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Pascale Besse *Editor*

Molecular Plant Taxonomy

Methods and Protocols

Second Edition

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Edited by

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Chapter 12

Amplified Fragment Length Polymorphism: Applications and Recent Developments

Thotten Elampilay Sheeja, Illathidath Payatatti Vijesh Kumar, Ananduchandra Giridhari, Divakaran Minoo, Muliyar Krishna Rajesh, and Kantipudi Nirmal Babu

Abstract

AFLP or amplified fragment length polymorphism is a PCR-based molecular technique that uses selective amplification of a subset of digested DNA fragments from any source to generate and compare unique fingerprints of genomes. It is more efficient in terms of time, economy, reproducibility, informativeness, resolution, and sensitivity, compared to other popular DNA markers. Besides, it requires very small quantities of DNA and no prior genome information. This technique is widely used in plants for taxonomy, genetic diversity, phylogenetic analysis, construction of high-resolution genetic maps, and positional cloning of genes, to determine relatedness among cultivars and varietal identity, etc. The review encompasses in detail the various applications of AFLP in plants and the major advantages and disadvantages. The review also considers various modifications of this technique and novel developments in detection of polymorphism. A wet-lab protocol is also provided.

Key words AFLP , cDNA, Epigenetics, Genetic diversity, Transcriptomics, MSAP , Restriction enzymes

1 Introduction

The AFLP technique is a patented technology first described by [1] and is applied widely in monitoring inheritance of agronomic traits in plants, pedigree analysis, parentage analysis, screening of DNA markers linked to genetic traits and genes of interest, etc. AFLP technique uses the entire genome for polymorphism and reproducibility and is recognized as a universal DNA fingerprinting system, universally accepted regarding origin and complexity of DNA samples and even small sequence variations that can be identified using a small quantity of DNA as low as 0.05 μg. A large number of

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fragments are detected on a gel that allows evaluation of a large number of loci at a time. It is much advantageous in terms of number of polymorphisms identified per reaction, reproducibility, ease, and cost of analysis.

1.1 Principle of AFLP

AFLP employs selective amplification of restriction fragments from a digested total genomic DNA using PCR. Genomic DNA is first digested by two restriction enzymes that cut the big molecules into a mixture of fragments enabling amplification by PCR. Usually in AFLP two restriction enzymes, a rare cutter like *Eco*RI (6-bp restriction site) and a frequent cutter like *Mse*I (4-bp restriction site), are used for restriction. Double-stranded oligonucleotide adapters consisting of a core sequence and a restriction enzyme-specific sequence homologous to one 5′ or 3′ end are then ligated to the DNA fragments using T4 DNA ligase. The ligated DNA fragments are amplified by PCR using primers complementary to the adapter and restriction site sequence with additional selective nucleotides at their 3′ end. Using selective primers reduces the complexity of the mixture, and those fragments complementary to nucleotides beyond restriction site will be amplified by these selective primers under stringent annealing conditions. Later, the polymorphisms are identified by a denaturing polyacrylamide gel electrophoresis and patterns between individuals are compared. In AFLP polymorphisms observed arise due to a mutation in the restriction site, a mutation in the regions complementary to primer extensions and adjacent to restriction site, or a deletion/insertion within the amplified region. Molecular polymorphisms are identified based on the presence or absence of particular DNA fragments of a given size among individuals (Fig. 1).

1.2 Basic Steps Involved in AFLP Analysis

A suitable DNA extraction protocol that yields good quality DNA without degradation may be employed for AFLP analysis. Quality of DNA needs to be ensured by an extra purification step; in case if the DNA extracts contain restriction or PCR inhibitors, an extra purification step may be incorporated. In AFLP, it is required to optimize the quantity of DNA for generating clear, intense AFLP patterns. These patterns may vary from species to species and depend on the genome size. Restriction fragments are generated using two restriction endonucleases, a rare cutting enzyme with 6–8 base recognition, in combination with a frequent cutting enzyme of four-base recognition. Enzymes are chosen based on the genome complexity and methylation status of the DNA. Complete digestion is to be ensured in order to avoid false polymorphisms due to amplification of fragments that are not fully digested. The AFLP protocol is designed to amplify and preferentially detect fragments with EcoRI cut at one end and MseI cut at the other. In AFLP different combinations of enzymes and multiple combinations of primers can be used for accessing hundreds of polymorphic markers.

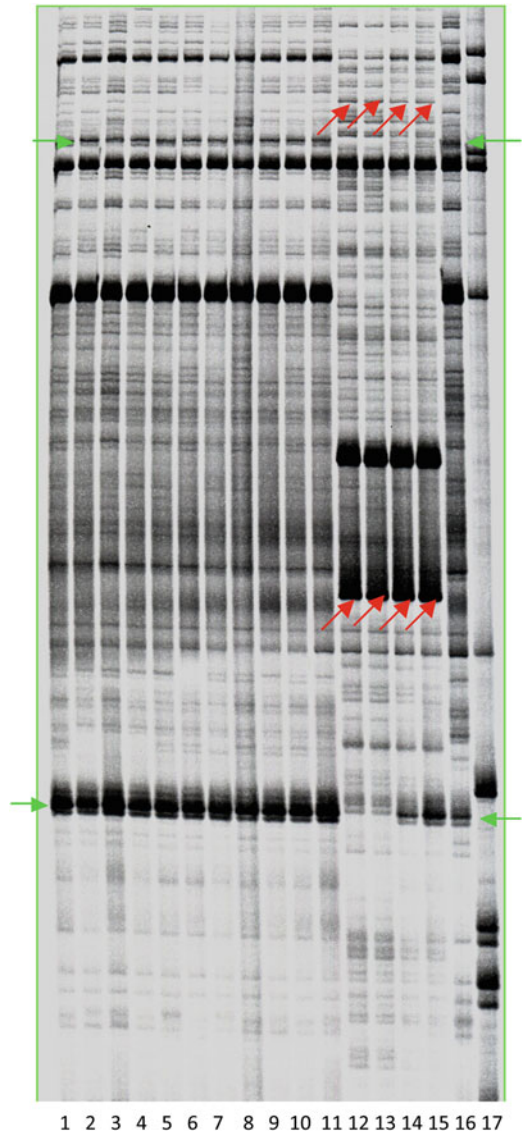

Fig. 1 AFLP profiles of *Vanilla* spp., seedling progenies, and interspecific hybrids, developed using primer combination EGG-MTG. Lanes 1–10: Seedling progenies of *V. planifolia* (1, V1; 2, V2; 3, V4; 4, V6; 5, V7; 6, V8; 7, V10; 8, V11; 9, V12; 10; V24); 11, *V. planifolia*; 12, *V. aphylla1*; 13, *V. aphylla2*; 14–16: interspecific hybrids of *V. planifolia* and *V. aphylla* (14, VH1; 15, VH4; 16, VH5); 17, Water Control. Arrows indicate species-specific bands (*V. planifolia* in green, *V. aphylla* in red)

1.3 AFLP Advantages and Applications

The AFLP technique is a robust tool due to the ability to generate quickly a large number of marker fragments without prior knowledge of the genomic sequence and can be multiplexed for analysis of hundreds of individuals at a time. It requires only a small quantity of DNA and is highly reproducible. Due to this reason, it is used in DNA fingerprinting of non-model organisms where no prior

sequence information is available. AFLP can be used for samples of any origin and complexity to detect sequence variations. Commercial AFLP primer sets are available which work on most organisms making this technique versatile. In-depth coverage of the genome is possible since large numbers of AFLP markers can be typed rapidly at a low cost. AFLP markers are largely independent since 90% of these reflect point mutations in the restriction sites. The co-migrating markers in AFLP are mostly homologous and locus specific and follow a Mendelian inheritance in plants [2, 3].

AFLP markers reveal a greater amount of diversity compared to other popular markers like RAPD, ISSR, SSR, RFLP, etc. (Chapters 11, 13, 14) and are highly reproducible and reliable due to the stringent hybridization conditions employed [4–6]. Due to these reasons, it can be upscaled, reproduced between different laboratories and conditions. These methods require very small quantity of DNA to generate huge amount of data.

AFLP differs from RFLP in that it employs PCR amplification to detect the polymorphisms on a denaturing PAGE while RFLP employs agarose or PAGE gels followed by hybridization. AFLP provides additional possibilities of detecting polymorphisms beyond the restriction site in comparison to RFLP wherein only the length variation within restriction sites is available and detects more point mutations, insertions, and deletions than RFLP to the tune of about 100–200 loci at a time. There is a scope of detecting unlimited polymorphisms by simply varying the restriction enzymes and the nature and number of selective nucleotides. AFLP fragments are mostly homologous and locus specific [7] except in polyploid species. Due to the above advantages, AFLP markers have proved effective in determining genetic differences among individuals, populations, and species. AFLP markers unravel cryptic genetic variation of closely related species which cannot be distinguished using conventional strategies. AFLP markers have the widest application in genetic variation analysis below species level for investigating population structure and differentiation and phylogenetic relationships based on genetic distances. They are highly instrumental in characterization of gene banks, fingerprinting, and estimation of genetic diversity for gene bank management. AFLP markers have been applied to evaluate gene flow and dispersal, outcrossing, introgression, and hybridization. The different applications of this versatile technique are detailed below.

1.3.1 Genetic Diversity Studies Using AFLP Markers

Analysis of genetic diversity and phylogenetic relationships is an important prerequisite for future breeding programs and conservation. It helps to understand evolutionary history of a species and the future risks to diversity. Evaluation of interpopulation variations indicates scope of geographic origin, dispersal of plant material, and gene flow between populations. Intraspecific genetic variability in

natural populations is an indicator of the potential to cope with changing environmental conditions and provides valuable inputs with respect to conservation and management of endangered and endemic plant taxa [8]. Diversity studies based on molecular markers are found to be more informative and reliable than that based on morphological and phenotypic traits. AFLP requires no prior sequence information and has a multi-locus and genome-wide nature, which makes it more popular than other molecular markers in DNA fingerprinting and genetic diversity analysis [6, 8–17].

In genus *Brassica*, several reports [4, 18–25] demonstrate the utility of AFLP in addressing important phylogenetic questions within the species and provide new insights for future breeding programs. In rice, AFLP analysis in four populations provided valuable insights regarding unique genes in Iranian native varieties, which will be useful for future breeding programs and stresses upon the need for conserving this unique diversity [26]. In *Jatropha*, AFLP analysis of five populations showed high intrapopulation variability, and this could identify promising genetic resources to be included in breeding programs [27]. Distribution of genetic variation in Illinois bundle flower was detected using AFLP markers, with a view to increase the efficiency of germplasm preservation and expedited plant breeding programs [28]. AFLP-based genetic diversity studies in *Pinus pinaster* populations provided important information on organization and subdivision of diversity, the genetic mechanisms underlying it, and sampling strategies to be adopted for species conservation [29].

Evidence for maintenance of genetic variability in Italian and Spanish durum wheat over the last century was revealed through AFLP marker-based analysis [30], which showed an enrichment of diversity in the cultivated pool and broadening of genetic background. In snap bean, AFLP-based genetic variability analysis exhibited a good level of variability and a possible relationship between bean growth habitat and the gene pool, which can be exploited for future breeding programs [31]. AFLP-based fingerprinting is a suitable technology for discovering genetic diversity in banana [32–36], and it also has an impact on conservation strategies and breeding ventures in banana. Phylogenetic and genetic diversity analysis of conserved endangered plant species has been successfully done through AFLP [37–40]. Diversity study within population and subpopulation of endangered sentry milk vetch (*Astragalus cremnophylax var. cremnophylax*) [41] through AFLP could estimate their adaptability to alien environments and also provides strategically important inputs for their conservation.

Germplasm collections have been characterized in *Jatropha curcas* [42] and *Rhodiola rosea* [43] using AFLP. Genetic diversity studies in natural populations of *Dendrobium thyrsiflorum* and radish [44] showed high interpopulation variations and correlation

of a few AFLP markers with the antioxidant activity [45] in case of the former. In teak, high genetic diversity could be observed within locations indicating importance of intensive location-wise collection of diverse superior genotypes for conservation and genetic improvement [46]. In lentil accessions genetic diversity and phylogenetic studies were conducted, and intraspecific genetic variability at high levels could be detected. An important outcome of this study was information on progenitor species of cultivated lentils [47]. Genetic diversity analysis in *Microlaena stipoides* using AFLP showed outcrossing and significant amount of variation within populations which can be used as a probable strategy for its propagation and for making microlaena more resilient in the long term [48].

Genetic relationships among different species of *Solanum* gave leads into the taxonomic resolution of this complex species and also provided insights into the origins/introductions of some of the important species [49]. Several other studies also have utilized AFLP for *Solanum* taxonomy [50–57].

In many cases AFLP analysis showed limited genetic diversity existing within germplasm collections, which indicates the need for conservation and also suggests that new accessions should be obtained from the center of origin of the species [58]. Intra-accession diversity studies in potato population showed lower levels of polymorphism within accessions of self-compatible when compared to self-incompatible taxa, thereby showing the high suitability of AFLP makers for evaluation of diversity between accessions in gene banks [59].

