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Characterization of Microsatellite DNA Loci in American Alligators

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Microsatellite loci of American alligators were identified from small insert DNA libraries. The average length of microsatellites (18.4 repeats) was similar to that observed in mammals. Polymerase chain reaction (PCR) primers were developed and tested for 20 microsatellite loci that contained at least 10 uninterrupted AC or AG repeats. Genotypes for the 15 loci that could be scored readily were obtained for alligators from Louisiana and Florida. Eleven of the 15 loci were polymorphic. For the polymorphic loci, the number of alleles per locus ranged from 4–17 (average = 8.5), and observed heterozygosity within populations ranged from 0.231–0.865 (average = 0.466). Heterozygosity of these loci is almost 20 times higher than values obtained using isozymes. Populations from Louisiana and Florida differed substantially at these loci (overall F_{ST} = 0.137, θ_p = 0.239, and R_{ST} = 0.387). The PCR primers also produced single amplicons from species of Alligatoridae, Gavialidae, and Crocodilidae, indicating that they may be useful for genetic studies in other crocodilians.

CROCODILIANS represent an ancient lineage of formerly common reptiles. Only eight of 124 described genera of Crocodilia survive, with 21 currently recognized species (Densmore, 1983). Many crocodilian species have been impacted severely by human activities. Of 32 regulated crocodilian species, subspecies, or populations, 19 are listed in CITES Appendix I, and the remaining 13 are listed in CITES Appendix II (U.S. Fish and Wildlife Service, 1994). The American alligator (*Alligator mississippiensis*) suffered severe population declines across its entire range during the 1960s and received full legal protection. This species is the most studied crocodilian, and basic research has assisted its recovery and management.

A need exists for polymorphic genetic markers for basic research in genetics, population biology, and reproductive ecology of crocodilians. Isozyme studies of alligators (Gartside et al., 1977; Menzies et al., 1979; Adams et al., 1980), however, have revealed low levels of intrapopulation variability. Population bottlenecks often are invoked as ad hoc explanations of low genetic variability (e.g., O'Brien et al., 1985), but alligators have maintained census population sizes of hundreds to thousands in several areas throughout historical times (Joanen and McNease, 1987). Although effective population size may differ substantially from census size, we believe it unlikely that effective population sizes were reduced in historical times to levels low enough to account for the observed level of protein variation (cf. Nei et al., 1975). Nile crocodiles also have been found to have low isozyme variability (Lawson et al., 1989), indicating that traditional genetic approaches may be of limited use in other crocodilians as well.

The primary goal of this research was to discover polymorphic loci that would be useful for genetic studies of American alligators. Our strategy was to isolate and characterize microsatellite loci for this purpose. Briefly, microsatellites are small tandem arrays of DNA sequences with unit repeat motifs of 1–5 base pairs (bp) and total array sizes of 10 to a few hundred bp (Charlesworth et al., 1994). Because most microsatellite loci are highly polymorphic and amenable to automated analysis, they are becoming the genetic markers of choice for addressing questions concerning genetic diversity and relatedness in wild and captive populations (Bruford and Wayne, 1993; Queller et al., 1993). Microsatellite loci described here display high levels of intra- and interpopulation variability, demonstrating their utility for studies of alligators. Many of the loci are conserved across crocodilian species, suggesting that some of the loci will be useful as genetic markers in other crocodilians.

MATERIALS AND METHODS

DNA was isolated from 19 American alligators sampled from marshes on the Rockefeller Wildlife Refuge (RWR), Cameron Parish, Louisiana (LA sample), and 14 alligators from the Everglades National Park, Dade County, Florida (FL sample). The FL sample consisted of wild-caught adults, whereas the LA sample consisted of adults maintained in breeding pens. The LA population was established from a sample of young alligators hatched from eggs collected

from wild nests on the RWR. DNA also was isolated from 10 alligators collected in Terrebonne Parish, Louisiana, as a group of hatchlings (Nest Site sample), and from at least one individual of each of 20 other crocodilian species (see Material Examined).

Blood was collected without injury to individuals from either an anterior dorsal sinus or caudal vein (Dessauer et al., 1996) and added directly to 10 volumes of extraction buffer. DNA was isolated from red cells by standard proteinase K digestion, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (Sambrook et al., 1989). DNA concentration, purity, and fragment length were assayed by ultraviolet absorption and electrophoresis through agarose gels stained with ethidium bromide (Sambrook et al., 1989).

