C-BANDING IN POLYTENE CHROMOSOMES OF SIX *SIMULIUM* SPECIES (DIPTERA: SIMULIIDAE) FROM DOI INTHANON NATIONAL PARK, NORTHERN THAILAND

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ABSTRACT

The polytene chromosomes of Simulium (Nevermannia) caudisclerum, S. (N.) feuerborni, S. (Simulium) fenestratum, S. (S.) rufibasis, S. (S.) nakhonense *and* S. (Montisimulium) sp. G *consistently showed C-banding of single thin to thick bands at centromere regions. telomeres and some interstitial sites. Despite the low amount of C-banded heterochromatin, these species showed intraspecific and interspecific differences in amount and distribution of heterochromatin. The larvae of* S. (S.) rufibasis *collected from different locations showed two different C-banding patterns. Pattern two had more C-banded materials in centromeres and interstitial regions than those in pattern one. In comparison with the C-banding pattern of* S. (N.) caudisclerum *which is presumed to be the most primitive species, the* Simulium *species in the present study were divided into three groups. Each group had cytological characteristic marked by differences in C-banding of centromeric heterochromatin. Therefore, the C-banding methods used are capable of demonstrating centromeric and interstitial hetrochromatin whose satellite DNA has been increased and/or decreased during chromosome evolution. Moreover, C-banding technique successfully revealed that band 84B2 on the chromosome arm IIIL of* S. (S.) nakhonense *was a sex-linked heterochromatic band which is involved in differentiation of genetic X-chromosomal and Y-chromosomal segments.*

INTRODUCTION

Blackflies belong to the family Simuliidae of the order Diptera. They are small, dark, stout-bodied and hump-backed flies. The Simuliidae is widely distributed in all zoogeographical regions where there is running water suitable as a habitat of the immature stage. In Thailand, there are only few studies of blackflies and is reported only on their morphology. Recently, a total of seventeen *Simulium* species consisting of thirteen known species, three unnamed species and one new species has been recognized at Doi Inthanon National Park, Chiang Mai Province, on the basis of external morphological characters of larvae and pupae (Kuvangkadilok, unpublished data). Most of the blackflies are recognized and defined on characters of their external morphology. However, the cytological studies which evaluate chromosome structure in larval salivary gland polytene chromosomes can be used for accurate identification.

The Giemsa C-banding techniques are believed to reveal areas of constitutive hetrochromatin[1,2] Heterochromatin contains a high amount of repetitive DNA or satellite DNA arranged tandemly in the vast majority of the genome.[3,4] The repeated DNA sequence represents noncoding DNA which has much faster sequence divergence than the unique DNA sequence of euchromatin.[5] It is believed that heterochromatin plays an important role in chromosome evolution,[6,7]

speciation[8,9] and identification of sex chromosomes.[10] Moreover, sex-linked and supernumerary heterochromatic bands or blocks which are involved in differentiation of genetic X and Y chromosomal segments were reported in many simuliids such as *S. ornatipes*[11], *S. ochraceum*[12], *S. metallicum*[13], *Cnephia dacotennis*[14] and *Prosimulium mixtum.*[15]

The Giemsa C-banding method stains not only the centromeres, but also interstitial and telomere chromosome bands. Differences in the quality, number, size or distribution of these C-bands were detected in some *Simulium* species such as *S. ornatipes* and *S. melatum.*[11] In a number of *Simulium* species the centromeres of the polytene chromosomes are not prominent and their locations are often uncertain. By a C-banding technique, the centromeric regions including interstitial and telomeric bands should be identifiable. Therefore, this study presents and compares the C-banding patterns of six *Simulium* species in different subgenera: *i. e., S. (N.) caudisclerum, S. (N.) feuerborni, S. (S.) fenestratum, S. (S.) rufibasis, S. (S.) nakhonense* and *S. (M.)* sp. G. from Doi Inthanon National Park, northern Thailand.

MATERIALS AND METHODS

Larvae of *S. (N.) caudisclerum, S. (N.) feuerborni, S. (S.) fenestratum, S. (S.) rufibasis, S. (S.) nakhonense* and *S. (M.)* sp. G collected from five localities at different altitudes of Doi Inthanon National Park, Chiang Mai Province (Fig. 1, Table 1). They were fixed in two changes of freshly prepared Carnoy's fixative (1:2, glacial acetic acid : absolute ethanol) and then store at -18°C for slide preparation.

Polytene chromosome squash preparations were made from penultimate instar larvae. Larvae were dissected and stained with 1.6% orcein (lactic : propionic acid : water, 2:2:1) for 15-20 minutes using the method described by Porter and Martin.[16]

For C-banding of polytene chromosomes, polytene chromosome squash preparations were made in 60% acetic acid using a siliconized coverslip. The slides were stored at -4°C and used for C-banding within 3 days of their preparation. Each slide was put in liquid nitrogen and then the coverslips were removed carefully using a razor blade. Chromosomes were dehydrated in 70%, 80% and absolute ethanol for 5 minutes each and dried on a slide warmer. Cytological techniques used for C-banding were adapted from Hadi *et al.*[17] Treatment times were slightly modified in order to achieve clear C-bands. Air-dried slides were treated in 0.2 N HCL at room temperature for 45 minutes and washed with distilled water. Slides were incubated in 5% $Ba(OH)_2$ at 65°C for 10 minutes and rinsed in distilled water. They were then incubated in 2xSSC (1xSSC consists of 0.15M sodium chloride and 0.015 M sodium citrate) at 65°C for

Table 1 List of six *Simulium* species from different localities of Doi Inthanon National Park used in this study.

Species	Localities (altitude)	Date of Collection
S. (N.) caudisclerum	Ang Kha (2460 m)	August 1997
S. (N.) feuerborni	Royal Project (1250 m)	May 1997
S. (S.) fenestratum	Siriphum waterfall (1270 m)	August 1997
S. (S.) nakhonense	Vang Kwai waterfall (410 m)	October 1997
S. (S.) rufibasis	Siriphum waterfall (1270 m)	October 1997
	Kew Mae Pan (2300 m)	May 1997
S. (M.) sp. G	Ang Kha (2460 m)	August 1997

