

PRIMER NOTES

Microsatellite markers in wood mouse and striped field mouse (genus *Apodemus*)

K. D. MAKOVA,* J. C. PATTON,*
E. YU. KRYSANOV,† R. K. CHESSER‡ and
R. J. BAKER*

*Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA, †A. N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, 117071, Russia, ‡Savannah River Ecology Laboratory, Aiken, SC 29802, USA

Keywords: *Apodemus*, Chelyabinsk, genetic markers, microsatellites, Murinae

Received 13 June 1997; revision accepted 19 August 1997

Correspondence: K. D. Makova, Tel. +01-806-742-2702; Fax. +01-806-742-2963; E-mail: bjkdm@ttacs.ttu.edu

To study possible effects of radiation and chemical pollutants on native rodents in highly polluted sites of the former Soviet Union we developed a system of microsatellite markers in mice (*Apodemus*). Microsatellites can provide information on mutation rate and population structure of the affected animals. Dubrova *et al.* (1996) reported that mutation rates at minisatellite loci in humans who lived in heavily polluted areas of Belarus after the Chernobyl accident were twice as high as that of the control group. No genetic studies have been done thus far on humans or animals in the vicinity of Chelyabinsk, Russia, one of the Earth's most radioactively and chemically polluted spots. Here, we report the development of 13 heterospecific microsatellites that amplify the DNA from both the wood mouse (*Apodemus sylvaticus*) and the striped field mouse (*A. agrarius*). Polymorphism of the microsatellite markers is described in animals collected near Chelyabinsk.

Mice of the genus *Apodemus* comprise a dominant group of murid rodents in the Palaearctic region (Corbet 1978). The two species we are studying (*A. agrarius* and *A. sylvaticus*) belong to different subgenera (*Apodemus* and *Sylvaemus*, respectively). Mice of this genus are commonly available at polluted sites and both *A. sylvaticus* and *A. agrarius* are widely distributed so that comparative studies are possible. According to several allozyme studies (e.g. Hartl *et al.* 1992), the genetic distance between *A. agrarius* and *A. sylvaticus* is much greater than is typical between rodent congeners.

Small-insert genomic libraries were constructed for *A. agrarius* and *A. sylvaticus* basically following the method described by Hillis *et al.* (1996). Total genomic DNA was isolated from two individual mice *A. agrarius* (TK44878) and *A. sylvaticus* (TK44936). Two libraries were constructed for each individual mouse: one after digesting the genomic DNA with *ApoI* (cloned into *EcoRI* site of pBluescript SK+), and the other after digesting the genomic DNA with *DpnII* (cloned into the *BamHI* site of pBluescript SK+). pBluescript SK+ (Stratagene) was dephosphorylated before ligation. XL-1 Blue ultracompetent cells (Stratagene) were used for transfection. All four libraries were hybridized with [γ - 32 P] end-labelled repeat-

containing oligonucleotides: (CA)₁₁, (GGT)₅, (GTT)₆, (GATA)₆ and (GACA)₅. Positive clones were archived for secondary screening. Secondary screening revealed the following results: 36 (CA)_n, seven (GGT)_n, 23 (GTT)_n, 17 (GATA)_n and eight (GACA)_n positive clones. From these clones we describe 12 primer pairs with expected product size ranges and amplification conditions (Table 1). Using amplifications with vector, insert and repeat-containing primers and other techniques described in Makova and Patton (1998), we increased the yield of acceptable microsatellites with conserved primer sites. Eleven tri- and tetranucleotide repeat microsatellite markers described here were developed by a single screening of only 5000 recombinant colonies. An additional primer pair was developed by aligning rat and mouse sequences of the promoter region of the Tumour Necrosis Factor gene (TNF) from GenBank, which contains (CA)_n repeat. Primers with conserved sites flanking the repeat were designed to amplify a 450 bp fragment from *Apodemus*. Sequence analyses of the PCR products were used to develop *Apodemus*-specific primers for TNF microsatellite (Table 1).

