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A comparative restriction endonuclease analysis of nuclear DNA from Austrian *Apodemus* species: Intraspecific variability in middle repetitive DNA families contrasts isoenzyme data

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Abstract

Studied middle repetitive DNA families in three Austrian *Apodemus* species (*Apodemus sylvaticus, Apodemus flavicollis* and *Apodemus microps*) using several type II restriction endonucleases and agarose gel electrophoresis. Three endonucleases, BstEII, EcoRI and HindIII revealed defined banding patterns due to specific repetitive DNA families. HindIII digested DNA samples from *Apodemus microps* differ from both *Apodemus sylvaticus* and *Apodemus flavicollis* DNA samples. Differences between the latter two species could be revealed with BstEII in only one of the populations under investigation. This finding indicates intraspecific variability even in the existance of middle repetitive DNAs in the latter species. This contrasts with the analysis of 16 isoenzyme loci, which exhibited only monomorphic patterns.

Introduction

The reconstruction and characterization of phylogenetic relationships is one of the most fascinating approaches of biology. The characterization of DNA by various means was found to be a reliable and helpful method in phylogenetic analysis. The large number of rodent species showing different degrees of divergence, together with high abundance, make them very useful for phylogenetic studies at the molecular level. For identification of sibling species, simple DNA analysis as presented in this paper may provide a powerful basis for finding new species characters and can help to elucidate the species state of the particular specimen.

The Austrian members of the Eurasian genus *Apodemus, Apodemus sylvaticus, Apodemus flavicollis* and *Apodemus microps,* belong to the most common small mammals in their area of distribution. Both similarity and high variability in their morphological characters have often led to a controversial discussion of their species state, possible introgression and natural hybridization (see Niethammer 1978a, b; Steiner 1978 for a review).

Chromosomal analysis (Engel et al. 1978; Csaikl 1980; Vujosevic et al. 1984) and isoenzyme studies (Engel et al. 1978; Csaikl et al. 1980, 1986; Gemmeke 1980, 1983) strengthen the picture of a very close phylogenetic relationship among these three species.

Studies on the nuclear DNA of these species are rather rare and mainly subject to molecular biology than to phylogenetic and systematic considerations (McLaren and Walker 1970; Cooke 1975; Brown and Dover 1979; Catzeflies et al. 1987). Furthermore, most of these studies are done with specimens of unknown origin or based on analysis of a single animal. This prompted us to study the nuclear DNA at the population level with various restriction endonucleases.

Table 1. Geographic area and external measurements of *Apodemus* species "Wiener Becken": 16° 20'–16° 25'/47° 53'–47° 56'; "Seewinkel": (16° 45'–17°/47° 45'–48°)*

	body			tail			hindfoot			ear		
	min	mean	max	min	mean	max	min	mean	max	min	mean	max
"Wiener Becken":												
A. s. (n=1)		83.0			73.0			21.0			14.3	
A. f. (n=7)	90.0	96.0	104.0	86.0	92.8	104.0	22.0	23.7	25.0	16.3	17.5	18.4
„Seewinkel":												
A. m. (n=9)	75.0	81.4	86.0	75.0	82.1	90.0	18.0	18.5	19.5	13.5	13.8	14.0
A. s. (n=5)	70.0	81.8	90.0	78.0	82.4	87.0	18.0	20.3	22.0	13.5	15.7	16.6
A. f. (n=3)	93.0	97.0	105.0	92.0	100.7	108.0	23.0	23.8	24.5	17.2	18.0	18.5

* Name and coordinates of the geographic area.
A. m.: *Apodemus microps*, *A. s.*: *Apodemus sylvaticus*, *A. f.*: *Apodemus flavicollis*. n-number of each species are given in brackets. Values are given in mm

Materials and methods

Animals

All animals used in this study were caught alive by help of wooden traps using peanut butter as bait. Most animals were kept alive in cages for at last six months with an offer of oats and apples ad libidum. Species determination was done by correlation of the length values of hind foot and ear. Values for external characters and geographical area are given in Table 1. Additionally, LDH isoenzymes were used to confirm the species-affiliation (Csaikl et al. 1980). All experiments were carried out with samples obtained from a single specimen.

Isoenzymes

The enzymes LDH (loci A and B, EC 1.1.1.27), NAD-MDH (loci s and m, EC 1.1.1.37), NADP-MDH (locus s, EC 1.1.1.40), 6-PGD (locus A, EC 1.1.1.42), G-6-PD (locus A, EC 1.1.1.49), SOD (locus A, EC 1.15.1.1), AAT (loci s and m, EC 2.6.1.1), CPK (loci A and B, EC 2.7.3.2), AK (locus A, EC 2.7.4.3), ACY-1 (locus A, EC 3.5.1.14), ADA (locus A, EC 3.5.4.4), and PGI (locus A, EC 5.3.1.9) were studied in homogenized muscle samples as described previously (Csaikl et al. 1980, 1986).

