AFLP genotyping and fingerprinting

Ulrich G. Mueller and L. LaReesa Wolfenbarger

Amplified fragment length polymorphisms (AFLPs) are polymerase chain reaction (PCR)-based markers for the rapid screening of genetic diversity. AFLP methods rapidly generate hundreds of highly replicable markers from DNA of any organism; thus, they allow high-resolution genotyping of fingerprinting quality. The time and cost efficiency, replicability and resolution of AFLPs are superior or equal to those of other markers [allozymes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellites], except that AFLP methods primarily generate dominant rather than co-dominant markers. Because of their high replicability and ease of use, AFLP markers have emerged as a major new type of genetic marker with broad application in systematics, pathotyping, population genetics, DNA fingerprinting and quantitative trait loci (QTL) mapping.

Ulrich Mueller and L. LaReesa Wolfenbarger are at the Dept of Biology, University of Maryland, College Park, MD 20742, USA (umueller@mail.utexas.edu; lw137@umail.umd.edu).

Empirical studies in ecology and evolution often depend on accurate assessment of genetic diversity[1,2] to address questions regarding genetic relatedness among individuals, population structure, phylogenetic relationships and mapping of quantitative trait loci (QTL). A series of techniques and genetic markers have been developed to estimate genetic diversity (Box 1), but no single technique is universally ideal; each available technique exhibits both strengths and weaknesses (Box 1). Therefore, the choice of technique is often a compromise that depends on the research question pursued and the genetic resolution needed, as well as on financial constraints and the technical expertise available.

A new technique that approaches an ideal is amplified fragment length polymorphism–polymerase chain reaction (AFLP–PCR), a relatively cheap, easy, fast and reliable method to generate hundreds of informative genetic markers[3,4]. The main disadvantage of AFLP–PCR is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states, such as heterozygosity analyses. However, because of the rapidity and ease with which reliable, high-resolution markers can be generated, AFLPs are emerging as a powerful addition to the molecular toolkit of ecologists and evolutionary biologists.

Analyzing genetic variation with AFLP markers

The key feature of AFLP–PCR is its capacity for the simultaneous screening of many different DNA regions distributed randomly throughout the genome. To achieve high reliability of the screen, genomic DNA is prepared in an ingenious, but technically straightforward, way that combines the strengths of two methods, the replicability of restriction fragment analysis and the power of the PCR (Refs 3,4). In essence, AFLP methods allow the detection of polymorphisms of genomic restriction fragments by PCR amplification (Boxes 1 and 2). AFLP markers have proved useful for assessing genetic differences among individuals, populations and independently evolving lineages, such as species.

Systematics, pathotyping and biodiversity surveys

For a wide range of taxa, including plants, fungi, animals and bacteria, AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species, that had been impossible to resolve with morphological or other molecular systematic characters[5–8]. For example, AFLP methods were shown to be superior to classic systematic methods in nematodes[9], have allowed finer differentiation of microorganisms[10,11] and have rapidly identified novel pathogens in epidemiological hospital surveys[12]. For closely related species, AFLP markers have also been used to infer phylogenetic relationships based on measures of genetic distance[5,7,9,11,13–15]. For higher taxonomic levels, phylogenetic inferences based on similarities of AFLP profiles become problematic, because the high variability of AFLP markers reduces similarities between distant taxa to the level of chance. Therefore, the usefulness of AFLP markers for systematics rests more on the rapid grouping of closely related lineages, which is crucial for biodiversity surveys[16] or epidemiological research[10,12].

Population and conservation genetics

AFLP markers have found the widest application in analyses of genetic variation below the species level, particularly in investigations of population structure and differentiation[5,8,17,18], including estimation of F_{ST} analogs[19,20] and genetic variation within populations[9,19,21,22]. Such analyses are crucial for conservation genetics, and the rapidity with which AFLP markers can be generated promises that these markers can deliver crucial information under the intense time constraints frequently demanded by pending conservation decisions[16,19,22]. Apart from problems of population structure and variation, AFLP markers have been applied to evaluate gene flow and dispersal[9,17,19,20], outcrossing[23], introgression[15] and cases of hybridization[7,17]. The high resolution of AFLP markers also enables testing for clonal identity between individuals (i.e. absence of recombination), and thus permits inferences about sexual versus asexual modes of reproduction[7,20,21].

