INTEGRATED SYSTEMATICS OF *Simulium multistriatum* SPECIES GROUP (DIPTERA: SIMULIIDAE) IN THAILAND

JIRAPORN THAIJARERN

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Mahasarakham University

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The examining committee has unanimously approved this dissertation, submitted by Miss Jiraporn Thaijarern, as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Mahasarakham University.

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TITLE Integrated systematics of *Simulium multistriatum* species group (Diptera: Simuliidae) in Thailand.

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ABSTRACT

A total of nine species of *Simulium multistriatum* species group were reported in Thailand. Members of this species group are morphologically very similar make it difficult for species identification and other related studies. The objectives of this study are to use an integrated approach based on morphology, cytology, and molecularly base on cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit II (COII) to examine taxonomy and systematics of *S. multistriatum* group in Thailand. A total 898 larvae from eight species (*S. bullatum, S. chaliowae, S. chainarongi, S. daoense, S. fenestratum, S. lampangense, S. malayense,* and *S. triglobus*) collected from 30 populations in Thailand, one from Malaysia and one from Vietnam were chromosomally examined. A total of 10 taxa were recognize based largely on unique chromosome rearrangements. *Simulium chainarongi, S. fenestratum,* and *S. triglobus* are homosequential species. Chromosomal analyses show that *S. daoense,* a species recently described from Vietnam, also occurs in Thailand. *Simulium malayense* consists of three cytoforms: A and B are sympatry in Thailand and C near the type locality in Malaysia. Identical chromosomes, minimal morphological differentiation, and low COI + COII differentiation (1.20%) suggests that *S. lampangense* is either a junior synonym or a homosequential cryptic species of *S. chaliowae*. High genetic diversity in the geographically widespread species, *S. fenestratum* suggests that it consists of at least two cryptic species. Chromosomal and molecular phylogenetic inference showed good agreement within the *S. multistriatum* species group.

Keywords: chromosomes, COI gene, COII gene, phylogeny, systematics

ชื่อเรื่อง ซิสเทมาทิกส์เชิงบูรณาการ ของแมลงริ้นดำกลุ่มสปีชีส์ *Simulium multistriatum* (Diptera: Simuliidae) ในประเทศไทย

ผู้วิจัย นางสาวจิราพร ไทยเจริญ
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บทคัดย่อ

แมลงริ้นดำกลุ่มสปีชีส์ *Simulium multistriatum* มีรายงานในประเทศไทย 9 สปีชีส์ การศึกษาก่อนหน้า พบว่าแมลงริ้นดำกลุ่มสปีชีส์นี้มีลักษณะสัณฐานวิทยาของตัวอ่อนคล้ายคลึงกันมาก ยากต่อการจำแนกชนิดและการศึกษาด้านอื่น การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาอนุกรมวิธาน และ ซิสเทมาทิกส์ของแมลงริ้นดำกลุ่มสปีชีส์ *Simulium multistriatum* โดยบูรณาการข้อมูลสัณฐานวิทยา พันธุศาสตร์เซลล์ และ พันธุศาสตร์โมเลกุล โดยใช้ลำดับนิวคลีโอไทด์ของยีน cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit II (COII) ศึกษาพันธุศาสตร์เซลล์ของแมลงริ้นดำกลุ่มสปีชีส์ *Simulium multistriatum* 8 สปีชีส์ (*S. bullatum*, *S. chaliowae*, *S. chainarongi*, *S. daoense*, *S. fenestratum*, *S. lampangense*, *S. malayense* และ *S. triglobus*) จำนวน 898 ตัวอย่าง จาก 30 ประชากรในประเทศไทย 1 ประชากรจากประเทศมาเลเซีย และ 1 ประชากรจากประเทศเวียดนาม พบว่าแมลงริ้นดำกลุ่มสปีชีส์ *S. multistriatum* ประกอบด้วย 10 cytoforms โดยใช้รูปแบบการจัดเรียงตัวของแบนด์พอลิทีนโครโมโซมที่จำเพาะในการแยกความแตกต่าง 3 สปีชีส์ (*S. chainarongi*, *S. fenestratum* และ *S. triglobus*) พบว่ามีรูปแบบโครโมโซมเป็น homosequential species การศึกษาพันธุศาสตร์เซลล์พบว่าตัวอย่างจากภาคเหนือของประเทศไทยมีลักษณะโครโมโซมเหมือนกับแมลงริ้นดำ *S. daoense* ที่พบในประเทศเวียดนาม ซึ่งเป็นรายงานการพบสปีชีส์นี้ครั้งแรกในประเทศไทย แมลงริ้นดำ *S. malayense* ประกอบด้วย 3 cytoforms (A, B และ C) cytoform A และ B พบในประเทศไทย cytoform C พบ ใกล้ type locality ในประเทศมาเลเซีย การศึกษา สัณฐานวิทยา พันธุศาสตร์เซลล์ พันธุศาสตร์โมเลกุล และนิเวศวิทยาบ่งชี้ว่า *S. chaliowae* และ *S. lampangense* อาจเป็นสปีชีส์เดียวกัน การศึกษาพันธุศาสตร์เซลล์ และ พันธุศาสตร์โมเลกุลโดยใช้ลำดับนิวคลีโอไทด์ของยีน COI และ COII และการกระจายทางภูมิศาสตร์ที่กว้างขวางของ แมลงริ้นดำ *S. fenestratum* บ่งชี้ว่าแมลงริ้นดำชนิดนี้ประกอบด้วยอย่างน้อย 2 สปีชีส์ การศึกษาสายสัมพันธ์ทางวิวัฒนาการโดยใช้พันธุศาสตร์เซลล์และพันธุศาสตร์โมเลกุลให้ผลที่สอดคล้องกันในแมลงริ้นดำกลุ่มสปีชีส์ *S. multistriatum* ในประเทศไทย

คำสำคัญ: โครโมโซม, cytochrome *c* oxidase subunit I, cytochrome *c* oxidase subunit II, สายวิวัฒนาการชาติพันธ์, ซิสเทมาทิกส์

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CHAPTER 1

INTRODUCTION

1.1 Background

Black flies are aquatic insects belonging to the family Simuliidae. There are 2,247 species reported globally (Adler and Crosskey, 2017). Adult females of many species feed on vertebrate blood including that of humans. Some black fly species are important vectors of human onchocerciasis or river blindness that is caused by the filarial nematode *Onchocerca volvulus* (Crosskey 1990). In addition, black flies transmit some viruses and arboviruses that cause diseases in economically important livestock and wild animals (Crosskey 1990). Black fly larvae are also important in stream ecosystems (Malmqvist *et al*., 2004).

In Thailand, 97 black fly species have been reported. These species are arranged in six subgenera of the genus *Simulium,* including *Asiosimulium*, *Daviesellum*, *Gomphostilbia*, *Montisimulium*, *Nevermannia*, and *Simulium*. The *Simulium multistriatum* species group belongs to subgenus *Simulium*. A total of 33 species have been reported globally for this species group (Adler and Crosskey, 2017). In Thailand, nine species are recorded including *S. bullatum, S. chainarongi, S. chanyae* (Takaoka and Choochote, 2007), *S. chaliowae* (Takaoka and Kuvangkadilok, 1999), *S. fenestratum* (Edwards, 1934), *S. lampangense* (Takaoka and Choochote, 2005a), *S. malayense* (Takaoka and Davies, 1995), *S. takense* (Takaoka and Choochote, 2005b), and *S. triglobus* (Takaoka and Kuvangkadilok, 1999). Members of the *S. multistriatum* species group in Thailand are distributed in diverse habitats. Some species are restricted to highly calcareous streams, such as *S. takense*, *S. chaliowae*, *S. lampangense*, and *S. triglobus* (Takaoka and Boonkemtong, 1999; Takaoka and Choochote, 2005c; Takaoka and Choochote, 2005a). *Simulium bullatum* is found in habitats at elevations >1,000 m above sea level. *Simulium fenestratum* is found in a wide range of habitats. *Simulium chainarongi* is geographically restricted to the northeastern part of Thailand.

Members of the *S. multistriatum* species group are morphologically very similar in the larval stage. Previous phylogenetic study based on two mitochondrial genes (COI, COII) and one nuclear gene (18S/ITS1) found that *S. takense* was genetically highly divergent (>20.3%) from other species. In addition, *S. chaliowae*, *S. fenestratum*, and *S. chainarongi* did not form a monophyletic clade. Thus, the taxonomy and systematics of this species group needs further investigation (Pramual and Kuvangkadilok, 2012). An additional complication is that some larvae of the members of the *S. multistriatum* species group are still unknown because they were recognized by minor differences in the adult or pupal stages (Takaoka and Choochote, 2005a; Takaoka and Choochote, 2005b).

Cytogenetic study using salivary gland polytene chromosomes is important for black fly taxonomy and systematics (Adler *et al*., 2004). However, this technique needs a high level of expertise to interpret chromosome banding patterns and typically can only work for the larval stage. Recently, molecular study has been used for black fly taxonomy and systematics. Molecular taxonomy based on the DNA barcode technique can be used to associate unknown life stage with the known species of black flies (Pramual and Wongpakam, 2014). However, different methods have both advantages and limitations. Thus, an integrated approach is preferable for black fly taxonomy and systematics (Adler and Huang, 2011; Pramual *et al*., 2015).Therefore, the objectives of this study are to use an integrated approach based on morphology, cytology, and molecular biology to examine the taxonomy and systematics of the *S. multistriatum* species group in Thailand.

1.2 Objective of the research

The objective is to investigate the taxonomy and systematics of black flies of the *Simulum multistriatum* species group in Thailand.

1.3 Scope of the research

Specimens of the *Simulium multistriatum* species group and associated ecological data were collected throughout Thailand. Specimens were preserved in Canoy's fixative. Banding patterns of polytene chromosome were examined and compared with the standard subgenus *Simulium* map of Rothfels *et al.* (1978) and Adler *et al.* (2016a). Sequences of cytochrome *c* oxidase subunit I (COI) and cytochrome *c* oxidase subunit II (COII) were used for phylogenetic analyses.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy of black fly

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Infraorder: Culicomorpha

Superfamily: Chironomidae

Family: Simuliidae

Subfamily: Simuliinae

Tribe: Simuliini

Genus: *Simulium* Latreille, 1802

Common name: black fly

Black flies belong to phylum Arthopoda, class Insecta, order Diptera, and family Simuliidae. Adults have a sturdy body shape and head slung low on the arched thorax. They have one pair of wings. Larvae are without true legs. This insect is almost everywhere except Antarctica and islands without flowing water. A total of 2,247 species (2,232 living and 15 fossil) have been reported globally (Adler and Crosskey 2017). Among 26 genera of black flies, most species (1,817 species) belong to genus *Simulium*. This genus is divided into 38 subgenera. The three most diverse subgenera of genus *Simulium* are *Simulium* (492 species), *Gomphostilbia* (240 species), and *Nevermannia* (236 species) (Adler and Crosskey, 2017).

2.2 Biology of black flies

Black flies are aquatic insects. The life cycle of the black fly is composed of four stages, including egg, larva, pupa, and adult (Figure 2.1). The immature stages (i.e. egg,

larva, and pupa) develop in flowing water and have a crucial role in circulation of nutrients and minerals in aquatic ecosystems (Malmqvist *et al*., 2004). Adults are in the terrestrial ecosystem. Both male and female adults feed on nectar and only females feed on blood from warm-blood vertebrates because they need blood proteins to complete their ovarian cycle (Crosskey, 1990).

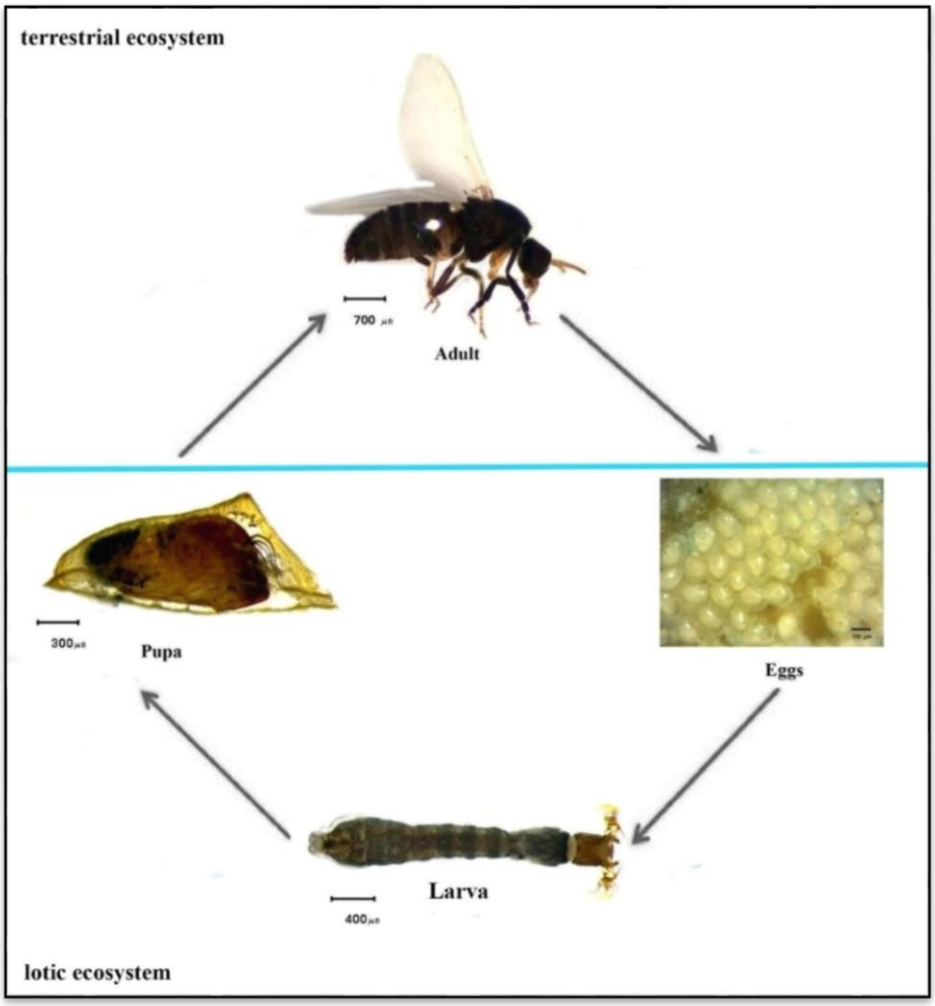

Figure 2.1 Life cycle of the black fly.
(Source: Crosskey, 1990)

2.2.1 Egg

Eggs of the black fly (Figure 2.2) are small (0.18–0.46 mm) and incomplete ellipses. The shell of eggs is smooth (Crosskey, 1990). After the female has been fertilized, it can spawn about 19-836 eggs and stick them together as a group to substrates in flowing water. Females can produce a pheromone while they are ovipositing (Malmqvist *et al*., 2004). The pheromone can help for other adult females to lay their eggs in the same area (McCall, 1997; Coupland, 1991). The shell of the eggs consists of mucopolysaccharides that help the eggs stick to rocks, leaves, or other objects in water. Egg colors varying from white to brown depend on age. Size of the eggs are about 0.18–0.46 mm. Egg sizes are related to egg numbers. Females that lay small numbers have eggs that are large and if they lay many eggs, the sizes are small (Welton and Bass, 2008). The hatching time varyies from one day to 10 months depending mostly on water temperature. At 20^{o}C – 25^{o}C, it take about 3–6 days to hatch but if the temperature is 10^{o}C-19^{o}C, it would take 1–2 weeks. Black flies in tropical zone need about 36–48 hours for hatching (Crosskey, 1990).

Figure 2.2 Eggs of the black fly *Simulium aureohirtum* in Thailand.

2.2.2 Larva

The larva of the black fly (Figure 2.3) consists of three parts (head capsule, thorax, and abdomen). The head capsule has two labral fans. Labral fans consist of a stalk and fan rays. The fan rays of larvae have microtrichia similar to a fine-tooth comb. These microtrichia are used for filtering nutrients in water. Numbers of fan ray and microtrichia are negatively related to the flow velocity. Fan rays are smaller in species occupying high current velocity (Zhang and Malmqvist, 1996; Palmer and Craig, 2000). On the thorax, the proleg is a prominent structure. The anterior end of the proleg has a ring of hooks called the anterior circlet, which helps larvae to attach to substrates for movement. Black spots on the thorax of the mature larva are gill histoblasts that will develop into the gills, the gas exchange organs in the pupa stage. The abdomen has rectal papilla dorsally and ventral papilla ventrally. Rectal papilla are an osmoregulatory organ. The ventral papilla, present in many black flies of the subgenus *Nevermannia* and *Gomphostilbia*, has no yet-known role but it has some value for taxonomy because this structure is present in

only some subgenera. The last segment of the abdomen has the anal sclerite and posterior circlet by which to attach to substrates. The larva of the black fly makes a silk pad of viscous fibers from its salivary glands to attach to substrates by means of its posterior circlet. Salivary glands that secrete silk to make cocoons are within the abdomen. The best quality polytene chromosomes that are crucial for black fly taxonomy are from these salivary glands. The growth phases of black fly larvae typically include 6-7 instars. The development time from larva to pupa stage is about 2 weeks in warm areas (Crosskey, 1990).

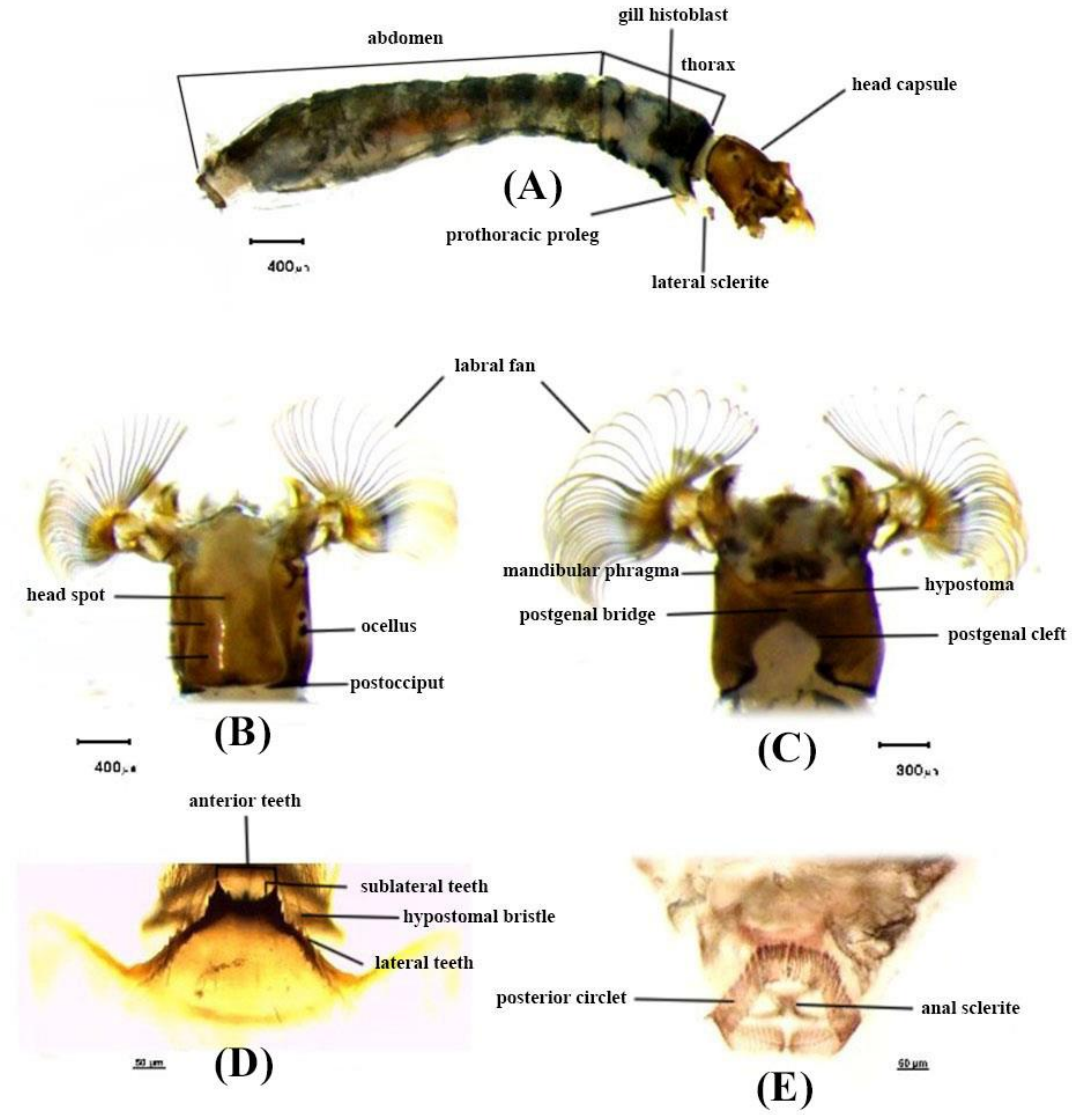

Figure 2.3 Structure of the black fly larva. (A) Mature larvae of *Simulium fenestratum* (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view), (D) Hypostoma, (E) Posterior circlet.

2.2.3 Pupa

The black fly pupa (Figure 2.4) is composed of two parts including the body and cocoon. Sizes of the pupal body range from 2 mm to 7 mm (Crosskey, 1990). The cocoon is made from protein fibers that are secreted from salivary glands of the larva. The pupal body is encapsulated within the cocoon. The pupal body is composed of three parts as in the larva: head, thorax, and abdomen. The head has cephalic trichromes. The thorax is slightly arched, and has thoracic trichomes and gill filaments. The role of gill filaments is gas exchange (Crosskey, 1990). Numbers and arrangements of gill filaments are valuable for black fly taxonomy. The pupa body is divided into nine segments. Segments 3 and 4 have recurved hooks and segments 5 to 9 have spine combs. The last segment typically has terminal spines. Pupal development time is about two to three days but can be longer depending mostly on the temperature (Adler *et al.*, 2004).

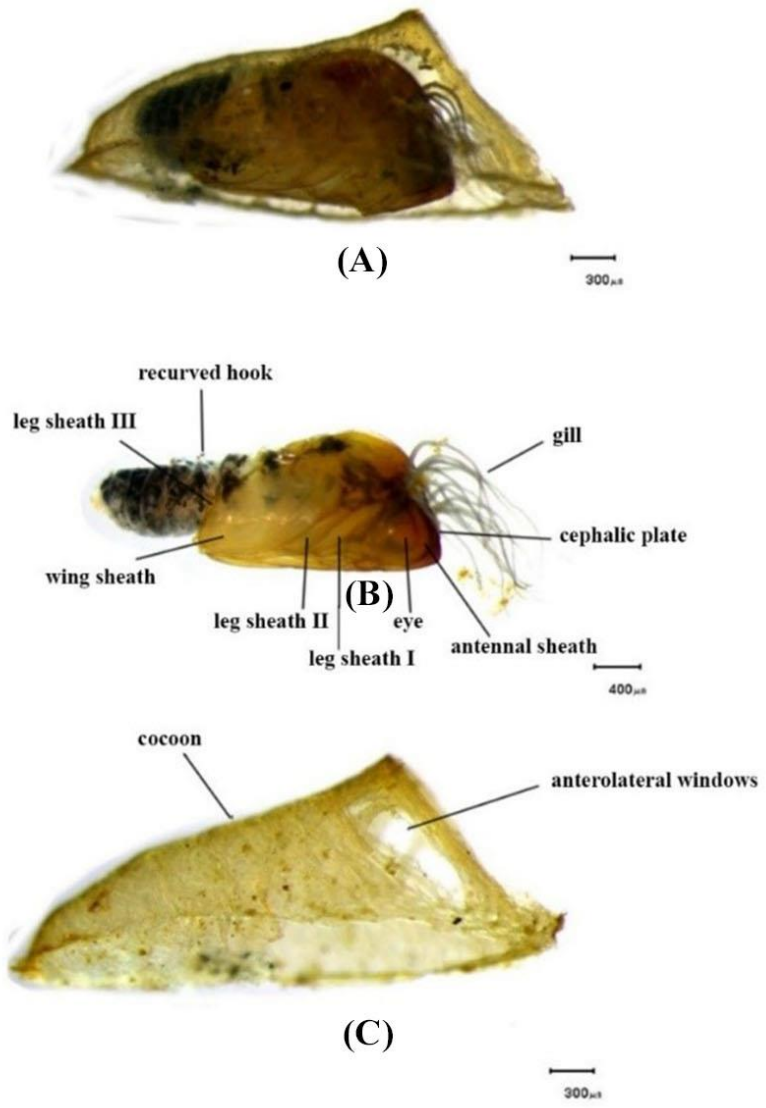

Figure 2.4 General structure of black fly pupa of *Simulium fenestratum*. (A) Pupa in cocoon, (B) Pupal (lateral view), (C) Cocoon (lateral view).

2.2.4 Adult

The adult black fly (Figure 2.5) has a body length between 1.2 and 6.0 mm and a wing length of 1.4 to 6.0 mm (Adler *et al*., 2004). The adult body consists of three parts, the head, thorax, and abdomen. The head comprises eyes that show sexual dimorphism. Male have two sizes of corneal facets. The upper corneal facets are about twice the size of the lower facets. Females have only one size of corneal facets. Other important structures of the adult head are the antenna, mouthparts, and clypeus. The maxillary laciniae of the mouthparts and the mandibles and hypopharynx are used to suck nectar and blood. The thorax consists of the scutum that helps protect the body of the black fly. The adult black fly has one pair of wings.

