

## PRIMER NOTES

### Isolation and characterization of microsatellite loci from the bush rat, *Rattus fuscipes greyii*

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The Australian bush rat, *Rattus fuscipes greyii*, possesses several characteristics which make it an ideal model organism for population and evolutionary genetic studies. The bush rat is one of several species belonging to Australia's endemic *Rattus*. Australia's endemic *Rattus* are a well characterized group for which detailed studies of karyotypic and protein evolution, interspecies hybrid fertility, and morphometric studies have been carried out (Baverstock & Adams 1987). Australia's endemic *Rattus* have an ideal hierarchical structure, allowing comparisons between populations, subspecies, and species. Furthermore, Australia's endemic *Rattus* form a monophyletic clade with respect to the black rat, *R. rattus*, and the laboratory rat, *R. norvegicus* (Taylor & Horner 1973), allowing comparisons at an even greater level of divergence.

In the present study we have successfully isolated microsatellite loci from a genomic DNA (gDNA) library using an enrichment technique described by Edwards *et al.* (1996). The library was created from gDNA extracted from the liver of a bush rat from Kangaroo Island (SCU Tissue ref: 2423) using standard protocols (Bothwell *et al.* 1990). The following oligonucleotides were used for enrichment: [GA]<sub>15</sub>, [GT]<sub>20</sub>, [ACT]<sub>14</sub>, [AGA]<sub>14</sub>, [CAA]<sub>14</sub>, [CTA]<sub>14</sub>, [CTT]<sub>14</sub>, [CTG]<sub>10</sub>, [CAG]<sub>10</sub> and [GAC]<sub>14</sub>, [AGC]<sub>14</sub>, [CAT]<sub>14</sub> and [ACA]<sub>14</sub>.

Ninety-four putative positive recombinants were picked from agar plates, individually suspended in 100 µL of 10 mM Tris (pH 8.0), and boiled for 5 min to lyse the cells and release the DNA. Following settling of the cell debris, 1 µL of supernatant was used as template for PCR amplification using universal M13 forward (5'-CGCCAGGGTTTCCAGTCACGAC-3') and reverse (5'-AGCGGATAACAATTCACACAGGA-3') primers (Pacific Oligos).

PCR reaction mixtures contained 1 µL of clone DNA, 50 mM KCl, 10 mM Tris (pH 8.0), 0.25 units of *Taq* DNA polymerase (Boehringer Mannheim), 1.5 mM MgCl<sub>2</sub>; 0.1 mM of each dNTP, 0.1 µM each of the universal M13 forward and reverse primers, and Milli-Q water to a total volume of 10 µL. All PCR reactions were performed using a PC960G

thermal cycler (Corbett Research, Sydney). PCR reactions were run under the following conditions: 1 min initial denaturation at 92 °C, followed by 30 cycles of 10 s denaturation at 92 °C, 30 s primer annealing at 50 °C, and 1 min extension at 75 °C, followed by a final extension step at 75 °C for 5 min. PCR products were electrophoresed on a 1% agarose gel, alongside amplified pUC containing no insert, to determine size of inserts. Gels were stained with ethidium bromide and visualized under UV light.

Of the 94 amplified clones, 46 clones with inserts ranging in size between 300 and 800 bp were selected for sequencing. Two microlitres of supernatant, from the lysed cells containing clones, was used as template for PCR amplification in a 20-µL total volume before sequencing. PCR conditions were the same as above. PCR products were purified by ethanol precipitation prior to sequencing (Sambrook *et al.* 1989). Purified products were resuspended in 20 µL of 10 mM Tris (pH 8.0), and 50 fmol of purified PCR product was used as template for sequencing.

Clones were sequenced in the forward direction using the universal M13 forward primer. Sequencing reactions were performed using the ABI Prism 'Big Dye' terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Extension products were separated on an ABI 377 automated DNA sequencer (Applied Biosystems). Sequences were edited using the program SeqEd™ version 1.0.3 (Applied Biosystems). Of the 46 sequenced clones, 31 contained microsatellites, 22 of which contained sufficient flanking sequence for primer design, which were then sequenced in the reverse direction using the universal M13 reverse primer. Forward and reverse sequences were aligned to determine a consensus sequence for primer design. Microsatellite primer pairs were designed using the program NAR (Rychlik & Rhoads 1989).

Of the 22 microsatellites for which PCR primers were designed, six loci (Table 1) were used to screen for genetic variation in island and mainland populations of *R. f. greyii* (F. Harriss *et al.* unpublished). For each locus, approximately 0.1 ng of gDNA, extracted from tail tissue (Sambrook *et al.* 1989), was used as template for PCR amplification. PCR reaction conditions were the same as above with the exception that magnesium concentrations and annealing temperatures for PCR reactions were optimized for each locus amplified (Table 1). A total of 81 alleles was detected from a sample of 324 individuals across 16 populations. All six loci were hypervariable with the number of alleles per locus ranging from 12 to 15 (Table 1).

The benefits of using enrichment techniques to rapidly identify large numbers of microsatellites for population and evolutionary genetics studies would be further enhanced by assessing the degree to which microsatellite loci are conserved across species. The microsatellite loci identified in the present study would be particularly suited to such tests because

**Table 1** Characterization, primer sequence, PCR conditions, levels of polymorphism, observed and expected heterozygosity, and GenBank accession no. for microsatellite loci isolated from a *Rattus fuscipes greyii* microsatellite library. Levels of heterozygosity are based on a sample of 23 individuals from Kangaroo Island

