An improved protocol for the production of AFLP™ markers in complex genomes by means of capillary electrophoresis

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Introduction

Among the variety of new methodologies for the production of molecular markers, amplified fragment-length polymorphism (AFLP) is gaining increasing attention among animal geneticists (Voss et al. 2001). This technique is based on the polymerase chain reaction (PCR) amplification of restriction fragments ligated to synthetic adapters and amplified using primers which carry selective nucleotides at their 3’ ends (Vos et al. 1995).

The AFLP technology was first successfully experimented in plant species and its application to animal science is quite recent (Otse et al. 1996; Vos & Kuiper 1996; Ajmone-Marsan et al. 1997).

Since the method was developed, AFLP products have mostly been separated using high resolution denaturing polyacrylamide gel electrophoresis and...
visualized by autoradiography, phosphoimager scanning (Vos et al. 1995) or silver staining (Terefeework et al. 2001).

A major analytical improvement has been recently obtained by the use of fluorescence labelled primers and the use of automatic sequencers in a range of (plant and animal) species (Ajmone-Marsan et al. 1997; Remington et al. 1999; Ovilo et al. 2000; Grando et al. 2003). In a number of applications, gel-based sequencers are being substituted by capillary sequencers, able to further increase throughput and precision of analyses (Lindstedt et al. 2000; Terefeework et al. 2001).

In this study, we show that traditional AFLP protocols produces poor results in capillary electrophoresis, and describe an optimized protocol for detecting AFLP markers in vertebrate species (fish, rodents and artiodactyles) using the CEQ 2000™ Beckman-Coulter DNA Analysis System (Beckman-Coulter, Fullerton, CA, USA). The effectiveness of Genographer elaboration software (Vers.1.6.0, J.J. Benham, Montana State University 2001) is also presented.

Materials and methods

DNA extraction and purification

A total of 526 genomic DNAs were extracted and purified from muscle tissue or blood of different fish species (the Salmonidae Salvelinus alpinus: 110 samples, two Mugilidae belonging to the genus Liza: 340 samples, the Gobiidae Knipowitschia panizzae: 35 samples), a laboratory strain of Mongolian gerbil (Meriones unguiculatus: 33 samples), a rodent largely used in laboratory bio-medical research, and domestic goats (Capra hircus: eight samples). DNA was extracted either according to the technique described by Moore (1999) or by means of the Aquapure genomic DNA kit (Biorad, Hercules, CA, USA).

Production of fluorescent AFLP markers

Fluorescent EcoRI/TaqI AFLPs were produced according to two protocols.

Protocol 1

Protocol 1 followed the traditional procedure described in Vos et al. (1995) – with modifications proposed by Vos & Kuiper (1996) and Ajmone-Marsan et al. (1997), to adapt the technology to the use of TaqI endonuclease – using 5 ng of fluorescent primer (Cy5) instead of the same amount of γ-33P-labelled EcoRI primer.

Samples were run under standard conditions for capillary electrophoresis, as proposed by Beckman-Coulter for fragment analysis: capillary temperature 50°C, template denaturation for 120 s at 90°C, injection time 30 s at 2 kV, fragment separation for 35 min at 6 kV.

Protocol 2

Protocol 2 was the result of a long optimization process aiming at increasing the efficiency of amplification of high-molecular-weight bands and the resolution of AFLP profiles detectable by CEQ 2000 (Figure 1). In particular, variables tested and optimized were: DNA amount, restriction and ligation temperatures and times, template dilution, primer concentrations and running conditions. We also assessed the effect of partial DNA degradation on amplification profiles.

Here, we describe only the modifications introduced in the original EcoRI/TaqI AFLP protocol described in Ajmone-Marsan et al. (1997). The complete detailed protocol is available at the address http://www.unicatt.it/zootecnica/aflp or upon request at nonnis@biol.unipr.it.

Restriction–ligation

TaqI and EcoRI restriction times were increased from 1 to 1.5 h and from 1 to 2 h, respectively; ligation conditions were modified from 3 h at 37°C to 16–18 h at 16°C. Following restriction–ligation, the 50 µl reaction volume was diluted four instead of 10 times in sterile apirogen water and processed further.

Pre-amplification

Pre-amplification was carried out in 50-µl volumes containing 15 instead of 5 µl of diluted ligated DNA. Pre-amplifications were diluted 30 instead of 10 times with sterile apirogen water and processed further.

