CHAPTER 5

Microsatellite markers for *Crocodylus*: new genetic tools for population genetics, mating system studies and forensics

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Questions of population identification and gene flow have become more important in the conservation and management of crocodilians. Additionally there has long been an interest in understanding crocodilian mating systems. To address such questions we designed nuclear microsatellite markers from the DNA of *C. acutus*, *C. porosus* and *C. johnstoni*. We report on the development and testing of 26 new microsatellite loci for *Crocodylus* spp., which represent the first microsatellite loci found in *Crocodylus*.

INTRODUCTION

GAINING an understanding of the genetic population structure of crocodilians and the complexities of their mating systems will contribute to the conservation and management of these species as well as addressing some persistent questions. In the most recent IUCN Action Plan for crocodiles (Ross 1998), for several species mention is made of the need for information on genetic diversity and relatedness. For example, population genetic surveys are needed of the critically endangered Orinoco crocodile (*C. intermedius*), to assess levels of genetic diversity to inform restocking and reintroduction programmes. In captive Cuban crocodiles (*C. rhombifer*), priority is given to determining the severity of genetic introgression from hybridization with either the Siamese crocodile (*C. simensis*) or the American crocodile (*C. acutus*). On a broader scale, high priority is given to investigate the taxonomy of freshwater crocodiles throughout SE Asia.

Sustainable use is seen as an important aspect of conservation programmes for many crocodilians (Ross 1998) and the use of genetic markers to determine the extent of gene flow among populations may contribute to more effective programmes. It is generally unknown what constitutes genetically discrete populations of a given species. To what extent is gene flow limited among the populations in different rivers or drainage basins? On what geographic scale does gene flow operate within management time frames and how does this vary among species? In making comparisons among species, are populations of freshwater species more genetically distinct than corresponding estuarine species due to greater dispersal by the latter between river systems? And how has gene flow been influenced by population loss and fragmentation? Few field studies have been able to address questions of migration between populations due to the difficulties of doing field work with dangerous animals in remote locations (Webb and Messel 1978). An increased understanding of the geographic limits of populations and the extent of gene flow among populations would provide basic information to guide management for conservation or to develop ecologically sustainable industries. In addition, using genetic markers as forensic tools can aid in identifying illegal harvest and trade.

There has long been an interest in understanding the complexities of crocodile mating systems, given the expectation of alpha male dominance and observed variations in mating behaviour (Ferguson 1985; Vliet 1989; Kofron 1991). Do alpha males have the highest reproductive success? What is the variation in male reproductive success and how is this influenced by density and sex ratios? Is there multiple paternity of clutches, and if so, how prevalent is it? To what extent does sperm storage (Ferguson 1985; Davenport 1995) and sperm competition influence paternity and hatching success? What are the relationships between mating system, alpha male dominance and migratory behaviour? Genetic markers

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have great potential for testing hypotheses about crocodilian biology built upon data from behavioural and mark-recapture studies.

Studies of gene flow and evolutionary history in vertebrates have mostly used genetic markers found within the mitochondrial DNA (mtDNA) genome. This is because mutation rates vary in different regions of the mtDNA and areas can be selected to have high enough variation to distinguish divergence, but not so high as to create "background noise". Because mtDNA is maternally inherited in vertebrates, such studies are limited to the history of female lineages within populations. In contrast, nuclear markers provide information on gene flow that is the result of mating behaviour by both males and females. Lately, an increasing number of studies are using a combination of mtDNA and nuclear markers to compare male *vs.* female gene flow. Nuclear markers can also be used in mating studies to assign parentage within a family and to estimate relatedness when pedigree relationships are unknown. One of the most promising classes of nuclear markers are microsatellite loci (Jarne and Lagoda 1996). These loci are found throughout the nuclear genome of eukaryotes and they are characterized by high mutation rates, great allelic diversity and high heterozygosity. This results in individuals having unique genotypes or "genetic fingerprints" which are needed for forensic-type studies of mating systems and are similarly useful for studies of gene flow among populations.

