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PERMANENT GENETIC RESOURCES NOTE

Development of microsatellite markers in the Australasian snake-necked turtle *Chelodina rugusa* and cross-species amplification

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Abstract

Seventeen microsatellite loci were developed for the snake-necked turtle, *Chelodina rugosa* (Ogilby, 1890). Sixteen of the loci were polymorphic but three of these loci had null alleles. One locus displayed linkage disequilibrium. These 17 markers were tested for amplification in eight congeneric species with varying success; 98% amplification in *Chelodina burrungandjii*, 72% in *C. canni*, 38% in *C. expansa*, 58% in *C. longicollis*, 67% in *C. mccordi*, 73% in *C. oblonga*, 81% in *C. parkeri*, and 68% in *C. pritchardi*. These microsatellite markers will be useful for population assignment, gene flow, mating systems and hybridization studies in the genus *Chelodina*.

Keyword: Australia, *Chelidae*, *Chelonia*, freshwater turtle, hybridization, wildlife management

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The genus *Chelodina* (Testudines: Chelidae) is an Australasian genus of snake-necked turtles comprising 13 species from Australia, Papua New Guinea, East Timor, Indonesian West Papua and Roti (Georges & Thomson 2006). *Chelodina rugosa* is found in parts of northern Australia, West Papua and southern Papua New Guinea. It is subject to legal harvest for the pet trade by the indigenous community of Maningrida in Arnhem Land, Northern Territory, Australia. We developed 17 microsatellite loci to test whether we could distinguish between legal collections of *C. rugosa* in Arnhem Land and illegal poaching activities. These loci were characterized for 76 individuals from two populations of *C. rugosa* from the Northern Territory that are 1.2 km apart (sample sizes of 41 and 35, respectively). We also tested the primers on eight other species: *Chelodina burrungandjii*, *C. canni*, *C. expansa*, *C. longicollis*, *C. mccordi*, *C. oblonga*, *C. parkeri* and *C. pritchardi* to better understand the complex patterns of hybridization that occur in this genus (Georges *et al*. 2002).

Total genomic DNA was extracted from skin tissue samples (taken from vestigial hind toe webbing) using standard salting-out protocol (Dethmers *et al*. 2006). A genomic library enriched for di- and trinucleotide repeats was constructed based on the fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) method (Zane *et al*. 2002). Modifications on the prescribed method are described below. DNA from a composite sample of four individuals (approximately 100 ng) was simultaneously digested with *Mse*I and ligated to *Mse*I AFLP adaptor (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′). The subsequent digestion–ligation mixture was amplified using polymerase chain reaction (PCR) under standard cycling conditions with the primer *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′). Amplified DNA was hybridized with a 'pool' of biotinylated probes [$(AAC)_8$ $(ACC)_8$ $(AGC)_8$, & $(ACG)_8$] by mixing preheated hybridization buffer (181 μL 6× SSC, 3 μL 10% SDS, 6 μL 50× Denhards) with a denatured solution containing 100 μL of amplified DNA and 10 μL of the probe pool. The total solution was incubated at 62 °C for 30 min. Hybridized DNA molecules were selectively captured using Streptavidin MagneSphere Paramagnet Particles (S-PMP) (Promega). Two hundred microlitres 6× SSC, 4 μL 50× Denhards and 2 μL 10% SDS, were added to the S-PMP, followed by the prepared DNA-probe hybridization and rotated for 20 min. The resultant S-PMP-probe-DNA conglomerate was then isolated using magnetic field separation. Removal of nonspecific DNA occurred through a sequence of two nonstringency washes followed

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Table 1 Characterization of 17 microsatellite loci for the Australasian snake-necked turtle, *Chelodina rugosa* for 76 individuals

