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Optimization of AFLP fingerprinting of organisms with a large-sized genome: a study on *Alstroemeria* spp.

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Abstract The recently introduced PCR-based DNA fingerprinting technique AFLP (amplified fragment length polymorphism) allows the selective amplification of subsets of genomic restriction fragments. AFLP has been used for multiple purposes such as the construction of linkage maps, marker saturation at specific genomic regions, analysis of genetic diversity and molecular phylogeny and cultivar identification. AFLP can be tailored by varying the number of selective nucleotides added to core primers and can allow accurate amplification, even in complex template mixtures generated from plant species with very large genomes. In this study *Alstroemeria*, a plant species with a very large genome, was tested for adapting the AFLP protocol. The results indicated that the estimated number of amplification products was close to the observed number when eight selective nucleotides were used but that seven selective nucleotides did not increase the number of amplification products fourfold. However, we found reproducibility in both +7 and +8 fingerprints. Various distributions of selective nucleotides over the various rounds of preamplifications were tested. Preamplification with four selective nucleotides followed by final amplification with eight selective nucleotides produced clear and reproducible AFLP patterns. The effects of GC content of primers and multiple preamplification steps were also discussed.

Key words AFLP genetic marker · *Alstroemeriaceae* · Inca lily · Multiple cascade PCR · Preamplification

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Introduction

In the last decade a large number of DNA marker techniques have been developed. These techniques are based either on DNA-DNA hybridization, like restriction fragment length polymorphism (RFLP) markers (Beckman and Soller 1983), or on the amplification of specific DNA fragments using specific or random primers like sequence-tagged sites (STS, Shin et al. 1990), sequence-characterized amplified region (SCAR, Paran and Michelmore 1993), random amplified polymorphic DNA (RAPD, Rafalski et al. 1991) and amplified fragment length polymorphism (AFLP, Vos et al. 1995). These marker techniques can be easily performed on species with a small to moderately large genome size, such as *Arabidopsis* (145 MB), barley (1,500 MB) (Bennett and Leitch 1995) and mammal genomes (3,000 MB). For Southern hybridization the detection limit is determined by the DNA binding capacity to membranes used for Southern blotting and labeling efficiency of the DNA probes. For polymerase chain reaction (PCR)-based marker techniques, however, the amount of template DNA needed for amplification is at least 1,000 times less than for Southern hybridization. The most important factor that might interfere with the amplification of specific fragments is the competition with other fragments that are amplified by random priming due to one or more mismatches.

The recently introduced PCR-based DNA fingerprinting technique AFLP (Vos et al. 1995) allows the selective amplification of subsets of genomic restriction fragments. AFLP has been used (1) for the construction of linkage maps, as in *Arabidopsis* (Alonso-Blanco et al. 1998), barley (Becker et al. 1995; Qi et al. 1998; Waugh et al. 1997) and potato (Van Eck et al. 1995); (2) for marker saturation at specific genomic regions in barley (Büschges et al. 1997), potato (Van Voort et al. 1997), rice (Maheswaran et al. 1997) and tomato (Thomas et al. 1997); (3) for the analysis of genetic diversity in

nematodes (Folkertsma et al. 1996) and for molecular phylogeny in potato (Kardolus et al. 1998); (4) for cultivar identification in barley (Ellis et al. 1997) and in potato (Milbourne et al. 1997). AFLP can be tailored according to the complexity of the pool of the restriction fragments by varying the number of selective nucleotides added to core primers which hybridize to adaptors ligated to the restriction fragments. Vos et al. (1995) tested the AFLP technique in middle-large human genomes and indicated that the AFLP technique could allow accurate amplification of subsets of restriction fragments, even in complex template mixtures generated from plant species with very large genomes, just by increasing the number of selective nucleotides added to the core primers.

An example of a plant species with a very large genome is *Alstroemeria*, also known as the Peruvian or Inca lily. It is a genus in the family of *Alstroemeriaceae* consisting of approximately 60 species of rhizomatous, herbaceous plants, most of which are endemic to Chile and Brazil (Bayer 1987; Ravenna 1988; Aker and Healy 1990). The nuclear DNA content (2C-value) ranges from 37 to 79 pg for Chilean species and from 50 to 56 pg for Brazilian species (Bharathan et al. 1994; Buitendijk et al. 1997). The haploid genome size (1C) of 25 pg, which equals approximately 25,000 Mbase pairs, will render approximately 12,000,000 different *Eco*RI/*Mse*I fragments, assuming random distribution of restriction sites, whereas only 36,000 fragments are expected from an *Arabidopsis* genome (1C = 0.04 pg).

