Chapter 10

Randomly 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 selecting core collections, determination of genetic distances, genome analysis, developing 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 and 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 AP-PCR, SCAR, DAF, SRAP, CAPS, RAMPO, and 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.

Key words RAPD, AP-PCR, SCAR, DAF, SRAP, CAPS, RAMPO, RAHM, DNA fingerprinting, Genetic diversity, Population and evolutionary genetics

1 Introduction

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,

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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 polymorphism (AFLPs) [2] (*see* Chapter 11), microsatellites/simple sequence repeats (SSRs) [3] (*see* Chapter 9), and single-nucleotide polymorphisms (SNPs) [4] (*see* Chapter 9). Although the sequencing-based molecular techniques provide better resolution at intra-genus and above level [5], it is 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.

RAPDs 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 fragments 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 radioactive detection. As RAPD require 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].

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, registration activities like cultivar identification [11], or control of seed purity of hybrid varieties [12]. The potential of RAPD for

varietal identification has been used varietal to know about the variety being exported or sold under various trade names, to settling a lawsuit involving unauthorized commercialization of a patented varieties [13], 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 high levels 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], 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 assumption of homology between bands of apparently the same molecular weight from the same primer is potentially another problem for RAPD surveys. Homology between co-migrating bands in different individuals is a good assumption when individuals are from the same population. This may not be true when individuals belong to different species or widely divergent populations [23]. Because the chance of co-migrating bands being homologous becomes less as populations diverge, it was suggested [1, 23] that RAPD analysis gives more accurate estimates between closely related populations and less accurate estimates for distantly related populations.

A disadvantage of RAPD markers is the fact that they are dominant markers and provides no information on heterozygosity. RAPD markers can be converted into codominant markers called SCAR markers (sequence characterized amplified regions) [24]. RAPDs also have shortcomings of reproducibility of data.

The reproducibility of different molecular markers, RAPD, AFLP, and SSR, tested in plants by a network of European laboratories [25] in which an optimal system (genetic screening package) was present was distributed to each of the laboratories. Different experiences were gained in this exchange experiment with the different techniques. RAPDs were found to be easy to perform by all groups, but reproducibility was not achieved to a satisfactory level.

For AFLPs, a single-band difference was observed in one track, while SSR alleles were amplified by all laboratories, but small differences in their sizing were obtained. Hence, RAPD marker identity might be established by fingerprinting a set of standard genotypes by RAPD to facilitate communication and the reproducibility among laboratories, which may be influenced by the independence of RAPD polymorphisms relative to each other and the distribution of polymorphism across genotypes [26].

The RAPD protocol is refined to techniques like SCAR, AP-PCR, DAF, SRAP, CAPS, RAMPO, and 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 [27] or by using a degenerate primer in the amplification reaction [28].

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 particular locus [29, 30]. 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 SSCP [31]. 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 it an extremely adaptable concept in the near future.

Arbitrary 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 [32], 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 [33] 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 5 bases to amplify DNA using polymerase chain reaction with high multiplex ratio [34]. 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 found to be promising in many plants for cultivar identification and sex determination [35] and for determination of genetic origin and diversity analysis [36].

The sequence-related amplified polymorphism technique (SRAP), a variation of RAPD, also uses arbitrary primers of 17–21 nucleotides to generate a specific banding pattern aimed to amplify coding sequences (ORFs) in the genome [37] and results in a moderate number of codominant markers. SRAP polymorphism 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, 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 of the RAPD-derived techniques 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) [38] or random amplified microsatellite polymorphism (RAMPO) [39]. 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 [38] (*see* 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 [39]. 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 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 data bank 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) are PCR- RFLP markers performed by digesting locus specific PCR amplicons with one or more restriction enzymes followed by separation of the digested DNA on agarose or polyacrylamide gels [40, 41]. 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.

2 Materials

2.1 Genomic DNA Isolation and Quantification

1. 2× extraction buffer: (2 % cetyltrimethylammonium bromide (CTAB), 100 mM Tris–HCl, pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8, 1.4 M NaCl, 1 % polyvinylpolypyrrolidone (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. 50× Tris-Acetate-EDTA (TAE) buffer (pH 8).
8. Agarose.
9. Ethidium bromide (10 mg/mL).
10. 6× loading dye (30 % glycerol, 5 mM EDTA, 0.15 % bromophenol blue, 0.15 % xylene cyanol).
11. MassRuler 1,000 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 2** and **3**).
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. 5× ligation buffer.
5. Sterile deionized water.
6. Overnight culture of E coli DH5α.
7. $CaCl_2$ (100 mM).
8. $MgCl_2$ (25 mM).
9. LB medium.
10. Sterile micro centrifuge 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 Arbitrary Primed Polymerase Chain Reaction (AP-PCR)

2.4.1 Genomic DNA Isolation and Quantification (See Subheading 2.1)

2.4.2 Reagents for PCR

1. Taq polymerase.
2. 10× PCR buffer.
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. 10× Tris-Borate-EDTA(TBE) buffer, pH 8.

