

## Restriction endonuclease digestion patterns of harvest mice (*Reithrodontomys*) chromosomes: a comparison to G-bands, C-bands, and *in situ* hybridization

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### Abstract

Constitutive heterochromatin of a karyotypically conserved species of harvest mouse was compared to that of three karyotypically derived species of harvest mice by examining banding patterns produced on metaphase chromosomes with three restriction endonucleases (*EcoRI*, *MboI* and *PstI*). Banding patterns produced by two of these restriction endonucleases (*EcoRI* and *MboI*) were compared to published G- and C-banded karyotypes and *in situ* hybridization of a satellite DNA repeat for these taxa. The third restriction endonuclease (*PstI*) did not produce a detectable pattern of digestion. For the most part, patterns produced by *EcoRI* and *MboI* can be related to C-banded chromosomes and *in situ* hybridization of satellite DNA sequences. Moreover, digestion with *EcoRI* reveals bands not apparent with these other techniques, suggesting that restriction endonuclease digestion of metaphase chromosomes may provide additional insight into the structure and organization of metaphase chromosomes. The patterns produced by restriction endonuclease digestion are compatible with the chromosomal evolution of these taxa, documenting that in the highly derived taxa not only are the chromosomes rearranged but the abundance of certain sequences is highly variable. However, technical variation and difficulty in producing consistent results even on a single slide with some restriction endonucleases documents the problems associated with this method.

### Introduction

Different chromosome banding procedures have revealed a longitudinal differentiation along the mammalian metaphase chromosome, and these procedures have allowed for considerable advances in mammalian cytogenetics (Hsu, 1973; Comings, 1978; Sumner, 1990). Although a precise understanding of what each chromosome band represents in terms of underlying DNA base composition and structure is not known for all cases, differential staining of metaphase chromosomes most likely relates to the underlying complexity known to exist in higher eukaryotic genomes (Britten & Kohne, 1968). One component of the mammalian chromosome that not only differentially stains but is also composed of specific combinations of nucleotide

sequences is constitutive heterochromatin (John, 1988). Treatment of mammalian chromosomes with alkaline or saline solutions reveals the location of constitutive heterochromatin in the form of C-bands (or C-positive material). These C-positive regions exhibit a wide range of distribution in mammals (centromeric, telomeric, interstitial, and whole arms) and in most cases these C-positive regions can be related directly to an underlying component of the genome known as satellite DNA (Singer, 1982; John, 1988). Satellite DNA sequences consist of tandem arrays of a monomer repeat that can vary in both size and complexity, and these sequences are common in most eukaryotic genomes (Singer, 1982).

One technique which has been used to examine longitudinal differentiation along mammalian chro-

mosomes with respect to genome complexity is the digestion of metaphase chromosomes with restriction endonucleases (Mezzanotte *et al.*, 1983; Lima-De-Faria *et al.*, 1980; Mezzanotte & Ferrucci, 1983, 1984; Bianchi *et al.*, 1985a, b; Babu, 1988; Zhang & Dong, 1989; Gosalvez *et al.*, 1989). This procedure reveals banding patterns which relate to the distribution of restriction endonuclease recognition sites along the nuclear DNA molecule (Miller, *et al.*, 1983; Bianchi *et al.*, 1985a, b). Although there is considerable debate as to why the distribution of digestion patterns is maintained in a species (structural domains or random distribution of sites), there is a definite correlation between digestion patterns and the distribution and chromosomal location of moderately and highly repetitive DNA sequences (Mezzanotte *et al.*, 1983, 1985; Miller *et al.*, 1983; Babu, 1986; Babu & Verma, 1986). Despite the fact that restriction endonucleases reveal consistent banding patterns in mammalian species, few comparative studies have examined the phylogenetic distribution of the patterns in closely related groups of mammals (for an exception see Bianchi *et al.*, 1985b).

