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Reveiw article

Application of AFLP markers in fishery and seafood

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ABSTRACT

Several sociological, health and conservation arguments request a correct labelling of seafood products and fish species. Nowadays, molecular genetics is a useful tool for food chain traceability, particularly in regards to species identification. Among the variety of PCRbased molecular markers, AFLPs (Amplified Fragment Length Polymorphisms) have recently been used to investigate genomes of different complexities. This paper assesses the potential use of the AFLP technology to determine fish and seafood species in processed commercial products, domestic stocks and research in the field of molecular ecology. In particular a species database of fish, molluscs and crustaceans has been created with the aim to identify species of origin of seafood products by previously defined AFLP patterns. Researchers in the field of molecular ecology and evolution require versatile and low-cost genetic typing methods. The AFLP method was introduced 20 years ago and shows many features that fulfil these requirements. With good quality genomic DNA at hand, it is relatively easy to generate anonymous multilocus DNA profiles in most species and the start-up time before data can be generated is often less than a week. These aspects include classical problems such as studies of population genetic structure and phylogenetic reconstructions, and also new challenges such as finding markers for genes governing adaptations in wild populations and modifications of the protocol that makes it possible to measure expression variation of multiple genes (cDNA-AFLP) and the distribution of DNA methylation. We hope this review will help molecular ecologists to identify when AFLP is likely to be superior to other more established methods, such as

microsatellites, SNP (single nucleotide polymorphism) analyses and multigene DNA sequencing.

1. Introduction

Several sociological, health and conservation arguments request the correct labelling of seafood products. The frequent practice of mislabelling involves such questions as truth in advertising, species substitution, consumer protection and management of depleted stocks and their monitoring (Marko et al., 2004). It is crucial that products be identified and examined in each step from fishing area and fish farms to trade and selling (Asaro, 2004). The precautionary measures are necessary due to the fact species substitution of food fish occurs frequently, particularly for imported products which are not recognizable by sight and are indistinguishable on the morphological base after processing and freezing. A recent paper by Marko et al. (2004) showed that some three-quarters of the fish sold in the U.S. such as the red snapper Lutjanus campechanus, belong to another species.

The AFLP (amplified fragment length polymorphism) method (Vos et al. 1995) has been heavily employed in research of plants, fungi and bacteria. The overwhelming majority of these studies have focused on crop species or otherwise economically important organisms. In contrast, relatively few studies have been done in animals, with a similar focus on domesticated and model species. These taxonomic and species biases suggest that there are other suitable genetic methods available for studies of wild animals. Alternatively, it could be that barriers for information flow between research fields, such as the tendency for plant and animal researchers to work in different buildings and publish in different journals, may have hampered the spread of the AFLP method to studies of wild animal species. If this is the case, AFLP might have been severely underused relative its potential in the research field of animal molecular ecology.

Before full genome sequencing of individuals will be feasible for population studies, researchers have to approximate the genome-wide variation based on information from a limited number of loci. Microsatellites, multigene DNA sequencing and SNPs provide high quality genetic information, but have the disadvantage of long start-up times and high costs of typing that will restrict the use of these markers to < 50 loci in most studies. The advantages of AFLP are that it requires comparatively short start-up time in most species and that numerous (> 1000) loci can be studied at moderate costs. The major disadvantage is, however, that the per-locus type of genetic information obtained by AFLP is relatively poor. Presence or absence of a DNA fragment can be detected at a given locus, but in most studies, it is impossible to separate between dominant homozygous (1/1) and heterozygous (1/0) genotypes.

2. Background to AFLP

The AFLP method was originally outlined and evaluated in detail in the study by Vos et al (1995). We will not repeat the protocol in this review, as there are many good sources of information on how to get started with AFLP (Blears et al. 1998; Mueller & Wolfenbarger 1999) but a brief summary is given in Fig 1. Here we also highlight some common technical problems often encountered. The AFLP is a very versatile toolbox and the original protocol can easily be modified in several ways to make it optimized to the species and problem in focus. The AFLP method produces bands (DNA fragments) that are separated based on differences in length using polyacrylamide gel electrophoresis (Fig. 1) or more recently sequencing robots. A band of a certain length represents a presence allele (scored 1) at such an AFLP locus. Individuals not having a band of that length instead have an absence allele (scored 0). The presence and absence character of the data cannot provide complete genotypic information for diploid organisms. This is because individuals with the band can either have two (1/1) or one (1/0) copy of the allele. Polymorphic (within the sample of study) AFLP loci are example of dominant genetic markers, and for each AFLP locus individuals with a band (the presence allele) are either homozygous (1/1) or heterozygous (1/0) and those without the band homozygous for the absence allele (0/0). Most population genetic analyses based on AFLP data assume that the absent allele really is absent from the data. This would happen if there was a base substitution (relative the present allele) in the sequence corresponding to the restriction sites for

