



RESEARCH ARTICLE

Morphological and genetic analyses of *Simulium (Gomphostilbia) okinawense* Takaoka and *S. (G.) tokarensis* Takaoka (Diptera: Simuliidae) from the Nansei Islands, Japan: redescription and transfer from the *S. ceylonicum* species-group to the *S. asakoe* species-group

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ARTICLE HISTORY

Received: 23 November 2022

Revised: 6 January 2023

Accepted: 6 January 2023

Published: 31 March 2023

ABSTRACT

Simulium (Gomphostilbia) okinawense Takaoka and *S. (G.) tokarensis* Takaoka, both from the Nansei Islands, Japan, were morphologically reexamined and genetically analysed by using the COI gene sequences. The female, male, pupa and mature larva of the two species are redescribed. Morphological reexamination shows that both species are more similar to species in the *S. asakoe* species-group than to those in the *S. ceylonicum* species-group, by having a medium-long female sensory vesicle, yellow tuft hairs (*S. (G.) okinawense*) or yellow tuft hairs mixed with a few to several dark hairs (*S. (G.) tokarensis*) at the base of the radial vein in the female and male, and medium-long larval postgenal cleft. However, the body of the male ventral plate (viewed ventrally) is parallel-sided (*S. (G.) okinawense*) or parallel-sided or slightly narrowed (*S. (G.) tokarensis*) and not emarginated basally, differing from those of most species in the *S. asakoe* species-group. Our genetic analysis shows that *S. (G.) tokarensis* is in the *S. asakoe* species-group, and *S. (G.) okinawense* formed a separate sister clade with other members of the *S. asakoe* species-group with high bootstrap support. From the results of morphological and genetic analysis combined, *S. (G.) okinawense* and *S. (G.) tokarensis* are transferred from the *S. ceylonicum* species-group to the *S. asakoe* species-group.

Keywords: Simuliidae; biodiversity; black fly; taxonomy.

INTRODUCTION

The Nansei Islands (or the Ryukyu Islands) are an arc of continental islands, located over 1,200 km between Kyushu, Japan and Taiwan (24–31°N), mostly in a subtropical climate zone. They are biogeographically of great interest because they are thought to have played a role as stepping stones for dispersal from the north by Palaearctic elements and from the south by Oriental elements, and also to be a cradle for many species isolated for long periods from the continents to evolve to become relict species under insular environments (Toda *et al.*, 2003).

The fauna of black flies of the Nansei Islands is represented by 18 species, which are placed in two small subgenera and 11 species-groups of three large subgenera of the genus *Simulium* Latreille: one in *Eusimulium* Roubaud, three in two species-groups of *Gomphostilbia* Enderlein, five in three species-groups of *Nevermannia* Enderlein, eight in six species-groups of *Simulium*, and one in *Wallacellum* Takaoka (Shiraki, 1935; Ogata, 1956, 1966; Takaoka, 1972, 1973, 1976a, 1976b, 1977; Takaoka *et al.*, 2021). It is,

thus, characterized by a relatively high diversity of lineages, though the number of species is small. It is also characterized by relatively high endemism (30% or six indigenous species of the 18 species recorded), and dominance of species represented in the north (eight species common to Kyushu, two species common to Taiwan, and one species common to both).

Information on their medical importance is scarce, though *S. (S.) japonicum* Matsumura was reported to bite humans and other animals in several of the Nansei Islands (Takaoka, 1977).

Among three species of the subgenus *Gomphostilbia*, which are all indigenous and are thought to have moved in from the south, *S. (G.) yaeyamaense* Takaoka was described as a new species belonging to the *S. batoense* species-group, after the species previously recorded as *S. (G.) batoense* Edwards in the Sakishima Islands, was reexamined (Takaoka, 1991). On the other hand, the two other species, *S. (G.) okinawense* Takaoka and *S. (G.) tokarensis* Takaoka, both described by Takaoka (1973, 1976b), were left unreviewed for their morphological characteristics, in particular, those used for the assignment to a species-group and comparison

with related species in the Oriental Region, although both species were provisionally placed in the *S. ceylonicum* species-group, redefined by Takaoka (2012).

In this study, we redescribe *S. (G.) okinawense* and *S. (G.) tokarensis* and reevaluate the species-group assignment of these two species by morphological reexaminations and genetic analysis using the COI gene sequences.

MATERIALS AND METHODS

Morphological analysis

Specimens of *S. (G.) okinawense* and *S. (G.) tokarensis* used for morphological observation and redescription were as follows:

S. (G.) okinawense. Two females and one male reared from pupae, with their associated pupal exuviae and cocoons, and 18 larvae collected from a stream (14°C, elevation 10 m, 28° 7' 59" N, 29° 14' 22" E), Tawara, Kakeromashima Island (just south of Amamioshima Island), Setouchi Township, Kagoshima Prefecture, Japan, 13-III-2016, by Y. Otsuka.

S. (G.) tokarensis. Three females and two males reared from pupae, with their associated pupal exuviae and cocoons, one pupa and six larvae, collected from Yarus River (17°C, elevation 100 m, 29° 49' 26" N, 129° 53' 49" E), Nakanoshima Island, Toshima Village, Kagoshima Prefecture, Japan, 29-X-2015, by Y. Otsuka.

All specimens were fixed in 80% ethanol. The methods of collection, description and illustration, and terms for morphological features, follow those of Takaoka (2003) and partially those of Adler et al. (2004).

Genetic analysis

S. (G.) okinawense. Five females and two larvae were used for DNA analysis: five females (No. 639, 668-671) captured by a sweeping net near Mt. Yuwan, Yamato Village, 18-V-2016, 28-VI-2018 by Y. Otsuka, and one larva (No. 614) collected from a small stream (width 1.0 m, 20°C, elevation 110 m, 28° 13' 32" N, 129° 19' 26" E), at Amurogama, Setouchi Township, 5-VII-2015, by Y. Otsuka, both in the southern part of Amamioshima Island, Kagoshima Prefecture; and one larva (No. 608) from Kakeromashima Island, same data as those for morphological analysis.

S. (G.) tokarensis. Three larvae (No. 629, 711 and 712) from Nakanoshima Island were used for DNA analysis: one larva (No. 629) from same data as those for morphological analysis, larva (No. 711) from Ookawa river (width 3.0 m, 16°C, elevation 160 m, 29° 50' 29" N, 129° 53' 17" E) 23-X-2018, by Y. Otsuka, one larva (No. 712) from Sato river (width 0.5 m, 18°C, elevation 50 m, 29° 50' 23" N, 129° 51' 11" E) 23-X-2018, by Y. Otsuka.

DNA was extracted from single larvae or adults using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Extracted DNA was dissolved in 200 µl AE buffer provided in the kit. The cytochrome c oxidase subunit I (COI) was amplified by polymerase chain reaction (PCR) using the following primers: LCO1490, 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198, 5'-TAACTTCAGGGTGACCAAAAATCA-3' for COI (Folmer et al., 1994). PCR was carried out using 20 µl volumes containing 0.5 units of *Ex Taq* (TaKaRa), 1X *Ex Taq* buffer, 2 mM of MgCl₂, 0.2 mM of each dNTP, 0.25 µM of each primer and 1 µl of the extracted DNA. The amplified products were electrophoresed through a 1% agarose gel. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and directly sequenced using the PCR primers. Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit and run on a 3130 Genetic Analyzer (Applied Biosystems). The sequence data for this paper have been deposited in the GenBank nucleotide sequence database under accession numbers LC731781- LC731790.

The sequences were aligned with those of twelve species in the *S. asakoae* species-group and three species in the *S. ceylonicum* species-group, all registered in GenBank, for reference. *Simulium (Simulium) tani* and *S. (S.) suzukii* were used as outgroups. The

Kimura two-parameter model was employed to calculate genetic divergence. By using the divergence values, construction of neighbor-joining (NJ) trees and the bootstrap test with 1,000 replications were performed with the MEGA version 11 program (Tamura et al., 2021).