The purpose of this study is to examine restriction endonuclease digestion patterns along metaphase chromosomes of four closely related species of harvest mice of the genus *Reithrodontomys* (*R. fulvescens*, *R. megalotis*, *R. montanus*, *R. sumichrasti*). This particular group of rodents was chosen for three reasons. First, these species have satellite DNA sequences with 350 base pair monomer repeats defined either by the restriction endonuclease *Pst*I (*R. fulvescens*) or *Eco*RI (*R. megalotis*, *R. montanus*, *R. sumichrasti*) (Hamilton *et al.*, 1990). Second, the chromosomal distribution of this satellite DNA is coincident with constitutive heterochromatin (Hamilton *et al.*, 1990). Third, species in this genus demonstrate considerable chromosome variation with respect to diploid number, fundamental number, and the distribution of constitutive heterochromatin with some species having a highly derived karyotype (Hood *et al.*, 1984).

## Materials and methods

**Chromosome preparations.** Cytological preparations followed the yeast stress, *in vivo* culture

method of Lee and Elder (1980). Chromosome preparations on microslides were prepared by air drying.

**Restriction endonuclease digestion.** The enzymes used for digestion were *Eco*RI, *Mbo*I, and *Pst*I (Bethesda Research laboratories, New England Biolabs, Sigma Chemical Co.). *Eco*RI was chosen because it produces an approximately 350 base pair satellite DNA repeat in three *Reithrodontomys* taxa (*montanus*, *megalotis*, and *sumichrasti*), for which the genome organization and chromosomal distribution has been well characterized by Southern blot analysis and *in situ* hybridization (Hamilton *et al.*, 1990). *Pst*I was chosen because it produces a similar 350 bp repeat in *R. fulvescens* (Hamilton *et al.*, 1990). Previous investigators have shown that *Mbo*I produces C-band-like patterns on metaphase chromosomes in some organisms (Babu, 1988; Gosalvez *et al.*, 1989). Therefore, this enzyme was chosen to compare the pattern produced by *Mbo*I with those produced by conventional C-banding techniques. The technique for digestion of metaphase cells with restriction endonucleases followed Lima-De-Faria (1980) as modified below. Enzymes were dissolved in an assay buffer specified by the supplier. Thirty microliters ( $\mu$ l) of the reaction mixture (3  $\mu$ l of specified buffer, 6  $\mu$ l of enzyme, and 21  $\mu$ l of ddH<sub>2</sub>O) were placed onto each slide, covered with a coverslip, sealed with rubber cement, and placed into a moist chamber and incubated at 37 °C for five hours. Initially, results of digestion times ranging from 2 to 24 h were compared. Because five hours gave similar results to 24 h we used five hours for all subsequent digestions. After incubation, the rubber cement was removed and coverslips were floated from the slide in a working solution of GKN (Lima-De-Faria *et al.*, 1980) to minimize scratching the preparations. Chromosomes were then stained with Giemsa (in phosphate buffer, pH 7.0) for seven minutes. Banding patterns were determined by examining 8-10 digested karyotypes per species.

It has been noted that some banding patterns produced by this technique may be due to the buffer conditions and not the restriction endonuclease (Bianchi *et al.*, 1985a). Therefore, control slides treated without the restriction endonuclease were incubated together with the experimental slides.

*Specimens examined.* TK numbers cross reference laboratory records and tissues to voucher specimens deposited in The Museum, Texas Tech University. *Reithrodontomys fulvescens*: TK22485 M; Oklahoma, Potowattomie co., 5.9 mi E 2.5 mi N Tecumseh; *R. fulvescens*: TK20599 F; Oklahoma, Hughes co., 4.5 mi E Wetumka; *R. sumichrasti*: TK20993 M; TK20995 F; Mexico, Oaxaca 3 mi by road Suchixtepx; *R. megalotis*: TK20996 M; Texas, Lubbock co., 1 mi N Loop 289 1 mi W University; *R. montanus*: TK22486 F; Texas, Wichita co., 1 mi N Lake Wichita.

