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TECHNICAL NOTE

Tetranucleotide microsatellite loci from the critically endangered hawksbill turtle (*Eretmochelys imbricata*)

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Abstract We describe isolation and characterization of 14 polymorphic tetranucleotide loci from the hawksbill turtle (Eretmochelys imbricata). We identified an average of 14.5 alleles per locus based on screening of 36 individuals captured on foraging grounds in Bocas del Toro Province, Panama, and 9.6 alleles in 13 individuals captured on foraging grounds in Bermuda. Observed heterozygosity ranged from 0.67 to 1.00, with a mean of 0.85 for the Panama foraging aggregation and 0.83 for the Bermuda foraging aggregation. This microsatellite suite has a combined non-exclusion probability of identity of 8.26 × 10–23. These markers should be informative in individual and population-focused analyses.

Keywords Eretmochelys imbricata · Hawksbill turtle · Microsatellite · Population

The hawksbill turtle (*Eretmochleys imbricata*) is a globally critically endangered marine species. Demand for the ornately patterned carapace scutes, known as tortoiseshell or *bekko*, by many cultures and over several centuries has significantly reduced the size and distribution of nesting populations of hawksbill turtles (Parsons 1972). In the Greater Caribbean region, several major rookeries were extinguished or severely depleted by overharvesting (Meylan 1999; McClenachan et al. 2006). Resolving connectivity among remnant rookeries and between nesting and foraging sites is essential for conservation planning and genetic data from both mitochondrial and nuclear markers are needed. Dinucleotide microsatellite loci have been previously isolated from hawksbill turtles (FitzSimmons et al. 1995; Lin et al. 2008; Miro-Herrans et al. 2008). In addition, several tetranucleotide microsatellite markers designed from loggerhead turtles amplify well and conform to Hardy–Weinberg equilibrium expectations in hawksbill turtles (Shamblin et al. 2009). However, additional markers are needed for fine-scale studies such as relatedness analyses. We address this need through development of 14 tetranucleotide markers to complement currently existing markers.

Genomic DNA was enriched for microsatellite loci using the methods described by Glenn and Schable (2005) with minor modifications. DNA was extracted from blood samples taken from two juvenile green turtles captured while foraging in the Indian River Lagoon east of Sebastian, Florida, USA using a Qiagen® DNEasy blood and tissue kit. DNA was digested using *RsaI* endonuclease (New England Biolabs). Double stranded SuperSNX linkers (Glenn and Schable 2005) were ligated to the digested genomic DNA overnight at 16 °C. Linker-ligated DNA was recovered using PCR on an Applied Biosystems 9700 thermal cycler and verified by agarose gel electrophoresis. Recovered DNA was hybridized to two different mixtures of biotinylated oligonucleotide probes (Integrated DNA Technologies, mix 1: $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$

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Table 1 Characterization of microsatellite loci amplified in 36 hawksbill turtles captured on foraging grounds in Panama

Locus	Primers	Repeat unit	A	Size range	H_O	H_E	P_{HW}	Null	Genbank
ERIM03F	**CAG**TTTTTGGTGTTGGCATAGAA	$(AGAT)^{9}$	11	180–228	0.72	0.85	0.253	0.080	JQ917149
ERIM03R	GTTTAACTGGGGCTATTTTTCCTT								
ERIM04F	**CAG**ATATGGGGTTTGAGGTTTGT	$(AGAT)^{11}$	10	141–179	0.89	0.88	0.199	−0.016	JQ917150
ERIM04F	GTTTAGGACACAAGACACTGTGGT								
ERIM08F	**CAG**CACCTTCTGCCTATCTATCC	$(AGAT)^{7}$	7	108–132	0.72	0.78	0.403	0.028	JQ917151
ERIM08R	GTTTCACACAGAAGGGCCCTAC								
ERIM18F	GTTTGATAGCACGTGAGGAAAGCC	$(AAAG)^{14}$	24	274–340	0.89	0.95	<0.001	0.025	JQ917152
ERIM18R	**M13**AAATTCCCAAAGTCTGCCGC								
ERIM19F	GTTTACGGTGCTCTATTGTTTCACTC	$(AAAG)^{16}$	14	187–247	0.92	0.92	0.954	−0.007	JQ917153
ERIM19R	**CAG**GCAGCATGGATTCAAACAATG								
ERIM21F	**CAG**AAGGGTGGAGAACAGTAAAGG	$(AAAG)^{8}$	10	237–285	0.92	0.84	0.131	−0.057	JQ917154
ERIM21R	GTTTGGGATTCCAGAGCACCAAAC								
ERIM22F	GTTTCAAGCTGCCATCACATCCTG	$(AAAG)^{16}$	11	354–406	0.83	0.88	<0.001	0.020	JQ917155
ERIM22R	**M13**GTTCATCCTTGTCTCTGGC								
ERIM23F	GTTTGCAAGCCTGAACCAAGAAC	$(AAAG)^{17}$	13	202–246	0.89	0.91	0.509	0.004	JQ917156
ERIM23R	**M13**ACCCAAATACACGCTACAGG								
ERIM24F	**CAG**GTCCCTGCCCTGAACTG	$(ATCT)^{6}$…	15	266–338	0.97	0.94	0.929	−0.024	JQ917157
ERIM24R	GTTTGTGTGTCTGAAGCTTTAGGTCC	$(AGAT)^{21}$							
ERIM25F	**CAG**TGGCGTTTTCATTTTGTAGA	$(AAAG)^{10}$	16	206–278	0.86	0.92	0.193	0.025	JQ917158
ERIM25R	GTTTAAGCTGTGAGTTTTCAGCAA								
ERIM27F	GTTTCTGCAAGAGCTCGTTTCTCT	$(AAAG)^{12}$	12	277–325	0.86	0.84	0.073	−0.022	JQ917159
ERIM27R	**M13**TTACACATTTTGCCCTTCAA								
ERIM28F	**CAG**CCATTGAAGTTTCTGCCTTT	$(ACAG)^{9}$	18	152–264	0.67	0.88	0.938	0.134	JQ917160
ERIM28R	GTTTTGGTGCATCAAATTTAACAGA								
ERIM29F	**CAG**ATCTCTGAAAAGAATGACAC	$(AAAC)^{7}$	10	121–163	0.94	0.92	0.656	−0.021	JQ917161
ERIM29R	GTTTACCCAGATGCTATAGTGA								
ERIM32F	**M13**TTGTTGAAAGTGCCGCCC	$(AGAT)^{12}$	10	190–238	0.75	0.79	0.375	0.024	JQ917162
ERIM32R	GTTTGGGAATAAGAGAGGCGACG								