RESULTS AND DISCUSSION

Redescription of *S. (G.) okinawense* and *S. (G.) tokarensis*

Simulium (Gomphostilbia) okinawense Takaoka, 1976

Female (n=2). Body length 2.2 mm. **Head**. Slightly narrower than width of thorax. Frons dark brown, densely covered with yellow scale-like recumbent short hairs interspersed with few dark longer hairs along each lateral margin; frontal ratio 1.57:1.00:2.10; frons:head ratio 1.0:4.75. Fronto-ocular area well developed, narrow, directed dorsolaterally. Clypeus dark brown, densely covered with yellow scale-like hairs interspersed with several dark longer hairs on each side. Labrum 0.57 times length of clypeus. Antenna composed of scape, pedicel and nine flagellomeres, dark brown except scape, pedicel and base of first flagellomere yellow. Maxillary palpus composed of five segments, light to medium brown, proportional lengths of third, fourth, and fifth segments 1.00:1.17:2.34–2.43; sensory vesicle (Figure 1A) ellipsoidal, medium-long (0.27–0.30 times length of third segment), with relatively large opening. Maxillary lacinia with 14 or 15 inner and 17 outer teeth. Mandible with 27 inner teeth and 6 or 7 outer teeth at some distance from tip. Cibarium (Figure 1B) medially forming sclerotized plate folded forward from posterior margin, with weakly sclerotized mediolongitudinal ridge with dark bifid apex. **Thorax**. Scutum brownish black except anterolateral calli ochreous, thinly pruinose and shiny when illuminated at certain angles, with three faint longitudinal vittae, densely covered with yellow scale-like recumbent short hairs even on median and two submedian longitudinal vittae. Scutellum dark brown, covered with yellow short hairs and dark brown long upright hairs along posterior margin. Postnotum brownish black, slightly shiny when illuminated at certain angles, and bare. Pleural membrane ochreous and bare. Katepisternum longer than deep, dark brown, shiny when illuminated at certain angles, moderately covered with fine yellow and brown short hairs. **Legs**. Foreleg: coxa yellow; trochanter light brown; femur light brown with apical cap medium brown (though extreme tip yellowish); tibia yellowish white except apical three-tenths brownish black; tarsus brownish black, with moderate dorsal hair crest; basitarsus moderately dilated, 6.10 times as long as its greatest width. Midleg: coxa medium brown except posterolateral surface dark brown; trochanter light brown except base yellow; femur light brown with apical cap medium brown (though extreme tip yellowish); tibia whitish yellow on basal one-third and light to dark brown on rest; tarsus dark brown to brownish black though basal half of basitarsus yellow (its border not well defined). Hind leg: coxa light to medium brown; trochanter yellow; femur medium brown with base yellow and apical cap dark brown (though extreme tip yellowish); tibia (Figure 1C) yellowish white on basal half and light brown to brownish black on rest; tarsus dark brown except basal two-thirds (though base light brown) and basal half of second tarsomere yellowish white; basitarsus narrow, nearly parallel-sided, though slightly narrowed apically, 5.44 times as long as wide, and 0.73 and 0.59 times as wide as greatest widths of tibia and femur, respectively; calcipala nearly as long as width at base, and 0.53 times as wide as greatest width of basitarsus; pedisulcus well developed; claw (Figure 1E) with large basal tooth 0.61 times length of claw. **Wing**. Length 2.0–2.1 mm. Costa with dark spinules and hairs except basal patch of yellow hairs mixed with few dark hairs. Subcosta with dark hairs except near apex. Base of radius with tuft of yellow hairs. Basal portion of radius fully haired. R₁ with dark spinules and hairs; R₂ with hairs only. Basal cell absent. **Halter**. White except basal portion darkened. **Abdomen**. Basal scale ochreous, with fringe of whitish yellow hairs. Dorsal surface of abdomen medium to dark brown

except segment 2 yellow except dark narrow area along posterior margin, moderately covered with dark short to long hairs; tergites of segments 2 and 6–9 shiny when illuminated at certain angles. Sternal plate on segment 7 undeveloped. **Terminalia.** Sternite 8 (Figure 1F) bare medially, with 17–28 medium-long to long hairs together with 5 slender short hairs on each side. Ovipositor valves (Figure 1F) triangular (though posteromedial corner rounded), thin, membranous, each moderately covered with microsetae interspersed with one short hair or without hair; inner margins roughly sinuous, somewhat sclerotized, and moderately separated from each other. Genital fork (Figure 1G) of usual inverted-Y form, with slender stem; arms of moderate width, moderately folded

medially, and with triangular lobe directed posteromedially. Paraproct in ventral view (Figure 1H) shallowly concave along anteromedial margin, with four or five sensilla on anteromedial surface; paraproct in lateral view (Figure 1I) somewhat produced ventrally beyond ventral tip of cercus, 0.54 times as long as wide, with 14–18 medium-long to long hairs on ventral and lateral surfaces. Cercus in lateral view (Figure 1I) short, rounded posteriorly, 0.42 times as long as wide. Spermatheca (Figure 1J) ellipsoidal, 1.6 times as long as its greatest width, well sclerotized and darkened except duct and small area near juncture with duct unpigmented, and with many fissures on outer surface; internal setae absent; both accessory ducts slender, slightly thicker in diameter than major one.

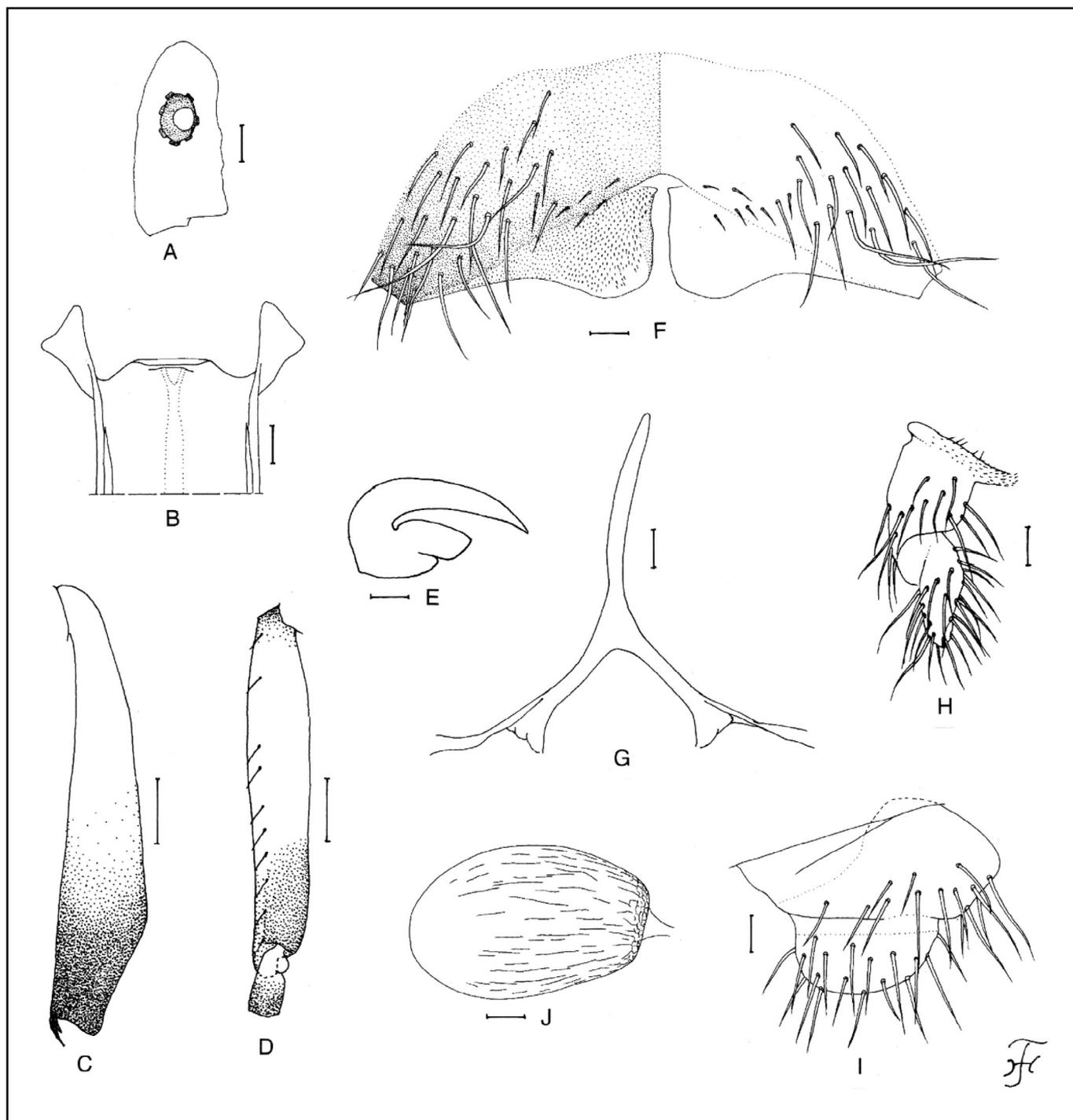


Figure 1. Female of *S. okinawense*. A, sensory vesicle (right side; front view); B, cibarium (front view); C, hind tibia (left side; outer view); D, hind basitarsus and second tarsomere (left side; outer view); E, claw; F, sternite 8 and ovipositor valves (ventral view); G, genital fork (ventral view); H & I, paraprocts and cerci (right side; H, ventral view; I, lateral view); J, spermatheca. Scale bars. 0.1 mm for C and D; 0.02 mm for A, B and F–J; 0.01 mm for E.

