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TECHNICAL NOTE

Tetranucleotide markers from the loggerhead sea turtle (*Caretta caretta*) and their cross-amplification in other marine turtle species

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Abstract The loggerhead sea turtle (*Caretta caretta*) is a federally threatened species and listed as endangered by the World Conservation Union (IUCN). We describe primers and polymerase chain reaction (PCR) conditions to amplify 11 novel tetranucleotide microsatellite loci from the loggerhead sea turtle. We tested primers using samples from 22 females that nested at Melbourne Beach, Florida (USA). Primer pairs yielded an average of 11.2 alleles per locus (range of 4–24), an average observed heterozygosity of 0.83 (range 0.59–0.96), and an average polymorphic information content of 0.80 (range 0.62–0.94). We also demonstrate the utility of these primers, in addition to primers for 15 loci previously described, for amplifying microsatellite loci in four additional species representing the two extant marine turtle families: olive ridley (*Lepidochelys olivacea*), hawksbill (*Eretmochelys imbricata*), green turtle (*Chelonia mydas*), and leatherback (*Dermochelys coriacea*).

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Extant marine turtles consist of seven species representing two families, the Cheloniidae and the Dermochelyiidae (Bowen and Karl 2007). With the exception of the data deficient flatback, the remaining six species of marine turtle are listed by the IUCN as vulnerable, endangered, or critically endangered. Mitochondrial DNA markers have been widely utilized to characterize marine turtle population structure, and microsatellite-based studies are becoming increasingly common (reviewed in Bowen and Karl 2007). The earliest surveys demonstrated conservation of a subset of microsatellite loci across marine turtle genera separated by greater than 100 million years of evolution (FitzSimmons et al. 1995). Despite this level of conservation, a need remains for additional, polymorphic loci to examine population genetics of marine turtles. Utilization of primers designed from the cheloniid turtles has proven particularly difficult with divergent leatherbacks, where null alleles or reduced polymorphism relative to the source species have been problematic (FitzSimmons et al. 1995; Rivalan et al. 2006). We describe the screening of 11 novel primer pairs in loggerheads as well as the cross-amplification of 26 primer pairs developed from loggerheads on 4 additional species of marine turtles: olive ridley, green turtle, hawksbill, and leatherback.

We identified 11 novel nuclear microsatellite loci by screening a microsatellite-enriched library for loggerhead turtles. Construction of the library and development of primers for 15 tetranucleotide loci for loggerhead turtle have been previously described (Shamblin et al. 2007). We screened primers from these 11 loci using samples from 22

Table 1 Characterization of 11 primer pairs amplifying tetranucleotide microsatellite loci in loggerhead turtles *Caretta caretta*

Locus	Primer Sequence (5′–3′)	GenBank Accession Number	Dye	Repeats in cloned allele	Clone size (bp)	*N*	A	Size range (bp)	H_0	H_E	P_{HW}	PIC
CcP2F11 F	***GGAAACAGCTATGACCAT***GTGCCTTAGGACTTGACTTG	EU125406	NED	$(AAAG)_{15}$	286	22	18	268–312	0.96	0.95	0.72	0.92
CcP2F11 R	GTTTGAAGAAAATAAATGAAACACTC											
CcP1F09 F	***GGAAACAGCTATGACCAT***AAATGTTGCCGTTTCTATTG	EU125405	FAM	$(ATCT)_{18}$	441	22	9	417–461	0.96	0.87	0.89	0.83
CcP1F09 R	GTTTCTCCCCCAACCTCTCAT											
CcP5C11 F	GTTTCTATTTGACACCACTCCACTCT	EU125396	HEX	$(AAAG)_7$	188	22	4	176–188	0.73	0.70	0.39	0.62
CcP5C11 R	***CAGTCGGGCGTCATCA***TTTGATTCTCTTCCCTTTACA											
CcP7D04 F	***CAGTCGGGCGTCATC***ATGAGCAAAGTAACCCTAACA	EU125400	FAM	$(AGAT)_{17}$	375	22	14	343–395	0.82	0.91	0.18	0.88
CcP7D04 R	GTTTGGAGCCAAATTAGAGATCAAC											
CcP7F06 F	***GGAAACAGCTATGACCA***TGACCACCCTCCAGTGAA	EU125402	HEX	$(ATCT)_{13}$	294	22	8	254–298	0.96	0.87	0.98	0.83
CcP7F06 R	GTTTGTGTTGGTTGATTTACTTCTATG											
CcP7C06 F	***CAGTCGGGCGTCATCA***TTAATTTCCTTCAGTTCAAGTG	EU125398	HEX	$(ATCT)_{15}$	306	22	10	274–314	0.86	0.89	0.29	0.86
CcP7C06 R	GTTTCAGCTTGAGCAAAGAGAGAG											
CcP7A08 F	***CAGTCGGGCGTCATCA***CTTGTGGCACCTTAGAAACTA	EU125397	FAM	$(AGAT)_8$	324	22	6	312–340	0.77	0.70	0.91	0.64
CcP7A08 R	GTTTAATTTTCCCTCTACTGTTATTC											
CcP7C08 F	***CAGTCGGGCGTCATCA***GAGCATTGGCAGCATAGA	EU125399	NED	$(ATCT)_6$	298	22	8	290–322	0.59	0.70	0.15	0.65
CcP7C08 R	GTTTAGTGAGTTCTAAAATGCCTAAC											
CcP7H10 F	***CAGTCGGGCGTCATCA***TCGAGACTCAATAAATACAGAGA	EU170009	NED	$(AGAT)_7$	215	22	6	203–223	0.73	0.78	0.94	0.72
CcP7H10 R	GTTTATAACAACACAGCATAAAATAAT											
CcP7E05 F	***CAGTCGGGCGTCATC***ACATAGAAACAGAGTTAAAAAATA	EU125401	HEX	$(AGAT)_{17}$	213	22	16	173–249	0.86	0.94	0.11	0.91
CcP7E05 R	GTTTGAAGAAATTAAACAGGATTATTA											
CcP8D06 F	***CAGTCGGGCGTCATCA***GAACTCGGTTGTGTCTTTGTA	EU125404	NED	$(CTTT)_{19} \cdots (CTTT)_4 \cdots (CTTT)_5$	390	21	24	291–395	0.95	0.97	0.08	0.94
CcP8D06 R	GTTTGTTCCCAGCATGAGAATGA											