Locus	Repeat Unit	Primer sequence (5'-3')	Annealing temp. (°C)	Mg [mM]	Size (bp)	No. of alleles	$H_O^*$	$H_E^*$	Accession no.
RfgB6	(CA) <sub>17</sub> (GT)(CA) <sub>4</sub> ... (CA) <sub>6</sub> (GT) <sub>4</sub>	F: CGCATTTGGTTCACAGCAT R: CCCCACCCAAATGTTTTGTGTT	58	1.5	210	12	0.76	0.82	AF110742
RfgC2	(GT) <sub>21</sub>	F: CCTCCACACTGCCCAAACA R: TGCCCTAAATTCATCCTTAGCA	56	2	155	14	0.79	0.82	AF110743
RfgD6	(GT) <sub>17</sub>	F: AAAACAGTTGCTGGTCTGGCAAA R: CGGCCCTTTCACGTCAT	58	2	280	15	0.92	0.85	AF110745
RfgE5	(CA) <sub>24</sub>	F: CATGAAGAAGGCTCAGCAAGCA R: GCTAGGCTCCTCTCTAAGCACTGA	55	2	135	14	0.36	0.8	AF110747
RfgL1	(CA) <sub>16</sub>	F: AGGCAGAGAGCAGTCAAGGACAT R: GGTGGGATGGTTCGGCAGA	58	1.5	320	13	0.74	0.81	AF110759
RfgL4	(GT) <sub>9</sub> AT(GT) <sub>4</sub> ... (GT) <sub>15</sub>	F: TCAGTTCCTGGGATTCACACTT R: GCGCGAACTACGGAAAATGA	56	2	320	13	0.72	0.88	AF110761

\*Levels of heterozygosity for Kangaroo island.

of the well structured phylogeny of Australia's endemic *Rattus* and of the genus *Rattus* in general. The cross-species transferability of the microsatellite loci identified in the present study is currently being investigated.

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### Microsatellite markers for American mink (*Mustela vison*) and ermine (*Mustela erminea*)

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The North Pacific coast of North America is a region of high mammalian endemism. There is concern about the conservation of many insular taxa because of limited distributions and the potential impact of disturbances such as clear-cut logging and introductions of exotic species (MacDonald & Cook 1996). Among these endemic taxa are American mink (*Mustela vison*) and ermine (*Mustela erminea*) subspecies that are considered 'potentially threatened' by the IUCN due to limited information available on their population status and taxonomic validity (Schreiber *et al.* 1989).

Microsatellite markers are useful tools for assessing genetic variability within and among populations (Haig 1998). We report here on the development of microsatellite markers to assess genetic differentiation among mink and ermine populations in southeast Alaska and coastal British Columbia.

Libraries were constructed using genomic DNA isolated by standard methods (Sambrook *et al.* 1989) from spleen and liver of a female mink and a male ermine, respectively. DNA partially digested with *BfaI* and *MseI* was separated by agarose gel electrophoresis after which fragments between 600 and 1200 nucleotides were excised and ligated into pGEM-5Zf(±) (Promega, Inc.).

Approximately 25 000 mink and 33 000 ermine colonies were screened using a [ $\gamma^{32}$ P]-dATP labelled (CA)<sub>15</sub> oligonucleotide probe as previously described (Ostrander *et al.* 1995). Fifty-five ermine and 68 mink clones were bidirectionally sequenced using the Taq DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer/ABI) with ABI 373A sequencers. Using PRIMER version 0.5 (Whitehead Institute, Cambridge, USA)

**Table 1** Characterization of mink (*Mvis*) and ermine (*Mer*) microsatellite loci. Unbiased estimates of heterozygosity ( $H_E$ ) were calculated following Edwards *et al.* (1992)

Locus	Repeat motif*	Primers (5'-3')	Size (bp)*	No. of alleles	$H_O$	$H_E$	$n$	Genbank Accession no.
Mvis002	CA <sub>11</sub>	TGGGAAAAATAGTGCTCCAAAG AAACAGCAGAGAGCATACAGCC	184	6	0.516	0.680	64	AF132100
Mvis020†	CA <sub>17</sub>	GGGTCAAGAGTTAGAGCCCC GACATGGTCAGATCCCCAC	178	7	0.421	0.659	19‡	AF132101
Mvis022†	CA <sub>13</sub>	ATCAAGTCCTGCATCAGGCT TGGGCTGTTTGTCCAGGT	283	8	0.397	0.621	63	AF132102
Mvis027	CA <sub>10</sub>	CCACAATAAGTTTCACTAACAC TGGAGGGAGGAACATAAG	184	5	0.476	0.706	63	AF132103
Mvis072	CA <sub>15</sub>	CTGCAAGCTTAGGAATGGAGA CCACTACACTGGAGTTTCAGCA	263	4	0.540	0.632	63	AF132104
Mvis075†	CA <sub>12</sub>	GAAATTTGGGGAATGCACTC GGCAGGATAGGATGTGAGCT	120	8	0.708	0.814	65	AF132105
Mvis099	CA <sub>16</sub>	TGAGGCAAGAGGAGCAAAAG TTTGCATTTCCCTGATGAGG	346	8	0.433	0.703	60	AF132106
Mer005	CA <sub>16</sub>	TCCTTGCCAGAGACACACAG GATGCCTCTCTCCGTCACCTC	296	12	0.839	0.901	56	AF132107
Mer009†	CA <sub>18</sub>	AGTGTCCCAAGCACAAATCC GATTTGGCTGCTTGCCTG	211	11	0.804	0.889	56	AF132108
Mer022†	CA <sub>15</sub>	CCATGCTTTGGGTAGGAGAA CCTTGTCTCAGGTGGTGG	255	16	0.857	0.897	56	AF132109
Mer030	CA <sub>14</sub>	TCCTTCCCTCAGTTCTCTGG GGGTGCCCTTCATAATTATC	222	13	0.786	0.827	56	AF132110
Mer041	CA <sub>11</sub>	TGTGTGATCTCTGGGAATTCTC TCTGCTCCCCAGATAAAAAGC	141	12	0.821	0.858	56	AF132111
Mer082	CA <sub>9</sub>	GCCCTGCTTGGGTGTAGAGT TTTGTAGTTCCCTGCACCAG	132	9	0.518	0.761	56	AF132112
Mer095	CA <sub>13</sub>	AGCTTTGCTGTTCTGCTTC CACCTCTATGTGCCATTTTGTC	149	9	0.607	0.763	56	AF132113

\*Based on clone sequence.

†Fluorescently labelled.

‡Females only.

 $H_O$ , observed heterozygosity;  $H_E$ , expected heterozygosity.

and Primers! for the Mac (Apple Pi, Inc.), primer pairs were designed with annealing temperatures of 60 °C, except for Mvis027 (53 °C). The first seven primer pairs in each species that yielded products which were polymorphic and easily scorable are shown in Table 1.