Amplification

The amount of labelled (Cy5) EcoRI primer was doubled (10 ng) compared with γ-33P-labelled primer; the amount of Taq polymerase (Roche Molecular Biochemical, Mannheim, Germany) increased from 0.4 to 1 U and the number of cycles increased from 36 to 43, still maintaining the original ‘touch-down’ amplification profile.

A 1.5-µl volume of PCR product and 0.5 µl of DNA internal size standards (CEQ DNA Size standard-600; Beckman-Coulter) were added to 40 µl of deionized formamide (J.T. Baker, Phillipsburg, NJ,
USA). A drop of mineral oil was added to samples, to allow eventual re-injection after the first run.

Samples were loaded into the CEQ™ 2000 DNA Analysis System equipped with a set of eight 33-mm separation capillary array. Running conditions were: capillary temperature 50°C, template denaturation for 120 s at 90°C, injection time 110 s at 2 kV, fragment separation for 55 min at 6 kV.

Fluorescent AFLP data analysis

Analysis of raw fluorescent AFLP data was performed by means of the ‘fragment analysis module’.

A minimum fluorescence threshold value of 2000 was chosen on the basis of several comparative analyses, but lower peaks have sometimes been considered, especially for high molecular weight fragments when their resolution was comparable to those of ladder fragments having similar size. Fragments useful to assess genetic differentiation within species were identified as those reaching the threshold peak and being well distinct from nearby peaks.

Analysed data were exported to Genographer software for further comparison and scoring. Data files referring to samples analysed with a same primer pair were exported as text files from the ‘Fragment results’ menu of CEQ2000 ‘Database manager’ in a same folder named after the primer pair. While exporting files, the ‘Header’ and ‘Result data’ frames had to be activated. No file name (default) was to be assigned during this process. At this point, Genographer was launched and single samples imported from selected folders into the main frame. During the files import procedure, ‘CEQ trace’ and D4 color were selected in the appropriate frames. After creation of a new file, Genographer constructs a virtual gel (Figure 2) with bands shaped on the base of peak height, resolution and mobility and permits the analysis of single fragments converting them in 0–1 binary matrices.

Following scoring, data were converted from Genographer into standard text format essential for phylogenetic analyses.

Primer pairs assayed

A total of six primer pairs (E32/T32; E32/T33; E33/T32; E33/T33; E40/T33; E40/T37) were tested.

Figure 1 Chromatogram of AFLP peaks obtained with protocol 2 and capillary electrophoresis. Red peaks correspond to internal size standards (CEQ WellRED fluorescent dye), and blue peaks represent Cy5-labelled AFLP fragments. In order to report a clear graphical view, only molecular sizes for some peaks are reported.
with protocol 1 and 9 with protocol 2 (E32/T32; E32/T49; E33/T32; E35/T37; E38/T54; E40/T32; E40/T33; E40/T37) were tested across protocols. Among these, four primer combinations (E32/T32; E33/T32; E40/T33; E40/T50; E42/T37; Table 1). Among protocols.

Table 1 Number of polymorphisms and total number of fragments (in parentheses) determined in each species per primer combination and all combinations together

