

ESTABLISHING MICROSATELLITE ANALYSIS FOR LOCALLY ENDANGERED POPULATIONS OF ROOT VOLE (*MICROTUS OECONOMUS*)

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The root voles (*Microtus oeconomus*) is endangered in Hungary; the monitoring of its fragmented and isolated populations is necessary for an effective nature conservation management. Analysing of microsatellite DNA has been shown to be an efficient method to assess genetic variability among a wide variety of organisms. The present paper describes how this technique can be used to study populations of root voles. Two different DNA sampling and extraction methods and 10 heterologous primer pairs were tested. From these 10 primer pairs, 6 were found appropriate for microsatellite analysis of this species. Sequence analysis of the amplified PCR products raised the problem that length polymorphism may be the result of variation of a compound microsatellite locus.

Key words: *Microtus oeconomus*, microsatellite analysis, sequence analysis, Hungary

INTRODUCTION

The probability of extinction of isolated populations shows negative correlation with population size (SOULE 1987). Natural demographic fluctuation and variation in environmental conditions may strongly affect the genetic parameters of populations and can lead to genetic erosion, hence making the structure of populations more fragile (GAINES & WHITTAM 1980, BIJLSMA & LOESCKE 1997). For this reason, the screening of genetic variation has a great importance in effective conservation measures of the targeted populations (CIOFI *et al.* 1998).

The root vole (*Microtus oeconomus* [PALLAS, 1776]) is the rarest vole species in Hungary according to the Red Data Book (RAKONCZAY 1989). The populations in the Carpathian Basin are enlisted as a local subspecies of root vole (*Microtus oeconomus méhelyi* [ÉHIK, 1928]) and their scattered populations are considered as a glacial relicts (JÁNOSSY 1986). The species has a Holarctic distribution with 23 distinguished subspecies (Online database <http://www.funet.fi/pub/sci/bio/life/mammalia/rodentia/arvicolidae/microtus>) and is a common rodent in Alaska, Siberia, and Northern Europe (TAST 1982). The variability of the North-

Mouse MapPairs™, Research Genetics, Inc., Huntsville, AL, USA), and one which had been previously described in the grey red-backed vole, *Clethrionomys rufocanus bedfordiae* (ISHIBASHI *et al.* 1995) all worked well in with meadow vole (*Microtus pennsylvanicus*) samples (MONCRIEF *et al.* 1997). Furthermore, we tested four of the eight primer pairs recently described in the Japanese field vole *Microtus montebelli* (ISHIBASHI *et al.* 1999). 'Mouse primers' were purchased from Research Genetics while the 'vole primers' were synthesized (EastPort Ltd., Csertex Ltd.).

Microsatellite analysis

PCR reactions were carried out using the thermal cycler PDR-91 (Biochemical Laboratory Service) in either 10 µl reaction mix covered with oil or in 50 µl mixture by Hot Start. The optimised mixtures for mouse/vole primers were respectively, as follows: 50 mM KCl, 10 mM Tris-HCl (pH8.3), 3 mM/1.5 mM MgCl₂, 300 µM/200 µM dNTP, 0.2 µM/0.15 µM of each primer, 0.5 U Taq DNA polymerase (Sigma), and 10–100 ng genomic DNA. After denaturation at 95°C/93°C for 5 min, the reaction was carried out in 30/35 cycles under the following conditions: mouse primers, 94°C for 45s, 57°C for 45s, 72°C for 60s; vole primers, 90°C for 30s, 54°C for 20s, 72°C for 20s; both finally ended by 72°C for 5 min. To check the presence of products, 10 µl of the amplicon was horizontally electrophoresed in 1.5% agarose gel.

PCR products were separated by size on 5% non-denaturing polyacrilamide gel (530×250×0.4 mm) and visualised by silver staining (MANIATIS *et al.* 1982). The DNA sequence of the purified PCR products (primer MSCRB-5) of three individuals was determined by using PRISM Ready Reaction Dye Deoxy Cycle sequencing protocol (Pelkin-Elmer) and an ABI 373A automated DNA sequencer (Applied Biosystems). Nucleotide sequences were assembled using the Lasergene program package (DNASTAR) and compared against GenBank sequences using the BLAST search program (ALTSCHUL *et al.* 1990).

RESULTS AND DISCUSSION

Feasibility of sampling methods

Tail-tips and tufts of hair were simultaneously tested as a useful source of DNA. As expected, hair produced a smaller yield of DNA (10–100 ng) compared to tail tips (1–10 µg). The amount of DNA from hair was just enough to produce an amplification product that resulted in a very weak signal on agarose-gel electrophoresis. Such results suggest that hair originated PCR products could not be analysed by the silver staining method. Still, if it was necessary, these amplicons could be separated in automated sequencers after fluorescent dye labelling.