In many of the genetic diversity and phylogenetic studies, grouping of individuals showed high correlation with taxonomic and molecular classifications, indicating that the observed variations could be due to genetic factors. However, in some cases morphological and agronomic traits did not correlate well with molecular classification due to genotype × environment interaction and polygenic nature of the traits [60].

Using AFLP markers genetic variation was detected among tea genotypes [61] that could not be distinguished using morphological and phenotypic markers. The grouping of populations in a dendrogram was consistent with the taxonomy, known pedigree of genotypes, and geographical origin. Valuable observations could be made regarding the origin/ancestry and genetic diversity of tea from this study. Analysis of genetic diversity using AFLP markers in jackfruit [58] showed that grouping of accessions correlated well with the taxonomic classifications. Through this study incorrect classifications could be rectified, and self-fertilization of clones in a hybridization material could also be detected. Genetic diversity studies using AFLP assigned genotypes into groups corresponding to origin and lineage relationships in cotton which can be exploited

in marker-assisted parental selection tool for plant breeders [62]. A study involving three species of Malvaceae depicted good congruence of AFLP-based clustering with earlier morphological and molecular investigations [63]. In pineapple cultivars from Thailand, AFLP-based clustering revealed moderate genetic diversity and congruence with earlier morphological characterization [64]. Phylogenetic relationship studies indicated that AFLP data correlated well with the taxonomic relationships among the cultivated lettuce and wild species, and the dendrogram generated was similar to the phenetic tree constructed using RFLP data [65]. In *Triticum aestivum* genotypes, a moderate correlation between AFLP and morphological markers was observed [66], while in olive cultivars, AFLP fingerprinting of core collection discriminated different cultivars, but clustering based on AFLP and fruit traits did not show significant correlation [67]. In azalea [68] and banana [32, 33] cultivars, no correlation between AFLP data and morphological traits existed, indicating that the majority of the polymorphisms did not contribute to phenotypic variation.

Genetic diversity and influence by environment could provide a better understanding of the natural variation and gene exchange that existed in a species with respect to its geographical location. This can help in preservation and development of germplasm resources especially in case of endangered species. In some studies a good correlation of AFLP data with the geographical origins and distance could be observed. In *Vigna* sp. [69], *Triticum* landraces [70], and banana [71], significant association was observed between AFLP data and geographic location. In *Hibiscus tiliaceus*, estimates of genetic diversity using AFLPs agreed well with the geographical distribution and life history traits [72]. AFLP analysis of Iranian potato germplasm [73] and *Lactuca* species [74] showed a high level of genetic diversity and clustering corresponding to the geographical origin of these varieties. In cowpea genetic distances were estimated in wild, weedy annuals, domesticated cowpea, perennial accessions, and wild subspecies, and AFLP markers could successfully uncover variation within both domesticated and wild accessions [75].

In alfalfa [76], soybean [77], and *Croton* sp. [78], AFLP was used to study genetic diversity of cultivated and natural populations, which showed no correlation between genetic and geographic distances. In betel vine cultivars, cluster analysis based on AFLP data showed that grouping of individuals was based on their genetic relatedness rather than place of collection [79]. In kale, landraces, cultivars, and wild populations exhibited higher levels of diversity among wild populations. The study indicated that genetic distance was not related to geographical distance and provided inputs on conservation strategies to be adopted [80]. Wild

populations of *Agave angustifolia* fingerprinted using AFLP showed a partial correlation with geographical distribution and variation between mother plants and vegetatively propagated mother rhizomes [81]. In the endangered *Glehnia littoralis*, AFLP analysis showed no obvious correlation between genetic and geographic distances, and the endangered status was attributed to the loss of wild habitats calling for ecological conservation strategies [16]. In black gram AFLP-based clustering of landraces indicated influence of soil pattern and topography in the genetic makeup and genetic distinctness [82].

1.3.2 Variety/Cultivar Fingerprinting, Kinship, and Genetic Fidelity

Lack of genetic identity is a serious problem in plant propagation and seed production of elite genotypes. For certification purpose, genotypes need to be characterized both at phenotypic and molecular level for identifying promising ones with outstanding agronomic, nutraceutical, and nutritional characteristics. Availability of informative molecular markers is an essential prerequisite for proprietary protection, establishing identity, early detection of seedlings in the nursery, and monitoring trade. AFLP being a dominant marker system and the availability of multi-locus and genome-wide marker profiles are the reasons that make it a preferred method for DNA fingerprinting [42]. Several studies endorse the utility of AFLP markers for discriminating between closely related individuals when compared to nuclear and chloroplast DNA markers [83, 84]. AFLPs are also the preferred method for establishing genetic fidelity in in vitro culture systems especially in commercial propagation [85] where soma clonal variation is a problem.

Along with genetic variability estimations in selected cultivars and lines of *Cornus florida,* a dichotomous key using specific AFLP markers was constructed to distinguish some of the popular cultivars and breeding lines [86]. Genomic fingerprints of elite genotypes of farmers were done using AFLP markers for the purpose of variety protection, seed certification, and future support to breeding programs in blackberry [87] and for detection of duplicates in germplasm collections of yam [88]. AFLP markers have the potential to resolve genetic differences at the level of "DNA fingerprints" for individual identification and parentage analysis [89].

In case of identification of clonally identical individuals, a large number of markers need to be screened to uncover existing genetic differences due to their extremely close nature. Clonally derived individuals in several plants could be delineated by AFLP making them suitable for analysis of relatedness, parentage, mating frequency, etc. due to low levels of co-migration of non-allelic fragments. AFLPs clearly established their utility for clonal differentiation and/or identification in *Vitis vinifera* ecotypes [90], and the profiles were well in congruence with those generated by ISTR (inverse sequence tagged repeat) markers. However, in

certain populations, ISTR revealed more polymorphism. Differences at the molecular level were identified between agave offsets and bulbils produced asexually from the same mother plant from different tissues using AFLP depicting the great potential of this method in plant cultivar identification [91]. In near-isogenic lines of soybean, distinguishing between individuals that differ at only a single small region in the entire genome was possible [9]. AFLP markers also enable testing of clonal identity between individuals and thus permit to make inferences about the sexual versus asexual reproduction modes [92].

AFLP markers have also been used to establish genetic fidelity in in vitro derived plants in several crops for confirming the commercial-scale plant production protocol [93, 94]. Clonal fidelity of micropropagated plants was established through AFLP in endangered *Arachis retusa* for germplasm storage and in *Dendrocalamus hamiltonii* [95]. In *Bambusa nutans*, AFLP revealed a high level of genetic stability in somatic embryo-derived plantlets [96]. AFLP successfully identified variations in cryopreserved in vitro shoot tips in *Rubus* [97].

1.3.3 QTL Mapping

AFLP markers have been used extensively for constructing linkage maps for QTL analysis of agronomic traits including disease resistance and salt tolerance [98–123]. AFLP markers have been widely used for map-based cloning of target genes linked to them, and SCAR markers for quality traits were developed in asparagus bean [124], alfalfa [125], tomato [126], eggplant [127], and maize [128].

1.3.4 Other Specific Applications of AFLP Marker Systems

In barley, AFLP assay and bulked segregant analysis involving selected individuals of a cross between water stress-tolerant and stress-sensitive genotypes identified a marker that was present only in the tolerant parent and tolerant bulk of F2 individuals [129]. In *Salvia miltiorrhiza* segregating sterile and fertile populations when subjected to bulked segregant analysis and AFLP marker analysis indicated several markers tightly linked to the drought stress genes. One of the markers was found to be identical to another marker tightly linked to male sterile gene with 95% identity [130]. Molecular tagging of male sterility locus was done using AFLP technique in a BC1 mapping population segregating for male sterility/fertility. Markers were identified for marker-assisted selection and genetic map constructed for the male sterility gene [131]. In *Piper betle*, a combination of bulked segregant analysis and AFLP screening identified two male sex-specific markers [80]. Bulked segregant analysis combined with AFLP identified markers linked to resistance to yellow rust disease in *Triticum aestivum* L [132]. AFLP coupled with bulk segregant analysis could identify markers linked to virus disease in tomato [133].

Species-specific AFLP fingerprints were generated and used for authentication in three species of *Zingiber*, which is proposed to help in resolving adulteration-related problems faced by commercial users [134]. In Andean blackberry, attempt was made to generate genomic fingerprints that will enable protection, seed certification, and future support to breeding programs [88]. In a study, AFLP genome scan was combined with environmental analysis for testing natural populations of *Liriodendron chinense* for signals of natural selection, and it identified a few outlier locus strongly associated with climatic factors [135]. AFLP investigation of 14 wild *D. glomerata* indicated that the genetic diversity and structure pattern of populations could be influenced by environmental factors like altitude, precipitation, latitude, and longitude [136]. In *Lactuca* sp. studies indicate that ecogeographical conditions can influence the genetic background of populations originating from them [137], and influence of biotic and abiotic stresses in the center of origin regions can lead to high genome-wide diversity in populations [138]. In rice, several high temperature responsive transcript-derived fragments (TDFs) were identified employing differential gene expression analysis coupled with AFLP [139]. Similar strategy in sugarcane identified several induced and repressed TDFs in response to infection by Sugarcane Mosaic Virus [140].

Isolation and characterization of differential genes in *Capsicum annuum* L. using AFLP indicated that space flight influenced main quality characters at genetic level, and induction of several novel genes was observed [141]. In *Spondias tuberosa* [142], outcrossing rates estimated using AFLP in a large population involving 12 families exhibited the open pollinated nature of the species and provided valuable inputs on strategies for conservation and breeding.

In Oregano, a high correlation between key chemotypic traits and AFLP markers could be established [143]. Genetic diversity assessments by AFLP markers in populations of *Amaranthus palmeri* was done to understand the distribution and development of herbicide resistance to glyphosate [144]. AFLP also helps to target other levels of diversity especially DNA methylation polymorphism and transcriptomic variation [145].

1.4 AFLP Versus Other Popular DNA Markers

In several species a greater degree of polymorphism was observed in AFLP-based diversity analysis compared to other popular markers like SNP, SSR, ISSR, and RAPD [146–151]. In vanilla RAPD and AFLP profiles coupled with morphological characters could successfully assess variability of genotypes and of successful interspecific hybridization and production of hybrids [152]. Genetic relationship studies in soybean genotypes [153] indicated a lower level of expected heterozygosity in case of AFLP markers in comparison with microsatellites and RAPD, in spite of the fact that AFLP generated the highest effective multiplex ratio as in other

studies. However, the marker index, a parameter involving expected heterozygosity and multiplex ratio, was much higher for AFLP markers indicating its superiority for detecting polymorphisms. The RFLP, AFLP, and microsatellite marker systems showed a good correlation in the present study. In *Brassica napus* hybrids, SSR was found to be more efficient than AFLP in evaluating genetic diversity, while AFLP was better for varietal identification and DNA fingerprinting [154]. In common bean SSR and AFLP showed a comparable accuracy in grouping genotypes according to their gene pool of origin [155]. AFLP was found to be the best molecular marker for fingerprinting and assessing genetic relationship among genotypes of *Dactylis glomerata* when compared to other markers like RAPD and ISSR [156].

In brinjal [157], *Jatropha* [158, 159], sugarcane [160], and *Miscanthus* sp. [161], the superiority of AFLP over RAPD in discriminating genotypes and estimation of genetic diversity was reported. In yet another study on *Aegilops* species, 50 populations analyzed using AFLP showed superiority of AFLP markers over RAPD as a tool for molecular variability studies in plant breeding programs [162]. AFLP turned out to be a better method for obtaining a more definitive grouping for study of genetic relationships both at species and cultivar level [35] in banana. AFLP was more efficient compared to SSR markers for detecting genetic variation among Ethiopian Arabica coffee genotypes [163], and on a small spatial scale, AFLPs outperformed SSRs in discriminating individuals and assigning them to population of origin [164] in *Eryngium*. In banana [36] estimates of genetic diversity did not show any significant correlation between microsatellite and AFLP markers. In maize [165], SSR and AFLPs were found to be equally suitable for genetic diversity studies. However, intrapopulation diversity studies in neem indicate a better efficiency of SAMPL markers over AFLPs in resolving differences between closely related accessions [166]. SRAP markers were found to be more informative than AFLP in giving high number of unique markers for identification of banana genotypes [167].

However, in the genus *Ocimum*, a combined analysis of morphological traits, volatile oil composition, and molecular markers is found to be an ideal strategy for taxonomical classification [168]. Genetic relationship study showed good correlation between AFLPs and RAPDs in potato and endorsed the application of a combination of marker systems like AFLP, SSR, and RAPD for better understanding of genetic relationship [169].

1.5 Disadvantages of AFLP Technique

AFLP is a cumbersome process involving several steps and requires reasonably large quantity (300–1000 ng per reaction) of good quality DNA and is a technically complicated procedure than simple markers like RAPD. AFLP employs polyacrylamide gels and silver staining and radioactivity of fluorescent probes for detection that

are laborious and expensive compared to agarose gels. It requires ligation and restriction enzymes and adapters, which adds to the extra cost compared to techniques like RAPD (Chapter 13). Post-run data analysis is lengthy and complex compared to RAPD. However, recently available kits and automation have made it more user-friendly. AFLP markers are dominant biallelic markers and polymorphic information content is low (maximum is 0.5). It is difficult to distinguish between heterozygous and homozygous individuals for the presence of allele, and precise estimation of heterozygosity is not possible, which limits its usage in population genetic analysis, genetic mapping, and marker-assisted selection. AFLP technique can produce artifacts in degraded samples like herbarium specimens, and to overcome this, fresh samples were included for comparison, thereby ensuring the presence of monomorphic fragments in the fresh samples as well as herbarium AFLPs [170].

