APPLICATION INFORMATION

Genetic Analysis

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM PROTOCOL FOR PLANT SCIENCE ON CEQ™ SERIES GENETIC ANALYSIS SYSTEM

Eiji Hayashi[1], Han-Chang Chi[2], Scott K. Boyer[2], David W. Still[1]
1. California State Polytechnic University, Pomona
2. Beckman Coulter, Inc.

Introduction

Amplified fragment length polymorphism (AFLP) has become a popular research tool for such diverse purposes as population genetics, molecular evolution, and breeding. Since the first protocol published by Keygene[1] to generate AFLP markers, many researchers have modified the protocol to suit their own detection systems and biological materials. Numerous differences exist between automated capillary-based AFLP and slab gel-based technology. Slab gel technology utilizes radioactively-labeled primers in the selective amplification procedure and denaturing polyacrylamide gel electrophoresis for separation of fragments. Banding patterns from autoradiographs are then visualized on an image analyzer. On the other hand, the CEQ 8000 Genetic Analysis System employs dye-labeled primers during selective amplification, which allows the use of this capillary electrophoresis instrument to replace slab gel systems. In addition, the CEQ's automated software facilitates the detection and categorization of the fragments produced in the AFLP reactions. These technological advances increase the sensitivity and resolution of the AFLP procedure, which necessitates optimizing the AFLP protocol for capillary electrophoresis. Many AFLP protocols have been published that differ in reaction and thermal cycling conditions, making it difficult for a researcher to decide which protocol should be used for their system. We have modified the original Vos *et al*.[1] protocol for the CEQ in order to develop genetic fingerprints and study phylogenetic relationships among both *Echinacea*[2] and *Ganoderma* species, and genetic mapping of lettuce and *Arabidopsis* recombinant inbred lines. Note: It is becoming increasingly clear that as the genetic distance increases among taxa, many AFLP fragments are not orthologous and therefore will not be suitable for determining phylogenetic relationships. However, AFLP markers excel in developing genetic fingerprints among closely related taxa and individuals. Because of the high number of individuals that must be genotyped in mapping projects, and the absolute necessity of accurately and reproducibly generating and detecting fragments, we revised the previously published *Echinacea* AFLP protocol of Kim *et al*., 2004.[2]

Several factors affect the reproducibility of AFLP. First, it is essential to begin with pure, intact, high-molecular-weight DNA. In plant (especially non-domesticated species) and fungi species, this often is a problem because of interfering phytochemicals and high-molecular-weight polysaccharides. Second, complete digestions must be assured, which may preclude simultaneous restriction and digestion reactions. Assuming the first two considerations are properly performed, the concentration of PCR components such as dNTPs, $MgCl_2$, primers, and DNA polymerase can have marked effects on the quality of the results. Finally, PCR cycling temperatures and ramping times can potentially affect results. In this publication, we show that by systematically examining each PCR component in selective amplification, a protocol can be easily optimized to improve the reproducibility of AFLP in terms of consistency of signal intensity, number of fragments generated, and improved resolution of the baseline.
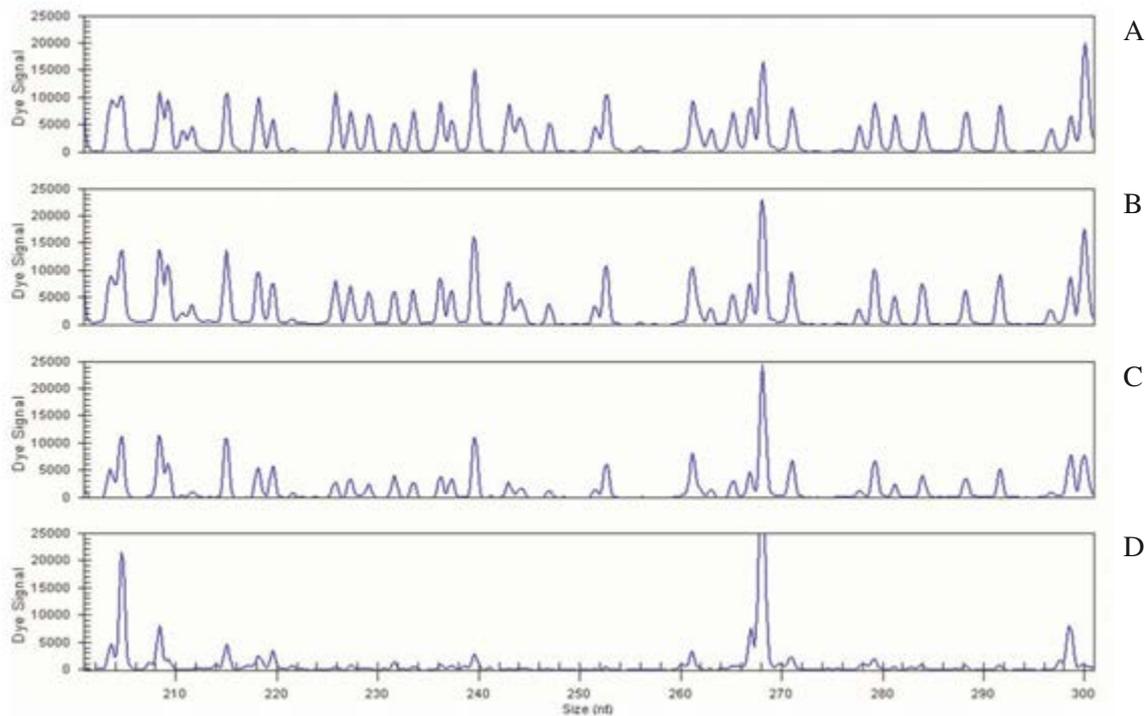
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Capillary Electrophoresis

Figure 1. The effect of different concentrations of dNTP (A) 0.2 mM, (B) 0.3 mM, (C) 0.4 mM, (D) 0.5 mM used in selective PCR reactions. Note that with increasing concentrations of dNTP, fewer fragments were generated and signal intensity tended to decrease. The data represented in each panel is from the same source plant and the same selective primers were used.