AFLP fingerprinting and kinship

AFLP markers have the potential to resolve genetic differences at the level of 'DNA fingerprints' for individual identification and parentage analysis. In the ideal case, a few primer combinations will suffice to generate an adequate number of polymorphic markers. In the worst case, many AFLP markers have to be generated with a series of primer combinations to reveal differences between closely related or inbred individuals, or to confirm a lack of differences for clonality. However, it is still unclear how many markers must be generated to ensure significant representation of hypervariable loci and to profile an individual at

 0169-5347/99/$ – see front matter PII: S0169-5347(99)01659-6

Box 1. Glossary

- **Types of genetic markers**

Multilocus marker: marker that screens many loci in the genome, as in the random amplified polymorphic DNA–polymerase chain reaction (RAPD–PCR), amplified fragment length polymorphism–PCR (AFLP–PCR) and minisatellite DNA fingerprinting.
Single-locus marker: marker that derives from a single locus in the genome, such as allozymes, most RFLPs and the typical microsatellite marker.
Dominant marker: marker that is scored as present or absent (null) and thus does not allow identification of homologous alleles (i.e. dominant markers fail to distinguish AA from Aa genotypes).
Co-dominant marker: marker that allows identification of homologous alleles and thus scoring of homozygote and heterozygote states. For many population genetic questions, co-dominant markers are clearly superior to dominant markers because (1) they allow estimation of allele frequencies; and (2) for a given level of analytical power, co-dominant markers require smaller sample sizes than dominant markers[27].

- **Technical terms**

Ligation: process of joining two pieces of DNA (or RNA) with enzymes called DNA (RNA) ligases.
Polymerase chain reaction (PCR): an enzyme-catalyzed, *in vitro* copying process of specific DNA sequences that uses extremely small amounts of template DNA. The PCR allows the selective amplification of specific DNA sequences.
Primer: short (usually 16–25 nucleotides) single-stranded sequence that can bind (anneal) to a complementary sequence and can serve as a starting point for DNA synthesis in a PCR reaction.
Quantitative trait loci (QTL): regions of the genome affecting variation in quantitative (phenotypic) traits. QTLs are identified by: (1) generating genotypic markers in individuals with a known pedigree; (2) creating a linkage map that shows the order of the markers and relative distance (in centimorgans; cM) between them; and (3) testing for statistical associations between markers (genotype) and phenotypic expression of the trait(s) of interest.
Simple sequence repeat (SSR): also known as microsatellite repeat, consisting of short nucleotide sequences (e.g. CAT) that are repeated many times in tandem (…CATCATCAT…). The number of SSR tandem repeats can vary in a sequence, and many such variants (alleles) can exist in a population.
Restriction enzyme: enzyme that cuts (restricts) double-stranded DNA at specific short sequences that are recognized by the enzyme. The most commonly used enzymes recognize sequences of four to eight nucleotides in length. Enzymes used in the AFLP protocol produce ends with an overhang of one DNA strand, as shown in the figure in Box 2 for the *Mse*I and *EcoR*I cuts.

the level of a true DNA fingerprint. This is crucial, for example, for identification of clonally identical individuals[7,20,21,24], where an insufficient number of fragments might not uncover existing genetic differences, and thus lead to an incorrect conclusion of clonal identity and asexual reproduction. However, in principle, any individual can be profiled with unique combinations of AFLP markers, because a large number of AFLP markers presumably include at least some that are hypervariable[4].

The capacity of AFLP markers to resolve extremely small genetic differences has been demonstrated in several studies. For example, AFLPs have been used to distinguish near-isogenic lines of soybean that differ at only a single, small region in the entire genome[25]. Studies using AFLPs have also delineated different sets of clonally descended individuals in several plants and fungi[7,20,21,24] and have detected the presence or absence of plasmids[13]. Theoretically, therefore, AFLP markers could be suitable for the analysis of relatedness, parentage, mating frequency or other genetic parameters in behavioral ecology, especially because AFLP markers are virtually free of artifacts[26] and because comigration of non-allelic fragments (which is an acute problem of anonymous markers for relatedness estimation[27]) occurs at extremely low levels[21,28]. For paternity analysis, for example, AFLP markers generated by only three AFLP primer systems sufficed to determine paternity of 96% of naturally pollinated seeds in a population of the shrub *Persoonia mollis*[29]. It remains unclear, however, whether the dominance of AFLP markers permits precise and unbiased estimation of relatedness[27,30]. AFLP relatedness analyses will need to be based on similarity indices developed for other dominant multilocus fingerprinting systems (using rare but not frequent AFLP markers[30]) or, alternatively, on co-dominant AFLP markers, if these can be identified with the help of known pedigrees[31–33].

QTL mapping

With the recent development of an arsenal of molecular markers that uncover population level polymorphisms (Box 3), mapping genes that affect quantitative variation (i.e. QTLs) has now become feasible for natural populations[34,35]. However, the application of QTL mapping remains difficult for many organisms, because the construction of a detailed linkage map requires the identification of many polymorphic loci that are dispersed throughout the genome. For most organisms, prior information about the genome is limited, and generating enough molecular markers through traditional restriction fragment length polymorphism (RFLP) techniques entails considerable time because few polymorphisms are generated with each RFLP probe (Box 3). Random amplified polymorphic DNA (RAPD)–PCR markers are used commonly to detect polymorphisms in organisms for which insufficient genetic information is available, but RAPD markers can also generate high levels of PCR artifacts[36]. Artifactual 'polymorphisms' are of less concern for linkage mapping, because mapping uses only polymorphic markers that are inherited in a mendelian fashion. However, repeatability of generated maps might remain problematic, because minor differences in RAPD–PCR thermocycling parameters can cause systematic changes in banding patterns and 'replicable polymorphisms' of unknown origin[36].