The animals were collected near Lake Berdenish (55°48'N, 60°52'E), 80 km from Chelyabinsk. DNA was isolated from livers of 26 wood mice (*A. sylvaticus*) and eight striped field mice (*A. agrarius*) following the protocol described in Longmire *et al.* (1997). DNA was amplified in a Perkin Elmer thermal cycler (model TC). One primer of each of the 13 primer pairs was labelled with one of the fluorochromes used in the ABI GeneScan™ system (ABI). Reaction mixtures contained the following: approximately 100 ng of genomic DNA, 2.5 units of *Taq* DNA polymerase (Promega), 10× Promega buffer, 1.5 mM MgCl₂, 0.6 μM of each primer (labelled and unlabelled), 250 μM dNTPs (Perkin Elmer), and water to the final volume of 25 μL. Thermal conditions were an initial 3-min denaturation at 94 °C (hot start), followed by 35 cycles at 94 °C for 1 min (denaturation), 30–45 s at the annealing temperature, 72 °C for 30–60 s (extension), concluding with a final 3-min extension at 72 °C. Exact conditions for each primer pair are given in Table 1. After amplification the 13 microsatellite loci for each mouse were analysed on an ABI 310 autosequencer. Thirteen loci were analysed in three runs for each animal. Results were compiled and analysed with GeneScan™ and Genotyper™ software (ABI).

To estimate the potential usefulness of the markers, we compared observed and expected heterozygosities for each microsatellite marker for populations of *A. agrarius* and *A. sylvaticus* near Chelyabinsk (Table 2). Twelve microsatellites appeared to be polymorphic in the population of *A. agrarius* and nine in the population of *A. sylvaticus*.

Each marker was also tested for amplification in other species: *A. flavicollis* (yellow-necked mouse), *Mus musculus* and *Rattus rattus* (Table 2). All 13 primer pairs amplified DNA from *A. flavicollis*, 11 (84.6%) amplified DNA from *M. musculus* and six (46.2%) amplified DNA from *R. rattus*. *Apodemus*, *Mus* and *Rattus* are all assigned to subfamily Murinae, family Muridae. Our study shows a higher percentage of heterospecific microsatellites within the subfamily Murinae than in the study of Kondo *et al.* (1993), where only 16% of *Rattus* primers

Table 1 Core repeat in sequenced clone, primer sequences (5' to 3'), PCR conditions (annealing temperature, annealing and extension time), product size range and GenBank accession number for 13 microsatellite loci in *Apodemus*. Locus GTTD9A was amplified with different reverse primers in *A. agrarius* (a) and *A. sylvaticus* (s). Nucleotide sequences for cloned fragments have GenBank accession numbers AF007198–AF007210

Locus	Cloned repeat	Primers	Annealing temperature (°C)	Annealing time (s)	Extension time (s)	Size range (bp)
CAA2A	(CA) ₂₁	F: AATTTGCCCTTAAAGTGAGGAAG R: GCAGTGACCCAGGAGAAAATTACC	58	30	45	94–118
GTTA1A	(GTT) ₇ GAA (GTT) ₉	F: TTTGATGCCTTGACTTTGATTACC R: AATGCCAGTGGTGATTTTATTTGG	55	45	60	250–283
GTTC4A	(GTT) ₆ T ₃ (GTT) ₅ T ₃ (GTT) ₂	F: GTAAATGGCTAGAAGGAGAGAAGTTTC R: TTCCTGGAAACTATTTGGTAAATCC	55	45	60	128–154
GTTD8S	(GTT) ₉	F: TCTGAACAGTGGTAGATAAATTAGAGCTTA R: GAAACCGTTTGGTAAGATACTACAAAA	55	45	60	101–110
GTTD9A	(GTT) ₁₄	F: CCCAAAATTGCCTTCCCTGTCC R: GGTCAGGATAGGCTGCATAGAAAAG (s) R: GAATTCTCAGGTCAGGATAGGCTG (a)	60	30	45	202–216
GTTF9A	(GTT) ₁₄	F: GGGTCCCAAGGGTAGTTTCAAAT R: CCCACCACAGCGTGTCTATAGG	55	45	60	95–120
GATAE10A	(GATA) ₉	F: GCAGGAGTTCAGCAGTCTGAGG R: GATGCCGAATGACAGGATTTGA	60	30	30	212–242
GACAA12A	(GA) ₁₁ (GACA) ₆	F: GTAAGTCTGCGAAGAGACACCAT R: TGTTAGGGGAAGTATGTTCAGTAGGAGT	55	30	45	252–268
GACAB3A	(GATA) ₂ (GACA) ₅ (GATA) ₁₀	F: AGGGGAACCTCACAAATATAGGAAA R: GGCTTCCAATTTTGAACACAGAGC	55	45	60	361–622
GACAE12A	(GACA) ₆ (CA) ₆ (GA) ₂₆	F: GTTTTGTGGGTTTCTGAGACTGAA R: ACTCGGCTCTTACTTGGTAATCTTCC	60	30	45	277–458
GACAD1A	(GA) ₂₀ (GACA) ₅	F: GCCCTGCATGAAGCTTACCA R: TCTAAGCTTTAACTTGTCTCCTCTCTG	55	30	45	168–209
GCATD7S	(CA) ₆ ... (GCAT) ₃ (GCAC) ₃	F: CTGGGCTTCTGCAGTGTCTTTTACC R: GCAGATGCCACCTTCTGTAAACAA	60	30	45	199–296
TNF (CA)	(CA) ₁₇	F: AGGAAATGGGTTTCAGTTCTCAGG R: GGTCCCCACCAGGATTTCTGTG	60	30	45	105–124

