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Determination of molecular diversity of *ex situ* conserved germplasm of *Piper longum* L. using RAPD markers

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Abstract
The genetic variation in *Piper longum* germplasm collected from North East states, India was investigated using Random Amplified Polymorphic DNA (RAPD) markers. A total of sixteen *P. longum* germplasms were considered for RAPD analysis and yielded appreciable amount of good quality DNA (A_{260}/A_{280}=1.8). The result indicated that the differential polymorphism in 16 different germplasm showing variation in percentage of polymorphism (25.01 to 60.00) in twenty decamer random primers. The maximum level of polymorphism was observed in OPB19 (60.00%), followed by OPB 1 (57.14%), OPA08 (50.5%), OPB02 (50.0%) OPB20 (50.0%) and OPA 11 (50.0%). Altogether one hundred and five (105) bands were amplified out of which forty one (41) bands were polymorphic. The UPGMA cluster analysis of RAPD banding pattern for correlation coefficient of similarity matrix showed that similarity value ranged from 0.587 to 0.862 indicating moderate genetic diversity among the germplasm studied. The dendrogram grouping pattern revealed that the germplasm formed four main clusters, cluster I (PLJ-18, PLJ-19, PLJ-03 and PLJ-20), Cluster II (PLJ-28, PLJ-29, PLJ-30 and PLJ-32), Cluster III (PLJ-09, PLJ-01, PLJ-22, PLJ-17 and PLJ-11) and cluster IV (PLJ-16 & PLJ-10). The check variety "Viswam" a collection from Kerala was considered as out group due to the originality of the variety.

Keywords: Northeast India, *Piper longum*, germplasm, genetic diversity, RAPD

Introduction
Among piper species known from earliest times, *P. longum* has been used for medicinal purposes. Ayurvedic publications including Sanskrit texts, have discussed in great detail the use of *P. longum* as flavoring agents in foods (Jayasinha, 1990) [3]. *P. longum* is an important drug capable of improving intellect and memory power and also to regain health by dispelling diseases. It has been found to possess antioxidant activity which neutralizes harmful effects of excessive free radicals produced in the body. *P. longum* possess several medicinal properties as well as pharmacological action such as antifungal, antiinflammatory, antioxidant and anti-cancer effect and it is known to have insecticidal activity against mosquitoes and flies (Atal *et al*., 1985; Miyakado *et al*., 1989) [1, 6]. Studies conducted on children revealed that long term use of fruit decreased (58.3%) severity of bronchial asthma. Molecular characterization of cultivars or germplasm is an urgent requirement in tune with the globalization of agriculture. Molecular markers offer means of identifying cultivars with much greater. To develop a more effective, accurate, reliable and sensitive technology for the authentication of medicinal plants, molecular markers are the best means to reveal variation in DNA level. Various molecular based markers have been developed in recent years that can be employed to analyze DNA for quality assurance, control and authentication of medicinal plant species reliability than the morphological traits which are governed by complex genetic interactions. Keeping the above facts in view the present experiment was conducted to study the genetic variation among the selected germplasm of medicinal plant *P. longum* using molecular marker.

Materials and Methods
The experimental materials comprised of twenty *P. longum* germplasms collected from different Northeastern states and planted at Horticultural experimental farm, Assam Agricultural University, Jorhat, Assam. Five samples from each experimental plot were considered for genetic diversity studies (Table 1).

Isolation of genomic DNA of *Piper longum* Germpalsm

Genomic DNA was extracted from fresh young leaves of sixteen *Piper longum* germplasm by using CTAB method (Jayasinha, 1990) [3]. DNA concentration was assessed by taking absorbance at 260 nm. Working samples were prepared to a concentration of 50 ngµl^{-1}. To estimate the quality of isolated DNA in terms of degree of shearing, the genomic DNA was subjected to agarose gel electrophoresis in 0.8% agarose gel.

