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PRIMER NOTE

Tetranucleotide microsatellites from the loggerhead sea turtle (*Caretta caretta*)

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Abstract

We describe primers and polymerase chain reaction conditions to amplify 15 tetranucleotide microsatellite loci from the loggerhead sea turtle (*Caretta caretta*). The primers were tested on 30 individuals that nested along the Georgia, USA coast. The primer pairs developed in this study yielded an average of 13.9 alleles per locus (range of 10–21), an average observed heterozygosity of 0.91 (range 0.79–1.00), and an average polymorphic information content of 0.88 (range 0.84–0.92).

Keywords: *Caretta caretta*, loggerhead turtle, microsatellites, primers, sea turtle, SSRs, tetranucleotide repeats

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The threatened loggerhead sea turtle (*Caretta caretta*) occurs globally in warm temperate and tropical ocean basins. Although extensive mitochondrial analyses aimed at characterizing phylogeography and the resolution of management units within this species have been conducted (Bowen *et al*. 1993, 1994; Encalada *et al*. 1998), complementary microsatellite analyses have received comparatively less attention until recently (Bowen *et al*. 2005). Despite demonstrated conservation of several microsatellite loci across all genera of extant cheloniid turtles (Fitzsimmons *et al*. 1995), there remains a need for additional, highly polymorphic loci to address questions of population genetics. We describe the development of a panel of species-specific, tetranucleotide microsatellite markers that will facilitate, in conjunction with previously developed dinucleotide microsatellite markers, the study of the relationship between kinship and spatial distribution in the loggerhead sea turtle.

We extracted DNA from blood obtained from two nesting (female) loggerheads using the DNAzol reagent (Invitrogen) followed by a phenol–chloroform extraction to remove remaining impurities. We digested DNA with *Rsa*I and *Bst*UI (New England Biolabs) and double-enriched for $(AAAG)_6$ $(ACCT)_6$ $(ACTC)_6$ $(AATC)_6$ $(ACAG)_6$ $(ACTG)_6$ $(AAAC)_6$ $(AATG)_6$ $(AGAT)_8$ $(AACT)_8$ $(AAGT)_8$ $(AAAT)_8$, and $(ACAT)_8$ (Glenn & Schable 2005). We ligated enriched product for tetranucleotide repeats into (polymerase chain reaction) PCR 2.1-TOPO vector, which was used to transform OneShot Top 10 Chemically Competent *Escherichia coli* cells (TOPO TA cloning kit, Invitrogen). We screened 672 colonies for inserts using the β-galactosidase gene and sequenced 672 positive (white) colony PCR products using BigDye (version 3.1, PE Applied Biosystems) chemistry and an ABI 3730 sequencer. Sequences were assembled and edited in SEQUENCHER 4.2 (Gene Codes) and exported to EPHEMERIS 1.0 (available at http://www.uga.edu/srel/DNA_Laboratory/dnacomputer_programs.htm) to search for microsatellite repeats. We designed 90 primer pairs for 200 contigs containing unique microsatellite repeats and added an M13-reverse or CAG tag to the 5′ end of one of each primer pair using OLIGO 4.0 (Molecular Biology Insights) to facilitate fluorescent size detection using ABI sequencers (Boutin-Ganache *et al*. 2001; Schable *et al*. 2002). Either a CAG or an M13-reverse tag was chosen for either the forward or reverse primer on the basis of minimizing self-complementarity, pair complementarity, and secondary structure of each primer or primer pair. We added GTTT 'pigtails' to the 5′ end of primers lacking either CAG or M13-reverse tag to facilitate the nontemplated addition of adenosine by *Taq* polymerase (Brownstein *et al*. 1996). We selected 48 primer pairs for testing based

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on microsatellite repeat number and primer characteristics used in choosing tags.