Protocols used to obtain and score microsatellites (Glenn, 1997) are available on the Internet at ftp://onyx.si.edu/protocols/MsatManV6.rtf. Briefly, 300-700 bp fragments of genomic alligator DNA were ligated into a plasmid. Portions were used for a random library or libraries enriched for microsatellites (Ostrander et al., 1992). Transformed bacterial colonies were screened by hybridization with radioactively labeled $d(AG)_{12}$ and $d(CA)_{12}$ or $d(AG)_{12}$ and $d(TG)_{15}$. Positive clones were sequenced according to the method of Meeker et al. (1993) or Rouer (1994) by using Sequenase 2.0 (US Biochemical). PCR primers were designed from sequences flanking the repetitive elements and designated by a number representing the order in which the loci were discovered. Microsatellites with AC repeats were numbered beginning with 1, and those with AG were numbered beginning with 101 [i.e., locus *AC-1* of Glenn et al. (1996) = *Amiμ1*, and *AG-1* = *Amiμ101*].

PCR amplifications had final concentrations of 50 mM KCl, 10 mM Tris-HCl pH 9, 1% Triton X-100, 1.5 mM $MgCl_2$, 150 μM of each dNTP, 0.5 μM of each primer, 1 unit *Taq* DNA polymerase and 50 ng of DNA. During optimization, annealing temperature was varied and/or bovine serum albumin (BSA; 250 μg/mL) was added. Thermocycling parameters were 94 C for 2 min, followed by 30 cycles of 94 C for 1 min, annealing temperature (Appendix 1) for 30 sec, and 72 C for 30 sec. During population surveys, 0.1 μM of one primer was radioactively labeled with ^{32}P, and 25 cycles were used. Radioactive PCR products were denatured and loaded onto large denaturing polyacrylamide gels. DNA sequencing ladders were used as size standards. After electrophoresis, gels were dried, autoradiographed at room temperature, and scored visually. Because allele sizes are difficult to compare across studies (Knowles et al., 1992), anyone wishing to compare these data with their own is encouraged to obtain reference samples from the authors.

We tested the ability of the primers to produce specific PCR products from DNA of other crocodilians in two separate experiments. PCR conditions were identical to those used for American alligators. Products were assayed on 2% agarose gels. Tests were considered positive when one or two bands of similar size and intensity to those from American alligators were produced. Decreasing stringency of PCR increased taxonomic breadth of taxa amplified, but it also tended to increase presence of extra nonspecific bands and smearing.

We used the following programs for data analysis: (1) Genestrut (Constantine et al., 1994) for calculation of allele frequencies, observed and expected heterozygosities (H_{obs} and H_{exp}), allele counts, and *F*-statistics; (2) GENEPOP 2 (Raymond and Rousset, 1995) for tests of Hardy Weinberg equilibrium (Rousset and Raymond, 1995), genotypic disequilibrium, θ_p, and probability tests of θ_p; (3) a Beta test version of Genetic Data Analysis (available via the Internet at http://chee.unm.edu/gda/) for θ_p estimation by the method of Weir and Cockerham (1984) and bootstrapping; (4) JMP 3.1.5 (SAS Institute) for statistical analyses; and (5) Excel 5.0 (Microsoft) for variance calculations used for estimation of R_{ST} (Slatkin, 1995). Tables of individual genotypes used for analyses are available from the authors (or at http://gator.biol.sc.edu/).

Results and Discussion

Thirty clones containing microsatellites were identified and sequenced. Five were obtained from the random library, 11 from the library enriched with $(AC)_{12}$ and $(AG)_{12}$, and 14 from the library enriched with $(AG)_{12}$ and $(TG)_{15}$. The average length of uninterrupted repeats from the random library was 16, whereas the average length of repeats for the two enrichments were 18.5 and 18.4 repeats, respectively. The distribution of the number of repeats in the cloned alleles (Fig. 1) was similar to distributions in other vertebrates (Ostrander et al., 1993; Slettan et al., 1993; FitzSimmons et al., 1995). Sequences of the cloned alleles were deposited in Genbank (accession numbers AF042037–AF042057).

PCR primers were designed from sequences of 21 of the 30 clones (Appendix 1). Nine clones were not studied further because there were inadequate lengths of flanking sequence to design primers, the sequence was of poor

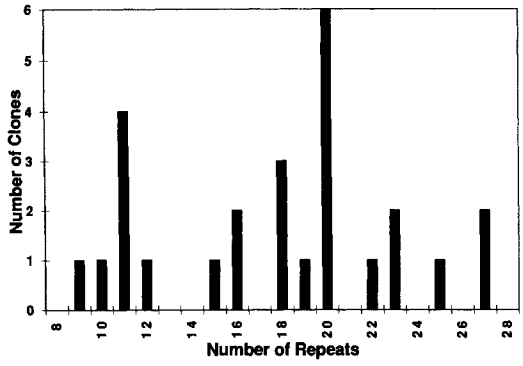

Fig. 1. Distribution of the number of repeats in microsatellite clones sequenced from American alligators. The longest uninterrupted run of tandem repeats from each clone is graphed.

