

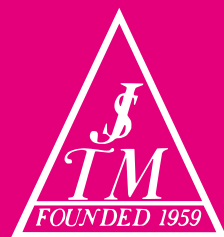
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TWO NEW AND THREE NEWLY RECORDED SPECIES OF BLACK FLIES (DIPTERA: SIMULIIDAE) FROM SABAH, MALAYSIA

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Abstract: Two new black-fly species, *Simulium (Gomphostilbia) guniki* sp. nov. and *S. (Nevermannia) borneoense* sp. nov., are described from adult flies emerged from pupae, collected from Sabah in Malaysia. *S. guniki* is assigned in the *ceyonicum* species-group, and is characterized by the darkened legs in the adults of both sexes, eight pupal gill filaments almost sessile, and the small postgenal cleft of the larva. *S. borneoense*, which is assigned to the *feuerborni* species-group, is most distinctive by having the four pupal gill filaments per side (in place of usual six filaments). In addition, three species of *Simulium (Gomphostilbia)*, i.e., *S. dentistylum*, *S. parahiyangum*, and *S. sheilae*, are newly recorded from Sabah.

Key words: Simuliidae, Black fly, Malaysia, Sabah, New species, Fauna

Edwards (1933) described five new species of black flies (Diptera: Simuliidae) from Sabah (formerly British North Borneo) in Malaysia, and then Smart and Clifford (1969) described additional six, one of which, *Simulium sabahense*, was found also in Sarawak, and another two, *S. tualanense* and *S. kiuliense*, were later synonymized with *S. aureohirtum* Brunetti and *S. nobile* de Meijere, respectively (Crosskey, 1973). Takaoka (1983) illustrated female genitalia of five known species (*S. aeneifacies* Edwards, *S. crassimanum* Edwards, *S. laterale* Edwards, *S. nigripilosum* Edwards, and *S. sabahense* Smart and Clifford), and Takaoka (1996) described one more new species, *S. beludense*, based on the pupal and emerged adult specimens, which were wrongly associated with female holotype of *S. nigripilosum* by Smart and Clifford (1969).

In 1998, I made faunistic surveys on Simuliidae in several localities of Sabah in Malaysia, and collected 12 species of *Simulium* s.l. including two new species and three newly recorded ones. I herein describe these two new species, of which one is assigned to the subgenus *Gomphostilbia* and the remaining one, to *Nevermannia*.

The morphological features and terms used herein follow mostly those of Crosskey (1969), and partially those of Takaoka (1983). All type specimens of new species will be in due course deposited at the Natural History Museum, London, U.K.

