



## Microsatellite DNA markers for New Zealand skinks

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Reptiles are a prominent part of the faunal diversity of New Zealand, but most species have suffered major range declines or extinctions in the past 2000 years. Nearly fifty percent of species (26 of 62) are now threatened or restricted to offshore islands and eight are subject to recovery programs (Towns and Daugherty 1994; Towns et al. 2001). Most declines can be attributed to loss or modification of habitat and predation by introduced mammals.

Previous genetic studies of New Zealand reptiles have increased the number of recognised species (e.g. Daugherty et al. 1990), but provided little autecological or population-level genetic information that could be applied to management. Here we present the first microsatellite DNA markers to be developed for a New Zealand lizard. We are using these markers to study dispersal in the grand skink, *Oligosoma grande*, a large rock-dwelling lizard endemic to sub-alpine grasslands in southern New Zealand. This formerly widespread species now has a restricted range, and is listed as 'vulnerable' under IUCN criteria (Hilton-Taylor 2000).

We isolated tetranucleotide and dinucleotide microsatellite DNA from *O. grande* using an enrichment protocol modified from Armour et al. (1994). A detailed protocol is available at <http://www.massey.ac.nz/~ofberry/MAKING%20A%20MICROSATELLITE%20LIBRARY.pdf>. Briefly, genomic DNA from 5 individuals was digested with *SAU 3A*, pooled, and the 300–600 base pair (bp) fraction ligated to *SAU* linkers (Royle et al. 1992). Fragments containing microsatellite DNA were selected by hybridisation to 3 mm<sup>2</sup> nylon membranes saturated with AAAG/TTTC, GATA/CTAT, GA/CT, or CA/GT target repeats. The enriched fraction was recovered by

washing with SSC (150 mM sodium chloride, 15 mM sodium citrate) and 0.1% SDS (sodium dodecyl sulphate), then stripped from the membranes (100 µl 50 mM KOH/0.01% SDS, followed by 100 µl 50 mM Tris HCl pH 7.5/0.01% SDS) and PCR amplified using *SAU LA* as the primer (95 °C, 5 min; 35 × (67 °C 30 secs, 72 °C 30 secs, 95 °C 30 secs); 70 °C 4 min).

*SAU* linkers were removed from the amplicons by digestion with *SAU 3A*. The DNA was purified (High-pure kit, Roche), ligated into pUC18 cut with *Bam* HI, and the plasmid transformed into Max Efficiency DH5α cells (Gibco BRL). Recombinant clones were identified by blue-white selection. Five hundred and fifty positive clones were cultured and screened for the presence of microsatellites by hybridisation to <sup>32</sup>P labeled (AAAG)<sub>n</sub>, (GATA)<sub>n</sub>, (GA)<sub>n</sub>, or (CA)<sub>n</sub> concatemers. Twenty-eight percent of clones successfully hybridised to the probes.

After sequencing 70 positive clones using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, PCR primers were designed using OLIGO version 4.0 (National Biosciences). Loci were co-amplified in groups of three (see Henegariu et al. 1997). Ten microlitre reactions consisted of 1 × PCR buffer (Roche), 2.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.4 µg/µL bovine serum albumin, 1 M Betaine, 0.5 U *Taq* polymerase (Roche), 20 ng skink genomic DNA, and primers. The PCR profile consisted of one cycle of 94 °C for 2 minutes, 30 cycles of (94 °C 30 secs, T<sub>annealing</sub> 20 secs, 72 °C 30 secs), and a final extension at 72 °C for 5 minutes. PCR was carried out on an iCycler thermal cycler (Biorad). Annealing temperatures and primer concentrations that gave the most even co-amplification are given in Table 1.

Table 1. Primers and PCR conditions used to amplify microsatellites in the grand skink, *Oligosoma grande*. Genbank accession numbers AF513228–AF513242

