GENOME ORGANIZATION IN THE GENUS *NOTOMYS*

BY

RODNEY WATT WARNOCK

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DECLARATION

The research carried out in the course of this investigation and the results presented in this thesis are, except where acknowledged, my original work.

Rod Warnock

Rodney Watt Warnock

September 1980.

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ABSTRACT

This thesis compares a variety of properties of the genomes in four closely related species (*N. mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus*) in the genus *Notomys* (Rodentia: Mammalia). A comparison of mitotic chromosome morphology using conventional G- and C-banded preparations indicates that there are only relatively minor differences in euchromatin arrangement. This applies both to automsomes and to the X chromosomes. On the other hand, each species has a unique distribution of constitutive heterochromatin as defined by C-banding. There are also differences in the frequency and distribution of chiasmata in male meiosis but these differences are not easily reconciled with any of the other observed differences in karyotype.

In three of the four species (*alexis*, *cervinus* and *fuscus*) the X chromosome is polymorphic and, in addition to the original-type X (approximately 5% of the haploid genome), accessory gonosomal heterochromatin may be present.

At the molecular level the genomes of all four species are characterized by the presence of similar satellite DNAs and there is a correspondence between the amount of satellite DNA and the amount of constitutive heterochromatin present in the genome. Using the technique of *in situ* hybridization the satellite DNA has been shown to be located within the constitutive heterochromatin and labelled RNAs obtained from the satellite of all four species cross-hybridize in all combinations. However, neither the accessory gonosomal heterochromatin nor the Y chromosome take up label and thus they are clearly differentiated from conventional constitutive heterochromatin.

Comparisons between the satellite DNAs of all four species using filter hybridization and restriction endonuclease digestion indicate that a close relationship exists between *N. mitchellii* and *N. alexis* whereas *N. cervinus* and *N. fuscus* are separated both from one another

and the other two species. This agrees with the conclusions derived both from comparisons of karyotype and body morphology.

Using tritiated thymidine as a marker for DNA replication it is shown that the four forms of chromatin present in *N. alexis* replicate in the following sequence - early, euchromatin - facultative heterochromatin - constitutive heterochromatin - accessory gonosomal heterochromatin, late - though there is a degree of asynchrony in the replication of each of these categories which leads to some overlap. The pattern of late labelling in two females heterozygous for an original-type X and one carrying accessory gonosomal heterochromatin indicates that the latter is more often late labelling. This may also indicate that it is preferentially inactivated.

There is no evidence that the observed differences in satellite DNA and heterochromatin content had any effect on meiotic pairing. Neither was there any simple relationship between the differences in heterochromatin content and the chiasma characteristics of a particular species. The significance of the differences in satellite DNA and heterochromatin content between the four species thus remains unresolved.

CHAPTER 1

GENERAL INTRODUCTION

1.1 *An introduction to the thesis*

Prior to 1961 it was assumed that the DNA of eucaryotes was uniform in character. This led to a major complication since clearly there was too much DNA to account for the structural gene loci which could be assumed to be present in any one genome. This difficulty came with the discovery that not all the DNA of a eucaryote genome is of the same kind. Thus, according to the number of copies of the DNA sequence which it contains, eucaryote DNA may be partitioned into three classes based on their relative rates of reassociation following thermal denaturation, namely:

(i) Highly repetitive DNA sequences which reassociate fastest (before Cot 10^{-2}) and have a copy number of greater than 10^{6}. This class includes a special category, satellite DNA, so called because in CsCl density gradients, it forms a density band separate from that of the major portion of the DNA. This separation has its basis in the differing base composition of the two DNAs. Satellites were first separated in this manner in 1961 by Kit in the mouse and Sueoka in the crabs *Cancer borealis* and *C. irroratus*. These satellites are termed 'patent' satellites (Skinner, 1977). A further category of highly repeated DNA may form cryptic satellites which can be detected only by using heavy metal ions, antibiotics or dyes in cesium density gradients when they form separate peaks. A third category of highly repeated DNA comprises that from some plants in which a proportion of the DNA does not form a conventional satellite, either patent or cryptic, but nevertheless has many of the characteristics of satellite DNA. Table 1.1 summarises some examples of the eucaryote genomes that have been characterized in terms of their highly repetitive DNA content.

Table 1.1: The highly repetitive DNA content of some eukaryote genomes.

p. sat. = patent satellite separable from main band DNA in CsCl density gradients,

c. sat. = cryptic satellite which are only detected and extracted by the use of heavy metal ions or other ligands.

ip. sat. =isopycnic satellites, i.e. two satellites with identical densities in CsCl which can be distinguished in alkaline CsCl gradients.

HR = highly repeated DNA which has the complexity and repetitive characteristics of satellite DNA but fails to band as satellite DNA

Organism	Buoyant density g/cm	HR DNA % of genome	Type of HR DNA	References
1. Plants: Dicots				
Cucumis melo	1.706	25	p. sat.	Bendich and Taylor (1977)
Cucumis sativus	1.702	28	p. sat.	Ingle *et al.* (1973)
	1.706	16	p. sat.	Ingle *et al.* (1973)
Lobularia maritima	1.688	23	p. sat.	Ingle *et al.* (1973)
	1.706	9	p. sat.	Ingle *et al.* (1973)
Linum unitatissimum	1.689	15	p. sat.	Ingle *et al.* (1973)
Passiflora antioquiensis	1.700	-	no sat.	Ingle *et al.* (1973)
Plants: Monocots				
Secale cereale	1.701	2-4	HR	Appels *et al.* (1978)
Scilla siberica	1.700	-	no sat.	Ingle *et al.* (1975)
2. Fungi				
Aspergillus nidulans	1.709	-	no. sat.	Timberlake, 1978
3. Animals: Invertebrates				
Pagurus pollicaris	1.725	1	ip. sat.	Skinner and Beattie (1973)
		19	c. sat.	Beattie and Skinner (1972)
Homarus americanus	1.715	7	p. sat.	Beattie and Skinner (1972)
Procambarus blandingii	1.682	11	p. sat.	Beattie and Skinner (1972)
4. Animals: Vertebrates				
Dipodomys ordii	1.702	15	p. sat.	Prescott *et al.* (1973)
	1.707	22	p. sat.	Fry *et al.* (1973)
	1.713	19)		
	1.713	11)	ip. sats	
Mus musculus	1.691	9	p. sat.	Corneo *et al.* (1968) Flamm *et al.* (1969)

(2) Middle repetitive DNA which reassociates slower than the highly repetitive sequences (between Cot 10^{-2} and Cot 10^{2}) and has a copy number of 10^{2} through to 10^{5}.

(3) Single copy DNA which reassociates slowest (after Cot 10^{2}) For example, in *Mus musculus* the highly repeated DNA comprises 10% of the genome while the middle repeat DNA sequences occupy 20% with the remaining 70% as single copy (Britten and Kohne, 1968 and their Fig. 12).

Components of the eucaryote genome which contain either satellite or highly repetitive DNAs have, in general, been found to correspond in location to constitutively heterochromatic regions of the chromosome complement (Pardue and Gall, 1970; Jones, 1970; Prescott, *et al.*, 1978). Such regions are heteropycnotic, i.e. differentially condensed during interphase and prophase of the cell cycle in all tissues at all times. Added to this constitutively heterochromatic regions of the chromosome characteristically, though not invariably, give a positive Giesma reaction following pretreatment with sodium hydroxide, barium hydroxide or other alkalis (Pardue and Gall, 1970; Arrighi *et al.*, 1971; Arrighi and Hsu, 1971; Vosa and Marchi, 1972) leading to the production of C-bands in such regions. Consequently there is good agreement between the presence of constitutive heterochromatin as determined by the C-banding technique and the occurrence of one or other of the forms of highly repetitive DNA (Table 1.2). Notice that in some instances, e.g. man, there is a marked discrepancy between the percentage C-band material and the percentage satellite DNA. Presumably this can be explained by the presence in the constitutively heterochromatic regions of other highly repetitively DNAs which have not yet been characterized (reviewed in Miklos and John, 1979).

Table 1.2: Species for which the C-band content and the satellite DNA content have been determined

Species	% C-band in genome	% satellite DNA in genome	References
Drosophila ezoana	33	0	Gatti *et al.* (1976)
D. hydei	30	3-4 in pupae 1 in salivary gland	Hennig *et al.* (1970) Hennig (1972)
D. melanogaster	22	28	Peacock *et al.* (1973)
D. simulans	30	22	Gatti *et al.* (1976) Travaglini *et al.* (1972)
D. virilis	52	41	Gatti *et al.* (1976) Gall *et al.* (1971)
D. texana	47	31	Gatti *et al.* (1976)
Muntiacus muntjak	15.4	4	Hsu and Arrighi (1971) Commings (1971)
Cricetulus griseus	23.5	1.3	Hsu and Arrighi (1971) Commings and Matoccia (1972)
Mus musculus	26.8	9	Hsu and Arrighi (1971) Corneo *et al.* (1968) Flamm *et al.* (1969) Schildkraut and Maio (1968)
Microtus agrestis	25.6	8	Hsu and Arrighi (1971) Yunis and Yasmineh (1971)
Homo sapiens	16.8	3.5	Hsu and Arrighi (1971) Corneo *et al.* (1972)
Cavia porcellus	18-20	7-11	Hsu and Arrighi (1971) Commings and Matoccia (1972)

Because of the very general correlation between C-band distribution, the presence of satellite DNA and regions which behave as a constitutively heterochromatin manner, it is perhaps not surprising that in searching for the functions of satellite DNAs many authors (Walker, 1968; Britten and Kohne, 1968; Yunis and Yasmineh, 1971; Bostock, 1971; Hsu, 1975; Hatch *et al.*, 1976) have turned their attention to the special properties which have been associated with constitutive heterochromatin (reviews in Darlington, 1947; Goldschmidt, 1955; Cooper, 1959).

Unfortunately many of these assumed properties are themselves suspect and few have been well documented. Recently, however, two hypotheses concerning the function of constitutively heterochromatic regions have received close attention and to the extent that one can equate constitutive heterochromatin with satellite DNA these two hypotheses have implications for the functional aspects of satellite DNA.

The first of these deals with the possibility that constitutive heterochromatin is involved in the regulation of chiasma frequency and distribution. This arises because a number of correlations have been found between the presence of constitutive heterochromatin, either as a component of the normal complement or else in the form of separate supernumerary chromosomes or chromosome segments and recombination (Rhoades, 1978; John and Miklos, 1979 and their Table XVII).

The second hypothesis attempts to relate changes in constitutive heterochromatin, and by implication changes in satellite DNA, to speciation mechanisms. Such arguments have taken two forms. On the one hand it has been suggested that alterations in the satellite DNA content of chromosomes may lead to pairing difficulties and so to infertility which then forms a component of reproductive isolation (Corneo, 1976, 1978; Fry and Salzer, 1977). No one, however, has offered direct evidence that such a phenomenon actually occurs. Indeed in several cases where there are undoubted differences in constitutive heterochromatin between subspecies or members of a polymorphic system it is clear that these differences do not lead to pairing problems (see General Discussion).

On the other hand, it has been assumed that the presence of constitutive heterochromatin predisposes a chromosome complement to structural rearrangements and that these structural rearrangements, and in particular pericentric inversions, provide a basis for subsequent reproductive isolation (Hatch *et al.*, 1976). These arguments are in fact extensions of the early proposition (Darlington, 1937) that heterochromatin has played a major role in karyotypic evolution. Again there is no direct evidence to support the hypothesis and the argument of Hatch *et al.* (1976) is one based on limited correlation which is open to criticism on other grounds (John and Miklos, 1979).

One of the major difficulties in attempting to define the role of heterochromatin and satellite DNA is that the evidence for any one group of species is largely fragmentary in nature. Rarely do we have available all the necessary information to evaluate the various components of the genome in relation to known genomic properties.

The Australian hopping mice, genus *Notomys* Lesson, offers a favourable system in which to carry out such an investigation. Four species, *Notomys mitchelli*, *N. alexis*, *N. cervinus* and *N. fuscus*, all with 2n = 48, have been examined here. A fifth species, *N. carpentarius* (= *N. aquilo*) was not available. The genus is described in detail in the next section. The karyotypes of *N. alexis*, *N. cervinus* and *N. fuscus* contain different amounts of C-band material, with *N. alexis* demonstrating autosomal polymorphism in its C-band material (Baverstock *et al.*, 1977b). However, no attempts have been made to apply the G-band technique to analyse the karyotypes of these four species or to characterize the DNA. *N. mitchellii* has not previously been C-banded. Consequently, this thesis focusses on defining the following characteristics in *Notomys*:

(i) analysis of the comparative structure of the mitotic karyotypes by C- and G-banding and silver staining,

(ii) comparison of the male meiotic chromosomes using air dried techniques and C-banding,

(iii) characterization of the DNA contents using density gradient, thermal denaturation, strand separation, filter hybridization and late labelling studies,

(iv) localization of the satellite DNAs both within and between species using *in situ* hybridization, and

(v) comparative analysis of the satellite DNAs using restriction endonucleases.

The aim of this thesis is to ask to what extent a knowledge of these several properties is able to provide information from which to question the relative merits of the above hypotheses. Firstly, does heterochromatin and satellite DNA have effects on the chiasma characteristics of these species? Secondly, do polymorphisms for C-band material contribute to pairing difficulties at meiosis? Thirdly, does the karyotypic data suggest that heterochromatin and satellite DNA have been involved in the speciation of *Notomys* by promoting chromosomal rearrangements such as pericentric inversions? In addition, a phylogeny for these four species of *Notomys* is proposed on both karyological grounds and satellite DNA affinity, and is compared with phylogenies derived from morphological studies.

1.2 *An introduction to the genus* Notomys

1.2.1 *The genus* Notomys *- taxonomy and phylogeny of the genus* Notomys

The rodent genus *Notomys* is included in the family Pseudomyinae (family Muridae) and has undergone marked adaptive radiation since arrival in Australia (Tate, 1951; Simpson, 1961; Keast, 1972). The predominent range of the genus *Notomys* is the arid areas of mainland Australia (Fig. 1.1a & b).

The order Rodentia includes families which have all undergone extensive adaptive radiation in different regions of the world. Some of these families, though differing greatly genetically, include species which have adapted to similar ecological niches by undergoing a remarkable convergent evolution. This convergence is particularly evident in the saltatorial, bipedal locomotion of rodents inhabiting desert and semi-arid regions. The rodent families which contain the species involved in this convergence are the Old World jerboas (*Dipodidae*) and jumping mice (*Cricetidae*), the North American kangaroo rats (*Heteromyidae*) and the Australian hopping mice *Notomys* (Muridae). These species have the following morphological features: enlargement of the eyes, increases in the size of the pinnae and/or the tympanic bullae - modification of the limbs so that the forelimbs are fore-shortened, while the hind limbs are lengthened and the side digits of the pes reduced - the tail is lengthened greatly and is terminated with a tuft of hair. The musculature of the back, flank and thigh is strongly developed and complex. The desert life has resulted in a fossorial habit (Stanley, 1971).

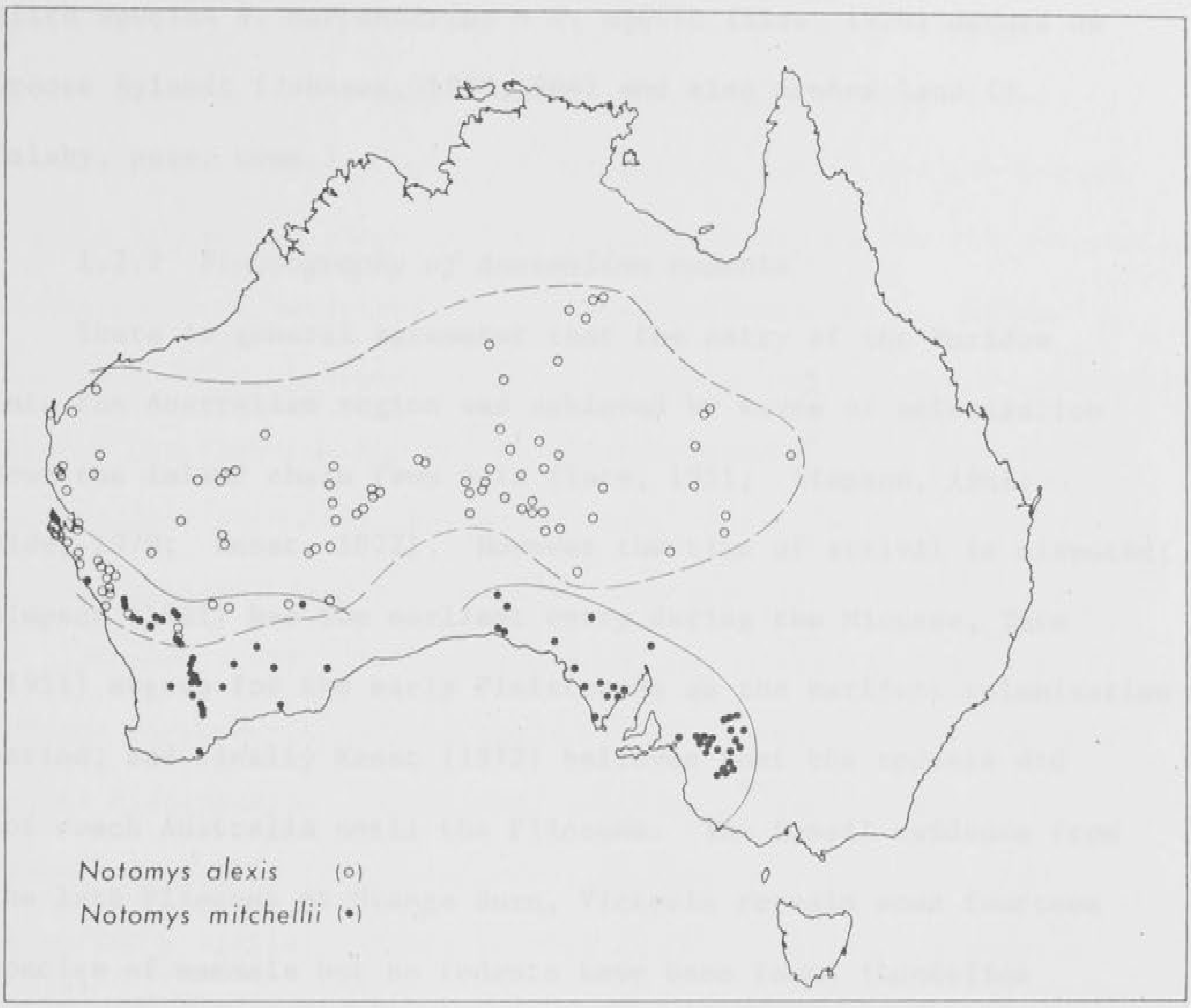
The five extant species of *Notomys* are: *N. mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus*. They inhabit the semi-arid desert environments on the Australian mainland (Fig. 1. 1a and b). The

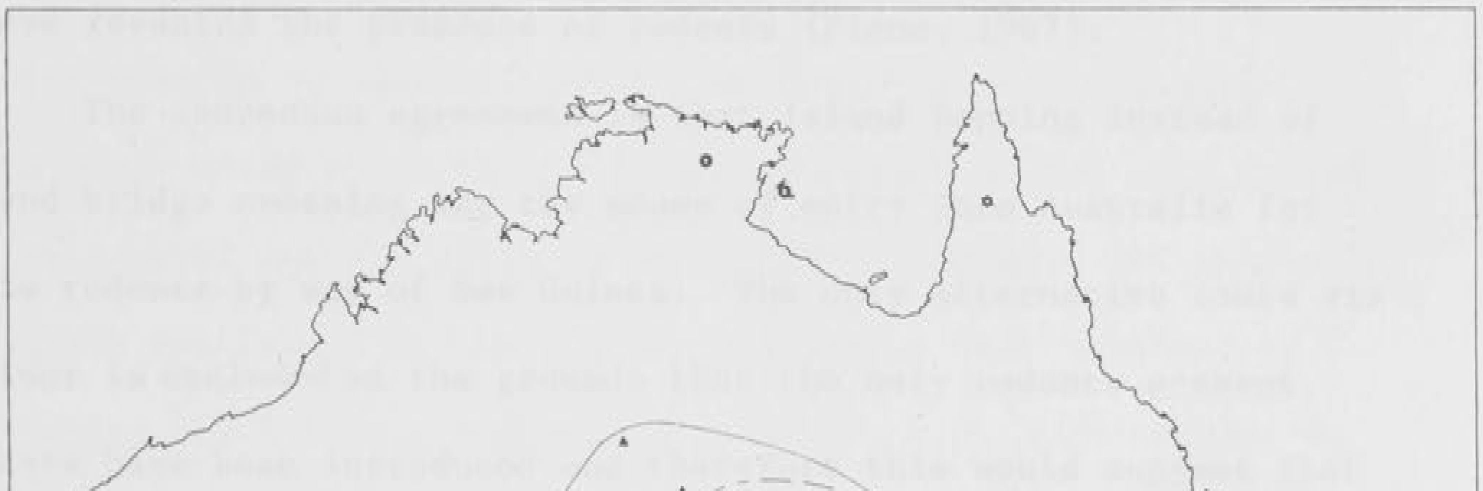
Figure 1.1 : The geographic distribution of the genus *Notomys*.

Upper map: The distribution of *N. alexis* and *N. mitchellii*.

Lower map: The distribution of *N. cervinus*, *N. fuscus* and *N. aquilo*. (= *N. carpentarius*).

The Cape York occurrence of *N. aquilo* is from an unspecified locality (Tate, 1961). The Groote Eylandt locality for *N. carpentarius* is from Johnson (1959,1964) and the single occurrence in Arnhem Land (J. Calaby, pers. comm.). The other species have had their distribution derived from museum records and the literature.


Notomys alexis (○)
Notomys mitchellii (•)


Notomys cervinus (▲)
Notomys aquilo (o)
Notomys fuscus (△)

fifth species *N. carpentarius* = *N. aquilo* (Ride, 1970) occurs on Groote Eylandt (Johnson, 1950,1964) and also Arnhem Land (J. Calaby, pers. comm.).

1.2.2 *Biogeography of Australian rodents*

There is general agreement that the entry of the Muridae into the Australian region was achieved by waves of colonisation down the island chain from Asia (Tate, 1951; Simpson, 1961; Ride, 1970; Keast, 1972). However the time of arrival is disputed; Simpson (1961) has the earliest entry during the Miocene, Tate (1951) argues for the early Pleistocene as the earliest colonisation period, and finally Keast (1972) believes that the rodents did not reach Australia until the Pliocene. The fossil evidence from the late Pliocene at Grange Burn, Victoria reveals some fourteen species of mammals but no rodents have been found (Lundelius and Turnbull, 1967), however, the Pliocene fossils of New Guinea have revealed the presence of rodents (Plane, 1967).

The concensus agreement is that island hopping instead of land bridge crossing was the means of entry into Australia for the rodents by way of New Guinea. The only alternative route via Timor is excluded on the grounds that the only rodents present there have been introduced and therefore this would suggest that the barriers to migration were too severe during the period of colonization (Simpson, 1961). Some birds did however use the East Indies-Timor-Australia route (Mayr, 1944).

Simpson (1961) believes that up to seven (but possibly fewer) stocks of murid rodents were already phylogenetically distinct either before or during their passage along the East Indies chain to Australia. The Australian rodents have been divided on taxonomic grounds into four groups: the Pseudomyinae, the Hydromyinae, the *Uromys/Melomys* group and finally the genus

Rattus (Tate, 1951; Simpson, 1961 and Ride, 1970). Simpson (1961) proposed a phylogeny of Australian rodents (Fig. 1.2a) which implies a closer relationship between *Rattus* and the *Uromys/ Meloyms* group than either has with the Pseudomyinae or the Hydromyinae. On the basis of karyotypic evidence, Baverstock *et al.* (197/a) have proposed a phylogeny (Fig. 1.2b) which shows closer relationship between the Pseudomyinae and the *Uromys/Melomys* group with their next nearest relatives the Hydromyinae. *Rattus* are shown arising from an ancestor at a much earlier time than in Simpson's model.

Johnson (1959, 1964) comments on the unexpected discovery of *Notomys carpentarius* on the humid Groote Eylandt whereas the more normal distribution is the arid interior of the mainland. However *N. aquilo* (= *carpentarius*) is recorded from Cape York which has a similar climate to that on Groote Eylandt (Thomas, 1921). Further Tate (1951) had earlier surmised that the genus *Notomys* may well have adapted during the Pleistocene in the extreme north of Australia when that region was arid and that the genus moved south to its present location as the arid region itself moved further south as the climate changed. The Groote Eylandt population may be a relic population left stranded by changing sea levels. Recently a specimen of *N. carpentarius* was caught just south of Maningrida, Arnhem Land (J. Calaby, pers. comm.) lending some strength to this suggestion.

1.2.3 *Previous literature on the genus* Notomys

There have been studies on aspects of the ecology, physiology and facets of general biology of some species of *Notomys*.

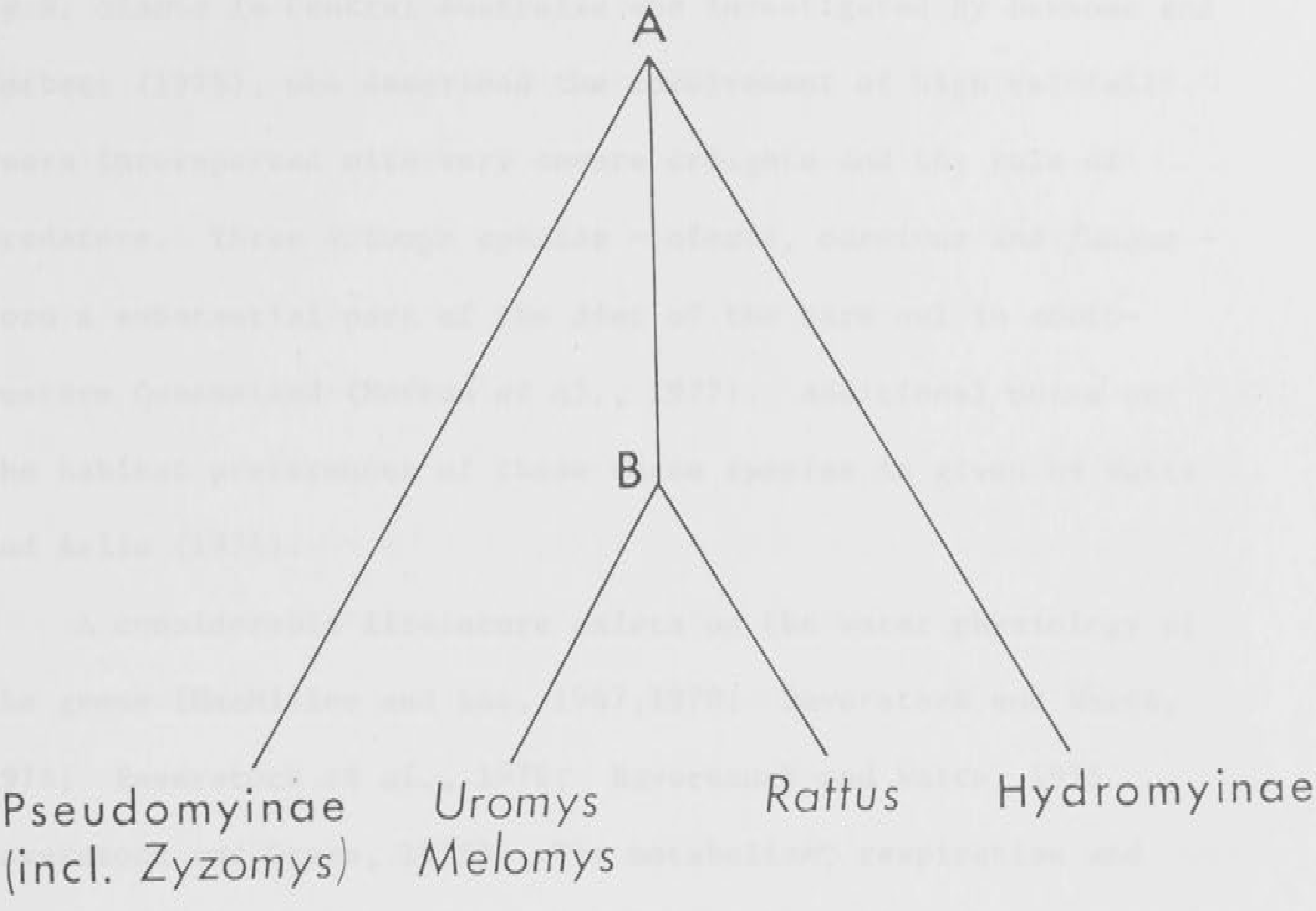
The detailed ethology of *N. alexis* (Stanley, 1971) includes a comparison with the jerboas, kangaroo rats and other desert adapted rodents and covers additional aspects of the general biology, physiology and ontogeny. The ecology of plague formation

Figure 1.2 : Phylogenies of Australian pseudomyid rodents.

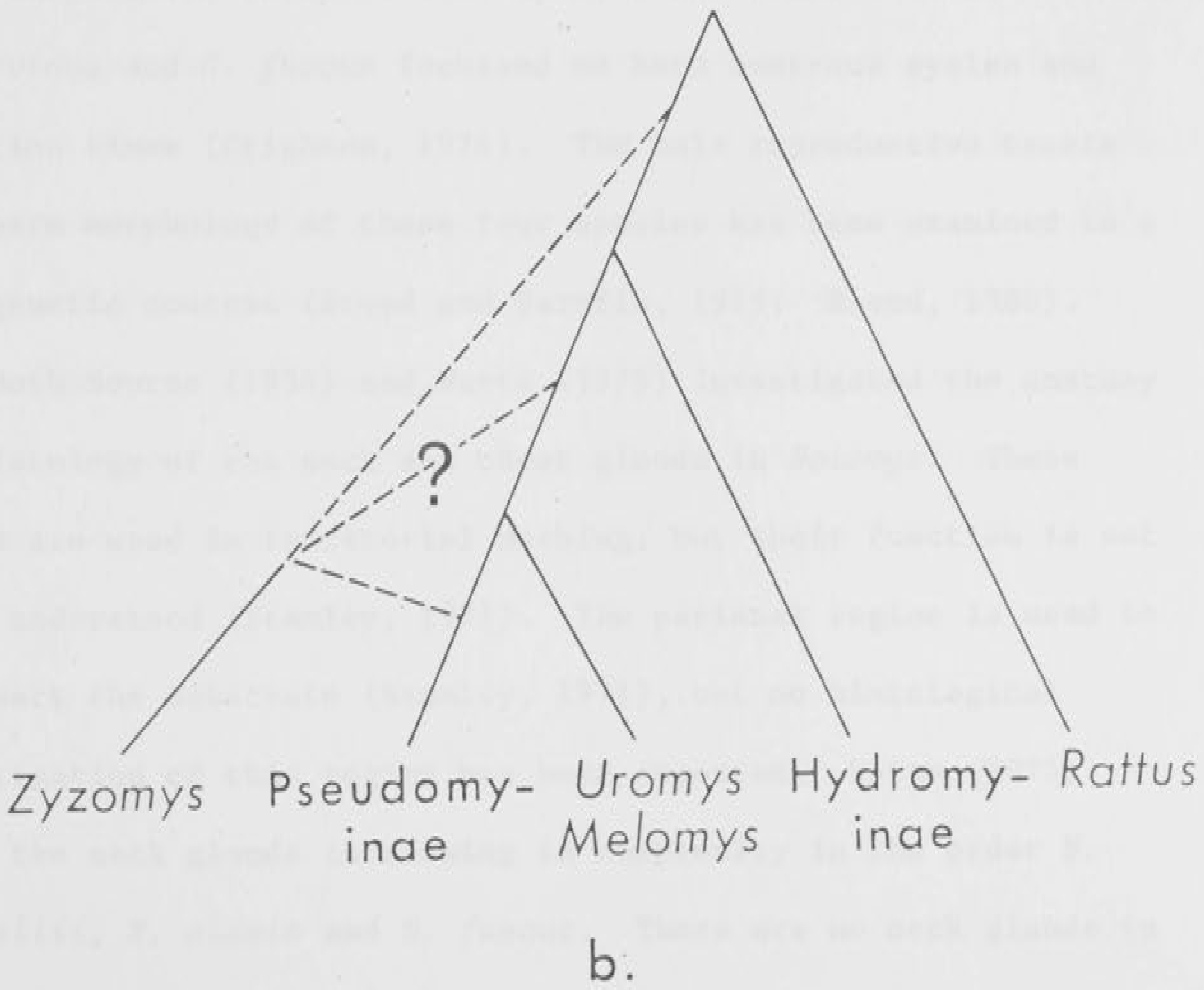
a. Phylogeny according to Simpson (1961).

b. Phylogeny after Simpson (1961) modified by Baverstock *et al.* (1977a) in view of karyotypic data.

Both diagrams from Baverstock *et al.* (1977a).


A
B
Pseudomyinae (incl. Zyzomys)
Uromys Melomys
Rattus
Hydromyinae

a.


?
Zyzomys
Pseudomy-inae
Uromys Melomys
Hydromy-inae
Rattus

b.

by *N. alexis* in Central Australia was investigated by Newsome and Corbett (1975), who described the involvement of high rainfall years interspersed with very severe droughts and the role of predators. Three *Notomys* species - *alexis, cervinus* and *fuscus* - form a substantial part of the diet of the barn owl in south-western Queensland (Morton *et al.*, 1977). Additional notes on the habitat preferences of these three species is given by Watts and Aslin (1974).

A considerable literature exists on the water physiology of the genus (MacMillen and Lee, 1967,1970; Baverstock and Watts, 1974; Baverstock *et al.*, 1976; Baverstock and Watts, 1975 Baverstock and Green, 1975). The metabolism, respiration and evaporative water loss at varying temperatures have been investigated in *N. alexis* (Withers *et al.*, 1979).

The reproductive biology of *N. alexis* females is well known (Breed, 1974,1975,1976,1977,1978,1979; Telfer and Breed, 1976) and a comparative study on this species and females of *N. mitchellii, N. cervinus* and *N. fuscus* focussed on both oestrous cycles and gestation times (Crighton, 1974). The male reproductive tracts and sperm morphology of these four species has been examined in a phylogenetic context (Breed and Sarafis, 1979; Breed, 1980).

Both Bourne (1934) and Watts (1975) investigated the anatomy and histology of the neck and chest glands in *Notomys*. These glands are used in territorial marking, but their function is not fully understood (Stanley, 1971). The perineal region is used to drag-mark the substrate (Stanley, 1971), but no histological investigation of this region has been reported. Watts (1975) found the neck glands increasing in complexity in the order *N. mitchellii, N. alexis* and *N. fuscus*. There are no neck glands in *N. cervinus* which had a sternal gland in males and in pregnant females in which the glands disappeared after lactation.

Inheritance studies of glucose phosphate isomerase (GPI, E.C. 5.3.19), transferrin and esterases in *N. mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus* revealed that GPI was inherited on Mendelian lines and electrophoretic variation existed in all species except *N. mitchellii* (Baverstock *et al.*, 1977d). Transferrin was shown to have a high intraspecific electrophoretic variability and a second pattern of transferrin variation in *N. alexis* was found to follow a Mendelian pattern of codominant inheritance. The considerable variability in electrophoretic forms of esterase activity in the four species was not elucidated.

Previous karyotypic studies on *Notomys* have been carried out by Kennedy (1969) who analysed both *Notomys alexis* and *N. cervinus* and concluded that their karyotypes were identical. This contrasts that these species have different karyotypes. The chromosomal evolution of the Pseudomyinae, the Hydromyinae and the *Uromys/Melomys* group and the position of the genus *Notomys* within it are discussed by Baverstock *et al.* (1977a). This work includes the standard karyotype of *Notomys alexis*, *N. fuscus*, *N. cervinus* and *N. mitchellii*, the C-banding karyotype of *N. cervinus*, sex chromosome polymorphism in the sex chromosomes of each species. The C-banded karyotype of *N. alexis*, *N. cervinus* and *N. fuscus* is described together with the extent of the polymorphism of the X and Y chromosomes and the autosomes in a subsequent paper (Baverstock *et al.*, 1977b).

The classification of the genus *Notomys* according to Tate (1951) and Simpson (1961) is as follows:

Order Rodentia

Family Muridae

Subfamily Pseudomyinae

Genus *NOTOMYS* Lesson

Notomys Lesson, 1842, Nouveau tableau du regne animal, mammiferes, p.129.
Podanomalus Waite, 1897, Proc. Roy. Soc. Victoria, new ser., 10: 117.
Thylacomys Waite, 1897, Proc. Roy. Soc. Victoria, new ser., 10: 121.
Ascopharynx Waite, 1900, Ann. Mag. Nat. Hist., ser. 8, 5: 222.

Notomys mitchellii (Ogilby)

Notomys mitchellii (Ogilby)

Dipus mitchellii OGILBY, 1838, Trans. Linnaean Soc. London, 18: 130.

Notomys alexis Thomas

Notomys alexis THOMAS, 1922, Ann. Mag. Nat. Hist., ser. 9, 9: 316.

Notomys cervinus (Gould)

Hapalotis cervinus GOULD, 1853, Proc. Zool. Soc. London, for 1851, p.127;
The mammals of Australia, pt.5 (vol.3), pl.10.
Podanomalus aistoni BRAZENOR, Mem. Natl. Mus. Melbourne, 8: 84.

Notomys fuscus (Jones)

Ascopharynx fuscus JONES, 1925, Rec. South Australian Mus., 3: 3.

Notomys carpentarius (Johnson)

Notomys carpentarius JOHNSON, 1959, Proc. Biol. Soc. Wash. 72: 186.

Notomys aquilo Thomas

Notomys aquilo THOMAS, 1921, Ann. Mag. Nat. Hist., ser.9, 8: 540.

CHAPTER 2

CHARACTERIZATION OF THE MITOTIC KARYOTYPES OF *NOTOMYS* BY C- AND G-BANDING

2.1 *Introduction*

Mammals in general, but rodents in particular, have undergone marked karyotypic change during their evolutionary history (White, 1973). Prior to the introduction of chromosome banding techniques in the late 1960's to early 1970's the only criteria which allowed identification of individual chromosomes within a genome were chromosome length, arm length ratios, the position of secondary constrictions and the presence of constitutively heterochromatic regions. These constraints meant that only a few karyotypes had sufficiently distinct chromosome morphologies to allow each member of the complement to be postively identified. Moreover even heterochromatic regions of chromosomes are isopycnic in condensed stages of the chromosome cycle, consequently such differences could only be exploited using prophase stages. The requirement existed for simple and reproducible procedures which would differentiate condensed chromosomes longitudinally and give some of the advantages available to those working with Dipteran polytene chromosomes to the study of mammalian chromosomes. The new chromosome banding techniques have partly fulfilled these requirements and have revolutionised karyotypic research. These techniques include:

(i) *Q-banding*. This procedure, was largely pioneered by Caspersson and his co-workers, and uses fluorochromes which bind differentially to DNA. It produces fluorescent banding patterns under ultra-violet illumination in both animal and plant chromosomes (Caspersson *et al.*, 1968; Caspersson *et al.*, 1969a,b; Caspersson *et al.*, 1970a,b,c).

(ii) *G- and R-banding*. During the early 1970's a simple reproducible Giesma staining technique was developed which, in vertebrate chromosomes, gives similar but not identical banding patterns to those obtained with Q-banding. However G-bands are visible using standard

Table 2.1 : Constitutive heterochromatin as determined from C-banding preparations and expressed as a percentage of the genome a. Hsu and Arrighi, 1971;b. Commings and Matoccia, 1972; c. Hatch *et al*., 1976; d. Deaven *et al*., 1977; e. Gatti *et al*., 1976; f. Arnason, 1974; g. Gamperl *et al*., 1976

Organism	Constitutive Heterochromatin (% of genome)
Carollia perspicillla a.	18.9
Cavia porcellus b.	18-20
Cricetulus griseus a.	23.5 ± 2.4
b.	18-20
g.	25
Cricetus cricetus g.	34
Coturnix coturnix japonica b.	9-15
Dipodomys 4 species unnamed c.	12.5-50
Equus caballus b.	10-12
Homo sapiens a.	16.8 ± 0.97
b.	10
Microtus agrestis a.	25.6 ± 0.94
b.	18-24
Mesocricetus auratus a.	33.6
Mus musculus a.	26.8 ± 1.98
	12-16
Muntiacus muntjak a.	15.4 ± 1.53
Oris ammon cyclocerus a.	22.8 ± 1.03
Peromyscus eremicus d.	35.8
P. maniculatus d.	5.7
P. crinitus d.	5.7
Drosophila melanogaster e.	33
D. simulans e.	30
D. virilis e.	52
D. texana e.	47
D. hydei e.	30
D. ezoana e.	33
Clobicephale macrorhyncha f.	10
Tursiops gilli f.	15
Balaenoptera borealis f.	25
B. acutorostrata f.	30
B. physalis f.	30

light microscopy without the need for ultra-violet illumination. The technique can involve a number of pre-treatments of chromsome preparations, however trypsin pre-treatment is perhaps the most common (Seabright, 1971, 1972; Drets and Shaw, 1971; Patil *et al.*, 1971; Schnedl *et al.*, 1971). R-banding results in a banding pattern which is the reverse of that given by the G-banding methods and is preferred by some laboratories (Duttrillaux *et al.*, 1971).

(iii) *C-banding*. Pardue and Gall (1970) were the first to observe that areas which contained satellite DNA, as indicated by *in situ* nucleic acid hydridization, stained heavily with Giemsa in mouse chromosome preparations. Subsequently it was found that when chromosome preparations were treated with alkali and then incubated in warm saline they stained differentially with Giemsa (Pardue and Gall, 1970, 1972; Arrighi *et al.*, 1971; Arrighi and Hsu, 1971; Vosa and Marchi, 1972). In particular constitutive heterochromatin stained deeply with this treatment whereas euchromatin and facultative heterochromatin stained only faintly. The technique is relatively simple, reproducible and is now widely used to estimate the percentage of constitutive heterochromatin in a wide range of organisms (Table 2.1). In the majority of cases C-banding indicates the extent and location of satellite DNAs within the genome though there remain some examples where C-banding does not indicate the presence of satellite or highly repeated DNA sequences (see Table 1.2). C-banding, however, lacks the sequence specificity of *in situ* hybridization. For example, in the human karyotype, while C-banding indicates the location of the four human satellite DNAs (Hsu and Arrighi, 1971), it does not differentiate between them, which is only possibility by using *in situ* hybridization (Gosden *et al.*, 1975). C-banding is of more general use in cytogenetics than G-banding since it is applicable to both plant and invertebrate chromosomes as well as to vertebrates.

The best example of the value of chromosome banding techniques is in studies on the primates in which these techniques have enabled the establishment of standards for nomenclature in human cytogenetics and these are presented in the Paris Conference (1971) and its 1975 Supplement. In humans, the clinical applications of chromosome banding facilitate both the detection and identification of the chromosomal elements involved in trisomics, inversions, translocations, deletions and additions. To obtain maximum resolution C-banding is used in addition to one or more of Q-banding, G-banding or R-banding techniques. For example, in a recent survey of 18 heteromorphisms only one, a 21p deletion, was detected using Orcein-staining, while six out of seven inversions were detected using G-banding. C-banding facilitated the detection of the remaining eleven heteromorphisms, ten of which were partial inversions of C-banding regions (Mayer *et al.*, 1978).

Banding techniques have also been used extensively in establishing a chromosomal phylogeny of the primates (Review: de Grouchy *et al.*, 1978). Indeed the history of primate chromosomal phylogeny is virtually the history of mammalian cytogenetics. Prior to the use of banding the only relevant observations were the chromosome numbers of the species under investigation coupled with descriptions of chromosome morphology based on centromeric index and overall size (Bender and Mettler, 1958; Chu and Bender, 1961; Klinger, 1963; Hamerton *et al.*, 1963; Bender and Chu, 1963; Ruffie *et al.*, 1970; Chiarelli, 1971). The initial application of banding technology to problems of primate cytogenetics began with the use of Q-banding (Pearson *et al.*, 1971). This was quickly followed by G-banding, C-banding and R-banding culminating in the development of standards of nomenclature to aid in the description and comparison of the karyotypes of the higher primates. These homologies of banding patterns in the higher primates have led to the investigation of whether similarity in the banding patterns corresponds to similarity in gene content. Some 40 enzymes for which the human chromosomal location was known have also been found in homologous regions in the karyotypes of the great apes with only one enzyme yielding an equivocal result (de Grouchy *et al.*, 1978). Evidently conservation of G-bands parallels conservation of structural gene loci though this does not indicate a direct relationship between the G-banding pattern of a chromosome and its sequences of genes as has been suggested by Mascarello *et al.* (1974a) on the basis of work by Miller *et al.* (1971). The research by Miller *et al.* was concerned with identification of structural abnormalities in human chromosomes using banding technology but it did not provide information on gene sequences. However, in *Mus musculus* chromosome banding techniques have not only enabled the identification of every chromosome in the karyotype, but also have allowed the assignation of 14 of the 19 linkage groups to 14 different chromosomes (Miller and Miller, 1972).

In the present study of the four taxonomically closely related species of *Notomys*, banding techniques have been used to probe the extent of interspecific variation in constitutive heterochromatin and the nature of the chromosomal rearrangements which distinguish these genomes. These data are important in elucidating the models of chromosomal evolution in the group and in addition impinge on recent hypotheses on speciation referred to in the introduction and which will be returned to in the general discussion at the end of this thesis.

2.2 *Methods*

2.2.1 *Origin of animals used for analysis*

The majority of the animals used in this study were obtained from laboratory stocks supplied generously by the Institute of Medical and Veterinary Science, Adelaide.

The species studied were:

Notomys mitchellii : 20 females and 23 males from I.M.V.S. laboratory stocks.

The laboratory stocks were bred from wild caught animals from Ooldea, Maralinga and Billiat locations in South Australia.

1 female and 2 males wild caught from Ooldea, S.A. supplied by I.M.V.S.

3 males from Billiat, S.A.

Notomys alexis : 69 females and 70 males from I.M.V.S. laboratory stocks established from Central Australia.

1 female and 2 males from 10 km north of Mt. Sarah Homestead, S.A.

8 males from Curtin Springs, N.T. supplied by Dr. W. Breed, Adelaide University, S.A.

Notomys cervinus : 15 females and 21 males from I.M.V.S. laboratory stocks derived from wild caught individuals from south-west Queensland during 1968-1972.

Notomys fuscus : 15 females and 16 males from I.M.V.S. laboratory stocks established from eight animals collected 10 km S.W. of Betoota, Queensland.

2.2.2 *Culture techniques*

2.2.2.1 *Lymphocyte cultures.* A whole blood sample (0.3 ml) was obtained by heart puncture and cultured in one of the following media for a total of 72 hours at 37°C.

(a)	Medium 199 (CSL)	4 ml
	Foetal calf serum or calf serum (CSL)	1 ml
	Heparin without antispetic 1000 i.u./ml	1 drop
	Phytohaemagglutinin solution (Burroughs Wellcome)	0.05 ml

or

(b)	Ham's F10 medium (CSL)	4 ml
	Foetal calf serum or calf serum (CSL)	1 ml
	Heparin without antiseptic 1000 i.u./ml	1 drop

Phytohaemagglutinin solution
(Burroughs Wellcome) 0.05 ml

Four hours prior to harvesting 0.2 ml of 0.02% colchicine in mammalian saline was added to the culture. At 72 hours the culture was transferred to centrifuge tubes and spun at 1000 rpm for 7 minutes. The supernatant was discarded and 6 ml of 0.56% potassium chloride was added at 37°C to the pellet which was gently shaken to suspend the cells. Incubation was continued at 37°C for 20 minutes. The cell suspension was then centrifuged again and all but 1.5 ml of the supernatant discarded. Five ml of fresh 3:1 methanol:glacial acetic acid fixative was added to the cell suspension and the mixture allowed to stand for ten minutes at room temperature. Following centrifuging and removal of the supernatant, fresh fixative was added and the cell pellet gently resuspended and allowed to stand a further 15 minutes. After a final centrifugation step the cell suspension was tested for mitotic chromosomal quality by means of a test slide and either stored in fresh fixative at 4°C or used to make further slides.

2.2.2.2 *Bone marrow cultures*. Bone marrow from femurs was cultured for 2 hours at 37°C in 4 ml of Ham's F10 media plus 0.3 ml of 0.02% colchicine solution. The culture was then centrifuged at 1000 rpm for 8 minutes. The supernatant was discarded and the pellet gently suspended in 6 ml of 0.56% potassium chloride solution and incubated for 20 minutes at 37°C. The cell suspension was again centrifuged and all but 1.5 ml of the supernatant removed and 5 ml of 3:1 methanol:glacial acetic acid fixative was added to the cell suspension and allowed to stand at room temperature for 10 minutes. Following another centrifuging and the addition of fresh fixative the cell suspension was again centrifuged, fresh fixative was added and an air dried test slide was made to determine a suitable cell concentration. In some cases slides were made immediately or alternatively fresh fixative was added to the cell suspension, which was then stored for future use at 4°C.

2.2.3 *Banding techniques*

2.2.3.1 *G-banding*. Slides were prepared by air-drying from cell suspensions in 3:1 methanol:glacial acetic acid fixative. The slides were then treated for 30 seconds in 0.25% trypsin (Difco brand 1:250) in 0.85% saline, then rinsed twice in 0.85% saline. Staining for 8 minutes in 2% Giemsa (Gurr's Improved R'66) in phosphate buffer pH 6.8 was followed by a rinse in buffer and then two rinses in deionised water. The slides were then air-dried and mounted. This method is a modified form of that used by Seabright (1971). An alternative G-banding method was used and is similar to that of Sumner *et al.* (1971). The slides were incubated for 60 minutes at 50°C in 2 x SSC (0.3 M sodium chloride plus 0.03 M trisodium citrate), then rinsed with deionised water, stained and then air-dried and mounted.

2.2.3.2 *C-banding*. Slides prepared in the manner for G-banding were treated using a modified method according to Scheres (1974, 1976). The slides were placed in saturated barium hydroxide solution at 60°C for 1-2 minutes, then rinsed with deionised water, followed by a rinse in distilled water, adjusted to pH 4 with hydrochloric acid. Staining was accomplished by immersion of the slides in a 0.005% solution of "Stains-all" (4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine-bromide, Serva) in a 1:1 formamide-water mixture (pH 8.2) for 10 minutes. Giemsa (Gurr's Improved R'66) 10% solution in phosphate buffer (pH 6.8) was also used as an alternative stain, but was judged to be a poorer stain than "Stains-all" on two grounds: (i) the staining of heterochromatin is less intense, (ii) Giemsa staining suffers from precipitation debris which soils some spreads.

2.2.4 *Chromosome measurement*

The direct comparison of linear measurements of chromosomes or segments of chromosomes at mitotic metaphase is complicated by differential contraction between constitutive heterochromatic and euchromatic regions. However if mitotic figures of similar degrees of contraction are selected for measurement much of the disadvantage can be avoided. Balicek *et al.* (1977) found that the variance in size of heterochromatic segments in human chromosomes with similar degrees of contraction had an error of less than 3% of the mean when 5 mitotic cells were measured.

Within the human karyotype there is a wide range in the variation of the size of the C-band regions of chromosomes 1, 9, 16 and the Y chromosome. A number of methods have been used to evaluate these size differences between homologues: (i) Comparison with particular euchromatic segments (various internal markers were used by Lubs and Ruddle, 1971; 9p by Madan and Bobrow, 1974; 21q by Muller *et al.*, 1975; 16p by Patil and Lubs, 1977), (ii) visual comparison of heterochromatic blocks (Craig-Holmes *et al.*, 1973; McKenzie and Lubs, 1975; Buckton *et al.*, 1976), (iii) linear measurement (Balicek *et al.*, 1977), area measurement (Mason *et al.*, 1975), (iv) densimetric measure (Geraedts *et al.*, 1974; Mason *et al.*, 1975; Sumner, 1977) and (v) arbitrary units (Madan and Bobrow, 1974; Van Dyke *et al.*, 1977). Many of these methods have been developed specifically for human karyotypic investigations. Because of its functional simplicity and relative accuracy linear measurements were used for determining the total content of the C-banding material both in the genomes and in individual chromosomes of *Notomys* in the present study.

2.3 *Results*

2.3.1 *Standard stained karyotypes*

Conventionally stained mitotic chromosome complements are shown in Fig. 2.1. Detailed analysis of such preparations indicates that while the four species share an identical diploid number nevertheless show a number of characteristic differences.

2.3.1.1 *Notomys mitchellii* (2n = 48) has a karyotype with two small pairs of metacentric autosomes, the remaining autosomes form a size-graded series. Some chromosomes show prominent short arms. Both the sex chromosomes are acrocentric. The X chromosome comprises 8.05% (Table 2.2) of the combined female haploid autosome length plus the euchromatic arm of the X chromosome (hereafter referred to as HEX), while the equivalent value for the Y chromosome is 4.58% HEX (Table 2.4). The X chromosome cannot be distinguished from chromosome 1 in standard stained preparations.

2.3.1.2 *Notomys alexis* (2n = 48), like *N. mitchellii*, has a karyotype with two small metacentric chromosome pairs with the remaining 21 pairs of autosomes forming a size-graded series. The sex chromosomes and autosomal pair 1 are all polymorphic. The X chromosome was found in three forms (Table 2.2):

(i) a metacentric (10.73% HEX),
(ii) a submetacentric (9.20% HEX), and
(iii) an acrocentric (7.19% HEX).

The Y chromosome was found in a range of forms from metacentric through to acrocentric. A sample of metacentric Y chromosomes comprised a mean 5.60% HEX, while in a sample of acrocentric Y chromosomes the equivalent figure was 4.84% HEX. Baverstock *et al.* (1977b) reported the occurrence of a number of polymorphisms involving chromosome 1. Their findings were confirmed in this study and indicate the presence of two distinct categories of polymorphism:

(i) a polymorphism for the presence or absence of a short arm, and
(ii) a polymorphism for the length of the long arm (Table 2.3).

2.3.1.3 *N. cervinus* (2n = 48) has a karyotype of 20 pairs of acrocentric autosomes, many of which have prominent short arms together with three small metacentric pairs. Both the sex chromosomes are polymorphic. The X chromosome was found in three forms (Table 2.2):

Figure 2.1 : Conventionally stained mitotic metaphase karyotypes of *Notomys*: (a) *N. mitchellii*; (b) *N. alexis*; (c) *N. cervinus* and (d) *N. fuscus*. The bar (——) = 10 µm. The arrows indicate the small metacentrics.

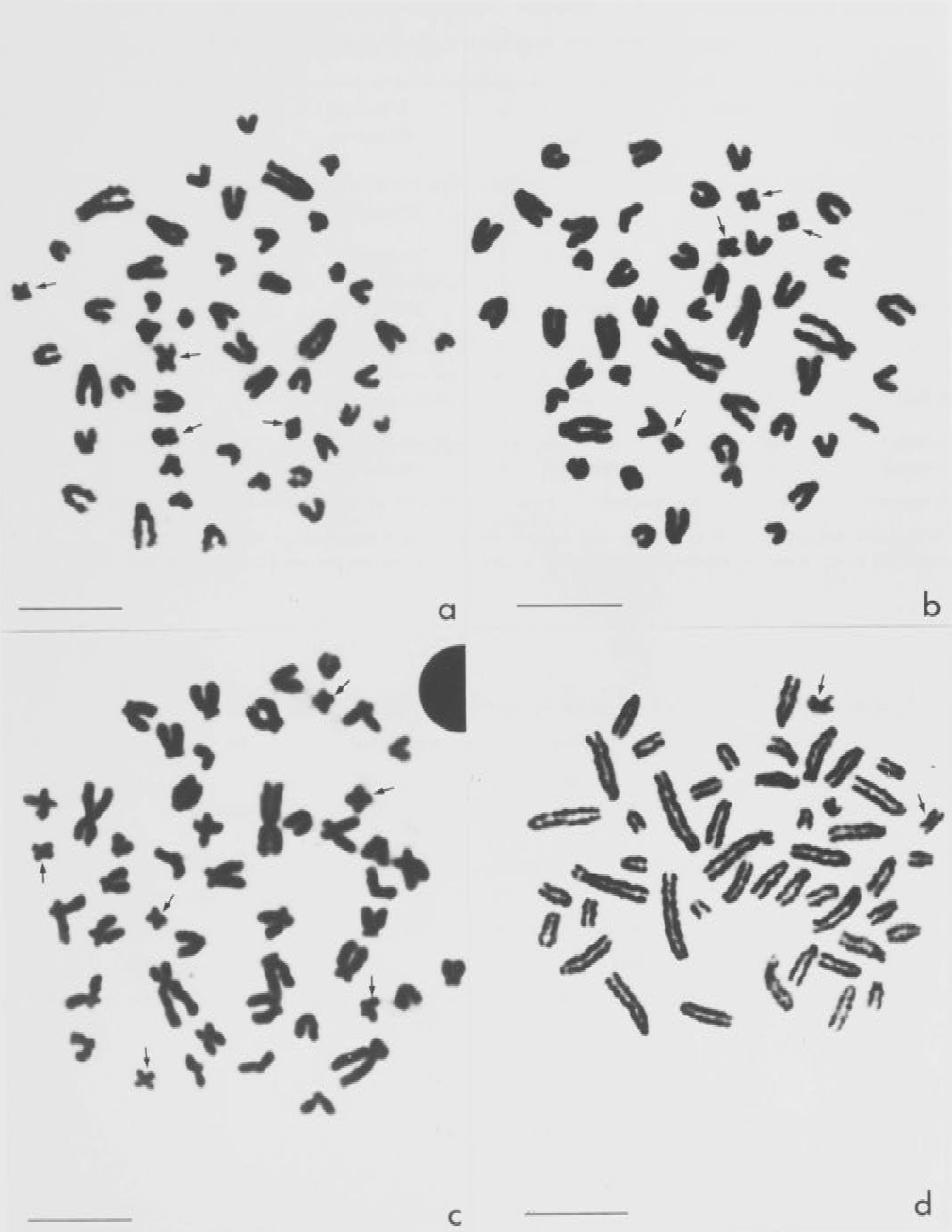

a
b
c
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Table 2.2: X chromosome forms in *Notomys* together with arm ratio characteristics and the percentage HEX calculated on a length basis. The values in the last three columns represent means and their standard errors, n = number of chromosome measured.

Species	Type of X Chromosome	n	Arm Ratio $\frac{\text{Longarm}}{\text{Shortarm}}$	Total X	Euchromatic Arm of X chromosome
Notomys mitchellii	Acrocentric	5	4.18±0.02	8.05±0.21	6.15±0.19
Notomys alexis	Metacentric	8	1.08±0.02	10.73±0.15	5.76±0.12
	Submetacentric	7	1.58±0.05	9.20±0.26	5.96±0.22
	Acrocentric	5	4.74±0.42	7.19±0.13	5.24±0.12
Notomys cervinus	Large metacentric*	7	0.78±0.01	10.54±0.17	5.20±0.15
	Medium metacentric	15	1.06±0.01	9.31±0.15	5.12±0.17
	Submetacentric	9	1.33±0.04	8.32±0.19	5.01±0.16
Notomys fuscus	Submetacentric	5	1.52±0.05	8.92±0.13	6.09±0.12
	Acrocentric	8	3.02±0.17	7.94±0.20	5.99±0.18

*In this case the ratio is short arm/long arm because this chromosome has its long arm composed of accessory gonosomal heterochromatin whereas as the other X chromosomes have long arms which are euchromatic.

Table 2.3 : Comparison of the sizes of the forms of Chromosome 1 in *Notomys alexis* in terms of the % HEX percentage on a length basis. n = number of chromosome measured.

Notomys alexis	n	Mean % HEX	Standard error of mean
Common form of Chromosome 1	10	8.36	0.09
Small form of Chromosome 1	10	6.57	0.05
Large form of Chromosome 1	10	9.45	0.04

(i) a metacentric (10.54% HEX),
(ii) a metacentric (9.31% HEX), and
(iii) a metacentric (8.32% HEX).

The Y chromosome was present in two forms a metacentric and an acrocentric both with 5.45% HEX (Table 2.4).

2.3.1.4 *N. fuscus* (2n = 48) has a sizegraded series of 22 pairs of telocentric and acrocentric autosomes and one small pair of metacentrics. The autosomes show little evidence of short arms. The X chromosome is found in two forms (Table 2.2):

(i) a submetacentric (8.92% HEX), and
(ii) a acrocentric (7.94% HEX).

The Y chromosome is acrocentric (7.54% HEX; Table 2.4). In addition, two of the automsomes are polymorphic. Pair 5 has a metacentric form and pair 8 has an acrocentric form.

The above observations largely agree with those of Baverstock *et al.* (1977b) on these species.

On the basis of size relationships each autosomal karyotype was divided into four size groups to facilitate scoring of chiasma characteristics in later meiotic studies (see Chapter 3):

(i) pair 1,
(ii) pairs 2 to 6,
(iii) pairs 7 to 20, and
(iv) pairs 21 to 23.

During meiosis, the sex chromosomes were found to form an association in male diakinetic figures which was highly distinctive allowing its immediate identification regardless of the precise forms of sex chromosomes involved.

Table 2.4: Y chromsomes expressed as a percentage of HEX on the basis of length measurement. The values are the means and their standard error. N = number of chromosomes measured.

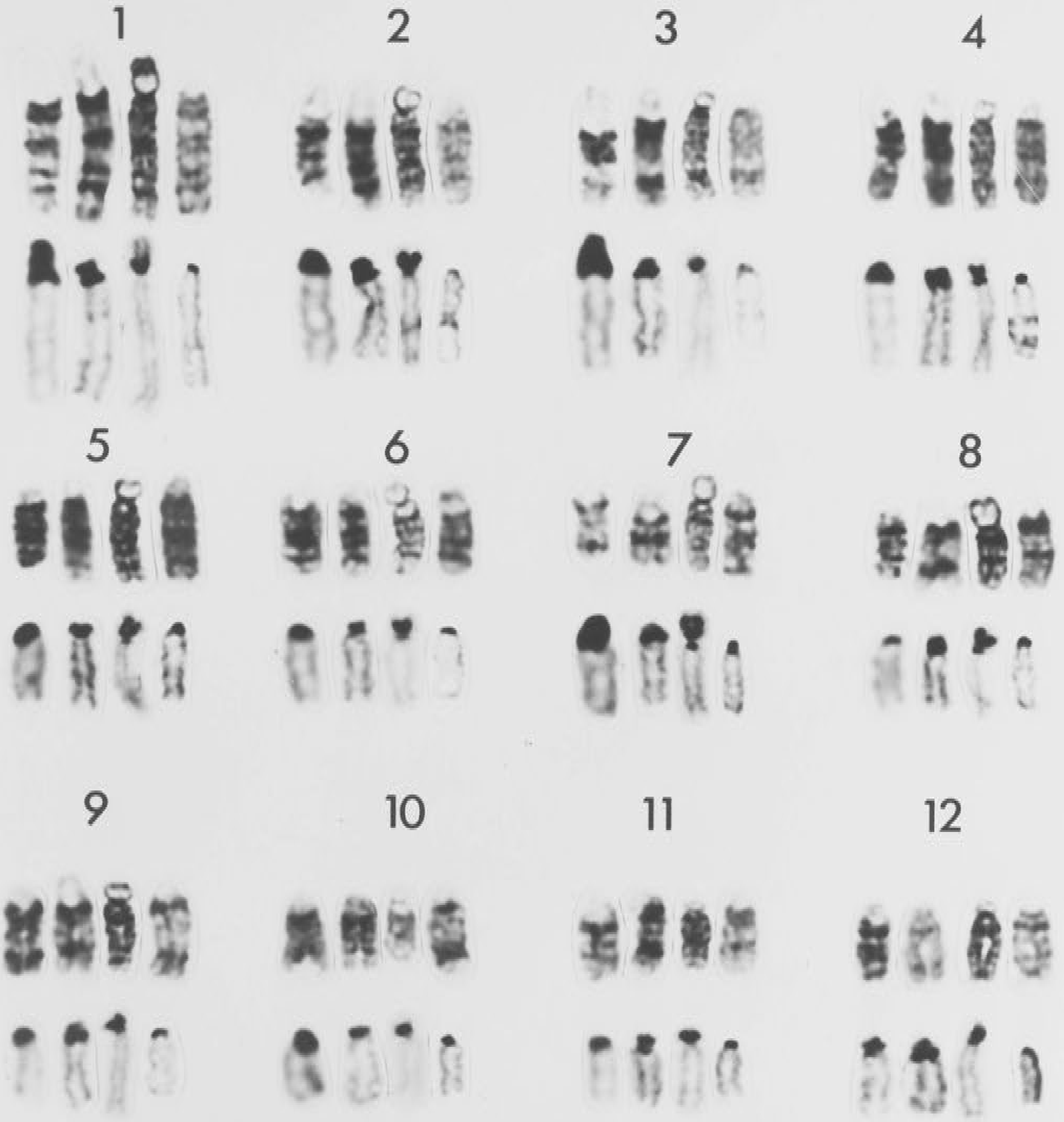
	Type of Y Chromosome	n	HEX ± S.E.
Notomys mitchellii	Acrocentric	5	4.58±0.03
Notomys alexis	Submetacentric	5	5.60±0.06
N	Acrocentric	5	4.84±0.10
Notomys cervinus	Metacentric to acrocentric	5	5.45±0.09
Notomys fuscus	Acrocentric	5	7.54±0.08

2.3.2 *C-banded karyotypes*

2.3.2.1 *Notomys mitchellii.* In this species the C-banding material in the autosomes is confined to the procentric regions, however, the non-homologous chromosomes show considerable variation in the amount of C-banding material (Fig. 2.2; Table 2.5). Chromosomes 1 and 19 have more short arm than long arm C-banding material, while chromosomes 3, 4, 6-10, 10, 13-17 have more long arm C-banding material. The remaining autosomes have approximately equal amounts of C-banding material in both long and short arms. In the two small metacentric chromosomes (pairs 21 and 22) this material is evenly distributed procentrically. Chromosomes 1, 3, 4, 18 and 19 have considerably more C-banding material than the remaining autosomes. Indeed chromosomes 18 and 19 would be placed higher in the karyotype sequence if only total chromosome length was considered since these chromosomes have more than 50% of their length in the form of C-banding material.

