Conservation Genet Resour (2011) 3:497–501
DOI 10.1007/s12686-011-9388-5

TECHNICAL NOTE

Development of ten polymorphic microsatellite loci for the sea snake *Hydrophis elegans* (Elapidae: Hydrophiinae) and cross-species amplification for fifteen marine hydrophiine species

Vimoksalehi Lukoschek · John C. Avise

Received: 5 January 2011 / Accepted: 17 January 2011 / Published online: 30 January 2011

Abstract We developed ten microsatellite loci for the elegant sea snake, *Hydrophis elegans*, from partial genomic DNA libraries using a repeat enrichment protocol. Eight loci had nine or more alleles per locus (maximum 20), while the other two had three and seven. All ten loci amplified successfully in 11 of the 15 additional hydrophiine sea snake species screened. Nine loci amplified successfully for three species and eight amplified successfully for the remaining species. Based on this highly successful cross-amplification we expect these ten loci to be useful markers for investigating population genetic structure, gene flow and parentage for all sea snake species from the *Hydrophis* group.

Keywords Microsatellite loci · Hydrophiinae · *Hydrophis* · Sea snakes · Connectivity · Parentage

Recently published IUCN Red List Assessments for all true sea snake species (Elapidae: Hydrophiinae) listed four of 54 species in Threatened or Near Threatened categories and 21 species as Data Deficient (IUCN 2010). Indeed, many aspects of the ecology and biology of sea snakes remain poorly understood and difficulties of direct observation in marine systems hinder significant progress. High-resolution molecular markers, such as nuclear microsatellites, provide compelling alternatives for addressing critical questions about population genetic structure, gene flow, dispersal, effective population sizes and mating systems. Microsatellite loci have only been developed for one sea snake species, *Aipysurus laevis,* (Lukoschek et al. 2005) and large-scale genotyping revealed relatively low polymorphism at most loci (Lukoschek et al. 2008). Moreover, true sea snakes comprise two evolutionary lineages (Lukoschek and Keogh 2006), the *Aipysurus* and *Hydrophis* groups, and microsatellites developed for *A. laevis* do not amplify in *Hydrophis* group species (Lukoschek 2008). The 39 *Hydrophis* group species are closely related (Lukoschek and Keogh 2006), so in order to obtain polymorphic markers for this group we developed microsatellite loci for *Hydrophis elegans* and conducted cross-species amplification trials for 13 *Hydrophis* group species.

We employed a modified version of a hybridization capture protocol using magnetic streptavidin beads and biotinylated probes (Hamilton et al. 1999; Hauswaldt and Glenn 2003) to enrich for microsatellites in a genomic library for *H. elegans*. Our protocol followed Mackiewicz et al. (2006) with one exception: we used a cocktail of biotinylated repeat probes comprising $(TG)_{12}$, $(AG)_{12}$, $(ATC)_8$, $(AAC)_8$, $(ACAG)_6$, $(ACTG)_6$, and $(AGAT)_7$. A total of 129 inserts were sequenced and microsatellite repeat regions detected by eye. Primers pairs were designed for all 24 inserts containing microsatellites using OligoAnalyzer 3.0 (Integrated DNA Technologies) and tested on *H. elegans* (n = 24). One primer from each pair was 5′ end labeled with a tag (5′-GGAAACAGCTATGACCATG-3′) for tailed PCR with an M13 primer labeled with a 6-FAM, HEX, or NED (Applied Biosystems) fluorophore. A subset of ten microsatellite loci amplified consistently and without multiple peaks. These were screened further in *H. elegans* and used in cross-species amplifications (Table 1).

