CHAPTER 7

# Discussion

Analysis of mitotic chromosomes revealed the exact nature of chromosomes of an individual. Mitotic metaphase as universally considered to be the most appropriate stage for studying the nature of chromosome, especially in relation to the position of the centromere and the size of the chromosome. All the four species investigated revealed that the size of the chromosomes were variable in each of the species. Of the four species investigated, the length of the mitotic chromosome complement of *Simulium* (*M*.) *dattai* measured 12.41µ and was the longest in comparison to other three species. The total chromosomal length of *Simulium* (*G*.) *williei* measured 11.52µ; *Simulium* (*N*.) *praelargum* "IIIL-1.2", 7.98µ, and 7.88µ in *Simulium* (*N*.) *praelargum* 'IL". The close range of measurements of *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium* (*N*.) *praelargum* "IL" also justified these two species belonging to the same group and species with variation only in inversions. Whereas the wide range in difference between *Simulium* (*M*.) *dattai* and *Simulium* (*G*.) *williei*, 12.41µ and 11.52µ justified their systematic status belonging to two separate genera, i.e., *Montisimulium* and *Gomphostilbia*. The closeness of the metrical data of each mitotic chromosome is also suggestive of the affinity

between the species. This fact of closeness of metrical data has been corroborated by the mitotic metrical data of *Simulium* (*M*.) *ghoomense* (Dey and Fumafartosok 1984b), where the measurements of the mitotic chromosome I and II showed much similarity but differing in mitotic chromosome III. Approximately 66% of similarity justifies the inclusion both the species under the same subgenus *Montisimulium* is justifiable. The difference of 33% amounts to their being two separate species.

Thorough investigation of the Simuliid population has been attempted through the larval mitotic and polytene chromosomes. The study was made covering the range between 26.935997°N to 27.051023°N of latitude and between 88.248367°E to 88.250685°E of longitude. The collections were made during the period 09/11/2008 to 27/07/2016. Total of 342 larval specimens were collected from selective spots having similar ecological conditions and type of the water body. Happy Valley, Dali, Bokshi Jhora, Gandhi Road, and Sonada were of the same type. In Dali two streams were designated as Stream 1 and Stream 2. Of the total specimens of 342 collected, 168 were males representing 49.12% and 174 comprising of 50.87%. The sex ratio in general appeared to be approximately 1:1.03. Total population of four species comprised the working material for understanding the diversity within a population, namely, *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2", *Simulium* (*Nevermannia*) *praelargum* "IL", *Simulium* (*Montisimulium*) *dattai* and *Simulium* (*Gomphostilbia*) *williei*.

