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CYTOGENETIC STUDIES ON GENUS MUS (RODENTIA, MURIDAE) WITH SPECIAL REFERENCES TO SPECIES FROM THAILAND

by

Amara Markvong

A Dissertation Submitted to the Faculty of the COMMITTEE ON GENETICS (GRADUATE) In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA

1977
I hereby recommend that this dissertation prepared under my direction by Amara Markvong entitled CYTOGENETIC STUDIES ON GENUS MUS (RODENTIA, MURIDAE) WITH SPECIAL REFERENCES TO SPECIES FROM THAILAND be accepted as fulfilling the dissertation requirement for the degree of DOCTOR OF PHILOSOPHY.

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

Final approval and acceptance of this dissertation is contingent on the candidate's adequate performance and defense thereof at the final oral examination.
STATEMENT BY AUTHOR

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SIGNED: Amara Markvong.
To my mother and father who

genetically

made it all possible
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Contemporary cytogenetic techniques including Q, G, C banding, Silver-NOR, etc., were applied to the chromosomes of 8 species of genus Mus to investigate phylogenetic relationships among members of this genus. These species were M. m. musculus (laboratory mouse), M. m. molossinus (Okinawa), M. caroli (Thailand), M. cervicolor (Thailand), M. cookii (Thailand), M. booduga fulvidiventris (Sri Lanka), M. dunni (India), M. pahari (Thailand), and M. shortridgei (Thailand). Other than M. pahari (subgenus Coelomys) and M. shortridgei (subgenus Leggadilla), all species belonged to the subgenus Mus.

Within the subgenus Mus, all species showed an identical diploid number (2n=40) and identical Q and G banding patterns, indicating very close relationships among them. However, differences in C band distribution and number and locations of nucleolus organizer regions were noted. Mus dunni possessed biarmed X chromosomes and a variable number of biarmed autosomes, one arm of each of which proved to be heterochromatic.

M. pahari had a diploid number of 48. Only a few chromosome pairs of this species had a banding pattern similar to those of the standard M. musculus, indicating that extensive rearrangement of genetic material must have
taken place between the subgenera, Mus and Coelomys. M. shortridgei had a basic diploid number of 46 but individuals sometimes possessed heterochromatic supernumerary chromosomes up to 5. Thus the diploid number varied from 46 to 51. No chromosomes of M. shortridgei had a banding pattern identical to any chromosomes in M. musculus, suggesting that the phylogenetic relationships between the two subgenera Leggadilla and Mus were very remote.

This investigation demonstrated that a cytogenetic approach to the phylogeny of Mus and related taxa should be enlightening and profitable, especially when more species, subspecies, and races are examined.
INTRODUCTION

For decades cytogeneticists have been using chromosomal features (number, size, morphology, behavior, etc.) as a group of characteristics to study species relationships, population structure, and evolutionary trends. Several reasons made this approach appealing: (1) the chromosomal characteristics are among those least affected by selection; (2) the chromosomes represent the vehicles of genetic material of an individual, a population, a subspecies, a species, or higher orders; and (3) the chromosomes can be analyzed directly from tissues of the specimens.

Voluminous literature has been written on cytology and chromosome evolution of both plant and animal materials, but plant cytogenetics was developed earlier than animal cytogenetics. There were several reasons for this:

1. Many plants are of economic importance. Cytogenetic investigations helped plant breeding programs.
2. A number of plants possessed large chromosomes. These plants were utilized for the studies on chromosome structure, chromosome behavior, responses of chromosomes to radiation and chemicals, and other cytological phenomena.
3. Plants can be artificially pollinated with relative ease to obtain interspecific hybrids. The meiotic behavior was extremely useful to analyze the genetic relationship of the taxa involved. It is not easy, even today, to achieve hybridization of animal species.

In the animal kingdom, insects were used as the principal material for cytogenetic analyses in the early period. The value of using insects for evolutionary studies was enhanced when polytene chromosomes were employed to increase resolution. In the second edition of his monumental book *Animal Cytology and Evolution*, White (1954) used much information from studies of lower animals. However, during the last 15 years, especially during the last five years, some very significant advances have been made in mammalian cytogenetics and human cytogenetics. The new edition of White's (1973) book incorporated a great deal of research data from mammals.

The recently developed banding techniques increased the cytogeneticist's ability to resolve somatic chromosomes, even though the bands were not as refined as those in the polytene chromosomes.

Genus *Mus* is one of the largest and most widely distributed genus of mammals. *Mus musculus* is one of the most important laboratory animals for studies in biomedical
sciences. Many wild species of Mus inhabit all of Eurasia, particularly Southeast Asia. The animals affect important crops and some are disease vectors.

Other than M. musculus, cytogenetic studies on the genus Mus have been deficient. The present investigation was suggested by Dr. J. T. Marshall. Dr. Marshall has been revising the taxonomy of this genus for a number of years, using specimens from Asia, particularly from Thailand. He has shipped live animals periodically to me in the United States for cytogenetic work for additional characterization of the species. Because of the paucity of specimens, the present investigation should not be regarded as a systematic work, but as a cytogenetic characterization of available specimens. Nevertheless, a number of interesting cytogenetic phenomena have been found to warrant further exploration, and the data suggest that in some cases chromosome analyses can be useful in systematics of Mus.
LITERATURE REVIEW

Although the study of mammalian chromosomes has a long history, technical difficulties hindered the progress until several methodological improvements were made in the 1950's. Real progress commenced with the finding that treatment of the cell with a hypotonic solution before fixation would spread the metaphase chromosomes. In this review, the discovery of hypotonic solution pretreatment in 1952 was arbitrarily used to divide early and recent developments in mammalian and human cytogenetics.

Prehypotonic Period

The book *An Atlas of the Chromosome Numbers in Animals* (Makino 1951) reported chromosome numbers of many species as extremely uncertain during early periods of cytological investigation. For example, human chromosome numbers were reported to vary from 8 to 48. A detailed historic account on the chromosome studies in man has been presented by Kottler (1974). The main reason for variable results reported in early vertebrate cytology was a technical one. In the metaphase stage, the chromosomes are crowded on the equatorial plate which makes counting difficult even in species with a low diploid number. An analysis of Figure 1 shows that low chromosome number does not insure
Figure 1. Metaphases of male Indian muntjac (2n=7) — a-c: Cells fixed in 45% acetic acid without hypotonic solution pretreatment. Note it is impossible to make an exact count of the chromosomes although the number is low. d: A cell pretreated with a hypotonic solution before fixation. Note excellent spread and good chromosome morphology. Cell stained with acetic orcein.
Figure 1. Metaphases of male Indian muntjac (2n=7).
an accurate count in Indian muntjac, 2n=7. Figures 1a to 1c were taken from a preparation without a hypotonic solution pretreatment. It is extremely difficult or even impossible to count seven chromosomes in these spreads. An extraneous material (probably nucleolar remnants) appears to coat the chromosomes and cause the individual chromosomes to become "glued" together. A hypotonic solution disperses the chromosomes as well as the coating material, so that a crisp outline of the chromosomes can be discerned (Fig. 1d).

Nevertheless, a considerable amount of credible work on mammalian cytology was carried out during this early period, primarily by S. Makino and R. Matthey. Makino (1951) surveyed a variety of species in the animal kingdom, but Matthey (1945) concentrated his effort on vertebrates, especially mammals. Matthey made a number of significant contributions, despite his use of classic cytological techniques. In the African pygmy mouse *Leggada*, a genus closely related to *Mus*, Matthey found that most populations had a basic diploid number of 36 monoarmed (acrocentric) chromosomes. In a few populations Robertsonian fusions (the fusion of two monoarmed chromosomes into one biarmed chromosome) had occurred. He found that even the sex chromosomes could be involved by Robertsonian fusions and in the extreme case, the diploid number was reduced to 18 biarmed chromosomes (for review, see Matthey, 1965).
These findings, based on chromosomal studies of Leggada, together with similar instances in other mammalian taxa, led Matthey (1945) to propose the term fundamental number (NF). The total number of chromosome arms constitutes the fundamental number for a given species. Robertsonian fusions may change the diploid number but not the NF. In the case of Leggada, the NF is 36, but the diploid number may change from 18 to 36. It was found in recent years that although Robertsonian fusion is the prevailing form of karyotypic evolution in mammals, other patterns of chromosomal changes in speciation are also common. Thus NF is not always a useful guide.

Posthypotonic Period

The discovery that a hypotonic environment causes the metaphase chromosomes to scatter constituted a significant advance in the studies on vertebrate cytogenetics. Both Hsu (1952) and Hughes (1952) used hypotonic solutions in conjunction with tissue culture cells instead of solid tissues. A tissue culture with many single cells has the advantage over cells in tissue masses for hypotonic treatment. Treatment is often ineffective in the latter.

In order to study the chromosomes of man, Tjio and Levan (1956) combined tissue culture and hypotonic solution pretreatment with colchicine pretreatment to arrest metaphases and the squash technique to flatten the cells. Their
revision of the human diploid number from the long-believed 48 to 46 suggested that many of the older investigations on mammalian cytogenetics might be erroneous. In the years to follow, only sporadic efforts were made to study mammalian cytogenetics. Before the mid-1960's, however, during this same time interval investigators concerned with human cytogenetics had scored numerous significant advances.

In the mid- and late-1960's a considerable amount of data on mammalian cytogenetics was already available from studies using the techniques of the posthypotonic period (Benirschke 1969). However, mammalian cytogenetics, as human cytogenetics, encountered the obstacle of morphological similarity of many chromosomes. In the human karyotype, for example, the 46 chromosomes could be classified into seven groups, but the only chromosomes which could be unequivocally identified in the best preparations were chromosomes 1, 2, 3, 16, and Y. In the mouse karyotype (2n=40), grouping was not possible because the chromosomes formed a continuous gradation of lengths with little distinction between adjacent pairs. The development of the various banding techniques in the late 1960's and early 1970's permitted longitudinal differentiations of chromosomes as an aid in identification and recognition and gave the field of mammalian cytogenetics added impetus.
Repetitive DNA Sequences and in situ Hybridization

In the 1960's, several advances were made in the analyses of eukaryotic genomes among which was the discovery of highly repetitive DNA fractions. Sueoka (1961) using the crab, *Cancer borealis*, discovered a satellite DNA fraction in neutral CsCl density gradient. Sometime later, Kit (1961) and Szybalski (1961) independently found a minor fraction in the DNA of the laboratory mouse (*Mus musculus*). Subsequent studies led to two important conclusions: (1) all eukaryotic genomes have minor "satellite" fractions, and (2) satellite DNA contains short but highly repeated base sequences (Flamm 1972).

In an effort to identify the chromosomal locations of certain types of nucleic acids, Pardue and Gall (1970) and Gall and Pardue (1971) developed the *in situ* nucleic acid hybridization procedure. These scientists isolated mouse satellite DNA and hybridized it back to the chromosomes and found that the satellite DNA fraction was localized in the centromeric areas of all chromosomes except the Y chromosome (Pardue and Gall 1970). The finding that highly repetitive DNA had a localized cytological distribution was quickly confirmed by many investigators using a variety of chromosomal DNA. These findings became cytologically important because of the correlation between.
repetitive DNA (satellite DNA) and constitutive heterochromatin (C band).

C Band Technique

The in situ nucleic acid hybridization procedure produced an extremely useful byproduct: a method for identifying constitutive heterochromatin (Arrighi and Hsu 1971, Chen and Ruddle 1971). The distribution of constitutive heterochromatin in mammalian chromosomes has been extensively studied by the C band technique (Hsu and Arrighi 1971; Bobrow, Madan, and Pearson 1972; Pathak, Hsu, and Arrighi 1973; Pathak, Hsu, Shirley, and Helm 1973). In many animals, C bands are located at or near the centromeres, but other patterns, such as interstitial, terminal, or even total arms, have also been found. Since C bands and highly repetitive DNA sequences are closely related, and since highly repetitive sequences do not contain genetic information (some investigators, e.g., Ohno 1972, even called them junk DNA), identification of C bands in studies on genomic evolution is of prime importance when interpreting phylogenetic results.

Chromosome Bands

As mentioned, cytogenetic studies in practically all organisms were hampered by the lack of methods to differentiate chromosomes with similar morphological characteristics. The polytene chromosomes have been useful in
genetic work not only because they are large but also because they have characteristic banding patterns which serve as unmistakable landmarks.

Caspersson et al. (1969) first reported that metaphase chromosomes could be differentiated into bright and dull fluorescent zones or bands when the cytological preparations were stained with quinacrine mustard and viewed with UV optics. These fluorescent bands (Q bands) are uniquely characteristic for each chromosome within a complement, and therefore allow identification of rearrangements such as translocations and inversions.

Several investigators, notably Sumner, Evans, and Buckland (1971) and also Seabright (1971) described methods for inducing chromosome crossbands with Giemsa stain (G bands). These G band techniques produced finer zoning of the chromosomes and required only bright-field microscopes instead of UV microscopes. Comparison between Q bands and G bands revealed that the major bands are nearly identical, i.e., bright Q bands are equivalent to dark G bands. At almost the same time, Dutrillaux and Lejeune (1971) devised a technique for staining the R bands which shows the reverse banding pattern of G banding.

One of the prominent landmarks in classic cytology is the achromatic secondary constrictions located in one or more chromosome pairs at specific loci. Cytologists have long associated these secondary constrictions with the
nucleolus, and the term nucleolus organizer (NOR) had been used. In many mammalian karyotypes, however, obvious NORs are absent. Hsu, Brinkley, and Arrighi (1967) speculated, from electron microscopic observations, that NORs of some species may be located at the telomeric ends and being achromatic, therefore escape notice.

Matsui and Sasaki (1973) used a special staining technique to reveal NORs (N banding). Later, Goodpasture and Bloom (1975) applied a silver-staining technique (Ag-AS) to identify the same structures. Both groups found that NORs in some animal species (such as the Chinese hamster) were indeed terminal. The locations of NORs have become an additional cytological characteristic in comparative cytogenetics and chromosomal evolution.

**Mammalian Cytogenetics and Cytotaxonomy**

Diploid Number and Genomic Size

As more and more species of mammals were examined cytologically, the range of the diploid number of this class has been expanded, ranging from 6 in *Muntiacus muntjak* (Wurster and Benirschke 1970) to 92 in *Anotomys leander* (Gardner 1971). The great majority of species exhibits a diploid number between 30 and 50.

The large spread in the diploid numbers does not mean that the variation in the genomic sizes is also enormous. When the chromosome size of various mammals are
compared, it becomes obvious that species with higher diploid number possess smaller chromosomes whereas species with lower diploid numbers possess larger chromosomes. Earlier, Ohno, Bečak, and Bečak (1964) measured the metaphase chromosomes of a number of mammalian species with diversified chromosome numbers and concluded that the total amount of chromatin of these species was similar. This piece of work indicates that a similar amount of DNA (genomic size) was packaged in "containers" of different sizes (chromosomes). Measuring DNA content per nucleus provided similar results (Rees and Jones 1972). Ohno et al. (1964) found variation in the size of the sex chromosomes in a number of species. These investigators found that the X chromosome in some species comprised approximately 5% of the haploid genome. In other species, the X may reach 10%, 15%, or even 20% of the haploid genome. Recently, all large X chromosomes have been found to contain a large amount of constitutive heterochromatin, while the euchromatic portion has remained at approximately 5% of the haploid genome.