Locus	Primer sequence (5' – 3')	Repeat array	PCR multiplex set	Primer conc. (mM)	T <sub>A</sub> °C	Size range of PCR product (bp)	Number of alleles	H <sub>obs</sub>	H <sub>exp</sub>	n
Oligr3	F CAGCCACCAGTATGTCAGTTG R TGCCTCTGCCACCAAATTAC	GATA <sub>19</sub>	1	0.25	57	223–317	17	0.89	0.79	286
Oligr4	F ACCACCAAAAAGGCTCTGCTC R ACCCATTAGCCATTATTAGCC	GATA <sub>15</sub>	1	0.30	57	197–258	15	0.80	0.77	288
Oligr10	F AGTGTGTTCTGACATTTGTGG R CCAGGATATACAGGATAACAG	GATA <sub>18</sub> (ATT)GATT <sub>9</sub>	1	0.50	57	250–294	12	0.85	0.82	285
Oligr2	F GGAGAGTTCATTGTGTTCCTA R AGAACCAACAACACCATCTGTA	AAAG <sub>21</sub> (GAAG)AAAG <sub>2</sub>	2	0.40	59	236–293	15	0.73	0.79	281
Oligr6	F TTTGGTGCCTTATTGCTTTG R GGTCTTTGGGTCTATGCTTTG	GATA <sub>18</sub>	2	0.50	59	126–220	18	0.81	0.80	287
Oligr13	F GGATCCCTGTGTTACCCACACA R CGTCCATCTGTCCATCTAATC	GATA <sub>23</sub> GATG <sub>5</sub>	2	0.30	59	200–326	26	0.77	0.80	285
Oligr7	F AAGCATCTTCGGTCCACGAC R CAAGAAAATGTAGGGCACGAC	GATA <sub>20</sub>	3	0.45	59	154–228	16	0.85	0.78	289
Oligr8	F CGGTCCAGCATCAGCCATCAC R GCACCACCCATTCAAAGATGTG	AAAG <sub>18</sub>	3	0.35	59	232–296	15	0.75	0.73	289
Oligr11	F CTTGAAGAGCAACGATGTCCA R GTTACTGATTGGCTGGGTGAC	GATA <sub>22</sub>	3	0.40	59	179–264	15	0.84	0.79	289
Oligr1	F TGTCATTCCCCGTA CTGTG R CCACCTCCCTCGGCATCATTC	AAAG <sub>28</sub>	4	0.35	52.5	168–219	15	0.80	0.81	246
Oligr14	F TCTGGTTAACAGAGATTCCAC R AGACAGTGGTGAAGTTGAAG	GT <sub>20</sub> (GATT)GT <sub>3</sub>	4	0.35	52.5	266–290	8	0.39	0.36	248
Oligr17	F GATCTGCAACCTATATGTACA R AGCAACCAAGAATACAAACAC	CA <sub>13</sub> (CG)CA <sub>4</sub>	4	0.45	52.5	264–305	13	0.82	0.80	248
Oligr15	F ACCTACAACCAGTCCACTTC R CTGGGCACTGAGTTGGTATGA	CA <sub>25</sub>	5	0.30	52.5	95–150	22	0.56	0.80	228
Oligr19	F CTGTCTGCTGCTAATGGAGAG R AAACACCCCTCTCGTTGTAC	CA <sub>18</sub>	5	0.30	52.5	148–182	11	0.77	0.76	250
Oligr20	F TTGCTGCTTCTATCCCTTCTC R TGGTGTGCCTTGCAATAGTC	CT <sub>4</sub> CA <sub>14</sub> (GAA)CA <sub>5</sub>	5	0.25	52.5	271–319	11	0.74	0.76	250

PCR multiplex set refers to loci that co-amplify. T<sub>A</sub> refers to annealing temperature, H<sub>obs</sub> and H<sub>exp</sub> refer to mean observed and expected heterozygosity respectively across groups of skinks.

Ten tetranucleotide and five dinucleotide loci were isolated and used to amplify DNA from grand skinks (Table 1). We genotyped individuals using DNA isolated from toeclips or tail-tips (*ca.* 2 mg tissue) by a salting-out procedure (Sunnucks and Hales 1996) or Aquapure tissue isolation kit (Biorad). Two hundred and ninety grand skinks were genotyped at 15 loci on an ABI PRISM 377 sequencer using GENESCAN version 3.1 software (Perkin-Elmer). These loci were highly variable. The average number of alleles per locus was 15.73 ( $\pm 1.16$  SE), and the average observed

heterozygosity was 0.76 ( $\pm 0.03$  SE). Tetranucleotide loci had significantly higher observed heterozygosity than dinucleotide loci ( $p < 0.01$ , two-sample permutation test with 9999 iterations).