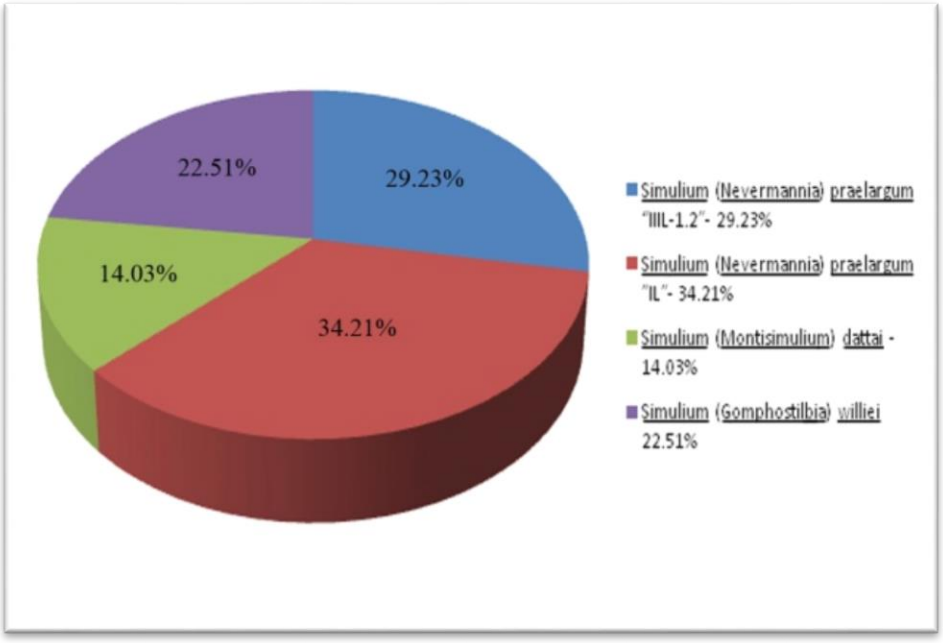


**FIGURE 42: A pie chart representing the composition of the *Simulium* larval populations.**

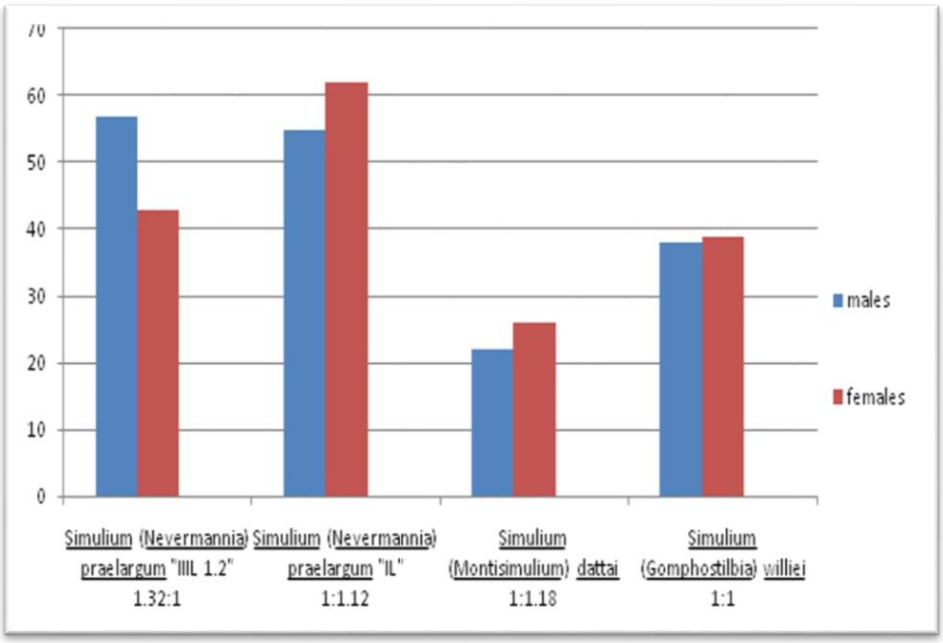


**FIGURE 43: A graphical representation of male: female ratio of individual species.**

Out of 342 total specimens, 100 were identified to be *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2", which made 29.23% of the total. 117 were *Simulium* (*Nevermannia*) *praelargum* "IL", which comprised 34.21% of the total. 48 were *Simulium* (*Montisimulium*) *dattai*, which comprised 14.03% of the total, and 77 were *Simulium* (*Gomphostilbia*) *williei*, comprising 22.51% of the total. Identification of *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2" and *Simulium* (*Nevermannia*) *praelargum* "IL" was possible as cytoforms after the polytene chromosome analysis, which otherwise morphologically larvae were identical to *Simulium* (*Nevermannia*) *praelargum*-st. A pie chart (Fig. 42) has been constructed to represent the composition of the *Simulium* larval populations collected from Happy Valley, Dali, Bokshi Jhora, Gandhi Road, and Sonada. A graphical representation (Fig. 43) of male: female ratio of individual species has been constructed. *Praelargum* seemed to be rich population. Overall and individual sex ratio between males and females seemed to be in approximately 1:1 ratio.

Well spread mitotic metaphase plates were selected and the metrical data of the chromosomes were derived for all the four species of black flies.

## I. Mitotic Chromosomes

The mitotic plates of all the four species investigated exhibited 2n=6. Chromosome I and II of all the four species investigated were metacentric and chromosome III was sub-metacentric. Total length

of the mitotic chromosomes in *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2" was 7.98µ while in *Simulium* (*Nevermannia*) *praelargum* "IL" was 7.88µ. The overall metrical data of the mitotic chromosomes of *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2" and *Simulium* (*Nevermannia*) *praelargum* "IL" were significantly similar. The total length of the mitotic chromosomes of *Simulium* (*Montisimulium*) *dattai* was found to be 12.41µ and 11.52µ in *Simulium* (*Gomphostilbia*) *williei*. Mean total length of the short arms of chromosome I, II, and III of *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2" was 3.31µ; Mean total length of the long arms of mitotic chromosome I, II, and III of *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2" was 4.67µ. Total length of short arms of mitotic chromosomes I, II, and III in *Simulium* (*Nevermannia*) *praelargum* "IL" was 3.26µ and that of the long was 4.62µ. In *Simulium* (*Montisimulium*) *dattai* the total length of short arm of all the mitotic chromosomes was 4.81µ and of long arms was 7.60µ. The length of the short arms of three mitotic chromosomes of *Simulium* (*Gomphostilbia*) *williei* was 4.61µ and that of long arms was 6.91µ. The metrical analysis of individual chromosomes of *Simulium praelargum* "IIIL-1.2" and *Simulium praelargum* "IL" showed significant similarities between these two species. These two species were metrically also different from *Simulium dattai* and *Simulium williei*. The difference in metrical chromosomal data of *Simulium dattai* and *Simulium williei* was significant hinting the distant species group.

