Using molecular marker technology in studies on plant genetic diversity

DNA-based technologies

PCR-based technologies

PCR with arbitrary primers

(RAPDs, DAF, AP-PCR)

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PCR with arbitrary primers

MAAP (multiple arbitrary amplicon profiling) is the acronym proposed to cover the three main technologies that fall in this category:

- Random amplified polymorphic DNA (RAPD)
- DNA amplification fingerprinting (DAF)
- Arbitrarily primed polymerase chain reaction (AP-PCR)

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Three main techniques fall within the category of PCR-based markers using arbitrary primers: RAPD, DAF and AP-PCR. MAAP is the acronym proposed, but not commonly used, by Caetano-Anollés *et al.* (1992) to encompass all of these closely related techniques. In this submodule, special attention will be given to RAPD, concluding with a comparison of RAPD with DAF and AP-PCR.

Reference

Caetano-Anollés, G., B.J. Bassam and P.M. Gresshoff. 1992. DNA fingerprinting: MAAPing out a RAPD redefinition? Bio/Technology 10 (9):937.

RAPD technology, step by step

- Main features
  - Uses a short random primer (usually 10 bases)
  - Amplifies anonymous stretches of DNA
- Laboratory steps are:
  - Isolating DNA
  - PCR reaction with a primer
  - Separating DNA fragments by gel electrophoresis
  - Visualising DNA fragments, using ethidium bromide

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The random amplified polymorphic DNA (RAPD) technique is a PCR-based method that uses a short primer (usually 10 bases) to amplify anonymous stretches of DNA. With this technique, there is no specific target DNA, so each particular primer will adhere to the template DNA randomly. As a result, the nature of the obtained products will be unknown. The DNA fragments generated are then separated and detected by gel electrophoresis.

Isolating DNA

Total, chloroplast or mitochondrial DNA can be used

- Tiny amounts of DNA are sufficient
- DNA must be clean and of high molecular weight

If minimal quality of DNA is not achieved, the reproducibility of results will be hard to ensure

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PCR reaction

- Components for a PCR
- Primers (10 bases long) are commercially available from different companies
- Addition of $MgCl_2$ is usual
- Annealing cycles are performed at low temperatures (about 40°C)

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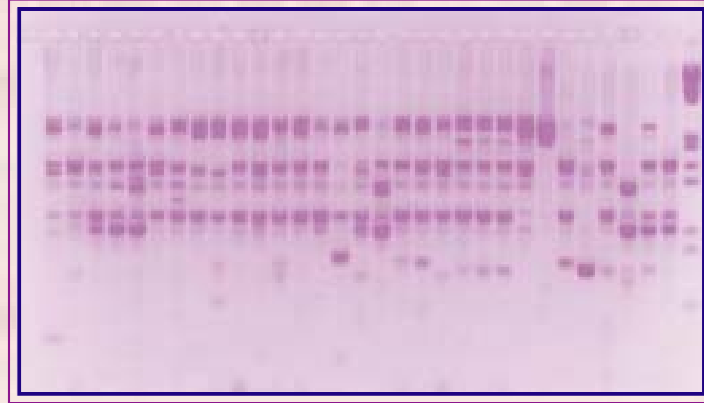
The PCR components needed are already discussed in the submodule "PCR basics". Only a short primer (usually 10 bp long) is used. Primers with these characteristics are commercially available under different brands. A concentration of $MgCl_2$ is often added to promote amplification of more bands through the reaction. However, care should be taken to find the suitable concentration for each case, to prevent the appearance of non-specific products.

PCR conditions usually include an annealing cycle at a low temperature (about 40°C), thus encouraging primer—DNA annealing and leading to a sufficient number of products. Again, the appearance of non-specific products must be prevented, which can be done by determining the appropriate temperature at which 'ghost' bands will not appear.

RAPD product detection

Agarose or acrylamide gel electrophoresis and visualisation with ethidium bromide



