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

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**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'-GGAACACGTATGACCATG-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).
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)_2(TCAC)_2(TC)_15(AC)_9 | M13-ATGGAGCAGCTCTGGAAGGACTGT CGTTTCTCCTGGCTGGATGTAT | 52      | 143                       | JF261162              |
| He730   | (GA)_18      | M13-GAGGGTTGGGTGGCTAACCAGTGGTT TGAAGACACACTCAAGG    | 52      | 179                       | JF261163              |
| He919   | (TAGA)_3(AA)AAGA(TAGA)_3 | M13-GGACTCTCCTGCTAACCAGGGAACAGCAGC  | 52      | 217                       | JF261161              |
| He967   | (GT)_3(AC)_9 | M13-ACCTTCTCCTTCAACGCCAACCAACCAT CAGTTTTGTCATCCTTGGTGA | 55      | 227                       | JF261165              |
| He953   | (TG)_10TTTGTA(TG)_8(AG)_7  | M13-GCCTGACAAATACATGGATGGCGT GGGACACCTTTAGGGGCTAGTT | 55      | 268                       | JF261166              |
| He978   | (AG)_3GTAT(GA)_3(CA)_10(CA)_9AGGAGAAGAGAAGCAGGAA  | CACCTGGGAATTCAAGGATCAACC | 55      | 177                       | JF261167              |
| He979   | (GATA)_3A(ATA)G_10 | M13-GTGTTGCTGCAACACATTTGAATGC CCACATTAGGAATCTGATGAAGGAGC | 55      | 227                       | JF261168              |
| He978   | (AC)_3AAACG(A)G_3(CAGAG)_3 | M13-GGCTCTACATACAAGGTCACATGC CCGCAGATGGATCAATGGTACG | 55      | 268                       | JF261169              |
| He962   | (CT)_11CATT(CT)_7TTAT(GT)_4 | M13-TGAGCTTCAGGGGACTGACCAGAAGTCTACATCAACAGGATGACCA | 55      | 254                       | JF261170              |
| He706   | (GT)_13(GA)_2(GGGA)_4AGGG(GA)_3(CA)_2(CAGA)_3 | M13-GGGTGAAAGCATGTGATTCTGCTGG TGATCCTGTAGCGGAGGTTGA | 52      | 325                       | JF261171              |

Amplifications of microsatellite loci were performed in a 10 μl volume containing 1× GoTaq reaction buffer (which included 1.5 mM MgCl₂), 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* ≥ 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 (*Hₐ*) ≥ 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 *Hₐ* < 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 ocellatus* (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  |
| He953 | 233–245         | 14 | 6  | 0.64 | 0.69 | N/A              | 0 | N/A|
| He958 | 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  | N/A | N/A | 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  |
| 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 |
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.

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**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. \( N_a \) is the number of alleles. \( H_0 \) and \( H_e \) 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 * (\( P = 0.05 \))

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