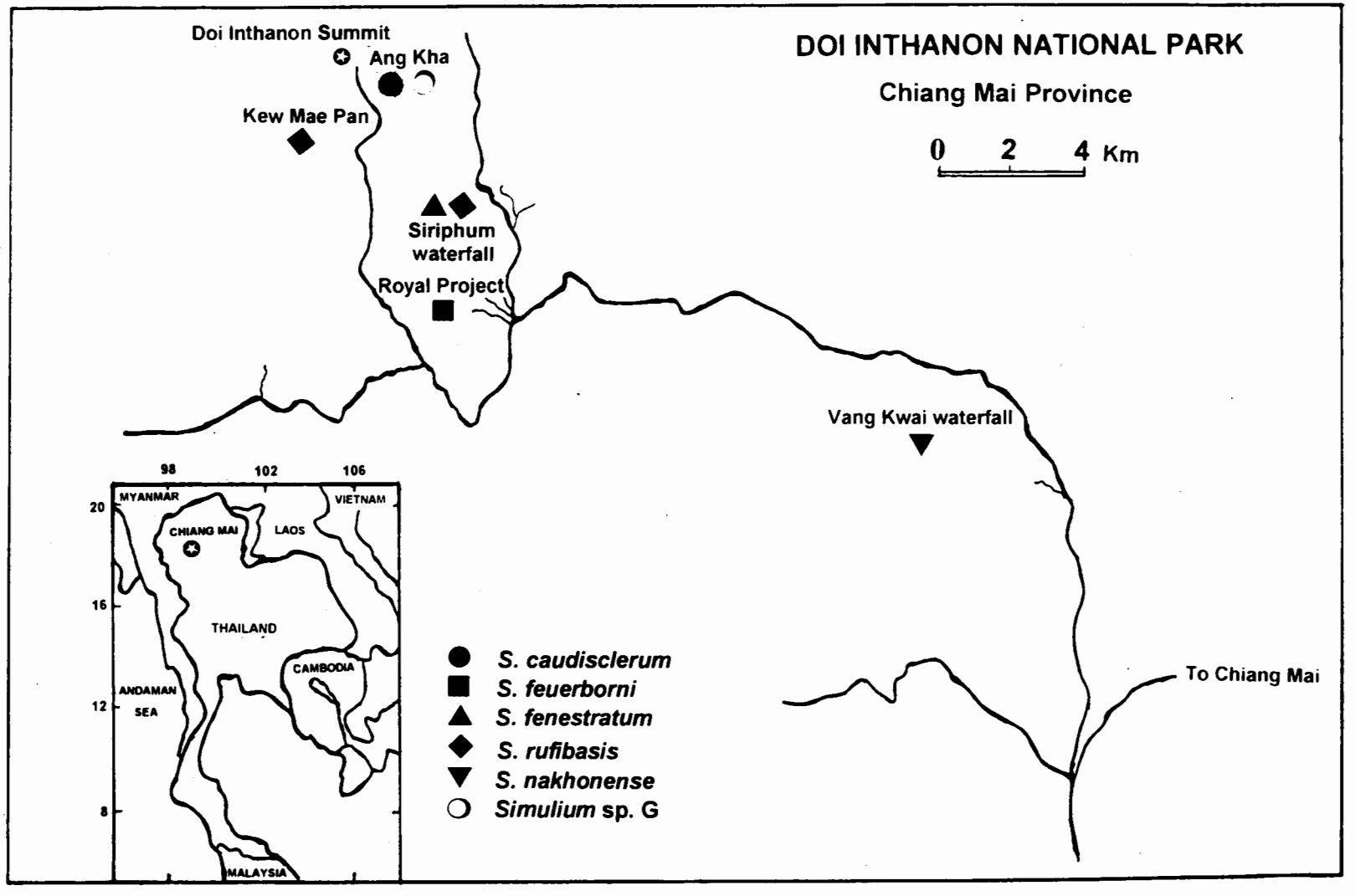

Fig.1 Map of Doi Inthanon National Park, Chiang Mai Province, showing the five localities where the six *Simulium* species were collected for this study.

3 hours. After being rinsed in distilled water and air dried, slides were stained with 4% Giemsa (Gurr's 66 improved solution, BDH) in phosphate buffer (pH 6.8) for 15-20 minutes. They were then rinsed in distilled water, air dried and mounted with DPX (BDH). C-banding in polytene chromosome is manifested as dark purple bands when viewed under a light microscope with a green filter. All slides were checked for C-banding appearance and thickness.

The remaining larval body was subsequently stained with Feulgen in order to reveal the gonads, employing a technique described by Bedo[18]. The male has a pair of small spherical testes while the female has elongate ovaries.

RESULTS

Distribution of C-banded material

By the C-banding technique, the characteristic polytene morphology was changed. However, C-bands were observed at centromeres, telomeres and in some interstitial regions. Distribution of C-bands is summarized in Table 2 and results from individual species are given in more detail below.

S. (N.) caudisclerum

Ocein stained and C-banded polytene chromosomes of *S. (N.) caudisclerum* are presented in Figure 2. The 10 larvae of this species had thin centromeric C-bands on all chromosomes and an interstitial C-band in section 20B of chromosome I (Fig. 2b, Table 2). The centromeric C-band on chromosome I was thicker than those on chromosome II and III. Interstitial C-band in section 54B adjacent to the centromere of chromosome II was observed in 1 of 10 larvae examined. Nucleolar and telomeric C-bands were not observed in this species.

S. (N.) feuerborni

The 18 larvae of *S. (N.) feuerborni* showed distinct centromeric C-bands nearly equal in thickness (Fig. 3b , Table 2). They were thicker than those of *S. (N.) caudisclerum.* Only 1 of 18 larvae examined had interstitial C-bands in sections 68B and 68C of chromosome II. Nucleolar and telomeric C-bands were not detected in any larvae.of this species.

S. (S.) fenestratum

All polytene chromosomes in 7 larvae showed thin centromeric C-bands (Fig. 4b , Table 2), which were similar to those of *S. (N.) caudisclerum.* The centromeric C-band on chromosome I was thicker than those on chromosome II and III. Interstitial, nucleolar and telomeric C-bands were not observed in this species.

S. (S.) nakhonense

The 22 females and 14 males showed thick centromeric C-bands in all three chromosomes (Fig. 5b, Table 2). Interstitial C-bands were observed only on chromosome III in sections 84B and 85A. A band in section 85A was found to be either homozygous or heterozygous in both males and females. In contrast, an interstitial C-band 84B2 was found to be sex-linked heterochromatin since all 10 males studied were homozygous for heavily stained bands which were fully synapsed in a pairing region of sections 84A-85A (Fig. 5c). On the other hand, the 13 females were heterozygous for asynapsed band 84B2 with one densely stained heterochromatin and one slightly stained in a non-pairing region of sections 84A-85A (Fig. 5d). However, the 3 females were homozygous for this band. Furthermore, band 84B2 was not C-banded in the 4 males and 6 females.

S. (S.) rufibasis

The larvae of this species were polymorphic for two C-banding patterns (Table 2). The first pattern was found in 27 larvae collected from Kew Pae Pan. All three chromosomes

Table 2 Distribution of C-bands on polytene chromosomes of six *Simulium* species.