Optimization of Selective Amplification on Lettuce

Effect of dNTP and MgCl$_2$ Concentrations

We first examined the effects of different concentrations of dNTP on the number of fragments generated and the intensity of the signal. The concentration of dNTPs was varied while MgCl$_2$ (2.0 mM) and selective primers (0.375 and 0.625 μM for *Eco*RI and *Mse*I primers, respectively) were held constant (Figure 1). In general, with an increasing concentration of dNTP, the signal intensity of most peaks gradually decreased (Figure 1A-D). At a dNTP concentration of 0.5 mM, the signal intensity of most peaks was too low to discriminate from background, although a few peaks were higher than those observed at 0.2 mM dNTP concentration (Figure 1 A *vs*. 1 D). An inverse relationship was observed between dNTP concentration and the number of fragments generated during selective amplification (Table 1). Higher dNTP concentrations (0.4 or 0.5 mM) produced far fewer fragments than lower concentrations. In addition, greater variability in the number of fragments produced was observed at higher dNTP concentrations as indicated by the larger coefficient of variation values (Table 1). No clear trend emerged in signal intensity with respect to dNTP concentration. These data indicate that as concentration increases, variability is greater and selective amplification efficiency decreases.

Table 1. Average Number of Fragments Generated and Average Signal Intensity at Various Concentrations of dNTPs During Selective Amplification.

dNTP (mM)	Fragments Detected	Average Signal Intensity (RFU)
0.1	121.7 (10.2)	8889.3 (5.0)
0.2	124.8 (3.1)	9147.8 (4.6)
0.3	107.0 (7.3)	8676.3 (10.8)
0.4	55.3 (24.3)	11688.7 (10.1)
0.5	23.1 (22.0)	6668.8 (14.3)

Data are averages of four individual recombinant inbred lettuce lines. Each line was replicated three times using the same capillary for each line. Coefficient of variation is given in parenthesis.

The concentration of $MgCl_2$ has the potential to greatly affect the efficiency of PCR reactions and it is often suggested as a starting point in optimizing PCR conditions. We compared the results of four different concentrations of $MgCl_2$ (1.0, 1.5, 2.0, 2.5 mM) used during selective amplification. No significant difference was observed among 1.5, 2.0, and 2.5 mM concentrations, but average intensity at 1.0 mM was clearly lower compared to all other conditions (data not shown).

In summary, based on the overall number of fragments, signal intensity, and reproducibility, we adopted the concentration of 0.2 mM dNTP and 2.0 mM $MgCl_2$ for selective amplification reactions. The overriding factor in choosing optimum concentrations is to generate as many clearly distinguishable fragments as possible to reduce automated scoring errors and the amount of time required to verify fragments by visual inspection of the electropherograms. In our experience, the majority of fragments generated are between 60 and 400 bp, and those over 400 bp are much less abundant. The high numbers of fragments that are generated within the 60 - 400 bp range are more than adequate for genetic fingerprinting and mapping studies.