2.3.2.2 *Notomys alexis.* The C-banding material in this species is also confined to the procentric region of all autosomes. Chromosomes 1-4 have more C-banding material than the other autosomes. In the majority of the autosomes in *N. alexis* the C-banding material is

Figure 2.2 : G- and C-banding haryotypes of *Notomys*. The chromosomes are presented in decreasing numerical order of size using the *N. fuscus* karyotype as the standard for the ordering of the chromosomes. Each group of eight cutouts represent the homologous chromosome from each of the four species. The upper four cutout chromosomes in each group represent the G-banding pattern in the order *N. mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus*. The lower four cutout chromosomes in each group represent the C-banding pattern in the same order. The 23 autosomes of each species are presented together with the acrocentric X chromosomes of *N. mitchellii*, *N. alexis* and *N. cervinus* and the large metacentric X chromosome of *N. cervinus*.



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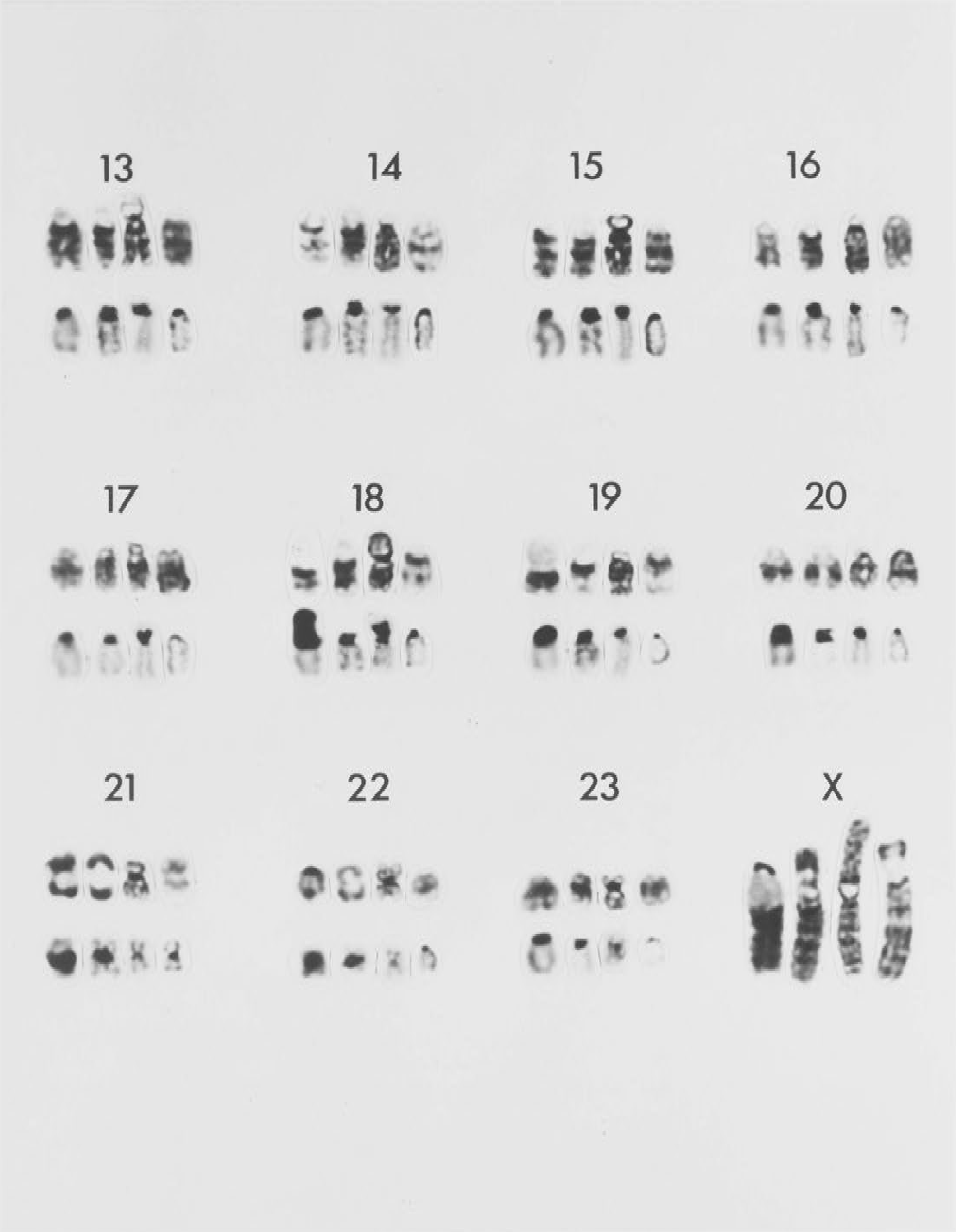
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23
X

Table 2.5 : Variation in the size of short arm (SA) and long arm (LA) constitutive heterochromatin in chromosomes 1 to 20 of *N. mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus*. Increases in number of + indicates increasing amounts of constitutive heterochromatin ranging from minium (min) to maximum (+++) while 0 = no detectable constitutive heterochromatin.

Chromosome		*N. mitchellii*	*N. alexis*	*N. cervinus*	*N. fuscus*
1	SA	+++	++	++	0
	LA	++	++	0	+
2	SA	+	++	++	+
	LA	+	++	+	+
3	SA	+	+	+	0
	LA	++	+	min	+
4	SA	+	+	++	min
	LA	++	++	+	+
5	SA	+	+	++	min
	LA	+	+	+	+
6	SA	min	min	++	min
	LA	+	+	+	+
7	SA	min	+	++	+
	LA	+	+	+	+
8	SA	min	+	++	min
	LA	+	+	+	+
9	SA	min	+	++	0
	LA	+	+	+	+
10	SA	min	min	+	0
	LA	+	+	min	+
11	SA	min	min	+	0
	LA	+	+	min	+
12	SA	++	++	+++	0
	LA	+	+	+	+
13	SA	min	+	++	0
	LA	+	+	min	+
14	SA	min	+	+	0
	LA	+	+	min	+
15	SA	min	+	++	0
	LA	+	+	min	+
16	SA	min	+	+	0
	LA	+	+	min	+
17	SA	0	0	+	0
	LA	+	+	+	+
18	SA	+++	+	+++	0
	LA	+++	+	+	+
19	SA	++	+	+	0
	LA	+	+	min	+
20	SA	+	+	+	0
	LA	+	+	+	+

evenly distributed between the long and short arms. However, there is more long arm heterochromatin in chromosomes 4, 6, 10 and 17.

As explained above chromosome 1 is polymorphic in length. Following C-banding these polymorphisms can be seen to involve different criteria (Fig. 3d i-iii).

(i) The 'common' form of chromosome 1 has both long arm and short arm constitutive heterochromatin (Fig. 2.3d i),

(ii) the 'small' form of chromosome 1 has long arm heterochromatin but lacks short arm heterochromatin (Fig. 2.3d ii) and is smaller than the other two forms of chromosome 1 (Table 2.3d), and

(iii) the 'large' form of chromosome 1 lacks any constitutive heterochromatin as well as lacking a short arm and is the largest of the three forms (Table 2.3).

In a breeding programme involving a male heterozygous for the 'common' and the 'small' forms of chromosome 1, the transmission of these types conformed to Mendelian expectations and there was no distortion of the sex ratio in the progeny (Table 2.6).

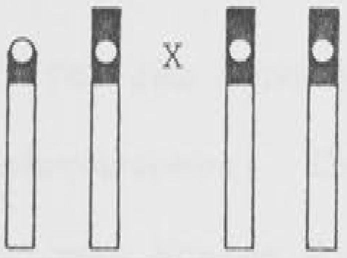
2.3.2.3 *Notomys cervinus*. The major feature of the C-banded karyotype of *N. cervinus* is the predominance of short arm heterochromatin in chromosomes 1, 2, 4, 5, 7-8, 9, 11, 13-15, 17 and 18. The remaining acrocentric autosomes have equal amounts of long and short arm heterochromatin. The three small metacentrics (pairs 21, 22 and 23) have less C-banding material than these elements in *N. mitchellii* or *N. alexis*. In chromosome 1 of *N. cervinus* the short arm is heterochromatic proximally with euchromatin located distally. This indicates a rearrangement due to either pericentric inverstion or translocation.

2.3.2.4 *Notomys fuscus*. The C-banded karyotype of *N. fuscus* differs markedly from those of the three other *Notomys* species. This

Table 2.6: Breeding data of a cross between a *Notomys alexis* male heterozygous for a chromosome 1 ('small form'/'common form') and 2 female *N. alexis* homozygous for the 'common form' of chromosome 1.

'small' form chromosome 1 = A1(-);
'common' form chromosome 1 = A1(+).

Male *N. alexis*
Heterozygous for
Chromosome 1
'Small form':
'Common form'
A1(+)/A1(-) XY



Female *N. alexis*
Homozygous for
Chromosome 1
'Common form'
A1(+)/A1(+) XX

Gametes		Female Gametes A1(+) X	 A1(+) X	Number of Progeny Obtained
A1	A1(+) Y	A1(+)/A1(+) XY	A1(+)/A1(+) XY	5 Homozgous Males
Male	A1(+) X	A1(+)/A1(+) XX	A1(+)/A1(+) XX	2 Homozygous Females
Gametes	A1(-) Y	A1(+)/A1(-) XY	A1(+)/A1(-) XY	2 Heterozygotes Males
	A1(-) X	A1(+)/A1(-) XX	A1(+)/A1(-) XX	4 Heterozygotes Females
			Progeny	7 Males 6 Females
			Composition	7 Homozygous 6 Heterozygous

Litter Characteristics of the above cross

Litter number	Litter size	Sex of Offspring Males	 Females
1	4	3	1
2	4	2	2
3	1	1	-
4	3*	-	1
5	4	2	2
Totals	16	8**	6

* 2 unsexed young eaten by mother

** 1 male not karyotyped

karyotype has much less C-banding material which is largely confined to the long arms of the autosomes. Only chromosomes 2, 4-8, 11 and 14 have detectable amounts of short arm heterochromatin and even these amounts are minimal (Fig. 2.2; Table 2.5).

2.3.2.5 *The sex chromosomes*

X chromosomes: The chromatin of the X chromosomes of the four species of *Notomys* stains differentially with the C-banding technique which allows three types of chromatin within the X to be distinguished (Fig. 2.3a i-viii):

(i) *Euchromatin* - which stains with a light intensity equivalent to that of the euchromatin of the autosomes

(ii) *Constitutive heterochromatin* - which stains intensely and is located at the centromere and adjacent to it

(iii) *Accessory gonosomal heterochromatin* - which stains variably in between the extremes of the euchromatin and the intense staining centric heterochromatin.

Y chromosomes: The Y chromosomes of the four species of *Notomys* vary in their response to C-banding (Fig. 2.3b, i-viii). These variations range through three forms:

(i) In some cases the Y chromosome stains almost as intensely as the constitutive heterochromatin of the pericentromeric regions of the autosomes and the X chromosomes (Fig. 2.3b, iii and ix).

(ii) In other cases, while the Y chromosome stains intensely, its centromeric heterochromatin stains even more intensely (Fig. 2.3b, i and v).

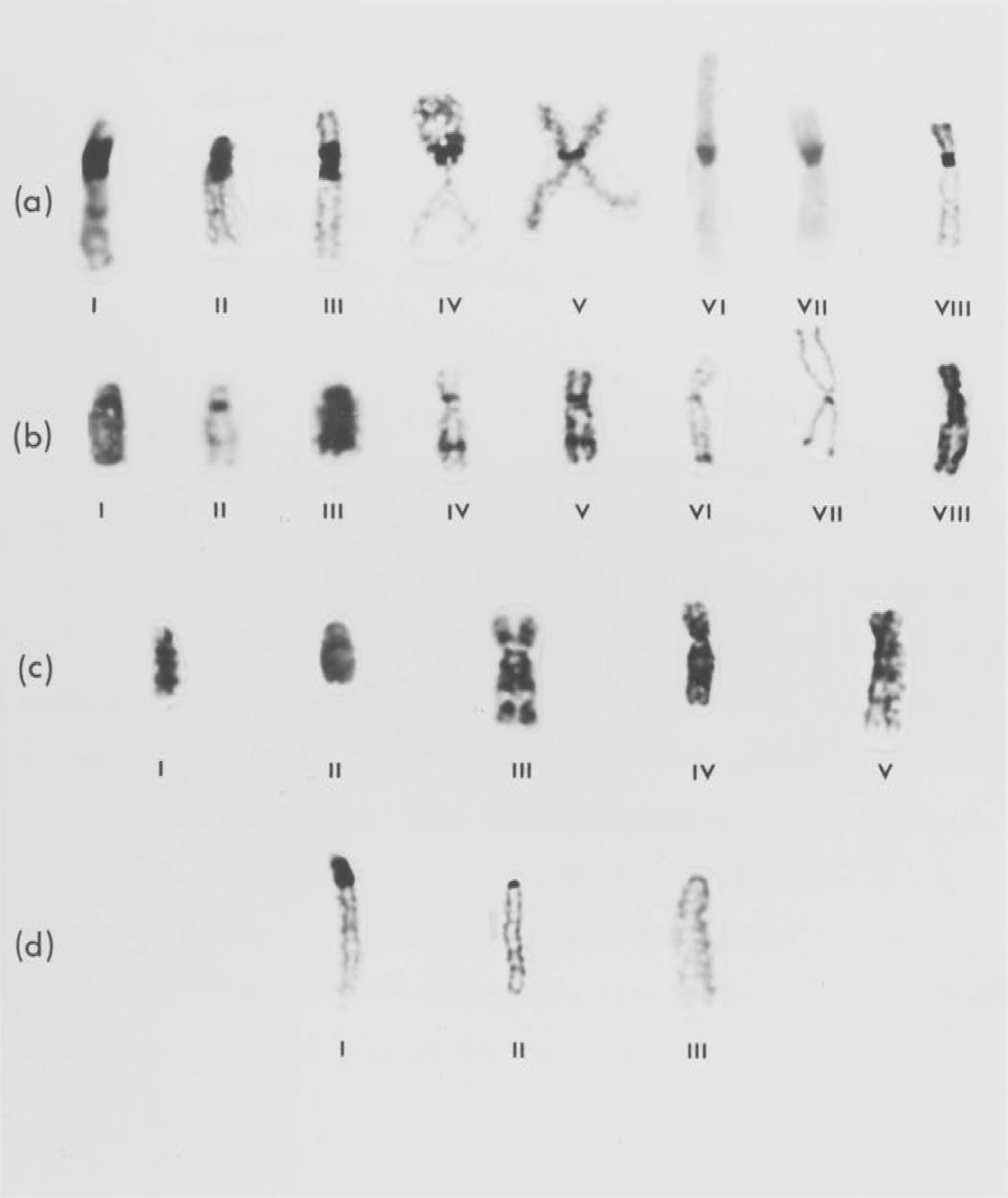
(iii) In still other cases the Y chromosome includes two distinct intensely stained C-band positive regions which are equal in intensity to the pericentromeric constitutive heterochromatin of the autosomes and the X chromosomes. These

Figure 2.3 (a): C-banding of the X chromosome of *Notomys*
(i) Acrocentric X chromosome of *N. mitchellii*
(ii) Acrocentric X chromosome of *N. mitchellii*
(iii) Acrocentric X chromosome of *N. alexis*
(iv) Metacentric X chromosome of *N. alexis* (from meiotic metaphase II cell)
(v) Submetacentric X chromosome of *N. cervinus* (from meiotic metaphse II cell)
(vi) Large metacentric X of *N. cervinus*
(vii) Submetacentric X of *N. cervinus*
(viii) Acrocentric X chromosome of *N. fuscus*

(b): C-banding of the Y chromosome of *Notomys*
(i) Acrocentric Y chromosome of *N. mitchellii*
(ii) Acrocentric Y chromosome of *N. mitchellii*
(iii) Acrocentric Y chromosome of *N. alexis*
(iv) Submetacentric Y chromosome of *N. alexis*
(v) Submetacentric Y chromosome of *N. alexis*
(vi) Submetacentric Y chromosome of *N. cervinus*
(vii) Submetacentric Y chromosome of *N. cervinus*
(viii) Acrocentric Y chromosome of *N. fuscus*

(c): G-banding of the Y chromosomes of *Notomys*
(i) Acrocentric Y chromosomes of *N. mitchellii*
(ii) Acrocentric Y chromosome of *N. mitchellii*
(iii) Submetacentric Y chromosome of *N. alexis*
(iv) Submetacentric Y chromosome of *N. cervinus*
(v) Acrocentric Y chromosome of *N. fuscus*

(d): C-banding of *Notomys alexis* chromosome 1 polymorphs
(i) 'Common' form
(ii) 'Small' form
(iii) 'Large' form


(a)
I
II
III
IV
V
VI
VII
VIII
(b)
I
II
III
IV
V
VI
VII
VIII
(c)
I
II
III
IV
V
(d)
I
II
III

cases involve the Y chromosomes of *N. alexis* and *N. cervinus*. In the submetacentric Y chromosome of *N. alexis* the pericentromeric region is C-band positive together with an interstitial region in the distal third of the long arm (Fig. 2.3b, iv-v). In the Y chromosome of *N. cervinus* there is a procentricC-band positive site situated in one arm only and this may involve either the long arm (Fig. 2.3b, vi) or the short arm (Fig. 2.3b, vii). In both these chromosomes the telomeres of the long arm and the short arm respectively are C-band positive (Fig. 2.3b, vi and vii).

2.3.2.6 *Genome content of C-banding material*

The total constitutive heterochromatin content of the genomes of the four species of *Notomys* was determined using chromosome length measurement (Table 2.7). The total length of the genome used for calculating the C-banding content includes the total length of the autosomes plus the euchromatic arm and pericentromeric C-banding material of the X chromosomes. It excludes the remaining portion of the various X chromosomes which is entirely accessory gonosomal heterochromatin. The exclusion of this material of the X chromosomes allows direct comparison of the constitutive heterochromatin content between species without the effects of variation in the size of the various X chromosomes both within and between species.

Table 2.7: The total constitutive heterochromatin content of the four species of *Notomys* expressed as a percentage of genome length based on the female haploid karyotype defined as the autosomes + the euchromatic arm of the X chromosome together with the pericentromeric heterochromatin but excluding the non-euchromatic arm of the X chromosome.

Species	n	Total constitutive heterochromatin in female haploid karyotype %	Standard error of mean
Notomys mitchellii	5	28.45	1.14
Notomys alexis	6	24.00	1.36
Notomys cervinus	8	25.51	1.01
Notomys fuscus	5	13.66	0.71

In order to facilitate the subsequent scoring of the chiasma characteristics in male meiosis across the four species, the distribution of constitutive heterochromatin in the genomes was considered in three categories (Table 2.8):

(i) Total constitutive heterochromatin in the 20 longest autosomes expressed as a percentage of their total length,

(ii) Total long arm constitutive heterochromatin present in the 20 longest autosomes expressed as a percentage of their total length, and

(iii) Total short arm constitutive heterochromatin present in the 20 longest autosomes expressed as a percentage of their total length.

The 20 largest autosomes in all these species are either telocentric or acrocentric chromosomes and with the exception of chromosome 1 of *N. cervinus* the short arms of the acrocentric chromosomes are all heterochromatic. Therefore the restriction of the comparison to the 20 largest autosomes avoids any complication arising from pericentric inversions.

Table 2.8 : Distribution of autosomal constitutive heterochromatin as revealed by C-banding in *Notomys mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus*, expressed as a percentage of autosomes 1-20 in each case. The values in the first three columns are the mean percentages together with the standard error of the mean. n = number of karyotypes measured.

Species	Total heterochromatic material in autosomes 1-20 expressed as a percentage	Percentage of long arm heterochromatin (LAH)	Percentage of short arm heterochromatin (SAH)	$\frac{SAH}{LAH}$
Notomys mitchellii	27.19±1.17 n = 5	13.38±0.86	13.81±0.52	1.04±0.06
Notomys alexis	22.82±1.28 n = 6	11.08±0.59	11.74±0.72	1.06±0.03
Notomys cervinus	24.39±0.78 n = 6	6.77±0.53	17.62±0.54	2.69±0.24
Notomys fuscus	13.13±0.63 n = 9	11.38±0.50	1.75±0.15	0.15±0.01

The three smallest autosomes on the other hand vary in structure (Fig. 2.1) - chromosomes 21 and 22 are metacentric and 23 is acrocentric in both *Notomys mitchelli* and *N. alexis;* in *N. cervinus* chromosomes 21, 22 and 23 are all metacentric while in *N. fuscus* chromosome 21 is metacentric and chromosomes 22 and 23 are both acrocentric. Consequently, these three chromosomes were also excluded from comparisons of chiasma position because of their varying structure in the different species. The chiasma data from these chromosomes were, however, included in chiasma frequency scoring (see Chapter 3).

2.3.3 *G-banded karyotype*

2.3.3.1 *General features of the G-banded karyotypes.* The G-banded karyotypes of the four species of *Notomys* (Fig. 2.2) reveal three clear features:

(i) On the basis of G-band pattern there appears to be a considerable degree of homology in the banding patterns of the euchromatic portions of the genomes (Fig. 2.2),

(ii) The heterogeneity in the distribution of constitutive heterochromatin both between and within species is clearly demonstrated with G-banding because regions which are C-banded positive, in general, show up as negative G-bands (Fig. 2.2), and

(iii) In *N. mitchellii, N. alexis* and *N. fuscus* all regions which were C-band positive are present as negative G-bands. However in *N. cervinus* all short and long arm heterochromatic regions are G-band negative, but the centromeric heterochromatin is G-band positive. This is especially clear in chromosomes 2, 6, 7, 10, 13, 17, 18 and 20 (see Fig 2.2) but is a general feature of this species.

Turning to the individual species there are a number of important points to note:

2.3.3.2 *Notomys mitchellii*. Technically *N. mitchellii* proved to be the most difficult karyotype of the four species to G-band. G-banding, however, proved vital for the correct identification of chromosome 18 and 19 within the karyotype. These chromosomes have in excess of 50% of their length as constitutive heterochromatin and in standard karyotype preparations would be placed much higher in the size order (Fig. 2.2). Of course C-banding would indicate the absence of euchromatin in one arm of these chromosomes but would not afford positive identification of the chromosome.

2.3.3.3 *Notomys alexis*. The G-banding karyotype of *N. alexis* reveals a close homology with that of *N. mitchellii* with respect to the euchromatic portion of the genome. The difference in the distribution of constitutive heterochromatin between *N. alexis* and *N. mitchellii* is as clearly revealed with G-banding as it is with C-banding (Fig. 2.2).

2.3.3.4 *Notomys cervinus*. The centromeric heterochromatin in *N. cervinus* is G-band positive in contrast to the condition in the other three species of *Notomys* in which the centromeric heterochromatin is G-band negative. The long and short arm heterochromatin which is contiguous with the centromeric heterochromatin is however G-band negative as it is in the other *Notomys* species.

Chromosome 1 is fixed for a rearrangement which Baverstock *et al.* (1977b) attributed to a pericentric inversion on the basis of C-banding. G-banding indicates that the long arms of chromosome 1 in *N. mitchellii*, *N. alexis* and *N. fuscus* have homologous band patterns to the long arm of *N. cervinus* (Fig. 2.2). Added to this, on the basis of length measurements *N. cervinus* pair 1 chromosomes consistently contains similar amounts of euchromatin in the long arm as does those of the other three *Notomys* species (Table 2.9). This implies that the euchromatin present at the telomeric region of the short arm in *N. cervinus* chromosome 1 must represent additional material and cannot be explained by a pericentric inversion within chromosome 1 of *N. cervinus*. Indeed the total euchromatin content of the *N. cervinus* chromosome 1 (i.e. long and short arm euchromatin) is 5.68% (20EAK (S.E. 0.19).

Table 2.9: Euchromatin present in the long arm of chromosome 1 of *Notomys mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus* expressed as a percentage of the total euchromatin in the 20 autosome karyotype (20EAK). n = number of chromosomes examined.

Species	n	Euchromatin in chromosome 1 expressed as a percentage of 20EAK (long arm only)	Standard error of mean
Notomys mitchellii	6	4.86	0.11
Notomys alexis	10	4.70	0.18
Notomys cervinus	12	5.22	0.10
Notomys fuscus	10	4.60	0.17

2.3.3.5 *Sex chromosomes*

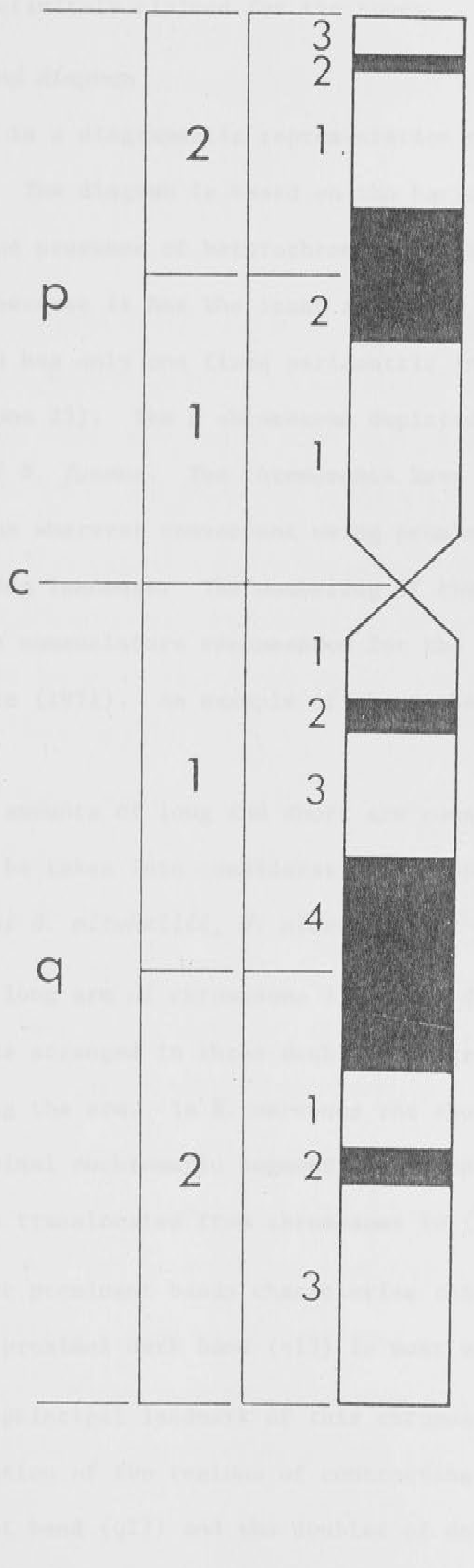
X chromosomes. The euchromatic arm of the X chromosome has a similar G-banding pattern in each of the *Notomys* species (Fig. 2.2) and this pattern is found in all the various polymorphic forms of the X chromosome. The non-euchromatic arm does not G-band clearly except at the proximal region which has two very distinct bands, a negative band adjacent to the centromere followed by a positive band distal to it.

Y chromosome. The Y chromosome in general G-banded inconsistently, particularly in *N. mitchellii*, though in some preparations relatively clear G-banding was obtained (Fig. 2.3c, i-v). The submetacentric Y of *N. alexis* gave consistently good G-banding and the pattern clearly shows the two C-band positive regions as G-band negative (compares Fig. 2.3b, iv with Fig. 2.3c, iii). There are certainly similarities in the patterns between the species but precise homo-

Figure 2.4 : Example to illustrate the convention for the numbering of the regions and the bands within those regions of *Notomys* G-banded chromosomes. The convention used follows that of the Paris Conference (1971).

c = centromere; p = short arm; q = long arm.

Explanation of the division of the short arm into two regions. The large positive G-band is taken as the landmark for this arm. In other cases a prominent negative band may be used. The centrepoint of the landmark band becomes the border between region 1 and 2. The landmark itself in its entirety becomes p12. The numbering of the bands within each region commences with 1 and proceeds from the centromere with each positive and negative band receiving consecutive numbers. Thus the small positive band in the short arm is numbered p22. Similarly in the long arm the landmark band is q14, whereas the proximal positive band is q12. No attempt has been made here to extend this system to include occasions where a landmark band is subsequently found to comprise a number of finer bands.


p
c
q
2
1
1
2
3
2
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2
1
1
2
3
4
1
2
3

Figure 2.4

logies cannot be definitely claimed for the bands.

2.3.3.6 *G-band diagram*

Fig. 2.5 is a diagrammatic representation of the G-bands in the genus *Notomys*. The diagram is based on the karyotype of *Notomys fuscus* excluding the presence of heterochromatic short arms. *Notomys fuscus* was chosen because it has the least amount of constitutive heterochromatin and has only one fixed pericentric inversion in its autosomes (chromosome 23). The X chromosome depicted represents the acrocentric form of *N. fuscus*. The chromosomes have been divided into two or three regions wherever convenient using prominent positive G-bands as the dividing landmark. The numbering of the bands is in accordance with the nomenclature recommended for the human karyotype by the Paris Conference (1971). An example of the numbering system is shown in Fig. 2.4.

The differing amounts of long and short arm constitutive heterochromatin needs to be taken into consideration in applying the diagram to the karyotypes of *N. mitchellii*, *N. alexis* and *N. cervinus*.

Chromosome 1: The long arm of chromosome 1 has six distinct dark bands arranged in three doublets distributed evenly along the arm. In *N. cervinus* the short arm has a terminal euchromatic segment which appears to have been translocated from chromosome 10 (Fig. 2.6).

Chromosome 2: Three prominent bands characterize this chromosome, the proximal dark band (q13) is most obvious.

Chromosome 3: The principal landmark of this chromosome is the adjacent location of two regions of contrasting intensity; the light band (q27) and the doublet of dark bands (q31) and (q33).

Chromosome 4: Four prominent dark bands are a distinctive feature of this chromosome, together with a doublet of medium bands (q22) and (q24), which result in a characteristic lighter region in the distal half of the chromosome.

Chromosome 5: Five distinct dark bands form the basis for the identification of the chromosome. The four proximal bands form two doublets consisting respectively of bands (q12) and (q14) and bands (q21) and (q23).

Chromosome 6: Four distinct dark bands are evenly distributed along this chromosome. Band (q14) is thinner than the remaining three bands in some preparations and it may also fuse with band (q21).

Chromosome 7: Four dark bands (q14, q21, q23 and q27) contribute to a diagnostic pattern which is, however, variable due to differing widths and coalescence of the bands. Bands (q21) and (q23) are usually present as a doublet but sometimes they coalesce. On the other hand, bands (q14) and (q27) vary in prominence.

Chromosome 8: This chromosome has a very distinct banding pattern due to two dark bands, the very large proximal band (q12) and the interstitial band (q21).

Chromosome 9: Two distinct dark bands (q12) and (q21) characterize this chromosome and result in dark regions both proximally and distally separated by a much lighter intervening region.

Chromosome 10: The landmark in this chromosome in *N. mitchellii*, *N. alexis* and *N. fuscus* is dark band (q21). In *N. cervinus*, however, some of this band together with the terminal segment have been translocated to form the short arm of chromosome 1 (Fig. 2.6).

Chromosome 11: Two doublets contribute to the distinctive pattern of this chromosome. The proximal doublet formed by dark bands (q12) and (q21) is very prominent and sometimes involves varying coalescence. The second doublet formed by bands (q26) and (q31), while always present, varies in intensity.

Chromosome 12: Five evenly spaced dark bands give a distinctive pattern to chromosome 12. That of (q21) is more prominent than the remaining four.

Chromosome 13: Three prominent dark, evenly spaced, bands of equal intensity identify this chromosome.

Chromosome 14: Here also three dark bands identify the chromosome, however, they lack both the prominence and the equality of width evident in those of chromosome 13.

Chromosome 15: In this chromosome the three dark bands which characterize it differ in width - interstitial band (q21) is much thicker than bands (q12) and (q23) which are approximately equal.

Chromosome 16: This chromosome includes four thin bands evenly spaced as its diagnostic feature.

Chromosome 17: Three bands, of which the middle one (q21) is greater in width than the remaining two dark bands, serve to identify this element.

Chromosome 18: Two prominent dark bands (q12) and (q14) provide a distinctive marker.

Chromosome 19: The prominent proximal dark bank (q12) is diagnostic in most preparations. However, differential contraction complicates identification in some cells and band (q12) may be present as a doublet of two dark bands.

Chromosome 20: The single interstitial dark band (q13) is clear and prominent in *N. mitchellii*, *N. alexis* and *N. fuscus*. This is not the case in *N. cervinus* where two distinct bands replace it.

Chromosome 21: In this metacentric element both arms have a similar pattern. Each arm has a single dark band - (q12) and (p12).

Chromosome 22: In *N. fuscus* this chromosome has two dark bands - (q12) which is very fine and (q15) which is the only major dark band. In *N. mitchellii*, *N. alexis* and *N. cervinus* it is clear that there has been a pericentric inversion.

Chromosome 23: Here a single dark band (q13) provides a convenient landmark. In *N. cervinus* this chromosome is metacentric, presumably as a result of pericentric inversion.

X chromosome: The acrocentric form has a short arm with a distinctive dark band (p12) which is also present in all other forms of the X chromosome. The long arm has two prominent dark bands (q16) and (q21) although bands (q12) and (q25) become more prominent in more contracted X chromosomes.

Y chromosome: The acrocentric form of the Y chromosome of *N. alexis* alone provided banding of sufficient quality to warrant a diagram. The landmarks are three light bands - (p12), (q12) and (q21).

Figure 2.5 : Diagram of G-band patterns of *Notomys* chromosomes. This diagram is based on the G-band karyotype of *Notomys fuscus*, but the short arm heterochromatin is excluded. The first band of the long arm represents the constitutive heterochromatin of that arm and is always a negative G-band. The Y chromosome depicted is that of the acrocentric form found in *N. alexis*. In addition the metacentric form of chromosome 22 present in *N. mitchellii*, *N. alexis* and *N. cervinus* is shown (see 22a) as too is the metacentric form of 23 from *N. cervinus* (see 23a). The banding intensity was graded into three categories and represented as follows: Black = dark G-band; striped = medium G-band; white = light G-band.

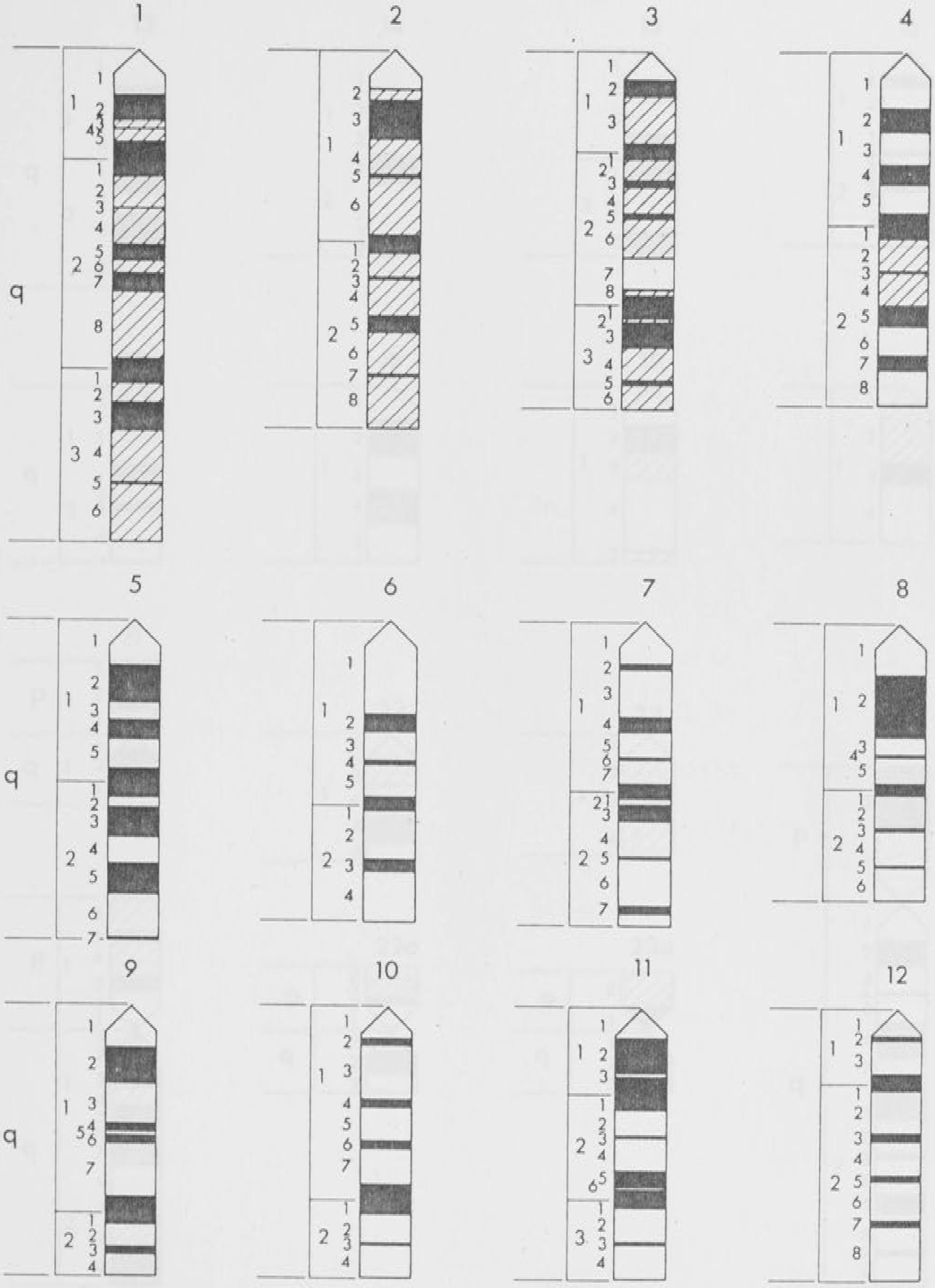
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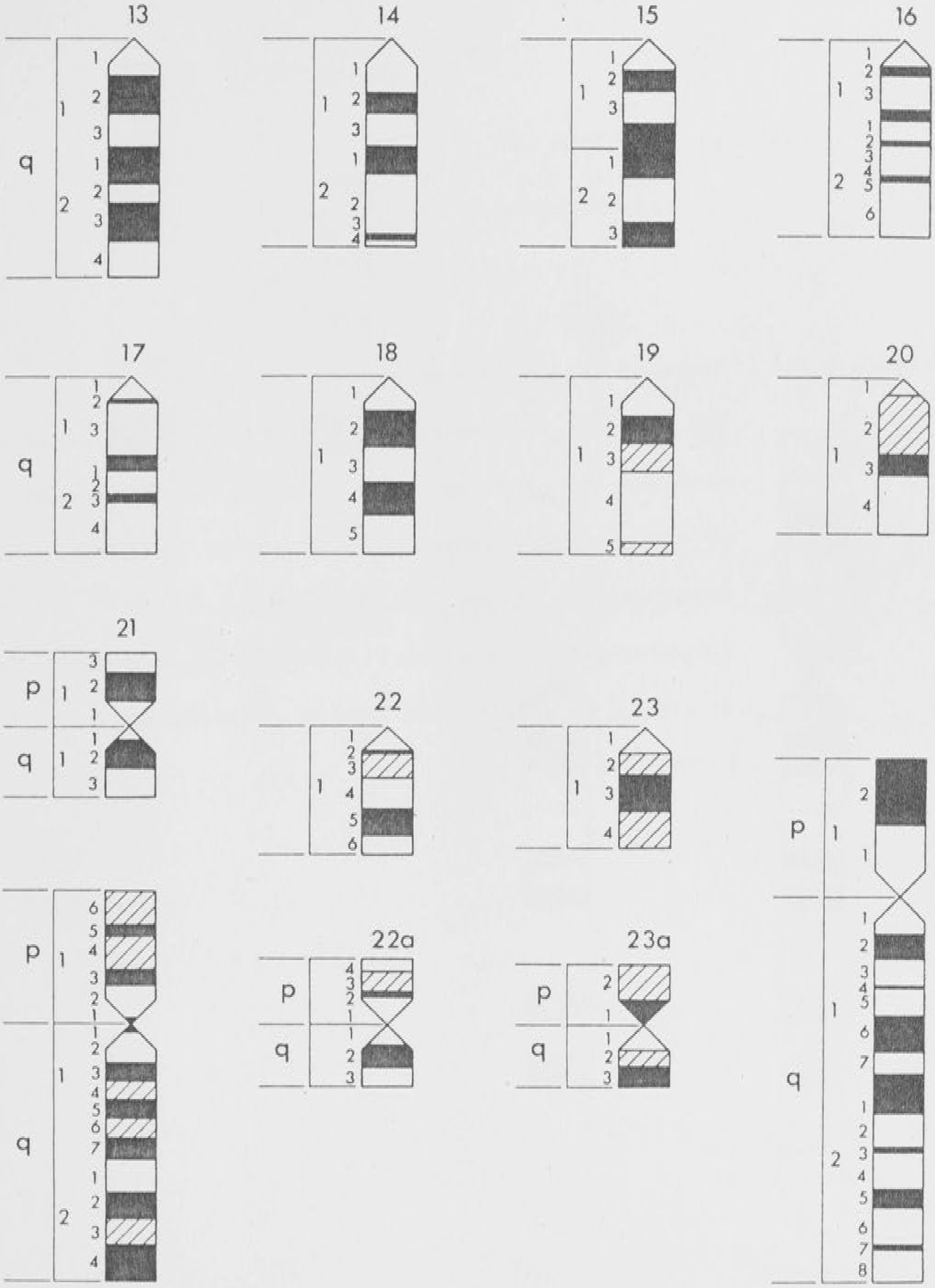
Figure 2.5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17
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19
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21
22
23
22a
23a
p
q

Figure 2.5 (cont)

Figure 2.6 : Proposed origin of *Notomys cervinus* chromosome 1. The original *N. cervinus* chromosome 10, which would have had the same banding pattern as the chromosome 10 common to the other *Notomys* species, undergoes translocation of its terminal segment to the telomere of the original *N. cervinus* chromosome 1 to result in the present form of *N. cervinus* chromosome 1.

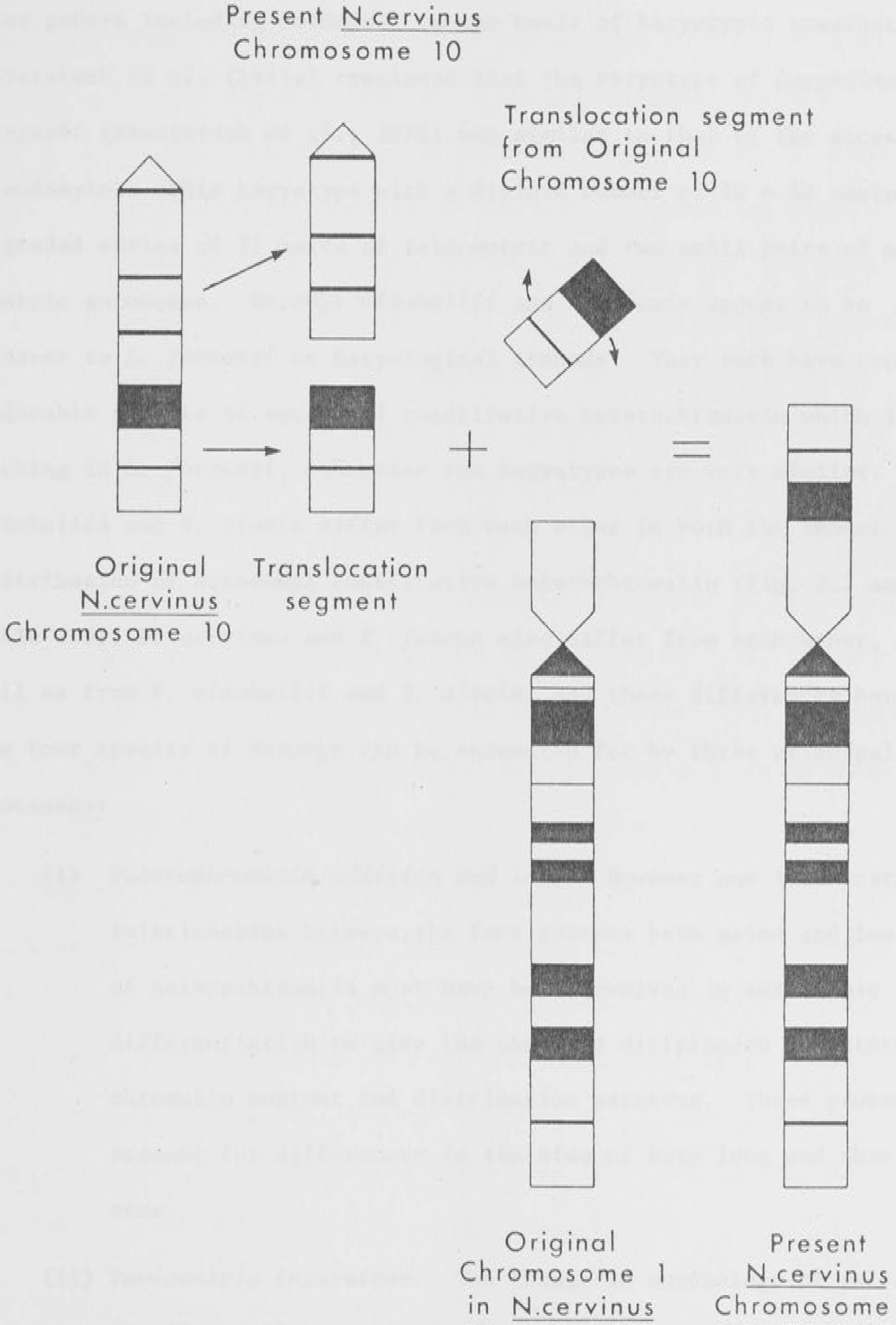

Present N.cervinus Chromosome 10
Translocation segment from Original Chromosome 10
+
=
Original N.cervinus Chromosome 10
Translocation segment
Original Chromosome 1 in N.cervinus
Present N.cervinus Chromosome 1

Figure 2.6

2.4 *Discussion*

The subfamily Pseudomyinae belongs exclusively to the Australian region (Tate, 1951; Simpson, 1961; Ride, 1970; Keast, 1972) and comprises nine genera including *Notomys*. On the basis of karyotypic examination Baverstock *et al.* (1977a) concluded that the karyotype of *Leggadina forresti* (Baverstock *et al.*, 1976) was similar to that of the ancestral pseudomyine. This karyotype with a diploid number of 2n = 48 contains a graded series of 21 pairs of telocentric and two small pairs of metacentric autosomes. *Notomys mitchellii* and *N. alexis* appear to be closest to *L. forresti* on karyological grounds. They each have considerable amounts of autosomal constitutive heterochromatin which is lacking in *L. forresti*, otherwise the karyotypes are very similar. *N. mitchellii* and *N. alexis* differ from each other in both the amount and distribution of autosomal constitutive heterochromatin (Fig. 2.2 and Table 2.5). *N. cervinus* and *N. fuscus* also differ from each other, as well as from *N. mitchellii* and *N. alexis*, and these differences between the four species of *Notomys* can be accounted for by three principal processes:

(i) *Heterochromatin addition and loss*. However one interprets the relationships between the four species both gains and losses of heterochromatin must have been involved in karyotypic differentiation to give the observed differences in heterochromatin content and distribution patterns. These processes account for differences in the size of both long and short arms.

(ii) *Pericentric inversions*. The change in morphology of pairs 21, 22 and 23 have all involved pericentric inversions. If, as Baverstock *et al.* (1977a) have concluded, *Leggadina* does indeed have the ancestral karyotype then inversions have converted an acrocentric to a metacentric in *N. cervinus*, while in *N. fuscus* one of the small metacentrics must have re-inverted. *N. fuscus* is also polymorphic for pericentric inversions in chromosomes 5 and 8.

(iii) *Presumptive translocation.* The G-banding of the long arms of pair 1 of all four species of *Notomys* suggests complete homology. The terminal euchromatic segment of the *N. cervinus* pair 1 short arm appears on the basis of G-banding homology to have arisen from the terminal section of the long arm of pair 10. Baverstock *et al.* (1977b), on the basis of C-banding, earlier proposed that a pericentric inversion followed by addition of heterochromatin to the proximal region was responsible for the production of the short arm of pair 1 of *N. cervinus*.

Length measurements of the euchromatin content of pair 1 chromosome from all four species of *Notomys* (Table 2.9) indicate that there is more euchromatin in *N. cervinus* pair 1 chromosomes than in the three other *Notomys* species. If this has not arisen by translocation then its origin remains unexplained.

These differences allow the construction of a phylogenetic tree which reflects the chromosomal relationships between the species (Fig. 2.7). These relationships suggest that *Notomys mitchellii* and *N. alexis* are more closely related to each other chromosomally than either is to *N. cervinus* or *N. fuscus*. *Notomys cervinus* and *N. fuscus* display a greater separation karyotypically from each other than either does from the duo of *N. mitchellii* and *N. alexis*.

2.4.1 *G-band comparisons*

Comparisons of the G-banded karyotypes of closely related species indicate that the arrangements of G-bands have often been highly conserved during evolution (Lejeune *et al.*, 1973; Pathak *et al.*,

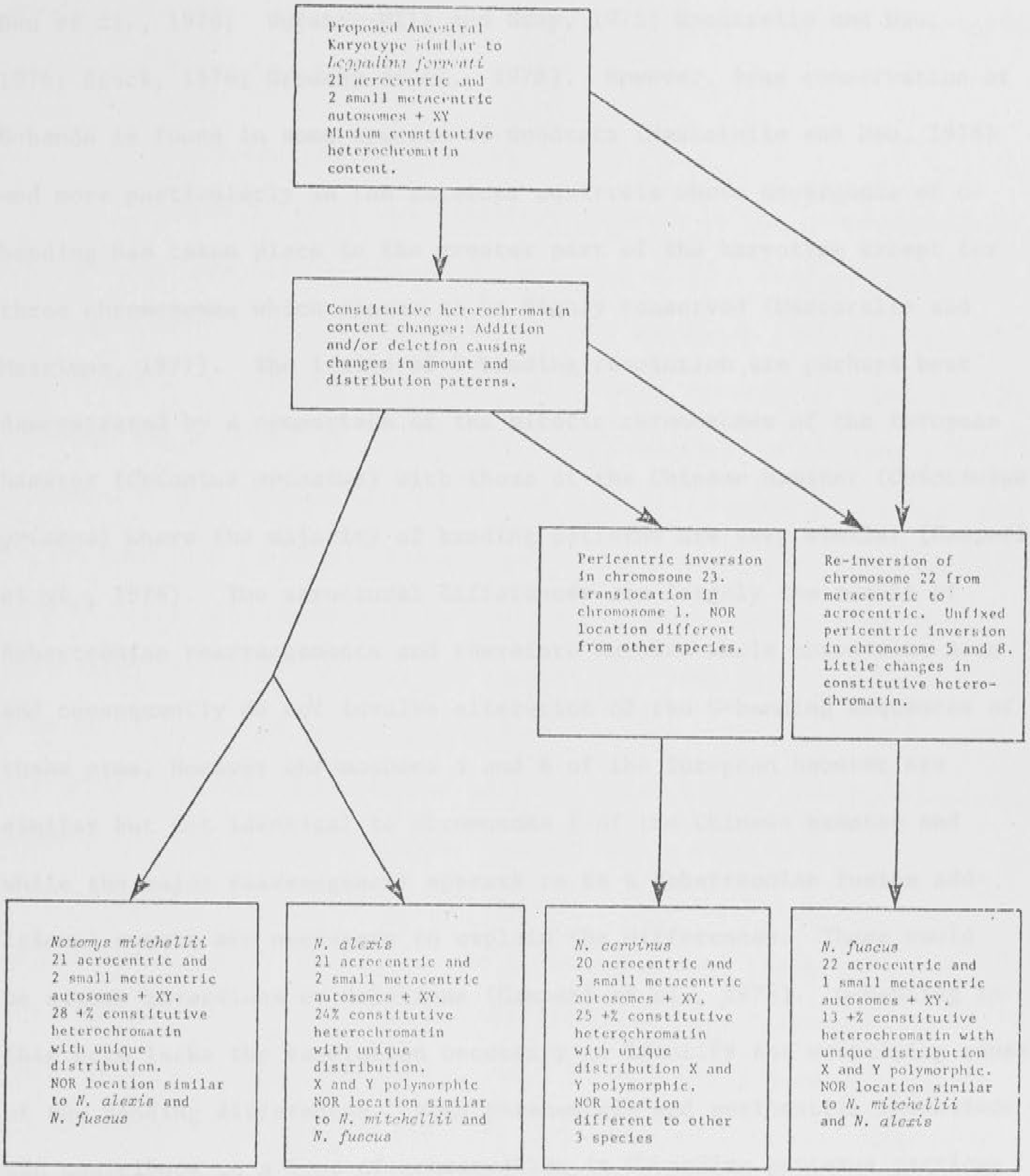

Proposed Ancestral Karyotype similar to *Leggadina forresti* 21 acrocentric and 2 small metacentric autosomes + XY Minium constitutive heterochromatin content.
Constitutive heterochromatin content changes: Addition and/or deletion causing changes in amount and distribution patterns.
Pericentric inversion in chromosome 23. translocation in chromosome 1. NOR location different from other species.
Re-inversion of chromosome 22 from metacentric to acrocentric. Unfixed pericentric inversion in chromosome 5 and 8. Little changes in constitutive hetero-chromatin.
Notomys mitchellii 21 acrocentric and 2 small metacentric autosomes + XY. 28 +% constitutive heterochromatin with unique distribution. NOR location similar to *N. alexis* and *N. fuscus*
N. alexis 21 acrocentric and 2 small metacentric autosomes + XY. 24% constitutive heterochromatin with unique distribution. X and Y polymorphic NOR location similar to *N. mitchellii* and *N. fuscus*
N. cervinus 20 acrocentric and 3 small metacentric autosomes + XY. 25 +% constitutive heterochromatin with unique distribution X and Y polymorphic. NOR location different to other 3 species
N. fuscus 22 acrocentric and 1 small metacentric autosomes + XY. 13 +% constitutive heterochromatin with unique distribution X and Y polymorphic. NOR location similar to *N. mitchellii* and *N. alexis*

Figure 2.7: A chromosomal phylogeny on the genus *Notomys* based on the karyotypic characteristics observed in G- and C-banded and silver stained preparations and assuming an ancestral karyotype similar to *Leggadina forresti* as proposed by Baverstock *et al.* (1977a).

1973a,b; Stock and Hsu, 1973; Arnason 1974a,b; Pathak and Stock, 1974; Hsu *et al.*, 1978; Wurster-Hill and Gray, 1975; Mascarello and Hsu, 1976; Stock, 1976; Grouchy *et al.*, 1978). However, less conservation of G-bands is found in some species of woodrats (Mascarello and Hsu, 1976) and more particularly in the antelope squirrels where divergence of G-banding has taken place in the greater part of the karyotype except for three chromosomes which appear to be highly conserved (Mascarello and Mazrimas, 1977). The limits of G-banding resolution are perhaps best demonstrated by a comparison of the mitotic chromosomes of the European hamster (*Cricetus cricetus*) with those of the Chinese hamster (*Cricetulus griseus*) where the majority of banding patterns are very similar (Gamperl *et al.*, 1976). The structural differences are largely the result of Robertsonian rearrangements and therefore involve whole chromosome arms and consequently do not involve alteration of the G-banding sequences of those arms. However chromosomes 5 and 6 of the European hamster are similar but not identical to chromosome 1 of the Chinese hamster and while the major rearrangement appears to be a Robertsonian fusion additional events are necessary to explain the differences. These could be either inversions or deletions (Gamperl *et al.*, 1976). G-banding in this case lacks the resolution necessary to identify the underlying cause of the banding differences. Both paracentric and pericentric inversions can contribute to a loss of conservation in G-banding patterns particularly if they involve small segments or if multiple inversions are involved.

In *Notomys*, while there is good agreement across the four species in G-banding patterns, some of the smaller acrocentric autosomes are difficult to match. This may be due to differential contraction, experimental variation or else it may represent real differences in the chromosome organization. Chromosomes 21, 22 and 23, which are those involved in pericentric inversions, are small chromosomes with minimal landmark features which make for difficulties in establishing homologies.

The large chromosomes provide more scope for detecting homology of banding patterns, and indeed there seems to be good agreement of patterns across all four species.

2.4.2 *X chromosome differences*

On the basis of the G-banding patterns in the X chromosomes of 46 mammalian species together, with a review of data from 32 additional mammalian species in the literature, Pathak and Stock (1974) conclude that there is a conservation of two major bands throughout these species, although the location of these bands is not necessarily the same. For example, in *Homo sapiens* one band is in each arm whereas in *Mus* both are in the same arm. However, the changes in the G-banding of the euchromatin of the X chromosome must be due to inversions whether paracentric, pericentric or both. Many of the changes in G-band pattern are too complex to be accounted for by a single event. Thus there are considerable differences in the G-banding and also the morphology of the X chromosomes between *Homo sapiens*, *Peromyscus*, *Mus* and cattle (Fig. 2.8).

In closely related groups on the other hand the G-banding pattern is certainly highly conserved. The X chromosomes of the great apes are a good example of this [Paris Conference (1971), Supplement (1975) their Fig. 4g]. Likewise, in the genus *Notomys* the euchromatic arm of the X chromosome in each of the four species is very similar in pattern. However there is little resemblance to the patterns of the X chromosomes of the great apes, *Mus*, *Peromyscus* or cattle.

The two major bands described by Pathak and Stock (1974) are resistant to both trypsin and urea treatments. There is, however, no way of determining whether or not the two bands observed in these 78 species referred to by Pathak and Stock are strictly homologous. Earlier, Ohno (1967) had proposed that the genetic content of the mammalian X chromosome is highly conserved and this proposal is supported by three additional facts:

Figure 2.8 : Comparison of the G-band idiogram of various mammalian X chromosomes; (a) *Notomys* acrocentric X chromosome, (b) *Mus musculus* X chromosome (adapted from Nesbitt and Franke, 1973), (c) *Homo sapiens* X chromosome (adapted from Paris Conference (1971), Supplement (1975)), (d) *Peromyscus* X chromosome (adapted from The Committee for Standardization of Chromosomes of *Peromyscus*, 1977), (e) Bovine x chromosome (adapted from Lin *et al.*, 1977).

< indicates the two major bands of the X chromosome after Pathak and Stock (1974).

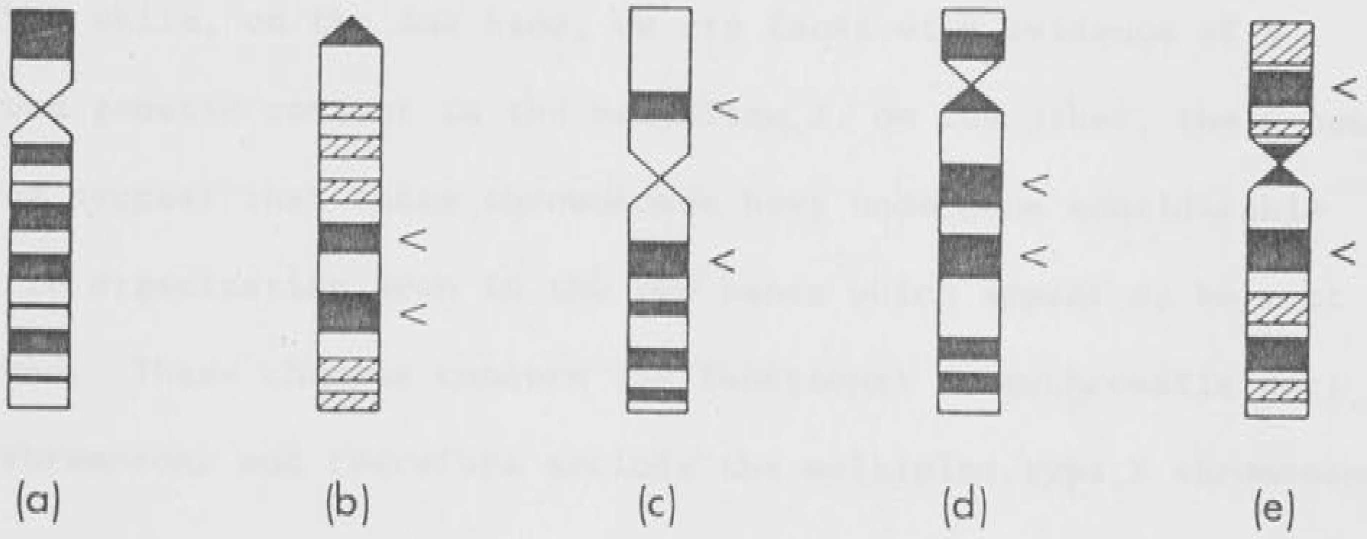


Figure 2.8

(i) The presence of the X-linked genes - G6PD, PGK, HGPRT and alpha-GAL in all those species for which genetic data are available,

(ii) The apparent constancy, at a level of 5-6%, of the euchromatic portion of the "original-type" or simplex X as a percentage of the haploid female genome, and

(iii) The system of dosage compensation which obtains in the X chromosomes of female placental mammals as a result of the facultative heterochromatinisation of one of the two X's.

Thus while, on the one hand, we are faced with evidence of a conserved genetic content in the mammalian X, on the other, the G-banding patterns suggest that these chromosomes have undergone considerable change in organization even in the two bands which appear to be most conserved. These changes concern the functional or euchromatic part of the X chromosome and therefore exclude the multiplex type X chromosomes.

2.4.3 *The origin of* Notomys *X chromosome variation*

Few mammals have been found that have polymorphic X chromosomes and those reported have all been rodents. For example, polymorphic X chromosomes occur in the short-tailed bandicoot rat, *Nesokia indica* (Jhanwar and Rao, 1973), in the West Malaysian house shrew, *Suncus murinus* (Yong, 1974), in *Bandicota bengalensis bengalensis* (Sharma and Raman, 1973) and in the woodrat, *Micropus phenax* (Mascarello *et al.*, 1974b). The genus *Notomys* has three species which show X chromosome polymorphism (Baverstock *et al.*, 1977b) and thus offers an unparalleled opportunity to shed some light on the origin of such polymorphisms.

The X chromosome of mammals often occupies 5-6% of the haploid chromosome complement and has been described as the "original-type" or simplex X (Ohno *et al.*, 1964). Larger multiplex X chromosomes were then termed "duplicate X's", if they approximate to twice the size of the "original-type X" (golden hamster), "triplicate-type X" when three times the "original-type X" (creeping vole) or "quadriplicate-type X" when four times the "original-type X" (common vole). This classification is unfortunate in two respects:

(i) It implies a precise multiplication of the genetic material of the X chromosome and, to the extent that this involves euchromatic material, of those genes in it. Very often the extra material on these chromosomes is heterochromatic which could, of course, result following a secondary inactivation of the extra material (Pathak and Stock, 1974), and

(ii) The actual size of many of the larger X chromosomes are anything but exact multiples of the "original X" (Wurster *et al.*, 1968; Pathak and Stock, 1974).

According to Ohno (1965) these larger X chromosomes may have arisen by either of two processes:

(i) Literal multiplication of the "original-type" X.

(ii) Addition of autosomal material to the "original-type" X. Matthey (1961) proposed that translocation of a complete or very large part of a pair of autosomes onto the original X and Y had occurred. This proposal would explain the formation of chiasmata in the sex bivalents of the Chinese hamster, *Cricetulus griseus*. It is not known whether these autosomal additions to the sex chromosomes were heterochromatic prior to translocation or whether they subsequently became heterochromatic (Fredga, 1970).

Evidence against literal duplication includes the observation that not all large X chromosomes are exact mutliples of the "original-type" X chromosome. For example, the X chromosome of the Patagonian hare, *Dolichotis patagonum* constitutes 8.4% of the haploid complement while in the bushy-tailed wood rat, *Neotoma cinerea* it occupies 8.1% and in the capybara, *Hydrochoeris hydrochaeris* 8.5%. Indeed, in the genus *Notomys*, of the nine forms of X chromosomes found in the four species, only two approximate to the "duplicate-type" X of Ohno *et al.* (1964). These two are the metacentric X of *N. alexis* and the large metacentric X of *N. cervinus*. The remaining seven X chromosomes are intermediate in size between the "original-type" X and the "duplicate-type" X (Table 2.2). Wurster *et al.* (1968) note that animals with "triplicate-type" X chromosomes, such as the creeping vole, *Microtus oregoni*, should show increases in the total DNA content but Ohno (1965) found no such increase in total DNA compared with other mammals. This last argument may not be valid since the known diploid genome of mammals ranges from 3-6 pg. Nevertheless it is clear that not all simplex X's do constitute 5-6% of the genome. Finally Wurster *et al.* (1968) argue that as there is no chiasma in the homologous regions of "original-type" X and Y then duplicate X chromosomes should also have no chiasma, but the Chinese and European hamsters both have large X chromosomes and have interstitial chiasmata. This would argue for an autosomal translocation as an origin rather than literal duplication. Wurster *et al.* (1968) thus support the claim of Schmid *et al.* (1965) that translocation of autosomal heterochromatin could provide a source of the large X chromosomes. Schmid *et al.* (1965) note that animals with the "original-type" X have most or all of their late replicating material on the autosomes, "duplicate-type" X bearing animals, such as the golden hamster, *Mesocricetus auratus*, have part of their heterochromatin on the X chromosomes and part on the autosomes, and finally "quadruple-type" X bearing animals, such as the common vole, *Microtus agrestis*, have other late replicating material predominently on the X chromosome with little on the autosomes.

