Using molecular marker technology in studies on plant genetic diversity

DNA-based technologies
Restriction fragment length polymorphisms

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RFLP technology

RFLP detection relies on the possibility of comparing band profiles generated after restriction <u>enzyme</u> digestion of target <u>DNA.</u>
The laboratory steps involved are as follows:

▶ Isolation of DNA
▶ Restriction digestion and gel electrophoresis
▶ DNA transfer by <u>Southern blotting</u>
▶ DNA <u>hybridisation</u>
  • The procedure
  • The DNA <u>probe</u>
  • Sources of probes
▶ Equipment

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Restriction fragment length <u>polymorphism</u> (RFLP) analysis was one of the first techniques to be widely used for detecting variation at the <u>DNA sequence</u> level. The principle behind the technology rests on the possibility of comparing band profiles generated after <u>restriction enzyme</u> digestion in DNA molecules of different individuals. Diverse <u>mutations</u> that might have occurred affect DNA molecules in different ways, producing fragments of variable lengths. These differences in fragment lengths can be seen after gel <u>electrophoresis,</u> hybridisation and visualisation.

Isolating DNA

▶ Total DNA is extracted from plant cells

▶ Alternatively, chloroplast and mitochondrial DNA can be used

▶ DNA must be clean and of high molecular weight

▶ Complications:
- Breakage during isolation
- DNA degraded by nucleases
- Joint isolation of polysaccharides
- Isolation of secondary plant metabolites

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Isolating DNA is the first step for many DNA-based technologies. DNA is found either in nuclear chromosomes or in organelles (mitochondria and chloroplasts). To extract DNA from its location, several laboratory procedures are needed to break the cell wall and nuclear membrane, and so appropriately separate the DNA from other cell components. When doing so, care must be taken to ensure the process does not damage the DNA molecule and that it is recovered in the form of a long thread.

Restriction digestion and gel electrophoresis

Hybridisation is needed to detect specific fragments
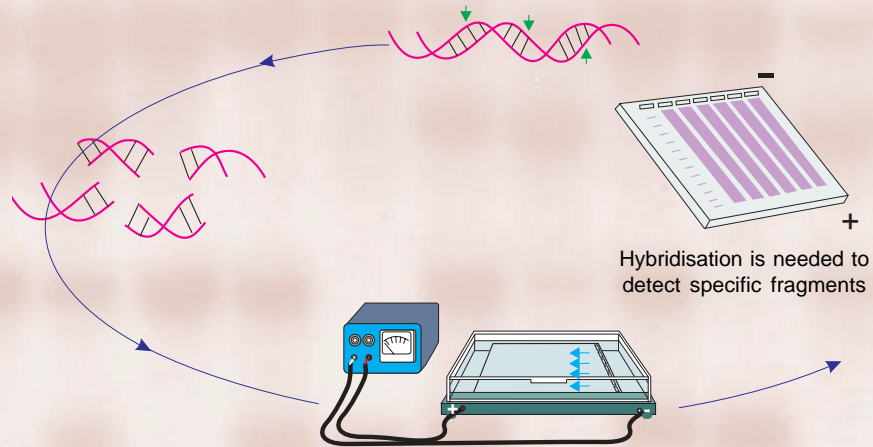
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Extracted DNA is digested with specific, carefully chosen, restriction enzymes. Each restriction enzyme, under appropriate conditions, will recognise and cut DNA in a predictable way, resulting in a reproducible set of DNA fragments ('restriction fragments') of different lengths.

The millions of restriction fragments produced are commonly separated by electrophoresis on agarose gels. Because the fragments would be seen as a continuous 'smear' if stained with ethidium bromide, staining alone cannot detect the polymorphisms. Hybridisation must therefore be used to detect specific fragments.

