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Conservation of Repetitive DNA Sequences in Deer Species Studied by Southern Blot Transfer

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Summary. The Cervidae show one of the largest variations in chromosome number found within a mammalian family. The five species of the deer family which are the subject of this study vary in chromosome number from 2n = 70 to 2n = 6. Digestion with the restriction enzymes EcoRI, HpaII, HaeIII and MspI reveals that there is a series of highly repetitive sequences forming similar band patterns in the different species. To obtain information on the degree of homology among these conserved sequences we isolated a HpaII restriction fragment of approximately 990 base pairs from reindeer DNA. This DNA sequence was ^{32}P-labelled and hybridized by the Southern blot technique to DNAs cleaved with HpaII and HaeIII from the reindeer and four other Cervidae species. Hybridization to specific restriction fragments was recorded in all species. The patterns of hybridization showed a higher degree of similarity between reindeer, elk and roe deer than between reindeer and the Asiatic species (fallow deer and muntjac). Homologies are still present between the highly repetitive sequences of the five species despite the drastic reorganization that led to a change in chromosome number from 6 to 70.

Key words: Chromosome evolution — Restriction enzymes — Nick translation — DNA blot transfer — Deer species — Repetitive DNA

Introduction

Repeated DNA sequences have provided a valuable tool for disclosing evolutionary relationships between chromosomes and between species. The distribution of such sequences has been found to follow rules in both animal and plant chromosomes (Peacock et al. 1977). The comparison of the organizations of the DNA satellites in ten species of the *Drosophila virilis* group has helped to elucidate the evolution of this genus (Gall and Atherton 1974), and the study of the evolution of repeated sequences is helping to clarify chromosome organization (Musich et al. 1978; Pech et al. 1979; Stephenson et al. 1981).

A central problem in chromosome organization and evolution is the elucidation of the mechanisms that allow a drastic change in chromosome number and still result in the formation of very similar species. The ordered molecular processes that result in the strong dispersion or regrouping of similar sets of genes and at the same time permit them to work in a coherent and organized fashion are not known.

The Cervidae are a unique family among the mammals. They exhibit the largest variation in chromosome number described so far. The species selected for the present investigation were: *Rangifer tarandus* (reindeer, 2n = 70), *Alces alces* (elk, 2n = 70), *Capreolus capreolus* (roe deer, 2n = 70), *Dama dama* (fallow deer, 2n = 68) and *Muntiacus muntjak* (Indian muntjac, 2n = 6, female). The group contains both species with similar karyotypes and others with sharply deviating chromosome numbers, yet the animals are so similar that they belong to the same family.

We report here on the cleavage of nuclear DNA obtained from these species. The restriction enzymes EcoRI, HpaII, HaeIII and MspI were used. Following agarose gel electrophoresis, the fragments of highly repetitive DNA form similar banding patterns among the five species. To obtain further in-

