Discussion

Chromosome number in black flies

Neuroblast cells contain 2n=6 chromosomes in all the Simulium species so far studied. Salivary gland nuclei have three polytene chromosomes with two homologues more or less intimately paired. Moreover, centromeric regions in most species form characteristic expanded regions dividing the polytene chromosome in the same proportions as the mitotic metaphase chromosomes. The major identifying landmarks of polytene chromosome of different species include Balbiani Ring, nucleolar organiser, puff, Parabalbiani and characteristic banding patterns. In the present investigation, the polytene chromosome of S. (S.) dentatum, S. (S.) singtamense, S. (S.) himalayense, S. (E.) praelargum and S. (E.) ghoomense and the ganglion chromosomes of S. (S.) himalayense and S. (E.) praelargum have been studied .

Idiograms (Fig. A) of five species were constructed from % TCL of each polytene chromosome. The position of centromere, Ring of Balbiani, nucleolar organiser region is shown in each of the idiograms. Inspection of idiograms of five species revealed that all the chromosomes are tightly paired and without any asynapsis. The chromosome I of S. (S.) singtamense is longer than that of other four species though the chromosome II and chromosome III of all the five species are more or less equal in length. In all the five

species, the Balbiani Ring is present in the terminal region of IIS chromosome. However, the position of NOR was found to be variable in those species. It was present in the IIIrd chromosome of S. (S.) singtamense and S. (S.) himalayense, while in S. (S.) dentatum, S. (E.) praelargum and in S. (E.) ghoomense it was in the Ist chromosome. However, in all the five species NOR was present near the centromere. In S. (S.) dentatum, the IIIL is characterised by flared end while no such distinctive character was encountered in other four species. Therefore, the comparison of the idiograms of five species of Simulium revealed that two species of the subgenus Eusimulium, S (E.) praelargum and S. (E.) ghoomense are very closely related, while the three species of the subgenus Simulium, S. (S.) dentatum, S. (S.) singtamense and S. (S.) himalayense differs from each other in respect of location of NOR, presence of flared end and in the length of the Ist chromosome. Furthermore, chromosome count, banding patterns, major morphological characters of these five species agree well with other Simulium species reported by earlier workers (Rothfels and Dunbar, 1953; Rothfels, 1956, 1979; Rothfels et al, 1978; Rothfels and Freeman, 1966, 1977; Rothfels and Mason, 1975; Rothfels and Nambiar, 1975; Bedo, 1975a,b, 1976, 1977a).

However, there is a deviation from the basic Simuliid complement in the genus Cnephia (Procunier, 1982b). Four of the five species of this genus revealed n=3, while in Cnephia lapponica, the basic chromosome complement is

reduced from n = 3 to n = 2 metacentric chromosomes as a result of fusion of chromosomes II and III.

General morphology of polytene chromosome

Centromere:

In black fly, centromere is an expanded region of polytene chromosome usually characterised by a heavy dark band (Dunbar,1962). The expanded region correspond in position to the centromeres in mitotic chromosomes. The expanded regions were joined to form a pseudochromocenter in Simulium pictipes A (Bedo,1975a) in S. melatum (Bedo,1976). True chromocenter, which is a regular phenomenon in Drosophila (White,1973) was also reported (Rothfels and Freeman,1976) in four species of Prosimulium, P. fontanum, P. saltus, P. approximatum and P. mysticum. In the present investigation, of the five species studied, three species S. (S.) dentatum, S. (S.) singtamense and S. (S.) himalayense showed prominent centromeres while in two species, S. (E.) praelargum and S. (E.) ghoomense, centromeres were difficult to recognise. The location of centromere in the polytene chromosomes of all the five species is summarised in Table 4.

Table : 4 : Distinguishing landmarks of three chromosomes of five species of Simuliidae.