Nuclear DNA extraction

Livers of freshly killed individual mice were minced into small pieces and homogenized with a Potter-Elvenhjem-homogenizer using icecold MSB-CA^{++} (Lansman et al. 1981). To avoid contamination with mitochondria, the crude nuclear pellet was washed three-times with MSB-EDTA (Lansman et al. 1981) and recollected by centrifugation at 700xg (5 min, 4 °C). This pellet was used for the actual DNA extraction according to Flamm et al. (1966). Quality and amount of DNA was checked both with a photometer and with agarose gel electrophoresis (Maniatis et al. 1982).

Restriction endonuclease analysis

All endonucleases used (AluI, BamHI, BstEII, EcoRI, HaeIII, HindIII, HpaII and MspI) were obtained from Bethesda Research Laboratories (BRL), Eggstein, FRG. Digestions were carried out using 40 µl reaction mixtures under conditions recommended by the vendor. A HindIII-digest of phage lambda DNA was used as molecular weight marker in every gel. Restricted DNA samples were electrophoresed on agarose (Seakem Co.) in the presence of ethidium bromide (Maniatis et al. 1982). The gels were photographed under UV-light with a Kodak Technikal Pan 2415 using a standard Leica/Elmar 65 Photographic equipment.

Results and discussion

With the discovery and purification of a number of type II restriction endonucleases, an easy and reliable method for coarse comparisons of nuclear DNA from species of interest is available. These enzymes cleave duplex DNA at a specific recognition site, which is typically four or six nucleotides of length. With the help of agarose gel electrophoresis it is possible to compare samples from different species but restricted with the same restriction endonuclease. DNA sequences existing to a higher than average degree with equal length boardered by the same recognition sequence, can be identified with the appropriate restriction endonuclease. They are revealed as a distinct band upon a smear of different sized DNA fragments in an ethidium bromide stained agarose gel (Fig. 1). Those bands are calculated to contain 20,000 copies per genome and are called middle repetitive DNA (Brown and Dover 1981). Therefore restriction endonuclease comparisons can give valuable information on repeated sequences, which seem to be a general feature of the eukaryote genome (Britten and Kohne 1968).

Nuclear DNA from 25 individual mice of the three *Apodemus* species, *Apodemus sylvaticus, Apodemus flavicollis* and *Apodemus microps* was digested with a panel of eigth restriction endonucleases: AluI, BamHI, BstEII, EcoRI, HaeIII, HindIII, HpaII and MspI. Different restriction endonuclease concentrations revealed the same DNA banding pattern only varying in the intensity of the bands. AluI and HaeIII produced fragment classes of lower molecular weight, which are difficult to resolve and to interprete under standard conditions. BstEII, EcoRI and HindIII produced reproducible predominant bands. No middle repetitive DNAs could be detected with the other restriction endonucleases used. A representation of the different digestions are shown in Fig. 1 and the results are summarized in Table 2. EcoRI digestions gave the same digestion pattern in all three species with bands of 1.8 kb, 1.3 kb, 0.5 kb and 0.4 kb. HindIII digestions revealed three bands of 1.2 kb, 0.75 kb and 0.40 kb in *Apodemus sylvaticus* and *Apodemus flavicollis* but only two (1.2 kb and 0.40 kb) appeared in *Apodemus microps*. Interestingly, BstEII revealed a weak DNA band of 2.4 kb only in the three individuals from the „Seewinkel"-population of *Apodemus flavicollis*. In specimens caught in the „Wiener Becken", this 2.4 kb band was absent. Additionally a comparison with published results reveals differences

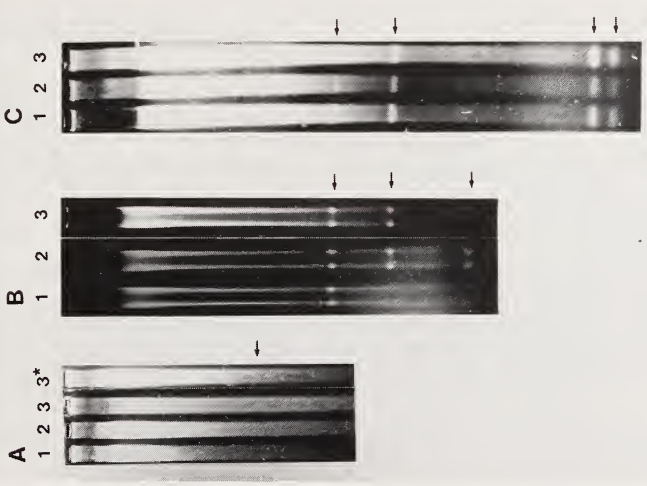

Fig. 1. Middle repetitive DNA in *Apodemus* species: Nuclear DNA was digested using A: BstEII; B: HindIII and C: EcoRI. Lanes 1: *Apodemus microps;* lanes 2: *Apodemus sylvaticus;* lanes 3: *Apodemus flavicollis* population I and II (II indicated by star)

