Modification of the AFLP Protocol Applied to Honey Bee (*Apis mellifera* L.) DNA

BioTechniques 26:704-709 (April 1999)

ABSTRACT

*The established amplified fragment-length polymorphism (AFLP) protocol was simplified and optimized for honey bee DNA (*Apis mellifera *L.). Compared to the original method, the following simplifications were made: (i) the digestion of DNA and ligation of the adapters are performed in one reaction vs. two, (ii) one restriction enzyme is used vs. two and (iii) amplification is accomplished in one reaction vs. two. PCR products are resolved in agarose-Synergel instead of polyacrylamide and are visualized by ethidium bromide staining rather than by autoradiography of labeled primers. Using the modified procedure for honey bee DNA, high reproducibility of the band patterns of PCR products and low sensitivity to the amplification conditions were seen. Analysis of honey bee DNA revealed considerable genetic variability within and between African and European bee samples. African- and European-specific fragments were found.*

INTRODUCTION

Amplified fragment-length polymorphism (AFLP) (17) is a recently developed polymerase chain reaction (PCR)-based technique that provides genetic markers for fingerprinting, mapping and studying genetic relationships among organisms (2,7,8,12,16, 18). This technique is popular because it detects a high amount of polymorphism and is reproducible.

The AFLP protocol developed by Vos et al. (17,18) involves digesting genomic DNA with two restriction enzymes: a frequent and an infrequent cutter. Following digestion, specially designed adapters for each restriction enzyme are ligated to the ends of the DNA fragments. Primers are made complementary to the adapter sequences, plus the enzyme sequences, and up to three randomly selected nucleotides at the 3′ end. The primers are used to amplify the DNA fragments generated by the restriction enzymes in two reactions: primers with one extra base at the 3′ end of the enzyme sequences are used in a pre-amplification, and primers with three extra bases are used in a second amplification. One of the primers in the second reaction is radioactively labeled. The amplified products are resolved in polyacrylamide gels and exposed to film for autoradiography.

Simplification of the original AFLP protocol would facilitate the analysis of large numbers of samples as, for example, in population genetic studies. Alternative procedures have been proposed to eliminate the use of radioactivity and polyacrylamide gels; however, DNA transfer to blots followed by a colorimetric or chemiluminescent detection are still required (11,13). In this report, we present an AFLP protocol simplified and optimized for honey bee (*Apis mellifera* L.) DNA. This procedure consists of three steps: (*i*) Digestion of genomic DNA with a single restriction enzyme and ligation to one type of adapter are accomplished together in one reaction. (*ii*) The digested-ligated DNA is amplified in a single reaction. (*iii*) The AFLP products are resolved in agarose-Synergel and visualized by ethidium bromide staining.

Amplification conditions were optimized for concentrations of honey bee template DNA, magnesium ions, primer and *Taq* DNA polymerase. This procedure reduces the number of steps and expensive reagents required in the original AFLP protocol. The modified method detects a considerable amount of polymorphism, which is sufficient for many purposes; although, it is not as much polymorphism as detected by the original procedure. With this modified AFLP procedure, useful honey bee DNA markers were found.

MATERIALS AND METHODS

DNA Samples

DNA was isolated from drone honey bees as described in Hall (5). Drones are haploid and thus display simpler banding patterns of amplified products than do workers (diploid females). European drone samples came from three locations in the United States (Florida, Kansas and Arizona), and African samples came from three locations in South Africa (Louis Trichardt, Johannesburg and Pretoria).

