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Cytogenetic comparison of the lesser mouse deer (*Tragulus javanicus*) and the greater mouse deer (*T. napu*).

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Abstract — Mouse deer is known as the smallest and one of the most primitive ruminants. The lesser and the greater mouse deer originated in three different parts of Malaysia were examined for cytogenetic study. The chromosome number of the mouse deer was 2n=32, consisted of 15 metacentric autosome pairs and X and Y sex chromosome pair. G-, R-band revealed polymorphisms of sex chromosomes among the mouse deer from different origins. X chromosome varied in length of short arm between the lesser and the greater mouse deer. Y chromosome showed polymorphisms not only between the lesser and the greater mouse deer, but also among the lesser mouse deer from different origins. The 18S-28S rRNA genes (18S-28S rDNA) were localized only in the X and Y chromosomes in both lesser and greater mouse deer by the Fluorescence *in situ* hybridization (FISH) method. The hybridization signals of 18S-28S rDNA showed polymorphisms not only in size, but also in their distribution patterns on the sex chromosomes. In addition to the general banding methods, the FISH analysis determined that the greater mouse deer from Tioman island would be remarkably different from the lesser mouse deer. The cytogenetic observations investigated in this study could be informative to characterize the lesser and the greater mouse deer. And it is also suggested that the chromosomal change would reflect that the relationship between the geographic distribution and speciation among the mouse deer.

Key words: chromosome, cytogenetic, FISH, mouse deer, *Tragulus*, 18S-28S rRNA.

INTRODUCTION

The mouse deer belongs to the order of Artiodactyla, which comprises 10 families in 3 suborders, Suiformes (Suidae, Tayassuidae and Hippopotamidae), Tylopoda (Camelidae) and Ruminantia (Tragulidae, Moschidae, Cervidae, Giraffidae, Antilocapridae and Bovidae). Since the Tragulidae (the mouse deer or chevrotain) has primitive features, it is distinguished from other ruminants that share a series of progressive features such as dental formula, present cannon bone and four-chambered stomach (Grubb 1993). Tragulidae are known as the smallest ruminant, with no horns or antlers, having tusk-like upper canines prominent in their males, various form of fused (cannon bone) or non fused metacarpals and poorly formed third stomachs (Vaughan *et al.* 2000). Tragulidae have only three extant genera and four species**,** *Hyemoschus* (*H. aquaticus*, African water mouse deer) in west and central Africa, *Moschiola* (*M. meminna*, Indian mouse deer) in Peninsula India and Sri Lanka, and *Tragulus* in southeastern Asia (Boonsong and Mcneely 1977; Payne *et al.* 1998; Vaughan *et al.* 2000). The genus *Tragulus* has two species, *T. javanicus* (lesser mouse deer) and *T. napu* (greater mouse deer). Lesser mouse deer, also called lesser oriental chevrotain, is one of the smallest ungulates in the world, weighing only 0.7-2.0kg. Greater mouse deer weigh 4.0-6.0kg. Both lesser and greater mouse deer range over southeastern Asia, and it is believed that they are sympatric in some part of the mainland and some neighboring islands. However, only lesser mouse deer distributes in Java (Boonsong and Mcneely 1977). These two species are distinguished by the pelage on the back

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and face, the white expanse on the chin and throat, and body size (Boonsong and Mcneely 1977; Payne *et al*. 1998). However, there are wide variation in size and coloration so that numerous races that present endemic features to each area in coloration and size have been described (Boonsong and Mcneely 1977; Payne *et al*. 1998). Thus the classifications of the two species remains ambiguous (Ellerman and Morrison 1951; Yong 1973b; Boonsong and Mcneely 1977). Cytogenetic studies have been useful in formulating phylogenies for many mammalian groups (Qumsiyeh and Baker 1988; Fechheimer 1989) and in distinguishing subspecies which are difficult to identify by phenotype only (Seuanez 1986; Benirschke and Kumamoto 1991; Hirai *et al.* 2002). The polymorphism of the distribution of the rDNA in the chromosomes has been also used as an effective cytogenetic marker for differentiation and classification of many mammals (Henderson and Bruere 1979; Mayr *et al*. 1985; Zijlstra 1997).

To investigate the differentiation of the lesser and greater Malay mouse deer from the cytogenetic standpoint, we examined the karyotype of these two species collected from three parts of Malaysia (Fig. 1) by G-, C- and R-band analysis and the chromosomal distribution of 18S-28S rRNA genes (18S-28S rDNA) in these mouse deers by the Fluorescence *in situ* hybridization (FISH) using mouse 18S-28S rDNA probe. We describe the cytogenetic observations of *Tragulus* revealed in this study and discuss the relationship between the geographical distribution and speciation in *Tragulus*.

MATERIALS AND METHODS

Animals - The lesser Malay mouse deer (*Tragulus javanicus*) and the greater Malay mouse deer (*T. napu*) were obtained individually from captive breeding stock in Malaysia (Summarized in Table 1 and Fig. 1). Three *T. javanicus* (two males, a female) originated in Pahang, Peninsular Malaysia, and one *T. napu* (a male) originated in Tioman island were kept at Universitiy Putra Malaysia, Selangor, Malaysia. Four *T. javanicus* (two males, two females) were obtained at a private breeding farm in Sabah, Borneo island, Malaysia (Fig. 1). Some of animals analyzed in this study have been studied for morphological analysis by Endo *et al*. (2002).

Cell Culture and Chromosome Preparation - Chromosome slides of the lesser and the greater Malay mouse deer were prepared from cultured lymphocytes or fibroblasts. Blood samples were collected in heparinized vacutainers. Lymphocytes were separated by centrifuge (300 x g, 15 minutes), and then transferred into culture flasks containing RPMI1640 medium supplemented with 20% fetal calf serum, 3μg/ml concanavalin A (type IV-S, Sigma), 10μg/ml lipopolysaccharide (Sigma), 2%HA-15 (Murex) and 50μM mercaptoethanol. These were incubated at 37°C in humidified atmosphere of 5%CO2 in air. Lymphocyte cultures for replication R-banding were established following methods for human lymphocytes (Takahashi *et al*. 1990) with slight modification. Mitogen-stimulated lymphocytes were synchronized by thymidine block (300μg/ml), and then Brd-U (25μg /ml) was incorporated during the late replication stage for replication R-band staining after release from excessive thymidine. R-bands were obtained by exposure of chromosome slides to UV light after staining with Höechst 33258. The fibroblasts from ear or lung were cultured in DMEM supplemented with 15% fetal bovine serum. For replication R-banding from fibroblasts, Brd-U (10μg/ml) was incorporated for 6 hours before harvesting. GTG-banding and sequential CBG-banding were obtained according to Sumner (1972) with slight modifications.

Chromosome of the lesser Malay mouse deer and the greater Malay mouse deer are numbered in order of size and each banding pattern.

Fluorescence in situ *hybridization (FISH)* - Of 8 Malay mouse deer surveyed in the present study, three male mouse deer from different origins were used to Fluorescence *in situ* hybridization (FISH) analysis. Two male lesser Malay mouse deer, one from Pahang (Tj-P2) and another from Borneo (Tj-B3), one greater Malay mouse deer from Tioman island (Tn-Tioman). For FISH

Table 1 — Specimens of lesser and greater Malay mouse deer collected in this study.

Species	Individual No.	Sex	Origin	†X	Y	††BW(kg)
Lesser Malay mouse deer	Tj-P1	male	Pahang, Selangor	SM	M	1.34
(*Tragulus javanicus)*	Tj-P2*	male	Pahang, Selangor	SM	M	1.12
	Tj-P3	female	Pahang, Selangor	SM	-	1.40
	Tj-B1	female	Sabah, Borneo	SM	-	2.80
	Tj-82	female	Sabah, Borneo	SM	-	2.50
	Tj-B3*	male	Sabah, Borneo	SM	ST	1.50
	Tj-B4	male	Sabah, Borneo	SM	ST	0.95
Greater Malay mouse deer	Tn-Tioman*	male	Tioman island	SM	ST	3.00
(T. napu)						

† X and Y, morphology of X and Y chromosome. SM, submetacentric. M, metacentric. ST, subtelocentric †† BV body weight. Asteric (*) indicates individual examined to localize 18S-28S rDNA.