Table 2 Polymorphism of 13 microsatellite loci in animals collected near Chelyabinsk as well as results from amplification of each microsatellite locus in other species. Number of alleles, observed (H_O) and expected (H_E) heterozygosities per locus are given for the population of *Apodemus agrarius* (eight animals) and the population of *A. sylvaticus* (26 animals)

Locus	<i>A. sylvaticus</i> (26 animals)			<i>A. agrarius</i> (8 animals)			Amplification in other species		
	No. of alleles	H_O (%)	H_E (%)	No. of alleles	H_O (%)	H_E (%)	<i>A. flavicollis</i>	<i>Mus musculus</i>	<i>Rattus rattus</i>
CAA2A	6	73	71	6	75	70	+	–	–
GTTA1A	1	0	0	4	63	70	+	+	+
GTTC4A	3	42	63	5	100	70	+	+	+
GTTD8S*	1	0	0	1	0	0	+	+	–
GTTD9A	5	65	64	3	75	51	+	+	–
GTTF9A	3	35	44	3	38	32	+	+	–
GATAE10A	1	0	0	4	88	73	+	+	+
GACAA12A†	–	–	–	4	50	55	+	+	+
GACAB3A	14	81	89	6	75	74	+	+	–
GACAE12A	6	42	39	9	100	84	+	+	–
GACAD1A	3	54	50	2	38	30	+	–	–
GCATD7S	9	65	77	2	13	12	+	+	+
TNF (CA)	7	92	81	6	88	77	+	+	+

+, successful amplification of microsatellite in other species.

*Preliminary results show that microsatellite GTTD8S is polymorphic in Chernobyl populations of *A. sylvaticus*.

†Presence of null alleles at locus GACAA12A in the population of *A. sylvaticus* made accurate scoring impossible.

amplified *Mus* DNA and 12% of *Mus* primers amplified *Rattus* DNA. Our results are in better agreement with the study by Moncrief *et al.* (1997), who showed that 28.8% of *Mus* primers amplified DNA of *Microtus* (subfamily Arvicolinae, family Muridae), suggesting a high level of conservation of microsatellite flanking regions in the family Muridae.

Acknowledgements

This study was supported by contract DE-FC09-96SR18546 between the USA Department of Energy and the University of Georgia and by funds from Texas Tech University. Sequences and microsatellites were run in TTU Biotechnology Core Facility by Dr Susan San Francisco. Amanda Wright helped in DNA isolation.

References

- Corbet GB (1978) *The Mammals of the Palaearctic Region: a Taxonomic Review*. Cornell University Press, Ithaca, NY.
- Dubrova YE, Nesterov VN, Krouchinsky NG *et al.* (1996) Human minisatellite mutation rate after the Chernobyl accident. *Nature*, **380**, 683–686.
- Hartl GB, Suchentrunk F, Willing R *et al.* (1992) Inconsistency of biochemical evolutionary rates affecting allozyme divergence within the genus *Apodemus* (Muridae: Mammalia). *Biochemical Systematics and Ecology*, **20**, 363–372.
- Hillis DM, Mable BK, Larson A *et al.* (1996) Nucleic acids IV: sequencing and cloning. In: *Molecular Systematics*, 2nd edn (eds Hillis D, Moritz C, and Mable B), pp. 321–381. Sinauer Associates Inc., Sunderland.
- Kondo Y, Wori T, Kuramoto T *et al.* (1993) DNA segments mapped by reciprocal use of microsatellite primers between mouse and rat. *Mammalian Genome*, **4**, 571–576.
- Longmire JL, Maltbie M, Baker RJ (1997) Use of 'lysis buffer' in DNA isolation and its implication for museum collections. *Occasional Papers of Museum of Texas Tech University*, **163**, 1–3.
- Makova KD, Patton JC (1998) Increased yield of heterospecific tri- and tetranucleotide microsatellites from small-insert libraries. *Biotechniques*, in press.
- Moncrief ND, Cockett NE, Neff AD *et al.* (1997) Polymorphic microsatellites in the meadow vole *Microtus pennsylvanicus*: conservation of loci across species of rodents. *Molecular Ecology*, **6**, 299–301.