The X chromosome polymorphism in *Notomys alexis* provides an opportunity to assess these two proposals of Ohno (1965). In *N. alexis* the polymorphism involves three different sized X chromosomes - an acrocentric X which at 7.2% of the female haploid chromosome length (FHCL) exceeds the "original-type" X chromosome length, a submetacentric X which falls between the "original-type" X and the "duplicate-type" X in length at 9.7% FHCL and finally the metacentric "duplicate-type" X at 10.7% FHCL. A third proposal not considered by Ohno could involve the addition and/or deletion of heterochromatin to give rise to these forms of X chromosomes in *N. alexis*. In the present context the three proposed origins can be summarized as follows:

(i) *Duplication or multiplication of the "original-type" X.* It could be argued that the "duplicate-type" *N. alexis* X arose by literal duplication as suggested by Ohno (1965) and following this the second arm became heterochromatinized. This would require a subsequent deletion in the heterochromatic arm of the "duplicate-type" X to explain the occurrence of the acrocentric and the submetacentric forms of the X chromosome in this species (Fig. 2.9).

(ii) *Translocation of autosomal heterochromatin onto the "original-type" X.* Certainly there is ample short arm constitutive heterochromatin available for translocation to the X chromosome in *N. alexis* (Fig. 2.10). However C-banding differentiates between the dark staining autosomal heterochromatin and the lighter staining heterochromatin of the X arm (as distinct from the X chromosome centromeric region which does stain dark). This difference could be due to differing condensation. However, the lack of demonstrable satellite in the accessory gonosomal heterochromatin (see Chapter 5) and the obvious presence of satellite DNA in the short arms of the autosomes would seem to argue against autosomal heterochromatin translocation since it would require the loss of either detectable satellite DNA or the loss of the satellite nature of the DNA in the translocated material.

Figure 2.9 : Duplication or multiplication of the "original-type" X chromosome with subsequent heterochromatinization of the added material resulting in the "duplicate-type" X found in *Notomys alexis*. Deletions could form both the acrocentric and submetacentric X chromosomes.

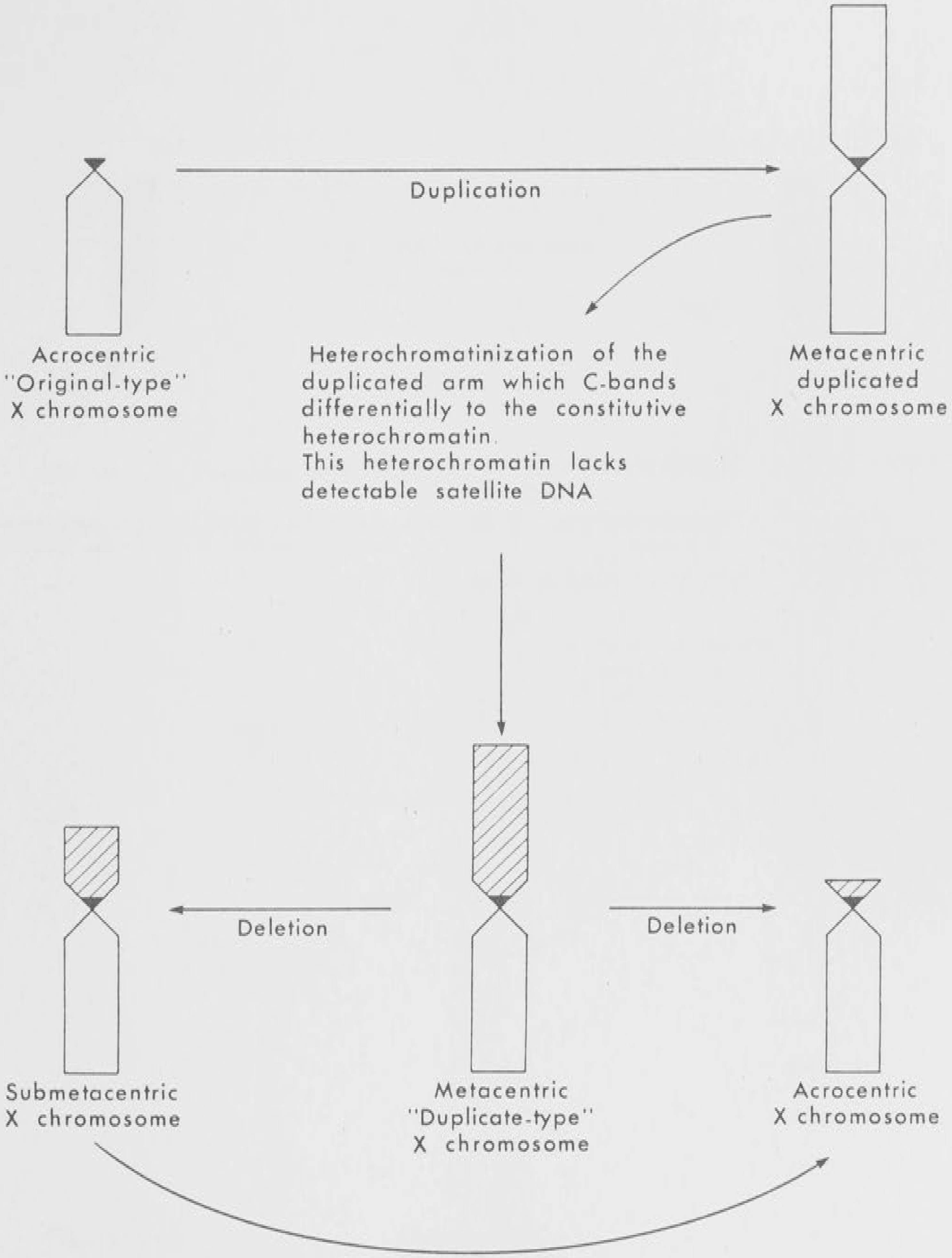

Duplication
Acrocentric
"Original-type"
X chromosome
Heterochromatinization of the duplicated arm which C-bands differentially to the constitutive heterochromatin.
This heterochromatin lacks detectable satellite DNA
Metacentric
duplicated
X chromosome
Deletion
Deletion
Submetacentric
X chromosome
Metacentric
"Duplicate-type"
X chromosome
Acrocentric
X chromosome

Figure 2.9

Figure 2.10 : Translocation of autosomal heterochromatin onto the "original-type" X chromosome. The translocation need not be a single event.

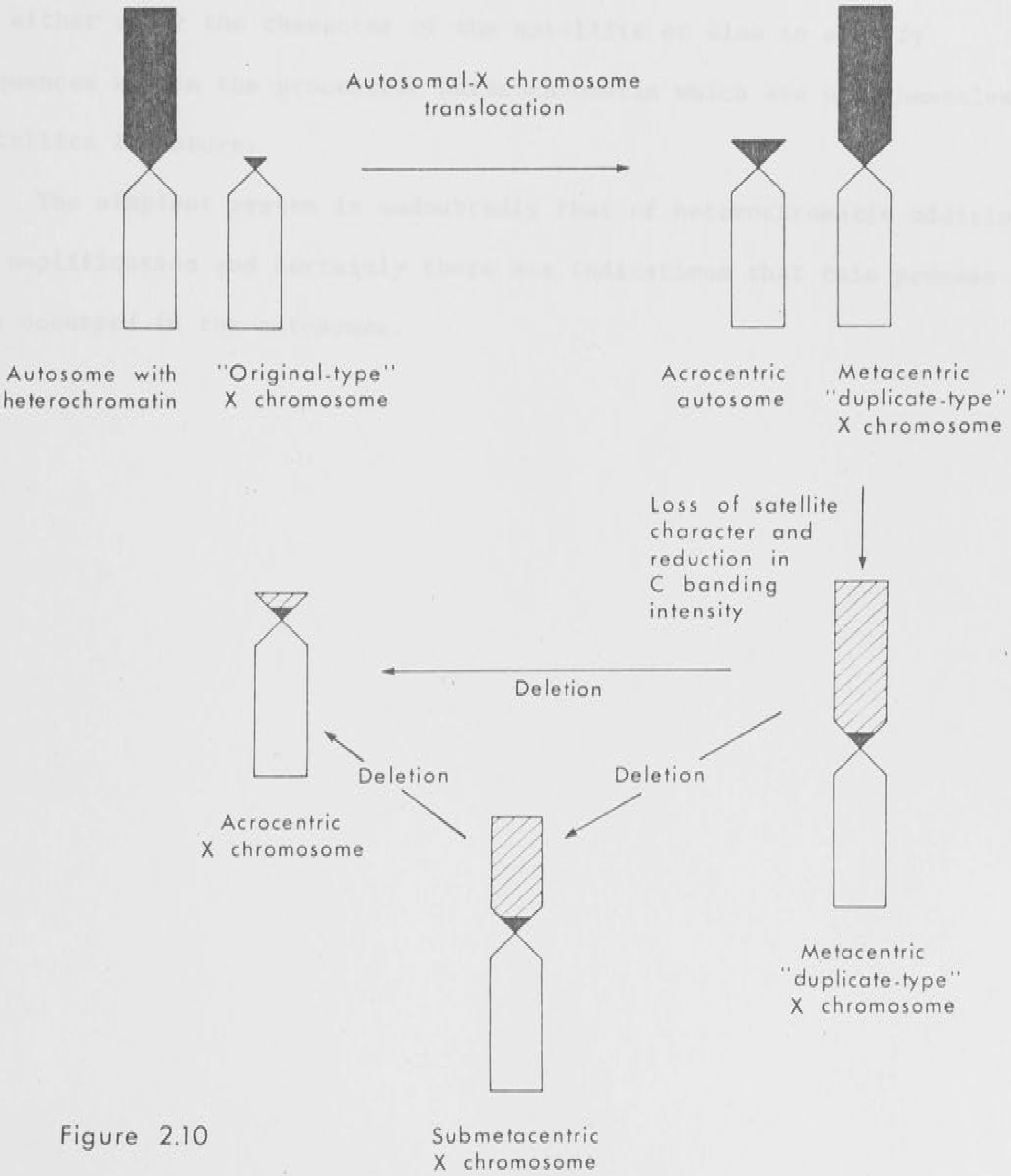

Autosomal-X chromosome
translocation
Autosome with heterochromatin
"Original-type" X chromosome
Acrocentric autosome
Metacentric "duplicate-type" X chromosome
Loss of satellite character and reduction in C banding intensity
Deletion
Deletion
Deletion
Acrocentric X chromosome
Metacentric "duplicate-type" X chromosome
Submetacentric X chromosome

Figure 2.10

(iii) *Arm growth by progessive amplification.* If the ancestral X chromosome of *N. alexis* was acrocentric, then all three X chromosome forms could arise by amplification from the procentromeric heterochromatin of such a chromosome (Fig. 2.11). Growth from the procentric heterochromatin,which contains satellite DNA,would have to include mechanisms to either alter the character of the satellite or else to amplify sequences within the procentric heterochromatin which are not themselves satellite in nature.

The simplest system is undoubtedly that of heterochromatin addition or amplification and certainly there are indications that this process has occurred in the autosomes.

Figure 2.11 : Arm growth by progressive amplification as a possible explanation of the origin of the three forms of X chromosomes in *N. alexis*.

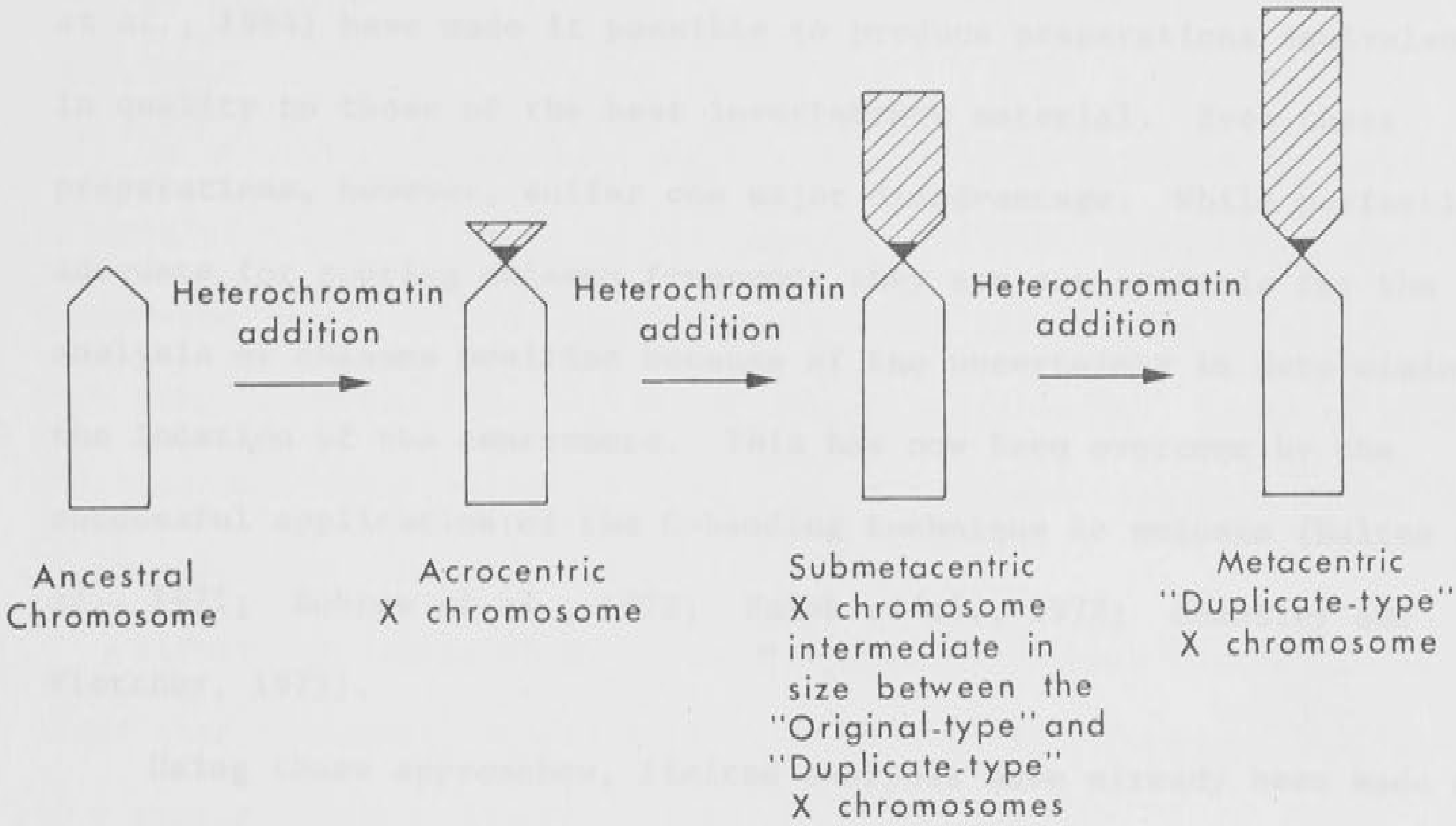

Heterochromatin addition
Heterochromatin addition
Heterochromatin addition
Ancestral Chromosome
Acrocentric X chromosome
Submetacentric X chromosome intermediate in size between the "Original-type" and "Duplicate-type" X chromosomes
Metacentric "Duplicate-type" X chromosome

Figure 2.11

CHAPTER 3

MEIOSIS, HETEROCHROMATIN AND CHIASMA CHARACTERISTICS IN MALE *NOTOMYS*

3.1 *Introduction*

Prior to the mid-1960's only limited data were available on meiosis in male mammals. Technical advances in the handling of meiotic material following the introduction of the air-drying technique (Evans *et al.*, 1964) have made it possible to produce preparations equivalent in quality to those of the best invertebrate material. Even these preparations, however, suffer one major disadvantage. While perfectly adequate for scoring chiasma frequency they are not suitable for the analysis of chiasma position because of the uncertainty in determining the location of the centromere. This has now been overcome by the successful application of the C-banding technique to meiosis (Hulten *et al.*, 1971; Bobrow *et al.*, 1972; Falek *et al.*, 1972; Chandley and Fletcher, 1973).

Using these approaches, limited analyses have already been made of the chiasma characteristics of mice and *Homo sapiens* (Polani, 1972; Forejt, 1973; Capanna *et al.*, 1976; Polani and Jagiello, 1976; Forejt and Gregorova, 1977; Speed, 1977; White *et al.*, 1978; Jagiello and Fang, 1979 on mice and Hulten, 1974 and Lange *et al.*, 1975) but few authors have attempted to extend meiotic analysis to other species. Indeed it is one of the great failings of mammalian cytogenetics that so little attention has been devoted to meiotic studies in contrast to the now enormous literature which exists on mitotic karyotypes.

The main chiasma characteristics of an organism are, without doubt, under genotypic control and, while the precise nature of this control remains to be clarified, it involves both major and polygenic loci (Rees, 1961; Shaw, 1972; Baker *et al.*, 1976). Two distinct patterns of control exist - random and localised. In the former there is a relationship between chromosome length and chiasma frequency, on the one hand, and between frequency and distribution on the other (Henderson, 1963; Southern 1967; Fox, 1973). In this system chiasma formation appears to be successive, usually commencing at the distal end of a chromosome or a chromosome arm. Following the production of the initial chiasma the position of further exchanges is a length dependent event that involves the operation of an interference factor which distributes chiasmata roughly evenly along the length of a chromosome or a chromosome arm.

In the case of localised chiasma distribution patterns different principles operate. There is not usually a simple relationship between chromosome length and chiasma frequency since all or most bivalents may form single chiasma irrespective of length differences. Added to this, all chiasmata occur either at distal or at proximal positions according to the patterns of localisation.

Within the limits of the overall system of chiasma control it is clear that in several respects the bivalents within a nucleus may behave in a semi-autonomous manner. Thus:

(i) in species with random distribution patterns neither Hewitt and John (1965), Southern (1967) nor Fox (1973) found any evidence for competition for chiasmata between bivalents co-habiting the same nucleus,

(ii) Parker *et al.* (1976) have demonstrated the occurrence of specific asynaptic mutants affecting only particular members of a given chromosome complement, and

(iii) even in species with markedly localised patterns individual bivalents may fail to conform with the norm (Klasterska *et al.*, 1974).

Indeed in metacentric elements the principle of autonomy may also extend to single chromosome arms. Thus Southern (1967) reports that, in metacentrics of four species of truxaline grasshoppers, chiasma interference does not extend across the centromere so that chiasma distribution in one arm is independent of that in the other arm.

Over and above these direct genotypic aspects of chiasma control three further factors exert some influence on the pattern of chiasma formation, namely the centromere, the presence of blocks of constitutive heterochromatin and the presence of structural rearrangement. Moreover these factors may interact with one another, and with the more conventional system of genotypic control, in a variety of ways:

(1) *The centromere effect* - It has been known for many years that in *Drosophila melanogaster* recombination decreases progressively when euchromatic sections are moved closer and closer to a centromere whether by inversion or by translocation. Recently Yamamoto and Miklos (1978) have been able to demonstrate that both the centromere and the heterochromatic blocks which occur next to the centromere are involved in this effect and that their influences may be confounded when they occur together.

(2) *The presence of heterochromatin* - Heterochromatin is important in regulating chiasma formation in three different ways. First, no chiasmata form in constitutively heterochromatic regions (John, 1976). Second, the presence of heterochromatic regions tends to inhibit chiamsa formation in adjacent regions (Miklos and Nankivell, 1976; John and King, 1980). Third, in a number of cases supernumerary heterochromatin has been found to produce inter-chromosomal effects in chiasma frequency (Review: John and Miklos, 1979).

(3) *The presence of structural rearrangements* - In grasshoppers pericentric inversion heterozygotes show straight pairing in the region of structural rearrangement. Consequently no chiasmata form in the non-homologously paired regions. In *Cryptobothrus chrysophorus* such heterozygous regions may accompany the presence of added distal blocks of heterochromatin. Such blocks normally lead to single chiasmata occupying proximal positions. In combination with a heterozygous inversion however chiasmata remain distal or at least interstitial (John and King, 1980).

Table 3.1 : Percentage of heterochromatin in the total genomes and in the 20 autosome karyotypes (= autosomes 1-20 = 20AK) of the four species of *Notomys* together with the distribution of heterochromatin in the total short and long arms of the 20AK and also its distribution in the chromosome groups

Species	% total hetero- chromatin	% total hetero- chromatin in 20AK	% short arm hetero- chromatin (= SAH)	% long arm hetero- chromatin (= LAH)
OVERALL GENOMIC:				
N. mitchellii	28.45	27.19	13.81	13.38
N. alexis	24.00	22.82	11.74	11.08
N. cervinus	25.51	24.39	17.62	6.77
N. fuscus	13.66	13.13	1.75	11.38
CHROMSOME 1:				
N. mitchellii		2.6	1.1	1.5
N. alexis		1.5	0.6	0.9
N. fuscus		0.7	0.0	0.7
N. cervinus		1.4	1.4	0.0
CHROMOSOMES 2-6:				
N. mitchellii		9.5	4.6	4.9
N. alexis		6.4	3.1	3.3
N. fuscus		4.2	1.1	3.1
N. cervinus		7.4	5.3	2.1
CHROMOSOMES 7-20:				
N. fuscus		8.2	0.6	7.6
N. mitchellii		15.1	8.1	7.0
N. alexis		14.9	8.0	6.9
N cervinus		15.6	10.9	4.7

As we have seen earlier in this thesis different species of *Notomys* have different amounts of heterochromatin and different patterns of heterochromatin distribution (see Chapter 2). Thus:

(i) *N. fuscus* has about a half of the total heterochromatin content per genome that is found in the other three species when both long and short arms are considered together (Table 3.1), and

(ii) if however we partition the heterochromatin between long and short arms it is *N. cervinus* that now appears anomalous since it has only about half of the content of long arm heterochromatin (= LAH) found in the other three species whereas *N. fucsus* has most of its heterochromatin exclusively in the long arm. On the other hand in terms of short arm heterochromatin (= SAH) *N. fuscus* is again anomalous, it has far less short arm heterochromatin than the other three species.

A comparison of the meiotic behaviour of these four species thus offers an opportunity to examine whether these differences are in any way related to chiasma frequency and chiasma distribution.

3.2 *Methods*

3.2.1 *Male meiotic preparations*

After removal from the animal the testis was placed in 1% sodium citrate. Following removal of the tunica, the testis was placed into fresh 1% sodium citrate solution, cut into quarters and allowed to stand at room temperature for 20 minutes. The tissue was then finely chopped, swirled gently and then allowed to settle. The supernatant was centrifuged at 1500 r.p.m. for 8 minutes and the pellet so obtained was gradually suspended in fresh 3:1 methanol:glacial acetic acid fixative and allowed to stand at room temperature for 10 minutes. The suspension was again centrifuged for 8 minutes and fresh fixative was added after removal of the supernatant and the pellet resuspended. The suspension was allowed to stand for 1-2 hours at room temperature. At least one hour at room temperature was critical at this stage if good quality spreads were to be obtained. Finally a third centrifugation was followed by suspension in a volume of fresh fixative necessary to achieve a suitable cell density on the slides. Slides were then prepared using an air dried technique which was a modification of that used by Evans *et al.* (1964) incorporating changes suggested by Chandley (personal communication).

The C- and G-banding techniques employed were the same as those used in the preparation of mitotic slides (see Chapter 2). G-banding was abandoned because it did not provide a satisfactory means of identifying the individual bivalents. While the centromeres were more accurately located with C-banding only bivalent 1 could be positively identified. The bivalents were therefore divided on the basis of size and morphology into five groups for meiotic analyses: Bivalent 1, Bivalents 2-6, Bivalents 7-20, Bivalents 21-23 and the sex XY bivalent. In the analyses of chiasma characteristics between the species in these groups bivalents 21-23 were excluded because they vary structurally across the species (Fig. 2.1). Thus chromosomes 21 and 22 are metacentric and 23 acrocentric in both *Notomys mitchellii* and *N. alexis*; in *N. cervinus*, on the other hand, all three are metacentric while in *N. fuscus* chromosome 21 is metacentric and chromosomes 22 and 23 both acrocentric. The structural differences alter both the lengths and the number of chromosome arms involved in these bivalents. Bivalents 21-23 were however included in the calculation of mean cell chiasma frequencies. The XY bivalent was not scored because there is some doubt as to whether the X and Y chromosomes are always associated in a chiasmate manner.

3.2.2 *Chiasma frequency analysis*

For the purpose of comparing chiasma frequency scores five male individuals of each of the four species were scored. These cells were also used in the chiasma distribution analysis. The number of cells scored for each individual appears in Table 3.2.

3.2.3 *Chiasma location analysis*

To facilitate the scoring of chiasma position the long arm of each chromosome was divided into three equal lengths on a visual basis regardless of the heterochromatin content (Figure 3.1). The short arm of each chromosome was not considered in this division as chiasmata were not observed in the short arms of any of the twenty autosomes scored in any of the animals of the four species of *Notomys*; this includes chromosome 1 of *N. cervinus* which does have a small segment of euchromatin in the short arm. The three smallest autosomes were excluded from the positional analysis because their differing morphology precludes meaningful comparative analysis. While this method of chiasma location is an arbitary, rather than a very exact one, it does give a means of rapidly assessing whether there are any marked differences in chiasma distribution patterns.

Representative diakinetic figures for each species of *Notomys* are shown in Figure 3.2, while Figure 3.3 shows examples of single, double and triple exchange bivalents and the position of the chiasmata in these bivalents using proximal, interstitial and distal location categories.

3.3 *Results*

3.3.1 *Chiasma frequency per cell*

Chiasma distribution patterns in the four species are largely random in character (Table 3.3). As expected, therefore, there is a relationship between chiasma frequency and chromosome length (Table 3.3).

Figure 3.1 : Division of the long arm of a *Notomys* chromosome into three equal lengths. P = proximal third, I = interstital third and D = distal third.

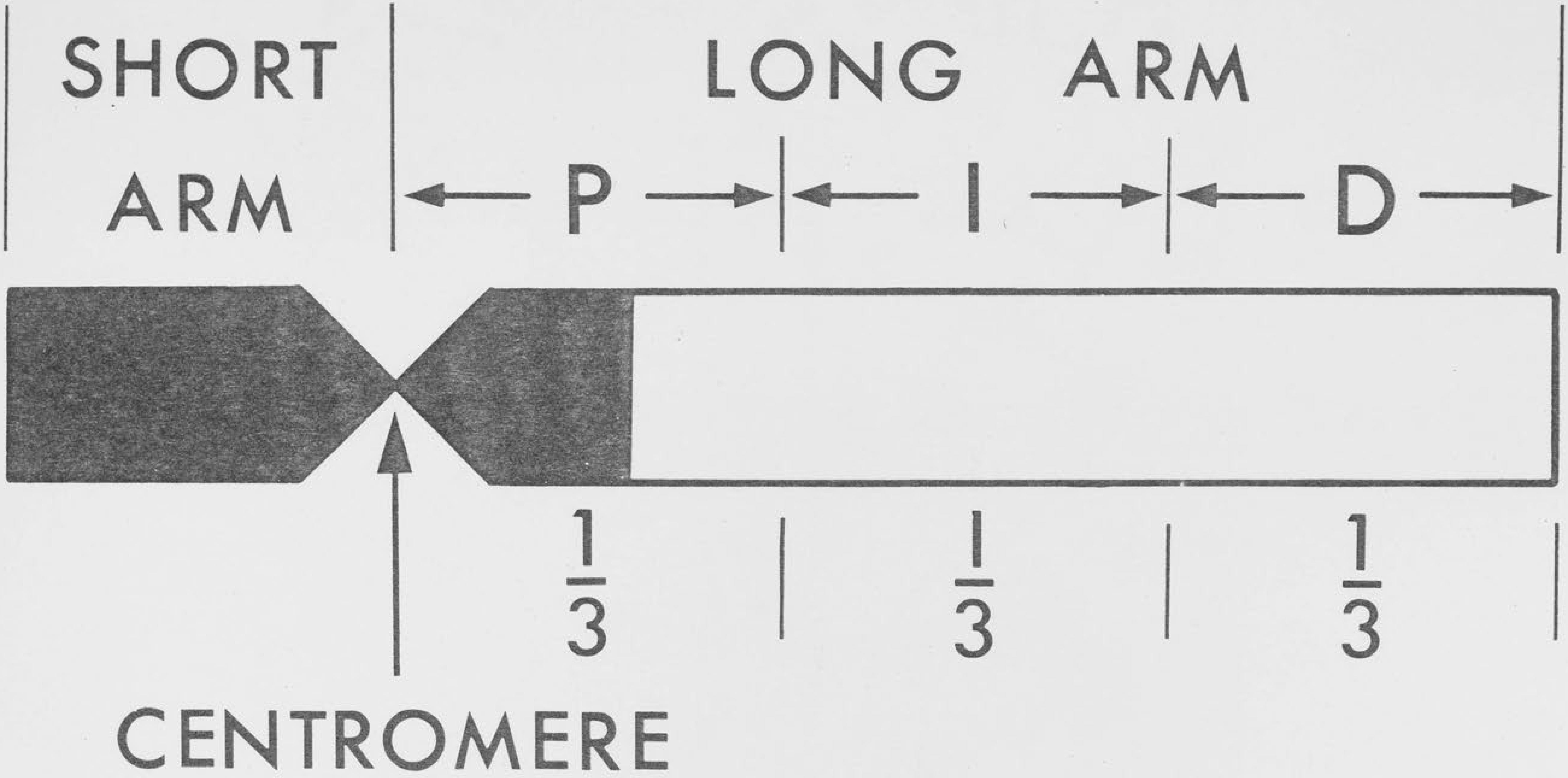

SHORT ARM
LONG ARM
P
I
D
1/3
1/3
1/3
CENTROMERE

Figure 3.2 : Diakinetic figures from male *Notomys*. (a) *N. mitchellii*, (b) *N. alexis*, (c) *N. cervinus* and (d) *N. fuscus*. Bar represents 10 µm. 1 indicates bivalent 1.

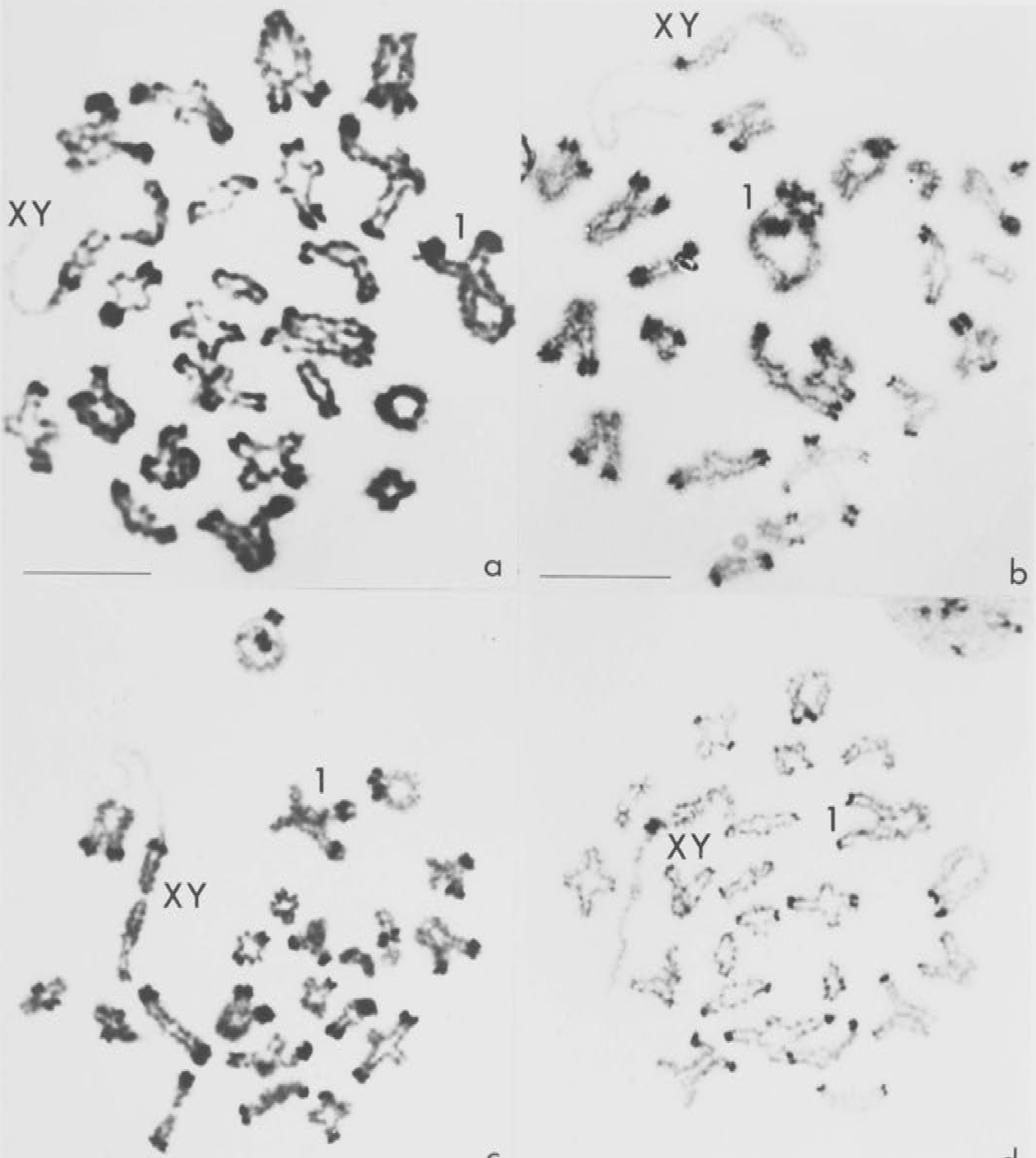

XY
1
a
XY
1
b
1
XY
c
XY
1
d

Figure 3.3 : Examples of single, double and triple exchange bivalents from *Notomys* male diakinetic figures together with the positional scoring as used in this study.

a-f : Single exchange bivalents

- a and b : Proximal chiasmata
- c and d : Distal chiasmata
- e and f : Interstitial chiasmata

g-l : Double exchange bivalents

- g : Proximal and distal chiasmata
- h : Interstitial chiasmata
- i : Proximal and distal chiasmata
- j-l : Interstitial and distal chiasmata

m-n : Triple exchange bivalents

- m and n : Proximal, interstitial and distal chiasmata.

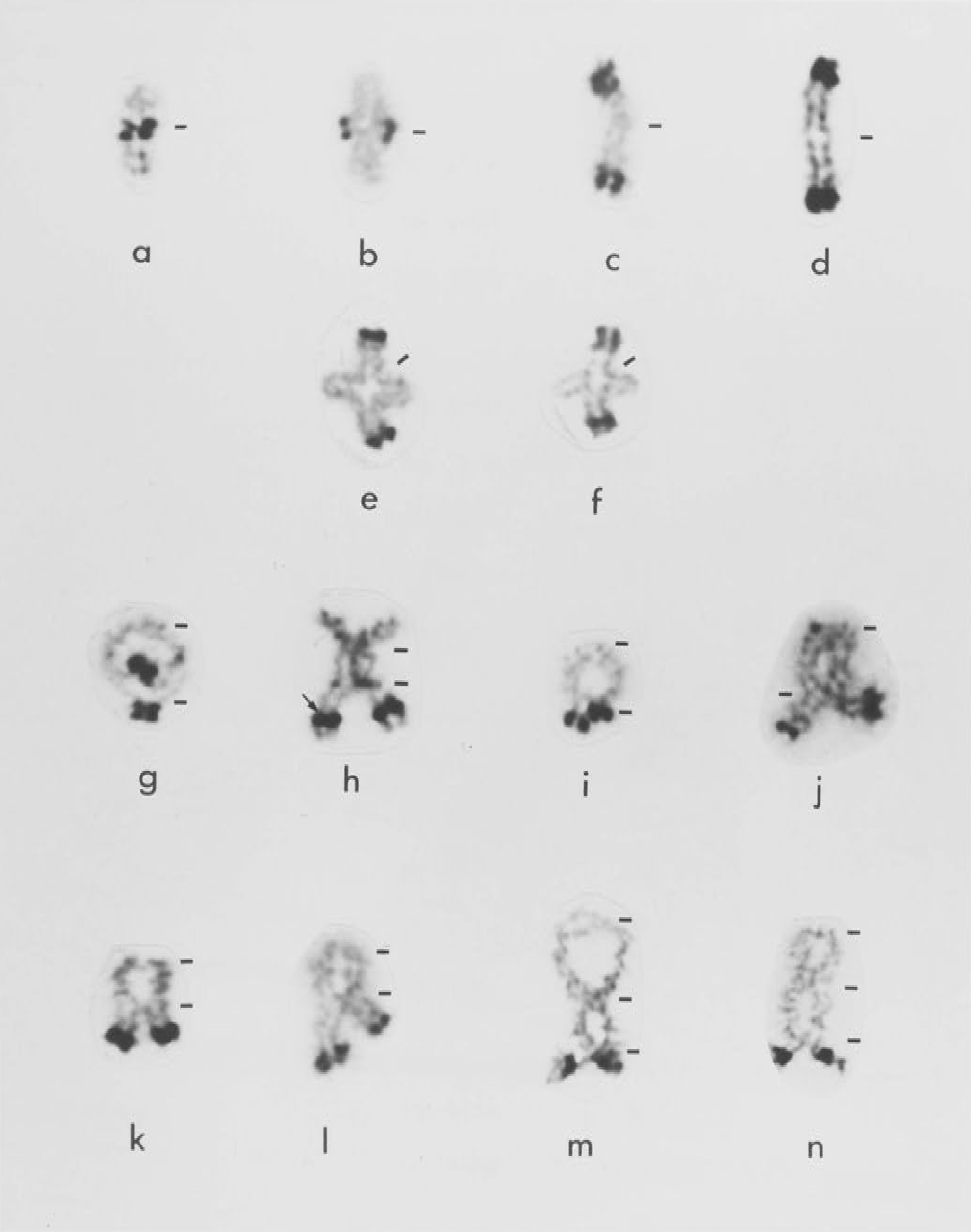

a
b
c
d
e
f
g
h
i
j
k
l
m
n

Table 3.2 : The number of cells scored for each individual male of the four species of *Notomys* used for both the chiasma frequency and distribution analyses

Species	Individual	Number of cells scored
N. mitchellii	1. Nm 183A	60
	2. Nm 172A	59
	3. Nm 177A	55
	4. Nm 140B	66
	5. Nm 186C	56
		296 = Total for species
N. alexis	1. Na 866C	75
	2. Na 127A	65
	3. Na 105	59
	4. Na 132A	55
	5. Na 200	55
		309 = Total for species
N. fuscus	1. Nf 24	60
	2. Nf 5C	63
	3. Nf 22	125
	4. Nf 20	59
	5. Nf 43A	21
		328 = Total for species
N. cervinus	1. Nc 241B	67
	2. Nc 30DE	78
	3. Nc 241A	75
	4. Nc 239C	79
	5. Nc 238A	81
		380 = Total for species

Table 3.3 : Mean chromosome length, mean euchromatin length, mean chiasma frequency per bivalent and the ratio of mean frequency per bivalent group to mean length. The chiasma frequency for 21-23 is invariably 1.0 per bivalent

Species	Mean total chromosomes length % 20Ak	Mean euchromatin length % 20EAK	Mean chiasma per bivalent	Mean chiasma frequency / Mean euchromatin length
BIVALENT 1:				
Notomys mitchellii	4.82	4.87	1.90	0.39
N. alexis	4.66	4.70	1.96	0.42
N. fuscus	4.30	4.60	1.85	0.40
N. cervinus	4.92	5.68	2.02	0.36
BIVALENTS 2-6:				
N. mitchellii	3.32	3.28	1.47	0.45
N. alexis	3.22	3.27	1.58	0.48
N. fuscus	3.27	3.30	1.43	0.43
N. cervinus	3.32	3.36	1.64	0.49
BIVALENTS 7-20:				
N. fuscus	2.10	2.06	1.05	0.51
N. mitchellii	2.04	2.05	1.11	0.54
N. alexis	2.09	1.98	1.12	0.57
N. cervinus	2.03	1.95	1.12	0.57

*20EAK = euchromatin in the 20 AK.

The reader will recall (Chapter 2) that there are short arms in all twenty largest autosomes in *N. mitchellii, N. alexis* and *N. cervinus* and that with the exception of pair 1 of *N. cervinus* all these arms are heterochromatic. *N. fuscus*, however, has short arms in only eight members of the complement; four of these occur in the 2-6 chromosome class and the remainder are in the 7-20 class. Where short arms are present they never form chiasmata. This applies even to the euchromatic section of the short arm of pair 1 of *N. cervinus*.

The mean cell chiasma frequency for each of the five individuals analysed per species are summarised in Table 3.4. If the variance of the mean cell chiasma number is transformed into a log variance then a one way analysis of variance indicates that there are no significant differences in the variability of chiasma frequency in the four species ($F_{3,16} = 2.98$, $P > .05$; Table 3.5).

A multilevel nested analysis of variance of the mean cell chiasma frequency data for five males of each species showed that the species differed ($F_{3,16} = 4.1$, $P < .05$; Table 3.6). The animals scored in this study all originate from inbred laboratory stocks, consequently they may not give a fair reflection of the differences which might obtain in the wild. Even so, an inspection of the individual means (Table 3.4) makes it clear that the occurrence of exceptional individuals within a given species may still be having disproportionate effects on the mean value (see for example individual 5 of *N. alexis* and *N. cervinus* in Table 3.4). Nevertheless the values for the five individuals of *N. fuscus* are consistently lower than those of any other species. Thus clearly, there is no simple relationship between total heterochromatin content per genome and mean cell chiasma frequency of the kind that has been observed in certain other species comparisons (e.g. *Atractomorpha*, Nankivell, 1976; *Caledia*, Shaw and Knowles, 1976; *Salvia*, Linnert, 1955). However *N. fuscus*, as we have seen, is exceptional in having very little

Table 3.4 : Chiasma frequency characteristics of *Notomys*

Mean cell chiasma frequency for each animal and for each species together with the percentage of total heterochromatin, long arm heterochromatin and short arm heterochromatin in the 20 largest autosomes

Total 20AK Het. = Total heterochromatin in the 20 largest autosomes

LAH 20AK = Long arm heterochromatin in the 20 largest autosomes

SAH 20AK = Short arm heterochromatin in the 20 largest autosomes

n = total cells scored

Species	% Total 20AK het.	% LAH 20AK	% SAH	Individual mean cell chiasma frequencies					Mean cell chiasma frequency
				1	2	3	4	5	
N. mitchellii	27.19	13.38	13.81	27.02	27.83	27.90	27.93	28.00	27.75
n = 296				±0.21	±0.25	±0.23	±0.25	±0.21	±0.10
N. alexis	22.82	11.08	11.74	26.87	28.17	28.37	28.99	31.11	28.67
n = 309				±0.24	±0.22	±0.30	±0.31	±0.42	±0.15
N. cervinus	24.39	6.77	17.62	27.67	28.05	28.62	28.97	30.81	28.85
n = 380				±0.22	±0.25	±0.22	±0.25	±0.24	±0.12
N. fuscus	13.13	11.38	1.75	26.67	26.73	26.76	27.29	27.57	26.91
n = 328				±0.16	±0.23	±0.29	±0.33	±0.26	±0.11

In the statistical analyses of the data the following abbreviations apply:

n.s.	not significant, $p > .05$
*	$.01 < p < .05$
**	$.001 < p < .01$
***	$p < .001$

Table 3.5 : A one way analysis of variance after logarithic transformation of the sample variance of the mean chiasmata for five males of each species of *Notomys* showing that the variation is homogeneous over the four species.

Source of variation	DF	SS	F
Between species	3	0.826	2.98 n.s.
Within species	16	1.476	
Total	19	2.302	

Table 3.6 : Multilevel nested analysis of variance for chiasma frequency per cell for five males of each species of *Notomys*.

Source of variance	DF	SS	$F_{16,1293}$	$F_{3,16}$
Between species	3	816.75		4.10^{*}
Between individuals within species	16	1063.62	16.2^{***}	
Between cells within individuals	1293	5309.69		
Total	1312	7190.06		

$^{*}0.1 < p < .05$

*** $p < .001$

short arm heterochromatin by comparison with the other three species and there exists a possibility that the extra genomic short arm heterochromatin in these species may in some way contribute to their higher chiasma frequencies. If this is the case then clearly it must depend upon some form of inter-chromosomal effect.

Broad comparisons between species are not likely to be especially meaningful in *Notomys* because the distribution of heterochromatin within a complement varies considerably. An added complication stems from the fact that with one exception the chromosomes within complements cannot be consistently distinguished on an individual basis. The exception is chromosome 1. The best one can achieve for the rest of the complement is to group chromosomes 2-6 on the one hand and 7-20 on the other though comparisons between these groups necessarily confound individual differences in the heterochromatic character of their component members.

3.3.2 *Chiasma frequency and distribution in bivalent 1*

Turning to chromosome 1, the four species form a graded series with respect to long arm heterochromatin content. *N. mitchellii* has most LAH and *N. cervinus* is devoid of LAH in this chromosome. Considering genomic short arm heterochromatin it is *N. cervinus* that has the most while *N. fuscus* has least (Table 3.7).

A comparison of the frequency of exchanges in bivalent 1 of *N. cervinus* and *N. fuscus* indicate that whereas single exchanges are least frequent in *N. cervinus* they are most frequent in *N. fuscus* with the two other species occupying intermediate positions (Table 3.7-8). Significantly *N. cervinus* and *N. fuscus* represent the extremes in the amount of short arm heterochromatin present within the genome, *N. cervinus* having some ten times more short arm heterochromatin than *N. fuscus*. At first sight this might suggest that genomes having higher amounts of short arm heterochromatin produce more chiasmata, a situation reminiscent perhaps of the inter-chromosomal effect which supernumerary heterochromatin is known to exert in a number of plants and animals (Review: John and Miklos, 1979). Double exchanges in bivalent 1 (Tables 3.7 and 3.9) also support this correlation. *N. fuscus* has significantly fewer double exchanges than the other three species, which have relatively increased genomic SAH. The situation in triple exchanges is more complex (Tables 3.7 and 3.10). *N. cervinus* has more triple exchanges in bivalent 1 than the other three species but in the case of *N. alexis* the difference is not significant. Thus overall in bivalent 1 increased chiasma frequency is associated with the increased genomic SAH.

Turning to chiasma distribution, there is one point of interest to note in the data for single exchanges in bivalent 1 (Fig. 3.4) namely that while distal chiasma are the most common type in both *N. alexis* and *N. cervinus*, this is not the case in *N. mitchellii* and *N. fuscus* where interstitial chiasmata predominate (Tables 3.11 and 3.12). This would imply that chiasmata do not always form first distally, contrary to the situation which has been proposed in *Schistocerca gregaria* (Henderson, 1963; Fox, 1973). In *Caledia*, too, some chromosomes have interstitial exchanges as the predominant class (Shaw and Knowles, 1976). Further, the distribution data for single exchanges in bivalent 1 in *Notomys* show no obvious relationship to long arm heterochromatin. The positional placement seems to be largely due to genotypic control. On the other hand, single exchange bivalents involving long chromosomes are less likely to reveal influences due to procentric long arm heterochromatin because much of the bivalent will be free of both the centromere effect and any effect stemming from the long arm heterochromatin.

When one turns to consider the distribution of double exchanges (Tables 3.11, 3.13 and Fig. 3.4), the pattern of both proximal and interstitial chiasmata differs in *N. cervinus* compared to all other three species whether one considers the overall distribution or the individual frequencies of proximal and interstitial chiasmata. This

Table 3.7 : Frequency of single, double, triple and quadruple chiasmate bivalents in the three bivalent groups (bivalent 1, bivalent 2-6 and bivalent 7-29) in *Notomys mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus*

*GSAH% = Percentage of genomic short arm heterochromatin relative to the total chromosomal length in the 20 autosome karyotype

**LAH% = Percentage of long arm heterochromatin relative to the total chromosomal length in the 20 autosome karyotype

BIVALENT 1

Species	LAH	GSAH	Singles		Doubles		Triples		Quads.		Bivs.
	%**	%*	No.	%	No.	%	No.	%	No.	%	No.
N. mitchellii	1.5	13.8	41	13.9	244	82.4	11	3.7	-	-	296
N. alexis	0.9	11.7	33	10.7	254	82.2	22	7.1	-	-	309
N. fuscus	0.7	1.8	67	20.4	242	73.8	19	5.8	-	-	328
N. cervinus	-	17.6	32	8.4	307	80.8	40	10.5	1	0.3	380

BIVALENTS 2-6

Species	LAH	GSAH	Singles		Doubles		Triples		Quads.		Bivs.
	%**	%*	No.	%	No.	%	No.	%	No.	%	No.
N. mitchellii	4.9	13.8	784	53.0	693	46.8	3	0.2	-	-	1480
N. alexis	3.3	11.7	648	42.0	892	57.7	5	0.3	-	-	1545
N. fuscus	3.1	1.8	933	56.9	701	42.7	6	0.4	-	-	1640
N. cervinus	2.1	17.6	716	37.7	1161	61.1	23	1.2	-	-	1900

BIVALENTS 7-20

Species	LAH	GSAH	Singles		Doubles		Triples		Quads.		Bivs.
	%**	%*	No.	%	No.	%	No.	%	No.	%	No.
N. fuscus	7.6	1.8	4349	94.7	243	5.3	-	-	-	-	4592
N. mitchellii	7.0	13.8	3705	89.4	439	10.6	-	-	-	-	4144
N. alexis	6.9	11.7	3820	88.3	506	11.7	-	-	-	-	4326
N. cervinus	4.7	17.6	4701	88.4	619	11.6	-	-	-	-	5320

Table 3.8 : Species comparison of the number of single chiasmate bivalents in the three bivalent groups analysed as 2 x 2 contingency tables. The probability given is that of the observed data, or more extreme data arising if the proportion of single chiasmate bivalents was the same for both species of the pair. Where the number in any cell of an analysis was less than 100, the probability has been calculated exactly. (See Table 3.4 for abbreviations of the statistical significance). The %LAH refer to the 20AK set as do the %GSAH

BIVALENT 1 SINGLES

Species	*N. mitchellii* 1.5% LAH 13.8% GSAH	*N. alexis* 0.9% LAH 11.7% GSAH	*N. fuscus* 0.7% LAH 1.8% GSAH	*N. cervinus* 0.0% LAH 17.6% GSAH
N. mitchellii 0.5% LAH 20AK		$X^2 = 1.42$ $p = .264$ n.s.	$X^2 = 4.70$ $p = .034$ *	$X^2 = 5.09$ $p = .025$ *
N. alexis 0.9% LAH 20AK			$X^2 = 11.4$ $p < .001$ ***	$X^2 = 1.02$ $p = .359$ n.s.
N. fuscus 0.7% LAH 20AK				$X^2 = 21.1$ $p < 0.001$ ***

BIVALENTS 2-6 SINGLES

Species	*N. mitchellii* 4.9% LAH 13.8% GSAH	*N. alexis* 3.3% LAH 11.7% GSAH	*N. fuscus* 3.1% LAH 1.8% GSAH	*N. cervinus* 2.1% LAH 17.6% GSAH
N. mitchellii 4.9% LAH 20AK		$X^2 = 36.9$ $p < .001$ ***	$X^2 = 4.82$ $p = .028$ *	$X^2 = 78.7$ $p < .001$ ***
N. alexis 3.3% LAH 20AK			$X^2 = 71.1$ $p < .001$ ***	$X^2 = 6.46$ $p = .011$ *
N. fuscus 3.1% LAH 20AK				$X^2 = 131$ $p < .001$ ***

BIVALENTS 7-20 SINGLES

Species	*N. fuscus* 7.6% LAH 1.8% GSAH	*N. mitchellii* 7.0% LAH 13.8% GSAH	*N. alexis* 6.9% LAH 11.7 GSAH	*N. cervinus* 4.7% LAH 17.6% GSAH
N. fuscus 7.6% LAH 20AK		$X^2 = 85.1$ $p < .001$ ***	$X^2 = 119$ $p < .001$ ***	$X^2 = 125$ $p < .001$ ***
N. mitchellii 7.0% LAH 20AK			$X^2 = 2.60$ $p = .107$ n.s.	$X^2 = 2.55$ $p = .111$ n.s.
N. alexis 6.9% LAH 20AK				$X^2 = 0.009$ $p = .926$ n.s.

Table 3.9 : Species comparison of the number of double chiasmate bivalents in the three bivalent groups (cf. Table 3.8).

BIVALENT 1 DOUBLES

Species	*N. mitchellii* 1.5% LAH 13.8% GSAH	*N. alexis* 0.9% LAH 11.8% GSAH	*N. fuscus* 0.7% LAH 1.8% GSAH	*N. cervinus* 0.0% LAH 17.6% GSAH
N. mitchellii 1.5% LAH 20AK		$X^2 = .001$ $p = 1.000$ n.s.	$X^2 = 6.76$ $p = .012$ *	$X^2 = .227$ $p = .689$ n.s.
N. alexis 0.9% LAH 20AK			$X^2 = 6.55$ $p = .013$ *	$X^2 = .162$ $p = .694$ n.s.
N. fuscus 0.7% LAH 20AK				$X^2 = 5.28$ $p = .023$ *

BIVALENTS 2-6 DOUBLES

Species	*N. mitchellii* 4.9% LAH 13.8% GSAH	*N. alexis* 3.3% LAH 11.8% GSAH	*N. fuscus* 3.1% LAH 1.8% GSAH	*N. cervinus* 2.1% LAH 17.6% GSAH
N. mitchellii 4.9% LAH 20AK		$X^2 = 36.1$ $p < .001$ ***	$X^2 = 2.24$ $p = .022$ *	$X^2 = 68.5$ $p < .001$ ***
N. alexis 3.3% LAH 20AK			$X^2 = 71.5$ $p < .001$ ***	$X^2 = 4.02$ $p = .045$ *
N. fuscus 3.1% LAH 20AK				$X^2 = 119$ $p < .001$ ***

BIVALENTS 7-20 DOUBLES - AS FOR BIVALENTS 7-20 SINGLES

Table 3.10 : Species comparison of the number of triple chiasmate bivalents in the three bivalent groups (cf. Table 3.8).

BIVALENT 1 TRIPLES

Species	*N. mitchellii* 1.5% LAH 13.8% GSAH	*N. alexis* 0.9% LAH 11.7% GSAH	*N. fuscus* 0.7% LAH 1.8% GSAH	*N. cervinus* 0.0% LAH 17.6% GSAH
N. mitchellii 1.5% LAH 20AK		X^2 = 3.40 p = .074 n.s.	X^2 = 1.47 p = .263 n.s.	X^2 = 11.1 p = .001 **
N. alexis 0.9% LAH 20AK			X^2 = .465 p = .522 n.s.	X^2 = 2.42 p = .141 n.s.
N. fuscus 0.7% LAH 20AK				X^2 = 5.16 p = .029 *

BIVALENTS 2-6 TRIPLES

Species	*N. mitchellii* 4.9% LAH 13.8% GSAH	*N. alexis* 3.3% LAH 11.7% GSAH	*N. fuscus* 3.1% LAH 1.8% GSAH	*N. cervinus* 2.1% LAH 17.6% GSAH
N. mitchellii 4.9% LAH 20AK		X^2 = .419 p = 0.727 n.s.	X^2 = .720 p = .512 n.s.	X^2 = 11.1 p = 0.001 **
N. alexis 3.3% LAH 20AK			X^2 = .004 p = 1.0000 n.s.	X^2 = 8.31 p = .004 **
N. fuscus 3.1% LAH 20AK				X^2 = 7.73 p = 0.008 **

Table 3.11 : Frequencies of proximal, interstitial and distal chiasmata within the three bivalent groups (bivalents 1, 2-6 and 7-20) in *Notomys mitchelli*, *N. alexis*, *N. cervinus* and *N. fuscus*

BIVALENT 1

Species	LAH %	SAH %		Singles			Doubles			Triples		
				Prox	Int	Dis	Prox	Int	Dis	Prox	Int	Dis
N. mitchellii	1.5	13.8	n =	0	24	17	97	171	220	10	12	11
			% =	0	58.5	41.5	19.8	35.0	45.2	30.3	36.4	33.3
N. alexis	0.9	11.7	n =	1	14	18	109	178	221	8	36	22
			% =	3.0	42.0	55.0	21.5	35.0	43.5	12.1	54.6	33.3
N. fuscus	0.7	1.8	n =	6	44	17	118	159	207	9	29	19
			% =	9.0	65.7	25.3	24.4	32.8	42.8	15.8	50.9	33.3
N. cervinus	-	17.6	n =	2	14	16	83	304	227	28	52	40
			% =	6.2	43.8	50.0	13.5	49.5	37.0	23.3	43.4	33.3

BIVALENTS 2-6

Species	LAH %	SAH %		Singles			Doubles			Triples		
				Prox	Int	Dis	Prox	Int	Dis	Prox	Int	Dis
N. mitchellii	4.9	13.8	n =	85	389	310	420	309	657	3	3	3
			% =	10.8	49.6	39.6	30.3	22.3	47.4	33.3	33.3	33.3
N. alexis	3.3	11.7	n =	25	265	358	487	465	832	4	6	5
			% =	3.9	40.9	55.2	27.3	26.1	46.6	26.7	40.0	33.3
N. fuscus	3.1	1.8	n =	129	491	313	437	308	657	3	9	6
			% =	13.8	52.6	33.6	31.2	22.0	46.8	16.7	50.0	33.3
N. cervinus	2.1	17.6	n =	33	276	407	558	762	1002	15	31	23
			% =	4.6	38.5	56.9	24.0	32.8	43.2	21.7	45.0	33.3

BIVALENTS 7-20

Species	LAH %	SAH %		Singles			Doubles			Triples		
				Prox	Int	Dis	Prox	Int	Dis	Prox	Int	Dis
N. fuscus	7.6	1.8	n =	561	1669	2119	172	79	235	-	-	-
			% =	12.9	38.4	48.7	35.4	16.3	48.3	-	-	-
N. mitchellii	7.0	13.8	n =	409	1278	2018	332	123	423	-	-	-
			% =	11.0	34.5	54.5	37.8	14.0	48.2	-	-	-
N. alexis	6.9	11.7	n =	384	1459	1977	318	209	485	-	-	-
			% =	10.0	38.2	51.8	31.4	20.6	48.0	-	-	-
N. cervinus	4.7	17.6	n =	588	2067	2046	278	395	565	-	-	-
			% =	12.5	44.0	43.5	22.5	31.9	45.6	-	-	-

Table 3.12 : Species comparison of the number of proximal, interstitial and distal chiasmata in single chiasmate bivalent 1 (cf. Table 3.8).

PROXIMALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		$X^2 = 1.26$ $p = .446$ n.s.	$X^2 = 3.89$ $p = .081$ n.s.	$X^2 = 2.63$ $p = .189$ n.s.
N. alexis 0.9% LAH 20AK			$X^2 = 1.19$ $p = .420$ n.s.	$X^2 = .383$ $p = .613$ n.s.
N. fuscus 0.7% LAH 20AK				$X^2 = .213$ $p = .720$ n.s.

INTERSTITIALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		$X^2 = 1.90$ $p = .242$ n.s.	$X^2 = .555$ $p = .539$ n.s.	$X^2 = 1.57$ $p = .244$ n.s.
N. alexis 0.9% LAH 20AK			$X^2 = 4.91$ $p = .033$ *	$X^2 = 0.005$ $p = 1.000$ n.s.
N. fuscus 0.7% LAH 20AK				$X^2 = 4.29$ $p = .050$ n.s.

DISTALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		$X^2 = 1.26$ $p = .350$ n.s.	$X^2 = 3.05$ $p = .092$ n.s.	$X^2 = .529$ $P = .488$ n.s.
N. alexis 0.9% LAH 20AK			$X^2 = 8.27$ $p = .007$ **	$X^2 = .183$ $p = .135$ n.s.
N. fuscus 0.7% LAH 20AK				$X^2 = 5.91$ $p = .022$ *

Table 3.13 : Species comparison of the number of proximal, interstitial and distal chiasmata in double chiasmate bivalent 1 (cf. Table 3.8).

PROXIMALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		$X^2 = .378$ $p = .539$ n.s.	$X^2 = 2.86$ $p = .091$ n.s.	$X^2 = 8.05$ $p = .005$ **
N. alexis 0.9% LAH 20AK			$X^2 = 1.20$ $p = .273$ n.s.	$X^2 = 23.8$ $p < .001$ ***
N. fuscus 0.7% LAH 20AK				$X^2 = 21.3$ $p < .001$ ***

INTERSTITIALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		$X^2 = 0$ $p = 1.000$ n.s.	$X^2 = .519$ $p = .471$ n.s.	$X^2 = 23.3$ $p < .001$ ***
N. alexis 0.9% LAH 20AK			$X^2 = .529$ $p = .467$ n.s.	$X^2 = 23.8$ $p < .001$ ***
N. fuscus 0.7% LAH 20AK				$X^2 = 30.8$ $p < .001$ ***

DISTALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		$X^2 = .251$ $p = .616$ n.s.	$X^2 = .528$ $p = .470$ n.s.	$X^2 = 7.42$ $p = .007$ **
N. alexis 0.9% LAH 20AK			$X^2 = .055$ $p = .815$ n.s.	$X^2 = 4.95$ $p = .026$ *
N. fuscus 0.7% LAH 20AK				$X^2 = 3.81$ $p = .051$ n.s.

Table 3.14 : Species comparison of the number of proximal, interstitial and distal chiasmata in triple chiasmate bivalent 1 (cf. Table 3.8).

PROXIMALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		X^2 = 4.89 p = .050 n.s.	X^2 = 2.64 p = .116 n.s.	X^2 = .673 p = .495 n.s.
N. alexis 0.9% LAH 20AK			X^2 = .346 p = .607 n.s.	X^2 = 3.43 p = .081 n.s.
N. fuscus 0.7% LAH 20AK				X^2 = 1.33 p = .323 n.s.

INTERSTITIAL

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20AK		X^2 = 2.91 p = .135 n.s.	X^2 = 1.78 p = .197 n.s.	X^2 = .517 p = .552 n.s.
N. alexis 0.9% LAH 20AK			X^2 = .165 p = .720 n.s.	X^2 = 2.15 p = .168 n.s.
N. fuscus 0.7% LAH 20AK				X^2 = .886 p = .420 n.s.

DISTALS

Species	*N. mitchellii* 1.5% LAH	*N. alexis* 0.9% LAH	*N. fuscus* 0.7% LAH	*N. cervinus* 0.0% LAH
N. mitchellii 1.5% LAH 20SK		X^2 = 0 p = 1 n.s.	X^2 = 0 p = 1 n.s.	X^2 = 0 p = 1 n.s.
N. alexis 0.9% LAH 20AK			X^2 = 0 p = 1 n.s.	X^2 = 0 p = 1 n.s.
N. fuscus 0.7% LAH 20AK				X^2 = 0 p = 1 n.s.

Figure 3.4 : The distribution of single, double and triple exchanges in bivalent 1 of each of the four species of *Notomys* expressed as a percentage.

Nm = *N. mitchellii*

Na = *N. alexis*

Nf = *N. fuscus*

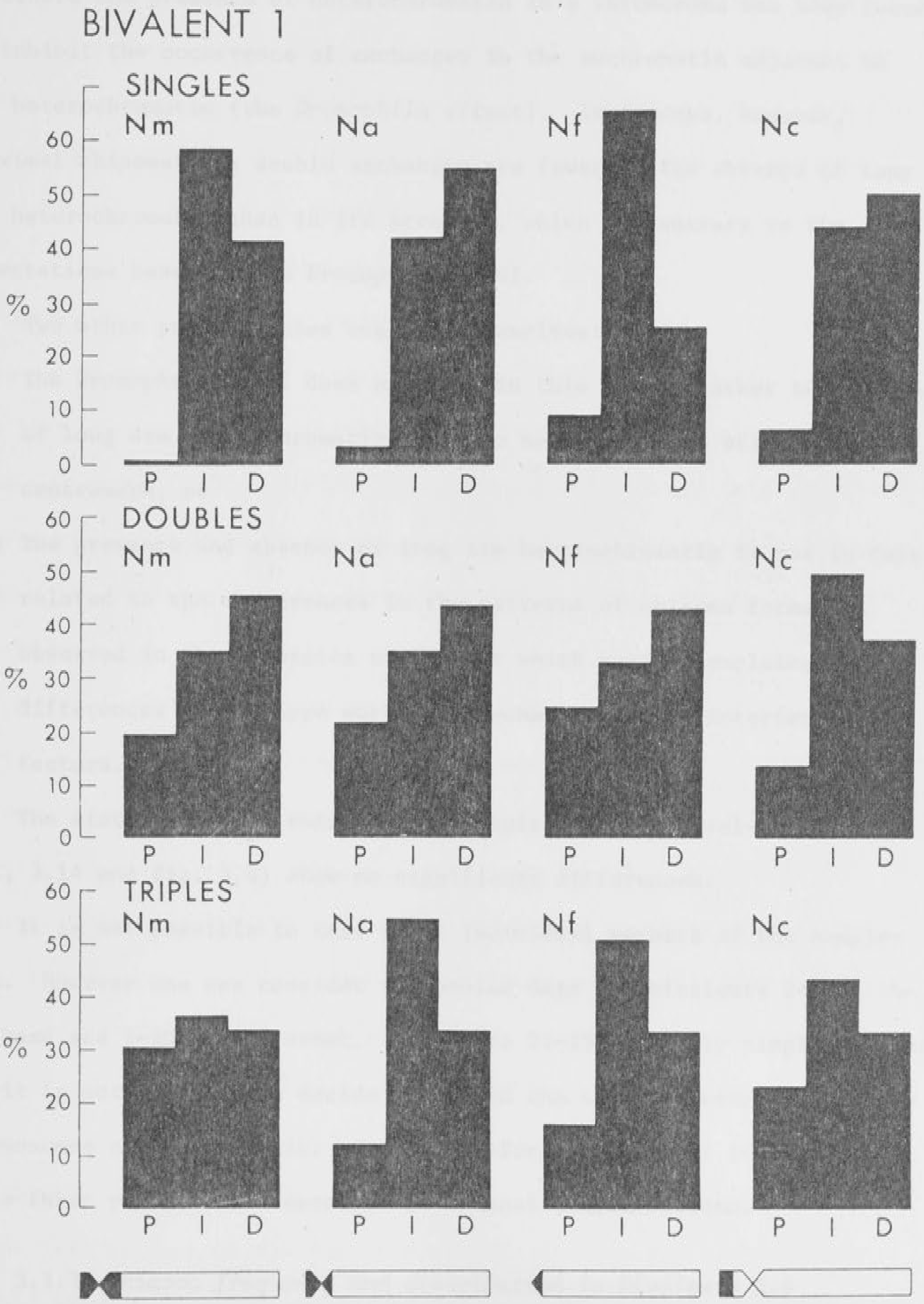
Nc = *N. cervinus*

P = Proximal chiasmata

I = Interstitial chiasmata

D = Distal chiasmata

Source data from Table 3.11.


