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SHORT REPORT

GENOTYPING FREE-RANGING RODENTS WITH HETEROLOGOUS PCR PRIMER PAIRS FOR HYPERVARIABLE NUCLEAR MICROSATELLITE LOCI

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ABSTRACT

The application of commercially available microsatellite primers, developed for genomic analysis of the laboratory rat (Rattus norvegicus) *and laboratory mouse* (Mus musculus), *to the genotyping of wild rodents provides a rapid and inexpensive alternative to cloning, sequencing and synthesizing species-specific primers de novo. The effort required to identify a useful panel of heterologous (interspecifically derived) primers for loci in genetically unknown rodents is illustrated with a study of three wild rodents in Thailand. Eight out of 13 (62%) of the rat primer pairs tested amplified polymorphic sequences in a representative of a closely related genus,* Maxomys surifer, *and all 8 of these primer pairs amplified polymorphic sequences in the wild congener,* R. rattus. *Of the mouse primer pairs 33% (10/30) amplified polymorphic sequences in a distantly related tree mouse,* Chiropodomys gliroides. *Preliminary surveys of wild* M. surifer *and* C. gliroides *in Thai forests revealed high levels of polymorphism at these microsatellite loci and up to 20 alleles segregating per locus. These loci are variable enough to be used to establish pedigree relationships and population structure, and monitor gene flow and genetic erosion. Multilocus genotyping based on noninvasive DNA sampling and PCR employing both homologous and heterologous microsatellite primers promises to revolutionize genetic analyses of free-ranging rodents.*

INTRODUCTION

The recent discovery of a class of genetic markers called microsatellites promises to revolutionize studies of mammal sociobiology, evolution, and conservation. Scientists and wildlife managers need genetic information to establish relationships among individuals and levels of variability in natural populations and captive colonies. Genetic data also permit the identification of hybrid individuals and of evolutionarily significant units for management purposes. Until recently, the necessity of collecting and freezing blood and tissue samples made such data difficult to acquire. Furthermore, analyses based on DNA fingerprinting suffered from problems of genetic interpretability and analyses based on DNA sequencing were rarely informative enough at the population level. Such technical problems have been eliminated by the development of the polymerase chain reaction (PCR) and the discovery of microsatellites. We are left, however, with the problem that the flanking sequences for each locus of interest

have to be known so that one may synthesize primer pairs capable of amplifying the genetic markers themselves by the PCR. There are two ways of determining these specific flanking sequences: one is laborious and involves sequencing the DNA in each species of interest, the other is to try to use known primers from closely related species. This paper describes the first use of such heterologous primers to study variation in rodents.

Microsatellites (MSATs) or simple sequence repeats (SSRs) are nuclear loci that are used as genetic markers in the human, mouse, and rat genome mapping projects. With up to 100,000 MSAT loci present in mammalian genomes, they represent the most informative source of polymorphic loci known to date.[1,2] They are relatively short (100–300 base pairs (bp)) sequences consisting of a 2–6 bp repeat (e.g.: di-, tri- or tetranucleotide repeats). The length of the repeat motifs often vary between individuals in a population and such sequence length variation is inherited in simple codominant Mendelian fashion. MSAT loci are often hypervariable and it is not unusual to find 6–10 alleles segregating in a small population of plants or animals. Loci are scattered evenly throughout the genomes of mammals and are flanked by unique sequences of DNA 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.

Woodruff[3] (1993) reviewed the clear advantages of microsatellite genotyping over traditional methods. For example, population surveys of microsatellite variation yield 5 or more times higher heterozygosity levels than can be detected using allozyme electrophoresis.[4-6] In contrast to multilocus minisatellite VNTRs resolved by whole genomic DNA fingerprinting (and even single locus minisatellite fingerprinting), the small size of microsatellite alleles permits genotyping from very small and even degraded samples of DNA. The need for organ, tissue, or blood samples, and the associated need to freeze the samples in the field, are thus obviated. Noninvasive genotyping based on DNA extracted from hair and even feces is now possible.[3,7-8]

The initial isolation and identification of useful microsatellite loci is a costly, time-consuming endeavor. The use of multilocus microsatellite genotyping has been hindered by the difficulties associated with the isolation of the loci and the determination of their unique flanking sequences. Unique oligonucleotides, called primers, which target such flanking sequences are essential for PCR amplification of each MSAT selected for study. A pair of primers (typically 20–30 bp long, one on either side of the MSAT) have to be synthesized for each locus. This can be done by preparing a genomic DNA library for the species of interest, screening with, for example, a single dinucleotide motif probe, and collecting and sequencing many positive clones. Although the protocols are now standard[6,9-10] this method can take 3–6 months of intensive laboratory work and cost a few thousand U.S. dollars.