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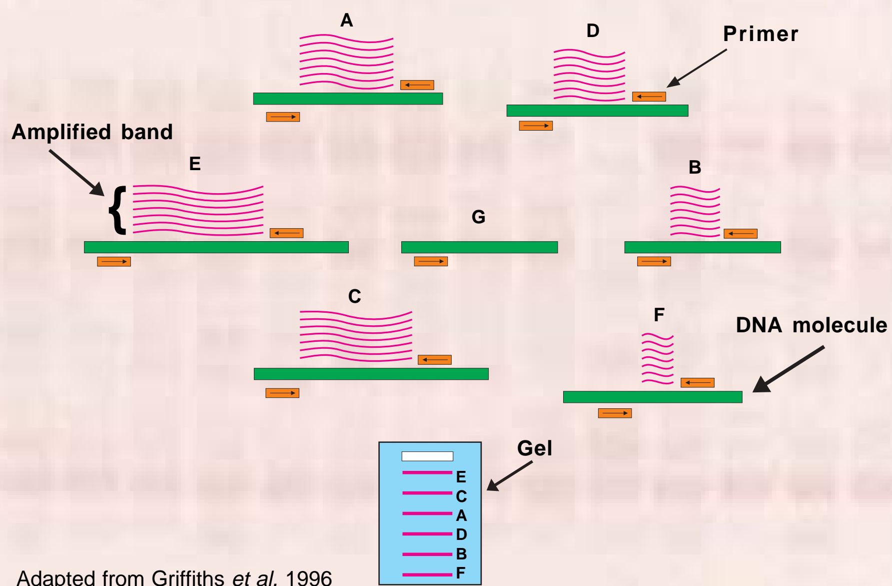
RAPDs can be detected by running PCR products through electrophoresis on an agarose or acrylamide gel. In both cases, the gel is stained with ethidium bromide.

The difference obtained by running RAPD products in acrylamide versus agarose lies only in the degree of resolution of bands. In most cases, agarose gel electrophoresis gives sufficient resolution.

Diagrammatic summary



Adapted from Griffiths *et al.* 1996

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Equipment

- Resources:
  - Distilled and/or deionised water
  - Reagents
- Equipment:
  - Refrigerator and freezer
  - Laminar flow hood
  - Centrifuge
  - Thermocycler
  - Power supply units
  - Hotplate or microwave
  - pH meter
  - Standard balance
  - Gel electrophoresis units
  - UV transilluminator

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Interpreting RAPD banding patterns (1)

DNA polymorphism among individuals can be due to:

- Mismatches at the primer site
- The appearance of a new primer site
- The length of the amplified region between primer sites

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Interpreting RAPD banding patterns (2)

Because of the nature of RAPD markers, only the presence or absence of a particular band can be assessed. Criteria for selecting scoring bands:

- Reproducibility—need to repeat experiments
- Thickness
- Size
- Expected segregation observed in a mapping population

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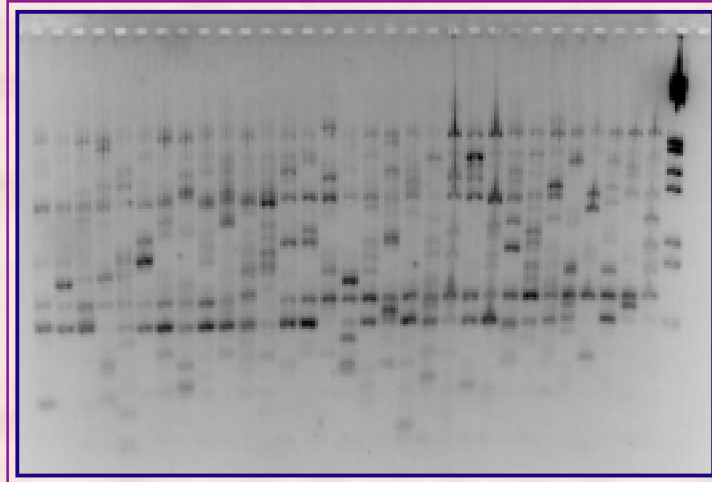
Interpreting RAPD banding patterns: Example of a bad RAPD gel



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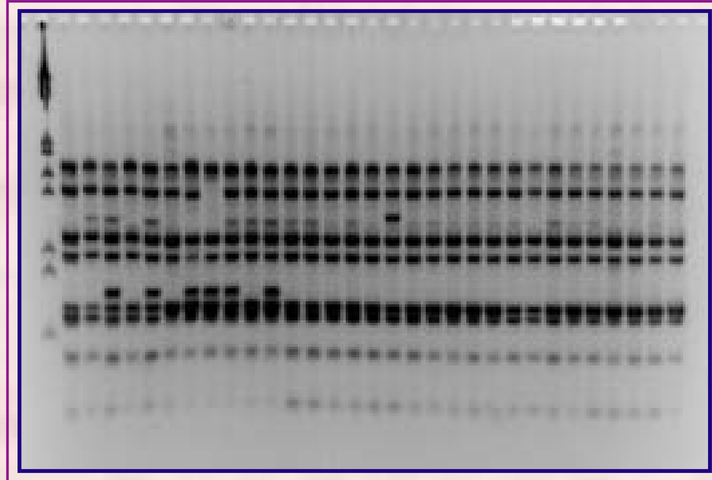
This picture shows an image of a bad quality RAPD gel. The bands are fuzzy. Those at the top have a smear starting from the well where the PCR product was loaded and many are observed only with difficulty. The hazy background makes observation difficult—whether, in certain cases, one band is found or two side by side. Certainly, some bands are clear and can be scored, but many other bands are dubious and their interpretation would be highly risky. Such difficulties raise questions of confidence in data collection.