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Liza saliens (n = 280)</th>
<th>Liza ramada (n = 60)</th>
<th>Knipowischia panizzae (n = 35)</th>
<th>Salvelinus alpinus (n = 110)</th>
<th>Meriones unguiculatus (n = 33)</th>
<th>Capra hircus (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E32/T32 AAC/AAC</td>
<td>15 (45)</td>
<td>19 (49)</td>
<td>18 (40)</td>
<td>–</td>
<td>6 (32)</td>
<td>–</td>
</tr>
<tr>
<td>E32/T38 AAC/ACT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E32/T49 AAC/CAG</td>
<td>15 (37)</td>
<td>13 (41)</td>
<td>–</td>
<td>2 (51)</td>
<td>3 (48)</td>
<td>–</td>
</tr>
<tr>
<td>E33/T32 AAC/AAC</td>
<td>–</td>
<td>–</td>
<td>2 (51)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E35/T37 ACA/ACG</td>
<td>8 (31)</td>
<td>–</td>
<td>2 (51)</td>
<td>1 (42)</td>
<td>3 (48)</td>
<td>–</td>
</tr>
<tr>
<td>E38/T54 ACT/CCT</td>
<td>13 (40)</td>
<td>–</td>
<td>1 (42)</td>
<td>3 (48)</td>
<td>4 (50)</td>
<td>–</td>
</tr>
<tr>
<td>E40/T32 AGC/AAC</td>
<td>25 (74)</td>
<td>28 (71)</td>
<td>21 (50)</td>
<td>5 (48)</td>
<td>2 (32)</td>
<td>–</td>
</tr>
<tr>
<td>E40/T33 AGC/AAG</td>
<td>18 (51)</td>
<td>–</td>
<td>20 (49)</td>
<td>9 (57)</td>
<td>1 (24)</td>
<td>–</td>
</tr>
<tr>
<td>E40/T50 AGC/CAT</td>
<td>15 (40)</td>
<td>22 (59)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E42/T37 AGT/ACG</td>
<td>4 (15)</td>
<td>–</td>
<td>2 (32)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E42/T48 AGT/CAC</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>113 (333)</td>
<td>82 (220)</td>
<td>81 (198)</td>
<td>16 (156)</td>
<td>17 (228)</td>
<td>28 (151)</td>
</tr>
<tr>
<td>% Polymorphic loci</td>
<td>33.93</td>
<td>37.27</td>
<td>40.90</td>
<td>10.26</td>
<td>7.46</td>
<td>18.54</td>
</tr>
</tbody>
</table>

Figure 2 Comparison of AFLP markers obtained by means of polyacrylamide autoradiography and CE (Genographer gel representation). For a better comprehension of Genographer banding pattern, only fragments in the size range 100–400 bp are illustrated.
Radioactive AFLP production and scoring

In goats, two primer combinations (E32/T38 and E42/T48) were used to compare radioactive and capillary electrophoresis and assess data reproducibility across protocols and technologies. Radioactive AFLPs were produced and run according to published protocols (Ajmone-Marsan et al. 1997) and binary scored visually.

Statistical analyses

Only descriptive statistics (average, standard deviation and range) on polymorphisms observed in the different species is reported, not being the objective of the present paper the discussion of population genetic data.

Results and discussion

Several papers have recently been published on the use of EcoRI/MseI AFLPs on CE for the detection of polymorphisms in simple genomes (Lindstedt et al. 2000; Terefowork et al. 2001). Conversely, this work focuses on the implementation of EcoRI/TaqI AFLPs on CE for the detection of polymorphisms in genomes of high complexity. Compared with EcoRI/MseI, EcoRI/TaqI AFLPs bring improvements in terms of polymorphisms and profile quality, particularly in mammals (Ajmone-Marsan et al. 1997), and their adaptation to CE represents an important step towards increasing sensitivity and precision of their assay and decreasing scoring time and errors.

The testing in CE of the protocol developed for AFLP production and detection on gel based sequencers (protocol 1; Vos et al. 1995; Ajmone-Marsan et al. 1997; Savelkoul et al. 1999) produced between 10 and 20 clearly detectable peaks per combination in the size range 50–250 bp, with fluorescence activity usually ranging between 10 000 and 200 000. A rapid signal decrease was observed for longer fragments (250–400 bp), with fluorescence activity hardly reaching 5000. Mean fluorescence values calculated in the whole fragments size range were 5000–8000 depending on the specific primer combination. In most cases, no fragments longer than 400 bp were observed. This protocol therefore does not perform appropriately on capillaries.

Methodological implementation was necessary to fulfill analytical needs of capillary electrophoresis. Sensitive steps to improve the AFLP methodology in connection with the use of CEQ 2000 capillary apparatus, were therefore identified.

Sufficiently purified high molecular weight genomic DNA is an important starting point to obtain reliable data and fragments larger than 300 bp. Approximately 200–400 ng of genomic DNA appeared to be a suitable quantity to be submitted to DNA restriction, as suggested by Vos et al. (1995). In our opinion, the observation made by Muller & Wolfenbarger (1999) about the use of partially degraded samples to obtain AFLP profiles should be somewhat reconsidered, at least using the protocol proposed and capillary electrophoresis. In fact, we observed that partially degraded DNA was suitable for analyses limited to short fragments (50–200 bp) even if larger fragments can be sometime obtained by increasing the amount of DNA to submit to restriction up to 600–700 ng.

