

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

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Abstract Olive ridleys, one of the widely distributed marine turtle species has undergone declines in recent years due to multiple anthropogenic factors warranting conservation efforts for which assessment of genetic variability in existing populations become critical. Here we describe development of ten new microsatellite markers from a short sequence repeat-enriched partial genomic DNA library, which are found to be highly informative for genetic studies. Eight of these markers when tested on 83 olive ridley turtles revealed high allelic diversity (4–27 alleles per marker), and high observed and expected heterozygosity estimates that ranged from 0.29 to 0.82 and 0.62 to 0.94, respectively. Two microsatellites were monomorphic in the tested olive ridley samples, but were found to be informative/polymorphic when tested on related marine turtle species. More importantly, nine of the new markers showed robust cross-species amplifications in three related species *Dermochelys coriacea*, *Chelonia mydas* and *Eretmochelys imbricata*. Thus, this study describes ten new microsatellite markers and also demonstrates their potential as efficient genetic markers in studies related to parentage analysis, population structure,

phylogeography and species relationships of olive ridleys and other marine turtle species.

Keywords *Lepidochelys olivacea* · Microsatellite · Conservation · Olive ridley marine turtle

Olive ridley is widely distributed and abundant of all marine turtles, but a large proportion of its population breeds synchronously at only a few rookeries in Pacific Mexico, Costa Rica and on the east coast of India (Pritchard 1997). Over 100,000 turtles have been reported to nest during the annual arribadas at Gahirmatha, while tens of thousands nest at Devi-River mouth and Rushikulya in Indian waters. However, these populations currently face a number of direct and indirect threats, warranting conservation strategies for which genetic assessment of existing populations becomes critical. Mitochondrial DNA diversity has been widely used to understand the phylogeography of marine turtles including olive ridleys (Bowen et al. 1998; Shanker et al. 2004) but there had been only few studies based on microsatellite markers to understand the population dynamics of this species (Jensen et al. 2006), mainly due to lack of such markers.

Microsatellites or Simple Sequence Repeats (SSR) markers are ideal for genetic diversity analysis because of their abundance, high polymorphism content, co-dominance, and more importantly being bi-parentally inherited these also help reveal patterns of male and female mediated gene flow. Although in recent years, several microsatellite markers have been developed for many species of marine turtles including six markers for olive ridleys (Aggarwal et al. 2004), but there is an urgent need to increase this repertoire for effective deployment of these highly useful markers in conservation genetic studies on olive ridleys and other marine turtle species.

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Table 1 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	N _A	H _O	H _E	PIC	GenBank Accession No.
OR-9	(GA) ₁₆	F: GCCCCACCCGAGGACAAAAG R: TTTTCACTCAACCTGTAATCCACCTCAT	FAM	55	150–174 (164)	83	11	0.293	0.762	0.72	EU162579
OR-11	(GA) ₂₂	F: TGAGCACTGCAAAATGGAGGATGGT R: AGTGCCTGATTCCTCGAGTTGCTGAG	FAM	55	182–242 (214)	83	27	0.728	0.939	0.93	EU162580
OR-12*	(GT) ₈ (GA) ₅ ..(GA) ₄	F: ATGGTTGGTCTGAGTATTATTTGGTGT R: ATACCCCTCCGTTTCTGTGTGTTCTTC	FAM	55	100	83	1	0	0		EU162581
OR-14	(GT) ₅ ..(GT) ₁₄ (GA) ₁₂	F: AGTGCCAGGGAGGGTGATTG R: CCTTGAGTTTTTCTGTTCCTCCCTA	FAM	55	147–201 (161)	83	21	0.725	0.941	0.93	EU162582
OR-16	(GT) ₅ (GA) ₇	F: CAAGGTTAGATATAGGAGGTGCTGATGT R: CCTGCTTTGAAATCCTGCCATAGTAATC	FAM	55	220–252 (224)	83	15	0.738	0.861	0.84	EU162583
OR-17**	(CA) ₇ ..(CA) ₆	F: CAAAAGATGATGGGGAGAAGAAAAT R: CCTCCCATACACACACTTCAACCTTT	FAM	55	234	83	1	0	0		EU162584
OR-18	(AC) ₁₁	F: AAACACCAGAAATAGAGGCTCAAACCT R: TCTCTGGGCTGCCTACTTTAATTC	FAM	55	108–120 (114)	83	7	0.593	0.649	0.58	EU162585
OR-19	(GT) ₁₁	F: CAGAAGGGCGAGTGTGCATGT R: GAATACATACACACGGCTCTACTC	HEX	55	148–156 (156)	83	4	0.647	0.681	0.59	EU162586
OR-20	(TG) ₁₄	F: TCACGAAACATCAGCAAAATTAAT R: GTCCCATCCCCCACCACA	FAM	57	110–124 (118)	83	6	0.529	0.649	0.54	EU162587
OR-22	(CT) ₇ (CA) ₆	F: AAGTCCTGTTGAATCCTGCCATAG R: GGTTAGATATAGGAGGTGCTGATGTTA	HEX	57	217–247 (219)	83	10	0.824	0.827	0.78	EU162588

