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Development of Microsatellite Markers for Siamese Crocodile (*Crocodylus siamensis*)

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ABSTRACT

New microsatellite markers were developed for Siamese crocodile (*Crocodylus siamensis*) by constructing a library for microsatellite DNA. Construction and characterization of the library are described in the present study. Twenty microsatellite markers were developed from a $(AC)_{15}$ enriched microsatellite DNA library. Among the twenty microsatellite loci, ten (50%) were polymorphic, where as the rest were monomorphic (with two to four alleles per locus). The microsatellite sequences obtained could be classified structurally into perfect repeats (80%) and imperfect repeats (20%). No incomplete repeat type was observed. These markers were tested in five individuals of the same species and these tests resulted in twenty new microsatellites markers for *C. siamensis*. Low number of alleles (1-4 alleles) with an average of 1.7 alleles per locus was observed. The average length of uninterrupted repeats from the selected clones was 12.3.

Key words: *Crocodylus siamensis*, Siamese crocodile, crocodile, microsatellite primer

INTRODUCTION

The Siamese crocodile (*Crocodylus siamensis*) is a critically endangered species of freshwater crocodiles. It was previously distributed throughout Southeast Asia. However, Thailand has extensive crocodile farms of *C. siamensis* and *C. porosus* breeds. Unintentional hybridization is often a more serious problem of conservation concern because it can occur undetected, particularly if hybrids do not have distinguishing morphological features. However, intentional hybridization could become a concern, if mixed species populations become the source for reintroduction efforts. In this case, hybridization may have dramatic effects for native endangered species e.g. Siamese crocodiles, if human-induce introductions arise from the population consisting a few or all hybrid offspring (Allendorf *et al.*, 2001).

In addition, developing a plan for preventing such hybridization for the Siamese crocodiles is limited since clear observation is rarely. Clear observation is rarely possible because mating occurs in the water and often involves groups of males and females which are difficult

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to differentiate (Lang, 1989). Even if a female is observed to be mounted by more than one male (Davis *et al*., 2000), it is unclear whether multiple males successfully copulate and inseminate her, resulting in fertilized eggs. Such observations have led to the supposition that female crocodiles may produce clutches of progenies by multiple males. Hybrids can be difficult to distinguish based on morphology, thus there is a need to develop molecular techniques to identify species status of individuals considered for release into the wild and to establish captive breeding programs for conservation.

Microsatellites have been the genetic tool of choice for DNA based parentage systems due to their highly polymorphic nature and have been employed for refined estimating of kinship and parentage (Bruford and Wayne, 1993) in many organisms including crocodiles (Glenn *et al.,* 1998; Davis *et al*., 2000). They are nuclear markers that consist of short tandem repeats, usually 1-5 bp in length, such as (AC)n or (ATT)n (Beckmann and Weber, 1992). They are found approximately every 10 kb in the eukaryotic genome and their repeat arrays are generally no longer than 300 bps (Stallings *et al*., 1991). Polymorphism arises through variation in the number of repeat units present, possibly owing to slipped-strand mispairing (Schlötterer and Tautz, 1992). Variation at microsatellite loci can be assayed by PCR amplification using primers complementary to unique sequences flanking specific repetitive arrays, followed by electrophoretic sizing of the PCR products (Tautz, 1989).

In the current study, microsatellite primers were developed to compare population genetic structure in crocodilian families (Glenn *et al*., 1996; FitzSimmons *et al*., 2001). Several sets of primers have been designed for three other species of Crocodylus (including the Cuban crocodile, *C. rhombifer*, the salt-water crocodile, *C. porosus* and the Australian freshwater crocodile, *C. johnsoni*). However, within this genus, most tests of cross-species PCR amplification indicated the presence of homologous microsatellite loci that were variable (FitzSimmons *et al*., 2001). Herein, we describe the development of new microsatellite DNA primers for the *C. siamensis*. This is an important first step that should help to establish conservation strategies and contribute to an understanding of the structure of wild, remnant populations for this species.

MATERIALS AND METHODS

Sample collection and DNA extraction

Whole blood (5ml) was collected without injury to individual from an anterior dorsal sinus of a live caught wild Siamensis crocodile. The sample was kept on ice and sent to laboratory for DNA extraction and used for constructing a microsatellite library. In addition, five individuals of the same species were selected from the Sriracha breeding farm. Blood samples were collected as indicated above for the analysis of designed microsatellite primers.

