Protocol L - An AFLP approach for assessment of maternal inheritance of gynogenetic sea bass [Pratical guide of protocols: methods of verification of the ploidy]

in

Felip A. (ed.), Carrillo M. (ed.), Herráez M.P. (ed.), Zanuy S. (ed.), Basurco B. (ed.). Advances in fish reproduction and their application to broodstock management: a pratical manual for sea bass

Zaragoza : CIHEAM / CSIC-IATS
Options Méditerranéennes : Série B. Etudes et Recherches; n. 63

2009
pages 75-80

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I - Introduction

Production of offspring with uniparental inheritance, either maternal by gynogenesis or paternal by androgenesis, requires the use of a reliable method to confirm the genetic origin of the individuals obtained. In this particular case, of the techniques described in triploidy research, karyotyping and NOR counting are routinely used to evaluate the restoration of the diploidy in gynogenetic and androgenetic fish, but they do not confirm the exclusive maternal or paternal transmission to offspring after chromosome set manipulation.

In gynogenesis induction, the use of biochemical markers (allozyme) has demonstrated to be a reliable method to confirm maternal inheritance in gynogenetic fish. However, the low genetic polymorphism of these markers has limited their application. Nevertheless, the development of a considerable amount of DNA markers has allowed their use in paternity tests to verify gynogenesis and other interesting purposes in aquaculture genetics (Liu and Cordes, 2004). Currently, the application of an Amplified Restriction Fragment Polymorphism (AFLP) approach (Felip *et al*., 2000) as well as the use of microsatellites (Peruzzi and Chatain, 2000; Francescon *et al.*, 2005) have evidenced their validity for monitoring maternal inheritance of gynogenetic offspring in sea bass.

Particularly, the AFLP technology is a variation of the DNA fingerprinting technique based on visualization of polymorphic amplified DNA restriction fragments between samples. Accordingly, the AFLP procedure involves three steps (Fig. L.1): the cutting of genomic DNA with two restriction endonuclease enzymes simultaneously (Step 1) followed by the selective amplification of a subset of genomic restriction fragments using the polymerase chain reaction (PCR) strategy (Step 2) and subsequently the electrophoretic separation of the DNA amplified fragments on denaturing polyacrylamide gels (Step 3) (Vos *et al*., 1995).

PCR amplification of restriction fragments is achieved by ligation of adapters to the genomic DNA fragments to generate target sites for primer annealing and amplification. The PCR amplification is performed in two consecutive reactions. In the first PCR reaction, called preamplification, DNA fragments are amplified with two AFLP primers, each having one selective nucleotide. These PCR products are diluted and used as templates for the second PCR reaction, called selective amplification. The selective amplification is performed using two AFLP primers, each having three selective nucleotides. Consequently, only those fragments in which the primer extensions match the nucleotides flanking the restriction sites will be amplified. For radioactive detection of the amplified fragments, one out of two selective primers is usually end-labeled with ^{32}P or ^{33}P. On the other hand, for fluorescent detection one out of two selective primers is usually labeled using fluorophore labels (i.e., Cy5, FAM, HEX, TET, ROX). Generally 50-100 restriction fragments between 50-500 bp are amplified and detected on denaturing polyacrylamide gels.

Currently, capillary electrophoresis systems have been developed in relation with genome projects. Alternatively to polyacrylamide gels, these DNA analysers can be used for separating and analysing DNA. They offer high speed and high resolution since higher electric fields can be applied due to high electric resistance and high efficiency of heat dissipation compared with slab gel electrophoresis.

At the present time, the AFLP technique has been used for many DNAs of any origin or complexity including DNAs from bacteria, yeasts, plants and animals. Since no prior sequence

knowledge of the target genome is required, it may be an advantage for using in fingerprinting of genomic DNAs from species with limited genomic information. AFLP technology is robust and reliable and requires only small amounts of DNA, although it requires specific equipment, as shown in Fig. L.2, and moderate expertise of the operator. In this Protocol the AFLP approach is shown as a reliable method for gynogenetic sea bass identification. Nevertheless, for a better understanding of this technique the paper of Vos *et al*. (1995) is recommended.