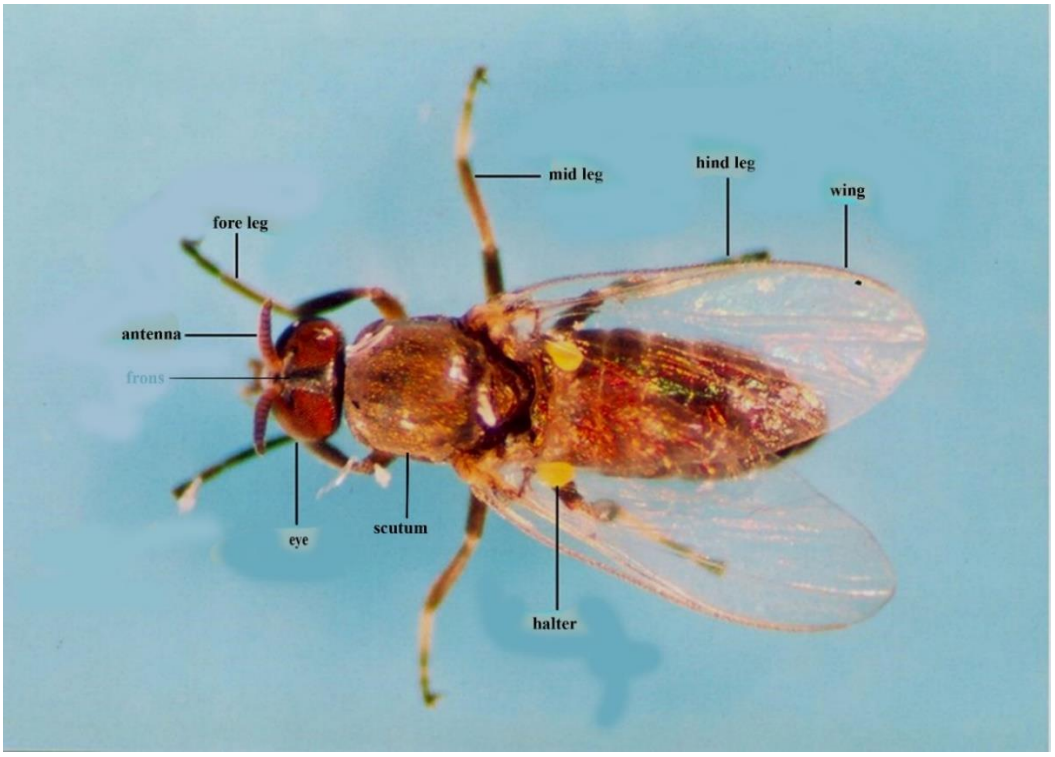

Figure 2.5 General structure of adult of the black fly *Simulium nakhonense*.
(Source: Pramual, 2014)

2.3 Ecology of black flies

Most ecological studies of black flies are about the immature stages. Ecological factors that play important roles in geographical distributions of black flies are stream width, depth, pH, velocity, water temperature, water conductivity, streambed particle sizes, canopy cover, and riparian vegetation (Adler *et al*., 2004; McCreadie *et al*., 2006; Pramual and Kuvangkadilok, 2009; Pramual and Wongpakam, 2010). Black fly larvae are sensitive to changes of physical characters of the stream. Changing of forest to agricultural land effects black fly community structure and diversity (Pramual and Kuvangkadilok 2009). Comparisons of the ecological factors between forest and agricultural streams found that agricultural areas are warmer, with higher conductivity and fewer riparian trees. Species richness in forest streams was significantly higher than in agricultural streams. Ecological conditions of the stream habitat could also contribute to black fly evolution and speciation. Pramual *et al*. (2012) found that stream velocity and elevation are the principal ecological factors differing between closely related species of subgenus *Gomphostilbia*, suggesting the possibility of speciation driven by ecological divergence.

In addition to individual species distributions, species assemblages are also influenced by stream ecology. Srisuka *et al*. (2015) found that seasonal biodiversity and ecological factors influence species distribution of black flies in Doi Pha Hom Pok National Park, Thailand. Ecological factors including elevation, canopy cover, riparian vegetation, and water velocity are important factors influencing species assemblages. Pangjanda and Pramual (2016) found that environmental conditions in the habitat play an important role in black fly community assemblages. Trait-based community structure analysis based on labral fan morphology found that most species (>78%) in the same community are more similar morphologically than expected by chance. As labral fans are related to habitat exploitation, habitat filtering is considered a major factor establishing community structure of larval black flies in Thailand.

2.4 Medical and economic importance of black flies

Adult black flies feed on plant nectar, but the female also needs protein in the blood to complete the ovarian cycle (Crosskey, 1990). More than 2,000 species of black flies feed on vertebrate blood (Figure 2.6) (Adler *et al*., 2010). Some of these species are vectors of parasites that cause diseases in humans and livestock. Black flies in the genus *Simulium* are vectors of *Onchocerca volvulus* that cause onchocerciasis or river blindness in humans. Adults of *O. volvulus* can live in the body of humans about fifteen years. They can move around the subcutaneous tissues in the body after 1-2 years. They also migrate to the eye, where they cause inflammation and other complications that can lead to onchocerciasis or river blindness (Andre *et al*., 2002). This disease has been found in Central and West Africa, Arabian Peninsula, North Yemen, Mexico, Guatemala, Venezuela, Colombia, and parts of Brazil (Ottesen *et al*., 2008). More than 100 million people were at risk and 18 million people were found with *O. volvulus* in the body, and 270,000 were blind (Boatin and Richards, 2006).

In addition, the larvae of *O. volvulus* also cause lymph node inflammation and lymphatic filariasis. Dead parasites cause a skin allergic reaction. They reduce pigmentation and vitamin D in the skin. Some people can feel sick and nauseous or asthma (black fly fever) after being bitten by black flies. Apart from this, onchocerciasis has an effect on the economy, requiring control and eradication of the disease. Onchocerciasis control was conducted for both black fly vectors and the parasite (i.e., *O. volvulus*). In 1946, the Onchocerciasis Control Programme (OCP) began using DDT to control black fly larvae by spraying flowing water in Kenya. DDT was used in 11 countries of Africa, but was unsuccessful because of high diversity and resistance.

Black flies also attack livestock. For example, in the United States, livestock deaths along river basins occur from black fly attacks, and cattle, horses, mules, hogs, turkeys, chickens, sheep, dogs, and cats have been killed (Adler *et al*., 2004). In 1974, because of the attack of adult black flies, weight and milk production were reduced about 15 % in Australia (Kettle, 1990).

In Thailand, although there are no reports on human onchocerciasis, biting of black flies can cause problems for people, particularly in tourist places. For example, in Doi

Inthanon National Park (northern Thailand) biting black flies cause black fly fever in humans (Kuvankadilok *et al*., 1999a). Fukuda *et al*. (2003) found three species of adult female black flies (*S. asakoae*, *S. nigrogilvum*, and *S. nakhonense*) are natural vectors of *Onchocerca* spp. that could be causal agents of some diseases in cattle.

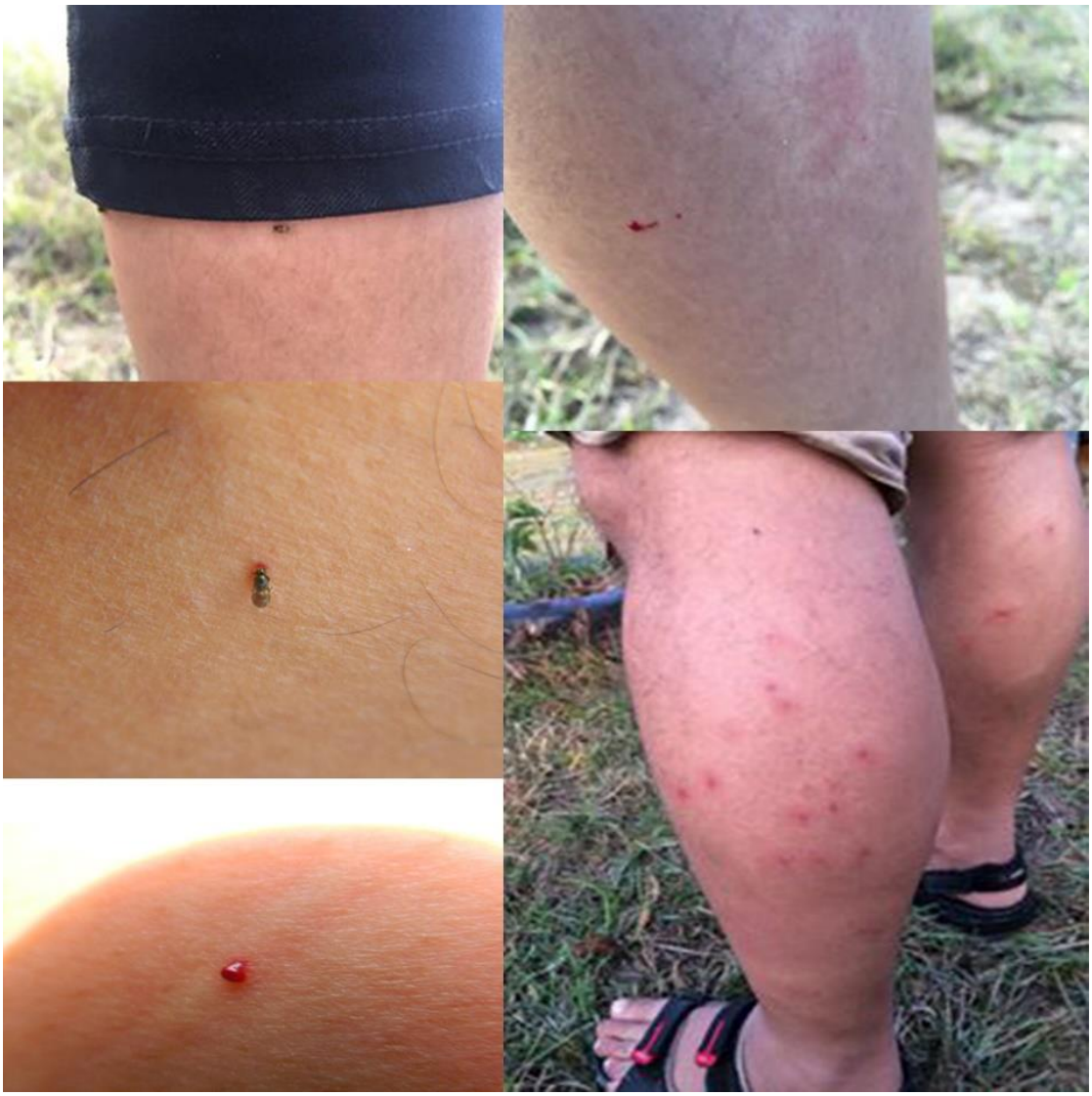

Figure 2.6 Black fly biting on humans.

2.5 Cytotaxonomy of black flies

Polytene chromosomes are found in the salivary glands of black fly larvae. All black flies have a haploid number of three chromosomes, except *Cnephia pallipes* in the Northern Palearctic region. Also, species in subgenus *Eusimulium* occurring in North America and elsewhere (Leonhardt, 1985) have a haploid number of two chromosomes. Some species of black flies have supernumerary chromosomes or B chromosomes that appear in the polytene complement as short, densely staining, usually banded, chromosomes, such as some species of genus *Cnephia* (Adler *et al*., 2004) and *Simulium feuerborni* Edwards in Malaysia (Pramual *et al*., 2015).

Polytene chromosomes play a significant role in the taxonomic study and systematics of black flies. Taxonomy of black flies can use the positions of chromosome landmarks including the Balbini ring (BR), nucleolar organizer (NO), and Parabalbiani (PB) for species identification (Adler *et al.*, 2004). However, the most important character of the polytene chromosomes for taxonomic study is the banding pattern (Adler *et al*., 2004). For example, *Simulium cauchense* Floch and Abonnenc and *S. quadrifidum* Lutz are highly similar morphologically in the larva and pupa. However, these species are readily distinguished by the position of the nucleolar organizer located on the short arm of chromosome I in *S. cauchense* and on the long arm of chromosomes III in *S. quadrifidum*. They also differ by three fixed inversions. On the long arm of chromosomes III of *S. quadrifidum* are 3 fixed inversions (IIIL-1, IIIL-2, and IIIL-3), on the short arm of chromosome III is one fixed inverstion (IIIS-1) (Aguilar *et al*., 2005).

In addition, polytene chromosomes can be used to recognize black fly species complexes. Criteria used to recognize cytological sibling species include presence of fixed chromosome inversion differences, different sex chromosome systems, and different frequencies of chromosome polymorphisms. Cytogenetic studies have shown that many morphological species are actually comprised of many cytological sibling species (e.g., Adler and Kachvorian, 2001; Tangkawanit *et al*., 2009; Pramual and Kuvangkadilok, 2012; Pramual *et al*., 2015).

In Thailand, cytotaxonomy of black flies has been reported in many species. Kuvangkadilok *et al*. (1999) examined larval polytene chromosomes of five species of black flies in northern Thailand, including *S.* (*Nevermannia*) *caudisclerum, S.* (*Simulium*) *fenestratum, S.* (*S.*) *nakhonense, S.* (*S.*) *rufibasis,* and *S.* (*Montisimulium*) sp. *G.* All the five species have three pairs of chromosomes (2n=6). Chromosome I of all species were metacentric while most of the chromosome II and III were submetacentric. All five species of genus *Simulium* have specific banding sequences which are different among species, although some banding sequences in chromosome arm IIIS show homology. Phasuk *et al*. (2005) used cytological criteria based on locations of chromosome landmarks to differentiate 12 species of black flies of subgenus *Gomphostilbia* in Thailand.

Jitklang *et al*. (2008) used cytogenetics to examine the *S. ceylonicum* species group in Thailand. The results indicated that this species group includes 10 cytoforms. Kuvangkadilok *et al*. (2008) and Pramual and Nanork (2011) found that *S. siamense* in Thailand, which belongs to the *S. batoense* species group, is composed of seven cytoforms. Different cytoforms seem to prefer different habitats: cytoform A and B were found in large streams with fast-flowing water and low conductivity. Cytoforms B, C, and D were found in warm streams with low dissolved oxygen and high conductivity. Cytoforms F and G were found in open streams. Cytogenetic study of *S. angulistylum* found that this species is composed of three cytoforms (A, B, and C). Distributions of these cytoforms were associated with ecology. Cytoforms A and B were found in low-elevation habitats (<600 m above sea level), whereas cytoform C occurred at high elevations (>1,000 m above sea level) (Pramual and Kuvangkadilok, 2012).

Cytogenetic study of *S. feuerborni* in Thailand revealed that this species is composed of two cytoforms (A and B). These cytoforms occurred in different geographic regions; Cytoform A was found in the north and northeast and cytoform B was found specifically in the north of Thailand (Pramual and Wongpakam, 2013). Cytotaxonomy indicates that the *S. tuberosum* species group is composed of seven morphospecies including *S. doipuiense*, *S. manooni, S. rufibasis, S. setsukoae, S. tani, S. yuphae,* and *S. weji*. The cytogenetic results indicate much greater diversity, with 17 cytoforms recognized.

Two species were shown to be species complexes, including *S. doipuiense* (two cytoforms, A and B), and *S. tani* 10 cytoforms (Tangkawanit *et al*., 2009).

2.6 Molecular systematics of black flies

Although polytene chromosomes have an indispensable role for black fly taxonomy and systematics, this technique has some limitations. Working with polytene chromosomes requires experience for reading the banding patterns and it is only workable typically for the larval stage. Molecular techniques, thus, have been applied to black fly taxonomy and systematics (e.g. Rivera and Currie, 2009; Pramual *et al*., 2005; Phayuhasena *et al*., 2010). DNA barcoding based on mitochondrial cytochrome oxidase I (COI) is a popular molecular technique successfully used for black fly identification (e.g. Rivera and Currie, 2009; Pramual *et al*., 2011; Pramual and Adler, 2014) and also association of black fly life stages (Pramual and Wongpakam, 2014). The COI sequences can also differentiate cytoforms of species complexes (Pramual and Adler, 2014).

Although COI sequences are successful as a marker for specimen identification, they can be limited for closely related species. For example, COI sequences failed to differentiate *S. doipuiense* and *S. rufibasis* (Sriphirom *et al*., 2014) and *S. nakhonense*, *S. chiangmaiense*, and *S. quinquestriatum* (Pramual and Adler, 2014). Therefore, multiple gene sequences are required for molecular taxonomy and systematics (Phayuhasena *et al*., 2010; Sriphirom *et al*., 2014; Moulton, 2000). The elongation complex protein 1 (ECP1) gene is a fast-evolving gene that successfully differentiates members of some species taxa such as members of the *S. jenningsi* species group (Senatore *et al*., 2014) and *S. tani* complex (Low *et al*., 2016). Thus, it is desirable that additional genes, such as ECP1, are used along with the COI standard barcoding gene for black fly taxonomy and systematics.

Although molecular genetic data can successfully delineate many black fly species, a number of studies found inconsistent results. Kruger *et al*. (2000) found that phylogenetic analysis based on mitochondrial 16s ribosomal RNA (rRNA) in

S. pandanophilum and the cytoform 'Kiwira' from East Africa, as distinct from the 'Sanje' group, does not agree with data from cytogenetic analyses. Pramual and Kuvangkadilok (2012) found that, based on COI barcoding sequences, species groups within subgenus *Gomphostilbia*, as currently recognized based on morphological criteria, are not monophyletic. Phayuhasena *et al*. (2010) found that the phylogenetic tree for 37 black fly species in Thailand is inconsistent with previous studies of morphology and cytology. Subgenus *Simulium* and *Gomphostilbia* were monophyletic in most analyses but the subgenus *Nevermannia* was paraphyletic because it included *Montisimulium*. Species groups were generally monophyletic except the *S. batoense* species group was always paraphyletic with regard to the other two species groups found in Thailand. All species were monophyletic except two specimens of *S. nakhonense* were paraphyletic with respect to *S. quinquestriatum*, although the relevant branch was very short and without bootstrap support. Sriphirom *et al*. (2014) found that molecular data supported the monophyletic status of most species of the *S. tuberosum* species group, but *S. doipuiense* and *S. rufibasis* were polyphyletic. Therefore, fully understanding taxonomy and systematics requires an integrated approach based on all available information from the molecular, cytological, morphological, and ecological levels.

2.7 Black flies in Thailand

Black flies in Thailand were first reported by Summer (1911). The first species was *S. nigrogilvum* (Summer, 1911). *Simulium hackeri* and *S. digrammicum* were discovered later and then there was no further study for 73 years. In 1984, a total of 19 species were reported (Takaoka and Suzuki, 1984) and seven more species were reported later (Takaoka and Saito, 1996). Recently, several aspects of black flies in Thailand have been investigated, including ecology (Pramual and Kuvangkadilok, 2009; Pramual and Wongpakam, 2010; Pangjanda and Pramual, 2016), cytology (Kuvangkadilok *et al*., 1999, 2008; Pramual and Wongpakam, 2011; Tangkawanit *et al*., 2011; Jitklang *et al*., 2008), population genetics (Pramual *et al*., 2005; Thaijarern *et al*., 2014; Pramual and Pangjanda, 2015; Chaiyasan and Pramual, 2016), and molecular systematics (Phayuhasena *et al*., 2010; Sriphirom *et al*., 2014). At present, 97 species, and 9 cytoforms of *S. tani*, 7 cytoforms of *S. siamense*, 3 cytoforms of *S.*

angulistylum, and 2 cytoforms each of *S. feuerborni* and *S. doipuiense* have been reported in Thailand (Adler and Crosskey, 2017). All species belong to genus *Simulium* and they are assigned to six subgenera, including *Asiosimulium* (3 species), *Davisellum* (2 species), *Gomphostilbia* (26 species), *Montisimulium* (6 species), *Nevermannia* (11 species) and *Simulium* (44 species).

2.8 *Simulium multistriatum* species group

The *Simulium multistriatum* species group is classified in the genus *Simulium*. This species group occurs in both the Palearctic and Oriental regions (Adler and Crosskey 2016). There are 32 species in the *S. multistriatum* species group including *S. barraudi* Puri, *S. bifengxiaense* Huang, Zhang and Chen, *S. demolaense* Takaoka and Somboon, *S. dentatum* Puri, *S. deothangense* Takaoka and Somboon, *S. digitatum* Puri, *S. hillycum* Maskey, *S. hirtinervis* Edwards, *S. kisapense* Takaoka, Sofian-Azirun and Ya'Cob, *S. konakovi* Rubtsov, *S. lacduongense* Takaoka and Ya'Cob, *S. laui* Takaoka, Sofian-Azirun, *S. lineothorax* Puri, *S multistriatum* Rubtsov, *S. novolineatum* Puri, *S. pulanotum* An, Guo and Xu, *S. sakishimaense* Takaoka, *S. subornatoides* Rubtsov, *S. tamorense* Takaoka and Shrestha, *S. tumidilfilum* Luo, Yang and Chen, *S. uncum* Zhang and Chen, *S. xanthogastrum* Rubtsov, and *S. xiaolongtanense* Chen, Luo and Yang (Adler and Crosskey, 2016). In Thailand, nine species of the *S. multistriatum* group have been recorded, including *S. bullatum* Takaoka and Choochote, *S. chainarongi* Kuvangkadilok and Takaoka, *S. chaliowae* Takaoka and Boonkemtong, *S. chanyae* Takaoka and Choochote, *S. fenestratum* Edwards, *S. lampangense* Takaoka and Choochote, *S. malayense* Takaoka and Davies, *S. takense* Takaoka and Choochote, and *S. triglobus* Kuvangkadilok and Takaoka (Table 2.1).

Table 2.1 List of species in *Simulium multistriatum* species group (Adler and Crosskey 2017).

Species	Geographical distribution
Simulium barraudi Puri, 1932	India (Kashmir, HP), China (Tibet), Pakistan
Simulium bifengxiaense Huang, Zhang and Chen, 2013	China (Sichuan)
Simulium bullatum Takaoka and Choochote, 2005	Thailand
Simulium chainarongi Kuvangkadilok and Takaoka, 1999	Thailand
Simulium chaliowae Takaoka and Boonkemtong, 1999	Thailand
Simulium chanyae Takaoka and Choochote, 2007	Thailand
Simulium demolaense Takaoka and Somboon, 2008	Bhutan
Simulium dentatum Puri, 1932	India (West Bengal, Assam, Me, Sik), Bhutan, China
Simulium deothangense Takaoka and Somboon, 2008	Bhutan
Simulium digitatum Puri, 1932	India (HP & Punjab), China (Gd, Tibet)
Simulium fenestratum Edwards, 1934	Indonesia (Sumatra), Laos, Thailand
Simlium hillycum Maskey, 1989	Nepal
Simulium hirtinervis Edwards, 1928	Malaysia (Malaya), Vietnam
Simulium kisapense Takaoka, Sofian-Azirun and Ya'Cob, 2012	Malaysia (Malaya)

Table 2.1 (Continued)

Species	Geographical distribution
Simulium konakovi Rubtsov, 1956	Siberia (Kurile Islands FE)
Simulium lacduongense Takaoka and Ya'Cob, 2015	Vietnam
Simulium lampangense Takaoka and Choochote, 2005	Thailand
Simulium laui Takaoka and Sofian Azirun, 2015	Vietnam
Simulium lineothorax Puri, 1932	India (Assam, TN)
Simulium malayense Takaoka and Davies, 1995	Malaysia (Malaya), Thailand, Vietnam
Simulium multistriatum Rubtsov, 1947	Tajikistan; Kazakhstan, Turkmenistan, Uzbekistan
Simulium novolineatum Puri, 1933	India (West Bengal, ArP, Ka, UP), Burma
Simulium pulanotum An, Guo and Xu, 1995	China (Tibet)
Simulium sakishimaense Takaoka, 1977	Japan (Nansei Islands), China, Taiwan.
Simulium subornatoides Rubtsov, 1947	Tajikistan
Simulium takense Takaoka and Choochote, 2005	Thailand
Simulium tamorense Takaoka and Shrestha, 2010	Nepal
Simulium triglobus Takaoka and Kuvangkadilok, 1999	Thailand
Simulium tumidilfilum Luo, Yang and Chen, 2010	China (Hubei)
Simulium uncum Zhang and Chen, 2001	China (Guizhou)
Simulium xanthogastrum Rubtsov, 1951	Tajikistan, Pakistan, Uzbekistan
Simulium xiaolongtanense Chen, Luo and Yang, 2007	China (Hubei), Nepal

2.8.1 *Simulium bullatum* Takaoka and Choochote, 2005

The female of *S. bullatum* ranges from 2.5 to 2.9 mm in length and wings are approximately 2.3 mm. The antenna is composed of 2+9 segments. The female has a simple claw. Pupa of *S. bullatum* (Figure 2.8) has a body length of 3.0-3.2 mm. Integument of the body is yellowish brown to medium brown color. Gill filaments consist of 8 filaments and a basal large unpigmented round organ. The cocoon is wall-pocket-shaped and it has anterolateral windows on each side (Takaoka and Choochote, 2005c). *Simulium bullatum* is closely related to *S. rotifilis* Chen and Zhang in Guizhou Province, China (Chen and Zhang, 1998).

The larva body of *S. bullatum* (Figure 2.7) is greyish brown or reddish brown to black. Body length is 5.6-6.2 mm. The head capsule has a yellowish brown cephalic apotome. The antenna is composed of three segments. The labral fans each have 44 main rays. The hypostoma has an anterior row of nine teeth. The postgenal cleft is large, deltoid, and not constricted basally. The abdominal segments do not have dorsolateral protuberances. The last abdominal segment does not have ventral papillae and the posterior circlet consists of 90-92 rows, each row including 12 hooklets (Pramual and Wongpakam, 2014).

This species is distinguished from other known species of this species-group in the pupal stage by an extraordinarily large unpigmented organ at the base of the gill filaments, as well as the arrangement of the eight gill filaments.

Simulium bullatum was first described from the female and pupa. Female adult and pupal specimens were collected in Northern Thailand. Pupae of this species were collected on slender roots of grasses trailing in a flowing stream, width ca. 1 m, depth 0.2 m, shaded, water temperature 20 C, elevation 1,420 m above sea level (Takaoka and Choochote, 2005c).

Cytogenetics have not yet been reported. Intraspecific genetic divergence based on COI sequences ranges from 0% to 0.86 %, with an average of 0.36% (Pramual and Wongpakam, 2014).