## Results and discussion

Digestion with *EcoRI* and *MboI* produced repeatable banding patterns on chromosomal regions whereas *PstI* did not produce detectable banding patterns. The *PstI* experiments were repeated numerous times with a variety of conditions and none produced banding. We do not have an explanation for this result.

Banding patterns produced by *in situ* digestion of chromosomes with *EcoRI* and *MboI* for the species examined are presented in Figures 1-4. Light staining portions of the chromosomes represent regions where the DNA has been cut by the restriction endonuclease and the DNA has been removed. The dark staining regions are areas of the chromosomal DNA which either have not been digested with the restriction endonuclease or represent areas in which the recognition sequences for the restriction endonuclease are far enough apart that the DNA cannot be removed from the chromosome. Table 1 compares the results of *MboI* digestion of metaphase chromosomes to the results obtained with C-banding and *in situ* hybridization of a satellite DNA probe. The overall restriction digestion patterns produced with *EcoRI* are not as clear in that this enzyme not only removes large blocks of DNA on some chromosomes, particularly in *R. montanus* and *R. megalotis* (Figs. 2 and 3), but also produces a G-banding-like pattern on other chromosomes (*R. fulvescens*, Fig. 1). Examination of the digestion patterns produced by *EcoRI* and *MboI* for *R. montanus*, and *R. megalotis* (Figs. 2 and 3) show that in general, the areas of the chromosomes digested away by *EcoRI* (light areas) appear as dark bands following digestion with *MboI*. Additionally, the

**Table 1.** Chromosomal distribution of C-band heterochromatin<sup>1</sup>, satellite DNA<sup>1</sup>, and *MboI* restriction site patterns for four taxa of *Reithrodontomys*. *MboI*-undigested refers to large blocks of DNA stained dark after digestion with *MboI* indicating that the DNA in this region remained on the chromosomes. All numbers listed indicate the number of pairs (unless stated otherwise) for each condition. The terms telomeric and centromeric indicate the presence of heterochromatin, hybridization, or undigested DNA near or at these structures. Other abbreviations are: LA = long arm; SA = short arm; X = X-chromosome; Y = Y-chromosome; and '-' refers to no darkly staining blocks of DNA in that region of the chromosomes.

Taxon	2n	C-band heterochromatin distribution						Highly repeated DNA distribution						Undigested blocks with <i>MboI</i>						
		Centromeric	Telomeric	Interstitial	Long arm block	Short arm block	Entire	Centromeric	Telomeric	Interstitial	Long arm block	Short arm block	Entire	Centromeric	Telomeric	Interstitial	Long arm block	Short arm block	Entire	
<i>R. fulvescens</i>	25	25	X	-	-	-	Y	25	-	-	-	-	-	-	21	5	-	-	-	X
<i>R. megalotis</i>	21-22	21	2(SA)	1(LA)	9 + X	9 + X	Y	21	2(SA)	1 + single	1 + single	8 + single	-	21	4	-	X	9	Y	
<i>R. montanus</i>	19	19	-	-	7	7	Y	19	-	-	-	6 + X + single	-	19	-	-	3 + X	7	-	
<i>R. sumichrasti</i>	20	20	2	-	10	10	Y	20	2	8 + single	-	10	-	20	2	-	X	10	Y	

<sup>1</sup> Hamilton et al. (1990)