The mitotic chromosomes were prepared form rudimentary testes in case of males and ovaries in case of females in addition to neural ganglia. The frequency of divisional plates in case of Neuroblast ganglia (neural ganglia) were very less in comparison to rudimentary gonads, which provided good mitotic plates from where the study has been made. Rudimentary ovaries provided more comport in working in comparison to rudimentary testes due to its size.

## II. Polytene Chromosomes

The polytene chromosomes from the salivary glands of the larvae were studied from the temporary slide preparations. The polytene chromosomes also exhibited n=3 as in case of all other Simuliid species of the world so far studied with an exception of *Cnephia* of having n=2. Polytene chromosomes were numbered as Chromosome I, II, and III on the basis of its relative length. Each chromosome was divided into short arm (S) and long arm (L) separated by the presence of a prominent heterochromatic centromere or centromeric band. In some cases the centromeres of all the three chromosomes were fused to form a large heterochromatic chromocenter, eg. *Simulium praelargum* "IIIL-1.2 (Thapa et al., 2014) and *Simulium dattai* (unpublished Thapa et al.). However in some cases partial fusion of only two of the three centromeres have been recorded giving rise to pseudo-chromocenter or partial putative chromocenter, e.g., *Simulium ghoomense* (Henry et al., 2011), where centromeres of chromosome II and III are involved. Each chromosome having short and long arm are assigned

the number of sections in accordance with the percentage derived from calculated total complementary length (TCL).

All the three polytene chromosomes had its distinguishing characteristic cytological landmarks, such as nucleolar organizer (NO), bulge (=double bubble) (B), ring of Balbiani (RB), parabalbiani (PB), trapezoid (T), capsule (Ca), and blister (Bl). The pattern of the telomeric ends was also of distinctive nature.

**Chromosome I**

The short of the chromosome I (IS) of all the species investigated exhibited a prominent nucleolar organizer (NO).

**Nucleolar organizer**

Chromosome of all the four species investigated had nucleolar organizer (NO) at the base of short arm near either centromere as in case *Simulium* (*N*.) *praelargum* 'IL" or *Simulium* (*G*.) *williei* or chromocenter as in case of case *Simulium* (*N*.) *praelargum* 'IIIL-1.2" and *Simulium* (*M*.) *dattai*. The nucleolar organizer (NO) appears as a non-staining region either interstitially or terminally located in different species. It is a site of heavy ribosomal RNA synthesis. In case of the members of *Simulium pictipes* group the nucleolus was terminal (Bedo 1975a). The NO region appears like a diffused body where the chromonemata on either side of the organizer appear to extend like a complex branching system with roots into the actual nucleolus. In the vicinity of NO region the bands appear to have

been disrupted. Nucleolar organizer (NO) is a diagnostic feature of the Simuliid chromosomes which is of utmost importance for the study of inter and intra specific relationships. NO may be difficult to identify unless counter stained with some other stains like light green. Nucleolar organizer (NO) in all the species studied here and other species described from Darjeeling Himalayan region such as *Simulium* (*Simulium*) *sintamense* (Dey et al., 1993), *Simulium* (*Simulium*) *himalayense*, *Simulium* (S*imulium*) *dentatum* (Henry et al., 2009), *Simulium* (*Nevermannia*) *praelargum*-st (Henry et al., 2010), *Simulium* (*Montisimulium*) *ghoomense* (Henry et al., 2011), the NO was readily identifiable without the need of counter staining. The NO in *Simulium* (*N*.) *praelargum* 'IIIL-1.2" and *Simulium* (*N*.) *praelargum* 'IL" was located in the section17 of chromosome IS. In *Simulium* (*M.*) *dattai and Simulium* (*G*.) *williei* the NO was located in section 18 of chromosome IS. Interstitial Nucleolar organizer (NO) had been reported 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). In addition to primary NO, a secondary NO was reported among the members of *S. ornatipes* (Bedo 1977) and *Simulium anatinum* (Rothfels and Golini 1983). However in the species under present investigation and others described earlier did not show the presence secondary NO. The NO in other Dipteran species is found to be located in the pairing segment of sex chromosome in different species of *Drosophila*. Whereas in *Chironomus* more than one NO was observed (White 1973). NO is

present in all the tissues in all stages of development and its location in the karyotype is the same be it polytene or mitotic nucleoli. Thw NO therefore, is an important landmark for cytotaxonomic study.

