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A Novel Set of Microsatellite Marker Loci Linkage-Mapped in the House Musk Shrew, Suncus murinus

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Abstract: *Ten microsatellite DNA loci developed for the white-toothed shrew (*Crocidura russula*) were tested for PCR amplification and for utility in linkage studies in the house musk shrew,* Suncus murinus*. Four primer pairs successfully yielded PCR amplicons and showed polymorphism between two mutant strains, BAN-*kc,oeb *and WZ. Cloning and sequencing of the PCR amplicons of all the four loci confirmed the presence of microsatellite sequences. Alleles segregating in an F_2 resource population constructed from the two strains ranged between two and five. Linkage analysis of the four loci together with 18 other polymorphic markers and three mutant loci resulted in five linkage groups containing three newly mapped microsatellite loci. This study reports the first microsatellite markers being registered in this species.*

Key words: Crocidura russula*, linkage map, microsatellite loci, polymorphism,* Suncus murinus

Introduction

The house musk shrew, *Suncus murinus*, an insectivorous species of a size comparable to the laboratory rat, has been domesticated since 1973 [16]. Several laboratory strains derived from Bangladesh, Japan, Nepal, Sri Lanka, and other localities [8, 11, 16, 17, 19] and mutant stocks showing morphological [8, 10, 18–20] and biochemical [20, 29] variants have been established. Genetic variability among different populations is evidenced in the diploid chromosome number ranging from 2n = 30 to 2n = 40 [8, 32, 33], with the latter proposed to be the basic karyotype since it occurs in majority of the populations [23].

In recent years, the utility of *Suncus murinus* as a model in biomedical research has increased, particularly in the areas of emesis research [30], motion sickness [31], female behavioral endocrinology [22], carcinogenesis [28], nutritional regulation of reproduction [27] and others. The growing importance of the musk shrew is further evidenced by the upsurge of articles indexed by PubMed (NLM, USA; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). However, the major obstacle in using the musk shrew for genetic studies is the current lack of a genetic map pertaining to its genome.

One of the most widely used and important genetic markers for constructing genetic maps are microsatellite

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DNA markers. Their highly polymorphic nature [26] and co-dominant mode of inheritance, coupled with the ease with which they can be typed using the PCR, have made them markers of choice in genome mapping [3]. The use of microsatellite loci in a species from which it was not originally developed, documented in several species, enables the construction of comparative maps between related species and may also be useful in QTL detection [5] particularly if a candidate gene is known from another species.

To this end and as part of our effort to develop markers to enhance the usefulness of *Suncus murinus* (Soricidae, Crocidurinae), we have assessed the utility of microsatellite DNA loci developed for the greater white-toothed shrew, *Crocidura russula* (Soricidae, Crocidurinae). In addition to amplification success, we checked for polymorphism using a three-generation resource population of *Suncus murinus* developed in our laboratory between two mutant strains, BAN-*kc,oeb*, fixed for the kinky coat (*kc*) [10] and open-eyelids at birth (*oeb*) [9] mutations and WZ, fixed for the waltzing (*wz*) mutation [19]. Together with comparative anchor tagged sequence (CATS) markers [13], previously found to be polymorphic between these two *Suncus murinus* strains [1], and three mutant loci (*kc, oeb* and *wz*), the polymorphic microsatellite DNA loci detected were used in linkage analysis.

Materials and Methods

Resource population

A three-generation resource population of *Suncus murinus* developed from BAN-*kc,oeb* and *wz* strains was used in this study. The characteristics, development and husbandry have been described previously [1, 9, 10, 19].

Characterization of Marker loci

Ten PCR primer pairs were synthesized (Sigma Genosys, Tokyo, Japan) according to the primer pair sequence of ten microsatellite DNA loci characterized for *Crocidura russula* [7]. The ten primer pairs were optimized using *Suncus murinus* genomic DNA obtained by standard phenol/chloroform extraction protocol [24]. Optimal PCR conditions were obtained by varying the annealing temperature between 48°C and 60°C after an initial trial at Tm –5°C (where Tm is the lower melting temperature of the primer pair) and under different concentrations of Mg^{2+}. PCR amplifications were conducted in a 14-μl reaction volume containing approximately 100 ng DNA, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5–2.5 mM $MgCl_2$, 1.0 mM dNTPs, 0.54 μM of each primer and 0.35 unit Taq DNA polymerase (Takara Bio Inc., Seta, Japan). Thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems) profiles were as follows: Initial denaturation at 94°C for 5 min, followed by 32 cycles at 94°C for 30 s; annealing at optimal primer temperature for 1 min and polymerization at 72°C for 1 min. The final polymerization step was for 10 min at 72°C. Electrophoretic sizing of the amplified products were done on 3.5% agarose gel made up of 1.5% Seakam® LE (BioWhittaker Molecular Applications, Rockland, ME, USA) and 2.0% Metaphor® (Cambrex Bioscience Rockland Inc., Rockland, ME, USA) in 1× TBE buffer using a Mupid electrophoresis unit (Cosmo Bio. Co. Ltd.,Tokyo, Japan) at room temperature. The PCR products of some loci were electrophoresed on 6% polyacrylamide gel using a *PAGERUN* electrophoretic unit (ATTO corporation, Tokyo, Japan). End products were stained in ethidium bromide and visualized under UV light. Microsatellite polymorphism and Mendelian co-dominant transmission of alleles were ascertained from the family of 26 individuals comprising four sires (BAN-*kc,oeb*), seven dams (WZ) and their $15F_1$ progeny (4 males, 11 females). To confirm the presence and the number of tandem repeats in all the detected alleles, cloning of the PCR amplicons and cycle sequencing were done as described previously [1]. Segregation data were obtained from $77F_2$ individuals by genotyping, and a chi-squared test was performed for the polymorphic markers to check for deviations from Mendelian segregation.

