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Variable Microsatellite Markers for Genotyping Tree Shrews, *Tupaia*, and their Potential Use in Genetic Studies of Fragmented Populations

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ABSTRACT We describe the sequences of six primer pairs for the PCR amplification of nuclear microsatellite markers in the tree shrews, *Tupaia glis* and *T. belangeri*. Multilocus genotyping based on non-destructive DNA sampling of live-trapped animals reveals high allelic variability (*A*) and heterozygosity (H_e) at these loci. Such characteristics make these genetic markers ideal for linkage mapping and comparative genomics, and for studies of pedigree relationships, population structure, and population genetic variability in wild populations. We illustrate another use of these markers in the study of genetic erosion in small recently fragmented natural populations of tree shrews in south Thailand. This method for detecting and monitoring genetic erosion in fragmented populations can be applied to larger taxa of traditional concern to wildlife managers.

KEYWORDS: genetic markers, microsatellites, mammals, tree shrews, genetic erosion.

INTRODUCTION

Tree shrews (Scandentia: Tupaiidae) have been studied extensively in captivity (>2000 published papers) because of interest in their possible resemblance of ancestral primates, as well as their behavior and physiology.[1,2] Wild tree shrews are less well known (see Emmons' recent book for the first detailed study)[3] but they are relatively common in both undisturbed forests and around villages in continental southeast Asia and the islands of the Sunda Shelf. The ease with which they can be live-trapped makes them attractive subjects for a variety of field investigations. Although some mitochondrial (mtDNA) and nuclear (nDNA) sequences and karyotype maps have been published in the context of establishing the phylogenetic position of the Scandentia among the Euarchonta mammals,[2, 4, 5] these are not variable enough for species or population level studies.

To prepare for a study on genetic changes in recently fragmented populations of small rainforest mammals in Thailand, we developed panels of microsatellite loci for individual genotyping of selected species of rodents and tree shrews. Although we found microsatellites in Thai rodents using heterologous primers discovered first in the laboratory rat and mouse,[6] in the case of tree shrews it was necessary to isolate, clone, and determine the unique flanking sequences of these genetic markers *de novo*. In this report we describe the primers needed to use these loci and illustrate their utility in a study of variability in natural populations.

Microsatellite loci are relatively short typically di-, tri-, and tetranucleotide sequence repeats in the nuclear genome. The length of the repeat motifs often varies between individuals in a population and such sequence length variation is inherited in simple co-dominant Mendelian fashion. These loci are often hypervariable and it is not unusual to find 6–10 alleles segregating in a small population of sexually reproducing plants or animals. They are scattered evenly throughout the genomes of mammals and are flanked by unique sequences of nDNA easily targeted for amplification using the polymerase chain reaction (PCR). These characteristics make them ideal as linkage map markers and for studies of pedigree relationships, population genetic variability, population structure and gene flow. Their use has revolutionized studies of sociobiology, microevolution, stock management and conservation genetics.[7-11]

Multilocus microsatellite genotyping may be used to monitor changes in a population's variability over time. We have been particularly interested in the effects of forest fragmentation on the genetic variability and viability of mammal populations isolated on small patches of remaining forest. Such populations are believed to experience genetic erosion, the

decrease in variation due to random genetic drift and inbreeding. Genetic erosion is both a symptom and a cause of endangerment of small isolated populations. The phenomenon has been long understood in terms of population genetic theory but the critical early stages of the process in nature have gone undocumented as the changes are rapid and difficult to monitor. In theory, average genetic heterozygosity decreases in isolated populations at a constant rate related to the genetic effective size (N_e), at $1/(2N_e)$ per generation.[12] A population with $N_e = 10$ may lose as much as 50% of its heterozygosity in the first 20 generations following isolation. The importance of such rapid genetic erosion on population viability remains unclear, as there are few studies of the process in nature.[13-15]

An opportunity to study genetic erosion empirically arose when 165 km^2 of lowland rainforest were flooded in 1987 following construction of a hydroelectric dam on the Khlong Saeng, Surat Thani Province, southern Thailand. More than 100 former hilltops became permanent islands in Chiew Larn reservoir and retained their original fauna of 10–12 species of small mammals. During years 5–8 post-fragmentation we monitored the demographic collapse of these communities,[16-18] and genetic variability in commoner species whose populations were effectively isolated on some islands.[19,20]