Robertsonian Fusion

In practically every taxon thus far studied, e.g., Equidae (Benirschke and Malouf 1974), Tupaiidae (Arrighi, Sorensen, and Shirley 1969), Muridae (Matthey 1965), etc., Robertsonian fusions appear to be the prevailing mechanism of karyological evolution.
According to the classic concept, a Robertsonian fusion requires a break in one of the acrocentrics at the long arm immediately distal to the centromere and a break in the other acrocentric at the "invisible" short arm. An asymmetrical translocation will result in a biarmed element plus a "bare" centromere which is subsequently lost. This scheme would suggest that karyotypes with more acrocentrics are more ancestral since a biarmed chromosome, once formed, cannot be split without acquiring a new centromere. Consequently, species with lower diploid numbers represent the karyologically more advanced forms.

Tandem Translocations

Robertsonian fusion requires a translocation of two acrocentric chromosomes at the centromeric regions. Thus the reduction of the diploid number of a given karyotype is determined by the number of acrocentrics present originally. As in the case of *Leggada* mentioned previously, the lowest diploid number that can be achieved from 36 acrocentrics is 18 metacentrics. A tandem translocation is a translocation that may fuse the centromere region of one chromosome and the telomere of another, or two telomeres. With tandem translocations, multiple translocations can combine several chromosomes into one chromosome. Even biarmed chromosomes can participate in the translocation process. Probably the first conclusive case of tandem translocation in mammals was
found by Stock and Hsu (1973) in comparing the G band patterns of the African green monkey, *Cercopithecus*, and the rhesus monkey, *Macaca*. These investigators suggested that an acrocentric of the green monkey may have fused with the telomere region of a biarmed chromosome to form a larger biarmed chromosome in the rhesus. In such case, a tandem translocation would reduce the fundamental number from three to two.

Perhaps tandem translocation is the most plausible mechanism to explain some unusual cases of karyotypic evolution. The most dramatic example was found in the barking deer genus *Muntiacus*. The diploid number of *M. reevesi* is 46, all acrocentric (Wurster and Benirschke 1967). With Robertsonian translocations, the lowest diploid number reaches 23. Yet in *M. muntjak* (Wurster and Benirschke 1970) the diploid number is 6. Therefore, tandem translocations may have been involved in the karyological evolution of this genus. Similarly, in the cotton rat, *Sigmodon hispidus*, Zimmerman and Lee (1968) found two distinct forms in terms of karyology, viz., one with a diploid number of 52 and the other, 22. Tandem translocation may have been involved.

Translocations involving centromere-telomere are not hypothetical cases in mammals. In human populations, a number of instances have been reported already, and a nomenclature system has been suggested (Hsu, Pathak, and Chen 1975).
Addition (or Deletion) of Heterochromatin

In a number of taxa, related species may possess the same diploid number but a varying number of chromosome arms. This is the opposite of Robertsonian translocation in which the diploid number changes but the number of chromosome arms remains constant. According to classic interpretations, there are several methods for increasing chromosome arms. In certain species where acrocentric chromosomes are common this may be accomplished by pericentric inversions and/or asymmetric reciprocal translocations. Hsu and Arrighi (1968) found that in 19 species of *Peromyscus* the diploid number was always 48, but the number of chromosome arms varies from 56 to 96. Reexamination by Pathak, Hsu, and Arrighi (1973) using C band preparations, revealed that in *P. eremicus* (96 arms) and *P. crinitus* (56 arms) all the short arms are heterochromatic, and the G band patterns of the long arms (euchromatic) of these two species are almost identical. Thus, the two species may differ only in the amount of heterochromatin, and heterochromatin can form new chromosome arms. This finding also indicates the danger of using NF for phylogenetic studies without differentiating euchromatin and heterochromatin. Instances similar to the situation in *Peromyscus* are found to be increasingly common.
Pericentric Inversions

Pericentric inversions can be positively identified only by the comparison of chromosomes which have been properly banded. At the present not many such preparations have been reported, but it is reasonable to conclude that pericentric inversion definitely exists as a force of karyological evolution in mammals. Arrighi, Stock, and Pathak (1976) compared the chromosomes of Peromyscus leucopus and P. crinitus and found several sets of pericentric inversions. Ward (1976) also found such a case in P. boylei. These findings indicate that in Peromyscus (or any other taxon) a variation in the number of chromosome arms may be due to the addition of heterochromatin, pericentric inversion, or both, in addition to Robertsonian fusions or tandem translocations. In Neotoma, Mascarello and Warner (1974) found a pericentric inversion involving a segment of heterochromatin.

Cytogenetics of Mus

The laboratory mouse (Mus musculus) has been extensively used in genetic studies and countless number of inbred strains are available. Although all 20 linkage groups in the lab mouse have been established correlation between linkage groups and chromosomes were not made because the chromosomes could not be distinguished by gross morphology using conventional staining techniques. Stich and
Hsu (1960) reported that the smallest autosome pair (No. 19) could be identified because there was a discontinuity in length gradation between the next smallest and No. 19, as well as a secondary constriction near its centromeric area. In the male somatic cells, three chromosomes of the size range of No. 19 were found. These investigators concluded that the third chromosome must be the Y chromosome. These characteristics have been used as cytological markers by biologists working on transplantation experiments. Levan, Hsu, and Stich (1962) presented a detailed analysis of the mouse karyotype and concluded that the X chromosome must be one of the largest chromosomes but there was no precise way of identifying it.

In general, Levan et al. (1962) found that mouse chromosomes were extremely poor for cytogenetic studies. However, some interesting discoveries were made despite the poor cytological quality of the lab mouse chromosomes. In valleys in the Swiss Alps, Gropp, Tettenborn, and von Lehmann (1970) found that the karyotype for *M. poschiavius* contained 14 biarmed chromosomes and 12 acrocentrics (2n=26). Thus, the NF remained the same as that of *M. musculus*. Presumably seven sets of Robertsonian fusions had occurred from the original 40 acrocentrics. Later, Gropp et al. (1972) and Capanna, Civitelli, and Cristaldi (1973) found that the *M. musculus* populations of the Alpine valleys displayed a varying number of biarmed chromosomes without
changing the NF. Yet in other parts of the world, Robertsonian fusion of the mouse chromosomes was rarely seen.

Banding techniques changed the gloomy picture of mouse cytogenetics into a bright one. Every chromosome of *Mus musculus* can now be readily identified and a standard banding nomenclature system has been proposed (Committee on Standardized Genetic Nomenclature for Mice 1972). All the fused chromosomes of *M. poschiavinus* have been identified by Q-banding (Zech et al. 1971). Tettenborn and Gropp (1970) found that individuals of *M. poschiavinus* crossed with *M. musculus* produced viable offspring, but trivalent formation in hybrid meiosis led to an increased frequency of aneuploidy, thus reducing fertility.

Cytogenetics of the lab mouse made a great stride forward after the banding innovations. Using these new techniques, linkage groups were identified in numerous live stocks which carry reciprocal translocations. By examining the banded karyotypes of different translocations, it was then possible to correlate linkage groups and chromosomes. At the present, practically all linkage groups have proper chromosome assignments (Miller and Miller 1972, Miller and Miller, 1975).

**Taxonomy of Mus**

The rodent family Muridae includes the Old World rats and mice. It is believed that Muridae did not emerge-
until late in Miocene. The members of this family are most abundant in the tropics and subtropics (Walker et al. 1964). The genus *Mus* apparently originated in the Old World, more specifically in Asia, where numerous indigenous taxa are found. However, *M. musculus* is now a cosmopolitan species distributed throughout the world by unintentional introduction by man.

The genus *Mus* Linnaeus, 1758, belongs to the Order Rodentia, Family Muridae, Subfamily Murinae. The mice in this genus have similar characteristics: upper incisors notched, first molar is more than half the length of molar row and has no postero-internal cusp, head and body about 100 mm in length, planter pad round rather than long and pointed, prelambdoidal fenestra exposes a slender para-occipital process at least in young, anterolateral corner of the parietal bone projects forward in a point, mammae 3+2 (Marshall 1969, 1972).

The taxonomy of *Mus* has been extremely complex and confusing. One of the latest attempts of classification was made by Ellerman (1947), who tried to define species and to group supposed species into subgenera by utilizing skull measurements as the principal criteria. However, he neglected the shape of the skull and other qualitative differences such as dentition. Taxonomy often results in confusion when one relies solely upon only one set of characteristics. For example, Allen (1927) confused *M.
caroli and M. musculus; Ellerman (1947) grouped M. nagarum
and M. cervicolor as one species.

Marshall (1969) classified the murid rats and mice of Thailand by using skin and skull morphology. His key provides good identification systems for rats, but not for mice since skull differences between sibling species are slight or wanting. He had to utilize other criteria, such as ectoparasites, gel electrophoretic patterns, serology, chromosomes, etc., to confirm the species and subspecies status of the mice studied. In a subsequent paper Marshall (1972) classified the entire genus Mus into three subgenera, viz., Leggadilla, Coelomys, and Mus (Appendix A). A recent article by Marshall (1975) added more data on some of the Mus species but did not change his classification system. In the present investigation, Marshall's classification is followed for convenience.

1. Subgenus Leggadilla (spiny mice), with supraorbital ridges for the attachment of masticatory muscles. Included are M. shortridgei from Thailand, M. platythrix, M. phillipsi, and M. fernandoni from India.

2. Subgenus Coelomys (shrew mice), with broad frontals and small eyes. Included are M. pahari from Thailand, M. mayorii from Sri Lanka, M. famulus from India, M. crociduroides from Sumatra, and M. vulcani from Java.
3. Subgenus *Mus* (house mice and commensal allies), with skull ridges or broad frontal bones, long incisive foramina, and no spines. Included are *M. caroli*, *M. cervicolor*, *M. cookii*, *M. musculus castaneus* from Thailand and Eastern Asia; *M. kakhyenensis*, *M. booduga*, *M. fulvidiventris* (which may be a subspecies of *M. booduga*), *M. dunni* from India; and some subspecies of *M. musculus* from Europe and the Middle East.

The laboratory mouse strains are considered to be derived from two subspecies of *M. musculus*, *M. musculus musculus* (Swiss mice) and *M. m. molossinus* (Marshall 1972). The former were found inside human dwellings and were introduced to the Americas, the latter were the progenitor of the small Asian laboratory mouse, which is common in Japan.

**Cytotaxonomy of Mus**

The karyology of the genus *Mus* is a complex one. In subgenus *Mus*, the diploid number appears to be 40 in all known cases analyzed in Southeastern Asia (Matthey and Petter 1968; Duncan 1973; Yosida et al. 1969; Markvong, Marshall, and Gropp 1973). With the exception of *Mus dunni*, all species have telocentric chromosomes. However, in Europe, individuals of *M. musculus* may have a varying number of biarmed chromosomes with a concomitant reduction of the diploid number. In the extreme case, the karyotype contains
18 biarmed chromosomes and four acrocentrics, two of which being the sex chromosomes. In *M. poschiavinus*, the karyotype consists of 14 biarmed chromosomes and 12 acrocentrics.

*Mus dunni* is karyologically unique. Matthey and Petter (1968) analyzed some 30 mouse specimens collected in Madras, India. Matthey found that the mice had two distinct karyotypes, one with the usual 40 telocentrics and the other with from 6 to 13 submetacentrics or subtelocentrics, but the diploid number remained 40. Moreover, he found that the short arms of these biarmed chromosomes varied in length, and that the biarmed X chromosome and telocentric Y chromosome were very large. When Petter examined the skin and skull specimens without knowing the karyological data, he separated them into two groups, and the cytological data and the taxonomic data completely coincided. These two investigators concluded that these mice represented two sympatric species, *M. booduga* (40 telocentrics) and *M. dunni* (40 chromosomes with a varying number of biarmed chromosomes). Later, cytological confirmation for *M. dunni* was made by Markvong et al. (1975) and Sharma and Garg (1975). These investigators found that the short arms of *M. dunni* are totally heterochromatic, and that the G band patterns of the euchromatic long arms are identical between *M. musculus* and *M. dunni*, a situation analogous to that of *Peromyscus*.

The diploid number of subgenus *Leggadilla* is highly variable. Specimens of *M. shortridgei* from Thailand
displayed a variable diploid number, from 46 to 49 (Gropp, Marshall, and Markvong 1973), two to three of these chromosomes are small metacentrics or submetacentrics and the remainder, acrocentric. The India species *M. saxicola gurkha* displays a diploid number of 22 with four biarmed chromosomes (Pathak 1976), but *M. s. sadha* had a diploid number of 24, 25, and 26 (Dhanda et al. 1973). *M. platythrix* had 2n=26 (Tsuchiya and Yosida 1972, Satya Prakash and Aswathanarayana 1972b). Unfortunately, no banding analyses have been made on these species.

Only one species of subgenus *Coelomys* has been karyologically analyzed. This is *M. pahari* from Thailand (Gropp et al. 1973), with a diploid number of 48.
MATERIALS AND METHODS

Animals

A total of 45 animals belonging to 8 species was used in this study, 11 of which were from laboratory colonies and the other 34 animals were trapped in their natural habitat in various parts of Thailand, India, and Sri Lanka. The number of specimens and their capture localities are shown in Appendix B. All wild-caught specimens were collected and identified on the basis of cranial and external morphology by Dr. Joe T. Marshall. The voucher specimens, with number corresponding to the karyogram, were deposited at the Smithsonian Museum, Washington, D.C., USA.

Reagents

Cell Culture

1. **Growth Medium.** Modified McCoy's 5a formula (Reference No. 7929 Grand Island Biological Co., Chagrin Falls, Ohio) supplemented with 20% fetal bovine serum (also from Grand Island).

2. **CO₂/Air Mixture.** The mixture (10% CO₂ and 90% air) was prepared by the Big Three Company, Houston, Texas, and was used to replace the regular air in culture flasks to maintain proper pH.
3. **Rinsing Solution.** The rinsing solution was Hanks' Balanced Salt solution without Ca$^{++}$ and Mg$^{++}$.

4. **Trypsin Solution.** Stock trypsin solution was 0.02% crystalline trypsin (3x crystalized, Worthington Biochemical Corp., Freehold, New Jersey) dissolved in rinsing solution, pH 7.8.

**Cell Harvesting**

1. **Colcemid Solution.** Colcemid was purchased from CIBA Pharmaceutical Company, Summit, New Jersey. The stock solution was 0.02% dissolved in distilled water.

2. **Proteolytic Enzymes.** Stock trypsin solution or 0.1% Pronase solution (Worthington Biochemical Corp., Freehold, New Jersey) was used to dislodge monolayer cells in cultures.

3. **Hypotonic Solution.** Two types of hypotonic solutions were used: 0.075 M KCl for bone marrow and a diluted medium consisting of one part of growth medium to 2.5 parts of distilled water for cell cultures.

