20°C [23-24].

2.2.2. Quantification of DNA.

DNA concentration and purity were measured by using UV–Vis spectrophotometer (UV 1601, Shimadzu, Japan) with TE buffer (pH 8.0) as blank. For further confirmation, the quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gel along with diluted uncut lambda DNA as standard. DNA is diluted to a concentration of 25ng/µl using TE buffer.

2.2.3. PCR Amplification using RAPD primers.

For RAPD analysis, PCR amplification of 30 ng of genomic DNA was carried out using 10 standard decamer oligonucleotide primers (Operon Tech., Alameda, CA, USA). The Primers with their sequence information are as follows:

PRIMER	SEQUENCE
OPA-01	5'-CAGGCCCTTC-3'
OPA-02	5'-TGCCGAGCTG-3'
OPA-03	5'-AGTCAGCCAC-3'
OPA-04	5'-AATCGGGCTG-3'
OPA-05	5'-AGGGGTCTTG-3'
OPB-01	5'-GTTTCGCTCC-3'
OPB-02	5'-TGATCCCTGG-3'
OPB-03	5'-CATCCCCCTG-3'
OPB-04	5'-GGACTGGAGT-3'
OPB-05	5'-TGCGCCCTTC-3'

Each amplification reaction mix of 25µl contained the 30ng template DNA, 2.5µl of 10X assay buffer (100mM Tris.Cl, pH 8.3; 0.5 M KCl; 0.1% gelatin), 1.5 mM $MgCl_2$, 200µM each of the dNTPs, 20ng primers, 1.0 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (TC- 312, Techne, UK) as follows:

I. Initial Denaturation- 94°C--------5 min

II. *Denaturation- 94°C for 2 min
*Annealing- 37°C for 1 min
*Elongation- 72°C for 2 min
45 Cycles

III. Final Elongation- 72°C-------5 min

IV. Final Hold- 10°C------- infinite

2.2.4. Electrophoretic analysis of amplified products.

The PCR products were separated on 1.4% agarose gel containing Ethidium bromide solution (@ 0.5µg/ml of gel solutions) in the submarine electrophoresis apparatus (Biotech,

Yercaud) using TAE (40mM Tris-acetate; 2mM EDTA) buffer at constant 50 V for about 4 hours. A gel loading buffer (20% Sucrose; 0.1 M EDTA, 1.0% SDS; 0.25% Bromophenol blue; 0.25% Xylene cyanol) was used as tracking dye. Amplified products were visualized by the Gel documentation system (Geldoc-XR, Bio-Rad, USA) and photographed. The size of the amplified products was determined using the 250bp ladder (Bangalore Genei Pvt. Ltd., Bangalore, India) as standard and TOTAL LAB SOLUTIONS (TL120) software.

2.3. Data scoring and analysis.

Each amplified fragment was considered as a unit character and, the unequivocally scorable and consistently reproducible amplified fragments were organized into 0-1 matrix and similarity coefficient of Jaccard [25-26] and genetic distance [27] were estimated from SSR marker data. The similarity matrix and genetic distance were used for the cluster analysis and construction of the dendrogram using UPGMA method using the NTSYS-pc version 2.02 [28]. Bootstrap analysis was carried out with Free Tree software [25-27] to evaluate the robustness of the nodes. Bootstrapping of the resulting dendrogram was calculated with 1000 replications [29-30].

3. Results and Discussion

3.1. Morphological variation among the four cultivars of Piper betle L.

The morphological variation among four cultivars of *Piper betle* L. is summarized in Table 1. Most of the characters showed different degrees of variation within each cultivar, in addition to a higher extent. The variation for leaf shape, leaf size, and flower color was higher between the cultivars tested here. The leaf indices measured, including leaf length, width, and petiole size. The leaf area showed a higher degree of variation between cultivars (Figure 1, Table 1). Hierarchical cluster analysis grouped these four cultivars into two major clusters (Figure 2), three cultivars (*P. betle* L. var. Bali, *P. betle* L. var. Chandrakana, and *P. betle* L. var. Kala Bangla) of *Piper* were represented in a cluster, and *Piper betle* L. var. Jhanji evidencing high genetic divergence among others tested here.

Table 1. Morphological variation among four cultivars of *Piper betle* L. by using SPSS 16.0.0v.