**Centromere**

The expanded region in the polytene chromosomes represents the centromere in black flies. In the polytene chromosome the centromere is usually characterized by a heavy dark band (Dunbar 1962). The position of centromere in polytene chromosome corresponds to the centromere in mitotic chromosome. The centromeres of the polytene chromosomes have fused together to form a chromocenter, which is a regular phenomenon in *Drosophila* (White 1973). In four species of *Prosimulium simulium, P. fontatum, P. saltus, P. approximatum,* and *P. misticum* (Rothfels and Freeman 1976) and in *Simulium* (*M.*) *ghoomense* (Henry et al., 2011) and *Simulium* (*N.*) *praelargum* "IIIL-1.2" (Thapa et al., 2014) the presence of chromocenter have been encountered. In some cases as in *Simulium pictipes* A the expanded regions were joined to form pseudo chromocenter in (Bedo 1975a). In *Simulium* (*M.*) *ghoomense* the centromere regions of chromosomes II and III have fused to form partial putative chromocenter (Henry et al., 2011), however, the first chromosome I had a centromere. In the present investigation of the four species, *Simulium* (*N.*) *praelargum* 'IIIL-1.2" and *Simulium* (*M.*) *dattai* the centromeres have been fused to form a chromocenter, whereas in *Simulium* (*N.*) *praelargum* 'IL" and *Simulium* (*G.*) *williei* have individual centromeres.

The centromere and chromocenter in case of four species studied marked the beginning of the long arm of all the three chromosomes which served as a partition between the short and the long arm. IL of all these four species did not show any major polytene chromosome cytological landmarks.

Centromeres or portion of the chromocenter of all the four species marked the beginning of the IL

**Chromosome II**

The short of the chromosome II (IIS) of all the species investigated exhibited a bulge (B), ring of Balbiani (RB), and Trapezoid or Triad or Trapezoidal (T) in the polytene chromosomes and parabalbiani (PB), trapezoid (T), DNA puff and jagged (jg) in long arm of chromosome II (IIL).

**Bulge (=double bubble)**

Bulge (=double bubble) (B) is also a puff which is a characteristic of IIS in most of the species of Simuliid polytene chromosome. In *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium (N*.) *praelargum* "IL", bulge is found in section 42. In *Simulium* (*M*.) *dattai* it was located in section 46, and in *Simulium* (*G*.) *williei* in section 47.

**Ring of Balbiani**

Ring of Balbiani (RB) is a large puff with a distinctive texture. Ring of Balbiani (RB) serves as an important landmark and is species specific. RB was identified in all the four species investigated. In *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium (N*.) *praelargum* "IL", RB was found in section 43. In *Simulium* (*M*.) *dattai* it was located in section 45, and in *Simulium* (*G*.) *williei* in section 48. In all the fours species investigated RB and B was closely associated with each other. In *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium (N*.) *praelargum* "IL", bulge (B) was located in section 42 before ring of Balbiani (RB) in 43. In *Simulium* (*M*.) *dattai* RB was located in section 45 before B in section 46 and in *Simulium* (*G*.) *williei*, B was located in section 47 before RB in section 48. RB was found to be present in section IIS 42 in all the members of *Simulium pictipes* (Bedo 1975a), while in species of *Simulium ornatipes* and *S. ruficorne* RB was reported in section IIS 43 (Bedo 1979, 1989), IIS 43 as in case of *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium (N*.) *praelargum* "IL". In *Simulium venustum*/*verecundum* RB is located in IIS 47 (Rothfels et al., 1978)

**Trapezoid**

There is a presence of quadrilateral with two (non adjacent parallel bands) deeply heterochromatic bands named as trapezoid (T). The trapezoids may also serve as important landmark for the identification of the species in Simuliids. The presence of trapezoid was observed in all the four species investigated. . In *Simulium* (*N*.)