If species with a large genome contain abundantly occurring repetitive sequences, the number of different amplification products will be reduced. Still, species with a large genome size are expected to generate more AFLP amplification products than species with small genomes. Until now little is known about the fine-tuning of AFLP for the analysis of species with such large genomes.

In the study presented here our aim was to adapt the AFLP protocol for *Alstroemeria*. Various numbers of selective nucleotides and preamplification steps were tested in order to obtain reproducible, clear and labor-saving fingerprints. Furthermore, the applicability of AFLP as a genetic marker in species with a large genome was discussed.

Materials and methods

Plant material

Sixteen accessions of in total seven diploid (2x = 2n = 16) non-inbred *Alstroemeria* species of Chilean or Brazilian origin were used to acquire general *Alstroemeria* AFLP fingerprints with a wide genotypic background. Several species commonly used as breeding parents were selected and crossed with each other (*A. aurea* × *A. inodora* and *A. inodora* × *A. psittacina*) (Table 1).

The AFLP protocol

Genomic DNA was isolated from fresh leaf tissue of greenhouse-grown plants using the CTAB method according to Rogers and Bendich (1988). The AFLP method was performed as described by Vos et al. (1995). A slight modification on this general protocol was made with respect to the number of selective nucleotides and the number of preamplification steps. The general protocol included four steps: (1) restriction of genomic DNA with *Eco*RI and *Mse*I, and ligation of adaptor sequences to the restriction fragments in order to generate the primary template, (2) selective preamplifications of this primary template with AFLP primers having various additional 3′ selective nucleotides, (3) selective amplification with ^{33}P-labeled *Eco*RI primers having three or four 3′ selective nucleotides and *Mse*I primers with four 3′ selective nucleotides, and (4) separation of labeled amplification products on a denaturing

Table 1 Accessions and origins of *Alstroemeria* species for AFLP analysis

Plant material	Accession/source[a]
Chilean species	
A. aurea R. Graham	A001, A002, A003, A004, A011, A017, 96A001-1
A. diluta Bayer	AD5 K
A. hookeri Loddiges spp. *hookeri*	AP6K
A. ligtu L. spp. *ligtu*	AL1 S
A. pelegrina L.	C100, C057-2
Brazilian species	
A. inodora Herb.	P002, P004-20, P008-1
A. pstittacina Lehm	D032
Interspecific hybrids (F_1)	
A. inodora × *A. psittacina*	PD4 (P002 × D032)
A. aurea × *A. inodora*	A1P2 (A001 × P002)
F_2	PD4⊗-2, PD4⊗-3, PD4⊗-4, PD4⊗-5, PD4⊗-6, PD4⊗-7, PD4⊗-8, PD4⊗-9
BC_1	SK004, SK011 ((A1P2) × P002), SK017 (P002 × (A1P2))

[a] Codes from accessions of species maintained at the Department of Plant Breeding, Wageningen Agricultural University

polyacrylamide sequencing gel. The anodal buffer was supplemented with 0.5 *M* sodium acetate to generate a salt gradient, which contributes to a better separation of the larger fragments. The gels were dried on Whatmann 3MM paper, and X-ray films (Konica, Tokyo, Japan) were exposed at room temperature for 1–7 days depending on the signal intensity.

Results and discussion

Reducing the number of amplification products

According to Vos et al. (1995) the number of amplification products generated by the AFLP technique is related to the size of the genome and the number of selective nucleotides added to the 3′ end of the *Eco*RI and *Mse*I core primers. In view of the large genome size of *Alstroemeria*, the number of selective nucleotides added to the core primers varied from six to eight. Theoretically, +6, +7 and +8 fingerprints named by their selective nucleotides should have roughly around 3,000, 720 and 180 bands, respectively, in consideration of the genome size of *Alstroemeria*. However, fingerprints with on average 109 (66–154) amplification products per lane were obtained using six selective nucleotides (*Eco*RI + 3/*Mse*I + 3) (Table 2). When using seven selective nucleotides (*Eco*RI + 3/*Mse*I + 4) the number of amplification products decreased significantly. The average number of amplification products per lane was 87 (47–158). Similarly, with eight selective nucleotides (*Eco*RI + 4/*Mse*I + 4), 91 products (36–135) were amplified. These results indicated that the expected number of amplification products was close to the observed number when eight selective nucleotides were used, always considering the rough estimation of the number of amplification products. However, the observed number of amplification products from six and seven selective nucleotides did not meet the expected number of amplification products. The number of amplification products dramatically reduced with six and seven selective nucleotides may be due to the presumed high frequency of repetitive DNA and due to technical limitations, such as the separation of nearly comigrating bands.