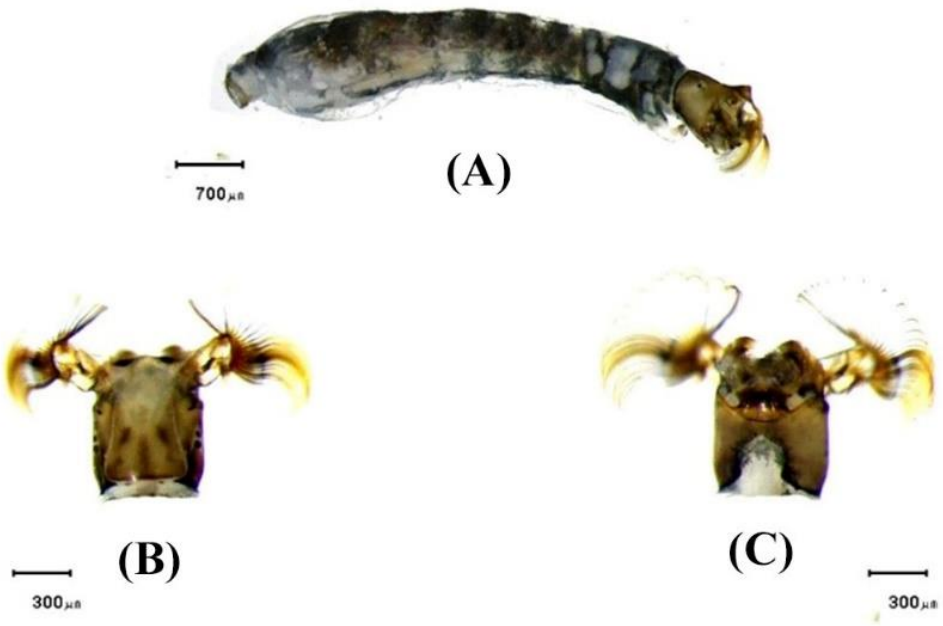

Figure 2.7 Larva of *Simulium bullatum*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

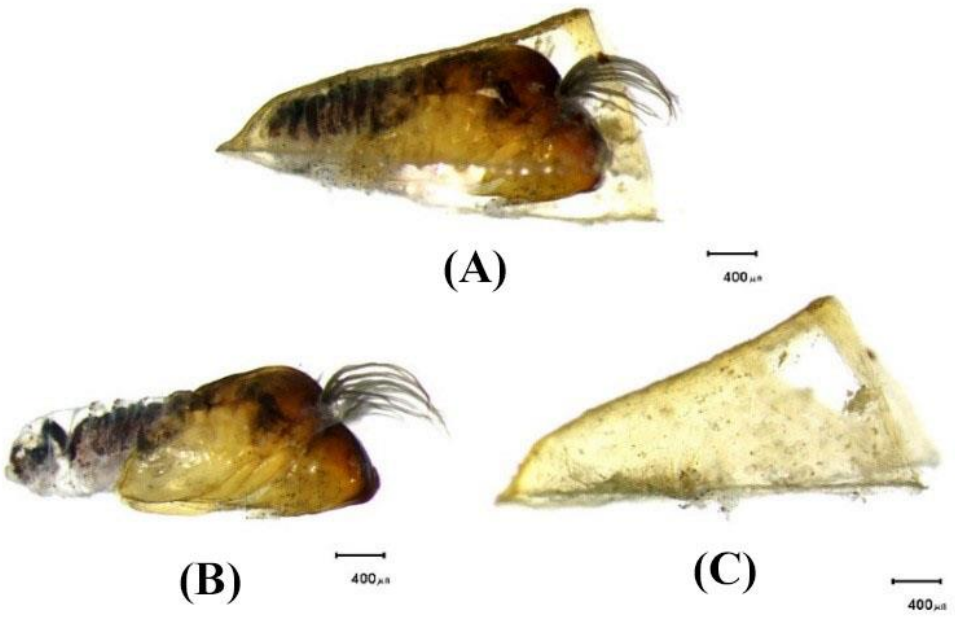

Figure 2.8 Pupa of *Simulium bullatum*. (A) Pupa in cocoon, (B) Pupa (lateral view), (C) Cocoon (lateral view).

2.8.2 *Simulium chainarongi* Takaoka and Kuvangkadilok, 1999

Adulst of *S. chainarongi* are 2.6 mm in length and wings are approximately 2.1 mm for females and 2.0 mm for males. The antenna is composed of 2+9 segments. The female has a simple claw. The pupa of *S. chainarongi* (Figure 2.10) has a body length of 3.0 mm. The integument of the head and thorax is yellowish brown to medium brown. Gill filaments consist of eight short and slender filaments arranged in pairs. The cocoon is shoe-shaped and with an anterior collar of variable heights without open windows on each side (Takaoka and Kuvangkadilok, 1999). The larva of *S. chainarongi* (Figure 2.9) is 4.8-5.0 mm in length with grayish black or greenish black color. The head capsule has a dark yellow to light yellowish brown cephalic apotome and positive head spots. The labral fans each have 42 main rays. The hypostoma has an anterior row of nine

teeth. The postgenal cleft is very large, rounded, and constricted basally. Abdominal segments 1-6 have a dorsolateral pair of protuberances. An accessory sclerite and ventral papillae are absent. The posterior circlet consists of 92 rows, and each row includes 16 hooklets (Takaoka and Kuvangkadilok, 1999). This species is easily distinguished from other species of the *S. multistriatum* species group by the color of the legs in the female and male. The larva of this species has the dorsal protuberances on the abdomen, similar to *S. hirtinervis* Edwards from Malaysia. But in the latter species these protuberances are present on abdominal segments 1-6, 1-7 and 1- 8 (Takaoka and Davies, 1995).

Simulium chainarongi was found in streams 2-10 m wide, at an elevation of 140 m. This species was first described from Kang Lum Duan waterfall, Ubonratchathani, northeastern Thailand. This species was found attached to trailing grasses, roots, and fallen leaves in fast flowing streams (Takaoka and Kuvangkadilok, 1999).

Cytogenetic study revealed that this species contains three (2n=6) polytene chromosomes (Kuvungkadilok *et al*., 2001). Intraspecific genetic divergence based on COI sequences ranges between 0.17% and 0.68 %, with an average of 0.54% (n=4) (Pramual and Adler, 2014).

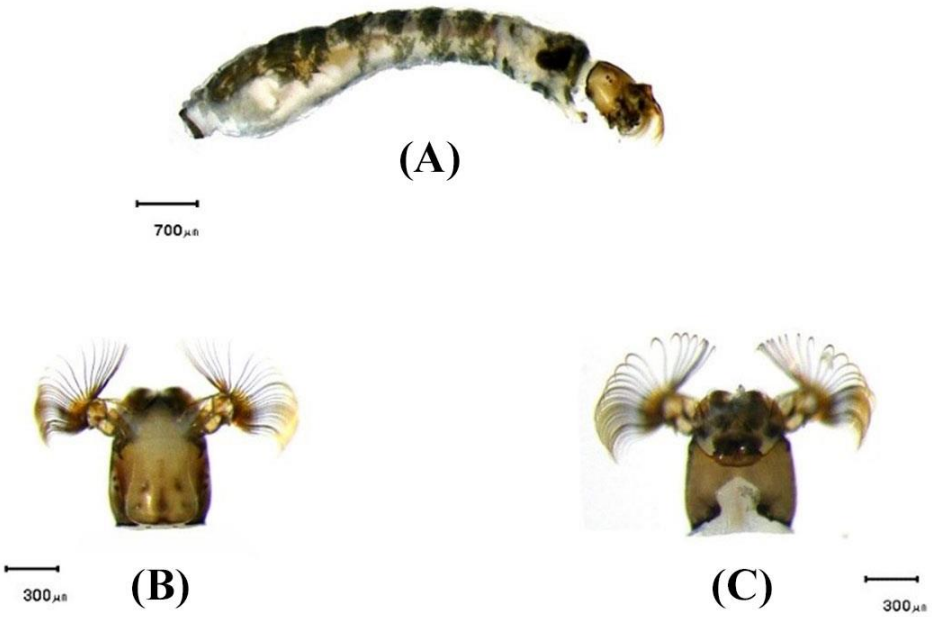

Figure 2.9 Larva of *Simulium chainarongi*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

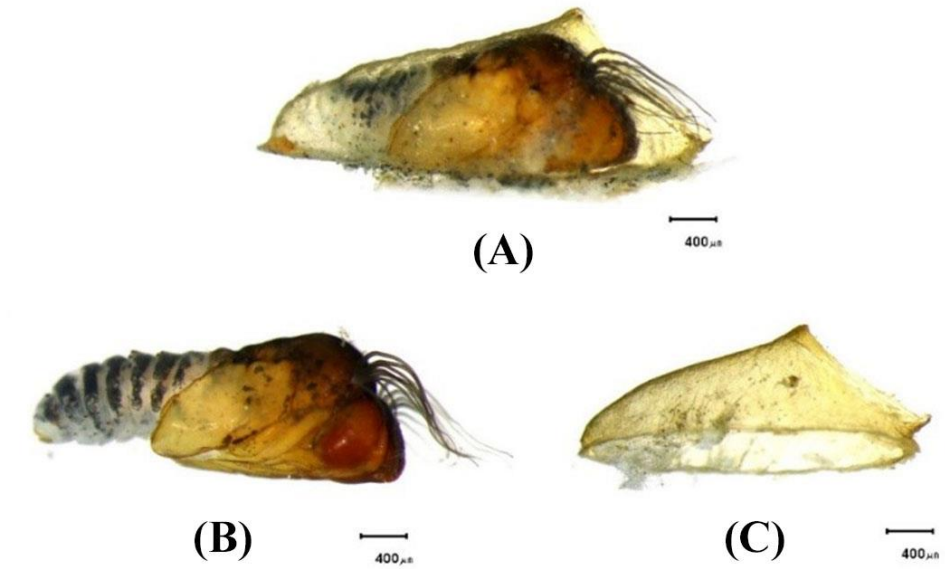

Figure 2.10 Pupa of *Simulium chainarongi*. (A) Pupa in cocoon, (B) Pupa (lateral view), (C) Cocoon (lateral view).

2.8.3 *Simulium chaliowae* Takaoka and Boonkemtong, 1999

The body length of *S. chaliowae* is 2.2 mm for females and 2.6 mm for males, and the wings are approximately 2.2 mm for females and 2.0 mm for males. The antenna is composed of 2+9 segments. The female has a simple claw. The pupa of *S. chaliowae* (Figure 2.12) is comprised of 3 parts, with body length 2.8 mm. The integument of the head and thorax is yellow. Gill filaments consist of 8 short and slender filaments in pairs. The cocoon is shoe-shaped with an anterior collar of moderate height. The cocoon consists of a thickly cemented silicaceous material because they are found in limestone streams with high conductivity (Takaoka and Kuvangkadilok, 1999).The larva of *S. chaliowae* (Figure 2.11) has a body length of 4.9-6.1 mm. The body is dark greenish or reddish brown on segment 1 of the thorax and light brown on the rest of the body. The head capsule has a light yellowish brown cephalic apotome and is narrowly dark brown along the lateral and posterior

margins. The labral fans each have 40 main rays. The postgenal cleft is large, rounded apically, and slightly constricted basally. Abdominal segments 2-6 each have a pair of small dorsolateral protuberance. The rectal organ consists of three lobes. The posterior circlet consists of 80 rows, each row including 17 hooklets (Pramual and Wongpakam, 2014). *Simulium chaliowae* can be distinguished from the other known species of this species-group by the shoe-shaped cocoon with an anterior collar of moderate height in place of the fenestrate cocoon. Females of this species are similar to those of *S. hirtinervis* from Peninsular Malaysia but can be separated by the much lighter coloring of the mid femur and tibia, the basal portion of the radial vein with a few hairs near the apical end, and the smaller spermatheca. The male of *S. chaliowae* is similar to that of *S. fenestratum* from Sumatra and Thailand (Edwards, 1934; Takaoka and Saito, 1996) but can be distinguish by the horn-like basal protuberance of the style with many teeth along the anterior margin. *Simulium chaliowae* was first descried from the adult and pupa by Takaoka and Kuvangkadilok (1999). The larva was reported later by Pramual and Wongpakam (2014). This species was found on fallen leaves in fast flowing water at an elevation of 290 m above sea level.

No cytogenetic data have been reported for this species. The COI barcoding sequences revealed that maximum intraspecific genetic divergence is 2.25% with an average of 0.60% (n=17) (Pramual and Adler, 2014).

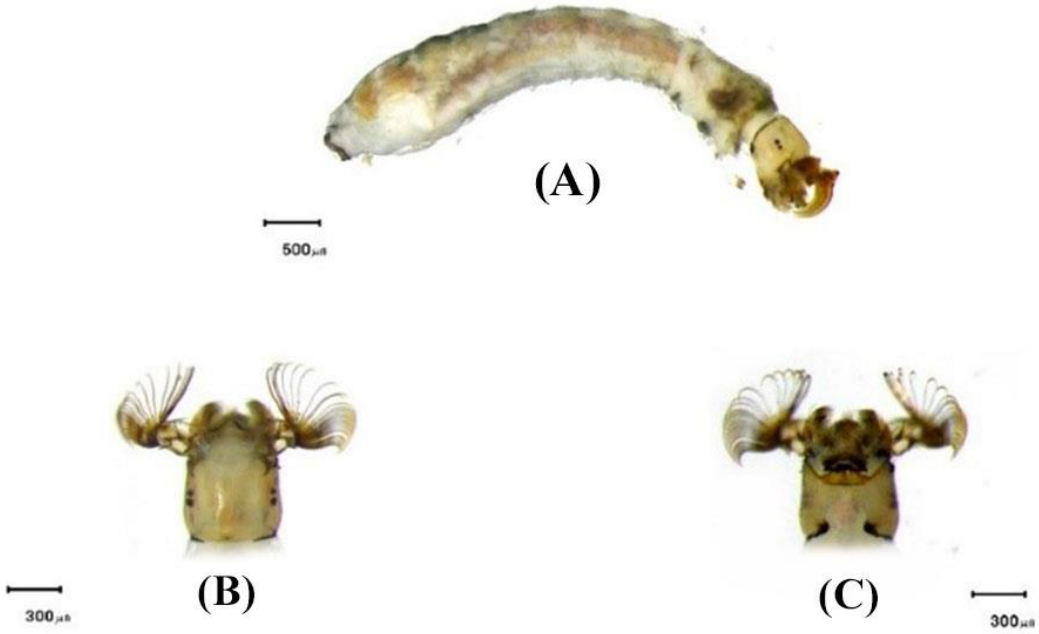

Figure 2.11 Larva of *Simulium chaliowae*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

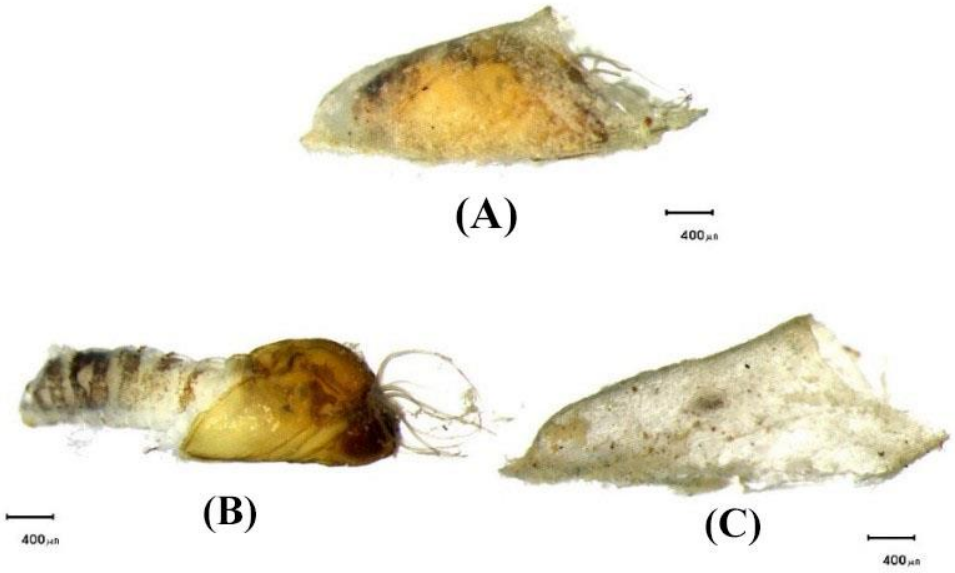

Figure 2.12 Pupa of *Simulium chaliowae*. (A) Pupa in cocoon, (B) Pupa (lateral view), (C) Cocoon (lateral view).

2.8.4 *Simulium fenestratum* Edwards, 1934

Simulium fenestratum was redescribed from the female, male, pupa, and mature larva by Takaoka and Suzuki (1984), while identifying this species as *S.* (*S.*) *sakishimaense* Takaoka, originally described from the Ryukyu Islands (Takaoka and Saito, 1996). The body length of the *S. fenestratum* is 3.2 mm for females and 2.4 mm for males, and the wings are approximately 2.3 mm for females and 2.1 mm for males. The antenna is composed of 2+9 segments. The female has a simple claw. The pupa of *S. fenestratum* (Figure 2.14) is comprised of 3 parts, with body length 2.8 mm. The integument of the head and thorax is yellow. Gill filaments consist of 8 slender filaments in pairs. The cocoon is shoe-shaped and consists of anterolateral windows on each side. The larva and pupa of *S. fenestratum* (Figure 2.13) were collected from Laos, Indonesia, and Thailand.

The body is dark greenish. The head capsule is dark yellow to light yellowish brown on the cephalic apotome, with positive head spots. The antenna is composed of 3 segments. The mandible has comb-teeth. The hypostoma has an anterior row of nine teeth. The postgenal cleft is large, rounded apically, and slightly constricted basally. Abdominal segments lack a pair of small dorsolateral protuberances. The rectal organ consists of 3 lobes. *Simulium fenestratum* can be distinguished from the other known species of this species-group by the abdominal segments lacking a pair of dorsolateral protuberances. The shoe-shaped cocoon has anterolateral windows on each side.This species was found on fallen leaves in fast flowing streams. Width of the streams was about 0.65-7.00 m, and the elevation 70-2,300 m. *Simulium fenestratum* is distributed in Indonesia, Laos, and Thailand (Adler and Crosskey, 2016). *Simulium fenestratum* has three (2n=6) polytene chromosomes (Kuvangkadilok et al., 2001). Five paracentric inversions were found in *S. fenestratum* (IS-1, IL-3, IIIL-1, IIIL-2, and IIIL-3) and a high frequency of a chromocenter (>80%) was found in a population from Loei Province, northeastern Thailand (Pramual and Nanork, 2012). Intraspecific genetic divergene based on COI sequences ranged between 0% and 2.98 %, with an average of 1.84% (n=8) (Pramual and Adler, 2014).

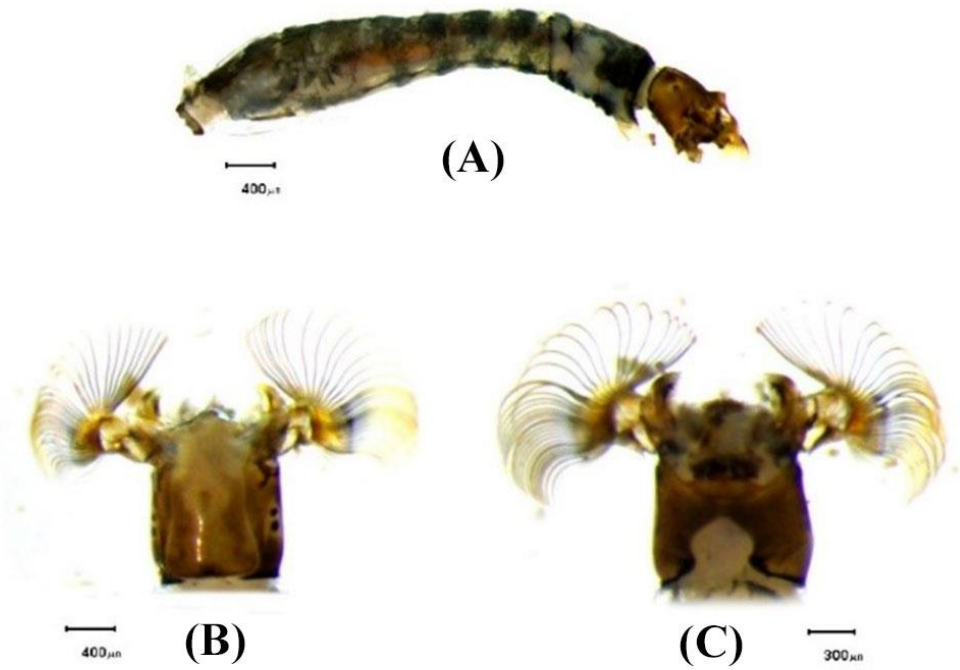

Figure 2.13 Larva of *Simulium fenestratum*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

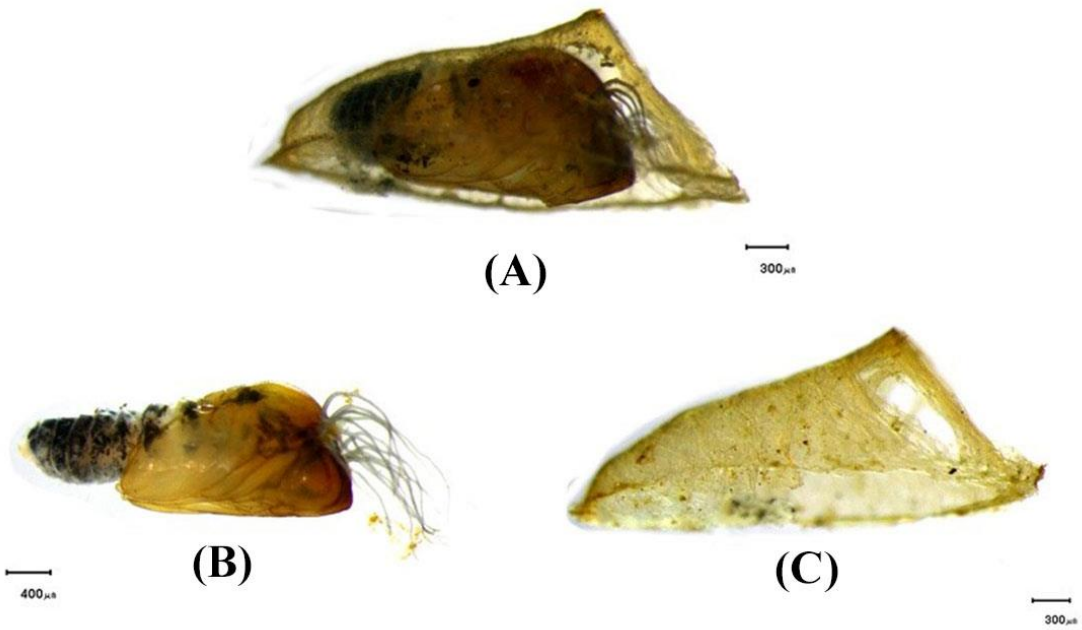

Figure 2.14 Pupa of *Simulium fenestratum*. (A) Pupa in cocoon, (B) Pupa (lateral view), (C) Cocoon (lateral view).

2.8.5 *Simulium lampangense* Takaoka and Choochote, 2005

In 2005, *S. lampangense* was described from the adult and pupa. Next, in 2015 we found the larval stage of *S. lampangense* but it has not been reported. This species was collected from the type locality (Wang Kaew and Wang Thong waterfall) in Lampang Province, which is a limestone stream.

The body length of *S. lampangense* ranges from 2.1 to 2.6 mm for females and 2.4-2.6 mm for males, and the wings are approximately 2.0-2.2 mm for females and 2.0 mm for males. The antenna is composed of 2+9 segments. The female has a simple claw. Pupae of *S. lampangense* were collected from fallen tree leaves in flowing water. Width of the streams was about 5-10 m, and the stream was rocky, shaded, and at an elevation of 570 m, with water temperature of 23.5 ^{o}C at Maekham waterfall.

Simulium lampangense was found together with *S. rudinicki* Takaoka and Davies and *S. weji* Takaoka (Takaoka and Choochote, 2005a).

The pupa of *S. lampangense* (Figure 2.15) has 3 parts, and the body length is 2.5-2.8 mm. The integument of the head including antennal sheaths is yellow to dark yellow. The gill consists of 8 short and slender filaments in 4 pairs. The cocoon is shoe-shaped with a very low anterior collar. The cocoon has anterolateral windows on each side (Takaoka and Choochote, 2005a).

Simulium lampangense is distinguished from other species of the *S. multistriatum* species group by the combination of the pupal characters of divergent gill filaments and fenestrated cocoon and some characters in the male and female. No cytogenetic data are available for *S. lampangense*. Intraspecific genetic divergence based on COI sequences ranges between 0% and 1.20 %, with an average of 1.20% (n=8), and the ECP1 gene ranges from 0% to 3.90% with an average of 3.90% (Thaijarern *et al.*, 2017).

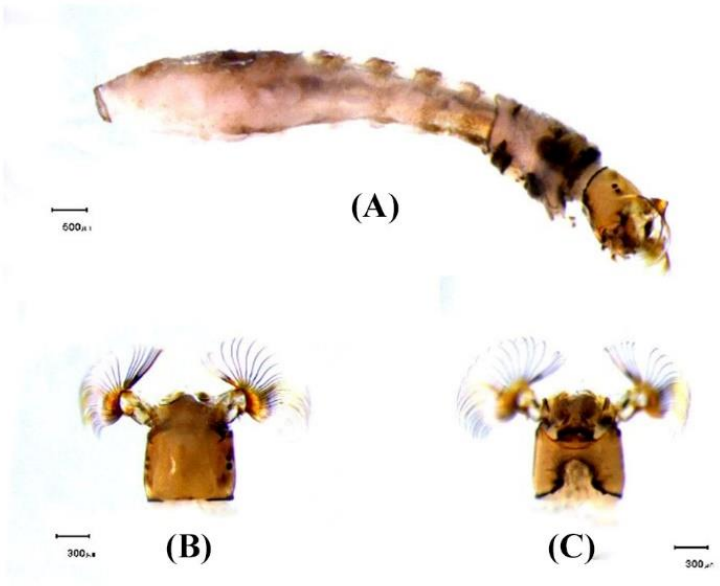

Figure 2.15 Larva of *Simulium lampangense*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

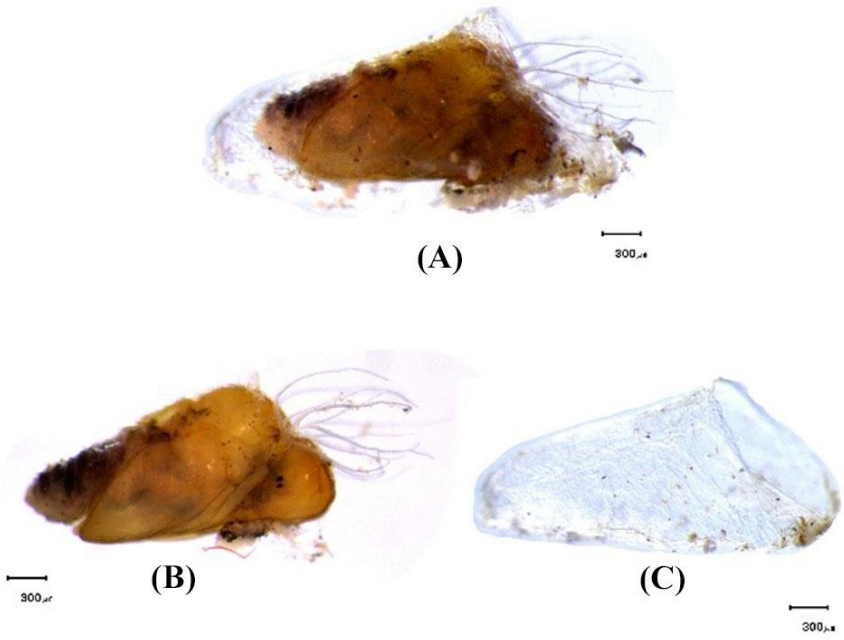

Figure 2.16 Pupa of *Simulium lampangense*. (A) Pupa in cocoon, (B) Pupa (lateral view), (C) Cocoon (lateral view).

