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Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications

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Amplified fragment length polymorphism (AFLP) is a novel molecular fingerprinting technique that can be applied to DNAs of any source or complexity. Total genomic DNA is digested using two restriction enzymes. Double-stranded nucleotide adapters are ligated to the DNA fragments to serve as primer binding sites for PCR amplification. Primers complementary to the adapter and restriction site sequence, with additional nucleotides at the 3′-end, are used as selective agents to amplify a subset of ligated fragments. Polymorphisms are identified by the presence or absence of DNA fragments following analysis on polyacrylamide gels. This technique has been extensively used with plant DNA for the development of high-resolution genetic maps and for the positional cloning of genes of interest. However, its application is rapidly expanding in bacteria and higher eukaryotes for determining genetic relationships and for epidemiological typing. This review describes the AFLP procedure, and recent, novel applications in the molecular fingerprinting of DNA from both eukaryotic and prokaryotic organisms.

Keywords: AFLP; molecular markers; genetic mapping; PCR; polymorphism; DNA fingerprinting

Introduction

Amplified fragment length polymorphism (AFLP) is a new molecular technique for fingerprinting DNAs of any origin and complexity. It has a number of potential applications such as monitoring inheritance of agronomic traits in plant and animal breeding, diagnostics of genetically inherited diseases, pedigree analysis, forensic typing, parentage analysis, screening of DNA markers linked to genetic traits and microbial typing. The AFLP technique has several advantages over other DNA fingerprinting systems. The most important of these are the capacity to inspect an entire genome for polymorphism and its reproducibility. AFLP can be applied to any DNA samples including human, animal, plant and microbial DNAs, giving it the potential to become a universal DNA fingerprinting system. The objectives of this review are to discuss, in detail, the AFLP procedure and its applications.

Principle of the method

Amplified fragment length polymorphism (AFLP) is the selective amplification of restriction fragments from a digest of total genomic DNA using the polymerase chain reaction (PCR). The technique, developed by Zabeau and Vos [46], is patented by Keygene NV (Wageningen, The Netherlands). With AFLP, molecular genetic polymorphisms are identified by the presence or absence of DNA fragments following restriction and amplification of genomic DNA. Figure 1 outlines the four steps of the AFLP technique: DNA digestion, ligation, amplification and gel analysis. Genomic DNA is first digested by two restriction enzymes. Double-stranded oligonucleotide adapters, homologous to one 5′- or 3′-end generated during restriction digestion, are ligated to the DNA fragments. The ligated DNA fragments are amplified by PCR using primers complementary to the adapter and restriction site sequence with additional selective nucleotides at their 3′-end. The use of selective primers reduces the complexity of the mixture. Only those fragments with complementary nucleotides

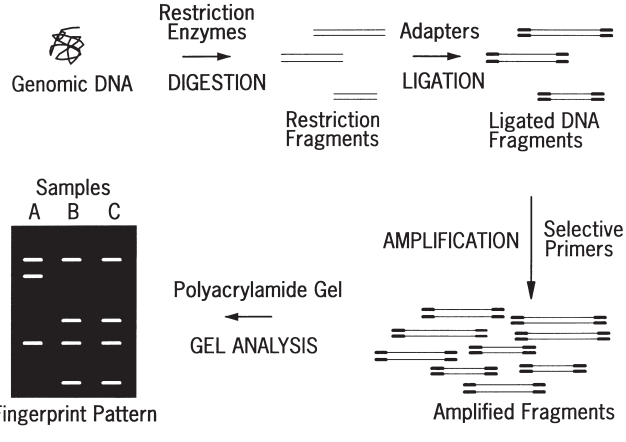

Figure 1 A schematic displaying the four basic steps of AFLP: digestion, ligation, amplification and gel analysis. Genomic DNA is digested by restriction enzymes and adapters are ligated to the restriction fragments. A subset of the ligated fragments are amplified by PCR, using primers with selective nucleotides at the 3′-end. Polymorphism is revealed by running the amplified products of various samples on a denaturing polyacrylamide gel.

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Abbreviations: A, adenine; AFLP, amplified fragment length polymorphism; ATP, adenosine triphosphate; bp, base pair(s); C, cytosine; cM, centimorgan(s); dCTP, deoxycytidine triphosphate; DNA, deoxyribonucleic acid; G, guanine; HG, hybridization group; ISTR, inverse sequence-tagged repeat; kbp, kilobase pair(s); Mb, megabases; P, phosphorus; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; T, thymine
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extending beyond the restriction site will be amplified by the selective primers under stringent annealing conditions. Polymorphisms are revealed by analysis of amplified fragments on a denaturing polyacrylamide gel, and comparison of the patterns generated for each sample.

Digestion of genomic DNA

Restriction fragments are generated using two restriction endonucleases, a 'rare'-cutting enzyme with 6- to 8-base recognition, in combination with a 'frequent'-cutting enzyme of 4-base recognition. The high degree of specificity of restriction enzymes results in production of a reproducible set of DNA fragments. The complexity of the genome and factors such as the methylation status of the DNA influence the choice of enzymes [1]. The use of two different enzymes allows the researcher to manipulate the number of fragments generated for amplification and produce fingerprint patterns of desired complexity. In addition, when the fragments are cut using two enzymes, a large number of different fingerprints can be produced using the combinations of only a few primers. Examples of rare-cutting enzymes employed in AFLP include *Eco*RI, *Ase*I, *Hin*dIII, *Apa*I and *Pst*I. *Mse*I and *Taq*I are the commonly used frequent-cutters. However, the DNA of most eukaryotes is AT-rich making *Mse*I (recognition sequence TTAA) the preferred frequent-cutter for AFLP [45]. The frequent-cutter generates small fragments within the desired size range of 100–1000 base pairs required for efficient PCR amplification and separation on denaturing polyacrylamide gels [1].

Three types of restriction fragments are generated following digestion: (i) fragments cut by the rare-cutting enzyme on both ends; (ii) fragments cut with the frequent-cutting enzyme on both ends; and (iii) fragments that have been cut by both the rare-cutter and frequent-cutter. Using *Eco*RI and *Mse*I restriction enzymes as examples, *Eco*RI-*Eco*RI, *Mse*I-*Mse*I and *Eco*RI-*Mse*I fragments would be produced during restriction enzyme digestion. More than 90% of the fragments are expected to have frequent-cutter sites on both ends (eg *Mse*I-*Mse*I fragments).

Ligation of adapters

AFLP is not dependent on prior sequence knowledge. Double-stranded nucleotide adapters (10–30 base pairs long), complementary to the sticky ends of the corresponding restriction site, are ligated to the restriction fragments using T4 DNA ligase (Figure 2). The sequence of the adapters and the adjacent restriction half-site serve as primer binding sites for subsequent PCR amplification. Adapters are composed of two synthetic oligonucleotides that are in part complementary to each other and form a double-stranded structure in solution under appropriate conditions. Ligation does not restore the original restriction enzyme site because of a base change incorporated into the adapter sequence (Figure 2). This change in the recognition site prevents restriction from taking place after ligation has occurred, enabling restriction and ligation reactions to be performed in the same tube. With these reactions occurring simultaneously, any fragment-to-fragment product is restricted.

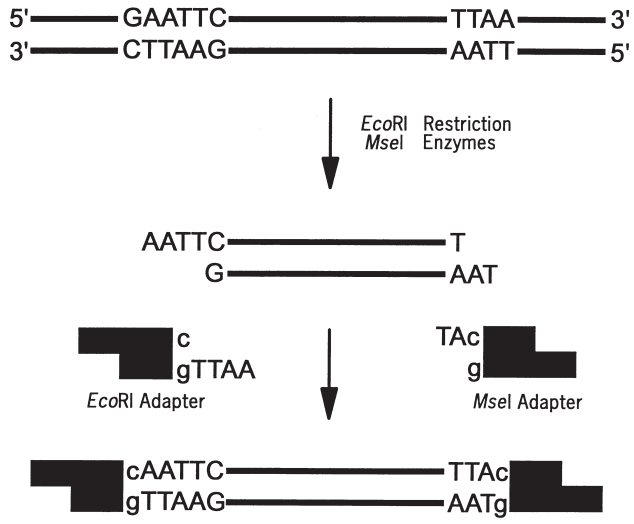

Figure 2 A schematic outlining the ligation of adapters to the ends of a restriction fragment. Genomic DNA is first restricted by *Eco*RI and *Mse*I. Double-stranded adapters, complementary to the short single-strand extension generated by the restriction enzymes, are ligated to the DNA fragment. The *Eco*RI and *Mse*I recognition sites are not restored by ligation because of a base change in the adapter sequence (shown in lower case).

In addition, adapter-to-adapter ligation is prevented by using nonphosphorylated adapters. Both of these features ensure that adapters are ligated to virtually all restriction fragments.

The complexity of the DNA mixture can be reduced prior to amplification using biotin-labeled adapters complementary to the rare-cutting restriction half-site (eg biotin-labeled *Eco*RI adapters). Biotinylated restriction fragments cut by the rare-cutting enzyme are selected using streptavidin-coated magnetic beads as described by Zabeau and Vos [46]. The use of biotin-labeled adapters separates fragments having at least one rare-cutting restriction site from the large number of fragments having two frequent-cutting restriction sites (eg *Eco*RI-*Eco*RI and *Eco*RI-*Mse*I fragments are separated from *Mse*I-*Mse*I fragments). This step is not essential but may be useful for decreasing background smears on gels.

Selective amplification

AFLP primers for selective amplification contain three types of DNA sequence: the 5′ region complementary to the adapter, the restriction site sequence and the 3′ selective nucleotides (Figure 3). Two AFLP primers are used; one primer is complementary to the adapter and adjacent rare-cutter restriction site sequence with one to three additional selective nucleotides at the 3′-end (eg *Eco*RI primer 3′-XXX, where X denotes the selective nucleotides), and the second primer is complementary to the adapter and frequent-cutter recognition site sequence with an additional selective one- to three-base extension (eg *Mse*I primer 3′-XXX).