Species	No. of larvae examined	Chromosome I			Chromosome II			Chromosome III		
		C	I	T	C	I	T	C	I	T
1. *S.* (*N.*) *caudisclerum*	10	++	+ (20B)	-	+	- or + (54B)	-	+	-	-
2. *S.* (*N.*) *feuerborni*	18	+++	-	-	++	- or + (68B), - or + (68C)	-	++	-	-
3. *S.* (*S.*) *fenestratum*	7	++	-	-	+	-	-	+	-	-
4. *S.* (*S.*) *nakhonense*, ♀	16	+++	-	-	+++	-	-	+++	+/-s or + (84B), + or +/- (85A)	-
	6	+++	-	-	+++	-	-	+++	+ or +/- (85A)	-
S .(*S.*) *nakhonense*, ♂	10	+++	-	-	+++	-	-	+++	+s (84B), + or +/- (85A)	-
	4	+++	-	-	+++	-	-	+++	+ or +/- (85A)	-
5. *S.* (*S.*) *rufibasis**	27	+++	-	-	++	- or + (57C)	-	++	-	- or +
S. (*S.*) *rufibasis***	12	+++	+ (23B)	-	+++	+ (56C),+ (57C)	-	+++	+ (86C)	+
6. *S.* (*M.*) sp. G	10	+++	-	-	+++	-	-	+++	-	-

C = centromere; I = interstitial; T = telomere; + = C-band present, homozygous; +/- = C-band present, heterozygous; - = C-band absent; s = sex-linked C-band; * = Kew Mae Pan population; ** = Siriphum waterfall population.

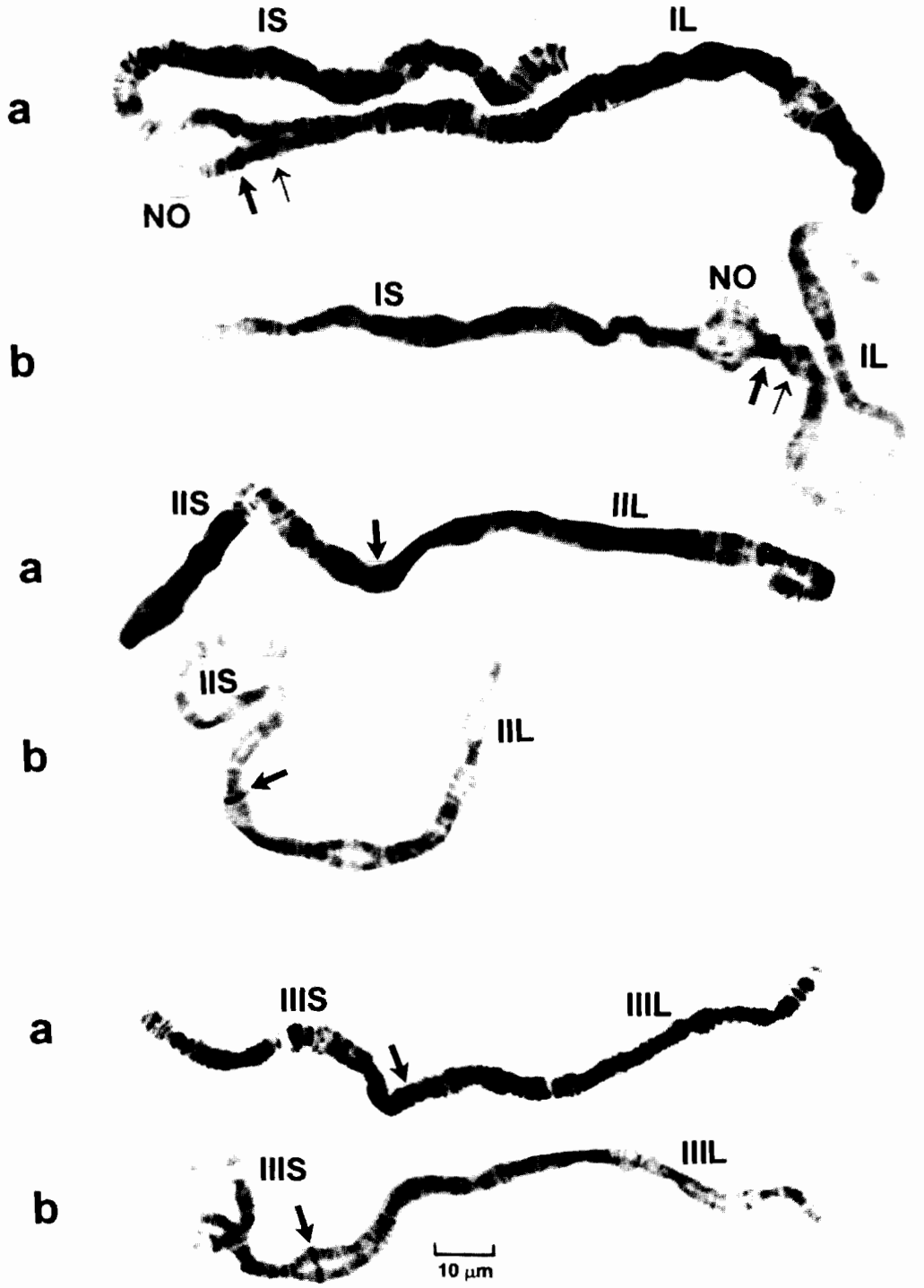

Fig.2 Orcein stained (a) and (b) polytene complement of *Simulium* (*Nevermannia*) *caudisclerum*. I, II, III = chromosomes I, II and III; S = short arm; L = long arm; NO = nucleolar organizer. Large arrows indicate centromeric-associated C-bands. Small arrows indicate interstitial C-bands.