Several questions about crocodilian history and biology have already been addressed using genetic tools. This has been particularly evident in the investigation of taxonomic questions, from the early use of chromosome variation (Cohen and Gans 1970), through to sequencing of regions of mtDNA (Densmore and Owen 1989; Densmore and White 1991). More recently, the tools of population genetics been applied to the study of crocodilians. Glen *et al.* (1996, 1998) developed nuclear microsatellite markers to address questions of genetic population structure and mating systems of American alligators (*Alligator mississippiensis*) as presented by Davis *et al.* (2000).

Many of these loci, originally found in the DNA of *A. mississippiensis,* can be amplified in Crocodylus spp. using the same techniques (Davis, FitzSimmons, T. Glenn and J. Gratten, unpubl. data). However, they do not appear to be variable in *Crocodylus* probably due to mutations within the repetitive region (Davis, FitzSimmons and J. Gratten, unpubl. data). This is in contrast to other ancient taxon in which microsatellite primers amplified loci which were variable across species with similar or longer expected divergence times (FitzSimmons *et al.* 1995; Rico *et al.* 1996). In order to study gene flow and mating systems in *Crocodylus* spp., we needed to develop crocodile-specific microsatellite primers.

We report on the development and testing of microsatellite markers that were identified from the nuclear DNA of *C. acutus*, *C. porosus* and *C. johnstoni*. These markers will be used initially i) to identify captive bred hybrids between *C. acutus and C. rhombifer*, ii) to identify the genetic contribution of Jamaican *C. acutus* in Florida, iii) to study mating systems of wild *C. johnstoni* and *C. porosus* and captive *C. porosus* in Australia and iv) to determine the genetic population structure (in combination with mtDNA analyses) of *C. porosus, C. johnstoni, C. novaguineae, C. siamensis* and *Tomistoma schlegelii*.

METHODS

Microsatellite loci were identified from three species of crocodiles, *Crocodylus acutus*, *C. porosus* and *C. johnstoni* by establishing and screening genomic DNA libraries. Most loci were found using the procedure outlined below but for some loci (Cj101, Cj104, Cj105, Cj107, Cj119, Cj122, Cj127, Cj128 and Cj131) we used the enrichment protocol of Paetakau (1999). DNA was extracted from tail scute or muscle samples following standard phenol/chloroform methods and digested with a combination of *Alu*I, *Hae*III, and *Rsa*I. Digested DNA was run on a 1.2% agarose gel and the 350–600 bp size fragments selected and purified using a QIAquick kit (QIAGEN). Crocodile DNA was blunt-end ligated into pUC18 *Sma*I/BAP plasmids and transformed into fresh competent *E. coli* cells by heat shocking (*C. porosus* and *C. johnstoni*) or electroporation (*C. acutus*). Colonies were grown on Hybond N+ membranes (Amersham, Sydney) and duplicate transferred to Zeta-Probe blotting membranes for hybridization. A synthetic oligonucleotide microsatellite probe (dA-dC)n-(dG-dT)n (Pharmacia) was radioactively labelled by random-priming (Feinberg and Vogelstein 1983) using a Mega-Prime kit (Amersham) and α^{32}PCTP. Filters were hybridized overnight at 65° and the final wash was done in 0.2 × SSC and 0.1% SDS at 65°. Filters were exposed to X-ray film and positive colonies were selected from the original Hybond colonies after alignment to autoradiography images. This library of potentially positive colonies was subjected to a second round of hybridization to confirm positive status, and the ligated target-plasmid DNA was isolated by a miniprep method (Sambrook *et al.* 1989) and screened for

microsatellites by PCR cycle sequencing with a primer end-labelled with $\gamma^{33}P$ (Murray 1989). Primers were designed for microsatellite loci containing at least 10 uninterrupted dinucleotide repeats, using OLIGO 4.0-s software (Rychlik 1992). PCR conditions were optimized for all loci and samples from different populations were screened for polymorphism using a $\gamma^{33}P$ end-labelled primer in the PCR reaction. Products were run on 6% denaturing sequence gels with a sequenced size standard and results were visualized by autoradiography. For reference to the protocol used in developing locus pCp-8h4 see Daglish (1998).