Locus	Primer sequences (5′–3′)	Repeat motif	PCR primer (μM)	T_a (°C)	$MgCl_2$ (mM)	Allele size range (bp)	N_A	N_E	H_O	H_E	Null freq.	GenBank Accession no.
T-11‡	F‡‡: CAGCCAAAAAAATGTAGGTCC R: TGTGACCACCTGATAACAGGC	$(CA)_{24}$	0.25	57	2	143–209	11	5.1	0.800	0.817	0.016	EU522102
T-12*	F††: GGGATCACTCGGCCACTCTGG R: ACCCAAGAATACCCGTCACCG	$(CAG)_9GAG$ $(CAG)_3$	0.40	57	2	157–163	3	2.4	0.514	0.591	0.011	EU522093
T-14*	F††: TAGGCTCAGGGATATGATAGC R: CTCCAGCGACAGTTGCAACAG	$(TGC)_8$	0.10	57	2	120–139	3	2.4	0.571	0.591	0.024	EU522094
T-15¶	F††: TGGTAAATAAGGGCTGCATGC R: CAGTTTCCTTACTTTGTCTGTC	$(AC)_{15}$	0.60	55	2	157–309	17	9.9	0.629	0.912	0.118	EU522103
T-17*	F‡‡: AACAGTATTATGGATGCAGAC R: GACACAAAAGGTACCATTCCC	$(TGC)_7$	0.40	57	2	118–136	4	1.5	0.229	0.339	0.049	EU522095
T-26†	F‡‡: CAGTGATTTTTGCTACCAAGG R: GCAAAACAGTATTATGGATGC	$(GCA)_7$	0.25	57	2	155–176	15	5.9	0.600	0.842	0.014	EU522096
T-27¶	F††: TTCTAGCCCAACCCATGTAGC R: GTGGTTATAAGGAAGTCATGC	$(TGC)_8$	0.40	55	2	140	1	—	—	—	—	EU522109
T-31‡	F**: GGGACCACTCATGGAACTAAG R: GGGATAGAATTGGGAATGTATG	$(AC)_{18}$	0.40	57	2	127–271	17	9.2	0.857	0.905	0.005	EU522104
T-39§	F**: AAGCAGGGAGTTGCAAATCAC R: ATCTGGCCTTTGGTCTTTCAG	$(CA)_{36}$	0.20	59	2.5	105–201	18	9.8	0.588	0.911	0.129	EU522105
T-41¶	F‡‡: TCCCTCACTTCTAGCTCTACC R: TCTTCTGTCTGGGTGGGTGTG	$(AC)_2C(AC)_{15}$ $(ACCC)_3$	0.75	55	2	125–207	11	3.6	0.606	0.734	0.079	EU522106
T-42*	F**: CCAAACTTGAACACTGCTGTG R: GGACTCCCAGATTATGGTCTC	$(ACC)_8$	0.15	57	2	155–164	2	1.2	0.171	0.205	0.022	EU522097
T-44†	F††: AAGGCAGTTGAGAACCAGGTG R: GTAGATGCCACCCATGTTGTC	$(AGC)_7$	0.20	57	2	133–145	4	3.7	0.743	0.740	0.001	EU522098
T-47§	F‡‡: CAATACTAGTCTGCTGTCACC R: CTAAGTTACCAATGCCTCC	$(CA)_{12}$	0.3	59	2.5	118–230	5	3.1	0.647	0.691	0.008	EU522107
T-58†	F**: TCCTGAAAGGGTGGGCAAAGG R: CTAGATGATTCTCAGTCTTTC	$(CAC)_7$	0.25	57	2	154–163	3	1.1	0.114	0.111	0.000	EU522101
T-67§	F††: TACCCTTTAGACTGAGGCAGG R: AGGAAGATGAATCAGGGTGAG	$(CA)_{27}$	0.25	59	2.5	106–184	18	10.3	0.676	0.916	0.098	EU522108
T-80*	F‡‡: CTCACCTGCAGCCTCTTTCTC R: AGGACCTTTCAGGACCCTCAC	$(TGC)_7$	0.30	57	2	144–168	3	2.1	0.457	0.527	0.027	EU522099
T-87*	F**: CAGCACTGATCTGCAAGTACC R: GCTACACCAGTTTCACTCTGC	$(TGC)_9$	0.30	57	2	124–154	3	2.4	0.600	0.593	0.006	EU522100

*–¶§Multiplex; **–‡‡primer labelled with WellRed Dye **D4, ††D3, ‡‡D2; N_A, number of alleles; N_E, effective number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

