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Isolation of microsatellite loci in *Akodon azarae* (Muridae, Sigmodontinae) and cross-amplification in other Akodontini species

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Introduction

Fragmentation of natural habitats is an increasing problem worldwide, having profound consequences on the genetic and demographic structure of natural populations (Mech and Hallet 2001). A representative example of a fragmentation process occurs in central Argentina, an area of approximately 500,000 km^2 formerly occupied by prairies, but currently consisting almost exclusively of crops and livestock pastures separated by fencelines, roads and railroads, along which native and introduced weeds grow: the 'border' habitats. One of the most important components of the fauna in this region are rodents of the subfamily Sigmodontinae, a highly speciose group of mostly South American distribution, assembled in seven different tribes (Reig 1984). Three of the most abundant Sigmodontine species in agroecosystems of central Argentina are *Calomys musculinus* and *C. venustus* (tribe Phyllotini), and *Akodon azarae*, pertaining to the tribe Akodontini, the second most important according to the number of species (Reig 1984). Previous studies have established that these species differ in several ecological and behavioural characteristics like habitat use (*A. azarae* and *C. venustus* preferentially use borders while *C. musculinus* inhabits both borders and crop fields; Mills *et al.* 1992; Polop and Sabattini 1993; Busch *et al.* 1997), space use (home range size vary by sex and breeding period in *A. azarae* and *C. musculinus*, and by population abundance in *C. venustus*; Priotto and Steinmann 1999; Priotto *et al.* 2002), mating system (promiscuous/polygynous in *C. venustus* and *A. azarae* and promiscuous in *C. musculinus*; Priotto and Steinmann 1999; Priotto *et al.* 2002; Steinmann *et al.* 2009) and competition (*A. azarae* is competitively dominant over *C. musculinus*; Busch *et al.* 2005). On these bases, it is predictable that these species will differentially perceive landscape fragmentation. Our research interest focusses on comparing the movement and the genetic structure of their populations in agroecosystems, as a model to assess the genetic consequences of fragmentation on the fauna of the region.

The development of codominant microsatellite DNA markers are an invaluable tool for studying fine-scale genetic differences. For this purpose we already isolated and characterized nine microsatellite loci in *C. musculinus* that also amplify in *C. venustus* (Chiappero *et al.* 2005, 2011). As part of our studies on population structure at a small spatial scale, we describe here results of the isolation and characterization of 10 microsatellite loci in *A. azarae*. We also investigated if the polymorphic primers developed amplify in other species of the tribe Akodontini.

This is, to our knowledge, the first report of microsatellite loci designed for the genus *Akodon*. Since this taxon has nearly 40 species distributed throughout South America from Colombia to southern Argentina and Chile, occupying a wide variety of habitats from 0 to 5000 m above sea level (Redford and Eisenberg 1984), our work has a potential utility for population genetic studies in the genus.

Materials and methods

The total genomic DNA was extracted from the heart of a single *A. azarae* (male no. 013) from a laboratory colony, using a standard phenol–chloroform procedure (Maniatis *et al.* 1982). For microsatellite isolation we followed the protocol of Glenn and Schable (2005). Briefly, genomic DNA

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Table 1. Characteristics of microsatellite loci isolated from *Akodon azarae*.