RESULTS

Electroporation efficiencies for the *C. acutus* library were 4.0×10^7 and approximately 20 000 colonies were screened for microsatellite loci. After the second screening, 31 colonies were identified as positives to be sequenced and from this, 25 microsatellite loci were found. PCR primers were designed for 18 loci, and 12 of these provided reliable amplifications (Table 1). Transformation efficiencies for the *C. porosus* and *C. johnstoni* libraries (excluding those libraries used in finding the pCp-8H4 locus; see Daglish 1998) ranged from 2.4×10^6 to 2.1×10^7 and approximately 16 000 colonies were screened for microsatellite loci. After the second screening, 21 colonies were identified as positives to be sequenced and from this, 15 microsatellite loci were found. PCR primers were designed for eight loci, and five of these (Cj16, Cj18, Cj35, Cp10) provided reliable amplifications (Table 1). Use of the enrichment protocol on *C. johnstoni* DNA yielded 41 microsatellite loci. Of these, 32 had ≥10 repeat units and primers were designed for 16 loci. Nine of those loci (Cj101, Cj104, Cj105, Cj107, Cj119, Cj122, Cj127, Cj128 and Cj131) are reported on here as being the most promising of the group to date. Five of the 26 loci reported on were compound microsatellites, the rest were simple repeats (Table 1).

Considering all loci, the number of alleles found was generally low, and when samples sizes were at least five individuals for a particular species at a given locus, the range in number of alleles was 1–12 locus (Table 2), with an average of only 3.4 alleles per locus. If the same analysis is restricted to only the species from which the particular loci originated, there is an similar lack of variation (3.9 alleles per locus). In all tests with at least five samples, 18.5% of loci were monomorphic for a particular species. Allele size often varied among species at a given locus and when more than one species was monomorphic, allele size was generally different. However, 1 bp shifts in allele lengths across species may be an artifact of the differences between gel procedures as the *C. porosus* and *C. johnstoni* samples were run independently.

DISCUSSION

Although we were successful at locating $(AC)_n$ microsatellite loci in *Crocodylus* spp., these loci were not as common in crocodiles as in other taxon. For example, of the 16 000 colonies screened from the *C. porosus* and *C. johnstoni* libraries, only 15 microsatellite loci were found. Given an average base pair length of crocodilian DNA within the colonies of 400 bp, then we found approximately 1 locus for every 42 700 bp of DNA. Other studies have found microsatellite loci on average every 5 000–50 000 bp (Jarne and Lagoda 1996). It was because of this limitation that the enrichment protocol was used and as expected, the yield in microsatellite loci was much higher.

In general the number of alleles per locus was fairly small, even in comparison to alligators. Of 10 polymorphic loci found in *A. mississippiensis*, the average number of alleles per locus was 8.3 (range of 4–16) for an average sample size of 9.4 (range of 4–17) animals (Glenn *et al.* 1996). In *C. acutus*, where large samples were available from multiple populations, numerous population-specific alleles were present (Davis, unpubl. data), thus suggesting that species with small sample sizes or limited sampling areas have more allelic variation than is apparent from the results in Table 2.

Even with these limitations, the initial applications of these primers are promising. Preliminary data from populations of *C. acutus*, *C. porosus* and *C. johnstoni* indicate that several loci will be quite useful in studies of population genetics. For example, significant allele frequency differences ($p < 0.05$) were found at three loci (Cj18, C391 and Cuj131) between two populations of *C. porosus* in Queensland located about 700 km apart (Table 3). In a captive population of *C. porosus* it appears that there will be enough genetic variation to determine paternal exclusion in clutches. For example, data from a single clutch at only 1 locus (CUD68) suggest that the alpha male of the system was either not the father, or that he shared paternity (Table 4). In contrast, a wild population of *C. johnstoni* in the Lynd River has such limited genetic variation that it may be too difficult to analyse the mating system. Of the 21 loci tested, 8 loci were monomorphic and of the remaining

Table 1. Microsatellite primers developed for *Crocodylus* spp.