4. **Fixative.** Carnoy's mixture (1 part glacial acetic acid and 3 parts methanol) was used.

**Giemsa Staining**

1. **Giemsa Stain.** Stock Giemsa solution was purchased from Gurr Bio/Medical Specialties, Los Angeles,
California, and made to 2% or 4% by diluting with Sorensen's buffer (0.01 M NaH$_2$PO$_4$ and 0.01 M Na$_2$HPO$_4$, pH 7.0). This stain was used for all staining procedures except autoradiography where a 10% Giemsa was used.

2. **Standard Saline Citrate (SSC).** A 10x concentrate SSC (0.15 M sodium citrate and 1.5 M NaCl) was diluted with distilled water to the desired concentration.

3. **HCl.** This solution was 0.2 N.

4. **NaOH.** This solution was 0.01 N in 2x SSC.

**Fluorescence Staining**

1. **Quinacrine Mustard (QM).** This compound was provided by D. G. Borgoankar, Johns Hopkins Hospital, Baltimore, Md. The concentration was 0.05% in distilled water.

2. **33258 Hoechst.** This fluorochrome was provided by Alfred Gropp, University of Lübeck, Germany. The stock solution was 50 µg/ml dissolved in Hanks' balanced salt solution and diluted to 0.05 µg/ml for staining.

3. **Buffer Solution.** The buffer solution was 0.2 M Na$_2$HPO$_4$ adjusted to pH 5.6 with 0.1 M citric acid.

4. **Krönig Cement.**
Silver-Impregnation Staining

1. **Silver Nitrate Solution.** This solution was 50% silver nitrate in deionized water, and filtered through a milipore filter (Type VC 0.10 μm).

DNA Synthetic Patterns

1. **3H-thymidine.** Purchased from New England Nuclear (Boston, Mass.) with a specific activity of 6 Ci/mM. Stock solution was prepared at 100 μCi/ml in rinsing solution, and diluted to 1 μCi/ml in growth medium.

2. **Autoradiographic Film.** Kodak AR-10 stripping film.

Procedures

Cytological Preparations

Slides were prepared by adding a few drops of distilled water to slides recently cleaned with acetone. Cells suspended in the Carnoy's fixative were allowed to drop onto the slide from a height of about 3 ft down to the prepared slide. The slide was then tilted slightly to drain the excess liquid and allowed to dry.

When bone marrow cells were used for cytological observations, the chromosome spreading was usually poor in air-dried preparations. This defect was remedied by dropping the cell-containing fixative on to the slide and immediately igniting the fixative until it was dry.
However, cells which were flame-dried often did not produce good banding results.

Bone Marrow Preparations

Each animal received an intraperitoneal injection of Colcemid solution in the amount of 0.003 ml/gm of body weight 1 hr prior to sacrificing. The animal was then anesthetized with methoxyfluorane, and the bone marrow from femurs and tibiae were flushed out with a small quantity of a hypotonic solution (0.075 M) injected by syringe. The marrow cells from one animal were pooled and suspended in approximately 5 ml of the KCl solution for 20-25 min before centrifugation at 200 g for 5 min. The cell pellet was then fixed in methanol-acetic acid (3:1) for 30 min. The cells were resuspended and centrifuged three times in freshly prepared Carnoy's mixture before being air-dried or flame-dried on slides for cytological observations.

Cell Culture

Biopsy or autopsy tissues were minced with sterile surgical blades into very fine fragments under aseptic conditions. When whole fetuses were used, fetuses were minced with small curved scissors in a Petri dish containing a small amount of growth medium. Tissue fragments were then transferred into either glass T-60 or plastic T-75 flasks. The amount of medium varied according to the amount of tissues used, usually not more than 20 ml. The air in the
flasks was replaced with sterile air-CO$_2$ mixture (9:1), and incubated at 37°C.

Generally, after an overnight incubation, fetal tissues began to attach to the floor of the flask and cellular growth could be observed. The flasks were shaken vigorously daily to dislodge the tissue fragments in order to achieve monolayer cultures. The medium was changed at least three times per week. The cultures were periodically observed with an inverted microscope. When the cellular growth was nearly confluent, the culture was again shaken by hand and the suspended tissues and cells were transferred into another flask. The attached cells in the original flask were refed until they were ready for subculture or harvest.

Subculturing was performed by decanting the growth medium and incubating the cells in the trypsin solution. As soon as the majority of the cells became free-floating, the cell suspension was transferred into a sterile conical centrifuge tube for centrifugation. Depending upon the size of the pellet, the cells were planted in one or two fresh flasks for subculture. The original flask, which still contained a sufficient number of cells, was usually saved for new growth after adding fresh medium.

For adult tissues, the initial growth period was usually much longer. Some tissues, e.g., ear, heart, etc., rarely attach to the floor of the culture flask, but would
shed cells as they floated. Actually this behavior gave a more evenly distributed monolayer culture and yielded less contact inhibition. Primary cultures were generally harvested for adult Mus tissue since there was little cellular growth.

Conventional Staining

Air-dried slides were placed in a 4% Giemsa solution in 0.01 M phosphate buffer (pH 7.0) for 5 to 7 min, rinsed with running deionized water, allowed to dry, and mounted with Permount or Eukit.

C Banding

The original C band procedure (Arrighi and Hsu 1971) simplified by Hsu (1973) was used throughout this study. This procedure employed the following steps:

1. Slides were treated with 0.2 N HCl for 10 to 30 min and rinsed with deionized water thoroughly.

2. Slides were treated with 0.01 N NaOH for 1 to 2 min and rinsed in 70% and 90% ethanol for 7 to 10 min each, and allowed to dry.

3. Moist chambers were prepared for incubation by wetting the bottom of 120 mm square Petri dishes with 10 to 15 ml of 2x SSC. Grommets were placed as stands for the slides which were placed horizontally with the cell side up. Three drops of
2x SSC were added onto the cell area of each slide and covered with a 22 x 50 mm coverslip.

4. The Petri dish was covered and incubated in an oven at 60°C overnight.

5. Slides were rinsed in 2x SSC three times for 5 min each.

6. Slides were dehydrated through 70% and 95% alcohol for 5 min each and air-dried.

7. Slides were stained in 4% Giemsa solution for 3 to 5 min.

G Banding

The G banding procedure followed that of Seabright (1971) with slight modifications: the air-dried slides were further dried in an oven at 60°C for at least 20 hr and cooled to room temperature before processing.

Five Coplin jars were arranged as follows: diluted trypsin solution containing stock trypsin solution diluted with rinsing solution (1:4 up to 1:1), rinsing solution, 70% ethanol, 95% ethanol, and Giemsa staining solution (2 to 4% in 0.01 M phosphate buffer).

Successful G banding depends on many factors and the duration of the trypsin treatment necessary to induce readable G bands was variable. Treatment time usually was from 10 to 60 sec for 1:1 dilutions and from 30 sec to 3 min for 1:4 dilutions. Each slide was then quickly passed
through the rinsing solution and then the alcohols, and finally was allowed to dry. The preparations were then stained in Giemsa solution for 4 to 7 min, rinsed in running deionized water, and air dried again.

Each preparation was microscopically examined to determine the best time for trypsin treatment. The criterion for best time was that the major G bands should be sharply defined and the minor bands should not be obliterated by overtreatment. Once such a suitable time treatment was found, several more slides were processed in the same manner. Under-treated preparations were re-dipped into trypsin solution for a few seconds and restained until proper differentiation was achieved.

Q Banding

In the present study, two fluorochromes, quinacrine mustard (QM) and the benzimidazole derivative 33258 Hoechst (H. 33258), were used. Although the Q banding patterns produced by the two compounds are very similar, one significant difference is well known; viz., the centromeric heterochromatin of Mus musculus shows a dull fluorescence with QM (Committee 1972) but bright fluorescence with H-33258 (Hilwig and Gropp 1972). In this present study, H-33258 produced generally superior results and therefore was used in most fluorescence preparations.
The staining procedure for both fluorochromes mainly followed that of Hilwig and Gropp (1972) slightly modified by Jalal, Markvong, and Hsu (1975). Each air-dried slide was refixed in Carnoy's mixture for 10 to 30 min, passed through 95% and 70% ethanol for 10 min each, and hydrated in two changes of distilled water for 2 min each, and air-dried. A few drops of the fluorochrome solution were added onto the slide to stain the cells and a coverslip was placed over the staining solution. The slide was then inverted for approximately 7 min after which the coverslip was removed by letting it slide off gently in distilled water and the slide was air-dried again.

The slide was then mounted in the buffer solution, covered with a coverslip, and sealed with Krönig cement. For H-33258 staining, the slides were stored in the dark at room temperature for at least two days and, in some cases, up to several months. Storing enhanced fluorescence stability under UV excitation. In many cases, the same cell could be repeatedly exposed without losing the ability to fluoresce. On the other hand, slides stained with QM had to be examined and photographed immediately. Therefore H-33258 proved to be much more convenient for Q banding than QM.
Silver Staining for Nucleolar Organizers

The ammoniacal silver staining technique, originally proposed by Howell and Denton (1974), specifically stains the nucleolus organizer regions (NOR) of metaphase chromosomes. This method is much simpler than the in situ hybridization technique and gives better microscopic resolution. Subsequently Bloom and Goodpasture (1976) further simplified the technique, which was the one used in this study.

Three or four drops of a 50% silver nitrate solution were placed over the cells of conventional air-dried preparations. A coverslip was placed over the AgNO₃ solution and the slide incubated in a moist chamber at either 60°C or 37°C. Usually incubation at 60°C required 2 to 4 hrs whereas incubation at 37°C required an overnight treatment. However, the time required for proper staining was variable and thus periodical monitoring was necessary. In properly silver-impregnated preparations, the nuclei and the metaphase chromosomes are light yellowish in color while the nucleoli and the NORs appear dark brown or black. The slides were washed in deionized water and left to dry. The preparation was sometimes lightly counter-stained with Giemsa.
DNA Replication Sequences

The experiments of the present study were limited to the analysis of terminal stages of the S phase. Tritiated thymidine (1 μCi/ml) was fed to actively growing cultures 3 hrs before harvest (the continuous labeling method). During the last hour, the cultures were treated with Colcemid. This procedure invariably yielded mitotic cells of two types, those originally in G₂ phase (unlabeled) and those in the late S phase (labeled during the terminal S phase).

Harvest and slide preparation procedures as well as the autoradiographic method followed those recommended by Schmid (1965). The slides, when thoroughly dried, were placed inside covered plastic slide boxes and stored under refrigeration to minimize background grains. Exposure time was 4 to 6 days.

The autoradiographs were developed in Kodak D19B developer at 20°C for 2 min, fixed in acid fixer for 2 min, and washed for 5 to 10 min in running water at 20°C. After drying, the autoradiographs were stained in 2% Giemsa solution for 7 min.

Meiotic Preparation for Diakinesis Cell Study

The procedure followed that of Evans, Breckon, and Ford (1964). Testes of adult males were excised, minced in 1% sodium citrate, and transferred to a 15 ml conical
centrifuge tube. After approximately 10 min at room temperature, the supernatant containing suspended cells was transferred to another centrifuge tube for an additional 5 to 10 min and centrifuged at 200 g for 5 min. The cell pellet was then fixed with the regular methanol-acetic mixture (3:1) and air-dried preparations were made. This method yielded a large number of cells in diakinesis, metaphase I and II.

The association of sex chromosomes can be seen clearly in the diakinesis stage. To identify the centromeric ends and heterochromatin, C banding was applied to these slides.

Meiotic Preparation for Pachytene Study

Testicular tissues of adult male mice were minced in a mixture consisting of 1 part trypsin solution (regular trypsin solution used for dislodging monolayer cells) and 9 parts growth medium. After mincing, the tissue fragments were placed in the trypsin medium and magnetically stirred for 4 hours at 4°C. The cellular material was washed twice with growth medium and then incubated at 37°C in growth medium for 3 to 4 hours.

After incubation, the cells were centrifuged, fixed in Carnoy's mixture, and air-dried. The preparations were then stained in 2% Giemsa for 4 min. This method yielded
good pachytene and diplotene cells with chromomeres arranged as if they were bands (Pathak, Hsu, and Markvong 1976).

Sister Chromatid Differential Staining

To demonstrate differential sister chromatid staining, the cells must be grown in medium containing 5-bromodeoxyuridine (BrdU) for two cell cycles. The present study was designed to show late replicating zones by using a 2+ cycle regimen. Cultures were incubated in medium containing BrdU (10 μg/ml) for 24, 30, 36, and 42 hours prior to harvest because the exact cell cycle time was unknown.

The harvest and slide preparation procedures were conventional. The prepared slides were treated in 1 M phosphate (Na₂HPO₄) buffer solution, pH 8.0, at 90°C for 90 min, rinsed in tap water, and stained in 4% Giemsa. Samples containing metaphases having 2+ cycle BrdU incorporation were selected for observation. Those with strictly two cycle BrdU incorporation displayed one lightly stained and one heavily stained chromatid in every chromosome and those with 2+ cycle BrdU incorporation showed areas with double-light segments.
RESULTS

Mus musculus

Karyotype

The chromosomes of M. musculus (2n=40) have been known to cytologists for decades. All chromosomes are acrocentric (the terms acrocentric and telocentric are used here interchangeably), and when arranged from largest to smallest, their lengths form a smooth gradation in size except for the shortest pair, pair 19. It is not feasible to group the chromosomes when stained by conventional methods (orcein, Giemsa, etc.) without banding (Levan et al. 1962). The X chromosome is among the longest of the chromosomes, and the Y is similar in length to the shortest autosomal pair. Figure 2a presents such a karyotype taken from an embryonic cell culture of the Swiss strain. The pairing of the chromosomes is by length only. The only morphological characteristic other than length is the presence of secondary constrictions which are located near the centromeres of several chromosomes (Figure 2a).

Application of the fluorescent stain quinacrine or Hoechst 33258 to the cytological preparations of the mouse has enabled significant advances to be made in murine karyology. Each chromosome has a characteristic
Figure 2. Karyotype of *Mus musculus* -- a: Conventional Giemsa stain, ♀ cell. b: Q banding, by Hoechst 33258, of a ♀ cell.
Figure 2. Karyotype of *Mus musculus*. 
fluorescence pattern which provides positive recognition (Figure 2b). The arrangement of the chromosomes in Figure 2b follows the standard system (Committee 1972). Throughout the present study, the 20 pairs of chromosomes were arranged in four rows and consequently from 1 to 19, with the sex pair (♀XX or ♂XY) placed last. The present data completely agree with the mouse standard banding patterns (Committee 1972, Nesbitt and Francke 1973). It should be pointed out, however, that in this study Hoechst 33258 was used instead of quinacrine. With H-33258, the centromeric areas of the chromosomes show bright fluorescence but dull fluorescence with quinacrine. However, the banding patterns (Q band) of the euchromatic arms are identical between the two fluorochromes.

For routine analysis and convenience, most cytogeneticists prefer Giemsa banding (G bands). Figure 3a presents a G banded karyotype of a male M. musculus. In general, the bright Q bands correspond to the dark G bands; but in G banded preparations more details (finer bands) can be observed. The Y chromosome is relatively dark throughout its length.

