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Full Length Research Paper

Eggshell membranes as a noninvasive sampling for molecular studies of Chinese alligators (*Alligator sinensis*)

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Noninvasive sampling is of prime essential on conservation genetics and molecular ecology. It is particularly preferred to use in the genetic identification of individuals and genetic analysis. A simple and efficient sampling is described for molecular studies from eggshell membranes in an endemic population of Chinese alligator (*Alligator sinensis*). This sampling strategy is fast, inexpensive and noninvasive to individuals. It could obtain large amounts of high-quality nucleic acids, isolated from different individuals. Amplification of mitochondrial and microsatellite DNA loci was successful from eggshell membrane in *A. sinensis*, after DNA extraction. These results show that eggshell membranes as a noninvasive sampling represent a reliable source of DNA for conservation genetics in endangered reptiles.

Key words: Eggshell membrane, noninvasive sampling, mtDNA, microsatellite genotyping, *Alligator sinensis.*

INTRODUCTION

The extraction and successful PCR amplification of DNA from shed hairs, buccal swabs, faeces, molted feathers, and reptile skin has increased the number of sampling techniques available for conservation genetics and molecular ecology research (Vigilant, 1999; Poschadel and Moller, 2004; Wehausen et al., 2004; Segelbacher, 2002; Tessier and Lapointe, 2003). Such noninvasive sampling techniques are preferred for studies that require the genetic identification of individuals as well as genetic analyses in general (Parsons et al., 2005; Valsecchi et al., 1998). With the exception of buccal swabs, subjects do not need to be captured, disturbed, or even observed; consequently, the number of studies using noninvasive sampling methods has increased dramatically. Conversely, other vertebrates could contaminate these biological materials and have an effect on the subsequent genetic analyses.

Eggshell membrane as a genetic sampling is desirable, especially when large populations or elusive species must be sampled. In the present study, we sought to confirm that eggshell membranes would yield enough quality DNA from Chinese Alligators (*Alligator sinensis*) for PCR amplification using different molecular markers and test its potential application in further conservation biology studies of large endangered reptiles.

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MATERIALS AND METHODS

Sample collection and DNA extraction

Eggs or shell fragments were collected after Chinese alligators had hatched from a total of four nests with 48 samples in the field and samples were stored in ethanol. Eggs or fragments were blotted on sterile paper. A piece of membrane was removed from the bottom of the egg with a pair of sterile tweezers. The membrane was placed into a labeled 1.5 ml microcentrifuge tube and crushed with tweezers until the fragments were 1 mm^2 in size. Samples were added PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4) buffer, mixed by vorteing for 5 min. Then, samples were washed twice with 700 µl STE (150 mM NaCl; 50 mM Tris HCl pH 8.0; 1 mM EDTA pH 8.0). The membranes were crushed with tweezers into pieces. We added 750 µl STE, 50 µl 20% SDS, and 20 µl of 20 mg/ml Proteinase K to the tubes, and the tubes were incubated at 55°C for 12 - 24 h after samples were vortexed for 1 min. Following the lysis phase, separation of solid and liquid com-

Table 1. PCR amplification and primers using in this study.

Molecular marker	Primer	Sequence (5'-3')	Product length (bp)	Reference
12S rRNA	L1091 H1478	AAACTGGGATTAGATACCCCACTAT GAGGGTGACGGGCGGTGTGA	460	Kocher et al. (1989)
Cyt *b*	Alli-M Alli-R	GCACTTCTCATCGGGTGAC ACGTGCTCTCGTGAAGGTAG	180	Yan et al. (2005)
D-loop	Con-mm p-Phe	CAATAGAGCATTTAGAGTATG AAAGCATAGCACTGAAAATG	530	Wang et al. (2003)