An alternative approach is to take advantage of MSATs characterized in the course of the various genome mapping projects: e.g., human, rodent, canine, bovine and porcine. Heterologous PCR primers, based on DNA sequences flanking microsatellite loci in one species and conserved in closely related species, have been reported in bovids, canids, cetaceans and primates[11-16] but not previously in rodents. In this paper we report the successful use of species-specific PCR primers developed for the laboratory rat and mouse genome projects to survey genetic variation in wild rodent species in Thailand.

In this demonstration we have investigated 44 commercially available laboratory rat and labboratory mouse MSAT primer pairs for their ability to amplify informative loci in free-

ranging Thai native rodents. We are using Thai rainforest rodents as indicator species in a project aimed at developing a new method of monitoring genetic erosion in small populations isolated on recently fragmented habitat patches.[3,8,17-20] Minimizing the time and effort invested in obtaining a useful panel of highly polymorphic loci with which to monitor genetic variability, structure and gene flow in natural populations was desirable. Our success with commercially available primer pairs suggests that others working in wild rodents might also circumvent the cloning and sequencing approach to identifying polymorphic MSATs in their study populations.

MATERIALS AND METHODS

Sample collection

Samples consisting of plucked hair and toe clips were obtained from 10 species of live rodents and other small mammals trapped and then released in the context of a study of faunal collapse on forested islands in Chiew Larn reservoir between Khao Sok National Park and Khlong Saeng Wildlife Sanctuary, Surat Thani province, south Thailand. The yellow-bellied rat, *Maxomys surifer,* pencil-tailed tree mouse, *Chiropodomys gliroides,* and roof rat, *Rattus rattus,* are abundant enough to use as genetic indicator species; others encountered included the noisy rat, *Leopoldamys sabanus,* Whitehead's rat, *Maxomys whiteheadi,* chestnut rat, *Niviventer bukit,* pencil-tailed rat, *N. cremoriventer,* ricefield rat, *Rattus argentiventer,* Muller's rat, *Sundamys muelleri,* and common treeshrew, *Tupaia glis.* All samples were preserved in 70% ethanol at the time of collection and, upon return to the laboratory, stored at 4° C for up to 3 years. In this report we describe results based on the study of the following samples: *Maxomys surifer* (4 populations of 24-30 individuals), *Chiropodomys gliroides* (4 pops./10-18 indiv.) and *Rattus rattus* (1 pop./10 indiv.).

DNA extraction

We combined the original method of Higuchi *et al.*[21] with a Chelex (BioRad Laboratories) extraction step[22] and a hair lysis buffer (Cristian Orrego, San Francisco State University, pers. comm.).[23] Hair follicles or 1 mm^3 pieces of toe clips were washed with 70% ethanol and double-distilled H_2O, dried, and immersed in hair lysis buffer and incubated overnight at 56°C. 10% Chelex solution was then added to the extraction mix and incubated for 15 min or overnight at 96° C.

PCR

Forty-four primers developed for laboratory rat, *Rattus norvegicus,* and laboratory mouse, *Mus musculus,* genome projects and marketed by Research Genetics, Inc. (Huntsville, Alabama USA.) were tested with DNA templates extracted from our samples for the presence of polymorphic microsatellite loci. Forward primers were end-labeled with radioactive ^{32}P according to manufacturer's specifications. Amplification was performed using a Hybaid Thermal Reactor and approximately 10–20 ng of DNA template in a 25 μl PCR reaction. Typically reactions involved 1 initial cycle of 3 min at 94°C, followed by 40 cycles with three phases (1 min denaturation at 94° C, 1 min annealing at 45–60° C, and 1 min extension at 72° C), and finally, 6 min at 72° C. It was necessary to modify this protocol for some loci and species depending on the conserved nature of the primer regions. Due to the degeneracy of the conserved PCR primer sites at some loci, reactions were optimized by scaling annealing temperatures and varying the number of cycles.

DNA visualization and interpretation

Reaction products were diluted four-fold in a formamide dye solution, denatured at 95° C for 5 min, and loaded on 8% formamide/urea acrylamide sequencing gels. M13 sequence was used as an external size standard. Genotypes were scored by eye; heterozygotes were clearly two-banded, homozygotes showed one major band.

