PRIMER NOTE

Isolation and characterization of novel microsatellite markers from the Australian tiger snakes (Elapidae: *Notechis*) and amplification in the closely related genus *Hoplocephalus*

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Abstract

We provide details of seven microsatellite loci (out of 20 primer pairs designed) that exhibited the cleanest and strongest banding patterns for the Eastern tiger snake *Notechis scutatus*. These loci were used to screen 76 individuals from across the geographical range of *N. scutatus* and a further 14 individuals of the closely related and endangered broadheaded snake *Hoplocephalus bungaroides*. We observed large numbers of alleles per locus (14–52) and relatively high levels of heterozygosity (0.270–0.696) within the *N. scutatus* sample. These markers are also likely to be informative for work on *H. bungaroides*.

Keywords: elapid, *Hoplocephalus*, microsatellite, *Notechis*, snake

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The Australian tiger snakes comprise either one or two very closely related species and are the only members of the genus *Notechis* (Cogger 2000). They represent one of the best studied groups of snakes in Australia with a considerable published knowledge base on their morphology, ecology, natural history, diet, behaviour, and distribution. They also represent one of the most famous examples of insular body size variation with both dwarf and giant forms (Schwaner & Sarre 1988). We are interested in levels of genetic diversity within and between populations of tiger snakes and, to this end, have discovered polymorphic microsatellites for this taxon. Tiger snakes are very closely related to the endangered broadheaded snake *Hoplocephalus bungaroides* (Keogh *et al.* 2000), and hence we have also tested the *Notechis* primers against a series of *H. bungaroides* samples to supplement microsatellites already developed for the species by Burns & Houlden (1999).

Notechis scutatus DNA was extracted from liver (South Australian Museum R31640) and a partial genomic library was made following methods we describe elsewhere (Scott *et al.* 2001), except that ligation products were transformed

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into chemically competent TOP10 cells (Invitrogen). Approximately 38 000 transformants were recovered, lifted and screened using methods described in Scott *et al.* (2001). A total of 160 putative positive recombinants were picked and DNA inserts amplified.

DNA amplification reactions were 40 μL in volume and contained 10 pmol of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 0.2 mM dNTPs and 1 unit of *Taq* DNA polymerase (Life Technologies). Double-stranded product was amplified using the step-down cycling profile described in Scott *et al.* (2001), which was performed on a Corbett PC-960C cooled thermal cycler (Corbett Research). Polymerase chain reaction (PCR) products were gel purified using the BRESAClean kit (Geneworks) following manufacturer's instructions.

A total of 107 amplified clones contained inserts ranging between 200 and 900 bp. Following second round screening (Scott *et al.* 2001), 55 recombinants were selected for sequencing. Clones were sequenced in both directions using universal M13 forward and reverse primers. Sequencing reactions were performed using the ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions. Extension products were separated and visualized on an ABI 377 automated

Table 1 Attributes of seven microsatellite loci developed for *Notechis scutatus*, trialled on this species and *Hoplocephalus bungaroides*. Forward primer sequences do not include the M13(−21) 5′ tail. Cloned allele length refers to product size generated from cloned microsatellite loci. The values for size range of alleles includes the additional 18 bp due to the M13(−21) tail. *N* refers to the number of samples scored at each locus

Locus (GenBank Accession no.)	Primers: 5′ to 3′	Repeats	Cloned allele length	Alleles (Range) *N* N. scutatus H. bungaroides	H_O, H_E N. scutatus H. bungaroides
Ns03	F: GCAGCCACCCGTATCAAC	$(GAT)_{11}$	250 bp	27 (207–328) **70**	0.514, 0.934
(AF321184)	R: GTTTGGCAAGCATTATCCGT			2 (224 & 243) **11**	0.000, 0.173
Ns05	F: ACAGCCTGCGCTTCTCTAAG	$(TG)_{12}T(TG)_3$	155 bp	33 (170–212) **69**	0.696, 0.936
(AF321185)	R: TGTAACCCAACCCAAGCAAT			2 (166 & 174) **14**	0.071, 0.071
Ns14	F: TATGTTCTATTTCCCCCCCC	$(CTTT)_{13}(CT)_{11}T(CT)_4$	285 bp	52 (275–423) **62**	0.339, 0.977
(AF321186)	R: CCAGAAGGCTTTTCCCTTTC			14 (312–372) **13**	0.538, 0.923
Ns32	F: ATTCAGATCCCCAGGAAAGA	$(CT)_{13}$	213 bp	14 (215–258) **63**	0.270, 0.823
(AF321187)	R: CTAGATTTTTACGAGCAGGT			3 (235–239) **14**	0.357, 0.524
Ns40	F: GGAGCAGACCATGAGCAGAT	$(TG)_{17}$	174 bp	24 (173–217) **66**	0.667, 0.940
(AF321188)	R: TCCCACATTGGGTCTGAAAT			2 (226 & 236) **10**	0.300, 0.395
Ns43	F: TCAGGTATTTTCCCACGTCA	$GACA(GA)_3CA(GA)_3TTG(GA)_{14}$	310 bp	29 (328–409) **68**	0.588, 0.798
(AF321189)	R: TAGCCCTTGTTTAGGAGCCA			7 (410–434) **13**	0.846, 0.858
Ns67	F: CTTCGACAAGTTGACAATGA	$(CT)_2TT(CT)_{16}(CA)_{21} ... (GA)_8$	224 bp	25 (216–265) **68**	0.647, 0.936
(AF321190)	R: ACTGTCTGCATGTGTTTGCT			2 (236 & 240) **13**	0.615, 0.492