A is the number of amplified alleles. HO is the observed heterozygosity. HE is the expected heterozygosity. PHW is the probability the locus is in Hardy–Weinberg equilibrium. Null is the null allele frequency estimate

CAG tag: CAGTCGGGCGTCATCA

M13 tag: GGAAACAGCTATGACCAT

$(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, (ACTC)6, $(ACTG)_6$; mix 2: $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$). Probes and hybridized DNA were captured using streptavidin-coated magnetic beads (DynaBeads®, Invitrogen®). Enriched DNA was washed and eluted at 95 °C and recovered through PCR. The enrichment procedure was repeated to increase the efficiency of microsatellite array capture. DNA from the second enrichment was used as template for amplification by two sequential rounds of PCR. The re-amplified PCR products were ligated into a pCR®2.1-TOPO® vector and transformed into TOP 10® competent cells (Invitrogen).

A total of 285 colonies were screened by colony PCR, treated with ExoSap-IT® (New England Biolabs) and sequenced using BigDye v3.1 (Applied Biosystems). Sequencing reactions were purified using Sephadex G-50 Fine (Sigma Aldrich) and analyzed on an Applied Biosystems 3730*xl* DNA Analyzer. Sequences were aligned and edited using Sequencher 4.2 (Gene Codes). Contigs were queried for microsatellite loci using MSATCOMMANDER (Faircloth 2008). BLAST alignments confirmed high sequence identities (>85 %) with loci previously isolated from loggerhead turtles (Cc7E11, Genbank DQ917773; Cc7G11, Genbank DQ917774; Shamblin et al. 2007; Cc2F11, Genbank EU125406; Cc7A08, Genbank EU125397; Shamblin et al. 2009), green turtles (CHMY30, Genbank JQ728662, Shamblin et al. 2012), and leatherback turtles (Derm13, Genbank GU592728; Derm30, Genbank GU592732; Genbank36, Genbank GU592736; Alstad et al. 2011). We designed primers for 37 novel loci using MSATCOMMANDER (Faircloth 2008) or manually with PRIMER3 (Rosen and Skaletsky 2000) when automated primer design failed.

Table 2 Characterization of microsatellite loci of 13 hawksbill turtles captured on foraging grounds in Bermuda

Locus	A	Size range	H_O	H_E	P_{HW}	Null
ERIM03	8	180–232	0.62	0.79	0.163	0.106
ERIM04	9	141–175	0.62	0.82	0.075	0.134
ERIM08	5	108–132	0.85	0.73	0.661	−0.105
ERIM18	16	286–332	0.92	0.96	0.474	0.000
ERIM19	11	195–247	0.92	0.90	0.931	−0.029
ERIM21	7	241–265	0.85	0.81	0.972	−0.050
ERIM22	7	360–390	0.85	0.87	0.310	−0.005
ERIM23	11	202–258	1.00	0.92	0.835	−0.061
ERIM24	11	266–318	0.85	0.92	0.320	0.021
ERIM25	9	222–278	1.00	0.86	0.394	−0.102
ERIM27	8	281–321	0.92	0.86	0.778	−0.059
ERIM28	14	152–260	0.92	0.94	0.232	−0.014
ERIM29	10	133–163	0.69	0.91	0.039	0.121
ERIM32	9	190–234	0.69	0.79	<0.001	0.049

A is the number of amplified alleles. H_O is the observed heterozygosity. H_E is the expected heterozygosity. P_{HW} is the probability the locus is in Hardy–Weinberg equilibrium. Null is the null allele frequency estimate

