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A new electrophoresis technique to separate microsatellite alleles*

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Analysis of large numbers of SSR (simple sequence repeats: microsatellites) reactions can be tedious, time-consuming and expensive. The objective of this study was to report a new electrophoresis method to analyze and visualize SSR data quickly and accurately and compare it to the ability of four other electrophoresis methods. Individual PCR reactions consisting of DNA from several *Cornus florida* L. (flowering dogwood) cultivars and two SSR primer pairs were assembled for analysis using the following three methods: agarose gel, polyacrylamide gel and QIAxcel System. Two separate PCR reactions consisting of the same components plus a fluorescent-labeled primer were set up for analyses using the CEQ[TM] 8000 Genetic Analysis System and ABI 3130xl DNA Sequencer. These five electrophoretic methods were assessed for advantages and disadvantages. Polyacrylamide gels had highest resolution of alleles, whereas agarose gels had the lowest. However, with both separation media, it was difficult to score the size of alleles. Capillary electrophoresis with the CEQ[TM] 8000 Genetic Analysis System and ABI 3130xl DNA Sequencer easily separated products and determined allelic size, but was more expensive than electrophoresis using either agarose or polyacrlamide gels. The QIAxcel System had lower resolution than CEQ[TM] 8000 Genetic Analysis System and ABI 3130xl DNA Sequencer. However, QIAxcel System was rapid and cost effective compared to the two widely used capillary sequencers, and also provided a computer generated gel image. For researchers in small to intermediate-sized laboratories, the QIAxcel System using a twelve channel, sieving-gel cartridge is an affordable device for SSR assays used for mapping and population diversity analysis.

Key words: SSR assay, capillary electrophoresis, polyacrylamide gel, *Cornus florida*.

INTRODUCTION

Traditional agarose and polyacrylamide gel electrophoresis were widely used in the earliest microsatellite (simple sequence repeats, SSRs) analyses. These media require simple devices and although the electrophoretic procedure can be done in most laboratories, it is labor-intensive (e.g., gel preparation, staining and photographing), especially for gene mapping and genomic linkage mapping, which requires larger population size and more molecular markers. The major limitations of these two protocols are they are not always amenable for accurately calculating the sizes of alleles and recording of data in an electronic format, making downstream analysis problematic.

Capillary electrophoresis technology is widely used in SSR and AFLP analyses (Guttman and Cook, 1991; Strege and Lagu, 1991; Huang et al., 1992; Mathies and Huang, 1992). The most widely utilized capillary electrophoresis devices are CEQ[TM] 8000 (CEQ 8000: Genetic Analysis System, Beckman Coulter, USA) and ABI 3100xl DNA Sequencer (ABI 3100xl: Applied Biosystems, USA). DNA analyses using capillary electrophoresis provides automated and accurate estimates of allele sizes. Capillary electrophoresis in combination with the use of fluorescently labeled primers provides high detection sensitivity of amplified DNA fragments (Ramachandran et al., 2003; Shi et al., 2003; Huang et al., 2006). However, the cost of the instrument, reagents,

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fluorescently labeled primers and sample preparation make the use of most capillary sequencers uneconomical for routine microsatellite analyses. In comparison, the QIAxcel System (Qiagen, USA) is a relatively inexpensive instrument that uses disposable micro-channel catridges containing sieving-gel matrix with ethidium bromide (EtBr) dye to generate both gel images and allele sizes (Amirkhanian and Liu, 2002; Liu et al., 1995; Lee, 2006). Most small- to mid- sized labs can afford this device for SSR assays. The QIAxcel does not require special primer labeling and provides comparable resolution as other capillary sequencers in one-tenth of running time (for 96 PCR samples).

Here, we report on a comparison of five methods for routine microsatellite array analyses using traditional agarose and acrylamide gels and the following three capillary electrophoresis systems: a compact, bench-top multi-capillary electrophoresis system, the QIAxcel System, the CEQ^{TM} 8000, and the ABI 3130xl. Microsatellites from flowering dogwood (*Cornus florida* L.) (Wang et al., 2007) were used to compare these five of electrophoresis systems.