by four stringency washes. Nonstringency washes were performed with gentle mixing for 30 s using 6× SSC, and 2× SSC, respectively. Stringency washes were performed using 0.99× SSC, 0.1% SDS; the third and fourth washes were rotated at 55 °C for 5 min and 30 min, respectively. Between each wash, DNA was recovered by magnetic field separation for 3 min. The enriched DNA was resuspended in 40 μL ddH_2O. One microliter of enriched DNA was amplified using the same conditions as for the prehybridization PCR. Fragments of 200–1000 bp were excised from a 1.2% agarose gel, purified and ligated into pGEM-T easy vector, using T4 DNA Ligase (Promega). Ligations were used to transform competent *Escherichia coli* cells (strain JM109, Promega) through electroporation. Following electroporation, the methods for cloning, identifying and subsequently extracting DNA from positive clone colonies followed those described in Hillyer *et al*. (2006). Sequencing of 90 clone colonies used the M13 F primer (5′-GTAAAACGACGGCCAGT-3′) (Amersham Pharmacia Biotech) and BigDye Terminators (PerkinElmer). Sequences were determined on an ABI PRISM 377 automated sequencer and edited by eye in BioEdit (Version 5.0.9: Hall 1999). Of the sequenced clones, 55 contained microsatellite arrays, 31 of which had sufficient flanking regions allowing for primer design.

Primer pairs for up to 17 loci were first tested on five individuals of *Chelodina rugosa* to select those that successfully amplified and were polymorphic. The remaining 14 loci were not tested for polymorphism in *C. rugosa*. PCR primers were designed with 5′ fluorescent modifications (WellRed dyes) to allow PCR multiplexing for up to six loci (Table 1). Each PCR amplification was performed in a 20-μL reaction containing 50–100 ng template, 2× PCR buffer,

Table 2 Cross-species amplification of *Chelodina rugosa* microsatellite primers for eight congeners

	Chelodina burrungandjii			*Chelodina canni*			*Chelodina expansa*			*Chelodina longicollis*		
Locus	Success	No. of alleles	Size range	Success†	No. of alleles	Size range	Success	No. of alleles	Size range	Success	No. of alleles	Size range
T-11	5/5	7	147–181	5/5	5	137–173	3/5	4	163–276	5/5	6	141–159
T-12	5/5	3	157–163	3/5	4	151–163	2/5	2	157–160	2/5	2	160–163
T-14	5/5	3	120–126	4/5	2	117–126	2/5	1	129	2/5	1	120
T-15	5/5	7	165–203	5/5	2	171–173	5/5	4	163–181	5/5	1	171
T-17	5/5	1	118	1/5	1	118	2/5	2	130–133	2/5	2	118–124
T-26	4/5	3	139–158	4/5	2	158–164	3/5	2	158–161	3/5	1	158
T-27	4/5	2	140–157	4/5	2	157–166	0/5	—	—	0/5	—	—
T-31	5/5	7	110–217	5/5	6	133–157	3/5	4	144–306	5/5	7	138–186
T-39	5/5	7	91–195	3/5	3	93–103	0/5	—	—	0/5	—	–
T-41	5/5	3	131–143	4/5	2	135–137	2/5	1	139	4/5	5	133–143
T-42	5/5	3	152–158	4/5	2	152–167	2/5	2	155–158	2/5	1	152
T-44	5/5	3	133–139	4/5	2	136–139	2/5	2	136–145	3/5	1	142
T-47	5/5	2	112–120	3/5	2	118–120	0/5	—	—	0/5	—	—
T-58	5/5	2	157–160	4/5	2	157–166	2/5	2	169–172	3/5	4	157–166
T-67	5/5	7	112–206	2/5	2	94–142	0/5	—	—	0/5	—	—
T-80	3/5	1	147	2/5	1	144	1/5	1	150	2/5	1	144
T-87	5/5	2	136–145	4/5	4	136–148	3/5	1	136	2/5	2	136–145

	Chelodina mccordi			*Chelodina oblonga*			*Chelodina parkeri*			*Chelodina pritchardi*		
Locus	Success	No. of alleles	Size range	Success†	No. of alleles	Size range	Success	No. of alleles	Size range	Success	No. of alleles	Size range
T-11	5/5	6	135–153	5/5	9	127–255	5/5	3	119–137	5/5	1	143
T-12	2/5	1	157	2/5	2	151–160	3/5	1	160	3/5	2	151–160
T-14	3/5	1	126	4/5	1	120	5/5	1	117	5/5	2	123–144
T-15	5/5	2	163–173	5/5	2	179–181	5/5	1	177	5/5	1	171
T-17	0/5	—	—	4/5	2	133–139	5/5	1	130	0/5	—	—
T-26	5/5	2	146–158	4/5	1	158	4/5	2	158–161	2/5	1	161
T-27	5/5	1	137	5/5	2	137–140	5/5	1	140	5/5	1	137
T-31	4/5	3	132–138	5/5	3	84–88	5/5	6	120–182	5/5	1	136
T-39	5/5	1	93	5/5	8	143–227	5/5	4	137–145	5/5	1	97
T-41	2/5	2	123–131	5/5	3	101–107	2/5	2	155–157	5/5	1	131
T-42	3/5	2	152–155	0/5	—	—	2/5	1	164	5/5	1	152
T-44	5/5	3	136–151	4/5	3	136–142	5/5	1	139	3/5	1	136
T-47	5/5	3	131–137	2/5	2	109–117	0/5	—	—	5/5	1	147
T-58	5/5	2	148–166	4/5	3	166–175	4/5	1	166	2/5	1	166
T-67	0/5	—	—	0/5	—	—	5/5	5	148–196	5/5	3	126–152
T-80	3/5	1	144	4/5	1	144	4/5	1	144	4/5	1	144
T-87	0/5	—	—	4/5	1	145	5/5	2	136–142	4/5	1	145