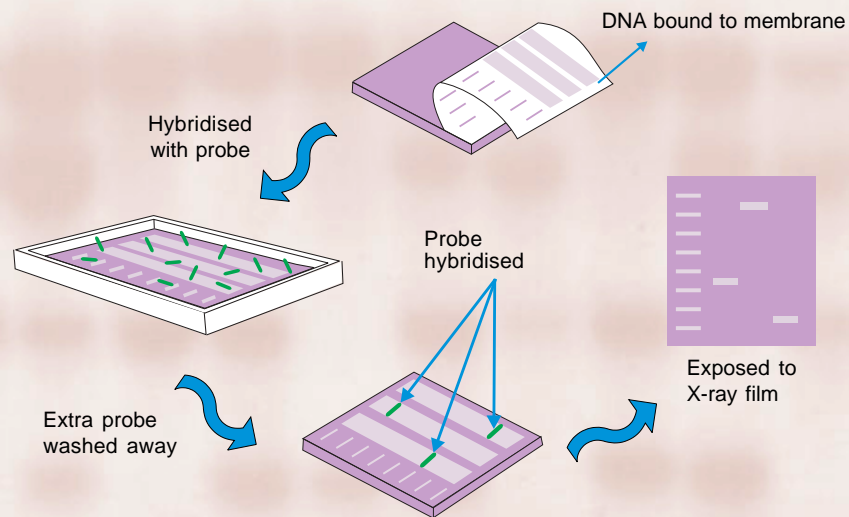## DNA transfer by Southern blotting

Weight

Membrane

Gel
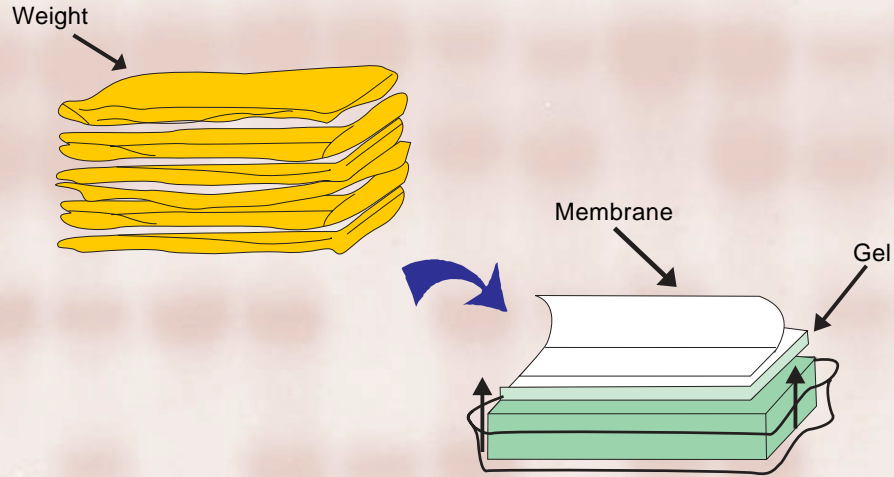
RFLPs 6

DNA transfer is called 'Southern blotting', after E.M. Southern (1975), who invented the technique. In this method, the gel is first denatured in a basic solution and placed in a tray. A porous nylon or nitrocellulose membrane is laid over the gel, and the whole weighted down. All the DNA restriction fragments in the gel transfer as single strands by capillary action to the membrane. All fragments retain the same pattern on the membrane as on the gel.

Reference

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by electrophoresis. J. Mol. Biol. 98:503-517.
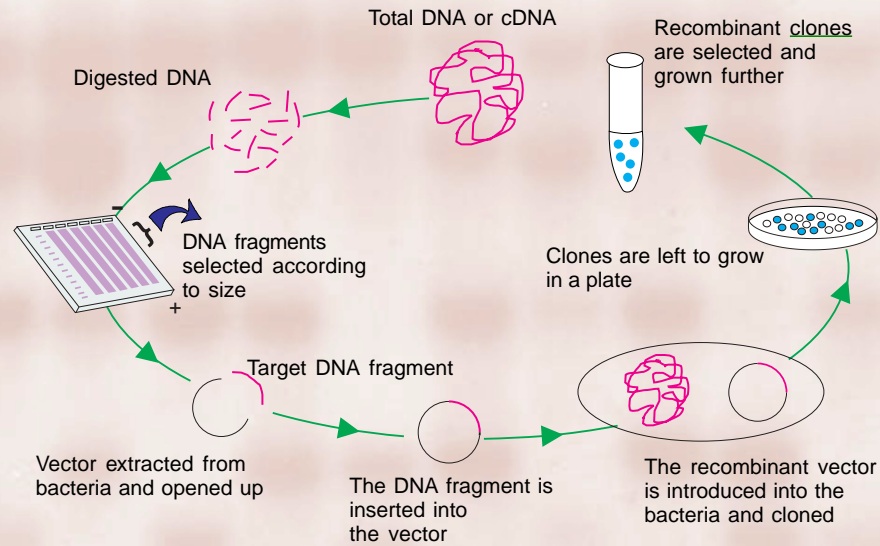
DNA hybridisation: The procedure

DNA bound to membrane

Hybridised
with probe

Probe
hybridised

Extra probe
washed away

Exposed to
X-ray film

Adapted from Griffiths *et al.* 1996

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The membrane with the target DNA is incubated with the DNA probe. Incubation conditions are such that if strands on the membrane are complementary to those of the probe, hybridisation will occur and labelled duplexes formed. Where conditions are highly stringent, hybridisation with distantly related or non-homologous DNA does not happen. Thus, the DNA probe picks up sequences that are complementary and 'ideally' homologous to itself among the thousands or millions of undetected fragments that migrate through the gel.