Because AFLP methods can generate many genome-wide polymorphic markers without prior sequence knowledge, AFLPs are a powerful tool for generating linkage maps. AFLP markers have been used extensively for constructing linkage maps for QTL analyses of agronomic plant traits, such as disease resistance and salt tolerance[28,37–39], and to identify QTLs associated with physiological traits in rats[40]. For maps constructed primarily with AFLP markers, distances between markers [measured in centimorgans (cM): corresponding to recombination frequency] are comparable to maps using RAPDs or RFLPs, and in one study ranged from 1 to 16 cM (average 4.3 cM)[41]. When added to existing maps, AFLP markers have greatly extended the total genomic coverage and decreased the average distance between markers[40,42,43]. Therefore, AFLPs are emerging as a marker of choice for genetic mapping for systems with few existing markers and for systems where additional markers are needed to augment existing RFLP and RAPD markers.

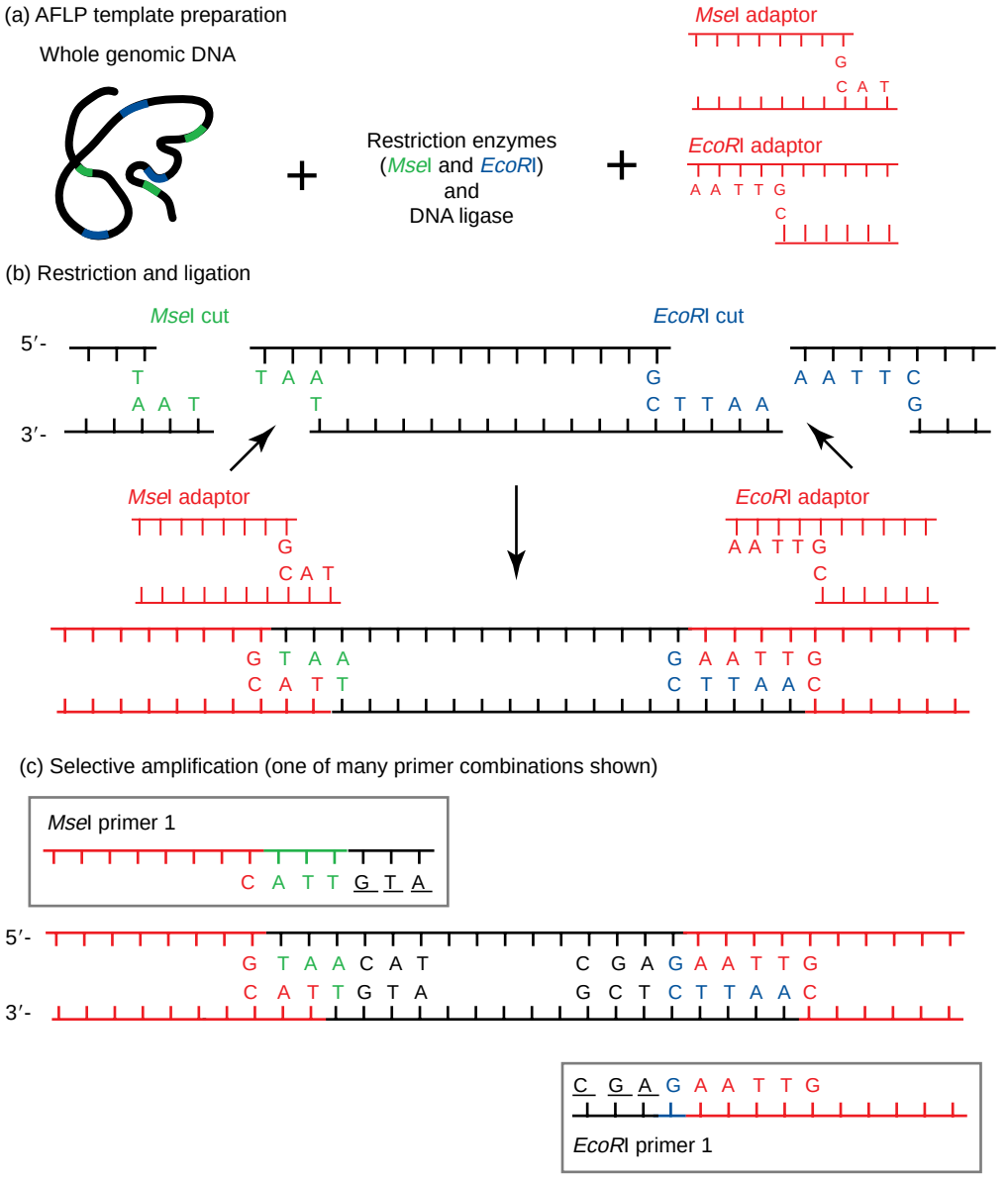
AFLP utility

The quantity of information generated, replicability, resolution, ease of use and cost efficiency of AFLP markers are at least as good, if not superior, to those of other standard molecular markers (Box 1; Box 3, Table I). AFLP markers offer the following six advantages.