Figure 3b presents a C banded karyotype of the mouse. Here the arrangement is arbitrary because the G bands of the euchromatic arms are not present. It can be seen that in M. musculus the constitutive heterochromatin is confined to the centromeric area of each chromosome, but
Figure 3. Karyotype of *Mus musculus*, ♀ cell -- a: G banding, the nomenclature system follows the standard system (Committee 1972). b: C banding.
Figure 3. Karyotype of *Mus musculus*, ♂ cell.
the amount of the C band material is somewhat variable (Figure 3b). One pair of medium-sized autosomes appears to have much smaller amount of C bands than other chromosomes.

DNA Synthetic Patterns

Only the terminal stages (late S) of DNA synthetic patterns were analyzed in the present study. The C band (centromeric) areas were found to be late replicating but were not the latest to complete replication. Figures 4a and 4b show late S and very late S patterns, respectively, of male cells, and Figures 4c and 4d, of female cells. Even in Figures 4a and 4c, some of the C band areas already showed very light grain distribution or no grain; but in the majority of cases both the centromeric areas and the euchromatic arms showed label. When the overall label is light (Figures 4b and 4d), indicating the very end of S phase, most centromeric areas are devoid of label and most of the grains are over the middle segments of several chromosomes.

The Y chromosome, as expected from data collected by investigators working on other mammals, was relatively late replicating. In 19 metaphases with light or very light grain patterns, the "hot Y" was found in 13 cases. Figure 4b depicts such a cell.

The identity of the late replicating X in the mouse cells without banding is of course conjectural, since the X
Figure 4. *Mus musculus*, autoradiographs showing late S phase patterns -- a: Moderately heavy label, ♀ cell. Identification of the Y is not certain. b: Light label, ♂ cell. Most label is in the euchromatic arms, not the heterochromatin in the centromeric areas. The Y is heavily labeled. c: Moderately heavy label, ♂ cell. Two long chromosomes are more heavily labeled (arrows). d: Very light label, ♀ cell. The presumed late-replicating X is more heavily labeled than other chromosomes.
Figure 4. *Mus musculus*, autoradiographs showing late S phase patterns.
is not morphologically unique. In a number of metaphases exhibiting late S grain patterns, one or more long chromosomes may be found to show more label than the others. In Figure 4c, two such chromosomes were noted, and in Figure 4d, one. The latter is presumed to be the hot X. Table 1 suggests that the hot X can be detected in the majority of cases.

Table 1. Tritiated labeled metaphase of Mus musculus (?) cells and the degree of chromosome labeling. Number of cells showing label intensity over the entire metaphase in relation to the number of long chromosomes labeled more heavily (hot X included ?) than the remaining chromosomes. Continuous $^3$H-thymidine labeling was 3 hr and autoradiographs were exposed for 4 days.

<table>
<thead>
<tr>
<th>Number of chromosomes more heavily labeled than others</th>
<th>Label over entire metaphase</th>
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<tr>
<td></td>
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<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>1</td>
<td>4</td>
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<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>&gt;2</td>
<td>7</td>
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</table>
Male Meiosis

Pathak et al. (1976) were successful in constructing a pachytene chromomere map of the male Chinese hamster and were able to show that the chromomere pattern of each bivalent matches its G band pattern of the somatic chromosomes. Since observations on the meiotic chromosomes should reveal cytological details in crosses between individuals carrying rearrangements and in interspecific hybrids, an attempt was made to apply the procedure of Pathak et al. to testicular cells of the mouse. The results were disappointing mainly because the pachytene bivalents of the mouse tended to adhere to one another at the centromeric ends. In a number of cases as many as three or four bivalents were found connected. Because of this undesirable phenomenon, the pachytene bivalents tended to overlap one another, and good spreads were extremely rare. Figure 5a shows the best spread obtained after scanning several hundred pachytene nuclei using a variety of technical modifications. When the sticking problem is resolved, pachytene bivalents are extremely useful in cytogenetic analysis. If indeed the pachytene pattern is equivalent to the G band pattern, then one may be able to identify every bivalent. However, Figure 5b shows that most of the pachytene cells have an inferior bivalent pattern. For example, in the upper right corner, three bivalents are attached to one
Figure 5. *Mus musculus* pachytene spermatocyte, showing the chromomere distribution patterns -- a: An optimal spread of chromosomes. b: Typical of most pachytene cells showing some bivalents clumped together. Note X-Y chromosomes forming a sex vesicle as a round dark structure.
Figure 5. *Mus musculus* pachytene spermatocyte, showing the chromomere distribution patterns.
another at their centromeres and, in the lower left corner, five bivalents are clumped together.

Since the results were not completely successful, further experimentation was discontinued. Nevertheless, the preliminary data suggested the possibility of employing pachytene bivalents for cytogenetic studies of the lab mouse when techniques can be improved to disperse the bivalents within the pachytene and diplotene nuclei.

Nucleolus Organizers and the Silver Stained Substances in Male Gametogenesis

The ammoniacal silver technique for staining NORs was applied by Goodpasture and Bloom (1975) to a number of mammalian species, including *Mus musculus*. In all cases, these investigators found that the chromosomal areas with special affinity for the silver stain (argentophilia) coincided with secondary constrictions in conventional preparations. Moreover, all the species analyzed by Goodpasture and Bloom had previously been investigated by means of *in situ* hybridization using $^3$H-rRNA as a probe. Thus silver staining of metaphase chromosomes appeared to be a good method for identifying NOR. In the lab mouse (Swiss strain) Goodpasture and Bloom (1975) found a maximum of six NORs in the somatic cells, all of which were located immediately distal to the C bands. The Goodpasture-Bloom conclusion was verified in the present study using an improved silver-staining technique (Bloom and Goodpasture 1976). Figure 6a
Figure 6. *Mus musculus*, *♂* cells, with silver staining for nucleolus organizer region (Ag-NOR) — a and b: Metaphase somatic cells showing six and four NORs (immediately distal to the centromeric C band) respectively. c: Interstitial cell with scattered silver-stained areas, probably representing nucleoli or remnants of nucleoli. d: Sertoli cell, only one large round nucleolus (N). The less deeply stained of one or two pieces representing heterochromatic material (H). e: Spermatogonial cells, the nucleoli are intensely stained with a structure of a wound cord, note the presumed C bands scattered over the nuclei (H).
Figure 6. *Mus musculus*, ♀ cells, with silver staining for nucleolus organizer region (Ag-NOR).
and 6b show two cells with six and four NORs respectively. It should be noted that the C band areas are also argenta-philic; but in better preparations the distinction between C band and NOR is more clear. C bands are at the tip of each chromosome whereas the NOR is distal to the C band and is usually represented by two round bodies, one in each chromatid.

To verify further that three pairs of chromosomes bear NORs an attempt was made to apply the same staining technique to testicular preparations. In this case one would expect three bivalents with silver staining. Unexpectedly a series of interesting phenomena were observed which are described in this section. Figure 6c shows a nucleus of an interstitial cell with scattered silver-stained areas, probably representing nucleoli or remnants of nucleoli. However, in the Sertoli cells, only one large nucleolus was generally observed (Figure 6d). The C band material, usually in one or two pieces, was less deeply stained with silver. The nuclei of the Sertoli cells were distinguished from those of the spermatogonial elements by the distribution of the C bands (less deeply stained chromatin). In each spermatogonial nucleus, there is usually one nucleolus. The nucleolus becomes intensely stained with a structure suggesting a wound cord or cords. The assumed C bands, however, are scattered (Figure 6e). These phenomena suggested that the orientation of
heterochromatin in interphase nuclei was cell specific or even stage specific.

As expected, three silver deposits were observed in each pachytene nucleus (Figures 7a, 7b), indicating the locations of NORs. These bodies with silver deposit were also found in early spermatids (round nucleus stage). In addition, C bands, when present, were less intensely stained (Figure 7c). As spermiogenesis proceeded, however, a series of phenomena were observed in silver-stained preparations. When the nucleus began to elongate, the relics of the NORs were still detectable (Figure 7d), but a necklace-like ring with intense silver staining developed outside of each nucleus. This ring closes up to form a line associated with the nucleus (Figure 7e and 7f). A complex configuration can be noted as spermiogenesis develops further (judged by the size and the shape of the spermatid nuclei). The silver-stained substance(s) are associated at one side of the nucleus but extending to the tail area, usually in the form of a hook (Figure 8a and 8b). This structure then loses its integrity, appears degenerated (Figures 8c and 8d), and finally disappears (Figure 8e). In mature spermatozoa (Figure 9) only a small argentaphilic spot is present at the junction between the nucleus and the tail.

Using cell fractionization techniques to purify various types and stages of cells in male gametogenesis, Platz et al. (1975) analyzed the proteins of the various
Figure 7. *Mus musculus, d* cells, Ag-NOR staining of testicular cells — a and b: Pachytene nuclei with three clusters of silver deposits, corresponding to three bivalents carrying NORs. c: Early spermatids with two to three silver stained NORs. d: An extranuclear ring of silver-stained substance appearing in the mid spermatid. e: The ring starting to fold in the later spermatid, f: The complete folding of the ring to a thick band as the spermatid develops further.
Figure 7. *Mus musculus*,♂ cells, Ag-NOR staining of testicular cells.
Figure 8. *Mus musculus* with Ag-NOR staining of sperm cells at late stage of development. a and b: Sperm showing silver stain substance(s) forming the lines along one end of nucleus to other end with the hook-like structures. c and d: Decomposition of the silver stained substance(s). e: No silver stained line along the sperms at later stages but the breakdown of the silver stained substance(s) is evident.
Figure 8. *Mus musculus* with Ag-NOR staining of sperm cells at late stage of development.
Figure 9. Mature spermatozoa of *Mus musculus*, Ag-NOR staining -- a: Entire mature spermatozoa. b and c: Enlargement of the head region, note only a small silver-stained spot (arrow).
cell fractions by means of polyacrylamide gel electrophoresis. These investigators found that between the early spermatid stage and the mature spermatozoa, new proteins were synthesized and discarded. Whether one or more of these newly synthesized proteins correspond to what was found in the silver-stained preparations of the present study remains unknown. This problem is of sufficient interest to warrant further exploration, especially the correlation between biochemical and cytological findings.

**Mus musculus molossinus**

It is easy to see from Figures 10 and 11 that the chromosomes of *M. m. molossinus* are not arranged in the exact size order as in the laboratory mouse. The reason is that in *M. m. molossinus* the distribution of heterochromatin is uneven among the chromosomes (Figure 11b). According to Q banding (Figure 10b) and G banding (Figure 11a), the euchromatic segments are the same as those of *M. musculus* previously described, but the C bands are not. The heterochromatic material (all centromeric) in some chromosomes forms long segments (e.g., chromosome No. 2) while in others it is barely visible in C band preparations. In Figure 11b, a C band karyotype, the pairing is again arbitrary. Polymorphism in C band distribution occurred even in homologous pairs since chromosome length and C band content did not always coincide for all chromosomes. Figure 12 further
Figure 10. Karyotype of Mus musculus molossinus (♀) -- a:
Conventional Giemsa. b: C banding with 33258 Hoechst.
Figure 11. Karyotype of Mus musculus molossinus -- a: G banding, ♀ cell. b: C banding, ♂ cell.
Figure 12. C banding of metaphase cell -- a: Laboratory mouse, Swiss strain. b: M. m. molossinus.
depicts the differences in C band distribution between *M. musculus* (Figure 12a) and *M. m. molossinus* (Figure 12b). In the former, the heterochromatic segments are more or less the same size in each chromosome, whereas in the latter the size difference is drastic. This different distribution of C band material between these two subspecies was also reported by Dev et al. (1975).

Figure 13 shows two metaphases of *M. m. molossinus* following silver staining. The maximum number of silver-NOR in *molossinus* is 8 (Figure 13a), two more than that of the Swiss mouse. In Figure 13b, there are 6 NORs. Even though the centromeric C bands also stain, NOR can be discerned by their characteristic double structure.

*Mus caroli*

The diploid number of *M. caroli* is 40, with all acrocentric chromosomes (Figure 14a). The Q band (Figure 14b) and the G band (Figure 15a) patterns are indistinguishable from those of *M. musculus*. The distribution of C bands among all chromosomes is relatively even (Figure 15b). Because of the long term tissue culture, a few cells may show chromosomal translocations. For example, in Figure 14b, the karyotype shows a translocation which has occurred between chromosome No. 12 and No. 19. This particular cell was selected because of its good Q bands and the translocation was not normal.
Figure 13. Ag-NOR staining of M. m. molossinus -- a: Metaphase cell showing 8 chromosomes with Ag-NOR. b: Six subcentromeric Ag-NORs (arrows).
Figure 14. Karyotype of *Mus caroli* -- a: Conventional Giemsa stain of a ♀ cell. b: Q banding of a ♀ cell, note the unusual translocation involved between chromosome 12 and 19 in this particular metaphase.
Figure 14. Karyotype of *Mus caroli*. 
Figure 15. Karyotype of *Mus caroli*, ♂ cells — a: G banding. b: C banding.
The number of silver-NOR characteristic for *M. caroli* was 9 to 11 which was higher than that for *M. musculus* (Figure 16a and 16b).

There was no particular karyotypic feature in meiosis to distinguish *M. caroli*. Figure 17a shows the sex chromosomes are associated end-to-end during diakinesis and metaphase I at the telomeric ends. A similar phenomenon was described in *M. musculus* by Hsu et al. (1971), Schnedl (1972), Polani (1972), and Kystikova and Forejt (1972). Using the C banding technique, the nucleolus of the Sertoli cells is frequently elongated (Figure 17b and 17c). In *M. musculus*, the nucleolus of the Sertoli cell is usually round (Figure 6d). In silver staining preparation, the silver was deposited outside the nuclei during spermatid maturation (Figure 17d-f) in a fashion similar to that described previously for *M. musculus* (Figures 7 and 8). This indicated that the phenomenon was not limited only to the Swiss mouse.

*Mus cervicolor*

The diploid number of *M. cervicolor* is 40 with all acrocentric chromosomes (Figure 18a). The Q band (Figure 18b) and the G band (Figure 19a) patterns are extremely similar to those of *M. musculus*. Dev, Miller, Miller et al. (1973), using quinacrine mustard, found a bright spot in the centromeric area of each chromosome of *M. cervicolor*. This
Figure 16. Ag-NOR staining of Mus caroli -- a: Metaphase cell showing 11 Ag-NORs. b: Metaphase cell with 9 Ag-NORs.
Figure 17. Testicular cells from *Mus caroli* -- a: Diakinesis, C band preparation. The sex bivalent showing telomere-telomere association. b and c: C band preparation of a Sertoli cell with an elongated nucleolus between the blocks of heterochromatin. d, e, and f: Ag-NOR staining of spermatid nuclei during spermiogenesis. The silver-stained substance forms a ring structure around the nucleus and disintegrates when the spermatid reaches maturity (f).
Figure 17. Testicular cells from *Mus caroli*. 
Figure 18. Conventional karyotype and Q banding of Mus cervicolor -- a: Conventional Giemsa stain of a \( \sigma \) cell. b: Q banding of a \( \varphi \) cell.
Figure 19. G and C banding karyotype of Mus cervicolor --
a: G banding of a ♂ cell. b: C banding of a ♀ cell, note telomeric C band (arrow) at one of the largest chromosomes.
is the only feature that can be used to distinguish the karyotypes of *M. cervicolor* and *M. musculus*. In the present study, which used Hoechst 33258 as the fluorochrome, the two species could not be distinguished.