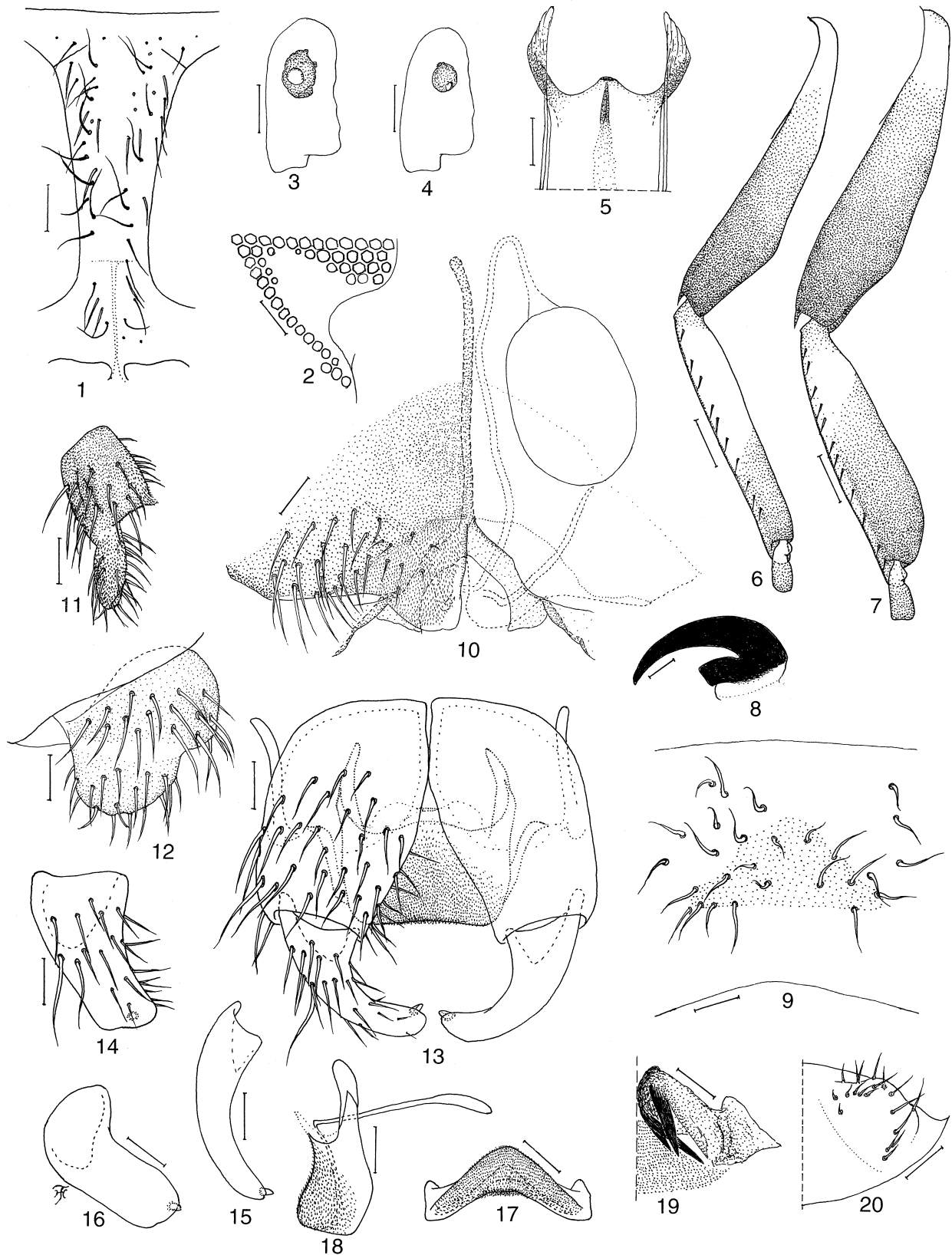
DESCRIPTION OF NEW SPECIES

Simulium (Gomphostilbia) guniki sp. nov.

Female. Body length ca. 2.1 mm. **Head.** Somewhat narrower than width of thorax. Frons (Fig. 1) narrow, dark brown, subshiny in certain angle of light, thinly white pruinose, covered with ca. 10 dark simple long hairs interspersed with 5 or 6 whitish-yellow scale-like recumbent hairs along each lateral margin; frontal ratio 1.9:1.0:3.5; frons-head ratio 1.0:6.1. Fronto-ocular area (Fig. 2) well developed, narrow. Clypeus dark brown, subshiny in certain angle of light, white pruinose, covered with 22-24 dark simple long hairs interspersed with ca. 12 whitish-yellow scale-like recumbent hairs on each side. Proboscis ca. 0.54 × as long as clypeus. Antenna composed of 2+9 segments, brownish black except scape, basal 1/2 of pedicel and base of 1st flagellar segment medium brown. Maxillary palp composed of 5 segments, light to medium brown, proportional lengths of 3rd, 4th and 5th segments 1.0:1.2:2.6; 3rd segment somewhat swollen; sensory vesicle (Fig. 3) ellipsoidal, ca. 0.33 × as long as 3rd segment, with medium opening medially. Maxillary lacinia with 10 or 11 inner teeth and 14 or 15 outer ones. Mandible with ca. 24 inner teeth and 7 or 8 outer ones. Cibarium (Fig. 5) medially forming round sclerotized plate folded forward from posterior margin, and with well sclerotized, medial longitudinal ridge near rounded tip. **Thorax.** Scutum brownish black, with 3 faint dark longitudinal vittae (1 medial and 2 subme-