Interpreting RAPD banding patterns: Example of a good RAPD gel



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This picture shows an image of a very high quality RAPD gel. Both, presence and absence of most bands are very clear and the background is transparent. The researcher would have no doubts while selecting bands and collecting data from this gel. Consequently, the interpretation of results can be very confident.

Advantages of RAPDs

- High number of fragments
- Simple
- Arbitrary primers are easily purchased, with no need for initial genetic or genomic information
- Only tiny quantities of target DNA are required
- Unit costs per assay are low

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Some comments:

- Many different fragments (corresponding to multiple loci dispersed throughout the genome) are normally amplified, using each single primer. The technique is therefore rapid in detecting polymorphisms. Although most commercially produced primers result in several fragments, some primers may fail to give amplification fragments from some material.

- The technique is simple. RAPD analysis does not require expertise to handle hybridisation of DNA or other highly technical activities.

Disadvantages of RAPDs

- Dominant
- Lack of a priori knowledge on the identity of the amplification products
- Problems with reproducibility
- Problems of co-migration

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RAPD markers are dominant. Amplification either occurs at a locus or it does not, leading to scores based on band presence or absence. This means that homozygotes and heterozygotes cannot be distinguished. In addition, the absence of a band through lack of a target sequence cannot be distinguished from that occurring through the lack of amplification for other reasons (e.g. poor quality DNA), contributing to ambiguity in the interpretation of results.

Nothing is known about the identity of the amplification products unless the studies are supported by pedigree analysis.

Problems with reproducibility result as RAPD suffers from sensitivity to changes in the quality of DNA, PCR components and PCR conditions, resulting in changes of the amplified fragments. Reproducible results may be obtained if care is taken to standardise the conditions used (Munthali *et al.*, 1992; Lowe *et al.*, 1996).

Problems of co-migration raise questions like 'Do equal-sized bands correspond to the same DNA fragment?'

- The presence of a band of identical molecular weight in different individuals is not evidence per se that the individuals share the same (homologous) DNA fragment.
- A band detected on a gel as being single can comprise different amplification products. This is because the type of gel electrophoresis used, while able to separate DNA quantitatively (i.e. according to size), cannot separate equal-sized fragments qualitatively (i.e. according to base sequence).

References

Lowe, A.J., O. Hanotte and L. Guarino. 1996. Standardization of molecular genetic techniques for the characterization of germplasm collections: the case of random amplified polymorphic DNA (RAPD). Plant Genet. Resour. Newsl. 107:50-54.

Munthali, M., B.V. Ford-Lloyd and H.J. Newbury. 1992. The random amplification of polymorphic DNA for fingerprinting plants. PCR Methods Appl. 1(4):274-276.

Applications

- Genetic diversity
- Germplasm characterisation
- Genetic structure of populations
- Domestication
- Detection of somaclonal variation
- Cultivar identification
- Hybrid purity
- Genome mapping

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References in purple are explained in detail in the following slides.

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Example: Meadow fescue

- Title:

  Fertilization and defoliation frequency affect genetic diversity of *Festuca pratensis* Huds. in permanent grasslands. Mol. Ecol. 1998. 7:1557-1567

- Objective:

  To assess the genetic variability of meadow fescue, and determine whether fertilisation and defoliation frequency influence genetic variability within natural populations

- Materials and methods:
  - Six natural populations and 3 cultivars of *Festuca pratensis* were studied, using 69 RAPD markers (13 primers) and 7 agronomic traits.
  - Samples of natural populations were taken from two unrelated long-term experiments, where treatments had been applied for 11 to 38 years

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Example: Meadow fescue (continued)

Results:

- Factor analysis of agronomic traits failed to separate cultivars from the other populations
- Cluster analysis of RAPD data resulted in a clear distinction between cultivars and natural populations
- Genetic variability within cultivars was lower than within natural populations
- Analysis of molecular variance (AMOVA) showed a significant effect of management on genetic variation

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Example: Meadow fescue (continued)

- Discussion:
  Significant genetic variation exists within cultivars and natural populations of *Festuca pratensis*. However, fertilisation and high cutting frequency may reduce it