the enzymes (e.g. 6 bp for Eco RI and 4 bp for Mse I) or in the sequence corresponding to the additional bases in the preamplification (1 bp + 1 bp) and selective (2 bp + 2 bp) amplification (Fig. 1). However, other types of mutations may result in a DNA fragment of a different length and hence a band at a different position in the gel. In this way two alleles at the same AFLP locus will mistakenly be scored as presence alleles at two different AFLP loci. Indel variation (insertions or deletions) between the primers, e.g. if the primers are spanning a polymorphic microsatellite region (e.g. Wong et al . 2001), may result in many such spurious AFLP loci. Also, a substitution that creates a new cut site for any of the two restriction sites between the primers may make the absent allele for one AFLP locus to be scored as a presence allele at another AFLP locus. Because AFLP gels are typically complex containing many polymorphic sites, it is rarely possible to find the alternative allele, unless segregation analyses of family data are conducted.

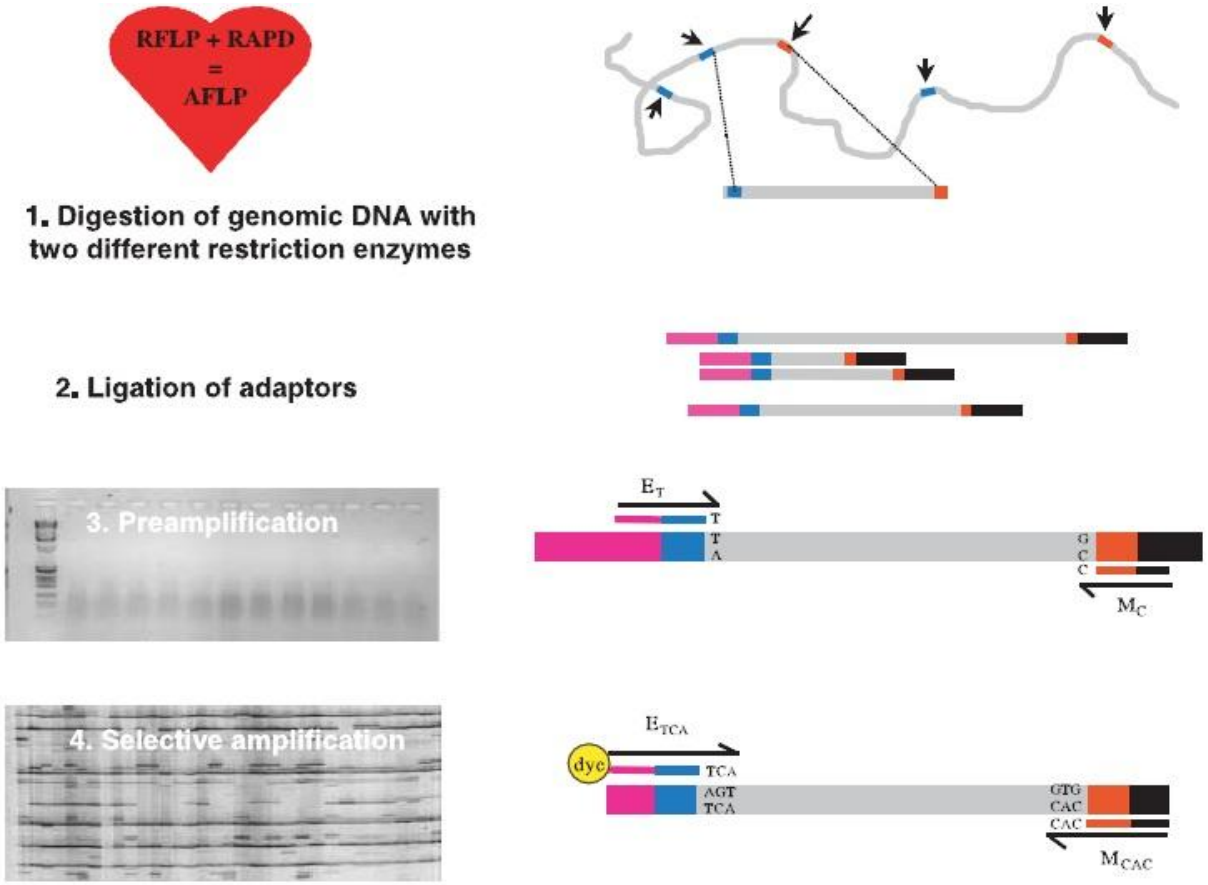

Fig. 1. AFLP is a clever combination of two older methods, RFLP and RAPD. (1) As with RFLP, genomic DNA is digested with restriction enzymes, in this case EcoRI (sites marked blue) and MseI (red). (2) Synthetically made short fragments of DNA (adaptors; pink for EcoRI and black for MseI) that have 'sticky ends' to the cut sites opened by the enzymes, are ligated (glued) to the thousands of anonymous DNA fragments. The adaptors used in the original protocol are designed in such a way that once ligated to the sticky ends of the fragments, the sequence is changed and no longer recognized by the restriction enzymes. Hence, restriction and ligation reactions can be run together. (3 and 4) Arbitrarily selected primers (similar to RAPD) used in PCRs will reduce the complexity in two steps. In the preamplification step, only fragments exhibiting the chosen bases inside the fragments will be amplified resulting in a reduction of fragment numbers by 1/16 (1/4 × 1/4) and these can be visualized as a smear when run on an ordinary agarose gel. In the selective amplification, a small aliquot of the preamplified fragments is used in a second PCR with two primers that extends additionally two bases inwards. This further reduces the number of fragments by 1/256 (1/4 × 1/4 × 1/4 × 1/4) with typical experiment showing about 100 different fragments. The fragments are then size separated and normally visualized by labelling the E-primer with a fluorescing dye.