Desired fragments may be detected after simultaneous exposure of the hybridised membrane and a photographic film.

DNA hybridisation: The DNA probe

Total DNA or cDNA

Recombinant clones are selected and grown further

Digested DNA

DNA fragments selected according to size

Clones are left to grow in a plate

Target DNA fragment

Vector extracted from bacteria and opened up

The DNA fragment is inserted into the vector

The recombinant vector is introduced into the bacteria and cloned

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To detect the subset of DNA fragments of interest from within all the fragments generated by restriction digestion, a probe is needed. The DNA probe usually comes from a DNA library (either genomic or cDNA), which is a collection of vectors (e.g. plasmids) that contain a representation of an original DNA molecule cut into pieces. Vectors may be transformed into bacteria and may multiply the piece of DNA they contain many times.

The DNA probe is also converted into a single-stranded molecule, conveniently labelled, using any standard method (e.g. a radioisotope or digoxygenin), and hybridised with the target DNA, which is stuck to the membrane.

DNA hybridisation: Sources of probes (1)

▶ Nuclear DNA:
  • Genomic libraries
  • cDNA

▶ Cytoplasmic DNA

▶ The species specificity of many single-locus probes requires that libraries be built when studying new species. However, probes from related genera can often be used

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Sources of DNA probes include:

- *Genomic libraries*—total plant DNA is digested with restriction enzymes and individual fragments cloned into a bacterial or viral vector. Suitable probes are selected from this 'anonymous' library for RFLP analysis.

- *cDNA (complementary DNA) libraries*—mRNA is isolated and transcribed into DNA, using the enzyme reverse transcriptase. The cDNA so obtained is cloned into vectors and used as a library for probes in RFLP analysis.

- *Cytoplasmic DNA*—mitochondrial and chloroplast DNA libraries.

As a result of the species specificity shown by many single-locus probes, genomic or cDNA libraries must often be built for studies on new species. This can be very time consuming. However, given current knowledge about common sequences and genes, probes from related genera can often be used.

Repetitive sequences of the minisatellite type also have their particular application in RFLP analysis. They are the repeat sequences of a basic 'motif'. They measure 10 to 60 bp, are found in tandem (i.e. head to tail) and occur at many loci on the genome.

Work on plant minisatellite markers resulted from pioneering studies on the human genome by Jeffreys *et al.* (1985a, b), which showed minisatellite markers to be highly variable in humans. Because polymorphisms are related to the number of repeated units, the sequences are also called variable number of tandem repeats (VNTRs; Nakamura *et al.* 1987). A carefully selected probe can detect restriction fragments that represent a large number of loci. The patterns of minisatellite-bearing restriction fragments on film (the so-called 'DNA fingerprint') allow clear discrimination between different individuals.

References

Jeffreys, A.J., V. Wilson and S.L. Thein. 1985a. Hypervariable 'minisatellite' regions in human DNA. Nature 314:67-73.

Jeffreys, A.J., V. Wilson and S.L. Thein. 1985b. Individual-specific "fingerprints" of human DNA. Nature 316:76-79.

Nakamura, Y., M. Leppert, P. O'Connell, R. Wolff, T. Holm, M. Culver, C. Martin, E. Fujimoto, M. Hoff, E. Kumlin and R. White. 1987. Variable Number of Tandem Repeat (VNTR) markers for human gene mapping. Science 235:1616-1622.

Rogstad, S.H., J.C. Patton, II and B.A. Schaal. 1988. M13 repeat probe detects DNA minisatellite-like sequences in gymnosperms and angiosperms. Proc. Natl Acad. Sci. U.S.A. 85:9176-9178.

Ryskov, A.P., A.G. Jincharadze, M.I. Prosnyak, P.L. Ivanov and S.A. Limborska. 1988. M13 phage DNA as a universal marker for DNA fingerprinting of animals, plants and microorganisms. FEBS Lett. 233:388-392.