Markers were tested on 65 mink and 56 ermine from Interior and southeast Alaska using both radioactive and fluorescent detection methods. For radioactive visualization, one primer of each pair was end-labelled with [ $\gamma^{32}$ P]-dATP using T4 polynucleotide kinase (Amersham Life Sciences). Polymerase chain reactions (PCR) were carried out utilizing 100 ng of genomic DNA, 10  $\mu$ M of each primer, 0.5 unit of *Taq* polymerase (Perkin-Elmer/ABI), 200  $\mu$ M of each dNTP, 10  $\mu$ M Tris-HCL (pH 8.3), 50  $\mu$ M KCl and 1.5  $\mu$ M MgCl<sub>2</sub> in 12  $\mu$ L volumes. Amplifications were performed with 9600 thermal cyclers (Perkin-Elmer/ABI) as follows: 1 min at 95 °C followed by 30 cycles of 20 s at 94 °C, 20 s at 60 °C, 20 s at 74 °C, and 30 min at 72 °C. Products were separated on

4–6% denaturing polyacrylamide gels and visualized using autoradiography. Allele sizes were assigned by comparison with the cloned sample. Ambiguous assignments were checked by adding fluorescently labelled dUTPs (Perkin-Elmer/ABI) to the reaction mix and visualizing the products by fluorescent detection (below).

For fluorescent detection, one primer in each pair (Table 1) was synthesized with either 6FAM, TET or HEX dyes (Perkin-Elmer/ABI). PCR products were run on an ABI 373A DNA sequencer and sized with internal lane standards (SC-TAMRA 350 or 500, Perkin-Elmer/ABI) using Genescan and Genotyper software (Perkin-Elmer/ABI).

The mean heterozygosity ( $\pm$ SEM) of mink loci was  $0.688 \pm 0.024$  (Table 1), similar to that described for other mink markers (0.623; O'Connell *et al.* 1996). The mean heterozygosity of ermine loci was  $0.842 \pm 0.023$ . Mvis020 appears to be X-linked as all males were homozygous. Single base pair differences were observed among some alleles for

**Table 2** Numbers of alleles detected using (a) mink- and (b) ermine-derived markers in other mustelids. Number of individuals tested is shown in parentheses

## a. Mink-derived markers

Species	Mvis002	Mvis020	Mvis022	Mvis027	Mvis072	Mvis075	Mvis099
Least weasel <i>Mustela nivalis</i>	6 (4)	2 (3)	2 (2)	3 (2)	3 (3)	5 (7)	mult.*
Ermine <i>Mustela erminea</i>	4 (10)	5 (10)	5 (10)	2 (10)	11 (10)	3 (10)	6 (10)
Wolverine <i>Gulo gulo</i>	1 (4)	2 (4)	4 (4)	2 (4)	3 (4)	4 (4)	1 (4)
American marten <i>Martes americana</i>	1 (8)	3 (5)	2 (4)	1 (8)	8 (8)	5 (7)	1 (8)
N. American river otter <i>Lontra canadensis</i>	3 (9)	2 (3)	2 (4)	1 (9)	4 (7)	3 (4)	2 (9)
Sea otter <i>Enhydra lutris</i>	1 (2)	2 (2)	2 (2)	2 (3)	1 (2)	3 (3)	1 (3)

## b. Ermine-derived markers

Species	Mer005	Mer009	Mer022	Mer030	Mer041	Mer082	Mer095
Least weasel <i>Mustela nivalis</i>	3 (2)	6 (7)	4 (7)	1 (2)	4 (3)	2 (2)	4 (2)
American mink <i>Mustela vison</i>	1 (10)	4 (10)	6 (10)	1 (10)	5 (10)	1 (10)	1 (10)
Wolverine <i>Gulo gulo</i>	1 (4)	2 (4)	3 (3)	1 (4)	1 (4)	1 (3)	1 (4)
American marten <i>Martes americana</i>	1 (4)	1 (3)	2 (3)	1 (8)	4 (6)	1 (6)	2 (8)
N. American river otter <i>Lontra canadensis</i>	5 (5)	3 (3)	6 (5)	1 (7)	n.a.*	1 (7)	3 (9)
Sea otter <i>Enhydra lutris</i>	1 (2)	1 (2)	1 (3)	1 (2)	n.a.*	1 (3)	1 (2)

\*n.a, no amplification; mult., multiple bands.

marker Mer082. For Mvis099, the sizes of the three smallest alleles, which were apparent on autoradiographs, could not be confirmed using fluorescent detection with labelled dUTPs.

Markers derived from one species were also tested in the other and in five other mustelids (Table 2). Cross-species amplifications were performed as above but using 2.5 mM MgCl<sub>2</sub> and 55 °C annealing temperatures for heterogeneric taxa (50 °C for Mvis027). Three loci were polymorphic in all species while only one locus was monomorphic, indicating the potential utility of these markers in studies of other mustelids.

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## Isolation and characterization of microsatellite loci in Atlantic haddock (*Melanogrammus aeglefinus*)

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The decline of haddock (*Melanogrammus aeglefinus*) populations due to overexploitation on both sides of the Atlantic Ocean is a problem for fishers and conservation managers. Current haddock stocks in the Northwest Atlantic are remnants of immense populations that were heavily fished by domestic and foreign fleets in the 1960s (Clark *et al.* 1982). These declines have had profound impacts on fishing communities along the United States and Canadian coasts. Fundamental insights into the population biology of commercial species are particularly critical when management decisions involve recovery plans for overexploited populations.

Molecular techniques (Shaklee & Bentzen 1998) implemented to determine population structure in Atlantic haddock have included the use of allozymes (Jamieson & Birley 1989; Giaever *et al.* 1995) and mitochondrial DNA (mtDNA) (Zwanenburg *et al.* 1992; Purcell *et al.* 1996) with some success. Recently, microsatellites have been exploited to infer population structure in Atlantic cod (*Gadus morhua*), a closely related gadid species (Ruzzante *et al.* 1998). Here, we describe the isolation and characterization of five polymorphic microsatellite loci in Atlantic haddock.

