Twelve new polymorphic microsatellite markers from the loggerhead sea turtle (Caretta caretta) and cross-species amplification on other marine turtle species

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Abstract We describe 12 new polymorphic dinucleotide microsatellite loci and multiplex Polymerase Chain Reaction conditions from the loggerhead sea turtle Caretta caretta. Levels of polymorphism were assessed in 50 individuals from the nesting population of the Cape Verde Islands. Number of alleles ranged from 3 to 13 (average of 7.33) and the values of observed heterozygosities from 0.32 to 0.80 (average of 0.61). Cross-species amplification on three other marine turtles, Chelonia mydas, Eretmochelys imbricata and Dermochelys coriacea, revealed polymorphism and variability at eight, eleven and three loci, respectively.

Keywords Microsatellites · Caretta caretta · Marine turtle · Dinucleotide repeats

The globally endangered loggerhead sea turtle Caretta caretta inhabits in warm temperate and tropical waters in all the ocean basins. Although the use of genetics as a tool for studying and understanding the marine turtles' biology is well established, most surveys have analysed the population structure revealed by mitochondrial DNA (mtDNA) (Encalada et al. 1998; Laurent et al. 1998; Kaska 2000; Bowen et al. 2004; Carreras et al. 2006). Nuclear markers such as microsatellites have allowed the identification of conservation and management units, the differences in the migratory behaviour of males and females, and the mating behaviour of several marine turtle species (Crim et al. 2002; Bowen et al. 2005; Jensen et al. 2006). However, these studies have been limited by the use of a few microsatellites, non-specific in many cases, which are conserved across all cheloniid turtles (Fitzsimmons et al. 1995). Recently, 15 specific tetranucleotide microsatellites for Caretta caretta have been published (Shamblin et al. 2007). Here, we present 12 new polymorphic specific dinucleotide microsatellite markers that could be useful in future population genetic studies from nesting and foraging areas of the loggerhead sea turtle. In addition, cross-species amplification of these C. caretta microsatellite primers was performed on three other marine turtle species using between 6 and 8 individuals per species.

Microsatellite markers were identified by the development of an enriched genomic library as described by Glenn and Schable (2003). Whole genomic DNA was extracted from tissue samples stored in ethanol 96% of four C. caretta individuals using Qiamp Kit (QIAGEN). We digested the isolated DNA with RsaI restriction enzyme (SIGMA) and the DNA fragments were ligated to SNX linkers using T4-DNA ligase (BioLabs). Ligated DNA was enriched with a biotinylated probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$. DNA fragments with repetitive sequences were captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. JM109 High Efficiency Competent Cells (Invitrogen) were transformed with the recovered enriched DNA using

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Table 1 Details of the 12 microsatellite markers developed in the study