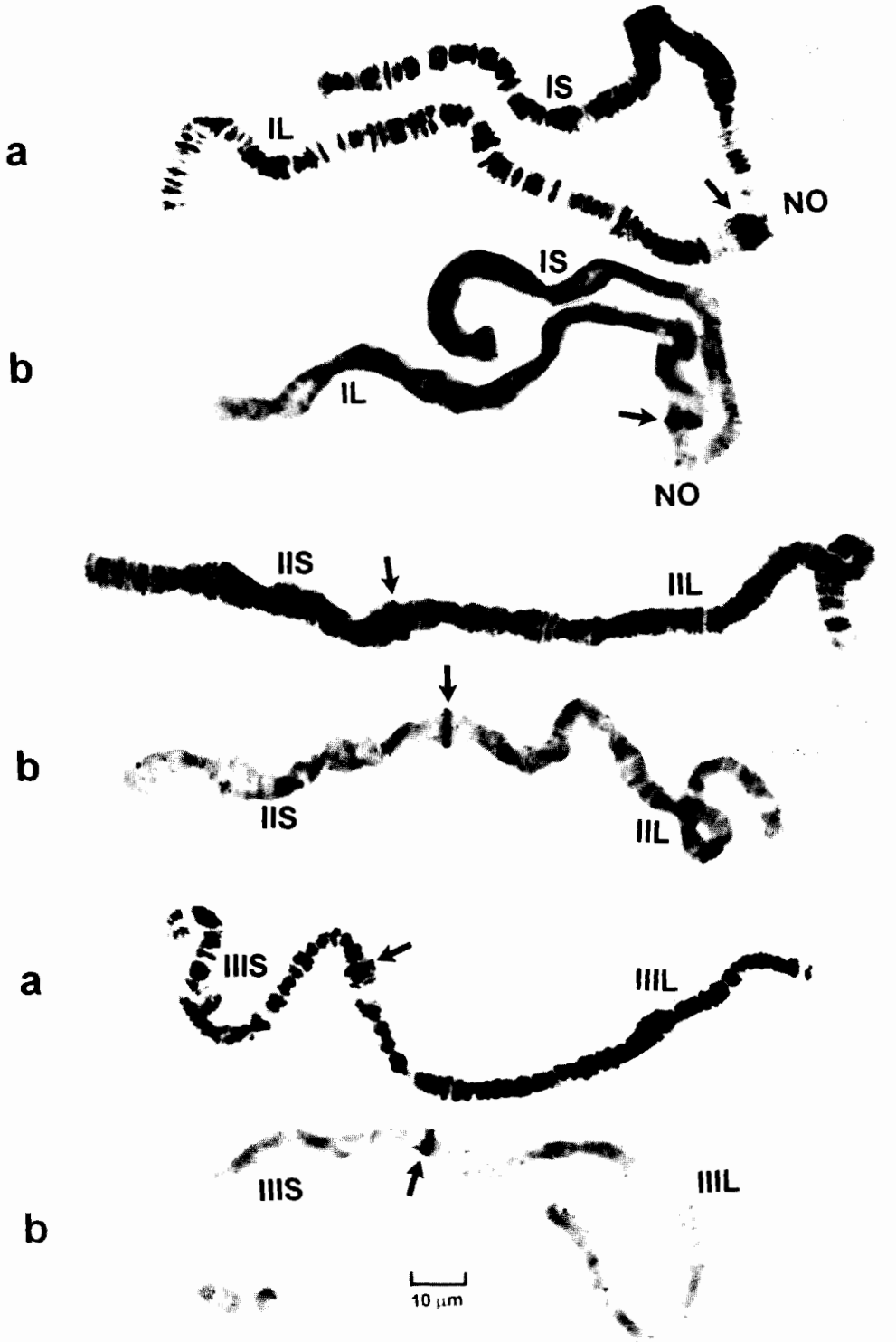

Fig.3 Orcein stained (a) and C-banded (b) polytene complement of *Simulium* (*Nevermannia*) *feuerborni*. Symbols and designations as in Fig. 2.

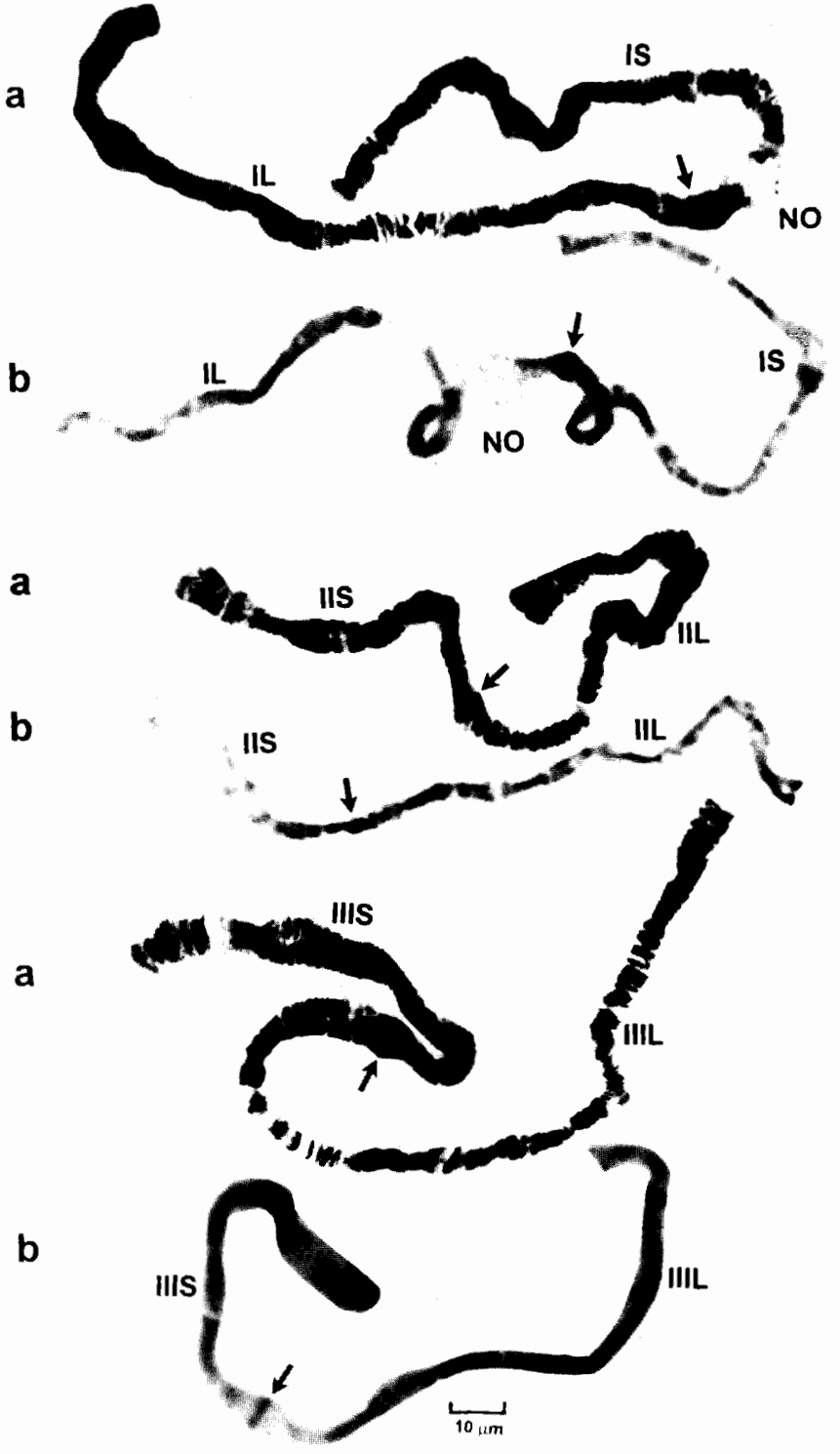

Fig.4 Orcein stained (a) and C-banded (b) polytene complement of *Simulium* (*Simulium*) *fenestratum*. Symbols and designations as in Fig.2.