Table 2. Microsatellite loci used for *Alligator sinensis.*

Locus	Primer sequences (5' to 3')	Repeat motif	Product size range (bp)	Annealing temperature (°C)
Amiµ8	F:CCTGGCCTAGATGTAACCTTC R:AGGAGGAGTGTGTTATTTCTG	(AC)33	159-169	56°C
Amiµ18	F:ATCTCCGAGGGGAAAAATACA R:AATAGATGGAGTGATGTTATAGTCAG	(AC)35	172-213	60°C

ponents was accomplished by centrifugation at room temperature at 12 000 rpm for 10 min. 300 µl supernatant was transferred into a 1.5 ml Eppendorf tube. All samples were extracted with phenol twice, chloroform once, and precipitated with 800 µl ethanol. The extracted DNA samples were stored at -20°C in 50-100 µl sterile water. DNA concentration was quantified at 260 nm using a BioPhotometer (Eppendorf Co.) to determine the amount and quality of obtained nuclear DNA.

PCR of mitochondrial and microsatellite loci

The suitability of these samples for PCR was assessed by amplification of both mitochondrial and microsatellite DNA loci. All extracts from eggshell membranes were used to amplify mtDNA 12S rRNA gene (Kocher et al., 1989), species-specific Cyt *b* gene (Yan et al., 2005), and mitochondrial D-loop region (Wang et al., 2003). All amplifications were performed using 25 mM $MgCl_2$, 1xNH_4 buffer, 1 mM of each primer, 0.5 mM of each dNTP, 0.25 unit of Taq polymerase (Takara Co.) and 10 ng of template DNA in a total volume of 30 µl. PCR conditions and primers are shown in Table 1. PCR was carried out on a peltier thermal cycler under the following cycling conditions: initial denaturation at 95°C for 4 min; 30 cycles at 94°C for 45 s, 54°C for 40 s, 72°C for 40 s, and final extension at 72°C for 7 min. PCR products were visualized on an 1.5% agarose gel by ethidium bromide staining and UV photography. In order to avoid any possible contamination, each step of DNA extractions and amplification included a negative control (without template). In addition, there was a physical separation of the lab rooms where pre- and post-PCR experiments were carried out. All steps were performed in separate places using aerosol resistant pipette tips.

To test for nDNA amplification, the Amiµ8 and Amiµ18 microsatellite markers developed for *Alligator mississipiensis* (Glenn et al., 1998) were tested in Chinese alligator (Table 2). PCR amplifications were carried out in 25 µl reactions that contained 50-100 ng of nuclear DNA, 0.4 µmol of forward primer, end-labelled with fluorescently labelled, 0.4 µmol of unlabelled reverse primer, 0.4 mM dNTPs, 1 U Taq polymerase (Takara Co.), 10X PCR buffer and various amounts of $MgCl_2$ (2 mM for Amiµ8 and 2.5 mM for Amiµ18). Thermal cycling protocols consisted of an initial denaturation at 94°C for 1 min, then 30 or 35 cycles of 94°C for 40 s, 56 or 60°C for 1 min (Table 2), 72°C for 45 s and a final extension at 72°C for 10 min. To determine base pair size and relative concentration for dilutions, PCR products were visualized in 2.5% agarose gels. Microsatellite profiles were obtained on an ABI 377 automatic sequencer using the software Genscan 3.7 (Applied Biosystems) and individually verified by eye. Measures of microsatellite variation were analyzed using Cervus 2.0 (Marshall et al., 1998).

RESULTS AND DISSCUSSION

In this paper, the authors successfully amplified fragments of 12S rRNA gene, species-specific primers and mtDNA D-loop from eggshell membranes in Chinese alligators with the expected size in the PCR reactions among four nests. DNA samples from eggshell membranes using three molecular markers were all amplified successfully (Figure 1).

The Amiµ8 and Amiµ18 markers presented amplification products and polymorphism for Chinese alligator. Measures for microsatellite variation among nests indicated high allelic variation (Table 3), with 5 and 8 alleles per locus for Amiµ8 and Amiµ18, respectively. Observed heterozygositics index were 0.54 and 0.56. Polymorphic information content (PIC) was 0.59 and 0.66. These variations allowed for the detection of multiple paternity of Chinese alligator (*A. sinensis*) hatchlings in the field. These results show that eggshell membranes provide a reliable source of DNA for determining microsatellite genotypes of alligator.