• Taxonomic scope: AFLP markers can be generated for any organism with DNA, and no prior knowledge about the genomic makeup of the organism is needed. Therefore, AFLPs have broad taxonomic applicability and have been used effectively in a variety of taxa, including bacteria[10,11,13], fungi[18,20,21,41], animals (nematodes[9], arthropods[8] and vertebrates[40,44,45]) and plants (cultivated crops[25,37,46] and trees[17,23,33]).

Box 2. Generating amplified fragment length polymorphism (AFLP) markers

Preparing the DNA: AFLP markers can be generated for DNAs of any organism, and no initial investment in primer/probe development or sequence analysis is required. Partially degraded DNA can be used, but DNA should be highly purified and free of polymerase chain reaction (PCR) inhibitors. Extremely small amounts of DNA (~50 ng) are digested with two restriction enzymes (a), and AFLP adaptors are joined (ligated) to these ends (b). Adaptor ligations are performed in the presence of restriction enzymes such that any fragment-to-fragment ligations are immediately recleaved by the restriction enzyme. The adaptor is designed so that ligation of a fragment to an adaptor does not reconstitute the restriction site. The end sequences of each adapted fragment now consist of the adaptor sequence (in red) and the remaining part of the restriction sequence (in blue and green). These known end sequences serve as priming sites in the subsequent AFLP–PCR.



Trends in Ecology & Evolution

(Online: Fig. I)

Selective amplification: Depending on genome size, restriction-ligation generates thousands of adapted fragments. For visualization after electrophoresis, only a subset of these fragments is amplified. To achieve selective amplification of a subset of these fragments, primers are extended into the unknown part of the fragments [underlined base pairs (bp)], usually one to three arbitrarily chosen bases beyond the restriction site (c, in black). A primer extension of one, two or three bases reduces the number of amplified fragments by factors of 4, 16 and 64, respectively. To minimize artifacts, most protocols incorporate two amplifications. The first is performed with a single-bp extension, followed by a more selective primer with up to a 3-bp extension. Because of the high selectivity, primers differing by only a single base in the AFLP extension amplify a different subset of fragments. Ideal extension lengths will vary with genome size and will result in an optimal number of products (bands), not too many bands to cause smears or high levels of band comigration during electrophoresis, but sufficient to provide adequate information. By using combinations of primers with different extensions, a series of AFLP amplifications can thus screen a representative fraction of the genome.

Scoring AFLP markers: AFLP–PCR products can be separated and scored with a variety of techniques, ranging from simple agarose gel electrophoresis to automated genotyping. Polyacrylamide gel electrophoresis (manual or with an automated sequencer) provides maximum resolution of AFLP banding patterns to the level of single-nucleotide length differences, whereas fragment length differences of less than ten nucleotides are difficult to score on agarose gels. Although agarose gels provide the least resolution, they are user-friendly, inexpensive and require minimal equipment.

AFLP reliability: Because AFLPs are dominant, multilocus markers that are scored as present or absent, artifactual amplification (or amplification failure) of a fragment will reduce AFLP reliability. Artifactual presence/absence of a band probably originates at the restriction-ligation step. For this step, it is crucial to ensure complete digestion (to prevent later amplification of uncut fragments); complete digestion is achieved by the use of high-quality DNA and an excess of restriction enzyme. Enzymes that are sensitive to DNA methylation can also cause incomplete digestion. PCR-generated artifacts are minimized by the high stringency (high annealing temperature) permitted by the long AFLP primers. High stringency ensures that the primer anneals only to perfectly matched template sequences and eliminates mispriming. Empirically, analysis of AFLP scores from duplicate test samples revealed average errors of 0–2% (Refs 17,22,26).

Box 3. Techniques to generate genetic markers

Allozymes: variants of the same enzyme, encoded by different alleles at the same locus. Because of amino acid charge differences, allozymes can be differentiated by their relative migration speed during gel electrophoresis. Many enzymes are invariant within populations (or even between species and higher taxa), and most polymorphic enzymes have only a few variants (generally two). Although this limits the power of allozyme analysis to resolve genetic differences, allozymes are time and cost efficient for research, requiring only a few polymorphic markers.