Equipment

▶ Resources:
- Distilled and/or deionised water
- Reagents

▶ Equipment:
- Refrigerator and freezer
- Laminar flow hood
- Centrifuge
- Power supply units
- Hotplate or microwave
- pH meter
- Standard balance
- Gel electrophoresis units
- Dark room
- UV transilluminator

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RFLP technology in pictures

The following slides illustrate the procedures of the RFLP technique

RFLPs   12

RFLPs   13

After agarose has been poured into the gel mould, combs are immediately inserted to form wells and left until the gel hardens. The combs are then removed and the gel placed in an electrophoresis chamber.
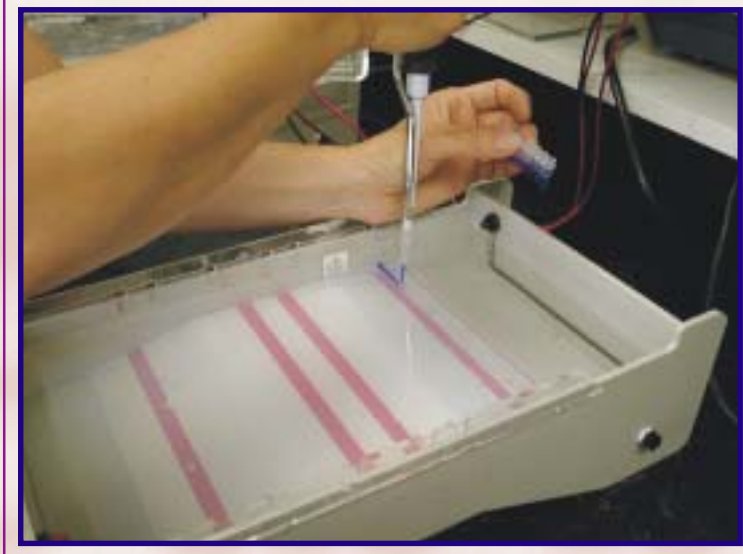
RFLPs 14

Samples of digested DNA, with bromophenol blue dye added, are loaded into the wells with a pipettor.

RFLPs   15

After electrophoresis, the gel is treated with NaCl to break the DNA <u>double helix</u> bonds and make it single-stranded. This allows later hybridisation with a single-stranded DNA probe.

RFLPs 16

The blotting tray is first prepared by saturating sponges with NaOH. Safety glasses and gloves are required, and a laboratory coat recommended. The safety regulations of the participating institution should be followed.

Absorbent paper is placed on top of the sponges to prevent direct contact with the gel.

Bubbles between the absorbent paper and sponges are removed by rolling a pipette or a glass rod across the paper. This ensures a complete transfer of the solution all through the gel.

RFLPs 19

The gaps left between the sponges and tray are covered with strips of plastic sheets to prevent evaporation, which would reduce the efficiency of transfer.

The treated agarose gel is placed on top of the absorbent paper.

Bubbles between the gel and paper are being squeezed out with a glass rod.

Membrane is cut into the appropriate size.

RFLPs 23

The membrane is placed on top of the gel, then covered with a piece of absorbent paper.

RFLPs 24

A stack of porous paper such as paper towels or newspaper is placed on the absorbent paper protecting the membrane.

The entire set-up is topped with a weight (here, a bottle of water standing on a piece of glass) to promote good transfer. After some hours the transfer is complete, the blotting paper is taken away, and the membrane stored until hybridisation with the probe.
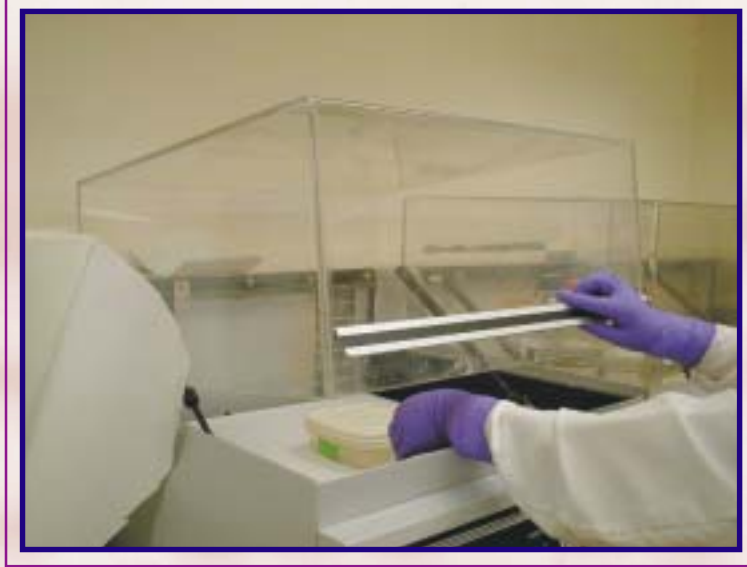
RFLPs 26

The process of hybridisation begins. A blocker DNA (to minimise background hybridisation with the membrane) is boiled to denature it to single strands.

RFLPs 27

The membrane is placed in a plastic container with the appropriate hybridisation solution and the blocker DNA, and pre-incubated.