PCR amplifications were consistently excellent with well preserved DNA for conservation genetic studies. Obtaining high quality nucleic acid is of prime important in DNA-based techniques. The simple and efficient ethanol storage solution presented here could be useful for mem-

Table 3. Genetic variation at two microsatellite loci, amplified from eggshell membranes among four nests. The expected (He) and observed (Ho) heterozygosity was calculated using Cervus 2.0 (Slate Marshall and Pemberton 2000).

Locus	N	No. of alleles	Ho	He	PIC
Amiμ8	48	5	0.542	0.663	0.592
Amiμ18	48	8	0.563	0.693	0.661

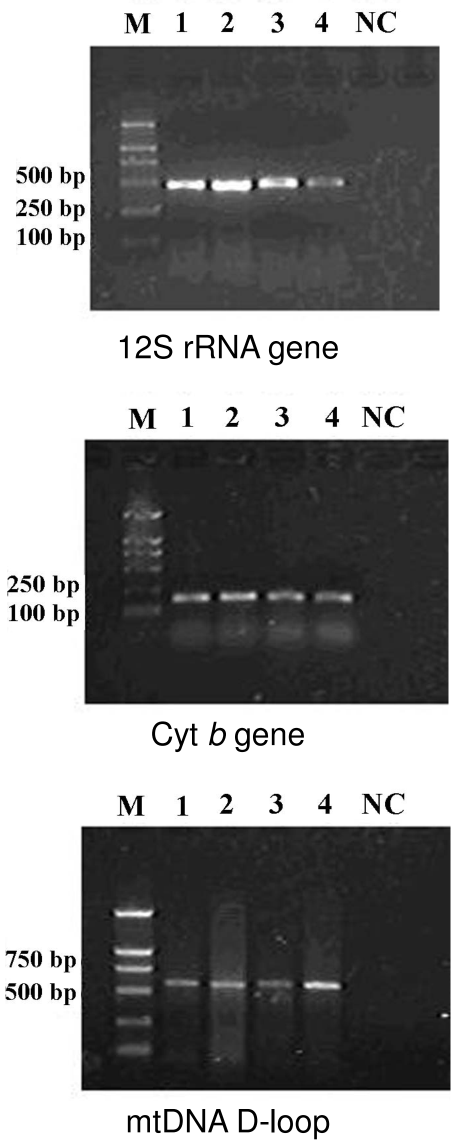

Figure 1. A 1.5% agarose gel showing the mtDNA 12S rRNA gene, Cyt *b* gene and D-loop for *Alligator sinensis* from eggshell membrane. Each lane represents one nest sample. MW: Molecular weight marker, DL 2000 (Takara Co.). NC: Negative Control without DNA template.

branes' preservation. Another improvement on the DNA isolation process was provided by a pretreatment with PBS buffer. Before cell lysis, we used STE buffer to wash the samples. The concentration and time/temperature for Proteinase K incubation were also very important to obtain high-quality DNA. After tissue digestion, a phenol-chloroformisoamyl alcohol purification step was utilized (Sambrook and Russell, 2001). The use of phenol-chloroform proved to be essential to obtaining pure DNA samples from eggshell membranes. Therefore, the extraction of DNA from membranes offers an extremely positive alternative to conventional DNA isolation techniques, representing a minimally destructive sampling approach.

We believe that the methodology described herein offers several positive features as compared to other sampling techniques such as faeces or buccal swabs. First, our new sampling methodology can provide high quality DNA while exerting little or no stress to researchers as one can collect eggs, or fragments thereof, relatively easily once alligators have hatched. Second, the technique has no detrimental effects on sampled reptiles, provided that eggs with viable embryos are not sacrificed. Finally, eggshell membrane sampling is simple to perform, fast, inexpensive, and could be routinely used for nest surveys. Our method could prove useful for other wildlife species with highly synchronized hatching or colonial nesters, such as sea turtles and other reptiles. The use of DNA amplified from eggshell membranes opens up important new possibilities for studying the occurrence and genetic makeup of endangered species.

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