BIVALENT 1
SINGLES
DOUBLES
TRIPLES
Nm
Na
Nf
Nc
%
0
10
20
30
40
50
60
P
I
D

difference depends upon fewer proximal and more interstitial chiasmata in *N. cervinus* which, it will be recalled, lacks long arm heterochromatin. Elsewhere the presence of heterochromatin in a chromosome has been found to inhibit the occurrence of exchanges in the euchromatin adjacent to the heterochromatin (the *Drosophila* effect). In *Notomys*, however, proximal chiasmata in double exchanges are fewer in the absence of long arm heterochromatin than in its presence, which is contrary to the expectations based on the *Drosophila* model.

Two other possibilities suggest themselves:

(i) The *Drosophila* model does not hold in this genus, rather the presence of long arm heterochromatin tends to neutralise the effect of the centromere, or

(ii) The presence and absence of long arm heterochromatin is not in fact related to the differences in the patterns of chiasma formation observed in these species of *Notomys* which must be explained on differences in genotype working presumably through interference factors.

The distribution of chiasmata in triple exchange bivalents (Tables 3.11, 3.14 and Fig. 3.4) show no significant differences.

It is not possible to test other individual members of the complement. However one can consider the pooled data for bivalents 2-6 on the one hand and 7-20 on the other. Bivalents 21-23 form only single chiasmata and it is not possible to decide which arm the chiasmata occupy when these chromosomes are metacentric. It is therefore impractical to deal with these three pairs with respect to positional considerations.

3.3.3 *Chiasma frequency and distribution in bivalents 2-6*

Turning to bivalents 2-6 (Table 3.7), there are only slight differences in the amount of long arm heterochromatin and it is the genomic short arm heterochromatin which provides the predominant difference in constitutive heterochromatin distribution. *N. fuscus* has minimal genomic SAH and *N. cervinus* the most. The frequency of single (Tables 3.7 and 3.8), double (Tables 3.7 and 3.9) and triple exchanges (Tables 3.7 and 3.10) shows *N. cervinus* to have significantly fewer singles and more doubles and triples than the other three species. *N. fuscus* has significantly more single and less double exchanges than the other three species. Here as in bivalent 1 high genomic SAH content is correlated with increased chiasma frequency.

Turning now to the data on distribution (Tables 3.11 and 3.15-17) the comparison between *N. alexis* and *N. fuscus* is particularly meaningful since these have the same percentage long arm heterochromatin. Despite this they show strikingly different patterns of distribution of single exchanges whether the distribution is considered wholely (Fig. 3.5) or individualy as proximal, interstitial or distal exchanges (Table 3.15). Clearly in this case long arm heterochromatin appears not to be exerting any effect.

Added to this while pair by pair comparisons do reveal some additional significant differences between species (Table 3.15) these differences are not consistently related to either heterochromatin amount or distribution. Rather they are most easily interpreted in terms of genotypic differences between species. For example, although *N. mitchellii* with the most long arm heterochromatin also has significantly more interstitial chiasmata than *N. cervinus* with the least long arm heterochromatin, *N. fuscus* and *N. alexis* with intermediate amounts do not conform to this pattern.

Double exchanges, however, do present a more consistent picture since their pattern of distribution (Table 3.16 and Fig. 3.5) is comparable in *N. mitchellii*, *N. alexis* and *N. fuscus* but differs from *N. cervinus*. There are significantly fewer proximals and more interstitials in *N. cervinus* than in any of the other three species which do not

Table 3.15 : Species comparison of the number of proximal, interstitial and distal chiasmata in single chiasmate bivalents 2-6 (cf. Table 3.8).

PROXIMALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		$X^2 = 24.9$ $p < .001$ ***	$X^2 = 3.48$ $p = .067$ n.s.	$X^2 = 20.1$ $p < .001$ ***
N. alexis 3.3% LAH 20AK			$X^2 = 43.2$ $p < .001$ ***	$X^2 = .471$ $p = .505$ n.s.
N. fuscus 31.% LAH 20AK				$X^2 = 38.9$ $p < .001$ ***

INTERSTITIALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		$X^2 = 10.9$ $p = .001$ ***	$X^2 = 1.54$ $p = .214$ n.s.	$X^2 = 18.6$ $p < .001$ ***
N. alexis 3.3% LAH 20AK			$X^2 = 21.1$ $p < .001$ ***	$X^2 = .783$ $P = .376$ n.s.
N. fuscus 3.1% LAH 20AK				$X^2 = 32.3$ $p < 0.001$ ***

DISTALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		$X^2 = 35.2$ $p < .001$ ***	$X^2 = 6.62$ $p = .010$ n.s.	$X^2 = 44.9$ $p < .001$ ***
N. alexis 3.3% LAH 20AK			$X^2 = 73.7$ $p < .001$ ***	$X^2 = .352$ $p = .553$ n.s.
N. fuscus 3.1% LAH 20AK				$X^2 = 89.4$ $p < .001$ ***

Table 3.16 : Species comparison of the number of proximal, interstitial and distal chiasmata in double chiasmate bivalents 2-6 (cf. Table 3.8).

PROXIMALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		X^2 = 3.45 p = .063 n.s.	X^2 = .246 p = .620 n.s.	X^2 = 17.6 p < .001 ***
N. alexis 3.3% LAH 20AK			X^2 = 5.72 p = .017 *	X^2 = 5.68 p = .017 *
N. fuscus 3.1% LAH 20AK				X^2 = 22.8 p < .001 ***

INTERSTITIALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		X^2 = 6.01 p = .014 *	X^2 = .043 p = .836 n.s.	X^2 = 46.8 p < .001 ***
N. alexis 3.3% LAH 20AK			X^2 = 7.17 p = .007 **	X^2 = 22.0 p < .001 ***
N. fuscus 3.1% LAH 20AK				X^2 = 50.2 P < .001 ***

DISTALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		X^2 = .184 p = .668 n.s.	X^2 = .082 p = .775 n.s	X^2 = 6.34 p = .012 *
N. alexis 3.3% LAH 20AK			X^2 = .016 p = .900 n.s.	X^2 = 4.96 p = .026 *
N. fuscus 3.1% LAH 20AK				X^2 = 4.87 p = .027 *

Table 3.17 : Species comparison of the number of proximal, interstitial and distal chiasmata in triple chiasmate bivalents 2-6 (cf. Table 3.8).

PROXIMALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		X^2 = .121 p = 1.000 n.s.	X^2 = .964 p = .628 n.s.	X^2 = .603 p = .676 n.s.
N. alexis 3.3% LAH 20AK			X^2 = .490 p = .674 n.s.	X^2 = .171 p = .736 n.s.
N. fuscus 3.1% LAH 20AK				X^2 = .223 p = .754 n.s.

INTERSTITIALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		X^2 = 1.07 p = 1.000 n.s.	X^2 = .675 p = .683 n.s.	X^2 = .435 p = .724 n.s.
N. alexis 3.3% LAH 20AK			X^2 = .235 p = .729 n.s.	X^2 = .121 p = .781 n.s.
N. fuscus 3.1% LAH 20AK				X^2 = .148 p = .793 n.s.

DISTALS

Species	*N. mitchellii* 4.9% LAH	*N. alexis* 3.3% LAH	*N. fuscus* 3.1% LAH	*N. cervinus* 2.1% LAH
N. mitchellii 4.9% LAH 20AK		X^2 = 0 p = 1.0 n.s.	X^2 = 0 p = 1.0 n.s.	X^2 = 0 p = 1.0 n.s.
N. alexis 3.3% LAH 20AK			X^2 = 0 p = 1.0 n.s.	X^2 = 0 p = 1.0 n.s.
N. fuscus 3.1% LAH 20AK				X^2 = 0 p = 1.0 n.s.

Figure 3.5 : The distribution of single, double and triple exchanges in bivalents 2-6 of each of the four species of *Notomys* expressed as a percentage.

Nm = *N. mitchellii*

Na = *N. alexis*

Nf = *N. fuscus*

Nc = *N. cervinus*

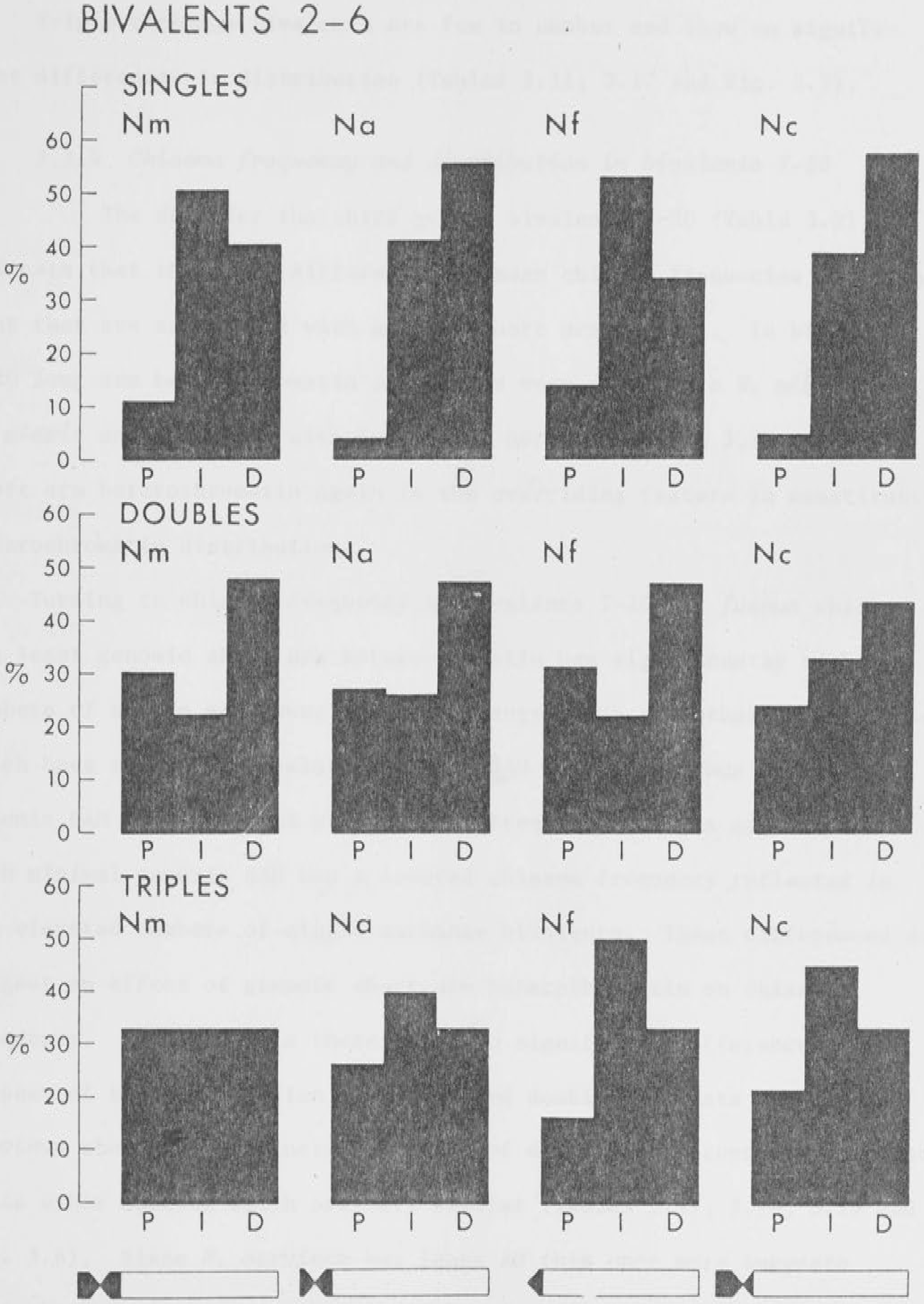
P = Proximal chiasmata

I = Interstitial chiasmata

D = Distal chiasmata

Source data from Table 3.11.

97


BIVALENTS 2–6
SINGLES
DOUBLES
TRIPLES
Nm
Na
Nf
Nc
%
0
10
20
30
40
50
60
P I D

differ from one another in these respects. These data are consistent with that of bivalent 1 doubles (Table 3.13 and Fig. 3.4).

Triple exchange bivalents are few in number and show no significant differences in distribution (Tables 3.11; 3.17 and Fig. 3.5).

3.3.4 *Chiasma frequency and distribution in bivalents 7-20*

The data for the third group, bivalents 7-20 (Table 3.3), indicate that there are differences in mean chiasma frequncies per bivalent that are correlated with genomic short arm content. In bivalents 7-20 long arm heterochromatin content is very similar in *N. mitchellii, N. alexis* and *N. fuscus* with less in *N. cervinus* (Table 3.7). Genomic short arm heterochromatin again is the overriding feature in constitutive heterochromatin distribution.

Turning to chiasma frequency in bivalants 7-20, *N. fuscus* which has least genomic short arm heterochromatin has significantly higher numbers of single and fewer double exchanges than the other three species which have relatively evelated genomic LAH contents. Thus here too genomic SAH is correlated with chiasma frequency changes and *N. fuscus* with minimal genomic SAH has a lowered chiasma frequency reflected in the elevated numbers of single exchange bivalents. These differences do suggest an effect of genomic short arm heterochromatin on chiasma frequency. Added to this there are also significant differences with respect of the distribution of single and double chiasmata with *N. cervinus* showing a distinctive pattern of distribution compared with the three other species which are very similar (Tables 3.11, 3.18, 3.19 and Fig. 3.6). Since *N. cervinus* has least AH this once more suggests that there is interaction between LAH and the centromere effect. *N. cervinus* has least LAH and presumably is sufficient to neutralise completely the centromere effect. Thus in double exchange bivalents of *N. cervinus* there are significantly fewer proximals than in the other three species (Tables 3.11, 3.19 and Fig. 3.6). The other species have very

Table 3.18 : Species comparison of the number of proximal, interstitial and distal chiasmata in single chiasmate bivalents 7–20 (cf. Table 3.8).

PROXIMALS

Species	*N. fuscus* 7.6% LAH	*N. mitchellii* 7.0% LAH	*N. alexis* 6.9% LAH	*N. cervinus* 4.7% LAH
N. fuscus 7.6% LAH 20AK		$X^2 = 6.54$ $p = .011$ *	$X^2 = 16.1$ $p < .001$ ***	$X^2 = .314$ $p = .576$ n.s.
N. mitchellii 7.0% LAH 20AK			$X^2 = 1.94$ $p = .163$ n.s.	$X^2 = 4.28$ $p = .039$ *
N. alexis 6.9% LAH 20AK				$X^2 = 12.6$ $p < .001$ ***

INTERSTITIALS

Species	*N. fuscus* 7.6% LAH	*N. mitchellii* 7.0% LAH	*N. alexis* 6.9% LAH	*N. cervinus* 4.7% LAH
N. fuscus 7.6% LAH 20AK		$X^2 = 13.0$ $p < .001$ ***	$X^2 = .029$ $p = .865$ n.s.	$X^2 = 29.2$ $p < .001$ ***
N. mitchellii 7.0% LAH 20AK			$X^2 = 11.1$ $p < .001$ ***	$X^2 = 77.7$ $p < .001$ ***
N. alexis 6.9% LAH 20AK				$X^2 = 29.0$ $p < .001$ ***

DISTALS

Species	*N. fuscus* 7.6% LAH	*N. mitchellii* 7.0% LAH	*N. alexis* 6.9% LAH	*N. cervinus* 4.7% LAH
N. fuscus 7.6% LAH 20AK		$X^2 = 26.4$ $p < .001$ ***	$X^2 = 7.47$ $p = .006$ **	$X^2 = 24.6$ $p < .001$ ***
N. mitchellii 7.0% LAH 20AK			$X^2 = 5.56$ $p = 0.18$ *	$X^2 = 99.4$ $p < .001$ ***
N. alexis 6.9% LAH 20AK				$X^2 = 57.3$ $p < .001$ ***

Table 3.19 : Species comparison of the number of proximal, interstitial and distal chiasmata in double chiasmate bivalents 7-20 (cf. Table 3.8).

PROXIMALS

Species	*N. fuscus* 7.6% LAH	*N. mitchellii* 7.0% LAH	*N. alexis* 6.9% LAH	*N. cervinus* 4.7% LAH
N. fuscus 7.6% LAH 20AK		X^2 = .788 p = .375 n.s.	X^2 = 2.25 p = .125 n.s.	X^2 = 30.3 p < .001 ***
N. mitchellii 7.0% LAH 20AK			X^2 = 8.51 p = .004 **	X^2 = 59.0 p < .001 ***
N. alexis 6.9% LAH 20AK				X^2 = 23.0 p < .001 ***

INTERSTITIALS

Species	*N. fuscus* 7.6% LAH	*N. mitchellii* 7.0% LAH	*N. alexis* 6.9% LAH	*N. cervinus* 4.7% LAH
N. fuscus 7.6% LAH 20AK		X^2 = 1.25 p = .263 n.s.	X^2 = 4.09 p = .043 *	X^2 = 42.9 p < .001 ***
N. mitchellii 7.0% LAH 20AK			X^2 = 14.3 p < .001 ***	X^2 = 89.0 p < .001 ***
N. alexis 6.9% LAH 20AK				X^2 = 35.9 p < .001 ***

DISTALS

Species	*N. fuscus* 7.6% LAH	*N. mitchellii* 7.0% LAH	*N. alexis* 6.9% LAH	*N. cervinus* 4.7% LAH
N. fuscus 7.6% LAH 20AK		X^2 = .004 p = .950 n.s.	X^2 = .024 p = .876 n.s.	X^2 = 1.04 p = .309 n.s.
N. mitchellii 7.0% LAH 20AK			X^2 = .012 p = .913 n.s.	X^2 = 1.33 p = .249 n.s.
N. alexis 6.9% LAH 20AK				X^2 = 1.17 p = .280 n.s.

Figure 3.6 : The distribution of single and double exchanges in bivalents 7-20 in all four species of *Notomys* expressed as a percentage.

Nf = *N. fuscus*

Nm = *N. mitchellii*

Na = *N. alexis*

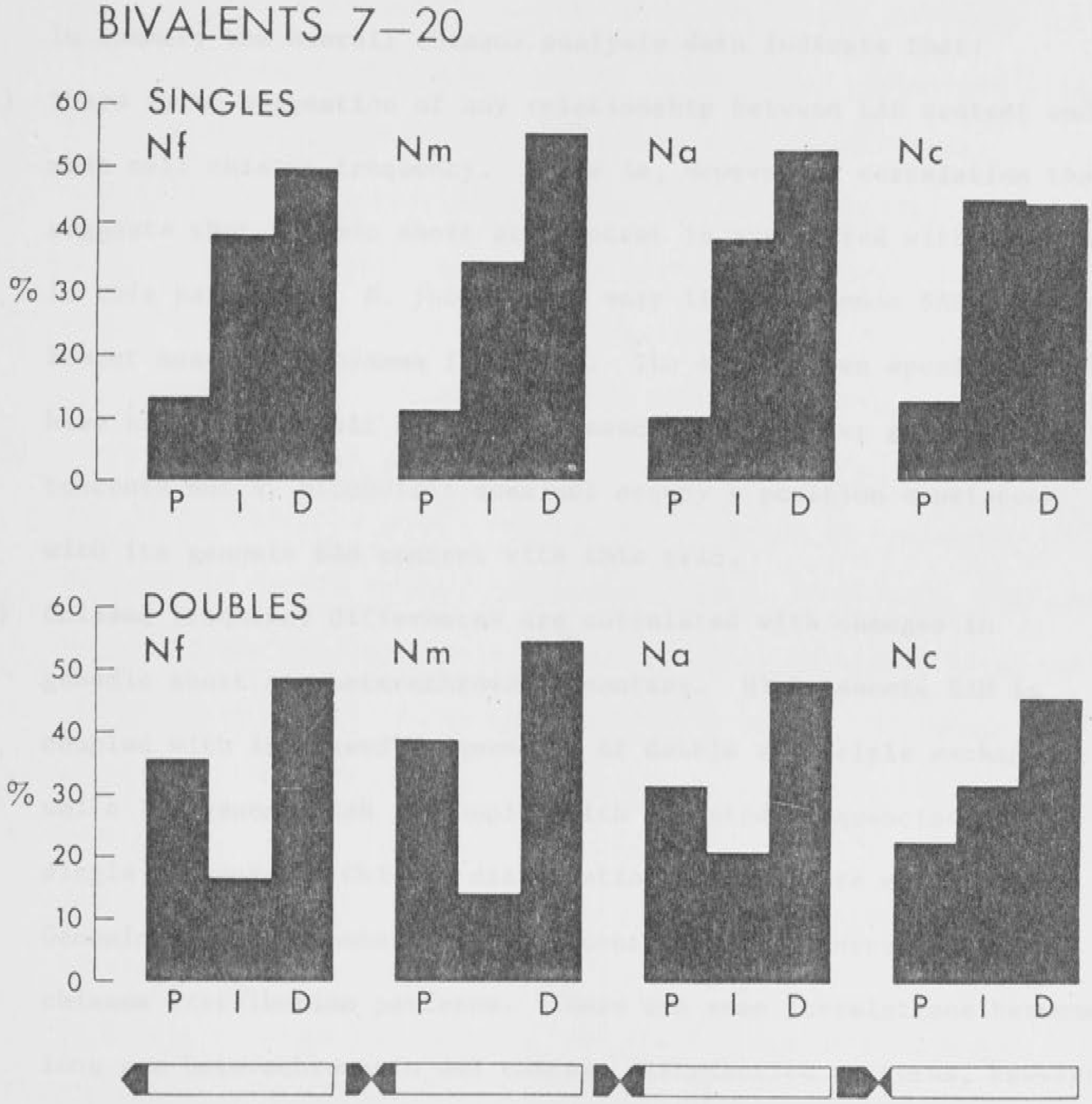
Nc = *N. cervinus*

P = Proximal chiasmata

I = Interstitial chiasmata

D = Distal chaismata

Source data from Table 3.11.


BIVALENTS 7–20
SINGLES
DOUBLES
Nf
Nm
Na
Nc
%
0
10
20
30
40
50
60
P I D

similar amounts of LAH and their distribution patterns of both single and double exchanges differ very little from one another (Tables 3.11, and Fig. 3.6). These data from double exchanges is similar to those from bivalents 1 and 2-6 and suggest that LAH content merely neutralises the centromere effect and has a negligible if any direct influence on chiasma position of its own.

In summary the overall chiasma analysis data indicate that:

(1) There is no suggestion of any relationship between LAH content and mean cell chiasma frequency. There is, however, a correlation that suggests that genomic short arm content is associated with changes in this parameter. *N. fuscus* with very little genomic SAH has the lowest mean cell chiasma frequency. The other three species all have higher mean cell chiasma frequencies and higher genomic SAH contents but *N. mitchellii* does not occupy a position consistent with its genomic SAH content with this trio.

(2) Chiasma frequency differences are correlated with changes in genomic short arm heterochromatin content. High genomic SAH is coupled with increased frequencies of double and triple exchanges, while low genomic SAH is coupled with elevated frequencies of single exchanges. Chiasma distribution patterns are more complex. Genomic short arm heterochromatin content is not correlated with chiasma distribution patterns. There are some correlations between long arm heterochromatin and chiasma distribution patterns, however, in single exchange bivalents in bivalent groups 1 and 2-6 there is no consistency in distribution patterns that can be linked to LAH content.

(3) The most consistent correlation involving heterochromatin content and chiasma frequency relates to that of genomic short arm heterochromatin and chiasma frequencies in these three bivalents classes.

Thus:

(i) Single exchange bivalents are most frequent in *N. fuscus* which has minimal genomic SAH in all chromosome classes (1, 2-6 and 7-20). In all cases these differences are significant in comparisons with all the remaining species.

(ii) Those species with elevated genomic SAH contents (*N. cervinus*, *N. mitchellii* and *N. alexis*) have more double exchanges than *N. fuscus* (little genomic SAH) in all three chromosome classes. In all comparisons these differences are significant.

(iii) Triple exchanges are more frequent in *N. cervinus* which has the most genomic SAH in both chromosome classes which form triples than the remaining species. The differences are significant in five out of the six comparisons.

(4) The most consistent chiasma distribution difference is that involving the distribution of double exchanges. This can be correlated with differences in LAH content and suggests that, in *Notomys*, LAH acts to neutralise the centromere effect resulting in higher frequencies of exchanges nearer to the heterochromatic blocks than would be expected. This, of course, is contrary to the situation in *Drosophila*. Thus, in double exchanges in bivalent 1 of *N. cervinus* which has no LAH there are significantly less proximals than in the other three species which do have LAH in this bivalent. Similarly in double exchange bivalents in classes 2-6 and 7-20, where *N. cervinus* has least LAH, there are fewer proximally located chiasmata.

Comparison of *N. mitchellii* with *N. alexis* with respect to chiasma frequency patterns reveal significant differences despite their having similar LAH and genomic SAH contents (Table 3.4). The bulk of these differences is found in bivalent group 2-6 where *N. mitchellii* has significantly more single and fewer double exchanges than *N. alexis*. Despite this difference in chiasma frequency *N. mitchellii* and *N. alexis* are still closer to one another than either is to *N. fuscus* or *N. cervinus*.

However the chiasma differences which exist between *N. mitchellii* and *N. alexis* seem greater than the differences which exist between them in both karyotype (Chapter 2) and satellite DNA (Chapter 6) would suggest. Of course, while the LAH and genomic SAH contents are very similar in *N. mitchellii* and *N. alexis*, the actual distribution of this material is unique in each case (see Fig 2.2 and Table 2.5). It is not possible to say if the difference between these two species is due to constitutive heterochromatin or to genotypic differences.

3.4 *Discussion*

From the chiasma analysis data it seems clear that there is no simple relationship between total heterochromatin content and mean cell chiasma frequency in *Notomys*. LAH is not correlated with mean cell chiasma frequency nor with chiasma frequency within the bivalent classes. Although LAH is implicated in chiasma distribution in a manner which suggests that it neutralizes the centromere effect, it does not appear to exert any direct effect on chiasma position as in *Drosophila* or *Atractomorpha*. Genomic SAH has no effect on chiasma distribution, but it may be correlated in some ill-defined way with mean cell chiasma frequency such that an increase in total genomic SAH increases this variable. Such a situation is without parallel in any system other than genomes which carry supernumerary heterochromatic material either in the form of B-chromosomes or supernumerary segments (John 1973; John and Miklos, 1979 their Table XVII).

Of course, even if the genomic SAH content is involved in the observed changes in mean cell chiasma frequencies, not all bivalents need be affected, and not all affected bivalents need to be changed to the same degree. In *Notomys*, frequency trends are consistent but they are not always significant statistically. High genomic SAH levels (e.g. *N. cervinus*) are accompanied by fewer singles and more doubles and triples; conversely low genomic SAH levels (e.g. *N. fuscus*) are correlated with high frequencies of singles and fewer doubles and triple exchanges. *N. fuscus* with least genomic short arm heterochromatin has significantly higher numbers of single and fewer double exchanges than the other three species in all bivalent groups. The correlation between genomic short arm heterochromatin content and chiasma frequency is consistent.

The absence of any simple effect in *Notomys* between long arm heterochromatin content and chiasma distribution may of course stem in part from the relatively crude method of defining chiasma location. Thus, in *Caledia*, Shaw and Knowles (1976) were able to demonstrate only a minimal influence of heterochromatin on distribution when they used a refined method of scoring chiasma position. A comparison between *Caledia captiva* and *C. species nova-1* (Table 3.20), the two species from the *Caledia* study which allow some degree of comparison to be made with the *Notomys* situation, suggest than even here the chiasma frequency changes are not evenly distributed throughout the genome. Only seven comparisons out of twenty-one reach significance (Table 3.21).

Another important factor which may contribute to the complexity of the situation in *Notomys* is the length of euchromatin available for exchange relative to the operation of the following four factors:

(i) The strength of the centromere effect.
(ii) The level of interference between exchanges.
(iii) The genotypic determinants of chiasma distribution.
(iv) The presence of any heterochromatin effects.

The distribution of single chiasma in *Notomys* indicate that proximal exchanges are infrequent in all chromosome classes (see Figs. 3.4-3.6). Consequently it would be unlikely that either the centromere or the long arm heterochromatin would exhibit any effect on the distribution of singles.

Double exchanges would seem to provide a better basis for determining whether there are centromere-heterochromatin effects in *Notomys*

Table 3.20 : Data on the frequency of single, double, and triple chiasma bivalents in *Caledia captiva* "Daintree" race and *Caledia species nova-1* in chromosomes 1 to 12 (data from Shaw and Knowles, 1976)

Chromosome number	*C. species nova-1*			*C. captiva* ("Daintree" race)		
	Singles	Doubles	Triples	Singles	Doubles	Triples
1	1	95	5	23	75	3
2	1	92	8	22	73	6
4	20	77	4	67	32	2
5	46	54	1	80	21	0
6	58	43	0	76	24	0
7	72	29	0	75	26	0
8	81	20	0	84	17	0
9	75	26	0	92	9	0
10	101	0	0	101	0	0
11	101	0	0	101	0	0
12	101	0	0	101	0	0

Table 3.21 : Chi-square analyses of the frequencies of single, double and triple chiasmate bivalents in Bivalents 1 through to 12 and *Caledia* species *nova 1* and *C. captiva* ("Daintree" race). (Date from Shaw and Knowles, 1976).

	Single chiasmate bivalents	Double chiasmate bivalents	Triple chiasmate bivalents
Bivalent number	Statistics	Statistics	Statistics
1	$X^2 = 18.20$ $p < 0.001$ ***	$X^2 = 1.28$ $p = 0.297$ n.s.	$X^2 = 0.481$ $p = 0.72$ n.s.
2	$X^2 = 17.365$ $p < 0.001$ ***	$X^2 = 1.21$ $p = 0.294$ n.s.	$X^2 = 0.267$ $p = 0.784$ n.s.
4	$X^2 = 18.23$ $p < 0.001$ ***	$X^2 = 12.32$ $p < 0.001$ ***	
5	$X^2 = 5.71$ $p = 0.022$ *	$X^2 = 10.741$ $p = 0.0011$ **	
6	$X^2 = 1.458$ $p = 0.264$ n.s.	$X^2 = 4.07$ $p = 0.049$ n.s.	
7	$X^2 = 0.0035$ $p = 0.914$ n.s.	$X^2 = 0.129$ $p = 0.76$ n.s.	
8	$X^2 = 0.003$ $p = 0.917$ n.s.	$X^2 = 0.207$ $p = 0.722$ n.s.	
9	$X^2 = 0.949$ $p = 0.347$ n.s.	$X^2 = 7.07$ $p = 0.009$ **	
10	all singles		
11	" "		
12	" "		

because the interference between the two exchanges results in one of the chiasma lying in the proximal region of the bivalent (Figs. 3.4-3.6). While there are no data from any of the three chromosome classes to suggest that the *Drosophila-Atractomorpha* type heterochromatin effect is detectable in *Notomys*. Double exchange bivalents in all three chromosome classes do exhibit significant differences in distribution pattern which suggest that, if anything, the presence of LAH neutralizes the centromere effect. Thus, in *Notomys*, exchanges are formed nearer to the proximal heterochromatic blocks in species with large blocks and further away in those species with smaller blocks.

In general terms, there are three possibilities for relating the distribution of chiasmata to the distribution of heterochromatin:

(i) Heterochromatin location may itself determine the chiasma distribution pattern as is the case in *Atractomorpha similis* (Miklos and Nankivel, 1976) and *Cryptobothrus chrysophorus* (John and King, 1980).

(ii) Chiasma siting determines heterochromatin localisation. While no documented instances of this phenomenon exist there remains the possibility that initial chiasma localisation could lead to the development of heterochromatic blocks in regions of the chromosome which are devoid of chiasma as was suggested by Nankivell (1967) for *Austroicetes interioris*.

(iii) A third possibility is that chiasma siting and heterochromatin sites are largely independent of one another, with the one qualification that chiasmata cannot form in a heterochromatic block. This is a difficult situation to identify since at face value there often appears to be a direct relationship between the two variables. Indeed White (1942, 1954) argued that chiasmata formed predominently at heterochromatin-euchromatin junctions.

Loidl (1979) working with *Allium flavum*, a species with distal chiasma localisation, found that there is a high correlation between the siting of C-bands and the occurrence of chiasmata, a situation not unlike that in rye. However as Jones and Rees (1964) and Jones (1967) have shown, it is possible through inter-specific hybridization to generate a rye genome in which the chiasma pattern is converted to a non-localized form. Since in this form the chromosome organization remains unchanged there is no reason to assume that the C-band pattern, which is also distal, will have altered. This example therefore shows quite clearly that the change in chiasma pattern is unrelated to the location of the heterochromatic segments and implies that the two are independent variables. Thus heterochromatin in rye does not, as might appear superficially, promote or attract chiasmata in its vicinity.

Indeed there is evidence, from a comparison of different species of *Allium*, that the same argument applies in Loidl's case too. Thus all species of *Allium* that have been C-banded have the bands located at or near the distal ends of the chromosome arms (Vosa, 1976a,b; Loidl, 1979). This applies not only in species with distally localized chiasmata, as in *A. flavum*, studied by Loidl, but in all the species so far examined, some of which have proximally localized chiasma, as is the case in *A. fistulosum* (Levan, 1933a), while others (*A. carinatum* - Levan, 1933a, 1937; *A. narcissiflorum*, *A. ammophilum* - Levan, 1935; *A. macranthum* - Levan, 1933b) clearly have a semi-localized distribution pattern. Clearly in the *Allium* species while the C-band pattern is reasonably conserved this is not the case with the chiasma pattern. Thus here, too, the overriding influence of the genotype appears to determine the distribution of chiasmata.

Given that a genome has heterochromatin, let us now consider the effects of heterochromatin content on chiasma frequency. Most of the cases where demonstrable effects have been shown to exist involve supernumerary heterochromatin. Here four kinds of relationships occur:

(i) The presence of supernumerary heterochromatin in the form of added supernumerary segments may increase chiasma frequency through inter-chromosomal effects (*Tolgardia infirma* - John and Freeman, 1976; *Stethophyma* species - Shaw, 1971a,b)

(ii) Alternatively, supernumerary heterochromatin in the form of B chromosomes may decrease chiasma frequency by inter-chromosomal effects (*Lolium perenne* - Cameron and Rees, 1967; *Triticum speltoides* - Zarchi *et al.*, 1972).

(iii) Heterochromatin in the form of introduced B chromosomes may have no effect on the mean cell chiasma frequency in *Secale cereale* but still increase the variance between cells (Jones and Rees, 1967,1969).

(iv) Finally, there are undoubtedly cases where supernumerary heterochromatin does not exert any demonstrable effect on either chiasma frequency or chiasma variance. Even here, however, it has not yet been ruled out that there is no effect on chiasma position independent of chiasma frequency.

If we turn to consider the behaviour of fixed heterochromatin then there is some evidence that the heterochromatin content of a genome can be correlated with changes in chiasma frequency. Though this evidence rests largely on between species comparisons, it can also be implicated with changes in chiasma position as is indicated by the following facts:

(i) There may be a direct negative effect of heterochromatic blocks on adjacent euchromatin. This has been both demonstrated in *Drosophila melanogaster*, by an experimental repositioning of heterochromatin (Miklos, unpublished observations in Miklos and John, 1979), and by a comparison of chromosomes races and their hybrids in *Cryptobothrus chrysophorus* (John and King, 1980). The first of these involves the experimental construction of a *Drosophila melanogaster* stock heterozygous for a telomeric block of heterochromatin. The heterozygotes show reduced recombination distally by comparison with the homozygote which lacks telomeric heterochromatin (see Fig. 6c of Miklos and John, 1979). The second example involves the use of chromosome races - one with fixed telomeric blocks the other without - to give hybrids heterozygous for the telomeric blocks. These situations are comparable with the situation found naturally in polymorphic populations of *Atractomorpha similis* (Miklos and Nankivell, 1976).

(ii) The only alternative is the situation we have already discussed in *Allium* and which obtains also in *Bryodema tuberculata* (Klasterska *et al.*, 1974). While this situation has been interpreted in terms of a positive effect of heterochromatin on chiasma siting the evidence when considered in toto argue strongly against this.

In *Notomys* as we have seen there are some grounds for arguing that LAH may neutralize the centromere effect in which case we may have an example of yet a third possible effect. Finally, a fourth possibility is suggested by the correlations which obtain between the genomic SAH content and chiasma frequency. However while the first of these is similar to that of *Drosophila melanogaster* X chromosome centric heterochromatin (Yamamoto and Miklos, 1978), the second is a novel effect. Both these effects in *Notomys* remain to be proven and it may well be that in this genus chiasma frequency is largely determined by the genotype.

CHAPTER 4

NUCLEOLAR ORGANIZING REGIONS IN *NOTOMYS*

4.1 *Introduction*

It has been shown in Chapter 2 that there are differences in the organization of the euchromatic regions in the karyotypes of the four species of *Notomys* as well as differences in heterochromatin content and chiasma characteristics. An additional source of variation between the complements of related species can often be found in the location sites of nucleolar organizing regions (NORs). To complete the chromosome comparison between the species of *Notomys*, therefore, this chapter examines the situation in respect of the number and distribution of NORs.

In most eucaryotes the 18S and 28S ribosomal RNA (rRNA) loci are found at the nucleolar organizing sites which are constant within a species. There are five ways in which the location of these sites can be determined:

(i) Conventional chromosome stains: In many instances the presence of secondary constrictions, which stain differentially, mark the position of NOR's. The human karyotype is a good example of this, having NOR's associated at the secondary constrictions of the short arms of chromosomes 13, 14, 15, 21 and 22 (Henderson *et al.*, 1974; Evans *et al.*, 1974). However not all chromosome complements have secondary constrictions, for example *Microtus agrestis* and *Carollia castanea* (Hsu *et al.*, 1975) and not all secondary constrictions necessarily define NOR's, for example *Littoria* sp. (King, 1980, in press).

(ii) In some cases nucleolar material is found associated with NORs (McClintock, 1934; John and Henderson, 1962; Given and Phillips, 1976). This is especially obvious at first meiotic prophase and is particularly evident in the lampbrush chromosomes of female amphibians (Gall, 1954; Callan, 1966; Mancino *et al.*, 1972; Macgregor and Kezer, 1973).

(iii) *In situ* hybridization techniques, using tritiated 18S and 28S ribosomal RNA (^{3}HrRNA), provide a particularly elegant method for locating sites of ribosomal genes (Barsacchi-Pilone *et al.*, 1974).

(iv) More recently silver staining techniques, such as the Ag-AS method (Goodpasture and Bloom, 1975; Bloom and Goodpasture, 1976) or the simpler 50% silver nitrate method (Varley, 1977), have been employed. In mammals these appear to stain the ribonucleic protein which surrounds those NORs (Schwarzacher *et al.*, 1978) that were functionally active in the last prophase (Miller, O.J., *et al.*, 1976; Miller, D.A., *et al.*, 1976).

The silver technique reveals that not all the NORs in a complement are active. In humans, for example (Varley, 1977) there is a variation in the number of NORs stained from individual to individual and also from cell to cell within individuals. Within an individual the modal number of active NORs is constant and persists over a three month test period. Cooke (1971) using associations of D group chromosomes also found agreement between replicates three months apart but replicates three years apart failed to agree. In different individuals there is variation in the number of active NORs which range from 3 to 5 through to 3 to 9 out of the possible 10 NORs in the human complement (Varley, 1977).

In the newt *Triturus cristatus carnifex* Varley and Morgan (1978) find that particular lampbrush loops which are clearly not NORs give a positive reaction with the silver staining technique. Thus while the silver staining technique does stain NORs in amphibians it is not completely specific to them. Likewise Nardi *et al.* (1977) report that in *T. vulgaris meridionalis* a series of sites in addition to the known NOR sites hybridized with ^{3}H 18S + 28S rRNA. Subsequently Nardi *et al.*,

(1978) found that these additional sites also stained with silver and some other sites, which had not given positive results with *in situ* hybridization, were silver positive.

(v) An N-banding technique, which utilizes Giesma staining following extraction of nucleic acids and histones, also stains NORs (Matsui and Sasaki, 1973). Although this technique was introduced before silver staining, it has been relatively little used and has now largely been superseded by the silver methods.

The two most commonly employed techniques, *in situ* hybridization and silver staining, differ in the information produced. The former provides data on the actual number of NOR sites whether active or not, while the latter indicates the number of active NOR sites. This appears to be true of mammals though obviously is not true of newts (see (iv) above).

4.2 *Methods*

Both the mitotic and the meiotic material from the four species of *Notomys* used for the location of NORs were samples of the slides used for G and C banding.

For silver staining, 3 to 4 drops of 50% w/v silver nitrate in distilled water were placed on the slide and a coverslip added. The slide was then sealed in a humid atmosphere at 37°C for 12 to 24 hours. If, necessary, counter staining was then carried out in 2% Giesma for 30 seconds. This method is adapted from Varley (1977). Combined silver staining and G banding, according to the method of Tantravahi *et al.* (1977), was attempted but did not prove successful.

4.3 *Observations and discussion*

4.3.1 *Mitotic silver staining*

Silver staining of mitotic preparations revealed the presence of NORs on two autosome pairs in *N. mitchellii*, *N. alexis* and *N. fuscus*

(Fig. 4.1a,b and d). In each of these species the NORs were located on the telomeres of the long arms of the chromosomes in question. In the absence of combined G banding and silver staining the chromosomes appear to be pairs 5 and 11 in each of these three species as determined on a length basis. Since this designation is necessarily tentative, it is not possible to be certain that the NOR bearing chromosomes in each species are indeed homologous.

In contrast to the situation in the above species. *N. cervinus* usually has 3 (Fig. 4.1c), but in some cells four pairs of autosomes on which NORs were seen. Added to this, their location is on the telomere of the heterochromatic short arms of what ~~previously~~ provisionally have been identified as chromosomes 2, 5, 10 and 11.

In *Notomys* there is some variation in the staining intensity from chromosome to chromosome within a cell (Fig. 4.2e). Furthermore, in some chromosomes, one chromatid either has a lighter stain than the other, or else completely lacks stain (Fig. 4.2d and a). Finally, in many cells NOR bearing chromosomes are found associated with silver staining material connecting two NORs (Fig. 4.2b,c).

The differential siting of NORs in *N. cervinus* represents a major karyotypic difference in the genus *Notomys*. Admittedly, it has not been determined that in the other three species the NORs are on homologous chromosomes, but the location of the NORs in *N. cervinus* is distinctive.

In the guinea pig, *Cavia cobaya* the NORs are usually located on the short arms of five pairs of autosomes; however, a polymorphism has been detected in which a NOR is located on the telomere of the long arm of one of these five chromosomes in some individuals (Zenzes *et al.*, 1977). This condition resembles that which exists between the species of *Notomys*, although of course in *Cavia* it is unfixed and intraspecific. Zenzes *et al.* (1977) attribute the change in *Cavia* to an inversion.

Figure 4.1 : Nucleolar organizer regions (NORs) in *Notomys* as revealed by silver-staining.

(a) *N. mitchellii*, (b) *N. alexis*, (c) *N. fuscus*, and *N. cervinus*.

Note the marked difference in NOR location in *N. cervinus* relative to the other three species.

Arrows indicate NORs.

Bar represents 10 µm.

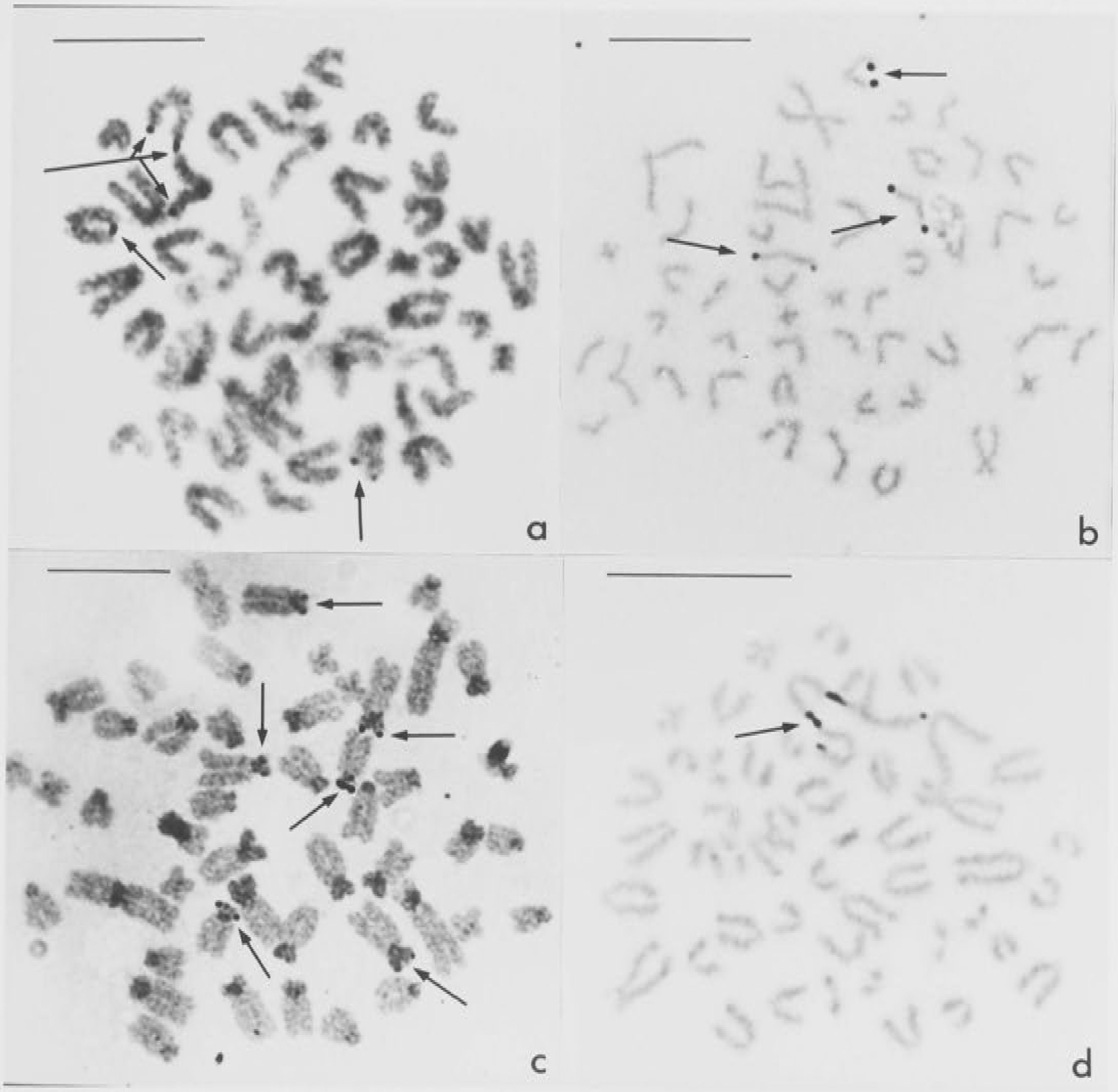

a
b
c
d

Figure 4.2 : NOR staining characteristics and variation in *Notomys*.

(a) The positive staining of one chromatid of a chromosome together with the absence of any staining in the other chromatid in *N. alexis*.

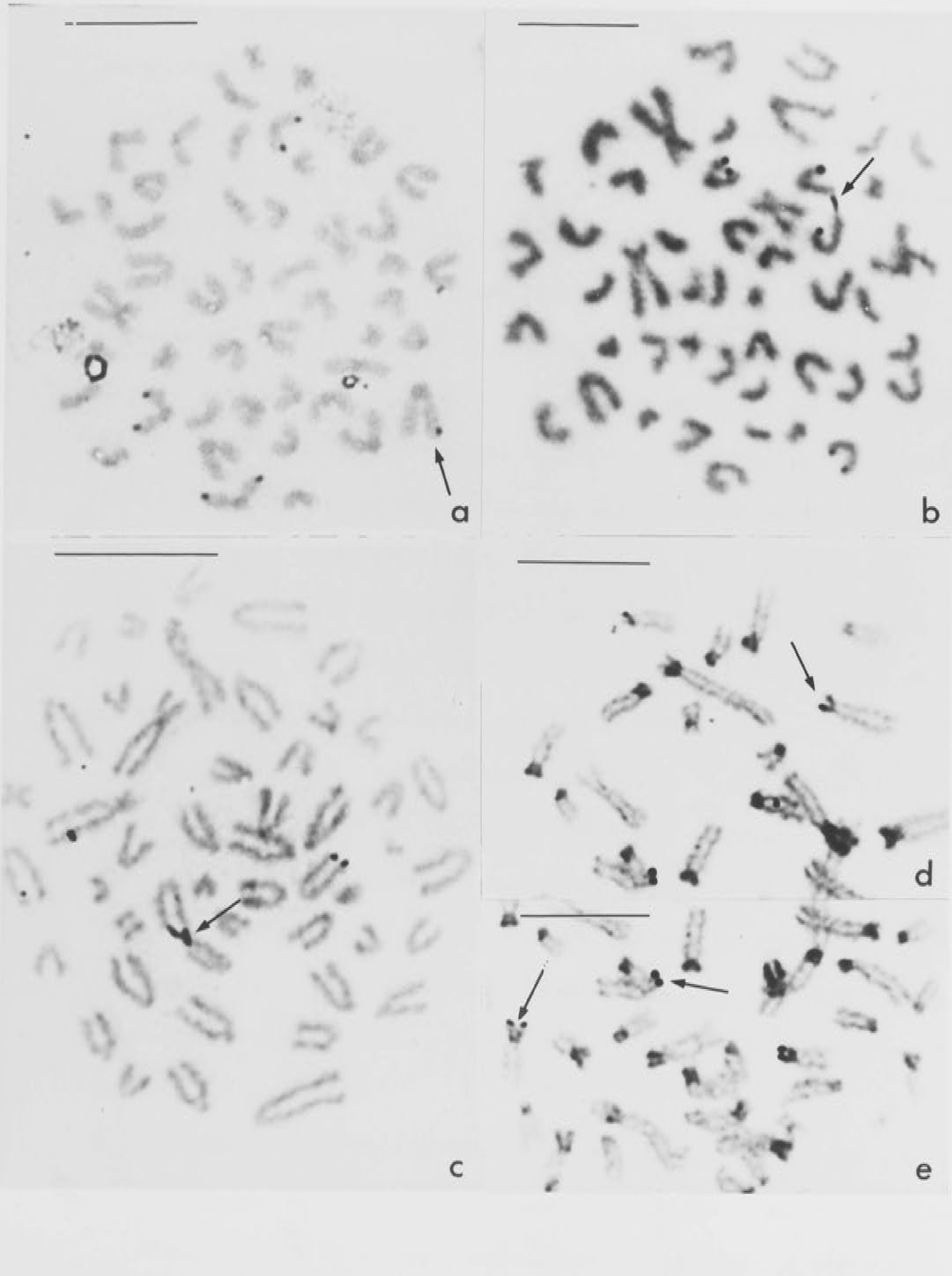
(b) Association of NOR bearing chromosomes with connecting material stained positively with silver nitrate solution in *N. alexis*.

(c) Connective material and the association of NOR bearing chromosomes stained positively with silver nitrate in *N. fuscus*.

(d) Variation of staining intensity within a NOR chromosome where one chromatid is more heavily stained with silver than the other (*N. cervinus*).

(e) Variation in staining intensity in one NOR chromosome compared to another (*N. cervinus*).

Bar represents 10 μm.


a
b
c
d
e

There are two possible explanations for the observed difference in NOR siting in *Notomys*:

(i) Activation - inactivation. In this case one would have to assume that multiple copies of NORs may be situated in the chromosome complement and that they become differentially activated or inactivated. In hybrid males from crosses of *Drosophila mullerii* females and *D. arizonensis* males, a latent NOR in the microchromosomes becomes activated (Bicudo and Richardson, 1977). This phenomenon suggests that latent NORs could account for some instances of apparent NOR shift. This mechanism is favoured by King (1980, in press) to account for the observed change in the position of NORs in the hylid frog genus *Littoria*. Further, in *Mus musculus* there is variation in the number and distribution of NORs both in inbred strains and in different sub-species (Henderson *et al.*, 1974; Elsevier and Ruddle, 1975; Dev *et al.*, 1977). This may have its basis in the activation-reactivation of NORs, since there is no change in the G banding pattern in these cases.

In *Notomys, in situ* hybridization using ^{3}H 18S + 28S rRNA could be used to test if there are NOR sites undetected by silver staining. This, however, presumes that latent sites necessarily have the full complement of rDNA which may not be the case. In this event activation would also involve an amplification process.

(ii) Chromosome rearrangements - here three possibilities can be envisaged:

a. Pericentric inversions involving one break point in the long arm region proximal to the organizer and one break point in the distal region of a short arm. This will produce a chromosome in which the organizer is now located terminally on the new short arm, while the G-band sequence of the new long arm appears unchanged.

b. A three break transposition in which the organizer is inserted from its terminal location in the long arm to an equivalent location in the short arm. This is complicated by the fact that the organizer will not be completely terminal in its new location.

c. Non-homologus exchanges. In this case NORs could be repositioned in the genome by non-homologous exchanges between the NOR and other regions which have similar DNA sequences. Heterochromatin associated with the nucleolus has been shown to contain repetitive DNA sequences (Pardue and Gall, 1970; Comings and Matoccia, 1972). In *Xenopus* spacer regions of rDNA have been found to contain internally repetitious simple sequence DNA (Wellaur *et al.*, 1976; Buongiorno-Nardelli *et al.*, 1977). If similar simple sequences of DNA occur in both NORs and other chromosome sites such as telomeres, then non-homologous exchange could be favoured. This type of repositioning has been suggested for *Triturus vulgaris meridionalis* by Nardi *et al.* (1978).

The difficulty with all three of these explanations is that contrary to the activation-inactivation hypothesis they provide no satisfactory basis for the difference in number of NORs which obtains between *N. cervinus* and the other three species.

Activation-inactivation would appear to be the most likely explanation for the situation found in *Notomys* because the difference in the number of NORs in *N. cervinus* compared with the other three species. Moreover, if the presumed chromosomal phylogeny of the pseudomyids suggested by Baverstock *et al.* (1977a) is correct then activation would again be the most likely explanation since they argue that *N. mitchellii* and *N. alexis* are closer to the ancestral stock from which the species of *Notomys* originated.

4.3.2 *Meiotic silver staining*

All four species respond identically to the application of silver staining to meiotic material with respect to the stages stained:

(i) There is a total absence of silver staining during the condensed stages of both meiotic divisions, i.e. from diakinesis to telophase II (Fig. 4.3i and ii).

(ii) A positive reaction was, however, obtained in Sertoli cells, in premeiotic nuclei, in early first prophase nuclei (leptotene to pachytene) and in post-meiotic nuclei up to and including the early stages of spermatid elongation (Fig. 4.3i).

(iii) Most of the observations were carried out on *N. cervinus* and here the Sertoli cells usually have from one to three large silver grains. Early spermatocytes ocassionally have from five to eight silver deposits but most usually these coalesce to give a smaller number of large clusters. In the other species of *Notomys* fewer grains are present reflecting the lower number of NORs in these species. The spherical spermatids have from 1 to 4 medium sized precipitates. In contrast to other mammals these are also found in the elongating spermatids (Fig. 4.i). However, during subsequent elongation and maturation there are no discrete grains although sperm heads may stain an even dark brown (Fig. 4.3iii). Human sperm reacts similarly with some acrosomal areas appearing darker (Hofgartner *et al.*, 1979).

The presence of silver staining in the post-meiotic spermatids raises the interesting question of whether there is haploid gene expression in the developing spermatozoa. This has been a contentious issue for some time (Monesi *et al.*, 1978).

In mammals spermatogenesis offers the only stage in the life cycle when the question of haploid gene expression can be investigated since following the production of haploid spermatids by meiosis there is a complex process of differentiation which leads to the development of the

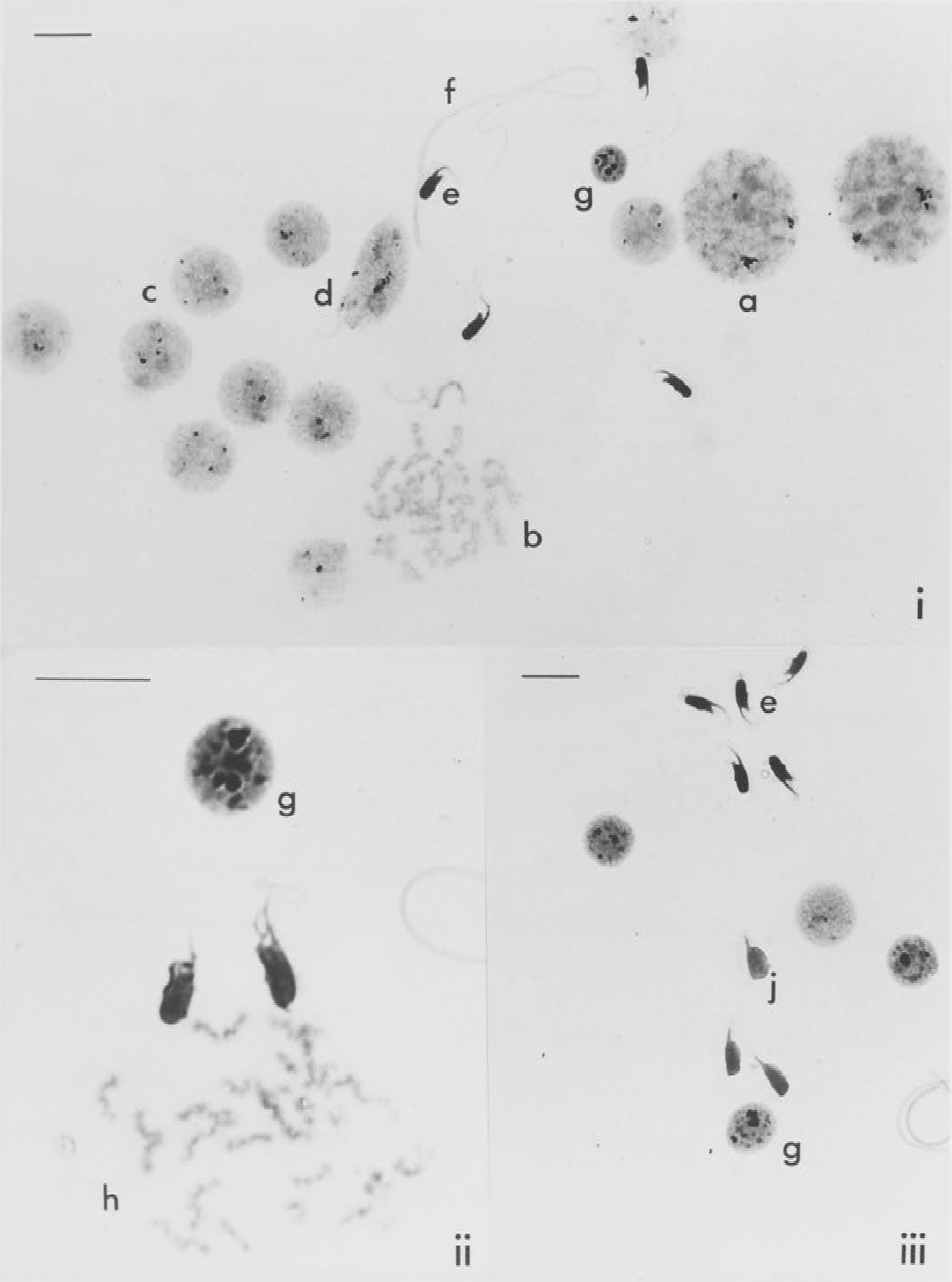
Figure 4.3 : Gametogenesis in a male *N. cervinus* using silver staining.

(i)
- a. Primary spermatocytes, note presence of 2-3 aggregates of silver grains.
- b. Diakinesis, note lack of silver staining.
- c. Spermatids, note the presence of up to 4 silver grains.
- d. Elongating spermatid still showing silver staining.
- e. Sperm heads, no grains but heads show a positive silver reaction.
- f. Sperm tail.
- g. Sertoli cell with 5 prominent silver grains.

(ii) Meiosis II metaphase in *N. cervinus*.
- g. Sertoli cell.

(iii) Sperm heads of *N. cervinus*.
- e. Mature sperm with overall dark staining but absence of silver grains.
- j. Maturing sperm showing absence of silver grains.

Bar represents 10 μm.


f
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mature sperm. In theory the development of the spermatozoon could be under the control of the diploid genotype, the haploid genotype or a combination of both. Diploid control would require the synthesis and accumulation of stable messenger RNAs (mRNAs) in the primary spermatocyte and their subsequent translation during spermiogenesis. With haploid control the spermatid nucleus would be responsible for synthesising the mRNAs required for spermiogenesis. Combined diploid and haploid control would involve both stable mRNA production in the primary spermatocyte as well as transcriptional activity of the haploid genome in the spermatid.

RNA polymerase activity has been shown to occur during early spermiogenesis (Moore, 1971) and autoradiographic studies of mammalian testis (Monesi, 1965; Utakoji, 1966; Kierszenbaum and Tres, 1975) show a small burst of post-meiotic nuclear RNA synthesis that declines once the spermatid embarks on maturation. However, this RNA consists of heterogeneous nuclear RNA while the ribosomal RNA is produced mainly in the primary spermatocyte (Kierszenbaum and Tres, 1975). Further, Betlach and Erickson (1976) present data that suggest that little or none of the 28S and 18S RNA found in the spermatozoa is transcribed in the maturing spermatozoa and that the majority is synthesised in the primary spermatocyte.

More recent work on *Mus musculus* (Geremia *et al.*, 1977,1978; Monesi *et al.*, 1978) demonstrates that the rate of RNA synthesis per DNA content is almost the same in both primary spermatocytes and round spermatids. The synthesis of RNA was found to occur at three stages of spermatogenesis:

(i) middle-late pachytene spermatocytes,

(ii) early pachytene and secondary spermatocytes, and in

(iii) round spermatids at stages 1-8 of spermiogenesis. No RNA synthesis took place in intermediate and late spermatids (steps 9-16 of spermiogenesis).

These data are in close agreement with earlier autoradiographic results of Monesi (1965). The round spermatids and the pachytene spermatocytes both synthesised polyadenylated RNA (presumptive mRNA) and ribosomal RNA. Further the poly $(A)^+$ RNA synthesised in the round spermatids is present in polysomes and is presumably mRNA (Geremia *et al.*, 1978). These results contrast, however, with those autoradiographic and electron microscopic observations which claim to show a lack of nucleolar activity in the spermatids of *Mus musculus* (Kierszenbaum and Tres, 1975).

Evidence obtained by silver staining of male gametogenesis in a range of vertebrates (Schmid *et al.*, 1977; Schwarzacher *et al.*, 1978; Hofgartner *et al.*, 1979; Pathak and Hsu, 1979) has been interpreted as a reactivation of the ribosomal genes in the haploid spermatid. This rests on the assumption that silver selectively stains transcriptionally active RNA genes in interphase nuclei at those rRNA sites on chromosomes that were transcribed during the preceding interphase (Miller *et al.*, 1976; Miller *et al.*, 1976). This assumption is supported by the fact that in some human-mouse hybrid cells only mouse 28S rRNA is synthesised and silver staining defines only the mouse NOR region (Eliceiri and Green, 1969). In male gametogenesis the silver staining pattern follows the pattern of rRNA synthesis (Galderi and Monesi, 1974; Tres, 1975). The silver-stainable material is an acidic protein component of the ribonucleic protein which accumulates around active NORs (Schwarzacher *et al.*, 1978). The vertebrates so far investigated represent a wide range of taxa and in male meiosis positive reactions to silver staining were obtained in both diploid and haploid cells. The positive reaction was detected in diploid cells through to late pachytene but not from diakinesis through meiotic metaphse I and II. The positive staining reappeared in haploid cells in early spermatids and persisted until elongation of the spermatids.

The results presented here for *Notomys* agree in general with these previous data, though here the staining persists clearly into the elongation stages of the spermatids. Taken at face value there would indeed appear to be evidence in *Notomys* for the haploid expression of NORs.

CHAPTER 5

SEX CHROMOSOME ASSOCIATION IN THE GENUS *NOTOMYS* AT MALE MEIOSIS

5.1 *Introduction*

At diakinesis and first meiotic metaphase of most mammalian species the X and Y chromosomes form a bivalent by end-to-end association (Solari, 1974). In these species this association has been interpreted in one of two ways:

(i) The association is a precocious terminalization of a chiasma that forms very close to a distal end of the bivalent (Ohno *et al.*, 1959; Fredga and Santesseon, 1964; Solari and Tres, 1970; Solari, 1974).

(ii) The association is non-chiasmate (Makino, 1941; Sachs, 1955). This possibility has recently been confirmed in the sand rat *Psammomys obesus* where chiasmata are never observed between the X and Y chromosomes with the light microscope and ultrastructure studies reveal a complete absence of synaptinemal complex (SC) formation between these chromosomes (Solari and Ashley, 1977). In this case the association is clearly non-chiasmate since a chiasma cannot form in the absence of a SC.