*praelargum* “IIIL-1.2” and *Simulium (N.) praelargum* “IL”, trapezoid (T) was located in section 52 of IIS. In *Simulium* (*M.*) *dattai* trapezoid (T) was located also in section 52 and in *Simulium* (*G.*) *williei*, trapezoid (T) was located in section 53. The presence of trapezoid in IIS of all the four species investigated is suggestive of the idea that it is an important polytene chromosomal landmark.

**Parabalbiani**

Parabalbiani (PB) is a characteristic of long arm of chromosome II (IIL). It is a darkly stained band with sharply defined and diffused edge. PB is unique to Simuliidae and is readily recognizable. PB was encountered in all the four species investigated. In *Simulium* (*N.*) *praelargum* “IIIL-1.2” and *Simulium (N.) praelargum* “IL”, PB was located in section 55. In *Simulium* (*M.*) *dattai*, PB was located in 68 and in *Simulium* (*G.*) *williei*, PB was located in 63. The location of PB in all the four species has been a distinguishing characteristic. In other species of *S. pictipes* (Bedo 1975a), *S. ornatipes* (Bedo 1977a), and *S. venustum*/*verecundum* complex (1978) and in *Metacnephia* (Procunier 1982a) PB has served as an important landmark for species identification.

Long arm of chromosome II (IIL) is characterized with some important banding pattern. Centromere marks the beginning of IIL followed by three sharp band in *Simulium* (*N.*) *praelargum* “IIIL-1.2” and *Simulium (N.) praelargum* “IL”, *Simulium* (*M.*) *dattai* and *Simulium* (*G.*) *williei*. A euchromatic region with an active gene

transcription, gray band (gb), has been observed in section 59 of *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium (N*.) *praelargum* "IL". However gb was not prominently observed in *Simulium* (*M*.) *dattai* and *Simulium* (*G*.) *williei*. Therefore gb has not been considered to be of much importance in the species identification. In *Simulium* (*N*.) *praelargum* "IL" DNA puff was prominent in section 58 which in other three species were not found to be prominent. A pair of band with rough texture was observed in section 62 of *Simulium* (*G*.) *williei* called jagged (jg). Centromeres or portion of the chromocenter of all the four species marked the beginning of the IIL

**Chromosome III**

The short of the chromosome III (IIIS) of all the species investigated is characterized by certain landmarks such as capsule (Ca) and blister (Bl).

**Capsule**

A swollen region named capsule (Ca) was located in section 72 in *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium (N*.) *praelargum* "IL". In *Simulium* (*G*.) *williei* was found to be present in section 79. In *Simulium* (*M*.) *dattai* capsule could not be confirmed. The position of the capsule in different species seemed to be variable and could not be considered as an important identification landmark.

**Blister**

A typical enlarged puffed region, blister (B) was found to be present in section 77 of *Simulium* (*N*.) *praelargum* "IIIL-1.2" and *Simulium (N*.) *praelargum* "IL". In *Simulium* (*M*.) *dattai* blister (B) was located in section 76 and in *Simulium* (*G*.) *williei* was located in section 74.

The long of chromosome III (IIIL) does not bear any characteristic landmarks. Centromeres or portion of the chromocenter of all the four species marked the beginning of the IIIL.

**Banding patterns**

Polytene is characterized by a series of heterochromatic and euchromatic bands throughout the length of the chromosomes. The banding pattern of each species is species specific. The linear bands are interspaced with enlarged swollen and loose structures called puffs. Puffs are genetic loci in the polytene chromosomes where intense RNA synthesis takes place. Appearance of puffs in general therefore depends upon the genetic activity of the chromosomes that vary overtime. However other structures such as bulge, ring of Balbiani, DNA puff, blister, and capsule have encountered in the Simuliids.

**Sex chromosome**

Heteromorphy of sex chromosome is known for many groups in animal kingdom. In most cases the Y-chromosome element is

smaller. The sex chromosome in Simuliidae is virtually unknown. Sex is determined primarily by a single locus or by a very short length of chromosome (Rothfels and Mason 1975). Many studies have revealed species in which the sex chromosomes have become differentiated by the linkage of inversions. One of such cases has been observed in *Simulium erythrocephalum* (Post 1985a). In *S. jenningsi* group the inversions played an important role in the determination of sex (Gorden 1984). In *Chironomous tetans* partial linkage of inversions to the X or Y-chromosomes was reported (Acton 1957). The species investigated so far from Darjeeling region did not reveal morphologically distinguishable sex chromosome. The sexes of the individual larvae were identified on the basis of nature of the gonads.

