High-throughput AFLP analysis using infrared dye-labeled primers and an automated DNA sequencer

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Abstract:

Amplified fragment length polymorphism (AFLP) analysis is currently the most powerful and efficient technique for the generation of large numbers of anonymous DNA markers in plant and animal genomes. We have developed a protocol for high-throughput AFLP analysis that allows up to 70 000 polymorphic marker genotype determinations per week on a single automated DNA sequencer. This throughput is based on multiplexed PCR amplification of AFLP fragments using two different infrared dyelabeled primer combinations. The multiplexed AFLPs are resolved on a two-dye, model 4200 LI-COR® automated DNA sequencer, and the digital images are scored using semi-automated scoring software specifically designed for complex AFLP banding patterns (AFLP-Quantar™). Throughput is enhanced by using high-quality genomic DNA templates obtained by a 96-well DNA isolation procedure.

Keywords: Amplified fragment length polymorphism (AFLP) | polymorphic marker | genotyping | automated DNA sequencers

Article:

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Research Report

High-Throughput AFLP Analysis Using Infrared Dye-Labeled Primers and an Automated DNA Sequencer

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ABSTRACT

Amplified fragment length polymorphism (AFLP) analysis is currently the most powerful and efficient technique for the generation of large numbers of anonymous DNA markers in plant and animal genomes. We have developed a protocol for high-throughput AFLP analysis that allows up to 70 000 polymorphic marker genotype determinations per week on a single automated DNA sequencer. This throughput is based on multiplexed PCR amplification of AFLP fragments using two different infrared dye-labeled primer combinations. The multiplexed AFLPs are resolved on a two-dye, model 4200 LI-COR® automated DNA sequencer, and the digital images are scored using semi-automated scoring software specifically designed for complex AFLP banding patterns (AFLP-Quantar™). Throughput is enhanced by using high-quality genomic DNA templates obtained by a 96-well DNA isolation procedure.

INTRODUCTION

The amplified fragment length polymorphism (AFLP) marker system (28,30) has the highest multiplex ratio of all PCR-based marker systems. More than 100 restriction fragments can be amplified simultaneously (25). The high multiplex ratio of AFLP analysis makes it the most efficient method for generating large numbers of anonymous DNA markers for use in genetic linkage and diversity studies. The efficiency and relatively low cost of AFLP analysis have enabled de novo genetic map construction in many species (5,9,19,20, 24,27), saturation of existing linkage maps (6,10,18,23,29), and high-resolution mapping of genomic regions of interest (3,4,8,11,22,26). In addition, the marker system has provided a fast, low-cost approach for studying genetic diversity (7,12,21) and obtaining molecular phylogenies (1,14,15,17).

Until recently, the potential throughput of AFLP analysis has been limited by the requirements of autoradiography and by inadequate gel analysis and scoring software. Fluorescent detection of AFLP fragments on gel scanners (12) alleviated the requirement for autoradiography but was still limited in the number of samples that could be resolved per gel run. Recently, it was demonstrated that automated DNA sequencers could be used to simultaneously collect images of multi-mixed or multiplexed AFLP fragments labeled with different fluorescent dyes. This approach made it possible to resolve more than one sample per lane and to increase the throughput of AFLP analysis several fold (2,13,16,25).

We have developed a modified AFLP protocol based on infrared detection of AFLP fragments on automated DNA sequencers as an alternative to autoradiography or fluorescent detection (24). Here, we describe several modifications to the original AFLP protocol (28) that collectively allow an average throughput of up to 14 000 polymorphic marker genotype determinations per gel.

MATERIALS AND METHODS

Plant Materials

Fresh leaf samples were collected from seedling trees of two interspecific backcross families of a hybrid of *Eucalyptus grandis* and *E. globulus*. All plant materials were collected and shipped to North Carolina State University by Shell Renewables, Paysandú, Uruguay.