Genomic DNA was digested with *Taq* I, in a final volume of 100 µl that composed of 10 µl of 10X *Taq* I buffer, 0.1 U/µl BSA, 0.25 U/µl of *Taq* I, and 1 µg/µl of genomic DNA. The mixture was incubated at 65°C for at least five hours or overnight. Digested DNA was run on a 0.8% agarose gel with ethidium bromide and visualized under ultraviolet (UV). DNA fragments with an average size of 500 to 1000 bp were isolated from agarose gel and purified by using QIAquick spin column (QIAGEN) followed by ethanol precipitation. This isolated DNA fragments were ligated into the *Cla* I site pBluescript II KS+ (Takara) and transformed into fresh competent XL1-Blue supercompetent cells (Stratagene) by heat shocking. Transformed cells were grown up overnight on LB agar plates containing 50 µg/ml ampicillin with X-Gal and IPTG. Recombinant colonies were transferred onto Hybond nylon membranes (Amersham, Sydney) and followed by

hybridizing with synthetic oligonucleotide microsatellite probe $d(AC)_{15}$. Prehybridization and hybridization were carried out at 42°C in 6X SSC (from a 20X stock = 3M NaCl, 0.3 M sodium citrate), 5X denhardt reagent (from a 50X stock = 1% BSA fraction V, 1% Ficoll and 1% polyvinylpropylene) and 0.1% SDS. After hybridization, it was washed twice (30 min) in a 6X SSC, 0.1% SDS solution. Filters were screened for microsatellite repeated by using Gene-Images random primer labelling kit (Amersham Pharmacia Biotech) and exposed to X-ray film. After alignment to autoradiography images, positive colonies were selected and a recombinant plasmid was isolated by using alkaline preparation and screened for recombinants DNA by restriction enzyme. The potentially positive recombinants were sequenced automatically (Fluorescent dye method, Applied Biosystems).

Primer design and genotyping

Primer pairs were designed to amplify the flanking regions of selected microsatellites using the program Genetyx software (GENETYX software development Co. Ltd., Tokyo, Japan). Primers were about 18-24 bp in length, with calculated annealing temperatures of 50-65°C with a maximum 4°C difference between each pair, and no primer dimer of hairpin formation. In the genotyping, DNA samples from 5 Siamese crocodiles were amplified in a 25 µl final volume of 1X PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 mM of each primer, 0.02 U/µl. Taq DNA polymerase, and 25 ng of DNA. The amplified conditions are started with denaturing step of 94°C for 3 min and followed by 35 cycles of 94°C for 1 min, the appropriate annealing temperature for 1 min, and amplification at 72°C for 1 min. Products were stored at 4°C until ready to be analyzed and scored. The alleles of the microsatellite primers were detected in 6% denaturing polyacrylamide gel electrophoresis and their expected sizes were compared with standard size of ϕ x *Hinf*I. The program Kodak 1D Digital Science V. 3.0.2: Scientific Imaging System (Eastman Kodak Company, New Haven, CT) was used for analyses of scientific images.

RESULTS AND DISCUSSION

Total 215 clones of transformant were constructed and hybridized with $d(AC)_{15}$ probe. It was found that 59 clones or 27.44% of total clones with tandem repeat nucleotide were recognized and selected. From these positive clones, 30 of them (50.7% of total $d(AC)_{15}$ positive clones) were selected for DNA sequencing. The sequencing result gave only 22 clones that can be selected to design primer. The other eight clones can not be studied further due to inadequate lengths of flanking sequence. Thirty-two microsatellite primers were designed from 22 positive clones and only 20 of these provided reliable amplifications (Table 1). The DNA sequences of the cloned alleles were submitted to Genbank (accession numbers EF413033-EF413054).

The microsatellite polymorphism in the farm population of Siamese crocodiles has been analysed by SDS-PAGE. Low number of alleles (1-4 alleles) with an average of 1.7 alleles per locus was observed (Table 1). The average length of uninterrupted repeats from the selected clones was 12.3. Ten (50 % of total microsatellite primers) of the 20 microsatellite loci were polymorphic. Information of all primers designed, including repeat motif, expected size, observed sizes, PCR conditions, and other characteristics, is presented in Table 1 and the illustrated of loci markers *CS*-4, *CS*-5 and *CS*-21 are presented in Figure 1.