Some mammals are exceptional, however, because there are distinct chiasmate associations between the X and Y chromosomes. Most of these species have X chromosomes which are considerably larger than the standard X. For example, *Apodemus mystacinus* (Wahrman and Ritte, 1963 and in John and Lewis, 1965 see their Fig. 155), the hamster species *Cricetus cricetus* and *Cricetulus griseus* (Vistorin *et al.*, 1977) all have a single visible chiasma between the X and Y. The presence of a chiasma has also been reported in the XY pair of the porcupine *Erethisum dorsatum* (Benirschke, 1968) and the goat *Capra hircus* (Datta, 1970), although in both cases the preparations lack the quality necessary for definitive diagnosis. Solari (1974) claimed that the association between the X and X in the African mouse *Rattus* (*Mastomys*) *natalensis* was chiasmate (Huang and Strong, 1962), however the authors themselves do not make such a claim and the accompanying illustration (their Fig. 4b) show the XY bivalent devoid of any suggestion of a visible chiasma.

Prior to air drying techniques, the identification of the actual end of the X and Y chromosomes involved in association at diakinesis in mammals was difficult for two reasons:

(i) Squash techniques did not yield meiotic preparations of sufficient clarity,

(ii) The precise identification of chromosome ends in the XY bivalent was difficult prior to the development of C-banding.

Perhaps the best documented case is that of the house mouse, *Mus musculus*. Both Makino (1941) and Matthey (1953) held that mouse X and Y chromosomes associated in a non-chiasmate manner by their long arms. However, Ohno *et al.* (1959) subsequently proposed that the short arms of the X and Y were homologous and that pairing took place between these arms with consequent formation of a chiasma. Using autoradiographic techniques Kofman-Alfaro and Chandley (1970) studied both early and late replication of mouse sex chromosomes at male meiosis. The late replication evidence showed that the X chromosome was associated with the Y chromosome by the telomere of the long arm, since the free end of the X was late replicating. Early replication data indicated that the centromeric end of the Y labels and this region was furthest away from the XY association. Thus the Kofman-Alfaro and Chandley (1970) study confirmed the long arm X to long arm of Y association suggested earlier by Makino (1941) and Matthey (1953). Under the electron microscope, the X and Y chromosomes of the mouse were found to be paired distally and a SC initially formed over almost the whole length of the Y chromosome but later reduced to a minute SC (Solari, 1970; Moses *et al.*, 1977). Use of C-banding clearly demonstrated that in the XY bivalent of mouse diakinetic figures, the centromeric end of the X was free at diakinesis (Hsu *et al.*, 1971; Schnedl, 1972; Polani, 1972). The centromere of the Y does not always C-band at meiosis (Polani, 1972), nevertheless, in the cells where the Y centromere does stain positively the correct orientation of the Y in the XY bivalent was obtained and invariably the Y was associated with the X by its non-centromeric end or long arm (Polani, 1972; Schnedl, 1972).

The use of C-banding techniques have revealed that the X and Y chromosomes of mammals may be associated at meiotic metaphase in a variety of ways:

(i) Telomere to telomere as found in *Mus musculus* (Hsu *et al.*, 1971; Kyslikova and Forejt, 1972; Schnedl, 1972; Polani, 1972) and in other *Mus* species such as *M. caroli*, *M. cookii* and *M. cervicolor* (Markvong *et al.*, 1976), as well as in both *Phodopus sungorus sungorus* and *Mesocricetus auratus* (Vistorin *et al.*, 1977).

(ii) Telomere to centromere as in *M. dunnii* (Markvong *et al.*, 1976). Here the precise arrangement of the X and Y chromosomes in the sex bivalent is somewhat confounded by the difficulty of distinguishing genuinely telocentric chromosomes from acrocentric chromosomes with small short arms.

(iii) Centromere to centromere as in *M. shortridgei* and *M. pahari* (Markvong *et al.*, 1976) with the same qualifications referred to in (ii) above.

(iv) Genuine interstitial chiasma are found in the hamster species *Cricetus cricetus* and *Cricetulus griseus* (Vistorin *et al.*, 1977), and in the field mouse *Apodemus mystacinus* (Wharman and Ritte, 1963). Even in such cases, however, interstitial chiasmata are not necessarily seen in every bivalent and end-to-end associations frequently also occur (e.g. in both *Cricetus cricetus* and *Cricetulus* *griseus*, and Fredga and Santesson, 1964).

5.2 *Methods*

The meiotic material from the four species of *Notomys* used in the study of sex chromosome behaviour were samples of the slides used in the analysis of chiasma characteristics (Chapter 3). Three staining methods are used: (i) 2% Giesma, (ii) C-banding as described in Chapter 2 (see 2.2), and (iii) silver-staining as used in Chapter 4 (see 4.2).

5.3 *Results and Discussion*

In mammalian spermatocytes the early meiotic stages are difficult to define precisely. This difficulty is, of course, confounded by the use of air drying techniques which tend to separate and mix different meiotic stages. Both these difficulties have contributed to the lack of agreement on the state of condensation and the mode of pairing of the X and Y chromosomes during first meiotic prophase (see Solari, 1974 for a review). A further factor which contributes to this problem is the presence of a chromatin sex vesicle (= XY body) which incorporates the sex chromosomes and to some extent obscures them. The core of the vesicle involves an association of the heterochromatic regions of the two sex chromosomes with their euchromatic portions external to this core and wound around it. Thus it is now generally agreed that this vesicle is formed during zygotene, that is, at the very time when the autosomes are engaged in pairing.

In silver-stained preparations of *Notomys* the grains which appear in early first meiocytes may show one of three relationships to the sex vesicle:

(i) They may be unassociated with it (Fig. 5.1).

(ii) They may be close to but external to it (Fig. 5.2).

(iii) They may be associated with the periphery of the vesicle (Fig. 5.3). In some cases the grains appear over the vesicle giving the illusion of being within it (Fig. 5.2).

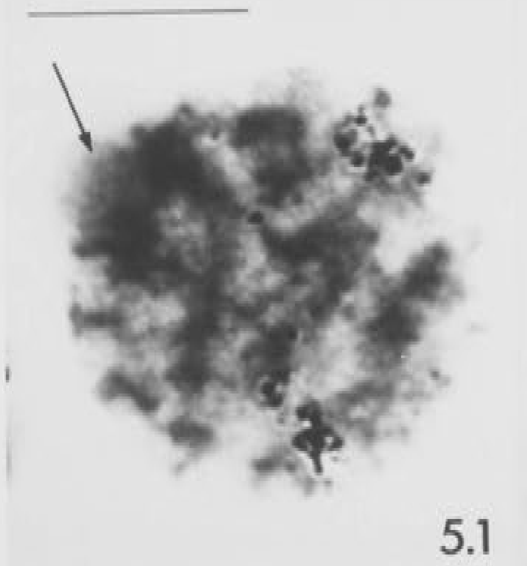
Figure 5.2 : Silver-stained early pachytene cell from *N. cervinus* with silver deposits associated with and over the sex vesicle (arrow). Bar = 10 μm.

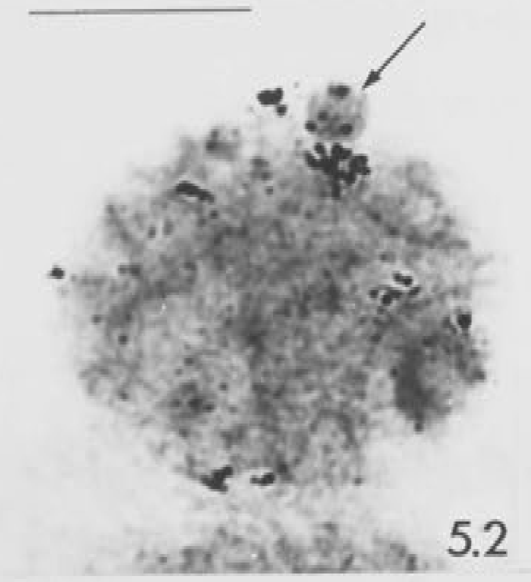
Figure 5.1 : Silver deposits showing no association with the sex vesicle (arrow) in *N. cervinus* at early pachytene. Bar = 10 μm. Silver-staining.

Figure 5.3 : Peripherally located silver deposits in assocition with the sex vesicle (arrow) in *N. cervinus* at early pachytene. Bar = 10 μm. Silver-staining.

Figure 5.4 : The XY body in *N. alexis* at pachytene showing the bipartite section (bs) peripherally located around the heterochromatic section (hs). Arrow indicates the XY body; bar = 10 μm.

Figure 5.5 : The bipartite section (bs) of the XY body clearly separated from the heterochromatic section (hs) in *N. alexis*. Bar = 10 μm. Giemsa staining.


5.1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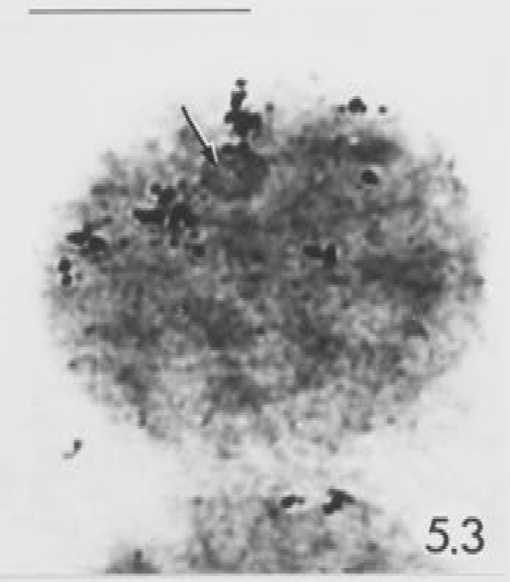
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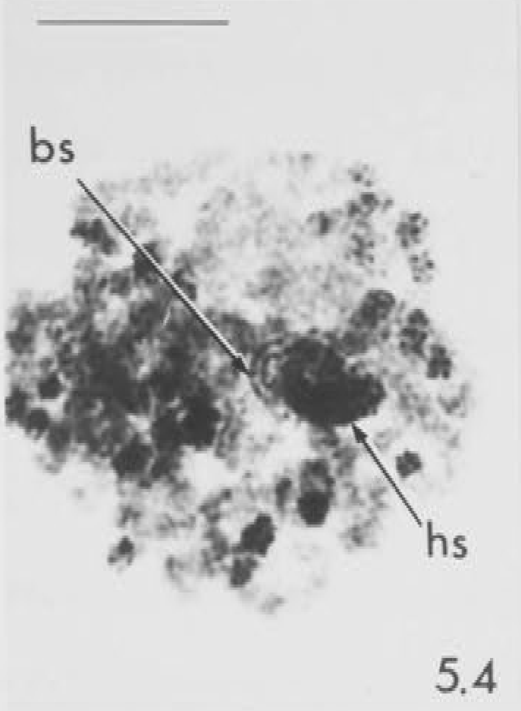

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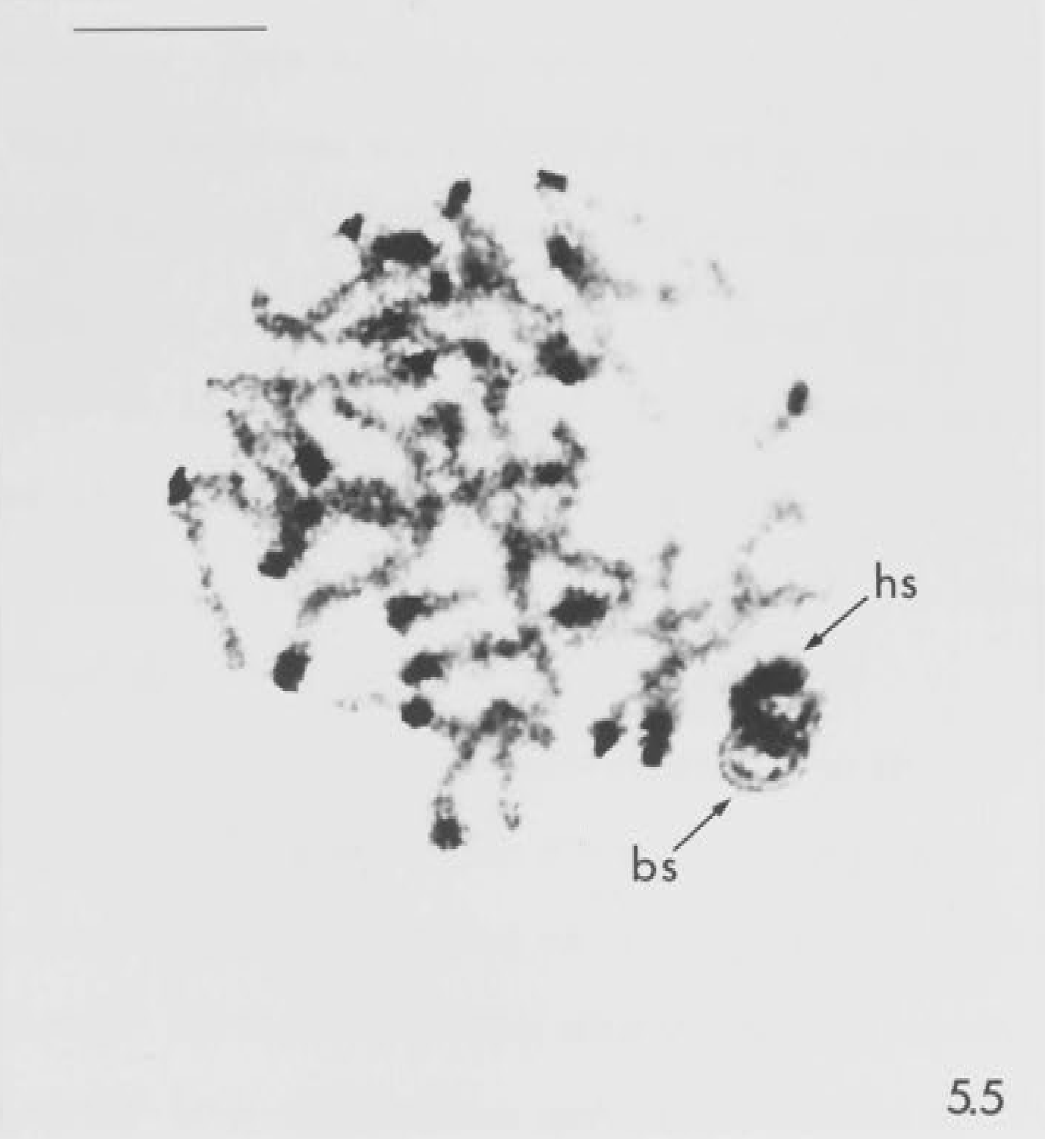


5.5

The relative frequency of these three types (Table 5.1) suggests that the grains are found at or near the sex vesicle more frequently than one would expect from a random relationship with it. Hofgartner *et al.* (1979) has made essentially similar observations in the mouse and he concluded that the grains were sometimes present within the sex vesicle as well as being commonly associated with it. They did not, however, observe this phenomenon in the rat, man or guinea pig. I find no evidence of the inclusion of silver deposits within the sex vesicle of *Notomys* and, as we have already seen, the NORs in *Notomys* are present on autosomes. In the mouse, Ohno (1957) initially concluded that the X and Y chromosomes were involved in organizing the nucleolus and excluded the autosomes from consideration. This position was maintained until quite recently (Solari, 1974). Henderson *et al.* (1974), using *in situ* hybridization, however, found that the NORs in the mouse are on autosome pairs 15, 18 and 19 and not on the X and Y. This was confirmed by Elsevier and Ruddle (1975). It is true however that in some mouse cell lines chromosome 12, 16 and 17, may also have NORs in addition to 15, 18 and 19 (Dev *et al.*, 1977). There is no evidence for NORs on the sex chromosomes of the mouse as detected by either *in situ* hybridization or silver-staining. Non-specific associations between regions of constitutive heterochromatin on different chromosomes do occur, however, (Hsu *et al.*, 1971; Natarajan and Gropp, 1972; Schmid *et al.*, 1975) and such associations has been attributed to satellite DNAs present in the constitutive heterochromatin of similar base composition and sequence (Hsu *et al.*, 1971; Schmid *et al.*, 1975). Similarly the association of silver-staining in association with the sex vesicle in *Notomys* may reflect similar DNA sequences in the NORs and in the sex chromosomes. Of course in *Notomys cervinus* the NORs are situated adjacent to large blocks of constitutive heterochromatin but in *N. alexis*, *N. mitchellii* and *N. fuscus* the NORs are situated on the telomeres of euchromatic arms and in these three species of *Notomys* there are no telomeric C-bands on the NOR bearing chromosome arms so that any association based on DNA homology would necessarily involve sequences within the NORs or the telomeres which are below the level of resolution expected from C-banding techniques.

Table 5.1 : Relationship of silver grains or deposits to the sex vesicle in *N. cervinus* early meiocytes which have been silver-stained. Number of cells scored = 100.

No association with sex vesicle	Near to the sex vesicle*	Peripheral to or over the sex vesicle*
26	52	22

*As these categories appear to be identical the silver deposits are found in association with the sex vesicle in 74% of the observed cells.

In early pachytene the XY body in *Notomys* retains both its dense heterochromatic region and its peripherally located lighter bipartite section (Fig. 5.4). By late pachytene the body has unravelled to the extent that one can now see the arrangement of the heterochromatic and euchromatic portions (Fig. 5.5).

At diakinesis C-banding reveals that in all four species of *Notomys* the heterochromatic arm of the X is located with the short arm of the Y (Fig. 5.6). In the vast majority of cases this association is terminal or end-to-end. On two occasions out of a total of several thousand observations, one in *N. cervinus* (Fig. 5.7) and one in *N. alexis* (Fig. 5.8), a chiasmate association has been seen between the heterochromatic arm of the X and one of the Y arms. While the precise arm of the Y chromosome involved could not be determined, it is thought to be the short arm because in both cases the Y chromosome involved was acrocentric and when

Figure 5.6 : XY association at diakinesis in *Notomys*.

a. *N. alexis* - short arm of the X chromosome associated with the short arm of the Y chromosome.

b. *N. alexis* - the heterochromatin short arm of the X chromosome associated with the short arm of the Y chromosome.

c. *N. fuscus* - the heterochromatic short arm of the X chromosome associted with the short arm of the Y chromosome.

d. *N. cervinus* - the heterochromatic short arm of the X chromosome associated with the short arm of the Y chromosome.

e. *N. cervinus* - the heterochromatic short arm of the X chromosome associated with the short arm of the Y chromosome.

f. *N. cervinus* - the heterochromatic short arm of the X chromosome associated with the short arm of the Y chromosome.

All examples are C-banded.

——— = 10 μm; Arrows indicate centromeres.

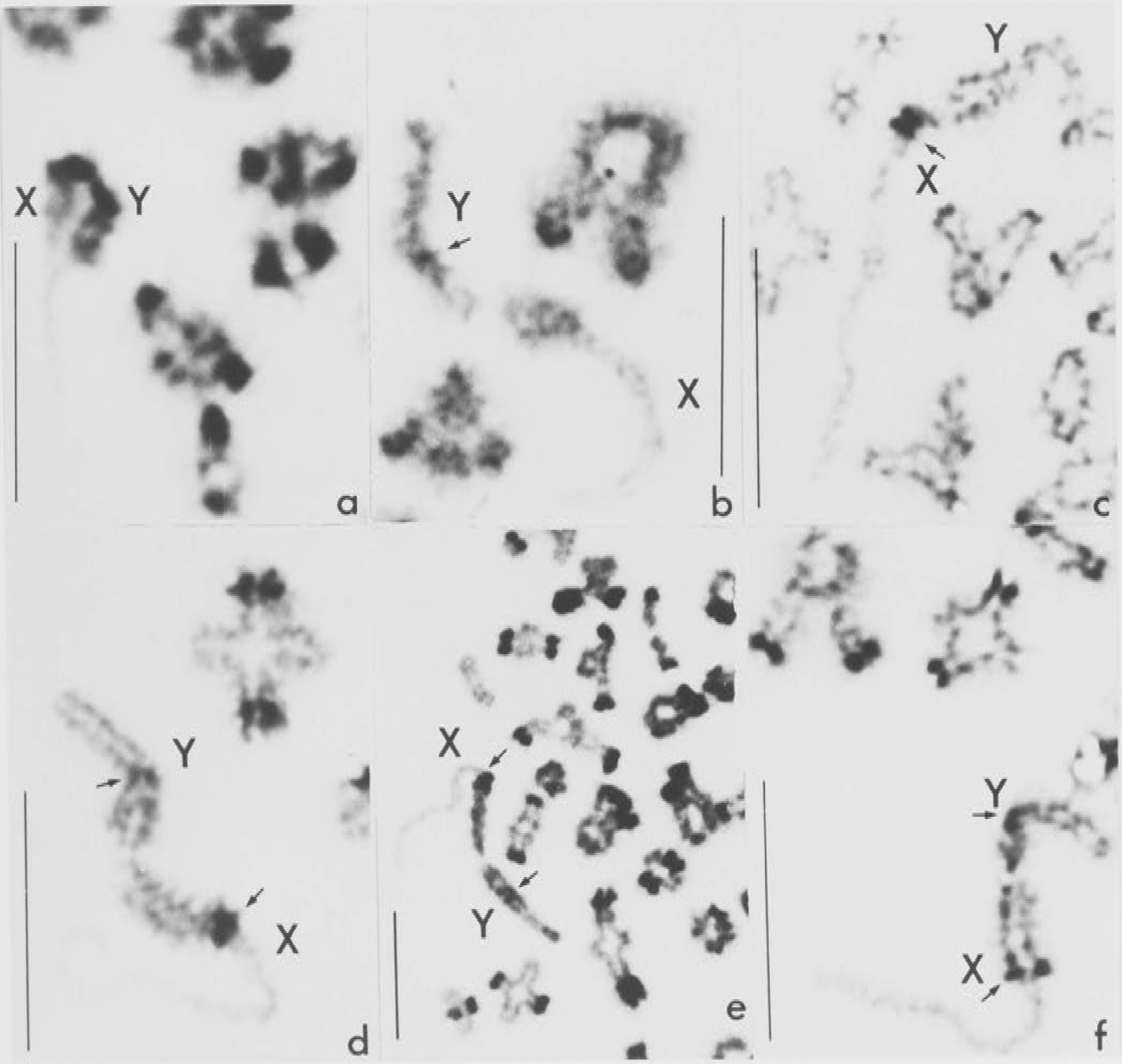
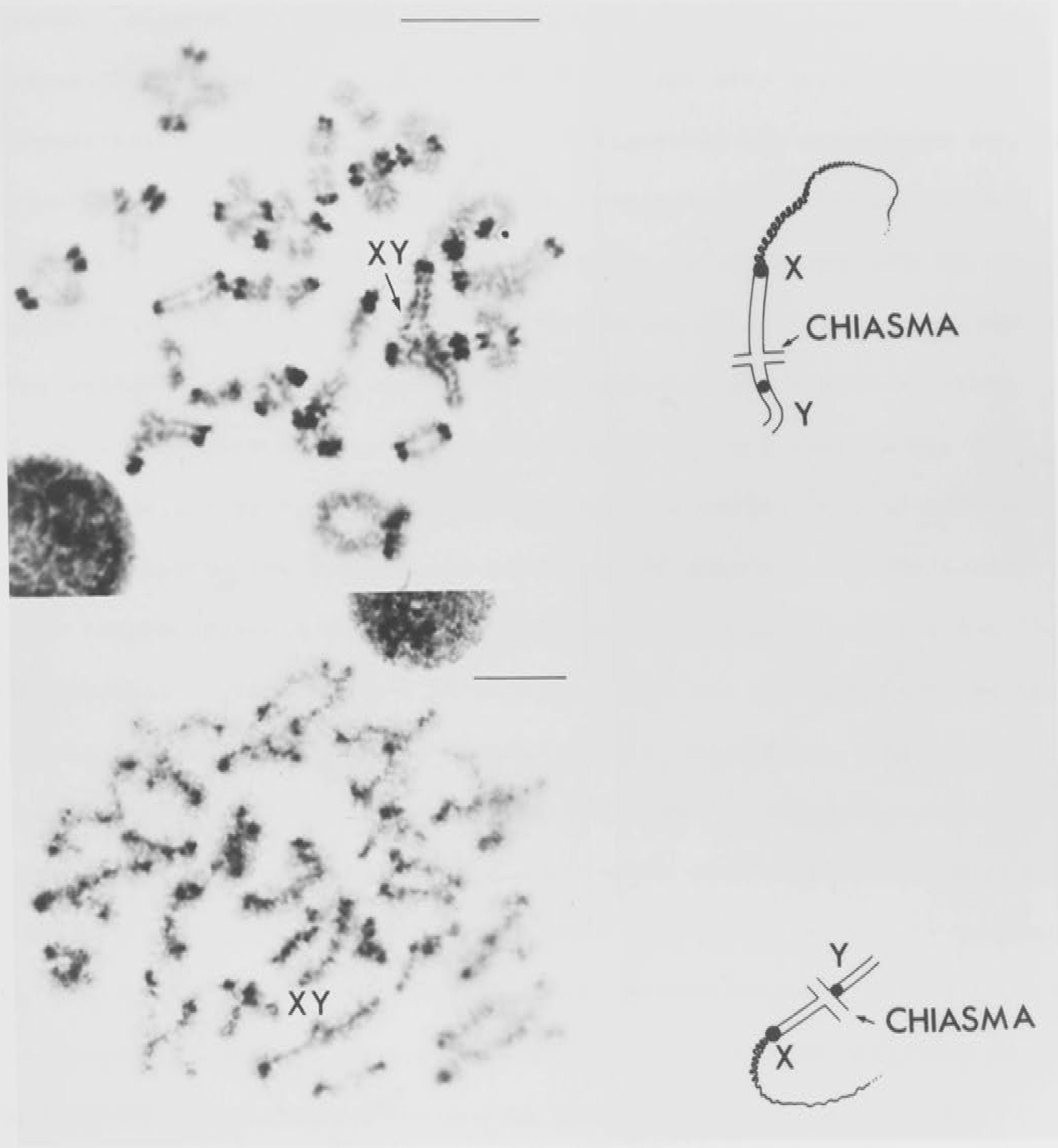

X
Y
a
Y
X
b
Y
X
c
Y
X
d
X
Y
e
Y
X
f

Figure 5.7 : A chiasmate association between the heterochromatic arm of the X chromosome and the Y chromosome in *N. cervinus*. Arrow = chiasma between X and Y; bar = 10 µm. C-banded preparation.

Figure 5.8 : A chiasmate association between the heterochromatic arm of the X chromosome and the Y chromosome in *N. alexis*. Arrow = chiasma; Bar = 10 µm. C-banded preparation.


XY
X
CHIASMA
Y
XY
Y
CHIASMA
X

such Y chromosomes are involved in end-to-end associations it is the short arm of the Y which forms an association with the X.

Some aspects of the cytology of *Notomys* sex chromosomes are analagous to the situation found in both *Cricetus cricetus* and *Critetulus griseus*. Firstly, there are similarities in C-banding behaviour. Earlier (Chapter 2) the X chromosomes of *Notomys* were found to C-band differentially. The euchromatin stained lightest, the centromere heterochromatin stained darkest while the non-euchromatic arm stained midway between these extremes. The situation in the *Cricetus cricetus* is very similar (Vistorin *et al.*, 1977). In *Critetulus griseus* both the centromeric region and the long arm of the X chromosome are C-band positive and while intensity differences exist they are slight and are not described in detail by Vistorin *et al.* (1977). Secondly, *in situ* hybridization indicates the presence of repeated DNA sequences in the centroemeric heterochromatin of the X chromosome of *Cricetulus griseus* but the heterochromatic long arms has no more repeated DNA than the euchromatic regions of the genome (Arrighi *et al.*, 1974). In *Notomys*, too, the X chromosome centromeric heterochromatin contains satellite DNA sequences whereas the non-euchromatic arm does not label (see Chapter 6). Thirdly, in *Notomys* rare chiasmate associations are found between the non-euchromatic arm of the X chromosome and the Y chromosome whereas in both species of hamsters chiasmate associations between the heterochromatic arm of the X chromosome and the Y chromosome are common though end-to-end associations also occur.

Vistorin *et al.* (1977) contrast the finding of chiasmata in the heterochromatin of the X and Y chromosomes of these hamster species with the exclusion of chiasmata from autosomal heterochromatin (John, 1976). It is questionable, however, if the heterochromatin involved in chiasma formation in these hamsters and also in *Notomys* can be considered as strictly equivalent. In each case it fails to C-band as intensely as the procentric heterochromatin and it apparently lacks repetitive DNAs. On both these grounds it may be considered atypical. In fact, Vistorin *et al.* (1977) note the proposal of Arrighi *et al.* 1974) that the Chinese hamster contains different kinds of constitutive heterochromatin and they add further that the lack of repetitive DNA may allow the formation of chiasma in these sex chromosomes.

Ultrastructural studies of the XY bivalent initially used sectioning (Solari, 1971), but later microspreading techniques were used (Counce and Meyer, 1973) in mammalian meiosis (Moses *et al.*, 1975). The application of this latter technique reveals that generally in mammals partial pairing of the X and Y chromosomes takes place in spermatocytes and synaptinemal complexes form in the paired region. This phenomenon has been demonstrated in humans (Solari and Tres, 1970; Moses *et al.*, 1975; Moses, 1977b); rat (Urena and Solari, 1970), the Armenian hamster, *Cricetus migratorius* (Solari, 1974), the golden hamster, *Mesocricetus auratus* (Moses, 1977b), the mouse (Moses, 1977b) and in both the dog and the lemur (unpublished results cited in Solari and Ashley, 1977). However in the sand rat *Psammomys obesus* either the ends of the X and Y chromosomes associate in a variety of ways or alternatively they do not pair at all and no synaptinemal complex has been found in any of these cases (Solari and Ashley, 1977).

In those mammals in which the X and Y pair and form synaptinemal complexes (SC) they do so during later zygotene and early pachytene at which the SC length is at its maximum (Moses, 1977b). The length of the SC varies from species to species and its behaviour during pachytene also varies. For example in the Chinese hamster it remains unchanged (Moses, 1977b) yet in humans it soon reduces to an end-to-end association (Moses *et al.*, 1975). Thus by late pachytene to early diplotene desynapsis of the X and Y is almost complete leaving only a small terminal or interstitial small segment of SC (Moses, 1977). As already pointed out in many mammals there is a synaptinemal complex present between at least parts of the X and Y pair though this may be reduced in length prior to the development of the final form of terminal XY association. While no ultrastructural evidence from *Notomys* is presented the observation of occasional chiasmate figures in both *N. cervinus* and *N. alexis* suggests that in some cases enough SC remains interstitially to allow a chiasma to form whereas in the majority of cases the SC is reduced to terminal associations. This latter view agrees with that of Moses (1977) but it is contrary to the assumption of Solari (1974), that the end-to-end connection invariably represents a chiasma between the terminal regions of the sex chromosomes.

CHAPTER 6

CHARACTERIZATION OF THE DNAS OF *NOTOMYS*

6.1 *Introduction*

The presence in the genomes of many eukaryotes of large amounts of highly repetitive DNA sequences poses the problem of whether these sequences have been selectively conserved because of their functional importance or whether they have been retained due to inherent amplification processes which result in large multiples of a particular sequence of DNA. A comparison of closely related species of relatively recent evolutionary divergence and speciation allows one to examine these possibilities in relation to other phylogenetic aspects of genome organization. As we have already seen the genus *Notomys* includes five extant species which appear to be closely related judged by morphological taxonomic criteria. The genus is endemic to Australia and is thought to have arisen from a common ancestor along with nine other rodent genera which gained entry into Australia sometime during the Miocene and Pliocene. The quantitative differences in constitutive heterochromatin content of the four species considered, combined with the common, though not exclusive, association of constitutive heterochromatin with satellite DNA, suggests that they may offer a good opportunity to probe the problems outlined above.

In the nineteen years since the discovery of satellite DNA in the mouse (Kit, 1961) and crabs (Sueoka, 1961), there has been a proliferation of methods used to characterize these DNAs. These various techniques, while complementary to one another, have provided increasing resolution as they have been refined. They include buoyant density analysis, thermal denaturation profiles, *in situ* and filter hybridization, restriction enzyme analysis and, finally, direct sequence analysis. The latter method, which is the most refined, was only available during the late stages of this study and consequently has not been used here. The other five methods of analysis have been employed to characterize the DNA in *Notomys*. It is appropriate therefore to introduce each of these techniques before describing the results obtained following their use.

6.1.1 *Buoyant density analysis*

The technique of buoyant density analysis which was used initially to characterize satellite DNA still provides valuable basic data. Analytical density gradient analyses of whole DNA allow one to estimate the satellite content of a sample and, to a certain extent, indicate whether the satellite is enriched in either G+C or A+T content. In addition to their analytical value density gradients are also used for the preparative isolation of satellite DNAs. In the case of cryptic satellites, ligands such as heavy metal salts, dyes and antibiotics are essential both for the detection and isolation of these DNAs in density gradients. Ligands, of course, may also enhance the isolation of non-cryptic satellites. Alkaline CsCl density gradients provide further information on the organization of satellite DNA molecules. In such gradients satellite DNAs which have more guanine and thymine bases in one strand of the molecule relative to the other are resolved as two separate bands - the heavier one contains a higher G+T content than the lighter, which has a higher A+C content. Satellite DNAs having a balanced distribution of guanine and thymine in each strand of the DNA molecule thus form a single density band in alkaline CsCl density gradients. For example, in the African green monkey the beta and gamma satellites have identical densities in neutral CsCl density gradients of 1.711 g/cm^3. However when these satellites are run in alkaline gradients the beta satellite is resolved as a single band while the gamma satellite is resolved as two distinct bands (Kurnit and Maio, 1974).

6.1.2 *Thermal denaturation*

Recent developments in thermal denaturation techniques permit comparative analyses of genomes (Ansevin *et al.*, 1976) and, in particular, expose regions of differing base compositions within them. Their development has involved improved methods of recording and controlling temperature increase, of hyperchromicity recording and of data smoothing all of which have led to increased resolution. Until recently, sigmoidal thermal denaturation curves with a transition of several degrees were used to characterize eukaryote DNAs. In these curves the midpoint temperature (Tm) of the sigmoidal curve, which represents the temperature at which 50% of the DNA is denatured, was most often used in thermal denaturation comparisons of DNAs. This criterion, however, represents only a single point on the melting curve and gives no measure of the slope of the transition or whether the curve is unimodal or plurimodal. The use of Tms in DNA characterization assumes that all melting transitions are both smooth and sigmoidal. Departures from symmetrical melting profiles have, however, been described in the DNAs from phage (Hirschmann *et al.*, 1967), protozoans (Mandel and Marmur, 1968), mammals (Pivec *et al.*, 1964) and plants (Huget and Jouanin, 1972). Recently high resolution thermal denaturation data on relatively simple naturally occurring DNA such as bacterial DNAs (Pivec, 1970), phage DNAs (Vizard, *et al.*, 1976) and also on the more complex DNAs of eucaryotes (Guttman *et al.*, 1977; Mayfield, 1977), have revealed considerably more detail than that of earlier data. One major contributing factor is the increased number of temperature and hyperchromicity recordings through the transition from less than 50 in traditional melts to in excess of 500 in high resolution melting profiles. The first derivative profiles of these thermal transitions, which apart from their inherently clearer graphic portrayal of data, reflect the increase in resolution due to these improvements compared to the older thermal transitions.

In eukaryotes, 'blocks' of DNA with base pair compositions different from the remainder of the genome are visible as distinct peaks in first derivative thermal denaturation profiles. In some eukaryote DNAs these features have been shown to correspond to the satellite components of the DNA, particularly in the case of the heavy GC rich satellites as in calf thymus DNA (Pivec *et al.*, 1972), but also in the case of both human and mouse satellite DNAs (Guttman *et al.*, 1977).

6.1.3 *Hybridization techniques*

Marmur and Lane (1960) and also Doty *et al.* (1960) found that DNA could be dissociated and reassociated *in vitro*. Inter-species hybrid molecules involving the DNA of two bacteria were first described by Schildkraut *et al.* in 1961 and this approach has then extended to include the production of *in vitro* DNA/RNA hybrid molecules (Schlildkraut *et al.*, 1961; Hall and Spiegelman, 1961). The DNA/RNA molecule formed when both components are from the same species are more thermally stable due to their complementarity than those formed when the two components are from different species; consequently when inter-species hybrids are formed their stability can be related to the degree of complementarity of their base sequences.

These initial investigations involved hybrid formation in solution (Hall and Spiegelman, 1961), coupled with the use of equilibrium density-gradient centrifugation to detect the hybrids so formed. In 1965, however, Gillespie and Spiegelman introduced a filter hybridization technique which involved the immobilization of denatured DNA on nitrocellulose filters to which an ^{3}HRNA (homologous hybrid), or a ^{3}HRNA complementary to the DNA of another species to be investigated (heterologous hybrid), was hybridized. The technique is convenient, sensitive and reliable. Surplus ^{3}H cRNA which is not hybridized can be removed by combined washing and RNAase treatment, which leaves the hybrid RNA/DNA molecule intact. The degree of relatedness or homology between the species can then be determined by comparing the thermal stability of the homologous RNA/DNA hybrid with that of the heterologous RNA/DNA hybrid. A 1% mismatching of base pairs results in an alteration of the thermal stability of the hybrid molecule by 1.6°C (Ullman and McCarthy, 1973). Filter hybridization has not been extensively used in studies of DNA relationship. However a number of recent studies have employed this technique as a probe in evolutionary investigations.

The technique of *in situ* hybridization employs similar methods to those of filter hybridization. Additionally, however, it enables one to locate cytologically the sequences used in the probe. To effect *in situ* hybridization, radioactively labelled fractions of either DNA or RNA, obtained either *in vivo* or transcribed from DNA templates *in vitro*, are hybridized to DNA denatured in chromosomal or other cytological preparations (John *et al.*, 1969; Pardue and Gall, 1969; Buongiorno-Nardelli and Amaldi, 1970). The sites of hybridization between the applied radioactive molecules and the *in situ* molecules can then be detected by means of autoradiography. The effectiveness of this technique has been confirmed using a number of criteria, namely molecular specificity, sensitivity, efficiency and site specificity. Investigations authenticating the molecular specificity of the technique have involved the hybridization of species specific satellite DNA sequences to cytological preparations. Initially these contained mixed populations of homologous and heterologous cells but subsequently metaphase preparations were employed (John *et al.*, 1969; Jones, 1970; Jones and Robertson, 1970). The homologous cells and chromosomes showed relatively enhanced RNase-resistant radioactive retention in contrast to the heterologous cells and chromosomes. When excess non-radioactive heterologous RNA is present in the *in situ* reaction there is no change in the formation of the radioactive *in situ* homologous hybrid (Pardue and Gall, 1969). In *Xenopus* the cytological sites of the ribosomal genes are known and the results of purified ^{3}H ribosomal RNA hybridized to cytological preparations of oocytes confirm the molecular specificity of the reaction (John *et al.*, 1969; Pardue and Gall, 1970).

The sensitivity of *in situ* hybridization is well demonstrated in the case of the chromosomal location of the four human satellite DNAs. These satellites occupy less than 4% of the total genomic DNA and the smallest detectable site is estimated to be less than 0.2% of the genome (Jones, 1973; Gosden *et al.*, 1975). In *Drosophila melanogaster* Wimber and Steffensen (1970) used *in situ* hybridization of radioactive 5S RNA to polytene chromosome to detect the chromosomal location of the 5S genes which represent 8 x 10^9 daltons of DNA. The efficiency of *in situ* hybridization has been estimated by Jones (1970,1973) at 8%, while Steffensen and Wimber (1972) estimated that, in the particular case of the 5S genes in *D. melanogaster* the efficiency was between 3 and 5%. As polytene development in their material was often only half that of full polytene development this suggests that a 10% efficiency, or higher, is more likely to be the true position in this case. The site specificity of *in situ* hybridization is demonstrated by the *Xenopus* example noted above but is also supported by the good agreement between the amount of satellite II in humans (2%) and the 1.9 to 2.4% of the genome occupied by sites indicated by *in situ* hybridization with ^{3}H cRNA from satellite II (Jones, 1973). However there are limitations to the *in situ* technique which must be observed. First, while hybridization may indicate several sites in a genome it cannot be concluded that each site contains identical DNA since the several sites may have sequences in addition to those present in the *in situ* hybridization probe or else they may act differently allowing only some sequence in the probe to hybridize at specific sites. Secondly, *in situ* hybridization gives only a minimal estimate of sites with similar sequences to the probe used. This limitation has been stated by Gosden *et al.* (1975) with respect to the chromosomal location of the four human satellite as follows "We must, however, point out that the pattern of distribution we find is a minimal one. It is possible that satellite DNA is located in other sites on the chromosomes which are not detected because DNA is lost from them during the experimental procedure. Chromosome denaturation with acids, as described here, rather than with alkali, minimises this loss, but it does still occur." These authors also emphasise that differences in hybridization efficiencies from one human satellite to another rule out quantitative measurements of absolute amounts of these satellites by *in situ* hybridization.

6.1.4 *Restriction enzymes*

The first restriction endonucleases were isolated from *Escherichia coli* and while they recognize specific nucleotide sequences they cleave DNA molecules non-specifically (Meselsen and Yuan, 1968; Linn and Arber, 1968). These enzymes have come to be known as Class I enzymes and are not useful in the analytical investigations of DNA molecules. Class II restriction endonucleases recognize nucleotide sequences and also cleave DNA molecules at specific nucleotide sequences. The first Class II restriction enzyme was isolated from *Haemophilus influenzae* by Smith and Wilcox (1970) and the recognition site sequence determined by Kelly and Smith (1970). This enzyme was then used to cleave SV40 DNA (Danna and Nathans, 1971) and this was quickly followed by the isolation of many restriction endonucleases from a wide range of bacteria and their subsequent use in the analytical investigations of DNAs from many organisms.

Restriction enzyme analysis of related DNAs offers advantages over hybridization techniques in that a single base change in a restriction site prevents cleavage of the DNA molecule at that site by the enzyme in use. This results in a change in fragment pattern when the digestion products are gel electrophoresed; such a small change would go undetected in hybridization experiments which would however detect 16-25% of these changes. However, the use of a single restriction endonuclease gives no idea of the sequence characteristics of the DNA intervening between two consecutive enzyme sites and as such does not afford a good measure of relatedness, yet information on these sequences may be revealed by hybridization techniques. The use of multiple restriction enzyme digests does however overcome this disadvantage to some degree.

6.2 *Methods*

6.2.1 *DNA preparation*

Fresh livers or brains were dissected directly into TES buffer. TES buffer consists of 30 mM Tris, 5 mM EDTA, 50 mM NaCl adjusted to pH 8.0. The total liver (or brain) from one animal plus 8 mls of TES buffer were hand homogenised on ice. Preparations were always made from a single tissue from an individual animal and kept separate thereafter. Sarkosyl 30% (Sarkosyl NL30 Geigy) was added to give a final concentration of 3% and lysis allowed to proceed for 3 minutes in ice. Ti50 polyallomer centrifuge tubes were loaded with 2 ml lysate, 3 ml TES buffer, 5 g caesium chloride, 0.26 ml ethidium bromide (5 mg/ml in TES) and run in a Ti50 rotor at 44,000 rpm for 40 hours at 15°C. The DNA was extracted from the tubes with a 19 gauge hypodermic needle using UV light to locate the DNA band. The ethidium bromide was removed from the DNA by eight iso-amyl alcohol extractions. The DNA was dialysed at 4°C in either TES or 1 x SSC for 12 hours. The DNA was then centrifuged in CsCl neutral gradients under the same conditions as before and then fractioned by 10 drop samples. The DNA was located by reading the optical density at A260 nm of the fractions. Satellite DNA was isolated using Hoescht 33258 dye according to the method of Manuelidis (1977).

6.2.2 *Analytical ultracentrifugation*

Analytical density gradients were run in a Beckman Model E ultracentrifuge. Two density markers were employed depending on the nature of the gradient. *Micrococcus luteus* (density 1.713 gm/cc) was provided by Dr. G.D. Clark-Walker while poly d (AT) density 1.6784 gm/cc, was donated by Dr P.M. Gresshoff.

6.2.3 *Thermal denaturation*

All thermal denaturations were carried out on unsonicated high molecular weight DNA in 0.1 x SSC at pH 7 using a Gilford 250 spectrophotometer fitted with a Gilford thermo-Programmer, Model 2527. The linear rate of temperature increase was 0.25°C per minute and the data were automatically plotted on a Gilford Model 6051 chart recorder with 10 readings per cuvette per degree centigrade. The cuvettes used have a capacity of 0.3 ml and are sealed with Teflon stoppers.

The thermal denaturation data are presented as:

i. Integral thermal denaturation profiles in which the ordinate is the percentage of total hyperchromicity (H) and the abscissa is the temperature (T).

ii. Modified first derivative thermal denaturation profiles in which the ordinate is the smoothed value of dH/dT for each temperature point. Smoothing is achieved by taking the derivative of the hyperchromicitiy at a temperature point and three adjacent points either side of the central point and averaging these seven data points by a programme written in BASIC-PLUS language using a Digital PDP 11/34 computer. The area under the resultant curve is set to 100 arbitary units. Plotting was achieved by the use of a flat bed plotter HP7200A.

6.2.4 *Synthesis of complementary* ^3H *cRNA*

Complementary RNA (cRNA) was synthesized in a *Escherichia coli* RNA polymerization reaction on templates of 1.712 satellite DNA from *N. mitchellii* and *N. alexis* and of 1.713 satellite DNA from *N. cervinus* and *N. fuscus*. The reaction mixture of 0.1 ml contained 10 µl RNA polymerase (*E. coli* holoenzyme), 4 µg satellite DNA, ^3H labelled uridine, cytidine and guanosine triphosphates at 75 μM, and cold adenine triphosphate 100 μM. The buffer contained 0.04 M Tris-HCl pH 7.9 M, 0.01 M $MgCl_2$, 0.15 M KCl and 0.002 M dithiothreitol. The reaction was carried out at 37°C for 2½ hours. DNase treatment was applied at room temperature following the addition of 250 μl Tris HCl 0.05 m pH 7.9, 15 μl yeast RNA (10 mg/ml), 10 μg/ml, DPFF Worthington RNase free and 5 μl 0.01 M $CaCl_2$ for 30 minutes. After phenol extraction, the cRNA was isolated by passage through a Sephadex G-75 column.

6.2.5 *Filter hybridization*

Nitrocellulose filters (Sartorius membrane filters Type SM, size 47, Pore size 0.45 μ) were washed in 100 ml of 4 x SSC, pH 7.2 for 20 minutes at room temperature on a shaker. The DNA solution (0.25 μg per filter) was denatured by placing 1 μg in 16 ml 4 x SSC and adding 600 μl of 1N potassium hydroxide. This solution was kept at room temperature for 5 minutes then placed on ice. Just prior to loading onto the filters the solution was neutralized with 600 μl of 1N hydrochloric acid. Loading was done by filtration without vacuum for 15 minutes, then washed twice with 5 ml of iced 3 x SSC at low vacuum and dried at room temperature for 30 minutes. The filters were placed in a vacuum for 3 hours at 80°C and then stored for use in a vacuum dessicator at 4°C. Immediately prior to use the filters were placed in vacuum at 80°C for 5 minutes before transferring into a hybridization solution consisting of 6 ml 3 x SSC/50% formamide, 5 μl yeast RNA, 5 μl cRNA *Notomys* satellite and 50 μl 10% SDS (sufficient for 4 filters). Hybridization was carried out for 3 hours at 37°C on a shaker. The filters were then washed twice in 3 x SSC/50% formamide for 5 minutes at 37°C, followed by three washes in 3 x SSC for 5 minutes each at 37°C. RNase treatment was continued for 30 minutes at 37°C in the following solution: 1.25 mls 3 x SSC, 5 μl RNase T1 (20 units/ml), 5 μl pancreatic RNase (from aliquot 2 mg/μl). The filters were then washed three times in 3 x SSC at 37°C followed by drying at room temperature.

The dry filters for Tm analysis were soaked in 3 x SSC/50% formamide then drained prior to being placed in a scintillation vial containing 0.6 mls 3 x SSC/50% formamide. This was held at 30°C for 3 minutes, and then transferred serially to other vials at temperature increments of 3°C for 3 minutes in each vial. After removal of the filter the vial and its contents were cooled on ice and 0.3 ml distilled water added followed by 9 ml Triton X-Toluene (TXT) scintillation fluid. This solution was assayed in a scintillation counter for ^{3}H cRNA content. The counts per minute (cpm) of each vial were plotted as cumulative cpm against temperature. The filters were retained after the final temperature treatment and assayed for residual radioactivity.

In these experiments 1.712 satellite DNA from *N. mitchellii* and *N. fuscus* and 1.713 satellite DNA from *N. cervinus* and *N. fuscus* were loaded onto filters. Hybridization was to ^{3}H cRNA obtained from *N. alexis* 1.712 satellite DNA or *N. fuscus* 1.713 satellite DNA.

6.2.6 In situ *hybridization*

The cytological slides used for *in situ* hybridization were prepared by the air drying technique described earlier for chromosome banding. The slides were treated with absolute alcohol for 2 minutes, rinsed twice with distilled water and then placed in 0.2 N HCl for 10 minutes at 37°C. Following two rinses in distilled water, then two rinses in 70% alcohol and two in 95% alcohol, the slides were allowed to dry. The hybridization was carried out at 41°C for 3 hours with 5 μl of hybridization solution. The hybridization solution was 25 μl formamide, 25 μl ^{3}H cRNA *Notomys* satellite in 6 x SSC and 50 μl 3 x SSC/50% formamide. During the hybridization the coverslip was sealed with soluble rubber to contain the reaction solution and prevent evaporation. After hybridization the coverslip was removed in 3 x SSC/50% formamide, incubated in 3 x SSC/50% formamide for 20 minutes and then rinsed three times in 2 x SSC for 10 minutes each at room temperature. RNase treatment was then carried out for 60 minutes at room temperature in a solution containing 100 ml 2 x SSC, 100 μl pancreatic RNase (from aliquot 2 mgm/ml) and 20 μl TI RNase. The slides were then rinsed 6 times in 2 x SSC at room temperature for 10 minutes each time, followed by two rinses in 70% ethyl alcohol, two rinses in absolute alcohol and then allowed to dry. The slides were dipped in a 50% solution of Ilford K2 emulsion in distilled water at 45°C, allowed to dry and then stored in a dry atmosphere in light-tight containers at 4°C. Exposure varied from seven days to six months and the slides were then developed for 2½ hours at 20°C in Kodak D19b and fixed in Ilford Rapid Fix (1:4 dilution) for 2½ minutes. The resulting autoradiographs were stained in 5% Geisma, differentiated, dried and coverslipped for scoring and photomicroscopy.

6.2.7 *Restriction endonuclease digests*

Digests were carried out on *Notomys* satellites DNAs, and pBR322 DNA. Four restriction enzymes were used: Hinf I, Hae III, Bam H were employed at 37°C for 1 hour, while Taq I was used for 1 hour at 65°C. The recognition sequences of the restriction enzymes used were as follows: Hinf I - G▼ANTC; Hae III - GG▼CC; Bam HI - G▼GATCC and Taq I - T▼CGA. Enzymes were purchased from Bio-Labs, U.S.A. In some experiments digestion was extended for longer periods of time and increased quantities of enzyme used.

The reaction mixture was:

1 μg DNA in 18 μl of 10 mM Tris, 0.1 mM EDTA pH 7.4	18 μl
Bovine serum albumin	1 μl
10 x enzyme buffer appropriate to enzyme used	2.2 μl
Enzyme	0.5-1 μl

6.2.8 *Gel electrophoresis*

6.2.8.1 *Acrylamide gels*: 10% polyacrylamide gels were made using Acrylamide:bisacrylamide 20:1 (Bio-Rad) in Tris-Borate buffer pH 8.3. The Tris-Borate buffer consisted of 100 mM Tris, 86 mM Boric Acid, 2 mM EDTA. The gels were electrophoresed vertically at 30 mA and 200 V at 4°C. Digests of pBR322-Hae III were used as fragment marker sizes. The lengths of the pBR322-Hae III digest fragments were taken from Sutcliffe (1978). The pBR322 DNA was a gift from Dr E. Dennis, Division of Plant Industry, Commonwealth Industrial and Research Organization.

6.2.9 *Base pair and molecular weight calibration*

The length of DNA fragments in a particular satellite DNA digest were estimated from the mobility of each fragment in the gel relative to the mobility of marker DNA fragments of known lengths. The stained gel was illuminated with ultra-violet light and photographed with Polaroid 4 x 5 Land Fil Type 55/Positive-Negative film through a Kodak yellow filter (no.15). The mobility of the various fragments was obtained by measuring photographic enlargements from the Polaroid negatives and also by measuring scans obtained from the Polaroid negative on a Joyce Loebl microdensitometer. The migration positions of the marker DNA fragments were plotted against the logarithm of the molecular weight using a Hewlitt-Packard 7200A Graphic-Plotter and the base pair length of the satellite DNA fragments was calculated from this plot using a programme written in BASIC-PLUS language on a Digital PDP 11/34 computer.

6.3 *Results*

6.3.1 *Buoyant density characterization*

Whole DNA from the four species of *Notomys* was resolved from two density peaks in neutral CsCl density gradients. The major peak, containing the main band DNA, had a density of 1.700 g/cm^3 in each case. The smaller peak, consisting of satellite DNA, had a density of 1.712 g/cm^3 in *N. mitchellii* and *N. alexis*, and 1.713 g/cm^3 in *N. cervinus* and *N. fuscus* (Fig. 6.1). The percentage of satellite in each genome was estimated from the relative areas in analytical ultracentrifuge traces. Expressed as a percentage of the total nuclear DNA the satellite DNA content and its S.D. for the four species is 28.06 ± 1.14% in *N. mitchellii*, 23.89 ± 1.41% in *N. alexis*, 24.45 ± 0.98% in *N. cervinus* 6.16 ± 1.45% in *N. fuscus*.

The density of the isolated satellite DNA from each species was confirmed by analytical neutral CsCl ultracentrifugation (Fig. 6.2) and the values obtained agreed with the densities indicated from whole DNA traces (Fig. 6.1).

In alkaline CsCl gradients the isolated satellite DNAs from each species were resolved into two peaks indicating an asymmetrical distribution of G+T bases between the strands of the DNA (Fig. 6.3). These results suggest minor differences in base composition of the four satellites. When the alkaline CsCl gradients were neutralized and re-run in analytical gradients all four satellites failed to return to their native density (Table 6.1). Unlike the alkaline gradient these data indicate considerable heterogeneity in these satellites.

Neutral CsCl gradients of the satellites following heat denaturation resulted in increased density giving the following values: *N. mitchellii* 1.726 g/cm^3; *N. alexis* 1.726 g/cm^3, *N. cervinus* 1.723 g/cm^3, and *N. fuscus* 1.728 g/cm^3. Moreover when these satellites were renatured in 2 x SSC at 65°C for 5 hours and then run in neutral CsCl density gradients none of the satellites returned to the density of the native satellites. The differences between the native density and that of the renatured satellites were *N. mitchellii* 0.009 g/cm^3, *N. alexis* 0.008

Table 6.1: Densities of native, heat denatured, alkali denatured and heat denature/reassociated satellite DNA from *Notomys mitchellii, N. alexis, N. cervinus* and *N. fuscus*

	N. mitchellii	*N. alexis*	*N. cervinus*	*N. fuscus*
Heat denatured satellite density	1.726	1.726	1.723	1.728
Native satellite density	1.712	1.712	1.713	1.713
$\Delta\rho$	0.014	0.014	0.010	0.015
Heat denatured/ reassociated in 2xSSC @ 65°C for 5 hours	1.720	1.721	1.717	1.723
Native	1.712	1.712	1.713	1.713
$\Delta\rho$	0.008	0.009	0.004	0.010
Neutralised alkaline gradient	1.728	1.729	1.726	1.730
Native	1.712	1.712	1.713	1.713
$\Delta\rho$	0.016	0.017	0.013	0.017

Figure 6.1 : Analytical ultracentrifugation of *Notomys* whole DNAs in CsCl.

(i) *N. mitchellii*

(ii) *N. alexis*

(iii) *N. cervinus*

(iv) *N. fuscus*

The density marker at 1.6784 g/cm^3 is poly d(AT).

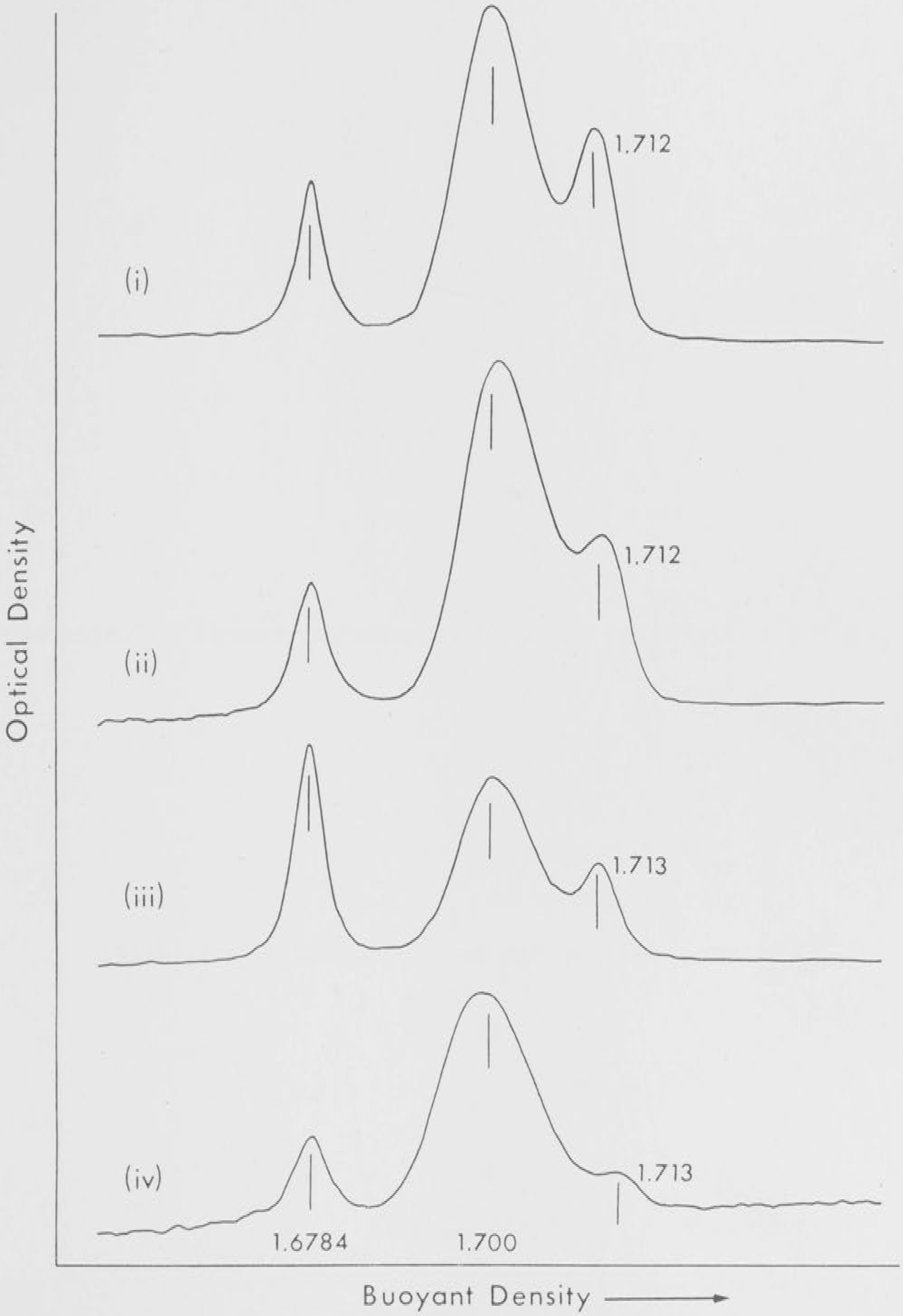

Optical Density
(i)
1.712
(ii)
1.712
(iii)
1.713
(iv)
1.713
1.6784
1.700
Buoyant Density

Figure 6.2 : Analytical ultracentrifugation of *Notomys* satellite DNAs in CsCl.

(i) *N. mitchellii*

(ii) *N. alexis*

(iii) *N. cervinus*

(iv) *N. fuscus*

The density marker at 1.6784 g/cm^3 is poly d(AT).

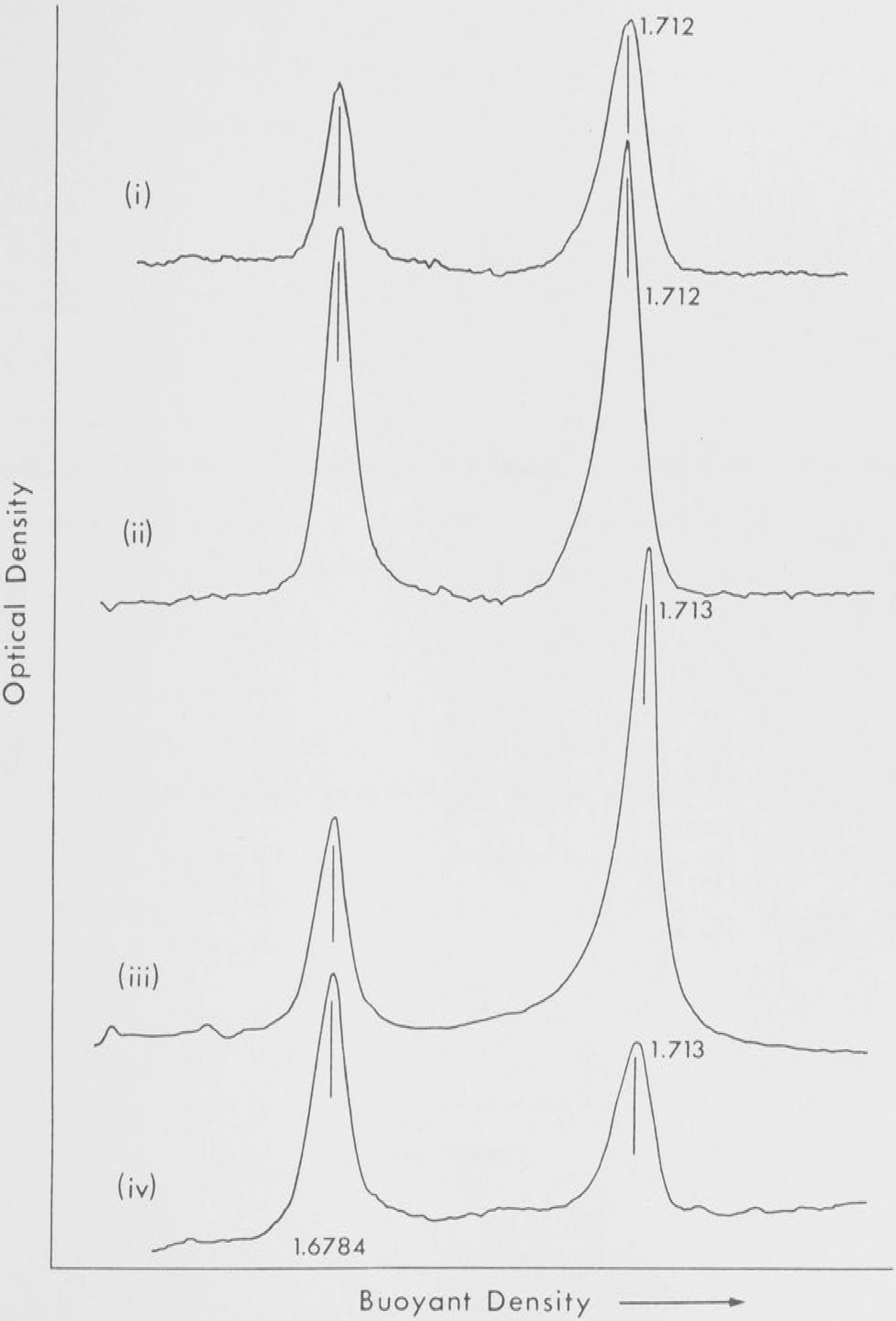

1.712
(i)
1.712
(ii)
1.713
(iii)
1.713
(iv)
1.6784
Optical Density
Buoyant Density

Figure 6.3 : Analytical ultracentrifugation of *Notomys* satellite DNAs in alkaline CsCl.