Male (n=1). Body length 2.0 mm. **Head.** Slightly wider than thorax. Upper eye medium brown, consisting of large facets in 15 vertical columns and 16 horizontal rows on each side. Clypeus brownish black, whitish pruinose, densely covered with yellow scale-like medium-long hairs (mostly directed upward) interspersed with several dark brown longer hairs near lower margin on each side. Antenna composed of scape, pedicel and nine flagellomeres, medium to dark brown except scape and pedicel light brown and base of first flagellomere whitish yellow; first flagellomere elongate, 1.57 times length of second. Maxillary palpus light to medium brown, with five segments, proportional lengths of third, fourth, and fifth segments 1.0:1.3:2.6; third segment (Figure 2A) slender; sensory vesicle (Figure 2A) small, ellipsoidal (0.14–0.17 times length of third segment), and with small opening. **Thorax.** Scutum brownish black except anterolateral calli dark brown, shiny widely along anterior margin, on shoulders, along both lateral margins and on prescutellar area when illuminated at certain angles, and densely covered with whitish-yellow scale-like recumbent short hairs. Scutellum dark brown to brownish black, covered with yellow short hairs and dark brown long upright hairs along posterior margin. Postnotum brownish black, slightly shiny when illuminated at certain angles, and bare. Pleural membrane ochreous and bare. Katepisternum dark brown, longer than deep, shiny when illuminated at certain angles, moderately covered with yellow and brown fine short hairs. **Legs.** Foreleg: coxa yellow; trochanter light brown; femur light brown except apical cap dark brown (though apical tip paler); tibia light to dark brown except medial portion of outer surface widely whitish; tarsus brownish black; basitarsus slightly dilated, 8.0 times as long as its greatest width. Midleg: coxa medium brown except posterolateral surface brownish black; trochanter light to medium brown; femur medium brown (though apical tip paler); tibia medium to dark brown except basal one-third whitish yellow; tarsus medium to dark brown though basal one-fourth or one-third of basitarsus somewhat paler. Hind leg: coxa medium brown; trochanter yellowish; femur medium brown with extreme base yellow and apical cap dark brown (though apical tip paler); tibia yellowish white on basal two-fifths and dark brown to brownish black on rest; tarsus dark brown except basal one-third to two-fifths of basitarsus and basal half of second tarsomere dark yellow; basitarsus (Figure 2B) enlarged, 3.2 times as long as wide, and 1.0 and 1.1 times as wide as greatest width of tibia and femur, respectively; calcipala (Figure 2B) slightly shorter than basal width, and 0.25 times as wide as greatest width of basitarsus; pedisulcus well developed. **Wing.** Length 1.9 mm. Other characteristics as in female including subcosta with hairs except near apex. **Halter.** Dull white except basal stem darkened. **Abdomen.** Basal scale dark brown, with fringe of light to dark brown hairs. Dorsal surface of abdomen medium brown to brownish black, covered with dark brown short to long hairs except parts of segment 2 with yellowish hairs; segments 2 and 5–7 each with pair of shiny dorsolateral or lateral patches. **Genitalia.** Coxite in ventral view (Figure 2C) nearly rectangular, 1.6 times as long as its greatest width. Style in ventral view (Figure 2C) bent inward, with single apical spine; style in ventrolateral view (Figure 2D) slightly tapered toward truncated or round apex, and 0.8 times length of coxite. Ventral plate in ventral view (Figure 2C) with body transverse, 0.6 times as long as wide, nearly parallel-sided, with anterior margin produced anteromedially, posterior margin somewhat concave ventromedially, and densely covered with microsetae on ventral surface; basal arms of moderate length, nearly parallel-sided, then slightly convergent apically; ventral plate in lateral view (Figure 2E) moderately produced ventrally; ventral plate in caudal view (Figure 2F) rounded ventrally, densely covered with microsetae on posterior surface. Median sclerite arising near anterior tip of ventral plate (Figure 2E) and broad, plate-like in caudal view (Figure 2G). Parameres (Figure 2H) of moderate size, each with three long and one medium-long stout hooks. Aedeagal membrane (Figure 2H) moderately setose; dorsal plate not defined. Ventral surface of abdominal segment 10 without

distinct hairs near posterolateral corners. Cercus small, rounded, with 20–22 hairs.

Pupa (n=3). Body length 2.5 mm. **Head.** Integument yellow, moderately covered with small round tubercles except antennal sheaths and ventral surface almost bare; frons with three pairs of unbranched long trichomes with or without coiled apices (Figure 3A); face with pair of unbranched long trichomes with coiled apices (Figure 3B); three frontal trichomes on each side arising close together, subequal in length to one another and longer than facial one. **Thorax.** Integument yellow, moderately covered with round tubercles, and with three long dorsomedial trichomes with coiled apices (gradually shortened posteriorly) (Figure 3C), two long anterolateral trichomes (anterior trichome more slender with straight apex, posterior one with coiled apex) (Figure 3D), one medium-long mediolateral trichome without coiled apex (Figure 3E), and three ventrolateral trichomes (one medium-long, two extremely short) with or without coiled apices (Figure 3F), on each side; all trichomes unbranched. Gill (Figure 3G) composed of eight slender thread-like filaments, arranged as [(1+2)+(1+2)]+2 or [3+(2+1)]+2 from dorsal to ventral, with medium-long common basal stalk having somewhat swollen transparent basal fenestra; common basal stalk 0.71–0.81 times length of interspiracular trunk; dorsal and middle triplets sharing short stalk, which is twice or little more as long as their primary stalks; dorsal triplet mostly composed of three individual filaments arising at same level, and dorsal and middle triplets typically composed of one individual and two paired filaments with extremely short secondary stalk; dorsal triplet also composed of three individual filaments arising at same level; stalk of ventral pair of filaments medium-long, 1.36–1.60 times length of common basal stalk, and 1.10–1.27 times length of interspiracular trunk, and somewhat thinner than common stalk of middle and dorsal triplets; primary stalk of dorsal triplet lying against that of ventral pair at angle of 30–45 degrees when viewed laterally; filaments of dorsal and middle triplets subequal in length (ca. 2.3 mm) and thickness to one another; two filaments of ventral pair subequal in length (ca. 2.9 mm) and thickness to each other and 1.1–1.2 times as thick as six other filaments of dorsal and middle triplets when compared basally; all filaments light brown, gradually tapered toward apex; cuticle of all filaments with well-defined annular ridges and furrows though becoming less marked apically, densely covered with two forms of microtubercles, larger ones on ridges smaller ones on interridges. **Abdomen.** Dorsally, all segments light yellow to yellow and without microtubercles; segment 1 with one unbranched slender medium-long hair-like seta (Figure 3H) on each side; segment 2 with one unbranched slender medium-long hair-like seta and five minute setae submedially (Figure 3I) on each side; segments 3 and 4 each with four hooked spines and one minute seta on each side; segment 5 lacking spine-combs and comb-like groups of minute spines on each side; segments 6–9 each with spine-combs in transverse row and comb-like groups of micro-spines on each side; segment 5 with four minute setae on each side; segments 6–8 each with two minute setae on each side; segment 9 with pair of triangular terminal hooks (Figure 3J), of which outer margin 1.3–1.7 times length of inner margin and weakly crenulated when viewed caudally. Ventrally, segment 4 with one unbranched hook (subequal in size to those on segments 5–7) and few slender short setae, on each side; segment 5 with pair of bifid hooks submedially and few short slender setae on each side; segments 6 and 7 each with pair of bifid inner and unbranched outer hooks somewhat spaced from each other and few short slender setae on each side; segments 4–8 each with comb-like groups of micro-spines. Each side of segment 9 with three grapnel-shaped hooklets. **Cocoon.** Wall-pocket-shaped, densely woven, widely extended ventrolaterally; anterior margin moderately or thickly woven medially; posterior three-fifths with floor roughly woven; individual threads invisible; 3.0 mm long by 2.1–2.6 mm wide.