The effect of reducing one extra selective nucleotide on the number of bands per lane was studied in greater detail by comparing the fingerprints generated with a total of seven selective nucleotides (the +7 fingerprint) with the four corresponding fingerprints generated with primers with eight selective nucleotides (corresponding +8 fingerprints) as well as with the fingerprint of a mixture of equal volumes of the corresponding +8 fingerprints. Likewise, the +6 fingerprints were compared with the corresponding four +7 fingerprints with seven selective nucleotides and their mixture (Fig. 1). Occasionally, we observed that a band found in the +7 fingerprint was amplified in two out of the corresponding four +8 fingerprints instead of the expected one lane (Fig. 1, indicated with arrowheads). Bands of +6 fingerprints were frequently found; two comigrating bands in the corresponding +7 fingerprints as well (Fig. 1, indicated with open arrowheads). This result, suggesting two comigrating alleles with one nucleotide difference, can explain the non-fourfold increase in bands. In addition, some bands of +8 fingerprints, that were missing on the lanes of the mixture and the preceding +7 fingerprint are indicated with arrows inside of the fingerprint (Fig. 1). The same holds

Table 2 Number of bands according to GC contents for six, seven and eight selective nucleotides

GC content effects	Primer combinations	Species	Average number of bands	Range	Standard deviation
Six selective nucleotides	16	7	109a[a]	66–154	17
33%	2	6	121a[b]	83–145	19
50%	8	7	109b	66–154	17
67%	4	6	110b	81–149	15
83%	2	5	94c	74–117	13
Seven selective nucleotides	24	7	87b	47–158	23
29%	3	6	111a	88–137	14
43%	9	7	98b	63–146	18
57%	8	6	81c	49–158	22
71%	4	6	73c	47–124	19
Eight selective nucleotides	80	2	91b	36–135	20
38%	4	2	84a	66–107	14
50%	16	2	90a	55–121	15
63%	28	2	87a	36–107	21
75%	24	2	94a	52–135	22
88%	8	2	97a	60–133	20

[a] Results of Duncan test ($\alpha = 0.05$) among band numbers of 6, 7 and 8 selective nucleotides
[b] Results of Duncan test ($\alpha = 0.05$) among band numbers of GC contents for each selective nucleotide class

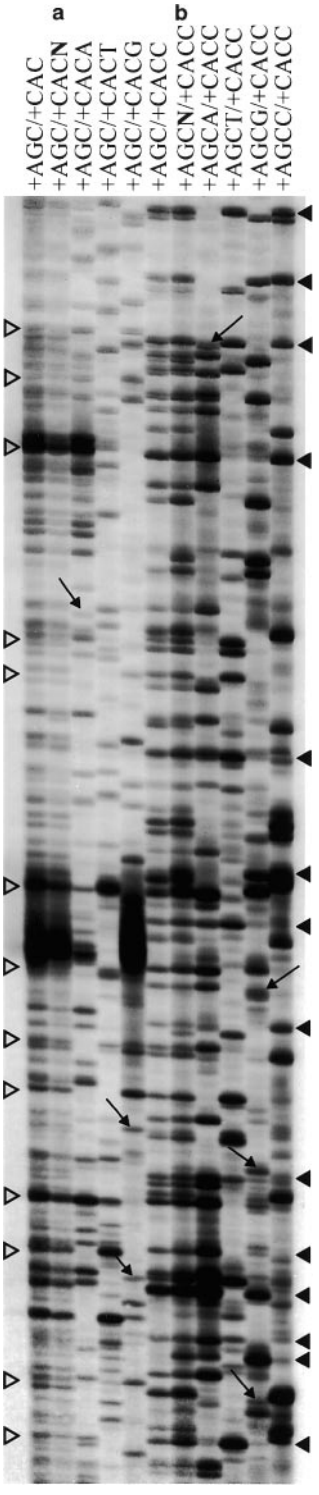

Fig. 1 The +6, +7 and +8 fingerprints of *A. inodora* (P002) and the mixture of corresponding fingerprints. *Arrows* indicate bands present but absent in the mixture and preceding fingerprints. *Arrowheads* indicate the homeologous bands present in two lanes.
[a]The mixture of +AGC/+CACA, +AGC/+CACC, +AGC/+CACG and +AGC/+CACT
[b]The mixture of +AGCA/+CACC, +AGCC/+CACC, +AGCG/+CACC and +AGCT/+CACC

true for the +7 fingerprints compared to the mixture and the preceding +6 fingerprints.

