APPLICATION INFORMATION

CEQ 2000XL

THE USE OF AFLP TECHNIQUES FOR DNA FINGERPRINTING IN PLANTS

James A. Saunders[1], Sue Mischke[1], and Alaa A. Hemeida[2]
[1]Alternate Crops and Systems Lab, Plant Sciences Institute, Beltsville Agricultural Research Center, USDA/ARS, Beltsville, MD
[2]Genetic Engineering & Biotechnology Research Institute (GEBRI), Sadat City, Minufiya University, Egypt

Introduction

In the last fifteen years, three devastating fungal diseases of *Theobroma cacao,* the chocolate tree, have enveloped virtually all areas of cocoa production in Central and South America. One of the common mechanisms for dealing with plant disease problems, particularly in areas with minimal resources for application of fungicides, has been the use of genetically resistant germplasm lines. Unfortunately, cocoa is a commodity that has an extended breeding of six years from seed to pod-bearing mature tree. Partially due to this long period, the tree has been maintained in a state of wild cultivation for almost 400 years without a great deal of crop improvement characteristic of other modern cultivated crops. As a result, there is very little information regarding the genetic diversity of the international cocoa germplasm collections.

DNA fingerprinting, a tool that has been widely used in forensic science, is also useful in a variety of applications with plants. It is used to identify genetic diversity within breeding populations, to positively identify and differentiate accessions, cultivars, and species that might be difficult to characterize due to similar morphological characteristics or indistinct traits, and to identify plants containing genes of interest such as the confirmation of transformation events. A number of molecular tools and procedures are being employed to establish DNA fingerprinting profiles and each of these procedures has its strengths and weaknesses. Amplified restriction Fragment Length Polymorphic (AFLP) DNA analysis, which is the focus of this report, is a useful procedure for DNA fingerprinting, especially when very little information is known about the genome of the plant under study.

AFLP DNA analysis of plants is a useful procedure for quickly assessing the genetic background of selected lines or populations. AFLP techniques produce a much higher percentage of polymorphic bands per analysis than the earlier procedures of RFLP (restriction fragment length polymorphisms) or RAPD analysis (random amplified polymorphisms). For example, in a comparative study of AFLP, RFLP, and RAPD analysis of inbred lines of soybean, Lin *et al.* (1996) reported that AFLP gave a number of discriminatory polymorphic bands or probes that was 8 to 10 times higher than the other two procedures (see Table 1).

AFLP Concept

AFLP DNA analysis is based on a multistep process (Figure 1) with the following steps: DNA digestion, fragment ligation to adapters, preselective PCR* amplification, selective PCR amplification, and fragment separation.

For reliability and consistency in AFLP analysis of DNA from plants, it is essential that each of the parameters of the AFLP process be understood and followed. Inconsistencies in results between laboratories are often due to small details involved in the incomplete digestion of the DNA, poor quality or quantity of the DNA, or the improper selection of the selective PCR primers. Optimization of these parameters is discussed below.

BECKMAN COULTER

Table 1. Comparisons of RFLP, RAPD and AFLP

Analysis Procedure	Number of Primers (Probes) Examined	Primers (Probes) with Polymorphisms	%	Number of Polymorphic Bands/ Primer (Probe)
RFLP	209	104	50	0.5
RAPD	245	85	35	0.7
AFLP	64	60	94	5.6

Adapted from Lin *et al.*, 1996.

Materials and Methods

DNA Isolation

DNA was isolated from fresh or frozen *T. cacao* leaf tissue (50 - 100 mg or less) by placing the plant material in a capped tube sandwiched between 500 mg of garnet and two ceramic beads (Lysing matrix, P/N 6540-401 from the FastDNA* kit, Qbiogene, Carlsbad, CA). Modified lysis buffer (400 µL) was added from either the DNeasy* Plant System (QIAGEN Inc., Valencia, CA), or from the D^2 BioTechnologies DNA X-Tract* Plus Isolation Kit (part number D2DNA02P). The plant tissue was completely macerated using a Fast Prep* 120 (Q-Biogene) shaker/basher at oscillation speed of 5.0 rpm for 40 seconds. Further DNA clean up followed manufacturer's instructions for the DNA isolation protocol used. The PicoGreen* (Molecular Probes, Inc., Eugene, OR) DNA quantitation kit was used to determine DNA content in the final preparation using fluorescence measurement from a Fluoroskan Ascent microplate reader equipped with 485/538 excitation/emission filter settings (Labsystems, Helsinki, Finland).