1.6 Modifications of AFLP

1.6.1 SAMPL

The selectively amplified microsatellite polymorphic loci (SAMPL) marker technique may be employed to detect higher levels of genetic variation within genotypes. SAMPL is a microsatellite-based modification of the AFLP assay and has all the advantages of the latter [171]. Due to its association with the hypervariable microsatellite region, this assay can detect high levels of polymorphism between closely related genotypes. Due to its ability to survey the hypervariable microsatellite region in the genome, it can detect higher levels of polymorphism per locus compared to AFLP. The SAMPL assay has been employed for analysis of genetic diversity in lettuce [172] and sweet potato [173] among other crops [174, 175]. The SAMPL assay revealed higher levels of polymorphism among *Withania somnifera* genotypes compared to the use of standard AFLP in all the genotypes tested. The AFLP markers and their modifications such as SAMPL are generally expensive to generate, technically tedious, and dominant in nature. This limits their large-scale application as diagnostic markers for species, cultivar, or varietal identification. For practical applications, these markers need to be converted to rapid, technically simple assays that can be used on crude DNA preparation. A fruitful attempt at converting SAMPL markers to useful diagnostic markers was one where *W. somnifera*-specific bands generated with SAMPL were used to develop a simple PCR-based assay [174]. All the tested genotypes can be distinguished at the seedling stage by the diagnostic markers generated.

1.6.2 M-AFLP

Microsatellite-amplified fragment length polymorphism (M-AFLP) is a modification of AFLP to detect intravarietal genetic differences and is known to be the most efficient system and generates the highest number of polymorphic bands compared to SSR, AFLP, and SAMPL [176]. Markers are anchored to the 5′-end of

microsatellite (e.g., SSR) loci in this new AFLP-derived marker system. M-AFLP combines the high heterozygosity of microsatellites (SSRs) with high multiplex ratio of AFLP-derived markers. Variation in the number of repeat units is the source of polymorphisms detected by the M-AFLP, and it is employed to develop SSR-type codominant markers from polymorphic M-AFLP bands. The technique does not require hybridization enrichment steps and provides substantial efficiency of SSR identification compared with conventional library procedures [177]. M-AFLP has been employed in cassava for genetic diversity analysis of cassava and other *Manihot* species [178], in grapevine for clone differentiation and varietal identification [176], in *Cynara cardunculus* for microsatellite locus identification [179], in *Poa pratensis* L. for genetic mapping of complex polyploids [177], and in *Lupinus angustifolius* L. for the isolation of sequence-specific PCR markers [180].

1.6.3 SSAP

Sequence-specific amplified polymorphism (SSAP) analysis [181] was one of the first retrotransposon-based barcoding methods based on AFLP. The BARE-1 LTR-RT is utilized by SSAP technique for molecular barcoding [181] using one primer complementary to an RT (e.g., 3′ LTR) and the other primer complimentary to the AFLP-like restriction site (usually MseI or PstI) adaptor. Primer pairs contain two or three selective nucleotides of MseI or PstI (or any restriction enzyme) adaptor primers and one selective nucleotide of either ^{32}P or fluorescently labeled retrotransposon-specific primers [179]. The primers in SSAP technique are designed based on the LTR region, but could also match to an internal sequence of the RT, like the polypurine tract (PPT), which is found internal to the 3′-LTR of retrotransposons [179]. When restriction enzymes have a long recognition site sequence, nonselective primers could also be used or when the copy number of the RTs is low. The type of SSAP primers used determines the quality of the SSAP pattern. SSAP usually exhibits higher level of polymorphism compared to AFLP and has been extensively used for diversity analysis studies in *Triticum* spp. [182], *Hordeum vulgare* [183], *Avena sativa* [184], *Aegilops* spp. [185], *Malus domestica* [186], *Cynara cardunculus* [187], *Lactuca sativa* [188], *Pisum sativum* and other Fabaceae species [179, 189], *Capsicum annuum*, *Solanum lycopersicum* [190], and *Ipomoea batatas* [191]. SSAP was also used for cladistic molecular barcodes to resolve evolutionary history in *Nicotiana* [192], *Vicia* [193], *Oryza* [194], *Triticum* [182], and *Zea* [195].

1.6.4 AIMS

The amplification of insertion mutagenized sites (AIMS) technique is mainly based on reducing the band complexity by specific PCR amplification of insertion mutagenized sites, by using a primer that is specific to *Mutator* transposon flanking sequences [196]. AIMS

procedure delivers possible gene candidates, but isolation of the gene has to be verified by another method. MuAFLP, another variant of AFLP, is similar to AIMS, and it targets amplification of *Mutator* transposon regions [197].

1.6.5 MSAP

The methylation-sensitive amplified polymorphism (MSAP) technique mainly involves cleavage with the methylation-sensitive restriction enzymes *HpaII* or *MspI*, followed by adapter ligation, amplification, and gel-based visualization [198, 199]. The methylation state of the external and internal cytosine residues strongly affects the cleavage capacities of *HpaII* and *MspI* within the recognized 5′-CCGG-3′ sequences. Thus, the methylation state is determined based on the ability of each enzyme to cleave the restriction site, for each of the specific bands. MSAP-based analyses can be performed for a range of species regardless of their genome size and availability of reference genome. Established in 1997 [198], MSAP has been effective in analyses of DNA methylation in various plant species [200–209]. This technique is widely used in non-model and model plants [210–214]. Being simple and useful, MSAP only provides a general overview of the methylation state and does not provide a specific sequence context. A novel technique called Methylation Sensitive Amplification Polymorphism Sequencing (MSAP-Seq) for the analysis of DNA methylation patterns in *Hordeum vulgare* based on the conventional MSAP analysis, with direct high-throughput sequencing using next-generation sequencing (NGS) and automated data analysis, was introduced [215]. MSAP-Seq allows for the global and direct identification of a large set of sequences that undergo DNA methylation changes without laborious band excisions, re-amplification, and subcloning, which are required for MSAP analysis.

1.6.6 AFLP-RGA

Resistance gene analog-anchored amplified fragment length polymorphism (AFLP-RGA) is a modified AFLP procedure first proposed in soybean (*Glycine max* L.) [216]. Here the degenerate RGA primers are used in combination with selective AFLP primer in the second round of amplification. The AFLP-RGA method combines the approach of AFLP with gene-anchored amplification and can provide more functional markers that are possibly distributed in other regions of the genome, thereby increasing the genome coverage.

1.6.7 TE-AFLP

The three endonuclease AFLP (TE-AFLP) technique reduces the number of amplified fragments not only by primer extension but also by selective ligation. Three endonucleases and two sets of adapters are used in a single reaction. As a consequence, the reduced number of potential amplifiable fragments diminishes competition during PCR, permitting stringent reaction conditions

and thus eliminating the need for a two-step amplification in fingerprinting complex genomes. TE-AFLP primer combinations generated a total of 12 and 48 polymorphic bands in 12 *Pongamia* accessions from different regions of Delhi [217].

1.6.8 SDAFLP

The secondary digest AFLP (SDAFLP) is a variation of MSAP technique wherein a restriction endonuclease site-specific single primer is used to amplify the digested template DNA and later digested with a methylation-sensitive enzyme. The fragments are re-amplified using a primer from previous amplification and a second primer specific to cleavage sites of methylation-sensitive primer [218].

1.6.9 MITE-AFLP

Miniature Inverted-repeat Transposable Elements (MITEs) were transposon elements discovered in plant genomes [219]. A successful application of conserved motif of a *Mite* element as a molecular marker in maize was demonstrated [220] with minor modifications of AFLP protocol.

1.6.10 RNA Fingerprinting Using cDNA-AFLP

cDNA-AFLP is a variation that combines RNA fingerprinting technique and AFLP wherein the standard AFLP protocol is applied on a cDNA template. This method is comparable with the northern blot analysis in studying gene expression [221]. This method is a useful modification to the RNA fingerprinting since it is possible to eliminate all nontarget bands. This modified method can be utilized in gene expression studies vis-a-vis biological pathways in plants. AFLP has also been used to generate mRNA fingerprints in hexaploid wheat and one of its deletion mutants, and the method was found useful for isolating sequences mapping to deleted chromosome segments in hexaploid wheat [222].

1.6.11 Nonradioactive DD-AFLP

It is a method of coupling differential display (DD) and AFLP for monitoring differentially expressed genes. Here double-stranded cDNA molecules are restricted and ligated to the defined adaptor sequences followed by amplification of a subset of ligation products with adaptor-specific primers carrying two or more arbitrary nucleotides and detection of bands representing gene of interest on a polyacrylamide gel. It is considered as a high-throughput method in functional genomics, and DD-AFLP patterns can be simulated for sequenced genomes by computer softwares, and information on undetermined genomes can be retrieved. Several modified methods that avoid use of radioisotopes were optimized and were widely used for detection of responsive genes in plants and tissues subjected to elicitors [223].

1.7 Patents and IPR Protection

Two patents regarding AFLP technology have been filed in the year 2018 and 2019. One patent is concerned with high-throughput detection of molecular markers based on AFLP and high-throughput sequencing. The invention relates to a high-

throughput method for the identification and detection of molecular markers wherein restriction fragments are generated and suitable adaptors comprising (sample-specific) identifiers are ligated. The restriction fragments which are adapter-ligated may be selectively amplified with adaptor-compatible primers carrying selective nucleotides at their 3′ end. The resulting fragments are sequenced at least partly using high-throughput sequencing methods, and the sequence parts of the restriction fragments together with the sample-specific identifiers serve as molecular marker. The other patent is titled as method for high-throughput AFLP-based polymorphism detection. The invention is mainly intended for discovery, detection, and genotyping of one or more genetic markers in one or more samples, comprising the steps of restriction endonuclease digest of DNA, adaptor ligation, optional pre-amplification, selective amplification, pooling of the amplified products, sequencing the libraries with sufficient redundancy, clustering followed by identification of the genetic markers within the library and/or between libraries, and determination of codominant genotypes of the genetic markers [224].

1.8 Conclusions

The wide popularity of AFLP technology is evident from the available literature. It has immense future prospects due to the versatility and flexibility especially in situations where no genomic information is available. The method is reliable both under sophisticated and ordinary conditions of processing and detection. While choosing an appropriate method for molecular marker analysis, the important factors into consideration are low cost, good throughput, convenience, and ease of operation and automation. RAPD, RFLP, SSR, etc. are popularly used markers and each one has its own advantage. However, many studies that we have mentioned in this chapter endorse the superiority of AFLP in diversity analysis, phylogenetic characterization, fingerprinting, etc. Despite the fact that AFLP provides a better coverage and estimate of genetic diversity, it is prudent to consider markers like SSR that are codominant and enable discrimination of heterozygous and homozygous individuals. Dominant AFLPs cannot be used to study heterozygosity. An integrated marker approach was found to be better in many studies for more accurate genotype characterization and taxonomy. It is prudent to use an appropriate marker considering the biological question and geographical scale investigated, last but not least the financial and resource constraints prevailing. More importantly results from molecular studies need to be integrated with knowledge on the morphological characteristics for a better understanding toward genetic improvement as well as germplasm conservation programs.

2 Materials

In case of AFLP ready-made chemicals are generally used. Uniformity in terms of chemical concentration needs to be maintained for all individuals to be analyzed in the AFLP experiments. All the reagents need to be stored at −20 °C. Some AFLP kits are currently available (*see* **Note 1**).

2.1 DNA Template Preparation

1. TE buffer (1): Dissolve 10 mM Tris–HCl and 1 mM EDTA in 1 L ddH_2O, and adjust to pH 8. Store at room temperature.

2.2 Restriction-Ligation (RL)

1. MseI restriction endonuclease (the "frequent cutter"—recognizes a four-base motif, i.e., 5′-TTAA). 1 U MseI is required for one reaction.
2. EcoRI restriction endonuclease (the "rare cutter"—recognizes a six-base motif, i.e., 5′-GAATTC). 5 U EcoRI is required for one reaction.
3. MseI-adaptor pair: 5′-GACGATGAGTCCTGAG and 5′-TAC TCAGGACTCAT. Stored at −20 °C as stock with concentration of 100 μM. Immediately prior to adding to the RL reaction, mix in proportion 1:1 (to obtain a concentration of 50 μM for each), then denature (i.e., heat up at 95 °C for 5 min) the required amount of combined MseI adaptors, and allow slow renature (let them cool slowly at room temperature for 10 min) to form double-stranded adaptor. Spin briefly.
4. EcoRI-adaptor pair: 5′-CTCGTAGACTGCGTACC and 5′-AAT TGGTACGCAGTCTAC. Store each adaptor primer individually at −20 °C as stock with concentration of 100 μM. Immediately prior to adding to the RL reaction, mix in proportion 1:1 (to obtain a concentration of 50 μM for each), then denature (i.e., heat up at 95 °C for 5 min) the required amount of combined EcoRI adaptors, and allow slow renature (let them cool slowly at room temperature for 10 min) to form double-stranded adaptor. Spin briefly.
5. T4 DNA ligase: 0.6 U T4 DNA ligase is required per ligation reaction.
6. T4 DNA ligase buffer.
7. BSA (bovine serum albumin). Stock solution of 10 mg/mL. Dilute prior to use (1 mg/mL).
8. 0.5 M NaCl.
9. TE 0.1 M buffer (1×): Dissolve 20 mM Tris–HCl and 0.1 mM EDTA in 1 L ddH_2O, and adjust to pH 8. Store at room temperature.
10. TBE buffer (stock solution 10×): Dissolve 108 g Tris base, 55 g boric acid, and 8.1 g Na_2EDTA in 1 L ddH_2O. Make up the pH to 8.2–8.3.

