Using molecular marker technology in studies on plant genetic diversity

DNA-based technologies

PCR-based technologies

Amplified fragment length polymorphisms (AFLPs)

Contents

- AFLP technology, step by step
  - Four steps
  - DNA digestion and ligation
  - PCRs and detection
  - Summarising the technology
- Equipment
- Interpreting AFLP bands
- Advantages and disadvantages of AFLPs
- Applications
  - *Limonium* sp.
  - *Stylosanthes* spp.
  - Indian mustard

AFLP* technology, step by step

Main features:

- A combination of the RFLP and PCR technologies
- Based on selective PCR amplification of restriction fragments from digested DNA
- Highly sensitive method for fingerprinting DNA of any origin and complexity
- Can be performed with total genomic DNA or with cDNA ('transcript profiling')

*The AFLP technique was developed by KeyGene (Netherlands), a private biotechnology company that has filed property rights on the technology. For more information, see KeyGene's home page: http://www.keygene.com

Reference

Zabeau, M. and P. Vos. 1993. Selective restriction amplification: a general method for DNA fingerprinting. European Patent Publication 92402629 (Publication No. EP0534858A1).

Four steps

- DNA is digested with two different restriction enzymes
- Oligonucleotide adapters are ligated to the ends of the DNA fragments
- Specific subsets of DNA digestion products are amplified, using combinations of selective primers
- Polymorphism detection is possible with radioisotopes, fluorescent dyes or silver staining

DNA digestion and ligation

- One restriction enzyme is a frequent cutter (four-base recognition site, e.g. MseI)
- The second restriction enzyme is a rare cutter (six-base recognition site, e.g. EcoRI)
- Specific synthetic double-stranded adapters for each restriction site are ligated to the DNA fragments generated

The DNA being examined is digested with two different restriction enzymes, one of which is a frequent cutter (the four-base restriction enzyme) and the other a rare cutter (the six-base restriction enzyme). Various enzyme/primer combinations can be used. MseI and EcoRI are best used in AT-rich genomes as they give fewer fragments in GC-rich genomes.

Specific synthetic adapters for each restriction site are then ligated to the digested DNA. Both the restriction and ligation steps can be performed in a single reaction.

PCRs and detection

- A first PCR (pre-selective) is performed, using oligonucleotide primers complementary to the adapter and restriction sites. A nucleotide is added to the primers to select only a subset of fragments
- Pre-selective amplification products undergo another PCR run, and again a subset of those fragments is selected. Usually, for the second selective amplification, two extra nucleotides are added to the primers
- Fragments are separated by denaturing polyacrylamide ('sequencing gels') or capillary gel electrophoresis

 AFLPs 6

Let's say an extra nucleotide A is added to pre-selective primers. Hence, only a subset of the fragments of the mixture is amplified (i.e. those in which the restriction site sequence is followed directly by an A). Amplification primers are usually 17 to 21 nucleotides in length, and anneal perfectly to their target sequences.

A second amplification is then carried out, using similar oligonucleotide primers but with two extra bases (e.g. AC). Therefore, only a subset of the first amplification reaction will undergo subsequent amplification during the second round of PCR (i.e. those in which the AC sequence follows the restriction site sequence).

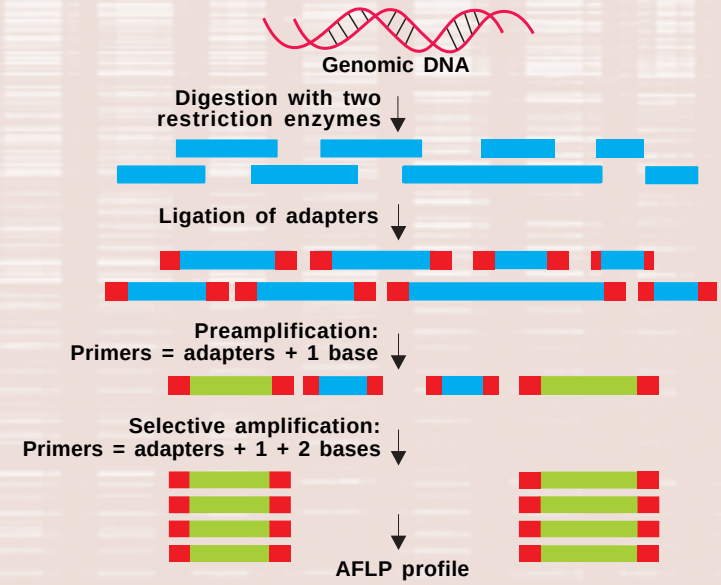
The subset of fragments are analysed by denaturing polyacrylamide gel electrophoresis to generate a fingerprint and DNA bands may be detected, using different methods.