Effect of Primer Concentration

Another factor that must be considered is the cost per sample. Dye-labeled primers are relatively expensive and add significantly to the cost of any project. Therefore, protocols should be optimized to use the lowest amount of dye-labeled primer while preserving fidelity and reproducibility of the fragments generated. To determine where this balance lay, we tested selective primers at various concentrations. Signal intensity increased in proportion to *Eco*RI and *Mse*I primers and we concluded that 0.625 µM of each primer was the optimum concentration (Figure 2). Varying the amount of *Mse*I primer appeared to have no effect on fragment numbers, size, or distribution (Figure 3). When the D4 (Beckman Coulter, Inc.) dye-labeled *Eco*RI primer was held at a constant concentration and the unlabeled *Mse*I primer was varied from 0.625 µM to 3.75 µM, virtually no differences were detected. The original protocol by Vos *et al*. suggests using a 6:1 ratio of *Mse*I (unlabeled): *Eco*RI (32P-labeled) primer for selective amplification in the autoradiography system. Our results, however, indicate equal concentrations of both unlabeled and D4 dye-labeled primers can be used with no sacrifice in quality and reproducibility in the CEQ™ 8000 System. The reaction volume and the units to describe the amount of
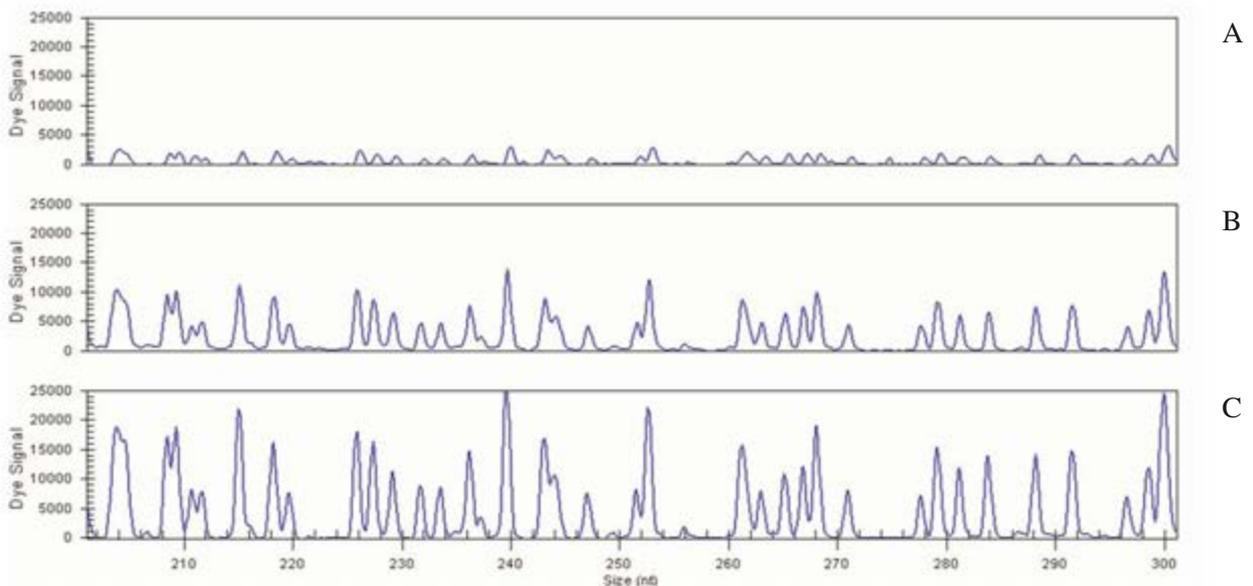

Figure 2. The effect of increasing concentrations of D4 dye-labeled EcoRI primer and unlabeled MseI primer during selective amplification. For each selective primer, the concentrations were (A) 0.125 µM, (B) 0.375 µM, (C) 0.625 µM. Lettuce was used as source DNA.

primers reported by Vos et al.[1] are different from those we used. Our protocol uses 8 μL as a reaction volume and units of μM for primer concentration while Vos et al. used 50 μL reaction volumes and ng units for the primers. We calculated the difference in concentration of primers between the two protocols. Our protocol uses 0.625 μM of D4 dye-labeled EcoRI primer, a concentration 14.5 times that of Vos et al.'s protocol which utilizes 32P-labeled EcoRI primer, while the concentration of unlabeled MseI primer in our protocol was approximately 2.4 times that reported by Vos et al. Although the concentrations are different between the two protocols, it is important to note that a single reaction contains approximately the same amount of primer, which we estimated as 4.76 pmol and 5.00 pmol, for the Vos et al. protocol and ours, respectively. Despite the lower signal intensity produced in our experiments by an EcoRI primer concentration of 0.375 μM relative to the higher level of 0.625 μM, the automated calling of fragments by the CEQ software was identical between the two (Fig. 2 B vs. Fig 2 C). Increasing the concentration of MseI while keeping the EcoRI primer at a constant concentration (0.625 μM) did not result in any further improvement in fragment resolution and the fragment composition was identical among all test conditions (Figure 3). From these results we concluded that a concentration of either 0.325 or 0.625 μM of D4 dye-labeled EcoRI primer, while 0.625 μM of unlabeled MseI primer is suitable during selective amplification. Optimization of different dye-labels may need to be performed.
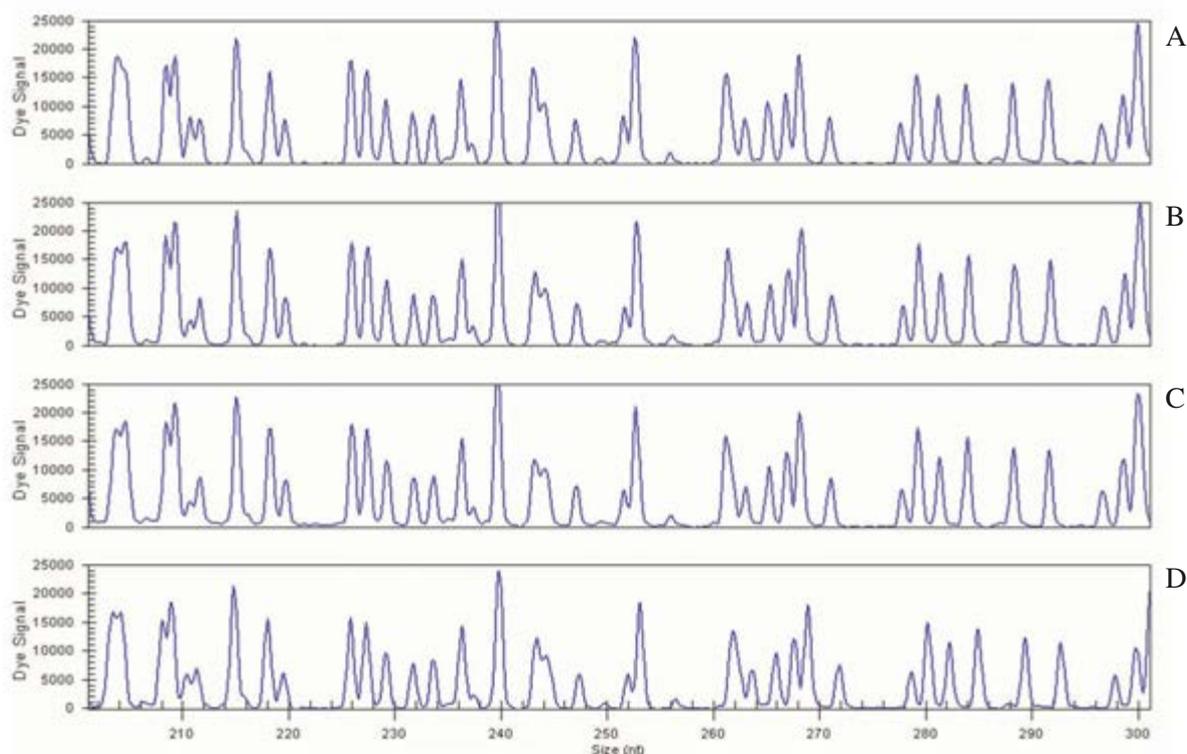