Total chromosomal complement of almost all the species of black flies is 2n=6, except for the members of the subgenus *Eusimulium* where 2n=4 (Leonhardt 1985). Usually three criteria reveals the presence of sibling species and cytotypes in a population through the study of polytene chromosomes viz, fixed inversion differences, the complement of floating inversions, and differential sex-determining mechanisms (Rothfels 1956). So, different cytoforms within a species can be distinguished by atleast one of these criteria. Paracentric inversions are usually responsible for the reorganization of chromosomes in black flies, the same inversions either become fixed, lost, a stable polymorphism, or a differentiated segment of an X or Y chromosome (Rothfels 1980). As in almost all flies, in most

species of black flies the males are heterogametic sex (XY) and females homogametic (XX) except for *S. siamense* where females are heterogametic and males homogametic as also in *S. yaeyamaense* (Hadi et al., 1995), *S. tahitiense* and *S. oviceps* (Rothfels 1979).

There are 33 nominal species of the *Simulium feuerborni* species group (Adler & Crosskey 2016), out of which only seven species namely, *S. feuerborni*, *S. praelargum*-st., *S. maeaiense* Takaoka & Srisuka, *S. sasai* (Rubtsov), *S. leigongshanense* Chen & Zhang, *S. mongarense* Takaoka & Somboon, and *S. fangense* Takaoka & Choochote, have a cocoon with a distinct anterodorsal projection. However the anterodorsal projection in the cocoon was absent in the two species cytologically described here, i.e., *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2" and *Simulium* (*Nevermannia*) *praelargum* "IL". Out of 33 species only two species from *Simulium feuerborni* species group have fewer than six gill filaments; *Simulium borneoense* Takaoka has four and *S. phami* Takaoka, Sofian-Azirun & Ya'cob has five gill filaments. Six gill filaments are characteristic feature of the *S. feuerborni* species-group, including this two new species. Morphologically larvae of *S. praelargum*-st, *S. praelargum* "IIIL-1.2", and *S. praelargum* "IL" are almost similar as in most of the sibling species or cryptic species, which are morphologically similar and indistinguishable. Though the chromosomal arrangement of some sections of the polytene chromosomes of these cytoforms are different. So in resolving the identification of these cytoforms as a sibling species or a new

species can be done only through morphological studies of adults or through molecular analysis and other ecological parameters as well.

The total chromosomal complement of this two new species, *S. praelargum* "IIIL-1.2" and *S. praelargum* "IL", as in most species of the Simuliidae (Rothfels 1979), consisted of three tightly paired homologues (2n = 6). Chromosome arms of IS, IIS, IIL, IIIS of *S. praelargum* "IIIL-1.2" is identical with that of *S. praelargum*-st and *S. praelargum* "IL", but differs from *S. praelargum*-st by two fixed inversions in IIIL and by four fixed inversions with *S. praelargum* "IL". Chromosome arms IS, IIS, IIL, and IIIS of *S. praelargum* "IL" are identical with those of *S. praelargum*-st and *S. praelargum* "IIIL-1.2". *S. praelargum* "IL" differs from these two species by four fixed inversions in IL: IL-1, IL-2, IL-3, and IL-4. IIIL of *S. praelargum* "IL" species is identical with IIIL of *S. praelargum*-st, but differs from *S. praelargum* "IIIL-1.2" by two fixed inversions. The nucleolar organizer (NO) of both *S. praelargum* "IIIL-1.2" and *S. praelargum* "IL" is in the short arm of chromosome I (IS) near the centromere in section 18, as in *S. praelargum*-st from Darjeeling, India. The *Simulium feuerborni* complex in Thailand also has the nucleolar organizer (NO) in IS near the centromere (Pramual & Wongpakam 2013). The centromeres of the two new species exhibit heavy heterochromatic bands, similar to those in *S. feuerborni* (Hadi et al., 1996) and *S. praelargum*-st (Henry et al., 2010), but *S. praelargum*-st and *S. praelargum* "IL" lacks a chromocenter, which is present only in *S. praelargum* "IIIL-1.2" (Thapa et al., 2014). B

chromosomes were not found in the mitotic or polytene complements of both *S. praelargum* "IIIL-1.2" and *S. praelargum* IL, although they have been observed in other species in the Darjeeling area, such as *S. gracile* Datta, *S. praelargum* (Dey & Fumafartosok 1984b), and a few unnamed species of the subgenus *Montisimulium* (unpublished). Nevertheless, B chromosomes have been observed in high frequency in cytoform C of *S. feuerborni* from Malaysia (Pramual et al., 2015). No rearrangements in the new species were linked to the X or Y chromosomes (X0X0, X0Y0), and no floating (polymorphic) inversions were found.