Genomic DNA was isolated from haddock muscle tissue (Sambrook *et al.* 1989) and digested with *Hind*III and *Xba*I (NE Biolabs). Restriction fragments of 200–1000 bp were isolated and ligated into phosphatase-treated pUC19 vector (Gibco BRL) in a 1:1 mass ratio. Plasmids were then used to transform competent DH5 $\alpha$  *Escherichia coli* bacteria (Gibco BRL) which were plated onto selective agar media.

Approximately  $3 \times 10^3$  insert-bearing clones were lifted and fixed onto nylon membranes (Magna). This partial genomic library was screened using an [ $\gamma^{32}$ P]-dATP end-labelled oligonucleotide probe (GT)<sub>15</sub>. Over 25 positive clones with varying levels of radioactive signal were isolated and glycerol stocks were made. Using universal pUC19 PCR primers, each of the inserts was amplified and products separated on a 2% agarose gel. Twenty positive clones with insert fragment sizes of approximately 200–1000 bp were sequenced on an automated sequencer (Perkin-Elmer). A total of nine sequences contained dinucleotide repeats. Primers were designed for these loci with the aid of the program PRIMER3 (Rozen & Skaletsky 1997).

Seven of these loci successfully amplified genomic haddock DNA. Microsatellites were confirmed in five of the remaining seven loci by directly sequencing of the PCR products with the locus-specific primers. Population screens were conducted to determine allelic diversity at these five loci using

genomic DNA from individuals collected from the Gulf of Maine (in spring, 1996) and from Tanafjorden, Norway (in autumn, 1992). The final concentrations of the PCR reagents in a volume of 25  $\mu$ L were as follows:  $\approx$  10 ng of genomic DNA, 1 $\times$  PCR buffer pH 9.5 (10 mM KCl, 20 mM Tris-HCl pH 8.3, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), 2 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP, 1  $\mu$ M unlabelled forward primer, 0.5  $\mu$ M unlabelled reverse primer, 0.5  $\mu$ M [ $\gamma^{32}$ P]-dATP radio-labelled reverse primer, and 0.75 units of *Taq* polymerase (Gibco BRL). PCR reactions were carried out in a PTC-100 thermal cycler (MJ Research) as follows: an initial denaturation of 4 min at 94  $^{\circ}$ C, followed by 35 cycles of 94  $^{\circ}$ C for 45 s, 55–60  $^{\circ}$ C for 1 min, 72  $^{\circ}$ C for 1 min with a final extension of 72  $^{\circ}$ C for 10 min. PCR products were resolved on 6% urea-polyacrylamide sequencing gels. Allelic diversity, primer sequences, and optimal observed annealing temperature for each locus are listed in Table 1.

Due to the promising levels of variability and heterozygosities shown for loci Mae111, Mae211 and Mae249, we designed and optimized a multiplex PCR reaction following the guidelines outlined by Henegariu *et al.* (1997). The final concentrations of reagents in a 25- $\mu$ L PCR cocktail were: 1  $\mu$ L  $\approx$  10 ng of genomic DNA, 1 $\times$  PCR buffer pH 9.5 [10 mM KCl, 20 mM Tris-HCl pH 8.3, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>], 2 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP, 0.2–1  $\mu$ M each forward primer, 0.2–1  $\mu$ M each reverse primer 5'-labelled with a TET, 6-FAM, or HEX ABI dye (Operon), and 0.75 units of *Taq* polymerase (Gibco BRL). PCR conditions were: initial denaturation of 4 min at 94  $^{\circ}$ C, followed by 35 cycles of 94  $^{\circ}$ C for 45 s, 54  $^{\circ}$ C for 1 min, 72  $^{\circ}$ C for 2 min with a final extension of 72  $^{\circ}$ C for 15 min. Fluorescent PCR products were visualized on an ABI377 automated DNA sequencer (Perkin-Elmer). Fluorescent peak data were analysed with Genescan (Version 2.1) and Genotyper (Version 2.1) software programs (Perkin-Elmer).

Based on the initial diversity screening it is likely that locus Mae110 contains an unusually high frequency of null alleles. Loci Mae206, Mae111, Mae211 and Mae249 conform to Hardy-Weinberg expectations when tested via chi-square in GENEPOP (Raymond & Rousset 1995). Locus Mae249 showed highly significant ( $P < 0.001$ ) and locus Mae111 showed near significant ( $P < 0.07$ ) genic and genotypic differentiation between the North American and European samples, suggesting that they may prove to be useful tools for population discrimination in Atlantic haddock.

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**Table 1** Characterization of five microsatellite loci in *Melanogrammus aeglefinus*

Locus	Repeat motif	Primer sequence (5'–3')	Temp (°C)	<i>n</i> *	Size range (bp)	No. of alleles	<i>H<sub>O</sub></i> *	<i>H<sub>E</sub></i> *
Mae206	(GT) <sub>9</sub> CT(GT) <sub>6</sub> (GC) <sub>3</sub> (CA) <sub>2</sub> TTGTGCA(TG) <sub>12</sub>	TGGCCCTCTCTCTGTCTTTC ATATCTGCTGCCCTTTCTGG	60	34	209–213	3	0.15	0.14
Mae110	(GT) <sub>27</sub> GA(GT) <sub>4</sub> (GA) <sub>2</sub> (GT) <sub>3</sub> (GA) <sub>2</sub> (GT) <sub>3</sub>	AAACCTGCCATCATTCTCC CCCGCACATAAATAAATGAAG	60	33	190–260	8	0.24	0.90
Mae111	(GT) <sub>53</sub> (GA) <sub>3</sub>	CCCCCTACAACAGTTTAGTTTG CGCACTCAGCACACACAGTA	60	81/67	217–349	36	0.93/0.96	0.94/0.95
Mae211	(GT) <sub>56</sub>	TGGGTTTACTACCAAGATGG AACGGAACATAAAACGAGAA	60	88/80	112–195	42	0.99/0.90	0.96/0.96
Mae249	[(CA) <sub>2</sub> CTA] <sub>3</sub> (CA) <sub>25</sub>	TCTCCCAATTCATTGCAAAAC ATGCTGCCGAGGTAGGTTG	55	88/76	113–213	37	0.95/0.97	0.95/0.95

GenBank Accession nos for the cloned sequences are AF129517–AF129521. Tests of cross-species amplification for Mae111, Mae211 and Mae249 in cod (*Gadus morhua*), winter flounder (*Pleuronectes americanus*) and pollock (*Pollachius virens*) proved unsuccessful.

