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Nectomys squamipes Microsatellites and Homologous Loci in Sigmodontine Rodents

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Three monomorphic and four highly polymorphic microsatellites of *Nectomys squamipes* were isolated and characterised in a sample of 141 specimens from eight different Brazilian localities. These seven microsatellites and four others previously described in this species were tested in seven other nonfocus sigmodontine species. At least three loci were successfully amplied in every species, but none was amplied in all species. All sequenced products in nonfocus species showed $(GT)_n$ motifs as in *N. squamipes*. Several loci were amplied in *Nectomys rattus* and *Oligoryzomys nigripes*, while absence of PCR products was observed more frequently in *Oxymycterus dasythricus* and *Akodon cursor*. Two of three monomorphic loci in *N. squamipes* were polymorphic in other species.

The majority of neotropical rodents and approximately 22% of all mammal species of South America belong to the family Sigmodontinae, a highly diverse group with a remarkable adaptative radiation including approximately 51 genera and 249 species (Reig 1984, 1986). Within this family, some species are particularly relevant, as is the case of *Nectomys squamipes*, a South American water rat that is a primary host of the parasite *Schistosoma mansoni* (D'Andrea et al. 2000; Rey 1993). *N. squamipes* is widely distributed along water streams in several regions of Brazil, but is also well adapted to peridomiciliar habitats (Bonvicino 1994). Because rodent fitness is not apparently reduced by infection (D'Andrea et al. 2000), infected rodents might eventually contribute to spreading the parasite by migrating to areas where secondary hosts (*Biomphalaria* species) are present.

Microsatellites are valuable tools for population studies, and five of these markers have been isolated and characterized in *N. squamipes* (Almeida et al. 2000). More markers are needed, however, for further assessing the genetic variability of several widely disperse populations and for studying migration patterns. Moreover, heterologous microsatellite primers can also be used in closely related species of different taxa (Schlötterer et al. 1991), probably because the origin and genomic dispersion of several microsatellites might have anteceded species diversification. Thus, microsatellites might be useful tools for population studies in nonfocus species, provided they are polymorphic and show Mendelian segregation. These conditions, however, might not be maintained across species, because polymorphic microsatellites in a focus species might be monomorphic in others (and vice versa), while evolutionary genomic rearrangements might result in deviations from Mendelian segregation patterns.

In this paper we describe seven new microsatellites in *N. squamipes*, thus increasing the number of molecular markers in this species. *N. squamipes* microsatellite primers were used for amplification in the congeneric species *Nectomys rattus* and representatives of six other genera to test the efficacy of cross-specific, heterologous microsatellite DNA amplification across a range of sigmodontine rodent taxa. This latter approach allowed us to test whether monomorphic loci in the focal species were consistently monomorphic across this taxonomic range.

Materials and Methods

One hundred forty-one *N. squamipes* were collected in eight different Brazilian localities: 8 from Fazenda União (16°50′S,

Table 1. Motifs and primer sequences of seven microsatellite loci of *Nectomys squamipes*

Locus	Repeat motif	Primer sequences (5′3′)	T_a°C	GenBank accession no.
Nec08	$(GT)_{25}$	F: GTGGATGGATTCATGTGATCTG	58	AF353184
		R: CAGACAGGGTCTCACTAAGTTGC		AF353185
Nec13	$(CA)_4GA(CA)_6GT(CA)_6$	F: TATCTGCTCAACATTCCAGGGT	58	AF283418
		R: TGGAGTAAGATGCTCAGAGTTG		
Nec17	$(CA)_8T(AC)_{22}$	F: TCCCTGGTTATCATACTTGAGG	58	AF283423
		R: GACAGTTCTCACTTTTCCATGG		AF283425
Nec19	$(CA)_{12}(TA)_5(TG)_{21}$	F: CCAAATGGTGCCTAAAAATCAG	58	AF353188
		R: TAGTAGAGAGCAACCAAAGGCC		AF353189
Nec23	$(GA)_4(GT)_9(GA)_{29}$	F: CTACCTCCAAAACAGAGAAAGG	55/57	AF353186
		R: CTGATTTCTGTGTATGATTGAGAT		AF353187
Nec24	$(CA)_5AA(CA)_6$	F: CTTCTGCCCTCCACAAATGATT	61	AF283427
		R: GTAGCAACTGCGTAACTTCCCC		
Nec29	$(CA)_{22}$	F: CAAATGTCCTCTGGTCTTCAC	60	AF353190
		R: CAACATTAGAGAAATTCAGGGC		AF353191

F, forward primer; R, reverse primer; T_a°C, annealing temperature.