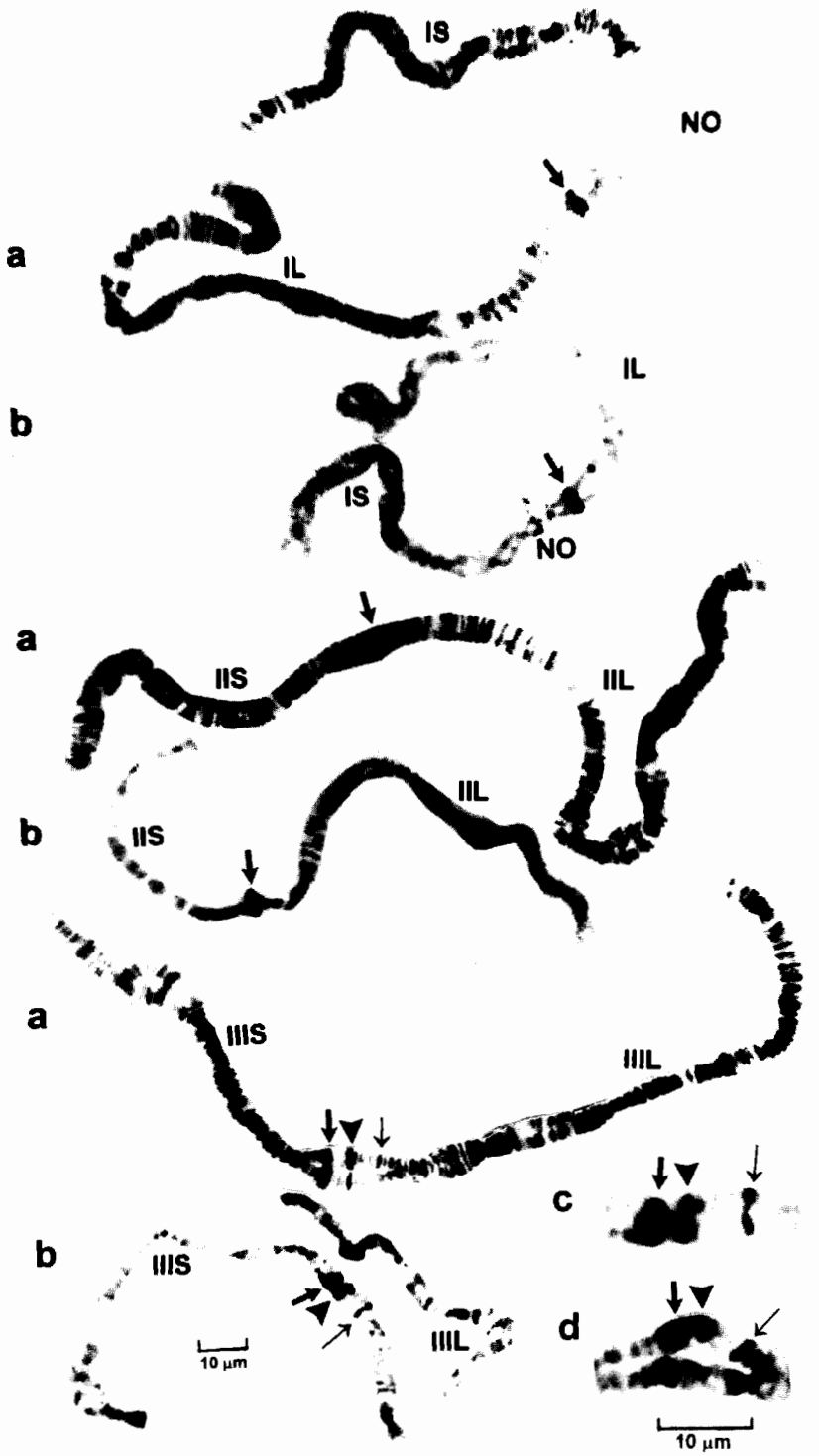

Fig.5 Orcein stained (a) and C-banded (b, c and d) polytene complement of *Simulium* (*Simulium*) *nakhonense*. Symbols and designations as in Fig. 2 except that large arrowheads indicate bands of sex-linked interstitial heterochromatin, homozygous in males (c) and heterozygous in females (d).

showed thin C-bands at the centromere (Fig. 6b). Telomeric C-bands on chromosome III were also observed in some larvae. An interstitial C-band was observed on chromosome arm IIL in section 57C in 7 of 27 larvae examined. In another pattern, which was observed in larvae collected from Siriphum waterfall, there was more C-banded material. The most prominent difference from the first pattern was the presence of interstitial C-bands on all chromosomes. Interstitial C-bands were present in section 23B of chromosome arm IL (Fig. 6c), sections 56C and 57C of chromosome arm IIL (Fig. 6d) and section 86C of chromosome arm IIIL (Fig. 6e). Telomeric C-bands were also observed at both ends of chromosome arms IIIS and IIIL of all examined larvae (Fig. 6e). The centromeric C-bands of chromosome II (Fig. 6d) was thicker than that of the first pattern (Fig. 6b).

S. (M.) sp. G

Simulium (*M.*) sp. G is a new species in subgenus *Montisimulium* found at Doi Inthanon National Park. It is unnamed because of lack of the adult and pupal specimens. All 10 larvae had strongly C-banded blocks at the centromeres of chromosome I, II and III (Fig. 7b). Centromeric C-bands of this species were thicker than those of *S.* (*N.*) *caudisclerum, S.* (*N.*) *feuerborni* and *S.* (*S.*) *fenestratum*. There were no interstitial, nucleolar and telomeric C-bands found in any larvae.

Comparison of C-banding patterns between species

In addition to C-banding variation within a species, *i.e., S.* (*S.*) *rufibasis,* there were also differences in C-banding patterns between species. The C-banding patterns of the *Simulium* species were apparent in the differences in thickness of centromeric and interstitial C-bands as described below.

Chromosome I

There were differences in thickness of centromeric C-bands between species (Table 2). The thicker centromeric C-bands were found in the larvae of *S.* (*N.*) *feuerborni, S.* (*S.*) *nakhonense, S.* (*S.*) *rufibasis* and *S.* (*M.*) sp. G. Similarly, there were also differences in the interstitial C-bands. The *S.* (*N.*) *caudisclerum* larvae and the larvae of *S.* (*S.*) *rufibasis* from Siriphum waterfall had more interstitial C-bands than the other species. The interstitial C-bands in sections 20B and 23B were present in all larvae of *S.* (*N.*) *caudisclerum* and *S.* (*S.*) *rufibasis* from Siriphum waterfall respectively. There were no any interstitial C-bands detected in this chromosome of the other species.