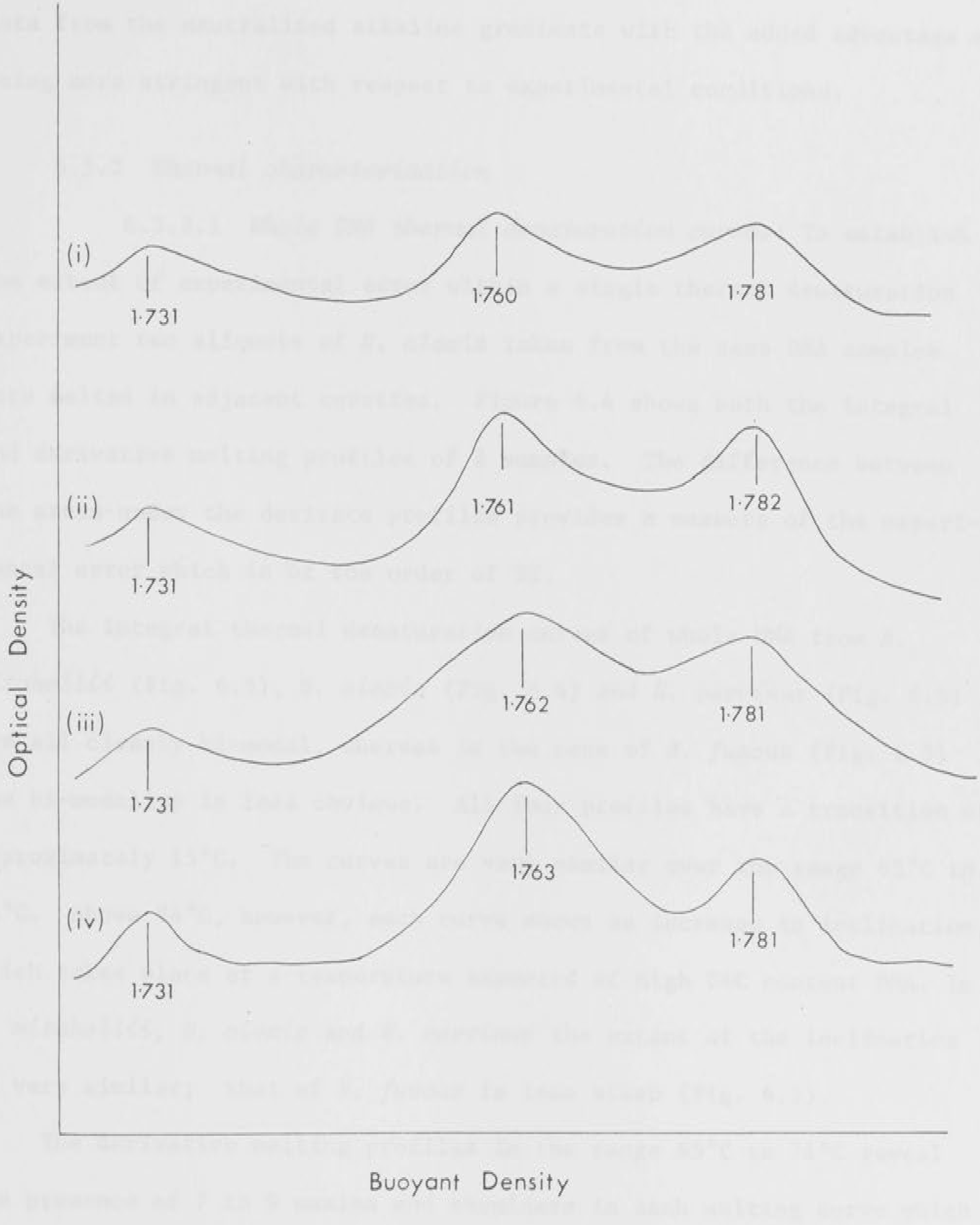
(i) *N. mitchellii*

(ii) *N. alexis*

(iii) *N. cervinus*

(iv) *N. fuscus*

The density marker at 1.731 g/cm^3 is poly d(AT).


(i)
1·731
1·760
1·781
(ii)
1·731
1·761
1·782
(iii)
1·731
1·762
1·781
(iv)
1·731
1·763
1·781
Optical Density
Buoyant Density

g/cm^3, *N. cervinus* 0.004 g/cm^3 and *N. fuscus* 0.010 g/cm^3. These data again suggest considerable sequence heterogeneity in all the satellites with that of *N. cervinus* showing least heterogeneity. They support the data from the neutralized alkaline gradients with the added advantage of being more stringent with respect to experimental conditions.

6.3.2 *Thermal characterization*

6.3.2.1 *Whole DNA thermal denaturation curves*: To establish the extent of experimental error within a single thermal denaturation experiment two aliquots of *N. alexis* taken from the same DNA samples were melted in adjacent cuvettes. Figure 6.4 shows both the integral and derivative melting profiles of 2 samples. The difference between the areas under the derivate profiles provides a measure of the experimental error which is of the order of 2%.

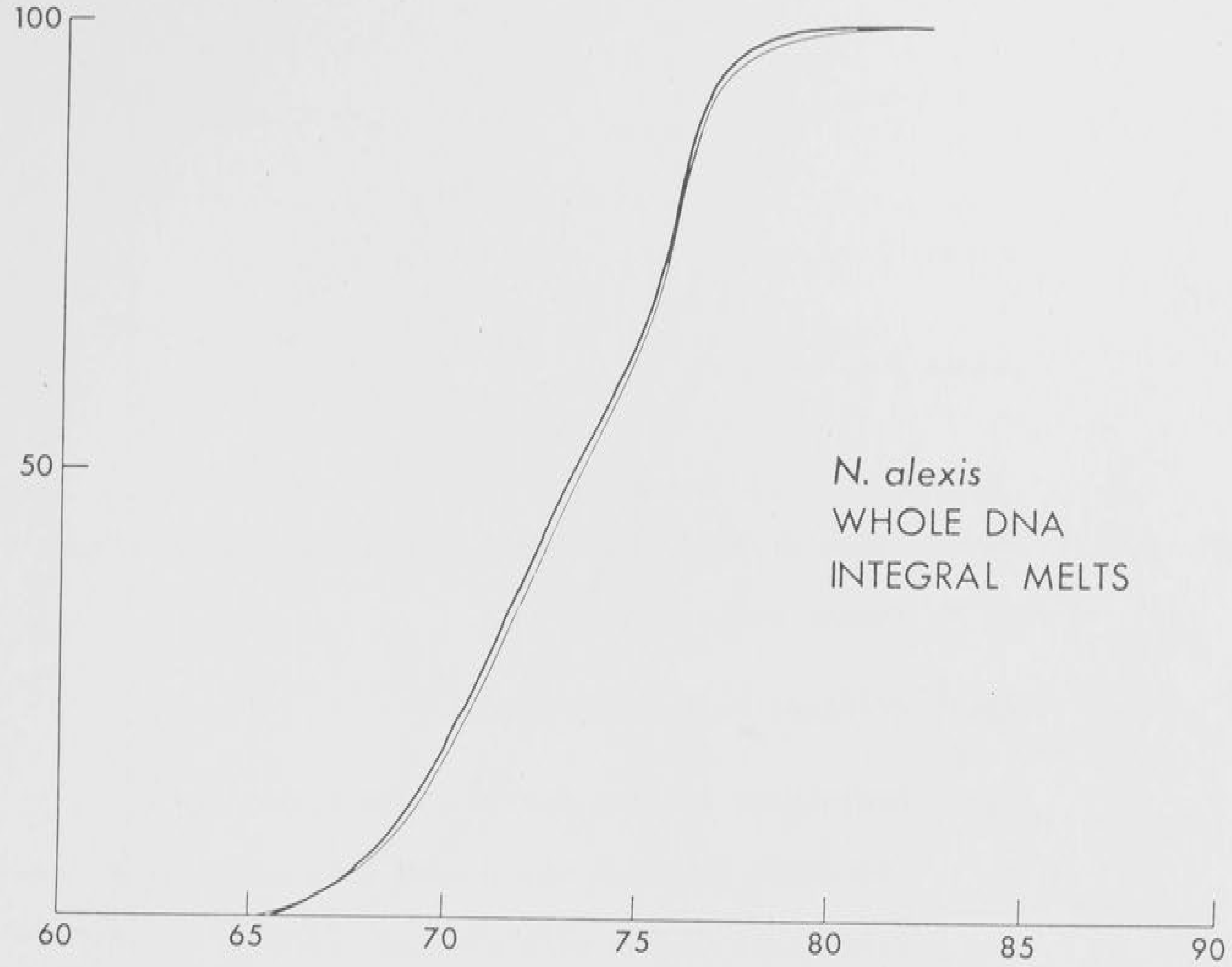
The integral thermal denaturation curves of whole DNA from *N. mitchellii* (Fig. 6.5), *N. alexis* (Fig. 6.4) and *N. cervinus* (Fig. 6.5) are all clearly bi-modal, whereas in the case of *N. fuscus* (Fig. 6.5) the bi-modality is less obvious. All four profiles have a transition of approximately 15°C. The curves are very similar over the range 65°C to 74°C. Above 74°C, however, each curve shows an increase in inclination, which takes place at a temperature expected of high G+C content DNA. In *N. mitchellii, N. alexis* and *N. cervinus* the extent of the inclination is very similar; that of *N. fuscus* is less steep (Fig. 6.5).

The derivative melting profiles in the range 65°C to 74°C reveal the presence of 7 to 9 maxima and shoulders in each melting curve which indicate compositional heterogeneity. These peaks are the result of blocks of DNA within the genome which have similar base composition and therefore have similar melting characteristics. Unfortunately this technique does not determine whether or not these blocks are widely separated from one another within the genome. The major feature of the derivative melting profiles of *N. mitchellii* (Fig. 6.5), *N. alexis*

Figure 6.4 : *Notomys alexis* whole DNA thermal denaturation of two aliquots from the same DNA sample to establish the experimental error (in the order of 2%).

Upper: *N. alexis* whole DNA integral curves. The Y axis is the percentage hyperchromicity.

Lower. *N. alexis* whole DNA derivative curves. The Y axis is a composite function which sets each curve to a total area of 100 arbitary units.


100
50
60
65
70
75
80
85
90
N. alexis
WHOLE DNA
INTEGRAL MELTS

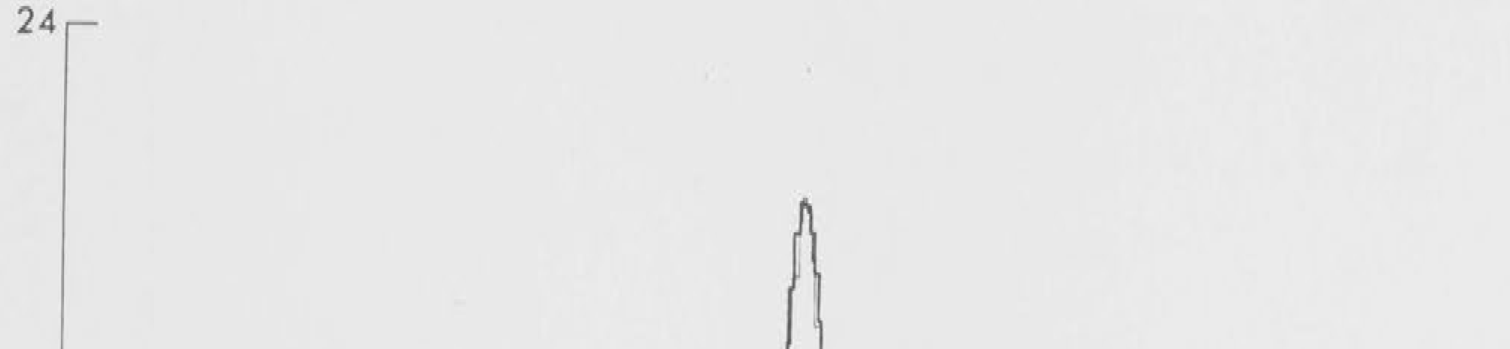
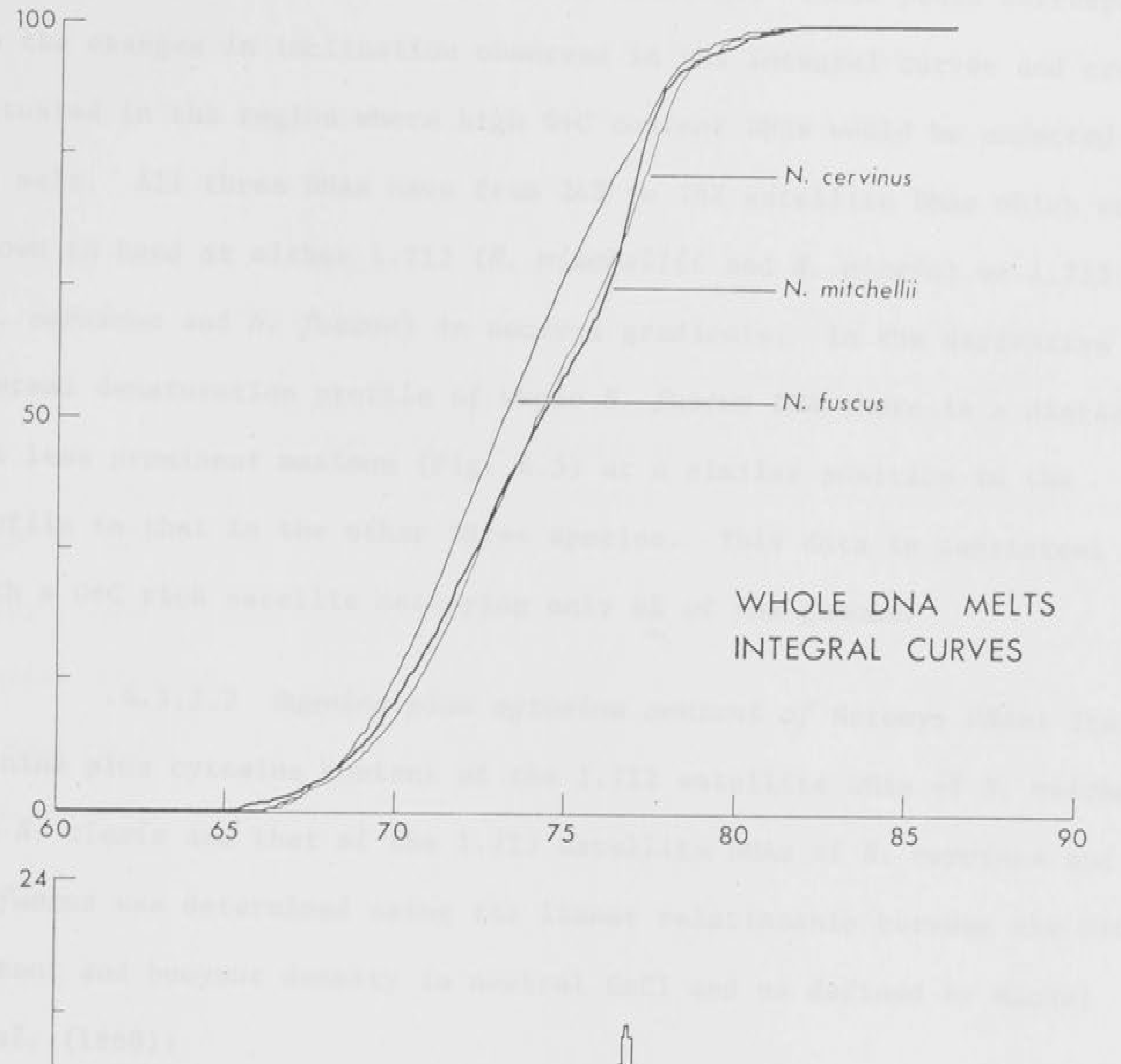

24
12
60
65
70
75
80
85
90
TEMPERATURE (°C)
N. alexis
WHOLE DNA
DERIVATIVE MELTS

Figure. 6.5 : Thermal denaturation of *Notomys mitchellii*, *N. cervinus*, and *N. fuscus* whole DNAs.

Upper: Integral melting curves.

Lower: Derivative melting curves. The Y axis is a composite function which sets each curve to a total area of 100 arbitary units.


100
50
0
% HYPERCHROMICITY
N. cervinus
N. mitchellii
N. fuscus
WHOLE DNA MELTS
INTEGRAL CURVES
60
65
70
75
80
85
90
24
12
0
N. mitchellii
N. cervinus
N. fuscus
WHOLE DNA DERIVATIVE
MELTS
60
65
70
75
80
85
90
TEMPERATURE (°C)

(Fig. 6.4) and *N. cervinus* (Fig. 6.5) whole DNA is the maxima which occupies the 74°C to 77°C temperature interval. These peaks correspond to the changes in inclination observed in the integral curves and are situated in the region where high G+C content DNAs would be expected to melt. All three DNAs have from 24% to 28% satellite DNAs which were shown to band at either 1.712 (*N. mitchellii* and *N. alexis*) or 1.713 (*N. cervinus* and *N. fuscus*) in neutral gradients. In the derivative thermal denaturation profile of whole *N. fuscus* DNA there is a distinct but less prominent maximum (Fig. 6.5) at a similar position in the profile to that in the other three species. This data is consistent with a G+C rich satellie occupying only 6% of the genome.

6.3.2.2 *Guanine plus cytosine content of* Notomys *DNAs*: The guanine plus cytosine content of the 1.712 satellite DNAs of *N. mitchellii* and *N. alexis* and that of the 1.713 satellite DNAs of *N. cervinus* and *N. fuscus* was determined using the linear relationship between the G+C content and buoyant density in neutral CsCl and as defined by Mandel *et al.* (1968):

$$(GC) = \frac{\text{Density in neutral CsCl} - 1.660\ \text{g/cm}^3}{0.098}$$

where (GC) = mole fraction of guanine plus cytosine in the DNA.

Additional determinations of the guanine plus cytosine content were made employing the relation between Tm and GC content of DNA samples in 0.1 x SSC represented by the equation of Mandel and Marmur (1968):

$$(GC) = (Tm - 53.9)\ 2.44$$

where (GC) = mole fraction of guanine plus cytosine in the DNA.

The G+C values obtained by both methods are summarised in Table 6.2.

6.3.2.3 *Satellite DNA thermal denaturation curves*: The satellite DNAs isolated from preparative density gradients of whole DNA from *N. mitchellii*, *N. alexis* and *N. cervinus* were thermally denatured using the same conditions as for the whole DNAs of these species. The

Table 6.2: Determination of guanine plus cytosine contents of the 1.712 satellite DNAs of *N. mitchellii* and *N. alexis* and the 1.713 DNAs of *N. cervinus* and *N. fuscus* using density in neutral CsCl gradients (Mandel *et al.*, 1968) and Tm value (Mandel and Marmur, 1968)

	Percentage of guanine plus cytosine in satellite DNA	
	Determination from density in neutral CsCl gradients	Determination from Tm
Notomys mitchellii	53	51.5
Notomys alexis	53	50.5
Notomys cervinus	54	50.5
Notomys fuscus	54	52.9

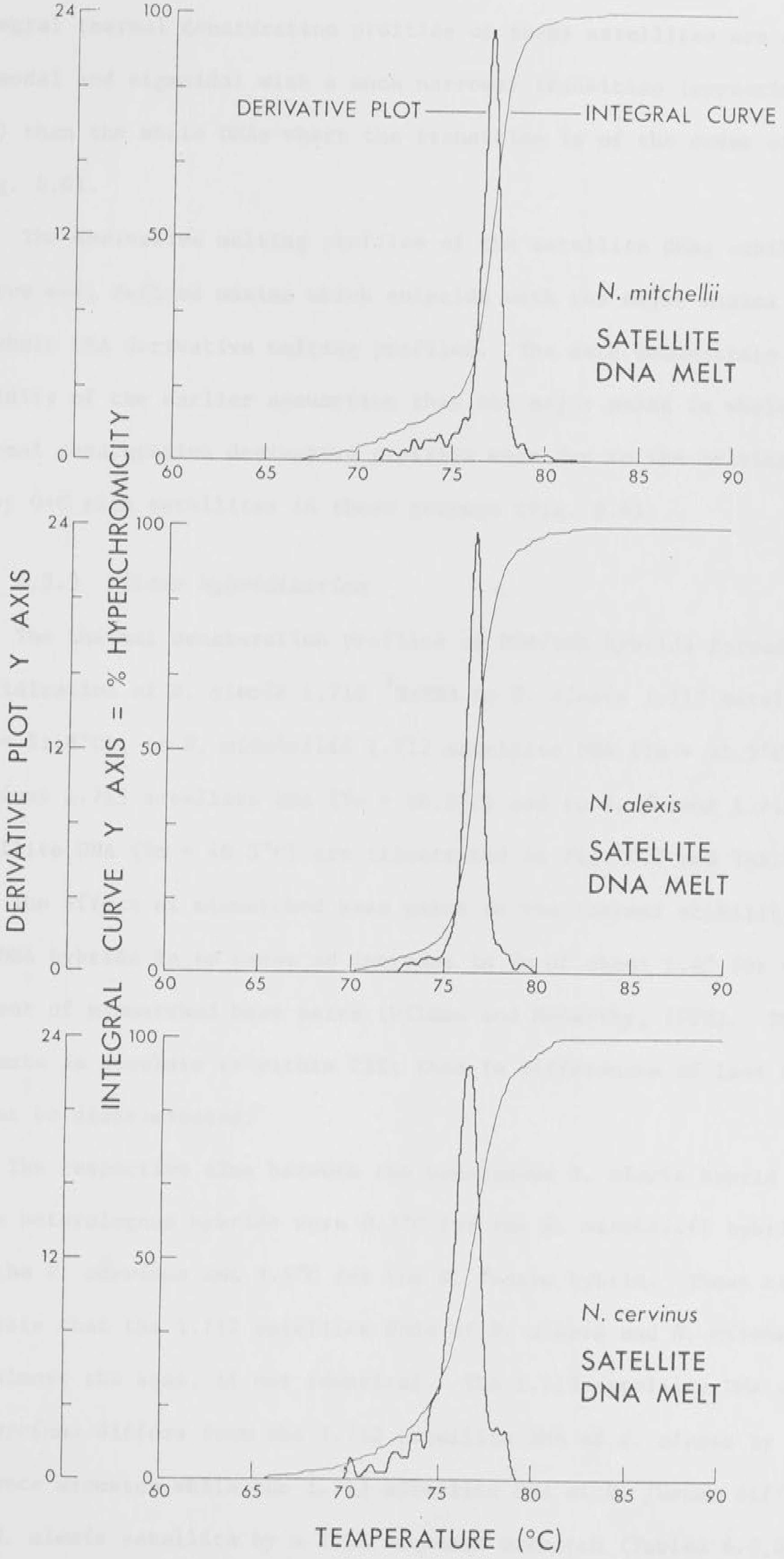
Figure 6.6 : Thermal denaturation of *Notomys* satellite DNAs.

Upper: *N. mitchellii* 1.712 satellite DNA integral and derivative melting curves.

Middle: *N. alexis* 1.712 satellite DNA integral and derivative melting curves.

Lower: *N. cervinus* 1.713 satellite DNA integral and derivative melting curves.

The Y axis in each derivative plot is a composite function which sets each curve to a total area of 100 arbitary units.


DERIVATIVE PLOT
INTEGRAL CURVE
N. mitchellii
SATELLITE DNA MELT
N. alexis
SATELLITE DNA MELT
N. cervinus
SATELLITE DNA MELT
DERIVATIVE PLOT Y AXIS
INTEGRAL CURVE Y AXIS = % HYPERCHROMICITY
0
12
24
0
50
100
60
65
70
75
80
85
90
TEMPERATURE (°C)

integral thermal denaturation profiles of these satellites are all unimodal and sigmoidal with a much narrower transition (approximately 8°C) than the whole DNAs where the transition is of the order of 15°C (Fig. 6.6).

The derivative melting profiles of the satellite DNAs exhibit narrow well defined maxima which coincide with the major maxima visible in whole DNA derivative melting profiles. The data demonstrate the validity of the earlier assumption that the major peaks in whole DNA thermal denaturation derivative profiles were due to the melting of heavy G+C rich satellites in these genomes (Fig. 6.6).

6.3.3 *Filter hybridization*

The thermal denaturation profiles of RNA/DNA hybrids formed by the hybridization of *N. alexis* 1.712 ^{3}HcRNA to *N. alexis* 1.712 satellite DNA (Tm = 51.8°C), to *N. mitchellii* 1.712 satellite DNA (Tm = 51.5°C), to *N. cervinus* 1.713 satellite DNA (Tm = 46.8°C) and to *N. fuscus* 1.713 satellite DNA (Tm = 48.3°C) are illustrated in Fig. 6.7 and Table 6.3.

The effect of mismatched base pairs on the thermal stability of RNA/DNA hybrids is to cause an increase in Tm of about 1.6° for each percent of mismatched base pairs (Ullman and McCarthy, 1973). This estimate is accurate to within 25%; thus Tm differences of less than 1°C cannot be discriminated.

The respective ΔTms between the homologous *N. alexis* hybrid and the three heterologous hybrids were 0.3°C for the *N. mitchellii* hybrid, 5°C for the *N. cervinus* and 3.5°C for the *N. fuscus* hybrid. These results indicate that the 1.712 satellite DNAs of *N. alexis* and *N. mitchellii* are almost the same, if not identical. The 1.713 satellite DNA of *N. cervinus* differs from the 1.712 satellite DNA of *N. alexis* by a 3.1% sequence mismatch while the 1.713 satellite DNA of *N. fuscus* differs from the *N. alexis* satellite by a 2.2% sequence mismatch (Tables 6.3,6.4).

Further thermal denaturation profiles of hybrids formed by the

Table 6.3: Tm differences between homologous RNA/DNA and heterologous RNA/DNA hybrids and the percentage of base pair mismatch in the satellite sequences tested for *Notomys alexis*, *N. mitchellii*, *N. cervinus* and *N. fuscus* satellites

Homologous hybrid	Heterologous hybrid	Tm difference in °C	% sequence mismatch
N. alexis-*N. alexis* RNA/DNA	*N. alexis*-*N. mitchellii* RNA/DNA	-0.3	nil
" " " " " "	*N. alexis*-*N. cervinus* RNA/DNA	-5.0	3.1
" " " " " "	*N. alexis*-*N. fuscus* RNA/DNA	-3.5	2.2
N. fuscus-*N. fuscus* RNA/DNA	*N. fuscus*-*N.alexis* RNA/DNA	-2.9	1.8
" " " " " "	*N. fuscus*-*N. mitchellii* RNA/DNA	-2.8	1.8
" " " " " "	*N. fuscus*-*N. cervinus* RNA/DNA	-4.0	2.5

Figure 6.7 : Thermal denaturation profiles of *Notomys alexis* 1.712 ^{3}HcRNA hybridized to *N. alexis* 1.712 satellite DNA (△— — — —△), to *N. mitchellii* 1.712 satellite DNA (■-------■), to *N. cervinus* 1.713 satellite DNA (○········○) and to *N. fuscus* 1.713 satellite DNA (●———●).

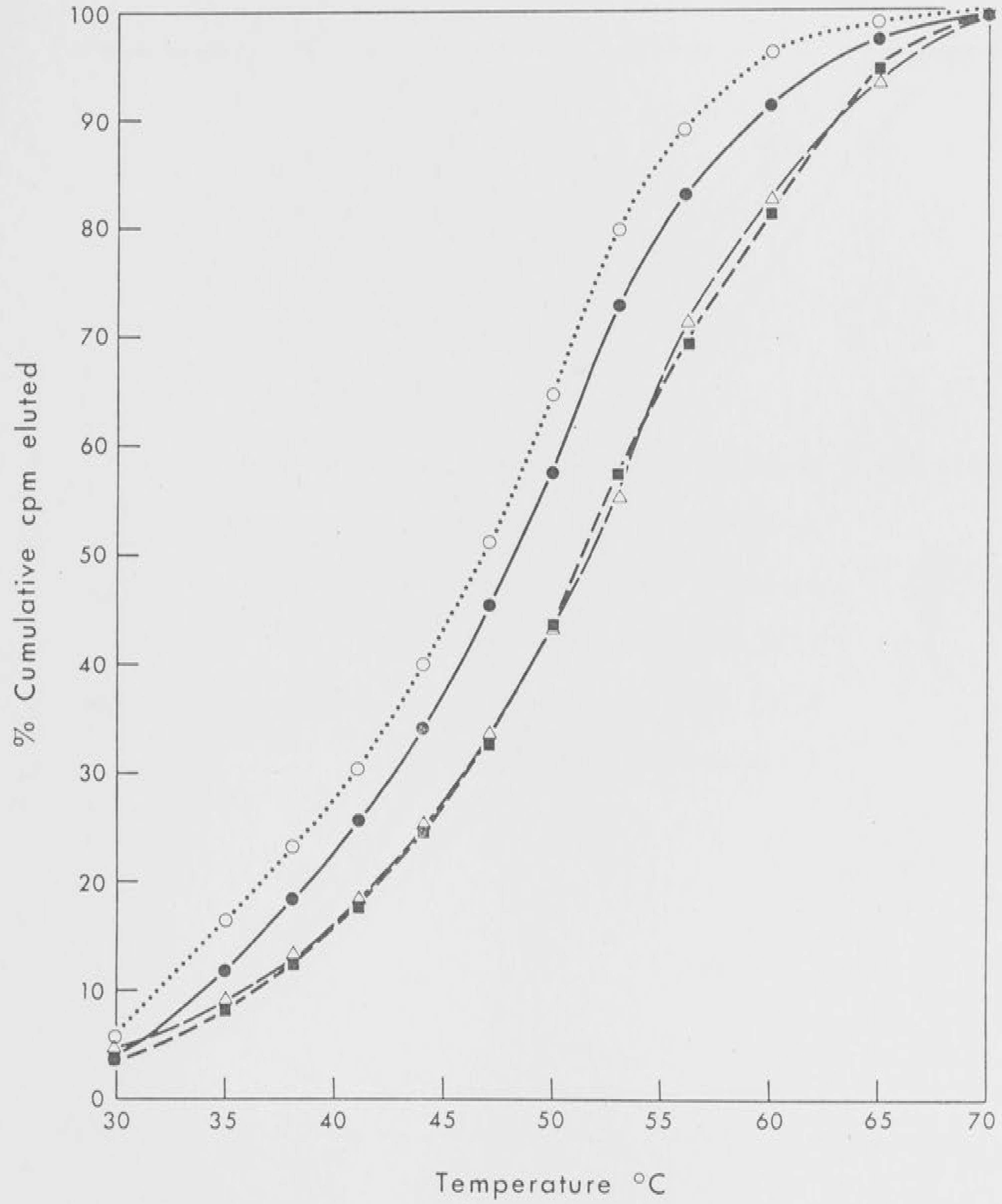

% Cumulative cpm eluted
0
10
20
30
40
50
60
70
80
90
100
30
35
40
45
50
55
60
65
70
Temperature °C

Figure 6.8 : Thermal denaturation profiles of *Notomys fuscus* 1.713 ^{3}HcRNA hybridized to *N. alexis* 1.712 satellite DNA (△– – –△), to *N. mitchellii* 1.712 satellite DNA (■------■), to *N. cervinus* 1.713 satellite DNA (○.......○) and to *N. fuscus* 1.713 satellite DNA (●——●).

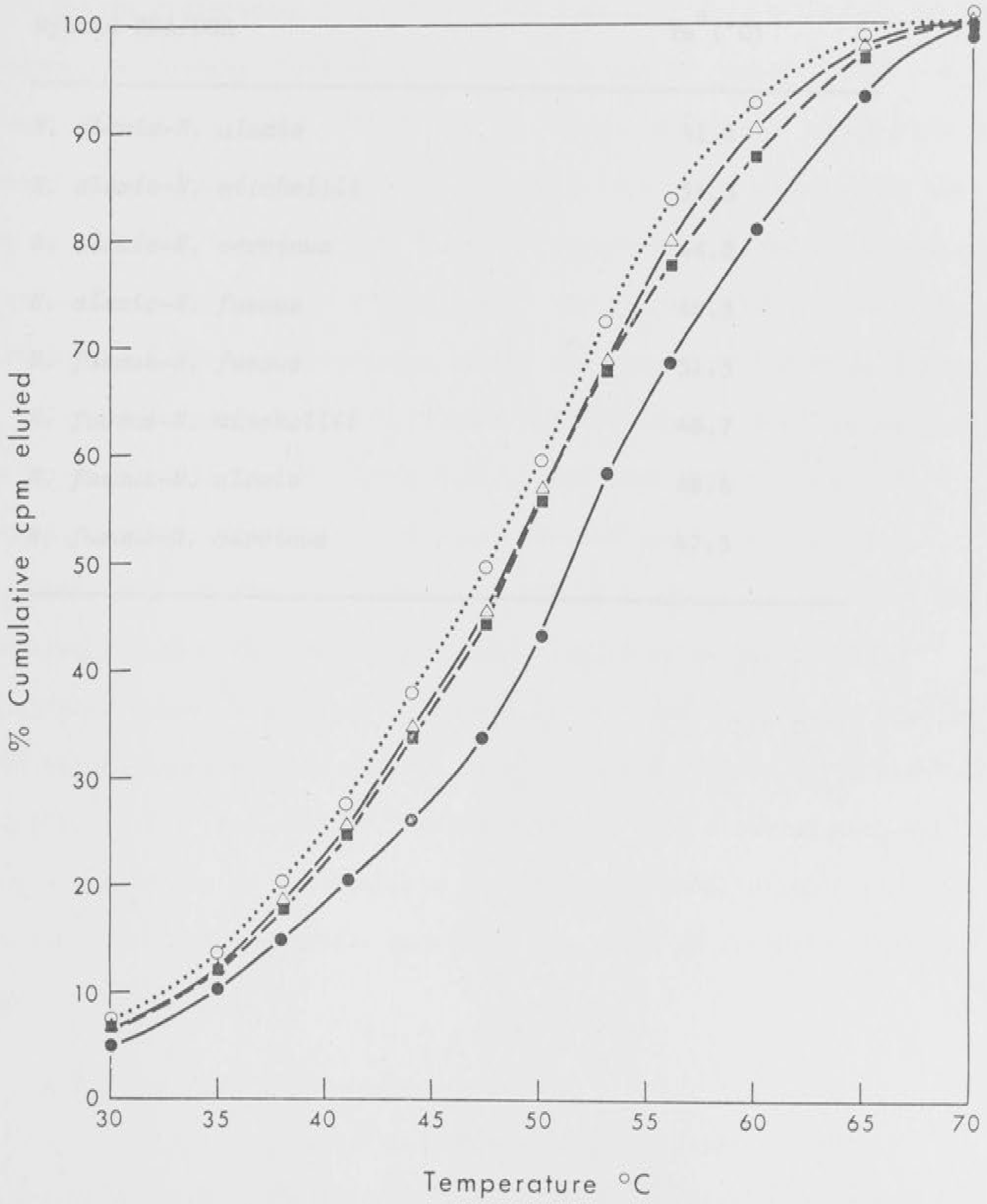

% Cumulative cpm eluted
100
90
80
70
60
50
40
30
20
10
0
30
35
40
45
50
55
60
65
70
Temperature °C

Table 6.4: Thermal stability of ^{3}HcRNA-DNA hybrids in filter hybridization of *Notomys* satellites.

*The temperature at which half the total radioactivity was eluted was taken to be the Tm.

Hybrid RNA/DNA	Tm* (°C)
N. alexis-N. alexis	51.8
N. alexis-N. mitchellii	51.5
N. alexis-N. cervinus	46.8
N. alexis-N. fuscus	48.3
N. fuscus-N. fuscus	51.5
N. fuscus-N. mitchellii	48.7
N. fuscus-N. alexis	48.6
N. fuscus-N. cervinus	47.5

hybridization of ^{3}HcRNA from *N. fuscus* 1.713 satellite DNA with the homologous *N. fuscus* 1.713 satellite (Tm = 51.5°) and the three heterologous satellite DNAs *N. alexis* 1.712 satellite (Tm = 48.6°C), *N. mitchellii* 1.712 satellite (Tm = 48.7°C) and *N. cervinus* 1.713 satellite (Tm = 47.5°C), are shown in Fig. 6.8 and Tables 6.3 and 6.4.

The Tm differences between the homologous *N. fuscus* RNA/DNA hybrid and the heterologous hybrids were 2.9°C for the *N. fuscus* ^{3}HcRNA - *N. alexis* DNA hybrid and 2.8°C for the *N. fuscus* ^{3}HcRNA - *N. mitchellii* DNA hybrid indicating a sequence mismatch of 1.8% in each case, while the ΔTm of 4°C with the *N. fuscus* ^{3}HcRNA - *N. cervinus* DNA hybrid indicates a sequence mismatch of 2.5% (Table 6.3). The almost identical Tm differences between the *N. fuscus* homologous hybrid and the heterologous *N. fuscus*-*N. mitchellii* and *N. fuscus*-*N. alexis* hybrids support the finding that the *N. mitchellii* and *N. alexis* 1.712 satellites are very similar in sequence. The 2.5% mismatch of bases between the *N. fuscus* 1.713 satellite DNA and the 1.713 satellite DNA of *N. cervinus* clarifies the difference suggested between these two satellites in the initial experiment where ^{3}HcRNA from *N. alexis* 1.712 satellite DNA was used to give heterologous hybrids with *N. cervinus* and *N. fuscus* 1.713 satellite DNA (Fig. 6.7). The earlier results indicate that although each differs from *N. alexis* it is not possible to guage the extent of difference between these two satellites purely on the basis of heterologous/heterologous comparisons.

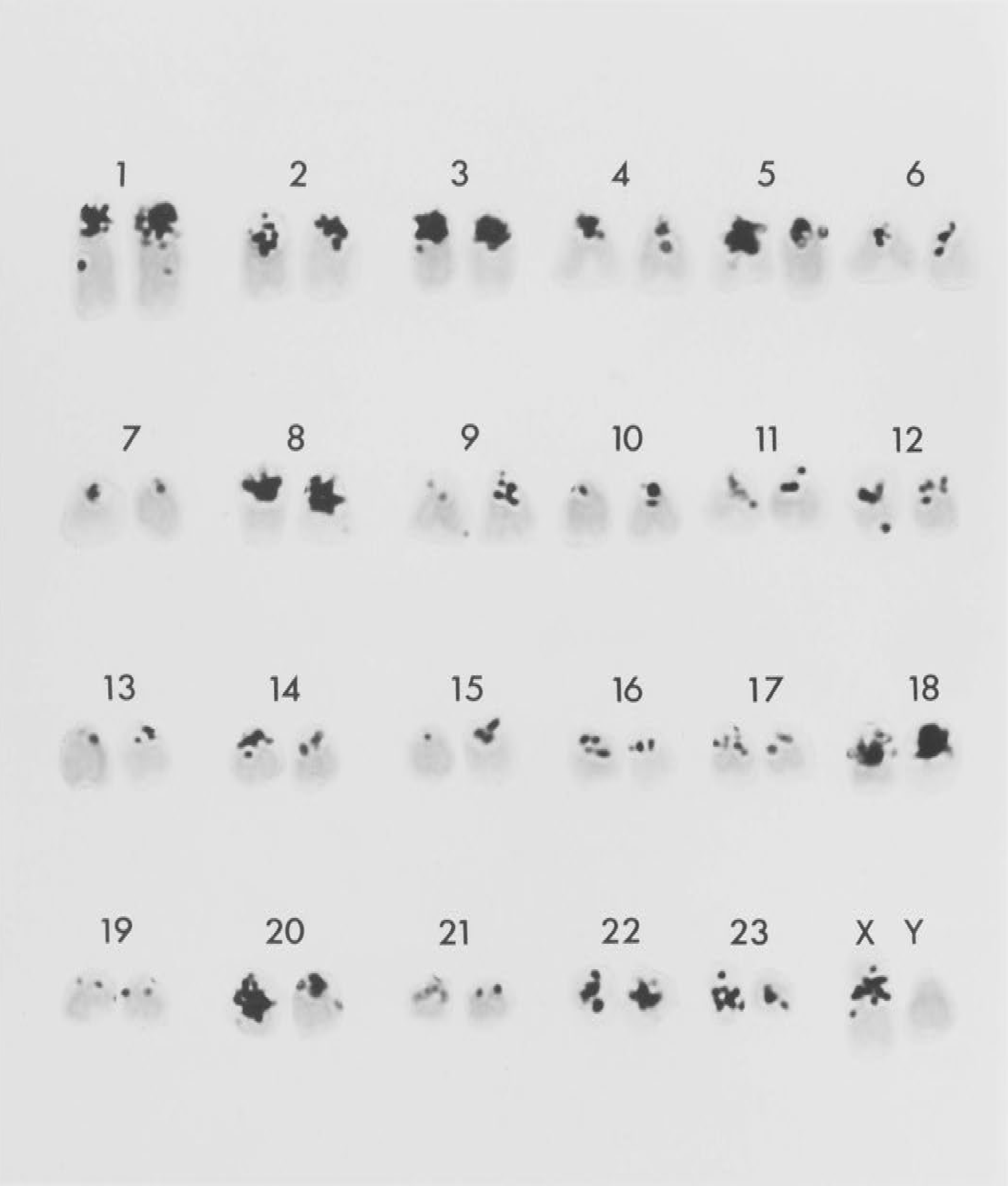
6.3.4 In situ *hybridization*

6.3.4.1 *Autosomal location of satellite DNA*

6.3.4.1.1 *^{3}HcRNA from satellite DNA hybridized to homologous karyotypes*:

^{3}HcRNA from the satellites of *N. mitchellii, N. alexis, N. cervinus* and *N. fuscus* when hybridized *in situ* to their homologous metaphase karyotypes revealed a chromosomal location of the satellites in the regions of the autosomes which were C band

Figure 6.9 : *In situ* hybridization of *Notomys cervinus* 1.713 ^{3}HcRNA to the mitotic chromosomes of a male *N. mitchellii*.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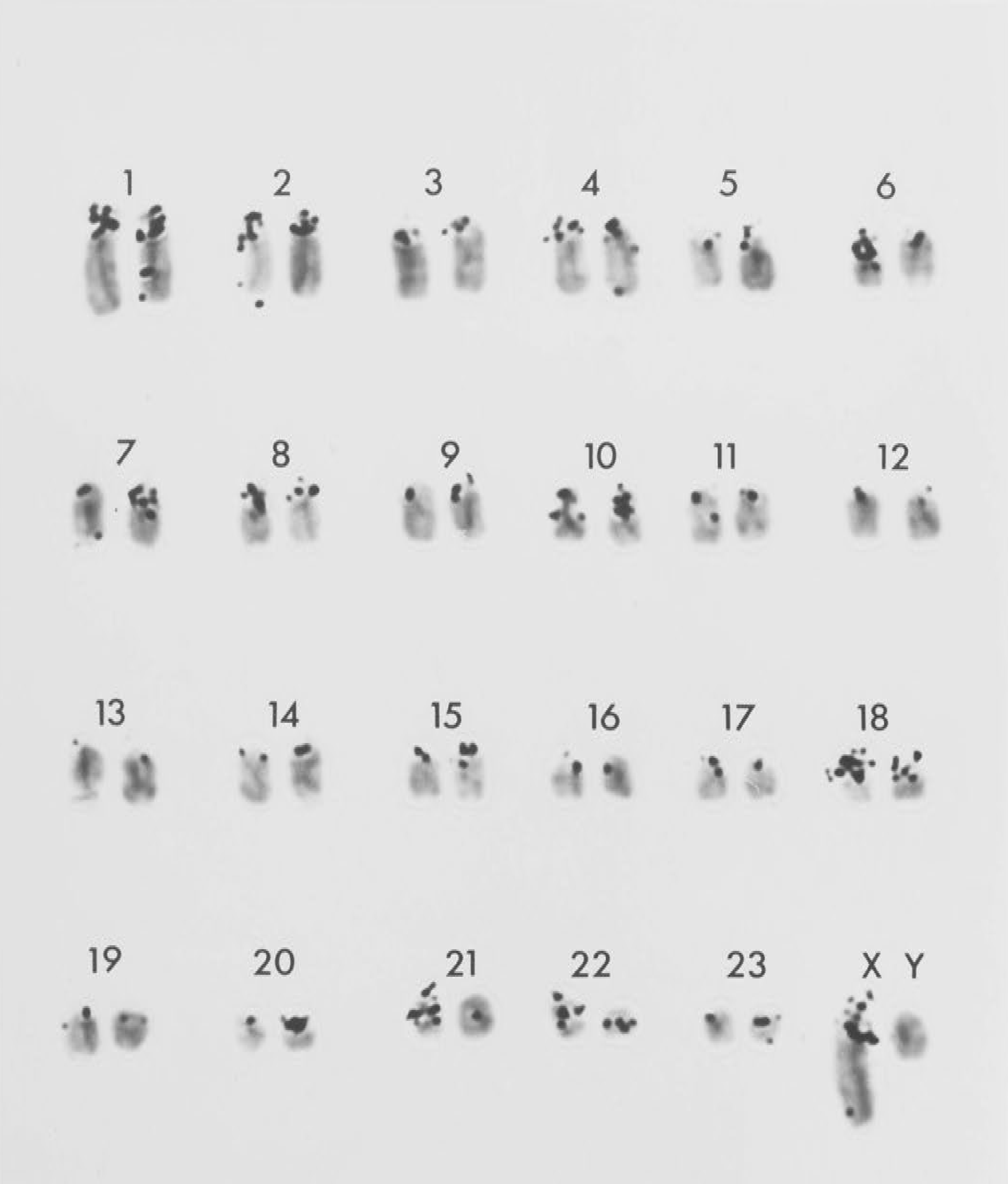
22
23
X
Y

Figure 6.10 : *In situ* hybridization of *Notomys cervinus* 1.713 ^{3}HcRNA to the mitotic chromosomes of a male *N. alexis*.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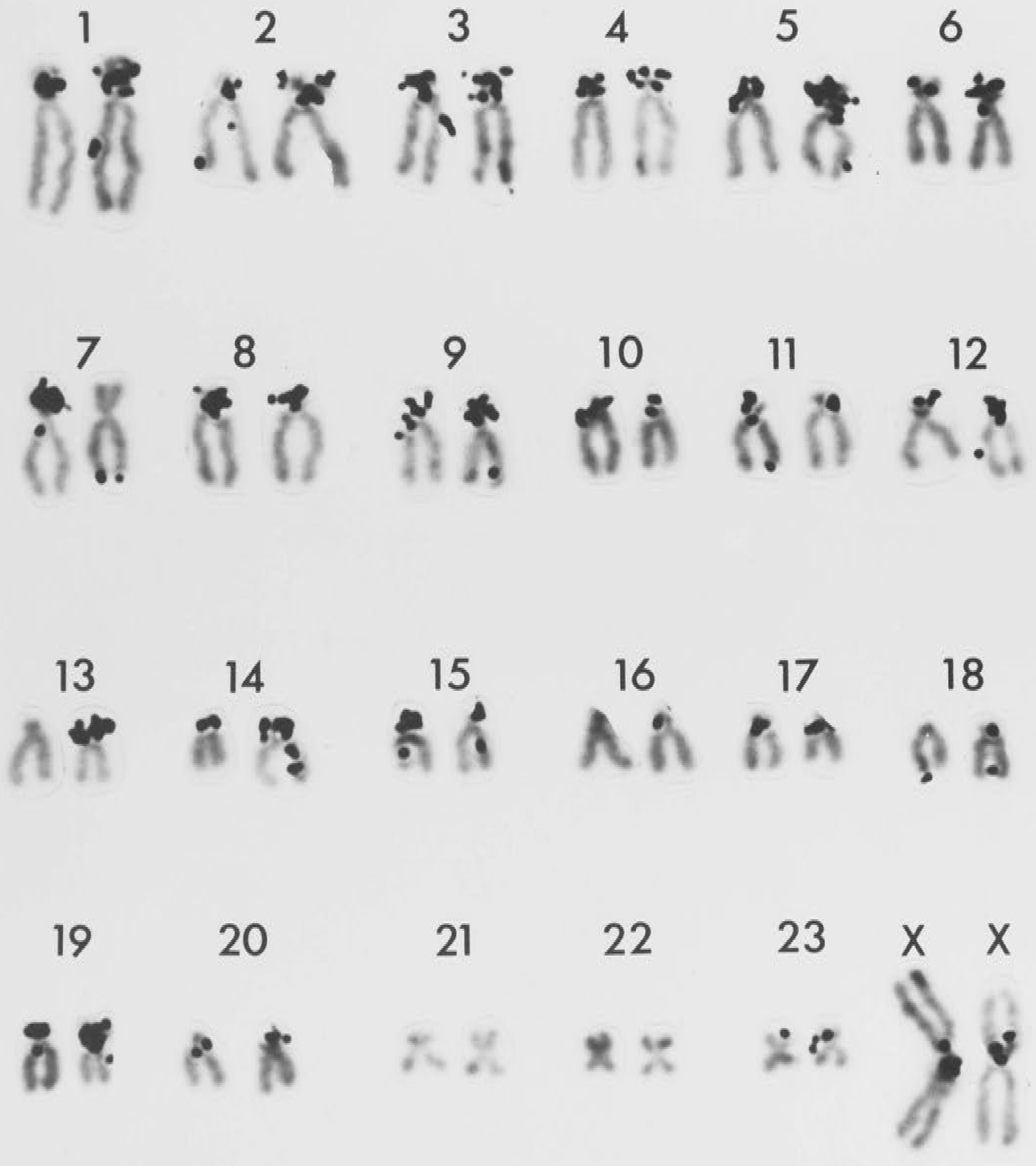
23
X
Y

Figure 6.11 : *In situ* hybridization of *Notomys cervinus* 1.713 ^{3}HcRNA to the mitotic chromosomes of a female *N. cervinus*.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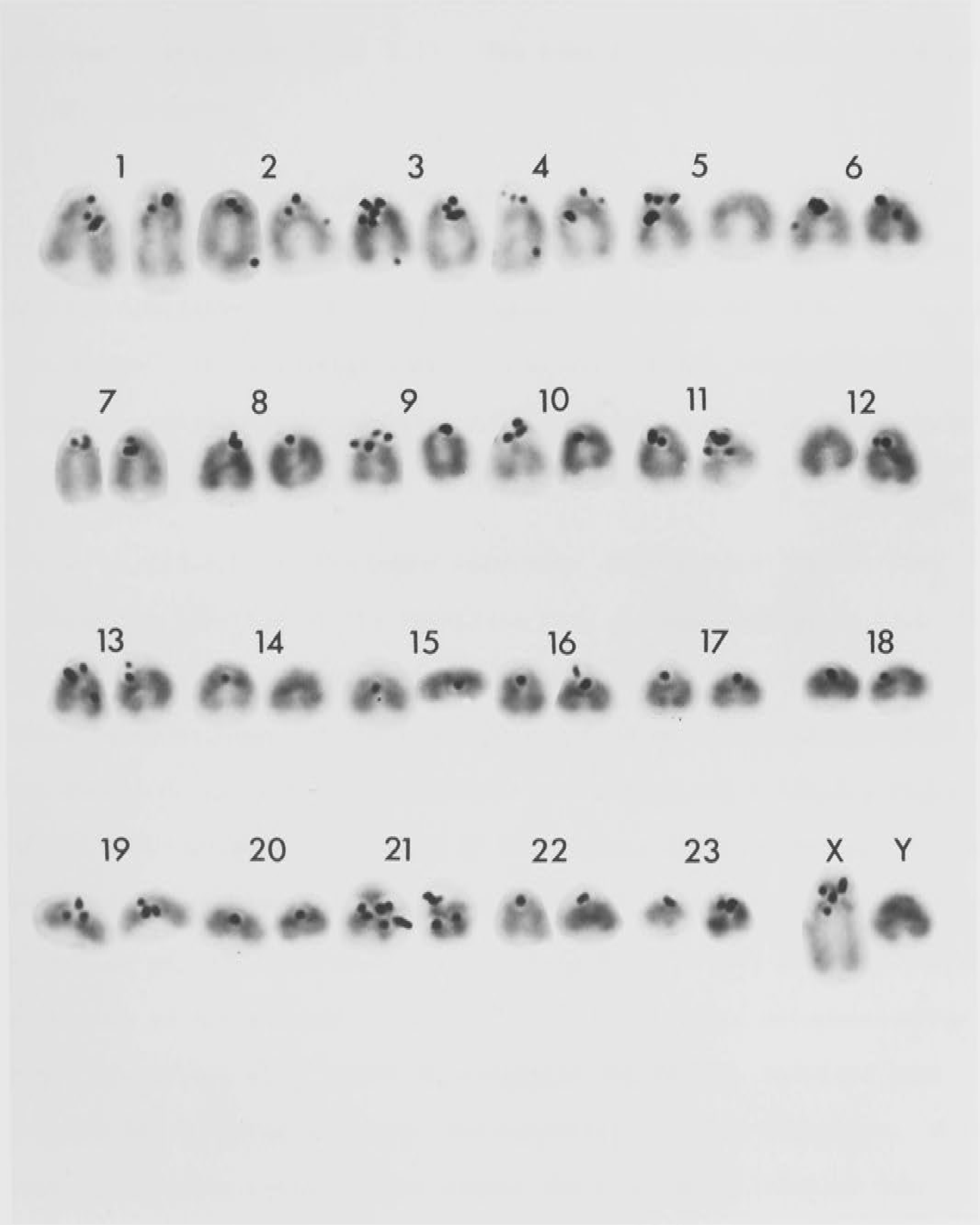
22
23
X
X

Figure 6.12 : *In situ* hybridization of *Notomys cervinus* 1.713 ^{3}HcRNA to the mitotic chromosomes of a male *N. fuscus*.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20
21
22
23
X
Y

positive (Figs. 6.9,6.10,6.11,6.12). In *N. cervinus* two of the three small metacentric pairs were lightly labelled while the remaining pair had negligible label after exposure for 6 months (Fig. 6.13). The metacentric autosomes do however have C band positive material located peri-centromerically (Fig. 2.2). The remaining three species show label on all autosomes.

6.3.4.1.2 *^{3}HcRNA from* Notomys *satellite DNAs hybridized to heterologous karyotypes*: ^{3}HcRNA from each satellite DNA was hybridized to the twelve possible combinations of heterologous karyotypes. The resultant labelling pattern in all heterologous hybridization paralleled that of the homologous cases indicating that there are similar sequences in all four *Notomys* satellite DNAs.

6.3.4.2 *X chromosome satellite DNA location*: In all four species the location of the satellite DNA, as indicated by *in situ* hybridization, was confined to the intense C band positive region immediately adjacent to the centromere but no satellite was detected in the heterochromatic but less intense non-centromeric C banding region of the X chromosomes. Material of this nature has been termed "accessory gonosomal heterochromatin" by Schmid (1967). As the *in situ* hybridization technique may lack the sensitivity necessary to detect the presence of sequences in minimal copy number, *N. cervinus* mitotic metaphase chromosomes hybridized with ^{3}HcRNA of satellite DNA from *N. cervinus* were exposed for 6 months to extend the sensitivity of the technique. Here also the intense C-band region around the centromere labelled heavily whereas in the majority of cells the euchromatic arm and the less intense C-banding heterochromatic arm of the X chromosome were devoid of label (Fig. 6.13a). In a few cases where the heterochromatic arm was very lightly labelled, the intensity was similar to the background label and the euchromatic arm too received similar small amounts of label. Hence it is concluded that the heterochromatic region of the X chromosomes in

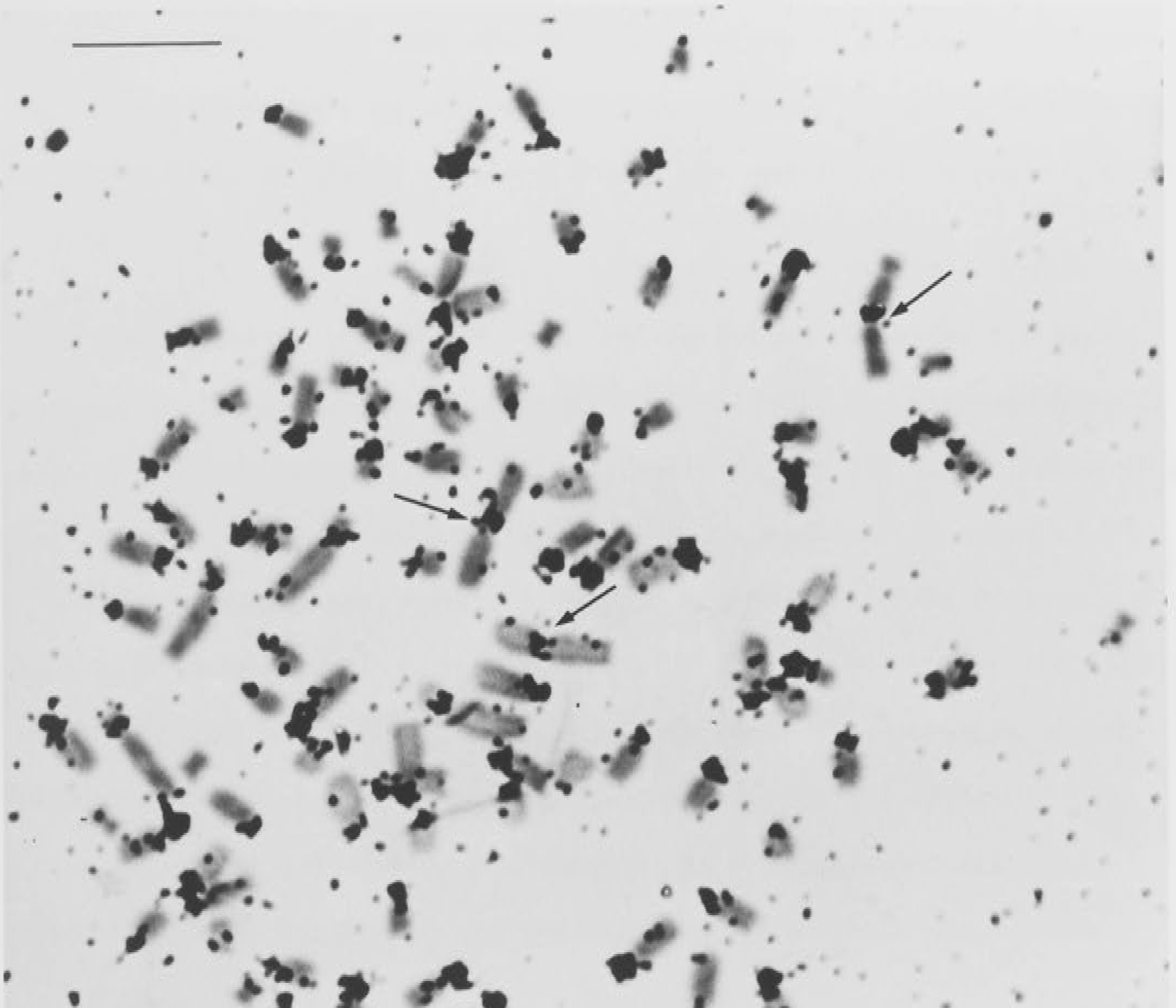
Figure 6.13 : (a) *In situ* hybridization of *N. cervinus* 1.713 ^{3}HcRNA to *N. cervinus* mitotic chromosomes. Two cells are included in this spread. Note the absence of label on both the euchromatin and the accessory gonosomal heterochromatin of the X chromosomes (arrowed). The small metacentric autosomes are either devoid of label or have label of similar intensity to that of the background. Exposure length was 6 months.

(b-e) Hybridization of *N. fuscus* (male) 1.713 ^{3}HcRNA to *N. alexis* male meiotic chromosomes.

(b-c) - Label on the XY bivalent is situated only at the centromeric region of the X chromosome. Exposure length 1 month.

(d-e) - Label on the XY bivalent is situated only at the centromeric region of the X chromosome. Exposure length 6 months.

Bar represents 10 μm.


a
Y
X
b
X
Y
c
X
Y
d
Y
X
e

Notomys, excluding the intense C-banding centromeric region, lack the nucleotide sequences complementary to those present in the ^{3}HcRNAs from these satellites or if they are present they are in minimal copy numbers. Of course, these data do not preclude the presence of an another satellite or other forms of highly repeated DNA as yet undetected in these genomes.

6.3.4.3 *Location of satellite sequences in Y chromosomes*: *In situ* hybridization to mitotic preparations failed to detect sequences similar to the satellites of any of the species in the Y chromosomes of either *N. mitchellii* (Fig. 6.9), *N. alexis* (Fig. 6.10) or *N. fuscus* (Fig. 6.12). However to avoid any ambiguity due to misidentification of the Y chromosome additional hybridization to the meiotic cells of a male *N. alexis* preparation was carried out (Fig. 6.13b-e). Here also the label was found only in the centromere region of the X chromosome in the XY bivalent; the Y chromosome was again completely devoid of label. The euchromatic and heterochromatic arms of the X chromosome were also devoid of label confirming the results from the mitotic preparations.

6.3.5 *Restriction endonuclease digestions*

6.3.5.1. *Hinf I digests*: Hinf I digests of the four *Notomys* satellites reveal a series based on a unit length of 22 base pairs (Diagram 6.1a; Fig. 6.14). In the fragment length range of 22 to 132 base pairs (monomer to hexamer) the four satellites have similar digestion patterns with Hinf I, in which the hexamer band is more prominent than any of the others. The prominence of the hexamer fragment in the satellites of all four *Notomys* species is most easily interpreted as an amplification event in the satellite of a common ancestor prior to the divergence of these species. The 22 base pair multimer series is maintained from the monomer through to the decamer in Hinf I-1.713 satellite DNA digests of both *N. cervinus* and *N. fuscus*. However, in both *N. mitchellii* and *N. alexis*, while the multimer series is preserved up to and including the hexamer fragment, a more complex pattern replaces

Diagram 6.1 : Fragment lengths resulting from restriction endonuclease digestion of *Notomys* satellite DNAs in the range 22 to 220 base pairs.

(a) Hinf I digestion of *N. mitchellii* 1.712 satellite DNA (Nm), *N. alexis* 1.712 satellite DNA (Na), *N. cervinus* 1.713 satellite DNA (Nc) and *N. fuscus* 1.713 satellite DNA (Nf).

(b) Hae III digestion of *N. mitchellii* 1.712 satellite DNA (Nm), *N. alexis* 1.712 satellite DNA (Na), *N. cervinus* 1.713 satellite DNA (Nc) and *N. fuscus* 1.713 satellite DNA (Nf).

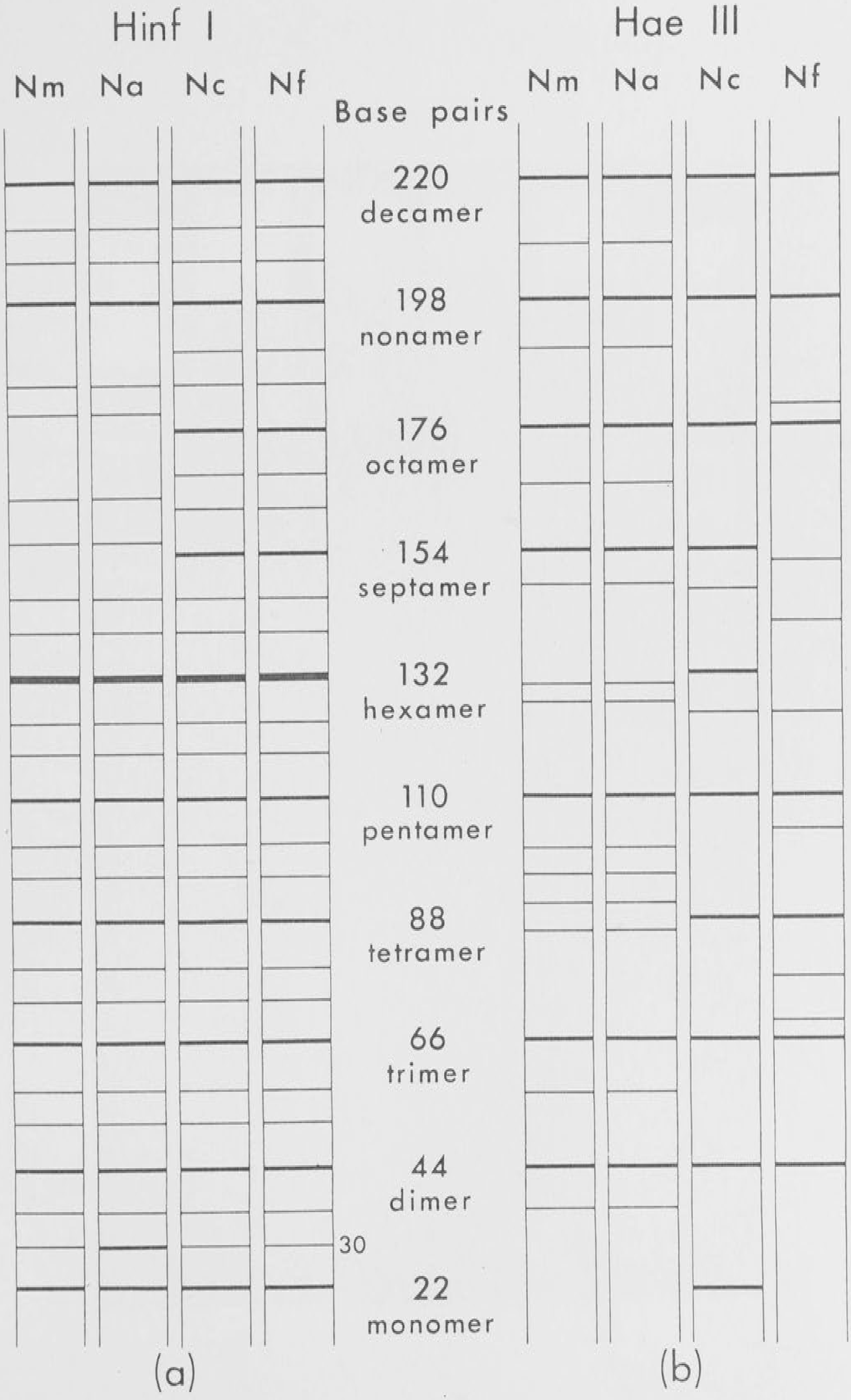
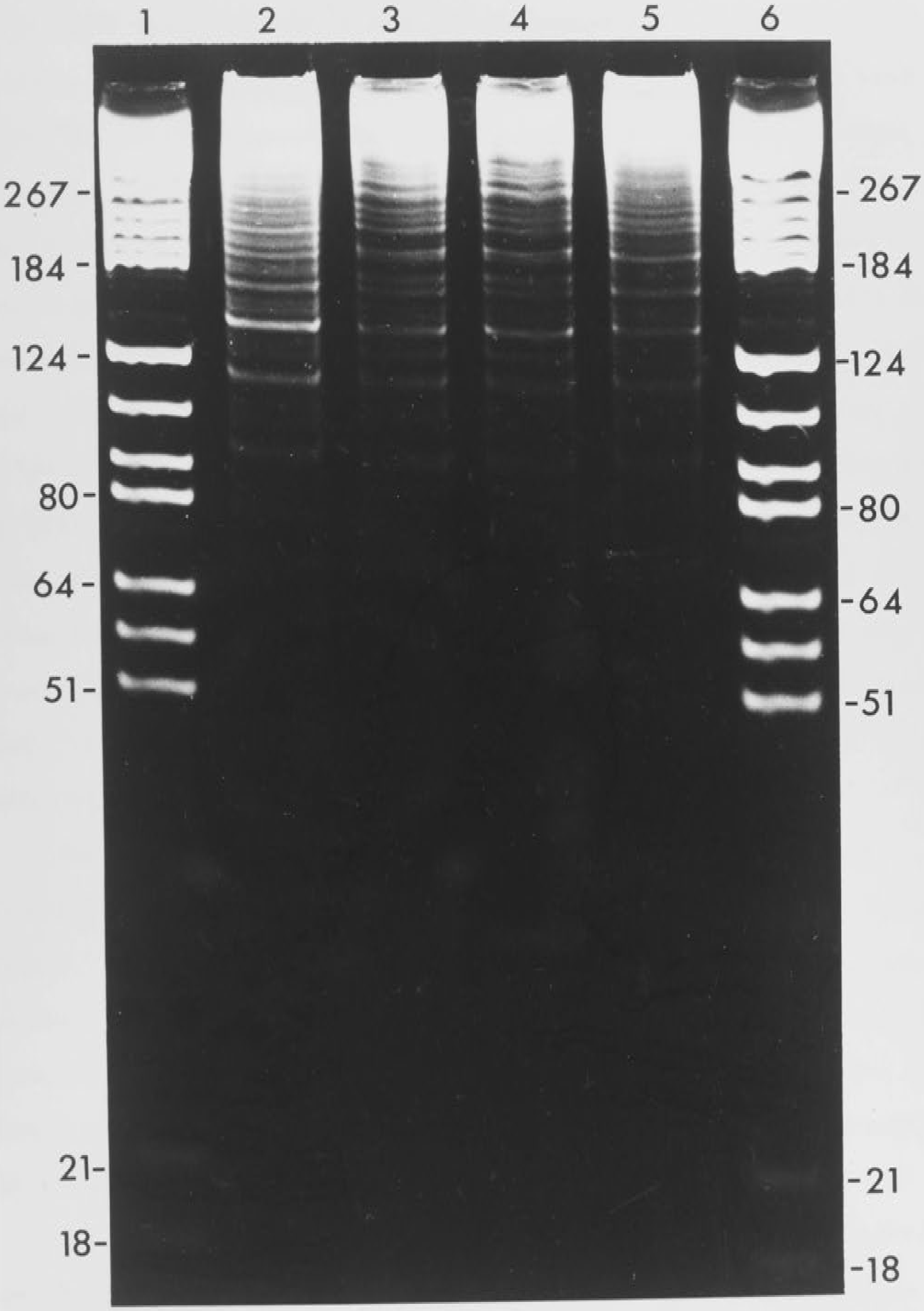

Hinf I
Hae III
Nm
Na
Nc
Nf
Base pairs
220
decamer
198
nonamer
176
octamer
154
septamer
132
hexamer
110
pentamer
88
tetramer
66
trimer
44
dimer
30
22
monomer
Nm
Na
Nc
Nf
(a)
(b)

Figure 6.14 : Digestion of *Notomys* satellite DNAs with Hinf I restriction endonuclease. Slots 1 and 6 contained pBR322 DNA digested with Hae III and coelectrophoresed with the digests in the other slots to act as a molecular weight marker.