96-Well DNA Isolation

Genomic DNA was extracted from leaves of *Eucalyptus* seedlings stored at 4°C for no longer than two weeks after collection. Approximately 50 mg leaf discs were homogenized to a fine paste in 2-mL screwcap tubes containing 600 µL buffer AP1 of the DNeasy® 96 Plant kit (Qiagen, Valencia, CA, USA) and lysing matrix 2 (1/4-inch sphere and 1/4-inch cylinder) of the FastDNA® Kit (QBiogene, Carlsbad, CA, USA). Twelve samples were homogenized at a

Table 1. Muliplexed AFLP Primer Combinations Tested in *Eucalyptus*

Multiplex group	IRDye label	*Eco*RI primer[a] selective nucleotides	*Mse*I primer[b] selective nucleotides	No. of fragments scored[c]	Total no. of fragments on gel image
A	700	ACA	CCA	49	74
A	800	ACG	CCA	33	52
B	700	ACA	CCA	47	90
B	800	ACC	CCA	30	61
C	700	ATT	CCA	-	-
C	800	ACC	CCA	31	53
D	700	ACT	CCA	34	81
D	800	ATC	CCA	44	107
E	700	ACT	CCA	36	78
E	800	ATG	CCA	52	100
F	700	ATT	CCA	39	109
F	800	ATC	CCA	51	107
G	700	ACA	CCC	42	69
G	800	AAC	CCC	32	55
H	700	AGC	CCG	24	68
H	800	AAA	CCG	36	70
I	700	ATT	CCT	30	91
I	800	AAC	CCT	34	68
J	700	ATT	CCT	53	96
J	800	AAG	CCT	50	104
K	700	ACA	CCT	25	61
K	800	AAG	CCT	38	89
L	700	ACA	CGG	15	31
L	800	AAG	CGG	20	46
			average =	37	76

[a]The *Eco*RI adapter primer sequence without selective nucleotides was 5′-GACTGCGTACCAATTC-3′
[b]The *Mse*I adapter primer sequence without selective nucleotides was 5′-GATGAGTCCTGAGTAA-3′
[c]Only polymorphic fragments that followed expected inheritance patterns and amplified reliably were scored (an average of 48.0% of the total number of fragments per gel image were scored).

time for 45 s in a FastPrep® FP120 Instrument (QBiogene) set at 4.5 m/s. The rest of the extraction procedure was performed in 96-well format using the DNeasy 96 Plant kit, as prescribed by the manufacturer. Genomic DNA was eluted into 1-mL collection microtube racks in 200 µL (2 × 100 µL) buffer AE (preheated to 70°C). This modified 96-well DNA extraction procedure allowed isolation of genomic DNA from 192 leaf samples in less than 6 h.

Multiplexed AFLP Procedure

Restriction, ligation, and preamplification reactions were performed essentially as described in the original AFLP protocol of Vos et al. (28), with modifications described elsewhere (20,24). Preamplification reactions were performed with standard *Eco*RI (E+A) and *Mse*I (M+C) adapter primers (28). The following thermocycling profile was used for selective preamplification: 28 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 60°C, and extension for 1 min at 72°C with a 1 s/cycle increase in the extension time. A final extension step of 2 min was performed at the end of the cycling program. The preamplification products were diluted 40-fold with low TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

The primer combinations used for final selective amplification were taken from a list of 24 primer combinations

previously selected by primer screening and used for linkage mapping in *Eucalyptus* (20). Twelve multiplex groups (two *Eco*RI primers and one *Mse*I primer each) were tested in two full-sib families of *Eucalyptus* trees (Table 1). Primer combinations that could not be multiplexed (unique *Eco*RI/*Mse*I combinations) were used in single-dye reactions and multi-loaded during gel electrophoresis (see below). Standard *Eco*RI and *Mse*I adapter primers with three selective nucleotides (i.e., E+ANN and M+CNN) were used for final selective amplification (Table 1). *Eco*RI primers were labeled with infrared dye IRDye™ 700 or IRDye™ 800 (LI-COR, Lincoln, NE, USA).

Multiplexed, selective amplifications were performed in 10-µL reaction volumes containing 3 µL diluted preamplification products, 2.5 ng IRDye 700-labeled *Eco*RI primer, 3.2 ng IRDye 800-labeled *Eco*RI primer, 30 ng *Mse*I primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM each dNTP, and 0.6 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA). Single-dye reactions were performed exactly as above but with only one IRDye-labeled *Eco*RI primer and 15 ng *Mse*I primer. PCR conditions were as follows: 13 cycles of 10 s at 94°C, 30 s at 65°C (reduced 0.7°C per cycle), and 1 min at 72°C, followed by 23 cycles of 10 s at 94°C, 30 s at 56°C, and 1 min (extended 1 s per cycle) at 72°C. All PCRs were performed in PTC-100™ thermal cyclers with heated lids (MJ Research, Waltham, MA, USA).