\*The *n*, *H<sub>O</sub>*, and *H<sub>E</sub>* for Mae206 and Mae110 are based on samples from North America; values for Mae111, Mae211, and Mae249 are based on samples from North America (first value) and from Europe (second value).

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## Isolation and characterization of microsatellite loci in European hake, *Merluccius merluccius* (Merlucidae, Teleostei)

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Genetic differentiation among marine fish stocks with planktonic egg and larval stages is generally low compared to that observed in freshwater and anadromous species (Ward *et al.* 1994). Differentiation in fish stocks, when observed, is generally over a macrogeographical scale and thus of little use in fisheries management (e.g. Rico *et al.* 1997). However, the development of codominant microsatellite DNA markers is proving an invaluable tool for studying fine-scale genetic differences (e.g. van Oppen *et al.* 1997a).

In this report we describe the isolation and character-

ization of six microsatellite loci from the European hake (*Merluccius merluccius*). This species is widely distributed in the northeast Atlantic and Mediterranean Sea. The range of this species extends from approximately 21°N off the coast of Mauritania to 62°N off the west coast of Norway and eastwards into the Mediterranean Sea (Anon 1977). The species is exploited throughout its range, commanding a high price on the European fish markets where it is sold almost exclusively for human consumption.

We have cloned and sequenced microsatellite loci from the hake genome. High-molecular-weight hake DNA from 10 individuals was pooled and 50 µg was digested to completion with *Sau3AI*. Selected fragments (between 200 and 600 pb) were isolated from a 1% agarose gel using the Gene Clean II kit (Bio 101) according to Rico *et al.* (1994), and ligated into the *Bam*HI site of puc18. The ligation products were used to transform XL-1 blue competent cells (Stratagene) which were plated out on selective Luria–Bertani (LB) media and cultured overnight. Colonies were lifted onto Hybond-N membranes (Amersham). Transformants were screened for inserts containing repeated motifs using synthetic polyAC/polyGT probes (Pharmacia Biotech) labelled by incorporation of [ $\alpha^{32}$ P]-dCTP in a newly synthesized polyAC strand as described in Rico *et al.* (1994). Positive clones were subjected to a secondary screening and 30 selected clones were sequenced in full by cycle sequencing using Thermo-sequenase™ polymerase (Amersham) and an ALF automatic sequencer (Pharmacia Biotech). Sequencing analysis of positive clones revealed six perfect repeated sequences of the dinucleotide GT (Table 1). In addition, several clones contained perfect and imperfect GT motifs smaller than five uninterrupted repeats, or had flanking sequences too small to be useful for primer design. Polymerase chain reaction (PCR) primers were designed from the sequence flanking the microsatellite using the computer program OLIGO™ Macintosh version 4.0 (National Biosciences).

To assess the level of polymorphism at these six microsatellite loci, genomic DNA was extracted from muscle or gill using a standard phenol–chloroform extraction protocol. PCR conditions were optimized for all six primer pairs by varying MgCl<sub>2</sub> concentrations and annealing temperatures. Once each primer pair showed one or two clear bands on agarose gels, PCR amplifications were conducted in 11 µL reactions containing 20–30 ng of template DNA, 1.1 µM of each primer (U-primer was fluorescein-labelled), 200 µM of each dNTP, MgCl<sub>2</sub> concentration given in Table 1, 0.2 µg of BSA (bovine serum albumin), 1×NH<sub>4</sub> buffer, 0.50 units BioTaq (Bioline) and Sigma ultrapure water to volume. Amplification was performed in a 96-well Perkin-Elmer 9700 thermocycler as follows: an initial denaturing at 94 °C for 2 mins, then seven cycles of 94 °C for 15 s, a locus-specific annealing temperature (Table 1) for 20 s and 72 °C for 15 s, followed by 23 cycles of 89 °C for 15 s, a locus-specific annealing temperature for 20 s and 72 °C for 15 s. Products were then diluted with Dextran blue loading buffer mixed with internal size markers prior to electrophoresis in an ALF automated sequencer (Pharmacia) as described in van Oppen *et al.* (1997b). Running conditions were as follows: 1000 V, 60mA, 50 W, 48 °C and 1.25 s sampling time. Alleles

**Table 1** Primer sequences, repeat unit structure, PCR conditions for [MgCl<sub>2</sub>] and annealing temperature (AT), expected and observed heterozygosities ( $H_E$  and  $H_O$ , respectively), mean allele length for all loci and GenBank Accession no.

Locus	Primer sequence (5'-3')	Type of repeat	[MgCl <sub>2</sub> ] (mM)	AT (°C)	$H_E$	$H_O$	Mean allele size	GenBank Accession no.
<i>Mmer-hk3b</i>	U - ACCCGGTCTCCTCACTAGCAGTTT	GT	1	52	0.69	0.50	332 bp	AF136627
	L - AGGCTTCAGGAACAGAATGCTATG							
<i>Mmer-hk7</i>	U - GCCCACAAACGCCACTGCTCACCT	AC	1.1	52	0.92	0.90	76 bp	AF136626
	L - CCAAGATGAGAAGAATGGTAGT							
<i>Mmer-hk9b</i>	U - CAAGCAAGACCTGGAGGGGAAAGAGATG	GA	1.5	53	0.96	0.90	145 bp	AF136628
	L - GTGCGTGCCTCCCAGGAGTTGCTTGTAG							
<i>Mmer-hk20</i>	U - TCGGCTGCAGGTCAGTTTCAGTTTTT	GT	1	50	0.91	0.78	222 bp	AF137595
	L - GTGCTGTTTTCTGCCATGCT							
<i>Mmer-hk29</i>	U - GGGTTGCCAGTGGTTACGGGTGTG	GT	1	50	0.88	0.72	161 bp	AF137597
	L - CGGCGACTGCGTGTCTCTGCTGCTGTG							
<i>Mmer-hk34b</i>	U - TATTGTGCWATCTCTATCGGTGA	GT	1	52	0.89	0.81	122 bp	AF137596
	L - AAAAAGATGCGAGGGACAGCTAAA							

were sized using the software program Fragment Manager™ version 1.2 (Pharmacia Biotech).