quality, or no PCR primers could be designed to meet minimal criteria for acceptability (Rychlik, 1993). One of the 21 clones (*Amiμ-14*) contained two microsatellites separated by several hundred bp. This locus was not studied further. A simple strategy for optimization of PCR conditions (Glenn, 1997) yielded amplification products from all primer pairs, although consistency of amplification and clarity of alleles varied considerably among loci (Appendix 1). Five loci were not studied further because they gave inconsistent amplification or patterns that were difficult to interpret. Thus, half of the microsatellite-positive clones yielded loci that were surveyed for polymorphism, a typical result for development of microsatellite loci.

Four of 15 microsatellite loci surveyed were monomorphic among 28 individuals examined (14 FL and 14 LA); the remaining 11 loci were polymorphic (Table 1), even among an initial survey of 10 individuals (5 FL and 5 LA). We made no attempt to discover or quantify nonamplifying (null) alleles (Pemberton et al., 1995). An average of four alleles per locus were found in both the FL and LA samples (Tables 1–2). If the Nest Site sample is included, 4–17 alleles per locus were found, and the average number of alleles increases to 6.8 for the 15 loci (8.9 if monomorphic loci are excluded). Observed heterozygosities at polymorphic loci were similar in the LA and FL samples, ranging from 0.23–0.86, and averaging 0.47 (Table 2). These values are similar to those observed in large populations of mammals but much higher than those reported for small populations of endangered mammals (Taylor et al., 1994; Gotelli et al., 1994). When all loci are combined, each individual in our sample had a unique genotype.

Genotypic disequilibrium values were calculated for all possible pairs of loci in the LA and FL samples. Nine of the 210 comparisons had *P*-values equal to or less than 0.05, and one pair of loci, *Amiμ6* and *Amiμ102*, had disequilibrium values equal to or less than 0.05 in both populations (LA $P = 0.05$; FL $P = 0.02$; combined $\chi^2 = 13.4$, 4 df, $P = 0.009$), suggesting that these loci are physically linked or otherwise correlated. No disequilibrium values were significant after correction for multiple comparisons (Rice, 1989). Thus, there does not appear to be significant linkage among these microsatellite loci.

Genotype frequencies were not consistent with the expectations of Hardy-Weinberg equilibrium (HWE) in two of 22 locus/population comparisons (Table 2). However, only one comparison, *Amiμ15* in the FL population, was significant after correcting for multiple comparisons (Rice, 1989). The deviation of *Amiμ15* in the FL population was due to an excess of homozygotes and may indicate the presence of null alleles. Even though our power to reject HWE is low (Rousset and Raymond, 1995), the LA population sample may contain siblings, and at least one locus may have null alleles. For the purpose of this paper, we accept that the two populations are in HWE.

Three previous studies used isozymes to survey genetic variation in alligators. Only three protein loci were polymorphic in alligators from LA (Gartside et al., 1977), two in animals from FL (Menzies et al., 1979), and five in a sample from South Carolina (Adams et al., 1980). Average heterozygosity ($\bar{H}$) also was very low: $\bar{H}$ for the alligators from LA was 0.021 (50 protein loci) and is among the lowest observed for a vertebrate species (Nevo et al., 1984).

Searches for DNA polymorphism also have shown relatively low diversity in the alligator genome. Examples include the M-13 DNA-fingerprinting probe (Vassart et al., 1987) where 10 alligators from the LA population were monomorphic (HCD, unpubl.) and the banded krait minor satellite DNA (Bkm) probe where only limited polymorphism was found (Demas and Wachtel, 1991). Even randomly amplified polymorphic DNAs (RAPDs; Bowditch et al., 1993) failed to detect high levels of variation (HCD, unpubl.). Although some of the RAPD amplicons were polymorphic, the percentage of arbitrary primers that revealed polymorphisms in alligator DNA (three of 20 tested on 20 individuals, HCD, unpubl.) was far lower than for other species that are thought to have large population sizes (Nusser et al., 1996).

Microsatellite loci reported here are the most polymorphic of any class of genetic marker used

TABLE 1. ALLELE FREQUENCIES AT POLYMORPHIC MICROSATELLITE LOCI IN POPULATIONS OF AMERICAN ALLIGATOR (*Alligator mississippiensis*) FROM LOUISIANA (LA) AND FLORIDA (FL). Sample sizes are in Table 2. Allele designations represent estimates of total allele size in base pairs.