Locus	GB Accession no.	Repeat motif	Primer sequence (5^{0}–3^{0})	N	Size Range (bp)	NA	H_O	H_E	PIC	MP	Mg (mM)	P (1M)
Cc-2	EF679214	$(TA)_4$ $(GA)_{13}$	F PETCCCCCATAACACCACATCTC	50	225–233	3	0.420	0.444	0.382	2	3.5	0.10
			R AGGTCACAAATGGAGCAAGC									
Cc-8	EF679216	$(GT)_8$ (AT) $(GT)_4$	F PETTGATGGAAACCCCTTCAAAC	50	182–204	6	0.560	0.561	0.527	2	3.5	0.10
			R TGTCACGGAGACACAAACATT									
Cc-10	EF679217	$(CT)_{13}$	F FAMTCCACATGGGGTTGTATGAA	50	415–431	4	0.700	0.684	0.630	2	3.5	0.10
			R TGCCCTCCTTGAGAATTCAG									
Cc-13	EF679218	$(CA)_{17}$	F NEDCACCATCTGGGGAGATGAAT	50	402–421	13	0.720	0.777	0.751	1	3	0.15
			R AGGCTTGCCTTTTTGAAGGT									
Cc-16	EF679219	$(CT)_{16}$	F NEDGTCTTGACTGACCTGGCACA	50	342–354	6	0.760	0.750	0.702	1	3	0.15
			R CCACCAAAAGGAGCAAAATG									
Cc-17	EF679220	$(AG)_{16}$	F FAMCCACTGGAAGTCTAAGAAGAGTGC	50	320–348	11	0.760	0.740	0.696	3	3	0.20
			R GGAATTGAAGGGATTTTGCT									
Cc-21	EF679221	$(TG)_{17}$ $(AG)_8$	F VICGGCTGTTCCTCTGAAACCTG	50	374–410	11	0.620	0.675	0.650	2	3.5	0.10
			R AGCAATGACAAGGGAGCATT									
Cc-22	EF679222	$(GA)_{17}$	F VICCCCCCACTGCTTTAACTTCA	50	218–236	6	0.500	0.543	0.493	1	3	0.15
			R TATTCCAACATGCCCACAGA									
Cc-25	EF679223	$(CT)_{15}$	F PETTTTTGCTTTCCCCATCTGAC	50	320–344	9	0.800	0.775	0.733	2	3.5	0.10
			R AGCCTCCAGCACAGCATTAT									
Cc-28	EF679224	$(CA)_{16}$	F FAMAGCCCATATGTTTCCCTTCA	50	191–211	7	0.520	0.539	0.486	3	3	0.20
			R TTGGCCCATCTTATTTCAGTG									
Cc-30	EF679225	$(CT)_{13}$ (CA) $(CT)_{21}$	F NEDCTTTTGGAGGCAGGCTAGTG	50	161–189	9	0.680	0.798	0.762	4	3	0.20
			R GAAGCCAGGTTGATCAGGAG									
Cc-32	EF679226	$(TC)_{11}$	F PETGCACATCTGGCTGTGAGAAG	50	386–390	3	0.320	0.381	0.325	4	3	0.10
			R CCCCCTCACCTCAGTAGCTT									

GB Acc. no.—GenBank Accession Number; N—number of samples analyzed; Size range—predicted size of the PCR product amplified; NA—number of alleles; H_o—observed heterozygosity; H_E—expected heterozygosity; PIC—polymorphic information content; MP—numbers indicate those loci which share multiplex PCR reactions; Mg—final $MgCl_2$ concentration and P—final primers concentration

Table 2 Summary of the cross-species amplifications of loggerhead sea turtle specific microsatellite markers tested on three marine turtle species: green turtle (Chelonia mydas), hawksbill turtle (Eretmochelys imbricata) and leatherback (Dermochelys coriacea)

Locus	Spp.	N	Status	NA	Size range (bp)	T^a (°C)	Mg (mM)	P (μM)
Cc-1	C. mydas	6	p	2	168–170	61	3	0.15
	E. imbricata	8	p	6	159–181	61	3	
	D. coriacea	7	m	1	175	55	3.5	
Cc-2	C. mydas	6	p	4	213–231	61	3.5	0.15
	E. imbricata	8	p	6	219–231	61	3.5	
	D. coriacea	7	m	1	221	61	3.5	
Cc-6	C. mydas	6	p	6	188–206	61	3	0.15
	E. imbricata	8	p	2	216–222	61	3	
	D. coriacea	7	m	1	160	52	3.5	
Cc-10	C. mydas	6	p	5	407–421	61	3.5	0.15
	E. imbricata	8	p	2	376–402	61	3.5	
	D. coriacea	7	na	0	–	(52–61)	3.5	
Cc-13	C. mydas	6	p	6	399–431	61	3	0.15
	E. imbricata	8	p	6	391–416	61	3	
	D. coriacea	7	m	1	388	61	3	
Cc-16	C. mydas	6	na	0	–	(52–61)	3	0.15
	E. imbricata	8	p	2	326–340	61	3	
	D. coriacea	7	na	0	–	(52–61)	3	
Cc-17	C. mydas	6	m	1	321	61	3	0.15
	E. imbricata	8	p	7	323–355	61	3	
	D. coriacea	7	p	2	316–318	61	3	
Cc-21	C. mydas	6	na	0	–	(52–61)	3	0.15
	E. imbricata	8	na	0	–	(52–61)	3	
	D. coriacea	7	m	1	388	61	3.5	
Cc-22	C. mydas	6	p	7	210–230	61	3	0.15
	E. imbricata	8	p	3	228–232	61	3	
	D. coriacea	7	p	2	219–223	61	3	
Cc-25	C. mydas	6	na	0	–	(52–61)	3.5	0.15
	E. imbricata	8	p	2	320–322	61	3.5	
	D. coriacea	7	m	1	322	61	3.5	
Cc-28	C. mydas	6	p	3	188–198	61	3	0.15
	E. imbricata	8	p	6	198–224	61	3	
	D. coriacea	7	p	2	194–196	61	3	
Cc-30	C. mydas	6	p	8	165–201	61	3	0.15
	E. imbricata	8	p	4	157–193	61	3	
	D. coriacea	7	m	1	154	61	3	
Cc-32	C. mydas	6	m	1	382	61	3	0.15
	E. imbricata	8	m	1	382	61	3	
	D. coriacea	7	m	1	357	61	3	