2.5 DNA Amplification Fingerprinting (DAF)

2.5.1 Genomic DNA Isolation and Quantification (See Subheading 2.1)

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. 10× Tris-Borate-EDTA(TBE) buffer, pH 8.
   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 mg).

2.6 The Sequence-Related Amplified Polymorphism Technique (SRAP)

Primers. The arbitrary primers consists of the following elements: core sequences, which are 13–14 bases long, where the first 10 or 11 bases start 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 for using the "CCGG" sequence in the core of the first set of SRAP primers was to target exons to open reading frame (ORF) regions (*see* **Note 4**).

2.6.1 Genomic DNA Isolation and Quantification (See Subheading 2.1)

2.6.2 Reagents for PCR (See Subheading 2.2)

2.6.3 PAGE Electrophoresis (See Subheadings 2.5.3 and 2.5.4)

2.7 Randomly Amplified Microsatellite Polymorphism (RAMPO)

2.7.1 Genomic DNA Isolation and Quantification (See Subheading 2.1)

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.

2.7.4 Autoradiography

2.8 Random Amplified Hybridization Microsatellites (RAHM)

1. 10- mer primers (Operon Technologies, Alameda, CA, USA)
2. Hybond-N+ filters (Amersham Inc.)
3. Oligonucleotide probes carrying simple sequence repeats (SSR) labeled with Digoxigenin-ddUTP (DIG Oligonucleotide 3'-End Labeling Kit, Boehringer Mannheim)
4. Gel purification - 'Double Geneclean' (BIO 101 Inc., USA)

2.8.1 Genomic DNA Isolation and Quantification (See Subheading 2.1)

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)

2.8.4 Autoradiography

2.9 Cleaved Amplified Polymorphic Sequences (CAPS)

1. Restriction enzymes: Mse I, Alu I, Mbo I, and Hae III.
2. Buffer 2 (NEB)—supplied at 10× concentration.
3. 50 mM NaCl.
4. 10 mM Tris–HCl.
5. 10 mM MgCl2.
6. 1 mM DTT pH 7.9 at 25 °C.
7. 100× BSA (10 mg/mL)—use at 1×.

2.9.1 Genomic DNA Isolation and Quantification (See Subheading 2.1)

2.9.2 Reagents for PCR Conditions (See Subheading 2.2)

2.9.3 Restriction Enzyme Digestion

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 [42])

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 with chloroform: isoamyl (24:1) and centrifuge at 12,378 × *g* for 10 min at room temperature.
3. Precipitate the DNA with 100 % ethanol or isopropanol and centrifuge at 19,341 × *g* for 10 min at 4 °C.

4. Wash the DNA with 70 % ethanol and 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 (*see* **Note 5**).

3.2.1 By Gel Electrophoresis

The comparison of an aliquot of the extracted sample with standard DNAs of known concentration (lambda *Hin* 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 *Hin* 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 *Hin* III digest marker bands as the top bands accounts 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 and 280 nm wavelength.
2. Add 2–5 μL of DNA mix properly and record the optical density at both 260 and 280 nm.
3. Estimate the DNA concentration employing the following formula:
   Amount of DNA (μg/μL) = (OD) 260 *50* dilution factor/1,000.
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 (See **Notes 2, 3, 6, and 7**)

1. 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 $MgCl_2$, and 0.125 mM each of dNTPs and 1× *Taq* DNA polymerase buffer (*see* **Note 6**).
2. 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** 7).

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

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.

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* **Notes 8–10**) (Fig. 1).

1. 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 (PAI).
   PAI was calculated by the formula PAI = No. of similar bands/Total no. of bands.
   The PAIs expressed as percentage indicated 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) [43] as the Dice coefficient/Jaccard coefficient assigns weights to matches rather than to mismatches and does take shared absences of bands into account.