Sl no	Cultivars name	Place of collection	Leaf length (in cm)	Leaf width (in cm)	Petiole size (in cm)
1	*Piper betle* L. var. Bali	Balasore	15.43 ± 0.20^{b}	11.36 ± 0.15^{b}	7.0 ± 0.20^{b}
2	*Piper betle* L. var. Chandrakana	Balasore	16.36 ± 0.15^{c}	13.36 ± 0.25^{c}	9.76 ± 0.15^{c}
3	*Piper betle* L. var. Jhanji	Ganjam	10.73 ± 0.15^{a}	8.40 ± 0.20^{a}	5.40 ± 0.20^{a}
4	*Piper betle* L. var. Kala Bangla	Puri	17.40 ± 0.20^{d}	14.93 ± 0.15^{d}	13.53 ± 0.25^{d}

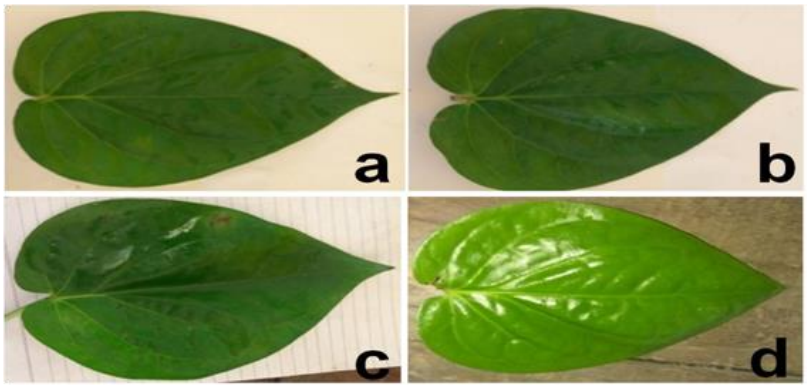

Figure 1. Morphological variations on leaves among four cultivars of *Piper betle.*

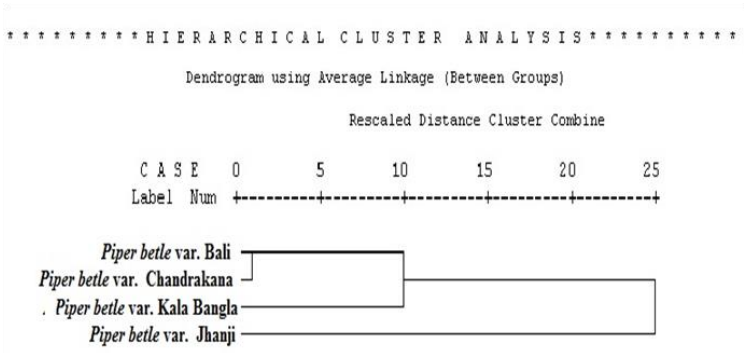

Figure 2. Hierarchical cluster analysis is based on morphometric parameters.

3.2. Genetic diversity analysis among four cultivars of Piper betle using RAPD marker analysis.

3.2.1. Optimization of amplification conditions.

Assays to optimize the template concentration were conducted over the range of 10 to 200ng. A constant banding pattern was obtained at a template concentration between 10 to 40ng. Ultimately, a template DNA concentration of 25ng/ 25µl was selected for PCR amplification. Furthermore, a low magnesium concentration (2.0mM) in the PCR reaction was found to be optimal for the purpose of producing clear and reproducible DNA fingerprints. To determine the degree of heterogeneity within the population, we extracted DNA from different individuals of the same accessions and bulked, and the bulked DNA was used for PCR amplification. The RAPD pattern was found to be identical for all cases.

3.2.2. Generation of RAPD markers.

For this purpose, the DNA isolated from four cultivars of *P. betle* was considered. A total of 10 decamer primers (Table 2) were used for RAPD analysis. Amplification of all 10 primers generated 43 unequivocal scorable bands; out of the 15 are polymorphic at the cultivar level during the present study. The size of the amplification products ranges from 280 bp to 2250 bp. A maximum of eight loci was amplified with primer OPB 04, whereas a minimum of one amplicon was observed with the primer OPB 01. Five species-specific amplicons were observed. The marker was designated as the operon primer number, followed by molecular weight in base pairs of the amplified band. Primer wise amplification and genetic identification of *P. betle* cultivars were enumerated as below:

Table 2. RAPD Genotyping data as revealed by the amplification of Ten decamer primers of OPA and OPB series.