AFLP Protocol

The AFLP protocol initially described by Vos *et al.* (1995), was performed using components from various commercial AFLP kits (*e.g.,* AFLP preamp primer mix I and AFLP core reagent kit from AFLP Analysis System I for plant genomes, Life Technologies, Rockville, MD; or AFLP amplification core kit, AFLP I preselective primer mix for regular genomes and AFLP *Eco*RI + *Mse*I adaptors from Applied Biosystems, Foster City, CA). Alternatively, adaptors and pre-selective primers can be synthesized as described in the literature (Vos *et al.*, 1995; Lin *et al.*, 1996; Waugh *et al.*, 1997; Reineke and Karlovsky, 2000; and Saunders *et al.*, 2001). The selective primers with the WellRED™ fluorescent dyes for the final PCR amplification in this procedure can be ordered from Research Genetics, Inc. (Huntsville, AL).

Digestion of Genomic DNA and Ligation of Oligonucleotide Adapters

Digestion of genomic DNA by the restriction enzymes *Eco*RI and *Mse*I and ligation of oligonucleotide adapters compatible with these endonucleases as shown in Figure 1 were accomplished in a single reaction mixture of 11 µL. Final concentrations of reagents were: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25.5 µg/mL bovine serum albumin, 5 mM NaCl, 45 µL/mL *Mse*I (900 U/mL of New England Biochemical 525CL), 45µL/mL of *Eco*RI (4500 U/mL of NEB 101CL), 30 µL/mL T4 ligase (6.1 x 105 Cohesive End Units/mL, or 6700 U/reaction, of NEB 202CL). Each 11-µL reaction aliquot contained approximately 50-200 ng of template DNA (at a final concentration of 5-20 µg/mL) in addition to *Eco*RI and *Mse*I adaptor pairs, at concentrations recommended by the manufacturer. Prior to each use, the adaptor pairs were preheated to 95ºC for 5 minutes, then allowed to cool slowly, over a ten-minute period, to room temperature. The mixture was incubated overnight at room temperature so that template DNA was completely digested, then each reaction was diluted 1:18 (11 µL + 189 µL) with 15 mM Tris (pH 8.0), 0.1 mM EDTA. The minimum time needed for complete digestion can be determined empirically by running a time-course experiment and visualizing the fragments on an agarose electrophoretic gel.