2.8.6 *Simulium malayense* Takaoka and Davies, 1995

Simulium malayense was first described from Malaysia. This species was also collected in southern Thailand (Kuvangkadilok and Takaoka, 2000). The female length of *S. malayense* is 2.4 mm and the wings are approximately 2.3 mm. The antenna is composed of 2+9 segments. The female has a simple claw. The pupa of *S. malayense* has a body length of 3 mm. The integument of the head and thorax is brownish. The gill consists of eight short and slender filaments in pairs. The cocoon is wall-pocket-shaped and without windows on each side (Takaoka and Davies, 1995). The larva of *S. malayense* (Figure 2.16) has a length of 5.6-6.0 mm. The body and thorax are greyish. The head capsule has a yellow cephalic apotome with indistinct head spots. The lateral and ventral surface is pale yellow. The antenna is composed of three segments. The labral fans have 43 main rays. The postgenal cleft is large, rounded apically, and slightly constricted basally.

Abdominal segments 1-8 each have a pair of small dorsolateral protuberances. The rectal organ consists of three lobes. The posterior circlet consists of 92 rows, each row including 16 hooklets (Takaoka and Davies, 1995). This species is easily distinguished from other species of the *S. multistriatum* species group by the simple cocoon without lateral windows anteriorly and the larva without dorsolateral protuberances on the abdomen.

No cytogenetic data are available for *S. malayense*. Intraspecific genetic divergence based on COI sequences ranges between 2.78% and 7.81%, with an average of 5.71% (Pramual and Adler, 2014).

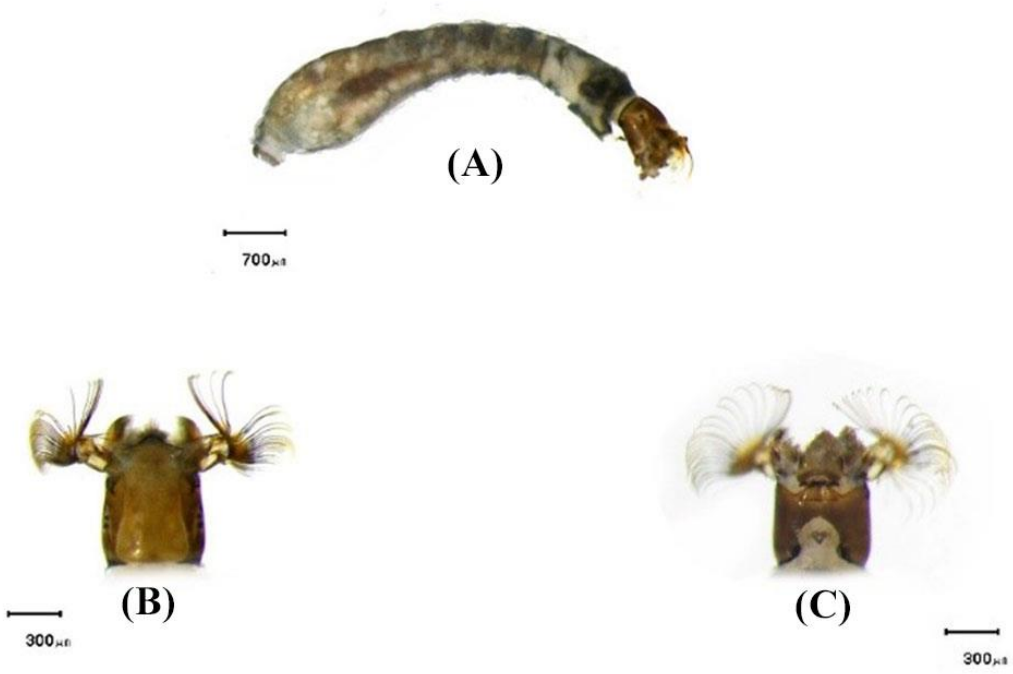

Figure 2.17 Larva of *Simulium malayense*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

2.8.7 *Simulium triglobus* Kuvangkadilok and Takaoka, 1999

Simulium triglobus was described from specimens collected from fallen leaves in Tontong waterfall, Nan Province. This species was collected in a small stream 0.5-1.0 m wide at an elevation of 500 m and 19℃ of water temperature. The female length of *S. triglobus* is 2.6 mm and the wings are approximately 2.5 mm. The antenna is composed of 2+9 segments. The female has a simple claw. The pupa (Figure 2.18) length is 2.8 mm. The integument of the head and thorax is dark yellow to light yellowish brown. The gill consists of eight short and slender filaments arranged in pairs. The cocoon is shoe-shaped with an anterior collar of moderate height without windows (Kuvangkadilok and Takaoka, 1999).

The body length of the larva of *Simulium triglobus* (Figure 2.17) is 5.4-6.0 mm and grayish brown or grayish black. The head capsule has a yellowish white cephalic apotome and is narrowly light brown along the posterior margin with indistinct head spots. The antenna is composed of three segments, plus an apical sensillum and it is longer than the stem of the labral fan. The labral fans have 56-60 main rays each and the mandible has comb-teeth. The hypostoma has an anterior row of nine teeth. The postgenal cleft is very large, rounded, and not constricted basally. The abdomen lacks dorsolateral protuberances. The accessory sclerite and ventral papillae are absent. The posterior circlet consists of 112-134 rows, each row including 22 hooklets (Kuvangkadilok and Takaoka, 1999).

Simulium triglobus is easily separated from the other known species of the same species-group by the cocoon of corbicular shape. The pupa also differs from those of *S. chaliowae* and *S. chainarongi*, described above, by the more branched thoracic trichomes, as well as the corbicular cocoon, and from *S. chainarongi* by the more slender gill filaments.

No cytogenetic data are available for *S. triglobus*. Intraspecific genetic divergence based on COI sequences ranges between 0% and 1.03 %, with an average of 0.29% (Pramual and Adler, 2014).

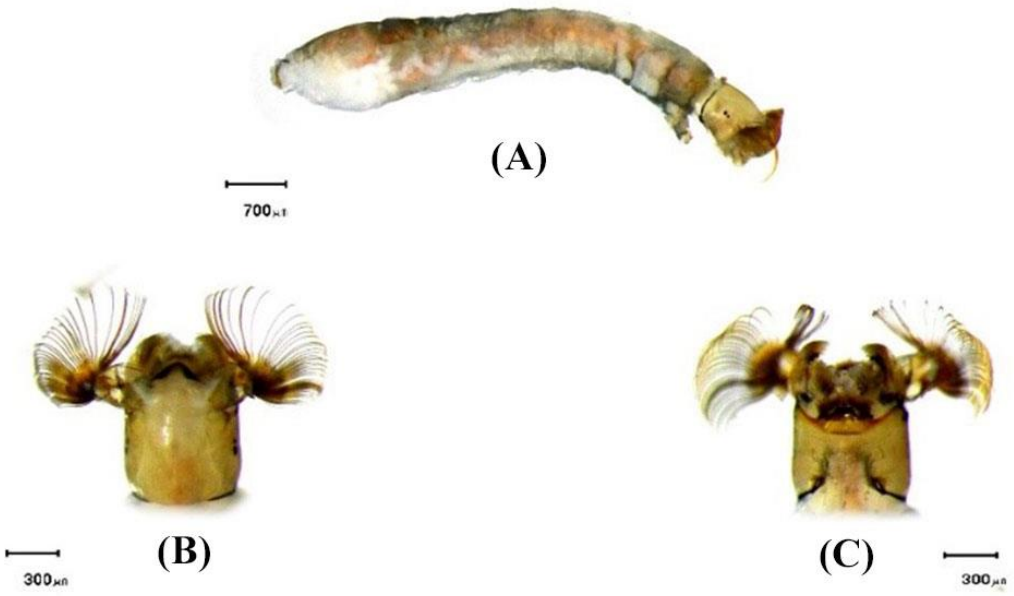

Figure 2.18 Larva of *Simulium triglobus*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

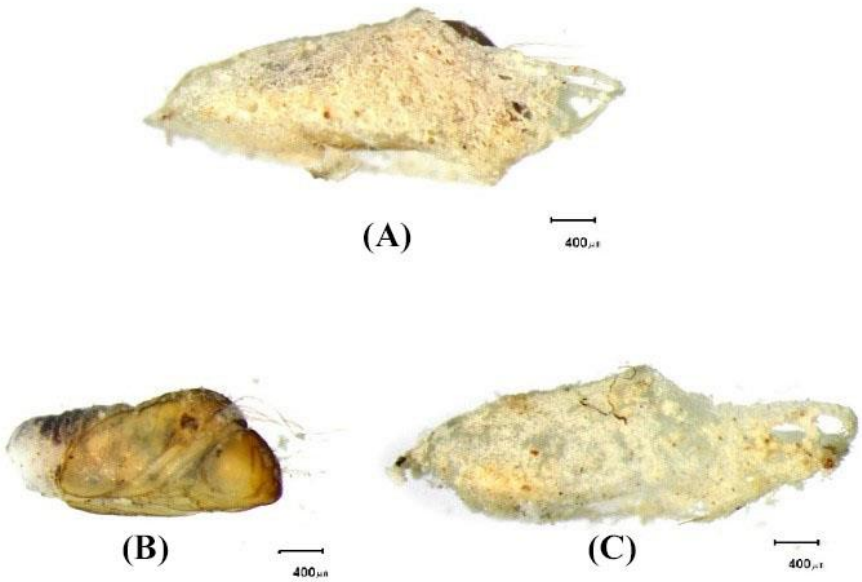

Figure 2.19 Pupa of *Simulium triglobus*. (A) Mature larva (lateral view), (B) Cephalic apotome (dorsal view), (C) Head capsule showing postgenal cleft (ventral view).

2.8.8 *Simulium chanyae* Takaoka and Choochote, 2007

Simulium chanyae was described only from the female. This species was collected while attracted to humans at Ban Mong Doi Pui Village, Doi Suthep-Pui National Park, Chiang Mai Province, and Northern Thailand at an elevation of 1,460 m above sea level. The male, pupa, and larva have not yet been reported. The female length of *S. chanyae* is 2.6 mm and the wings are approximately 2.4 mm. The antenna is composed of 2+9 segments. The female has a simple claw.

Simulium chanyae seems to be most closely related to *S. novolineatum* Puri from India but is distinguished from the latter species by the basitarsus of the

middle leg of *S. chanyae* being yellowish-white on the basal 2/3 but yellowish on the basal 1/2 in *S. novolineatum*.

2.8.9 *Simulium takense* Takaoka and Choochote, 2005

Simulium takense was described from male, female, and pupal specimens collected from Nang Kruan Waterfall, Tak Province. The larva of this species has not yet been reported. Pupa of this species occur in highly calcareous streams with high conductivity (Pramual and Nanork 2012). The female length of *S. takense* is 2.5-3.0 mm and the wings are approximately 2.3 mm. The antenna is composed of 2+9 segments. The female has a simple claw. The pupa of *S. takense* was collected on rocks in a waterfall. The gill has 8 short filaments in 4 widely divergent pairs. The cocoon is shoe-shape, with a high anterior collar, and is tightly woven. *Simulium takense* was found together with *S. prayongi*. This species is closely related to *S. chaliowae* Takaoka and Boonkemtong, *S. chainarongi* Takaoka and Kuvangkadilok, and *S. triglobus* Kuvangkadilok and Takaoka.

Simulium takense is distinguished from these 3 species by the paraproct in the female and ovipositor with several stout hairs, and in the pupa by the gill filaments being widely divergent and the presence of distinct spine-combs and terminal hooks on abdominal segment 9. The male of *S. takense* can be separated from that of *S. chaliowae* by the bicolored hind basitarsus and from that of *S. chainarongi* by the ventral plate with a concave posterior margin (Takaoka and Choochote, 2005b).

There is no cytogenetic data for *S. takense*. Intraspecific genetic divergence based on COI sequences ranges between 0.34% and 2.43 %, with an average of 1.16 % (Pramual and Adler, 2014).

Morphological characters of some developmental stages can be readily used to distinguish members of the *S. multistriatum* species group. However, larvae of this species group are morphologically highly similar. Therefore, it is necessary to use an integrated approach based on multiple characters including

morphology, cytology, molecular biology, and ecology for the taxonomy and systematics of the *S. multistriatum* group in Thailand.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Specimen collection and identification

Larvae of the *Simulium multistriatum* species group were collected by forceps from 31 stream sites in Thailand (Table 3.1, Figure 3.1) and one site each in Malaysia and Vietnam. Larvae and pupae were preserved in Carnoy's solution (1:3 glacial acetic acid: absolute ethanol) and stored at -20 °C until use. Ecological factors of the stream habitat including stream width, depth, velocity, pH, water temperature, and conductivity were measured. In addition, physical parameters including streambed particle size, riparian vegetation, and canopy cover were recorded following the method described in McCreadie *et al*. (2006) (Table 3.2-3.4). These physical parameters were visually estimated. Ranked measurements of the dominant substrate can be used in statistical analyses. Species were identified following keys and descriptions of Takaoka and Davies (1995), Takaoka and Kuvangkadilok (1999), Takaoka and Choochote (2004), Takaoka and Choochote (2005a), and Takaoka and Choochote (2005b).

Table 3.1 Collection sites for larvae of the *Simulium multistriatum* species group in Thailand.

Site	Location	Province	Elevation (m asl)	Latitude Longitude	Date	Larvae prepared (completely analyzed)	Taxon
Thailand							
270	Lert Pop waterfall	Loei	1,152	17º 30'N 101º 20'E	27 Nov 2013	17 (17)	*S. bullatum*
279	Phu Suan Sai	Loei	918	17º 30'N 101º 56'E	28 Nov 2013	9 (9)	*S. bullatum*
396	Lert Pop waterfall	Loei	1,123	17º 30'N 101º 20'E	20 Sep 2015	2 (2)	*S. bullatum*
						17 (12)	*S. fenestratum*
287	Na Ku Ha waterfall	Phrae	556	18º 07'N 100º 17'E	6 Dec 2013	71 (59)	*S. chaliowae*
294	Huai Ton Phueng waterfall	Phayao	361	19º 16'N 100º 53'E	7 Dec 2013	89 (69)	*S. chaliowae*
244	Pha Luang waterfall	Ubon Ratchathani	166	15º 36'N 105º 22'E	2 Dec 2013	43 (28)	*S. chainarongi*
253	Huai Sai Yai waterfall	Ubon Ratchathani	218	14º 55'N 105º 30'E	3 Dec 2013	18 (7)	*S. chainarongi*
254	Huai Luang waterfall	Ubon Ratchathani	240	14º 25'N 105º 24'E	3 Dec 2013	51 (29)	*S. chainarongi*
255	Kaeng Lam Duan waterfall	Ubon Ratchathani	179	14º 26'N 105º 06'E	3 Dec 2013	71 (57)	*S. chainarongi*
350	Wang Yai waterfall	Si Saket	219	14º 26'N 104º 29'E	16 Oct 2014	16(12)	*S. chainarongi*
298	Siribhumi waterfall	Chiang Mai	1,304	18º 32'N 98º 30'E	8 Dec 2013	9 (9)	*S. daoense*

***** IIIL-2 was not analyzed for all larvae; for each site, the number analyzed is given in parentheses: Site 264 (4), 268 (7), 275 (9), 276 (7), 280 (18), 401 (3), 297 (26), 320 (4), 342 (4), 434 (30). ****** Of 16 larvae analyzed from this collection, 14 were *S. fenestratum* and 2 were *S. malayense* A; larvae of *S. daoense* were not found on this date. Larvae of *S. fenestratum* were cytologically similar to those of 8 Dec 2013 and are not included in analysis.

Table 3.1 (Continued)

Site	Location	Province	Elevation (m)	Latitude Longitude	Date	Larvae prepared (completely analyzed)	Taxon
264	Piang Din waterfall	Loei	627	17º 03'N 101º 44'E	26 Dec 2013	16 (8*)	*S. fenestratum*
268	Song Khon waterfall	Loei	743	17º 21'N 101º 24'E	27 Dec 2013	14 (12*)	*S. fenestratum*
275	Than Sawan waterfall	Loei	506	17º 29'N 101º 3.5'E	28 Dec 2013	14 (10*)	*S. fenestratum*
276	Khring waterfall	Loei	643	17º 28'N 101º 58'E	28 Dec 2013	16 (8*)	*S. fenestratum*
280	Tad Huong waterfall	Loei	560	17º 33'N 100º 59'E	28 Dec 2013	29 (19*)	*S. fenestratum*
401	Huai Khamin Noy waterfall	Loei	1,208	16º 59'N 101º 00'E	26 Oct 2015	7 (4*)	*S. fenestratum*
363	Than Ngam waterfall	Udon Thani	278	17º 09'N 102º 44'E	7 Dec 2014	10 (3)	*S. fenestratum*
364	Koi Nang waterfall	Udon Thani	416	17º 07'N 102º 43'E	7 Dec 2014	26 (16)	*S. fenestratum*
297	Ban Pang Faen	Chiang Mai	615	19º 00'N 99º 18'E	7 Nov 2013	46 (29*)	*S. fenestratum*
372	Huai Mae Om Long	Chiang Mai	852	18º 09'N 98º 13'E	16 Jan 2015	23 (15)	*S. fenestratum*
384	Mae Taeng District	Chiang Mai	703	19º 07'N 98º 45'E	16 Jan 2015	11 (9)	*S. fenestratum*
402	Phu Soi Dao waterfall	Uttaradit	654	17º 44'N 100º 59'E	27 Oct 2015	23 (15)	*S. fenestratum*
320	Mae Sot	Tak	303	16º 48'N 98º 59'E	19 Feb 2014	7 (6*)	*S. fenestratum*

***** IIIL-2 was not analyzed for all larvae; for each site, the number analyzed is given in parentheses: Site 264 (4), 268 (7), 275 (9), 276 (7), 280 (18), 401 (3), 297 (26), 320 (4), 342 (4), 434 (30).****** Of 16 larvae analyzed from this collection, 14 were *S. fenestratum* and 2 were *S. malayense* A; larvae of *S. daoense* were not found on this date. Larvae of *S. fenestratum* were cytologically similar to those of 8 Dec 2013 and are not included in analysis.

Table 3.1 (Continued)

Site	Location	Province	Elevation (m)	Latitude Longitude	Date	Larvae prepared (completely analyzed)	Taxon
342	Klong Na Rai waterfall	Chan Tha Buri	43	12º 34'N 102º 10'E	5 Mar 2014	43 **(**30*)	*S. fenestratum*
343	Pliw waterfall	Chan Tha Buri	46	12º 31'N 102º 10'E	5 Mar 2014	66 **(**63*)	*S. fenestratum*
345	Klong Gaew waterfall	Trat	65	12º 37'N 102º 34'E	5 Mar 2014	7 **(**6)	*S. fenestratum*
447	Siribhumi waterfall	Chiang Mai	1,304	18º 32'N 98º 30'E	13 Oct 2017	14 **(**7)**	*S. fenestratum*
408	Wang Kaew waterfall	Lampang	626	19º 18'N 99º 39'E	18 Dec 2015	26 **(**19)	*S. lampangense*
409	Wang Thong waterfall	Lampang	510	19º 16'N 99º 39'E	18 Dec 2015	27 **(**23)	*S. lampangense*
298	Siribhumi waterfall	Chiang Mai	1,304	18º 32'N 98º 30'E	8 Dec 2013	7 **(**5)	*S. malayense* A
						1 (1)	*S. malayense* B
					13 Oct 2017	2 (2)**	*S. malayense* A
291	Ton Tong waterfall	Nan	1,027	19º 12'N 101º 04'E	6 Dec 2013	43 (28)	*S. triglobus*
Malaysia							
MYC	Chenderiang waterfall	Perak	129	04º 21'N 101º 14'E	23 Feb 2015	11 (6)	*S. malayense* C
Vietnam							
Dao	Sapa	Lao Cai	1,315	22˚23'N 103˚50'E	22 Dec 2014	3 (3)	*S. daoense*

***** IIIL-2 was not analyzed for all larvae; for each site, the number analyzed is given in parentheses: Site 264 (4), 268 (7), 275 (9), 276 (7), 280 (18), 401 (3), 297 (26), 320 (4), 342 (4), 434 (30). ****** Of 16 larvae analyzed from this collection, 14 were *S. fenestratum* and 2 were *S. malayense* A; larvae of *S. daoense* were not found on this date. Larvae of *S. fenestratum* were cytologically similar to those of 8 Dec 2013 and are not included in analysis.

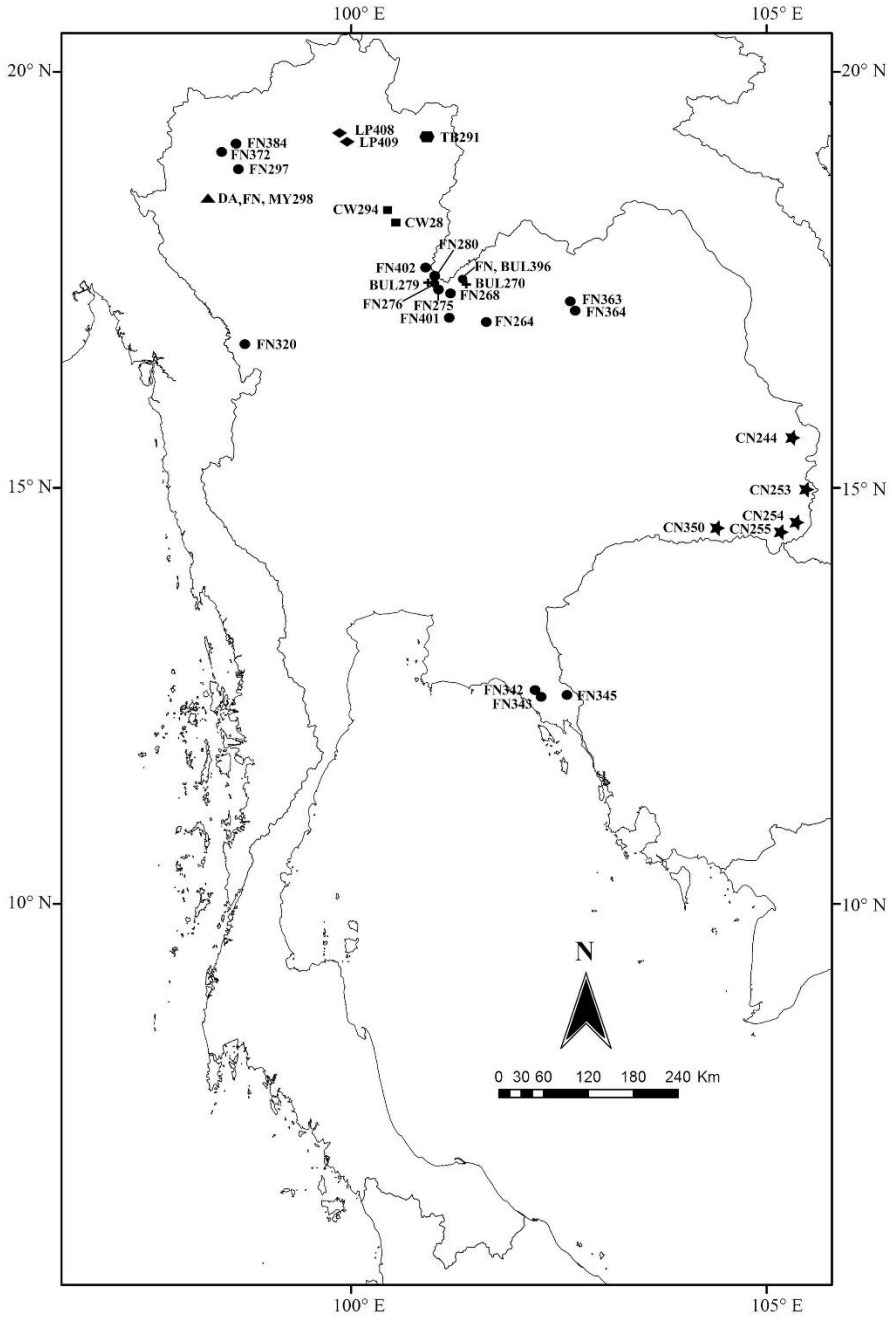

Figure 3.1 Sampling sites for eight nominal species of *Simulium multistriatum* species group in Thailand. BUL= *Simulium bullatum*, CN= *Simulium chainarongi*, CW= *Simulium chaliowae*, DA= *Simulium daoense*, FN= *Simulium fenestratum*, LP= *Simulium lampangense*, MY= *Simulium malayense*, TB= *Simulium triglobus*. Details for code numbers are shown in Table 3.1

3.2 Cytological study

The last or penultimate-instar larvae were used for salivary gland polytene chromosome preparation. Polytene chromosomes were prepared using the Feulgen method of Rothfels and Dunbar (1953). The head and thorax were cut off and transferred from Carnoy's fixative to 80% ethanol and stored at -20 °C for molecular study.