11. Size ladder of 1500 bp.
12. Loading buffer for electrophoresis.

2.3 Pre-Selective PCR Amplification (See Note 1)

1. AmpliTaq or RedTaq.
2. Taq DNA polymerase buffer.
3. Deoxynucleotide mix (dNTPs) in concentration 10 mM each dATP, dCTP, dGTP, dTTP. Ready-made mix (e.g., GeneAmp dNTP Blend, 10 mM, from Life Technologies) is recommended.
4. EcoRI primer: 5′-GACTGCGTACCAATTCA. Store as stock solution at 100 μM.
5. MseI primer: 5′-GATGAGTCCTGAGTAAC. Store as stock solution at 100 μM.
6. TE 0.1 M buffer (1×) (prepared as above).
7. 1000 bp ladder.

2.4 Selective PCR Amplification

1. RedTaq (1 unit).
2. RedTaq buffer (10×).
3. dNTPs (10 mM).
4. EcoRI primers: 5-GACTGCGTACCAATTCXXX where X stands for selective nucleotides. These primers are fluorescently labeled, and the working concentration of the EcoRI primer is 1 μM. Store as stock solution (100 μM) for several years and as working solution (1 μM) for several months (*see* **Note 2**).
5. MseI primers: 5-GATGAGTCCTGAGTAAXXX where X stands for selective nucleotides. The working concentration of the MseI selective primer is 5 μM. Store as stock solution (100 μM) and as working solution (5 μM) (*see* **Note 2**).
6. Thermal cycler.

2.5 Separation and Visualization of Fragments (See Note 3)

1. Sephadex G-50 Fine or Superfine. Weigh 10 g of the powder and mix with 120 mL ddH_2O and 100 μL 100× TE buffer. Let it stand for a couple of hours. Store at room temperature and use within 1 week. The solution of Sephadex settles out, and it should be resuspended before using.
2. MultiScreen HV plates. Store at room temperature.
3. GeneScan ROX or another fluorescently labeled, internal ladder suitable for sequencers. Store at 4 °C.
4. Hi-Di formamide.
5. LI-COR DNA Analyzer used for visualization of fragments.
6. Polymer and buffers, specific for the type of sequencer used. Usually stored at 4 °C.

3 Methods

In addition to the described methods, recent AFLP modifications in procedure and detection are also available (*see* **Note 3**).

3.1 DNA Template Preparation

The AFLP procedure requires genomic DNA stored in 1× TE buffer.

3.2 RL (Restriction-Ligation)

1. Heat the required amount of MseI (50 μM) and EcoRI (5 μM) of each adaptor pairs at 95 °C for 5 min, each pair in a separate vial. Allow them to cool gradually to room temperature for 10 min. Spin briefly in a microcentrifuge for 10 s (*see* Subheading 2.2, **item 3**).
2. Master mix for all samples is to be prepared, which is planned to be analyzed in one batch, starting with ddH_2O, T4 ligase buffer (contains 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM dithiothreitol in a solution of pH 7.5 at room temperature), T4 ligase (0.6 units), NaCl (0.5 M), BSA (1 mg/mL), both adaptor pairs, and finishing with the three enzymes. Spin briefly (*see* Subheading 2.2, **item 4**).
3. Aliquot 5.5 μL of the master mix in individual tubes.
4. For each sample, add 5.5 μL DNA in one tube. The final reaction volume will be 11 μL. Vortex and centrifuge briefly.
5. The reaction is incubated at 37 °C for at least 3 h in a thermal cycler with a heated cover. The incubation is continued at 17 °C overnight, or at least for 3 h (17 °C is the optimum temperature for ligation activity).
6. The efficiency of the restriction reaction can be tested by running 5 μL of several of the reactions on 1.5% agarose gel prepared in 1× TBE buffer for 20 min at 90 V (*see* **Note 4**).
7. The reaction is stopped by diluting it 20-fold with 1× TE 0.1 M buffer.
8. The RL reactions can be stored for longer periods at −20 °C.

3.3 Pre-selective PCR Amplification

1. Dilute and mix pre-selective primers in proportion of 1:1:18 with ddH_2O to result in a working concentration of 5 μM each primer (*see* Subheading 2.3, **items 4** and **5**).
2. Prepare a master mix for all samples that you plan to analyze in one batch, starting with ddH_2O, 10× Taq buffer (2.5 μL for each reaction), dNTPs (10 mM), primers (5 μM each), and Taq polymerase (1 unit for each reaction). The quantities of various components are according to manufacturer's instruction.
3. Aliquot 8 μL of the master mix in individual 1.5 mL Eppendorf tubes.

4. Add 2 μL of the diluted RL product to each tube. The final reaction volume will be 10 μL. Vortex and centrifuge (1500 × *g*) briefly.
5. Use a thermal cycler with heated cover and run the following program: one hold of 72 °C for 2 min; 20 cycles of 94 °C for 1 s, 56 °C for 30 s, and 72 °C for 2 min; and finish with a hold of 60 °C for 30 min.
6. The efficiency of the pre-selective amplification can be tested by running 5 μL of several of the reactions on a 1.5% agarose gel in 1× TBE buffer, for 20 min at 90 V. If the RedTaq polymerase is used, no loading buffer is to be used. A smear product with few brighter bands in the 100–1500 base pair range should be visible (*see* **Note 4**).
7. Dilute the pre-selective reactions 20-fold with 1× TE 0.1 M buffer. Mix thoroughly. For the samples for which an aliquot of the PCR product has been run on agarose gel, reduce the dilution volume.
8. Store the diluted pre-selective reactions in the fridge for 1 day and at −20 °C for months.

3.4 Selective PCR Amplification (See Note 5)

1. Prepare a master mix for all samples that is planned to be analyzed in one batch, starting with ddH_2O, 10× Taq buffer (2.5 μL for each reaction), dNTPs (10 mM), primers (EcoRI primer 1 μM and Mse I primer 5 μM), and finishing with the Taq (1 unit). The components were added based upon manufacturer's instruction. Spin briefly.
2. Aliquot 8 μL of the master mix in individual 1.5 mL Eppendorf tubes.
3. Add 2 μL of the diluted pre-selective product to each tube. The final reaction volume will be 10 μL. Vortex and centrifuge (1500 × *g*) briefly.
4. Use a thermal cycler with heated cover and run the following program (90% ramp time): one hold of 94 °C for 2 min; nine cycles of 94 °C for 1 s, 65 °C—1 °C every cycle for 30 s, and 72 °C for 2 min; followed by 23 cycles of 94 °C for 1 s, 56 °C for 30 s, and 72 °C for 2 min; and finish with a hold of 60 °C for 30 min. Program the cycler to keep the reactions at 4 °C until they are removed.
5. Freezing the selective reactions is recommended as soon as possible. They can, however, be kept for 1 day in the fridge.

3.5 Separation and Visualization of Fragments (See Note 6)

1. Apply 200 μL of mixed Sephadex solution to each well of a MultiScreen (MS) HV plate. Place the MS plate on top of a microtiter plate to collect water. Pack the Sephadex by spinning at 600 × *g* for 1 min. Discard water that has been collected in the microtiter plate.

2. Repeat **step 1**.
3. Repeat **step 1** by packing the Sephadex by centrifuging at 600 × *g* for 5 min.
4. The MS plate is placed along with the Sephadex filter on top of a fresh microtiter plate to collect the filtered selective product.
5. Mix together the selective reactions of up to three primer combinations corresponding to one individual sample, by applying 5 μL of each selective PCR product, and the PCR product was labeled separately for easy identification (e.g., labeled green, yellow, and blue). Spin the MS plate (on top of the clean microtiter plate) at 600 × *g* for 5 min (*see* **Note 6**).
6. Discard the Sephadex filter. The HV plate can be reused for up to ten times after washing.
7. Make up the loading mixture for the number of samples to be loaded on the sequencer using 9.8 μL Hi-Di formamide and 0.2 μL of GeneScan ROX per sample. Do not forget to account also for two more samples as a tolerance for potential pipetting inaccuracies.
8. Aliquot 10 μL of loading mixture to each well of a clean microtiter plate.
9. Add 1.2 μL of the filtered, combined selective products to each well. Vortex and centrifuge briefly.
10. Cover the microtiter plate containing loading mixture and sample; heat it up at 95 °C for 5 min and cool the plate on ice immediately to denature the AFLP fragments.
11. Load the plate containing the denatured samples onto the sequencer.

4 Notes

1. PE Applied Biosystems (Foster City, CA, USA) has developed an AFLP™ Plant Mapping Kit based on the AFLP procedure patented by Keygene NV (Wageningen, The Netherlands). Two modules are available depending on the genome size. The Small Plant Genome Kit is used for genomes ranging from 50 to 500 megabases, and the Regular Plant Genome Kit is for genomes of 500–5000 megabases. Restriction fragments are generated using EcoRI and MseI restriction enzymes. For pre-amplification, both pre-selective primers in the Regular Plant Genome Kit have an additional selective nucleotide at the 3′-end. However, only the MseI pre-selective primer has a selective base in the Small Plant Genome Kit. AFLP Analysis System II, a kit developed by Thermo Fisher Scientific, is designed for use with plants having genomes ranging in size from 1×10^8 to 5×10^8 bp. The

AFLP Analysis System I is designed for plants having genome size of 5×10^8 to 6×10^9 bp range. AFLP kits developed by Li-COR Biotechnologies also helps to genotype individuals in certain populations with less genetic variability.

2. The number of selective nucleotides of the primers can be increased or decreased based on the genome size and the availability of restriction sites in the genomes that are to be analyzed. Longer pre-selective and selective primers are used for large genomes and shorter selective primers, with only two selective nucleotides for smaller genomes. The use of a different combination of restriction enzymes results in fine-tuning of the number of AFLP fragments generated as a result.
3. Since the original AFLP protocol was published (1), numerous variants have been introduced. The major improvements in the main protocol include (1) the use of IRDye® infrared dye (IRD) or other fluorescently labeled oligonucleotide primers instead of radioactive ones and (2) fragment analysis with an automated DNA sequencer instead of polyacrylamide gel electrophoresis. AFLP markers generated using IRD primers and visualization of fragments by a gel-based sequencer such as a LI-COR DNA Analyzer produced successful results for plant species with genomes of varying complexities [225–227].
4. A smear product in the 100–1500 base pair range should be visible. Make sure the genomic DNA is fully restricted, so no high-weight DNA molecules are present.
5. Another modified protocol wherein which genomic DNA was digested with 5 units of EcoRI and 5 units of TruI (an isoschizomer of MseI). Selective PCR reaction was done with fluorescently labeled EcoRI+NNN and 1 mM un-labeled MseI+CTT [228].
6. A modified protocol in amaranth [229] involved the analysis of AFLP products in ABI PRISM 310 Genetic analyzer (Applied Biosystems), and GeneScan software program was also used in the analysis. Modification in the analysis of AFLP fragments was introduced for AFLP marker study of the wild species of lettuce crop, *Lactuca aculeata*, resistant against downy mildew pathogen [230]. AFLP analyses were performed using the commercial IRDye® Fluorescent AFLP® Kit designed for large plant genome analysis. The results were visualized using an automated AFLP analysis program (LI-COR SAGAMX v.3.3) [78]. In another modification of the protocol (1), Pst1 and EcoR1 and the 4-bp cutting enzyme Mse1 were used. PCR reactions were set up in Beckman Biomek 2000 liquid handling device. Electrophoresis was carried out on the Bio-Rad Sequi-Gen GT system. A Promega *fmol* DNA Cycle Sequencing System marker (Promega Q4100) was run to estimate the

product size and "control lanes" of standard potato genotypes. Gels were dried onto paper exposed to X-ray film which was then developed using a Konica Minolta film processor (SRX-101A 2006) [59]. A modified protocol was followed for AFLP fingerprinting [231], wherein which the primer combinations with highest polymorphic index were selected to investigate the genetic variability in separate sets of analysis for wild population of two important medicinal plant species. In a modified protocol developed [232], fluorescently labeled AFLP primer combinations were used, and PCR products were separated using capillary electrophoresis.