The labelled probe is added to the container with the hybridisation solution and membrane, and incubated overnight in an oven. The following day, the membrane is removed from the hybridisation set up, and washed with the appropriate stringency solution.

(*Note*: Institutions vary with respect to required safety practices; please check with your host institution.)

The membrane is then blotted dry and put into a cassette for holding X-ray film.

RFLPs 30

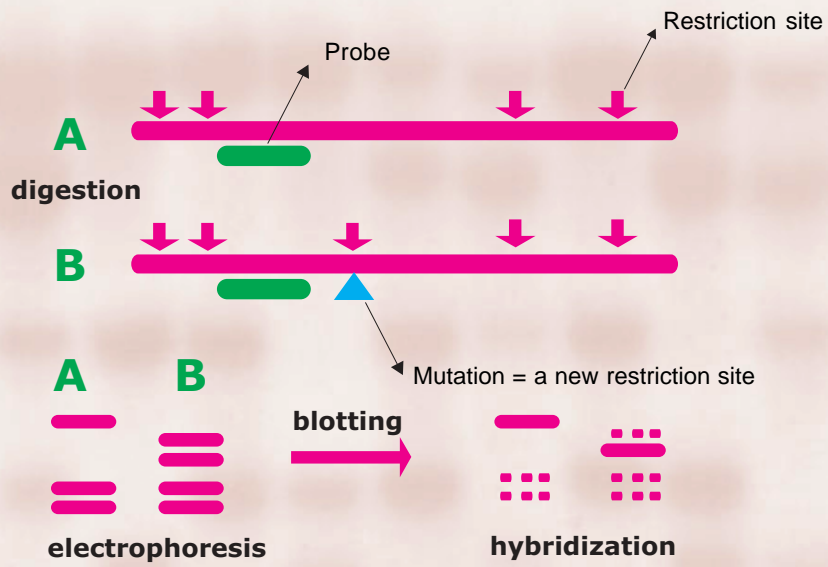Inside a dark room, an X-ray film is also inserted into the cassette.

RFLPs 31

The cassette is wrapped, or sealed with tape, and stored in a freezer until the film is sufficiently exposed, usually 1 to 4 days.
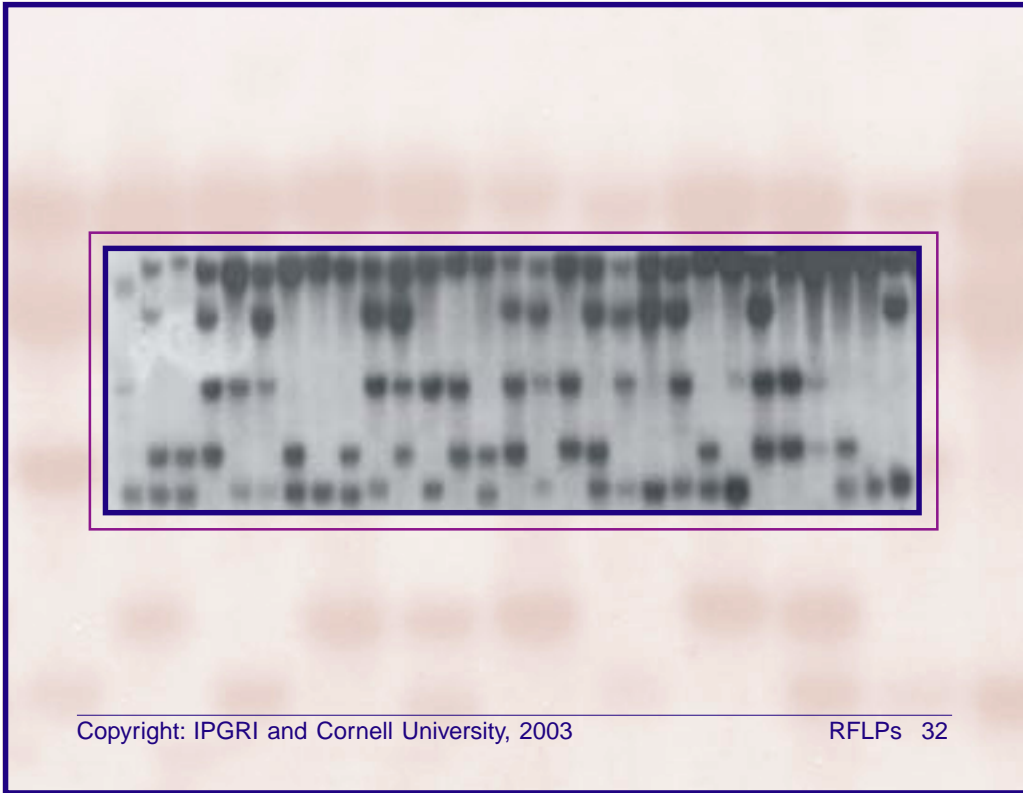
RFLPs 32