Following the restriction-ligation reaction, a limited number of ligated restriction fragments are selectively amplified by the AFLP primers (Figure 3). Only a subset of the template fragments, with complementary nucleotides

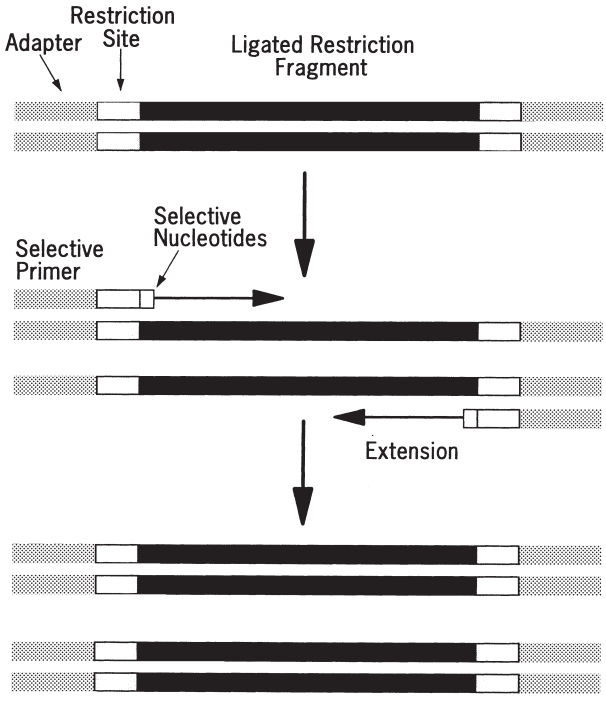

Figure 3 A schematic depicting the PCR amplification of a ligated restriction fragment. The AFLP primers have three regions, the 5′-end complementary to the oligonucleotide adapter sequence, the restriction site sequence and the 3′ selective nucleotides. The horizontal arrows indicate the direction of DNA synthesis. With each PCR cycle, the amount of DNA is doubled.

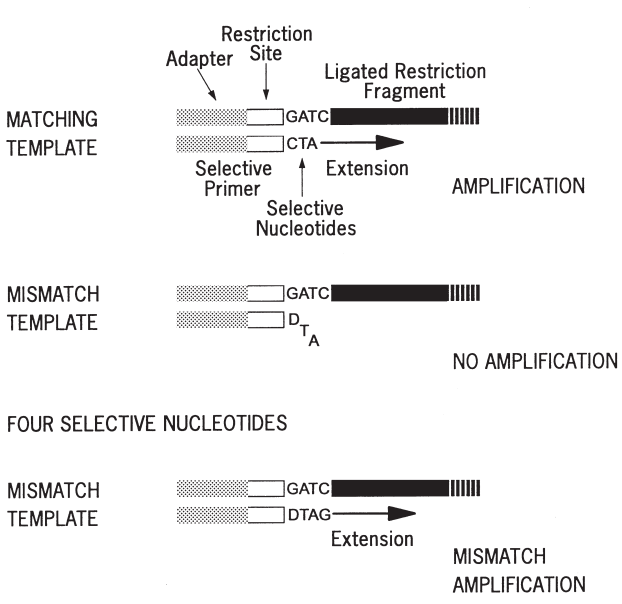

Figure 4 A schematic illustrating the selectivity of AFLP primers. The first example shows successful extension of a primer with three selective nucleotides matching the template sequence. The arrow indicates the direction of DNA synthesis. In the second example, there is a mismatch between the three selective bases and the restriction fragment preventing extension of the primer. The final example illustrates the mismatch amplification that can occur with primers having four selective bases. D = G, A or T.

extending beyond the restriction site, will be amplified under stringent annealing conditions (Figure 4). The nucleotide extensions on the 3′-end of the primers serve two purposes: (i) they allow a variety of restriction fragment subsets to be amplified; and (ii) they provide additional possibilities for polymorphisms to be detected beyond the restriction site itself.

Although fragments cut only by the frequent-cutter (eg *Mse*I-*Mse*I fragments) are the predominant species (>90%), fragments cut by both enzymes (eg *Eco*RI-*Mse*I fragments) are preferentially amplified. There are two reasons for the efficiency: (i) the primer complementary to the rare-cutting restriction site and adapter (eg *Eco*RI primer) has a higher annealing temperature than the primer of the frequent-cutter (eg *Mse*I primer); and (ii) the fragments cut by both enzymes (eg *Eco*RI-*Mse*I fragments) are amplified using two primers (eg *Mse*I and *Eco*RI primers) preventing the formation of an inverted repeat at the ends [45]. Base-pairing of the ends of the fragment forms a stem-loop structure which competes with primer annealing. The PCR conditions are an important feature of the AFLP technique. Most restriction digestion techniques generate such complex patterns that differences cannot be discerned. However, by increasing the efficiency of the primer complementary to the rare-cutter, only a subset of the fragments (<10%) are efficiently amplified (eg *Eco*RI-*Mse*I fragments) to allow polymorphism to be revealed.

The number of amplified fragments is determined by the complexity of the genomic DNA, the choice of enzymes, as well as the number and type of selective nucleotides in the PCR primers. There is an almost linear correlation between the number of amplified fragments and the genome size [45]. As the number of selective nucleotides is increased, the complexity of the DNA fingerprint decreases. The number of amplified fragments is reduced approximately four-fold with each additional selective base, assuming random base distribution [45]. Although there is no standard method for chosing selective nucleotides, nucleotide extensions composed of rare di- or tri-nucleotides will greatly reduce the number of amplified fragments. The length and nature of the base extension on the 3′-end of the primers can be manipulated to generate fingerprints of desired complexity.

For small genomes of 10^6–10^7 base pairs (bp), one to two selective nucleotides on the 3′-end of each primer may be sufficient to reveal polymorphism. More complex genomes ranging from 10^8–10^9 bp will require additional selective nucleotides to yield the desired number of amplified fragments. Typically, the ideal number of amplified restriction fragments ranges from 50–100 [45].

Although the complexity is reduced with each additional selective nucleotide, the selectivity is maintained with nucleotide additions to a maximum of three selective bases. The fingerprints generated are merely a subset of the previous pattern. However, selectivity is lost with a 4-base extension. This loss of selectivity was demonstrated by Vos *et al* [45] when additional bands were amplified using four selective bases, which had not been detected in the corresponding fingerprint with primers having three selective bases. This indicates a tolerance of mismatches during amplification by primers with 4-base extensions (Figure 4).

Gel analysis

Prior to loading the amplified products into polyacrylamide gels, the DNA samples are denatured by heating at 90–95°C for 3–5 min. To prevent the occurrence of double bands or 'doublets' on the gel due to unequal mobility of the two strands of an amplified fragment, only one strand is labeled. Two labeling techniques, using either a radioactive label or a fluorescent dye are employed. Typically, the primer corresponding to the rare-cutting restriction enzyme is labeled by phosphorylating the 5′-end with [γ-^{32}P or -^{33}P]ATP using T4 polynucleotide kinase. A relatively new labeling technique involves the use of fluorescent dyes as described below.

Following electrophoretic separation of the amplified fragments on a 4.5% or 5% polyacrylamide gel, typical AFLP patterns are visualized by autoradiography. The resulting banding patterns may be visualized by eye, or captured by high-resolution densitometric scanning for computer-assisted analysis (eg Gel Compar 3.1 software, Applied Maths, Kortrijk, Belgium).

Chalhoub *et al* [8] evaluated silver staining for the visualization of AFLP products in polyacrylamide gels (Table 1). Comparison of AFLP fingerprints visualized by autoradiography and silver staining revealed similar sensitivity and resolution. The silver staining procedure does not include labeled primers, thus both strands of amplified fragments are visualized. Regardless of the presence of double bands, no complexities in the banding patterns were observed. An advantage of the silver staining technique is the possible recovery of fragments from the dried gels at a later date by simple rehydration and direct transfer to a PCR tube for amplification. This is particularly useful in cases where further characterization of specific fragments is required such as cloning, sequencing, preparation of probes or development of sequence-characterized amplified regions.

Similar results were obtained by Cho *et al* [9] in a study evaluating the cloning and mapping of AFLP fragments from silver-stained polyacrylamide gels (Table 1). Similar banding patterns were obtained using ^{32}P and silver staining. However, the resolution of silver-stained gels was found to be greater than that of ^{32}P-labeled gels particularly in the region of the gel containing fragments greater than 300 bp. The silver staining method is advantageous in that it does not involve the use of radioactive isotopes. In addition, the high resolution and ability to excise bands directly from the gel make silver staining a more useful and versatile detection method for AFLP amplification products.

Inclusion of PCR preamplification for complex genomes

A two-step amplification strategy was developed for complex genomes (10^8-10^9 bp) requiring three selective nucleotides on one or both primers. The first PCR amplification, called preamplification, utilizes primers having a single or no selective nucleotide. PCR products from preamplification are diluted and used as templates for a second amplification reaction using primers with full base extensions.