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

female loggerheads that nested in the Archie Carr National Wildlife Refuge (ACNWR), Florida (USA) in 2006 and the two Georgia females used for library construction. After confirming clone size, we excluded the Georgia samples from analyses. To identify loci with potential utility in other marine turtle population studies, we screened the 11 novel primer pairs and the 15 previously developed for loggerhead turtles in four other marine turtle species. We collected the green and leatherback samples from females that nested at ACNWR. The hawksbill samples represent two Caribbean rookeries, and the olive ridley samples represent foraging animals from the eastern tropical Pacific.

We extracted DNA from the ACNWR samples, as well as the hawksbill samples using the DNeasy Tissue kit (QIAGEN). We extracted DNA from the olive ridley samples using a blood lysis protocol (Grimberg et al. 1989). PCR amplifications were performed in 10 µl volumes using GeneAmp® PCR System 9700 thermal cyclers (PE Applied Biosystems). Reactions consisted of 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 labeled tag (HEX, FAM, or NED + CAG or M13-reverse) (Glenn and Schable 2005), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 U Taq DNA Polymerase, and approximately 10 ng DNA. We labeled M13 and CAG universal primers with HEX, FAM, or NED fluorescent dyes. Cycling parameters, utilizing a touchdown protocol (Don et al. 1991), were: 95°C for 5 min, 20 cycles of 95°C for 20 s; 60°C for 20 s minus 0.5°C per annealing cycle; and 72°C for 30 s followed by 20 cycles of 95°C for 20 s; 50.0°C for 30 s; 72°C for 30 s; and a final extension period of 10 min at 72°C.

PCR products and size-scored fragments were analyzed using an ABI 3730xl DNA Analyzer with Genescan Rox500 fluorescent size standard (PE Applied Biosystems)

Table 2 Cross-species amplification of microsatellite primers developed for *Caretta caretta*