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Chapter 13

Random Amplified Polymorphic DNA (RAPD) and Derived Techniques

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Abstract

Understanding biology and genetics at molecular level has become very important for dissection and manipulation of genome architecture for addressing evolutionary and taxonomic questions. Knowledge of genetic variation and genetic relationship among genotypes is an important consideration for classification, utilization of germplasm resources, and breeding. Molecular markers have contributed significantly in this respect and have been widely used in plant science in a number of ways, including genetic fingerprinting, diagnostics, identification of duplicates and selection of core collections, determination of genetic distances, genome analysis, development of molecular maps, and identification of markers associated with desirable breeding traits. The application of molecular markers largely depends on the type of markers employed, distribution of markers in the genome, type of loci they amplify, level of polymorphism, and reproducibility of products. Among many DNA markers available, random amplified polymorphic DNA (RAPD) is the simplest, is cost-effective, and can be performed in a moderate laboratory for most of its applications. In addition, RAPDs can touch much of the genome and has the advantage that no prior knowledge of the genome under research is necessary. The recent improvements in the RAPD technique like arbitrarily primed polymerase chain reaction (AP-PCR), sequence characterized amplified region (SCAR), DNA amplification fingerprinting (DAF), sequence-related amplified polymorphism (SRAP), cleaved amplified polymorphic sequences (CAPS), random amplified microsatellite polymorphism (RAMPO), and random amplified hybridization microsatellites (RAHM) can complement the shortcomings of RAPDs and have enhanced the utility of this simple technique for specific applications. Simple protocols for these techniques are presented along with the applications of RAPD in genetic diversity analysis, mapping, varietal identification, genetic fidelity testing, etc.

Key words AP-PCR, SCAR, DAF, SRAP, CAPS, RAMPO, RAHM, DNA fingerprinting, Genetic diversity, Population and evolutionary genetics, Mapping, Genetic fidelity, Cultivar identification, Bulked segregant analysis

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1 Introduction

1.1 RAPD Technique

The advent of polymerase chain reaction (PCR) and subsequent emergence of DNA-based markers have provided plant taxonomists easy and reliable techniques to study the extent and distribution of variation in species gene pools and to answer typical evolutionary and taxonomic questions which were not previously possible with only phenotypic methods. Properties desirable for ideal DNA markers include highly polymorphic nature, codominant inheritance, and frequent occurrence in the genome, easy access, easy and fast assay, and high reproducibility. DNA marker systems based on PCR include random amplified polymorphic DNAs (RAPDs) [1], amplified fragment length polymorphisms (AFLPs) [2] (Chapter 12), microsatellites/simple sequence repeats (SSRs) [3] (Chapter 11), and single nucleotide polymorphisms (SNPs) [4] (Chapters 9 and 10). Although the sequencing-based molecular techniques provide better resolution at intra-genus and above level [5], they are expensive and laborious. Frequency data from markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites provide the means to classify individuals into nominal genotypic categories and are mostly suitable for intraspecies genotypic variation study. Compared to other PCR-based techniques which vary in detecting genetic differences and applicability to particular taxonomic levels, RAPD is a cost-effective tool for taxonomic studies.

RAPD is an adaptation of the PCR which relies on the rationale that at low stringency, a given synthetic oligonucleotide primer is likely to find a number of sequences in the template DNA to which it can anneal when these sites are close to each other and lie in opposite orientations and the DNA sequence between the sites will be amplified to produce a DNA fragment characteristic of that genome. Multiple bands of different sizes produced from the same genomic DNA constitute a "fingerprint" of that genome [1]. Patterns from different individuals and species will vary as a function of how similar the genomic DNA sequences are between samples. RAPD polymorphisms result from either chromosomal changes in the amplified regions or base changes that alter primer binding. This assay has the advantage of being readily employed, requiring very small amounts of genomic DNA, and eliminating the need for blotting and radio-active detection. As RAPD requires initial genome information, it provides markers in regions of the genome previously inaccessible to analysis. RAPD-derived estimates of genetic relationships are in good agreement with pedigree, RFLP, and isozyme data [6, 7].

1.2 Recent Applications of RAPD and Its Derived Techniques

DNA fingerprinting for cultivar or varietal identification has become an important tool for estimating genetic diversity for plant breeding, germplasm management, utilization [8], monitoring genetic erosion, and removing duplicates from germplasm collections [9]. As RAPD markers could gain information about genetic similarities or differences that are not expressed in phenotypic information, RAPD analysis becomes an inexpensive tool to characterize germplasm collections [10], to understand the pattern of evolution from wild progenitors, and to classify them into appropriate groups.

RAPDs have been successfully applied in estimation of varietal distinctiveness and relatedness of commercially important crops and registration activities like cultivar identification [11] and hybrid verification [12]. The potential of RAPD for varietal identification has been used to know about the variety being exported or sold under various trade names, for settling a lawsuit involving unauthorized commercialization of patented varieties [13], and to identify the cases of adulteration and even the level of adulteration [14].

As RAPDs make use of arbitrary primers, some of them amplify DNA at highly conserved region, leading to generate polymorphisms at a high level of classification, whereas some will amplify at highly variable region, useful for classification and analyses at and below the species level. This property of RAPD is taxonomically useful at subgeneric level [15] and species level [16] and for the analysis of geographic variation. Another application of RAPD is for evaluation of the genetic integrity of somatic embryo-derived plants [17].

RAPDs have significant use in ecology in studying mating systems and assigning paternity. In plants, insect pollination might be studied by fingerprinting all the potential pollen sources by RAPDs and comparing the dominant RAPD bands seen in the resulting seeds [18]. RAPDs are useful in hybridization studies to document intergeneric hybridization [19] to identify species specific bands as well as interspecific hybridization and detection of introgression in both natural and cultivated plant populations [20]. RAPDs may provide insights into organismal evolutions that are overlooked by single-gene comparisons [21].

The RAPD technique has received a great deal of attention from population geneticists [22] because of its simplicity and rapidity in revealing DNA-level genetic variation.

The RAPD protocol is refined to techniques like sequence characterized amplified region (SCAR), arbitrarily primed polymerase chain reaction (AP-PCR), DNA amplification fingerprinting (DAF), sequence-related amplified polymorphism (SRAP), cleaved amplified polymorphic sequences (CAPS), random amplified microsatellite polymorphism (RAMPO), and random amplified hybridization microsatellites (RAHM) so that some of the current problems such as lack of reproducibility and codominant nature of

inheritance will be overcome. Using several strategies, various modifications have been developed in conjunction with RAPD to enhance the ability to detect polymorphism either by using more than one arbitrary primer [23] or by using a degenerate primer in the amplification reaction [24].

Sequence characterized amplified region (SCAR) markers are generated by sequencing RAPD marker termini and designing longer primers (22–24 nucleotide bases long) for specific amplification of a particular locus [25, 26]. SCARs are usually dominant markers; however, some of them can be converted into codominant markers by digesting them with tetra cutting restriction enzymes, and polymorphism can be deduced by either denaturing gel electrophoresis or single-strand conformation polymorphism (SSCP) [27]. Besides higher specificity, it is based on the presence/absence of a single specific amplicon, considerably simplifying the interpretation of the results, especially when a large number of samples are checked. SCARs also allow comparative mapping or homology studies among related species, thus making them an extremely adaptable concept in the near future.

Arbitrarily primed polymerase chain reaction (AP-PCR) is a special case of RAPD, wherein discrete amplification patterns are generated by employing single primers of 10–50 bases in length in PCR of genomic DNA. Unlike RAPDs, the oligonucleotide length and primer concentrations are tenfold higher [28], and two cycles of low-stringency annealing conditions to allow mismatches followed by PCR at high stringency and the newly synthesized fragments are radiolabeled using dCTP. AP-PCR generated fragments are analyzed as plus/minus DNA amplification-based polymorphism [29] due to either sequence divergence at one of the priming sites or insertion/deletion within the amplification region.

DNA amplification fingerprinting (DAF) uses single arbitrary primers as short as five bases to amplify DNA using polymerase chain reaction with high multiplex ratio [30]. This marker shares those features common to AP-PCR and RAPDs—namely, it results in plus/minus heritable amplification polymorphism, a preponderance of dominant marker loci, and unknown allelism between fragments of equivalent molecular weight. DAF bands contain many more bands than AP-PCR and RAPD patterns, and the likelihood is increased for observing polymorphism between samples. DNA amplification fingerprinting (DAF) has been found to be promising in many plants for cultivar identification and sex determination [31] and for determination of genetic origin and diversity analysis [32].

The sequence-related amplified polymorphism (SRAP) technique, a variation of RAPD, also uses arbitrary primers of 17–21 nucleotides to generate a specific banding pattern aimed to amplify coding sequences (open reading frames (ORFs)) in the genome [33] and results in a moderate number of codominant markers.

SRAP results from two events: fragment size changes due to insertions and deletions, which could lead to codominant markers, and nucleotide changes leading to dominant markers. It has several advantages over other systems: simplicity, reasonable throughput rate, and it allows easy isolation of bands for sequencing, discloses numerous codominant markers, and allows screening thousands of loci shortly to pinpoint the genetic position underlying the trait of interest. The primers and primer concentration vary for each RAPD derived technique which increases its utility in various applications (*see* **Note 1**).

To derive greater information from RAPD patterns, the strategy of hybridizing SSR repeat primers to RAPD amplification patterns has been described. The method has been called either random amplified hybridization microsatellites (RAHM) [34] or random amplified microsatellite polymorphism (RAMPO) [35]. In RAHM, RAPD amplification and oligonucleotide screening are combined for detection of microsatellites to provide more information from RAPD gels and also help to reveal microsatellite genomic clones without the time-consuming screening of genomic libraries [34] (Chapter 9). RAMPO combines arbitrarily or semi-specifically primed PCR with microsatellite hybridization to produce several independent and polymorphic genetic fingerprints per electrophoretic gel. In this approach, the amplified products resolve length polymorphism that may be present either at the SSR target site itself or at the associated sequence between the binding sites of the primers [35]. The RAPD binding site actually serves as an arbitrary end point for the SSR-based amplification product, and therefore, the products obtained are not as restricted by the relative genomic positions of a specific SSR.

Another strategy is referred to as cleaved amplified polymorphic sequences (CAPS), in which sequence information from cloned RAPD bands can be used for analyzing nucleotide polymorphisms. CAPS markers rely on differences in restriction enzyme digestion patterns of PCR fragments caused by nucleotide polymorphism between ecotypes. Sequence information available in databank of genomic DNA or cDNA sequences or cloned RAPD bands can be used for designing PCR primers for this process. Cleaved amplified polymorphic sequences (CAPS) [36] are analogous to RFLP markers in that a region of DNA containing a restriction enzyme site unique to an allele is amplified, cleaved, and compared for their differential migration [36, 37]. The sizes of the cleaved and uncleaved amplification products can be adjusted arbitrarily by the appropriate placement of the PCR primers. Critical steps in the CAPS marker approach include DNA extraction, PCR conditions, and the number or distribution of polymorphic sites.

RAPD has gained a lot of popularity over the last decades due to its ease of operation, low cost, and versatility. It has been extensively used in cultivar identification, genetic diversity analysis, population studies, mapping, molecular breeding and gene tagging, genetic fidelity establishment, etc. RAPD-based identification and characterization of plant genetic resources have helped in attaining goals of conservation of plant resources and in understanding extent and distribution of variation in species gene pools to sort out evolutionary and taxonomic ambiguities. Frequency data from RAPD helps to classify individual into genotypic classes and thus is appropriate for intraspecies genotypic variation studies. RAPD either alone or in combination with other markers like RFLP and SSR provides essential start points for physical isolation of genes of interest, which may further be exploited through marker- assisted selection, gene pyramiding, and transfer to other species. Especially in gene tagging, RAPDs are a preferred method in self-pollinated crops wherein variations between individuals within a species or related breeding material is sought [38]. RAPD is a preferred method for detecting genetic variations induced by somaclonal variation in micro-propagated as well as cryopreserved plants [39]. However, the usage of RAPD has shown a decline in the past few years owing to several factors including the lack of reliability and reproducibility of the technique, advent of novel and derived strategies, and cost-effective means of next-generation sequencing methods. Hence, in the recent references, we could find a trend wherein RAPD analysis was done using very high number of primers [40] or was used along with other markers like ISSR (inter-simple sequence repeat), SSR, AFLP [41, 42], etc., for improving reliability of results. The various applications of RAPD and its derived techniques in plants are extensively dealt in earlier reviews [43–52]. Here, we have compiled only the recent important references on applications of RAPD and its derived techniques as detailed below.

1.2.1 Cultivar Identification

Traditionally, grapevine cultivars have been identified based on the morphological characteristics, but because of the similar pedigree backgrounds, the identification of closely related cultivars has been difficult. Identification of 37 different grapevine cultivars was done using 16 SCAR markers developed from RAPD marker [53]. For identifying cultivars based on random amplified polymorphic DNA (RAPD) markers, cultivar identification diagrams (CIDs) provide a rapid and efficient approach. About 64 tomato cultivars were identified using CID [54]. About 22 onion cultivars were identified using RAPD markers. The cultivars could be easily distinguished based on the polymorphic bands produced by various RAPD primers [55]. Ten autochthonous cultivars of sweet cherry (*Prunus avium*) were validated using 30 RAPD markers. It was also possible to distinguish two important cultivars of tremendous market value

based on the markers [56]. In olive, cultivars sampled from different countries in the Mediterranean region exhibited high resolving power for cultivar identification using RAPD [57]. RAPD technique was used for rapid characterization of Indian medicinal plant *Strychnos minor* Dennst of 16 different localities of Coromandel Coast of Tamil Nadu [58].