Figure 3. *The effect of varying concentrations of unlabeled MseI primer with a constant concentration of D4 dye-labeled EcoRI primer during selective amplification. The concentration of the MseI primer was (A) 0.625 μM, (B) 1.66 μM, (C) 2.71 μM, (D) 3.75 μM while the the EcoRI primer was 0.625 μM for the data presented in panels A-D. Source DNA is lettuce.*

Effect of DNA Polymerase

A number of DNA polymerases are available on the market, each of which may vary significantly in cost, and, potentially, in their suitability for AFLP. Accordingly, we screened several popular DNA polymerases (*Taq* and *Tfl*), two hot-start polymerases, and four regular (*i.e.*, not hot-start) polymerases to determine which gave the highest signal intensity and sharpest signals. Both hot-start polymerases use thermolabile neutralizing antibodies to *Taq* polymerase, in which activation of the polymerase occurs during the first denaturation step (Figure 4 A, Sigma Jumpstart;* 4 B, Invitrogen* Platinum*). Within the group of DNA polymerases tested in the experiments presented herein, the hot-start polymerases and one of the four regular DNA polymerases appeared to give nearly identical, and clearly superior results (Figure 4 A, B, C) while two regular polymerases (Figure 4 E, F) produced unacceptable results. The polymerase represented in Figure 4 D generated fragments that were not present in Figure 4 A, B, and C, and therefore would present scoring problems in mapping populations. In this experiment, each DNA polymerase used the same PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), but none of the polymerases required unique concentrations or buffer components to function properly. A separate experiment showed little improvement when
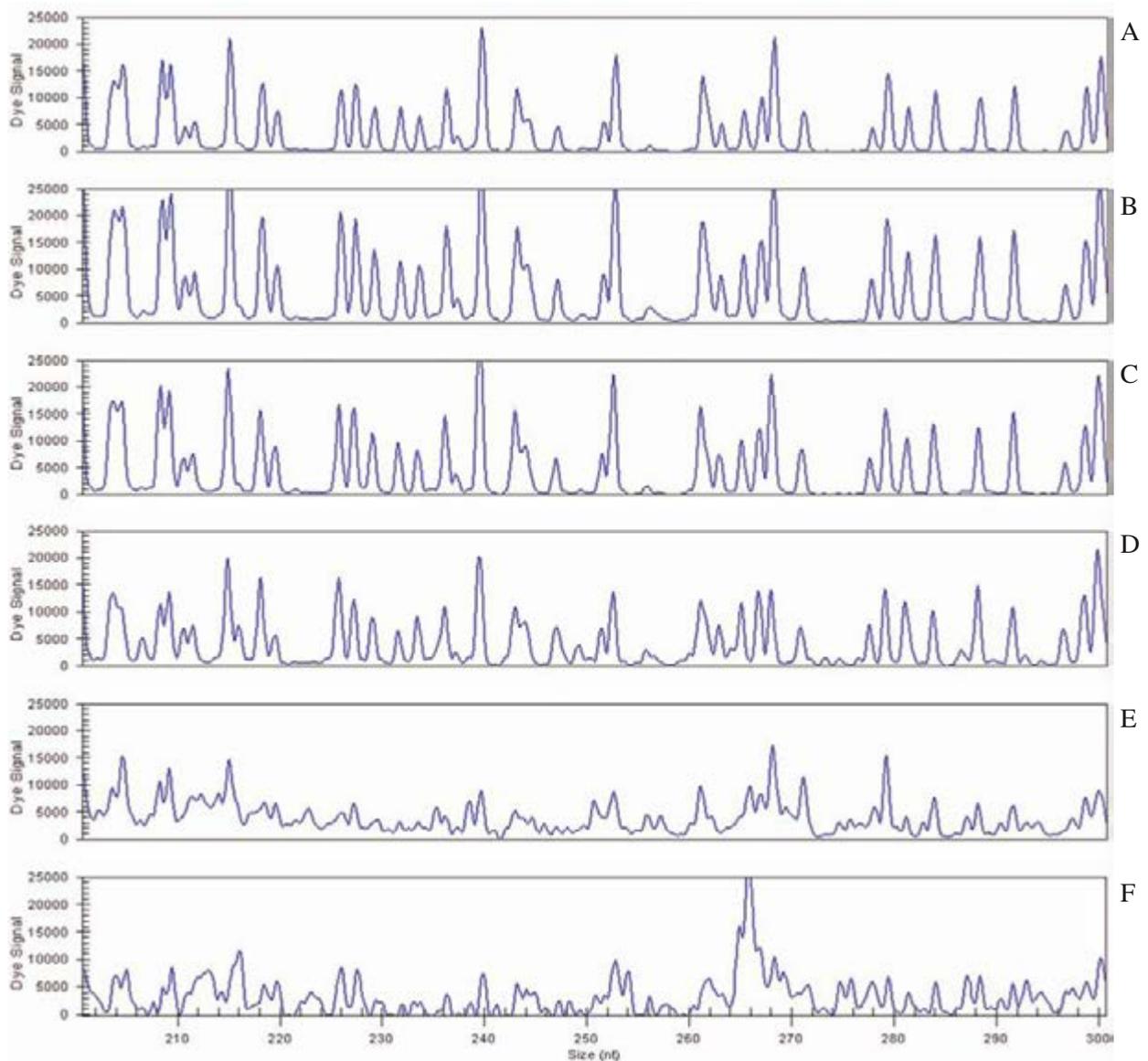