Restriction fragment length polymorphisms (RFLPs): sequence variation revealed by differences in DNA fragment lengths after treatment with a restriction enzyme (Box 1) and electrophoretic fragment analysis. Two sources contribute to RFLPs: (1) presence and/or absence of restriction sites that determine the number of fragments generated; and (2) length variation caused by insertions or deletions between restriction sites. RFLPs can be generated by restricting single polymerase chain reaction (PCR) products or, more commonly (e.g. in mapping), by restricting whole genomic DNA and probing with labeled sequences (probes).

Microsatellites: a simple sequence repeat (SSR; Box 1) consisting of two to six, but usually two or three, nucleotides that are repeated many times in tandem (e.g. CACACA…) and that show high variation in repeat number between individuals. By developing PCR primers for the regions flanking a microsatellite repeat, microsatellite allele variation at this site can be screened through high-resolution electrophoresis of microsatellite PCR products. Development of a sufficient number of microsatellite primers requires considerable molecular skills (i.e. cloning and sequencing) and patience (involving a minimum of several months of work). Microsatellite primers developed for one species can rarely be used beyond the very closest relatives; practically, therefore, microsatellite primers need to be developed *de novo* for each new species. The analytical strengths of microsatellite markers are co-dominance and hypervariability (the typical microsatellite locus has more than two alleles, if not dozens).

Random amplified polymorphic DNA (RAPD): RAPD markers are generated by amplification of random DNA segments with short primers (usually about ten nucleotides long) of arbitrary nucleotide sequence. The shortness of the primer demands that the PCR amplification occurs under relatively low selectivity (stringency), which increases the chance of nonspecific priming (primer mismatches) and thus artifactual 'polymorphisms' (up to 60% 'error' bands[36]). Short primers are necessary to obtain matches with complementary sequences in the genome and thus any amplification. Amplification products might be present or absent between individuals (presence or absence of priming sites) and, if present, they can differ in length (length variation between priming sites). RAPDs are dominant markers, but homologous alleles can sometimes be identified with the help of pedigrees.

The ideal technique to assess genetic diversity should meet the following criteria: (1) be cheap and time efficient; (2) generate multiple, independent markers; (3) provide adequate resolution of genetic differences; (4) be reliable (replicable); (5) use extremely small tissue and DNA samples, even partially degraded samples; (6) require little molecular expertise; and (7) require no prior information about an organism's genome. No existing technique meets all these criteria, and techniques scoring high in some criteria invariably score low in others. Five popular genetic markers are compared in the following table.

Criterion[a]	AFLP	RAPD	SSR	RFLP	Allozymes
Quantity of information	High	High	High	Low	Low
Replicability	High	Variable	High	High	High
Resolution of genetic differences	High	Moderate	High	High	Moderate
Ease of use and development	Moderate[b]	Easy	Difficult	Difficult	Easy
Development time	Short	Short	Long	Long	Short

[a]The scoring scheme follows closely those in Hillis *et al.*[2] and Karp and Edwards[49].

[b]Analysis of amplified fragment length polymorphism (AFLP) markers is easy with the help of an automated genotyper, or when using low-resolution agarose gel electrophoresis[24], but manual polyacrylamide electrophoresis requires a certain amount of experience. (Online:Table I)

• Error levels: AFLP amplifications are performed under conditions of high selectivity (at high stringency), thus eliminating the artifactual variation that is seen routinely in RAPD–PCR (Box 3). Repeated AFLP amplifications show near perfect replicability[4,26], and overall errors (including mispriming and scoring error) generally amount to less than 2% (Refs 10,11,15,17,22). In a careful and rigorous experiment, Jones *et al.*[26] tested the replicability of AFLP markers by comparing AFLP analyses, conducted on the same samples in eight different European laboratories, and found only a single scoring difference (absence of one band among a total of 172 in the AFLP profiles). The between-laboratory error for AFLP markers, therefore, was less than 0.6%, which was at the level of microsatellite scoring errors estimated in the same study.

• Quantity of tissue or DNA: AFLP analysis requires minimal amounts of DNA and partially degraded samples can be used. Therefore, extremely small samples and very small organisms can be examined with AFLP–PCR. As an extreme case, Rosendahl and Taylor[21] succeeded in generating AFLP markers from single spores of mycorrhizal fungi (each spore yielding ~0.1–0.5 ng DNA).

• Time efficiency: AFLP markers can be generated at great speed, as illustrated by the high ratio of polymorphisms generated per PCR experiment (multiplex ratio) and by the high percentage of polymorphism in all markers generated (% polymorphism; U.G. Mueller and L.L. Wolfenbarger, unpublished). Studies routinely report the screening of hundreds or even thousands of markers[25,44,45]. For example, Mackill *et al.*[46] estimate that a single researcher could assay thousands of loci per month, of which at least 30% are polymorphic.

• Mendelian inheritance: AFLP markers segregate in a mendelian fashion[4,25,45] and can be used for population genetic and QTL analyses.