Name of the species	Position of centromere	Position of BR	Position of PBR	Position of NOR	Position of Puffs
S. (S.) dentatum (Female)	IL - 21(A) IIL - 55 IIIL - 84	IIS - 41B	IIL - 64B	IS - 19	IS - 12 IIS - 42
S. (S.) singlamense (Female)	IL - 23 IIL - 59 IIIL - 85A	IIS - 47	---	IIIL - 86	IS - 14 IIS - 52
S. (S.) himalayense (Female)	IL - 21 IIL - 54 IIIL - 83	IIS - 43	IIIL - 93C	IIIL - 87	IS :2B,13A IL :32,39B IIS:44,49,51 IIIL :91,92
S. (E.) praelarqum (Male)	IL - 21A,B IIL - 54A,B IIIL - 85	IIS - 45B	IIL - 57B IIIL-89A	IL - 22	IS :7B,8B IL :34B,35A IIS :48B IIIS - 76B
S. (E.) ghoomense (Female)	IL - 18 IIL - 54 IIIL - 83	IIS - 45A	IIL - 69	IL - 21	IS:14.IL:29A, 30A.IIS:46A. IIL:59B,61C. IIIS:78B/79B

It has been observed that there was little variation in the location of centromeres in all the five species studied here. Dark heavy centromeric bands of S. (S.) singtamense and S. (S.) himalayense are comparable to that of the species belonging to S. pictipes group (Bedo,1975a), while the centromere of S. (S.) dentatum did not reveal any heavily stained band, but rather granular in appearance with irregular bands similar to that of the members of S. venustum/verecundum complex (Rothfels et al., 1978),S. fibrinflatum and S. luggeri (Gordon,1984). On the other hand, the centromeres of two species, S.(E.) praelargum and S. (E.) ghoomense were difficult to recognise. In the former species,the centromere was not typically expanded region though it is characterised by dark heavily stained bands. On the other hand, the centromere of chromosome I of S. (E.) ghoomense is easily identifiable while it needs a little effort to identify centromere in case of chromosomes II and III. It is interesting to note that the centromere of S. (E.) ghoomense is comparable to that of Metacnephia sp.(Procunier,1982a). Moreover, centromeres of polytene chromosomes could also be identified by C and fluorescent banding techniques. The centromeric regions of the members of S. pictipes group displayed bright fluorescence with Quinacrine (Bedo,1975 a),while polytene chromosome of S. ornatipes and S. melatum(Bedo,1975b) found to be C band positive. Therefore, banding technique is very useful in the identification of centromere when it was not a typical expanded region.

Nucleolar organising region:

The nucleolar organiser appears as a gap in the polytene chromosomes surrounded by nucleolus. The bands in the immediate vicinity of NOR are somewhat disrupted and the chromonemata on either side of the organiser appear to extend like a complex branching system with roots into the actual nucleolus. Moreover, study of nucleolar relations in Simuliidae promises to be diagnostically useful, because of the intra-specifically constant and inter-specifically variable attachment of the main nucleolus. In Simulium, the terminal nucleolus which was reported in the members of S. pictipes group (Bedo, 1975 a) was difficult to identify without counterstaining by light green. However, nucleolar organiser of interstitial region could easily be identified without subjecting it to any specialised staining technique. In all the five species investigated here, NOR was located in the interstitial region. In S. (S.) dentatum, S. (E.) praelargum and S. (E.) ghoomense, NOR was located in IS 19, IL 22 and IL 21 respectively, while in S. (S.) singtamense and S. (S.) himalayense it was found in IIIL 86 and IIIL 87 respectively (Table 4). Therefore, except S. (S.) dentatum, where NOR is located in IS arm, the location of NOR is similar in other two species of the subgenus Simulium. Furthermore, in two species of the subgenus Eusimulium, the NOR is also found in the same arm. Hence, the location of NOR could be used effectively in the characterisation at subgeneric level. Interstitial NOR was also reported earlier

in S. venustum and verecundum complex (Rothfels et al.,1978),Cnephia(Procunier,1982b), species of Prosimulium (Rothfels and Freeman,1977),and Simulium (Bedo, 1977; Gordon,1984). Besides primary nucleolus, a secondary nucleolus was also reported among the members of S. ornatipes(Bedo, 1977). The secondary nucleolus frequently fused with the main nucleolus in S. ornatipes. However, in the species investigated here, no such secondary nucleolus was observed. Among other dipteran species, the NOR is found to be located in the pairing segment of sex chromosome in different species of Drosophila, while in some species of Chironomus, more than one nucleolus was observed (White,1973). Furthermore, nucleolus is present in all the tissues in all stages of development and its location in the karyotype is the same in both polytene and mitotic nucleoli. Therefore, nucleolar organiser region naturally serves as an important character for cytotaxonomic study.