Table 1: Place of collection, name of accessions and code of *P. longum*

Sl. No	Place of collection	*Piper longum* code	Name of the accession
1	Banderdewa, Arunachal Pradesh	G_1	PLJ-18
2	Bokakhat, Assam	G_2	PLJ-19
3	East Kameng, Arunachal Pradesh	G_3	PLJ-17
4	Umrangso, Meghalaya	G_4	PLJ-09
5	Khetri, Assam	G_5	PLJ-01
6	Namgui, Arunachal Pradesh	G_6	PLJ-03
7	Kadamtal, Assam	G_7	PLJ-22
8	Jagirod, Assam	G_8	PLJ-20
9	Kahikuchi, Assam	G_9	PLJ-10
10	Sarupathar, Assam	G_{10}	PLJ-11
11	Barpeta, Assam	G_{11}	PLJ-29
12	Mirza, Assam	G_{12}	PLJ-28
13	Dharmanagar, Tripura	G_{13}	PLJ-16
14	Boxirhat, Assam	G_{14}	PLJ-30
15	Siang, Arunachal Pradesh	G_{15}	PLJ-32
16	Check var. from Kerela	G_{16}	Viswam

RAPD analysis

RAPD primers used in the present study were random sequence, 10-base, oligonucleotide primers (Table2). Protocol for PCR was optimised by varying the concentration of $MgCl_2$, dNTP, primers, *Taq* polymerase and template genomic DNA (Khan *et al.*, 2007) [4]. PCR reactions (20µl) contained 20ng of genomic DNA, 1 unit of *Taq* DNA polymerase (TAKARA Biotechnology (Dalian) Company LTD.), 2µl of 10× *Taq* DNA polymerase buffer, 2mM MgCl2, 200µM of each dNTPs and 0.2µM of random primer. DNA amplification was carried out in a Gene Amp. PCR System 2400 or 2700 (Perkin-Elmer) for 40 cycles.

Denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 4 min, annealing at 37 °C for 45sec and extension at 72 °C for 90 sec, finished with final extension at 72 °C for 7minutes were considered under thermal profile. Approximately 20µl of completed amplification reaction was run in 1.5% agarose gels containing ethidium bromide (0.5 µgml^{-1}) and photographed under UV light in a gel documentation system then pair wise genetic similarity index was calculated as per Jaccard's coefficient of similarity (Jaccard, 1908) [2].

Statistical analysis

RAPD primers which gave consistent profiles across the populations and also those that appeared to have diagnostic markers were chosen for further analysis. The presence and absence of bands were scored as 1 or 0 respectively. The presence and absence of product in certain germplasm was designated as '1' and '0', respectively. Faint bands were not recorded for analysis and the data were analyzed using the method [5]. The pair wise genetic similarity index was calculated as per Jaccard's coefficient of similarity. Cluster analysis was then performed to create a dendrogram using UPGMA by the software NTSYS PC (Rohlf, 2000) [9].

Jaccard's coefficient of similarity

$$F=N_{ABI}/(N_{T}-N_{ABO})$$

Where,

F= Similarity index

$N_{ABI=}$ No. of bands present (Scored) in both accession A&B

$N_{ABO=}$ No. of bands present in all test entries but not present in accession A or B.

$N_{T=}$ Total number of bands scored in the study.

Results and Discussion

Identification of genetic variation in selected *P. longum* germplasm: The protocol employed in this investigation yielded appreciable amount of good quality DNA as represented by A_{260}/A_{280} ratio was found to be more than 1.8. The extracted genomic DNA was run in 0.8% agarose gel for gel analysis and results revealed good quality DNA as confirmed by intactness and minimal shearing.

Polymorphism of *Piper longum* germplasm using RAPD

Twenty decamer random primers were used to generate RAPD fingerprints of the studied germplasm (Table 2). The observed result indicated that out of the amplified one hundred and five (105) bands, forty one (41) bands were polymorphic. The average polymorphism was found to be 43.26%. The maximum level of polymorphism was recorded by OPB19 (60.00%), followed by OPB 1 (57.14%), OPB2 (50.00%), OPB20 (50.00%), OPA11 (50.00%) and OPA08 (50.5%). The minimum level of per cent polymorphism was observed in OPA06 (25.01) followed by OPB 03(25.05), OPB05 (33.30) and OPB06 (33.30) (Table 2 and fig 1 to 3). The RAPD banding pattern generated by different primers were shown in table 2. The polymorphism found in this study might be due to genetic diversity within these germplasm. Differential polymorphism was observed in 16 different germplasm showing variation in percentage of polymorphism ranges from 25.01 to 60% determined by using 20 primers. There are chances of ambiguity in the homology of RAPD bands especially in the context of varying chromosome number among piper species. Similar results were reported in selection of 20 micro-propagated plants of *Piper longum* that has shown 56% polymorphism [7]. The present results demonstrated the utility of RAPD markers to characterize genetic diversity and similarity among the 16 germplasm of *Piper longum*. Morphological characters are altered by various environmental factors and variation shown may not be true at the genetic level. So RAPD is found to be successful in finding out the variability between germplasm.