II - Objective

The objective of this Protocol is to demonstrate the potential use of AFLP markers for determining the maternal inheritance of gynogenetic offspring in sea bass.

III - Procedure

A schematic flow chart of AFLP procedure is shown in Fig. L.1. Genomic DNA was obtained from blood samples according to the Protocol previously described by Martínez *et al*. (1998). Subsequently, the methodology of Vos *et al*. (1995) was adapted to the analysis of sea bass samples (Felip *et al.*, 2000) and according to the manufacturer´s instructions for the use of AFLP Analysis System I and AFLP Starter Primer Kit (Cat Nos. 10544-013 and 10483-014; Invitrogen). The AFLP procedure used in the sea bass was based on radioactive detection. Accordingly, some specific requirements related to the use of radioactivity are included throughout this Protocol.

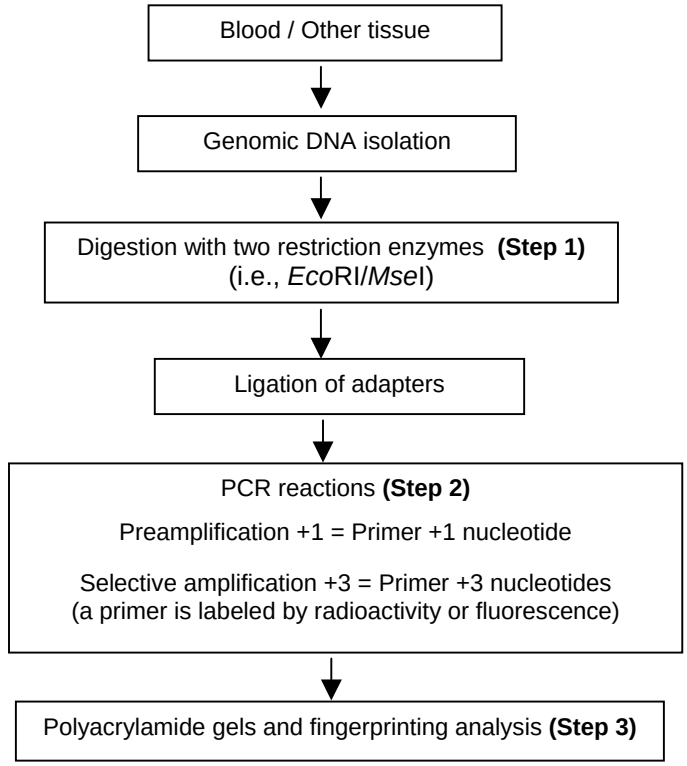

Fig. L.1. Summary of AFLP technology.