MATERIALS AND METHODS

Specific microsatellite primers were developed for *Tupaia glis* based on a voucher specimen collected near Ban Ta Khun village at Chiew Larn Dam, about 50 km west of Surat Thani, Thailand. Total DNA was extracted from liver and heart tissue of one adult female preserved in 70% ethanol, using the phenol-chloroform extraction method. A 300–600 bp partial genomic library was constructed by ligation of size-selected *Mbo*I digested total DNA into a pTZ18U/BamHI vector. Transformations were performed by electroporation into JS5 *E. coli* electro-competent cells (BIORAD). Recombinant colonies were concentrated on 30 mm pore nylon filters (Millipore, Bedford) and probed with dinucleotide repeat polymers ($(CA)_{15}$ and $(GA)_{15}$). High-stringency positive clones were picked and sequenced using pTZ18U primers. Thirteen primer pairs (12 $(CA)_n$ repeats and one GA repeat) were designed using the program PRIMER (version 5.0, Whitehead Institute for Biomedical Research). PCR reaction conditions for each primer pair were determined by first performing amplification using 3 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 6 min. The 25 µl amplification reaction contained 10X *Taq* polymerase reaction buffer (10mM Tris, pH 8.3, 0.01% gelatin, 1.5mM $MgCl_2$, 100 mM KCl), 0.2 mM each dNTP, 10 pmol unlabeled forward primer, 10 pmol unlabeled reverse primer, 2 pmol ^{32}P end-labeled forward primer, 1–2 units *Taq* polymerase (Promega), and 10 µl of DNA (approximately 1–10 ng). Products were electrophoresed on 8% super-denaturing formamide/urea acrylamide sequencing gels along with the M13 sequence as an external size standard. For loci where no product was observed, the reactions were repeated and the annealing temperatures were raised or lowered until the optimal conditions were achieved.

To assess variability at each locus in wild tree shrews, we genotyped 19 individuals live-trapped and released in 1993 from one study population (Mainland R, 110 ha of continuous forest)[20] at Khlong Saeng Wildlife Sanctuary, using DNA extracted and amplified from ethanol preserved ear- and toe-clips. Allelic variability, *A*, (mean alleles/locus), and unbiased expected heterozygosity (H_e) were measured and calculated. Genotype frequencies were compared to those expected under panmixia and a probability test was performed to estimate linkage disequilibrium using the program GENEPOP.[21] Seven out of the thirteen loci gave specific amplification products in the wild study population; the GA repeat was monomorphic, six CA repeat loci were polymorphic in this population. These six microsatellite loci were subsequently amplified from 95 additional individuals sampled from this mainland population in 1994 (n = 8) and 1995 (n = 12) and from two populations isolated on islands in the reservoir: Island R (110 ha; sample sizes from year 1992 = 5, 1993 = 33, 1994 = 9) and Island M (10 ha; sample sizes from year 1992 = 8, 1993 = 6, 1994 = 8, 1995 = 6). Genetic variability on the islands was compared annually with that in the mainland population, which served as a control representative of the undisturbed populations living in this area before the valley was flooded in 1987. Wilcoxon signed ranks tests were used to test for differences in A or H_e significant at the $p<0.05$ level.

The six primer pairs were also tested for their ability to amplify orthologous loci in the related Thai species, *T. belangeri,* based on tissue samples of two individuals provided by the Zoological Society of San Diego.

RESULTS

Six out of thirteen cloned loci were polymorphic and are useful in characterizing genetic variability in *T. glis*. These primers were also found to amplify orthologous loci in *T. belangeri*. Figure 1 shows a typical autoradiograph of a gel with eight scorable individuals, all heterozygotes. The primer sequences, repeat motif, allele sizes, and PCR reaction conditions are described in Table 1. Linkage was not expected as *T. glis* has 30 pairs of chromosomes;[22] in our tests of linkage disequilibrium within each sample no significant linkage was detected.

Variability of these loci in a large undisturbed natural population (Mainland R, 1993) of *T. glis* was high (Table 1: 6–14 alleles/locus, H_e = 0.76–0.93). Allele frequencies at each locus are compared between Mainland R and large Island R 1993 samples (Figure 2). The patterns in the two samples are not significantly different. Genotype frequencies deviated slightly from Hardy-Weinberg expectations at most loci and

