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RESEARCH ARTICLE

A new species of black fly, formerly cytoform C of the *Simulium angulistylum* complex (Diptera: Simuliidae), from a high mountain in northeastern Thailand

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ABSTRACT

Cytoform C, one of three cytoforms in the *Simulium angulistylum* Takaoka & Davies complex from a high mountain in northeastern Thailand was morphologically and molecularly investigated. All known life stages (larva, pupa, adult male and female except egg) were morphologically similar to, but distinguishable from *S. angulistylum* s. str. and *S. isanense* Takaoka, Srisuka & Saeung in the adults by the relative length of the fore and hind basitarsi and relative length of the tooth to the claw. It is also morphologically distinct from other species of the *S. epistum* species-group. Here, it is formally described as a new species, *S. prayooki*. Molecular genetic data based on mitochondrial cytochrome c oxidase subunit I (COI) also supported the morphological similarity between the new species and the two related known species (*S. angulistylum* s. str. and *S. isanense*) as phylogenetic analysis retrieved them all from a single clade and with a low level of interspecific genetic divergence (1.74%). This might possibly have resulted from incomplete lineage sorting as they are likely to share a recent common ancestor. Despite limitation of molecular genetic differentiation, the new species was distinctly different from two other cytoforms of *S. angulistylum* complex based on polytene chromosome banding patterns and ecology of the immature stages. Thus, this study highlights the necessity of using an integrated approach for fully understanding black fly biodiversity.

Keywords: cytoform; DNA barcode; species complex; taxonomy.

INTRODUCTION

Black flies (Diptera: Simuliidae) are insects of medical and veterinary importance. Many species are vectors of disease agents transmitted to humans and other animals. These blood-sucking insects are vectors of *Onchocerca volvulus*, the filarial nematode that is the causative agent of human onchocerciasis or river blindness. More than one million people have visual loss as a result of this disease (WHO, 2019). In addition to *O. volvulus*, there are at least 11 other *Onchocerca* species in wild animals that are transmitted by 20 black fly species (Adler & McCreadie, 2019). Occasionally, these *Onchocerca* species have been transmitted to human and cause disease "zoonotic onchocerciasis" (Takaoka *et al.*, 2012). Black flies also transmit other disease agents such as viruses and protozoa including haemosporidian parasites of the genus *Leucocytozoon* and *Trypanosoma*, thereby causing economically significant disease in poultry (Adler & McCreadie, 2019).

Given the medical and veterinary significance, accurate taxonomy is of paramount importance for every aspect of black fly studies. Accurate taxonomy of black flies often requires an integrated approach using morphology, cytology (i.e., banding patterns of polytene chromosomes) and molecular biology (Adler & Huang, 2011; Low *et al.*, 2016; Ya'cob *et al.*, 2017). Many morphological species are found to be a species complex when following cytogenetic or molecular examination (e.g., Pramual & Kuvangkadilok, 2012; Pramual *et al.*, 2015; Adler *et al.*, 2016). In Thailand, at least 10 morphological species have been recognized as cytologically species complexes (Pramual, 2021). Many cytological distinct forms of these species complexes have not yet been fully investigated.

The *Simulium angulistylum* Takaoka & Davies complex is one of the species complexes recorded in Thailand (Pramual & Kuvangkadilok, 2012). *Simulium angulistylum* s. str. was described from Malaysia by Takaoka and Davies (1995) and has also been recorded in Thailand (Takaoka *et al.*, 2019; Adler, 2022). It was placed in the *S. epistum* species-group in the subgenus *Gomphostilbia* Enderlein defined by Takaoka (2012). Based on banding patterns of the polytene chromosomes, populations of *S. angulistylum* in Thailand were cytologically identified into three cytoforms (A, B, and C) (Pramual & Kuvangkadilok, 2012). These cytoforms are shown to be molecularly and ecologically different (Pramual & Kuvangkadilok, 2012). Cytoform A was geographically widespread being recorded throughout Thailand while cytoform B was restricted to lower part of the northeastern region and cytoform C was only found on a high

elevation mountain (>1,000 m above sea level) in Loei province, also from the northeast Thailand (Pramual & Kuvangkadilok, 2012).

In this study, morphological characteristics of the female, male, pupa and mature larva of cytoform C were examined. Comparisons with other known species of the *S. epistum* species-group revealed that cytoform C is morphologically different. Therefore, cytoform C is formally described as a new black fly species. Molecular genetic data based on the mitochondrial cytochrome c oxidase I (COI) DNA barcoding region was also used to investigate molecular genetic differentiation between this new species and other members of *S. epistum* species-group.

MATERIALS AND METHODS

Sample collection

Black fly specimens of the novel species were sampled from a small stream in a natural forest at Phu Ruea mountain (17° 29′ 59″ N/ 101° 20′ 09″ E, elevation 1,141 m above sea level), Phu Ruea District, Loei Province, northeastern Thailand. Larvae and pupae were collected using fine forceps and fixed in 80% ethanol. Some pupae were reared to adults in plastic bottles. Adult specimens were fixed in 80% ethanol for further morphological examination.

Morphological examination and descriptions

Morphological characteristics of larval, pupal and adult specimens were examined under a stereomicroscope and a compound microscope. Dissected parts were cleared in hot 85% lactic acid, transferred to glycerin and then examined under a compound microscope. Morphological characteristics were measured using a microscopic micrometer. Illustrations were drawn using a camera lucida attached to a Leica DM 750 compound microscope. Descriptions of morphological characteristics followed the terminology of Takaoka and Suzuki (1984) and Adler *et al*. (2004). Type specimens were deposited in the Department of Biology, Mahasarakam University, Mahasarakam Province, Thailand.

DNA extraction, polymerase chain reaction (PCR), and sequencing.

DNA was extracted from the whole body of eight individuals using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn. Bhd, Malaysia). Polymerase chain reaction (PCR) of the COI gene barcoding region was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAA AGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*., 1994). PCR reaction conditions followed those of Tangkawanit *et al*. (2018). PCR products were checked with 1% agarose gel electrophoresis and purified using a PureDireX PCR CleanUp & Gel Extraction Kit (Bio-Helix, Taiwan). DNA sequencing was performed at ATCG Company Limited (Thailand Science Park (TSP), Pathumthani, Thailand) using the same primers as for PCR.

DNA sequence analysis

A total of eight sequences of the new species were obtained from three developmental stages, i.e., adult (4 specimens, 2 males and 2 females), pupa (2 specimens) and larva (2 specimens). In total, 101 COI sequences from closely related species of the *S. epistum* species-group were retrieved from GenBank and, together with eight sequences from the new species (GenBank accession numbers: ON351561-ON351568), were used for genetic relationship analyses. Intraspecific and interspecific genetic divergences were calculated based on the Kimura-2-parameter (K2P) model in TaxonDNA 1.7.8 (Meier *et al*., 2006). Phylogenetic relationships were inferred using maximum likelihood (ML) method. The ML tree analysis was performed in the RAxML web server version (https://raxml-ng.vital-it.ch) (Kozlov *et al*., 2019). The generalized time-reversible nucleotide substitution model with gamma correction and proportion of invariant sites with 100 replicate bootstrapping, were selected for ML tree analysis. Sequences of *S. chumpornense* (HM775279, MT262570) and the *S. siamense* complex (HQ738668) were used as outgroups.