Primer	Sequence (5'-3')	GC content (%)	Number of bands amplified	Number of polymorphic bands	%age of polymorphism	Range of amplified products (in bp)	Species-specific bands (if any)	Remarks
OPA-01	CAGGCCCTTC	70	4	1	25%	670-1760	-	-
OPA-02	TGCCGAGCTG	70	4	1	25%	520-1160	$OPA02_{780}$	var. Kala Bangla
OPA-03	AGTCAGCCAC	60	2	1	50%	310-640	-	-
OPA-04	AATCGGGCTG	60	5	4	80%	268-1363	$OPA05_{730, 680}$	var. Chandrakanta
OPA-05	AGGGGTCTTG	60	4	1	25%	444-1500	-	-
OPB-01	GTTTCGCTCC	60	1	0	0%	780	-	-
OPB-02	TGATCCCTGG	60	6	1	16.66%	500-1250	$OPB02_{500}$	var.Bali
OPB-03	CATCCCCCTG	70	5	2	40%	380-1250	$OPB03_{1250, 380}$	var. Kala Bangla
OPB-04	GGACTGGAGT	60	8	1	12.5%	280-2250	$OPB04_{580}$	var. Chandrakanta
OPB-05	TGCGCCCTTC	70	4	3	75%	260-810	-	-
Total			43	15	34.88%	260-1760		

3.2.3. Amplification with Primer OPA 01.

This primer has amplified four scorable amplicons in the range from 670 to 1760bp (Figure 3), and three are monomorphic, and one is polymorphic in nature. All the collected genotypes were responded to Primer OPA 01 for amplification.

3.2.4. Amplification with Primer OPA 02.

This primer has amplified 4 scorable amplicons in the range from 520 to 1160 bp (Figure 3), and 3 of those were polymorphic in nature. OPA02 primer generated unique amplicons for *P. betle var. Kala Bangla* ($OPA02_{780}$) cultivars (Table 3).

3.2.5. Amplification with Primer OPA 03.

This primer has amplified two scorable amplicons in the range from 310 to 640 bp (Figure 3), and one was polymorphic, and one was monomorphic in nature.

3.2.6. Amplification with Primer OPA 04.

This primer has amplified five scorable amplicons in the range from 460 to 1180 bp (Figure 3), out of which four polymorphic, one is monomorphic in nature.

3.2.7. Amplification with Primer OPA 05.

This primer has amplified four scorable amplicons in the range from 540 to 1480 bp (Figure 3), and one of those was polymorphic in nature.

3.2.8. Amplification with Primer OPB 01.

This primer has amplified one scorable amplicon of 780 bp (Figure 3).

3.2.9. Amplification with Primer OPB 02.

This primer has amplified five scorable amplicons in the range from 500 to 1250 bp (Figure 3), and one of those was polymorphic in nature. $OPB02_{500}$ unique amplicon for *P. betle var.* Bali (Table 2).

3.2.10. Amplification with Primer OPB 03.

This primer has amplified five scorable amplicons in the range from 380 to 1250 bp (Figure 3), and two of those were polymorphic in nature. OPB 03 primer generated two unique amplicons ($OPB\ 03_{1250,\ 380}$) for *Piper betle* L. var. Kala Bangla (Table 2).

3.2.11. Amplification with Primer OPB 04.

This primer has amplified eight scorable amplicons in the range from 280 to 2250 bp (Figure 3), and one of those was polymorphic in nature. OPB 04 primer generated one unique amplicon ($OPB\ 04_{580}$) for *Piper betle* L. var. Chandrakanta (Table 2).

3.2.12. Amplification with Primer OPB 05.

This primer has amplified four scorable amplicons in the range from 260 to 810 bp (Figure 3), and three of those were polymorphic in nature. OPB 05 primer generated one unique amplicon (OPB 05_{420}) for *Piper betle* L. var. Kala Bangla (Table 2).