The C bands of *M. cervicolor* are centromeric (Figure 19b). The amount of C band material is less than that of *M. musculus* in most pairs. However, in one chromosome (arrow, Figure 19b), a telomeric C band is present. A similar C band chromosome was found in cells of the embryos of a particular female as well as in her bone marrow cells. Other cell lines were not tested.

Testicular preparation of a male *cervicolor* were also examined. There were no unique features of the meiotic divisions of this species except that the pachytene bivalents were not as difficult to spread as those of *M. musculus*. The sex chromosomes were found paired at the telomeric ends, as in *M. musculus* and *M. caroli*.

**Mus cookii**

The diploid number is 40 with all acrocentric chromosomes. Its karyotype (Figure 20a), Q banding (Figure 20b), G banding (Figure 21a), and C banding (Figure 21b) patterns are all indistinguishable from those of the species of the subgenus *Mus* which were described earlier.

The NORs are located, as in other *Mus* species, at positions distal to the centromeric C bands (Figure 22a).
Figure 20. Conventional karyotype and G banding of *Mus cookii* -- a: Conventional Giemsa stain of a ♂ cell. b: Q banding of a ♀ cell.
Figure 21. G and C banding karyotype of *Mus cookii* -- a: G banding of a ♂ cell. b: C banding of a ♀ cell.
Figure 22. Metaphase cells of *Mus cookii* -- a: Metaphase plate of *Mus cookii* after Ag-NOR staining, showing 5 chromosomes with Ag-NORs. b: Diakinesis taken from a C banding preparation, showing telomere-telomere association of the sex bivalent.
Figure 22. Metaphase cells of *Mus cookii*. 
Usually 5 or 6 silver NORs were present. The meiotic divisions exhibited no unique characteristics, and the sex chromosomes were associated during meiotic prophase and metaphase I at the telomeres (Figure 22b).

*Mus booduga fulvidiventris*

The diploid is 40 with all acrocentric chromosomes. Figure 23 presents the G band (Figure 23a) and C Band (Figure 23b) patterns of this species. It can be seen that the major G bands are again similar to those of other species described thus far. The Y chromosome is considerably shorter than that of other species. In most *Mus* species the Y is similar in length to the shortest autosomal pair, No. 19. In *M. booduga fulvidiventris*, the Y is approximately one-third of the length of No. 19. The distribution of C bands is more uneven than other species except *M. musculus molossinus*. In some chromosomes there appears to be a lightly stained gap cutting the C band into two segments (Figure 23b). Whether this light gap represents the nucleolus organizer region is not known. *M. booduga fulvidiventris* was the first species analyzed in this series and the silver NOR technique was not available at that time.

In view of the fact that some mammalian species (such as the ocelot, a number of bats, several species of marmosets) possess a tiny Y chromosome, their necessary
Figure 23. Karyotype of *Mus booduga fulvidiventris* (♂) --
  a: G banding.  b: C banding.
genes are apparently packed in a small functional part of the Y. Since the Y chromosome in most mammalian species is longer as well as late replicating due to the addition of heterochromatin, the very small Y chromosome in *M. b. fulvidiventris* might be useful in testing this phenomenon by observing its replication time relative to other chromosomes. The present study showed that the Y chromosome was not the last to complete its replication. Figures 24a and 24b show two autoradiographs taken from sample labeled continuous with $^{3}$H-thymidine for three hours. It can be noted that the Y chromosome in Figure 24b, representing very late S phase, is devoid of silver grains while the centromeric areas (C band) still carry heavy label. In *M. booduga fulvidiventris*, the centromeric C bands, not the Y, are the last to complete DNA replication.

Additional metaphase figures showing label after three hours of continuous $^{3}$H-thymidine treatment were also prepared. The metaphases were roughly classified into four categories with respect to the overall label intensity: heavy, moderate, light, and very light. The label over the Y chromosome was classified into no label, light (1 to 3 grains), moderate (4 to 6 grains), and heavy (grains too numerous to count). Table 2 summarizes the observational results. It can be seen that when the overall label is very light (very late S), the Y chromosome was invariably not labeled. Only when the overall label was moderate or heavy
Figure 24. *Mus booduga fulvidiventris*, autoradiographs showing late S phase patterns,♂ cells — a: Moderately heavy label. Note some distinctly banded label along some chromosomes (arrows). The Y chromosome is heavily labeled. b: Very late S phase. Centromeric heterochromatin areas show heavy label. The Y chromosome is not labeled (arrow).
Figure 24. *Mus booduga fulvidiventris*, autoradiographs showing late S phase patterns, ♂ cells.
Table 2. Tritiated labeled metaphase of male *Mus booduga fulvidiventris* cells and the degree of Y chromosome labeling. Number of cells showing label intensity over the entire metaphase in relation to label over the Y chromosome. Continuous $^3$H-thymidine labeling was 3 hr and autoradiographs were exposed for 4 days.

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<th>Label over Y</th>
<th>Label over entire metaphase</th>
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<td>No label</td>
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was the label over the Y. These observations suggested that the Y of *M. booduga fulvidiventris* is late replicating but not among the latest to finish DNA synthesis. Using the marmoset *Callithrix jacchus* whose Y chromosome is also very small, McLaren (1974) reached a similar conclusion. She observed two phenomena related to late replication: (1) the Y chromosome was not the latest to complete replication, and (2) the property of late replication, commonly attributed to the mammalian Y, is probably related to the constitutive heterochromatin of the Y.

**Mus dunni**

Karyotype

The diploid number of *M. dunni* is 40 but unlike the karyotypes of other species in the subgenus *Mus*, most autosomes of the present study's specimen were subteloctentric, with short second arms (Figure 25a). The X chromosome is a very long submetacentric, and the Y is a long telocentric. The sex chromosomes were very similar in morphology to those described by Matthey and Petter (1968). The autosomes, however, were not the same. In the specimens described by Matthey and Petter, most autosomes were telocentric, but 7 to 10 of them were biarmed, and the second arms were considerably longer than those of our specimens.
Figure 25. Karyotype of Mus durni (♂) -- a: Conventional Giemsa stain. b: C banding.
C Banding

Figure 25b presents a C band karyotype of *M. dunni*. It is obvious that all the short arms of the autosomes are heterochromatic. The short arm of the X chromosome is likewise heterochromatic, but its stainability is not uniform throughout its length. Furthermore, the most distal segment of the long arm of the X is also heterochromatic. In conventionally stained preparations, the two chromatids of the X at metaphase were frequently seen to be more tightly held together at the distal long arm segment (Figure 25a). Matthey and Petter (1968) suggested that the extraordinary length of the X in *M. dunni* was the result of translocation. The C band distribution of the present study showed this was not the case (Figure 25b).

The Y chromosome is totally heterochromatic. The stainability is not uniform throughout its length. In species of the subgenus *Mus* thus far observed, the Y chromosome of *M. dunni* is the longest.

G Banding

If only the long arms of *M. dunni* autosomes are examined, their gross G band patterns are identical to those of *M. musculus* or any other species in the subgenus *Mus* (Figure 26a). However, because of the heterochromatic short arms, the arrangement of *M. dunni* chromosomes according to the *M. musculus* size nomenclature may give misleading
Figure 26. G band karyotype of *Mus dunni* and artificial G band karyotype of *Mus dunni* and *Mus musculus* —
a: *Mus dunni* (♂). b: Comparing *Mus dunni* (left side of each pair) and *M. musculus* (right side of each pair).
Figure 26. G band karyotype of Mus dunni and artificial G band karyotype of Mus dunni and Mus musculus.
impressions. For example, chromosome 16 is now considerably shorter than chromosome 17. The short arm of the X and most of the Y stain relatively deeply in G band preparations, but the terminal segment of the X long arm and that of the Y are completely G−. These segments are very difficult to demonstrate in photomicrographs suitable for showing regular G bands. G− terminal segments are more easily seen with phase-contrast optics.

Figure 26b presents additional evidence that the G band pattern of M. dunni and M. musculus (other than the short arms of M. dunni) are similar, at least in terms of major bands. In each pair the left side belongs to M. dunni and the right side to M. musculus. It should be noted that this artificial karyotype was constructed from two original karyotypes. Minor discrepancies in banding patterns of some "chromosome pairs" are due to differences in preparation.

Q Banding

The Q band patterns of the euchromatin confirmed the conclusion reached from G band analyses; viz., the gross arrangements of bands along long arms of M. dunni were similar to those of M. musculus. All short arms of the autosomes were dully fluorescent. The short arm of the X and most of the Y were brightly fluorescent, whereas the tip of the X long arm and the tip of the Y were Q−. These findings suggested that the molecular composition of the
heterochromatin of *M. dunni* sex chromosomes may be of several different kinds.

**DNA Synthetic Patterns**

At the terminal or near-terminal stages of DNA replication, the label over the chromosomes is distinctly localized and banded. Practically every labeled metaphase in our autoradiographs showed such a pattern. Figure 27a and 27b show two such metaphases. The chromosomes were paired by length and by labeling pattern.

It is clear that the short arms (heterochromatin) are not the latest to finish replication. At the very late S phase, scattered grains were observed over the short arm of the X, the Y, and some euchromatic arms. The replication of the sex chromosomes appears to follow a certain sequence. The terminal segment of the X long arm (heterochromatin) finishes replication first, followed by the centromeric segments of the X, the long arm of the X, and the terminal segment of the Y, while the short arm of the X and a few regions in the Y are among the last to finish replication.

**Sister Chromatid Differential Staining**

Recently a number of investigators, notably Latt (1973), suggested a staining procedure to demonstrate sister chromatid exchanges. He took advantage of the fact that fluorescence by 33258 Hoechst is quenched when both DNA
Figure 27. *Mus dunni* (♂) autoradiographs (a and b) showing late S phase patterns -- Chromosomes were matched by length and grain pattern. Note the lack of label over many short arms (C band), indicating that heterochromatin of the autosomes finishes DNA synthesis before some areas of euchromatic arms. Heterochromatin of the sex chromosomes is late replicating.
Figure 27. Mus dunni (♂) autoradiographs (a and b) showing late S phase patterns.
strands of a chromatid contain bromouracil. Cells were grown in a medium containing 5-bromodeoxyuridine (BrdU) for two cell cycles. Each metaphase chromosome then consisted of one chromatid with BU-substitution for thymine in both DNA strands (dull fluorescence) and the other chromatid with BU-substitution in only one DNA strand (bright fluorescence). Sister chromatid exchanges were extremely clear in such preparations. The fluorescence method was later modified for Giemsa staining (Perry and Wolff 1974, Wolff and Perry 1974, Korenberg and Freedlender 1974). In such preparations the chromatid with double BU-substitution stains lightly and the chromatid with a single BU-substitution stains deeply.

These newer methods can be used to analyze DNA synthetic time sequences of chromosomes by allowing the cells to incorporate BrdU for more than two, but less than three, cell cycles. In such cell populations the late replicating chromosomes or chromosome segments should be "double-light" with Giemsa staining (three cycles) while the rest of the chromosomes should contain one deeply-stained chromatid and one lightly stained chromatid (two cycles). Thus by tracing the appearance of double-light segments in cells grown in BrdU medium for 2+ cell cycles, investigators should be able to determine the DNA synthetic time sequences by staining, the results of which should
complement the data obtained from $^3$H-thymidine autoradiography.

Since the euchromatic long arms of *M. dunni* appear to have discrete late replicating zones and the heterochromatic short arms of the autosomes appear to finish replication before some zones in the long arms, autosomes of *M. dunni* cells with 2+ cycles of BrdU incorporation are expected to show one lightly stained chromatid and one heavily stained chromatid with double light gaps. Most of the heterochromatic short arms should be double light. *M. dunni* embryonic cell cultures therefore were grown in medium containing BrdU (5 μg/ml) for this series of experiments. All cultures were incubated in complete darkness when BrdU was present. Because the cell cycle time of these cells was not yet determined, the experiment was done with a series of BrdU incubation periods of 24, 30, 36, 42, and 48 hours, hoping that one or more samples would give the desired 2+ cell cycles. All cultures of this series of experiments received a one-hour Colcemid treatment prior to harvest.

The procedure for differentiating chromatids with Giemsa staining followed that of Korenberg and Freedlender (1974) with one modification, viz., treating the slides in hot Na$_2$HPO$_4$ solution (pH 8.0) for 90 minutes instead of 10 minutes. Figure 28a (taken from a 30-hr sample) shows a metaphase after two cycles in the presence of BrdU. Every chromosome, including the short heterochromatic second arm,
Figure 28. *Mus dunnii* (♂) sister chromatid differential staining with Giemsa -- a: BrdU-substitution for two cell cycles. Each chromosome, including the heterochromatic short arm, shows one heavily stained and one lightly stained chromatid. b: BrdU-substitution for 2+ cell cycles. The late-replicating chromosome segments are double-light with Giemsa staining (three cycles) while the rest of the chromosome segments contain one deeply-stained and one lightly-stained chromatid (two cycles). The late-replicating segments include short arm of the X, some short arms of the autosomes, and light gaps in the long arm of the X, the Y, and a number of autosomes (arrows).
Figure 28. *Mus dunni* (♂) sister chromatid differential staining with Giemsa.
shows one lightly stained and one deeply stained chromatid. Figure 28b shows a metaphase with 2+ cycle of BU-incorporation. The late replicating short arm of the X and the short arms of several autosomes are double-light and the long arms of a number of autosomes as well as the sex chromosomes show light gaps in the heavily-stained chromatid (arrows).

The present data demonstrate that DNA synthesis along a chromosome may initiate and terminate in discrete segments. Certainly such segments are too large for replicons; but as Stubblefield (1974) suggested, the G bands may represent clusters of replicons which initiate and finish replication almost in unison. Crossen, Pathak, and Arrighi (1975) used 1+ cell cycle of BrdU incorporation to trace the replication patterns of the Chinese hamster chromosomes and found that the "units" corresponded well to G bands.

Meiosis

The meiotic divisions of M. dunni were studied by Pathak and Hsu (1976) in some detail. Using heterochromatic segments as a guide, these authors were able to identify early meiotic prophase states (leptotene, zygotene, and pachytene) in C banded preparations by counting the number of heterochromatic bodies. The behavior of the sex chromosomes during these stages could therefore be observed. The
sex chromosomes were found to be separated in leptotene, but they moved toward each other in zygotene and formed a sex vesicle after the completion of autosomal synapsis by early pachytene.