RESULTS

Taxonomy

This new species is also placed in the *S. epistum* species-group in the subgenus *Gomphostilbia* by having the following diagnostic characteristics: in the female and male by the antenna with nine flagellomeres and pleural membrane bare; in the female by the claw with a large basal tooth, hair tuft on the base of the radial vein yellowish, and hind tibia yellow on the basal half with a subbasal dark spot; in the male by the hind basitarsus slender and nearly parallel-sided, ventral plate transverse, much produced ventrally; in the pupa by the gill with eight slender short filaments and terminal hook widened with its outer margin crenulated; and in the larva by the postgenal cleft long, approaching to the posterior margin of the hypostoma, and abdominal segments 5–8 densely covered with dark minute setae each with 4–8 branches.

***Simulium* (*Gomphostilbia*) *prayooki* Pramual, Jomkumsing, Thongyan, Wongpakam & Takaoka sp. nov.**

Female

Body length 2.3–2.6 mm (n = 3). Head: Slightly narrower than width of thorax. Frons brownish black, moderately covered with yellow fine hairs intermixed with several longer hairs along each lateral margin; frontal ratio 1.7:1.0:2.8; frons:head ratio 1.0:5.2–5.4. Fronto-ocular area well developed, directed dorsolaterally. Clypeus brownish black, densely covered with yellowish-white scale-like hairs interspersed with several dark longer hairs on each side of lower half. Labrum 0.7 as long as clypeus. Antenna composed of scape, pedicel and 9 flagellomeres; scape, pedicel and basal half of first flagellomere light brown, other flagellomeres medium brown. Maxillary palpus composed of 5 segments, medium brown, proportional length of third, fourth, and fifth articles segments 1.00:1.1–1.2:2.4–2.6; sensory vesicle (Figure 1A) ellipsoidal, 0.4 times as long as third segment, and with moderate-sized opening near apex. Lacinia with 11 inner and 13–15 outer teeth. Mandible with 20–25 inner teeth and 11–15 outer teeth. Cibarium (Figure 1B) with blunt short and broad median projection on posterior margin and weakly sclerotized mediolongitudinal ridge.

Thorax. Scutum brownish black, shiny when illuminated in front and viewed dorsally, densely covered with whitish-yellow recumbent short hairs, and with several dark brown long upright hairs on prescutellar area. Scutellum dark brown, covered with short yellow hairs and long dark upright hairs. Postnotum dark brown, bare. Katepisternum dark brown, longer than deep, moderately covered with fine short hairs. ***Legs***. Foreleg (Figure 1C): coxa and trochanter yellow except apical half of trochanter light brown; femur light brown with apical cap medium brown though extreme tip yellow; tibia whitish yellow on extreme base, light brown on basal half with medium brown marking near base, median area of outer surface whitish yellow, apical 1/3 medium brown though extreme apical tip whitish yellow; tarsus brownish black with moderate dorsal hair crest; basitarsus moderately dilated, 6.6 times as long as its greatest width. Midleg (Figure 1D): coxa light brown; trochanter whitish yellow on basal 1/2 and brown on rest; femur light brown with medium brown apical cap; tibia light brown on basal 1/4 with medium brown subbasal spot, and medium brown on apical 3/4; tarsus dark brown except basal half of basitarsus and base of first tarsomere light brown. Hind leg (Figure 1E): coxa yellow except anterior and posterior surface medium brown; trochanter yellow; femur light brown except base yellow and apical cap medium brown though extreme tip whitish yellow; tibia yellow on basal half with

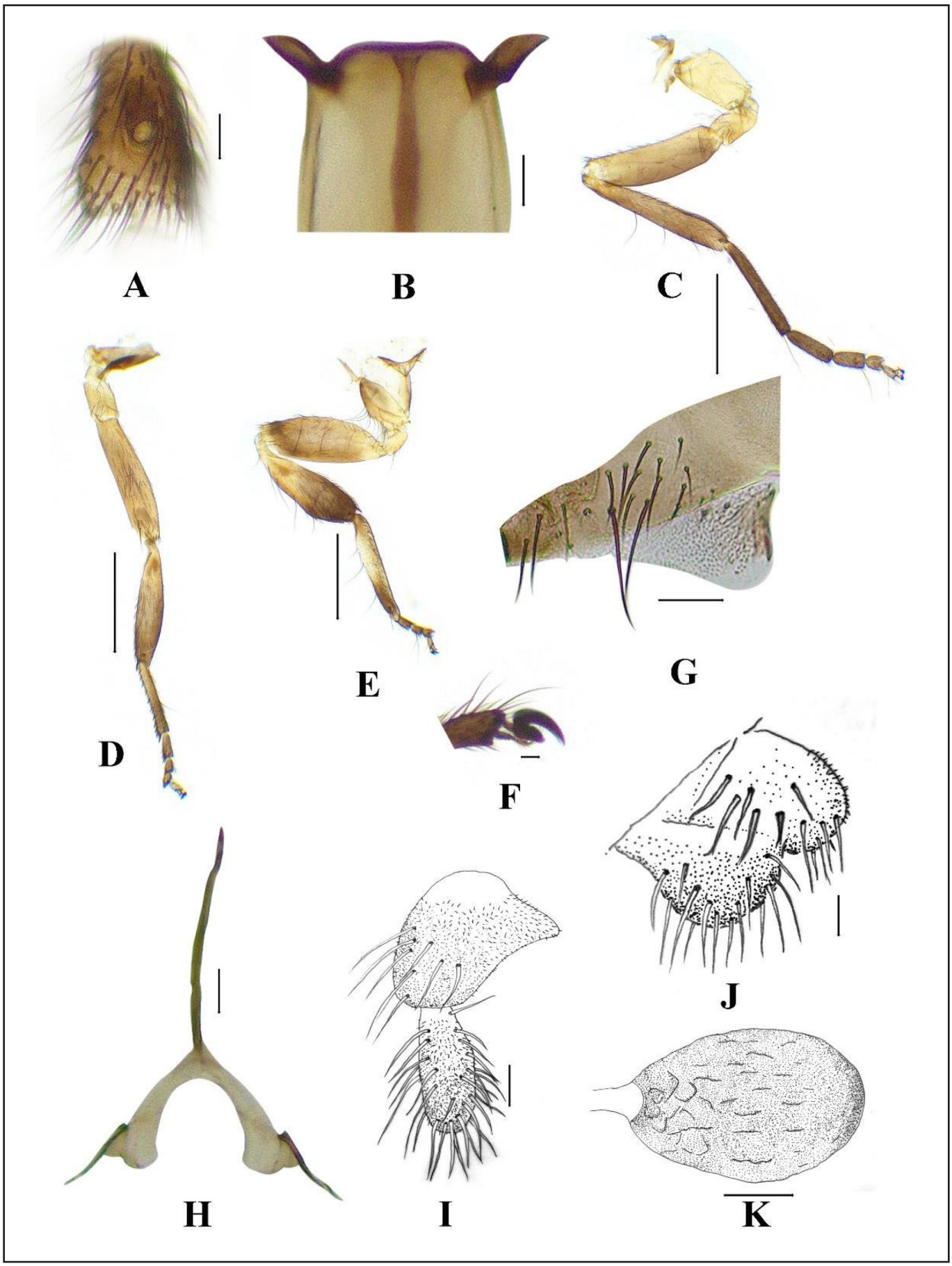

Figure 1. Female of *Simulium prayooki* sp. nov. (A) Third segment of maxillary palpus with sensory vesicle (left side; front view). (B) Cibarium (only half was shown; front view). (C) Fore leg. (D) Mid leg. (E) Hind leg. (F) Claw. (G) Sternite 8 and ovipositor valve (ventral view; right half). (H) Genital fork (ventral view). (I) and (J) Paraprocts and cerci (I, ventral view; J, lateral view). (K) Spermatheca. Scale bars: 0.5 mm for C–E; 0.02 mm for A–B, G–K; 0.01 mm for F.