The larvae were slit open on the posteroventral portion of the abdomen in fixative using dissecting needles and placed in a vial of distilled water for 20 minutes at room temperature. The water was removed from the vial by a pipette dropper; 5N HCL was added to replace the water for 30 minutes at room temperature. The larva was then transfered to a vial containing Feulgen stain for one hour after which the Feulgen was discarded and replaced with sulfur water for 10 minutes. The sulfur water was then removed and after being washed twice with cold tap water, the larva was ready for chromosome preparation. One drop of 50% acetic acid was placed on the middle of a microscope slide and the larva was placed in a drop of cold water at one side of the slide. The needles were used to pull out the salivary gland from the posterior portion of the abdomen and place it in the 50% acetic acid. All large pieces of tissues were removed and a cover slip applied and pressed with the thumb. The polytene chromosomes that were well spread were used for cytotaxonomy.

3.3 Molecular study

Genomic DNA was extracted using Vivantis GF-1 Nucleic Extraction kit. Polymerase chain reaction (PCR) was used to amplify a fragment of cytochrome *c* oxidase subunit I (COI) gene using primers LCO1490 (5′ GGTCAACAAATCATA AAG ATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*., 1994). PCR condition for COI gene were 2 min at 94 °C, 1 min at 45 °C, and 45 s at 72 °C, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 45s, extension at 72 °C for 45s, and a final extension at 72 °C for 4 min (Rivera and Currie, 2009). Cytochrome c oxidase subunit II (COII) was amplified using the primers TL2-J-

3034 (5′-ATTATGGCAGATTAGTGCA-3′) and TK-N-3785 (5′-GTTTAAGAGACCAGTACTTG-3′) (Conflitti *et al*., 2010). Polymerase chain reaction (PCR) conditions was used 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 45 °C for 60s, extension at 72 °C for 60s, and a final extension at 72 °C for 7 min (Conflitti *et al.*, 2010).

PCR products were checked by 1% agarose gel electrophoresis and purified using a High Yield Gel/ PCR DNA Fragment Extraction kit (RBC BioScience, Taiwan). Sequencing will be performed at the Macrogen DNA sequencing service (Seoul, Korea) using the same primers as in the PCR.

3.4 Data analysis

3.4.1 Cytological analysis

Representative chromosome preparations were photographed under oil immersion on an Olympus BX40 microscope (*Simulium daoense*) or a BH-2 Olympus microscope with a Jenoptik ProgRes® SpeedXT Core 5 digital camera (all other taxa). The images were imported into Adobe® PhotoShop® Elements 8 or PhotoShopCS6 to construct chromosomal maps. The standard map for the *S. multistriatum* species group was based on the most central sequence among the group members, relative to the standard sequence identified by Rothfels *et al.* (1978) and Adler *et al.* (2016a) for the subgenus *Simulium*.

Chromosomal terminology and section numbering for the three chromosomes (I, II and III) followed the practice of Rothfels *et al*. (1978) and Adler *et al.* (2016a). Fixed inversions are italicized in the text and underlined on the chromosome maps; polymorphic rearrangements are given in regular type. Additional rearrangements are coded in the text and on the maps as follows: heteroband (hb), diffuse centromere band (d); thickened of heteroband in centromere region (CII), additional (insertion) band (i), flocculent band expression (fl), and band enhancement (+).The following centromere associations were recognized: i) chromocenter—all centromeres of all chromosomes in each nucleus are attached to a central mass of heterochromatin, ii) partial chromocenter—the centromeres of

any two chromosomes of each nucleus are attached to a heterochromatic mass, and iii) ectopic pairing (pseudochromocenter)—two or more centromeres are associated with one another in at least some, but typically not all, nuclei; additional heterochromatin is absent from the centromere associations (Adler *et al.*, 2004).

The maximum width of the terminal flare for each chromosome arm of *S. bullatum*, *S. fenestratum*, and *S. triglobus* was measured against the width of a subterminal indicator band, and grouped into size categories as follows: 0 (< width of the indicator band), 1 (≤ 1.5 times width of the indicator band, 2 (>1.5 to ≤ 2.0 times width of the indicator band, and 3 (> 2.0 times width of the indicator band). A Mann-Whitney U test was used to evaluate ranked characteristics. Polymorphic inversions were tested for Hardy-Weinberg equilibrium when samples were large enough to provide at least five larvae for each state (homozygous standard, heterozygous, and homozygous inverted).

3.4.2 DNA sequence analysis

A total of 69 sequences from ten cytologically distinct taxa of the *S. multistriatum* species group was included in the analyses (GenBank accession numbers: MG733997 – MG734134). Genetic distances were calculated using MEGA7 (Tamura *et al*., 2013), based on the Kimura 2-parameter (K2P). Phylogenetic analyses were conducted separately for the COI and COII sequences and for the combined dataset, and included neighbor-joining (NJ), maximum likelihood (ML), maximum parsimony (MP), and Bayesian methods. The NJ method inferred phylogenetic trees in MEGA 7 (Tamura *et al*., 2013), and MP was calculated separately for each gene in PAUP* v.4.10b (Swofford, 2002). Branch support for NJ and MP was calculated using the bootstrapping method with 1000 replicates. Maximum likelihood (ML) was performed with PhyML 3.0 (Guindon *et al*., 2010). Node support was determined using an approximate likelihood-ratio test (Anisimova & Gascuel 2006; Guindon *et al.*, 2010).

Bayesian inference was performed with MrBayes 3.04b and run for 2,000,000 generations with a sampling frequency of 100 generations (Huelsenbeck & Ronquist, 2001). *Simulium nodosum*, a member of the *S. nobile* species group, was used as an

outgroup in all molecular phylogenetic analyses. Efficiency of specimen identification was obtained using the best match method in TaxonDNA (Meier *et al.*, 2006).

CHAPTER 4

RESULTS

4.1 Cytogenetics

4.1.1 *Simulium multistriatum* species group: standard map

The chromosomes of 898 larvae of the *Simulium multistriatum* species group were prepared, and the banding patterns of 74.1% (655) of the larvae were analyzed completely (Table 3.1), with the exception of the IIIL-2 sequence of *S. fenestratum*, for which 184 (67.6%) of 272 otherwise fully evaluated individuals were analyzed.

All larvae of the *Simulium multistriatum* species group had three pairs of tightly synapsed homologues ($2n = 6$). The nucleolar organizer (NO) was in the base of IS, near the expanded centromere region. The sex chromosomes of all species were microscopically undifferentiated.

The standard banding sequence of the *S. multistriatum* species group differed from the *Simulium* subgeneric standard by having the nucleolar organizer in the base of IS (rather than in IIIL) and by 7 fixed inversions, as follows:

IS: The basic sequence of this arm was identical to the subgeneric standard sequence of Rothfels *et al.* (1978).

IL: The standard sequence for the *S. multistriatum* species group was identical to the subgeneric standard of Rothfels *et al.* (1978).

IIS: The IIS sequence for all members of the *S. multistriatum* species group was removed from the subgeneric standard of Adler *et al*. (2016) by three fixed inversions. These three inversions divided the arm into six fragments. The most parsimonious reassemblage of fragments to produce the subgeneric standard, one inversion at a time, is shown below, and the order of the fragments is represented by the letters a to j (Figure 4.6), where slashes represent inversion breakpoints and brackets represent the inversion in each sequence.

Inversion 2: a / g f / b c [i h / e d] j (= *Simulium multistriatum* species group standard)

Inversion3: a / g f / [b cd e] hij

Inversion 1: a [g fe dc b] hij

a b c d e f g h i j (= *Simulium* subgeneric standard)

IIL: All members of the *S. multistriatum* species group could be derived by one fixed inversion (*IIL-1*; Figure 4.7) from the subgeneric standard sequence of Rothfels *et al.* (1978).

IIIS: The basic sequence of this arm (Figure 4.12) was identical with the subgeneric standard sequence of Rothfels et al (1978).

IIIL: The basic IIIL sequence for the *S. multistriatum* species group was removed from the standard sequence of Adler *et al.* (2016) by three inversions (*IIIL-3*, *IIIL-5*, and *IIIL-6*) (Figure 4.10).

4.1.2 Cytogenetics of *Simulium bullatum* Takaoka and Choochote

Three populations of *S. bullatum* from Loei Province were studied, and all 28 larvae prepared for analysis were analyzed completely (Table 3.1). *Simulium bullatum* differed from the standard for the *S. multistriatum* species group by one fixed inversion (*IIIL-10*) (Figure 4.14) and a true chromocenter characterized by a glassy or darkly staining heterochromatic mass (Figure 4.1). Flaring at the end of IIIS was 3.0 times greater than the width of the indicator band, whereas the ends of IS, IL, IIS, IIL, and IIIL were ≤ 1.5 times the width of the respective indicator bands (*cf.* Figure 4.16B). Autosomal polymorphisms were not found.

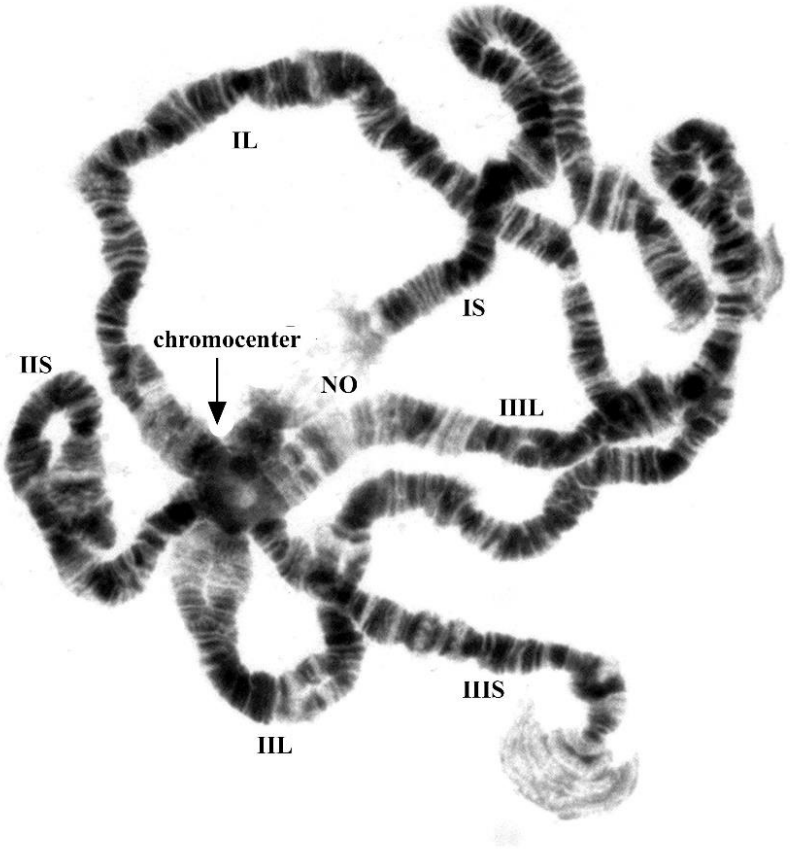

Figure 4.1 Complete chromosomal complement of *Simulium bullatum* (male larva) showing all centomeres attached to a chromocenter, NO = nucleolar organizer.

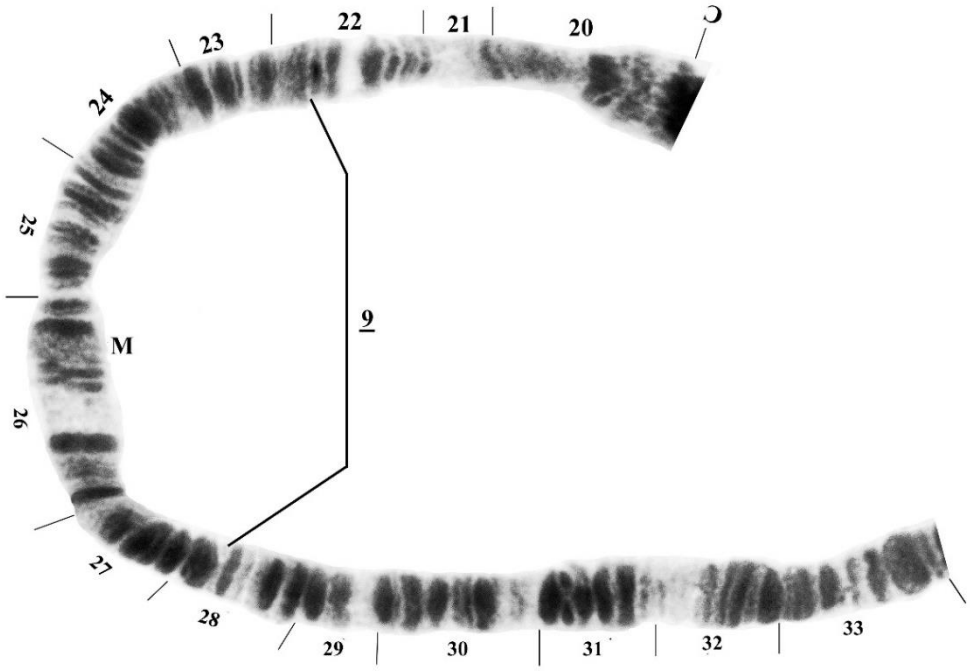

Figure 4.2 IL arm of *Simulium bullatum* (female larva), corresponding to the *Simulium* subgeneric standard of Rothfels *et al.* (1978) and the standard IL sequence of the *Simulium multistriatum* species group. Limits of fixed inversion *IL-9* are indicated with brackets. C = centromere, M = marker.

4.1.3 Cytogenetics of *Simulium chainarongi* Takaoka and Kuvangkadilok

Simulium chainarongi was found only in northeastern Thailand. A total of 133 (66.8%) larvae of 199 prepared from five populations was analyzed completely. The banding pattern of *S. chainarongi* differed by two inversion (*IL-9*, *IIIL-4*) (Figure 4.2, 4.10C) from the group standard. Its banding pattern did not, however, differ from that of four other group members (*S. chaliowae*, *S. fenestratum*, *S. lampangense*, and *S. triglobus*), although its centromeric characteristics were unique. The centromere bands of all chromosomes were typically more darkly stained and well defined than in any other group members (Figure 4.3A). One autosomal inversion (IIIS-1) was found heterozygously (Figure 4.13) in one male. Four band polymorphisms were found heterozygously in the centromere region of chromosome II: a diffuse centromere band in one male, a band

enhancement in the base of IIL (4 males and 1 female), a heterochromatic insert in the base of IIS (eight males and two females), and an expanded and flocculent IIL base in one male (Table 4.1, Figure 4.4).

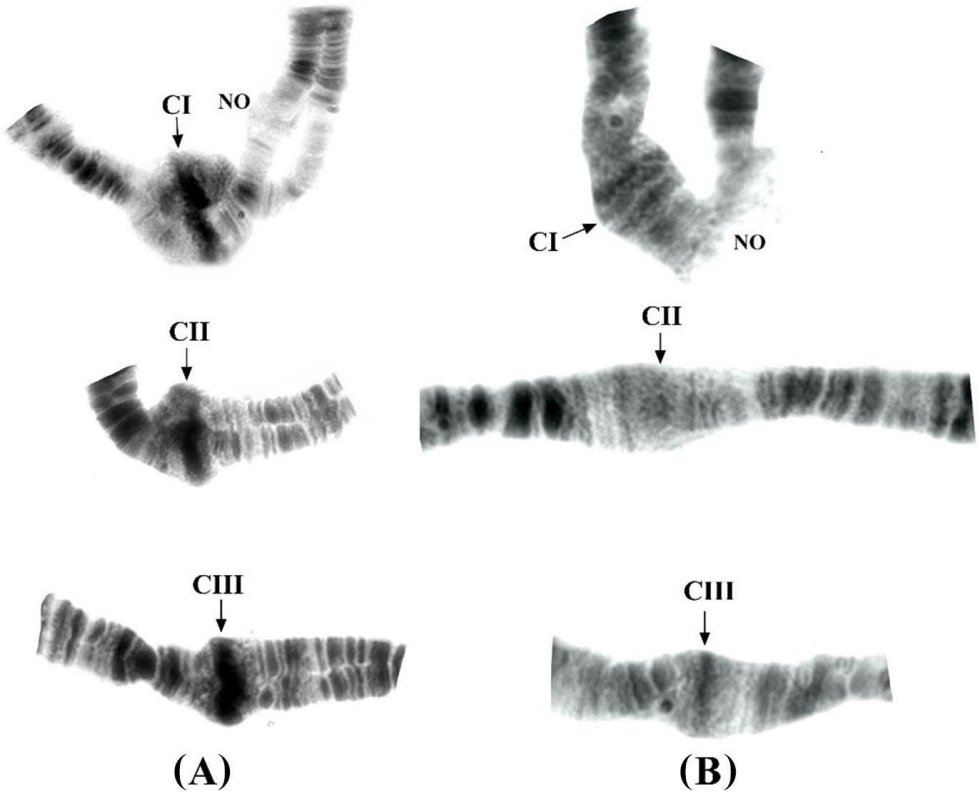

Figure 4.3 Characteristics of centromere regions. (A) *Simulium chainarongi* (B) *Simulium fenestratum*. C = centromere, NO = nucleolar organizer.

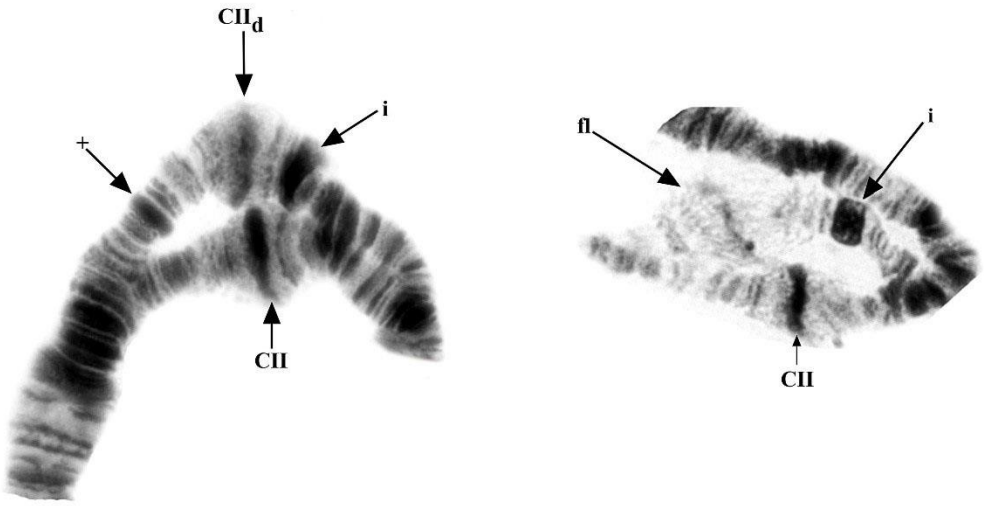

Figure 4.4 Basal rearrangements in chromosome II of *Simulium chainarongi*. CII = typical centromere band, CII_d = diffuse centromere band, i = heterochromatic insert in the base of IIS, + = band enhancement in the base of IIL, fl = expanded and flocculent IIL centromere region.

4.1.4 Cytogenetics of *Simulium chaliowae* Takaoka and Boonkemtong

Simulium chaliowae was found only in limestone streams in northern Thailand. The chromosomes of 128 (80%) of 160 prepared larvae from two sites were analyzed completely. This species differed from the *S. multistriatum* species group standard by three fixed inversions (*IL-9*, *IIIL-2*, and *IIIL-4*). Two floating inversions were found: IS-1 (Figure 4.9) and IIIL-8 (Figure 4.10), each in a separate female (frequency = 0.01). The "2 blocks" marker in IS (section 4) was found in two configurations, either standard (Figure 4.5) or with the first heavy block divided by a gap (Figure 4.5). Based on 10 larvae per location (10 nuclei per larva), an average of 58% of nuclei per larva from site 287 and 57% of nuclei per larva from site 294 had the first block divided by an unstained gap.

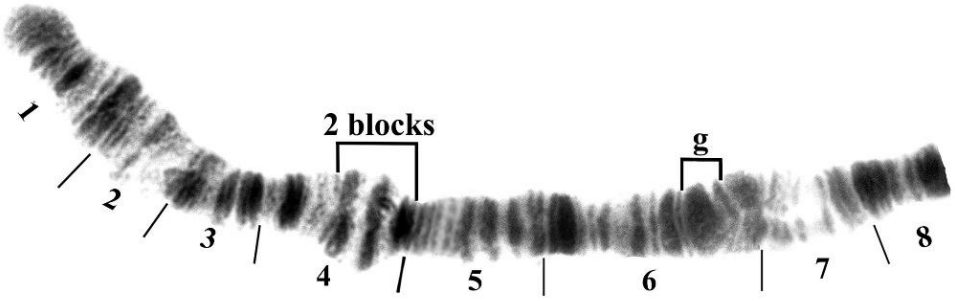

Figure 4.5. IS (sections 1 to 8) arm of *Simulium chaliowae* (male larva); g, glazed.

4.1.5 Cytogenetics of *Simulium daoense* Takaoka & Adler

All 9 larvae of *S. daoense* from Siribhumi Waterfall, Chiang Mai Province (site 298) were analyzed completely. This species differed from the *S. multistriatum* species group standard by having fixed inversions *IL-9* and *IIIL-4* (Figure 4.2, 4.10C) and *IIIL-1* (Figure 4.8). Autosomal polymorphisms were not found. The identity of this species was confirmed by chromosomal analysis of two larvae collected from the type locality of *S. daoense* at the same time that the type series was collected by Takaoka *et al*. (2017).

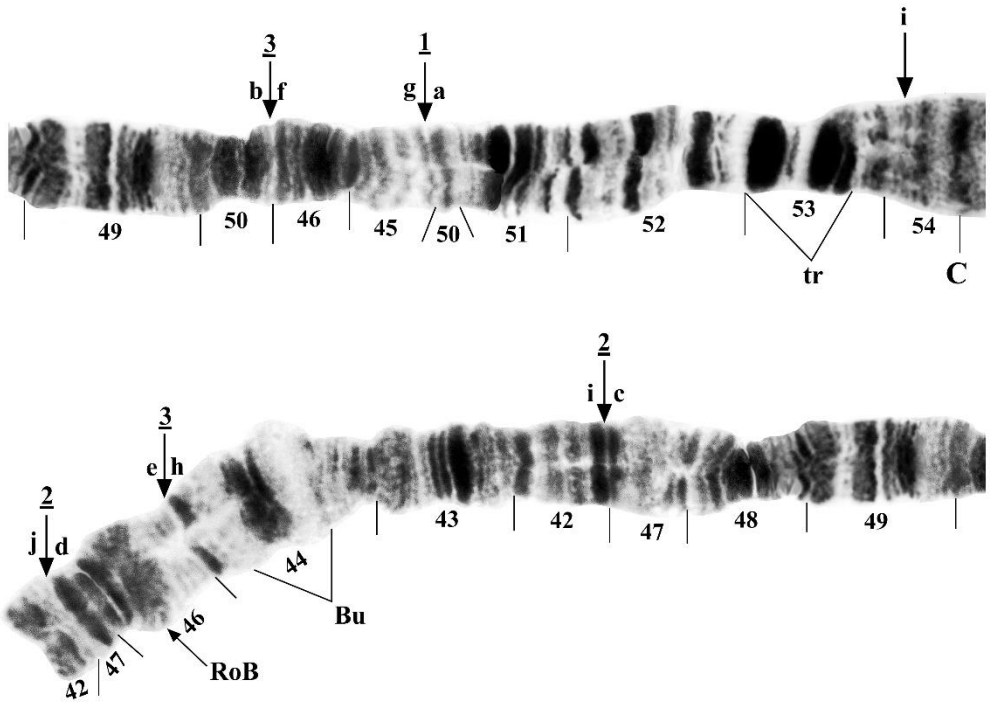

Figure 4.6 IIS arm of *Simulium daoense* (male larva), showing the standard sequence for the *Simulium multistriatum* species group relative to the *Simulium* subgeneric standard of Adler *et al*. (2016a). Breakpoints of three fixed inversions are indicated by numbered arrows 1–3. The letters a to j, when alphabetized, produce the subgeneric standard sequence. Fl = expanded and flocculent IIL base, + = location of band enhancement in *Simulium chainarongi.* C = centromere.

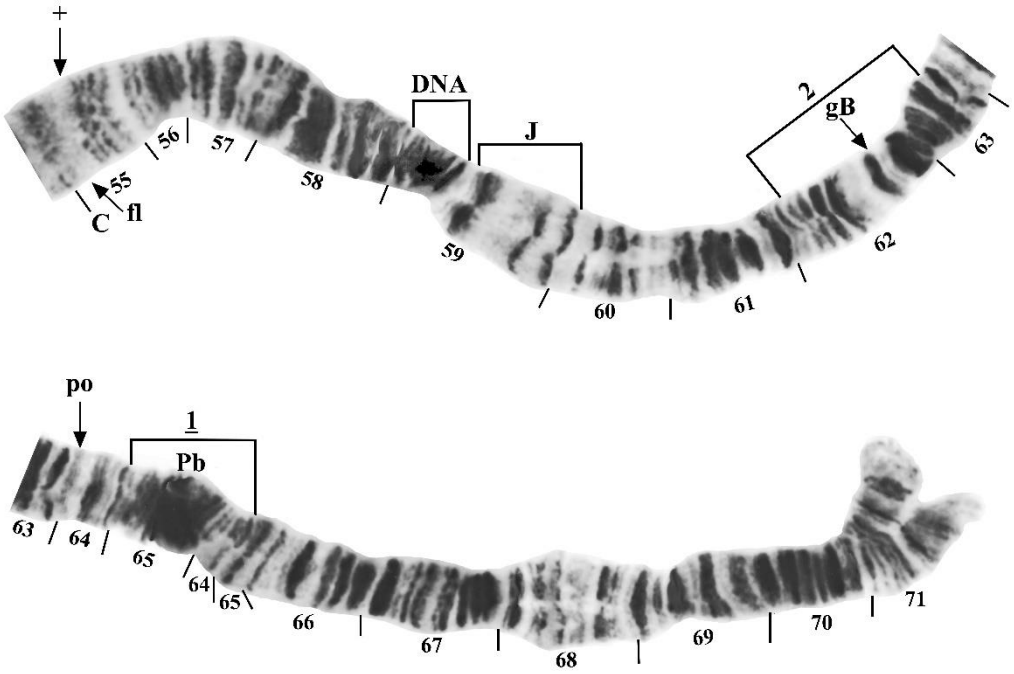

Figure 4.7 IIL arm of *Simulium daoense* (male larva), showing the standard sequence for the *Simulium multistriatum* species group, including fixed inversion *IIL-1*. Breakpoints of floating inversion IIL-2 of *Simulium fenestratum* are indicated by a bracket. C = centromere, gB = gray, J= jagged, band, Pb = parabalbiani, po = polar.