Electrophoresis and Detection of AFLP Fragments

AFLP fragments were resolved in 25-cm gels (0.25 mm spacer thickness) containing 8% Long Ranger™ polyacrylamide gel solution (BMA, Rockland, ME, USA), 7.0 M urea and 0.8× TBE (71.2 mM Tris, 71.2 mM boric acid, and 1.6 mM EDTA). Final AFLP reaction products were dried under vacuum and resuspended thoroughly with one volume of loading buffer (95% deionized formamide, 20 mM EDTA, pH 8.0, and 1 mg/mL bromophenol blue), denatured for 3 min at 90°C and then transferred to ice before loading. Disposable, 96-well KB^{Plus} paper combs (LI-COR) were used for gel loading. Sample loading volume was set to between 0.6 and 0.8 µL, depending on the band intensity produced by the specific primer combination. For single-dye reactions, IRDye 800-labeled AFLP products were loaded and run into the gel for 5 min before loading IRDye 700-labeled products. At least one lane per gel loading was loaded with 50–700 bp sizing standard (LI-COR) labeled with the corresponding IRDye.

Electrophoresis and detection were performed on a two-dye, model 4200 LI-COR® automated DNA sequencer. Run parameters were as follows: 1500 V, 35 mA, 42 W, 48°C, signal channel 3, and motor speed 3. A total of eight frames of image were collected per gel loading with the pixel depth set to 16-bit. Approximately 3.5 h after the first loading, electrophoresis and image collection were stopped, and a second set of samples was loaded using the same gel and 96-well comb used for the first loading.

Scoring of Digital AFLP Gel Images

Digital AFLP gel images were scored to obtain binary (band presence/absence) data using the AFLP-Quantar™ software program (version 1.05; KeyGene products B.V., Wageningen, The Netherlands). Lane definition and band sizing were performed as described in the user manual. The minimum band ratio for scoring a "+" (band present) was set to 0.22, and the minimum band ratio for scoring a "?" (unsure/missing data) was set to 0.18. All lane positions with intensity ratios of less than 0.18 of the maximum band intensity (set to 1.0) within the particular size class were automatically scored as "-" by the software. Semi-automated scoring was performed by manually clicking on polymorphic fragments present in either of the two parents (included in lanes 1 and 2 of each image). Subsequent images with AFLP lanes produced with the same primer combination were automatically scored using