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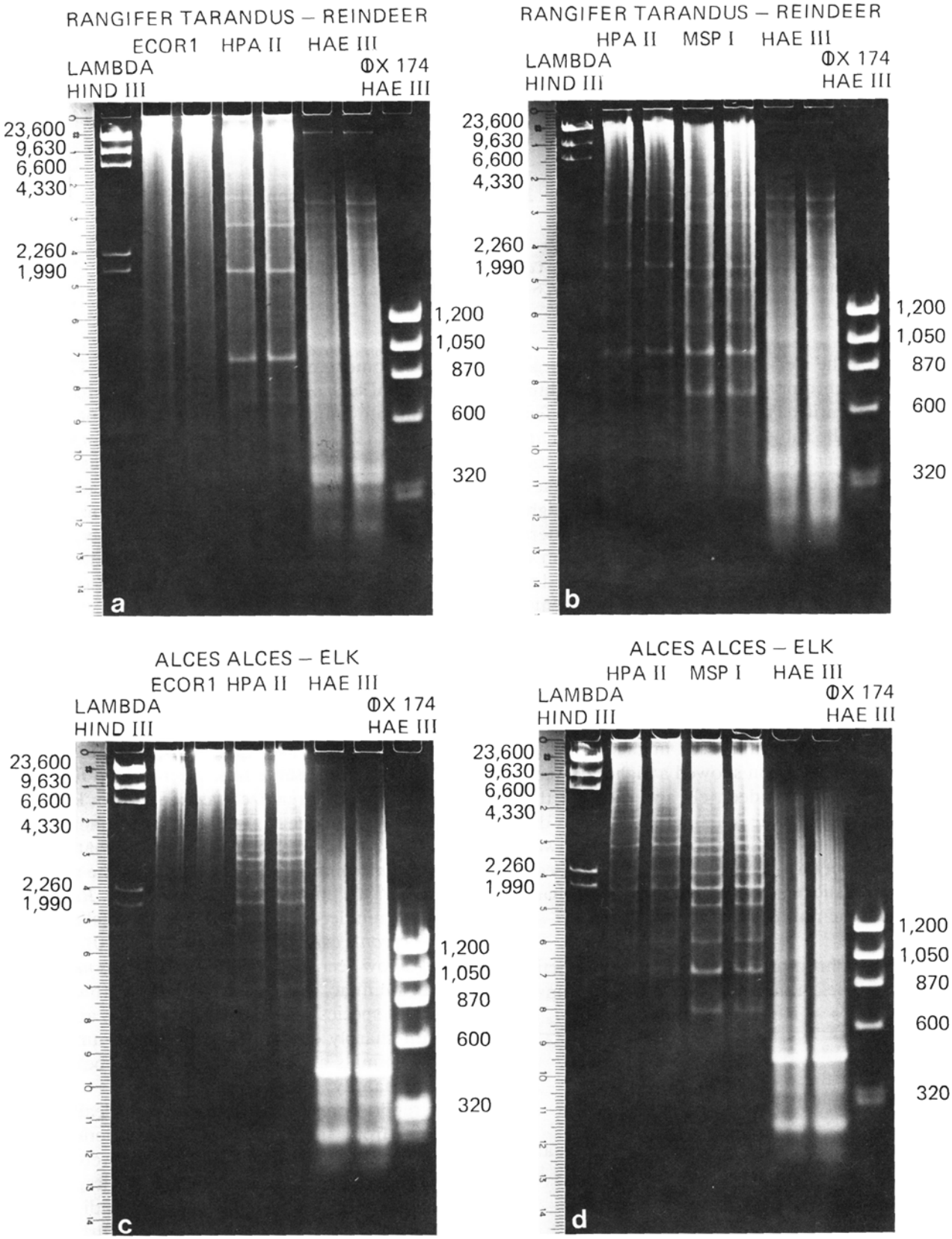
RANGIFER TARANDUS – REINDEER
ECOR1 HPA II HAE III
LAMBDA HIND III
ΦX 174 HAE III
23,600
9,630
6,600
4,330
2,260
1,990
1,200
1,050
870
600
320
a
RANGIFER TARANDUS – REINDEER
HPA II MSP I HAE III
LAMBDA HIND III
ΦX 174 HAE III
23,600
9,630
6,600
4,330
2,260
1,990
1,200
1,050
870
600
320
b
ALCES ALCES – ELK
ECOR1 HPA II HAE III
LAMBDA HIND III
ΦX 174 HAE III
23,600
9,630
6,600
4,330
2,260
1,990
1,200
1,050
870
600
320
c
ALCES ALCES – ELK
HPA II MSP I HAE III
LAMBDA HIND III
ΦX 174 HAE III
23,600
9,630
6,600
4,330
2,260
1,990
1,200
1,050
870
600
320
d

formation on the degree of homology of these sequences, we carried out DNA–DNA hybridization between an isolated DNA sequence from reindeer and the nuclear DNAs of the five species.

Materials and Methods

DNA Preparation and Electrophoresis in Slab Gels. DNA was prepared by different procedures involving nuclear extraction as in Lima-de-Faria et al. (1975) and Yasmineh and Yunis (1970). The DNA was of a molecular weight exceeding 30×10^6 daltons. Varying amounts of DNA (0.5–9 μg) were used in different experiments to assess the effect of the enzyme. Different amounts of enzyme were also employed (1.5–7.0 units). In every experiment each DNA was run in duplicate on the gels to check whether the banding pattern observed was reproducible. Four molecular-weight markers were employed: (1) phage lambda cleaved by EcoRI, (2) lambda *rif* cleaved by EcoRI, (3) lambda cleaved by HindIII, and (4) ΦX174 cleaved by HaeIII. Together they furnish fragments ranging in length from 23.6×10^3 to 73 nucleotide pairs. The enzyme activity was checked by cleaving lambda DNA with the enzymes used for cleaving total nuclear DNA. Repeated freezing and thawing of the enzymes and DNAs was avoided. Gelatin or bovine serum albumin was added to stabilize the enzymes and enhance their activity. All glassware was sterilized by autoclave (Lima-de-Faria et al. 1980). The agarose powder and the electrophoresis buffer were autoclaved for 20 min at 1 atm. The concentration of the agarose was 1.4%. Ethidium bromide (0.5 μg/ml) was added to the gel. The electrophoresis buffer for slab gels was 0.05 M Tris base, 0.02 M sodium acetate ($3H_2O$), 0.002 M EDTA, 0.018 M NaCl, pH 8.05, to which ethidium bromide was added to a final concentration of 0.5 μg/ml. The gels were run at 18 mA for 17 h. Each enzyme was diluted in its specific reaction mixture together with the DNA, and the mixtures were incubated for 2 h at 37°C. The reaction was stopped by putting the incubation tubes at 65°C for 5 min, and then on ice for 5 min. Then 10 μl 0.1 M EDTA was added. Sucrose (10 mg) and 5 μl bromophenol blue at a concentration of 5 mg/ml were also added. The enzymes were obtained from New England Biolabs (USA) and Bethesda Research Laboratories (USA).