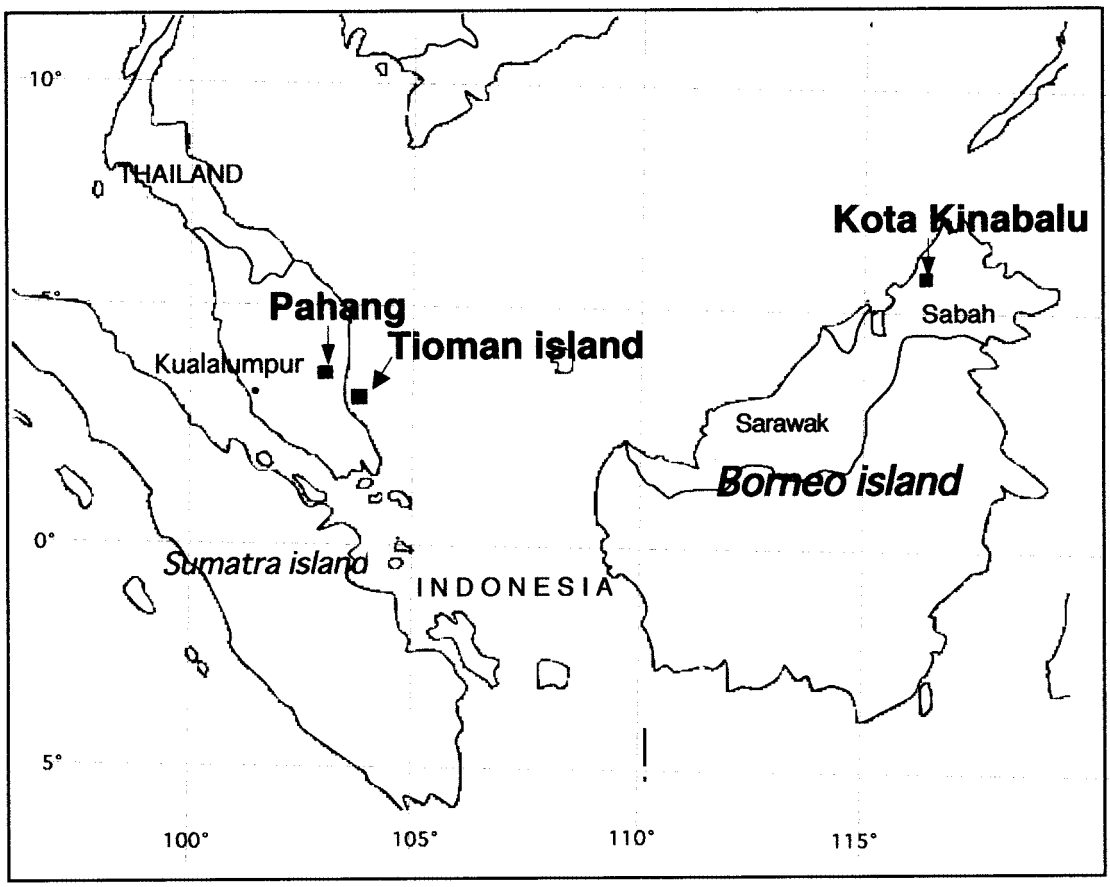


Fig. 1 — Map of the samples of Malay mouse deers originated. Tioman island is located in the South China Sea and east of the coast of Pahang.

analysis, 6.6 kb mouse 18S-28S rDNA clone (Kominami *et al.* 1982) was used as probe. For the 18S-28S rDNA probe, the chromosome slides were pretreated with RNase and then denatured at 68ºC for 2 minutes in 70% formamide / 2 x SSC solution. Probe DNA were labeled with biotin-16- dUTP using a nick translation kit (Roche) and ethanol precipitated with salmon sperm DNA and *E.coli* tRNA. Hybridization and detection of fluorescence signals were performed according to Matsuda and Chapman (1995). Probe DNA were hybridized for 15 hours at 37ºC. The hybridized 18S-28S rDNA probes were stained with Cy2-conjugated Streptavidin (Jackson). The slides were stained with 0.75 μg/ml propidium iodide (PI) for observation. Fluorescent images were observed by an Olympus BX-60 epifluorescence microscope with Olympus filter set U-MWIB (excitation at 470-490 nm), U-MSWG (480-550 nm) U-MWU (330-385 nm) and photographed with Kodak Ektachrome ISO 100 films.

RESULTS

*Karyotype of Mouse deer (*Tragulus*)* - The karyotype of the mouse deer studied was 2n=32, and consists of 15 pairs of autosomes and one pair of sex chromosomes. The GTG-, CBG- and RBG-banded karyotype were shown in Figures 2 (a, b), 3 (a, b) and 4 (a, b, c). Chromosomes are numbered in order of size. All autosomes were metacentric and the basic banding patterns were overall similar in all animals analyzed in this study. However, the difference in sex chromosomes was found between the lesser mouse deer and the greater mouse deer as well as among the lesser mouse deer from Pahang and from Borneo island. All of the mouse deer surveyed in this study have large submetacentric X chromosomes, which have a second constriction region in its short-arm, negatively stained with G-, C- and R-band (Fig. 5). The size of the short-arm of X chromosome of the greater mouse deer (Tn-Tioman) was much shorter than that of the lesser mouse deer, because of the decrease of the second constriction region on its short arm. Y chromosome varied in morphology not only between the lesser and the greater mouse deer, but also among the lesser mouse deer. The Y chromosome showed metacentric in the lesser mouse deer from Pahang (Tj-P2), subtelocentric in the lesser mouse deer from Borneo island (Tj-B3) and in the