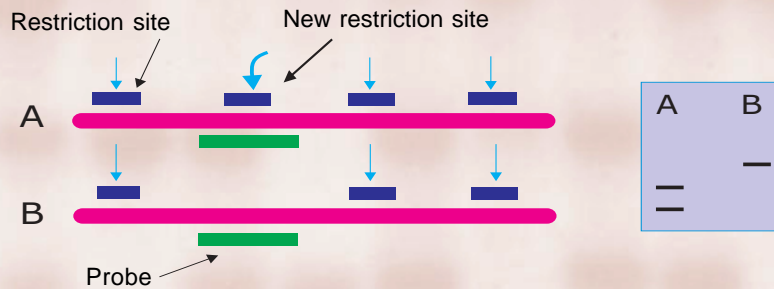This is an RFLP autoradiogram.

RFLP in pictures: summary

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Interpreting RFLP bands (1)

A mutation creates a new restriction site within the target region. Two smaller bands are therefore detected on the film
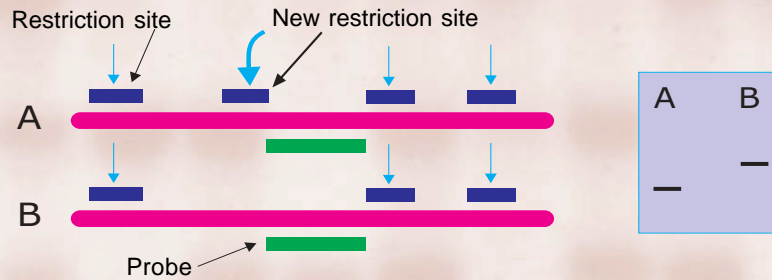
RFLPs 34

RFLP technology ideally results in a series of bands on a gel, which can then be scored either for presence or absence of particular bands, or as codominant markers. Differences between genotypes are usually visualised as a diverse pattern of DNA restriction fragments.

The different mutational events responsible for the polymorphisms detected by RFLP analysis are presented in this and following slides.

In the diagram above, a mutation creates a new restriction site in segment A, just at the location of the probe's recognition. As a consequence, the probe will simultaneously hybridise with the two segments created by the enzyme. In segment B, where no mutation has occurred, only one segment will hybridise with the probe. During electrophoresis, the two segments from A will migrate farther through the gel than the segment hybridised on B and, as such, the polymorphism will be observed in the gel as shown in the inset (at right).

Interpreting RFLP bands (2)

A mutation creates a new restriction site between flanking restriction sites, creating a smaller restriction fragment

In this case, a new restriction site is again created by mutation in segment A. However, the new site appears to one side of the location of the probe's recognition. Hence, only one fragment will hybridise in segment A and one in segment B. The polymorphism will be shown as a shorter fragment hybridised in A than in B. The shorter fragment will also migrate farther in the gel (see inset).

Interpreting RFLP bands (3)

An insertion of a DNA sequence between the flanking restriction sites creates a larger restriction fragment
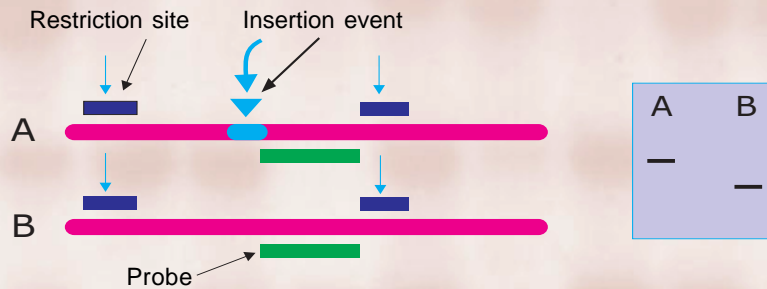
If an <u>insertion</u> event takes place between two restriction sites (segment A), the hybridised fragment will be longer. As a result, a polymorphism will be observed between individuals A and B, in that the fragment hybridised in A will be longer than that in B and its migration distance shorter (see inset).