- Conclusions:
  Unfertilised and rarely cut populations of meadow fescue should be conserved as a gene pool. Further studies of other species are needed to learn more about how management systems influence the diversity of plant communities and the genetic architecture of species

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Example: Bell pepper

- Title:

  Variation among and within *Capsicum* species revealed by RAPD markers. Theor. Appl. Genet. 1999. 99:147-156

- Objective:

  To characterize germplasm of *Capsicum* species

- Materials and methods:

  A total of 134 accessions from six *Capsicum* species, maintained at the Asian Vegetable Research and Development Center (AVRDC) genebank, were characterised with 110 RAPD markers (25 primers)

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Example: Bell pepper (continued)

- Results and discussion:
  - Ten pairs of potentially duplicate accessions documented
  - Diagnostic RAPDs were identified and employed to improve taxonomic identification. They could also be used to monitor natural and artificial hybridisation
  - Three *Capsicum* accessions, misclassified on morphological traits, were re-identified through diagnostic RAPDs
  - Three accessions, previously unclassified, were assigned to a species based on diagnostic RAPDs
  - *Capsicum annuum* accessions from the genebank did not differ from lines in the breeding program for RAPD variation or diversity

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Example: Bell pepper (continued)

Conclusions:

The AVRDC's pepper breeding program seems to be working with a diversity that is representative of its pepper collection in the genebank. Molecular markers can be useful for rationalising the management of an *ex situ* collection (e.g. identifying duplicates or taxonomic errors)

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Example: Modern rose

- Title:

  The domestication process of the Modern Rose: genetic structure and allelic composition of the rose complex. Theor. Appl. Genet. 2001. 102:398-404

- Objective:

  To evaluate the genetic variability among cultivated rose varieties and investigate the history of rose breeding

- Materials and methods:

  From 13 horticultural groups, 100 old varieties of cultivated rose were selected for the way they marked successive stages of domestication. They were studied with AP-PCR, using five long (20-mer) primers

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Example: Modern rose (continued)

Results and discussion:

- Fifty-eight polymorphic DNA fragments were produced, 55 being informative and discriminatory, allowing differentiation among almost all 100 cultivars

- A dendrogram showed the relationships between Chinese and European founder roses, hybrid groups of the first and second generations, and the most modern hybrid Teas produced during domestication

- Principal component analysis demonstrated the occurrence of a continuous gradient of the European/ Chinese allele ratio, and considerable reduction in genetic variability over the course of domestication

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Example: Modern rose (continued)

Conclusions:

The effect of selection for a limited array of morphological traits resulted in the retention of only a small number of alleles during the domestication of rose. Rose has much more genetic variation than is so far used. Selecting for different variants to generate varieties with new and valuable aesthetic traits should be possible

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Differences: DAF and RAPD technologies

For DAF:

- Primer concentrations are higher
- Shorter primers are used (5 to 8 nucleotides)
- Two-temperature cycle vs. the 3-temperature cycle used in RAPD
- Highly complex banding patterns

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References

Caetano-Anollés, G., B.J. Bassam and P.M. Gresshoff. 1991a. DNA amplification fingerprinting: a strategy for genome analysis. Plant Mol. Biol. Rep. 9(4):294-307.

Caetano-Anollés, G., B.J. Bassam and P.M. Gresshoff. 1991b. DNA amplification using very short arbitrary oligonucleotide primers. Bio/Technology 9:553-557.

Differences: AP-PCR and RAPD technologies

In AP-PCR:

- The amplification is in three parts, each with its own stringency and concentration of constituents
- High primer concentrations are used in the first PCR cycles
- Primers of variable length, and often designed for other purposes, are arbitrarily chosen for use (e.g. M13 universal sequencing primer)

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Reference

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18(24):7213-7218.

In summary

- The RAPD technology is based on a simple PCR with a single short arbitrary primer
- RAPD easily produces a significant number of bands, but markers are dominant and reproducibility problems are common
- DAF and AP-PCR are alternative, more complex technologies, of RAPD

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By now you should know

- Main features of the RAPD procedure
- Causes of polymorphisms in the DNA molecule that can be detected with RAPD
- Criteria for selecting RAPD bands for genetic diversity analysis
- Advantages and disadvantages of RAPD technology
- Main differences of DAF and AP-PCR with RAPD technology

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Basic references

Caetano-Anollés, G., B.J. Bassam and P.M. Gresshoff. 1991a. DNA amplification fingerprinting: a strategy for genome analysis. Plant Mol. Biol. Rep. 9(4):294-307.

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PCR-based technologies
Amplified fragment length polymorphisms

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