MATERIALS AND METHODS

Materials and PCR

Genomic DNA was isolated from 12 *C. florida* cultivars using the Qiagen DNeasy Kit (QIAGEN, USA) and quantified with ND-Spectrophotometer (Nanodrop Technologies, Inc. Wilmington, DE, U.S.A). All genomic DNA isolations were diluted to 2 ng/μl with 0.1× TE buffer. Twenty μl PCR reactions consisted of 2 μl of genomic DNA (4 ng), 2 μl GeneAmp 10× PCR Buffer II (Applied Biosystems, USA), 2 μl 20 mM MgCl$_2$, 2 μl 2 mM dNTPs, 0.06 μl 5U AmpliTaq Gold® DNA polymerase (Applied Biosystems, USA), 2 μl of 2.5 μM SSR primer mix (both forward and reverse primers) and 9.94 μl of sterile water. Two duplicate 20 μl PCR reactions were prepared except that the fluorescent-labeled forward SSR primers were substituted for CEQ 8000 (WellRed D4 labeling primer) and ABI 3130 x l (FAM labeling primer). The PCR program for all reactions was 95°C for 3 min, followed by 35 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 40 s, and a final extension at 72°C for 1 min and was conducted in 0.2 ml volume 12-strip tubes using Mastercycler Eppendorf Gradient (Eppendorf, Germany).

Additionally, DNA with and without fluorescent labels from the PCR reactions were quantified with the ND-Spectrophotometer and serially diluted (1:2- to -1:2048) with 0.1× TE buffer. Dilutions were electrophoresised to determined detection limits of the QIAxcel, CEQ 8000 and ABI 3130xl electrophoretic systems. Detections limits were determined in ng DNA/lane for the three systems based on the dilutions of the DNA from the PCR reactions.

Sample separation

Ten μl PCR samples from the non-labeled primer reactions were loaded into each well of a horizontal 3% MetaPhor® agarose gel (Cambrex Bio Science, Rockland, Maine, USA) made with TBE (Tris-Boric acid- EDTA) buffer. Products were separated at 100 V for 2 h and visualized using EtBr. 3 μl of the same PCR products were also separated vertically on 7 M urea passive denaturing 10% polyacrylamide gels with 1× TBE buffer at 100 V for 60 min (Caetano-Anolles et al., 1991) and stained with silver (Bassam et al., 1991). A 25bp DNA ladder was used to estimate allele sizes in
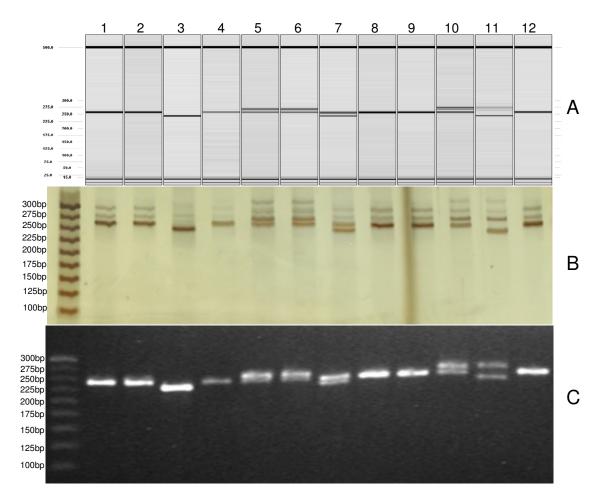
base pairs (bp) for both gels. The same PCR products in 0.2 ml, 12-tube strips, were transferred directly from the thermocycler into the sample tray of the QIAxcel System. Separation was performed using the OL700 method (sample injection voltage 8 KV, 20 s, separation voltage 3 KV and separation time 700 s) in a 12-channel gel cartridge (GCK5000) purchased from eGene Inc. (currently Qiagen, USA). The size of the alleles resolved from the subsequent separation were automati-cally calculated in bp and exported using the BioCalculator^{TM} software, which provides a gel view and an electro-pherogram of the separation, for comparison, the PCR products with WellRed-labeling primer and FAM-labeling primer were respectively run on the CEQ 8000 (capillary temperature 50°C, inject voltage 2.0 KV, 30 s, denature 90°C, 120 s, separate voltage 4.8 KV, 60 min) and ABI 3130xl (capillary temperature 60°C, prerun at 15.0 KV for 3 min, inject samples at 1.2 KV for 23 s, separation at 15.0 KV for 12.5 min).
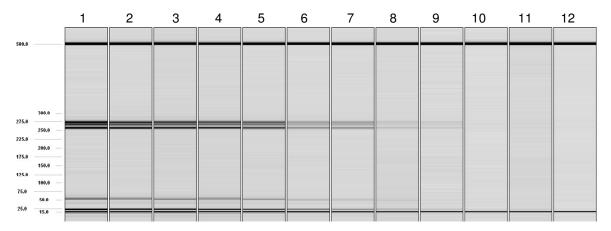
RESULTS AND DISCUSSION