Slot 2 : *N. cervinus* 1.713 satellite DNA-Hinf I digest.
Slot 3 : *N. mitchellii* 1.712 satellite DNA-Hinf I digest.
Slot 4 : *N. alexis* 1.712 satellite DNA-Hinf I digest.
Slot 5 : *N. fuscus* 1.713 satellite DNA-Hinf I digest.
The Gel used in the analysis was 10% acrylamide.


1
2
3
4
5
6
267
184
124
80
64
51
21
18
267
184
124
80
64
51
21
18

the multimer series from the septamer to monomer fragment lengths. From the monomer through to the decamer in the multimer series both *N. cervinus* and *N. fuscus*, and from the monomer through to the hexamer in both *N. mitchellii* and *N. alexis*, a doublet of fragments occurs in the interval between those forming the multimer series. The electrophoretic bands which define the fragments forming the doublet are less than a third as intense as the bands produced by the multimer series. The multimer plus doublet series form a regular spaced pattern. The doublet fragments may be explained by the appearance of a secondary or additional Hinf I site between the two primary sites which contribute to the multimer series. The secondary site, positioned 8 base pairs from one primary site, would place it 14 base pairs from the next primary site. This situation must initially have been repeated throughout sections of the satellite due possibly to subsequent amplification. Subsequent elimination of adjacent primary and secondary Hinf I sites in differing multiples would then result in fragments giving the doublet series observed here. A site could be eliminated either by mutation or else by methylation of base pairs within it.

The *N. alexis* and *N. mitchellii* Hinf I digests differ in the prominence of the 30 base pair fragment which is very prominent in *N. alexis*. This fragment is also involved in the doublet series. However, as the other member of the doublet is not of equal intensity, the added intensity of the 30 base pair fragment must be due to a population of satellite DNA fragments which is entirely separate from those resulting in the doublet series.

In summary, Hinf I digestion discriminates between *N. mitchellii* and *N. alexis* satellites and also between these satellites and those of *N. cervinus* and *N. fuscus*. It does not distinguish between the satellites of *N. cervinus* and *N. fuscus*.

6.3.5.2 *Hae III digests*: Hae III digest of *Notomys* satellites discriminates between *N. cervinus* and *N. fuscus* and also between these satellites and those of *N. mitchellii* and *N. alexis* (Diagram 6.1b; Fig. 6.15). However, Hae III digests of *N. mitchellii* and *N. alexis* appear to be indistinguishable. Here also, the major fragment sizes are multiples of 22 base pairs but there are rearrangements affecting this pattern. The Hae III - *N. cervinus* digest has a relatively simple pattern which is based essentially on a 22 base pair monomer and multiples of this unit, although there are minor fragments of 125 and 147 base pairs in length. The Hae III - *N. fuscus* digest pattern is more complex but contains all elements in the multimer series except for the hexamer and septamer fragments. Additional fragments are 70, 78, 104, 125, 141, 152 and 180 base pairs in length. None of these fragments appear as though they are part of a multiple series between the major sites which produce the observed doublet pattern. These additional fragments could be from sites in the satellite non-contiguous with those resulting in the multimer series or alternatively they could have arisen from a section of satellite originally containing major sites which were subsequently changed by mutation or methylation.

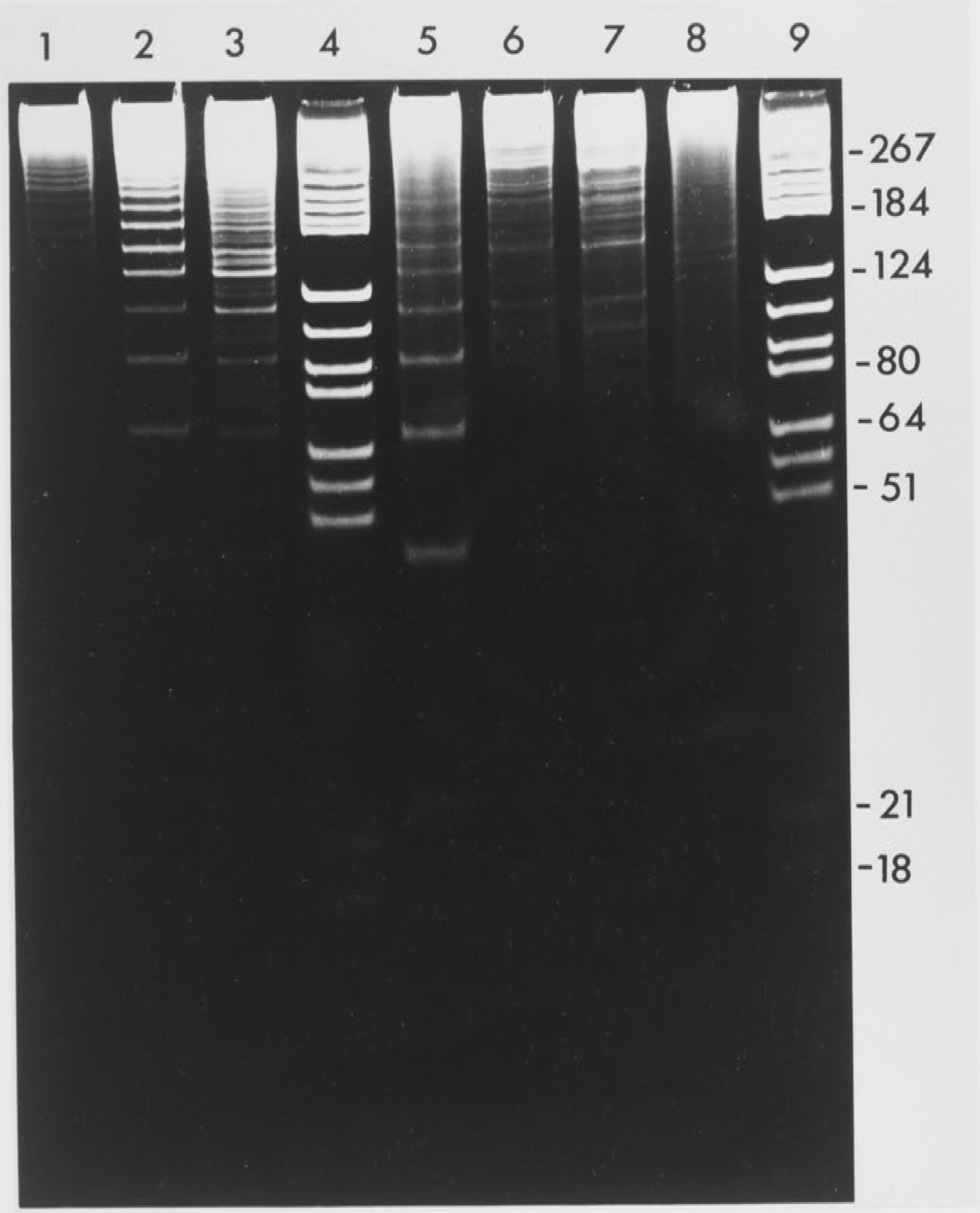
N. mitchellii and *N. alexis* have more complex patterns from the *N. cervinus* Hae III digest. In both of these the 22 base pair multimer series is present but has undergone some alteration. The tetramer and hexamer fragments are both absent and the septimer fragment is reduced in both digests. In addition there are a number of fragments all of lesser intensity than those of the multimer series. The additional fragments cannot be explained in terms of multimer fragments.

6.3.5.3 *Bam HI digests*: In the range of fragment sizes from 22 through to 220 base pairs all four Bam HI satellite DNA digests are strikingly similar due to the multimer series of 22 base pairs (Diagram 6.2a; Fig. 6.16). In these digests the major fragments form a complete

Figure 6.15 : Digestion of *Notomys* satellite DNAs with restriction endonucleases. Slots 4 and 9 contain pBR322-Hae III digests which have been coelectrophoresed with the digests in the other slots and used as molecular weight markers.

Slot 1 : *N. cervinus* 1.713 satellite DNA-Taq I digest.
Slot 2 : *N. cervinus* 1.713 satellite DNA-Hinf I digest.
Slot 3 : *N. cervinus* 1.713 satellite DNA-Hae III digest.
Slot 4 : pBR322 DNA-Hae III marker digest.
Slot 5 : *N. cervinus* 1.713 satellite DNA-Hae III digest.
Slot 6 : *N. mitchellii* 1.712 satellite DNA-Hae III digest.
Slot 7 : *N. alexis* 1.712 satellite DNA-Hae III digest.
Slot 8 : *N. fuscus* 1.713 satellite DNA-Hae III digest.
Slot 9 : pBR322 DNA-Hae III marker digest.

The gel analysed was 10% acrylamide.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5
6
7
8
9
-267
-184
-124
-80
-64
- 51
- 21
-18

Figure 6.16 : Digestion of *Notomys* satellite DNAs with restriction endonucleases.

(a) Bam HI digestions.

Slot 1 : *N. cervinus* 1.713 satellite DNA-Bam HI digest used as a molecular weight marker.

Slot 2 : *N. mitchellii* 1.712 satellite DNA-Bam HI digest.

Slot 3 : *N. alexis* 1.712 satellite DNA-Bam HI digest.

Slot 4 : *N. fuscus* 1.713 satellite DNA-Bam HI digest.

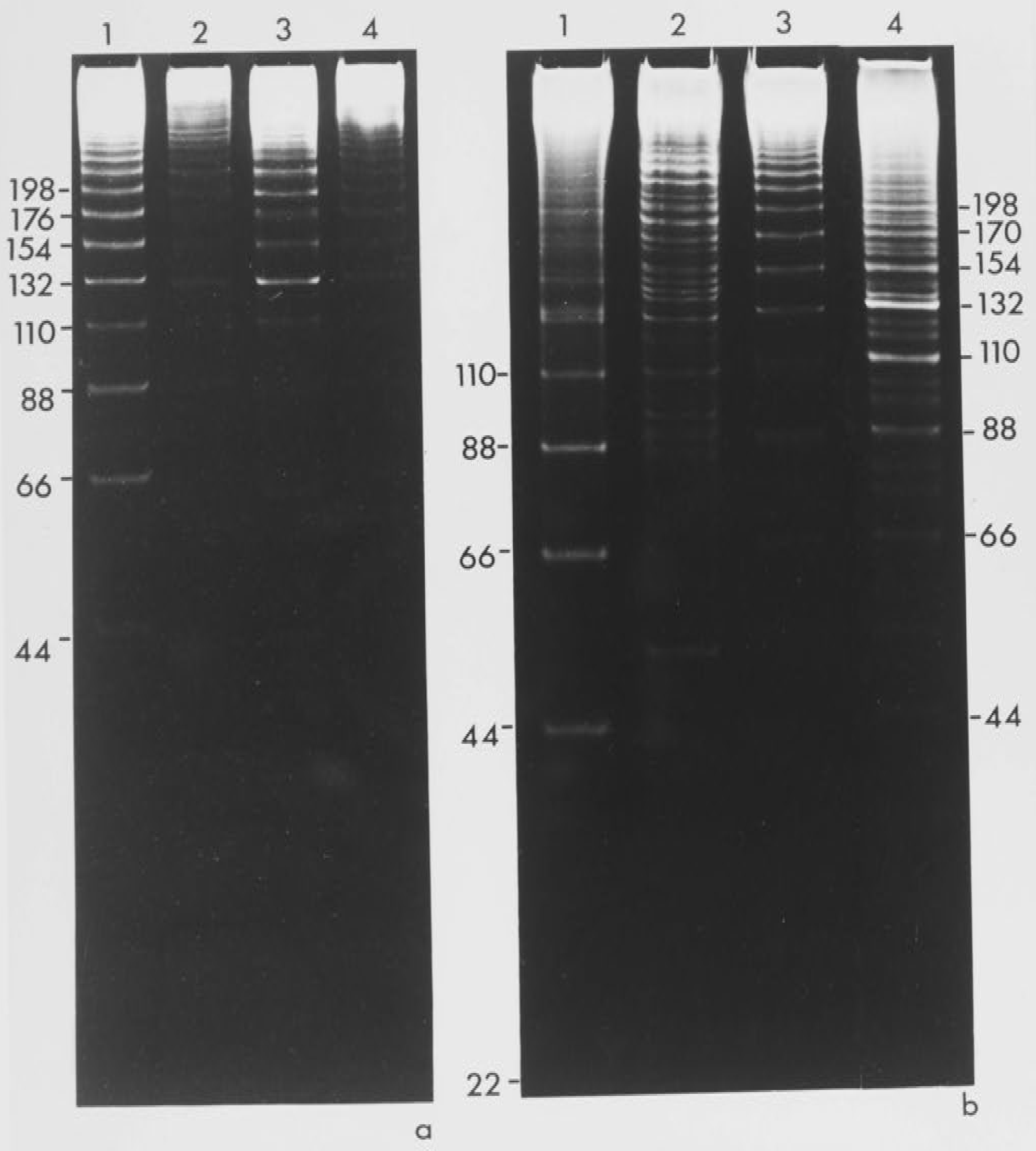
(b) Digestion of *Notomys* satellite DNAs with Hae III, Taq I, Bam HI and Hinf I restriction endonucleases.

Slot 1 : *N. cervinus* 1.713 satellite DNA-Hae III digest used as a molecular weight marker.

Slot 2 : *N. alexis* 1.712 satellite DNA-Taq I digest.

Slot 3 : *N. cervinus* 1.713 satellite DNA-Bam HI digest.

Slot 4 : *N. cervinus* 1.713 satellite DNA-Hinf I digest used as molecular weight marker. Slot 4 is from the same gel as slots 1-3 but is has been cut from the gel photograph and moved adjacent to them for simplicity of presentation. Both gels were 10% acrylamide.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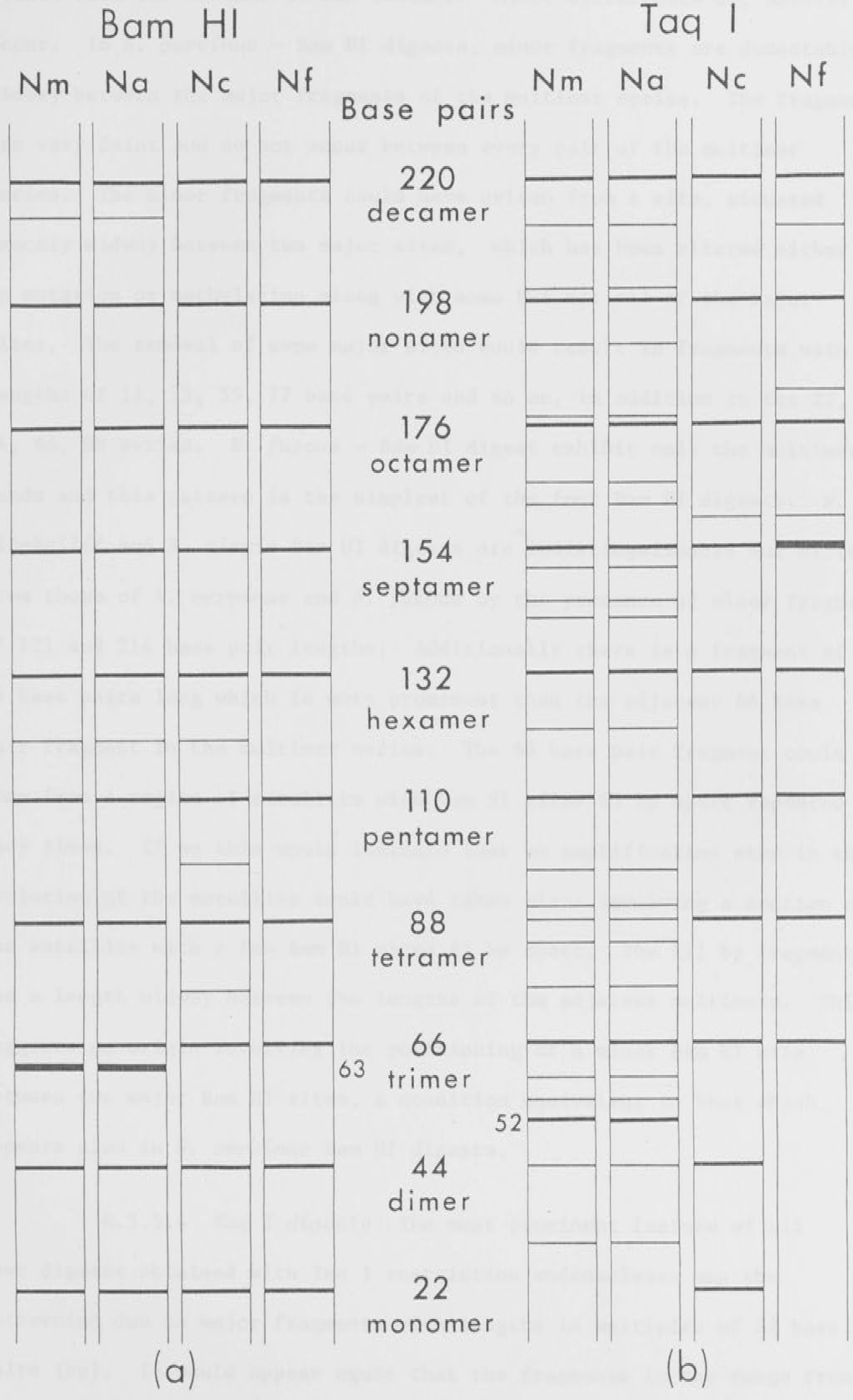
198
176
154
132
110
88
66
44
a
1
2
3
4
110
88
66
44
22
198
170
154
132
110
88
66
44
b

Diagram 6.2 : Fragment lengths resulting from restriction endonuclease digestion of *Notomys* satellite DNAs in the range 22 to 220 base pairs.

(a) Bam HI digestion of *N. mitchellii* 1.712 satellite DNA (Nm), *N. alexis* 1.712 satellite DNA (Na), *N. cervinus* 1.713 satellite DNA (Nc) and *N. fuscus* 1.713 satellite DNA (Nf).

(b) Taq I digestion of *N. mitchellii* 1.712 satellite DNA (Nm), *N. alexis* 1.712 satellite DNA (Na), *N. cervinus* 1.713 satellite DNA (Nc) and *N. fuscus* 1.713 satellite DNA (Nf).


Bam HI
Taq I
Nm
Na
Nc
Nf
Nm
Na
Nc
Nf
Base pairs
220
decamer
198
nonamer
176
octamer
154
septamer
132
hexamer
110
pentamer
88
tetramer
66
63
trimer
52
44
dimer
22
monomer
(a)
(b)

series from the monomer to the decamer. Minor differences do, however, occur. In *N. cervinus* - Bam HI digests, minor fragments are detectable midway between the major fragments of the multimer series. The fragments are very faint and do not occur between every pair of the multimer series. The minor fragments could have arisen from a site, situated exactly midway between two major sites, which has been altered either by mutation or methylation along with some but not all of the major sites. The removal of some major sites would result in fragments with lengths of 11, 33, 55, 77 base pairs and so on, in addition to the 22, 44, 66, 88 series. *N. fuscus* - Bam HI digest exhibit only the multimer bands and this pattern is the simplest of the four Bam HI digests. *N. mitchellii* and *N. alexis* Bam HI digests are indistinguishable but differ from those of *N. cervinus* and *N. fuscus* by the presence of minor fragments of 121 and 214 base pair lengths. Additionally there is a fragment of 63 base pairs long which is more prominent than the adjacent 66 base pair fragment in the multimer series. The 63 base pair fragment could come from a region of satellite with Bam HI sites 63 bp apart repeated many times. If so this would indicate that an amplification step in the evolution of the satellite could have taken place involving a section of the satellite with a few Bam HI sites 63 bp apart. The 121 bp fragment has a length midway between the lengths of the adjacent multimers. This suggests an origin involving the positioning of a minor Bam HI site between two major Bam HI sites, a condition equivalent to that which appears also in *N. cervinus* Bam HI digests.

6.3.5.4 *Taq I digests*: The most prominent feature of all four digests obtained with Taq I restriction endonuclease was the patterning due to major fragments with lengths in multiples of 22 base pairs (bp). It would appear again that the fragments in the range from 22 to 220 bp in *N. cervinus* - Taq I digests give evidence of doublets which are approximately midway between the multimer pairs (Diagram 6.2b;

Figure 6.17 : Digestion of *Notomys* satellite DNAs with restriction endonucleases.

(a) Taq I digests.

Slot 1 : pBR322 DNA-Hae III digest which was coelectrophoresed with the remaining digests to act as a molecular weight marker.

Slot 2 : *N. cervinus* 1.713 satellite DNA-Taq I digest.

Slot 3 : *N. mitchellii* 1.712 satellite DNA-Taq I digest.

Slot 4 : *N. alexis* 1.712 satellite DNA-Taq I digest.

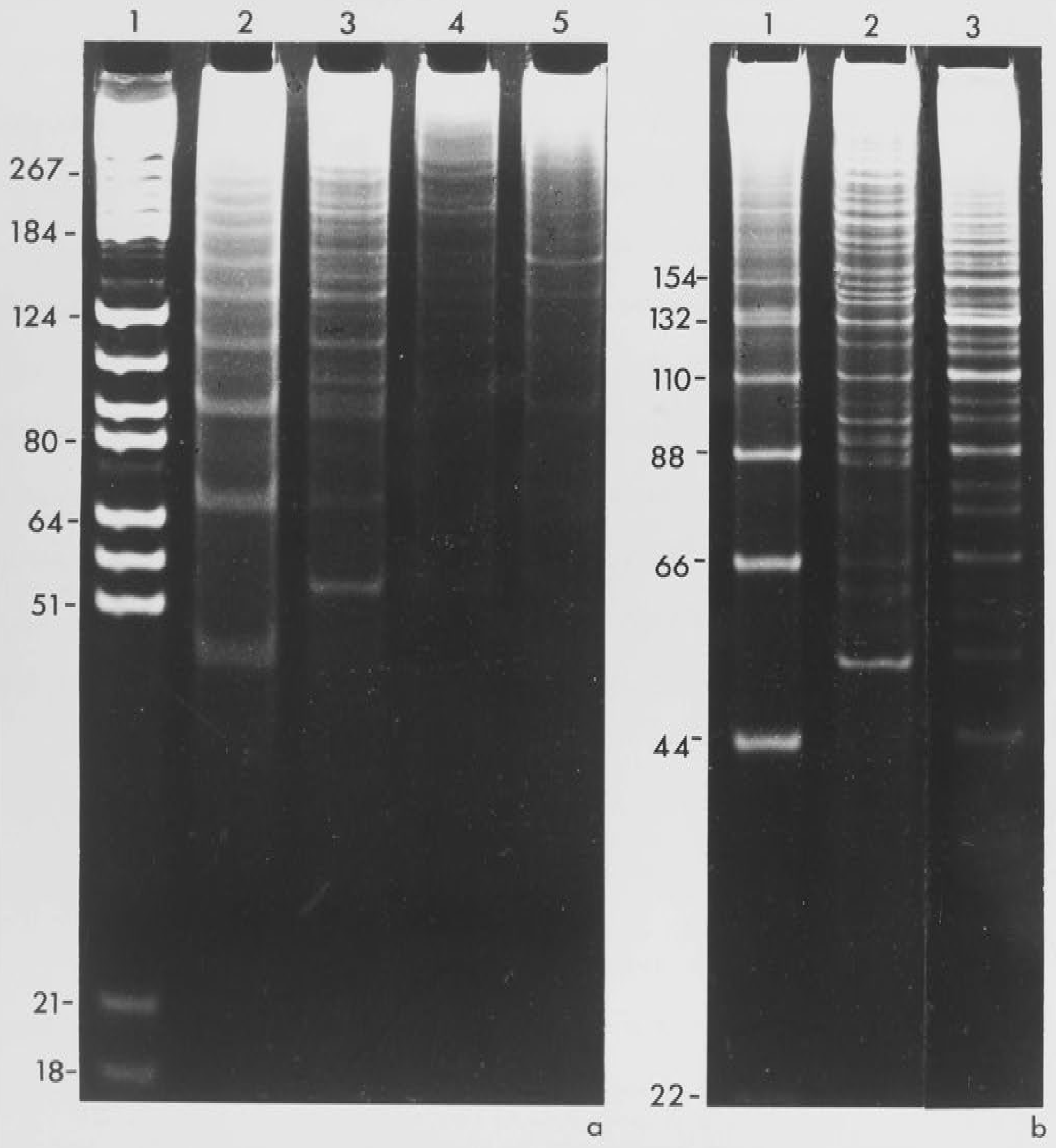
Slot 5 : *N. fuscus* 1.713 satellite DNA-Taq I digest.

(b) Digestion of *Notomys alexis* 1.712 satellite DNA with Taq I restriction endonuclease digests.

Slot 1 : *N. cervinus* 1.713 satellite DNA-Hae III digest coelectrophoresed with the other slots and used as a molecular wight marker.

Slot 2 : *N. alexis* 1.712 satellite DNA-Taq I digest.

Slot 3 : *N. cervinus* 1.713 satellite DNA-Hinf I digest also used as a molecular weight marker. Slot 3 is from the same gel as slots 1 and 2 but it has been cut from the gel photograph and moved adjacent for simplicity of presentation.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267-
184-
124-
80-
64-
51-
21-
18-
a
1
2
3
154-
132-
110-
88-
66-
44-
22-
b

Fig. 6.17). The microdensitometer tracing reveals the presence of these doublets between the members of the major fragments from the trimer through to the decamer fragments. The poor resolution of the tracing prevents accurate length estimates being made. The *N. fuscus* - Taq I digests have a similar pattern to that of *N. cervinus* - Taq I digests; however, the 154 bp fragment is more intense than the members of the multimer series in contrast to the *N. cervinus* digests where the multimer series are even. The increased prominence of the 154 bp fragment suggests that a region of the satellite may exist in which there are repetitive Taq I sites 154 bp apart. *N. mitchellii* - Taq I and *N. alexis* - Taq I digests are indistinguishable from one another but differ from those of *N. cervinus* and *N. fuscus*. In these digests the 22 bp multimer series persists, but several fragments in the series show reduced presence (e.g. trimer, tetramer, septimer and octomer fragments. A 52 bp fragment is prominent in both *N. mitchellii* and *N. alexis* digests and is absent from both *N. cervinus* and *N. fuscus* digests.

6.4 *Discussion*

6.4.1 *Buoyant density analyses*

The similarity of the buoyant densities of the four *Notomys* satellite DNAs suggested that these satellites may have similar sequences. This possibility is supported by the morphological similarity of the species and the relatively recent origin of the genus from the ancestral pseudomyid stock. The genus *Notomys* thus provides an interesting system to investigate the variation in both the content and the distribution of satellites within the genomes. The variation in the satellite DNA content from 6% in *N. fuscus* to 28% in *N. mitchellii*, as estimated from whole DNA density gradients, plus the suggested distributional variation of these satellites within the genomes, as evidenced by the C-band variation within the karyotypes, further suggests that the sequence of these satellites may be conserved.

On the basis of their buoyant density in CsCl the 1.712 satellite DNAs of *N. mitchellii* and *N. alexis* were determined to have a G+C content of 53% while the 1.713 satellites of *N. cervinus* and *N. fuscus* have a G+C content of 54% (Table 6.2). The linear relationship between buoyant density of DNA in CsCl gradients and G+C content (Schildkraut *et al.*, 1962; Mandel *et al.*, 1968) holds for the majority of eukaryote DNAs in the range of 40% to 70% G+C contents (De Ley, 1970), but there are notable exceptions, for example, the HS alpha and beta satellites of the kangaroo rat (Hatch and Mazrimas, 1970). The hermit crab, *Pagurus pollicaris*, has two satellite DNAs (I and II) which have identical densities in CsCl of 1.724 g/cm^3 but have G+C contents of 62% and 51%. (Skinner and Beattie, 1974; Skinner, 1977). Therefore, while G+C content as determined by density values does provide a comparison between DNAs, such comparisons need to be assessed by additional criteria such as Tm or else by direct sequencing. The Tm determined G+C percentage contents for *Notomys* satellites were *N. mitchellii* 51.5%, *N. alexis* 50.5%, *N. cervinus* 50.3% and *N. fuscus* 52.9%. The accuracy of G+C content estimates from density gradients has been given at ± 0.7 to 1.0% by Mandel *et al.* (1968), while that determined from Tm data is ± 0.4 to 1.0% (Mandel and Marmur, 1968). The less stringent criteria would place the G+C contents of *N. mitchellii* and *N. fuscus* within the error of the experimental procedures, while the application of the more stringent criteria indicate that the estimates of G+C content differences for *N. mitchellii*, *N. alexis* and *N. cervinus* represent real differences and *N. fuscus* falls precisely on the limit. These differences could be due to the differing content of methylated bases (methylcytosine and/or methyladenine) in these satellites or the presence of unusual bases (Mandel and Marmur, 1968). The G+C content determination of DNA by either density gradient or Tm measurements of DNA fails to give information on the individual percentage contents of the bases which can be provided by chemical methods.

Where two density bands are formed by satellite DNA in alkaline CsCl gradients, strand differences in G+T content are present so that this method offers a simple probe to discriminate satellite DNAs. A classical example of its use is in the case of the satellite DNAs of the African green monkey as noted earlier. In the rat kangaroo the HS alpha and HS beta satellites each have a density of 1.713 g/cm^3 and while each undergo strand separation in alkaline CsCl gradients they do so to differing extents. The HS alpha satellite forms density bands at 1.709 and 1.796, while the HS beta satellite forms bands at 1.762 and 1.771 (Fry *et al.*, 1973). All four *Notomys* satellites undergo considerable strand separation in alkaline CsCl gradients indicating a strand bias in G+T content. In each case the strand separation is of a similar magnitude. These data obviously rule out gross differences such as those described for the African green monkey or for the kangaroo rat satellites but they do not rigorously exclude the occurrence of minor differences in base composition.

When alkaline CsCl gradients of the *Notomys* satellite DNAs are neutralised and re-run in the analytical ultracentrifuge they form well defined single bands at densities considerably higher than the native satellites. The $\Delta\rho$s observed were as follows: *N. cervinus* 0.013 g/cm^3, *N. mitchellii* 0.016 g/cm^3 and for both *N. alexis* and *N. fuscus* 0.017 g/cm^3. These data suggest sequence heterogeneity in these satellites. A similar conclusion has been arrived at in respect of both mouse and guinea pig satellite (Corneo *et al.*, 1968; Flamm *et al.*, 1969).

When *Notomys* satellites are heat denatured they have densities in neutral CsCl gradients well above those of the native satellites. The $\Delta\rho$s are 0.010 g/cm^3 for *N. cervinus*, 0.014 g/cm^3 for both *N. mitchellii* and *N. alexis* and 0.015 g/cm^3 for *N. fuscus*. When heat denatured *Notomys* satellites are reassociated in 2 x SSC for 5 hours at 65°C and then run in neutral CsCl gradients the densities are closer to those of the native satellites. The Δρs between the native satellites and the heat denatured-renatured satellites were *N. cervinus* 0.004 g/cm^3, *N. mitchellii* 0.008 g/cm^3, *N. alexis* 0.009 g/cm^3 and *N. fuscus* 0.010 g/cm^3. These data support earlier results with mouse (Bond *et al.*, 1967), guinea pig (Flamm *et al.*, 1969) and human satellites (Corneo *et al.*, 1968) which indicated that extended incubation allowed better matching of the base pairs due to the more stringent conditions which produced renatured products closer to native satellite DNA. However the renatured *Notomys* satellites still differ in density from the native satellites indicating considerable heterogeneity.

In each of these experiments the *N. cervinus* satellite displayed less evidence of sequence heterogeneity compared to those of the other three species.

6.4.2 *Thermal characterization*

The derivative thermal denaturation profiles of whole DNA from the four species of *Notomys* clearly indicate the presence of a component which melts at higher temperatures relative to the remainder of the DNA. This is particularly so in the case of *N. mitchellii*, *N. alexis* and *N. cervinus* but it is also true in *N. fuscus*. These components are prominent features of the derivative profile and similar features in other eukaryote DNAs (Mayfield, 1977) have been attributed to the presence of large amounts of repetitive DNA in eukaryotes (Britten and Kohne, 1968). The integral thermal denaturation profiles do reveal the presence of these components, but they are less prominent and do not demonstrate the presence of other maxima which are present in the derivative profiles.

The derivative thermal denaturation profiles of the satellite DNAs of *N. mitchellii*, *N. alexis* and *N. cervinus* isolated by CsCl density gradient fractionation clearly demonstrate that the high melting component in the whole DNA thermal denaturation profiles are due to the presence of these satellite components in the whole DNA. In the derivative thermal denaturation profiles of *Notomys* whole DNAs other distinct maxima occur which are reproducible and are probably due to blocks of DNA with base compositions different from the average base pair composition of the remainder of the genome. Mayfield (1977) attributes these maxima to the presence of blocks of intermediately repetitive DNA. The maxima were not further investigated in this study and so it is not possible to assess the relevance of Mayfield's claim.

6.4.3 *Filter hybridization*

A number of authors have used the filter hybridization technique to assess the phylogenetic relationships of various species groups and also to provide data on the degree of sequence conservation in these groups. These include Macgregor *et al.* (1973) on the salamander genus *Plethodon*, Dunsmuir (1976) on kangaroos and wallabies, Venolia (1977) on kangaroo, wallaroos and wallabies, Lohe (1977) on the sibling species *Drosophila simulans* and *D. melanogaster* and Arnason *et al.* (1978) on the whale genus *Balaenoptera*. In the main all these authors find a good relationship between the degree of hybridization and the phylogenetic relatedness as assessed by other taxonomic criteria. The data from these studies are summarised in Table 6.5.

Macgregor *et al.* (1973) found that the data from filter hybridizations, using ^{3}HcRNA from the 1.728 satellite of *Plethodon cinereus cinereus* as a probe, agreed largely with the taxonomic arrangement of seven species of *Plethodon* by Highton (1972). These authors differ from Highton only in the placing of *P. nettingi shenandoah*. Highton (1972) considers *P. n. nettingi*, *P. n. hubrichti* and *P. n. shenandoah* as subspecies of *P. nettingi* which belongs to a species group including *P. cinereus*, *P*, *richmondi* and *P. hoffmani* with *P. dorsalis* in a more distally related species group (Fig. 6.18a). Macgregor *et al.* (1973) state that

Table 6.5: Representative studies involving filter hybridization of ^{3}HcRNA from satellite DNAs to probe the relatedness of satellite DNAs from closely related species in different taxa

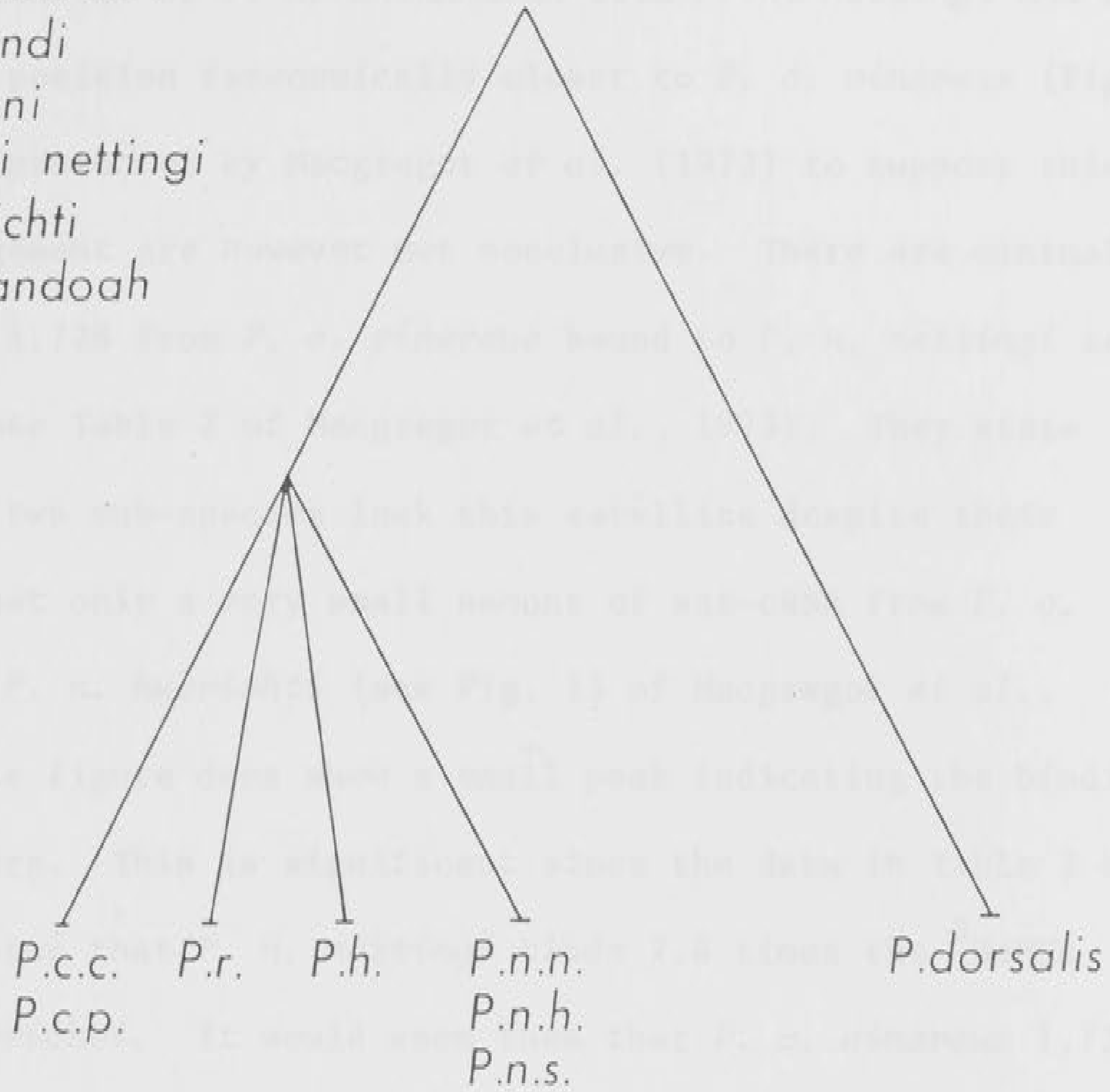
Genus	Species	Hybrid type	Probe ^{3}HcRNA	Tm (°C)	ΔTm (°C)	% mismatch	Reference
Drosophila	*D. simulans*	Homologous	1.672	32.3	0	0	Lohe (1977)
	D. melanogaster	Heterologous		32.2	-0.1	0	
	D. simulas	Homologous	1.707	61.3	0	0	
	D. melanogaster	Heterologous		61.5	0.2	0	
	D. simulans	Homologous	1.695	43.5	0	0	
	D. melanogaster	Heterologous		43.3	-0.2	0	
	D. simulans	Homologous	1.696	44.0	0	0	
	D. melanogaster	Heterologous		44.5	0.5	0	
Whales	*B. physalus* (fin whale)	Homologous	1.711	62.5	0	0	Arnason *et al.* (1978)
Balaenoptera	*B. acutorostrate* (minke)	Heterologous		64.1	1.6	1.0	
	B. borealis (sei whale)	Heterologous		65.7	3.2	2.0	
Balaenoptera	*B. acutorostrata*	Homologous	1.702	58.3	0	0	Arnason *et al.*(1978)
	B. physalus	Hetero'ogous		60.0	1.7	1.0	
Salamanders	*P. cinereus cinereus*	Homologous	1.728	76.5	0	0	Macgregor *et al.* (1973)
Plethodon	*P. c. polycentratus*	Heterologous		78.0	1.5	1.0	
	P. richmondi	Heterologous		73.5	-3.0	2.0	
	P. hoffmani	Heterologous		76.0	-0.5	0	
	P. nettingi shenandoah	Heterologous		72.3	-4.2	2.6	
	P. nettingi	Heterologous		*			*data not given
	P. nettingi hubrichti	Heterologous		*			
	P. dorsalis	Heterologous		*			
Kangaroos	*Macropus rufogriseus*	Homologous	1.708	55.0	0	0	Dunsmuir (1977)
Macropus	*M. giganteus*	Heterologous		50.0	-5.0	3.5	
Megaleia and	*Megaleia rufa*	Heterologous		49.5	-4.5	3.0	
Wallabia	*Wallabia bicolor*	Heterologous		49.0	-6.0	4.0	
Macropus	*Macropus rufogriseus*	Homologous	1.698	57.5	0	0	Dunsmuir (1976)
Megaleia	*Megaleia rufa*	Heterologous		57.0	-0.5	0	
Macropus,	*Macropus macropus robustus*	Homologous	1.710	52.5	0	0	Venolia (1977)
Megaleia and	*M. m. erubescens*	Heterologous		53.0	0.5	0	
Wallabia	*M. antilopinus*	Heterologous		52.5	0	0	
	M. rufogriseus	Heterologous		49.1	-3.4	2.1	
	M. fuliginous	Heterologous		48.2	-4.3	2.7	
	M. giganteus	Heterologous		49.1	-3.4	2.1	
	M. eugenii	Heterologous		47.3	-5.2	3.3	
	Megaleia rufa	Heterologous		48.4	-4.1	2.6	
	Wallabia bicolor	Heterologous		49.1	-3.4	2.1	

Figure 6.18 : a. Phyogenetic tree based on morphological and geographic data from Highton (1972).

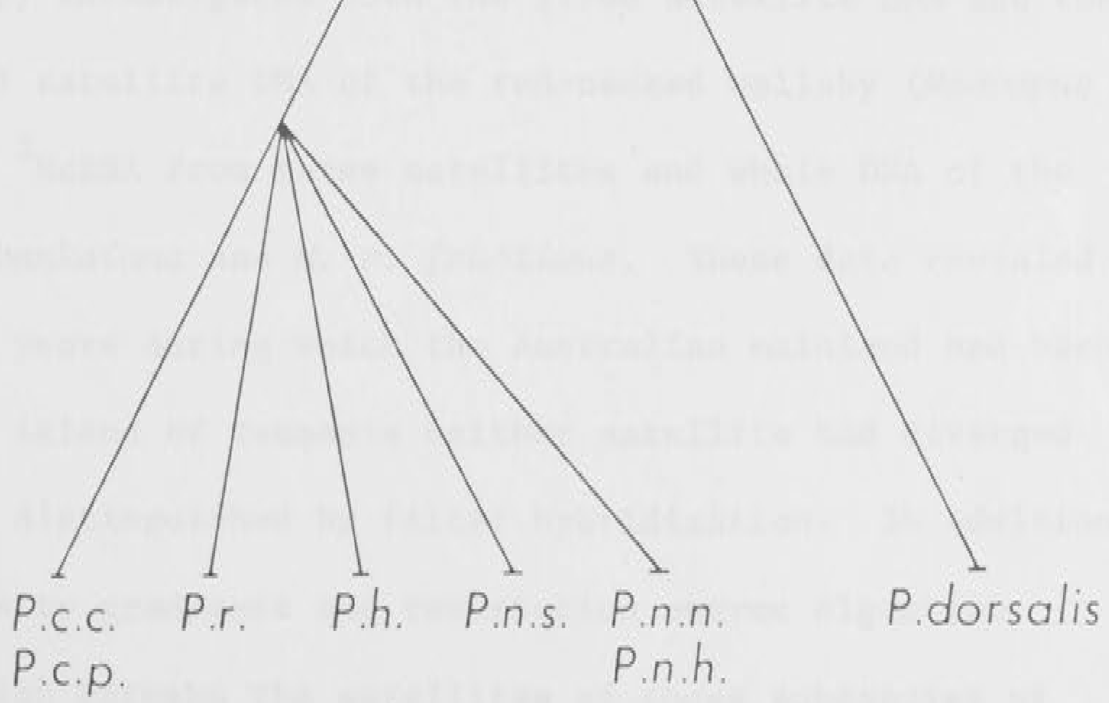
b. Phylogenetic tree based on filter hybridization data from Macgregor *et al.* (1973).

P.c.c. = Plethodon cinereus cinereus
P.c.p. = P.c.polycentratus
P.r. = P.richmondi
P.h. = P.hoffmani
P.n.n. = P.nettingi nettingi
P.n.h. = P.n.hubrichti
P.n.s. = P.n.shenandoah

a.


P.c.c.
P.c.p.
P.r.
P.h.
P.n.n.
P.n.h.
P.n.s.
P.dorsalis

b.

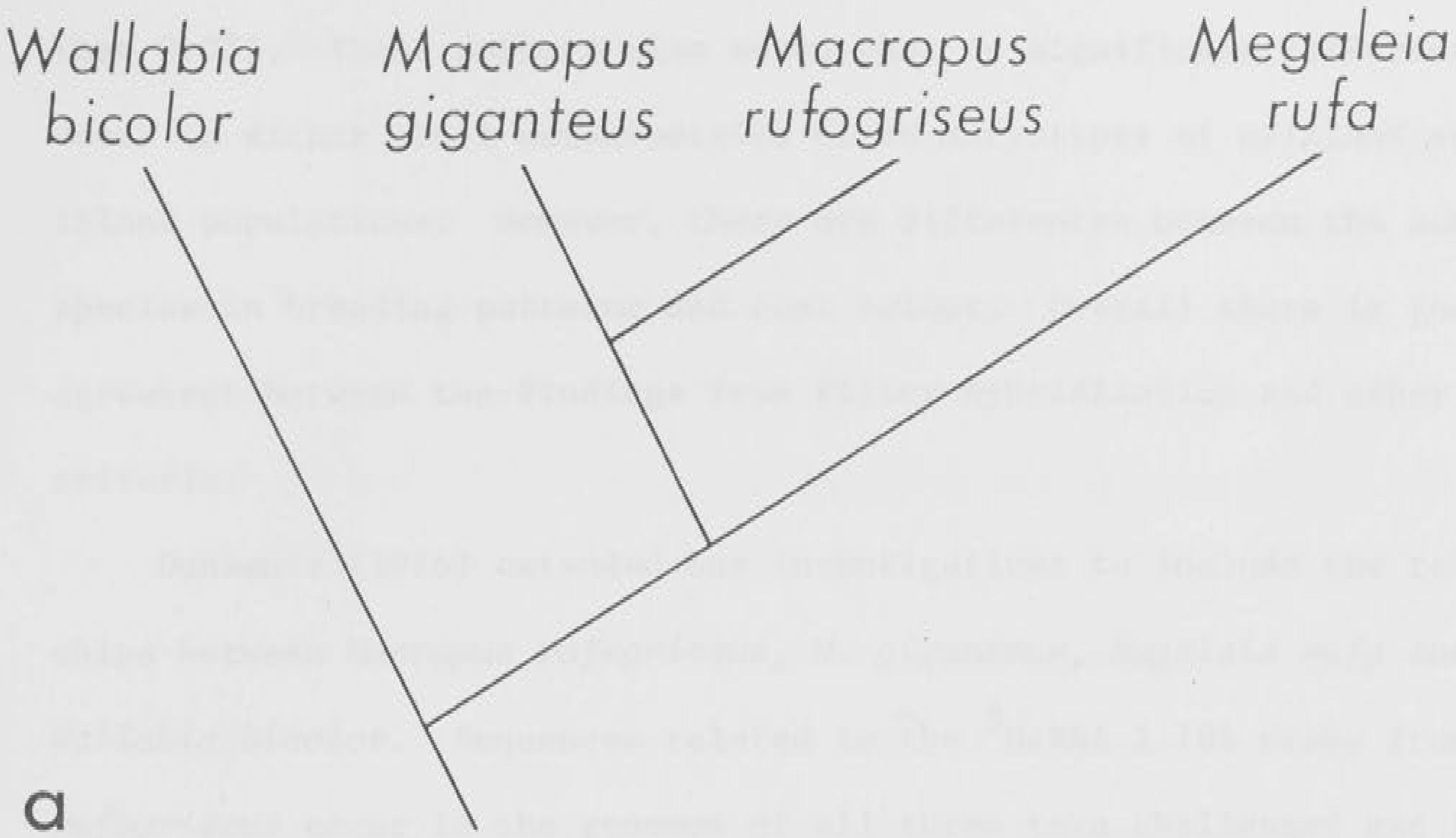

P.c.c.
P.c.p.
P.r.
P.h.
P.n.s.
P.n.n.
P.n.h.
P.dorsalis

there are *P. c. cinereus* related sequences present in the genome of *P. n. shenandoah* but not in *P. n. nettingi* or *P. n. hubrichti*. Consequently they suggest the removal of *P. n. shenandoah* from *P. n. nettingi* and *P. n. hubrichti* to a position taxonomically closer to *P. c. cinereus* (Fig. 6.18b). The data presented by Macgregor *et al.* (1973) to support this taxonomic rearrangement are however not conclusive. There are minimal amounts of ^{3}HcRNA 1.728 from *P. c. cinereus* bound to *P. n. nettingi* and *P. n. hubrichti* (see Table 2 of Macgregor *et al.*, 1973). They state that these latter two sub-species lack this satellite despite their earlier comment that only a very small amount of sat-cRNA from *P. c. cinereus* binds to *P. n. hubrichti* (see Fig. 13 of Macgregor *et al.*, 1973). Indeed this figure does show a small peak indicating the binding noted by the authors. This is significant since the data in Table 2 of their paper indicates that *P. n. nettingi* binds 2.8 times the ^{3}HcRNA bound by *P. n. hubrichti*. It would seem then that *P. c. cinereus* 1.728 satellite sequences are present in both *P. n. nettingi* and *P. n. hubrichti* as well as in *P. n. shenandoah* in low copy number. Thus it is possible that the phylogenetic placing of these eight Plethodon species by Highton (1972) is in even closer agreement than Macgregor *et al.* (1973) suggest.

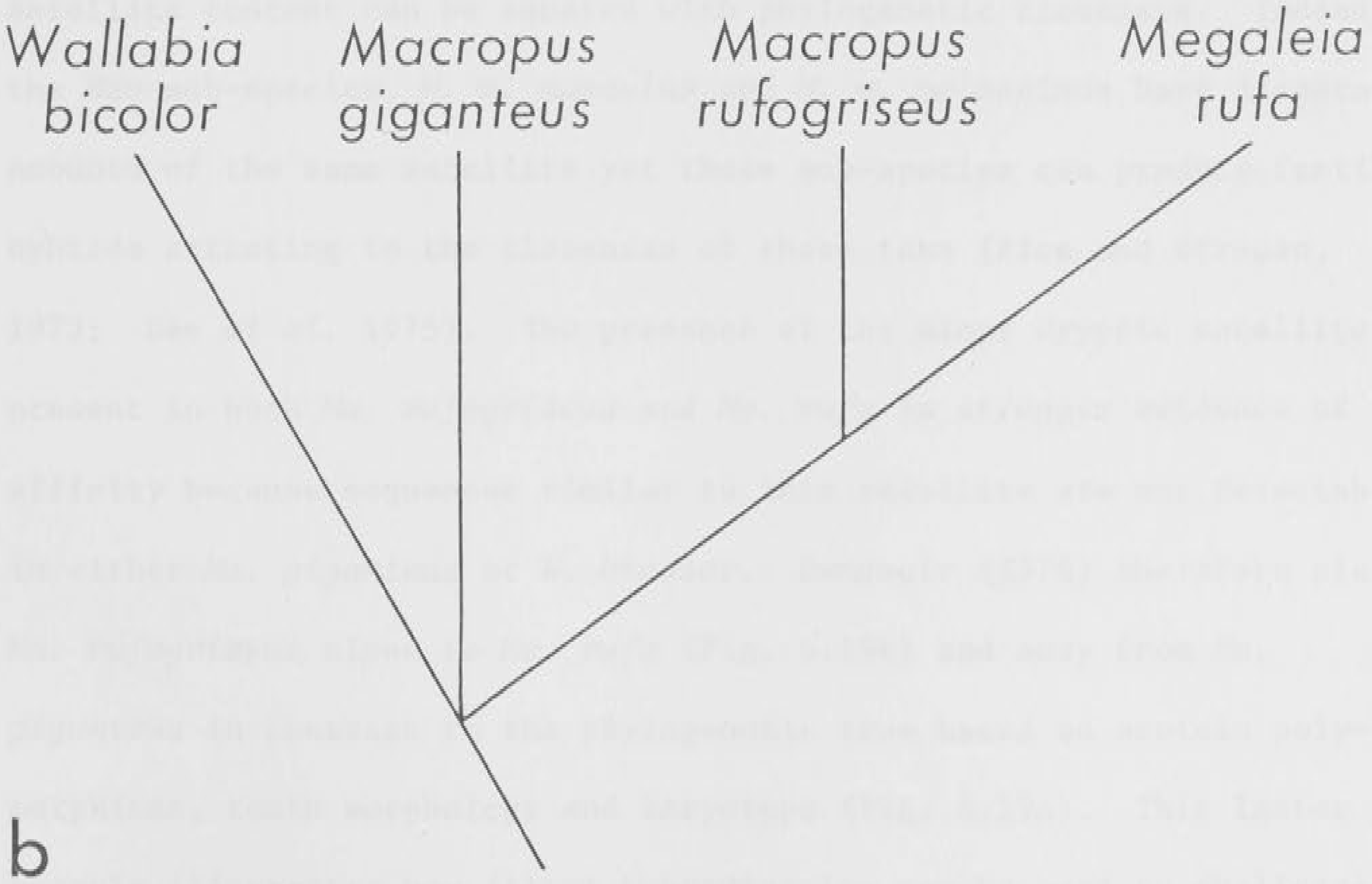
Dunsmuir (1976) investigated both the 1.708 satellite DNA and the minor cryptic 1.698 satellite DNA of the red-necked wallaby (*Macropus rufogriseus*) using ^{3}HcRNA from these satellites and whole DNA of the sub-species *M. r. banksiana* and *M. r. fructicus*. These data revealed that in the 10,000 years during which the Australian mainland had been separated from the island of Tasmania neither satellite had diverged sufficiently to be distinguished by filter hybridization. In addition, analyses using density gradients and restriction enzyme digestion failed to distinguish between the satellites of these subspecies of

Figure 6.19 : a. Phylogeny of some macropod species based on protein polymorphisms, tooth morphology and chromosome morphology.

b. Phylogeny based on satellite DNA sequences (after Dunsmuir, 1976).


Wallabia bicolor
Macropus giganteus
Macropus rufogriseus
Megaleia rufa

a


Wallabia bicolor
Macropus giganteus
Macropus rufogriseus
Megaleia rufa

b

M. rufogriseus. Further evidence from protein polymorphisms provided by Johnson and Sharman (1979) indicate a lack of variation between mainland and island populations and a coefficient of genetic similarity greater than 0.925. These authors also noted that no significant differences occur in either skull morphometrics or in karyotypes of mainland and island populations. However, there are differences between the subspecies in breeding patterns and coat colour. Overall there is good agreement between the findings from filter hybridization and other criteria.

Dunsmuir (1976) extended her investigations to include the relationships between *Macropus rufogriseus, M. giganteus, Megaleia rufa* and *Wallabia bicolor*. Sequences related to the ^{3}HcRNA 1.708 probe from *Ma. rufogriseus* occur in the genomes of all three taxa challenged and the sequence divergence in all cases is around 3%. Dunsmuir (1976) stresses that *Me. rufa* has more 1.708 satellites than either *Ma. giganteus* or *W. bicolor* but it is questionable whether quantitative similarities in satellite content can be equated with phylogenetic closeness. Indeed the *Mus* sub-species, *M. m. musculus* and *M. m. molossinus* have disparate amounts of the same satellite yet these sub-species can produce fertile hybrids attesting to the closeness of these taxa (Rice and Strauss, 1973; Dev *et al.* 1975). The presence of the minor cryptic satellite present in both *Ma. rufogriseus* and *Me. rufa* is stronger evidence of affinity because sequences similar to this satellite are not detectable in either *Ma. giganteus* or *W. bicolor*. Dunsmuir (1976) therefore places *Ma. rufogriseus* close to *Me. rufa* (Fig. 6.19b) and away from *Ma. giganteus* in contrast to the phylogenetic tree based on protein polymorphisms, tooth morphology and karyotype (Fig. 6.19a). This latter example illustrates how filter hybridization may be used to challenge phyllogenies based on other criteria. In addition Dunsmuir's study highlights the increased resolution afforded by filter hybridization in the detection of the 1.708 satellite DNA in *Me. rufa, Ma. giganteus* and

W. bicolor which CsCl density gradients failed to identify in these genomes.

Venolia (1977) investigated the satellite DNA relationship between the wallaroos (*Macropus robustus robustus, M. r. erubencens* and *M. antilopinus*) and five other species of *Macropus* and *Megaleia rufa*. The three wallaroos are taxonomically closer than the other species and Venolia's data from filter hybridization support this situation. The wallaroos in homologous and heterologous filter hybrids have almost identical Tms but differ from the remaining six species by Tms of 3.4°C to 5.2°C which indicates a sequence divergence of 2.1-3.3% (Table 6.5). The wallaroos display a high degree of sequence conservation with a lesser degree of conservation in the other *Macropus* species and *Megaleia rufa*. Apart from the taxonomic closeness of the wallaroo group a shared chromosomal polymorphism, which involves the absence of C banding material in chromosome 5, has been found in each member of the trio but is absent from the remaining taxa. These data support the findings from filter hybridization.

Homologous and heterologous hybrids involving the 1.672, 1.695, 1.696 and 1.707 satellite DNAs of the sibling species of *Drosophila simulans* and *D. melanogaster* reveal a nucleotide difference of 1 bp in 200 (Lohe, 1977) and this supports the closeness of these species taxonomically and also the conservation of these sequences in each species.

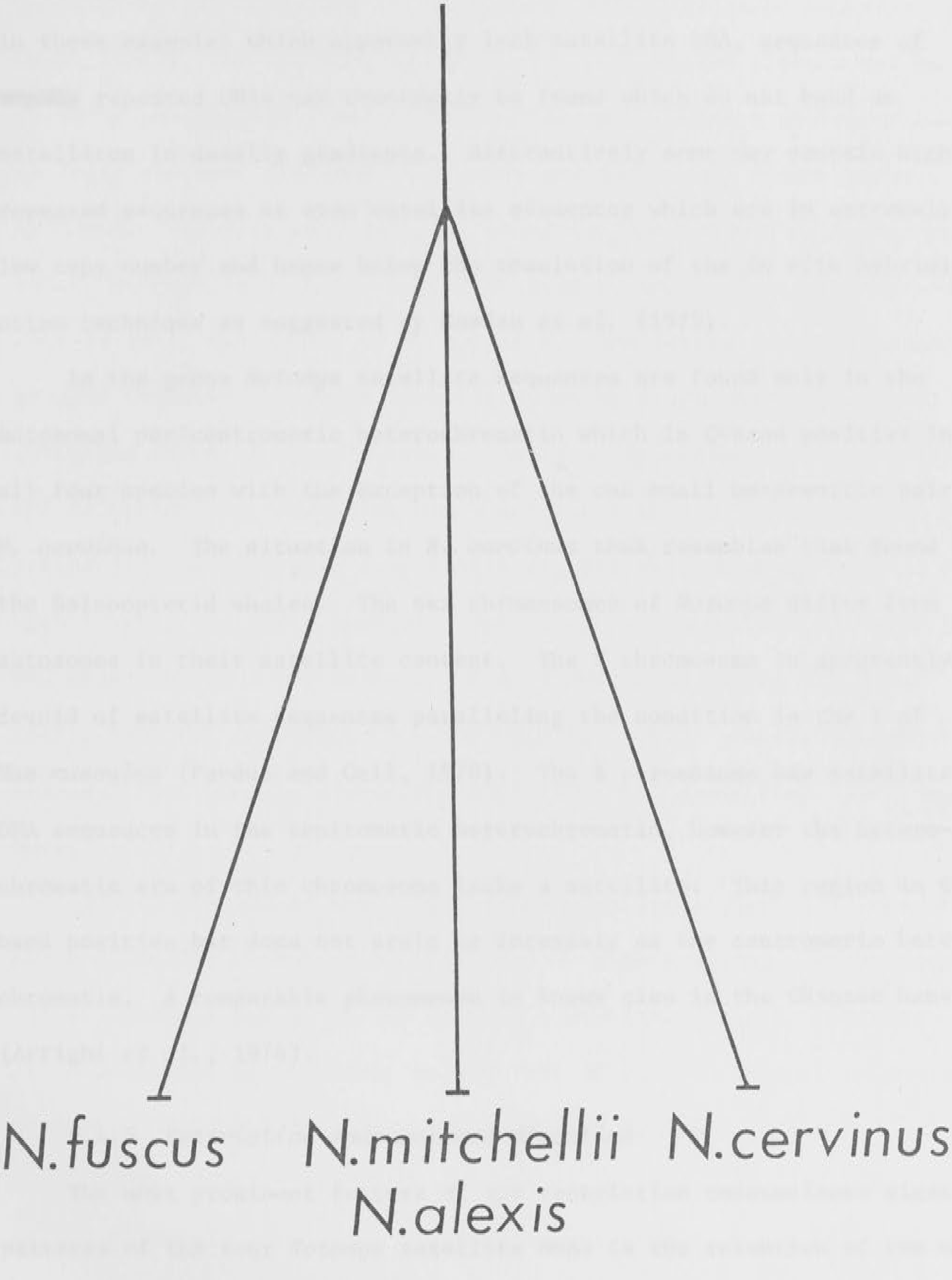
In the whale genus *Balaenoptera* Arnason *et al.* (1978) state that the melting curves of both homologous and heterologous hybrids between the fin whale ^{3}HcRNA 1.711 satellite DNA probe in fin, sei and in minke whale DNA are 'virtually identical'. Whereas the data provided indicates ΔTms of 2.6 C for the homologous fin/fin hybrid compared to the heterolgous fin/sei hybrid and 1.3°C for the fin/fin hybrid compared with the fin/minke hybrid (see Fig. 4 of Arnason *et al.*, 1978). The ΔTms represent 1.7% and 1% sequence divergence respectively. Similary ^{3}HcRNA 1.702 minke whale probe used with minke whale DNA and also with fin whale DNA gave a ΔTm of 1.3°C with a sequence divergence of 1%. The data therefore suggest some divergence rather than the complete conservation claimed by the authors.

In the genus *Notomys* the divergence of satellite DNA sequences as indicated by filter hybridization is within the limits established for the above genera. The closeness of *N. mitchellii* and *N. alexis* on both morphological and karyological grounds is supported by the filter hybridization data, as indeed, are the separations of *N. cervinus* and *N. fuscus* from one another and from both *N. mitchellii* and *N. alexis.* Figure 6.20 represents the phylogenetic tree constructed on the basis of data from the filter hybridizations.