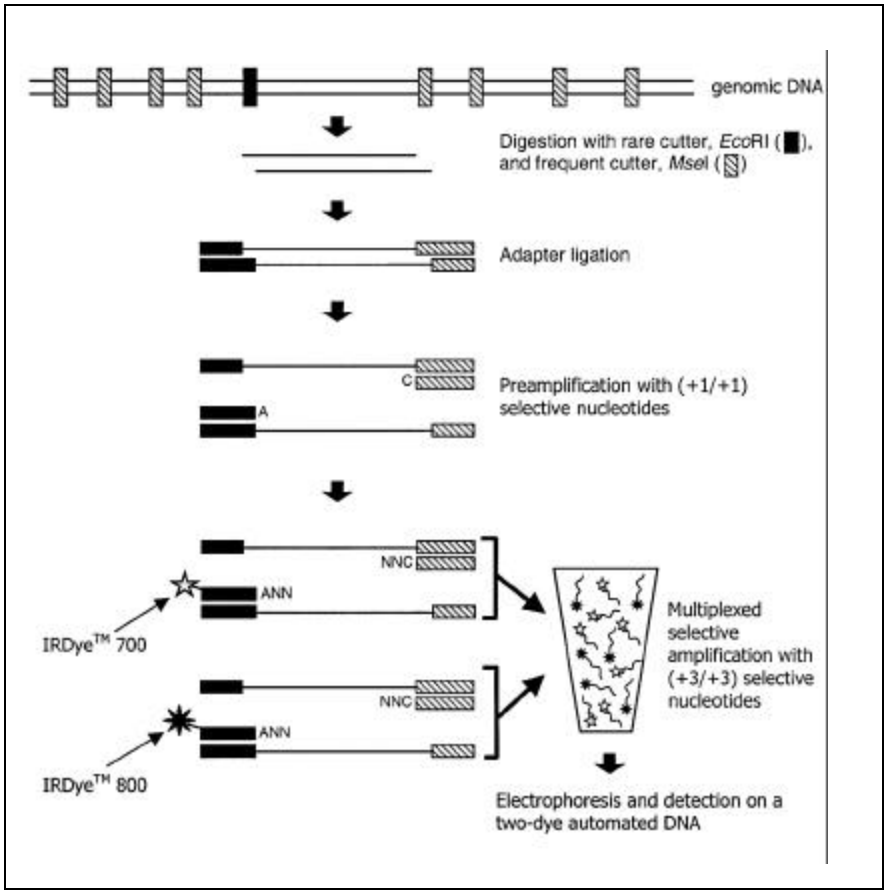

Figure 1. Outline of multiplexed AFLP analysis using infrared dye-labeled primers.

Table 2. Reproducibility of Semi-Automated Scoring Using AFLP-Quantar

Reproducibility	Total[a]	Percentage[b]
Number of identical marker genotype determinations (++ or --)	5579	97.28
Number of bands scored absent in first and present in second repeat	22	0.38
Number of bands scored present in first and absent in second repeat	44	0.77
Total number of non-reproducible data points	66	1.15
Number of marker determinations (scored either + or -) in both repeats	5645	98.43
Unsure/missing data	**Total**	**Percentage**
Number of marker determinations (scored either + or -) in both repeats	5645	98.43
Number scored "?" (unsure) in both repeats	6	0.10
Number scored present (+) in one and unsure (?) in other repeat	37	0.65
Number scored absent (-) in one and unsure (?) in other repeat	47	0.82
Total number of unsure (?) data points in either repeat	90	1.57
Total number of AFLP marker genotype determinations evaluated	5735	100

[a]A total of 185 AFLP lanes across two images were each scored twice for the same 31 polymorphic AFLP fragments using the same scoring procedure.

[b]All percentages are calculated as fractions of the total number of AFLP marker genotypes evaluated (5735).

the CopyScore command of AFLP-Quantar. All the software-assigned scores were reevaluated using the Zoom Window function of AFLP-Quantar. Band size, presence and absence data, and lane names were exported directly to a spreadsheet program and reformatted for use in mapping software. Two 96-well gel images were each scored twice by independently repeating the whole scoring procedure in AFLP-Quantar. These results (Table 2) were used to establish the reproducibility of semi-automated band scoring using AFLP-Quantar.

RESULTS AND DISCUSSION

There are three main requirements for a high-throughput AFLP genotyping system: (*i*) a high-throughput DNA extraction procedure that produces high-quality DNA suitable for the restriction and ligation steps of AFLP analysis, (*ii*) a robust, high-throughput amplification and fragment analysis system, and (*iii*) accurate, automated scoring software. The development of this high-throughput genotyping protocol relied heavily on meeting these requirements with the best combination of technologies available.

96-Well DNA Isolation

The high-throughput DNA isolation procedure yielded consistent amounts (between 5 and 10 µg) of high-quality DNA. The high quality of the DNA samples significantly reduced the observed failure rate (to less than 2%) during the restriction and ligation steps of AFLP analysis. This increased the overall throughput of the genotyping protocol by reducing the need to re-array or fill in failed samples. Most DNA samples were kept in the same 96-well order through DNA isolation, AFLP analysis, and gel electrophoresis. This made sample tracking and record keeping easier and less prone to error.

Multiplexed AFLPs Using Infrared Dye Detection on Automated Sequencers

The detection of AFLP fragments using infrared technology offers several advantages over conventional detection using autoradiography. In particular, the use of radioactivity is eliminated, and gel images are available for analysis immediately after gel electrophoresis. Our multiplexed, selective amplification procedure (Figure 1) was designed to complement the two-dye detection capability of LI-COR automated sequencers. The ability to collect gel images in two channels and to load gels twice allowed us to resolve and capture 384 lanes of AFLPs on a single 25-cm acrylamide gel (Figure 2).