dial), shiny, whitish pruinose, densely covered with yellowish-white or golden-yellow scale-like recumbent hairs intermixed with dark similar hairs; scutellum dark brown, covered with yellowish short hairs as well as dark long upright hairs along posterior margin. Postscutellum dark brown, shiny, white-pruinose, bare. Pleural membrane bare. Katepisternum dark brown, longer than deep, moderately covered with dark hairs. **Legs.** Light to dark brown except base of mid and hind tibiae, basal 1/2 of hind basitarsus and basal 1/2 of 2nd tarsal segment whitish (Fig. 6); all tibiae densely covered with whitish fine hairs (shiny in light) on outer surface (fore tibia), or on posterior and outer surfaces of a little less than basal 1/2 (mid tibia), or on posterior and outer surfaces of basal 3/5 (hind tibia); fore tarsus with moderate dorsal hair crest; fore basitarsus moderately dilated, ca. $6.6\times$ as long as its greatest width; hind basitarsus (Fig. 6) narrow, nearly parallel-sided, ca. $6.3\times$ as long as wide, and ca. $0.7\times$ and ca. $0.6\times$ as wide as greatest width of tibia and femur, respectively; calcipala ca. $1.2\times$ as long as wide, and ca. $0.6\times$ as wide as distal portion of basitarsus. All femora, tibiae and parts of tarsus densely covered with dark (and also pale) scale-like hairs. Claws (Fig. 8) each with large basal tooth, ca. $0.4\times$ as long as claw. **Wing.** Costa with dark spinules as well as dark hairs; subcosta with dark hairs except near apex bare; basal portion of radius fully haired; tuft hairs at base of radial vein dark brown; basal cell absent; length ca. 2.0 mm. **Abdomen.** Basal scale dark brown, with fringe of dark hairs with apical portion pale. Dorsal surface of abdomen brownish black except tergites 2, 3, 4 and 5 medium brown, moderately covered with short dark hairs; tergites of segments 2, 6, 7 and 8 wide and shiny, while those of segments 3, 4 and 5 narrow, nearly quadrate, subequal in size to one another, and all dull; ventral surface brownish black except median large portion of segment 2 pale; sternal plate on segment 7 (Fig. 9) weakly developed, triangular. **Genitalia** (Figs. 10-12). Sternite 8 bare medially, with 18 or 19 long stout hairs and 2 or 3 short hairs on each side. Anterior gonapophyses nearly triangular, with round medioposterior corner, thin, membranous, covered moderately with microsetae, interspersed with 4 or 5 short setae; inner margins sinuous, moderately sclerotized, close together. Genital fork of usual inverted-Y form, with arms of moderate width; arm moderately folded medially. Paraproct moderately produced ventrally, with 17 long hairs on ventral and lateral surfaces, and with 6-8 short hairs on inside surface. Cercus ca. $0.5\times$ as long as wide, rounded. Spermatheca ellipsoidal, ca. $1.5\times$ as long as wide, well sclerotized except tube, and with many fissures on surface; internal setae appear to be absent; both accessory tubes slender, subequal in diameter to major one.

Male. Body length 2.1 mm. **Head.** Slightly wider than thorax. Holoptic; upper eye consisting of 14 vertical columns and 16 or 17 horizontal rows of large facets. Face brownish black, white pruinose. Clypeus brownish black, shiny, thinly white pruinose, moderately covered with dark simple long hairs. Antenna composed of 2+9 segments, dark brown except base of 1st flagellar segment somewhat paler; 1st flagellar segment elongate, ca. $1.7\times$ as long as 2nd flagellar segment. Maxillary palp with 5 segments, light to medium brown, proportional lengths of 3rd, 4th and 5th segments 1.0:1.1:2.9; sensory vesicle (Fig. 4) nearly globular, small, ca. $0.2\times$ as long as 3rd segment, and with small opening. **Thorax.** Scutum brownish black, shiny (in certain angles of light, white pruinose broadly on shoulders, along lateral borders, and on prescutellar area), and densely covered with whitish-yellow (or golden-yellow in light) scale-like recumbent hairs, intermixed with brown ones; other features as in female. **Legs.** Light to dark brown except extreme base of mid and hind tibiae and basal 1/3 of hind 2nd basal segment whitish yellow, and basal 1/3 of hind basitarsus somewhat paler; all tibiae densely covered with whitish fine hairs (shiny in light) on outer surface (fore tibia), or on posterior and outer surfaces near base (mid and hind tibiae); fore tarsus with moderate dorsal hair crest; fore basitarsus somewhat dilated, ca. $7.6\times$ as long as its greatest width; hind basitarsus (Fig. 7) enlarged, gradually widened toward midpoint, then parallel-sided, and narrowed toward apex, ca. $3.91\times$ as long as wide, and ca. $0.85\times$ and ca. $0.96\times$ as wide as greatest width of tibia and femur, respectively; calcipala small, nearly as long as wide, and ca. $0.27\times$ as wide as greatest width of basitarsus. All femora, tibiae and parts of tarsus moderately or densely covered also with dark scale-like recumbent hairs. **Wing.** Length ca. 1.9 mm; other features as in female except subcosta bare. **Abdomen.** Basal scale brownish black with fringe of dark hairs. Dorsal and ventral surfaces of abdomen almost brownish black to black except median portion of ventral surface of 2nd segment pale, and covered with dark hairs; segments 2, 5, 6 and 7 each with pair of shiny dorsolateral patches, of which those on segment 2 broadly connected in middle to each other, and those on other segments not connected to each other. **Genitalia** (Figs. 13-20). Coxite in ventral view ca. $1.6\times$ as long as wide. Style in ventral view ca. $0.8\times$ as long as coxite, gently bent inward, with apical spine having median slit; style tapered from base to midpoint, then nearly parallel-sided apically when viewed ventrolaterally; style with round apex (though small incision present in right style) when viewed posteriorly. Ventral plate in ventral view transverse, nearly parallel-sided (though posterior 1/2 slightly narrowed), with posterior margin somewhat concave medially, and densely cov-