medium brown subbasal spot, and medium brown on apical half; tarsus brownish black except basal 2/3 of basitarsus whitish yellow; basitarsus widened from base to basal 1/3, then gradually narrowed to apex, 7.5–8.0 times as long as wide; calcipala well developed, as long as width at base; pedisulcus well developed; claw (Figure 1F) with large basal tooth 0.63–0.66 times length of claw. ***Wing***. Length 2.0–2.2 mm. Costa with dark spinules and hairs. Subcosta haired except near apex where bare. Basal section of radius fully haired; R_1 with dark spinules and hairs; R_2 with hairs. Basal cell absent. ***Halter***. White except basal portion darkened. ***Abdomen***, Basal scale light brown, with fringe of whitish yellow hairs. Dorsal surface of abdomen medium brown to dark brown except basal half of segment 2 yellow, moderately covered with dark medium to long hairs and short yellow hairs; ventral surface of segments 2–3 white, and those of other segments medium to dark brown. ***Terminalia***. Sternite 8 (Figure 1G) bare medially, with 14 or 15 dark short to long hairs on each lateral surface. Ovipositor valve (Figure 1G) rounded posteomedially, membranous but sclerotized on the inner margin, each moderately covered with microsetae and several short to medium-long hairs, except portion along inner and posterior margins narrowly bare, inner margin slightly concave medially. Genital fork (Figure 1H) of inverted-Y form, with slender dark stem, and arms of moderate width, each broadened from base to apex without projection directed posteromedially. Paraproct in ventral view (Figure 1I) nearly triangular, with anterior surface bare, and anteromedial surface well sclerotized; paraproct in lateral view (Figure 1J) markedly produced ventrally beyond ventral tip of cercus, 0.6 times as long as wide, and with 15–17 medium-long to long hairs on lateral and ventral surfaces. Cercus in lateral view (Figure 1J) short, rounded posteriorly, 0.6 times as long as its greatest width. Spermatheca (Figure 1K) ellipsoidal, 1.5 times as long as its greatest width, well sclerotized and pigmented except junction with duct, without internal setae, and accessory ducts unpigmented, subequal in thickness to each other and to major duct.

Male

Body length 2.2–2.5 mm (n = 3). ***Head***. Slightly wider than thorax. Upper eye medium brown, consisting of large facets in 11 or 12 vertical columns and 11 or 12 horizontal rows. Clypeus brownish black, white pruinose when illuminated dorsally and viewed anteriorly, densely covered with yellow hairs interspersed with several dark simple long hairs. Antenna as in female. Maxillary palpus light to medium brown, composed of five segments, proportional lengths of third, fourth and fifth segments 1.0:1.2–1.3:2.7–2.8; third segment (Figure 2A) widened apically; sensory vesicle (Figure 2A) ellipsoidal, 0.4 times as long as third segment with moderate-sized opening near apex. ***Thorax***. As in female. ***Legs***. (Figure 2B–D). Color as in female. Fore basitarsus (Figure 2B) moderately dilated, 7.5–7.6 times as long as its greatest width. Hind basitarsus (Figure 2D) nearly parallel sided, 5.5 times as long as wide; calcipala slightly longer than basal width. Pedisulcus well developed. ***Wing***. Length 1.9–2.0 mm. Other characteristics as in female except subcosta bare. Halter pale grayish except basal stem darkened. ***Abdomen***. Basal scale light brown, with fringe of yellow hairs. Dorsal surface of abdomen brownish black except entire surface of segment 2 and anterior 1/4 of segment 3 yellow, moderately covered with dark brown unbranched hairs; ventral surface of segment 2 whitish, and those of other segments light to medium brown. ***Genitalia***. Coxite in ventral view (Figure 2E) nearly rectangular, 1.5 times as long as its greatest width. Style in ventral view (Figure 2E) broad on basal 2/3 and tapered apically, bent inward, and with a spine at apex; style in ventrolateral view (Figure 2F) broad and nearly parallel-sided from base to apical 1/3, then abruptly bent inward. Ventral plate in ventral view (Figure 2E) with body transverse, 0.5 times as long as wide, anterior margin produced anteromedially, posterior margin nearly straight, lateral margins much narrowed posteriorly from middle, densely covered with microsetae on ventral surface except anterolateral areas bare; basal arm of moderate length, nearly parallel-sided; ventral plate in lateral view (Figure 2G) moderately produced ventrally and dorsally; ventral plate in caudal view (Figure 2H) with ventral margin rounded and dorsal margin nearly straight medially, densely covered with microsetae on posterior surface except dorsolateral areas bare. Median sclerite (Figure 2I) weakly sclerotized medially. Paramere (Figure 2J) of moderate size, with four long hooks and several short hooks. Aedeagal membrane moderately covered with microsetae.

Pupa

Body length 2.5–3.1 mm (n = 4). ***Head*.** Integument ochreous, densely covered with small round tubercles except antennal sheaths and ventral surface almost bare; antennal sheath without protuberances; frons with three long unbranched trichomes arising close together on each side; face with one long unbranched trichome on each side. ***Thorax*.** Integument ochreous, densely covered with round tubercles, except posterior half moderately covered with tubercles on dorsal surface and almost bare on lateral surface, and with six thoracic trichomes (three long anterodorsally, two long anterolaterally, the anterior of trichome is slightly shorter than the posterior trichome and one medium-long mediolaterally) on each side. All thoracic trichomes unbranched. Gill (Figure 3A) composed of eight slender filaments arranged as 3+(1+2)+2 from dorsal to ventral; two triplets and one ventral pair arising close together but independently at same level from short common basal stalk; each with short primary stalk; dorsal and middle triplets not sharing primary stalk; dorsal triplet composed of three individual filaments arising at same level or one individual and two paired filaments; middle triplet composed of one individual and two pair filaments with very short secondary stalk; ventral pair with very short stalk; all filaments subequal in length although one of the three filaments of middle triplet slightly longer the others; ventral filament of ventral pair relatively thicker than others, relative thickness of eight filaments, when measured at basally, from dorsal to ventral 1.0:1 .0:1.0:1.4:1.0:1.4:1.2:1.8; all filaments tapering toward apex, with numerous transverse ridges and furrows, and densely covered with minute tubercles. ***Abdomen***. Dorsally, segment 1, 2 and 9 almost entirely light brown, segments 3, 4 and 5 each with narrowly partially light brown narrowly along anterior margins, segments 6–8 unpigmented; segment 1 with unbranched hair-like seta on each side; segment 2 with one unbranched slender short hair-like seta and five minute setae near posterior margin; segments 3 and 4 each with one colorless minute seta submedially near anterior margin and four darkened hooked spines, one minute colorless seta near posterior margin one each side; segment 5 with four minute colorless setae near posterior margin on each side and without spine-combs; segments 6–9 each with spine-combs in transverse row and comb-like groups of minute spines on each side; segments 6–8 each with two minute colorless setae near posterior margin on each side; segment 9 with a pair of wide terminal hooks, of which outer margins are about 2.5 times as long as inner margins, and crenulated (Figure 3B). Ventrally, segment 4 with one unbranched hook and three minute colorless setae on each side; segment 5 with a pair of bifid and trifid hooks submedially and two minute colorless setae on each side; segments 6 and 7 each with a pair of bifid (inner) and unbranched (outer) hooks and three minute colorless setae on each side; segment 4–9 each with comb-like groups of microspines; segment 9 with three grapnel-shaped hooklets on each lateral surface. ***Cocoon*.** Wall-pocket shaped, thickly woven, without anterodorsal projection.