• Resolution: Because of the nearly unlimited number of markers that can be generated with AFLP–PCR, using a series of different primer combinations, at least some AFLP markers will be located in variable regions[4] and thus reveal even minor genetic differences within any given group of organisms. Single-nucleotide differences between AFLP fragments can be resolved with either manual polyacrylamide gel electrophoresis or with the help of automated genotypers (Box 2). These high-resolution methods do require training and some laboratory setup costs, but it is also possible to analyze AFLP markers with technically simpler agarose gel electrophoresis (although simplicity is bought at the cost of lower resolution; U.G. Mueller and L.L. Wolfenbarger, unpublished).

Co-dominant AFLP markers

Compared with the popular co-dominant microsatellite (SSR) markers, AFLP markers suffer from their general dominant nature (Boxes 1 and 3). However, detailed pedigree information allows the identification of co-dominant AFLP markers, which permit precise estimation of allele frequencies and more powerful population genetic analysis. Co-dominant AFLP markers have been found, for example, at frequencies of 4–15% (Refs 31,32,47) among all polymorphic AFLP markers; thus, 200 polymorphic markers will contain co-dominant markers from at least 5–30 loci. Moreover, a novel extension of the AFLP technique called 'microsatellite AFLP' or SAMPL (selective amplification of microsatellite polymorphic DNA) appears to generate co-dominate markers at even higher frequencies[33,48]. SAMPL methods combine AFLP primers with anchored, compound SSR primers, and merge AFLP user-friendliness with the

analytical power of microsatellite co-dominance[33,48]. SAMPL might be an important extension of AFLP technology if it can be shown that SAMPL methods uncover adequate variation, that the microsatellite anchoring is reliable and that SAMPL markers are free of PCR artifacts. If a sufficient number of co-dominant markers can be identified, standard AFLPs or microsatellite AFLPs might approach the power of microsatellites, while circumventing the time-consuming setup costs in developing SSR primers.

Conclusions

Because of their unparalleled sensitivity to minor genetic differences, PCR-based markers such as AFLPs and microsatellites are likely to remain key molecular tools for some time to come. The high reliability of AFLP markers could lead to the displacement of RAPD markers, and the user-friendliness of AFLP markers might cause a partial replacement of other high-resolution markers such as RFLPs and microsatellites, at least for some research problems (e.g. QTL mapping and possibly population differentiation). However, because of their largely dominant nature, AFLP markers are unlikely to outcompete co-dominant markers, such as microsatellites or allozymes, which clearly allow more powerful population-genetic analyses. Thus, AFLP and microsatellite markers, coupled with sequencing information for systematic analyses, could surface as the main tools for the analysis of genetic variation. Although AFLP–PCR is not a panacea for all molecular problems in ecology and evolution, it offers many advantages and therefore will probably replace several standard techniques. Researchers interested in genetic diversity, population structure, phylogeny or QTL mapping should carefully examine the relative strengths of AFLP markers (Box 3) – and their limits – in the context of the particular research question pursued.

Acknowledgements

We thank Rachelle Adams, Eduardo Eizirik, Dina Fonseca, John McCarty, David Queller, Gerald Wilkinson and Tim Wright for comments. We are grateful to the NSF (DEB-9707209) and the NSF/Sloan Postdoctoral Fellowship Program in Molecular Evolution for support during the writing of this review.