The Ring of Balbiani (RB):

Since the site of RB or Balbiani Ring is species specific, therefore, this structure also serves as an important landmark for the identification of Simulium species. It is identifiable in all the species studied in the present investigation. In all the five species, the RB is present in IIS chromosome. In S. (S.) dentatum, S. (S.) singtamense and S. (S.) himalayense, they were encountered in IIS 41B, IIS 47 and IIS 43 respectively, while in S. (E.) praelargum and S. (E.) ghoomense, it was observed in IIS 45B

and IIS 45A respectively (Table 4). Therefore, it seems that the location of RB is species specific in three species of the subgenus Simulium, while in two species of Eusimulium no such specificity was perceptible. Furthermore, a comparison with other Simulium species revealed that the RB is present in the section IIS 42 in all the members of the Simulium pictipes group (Bedo, 1975a), while in species of Simulium ornatipes and S. ruficorne, it was reported in section IIS 43 (Bedo, 1977,1989), comparable to that of S. (S.) himalayense. On the other hand, RB is located in IIS 47 in S. venustum / verecundum (Rothfels et al.,1978), in S. jenningsi, S. fibrinflatum and S. luggeri,(Gordon, 1984) similar to that of S. (S.) singtamense.

Moreover, in the genus Cnephia, the RB was also located in IIS 47 (Procunier, 1982b), indicating a close relationship between Simulium and Cnephia with respect to the position of Balbiani Ring. Therefore, the present study clearly shows that BR could be used as an important landmark for the characterisation at generic and subgeneric levels.

Puffing in polytene chromosome At particular stages of development, some of the genetic loci in the polytene chromosome undergo a spectacular change in appearance. They become converted into large swellings or puffs. It has been generally believed that a process of puffing is due to the biosynthetic activity of a particular loci concerned. Therefore, study of the puffing pattern is of great

importance for understanding gene action. Puffs were encountered in various members in all the species under present investigation. Position of puffs in polytene chromosomes of different species is summarized in Table 4.

In S.(S.) dentatum and S. (S.) singtamense two large and clear puffs were seen in IS and IIS respectively. On the other hand, in S. (S.) himalayense, S. (E.) praelargum and in S. (E.) ghoomense, a large number of puffs were encountered in Ist. 2nd. and 3rd. chromosomes. Puffs were also reported in Simulium pictipes (Bedo, 1975a), S. ornatipes (Bedo, 1977) and in members of S. venustum/verecundum complex (Rothfels et al.,1978). However, no attempt has yet been made to study the detailed puffing pattern of different species of Simulium. However, in other dipterans, puffing patterns have been extensively studied. In Chironomus tentans and C. pallidivittatus, puffing patterns have been studied (Grossbach,1968,1969) in detail in relation to the synthesis of specific silk like proteins which are produced in large amount in the cells of salivary gland. In Drosophila melanogaster, the puffing pattern of X chromosome loci is similar in both the sexes. But a group of puffs were active for a longer time in male than in female (Ashburner,1967,1969a). While studying the puffing patterns of D. simulans and D. melanogaster, Ashburner (1969a,b) observed that X chromosome of D. simulans form two puffs which were absent in D. melanogaster. Moreover,D. melanogaster has one autosomal puff (46A) which was not

present in D. simulans. It is also interesting to note that hybrid between these species show a heterozygous puff. Furthermore, differences in time of puffing and size of puff also exists between different strains of D. melanogaster and certain puffs are active in some strains,but are not seen in others (Ashburner,1969 c). Therefore,the study of puffing patterns may be a helpful guide in demarcating congeneric as well as sibling species. Studies of the puffs in chromosomes of five species under present investigation also revealed the difference in location and number of puffs between congeneric species. Hence,it seems that, in the Simuliidae,the study of the puffing pattern could also be used as an important landmark for the comparison of the polytene chromosome of different species.

Parabalbiani Ring (PBR) Parabalbiani is darkly stained band with one sharply defined and one diffused edge. This structure serves as an useful polarised marker. PBR is unique to the Simuliidae and readily recognised in the species of different genera. Of the five species studied in the present investigation ,PB was encountered in all the species except S. (S.) singtamense. In S. (S.) dentatum, the PB was found in IIL 64B in both male and female individuals while in S. (S.) himalayense and S. (E.) praelargum, it was located in IIIL 93C and IIIL 89A, IIL 57B respectively. It was found in IIL 69 in S. (E.) ghoomense (Table 4). Therefore, the distribution of PB in all the four species indicates that this landmark could be effectively used in

distinguishing the species. PB, comparable to the above named species, was also reported in other species of Simuliidae. In each species, it serves as an important landmark such as in S. pictipes (Bedo,1975a), S. ornatipes(Bedo 1976) ,S. venustum/verecundum complex (Rothfels et al.,1978) and in Metacnephia (Procunier,1982a).