Figures 1-20 Morphological characters of *S. guniki* sp. nov. 1, female frons; 2, female fronto-ocular area; 3 and 4, 3rd segment of maxillary palp with sensory vesicle (3, female and 4, male); 5, female cibarium; 6 and 7, tibia, basitarsus and 2nd tarsal segment of hind leg (6, female and 7, male); 8, female claw of hind leg; 9, sternite 7 of female abdomen (ventral view); 10, sternite 8, anterior gonapophyses, genital fork and spermatheca of female genitalia *in situ* (ventral view); 11 and 12, paraproct and cercus *in situ* (11, ventral view and 12, lateral view); 13, coxites, styles, and ventral plate of male genitalia *in situ* (ventral view); 14-16, right style (14, ventrolateral view, 15, medial view and 16, end view); 17, ventral plate (end view); 18, ventral plate and median sclerite (lateral view); 19, left paramere and aedeagal membrane (end view); 20, left cercus (end view). Scale bars: 0.1 mm for figs. 6 and 7; 0.04 mm for figs. 1 and 2; 0.03 mm for figs. 3-5 and 9-20; 0.01 mm for fig. 8.

ered with microsetae on ventral surface; basal arms of moderate length, somewhat diverged and then slightly converged; ventral plate in lateral view somewhat produced ventrally; ventral plate in end view produced ventrally, with ventrolateral margins nearly straight, with dorsal margin much depressed, and densely covered with microsetae on posterior surface. Parameres of moderate size, each with 3 distinct stout hooks (2 long and 1 medium) and several smaller ones close together near apex; aedeagal membrane moderately setose. Median sclerite thin, plate-like, wide. Ventral surface of 10th abdominal segment moderately sclerotized anteriorly forming dorsal plate, without short setae near posterior margin on each side. Cerci rounded, each encircled with 18 or 19 setae.