V. Lukoschek · J. C. Avise
Department of Ecology and Evolutionary Biology,
University of California at Irvine, Irvine, CA 92697, USA

V. Lukoschek (✉)
ARC Centre of Excellence for Coral Reef Studies,
James Cook University, Townsville, QLD 4811, Australia
e-mail: vimoksalehi.lukoschek@jcu.edu.au

Table 1 Characteristics of ten microsatellite loci developed for *Hydrophis elegans*

Locus	Repeat motif	Primer sequence (5′–3′) (F/R)	Tm (°C)	Expected product size (bp)	GenBank accession no.
He792	$(AC)_3(TCAC)_2(TC)_{15}(AC)_9$	M13-ATTGGAGCAGCTCTGAAGGACTGT	52	143	JF261162
		CGTTTCTCCTTGGCCTGGATGATT			
He730	$(GA)_{18}$	M13-GAGTGTTTGTGGCTGAACCAGTTTG	52	179	JF261163
		TGAGACACCTCACAAGGG			
He919	$(TAGA)_4TAGG(TAGA)_8AAGA(TAGA)_3$	M13-GGACTTCCTGCTCAGTACTTGTGT	52	217	JF261164
		GCATTGGCTAGAGCAAGTGCATGT			
He967	$(GT)_3AT(GT)_9(GA)_{19}$	M13-ATCCTTCCTACCAGCCACAACCAT	55	227	JF261165
		CAGGTTGTTGTTGATCCTTGGTGA			
He953	$(TG)_{10}TTTGTA(TG)_8(AG)_7$	M13-GCTCTGACAATACATGGATGGCGT	55	268	JF261166
		GGCGACTTTAAGGCAGCATAGGTT			
He778	$(AG)_8GTAT(GA)_6CA(GA)_5CAGAGG(GA)_4AA(GA)_6$	M13-AAGGAAGGAGACAGAAGCGAACCA	55	177	JF261167
		CACCTGGGAATTCTAGGATCAAGC			
He793	$(GATA)_9A(ATAG)_{10}$	M13-GTGGTCTTGACACAACTTGAATGC	55	227	JF261168
		CCAGCATTAGGAATCTGATGAAGGGAGC			
He978	$(AC)_5AAAC(AG)_9(ACAG)_3(ACAGAG)_3$	M13-GGGCTTCATCATAAAGGTCACAATGC	55	268	JF261169
		CGCAGAAGTAGGATCAATGGTAGC			
He962	$(CT)_{11}CATT(CT)_7TTAT(GT)_4$	M13-TGAGCTTCAAGGGAGCTGACCATA	55	254	JF261170
		GGTGCATTAGACTCATCAAGAGTACCAC			
He706	$(GT)_8(GA)_{12}(GGGA)_4AGGG(GA)_5CA(GA)_2(CAGA)_5$	M13-GGGTGAAGCATCTGATAGTCTGTG	52	335	JF261171
		AGTCACTGTACGAGGCAGTTGTGA			

Amplifications of microsatellite loci were performed in a 10 µl volume containing 1× GoTaq reaction buffer (which included 1.5 mM $MgCl_2$), 0.25 µg bovine serum albumin, 0.2 mM each dNTP, 0.25 µM M13-labeled primer, 0.25 µM locus-specific primer, 0.025 µM tailed locus-specific primer, 0.4 U GoTaq DNA polymerase (Promega), and 1 µl genomic DNA. Amplifications were conducted using an initial denaturation step at 95°C for 5 min, followed by 32 cycles of 95°C for 40 s, locus specific annealing temperatures (Tm in Table 1) for 40 s, and 72°C for 1 min. PCR products were pooled into two groups, diluted ten-fold and electrophoresed on an ABI 3130xl automatic sequencer. Alleles were sized using a ROX labeled GS500 internal standard and scored using GeneMapper 4.0 (Applied Biosystems).

We screened 71 adult *H. elegans* from three regions around Australia. Cross-species amplifications were conducted for 13 *Hydrophis* group species plus two 'primitive' species (Lukoschek and Keogh 2006). For one species, *Lapemis curtus,* we screened 76 individuals while sample sizes for the remaining 14 species ranged from 1 to 14 (Table 2), typically from one or two locations per species. Samples were mostly obtained from trawler by-catch but also museum collections. Genotypic frequencies for species with $N \geq 10$ were tested for conformance to Hardy–Weinberg equilibrium (HWE) using the exact test (Guo and Thompson 1992) while linkage disequilibrium (LD) was tested in *H. elegans* and *L. curtus* using the exact test implemented in GenePop Web Version 4.0.10 (Raymond and Rousset 1995; Rousset 2008).