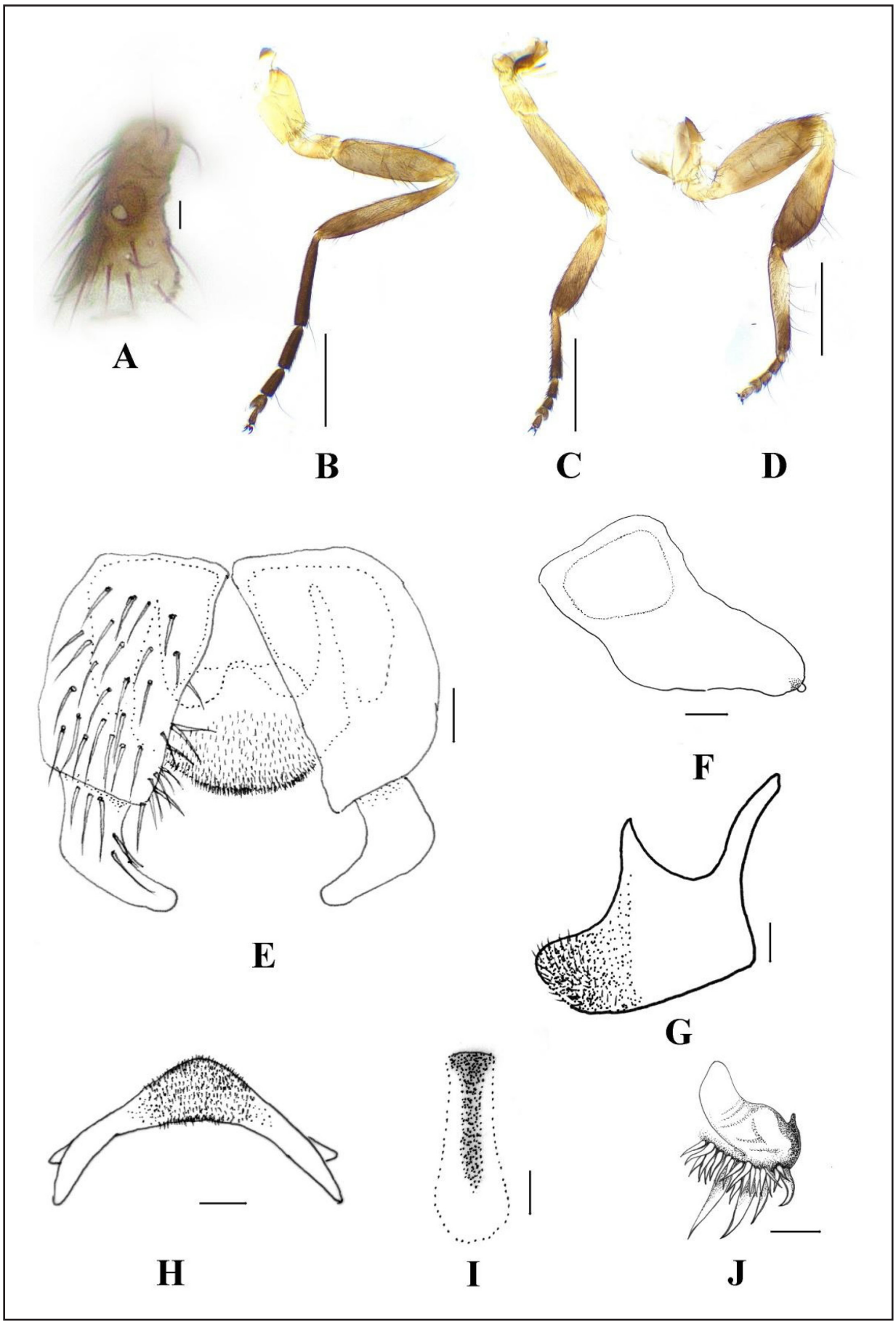

Figure 2. Male of *Simulium prayooki* sp. nov. (A) Third segment of maxillary palpus with sensory vesicle (left side; front view). (B) Fore leg. (C) Mid leg. (D) Hind leg. (E) Coxite, style (right side) and ventral plate (ventral view). (F) Style (right side, ventrolateral view). (G) Ventral plate (lateral view). (H) Ventral plate (caudal view). (I) Median sclerite (caudal view). (J) Paramere (left side, caudal view). Scale bars: 0.5 mm for B–D; 0.02 for A, F–J.

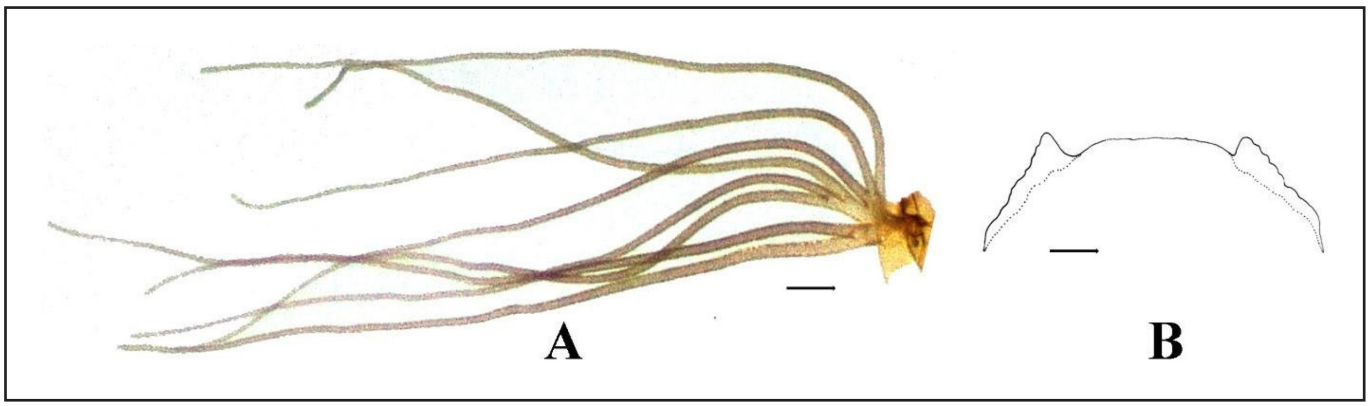

Figure 3. Pupa of *Simulium prayooki* sp. nov. (A) Gill filaments (left side; outer view) (B) Terminal hooks (caudal view). Scale bars: 0.1 mm for A; 0.02 mm for B.