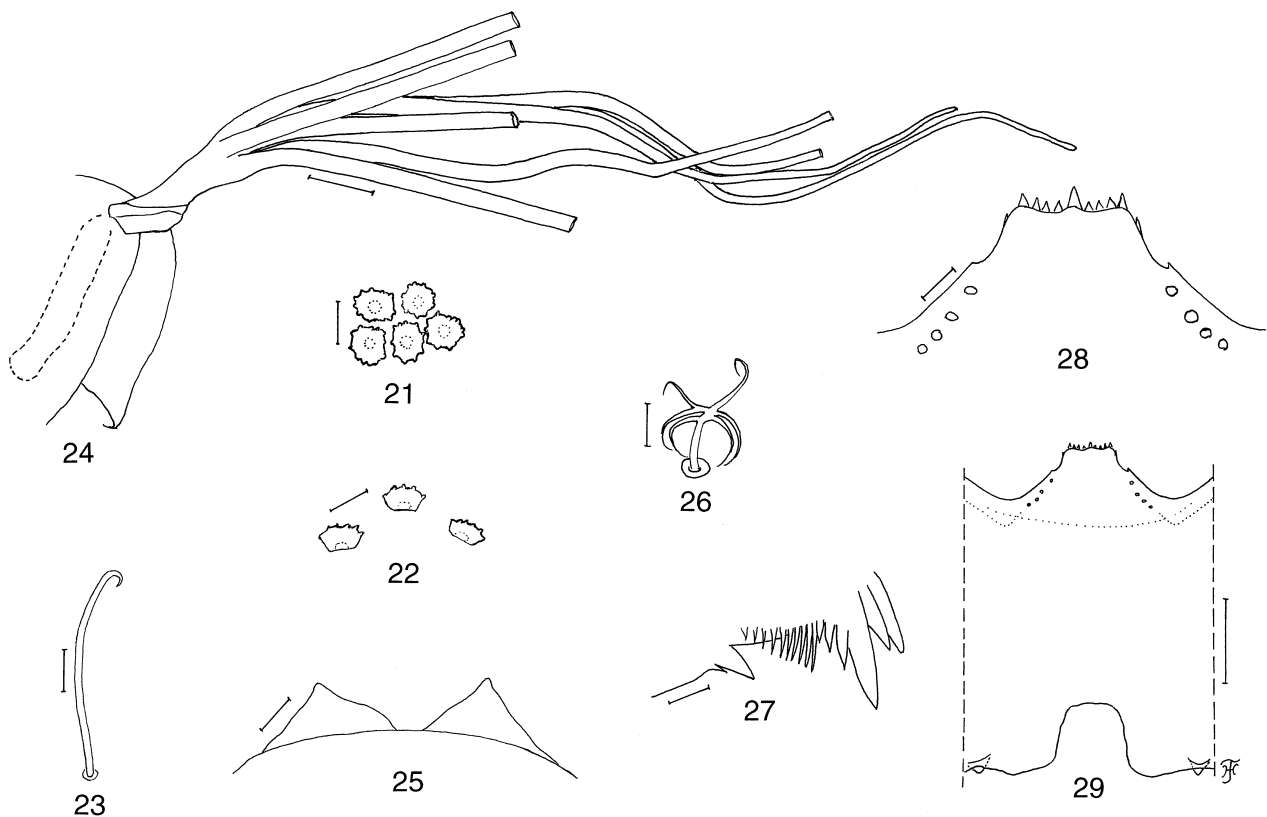
Pupa. Body length 2.5 mm. **Head.** Integument light to medium brown, moderately or densely covered with large tubercles, each having minute tubercles on surface (Figs. 21 and 22); antennal sheath normal, with no spinous projections, and almost bare; face with 1 pair of simple long very stout dark trichomes with coiled apex (Fig. 23), and frons with 3 pairs of similar trichomes; 3 frontal trichomes on each side arising close together, subequal in size to one another. **Thorax.** Integument light to medium brown, moderately covered with large tubercles similar to those on frons, and with 9 pairs of trichomes (3 dorsally, 2 anterolaterally, 1 posterolaterally, and 3 ventrolaterally), all similar to those on face and frons except 2 of 3 ventrolateral pairs medium and slender, and 1 of these 2 trichomes with uncoiled apex. Gill (Fig. 24) composed of 8 slender filaments, all arising almost directly from short basal common stalk, which has somewhat swollen transparent portion ventrally at base (though partially broken); apical portions of filaments lost except 2 filaments which are intact, and ca. 1.6 mm long; all filaments light to medium brown, subequal in thickness and also probably in length to one another, and all nearly the same size along basal 1/2, then gradually tapered toward apex; outer surface of filaments lacking annular ridges (though furrows present irregularly), and densely covered with minute tubercles. **Abdomen.** Tergum 1 pale yellow, bare, with 1 simple slender medium-long seta on each side; tergum 2 pale yellow anteriorly, bare, with 1 simple slender medium-long seta and 5 short setae on each side; terga 3 and 4 mostly pale, each with 4 hooked spines and 1 short seta on each side; tergum 5 lacking spine-combs; terga 6-8 each with distinct spine-combs in transverse row, together with comb-like groups of minute spines on each side; tergum 9 with comb-like groups of minute spines, and 1 pair of distinct flattened terminal hooks, which are nearly triangular when viewed posteriorly (Fig. 25). Sternum 4 with a few simple slender minute setae on

each side. Sternum 5 with 1 pair of bifid hooks submedially and a few short simple slender setae on each side; sternum 6 and 7 each with 1 pair of bifid inner and simple outer hooks somewhat spaced from each other, and a few simple short slender setae on each side. Each side of segment 9 with 3 grapnel-like hooklets (Fig. 26). **Cocoon.** Wall pocket-shaped, neatly and thickly woven; anterior margin somewhat thickly woven, and narrowly connected ventrally; posterior 1/2 with floor roughly woven; individual threads visible; 2.5 mm long \times 0.9 mm wide.