^a Fluorescence label at 5'-end; ^b Figures in parenthesis: size of the most frequent allele; T_a: Locus specific annealing temperature; N: Number of samples analyzed; N_A: 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; ** Microsat locus was found to be polymorphic for all the other three species tested

Table 2 Cross-species amplification status of olive ridley turtle-specific microsatellite markers in three related marine turtle species

Locus	Dermochelys coriacea (Leatherback)				Chelonia mydas (Green)				Eretmochelys imbricata (Hawksbill)			
	Status ^b	Size range	N _A	T _a /Mg ⁺⁺	Status ^b	Size range	N _A	T _a /Mg ⁺⁺	Status ^b	Size range	N _A	T _a /Mg ⁺⁺
OR-9	P	136–138	2	55/2	P	161–165	3	55/2	P	146–168	6	55/2
OR-11	M	199	1	55/2	P	183–197	4	55/2	P	187–213	2	55/2
OR-12	M	87	1	55/2	P	95–111	4	55/2	P	96–118	3	55/2
OR-14	P	130–138	2	55/2	P	122–128	2	55/2	P	121–133	4	55/2
OR-16	P	229–249	4	55/2	P	258–280	4	55/2	P	198–258	4	55/2
OR-17	P	335–341	4	55/2	P	255–335	6	55/2	P	221–271	4	55/2
OR-18	No amplification				P	111–115	3	55/1.5	P	119–125	2	55/1.5
OR-19	P	154–156	2	55/1.5	P	150–164	8	55/1.5	P	150–156	3	55/1.5
OR-20	No amplification				No amplification				No amplification			
OR-22	No amplification				P	252–262	5	57/2	P	195–255	6	57/2

^a Six unrelated samples were analyzed for each of the three turtle species; ^bStatus of the amplified alleles; P = Polymorphic; M = Monomorphic; N_A: Number of alleles; T_a: Locus specific annealing temperature in °C; Mg⁺⁺: Magnesium ion concentration in mM used for PCR amplifications

Microsatellite markers described in this study were developed from an SSR-enriched genomic library constructed using the method described earlier (Aggarwal et al. 2004). Briefly, it involved: digestion of total genomic DNA of olive ridley with *RsaI* and *HaeIII* restriction endonucleases; agarose gel fractionation and gel-elution of 0.5–1.5 kb fragments followed by ligation with *MluI* adaptors (Edwards et al. 1996). The adaptor-ligated SSR-rich DNA fragments were then 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 genomic library was constructed by cloning the selected DNA fragments into pMOS vector using *Escherichia coli* DH5α cells. Plasmids were isolated and cloned inserts were amplified/sequenced using M13 universal primers. SSR-positive sequences were then identified using MISA (<http://pgrc.ipk-gatersleben.de/misa/>) and primers were designed from sequences that had >18 bp long repeat regions, using the program GENETOOL version 1.0 (<http://www.doubletwist.com>). Each forward primer was labeled with fluorescent dye (FAM or HEX) for amplicon detection. The utility of the working primer-pairs as genetic markers was tested using a panel of 83 unrelated samples from a large population of olive ridley turtles, and also six samples each of three other marine turtle species (*Dermochelys coriacea*, *Chelonia mydas* and *Eretmochelys imbricata*). Microsatellite alleles PCR-amplified using genomic DNA of test samples (isolated from muscle tissues) were resolved by GeneScan analysis on ABI 3700/3730 automated DNA sequencers and precisely sized using the software GeneMapper ver3.7 (Applied Biosystems) as per Aggarwal et al (2004). The marker attributes like Polymorphism Information Content (PIC), observed heterozygosity (*H*_O), expected heterozygosity (*H*_E), deviations from Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD) were calculated using ARLEQUIN ver3.01 (<http://cmpg.unibe.ch/software/arlequin3>) and CERVUS ver2.0 (<http://www.fieldgenetics.com>).

The details of the new markers viz., locus designation, repeat motifs, primer sequences, allele attributes are summarized in Table 1, while their cross-species transferability status is shown in Table 2. In general, all the new markers were highly polymorphic and informative. Eight of the ten new microsatellites revealed 4–27 alleles, and 0.541–0.939 PIC across the tested samples. All the markers except OR-9, followed HWE, showed no LD, and their *H*_O and *H*_E estimates were almost comparable, strongly suggesting the sampled olive ridley population to be genetically unstructured and healthy (our unpublished data). Moreover, all the new markers except OR-20 showed highly robust amplifications and considerable polymorphism in three related marine

species. Most interestingly, the two markers OR-12 and OR-17 that were monomorphic in olive ridleys, were found to be highly polymorphic for the other three turtle species (Table 2).

Many conservation studies are limited by the availability of suitable genetic markers that can work consistently and reliably across populations/species of interest. It is expected that the highly informative microsatellite primers developed in this study would greatly facilitate the studies on assessment of genetic variability in natural populations, understand their population structure, reproductive behavior, phylogeography and species relationships, of not only the olive ridley turtles but equally of the other related marine turtles, thus helping formulation of efficient management strategies and their conservation.

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