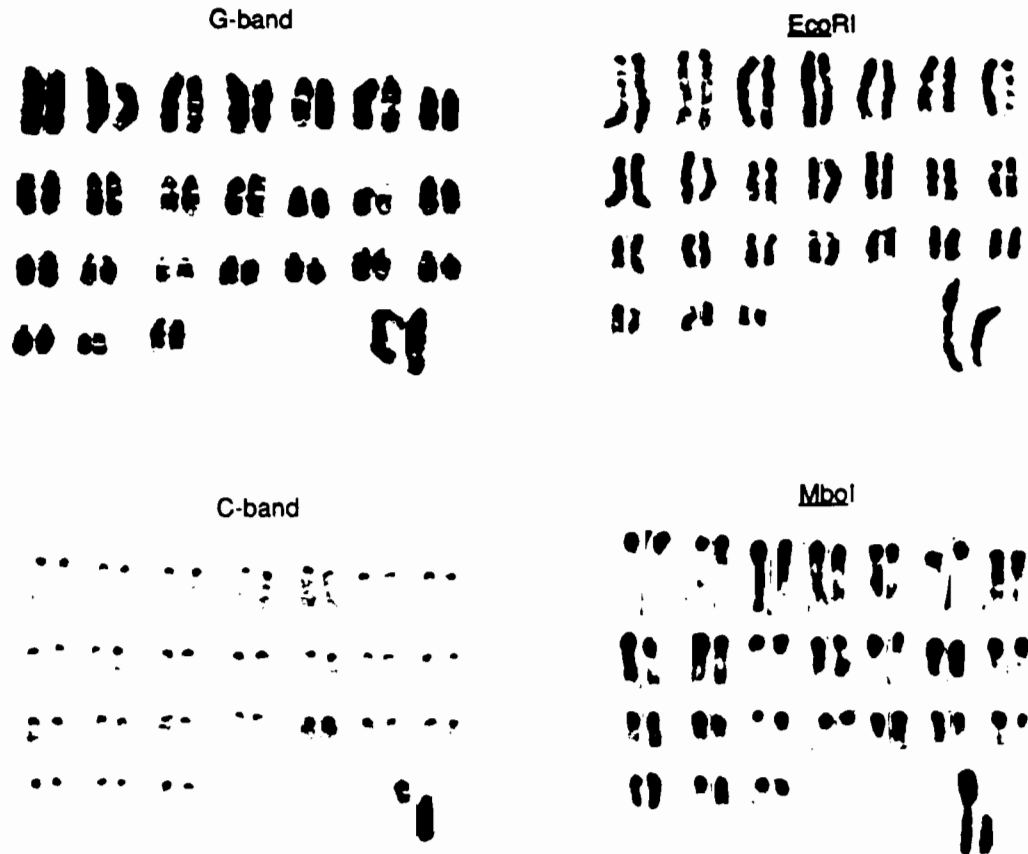


Fig. 1. Comparison of banding patterns produced in *Reithrodontomys fulvescens* by G-banding, *EcoRI*, C-banding, and *MboI*. G- and C-banded preparations are from published karyotypes (Robbins & Baker, 1980).

patterns of restriction endonuclease digestion with *MboI* for all taxa can be related for the most part to C-banded chromosomes. For instance, *R. fulvescens* has heterochromatin localized in the centromeric regions of all chromosomes plus the telomeric region of the X chromosome, and most of the Y chromosome. *MboI* digestion of chromosomes from *R. fulvescens* produces a similar pattern, with most centromeric regions staining darker than that seen from conventional C-banding (Fig. 1). The only major difference is the extent of digestion seen on the Y chromosome and the large staining block seen on the short arm of the X chromosome. *MboI* digestions in the remaining three species are also similar to C-banding patterns with both centromeric and heterochromatic short arms staining dark

following digestion with *MboI* (Figs. 2-4).

*EcoRI* digestion of chromosomes from *R. montanus*, *R. megalotis* and *R. sumichrasti* (Figs. 2-4) gave essentially the same results as those revealed by *MboI* digestions. The primary differences in banding patterns produced by these two enzymes for all four taxa is that *EcoRI* produced some G-band like patterns in *R. fulvescens* and *R. sumichrasti* and also removed the large blocks of DNA that stained darkly after *MboI* digestion in *R. montanus* and *R. megalotis*. The pattern of *EcoRI* digestion seen in *R. fulvescens* is similar to G-banding in that there are differentially dark and light staining regions dispersed throughout most chromosomes, with dark staining regions in the C-band positive regions (Fig. 1). The patterns seen in *R. megalotis*,