In addition to the advantage of not requiring radioisotopes, fluorescent primers can be loaded as sets of three, each labelled with a different coloured dye, into the same gel lane, thus maximising the number of data points gathered per gel (Zhao *et al.*, 2000).

Reference

Zhao, S., S.E. Mitchell, J. Meng, S. Kresovich, M.P. Doyle, R.E. Dean, A.M. Casa and J.W. Weller. 2000. Genomic typing of *Escherichia coli* 0157:H7 by semi-automated fluorescent AFLP analysis. Microbes Infect. 2:107-113.

Summarising the technology*



*Adapted from KeyGene's Web site: http://www.keygene.com

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
  - Vertical gel electrophoresis units
  - UV transilluminator
  - Automatic sequencer

Interpreting AFLP bands

The AFLP technique detects polymorphisms arising from changes (presence or size) in the restriction sites or adjacent to these

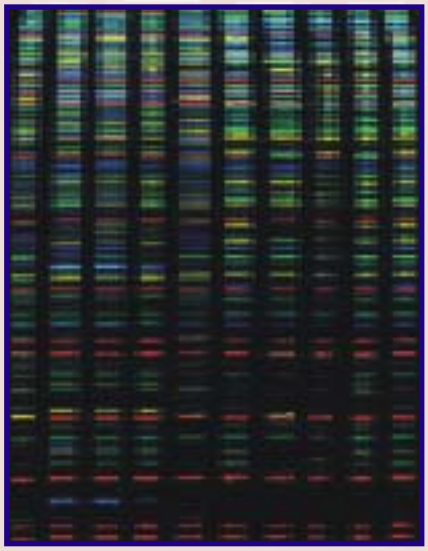
- Different restriction enzymes can be used, and different combinations of pre- and selective nucleotides will increase the probability of finding useful polymorphisms
- The more selective bases, the less polymorphism will be detected
- Bands are usually scored as either present or absent
- Heterozygous versus homozygous bands may be detected, based on the thickness of the signal, although this can be tricky

 AFLPs 9

The molecular basis of AFLP polymorphisms will usually be caused at the nucleotide level. Single nucleotide changes will be detected when (1) the actual restriction sites are affected; and (2) nucleotides adjacent to the restriction sites are affected, which cause the primers to mispair at the 3' end and prevent amplification.

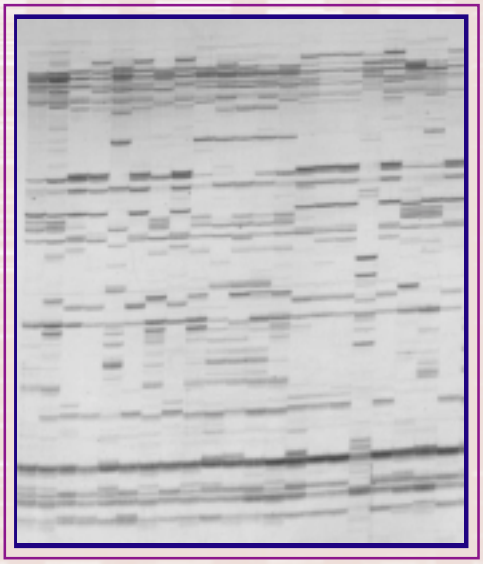
Most AFLP markers will be mono-allelic, meaning that only one allele can be scored and the corresponding allele is not detected. At a low frequency, bi-allelic markers will be identified, as a result of small insertions or deletions in the restriction fragments.

An AFLP gel run with fluorescent dyes



This image shows an AFLP gel run in an automatic sequencer. Before loading the gel, samples were labelled with one of three fluorescent dyes (yellow, blue or green). Red marks a control sample that was included with the other samples to monitor the performance of the electrophoresis. Ascertaining the presence or absence of particular bands directly from the gel is almost impossible because of the high number of bands normally obtained through the AFLP procedure, and because fluorescent dyes as such cannot be seen by eye. Bands are determined with a laser, and data collection with the help of specialised computer software.

AFLPs detected with silver staining



This image shows an AFLP gel run in a manual device (vertical electrophoresis unit) and stained with silver nitrate. The picture shows that certain regions of the gel are crowded with bands, whereas others are emptier. Collecting data from silver-stained gels can be done by eye, or with the help of a computer after the gel is appropriately scanned.

Advantages of AFLPs

- AFLPs allow a quick scan of the whole genome for polymorphisms
- Because of the large number of bands generated, each marker gives a highly informative fingerprint
- They are also highly reproducible
- No prior sequence information or probe generation is needed
- Extremely useful in creating quick genetic maps
- “Transcript profiling”

 AFLPs 12

The AFLP technology can be applied to any DNA sample, including human, animal, plant and microbial DNA, giving it the potential to become a universal DNA fingerprinting system.

