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PRIMER NOTE

Development and characterization of novel microsatellite markers from the olive ridley sea turtle (*Lepidochelys olivacea*)

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Abstract

Olive ridley turtles, although widely distributed globally and in Indian coastal waters, have undergone declines in recent years due to anthropogenic factors, particularly fishery-related mortality. Assessment of genetic variability in existing populations is critical to the development of effective conservation strategies. Here we describe the development of six highly polymorphic microsatellite loci from a simple sequence repeat-enriched genomic DNA library of olive ridley turtle. Characterization of five of these loci using 83 individual olive ridley turtles revealed eight to 24 alleles per locus, high observed and expected heterozygosity values and broad cross-species amplifications. The sixth microsatellite was found to be monomorphic in the olive ridley samples but was polymorphic in two related marine turtle species. These microsatellites thus provide efficient genetic markers to understand the population structure, phylogeography and species relationships of olive ridley and other marine turtle species.

Keywords: conservation, *Lepidochelys olivacea*, microsatellite, olive ridley

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Olive ridley turtles (*Lepidochelys olivacea*) are globally distributed and nest in large numbers at a few sites (Pritchard 1997), including Orissa on the east coast of India (Shanker *et al*. 2003). In recent years, there has been a decline in these nesting populations due mainly to anthropogenic threats (Shanker *et al*. 2003). Given the conflicts between development and conservation, the prioritization of populations and habitats becomes a key component of management and genetic data help in these assessments. The extent and distribution of genetic variation within a species are of fundamental importance to its evolutionary potential and to determining its chances of survival. In marine turtles, mitochondrial DNA diversity has been widely used to understand their population structure and migration patterns (Bowen & Karl 1997) but the use of microsatellites for population level analysis remains lacking mainly due to the availability of few such markers. Microsatellites or simple sequence repeat (SSR) markers are ideal for genetic diversity and mapping studies because of their abundance, high polymorphism content, codominance, easy detection and transferability across studies. As microsatellite markers are biparentally inherited, they also help to reveal patterns of male- and female-mediated gene flow which provide efficient markers for understanding genetic relationships between populations of a species. In recent years, several microsatellite markers have been developed for other species of marine turtles [*L. kempii* (Kichler *et al*. 1999), *Chelonia mydas* (Fitzsimmons *et al*. 1995) and *Dermochelys coriacea* (Crim *et al*. 2002)] but none has been described for olive ridleys. Although few cross-species microsatellites can be used for olive ridley analysis (our unpublished data), the availability of species-specific markers is highly desirable for population structure assessments.

An SSR-enriched genomic library was constructed following a modified protocol based on the methods of Bloor *et al*. (2001) and Edwards *et al*. (1996). Approximately 10 μg total genomic DNA of olive ridley was isolated from

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Table 1 Cross-species amplification status of olive ridley turtle-specific microsatellite markers in three related marine turtle species

Turtle Species@	*Dermochelys coriaceae (Leatherback)*					*Chelonia mydas (Green Turtle)*					*Eretmochelys imbricata (Hawksbill)*				
Locus	Status#	NA	Size range	T_a	Mg^{++}	Status#	NA	Size range	T_a	Mg^{++}	Status#	NA	Size range	T_a	Mg^{++}
OR-1	No amplification					M	1	138	55	2	M	1	146	57	2
OR-2	P	3	172–180	55	1.5	P	7	154–180	55	2	P	5	162–182	55	1.5
OR-3	M	1	142	55	1.5	P	4	162–174	55	2	P	3	140–178	55	1.5
OR-4	No amplification					P	3	100–125	52	2	P	2	100–110	52	2
OR-7	M	1	204	57	2	P	3	222–230	55	2	P	4	162–222	57	1.5
OR-8	P	6	164–192	55	1.5	P	5	140–170	55	2	P	6	148–162	57	1.5

@: Six unrelated samples were analyzed for each of the three turtle species;
#: Status of the amplified alleles; P = polymorphic; M = monomorphic; NA: Number of alleles;
T_a: Locus specific annealing temperature in °C; Mg^{++}: Magnesium ion concentration in mM used for PCR amplifications.

blood (Lang *et al*. 1993), digested with Rsa*I* and Hae*III* restriction endonucleases and fractionated on 1.5% agarose gel. Fragments of 0.5–1.5 kb were gel-eluted and ligated with Mlu*I* adaptors (Edwards *et al*. 1996). The adaptor-ligated SSR-rich DNA fragments were selected by hybridization to biotinylated oligonucleotides [$(GA)_{15}$, $(CA)_{15}$, $(AGA)_{10}$, $(CAA)_{10}$] and capturing with streptavidin-conjugated magnetic beads (Dynabeads, DYNAL). The SSR-enriched DNA fragments were cloned into pMOS vector and transformed into competent *Escherichia coli* DH5α cells. Plasmids were isolated from 50 random clones using an alkaline-lysis method (Sambrook *et al*. 1989). Cloned inserts were amplified and sequenced using M13 universal primers and a Bigdye terminator sequencing kit on an ABI-PRISM 3700 automated DNA sequencer (Applied Biosystems) for both strands. Twenty sequences with SSR motifs were identified and primers were designed and synthesized for 11 sequences that had > 18 bp long repeat regions, using the program GENETOOL version 1.0 (http://www.doubletwist.com) and DNA/RNA Synthesizer-394 (Applied Biosystems).