SCAR markers based on species-specific RAPD amplicons were developed in four species of the medicinal tuber, *Pinellia ternata*, *Pinellia tripartita*, *Pinellia pedatisecta*, and *Typhonium flagelliforme*, for verification through multiplexing [59]. RAPD-PCR-amplified fragments were used to develop SCAR markers for identification of medicinal plant *Lonicera japonica* [60] and in longan fruits [61]. RAPD fragments from *Litchi chinensis* were cloned, sequenced, and converted into stable SCAR markers for authentication and validation of *L. chinensis* cultivars [62]. Certification of the two maple species, red maple (*Acer rubrum*) and silver maple (*A. saccharinum*), and their hybrids was done through the development of SCAR markers. The information obtained can be used for tracking the introgression of *A. rubrum* and *A. saccharinum* DNA in other hybrid trees or their populations [63]. RAPD-DAF markers were used to discriminate between jalapeño peppers with little phenotypic difference [64]. In yet another study, RAMP-PCR-amplified fragments were used to develop four novel SCAR markers for the genetic authentication of *L. japonica* from its substitutes [65]. RAMP-PCR was found to be better than traditional RAPD-PCR when employed to study genetic diversity and varietal authentication of the herb *Angelica sinensis* (Oliv) [66].

1.2.2 Genetic Mapping and Tagging

For genetic mapping applications, RAPD has been known as a non-biased and neutral marker. It does not require information about a particular sequence in the genome [67]. In RAPD analysis, the entire plant genome is targeted for primer annealing which facilitates development of a higher density map. RAPD does not require DNA probes, blotting and hybridization, and primer designing procedures. Small amounts of DNA are required, and high-throughput sampling can be obtained. RAPD generated DNA fragments possessed many of the DNA sequences that are related to chromosome size changes as it is reported in many studies that the amplified fragments in an RAPD reaction were preferentially amplified from species containing a common genome consisting of large chromosomes [68]. The above advantages make RAPD a preferred choice in gene tagging involving several different types of populations like backcross selection progenies, recombinant inbred lines, near-isogenic lines, etc. Bulked segregant analysis was also employed to tag traits from populations having contrasting characters [38].

In *Saccharum officinarum* L., an RAPD marker was found to be linked to eyespot susceptibility, and it also helped to identify additional linkage groups. This particular work showed that linkages identified in this map could potentially be used for marker-assisted selection [69]. Molecular evaluation of two guava mapping populations (MP), MPI comprising 94 F1 progenies and MPII comprising 46 F1 progenies, was carried out using random amplified polymorphic DNA (RAPD) markers. Genotypic data thus generated can be further exploited for constructing genetic linkage maps and mapping complex Quantitative Trait Loci (QTLs) governing fruit quality traits in guava [70]. A reference genetic map for *Capsicum baccatum* was constructed based on RAPD molecular markers [71]. Using SRAP markers, a molecular genetic map for hawthorn, a medicinal plant, was constructed which can be used for marker-assisted selection in the particular plant species [72].

1.2.3 Assessment of Outcrossing Rates

Outcrossing rates in sweet passion fruit were assessed using RAPD molecular markers. The results showed that all the progenies assessed were derived as a result of outcrossing [73]. RAPD was used to study outcrossing in *Agave schottii*, and it was found that RAPD markers are useful tools for assessing ecological phenomena like outcrossing [74]. RAPD markers were used to estimate the outcrossing rate in Ethiopian mustard (*Brassica carinata*). It was analyzed by looking into the banding pattern of offsprings of two parental lines grown in open pollinated isolation lines [75]. The rate of outcrossing in orchards containing 'Hass' avocado (*Persea americana* Mill.) was determined using RAPD markers. The data included 2393 fertilization events taken from two areas of southern California of different climate over a period of 4 years. Three potential pollen sources were also investigated using RAPD markers specific to each pollen source [76]. RAPD markers were found to be useful in understanding breeding patterns in faba beans [77]. In *B. carinata*, RAPD markers helped in estimating outcrossing rate and the opportunity for exploiting heterosis through synthetic and/or hybrid cultivar breeding [75].

1.2.4 Genetic Fidelity Testing

Genetic fidelity testing of in vitro propagated *Araucaria excelsa* R. Br. var. *glauca* plantlets was done using RAPD technique. A total of 1676 fragments were generated with 12 RAPD primers in micro-propagated plants and mother plants [78]. RAPD was employed to test the genetic fidelity among the regenerants in *Spilanthes calva* DC [79]. Genetic fidelity was confirmed in micro-propagated *Drosera* plantlets using RAPD [80]. Assessment of genetic fidelity through RAPD analysis was done in in vitro raised plants (*Swertia chirayita*), and the plants showed high clonal fidelity [81]. In vitro regeneration of *Guizotia abyssinica* Cass and

evaluation of genetic fidelity through RAPD markers showed the presence of somaclonal variation in the plantlets arising from direct regeneration as well as from indirect regeneration [82]. Some studies endorse utilizing one more marker like ISSR in conjunction with RAPD for better analysis of genetic fidelity in banana [83], grapes [84], and mango ginger [85]. Genetic stability of in vitro propagated potato micro-tubers examined using AFLP, SSR, and ISSR indicated them to be superior to RAPD [86]. In endemic medicinal plants *Pittosporum eriocarpum* Royle [87] and *Rauvolfia tetraphylla* L., [41], RAPD was used to validate the genetic homogeneity of in vitro raised plantlets in conjunction with SCoT and ISSR markers. In *Salvia hispanica* L., a reasonably good number of RAPD and ISSR primers were employed for confirming genetic fidelity of in vitro regenerated plantlets [88]. The genetic uniformity of blackberry plants (*Rubus fruticosus* L.) obtained by micropropagation was analyzed by RAPD and SRAP markers [89]. ISSR and RAPD analysis was used to assess genetic uniformity of transgenic cotton containing Bt and chitinase genes [42].

1.2.5 Inter and Intraspecies Variations and Genetic Diversity

RAPD is found to be more suitable in large-scale screening of closer populations found in similar habitats. However, the discrimination capacity decreases relatively when populations from distant locations are analyzed. RAPD may not be much suitable for genetic diversity analyses of populations in wide geographic areas. RAPD includes some deflections in the genetic discrimination of populations having high genetic diversity in different habitats. Combining RAPD and SCAR markers provides a simple and reliable tool for genetic characterization of plant species. Genetic diversity of 21 aromatic rice genotypes (*Oryza sativa* L.) was assessed using about 38 RAPD primers [90]. The RAPD profile helps to identify variations of the diagnostic markers on aromatic rice genotypes [91], identification of rice at the level below species [92]. For the identification and protection of natural resources, genetic tracking of aromatic rice germplasm is essential. Genetic variation in *Ocimum* species was studied using RAPD markers. Many unique species-specific alleles were amplified by RAPD in *Ocimum* species [93]. In bamboo, RAPD-RFLP analysis was able to generate a low-cost and fast screening method for genetic characterization of genera and species of bamboo [94]. In *Miscanthus* spp., genetic diversity and relationships based on RAPD and AFLP indicated significant genetic differentiation among accessions due to geographic distance [95].

Genetic diversity analysis in sweet potato [96] and *Elymus* spp. [97] indicated a close correspondence of RAPD and ISSR markers in detecting variability. Genetic diversity studies in *Harpagophytum* species using ISSR and RAPD markers indicated evidences of introgression and interspecific gene flow [98]. Genetic diversity analysis

of cumin genotypes based on sequence-related amplified polymorphism (SRAP) markers was conducted, and it was found that there is a need for enhancing the genetic base of cumin germplasm using different breeding approaches, viz., mutagenesis, wide hybridization or somaclonal variation, and germplasm introduction [99]. Genetic diversity and population structure study within and among six natural populations of *Limonium sinense*, a plant which has medicinal and ornamental values, was conducted using SRAP markers, which could develop insight and useful strategies for its conservation [100]. A highly efficient and economical technology of sequence-related amplified polymorphism (SRAP) molecular markers with an automated fragment analyzer ABI 3500xL was developed, to detect genetic diversity in upland cotton [101]. Genetic diversity studies in strawberry cultivars in Indonesia using CAPS molecular markers resulted in the grouping of the cultivars into four clusters [102].

Cleaved amplified polymorphic sequence (CAPS) marker analysis of four chloroplast DNA regions, rbcL-ORF106, trnF-trnV, trnV-rbcL, and trnK2-trnQ, in 42 citrus accessions including mandarins and their close relatives showed their close relationship and low variation in chloroplast DNA of mandarins [103].

1.2.6 Others

RAPD was used to evaluate genotoxic effects in many studies to identify DNA damage induced due to harmful agents like heavy metals [104–106]. RAPD was successfully applied to whole germplasm collections of flax to identify redundant and distinct accessions and associated traits useful in future breeding programs [107] and to identify duplicates in germplasm collections of rice at International Rice Research Institute, Philippines [108]. RAPD is a preferred choice for the detection of adulteration in medicinal plants and successfully used especially when the adulterant is a different species [109, 110]. An interesting study has been reported that utilizes commercial RAPD analysis beads in differentiating about 63 different food and feed legume species for establishing authenticity and correct labeling of raw material used in food or feed samples [111]. Similarly in medicinally important *Ocimum* spp., diagnostic RAPD markers were useful in identifying raw materials for herbal drugs [112]. RAPD markers linked to disease-resistant genes in plants like the rpg4 gene responsible for stem rust resistance in barley [113] and heat smut resistance [114] have been identified. Dwarfism gene has been located by an RAPD marker in barley [115]. RAPD markers were exploited in identifying somatic hybrids [116]. RAPD was successfully used to reveal polymorphism in mutant potato [117] and chrysanthemum [118] obtained via gamma irradiation.

1.3 Disadvantages of RAPD Technique and Solutions

The main concern about RAPD is its lack of reproducibility within and between laboratories. Differences in amplification patterns based on type of thermocycler and primers used and also concentration of Taq polymerase and amplification conditions are the commonly reported issue. The most important factor affecting reproducibility is the low quality of DNA template [28]. Differences between template DNA concentrations of individual samples can also affect the amplification profile [45]. It is a dominant marker and presence of a band of apparently identical molecular weight in two different individuals cannot be considered as identical loci and thus gives more accurate estimates between closely related populations than the distant ones (1). A single RAPD band can be comprised of a number of co-migrating amplification products. However, it is suggested that RAPD polymorphisms can be successfully reproduced among laboratories when standard reaction conditions are used and similar temperature profiles in tubes are followed [119]. Some authors also report that when more samples and primers are included in the study, the fingerprint and phylogeny are more accurate [120]. A preliminary pedigree analysis is a prerequisite to assign markers to specific loci. To get comparable results with other codominant markers, two to ten times more individuals need to be sampled per locus, and marker alleles for these loci should be in low frequencies [121]. Many studies indicate that RAPD shows significant difficulties in cultivar characterization due to low polymorphism, irreproducibility, and weak grouping due to artifacts [122, 123]. RAPD marker identity might be established by fingerprinting a set of standard genotypes by RAPD to facilitate communication and the reproducibility among laboratories. In cases where a single primer is unable to distinguish all cultivars in a study, a combination of polymorphic bands generated by various primers can be utilized. Converting RAPD markers to

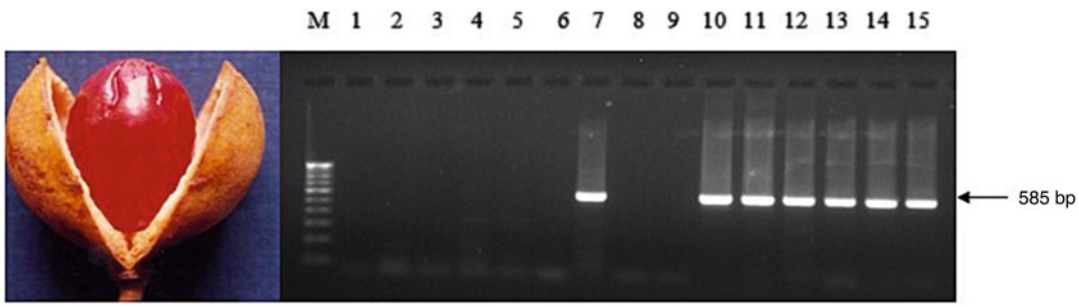

Fig. 1 Unique RAPD-derived SCAR marker for identification of an endangered and endemic species of *Myristica*, viz., *Knema andamanica*. (**a**) Fruits of *K. andamanica* with unique fused mace. (**b**) RAPD derived SCAR marker showing amplification of a marker of 585 bp in *K. andamanica* accessions absent in other wild and related genera of *Myristica*. Lanes M- 100 bp marker. 1–8: *M. fragrans*, *M. beddomei*, *M. malabarica*, *M. prainii*, *M. fatua*, *M. andamanica*, *K. andamanica*, *M. amygdalina*, 9: Control, lanes 10–15: Different germplasm accessions of *K. andamanica* from the repository at ICAR-IISR

more reliable SCAR markers and also using one or two other marker methods in conjunction with RAPD are some useful tips to improve reliability and reproducibility of results (Fig. 1).