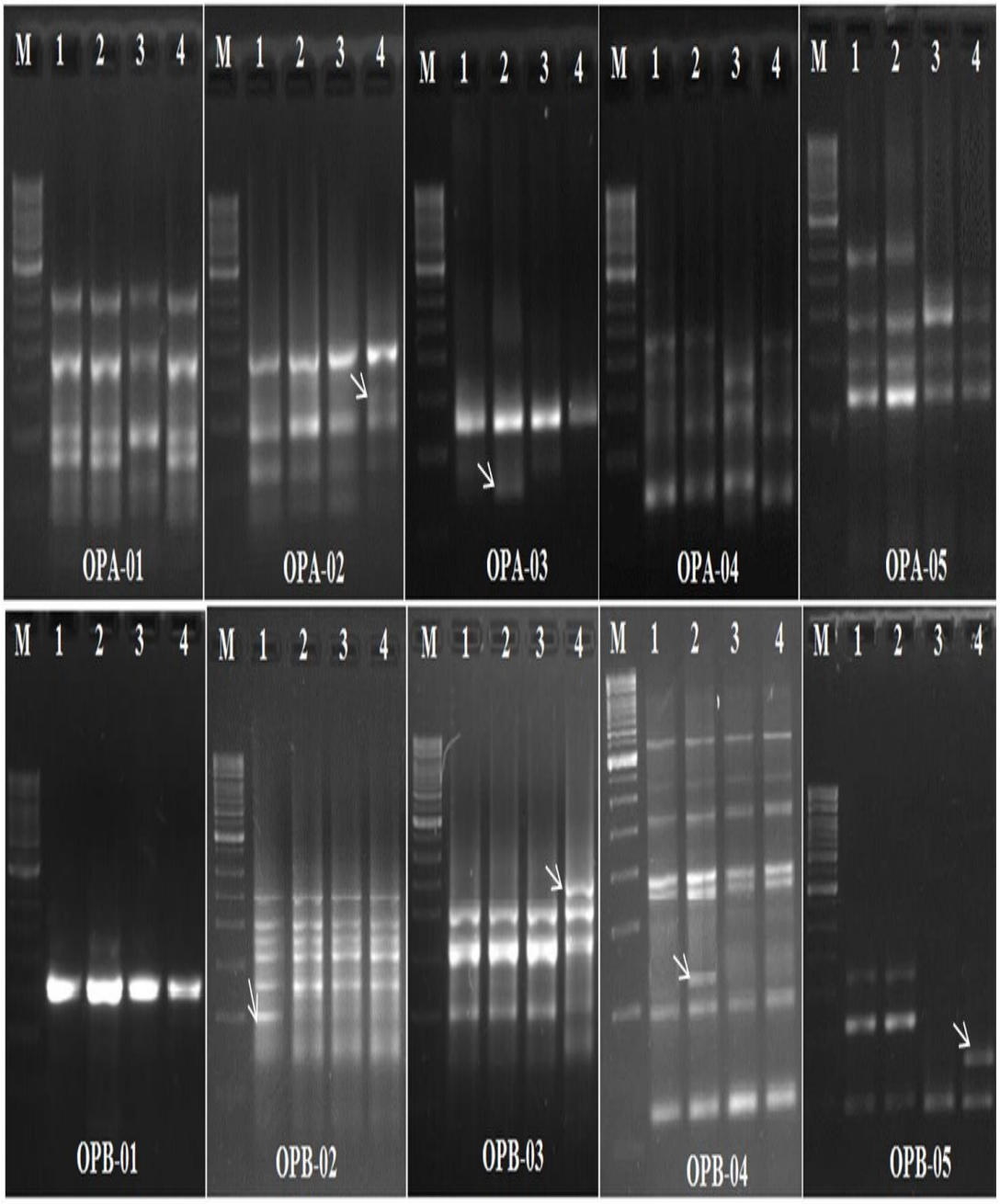

Figure 3. Electrophoretic banding pattern of amplified products obtained with four different genotypes (Lane 1-4: *Piper betle* L. var. Bali, *Piper betle* L. var. Chandrakana, *Piper betle* L. var. Jhanji, *Piper betle* L. var. Kala Bangla; M: Mol wt. marker) by using primer OPA01, OPA02, OPA03, OPA04, OPA05 OPB01, OPB02, OPB03, OPB04, and OPB05).

3.3. Genetic relationship among the Piper betle L. cultivars based on RAPD data analysis.

The Jaccard's similarity matrix indices were estimated among the four cultivars of *Piper betle* L. to assess the inter-genomic variation and genetic divergence. Similarity matrices indices among the four were in the range of 0.76 to 0.97 (Table 3). Among the cultivars, *Piper betle* L. var. Bali and *Piper betle* L. var. Chandrakanta is pretty close to each other (Similarity

matrices value 0.97) while, *Piper betle* L. var. Bali and *Piper betle* L. var. Kala Bangla (Similarity matrices value 0.82), furthermore *Piper betle* L. var. Bali and *Piper betle* L. var. Jhanji (Similarity matrices value 0.78) are distantly related to each other. Nei's genetic distance was also calculated and shown in Table 4. A dendrogram was constructed from the similarity matrix indices values (Figure 4). Dendrogram revealed *Piper betle* L. var. Bali, *Piper betle* L. var. Chandrakanta and *Piper betle* L. var. Kala Bangla is in a cluster, and *Piper betle* L. var. Jhanji apart from them.