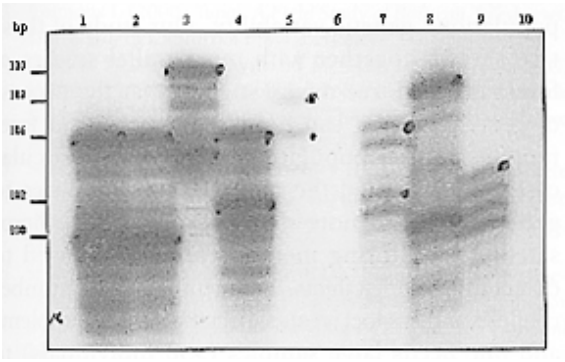

Fig 1. Autoradiograph of PCR amplification products at locus TG 21. Lanes 1-9: PCR products from nine *T. glis* sampled in 1994 from mainland site (R; 106 ha), Khlong Saeng, Surat Thani, Thailand. Lane 10: negative control for PCR amplification. The sizes of the PCR products were estimated using M13 sequence as an external size standard.

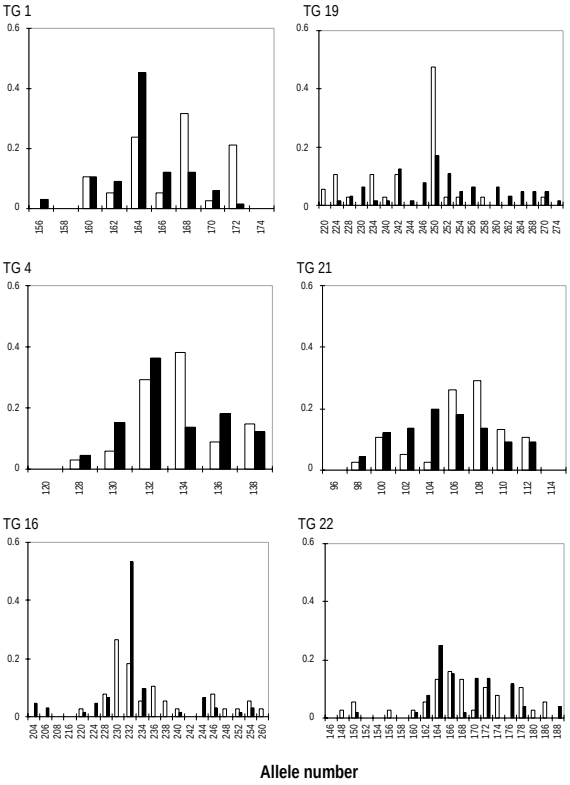

Fig 2. Distribution of allele frequencies for six microsatellite loci in the control population (mainland R, 1993, open bars) and a population isolated six years previously (island R, 106 Ha, shaded bars). Frequencies are plotted against the different alleles, designated in order of increasing size (number of base pairs).

Table 1. Primer sequences and characteristics of *Tupaia glis* polymorphic microsatellite loci.*

Locus	Primer pair sequences (5' to 3')	Repeat size	Size range (bp)	*nA*	H_e	H_o	T_A	GenBank No.
TG 1	F: TTCCTGTTACTCTGTTTTTTTCAGG	$(CA)_{19}$	160–172	7	0.80	0.74	52	AY064160
	R: ACATGGCTAACTGTGTGCTTTG							
TG 4	F: TGAAAACTGGCAATTCATATGC	$(CA)_9TA(CA)_2TA(CA)_3$	128–138	6	0.76	0.77	52	AY064161
	R: CAATCCTTTTTCGTTAGTTTTGTG							
TG 16	F: AAGTTTAATACCGGGCTGTTGA	$(CA)_{10}$	228–260	13	0.89	0.68	55	AY064162
	R: CAAGTCGCTGTATCGGTCAATA							
TG 19	F: ACCCCTCCCTAAAGGAACT	$(CA)_7TAAA(CA)_8$	220–270	11	0.76	0.26	50	AY064163
	R: CGCCCTATAGAAACCTCTCC							
TG 21	F: TTTTAGACGACAAAACCCC	$(CA)_{12}$	98–112	8	0.83	0.63	53	AY064164
	R: TAAAAAGACATAAAACACGTCA							
TG 22	F: GTGAGTGCACTTGCCCTGTA	$(CA)_8A(CA)_{10}$	150–186	14	0.93	0.79	55	AY064165
	R: TCCTGAACCTGGTGGCTAAC							

* *nA*, no. of alleles; H_e and H_o, expected and observed heterozygosity (in Mainland R study site, 1993 sample (N = 19); T_A, annealing temperature (°C).

in most samples, but the observed heterozygote deficiencies are statistically insignificant and probably attributable to the large number of alleles segregating and the small sample sizes.