Digestion/Ligation Reactions

Double-stranded *Eco*RI adapters (17) were prepared by first synthesizing oligonucleotides corresponding to each strand. The strands were mixed in equimolar amounts and annealed at 90°C for 1 min followed by a reduction of 1°C every 20 s until room temperature was reached. Digestion of the genomic DNA and ligation with the adapters was done in one reaction. Two micrograms of genomic DNA were added to 20 µL of 10 mM Tris-HAc, pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM dithiotreitol (DTT), 50 ng/µL bovine serum albumin (BSA) and 1 mM ATP as suggested by Vos et al. (17). Twenty units of *Eco*RI, 1 U of T4 DNA ligase and 50 pmol of the *Eco*RI adapter were added, and the mixture was incubated at 37°C for 5 h. To remove unincorporated adapters and residual enzymes, 80 µL of water, 100 µL of 7.5 M NH_4-Ac and 600 µL of 95% ethanol were added. The annealed adapter was precipitated, the pellet washed with 500 µL of 75% ethanol, air-dried for 10 min, resuspended in 100 µL of 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA (TE) and stored at -20°C. Enzymes were obtained from Boehringer Mannheim (Indianapolis, IN, USA). Primers were synthesized by Life Technologies (Gaithersburg, MD, USA).

DNA Amplification and Electrophoresis

As described in the Results section, amplification conditions were optimized for concentrations of four reagents: template honey bee DNA, magnesium ions, primer and *Taq* DNA polymerase. PCRs were performed in 50 µL total volume in thin-walled, 0.5-mL microcentrifuge tubes in a PTC-100™ Thermal Cycler (MJ Research, Watertown, MA, USA). The PCR

profile consisted of one step at 95°C for 1 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min with a final extension at 72°C for 5 min. After optimization of the PCR conditions, African and European honeybee drone DNA samples were amplified with three different primers (*Eco*RI + AGT, *Eco*RI + ATA and *Eco*RI + AGC), and PCR products were resolved in a 2.5% agarose-Synergel™ (0.7% agarose and 0.9% Synergel; Diversified Biotech, Boston, MA, USA) in 0.5× TPE buffer or 1× TBE buffer