Some 480 adult hake from six North Atlantic and Mediterranean Sea samples were screened to assess polymorphism for all loci, with the exception of locus *Mmer*-UEAHk7 for which only 10 individuals were analysed. The six microsatellite loci revealed extensive allelic polymorphism, with the number of alleles varying from 12 to 47 depending on the locus and origin of the sample (Table 1). Alleles at all these loci were found to vary in size by multiples of two nucleotides, suggesting that mutations primarily involved loss or gain of repeated units.

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## Tri- and tetranucleotide microsatellite loci in honey bees (*Apis mellifera*) — a step towards quantitative genotyping

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Honey bees are polyandrous and a large number of worker bees need to be genotyped in order to characterize each colony of interest. The analysis of pooled bee or sperm samples by means of quantitative polymerase chain reaction (PCR) would therefore be highly desirable. However, PCR amplification of dinucleotide repeat loci typically results in several PCR products for single alleles due to polymerase slippage and/or nontemplated nucleotide addition, which complicate an analysis of pooled samples. Microsatellite loci with longer repeat units tend to produce less extraneous PCR products and have been shown to be suitable for quantitative genotyping with pooled samples (e.g. Pacek *et al.* 1993). Here, we

**Table 1** Characteristics of 14 microsatellite loci of *Apis mellifera*. GeneBank Accession nos are AF140066, AF140067, AF140069–AF140080. The number of observed alleles refers to nine different chromosomes at most (five worker bees from one colony, one worker from another colony). The number of bands (PCR products) per allele was determined from autoradiographs, but only bands showing approximately more than 10% of signal intensity of the main band were considered (assumed original + nucleotide addition + slippage bands)

Locus	Core sequence of cloned allele	Primer sequences (5'–3') F: forward; R: reverse	Size of allele in clone (bp)	Annealing temp. (°C)	No. of observed alleles	No. of bands per allele
4A1	att((att) <sub>2</sub> atc) <sub>2</sub> ...(att) <sub>8</sub> *	F: aag caa gct agg taa tca tca R: cga ttc gtc tca caa tgt act	218	60	5	1 + 1 + 0
4A3	tta((tta) <sub>8</sub> cta) <sub>2</sub> (tta) <sub>2</sub> *	F: cga tct ccg ccg ttt tat atc R: gcg cgg gta tcg tcg ata gtg	145	47	3	1 + 0 + 2
4A18	(gtc) <sub>7</sub>	F: ctc ccc tcc ctt cgc ggt gtc R: ggg tcc gtg ccg cgt tct cac	149	61	2	1 + 0 + 1
4A31	(ttg) <sub>9</sub>	F: gaa gga tcg cag aat aac tag R: gca tca tgt aaa ctg gga gag	189	52	3	1 + 1 + 1
4A42	(ttg) <sub>10</sub>	F: acg gtg tta tgg ctg cta gtc R: gtc cgt gat gga acg atc ttt	89	50	3	1 + 1 + 1
4A43	(atc) <sub>9</sub>	F: ctc gaa ttc taa tcc aac aac R: aaa cga gca cga aga att aac	187	49	3	1 + 1 + 0
4A44	(ctt) <sub>8</sub> (cat) <sub>4</sub> ct <sub>4</sub> (ttc) <sub>4</sub>	F: aat ttc gca agt tca tca tca R: aat tca tgc tgc ttc aac tag	201	48	2	1 + 1 + 0
4A45	(atc) <sub>6</sub>	F: ttc gca gaa agc gag aaa tta R: ctg gac ccc tgt gaa cgt gat	87	49	2	1 + 1 + 1
4A46	(ttg) <sub>6</sub> ...(att) <sub>20</sub> ...(att) <sub>12</sub> *	F: gaa ata tct ttt att ttg ttg R: ggc gat aat gac agt aat gac	191	40	3	1 + 4 + 3
4A48	(aag) <sub>6</sub> ...(atc) <sub>5</sub>	F: tgc cag aaa aaa aga aga R: agg gga atg ttg att gaa aaa	193	48	3	1 + 1 + 0
4A49	(cca) <sub>6</sub> (ccg) <sub>3</sub> (cca) <sub>5</sub>	F: cat cct gcc cca cta cct act R: tgc cga tgg aca gac tgt tac	115	57	2	1 + 0 + 0
4A64	(cat) <sub>2</sub> cgt(cat) <sub>19</sub>	F: acg gaa ctg aaa gag gga aac R: cgg aaa atc ata cac gtt ctt	156	50	4	1 + 1 + 1
4A110	atcg(atcc) <sub>4</sub> (atct) <sub>2</sub> atcc	F: cgc tcg cgg tgg att tca ttt R: ggc aaa agt ggc gga gaa aga	160	56	2	1 + 2 + 1
4A112	(tcg) <sub>11</sub>	F: gcg cgg aaa gaa gat aat aaa R: aca aca ccc acg tcg agt tac	107	53	2	1 + 1 + 0

\* denotes complex core sequences which are not shown in full detail here.

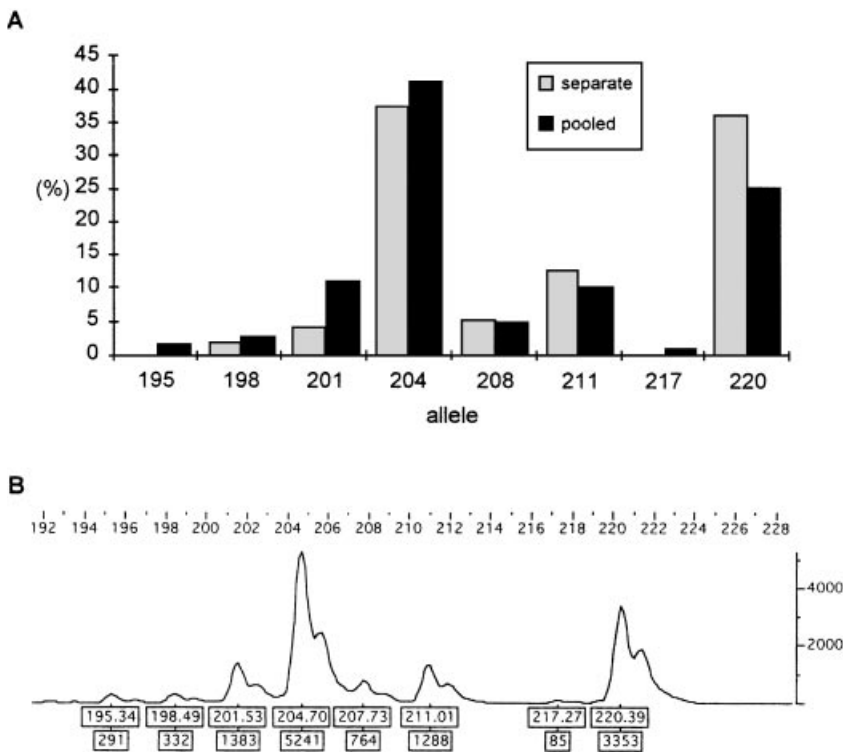
report on microsatellite loci in honey bees with tri- and tetranucleotide repeat units.