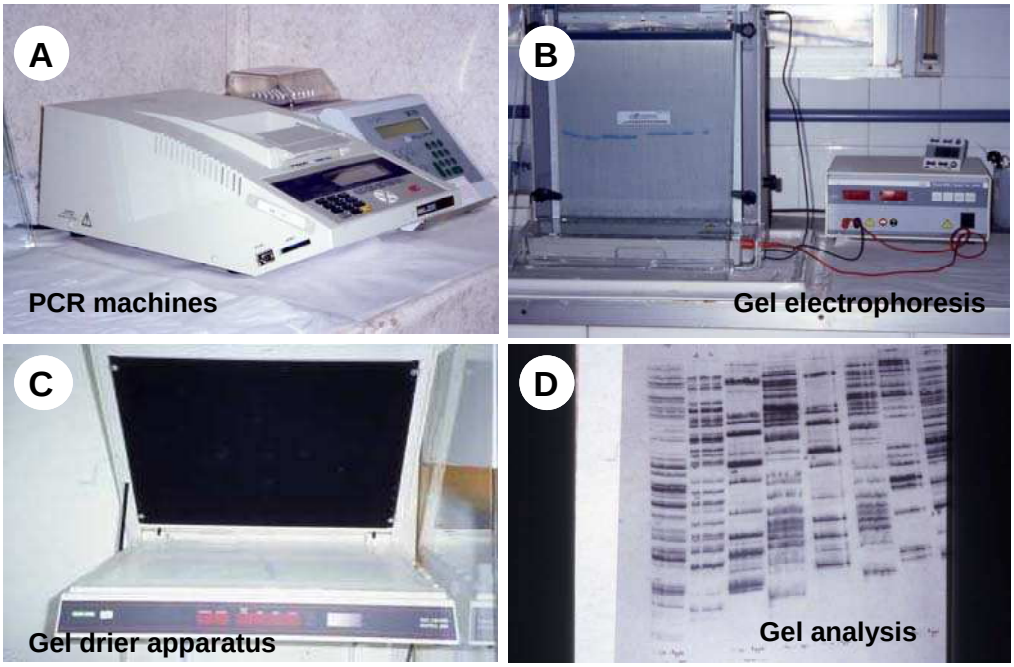

Fig. L.2. **Equipment for AFLP technology based on radioactive detection. (A) PCR machines for DNA fragment selective amplification. (B) A 6% polyacrylamide gel running in an electrophoretic apparatus (C) A gel drier apparatus (D) A dried gel exposured to X-ray film for visualizing fingerprint patterns.**

IV - Materials and equipment

The components of the AFLP Analysis System I (Cat No. 10544-013) include the AFLP Core reagent Kit to perform the restriction digestion of genomic DNA, the ligation of adapters and the preamplification reaction. The restriction endonucleases provided by the kit are *Eco*R I and *Mse* I. *Taq* DNA polymerase is not included with this system.

The components of the AFLP Starter Primer Kit (Cat No. 10483-014) include 8 *Mse* I primers (CAA, CAC, CAG, CAT, CTA, CTC, CTG, and CTT) and 8 *EcoR* I primers (AAC, AAG, ACA, ACC, ACG, ACT, AGC, and AGG) to perform the selective amplification reactions. Accordingly, a total of 64 different primer pairs (i.e., 8 *Mse* I x 8 *EcoR* I = 64 primer combinations) can be analysed. *Taq* DNA polymerase is not included with this system.

It should be noted that for radioactive detection of AFLP amplifications, the laboratory requires a specific radioactive workspace for authorized personnel only to conduct these analyses and a dark room for developing X-ray films.

V - Reagents and solutions

- *Taq* DNA polymerase
- 1.5-ml microcentrifuge tubes
- 0.2-ml thin-walled microcentrifuge tubes
- Automatic pipettes and tips

- A microcentrifuge
- Water bath
- A programmable thermal cycler
- Denaturing polyacrylamide gel electrophoresis solution
- 10X TBE buffer
- Formamide dye
- 10% APS
- TEMED
- Sequencing gel caster
- Nucleic acid sequencing system (electrophoretic apparatus)
- Glass plates
- Gel drier apparatus (for radioactive detection)
- X-ray film (for radioactive detection)
- Film cassette
- Developer and fixer solutions (for X-ray film developing)
- Light box (for X-ray film analysis)
- [Gamma-^{33}P or ^{32}P] ATP (for radioactive detection)

VI - Results and discussion

This procedure is an example that illustrates the importance of the use of DNA molecular markers in aquaculture genetics. In recent years, the application of several molecular markers (e.g., microsatellites, AFLPs, RFLPs, SNPs) has been revolutionary in different researching areas, including fish research. Currently, the application of DNA marker technologies involves different topics in aquaculture genetics including paternity tests, linkage mapping, identification of quantitative trait loci (QTL), marker-assisted selection (MAS) and identification of genes associated to productive traits among others (Liu and Cordes, 2004). Particularly, the use of this molecular tool confirmed that it was a reliable and accurate technique to verify the exclusive maternal genomic contribution of meiogynogenetic fish in sea bass (Fig. L.3).