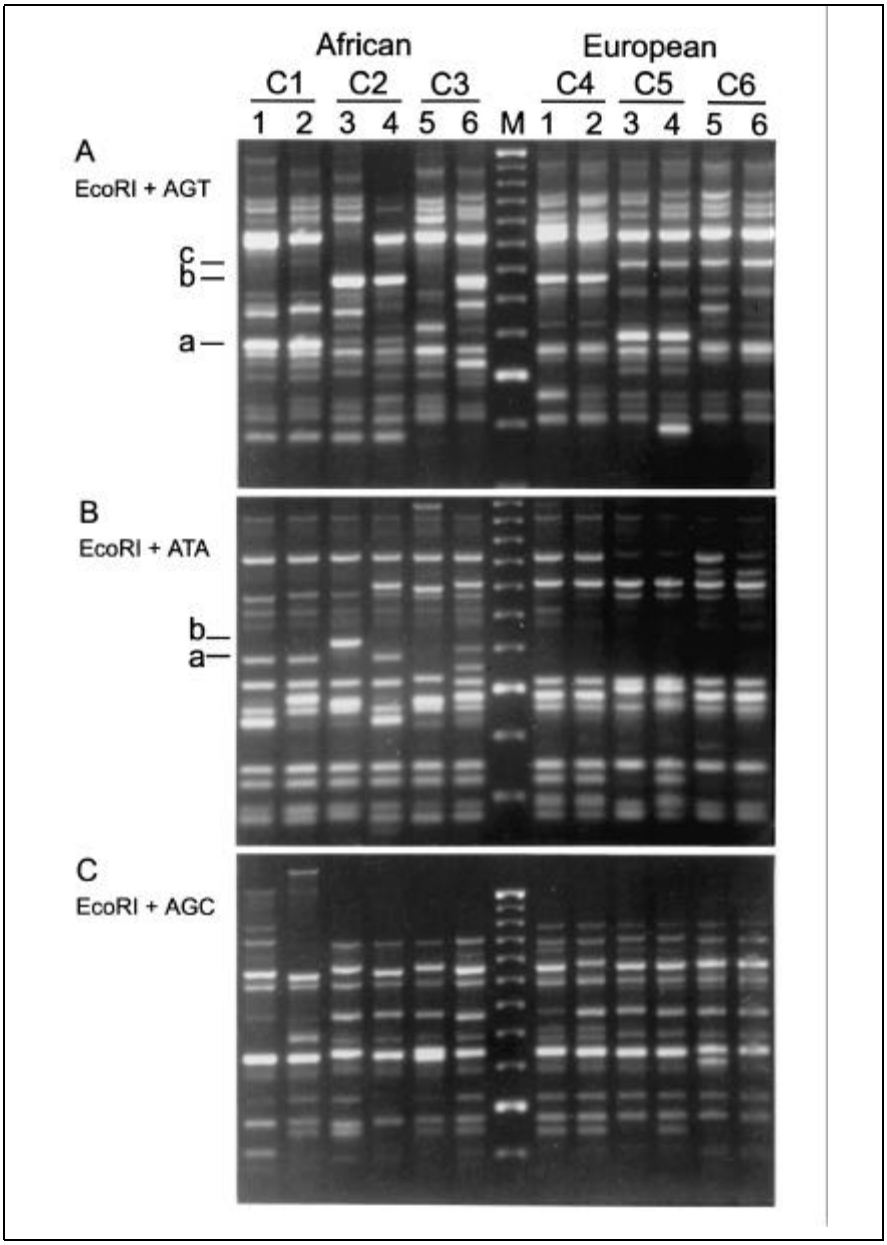

Figure 1. Amplification of African and European honey bee drone samples with primers *Eco*RI + AGT (A), EcoRI + ATA (B) and *Eco*RI + AGC (C). M = molecular size standard (100-bp ladder; Life Technologies). Two drones per colony (lanes 1–6) and three colonies of each bee type (C1–C3 for the African bees and C4–C6 for the European bees) were used. Note the few monomorphic bands and the substantial amount of polymorphism found in the amplification products for all the primers. Bands characteristic of some colonies were found, for example, band "a" for colony 1 and band "b" for colonies 2 and 4 using primer *Eco*RI + AGT. African- or European-specific bands were also found, for example, band "c" using primer EcoRI + AGT is European-specific, and bands "a" and "b" using primer *Eco*RI +ATA are African-specific.

[buffer formulas in Sambrook et al. (14)]. Gels were run for at 4 V/cm for 14 h, stained with ethidium bromide and viewed over UV light to visualize the PCR products.

RESULTS

Optimization of PCR Reagents

DNA concentration was tested from 1–100 ng in a 50-µL reaction volume (in triplicate). Above 10 ng, high reproducibility with no variability in the banding pattern was seen, but intensification of some bands was apparent as the DNA concentration was increased. Magnesium ion concentrations were tested from 0.5–3.5 mM (in duplicate). At 0.5 mM, no bands or a very small number of bands were seen. At 1.5 mM, the number of bands increased, and differences in band intensity were seen. Results were reproducible between 2.0 and 2.5 mM. As the concentration increased above that level, no new bands were formed, and bands began to disappear, until at 3.5 mM, no bands were seen. Primer concentrations from 100 to 500 nM were tested in 100-nM increments. Low-molecular-weight bands were not amplified at 100 nM. As primer concentration increased, more bands were visible, and low-molecular-weight bands increased in intensity. No differences in banding patterns were seen when primers at 400–500 nM were used. *Taq* DNA polymerase concentration was tested from 0.5–3.5 U in a 50-µL reaction volume. No amplification was seen at 0.5 U. Amplification results were consistent at enzyme concentrations between 1.0 and 3.0 U. At 3.5 U, a small amount of smear was formed. Nucleotide concentrations from 200–400 µM did not result in differences in the amplification when 2.5 mM magnesium ions were used. Thus, the optimized conditions for amplification using honey bee DNA were set at: 2.0 mM $MgCl_2$, 2.0 U *Taq* DNA polymerase, 50 ng DNA, 400 nM primer and 250 µM of each dNTPs in 10 mM Tris-HCl, pH 8.3, 50 mM KCl in a 50-µL reaction volume.