Microsatellite loci were essentially isolated as described in Tautz (1989). Genomic DNA of *Apis mellifera carnica* was digested with *Hae*III, *Dra*I, *Hinc*II and *Pvu*II. Size-selected fragments in the range of about 300–500 bp were ligated into M13 mp19 phage vector and transformed into *Escherichia coli* XL1-Blue MRF' cells (Stratagene). The resulting library was screened with the oligonucleotides (ATT)<sub>5</sub> (CCA)<sub>5</sub> (GTT)<sub>5</sub> (TAC)<sub>5</sub> (TCA)<sub>5</sub> (TCT)<sub>5</sub> (TCC)<sub>5</sub> (TCG)<sub>5</sub> (TGC)<sub>5</sub> (ATCC)<sub>4</sub> and (GATA)<sub>4</sub>, and for 14 of the 128 positive clones that were sequenced, primers flanking the repeat sequence were designed (Table 1).

Individual PCRs were performed in 10 µL of a solution of 50 mM KCl, 10 mM Tris (pH 8.3), 100 µg/mL gelatine, 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 1 µM of each primer, about 5 nM of one primer 5'-end-labelled with <sup>33</sup>P, 0.5 U *Taq* polymerase and about 50 ng of bee DNA. The PCR profile comprised 25 cycles of 1 min at 92 °C, 1 min at the respective annealing temperature (Table 1) and 1 min at 72 °C on DNA Thermal Cyclers 480 (Perkin-Elmer). PCR products were separated on denaturing 6% acrylamide/bis-acrylamide

gels together with sequencing reactions as a size standard, and visualized by autoradiography. All of the 14 loci proved to be polymorphic, but all loci except one proved to produce more than one amplification product per allele in PCR (Table 1). Despite PCR optimization efforts (type and concentration of polymerase, cycling profile, concentration of primers, dNTPs, MgCl<sub>2</sub> and template DNA, pH) the amplification product pattern could not be changed substantially.

We further concentrated on locus 4A1 that is quite polymorphic, very robust in PCR conditions and that displays only one extraneous band. From a sample of 100 honey bee worker pupae, DNA was isolated separately from individual heads, while the corresponding thoraces were pooled for DNA extraction. Separately isolated DNAs were genotyped separately. The pooled DNA sample was genotyped multiple times using fluorescent dye-labelled primers, an automated electrophoresis instrument (ABI PRISM 377) and ABI software (PE Applied Biosystems). By counting the numbers of each allele obtained by separate analyses an expectation for the result of the pooled DNA sample was derived assuming that DNA from all individual bees was present in the pool in equal amounts and that PCR is quantitative (Fig. 1).



**Fig. 1** (A) Relative allele frequencies of locus 4A1 as determined by PCR of separate and pooled DNA extractions. Allele frequencies for the pooled sample were calculated by averaging over results from three PCR replicates. (B) Electrophoretogram of PCR products of locus 4A1 from a pooled DNA extraction of 100 honey bee workers. Horizontal scale: number of nucleotides as calculated by the programme GeneScan. Vertical scale: relative signal intensity. Estimated size in nucleotides and relative peak height is shown below each peak used in the calculation.

Generally, the amount of PCR products of the pooled sample as measured by peak height was more or less representative. Even a rare allele that was present in the pooled sample in a frequency of only 2% (allele 198; 4 out of 200 alleles) could clearly be detected (but see below). However, alleles of large size were represented in lower frequencies than expected, whereas short alleles were over-represented. This is probably a result of template competition, i.e. shorter fragments have a higher probability to be produced in total length than longer fragments. Additionally, two alleles (195; 217) that were not present in the original samples showed up in the PCR products of the pooled sample. These fragments must have been produced *de novo* during PCR, probably by slippage. However, the results of the pooled sample were sufficiently similar to those obtained from individual amplifications to make this a promising tool for future studies.

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## Polymorphic microsatellite markers in the perennial herb *Heloniopsis orientalis* (Thunb.) C. Tanaka

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*Heloniopsis orientalis* (Thunb.) C. Tanaka (Liliaceae), an evergreen perennial herb, is distributed widely in Japan and also grows on the Korean Peninsula. This species occurs from temperate woodlands to alpine meadows (Kawano & Masuda 1980), and grows in wet or moist places on the forest floor or the edge of evergreen and deciduous forests. It is also found in open moist habitats in temperate regions.