One primer combination, E+ATT/M+CCA, did not produce scorable banding patterns when multiplexed with E+ACC/M+CCA (Table 1, multiplex group C) but did produce reliable results when multiplexed with E+ATC/M+CCA (multiplex group F). The apparent competition effect between E+ATT and E+ACC may be due to differences in the melting temperature (T_m) of the selective nucleotides, which offers a selective advantage to the E+ACC primer during the "touch down" phase of the PCR program. This suggests that multiplex pairs should be chosen on the basis of similar predicted T_m of the labeled primers.

Banding patterns resulting from multiplexed amplification were identical to those obtained with single-dye reactions, except for a very infrequent occurrence of additional fragments observed in multiplexed reactions (results not shown). These bands may result from *Eco*RI-800/*Eco*RI-700 amplification, although these events are expected to occur at a very low frequency.

To test the reproducibility of the AFLP banding patterns, two independent DNA samples were obtained from each of two *Eucalyptus* trees. These DNA samples were analyzed independently using the protocol described above. The final amplification products of the repeats were loaded side by side and resolved on a LI-COR automated sequencer. The resulting banding patterns (Figure 3) were analyzed to determine the proportion of reproducible AFLP bands. Of a total of 1465 AFLP bands evaluated, 1452 (99.11%) were identical between the two repeats of each genotype, while 13 (0.89%) were present in only one of the two repeats.

The primer combinations used to construct the multiplex groups given in Table 1 were restricted to a previously published set of primer combinations for *Eucalyptus* (20). A much larger number of multiplex groups is possible when considering all E+ANN/M+CNN combinations.

Semi-Automated Scoring of AFLP Images

Infrared detection of labeled AFLP fragments on LI-COR automated sequencers produced clear, discrete banding patterns (Figure 4) that were suitable for semi-automated scoring using dedicated scoring software. AFLP-Quantar is the first commercially available software package specifically developed for the analysis and scoring of complex AFLP banding patterns. In our experience, lane definition, band sizing, and semi-automated scoring of 96-well images (approximately 3500 marker genotype determinations each) could be completed within 20 min using AFLP-Quantar. However, an additional 20 min per gel image was required to reevaluate all the software-assigned scores.

The reproducibility of semi-automated scoring was high. More than 97% of marker genotype determinations were identical in two repeats (Table 2). The percentage of non-reproducible marker genotype determinations (1.15%) provides an upper boundary for the true error rate of scoring because some proportion of these scores is expected to be correct. The rest of the non-reproducible marker genotypes represent a low amount of missing (unsure) data (less than 1.0% within each repeat) and is not expected to significantly reduce the accuracy of the mapping data.

Large mapping or genotyping projects often require the analysis of AFLP banding patterns across several gel images. This process can be time consuming and prone to error because of gel-to-gel variation in band migration. The CopyScore command of AFLP-Quantar increased throughput and accuracy of scoring by allowing automatic scoring of the same set of markers on all subsequent images of the same primer-enzyme combination. This was achieved by visually aligning link lanes (identical samples) run on all the gels of the same primer-enzyme combination. This procedure reduced analysis time to less