†Success is the total number of individuals that successfully amplified over the total number of individuals tested.

2–2.5 mM $MgCl_2$ (Table 1), 0.2 mM each dNTP (Bioline), 0.10–0.75 μM of each primer (Table 1) and 1 U of *Taq* DNA polymerase (Bioline Red*Taq*). All reactions had an initial 5 min denaturation at 94 °C, followed by 30 cycles of 94 °C for 1 min, annealing temperature (T_a) for 30 s (Table 1), and 72 °C for 1 min, with a final extension of 4 min at 72 °C. DNA fragments were separated on a Beckman Coulter CEQ 8000 Genetic Analysis System and sized with Beckman Coulter version 9.0 CEQ software using 400 DNA ladder as an internal size standard.

Characteristics of the 17 loci are summarized in Table 1. The locus T27 was monomorphic for *Chelodina rugosa* but was polymorphic in other species (Table 2). Expected and observed heterozygosities, and the number of alleles per locus were generated using PopGene 1.31 (Yeh *et al*. 1999). Each locus was tested for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium using GenePop 3.4 for each population separately and all samples combined (Raymond & Rousset 1995). Micro-Checker 2.2.3 (Van Oosterhout *et al*. 2004) was used to detect null alleles

in each population and the program FreeNA (Chapuis & Estoup 2007) was used to estimate the frequency of the null alleles. Linkage disequilibrium was found between loci T26 and T41, and between T26 and T17 for both populations. Significant deviations from Hardy–Weinberg equilibrium were found for loci T15, T39 and T67 ($P < 0.05$), in which observed heterozygosity was lower than expected heterozygosity. Micro-Checker analyses detected possible evidence for null alleles, and null allele frequencies of 11.8% 12.9% and 9.8%, respectively, suggesting there are null alleles at these loci (Table 1). Dinucleotide microsatellite loci displayed greater allelic richness (mean $N_A = 13.8$) compared to trinucleotide loci (mean $N_A = 4.4$) but the levels of expected heterozygosity were both high (mean $H_E = 0.69$ and 0.43 for dinucleotides and trinuceotides, respectively) suggesting that both types of markers will be informative genetic markers.

Primers were tested on five individuals of eight additional species: *C. burrungandjii* (four populations), *C. canni* (two populations), *C. expansa* (two populations), *C. longicollis* (four populations), *C. mccordi* (one population), *C. oblonga* (one population), *C. parkeri* (one population) and *C. pritchardi* (one population). The details of the cross-species amplification of the primers are shown in Table 2. The rate of amplification success varied across these eight species: 98% in *C. burrungandjii*, 72% in *C. canni*, 38% in *C. expansa*, 58% in *C. longicollis*, 67% in *C. mccordi*, 73% in *C. oblonga*, 81% in *C. parkeri*, and 68% in *C. pritchardi*. Tests of the transferability of the primers suggest that they are (i) highly suitable for *C. burrungandjii* and *C. canni* (15/17 loci polymorphic); (ii) have limited suitability for *C. oblonga* (11/17 loci polymorphic), *C. mccordi* and *C. expansa* (9/17 loci polymorphic), *C. longicollis* and *C. parkeri* (7/17 loci polymorphic); and (iii) are not suitable for *C. pritchardi* (3/17 loci polymorphic) for population genetic, gene flow and hybridization studies.

In conclusion, these new microsatellite loci will contribute to the genetic monitoring of trade in *C. rugosa*. They will also be used for studies of mating systems, population genetics, gene flow and hybridization in the genus *Chelodina*.

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