RESULTS

To date, we have tested 44 commercially available rat and mouse MSAT primer pairs for their ability to amplify loci in other rodents. Typical results are shown in Figure 1. Alleles were recognized as the highest intensity band among weak shadow bands in homozygous individuals or the two highest intensity bands in case of hetorozygous individuals. DNA extracted from *Chiropodomys gliroides* was tested with 30 mouse primer pairs. 10 loci were successfully amplified, 3 monomorphic and 7 polymorphic in the populations sampled (Table 1). In attempts to amplify *Maxomys surifer* MSATs with 14 rat primer pairs and 1 mouse primer pair we found 9 polymorphic MSAT loci in the Thai rat. These comprised 8 loci detected with rat primers and one locus, D18Mit17, amplified with a mouse primer pair (Table 2). All eight of these *R. norvegicus* primer pairs amplified polymorphic products in *R. rattus*. Finally, preliminary screening of six additional Thai rodents (*Leopoldamys sabanus, M. whiteheadi, Niviventer bukit, N. cremoriventer, R. argentiventer, Sundamys muelleri*) with these heterologous primers is underway and giving promising results (data not shown). We have not yet found any rodent primers capable of amplifying microsatellite loci in the far more distantly related treeshrew, *Tupaia glis*.

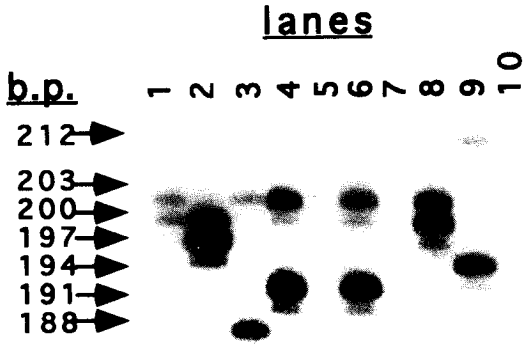

Fig. 1. Autoradiogram of microsatellite locus D18Mit17 amplified from toe-clip tissue specimens of eight yellow-bellied rats (*Maxomys surifer*) from mainland site Q, Khlong Saeng, Thailand. PCR products were generated with primers D18Mit17R/D18Mit17L and separated on a polyacrylamide gel. Each lane shows one (homozygote) or two (heterozygote) strong bands, fainter bands are artifactual stutter bands generated in the PCR. Lanes #1-4 and 6-9: 8 specimens of yellow-bellied rats; lane #5: negative control of DNA extraction; lane #10: negative control of PCR. Genotypes are as follows: 1: 203/200, 2: 200/197, 3: 203/188, 4: 203/191, 6: 203/191, 7: no product, 8: 203/200, 9: 212/194, where alleles are identified by their length in base pairs.

TABLE 1. List of mouse microsatellite loci that gave specific ptoducts in *Chiropodomys gliroides*.

No.	Locus name	repeat sequence	PCR primers(5'-3')	Product length (bp)	allele number
1	D1Mit 4	CA	gctactgctttggagtcagt atgacttgagctcagtctctg	302-246	14
2	D1Mit24	CA	ccaatccatcttgggcag attggttttgctgaaccagg	118-214	14
3	D2 Mit29	TAG	cggtgacgaagcttctgag ctttgaatatgaactctcaccttcc	79	1
4	D3Mit21	GCA	aagctctacagcggaagcac ctggggagtttcaggttcct	132	1
5	D3Mit22	GCT	aaggattgaagaatggttggg aatcagcgatttcagcacg	74-247	6
6	D11Mit4	TG	cagtgggtcatcagtacagca aagccagcccagtcttcata	288-210	20
7	D11Mit36	TG	ccagaacttttgctgcttcc gtgagccctaggtccagtga	340-286	11
8	D12Nds2	unknown	acatggtaatttatgggcaa ctggatacctgcaatagtaga	170-250	19
9	D17Mit20	ATAC	agaacaggacaccggacatc tcataagtaggcacaccaatgc	148-195	19
10	D18Mit17	AGC	tcaggcagattccaagcag ctgtgggtagcccaagtcat	183	1

TABLE 2. List of rodent microsatellite loci that gave specific ptoducts in *Chiropodomys gliroides*.

No.	Locus name	repeat sequence	PCR primers(5'-3')	Product length (bp)	allele number
1	ACE	unpublished	ataacaccaacattaccatagaggg atactcagttcagacttttcaccaatttt	187-159	19
2	MHCG	unpublished	cctgaacataaggaaacatcacc ctctgacttcaacacatgaggtg	111-190	10
3	RR1023	unpublished	agcctcactgatgctcctgt ccaagagctacctgcactcc	259-212	15
4	RR24	unpublished	ctctttgggatgaaccggta aatgggaagcaacagcattc	250-164	20
5	RR360	unpublished	atgagaggtcaaagcttctca agacctgggacagggtcct	118-82	14
6	RR1150	unpublished	agcctaaaatttgactctctttgc ggttcacaccatggtgattg	140-127	6
7	D18Mit17*	unpublished	tcaggcagattccaagcag ctgtgggtagcccaagtcat	223-193	14
8	RR514	unpublished	ttctgtattaaccacagaaagaagc aagccagcccaaagtaaatg	300-220	11
9	RR53	unpublished	tgttctacctccctcccctt ttctgccaagataccccaac	140-127	7

* D18Mit17 was developed for the laboratory mouse, all others for the rat.