Interpreting RFLP bands (4)

A deletion of a DNA sequence between the flanking restriction sites creates a smaller restriction fragment

Restriction site   Deletion event

A

B

Probe

A   B
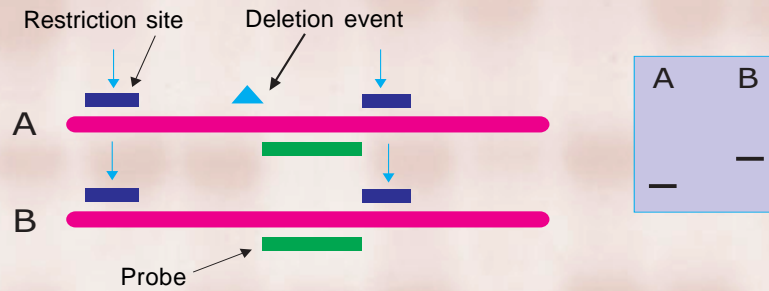
RFLPs   37

If a deletion occurs between flanking restriction sites, the probe will hybridise to a shorter segment. This will be observed as a polymorphism in the gel with a fragment that migrated farther in the gel for individual A (see inset).

Interpreting RFLP bands (5)

One of the flanking restriction sites is changed or lost through mutation or deletion. Consequently, the restriction fragment is altered

Restriction site   Lost site

A

B

Probe

A   B
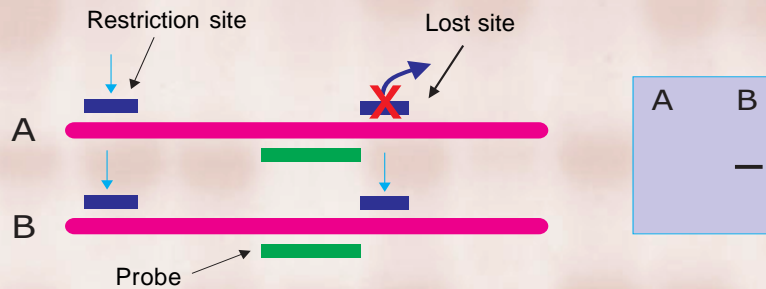
RFLPs 38

If only one restriction site remains, no restriction fragment is generated, and no hybridisation can occur. If a new site has been generated by the change, the new fragment will hybridise, and will show a different band pattern than an individual not having the change.

Advantages of RFLPs

▸ Highly robust methodology with good transferability between laboratories

▸ Codominantly inherited and, as such, can estimate heterozygosity

▸ No sequence information required

▸ Because based on sequence homology, highly recommended for phylogenetic analysis between related species

▸ Well suited for constructing genetic linkage maps

▸ Locus-specific markers, which allow synteny studies

▸ Discriminatory power—can be at the species and/or population levels (single-locus probes), or individual level (multi-locus probes)

▸ Simplicity—given the availability of suitable probes, the technique can readily be applied to any plant

RFLPs   39

Disadvantages of RFLPs

▶ Large amounts of DNA required

▶ Automation not possible

▶ Low levels of polymorphism in some species

▶ Few loci detected per assay

▶ Need a suitable probe library

▶ Time consuming, especially with single-copy probes

▶ Costly

▶ Distribution of probes to collaborating laboratories required

▶ Moderately demanding technically

▶ Different probe/enzyme combinations may be needed

RFLPs   40

Applications

▶ Genetic diversity

▶ Genetic relationships

▶ History of domestication

▶ Origin and evolution of species

▶ <u>Genetic drift</u> and selection

▶ Whole genome and comparative mapping

▶ Gene tagging

▶ Unlocking valuable genes from wild species

▶ Construction of exotic libraries

RFLPs 41

References marked in purple are discussed in detail in the following slides.

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Tanksley, S.D., M.W. Ganal, J.P. Prince, M.C. de Vicente, M.W. Bonierbale, P. Broun, T.M. Fulton, J.J. Giovannoni, S. Grandillo, G.B. Martin, R. Messeguer, J.C. Miller, L. Miller, A.H. Paterson, O. Pineda, M.S. Röder, R.A. Wing, W. Wu and N.D. Young. 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141-1160.

Tanksley, S.D. and S.R. McCouch. 1997. Seed banks and molecular maps: unlocking genetic potential from the wild. Science 277:1063-1066.

Zamir, D. 2001. Improving plant breeding with exotic genetic libraries. Genetics 2:983-989.