43°35′ W), 26 from Glicério (21°14′S, 42°03′ W), 49 from Sumidoro (22°03′S, 42°40′ W), 21 from Tarituba (23°02′S, 44°35′ W), 8 from Ilha da Marambaia (23°03′S, 43°58′ W), 13 from Ilha Grande (23°11′S, 44°11′ W), 10 from Pedreiras (22°43′S, 46°55′ W), and 6 from Fazenda Canoas (16°50′S, 43°35′ W).

Genomic DNA of rodent specimens was extracted from livers by standard proteinase-K/phenol-chloroform procedures (Sambrook et al. 1989). A genomic DNA library was prepared following *Alu*I digestion of one *N. squamipes* DNA sample. Size-selected fragments of 200–700 bp were ligated into *Sma*I-digested, dephosphorylated pUC18 and transferred to *E. coli* DH5α competent cells. A total of 18,210 recombinant colonies were transferred to nylon membranes (NEN) and hybridized with [γ^{32}P]-ATP labeled $(GT)_{10}$, $(CT)_{10}$, $(AGG)_7$, $(GAA)_7$ and $(GATA)_5$ oligonucleotides. DNA samples from 111 positive colonies were labeled with Big Dye™ Terminator Cycle Kit (Applied Biosystems) and sequenced with an ABI Prism 377. Twenty-six clones showed CA repeat motifs, and primers were designed for amplifications of genomic DNA. Eleven primer pairs resulted in successful amplifications; four primer sets amplified *Nec12* (GenBank accession no. AF283417), *Nec14* (AF283420 and AF283419), *Nec18* (AF283426 and AF283424), and *Nec28* (AF283428; see Almeida et al. 2000), and seven other primer sets amplified the microsatellites herein reported. Sequence data of cloned *N. squamipes* microsatellites were deposited in GenBank.

The seven new microsatellite loci were described and analyzed in *N. squamipes* specimens. Monomorphism was confirmed in at least 36 individuals of different geographic origin. PCR reactions were carried out in final volumes of 15 μl with ~10 to 40 ng of genomic DNA, 10 m*M* Tris–HCl (pH 9.0), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 7 pmol of fluorescence-labeled forward primer, 10 pmol of reverse primer, 300 μmol of each dNTP, and 1 U of *Taq* DNA polymerase (Pharmacia). An initial denaturation period of 5 min at 94°C was followed by 30 cycles of 30 s at 94°C, 30 s at T_a°C [see Table 1 and Almeida et al. (2000) for previously reported annealing temperatures], 30 s at 72°C, and a final extension period of 4 min at 72°C. Amplification of *Nec23* was carried out with seven cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C.

Fragment analyses were conducted with an ABI Prism 377 with standard loading and electrophoresis conditions.

Table 2. Genetic variation of seven microsatellite loci in *N. squamipes*

Locus	*N*	A	*F*	He	Ho	ASR
Nec08	141	11	0.271	0.870[c]	0.657	215–273
Nec13	72	1	1	0	0	209
Nec17	73	1	1	0	0	239
Nec19	141	17	0.318	0.770[a]	0.685	221–311
Nec23	139	31	0.214	0.913[b]	0.783	345–463
Nec24	36	1	1	0	0	117
Nec29	78	12	0.365	0.816[c]	0.462	275–305

Exact *P*-values obtained with Markov chain method for difference between He and Ho: *N*, number of examined animals; A, number of alleles per loci; *F*, frequency of the most common allele; He, expected heterozygosity; Ho, observed heterozygosity; ASR, allele size range (in base pairs).