Table 2: Name of the primers with the number of amplified products and Percentage polymorphism generated by RAPD

Primers	Sequence	Total no. of bands	No. of monomorphic bands	No. of polymorphic bands	Polymorphism (%)
OPB 1	GTTTCGCTCC	7	3	4	57.10
OPB 2	TGATCCCTGG	4	2	2	50.00
OPB 3	CATCCCCCTG	4	3	1	25.50
OPB 4	GGACTGGAGT	7	4	3	43.50
OPB 5	TGCGCCCTTC	3	2	1	33.30
OPB 6	TGCTCTGCCC	3	2	1	33.30
OPB 8	ATCCACACGG	9	5	4	44.00
OPB 15	GGAGGGTGTT	3	2	1	33.33
OPB 17	AGGGAACGAG	6	4	2	33.33
OPB 19	ACCCCCGAAG	5	2	3	60.00
OPB 20	GGACCCTTAC	4	2	2	50.00
OPA 6	GGTCCCTGAC	4	3	1	25.01
OPA 7	GAAACGGGTG	5	2	3	41.17
OPA 8	GTGACGTAGG	4	2	2	50.50
OPA 9	GGGTAACGCC	7	4	3	43.00
OPA 11	CAATCGCCCT	4	2	2	50.00
OPA 12	GACCGCTTGT	5	3	2	40.00
OPA 13	CAGCACCCAC	3	2	1	33.33
OPA 15	TCGTTGCTGG	7	4	3	40.00
OPA 17	TCGGCGCCGT	10	6	4	40.22
Total		104	59	45	43.26