Example: Maize

▶ Title:

Relationships among maize inbred lines and populations from European and North American origins as estimated using RFLP markers. Theor. Appl. Genet. 1999. 99:473-480

▶ Objective:

To examine the genetic relationships among inbred lines from known heterotic groups and landraces of great historical importance in the development of elite material

▶ Materials and methods:

Sixty-two inbred lines of known heterotic groups and 10 maize populations (about 30 individuals per population) were assayed at 28 RFLP loci (29 different probe/enzyme combinations)

RFLPs   42

Example: Maize (continued)

Results:

Comparing <u>alleles</u> specific to each type of <u>germplasm</u> showed a deficit of alleles within lines accounting for about 22% of the total allelic richness of the populations:

- Associations among inbreds and populations proved consistent with <u>pedigree</u> data of the inbreds and provided new information on the genetic basis of heterotic groups

- European flint inbreds were revealed to be as close to the north-eastern U.S. flint population studied as to the typical European populations

RFLPs   43

Examples: Maize (continued)

▶ Discussion:

The populations represent significant reservoirs of diversity, and elite germplasm is not likely to contain all useful alleles

Question: How can the European heterotic group be closer to the north-eastern U.S. flint 'Compton's Early' populations than to other U.S. populations?

▶ Conclusions:

Results suggest that a larger set of populations should be studied with molecular markers to develop appropriate strategies for the most effective use of these genetic resources in breeding programs

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Example: Wheat

▶ Title:

Molecular markers to study genetic drift and selection in wheat populations. J. Expl. Bot. 1999. 50(332):283-290

▶ Objective:

To compare allelic frequencies in wheat populations that have been subjected to natural selection

▶ Materials and methods:

Two initial populations and six derived subpopulations cultivated for 10 years in contrasting sites were studied at 30 loci

RFLPs   45

Examples: Wheat (continued)

▶ Results and discussion:

Differentiation between subpopulations based on RFLP diversity was highly significant:

- Allelic frequency variation was found to be much greater than expected under genetic drift only. Selection greatly influenced the evolution of populations

- Some loci revealed higher differentiation than other. This may have indicated that they were genetically linked to other polymorphic loci involved in adaptation

▶ Conclusions:

Variations of allelic frequencies observed for the RFLP markers cannot be explained by evolution under genetic drift only but by direct or indirect effects of selection

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Examples: Scots pine

▸ Title:

The postglacial history of Scots pine (*Pinus sylvestris* L.) in western Europe: evidence from mitochondrial DNA variation. Mol. Ecol. 1999. 8:83-888

▸ Objective:

To investigate the geographical structure of mitochondrial DNA variants in western European populations of Scots pine

▸ Materials and methods:

Twenty populations of *P. sylvestris* from Scotland and 18 from continental Europe (an average of 21 individuals per population) were studied, using RFLP analysis of total DNA

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Example: Scots pine (continued)

▶ Results:

Three major <u>mtDNA</u> RFLP patterns (mitotypes a, b, and d) were detected:

- Within Spain, all three mitotypes were found. Gene diversity was high, being distributed predominantly among, rather than within, populations. Mitotype d was present only in the southernmost population (Sierra Nevada, Spain)

- Italian populations were fixed for mitotype b. Populations from northern France, Germany, Poland, Russia and southern Sweden were fixed for mitotype a. Populations in northern Fennoscandia (Norway, Sweden and Finland) were fixed for mitotype b

- In Scotland, mitotype a was largely fixed. Mitotype b was present in some polymorphic populations

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Example: Scots pine (continued)

▶ Discussion:
  - The detection of different mitoypes was unequivocal proof of genetic differences in the mtDNA genome. The diversity of mitotypes in Spanish populations indicates that these either descend from survivors in refugia during the last glaciation or represent Tertiary relics that have survived as isolated populations

  - In Europe, after glaciation, *P. sylvestris* apparently began developing in at least three evolutionary directions, each of which had a different origin—Spain, north central Europe and northern Fennoscandia

▶ Conclusions:
  Studies of maternally inherited mtDNA markers can provide useful insights into the history of species and help define geographical areas with germplasm that may be conserved

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In summary

▸ The RFLP technology detects length changes in target DNA molecules after restriction enzyme digestion

▸ RFLP bands are detected by hybridising the target DNA with a DNA probe

▸ RFLP banding patterns reflect different mutational events at the hybridisation site of the probe or its neighbouring region

▸ RFLP is a highly robust technology, but time consuming and technically demanding

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By now you should know

▶ Main steps required for detecting RFLPs

▶ Different sources of probes

▶ The effect of different mutational events on detecting RFLP banding patterns

▶ The advantages and disadvantages of the RFLP technology for genetic diversity analysis

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Basic references

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DNA-based technologies
PCR-based technologies
PCR basics

▶ DNA-based technologies
- PCR-based technologies
  - PCR with arbitrary primers
  - Amplified fragment length polymorphisms (AFLPs)
  - Sequences-tagged sites (STS)
  - Latest strategies

▶ Complementary technologies

▶ Final considerations

▶ Glossary

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