	Amiμ3			*Amiμ5*			*Amiμ13*			*Amiμ16*	
Allele	LA	FL	Allele	LA	FL	Allele	LA	FL	Allele	LA	FL
300	0.059	0	253	0	0.071	232	0.517	0.346	232	0.737	0
308	0	0.296	257	0	0.179	236	0.103	0	236	0.026	0
310	0.588	0.222	259	0.474	0.071	238	0.379	0	240	0	0.429
312	0.353	0.333	261	0.395	0.679	240	0	0.654	242	0	0.036
314	0	0.148	263	0.132	0				244	0.237	0.536
	Amiμ6			*Amiμ15*			*Amiμ18*			*Amiμ20*	
Allele	LA	FL	Allele	LA	FL	Allele	LA	FL	Allele	LA	FL
114	0	0.179	149	0	0.107	162	0.237	0	140	0.429	0.143
122	0	0.143	151	0.026	0.464	170	0.184	0	142	0.057	0
126	0.216	0	153	0	0.143	178	0.184	0	144	0.314	0.107
128	0.135	0.679	159	0.789	0.107	182	0.237	0.571	146	0.086	0.107
132	0.378	0	161	0.079	0.036	186	0	0.25	160	0	0.036
134	0.027	0	163	0.105	0.036	188	0.158	0.143	162	0.086	0.357
136	0.243	0	165	0	0.107	190	0	0.036	164	0.029	0.071
									168	0	0.179
	Amiμ8			*Amiμ17*			*Amiμ102*				
Allele	LA	FL	Allele	LA	FL	Allele	LA	FL			
130	0	0.107	218	0	0.036	221	0	0.462			
132	0	0.036	230	0.056	0	223	0	0.077			
134	0.289	0.321	238	0	0.143	227	0.027	0			
136	0	0.357	242	0	0.464	231	0	0.077			
138	0.5	0.107	246	0	0.214	233	0	0.038			
140	0.026	0.036	250	0	0.036	235	0	0.038			
144	0.053	0.036	254	0.056	0.071	237	0	0.038			
148	0.026	0	258	0.028	0.036	243	0.27	0			
152	0.026	0	260	0.056	0	245	0.216	0			
154	0.079	0	262	0.278	0	247	0.135	0.038			
			264	0.083	0	249	0.108	0.077			
			266	0.306	0	251	0	0.154			
			272	0.056	0	253	0.027	0			
			276	0.083	0	255	0.081	0			
						257	0.027	0			
						261	0.108	0			

thus far to characterize variation in alligators. The number of alleles and heterozygosity detected at polymorphic loci is similar to those in large outbred vertebrate populations (Glenn, 1997). However, alligator microsatellites have the lowest proportion of polymorphic loci thus far reported in vertebrates: birds (e.g., Crooijmans et al., 1995), turtles (e.g., FitzSimmons et al., 1995), mammals (e.g., Gottelli et al., 1994), and fish (e.g., Slettan et al., 1993). This pattern of variability supports the view that recent population bottlenecks of alligators in the LA and FL populations have not affected their genetic variation (i.e., number of alleles and heterozygosity at polymorphic loci). It is, however, consistent with a prehistorical (i.e., pre-Columbian) population bottleneck (or selective event) of larger magnitude that eliminated variation from much of the genome. Because variation would be regained first at loci with the highest mutation rates, a prehistorical bottleneck (or selective event) hypothesis also is consistent with the low level of protein polymorphism and DNA diversity in regions of the genome with lower mutation rates. A population bottleneck that reduced the American alligator population to numbers far below historical levels, for example at the peak of North American glaciation, is consistent with the genetic data.

Alligators from the FL and LA populations differed substantially in allele frequencies at microsatellite loci (Table 1). Allele frequencies

TABLE 2. SAMPLE SIZE (n), NUMBER OF ALLELES (A), OBSERVED HETEROZYGOSITY (H_o), EXPECTED HETEROZYGOSITY (H_e), AND TOTAL EXPECTED HETEROZYGOSITY (H_t) FOR POLYMORPHIC MICROSATELLITE LOCI.