Table 3. Jaccard's similarity matrix is based on 1-0 binary matrix of RAPD marker data generated for four cultivars of *Piper betle* L.

	var. Bali	**var. Chandrakana**	**var. Jhanji**	**var.Kala Bangla**
var.Bali				
var.Chandrakana	0.97			
var. Jhanji	0.78	0.76		
var.Kala Bangla	0.82	0.80	0.76	

Table 4. Nei's genetic distance is based on 1-0 binary matrix of RAPD marker data generated for four cultivars of *Piper betle* L.

	var.Bali	**var. Chandrakana**	**var. Jhanji**	**var. Kala Bangla**
var.Bali		0.01	0.13	0.10
var.Chandrakana	0.01		0.14	0.12
var. Jhanji	0.13	0.14		0.14
var.Kala Bangla	0.10	0.12	0.14	

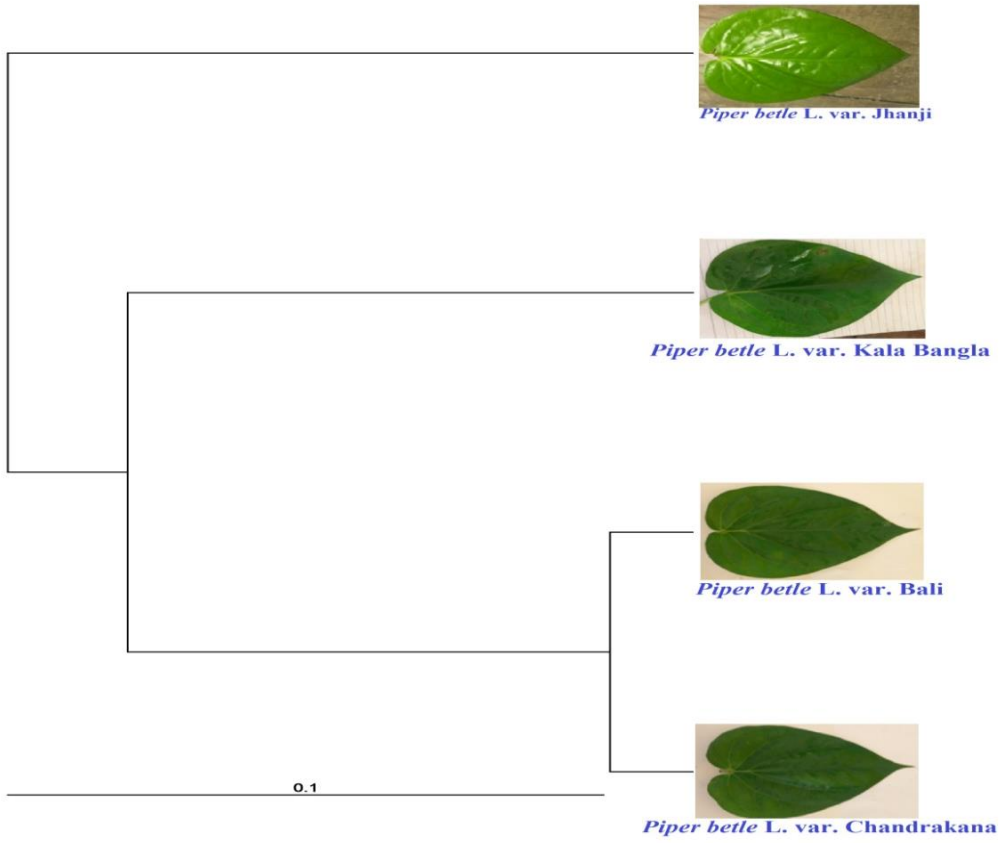

Figure 4. A dendrogram derived from the RAPD marker data of four cultivars of *Piper betle* L.

4. Conclusions

Assessment of genetic variability within a cultivated crop has important consequences in plant breeding and the conservation of genetic resources [31]. 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 [32-37]. Our outcomes indicated the presence of wide genetic variability despite being a vegetatively grown crop, which reflects a high level of polymorphism at the DNA level [38-39]. Variations in DNA sequences lead to polymorphism, and greater polymorphism is an indication of greater genetic diversity [40]. The ISSR profiles could reveal variability among the cultivars, and nomenclature can smoothly be verified in this study. As leave varieties were collected from different geographical regions, and sometimes it is observed as morphologically similar, our results clearly state that cultivars have some genetic similarity, but they are not the same [36, 37]. The observed variations among four varieties could be assigned to the fluctuating macro and microclimatic conditions of habitat [41]. Our outcomes also help researchers to create hybrid variety by crossing from various groups. RAPD analysis revealed a high degree of genetic diversity among the species analyzed in the study, which can contribute to crop improvement.

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Conflicts of Interest

The authors declare no conflict of interest.

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