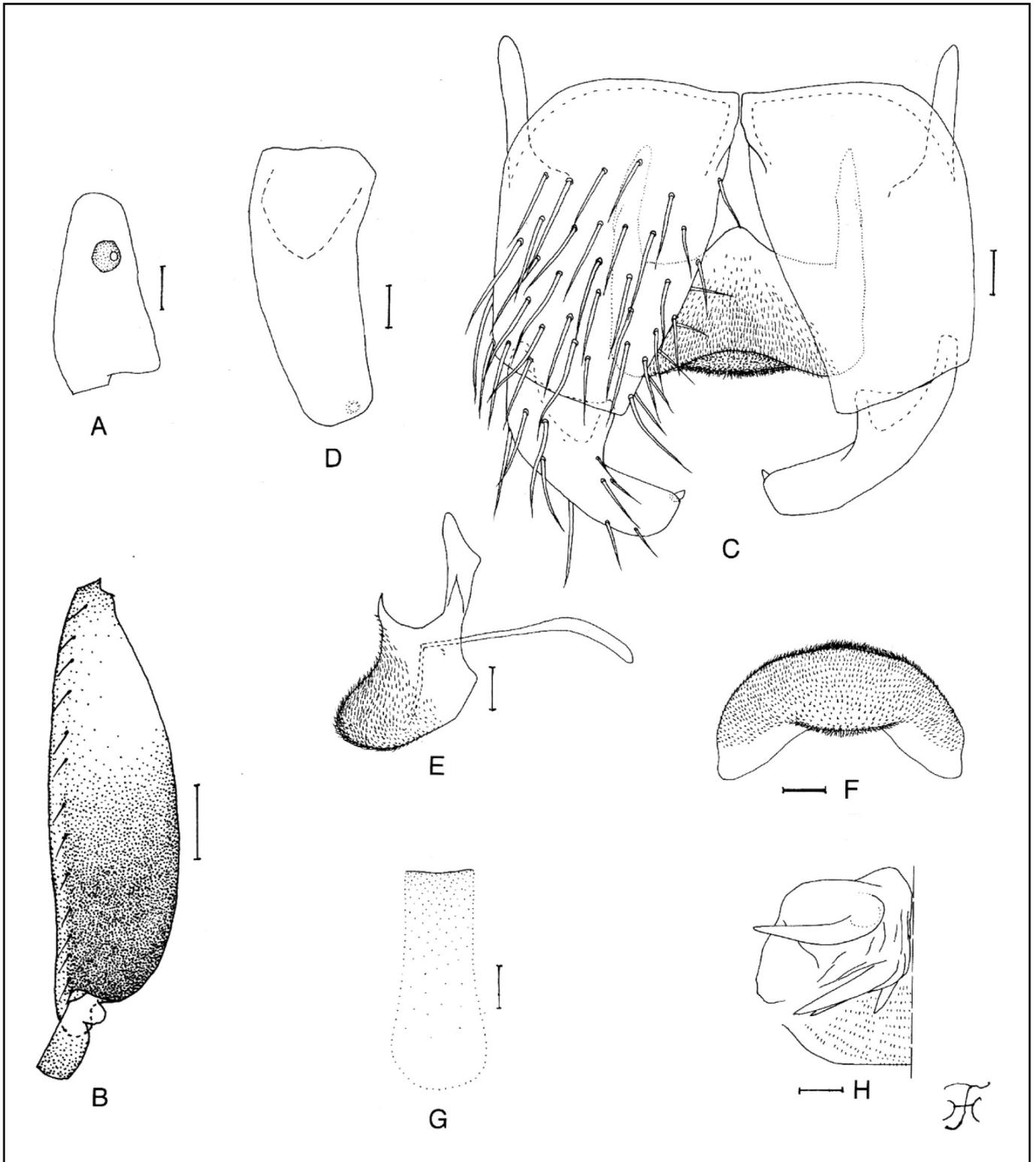


Figure 2. Male of *S. okinawense*. A, sensory vesicle (right side; front view); B, hind basitarsus and second tarsomere (left side; outer view); C, coxites, styles and ventral plate (ventral view); D, style (right side; ventrolateral view); E, ventral plate and median sclerite (lateral view); F, ventral plate (caudal view); G, median sclerite (ventral view); H, paramere and aedeagal membrane (right half; caudal view). Scale bars. 0.1 mm for B; 0.02 mm for A and C–H.

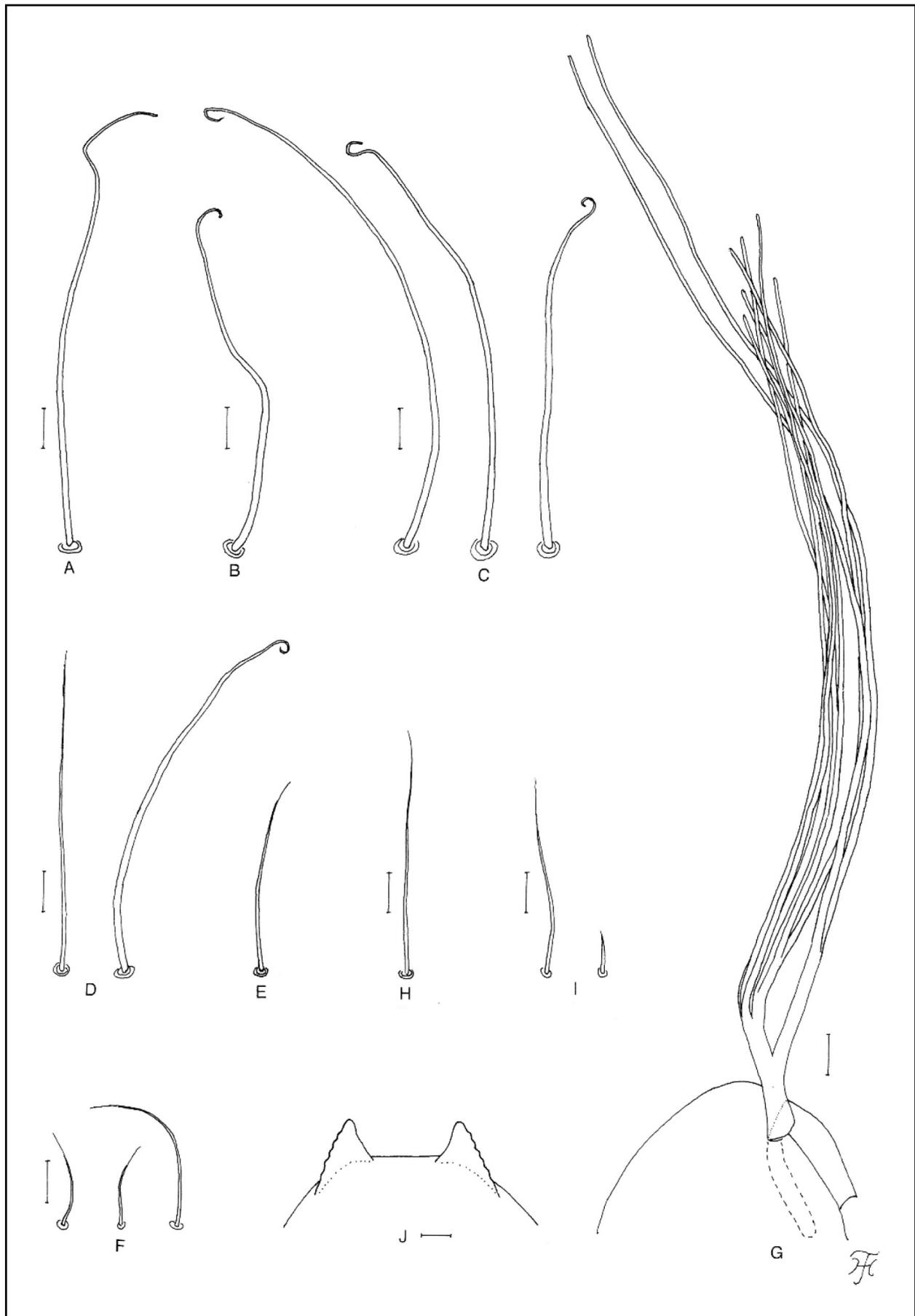


Figure 3. Pupa of *S. okinawense*. A, frontal trichome; B, facial trichome; C–F, thoracic trichomes (C, mediodorsal; D, anterolateral; E, mediolateral; F, ventrolateral); G, gill filaments (right side; outer view); H, hair-like seta on dorsum of abdominal segment 1; I, hair-like seta and minute seta on dorsum of abdominal segment 2; J, terminal hooks (caudal view). Scale bars. 0.1 mm for G; 0.02 mm for A–F, H, and I; 0.01 mm for J.

Mature larva (n=16). Body length 4.6–5.0 mm. Body grayish (though thoracic segment 3 often paler and its ventral surface ochreous, and ventral surface of abdominal segments 5–9 whitish), with reddish brown transverse band on dorsum of abdominal segments 5 and 6 (though that on segment 6 usually less distinct and sometimes disappeared). **Head.** Head capsule whitish yellow except eye-spot region whitish; head spots positive, of which anterior mediolongitudinal spots and mediolateral spots on dorsal surface, and isolated spot below eye on lateral surface usually faint. Antenna composed of three articles and apical sensillum, longer than stem of labral fan; proportional lengths of first, second, and third articles 1.00:0.87–0.89:0.93–1.96. Labral fan with 35 or 36 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); major tooth at acute angle against mandible on apical side; supernumerary serrations absent. Hypostoma (Figure 4B) with row of nine apical teeth, of which median tooth slightly longer than each corner tooth; lateral margin smooth; four or five hypostomal bristles per side lying nearly parallel to lateral margin. Postgenal cleft (Figure 4C, 4D) medium-long, 1.2–2.6 times as long as postgenal bridge, and rounded or pointed apically. **Thorax and Abdomen.** Thoracic and abdominal cuticle almost bare except abdominal segments 5–8 sparsely to moderately covered with dark unbranched minute setae dorsally and dorsolaterally; last abdominal segment densely covered with colorless unbranched minute setae on dorsolateral and lateral surfaces of each side of anal sclerite. Rectal organ (Figure 4E) with 4–6 secondary finger- or thumb-like lobules per lobe. Anal sclerite of usual X-form, with anterior arms 1.0–1.2 times as long as posterior ones, broadly sclerotized at base; accessory sclerite absent. Last

abdominal segment with pair of large conical ventral papillae. Posterior circlet with 80–85 rows of hooklets with up to 13 or 14 hooklets per row.

Remarks. *Simulium* (*G.*) *okinawense* was described from females, males, pupae and larvae collected from Okinawa Island and was also recorded from other islands in the Nansei Islands such as Ishigaki, Iriomote, Amamioshima, Uke and Yoro (Takaoka, 1976b).

In this study, we used specimens collected from Kakeromashima Island, just south of Amamioshima Island.

Newly described features in the present study include color of tuft hairs at the base of the radial vein in the female and male; frons:head ratio, cibarium (Figure 1B), relative length of the labrum against the clypeus, relative length of the fore basitarsus, and relative length of the tooth against the claw (Figure 1E) in the female; relative length of the trichomes on the frons, face and thorax (Figure 3A–3F), and terminal hooks (Figure 3J) in the pupa.

The characteristics differing from those in the original description (in parentheses) are number of upper-eye (large) facets in 15 vertical columns and 16 horizontal rows on each side (ca. 13 horizontal rows), relative length of the fore basitarsus against its greatest width 8.0 (6.5), ventral plate parallel-sided (slightly narrowed posteriorly), paramere with three long and one medium-long hooks (three long hooks) in the male; abdominal segments 1–8 grayish with reddish-brown transverse band on segments 5 and 6 (abdominal segments 1–5 pale greenish, and segments 6–8 pale yellow). Whether these differences are intraspecific or interspecific variation should be studied in the future by comparing specimens from various localities since this species is widely distributed in the Nansei Islands (Takaoka, 1976b).