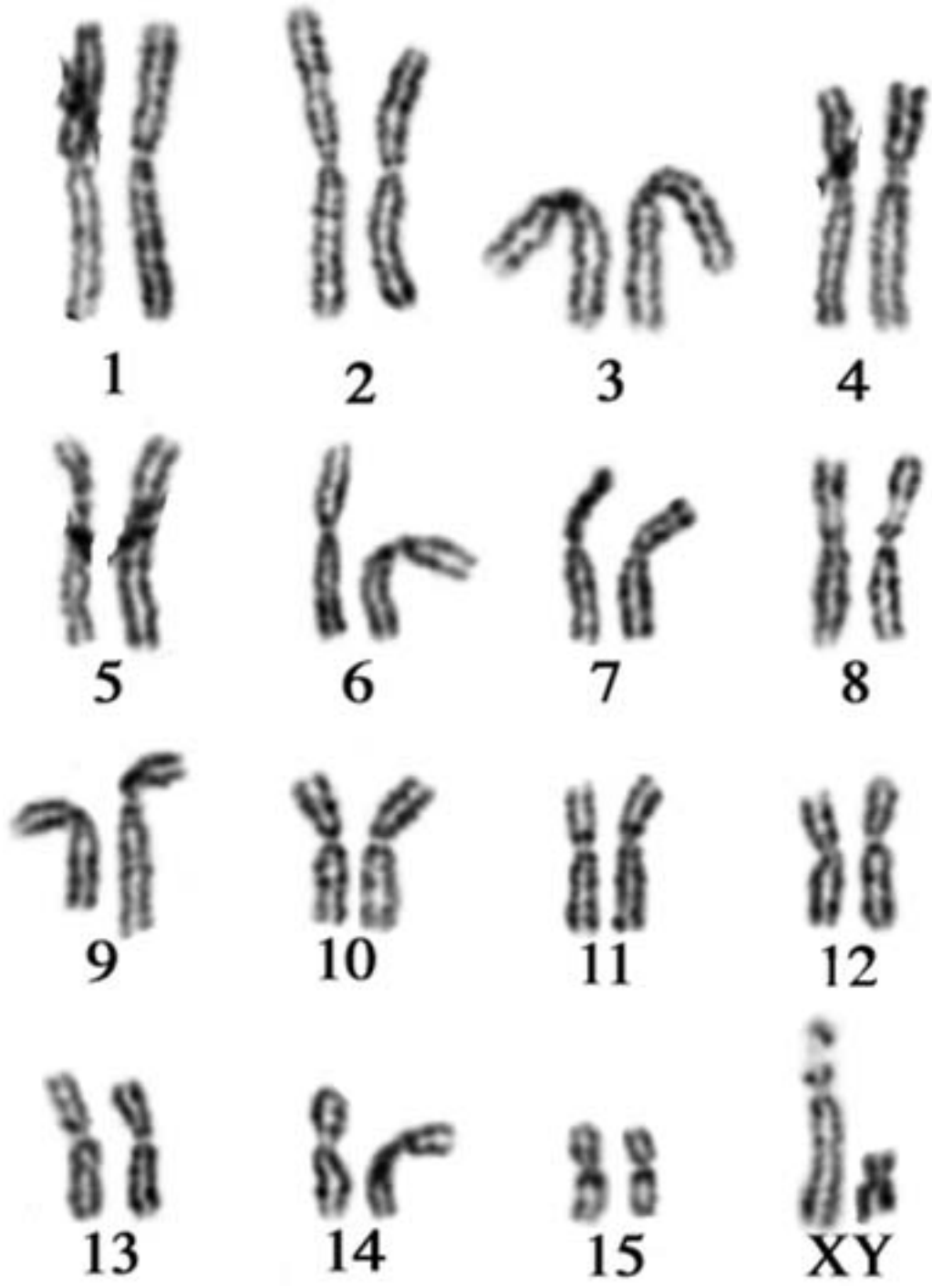
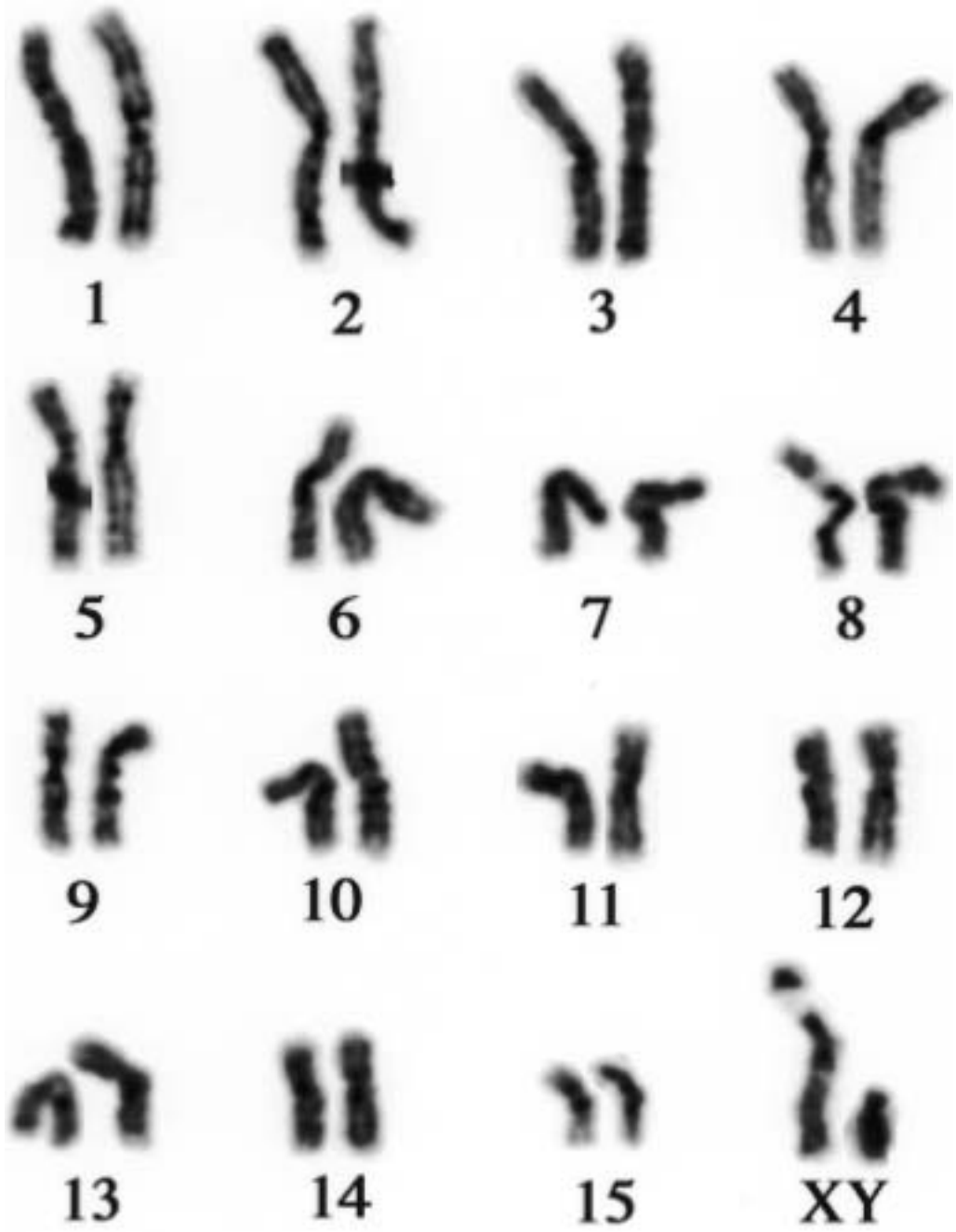


Fig. 2 — GTG-banding karyotype of the lesser.Malay mouse deer from Pahang (a) and from Borneo (b).

greater mouse deer (Tn-Tioman), respectively (Fig. 5). The Y chromosome of the greater mouse deer (Tn-Tioman) was apparently larger than that of the lesser mouse deer, which is larger than chromosome15 in size (Fig. 4c), while the Y chromosome of the lesser mouse deer from both Pahang and Borneo is the smallest of any other autosomes (Figs. 2a and 2b, 3a and 3b and 4a, 4b and 4c).

CBG-band revealed the centromeric constitutive heterochromatin in all chromosomes (Figs. 3a and b). Some chromosomes have additional telomeric and / or interstitial positive C-bands. The distribution of those interstitial C-bands corresponded to the second constriction regions (See figures 2a and 2b; 3a and 3b; 4a, 4b and 4c). The Y chromosome shows entirely C-band positive. There are polymor-



phisms of C-band pattern among the lesser mouse deer. All of the autosomes of Tj-P2 have pronounced centromeric C-bands (Fig. 3a), while some autosomes of Tj-B3 have quite weak centromeric C-bands (Fig. 3b).

Unfortunately, GTG and CBG-band of Tn-Tioman could not obtained in this study because of the poor cultivation.