Linkage Analysis

Linkage groups were determined under linkage criterion of P=0.001 and Kosambi mapping function, with the Macintosh version of Map Manager QTXb20 [14]. Significant linkage was determined at LOD ≥ 3.0 in all cases.

The microsatellite loci developed in this study were named using the prefix, *NGA* (corresponding to the code for our laboratory) followed by an Arabic numeral.

Results

Microsatellite markers

We have explored the possibility of utilizing microsatellite loci developed in a different but closely related species for linkage analysis in *Suncus murinus*. The four loci (*NGA4, NGA5, NGA6* and *NGA7*) that were successfully amplified showed length polymorphism between the two mutant strains. The remaining six primer pairs (namely 3, 9, 23, 45, 53 and 54; [7]) yielded multiple PCR products. The inheritance pattern of the bands of these six primer pairs did not conform to Mendelian fashion in the resource family. It was concluded that these primer pairs could not be utilized in *Suncus murinus*.

Cloning and sequencing of the PCR amplicons of the four loci obtained from representative parents revealed the presence of microsatellites and also confirmed the size variants observed on the electrophoretic gel (Table 1). Size differences observed at each of the four loci for both strains were based exclusively on the core repeat sequence. The four loci also exhibited the co-dominant mode of inheritance ascertained from the family of 26 individuals (7 grandparents and 15 F_1 individuals). The number of segregating alleles identified for both strains varied between two and five (Table 1).

Genotyping data for each of the four polymorphic loci obtained from the 77F_2 mapping population was used to test whether genotypic frequencies agreed with Mendelian segregation. No deviation from Mendelian segregation was observed for *NGA4*, *NGA5* and *NGA7*. The locus *NGA6*, however, was not initially fully informative in some of the F_2 individuals because both alleles are of the same size (i.e. shared alleles) in the two strains (Table 1), thus appearing homozygous in three F_1 individuals. Once the F_2 dataset associated with these three F_1 individuals were removed, the deviations fell in line with Mendelian segregation (Table 2).

Linkage mapping

Linkage analysis based on the segregation data in our resource population was carried out using all four microsatellite loci together with 18 polymorphic CATS markers and three mutant loci (*kc*, *oeb* and *wz*) previously genotyped in this resource population [1]. Three microsatellite DNA loci (*NGA4*, *NGA5* and *NGA7*) and two CATS markers (*NRAS* and *GJB2*) were newly mapped in this study. *NGA5*, *NGA7* and *NRAS* are in linkage group IV with LOD scores of 11.7 and 6.3 for *NGA5-NGA7* and *NGA5-NRAS*, respectively. In linkage group V, *NGA4* and *GJB2* are linked with a LOD score of 4.7. The current linkage map of *Suncus murinus* comprises seven CATS markers, three microsatellite DNA markers and one mutant gene, covering a total distance of 96.6 cM in five linkage groups (Fig. 1). The locus *NGA6* was not assigned to any group.

Table 1. Characteristics of the four polymorphic microsatellite loci in the two strains of *Suncus murinus*, BAN-*kc,oeb* (BAN) and WZ

Locus[a]	Primer pair sequence (5' => 3')[b]	PCR conditions[c]		Repeat motif[d]	Allele size in bp (number of repeats)	
		T_A	Mg^{2+}		BAN	WZ
NGA4 (17)	F: tccatcatcttcttagggttgc R: acaggatttttctgtcccatg	48	2.5	$(GT)_n$	124 (27), 130 (30)	114 (22), 116 (23)
NGA5 (30)	F: gaaacaaaagtgtatgtgcgc R: ctaagcaggattcattttcgg	48	2.0	$(GT)_n$	63 (7), 67 (9)	73 (12), 79 (15)
NGA6 (57)	F: acaattgtctatggttattggcg R: tctggaagaaacccatttgg	48	2.0	$(CA)_n$	118 (23), 126 (27)	118 (23), 126 (27)
NGA7 (72)	F: tccctccttggttacttcataac R: gagtaattcttgagcgctgc	54	1.5	$(TC)_4TATCT(TC)_n$ $(TC)_2TATCT(TC)_n$	217 (32), 249 (48) -	197 (22) 193 (22), 207 (27)