To reduce background noise, DNAdigestion and ligation time was increased. Also, an improved signal-to-noise ratio and a higher relative signal of longer fragments was observed when increasing the concentration of template DNA in the pre-amplification step, the dilution of pre-amplified DNA used in the AFLP amplification and the number of final amplification cycles. In addition, two-fold increased quantity of Cy5-labelled EcoRI primers had the effect to reduce competition between fragments during amplification, allowed a better amplification of high-molecular weight molecules and permitted a better resolution of the fragments in the automatic capillary sequencer. Protocol 2, improved with the modifications described, produced on average 50 fragments per primer combination (range 30–80). Fragments amplified ranged between 50 and 650 bp in most species. Highest peak heights were generally around 150 000–200 000 (Figure 1), and mean values for the whole size range were 25 000–30 000. Bands amplified by protocol 1 matched the lower (40–60%) molecular weight subset of bands produced by protocol 2, depending on primer pair.

Maximum detectable dimensions of AFLP fragments is related to signal to noise ratio and resolution of higher molecular weight bands and by the instrument separation time set by the operator. We suggest the use of a separation time of 55 min, as a best compromise between detectable dimensions (650 bp) and peak resolution. In fact, peak resolution is important for the critical evaluation of fragments differing in just one base, and to avoid erroneous interpretation of polymorphisms.

The transformation of the chromatogram into a banding pattern gel representation by means of Genographer software represents an important aid to score AFLP marker in a semi-automated manner.
Genographer allows a direct evaluation of single peaks in several individuals by means of specific frames and detects the presence/absence of the fragment in relation to the peak height and resolution thresholds. It also normalizes a gel in relation to the strength of total signal in each lane. The lanes are normalized so that the total signals are equal to the average signal in the gel. This will affect all of the aspects of the program, the gel view, the trace, the graph, and the analysis.

Hence, the simultaneous evaluation of several individuals gives a valuable representation of polymorphic fragments. Finally, binary 0–1 matrices can be organized for phylogeny softwares by exporting Genographer binary files as text files having format compatible with phylogenetic or mapping softwares.

Particular attention must be deserved to correct band alignment. We observed that appropriate alignments generally begins around 70 bp and that artefact polymorphisms at lower molecular weight may occur (Figure 2). Short fragments are to be considered with caution also because they show a higher risk of homoplasy (O'Hanlon & Peakall 2000; Vekemans et al. 2002). We considered those fragments having molecular weight higher than 75–80 bp as informative.

Following data analysis and scoring, the average percentage of polymorphic loci observed per primer pair was 34 ± 7% (range 27–41%) in Liza saliens (sharp-nose mullet); 37 ± 8% (range 32–39%) in Liza ramada (thin-lipped mullet); 41 ± 7% (range 36–45%) in K. panizzae (lagoon goby); 10 ± 6% (range 4–16%) in S. alpinus (naturally inbred Arctic char population); 7 ± 2% (range 2–19%) in M. unguiculatus (artificially inbred rodent strain); 19 ± 2% (range 17–20%) in Capra hircus (domestic goat) (Table 1).

Levels of AFLP variability depend on the primer combination, and inbreeding level of taxa analysed. Inbred animals, as S. alpinus and M. unguiculatus, had polymorphisms levels lower than other panmictic species due to enhanced population consanguinity (Frankham 1995; Neumann et al. 2001; Keller & Waller 2002). Although a strict comparison of AFLP polymorphisms in different species depends on such variables as the species itself, the population, the intra-population sampling, the restriction enzymes, the primer combinations, and the scoring criteria for mono/polymorphic fragments, polymorphism of AFLP data previously reported in the literature in vertebrate species (Otsen et al. 1996; Seki et al. 1999; Zhanjiang et al. 1999) are in agreement with our results.

Protocol reliability and data quality was confirmed by comparison with radioactive AFLPs in goats. In this species, 107 fragments generated by two primer combinations with either fluorescent or radioactive technologies were attributed the same scoring in the size range 100–500 bp (Figure 2). It must be remarked that compared with AFLP radioactivity and visual scoring, capillary systems and semi-automated analysis supported by Genographer elaboration, increased data throughput and scoring reliability, decreasing the overall experimental effort.

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References