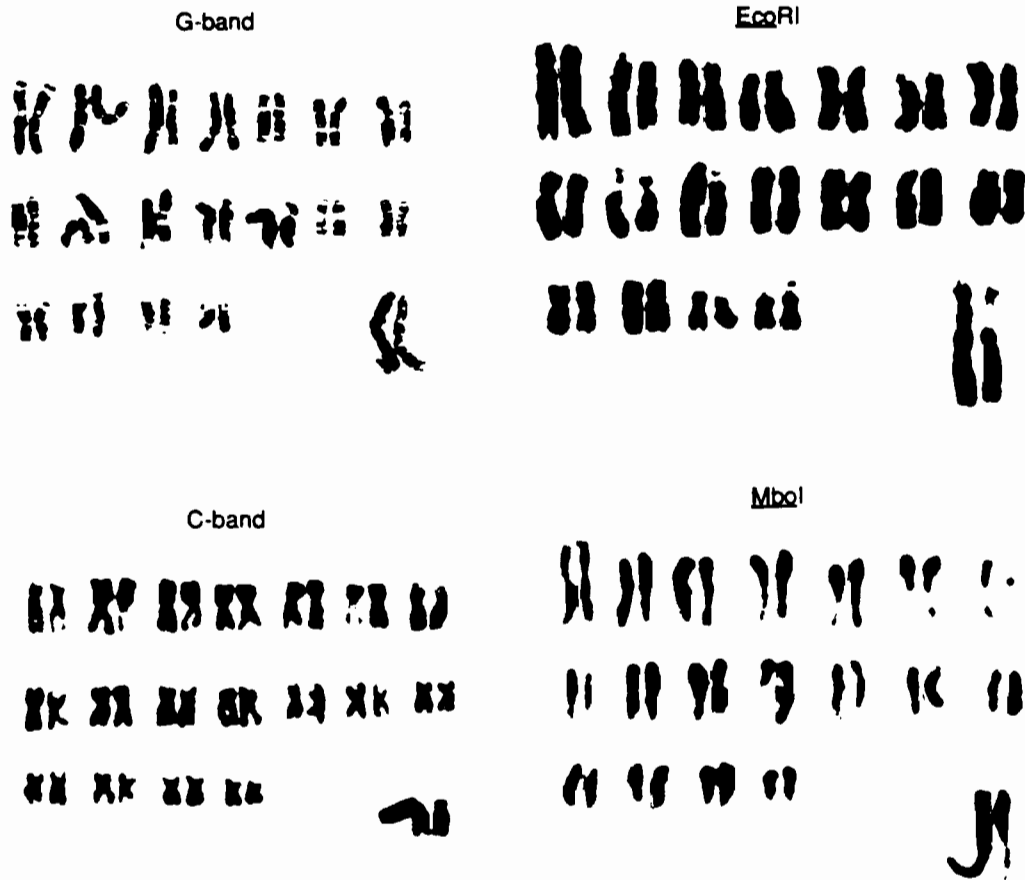


Fig. 2. Comparison of banding patterns produced on fixed metaphase chromosomes of *Reithrodontomys montanus* by G- and C-banding (Robbins and Baker, 1980) with *EcoRI* and *MboI* restriction endonuclease digestion. Chromosome pairs 7, 10, 17, and 18 are shown upside down because they were originally mounted upside down in the C-banded preparation of Robbins and Baker, 1980.

*R. montanus*, and *R. sumichrasti* appear quite different from those seen in *R. fulvescens*. Although G-band-like regions appear on some chromosomes, the digestion patterns seen in *R. montanus* and *R. megalotis* coincide with the removal of C-positive regions corresponding to heterochromatin. In the case of *R. montanus*, most regions corresponding to short arm heterochromatin were removed by restriction endonuclease digestion on both the autosomes and the X chromosome. In *R. megalotis*, five pairs of chromosomes have lightly staining areas on the short arms, and four pairs have light interstitial bands (Fig. 3). Similar results can also be seen in *R. sumichrasti*, with interstitial and telomeric blocks of heterochromatin removed as well as the production of lightly staining whole chromo-

somes that are known to be C-band positive (Fig. 4).