Nick Translation and Southern Blot Transfer. The horizontal gel electrophoresis systems H3 and H0 and the blot transfer system from Bethesda Research Laboratories were employed. Deoxyadenosine 5′-[α^{32}P]triphosphate, triethylammonium salt, specific activity approximately 3000 Ci/mmol, was used together with the solutions from the nick translation kit from the Radiochemical Center (Amersham, England) (Maniatis et al. 1975; Rigby et al. 1977; Jeffreys and Flavell 1977). The specific activity of the reindeer DNA labelled by nick translation was 0.5×10^8 cpm/μg. The DNA blot transfer was carried out according to the method of Southern (1975). Prehybridization took place in 10× Denhart solution in 3× SSC and unlabelled sea trout DNA was added to reduce background. After both DNAs were denatured, the hybridization was carried out at 64°C in 1× Denhart solution in 3× SSC for 3 h, followed by washing of the filter in 2× SSC (3 times) at 64°C. Kodak X-Ray film X-omat RP was exposed for 16 h.

Results

The nuclear DNA of each of the five species was cleaved with the restriction enzymes EcoRI (recognition site, 5′-GAATTC-3′), HpaII (5′-^{m}CCGG-3′), HaeIII (5′-GGCC-3′) and MspI (5′-C^{m}CGG-3′). After incubation with the enzymes, the DNA was submitted to electrophoresis in agarose gels (Figs. 1–3). Highly repetitive sequences form bands in the gels. Following EcoRI digestion, few bands appeared in the DNA of these species except in the Indian muntjac. However, the cleavages with HpaII and HaeIII produced many bands in all DNAs. Moreover, some of the bands seen after HpaII appear well separated from the main bulk of the DNA fragments. This is a feature that allows them to be isolated in relatively pure form.

The cleavage with HpaII produced the following patterns (Figs. 1 and 2). The sequences that form the reindeer bands have lengths of 990, 1920, 2850, 3850, 4600 and 5690 base pairs (bp). These segments are two to six times the length of the 990-bp segment. The elk DNA showed a large number of bands after HpaII digestion (Fig. 1c and d). One series of bands corresponded to the reindeer series just mentioned with respect to fragment size. Moreover, in this species there are two other series of bands starting with fragments of 1155 and 1716 bp and shared with the MspI, and partly with the HpaII, digestion of reindeer DNA. Roe deer DNA has two of the bands present in the reindeer and elk, namely those of 1920 and 3850 bp (Figs. 2a and b and 3). There are other bands present that have no