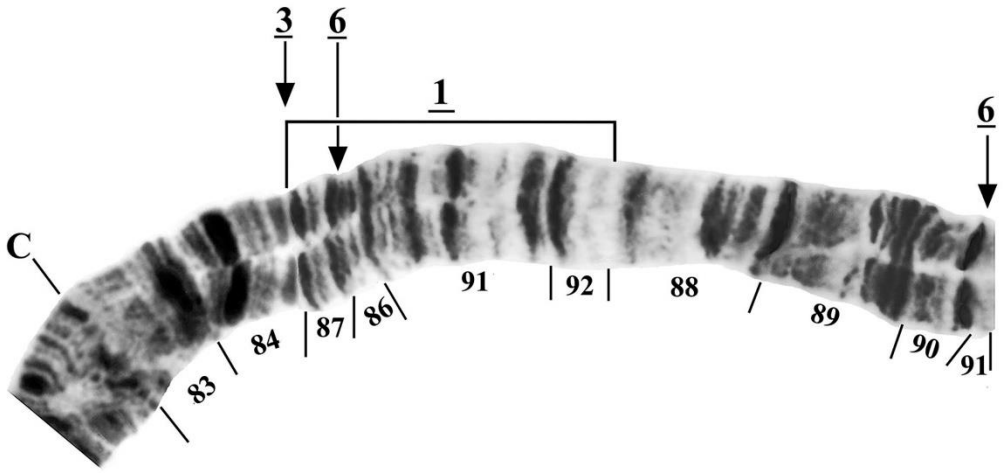

Figure 4.8 IIIL arm (sections 83 to 91) of *Simulium daoense* (male larva) showing the *IIIL-1* sequence on top of *IIIL-3, 6.*

4.1.6 Cytogenetics of *Simulium fenestratum* Edwards

This species was the most common and widely distributed member of the *Simulium multistriatum* species group in Thailand. The chromosomes of 272 (71.2%) of 382 prepared larvae of *S. fenestratum* were analyzed completely, except for inversion IIIL-2, which was evaluated in 67.6% of the 272 fully analyzed larvae (Table 4.2, footnote). The banding sequence differed from the group standard by two fixed inversion (*IL-9*, *IIIL-4*). The centromere bands of all chromosomes were diffuse and weakly stained (Figure 4.3B). *Simulium fenestratum* had 12 autosomal floating inversions (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IIL-2, IIIL-2, IIIL-7, and an unresolved compound inversion in IIIL) (Table 4.2; Figure 4.10, 4.11), plus one band insert in the expanded centromere region of chromosome I (IL 20i) and a heteroband (IL 21hb) in one female (Figure 4.11)

All floating inversions were in low frequency (typically < 0.10; 0.17 for IL-6 in one small sample of 3 larvae), except IIIL-2 (Figure 4.12), which varied in frequency with location (Table 4.2). IIIL-2 was found in all populations, except two in Chan Tha Buri province (sites 342 and 343). All specimens from Loei province, Udon Thani province, and

one site (402) in Uttaradit province were homozygous for IIIL-2, whereas other populations varied in frequency from 0.07 to 0.66. One population (Chiang Mai Province, site 297) large enough for statistical evaluation was in Hardy-Weinberg equilibrium for IIIL-2 (χ^2 = 0, P > 0.05). A small sample of six larvae from Trat province (site 345) had a deficiency of heterozygotes: 4 homozygous inverted (3 female: 1 male), 1 homozygous standard larva (male), and one heterozygote (male).

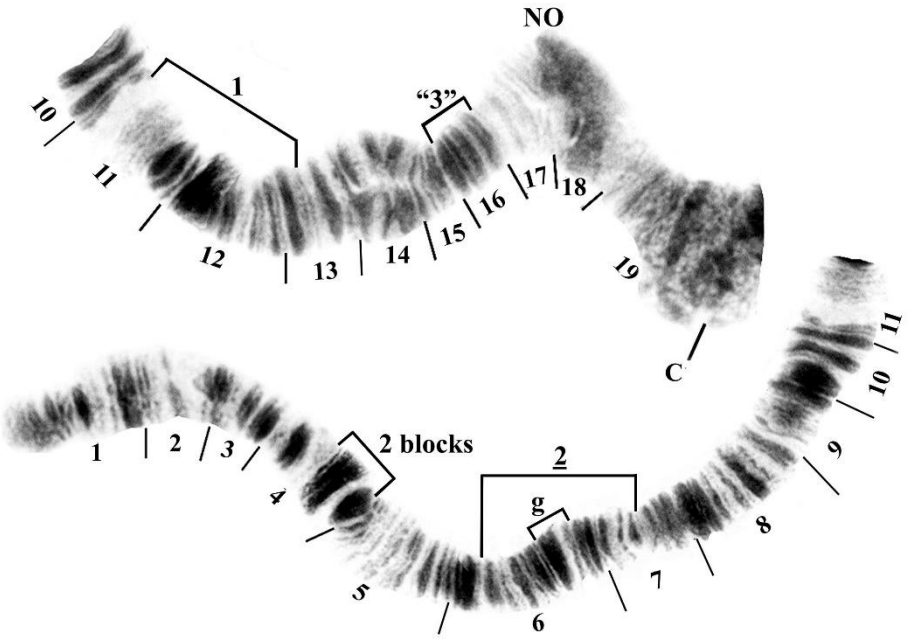

Figure 4.9 IS arm of *Simulium fenestratum* (female larva), corresponding to the *Simulium* subgeneric standard of Rothfels et al. (1978). Breakpoints of floating inversion IS-1 and fixed inversion *IS-2* are indicated. C = centromere, g = glazed, NO = nucleolar organizer.

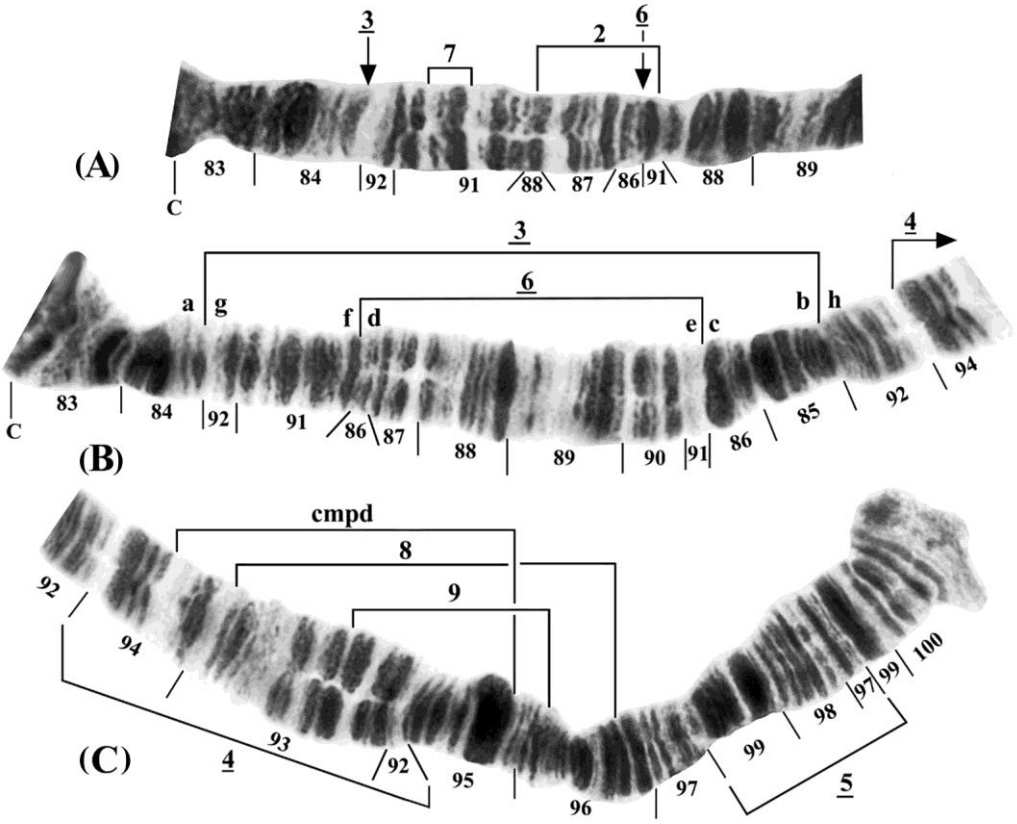

Figure 4.10 A, Basal sections (female larva, site 402) showing the IIIL-2, *IIIL-3*, *6* sequence; breakpoints of floating inversion IIIL-7 are indicated by a bracket. B, C. Entire arm (female larva, site 343), showing the *IIIL-3*, *4*, *5*, *6* sequence; ordering the letters a–h produces the *Simulium* subgeneric sequence of Adler et al. (2016a). Breakpoints of three floating inversions (IIIL-8, IIIL-9, and compound (cmpd) inversion) are indicated by brackets. C, centromere.

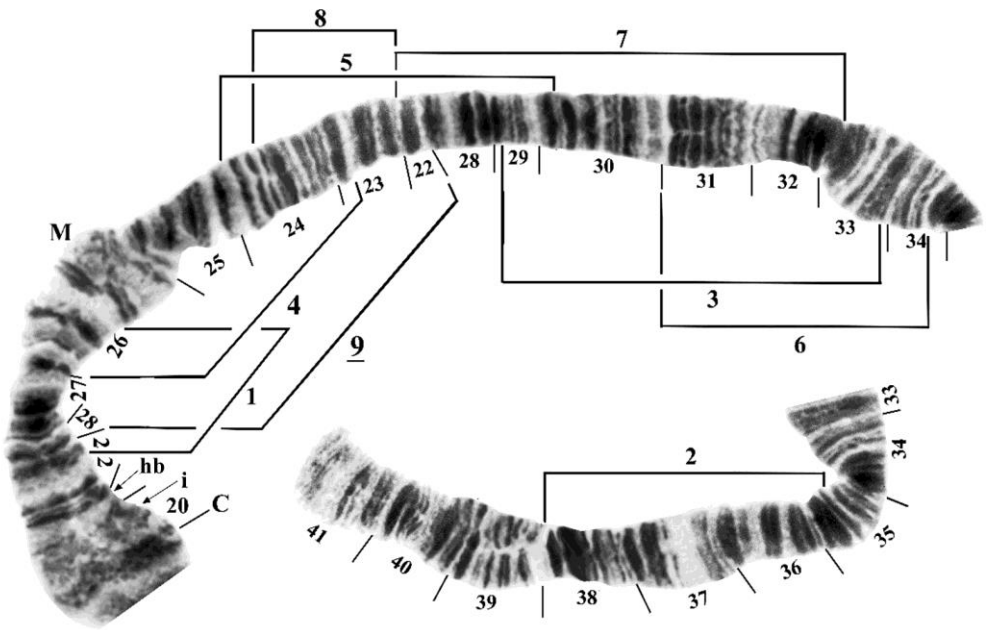

Figure 4.11 IL arm of *Simulium multistriatum* species group. *Simulium daoense* (male larva, sections 20–32) and *Simulium fenestratum* (female, sections 33–41), showing the *IL-9* sequence. Breakpoints of floating inversions of *Simulium fenestratum*. Arrows indicate location of a heterochromatic band insertion in the *S. fenestratum*, hb = heteroband, i = heterochromatic insert, M = marker.

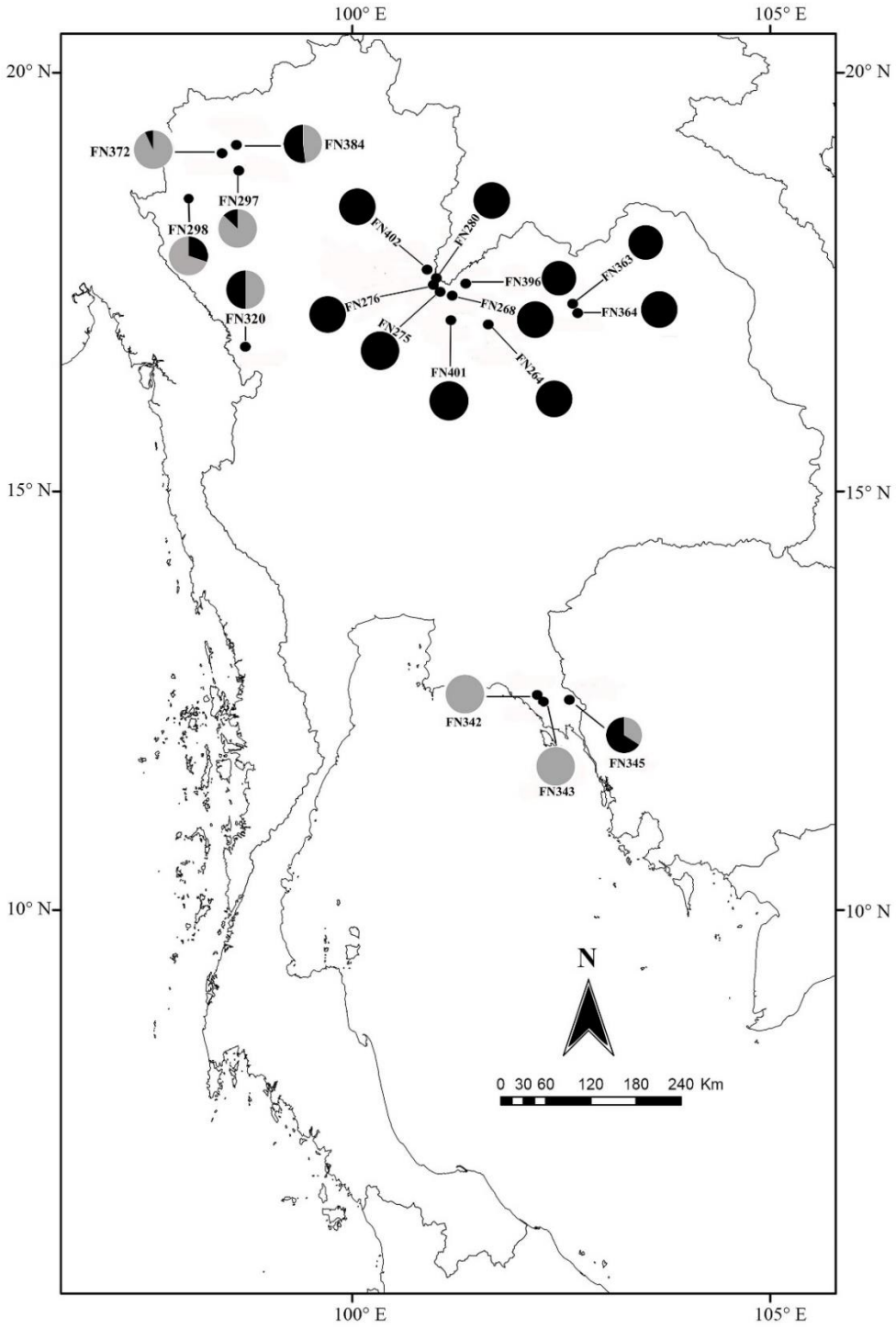

Figure 4.12 Frequency of IIIL-2 inversion (black = inverted, gray = standard) in 18 populations of *Simulium fenestratum* in Thailand. Details for code numbers are shown in Table 3.1

4.1.7 Cytogenetics of *Simulium lampangense* Takaoka and Choochote

Simulium lampangense was restricted to limestone streams in Northern Thailand. Of 53 larvae from two populations (Wang kaew Waterfall, WangThong Waterfall) in Lampang Province, 42 (79.2%) were analyzed completely. The banding pattern of this species differed from that of the *S. multistriatum* species group standard by two fixed inversions (*IL-9* and *IIIL-2*). The banding pattern of *S. lampangense* differed from that of *S. fenestratum* only by the "2 blocks" marker. Based on 9 larvae per location (7 to 18 nuclei per larva), an average of 46.7% of nuclei in larvae from Wang Kaew waterfall (site 408) and 30.1% from Wang Thong waterfall (site 409) had the "2 blocks" marker with an unstained gap in the first block—the same condition as in *S. chaliowae*. Presence of the gap was positively related to the degree of polytenization. Thus, larger larvae, which exhibited a greater degree of polytenization had a higher proportion of the "2 blocks" marker with an unstained gap (Figure 4.5). The chromosomal banding pattern of *S. lampangense* was, therefore, identical to that of *S. chaliowae* in all respects.

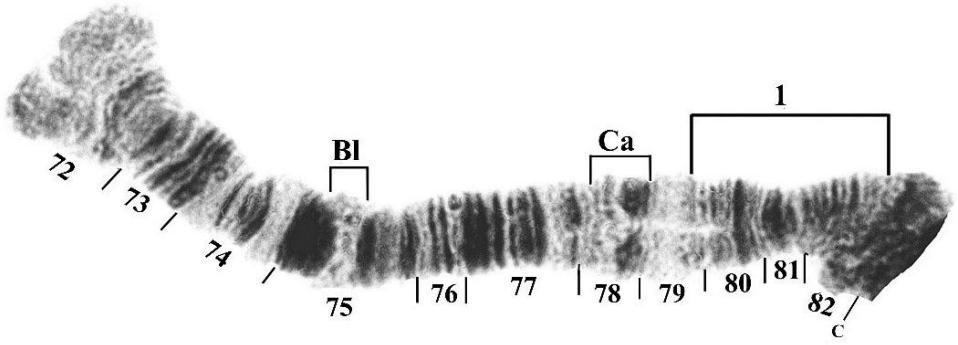

Figure 4.13 IIIS arm of *Simulium lampangense* (male larva), representing the standard sequence for the *S. multistriatum* species group, which is identical to the *Simulium* subgeneric standard sequence of Rothfels et al. (1978). Breakpoints of floating inversion IIIS-1 of *Simulium chainarongi* are shown. Bl= blister, Ca= capsule, C = centromere.

4.1.8 Cytogenetics of *Simulium malayense* Takaoka and Davies

A total of 12 specimens of *S. malayense* were analyzed completely: 8 (80.0%) of 10 larvae from Thailand and 6 (54.5%) of 11 larvae from Malaysia. The banding pattern of *S. malayense* was distinguished from that of the *S. multistriatum* species group standard by one fixed inversion (*IIIL-10*) (Figure 4.14). Three cytoforms were found in *S. malayense*. Cytoforms A (7 larvae) and B (1 larva) were found sympatrically in Thailand and cytoform C (6 larvae) was collected in Malaysia. Cytoform A was uniquely characterized by *IS-2* (Figure 4.9) and ectopic pairing of CI and CIII in 1% to 81% of nuclei per larva. Cytoform B, consisting of only one male larva, expressed ectopic pairing that involved all combinations of the three centromere bands. It was collected in the same sample as cytoform A but was homozygous standard for IS. Cytoform C was characterized by a partial chromocenter involving CI and CII (Figure 4.15). CIII was well defined and darkly staining, but did not participate in the chromocentric association.

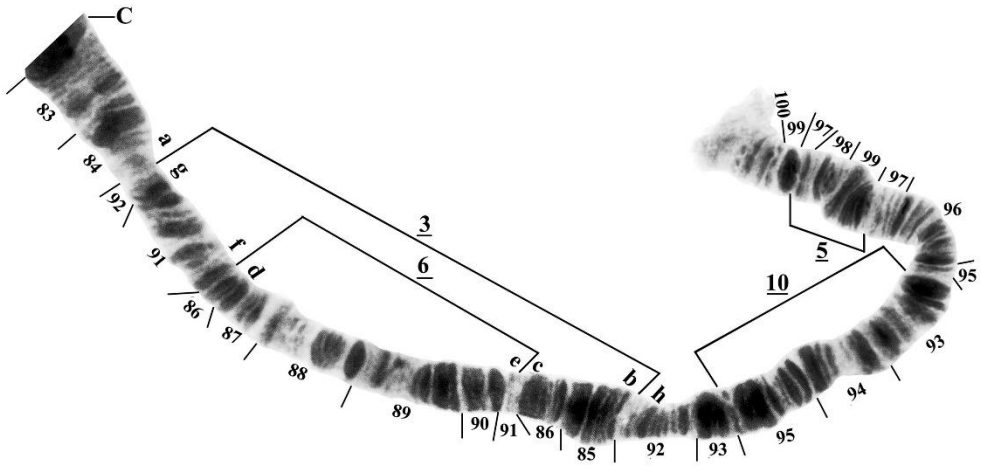

Figure 4.14 IIIL of *Simulium malayense* cytoform B (female larva) showing the *IIIL-3, 5, 6, 10* sequence; ordering the letters a–h produces the *Simulium* subgeneric sequence.

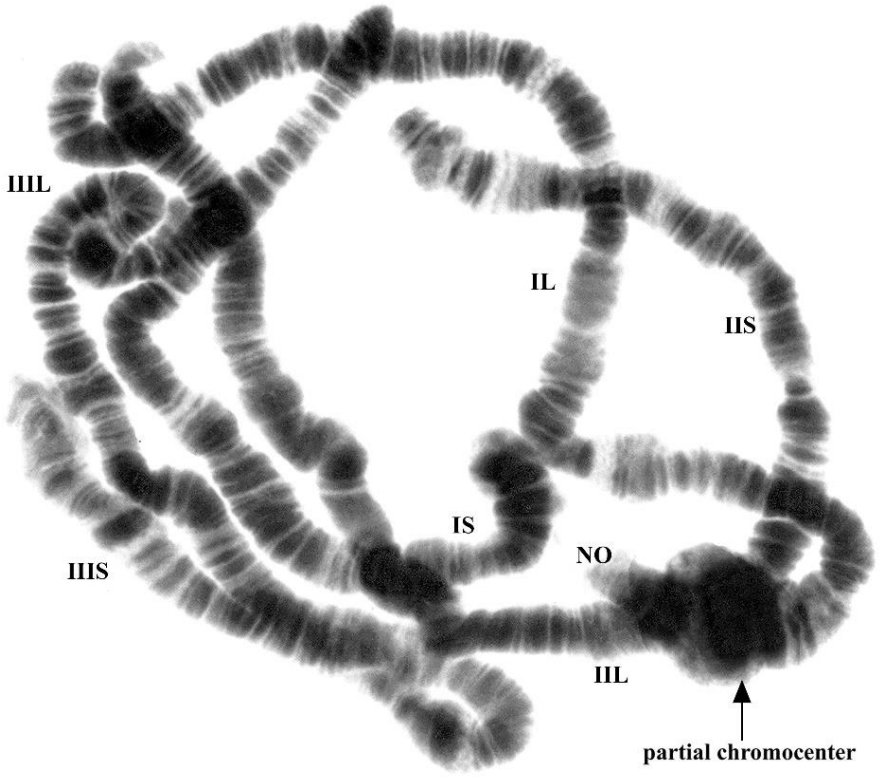

Figure 4.15 Complete chromosomal complement of *Simulium malayense* cytoform B (female larva) showing partial chromocenter (CI + CII), NO = nucleolar organizer.

4.1.9 Cytogenetics of *Simulium triglobus* Kuvangkadilok and Takaoka

This species was found at only one site, that the type locality in Nan Province. A total of 28 (65.1%) of 43 larvae were analyzed completely. All larvae had two fixed inversion (*IL-9, IIIL-4*), but otherwise had the standard banding sequence for the group. However, the degree of flaring of the ends of chromosome arms IL, IIL, IIIL, and IIIS was significantly greater for *S. triglobus* (14 larvae, 4–10 nuclei per larva) than for *S. fenestratum* (13 larvae, 4–10 nuclei per larva), which represented the standard flaring condition for the group (Mann-Whitney U test, $P < 0.01$, $df = 25$) (Table 4.3, Figure

4.16B). One floating inversion (IIIL-9) (Figure 4.10C) appeared heterozygously in one male larva.

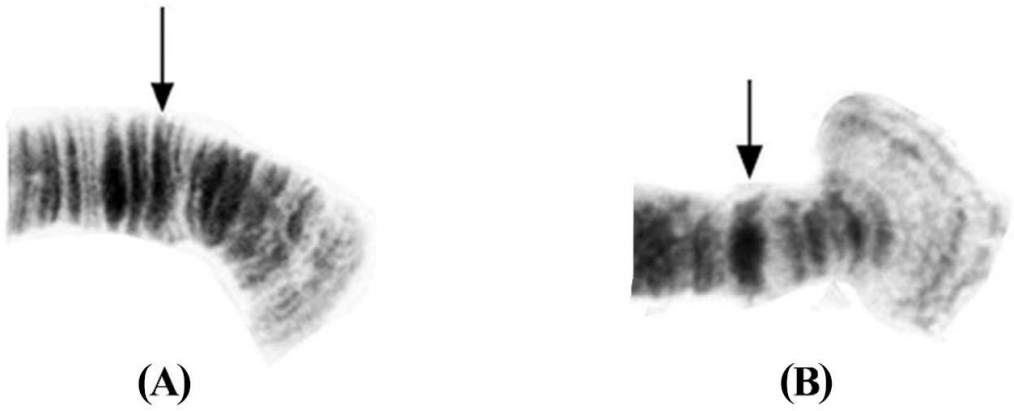

Figure 4.16 Degree of terminal flaring of chromosome arms (A) IIL of *Simulium fenestratum*, (B) IIIL of *Simulium triglobus*. Arrows show indicator bands against which the width of the terminal flare was measured.

4.1.10 Chromosomal relationships of *Simulium multistriatum* species group in Thailand.

Our outgroup analysis indicated that the basic IS and IIIS sequences of the *S. multistriatum* species group (Table 4.4), which are standard for the subgenus *Simulium*, carry no shared inversions with other analyzed species groups of the subgenus. Although the *S. striatum* species group has multiple inversions in the base of IL, as do a number of other species groups in the subgenus *Simulium* (Adler *et al.* 2016b), none of the breakpoints are shared with the *S. multistriatum* species group. Thus, all rearrangements in IL are unique to the *S. multistriatum* species group. *IIS-1* is shared with the *S. malyschevi-reptans* and *S. striatum* species groups, and *IIS-3* is shared with the *S. striatum* species group. The *S. striatum* and *S. multistriatum* species groups each have one additional, but distinctly different, fixed inversion in IIS. *IIL-1* of the *S. multistriatum* species group is shared with the *S. striatum* group. *IIIL-5* of the *S. multistriatum* species group is shared with the *S. nobile* species group, in which it is referred to by Tangkawanit *et al.* (2011) as *IIIL-b*, and

with the *S. striatum* species group. All other fixed IIIL inversions are unique to the *S. multistriatum* and *S. striatum* species groups. The position of the nucleolar organizer in the base of IS is shared with the *S. striatum* species group.