Do patterns of restriction endonuclease digestions of metaphase chromosomes reveal information pertaining to the underlying structure of chromosomes? Miller *et al.* (1983) and Bianchi *et al.* (1985a) have suggested that chromosome structure *per se* plays no role in the action of restriction endonuclease banding patterns, with DNA from all regions being equally accessible to restriction endonuclease digestion. Others, however, suggest that differential organization of chromosomes may influence the activity of restriction endonuclease digestion (Lica & Hamkalo, 1983; Mezzanotte *et al.*, 1985; Burkholder, 1989; Gosálvez *et al.*, 1989). If DNA sequence is the sole factor governing band-

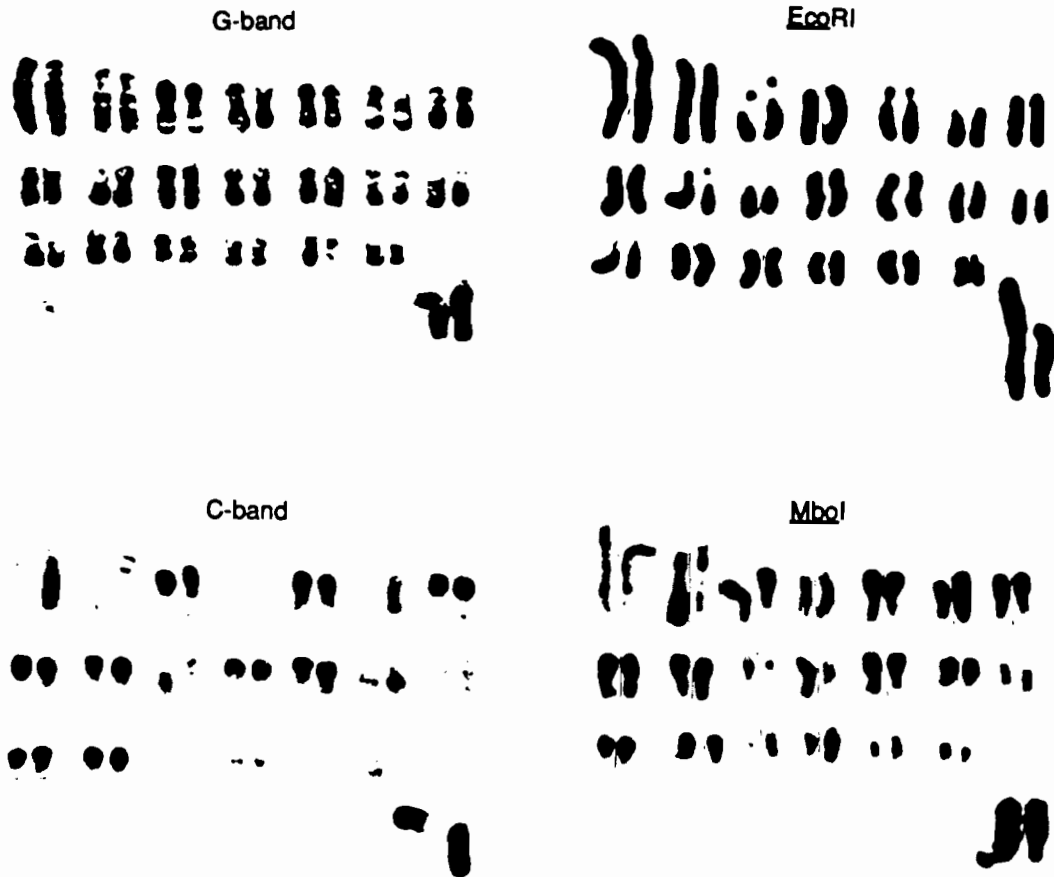


Fig. 3. Banding patterns on fixed metaphase chromosomes of *Reithrodontomys megalotis* produced by G- and C-banding (Robbins and Baker, 1980) compared with patterns produced by the restriction endonucleases *EcoRI* and *MboI*.