Positive and negative controls were used for all amplifications. The positive controls included undigested, unligated DNA with all the other reagents, which checked for any possible bands that could result from primer annealing to a genomic DNA region and not to the adapters. The negative control was a reaction with only the reagents without template DNA and was intended to detect possible artifacts generated from the primers. No visible bands were found in either positive or negative controls; therefore, all the bands were produced from the annealing of primers to the adapters. Artifactual bands were seen in the negative control when more than 35 cycles were used in the PCR profile but were not seen in any of the reactions that included template DNA.

AFLP Using Honey Bee DNA

Three primers were used to amplify DNA of samples from African and European honey bees. The DNA of two drones from each of six colonies, three African and three European, was amplified using the optimized conditions. Figure 1 shows the amplified products, separated by electrophoresis and stained with ethidium bromide. Substantial amounts of polymorphism were seen for all the primers used. A few monomorphic bands (one or two per primer) were produced. Each sample had a unique banding pattern, thereby showing the effectiveness of the method for individual identification (i.e., fingerprinting). For example, with one primer, individuals from different colonies could be distinguished by the presence of unique bands (Figure 1A). African- or European-specific bands were also found (Figure 1, A and B). Determining the frequencies of the bands in larger African and European honey bee populations is being investigated (unpublished).

DISCUSSION

Population genetic studies have been enhanced with genetic markers detected through methods based on the PCR, such as microsatellites (15), restriction fragment-length polymorphisms in PCR products (PCR-RFLP) (6,9,10), random-amplified polymorphic DNA (RAPD) (19) and, more recently, AFLP (17). AFLP uses arbitrary sequences to generate polymorphic amplified fragments from restriction-enzyme-digested DNA. Thus, this method combines the simplicity of RAPDs with the reliability of RFLPs. As with RAPD analysis, AFLP does not require prior knowledge of the DNA sequence. The longer primers used for AFLP require higher annealing temperatures, and binding is more specific and stable.

The simplified AFLP procedure described here generates a substantial amount of polymorphism, although not at levels as high as those generated with the original procedure. The amount of polymorphism can be increased by making primers less specific (using two extra bases instead of three) and by changing the enzyme (using a four- instead of six-base cutter). The digestion of DNA and ligation of adapters is completed in 5–8 h. After the digestion-ligation step, the same sample can be used with different primers, in which case, the remaining procedure can be completed in less than 18 h.

In optimizing the reaction, we found a low sensitivity to the concentrations of DNA and other PCR reagents. High DNA concentrations in AFLP do not affect the reproducibility of the reaction, but the reaction may need to be optimized for DNA from different organisms. In negative controls (all the reagents except DNA), artifactual bands were detected when more than 35 cycles were used. When genomic DNA was amplified, these bands did not appear even after 35 cycles. Possible problems with AFLP include the co-amplification of DNA from endosymbionts such as bacteria or other microorganisms associated with the organism of interest. Partial digestion of the DNA in the digestion-ligation reaction can lead to erroneous results and, therefore, an excess of restriction enzyme to digest the DNA to completion should be used. Also, co-migration of amplified products can make patterns difficult to interpret.

Markers found with AFLP are, in general, dominant. Co-dominant markers are more valuable for population genetic studies because homozygous and heterozygous individuals can be distinguished. Co-dominant markers can be found with AFLP if the polymorphic restriction sites fall within

fragments sufficiently short to be amplified. Thus, the fragments lacking the sites and the fragments resulting from cuts at the sites can be seen. By modifying this AFLP procedure to amplify longer DNA fragments (1,3,4), the possibility of finding co-dominant markers would increase.

The AFLP procedure typically displays multiple polymorphisms that can serve as genetic fingerprints of individuals. However, the system can also be used as a source of single genetic markers. DNA fragments containing polymorphic sites can be isolated from the agarose-Synergel, cloned, sequenced and used to design primers that amplify only one polymorphic region.

ACKNOWLEDGMENTS

This work was supported by a USDA-NRI Competitive Research Grant to H.G.H. This is Florida Agricultural Experiment Station Journal Series No. R-06566.

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Received 4 August 1998; accepted 2 December 1998.

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