Figure 29 is reproduced here with the permission of Dr. Sen Pathak to illustrate the association of the sex chromosomes in diakinesis. The heterochromatic short arm, the euchromatic long arm, and the heterochromatic terminal segment of the X chromosome are clearly visible in Figures 29a and 29b. The Y chromosome, almost totally heterochromatic, appears to attach to the terminal C band of the X at its centromeric end. Thus, cytologically *M. dunni* can be distinguished from the rest of the species of subgenus *Mus* by two characteristics: (1) the presence of the heterochromatic short arms, and (2) the association points of the sex chromosomes in meiotic prophase.

Theoretically, telomeric association of the sex chromosomes during meiosis (as in *M. musculus* and other *Mus* species) should be most convenient for anaphase disjunction. The centromere-telomere association found in *M. dunni* is a unique situation. However, there appears to be no difficulty for orderly disjunction since most cells in the second meiotic division (Figures 29c and 29d) contain either the X or the Y chromosome.
Figure 29. Meiotic cells of *Mus dunni* with C banding —
a: Diakinesis. b: Cut-outs of sex bivalents from four metaphase I stage showing centromere-telomere association between the Y and the long arm of the X, respectively. c and d: Metaphase II with an X chromosome and a Y chromosome, respectively. (Photos courtesy of Dr. Sen Pathak.)
Figure 29. Meiotic cells of *Mus dunni* with C banding.
Comparative Karyotype of Subgenus Mus

It appears that the G banding patterns of all the species of subgenus Mus thus far described are very similar. To illustrate this point, haploid sets of M. musculus, M. m. molossinus, M. caroli, M. cervicolor, M. cookii, and M. booduga fulvidiventris were compared side by side in a chromosome composite (Figure 30). Since a comparison between M. musculus and M. dunni has already been made (Figure 26), the last species is not included in Figure 30. It can be seen that the major G bands of all chromosomes are similar for all species. The main difference appears to be the amount and distribution of C bands.

The current theory regarding the significance of comparative banding patterns is that similarity suggests evolutionary conservatism in the arrangement of genetic material (Mascarello, Stock, and Pathak 1974). If this explanation is correct, then rearrangement of genetic material has not played a significant role in the evolution of subgenus Mus. This is in contrast to other subgenera of Mus where the banding patterns are different. For example, M. pahari and M. shortridgei show very little homology when their chromosomes are compared with those of the subgenus Mus (see below).
Figure 30. G banding comparison of 6 species of mice belonging to subgenus Mus -- One haploid set of each species is arranged such that the row number represents the chromosome number and the column letter represents each species, a = M. musculus, b = M. m. molossinus, c = M. caroli, d = M. cervicolor, e = M. cookii, f = M. booduga fulvidiventris.
Figure 30. G banding comparison of 6 species of mice belonging to subgenus Mus.
**Mus pahari**

*M. pahari* has 48 acrocentric chromosomes (Gropp et al. 1973). One pair of long chromosomes shows an obvious secondary constriction at the vicinity of the centromere (Figure 31a). All chromosomes have centromeric C bands (Figure 31b).

Since embryonic tissue was not available, G banding was prepared from bone marrow, which proved less satisfactory. The karyotype presented in Figure 32a is the best. However, it is clear that most chromosomes have G band patterns that cannot be identified on the basis of the *M. musculus* chromosomes. Numbers representing *Mus musculus* chromosomes were placed under a few pairs of the *Mus pahari* chromosomes where presumed similarities existed. The rest of the chromosome pairs appeared unique.

In meiotic divisions, 24 bivalents are present in diakinesis (Figures 32b and 32c). Unlike the mouse species previously described, the sex chromosomes associated with each other at their centromeric ends. This was particularly clear when the preparations were C band treated (Figure 32b).

**Mus shortridgei**

Gropp et al. (1973) reported that the basic diploid chromosome number of *M. shortridgei* is 46. The autosomes consist of 21 pairs of acrocentrics and one pair biarmed.
Figure 31. Karyotype of *Mus pahari* (♀) with 2n=48 -- a: Conventional Giemsa stain.  b: C banding.
Figure 32. Karyotype of *Mus pahari* -- a: G banding. The numbers represent the chromosome pairs which seem to correspond to those of *Mus musculus*. b and c: Diakinesis with C banding and conventional Giemsa stain respectively. Note centromere-centromere association of the sex bivalent.
Figure 32. Karyotype of *Mus pahari*.
chromosomes which can be either two metacentric, two submetacentric chromosomes, or one of each. The sex chromosomes are acrocentrics. Individuals may possess a variable number of supernumerary chromosomes, raising the total diploid number as high as 49. Gropp (1976) has recently observed that the chromosome number in somatic cells may reach 51, 5 above the basic number. Within an individual, the diploid number does not vary. This situation is similar to B chromosomes of maize and supernumerary chromosomes of many other life forms reported in the literature.

As in the case of *M. pahari*, adult bone marrow cells were used for chromosome analyses because embryonic tissues were not available. The chromosome number of the individual presented in Figure 33a is 47. The autosomes consist of 21 pairs of acrocentric chromosomes, one pair of medium-sized biarmed chromosomes (one metacentric and the other submetacentric) indicating dimorphism, and one small metacentric chromosome. The X chromosome is acrocentric. In C banded preparations (Figure 33b), all acrocentric chromosomes had only centromeric C bands. The three biarmed chromosomes, however, had different patterns. The submetacentric had a broad centromeric C band with heterochromatin in almost the entire long arm except a narrow zone near the centromere. The terminal segment of its short arm is euchromatic. The larger metacentric was euchromatic at the terminal segment of one of its arms, and
Figure 33. Karyotype of *Mus shortridgei* (♀) -- This individual has a diploid number of 47 with one metacentric supernumerary chromosome and a pair of heteromorphic biarmed chromosomes which contain a substantial amount of C band. a: Conventional Giemsa stain. b: C banding.
Figure 33. Karyotype of *Mus shortridgei* (♀).
the second arm was totally heterochromatic. The small metacentric was totally heterochromatic.

It would appear that the two larger biarmed chromosomes were really a pair, but had a large amount of heterochromatin added to them. An inversion might have occurred to cause the morphological difference. The third one, being totally heterochromatic, was the supernumerary chromosome of this specimen.

Since sufficient specimens were not available for the studies of chromosome variability and G band patterns of *M. shortridgei*, additional cytological data and photos were obtained from Alfred Gropp in Lübeck. Gropp's cytological analysis of *M. shortridgei* shows a diploid number of 51, the highest chromosome number recorded thus far (Figure 34a). All of the five supernumerary chromosomes proved to be C band positive. The last autosomal pair was somewhat heteromorphic, similar to the one presented in Figure 33b. It is possible that the last autosomal pair (No. 22) was originally a telocentric but later added a heterochromatic arm. Subsequent inversions could have resulted in morphological variability.

From the G band karyotype presented in Figure 34b it is easy to conclude that very few chromosome pairs of *M. shortridgei* show any banding patterns similar to those of *M. musculus*, except the X chromosome which possesses the two characteristic bands in most mammals (Pathak and Stock 1974).
Figure 34. Karyotype of *Mus shortridgei* -- a: Female C band karyotype with a diploid number of 51 including a pair of subtelocentric chromosomes and 5 small supernumerary chromosomes. b: Male G band karyotype with a diploid number of 48 including a pair of metacentric and two small supernumerary chromosomes. (Photos courtesy of Professor Alfred Gropp.)
Figure 34. Karyotype of *Mus shortridgei*. 
In male meiosis, the characteristic feature found in *M. shortridgei* is the centromeric association between the X and the Y chromosomes (Figure 35). This behavior has been found in *M. pahari* (Figures 32b and 32c). Silver staining revealed at least three heavy clusters in pachytene, suggesting the locations of three NORs (Figure 36). It is interesting to note that the sex vesicle invariably contained two thread-like structures, one longer than the other. The longer one was usually in the form of a horseshoe and terminally associated with the short one (Figure 36c, 36d). The association is particularly well shown in Figure 36b in which the longer element is twisted into an S-shaped structure. Presumably these represent the X and the Y chromosomes.

The developing spermatids, when stained with silver also showed extra nuclear material which undergoes a series of changes until it is finally discarded as the sperm matures (Figures 37a and 37b). This phenomenon was described in more detail with *M. musculus* (Figures 7, 8, and 9).
Figure 35. Diakinesis of *Mus shortridgei* (♂) with a diploid number of 46 —- a: Conventional Giemsa stain. b: C banding. Note centromere-centromere association of the sex bivalent.
Figure 36. Pachytene of *Mus shortridgei* (♂) with Ag-NOR staining -- a and b: Early pachytene with moderately condensed chromosomes. c and d: Late pachytene with very diffused chromosomes. Note the visible structure inside the sex vesicle.
Figure 37. The developing spermatids of *Mus shortridgei* with Ag-NOR staining —
a: The argentophilic substance forming a ring structure around the nucleus.  
b: Further maturation of the spermatids with discarded silver-stained substances.
DISCUSSION

It should be emphasized at the beginning of this discussion that chromosome features constitute one set of phenotypes. This set of phenotypes probably does not have as much adaptive value as others, and the phylogenetic relationships obtained from karyologic data would, therefore, be more objective. However, because of the probable lack of adaptive value, karyologic evolution does not necessarily parallel the general trend of organic evolution. That is to say, two species with vastly different karyotypes do not necessarily show extensive evolution in other characteristics. The Mus situation is a good example. Karyological differences between M. musculus and M. poschiavinus are so great (40 telocentrics vs. 14 metacentrics and 12 telocentrics) that on this basis alone one might conclude that these two species were quite unrelated. And yet only seven sets of Robertsonian fusions can account for the karyological difference. Conversely, the identical karyotypes of two species do not indicate that profound evolution of other characteristics has not occurred. Species characterized on the basis of differences in gross morphology (such as M. booduga and M. caroli) may possess identical karyotypes, including the G banding patterns. Therefore, karyological evolution should not be relied upon
to interpret phylogeny as a whole, although it may be
extremely valuable in many instances.

Caution should also be exercised if the judgment
involves interpretations of organic evolution in general
based on ancestral or more evolved karyologic features. A
species with a more ancestral form of karyotype is not
necessarily more primitive in phylogeny, and conversely, a
species with highly evolved karyotype may not be advanced in
other characteristics. In other words, karyologic evolution
may be independent of evolution of other characteristics of
the organism.

With these limitations, karyologic characteristics
are highly useful. In a number of instances, they have
provided important taxonomic clues. For example, the
Arizonan "subspecies" of *Sigmodon hispidus* (2n=22) is
phylogenetically remote from the rest of the cotton rats
belonging to the species *S. hispidus* (2n=52). Considering
Robertsonian fusions to occur for all chromosomes, the
greatest reduction one could expect from a diploid number
of 52 would be to 26. Thus the Arizona form must have
undergone profound chromosome rearrangements. Interspecific
hybrids, if produced, are expected to be sterile and the
isolation between the two forms is probably complete. Yet
morphologically they are so similar that taxonomists placed
them in the same species until Zimmerman and Lee (1968)
suggested species status for *S. arizonae* based on chromosome analysis.

When closely related taxa are compared, chromosomes are excellent objects for tracing genetic relationships. After the perfection of banding techniques, a great deal of precision has been gained and many misinterpretations have been avoided. For example, Pathak, Hsu, Shirley, and Helm (1973) found a pair of large metacentrics in the Mexican species of the climbing rats, *Tylomys*. Without banding, this chromosome would have been considered homologous in all species in which it appeared, but G banding showed that different sets of Robertsonian fusion had occurred.

One of the most difficult questions in the karyologic evolution of mammals is "What is the most ancestral karyotype of mammals?" With a spectrum of diploid numbers ranging from 6 to 92 in the class, and with the fact that each higher taxon (family, for example) always contains a heterogeneity of diploid numbers no general conclusion can be made in this respect. However, within a family or a genus, some conclusions can be drawn if a large number of species and subspecies are analyzed.

**The Genomic Size of Mammals**

As mentioned previously (see Literature Review), the genomic size of mammals is probably constant as far as euchromatic material is concerned. With the aid of flow
microfluorometer (FMF) to measure DNA content per cell, Deaven and Pettersen (1974) found that regardless of the diploid number the genomic size of many mammals is more or less the same. Thus, chromosomes are merely packages of a given amount of DNA. More chromosomes means smaller packages and fewer chromosomes means larger packages. No concrete data are available to suggest whether there is an optimal diploid number for a given genomic size. In mammals, the prevailing diploid numbers are between 30 and 50. Whether this phenomenon happens by chance or by selection is not known.

Nevertheless, when heterochromatin content is considered, the genomic size of certain mammals becomes larger than the standard. A good example is found in the deer mouse genus *Peromyscus*. Various investigators have found, from more than 20 species thus far analyzed, 48 as the diploid number for this genus, but the number of chromosome arms (NF) may vary from 56 (*P. boylei* and *P. crinitus*) to 96 (*P. eremicus* and *P. collatus*). Pathak, Hsu, and Arrighi (1973) reported that the short arms of both *P. eremicus* and *P. crinitus* are heterochromatic and the long arms of the two species were essentially the same. Using a relatively crude method of measuring the total length of the metaphase chromosomes, these investigators found that *P. eremicus* possessed approximately 32% more chromatin material than *P. crinitus*. A similar difference, 36%, was obtained by FMF
measurements of DNA content (Deaven 1976). Thus, the proportion of the genomic size of *P. crinitus* as compared to *P. eremicus* is roughly in the proportion 100:135 and all additional *P. eremicus* DNA is presumably of the type that forms heterochromatin. More striking was the case found by Mascarello (1976) when he compared the chromosomes of two groups of ground squirrels, *Spermophilus* and *Ammospermophilus*. The members of the former had only a small piece of C band at each centromere, but members of the latter had large blocks of heterochromatin distributed in all chromosomes. DNA measurements by FMF between the two genera approximates 100:200. The present study data on *Mus dunnii* are another good example. The extra short arms were composed of heterochromatin while the long arm G bands resembled those of the standard *M. musculus*. Thus in interpreting genetic relationships by chromosome constitution, C bands must be considered,

**Cytogenetic Evolution in Mammals**

From available information, karyologic evolution in mammals seems to proceed in several different directions. As these are closely related to the discussion on genus *Mus*, they will be discussed here by way of an introduction to the *Mus* problem.
Robertsonian Translocation

Robertsonian translocation appears to be the prevailing mechanism for karyologic evolution in mammals (White 1973). One can find conclusive evidence for the Robertsonian process in practically every family (Benirschke and Malouf 1974). One aspect of the Robertsonian process deserves some discussion. Most cytogeneticists consider the fusion of two monoarmed chromosomes (telocentrics or acrocentrics) into one biarmed chromosome as the mechanism of Robertsonian translocation. However, the reverse process, fission of a biarmed into two acrocentrics, may also occur in karyologic evolution. Todd (1970) proposed an extensive scheme to show how fission has operated in Carnivora. His main point is that more recent families such as Ursidae and Canidae possess a higher diploid number than families emerging at an earlier geologic time such as Felidae. Therefore, Todd considers that fission, instead of fusion, is the major force in karyologic evolution and that karyotypes with more biarmed chromosomes are more primitive.