The evolutionary relationships of in the *Simulium multistriatum* species group, based on shared rearrangements, show two main lineages (Figure 4.17). The first lineage, consisting of 6 formally described species, was defined by *IL-9* and *IIIL-4*. Within this lineage, a group of three nominal species (*Simulium chainarongi*, *S. fenestratum*, and *S. triglobus*) is defined by IIIL-2, which was fixed in *S. chaliowae* and *S. lampangense* but polymorphic in *S. fenestratum*. *Simulium bullatum* and the three cytoforms of *S. malayense* formed the second primary lineage, defined by *IIIL-10*.

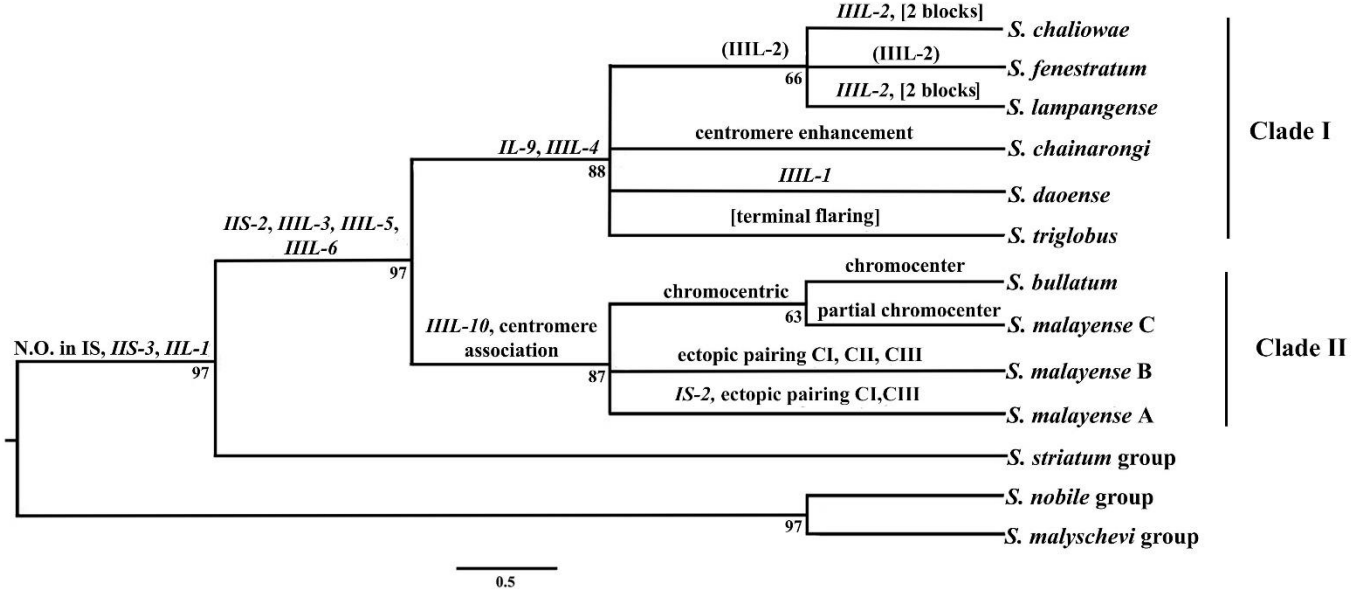

Figure 4.17 Cytophylogeny of 10 taxa in the *Simulium multistriatum* species group in Thailand (and one taxon in Malaysia). Characters in brackets indicate possible environmental influence. C = centromere. Bootstrap values for maximum parsimony are shown above or near the branches.

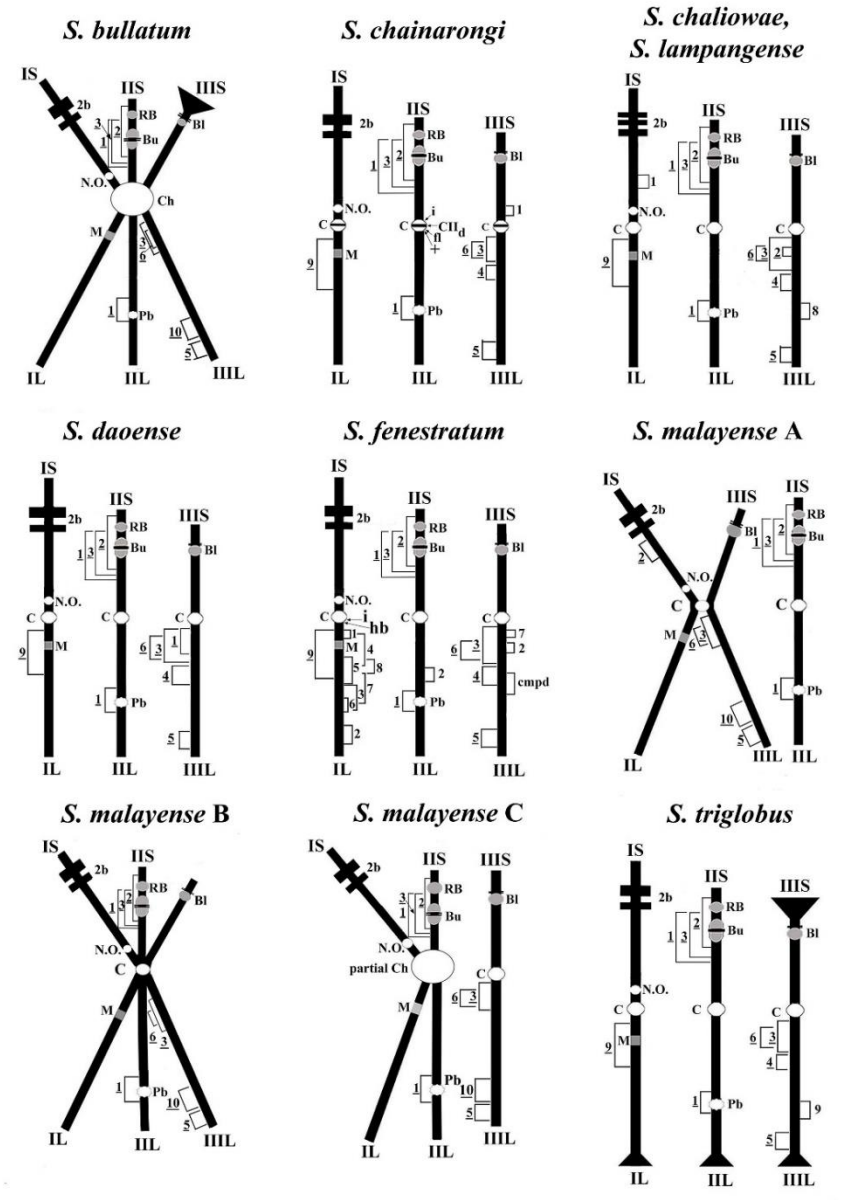

Figure 4.18 Idiograms of 10 cytoforms in the *Simulium multistriatum* species group, summarizing all chromosomal features relative to the *Simulium* subgeneric standard, including all fixed inversions (underlined and bracketed on the left side of the chromosomes) and autosomal polymorphisms (bracketed on the right side). C = centromere, Ch = chromocenter, M = marker, NO = nucleolar organizer, Pb = parabalbiani.

Table 4.1 Frequency of homologues with chromosome rearrangements in seven nominal species of the *Simulium multistriatum* group in Thailand and Malaysia.

Species	***S. chainarongi***					***S. chaliowae***		***S. lampangense***		***S. bullatum***			***S. triglobus***	***S. malayense***			***S. daoense***
														A	**B**	**C**	
Site	244	253	254	255	350	287	294	408	409	270	279	396	220	298	298	MYC	298
(male:female)	15:13	2:5	13:16	29:28	5:7	31:28	31:38	13:6	14:9	15:2	7:2	1:1	20:8	2:5	1:0	2:4	4:5
CII_d	0.02																
IIL+	0.02		0.02	0.03													
IIS 54i	0.07			0.05													
IIL 54fl	0.02																
IS-1						0.01											
IS-2														1.00			
IL-9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00				1.00				1.00
IIIS-1	0.02																
IIIL-1																	1.00
IIIL-2						1.00	1.00	1.00	1.00								
IIIL-4	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00				1.00				1.00
IIIL-8							0.01										
IIIL-9													0.02				
IIIL-10										1.00	1.00	1.00		1.00	1.00	1.00	
Chromocenter										1.00	1.00	1.00					

CII_d , diffuse centromere band of chromosome II ; IIL+ , band enhancement in the long arm of chromosome II; IIS 54i, additional (insertion) band in section 54; and IIL 54fl, flocculent band expression in section 54 of the long arm of chromosome II.

Table 4.2 Frequency of homologues with chromosome rearrangements in *Simulium fenestratum* at 18 sites in Thailand.

	Simulium fenestratum																	
Site	396	264	268	275	276	280	401	363	364	297	298*	372	384	402	320	342	343	345
(male: female)	9:3	2:2	2:5	5:4	3:4	8:10	3:0	2:1	6:10	15:11	4:3	4:11	6:3	1:3	0:4	1:3	13:17	3:3
IL-1										0.02							0.02	
IL 20i										0.02								
IL 21hb										0.02								
IL-2																		0.08
IL-3														0.03				
IL-4										0.02								
IL-5										0.02								
IL-6								0.17		0.02								
IL-7	0.04																	
IL-8	0.04																	
IL-9	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
IIL-2	0.04																	
IIIL-2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.13	0.43	0.07	0.55	1.00	0.50			0.66
IIIL-4	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
IIIL-7												0.06						
IIIL-cmpd[1]																		0.08

IL 20i, additional (insertion) band in section 20 of the long arm of chromosome I; IL 21hb, heteroband in section 21 of the long arm of chromosome I; IIIL-cmpd, compound inversion: two overlapping inversions with unresolved inner breakpoints between outer section limits 94–96 of the long arm of chromosome III. * 8 Dec 2013 only.

Table 4.3 Size classes (mean ± SE) for degree of terminal flaring of chromosomes of *Simulium triglobus* and *Simulium fenestratum*.

Species (n larvae)	Size of flared end			
	IL	**IIL**	**IIIS**	**IIIL**
Simulium triglobus (14)	1.4 ± 0.13	1.4 ± 0.18	2.9 ± 0.05	1.8 ± 0.11
Simulium fenestratum (13)	0.2 ± 0.10	0.4 ± 0.14	1.0 ± 0.11	0.9 ± 0.11

All means differed between species, except for IS and IIS (Mann Whitney U test, $P < 0.01$, $df = 25$).

Table 4.4 Matrix of 14 chromosomal rearrangements of the *Simulium striatum*, *S. malyschevi-reptans*, and *S. nobile* speies groups (out groups) and 10 species in the S. multistriatum group in Thailand and Malaysia.

Rearengements	***IIS - 1***	***IIS-2***	***IIS-3***	***IIL-1***	***IIIL-3***	***IIIL-4***	***IIIL-5***	***IIIL-6***	***IL-9***	***IIIL-2***	***IIIL-10***	**Chromocentric**	**Centromere association**	**Position of No. on IS**
S. striatum group	1	0	1	1	0	0	1	0	0	0	0	0	0	1
S. malaschevi-reptans group	1	0	0	0	0	0	0	0	0	0	0	0	0	0
S. nobile group	0	0	0	0	0	0	1	0	0	0	0	0	0	0
S. bullatum	1	1	1	1	1	0	1	1	0	0	1	1	1	1
S. chaliowae	1	1	1	1	1	1	1	1	1	1	0	0	0	1
S. chainarongi	1	1	1	1	1	1	1	1	1	0	0	0	0	1
S. daoense	1	1	1	1	1	1	1	1	1	0	0	0	0	1
S. fenestratum	1	1	1	1	1	1	1	1	1	1	0	0	0	1
S. lampangense	1	1	1	1	1	1	1	1	1	1	0	0	0	1
S. malayense A	1	1	1	1	1	0	1	1	0	0	1	0	1	1
S. malyense B	1	1	1	1	1	0	1	1	0	0	1	0	1	1
S. malayense C	1	1	1	1	1	0	1	1	0	0	1	1	1	1
S. triglobus	1	1	1	1	1	1	1	1	1	0	0	0	0	1

4.1.11 Chromosomal identification key for members of the *Simulium multistriatum* species group in Thailand, Malaysia and Vietnam.

1. Centromere bands associated in >50% of nuclei (e.g., Fig. 10). *IL-9* absent (Figure 4.11), *IIIL-10* present (Figure 4.9B). ……...……2
   Centromere bands associated in <10% of nuclei. *IL-9* present (Figure 4.2), *IIIL-10* absent…….…….…….5

2. True chromocenter with extra heterochromatin present (Figure 4.1) ……...***Simulium bullatum***
   Partial chromocenter (Figure 4.15) or ectopic pairing present ……..…..3

3. Ectopic pairing of all combinations of centromere bands (CI, CII, and CIII) ……..... ***Simulium malayense* cytoform B (Thailand)**
   Partial chromocenter or ectopic pairing of only 2 centromere bands…….4
4. *IS-2* present (Figure 4.9). Ectopic pairing involving centromeres I and III in 1% to 60% of nuclei.
   …… ***Simulium malayense* cytoform A (Thailand)**
   IS-2 absent. Partial chromocenter (Figure 4.15) present, with extra heterochromatin, involving centromeres CI and CII.
   ……***Simulium malayense* cytoform C (Malaysia)**
5. Centromere bands well defined, darkly stained (Figure 4.3A) ……..……. ***Simulium chainarongi***
   Centromere bands weakly defined, lightly stained (Figure 4.3B) ……..……6
6. Flaring of ends of IL, IIL, IIIL, and IIIS >1.5 times wider than respective indicator bands (Figure 4.16B) ……***Simulium triglobus***
   Flaring of ends of all chromosome arms <1.5 times wider than respective indicator bands (Figure 4.16A). ……...…..……7

7. “2 blocks” marker appearing as 3 blocks in at least some nuclei (Figure 4.5). *IIIL-2* present (Figure 4.10A)
   ……***Simulium chaliowae, Simulium lampangense***
   “2 blocks” marker standard (Figure 4.9). *IIIL-2* absent.
   ……8
8. *IIIL-1* present (Figure 4.8)……..…..…….…….***Simulium daoense***
   IIIL-1 absent…….……..***Simulium fenestratum***

4.2 Molecular genetic variation

4.2.1 Intraspecific and interspecific genetic divergences

The COI and COII gene sequences were analyzed 69 specimens from 10 taxa of the *S. multistriatum* species group in Thailand (Table 4.5, 4.6). The sequence length of the COI gene was 581 bp. There were 168 variable sites, of which 143 were parsimony-informative. The maximum intraspecific genetic divergence based on the COI sequence (Table 6) was in *S. daoense* (5.60%), and the minimum values were in *S. bullatum*, *S. chainarongi*, and *S. chaliowae* (0.10%). The minimum interspecific genetic divergence was between *S. chaliowae* and *S. lampangense* (1.30%). The maximum interspecific genetic divergence was between *S. daoense* and *S. malayense* cytoform C from Thailand (14.70%).

The sequence length of the COII gene was 697 bp, with 235 variable sites, of which 184 were parsimony-informative. Maximum intraspecific genetic divergence based on the COII sequence was in *S. daoense* (3.30%), and the lowest intraspecific genetic divergence was in *S. chaliowae* (0.40%). The maximum interspecific genetic divergence was between *S. lampangense* and *S. malayense* cytoform A, and *S. malayense* cytoform A and *S. malayense* cyotform C (16.00%), and the minimum interspecific genetic divergence was between *S. chaliowae* and *S. lampangense* (1.20%).

The COII sequences were more effective than COI for differentiating members of the *S. multistriatum* species group. Identifications based on best match for COI were 73.91% correct (51 of 69 sequences), with 14.49% (10 of 69 sequences) misidentifications

and 11.59% (8 of 69 sequences) ambiguous identifications. Identifications based on COII were 97.10% (67 of 69 sequences) correct, with 2.89% (2 of 69 sequences) misidentifications and no ambiguous identifications (Table 4.7).

Table 4.5 Range (and mean) of intraspecific and interspecific genetic distances for mitochondrial cytochrome *c* oxidase I (COI) sequences of nine nominal species of the *Simulium multistriatum* species group in Thailand, Malaysia and Vietnam based on Kimura 2-parameter.

Species	**BUL**	**CN**	**CW**	**Da**	**FN**	**LP**	**MYA**	**MYC**	**TB**
***S. bullatum* (BUL)**	0.000-0.004 (0.001)								
***S. chainarongi* (CN)**	0.088-0.090 (0.088)	0.00-0.002 (0.001)							
***S. chaliowae* (CW)**	0.092-0.097 (0.094)	0.036-0.042 (0.038)	0.000-0.004 (0.001)						
***S. daoense* (DA)**	0.128-0.166 (0.145)	0.077-0.158 (0.112)	0.066-0.170 (0.112)	0.000-0.101 (0.056)					
***S. fenestratum* (FN)**	0.083-0.095 (0.090)	0.021-0.042 (0.033)	0.006-0.032 (0.016)	0.058-0.202 (0.110)	0.004-0.026 (0.015)				
***S. lampangense* (LP)**	0.086-0.095 (0.092)	0.028-0.038 (0.033)	0.004-0.019 (0.013)	0.056-0.170 (0.106)	0.002-0.026 (0.013)	0.000-0.016 (0.009)			
***S. malayense* Cytoform A (MYA)**	0.063-0.073 (0.068)	0.101-0.110 (0.105)	0.103-0.115 (0.109)	0.125-0.161 (0.144)	0.101-0.147 (0.110)	0.103-0.117 (0.108)	0.014		
***S. malayense* Cytoform C (MYC)**	0.021-0.050 (0.026)	0.083-0.110 (0.097)	0.088-0.114 (0.096)	0.123-0.187 (0.147)	0.077-0.112 (0.091)	0.081-0.112 (0.092)	0.058-0.097 (0.071)	0.004-0.031 (0.014)	
***S. triglobus* (TB)**	0.092-0.094 (0.093)	0.093-0.102 (0.097)	0.093-0.102 (0.097)	0.093-0.172 (0.124)	0.088-0.106 (0.097)	0.091-0.102 (0.096)	0.110 -0.115 (0.113)	0.090-0.118 (0.104)	0.002-0.012 (0.007)

Table 4.6 Range (and mean) of intraspecific and interspecific genetic distances for mitochondrial cytochrome c oxidase II (COII) sequences of nine nominal species of the *Simulium multistriatum* species group in Thailand, Malaysia and Vietnam based on Kimura 2-parameter.

Species	**BUL**	**CN**	**CW**	**Da**	**FN**	**LP**	**MYA**	**MYC**	**TB**
***S. bullatum* (BUL)**	0.000-0.012 (0.007)								
***S. chainarongi* (CN)**	0.092-0.137 (0.105)	0.002-0.038 (0.019)							
***S. chaliowae* (CW)**	0.074-0.087 (0.081)	0.034-0.074 (0.048)	0.000-0.006 (0.004)						
***S. daoense* (DA)**	0.121-0.147 (0.134)	0.054-0.090 (0.067)	0.082-0.135 (0.107)	0.029-0.060 (0.033)					
***S. fenestratum* (FN)**	0.081-0.119 (0.095)	0.016-0.063 (0.033)	0.010-0.053 (0.029)	0.044-0.118 (0.072)	0.004-0.040 (0.019)				
***S.lampangense* (LP)**	0.084-0.103 (0.095)	0.018-0.067 (0.038)	0.003-0.020 (0.012)	0.046-0.083 (0.061)	0.014-0.044 (0.025)	0.000-0.028 (0.010)			
***S. malayense* cytoform A (MYA)**	0.094-0.115 (0.105)	0.114-0.134 (0.123)	0.148-0.167 (0.158)	0.145-0.170 (0.158)	0.116-0.168 (0.138)	0.154-0.167 (0.160)	0.012		
***S. malayense* Cytoform C (MYC)**	0.032-0.055 (0.046)	0.096-0.137 (0.110)	0.098-0.119 (0.109)	0.072-0.089 (0.081)	0.094-0.123 (0.106)	0.092-0.116 (0.105)	0.154-0.167 (0.160)	0.010-0.034 (0.020)	
***S. triglobus* (TB)**	0.114-0.152 (0.127)	0.072-0.105 (0.090)	0.098-0.132 (0.111)	0.072-0.098 (0.083)	0.078-0.123 (0.098)	0.087-0.130 (0.106)	0.132-0.161 (0.144)	0.114-0.146 (0.127)	0.002-0.026 (0.015)

Table 4.7 Nucleotide sequence statistics based on COI and COII of black flies in the *Simulium multistriatum* species group in Thailand. Percent correct identification is based on best match method in TaxonDNA (Meier *et al.* 2006).

	Number of species	**Number of sequences**	**Variable sites (V)**	**Parsim-info sites (Pi)**	**Length of sequences (bp)**	**Percent correct identification (n)**	**Percent misidentification (n)**	**Percent ambiguous (n)**
COI	9	69	168	143	581	73.91% (51)	14.49% (10)	11.59% (8)
COII	9	69	235	184	697	97.10% (67)	2.89%(2)	-

4.2.2 Molecular phylogenetic relationships of *Simulium multistriatum* species group in Thailand.

Phylogenetic analyses were conducted for COI, COII, and the combined dataset (COI + COII). All phylogenetic analysis methods (NJ, MP, ML, and Bayesian) revealed similar tree topologies; thus, only NJ trees are shown. The NJ tree based on COI sequences revealed two major clades (Figure 4.19). *Simulium chainarongi, S. chaliowae, S. daoense, S. fenestratum, S. lampangense,* and *S. triglobus* formed clade I. *Simulium fenestratum* and *S. lampangense*, were paraphyletic, whereas *S. chainarongi* and *S. chaliowae* were monophyletic within this clade I. All members of *S. daoense* from Thailand and Vietnam were clustered in the same clade with moderate bootstrap support (>71%). Clade II comprised *S. bullatum, S. malayense* cytoform A from Thailand, and *S. malayense* cytoform C from Malaysia. *Simulium bullatum* and *S. malayense* cytoforms A and C in clade II were monophyletic with strong support. *Simulium malayense* cytoform B was not available for molecular analysis.

The phylogenetic analysis based on the COII gene (Figure 4.20) provided better resolution than did the COI gene. The COII sequences revealed two main clades. Clade I comprised of six species including *S. chaliowae, S. chainarongi, S. daoense, S. fenestratum, S. lampangense* and *S. triglobus*. All species in this clade were monophyletic. All specimens of *S. lampangense* formed a single well-supported clade with *S. chaliowae*. Clade II was composed of *S. bullatum, S. malayense* cytoform A from Thailand, and cytoform C from Malaysia.

The phylogenetic tree based on the combined data (Figure 4.21) showed two main clades similar to the trees derived from the COI and COII sequences. *Simulium chainarongi, S. chaliowae, S. daoense, S. fenestratum, S. lampangense* and *S. triglobus* each formed a monophyletic clade within clade I. *Simuliuum lampangense* formed a single well-supported subclade with *S. chaliowae* within clade I. *Simulium bullatum*, and *S. malayense* cytoforms A and C formed a monophyletic clade within clade II.

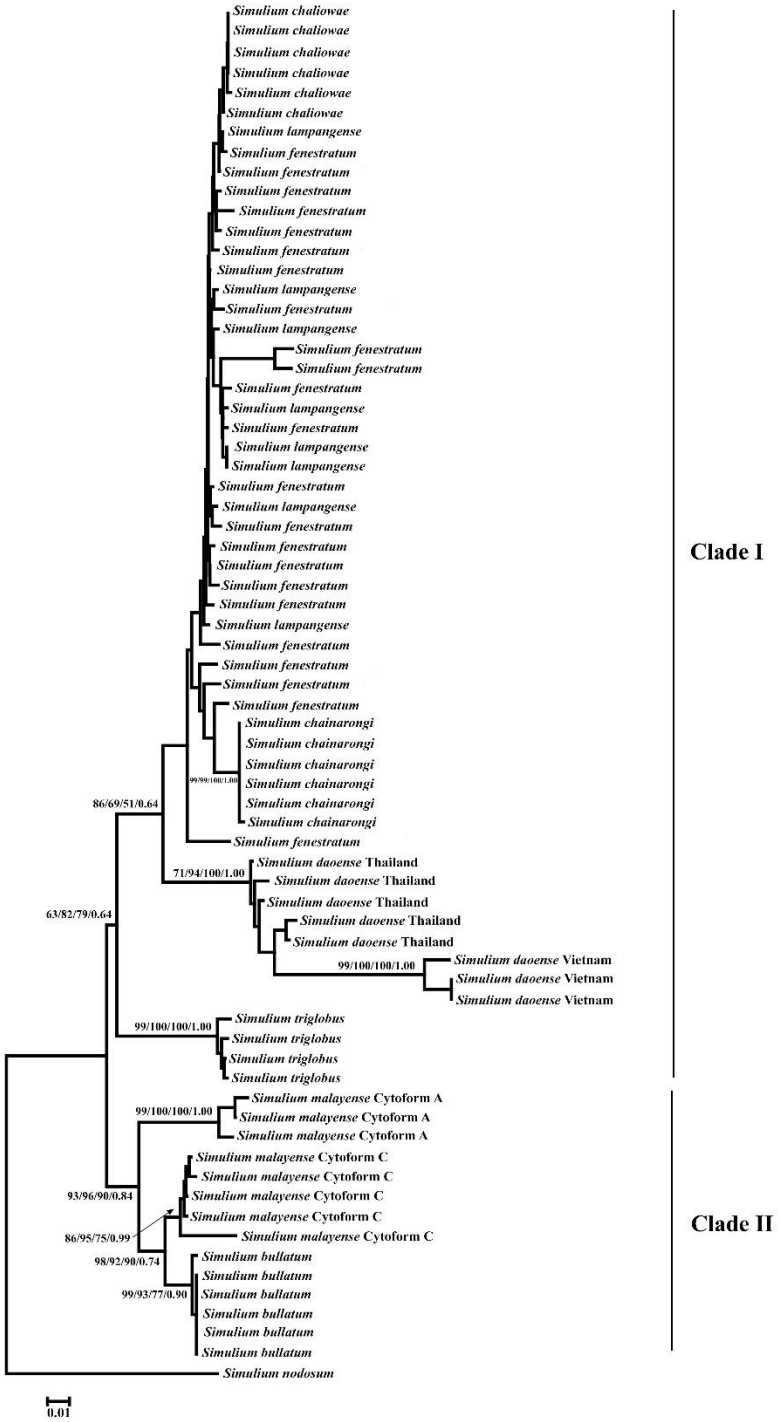

Figure 4.19 Neighbor-joining (NJ) tree for nine species in *Simulium multistriatum* species group from Thailand, based on COI sequences. Bootstrap values of neighbor-joining, maximum parsimony (MP), and maximum likelihood (ML), and posterior probabilities of Bayesian analysis (BA) are shown above or near branches. Scale bar represents 0.01 substitutions per nucleotide position.

