AFLP Fragment Isolation Technique as a Method to Produce Random Sequences for Single Nucleotide Polymorphism Discovery in the Green Turtle, *Chelonia mydas*

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The green sea turtle, *Chelonia mydas*, was used as a case study for single nucleotide polymorphism (SNP) discovery in a species that has little genetic sequence information available. As green turtles have a complex population structure, additional nuclear markers other than microsatellites could add to our understanding of their complex life history. Amplified fragment length polymorphism technique was used to generate sets of random fragments of genomic DNA, which were then electrophoretically separated with precast gels, stained with SYBR green, excised, and directly sequenced. It was possible to perform this method without the use of polyacrylamide gels, radioactive or fluorescent labeled primers, or hybridization methods, reducing the time, expense, and safety hazards of SNP discovery. Within 13 loci, 2547 base pairs were screened, resulting in the discovery of 35 SNPs. Using this method, it was possible to yield a sufficient number of loci to screen for SNP markers without the availability of prior sequence information.

Key words: AFLP, *Chelonia mydas*, green turtle, single nucleotide polymorphism, SNP

Single nucleotide polymorphisms (SNPs) are the most common type of polymorphism in many genomes and show great promise as genetic markers for improving data quality and genotyping efficiency for population and evolutionary studies (Aitken et al. 2004; Morin et al. 2004). SNPs are the result of a single nucleotide mutation; they typically occur every 200–1000 bp in most genomes (Wang et al. 1998; Brumfield et al. 2003; Aitken et al. 2004). The ubiquity of SNPs throughout genomes in addition to their associated simple mutation models render them appealing markers for a broad range of applications including association and population studies (Brumfield et al. 2003; Smith et al. 2005).

Due to the small amount of nuclear sequence data available for most species as well as the current labor- and cost-intensive processes involved in SNP discovery, the identification of SNP markers in nonmodel organisms is quite recent and still relatively uncommon. The prediction that SNPs will become the marker of choice in evolutionary, ecological, and conservation studies is beginning to come true, and studies of SNPs in nonmodel organisms are becoming more common. Nevertheless, a search of several public databases and journals focusing on molecular methods and nonmodel organisms resulted in fewer than 15 publications (Primmer et al. 2002; Nicod and Largiader 2003; Aitken et al. 2004; Fang et al. 2005; Seddon et al. 2005; Smith et al. 2005; Cenadelli et al. 2007; Lin et al. 2007; Morin et al. 2007; Rosenblum et al. 2007).

Sea turtles are animals of considerable interest in conservation genetics due to their complex life histories, which challenge conventional definitions of stock structure (Bowen and Karl 2007). Due to female nesting site fidelity, analyses of maternally inherited mitochondrial DNA (mtDNA) can provide a different image of population structure than biparentally inherited nuclear DNA (nDNA) that also reflects patterns of male-mediated gene flow (Bowen and Karl 2007). Most studies have used mtDNA sequence data to address evolution, phylogeny, and population dynamics in green turtles (Bowen et al. 1992; Karl et al. 1992; Dutton et al. 1996, 2008; Encalada et al. 1996). It is increasingly apparent that genetic surveys should address all life stages and employ both mtDNA and multiple

nuclear loci to accurately identify management units in migratory marine animals (Bowen and Karl 2007). Although there has been limited use of microsatellites for green turtles, more nDNA data are needed (Karl et al. 1992; FitzSimmons et al. 1997; Roberts et al. 2004; Dutton and Frey 2009). An additional class of nuclear markers like SNPs could add to previous data and improve our understanding of marine turtle biology and evolution.

Conserved polymerase chain reaction (PCR) primers designed primarily from primates and rodents, developed for comparative genomics research, have been applied to a range of mammals. These primers have exhibited reduced amplification success with increased phylogenetic distance from the target species (Aitken et al. 2004). These data have led to an inference that PCR primers designed from mammalian genomes will not yield a significant number of sequences for SNP marker discovery in more divergent taxa such as reptiles. Therefore, for our ongoing sea turtle studies, alternate SNP discovery methods were used. Like mammals, once screening primers have been developed for one species of sea turtle, it is likely that they can be applied to other species of turtles or reptiles for SNP discovery. Indeed, previous work with microsatellites show cross-taxa amplification indicating the conservation of flanking sequences spanning 300 million years of the divergent evolution of marine turtles (FitzSimmons et al. 1995).