2 Materials

2.1 Genomic DNA Isolation and Quantification

1. Extraction buffer (2×): 2% cetyltrimethylammonium bromide (CTAB), 100 mM Tris HCl, pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8, 1.4 M NaCl, 1% polyvinyl polypyrrolidone (PVPP).
2. Chloroform: isoamyl alcohol (24:1).
3. 100% Ethanol or isopropanol.
4. 70% Alcohol.
5. TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8).
6. RNase A (10 mg/mL).
7. Tris-acetate-EDTA (TAE) buffer (pH 8) (50×).
8. Agarose.
9. Ethidium bromide (10 mg/mL).
10. Loading dye (6×): 30% glycerol, 5 mM EDTA, 0.15% bromophenol blue, 0.15% xylene cyanol.
11. MassRuler 1000 bp DNA ladder.

2.2 Reagents Used for RAPD-PCR

1. Taq DNA polymerase with 10× buffer.
2. 10 mM dNTPs: 10 mM each of dATP, dCTP, dGTP, and dTTP.
3. 25 mM $MgCl_2$.
4. 10 μM Primers (operon primers are the most commonly used RAPD primers) (*see* **Notes 1** and **2**).
5. Milli-Q water.

2.3 Sequence Characterized Amplified Region (SCAR)

2.3.1 Genomic DNA Isolation and Quantification

(*See* Subheading 2.1).

2.3.2 Reagents for PCR

(*See* Subheading 2.2).

2.3.3 Gel Extraction

1. QIAquick gel extraction kit, Qiagen, Germany.

2.3.4 Cloning of PCR Amplified Gene

1. PCR amplified and purified product.
2. PCR cloning vector.
3. T4 DNA ligase.
4. Ligation buffer (5×).
5. Sterile deionized water.
6. Overnight culture of *E. coli* DH5∝.
7. $CaCl_2$ (100 mM).
8. Mg Cl_2 (25 mM).
9. LB medium.
10. Sterile microcentrifuge tubes and tips.
11. Sterile glycerol (80%).
12. LB agar with ampicillin (100 µg/mL), X gal (20 µg/mL), and IPTG (40 µg/mL).

2.4 Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

(*See* Subheading 2.1).

2.4.1 Genomic DNA Isolation and Quantification

2.4.2 Reagents for PCR

1. Taq polymerase.
2. PCR buffer (10×).
3. 25 mM $MgCl_2$.
4. 10 mM each of dNTPs.
5. 50 µCi α-[^{32}P] dCTP.
6. 10 µM of each primer.

2.4.3 Electrophoresis

1. 40% Acrylamide-bis-acrylamide.
2. 7.5 M Urea.
3. Tris-borate-EDTA (TBE) buffer, pH 8 (10×).

2.5 DNA Amplification Fingerprinting (DAF)

(*See* Subheading 2.1).

2.5.1 Genomic DNA Isolation and Quantification

2.5.2 Reagents for PCR

(*See* Subheading 2.2).

2.5.3 PAGE Reagents

1. 40% Acrylamide-bis-acrylamide.
2. 7.5 M Urea.
3. Tris-borate-EDTA (TBE) buffer, pH 8 (10×).
   Cover the bottle with aluminum foil and store at 4 °C and use before 1 month.
4. 10 bp MassRuler.
5. 100 bp MassRuler.

2.5.4 Silver Staining Reagents

1. Acetic acid, glacial.
2. Silver nitrate crystal, AR (ACS) ($AgNO_3$).
3. Formaldehyde solution, AR (ACS) (HCHO).
4. Sodium thiosulfate (Na_2S_2O).
5. Sodium carbonate powder, ACS reagent (Na_2CO_3).
6. Ethanol.
7. Silver staining solution (250 mg silver nitrate and 375 μL formaldehyde and 50 μL sodium thiosulfate).
8. Ice-cold developer solution (10 °C) (7.5 g sodium carbonate, 375 μL formaldehyde, and 50 μL sodium thiosulfate (10 mg in 1 mL water) in 250 mL water).
9. Formamide loading dye: 80% formamide, 10 mM EDTA, pH 8.0, 1 mg/mL xylene cyanol, 1 mg/mL bromophenol blue, 50% glycerol in a final volume of 10 mL.

2.6 The Sequence-Related Amplified Polymorphism (SRAP) Technique

2.6.1 Genomic DNA Isolation and Quantification

(*See* Subheading 2.1).

2.6.2 Reagents for PCR Conditions

(*See* Subheading 2.2 but using different primers in **step 4**).

1. *Primers*: The arbitrary primers consist of the following elements: core sequences, which are 13 to 14 bases long, where the first ten or 11 bases starting at the 5′ end are sequences of no specific constitution ("filler" sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The purpose of using the "CCGG" sequence in the core of the first set of SRAP primers was to target exons to open reading frame (ORF) regions.

2.6.3 PAGE Electrophoresis

(*See* Subheadings 2.5.3 and 2.5.4).

2.7 Random Amplified Microsatellite Polymorphism (RAMPO)

(*See* Subheading 2.1).

2.7.1 Genomic DNA Isolation and Quantification

2.7.2 Reagents Used for RAPD and Microsatellite-Primed PCR (MP-PCR)

(*See* Subheading 2.2).

2.7.3 Hybridization with Microsatellite-Complementary Probes

1. Nylon membrane (Hybond, Amersham).
2. ^{32}P-labeled microsatellite-complementary oligonucleotide probes.
3. 5 mM EDTA.

2.8 Random Amplified Hybridization Microsatellites (RAHM)

(*See* Subheading 2.1).

2.8.1 Genomic DNA Isolation and Quantification

2.8.2 Reagents Used for RAPD-PCR

(*See* Subheading 2.2).

2.8.3 Hybridization with Microsatellite-Complementary Probes

(*See* Subheading 2.7.3).

2.9 Cleaved Amplified Polymorphic Sequences (CAPS)

(*See* Subheading 2.1).

2.9.1 Genomic DNA Isolation and Quantification

2.9.2 Reagents for PCR Conditions

(*See* Subheading 2.2).

2.9.3 Restriction Enzyme Digestion

1. Restriction enzymes: Mse I, Alu I, Mbo I, Hae III.
2. Buffer 2 (New England Biolabs (NEB), UK)—supplied at 10× concentration.
3. NEB buffer 2 (1×).

4. 50 mM NaCl.
5. 10 mM Tris–HCl.
6. 10 mM $MgCl_2$.
7. 1 mM DTT, pH 7.9 at 25 °C.
8. 100× BSA (10 mg/mL)—use at 1×.

2.9.4 PAGE Reagents (*See* Subheading 2.5.3).

2.9.5 Silver Staining Reagents (*See* Subheading 2.5.4).

3 Methods

3.1 Isolation of Genomic DNA (Modified Doyle and Doyle, 1990) [124]

1. Grind 2 g of clean young leaf tissue to fine powder with a pestle and mortar after freezing in liquid nitrogen; transfer it to 10 mL CTAB extraction buffer and incubate at 60 °C for 1 h.
2. Extract the supernatant with chloroform: isoamyl alcohol (24:1) and centrifuge at 12,378 × *g* for 10 min at room temperature.
3. Precipitate the DNA with 100% ethanol or isopropanol; centrifuge at 19,341 × *g* for 10 min at 4 °C.
4. Wash the DNA with 70% ethanol; centrifuge at 19,341 × *g* for 5 min at 4 °C.
5. Dry the pellet and dissolve the DNA in 1× TE buffer.
6. Treat the DNA in solution with RNase (10 μg/mL) at 37 °C for 30 min.
7. Wash with chloroform: isoamyl alcohol (24:1) and centrifuge at 12,378 × *g* for 10 min at room temperature.
8. Precipitate with 100% ethanol and dissolve in 1× TE buffer. Store frozen at −20 °C.

3.2 DNA Quantification

It is an essential step in many procedures where it is necessary to know the amount of DNA that is present when performing techniques such as PCR and RAPDs.

3.2.1 By Gel Electrophoresis

The comparison of an aliquot of the extracted sample with standard DNAs of known concentration (Lambda *Hind* III) can be done using gel electrophoresis.

1. 5 μL of the DNA is mixed with 1 μL of 6× loading dye and loaded onto a 0.8–1% agarose gel along with 500 ng of Lambda *Hind* III digest marker and electrophoresed at 90 V for 30 min.
2. The quantity of extracted DNA is estimated based on the intensity of Lambda *Hind* III digest marker bands as the top

bands account for half amount (250 ng) of total loaded amount.

3. The quality of genomic DNA is confirmed for its integrity.

3.2.2 Using UV Spectrophotometer

1. Take 1 mL of TE buffer in a cuvette and calibrate the spectrophotometer at 260 nm and 280 nm wavelength.
2. Add 2 to 5 μL of DNA, mix properly, and record the optical density at both 260 nm and 280 nm.
3. Estimate the DNA concentration employing the following formula:

$$\text{Amount of DNA } (\mu g/\mu L) = (\text{OD})\ 260 \times 50 \times \text{dilution factor}/1000$$

4. Judge the quality of DNA from the ratio of OD values recorded at 260 and 280 nm. Pure DNA has values close to 1.8.
5. Dilute the DNA sample to get 20 ng/μL.

3.3 RAPD

3.3.1 PCR Amplification of Genomic DNA with Primers

Amplify 20–50 ng of genomic DNA in a reaction mix containing 1.0 U *Taq* DNA polymerase, 1 μM primer, 1.5–2.0 mM $MgCl_2$, 0.125 mM each of dNTPs, and 1× *Taq* DNA polymerase buffer (*see* **Note 1**).

1. The amplification profile consists of an initial denaturation of 3 min at 94 °C followed by 35–40 cycles of denaturation for 1 min at 94 °C, annealing for 37 °C for 1 min and extension at 72 °C for 2 min and final extension for 6 min at 72 °C (*see* **Note 2**).

3.3.2 Gel Electrophoresis

1. Amplified RAPD products are separated by horizontal electrophoresis in 1.5% (w/v) agarose gel, with 1× TAE buffer, stained with ethidium bromide (0.5 μg/mL) and analyzed under ultraviolet (UV) light. The length of the DNA fragments is estimated by comparison with DNA ladder.

*3.3.3 Scoring and Interpretation of RAPD Banding Patterns (See **Note 3**)*

Variability is then scored as the presence or absence of a specific amplification product.

Polymorphism usually results from mutations or rearrangements either at or between the primer binding sites, due to appearance of a new primer site, mismatches at the primer site, and difference in the length of the amplified region between the primer sites due to deletions or insertions in the DNA.

1. Each gel is analyzed by scoring the present (1) or absent (0) polymorphic bands in individual lanes. The scoring procedure is based on the banding profiles which are clear, transparent, and repeatable (*see* **Note 4**).

   The RAPD profiles are compared between the genotypes to estimate the similarity index. Studies are initiated to assess the similarity/differences between the genotypes using RAPD polymorphism as estimated by Paired Affinity Indices (PAIs).

   PAI is calculated by the formula PAI
   = no.of similar bands/total no.of bands

   The PAIs expressed as percentage indicate the similarity (%) between any two genotypes.
2. The binary matrix is transformed into similarity matrix using Dice similarity (NTSYS-PC 2.01; Numerical Taxonomy System of Multivariate Programs) [125]. The Dice coefficient is preferred to the Jaccard coefficient because it assigns weights to matches rather than to mismatches and does take shared absences of bands into account (*see* **Notes 5** and **6**).
3. The similarity matrix is subjected to a clustering analysis using the unweighted pair group method with arithmetic means (UPGMA; NTSYS-PC 2.0) [125].
4. The RAPD matrix can also be analyzed using the neighbor-joining (N-J) method. Evaluate statistical support for the clusters recovered both in the UPGMA and N-J trees by generating 1000 bootstrap pseudoreplicates (*see* **Note 7**) (Fig. 2).

3.4 Sequence Characterized Amplified Region (SCAR)

1. Genomic DNA is isolated, quantified, and diluted (*see* Subheading 3.1).
2. 20–50 ng of genomic DNA is amplified using random primers (*see* Subheading 3.3.1).

3.4.1 Amplification

3. Aliquots (5.0 μL) of RAPD products are separated by horizontal electrophoresis in 1.5% (w:v) agarose gel, with 1× TAE buffer, stained with ethidium bromide (0.5 μg/mL) and analyzed under ultraviolet (UV) light. The length of the DNA fragments is estimated by comparison with DNA ladder.

3.4.2 RAPD Fragment Selection and Cloning

1. From obtained RAPD fingerprints, the polymorphic RAPD marker bands are selected.
2. These bands are cut, eluted, and purified using QIAquick gel extraction kit, cloned and sequenced.
4. Primer design: New longer and specific primers of 15–30 bp are designed for the DNA sequence, which is called the SCAR (*see* **Note 8**).