Table 1 Exprimental protocols of studies utilizing the AFLP technique for analysis of genetic diversity in eukaryotes

Reference	Organism studied	Preselective primers	Selective primers	No. of primer pairs tested
[8]	*Pisum sativum* L. (pea)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[ab]	N/A
[9]	*Oryza sativa* (rice) *japonica* and *indica* cultivars	Not utilized	*Taq*I 3′-3 *Pst*I 3′-2[bc]	1
[12]	*Globodera rostochiensis* and *G. pallida* (potato cyst nematodes)	*Mse*I 3′-0 *Eco*RI 3′-0	*Mse*I 3′-2 *Eco*RI 3′-2[a]	12
[13]	*Miscanthus* spp (perennial grass)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	6
[15]	*Lactuca* spp (lettuce)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	3(4)
[28]	Symbiotic fungi of fungus-growing ant *Cyphomyrmex minutus*	Not utilized	*Pst*I 3′-2	6 primers
[32]	*Camellia sinensis* (L.) O. Kuntze (Indian and Kenyan tea)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	5
[35]	*Glycine max* and *Glycine soja* (soybean)	Not utilized	*Taq*I 3′-2/3[a] *Pst*I 3′-2/3 *Hin*dIII 3′-2/3	6
[36]	*Hordeum vulgare* (barley)	*Mse*I 3′-1 *Eco*RI 3′-1	12 *Mse*I 3′-3 8 *Eco*RI 3′-3[a]	96
[38]	*Vitis vinifera* L. Sangiovese and Colorino (grapevine cultivars)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	8
[39]	*Lens* (lentil)	Not utilized	*Mse*I 3′-2/3 *Pst*I 3′-2/3[a]	4
[41]	*Astragalus cremnophylax* var *cremnophylax* (endangered sentry milk-vetch)	*Mse* 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	9

N/A = information not available.
[a]5′-end of primer labeled using [γ-^{32}P or γ-^{33}P]ATP.
[b]Selective primers not labeled for silver staining.
[c]5′-end of primer labeled using [^{32}P]dCTP.

Preamplification reduces the overall complexity of the mixture up to 16-fold, allowing the target sequence to become the predominant species. The inclusion of preamplification reduces background smears in the fingerprint pattern resulting from mismatched amplification, that is tolerated at a low level, by primers with three selective bases (Figure 4).

AFLP™ kits available

AFLP™ Plant Mapping Kit

PE Applied Biosystems (Foster City, CA, USA) has developed an AFLP™ Plant Mapping Kit based on the AFLP procedure patented by Keygene NV (Wageningen, The Netherlands). Two modules are available depending on

the genome size. The Small Plant Genome Kit is used for genomes ranging from 50–500 megabases, and the Regular Plant Genome Kit is for genomes of 500–5000 megabases. Restriction fragments are generated using *Eco*RI and *Mse*I restriction enzymes. For preamplification, both preselective primers in the Regular Plant Genome Kit have an additional selective nucleotide at the 3′-end. However, only the *Mse*I preselective primer has a selective base in the Small Plant Genome Kit.

There are 16 selective primers available, eight *Mse*I primers and eight *Eco*RI primers. The *Mse*I primers have a total of three selective nucleotides at the 3′-end. The *Eco*RI primers have two selective nucleotides (for the Small Plant Genome Kit) or three selective nucleotides (for the Regular Plant Genome Kit) at the 3′-end. Any combination of one *Eco*RI and one *Mse*I primer can be used, providing 64 possible primer pair combinations.

AFLP™ Microbial Fingerprinting Kit

A similar AFLP kit using *Eco*RI and *Mse*I restriction enzymes is offered by PE Applied Biosystems for microbial fingerprinting. For the less complex genomes of microorganisms, preselective amplification is performed using primers composed of only the core sequence (adapter and restriction site sequence) without the addition of selective nucleotides. Selective nucleotides are introduced in the second amplification step. Eight *Mse*I and eight *Eco*RI selective primers are available, four of each type have one selective nucleotide and four have two selective nucleotides at the 3′-end.

An unique feature of the AFLP™ Plant Mapping and AFLP™ Microbial Fingerprinting Kits is the 5′-end label on the *Eco*RI selective primers. Instead of a 5′-radioactive label, the *Eco*RI primers have a fluorescent dye label at the 5′-end. The fluorescent dyes are excited by laser radiation and are visualized by their characteristic absorption-emission frequencies. Thus, only the fragments containing an *Eco*RI restriction site are detected using systems such as ALFexpress™ DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden) and ABI PRISM™ DNA Sequencer (Perkin Elmer Corporation, Foster City, CA, USA).

Three types of fluorescent dyes are used to label the *Eco*RI selective primers, carboxyfluorescein (FAM), carboxytetramethyrhodamine (TAMRA), and carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (JOE). A fourth fluorescent dye, carboxy-X-rhodamine (ROX), is loaded along with each sample to serve as an internal size standard. The standard ensures that all amplification fragments are accurately sized. For high throughput, PCR products from up to three reactions, labeled with different coloured dyes, can be loaded into a single lane because each dye label has its characteristic absorption-emission frequency [29]. The fluorescent dye labels are detected with high sensitivity by the DNA sequencers. Results are analyzed with GeneScan Analysis software (PE Applied Biosystems) and displayed in any combination of electropherograms and tabular data. GeneScan Analysis software can be used to prepare data for further analysis using GenoTyper™ software (PE Applied Biosystems). GenoTyper converts data into the format required by downstream applications, such as linkage analysis, databases, or spreadsheets. Although there is no software currently available to conduct cluster analysis using GeneScan files directly, it is possible to construct dendrograms using software packages such as TREECON [44] if the AFLP data are first converted to a binary file. Instead of manually scoring the presence or absence of fragments to generate a binary file, AFLPapp software [4] can be used to convert GeneScan data directly to a binary file.

Factors affecting reproducibility

Genomic DNA of high purity is required for AFLP to ensure complete digestion by the restriction endonucleases. Incomplete restriction of DNA generates partial fragments, predominantly of high molecular weight. Amplification of fragments that are not fully digested generates an altered banding pattern, and may be misinterpreted as false polymorphisms [45].

Although the AFLP procedure is affected by DNA quality, it is insensitive to the template DNA concentration. The protocol is optimized such that the amplification reaction ceases when the labeled primer is consumed [45]. This ensures that fingerprints of equal intensity are produced despite variations in template concentration. However, at very high template dilutions (picogram quantities), the nucleotide sequences flanking the restriction site will no longer be random for a small pool of restriction fragments and variations in the banding patterns may be observed. Typically, 0.05 μg of genomic DNA is required for small genomes ranging from 50–500 megabases and 0.5 μg for genomes of 500–5000 megabases.

Advantages of AFLP

The AFLP technique can be used for DNA samples of any origin or complexity. Small sequence variations can be detected using only small quantities of genomic DNA (0.05–0.5 μg). The capacity to reveal many polymorphic bands in one lane is a major advantage of AFLP markers. The numerous bands on a gel are analyzed simultaneously making AFLP an extremely efficient technique. AFLP has the capacity to inspect a much greater number of loci for polymorphism than other currently available PCR-based techniques, such that the number of polymorphisms detected per reaction are much higher. AFLP is superior in terms of the number of sequences amplified per reaction and their reproducibility. The markers produced are reliable and reproducible within and between laboratories, and are relatively easy and inexpensive to generate. A virtually unlimited number of markers can be generated by simply varying the restriction enzymes, and the nature and number of selective nucleotides.

Comparison to other molecular-based techniques

AFLP has advantages over other molecular-based techniques for DNA fingerprinting including restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). The technique of RFLP includes digestion of genomic DNA with restriction enzymes. The pattern of restriction fragments generated for

each sample is compared, following Southern hybridization, to reveal polymorphism.

The major difference between AFLP and the traditional RFLP technique is that PCR amplification is used for the detection of restriction fragments in AFLP analysis. However, in a modified RFLP technique, called PCR-RFLP, PCR amplification is followed by restriction digestion. Furthermore, AFLP fragments are run on a denaturing polyacrylamide gel to detect the presence or absence of restriction fragments as opposed to RFLP which displays length differences of restriction fragments on agarose or polyacrylamide gels following hybridization. Due to the nature of the RFLP technique, only the restriction site is scanned for differences in DNA sequence. The selective nucleotides included in AFLP provide additional possibilities for polymorphisms to be detected beyond the restriction site itself. AFLP has the capacity to detect more point mutations than RFLP [3]. Insertions and deletions are detected at approximately the same frequency [3]. In a single hybridization experiment, RFLP can detect, at most, a few genetic loci compared to 100–200 loci detected using AFLP [26]. In addition to a greater number of polymorphisms per reaction, AFLP is also superior in terms of efficiency as it does not require template DNA sequencing. Fingerprints are produced without prior sequence knowledge.

RAPD is a PCR-based technique similar to AFLP. However, AFLP uses primers specific to the adapter and restriction site sequence, whereas RAPD utilizes arbitrary primers. The arbitrary primers, having no known homology to the target sequence, are used to randomly amplify segments of the target DNA. Fragments of various sizes are generated during PCR. To allow the primer to anneal to several locations on the two strands of target DNA, the amplification is performed at low stringency (annealing at 36–45°C) [33]. RAPD analysis is easier to perform than AFLP [3]. However, the RAPD technique is very sensitive to the reaction conditions, template DNA concentration and purity, and PCR temperature profiles, limiting its application. AFLP analysis uses stringent annealing conditions which guarantee a better reproducibility [12].

For PCR-based techniques, such as RAPD and PCR-RFLP, the term AFLP is sometimes used in a general sense to refer to a unique pattern of amplifed products separated by gel electrophoresis. However, these techniques are not based on the patented procedure of Zabeau and Vos [46], and do not employ adapters or selective nucleotides.

Applications

AFLP has practical applications for DNA fingerprinting of prokaryotes and eukaryotes. It has been used for marker-assisted plant breeding, the construction of high-density molecular linkage maps, and for the positional cloning of genes of interest. AFLP also has important applications for strain identification and characterization of bacteria and fungi, as well as eukaryotic pathogens of plants and animals. In a modified procedure, AFLP has also been used to generate RNA fingerprints using cDNA as template.