Mature larva. Body length 4.2 mm. Body entirely greyish black. Head capsule dark brown except eye-spot region on each side. Cervical sclerite composed of 2 small rod-like pieces, not fused to occiput, widely separated medially from each other. Antenna consisting of 3 segments and apical sensillum, longer than stem of labral fan; proportional lengths of 1st, 2nd and 3rd segments 1.0:1.0:0.8. Labral fan with ca. 40 main rays. Mandible (Fig. 27) with 2 usual mandibular serrations; comb-teeth composed of 3 teeth, gradually decreasing in size from 1st to 3rd; supernumerary serrations absent. Hypostomium (Fig. 28) with a row of 9 apical teeth; median and corner teeth well developed; 3 intermediate teeth on each side subequal in size to one another; lateral serrations undeveloped; 4 hypostomal bristles lying parallel to, or slightly divergent posteriorly from, lateral margin on each side. Postgenal cleft (Fig. 29) small, nearly quadrate in shape, and ca. 0.4 \times as long as postgenal bridge. Thoracic cuticle very sparsely covered with simple minute setae. Abdominal cuticle covered with simple setae very sparsely on segments 1-5, sparsely on other posterior segments except last segment densely covered with colorless simple (and a few bifid) setae on each side. Rectal papilla (retracted) appearing to be composed of 3 simple lobes. Anal sclerite of usual X-form, with posterior arms nearly as long as anterior ones; basal portion of arms widely sclerotized. Accessory sclerite absent. Ventral papillae large, conical, placed ventrally, then, well discernible when the larva is viewed laterally. Posterior cirlet with ca. 80 rows of up to 14 hooklets per row.

TYPE SPECIMENS. Holotype, female, reared from pupa, collected at Timpohon, at alt. 1,970 m, upstream of the water reserve just above the Carson Fall, in Kinabalu National Park, Sabah State, 13.III.1998. Paratype 1 male, reared from pupa, 1 mature larva, same data and date as holotype.

ECOLOGICAL NOTES. Two pupae were collected from slender tree sticks in water of a small clean stream flowing down on the rocky streambed, shaded, in natural forest,



Figures 21-29 Morphological characters of *S. guniki* sp. nov. 21-26, pupa, and 27-29, larva. 21 and 22, tubercles on thoracic integument (21, dorsal view and 22, lateral view); 23, facial trichome; 24, right gill filaments (lateral view); 25, terminal hooks (end view); 26, grapnel-like hooklet; 27, apical portion of mandible; 28, hypostomium (bristles omitted, ventral view); 29, ventral surface of head capsule showing hypostomium and postgenal cleft. Scale bars: 0.1 mm for fig. 24; 0.2 mm for fig. 29; 0.02 mm for figs. 23 and 28; 0.01 mm for figs. 21, 22, 25, 26 and 27.

with water temperature of 13 °C. This species was collected together with *S. aeneifacies*, *S. borneoense* sp. nov. and *S. nigripilosum*.

DISTRIBUTION. Sabah.

ETYMOLOGY. The species *guniki* is named after Dr. Gunik Gunsalam, Chief entomologist, Research and Education Division, Kinabalu Conservation Center, Sabah Parks (Kinabalu Park), Sabah.