**Table 1** Characterization of 14 microsatellites loci in *Heloniopsis orientalis*. EMBL accession numbers for the cloned sequences are AJ131503–AJ131516

Locus	Primer sequences (5'–3')	Repeat structure*	Annealing temp. (°C)	Size (bp)	No. of alleles	$H_O$	$H_E$
HO51	5'-TCAAAAGGTCAGCAGATACA-3' 5'-TCCCAGAAATGAACCCATAA-3'	(TC) <sub>20</sub>	47.6	116	3	0.263	0.194
HO53	5'-TGGCGGACGGTTGGAGAATC-3' 5'-GGTGGGAGCAAAGGATGAAG-3'	(GA) <sub>12</sub>	53.7	370	4	0.200	0.628
HO54	5'-AAAGTTTGCTCCACAGTATC-3' 5'-GATCTTCTTCACCAATAAGG-3'	(AG) <sub>13</sub>	47.4	290	6	0.600	0.735
HO59	5'-GATCAGCGGCTTGTATAT-3' 5'-TTTGCTTTCTCTTTCACC-3'	(GT) <sub>37</sub>	50.4	199	5	0.050	0.584
HO68	5'-GTAGCCAITTAAGGATGTGTC-3' 5'-CTCCCACAGCTACTTACTTT-3'	(GA) <sub>14</sub>	48.8	121	7	0.900	0.813
HO69	5'-GGCGAGGAATGGTTGTGTCA-3' 5'-GGGCTCCCCCTTGAGTTATT-3'	(CT) <sub>41</sub>	54.8	235	13	0.400	0.848
HO70	5'-TGGTGATACGGCTAAGTAAA-3' 5'-AACCCCTGGTACATGAAT-3'	(CT) <sub>27</sub>	50.4	210	10	0.550	0.878
HO71	5'-ATGGACAAGGTCTACGAATA-3' 5'-CAGAATAAATGAATGAGTGA-3'	(CT) <sub>24</sub>	48.8	137	10	0.500	0.848
HO72	5'-AAGCCAAATTAGAGTTCAGA-3' 5'-TTCAAATATTAGACCATCAA-3'	(GA) <sub>20</sub>	48.1	242	8	0.450	0.783
HO81	5'-GGTCGGGACTGCTGAAGAAG-3' 5'-TGAATGAGTGAGGAGGAGAG-3'	(CT) <sub>24</sub>	53.5	205	9	0.400	0.836
HO83	5'-GGGCATTATGTCCACTTGTA-3' 5'-ATCTGGTACGAACACTTCTT-3'	(CT) <sub>16</sub>	49.6	172	5	0.550	0.698
HO85	5'-GAAAATTATTTCATCAACACC-3' 5'-CCCTTCTTCTCTCCCTATCC-3'	(AG) <sub>18</sub>	48.0	121	5	0.350	0.476
HO201	5'-TCGCAAGGCGAAAGTCTCT-3' 5'-CGCCGTCGTCGGTGTAGTCA-3'	(GA) <sub>14</sub>	59.1	266	5	0.450	0.628
HO206	5'-ACAAGCCCTATTCTGCTACT-3' 5'-CGATGGATATGTAAGAGATG-3'	(GA) <sub>36</sub>	48.9	219	6	0.700	0.864

\*Cloned sequence.

 $H_O$ , observed heterozygosity. $H_E$ , expected heterozygosity.

Reproduction in *Heloniopsis orientalis* is by sexual or vegetative means. A variety of insects have been reported appearing to visit this species (Takahashi 1988), but it blooms in very early spring when the supply of pollinators is limited. Almost all flowers in cross- and self-pollination experiments produced fruits (Takahashi 1988). Moreover, it was reported that the number of seeds produced per plant is very high in the field (Kawano & Masuda 1980). These findings suggest that self-pollination is common (Takahashi 1988). *H. orientalis* also reproduces vegetatively by buds formed at the tips of leaves (Kumazawa 1960). When the buds on leaf-tips touch moist ground, roots are initiated and independent plants are produced. 'Individual' can be recognized at two different

levels in clonal plants: genet and ramet. We must pay attention to the genet level to understand the population structure and dynamics of clonal plants, because genetic variation occurs mainly at this level (Eriksson 1993). It is often difficult to identify genets of *H. orientalis* in the field.

We have developed 14 polymorphic microsatellite markers in *H. orientalis* to analyse the genetic population structure. The microsatellite loci were isolated through the screening of short fragments of genomic DNA of *H. orientalis* (Queller *et al.* 1993).

Genomic DNA was extracted from leaves of *H. orientalis* collected in Kyotanabe City, Kyoto Prefecture, Central Japan, by the modified CTAB (cetyl trimethyl ammonium bromide)

method (Milligan 1992). It was then digested with *Mbo*I (NEB). DNA fragments ranging between 400 and 600 bp were ligated to pUC19 which had been digested with *Bam*HI (Takara), and transformed into JM109 *Escherichia coli* cells. Recombinant colonies formed on agar plates were lifted with nylon membranes (Hybond-N<sup>+</sup>, Amersham Pharmacia), and screened using two synthetic oligonucleotides (GA)<sub>20</sub> and (CA)<sub>20</sub>, labelled with a DIG oligonucleotide tailing kit (Roche Diagnostics). The pUC19 vectors from hybridization-positive colonies were extracted by a kit for miniprep (Wizard plus minipreps DNA purification system, Promega), and were cycle sequenced (ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction kit, Perkin-Elmer) with an auto-sequencer (ABI 377, Perkin-Elmer).

We designed 18 pairs of polymerase chain reaction (PCR) primers using a computer program (OLIGO, National Bioscience). PCR amplifications were performed by a thermal cycler (GeneAmp PCR System 9600, ABI). The volume of each reaction mixture was 10 µL and contained 10 ng of DNA from *H. orientalis*, 5 pmol of primers labelled with fluorescent phosphoramidites (TET or 6-FAM), 0.25 U of *Taq* polymerase (Ampli Taq Gold, ABI), 200 µM of dNTP, 5 µM of Biotin-16-dUTP, 1.5 mM of MgCl<sub>2</sub>, 10 mM of Tris-HCl pH 8.3, 50 mM of KCl and 0.001% of gelatin. An initial denaturation at 94 °C for 9 min prior to 30 cycles of denaturation at 94 °C for 30 s. This was followed by annealing for 30 s, extension at 72 °C for 1 min, and a final incubation at 72 °C for 7 min. The PCR products were resolved in 6% denaturing polyacrylamide gels, and the sizes were determined by automated fluorescent scanning detection on the ABI 377 instrument with the GeneScan™ analysis software (ABI).

The target sequences were successfully amplified by 14 out of 18 designed PCR primer pairs for the DNA samples extracted from 40 individuals of the species. Each locus was polymorphic; the number of alleles per locus was between three (HO51) and 13 (HO69), and the observed heterozygosity ranged from 0.050 (HO51) to 0.900 (HO68) (Table 1).

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