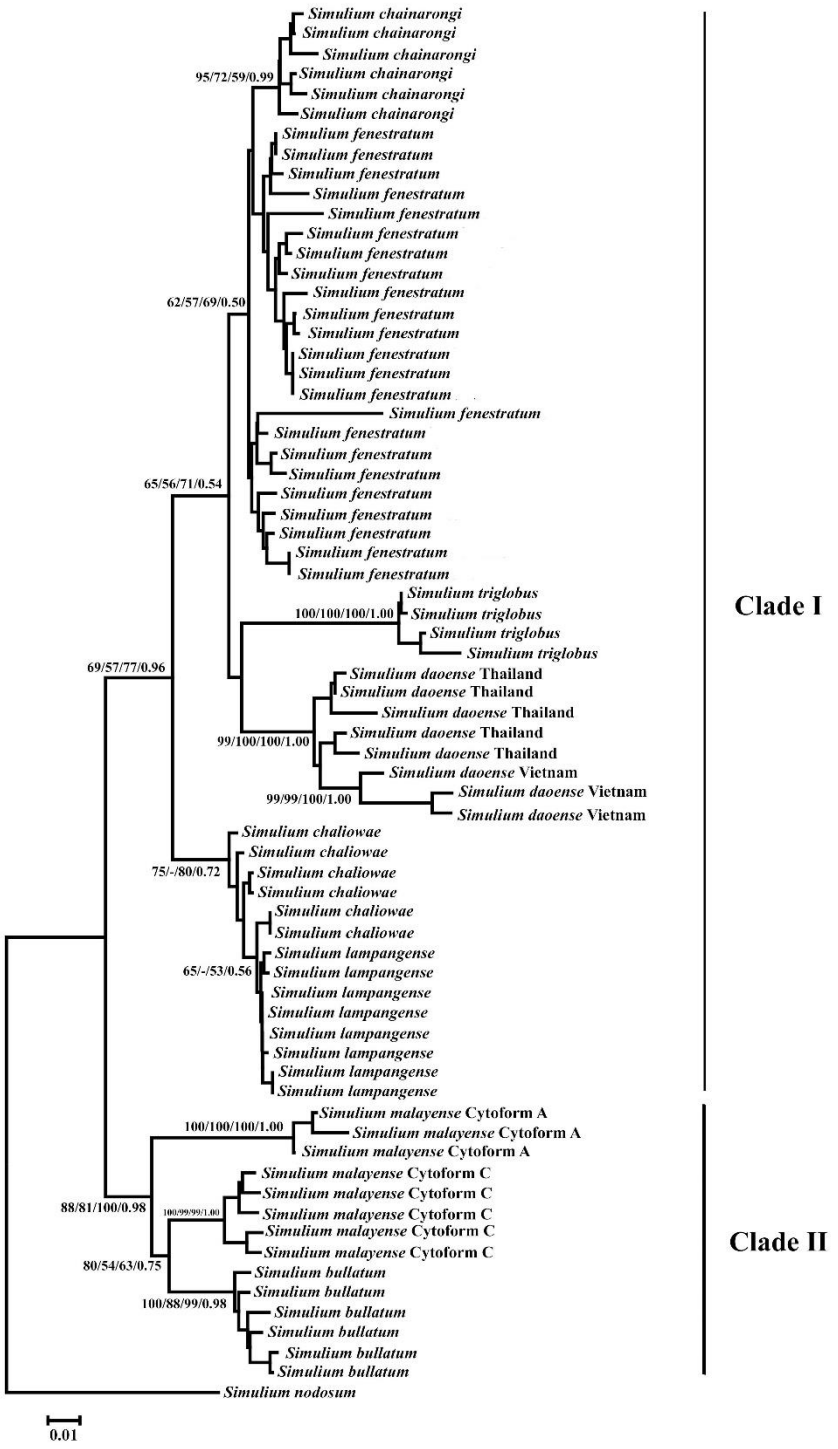

Figure 4.20 Neighbor-joining (NJ) tree for nine species in *Simulium multistriatum* species group from Thailand, based on COII sequences. Bootstrap values of neighbor-joining, maximum parsimony (MP), and maximum likelihood (ML), and posterior probabilities of Bayesian analysis (BA) are shown above or near branches. Scale bar represents 0.01 substitutions per nucleotide position.

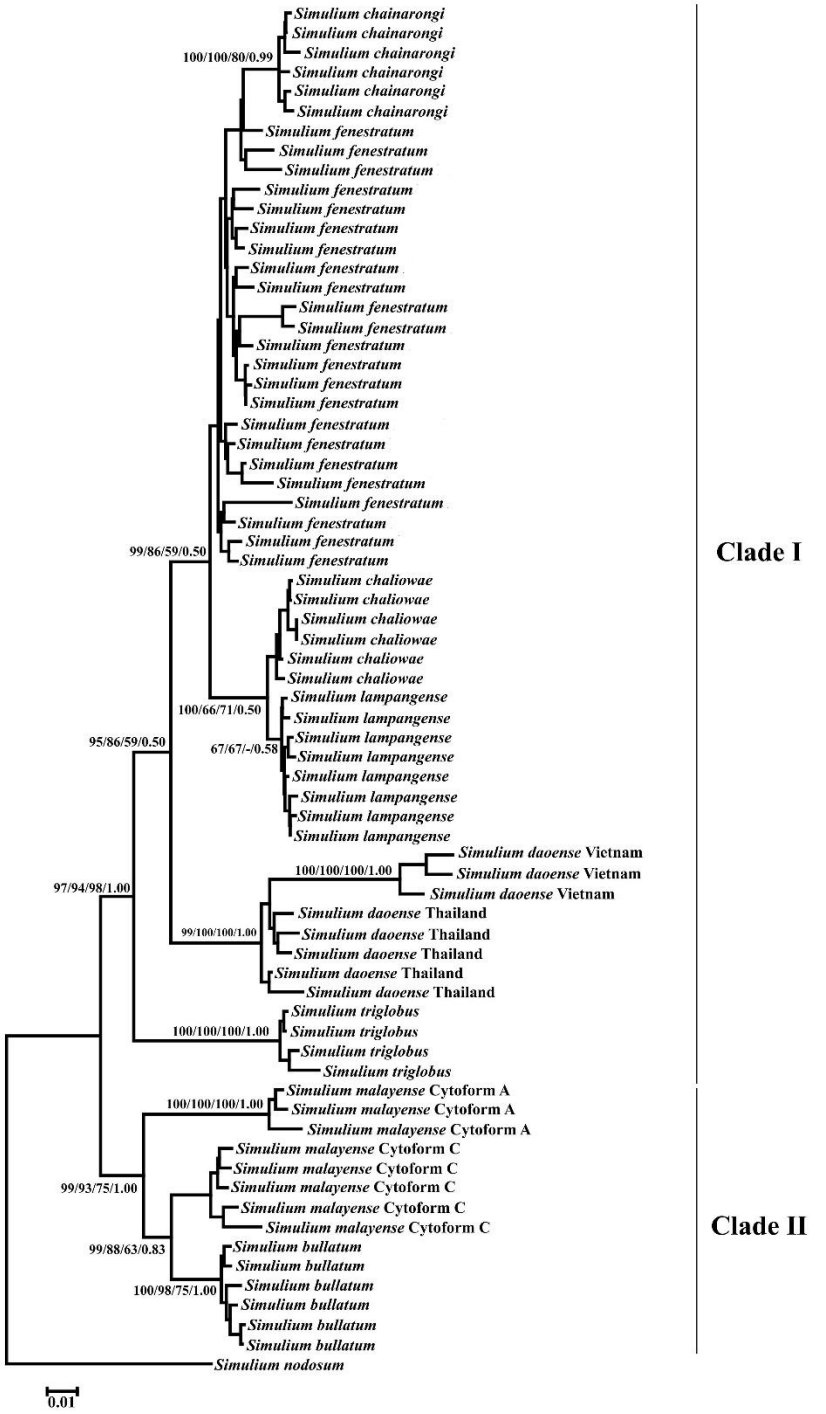

Figure 4.21 Neighbor-joining (NJ) tree for nine species in *Simulium multistriatum* species group from Thailand, based on combined (COI + COII) data set. Bootstrap values of neighbor-joining, maximum parsimony (MP), and maximum likelihood (ML), and posterior probabilities of Bayesian analysis (BA) are shown above or near branches. Scale bar represents 0.01 substitutions per nucleotide position.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

5.1.1 Taxonomic status of chromosomal entities

The chromosomal study recognizes 10 distinct taxa among eight nominal morphospecies in the *S. multistriatum* species group. Chromosomal analyses confirm reproductive isolation (species status), via absence of hybrids, of eight species, reveal two undescribed taxa (*S. malayense* cytoforms A and B), and suggest possible conspecificity of at least two nominal species (*S. chaliowae* and *S. lampangense*) and possible cryptic species in another (*S. fenestratum*).

The species status of *S. bullatum* separate from all other studied species is supported chromosomally by the presence of a large chromocenter. Morphologically, the large unpigmented organ of its pupal gill is unique (Takaoka & Choochote, 2005b). Molecular analyses also support its distinction from other species, with K2P genetic distances ranging from 2.60% to 14.50% for COI and 4.60% to 15.60% for COII. Molecular phylogenetic analysis showed that all specimens of *S. bullatum* form a monophyletic clade, in agreement with a previous molecular study based on the COI and ECP1 genes (Thaijarern *et al*., 2017).

Species status is tentatively suggested for the three cytoforms of *S. malayense*. The presence of *IS-2* in homozygous and standard arrangements, coupled with unique centromeric associations, in sympatry suggests that cytoforms A and B are distinct species. This suggestion, however, is tempered by an inadequate sample size and by lack of material of cytoform B for molecular analysis.

Phylogenetic analysis corroborates chromosomal data that *S. malayense* cytoform C is genetically distinct from A and B. However, the allopatric distribution of A and B relative to C does not permit a strict evaluation of species status; A and B are about 1,605 km distant from C. The collection site of cytoform C is less than 100 km from the type locality of *S. malayense* and, therefore, is the best candidate for typical *S. malayense*. *Simulium chainarongi* is homosequential with *S. fenestratum* and *S. triglobus* but has unique centromeric expression and polymorphisms. Differential expression of centromere

bands is not uncommon in closely related species of black flies (Bedo, 1975). Although, *S. chainarongi* is difficult to separate morphologically from some species in the *S. multistriatum* species group (Takaoka & Kuvangkadilok, 1999), its unique centromeric expression among studied group members, coupled with molecular and ecological data (i.e., low-elevation habitats: < 250 m) support *S. chainarongi* as a distinct species. Gene sequences indicate that *S. chainarongi* is monophyletic, with interspecific genetic distances ranging from 3.30% to 11.20% for COI and 3.30% to 12.30% for COII.

Simulium daoense differs by only one fixed inversion (*IIIL-1*) from *S. chainarongi*, *S. fenestratum*, and *S. triglobus*. Our chromosomal analysis revealed the presence of *S. daoense* in Thailand (Chiang Mai Province) for the first time. The Thai population is chromosomally identical with *S. daoense* from the type locality in Vietnam about 700 km distant. The type locality of *S. daoense* is at high elevation (1,315 m), similar to the location in Thailand (1,304 m). The stream width at the type locality is small (0.5 m) and the water temperature is cold (7.0 °C), whereas in Thailand, the stream is wider (5 m) and the water temperature warmer (15.7˚C). The COI and COII sequences place *S. daoense* from Thailand and Vietnam together, with maximum intraspecific genetic divergence of 5.60% for COI and 3.30% for COII. The chromosomes suggest that the two populations are a single species, but environmental effects and geographic distance might be driving the molecular differentiation between the two populations. Geographic distance is the main factor limiting gene flow between populations of many black fly species (Pramual *et al.* 2005), particularly those inhabiting high-elevation areas (Finn & Adler, 2006; Finn *et al.* 2006; Pramual & Wongpakam, 2013).

The banding pattern of *S. triglobus* is homosequential with that of *S. chainarongi* and *S. fenestratum*, but differs from that of *S. chainarongi* by standard centromere band expression and from both species by enhanced terminal flaring of the chromosome arms. Typically, the chromosomal ends would be heterochromatinized via duplication processes related to repeat DNA elements and perhaps heterochromatic genes. Thus, when the ends flair, some form of expression is probably occurring with regard to these elements or genes. Gene expression is necessary to produce the appropriate proteins for development in particular environments (Gottlieb, 1998). The enhanced flaring of *S. triglobus* might be associated with conditions in calcareous streams, such as high calcium carbonate, pH, and

conductivity. Although the effect of calcium carbonate on genomic expression has not been investigated for black flies, some evidence suggests an indirect effect on the structure of polytene chromosomes in larval chironomids via surface adsorption of heavy metals on marl surfaces (Jabłońska-Barna *et al.*, 2013).

If terminal flaring of chromosomes is environmentally influenced, no chromosomal evidence is available to support species status of *S. triglobus*. Morphological support for the species status of *S. triglobus* separate from *S. chainarongi* and *S. fenestratum* is based primarily on the more branched thoracic trichomes and corbicular cocoon of the pupa, lack of dorsolateral protuberances on the larval abdomen, and three (versus one) spermathecae in females of *S. triglobus* (Takaoka & Kuvangkadilok, 1999). Phylogenetic analysis based on COI and COII support the unique, albeit weak chromosomal feature (flaring); all specimens of *S. triglobus* from the type locality form a monophyletic clade with strong support (100%), agreeing with a previous DNA barcode tree by Pramual and Wongpakam (2014). This species also shows a high level of interspecific molecular genetic differentiation (K2P genetic distance: 9.30% to 12.40% for COI and 8.30% to 14.40% for COII).The banding patterns and chromosomal characteristics of *S. chaliowae* and *S. lampangense* are identical, and both species are known only from limestone streams. The unique expression of the “2 blocks” marker is positively related to the degree of polytenization, and might represent gene expression related to environmental influence from the calcareous streams they inhabit, as discussed for *S. triglobus*. The COII sequence and the combined COI + COII data set show a close relationship between *S. chaliowae* and *S. lampangense*, with low intrespecific genetic divergence (1.20% for COII). The results agree with those based on the ECP1 gene, which found that *S. chaliowae* and *S. lampangense* are closely related but fall into separate clusters (Thaijarern *et al*., 2017). The molecular difference might be a site effect, rather than a species effect. Known locations of *S. chaliowae* are about 130 to 150 km from those of *S. lampangense*. If these taxa are restricted to calcareous streams, which have patchy distributions (Pramual & Pangjanda, 2015), the implication is that females tend to return to their natal streams to oviposit, enhancing the buildup of location-specific genetic differences. Morphologically, the larvae differ only by the presence of dorsal protuberances on abdominal segments 2 to 6 in *S. chaliowae* versus 3 to 7 in *S. lampangense* (Thaijarern *et al.*, 2017); however, this character

varies intraspecifically in some members of the *S. multistriatum* species group (Takaoka & Kuvangkadilok, 1999; Takaoka & Choochote, 2005a). For example, *S. chainarongi* has dorsal protuberances not only on abdominal segments 1-6 but also segments 7 and 8 (Takaoka & Kuvangkadilok, 1999), and the observation found some populations of *S. fenestratum* have dorsal protuberances whereas others do not.

The cocoon of *S. lampangense* is fenestrated and either slipper- or shoe-shaped, whereas that of *S. chaliowae* is unfenestrated and shoe-shaped (Takaoka & Kuvangkadilok, 1999; Takaoka & Choochote, 2005a). However, the presence of windows in the cocoon can vary intraspecifically in other simuliid species (Adler *et al.* 2004). Differences in the adults are based on minor characteristics, such as the length to width ratio of the anal lobe (Takaoka & Kuvangkadilok, 1999; Takaoka & Choochote, 2005a). Morphological differences might also be location-specific. Thus, the evidence that *S. chaliowae* and *S. lampangense* are distinct species is weak, suggesting that *S. lampangense* might be a junior synonym of *S. chaliowae*. Until evidence to the contrary can be adduced, we continue to recognize *S. chaliowae* and *S. lampangense* as separate species, based on recovery of distinct clusters in molecular phylogenies using the ECP1 (Thaijarern *et al.*, 2017) and COII genes, and minor morphological differences. Thus, they would be nearly homosequential cryptic species. The chromosomes of *S. chaliowae* and *S. lampangense* are fundamentally the same as those in populations of *S. fenestratum* homozygous for the IIIL-2 inversion. Larvae and pupae of *S. chaliowae* and *S. lampangense* have conventionally been distinguished from *S. fenestratum* by body color, dorsal protuberances on the abdomen, and shape of the cocoon (Takaoka & Boonkemtong, 1999). These characters, however, are subject to intraspecific variation. The male of *S. chaliowae* is similar to that of *S. fenestratum* but is distinguished by the horn-like basal protuberance of the gonostylus with many teeth along the anterior margin (Takaoka & Kuvangkadilok, 1999), and *S. lampangense* can be distinguished from *S. fenestratum* by having a bare radial vein in females and several spines on the basal protuberance of the gonostylus (Takaoka & Choochote, 2005a); *S. fenestratum* has one apical spine on the basal protuberance (Takaoka, 1977).

Although the ECP1 gene separates *S. fenestratum* from *S. chaliowae* and *S. lampangense* with strong support, the COI gene groups some populations of *S. fenestratum*

in a clade with *S. chaliowae* (Pramual & Wongpakam, 2014; Thaijarern *et al*. 2017). The chromosomal results provide some indication that *S. fenestratum* consists of cryptic species. A previous report of cryptic species (i.e., chromocentric individuals) in *S. fenestratum* (Pramual & Nanork, 2012) actually pertains to *S. bullatum*. The evidence for cryptic species of *S. fenestratum* involves the IIIL-2 inversion. The existence of populations homozygous standard, homozygous inverted, and polymorphic for this inversion presents four hypotheses: i) a single polymorphic species, with IIIL-2 perhaps reflecting local adaptation; ii) two species, one fixed for IIIL-2 and one polymorphic for IIIL-2; iii) two species, one homozygous standard and one polymorphic; and iv) three species, one fixed for IIIL-2, one fixed for standard, and one polymorphic. This same scenario has been found in the *S. tani* complex in Thailand, with all four possibilities (although involving other inversions), representing different cytoforms (Tangkawanit *et al.,* 2009). A population in Trat Province where individuals homozygous for IIIL-2 and for standard were found, with a dearth of heterozygotes, might provide a test for cryptic species if larger samples are available to test the inversion for Hardy-Weinberg equilibrium or to conduct a molecular evaluation. The biogeographic pattern for IIIL-2 in *S. fenestratum* is similar to that for inversions in *S. aureohirtum* and *S. tani* s. l., which increase in frequency with latitude (Pramual *et al*. 2005, 2008).

5.1.2 Phylogenetic relationships

Monophyly of the *S. multistriatum* species group, based on species from Thailand, is demonstrated on the basis of four shared chromosomal rearrangements (*IIS-2*, *IIIL-3*, *IIIL-5*, and *IIIL-6*), providing a framework for further testing of the other 25 known species (Adler & Crosskey, 2017) in the group. The chromosomal characters should prove especially useful for evaluating group membership of species such as *S. takense*, which does not cluster with the *S. multistriatum* species group on the basis of molecular evidence (COI, COII, and 18S/ITS) (Pramaul & Nanork, 2012; Pramual & Adler, 2014).

Chromosomal characters suggest that the *S. multistriatum* species group is most closely related to the *S. striatum* species group and these two groups, in turn, are related to the *S. malyschevi-reptans* and *S. nobile* species groups. This cluster of species groups finds morphological support based on the presence of fenestrated cocoons. Although, the fenestra

can be lost in some species (Moulton & Adler, 1995), their presence is strongly associated with these groups. If this structural character has phylogenetic value, we would expect the *S. griseifrons* species group *s. l.* (including its recent divisions; Takaoka, 2017) also to be a member of this clade; to date, no chromosomal information is available for *S. griseifrons* and its relatives. The results are further supported by molecular evidence (Thanwisai *et al*., 2006; Phayuhasena *et al*., 2010; Pramual & Adler, 2014).

The chromosomal phylogeny showing that species of the *S. multistriatum* species group in Thailand fall into two distinct groups based on three fixed chromosome inversions (*IL-9, IIIL-4, IIIL-10*) and centromere associations agrees with phylogenetic analysis based on COI and COII gene sequences: *S. bullatum* and *S. malayense* cytoforms A, B, and C form one clade, and *S. chainarongi*, *S. chaliowae*, *S. daoense*, *S. fenestratum*, *S. lampangense*, and *S. triglobus* form another clade. These results agree with a previous DNA-barcode tree (Pramual & Wongpakam, 2014). A paucity of shared structural characters does not permit inference of a morphological phylogeny. The chromosomal evidence that the *S. multistriatum* group is most closely related to the *S. striatum* group does not agree with molecular analyses based on multiple genes (Otsuka *et al.*, 2003; Thanwisai *et al.*, 2006; Phayuhasena *et al*., 2010; Pramual & Adler, 2014).

5.1.3 Speciation in the *Simulium multistriatum* group

Limited morphological differentiation is found among the species in the *S. multistriatum* species group, consistent with the low levels of cytogenetic differentiation. Only 30 rearrangements, other than those common to the basic sequence, have been found in this species group in Thailand. This number is low compared with other Southeast Asian species groups, such as the *S. tuberosum* group in Vietnam, with 88 rearrangements (Adler *et al.*, 2016a). Speciation in the Simuliidae is typically associated with chromosomal phenomena, particularly coadaptation of sex chromosomes, cooption of individual rearrangements for different roles in different lineages, and more rarely, larger genomic restructuring events (Rothfels, 1989; Adler *et al.*, 2016).

Speciation in the *S. multistriatum* group, however, is largely not associated with the typical chromosomal phenomena in the Simuliidae. The sex chromosomes, for example, are undifferentiated in all taxa in our study. Only one inversion, IIIL-2, functions

in multiple roles: fixed in *S. chaliowae* and *S. lampangense* and polymorphic in *S. fenestratum*. Sharing of the IIIL-2 inversion suggests that *S. fenestratum*, *S. chaliowae*, and *S. lampangense* are derived from a common ancestor. Accordingly, this inversion would have been polymorphic in the ancestor, remained so in *S. fenestratum*, and become fixed in *S. chaliowae* and *S. lampangense*. The latter two species occur only in a specific habitat, calcareous streams; thus, fixation of *IIIL-2* might be associated with habitat specialization. Chromosomal and molecular study of another calcareous stream specialist, *S. weji*, in Thailand suggests that females return to their natal sites to oviposit (Pramual & Pangjanda, 2015). If this scenario also applies to *S. chaliowae* and *S. lampangense*, inbreeding could occur and facilitate the fixation of *IIIL-2* in the population. The low molecular diversity in the COI and COII genes of *S. chaliowae* and *S. lampangense* supports this possibility.

Rather than the typical chromosomal rearrangements associated with simuliid speciation, (e.g., inversions), other chromosomal phenomena occur in the *S. multistriatum* species group, including centromere associations with and without heterochromatinization (*S. bullatum* and *S. malayense* cytoforms A, B, and C). Centromere associations occur throughout the Simuliidae in more than 12% of all species (Adler *et al.*, 2010). Heterochromatinization within the genome can serve as a driving force in speciation (Ferree & Barbasha, 2009). The chromosomal and molecular phylogenetic clustering of the three types of centromeric associations (i.e., ectopic pairing, partial chromocenter, and chromocenter) suggests a common origin. The simplest form of centric association, ectopic pairing, is a frequent phenomenon in diverse species, and is often restricted to certain populations of a species, although it is not necessarily expressed in all individuals or even in all nuclei of an individual (Rothfels & Freeman, 1977). The origin of heterochromatinization is inferred to be a result of over-replication of repetitive elements (Thapa *et al.*, 2014). An occasional tendency for centromeres to associate ectopically might represent the first step in acquisition of a permanent, species-specific chromocenter or partial chromocenter, with the next step being addition of heterochromatin. A similar progression from a chromocentric to fully chromocentric, via ectopic pairing (pseudochromocenter) has also been proposed for some members of the *S. vernum* species group, of which at least one species exhibits chromocenter polymorphism (Brockhouse *et al.*, 1989). The partial chromocentric state, which is typically a species-specific trait, might

result from the loss of repetitive DNA sequences responsible for the chromocenter from only one of the three chromosomes (Brockhouse *et al.*, 1989).

5.2 Conclusion

A total of 10 cytologically distinct taxa were discovered among eight nominal morphospecies. Chromosomal analyses confirm reproductive isolation (species status) of these species, via absence of hybrids, of 10 species, reveal two undescribed taxa (*S. malayense* cytoforms A and B), and suggest conspecificity of at least two nominal species (*S. chaliowae* and *S. lampangense*) and possible cryptic species in another (*S. fenestratum*). Chromosomal analysis revealed the presence of *S. daoense* in Thailand (Chiang Mai Province) for the first time. Molecular analysis based on COI and COII gene supported two cytoforms of *S. malayense*. *Simulium bullatum, S. chainarongi,* and *S. triglobus* were clearly separated from other species based on the unique characteristics of the chromosomes. Separation of these species also is supported by molecular study based on the COI and COII sequences. Molecular study based on COI and COII supported chromosomal results and suggested that *S. lampangense* is possibly a synonym of *S. chaliowae*. Chromosomal results provide some indication that *S. fenestratum* consists of cryptic species. This possibility is also supported by a high level of genetic divergence within this species. Therefore, an integrated approach based on morphology, cytology, and molecular data can be used to examine the taxonomy and systematics of the *S. multistriatum* species group in Thailand.

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