There are four cytoforms documented in *S. feuerborni* viz, cytoform A and cytoform B from Thailand (Pramual & Wongpakam 2013), cytoform C from Peninsular Malaysia, and cytoform D from Indonesia (Java) (Pramual et al., 2015). The characteristic of cytoform A is the standard polytene chromosome banding sequence and undifferentiated sex chromosomes (Pramual and Wongpakam 2013). There are more than six fixed inversions in cytoform B. A fixed inversion on the long arm of chromosome III, undifferentiated sex chromosomes and a high frequency of B chromosomes is the characteristic of Cytoform C (Pramual et al., 2015). Cytoform D is characterized by a fixed inversion on IIL and undifferentiated sex chromosomes (Pramual et al., 2015). But none of these inversions found in cytofoms A, B, C, or D of *S. feuerborni* are found in either of the two new species investigated, *S. praelargum* "IIIL-1.2" or *S. praelargum* "IL". At least some speciations in the Simuliidae are

accounted by complex karyotypic and genomic changes (Adler et al., 2016). These changes involve at least three chromosomal restructuring phenomena: i) a whole-arm interchange, ii) differential expression (e.g., fixed vs floating) of one and the same rearrangement in different species, and iii) taxon-specific differentiation of sex chromosomes. The *S. feuerborni* group is atypical in that no taxa to date have been found with sex-chromosome differentiation, all taxa being X0X0, X0Y0. It would appear that in the *S. praelargum* complex, heterochromatic polymorphisms for centromere and telomere band enhancements and chromocenters (Thapa et al., 2014) occurred in an ancestral intermediate and might be associated with present-day speciation of the three extant taxa in the complex.

In sympatric situations, the absence of heterozygotes attests directly to reproductive isolation of sibling species (Rothfels 1979). In general, this taxonomic status can be ascribed to a larval *Simulium* population, demonstrating the lack of appropriate heterozygote(s) for any particular salivary gland autosomal inversion polymorphism (Procunier 1984). Larval populations occur in sympatry, e.g., "IIIL-1.2" and IIIL-st occur in the same stream at the Happy Valley and Sonada sites. *S. praelargum*-st, *S. praelargum "*IIIL-1.2", and *S. praelargum "*IL", occur in close proximity with respect to dispersal, they were found in two or three different streams but within 1 m of each other at one place and within 100 m of each other at the other place at the Dali site. Adult emergence times do overlap for the

distinct cytological entities described here; consequently, these populations can be considered sympatric. This site is .particularly instructive for providing information on conclusions reached that three distinct entities occur in the Darjeeling area and that they are reproductively isolated.

As in most species of the Simuliidae (Rothfels 1979), *Simulium* (*Montisimulium*) *dattai* also has three pairs of polytene chromosomes (2n = 6). Few nominal species like *S. inflatum* (Rubtsov), *S. jasgulemum* (Chubareva), and *S. octofiliatum* (Rubtsov) of the subgenus *Montisimulium* have B chromosomes. (Chubareva & Petrova 2008) but however it was not found in the mitotic or polytene complements of *Simulium* (*Montisimulium*) *dattai*. As in most species of the sub genus *Montisimulium* like, *S. bartangum, S. jasgulemum, S. obichingoum, S. quattuordecimfilum, S. vantshum* ( Chubareva 2000) and *S. ghoomense* (Henry et al., 2011), the nucleolar organizer (NO) is the short arm of chromosome I near the centromere in section 18. However in *S. asulcatum* (Rubtsov) the nucleolar organizer is in the base of IL (Chubareva & Petrova 2008). *Simulium dattai* has been found to exhibit a large and prominent chromocenter as found in about 12% of black flies (Adler *et al.*, 2010), and about half of the chromosomally mapped species of the subgenus *Montisimulium* (Chubareva & Petrova 2008). But *Simulium ghoomense* mapped form this region is the only member under this subgenus to have a putative partial chromocenter, which involves only chromosome II and chromosome III. The terminal

third or more of IS of *Simulium dattai* is homologous with that of species such as *Simulium montium* and *Simulium ghoomense*. IL of *Simulium dattai* shows terminal homology with *Simulium ghoomense*. IIS and IIIS of *Simulium dattai* are almost similar and homologous to some extent with that of *Simulium ghoomense.* In the mapped species of the subgenus *Montisimulium* the banding pattern of the short arms of chromosomes II and III are conserved and appear similar if not exact to those of *Simulium dattai*. IIL of *Simulium dattai* also shows terminal homology with *Simulium ghoomense* and other species, such as *S. alizadei* and *S. octofiliatum*, atleast in the presence of parabalbiani which is located distally. The terminal sections of IIIL of *Simulium dattai* also show terminal homology with that of *Simulium ghoomense.*

