Application of RAPD fingerprinting in selection of micropropagated plants of *Piper longum* for conservation

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Random amplified polymorphic DNA fingerprints of twenty micropropagated plants and the mother plant were analysed by polymerase chain reaction of genomic DNA using ten random 10-mer primers. The RAPD fragments were scored for presence/absence to calculate Jaccard's similarity index. Clustering based on similarity index was done following unweighted pair group with arithmetic mean method and a dendrogram was constructed. The dendrogram showed eighteen micropropagated plants forming a major cluster along with the mother plant. The other two micropropagated plants could be regarded as molecular off-types (putative somaclonal variants) as they have shown less than 80% similarity to the mother plant and other micropropagated plants. Among the eighteen micropropagated plants of the major cluster the order of preference to maintain maximum fidelity to the elite genotype (the mother plant) for conservation was established.

THE widespread loss of the world's biological wealth is one of the most serious global crises today[1]. Regarding plants, systematic collection and preservation of as many species as possible, particularly the endangered plant species, are the most effective means of preventing the crisis from reaching the point of no return. When an endangered vegetatively propagated species is located in a state of collection of few genotypes, collection of individuals for conservation purpose becomes critical. In this context, the technology for *in vitro* multiplication is of great use and application. Micropropagation *in vitro* and re-introduction into the original or favourable habitats is one strategy for conservation of critically endangered, vegetatively propagated species. Screening the micropropagated plants, before re-introduction, using molecular markers is desirable to reduce the chances for inclusion of variable genotypes. There are a number of molecular markers available for such screening, however, DNA-based markers are preferable as they are not influenced by developmental or environmental effects. Random amplified polymorphic DNAs (RAPDs[2,3]) is one such DNA-based marker system which could be used for screening by DNA fingerprinting. The RAPD markers, which are practically unlimited in number and portray variation in the whole genome analysed, are cost effective and easy to do with large number of samples. In this paper, we describe RAPD analysis of a population of micropropagated plants of *Piper longum* to demonstrate its application in exercising selection at molecular level to maintain genetic fidelity for conservation of elite genotypes by micropropagation.

For the present study, a mother plant of *Piper longum* (an elite genotype from which the explants were collected) and its twenty randomly selected micropropagated progenies were used. Genomic DNA from the leaves of the adult mother plant and the selected micropropagated plants before the hardening stage were isolated following the CTAB method[4]. DNA fingerprinting by RAPD was performed by polymerase chain reaction (PCR) amplification of genomic DNA with ten, 10-mer random oligonucleotides as primers (Operon Tech, CA, USA). A typical 25 µl reaction mixture included 10 ng genomic DNA, 2.5 µl 10x assay buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl and 0.01% gelatin], 2 mM $MgCl_2$, 100 µM each dNTPs (dATP, dGTP, dCTP and dTTP), 15 ng primer and 1.0 u *Taq* DNA polymerase (US Biochemicals, USA). The reaction mixture was overlaid with an equal volume of mineral oil, and DNA amplification was performed in a Perkin Elmer DNA Thermal Cycler 480. The amplification condition included a total of 45 cycles with 1 min (3 min for the first cycle) at 94°C for template denaturation, 1 min at 37°C for primer annealing and 2 min (10 min for the final cycle) at 72°C for primer extension.

The PCR amplification products were separated in 1.3% agarose gels (in 1x TAE buffer) and the resultant amplified DNA profile, called RAPD fingerprints of individual plants for different primers were photographed. PCR amplifications for the ten primers were performed three times and only those amplification products which were consistently produced in two successive amplifications were scored for presence/absence. Percentage of polymorphism was calculated as the proportion of amplification products which were polymorphic across all the genotypes to the total number of amplified products. Jaccard's similarity index was calculated as [2 × no. of shared fragments)/total no. of fragments] and clustering was done following the unweighted pair group with arithmetic mean (UPGMA) method[5].

Table 1. Details of the RAPD analysis of 20 micropropagated plants and a mother plant of *Piper longum*

No.	Primer	Sequence	No. of amplified fragments	
			Total	Polymorphic
1	OPA05	5'AGGGGTCTTG	5	4
2	OPA08	GTGACGTAGG	8	4
3	OPA15	TTCCGAACCC	7	5
4	OPA18	AGGTGACCGT	6	3
5	OPA20	GTTGCGATCC	6	5
6	OPD01	ACCGCGAAGG	8	8
7	OPD11	AGCGCCATTG	8	3
8	OPD12	CACCGTATCC	4	2
9	OPD13	GGGGTGACGA	7	0
10	OPD14	CTTCCCCAAG	6	3