Applicability of primers

Two out of five of the 'mouse primers' yielded characteristic PCR products with root vole samples. These primers were D8Mit13 and D5Mit 10 and they worked in a very narrow range of reaction conditions. The other three 'mouse primer pairs' (D9Mit64, D9Mit248, DXMit64) did not give distinct PCR products, although they amplified house mouse (*Mus musculus*) and striped field mouse (*Apodemus agrarius*) which were used as reference samples (PAPP & GUBÁNYI unpubl. data).

Amplification of root vole samples by 'vole-primers' MSCRB-5, MSMM-2, MSMM-3 and MSMM-4 also yielded specific products. These primers worked in a wider range of reaction conditions and most of them gave products with bank vole (*Clethrionomys glareolus*) samples as well. Primer pair MSMM-1 did not give a distinct PCR product for the examined species, nor did they amplify bank vole reference samples (PAPP & GUBÁNYI unpubl. data).

Presently, the number of alleles in root voles at these microsatellite loci has not been completely determined, although we have shown two loci MSCRB-5 and MSMM-2 to be polymorphic. Investigations continue to estimate the extent of variability of these loci.

Sequence analysis

The sequence analysis of PCR products obtained by primer MSCRB-5 (Fig. 1) has revealed a possible problem. The polymorphism of a locus might be the result of the repeat variation of more than one repetitive region close to each other. In the GenBank Report (ISHIBASHI *et al.* 1995) eight repetitive regions of dimers and tetramers were described from the identical locus of the grey red-backed vole

<u>Crb (GenBank)</u>	<u>CTCCTGGTAA TTTTCATCTT ACCTATATGT GTAAGTTTTA ACGTCTGGGT</u>	<u>50</u>
(98) MoL2	TCTGGGT	50
(97) MoV8	CTCCTGGTAA TTTTCATCTT ACCMGN GTGT GTATGTTTTA AAGTCTGGGT	50
(98) MoS4	CTCCTGGTAA TTTTCATCTT ACCGG GTGT GTATGTTTTA AAGTCTGGGT	50
	<u>TTTACATCTG AGAGAAAACA TGCAGTGTGT GTATGTGTAT GTGTGTACGT</u>	<u>100</u>
	TTTACATCTG AGAGAAAACA TGC AGTGTGT GTGTGTGTGT ATGT --ACAT	98
	TTTACATCTG AGAGAAAACA TGC AGTGTGT GTGTGTGTGT ATGT --ACAT	98
	TTTACATCTG AGAGAAAACA TGC AGTGTGT GTGTGTGTGT N?N?N?AC	100
	<u>ACATAATACA TTTTTGCACG TACGTATATT TGTGTGTGTG TGTGTGTATG</u>	<u>150</u>
	ACAC-ATAT- GTAC-GCATA CATATATATT T----- ----GCATG	131
	ACAC-ATAT- GTAC-GCATA CATATATATT T----- ----GCATG	131
	ACAN-ATNT- N?N?C-ACACA NATATATATA T----- ----GCN?N	133
	<u>TATGTATGTG TGTATGTATG TATGTATTTC CTAAATGCAA ACACCAACC</u>	<u>199</u>
	CAT ----- ---- GTATG TATGTATTTC CTAAATGCAA ACACCAACCA A	170
	CAT ----- ---- GTATG TATGTATTTC CTAAATGCAA ACACCAACCA A	170
	CMN ----- ---- GTGTA TATN?N?ATC CCAAANGCN? NACNANANA CA	173

Fig. 1. The alignment of three root vole PCR products (primer MSCRB-5) in comparison with the most homologous sequence of GenBank, grey red-backed vole (Crb) microsatellite locus MSCRB-5. The repeat motifs are printed in bold face, the one responsible for length polymorphism is shown in the small box. Individuals (98)MoL2 and (97)MoV8 are homozygous and identical for this locus. While (98)MoS4 is heterozygous and thus read sequence is the result of two overlapping products. N=uncertain, - deletion/insertion.

(GenBank accession number D37836). We have found six presumptive microsatellite repeat motif close to each other in this region. These are the following (with positions of starting base): (27)GT 3 times, (60)GA 3 times, (75)GT 8 times, (116)AT 4 times, (127)GCAT 2 times, (135)GTAT 3 times. Thus, similar allele lengths may arise from different combinations of repeat motif patterns. Obviously, we had to determine which one of the above mentioned motifs is responsible for the polymorphism of our PCR products. Fortunately, the animal coded (98)MoS4 was a heterozygote for this locus. By analysing the DNA sequence and separating the two alleles, it became apparent that the '(75)GT n times' was the repeat mainly responsible for length polymorphism [in the case of (98)Mos4 n=8 and n=11 on homologous chromosomes]. Therefore, locus MSCRB-5 is applicable for population genetic studies, since allele length depends only on the repeat number of one satellite. Nevertheless, situations such as this indicate the importance of sequencing at least a few PCR products of each locus to avoid misinterpretation of allele length polymorphism. The possibility of the presence of null alleles should be considered as well (PEMBERTON *et al.* 1995).

In conclusion, we have successfully extracted and amplified root vole DNA samples with the help of six primer pairs. The analysis of length polymorphism for each locus and the evaluation of the variability at these loci are both in progress.

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