FISH analysis - The results of FISH analysis are shown in Figure 6-1. The cluster of 18S-28S rRNA genes (18S-28S rDNA) was present only in X and Y chromosomes, no other signals were found in any autosomes. The fluorescent signals of 18S-28S rDNA showed polymorphisms in both size-intensity and location among the three mouse deer (Fig. 6-2). The strong hybridization signals were found in the short-arm of the X chromosome, that is the second constriction region; those intensity were strong in the lesser mouse deer (Tj-P2, Tj-B3), while those of the greater mouse deer (Tn-Tioman) were weaker. These intensity of hybridization signals corresponded to the size of the constriction region ob-

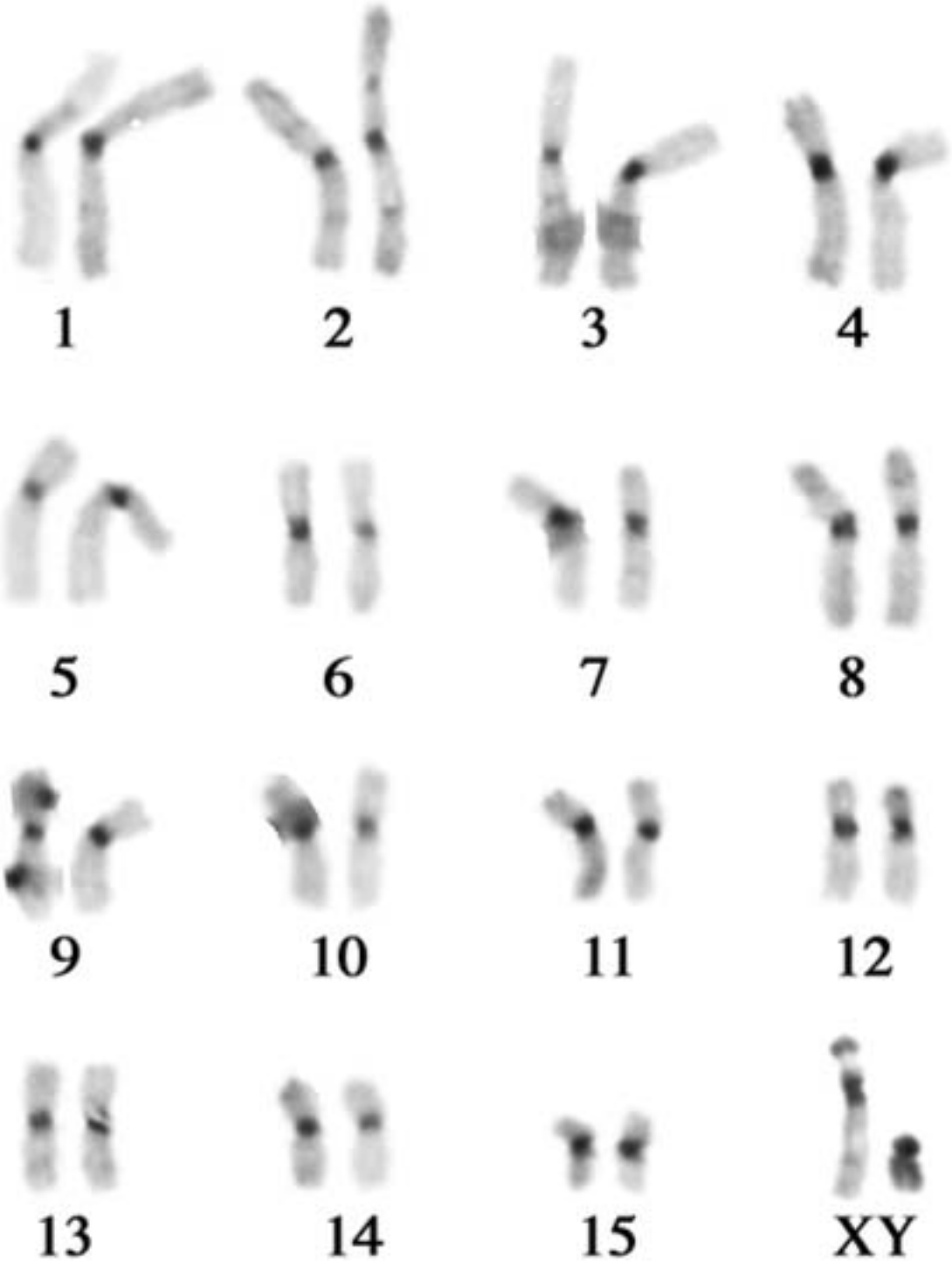
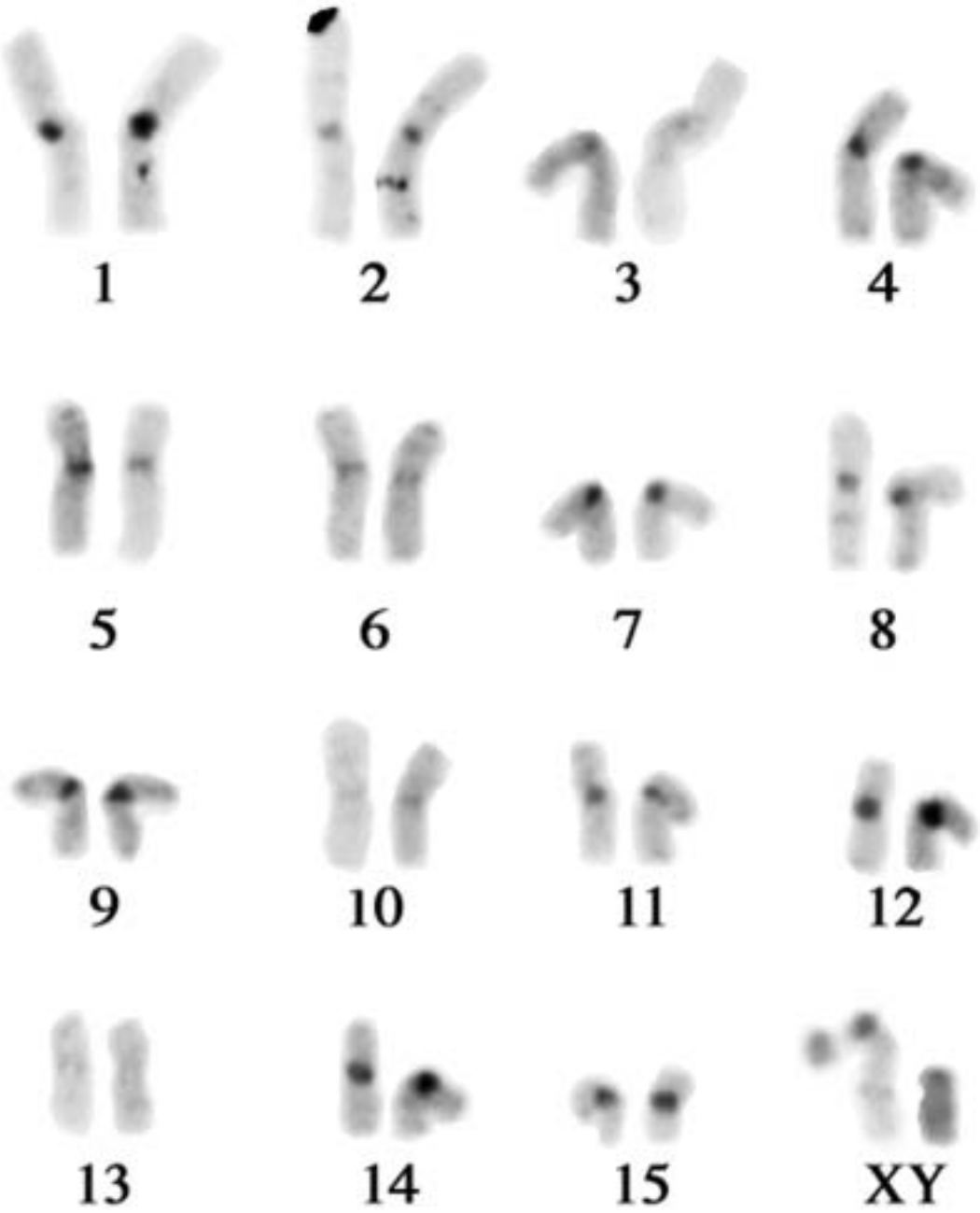


Fig. 3 — CBG-banding karyotype of the lesser Malay mouse deer from Pahang (a) and from Borneo (b). (a):all chromosomes present same intensity of C-band. (b):chromosome 1 shows pronounced C-band from other autosomes, and chromosome 3,6,10, and 13 shows pale C-bands.

served in GTG- and RBG-band (Fig. 5). Regarding the hybridization signals on the Y chromosome, they were located at distal end of long-arm in the lesser mouse deer (Tj-P2, Tj-B3), whereas, in the greater mouse deer (Tn-Tioman), the hybridization signals were located at the proximal region of long-arm of the Y chromosome, that separated into two blocks. And the intensity of those two signals on the long



arm of the Y chromosome in the greater mouse deer (Tn-Tioman) was stronger than that of the lesser mouse deer (Tj-P2, Tj-B3) (Figs. 6-1 and 6-2).