Spp.—species name; N—number of samples analyzed; NA—number of alleles; Size range—predicted size of the PCR product amplified; T^a—annealing temperature, in brackets range of temperature used; Mg—final $MgCl_2$ concentration; P—final primers concentration. In the column Status we show: na—no amplification; p—polymorphic; and m—monomorphic

the cloning vector pGEM-T Easy Vector II (Promega). We amplify a total of 223 positive colonies (white) using the universal M13 primers. PCR products ranging between 500 and 1000 base pair (bp) were sequenced using Big Dye Terminator ver. 3.1 chemistry (Applied Biosystems) in an ABI 3100 automated sequencer. Chromatograms were analyzed and edited using BioEdit Sequence Alignment Editor ver. 7.0.9 (Hall 1999)

screening for microsatellite repeats. We designed 23 primer pairs using Primer3 v.0.3.0 online software (Rozen and Skaletsky 2000) out of which 12 consistently amplified and were polymorphic.

Four multiplex PCR amplifications were performed in a 20 μl total volume with 40 ng of genomic DNA. Final concentrations for primers and $MgCl_2$ for each reaction are shown in Table 1. Final concentrations for optimizing reactions were PCR buffer 1× (Bioline), 20 μM BSA, 0.25 μM dNTPs, and 0.5 U Taq DNA polymerase (Bioline). Reactions for all loci were carried out using the same thermal cycling program: an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s 61°C for 30 s and 72°C for 30 s, and a final extension period of 10 min at 72°C. Following this procedure, we genotyped 50 individuals of C. caretta from the Cape Verde population. Fragment sizes were scored using an ABI 3100 automated sequencer with LIZ 500 (Applied Biosystems) as internal fluorescent size standard. The results were analysed using GENEMAPPER ver. 3.5. We calculated observed and expected heterozygosities, number of alleles per locus and polymorphic information content (PIC) with Cervus ver. 3.0 (Marshall et al. 1998). We also tested for linkage disequilibrium and deviations from Hardy-Weinberg equilibrium using Arlequin 3.0 program (Excoffier et al. 2005). Sequential Bonferroni corrections were further conducted using a global P value of 0.05 (Rice 1989).

In addition to the 12 polymorphic loci obtained for the loggerhead sea turtle (Table 1), two more loci Cc-1 (GenBank Accession Number EF679213) and Cc-6 (GB Acc. Num. EF679215) were tested in 16 loggerhead sea turtle samples from the Canary Islands feeding ground, a mixed stock of different populations. We found only one allele for Cc-1 and two alleles, one of them in a frequency of 94%, for Cc-6. However, these two loci resulted to be polymorphic for other marine turtle species (Table 2). Table 1 shows locus designation, GenBank Accession Numbers, repeat motifs, primer sequences, allele attributes, PIC estimates and information about PCR conditions. In spite of being a dinucleotide marker, Cc-13 presented alleles that differ only by one base. This could be caused by the fact that this locus contains two additional STR sequences of a multiple adenine in one and a multiple cytosine in the other. After sequential Bonferroni corrections, no loci showed significant deviations of Hardy-Weinberg equilibrium, but two pair of loci yielded significant linkage disequilibrium (Cc-21 with Cc-28 and Cc-21 with Cc-32). Table 2 summarizes the polymorphic microsatellite results of the cross-species amplification tests. In conclusion, we identified 12 new specific polymorphic microsatellite markers for the loggerhead sea turtle which should prove useful in population and parentage analysis in this and other marine turtle species.

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