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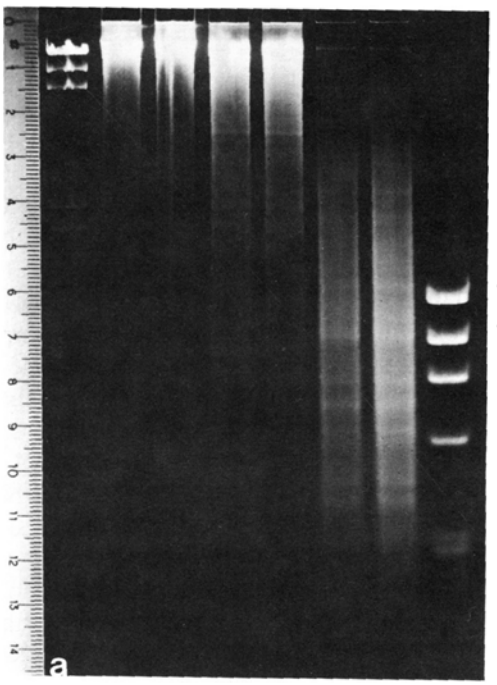
Fig. 1. Nuclear DNAs of *Rangifer tarandus* (reindeer) and *Alces alces* (elk) treated with the restriction enzymes EcoRI, HpaII, HaeIII and MspI. After digestion, the DNAs were electrophoresed in agarose gels. Two wells were used for each enzyme to show that the pattern of cleavage was reproducible. Lambda DNA cleaved with HindIII was used as a molecular weight marker (left side, only one well). On the right side can be seen ΦX174 DNA cleaved with HaeIII (one well), also used as a marker. The molecular weight values are in base pairs (bp). HpaII and HaeIII cleave CCGG and GGCC, respectively, but they produce quite different general patterns. HpaII forms a gradient of mainly large sequences in the range of 23.0×10^3 to 600 bp, whereas HaeIII is characterized by mainly small segments in the range 4.0×10^3 to 100 bp. HpaII and MspI both cleave CCGG, but MspI cleaves C^{m}CGG or CCGG whereas HpaII cleaves ^{m}CCGG (m = methylation). **a** EcoRI shows no bands but HpaII displays a "ladder" of bands consisting of fragments with molecular weights of 990, 1920, 2850, 3850, 4600 and 5690 bp. It is the 990 band that was used as a probe in hybridization. HaeIII shows a series of bands. **b** Comparison of the HpaII pattern with the MspI pattern. Two new band series forming "ladders" appear distinctly in the MspI digestion, indicating that methylation is involved in this case. These series start with segments with 1155 and 1716 bp. **c and d** The elk DNA shows no difference between the MspI and HpaII patterns and has the same series of bands as is found in the MspI digestion of reindeer DNA. A strong band appears at 460 bp in HaeIII