in HindIII digested DNA of *Apodemus microps* (Cooke 1975). While in *Apodemus microps* samples from Czechoslovakia, the same three bands as in *Apodemus sylvaticus* and *Apodemus flavicollis* exist, whereas in our material from the „Seewinkel", the 0.75 kb band is absent. This points to a geographical variability in the existance of middle repetitive DNA families. To our knowledge this is the first description of diversity in the appearance of middle repetitive DNAs in different populations of a species. Analysis of mitochondrial DNA from the same *Apodemus* populations in Eastern Austria revealed also a high degree of intraspecific variability (Csaikl et al. in prep.). Additional support for this findings comes from cytogenetic results. With C-banding techniques several authors (Engel et al. 1978; Vujosevic et al. 1984; Habenicht 1978) could detect variability in the heterochromatin of *Apodemus flavicollis* chromosomes from different geographic populations. In contradiction to cytogenetic, mitochondrial and nuclear DNA data, isoenzyme analysis of this species from us and others (Csaikl et al. 1980; Gemmeke et al. 1987) showed no geographic variation. The analysis of 16 different enzymatic loci revealed no intraspecific variability in the specimes studied for middle repetitive DNA. All samples were homozygous for the same „frequent" allele described previously (Csaikl et al. 1980, 1986).

We consider our results on middle repetitive DNA presented in this paper to be a useful tool to distinguish between *Apodemus microps* on one hand and *Apodemus sylvaticus* and *Adpodemus flavicollis* on the other in Eastern Austria. A separation of *Apodemus sylvaticus* vs. *Apodemus flavicollis* is only possible in the „Seewinkel"-population, thus indicating that intraspecific variability is reflected also in nuclear DNA families. Our results indicate also that this type of nuclear DNA families are subjected to intraspecific variation.

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Zusammenfassung

Restriktionsendonuclease-Vergleich von Kern-DNA in Österreich vorkommender Apodemus Arten: Nachweis von intraspezifischer Variabilität in mittelrepetitiven DNA-Familien bei fehlender intraspezifischer Isoenzymvariabilität

Untersuchungen über mittelrepetitive DNA Familien der drei in Österreich vorkommenden *Apodemus* Arten, *Apodemus sylvaticus, Apodemus flavicollis* und *Apodemus microps* werden vorgestellt.

Table 2. **Middle repetitive DNA classes of *Mus musculus (M. m.), Apodemus sylvaticus (A. s.), Apodemus flavicollis (A. f.)* and *Apodemus microps (A. m.)* appearing after digestions with some restriction endonucleases (R. E.)**

species / R.E.	*M. m.*	*A. s.*	*A. f.* I	*A. f.* II	*A. m.*
EcoRI	–	1.80 kb	1.80 kb	1.80 kb	1.80 kb
	1.30 kb	1.30 kb	1.30 kb	1.30 kb	1.30 kb
	–	0.50 kb	0.50 kb	0.50 kb	0.50 kb
	–	0.40 kb	0.40 kb	0.40 kb	0.40 kb
HindIII	–	1.20 kb	1.20 kb	1.20 kb	1.20 kb
	–	0.75 kb	0.75 kb	0.75 kb	–
	–	0.40 kb	0.40 kb	0.40 kb	0.40 kb
BstEII	–	–	–	2.40 kb	–

Type *A. f.* I was found in the "Wiener Becken", type *A. f.* II in the "Seewinkel".

Unter Verwendung mehrerer Typ II Restriktionsendonucleasen konnten definierte Bandenmuster auf Grund spezifischer mittelrepetitiver DNA Familien festgestellt werden. HindIII verdaute DNA von *Apodemus microps*, unterscheidet sich von DNA der beiden anderen Arten *(Apodemus sylvaticus, Apodemus flavicollis)*. Ein Unterschied in BstEII verdauter DNA zwischen beiden letzteren Arten konnte nur in einer der untersuchten Populationen nachgewiesen werden. Dieses Ergebnis belegt die Existenz von intraspezifischer Variabilität von mittelrepetitiver DNA bei *Apodemus flavicollis*. Die Untersuchung von 16 Isoenzymloci ergab hingegen nur monomorphe Muster.

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