	Everglades, FL				Rockefeller, LA				Overall
Locus	n	A	H_o	H_e[a]	n	A	H_o	H_e[a]	H_t
Amiμ3	14	4	0.815	0.758	17	3	0.471	0.542	0.700
Amiμ5	14	4	0.500	0.516	19	3	0.632	0.619	0.633
Amiμ6	14	3	0.500	0.505	19	5	0.595	0.752	0.770
Amiμ8	14	7	0.714	0.770	19	7	0.579	0.673	0.787
Amiμ13	14	2	0.231	0.471	16	3	0.759	0.599	0.677
Amiμ15	14	7	0.571	0.754	19	4	0.368	0.368	0.743
Amiμ16	14	3	0.786	0.548	19	3	0.421	0.411	0.674
Amiμ17	14	7	0.786	0.735	18	9	0.722	0.825	0.888
Amiμ18	14	4	0.571	0.611	19	5	0.789	0.817	0.779
Amiμ20	14	7	0.857	0.820	17.5	6	0.686	0.719	0.816
Amiμ102	13	9	0.769	0.769	19	9	0.865	0.853	0.898
Mean[a]		4	0.473	0.484		4	0.459	0.479	0.760

[a] All probabilities of Hardy Weinberg equilibrium except *Amiμ15* FL, were not significant following corrections for multiple tests (Rice, 1989). Means include monomorphic loci (*Amiμ1, Amiμ12, Amiμ19,* and *Amiμ101*; n = 28; 14 FL and 14 LA for each locus).

were especially distinct at *Amiμ16, Amiμ17,* and *Amiμ102,* where the most common allele in the LA sample was not present in the FL sample (Table 1). Both F_{ST}-values and θ_p-values were relatively high at all 11 polymorphic loci, averaging 0.137 and 0.239, respectively (Table 3). Chi-square tests of F_{ST}, and probability tests or bootstrapping of θ_p, indicated that the two populations differed significantly ($P < 0.05$) at all 11 polymorphic loci. Although θ_p and F_{ST} are based on different models, with θ_p having theoretical advantages (Cockerham and Weir, 1993), our estimates of the two are highly correlated ($r^2 = 0.999$; rho = 0.998, $P < 0.001$), and differ only in absolute magnitude (Table 3).

We also estimated population divergence by using R_{ST} (Slatkin, 1995). The model used to construct R_{ST} is distinct from those underlying F_{ST} and θ_p estimates in that R_{ST} incorporates the concept that alleles of similar size are more closely related than alleles of very different size. As expected (Slatkin, 1995), the estimate of R_{ST} across all polymorphic loci (0.387) was greater than that for θ_p or F_{ST} (Table 3). However, there was no significant correlation between individual locus estimates of θ_p and R_{ST} or F_{ST} and R_{ST}.

The differences among these estimators of population divergence are illustrated by locus *Amiμ17* where many alleles differ substantially in size (Table 1). Because allele size at this locus differs substantially between LA and FL populations, *Amiμ17* has the largest estimate of R_{ST} (Table 3). However, because both populations have high within-population heterozygosity, the estimate of F_{ST} is intermediate in comparison to other loci (Table 3). This underscores the differences between the models used for F_{ST} and R_{ST} and emphasizes that comparisons between

TABLE 3. GENETIC DIVERGENCE[a] OF LA AND FL ALLIGATOR POPULATIONS.

Locus	F_{IS}	F_{IT}	F_{ST}	f	F	θ_p	R_{ST}
Amiμ3	0.008	0.082	0.074	0.042	0.174	0.137	0.022
Amiμ5	0.003	0.106	0.103	0.001	0.184	0.185	−0.060
Amiμ6	0.134	0.289	0.180	0.166	0.415	0.299	0.296
Amiμ8	0.106	0.178	0.081	0.112	0.245	0.151	0.201
Amiμ13	0.072	0.269	0.213	0.077	0.401	0.351	0.096
Amiμ15	0.168	0.368	0.240	0.148	0.476	0.386	0.218
Amiμ16	−0.271	0.105	0.295	0.238	0.333	0.461	0.489
Amiμ17	0.034	0.151	0.121	0.048	0.250	0.213	0.586
Amiμ18	0.049	0.126	0.081	0.046	0.187	0.147	0.373
Amiμ20	−0.004	0.055	0.058	0.018	0.128	0.112	0.281
Amiμ102	−0.009	0.090	0.098	0.008	0.184	0.177	0.365
Overall	0.031	0.164	0.137	0.044	0.273	0.239	0.387

[a] F_{IS}, F_{IT}, and F_{ST} are estimators of Nei and Chesser (1983); f, F, and θ_p are estimators of Weir and Cockerham (1984) and are analogous to Wright's F_{IS}, F_{IT}, and F_{ST}, respectively. R_{ST} is Slatkin's (1995) estimator of population differentiation. All values of F_{ST} and θ_p are highly significant ($P \leq 0.01$).

estimates of F_{ST} and R_{ST} may be misleading unless a large number of polymorphic loci (e.g., ≥ 20) are assessed.