Generation of high-resolution genetic maps in plants

In a relatively well-characterized mapping population of potato, van Eck *et al* [43] studied the localization of AFLP markers relative to a mapping population of 197 RFLP, nine isoenzyme, and 11 morphological markers. Six primer combinations generated 264 segregating AFLP amplification products in a diploid backcross population from non-inbred potato parents. More details on experimental protocol are outlined in Table 2. The segregating patterns of the AFLP amplification products observed in the offspring were used to evaluate the inheritance of putative AFLP loci. AFLP mapping generated more than double the number of genetic markers when compared to RFLP. Athough the AFLP markers were randomly distributed, they targeted regions already mapped by RFLP markers such that the total map length increased only 5% from 1120 to 1170 centimorgans (cM). van Eck *et al* [43] concluded that AFLP may substitute for other marker systems. The AFLP amplification products mapped by van Eck *et al* [43] will enable the chromosomal identity of linkage groups to be established in future mapping studies because of the locus specificity of AFLP markers. In addition, the identification and mapping of comigrating AFLP fragments from other genomes will be facilitated.

In a similar study on barley, Becker *et al* [3] generated 118 AFLP markers using 16 primer combinations (Table 2). Two of the linked AFLP markers could not be assigned to one of seven linkage groups. The remaining 116 markers were mapped to an existing barley RFLP map including five microsatellite and four protein marker loci. The AFLP markers mapped to all regions of the genome including three gaps, 2L, 4L and 6, in which no RFLP loci had been mapped. Addition of markers at the chromosome tips, and bridging the three gaps in the original RFLP map, increased the barley map of 1096 cM to a length of 1873 cM. A 58% increase in the barley map length was determined and is quite dramatic in comparison to the 5% increase observed by van Eck *et al* [43] in potato. The results of Becker *et al* [3] indicate that AFLP is very useful for marker enrich-

Table 2 Experimental protocols employed in studies utilizing the AFLP technique for generation of high-resolution maps

Reference	Organism studied	Preselective primers	Selective primers	No. of primer pairs tested
[3]	*Hordeum vulgare* L. (barley)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	16
[25]	*Oryza sativa* (rice) *japonica* and *indica* cultivars	*Mse*I 3′-0/1 *Eco*RI 3′-0	*Mse*I 3′-3 *Eco*RI 3′-3/2[a]	18
[30,31]	*Rattus norvegicus* (rat)	*Mse*I 3′-1 *Sse*I 3′-1	*Mse*I 3′-3 *Sse*I 3′-2[a]	4
[37]	*Beta vulgaris* L. (sugar beet)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	4
[43]	*Solanum tuberosum* (potato)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	6

[a]5′-end of primer labeled using [γ-^{32}P or γ-^{33}P]ATP.

ment and in some cases may extend the length of existing linkage maps.

In a study on rice, Mackill *et al* [25] compared the levels of polymorphism for AFLP, RAPD, and microsatellite markers on 12 *japonica* and two *indica* rice cultivars. Using 18 AFLP primer pairs, 143 of a total of 539 generated bands were polymorphic (Table 2). Twenty-one RAPD primers produced 103 bands, 43 of which were polymorphic. The number of alleles detected using 14 microsatellite primers ranged from one to six. Although the rice accessions were classified into the same subspecies using all marker types, the level of polymorphism varied considerably. Within *japonica* cultivars, the average percent polymorphism between any two accessions was 22% for AFLP, 24% for RAPD and 36% for microsatellite markers (monomorphic bands excluded). The average percent polymorphism between *indica* and *japonica* accessions was highest for microsatellite (76%) and AFLP (65%) markers as compared with RAPD (35%) markers. Although microsatellite markers produced the highest level of polymorphism, the total number of polymorphic bands was much higher for AFLPs with an average of eight per gel. Only one locus per gel can be scored for microsatellite analysis unless multiplexing is employed. To evaluate the genetic mapping of AFLP markers, 80 F2 plants of an *indica* × *japonica* cross were analyzed using seven primer combinations. An existing RFLP-RAPD map containing 115 RFLP, 10 RAPD markers and one morphological trait was used to map 50 of 54 scored AFLP bands to specific chromosomes. The markers were distributed throughout the rice genome revealing the potential of AFLP markers for mapping important genes in rice.

AFLP and RFLP markers were used by Meksem *et al* [26] to construct a high-resolution genetic map of the *R1* locus on chromosome V of potato. The *R1* allele confers, on potato, a race-specific resistance to *Phytophthora infestans*, a fungal pathogen. Bulked segregant analysis and AFLP markers were used to select markers mapping less than 1 cM from the *R1* gene. Of 3200 informative AFLP loci, 29 displayed linkage to the *R1* locus. These 29 fragments were amplified by 21 of a total of 108 primer combinations tested. The AFLP protocol used by Meksem *et al* [26] is outlined in Table 3. Genotypic analysis of 461 F1 plants revealed that eight loci mapped within a genetic distance of 6 cM as defined by two marker loci, *GP21* and *GP179*, flanking the *R1* locus. Only six were linked in coupling with the resistance allele *R1*. The DNA markers generated in the study by Meksem *et al* [26] will aid in the further characterization of the *R1* gene and may help to elucidate the mechanism of race-specific resistance to *P. infestans*.

A high-resolution map of the *Gro1* locus on potato chromosome VII was constructed by Ballvora *et al* [2] using RFLP, RAPD and AFLP markers. The dominant allele, *Gro1*, confers resistance to a root cyst nematode, *Globodera rostochiensis*. The AFLP protocol of Meksem *et al* [26] was employed for this study (Table 3). AFLP patterns were generated using 24 primers for a total of 144 primer combinations tested. Bulked segregant analysis was used to select RAPD and AFLP markers closely linked to *Gro1* in a segregating population of 1105 plants. Approximately 3000–3300 informative loci were analyzed. Three RFLP, one RAPD and two AFLP markers were linked to the *Gro1* locus. The RFLP and RAPD markers were inseparable from the *Gro1* locus, while the two AFLP markers flanked *Gro1* at genetic distances of 0.6 cM and 0.8 cM (where 1 cM = 100 kilobase pairs (kbp)). The closely linked AFLP markers identified will facilitate molecular cloning of the *Gro1* gene based on map position. Cloning the *Gro1* gene may contribute to the elucidation of the mechanism of resistance, and the *Gro1* gene can be directly transformed into susceptible potato cultivars that are agronomically sound.

Table 3 Experimental protocols employed in studies utilizing the AFLP technique for positional cloning of genes of interest

Reference	Organism studied	Preselective primers	Selective primers	No. of primer pairs tested
[2]	*Solanum tuberosum* (potato)	*Mse* 3′-1	*Mse*I 3′-3	144
[26]		*Hin*dIII3′-1	*Hin*dIII 3′-3[a]	108
[5]	*Solanum tuberosum* (potato)	Not utilized	*Mse*I 3′-3 *Pst*I 3′-2[a]	216
[6,7]	*Populus* spp (poplar tree)	*Mse*I 3′-1 *Eco*RI 3′-1	*Mse*I 3′-3 *Eco*RI 3′-3[a]	144
[10]	*Arabidopsis thaliana*	*Mse*I 3′-0 *Eco*RI 3′-0	*Mse*I 3′-3 *Eco*RI 3′-2[a]	112
		*Sac*I 3′-0	*Mse*I 3′-2 *Sac*I 3′-2[a]	192
[40]	*Lycopersicon esculentum* (Cf-9) × *L. pennellii* (tomato)	Not utilized	*Mse*I 3′-3 *Pst*I 3′-2[a]	728

[a]5′-end of primer labeled using [γ-^{32}P or γ-^{33}P]ATP.

Using bulked segregant pools from F2 progeny, of the cross *Lycopersicon esculentum* (Cf-9) × *L. pennellii*, and AFLP, Thomas *et al* [40] screened approximately 42 000 AFLP loci for tight linkage to the tomato *Cf-9* gene. The *Cf-9* gene, on chromosome 1 of tomato, codes for resistance to the leaf mould pathogen, *Cladosporium fulvum*. A total of 728 primer combinations were tested using 13 *Pst*I and 56 *Mse*I primers. Additional details of the experimental protocol are outlined in Table 3. Of the 42 000 AFLP loci inspected for polymorphism, approximately 14 000 were potentially informative. This equates to one AFLP marker every 70 kbp if the markers are distributed at random over the tomato genome estimated at 10^9 bp. Analysis of F2 recombinants identified three markers co-segregating with *Cf-9*. Further analysis of plasmid clones containing the gene allowed the position of the markers to be determined. Two of the markers were found to be located on opposite sides of the *Cf-9* gene, separated by 15.5 kbp.

The AFLP technique was used by Cnops *et al* [10], in a chromosome landing strategy, for the isolation of the *TRN1* gene. The *tornado1* (*trn1*) mutation was identified in a T-DNA transgenic line of *Arabidopsis* (ecotype C24) and is located 5 cM from a T-DNA insertion. Visible markers and RFLP markers were used to map the *TRN1* locus to the bottom half of chromosome 5. The AFLP technique

was applied to recombinant classes, within a 3-cM region around the *TRN1* locus, to build a high-resolution map in this region. Two different restriction enzymes, *Sac*I and *Eco*RI, were used in combination with *Mse*I, to generate template DNA for amplification (Table 3). Approximately 26 000 AFLP fragments were generated using 192 *Sac*I-*Mse*I primer combinations and 112 *Eco*RI-*Mse*I primer combinations. Seventeen AFLP markers were identified, four of which were so closely linked to the *TRN1* locus that no recombination event was detected. Three AFLP fragments were purified from the dried gel and cloned: two *trn1* co-segregating markers and one marker located immediately below *trn1*. To facilitate the recovery of the fragments, a simplified banding pattern (with 16-fold fewer bands) was generated using *Sac*I and *Mse*I primers with an additional selective nucleotide on the 3′-ends. The three tightly linked and co-segregating AFLP markers were used as probes to identify two yeast artificial chromosomes spanning the *TRN1* locus. Chromosome landing of the *TRN1* gene in *Arabidopsis* was proven to be successful using an AFLP-based strategy. Isolation and characterization of the *TRN1* gene will allow the process leading to dwarfism and twisting to be investigated on a molecular level.