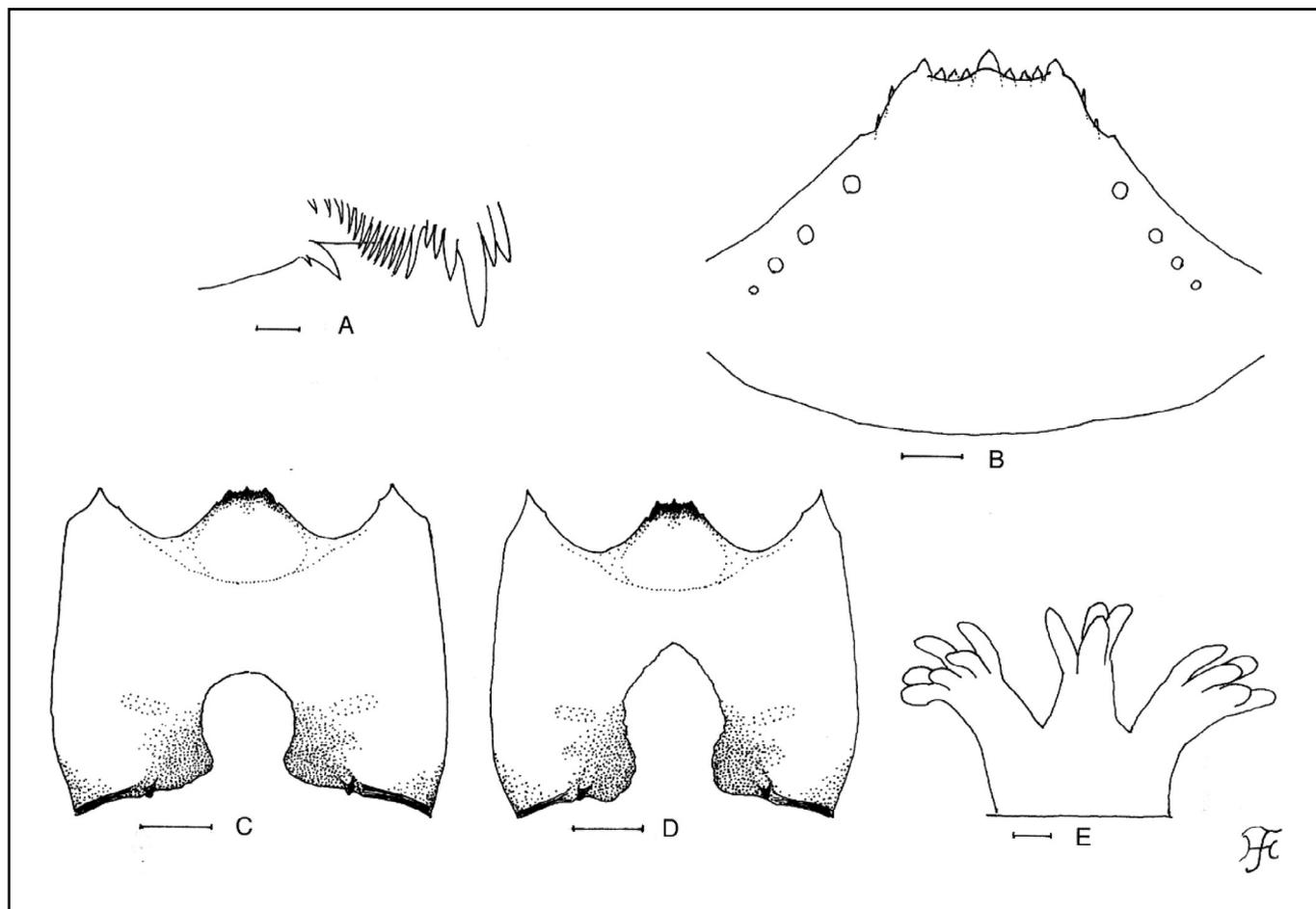


Figure 4. Mature larva of *S. okinawense*. A, tip of mandible; B, hypostoma; C and D, head capsules (ventral view). Scale bars. 0.1 mm for C and D; 0.05 mm for E; 0.02 mm for B; 0.01 mm for A.

This species is characterized in the female and male by the haired subcosta and yellow tuft hair at the base of the radius, in the female by the medium-long sensory vesicle (Figure 1A), and genital fork with a triangular projection directed posteromedially (Figure 1G); in the male by the large number of upper-eye (large) facets in 15 vertical columns and 16 horizontal rows, ventral plate parallel-sided when viewed ventrally, and hind basitarsus (Figure 2B) enlarged, 3.2 times as long as wide, and 1.0 and 1.1 times as wide as the greatest width of the hind tibia and femur, respectively; in the pupa by the gill with eight slender filaments, of which two filaments of the ventral pair are slightly longer and thicker than six other filaments (Figure 3G), and terminal hooks plate-like, with an outer margin 1.3–1.7 times length of inner margin and weakly crenulated when viewed caudally (Figure 3J); and in the larva by the abdomen grayish with reddish-brown markings on segments 5 and 6, and postgenal cleft medium-long, 1.2–2.6 times as long as the postgenal bridge (Figure 4C, 4D).

This species is similar to *S. (G.) taitungense* Huang & Takaoka (in the *S. asakaoe* species-group) from Taiwan (Huang et al., 2011), but it is barely distinguished from the latter species in the male by the hind basitarsus dark brown except the basal one-third to two-fifths dark yellow (hind basitarsus dark brown to brownish black except the basal half yellow in *S. (G.) taitungense*), hind basitarsus 3.2 times as long as its greatest width, and 1.0 and 1.1 times as wide as the hind tibia and femur (hind basitarsus 3.95–4.13 times as long as its greatest width, and 0.83–0.84 and 0.87–0.89 times as wide as the hind tibia and femur in *S. (G.) taitungense*), and lateral margins of the ventral plate parallel-sided (somewhat concave medially in *S. (G.) taitungense*), in the pupa by the angle between the stalk of the dorsal triplet and that of the ventral pair of gill filaments (45° in this species versus 90° in *S. (G.) taitungense*), and in the larva by the head capsule lacking a dark X-shaped marking on the ventral surface (head capsule with a dark X-shaped marking in *S. (G.) taitungense*).

Simulium (Gomphostilbia) tokarensis Takaoka, 1973

Female (n=3). Similar to that of *S. (G.) okinawense* except following characteristics. Body length 2.2–2.5 mm. **Head.** Frontal ratio 1.88–1.90:1.00:2.32–2.46; frons:head ratio 1.00:4.09–4.70. Labrum 0.57–0.65 times length of clypeus. Antenna. Maxillary palpus: proportional lengths of third, fourth, and fifth segments 1.00:1.09–1.20:2.69–3.12; sensory vesicle (Figure 5A) ellipsoidal, medium-long (0.25–0.30 times length of third segment), with relatively large opening. Maxillary lacinia with 11–13 inner and 15–17 outer teeth. Mandible with 24–26 inner teeth and 6–8 outer teeth at some distance from tip. **Thorax.** Scutum medium to dark brown except anterolateral calli ochreous, thinly pruinose and shiny when illuminated at certain angles, with three faint dark longitudinal vittae, densely covered with yellow scale-like recumbent short hairs. Scutellum dark brown, covered with yellow short hairs and dark brown long upright hairs along posterior margin. Postnotum dark brown, slightly shiny when illuminated at certain angles, and bare. **Legs.** Foreleg: coxa and trochanter yellowish white; basitarsus moderately dilated, 6.09–6.26 times as long as its greatest width. Midleg: coxa light brown except posterolateral surface dark brown; trochanter yellowish white; femur light brown with base yellowish and apical cap medium brown (though extreme tip yellowish); tibia yellowish white on basal two-fifths and light to dark brown on rest; tarsus dark brown though basal one-third of basitarsus dark yellow (its border not well defined). Hind leg: coxa light brown; trochanter yellow; femur medium brown with base yellow and apical cap dark brown (though extreme tip yellowish); tibia yellowish white on basal half or little more and light brown to brownish black on rest; basitarsus (Figure 5C) 5.88–6.13 times as long as wide, and 0.65–0.76 and 0.56–0.62 times as wide as greatest widths of tibia and femur, respectively; calcipala nearly as long as width at base, and 0.50 times as wide as greatest width of basitarsus; pedisulcus well developed;

claw with large basal tooth 0.56 times length of claw. **Wing.** Length 2.1–2.2 mm. Base of radius with tuft of yellow hairs mixed with few dark hairs. **Abdomen.** Basal scale ochreous, with fringe of whitish yellow hairs. Dorsal surface of abdomen medium to dark brown except segment 2 ochreous to light brown except dark narrow area along posterior margin. **Terminalia.** Sternite 8 bare medially, with 15–19 medium-long to long hairs together with 3–5 slender short hairs on each side. Ovipositor valves each moderately covered with microsetae interspersed with two short hairs. Genital fork (Figure 5E) of usual inverted-Y form, with slender stem; arms of moderate width, moderately folded medially, and without triangular lobe directed posteromedially. Paraproct in ventral view with five or six sensilla on anteromedial surface; paraproct in lateral view 0.60 times as long as wide, with 17–21 medium-long to long hairs on ventral and lateral surfaces. Cercus in lateral view 0.50 times as long as wide. Spermatheca 1.48 times as long as its greatest width.