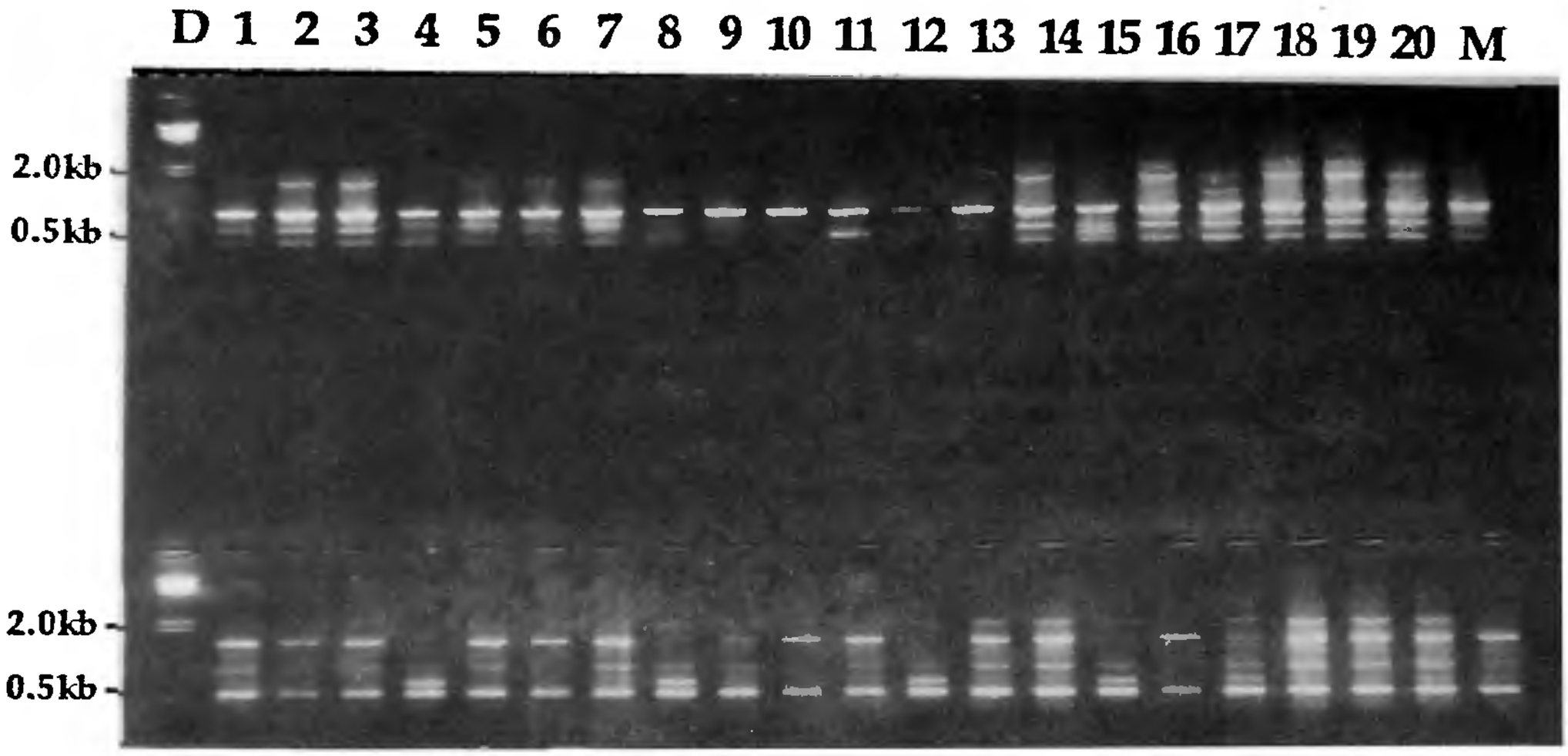

Figure 1. RAPD fingerprints of twenty micropropagated plants and the mother plant obtained by PCR amplification of genomic DNA with primer OPA15 (Top) and primer OPA20 (Bottom). D is the lane loaded with *Hin*dIII digested λ phage as DNA size marker, followed by amplified products of 20 micropropagated plants and the mother plant (M).

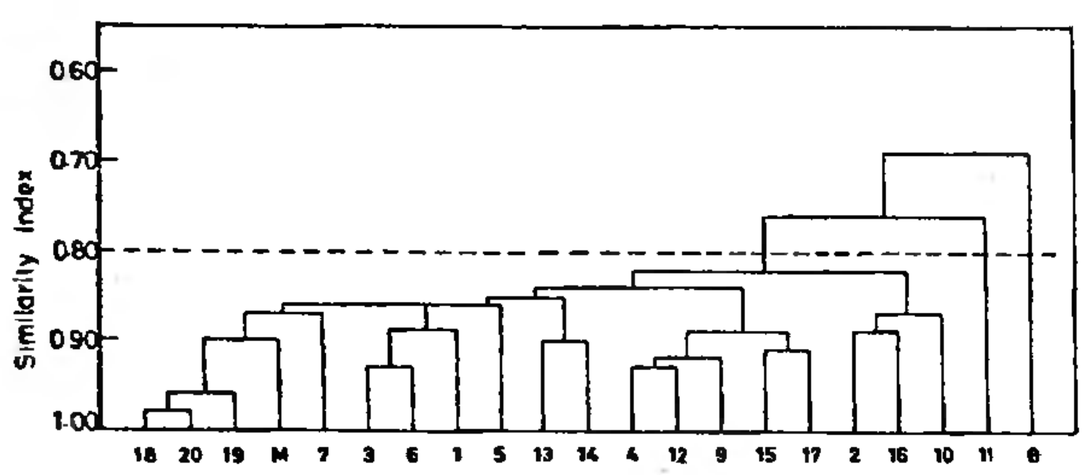

Figure 2. Dendrogram obtained by cluster analysis of the genotypes based on similarity index. 1 to 20 are the micropropagated plants and M is the mother plant.