We optimized primer pairs using DNA samples obtained from loggerheads nesting on several Georgia barrier islands. DNA was extracted from samples using the DNeasy Kit (QIAGEN). PCR amplifications were performed in 10 μL volumes using GeneAmp PCR System 9700 thermal cyclers (PE Applied Biosystems). Final concentrations for optimizing reactions were 10 mM Tris pH 8.4, 50 mM KCl, 0.5 μM 'pigtailed' primer, 0.05 μM CAG or M13-reverse tagged primer (CAG or M13-reverse + primer), 0.45 μM dye-labelled tag (HEX or FAM + CAG or M13-reverse), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 U *Taq* DNA polymerase, and 50 ng DNA. M13 and CAG universal primers were labelled with FAM or HEX fluorescent dyes. Reactions were optimized with six individuals using two touchdown thermal cycling programs (Don *et al*. 1991), each encompassing a 10.5 °C span of annealing temperatures (ranges: 60–49.5 °C, 65.0–54.5 °C). Cycling parameters were: 21 cycles of 95 °C for 20 s; highest annealing temperature for 20 s at –0.5 °C per annealing cycle; and 72 °C for 30 s, followed by 14 cycles of 95 °C for 20 s; 49.5 °C or 54.5 °C, respectively, for 30 s; 72 °C for 30 s; and a final extension period of 10 min at 72 °C.

PCR products were checked for amplification and scored using an ABI 3730 sequencer with GENESCAN Rox500 fluorescent size standard (PE Applied Biosystems). Results were analysed using GENEMAPPER software (PE Applied Biosystems) and optimal touchdown cycling schemes were identified. Following optimization, 24 additional individuals were genotyped. We calculated observed and expected heterozygosities and polymorphic information content for each locus using CERVUS 2.0 (Marshall *et al*. 1998). GENEPOP 3.4 (Raymond & Rousset 1995) was used to test for Hardy–Weinberg equilibrium and genotypic linkage disequilibrium. We conducted a posteriori sequential Bonferroni correction (Rice 1989).

Table 1 summarizes the characteristics of 15 primer pairs developed from the loggerhead sea turtle. The number of alleles per locus ranges from 10 to 21, averaging 13.9. Total exclusionary power with both parents unknown is 1.000000. No deviations from Hardy–Weinberg equilibrium or significant linkage disequilibrium were detected after sequential Bonferroni correction.

Table 1 Characterization of 15 primer pairs amplifying microsatellite loci from loggerhead sea turtles (*Caretta caretta*) collected along the Georgia, USA coast

Locus	Primer sequence (5′–3′)	GenBank Accession no.	Dye	Annealing start temp. (°C)	Repeats in cloned allele	Clone size (bp)	N	A	Size range (bp)	H_O	H_E	P_{HW}	PIC
Cc1B03 F	***CAGTCGGGCGT***CATCATCAATAGCT	DQ917761	FAM	60	$(ATCT)_{15}$	304	30	13	264–314	0.87	0.87	0.30	0.84
	CAGTCATAGGT												
Cc1B03 R	GTTTTAGCAGACATAGGGGAGTG												
Cc1F01 F	***CAGTCGGGCGTCATCA***GTGTGAAG	DQ917762	FAM	60	$(ATCT)_{15}$	332	30	13	308–360	0.93	0.90	0.73	0.88
	GCTCTAAAACTAAT												
Cc1F01 R	GTTTTATACTGGGACGATAGGATAAA												
Cc1G02 F	***GGAAACAGCTATGACCAT***AGGTG	DQ917763	FAM	60	$(ATCT)_9$	286	30	14	262–322	0.90	0.94	0.15	0.92
	CCTAAACATTGATAGT												
Cc1G02 R	GTTTTATACTGTGCTCTTTCGTGTAAT												
Cc1G03 F	***CAGTCGGGCGTCATCA***GGAATGT	DQ917764	FAM	60	$(AGAT)_{20}$	324	30	12	276–328	0.93	0.91	0.91	0.89
	GCAGAATGTATGT												
Cc1G03 R	GTTTTAACGAGCATGTATCTAAAGTAA												
Cc1H11 F	***GGAAACAGCTATGACCA***TAACTTT	DQ917765	HEX	60	$(ATCT)_{16}$	249	29	12	215–263	0.93	0.89	0.33	0.86
	TGACTATCTCCCCCTAC												
Cc1H11 R	GTTTGACGTGATGCCTGGTGAG												