Male (n=2). Body length 2.5–2.7 mm. **Head.** Slightly wider than thorax. Upper eye medium brown, consisting of large facets in 12 or 13 vertical columns and 14 horizontal rows on each side. Clypeus dark brown, whitish pruinose, densely covered with yellow scale-like medium-long hairs (mostly directed upward) interspersed with several dark brown longer hairs near lower margin on each side. Antenna composed of scape, pedicel and nine flagellomeres, medium to dark brown except scape, pedicel and base of first flagellomere whitish yellow; first flagellomere elongate, 1.77 times length of second. Maxillary palpus: proportional lengths of third, fourth, and fifth segments 1.00:1.14–1.19:3.09–3.25; third segment (Figure 5B) widened apically; sensory vesicle (Figure 5B) small, ellipsoidal (0.20 times length of third palpal segment), and with small opening. **Thorax.** Scutum dark brown except anterolateral calli light brown, with three faint dark longitudinal vittae, shiny widely along both lateral margins and on prescutellar area when illuminated at certain angles, and densely covered with yellow scale-like recumbent short hairs. Scutellum dark brown, covered with yellow short hairs and dark brown long upright hairs along posterior margin. Postnotum dark brown, slightly shiny when illuminated at certain angles, and bare. Pleural membrane ochreous and bare. Katepisternum dark brown, longer than deep, shiny when illuminated at certain angles, moderately covered with yellow and brown fine short hairs. **Legs.** Foreleg: coxa and trochanter whitish yellow; femur light brown except apical cap dark brown (though apical tip yellow); tibia whitish widely on outer surface except inner surface light brown and apical cap dark brown; tarsus brownish black; basitarsus slightly dilated, 7.10–7.68 times as long as its greatest width. Midleg: coxa medium brown except posterolateral surface brownish black; trochanter whitish yellow; femur medium brown (though basal and apical tips yellow); tibia medium to dark brown except basal one-third whitish yellow; tarsus medium to dark brown though base of basitarsus dark yellow. Hind leg: coxa light to medium brown; trochanter whitish yellow; femur medium brown with base whitish yellow and apical cap dark brown (though apical tip paler); tibia dark brown except little more than basal one-third whitish yellow; tarsus medium brown except basal half of basitarsus and basal half of second tarsomere yellow; basitarsus (Figure 5D) enlarged, 3.16–3.23 times as long as wide, and 1.07 and 1.28–1.29 times as wide as greatest width of tibia and femur, respectively; calcipala (Figure 5D) slightly shorter than basal width, and 0.29 times as wide as greatest width of basitarsus; pedisulcus well developed. **Wing.** Length 2.2 mm. Other characteristics as in female except subcosta bare, and base of radial vein with tuft of yellow hairs mixed with several dark hairs. **Halder.** Dull white to gray except basal stem darkened. **Abdomen.** Basal scale dark brown, with fringe of light to dark brown hairs. Dorsal surface of abdomen medium brown to brownish black, covered with dark brown short to long hairs except parts of segment 2 with yellowish hairs; segments 2 and 5–8 each with pair of shiny dorsolateral or lateral patches. **Genitalia.** Coxite

in ventral view (Figure 5F) nearly rectangular, 1.7 times as long as its greatest width. Style in ventral view (Figure 5F) bent inward, with single apical spine; style in ventrolateral view (Figure 5G) slightly tapered toward middle, then nearly parallel-sided to apex, and 0.77 times length of coxite. Ventral plate in ventral view (Figure 5F) with body transverse, 0.51 times as long as wide, nearly parallel-sided or slightly narrowed posteriorly, with anterior margin produced anteromedially, posterior margin slightly concave ventromedially, and densely covered with microsetae on ventral surface; basal

arms of moderate length, nearly parallel-sided, then somewhat convergent apically; ventral plate in lateral view (Figure 5H) moderately produced ventrally; ventral plate in caudal view (Figure 5I) rounded ventrally, densely covered with microsetae on posterior surface. Median sclerite, parameres and aedeagal membrane as in male of *S. (G.) okinawense*. Ventral surface of abdominal segment 10 without distinct hairs near posterolateral corners. Cercus small, rounded, with 14 hairs.

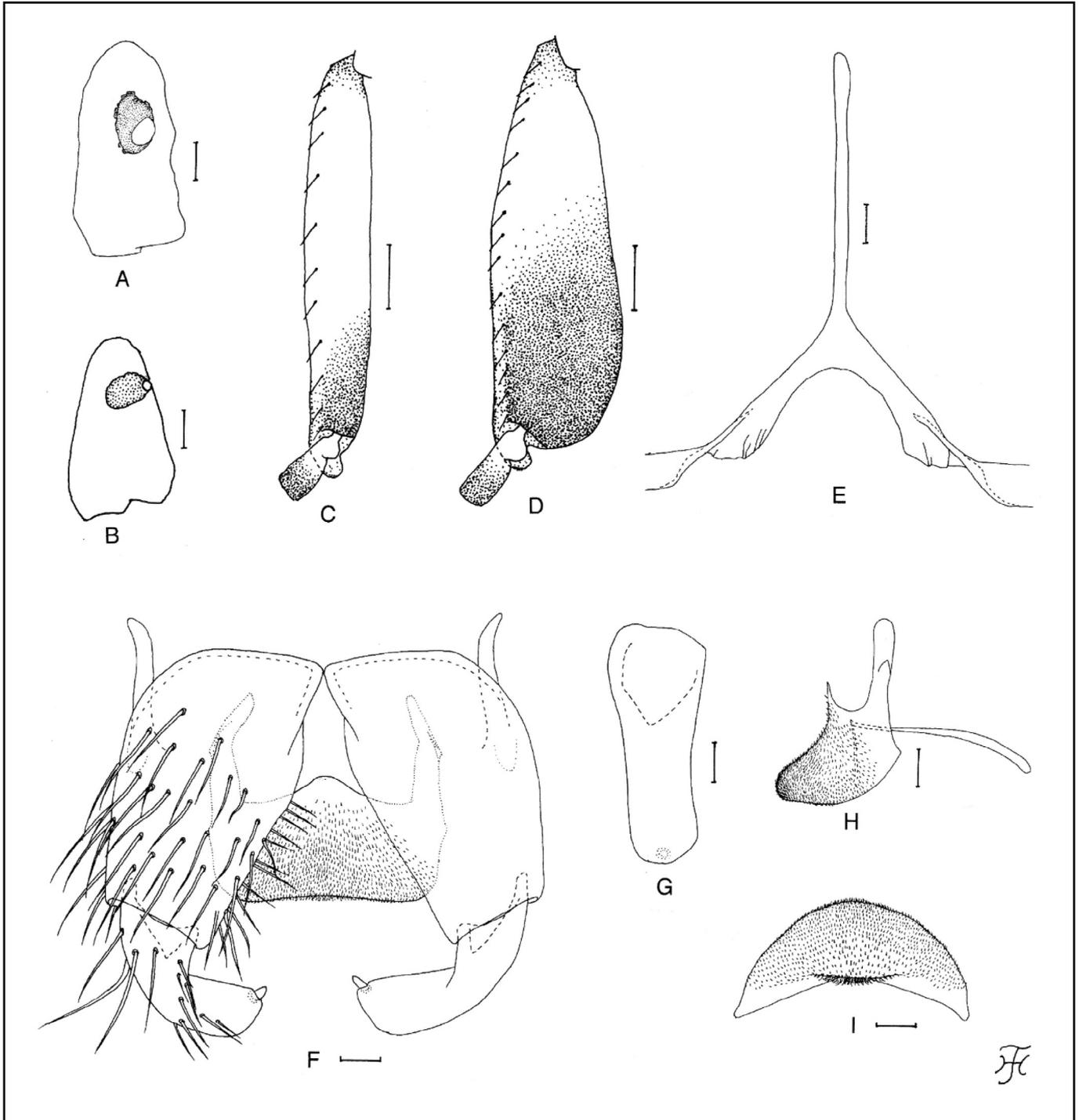


Figure 5. Female and male of *S. tokarensis*. A and B, sensory vesicles (right side; front view; A, female; B, male); C and D, hind basitarsi and second tarsomeres (left side; outer view; C, female; D, male); E, female genital fork; F, coxites, styles and ventral plate of male genitalia (ventral view); G, style (right side; ventrolateral view); H, ventral plate and median sclerite (lateral view); I, ventral plate (caudal view). Scale bars. 0.1 mm for C and D; 0.02 mm for A, B and E-I.