References

1 Avise, J.C. (1994) *Molecular Markers, Natural History and Evolution*, Chapman & Hall
2 Hillis, D., Moritz, C. and Mable, B. (1996) *Molecular Systematics*, Sinauer
3 Vos, P. and Kuiper, M. (1997) **AFLP analysis,** in *DNA Markers: Protocols, Applications, and Overviews* (Caetano-Anollés, G. and Gresshoff, P.M., eds), pp. 115–131, Wiley
4 Vos, P. *et al.* (1995) **AFLP: a new technique for DNA fingerprinting,** *Nucleic Acids Res.* 23, 4407–4414
5 Heun, M. *et al.* (1997) **Site of einkorn wheat domestication identified by DNA fingerprinting,** *Science* 278, 1312–1314
6 Russell, J.R. *et al.* (1997) **Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs,** *Theor. Appl. Genet.* 95, 714–722
7 Beismann, H. *et al.* (1997) **AFLP analysis sheds light on distribution of two *Salix* species and their hybrid along a natural gradient,** *Mol. Ecol.* 6, 989–993
8 Triantaphyllidis, G.V. *et al.* (1997) **International study on *Artemia*. LVII. Morphological and molecular characters suggest conspecificity of all bisexual European and North African *Artemia* populations,** *Mar. Biol.* 129, 477–487
9 Semblat, J.P. *et al.* (1998) **High-resolution DNA fingerprinting of parthenogenetic root-knot nematodes using AFLP analysis,** *Mol. Ecol.* 7, 119–125
10 Janssen, P. *et al.* (1997) **Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting,** *Int. J. Syst. Bacteriol.* 47, 1179–1187
11 Huys, G. *et al.* (1996) **High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting,** *Int. J. Syst. Bacteriol.* 46, 572–580
12 Koeleman, J.G.M. *et al.* (1997) **Nosocomial outbreak of multi-resistant *Acinetobacter baumannii* on a surgical ward: epidemiology and risk factors for acquisition,** *J. Hosp. Infect.* 37, 113–123
13 Keim, P. *et al.* (1997) **Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers,** *J. Bacteriol.* 179, 818–824
14 Powell, W. *et al.* (1996) **The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis,** *Mol. Breed.* 2, 225–238
15 Tohme, J. *et al.* (1996) **AFLP analysis of gene pools of a wild bean core collection,** *Crop Sci.* 36, 1375–1384
16 Karp, A., Seberg, O. and Buiatti, M. (1996) **Molecular techniques in the assessment of botanical diversity,** *Ann. Bot.* 78, 143–149
17 Arens, P. *et al.* (1998) **Molecular genetic analysis of black poplar (*Populus nigra* L.) along Dutch rivers,** *Mol. Ecol.* 7, 11–18
18 Gonzalez, M. *et al.* (1998) **Characterization of Mexican isolates of *Colletotrichum lindemuthianum* by using differential cultivars and molecular markers,** *Phytopathology* 88, 292–299
19 Travis, S., Maschinski, J. and Keim, P. (1996) **An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers,** *Mol. Ecol.* 5, 735–745
20 Majer, D., Lewis, B.G. and Mithen, R. (1998) **Genetic variation among field isolates of *Pyrenopeziza brassicae*,** *Plant Pathol.* 47, 22–28
21 Rosendahl, S. and Taylor, J.W. (1997) **Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP,** *Mol. Ecol.* 6, 821–829
22 Winfield, M.O. *et al.* (1998) **A study of genetic diversity in *Populus nigra* subsp. *betulifolia* in the Upper Severn area of the UK using AFLP markers,** *Mol. Ecol.* 7, 3–10
23 Gaiotto, F.A., Bramucci, M. and Grattapaglia, D. (1997) **Estimation of outcrossing rate in a breeding population of *Eucalyptus urophylla* with dominant RAPD and AFLP markers,** *Theor. Appl. Genet.* 95, 842–849
24 Mueller, U.G., Rehner, S.A. and Schultz, T.R. (1998) **The evolution of agriculture in ants,** *Science* 281, 2034–2038
25 Maughan, P. *et al.* (1996) **Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis,** *Theor. Appl. Genet.* 93, 392– 401
26 Jones, C.J. *et al.* (1997) **Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories,** *Mol. Breed.* 3, 381–390
27 Lynch, M. and Milligan, B.G. (1994) **Analysis of population genetic-structure with RAPD markers,** *Mol. Ecol.* 3, 91–99
28 Rouppe Van Der Voort, J.N.A.M. *et al.* (1997) **Use of allele specificity of comigrating AFLP markers to align genetic maps from different potato genotypes,** *Mol. Gen. Genet.* 255, 438–447
29 Krauss, S.L. (1999) **Complete exclusion of nonsires in an analysis of paternity in a natural plant population using amplified fragment length polymorphism (AFLP),** *Mol. Ecol.* 8, 217–226
30 Reeve, H.K., Westneat, D.F. and Queller, D.C. (1992) **Estimating average within-group relatedness from DNA fingerprints,** *Mol. Ecol.* 1, 223–232
31 Waugh, R. *et al.* (1997) **Homology of AFLP products in three mapping populations of barley,** *Mol. Gen. Genet.* 255, 311–321
32 Lu, Z.X. *et al.* (1998) **Construction of a genetic linkage map and identification of AFLP markers for resistance to root-knot nematodes in peach rootstocks,** *Genome* 41, 199–207
33 Paglia, G. and Morgante, M. (1998) **PCR-based multiplex DNA fingerprinting techniques for the analysis of conifer genomes,** *Mol. Breed.* 4, 173–177
34 Lynch, M. and Walsh, B. (1998) *Genetic Analysis of Quantitative Traits*, Sinauer

35 Mitchell-Olds, T. (1995) **The molecular basis of quantitative genetic variation in natural populations,** *Trends Ecol. Evol.* 10, 324–327

36 Pérez, T., Albornoz, J. and Domínguez, A. (1998) **An evaluation of RAPD fragment reproducibility and nature,** *Mol. Ecol.* 7, 1347–1358

37 Jin, H. *et al.* (1998) **Identification of quantitative loci for tolerance to barley yellow dwarf virus in oat,** *Phytopathology* 88, 410–415

38 Quarrie, S.A. *et al.* (1997) **QTL analysis to study the association between leaf size and abscisic acid accumulation in droughted rice leaves and comparisons across cereals,** *Plant Mol. Biol.* 35, 155–165