Banding pattern

The pattern of dense bands and less dense interbands that characterise the most polytene chromosomes is a feature that has been of the utmost value to geneticists, particularly since the demonstrations by Bridges (1937) that the genetic and polytene chromosome maps are colinear. In the present investigation, of all the five species studied, the distribution of heavily and lightly stained bands in the polytene chromosome complement is not random, but reflects some overall organisation. In S. (S.) dentatum, both in male and female sexes, there was a preponderance of dark bands in all the three chromosomes. There were some fine bands near the nucleolar organiser region in IS while in IL, series of dark bands distributed throughout the chromosomes. Though the diameter of chromosome I was uniform, constricted necks were encountered in IIL 59 and in IIIL 95. Fine lightly stained bands were found near the centromeres of chromosome I, II and III. Unlike the former species, all three chromosomes of S .(S.) singtamense are of uniform diameter and are characterised by some specific banding patterns such

as groups of heavy bands in IS 2-5; IS 7-10. Moreover,there was a shield - like band in IL 34. There were two groups of darkly stained bands, IIS 50C - 52A and IIS 54-55A. In all three chromosomes, fine lightly stained bands were found near the centromeric regions. In S. (S.) himalayense,the distribution of light and deeply stained bands were more or less uniform. However, as in other two species,lightly stained bands were present near the centromeric regions in all the three chromosomes. The specific banding character of this species includes band in IS 4A, a group of deeply stained bands in IS- 16 and 17; heavy band in IL- 24C, a group of four bands in IL- 38 and 39A. Dark marker bands were also present in IIS 45C - 47A. In the IIIrd chromosome, there was a group of heavy bands in IIIS- 78 and a group of deeply stained bands in IIIS- 75. In S. (E.) praelargum, on the other hand, dark and light bands were distributed uniformly throughout the length of the chromosome as in S. (S.) himalayense. The diameter of the chromosome was not uniform. Constricted neck, one each in IS- 11, IL- 32 and IIIS- 82 was also encountered in S. (E.) praelargum which were comparable to those of IIL- 59 and IIIL- 95 in S. (S.) dentatum. Moreover,in S.(E.) praelargum, deeply stained group of bands were found in all the three chromosomes. Besides the marker band, shield -like band was also encountered in IL- 29. Comparable shield - like band was also found in IL- 34 of S. (S.) singtamense. In S. (E.)

ghoomense, on the other hand,there was a preponderance of lightly stained bands as in S. (E.) praelargum and S. (S.) dentatum. Moreover,specific group of marker bands were also found in all the three chromosomes, especially in chromosome I. In some individuals of this species, an inversion involving the sections 33-37A was encountered in IL. However, no such inversion was found in other species under present investigation. Therefore, similarity and dissimilarity between the banding patterns of these five species suggest that there is an interrelationship between all these five species and each species is characterised by specific marker bands.

Sex Chromosome :

Sex chromosome heteromorphy is known from many groups of animals and plants, and in most cases it is the Y chromosome which is the smaller element. Presumably such systems evolve from morphologically identical and freely recombining sex chromosomes. Size difference between the sex chromosomes of Simuliidae is virtually unknown and in most species they are indistinguishable in the polytene nucleus (Post, 1985). Therefore, sex is determined primarily by a single locus or by very short length of chromosome (Rothfels and Mason, 1975). However, many studies have revealed species in which the sex chromosomes have become differentiated by the linkage of inversions as observed in Simulium erythrocephalum (Post, 1985). Such inversions may be fixed or polymorphic on either of the sex chromosomes or occur on

both but at different frequencies (Post, 1982a). All the five spcies namely, S. (S.) dentatum, S. (S.) singtamense, S. (S.) himalayense, S. (E.) praelargum and S. (E.) ghoomense under present investigation did not reveal any morphologically distinguishable sex chromosomes in male or female and only larval sex was identified from the structure of the gonads. Except S. (E.) ghoomense, where an inversion was encountered in IL of some of the individuals, no inversion or sex specific heteroband was encountered in any of these species. Therefore, detail study of the population of each species might throw some light on the sex chromosome in these species which is not within the scope of present investigation. However, the presence of sex chromosomes was reported in other members of Simuliidae. Typically, in black flies, sex chromosome become differentiated by linkage of chromosomal rearrangements to either the genetic X of genetic Y chromosome. Species of Prosimulium (Ottonen, 1966; Rothfels, 1956) show increase in differentiation of sex chromosomes by addition of inversions resulting in complex configuration involving whole arms. Inversion played an important role in the differentiation of Y chromosome in S. longistylatum while S. pictipes revealed 'heteroband' in IIL (Bedo, 1975a). Inversions also played an important role in the determination of sex in the species of S. jenningsi group (Gordon, 1984). This type of sex chromosome system was also found in Chironomidae (Beermann, 1955). A partial linkage of inversion to the X or Y chromosome was reported