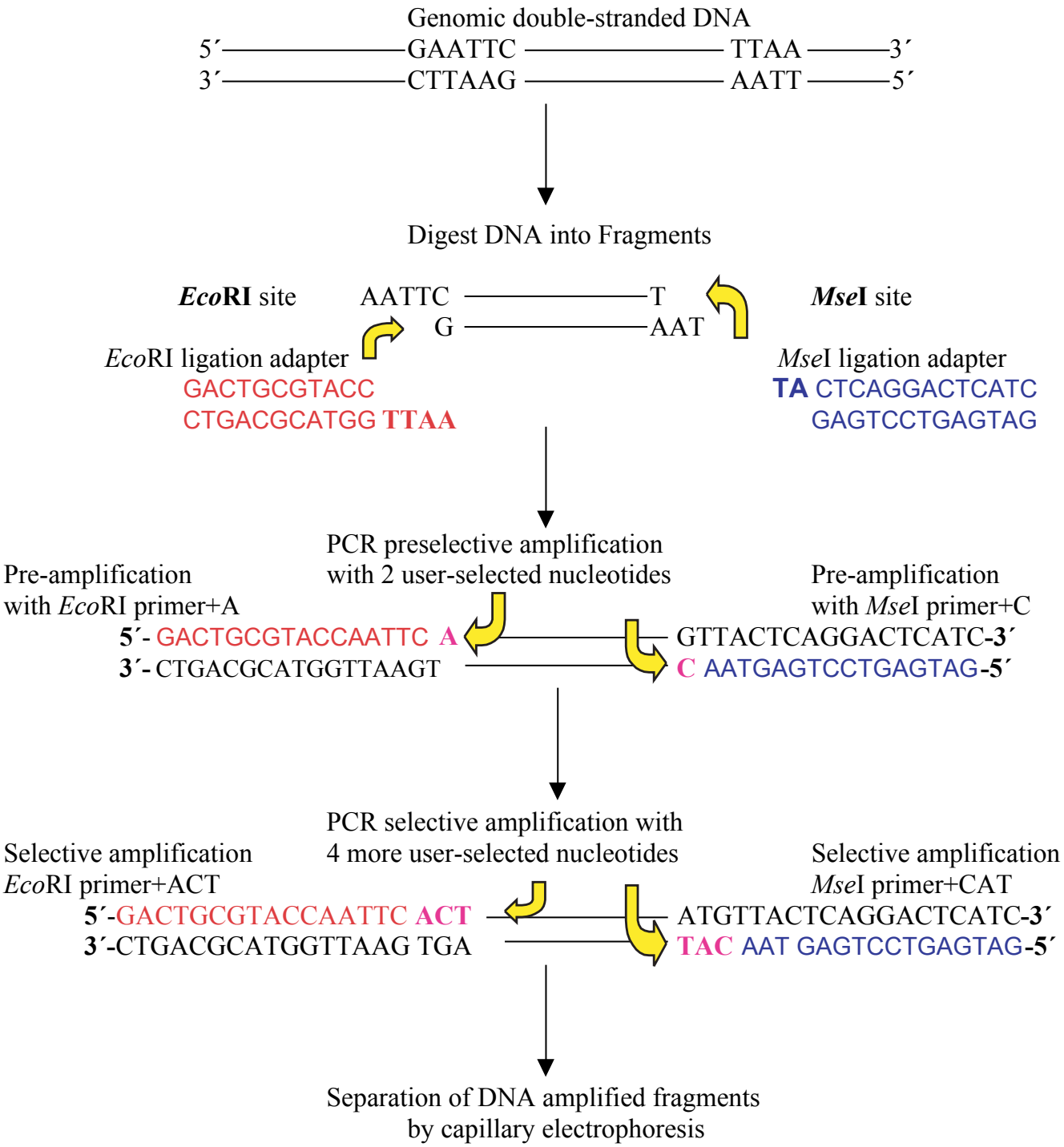

Figure 1. *The AFLP Concept.*

Table 2. Primer-Pair Combinations								
-ψ = Dye	*M-CAA*	*M-CAC*	*M-CAG*	*M-CAT*	*M-CTA*	*M-CTC*	*M-CTG*	*M-CTT*
E-AAC-ψ D2-labeled	Primer Pair #1	Primer Pair #2	Primer Pair #3	Primer Pair #4	Primer Pair #5	Primer Pair #6	Primer Pair #7	Primer Pair #8
E-AAG-ψ D3-labeled	Primer Pair #9	Primer Pair #10	Primer Pair #11	Primer Pair #12	Primer Pair #13	Primer Pair #14	Primer Pair #15	Primer Pair #16
E-ACA-ψ D4-labeled	Primer Pair #17	Primer Pair #18	Primer Pair #19	Primer Pair #20	Primer Pair #21	Primer Pair #22	Primer Pair #23	Primer Pair #24
E-ACC-ψ D2-labeled	Primer Pair #25	Primer Pair #26	Primer Pair #27	Primer Pair #28	Primer Pair #29	Primer Pair #30	Primer Pair #31	Primer Pair #32
E-ACG-ψ D3-labeled	Primer Pair #33	Primer Pair #34	Primer Pair #35	Primer Pair #36	Primer Pair #37	Primer Pair #38	Primer Pair #39	Primer Pair #40
E-ACT-ψ D4-labeled	Primer Pair #41	Primer Pair #42	Primer Pair #43	Primer Pair #44	Primer Pair #45	Primer Pair #46	Primer Pair #47	Primer Pair #48
E-AGC-ψ D2-labeled	Primer Pair #49	Primer Pair #50	Primer Pair #51	Primer Pair #52	Primer Pair #53	Primer Pair #54	Primer Pair #55	Primer Pair #56
E-AGG-ψ D3-labeled	Primer Pair #57	Primer Pair #58	Primer Pair #59	Primer Pair #60	Primer Pair #61	Primer Pair #62	Primer Pair #63	Primer Pair #64

Preselective PCR Amplification

Preselective PCR amplification as depicted in Figure 1, was performed using either of two commercial AFLP kits. For the Applied Biosystems AFLP kit, the 20 µL reaction contained 4 µL of the diluted restricted/ligated DNA and 16 µL of a mixture with 1 µL of *Eco*RI+A and *Mse*I+C AFLP preselective primers with 15 µL of AFLP core mix. The Life Technologies AFLP kit contained 50 µL of the following: 5 µL of diluted restricted-and-ligated DNA and 45 µL of a cocktail made with 40 µL preamp primer mix, 5 µL 10X PCR buffer for AFLP and 0.2 Units Taq polymerase. The PCR program for the preselective amplification was: 72ºC for 3 minutes, followed by 20 repetitive cycles of 94ºC for 20 seconds, 56ºC for 30 seconds, and 72ºC for 2 minutes, with a final hold at 60ºC for 30 minutes. All samples were stored at 4ºC following amplification on a GeneAmp® 9700 PCR system (Applied Biosystems). The amplified product was diluted 20-fold using 15 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA.