The populations from FL and LA also differed significantly in isozyme frequencies. Based on data in Adams et al. (1980), and assuming HWE, we estimated overall values of $F_{ST} = 0.060$ and $\theta_p = 0.115$. These estimates, however, are based on only three polymorphic loci: *Alb*, $F_{ST} = 0.026$, $\theta_p = 0.060$, $P > 0.05$; *Ldh-2*, $F_{ST} = 0.054$, $\theta_p = 0.112$, $P < 0.05$; and *Pep-1*, $F_{ST} = 0.084$, $\theta_p = 0.137$, $P < 0.01$). Assessment of divergence at these protein loci thus relies largely upon a single polymorphic locus (i.e., *Pep-1*).

Major advantages of using microsatellites rather than isozymes to estimate population divergence include the following: (1) more polymorphic loci upon which to base estimates of genetic divergence; (2) rapid generation of alleles at microsatellite loci such that divergence may occur faster than by drift alone (Slatkin, 1995); and (3) assuming a stepwise mutation model, higher information content because ancestry of alleles at microsatellite loci may be inferred from size (Slatkin 1995). Major disadvantages of using microsatellites to estimate population divergence include the following: (1) high mutation rates that may lead to saturation more quickly than other molecular markers; (2) the potential for homoplasious mutations (Glenn, 1997); and (3) potential limits to divergence in large populations if the number of possible allelic states is small (Nauta and Weissing, 1996). However, with respect to the last, the number of possible allelic states is rarely as restricted as in the models used by Nauta and Weissing (1996). Consequently, allopatric populations are unlikely to converge upon the same microsatellite alleles (or frequencies of those alleles) at all loci. Homoplasious mutations also will cause estimates of divergence to be conservative. This suggests that, although the time that microsatellite divergence increases linearly may be only a few hundred to a few thousand generations (Goldstein et al., 1995), microsatellites may detect divergence beyond that time scale.

To examine cross-species utility of our primers, we tested them for their ability to amplify microsatellites in other crocodilians (Appendix 2), including species of the genus *Crocodylus* (Appendix 3). Sixty percent of all heterologous species/locus tests were positive or weakly positive. Positive tests were more common with species that are closely related to American alligators. Examples include *A. sinensis* (positive in 94% of tests) and the alligatorid genera *Caiman*, *Melanosuchus*, and *Paleosuchus* (positive in 72% of tests). Species from other families (genera *Tomistoma*, *Gavialis*, and *Crocodylus*) yielded positive amplification less often (Appendices 2–3). Some loci appear more highly conserved than others: *Amiμ1* and *Amiμ101* were positive for all crocodilians tested, whereas *Amiμ5* was positive only for American alligators. R. B. Zucoloto (pers. comm.) recently amplified and scored microsatellites from *Caiman latirostris* by using the conserved and polymorphic loci identified in this study.

Interestingly, there is a negative correlation between evolutionary conservation of amplifiability and allelic diversity in these loci (Glenn et al., 1996). Initial data (N. N. FitzSimmons pers. comm.) indicate that most of the loci described here either are not conserved or are not polymorphic in species of the genus *Crocodylus*. We suspect that the alligator primers will be quite useful in studies of other species of Alligatoridae and Gavialidae but less useful for studies of species of Crocodilidae (Glenn et al., 1996).

Material Examined

Samples with HCD numbers are in the frozen tissue collection of the Louisiana State University Museum of Natural Science; samples with LD (Llewellyn Densmore III) numbers are in the collections of Texas Tech University.

Alligatoridae.—Alligator mississippiensis, Rockefeller Wildlife Refuge, Cameron Parish, LA: HCD 5990A-S (19 individuals); Everglades National Park, Dade County, FL: HCD2589, 2625, 2597, 2626, 2604, 2638, 2611, 2640, 2615, 2647, 2618, 2649, 2620, 2650; Terrebonne Parish, LA; Marsh south of Houma:, HCD5904, 5905, 5906, 5907, 5909, 5910, 5911, 5912, 5913, 5914. *Alligator sinensis*, LD31387-5. *Caiman crocodilus*, HCD3380, HCD4236. *Caiman latirostris*, LD31587-5. *Melanosuchus niger*, LD93087-1. *Paleosuchus palpebrosus*, LD100187-8, LD100187-9.