For *H. elegans*, nine microsatellite loci had moderate to high numbers of alleles (7–20) per locus and six had expected heterozygosities (H_e) ≥ 0.80 (Table 2). Genotype frequencies at four loci departed from Hardy–Weinberg equilibrium (HWE) at $P < 0.05$ (Table 2), but none remained significant after Bonferroni correction. Two pairs of loci were in linkage disequilibrium (LD) at $P < 0.05$ but only one pair (He978 and He706) remained significant after Bonferroni correction. This locus-pair was not in LD for *L. curtus* (see below), suggesting a sampling effect rather than physical genetic linkage. For *Lapemis curtus*, numbers of alleles per locus ranged from 3 to 16 with three loci having more alleles than for *H. elegans* (Table 2). However, expected heterozygosities in *L. curtus* typically were lower than in *H. elegans*, with eight loci having $H_e < 0.80$ (Table 2). Genotype frequencies at two loci departed from HWE at $P < 0.05$ (Table 2) but none remained significant after Bonferroni correction. Six pairs of loci were in LD at $P < 0.05$ but did not include He978 and He706, and none remained significant after Bonferroni correction.

All ten loci amplified successfully in ten of the 14 additional sea snake species screened while nine loci amplified successfully for three species and eight for the

Table 2 Attributes of ten microsatellite loci developed for *Hydrophis elegans* and the results of cross-species amplification trials for 15 hydrophiine sea snake species

Locus	*Hydrophis elegans* (N = 71)					*Lapemis curtus* (N = 76)				
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na	Ho	He
He792	148–180	69	14	0.77	0.86	142–162	75	8	0.40	0.43
He730	188–230	61	20	0.77	0.80	184–208	73	6	0.68	0.62
He919	212–248	60	9	0.83	0.80	192–276	73	16	0.86	0.88
He967	225–267	41	18	0.76*	0.90	215–251	67	8	0.57*	0.67
He953	270–298	65	11	0.82	0.81	282–296	73	3	0.08	0.08
He778	194, 196, 198	68	3	0.24	0.23	188–212	76	4	0.50	0.47
He793	204–248	67	12	0.85	0.85	210–268	71	13	0.85*	0.87
He978	270–290	60	7	0.62*	0.66	270–292	72	3	0.07	0.07
He962	266–298	69	10	0.26*	0.41	272–300	73	6	0.34	0.34
He706	344–360	63	9	0.71*	0.75	346–362	54	4	0.09	0.09

Locus	*Hydrophis occellatus* (N = 14)					*Astrotia stokesii* (N = 2)		
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na
He792	156–164	14	4	0.43	0.53	148, 152	2	2
He730	196–218	13	9	0.92	0.85	194	2	1
He919	220–244	12	7	0.83	0.81	232	1	1
He967	233–245	14	6	0.64	0.69	N/A	0	N/A
He953	270–286	13	7	0.85	0.79	288	2	1
He778	162–210	12	6	0.67	0.70	186, 196	2	2
He793	220–248	13	8	0.77	0.76	214–242	2	4
He978	278, 286	11	2	0.18	0.17	280	2	1
He962	282, 288, 290	14	3	0.43*	0.61	286, 290	2	2
He706	344	10	1	N/A	N/A	348, 358, 370	2	3

Locus	*Hydrophis lapemoides* (N = 11)					*Hydrophis macdowelli* (N = 10)				
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na	Ho	He
He792	142–156	11	6	0.82	0.69	140–150	9	2	0.56	0.40
He730	192–204	11	4	0.18*	0.58	184, 194, 204	10	3	0.90	0.62
He919	212–240	11	7	0.73	0.79	236–256	7	6	0.71	0.76
He967	231–249	10	6	0.80	0.75	229–241	9	4	0.33	0.38
He953	286–296	10	5	0.60	0.63	272–292	9	5	0.67	0.64
He778	188, 190, 196	9	3	0.22	0.20	186, 198	9	2	0.56	0.48
He793	214–230	11	5	0.82	0.78	224–260	10	7	1.00	0.83
He978	278	11	1	N/A	N/A	286, 290, 294	9	3	0.78	0.55
He962	282–292	10	4	0.30	0.35	270, 272	10	2	0.10	0.10
He706	348–354	11	4	0.27*	0.43	334	10	1	N/A	N/A