CAPREOLUS CAPREOLUS – ROE DEER

ECOR1 HPA II HAE III

LAMBDA HIND III

ΦX 174 HAE III

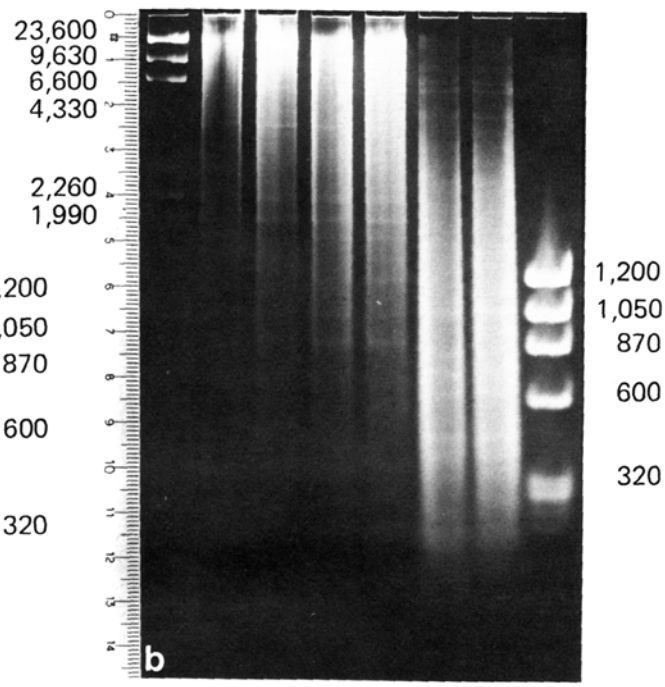


CAPREOLUS CAPREOLUS – ROE DEER

HPA II MSP I HAE III

LAMBDA HIND III

ΦX 174 HAE III

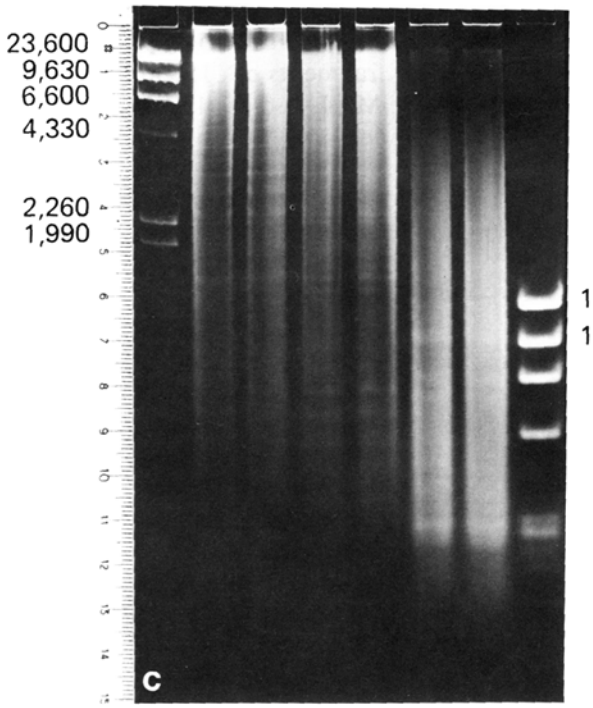


DAMA DAMA – FALLOW DEER

HPA II MSP I HAE III

LAMBDA HIND III

ΦX 174 HAE III

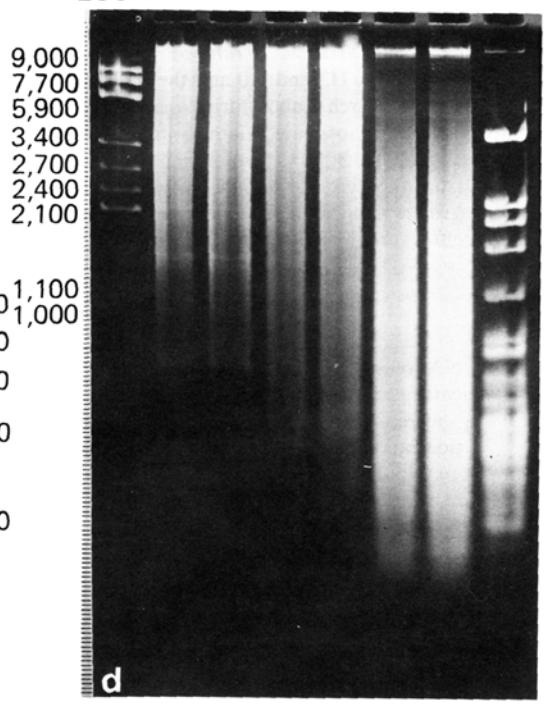


MUNTIACUS MUNTJAK – INDIAN MUNTJAC

ECOR1 HPA II HAE III

LAMBDA RIF ECOR1

LAMBDA HAE III



obvious correspondence with elk and reindeer DNA. Muntjak DNA forms a large number of bands, most of them different from those in reindeer, elk and roe deer DNA, except for the 3850-bp band, which is common to all five species. Fallow deer DNA occupies an intermediate position but most of its bands are common to muntjak DNA (Figs. 2c and d and 3).

The treatment with MspI produced bands of similar size and position to those observed after HpaII digestion in the species analysed (Figs. 1b and d and 2b and c). However, in the reindeer two series of highly repetitive DNA fragments were very conspicuous after MspI digestion but appeared only as faint bands after HpaII digestion (Figs. 1b and 3). These series consisted of the segments which have as basic units the fragments with 1155 and 1716 bp.

The cleavage with HaeIII gave a picture characterized by DNA segments mainly less than 4.0×10^3 bp in length. Only in muntjak and roe deer DNAs were there distinct bands consisting of segments of larger size. Two other features are an 1120-bp band common to all species and a thick 460-bp band in elk DNA (Fig. 3).

The similarities among the band patterns of the five species after HpaII and HaeIII digestion led us to investigate the degree of homology between the highly repetitive sequences. For this purpose we isolated the most distinct band of the reindeer DNA which was also far away from the bulk of the DNA fragments. This was the 990-bp HpaII band. We isolated these fragments by preparative electrophoresis and nick translated them using ^{32}P (Maniatis et al. 1975; Jeffreys and Flavell 1977; Rigby et al. 1977). The radioactive DNA was hybridized with the HpaII- and HaeIII-digested DNAs of the five species after they had been transferred to a filter by the Southern blot method (Southern 1975).

The labelled reindeer probe hybridized with all HpaII fragments in the reindeer series of bands (Fig. 3). In the elk, hybridization was recorded in the bands corresponding to the reindeer series. However, the labelling was somewhat weaker in the band at 990 bp. Hybridization with the components of the other multiple series was also recorded. In the roe deer, the hybridization occurred mainly with the 1920- and 3850-bp fragments. In fallow deer and muntjac the labelling occurred in several bands. Of these, one appears to be common to all of the species (3850 bp). Limited hybridization was observed with the HaeIII digest products in all species except roe deer. In this species considerable hybridization occurred, with four well-separated bands (Fig. 3). In the elk labelling appeared in bands located between 1100 and 400 bp. In the Indian muntjac, hybridization occurred to a lesser extent in a band at 2800 bp and in several other bands of lower molecular weight.