Although maps are available for some species in the subgenus *Gomphostilbia* but other species in this subgenus have been inadequately studied chromosomally. This is the first available map of *Simulium* (*Gomphostilbia*) *williei* in the *Gombakense* species-group from the subgenus *Gomphostilbia* from Darjeeling region. As a rule as in most of the species of the Simuliidae (Rothfels 1979), *Simulium* (*Gomphostilbia*) *williei* also consists of three pairs of polytene chromosomes (2n = 6). As in most species from the subgenus *Gomphostilbia*, the nucleolar organizer (NO) of *Simulium* (*G.*) *williei* is also at the base of IS of chromosome one near the centromere. The fore and terminal end of IL of *S. williei* shows homology with that of standard map of *Simulium ceylonicum* species

group. The centromeric regions were not expanded and the major landmarks, the ring of Balbiani and bulge in the IIS arm, were sub terminal in *Simulium gombakense* from Thailand (Phasuk et al., 2005) which is located somewhere near the middle of the mapped polytene chromosome of *Simulium williei* from Darjeeling. But in standard map of *Simulium ceylonicum* from the *Ceylonicum* species group the ring of Balbiani and bulge were more towards the telomeric side (Jitklang et al., 2008). Whereas the bulge in *S. trangense* was more towards the telomeric side and the ring of Balbiani was towards the centromeric side (Jitklang et al., 2008). The sections housing the major landmarks, jagged (jg) and parabalbiani (PB) in IIL of chromosome two of *Simulium williei* show homology with the standard map of *Simulium ceylonicum* species group, however, the position of jagged (jg) and parabalbiani (PB) are reversed in *S. trangense*. The last few sections of IIIS near the centromere of chromosome three of *Simulium williei* shows homology with the standard map of *Simulium ceylonicum* species group in having a series of deep bands. The deeply stained post centromeric bands of *Simulium williei* are comparable to that of standard map of *S. asakoae,* and other species of the same group, *S. curtatum* n. sp., *S.* nr. *asakoae* 2, *S.* nr. *asakoae* 3, *S.* nr. *asakoae* 4, *S. inthanonense*, *S. sheilae*, *S. trangense* n. sp., and *S. doisaketense* n. sp.

**Status *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2":** This new species differ from those previously described *S. praelargum*-st by a

two step fixed included inversion (IIIL-1.2) in chromosome IIIL and by a large enhanced chromocenter (Fig. 16). The limits for IIIL-1 encompass sections 85–94. The second included inversion (IIIL-2) re-inverts the segment 85–92 thereby transposing sections 94 and 93, leaving them proximally closer to the chromocenter. The new species is informally designated here as *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2".

**Status *Simulium* (*Nevermannia*) *praelargum* "IL":** This new species differ from *S. praelargum*-st and *S. praelargum* IIIL-1.2 by four fixed inversion in long arm of chromosome IL: IL-1, IL-2, IL-3, and IL-4. The new species is informally designated here as *S.* (*Nevermannia*) *praelargum* "IL".

All the three species *S. praelargum*-st, *S. praelargum* "IIIL-1.2", and *S. praelargum* "IL" have been found to be sympatric

**Status of *Simulium* (*Montisimulium*) *dattai*:** This species was first reported from Darjeeling by Datta et al., 1975 from Kurseong, Darjeeling, on the basis of the pupal characters only as *Simulium* (*Eusimulium*) sp. B. The same species was described morphologically as from Bhutan basing on the larval characters and renamed as *Simulium* (*Montisimulium*) *dattai*. The mitotic data and the polytene chromosome map of this species supported this species at the cytological level.

**Status of *Simulium* (*Gomphostilbia*) *williei*:** This species was described based on larval and pupal characters only from Dali, Darjeeling. The polytene chromosome map constructed here along with other mitotic data provides additional characteristics to the description of this newly described species and adds to the diversity richness in the subgenus *Gomphostilbia*.