Selective PCR Amplification

For selective PCR amplification of restriction fragments, custom primers were prepared for recognition of *Eco*RI and *Mse*I adopters. The *Eco*RI selective amplification primer had a sequence of 5´-GACTGCGTACCAATTCA*NN. For recognition of the *Mse*I adaptor on the other side of the DNA fragment, primers were synthesized with a sequence of 5´-GATGAGTCCTGAGTAAC*NN (Figure 1). The A* and C* bases represent bases selected for primers in the initial preselective amplification and the N´s represent user-selected bases amplified in the second selective PCR amplification. We routinely use the subset of the 64 primer combinations provided in commercial AFLP kits made by either Life Technologies or Applied Biosystems as shown in Table 2. Fragments are visualized by attaching a D4, D3 or D2 WellRED™ dye to the 5´ end of each *Eco*RI selective amplification primer with no modification made to the *Mse*I primer. Note that not every primer pair in Table 2 was used since 3 to 7 pairs were usually sufficient to distinguish varieties of plants.

The second, selective, PCR amplification reaction requires a DNA template from the preselective PCR reaction, Taq polymerase, dNTPs, a dye-labeled primer as described above, and the standard buffers and salts optimized for the reaction. The PCR reaction mixture consists of 15 µL AFLP reaction buffer (Applied Biosystems AFLP kit), 1 µL *Eco*RI selective primer which is dye labeled and contains 3 user-selected nucleotides (see Table 2), 1 µL *Mse*I selective primer without label that contains 3 user-selected nucleotides (at 5 µM to give final 0.25 µM final concentration), and finally 3 µL of diluted amplified product from preselective amplification.

The PCR program for the selected amplification consisted of an initial warm-up at 94ºC for two minutes, one cycle of 94ºC for 20 seconds, 66ºC for 30 seconds, 72ºC for 2 minutes, followed by ten subsequent cycles, each with a 1ºC lowering of the annealing temperature, followed by 25 cycles of 94ºC for 20 seconds, 56ºC for 30 seconds and 72ºC for two minutes and finally a hold at 60ºC for 30 minutes before storing the samples at 4ºC.

When PCR primers for selective amplification anneal to the restriction fragment, the addition of the three specific nucleotides at the 3´ ends (which extend into the restriction fragment) results in recognition and amplification of only a subset of the DNA. If eight different primers of each type (*Eco*RI and *Mse*I) are synthesized, 64 combinations of primer pairs are possible. "E-NNN" is the primer for recognition of the *Eco*RI side of the fragment and "M-NNN is primer for the *Mse*I side. Only the E-NNN primers are labeled with a fluorescent dye; the M-NNN primers are not. Individual dye colors for each primer can be specified.

Preparation of DNA Amplification Fragments for Separation by Capillary Electrophoresis

To prepare DNA fragments for separation by capillary electrophoresis, sample loading solution was prepared with a 400-base-pair (bp) DNA size standard labeled with WellRED™ dye D1 (approximately 100:1; Beckman Coulter 608082 and 608098). This solution was thoroughly mixed by vortexing for a minimum of two minutes. A 30-µL aliquot of this cocktail was added to 1.5 µL of the selective amplification product. Each well was overlaid with a drop of Sigma mineral oil (M5904) and samples were analyzed in the CEQ™ 2000XL from Beckman Coulter.

Results and Discussion

DNA fingerprinting in plants can be applied to a number of applications and uses. The example shown here involves a study of genetic diversity in an important crop plant that has a special place in the hearts and stomachs of many of us.

The United States Department of Agriculture has begun a program of genetic characterization of cocoa via DNA fingerprinting procedures. Our laboratory has been involved in DNA fingerprinting of a number of crops using different molecular procedures (Saunders *et al.*, 1999; Degani *et al.*, 2001; Matthews *et al.*, 2001; Saunders *et al.*, 2001). We have evaluated the use of AFLP DNA analysis as a potential method for germplasm identification for this project.