DNA sequencer (Applied Biosystems). Sequences were aligned and edited using SEQUENCHER version 3.1.1 (Gene Codes Corporation). Of the 55 sequenced clones, 20 yielded sequence of appropriate length and sufficient quality for primer design. Primer pairs for amplification of microsatellite loci were designed using PRIMER version 0.5 (Whitehead Institute of Biomedical Research). Primer pairs were designed to yield products in three size classes in order to facilitate multiplex analysis of microsatellite loci.

A panel of seven *N. scutatus* samples was used to screen each of the 20 primer pairs. The step-down protocol described by Scott *et al.* (2001) was modified (denaturation 30 s, annealing 30 s, extension 45 s; annealing temperature 'stepped-down' by 5 °C on every second cycle from 65° to 35 °C; 25 cycles at final annealing temperature of 30 °C) and used to amplify microsatellite fragments, which were then visualized on 2% agarose gels. Based on the strength and clarity of banding patterns, eight loci were then selected for further investigation. These loci were used to screen the same panel of samples. The same step-down protocol was used to generate fluorescent microsatellite fragments through [F]dUTP incorporation (PE Biosystems) according to manufacturer's instructions. Extension products were separated and visualized on an ABI 377 autosequencer. Data were analysed using GENESCAN Analysis 3.1 (PE Biosystems).

Seven loci exhibited variable banding patterns and forward primers were redesigned by adding an M13(−21) tail (5′-TGTAAAACGACGGCCAGT-3′) to their 5′ ends (Schuelke 2000). A panel comprising 76 samples of *N. scutatus*

from across the species range was then used to evaluate each of the seven loci. An additional 14 samples of the closely related and endangered *H. bungaroides* (Keogh *et al.* 2000) were screened to assess cross-species amplification of these loci.

Amplification reactions were 20 μL in volume and contained approximately 100 ng of template, 2.5 pmol of the M13(−21) tailed sequence-specific forward primer, 10 pmol of the sequence-specific reverse primer, 10 pmol of a fluorescent dye-labelled M13(−21) universal primer (either FAM, HEX or TET, Life Technologies), 2 μL each of 10× PCR_x Amplification Buffer and 10× PCR_x Enhancer Solution (Life Technologies), 2 mM $MgSO_4$, 0.2 mM dNTPs, and 1 unit of PLATINUM® *Taq* DNA polymerase (Life Technologies). The cycling profile mentioned previously was further modified (annealing temperature 'stepped-down' by 5 °C on every second cycle from 65° to 55 °C; 25 cycles at final annealing temperature of 50 °C) and used to amplify target fragments. Fluorescent dye-labelled fragments were separated and visualized as before and data were analysed using GENESCAN Analysis 3.1 (PE Biosystems). Allele sizes were 18 bp longer than those labelled using F[dUTPs] due to the additional 18 bp of the M13(−21) sequence.

Table 1 shows attributes of seven microsatellite loci in 76 samples representing the geographical range of *N. scutatus* in Australia and in 14 samples of *H. bungaroides*, representing four geographically close (within 5 km) populations in south-eastern New South Wales. These seven microsatellite loci exhibited large numbers of alleles per locus (14–52) in *N. scutatus* while fewer alleles (2–14) were observed

in *H. bungaroides*. Given the distribution of the *Notechis* samples, including several islands of the southern Australian coast, Hardy–Weinberg assumptions are violated. The trend to heterozygote deficit observed in *N. scutatus* (Table 1) is most likely due to a Wahlund effect, although the presence of null alleles cannot be discounted. This trend is also observed in *H. bungaroides*, although three loci (Ns40, Ns43, Ns67) met Hardy–Weinberg expectations. The *Hoplocephalus* panel comprised animals from four localities within a relatively small area but population subdivision is likely due to low dispersal and the fragmented nature of appropriate habitat (Webb & Shine 1997).

In summary, these seven tiger snake microsatellite loci exhibited large numbers of alleles per locus and high heterozygosity (mean = 0.53, SE ± 0.06) relative to other reptiles (Burns & Houlden 1999). This suggests that they will be extremely useful for investigating the population genetics of these snakes across their range. Several of these microsatellites are also highly polymorphic in the endangered broadheaded snake and so will be useful in assessing genetic diversity and other facets of population biology.

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