For this study, a total of 64 primer pairs were analysed in three different meiogynogenetic sea bass offspring and their male and female parents. The analysis of four primer pairs in each of the three offspring (i.e., 1, 2 and 3 groups) yielded 11, 9 and 4 diagnostic polymorphisms, respectively. Diagnostic polymorphisms were based on the evidence of solely male-specific bands in each offspring, which were absent in the female parent for each group. According to our data, results showed that the probability of identifying a nongynogenetic fish in our analysis was 72% using 4 diagnostic markers, 94% using 9 and 97% using 11. Paternity testing revealed that gynogenesis induction in sea bass using the optimal conditions of chromosome manipulation previously described in this specie (Felip et al., 1999; see Protocol *H*) yielded on 90-100% meiogynogenetic sea bass with 30-35% survival of the diploids at hatching (Felip *et al.*, 2001).

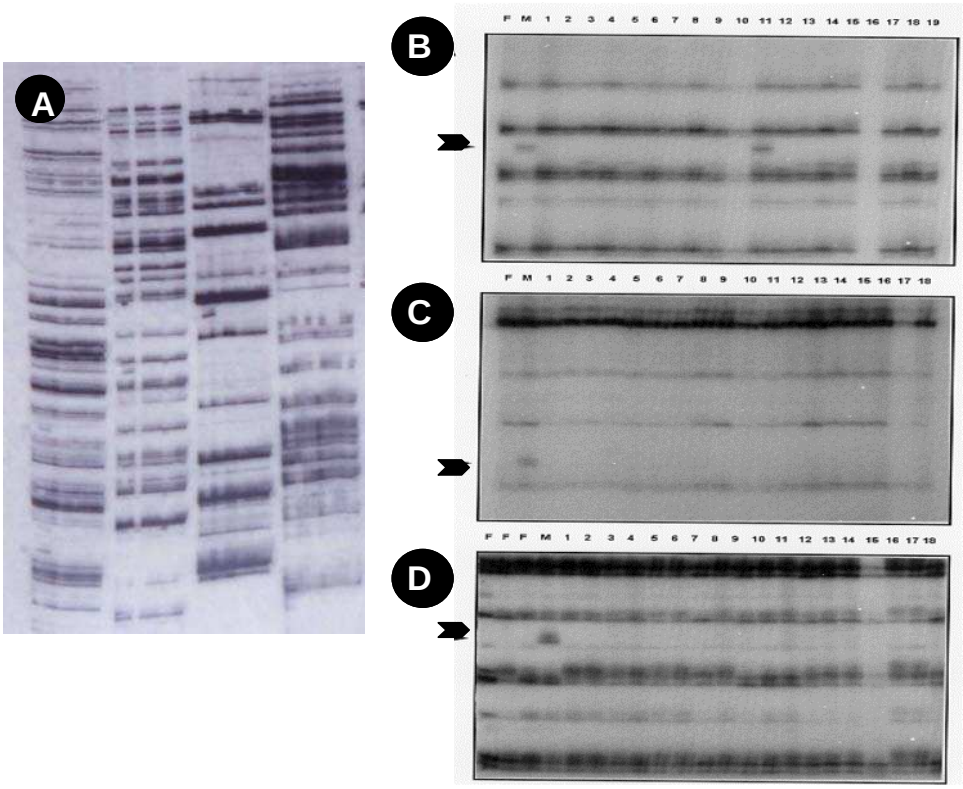

Fig. L.3. AFLP fingerprinting of gynogenetic sea bass. (A) An overview of a fingerprint pattern for scoring polymorphic markers. (B, C, and D) Scoring of gynogenetic fish in three different groups of progenies. Female and male parents are presented as lanes F and M, respectively. Offspring are shown in lanes 1 through 18-19. With the exception of the fish in lane 11 in B, which shows paternal inheritance thus indicating that it was not a true gynogen, all analysed fished show maternal inheritance. Modified from Felip *et al*. *(2000).*

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