Chromosome II

There was variation in thickness of the centromeric C-bands between species (Table 2). The centromeric C-bands of larvae of *S.* (*N.*) *feuerborni* and *S.* (*S.*) *rufibasis* from Kew Mae Pan were slightly thicker than those of *S.* (*N.*) *caudisclerum* and *S.* (*S.*) *fenestratum*. However, the thickest centromeric C-bands were detected in the specimens of *S.* (*M.*) sp. G, *S.* (*S.*) *nakhonense* and *S.* (*S.*) *rufibasis* from Siriphum waterfall. There were also differences in the interstitial C-bands. Two interstitial C-bands in sections 56C and 57C were detected in all larvae of *S.* (*S.*) *rufibasis* from Siriphum waterfall.

Chromosome III

The thickness of centromeric C-bands of six species were similar to those of chromosome II (Table 2). However, there were differences in interstitial and telomeric C-bands. An interstitial C-band in section 86C and telomeric C-bands at both ends of this chromosome were found in all larvae of *S.* (*S.*) *rufibasis* from Siriphum waterfall. *S.* (*S.*) *nakhonense* had an interstitial C-band in section 85A which was either homozygous or heterozygous in both males and females. Moreover, *S* (*S.*) *nakhonense* showed a sex-linked heterochromatin in the band 84B2 while the other species did not show any sex-linked bands.

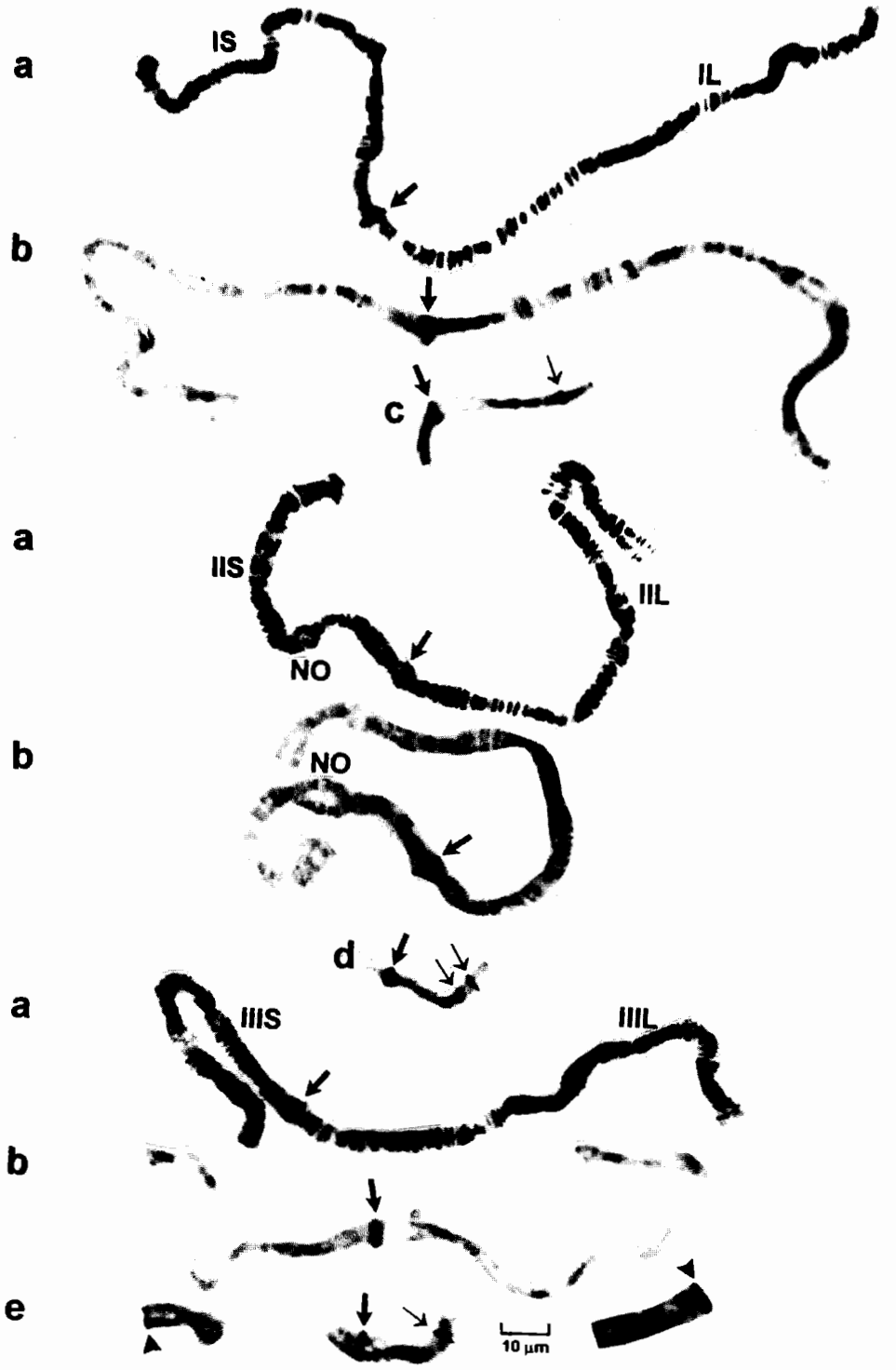

Fig.6 Orcein stained (a) and C-banded (b, c, d and e) polytene complement of *Simulium* (*Simulium*) *rufibasis*. Centromeric and interstitial C-bands differences between populations of Kew Mae Pan (b) and Siriphum waterfall'(c, d and e) are shown. Symbols and designations as in Fig. 2 except that small arrowheads indicate telomeric C-bands.