Mature larva

Body length 5.4–5.9 mm (n = 5). Body grayish, mottled with reddish-brown pigments. ***Head***. Head capsule moderately covered with colorless unbranched minute setae dorsally; cephalic apotome light brown; two weakly delimited mediolongitudinal head spot groups; posterior margin of lateral surface dark brown; area around eye-spot region pale yellow to white; eyebrow well defined; anterolateral surface light brown; ventral surface of head capsule light brown except each basal side of postgenal cleft dark brown. Antenna composed of three articles and apical sensillum, longer than stem of labral fan; proportional lengths of first, second and third articles 1.00:1.14:0.86. Labral fan with 38–40 primary rays. Mandible (Figure 4A) with three comb-teeth decreasing in length from first tooth to third; mandibular serration composed of two teeth (one medium-sized, one small-sized); supernumerary serrations absent. Hypostoma (Figure 4B) with row of nine apical teeth, median tooth is slightly longer than each corner tooth; three intermediate teeth on each side shorter than corner tooth; lateral margin smooth, with five hypostomal bristles per side, lying nearly parallel to each lateral margin. Postgenal cleft (Figure 4C) long, nearly reach postgenal bridge, widen to middle then narrowed to apex. ***Thorax*** and ***Abdomen***. Thorax and abdominal segments 1–4 sparsely covered with unbranched minute colorless setae dorsally; segments 5–8 densely covered with dark setae each with 4–8 branches (mostly 7 and 8 branches) (Figure 4D) on dorsal and dorsolateral surfaces; last abdominal segment moderately covered with unbranched colorless setae on dorsolateral and lateral surfaces. Rectal organ compound, with 16–18 finger-like secondary lobules per lobe. Anal sclerite of X-form, with anterior arms 0.9 times as long as posterior ones; broadly sclerotized at base; accessory sclerite absent. Last abdominal segment with a pair of conical ventral papillae. Posterior circlet with 85–90 rows of hooklets each with 16–20 hooklets.

Type materials

HOLOTYPE: Female adult with its associated pupal exuviae and cocoon (in 80% ethanol): THAILAND, Loei Province, Phu Ruea District, Phu Ruea Mountain (17°29′59″ N, 101°20′09″ E), collected from a small (width 2 m), shallow (depth 4 cm), moderate flow (0.94 m/s) stream, with boulder and rocky bottom in a completely shaded area, 1,141 m, 28-V-2017, by P. Pramual. Deposited in Department of Biology, Faculty of Science, Mahasarakham University, Thailand.

PARATYPES. Adults: Two females, three males (all with their associated pupal exuviae and cocoons), four pupae and five mature larvae (in 80% ethanol), same data as those of holotype.

Biological notes. Immature stages (i.e., larva and pupa) of this species co-exist with *S*. nr. *feuerborni*, *S*. nr. *asakoae* and *S. yuphae*. The biting habit of this species is unknown.

Etymology. The species name *prayooki* is in honor of Associate Professor Dr. Prayook Sriwilai, President of Mahasarakham University, who has continuously supported the senior author to work on biodiversity of black flies in Thailand.

DNA sequence variation and DNA barcode tree

Ten COI sequences reported previously as *S. angulistylum* cytoform C by Pramual and Kuvangkadilok (2012) were included as members of the new species. Therefore, a total of 18 COI sequences were included in data analysis for *S. prayooki* sp. nov. The intraspecific genetic divergence based on the K2P model ranged between 0 and 3.34% (Table 1). Interspecific genetic divergence between the new species and other members of the *S. epistum* species-group ranged between 0.69% (when compared to *S. angulistylum* cytoform B) and 15.19% (when compared to *S. atratum* De Meijere and *S. cheongi* Takaoka & Davies) (Table 1). Maximum intraspecific genetic divergence for other members of the *S. epistum* species-group was generally low (<2.31%) except for *S. angulistylum* cytoform B, which showed greatest divergence (5.55%). Minimum interspecific genetic divergence was lowest between *S. cheongi* and *S. whartoni* Takaoka & Davies with no genetic differentiation. Greatest minimum interspecific genetic divergence was between *S. isanense* Takaoka, Srisuka & Saeung and *S. whartoni* (14.20%) (Table 1).

The maximum likelihood tree (Figure 5) inferred from COI sequences divided six taxa members of the *S. epistum* species-group into two clades (I, II). The *S. angulistylum* s. str. from Malaysia, cytoform A, B, *S. isanense* and *S. prayooki* sp. nov. formed one clade (I) and *S. cheongi*, *S. atratum* and *S. whartoni* formed another clade (II). All sequences of *S. prayooki* sp. nov. obtained in the present study from all three known life stages (larva, pupa and adult) and those of *S. agnulistylum* cytoform C formed a subclade sister to the *S. angulistylum* s. str. from Malaysia, cytoform A and B, although with weak support (50%). Two sequences of *S. angulistylum* 'B' reported by Pramual and Kuvangkadilok (2012) were also included in this clade. Remaining sequences of *S. angulistylum* 'B' formed a subclade sister to *S. isanense*. In the clade II, *S. whartoni* and *S. atratum* each formed monophyletic clades but were included in the clade of *S. cheongi*.