[a]The nucleotide sequence data for all the 15 alleles of the four loci reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB255387 and AB255586 to AB255599. The names of *Crocidura russula* loci [7] corresponding to the *Suncus murinus* loci are given in parenthesis. [b]Primer pair sequence as published by Favre & Balloux [7]. [c]Conditions optimized for *Suncus murinus*. T_A is annealing temperature in °C while Mg^{2+} concentration is in mM. [d]Based on the strand amplified according to the primer pair order shown. n denotes the number of repeats. In locus *NGA7*, two imperfect repeat types were detected in the *Suncus murinus* sequence region corresponding to the 17bp deleted region in the *Crocidura russula* sequence as shown in Fig. 2.

Table 2. Genotyping data of the four polymorphic loci segregating in F_2 progenies obtained by an intercross between sires of BAN-*kc,oeb* (B/B genotype) and dams of WZ (W/W genotype) strains of *Suncus murinus*

Locus	Number of F_2 individuals genotyped[a]	Number of observed (expected) genotypes			χ^2 value[b]
		B/B	B/W	W/W	
NGA4	77	11 (19.25)	46 (38.5)	20 (19.25)	5.03
NGA5	77	22 (19.25)	34 (38.5)	21 (19.25)	1.08
NGA6	46[c]	11 (11.5)	17 (23.0)	18 (11.5)	5.26
NGA7	77	13 (19.25)	40 (38.5)	24 (19.25)	3.26

[a]All genotyped data will be made available upon request. [b]All values are not significantly different from Mendelian expectations at $P>0.05$ (d.f. = 2). [c]The genotype data of 31 individuals were discarded because they were not fully informative (see text for details).

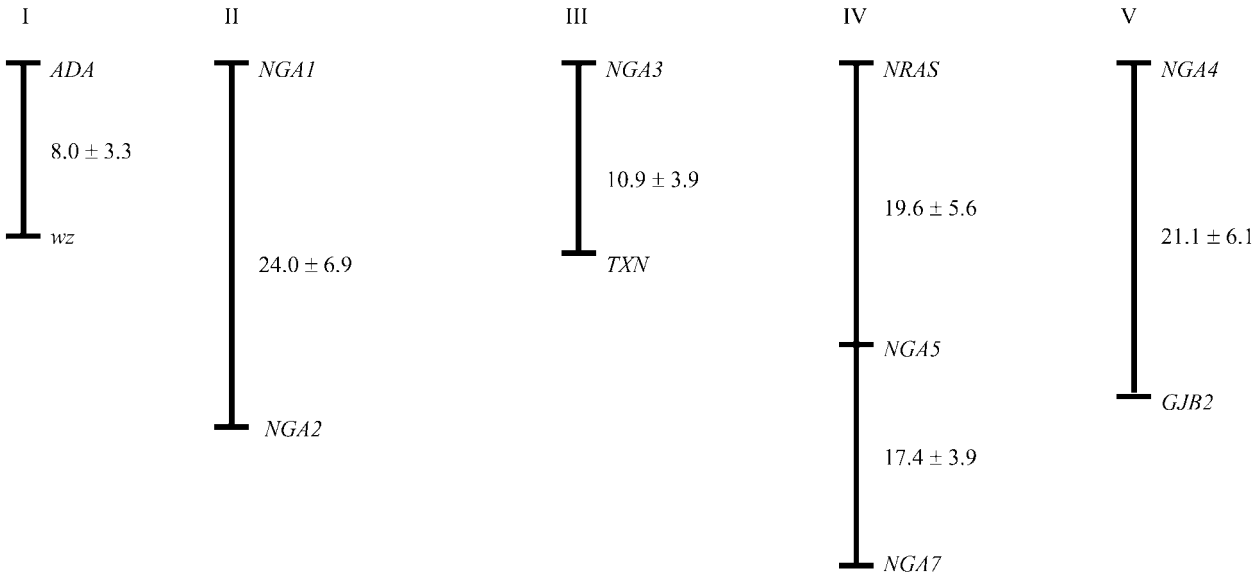

Fig. 1. The current linkage map of *Suncus murinus*. The map was constructed with Map Manager QTXb20 (Macintosh version) using the P=0.001 criterion. Distances (± SE) were computed with the Kosambi mapping function and are listed in cM to the right of each linkage group. All loci in linkage groups IV and V were newly mapped in this study while the others had been previously mapped [1].