Discussion

MspI is an enzyme that cleaves the same sequence as HpaII, but MspI cleaves at CCGG sites irrespective of the presence of a 5-methyl group at the internal C residue, whereas HpaII cleaves only those CCGG sites that are unmethylated at this position (Waalwijk and Flavell 1978). There were no obvious differences between the patterns produced by MspI and HpaII digestion in the elk (Fig. 1d), roe deer (Fig. 2b) and fallow deer (Fig. 2c). This indicates that methylation was not present in the CCGG sequences of the highly repetitive DNA. In the reindeer the electrophoretic pattern showed that methylation affected the cleavage in two classes of fragments (Fig. 1b). These build two "ladders" of repeated sequences starting with the fragments with 1155 and 1716 bp. However, the band of 990 bp, which was chosen for hybridization with the other DNAs, has the same size and position after both MspI and HpaII treatments. This indicates that the DNA is not methylated at this site.

The degree of hybridization was estimated by comparing the intensity of ^{32}P-labelling with the amount of DNA present in the different bands. Some equally stained bands showed different degrees of hybridization. This was taken to mean that there is a higher sequence homology in the heavily labelled bands. In other cases, the degree of hybridization directly correlated with the amount of DNA present in the gels.

Highly repetitive sequences have been used to elucidate chromosomal and species evolution in the genus *Drosophila*. Gall and Atherton (1974) were able to build an evolutionary tree for four *Drosophila* species based on the properties of their DNA satellites. In this invertebrate group the chromosome number did not vary appreciably. Despite the extreme variation in chromosome number, the higher mammals that we investigated show similarities among their highly repetitive sequences. These similarities are indicated by the following fea-

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Fig. 2. The same markers were used as in Fig. 1 except for lambda *rif* DNA cleaved with EcoRI in the Indian muntjac. **a and b** *Capreolus capreolus* (roe deer). There is no difference between the HpaII and MspI patterns. No bands are seen in EcoRI, whereas a few appear in the HaeIII lane. **c** *Dama dama* (fallow deer). HpaII and MspI produce identical patterns. HaeIII shows a few bands. **d** *Muntiacus muntjak* (Indian muntjac). There are distinct bands in EcoRI and HpaII, but few in HaeIII