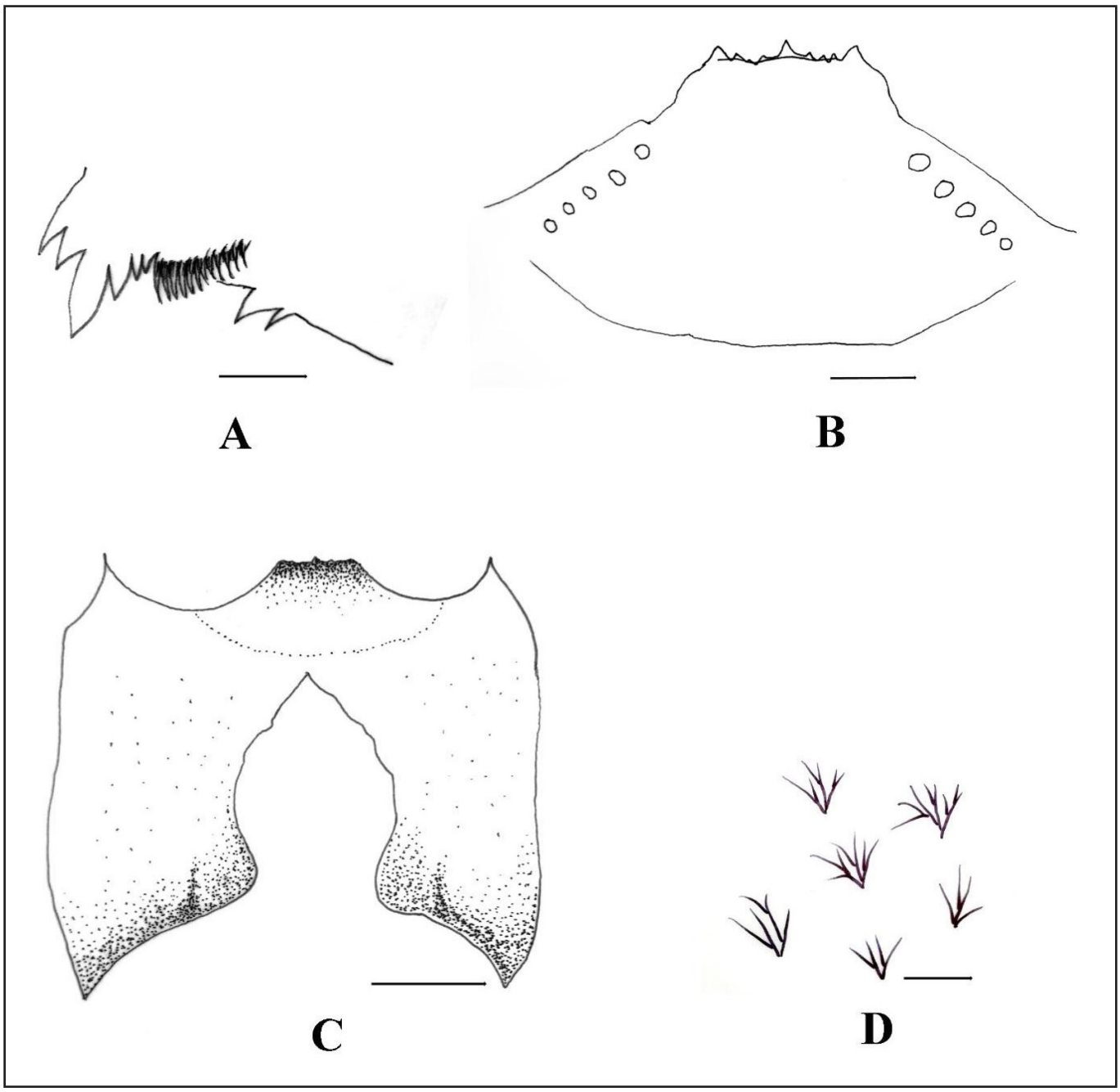

Figure 4. Larva of *Simulium prayooki* sp. nov. (A) Mandible. (B) Hypostoma. (C) Head capsule showing postgenal cleft (ventral view). (D) Dark multi-branched setae on dorsal surface of abdominal segment 8. Scale bars: 0.1 mm for C; 0.02 mm for A, B and D.

Table 1. Intraspecific (bold) and interspecific genetic divergences (%) of *Simulium prayooki* sp. nov. and other members of *S. epistum* species-group

	S. prayooki (n = 18)	*S. angulistylum* s. str. (n = 3)	*S. angulistylum* cytoform A (n = 13)	*S. angulistylum* cytoform B (n = 13)	*S. isanense* (n = 3)	*S. atratum* (n = 4)	*S. whartoni* (n = 3)	*S. cheongi* (n = 45)
S. prayooki	**0–3.34**							
S. angulistylum s. str.	1.74–3.31	**0–0.21**						
S. angulistylum cytoform A	1.21–3.53	0.43–1.74	**0–2.27**					
S. angulistylum cytoform B	0.69–5.75	1.30–5.40	1.03–5.57	**0–5.55**				
S. isanense	4.32–6.32	5.23–5.70	4.68–5.86	2.40–5.84	**0.20–0.63**			
S. atratum	12.19–15.19	13.70–14.73	13.60–15.46	12.55–16.02	13.95–5.26	**0.00–1.05**		
S. whartoni	13.19–15.17	14.21–15.23	14.11–15.98	12.55–15.75	14.20–14.99	2.56–4.10	**0.42–1.05**	
S. cheongi	12.06–15.19	13.47–15.28	12.83–15.74	11.66–15.75	13.22–15.30	2.56–4.12	0.00–2.34	**0–2.31**

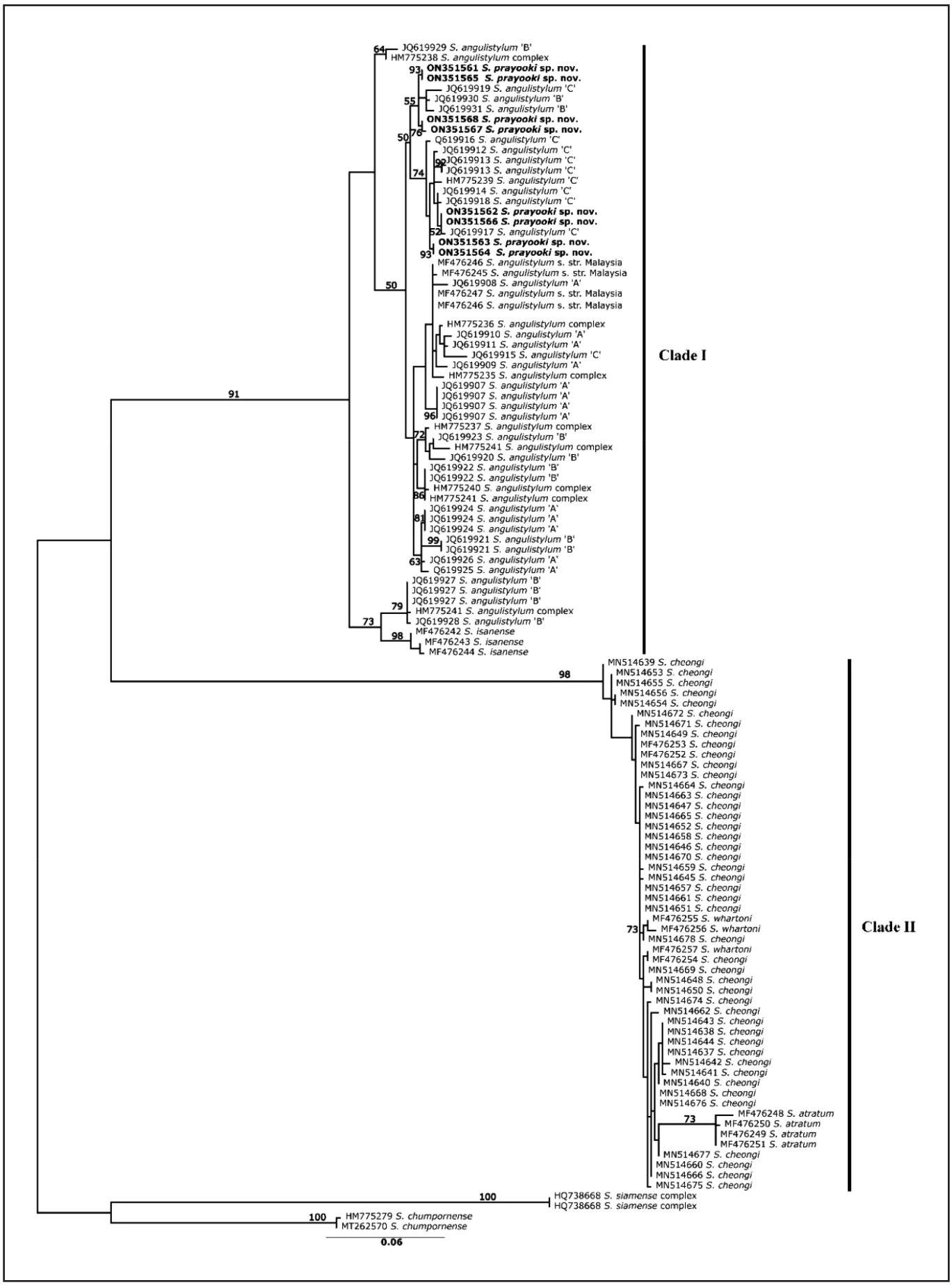

Figure 5. Maximum likelihood tree inferred from mitochondrial COI sequences of five taxa members of the *S. epistum* species-group and the new species. Bold characters indicate specimens obtained in this study. Names of the species and cytoforms of *Simulium angulistylum* complex are listed following the GenBank accession number. Sequences for which cytoform is unknown are indicated as *S. angulistylum* complex.