Locus	Core motif	Primers pair sequences (5′-3′)	*T* (°C)	$MgCl_2$ (mM)	Number of cycles	Gel%	Range of allele size	No. of Alleles	H_o	H_e	GeneBank accesion no.
*Aaz1**	$(ACAT)_8$	F:GCACAGGACCTTTGTGCT R:GGTCTAGGGTATCCAACACCT	52	2	28	7	170–194	13	0.764	0.869	HM046488
*Aaz2**	$(GA)_{19}$	F:GCAGAATCCCAGCCTCGTCTAC R:TCAGAGGCCTCATCAATCTTGCCAC	52	2.5	28	8	156–194	16	0.445	0.843	HM046489
*Aaz3**	$(AC)_{15}$	F:CTAGCAGAATCCCTCTGTGTGAAA R:GCCTCTCTAAAAAACGAGATTTGC	48	1.2	36	8	146–170	12	0.859	0,888	HM046490
*Aaz4**	$(GT)_2T(GT)_5ATGCGAG$ $TAT(GT)_4AT\ (GT)_{18}$	F:GATCTTCTTTCTGGTAAGGATAAAAC R:TGATTGACATACTTGAACAATGT	50	2.5	30	6	180–242	13	0.775	0.913	HM165271
*Aaz5**	$(AGAC)_6(AC)_{17}$	F:GCAATGATTCTTTAGAAATATCACCA R:CAAACAATTAAGGATTTTTGGTC	50	2.5	28	8	116–142	11	0.614	0.886	HM046491
*Aaz6**	$(AC)_{25}$	F:CCCAGGAAAACAGAGCATCGT R: TGCACACTGCNTGAGTGA	54	1.5	30	7	160–188	15	0.473	0.919	HM046492
Aaz7	$(GA)_{22}$	F: GATAAATCCATATTCAAACTAACC R: GATTCTGTTGATTTGGAGACTT	45	2.5	32	8	130	1	0	0	HM560015
*Aaz8**	$(GT)_6(GAGT)_2(GTGA)_2$ $(GT)_{11}(GA)_{11}AA(GA)_{10}$	F:CTTTACAGTGTGCTTTTCA R: TGCTTTCAAGGAGACATT	48	1.5	30	8	122–166	16	0.618	0.895	HM046493
Aaz9	$(AC)_{24}$	F: GGAGACTGTTCTGACTCCCACAA R: AGAGACCCCGTGTCAGGG	48	1.5	30	7	120	1	0	0	HM560016
*Aaz11**	$(CA)_{16}$	F: GCCTATAGCACACATGTGCA R: AGCAGAATCTTTTCATTTTCCAAGA	52	2	33	8	112–138	15	0.764	0.883	HM046494

(Mean observed (H_o) and expected (H_e) heterozygosities are an average over two populations. F, forward primer; R, reverse primer; *T*, annealing temperature,*, indicates those loci that were polymorphic.)

was digested with *Hae*III (New England Biolabs, Ipswich, England) and separated by agarose gel electrophoresis. Fragments of sizes ranging from 300 to 1000 bp were isolated from gel and ligated to the linker-oligonucleotides SNX-F and SNX-R (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′; 5′-pGATTCTGCTAGCTAGGCCTTAAACAAAA-3′). Enrichment of the selected fragments was performed by hybridization at 50°C with biotinylated oligonucleotides containing different microsatellite repeats ($(AG)_{12}$, $(TG)_{12}$, $(ACAG)_6$, $(ACTC)_6$, and $(ACTG)_6$) and captured on streptavidin beads. The recovered DNA was amplified by polymerase chain reaction (PCR) using SNX-F as the primer, ligated into the Pcr2.1-Topo Vector (Invitrogen, Buenos Aires, Argentina) and transformed into *Escherichia coli* DH5α competent cells.

Approximately 494 recombinant colonies were picked from agar plates, individually suspended in 50 μL of distilled water and boiled for 5′ to release DNA. The molecular size of the inserts was checked by a PCR reaction, using M13 forward and reverse primers. Forty one colonies, which generated PCR products between 500 bp and 800 bp, were sequenced using M13 universal primers at Macrogen (Rockville, USA). Of them, 33 contained unique inserts with microsatellite repeats. Primers were designed for 13 sequences showing adequate microsatellite repeats and flanking sequences, using the software FASTPCR (Kalendar 2004). Loci were named using the prefix Aaz followed by a number.

Amplification conditions for each primer were optimized using as template DNA extracted from four individuals captured in two natural populations. Amplifications were performed using Biometra *Uno*II thermal cycler (Biometra, Göttingen, Germany), varying the annealing temperature, number of cycles and $MgCl_2$ concentration in order to obtain amplification of neat bands. The final cycling condition consisted in an initial denaturation step of 5 min at 92°C, followed by 30 cycles of 30 s at 92°C, 30 s at the annealing temperature (table 1) and 30 s at 72°C, ending with 5 min at 72°C. The optimized PCR mix contained 1× reaction buffer (10 Mm Tris-HCL, 50 Mm KCl, pH 8.3), 60 μM of each dNTP (Invitrogen), 0.4 μM of each primer, 1.3 M of betaine (Sigma, Buenos Aires, Argentina), 1 U of *Taq* polymerase (Fermentas Life Sciences, Buenos Aires, Argentina), 10 ng of template ADN, and $MgCl_2$ varied according to each primer (table 1) in a total volume of 10 μL. Amplified alleles were separated by electrophoresis using the Tris–Glycine buffer system (White *et al.* 2000) on 20-cm long, 8% native polyacrylamide gels along with a 10-pb molecular marker (Invitrogen, Buenos Aires, Argentina) and stained with silver nitrate (Neilan *et al.* 1994).