DNA Isolation

To utilize AFLP DNA analysis to its full potential, it is critical to successfully optimize several steps in the process. One of the first and most important of these steps is the isolation of the DNA for the genetic analysis. AFLP techniques require 100-500 ng of relatively pure DNA. The quality of the DNA is important in view of the fact that the process is dependent upon the complete enzymatic digestion of the DNA via endonucleases. Because these fragments are subsequently used in PCR amplification, it is necessary to have complete digestion of the DNA to get reproducible patterns between replicate samples. We have found that many plants have high levels of phenolics which sometimes interfere with DNA isolation and, as luck would have it, cocoa is one of those problem crops. Although a number of DNA isolation procedures and kits have been used successfully in our laboratory, we have found that the convenience, reliability, and cost effectiveness of two commercially available DNA isolation kits to be particularly desirable. Each of those DNA isolation kits comes with detailed manufacturer's instructions that are cited in the materials and methods. Further, we have found that the use of mortars and pestles for DNA isolation raises the potential for cross contamination of samples during processing. As an alternative, we have used the Q-Biogene Model 120 FastPrep system (web site www.qbiogene.com) which we have renamed the "shaker/basher") for homogenizing plant leaf samples in self-contained microfuge tubes.

DNA Digestion

Incomplete digestion of the DNA, either because the DNA is under-digested or because the purity of the DNA prevents complete digestion, yields highly variable banding patterns of little use in DNA profiling studies. We recommend an overnight digestion at room temperature to ensure complete digestion of DNA. This process can be monitored easily by agarose gel electrophoresis.

Several commercial plant AFLP kits are available that yield very good results. Fortunately, most

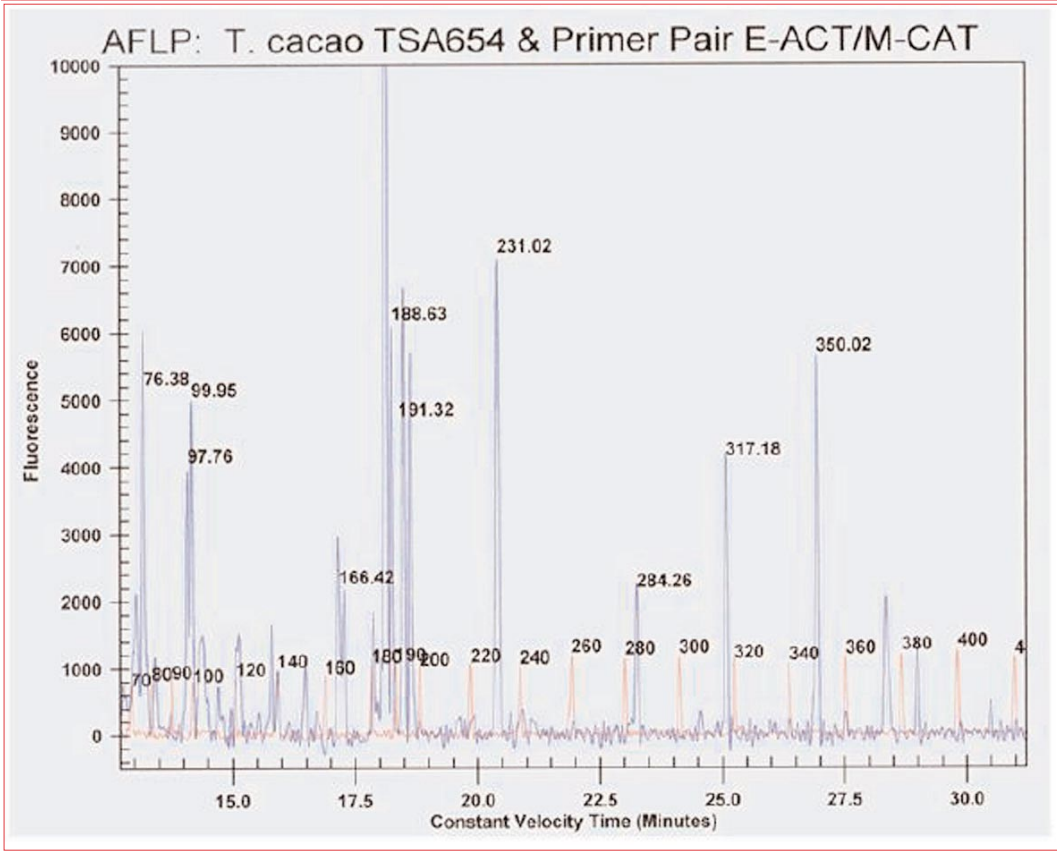

Figure 2. *AFLP DNA fingerprint of* T. cacao *TSA654 and primer pair E-ACT/M-CAT labeled with the D4-WellRED™ dye.*