DISCUSSION

Comparison of general band - The diploid number of lesser mouse deer and greater mouse deer are both 2n=32, that was same as the previous reports for the lesser Malay mouse deer (YONG 1973a; GALLAGHER *et al*. 1996) and Yunnan mouse deer (LIMING and YUZE 1989). The karyotype of the mouse deer consists of 15 pairs of metacentric autosomes and one pair of sex chromosomes. This complement comprises of all metacentric autosomes in *Tragulus* is different from those of most species in Bovidae and Cervidae comprise of acrocentric chromosomes (WURSTER and BENIRSCHKE 1967; GALLAGHER and WOMACK 1992; GALLAGHER et al. 1994). It is believed that the chromosomal evolution in the superfamily Bovidea occurred by Robertsonian centric fusion (WURSTER and BENIRSCHKE 1968). The findings that all autosomes of *Tragulus* comprise of metacentric-type would suggest that the karyotype of *Tragu-*

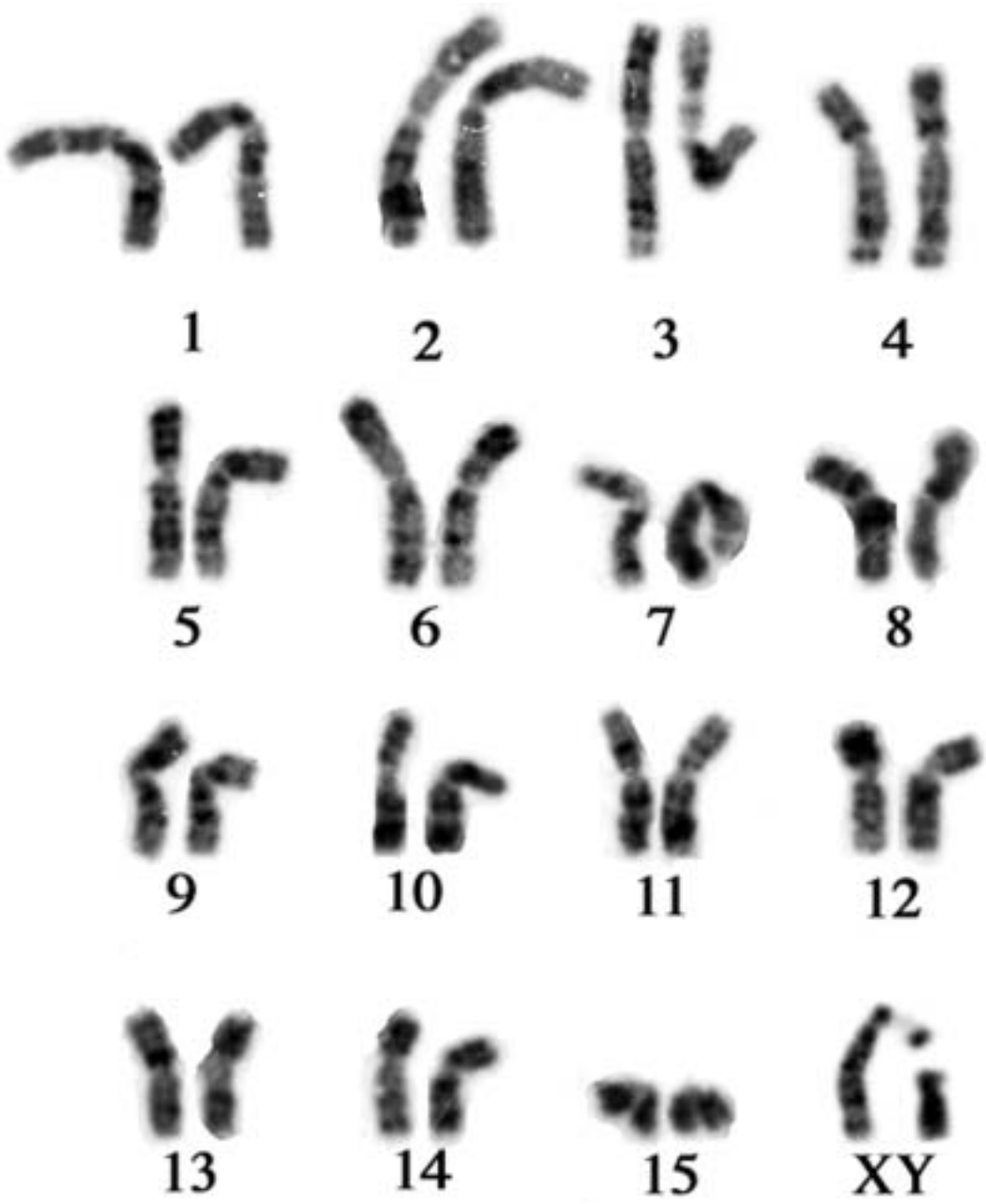
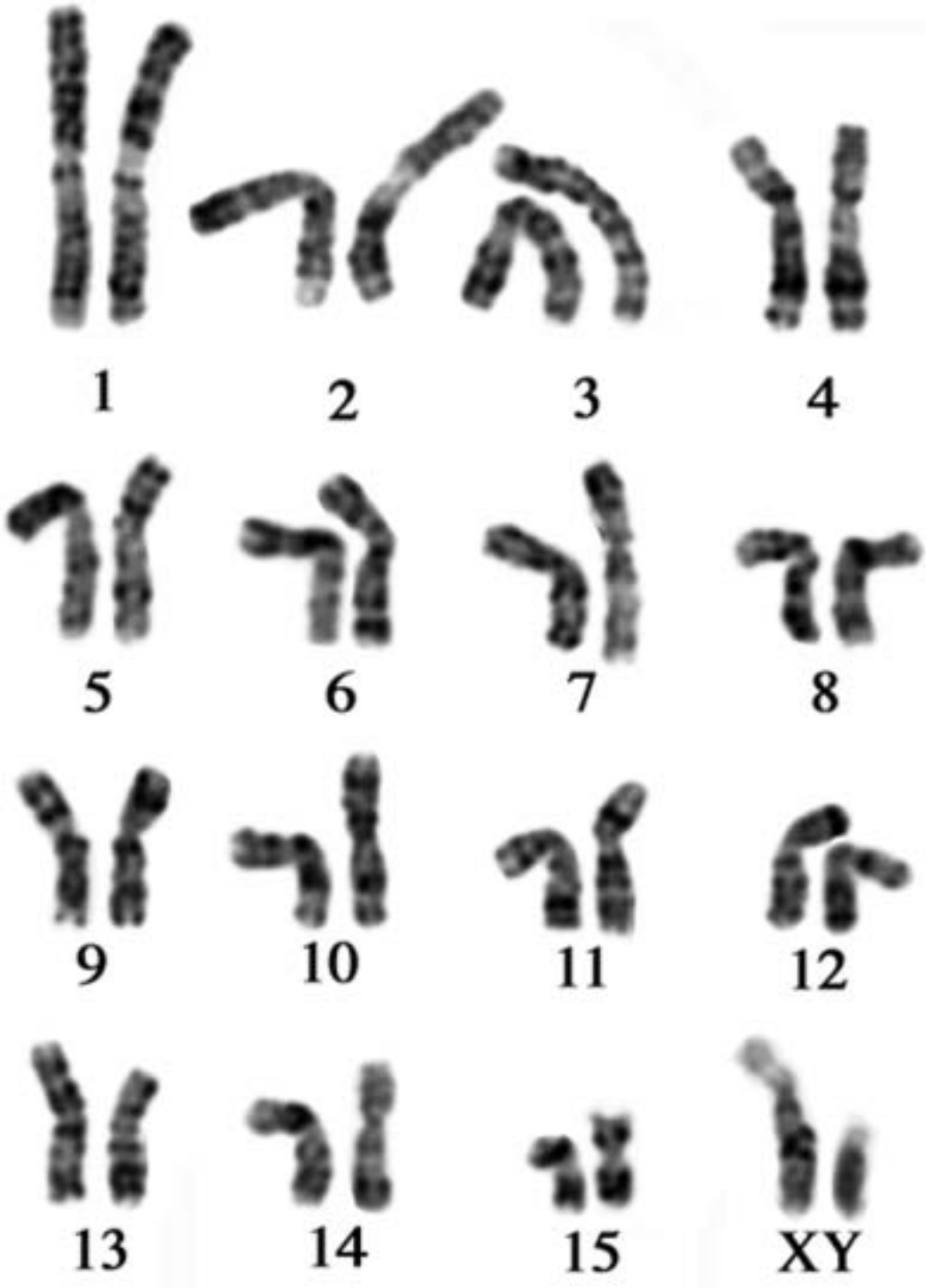


Fig. 4 — RBG-band pattern of the lesser Malay mouse deer from Pahang (a; Tj-P2) and from Borneo (b; Tj-B3), and the greater Malay mouse deer from Tioman island (c).