Amplified fragment length polymorphism (AFLP) identifies DNA polymorphisms without prior sequence knowledge by the amplification of random genomic DNA fragments (Vos et al. 1995). This technique generates a select subset of DNA fragments from genomic DNA with the use of restriction enzymes, oligonucleotide adaptors, and generic PCR primers. Individual AFLP fragments have the potential to be sequenced after electrophoretic separation and excision due to known adaptor sequences. Nucleotide sequence data obtained from AFLP fragments can be used to design internal locus-specific primers to amplify the DNA fragment internal to the AFLP marker (Meksem et al. 2001; Bensch et al. 2002; Brugmans et al. 2003; Nicod and Largiader 2003). These locus-specific primers can then be used to amplify particular fragments directly from multiple specimens, generating nDNA sequence for phylogenetic or population genetic analysis, or for SNP discovery. We describe methods for generation of a moderate number of nuclear fragments from green turtle DNA and the results of SNP discovery from those fragments in population samples. These methods can result in development of a sufficient number of SNP loci for most types of population genetic studies in any organism, regardless of prior availability of genomic information.

Materials and Methods

DNA was extracted from green turtle blood and tissue samples using silica-based extraction columns (DNeasy, Qiagen, Inc., Valencia, CA). DNA was extracted from 5 μl of blood or approximately 20 mg of skin. nDNA quality and approximate concentration were checked by electrophoresis of a 3-μl extracted sample in a 1% agarose gel containing ethidium bromide. The concentration of samples visually confirmed as containing undergraded genomic DNA were quantified by ultraviolet (UV) spectrophotometry.

AFLP techniques (Vos et al. 1995) were performed on 4 DNA samples using AFLP Analysis System I and AFLP Starter Primer Kit (Invitrogen Corporation, Carlsbad, CA) with some method modifications. Approximately 200 ng of genomic DNA was digested for 2 h at 37 °C with 2 μl of an EcoRI/MseI enzyme mixture (1.25 units/μl each enzyme in 10 mM Tris–HCl [pH 7.5], 50 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol [DTT], 0.1 mg/ml bovine serum albumin, 50% [v/v] glycerol, and 0.1% Triton X-100) and 5 μl of a 5× reaction buffer (50 mM Tris–HCl [pH 7.5], 50 mM Mg-acetate, and 250 mM K-acetate) in a total reaction volume of 25 μl. After digestion, the mixture was incubated for 15 min at 70 °C and placed on ice. Next, 24 μl of adaptor ligation solution (0.2 μM EcoRI adaptor and 2 μM MseI adaptor in 0.4 mM ATP, 10 mM Tris–HCl [pH 7.5], 10 mM Mg-acetate, and 50 mM K-acetate), and 1 μl T4 DNA ligase (1 unit/μl in 10 mM Tris–HCl [pH 7.5], 1 mM DTT, 50 mM KCl, and 50% glycerol) were added to the restriction digest mixture, and the mixture was incubated for 2 h at 20 °C for ligation.

Preamplification of the primary template utilized AFLP primer sequences and reaction conditions as described in Vos et al. (1995). The set of primers included EcoRI core sequence E_A (5′-GACTGCGTACCAATTC-3′) with an additional selective base (A) and MseI core primer sequence M_C (5′-GATGAGTCCTGAGTAA-3′) with a 1-base extension (C). Preamplification conditions included 5 μl of undiluted template DNA with ligated adaptors, 0.74 μg/ml of each primer (E_A and M_C), 7.8 mM deoxynucleotide triphosphates (dNTPs), 20 mM Tris–HCl (pH 8.4), 1.5 mM MgCl$_2$, 50 mM KCl, and 5 units of HotStar Taq Polymerase (Qiagen, Inc.) in a total volume of 51 μl. Cycling parameters for preamplification included a 15-min initial denaturation step at 94 °C, followed by 30 cycles of 94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s, and a final elongation step at 72 °C for 10 min. Preamplification reaction products were diluted 1:1 in deionized water.

For each primer, 3 selective nucleotides were used to amplify the preamplification product to generate the AFLP fragments. Selective amplification reactions were performed in a 20-μl total volume using modified MseI and EcoRI core primers with 2 additional selective nucleotide bases (NN). Reaction conditions included 0.695 ng E_{ANN}, 1.5 ng M_{CNN}, 2.25 mM dNTPs, 1× Qiagen HotStar Taq PCR Buffer (with 1.5 mM MgCl$_2$) (Qiagen, Inc.), 5 μl diluted preamplified template DNA, and 0.5 U HotStar Taq Polymerase (Qiagen, Inc.). PCR cycling conditions included an initial denaturation step at 95 °C for 10 min, a touchdown cycle of 95 °C for 30 s, 65 °C for 30 s (decreasing 0.7 °C for 13 cycles), 72 °C for 60 s, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, and a final elongation step of 72 °C for 10 min.