Using *Populus* spp as a model tree, Cervera *et al* [6] showed and discussed three possible applications of AFLP™ in forest tree breeding. The first application, which is described in more detail by Cervera *et al* [7], involves the use of AFLP to identify molecular markers tightly linked to the *Mer* locus conferring resistance against three races (E1, E2 and E3) of the leaf rust fungus, *Melampsora larici-populina*. This disease causes premature defoliation, leaving the tree susceptible to secondary pathogen infection and environmental stress, and can reduce growth by more than 20%. The study was carried out using a hybrid progeny from a cross between a resistant *P. deltoides* female and a susceptible *P. nigra* male. Data suggested that resistance is determined by a single dominant marker. AFLP analysis using 144 primer combinations (Table 3) and bulked segregant analysis, identified three AFLP markers tightly linked to the *Mer* locus. These markers can be used in current breeding programs and for future cloning of the resistance gene. The second application involves the generation of genomic fingerprints for each species of *Populus*, using AFLP, for identification and taxonomic analysis. The results for the study on the diversity among *Populus* spp have not yet been reported. The final application involves the generation of high-density linkage maps of *P. deltoides*, *P. nigra* and *P. trichocarpa* using the 'two-way pseudo-testcross' strategy in combination with AFLP analysis. The 'two-way pseudo-testcross' mapping strategy is based on linkage analysis of those markers that are heterozygous in one parent and null in the other parent. These results have also not yet been published. The multiple studies on *Populus* spp using AFLP reveals the versatility of the technique for a variety of applications.

Brigneti *et al* [5] combined AFLP and bulked segregant analysis of an F1 tetraploid potato population to identify AFLP markers tightly linked to the Ry_{sto} gene conferring resistance to all strains of the potato virus Y. Ry_{sto} is a dominant gene and was mapped to chromosome XI of the potato genome. Preliminary AFLP analysis using 216 primer combinations identified three markers linked to and on either side of Ry_{sto}. Four additional AFLP markers were identified in a population of 360 segregating progeny of a potato cross between a resistant (Ry_{sto}) and a susceptible parent. Two markers co-segregated with Ry_{sto} and the other two were located on either side of Ry_{sto}, separated by one recombination event. AFLP analysis was successfully used to map the Ry_{sto} gene, however the closest flanking markers were estimated to be 0.6 cM apart (where 1 cM = 1 Mb). Additional markers around the resistance gene are necessary for positional cloning.

The linkage map of sugar beet (*Beta vulgaris* L.) was extended by Schondelmaier *et al* [37] using the AFLP technique on an F2 population consisting of 94 individuals. A total of 120 AFLP markers, generated using four primer combinations (Table 2) were integrated into an existing linkage map based on 207 RFLP loci. The AFLP markers were evenly distributed over all nine linkage groups with the exception of linkage group V. The low number of AFLP loci on linkage group V was thought to be due to the low number of primer combinations tested. Integration of AFLP loci served to extend the sugar beet map from 544 cM to 557 cM. The increase in genetic length was brought about through the addition of AFLP markers at the ends of chromosomes II, IV and VII, as well as AFLP loci filling some gaps in the RFLP map. AFLP technology proved to be a promising tool for estimating the genetic diversity of sugar beet breeding lines because of the even distribution of AFLP markers throughout the genome.

Analysis of genetic diversity in plants

The variation in AFLP patterns within barley species was investigated by Qi and Lindhout [36]. Two barley lines, L94 and Vada, were used to screen 96 primer combinations (Table 1). Forty-eight of the best primer pairs were selected and used to generate AFLP profiles for 16 representative barley lines, including L94 and Vada. The data from 24 primer combinations were evaluated to study the polymorphism rates. Of 2188 clearly visible bands within the range of 80–510 bp, 55% showed some degree of polymorphism among the 16 lines. The large number of bands and the high rate of polymorphism indicate the efficiency of the AFLP technique for marker generation in barley. Comparison of 48 AFLP profiles of parent lines revealed more than 100 common markers (possibly locus-specific) among populations or crosses. These markers will greatly facilitate the merging of existing data into one integrated genetic map of barley.

The genetic diversity within and among populations of the endangered sentry milk-vetch, *Astragalus cremnophylax* var *cremnophylax*, was assessed by Travis *et al* [41] using AFLP. Three populations in the Grand Canyon National Park (Arizona, USA) comprise all known individuals remaining in the wild. The oldest population on the South Rim (site 1) consists of approximately 500 individuals. A second population on the South Rim (site 2) consists of only two individuals. The third population, located on the North Rim, is estimated to consist of 1000 individuals who are distributed into four distinct subpopulations (A, B, C and D). A total of 220 polymorphic fragments were scored using nine primer combinations (Table 1).

Diversity was measured within each population and subpopulation on the basis of average heterozygosity and the proportion of polymorphic genes. Genetic diversity within each population varied directly with population size, with the largest population having the greatest diversity. There was no significant difference in the diversity among the subpopulations at the North Rim. Comparisons of AFLP patterns revealed greater similarity among plants collected from the same population than among plants collected from separate populations. The genetic relationship among the three populations, as revealed by the AFLP results, will be very important for conservation strategies. The information can be used to determine the potential of individuals to adapt to other locations if wild populations are to be augmented.

Sensi *et al* [38] compared AFLP and inverse sequence-tagged repeat (ISTR) analyses in their potential to reveal polymorphisms in a group of 19 *Vitis vinifera* L. accessions, including 13 Sangiovese and six Colorino grapevines. ISTR analysis is based on the selective PCR amplification of genomic DNA using primers derived from *copia*-like repetitive elements. It is similar to AFLP in terms of the number of loci detected and percentage of polymorphic bands, but it has the added advantage of not requiring any manipulation of DNA following isolation. A total of 264 polymorphic DNA fragments (57.6% polymorphism) were generated by AFLP using eight primer combinations (Table 1). Five ISTR primer combinations generated 249 polymorphic markers (71% polymorphism). The AFLP and ISTR results were used to construct dendrograms based on cluster analysis. Comparable results were obtained by AFLP and ISTR. The Colorino accessions were found to be genetically distinct from Sangiovese, and the Colorino americano ecotype significantly diverged from both groups. However, a higher proportion of polymorphism was detected within the Sangiovese group by ISTR analysis. The genetic variability within and among cultivars was explained by the putative polyclonal origin and the variation in selection pressures in different locations. Both techniques were useful for investigating genetic diversity among *Vitis vinifera* ecotypes and indicated the potential application for clonal differentiation and/or identification.

Genetic relationships among 12 soybean genotypes, including ten cultivated (*Gycine max*) and two wild soybean (*G. soja*) accessions, were determined by Powell *et al* [35] using RFLP, RAPD, AFLP and microsatellite markers. The AFLP protocol employed is outlined in Table 1. The four marker assays were compared using three features: (1) information content (expected heterozygosity); (2) number of loci (or bands) simultaneously analyzed per experiment (multiplex ratio); and (3) effectiveness in assessing relationships among accessions. The expected heterozygosity is a function of a marker system's ability to distinguish among genotypes. The highest levels of polymorphism (expected heterozygosity) were detected with microsatellite analysis and the lowest with RAPD and AFLP (not significantly different). However, AFLP markers generated the highest effective multiplex ratio. The AFLP assay provides a high degree of flexibility because the restriction enzymes, and nature and number of selective nucleotides can be manipulated to adjust the multiplex ratio. The expected heterozygosity and multiplex ratio were multiplied to generate a single parameter, termed marker index, used to evaluate the marker system's overall efficiency in detecting polymorphism. Although the expected heterozygosity was among the lowest for AFLP, the marker index for AFLP markers was almost an order of magnitude higher than the other assays because of the contribution of the large effective multiplex ratio. Comparison of genetic relationships involving both cultivated (*G. max*) and wild (*G. soja*) accessions revealed a high correlation of RFLP, AFLP and microsatellite analyses. However, correlations of RAPD data were lower because RAPDs are less effective in resolving interspecific similarities. The correlations between marker systems were significantly lower when the comparisons involved only the 10 *G. max* accessions. Within *G. max*, RAPD and AFLP similarity estimates were closely correlated as were AFLP and RFLP similarities. The microsatellite similarities were uncorrelated for *G. max* comparisons. The AFLP assay was determined to have the highest marker index value, indicating its superiority in detecting polymorphism in soybean genotypes.

AFLP analysis was employed by Paul *et al* [32] to detect genetic variation among 32 genotypes of tea (*Camellia sinensis* (L.) O. Kuntze) collected from India and Kenya. AFLP analysis, using five primer combinations, discriminated all of the genotypes including those that could not be distinguished by morphological or phenotypic traits. The AFLP results were used to construct a dendrogram by cluster analysis. The dendrogram separated the tea populations into China type (*sinensis*), Assam type (*assamica*) and Cambod type (*assamica* spp *lasiocalyx*). The grouping of populations in the dendrogram was, in most cases, consistent with the taxonomy, known pedigree of some of the genotypes, and their geographical origin. However, six of the 19 Assam genotypes (two Indian and four Kenyan) have been previously classified as China type using morphological traits, which may be subject to substantial environmental changes. In addition, the dendrogram grouping of the four Assam genotypes from Kenya has been supported by RAPD analysis. For the three Cambod genotypes, one was previously reported as a China type by RAPD analysis. No explanation was offered for this discrepancy. Similar grouping of the genotypes into Assam, China and Cambod types was achieved by principal coordinate analysis. The Assam clones from India and Kenya clustered closely on the principal coordinate analysis plot showing common ancestry. This provides support for the belief that Kenyan teas originated from India. The China types were more dispersed on the plot indicating greater genetic variation. However, clones collected from the same region exhibited less overall genetic variation because of similar selection pressures. Calculations on genetic diversity revealed that 79% was detected within populations and 21% between Indian and Kenyan populations. Estimates of diversity within populations showed that the China-type teas were the most variable and the Cambod population exhibited the lowest variability. However, the Cambod population size was the smallest. Successful discrimination of genotypes revealed that AFLP analysis can be used as an additional

molecular marker assay for studying genetic diversity and population genetics in tea.