[a] $P < 0.01$.

[b] $P < 0.001$.

[c] $P < 0.0001$.

Table 3. Allele range in base pairs, number of alleles (in parentheses), and number of analyzed individuals per species (*N*)

	Oryzomyni						Phyllotyni		*Insertae sedis*		Akodontini			
Locus	*N. rattus*	*N*	*O. megacephalus*	*N*	*O. nigripes*	*N*	*C. tener*	*N*	*D. collinus*	*N*	*O. dasythricus*	*N*	*A. cursor*	*N*
Nec 8	229–249 (7)	5	311–325 (7)	5	227–271 (7)	5	—	5	293–328 (6)	5	326–344 (5)	5	**300 (1)**	5
Nec 12	225–237 (4)	5	218–230 (6)	5	—	5	—	5	—	5	—	5	—	5
Nec 13	**209 (1)**	5	352–434 (5)	5	233–237 (3)	5	290–310 (3)	4	305–400 (6)	5	241–244 (4)	5	—	5
Nec 14	200–234 (7)	5	—	5	218–246 (3)	5	—	5	207–224 (3)	5	215–223 (2)	5	215–223 (2)	3
Nec 17	—	4	—	5	—	3	—	4	—	5	—	5	—	3
Nec 18	121–203 (8)	5	—	5	220–263 (7)	5	?	3	180–244 (5)	5	—	5	214–228 (4)	5
Nec 19	232–242 (6)	5	187–221 (4)	4	**194 (1)**	5	**209 (1)**	4	—	5	NT	5	NT	5
Nec 23	347–411 (8)	5	—	5	337–371 (6)	5	—	5	353–421 (8)	5	—	5	—	5
Nec 24	**117 (1)**	5	—	5	117–141 (6)	5	—	5	—	5	—	2	—	5
Nec 28	135–153 (6)	5	—	2	199–279 (6)	5	139–199 (5)	5	123–145 (5)	5	—	3	—	5
Nec 29	274–304 (7)	5	—	5	—	5	288–304 (6)	5	—	5	—	5	—	5

Monomorphic loci are indicated in bold. —, no amplification; ?, lack of resolution; NT, not tested.

Alleles were sized relative to an internal size standard and analyzed with GeneScan 2.1 (Applied Biosystems). Linkage disequilibrium between loci was tested with Fisher's exact test using Markov chain (3,000 dememorizations; 450,000 iterations) under a null hypothesis postulating lack of genotype association between different loci with GENEPOP 3.2a (Raymond and Rousset 1995). Deviations from Hardy–Weinberg equilibrium at each locus were tested by a Markov chain approximation (3,000 dememorizations; 450,000 iterations) of unbiased exact P-values (Guo and Thompson 1992) with GENEPOP 3.2a.

These seven microsatellites and four previously described microsatellites (*Nec12, Nec14, Nec18*, and *Nec28*) were tested in a maximum of five randomly selected individuals of *N. rattus* and representatives specie of three sigmodontine tribes: *Oryzomys megacephalus* and *Oligoryzomys nigripes* (tribe Oryzomyini), *Calomys tener* (tribe Phyllotyni), *Oxymycterus dasythricus* and *Akodon cursor* (tribe Akodontini), and *Delomys collinus* (*insertae sedis*). Dubious products showing smears were disconsidered. The following amplified products of nonfocus species were sequenced for confirming homology: *Nec08* in *N. rattus* and *D. collinus*, *Nec13* in *O. nigripes* and *O. dasythricus*, *Nec14* and *Nec18* in *O. nigripes*, and *Nec19* in *O. megacephalus* (AY071917) and *O. nigripes* (AY071918). Only sequence data containing microsatellite flanking regions were deposited in GenBank.