Comparison of the three annual samples from the control population (Mainland R) revealed no statistically significant differences in A or H_e. Comparison of the two island populations, isolated from the mainland in 1987, with the control samples (matched by year) revealed no statistically significant loss of genetic variability in fragmented populations until 1995, the last year of observations. In 1995 (year 8 post-fragmentation), the small Island M population (10 ha) exhibited reduced genetic variation: heterozygosity (0.475 vs 0.76–0.93 in the control population) and allelic diversity (A = 3.17 vs 5.67–9.83) as reported in detail elsewhere.[20] Changes in allele frequencies in two of the six loci monitored in the 10 ha island population illustrate the pattern whereby common alleles became more common and rare alleles dropped out (Figure 3).

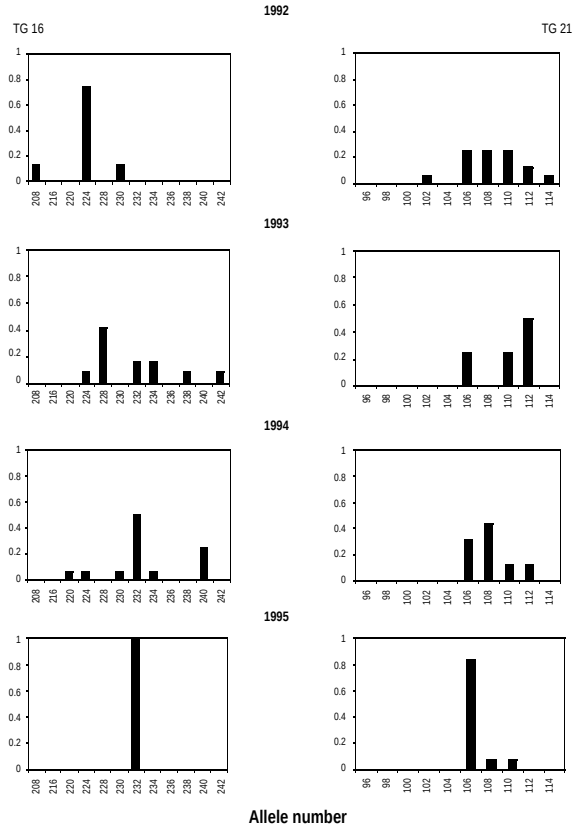

Fig 3. Changes in allele frequencies at two microsatellite loci in a population isolated on small island M over the period 5-8 years post-isolation (1992-1995). Frequencies are plotted against the different alleles, designated in order of increasing size, shown in base pairs.

DISCUSSION

The provision of primers for these hypervariable loci should facilitate studies of mating systems, population structure and gene flow and also facilitate the mapping of the tree shrew genome. The results with *T. belangeri* indicate that these primers may be also useful in studies of other tree shrews and in comparative genomics. Currently 16–18 species of tree shrews are recognized and their genetic characterization is of interest in several laboratories (including Y Kawamoto, Kyoto; M Ramon, M Sorenson, Boston; F Sheldon and K H Han, Louisiana State). This work is relevant to the Thai tree shrews, as recent studies have revealed unexpected diversity among animals traditionally referred to *T. glis*.[23-26]

This panel of microsatellites is clearly variable enough to permit the measurement of a natural populations' genetic variability. Our study of these tree shrews, together with our parallel studies of forest rats and tree mice[20] suggest that the process of genetic erosion can now be monitored in free-ranging natural populations. In these particular cases we attributed the observed loss of alleles to genetic drift but note that the multilocus microsatellite monitoring method can also be used to detect inbreeding effects. Unfortunately, the number of alleles at these loci creates special statistical problems and one needs large samples (>50 individuals) to prove that allele frequencies are changing significantly. In retrospect, most of our samples were too small. Alternatively, one can increase statistical power by increasing the number of loci surveyed.

Nevertheless, with appropriate experimental design and larger samples it should be possible to use multilocus microsatellite variability to monitor genetic erosion in fragmented populations of animals. The method can easily be up-scaled to larger mammals like elephants, [27] tigers and hornbills[28] of conventional concern to Thai conservationists. This would provide wildlife managers with a new tool to assess a population's viability and opens up numerous research opportunities, as habitat fragmentation is such a ubiquitous phenomenon. Although monitoring genetic erosion in long-lived species may not be practical, much can be learned immediately by comparing isolated populations to those still more continuously distributed. Multilocus microsatellite monitoring can thus contribute to the larger goals of biodiversity conservation in Thailand.[29, 30]

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