Figure 4. *Electropherogram of AFLP fragments generated by different types of DNA polymerase. (A) Sigma JumpStart Taq; (B) Invitrogen Platinum Taq; (C) Promega GoTaq*; (D) Promega Tfl; (E) Stratagene TaqPlus* Maxx; (F) Takara Ex Taq. For each selective amplification reaction, 0.025 U/μL DNA polymerase was used. Each reaction used the same PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl) and the source DNA was lettuce.*

using the manufacturer's buffers and recommended polymerase concentrations (Figure 5). Using the hot-start polymerase from Figure 4 A, we increased the concentration of this polymerase from 1x to 3x (Figure 6). No significant differences in the signal intensity or clarity of the base line were observed among the four different concentrations. Thus, we concluded: a) hot-start polymerases are suitable, and are at least comparable to regular polymerases during selective amplification; b) significant differences exist among polymerases presumably due to the inherent properties of the polymerase itself; c) a concentration of 0.025 U/μL of polymerase is sufficient to generate high-quality and reproducible fragments. Although higher concentrations (up to 0.075 U/μL tested) of polymerase do not impact the performance of AFLP analysis on the CEQ™ 8000 System, they are not needed and add to the cost-per-sample expense.
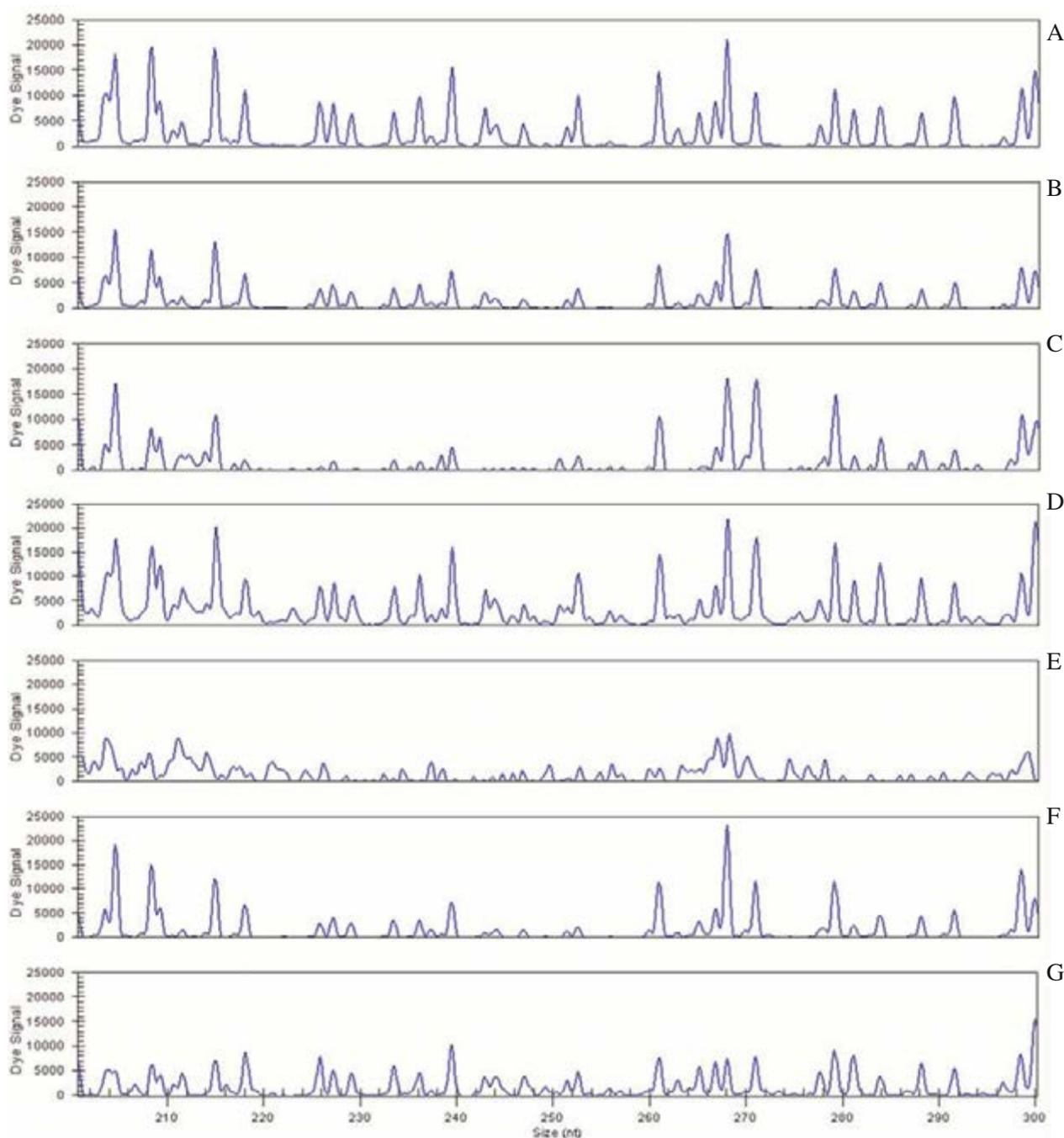