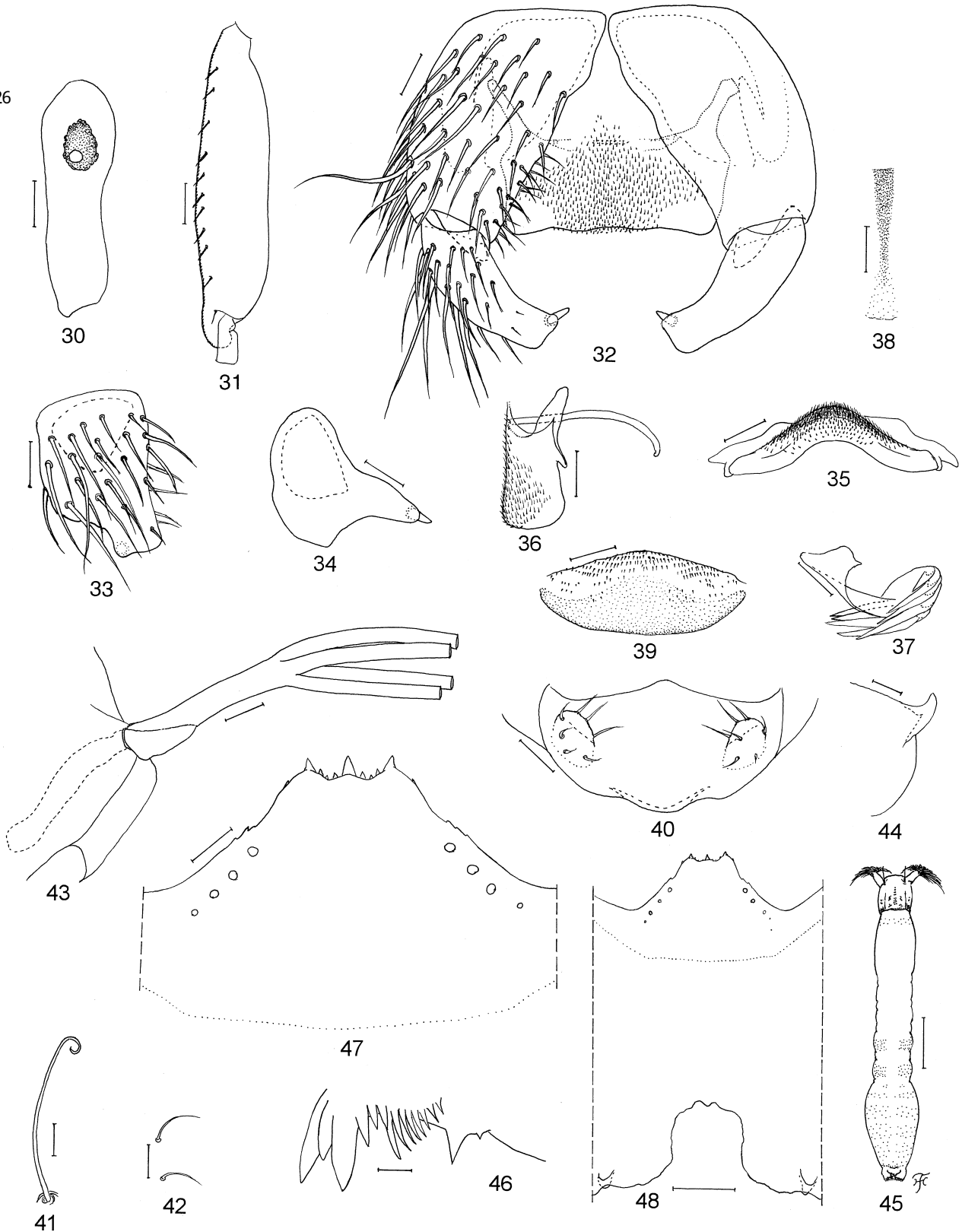
REMARKS. This new species is assigned in the *ceylonicum* species-group, defined by Takaoka and Davies (1996), by having the enlarged male hind basitarsus (Fig. 7). Within this species-group, it is easily separated from the other known species by having the darkened legs in the adults of both sexes, narrow frons in the female (Fig. 1), enlarged tubercles on the frons and thorax (Figs. 21 and 22), gill filaments almost sessile (Fig. 24), and triangular terminal hooks in the pupa (Fig. 25). The larva of this new species is

also very distinctive by having the small postgenal cleft (Fig. 29) and very darkened unicolorous body.

***Simulium (Nevermannia) borneoense* sp. nov.**

Female. Unknown.

Male. Body length 3.1 mm. **Head.** Slightly wider than thorax. Holoptic; upper eye consisting of large facets in 15 vertical columns and 17 horizontal rows. Clypeus medium brown, dull, thinly whitish grey pruinose, moderately covered with dark simple hairs. Antenna composed of 2+9 segments, dark brown except base of 1st flagellar segment pale yellow, and scape and pedicel light to medium brown; 1st flagellar segment much elongate, ca. 2.4 × as long as 2nd flagellar segment. Maxillary palp brown, composed of 5 segments, proportional lengths of 3rd, 4th and 5th segments 1.0:1.0:1.6; 3rd segment (Fig. 30) of moderate size; sensory vesicle small, ellipsoidal, ca. 0.22 × as long as 3rd segment. **Thorax.** Scutum medium to dark brown, subshiny, thinly whitish-grey pruinose, densely covered with yellow recum-



Figures 30-48 Morphological characters of *S. borneoense* sp. nov. 30-40, male, 41-44, pupa and 45-48, larva. 30, 3rd segment of maxillary palp with sensory vesicle; 31, basitarsus and 2nd tarsal segment of hind leg; 32, coxites, styles, and ventral plate of genitalia *in situ* (ventral view); 33 and 34, right style (33, ventrolateral view, 34, end view); 35, ventral plate (end view); 36, ventral plate and median sclerite (lateral view); 37, left paramere (anterodorsal view); 38, median sclerite (end view); 39, aedeagal membrane and dorsal plate (posterodorsal view); 40, cerci (end view); 41, facial trichome; 42, frontal trichomes; 43, right gill filaments (only basal portion shown, lateral view); 44, left terminal hook (lateral view); 45, whole body of larva showing faint brownish markings on thorax and abdomen (dorsal view); 46, apical portion of mandible; 47, hypostomium (bristles omitted, ventral view); 48, ventral surface of head capsule showing hypostomium and postgenal cleft. Scale bars: 1.0 mm for fig. 45; 0.1 mm for figs. 31, 43 and 48; 0.03 mm for figs. 30, 32-40 and 47; 0.02 mm for figs. 41, 42 and 44; 0.01 mm for fig. 46.