Crocodylidae.—Crocodylus acutus, HCD3071, HCD3073. *Crocodylus cataphractus*, LD31787-2. *Crocodylus intermedius*, LD31787-1. *Crocodylus johnsoni*, LD93087-5. *Crocodylus mindorensis*, LD3791-2. *Crocodylus moreletii*, LD31587-10. *Crocodylus niloticus*, LD60292-11. *Crocodylus novaeguineae*, LD3791-1. *Crocodylus palustris*, LD51091-1. *Crocodylus porosus*, LD52291-3, HCD6011. *Crocodylus rhombifer*, LD31387-6, LD110989-9. *Crocodylus siamensis*, LD61591-1, LD52291-2. *Osteolamus tetraspis*, LD71390-1, LD52291-1.

Gavialidae.—Gavialis gangieticus, HCD4219, *Tomistoma schlegelli*, HCD3361, LD31787-4.

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APPENDIX 1. CHARACTERISTICS OF PRIMERS USED TO AMPLIFY 20 ALLIGATOR MICROSATELLITE LOCI. Forward primers are given above and reverse primers are given below.

Locus	Primer sequence	Tm[a]	Repeat[b]	Temp[c]	Size[d]	Scoring[e]	Consistency[f]
Amiμ1	TTCCCTAACTCGGCAACCAA	55	$(AC)_{11}$	50	194	OK	OK
	CAGCTCAGCATTTATCAGACCA	52					
Amiμ2	CATCATGACCACCTCTGGAGATGACC	62	$(AC)_{20}$	53	99	Poor/OK	Poor
	AAAGGTGAATGTGTTGGTGCCT	55					
Amiμ3	ACTGTTGCTTTTCCTAATCTGTTT	51	$(AC)_{16}$	50	240	Good	OK
	CATAAAATTGTGCTAATGTCCC	49					
Amiμ5	CCGACCCTACTAACTATCAAA	46	$(AC)_{20}$	50	265	OK	OK/Poor
	TATCTCACAATCTCCTACCTT	43					
Amiμ6	TTCTTTCCCAGATACACACTT	46	$(AC)_{20}$	55	126	OK	OK
	AGTAGAAGGGGACAGGTTATT	46					
Amiμ7	CATTTACTGGCTCTTGGTTTA	47	$(AC)_{11}$	47	138	OK	OK[g]
	TCTTCCATCAGCAGNATTCTT	46					
Amiμ8	CCTGGCCTAGATGTAACCTTC	50	$(AC)_{>10}$	55	>115	OK	OK
	AGGAGGAGTGTGTTATTTCTG	45					
Amiμ9	GCAGTGCCAGCGTCAGGAG	57	$(AC)_{18}$	60	152	Poor	Poor
	CTGGGGGTGCAGAGGCAACA	60					
Amiμ10	GTGCCCATTAGACCCTCCAT	53	$(AC)_{18}$	55	125	Poor	Poor
	GTTATTCCTGCTCCCCTTCC	52					
Amiμ11	AAGAGATGTGGGTGCTGCTG	53	$(AC)_{23}$	64	246	OK	OK[g]
	TCTCTGGGTCCTGGTAAAGTGT	52					
Amiμ12	CTTTCACTCCCTACACTCCTACTT	50	$(AC)_{13}(AT)_9(AT)_7$	47	237	OK	OK
	CACATATCACTAATTCGTATATCA	44					
Amiμ13	CCATCCCCACCATGCCAAAGTC	62	$(AC)_{>16}$	60	>170	Good	Good
	GTCCTGCTGCTGCCTGTCACTC	59					
Amiμ14	TCTTTGTGTATGGCAGTGCAAGC	57	$(AC)_{15}$	ND[h]	ND[h]	ND[h]	ND[h]
	CACCCCCCACCTGGAATTGT	58					
Amiμ15	CACGTACAAATCCATGCTTTC	50	$(AC)_{16}$	60	159	OK/Good	Good
	GGGAGGGTTCAGTAAGAGACA	50					
Amiμ16	TTTGGGCTGTGAAACAAGTATT	51	$(AC)_{27}$	60	249	OK	Good
	TCCCCTGATAGTCTTCTATAAAC	46					
Amiμ17	GCTGACCTTGGTTGGAAACTCTA	54	$(AC)_{22}(AT)_{7-}$	55	234	Good	Good
	CCTGTTCTTGCATAAANCTGATA	49	$(ATGT)_{12}(AGAT)_9$				
Amiμ18	ATCTCCGAGGGGAAAAATACA	52	$(AC)_{23}$	60	188	Good	OK/Poor
	AATAGATGGAGTGATGTTATAGTCAG	48					