Primer name	Primer sequence 5'—3'	Species	Repeat motif
C391F	ATGAGTCAGGTGGCAGGTTC	*C. acutus*	$(CA)_{22}$
C391R	CATAAATACACTTTTGAGCAGCAG		
CE4-47F	AGTCCCTCTCCCCCACTC	*C. acutus*	$(CA)_{14}$
CE4-47R	TGCCATCTGATCTCAACCTC		
Cj16F	CATGCAGATTGTTATTCCTGATG	*C. johnstoni*	$(CA)_{20}$
Cj16R	TGTCATGGTGTCAATTAAACTC		
Cj18F	ATCCAAATCCCATGAACCTGAGAG	*C. johnstoni*	$(CA)_{21}$
Cj18R	CCGAGTGCTTACAAGAGGCTGG		
Cj35F[1]	GTTTAGAAGTCTCCAAGCCTCTCAG	*C. johnstoni*	$(CT)_7TA(CA)_{17}(CT)_{12}$
Cj35R	CTGGGGCAAGGATTTAACTCTC		
Cj101F	ACAGGAGGAATGTCGCATAATTG	*C. johnstoni*	$(CA)_{12}$
Cj101R[1]	GTTTATACCGTGCCATCCAAGTTAG		
Cj104F	TCCTTCCATGCATGCACGTGTG	*C. johnstoni*	$(CA)_{12}$
Cj104R[1]	GTTTCAGTGTCTGGTATTGGAGAAGG		
Cj105F	CAACAGAAAGTGCCACCTCAAG	*C. johnstoni*	$(CA)_{14}$
Cj105R[1]	GTTTGATTATGAGACACCGCCACC		
Cj107F	ACCCCGCATTCTGCCAAGGTG	*C. johnstoni*	$(CA)_{16}$
Cj107R[1]	GTTTATTGCCATCCCCACTGTGTC		
Cj119F[1]	GTTTGCTGTGGAATGTTTCTAC	*C. johnstoni*	$(CA)_{14}$
Cj119R	CGCTATATGAAACGGTGGCTG		
Cj122F[1]	GTTTCATGCTGACTGTTTCTAATCACC	*C. johnstoni*	$(CA)_{18}$
Cj122R	GGAACTACAATTGGTCAACCTCAC		
Cj127F	CCCATAGTTTCCTGTTACCTG	*C. johnstoni*	$(CT)_7TT(CT)_{12}$ $(CA)_{16}$
Cj127R[1]	GTTTCCCTCTCTGACTTCAGTGTTG		
Cj128F	ATTGGGGCAGATAAGTGGACTC	*C. johnstoni*	$(CA)_{22}$
Cj128R[1]	GTTTCTGCTTCTCTTCCCTACCTGG		
Cj131F[1]	GTTTGTCTTCTTCCTCCTGTCCCTC	*C. johnstoni*	$(CA)_{14}$
Cj131R	AAATGCTGACTCCTACGGATGG		
Cp10F	GATTAGTTTTACGTGACATGCA	*C. porosus*	$(CA)_{15}$
Cp10R	ACATCAAGTCATGGCAGGTGAG		
CU4-121F	GGTCAGCTAGCAGGGTG	*C. acutus*	$(CA)_{11}$
CU4-121R	TGGGGAAATGATTATTGTAA		
CU5-123F	GGGAAGATGACTGGAAT	*C. acutus*	$(CA)_{26}$
CU5-123R	AAGTGATTAACTAAGCGAGAC		
CUC20F	GATCTGCAGTGCAAGAAAAG	*C. acutus*	$(CA)_{13}$
CUC20R	GGTTTAGCGGTCACAGTAAC		
CUD68F	GCTTCAGCAGGGGCTACC	*C. acutus*	$(CA)_{15}$
CUD68R	TGGGGAAACTGCACTTTAGG		
CUD78F	GAAGTGAATGCCATCTATCA	*C. acutus*	$(CA)_{10}(TA)_9$
CUD78R	AATTGCATCCCCTTTTG		
CUI108F	ACTGGCCACAGCTGGGGTA	*C. acutus*	$(CA)_{20}$
CUI108R	CCAGCAGCGTGGAGAGCTG		
CUJ131F	GTCCCTTCCAGCCCAAATG	*C. acutus*	$(CA)_7(TA)_3(CA)_{10}$
CUJ131R	CGTCTGGCCAGAAAACCTGT		
CUJ-B131F	CCTGCCCAAGCCCATCAAT	*C. acutus*	$(CA)_{18}(TA)_9$
CUJ-B131R	CCCTTTTGGCATGGCACAGT		
CUI99.2F	CACTGTGGGGGCCTCAATCTG	*C. acutus*	$(CA)_{18}$
CUI99.2R	AGGCAGGTGGTAGGACCCTAGCAAT		
CR52F	GATCAAAATGAAACACCACA	*C. acutus*	$(CA)_{12}$
CR52R	CATAAATACACTTTTGAGCAGCAG		
pCp-8H4F	AAGGGCAAACGATGTGAAAAGT	*C. porosus*	$(CA)_{16}$
pCp-8H4R	CTTTCCTGAGGTTGCATCATGA		