Such alternative presence alleles are not independent and thus violate an important assumption in analyses of population structure and estimates of population genetic diversity. The introduced bias is however assumed to be negligible as long as AFLP-length codominance is rare (< 10%) and a large number of informative bands (> 100) have been studied (Parsons & Shaw 2001). Another problem is size homoplasy, i.e. that bands of the same length are not homologous and thus representing two or more different AFLP loci, and this is of particular concern in studies of genetic diversity and phylogenetic reconstructions (O'Hanlon & Peakall 2000; Vekemans et al . 2002). Size homplasy has been found to increase with the density of amplified fragments and decrease with the length of the amplified fragments (Vekemans et al. 2002). A simple protocol to detect size homoplasies has been developed by O'Hanlon & Peakall (2000).

3. History of application

In the study by Vos et al. (1995) the method was evaluated by using organisms with genomes widely differing in complexity (bacteria, yeast, plants and humans) demonstrating its broad applicability. Animal researchers did not follow the rapid and wide acceptance of the AFLP technique as it was in studies of plants. Altogether, there are only 115 studies of mammals, birds, fish and insects 2b), of which 33% involved domesticated species. It is notable that there is only one AFLP study in Drosophila (Luckinbill & Golenberg 2002) despite the wide use of Drosophila in genetic research. Similarly, there are only eight AFLP studies of humans. We are interpreting this pattern such that the incitement of using AFLP is declining with the overall knowledge of the genome of the studied organism. The nonrandom distribution of the AFLP method relative to organism group and research tradition merits some thoughts. Around 1995, microsatellites were the prevailing molecular markers used by animal researchers, despite problems associated with isolation and transferability of markers between species (Queller et al. 1993). Similar to many molecular methods used in molecular ecology, the microsatellite technology was first developed for studies of human genetics (Litt & Luty 1989; Weber & May 1989). Although plant researchers also used microsatellites in the early 1990s, the much simpler and less expensive RAPD (random amplified polymorphic DNA) technique (Williams et al. 1990) rapidly became the method of choice (Ritland & Ritland 2000). By being familiar with RAPD, plant researcher learned that the disadvantage of dominant markers can often be more than compensated for by the relative ease of which large number of variable loci can be found and typed, and for this, AFLP is much more powerful than RAPD. Animal researchers might have been further discouraged to start working with dominant markers due to frequent reports on problems with repeatability with RAPD (Peréz et al. 1988).