ing patterns produced by restriction endonuclease digestion, then a restriction endonuclease like *MboI* which recognizes four bases will have a higher probability of digesting larger blocks of chromosomal DNA as opposed to an enzyme like *EcoRI* which recognizes six bases. On the other hand, if differential organization of chromosomes influences the activity of restriction endonuclease digestion, then it might be expected that euchromatin would be more accessible to digestion of large blocks of DNA than heterochromatin because the latter is more tightly coiled and presumably has an under-representation of restriction endonuclease sites as a result of reduced sequence complexity.

Results of this study are interpreted as suggesting that both the distribution of recognition sites for

a restriction endonuclease and the differential organization of the chromosomes may be factors effecting restriction endonuclease patterns seen in these taxa. The G-banded patterns produced by *EcoRI* are compatible with the view that regions have an equal probability of being digested with enzymes, with the extent of digestion being limited by the random distribution of recognition sites in the genome and the distance between sites. This may be why one sees G-band-like patterns with *EcoRI* and the elimination of entire regions with *MboI*. Nevertheless, the *EcoRI* and *MboI* patterns of digestion cannot be explained entirely by the random distribution of sites. Hamilton *et al.* (1990) reported a correspondence between the distribution of constitutive heterochromatin and satellite DNA

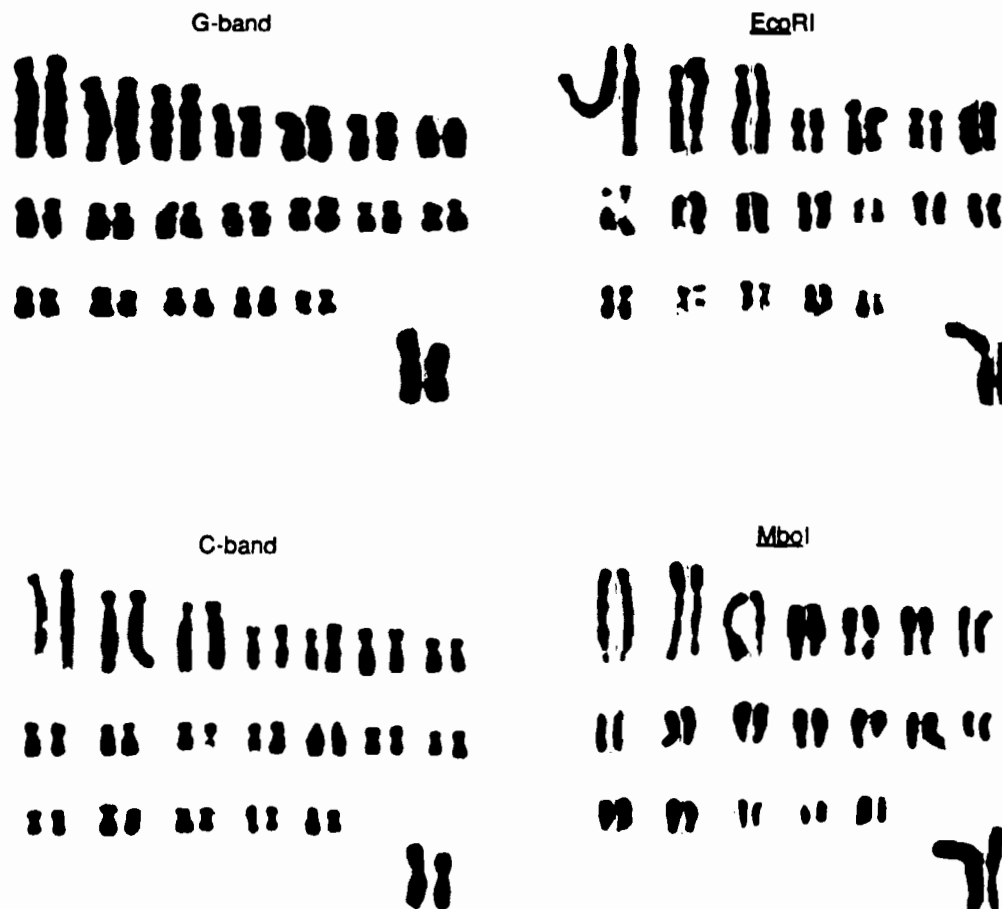


Fig. 4. G- and C-banded karyotypes of *Reithrodontomys sumichrasti* (Hood *et al.*, 1984) compared to the banding patterns produced by the restriction endonucleases *EcoRI* and *MboI*.

sequences within the chromosomes of *Reithrodontomys* (Table 1). In the case of *R. fulvescens*, the satellite sequence was found to be located centromerically by *in situ* hybridization with both the monomer repeat isolated from *R. fulvescens* (defined by *PstI*) and the repeat isolated from the other three taxa (defined by *EcoRI*) under study (Hamilton *et al.*, 1990). This indicates that although these repeats are defined by two different restriction endonucleases in these taxa, the DNA sequences have a high degree of sequence identity. *Reithrodontomys megalotis*, *R. montanus*, and *R. sumichrasti*, on the other hand, have satellite sequences distributed in centromeric regions, chromosome short arms, interstitial regions, and throughout entire chromosomes (Table 1). In all three species, the monomer