Figure 5. *Comparison of different DNA polymerases (0.025 U/μL) during selective amplification. (A) Sigma JumpStart Taq; (B) Invitrogen Platinum Taq; (C) Stratagene TaqPlus Maxx; (D) Takara Ex Taq; (E) Stratagene PfuUltra-High Fidelity ; (F) Promega GoTaq; (G) Promega Tfl. Each reaction used the manufacturer-supplied buffer and lettuce was used as the source DNA.*
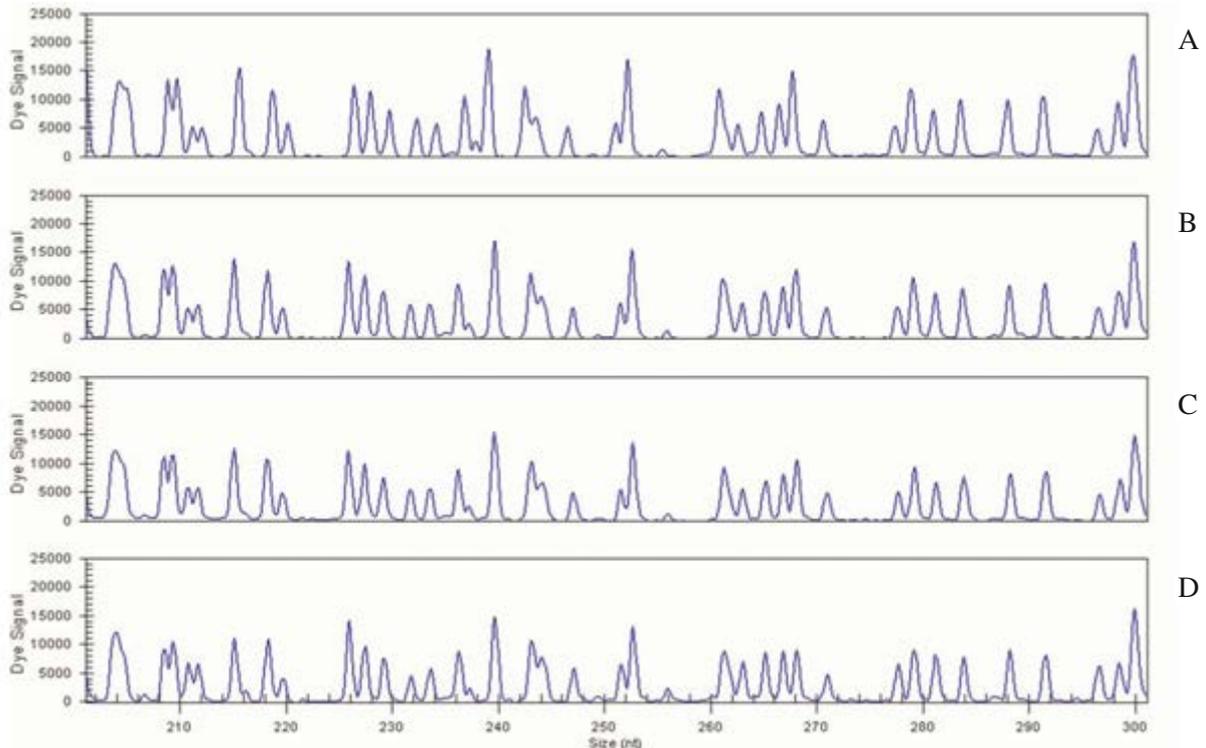
Figure 6. The effect of increasing concentrations of DNA polymerase (Sigma JumpStart Taq DNA polymerase). A), 0.025 U/µL; B) 0.0375 U/µL; C) 0.0625 U/µL; D) 0.075 U/µL. Lettuce was used as source DNA.

PCR Cycling Program

The potential exists for thermal cycling programs to affect the AFLP results. Two sources of variability exist, the first being a simple difference in protocol (*e.g.*, cycling temperatures or length of cycle) while a second source arises from differences in ramping times between instruments. In order to test these potential sources of variation, we compared a water-cooled thermal cycler against a peltier cooled thermal cycler using the cycling program as described by Vos *et al*.[1] To this, we compared a substantially different program, as described in our previous protocol.[2] Using our improved AFLP protocol, we generated fragments under the different combinations of preamplification and selective amplification cycling programs, using the two previously described machines. We did not observe significant differences between the two machines, indicating that differences in ramping times have negligible effects. We did, however, detect significant differences between the two thermal cycling protocols. Fragments generated from our modified thermal cycling protocol (described herein) had higher signal intensity relative to those produced by the original Vos *et al*.[1] protocol, especially in the 350-500 nucleotide range. This would allow the researcher to more easily and reproducibly identify polymorphic fragments in the higher fragment size range (data not shown). These results suggest that although the focus in improving a protocol should be directed towards examining component concentrations and assessing DNA polymerase performance, significant improvements can be realized by altering the thermal cycling protocol.