As was mentioned earlier, karyologic evolution does not necessarily parallel evolution of other phenotypic characteristics and organic evolution in general. Todd's argument neglected this point. One may just as easily develop a scheme to show that the ancestral forms from which Ursidae and Canidae were derived retained a high diploid number with many acrocentrics while the felid branch evolved
with many fusions. These ancestral forms have become extinct. When one surveys the karyotypic patterns in mammals, one finds more evidence in favor of fusion than of fission. The European mouse populations which have been widely introduced by humans, have a relatively short evolutionary history. Yet in certain Alpine valleys, populations have been found with varying numbers of biarmed chromosomes. This could mean that fusion has occurred repeatedly. In the human population, which have been intensively analyzed cytologically, fusions have been repeatedly discovered. The human karyotype consists of 10 acrocentrics and 36 biarmed chromosomes. Carriers with D/D, D/G, and G/G translocations (Robertsonian fusion) are common. Yet only in one recent report was a possible fission noted (Hansen 1975). This individual had 47 chromosomes with only one regular No. 7 chromosome. The homolog was represented by two telocentrics with the banding patterns identical to 7p and 7q. If fission is the major force of karyologic evolution in mammals, one would expect more individuals with 47 chromosomes than with 45 chromosomes (trisomy and monosomy excluded in the human populations). But this is not the case.

The classic interpretation for Robertsonian fusion is that a break occurs in the long arm of an acrocentric immediately distal to the centromere and another break occurs at the "invisible" short arm of the second chromosome. An asymmetrical translocation would result in a biarmed
chromosome and a bare centromere which is subsequently lost. The difficulty in postulating fission is that a split of a biarmed chromosome at the centromeric region would result in an acentric fragment, unless there is another chromosome which is broken simultaneously at the centromere and donates its centromeric fragment to the acentric fragment. Even with the "centromere donor" hypothesis, an acentric fragment (the donor) will result. But the death of one of the daughter cells after cell division probably would not be important as far as the organism is concerned.

Tandem Translocation

Recent literature in human cytogenetics indicates that translocations between the centromeric area of one chromosome and the telocentric area of another, or translocations between two telomeric ends of two different chromosomes may be more common than cytogeneticists have suspected. In natural populations, good evidence of such tandem translocations have been presented by Lejeune et al. (1973) when they compared the banding patterns of human chromosomes with those of chimpanzee. Stock and Hsu (1973) also found several sets of tandem translocations between the chromosomes of the rhesus monkey (Macaca mulatta) and those of the African green monkey (Cercopithecus aethiops). Such translocations are expected to result in dicentric chromosomes, but apparently some unknown mechanism operates
causing one of the centromeres to be inactive. Thus a dicentric chromosome behaves as if it has only a single centromere (Hsu et al. 1975). This mechanism can reduce a karyotype with a high diploid number to a low diploid number because repeated tandem translocations may bring more than two chromosomes into a single chromosome. Most likely the muntjac situation (from 46 to 6) and the Sigmodon situation (from 52 to 22) was accomplished by tandem translocations as well as centric fusions.

When the diploid numbers within a taxon are examined, it can be inferred that tandem translocations may have occurred. Probably many large chromosomes represent the products of tandem translocations. Most bats have a low diploid number between 20 and 30, but Vespertilionids and Tadarids have species with diploid numbers above 50. Similarly, in microtine rodents, the diploid number ranges from 56 in Clethrionomys to 18 in Microtus oregoni. Unless some genetic material is discarded in the process of evolution, tandem translocation should be the logical mechanism.

It is not known whether a chromosome representing tandem translocations can be split into two or more components with the reactivation of the latent centromeres. Pathak, McGill, and Hsu (1975) treated the Indian muntjac cells with the antibiotic actinomycin D, and observed in the recovering cell populations a split of the X chromosome at the nucleolus organizer region. The fragment, which should
be acentric, appeared to possess a centromere. Very possibly this represented an experimentally induced fission of an original tandem translocation. If latent centromeres indeed exist, and if they can be reactivated after severing from the compound chromosome, then fission of chromosomes is not restricted to the Robertsonian process. However, concrete evidence supporting such a contention is still lacking.

Pericentric Inversions

When Matthey and Petter (1968) found a variable number of short arms in the karyotypes of Mus dunni, they postulated that the karyotypic changes were accomplished by pericentric inversions. Hsu and Arrighi (1968) made the same interpretation to explain the differences in the number of chromosome arms in Peromyscus. These interpretations proved to be erroneous because the short arms of the M. dunni chromosomes represent additions of heterochromatic material (Markvong et al. 1975). In Peromyscus, addition of heterochromatic arms is also the prevailing mechanism for the change in the number of short arms, but evidence has been obtained to show that pericentric inversions indeed exist (Arrighi et al. 1976, Ward 1976).

Heterochromatin

In most mammalian species, C bands are usually centromeric, and the amount is not excessive. However, in
some species within a genus, or sometimes the entire genus, a large amount of heterochromatin may accumulate in chromosomes, particularly the sex chromosomes. Why some taxa acquire an excessive amount of heterochromatin while others do not is an unanswered question. The fact that such instances are scattered within different families and orders suggests that the process of acquisition of highly repetitive DNA has occurred repeatedly and independently. In addition to the Peromyscus and Ammospermophilus cases mentioned previously, widely distributed C bands have been found in some species of the kangaroo rats (Dipodomys), pocket gophers (Thomomys), the Syrian hamster (Mesocricetus auratus), a number of species of Microtus, many whales, several species in Bovidae and Cervidae, and others. If repetitive DNA is useful for the organisms, why do some species keep only a minimum amount while others acquire a large amount? Why in some species (e.g., Microtus agrestis) do the sex chromosomes carry the bulk of the C band material while in other species the C bands form blocks in all chromosomes? How is an excessive amount of repetitive DNA generated? What would happen in meiosis of hybrids from crosses between one parent with a large amount of heterochromatin in all chromosomes and the other with a very small amount only at the centromeres?

There are no good answers to these questions at the present time. In fact the precise identification of C bands
has had a history of only a few years. But in phylogenetic studies of whatever taxa, including plants and insects, the amount, the distribution, and (if feasible), the biophysical properties of the C band material become important objects of inquiry.

The Nucleolus Organizer Regions

Prior to the innovations of the banding techniques, one of the morphologic landmarks in karyology was the secondary constriction or nucleolus organizer region (NOR). In some karyotypes, the NOR is located in one pair of chromosomes and in others NORs may be located in more than one pair. The positions of NOR may vary from species to species, but within a species, they were considered to be consistent. However, karyotypes of many mammalian species show no suggestions of NORs in any chromosome. Recent cytochemical techniques, especially the silver straining method (Goodpasture and Bloom 1975) has revealed that in karyotypes where consistent secondary constrictions are absent, NORs are located at the telomere regions.

Because NORs represent the locations of ribosomal genes, they become an important cytogenetic probe for the 28S+18S ribosomal genes (plus spacers) which all eukaryotes possess. Ribosomal genes are moderately repetitious. Apparently in many species all the genes clustered in one chromosome segment. Cytologically, then, they display one
deep secondary constriction in the haploid genome. Probably this represents a more ancestral form because conceivably repetitive sequences do not arise sporadically and independently. Presumably gene duplication occurs at the site of the original gene, and the dispersion of the duplicated genes to other sites is the result of subsequent rearrangements. Thus multiple NORs may be a more evolved pattern. Again, the evolution of these particular gene sets should be independent of the organic evolution as a whole, so that a species with primitive NORs is not necessarily primitive in other aspects. Many primates have a single NOR for each haploid complement, whereas lower forms of mammals may possess multiple NORs. However, the number and the distribution of NORs can be effectively utilized to decipher species and populational relationships.

Supernumerary Chromosomes

A few reports are available to show that in some mammalian species supernumerary chromosomes have been recorded in natural populations. Shellhammer (1967) found that in Californian populations of the harvest mouse, Reithrodontomys minimus, the chromosome number may vary from 42 to 48. All the extra chromosomes above 42 were tiny centric fragments. In black rats (Rattus rattus), Yosida and Sagai (1975) found supernumerary chromosomes in many Asian populations. Unlike those of the harvest mouse, the
supernumerary chromosomes of the black rat are the same size as the small metacentrics. They further demonstrated by C bands that all the supernumerary chromosomes were heterochromatic. Gropp et al. (1973) also reported supernumerary chromosomes in *Mus shortridgei*. The present study also demonstrated that the supernumerary chromosomes of this species were made of heterochromatin. Although no C banding was carried out in Shellhammer's (1967) material, it is reasonable to assume that the minute centric fragments are also heterochromatic. Probably supernumerary chromosomes represent a special way of increasing heterochromatin. Here the special feature is the presence of centromeres. It is possible that these supernumerary chromosomes can serve as receptors of chromosome segments in reciprocal translocations. Although unproven, this mechanism may provide one way of increasing chromosome numbers.

**Cytogenetic Evolution of *Mus***

The cytogenetic evolution of *Mus* appears to be a complex one and the present data merely represent only a small portion of the overall picture. Nevertheless, it is already possible to detect a trend.

**Robertsonian Translocations**

It is interesting to note that Robertsonian translocations have been the major force in karyotypic evolution in *Leggad*a, an African genus closely related to *Mus*, but in
Southeast Asia, all *Mus* species possess only telocentric chromosomes in their karyotypes without any indication of fusions. Furthermore mice have been universally used as the major experimental animals and numerous inbred strains have been established. Yet detection of fusion has been a rare occurrence. It is inconceivable that Robertsonian fusions have never occurred in subgenus *Mus* because individuals of *M. musculus* in the Alps have provided ample evidence for Robertsonian fusions. Perhaps in the large Asian continent where the species originated chromosome mutants are difficult to establish because of large population sizes and partial sterility due to nondisjunction in meiosis. Perhaps in isolated pockets, such as valleys found in the Alps, oases of the great deserts, and small, inaccessible islands, chromosome races with fusions and/or other rearrangements can be found in Asia also.

The lack of Robertsonian fusions does not apply to subgenus *Leggadilla* in India, since diploid numbers of the *saxicola-platythrix* complex show an apparent correlation between the reduction of diploid number and the concomitant increase of biarmed chromosomes.

NonRobertsonian Translocations

A synopsis of the diploid chromosome constitution of the members of genus *Mus* reveals the following synopsis:
Thus, there are four known karyotypes, \(2n=48, 46, 40,\) and \(26\) telocentrics. The taxa of the saxicola group with diploid numbers lower than 26 have undergone centric fusions. But the karyotypes with a diploid number higher than 26 showed only telocentrics. This indicates that Robertsonian translocations were not involved in the reduction or increase of the diploid numbers. The difference, therefore, must have been accomplished by means other than centric fusion, most likely tandem translocations. It would be interesting and revealing to employ banding techniques to determine the homology of all these karyotypes from which phylogenetic relationships can be established.

**Repetitive DNA and Heterochromatin**

Accumulated Mus data suggest that this genus would be an ideal model for further exploration of the evolutionary process of repetitive DNA and heterochromatin. In
terms of heterochromatin, the following facts are known:

1. All species possess centromeric heterochromatin and, in most cases, only centromeric heterochromatin.

2. The amount of heterochromatin in each chromosome may be significantly different between subspecies (Hsu and Markvong 1975, Dev et al. 1975) and between species (Markvong et al. 1975).

3. The amount of heterochromatin in each chromosome may vary from individual to individual and from one inbred line to another (Dev, Miller, and Miller 1973; Forejt 1973).

4. The amount of heterochromatin may even be drastically different between homologous chromosomes. This is particularly obvious in *M. m. molossinus* (Dev et al. 1975).

5. In *M. cervicolor*, telomeric heterochromatin has been found.

6. In *M. dunni*, a variable number of heterochromatic second arms has been reported (Matthey and Petter 1968, Markvong et al. 1975, Sharma and Garg 1975). Also, the X chromosome of *M. dunni* invariably has a long, heterochromatic second arm and a terminal heterochromatic segment in the long arm.

7. The Y chromosome of *Mus*, with the exception of *M. dunni*, is usually a small chromosome. The Y of *M.*
**dunni** is a very long element. All the Y chromosomes are mainly heterochromatic.

8. In *M. shortridgei*, a variable number of heterochromatic supernumerary chromosomes may be present in the karyotype.

One may hypothesize that translocations might have occurred repeatedly in the past to give rise to the variation in the amount and the distribution of heterochromatin noted. However, an alternative hypothesis may be proposed without resorting to translocations. The main component of heterochromatin is the repetitive DNA sequences which follow one another tandemly in a DNA molecule. A piece of heterochromatin may contain thousands of copies of the reiterated DNA sequences. Electron microscopic observations have shown that synaptinemal complexes are present in all bivalents from one end to the other, including the heterochromatic segments (Hsu et al. 1971; Moses, Counce, and Paulson 1975). This suggests that pairing of repetitive DNA sequences between homologous chromosomes takes place during early meiosis. Since the repetitive sequences are the same one after another, pairing between an earlier sequence in one chromosome with an identical but later occurring sequence in the homolog would still result in a perfect match, but an unpaired loop may be formed. A crossover within this paired segment could easily transfer a segment of the repetitive
DNA from one homolog to another, thus increasing the length of C band in one and decreasing it in the homolog without affecting the euchromatin material.

Figure 38 diagrammatically presents this concept. Repetitive DNA (dashed lines) is present on both sides of the acrocentric chromosome. If meiotic pairing is perfect (top figure), crossover within the heterochromatic segment does not change the structure nor the morphology of this chromosome pair. But if pairing slips, as indicated in the middle (long arm) and the bottom (short arm) figures, crossing over may change the length of the heterochromatic segments. This scheme may explain the C band polymorphism observed in nature (Craig-Holmes and Shaw 1971; Pathak, Hsu, and Arrighi 1973), and the differences between M. musculus musculus and M. m. molossinus. It also explains how M. dunni can acquire heterochromatic second arms.

However, this scheme is purely speculative. It may be possible to use whole-mount electron microscopy of pachytene chromosomes (Counce and Mayer 1973) to observe whether "slip-pairing" does occasionally take place, especially in materials with large segments of interstitial heterochromatin. Recently Mascarello (1976) found that the ground squirrel Ammospermophilus possessed a large amount of heterochromatin distributed in all chromosomes with long interstitial segments in some chromosome pairs. He further observed that pairing between homologous heterochromatin
Figure 38. Diagrammatic representation of the proposed mechanism for the variability in the amount of centromeric heterochromatin — Thin continuous line, euchromatin; dashed line, heterochromatin containing repetitive DNA sequences. Each bar represents one sequence, all reiterations. Circles are centromeres; vertical lines are crossing-over points.

Upper panel: Perfect synopsis between homologous chromosomes. Crossing over on both sides of the centromere results in no change.

Middle panel: Mismatching of repetitive sequences in the long arm. Crossing over results in one homologous chromosome having a shorter C band and the other a longer C band.