We used the randomisation approaches implemented in FSTAT 2.9.3 (Goudet 1995) to test for departures from Hardy-Weinberg and linkage equilibrium. Grand skinks live on rock outcrops in groups of *ca.* 20 related individuals and isolated from other groups by 50–100 m of non-habitat matrix vegetation. This situation is likely to produce non-random

Table 2. Amplification of microsatellite DNA loci in New Zealand skinks (Genera: *Oligosoma* and *Cyclodina*). ● indicates amplification of a product of comparable size to that amplified from *O. grande*. ○ indicates no amplification of any products. One individual of each species was tested per locus. \* denotes species with high conservation priority (Townsend et al. 2001)

Species	Locus	Oligr1	Oligr2	Oligr3	Oligr4	Oligr6	Oligr7	Oligr8	Oligr10	Oligr11	Oligr13	Oligr14	Oligr15	Oligr17	Oligr19	Oligr20
<b><i>Oligosoma</i></b>																
<i>grande</i> *		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
<i>acrinasum</i>		●	●	●	●	○	○	●	●	○	○	●	●	●	●	○
<i>chlaronoton</i>		●	●	●	●	●	●	●	○	●	●	○	●	●	●	●
<i>homanolotum</i> *		○	●	●	●	●	○	●	●	○	●	●	●	●	●	●
<i>infrapunctatum</i> *		●	●	●	●	●	●	●	○	●	●	●	●	●	●	○
<i>lineocellatum</i>		●	●	●	●	●	●	●	○	●	●	●	●	●	●	●
<i>longipes</i> *		●	●	●	○	○	●	●	○	○	●	○	○	●	●	●
<i>moco</i>		●	○	●	●	●	○	●	●	○	●	●	●	●	●	●
<i>otagense</i> *		○	○	●	●	●	●	●	○	○	●	●	○	○	●	○
<i>smithi</i>		○	●	○	○	●	●	●	○	○	●	●	○	○	●	●
<i>suteri</i>		●	●	●	●	○	○	●	○	○	●	○	○	○	●	●
<i>waimatense</i> *		●	●	●	●	○	○	●	○	○	●	●	●	●	●	○
<i>zelandicum</i>		●	○	●	○	●	○	●	○	○	●	●	●	○	●	●
<b><i>Cyclodina</i></b>																
<i>aenea</i>		●	○	●	●	●	●	●	○	●	●	○	●	●	●	●
<i>alani</i> *		●	●	●	●	●	●	●	○	●	●	●	●	●	●	●
<i>oliveri</i>		●	●	●	●	○	○	●	○	○	●	●	●	●	●	●
<i>whitakeri</i> *		●	●	●	●	●	●	●	○	○	●	●	○	●	●	●
<i>Lampropholis delicata</i>		○	○	○	○	○	○	●	○	○	●	○	○	○	○	○
<i>Leiolopisma telfairi</i>		○	○	○	●	○	○	●	○	○	○	○	●	○	●	○

associations between alleles. To account for Wahlund effects, Hardy-Weinberg tests were performed by randomising alleles among individuals on each rock outcrop. Using a global test, we found no significant overall departure from Hardy-Weinberg equilibrium ( $p > 0.05$ ). However, tests per locus and per rock outcrop showed that on some rock outcrops the loci Oligr2 and Oligr15 had significant deficits of heterozygotes ( $p < 0.01$ ; Oligr2 average  $F_{IS} = 0.06$ ; Oligr15 average  $F_{IS} = 0.30$ ), and null homozygotes indicating the existence of null alleles. Attempts to correct for null alleles at locus Oligr15 by reducing annealing temperature were unsuccessful. Loci were not linked ( $p < 0.001$ ).

New Zealand has two endemic skink genera, *Oligosoma* (~22 spp.), and *Cyclodina* (~7 spp.), which are members of the *Eugongylus* Group of Indo-Pacific skinks (Hutchinson and Donnellan 1993). Cross-species amplification of loci was tested on 16 of these 29 species and the related species, *Leiolopisma telfairi*, from Mauritius in the western Indian Ocean, and *Lampropholis delicata* from eastern Australia. Amplification fragments were considered homologous to *O. grande* microsatellites if they amplified fragments of similar size to those expected from *O. grande* ( $\pm 100$

bp). We tested one individual of each species for each locus using the PCR profile optimised for *O. grande*. An average of 11.8 loci amplified in each New Zealand species tested (range 9–14; Table 2). Presumptive homologous loci also amplified in *L. telfairi* and *L. delicata* suggesting that these markers could also be applied to other *Eugongylus* Group skinks.

Though their use is now widespread, there have been few applications of microsatellite DNA in the conservation of New Zealand's biodiversity. The markers described here are highly variable in the grand skink and should be suitable for high-resolution genetic studies on other New Zealand skinks, including studies of dispersal, mating systems, and assigning provenance to smuggled animals. They are likely to find applications in ecological and conservation-related research on this diverse but imperiled fauna.

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