The primers used, sequence of the primers, the number of amplification products and the number of polymorphic fragments are given in Table 1. A total of 65 fragments were amplified and among them 37 were polymorphic. The number of amplification products per primer ranged from four (OPD12) to eight (OPD01, OPD08, OPD11). The number of polymorphic amplification products per primer was zero (OPD13) to eight (OPD08). The RAPD fingerprints of 20 micropropagated plants and the mother plant amplified by the primer OPA15 and OPA20 are shown in Figure 1. The cluster diagram of the genotypes is shown in Figure 2.

Propagation from the vegetative cells under normal condition is expected to yield progenies genetically identical to the mother plant. However, *in vitro* propagation through callus or direct organogenesis frequently results in variation. Therefore, to reduce genetic instability in micropropagated plants, meristematic tissues are often used as explants[6,7], as the organized tissues are believed to be immune to genetic changes *in vitro*[8]. Despite that, morphological variations in *in vitro* cultured plants of meristematic tissue have also been documented in a number of crops like strawberry[9], grapevine[10] and pineapple[11]. Variations at molecular level were reported in *Populus deltoides* using RAPD markers[12]. These reports indicate that regardless of the kind of explant tissue used for *in vitro* multiplication, genetic variation due to culture conditions is bound to occur. Therefore, a system of preliminary screening of micropropagated plants to identify the variants is particularly important in case of perennials like *P. longum*. In this regard, screening by RAPD fingerprinting has been useful in detecting variants at an early stage of plant development[13].

In the present study, comparison of RAPD fingerprints of 20 micropropagated plants and the mother plant showed 56% polymorphism. The observed variation was not confined to a few individuals but spread through all the micropropagated plants. Complete homology between the mother plant and any micropropagated plant was not observed. Unlike in other cases (e.g. *Populus*[12]), the polymorphic fingerprints were rather complex and required statistical analysis to find out the degree of homology. Calculation of similarity for all possible combinations and construction of dendrogram, based on clustering by the UPGMA method, showed 18 micro-

propagated plants, forming a major cluster along with the mother plant at 80% similarity level. It could be seen from the dendrogram that three plants (18, 19, 20 in Figure 2) showed highest similarity to the mother plant followed by the plants numbered 7, (3, 6, 1, 5), (13, 14), (4, 12, 9, 15, 17) and (2, 16, 10) in the descending order of similarity. The plant numbers given in brackets are members of sub-clusters having the same level of similarity to the mother plant as they share a common node. However, the plants within a sub-cluster may have varying level of similarity among them (see Figure 2). The other two plants (11 and 8), showing less than 80% similarity to the mother plant, are considered as molecular off-types and therefore, not favoured in a selection process aimed at preservation of genetic characteristics of an elite genotype among its micropropagated progenies.

Thus, the present study establishes the potential use of RAPD fingerprinting in the selection of genotypes from micropropagated plants for conservation. This selection would otherwise not be possible at early stages of plants development. Further, the causes of variation observed between the mother plant and micropropagated plants are being analysed to enable manipulation as required.

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Cyanobacterial mats from the Neoproterozoic Vaishnodevi Limestone, Jammu and Kashmir

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Coccoid, crustose cyanobacterial mats have been discovered from cherts associated with stromatolites around Bidda in the Riasi Inlier of the Vaishnodevi Limestone, Jammu and Kashmir. *Eoentophysalis belcherensis*, *Palaeopleurocapsa wopfnerii* and *Myxococcoides cantabrigiensis* populate the mat assemblage. Oscillatorian filamentous sheaths assigned to *Eomycetopsis robusta* occur as a secondary mat builder. *Eoentophysalis belcherensis* and *Palaeopleurocapsa wopfnerii* have extant mat-forming counterparts. This cyanobacterial mat proliferated in warm, shallow marine, hypersaline environment as deduced from comparison with modern analogues.

THE Proterozoic Eon is the age of cyanobacteria. The group during this period reached their zenith and amplified improvizing various ramifications in its morphological and structural modifications to enhance their physiological efficiency. Functional modifications had priority over structural development. They were protean and environment-responsive and conquered all conceivable aquatic ecological realms, including restricted coastal environments such as sabkhas and hypersaline embayments forming microbial mats. They are also known to inhabit hot acid, alkaline and sulphide springs, forming chemolithotrophic biofilms and mats as well as deep sea hydrothermal vents. These environments are not too congenial but repugnant. In these niches they are extant even today after extensive proliferation for over 2 billion years, though their beginnings are to be sought in the 3.5 billion-year-old Archaean sequences. Hypobradytely the group explicitly displays, as opposed to prevalent tachytelic, horotelic and bradytelic evolution, verily endowed the organism elasticity and tolerance to changing environments. The group did not develop ecologic-specific specialization that restricted them to grow and inhabit only certain environments while