A total of 20 microsatellite loci were structural analysis as indicated by Weber (1990) and 16 loci exhibited perfect microsatellite motifs without any interruption in the repeats, where as 4 loci contained compound microsatellite repeats with a run of CA or GT repeats. No imperfect

Table 1 Primer parameter and observed values for *Crocodylus siamensis*

Locus (GenBank accession no)	Primer: (5' —> 3')	Lenght	%GC	TM	Repeats	Expected size (bp)	Observed allele size range (bp)	Alleles per locus	PCR condition			Polymorphism
									Annealing (°C)	Mg^{2+} (mM)	Cycle	
CS-2	F: GCAAACCTATCACCAGTTCAAGC	23	47.82	58.76	(CA_8)	230	230-250	2	68	1.5	40	Yes
(EF4130039)	R: TCTCTGTCACCTGAACCTTCCTC	23	52.17	58.80								
CS-4	F: CCATGCCCTACCACACAACTG	21	57.14	58.79	(AC_{13})	200	200-210	2	65	1.5	35	Yes
(EF4130051)	R: CAAACACAAAGGCATTCAAAGATG	24	37.50	58.69								
CS-5	F: TCTCTCCTTCTTTCTGCTTGTGC	23	47.82	58.76	(AC_{16})	200	190-210	2	65	1.5	35	Yes
(EF4130052)	R: GTGCAAGGTCTGTACCTGTGTGA	23	52.17	58.80								
CS-10	F: TGACAGTGGCTTTATTGAACAGG	23	43.47	58.72	(GC_5AC_{17})	240	240-250	2	65	1.5	30	Yes
(EF4130033)	R: TGGACTCTCTCCTCCTGGACTTC	23	56.52	58.85								
CS-12	F: GGACAGCAACAGAAAAGACAGG	22	50.00	58.75	(AG_8)	200	200	1	60	1.5	35	No
(EF4130034)	R: ATAGGAAGCGTTCGTGCTGATG	22	50.00	58.75								
CS-14	F: CCTTCATGTGGATTAGGAACAGG	23	47.82	58.76	(AC_{10})	270	270	1	60	1.5	35	No
(EF4130035)	R: TAGCAGCTTGAAGTGGGTAGCAG	23	52.17	58.80								
CS-15	F: TCACCTGCATATTTCTCCTTCCA	23	43.47	58.72	(TC_6)	220	210-230	4	65	1.5	35	Yes
(EF4130036)	R: GACCGGGGCTCAGTAAAACAC	21	57.14	58.79								
CS-17	F: GATCCCTCCTACACACACACG	21	57.14	58.79	(GC_5AC_{12})	180	170-200	2	60	1.5	35	Yes
(EF4130037)	R: TATTAGGGCTGGACAGTCAAAGG	23	47.82	58.76								
CS-18	F: GCAGAACCATAACACATGCACAG	23	47.82	58.76	(A_{19})	240	240	1	60	1.5	35	No
(EF4130038)	R: GCATTCCATAGTCCCCTCCATAG	23	52.17	58.80								
CS-20	F: CCTTCCGCCCAAAATAATTGC	21	47.61	58.69	$(C_{10}AC_5)$	249	249	1	60	1.5	35	No
(EF4130040)	R: CCCTGCACTAACAAGGAGCAG	21	42.85	58.79								