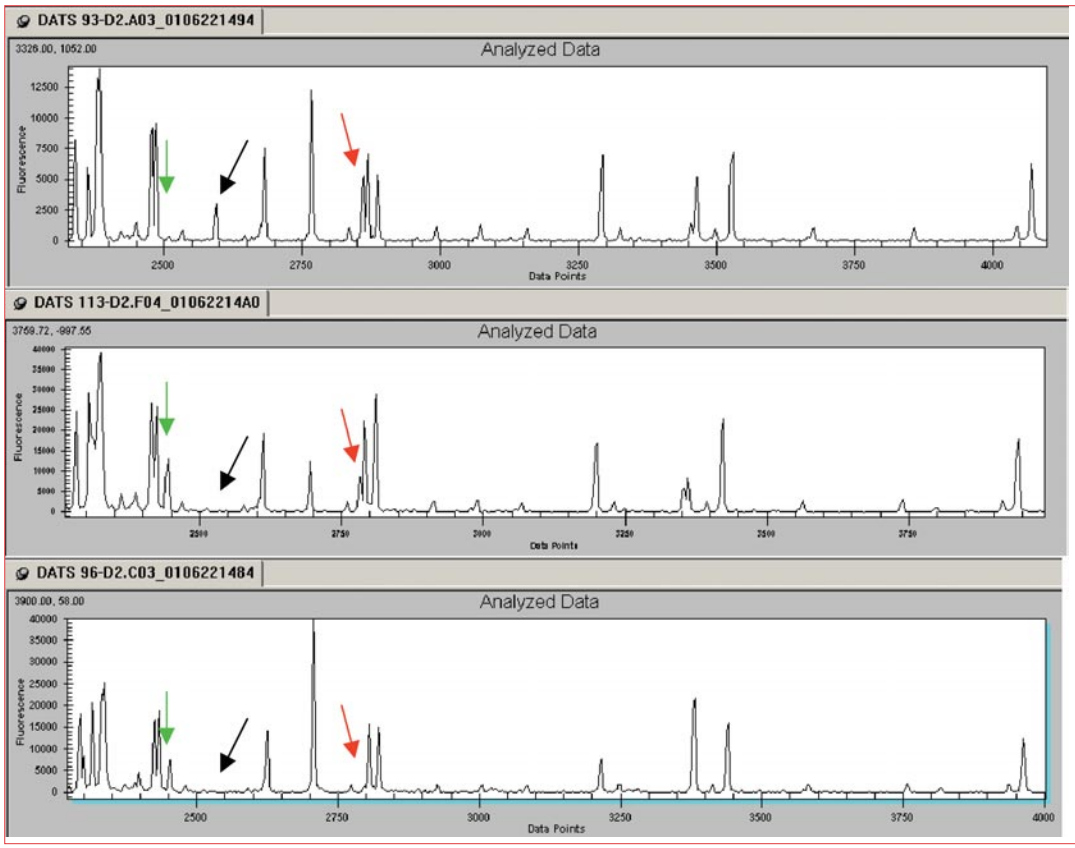

Figure 3. *Example of AFLP DNA fingerprints from some Cocoa samples using E-ACC primers labeled with D2-WellRED dye. Note: some peaks, as indicated by green, black, and red arrows, are present in some samples but not in others.*

manufacturers of these kits have followed a common protocol for the AFLP process which involves the digestion of genomic DNA by two commonly available endonucleases: *Eco*RI and *Mse*I restriction enzymes. *Mse*I is a 4-bp frequent cutting endonuclease which is typically mixed together with the less frequent 6-bp cutting endonuclease known as *Eco*RI (see Figure 1 for restriction cut site details). The use of these two restriction enzymes typically digests plant genomic DNA into fragments with a size range of 50-2000 bp.