APPENDIX 1. CONTINUED

Locus	Primer sequence	Tm[a]	Repeat[b]	Temp[c]	Size[d]	Scoring[e]	Consistency[f]
Amiμ19	GCTCTGCTGGGTGTGATACTT	51	$(AC)_{11}(CT)_{11}$	60	143	OK	Good
	GCATCCACCCCTGTTCTCTGT	55					
Amiμ20	TTTTTCTTCTTTCTCCATTCTA	45	$(AC)_{25}$	55	162	Good	Good
	GATCCAGGAAGCTTAAATACAT	47					
Amiμ101	GATCCCAGCGCTCTCTCT	49	$(AG)_{15}$	50	131	OK	Good
	CCCTGGTTTCACTAAGTATTTGG	52					
Amiμ102	TTTGGAACTTTTTGAGACTTTTAC	49	$(AG)_{20}(AT)_{20}$	57	221	OK	Good
	TCCAGAAAGGCATTAAAAACCTA	52					

[a] Predicted melting temperature of oligonucleotides under standard PCR conditions (Rychlik, 1993).
[b] Repeat structure of allele initially cloned.
[c] Optimal annealing temperature in C (determined empirically).
[d] Expected size (bp) of amplicon derived from clone sequenced.
[e] Ease with which microsatellite genotypes can be assigned.
[f] Robustness of PCR conditions to amplify reliably a single product.
[g] Duplicated locus (2–4 alleles detected per individual).
[h] Not determined.

APPENDIX 2. AMPLIFICATIONS OF FRAGMENTS OF SIMILAR SIZE FROM OTHER CROCODILIANS.

	Alligatoridae					Gavialidae		Crocodilidae			
Locus	MIS	SIN	CAI	MEL	PAL	TOM	GAV	OST	CAT	NIL	RHO
Amiμ1	+	+	+	+	+	+	+	+	+	+	+
Amiμ3	+	+	+	+	+	+	+	−	+	+	+
Amiμ5	+	−	−	−	−	−	−	−	−	−	−
Amiμ6	+	+	−	−	−	+	+	−	−	−	−
Amiμ7	+	+	+	+	−	−	−	−	−	−	−
Amiμ8	+	+/−	+/−	+	+	+/−	+/−	−	−	−	+
Amiμ10	+	+	+	+	+	−	−	−	−	+/−	−
Amiμ11	+	+	+	+	+	−	−	−	−	−	−
Amiμ12	+	+	+	+	+	+	+	−	+	+	+
Amiμ13	+	+	+/−	−	+	+	+	−	+	−	−
Amiμ15	+	+	−	+	−	−	−	−	−	−	−
Amiμ16	+	+	+	+	+	+	+	−	−	−	−
Amiμ17	+	+	−	+/−	+	+	+	−	+	−	−
Amiμ18	+	+	−	+	+	+	+/−	−	+/−	−	−
Amiμ19	+	+	−	+	+	+	+	−	+/−	+/−	+/−
Amiμ20	+	+	+	+	+	+	+	−	+	+/−	+
Amiμ101	+	+	+	+	+	+	+	+	+	+	+
Amiμ102	+	+	+	−	−	−	−	−	−	−	−

MIS = *Alligator mississippiensis*; SIN = *Alligator sinensis*; CAI = *Caiman crocodilus*; MEL = *Melanosuchus niger*; PAL = *Paleosuchus palpebrosus*; TOM = *Tomistoma schlegelli*; GAV = *Gavialis gangieticus*; OST = *Osteolamus tetraspis*; CAT = *Crocodylus cataphractus*; NIL = *C. niloticus*; RHO = *C. rhombifer.*

APPENDIX 3. AMPLIFICATIONS OF FRAGMENTS OF SIMILAR SIZE FROM SPECIES OF *Crocodylus.*

	Crocodiliaae										
Locus	ACU	CAT	INT	JON	MIN	NIL	NOV	PAU	POR	ROM	SIA
Amiμ1	+	+	+	+	+	+	+	+	+	+	+
Amiμ3	−	+	−	−	−	+	+	+/−	+/−	+	−
Amiμ5	−	−	−	−	−	−	−	−	−	+	−
Amiμ8	−	−	+/−	+/−	+/−	−	+	−	+	−	+/−
Amiμ10	−	−	−	+/−	+/−	+/−	−	+/−	+/−	−	+/−
Amiμ20	−	+	+	+/−	+	+/−	−	+/−	+	+	+
Amiμ101	+	+	+	+	+	+	+	+	+	+	+

ACU = *Crocodylus acutus*; CAT = *C. cataphractus*; INT = *C. intermedius*; JOH = *C. johnsoni*; MIN = *C. mindorensis*; NIL = *C. niloticus*; NOV = *C. novaeguineae*; PAU = *C. palustris*; POR = *C. porosus*; ROM = *C. rhombifer*; and SIA = *C. siamensis.*

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