4. Genetic diversity of species or populations

The level of genetic diversity may reveal information about historical population sizes and structure (Kliman et al. 2002; Sabeti et al. 2002; Wang et al. 2002; Sivasundar & Hey 2003). For managing rare and threatened species, knowledge of intraspecific genetic variation may help to assess extinction risks and evolutionary potential in a changing world (Hedrick 2001). Measuring intraspecific genetic diversity is complicated by the fact that the pattern of diversity may vary substantially across the genome (Sachidanandam et al. 2001) due to gene-specific variation in mutation rates, recombination and mode of selection (Lynch 2002; de Massy 2003; Luikart et al . 2003). Ideally, we want a measure of overall genetic diversity that easily can be compared between studies. The traditional ways to measure genetic diversity, e.g. as the average level of heterozygosity at codominant markers, such as allozymes or microsatellites, are problematic in this respect. The mutation rate at the studied loci will affect the heterozygosity estimate, and microsatellites are particularly sensitive to this sort of bias. Even more important is that these methods normally restrict the user to examine less than a few dozen of loci, for most species corresponding to less than one marker per chromosome.

5. Population structure

On the top of the agenda for many molecular ecologists is to study genetic structure of populations. In a recent review about genetic structure in plants, Nybom (2004) demonstrated that FST values (and FST analogues) obtained from dominant markers (AFLP and RAPD) were overall similar to estimates obtained from microsatellites and allozymes. However, from simulated data it has been shown that 4 to 10 times as many loci have to be used for dominant compared with codominant markers in order to achieve the same precision (Mariette et al. 2002).

Some useful programs for calculating FST analogous from AFLP data are given in Table 2. In studies of wild animals, obtained AFLP-based FST values have been found to be both significantly higher (Mock et al. 2002; Whitehead et al. 2003) and lower (Yan et al. 1999) than estimates for simultaneously investigated codominant markers. However, the numbers of codominant markers used in these studies were relatively few (< 10) and showed large differences in the per-locus estimates of FST values, which put quite a lot of measurement errors into these estimates. Populations exchanging migrants at rates observable for ecologists might have FST < 0.05, and to exclude such values from panmictic situations (FST = 0) data from many loci and individuals are required. AFLP should therefore be very suitable for such situations, as exemplified by a few studies of birds (Wang et al. 2004), fish (Whitehead et al. 2003; Campbell & Bernatchez 2004), insects (Miller et al. 2002; Svensson et al. 2004) and molluscs (Wilding et al. 2001) revealing subtle but significant population structures.

6. Finding genes affecting phenotypes

Selection may shape phenotypic differences between populations even in situations when they exchange genetic material. However, alleles of neutral loci will move more freely than loci linked to the genes contributing to the population-specific phenotypes because the latter may be selected against in the alternative population (Luikart et al. 2003; Beamont & Balding 2004). A few recent studies have taken this notice as a starting point for finding genes that matter, or rather markers for such genes, following a strategy called 'genome scans' (Stortz 2005).

Periwinkles of the species Littorina saxitilis have two different shell morphs. Thick-shelled individuals occur in the lower shore that apparently give them protection against crab predation, and thinner-shelled individuals with greater foot area in wind- and wave-exposed shores where the rate of crab predation is absent (Wilding et al. 2001). By studying four pairs of parapatric populations (5 to 300 m apart), FST was estimated for each of 306 AFLP loci, and the result was compared with a distribution of simulated FST values obtained from parameters of migration and mutation in the data (Wilding et al. 2001). With this approach Wilding et al. (2001) identified that about 5% of the loci were under differential selection in the two morphs. A striking result was also that a neighbour-joining tree based on the full data set put the morphs in two different clusters, but after removing the 15 loci identified to be under selection, populations clustered by sampling site. Hence, there were some common morph genes in the different locations, which when removed, unravelled a phylogeographic pattern presumably shaped by isolation by distance. A study on population divergence on the lake whitefish Coregonus clupeaformis, comparing dwarf and normal ecotypes, used a similar approach and found that about 1.4% of loci might have been under directional selection (Campbell & Bernatchez 2004).

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