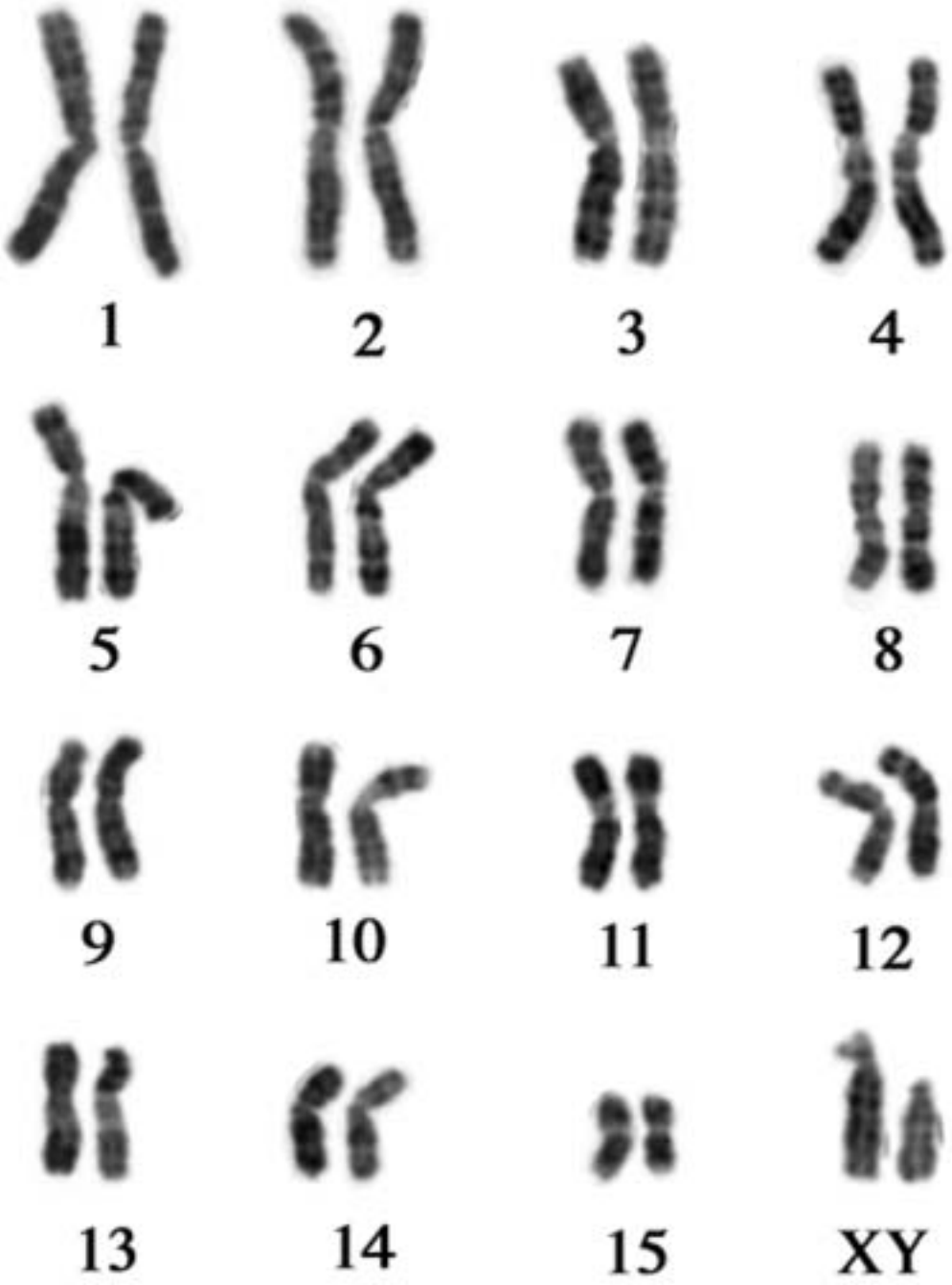
lus could be an exceptional condition among Ruminants.

The polymorphisms of X and Y chromosomes are shown in both the size and the morphology. The differences of sex chromosomes were present not only between the lesser mouse deer and the greater mouse deer, but also among the lesser mouse deer from Phang, Peninsular Malaysia (Tj-P2), and from Borneo island (Tj-B3) (Fig. 5). The similar interspecific or intraspecific variations of sex chromosomes in voles and tree shrew have been reported (IWASA *et al.* 1999; IWASA and SUZUKI 2002; HIRAI *et al.* 2002). The differences in the size of the short arm of X chromosome and the Y chromosome would be explained to be due to the amount of heterochromatin. The significance of such differences in the sex chromosomes has demonstrated by the analysis of the synaptonemal complex-axis (SC-axis) length in homologous autosomes and sex chromosomes during mitosis (IWASA *et al.* 1999). So, these findings, the polymorphism of X and Y chromosomes among the



mouse deer, could be informative in differentiating these two species or subspecies in cases where it is difficult to distinguish by appearance.

C-band positive region reflects the constitutive heterochromatin, highly repeated satellite DNA (PARDUE and GALL 1970; MODI *et al.* 1988). There were polymorphisms of C-band pattern among the lesser mouse deer. The result of C-band pattern of the lesser mouse deer in this study (Fig. 3a) was same as the previous reports of the lesser Malay mouse deer and Yunnan mouse deer (YONG 1973a; LIMING and YUZE 1989; GALLAGHER *et al.* 1996). Centromeric heterochromatin was present in all autosomes and Y chromosome was entirely C-band positive in the lesser mouse deer (Figs. 3a and b). Additional telomeric or interstitial heterochromatin was seen in



some autosomes (Figs. 3a and b). It corresponds that C-band reveals constitutive heterochromatin at centromeric, interstitial and telomeric region. Regarding the lesser mouse deer from Borneo, the centromeric C-bands of chromosome 3, 6, 10 and 13 were very faint and that of chromosome 1 was stronger than other autosomes (Fig. 3b). It is known that C-band is often variable in banding pattern or size between species, interspecies, and sometimes even within homologous chromosomes (DEV *et al.* 1975; MAYR *et al.* 1985, 1987). The differences in the heterochromatin pattern (C-band) in Bovidae and Cervidae have often been reported, and they permit the characterization of each individual. The polymorphisms of C-band pattern among the lesser Malay mouse deer observed in this study might facilitate the characterization of origins or individuals of lesser Malay mouse deer as the case of Bovine species.