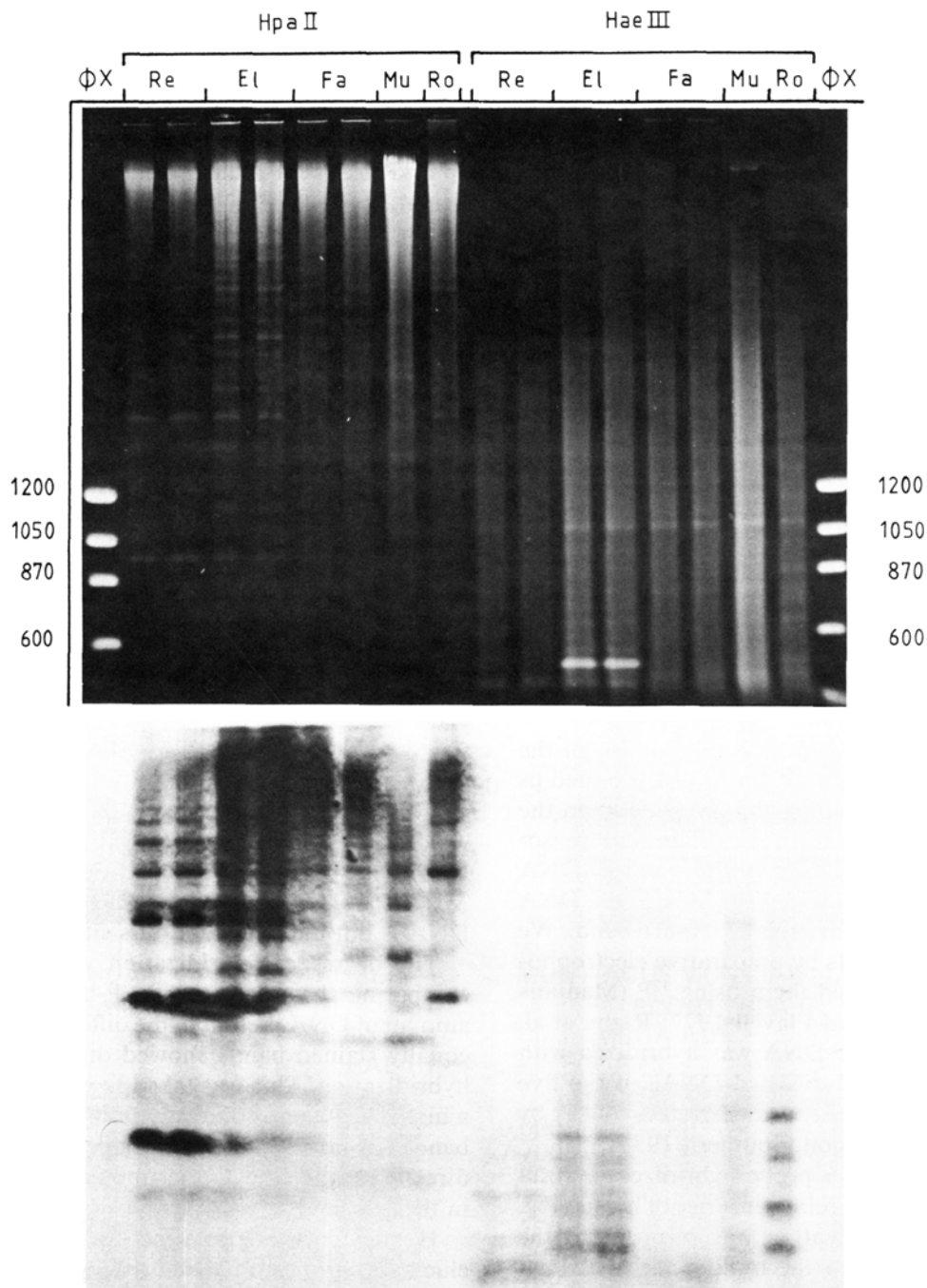

Fig. 3. **Upper part** Nuclear DNA of five species of Cervidae was cleaved with the restriction endonucleases HpaII and HaeIII and separated by electrophoresis in a 1.0% agarose gel. *Re,* reindeer (two wells); *El,* elk (two wells); *Fa,* fallow deer (two wells); *Mu,* Indian muntjac (one well); *Ro,* roe deer (one well); ΦX, ΦX174 DNA cleaved with HaeIII. After HpaII cleavage bands appear in the DNA of all species. In the reindeer the bands comprise a series with fragment sizes of 990, 1920, 2850, 3850, 4600 and 5690 bp. The molecular weight determinations were made on individual gels in which lambda DNA cleaved with HindIII and ΦX174 DNA cleaved with HaeIII were used as markers (Figs. 1 and 2). In the elk the series of bands found in the reindeer was accompanied by a number of bands of intermediate sizes. The roe deer shows several bands, two of them also present in the reindeer and elk DNAs (1920 and 3850 bp). The band pattern of muntjac mainly resembles that of fallow deer. Following HaeIII digestion there is a band common to all species (1120 bp) and a strong band in the elk DNA (460 bp). **Lower part** The DNA of the 990-bp reindeer band (located between the markers for 870 and 1050 bp) was isolated and nick translated with ^{32}P. This DNA was hybridized with the DNAs of the gel shown in the upper part of the figure after they had been transferred to a filter by the Southern blot method. Hybridization with the HpaII digest products of reindeer, elk and roe deer DNA occurred mainly in DNA present in the series of bands mentioned above. Some degree of hybridization was also seen with muntjac and fallow deer DNAs. The HaeIII digestion revealed hybridization mainly with roe deer, elk and reindeer DNA fragments present in the lower part of the gel, except for some bands in the muntjac DNA which appear in the middle region. There was no hybridization between the reindeer probe and the ΦX174 DNA