in Chironomus tentans (Acton, 1957) and in C. intertinctus (Martin, 1962). The simplest kind of sex chromosome differentiation involves a failure of polytene chromosome pairing between specific sites of the X and Y chromosomes, the inclusion of heterochromatic supernumerary bands (Basrur, 1959) or alteration in size and composition of existing bands (Bedo, 1975a). Bedo (1977) also reviewed the distribution of para- and pericentric inversions in 65 black fly species from five genera namely, Prosimulium, Twinnia, Cnephia, Eusimulium and Simulium and found significantly higher frequency of pericentric inversions in the sex chromosome systems. Therefore, inversions play an important role in the differentiation of sex chromosomes in Simuliidae.

Cytophylogeny in black flies

Detailed studies of the polytene chromosome of numerous species of black flies now mainly confined to Canadian and European species, and little work has been done on the chromosome of Oriental black flies. Dey and Wangdi(1984 a) reported the mitotic chromosomes of four species namely, Simulium (Simulium) dentatum, S. (S.) ramosum, S. (E.) aureohirtum and S. (E.) purii from the Darjeeling and adjoining hill areas which is a part of Eastern Himalayas. They have found the diploid count of 2n=6 in each of the four species and chromosomes are metacentric in nature .Moreover, Dey and Wangdi (1984 b) have also reported

the presence of supernumerary chromosomes in the neuroblast metaphase chromosomes of two other species, S. (E.) gracilis and S. (E.) ghoomense. This work has been further extended by studying the polytene chromosomes of five species, reported in the present investigation. A comparison of the polytene chromosomes of these five species with those of European and North American species did not reveal any remarkable difference. However, further studies are required on the species of Eastern Himalayan region to make any meaningful linkage between the species of these continents. Rothfels et al., (1978) constructed an outline chromosome phylogenies that extended through the Prosimuliinae and encompassed most of the known species in Gymnopais, Twinnia, Heloden and Prosimulium. Less extensive phylogenies was also reported in Cnephia, Metacnephia, Eusimulium (Bedo 1977; 1979 a,b,c; Dunbar, 1965, 1967) and Simulium (Rothfels et al., 1978; Bedo, 1977, 1989). However, no serious effort has been made to link up those phylogenies though certain chromosome ends (IS) and middles are shared by members of all genera of the family. For the IIS arm, Dunbar (1967) claimed that the closest members of Cnephia, and Eusimulium, C. dacotensis and Eusimulium anatinum differ in but a single inversion. Furthermore, it has been suggested (Dunbar, 1967) that the IIS sequence of some Cnephia species can probably be successfully compared to that of of some Prosimulium, and that of Eusimulium to that of some Simulium. Therefore, it seems that there is

possibilities in linking up different phylogenies. Moreover, the chromosomal rearrangements like inversion plays an important role in the speciation process. Fixation of inversions and acquisition of unique floating inversions suppose to be one of the important processes in the evolution of Simuliidae (Bedo,1977). Chubareva (1977) also proposed a chromosome phylogeny for Simuliidae where Gymnopais has been considered as base on the basis of morphological dogma and not by cytological analysis. Rothfels (1979) also assumed that Gymnopais is the most primitive of all cytologically studied black flies, though Helodon, Gymnopais, Twinnia and Prosimulium are cytologically closely related genera. However, nucleic acid hybridisation studies (Sohn et al.1975,Teshima,1972) suggested that some species within Simulium appear to be as remote from each other as they are from species of the genus Cnephia. Moreover, at an interfamily level this type of study hold the promise of allowing the identification of most closely related genera of different families and thereby providing clues to ancestry. Therefore, integration of the cytological data from different areas and continuous interaction among cytologists,molecular biologists and taxonomists will certainly contribute towards elucidating phylogeny in Simuliidae.