Because of the nature of AFLP primers, the markers obtained are highly reliable and robust, unaffected by small variations in the amplification process.

A typical AFLP fingerprint contains between 50 and 100 amplified fragments, many of which, or even most, may serve as genetic markers.

The generation of transcript profiles using AFLPs with cDNAs is an efficient tool for identifying differentially expressed mRNAs. This tool has several advantages that can be useful for discovering genes in germplasm.

Disadvantages of AFLPs

- AFLPs generate huge quantities of information, which may need automated analysis and therefore computer technology
- AFLP markers display dominance
- In genetic mapping, AFLPs often cluster at the centromeres and telomeres
- They are technically demanding in the laboratory and, especially, in data analysis

A further drawback of AFLP technology is perhaps the lack of guarantee of homology between bands of similar molecular weight (MW), thus creating difficulties for some types of studies such as phylogenetic analyses. However, while non-homologous bands with similar weight are also found with other markers such as RAPDs, they may, in fact, be less common with AFLP technology because gel resolution is very high and, consequently, the likelihood of non-homologous bands being coincidentally of the same molecular weight is low.

Applications

- Genetic diversity assessment
- Genetic distance analysis
- Genetic fingerprinting
- Analysis of germplasm collections
- Genome mapping
- Monitoring diagnostic markers

 AFLPs 14

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

Badr, A., K. Müller, R. Schäfer-Pregl, H. El Rabey, S. Effgen, H.H. Ibrahim, C. Pozzi, W. Rohde and F. Salamini. 2000. On the origin and domestication history of barley (*Hordeum vulgare*). Mol. Biol. Evol. 17(4):499-510.

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Palacios, C. and F. González-Candelas. 1999. AFLP analysis of the critically endangered *Limonium cavanillesii* (Plumbaginaceae). J. Hered. 90(4):485-489.

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Sawkins, M.C., B.L. Maass, B.C. Pengelly, H.J. Newbury, B.V. Ford-Lloyd, N. Maxted and R. Smith. 2001. Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment length polymorphism. Mol. Ecol. 10:1947-1958.

Srivastava, A., V. Gupta, D. Pental and A.K. Pradhan. 2001. AFLP-based genetic diversity assessment amongst agronomically important natural and some newly synthesized lines of *Brassica juncea*. Theor. Appl. Genet. 102:193-199.

Vuylsteke, M., R. Mank, B. Brugmans, P. Stam and M. Kuiper. 2000. Further characterization of AFLP (R) data as a tool in genetic diversity assessment among maize (*Zea mays* L.) inbred lines. Mol. Breed. 6(3):265-276.

Example: *Limonium* sp.

- Title:

  AFLP analysis of the critically endangered *Limonium cavanillesii* (Plumbaginaceae). J. Hered. 1999. 90(4):485-489

- Objective:

  To compare the performance of AFLPs for the genetic diversity analysis of *L. cavanillesii* and their efficiency against a previous RAPD study

- Materials and methods:

  DNA was extracted from 29 wild individuals. These individuals were the same as those employed in a previous RAPD study*. Three primer combinations were selected

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*Palacios, C and F. González-Candelas. 1997. Lack of genetic variability in the rare and endangered *Limonium cavanillesii* (Plumbaginaceae) using RAPD markers. Mol. Ecol. 6:671-675.

Example: *Limonium* sp. (continued)

- Results:

  A total of 231 fragments were generated: on average, 223 per individual and 77 per primer combination. Only 6% of AFLP markers were polymorphic. With these, 11 different AFLP profiles could be distinguished in the species, while in a previous RAPD* study, no polymorphic markers were obtained

- Discussion:

  AFLPs proved to be a suitable marker type for gathering the information critical to identifying natural populations at risk and planning recovery strategies for *L. cavanillesii*. The low levels of genetic variability found in *L. cavanillesii* could be explained as a consequence of either its apomictic reproductive system or a recent and severe bottleneck event

* Palacios, C and F. González-Candelas. 1997. Lack of genetic variability in the rare and endangered *Limonium cavanillesii* (Plumbaginaceae) using RAPD markers. Mol. Ecol. 6:671-675.

Example: *Stylosanthes* spp.

- Title:

  Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment length polymorphism. Mol.Ecol. 2001. 10:1947-1958

- Objective:

  To assess genetic variation in two species of the tropical genus *Stylosanthes* Sw.