6.4.4 In situ *hybridization*

In many species satellite DNA is located within those regions which stain positively with the C-banding technique (Pardue and Gall, 1970; Jones, 1970; Arrighi and Hsu, 1971). There are a few species, however which have C-banded regions which apparently lack satellite DNA sequences. Indeed the initial organism used to detect the cytological location of satellite DNA within the genome, *Mus musculus*, apparently lacks satellite DNA in its Y chromosome which however is C-band positive (Pardue and Gall, 1970). In the Chinese hamster the X chromosome has C-banding material in the long arm which lacks repetitive DNA and here too the Y chromosome also lacks repititive DNA (Arrighi *et al.*, 1974) although Kurnit (1979) considers that this latter case is in need of re-investigation in view of the discovery of repetitive restriction fragments in the Chinese hamster genome which may represent a cryptic satellite (Wolgemuth *et al.*, 1978). Nevertheless, it has not been demonstrated that this cryptic satellite is present in the sex chromosome pair. In the human karyotype six chromosomes apparently lack any of the four human satellites despite having considerable C-banding material (Gosden *et al.*, 1975; reviewed in Miklos and John, 1979). The Balenopterid

Figure 6.20 : Phylogenetic tree for the four species of *Notomys* based on filter hybridizations.


N.fuscus
N.mitchellii
N.alexis
N.cervinus

whales, apart from having two satellite DNAs which occupy two distinctly different cytological sites have a number of C-banded centromeric regions which apparently lack satellite DNAs (Arnason *et al.*, 1978). Of course in these examples which apparently lack satellite DNA, sequences of [redacted] repeated DNAs may eventually be found which do not band as satellites in density gradients. Alterantively some may contain highly repeated sequences or even satellite sequences which are in extremely low copy number and hence below the resolution of the *in situ* hybridization technique as suggested by Gosden *et al.* (1975).

In the genus *Notomys* satellite sequences are found only in the autosomal pericentromeric heterochromatin which is C-band positive in all four species with the exception of the one small metacentric pair in *N. cervinus*. The situation in *N. cervinus* thus resembles that found in the Balenopterid whales. The sex chromosomes of *Notomys* differ from the autosomes in their satellite content. The Y chromosome is apparently devoid of satellite sequences paralleling the condition in the Y of *Mus musculus* (Pardue and Gall, 1970). The X chromosome has satellite DNA sequences in the centromeric heterochromatin, however the heterochromatic arm of this chromosome lacks a satellite. This region is C-band positive but does not stain as intensely as the centromeric heterochromatin. A comparable phenomenon is known also in the Chinese hamster (Arrighi *et al.*, 1974).

6.4.5 *Restriction endonuclease digestion*

The most prominent feature of the restriction endonuclease digestion patterns of the four *Notomys* satellite DNAs is the retention of the multimer series based on 22 base pairs. There are, however, alterations to this pattern both in the digests of a single enzyme on an individual satellite and also in the patterns obtained using a single enzyme across the four satellites. Indeed, it is the variation in pattern that allows the use of restriction enzyme digestion to arrange the four species into a phylogenetic tree.

The pattern of the digests obtained in *Notomys* is complex and, despite the limited approach adopted in this study, offers information in both the phylogeny and the structural organization of the satellites. Cooke (1975), for example, has shown how restriction digests can be used to infer phylogenetic relationships. He constructed a phylogeny based on EcoR1 and Hind III digests of five species of the rodent genus *Apodemus*. The resultant grouping shows *A. flavicollis, A. sylvaticus* and *A. microps* to be more closely related to one another than either of them is to *A. mystacinus* or *A. agrarius*. The latter two species are grouped together. Further investigations by Brown and Dover (1979) involving three of these species, namely *A. sylvaticus, A. flavicollis* and *A. mystacinus*, and using Alu I and Taq I in single digests and Taq I/Hind III and Taq I/Alu I in double digests confirmed the work of Cooke with respect to these species. The close correspondence of satellite DNA sequences in *A. sylvaticus* and *A. flavicollis* (Brown and Dover, 1979), however, contrasts with the relatively high genetic distances assessed from protein electrophoresis (Benmehdi pers. comm. in Brown and Dover, 1979).

Hinf I digests of the four *Notomys* satellites gives evidence of a clear dichotomy with *N. mitchellii* and *N. alexis* having similar patterns and *N. cervinus* and *N. fuscus* sharing a distinct pattern. Further differentiation is indicated because the 30 bp in *N. alexis* fragment is much more prominent than in *N. mitchellii*. *N. mitchellii* and *N. alexis* share a very similar digestion pattern in the range of 22 to 220 bp and, it is suggested, arose from a common ancestor which would have had a pattern common to both species. Whether the prominent 30 bp fragment in *N. alexis* was also present in the ancestral genome is not clear. If it was then one would have to conclude that it has been lost in *N. mitchellii, N. cervinus* and *N. fuscus*. This would have had to involve two separate deletions since *N. alexis* and *N. mitchellii* share a common ancestor. On the other hand a single amplification in *N. alexis* involves only one step. The 30 bp fragments in *N. mitchellii*, *N. cervinus* and *N. fuscus* are of equal intensity with their 36 bp doublet partners and therefore provide no evidence for alteration of the kind provided by the 30 bp fragment in *N. alexis*. The major differences in Hinf I digestion patterns, which separate *N. cervinus* and *N. fuscus* on one hand from *N. mitchellii* and *N. alexis* on the other, exists in the range between the hexamer and the nonamer. This difference, plus the identical patterns of *N. cervinus* and *N. fuscus*, suggests that these species shared a common ancestor different from that of *N. mitchellii* and *N. alexis*. The phylogeny based on these data would require a stem ancestor giving rise to two species. One of these in turn produced *N. mitchellii* and *N. alexis* while the other was the common ancestor of *N. cervinus* and *N. fuscus*. Figure 6.21a depicts this arrangement from the Hinf I digestion data.

Hae III digest fail to distinguish between *N. mitchellii* and *N. alexis* which have more complex patterns than either *N. fuscus* or *N. cervinus*. *N. cervinus* has the more simple pattern with very little alteration to the multimer series. The Hae III patterns suggest three groups in which *N. mitchellii* and *N. alexis* are close together with *N. cervinus* and *N. fuscus* each separate. These data suggest a greater difference between *N. cervinus* and *N. fuscus* than was indicated by either Hinf I or Bam HI digests. Figure 6.21b depicts the phylogeny based on Hae III digest data in the monomer to decamer range of fragment sizes.

The most prominent feature of the Bam HI digests is the complete multimer series from monomer to decamer in all four species. Both *N. mitchellii* and *N. alexis* Bam HI digests have a 63 bp fragment which is more prominent than the 66 bp or triple fragment. Neither *N. cervinus* or *N. fuscus* digest have this feature so that here also a division can be made. Further a minor fragment of 214 bp distinguishes *N. alexis*

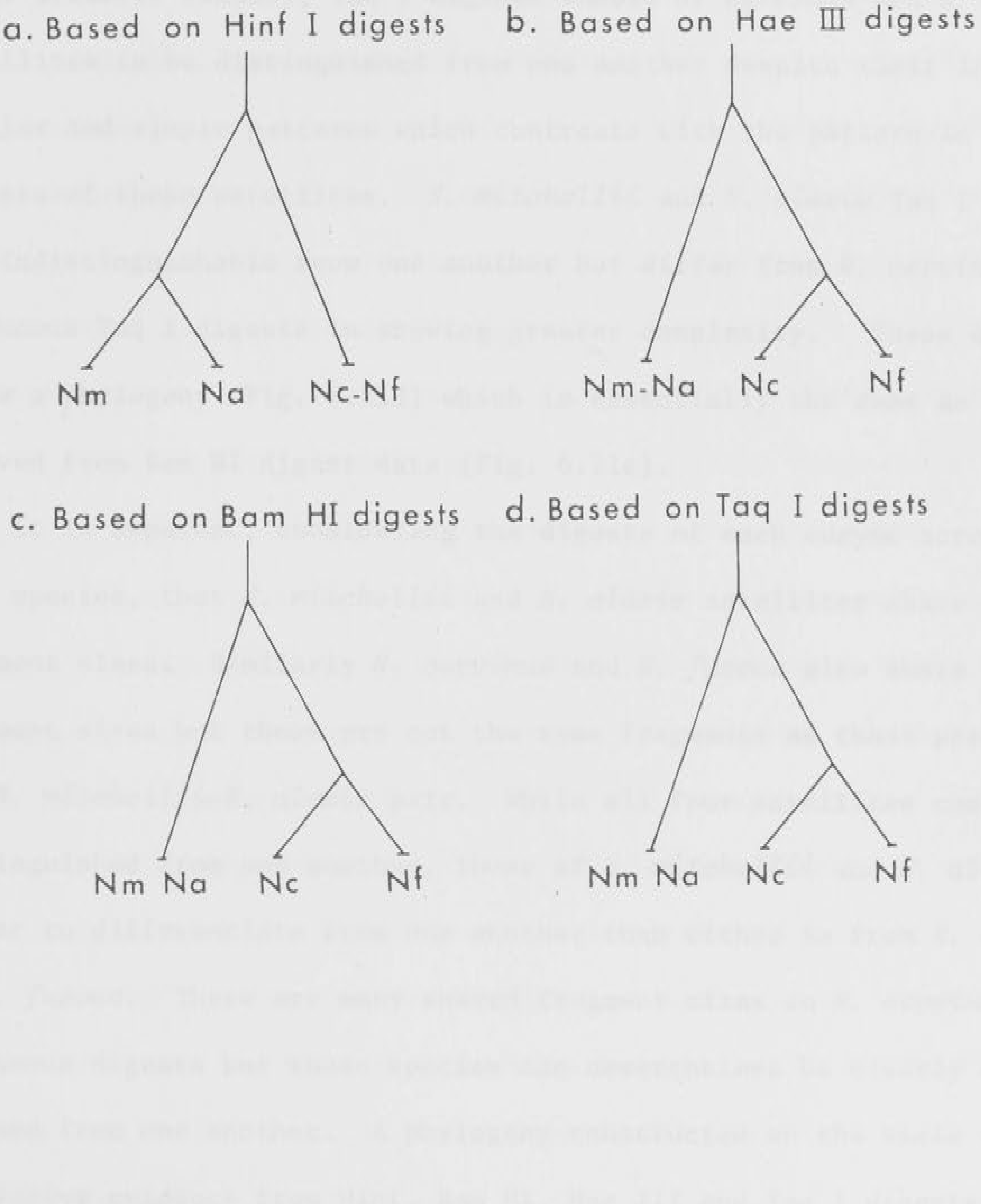
Figure 6.21 : Phylogenetic trees for *Notomys mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus* based on the similarities of restriction enzyme digest considered separately.

a. Based on Hinf I digests.

b. Based on Hae III digests.

c. Based on Bam Hi digests.

d. Based on Taq I digests.



a. Based on Hinf I digests

b. Based on Hae III digests

c. Based on Bam HI digests

d. Based on Taq I digests

Nm
Na
Nc-Nf
Nm-Na
Nc
Nf
Nm Na
Nc
Nf
Nm Na
Nc
Nf

from *N. mitchellii*, while a series of faint bands midway between the major bands of the multimer series in *N. cervinus* distinguishes between the *N. cervinus* and *N. fuscus* digests. These characteristics suggest a phylogeny similar to that derived from Hinf I digests but with the additional property of subdividing the *N. cervinus*-*N. fuscus* coupling (Fig. 6.21c). Finally, Taq I digests enable *N. cervinus* and *N. fuscus* satellites to be distinguished from one another despite their largely similar and simple patterns which contrasts with the pattern in Hae III digests of these satellites. *N. mitchellii* and *N. alexis* Taq I digests are indistinguishable from one another but differ from *N. cervinus* and *N. fuscus* Taq I digests in showing greater complexity. These data allow a phylogeny (Fig. 6.21d) which is essentially the same as that derived from Bam HI digest data (Fig. 6.21c).

It is apparent, considering the digests of each enzyme across the four species, that *N. mitchellii* and *N. alexis* satellites share many fragment sizes. Similarly *N. cervinus* and *N. fuscus* also share many fragment sizes but these are not the same fragments as those present in the *N. mitchellii*-*N. alexis* pair. While all four satellites can be distinguished from one another, those of *N. mitchellii* and *N. alexis* are harder to differentiate from one another than either is from *N. cervinus* or *N. fuscus*. There are many shared fragment sizes in *N. cervinus* and *N. fuscus* digests but these species can nevertheless be clearly distinguished from one another. A phylogeny constructed on the basis of the cumulative evidence from Hinf, Bam HI, Hae III and Taq I digests of the satellite DNAs of the four *Notomys* species (Fig. 6.22) gives a similar relationship to those suggested on morphological (Tate, 1951) or karyological grounds.

It is also possible to gain some indication of the structural organization of the satellite DNAs in *Notomys* from the restriction data although precise determinations require more extensive analyses than were attempted in this study. The persistence, to varying degrees, of the major multimer series in all digests suggests that restriction enzyme sites existed 22 bp apart in the ancestral satellite. Of course the ancestral satellite with its highly repetitive nature would also have sequence spots which are one base removed from being an active site for a specific enzyme. These sequence spots would be repeated many times in the satellite at regular intervals and mutation could convert these sequence spots into an active site. This explanation emphasises the production of new sites rather than the elimination of existing sites. For the purposes of simplicity I have concentrated on the latter. Obviously both the creation and elimination of sites takes place over a period of time. As only single enzyme digests were used in this study it is not possible to assess the spatial relationshp of Hinf I, Bam HI, Hae III and Taq I sites within a given satellite.

Further investigations using combinations of these enzymes are necessary to provide data bearing on this problem. Such additional data should also reveal the steps involved in the evolution of these satellites. Prominent fragments such as the hexamer in Hinf digests need to be investigated to determine their source within the satellite, because at present, it is not known if they arise from sites interspersed within the DNA involved in the remainder of the multimer series. Similar problems are posed by the 30 bp fragment in *N. alexis* Hinf I digests and the 63 bp fragments in both *N. mitchellii* and *N. alexis* Bam HI digests.

The complex multimer and doublet series obtained in Hinf I digests of *Notomys* places some constraints on the sequence composition of the basic 22 bp unit. In order to produce the regular pattern observed some lengths of the satellite will have Hinf I sites arranged as follows:

```
      ▼             ▼       ▼
5' ..GANTC?????????GANTC???GANTC .. 3'

3' ..CTNAG?????????CTNAG???CTNAG .. 5'
         ▲             ▲       ▲
```

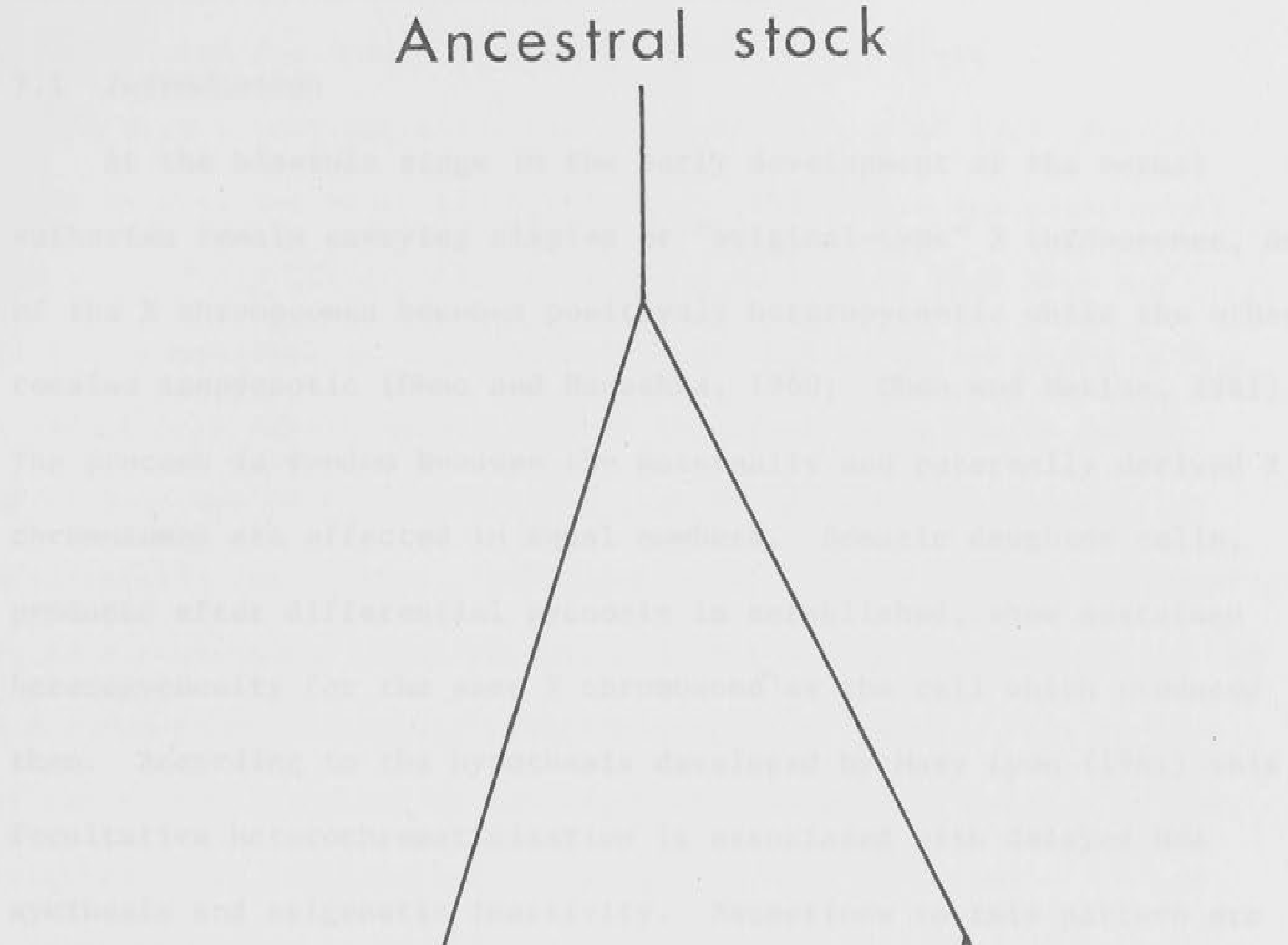
Similarly *N. cervinus*-Bam HI digests which show evidence of ½-mer fragments in addition to the multimer series, suggests lengths of satellite DNA with the following arrangement of Bam HI sites:

```
      ▼          ▼          ▼
5' ..GGATCC?????GGATCC?????GGATCC .. 3'

3' ..CCTAGG?????CCTAGG?????CCTAGG .. 5'
          ▲          ▲          ▲
```

Obviously for the production of the complex but ordered patterns observed considerable sequence change is required as the full series of multimers demands DNA of various lengths each in multiples of 22 bp. However despite these sequence changes the short order periodicity is maintained relatively well. This is also the case in *Apodemus* (Cooke, 975), athough *Notomys* unquestionably presents a more complex system.

Figure 6.22 : Phylogeny for *N. mitchellii*, *N. alexis*, *N. cervinus* and *N. fuscus* based on similarities between digests of satellite DNAs with Hinf I, Bam HI, Hae III and Taq I restriction endonucleases considered overall.


Ancestral stock
N.mitchellii
N.alexis
N.cervinus
N.fuscus

CHAPTER 7

LATE REPLICATION OF DNA IN *NOTOMYS ALEXIS*

7.1 *Introduction*

At the blastula stage in the early development of the normal eutherian female carrying simplex or "original-type" X chromosomes, one of the X chromosomes becomes positively heteropycnotic while the other remains isopycnotic (Ohno and Hauschka, 1960; Ohno and Makina, 1961). The process is random because the maternally and paternally derived X chromosomes are affected in equal numbers. Somatic daughter cells, produced after differential pycnosis is established, show sustained heteropycnosity for the same X chromosome as the cell which produced them. According to the hypothesis developed by Mary Lyon (1961) this facultative heterochromatinization is associated with delayed DNA synthesis and epigenetic inactivity. Exceptions to this pattern are found in females of the Indian house shrew *Suncus murinus* in which the facultative X chromosome completes replication prior to both its homologue and the autosomes (Rao *et al.*, 1970). Thus it may be more appropriate to consider the replication pattern of the facultative heterochromatic X as being asynchronous with respect to the bulk of the euchromatin (Comings, 1972; Gartler and Andina, 1976).

The Lyon hypothesis can, of course, be tested only under circumstances where the two X chromosomes are individually and consistently able to be distinguished from one another. Two such situations have been employed. First, cases where one of the X chromosomes is structurally abnormal. Second, cases involving hybrids between forms with different X chromosomes. The results from both these test situations have been equivocal. In mice, for example, it is possible to synthesize females carrying one normal X chromosome and a translocated X-autosome. Two such translocations have been used - Cattanach's, which is probably a non-reciprocal translocation (Evans *et al.*, 1965) and Searle's, which is a conventional reciprocal translocation (Lyon *et al.*, 1964). While Cattanach's translocation gives a random inactivation pattern that of Searle is clearly non-random with the normal X being preferentially affected.

The second test situation has involved the use of inter-specific hybrids between the horse and the donkey. These have sex chromosomes which are clearly differentiated from one another so that they are easily distinguished in the hybrid female. Mukherjee and Sinha (1964) claimed to have demonstrated random X inactivation in female mules whereas Hamerton *et al.* (1969) find that the donkey X chromosome is preferentially inactivated. Mukherjee *et al.* (1970) explain the discrepancy as a consequence of differential selection and distribution of the two X chromosomes during differentiation of the tissue. This implies that the results obtained do not represent the pattern earlier in the development when inactivation is assumed to have been random. A more pertinent criticism is that it is invalid to use inter-specific hybrids to test the Lyon hypothesis because other factors may play a role in determining the pattern of inactivation (Hamerton and Gianelli, 1970).

Notomys alexis offers yet a further possibility for testing the Lyon hypothesis since here there are three distinct X chromosomes differentiated by the presence of additional material. There are however three complications:

(i) There are no known gene markers which can be used in this case to test directly for inactivation.

(ii) Additional material of an unconventional kind compared with the "original-type" X situation is present in some *Notomys* X chromosomes. Unconventional material is also known in a number of rodents and ungulates and Schmid (1967) has termed it "accessory gonosomal heterochromatin" (Table 7.1). He has shown that in both *Microtus agrestis* and *Mesocricetus auratus* this additional heterochromatin replicates later than the faculta-

Table 7.1 : Comparison of constitutive, facultative and accessory gonosomal heterochromatin.

	Constitutive heterochromatin	Facultative heterochromatin	Accessory gonosomal heterochromatin
Location	On both homologues	On only one homologue or one set of homologues	On both homologues of multiplex-type X chromosomes
C-banding	+	-	C-bands of intermediate intensity
Time of DNA replication relative to euchromatin	Asynchronous usually late	Asynchronous usually late	Asynchronous usually late
Genetic state	Inactive	Inactive	Inactive?
Gene content	Genes, if present, occur at a low density relative to euchromatin	Gene content as in euchromatic homologue	Unknown
Heteropycnosis	+	+	+
Satellite DNA	Usually present	If present only in low copy number	Has not been found in *Cricetulus griseus* *Notomys* spp.
Presence of chiasmata	No	Yes	Yes

tive heterochromatin, an observation which has been confirmed in *Rattus (Mastomys) natalensis* (Huang, 1968) and *Rangifer tarandus* (Fraccaro *et al.*, 1968). The accessory genosomal heterochromatic is not only the last to replicate but a degree of asynchrony is found between the two X chromosomes in *Mesocricetus auratus* (Galton and Holt, 1964), *Microtus agrestis* (Schmid, 1967) and *Rattus (Mastomys) natalensis* (Huang, 1968). However, as this asynchrony is observed only in cells at the very end of S phase when only the accessory gonosomal heterochromatin is labelled, it is not possible to determine if the heavy label is on the facultative X chromosome.

(iii) Late replication as such is not always associated with facultative heterochromatin. Thus, constitutively heterochromatic regions also commonly show late replication (e.g. *Suncus murinus* Rao *et al.*, 1970). Additionally, there are regions within the euchromatin of the human genome which are late replicating but are not constitutively heterochromatic when judged by either C-banding or *in situ* hybridization techniques (Comings, 1972; Farber and Davidson, 1977; Schmid, 1963). Similarly, the last regions to complete replication in the Indian muntjac *Muntiacus muntjak* are the positive C-bands (Sharma and Dhaliwal, 1974). The tendency towards asynchrony is also revealed in the differing replication patterns observed in human fibroblasts compared with human leucocytes (Slesinger *et al.*, 1974; Prokofieva-Belgovskaya *et al.*, 1976) and between lymphocytes and amniotic epithelial cells (German and Aronian, 1971). Asynchrony may also occur between homologous autosomes (Schmid, 1963; Bianchi and Bianchi, 1965; Atkins and Santesson, 1966; Farber and Davidson, 1977).

This asynchrony of replication in euchromatin appears to be dependent upon its AT/GC content. AT rich euchromatin replicates late, GC rich euchromatin does not (Tobia *et al.*, 1972; Bostock *et al.*, 1976). This is also the case for the satellite DNA of the mouse which is AT rich and replicates late (Flamm *et al.*, 1971). On the other hand other satellite DNAs show no such simple relationship. Thus the HS-beta of *D. ordii* with a GC content of 66.1% is not early replicating, rather it replicates throughout S phase. Similarly the HS-alpha satellite (GC content = 43.4%) replicates mostly at the very end of S phase compared with main band (GC content = 38.5%). While the heterogeneity of replication behaviour of constitutive heterochromatin is thus not unexpected, because of its structural heterogeneity, it is also unexplained in terms of the known differences in molecular structure (Bostock *et al.*, 1976).

To test the Lyon hypothesis, the pattern of late replication in two *N. alexis* females heterozygous for submetacentric/acrocentric X chromosomes has been examined. In such combinations the submetacentric X carries a substantial segment of accessory gonosomal heterochromatin (see Section 2.3.2.5).

7.2 *Methods*

Standard leucocyte cultures (see Section 2.2) were used for the late replication studies. A continuous labelling method was established using tritiated thymidine at a concentration of 1 μCi/ml of culture medium (methyl-^{3}H thymidine 19 Ci/mmol, Amersham) which was added 6 hours prior to harvesting. Colchicine (0.2 ml of 0.02% solution) was added 2 hours prior to harvesting and followed the regime described earlier (see Section 2.2). Slides were dipped in Ilford K2 emulsion and stored in a dry atmosphere at 4°C for 28 days then developed in D19b and stained with Giesma. Following photomicroscopy the slides were degrained and re-photographed to check the identification of the X chromosomes and the regions found to be late replicating on the autosomes.

7.3 *Observations and Discussion*

7.3.1 *Late replication patterns*

Studies in a variety of other mammals (Schmid, 1963; Mukherjee and

Sinha, 1963,1964; Fraccaro *et al.*, 1968; Cohen and Rattazzi, 1971; McLaren, 1972; Therman *et al.*, 1974) have established that the last half of S phase is best defined in leucocyte cultures if label is added 5 to 6 hours before harvesting. My own observations on *Notomys* confirm that 6 hours of label provide adequate numbers of suitably labelled cells.

Female cells were judged to be late replicating when the label was confined to the X chromosomes and the known constitutive heterochromatic pericentromeric regions of the autosomes, leaving the euchromatic regions relatively free of label (Fig. 7.1). In late labelled metaphases from a polymorphic female *Notomys alexis* the following patterns were observed:

(i) The pericentromeric regions of the majority of autosomes which are also C-band positive, are late replicating (Fig. 7.1b and b'). Exceptions to this pattern include cells where one of a pair of acrocentric autosomes and one of the small metacentric autosomes are devoid of label (Fig. 7.1b and b'). This observation suggests that there is a degree of asynchrony within the constitutively heterochromatic regions of the genome and that homologous chromosomes can also be asynchronous in replication with respect to their constitutive heterochromatin.

(ii) The facultative arm of the late replicating X chromosome is sometimes differentially affected with the telomeric half of this arm labelled and the proximal half entirely free of label (Fig. 7.1b and b'). This suggests that the proximal arm replicates earlier than do the telomeric arm, the non-euchromatic arms of both X chromosomes and also the bulk of the constitutive heterochromatin of the autosomes.

(iii) The non-euchromatic arms of both X chromosomes (i.e. the accessory gonosomal heterochromatin in terms of Schmid, 1967) are invariably late replicating (Figs. 7.1a and b). This is also found in a number of other animals which have multiplex-type X chromosomes, namely *Microtus agrestis* (Wolf *et al.*, 1965; Schmid, 1967), *Mesocricetus auratus* (Galton and Holt, 1964; Hill and Yunis, 1967; Schmid, 1967)

Figure 7.1 : Late DNA replication patterns in *Notomys alexis* females heterozygous for submetacentric X and acrocentric X chromosomes.

a. Late DNA replication pattern showing the submetacentric X as the late labelled element.

a'. Same cell re-photographed with the silver grains removed.

b. Late DNA replication pattern showing the submetacentric X later labelled than the acrocentric X chromosome.

Note: (i) The absence of label on some small autosomes indicating that in these chromosomes the constitutive heterochromatin has replicated earlier than this material on the other chromosomes.

(ii) Some euchromatin telomeric regions are shown to be late-labelling by the presence of silver grains.

b'. Same cell as in (b) re-photographed after removal of the silver grains.

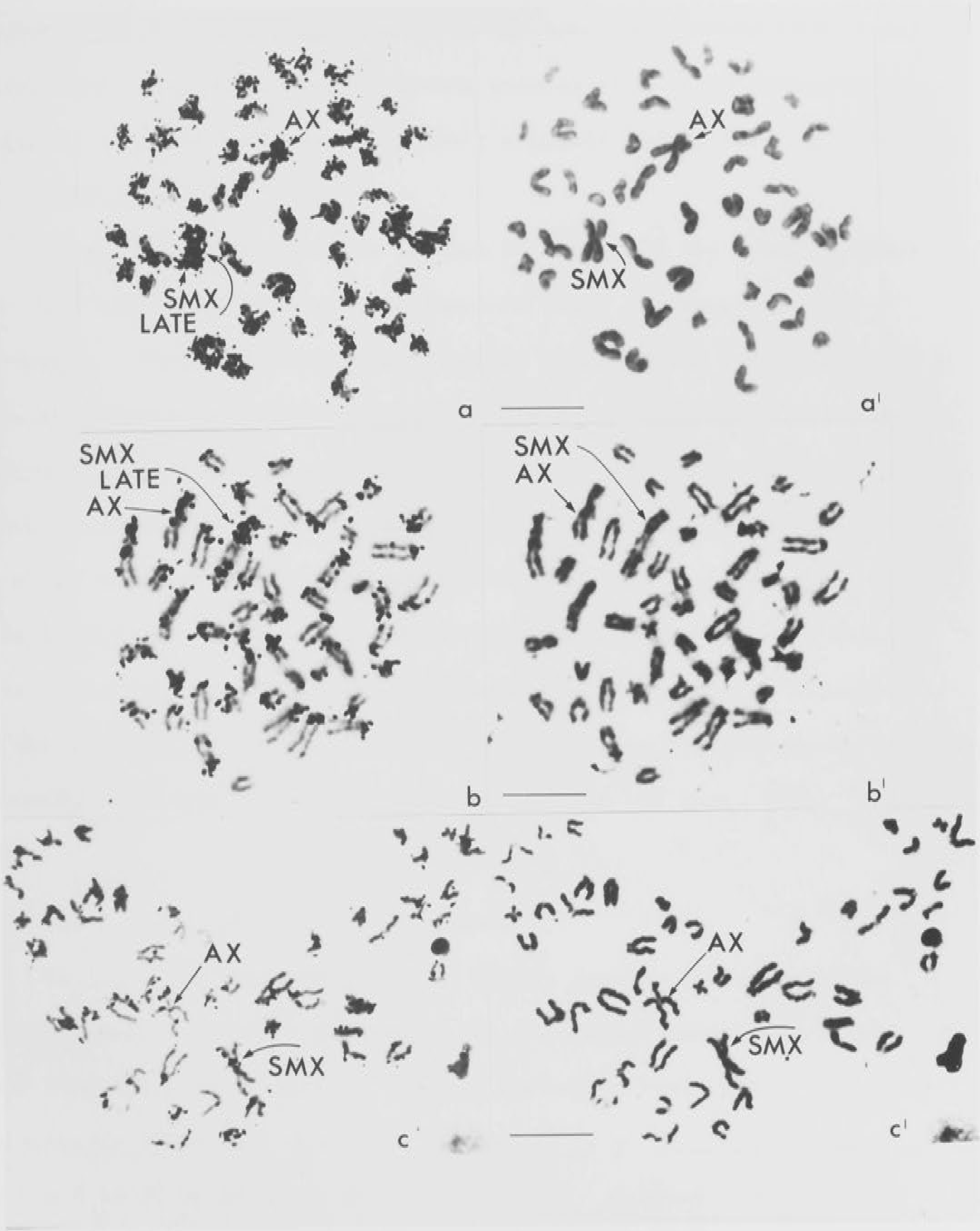
c. Late DNA replication pattern in which both X chromosomes are equally labelled.

c'. Same cell as that shown in (c) with the silver grains removed prior to photography.

The bar in each photograph represents 10 μm.

SMX = submetacentric X chromosome.

AX = acrocentric X chromosome.


AX
SMX
LATE
a
AX
SMX
a'
SMX
LATE
AX
b
SMX
AX
b'
AX
SMX
c
AX
SMX
c'

and *Cricetulus griseus* (Hsu, 1964).

(iv) The bulk of the euchromatin of the autosomes replicates earlier than all the above categories. However, some euchromatic regions, particularly some telomeres, are late replicating (Fig. 7.1c). Still other autosomes, despite having constitutive heterochromatic regions, complete replication in their entirity before the remainder of the complement (Fig. 7.1b).

Thus, it is clear that in females of *N. alexis* the order in which the four categories of chromatin complete their replication is euchromatin - facultative heterochromatin - constitutive heterochromatin - accessory gonosomal heterochromatin. Within the facultatively heterochromatic X of *N. alexis* the telomeric half of the facultative arm is later replicating than the proximal half. In very late labelled cells, in which only the accessory gonosomal heterochromatin is labelled on both X chromosomes, there is a definite asynchrony between the two. Here it is not possible to distinguish which late labelling chromosome is the facultative element. This is comparable to *Microtus agrestis* (Schmid, 1967) and *Mesocricetus auratus* (Galton and Holt, 1964; Schmid, 1967).

7.3.2 *Late labelled X chromosomes*

In leucocyte cultures of two *N. alexis* females heterozygous for morphologically distinct X chromosomes, the submetacentric form was most frequently late labelled (Figs. 7.1a and b; Table 7.2). The submetacentric chromosome was late labelled in 42 of 46 cells in female *Na 4* and in 37 of 54 cells in female *Na 800*. Neither result is consistent with the hypothesis of random inactivation ($X_1^2 = 31.4$, $p < .001$; $X_1^2 = 7.41$, $.001 < p < .01$; respectively).

In both experiments a considerable number of cells had both X chromosomes with similar patterns of label. The *Na 4* culture had 10 cells (18%) in this category, while in the *Na 800* culture 9 cells (14%) had

Table 7.2 : Late replicting X chromosome behaviour in females of *Notomys alexis* heterozygous for a submetacentric and an acrocentric form of the X chromosome.

Female		Labelling pattern observed in X chromosomes			Total
		Late submetacentric	Late acrocentric	Both with equal label	
Na 4	n	42	4	10	56
	%	75.0	7.1	17.9	100
Na 800	n	37	17	9	63
	%	58.7	27.0	14.3	100

equally labelled X chromosomes (Fig. 7.1c and c'). Schmid (1967) describes a similar occurrence where both X chromosomes in *Microtus agrestris* had equal labelling in 20% of the metaphase examined. In *Notomys* the euchromatic arms were free of label in contrast to the accessory gonosomal heterochromatin of these chromosomes. Schmid (1967) also reported that the X chromosomes in *Mesocricetus auratus* behave in a similar manner. As shown by its later replication than the acrocentric form, the submetacentric X in *N. alexis* is preferentially inactivated. This contrasts with the heteromorphic X chromosomes that randomly late label in the Indian gerbil, *Tatera indica cuverii* (Rao *et al.*, 1968). The nature of the size difference in the X chromosomes in *T. i. cuverii* is however unknown since no C-banding studies have been carried out in this species. Thus if the pattern of late replication in the two morphologically distinct forms of X chromosomes of *N. alexis* are indeed indicative of differential gene activity then it is clear that X inactivation in this case is not at random.

There are a number of earlier human studies of normal and 'abnormal' X chromosomes which coexist in the same female. In these cases it is evident that the two types of cell expected on the basis of random inactivation and in the absence of cell selection, are not equally represented in the adult female (Table 7.3). There are at least four interpretations:

(i) The original inactivation event may well have been random and subsequent cell selection may account for the observed non-randomness. This explanation has been invoked, in part, by Mukherjee *et al.* (1970) to explain the non-random results in female mules studied by Hamerton *et al.* (1969). Different tissues may have different patterns of X chromosome late labelling. The data from female mules show preferential expression of donkey G6PD enzyme in liver, of horse G6PD in several other tissues whereas in the lung and thyroid the expression is random (Hook and

Table 7.3 : Late replication behaviour in human females with an 'abnormal' X chromosome.

Type	Late-replicating chromosome	References
X,Xr	Xr	Lindsten and Tillinger, 1962
X,Xr	Xr	Rowley *et al.*, 1964
X,Xd	Xd	Atkins and Santesson, 1965
X,Xd	Xd	Hsu and Hirschhorn, 1970
X,Xd	Xd	Buckton *et al.*, 1971
X,Xiso	Xiso	Muldal *et al.*, 1963
X,Xiso	Xiso	Taft and Brooks, 1963
X,Xiso	Xiso	Miller *et al.*, 1963
Balanced X-autosomes translocations		
X,t(Xg-;5q+)	Xn + X part of Xt	Mann and Higgins, 1974
X,t(Xq-;Cq+)	Xn	Thorburn *et al.*, 1970
X,t(Xq-;9p+)	Xn 68% Xn + X part of Xt 32%	Cohen *et al.*, 1972
X,t(Xq-;9q+)	Xn 84% Xn + X part of Xt 16%	Leisti *et al.*, 1975
X,t(Xq-;12q+)	Xn	Sarto *et al.*, 1973
X,t(Xq-;14q+)	Xn	Allderdice *et al.*, 1971
X,t(Xq-;15p+)	Xn	Lucas and Smithies, 1973
X,t(Xq-;19q+)	Xn	Gerald *et al.*, 1973
X,t(Xq-;21p+)	Xn	Summitt *et al.*, 1974
X,t(Xp-;14q+)	Xn	Buckton *et al.*, 1971
X,t(Xq+3q-)	Xn	De La Chapelle and Schroder,1973
X,t(Xq+8q-)	Xn	Tipton pers. comm. in Leisti *et al.*, 1975
X,t(Xp+;18q-)	Xn 22% Xt 78%	Thelen *et al.*, 1971
Unbalanced X-autosomes translocations		
X,t(Xp+;?)	Xt	Wie Lie *et al.*, 1964
X,t(Xq+;?)	Xt	Hugh-Jones *et al.*, 1965
X,t(Xq+;?)	Xt	Thorburn *et al.*, 1967
X,t(Xp+;3q-), r3+	Xt	Mukherjee and Burdette, 1966
X,t(Xp+;C)c-	X part of Xt only	Neuhauser and Back, 1967
X,t(Xq+;?)	X part of Xt only	German, 1967
X,t(Xq8q)	X part of Xt only	Cohen *et al.*, 1972
X,t(Xq+;15q-)	Xt 75% Xn 11% both Xt and Xn 10%	Engel *et al.*, 1971
X,t(Xq+8q) distal segment Xq lost	Xt	Tipton pers. comm. in Leisti *et al.*, 1975

Table 7.3 Contd.

X,t(Xq+;13q	X part of Xt 75% Xt 25%	Crandall *et al.*, 1973 Crandall *et al.*, 1974
XX,21p	Xn 6% Xn + X part of Xt 10% Xn + Xt 84%	Summitt *et al.*, 1974
X,-X,+der(9)	Almost all Xt 95% Xt + Xn 5%	Leisti *et al.*, 1975

Brustman, 1970,1971).

(ii) Cell differentiation may contribute to the final inactivation picture in that the stem cells which were randomly inactivated will each give rise to daughter cells having the same X inactivated. Thus a single stem cell may give rise to a tissue with a pattern of inactivation bearing no resemblance to the stem stock considered *in toto*. Of course the interplay of both (i) above and cell differentiation is most likely to represent the true picture.

(iii) The paternal or maternal origin of the X chromosome may have some influence on which X chromosome is late labelling. Alternatively, in mules and hinnies Giannelli and Hamerton (1971) suggest that either the donkey X may be more prone to heterochromatinization than that of the horse or that the donkey X may commence heterochromatinization earlier than does the horse X and that this property is retained in the hybrids.

(iv) Giannelli and Hamerton (1971) have cited inter-animal variation as a possible cause of the difference between their observations on female mules and those of Mukherjee and Sinha (1964) and Mukherjee *et al.* (1970).

In *N. alexis* it is possible that the accessory gonosomal heterochromatin present in the submetacentric form of the X chromosome may influence the pattern of replication. This may be tested by the use of the metacentric form of the X chromosome in heterozygous combination with either the acrocentric or submetacentric forms of the X in *N. alexis*. These steps would establish if those chromosomes with increased amounts of accessory gonosomal heterochromatin are preferentially inactivated. The presence of polymorphic X chromosomes in *N. cervinus* and *N. fuscus* would allow further tests along these lines. As all three forms of the X chromosome of *N. alexis* have been found in wild caught animals from within a 1 km radius and two forms of the X from another locality some hundreds of kilometres away but within a 2 km radius (Baverstock *et al.*, 1977b) the polymorphism in a does not suggest marked clinal variation.

CHAPTER 8

GENERAL DISCUSSION

By using a variety of biochemical and cytological techniques the structure of the genome of the genus *Notomys* has been analysed from several different, but complementary, aspects. It has provided considerable new information on genomic organization and differentiation within a single genus and in particular has given a new perspective on the relationships between the four species.

The four species of the genus *Notomys* are taxonomically quite distinct from all other pseudomyids. However as early as 1951 Tate, working on skeletal characters, had concluded that *N. mitchellii*, *N. alexis* and *N. fuscus* form a closely related trio of species while *N. cervinus* is somewhat more separated from them. The distinctive nature of *N. cervinus* was subsequently reinforced by the studies of Crichton (1974), using female reproductive characteristics and Watts (1975), using neck gland morphology and histology. A comparative study of the male reproductive organs (Breed and Sarafis, 1979; Breed, 1980) likewise offers good grounds for separating *N. cervinus* from its congeneric sister species.

The phylogenetic relationships between *N. mitchellii*, *N. alexis* and *N. fuscus* vary according to the technique used for comparative assessment. For example on skeletal characteristics *N. mitchellii* and *N. alexis* appear to be closer to one another than either is to *N. fuscus*, whereas on the basis of sperm morphology, *N. mitchellii* is readily distinguished from both *N. alexis* and *N. fuscus* (Breed and Sarafis, 1979). If one considers the structure of the neck glands, there are grounds for arguing that *N. mitchellii* has the simplest structure with *N. alexis* intermediate between this species and *N. fuscus*. *N. cervinus* is again quite distinct since it lacks neck glands (Watts, 1975) though it has a sternal gland, both in males and in lactating females, which is not found in the other three species. The somewhat variable relationships which emerge on the basis of these different character combinations clearly emphasise the difficulty of dealing with related species which have a relatively short evolutionary history and are few in number.

Cytogenetic data can be brought to bear on the relationships between the four species of *Notomys*. If we examine the observations made in this study (Table 8.1), then at the gross karyotypic level, *N. mitchellii* and *N. alexis* are obviously more closely related to one another than either is to *N. cervinus* or *N. fuscus*, which clearly differ from one another. The distribution of constitutive heterochromatin reinforces this observation. While *N. mitchellii* and *N. alexis* have very similar amounts of total heterochromatin, they do show distinctive differences in the distribution of both LAH and SAH. *N. fuscus* is distinctive because it has relatively little constitutive heterochromatin and satellite DNA, and also relatively few members of its complement carry heterochromatic short arms. *N. cervinus*, on the other hand, is distinctive due to the large number of chromosomes which carry heterochromatic short arms. If one excludes the rearrangements which characterize the karyotypes then the G-banding patterns are very similar. This is not surprising when one considers the relatively recent divergence of the *Notomys* species.

The location of the nucleolar organizers in the *N. cervinus* karyotype is also distinctive. In *N. cervinus* the NORs are located on the heterochromatic short arms, in contrast to *N. mitchellii, N. alexis* and *N. fuscus* where the NORs are found on the telomeres of the euchromatic long arms. It is not known if the chromosomes involved are homologous but they are of similar size however *N. cervinus* has more NORs than the other species as shown by silver staining, but the reason for this is not apparent.

Table 8.1: Summary of the relationship between the four species of *Notomys* from the data presented in this thesis

Species	*N. mitchellii*	*N. alexis*	*N. cervinus*	*N. fuscus*
Chromosomal characteristics				
Diploid chromosome number	48	48	48	48
Autosomal structure A = acrocentric M = metacentric	42A 4M	42A 4M	40A 6M	44A 2M
Relationship to presumed ancestral karyotype of 42A + 4M**	Basically similar plus added heterochromatin	Basically similar plus added heterochromatin	Differs by one pericentric inversion, one translocation plus added heterochromatin	Differs by one pericentric inversion plus changes in heterochromatin and two unfixed pericentric inversions
Distribution of constitutive heterochromatin				
% total constitutive heterochromatin in 20AK	27.19	22.82	24.39	13.13
% LAH 20AK	13.38	11.08	6.77	11.38
% SAH 20AK	13.81	11.74	17.62	1.75
% satellite DNA in genome	28.06	23.89	24.45	6.16
Buoyant density of satellite DNA g/cm^3				
Neutral CsCl	1.712	1.712	1.713	1.713
Alkaline CsCl	Two density peaks 1.760&1.781	Two density peaks 1.761&1.782	Two density peaks 1.762&1.781	Two density peaks 1.763&1.781
Neutralised alkaline CsCl	1.728	1.729	1.726	1.730
Heat denatured	1.726	1.726	1.728	1.728
Reassociated heat denatured	1.720	1.721	1.717	1.723
% G + C in satellite DNAs				
Based on buoyant density	53	53	54	54
Based on Tm	51.5	50.5	50.3	52.9
Satellite DNA Relatedness expressed as % divergence derived from filter hybridization				
H^3cRNA from *N. alexis* satellite DNA as probe	0	Homologous hybrid	3.1	2.2
H^3cRNA from *N. fuscus* satellite DNA as probe	1.8	1.8	2.5	Homologous hybrid
Restriction endonuclease digestion of satellite DNAs				
Hinf	Similar to *N. alexis* differs from *N. cervinus* & *N. fuscus*	Similar to *N. mitchellii* 30 bp band prominent differs from *N. cervinus* & *N. fuscus*	Identical to *N. fuscus* differs from *N. mitchellii* & *N. alexis*	Identical to *N. cervinus* differs from *N. mitchellii* & *N. alexis*
Hae III	Identical to *N. alexis* differs from *N. cervinus* & *N. fuscus*	Identical to *N. mitchellii* differs from *N. cervinus* & *N. fuscus*	Different from all others	Different from all others
Bam HI	Identical to *N. alexis* differs from *N. cervinus* & *N. fuscus*	Identical to *N. mitchellii* differs from *N. cervinus* & *N. fuscus*	Differs from all others	Differs from all others
Taq I	Identical to *N. alexis* differs from *N. cervinus* & *N. fuscus*	Identical to *N. mitchellii* differs from *N. cervinus* & *N. fuscus*	Similar to *N. fuscus* but with minor band differences differs from both *N. mitchellii* & *N. alexis*	Similar to *N. cervinus* but with minor band differences differs from both *N. mitchellii* & *N. alexis*

Note: The major feature of all the above restriction endonuclease digests of *Notomys* satellite DNAs is the 22 bp multimer series which is, however, incomplete in some digests.

Table 8.1 contd.

Chiasma frequency characteristics				
Mean chiasma frequency per cell	27.75	28.67	28.85	26.91
Bivalent 1	Singles intermediate between *N. cervinus* & *N. fuscus*	Singles intermediate between *N. cervinus* & *N. fuscus*	Single exchanges reduced. Triples higher than other 3 species	Single exchanges more than in all other species
Bivalents 2-6	Intermediate between *N. cervinus* & *N. fuscus*	Intermediate between *N. cerminus* & *N. fuscus*	Fewer singles & more doubles & triples than other 3 species	More singles & few doubles than other 3 species
Bivalents 7-20	Less singles than *N. fuscus* more doubles than *N. fuscus*	Less singles than *N. fuscus* more doubles than *N. fuscus*	Less singles than *N. fuscus* more doubles than *N. fuscus*	More singles and less doubles than other 3 species
Chiasma distribution characteristics				
Bivalent 1			Fewer proximals than other species in double exchanges No LAH	
Bivalents 2-6			Fewer proximals more interstitials than other species in double exchanges Least LAH	
Bivalents 7-20			Fewer proximals more interstitials than other species in double exchanges Least LAH	

**Baverstock *et al.* (1977a).

Filter hybridization using ^{3}HcRNA from satellite DNA in homologous and heterologous combinations shows the affinity between the satellite of *N. mitchelii* and *N. alexis* and the differences between them and those of *N. cervinus* and *N. fuscus*. These two latter species clearly differ from each other on this criterion by approximately the same amount as each does from the *N. mitchellii-N. alexis* duo. The restriction endonuclease digests give patterns which generally support the data from the filter hybridizations but additionally they do allow the separation of *N. mitchellii* from *N. alexis*. The data from the restriction endonuclease digests allow a phylogeny to be drawn (Fig. 6.22) which agrees with the relationships derived by Tate (1951) from morphological criteria and also those derived from karyotypic data.

Apart from these general insights into genomic organization and relatedness in the species within the genus the observations reported here have a bearing on the two recent hypotheses on to the functional aspects of heterochromatin differentiation which were referred to in the general introduction. The first of these concerns the possible role of heterochromatin variation in regulating recombination within genomes (Rhoades, 1978; John and Miklos, 1979). The second is the possible involvement of heterochromatin variation in the process of speciation (Corneo, 1976, 1978; Fry and Salzer, 1977; Hatch *et al.*, 1976).

It is clear that in *Notomys* there is no simple correlation between either total heterochromatin content or total long arm heterochromatin and mean cell chiasma frequency of the type which has been reported in *Atractomorpha* (Nankivell, 1976), *Caledia* (Shaw and Knowles, 1976) and *Salvia* (Linnert, 1955). In part this may be due to the actual location of the heterochromatin. Thus given the absence of chiasma interference across the centromere, short arm heterochromatin will obviously not influence chiasma distribution since chiasmata do not form in the short arms. Similarly, since the long arm heterochromatin is located procentrically its action will possibly be overridden by that of the centromere. The only correlation which does obtain is that between total genomic short arm heterochromatin content and mean cell chiasma frequency. Large increases in short arm heterochromatin are positively correlated with increases in mean cell chiasma frequency. Even here, however, it is not possible to rule out genotypic differences between the species as the causal basis for the difference between them, and similar qualifications apply to other cases of interspecific comparisons (*Atractomorpha* species, Miklos and Nankivell, 1976; the hedgehogs, *Erinaceus europaeus* and *Aethechinus algirus*, Natarajan and Gropp, 1971).

Interspecific comparisons in *Notomys* indicate that long arm heterochromatin may influence chiasma distribution by "neutralizing" the centromere effect in all bivalent groups which have double exchanges. Even so it is apparent that in *Notomys* recombination modification by long arm heterochromatin is minimal. This is not surprising when one considers that the heterochromatic blocks which have most effect on chiasma distribution in other species are terminally located on otherwise euchromatic chromosome arms (*Chorthippus jucundus*, John, 1973; *Cryptobothurus chrysophorus*, John and King, 1980; *Atractomorpha similis* Miklos and Nankivell, 1976). Of course in *Notomys* no attempt has been made to examine chiasma variation during female meiosis where differences from the situation found in the male might be expected since sex differences in chiasma frequency and distribution have been reported in *Stethophyma grossum* (White, 1936, 1973; Perry and Jones, 1974), *Fritillaria meleagris* (Newton and Darlington, 1930; Fogwill, 1958) and *Triturus helveticus* (Watson and Callan, 1963). In other species the chiasma patterns are very similar in both sexes and included in this group are the grasshopper *Chorthippus brunneus* (Jones *et al.*, 1975), rye (Davies and Jones, 1974) and barley (Bennett *et al.*, 1973). Clearly the female meiotic patterns in test situations such as those cited for *Atractomorpha similis* and *Cryptobothrus chrysophorus* will be of interest.

In *Lolium* and *Festuca*, there is evidence that chiasma frequencies are under genotypic control (Rees and Dale, 1974). Low chiasma frequencies have mostly distal chiasmata, while high chiasma frequencies are accompanied by increases in the number of interstitial chaismata. From these data, Rees and Dale (1974) have proposed a model to explain the effects of chiasma frequency variation and positional changes on the release of genetic variability. In *Lolium* and *Festuca* the short lived species have higher chiasma frequencies than perennials; high chiasma frequencies are associated with low phenotypic and genetic variance for characters known to be under polygenic control. In *Notomys* detailed studies of this nature have not been undertaken but the following comparison suggests that they too may show a similar pattern.

Comparison of the ecologies of *N. alexis* and *N. mitchellii* although incomplete, does indicate important differences. Thus, *N. mitchellii* inhabits the southern regions of Australia (Fig. 1.1a) which have a low, but regular winter rainfall. These conditions permit an annually recurrent breeding season during which several litters may be produced (Crichton, 1974). Plague formation has never been reported for *N. mitchellii*. *N. mitchellii* has a lower mean cell chiasma frequency with fewer interstitial chiasmata than is found in *N. alexis*. These data fit the first model of Rees and Dale (1974, their Fig. 10) which suggests that low chiasma frequencies and fewer interstitial chiasmata favour the segregation of interstitially located genes as a tightly linked block. In contrast *N. alexis* inhabits a large proportion of the semi-arid to arid centre of Australia (Fig. 1.1a) and undergoes spasmodic plague formation in response to the erratic production of abundant food resources (Newsome and Corbett, 1975). The periods of abundant food supply result from rare but extensive local rains or floods which have their source of water supply well away from the region. *N. alexis* also differs from other *Notomys* species by its ability to conceive during lactation and to produce large litters (Crichton, 1974). This enables *N. alexis* to function as a rapid opportunistic breeder when the rare but favourable conditions prevail. Compared to *N. mitchellii*, *N. alexis* has a high mean cell chiasma frequency and ~~proportially~~ Proportionally more interstitial chiasmata. These data fit the second model suggested by Rees and Dale (1974, their Fig. 10) which provides for the release of variability due to the disruption of gene complexes by increased numbers of interstitial chiasmata.

N. cervinus and *N. fuscus* also provide an interesting comparison as they occur sympatrically (Fig. 1.1b), however, *N. cervinus* is found on open gibber plains or gibber plains with alluvial flats, while *N. fuscus* is found on sand dunes and rarely on the gibber areas (Watts and Aslin, 1974). There is a marked difference in the chiasma characteristics of the two species. *N. cervinus* has a higher mean cell chiasma frequency (28.85) and proportionally more interstitial chiasmata than *N. fuscus* (26.91). Crichton (1974) suggest that delayed implantation in *N. cervinus* may enhance its ability to breed under adverse nutritional conditions which prevail in the harsh gibber microclimate. Little is known of the breeding biology of *N. fuscus* so that comparisons with *N. cervinus* are impossible at this point. Field population studies of *Notomys* species will be necessary to establish if the broad agreement of these data for *N. mitchellii* and *N. alexis* with the models proposed by Rees and Dale (1974) is to be substantiated. Of particular interest would be studies on populations of these species in Western Australia where their distributions are parapatric and where their morphology is very similar (Dr D. Kitchener, pers. comm.).

Turning now to the relevance of heterochromatin to processes of speciation there are two relevant points to note. Firstly, the presence of a polymorphism for constitutive heterochromatin in chromosome 1 of *N. alexis* does not lead to any disruption of meiotic pairing which might serve to produce a measure of infertility (see Section 2.3.2.2.; Table 2.6). Comparable situations exist in many other species (Table 8.2). For example, *Mus musculus* (Dev *et al.*, 1973,1975; Forejt, 1973), *Peromyscus maniculatus* (Waterbury, 1972), *Neotoma micropus* (Mascarello and Warner, 1974; Warner, 1976) and *Neotoma lepida* (Mascarello and Hsu, 1974) have all been used in breeding experiments which involve homologues with differing amounts of constitutive heterochromatin and none have shown failure of pairing in meiosis or reduced fertility.

Secondly, differences in the pattern of heterochromatin distribution between the species of *Notomys* are not associated with structural differences between the euchromatic components of their respective karyotypes. The differences in satellite DNA and constitutive heterochromatin content in *Notomys* can be used to distinguish two groups, one with a satellite DNA content of 23-28% (*N. mitchellii*, *N. alexis* and *N. cervinus*) and the other, (*N. fuscus*) with only 6%. If, as Hatch *et al.* (1976) have proposed, large amounts of satellite DNA promote chromosomal rearrangement, especially pericentric inversions, then one might expect fewer pericentric inversions in *N. fuscus*. This is not the case (Table 2.7) and quite clearly the evidence in *Notomys* does not support the hypothesis. By using G- and C-band comparisons both *N. cervinus* and *N. fuscus* have more inferred rearrangements than either *N. mitchellii* or *N. alexis*. Yet *N. cervinus* and *N. fuscus* differ markedly in their content of satellite DNA and constitutive heterochromatin. Additionally, *N. fuscus* has least satellite DNA but the highest number of rearrangements. If only the three species with high satellite DNA contents are considered then *N. cervinus* shows

Table 8.2: Organisms with C-band polymorphisms which suggest there can be no effect of heterochromatic difference on chromosome pairing.

Organism	Polymorphism of C-band	References
Allium flavum	7 autosomes	Loidl (1979)
Scilla subirica	6 autosomes	Vosa (1973)
Hybrids of *Mus m. musculus* & *M. m. molossimus*	15 autosomes	Dev *et al.* (1975)
Homo sapiens	Chromosomes 1,3, 4,9,13,14,15,16, 21,22,Y but mainly 1, 9 & 16	Angell and Jacobs (1978) Buckton *et al.* (1976) Craig-Holmes *et al.* (1973) Craig-Holmes *et al.* (1975) Evans (1977) Geraedts *et al.* (1974) Geraedts *et al.* (1975) Hoehn *et al.* (1977) Lubs and Ruddle (1971) Madan and Bobrow (1974) McKenzie and Lubs (1975) Muller *et al.* (1975) Muller and Klinger (1976) Phillips (1977) Robinson *et al.* (1976) Carnevale *et al.* (1976)
Rattus rattus tanezumi	Chromosomes 3,4, 7,9,11, & 13	Yosida and Sagai (1975)
Erinaceus europaeus	Chromosomes 13, 14,15,20 & 21	Mandahl (1978) Gropp and Natarajan (1972) Natarajan and Gropp (1972) Gropp *et al.* (1969).

more chromosomal rearrangement than either *N. mitchellii* or *N. alexis*. All these comparisons are contrary to the expectations of the Hatch hypothesis. Furthermore the pattern observed in *Notomys* is not unique.

Similarly data from the genus *Peromyscus*, all species of which have 2n = 48, do not support the Hatch *et al.* (1976) hypothesis. For example Arrighi *et al.* (1976) show that *P. crinitus* (FN = 56) has 4 heterochromatic short arms whereas *P. eremicus* (FN = 96) has 46 heterochromatic short arms. *P. eremicus*, however, has 35.8% C-banding material compared with only 5.7% in *P. crinitus* (Deaven *et al.* 1977) and it is reasonable to assume that the C-bands of *P. crinitus* contain satellite DNA as do those of *P. eremicus* (Hazen *et al.*, 1977). The observed karyotypic changes thus provide an increase in the number of heterochromatic short arms; there is no evidence for the increase in chromosome arms due to pericentric inversions as the Hatch hypothesis would predict. Also there are some genera with little satellite DNA content but still showing considerable karyotypic change. Miklos *et al.* (1980) have pointed out that in Australian species of *Rattus*, *R. sordidus* (2n = 32) differs from *R. villosissimus* (2n = 50) by 11 fission or fusion events yet both have negligible satellite DNA (Baverstock *et al.*, 1977c; Miklos *et al.*, 1980). Obviously large amounts of satellite DNA are not a necessary prerequisite for chromosomal rearrangements at least in this genus.

It is clear from the above examples and also in *Notomys* that satellite DNA is an unlikely candidate for involvement in speciation in the manner proposed by Hatch. Nor is there any evidence to support the suggestion by Hatch that pericentric inversions lead to fertility problems which provide an isolating mechanism. There is no evidence to support the arguments that changes in satellite DNA and hence constitutive heterochromatin disrupt pairing at meiosis resulting in infertility and therefore reproductive isolation (Corneo, 1976,1978; Fry and Salzer, 1977).

In the grasshopper *Caledia captiva*, F1 hybrids between chromosomally differential sub-species are heterozygous for 7 pericentric rearrangements without impairment of meiosis (Moran, 1978). These hybrids are both viable and fertile (Shaw *et al.*, 1976). When heterozygous, the pericentric rearrangements impose no mechanical restraints on meiosis because there is no crossing over between the inverted region and it normal homologous region (Shaw *et al.*, 1977; Moran, 1978). The heterozygosity for pericentric rearrangements does however induce a major change in the pattern of recombination. Thus chiasmata occur exclusively outside the rearranged regions of the chromosomes thus generating novel recombinants (Shaw and Wilkinson, 1980). In this case if there is any reduction in fitness due to the presence of these rearrangements it is not due to mechanical breakdown of meiosis but is more likely to arise from genotypic imbalance among the F2 and backcross progeny (Shaw and Wilkinson, 1980).

The biological relevance of the observed difference in heterochromatin content between the karyotypes of the four species of *Notomys* remains unclear. The problem is not unique to *Notomys*; Recent reviews have discussed the biological implications of large amounts of repeated DNAs in the genomes and suggested that this DNA may not have specific functions. Orgel and Crick (1980) define 'selfish DNA' repeated sequences as having two distinct characters. Firstly, it arises when a DNA sequence spreads by forming additional copies of itself within the genome. Secondly, it makes no specific contribution to the phenotype. In general, they address their remarks to middle repetitive DNA but much of their discussion refers also to highly repeated DNA. Orgel and Crick (1980) and also Doolittle and Sapienza (1980) point to the possible error of ascribing function to all segments of repetitive DNA. Orgel and Crick (1980) suggest that if a particular DNA sequence acquires a function it does not necessarily follow that all copies of the sequence will have the same function. For example, some copies of the sequence might not be favourably positioned to function correctly. The known distribution pattern of most highly repeated DNA is not random in the genome but is largely although not exclusively found in constitutive heterochromatin most often located near centromeres, to a lesser extent located at the telomeres and rarely found at interstitial sites. Further, the effects of heterochromatin on chiasma frequency and distribution appear to have their basis in amount and position of the heterochromatin rather than in the precise sequence of the highly repeated DNA present (Dover, 1980). Of course, the molecular and mechanical bases behind the correlated changes in chiasma characteristics and heterochromatin content are unknown and the correlation is not uniform or as marked in all species so far studied.

It is worth remembering that until recently heterochromatin was generally thought to lack genes and the highly repeated DNAs contained in it were thought to be non-transcriptional. This is now known to be incorrect and in *Drosophila melanogaster* vital and non-vital gene loci have been found in heterochromatin (Hilliker and Appels, 1980), and satellite DNAs are transcribed in the newt *Triturus cristatus carnifex* (Varley *et al.*, 1980). It is possible that functions will be indicated when the molecular structure of heterochromatin is better known. For example, it has already been suggested that total DNA content might exert effects on such parameters as generation time and cell cycle duration (Bennett, 1972) or cell volume and nuclear volume (Cavalier-Smith, 1978) and the amount of heterochromatin may play a role in these effects.

For the present there are insufficient data available to critically assess whether repeated sequence DNAs have a general biological function but the patterns of occurrence within karyotypes do provide an obstacle to the general acceptance of the selfish DNA hypothesis.

The results of this study on *Notomys* suggest at least three avenues for future research which would provide data of relevance to some important questions:

(i) To what extent are *Notomys* satellite sequences conserved? While some aspects of the satellite DNA analyses in this study suggest a degree of conservation across the four species (for example, filter and *in situ* hybridization and the retention of the 22 bp multimer series in restriction digests). Other data suggest some heterogeneity; for example, the failure of the denatured satellite DNAs to return to their native density following renaturation and the presence of some bands in some restriction enzyme digests of a satellite DNA and their absence in others. To extend the satellite DNA study both cloning and sequencing techniques could be used. The isolation of the DNA from one band of the multimere series, for example the decamer band, could be subjected to cloning then sequencing to establish the degree of heterogeneity within the fragment population of this band. The degree of sequence divergence between the smaller fragments of the multimer series and the decamer band could be established. Both these approaches could be extended to the satellite DNAs each of the four species of *Notomys* to provide better information on the degree of divergence between the satellite sequences.

(ii) Is the X chromosome with most accessory gonosomal heterochromatin preferentially inactivated? The effect of accessory gonosomal heterochromatin on X chromosme inactivation could be investigated by comparing females of *N. alexis*, *N. cervinus* and *N. fuscus* heterozygous for X chromosomes with differing amounts of accessory gonosomal heterochromatin. Concurrently, field populations of *Notomys* could be surveyed for polymorphic X-linked enzyme markers to allow genetic testing of the X inactivation of these species.

(iii) If inter-specific hybrids are produced, differing in heterochromatin content on each chromosome, is there any rearrangement in chiasma patterns? In this study inter-specific pairings of *Notomys* were unsuccessful. However artificial insemination might overcome pre-mating isolation and permit the investigation of inter-specific crosses. Meiosis could then be investigated in hybrids heterozygous for widely differing heterochromatin content on each chromosome (for example, *N. fuscus-N. mitchellii*) and the frequencies and distributions of chiasmata examined.

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