[1]These primers were designed to have a GTTT tail on the 5' end by either adding GT or GTT. This was done to optimize the quality of PCR products while using a complementary fluorescent-labelled primer.

Table 2. Number of alleles and their range in base pair (bp) length found at microsatellite loci in *Crocodylus* spp. and *Osteolaemus tetrapis (O.t.)*. These loci were originally found in DNA extractions from *C. acutus, C. porosus or C. johnstoni* (see Table 1). The number of individuals sampled is shown in parentheses. *Crocodylus* spp. are as follows: *C. acutus (C.a.), C. johnstoni (C.j.), C. mindorensis (C.mi.), C. moreleti (C.mo.), C. niloticus (C.n.), C. palustris (C.pa.), C. porosus (C.po.),* and *C. rhombifer (C.r.)*.

	Species								
Locus	*C.a.*	*C.j.*	*C.mi.*	*C.mo.*	*C.n.*	*C.pa.*	*C.po.*	*C.r.*	*O.t.*
C391									
# alleles	5(45)	10(41)	2(2)	5(6)	2(2)	2(1)	12(51)	1(10)	1(3)
bp range	163–215	164–192	151–159	165–207	129–137	215–221	226–270	127	137
Ce4-47									
# alleles	—	3(41)	—	—	—	—	no	—	—
bp range		143–149							
Cj16									
# alleles	5(37)	2(42)	2(2)	1(6)	1(2)	1(1)	7(68)	3(6)	1(3)
bp range	138–168	154–160	148–156	138	148	156	144–182	146–162	144
Cj18									
# alleles	2(37)	4(42)	1(2)	1(6)	3(2)	1(1)	9(27)	3(8)	2(3)
bp range	204–206	205–213	200	194	204–218	208	206–230	204–214	196–198
Cj35									
# alleles	—	1(9)	—	—	—	—	—	—	—
bp range		174							
Cj101									
# alleles	—	4(29)	—	—	—	—	6(30)	—	—
bp range		343–349					349–371		
Cj104									
# alleles	—	7(68)	—	—	—	—	3(39)	—	—
bp range		198–214					206–210		
Cj105									
# alleles	—	5(30)	—	—	—	—	4(30)	—	—
bp range		365–377					365–385		
Cj107									
# alleles	—	7(35)	—	—	—	—	4(29)	—	—
bp range		225–239					229–239		
Cj119									
# alleles	—	3(29)	—	—	—	—	—	—	—
bp range		182–186							
Cj122									
# alleles	—	7(49)	—	—	—	—	3(31)	—	—
bp range		365–389					377–389		
Cj127									
# alleles	—	4(13)	—	—	—	—	—	—	—
bp range		341–367							
Cj128									
# alleles	—	5(43)	—	—	—	—	3(9)	—	—
bp range		226–252					246–254		
Cj131									
# alleles	—	1(28)	—	—	—	—	6(19)	—	—
bp range		232					228–244		
Cp10									
# alleles	—	2(41)	—	—	—	—	3(28)	—	—
bp range		194–204					194–202		
CU4-121									
# alleles	—	2(31)	—	—	—	—	3(32)	—	—
bp range		174–182					178–184		
CU5-123									
# alleles	5(30)	—	2(2)	3(6)	no	no	—	3(6)	1(3)
bp range	220–224		198–200	204–226				200–210	198
CUC-20									
# alleles	2(38)	2(41)	—	2(6)	1(2)	no	1(20)	1(10)	no
bp range	164–168	174–176		164–176	164		167	164	
CUD-68									
# alleles	3(44)	1(24)	2(2)	no	1(2)	2(1)	6(20)	2(10)	1(3)
bp range	127–131	129	131–133		125	155–165	123–145	127–131	123