In conclusion, on the one hand, we recognized that the preceding fingerprint and the fingerprint of the mixture were largely identical, suggesting that a reduction in selective nucleotides did not generate artifacts. On the other hand, we failed to recognize a fourfold increase due to one less extra selective nucleotide. These results indicate that the AFLP technique is reproducible, allowing us to work with +7 and +8 selective nucleotides regardless of the theoretical expectation of the amplification products.

The GC content effect was shown on 120 primer combinations with 16 accessions (Table 2). The data were analyzed with the Duncan test ($\alpha = 0.05$). In +6 fingerprints, the average band numbers of 33% and 83% GC contents showed significant differences, 121 and 94 bands, respectively. In +7 fingerprints higher GC contents significantly reduced band numbers: 29%, 43%, 57% and 71% GC contents showed 111, 98, 81 and 73 bands, respectively. This indicates the importance of determining the primer combinations. However, the GC contents of +8 fingerprints did not influence band numbers. In soybean, which has an unusually high AT content, the number of AFLP bands generally increased for primer combinations with AT-rich selective nucleotides. Inversely, GC-rich selective nucleotides reduced the number of bands and increased the quality of bands (Keim et al. 1997). The determination of the quality of the bands was based on band intensity and separation from fragments of similar size. *Alstroemeria* has been shown to be AT rich in repetitive DNA in the case of *A. aurea* (56%) (De Jeu et al. 1997) and Brazilian species (61%) (Kuipers, personal communication). Our data support that *Alstroemeria* is AT-rich and that the GC contents of primers influence the number of bands.

We conclude that a total of six, seven or eight selective nucleotides could produce clear and reproducible AFLP patterns (less than 100 bands) depending on the primer combinations and species.

The effect of preamplification steps on AFLP fingerprints

In the previous paragraph we determined the influence of the number of selective nucleotides used during the final PCR on the amplification products. In this section we focus on (1) the influence of the number of selective bases of the primers on the preamplification and (2) the influence of the number of preamplification steps on the accuracy of amplification, which affects the reproducibility and the clearness of the bands.

For plant species with a large genome we should include a selective preamplification of the template with moderate selectivity of the primers to avoid mismatches. In the case of average-sized genomes, preamplification steps with *Eco*RI + 1/*Mse*I + 1 have been commonly used to avoid mismatches (Vos et al. 1995; Qi and Lindhout 1997). Next to that, the final selective PCR amplification with the radioactively labeled primer combination should also have moderate selectivity. In this experiment we applied various distributions of the selective nucleotides across the

various rounds of preamplifications on 11 related genotypes (parents: P002, D032; F_1 plant: PD4; F_2 plants: PD4⊗-2, PD4⊗-3, PD4⊗-4, PD4⊗-5, PD4⊗-6, PD4⊗-7, PD4⊗-8, PD4⊗-9) (Table 1). Eight selective nucleotides (*Eco*RI + 4/*Mse*I + 4) at the final PCR rendered 14 different preamplification steps (Table 3). Previous experiments on small genomes have shown that the selective amplification of subsets of fingerprints was accurate when no more than three selective nucleotides were added onto the core primer. Application of four selective nucleotides without any preamplification was no longer accurate because mismatches were tolerated at the first selective base (Vos et al. 1995).

The overall band intensity of the ST0 (PT → + AGCC/ + CACG) fingerprint was very weak resulting in the absence of bands which were amplified in other preamplification steps (Fig. 2). This is possibly because of the extreme template competition. However, only a few mismatches were accompanied as a result of the eight selective nucleotides used in our study (Data not shown). Unexpectedly, the overall band intensity of the ST1 (PT → + A/ + C → + AGCC/ + CACG) fingerprint was increased, producing an AFLP pattern with extra bands and smears. We are uncertain as to whether these extra bands and smears should be considered as background until genetic studies are carried out. ST2 (PT → + AG/ + CA → + AGCC/ + CACG) fingerprint produced a clear and reproducible AFLP pattern (Figs. 2 and 3). A severe disappearance of bands was detected on the ST3 (PT → + AGC/ + CAC → + AGCC/ + CACG) fingerprint, resulting in an increase in the intensity of some bands. A contrary AFLP pattern was noticeable between the ST1 and ST3 steps because both steps had six selective nucleotides jump. The distinctive differences in fingerprints due to the preamplification steps proved the importance of choosing them carefully. In conclusion, we chose the ST2 fingerprint for use in further studies because of its reproducibility and efficiency.