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Table 1 *Continued*

Locus	Primer sequence (5′–3′)	GenBank Accession no.	Dye	Annealing start temp. (°C)	Repeats in cloned allele	Clone size (bp)	*N*	*A*	Size range (bp)	H_O	H_E	P_{HW}	PIC
Cc2G10 F	***CAGTCGGGCGTCATCA***GTGGCAAG GTCAAATACAG	DQ917766	HEX	60	$(CTTT)_{16}$	301	30	21	261–326	0.90	0.94	0.89	0.92
Cc2G10 R	GTTTGCCCTTATTTGGTCACAC												
Cc2H12 F	***CAGTCGGGCGTCATCA***TCTTCAGG AGTTTTTGACTTG	DQ917767	FAM	60	$(AAAG)_8$ … $(AGGC)_9$	325	30	13	294–357	0.97	0.88	0.90	0.85
Cc2H12R	GTTTCCACACCCCTGTTTCAGA												
Cc5C08 F	GTTTCTTTGATGGTTTTTCTGTTATC	DQ917768	FAM	60	$(CTTT)_{18}$	325	30	13	281–333	0.93	0.93	0.89	0.90
Cc5C08 R	***CAGTCGGGCGTCA***TCAGTCTTCA GGGTATCGTGTAAT												
Cc5F01 F	GTTTAAAGGATTTGAGATGTTGTATG	DQ917769	HEX	60	$(CTTT)_{13}$	161	30	21	137–191	0.93	0.93	0.20	0.91
Cc5F01 R	***CAGTCGGGCGTCATCA***CCAGTTGTC TTTCTCCAGTG												
Cc5H07 F	***CAGTCGGGCGTCATC***AAGGAAGG AGAGGCTTATTAC	DQ917770	FAM	60	$(CTTT)_{16}$	239	29	11	215–263	1.00	0.89	0.20	0.86
Cc5H07 R	GTTTGGGCAATGAGACTGGAACTA												
Cc7B07 F	GTTTATAATGTTGGTGAGCAATATAG	DQ917771	HEX	65	$(ATCT)_{18}$	266	27	16	223–299	0.96	0.92	0.65	0.89
Cc7B07 R	***CAGTCGGGCGTCATCA***GGAGTTAA ACCAGGCACAGT												
Cc7C04 F	GTTTCCTAACCAACGGAGAAACA	DQ917772	HEX	60	$(AGAT)_{11}$	226	26	14	202–250	0.96	0.90	0.94	0.87
Cc7C04 R	***CAGTCGGGCGTCATCA***CTCCTTCAG AAGTCTTCACAT												
Cc7E11 F	GTTTGAAGAGCTGACCCCATATAG	DQ917773	FAM	60	$(AGAT)_{11}$	320	29	11	304–360	0.79	0.87	0.05	0.84
Cc7E11 R	***GGAAACAGCTATGACCAT***AAACAC AGAAATGAGGGATAG												
Cc7G11 F	GTTTCTCAGCATGAAAGTGTAATAC	DQ917774	FAM	60	$(AGAT)_{14}$	310	30	11	282–322	0.83	0.89	0.61	0.86
Cc7G11 R	***CAGTCGGGCGTCAT***CATGGATTTGT TTCAGTAATAG												
Cc8E07 F	GTTTAGCACTGGGTTGTGTGATTA	DQ917775	FAM	60	$(CTTT)_{12}$	285	30	12	273–321	0.80	0.89	0.11	0.86
Cc8E07 R	***GGAAACAGCTATGACCAT***ACCACC ATTCTGATTTGTTAG												

Sequences used to introduce sites for the universal primer are in bold italics.
Underlined bases indicate sharing of nucleotides between CAG (5′-CAGTCGGGCGTCATCA-3′) tag, M13R (5′-GGAAACAGCTATGACCAT-3′) tag, or GTTT 'pigtail' and the locus-specific primer binding site.
Clone size refers to the predicted size of the PCR product amplified from the clone used to develop each locus; *N*, number of individuals genotyped at each locus; *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HW}, probability that genotype proportions conform to Hardy–Weinberg equilibrium; and PIC, polymorphic information content.

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