Table 1 Primer parameter and observed values for *Crocodylus siamensis*

Locus (GenBank accession no)	Primer: (5' —> 3')	Lenght	%GC	TM	Repeats	Expected size (bp)	Observed allele size range (bp)	Alleles per locus	PCR condition			Polymorphism
									Annealing (°C)	Mg^{2+} (mM)	Cycle	
CS-21	F: ATTTCCACTCACAGCTTCAAACC	23	43.47	58.72	(AC_5)	260	260-270	2	60	1.5	35	Yes
(EF4130041)	R: GGATGCTTGTAGGTCTTGTTTAGC	24	45.83	58.77								
CS-22	F: CTGTAGGCTGTGACAAAATCCTG	23	47.82	58.76	(AC_7)	180	180	1	60	1.5	35	No
(EF4130042)	R: CATAACCAACCAGAATGTGACTGC	24	45.83	58.72								
CS-24	F: GAACCCAGGAGGCAGGAGAG	20	65.00	58.83	(AC_{26})	270	270	1	68	1.5	35	No
(EF4130043)	R: CACATACACAGAACCCAGGTGTG	23	52.17	58.80								
CS-25	F: ATCCTCAGTGTCATCTGCTCACC	23	52.17	58.80	(AC_9)	180	180	1	65	1.5	35	No
(EF4130044)	R: TCTTCCCTGCTCACCTTTCTTTC	23	47.82	58.76								
CS-26	F: GCCATGTGTACTAACTGGGAAGTC	24	50.00	58.81	(AC_{17})	240	240	1	65	1.5	35	No
(EF4130045)	R: GCCATTTTTAGTCAGGTTGTTGC	23	43.47	58.72								
CS-28	F: CAGTCTCCAGCACTTGGGGATAG	23	56.52	58.85	(AC_{15})	200	180-200	2	68	1.5	35	Yes
(EF4130046)	R: TTGGCTACAAAGGACCAACTCAC	23	47.82	58.76								
CS-30	F: ACTCACACTACCATCTCCAGCAC	23	52.17	58.80	(AC_{15})	210	210	1	68	1.5	35	No
(EF4130047)	R: TGTGTGTCATGTGCGTGAACC	21	52.38	58.74								
CS-32	F: GTACCAAGCCCCTTTAACACCTG	23	52.17	58.80	(AC_{17})	245	245	1	68	1.5	35	No
(EF4130048)	R: GGGGAGAAGGAAACTAGGAGAGG	23	56.52	58.85								
CS-33	F: ATCAACTTTCAGCCTGGGATAGG	23	47.82	58.76	(AC_{12})	200	200-210	2	68	1.5	40	Yes
(EF4130049)	R: ATGTCTTATTGCTTCCCTTGCAC	23	43.47	58.72								
CS-35	F: GTCTGAAAAGGGTGTTTGTG	20	45.00	58.63	(AC_{17})	230	230-240	2	60	1.5	35	Yes
(EF4130050)	R: CCTAAACTGTTAGAAGCCAAG	21	42.85	58.64								

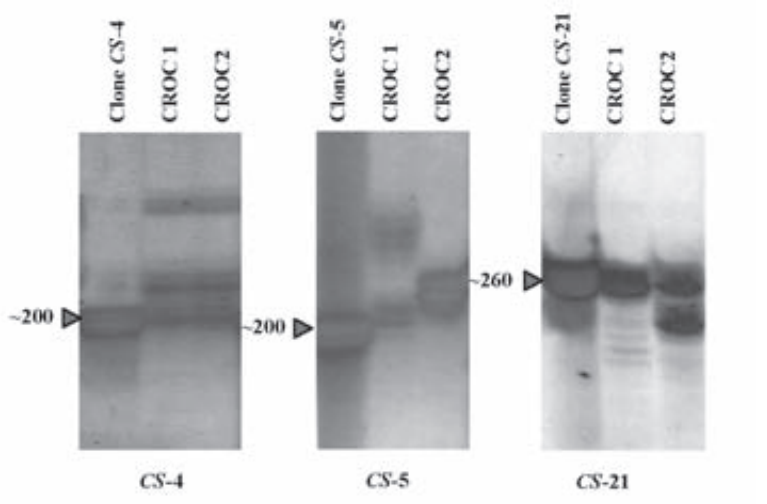

Figure 1 PCR product from locus *CS*-4, *CS*-5, and *CS*-21 showed polymorphic pattern.

repeat locus was observed in the study.

The enrichment procedure that we employed was successful. The number of microsatellite library obtained in our study was di-nucleotide repeats which are common in Crocodylidae and Alligatoridae as previously reported by Glenn *et al*. (1998) for *A. mississipiensis*, FitzSimmons *et al*. (2001) for *C. porosus* and *C. johnstoni*, and Zucoloto *et al*. (2002) for *Caiman latirostris*.

Regarding to the former, captive colonies could be more efficiently managed by establishing individual pedigrees that would help to keep inbreeding coefficient as low as possible. With respect to the latter, genetic studies of the behavioral ecology of remnant populations will allow assessment of mating systems and dispersal patterns of wild individuals, helping researchers to understand how the remnant populations use the landscape.

In future studies, we will further characterize the primers obtained by verifying segregation and heterozygosity. The development of these new microsatellite markers significantly increases our capability to assess the diversity of *C. siamensis* in Thailand. These new markers will improve exclusion power for maternity tests and the resolution of parentage identification among wild individuals in *C. siamensis*.

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