Locus	*Pelamis platurus* (N = 10)					*Hydrophis brooki* (N = 1)		
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na
He792	146–156	10	5	0.70	0.74	154	1	1
He730	182–200	10	6	0.60	0.64	N/A	0	0
He919	216–240	5	4	0.80	0.70	224, 228	1	2
He967	227–245	7	7	0.86	0.76	247, 249	1	2
He953	284–294	9	6	0.44*	0.78	288, 292	1	2
He778	176–198	10	4	0.30	0.27	208	1	2

Table 2 continued

Locus	*Pelamis platurus* (N = 10)					*Hydrophis brooki* (N = 1)				
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na		
He793	228–252	9	7	0.78	0.81	232, 236	1	2		
He978	278, 280	10	2	0.10	0.10	274, 282	1	2		
He962	274–292	10	8	0.80	0.75	270, 288	1	2		
He706	320–348	9	5	0.67	0.52	348	1	1		
Locus	*Hydrophis major* (N = 13)					*Acalyptophis peronii* (N = 8)				
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na	Ho	He
He792	146–166	13	6	0.69	0.54	156, 160, 164	8	3	0.75	0.62
He730	N/A	0	0	N/A	N/A	194, 196, 200	8	3	0.13	0.32
He919	208–238	8	10	0.75*	0.88	228–272	5	8	1.00	0.86
He967	225–239	9	4	0.33*	0.51	241, 247	6	2	0.33	0.28
He953	272–290	12	4	0.42	0.63	288, 290, 292	8	3	0.38	0.32
He778	186–208	13	4	0.54	0.43	188, 198, 210	8	3	0.38	0.32
He793	200–252	12	8	0.75	0.81	228–248	7	5	0.29	0.65
He978	280, 282, 286	7	3	0.71	0.52	278	8	1	N/A	N/A
He962	272–294	13	7	0.62	0.72	276–286	8	4	1.00	0.73
He706	348–358	8	4	0.38	0.65	350	7	1	N/A	N/A
Locus	*Hydrophis ornatus* (N = 7)					*Hydrelaps darwiniensis* (N = 1)				
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na		
He792	146, 156, 160	7	3	0.43	0.36	144, 162	1	2		
He730	192–216	7	7	0.86	0.82	N/A	0	N/A		
He919	228, 232, 236	3	3	0.67	0.61	250, 262	1	2		
He967	237–247	5	4	0.40	0.48	217, 219	1	2		
He953	282, 294	2	2	0.00	0.50	288	1	1		
He778	196	2	1	N/A	N/A	234, 236	1	2		
He793	224–240	7	5	0.86	0.76	232, 234	1	2		
He978	278	7	1	N/A	N/A	N/A	0	N/A		
He962	270	6	1	N/A	N/A	282	1	1		
He706	342, 348	4	2	0.25	0.22	248	1	1		
Locus	*Hydrophis kingii* (N = 9)					*Hydrophis pacificus* (N = 6)				
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na	Ho	He
He792	138–152	9	4	0.67	0.66	152–162	6	4	0.83	0.65
He730	182–190	9	4	0.22	0.30	184–222	6	5	0.67	0.75
He919	244–278	8	7	0.63	0.80	216–232	4	5	0.75	0.78
He967	235, 237	8	2	0.13	0.12	249, 251	4	2	0.25	0.22
He953	278, 280, 286	9	3	0.44	0.54	286	6	1	N/A	N/A
He778	176, 194, 196	9	3	0.33	0.51	196, 204, 208	6	3	0.50	0.40
He793	214–238	9	6	0.89	0.77	214–230	6	5	0.83	0.67
He978	286, 288	6	2	0.50	0.49	270	4	1	N/A	N/A
He962	282, 286, 290	8	3	0.88	0.59	276, 280	6	2	0.17	0.15
He706	340, 344	8	2	0.13	0.12	346	5	1	N/A	N/A