Sequence comparison between Suncus murinus and Crocidura russula

Using the sequence data of the four loci that were used for linkage analysis, (i.e. *Crocidura russula* loci 17, 30, 57 and 72 with GenBank accession numbers Y09414, Y09408, Y09410 and Y09411 respectively, corresponding to *NGA4–7*), we compared them with the allele having the longest repeat sequence in *Suncus murinus* (Fig. 2). Most of the sequences were essentially conserved with only some point mutations and insertions or deletions of few base pairs. Two exceptions are *NGA4* and *NGA7*, for which an 18-bp deletion in the *Suncus murinus* sequence and a 17-bp deletion in the *Crocidura russula* sequence, respectively, differentiate between the two species. Also, except for locus *NGA7* showing two imperfect repeat types (Table 1), the repeat motif obtained for *Suncus murinus* corresponded well with that of *Crocidura russula*.

Discussion

This study was designed to develop microsatellite DNA markers to enhance the utility of the house musk shrew, *Suncus murinus* taking advantage of ten

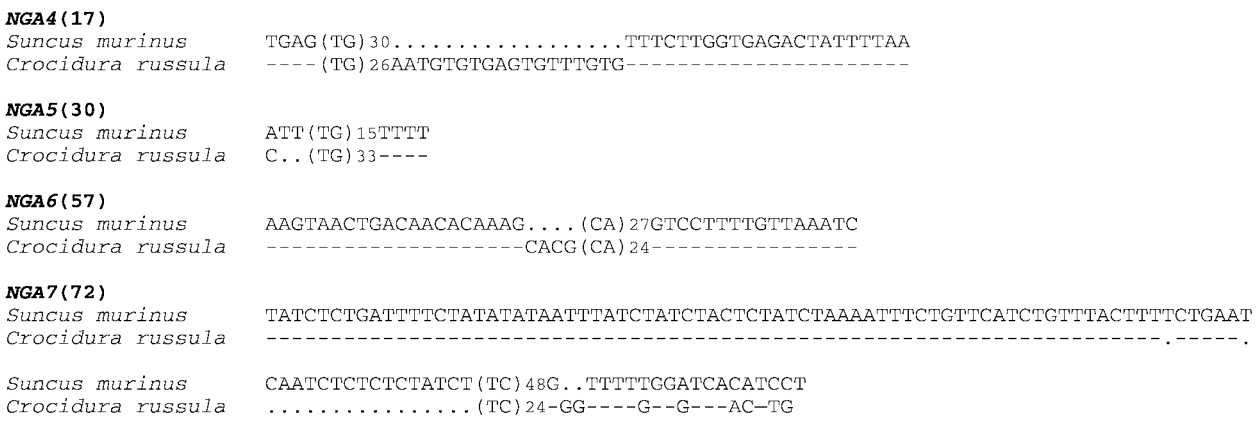

Fig. 2. Nucleotide sequences alignment between *Suncus murinus* (NGA4–7) and *Crocidura russula* (17, 30, 57 and 72; [7]). Dashes (-) correspond to identical nucleotides while dots (.) have been placed to show location of insertions or deletions.

microsatellite DNA markers available in *Crocidura russula*. We had successful amplification in four, all of which contained microsatellites and exhibited length polymorphism as well as co-dominance in the two mutant strains of *Suncus murinus* used in this study. Utilizing microsatellite DNA loci from non-source species can obviate the time consuming work of constructing a genomic library and screening it for microsatellite containing sequences; and this strategy has been successfully used for a number of species [6, 12, 21, 25]. The possibility of amplifying a microsatellite locus in another species depends on the conservation of its flanking regions, which is a function of the phylogenetic distance between the taxa [2]. In the work of Favre and Balloux [7], 70%, 60% and 0% of the same ten primer pairs amplified polymorphic end product in *Crocidura leucodon*, *Crocidura suaveolens* and *Sorex araneus*, respectively, as compared to the 40% obtained for *Suncus murinus*. These percentage differences in amplification must reflect the phylogenetic relationship among the three genera based on similarities in anatomy, physiology, distribution and evolutionary history [4]. *Suncus murinus* and *Crocidura russula* are classified under the subfamily Crocidurinae while *Sorex araneus* is placed in the subfamily Soricinae.

Of the four microsatellite loci developed in this study, three have been assigned to the *Suncus murinus* linkage map. The three newly mapped microsatellite loci co-segregated with two CATS markers, *NRAS* (Human: 1p13) and *GJB2* (Human: 13q11-q12), also newly mapped in this study. This study reports the first microsatellite markers being registered in this species.

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