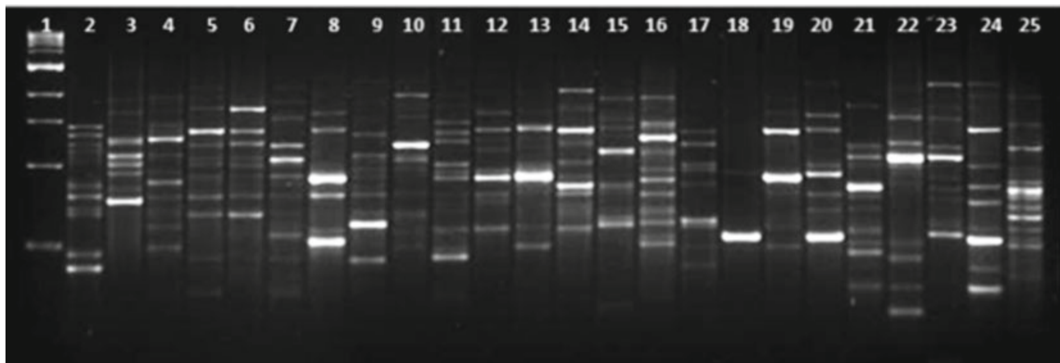

Fig. 1 RAPD polymorphism expressed by "operon primers" OPC-09 in wild and related species of black pepper (*Piper nigrum*) (1) 1 kb ladder, (2) *P. longum*, (3) *P. hapnium*, (4) *P. mullesua*, (5) *P. attenuatum*, (6) *P. argyrophyllum*, (7) *P. hymenophyllum*, (8) *P. bababudani*, (9) *P. trichostachyon*, (10) *P. galeatum*, (11) *P. sugandhi*, (12) *P. psuedonigrum*, (13) *P. nigrum*, (14) *P. schimdti*, (15) *P. wightii*, (16) *P. silentvalyensis*, (17) *P. barberi*, (18) *P. betel*, (19) Cultivated black pepper cv. Karimunda, (20) *P. chaba-1*, (21) *P. chaba-2*, (22) *P. colubrinum-1*, (23) *P. colubrinum-2*, (24) *P. arboreum*, and (25) *P. ornatum*

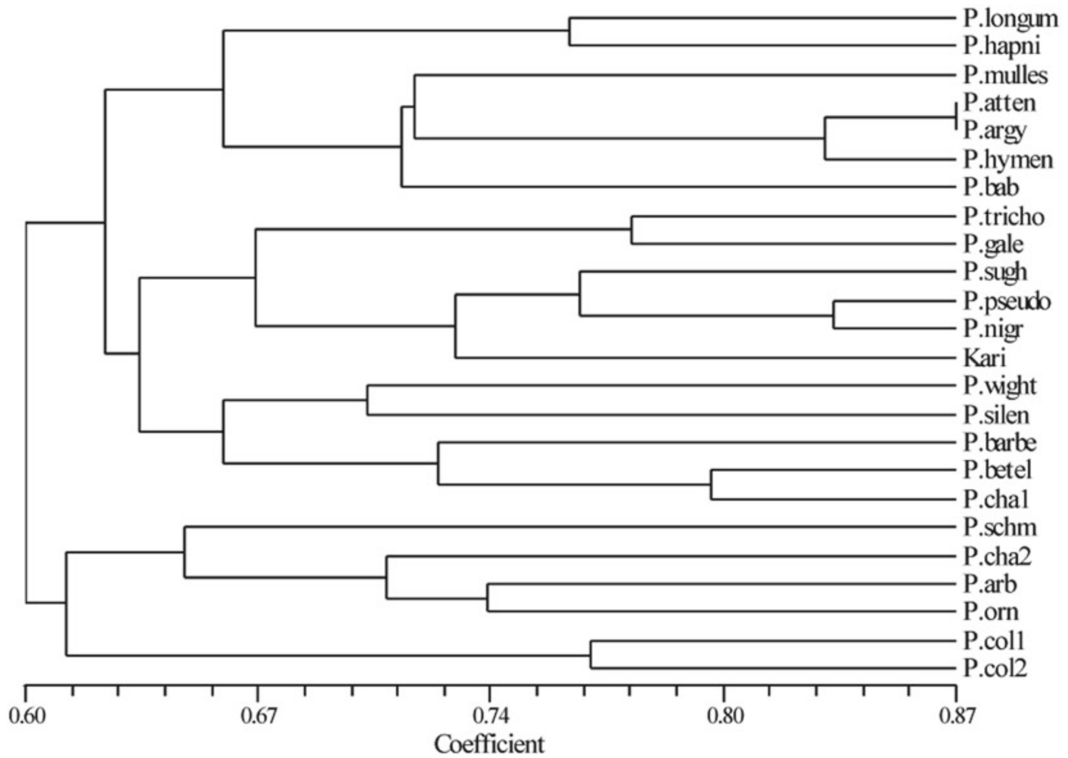

Fig. 2 Dendrogram of interrelationships among wild and related species of black pepper (*Piper nigrum*) (1) *P. longum*, (2) *P. hapnium*, (3) *P. mullesua*, (4) *P. attenuatum* (5) *P. argyrophyllum*, (6) *P. hymenophyllum*, (7) *P. babadani*, (8) *P. trichostachyon*, (9) *P. galeatum*, (10) *P. sugandhi*, (11) *P. psuedonigrum*, (12) *P. nigrum*, (13) *P. schimdti*, (14) *P. wightii*, (15) *P. silentvalyensis*, (16) *P. barberi*, (17) *P. betel*, (18) Cultivated black pepper cv. Karimunda, (19) *P. chaba-1*, (20) *P. chaba-2*, (21) *P. colubrinum-1*, (22) *P. colubrinum-2*, (23) *P. arboreum*, and (24) *P. ornatum*