Table 2 continued

Locus	*Hydrophis cyanocinctus* (N = 3)					*Parahydrophis mertoni* (N = 1)		
	Size range (bp)	N	Na	Ho	He	Size range (bp)	N	Na
He792	144–150	3	3	0.33	0.50	154	1	1
He730	190, 194	3	2	0.33	0.28	184	1	1
He919	216–232	2	3	1.00	0.63	228	1	1
He967	235–251	3	4	0.67	0.67	N/A	0	N/A
He953	286, 288	3	2	0.67	0.44	284, 294	1	2
He778	192, 208	3	2	0.33	0.50	196	1	1
He793	218–234	3	5	1.00	0.78	226, 230	1	2
He978	294	1	1	N/A	N/A	282	1	1
He962	276, 278	3	2	0.33	0.28	294	1	1
He706	342, 348	3	2	0.33	0.28	356	1	1

N is the number of samples that successfully amplified and were scored for each locus. *Na* is the number of alleles. *Ho* and *He* refers to observed and expected heterozygosity calculated by GenAlEx (Peakall and Smouse 2006). Hardy–Weinberg Equilibrium was evaluated for the seven species with sample sizes of ten or more snakes

Loci that deviated from HWE are indicated by * ($P = 0.05$)

remaining species (Table 2). Allele sizes and frequency distributions varied considerably among species (Table 2). A few loci departed from HWE for the five species with $N \geq 10$ (Table 2) but different loci typically were involved suggesting sampling artefacts. Only two of 225 tests showed departures from LD ($P < 0.05$) and none remained significant after Bonferroni correction. These highly successful cross-amplifications indicate that these ten loci will be useful for investigating population genetic structure, gene flow and parentage for all *Hydrophis* group species, plus the three 'primitive' Australian endemics.

Acknowledgments This work was supported by the Sea World Research and Rescue Foundation; the University of California, Irvine; and the ARC Centre of Excellence for Coral Reef Studies. We thank Andrei Tatarenkov, Felipe Barreto and Rosemary Byrne for lively discussions about microsatellite development.

References

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361–372

Hamilton MB, Pincus EL, Di Fiore A, Flescher RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. BioTechniques 27:500–507

Hauswaldt JS, Glenn TC (2003) Microsatellite DNA loci from the diamondback terrapin (*Malaclemys terrapin*). Mol Ecol Notes 3:174–176

IUCN (2010) IUCN red list of threatened species. Version 2010.4. www.iucnredlist.org

Lukoschek V (2008) Molecular ecology, evolution and conservation of hydrophiine sea snakes. Ph.D. Thesis, James Cook University, Townsville

Lukoschek V, Keogh JS (2006) Molecular phylogeny of sea snakes reveals a rapidly diverged adaptive radiation. Biol J Linn Soc 89:523–539

Lukoschek V, Waycott M, Dunshea G (2005) Isolation and characterization of microsatellite loci from the Australasian sea snake, *Aipysurus laevis*. Mol Ecol Notes 5:875–881

Lukoschek V, Waycott M, Keogh JS (2008) Relative information content of polymorphic microsatellites and mitochondrial DNA for inferring dispersal and population genetic structure in the olive sea snake, *Aipysurus laevis*. Mol Ecol 17:3062–3077

Mackiewicz M, Tatarenkov A, Perry A, Martin R, Elder JFJ, Bechler DL, Avise JC (2006) Microsatellite documentation of male-mediated outcrossing between inbred laboratory strains of the self-fertilizing mangrove killifish (*Kryptolebias marmoratus*). J Hered 97:508–513

Peakall R, Smouse PE (2006) genalEx 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86: 248–249

Rousset F (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol Ecol Res 8:103–106