Primer pairs producing good quality amplification patterns were tested for polymorphism using 21 individuals from two natural populations in central Argentina: Chucul (33°0′35″S, 64°10′17″W) and Piamonte (32°7′60″S, 61°58′60″W). Polymorphic primer pairs for these loci were

Table 2. Cross amplification of eigth microsatellite loci designed for *Akodon azarae*. The number of individuals showing a successful amplification versus the total number of individuals analysed per species (N/T), the allele range in base pairs and the total number of alleles (in parentheses) are indicated; '-' indicates no amplification.

Locus	N/T	*A. dolores*	N/T	*A. polopi*	N/T	*A. simulator*	N/T	*A. espegazini*	N/T	*O. rufus*	N/T	*Necromys* sp.
Aaz1	4/4	166–180 (4)	2/2	180–188 (2)	2/2	190–210 (4)	2/2	164–180 (3)	4/4	180 (1)	4/4	198–208 (3)
Aaz2	2/4	156–170 (3)		–		–	2/2	168–170 (2)		–		–
Aaz3	3/4	164–180 (5)	2/2	176–190 (2)		–	2/2	184–196 (2)	1/4	168–190 (2)		–
Aaz4	3/4	200–226 (5)	1/2	208–214 (2)		–	1/2	200–210 (2)	4/4	200 (1)	3/4	200–214 (3)
Aaz5	3/4	122–142 (5)	2/2	116–120 (2)	2/2	130–136 (4)	2/2	126–150 (4)	4/4	148–152 (2)	1/4	116 (1)
Aaz6	2/4	160–168 (2)		–	1/2	164 (1)	2/2	168–176 (2)		–		–
Aaz8		–		–		–		–		–		–
Aaz11	3/4	104–122 (5)	1/2	132–142 (2)	2/2	132–146	2/2	104–140 (3)		–		–

also tested for amplification in individuals of the following species of the tribe Akodontini: *A. dolores*, *A. simulator*, *A. polopi, A. spegazini, Oxymycterus rufus* and *Necromys* sp. Conditions for amplification were the same as those for *A. azarae*.

Conformance to Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using FSTAT version 2.9.3.2 (Goudet 2002).

Results and discussion

Primers for 10 loci produced good quality amplification patterns; eight of them (*Aaz1, Aaz2, Aaz3, Aaz4, Aaz5, Aaz6, Aaz8* and *Aaz11*) were polymorphic. Characteristics of the loci (locus name, GenBank accession no., repeat core, primers sequences, annealing temperature, range of allele size, number of alleles amplified, average observed and expected heterozygosities and $MgCl_2$ concentration) are provided in table 1.

Locus *Aaz6* deviated from HWE in both populations, and loci *Aaz2* and *Aaz5* deviated only in Chucul and Piamonte populations, respectively. Since no null homozygotes were observed, the presence of null alleles in a low frequency can not be discarded. Number of alleles per locus ranged from 11 to 16. Levels of heterozygosity were high (table 1) and were similar to those observed in other Sigmodontinae rodents, for example: H_o between 0.278 and 0.889 in *C. musculinus* (tribe Phyllotini, Chiappero *et al.* 2005), between 0.371 and 0.896 in *Oligoryzomys longicaudatus* (tribe Oryzomini, González-Ittig *et al.* 2008), between 0.462 and 0.783 in *Nectomys squamipes* (tribe Oryzomini, Maroja *et al.* 2003). There was no evidence of LD between pairs of loci in any population.

Results of cross-species amplifications are shown in table 2. All primers produced successful amplifications in at least one of the species of the tribe Akodontini assayed, with the exception of locus *Aaz8* that failed to amplify in all of them.

The high levels of polymorphism detected indicate that the loci here developed would provide enough information for the study of the microgeographic genetic structure in *A. azarae*. Also, it is worth noting that these primers cross-amplified successfully in other species of the tribe Akodontini and appear to be highly polymorphic, given the high number of alleles amplified in relation to the number of individuals of each species tested (table 2). The cross amplification of these primers in other species of the tribe Akodontini highlight their potential usefulness for population genetic studies in those species.

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