39 Voorrips, R.E., Jongerius, M.C. and Kanne, H.J. (1997) **Mapping of two genes for resistance to clubroot (*Plasmodiophora brassicae*) in a population of doubled haploid lines of *Brassica oleracea* by means of RFLP and AFLP markers,** *Theor. Appl. Genet.* 94, 75–82

40 Otsen, M. *et al.* (1996) **Use of AFLP markers for gene mapping and QTL detection in the rat,** *Genomics* 37, 289–294

41 Van Der Lee, T. *et al.* (1997) **AFLP linkage map of the oomycete *Phytophthora infestans*,** *Fungal Genet. Biol.* 21, 278–291

42 Cho, Y.G. *et al.* (1998) **Integrated map of AFLP, SSLP and RFLP markers using a recombinant inbred population of rice (*Oryza sativa* L.),** *Theor. Appl. Genet.* 97, 370–380

43 Powell, W. *et al.* (1997) **Analysis of quantitative traits in barley by the use of amplified fragment length polymorphisms,** *Heredity* 79, 48–59

44 Ajmone-Marsan, P. *et al.* (1997) **AFLP™ markers for DNA fingerprinting in cattle,** *Anim. Genet.* 28, 418–426

45 Liu, Z. *et al.* (1998) **Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2, and backcross hybrids,** *Mol. Gen. Genet.* 258, 260–268

46 Mackill, D.J. *et al.* (1996) **Level of polymorphism and genetic mapping of AFLP markers in rice,** *Genome* 39, 969–977

47 Boivin, K. *et al.* (1999) **Towards a saturated sorghum map using RFLP and AFLP markers,** *Theor. Appl. Genet.* 98, 320–328

48 Witsenboer, H., Vogel, J. and Michelmore, R.W. (1997) **Identification, genetic localization, and allelic diversity of selectively amplified microsatellite polymorphic loci in lettuce and wild relatives (*Lactuca* spp.),** *Genome* 40, 923–936

49 Karp, A. and Edwards, K.J. (1997) **DNA markers: a global overview,** in *DNA Markers: Protocols, Applications, and Overviews* (Caetano-Anollés, G. and Gresshoff, P.M., eds), pp. 1–13, Wiley

Mitogenomics: digging deeper with complete mitochondrial genomes

Jason P. Curole and Thomas D. Kocher

Mitochondrial genomes are being used to study increasingly ancient divergences among animal groups. Recent studies of complete mitochondrial DNA sequences have arrived at somewhat heretical conclusions, raising questions about the use of mitochondrial gene sequences for studying the relationships among highly divergent lineages. Other studies have documented convergent evolution of mitochondrial gene order, casting doubt on the use of these characters for phylogenetic analysis. The use of mitochondrial genomes for studying such deep divergences is coming under increased scrutiny, and these novel results need to be confirmed with data from nuclear genes.

Jason Curole and Thomas Kocher are at the Dept of Zoology and Program in Genetics, University of New Hampshire, Durham, NH 03824, USA (jcurole@cisunix.unh.edu; Tom.Kocher@unh.edu).

Mitochondria contain their own genome, a circular DNA molecule present in several copies per organelle. Animal mitochondrial DNA (mtDNA) offers distinct advantages over other genes for phylogenetic analysis[1]. Because mitochondrial genes are effectively single copy, comparisons of paralogous genes is generally not a concern. The clonal pattern of maternal inheritance typical of most animals allows direct reconstruction of a bifurcating tree topology. Uniparental inheritance also reduces the effective population size of mitochondrial genes, which means that variants are fixed more quickly between speciation events. Mitochondrial DNA also has a much higher rate of base substitution than most nuclear genes. These characteristics have made mtDNA a popular genetic marker for evolutionary studies.

Almost ten years have passed since the polymerase chain reaction (PCR) enabled the rapid sequencing of animal mitochondrial genes[2]. At that time, few people believed that mtDNA sequences would be a useful phylogenetic marker for clades that diverged more than a few million years ago. Continued technological improvements have made possible the rapid sequencing of complete mitochondrial genomes. New genome sequences are being published at a rate of one per month (Fig. 1). These longer sequences have encouraged attempts to reconstruct relationships among more divergent lineages, but the surprising results of several recent papers raise questions about the validity of this approach.

Vertebrate relationships

An accurate phylogeny is the foundation for a complete understanding of vertebrate evolution. Therefore, questions of vertebrate relationships have been a primary focus in the field of molecular systematics. Although fossils provide evidence of morphological change, the genetic changes associated with evolution can only be discerned from comparisons among extant taxa. In Fig. 2, we present the traditional hypothesis of relationships of chordate taxa discussed in this review.

Tetrapod origins

The extant sister group to tetrapods is one of the most important questions of vertebrate relationships. Based on paleontological evidence, the Actinopterygii (ray-finned

 0169-5347/99/$ – see front matter PII: S0169-5347(99)01660-2