Table 2 — *continued*

Locus	Species								
	C.a.	*C.j.*	*C.mi.*	*C.mo.*	*C.n.*	*C.pa.*	*C.po.*	*C.r.*	*O.t.*
CUD-78									
# alleles	2(30)	1(12)	1(2)	2(6)	1(2)	no	3(60)	1(8)	1(3)
bp range	211–213	210	209	213–215	213		216–228	211	207
CUI-108									
# alleles	2(34)	—	2(7)	1(2)	no	no	—	—	1(9)
bp range	75–77		75–77	77					75
CUJ-131									
# alleles	5(38)	3(40)	2(2)	1(7)	1(2)	1(1)	3(40)	2(8)	no
bp range	240–254	165–177	262–264	250	246	264	202–212	256–260	
CUJB-131									
# alleles	3(40)	4(37)	1(2)	1(6)	1(2)	1(1)	3(20)	1(13)	2(3)
bp range	161–167	168–182	179	167	161	175	160–190	161	189–201
CUI-99.2									
# alleles	5(38)	1(8)	11(2)	4(7)	no	1(1)	2(8)	4(6)	no
bp range	162–178	159	164	174–182		162	159–165	184–216	
CR52									
# alleles	5(41)	—	2(2)	2(6)	no	no	—	2(10)	no
bp range	139–147		109–151	137–145				167–171	
pCp-8H4F									
# alleles	—	—	—	—	—	—	3(11)	—	—
bp range							100–104		

Note: "—" signifies not tested, "no" signifies that the primers did not amplify a reliable locus.

Table 3. Preliminary data on allele frequencies at three loci in two populations of *C. porosus* located in Queensland at Lakefield National Park (LNP, n = 18) and the Central coast region (C, n = 15) near Townsville.

Locus									
Cj18			C391			Cuj131		
alleles	frequency		alleles	frequency		alleles	frequency	
	C	LNP		C	LNP		C	LNP
184	0	0.22	236	0.04	0	202	0.1	0.48
206	0.06	0.11	242	0.22	0.06	204	0.87	0.42
212	0	0.19	244	0	0.38	206	0	0
216	0.61	0.28	246	0.11	0.06	208	0.03	0.02
219	0	0.08	248	0	0.06	210	0	0.08
220	0	0.03	250	0.59	0.31	212	0	0
224	0.33	0	252	0	0			
228	0	0.06	256	0	0.13			
230	0	0.03	260	0.04	0			

polymorphic loci, the average number of alleles was 2.6 (n = 15–24 individuals tested).

It is interesting to note that at some loci there is a large shift in allele size between species, as in the case of *C. porosus* and *C. johnstoni* at the C391 and Cuj131 loci. Such variation in allele size or in the number of alleles, suggests relatively dynamic loci that may be of interest in the study of microsatellite evolution. Forensic identification can also take advantage of unique allele sizes, or ranges as observed among species, particularly if a multi-locus analysis is undertaken on the specimens of interest.

It is hoped that these primers will enable the application of genetic investigations in other studies of *Crocodylus* spp. As in other species, these genetic markers will offer the most powerful insights when they are combined with demographic or behavioural data when available. Additionally, a combined approach using both mtDNA and microsatellite markers will be the most effective for elucidating questions of gene flow and

Table 4. Genotypes at the CUD68 locus of the surviving offspring from a single clutch, hypothesized parents and the alpha male in a captive population of *C. porosus*. Probable parental genotypes were deduced from offspring genotypes. The alpha male could only have fathered offspring under a situation of multiple paternity as yet undetected.

Genotype							
Offspring (n)			Probable parents		alpha male	
143	141	(4)	123	143	137	143
143	143	(3)	141	143		
123	141	(6)				
123	143	(5)				

population history. We hope that the availability of these microsatellite markers will allow comparative studies of mating systems and population structure among Crocodilians so we can move forward with the next set of questions.

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