Development of microsatellite markers for parentage typing of chicks in the ostrich *Struthio camelus*

C. N. KIMWELE,* J. A. GRAVES,* T. BURKE,† and O. HANOTTE

*School of Biological and Medical Sciences, University of St. Andrews, St. Andrews, KY16 9TS, †Department of Zoology, University of Leicester, Leicester LE1 7RH

Keywords: communal nesting, microsatellite, ostrich, PCR, ratites

Received 16 May 1997; revision received 26 August 1997; accepted 27 August 1997

Correspondence: J. A. Graves. Fax: +44 1334 463 600; E-mail: jag@st-and.ac.uk

The ostrich has a unique breeding organization based around

a communal nesting system. The female that first lays in a nest, the major female, allows other females to lay in the nest, but only she and the male incubate the eggs, provide care for the chicks and incur the costs and risks involved (Sauer & Sauer 1966; Hurxthal 1979; Bertram 1992). Female ranges overlap with several male territories, and males solicit copulations with any female entering their territory so that all females mate with several males. Some females lay in several nests belonging to different males (Hurxthal 1979; Bertram 1992). Thus, any chick hatched from this system has several potential mothers and fathers that it is necessary to screen. As microsatellite repeat sequences are highly polymorphic and can be used to genotype possible parents from very small amounts of DNA, they are ideal for this (Tautz 1989). We have developed a set of seven primer pairs that are specific for polymorphic microsatellite repeats in the ostrich, and we have found that we can genotype chicks using DNA isolated from the membrane and blood left in the hatched egg. We have also been able to get samples from the adults using a biopsy dart while they are away from the nest, thereby minimizing interference with nesting activity.

The microsatellite loci were isolated by screening a library of size-selected fragments of genomic DNA of *Struthio camelus massaicus*, collected from farmed ostriches originally from Nairobi National Park, Kenya. Genomic DNA from 10 individuals (five males and five females) was pooled and digested with *HaeIII*, *AluI* and *RsaI* (Gibco BRL). The digested DNA was size-selected for 250–800 bp on a 0.8% low-melting-point agarose gel. After recovery by phenol/chloroform extraction, the DNA was ligated into the vector pBS KS+ (Stratagene) that had been restricted with *SmaI* (Gibco BRL) and dephosphorylated with calf intestinal phosphatase (Pharmacia) in accordance with the manufacturer's instructions. After transformation in *Escherichia coli* XL1 competent cells (Stratagene) and plating onto agar plates with ampicillin, the colonies were transferred onto nylon filters (Hybond-N+ Amersham) and hybridized with (AC)₂₃ oligonucleotide which had been end-labelled with [γ -³²P]-dATP (Amersham) using T₄ polynucleotide kinase (Pharmacia). Positive colonies were rescreened and 25 positive clones were sequenced using either a T7 sequencing kit (Pharmacia) or an ABI 377 PRISM automated sequencer. Seven of these clones had both uninterrupted repeats of 15 or more dinucleotide pairs and suitable flanking regions for primers. Ten sequences had less than 15 pairs of repeats and showed little or no polymorphism, two did not have suitable flanking regions and six had no repeat sequences or very dispersed repeats.

PCR amplifications for evaluation of heterozygosity were carried out in a GRI Minicycler thermal cycler using DNA from 14 individuals (seven males and seven females). The amplification reaction contained 1–10 ng of template DNA, 50 mM KCl, 10 mM Tris pH 9.0 (at 25 °C), 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 12 pmol of each primer and 0.5 U of *Taq* polymerase in a volume of 25 μ L under mineral oil. The amplification program was 94 °C for 4 min, then 35 cycles of a denaturation temperature of 94 °C for 10 s, an annealing temperature of 56–59 °C for 30 s depending on the primers used (Table 1) and primer extension at 72 °C for