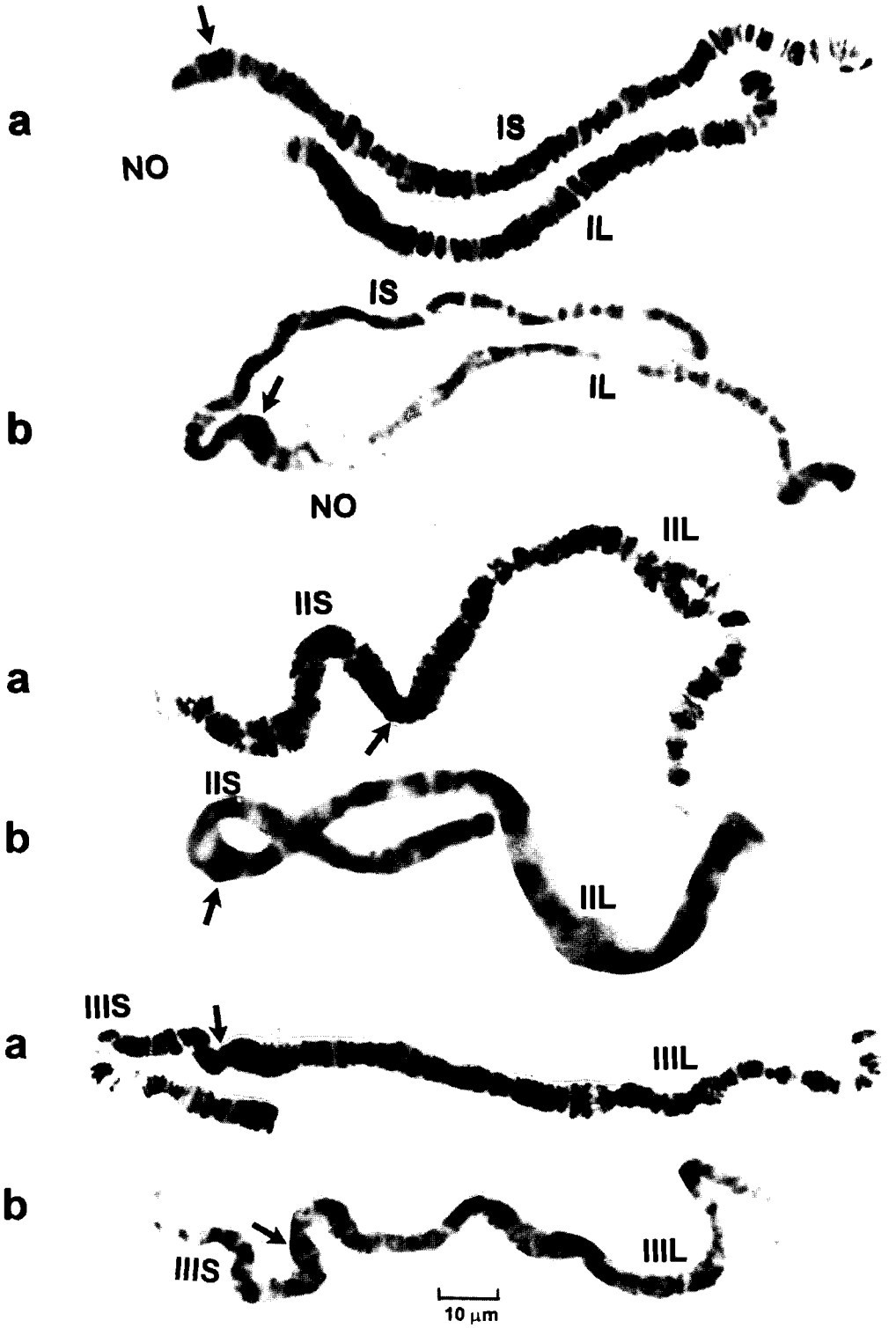

Fig.7 Orcein stained (a) and C-banded (b) polytene complement of *Simulium* (*Montisimulium*) sp. G. Symbols and designations as in Fig. 2.

DISCUSSION

Intraspecific C-banding differences

The geographic polymorphism for C-banding pattern between two populations of *S.* (*S.*) *rufibasis* was shown clearly. The thickness of centromeric C-bands and the presence of interstitial and telomeric C-bands in C-banding patterns of larvae from Kew Pae Pan seem to be the most distinctive result in this study. It has already been known that the amount of heterochromatin may vary from individual to individual within species.[19] Moreover, heterochromatin polymorphisms within one species have been reported in some other *Chironomus* species[7] and in a variety of organisms including ants[20], grasshoppers[21], mice[22], rat[6], chicken[23] and humans.[24,25] Lentzios *et al.*[6] reported seasonal polymorphism for prominent C-bands adjacent to the centromeric bands of chomosome I and III in *Chironomus tepperi*. In addition, two different C-banding patterns were also observed in *C. nepeanensis*. Craig-Holmes *et al.*[25] studied the frequency of normal cell-to cell variability of C-bands in humans, in which variation per chromosome pair per cell ranging from 2 to 16% was found. They assumed this to be artifactural resulting from technical variables. However, they discovered 31 different C-band variants in 20 unrelated individuals using the criterion of appearance of a C-band variant in at least 70% of the cells examined per individual.

In the present study, it is not certain whether intraspecific C-band variation found in two populations of *S. rufibasis* collected from different locations is the result of technical variables or rather, the result of real differences in heterochromatin content. A molecular study along with the C-banding study needs to be done to clarify the characteristics of the differentially homozygous banding regions among individuals and among cells from the same individual, as has been studied in *Rhynchosciara hollaenderi*.[26] Furthermore, the intraspecific difference in the DNA content of homologous C-bands with different thickness among cells and individuals should be determined by the method of cytophotometry to see whether they have an equal amout of DNA content.

Interspecific C-banding differences

Study of C-banding patterns of polytene chromosomes of six *Simulium* species, *S.* (*N.*) *caudisclerum, S.* (*N.*) *feuerborni, S.* (*S.*) *fenestratum, S.* (*S.*) *rufibasis, S.* (*S.*) *nakhonense* and *Simulium* (*M.*) sp. G, using C-banding technique revealed interspecific differences. These differences were in respect to the amount and distribution of constitutive heterochromatin in centromere, interstitial and telomeric bands. Based on the thickness of centromeric C-bands, the *Simulium* species in the present study can be divided into three groups. Group 1, including *S.* (*N.*) *caudisclerum* and *S.* (*S.*) *fenestratum*, has only small amounts of centromeric heterochromatin. Chromosome I has thicker centromeric heterochromatin as compared with chromosome II and III, which are equal in thickness. Group 2 consists of *S.* (*N.*) *feuerborni* and *S.* (*S.*) *rufibasis* from Kew Mae Pan. The larvae of these species have more centromeric heterochromatin in all three chromosomes than those of the first group. Chromosome I also has more centromeric heterochromatin than chromosome II and III. Group 3 consists of *S.* (*S.*) *rufibasis* from Siriphum waterfall, *S.* (*M.*) sp. G and *S.* (*S.*) *nakhonense*. This group exhibits the most thickness and distinctive centromeric heterochromatin. Centromeric heterochromatin of all three chromosomes in this group is almost equal.