Transferability to Other Plant and Fungi Species

The great utility of the AFLP procedure lies in its application to any non-model species. We were interested in knowing if after optimization for lettuce, the protocol could be applied equally effectively and without further development to other plant species. We applied the protocol to the plant species *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Echinacea angustifolia* and a fungus, *Ganoderma applanatum* (Figure 7). In each case the results were reproducible when multiple samples (3 per species) were utilized. The number and nature of the selective nucleotides were optimized when applied to these species (as indicated in the Figure 7 caption), but no other changes were necessary. It is important to note that when working with a new species, a large number of selective primer combinations will need to be screened; it is not possible to determine *a priori*, which combinations will produce the largest number of fragments or polymorphisms in genetic marker studies.
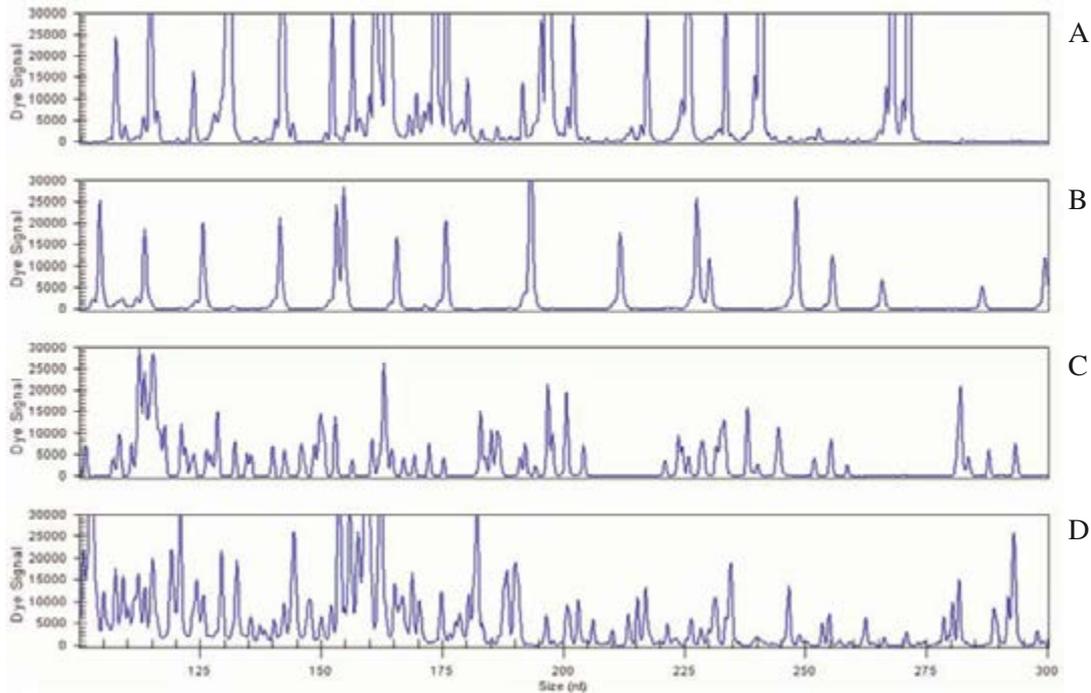
Figure 7. *Electropherogram of AFLP fragments from one fungus and three plant species using the improved protocol described herein. (A) Ganoderma applanatum Taq*I-AC*, Hin*dIII-TA*; (B) Arabidopsis thaliana (Ler), Eco*-AG*, Mse*-CTA*, (C) Lycopersicon esculentum, Eco*-AG*, Mse*-CTA*, (D) Echinacea angustifolia, Eco*-ACA*, Mse*-CAG.*

In summary, the most important aspect of any AFLP project lies in generating a high number of fragments in a reproducible manner. Improving the quality of the electropherogram, *i.e.* sharpness, signal intensity, and base line resolution, is an important process prior to the undertaking of a project, as the generated data will be more reliable. In mapping studies, this becomes particularly important, because markers that exhibit low intensity increase the likelihood of scoring errors. A miscoded fragment decreases the probability of finding segregating markers and can contribute to marker distortions in segregating populations.

We have shown results from our approach to optimize the quality of the AFLP fragments generated by examining a number of different reaction conditions in selective amplification, including different species of DNA polymerase, different concentrations of dNTP, $MgCl_2$, DNA polymerase and selective primers, as well as different thermal cycling programs. The protocol as described herein was optimized with respect to generating sharp and intense signals, with high reproducibility, between samples in our mapping project in lettuce. We have also applied the same protocol to other commonly used plant species with equal success.