repeat of the satellite sequence is approximately 350 base pairs long and defined by *EcoRI*. Therefore, it is not coincidental that *EcoRI* removes most constitutive heterochromatin from the chromosomes of these three species, whereas it demonstrates a more random distribution in *R. fulvescens*. However, the observation that *EcoRI* (a six base cutter) is removing large blocks of heterochromatic material from *R. megalotis*, *R. montanus*, and *R. sumichrasti* does not reflect the suggestion that chromosome structure affects restriction endonuclease digestion of metaphase chromosomes. It is probable that a deficiency of *MboI* sites in the tandemly repeated satellite DNA sequences accounts for the fact that *MboI* digestion produces patterns similar to those seen with C-banding.

Restriction endonuclease digestions of chromosomes can also reveal patterns not seen with conventional G- and C-banding techniques. For instance, Miller *et al.* (1983) have shown that reduced staining on the short arm of human chromosome 14 by *MspI* was indicative of this chromosomal region containing the 18S and 28S rDNA genes. In the case of all species of *Reithrodontomys* examined, unique patterns were revealed with restriction endonucleases, and many of these patterns demonstrated considerably more chromosomal differentiation than that revealed by conventional staining procedures. Additionally, although *in situ* hybridization gives the appearance that the blocks of heterochromatin are homogeneous (Hamilton *et al.*, 1990), restriction endonuclease digestion of these areas produces a repeatable banding pattern in heterochromatic blocks that we interpret as indicating some divergence within the subunits of these major blocks. These may represent divergent regions in which *EcoRI* sites are missing, producing dimers, trimers, etc., too large to be removed with restriction endonucleases because of chromosomal proteins associated with the DNA. Restriction endonuclease digestion also provides additional resolution concerning the X and Y chromosomes that was not detected by either *in situ* hybridization or conventional staining techniques. The Y chromosome in *R. fulvescens* is entirely heterochromatic using C-banding techniques. *In situ* hybridization produces a fluorescent signal from only a small portion of the Y (Hamilton *et al.*, 1990). *MboI* completely digests the entire Y of *R. fulvescens* although this same restriction endonuclease does not digest autosomal heterochromatin (Table 1). Additionally, both the X and Y chromosomes revealed unique patterns upon digestion with either *EcoRI* or *MboI*. Taken together, these data are interpreted as indicating that in *R. fulvescens* the heterochromatic repeats on the sex chromosomes are not identical to each other and that the heterochromatic repeats on the sex chromosomes have a different evolutionary history than those found near the autosomal centromere.

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