The general trend of chromosome evolution of eukaryote tends toward a gain in the heterochromatin.[27] In this view, *S.* (*N.*) *caudisclerum*, which is presumed to be the most primitive species from the most primitive subgenus *Nevermannia*, seems to have the least C-banded heterochromatin among the *Simulium* species, with the exception of *S.* (*S.*) *fenestratum*. In

contrast, the other species, *S.* (*S.*) *rufibasis* and *S.* (*S.*) *nakhonense*, which are classified into subgenus *Simulium*, the most specialized group, have more heterochromatin. Therefore, it may indicate that an increase in C-banded material may occur during chromosomal evolution in the species of *Simulium*. However, the C-banding pattern of *S.* (*S.*) *fenestratum* in the subgenus *Simulium* is similar to that of *S.* (*N.*) *caudisclerum*. It is possible that a decrease in C-banded material may also be involved in the evolution of chromosomes in the *Simulium* species. Therefore, increase or decrease in heterochromatin may play an important role in evolution of chromosomes in *Simulium* species as found in the forms of *Chironomus oppositus*.[28] Increase in the amount of C-banded heterochromatin may be involved when it has specific functions in particular regions.[7] An interspecific variation in amount and pattern of C-banding has also been reported among members of a group of Australian *Chironomus* species[7] and between related species of some organisms, for example, between the gorilla and humans.[29] Thus it seems that amounts and locations of C-banded heterochromatin may vary relatively rapidly in evolution.

Compared with *S. ornatipes* and *S. melatum*[11], the six *Simulium* species in this study had small amounts of C-banded heterochromatin. Constitutive heterochromatin is composed of highly repeated DNA sequences including satellite DNA.[3,4] Satellite DNA is usually localized to C-bands in some insects, for example, in *Drosophila*[30] and *Rynchosciara*.[31] Britten and Kohn[3] suggest that satellite DNA can increase the evolutionary potential of an organism. On the other hand, Hatch *et al.*[32] and Fry and Salser[33], by comparing amounts and locations of satellite DNA in various species of rodents, suggested that satellite DNA may function in speciation. Molecular studies on the European *Chironomus* species, *C. melanotus* with extremely large centromeric C-bands[34] show 15% satellite DNA.[35] Thus, it is likely that satellite DNA can be detected from the large size and number of C-bands in *Simulium* species. However, satellite DNA may be not detected from small size C-bands, unless more sophisticated techniques are developed for the analysis of satellite DNA. Therefore, it is possible to obtain more information on phylogenetic relationships between the species of *Simulium* by comparing the amount and base content of DNA contained in individual C-bands as has been done in the *melanogaster* group of *Drosophila*.[36]

Sex-linked C+heterochromatin

Sex-linked C$^+$heterochromatin bands are common in lower Diptera and may mark the female or male sex chromosome, X or Y chromosome. In this study, chromosome III of *S.* (*S.*) *nakhonense* seems to be a sex chromosome, since band 84B2 adjacent to the centromere of the long arm appears to be sex-linked. This band was heterozygous for one densely stained heterochromatin and one slightly stained in a non pairing region of sections 84A-85A in 13 females and homozygous for two heavily stained bands in 10 males and 3 females. The heavily stained C-band of 84B2 might correspond to X and the slightly stained one, to Y. Thus the sex determining system in *S.* (*S.*) *nakhonense* seems to be heterogametic in the female and homogametic in the male, as found in gall-form Tephritidae.[37] It is possible that a non-pairing region of sections 84A-85A occurred due to genetic differences between the X and Y chromosome and may represent the primary differential region of the sex chromosome as suggested by Post[38] for *S. erythrocephalum*. The three homozygous females found in this study were unexpected and they may be the result of some sex mutation or missexing. On the other hand, band 84B2 of some males (4 males) and females (6 females) was not a sex-linked C$^+$heterochromatic band. This indicates that these individuals have no cytologically obvious sex differential segments and they have undifferentiated and indistinguishable sex chromosomes. This is presumably the

ancestral condition. Heterobands included or not included on the centromere, which are involved in differentiation of genetic X-chromosomal and Y-chromosomal segments, have been reported in many *Simulium* species. These are band 84A, the centromere of chromosome III in *S. sundaicum*;[17] band 21A, the centromere of chromosome I and one band in section 20B in *S. eximium*;[39] and band 31B1 in *S. ochraceum.*[12] Thus sex determination of species in the subgenus *Simulium* is not simple but associated with different chromosomes in different species.

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บทคัดย่อ

โพลีทีนโครโมโซมของริ้นดำชนิด *Simulium (Nevermannia) caudisclerum, S. (N.) feuerborni, S. (Simulium) fenestratum, S. (S.) rufibasis, S. (S.) nakhonense* และ *S. (Montisimulium)* sp. G แสดงแถบสีซีที่เป็นแถบบางและหนา บริเวณเซนโทรเมียร์ ทีโลเมียร์ และบริเวณอื่นบางแห่งบนโครโมโซม แม้ว่าริ้นดำเหล่านี้มีปริมาณเฮเทโรโครมาทินค่อนข้างน้อย แต่พบความแตกต่างของปริมาณและการกระจายของเฮเทโรโครมาทินในริ้นดำที่อยู่ต่างสปีชีส์และภายในสปีชีส์เดียวกัน ตัวอ่อนริ้นดำชนิด *S. (S.) rufibasis* ที่เก็บจากสถานที่ต่างกันแสดงแบบแผนของแถบสีซีสองแบบ แถบสีซีแบบที่สองมีปริมาณเฮเทโรโครมาทินบริเวณเซนโทรเมียร์ และบนแขนโครโมโซมบางแห่งมากกว่าแบบที่หนึ่ง จากการเปรียบเทียบแบบแผนแถบสีซีของริ้นดำชนิดต่างๆกับริ้นดำชนิด *S. (S.) caudisclerum* ซึ่งคาดว่าเป็นชนิดที่ใกล้เคียงกับบรรพบุรุษมากที่สุด สามารถแบ่งกลุ่มริ้นดำออกเป็น 3 กลุ่ม ซึ่งแต่ละกลุ่มมีปริมาณเฮเทโรโครมาทินบริเวณเซนโทรเมียร์ต่างกัน ดังนั้นวิธีการย้อมแถบสีซี สามารถแสดงปริมาณเฮเทโรโครมาทินบริเวณเซนโทรเมียร์และบางแห่งบนโครโมโซม เฮเทโรโครมาทินประกอบด้วยแซทเทลไลต์ดีเอนเอซึ่งมีการเพิ่มและ/หรือลดปริมาณระหว่างการวิวัฒนาการของโครโมโซม นอกจากนี้การย้อมแถบสีซียังแสดงให้เห็นว่า แบนด์ 84B2 ซึ่งอยู่บนโครโมโซมแขน IIIL ของ *S. (S.) nakhonense* เป็นเฮเทโรโครมาทินที่มีความสัมพันธ์กับเพศและเกี่ยวข้องกับการดิฟเฟเรนทิเอชันของส่วนของโครโมโซม X และ Y