Since there are prominent differences (the shortened second constriction region of X chromosome and the lengthened Y chromosome in Tn-Tioman), it is supposed that there should be remarkable cy-

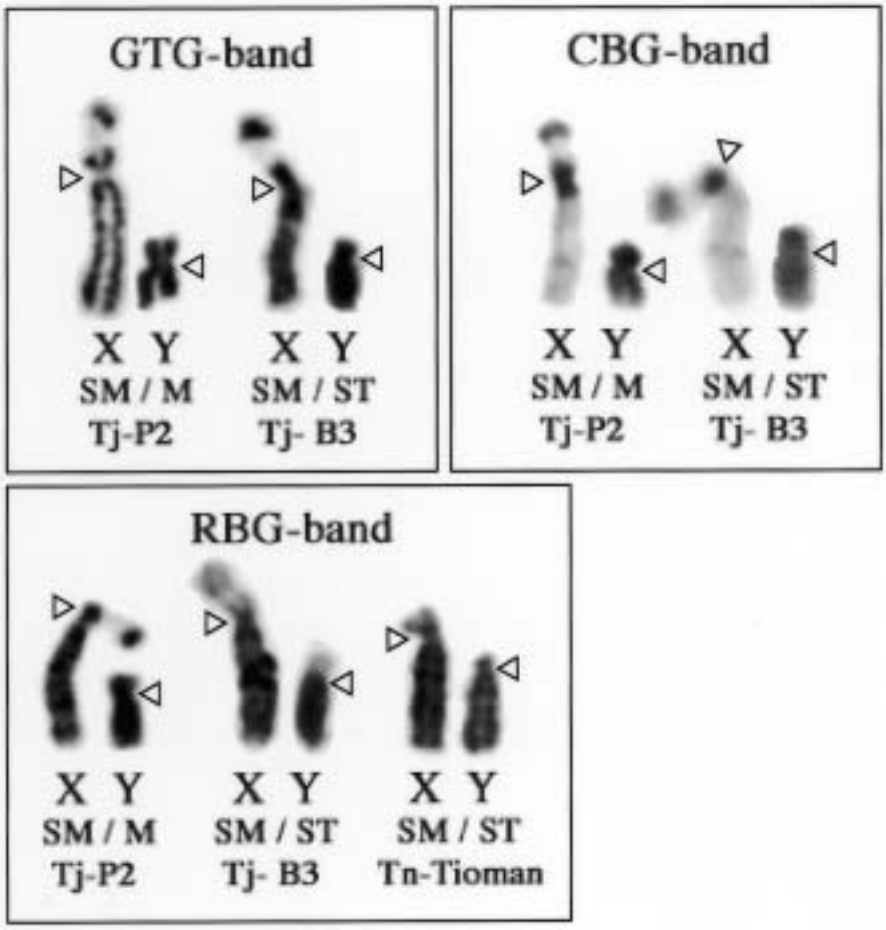


Fig. 5 — Conventionally stained X and Y chromosomes of the lesser mouse deer from Pahang (Tj-P2) and from Borneo island (Tj-B3), and the greater mouse deer from Tioman island (Tn-Tioman). SM, sabmetacentric; M, metacentric; ST, subtelocentric. Arrowheads indicate the centromeric regions.

togenetic differences between the greater mouse deer and the lesser mouse deer. FISH analysis using 18S-28S rDNA probe in present study resulted in confirmation of this suggestion.

FISH analysis - 18S-28S rDNA is detected only in sex chromosomes in all metaphase applied to FISH analysis in this study. This distribution pattern of 18S-28S rDNA such as restricted in sex chromosomes is quite unique, since most species of mammal have18S-28S rDNA at multiple loci and they are on autosomes. This distribution pattern is also exceptional among Ruminants. Since nucleolar organizer regions (NORs) are distributed over four or five autosome pairs in most species of ruminants (Mayr *et al.* 1985). The Y chromosome is believed to be mostly heterochromatic and containing a little number of genes, and only a few species of mammals have NORs on Y chromosome (i.e. *Carollia castanea, Hylobates concolor, H. syndactylus*) (Hsu *et al.* 1975; Ledbetter 1981; Tuinen and Ledbetter 1982). In most species of Ruminants, NORs distribute on several autosomal chromosomes; four or five autosome pairs among most Bovidae and two autosome pairs among most Cervidae (Henderson and Bruere 1979; Tuinen *et al.*1983; Mayr *et al.* 1985, 1987). These previous investigations indicate that there has been a high conservatism of the number and location of NORs in each family of Ruminants during evolution. There is an exception such as Indian muntjac (2n=♀*6,* ♂*7 Muntiacus muntjak vaginalis),* which is known for the lowest chromosome number among mammals. It is believed that Indian muntjak went through exceptional karyological evolution and has NORs on chromosome 1 and X chromosome (Wurster and Benirschke 1970). The X chromosome of Indian muntjac was formed by a fusion between an autosome and ordinal X chromosome. The NORs in the Indian muntjak is actually localized on real autosomal region of the compound X chromosome. As far as we know, mammalian species that have 18S-28S rDNA or NORs on X chromosome are Indian muntjac, Seba's fruit bat, (2n=♀*20,* ♂*21 Carollia perspicillata*), *Carollia castanea* (2n=22), Rat kanga-

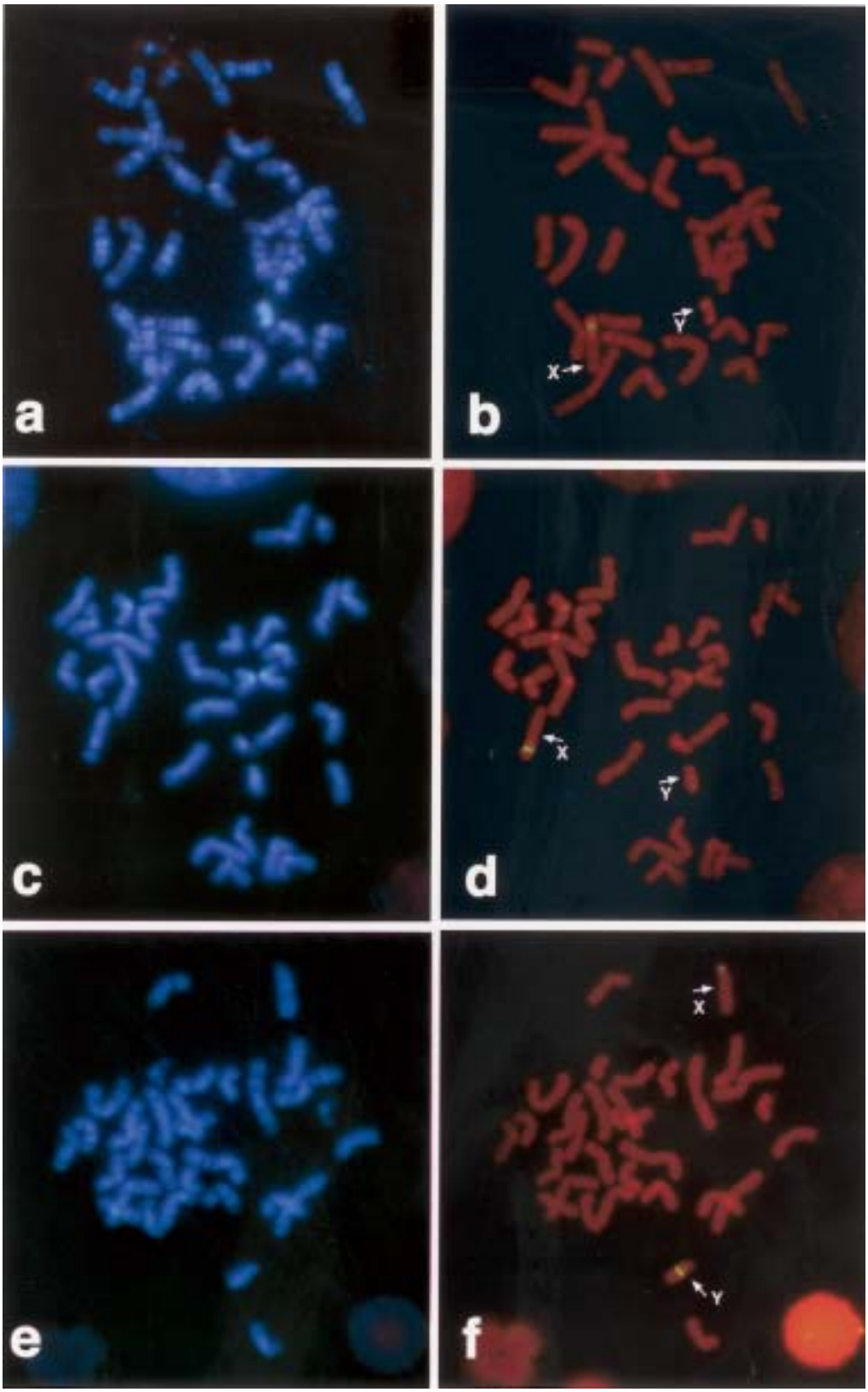


Fig. 6-1 — Metaphase chromosomes of the mouse deer after direct R-banding FISH with the mouse 18S-28S rDNA. Q-band of Tj-P2 (a), Tj-B3 (c), and Tn-Tioman (e). The hybridyzation signals on R-banded chromosomes of Tj-P2 (b), Tj-B3(d), and Tn-Tioman (f). Arrowheads on the left side indicate the centromeric regions.