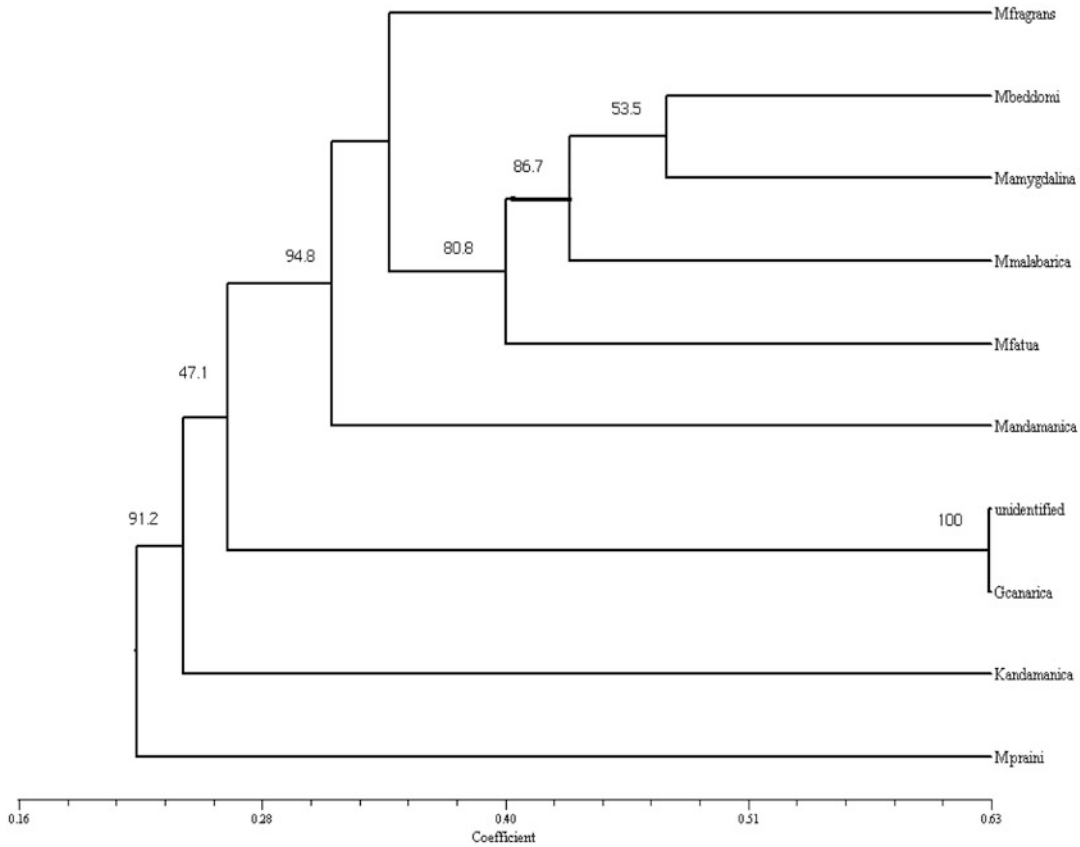

Fig. 2 Dendrogram generated using UPGMA using RAPD marker data in wild and related genera of *Myristica*. Number of forks indicates confidence limits for grouping of those species in a branch occurred, based on 2000 cycles in bootstrap analysis, using Winboot program

3. PCR amplification: For the verification of primers ability to amplify predicted fragment length, primers are tested with isolated DNA.

3.5 Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

3.5.1 Amplification

1. Amplify 20 ng genomic DNA in a PCR reaction mix containing 0.025 U Taq polymerase and 1× buffer (Stratagene) with 4 mM $MgCl_2$, 0.2 mM of each dNTP, and 10 μM primer.
2. Amplification profile consists of an initial denaturation of 94 °C for 5 min followed by 40 °C for 5 min for low stringency annealing of primer and 72 °C for 5 min for extension for two cycles. This temperature profile is followed by ten high stringency cycles: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min for ten cycles.
3. At the end of this reaction, add 90 μL of a solution containing 2.25 U Taq polymerase in 1× buffer, 0.2 mM dNTPs, and 50 μCi α-[^{32}P] dCTP, and the high stringency cycles are continued for an additional 20 or 30 rounds.

3.5.2 Electrophoresis

1. Prepare the 40% stock 19:1 acrylamide bis-acrylamide solution and store it in dark bottles at 4 °C.
2. Prepare 5% working solution containing 7.5 M urea, 40% acrylamide bis-acrylamide. Assemble electrophoresis unit by adding 0.5× TBE buffer to upper tank and lower tank.
3. Add 4 μL of the loading buffer to 8 μL of the final amplified reaction mix.
4. Load this sample into the gel and conduct electrophoresis at 200 V for 55 min.
5. The AP-PCR generated fragments are size separated on polyacrylamide and visualized via radiography.

3.6 DNA Amplification Fingerprinting (DAF)

3.6.1 Amplification

1. Amplify 20 ng of genomic DNA in a 10 μL PCR reaction mix containing 0.5 U of Taq polymerase, 200 μM each dNTP, 0.5 μM primer, and 1× PCR buffer with 2 mM $MgCl_2$ overlaid with a drop of mineral oil.
2. The amplification profile consists of an initial denaturation at 5 min of 94 °C followed by 40 cycles of denaturation for 5 s at 94 °C, annealing at either 35 °C or 45 °C and 30 s at 72 °C.
3. The amplification products are separated in a vertical electrophoresis system using 5% non-denaturing polyacrylamide gel of 0.5 mm thickness to separate DNA fragments according to their molecular weight.
4. Gel preparation (*see* Subheading 3.5.2).

3.6.2 Silver Staining for DNA Visualization

1. Gently place the gel in 10% (v/v) glacial acetic acid for 30 min at room temperature.
2. Rinse the gel in deionized water twice for about 2 min each.
3. Immerse the gel in silver staining solution for 20 min.
4. Pour out the silver stain solution and wash the gel quickly with deionized water within 10 s.
5. Immerse the gel in an ice-cold developer solution (10 °C) until optimal image intensity is obtained. Stop the developing process by immersing the gel in 7.5% ice-cold glacial acetic acid.
6. Transfer gel onto the Whatman paper.
7. Air-dry the gel or dry using gel drier at 70 °C for 30 min.

3.6.3 Gel Interpretation

Scoring can be done by the presence or absence of band. Bands are sized and matched directly on gels, autoradiographic or photographic films, or photocopies on transparency overlays.

3.7 Sequence-Related Amplified Polymorphism (SRAP) (See Note 9)

3.7.1 Amplification

1. Amplify 20 ng of genomic DNA in a PCR reaction mix containing 1 U of *Taq* polymerase, 200 μM each dNTP, 0.1 mM each forward and reverse primer, and 1× PCR buffer with 1.5 mM $MgCl_2$.
2. The amplification profile consists of an initial denaturation at 2 min of 94 °C followed by five cycles of denaturation for 1 min at 94 °C, annealing at 35 °C for 1 min and 72 °C for 1 min; followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; followed by 7 min at 72 °C.
3. Polyacrylamide gel electrophoresis (*see* Subheading 3.5.2).
4. Marker analysis: Each polymorphic band can be scored as a single dominant marker.

3.7.2 Sequencing of SRAP Marker Bands

1. After electrophoresis, the gel is exposed overnight to a high-sensitivity film (Kodak BioMax).
2. Using the exposed film as a blueprint, the gel pieces containing the polymorphic bands are cut and introduced into a dialysis tube.
3. The dialysis tube is placed into the buffer tank of a sequencing-gel apparatus, and the DNA is electro-eluted in 1× TBE buffer. The application of 2000 V, which is the same voltage used for running sequencing gels, results in the complete electro-elution of DNA into buffer from the gel fragment.
4. After ethanol precipitation and TE buffer suspension, the DNA can be used for direct sequencing.

3.8 Random Amplified Microsatellite Polymorphisms (RAMPO)

(*See* Subheadings 3.1 and 3.2).

3.8.1 Genomic DNA Isolation

3.8.2 Amplification of Genomic DNA with RAPD Primers/Microsatellite Primers

1. The DNA is first amplified with a single arbitrary (*see* Subheading 3.3.1) or microsatellite-complementary PCR primer (MP-PCR) (*see* **Note 10**).
2. The products are separated on agarose gel (1.4%), stained with ethidium bromide, and photographed.

3.8.3 Hybridization with Microsatellite-Complementary Probes

1. The gel is either dried or blotted onto a nylon membrane.
2. Hybridize to a [^{32}P]-labelled, microsatellite-complementary oligonucleotide probe.
3. Hybridization was done overnight at 42 °C containing 20–40 ng/mL of the probe.

4. Filters are washed twice for 5 min at room temperature in 2× SSC, 0.1% SDS followed by two final washing steps (2× 15 min) at different stringencies.
5. The stringency can be varied through temperature (50–65 °C) and salt concentration (1× SSC; 0.1% SDS to 0.1× SSC; 0.1% SDS).
6. Positive signals are detected by chemiluminescence system and documented by exposure to X-ray film for 1–2 h.

3.9 Random Amplified Hybridization Microsatellites (RAHM)

1. Amplify the DNA using RAPD primers (*see* Subheading 3.3.1).
2. The amplified products are separated by gel electrophoresis (*see* Subheading 3.3.2).
3. The polymorphisms on the agarose gel are identified and scored (*see* Subheading 3.3.3).
4. The amplified DNA is then transferred onto Hybond-N+ filters using Southern blot procedures.
5. The filters are then hybridized with radiolabeled oligonucleotide probes carrying simple sequence repeats (SSR).
6. The luminescent signals produced are detected by autoradiography. Hybridizing bands are named random amplified hybridization microsatellites (RAHM).

3.10 Cleaved Amplified Polymorphic Sequences (CAPS)

1. Genomic DNA is isolated (*see* Subheadings 3.1 and 3.2).
2. Amplify the different CAPS marker locus by PCR (*see* Subheading 3.3.1).
3. Analyze the PCR by gel electrophoresis to confirm amplification of DNA and the yield.
4. Mix 5 μL PCR reaction and 10 μL digest mix. The reaction mixture for the enzyme digestion contained 5 μL PCR product, 9 μL ddH_2O, and 0.3 μL restriction enzyme (10 U/μL), which were then incubated at 37 °C for 5 h and then heated to 65 °C for 5 min.
5. Mix equal parts of digest mix and formamide loading dye. Denature sample by heating at 94 °C for 5 min and then placing tube on ice.
6. Resolve restriction fragments using 1× TBE, 8.25% polyacrylamide gel.
7. Load 2.5 μL of the denatured sample per lane.
8. Denature by heating at 94 °C for 5 min and then placing tube on ice.
9. Load 3.5 μL of the denatured ladder per lane, equivalent to 117 ng DNA.

10. Run gel at 80 W for approximately 80 min or until the bromophenol blue dye front has reached the bottom of the gel.
11. Follow usual silver staining protocol to stain gel (*see* Subheading 3.6.2).

4 Notes

1. RAPD reaction is far more sensitive than conventional PCR because of the length of a single and arbitrary primer used to amplify anonymous regions of a given genome. Optimization of reaction conditions should precede the actual RAPD analysis to get consistent and reproducible results. The following optimizations are essential: template DNA concentration and quality, *Taq* DNA polymerase concentration, Mg^{2+} ion concentration, primer concentration and annealing temperature, and primers suitable for detection of polymorphic loci in the taxa to be analyzed [126].
2. Too many RAPD cycles can increase the amount and complexity of nonspecific background products, while too few cycles give low product yield. The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized. Although the sequences of RAPD primers are arbitrarily chosen, two basic criteria must be met: a minimum of 40% GC content (50–80% GC content is generally used) and the absence of palindromic sequence (a base sequence that reads exactly the same from right to left as from left to right). Because G-C bond consists of three hydrogen bridges and A-T bond consists of only two, a primer-DNA hybrid with less than 50% GC will probably not withstand the 72 °C temperature at which DNA elongation takes place by DNA polymerase [1].
3. Data from at least ten primers with a total of 100 RAPD bands are needed to produce a stable classification [127].
4. The probability of a scored RAPD band being scored in replicate data is strongly dependent on the uniformity of amplification conditions between experiments, as well as relative amplification strength of the RAPD band [128]. The criteria for selecting scoring bands include reproducibility and consistency (the experiments need to be repeated to achieve reproducible results) and thickness and size of the bands.
5. Deleting inconsistent or faint bands or using only those bands that are reproducible introduces false negatives, and simply ignoring RAPD artifacts and using all bands introduces false positive into RAPD data [129].

6. If estimates of the percent of false-positive and false-negative bands in the RAPD data are available (such as when replicate runs have been made), equations described earlier [130] can be used to determine the actual bias by subtracting the true value from the estimated value. Once the bias is known, it can be used to determine whether the RAPD protocol has been optimized sufficiently to provide accurate enough estimates of the similarities.
7. Other softwares like PAUP, PHYLIP, CLINCH, MaClade, PopGene, and Arlequin can also be used to accomplish the cluster algorithms and for phylogenetic analysis.
8. In SCAR, the longer primer sequence increases the specificity of the PCR reaction and produces results less sensitive to changes in reaction conditions. SCAR is thus more reproducible than RAPD [131].
9. The rationale behind primer designing in SRAP is based on the fact that exons are normally in GC-rich regions. The core is followed by three selective nucleotides at the 3′ end. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long.
10. If RAPD gels were used for RAMPO analysis, banding patterns are generally less complex, less variable, and easier to interpret than those derived from MP-PCR gels [132].

References

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