Pupa (n=6). Body length 2.5–3.0 mm. **Head.** Integument yellow to ochreous, densely and neatly covered with small round tubercles except antennal sheaths and ventral surface almost bare; frons with three pairs of unbranched long trichomes with or without coiled apices (Figure 6A); face with pair of unbranched long trichomes with coiled apices (Figure 6B); three frontal trichomes on each side arising close together, subequal in length to one another and longer than facial one. **Thorax.** Integument yellow to ochreous, densely and neatly covered with round tubercles, and with three long dorsomedial trichomes with coiled apices (gradually shortened posteriorly) (Figure 6C), two long anterolateral trichomes (anterior trichome more slender with straight apex, posterior one with coiled apex) (Figure 6D), one medium-long mediolateral trichome without coiled apex (Figure 6E), and three ventrolateral trichomes (two medium-long, one extremely short) without coiled apices (Figure 6F), on each side; all trichomes unbranched. Gill (Figure 6G, 6H, 6I) composed of eight slender thread-like filaments, arranged as [(1+2)+(1+2)]+2 (dorsal and inner triplets and ventral pair), arising close together from short common basal stalk having somewhat swollen transparent basal fenestra; common basal stalk 0.63–0.73 times length of interspiracular trunk; each triplet composed of one individual and two paired filaments with extremely short secondary stalk; one individual filament of inner triplet and one individual filament and two paired filaments of dorsal triplet lying almost horizontally from inside to outside when viewed dorsally (Figure 6H), and two filaments of inner triplet and two filaments of ventral pair lying obliquely nearly side by side when viewed ventrally (Figure 6I); stalk of ventral pair of filaments short, 0.63–0.68 times length of common basal stalk, and 0.42–0.46 times length of interspiracular trunk; six filaments of two triplets subequal in length (1.8–2.0 mm) and thickness to one another; two filaments of ventral pair subequal in length (2.3–2.6 mm) and thickness to each other and 1.2–1.5 times as thick as six other filaments of dorsal and inner triplets when compared basally; all filaments light brown, gradually tapered toward apex; cuticle of all filaments with well-defined annular ridges and furrows though becoming less marked apically, densely covered with microtubercles. **Abdomen.** Similar to that of *S. (G.) okinawense* except following characteristics. Dorsally, all segments light yellow; segments 1 and 2 each moderately covered with microspines (Figure 6J); segment 5 with or without spine-combs (one or two spines present on each side of two pupae); segment 9 with pair of widened plate-like terminal hooks (Figure 6M), of which outer margin 2.4–3.5 times length of inner margin and weakly crenulated when viewed caudally. Ventrally, segment 4 with pair of unbranched or bifid hooklets. **Cocoon.** Similar to that of *S. (G.) okinawense* except size: 3.0–3.5 mm long by 2.0–2.4 mm wide.

Mature larva (n=3). Body length 5.0–5.5 mm. Body grayish, with or without faint reddish-brown markings on abdominal segments 5–9. **Head.** Head capsule whitish yellow to yellow except eye-spot region whitish, and ventral surface somewhat darkened around postgenal cleft; head spots faintly positive. Antenna composed of three articles and apical sensillum, longer than stem of labral fan; proportional lengths of first, second, and third articles 1.00:0.81–0.88:0.81–0.86. Labral fan with 24–28 primary rays. Mandible and hypostoma similar to those (Figure 4A, 4B) of *S. (G.) okinawense* except five or six hypostomal bristles per side. Postgenal cleft medium-long, 1.1–1.4 times as long as postgenal bridge, and rounded anteriorly (similar to that (Figure 4C) of *S. (G.) okinawense*). **Thorax and Abdomen.** Thoracic and abdominal cuticle nearly bare except abdominal segments 5–8 moderately covered with dark unbranched, bifid and trifid minute setae dorsally and dorsolaterally; last abdominal segment densely covered with colorless unbranched minute setae on dorsolateral and lateral surfaces of each side of anal sclerite. Rectal organ simple, without secondary lobules. Anal sclerite of usual X-form, with anterior arms nearly as long as posterior ones, broadly sclerotized at base; accessory sclerite absent. Last abdominal segment with pair of large conical ventral papillae. Posterior circler with 106–108 rows of hooklets with up to 13 or 14 hooklets per row.

Remarks. *Simulium (G.) tokarensis* was described from females, males, pupae and larvae collected from Nakanoshima Island, Tokara Islands (Takaoka, 1973). It is not recorded from other islands of the Nansei Islands.

Morphological features newly described in the current study include relative length of the sensory vesicle against the third palpal segment, color of tuft hairs at the base of the radial vein in the female and male; frontal ratio, frons:head ratio, cibarium, relative length of the labrum against the clypeus, relative length of the hind basitarsus against its greatest width, and relative length of the tooth against the claw in the female; number of vertical columns of upper-eye (large) facets, abdomen with or without a pair of shiny spots in the male; relative length of the trichomes on the frons, face and thorax (Figure 6A–6F); terminal hooks (Figure 6M) in the pupa; and color of abdomen in the larva.

The characteristics differing from those in the original description (in parentheses) are relative length of the fore basitarsus against its greatest width 6.1–6.3 (ca. 5.0) in the female; fore coxa whitish yellow (yellowish brown), paramere with three long hooks and one medium-long hook (three large hooks and two or three incomplete small ones) in the male. These differences are thought to be intraspecific variation or an error of observation in the past.

This species is characterized in the female and male by the base of the radius with the tuft of yellow hairs mixed with dark hairs, in the female by the medium-long sensory vesicle (Figure 5A), and genital fork without a triangular projection directed posteromedially (Figure 5E); in the male by the number of upper-eye (large) facets in 12 or 13 vertical columns and 14 horizontal rows, hind basitarsus (Figure 5D) enlarged, 3.16–3.23 times as long as wide, and 1.07 and 1.28–1.29 times as wide as the greatest width of the hind tibia and femur, respectively, and ventral plate parallel-sided or slightly narrowed posteriorly when viewed ventrally (Figure 5F); in the pupa by the gill with eight slender filaments directed forward close together (Figure 6G), dorsum of abdominal segments 1 and 2 each moderately covered with microspines (Figure 6J), and terminal hooks plate-like, with the outer margin 2.4–3.5 times the length of the inner margin and weakly crenulated when viewed caudally (Figure 6M); and in the larva by the abdomen grayish, with or without faint reddish-brown markings on abdominal segments 5–9, postgenal cleft medium-long, 1.1–1.4 times as long as the postgenal bridge, and rectal organ simple, without secondary lobules.

This species is remarkable in having the simple larval rectal organ, which is very rare among species in the *S. ceylonicum* species-group of the subgenus *Gomphostilbia*. Only two other species from Sri Lanka, *S. (G.) ceylonicum* (Enderlein) and *S. (G.) elae Davies & Györkös*, have a simple rectal organ (Davies & Györkös, 1987). However, these species are readily distinguished from *S. (G.) tokarensis* by the female genital fork with a triangular projection directed posteromedially, male hind tibia whitish only on the base, stalk of the ventral pair of the pupal gill filaments much longer than the common basal stalk, and larval postgenal cleft long, 4.0 times as long as the postgenal bridge.

Morphological evaluation of a few diagnostic characteristics for assignment to species-group

Both *S. (G.) okinawense* and *S. (G.) tokarensis* were provisionally placed in the *S. ceylonicum* species-group redefined by Takaoka (2012) when the former *S. ceylonicum* species-group was split into three groups: *S. darjeelingense* species-group, *S. asakaoe* species-group and *S. ceylonicum* species-group s. str. The former *S. ceylonicum* species-group was characterized by having the enlarged male hind basitarsus (Takaoka & Davies, 1996). The *S. darjeelingense* species-group is separated from both the *S. asakaoe* species-group and *S. ceylonicum* species-group s. str. by the darkened fore coxae, and pupal gill of eight short filaments with their smooth surface, lacking annular ridges and furrows, whereas the latter two species-groups are distinguished from each other by the shape of the ventral plate and color of tuft hairs at the base of the radial vein in the