Amplification was performed in 10 µl reactions consisting of 10 mM Tris pH 8.4, 50 mM KCl, 0.5 µM GTTT "pig-tailed" locus specific primer, 0.05 µM CAG (CAG TCGGGCGTCATCA) or M13 (GGAAACAGCTATGAC CAT) tagged locus specific primer, 0.45 µM fluorescently labeled CAG or M13 tag (Boutin-Ganache et al. 2001), 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.5 U Taq polymerase, and approximately 2–5 ng of template DNA. Fluorophores used were VIC, PET, NED (Applied Biosystems) and FAM (Integrated DNA Technologies). Touchdown cycling parameters (Don et al. 1991) 95 °C for 5 min, 20 cycles of 95 °C for 30 s, 60 °C minus 0.5 °C per cycle for 30 s and 72 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 10 min. Amplicon fragment sizes were analyzed on an Applied Biosystems 3730*xl* DNA Analyzer using LIZ500 size standard (Applied Biosystems). Allele sizes were scored using GENEMAPPER (Applied Biosystems).

Thirty-seven primer pairs were screened for amplification consistency and polymorphism in 36 hawksbill turtles captured on foraging grounds in Bocas del Toro Province, Panama, and 13 hawksbills captured while foraging in Bermuda waters. Fourteen loci amplified consistently and were polymorphic (Tables 1 and 2). Data were formatted for analysis using GMCONVERT (Faircloth 2006), and CERVUS 3.0 (Kalinowski et al. 2007) was used to calculate allelic richness (k), observed heterozygosity (H_O), expected heterozygosity (H_E), and deviations from Hardy–Weinberg equilibrium (HWE). We calculated linkage disequilibrium (LD) using GENEPOP (Raymond and Rousset 1995). ERIM18 and ERIM22 were out of HWE in the Panama foraging aggregation, and ERIM32 was out of HWE in the Bermuda foraging sample. No loci pairs exhibited linkage disequilibrium in either foraging aggregation. The number of alleles per locus ranged from 7 to 25, with an average of 15 alleles per locus across both foraging aggregations. This microsatellite suite has a combined non-exclusion probability of identity of 8.26×10^{-23}. These markers will complement those already available for use in individual identification and other fine-scale genetic analyses.

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References

Alstad TI, Shamblin BM, Bagley DA, Ehrhart LM, Nairn CJ (2011) Isolation and characterization of tetranucleotide microsatellites from the leatherback turtle (Dermochelys coriacea). Conserv Genet Resour 1:213–215

Boutin-Ganache I, Raposo M, Raymond R, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele sizing methods. Biotechniques 31:24–28

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Faircloth BC (2006) GMCONVERT: file conversion for GENEMAPPER output files. Mol Ecol Notes 6:968–970

Faircloth B (2008) MSTACOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol Ecol Resour 8:92–94

FitzSimmons NN, Moritz C, Moore SS (1995) Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. Mol. Biol Evol 12:432–440

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. In: Zimmer EA, Roalson EH (eds) Methods in enzymology 395, molecular evolution: producing the biochemical data, part B. Academic Press, San Diego

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol 16:1099–1106

Lin G, Chang A, Yap HW, Yue GH (2008) Characterization and cross-species amplification of microsatellites from the endangered hawksbill turtle (*Eretmochelys imbricata*). Conserv Genet 9:1071–1073

McClenachan L, Jackson JBC, Newman MJH (2006) Conservation implications of historic sea turtle nesting beach loss. Front Ecol Environ 4:290–296

Meylan AB (1999) Status of the hawksbill turtle (*Eretmochelys imbricata*) in the Caribbean region. Chelonian Conserv Biol 3:177–184

Miro-Herrans AT, Velez-Zuazo X, Acevedo JP, McMillan WO (2008) Isolation and characterization of novel microsatellites from the critically endangered hawksbill sea turtle (*Eretmochelys imbricata*). Mol Ecol Resour 8:1098–1101

Parsons JJ (1972) The hawksbill turtle and the tortoise shell trade. In: Études de Géographie Tropicale Offertes á Pierre Gourou. Mouton, Sorbonne, pp 45–60

Raymond M, Rousset F (1995) An exact test for population differentiation. Evolution 49:1280–1283

Rosen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Shamblin BM, Berry BE, Lennon DM, Bagley DA, Ehrhart LM, Nairn CJ (2012) Tetranucleotide microsatellite loci from the endangered green turtle (Chelonia mydas). Conserv Genet Resour. doi:10.1007/s12686-012-9643-4

Shamblin BM, Faircloth BC, Dodd M, Wood-Jones A, Castleberry SB, Carroll JP, Nairn CJ (2007) Tetranculeotide microsatellites from the loggerhead sea turtle (*Caretta caretta*). Mol Ecol Notes 7:784–787

Shamblin BM, Faircloth BC, Dodd MG, Bagley DA, Ehrhart LM, Dutton PH, Frey A, Nairn CJ (2009) Tetranucleotide microsatellites from the loggerhead sea turtle (*Caretta caretta*) and their cross-amplification in other marine turtle species. Conserv Genet 10:577–580