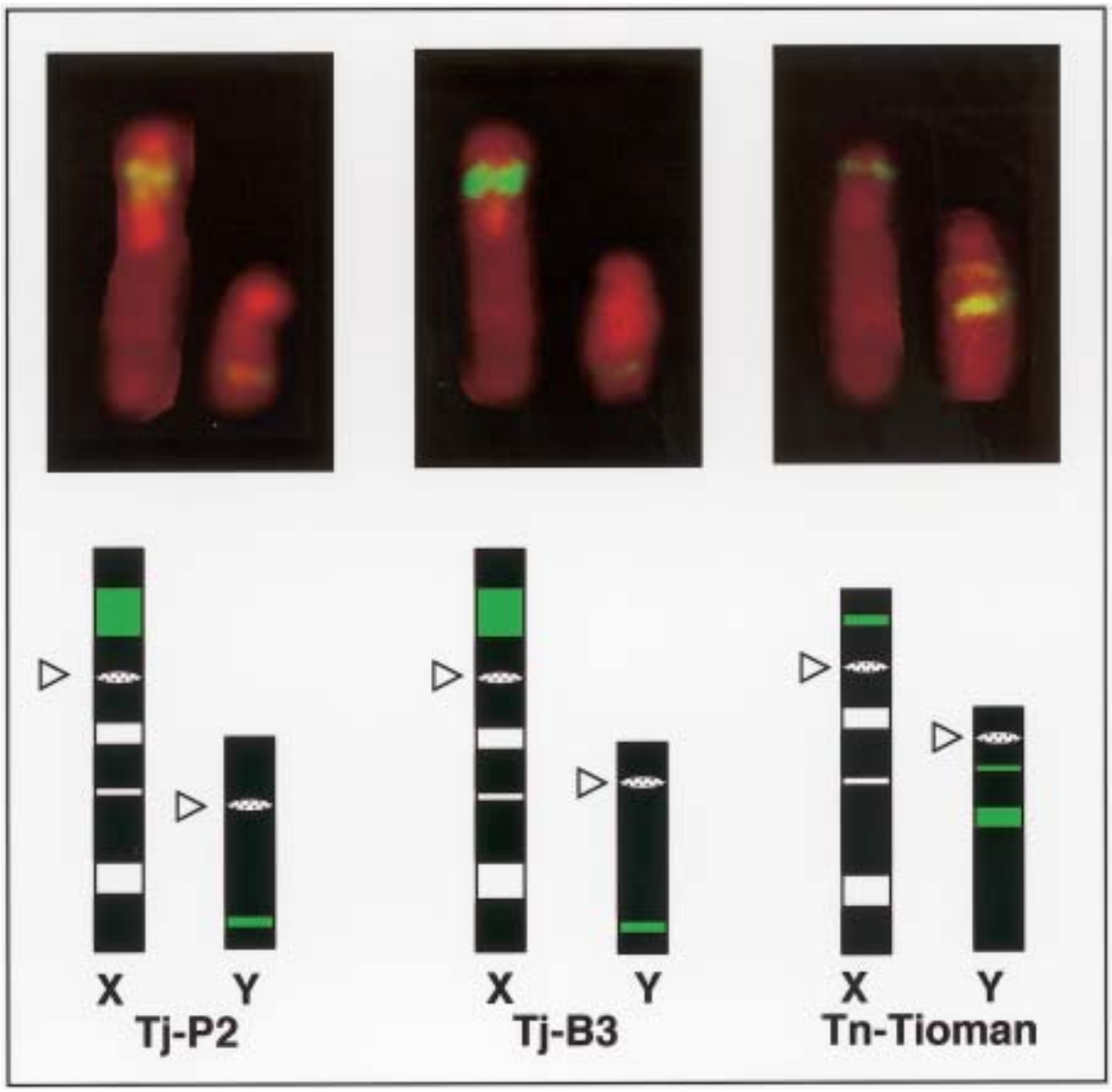


Fig. 6-2 — Schematic illustration of the hybridiztion signals (green) in X and Y chromosomes on R-banded (black) chromosomes of Tj-P2, Tj-B3 and Tn-Tioman. Arrowheads on the left side indicate the centromeric regions.

roo (2n=♀*12,* ♂*13 Potorous tridactylis*) and Chinese hamster (2n=22 *Cricetulus griseus*) (Wurster and Benirschke 1970; Hsu *et al.* 1975; Goodpasture and Bloom 1975). Of these animals, the X chromosome of Indian muntjac, Seba's fruit bat and rat kangaroo have a translocated autosome. So the NORs in their X chromosomes are really localized in the translocated autosomal region of X chromosome (Hsu *et al.* 1975; Goodpasture and Bloom 1975). Only *Carollia perspicillata* has 18S-28S rDNA in real sex chromosome, similar to the Malay mouse deer in this study. The prominent fluorescent signals at second constriction region of X chromosome in this study (Fig. 6) supports the previous cytological literature that secondary constrictions of chromosomes are usually associated with nucleoli, referred to as NORs. The weaker fluorescent signals of short arm of X chromosome in Tn-Tioman could be explained by the decrease of 18S-28S rRNA genes. Another site of 18S-28S rDNA in Malay mouse deer is in the Y chromosome, showing polymorphism of distribution pattern. Single block fluorescent signal was present at telomeric region of long arm of Y chromosome in the lesser mouse deer (Tj-P2 and Tj-B3), whereas two separated block signals were found in the internal region of the long arm of Y chromosome in the greater mouse deer (Tn-Tioman) (Fig. 6). It is believed that only one species of mouse deer,

which is called *T. napu rufulus,* distributes in Tioman island (YONG 1973b). In addition to the decrease of short arm of the X chromosome, the distribution patterns of 18S-28S rRNA genes on the Y chromosome of Tn-Tioman indicate that there have been several drastic chromosomal rearrangements through speciation between the lesser and the greater mouse deer (Fig. 6-2).

LIMING and YUZE (1989) reported that the Yunnan mouse deer in China (*Tragulus javanicus williamsoni*), which is regarded as one of the subspecies of lesser mouse deer, have the submetacentric Y chromosome and it is larger than that of the lesser Malay mouse deer (*T. javanicus angustiae*). They also reported that the NORs in Yunnan mouse deer located on the short arm of chromosome 4 and long arm of the Y chromosome. However, no autosome carried 18S-28S rDNA in our present results. Since the chromosome 4 and X have close resemblance in size and shape, X chromosome may have been taken for the chromosome 4 in that report (LIMING and YUZE 1989). Indeed, only one mate of the chromosome 4 had NOR in their results. However, the possibility of existence of 18S-28S rDNA polymorphisms among lesser Malay mouse deer and Yunnan mouse deer still cannot be denied. These polymorphisms of sex chromosomes and 18S-28S rDNA distribution patterns among the genus *Tragulus* from different origins might reflect the geographical characteristics. The cytogenetic observations revealed in this study have demonstrated that the chromosome study would be valuable in characterizing two species of the genus *Tragulus* and informative for the relationships between the geographical distribution and their speciation. This also means that the chromosome study could provide the insights into phylogenetic relationships.

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