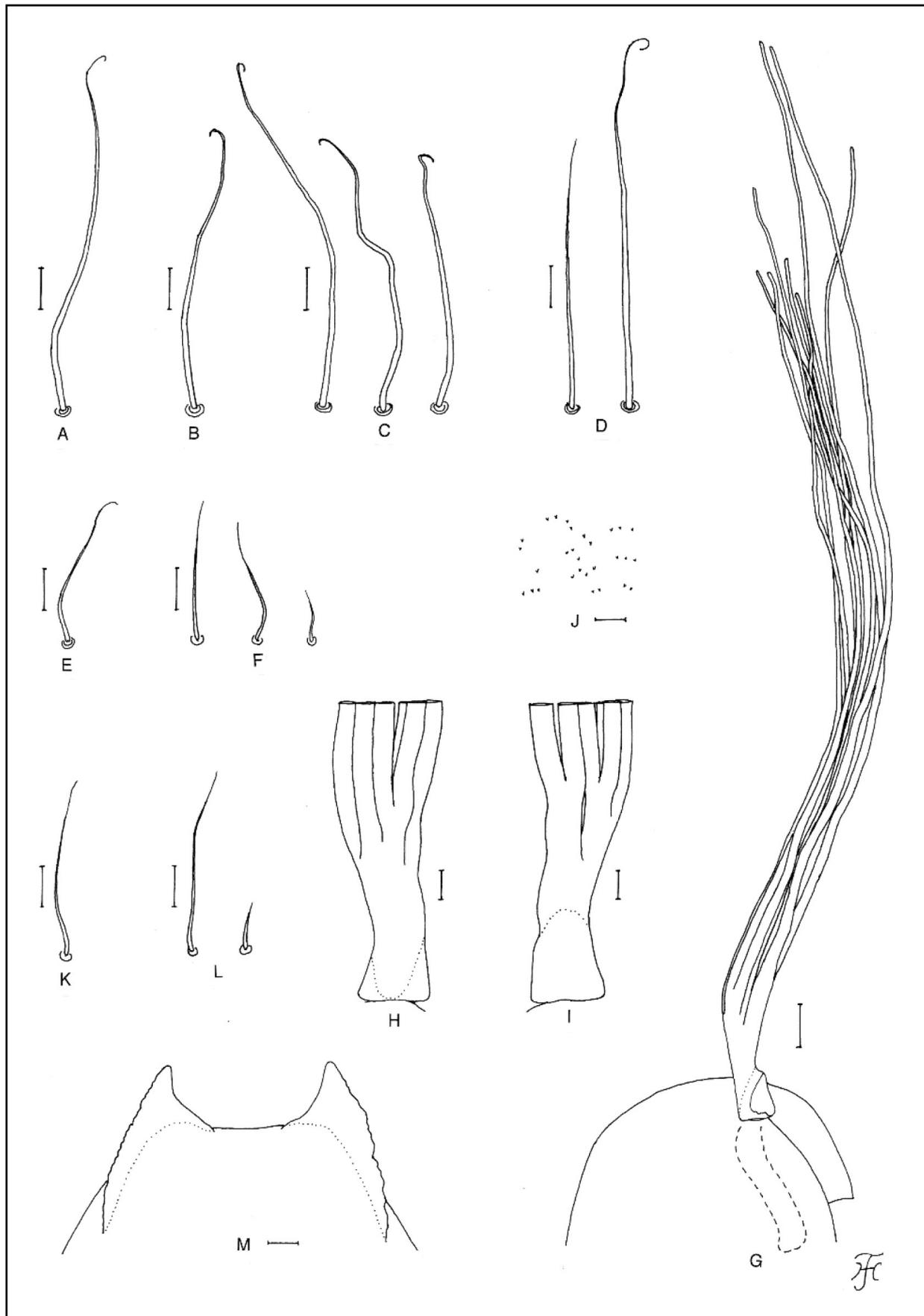


Figure 6. Pupa of *S. tokarensis*. A, frontal trichome; B, facial trichome; C–F, thoracic trichomes (C, mediodorsal; D, anterolateral; E, mediolateral; F, ventrolateral); G, gill filaments (right side; outer view); H and I, basal portions of gill filaments (right side; H, dorsal view; I, ventral view); J, microspines on dorsum of abdominal segment 1; K, hair-like seta on dorsum of abdominal segment 1; L, hair-like seta and minute seta on dorsum of abdominal segment 2; M, terminal hooks (caudal view). Scale bars. 0.1 mm for G; 0.04 mm for H and I; 0.02 mm for A–F, K and L; 0.01 mm for J and M.

female and male. When viewed ventrally, the ventral plate has the lateral margins somewhat emarginated basally or medially in the *S. asakoa* species-group, but narrowed posteriorly from the base or from the middle in the *S. ceylonicum* species-group s. str., and the tuft hairs are yellow in the *S. asakoa* species-group and dark in the *S. ceylonicum* species-group (though a few exceptions are known) (Takaoka, 2012).

In the current study, it is confirmed that the ventral plate has lateral margins parallel-sided in *S. (G.) okinawense* or parallel-sided or slightly narrowed posteriorly in *S. (G.) tokarensis*. The shape of the ventral plate of both species is, thus, somewhat similar to those of the *S. ceylonicum* species-group but differs from those of the *S. asakoa* species-group.

On the other hand, yellow tuft hairs (though mixed with dark hairs in *S. (G.) tokarensis*) at the base of the radius in the female and

male fit to the diagnosis of the *S. asakoa* species-group. In addition, the medium-long female sensory vesicle and medium-long larval postgenal cleft in both species are shared by most species of the *S. asakoa* species-group (female sensory vesicles and larval postgenal clefts are long in most known species in the *S. ceylonicum* species-group) (Takaoka, 2012; Takaoka et al., 2020).

Genetic analysis

Our genetic analysis (Figure 7) shows that *S. (G.) tokarensis* is closest to *S. (G.) songense*, *S. (G.) loeiense* and *S. (G.) phapeungense*, all in subgroup IV in the *S. asakoa* species-group. *Simulium (G.) okinawense* formed a sister clade with other members of the *S. asakoa* species-group with a high bootstrap value (80%). Their sister relationship is also supported by shorter genetic distances when compared with *S. ceylonicum* species-group (9.5% versus 12.1%).

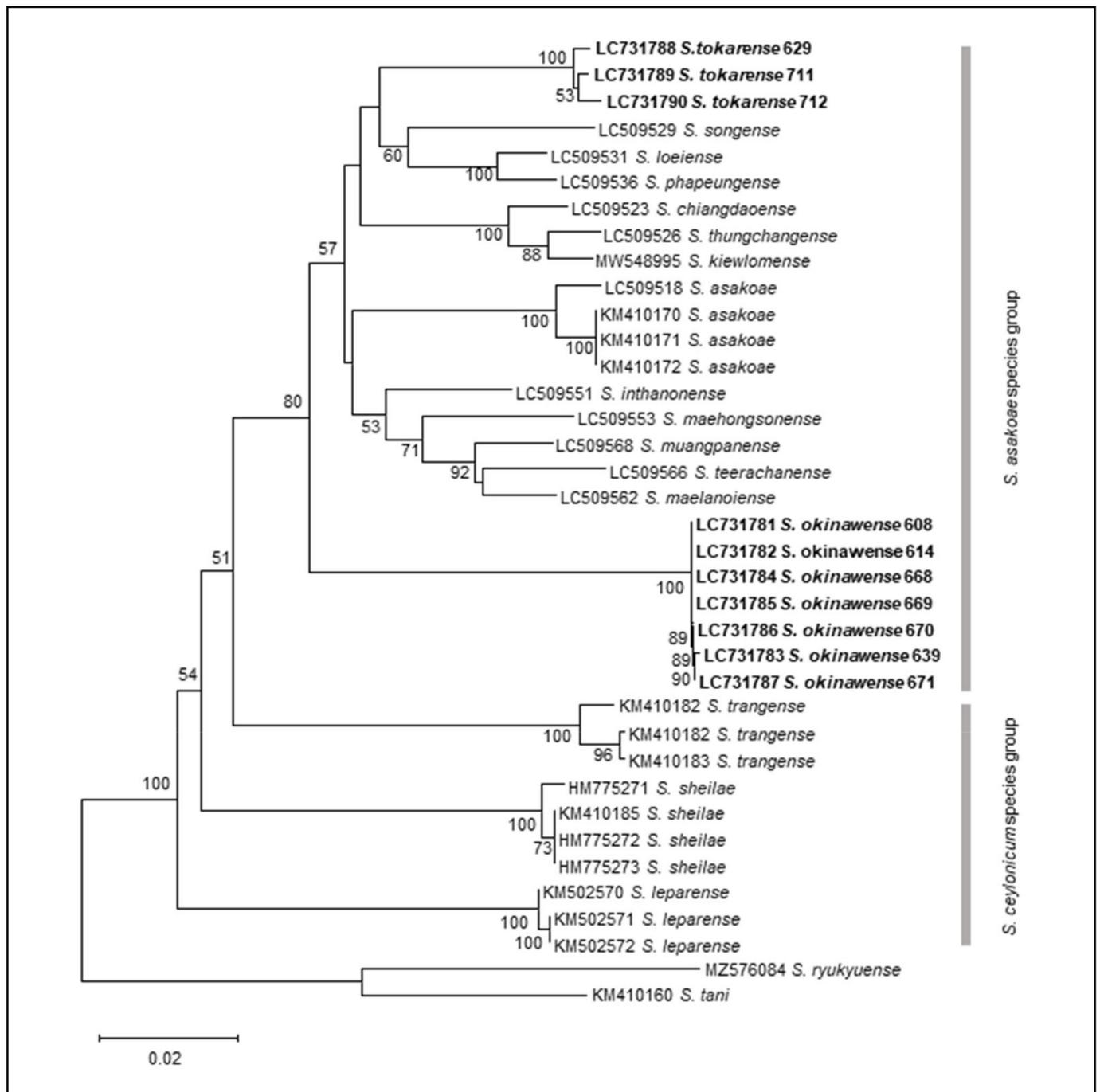


Figure 7. Neighbor-joining tree of species of the *Simulium asakoa* species-group and the *S. ceylonicum* species-group based on 658 bp COI gene sequences. Bootstrap values above 50% are shown near the branches. Branch lengths are proportional to genetic distance (scale bar). Bold characters represent the sequences obtained in the present study.

CONCLUSION

From the results of morphological and genetic analysis combined, *S. (G.) okinawense* and *S. (G.) tokarensis* are transferred from the *S. ceylanicum* species-group to the *S. asakoe* species-group.

ACKNOWLEDGEMENTS

We are grateful to Dr. Peter H. Adler (Professor Emeritus, Clemson University, Clemson, SC, USA) for reading the current manuscript and providing valuable comments. We acknowledge funding from the Ministry of Higher Education, Malaysia, under the Higher Institution Centre of Excellence (HiCoE) niche area vector and vector-borne diseases (project no. MO002-2019).

Declaration of Competing Interest

We declare that this is our original work. It has not been published elsewhere and we have no conflicts of interest concerning the work reported in this paper. All authors have contributed to this study throughout the study design, field work, data collection, data analyses and data interpretation. The authors have read and approved the manuscript.

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