DNA Ligation and PCR Amplification

The crux of the AFLP process is the selective amplification of portions of the genomic DNA using PCR primers ligated onto the endonuclease cut sites of the DNA. Since the sequence of the restriction sites are known, additional selectivity can be gained by adding specific nucleotides to the PCR primer sequence. The primers are designed to amplify only DNA fragments with an *Eco*RI restriction site on the 5´ end of the double-stranded DNA and an *Mse*I restriction site on the 3´ end. By convention, the addition of an adenine (A) to the PCR primer increases the selectivity of the *Eco*RI site and the addition of a cytosine (C) to the PCR primer increases the selectivity of the *Mse*I site during preselective PCR amplification. Once the preselective PCR amplification is accomplished, aliquots of the preselective amplification process can be used for a second more selective amplification using a variety of 64 different combinations of additional user selected PCR primers provided in commercially available kits as shown in Table 2. For example in Figure 1, the *Eco*RI primer is extended by two additional base pairs (CT) and the *Mse*I site is also is extended by two base pairs (AT). The strength of this technique lies in the fact that multiple primers can be run from the preselective amplification mixture to determine if they can distinguish between unknown samples. In our experience, we can typically detect differences in AFLP DNA fragments patterns when more than 3 to 7 primer pairs are used. Note that there are total of 256 potential PCR primer combinations that are possible by varying only the last two base pairs of each restriction site, but somebody has used the good sense to limit commercially available primers to 64 unique combinations (Table 2).

DNA Separation

Although the fragmented DNA from AFLP protocols covers a fairly broad range of sizes, a one-base-pair separation of DNA fragments is often necessary to distinguish between samples. We can achieve this degree of separation reliably on DNA fragments that fall within the general range of 60 to 350 base pairs. The PCR parameters are usually optimized so that larger fragments are not amplified. Capillary electrophoresis systems have the advantage of low operator input, automated sample injection and processing, and one-base-pair separation of DNA fragments within a moderate working range of 60 to 350 bp. The CEQ™ 2000XL DNA fragment analyzer from Beckman Coulter offers the convenience of the capillary electrophoresis systems along with reasonably high-throughput operations by processing eight samples at once.

These types of DNA analyzers separate DNA profiles extracted from each plant into specific fragments that can be evaluated on an electropherogram. The electropherogram (Fig. 2) shows standard DNA fragments (in red) that are injected into the same separation run as the samples (peaks in blue). The sizes of various sample DNA fragments are determined and the position of the individual peaks that are detected for each sample represent the DNA fingerprint that is unique to that type of plant. The DNA fingerprints (Fig. 3) not only identify one plant from another, they also help to establish the genetic relationship between plants so that individuals that are closely related are linked together and plants that are more distantly related are identified.

AFLP analysis of *Theobroma cacao* with a single PCR primer set based on the *Eco*RI and the *Mse*I cut site plus three selective nucleotides from each end of the DNA template. Separation of individual peaks is achieved at the one-base-pair resolution within the range of 50 to 350 bp. In AFLP analysis, each peak is either present or it is absent and the output text allows for automatic scoring of the AFLP fingerprints using a bioinformatics tool such as AFLP Dominant Scoring software available from Beckman Coulter's web site (http://www.beckmancoulter.com/Beckman/biorsrch/prodinfo/autodna/aflpsoftinst.asp). An example of a portion of data output from this software with "1" representing the presence and "0" the absence of a peak is illustrated in Table 3.

In view of this, minimal threshold levels for peak analysis should be set high enough to clearly distinguish the presence of a true peak from irregular background noise.

Table 3. An Example of a Portion of Data Output from AFLP Dominant Scoring Software

Marker Size	*A01*	*B01*	*C01*	*D01*	*E01*	*F01*	*G01*	*H01*	*A02*	*B02*	*C02*	*D02*	*E02*	*F02*	*F01*
59	0	0	0	0	1	0	1	1	1	1	1	0	0	1	1
62	1	1	1	0	1	1	1	0	1	1	0	1	0	1	1
84	1	1	1	0	1	0	1	0	1	1	0	1	1	1	1
87	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0
98	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
104	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0
118	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
134	0	0	0	0	1	0	1	0	1	1	0	0	0	1	1
136	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1
138	0	1	1	1	0	0	0	0	1	0	0	0	1	0	0
140	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1
148	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0
173	0	0	0	0	1	1	0	1	1	1	1	0	0	0	1
178	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0
188	0	1	1	1	0	0	0	0	1	0	1	0	0	0	0
211	0	0	1	0	0	1	0	1	1	0	1	0	1	1	1
215	1	0	1	0	1	1	1	1	0	0	0	1	1	1	1
218	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0
226	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
228	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0
258	0	1	1	1	0	0	0	0	1	0	1	0	0	0	0
285	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1
287	0	0	1	0	0	1	1	1	1	0	0	0	0	1	1
298	0	1	1	1	0	0	0	0	1	0	1	0	0	0	0
305	0	0	0	0	1	1	0	1	1	0	1	0	1	0	0
318	0	1	1	1	0	1	0	0	1	0	1	0	0	0	0
326	0	0	1	0	0	0	0	1	1	0	1	0	1	0	1
331	1	1	0	0	1	0	0	0	0	1	0	1	1	1	0
378	0	1	1	1	0	1	0	0	1	0	1	0	0	0	0