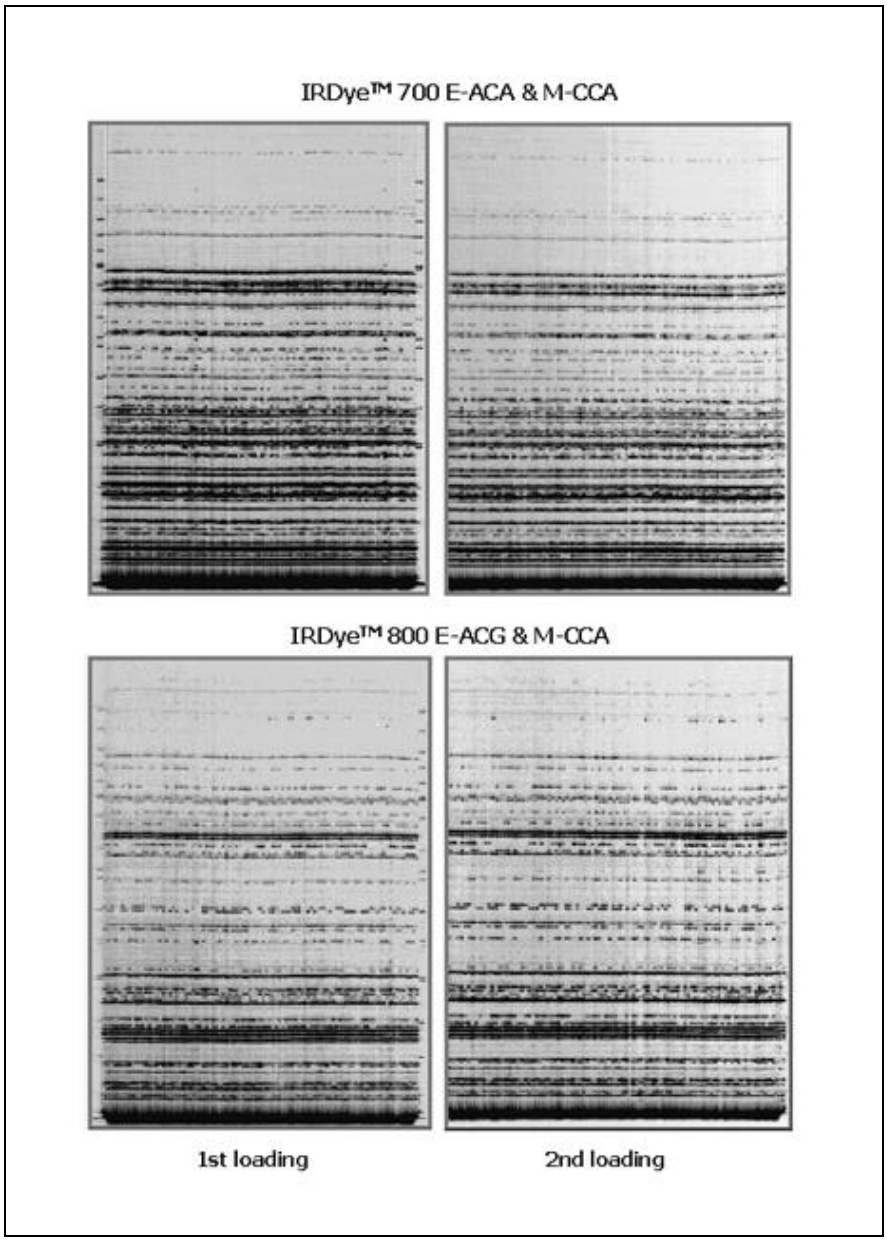

Figure 2. LI-COR TIFF images containing 384 lanes of AFLP fragments resolved on a single 25-cm acrylamide gel. The gel images were collected in the IRDye 700 (top) and IRDye 800 (bottom) channels of a two-dye, model 4200 LI-COR automated sequencer. The AFLP fragments were the products of multiplexed final amplification reactions containing *Eco*RI-adapter primers E+ACA (IRDye 700) and E+ACG (IRDye 800) and *Mse*I-adapter primer M+CCA. Gel images on the left were collected after the first loading. Gel images on the right were collected after the second loading.