A comparison of the actual gel images obtained from agarose and polyacrylamide electrophoresis and the digitally constructed image from the QIAxcel System of PCR amplified samples with one SSR primer pair from 12 flowering dogwood cultivars are shown in Figure 1. Alleles are clearly separated in the constructed gel from the QIAxcel System (Figure 1A) in contrast to those from the other two traditional gel views (Figures 1B and C). The agarose gel (Figure 1C) had lower resolution than the polyacrylamide gel (Figure 1B). However, due to the higher sensitivity of silver staining compared to EtBr fluorescence employed by QIAxcel System and the agarose method, more background noise and some non-specific bands in the polyacrylamide gels were detected. Bassam and Caetano-Anolles (1993) reported that 1 pg DNA /mm^2 of band could be detected on polyacrylamide gels (Bassam et al., 1993), whereas about 2 ng of DNA can be visualized on 3% high resolution Metaphore agarose gel. We determined from dilution experiments (data not shown) that about 0.6 ng of DNA can be reliably detected using the QIAxcel System (Figure 2) and both the CEQ 8000 and ABI 3130xl had DNA detection limits of less than 0.1 ng DNA.

Detection of minor and nonspecific products increases the difficulty of legitimate allele identification. Furthermore, it is difficult to accurately calculate allele sizes from both types of traditional gels because of differences in migration between lanes in the gels, especially the size markers in the outer lanes (Figure 1B and C). While agarose and polyacrylamide gels are easy to prepare, both methods present problems with reproducibility and accurately determining allele size.
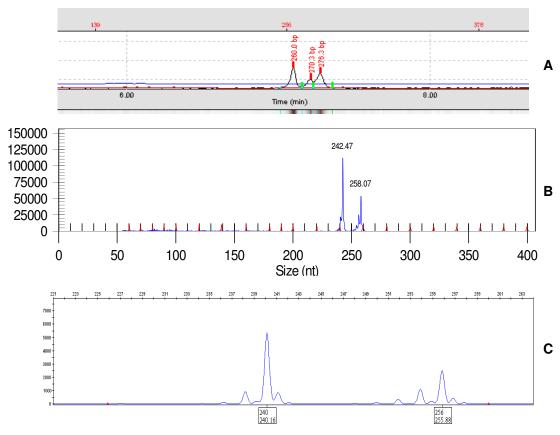
The same samples with fluorescently labeled primers were separated on the CEQ 8000 and ABI 3130xl and showed slightly higher (1 - 2 bp) resolution than QIAxcel System (Figure 3). All five SSR assays presented very similar allele separation (Figure 1). The QIAxcel System (Figure 3A), CEQ 8000 (Figure 3B) and ABI 3130xl (Figure 3C) automatically generated tables of allele size. Although the QIAxcel System had relatively lower resolu-tion than the CEQ and ABI platforms, it is less expensive

Figure 1. Alleles amplified using SSR primer set CF11 (GenBank accession number ED652034) for 12 flowering dogwood cultivars. Gel images are shown from the analyses using QIAxcel System-GT12™ genetic analyzer (A), 10% polyacrylamide gel (B), and 3% MetaPhor® agarose gel (C) and lanes 1-12 represent different flowering dogwood cultivars. Note that one broad, diffuse band in lanes 5 and 6 are shown in the agarose gel (C), whereas two products are clearly resolved by both the QIAxcel System-GT12™ genetic analyzer (A) and the silver stained polyacrylamide gel (B). The polyacrylamide gel also shows some non-specific or spurious background staining.

Figure 2. The minimum DNA (sample 10 was amplified with SSR primer CF11) detected by the QIAxcel system. Approximately 600 ng of PCR products could be diluted to 0.6 ng and still be detected. The undiluted PCR products are in lane 1; lanes 2 -12 are serial dilution with 0.1× TE,1:2, 1:4, ……, 1:2048. The 15 and 500 bp line is the alignment marker that indicates the electrophoresis started and completed.