Conclusion

The use of AFLP DNA analysis as a molecular marker to determine the genetic diversity of a plant population is a reliable procedure when coupled with a separation system capable of resolving DNA fragments of 50 to 350 bp with one-base-pair resolution. High-throughput sample processing can be achieved with the multi-channel CEQ 2000XL capillary electrophoresis system by virtue of its ability to process eight samples at a time with a 30- to 40-minute cycle time and unattended sample loading between runs. AFLP DNA analysis can also be used in a variety of other DNA fingerprinting and genetic mapping procedures to provide markers for traits of interest in plants when very little preliminary knowledge of gene sequence is available. This type of information is especially important in the genetic analysis of tree crops due to the long lead times required to determine actual field performance of breeding results.

References

1. Lin, J. J., Kuo, J., Ma, J., Saunders, J. A., Beard, H. S., MacDonald, M. H., Kenworthy, W., Ude G. N., Matthews, B. F. Identification of molecular markers in soybean comparing RFLP, RAPD and AFLP DNA mapping techniques. *Plant Molecular Biology Reporter 14*(2)*:* 156-169 (1996)
2. Saunders, J. A., Pedroni, M. J., Daughtry, C. S. DNA Fingerprinting of Marijuana by the AFLP Technique. *Focus 20:* 10-11 (1999)
3. Degani, C., Rowland, L. J., Saunders, J. A., Hokanson, S. C., Ogden, E. L., Golan-Goldhirsh, A., Galletta, G. J., A comparison of genetic relationship measures in strawberry (*Fragaria ananassa* Duch.) based on AFLP's, RAPD's, and pedigree data. *Euphytica 117:* 1-12 (2001)
4. Saunders, J. A., Pedroni, M. J., Penrose, L., Fist, A.J. AFLP DNA analysis of opium Poppy. *Crop Science 41:* number 5, (Sept. 2001)
5. Matthews, B. F., Devine, T. E., Weisemann, J. M., Beard, H. S., Lewers, K. S., MacDonald, M. H., Park, Y. B., Maiti, R., Lin, J. J., Kuo, J., Pedroni, M. J., Cregan, P. B., and Saunders, J. A. Incorporation of sequenced cDNA and genomic markers into the Soybean genetic map. *Crop Science 41:* 516-521 (2001)
6. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kulper, M., Zabeau, M. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research 23:*(21), 4407-4414, (1995).
7. Waugh, R., Bonar, N., Baird, E., Thomas, B., Graner, A., Hayes, P., Powell, W. Homology of AFLP products in three mapping populations of barley. *Mol. Gen. Genet. 255:* 311-321 (1997)
8. Reineke, A., Karlovsky, P. Simplified AFLP protocol: Replacement of primer labeling by the incorporation of µ-labeled nucleotides during PCR. *Biotechniques 28:* 622-623 (2000)

I.S. EN ISO 9001

BECKMAN COULTER™

Developing innovative solutions in genetic analysis, drug discovery, and instrument systems.

Innovate SIMPLIFY Automate

Beckman Coulter, Inc. • 4300 N. Harbor Boulevard, Box 3100 • Fullerton, California 92834-3100
Sales: 1-800-742-2345 • Service: 1-800-551-1150 • Telex: 678413 • Fax: 1-800-643-4366 • www.beckmancoulter.com

Worldwide Bioresearch Division Offices:
Australia (61) 2 9844-6000 **Canada** (905) 819-1234 **China** (86) 10 6515 6028 **Eastern Europe, Middle East, Africa** (41) 22 994 07 07 **France** 01 49 90 90 00 **Germany** (89) 358700 **Hong Kong** (852) 2814 7431 / 2814 0481 **Italy** 02-953921 **Japan** 03-5404-8359 **Mexico** 525-559-16-35 **Netherlands** 0297-230630 **Singapore** (65) 339 3633 **South Africa** (27) 11-805-2014/5 **Spain** (34) 91 3836080 **Sweden** 08-564 85 900 **Switzerland** 0800 850 810 **Taiwan** (886) 2 2378 3456 **Turkey** 90 216 309 1900 **U.K.** 01494 441181 **U.S.A.** 1-800-742-2345

B2001-4594