Using molecular marker technology in studies on plant genetic diversity

Complementary technologies

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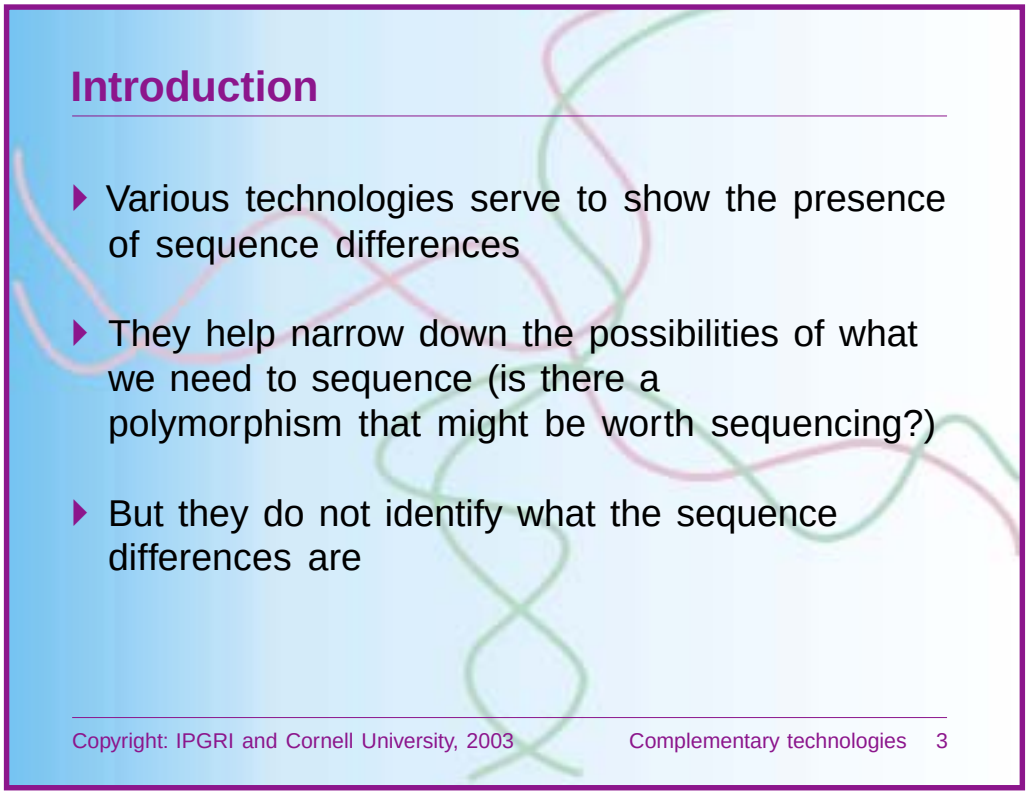

When looking at differences in the DNA sequence, we need to be able to separate specific DNA segments from a mixture such as from the whole genome.

Electrophoresis separates molecules in an electrical field on the basis of charge, size and shape. If a DNA molecule is cut into small sections and placed in a well at one end (cathode) of an agarose gel, the DNA fragments will move through the gel towards the anode. Their speed will depend on their individual sizes, so they end up forming bands located at different positions in the gel. The bands can then be visualised with ethidium bromide staining, which causes the DNA to fluoresce under UV light.

The same result is achieved by electrophoresis in an acrylamide gel, the difference being a matter of resolution. The acrylamide is able to discriminate smaller differences in fragment size.

Electrophoresis is a diagnostic procedure that allows us to identify molecules of different sizes. When used as such, electrophoresis is itself a means of showing polymorphisms and, consequently, genetic variation between genotypes.

But electrophoresis can also be useful as a first step towards identifying and isolating specific DNA molecules that, even if the same size, differ in sequence composition.

Denaturing gradient gel electrophoresis (DGGE) (1)

- Permits detecting very small DNA polymorphisms or mutations
- Can be applied to long DNA fragments, measuring hundreds of base pairs
- Does not require previous knowledge on the existence of a polymorphism
- Based on the renaturation properties of DNA strands
- DNA denaturation takes place through electrophoresis

Denaturing gradient gel electrophoresis (2)

- Small DNA fragments are subject to electrophoresis through a polyacrylamide gel under increasingly denaturing conditions (formamide/urea concentrations)
- The DNA 'melts' and becomes single stranded
- DNA molecules change their shape and stop moving
- Differences in DNA sequence are identified

The DNA fragments migrate first as double-stranded molecules. Later, because of the gel's changing composition, the molecules denature and become single stranded, forming a branched structure. This changed structure results in the molecules' diminished ability to move through the gel.

The point at which the DNA melts depends on the nucleotide sequence in the melted region. The final location of the molecules in the gel thus depends on the nucleotide sequence of the fragments.

Denaturing gradient gel electrophoresis (3)

DGGE can be a two-step process:

- Samples are run to separate fragments according to size
- Fragments are separated under DGGE conditions by the 'melting point' process

When samples are mixed, double bands indicate sequence differences between bands of the same size

At least 95% of differences in sequence composition is estimated as being detected with this procedure.