The application of two (ST4, ST5, ST6, ST7, ST8, ST9) or three (ST 10, ST11, ST12, ST13) preamplification steps resulted in a general decrease in the intensity of fragments larger than 350–400 nucleotides compared to one preamplification step, indicating that smaller fragments have an advantage over larger fragments during PCR (data not shown). The intensity of several products had minor differences. In few cases the intensity changed in such a way that the band was present or absent owing to the preamplification steps (Fig. 2). The independent AFLP pattern was detected on the basis of the preamplification steps even though the final selective bases were identical. However, a multiple preamplification (2–4 times) in general was reproducible although it was not preferable because of the loss of large fragments and extra labor. It provide the opportunity to increase the selective bases as long as one or two selective nucleotides for both primers are used.

Are AFLP products good genetic markers?

Both of the two segregating populations (F_2 and BC_1) used to choose the optimized AFLP protocol are genetically related. Therefore, the markers obtained in these progenies could be used to validate the accuracy of AFLP fingerprinting, because fragments observed in offspring genotypes should be present in at least one of the parental genotypes (Table 1). The theoretically expected segregation patterns were demonstrated,

Table 3 Fourteen various preamplification steps with final +4/+4 selective nucleotides on 6 genotypes (P002, D032, PD4, PD4⊗-2, PD4⊗-8, PD4⊗-9)

Code	Preamplification steps	Example
ST0	PT[a] → + 4/ + 4	PT → + AGCC/ + CACG
ST1	PT → + 1/ + 1 → + 4/ + 4	PT → + A/ + C → + AGCC/ + CACG
ST2	PT → + 2/ + 2 → + 4/ + 4	PT → + AG/ + CA → + AGCC/ + CACG
ST3	PT → + 3/ + 3 → + 4/ + 4	PT → + AGC/ + CAC → + AGCC/ + CACG
ST4	PT → + 1/ + 1 → + 2/ + 2 → + 4/ + 4	PT → + A/ + C → + AG/ + CA → + AGCC/ + CACG
ST5	PT → + 1/ + 1 → + 3/ + 3 → + 4/ + 4	PT → + A/ + C → + AGC/ + CAC → + AGCC/ + CACG
ST6	PT → + 2/ + 2 → + 3/ + 3 → + 4/ + 4	PT → + AG/ + CA → + AGC/ + CAC → + AGCC/ + CACG
ST7	PT → + 2/ + 2 → + 2/ + 3 → + 4/ + 4	PT → + AG/ + CA → + AG/ + CAC → + AGCC/ + CACG
ST8	PT → + 2/ + 2 → + 3/ + 2 → + 4/ + 4	PT → + AG/ + CA → + AGC/ + CA → + AGCC/ + CACG
ST9	PT → + 2/ + 2 → + 3/ + 3 → + 4/ + 4	PT → + AG/ + CA → + AGC/ + CAC → + AGCC/ + CACG
ST10	PT → + 1/ + 1 → + 2/ + 2 → + 2/ + 2 → + 4/ + 4	PT → + A/ + C → + AG/ + CA → + AG/ + CA → + AGCC/ + CACG
ST11	PT → + 1/ + 1 → + 2/ + 2 → + 2/ + 3 → + 4/ + 4	PT → + A/ + C → + AG/ + CA → + AG/ + CAC → + AGCC/ + CACG
ST12	PT → + 1/ + 1 → + 2/ + 2 → + 3/ + 2 → + 4/ + 4	PT → + A/ + C → + AG/ + CA → + AGC/ + CA → + AGCC/ + CACG
ST13	PT → + 1/ + 1 → + 2/ + 2 → + 3/ + 3 → + 4/ + 4	PT → + A/ + C → + AG/ + CA → + AGC/ + CAC → + AGCC/ + CACG