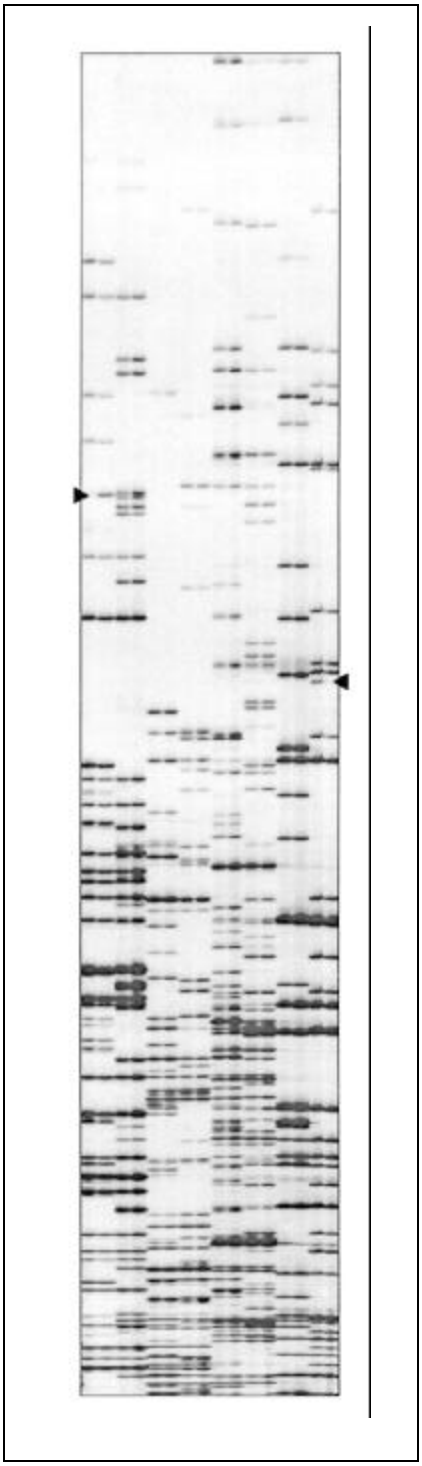

Figure 3. Reproducibility of AFLP profiles produced with infrared dye-labeled selective primers. Section of a gel image (IRDye 800 channel) containing AFLP profiles obtained with four primer combinations. Each pair of lanes contains two independent repeats of the same *Eucalyptus* genotype (samples were analyzed independently from DNA extraction through final selective amplification). Two genotypes were tested with each primer combination. Solid arrowheads indicate differences between the two repeats.

than 15 min per 96-well image (not including reevaluation and editing).

AFLP Marker Throughput Achieved

An average of 76 AFLP fragments were detected per lane, of which 37 (48%) were polymorphic (Table 1). Therefore, the average throughput achieved was 29 184 marker genotype determinations per gel (76 fragments per lane × 96 lanes per image × 4 images per gel), of which 14 208 were polymorphic. The average run time for two loadings on a single 25-cm gel was 7 h. Allowing for one gel (two loadings) per day, a total of approximately 146 000 marker genotypes (70 000 polymorphic marker genotypes) can be evaluated in a five-day week using this protocol. The same throughput can be maintained with single-dye reactions that are multiloaded, although this approach requires twice the number of PCRs.

We have therefore succeeded in doubling the multiplex ratio of AFLP analysis and quadrupling the throughput of the marker system, while significantly reducing the cost per marker genotype determination. This throughput, in terms of total number of fragments analyzed per gel, should be applicable to most other species. The total number of AFLP fragments per lane will depend on the genome size and on the number and composition of selective nucleotides used. The number of polymorphic fragments per lane, however, will depend on the genetic diversity among the parents of mapping populations or samples analyzed.

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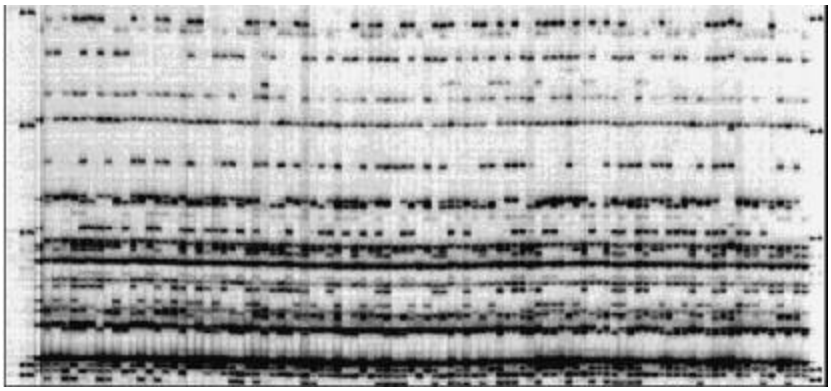

Figure 4. Section of a 96-well LI-COR TIFF image showing details of AFLP fragments in the range of 200–350 bp. The first two lanes contain AFLP fragments of the parents of a full-sib family of *Eucalyptus* trees. The other 94 lanes contain AFLP products of their full-sib progeny. The two outside lanes on each side contain IRDye 800-labeled molecular weight marker (LI-COR).

tional Needs Graduate Fellowship Program. Note that the AFLP® technique is covered by patents or patent applications owned by Keygene N.V. AFLP® is a registered trademark of Keygene N.V. Trademark registration for AFLP-Quantar™ has been filed.

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