Improved AFLP Protocol for the CEQ™

Described below is a modification of the protocol described by Vos *et al*.[1] 500 ng of DNA was double-digested by *Eco*RI and *Mse*I, and, simultaneously, adapters were attached to the end of the restricted fragments in a final volume of 11 μL restriction/ligation buffer: 1x T4 ligase buffer (New England Biolab, NEB), 0.05M NaCl, 0.045 mg/ml BSA, 1μM *Eco*RI adaptor, 5 μM *Mse*I adaptor, 5 U *Eco*RI (NEB), 5 U *Mse*I (NEB) and 1 U T4 DNA ligase (NEB). Preselective amplification was performed in a final volume of 13 μL with the buffer consisting of: 1x *Ex Taq*ˣ buffer with 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM *Eco*RI primer (+1), 0.3 μM *Mse*I primer (+1), 0.5 U *Ex Taq* DNA polymerase (Takara) and 3 μL of 10-fold diluted restriction-ligation product. Selective amplification was performed in a 8 μL reaction mix consisting of: 1x PCR buffer (Sigma) included with JumpStart polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.625 μM D4 dye-labeled *Eco*RI primer (+2 or +3), 0.625 μM *Mse*I primer (+3), 0.025 U/μL JumpStart *Taq* DNA polymerase (Sigma) and 2 μL of 50- fold diluted preselective amplification product. The thermal cycling program reported by Vos *et al*.[1] was modified as described in Part V of the AFLP method. Fragment analysis was performed on a Beckman Coulter, Inc. CEQ 8000 Genetic Analysis System.

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Table 2. Restriction and Ligation

Reagent	Stock Concentration	Final Concentration	µL/one Rxn
10 X T4 Ligase buffer (with ATP)	10X	1X	1.10
NaCl	0.5 M	0.05 M	1.10
BSA	1 mg/mL	0.045 mg/mL	0.50
*Eco*RI adapter	5 µM	1 µM	2.20
*Mse*I adapters	50 µM	5 µM	1.10
*Eco*RI	20 U/µL	5 U	0.25
*Mse*I	10 U/µL	5 U	0.50
T4 DNA Ligase	6 U/µL	1 U	0.17
Genomic DNA	500 ng/µL	500 ng	1.0
di-H_2O	--	--	3.08
Total Volume	--	--	11.0

AFLP Method

I. Restriction and Ligation (RL)

1. Heat *Eco*RI and *Mse*I adapters (Mix of Adapter 1 and 2) at 95°C for 5 minutes and then cool down to room temperature.
2. Make RL mix solution in order of above Table.
3. RL mix 6.92 µL + 500 ng/µL of Genomic DNA. Add dH_2O up to final volume of 11 µL.
4. Incubate the mix at 37°C for 3 hours in a water bath or thermal cycler.
5. Check success of RL products on agarose gel:
   a. Run 5 µL of RL product on a 1.2% agarose gel containing EtBr.
   b. Photograph gel using UV transillumination.
6. Dilute RL product 10x with TE 0.1 buffer.

II. Preselective Amplification (PS)

Following preselective amplification, dilute reaction products 10-50 fold with TE 0.1 buffer.

Table 3. Preselective Amplification

Reagent	Stock Concentration	Final Concentration	µL/one Rxn
Template DNA (10x-diluted RL product)			3.0
10 x PCR buffer (w/ 20 mM $MgCl_2$)	10X	1X	1.30
dNTPs each (Takara)	2.5 mM	0.2 mM each	1.04
*Eco*RI PS primer (*Eco*RI + 1)	10 µM	0.3 µM	0.39
*Mse*I PS primer (*Mse*I + 1)	10 µM	0.3 µM	0.39
Ex Taq DNA polymerase (Takara)	5 U/µL	0.5 U	0.10
di-H_2O	—	—	6.78
Total Volume			13.0

III. Selective Amplification (SA)

Table 4. Selective Amplification

Reagent	Stock Concentration	Final Concentration	µL/one Rxn
Template DNA (10-50x diluted PS product)			2.0
10 x PCR buffer (Sigma)	10X	1X	0.8
MgCl$_2$	25 mM	2.0 mM	0.64
dNTPs each (Takara)	2.5 mM	0.2 mM each	0.64
*Eco*RI labeled selective primer (*Eco*RI + 3)	10 µM	0.625 µM	0.5
*Mse*I selective primer (*Mse*I + 3)	10 µM	0.625 µM	0.5
di-H$_2$0	—	—	2.84
Sigma JumpStart *Taq* DNA polymerase	2.5 U/µL	0.025 U/µL (0.2 U)	0.08
Total Volume			8.0

IV. Sample Run Conditions

1. Sample preparation for the CEQ™ 8000.
   a. Add 1 µL of selective amplified reactions to sample plate containing 35 µL of sample loading solution (Beckman Coulter, Inc.) + 0.66 µL of DNA size standard (600) in each well.
2. CEQ run conditions:
   a. Denaturation at 90°C for 120 seconds, injection for 30 seconds at 1 kV, and separation at 5 kV for 55 minutes.

V. Thermal Cycler Programs

Table 5. Preselective Amplification Program

1.	72°C - 2 minutes	1 cycle
2.	94°C - 20 seconds	
	56°C - 30 seconds	20 cycles
	72°C - 2 minutes	
3.	72°C - 2 minutes	1 cycle
4.	60°C - 30 minutes	1 cycle

Table 6. Selective Amplification Program

1.	94°C - 2 minutes	1 cycle
2.	94°C - 20 seconds	
	66°C - 30 seconds — decrease 1.0°C/cycle	10 cycles
	72°C - 2 minutes	
3.	94°C - 30 seconds	
	56°C - 30 seconds	25 cycles
	72°C - 3 minutes	
4.	60°C - 30 minutes	1 cycle

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