[a] Primary templates. + 1/ + 1, + 2/ + 2, + 2/ + 3, + 3/ + 2, + 3/ + 3 and + 4/ + 4 indicate the selective nucleotides for each primer. Comparable procedures were followed for other primer combinations (+ AGCA/ + CACA, + AGCA/ + CACC, + AGCA/ + CACG, + AGCA/ + CACT, + AGCA/ + CCCA, + AGCA/ + CCCC, + AGCA/ + CCCG, + AGCA/ + CCCT, + AGCC/ + CACC, + AGCG/ + CACC and + AGCT/ + CACT)

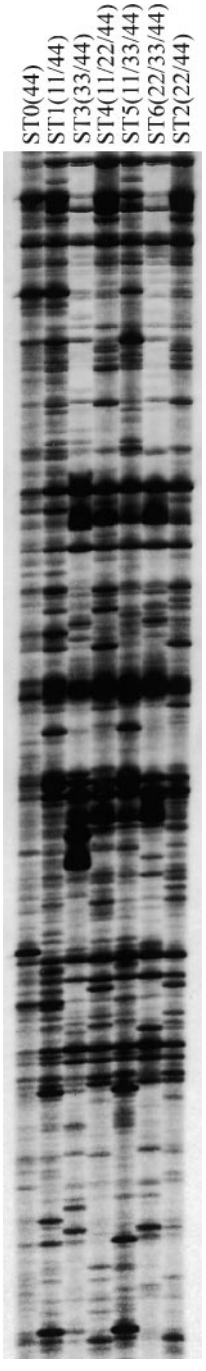

Fig. 2 The +8 fingerprint of F_2 (PD4⊗-9) with seven different preamplification steps (See Table 2 for details)

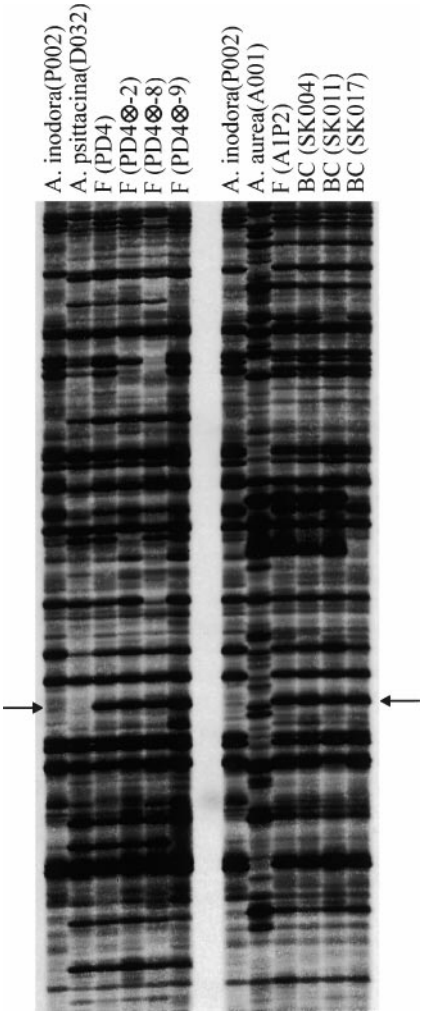

Fig. 3 An example of the AFLP segregation pattern of the two populations (F_2 and BC_1) by +AGCA/+CACC primer combination. The *arrows* indicate the only bands that are not explainable

and most of the bands from progenies were traceable through their parents when performed with the +2/+2 preamplification step (ST2) (Fig. 3). In the F_2 population from the *A. inodora* × *A. psittacina* cross, bands were evenly derived from each parent and the bands of backcross progenies were mostly from the recurrent parent (P002). Only 1 band from the progenies was not present in the parents' fingerprints suggesting possible mismatches (Fig 3. arrow indicated). Further linkage analysis will allow the verification of true AFLP markers. Furthermore, the intensity difference in some bands indicated the possibility of zygosity determination as a codominant marker (Van Eck et al. 1995; Staub and Serquen 1996).

In conclusion, various primer combinations of +7 and +8 fingerprints should be tested before any investigation depending on the species and the purpose of the study. The ST2 preamplification step (PT → +2/+2 → +3/+4 or +4/+4) was suitable for genetic studies in *Alstroemeria* species because of its robustness and efficiency. Consequently, AFLP markers can be applied for species with large genomes as long as the preamplification step and the final selective nucleotides are well defined by the users.

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