DISCUSSION

This new species is characterized in the female by the hind basitarsus relatively narrow, 7.5–8.0 times as long as its greatest width and claw with a relatively long basal tooth 0.63–0.66 times as long as the claw; in the male by the number of upper-eye (large) facets in 11 or 12 vertical columns and 11 or 12 horizontal rows; in the pupa by the gill having eight filaments arranged in three groups that arise independently at the same level from a short common stalk, the ventral filament of the ventral pair is 1.5 times thicker than others, and dorsal surface of abdominal segments 1–5 and 9 entirely or partially light brown; and in the larva by the postgenal cleft long, approaching to the posterior margin of the hypostoma, abdominal segments 5–8 being densely covered by dark spinous setae each with 4–8 branches (mostly 7 and 8 branches), and the rectal organ with three lobes, each with 16–18 finger-like secondary lobules.

The larval stage of this new species was formerly recognized as one cytoform of the *S. angulistylum* complex (cytoform C) (Pramual & Kuvangkadilok, 2012) and several morphological characteristics of the larval stage are shared with *S. angulistylum* s. str. including the long postgenal cleft nearly reaching the posterior margin of the hypostoma and abdominal segments 5–8 densely covered with dark setae each with 4–8 branches.

This new species is morphologically similar to *S. angulistylum* s. str. and *S. isanense* in many characteristics including color of adult legs, wide and inwardly twisted style, arrangement of the pupal gill, and larval abdomen densely covered with multibranched dark setae dorsally, but is distinguished from the latter two species in the female by the relatively slender hind basitarsus 7.5–8.0 times as long as its greatest width (6.4–6.5 times in the latter two species), claw with a relatively long basal tooth 0.63–0.66 times as long as the claw (0.50–0.51 in the latter two species), and genital fork lacking a projection directed posteromedially (present in the latter two species), and in the larva by the greater number (16–18) of secondary lobules per lobe of the rectal organ (8 or 9 and 9–14 in *S. angulistylum* s. str. and *S. isanense*, respectively) (Takaoka & Davies, 1995; Takaoka *et al*., 2017). Further it is distinguished from *S. angulistylum* s. str. in the male by the relatively slender fore basitarsus 7.5–7.6 times as long as its greatest width (5.5 times in *S. angulistylum* s. str.), and in the pupa by the dorsum of abdominal segments 1–5 entirely or partially darkened (not darkened in *S. angulistylum* s. str.); and from *S. isanense* in the female by the relatively dilated fore basitarsus 6.6 times as long as its greatest width (7.3–7.4 times in *S. isanense*) (Takaoka & Davies, 1995; Takaoka *et al*., 2017).

Molecular genetic analysis supported the conclusion based on morphology that *S. prayooki* sp. nov. is closely related to the other cytoforms of the *S. angulistylum* complex and to *S. isanense* as revealed by the ML tree. Lowest interspecific genetic divergence was found between *S. prayooki* sp. nov. and the *S. angulistylum* cytoform B (0.69%) that overlapped with intraspecific genetic divergence. No or very low interspecific genetic divergence is not uncommon for tropical black fly species, particularly for members of the subgenus *Gomphostilbia* (Pramual *et al*., 2011, 2021; Pramual & Adler, 2014; Takaoka *et al*., 2020; Srisuka *et al*., 2021). Recent DNA barcoding analysis found that 12 species of black flies in Thailand have no genetic differentiation based on their COI sequence (Pramual *et al*., 2021). In the *S. epistum* species-group, no genetic differentiation was found between *S. whartoni* and *S. cheongi* based on COI sequences. Low genetic variation between closely related species could be due to misidentification, incomplete lineage sorting or inadequate phylogenetic signal for the COI sequences because they had possibly diverged from a common ancestor only recently (Takaoka *et al*., 2020). Thus, more variable molecular markers are needed, such as big zinc finger (BZF) and the elongation complex protein 1 gene (ECP1) that have been successfully used for differentiation of the closely related black fly species in Thailand (Thaijarern *et al*., 2017; Aupalee *et al*., 2020).

Despite close molecular similarity to other cytofoms of the *S. angulistylum* complex, the new species is readily distinguishable based on polytene chromosome banding patterns. As has been reported previously, the new species is cytoform C of the *S. angulistylum* complex that has two fixed chromosome inversions, one on the long arm of chromosome II (*IIL-2*) and another on the short arm of chromosome III (*IIIS-1*) (Pramual & Kuvangkadilok, 2012). However, chromosomes of *S. angulistylum* s. str. from Malaysia have not yet been investigated, although based on COI sequences, *S. angulistylum* s. str. was genetically most similar to *S. angulistylum* cytoform A with minimum genetic divergent of 0.43%. Therefore, it will be useful to examine whether *S. angulistylum* s. str. is chromosomally the same as cytoform A reported in Thailand or not. Further studies are also needed to examine whether this new species differs morphologically from cytoforms A and B of the *S. angulistylum* species-complex.

In addition to cytogenetic differences, the new species is also ecologically different from the other cytoforms of the *S. angulistylum* complex. This new species was found only at high elevation (>1,000 m above sea level) area while the other cytoforms of the the *S. angulistylum* complex and *S. isanense*, both occur at lower (<600 m) elevations (Pramual & Kuvangkadilok, 2012; Takaoka *et al*., 2017).

In conclusion, the *S. angulistylum* cytoform C recorded from a high mountain in northeastern Thailand was formally described as a new species, *S. prayooki* sp. nov. This new species is molecularly similar to *S. angulistylum* s. str. and *S. isanense*. However, there are certain morphological characteristics in the female, male, pupa and larva that distinguish this new species from the closely related taxa and all other members of *S. epistum* species-group. In addition, the new species is ecologically unique among members of this species-group because the immature stages (i.e., larva and pupa) are restricted to only high (>1,000 m) mountainous streams. Two remaining cytoforms (A and B) of the *S. angulistylum* complex in Thailand have not yet been morphologically examined. Thus, further investigation will be useful for species status evaluation of these cytoforms.

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Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Adler, P.H. (2022). World blackflies (Diptera: Simuliidae): a comprehensive revision of the taxonomic and geographical inventory [2021]. https://biomia.sites.clemson.edu/pdfs/blackflyinventory.pdf. Accessed 5 April 2022.

Adler, P.H., Currie, D.C. & Wood, D.M. (2004). The Black Flies (Simuliidae) of North America. New York: Cornell University Press. pp. xv+941.