The phylogenetic relationships among 44 morphologically diverse lines of cultivated lettuce, *Lactuca sativa*, and 13 accessions of the wild species *L. serriola*, *L. saligna*, *L. virosa*, *L. perennis*, and *L. indica* were investigated by Hill *et al* [15] using AFLP markers (Table 1). The same genotypes (excluding cv 'Lakeland') were analyzed by RFLP markers in a previous study. The AFLP data were used to construct a dendrogram by cluster analysis that was found to be consistent with known taxonomic relationships, and was similar to the phenetic tree constructed with RFLP data. Although the dendrograms were similar, the overall genetic distance among taxa was genetically higher with RFLP markers. The difference was explained by the method used to select probes for RFLP analysis, in that probes known to detect polymorphism are chosen. AFLP data revealed that accessions of *L. serriola* L. had the highest mean intra-specific distance and clustered on a sister branch of the *L. sativa* complex in the dendrogram. The close relationship provides additional support that *L. serriola* is the likely progenitor species of cultivated lettuce. With the exception of *L. serriola* and *L. sativa*, all species formed their own major branch with a distance value greater than 1.0. The 44 accessions of *L. sativa* were further subdivided as discrete branches according to their plant type: butterhead, crisphead, romaine, and looseleaf. AFLP and RFLP distance matrices were compared to test the ability of the marker systems to distinguish the *L. sativa* accessions. It was determined that AFLP analysis provided a more definitive grouping of the accessions. AFLP was clearly shown to be a reliable technique for studying genetic relationships, both at the species and cultivar level.

AFLP was used by Sharma *et al* [39] to analyze the genetic diversity and phylogeny of 54 lentil accessions including 26 cultivated genotypes of *Lens culinaris* (13 each of var. *macrosperma* and *microsperma*), and seven genotypes of the wild taxa, *L. culinaris* spp *orientalis*, *L. odemensis*, *L. nigricans* and *L. ervoides*. Results of AFLP analysis were compared with RAPD results previously obtained using the same material. A total of 148 AFLP markers were generated using four primer pairs with an average of 37 informative bands per primer combination. RAPD analysis using 100 10-mer primers produced 10-fold fewer informative bands per primer. The 23 AFLP markers produced by one primer pair were used to generate a dendrogram of the six *Lens* populations and a second dendrogram of the 54 genotypes. Similar results were obtained with the three other primer combinations. Information was also provided by principal coordinate analysis and construction of an unrooted phylogenetic tree using the 23 AFLP markers. A second unrooted tree was prepared using all of the 148 markers to provide further discrimination at the varietal or subspecies level. The greatest intra-specific genetic variability was detected for the *L. nigricans* accessions and the least diverse group was var *microsperma*. Subspecies *orientalis* and var *macrosperma* showed the greatest inter-specific similarity. A high degree of genetic identity was also exhibited between spp *orientalis* and var *microsperma* providing strong support that spp *orientalis* is the progenitor species of cultivated lentils. RAPD results differed from AFLP analysis in that the genetic identity between spp *orientalis* and *macrosperma* was comparable with *microsperma* suggesting a near simultaneous evolution. Among the wild taxa, the most closely related species/subspecies were *L. nigricans* and *L. ervoides*. There was additional inconsistency between RAPD and AFLP analysis with the phylogenetic placement of the wild population, *L. odemensis*. Although the two methods provided similar conclusions for the phylogeny of *Lens*, RAPD analysis was unable to discriminate among several genotypes. However, using the 148 AFLP markers, it was possible to differentiate among all the genotypes. AFLP analysis was shown to provide a higher degree of resolution in discerning the phylogeny of *Lens*.

The genetic diversity of 48 samples of European *Miscanthus* species (perennial grass), including 11 clones of *M. sinensis*, two clones of *M. sacchariflorus*, 31 accessions of *M.* × *giganteus* and four hybrids created by crossing *M. sinensis* and *M. sacchariflorus* clones was analyzed by Greef *et al* [13] using the AFLP technique. Approximately 250 polymorphic markers were generated using six primer combinations (Table 1). Genetic variation was calculated using cluster analysis and principal coordinate analysis. AFLP revealed two main groups represented by *M. sinensis* and *M. sacchariflorus* clones. The *M.* × *giganteus* accessions were clustered under the *M. sacchariflorus* group with a relatively high distance of about 0.3 units. The divisions, based on AFLP analysis, were found to agree with taxonomic classification. The genetic diversity was highest among the *M. sinensis* pool and low in the *M.* × *giganteus* pool. The *M.* × *giganteus* accessions were divided into two clusters with a high genetic similarity. Only three of the 31 accessions could be differentiated from the rest. No polymorphism was detected between micro- and rhizome-propagated *M.* × *giganteus* accessions. However, more polymorphism may be detected among *M.* × *giganteus* accessions using additional primer combinations. The AFLP technique was also useful for classifying many samples that were incorrectly named by botanical gardens as *M. saccariflorus* clones, the majority belonging to the *M.* × *giganteus* pool and one clone clustered in the *M. sinensis* group. In addition to evaluating genetic variation, and detecting incorrect classification, AFLP was used to determine self-fertilization of the *M. sinensis* clones in the hybridization of *M. sinensis* and *M. sacchariflorus* material.

Generation of high-resolution genetic maps in animals

AFLP analysis by Otsen *et al* [30] contributed a total of 18 AFLP markers to the linkage map of the rat, and showed the potential of AFLP markers for the detection of quantitative trait loci. Details of the experimental protocol are outlined in Table 2. The 112 progeny of a (BN × ACI)F1 × ACI backcross were subjected to AFLP analysis, revealing eight polymorphic markers. A genetic linkage map constructed in the backcross experiment, using 12 biochemical, two immunological, one RFLP and 55 simple sequence length polymorphism markers, was successful in localizing seven of the eight AFLP markers to a specific chromosome. A panel of 34 H × B/B × H recombinant

inbred strains was also subjected to AFLP analysis. Eleven of 15 identified polymorphic bands could be assigned to specific chromosomes by comparison with 450 loci of the recombinant inbred strain linkage map. Genotypes of the 11 AFLP markers were tested for correlation with blood pressure data available for 32 of the recombinant inbred strains. A suggestive correlation was observed between the mean arterial pressure and two closely linked AFLP markers on chromosome 20. The three AFLP markers assigned to chromosome 20 may facilitate the mapping of the putative blood pressure regulatory gene to a more restricted region.

Characterization of mammalian genotypes

The AFLP technique was used by Otsen *et al* [31] for genetic characterization of 12 rat inbred strains. AFLP analysis, using four primer combinations (Table 2), revealed considerable variation among strains. However, identical banding patterns were generated for animals of the same strain using the four primer pairs. AFLP profiles were used to estimate the genetic relationship among the 12 strains. A dendrogram was constructed, based on cluster analysis, using the AFLP data. Interpretation of strain relationship was not attempted because the chromosomal location of the AFLP markers was unknown. However, the linkage map constructed by Otsen *et al* [30] may assist in this matter. Nevertheless, the high degree of inter-strain variation detected by AFLP analysis indicates its utility for characterizing rat inbred strains used in biomedical research.

Genotypic analysis of bacteria

The AFLP technique was evaluated by Huys *et al* [16] for its usefulness in the genotypic classification of aeromonads. A total of 249 presumptive aeromonads were isolated from water samples collected from drinking water production plants in Flanders, Belgium. Gas-liquid chromatographic analysis of total cellular fatty acids classified 228 isolates in the genus *Aeromonas* using a commercial database. Huys *et al* [16] established an extended database, based on fatty acid analysis, using 70 *Aeromonas* reference and type strains representing the 13 known hybridization groups (genomic species). All but 42 isolates were identified into phenotypically defined groups, also called phenospecies, using the extended database. Of the 186 identified isolates, 125 were further allocated to an existing hybridization group. AFLP analysis was performed by Huys *et al* [16] using the protocol of Zabeau and Vos [46] (Table 4). The resulting banding patterns enabled the 125 *Aeromonas* strains to be characterized to one particular hybridization group. Even strains within one hybridization group could be differentiated from each other using the fingerprint patterns. Huys *et al* [16] found that the AFLP data confirmed the results of fatty acid analysis. Thus the study revealed the usefulness of AFLP for the classification of *Aeromonas* isolates.

In a subsequent study, Huys *et al* [17] investigated the ability of AFLP to differentiate the 14 defined DNA hybridization groups in the genus *Aeromonas*. A total of 98 *Aeromonas* type and reference strains representing the 14 hybridization groups were included in the study, as well as four phenospecies not yet allocated to a particular hybridization group. Analysis of the banding patterns revealed 13 AFLP clusters. In many cases, the fingerprints enabled isolates within a hybridization group to be differentiated at the strain level. The AFLP protocol of Huys *et al* [17] included only one primer pair (Table 4). Performing a second AFLP reaction with other primer combinations may differentiate those isolates producing very similar patterns. The high level of correlation between AFLP results and previously published DNA hybridization data showed AFLP to be a valuable technique for classification of *Aeromonas* species.

The AFLP patterns produced by Huys *et al* [17] were entered as representative library entries to generate a new hybridization group-specific genotypic database called AERO94. This database was employed in a subsequent study by Huys *et al* [18] to determine the genotypic diversity among 168 *Aeromonas* isolates obtained from drinking water production plants in Flanders, Belgium. Of the 168 isolates, 86% (144) could be allocated to one of the 14 recognized DNA hybridization groups. A fatty acid analysis database constructed using the same collection of type and reference strains used to generate the AFLP library, AERO94, was only able to classify 55 of the 144 *Aeromonas* strains. The results of Huys *et al* [18] clearly indicate the higher potential of AFLP for taxonomic identification and classification of unknown aeromonads compared to chemotaxonomic methods.