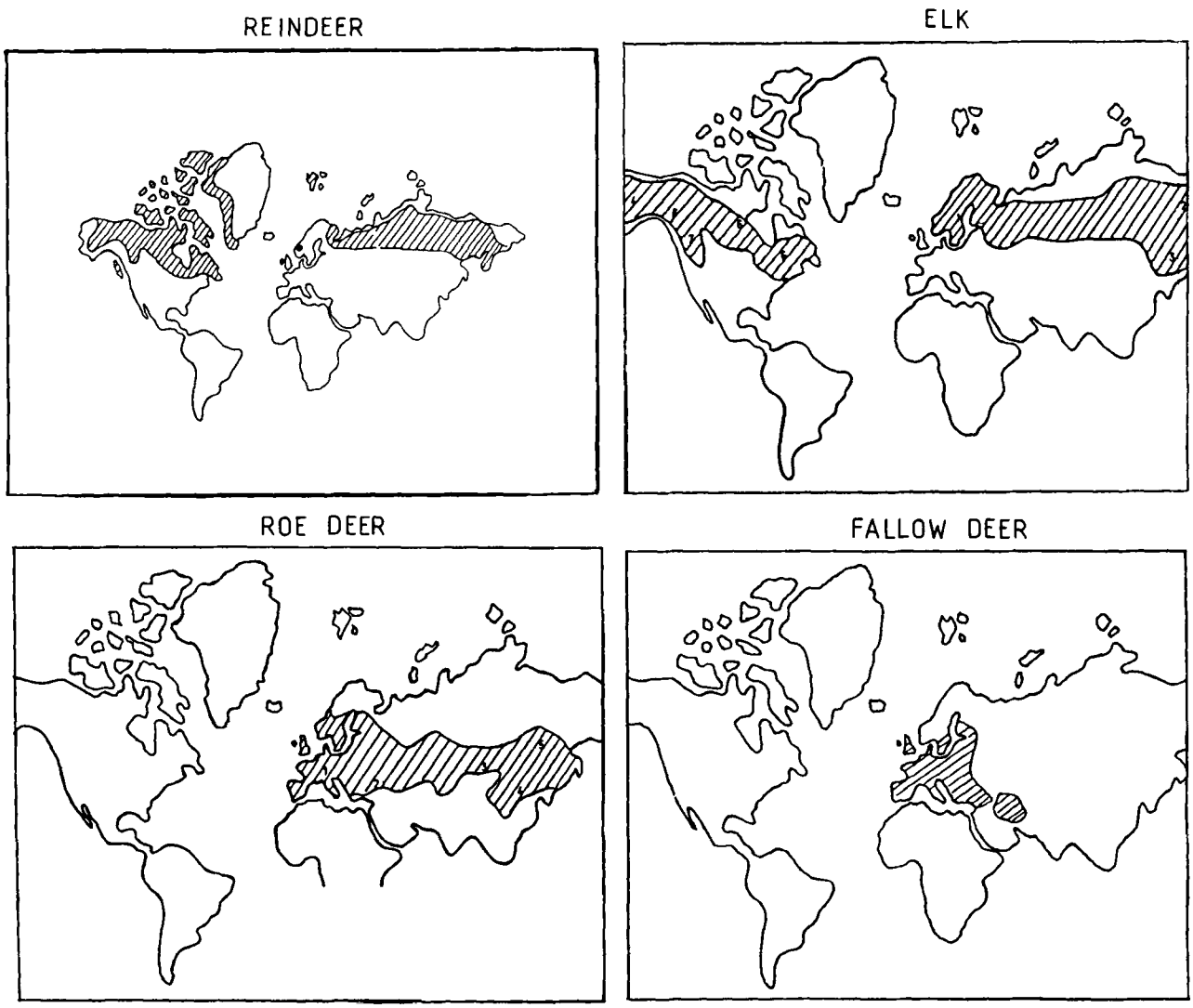

Fig. 4. Geographic distribution of the reindeer, elk, roe deer and fallow deer. The reindeer occupies the arctic region (tundra), the elk the belt of coniferous forests, the roe deer mixed forest and the fallow deer mainly the Mediterranean region and parts of the Middle East

tures: (1) Bands with similar molecular weights were common to some or all of the five species. (2) Hybridization homologies exist between the reindeer band used as a probe and the DNA fragments of the other species. Although the female of the Indian muntjac has only 6 chromosomes and the reindeer 70, the DNA–DNA hybridization revealed homologies between the highly repetitive sequences of these two deer species. (3) The homologies are evident after both HpaII and HaeIII digestion.

The results show a greater degree of homology between the reindeer, elk and roe deer than between fallow deer and Indian muntjac, the Asiatic species. This agrees with their present geographic distribution (Fig. 4) and the genetic analysis carried out by Baccus et al. (1983).

The question arises as to what rules of chromosome organization allow the genome to reorganize so drastically while at the same time conserving certain repeated sequences and producing animals so similar as to be included in the same family (Lima-de-Faria 1973, 1980). The conservatism of repeated sequences and of series of electrophoretic bands found in the Cervidae is likewise found in other animal and plant groups investigated by similar methods (Flavell et al. 1977; Peacock et al. 1977; 1978; Brykov et al. 1979; Bedbrook et al. 1980). The weak hybridization to fragments located at intermediate positions between the series with heavy labellings may indicate an organization of highly repetitive deer DNA similar to that of *Bos taurus* (Pech et al. 1979). This hybridization would result from similar short-interval repeats of a few nucleotide pairs present in different repetitive DNAs.

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