After the amplification, 6 μl of each reaction product was mixed with 2 μl of 5× loading buffer and electrophoresed

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in a 3% agarose gel containing 0.4 μg/ml ethidium bromide to assess amplification success. Sample lanes with multiple bands ranging in size between 50 and 500 bp and clean negative controls were chosen as candidates to isolate bands for sequencing.

Successful AFLP reaction samples were electrophoresed on Spreadex® EL 600 mini gels (Elchrom Scientific, Cham, Switzerland). Ten microliters of each selective PCR product were electrophoresed along with 4 μl of a 50–1000 bp DNA marker (Cambrex Bio Science, Rockland, ME) as a size standard to estimate AFLP band sizes. Spreadex® gels were used on their plastic backing in a regular submarine unit in a 0.75× dilution of standard Tris-acetate EDTA (TAE) buffer. After a preelectrophoresis step for 10 min at 50 V, samples were loaded and immediately electrophoresed at 100 V for 10 min followed by 80 V for 260 min. Gels were stained on a shaker for 2 h in SYBR green II (1:10,000 in 0.25× TAE; Molecular Probes, Eugene, OR) with the plastic backing removed. Immediately after staining, bands were viewed on a DR45M Dark Reader® Transilluminator (Clare Chemical Research, Dolores, CO). AFLP fragments ranging in size from 100 to 1000 bp were excised using a 1000-μl pipette tip. Fragments were selected based on whether they were clearly separate from other fragments and were only isolated from a single individual to use for sequencing and subsequent primer design. The gel pieces were eluted individually in 25 μl of deionized water for at least 24 h prior to use for PCR amplification.

Excised AFLP fragments were amplified with primers designed to anneal to the common adaptor sequences flanking the variable genomic regions of the generated restriction fragments (*Eco*RI + 0 and *Mse*I + 0). These fragments were sequenced in both directions using the same primers (Brugmans et al. 2003).

Results and Discussion

A total of 41 selective amplification base combinations from the AFLP Starter Kit were used on between 1 and 4 representative *Chelonia mydas* samples. One hundred and eleven bands were excised from 4 gels. Of those, 78 amplified as a single band and 23 of those (29%) resulted in clean sequence that was used for primer design. Based on these sequences, 21 pairs of primers were designed to amplify homologous copies of loci across multiple green turtles for SNP discovery. Thirteen of the assays (62%) successfully produced sequences adequate for SNP discovery ranging in size from 117 to 534 bp. We were able to identify 35 SNPs from a total of 2547 bp of turtle DNA sequence. SNPs were present in 10 of 13 screened loci (Roden et al. 2009; GenBank Accession Nos FI276987–FI276999). Presumably, additional loci could be screened after redesign of some primers to allow reliable resequencing from the ascertainment panel of samples.

Previously, AFLPs have been used to generate and isolate individual products, but all methods have relied on polyacrylamide gels and either fluorescent or radioactively labeled primers for detection using DNA transfer and autoradiographs or marked glass plates to recover bands (Meksem et al. 2001; Bensch et al. 2002; Brugmans et al. 2003; Nicod and Largiader 2003; Fang et al. 2005). Our results were accomplished without the use of cloning, labeled primers, or DNA transfer and hybridization methods. As a result, multiple bands could be detected and excised in a single experiment. Ascertainment bias was avoided by sequencing AFLP fragments from a geographically diverse set of ascertainment samples for SNP discovery.

Several key components were necessary for the success of this approach. AFLP reactions amplified substantially better with the use of HotStar *Taq* DNA Polymerase (Qiagen, Inc.) compared with standard *Taq* polymerase. In addition, due to the small size of Spreadex® gels, the original protocol of UV illumination to perform gel cuts was unsuccessful. These extracts often contained multiple comigrating fragments of similar size resulting in overlapping sequence data. Introduction of a Dark Reader® Transilluminator (Clare Chemical Research) allowed more time and physical manipulation of the gel for isolation of single AFLP fragments without the detrimental UV exposure to the DNA sample and user. The use of SYBR Green II eliminated the use of costly and time consuming labeling methods, like radioactivity, to detect AFLP bands. SYBR Green I would have been the preferred reagent. However, SYBR Green II was readily available and succeeded in binding to the DNA during the staining process.

The development of a set of SNP loci for marine turtles promises to be a rapid way of genotyping individuals. In addition, SNPs can eliminate the variability of microsatellite genotypes due to differences in technology and scoring methods between laboratories (Aitken et al. 2004). These methods can be applied to a broad range of taxa to aid in a variety of applications including ecological, behavioral, forensic, and population structure studies.

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