Results and Discussion

Sequence data of the seven presently identified *N. squamipes* microsatellites are shown in Table 1; three of these seven loci were monomorphic in this species (Table 2). The four polymorphic loci showed several alleles with high heterozygosity per locus, attesting their suitability for assessing genetic variability within and between water-rat populations. Three polymorphic loci (*Nec08, Nec19*, and *Nec29*) showed a continuous spectrum of allele size, while one of them (*Nec23*) presented a size gap between 393 and 445 bp. There was no evidence of linkage disequilibrium between loci ($P > .05$ with Bonferroni correction). Expected heterozygosity was significantly higher than observed heterozygosity for all polymorphic loci (Table 2), but this was probably a result of the Wahlund effect (Hartl and Clark 1997), due to pooling *N. squamipes* specimens of different populations.

When tested in nonfocus, sigmodontine species, at least three loci were successfully amplified in each species, but none was amplified in all species (Table 3). In *N. rattus* and *O. nigripes* most heterologous amplifications were successful (10 and 8, respectively), while in the five other nonfocus species no more than six heterologous amplifications were observed (Table 3). Similarly, Moncrief et al. (1997) reported that only 28.8% of *Mus musculus* primers could be successfully used for microsatellite amplifications in *Microtus pennsylvanicus*. Conversely, similar studies in other mammalian orders were more successful; in Cetacea, Schlötterer et al. (1991) reported that 100% of *Globicefala melas* primers could be used for amplifying several species of the major groups of this order, while in artiodactyls, Pépin et al. (1995) showed that 40% of bovine microsatellite primers could be used in goats. Poorer results with heterologous rodent primers might be a consequence of the higher rate of evolutionary change of the rodent genome in respect to other mammals (Martin and Palumbi 1993).

We did not estimate heterozygosity in nonfocus species, due to small sample size (a maximum of five individuals per species), although the number of alleles per polymorphic locus ranged from two to eight. All sequenced products in nonfocus species showed $(GT)_n$ motifs as in *N. squamipes*. This was coincident with previous comparisons of microsatellite sequence data showing across-species homologies (Ezenwa et al. 1998). Moreover, two *N. squamipes* microsatellites (*Nec08* and *Nec14*) with "perfect" (or "pure") motifs were also found to be "perfect" in nonfocus species (in *N. rattus* and *Delomys collinus*, and in *O. nigripes*, respectively), while one *N. squamipes* microsatellite (*Nec13*) with "interrupted" (or "imperfect") motifs was also "interrupted" in *O. nigripes* and *O. dasythricus*. However, *Nec18*, with a "perfect" motif in *N. squamipes*, showed an "interrupted" motif in *O. nigripes*.

Heterologous primers were generally more effective in phylogenetically closer species (Table 3). All but one

microsatellite were successfully amplified in *N. rattus*, a congeneric species of *N. squamipes*. Moreover, PCR amplification products were frequently observed in another species belonging of the same focus-species tribe (Oryzomyni), *O. nigripes*. Six microsatellite loci were amplified in *D. collinus*, a species of controversial taxonomic position [in Oryzomyni *sensu* (Reig 1986) or in Thomasomyni *sensu* (Musser and Carleton 1993)]. Four microsatellites were amplified in *C. tener* (Phyllotyni), and three in the representative species of the tribe Akodontini (*O. dasythricus* and *A. cursor*).

The observed monomorphism of *Nec13* and *Nec24* shared by *N. squamipes* and *N. rattus* (Tables 2 and 3) might be due to fixation of one allele with a small number of imperfect repeats (Goldstein and Clark 1995; Levinson and Gutman 1987). The other monomorphic locus (*Nec17*), amplified only in *N. squamipes*, showed a high number of repeats similar to polymorphic loci (Table 1). This observation was contrary to the common finding that monomorphic loci contain low numbers of repeats (Blanquer-Maumont and Crouau-Roy 1995).

Two of the three monomorphic loci of *N. squamipes* (*Nec13* and *Nec24*) were polymorphic in other species. On the other hand, *Nec19*, a polymorphic locus in *N. squamipes*, *N. rattus*, and *O. megacephalus*, was monomorphic in *O. nigripes* and *C. tener*. Similarly, *Nec8* was polymorphic in five nonfocus species and monomorphic in *A. cursor*. These findings show that monomorphic loci might be useful in nonfocus species despite the fact that they might not be reported or tested (Amos and Hardwood 1998; Garza et al. 1995; Pépin et al. 1995).

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