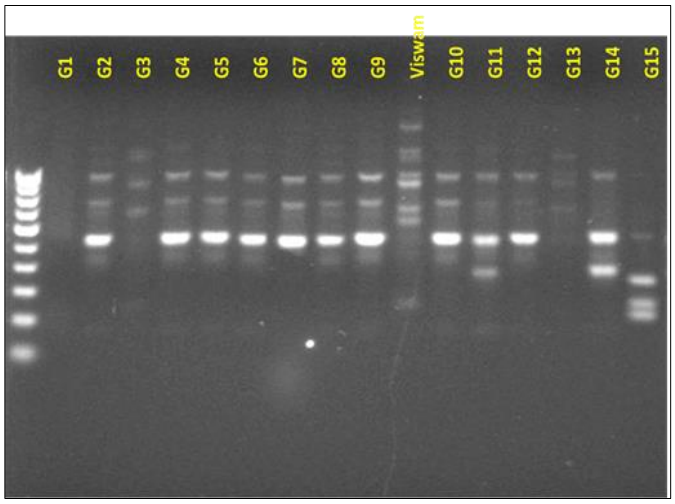

Fig 1: Amplification profile of *P. longum* germplasm with RAPD primer OPB19

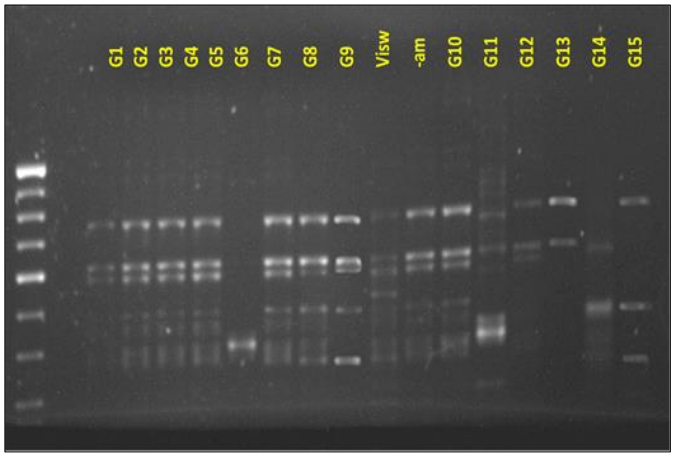

Fig 2: Amplification profile of *P. longum* germplasm with RAPD primer OPA08

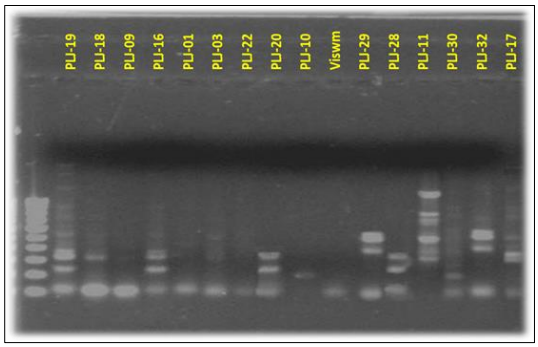

Fig 3: Amplification profile of *P. longum* germplasm with RAPD primer OPB1

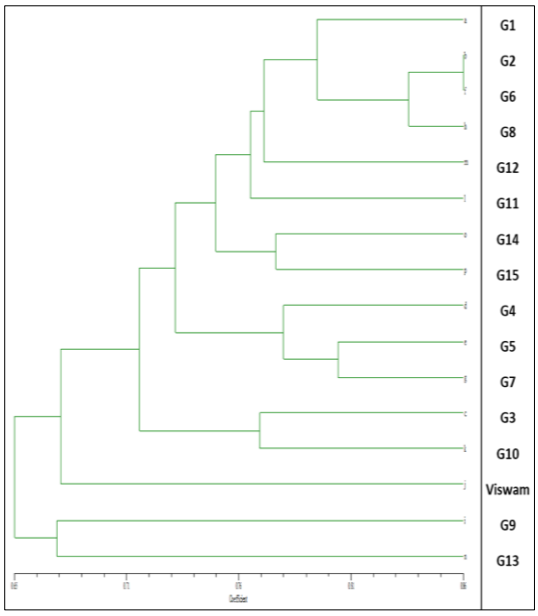

Fig 4: UPGMA dendrogram of sixteen *P. longum* germplasm based on 20 random decamer primers

Genetic diversity and Cluster analysis among *P. longum* germplasm

Twenty decamer random primers were used to generate RAPD fingerprints of the studied germplasm. The PCR products were subjected to 1.8% agarose gel electrophoresis to separate the amplified DNA fragments according to molecular size and visualized under UV light after stained with ethidium. In this present investigation UPGMA cluster analysis of RAPD banding pattern was performed and the correlation coefficient of similarity matrix was noted (Fig.4). The similarity value ranged from 0.587 to 0.862 indicating moderate genetic diversity among the germplasm studied. Similar results were reported in *Piper nigrum* that similarity ranged lies in between 0.20 and 0.66 (Kumar *et al*., 2003) [5]. A dendrogram using average linkage between groups was generated. Grouping pattern revealed that the germplasm formed four main clusters. The major cluster I consisted of germplasm PLJ-19, PLJ-18, PLJ-3 and PLJ-20. Out of which PLJ-19 and PLJ-3 were very close. The germplasm PLJ-19 was a collection from Bokakhat, Assam and PLJ-03 from Namgui, Arunachal Pradesh. Cluster II consisted of PLJ-30, PLJ-32, PLJ-28 and PLJ-29. Cluster III also comprises of germplasm PLJ-09, PLJ-01, PLJ-22, PLJ-17 and PLJ-11.The germplasm PLJ-01 and PLJ-22 were very close. Both of them are local collections Khetri and Kadamtal, respectively. The major cluster IV consisted of two germplasm PLJ-16 & PLJ-10. The germplasm "Viswam" considered as out group due to non-formation of cluster. The originality of the germplasm may be the reason of its out grouped. Cluster analysis showed the grouping of germplasm mainly on the basis of geographical location with a few exceptions. Similar results were also reported in selection of 20 micro-propagated plants of *Piper longum* that has shown 56% polymorphism (Parani *et al*., 1997) [8].

Conclusion

The genetic variation in *Piper longum* germplasm collected from North East states, India was investigated using Random Amplified Polymorphic DNA (RAPD) markers. The result indicated that the differential polymorphism in 16 different germplasm showing variation in percentage of polymorphism in twenty decamer random primers. The maximum level of polymorphism was observed in OPB19 (60.00%), followed by OPB 1 (57.14%), OPA08 (50.5%), OPB02 (50.0%) OPB20 (50.0%) and OPA 11 (50.0%). The UPGMA cluster analysis of RAPD banding pattern for correlation coefficient of similarity matrix showed that similarity value ranged from 0.587 to 0.862 indicating moderate genetic diversity among the germplasm studied. Cluster analysis showed the grouping of germplasm mainly on the basis of geographical location with a few exceptions and varies each other with geographical location.

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