Figure 3. The graphs of the allele sizes of one flowering dogwood cultivar (sample 7 in Figure 1) with SSR primer CF11 (GenBank accession number ED651977) presented by QIAxcel System-GT12™ genetic analyzer (A), CEQ ^{TM} 8000 genetic analysis system (B) and ABI 3130xl Sequencer (C).

and more convenient for routine SSR assay, especially for mapping and large-scale population analysis. This device does not require labeled primers and can analyze 96 samples in less than two hours. The QIAxcel System also provides both a gel image (Figure 1A) and an electropherogram (Figure 3A).

A weakness of the QIAxcel System is that the instrument software calculates allele sizes based on the DNA size marker table generated from an earlier analysis on the same cartridge, whereas both the CEQ and ABI instruments use an internal standard in each sample and allelic samples are calculated individually for each capillary. When allelic sizes that were determined on the three capillary systems are compared, the data generated by the QIAxcel System were typically 5 - 10 bp higher than that shown by the CEQ 8000 or ABI 3130xl. However, when size markers are included in all of the samples for the QIAxcel System, the calculated allelic sizes were within 1 or 2 bp of that determined on the CEQ 8000 and ABI 3130xl (data not shown). It is not unusual to see slight variation in allele sizing between different instruments or even when using different reagents on the same machine. As long as there is adequate resolution and control samples, allele sizes from one machine can be adjusted and combined with data generated by another instrument. This is not an issue for application where allele sizes are converted to binary (1, 0) output before analysis.

We analyzed 12 flowering dogwood cultivars using two SSR primer sets and five electrophoresis methods. The greatest number of alleles was detected by ABI3100xl (19), following by QIAxcel System (13), CEQ 8000 (11), polyacrylamide gel (8) and MetaPhor gel (7) (Figure 3; Table 1). Although the polyacrylamide gel had higher resolution than agarose gels because of the sensitivity of the silver stain and smaller gel pore size, it was difficult to analyze many samples in one gel and accurately score the allele size. ABI 3130xl (1 - 2bp) had higher resolution than QIAxcel System and CEQ 8000 and the other two macro gel-based separation techniques. Furthermore, the primer-labeled capillary systems are able to detect single nucleotide polymorphisms (Amirkhanian and Liu, 2002; Liu et al., 1995; Lee, 2006), and performed automated generation of allele size tables.

Conclusion

In our study, the material cost for sample analysis using the QIAxcel System with 96 samples was very similar to

Table 1. Number of alleles detected by five different electrophoresis methods in 12 *Cornus florida* cultivars using two SSR primer pairs.

SSR primers	CF11	CF49	Total	Mean
QIAxcel System	6	7	13	6.5
3% Agarose (MetaPhor®) gel	4	3	7	3.5
10% Polyacrylamide gel	4	4	8	4.0
CEQ8000	4	7	11	5.5
ABI 3130xl	5	14	19	9.5

those using agarose or polyacrylamide gels, which was much lower than other capillary electrophoreses. However, the QIAxcel System was less labor-intensive since it could automatically analyze 96 samples without the need for manual sample and gel preparations. Furthermore, the gel separation time for 12 samples was less than 12 min compared to more than two hours using traditional agarose or polyacrylamide gels (including staining) and also less than other capillary electrophoresis assays (two hours for CEQ 8000 with a four capillary array), 35 min for ABI 3130xl with a 16 capillary array. This advantage is more obvious when larger samples (for example 96 samples) with more markers are analyzed. The QIAxcel System cost is about 20% of the price for the CEQ 8000 and ABI 3130xl. The relatively lower resolution of the QIAxcel System compared to the CEQ and ABI instruments does not affect most DNA analysis for SSR mapping and population diversity since allele sizes are generally greater than two base pair, the maximum resolution of the QIAxcel System. Another advantage to the QIAxcel System is that fluorescently labeled primers are not required because the allele detection is accomplished using EtBr present in the gel matrix. The software automatically stores the digital data and the calculated allele size data can be exported. One disadvantage of the QIAxcel System is that the allele data are exported in base pairs. If binary data are needed for analysis, conversion is necessary and can be accomplished in Microsoft Excel™ using a free macro (Rinehart, 2004). The CEQ and ABI software provide similar quality data and can export binary data, but a "traditional" gel image is not provided. If only a few samples are to be run, the CEQ and ABI instruments are well-suited to SSR detection and analysis. The cost savings resulting from the labor, and time saved, lower instrument price, and fewer reagents make the use of the QIAxcel System a fast, cost-effective and accurate method for performing routine or large-scale SSR assays.

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