Bottom panel: Same as middle panel but the mismatching occurs in the heterochromatic second arm. Crossing over results in one homologous chromosome having a longer second arm and the other a shorter second arm.
Figure 38. Diagrammatic representation of the proposed mechanism for the variability in the amount of centromeric heterochromatin.
segments takes place in early pachytene, but the heterochromatic segments separate before the euchromatin segments in early diplotene stage, forming interstitial loops. It may be useful to observe a large number of diploid figures to determine if the length of the loops between the homologs always remains the same. An unequal loop in different cells would strongly suggest slip-pairing and, hence, unequal crossover.

Repetitive DNA and Speciation

Mascarello et al. (1974) showed, by comparing banding patterns of many species of rodents, that the euchromatic chromosomes are highly conservative in their gene arrangements. Yet the repetitive DNA sequences appeared to be highly variable. Even related species may possess extremely different satellite DNA sequences (Sutton and McCallum 1972). These data support the idea that structural genes must be conservative because mutations would result in nonfunctional enzyme products which may be lethal to the individual, while mutations in the repetitive sequences, being nontranscriptional, are tolerated. However, it is still puzzling how mutational events, which changed a repetitive sequence in one chromosome, could be transferred to other chromosomes and could even replace the original one. A scheme is proposed in Figure 39 to explain this phenomenon. Since all heterochromatic pieces contain
Figure 39. Diagrammatic representation of proposed mechanism for transferring heterochromatin from one chromosome to another — Continuous straight and wavy lines, euchromatin of two different chromosomes; dashed line, heterochromatin containing repetitive DNA sequences. Each bar represents one sequence, all reiterations. Circles are centromeres; vertical lines are crossing-over points; stars (*) are ribosomal genes.

Upper panel: Two pairs of chromosomes synapse along repetitive DNA sequences in the long arms. Crossing over may result in shortening or lengthening of heterochromatin as well as transferring ribosomal genes from one chromosome to another.

Lower panel: Two pairs of chromosomes synapse along repetitive DNA sequences in the short arm. Crossing over may result in shortening or lengthening of short arms.
Figure 39. Diagrammatic representation of proposed mechanism for transferring heterochromatin from one chromosome to another.
the same repetitive DNA sequence or sequences, meiotic pairing of nonhomologous chromosomes in the C band region could take place. Figure 39 shows how loop formation and crossover between similar repetitive sequences may occur between nonhomologous chromosomes. Thus a new sequence may be passively transferred from one chromosome to another. The fact that in M. musculus bivalents seem to adhere to one another at the C band regions during pachytene suggests that nonhomologous chromosomes may indeed pair by mistake at the heterochromatic site.

The Role of Heterochromatin in Speciation

A number of hypotheses have been proposed for the function of repetitive DNA or heterochromatin by biologists (Walker 1972, Yunis and Yasmineh 1971, Britten and Davidson 1971, Sutton and McCallum 1972, Hsu 1975). Differences in the amount, distribution, and composition of heterochromatin of eukaryotes have been observed but the exact role that heterochromatin plays in speciation is still unknown.

The Nucleolus Organizer Regions

Dev et al. (1971) found that the locations of the secondary constrictions (usually immediately distal to the centromeric heterochromatin) of the mouse chromosomes may vary from one inbred strain to another. These investigators used Q banding to identify the chromosomes. Later, Henderson et al. (1974) employed in situ hybridization
techniques with ribosomal RNA as a probe, identified the rDNA sites on three pairs of chromosomes, 16, 18, and 19. Elsevier and Ruddle (1975), also employing in situ hybridization techniques, found that the rDNA sites were in chromosomes 15, 16, and 18. The discrepancy apparently can be attributed to the use of different inbred mouse strains, a conclusion supporting the one arrived at by Dev et al. (1971).

As earlier discussed, ribosomal genes (18S + 28S + spacer) are moderately repetitive, with approximately 400 to 500 copies per mammalian genome. Fortunately, in most eukaryotes, ribosomal genes are clustered making detection by cytochemical method or in situ hybridization procedures possible. Elsevier and Ruddle (1975) postulated that ancestral mice might have rDNA in all five chromosome pairs (12, 15, 16, 18, and 19), but inbreeding has condensed the clusters into different chromosome pairs. If this were the case, then karyological evolution could be seen in a matter of decades.

The shifting of NORs in chromosomes is not found only in the inbred strains of Mus musculus. Four pairs of silver NORs were found in Mus m. molossinus, and a large chromosome bearing secondary constriction in Mus cervicolor. Similarly, Ward (1976) found different numbers of NORs in different subspecies of Peromyscus boylei. Thus the changes
in NOR appear to be excellent markers in comparing cytogenetic features for phylogenetic studies.

Cytotaxonomy of *Mus*

Cytotaxonomy of *Mus* offers excellent possibilities in certain areas but is disappointing in others. Most species in subgenus *Mus*, with the exception of those in the Alps, seem to keep the same karyotype. Thus cytological analyses, even with banding, help systematists little. On the other hand, the complex karyological pictures of *Leggadilla* and *Coelomys* appear to be a useful model for cytotaxonomy studies.

**The Outlook**

Mammalian cytogenetics has been one of the most rapidly developing biological disciplines during recent years. Technical innovations have greatly improved the resolution of microscopic observations while other methods such as biophysical analyses of macromolecules, somatic cell hybridization, etc., have complemented cytogenetic analyses. The present data suggest that there is sufficient diversity among subgenera of the genus *Mus* to encourage further investigations both in cytology and in molecular biology.
SUMMARY

Chromosomal characteristics of eight species of the genus Mus were analyzed using a number of contemporary techniques in order to interpret their phylogenetic relationships on a cytogenetic basis. These species were:

Subgenus Mus

Mus musculus musculus (Laboratory strain)
Mus m. molossinus (Okinawa)
Mus caroli (Thailand)
Mus cervicolor (Thailand)
Mus cookii (Thailand)
Mus booduga fulvidiventris (Sri Lanka)
Mus dunni (India)

Subgenus Coelomys

Mus pahari (Thailand)

Subgenus Leggadilla

Mus shortridgei (Thailand)

The cytogenetic characteristics employed in the present study were the following:

1. Conventional staining, including diploid number and gross chromosome morphology.
2. Fluorescent (Q) banding.
3. Giemsa (G) banding.
4. Constitutive heterochromatin (C banding).
5. Silver staining for nucleolar organizer regions (NOR).

With the exception of M. dunnii, all species in subgenus Mus showed similar karyological characteristics. The diploid number is 40, all chromosomes being telocentric. The Y chromosome is usually the shortest or one of the shortest chromosomes of the complement. The Q band and the G band patterns are extremely similar, indicating that major genic arrangements have been conserved during the course of speciation. However, two characteristics, the distribution of C bands and the distribution of NORs, may vary. In all these species, C bands are located in the centromeric areas, but the amount for an individual chromosome is not always constant. The most striking difference is found between the two subspecies of Mus musculus, namely, M. m. musculus and M. m. molossinus. In M. m. musculus, the C bands are more or less evenly distributed but in M. m. molossinus they are highly uneven. Some chromosomes may not demonstrate C bands while others may possess large amounts. The number and locations of NORs vary not only from species to species but also among inbred strains of laboratory mice, M. musculus.

The sex chromosomes of all these species associate telomERICALLY during meiotic prophase and metaphase. Silver
staining revealed extra nuclear argentophilic material during spermatid development.

_M. dunni_ is unique in three respects:

1. The X chromosome is biarmed, with a completely heterochromatic short arm and a terminal C band in the long arm. The Y chromosome is as long as the long arm of the X.

2. The autosomes possess a variable number of biarmed chromosomes (up to 38). The short arms are invariably heterochromatic.

3. In meiosis, the centromeric end of the Y is associated with the telomeric end of the long arm of the X chromosome. When C bands are disregarded, the banding pattern of the _M. dunni_ karyotype is identical with those of other species in the subgenus _Mus._

_M. pahari_ has a diploid number of 48, all chromosomes being telocentric. The Q or C banding patterns are considerably different from those of subgenus _Mus._ Only a few chromosome pairs can be tentatively identified as equivalent to those of _M. musculus_ which is used as a standard. In meiosis, the sex chromosomes are associated with each other at the centromeric ends.
M. shortridgei is unique in several respects.
1. The basic diploid number is 46, with 44 telocentric chromosomes and two biarmed chromosomes. The two biarmed chromosomes are polymorphic because of the distribution of C bands.
2. Different individuals may carry heterochromatic supernumerary chromosomes (up to 5). Thus the diploid chromosome number may vary from 46 to 51.
3. The G band karyotype is so different from that of M. musculus that no definitive homology can be assigned.
4. In meiosis, the sex chromosomes associate with each other at the centromeric ends.

It appears that karyological changes in genus Mus may be a useful tool for phylogenetic studies of this genus. In Europe, M. musculus populations exhibit a varying number of Robertsonian fusions, while in Asia, where Mus originated, fusion is rarely found. Differences in C band (repetitive DNA) and NOR distribution appear to be the most frequent changes in subgenus Mus. However, the changes in gene arrangement between subgenus Mus and other subgenera seem very profound. Apparently tandem translocations and other rearrangements, e.g., inversions, have occurred in karyotypic evolution of Mus. If intermediate forms between the karyotype of M. musculus and those of the subgenera
Coelomys and Leggadilla can be found, tracing the phylogenetic relationships by modern cytogenetic techniques should prove very profitable.
APPENDIX A

EURASIAN SPECIES OF THE GENUS MUS*
<table>
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<th>Y</th>
<th>Ref.</th>
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<th>Distribution</th>
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<td>0</td>
<td>38</td>
<td>0</td>
<td>A</td>
<td>A</td>
<td></td>
<td>in side building</td>
<td>Europe; intro to Americans, some Pacific Islands, and Australia</td>
</tr>
<tr>
<td>(large European laboratory strains)</td>
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<td>M. m. poschiavinus</td>
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<td>26</td>
<td>0</td>
<td>10</td>
<td>14</td>
<td>A</td>
<td>A</td>
<td>3</td>
<td></td>
<td>Valle di Poschia vo, Switzerland</td>
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<td>(NF=40) May be distinct species?</td>
<td></td>
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<tr>
<td>II. Molossinus gr.</td>
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<tr>
<td>M. m. wagneri</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>M. m. molossinus</td>
<td></td>
<td>40</td>
<td>0</td>
<td>38</td>
<td>0</td>
<td>A</td>
<td>A</td>
<td></td>
<td>building and outdoor</td>
<td>Japan</td>
</tr>
<tr>
<td>Subgenus</td>
<td>Scientific name</td>
<td>2n</td>
<td>ST</td>
<td>A</td>
<td>M</td>
<td>X</td>
<td>Y</td>
<td>Ref.</td>
<td>Habitat</td>
<td>Distribution</td>
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</tr>
<tr>
<td>M. m. castaneus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40</td>
<td>0</td>
<td>38</td>
<td>0</td>
<td>A</td>
<td>A</td>
<td>2,5</td>
<td>warehouse, building</td>
<td>(Eastern Asia) Sri Lanka; Calcutta, India; Sikkim; Burma; Yunnan, Kweichow, Fukien, China; Taiwan; Vietnam; Thailand; Malaya; Singapore; Indonesia; Philippines; and introduce to some Pacific Islands</td>
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<tr>
<td>M. kakhyenensis</td>
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<td></td>
<td></td>
<td>Poona, India; Nepal; Sikkim; Assam; Burma; Yunnan, China</td>
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<tr>
<td>M. booduga</td>
<td>40</td>
<td>0</td>
<td>38</td>
<td>0</td>
<td>A</td>
<td>A</td>
<td>7,8,10</td>
<td>rice field</td>
<td>Nepal; India; Burma</td>
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<tr>
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<td>40</td>
<td>0</td>
<td>38</td>
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<td>A</td>
<td>A</td>
<td>6</td>
<td></td>
<td>Poona, Madras, India; Sri Lanka</td>
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<tr>
<td>Subgenus</td>
<td>Scientific name</td>
<td>2n</td>
<td>ST</td>
<td>A</td>
<td>M</td>
<td>X</td>
<td>Y</td>
<td>Ref.</td>
<td>Habitat</td>
<td>Distribution</td>
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<td>31</td>
<td>0</td>
<td>SM</td>
<td>A</td>
<td>7</td>
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<td>&quot; &quot; &quot;</td>
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<td></td>
<td>8</td>
<td>30</td>
<td>0</td>
<td>SM</td>
<td>A</td>
<td>7</td>
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<td>9</td>
<td>29</td>
<td>0</td>
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<td>A</td>
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<td></td>
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<td>10</td>
<td>28</td>
<td>0</td>
<td>SM</td>
<td>A</td>
<td>7</td>
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<td></td>
<td></td>
<td>38</td>
<td>Khajuraho, India</td>
<td>&quot; &quot; &quot;</td>
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<td>0</td>
<td>&quot; &quot; &quot;</td>
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</tr>
</tbody>
</table>

^Species occur in Thailand.

^ST = subtelocentric chromosome.

SM = Submetacentric chromosome.

A = Acrocentric chromosome.

M = Metacentric chromosome.

CReference code: (1) Dhanda et al. (1973); (2) Duncan (1973); (3) Gropp et al. (1970); (4) Gropp et al. (1973); (5) Markvong et al. (1973); (6) Markvong et al. (1975); (7) Matthey and Petter (1968); (8) Mittal and Kaul (1974); (9) Pathak (1976); (10) Satya Prakash and Aswathanarayana (1972a); (11) Satya Prakash and Aswathanarayana (1972b); (12) Tsuchiya and Yosida (1972); (13) Yosida et al. (1969).
APPENDIX B

SPECIES EXAMINED

Specimens of mice (except *Mus musculus musculus*) used in the present investigation are deposited at the Smithsonian Museum, Washington, D.C., USA. Each specimen is labeled with Dr. T. C. Hsu's laboratory numbers.

*Mus musculus musculus* Linnaeus, 1758 (House mouse).
Laboratory Swiss strain (3 males, 4 females).

*Mus musculus molossinus* Temminck, 1843 (House mouse).
Laboratory colony was kindly provided by Dr. Alfred Gropp. Parents of the colony collected by Dr. Marshall were native to Okinawa, Japan (2 males, 2 females: 2388, 2389, 2899, 2710).

*Mus caroli* Bonhote, 1902 (Ryukyu mouse). Thailand. Chonburi Province, Sriracha District; Rayong Province (2 males, 4 females: 2808, 2872, 2873, 2916, 2921, 2922).

*Mus cervicolor cervicolor* Hodgson, 1845 (Fawn-colored mouse). Thailand. Chonburi Province, Sriracha District; Rayong Province (3 males, 4 females: 2689, 2690, 2881, 2882, 2909, 2929, 3008).

*Mus cookii* Ryley, 1914 (Cook's mouse). Thailand. Loei Province, Muang District; Tak Province, Muang District (2 males: 2890, 3013).


Mus pahari Thomas, 1916 (Shrew-mouse). Thailand. Tak Province; Chiangmai Province, Chomtong District, Doi Inthanon (1 male, 2 females: 3032a, 3032b, 3034).

LITERATURE CITED


Deaven, L. L. 1976. Cellular and Molecular Radiobiology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico, personal communication.


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