All primer pairs were used to standardize the polymerase chain reaction (PCR) amplification conditions and the utility of the working pairs as genetic markers was tested on a panel of 83 individuals representing different nesting populations of olive ridley turtles along the east coast of India and 18 samples belonging to three other marine turtle species (leatherbacks, green turtle and hawksbill; Table 1). DNA of test samples was isolated from muscle tissues originally collected and stored in 90% ethanol using the proteinase-K/sodium perchlorate method (Sambrook *et al*. 1989). The PCR amplifications were carried out in 15-μl reactions with 5 ng of genomic DNA, 1 × PCR buffer-I, 100 μM of dNTPs, 2 pM of each primer and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems) on a PTC-200 Thermal-Cycler (MJ Research). Thermal cycling parameters were: initial denaturation at 94 °C for 10 min followed by 35 cycles of denaturation at 94 °C for 1 min, primer-specific annealing temperature for 1 min (Table 2), extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Amplified products were first checked in 1.5% agarose gel and then analysed on an ABI-Prism 377 automated DNA sequencer, as per the manufacturer's instructions. Resolved PCR products were precisely sized using the software GENOTYPER 2.1 (Applied Biosystems) to calculate the number, range and distribution of amplified microsatellite alleles. The observed and expected heterozygosity estimates were calculated using ARLEQUIN version 2.0 (Schneider *et al*. 2000) and POLYMORPHISM INFORMATION CONTAIN (PIC) using online tools (http://www.agri.huji.ac.il/~weller/Hayim/parent/PIC.htm).

Six of the 11 primer pairs could be validated as highly polymorphic and informative microsatellite markers useful for genetic studies on olive ridley turtles as well as other marine turtle species tested in the study. The details of these markers, namely locus designation, repeat motifs, primer sequences, allele attributes, PIC estimates and GenBank Accession number, are summarized in Table 2 whereas their cross-species status is shown in Table 1. Interestingly, the marker OR-3, that was monomorphic in olive ridley turtles, was found to be polymorphic for other turtle species. Hardy–Weinberg equilibrium (tested using the Markov chain algorithm and Fisher's exact test in ARLEQUIN version 2.0; Schneider *et al*. 2000) indicated no null allele in olive ridley turtles for any of the six microsatellite loci described in this study.

In conclusion, the six microsatellite loci described here provide potentially useful markers for the assessment of genetic variability within and across populations of olive ridley and other turtle species to understand their population structure, reproductive behaviour, phylogeography and species relationships, paving the way for their

Table 2 Details of the microsatellite markers developed in the study

Locus	Repeat motif	Primer sequence (5′–3′)	Tag[a]	T_a (°C)	Size range (bp)[b]	N	NA	H_O	H_E	PIC	GenBank Accession
OR-1	$(CAAA)_{16}$	F: CCCCTTGTGTTCTGAAATCCTATGA R: CAGGCATAGGGAAAAATCAGAGGTA	FAM	55	150–202 [160, 162]	83	24	0.71	0.94	0.92	AY325422
OR-2	$(GT)_8GCC(GT)_5$	F: GCTCCTGCATCACTATTTCCTGTT R: TGCTGCCCCCACACCCTCTG	FAM	55	153–185 [169]	83	12	0.86	0.85	0.84	AY325423
OR-3*	$(TC)_9(AC)_6GC(AC)_2$	F: TTGTTTTATTTTTATTGGTCATTTCAG R: GCACCTTTTCACGTTGTCCACATGT	FAM	55	146	83	1	0	0		AY325424
OR-4	$(TG)_9..(TG)_{23}$	F: AGGCACACTAACAGAGAACTTGG R: GGGACCCTAAAATACCACAAGACA	HEX	52	122–172 [128, 158]	83	18	0.72	0.91	0.92	AY325425
OR-7	$(GT)_6(GA)_7$	F: GGGTTAGATATAGGAGGTGCTTGATGT R: TCAGGATTAGCCAACAAGAGCAAAA	FAM	55	185–219 [187]	83	16	0.68	0.85	0.84	AY325427
OR-8	$(TC)_{23}$	F: GCACTGGTGGGAAAATATTGTTGT R: GCTGGGCTAATAAAATGTTGTGCA	FAM	55	148–166 [148, 154]	83	8	0.93	0.78	0.76	AY325428

[a]: Flourescence label at 5′-end; [b]: Figures in paraenthesis: size(s) of the most frequent allele(s); T_a: Locus specific annealing temperature; N: Number of samples analyzed; NA: Number of alleles; H_O: Observed heterozygosity; H_E: Expected heterozygosity; *: Microsat locus was found to be polymorphic for Green turtle and Hawksbill turtle species.

conservation and efficient management strategies. These markers will be most useful for studying the reproductive behaviour of these species, e.g. multiple paternity, a highly debated aspect of sea turtle biology.

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