- Materials and methods:

  For analysis, 111 accessions were selected: 59 *S. viscosa* and 52 *S. humilis*, representing the geographical distribution of both species. Five primer combinations were used for *S. humilis* and four for *S. viscosa*

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Example: *Stylosanthes* spp. (continued)

Results:

- *S. humilis*: 316 out of 417 total bands were polymorphic (75%). The average similarity among accessions was 0.71, and the average genetic distance was 0.17 (Jaccard's coefficient)
- *S. viscosa*: 312 out of 373 total of bands were polymorphic (83%). The average similarity value among accessions was 0.67, and the average genetic distance was 0.20
- Cluster analysis and PCA grouped accessions from both species by geographical origin, with some exceptions. One explanation may be the incidence of long-range dispersal events or introductions of genotypes from one area to another by humans

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Example: Stylosanthes spp. (continued)

- Discussion:

  These results demonstrate the usefulness of AFLP technology, not only to detect genetic diversity within species of *Stylosanthes*, but also to identify individuals that might have been misclassified

- Conclusions:

  This study shows the ability of AFLP to detect geographic patterns in genetic variation, which information is necessary for developing a strategy for optimal conservation

Example: Indian mustard

- Title:

  AFLP-based genetic diversity assessment amongst agronomically important natural and some newly synthesized lines of *Brassica juncea*. Theor. Appl. Genet. 2001. 102:193-199

- Objectives:
  - Study the genetic variation among *B. juncea* accessions
  - Identify the AFLP primer combinations that would be informative for varietal identification
  - Search polymorphic markers for eventual tagging of putative agronomic traits available in the germplasm studied

- Materials and methods:

  The study used 30 *B. juncea* accessions, representing 21 established natural populations and 9 synthetic varieties and lines. Selective amplification was carried out with 21 EcoRI/ MseI primer pair combinations

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Example: Indian mustard (continued)

Results:

- For the 30 genotypes, 1251 fragments were scored. On average, 37 bands per primer combination were polymorphic
- No single primer pair could distinguish all 30 genotypes on the basis of the presence or absence of variety specific bands
- Four primer pairs were found to be most informative and had 100% discriminatory power
- Cluster analysis based on these four informative primers broadly agreed with results of earlier studies based on morphological traits* and RAPD data**

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**Jain, A., S. Bhatia, S.S. Banga, S. Prakash and M. Lakshmikumaran. 1994. Potential use of the random amplified polymorphic DNA (RAPD) technique to study the genetic diversity in Indian mustard (*Brassica juncea*) and its relationship to heterosis. Theor. Appl. Genet. 88:116-122.

*Pradhan, A.K., Y.S. Sodhi, A. Mukhopadhyay and D. Pental. 1993. Heterosis breeding in Indian mustard (*Brassica juncea* (L.) Czern. & Cross): analysis of component characters contributing to heterosis for yield. Euphytica 69:219-229.

Example: Indian mustard (continued)

- Discussion and conclusions:

  In a previous RAPD* study with 32 primers, an average of 12 polymorphic loci per primer had been obtained. Thus, the AFLP technique provided a higher degree of resolution for discriminating among closely related germplasm than did RAPDs. AFLP analysis therefore has the potential to complement both conventional and other types of molecular marker data

*Jain, A., S. Bhatia, S.S. Banga, S. Prakash and M. Lakshmikumaran. 1994. Potential use of the random amplified polymorphic DNA (RAPD) technique to study the genetic diversity in Indian mustard (*Brassica juncea*) and its relationship to heterosis. Theor. Appl. Genet. 88:116-122.

In summary

- The AFLP technology is based on the selective amplification of restriction fragments after DNA digestion
- It detects polymorphisms caused by changes in the restriction sites or their neighbouring regions
- This technology yields a large number of bands per run, but they are dominant and the procedure technically demanding

By now you should know

- The different steps involved in the generation of AFLPs
- Main considerations for interpreting AFLP bands
- Advantages and disadvantages of AFLPs for genetic diversity analysis

Basic references

Brown, S.M. and S. Kresovich. 1996. Molecular characterization for plant genetic resources conservation. Pp. 85-93 in Genome Mapping in Plants (H. Paterson, ed.). RG Landes Company and Academic Press, Austin, TX.

KeyGene, N.V. 2001. Empowering genomics. http://www.keygene.com (30 June 2002)

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Zabeau, M. and P. Vos. 1993. Selective restriction amplification: a general method for DNA fingerprinting. European Patent Publication 92402629 (Publication No. EP0534858A1).

Zhao, S., S.E. Mitchell, J. Meng, S. Kresovich, M.P. Doyle, R.E. Dean, A.M. Casa and J.W. Weller. 2000. Genomic typing of *Escherichia coli* 0157:H7 by semi-automated fluorescent AFLP analysis. Microbes Infect. 2:107-113.

Next

DNA-based technologies
PCR-based technologies
Sequences-tagged sites

- DNA-based technologies
  - PCR-based technologies
    - Latest strategies
- Complementary technologies
- Final considerations
- Glossary