To evaluate the potential of AFLP to characterize bacterial strains at the subgeneric level, 36 *Xanthomonas* strains (including 29 pathovars), one *Stenotrophomonas* strain, and 90 *Aeromonas* strains (representing the 14 hybridization groups) were subjected to AFLP by Janssen

Table 4 Experimental protocols of studies utilizing the AFLP technique for the genotypic analysis of bacteria

Reference	Organism studied	Preselective primers	Selective primers	No. of primer pairs tested
[14]	*Paenibacillus larvae* and *P. pulvifaciens* (honeybee pathogens)	Not utilized	*Mse*I 3′-1 *Eco*RI 3′-1[a]	1
[16]	*Aeromonas*	N/A	N/A[a]	N/A
[17,18]	*Aeromonas*	Not utilized	*Taq*I 3′-1 *Apa*I 3′-1[a]	1
[19]	*Xanthomonas* *Stenotrophomonas* *Aeromonas* *Clostridium* *Bacillus* *Acinetobacter* *Pseudomonas* *Vibrio*	Not utilized	*Taq*I 3′-1/2 *Apa*I 3′-1[a] *Mse*I 3′-1 *Eco*RI 3′-0/1[a] *Hin*dIII 3′-1[a]	14
[21]	*Bacillus anthracis*	*Mse*I 3′-0 *Eco*RI 3′-0	*Mse*I 3′-1 *Eco*RI 3′-1[a]	16
[22]	*Aeromonas*	Not utilized	*Taq*I 3′-1	1
[23]			*Apa*I 3′-1[a]	3
[24]	*Escherichia coli* and *Agrobacterium tumefaciens*	*Mse*I 3′-0 *Eco*RI 3′-0	*Mse*I 3′-1 *Eco*RI 3′-1[a]	N/A

N/A = information not available.
[a]5′-end of primer labeled using [γ-^{32}P or γ-^{33}P]ATP.

et al [19]. DNA template for AFLP was prepared using *Taq*I and *Apa*I restriction endonucleases (Table 4). The high discriminatory power of AFLP enabled individual strains within the pathovars of *Xanthomonas* species to be differentiated based on their AFLP banding patterns. AFLP analysis of the *Aeromonas* isolates revealed similar results. Strains of the same hybridization group clustered together and most strains within a given hybridization group could be differentiated from each other. In both cases, there was good correlation between the AFLP data and existing taxonomic data. To determine if the use of other restriction enzymes would influence the AFLP-based grouping of bacterial strains, AFLP analysis of the 37 *Xanthomonas* and 12 *Aeromonas* strains (representing hybridization groups 1, 2 and 3) was repeated with DNA fragments generated using *Eco*RI and *Mse*I restriction endonucleases (Table 4). For both *Xanthomonas* and *Aeromonas*, the generation of fewer restriction fragments resulted in unevenly distributed banding patterns with a higher concentration of high molecular weight fragments. This was attributed to the lower cleavage frequency of *Eco*RI and *Mse*I with G+C-rich *Xanthomonas* and *Aeromonas* DNA. Although the grouping of *Xanthomonas* strains was highly similar to the clusters achieved with *Apa*1-*Taq*1 templates, the linkage levels were 5–10% lower among the various clusters and among the individual strains. For the *Aeromonas* strains, the linkage levels of the three hybridization groups dropped considerably with hybridization groups 1 and 2 grouping together in one cluster. This clearly shows that the choice of restriction enzymes can affect the accuracy of the AFLP analysis. The best combination of endonucleases should be determined empirically for each organism to ensure accurate results.

In the same study, Janssen *et al* [19] tested the general applicability of the AFLP technique in bacterial taxonomy using four strains belonging to the genera *Clostridium*, *Acinetobacter*, *Bacillus*, *Pseudomonas* and *Vibrio*. Of the four strains investigated for each genera, three belonged to the same species, or were highly related species as with *B. cereus* and *B. thuringiensis*. In all cases, the four isolates could be discriminated. However, the three related strains had very similar banding patterns with the fourth strain having a much different pattern. Use of the AFLP technique for DNA of any source and complexity was demonstrated in this comprehensive study. The genomes of the genera investigated vary both in size and base composition, yet the superior discriminative power of the technique for differentiating highly related strains was maintained.

To gain a better understanding of the mechanism of virulence of *Aeromonas* spp, Kuhn *et al* [22] analyzed 120 isolates including 80 fecal isolates from patients in Bangladesh (69 from patients with diarrhea and 11 from healthy controls), and 40 environmental isolates from surface water. All isolates were phenotyped using a high-resolution biochemical fingerprinting system (the PhenePlate system). Most (106) were assigned to hybridization groups by fatty acid profiles. However, for 29 isolates requiring further characterization, or of special interest, AFLP analysis was employed (Table 4). In addition, all isolates were assayed for the production of hemolysin and cytotoxin. The aim of the study was to determine whether pathogenicity can be associated with certain phenospecies and/or DNA hybridization groups, and whether the production of virulence factors is prevalent among human isolates. Isolates of the phenotype BD-2 and hybridization group 1 predominated in patients. In addition, these isolates produced relatively high levels of virulence factors suggesting that the HG1/BD-2 type may represent a true human pathogenic *Aeromonas*.

In a second study by Kuhn *et al* [23], the AFLP technique was used to study the diversity and persistence of coliforms and *Aeromonas* isolated from a Swedish drinking water well. A total of 40 water samples were collected over a 4-year study period. When available, 32 bacterial colonies were isolated from each water sample. Preliminary biochemical characterization of the isolates was performed using the PhenePlate fingerprinting system. A total of 170 different phenotypes were identified among 1143 studied isolates. Isolates that were suspected of representing predominant clones in the well water were further characterized using the API 20E system, cellular fatty acid analysis, and AFLP. Most phenotypes were only represented by a few isolates and (or) were restricted to one or two sampling occasions. Only one phenotype, identified as *A. hydrophilia*, was identified in more than three water samples. Thirty-nine percent of the isolates were found to belong to this phenotype and were present in 28 samples distributed over the whole study period. Eleven representative *A. hydrophilia* isolates from 11 different sampling occasions were subjected to AFLP analysis. Nearly identical banding patterns were generated for each of the three different *Apa*I-*Taq*I selective primer pairs (Table 4). Cluster analysis of the banding patterns revealed that all 11 *A. hydrophilia* isolates grouped within the same hybridization group, HG3, indicating that they were most probably of the same clonal origin. Although most strains were only transient inhabitants of the well, the AFLP results suggest that the *A. hydrophilia* phenotype represents a genetically stable *Aeromonas* clone which persisted in the well water for the entire 4-year study.

Keim *et al* [21] used AFLP to analyze 79 *Bacillus anthracis* isolates and isolates of six related *Bacillus* species (*B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. subtilis*, *B. polymyxa* and *B. stearothermophilus*) for molecular variation (Table 4). *B. anthracis* has two large plasmids, pX01 and pX02, that carry essential genes for pathogenesis. To focus on chromosome-based relationships, purified plasmid DNA of *B. anthracis* was analyzed to exclude plasmid-specific AFLP fragments from the fingerprints. Great AFLP diversity was observed among the *Bacillus* taxa. Cladistic and phenetic analysis of the banding patterns revealed the phylogenetic relationships among the related *Bacillus* species, and among the *B. anthracis* isolates. *B. cereus* and *B. thuringiensis* were determined to be the closest taxa to *B. anthracis* with *B. mycoides* being slightly more distant. An extremely low level of molecular variation was detected among the isolates of *B. anthracis*, which is thought to be one of the most genetically uniform bacterial species. Of a total of 1221 amplified fragments, 97% (1184) were monomorphic. In contrast, only 40% of the fragments were similar between *B. anthracis* and its closest relatives, *B. cereus* and *B. thuringiensis*. Although there was little variation, cluster analysis identified two well-defined groups of *B. anthracis* isolates. AFLP proved to be an effective tech-

nique for molecular typing of *B. anthracis*, a relatively monomorphic bacterial species, because of its capacity to inspect a large percentage of the genome for genetic variation.

Lin *et al* [24] used AFLP to analyze different strains of *Escherichia coli* and *Agrobacterium tumefaciens*. The *E. coli* strains tested included BL21, BL21F′IQ, DH5α, DH5αF′IQ, HB101 and W. The AFLP technique employed (Table 4) was successful in differentiating the *E. coli* strains. Polymorphic bands found between BL21F′IQ and BL21 as well as between DH5αF′IQ and DH5α demonstrated that AFLP is able to differentiate fragments as small as 100 kb, since the F′IQ is the only difference between the strains. The *A. tumefaciens* strains tested included the octopine-like strain LBA4404 and napoline-like strains C58 and EHA101. AFLP was shown to be a superior molecular typing system for analysis of *A. tumefaciens* than traditional phenotypic ribotyping. This further demonstrates the general applicability of AFLP for determining genotypic relationships in bacterial genera.

Epidemiological typing of bacteria

AFLP analysis, in addition to ribotyping, biotyping, cell envelope protein electrophoretic typing and antibiogram typing, were used by Dijkshoorn *et al* [11] to compare 31 *Acinetobacter baumannii* strains. Fourteen strains from nosocomical outbreaks in different northwestern European cities, and 17 sporadic strains apparently not associated with outbreaks were investigated. The AFLP procedure employed is provided in Table 5. A dendrogram, based on AFLP data, revealed 18 clusters and single strains at a delineation level of 89.0% similarity. Twelve of the 14 outbreak strains were grouped into two clusters. The first of these included nine outbreak strains with 89.9% similarity and the second cluster comprised three outbreak strains and one sporadic strain linked at 92.8%. The AFLP patterns of the two clusters differed by only one or two bands. Combination of all typing results linked 18 of the 31 strains into four groups. Only two outbreak strains and eleven nonoutbreak strains failed to cluster. The strains in AFLP clusters one and two comprised Groups I and II, respectively. The uniformity of typing characters of the two sets of outbreak strains suggests that each cluster has a common clonal origin.