DGGE also serves to distinguish homozygous versus heterozygous genotypes for a particular DNA fragment. To take advantage of this capacity, a cycle of denaturation and renaturation must be conducted after the last PCR cycle. Homoduplexes and heteroduplexes are formed as alleles reassociate. In the DGGE gel, fast-migrating homoduplex combinations will indicate homozygous genotypes. Heterozygous genotypes will show both homoduplex and heteroduplex combinations. Heteroduplexes are formed through mispairing and rapid denaturation in the gel, which will stop the migratory course of these molecules.

Thermal gradient gel electrophoresis (TGGE)

- Similar to DGGE
- Increasingly denaturing conditions are achieved by a temperature gradient instead of by changing reagent concentrations
- Can also be used for analysing single-stranded RNA and proteins

Basic reference

Myers, R.M., N. Lumelsky, L.S. Lerman and T. Maniatis. 1985. Detection of single base substitutions in total genomic DNA. Nature 313:495-498.

Single-stranded conformational polymorphism (SSCP) (1)

- SSCP is based on the electrophoretic behaviour of a single-stranded DNA molecule through a non-denaturing acrylamide gel
  - A single-stranded molecule has the property of forming secondary structures through internal base pairings
  - These secondary structures are sequence-dependent and result in particular shapes for each single-stranded molecule
- Differences in secondary structures cause the DNA strands to migrate differentially on the gel

SSCP can distinguish between two very similar DNA sequences only on the basis of the particular shape of their single-stranded structures. In principle, then, even two alleles of the same gene can be discriminated.

SSCP (2)

- The sequence of interest goes through PCR
- Next, the PCR product is denatured at 94°C and rapidly cooled down on ice. Single-stranded molecules do not pair but form stable secondary structures
- The reassociated fragments are then subject to electrophoresis:
  - In an homozygous case, two bands can be observed, each corresponding to a slightly different secondary structure
  - In a heterozygous case, at least four bands can be observed

SSCP is a simple technique, but has at least two major disadvantages:

- The electrophoretic behaviour of the single-stranded molecules is unpredictable, depending very much on temperature and running conditions.
- In the case of long DNA fragments (> 200 bp), the method becomes insensitive to some mutations. In principle, SSCP seems to work better for small insertions and/or deletions.

Basic reference

Hayashi, K. 1992. A method for the detection of mutations. Genet. Anal. Tech. Appl. 9:73-79.

Heteroduplex analysis

- Two PCR-amplified products are mixed in equal quantities, denatured at 95°C and allowed to cool
- During cooling, DNA strands reanneal to form heteroduplex DNA
- Any mismatches in the heteroduplex will cause it to have a different three-dimensional structure, with a reduced mobility that is proportional to the degree of divergence of the sequences

Basic reference

Delwart, E.L., E.G. Shpaer, J. Louwagie, F. McCutchan, M. Grez, H. Rübsamen Waigmann and J.I. Mullins. 1993. Genetic relationships determined by a heteroduplex mobility assay: analysis of HIV env genes. Science 262:1257-1261.

Denaturing high-performance liquid chromatography (DHPLC): methodology

- This method can detect sequence differences of a single base pair as well as insertions and/or deletions
- Works with crude PCR products and does not require prior DNA sequencing
- Based on the differential elution of homoduplex and heteroduplex DNA when run through a column

DHPLC is a high-performance liquid chromatography method in which DNA fragments are separated according to size and/or presence of heteroduplexes (reannealed DNA strands) during their passage through a gradient in a column.

In double-stranded amplified DNA, nucleotides that are mismatched through mutations and polymorphisms become evident after heteroduplex formation. The presence of these polymorphisms creates a mixed population of heteroduplexes and homoduplexes during reannealing of wild type and mutant DNA. If this mixture of fragments is run under partially denaturing conditions by HPLC, heteroduplexes elute from the column earlier than the homoduplexes because of their lower melting temperature.

Analysis can be performed to detect sequence variation between individuals or determine heterozygosity.

DHPLC: applications

- Finding new mutations and polymorphisms in any DNA fragment or particular gene sequence
- Screening clones to identify candidate fragments for sequencing
- Evaluating the fidelity of amplified fragments
- Diagnosing for the presence of known mutations

Basic reference

Oefner, P.J. and P.A. Underhill. 1998. DNA Mutation Detection Using Denaturing High-Performance Liquid Chromatography (DHPLC). Current Protocols in Human Genetics, supplement 19, 7.10.1-7.10.12. Wiley & Sons, NY.

In summary

- Several technologies help identify the presence of sequence differences
- While they cannot tell what the variant is, they can help narrow the range of strategies to use for detecting it

By now you should know

The basic principles of:

- Denaturing gradient gel electrophoresis
- Thermal gradient gel electrophoresis
- Single-stranded conformational polymorphism
- Heteroduplex analysis
- Denaturing high-performance liquid chromatography

Next

Final considerations

- Glossary