3. The similarity matrix is subjected to a clustering analysis using the unweighted pair group method with arithmetic means (UPGMA; NTSYS-PC 2.0) [43] (*see* **Notes 11** and **12**).
4. The RAPDs matrix can be analyzed using the neighbor-joining (N-J) method and evaluated statistical support for the clusters recovered both in the UPGMA and N-J trees by generating 1,000 bootstrap pseudoreplicates.
5. Dendrograms are then constructed according to the UPGMA, using NTSYS-PC 2.01 [43] (Fig. 2).

3.4 Sequence Characterized Amplified Region (SCAR)

3.4.1 Amplification

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 3.2).
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 Fragments 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.
3. PCR amplification: For the verification of primers ability to amplify predicted fragment length, primers are tested with isolated DNA.
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 13**).

3.5 Arbitrary Primed Polymerase Chain Reaction (AP-PCR)

3.5.1 Amplification

1. Amplify 20 ng genomic DNA in a PCR mix containing 0.025 U Taq polymerase and 1× buffer (Stratagene) adjusted to 4 mM with $MgCl_2$, 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 10 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 store it in dark bottles at 4 °C.
2. Prepare 5 % working solution containing 7.5 M urea, 40 % acrylamide bis-acrylamide, TBE buffer, and 10× TBE buffer. 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 18 W 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 mix containing 0.5 U of Taq polymerase, 200 μM each dNTPs, 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 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)

3.7.1 Amplification

1. Amplify 20 ng of genomic DNA in a PCR mix containing 1 U of Taq polymerase, 200 μM each dNTPs, 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 5 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; and 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 was electroeluted in 1× TBE buffer. The application of 2,000 V, which is the same voltage used for running sequencing gels, resulted in the complete electroelution 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 Randomly Amplified Microsatellite Polymorphisms (RAMPO)

3.8.1 Genomic DNA Is Isolated (See Subheadings 3.1 and 3.2)

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 14**).
2. The products are separated by on 1.4 % agarose gels, stained with ethidium bromide, and photographed.
3. Before hybridization to a new probe, membranes are stripped by washing in 5 mM EDTA at 60 °C (2× 30 min).

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]-labeled, microsatellite-complementary oligonucleotide probe.
3. Hybridization is 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; 0.1I SDS followed by two final washing steps (2 × 15 min) at different stringency.
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 either chemiluminescence system and documented by exposure to X-ray film for 1–2 h.

3.9 Random Amplified Hybridization Microsatellites (RAHM)

1. The DNA is amplified 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. Amplifying the different CAPS marker locus by PCR.
3. Analyzing the PCR by gel electrophoresis to confirm amplification of DNA and the yield.

4. Mix 5 μL PCR and 10 μL digest mix, incubate at 37 °C for 5 h, and then heat 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. Randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR) use relatively low concentrations (e.g., 0.2 μmol/L) of single short oligonucleotide primers in the PCR with annealing temperatures ranging from 37 to 40 °C, and up to 20 markers can be simultaneously amplified and detected. DNA amplification fingerprinting (DAF) also implements a single short oligonucleotide primer but at a higher concentration (5 μmol/L), and higher annealing temperatures (53–57 °C) using DNA polymerase Stoffel Fragment in PCR.
2. 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 the A–T bond 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 10 primers with a total of 100 RAPD bands are needed to produce a stable classification [44].
4. 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.

5. The most important factor for reproducibility of the RAPD profile has been found to be the result of inadequately prepared template DNA which could be overcome through choice of an appropriate DNA extraction protocol to remove any contaminants [45]. Differences between the template DNA concentrations of two DNA samples will result in the loss or gain of some bands.
6. 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. 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 [46].
7. 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.
8. 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 [26].
9. 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 [47].
10. The criteria for selecting scoring bands include reproducibility and consistency—the experiments need to be repeated to achieve reproducible results, thickness, and size of the bands.
11. 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 [48] 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.
12. Other softwares like PAUP, PHYLIP, CLINCH, MaClade, PopGene, and Arlequin can also be used to accomplish the cluster algorithms and for phylogenetic analysis.

13. In SCAR, the longer primer sequence increases the specificity of the PCR and produces results less sensitive to changes in reaction conditions and thus more reproducible than RAPD [49].
14. If RAPD gels are used for RAMPO analysis, banding patterns are generally less complex, less variable, and easier to interpret than those derived from MP-PCR gels [50].

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