Table 5 Experimental protocols of studies utilizing the AFLP technique for epidemiological typing of bacteria

Reference	Organism studied	Preselective primers	Selective primers	No. of primer pairs tested
[11]	*Acinetobacter baumannii*	Not utilized	*Taq*I 3′-2 *Hin*dIII 3′-1[a]	1
[20]	*Acinetobacter*	Not utilized	*Taq*I 3′-2 *Hin*dIII 3′-1[a]	1
[34]	*Mycobacterium kansasii*	Not utilized	*Pst*I 3′-2 *Pst*I 3′-5	N/A
[42]	*Legionella pneumophilia*	Not utilized	*Pst*I 3′-1 *Pst*I 3′-2	16

N/A = information not available.
[a]5'-end of primer labeled using [γ-^{32}P or γ-^{33}P]ATP.

AFLP was evaluated by Janssen *et al* [20] for its usefulness in the epidemiological typing of 25 *Acinetobacter* strains isolated during five hospital outbreaks in three countries (Table 5). A dendrogram, constructed by cluster analysis of AFLP data, revealed five clusters with a minimum of 94% similarity. Each cluster comprised strains from one particular outbreak and shared identical banding patterns. The AFLP data were verified by earlier published typing data including antibiogram typing, biotyping, cell envelope protein electrophoretic profiling and ribotyping. AFLP proved to be a valuable alternative in epidemiological typing.

Characterization of water and human isolates of *Mycobacterium kansasii* using a variety of typing methods, including RFLP analysis with the major tandem repeat probe and the IS*1652* probe, pulsed-field gel electrophoresis (PFGE), PCR restriction analysis of the *hsp-65* gene and AFLP analysis, allowed Picardeau *et al* [34] to establish an epidemiological relationship between environmental and clinical isolates. The isolates investigated included the type strain and 62 *M. kansasii* isolates including 38 clinical strains (14 from AIDS patients) and 24 strains from tap water samples, all recovered in France. AFLP analysis involved the use of only one restriction endonuclease, *Pst*I and amplified products (3–8 products of 0.5–2.5 kb) were separated by gel electrophoresis using a 2% agarose gel (Table 5). Although the five methods reveal independent polymorphisms and provide different levels of strain characterization, the results indicated five homogeneous clusters suggesting the existence of five distinct subspecies within *M. kansasii*. The PFGE and AFLP data were particularly informative because they revealed polymorphisms within each cluster. Mycobacterial infections are considered to be acquired from the environment and *M. kansasii* has been almost exclusively recovered from tap water. All clusters contained clinical strains (both from AIDS and non-AIDS patients) and environmental strains providing further evidence for the involvement of tap water in human infections.

The AFLP technique was evaluated by Valsangiacomo *et al* [42] for the molecular typing of 28 strains of *Legionella pneumophilia* including clinical and environmental strains from different regions of Switzerland. A single restriction endonuclease, *Pst*I, was used to generate restriction fragments. Following selective amplification, the fragments (5–10) were separated by gel electrophoresis on a 1.5% agarose gel (Table 5). AFLP analysis of the 10 clinical strains isolated from patients and their living environments (from three cases of legionellosis) made it possible to identify the origin of infection. The banding patterns of the 18 environmental isolates revealed genetic heterogeneity. The potential of AFLP for epidemiological studies was revealed in this study.

Genotypic classification of fungi

A simplified AFLP protocol, using only *Pst*I restriction endonuclease, was used by Mueller *et al* [28] to detect genetic differences among 14 symbiotic fungi of the fungus-growing ant, *Cyphomyrmex minutus* (Table 1). The isolates

were grouped into four distinct fingerprint 'types' by each of six *Pst*I primers. The fungi within each type showed identical banding patterns. It was suggested that each fungal type may represent a distinct clone propagated vegetatively by the ant, or may correspond to a free-living species of fungus acquired by the ant. The genetic differences among the 14 isolates predicted by vegetative-compatibility assays corresponded to the fungal types revealed by AFLP analysis, suggesting that vegetative compatibility can be used as a crude indicator of genetic differences among dikaryotic fungi.

Characterization and classification of pathogens

AFLP analysis was used by Heyndrickx *et al* [14], in addition to other morphological and biochemical tests, a variety of chemotaxonomic and genomic fingerprints, and DNA relatedness measurements, to support the reclassification of the honeybee pathogens *Paenibacillus larvae* and *P. pulvifaciens* as *P. larvae* subsp *larvae* and *P. larvae* subsp *pulvifaciens*. *Paenibacillus larvae* is an obligate pathogen of honeybee (*Apis mellifera*) larvae causing American foulbrood disease. *P. pulvifaciens* causes a rare disease called powdery scale characterized by a powdery decay of the larvae. A total of eight strains (four of each species) were analyzed by the AFLP procedure outlined in Table 4. The AFLP patterns of *P. larvae* and *P. pulvifaciens* strains grouped into two distinct clusters, indicating the existence of two subgroups. The AFLP technique proved to be very useful for Heyndrickx *et al* [14] for the characterization of bacteria at the subgeneric level and allowed differentiation within the range of biovar to genomic species.

Folkertsma *et al* [12] used AFLP to characterize a total of 24 potato root cyst nematode populations including nine *Globodera rostochiensis* and 15 *G. pallida* populations. A total of 987 marker-loci were screened using 12 primer combinations. More details on experimental protocol are outlined in Table 1. The polymorphic DNA fragments for the nine *G. rostochiensis* populations were separated into two subsets based on presence or absence polymorphisms and band intensity polymorphisms. There was close agreement between the dendrograms generated using both AFLP data sets. The nine populations branched into three similar groups containing three, five and one population(s), respectively. The pathotype classification of the nine *G. rostochiensis* populations corresponded to the dendrograms generated using AFLP. Although all *G. pallida* populations were differentiated using AFLP, band intensity polymorphisms could not be identified. The banding patterns generated for the 15 *G. pallida* populations were very complex, with the primer combinations used providing only qualitative data. Unlike the *G. rostochiensis* populations, the clustering of *G. pallida* populations by AFLP, did not resemble their pathotype classification. The possible explanations provided by Folkertsma *et al* [12] included the inadequacy of the pathotype scheme for *G. pallida* and the structure of the genetic variation among *G. pallida* populations (ie the distribution of the polymorphic DNA fragments). A large proportion of the polymorphic DNA fragments were scattered, hindering the classification of the *G. pallida* populations. Further testing with additional primer combinations may permit the clear clustering of *G. pallida* populations.

RNA fingerprinting using cDNA-AFLP

A novel study by Bachem *et al* [1] combined an RNA fingerprinting technique based on AFLP, called cDNA-AFLP, with a highly synchronous *in vitro* potato tuberization system to analyze transcriptional changes at and around the time of tuberization. The cDNA-AFLP technique uses the standard AFLP protocol on a cDNA template (Table 6). The expression of two genes expressed during tuberization was analyzed. One gene codes for the major potato storage protein, patatin, and a second codes for ADP-glucose pyrophosphorylase, a key enzyme in the starch biosynthetic pathway. The kinetics of expression revealed by cDNA-AFLP were comparable to those found in Northern analysis, the traditional fingerprinting method. The verification of band identity is typically a difficult procedure in RNA fingerprinting. However, by using selective primers with nucleotide extensions of three bases in the cDNA-AFLP procedure (Table 6), it was possible to eliminate virtually all non-target bands. The results of Bachem *et al* [1] revealed the use of cDNA-AFLP for identifying developmentally regulated genes. The technique may allow the detailed characterization of gene expression in a wide range of biological processes.

Money *et al* [27] investigated the use of AFLP to generate mRNA fingerprints in hexaploid wheat and one of its deletion mutants. Messenger RNA was extracted from wheat cv Chinese Spring and a mutant of this variety with deletions on chromosomes 3A and 3B. The standard AFLP procedure was performed using double-stranded cDNA synthesized from the extracted mRNA (Table 6). Comparison of the AFLP fingerprints revealed 16 polymorphic fragments. These fragments were excised and reamplified. Purified reamplified products were labeled and hybridized with various genomic DNA digests from Chinese Spring and the deletion mutant. Five products with similar hybridization patterns were found to cross-hybridize with the 18S-5.8S-28S rRNA genes of wheat. The remaining 11 probes hybridized to single or low copy sequences. Two of these fragments, present in Chinese Spring, were found to be located on chromosome 3A. Generation of mRNA fingerprints using AFLP proved to be useful for isolating sequences mapping to deleted chromosome segments in hexaploid wheat.

Table 6 Experimental protocols of studies utilizing the cDNA-AFLP technique for RNA fingerprinting

Reference	Organism studied	Preselective primers	Selective primers	No. of primer pairs tested
[1]	*Solanum tuberosum* (potato)	*Taq*I 3′-0 *Ase*I 3′-0	*Taq*I 3′-2 *Ase*I 3′-2[a]	N/A
[27]	*Triticum* (wheat)	*Mse*I 3′-0 *Pst*I 3′-0	*Mse*I 3′-2/3 *Pst*I 3′-0[a]	49

N/A = information not available.
[a]5′-end of primer labeled using [γ-^{32}P or γ-^{33}P]ATP.

Future perspectives

The AFLP technique can be applied to DNA of any source or origin. There is an extensive number of applications for this virtually universal marker system. Since its development in 1993, it has been widely used in the field of plants. Its application for other eukaryotes and prokaryotes is expected to increase. Although all publications to date utilize radioactive isotopes for the 5′-end labeling of one primer of the pair, the use of fluorescent dye-labeled primers will become more prevalent. The many advantages of the technique, primarily its reproducibility and number of loci detected per reaction, will enhance its overall application.

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