Adler, P.H. & Huang, Y.T. (2011). Integrated systematics of the Simuliidae (Diptera): evolutionary relationships of the little-known Palearctic black fly *Simulium acrotrichum*. *The Canadian Entomologist* **143**: 612-628. https://doi.org/10.4039/n11-035

Adler, P.H. & McCreadie, J.W. (2019). Black flies (Simuliidae). In: Medical and Veterinary Entomology, Mullen, G.R. & Durden, L.A. (editors), 3rd edition. San Diego, CA: Elsevier, pp. 237-259. https://doi.org/10.1016/B978-0-12-814043-7.00014-5

Adler, P.H., Takaoka, H., Sofian-Azirun, M., Low, V.L., Ya'cob, Z., Chen, C.D., Lau, K.W. & Pham, X.D. (2016). Vietnam, a hotspot for chromosomal diversity and cryptic species in black flies (Diptera: Simuliidae). *PLoS One* **11**: e0163881. https://doi.org/10.1371/journal.pone.0163881

Aupalee, K., Saeung, A., Srisuka, W., Fukuda, M., Junkum, A., Pitasawat, B. & Takaoka, H. (2020). Three new species of the *Simulium* (*Simulium striatum* species-group (Diptera: Simuliidae) from Thailand, with their phylogenetic relationships. *Acta Tropica* **211**: 1-14. https://doi.org/10.1016/j.actatropica.2020.105625

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294-299.

Kozlov, A.M., Darriba, D., Flouri, T., Morel, B. & Stamatakis, A. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* **35**: 4453-4455. https://doi.org/10.1093/bioinformatics/btz305

Low, V.L., Takaoka, H., Pramual, P., Adler, P.H., Ya'cob, Z., Huang, Y.T., Da Pham, X., Ramli, R., Chen, C.D., Wannaket, A. *et al*. (2016). Delineating taxonomic boundaries in the largest species complex of black flies (Simuliidae) in the Oriental Region. *Scientific Reports* **6**: 1-8. https://doi.org/10.1038/srep20346

Meier, R., Shiyang, K., Vaidya, G. & Ng, P.K. (2006). DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Systematic Biology* **55**: 715-728. https://doi.org/10.1080/10635150600969864

Pramual, P. (2021). Black fly diversity and impacts on human welfare in Southeast Asia. In: Biodiversity of Southeast Asian Parasites and Vectors causing Human Disease, Petney, T.N., Saijuntha, W. & Mehlhorn, H. (editors), Parasitology Research Monographs, 14. Springer, Cham. pp. 143-164. https://doi.org/10.1007/978-3-030-71161-0_7

Pramual, P. & Adler, P.H. (2014). DNA barcoding of tropical black flies (Diptera: Simuliidae) of Thailand. *Molecular Ecology Resources* **14**: 262-271. https://doi.org/10.1111/1755-0998.12174

Pramual, P., Jomkumsing, P., Wongpakam, K. & Wongwian, P. (2021). DNA barcoding of tropical black flies (Diptera: Simuliidae) in Thailand: One decade of progress. *Acta Tropica* **224**: 106116. https://doi.org/10.1016/j.actatropica.2021.106116

Pramual, P. & Kuvangkadilok, C. (2012). Integrated cytogenetic, ecological, and DNA barcode study reveals cryptic diversity in *Simulium* (*Gomphostilbia*) *angulistylum* (Diptera: Simuliidae). *Genome* **55**: 447-458. https://doi.org/10.1139/g2012-031

Pramual, P., Thaijarern, J., Sofian-Azirun, M., Ya'cob, Z., Hadi, U.K. & Takaoka, H. (2015). Cytogenetic and molecular evidence of additional cryptic diversity in high elevation black fly *Simulium feuerborni* (Diptera: Simuliidae) populations in Southeast Asia. *Journal of Medical Entomology* **52**: 829-836. https://doi.org/10.1093/jme/tjv080

Pramual, P., Wongpakam, K. & Adler, P.H. (2011). Cryptic biodiversity and phylogenetic relationships revealed by DNA barcoding of Oriental black flies in the subgenus *Gomphostilbia* (Diptera: Simuliidae). *Genome* **54**: 1-9. https://doi.org/10.1139/G10-100

Srisuka, W., Aupalee, K., Low, V.L., Ya'cob, Z., Fukuda, M., Saeung, A. & Takaoka, H. (2021). A new species of *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) from Northern Thailand, with its genetic relationship in the *S. asakoae* species-group. *Acta Tropica* **218**: 105889. https://doi.org/10.1016/j.actatropica.2021.105889

Takaoka, H. (2012). Morphotaxonomic revision of *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) in the Oriental Region. *Zootaxa* **3577**: 1-42. https://doi.org/10.11646/zootaxa.3577.1.1

Takaoka, H. & Davies, D.M. (1995). The black flies (Diptera: Simuliidae) of West Malaysia. Fukuoka: Kyushu University Press, pp. viii+175.

Takaoka, H., Fukuda, M., Otsuka, Y., Aoki, C., Uni, S. & Bain, O. (2012). Blackfly vectors of zoonotic onchocerciasis in Japan. *Medical and Veterinary Entomology* **26**: 372-378. https://doi.org/10.1111/j.1365-2915.2012.01023.x

Takaoka, H., Srisuka, W., Fukuda, M. & Saeung, A. (2020). Twenty-one species of the *Simulium* (*Gomphostilbia*) *asakoae* species-group (Diptera: Simuliidae) in Thailand. *ZooKeys* **950**: 51-152. https://doi.org/10.3897%2Fzookeys.950.51298

Takaoka, H., Srisuka, W., Low, V.L., & Saeung, A. (2017). A new black fly species of the *Simulium* (*Gomphostilbia*) *epistum* species-group (Diptera: Simuliidae) from Thailand. *Acta Tropica* **176**: 373-379. https://doi.org/10.1016/j.actatropica.2017.09.006

Takaoka, H., Srisuka, W. & Saeung, A. (2019). Checklist and keys for the black flies (Diptera: Simuliidae) in Thailand. *Medical Entomology and Zoology* **70**: 53-77. https://doi.org/10.7601/mez.70.53

Takaoka, H. & Suzuki, H. (1984). The blackflies (Diptera: Simuliidae) from Thailand. *Medical Entomology and Zoology* **35**: 7-45. https://doi.org/10.7601/mez.35.7

Tangkawanit, U., Wongpakam, K. & Pramual, P. (2018). A new black fly (Diptera: Simuliidae) species of the subgenus *Asiosimulium* Takaoka Choochote from Thailand. *Zootaxa* **4388**: 111-122. https://doi.org/10.11646/zootaxa.4388.1.8

Thaijarern, J., Pramual, P. & Adler, P.H. (2017). Life-stage association of black flies, using a fast-evolving nuclear gene sequence, and description of the larva of *Simulium lampangense* Takaoka & Choochote (Diptera: Simuliidae) from Thailand. *Zootaxa* **4299**: 263-270. https://doi.org/10.11646/zootaxa.4299.2.6

WHO (World Health Organization) (2019). Onchocerciasis. https://www.who.int/news-room/fact-sheets/detail/onchocerciasis. Accessed 25 April 2022.

Ya'cob, Z., Takaoka, H., Low, V.L. & Sofian-Azirun, M. (2017). First description of a new cryptic species, *Simulium vanluni* from Peninsular Malaysia: an integrated morpho-taxonomical and genetic approach for naming cryptic species in the family Simuliidae. *Acta Tropica* **167**: 31-39. https://doi.org/10.1016/j.actatropica.2016.12.009