DISCUSSION

The rat and the mouse genomes have been diverging for 15 (10–30) Myr,[23] yet this study demonstrates that there are probably significant numbers of commercially available PCR primers conserved across rodent species and genera. Based on this survey of *Rattus norvegicus* primer pairs, 100% of the primers tested amplified MSATs in the congener *R. rattus* and 60–65% amplified MSATs in the closely related genus *Maxomys*. Predictably fewer (33%) of the *Mus musculus* primers tested amplified MSATs in the distantly related *Chiropodomys gliroides*. To date we have found a single pair of *Mus musculus*-derived primers that cross-amplified microsatellites in the unrelated rat *Maxomys surifer*.

Many of the microsatellites loci displayed significant numbers of variable alleles. All nine *Maxomys surifer* microsatellite loci tested are polymorphic and 6–20 alleles were detected during this preliminary screening of 20-30 rats per locus. In the case of *Chiropodomys gliroides*, 27% of the amplified microsatellites were polymorphic in an initial screening of 10-20 individual mice per locus. Comparable data from wild populations of the laboratory rat and mouse species from which these loci were derived are not yet available. Nevertheless, these levels of polymorphism and heterozygosity exceed those revealed by allozymes and bode well for microsatellite based surveys of genetic variation.

Commercially available primers can be screened for their ability to amplify microsatellite loci in a species of interest in a few days. Accordingly, our results suggest that it may be faster and less expensive in specific cases to buy and screen a number of primers than developing species-specific primers by cloning, sequencing and synthesizing oligonucleotides *de novo*. This alternative approach would appear particularly suited for studies of rodents as the Research Genetics, Inc., catalog now lists more than 400 mapped polymorphic mouse microsatellite primer pairs (mouse MapPairs™) and over 450 Rat MapPairs™. Deitrich *et al.* (1992) and Serikawa *et al.* (1992) have described many of these microsatellites in the mouse and rat,[24,25] respectively, and by late 1994 the mouse map developed at the Whitehead Institute/MIT Genome Center had identified over 5,000 such markers with an average spacing of less than 1 cM. Primers may be selected for testing based on the nature of the simple sequence repeat motif, the total length of the microsatellite in previously studied populations, and chromosomal location. Microsatellites on the sex chromosomes can be especially informative in some research.

Despite the advantages of this approach the interpretation and analysis of results have to be done with caution. Firstly, microsatellite loci are among some of the most rapidly evolving sequences in mammalian nuclear genomes. Heterologous primers do not necessarily amplify homologous sequences between species, and may not share the same chromosomal location. Our preliminary results, for example, suggest that one locus reported to be a trinucleotide in *Rattus norvegicus* might be expanding as a dinucleotide repeat in *Maxomys surifer*. (This hypothesis can be tested by direct sequencing.) Secondly, primer sites themselves may diverge during the evolution of a group of related species. Thirdly, one must be aware that nonamplification of one of the two alleles present in a heterozygote can occur by chance when the DNA source or template contains only 1-3 copies of the nDNA genome.[26,27] Although we have not experienced this problem of false homozygotes in our rodent studies it may be a significant problem in surveys based on shed hair and feces. Finally, because of the less stringent reaction conditions used to amplify these heterologous loci, the number of non-specific PCR products and shadow bands may increase and can confound the scoring of alleles of similar

size. For some studies the traditional alternative approach of developing species-specific primers *de novo* may be the preferred option from the outset.

The application of MSAT to studies of mammalian population ecology and evolutionary genetics is in its infancy. Initial reports of the use of MSAT genotyping in wild mammals have involved chimpanzees,[13,14] canids,[15] bears,[6] whales[28] and wombats.[29] Other studies of nonhuman primates[30] have taken appropriate advantage of the 3617 microsatellites reported in the context of the Human Genome Project.[9] The genome mapping underway for commercially important animals like the cow, pig, and dog[31-33] is identifing numerous MSAT loci that will facilitate studies of the genetics of related species. With a multilocus panel of about 10 suitably polymorphic microsatellite loci it should be possible to uniquely identify individuals in a population, establish paternity and other relationships, establish population structure, and levels of historical and comtemporary gene flow.[3,4,13-14,34] Rodents provide ideal subjects for the investigation of a number of important population, ecological and sociobiological issues and hypervariable microsatellites provide the key to the resolution of many long-standing problems. The fact that individuals can now be genotyped noninvasively and uniquely will permit the long-term monitoring of free-ranging study populations.

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