Locus	*Lepidochelys olivacea* ($n = 23$)					*Eretmochelys imbricata* ($n = 22$)					*Chelonia mydas* ($n = 23$)					*Dermochelys coriacea* ($n = 22$)				
	N	A	Size (bp)	H_O	H_E	*N*	A	Size (bp)	H_O	H_E	*N*	A	Size (bp)	H_O	H_E	*N*	A	Size (bp)	H_O	H_E
CcP2H12	23	20	336–388	0.87	0.93	13	20	326–372	0.75[a]	0.92	22	15	325–379	0.91	0.93	20	9	337–369	0.90	0.85
CcP2G10	23	15	269–345	1.00	0.92	16	10	280–324	0.94	0.90	22	10	281–345	0.73	0.86	–	–	–	–	–
CcP2F11	23	18	258–344	0.91	0.95	21	11	254–298	0.76[a]	0.91	22	13	260–324	0.86	0.85	–	–	–	–	–
CcP1H11	23	11	208–281	0.87	0.82	–	–	–	–	–	–	–	–	–	–	22	1	165	0.00	0.00
CcP1F01	23	16	313–385	0.96	0.93	22	4	295–307	0.64	0.65	8	6	320–352	0.38[a]	0.86	–	–	–	–	–
CcP1F09	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CcP1G03	22	3	270–278	0.59	0.57	21	2	274–282	0.05	0.05	23	1	270	0.00	0.00	–	–	–	–	–
CcP1B03	23	1	264	0.00	0.00	21	1	264	0.00	0.00	16	5	271–295	0.69	0.63	–	–	–	–	–
CcP1G02	22	13	270–322	0.86	0.91	19	11	273–329	0.84	0.90	23	8	259–303	0.78	0.72	20	9	269–317	0.75	0.85
CcP5C08	22	17	292–368	0.96	0.93	21	13	294–358	0.95	0.92	–	–	–	–	–	7	9	296–336	1.00	0.93
CcP5H07	22	11	235–279	1.00	0.90	20	10	231–275	0.80	0.87	22	11	227–275	0.91	0.89	22	10	218–254	0.91	0.89
CcP5F01	23	7	116–160	0.57	0.63	19	18	146–192	0.95	0.94	16	7	126–170	0.31[a]	0.76	19	9	143–189	0.84	0.85
CcP5C11	22	2	176–180	0.09	0.09	20	2	188–192	0.15	0.30	23	1	204	0.00	0.00	22	10	211–251	0.77	0.79
CcP7D04	23	20	336–418	1.00	0.95	20	6	323–351	0.80	0.70	22	12	327–383	0.91	0.91	22	1	322	0.00	0.00
CcP7F06	20	13	282–402	0.70[a]	0.92	16	8	271–311	0.63[a]	0.83	23	3	263–279	0.22	0.20	–	–	–	–	–
CcP7G11	21	12	285–337	0.95	0.89	21	5	288–304	0.67	0.76	22	2	265–271	0.46	0.41	21	4	296–312	0.71	0.65
CcP7C06	22	13	293–343	0.72[a]	0.91	22	6	271–286	0.64	0.65	22	17	345–475	0.64[a]	0.94	20	11	289–329	0.85	0.87
CcP7C04	22	13	209–265	0.86	0.91	22	7	192–224	0.50	0.50	23	1	193	0.00	0.00	–	–	–	–	–
CcP7B07	23	4	206–218	0.83[a]	0.64	18	9	223–263	0.61	0.75	23	11	215–259	0.96	0.91	22	1	198	0.00	0.00
CcP7E11	22	13	295–347	0.82	0.92	21	10	283–323	0.90	0.90	21	5	280–300	0.67	0.64	22	5	287–307	0.82	0.73
CcP7A08	23	18	316–400	0.87	0.93	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CcP7C08	23	3	288–298	0.30[a]	0.47	21	4	280–294	0.48	0.60	22	1	274	0.00	0.00	21	1	276	0.00	0.00
CcP7H10	22	5	213–237	0.32	0.29	22	10	207–251	0.86	0.83	20	13	227–283	0.90	0.90	–	–	–	–	–
CcP7E05	23	2	164–168	0.26	0.23	21	17	182–250	0.86	0.93	23	14	181–241	0.96	0.93	–	–	–	–	–
CcP7E05	23	11	320–364	0.91	0.91	15	12	279–365	0.87	0.85	22	8	269–321	0.68	0.62	22	9	296–332	0.96	0.89
CcP8E07	23	17	259–333	0.91	0.91	19	12	264–312	0.84	0.89	23	12	272–314	0.87	0.89	21	10	255–327	0.95	0.89

N, Number of samples successfully amplified; A, number of alleles; Size, base pair length of amplicon; H_O, observed heterozygosity; H_E, expected heterozygosity

[a] Indicates deviation from Hardy–Weinberg equilibrium; – indicates amplification failure

and GeneMapper 4.0 software (PE Applied Biosystems). We calculated observed and expected heterozygosity and polymorphic information content for each locus using CERVUS 2.0 (Marshall et al. 1998). We tested Hardy–Weinberg equilibrium and genotypic linkage disequilibrium using GENEPOP 3.4 (Raymond and Rousset 1995) and conducted a posteriori sequential Bonferroni correction (Rice 1989).

Table 1 summarizes the characteristics of 11 new primer pairs developed from the loggerhead sea turtle. The number of alleles per locus ranges from 4 to 24, averaging 11.2. Total exclusionary power with both parents unknown is 1.000000. No deviations from Hardy–Weinberg equilibrium or linkage disequilibrium were detected after sequential Bonferroni correction.

Cross-species utilization tests indicate that despite millions of years of divergent evolution, a number of loci are conserved across marine turtle taxa and are potentially useful in studies examining population genetics of the respective species (Table 2). Null alleles and amplification failures increased with phylogenetic distance, while polymorphism generally decreased with increased phylogenetic distance.

Genetic analyses have been instrumental in elucidating many aspects of marine turtle ecology and population structure. However, genetic resources are relatively limited for marine turtle species, particularly molecular markers for bi-parentally inherited nuclear loci. Novel primers reported herein compliment previously published loggerhead microsatellite suites (Shamblin et al. 2007; Monzón-Argüello et al. 2007). Cross-utilization of loggerhead-derived markers in other marine turtle species expands the genetic resources available for research strategies that will advance our understanding of marine turtle genetics and ecology.

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