bent hairs, and with several black upright hairs on prescutellar area. Scutellum light brown, with many dark upright hairs as well as yellow recumbent hairs. Postscutellum medium to dark brown, not shiny, and bare. Pleural membrane bare. Katepisternum medium to dark brown, longer than deep, and bare. **Legs.** Foreleg: coxa and trochanter dark yellow to light brown; femur light brown with apical cap medium to dark brown; tibia medium brown with apical cap dark brown; tarsus dark brown; basitarsus slender, slightly dilated, ca. $10.0\times$ as long as its greatest width at apex. Midleg: coxa medium brown; trochanter yellow or a little darker; femur light brown with apical cap medium brown; tibia medium brown with apical cap dark brown; tarsus dark brown. Hind leg: coxa medium brown; trochanter yellow; femur light brown with apical cap dark brown; tibia medium brown with base light brown and apical cap dark brown; basitarsus medium brown, and other tarsal segments dark brown except basal 1/3 of 2nd segment dark yellow or light brown; basitarsus (Fig. 31) enlarged, ca. $3.87\times$ as long as its greatest width, ca. $1.07\times$ and ca. $1.11\times$ as wide as hind tibia and femur, respectively. Calcipala well developed, ca. $0.9\times$ as long as wide; pedisulcus well developed. All femora moderately covered with yellow hairs on outer and/or posterior surface of almost all of shaft, and fore tibia moderately covered with yellow hairs on outer surface of basal 4/5 of shaft, and mid and hind tibiae also moderately covered with yellow hairs on posterior and outer surface of basal 3/4 of shaft. **Wing.** Costa with dark spicules and hairs; subcosta with several hairs on basal 2/3; basal portion of radial vein fully haired; tuft hairs at base of radial vein dark; basal cell absent; length 2.6 mm. **Abdomen.** Basal scale dark brown, with fringe of dark long hairs. Dorsal surface of abdominal segments dark brown, with dark simple hairs; ventral surface light to medium brown. **Genitalia** (Figs. 32-40). Coxite subquadrate, much longer than wide. Style much shorter than coxite, broad, nearly parallel-sided from base to near apex, then abruptly tapered apically and bent inwards, and with distinct apical spine directed inward and forward. Ventral plate lamellate, much shorter than wide, well sclerotized except along anterior margin thin, membranous, with posterior margin sinuous (when viewed ventrally), and moderately covered with fine short setae on ventral and posterior surfaces; arms of moderate length, stout, and curved outwardly and dorsally. Parameres of normal form, each with 8 distinct hooks. Median sclerite simple, club-shaped, narrow medially. Dorsal plate well developed, broad, thin, with medial portion widened. Aedeagal membrane moderately covered with spinous microsetae. Cerci small, rounded, each with 6 or 7 simple hairs.

Pupa. Body length 3.2 mm. **Head.** Integument yellow,

sparsely covered with round tubercles; face with 1 long somewhat stout simple trichome with coiled apex (Fig. 41) on each side, while frons with 2 short slender simple trichomes (Fig. 42) on each side. **Thorax.** Integument yellow, sparsely covered with round tubercles, with 3 long slender simple trichomes mediodorsally, 2 slender simple-long trichomes (1 long, and 1 medium-long) mediolaterally, 1 medium slender simple trichome posterolaterally, and 3 short slender simple trichomes ventrolaterally, on each side. Gill (Fig. 43) with 4 long slender filaments arranged in inner and outer pairs, each pair sessile or with very short stalk, arising from medium-long basal common stalk; all filaments lying close together, directed forward, yellowish or yellowish brown, subequal in length and thickness to one another (length 3.8-4.1 mm), and much longer than pupal body; cuticular surface with distinct annular ridges and furrows (though ridges becoming indistinct on apical 1/2), and densely covered with minute tubercles. **Abdomen.** Terga 1 and 2 yellowish, almost bare; tergum 1 with 1 medium slender seta on each side; tergum 2 with 1 medium-long slender seta and 5 short spinous setae on each side; terga 3 and 4 each with 4 hooks and a few spinous setae on each side; terga 5-8 each with a transverse row of spine-combs and comb-like groups of minute spines directed backward on each side (though those on tergum 5 small in number); tergum 9 with 1 pair of distinct horn-shaped terminal hooks (Fig. 44), and comb-like groups of minute spines. Sternum 4 with a few slender setae on each side; sternum 5 with 1 pair of bifid hooks submedially and a few slender setae on each side; sterna 6 and 7 each with 1 bifid hook submedially, 1 simple hook laterally, and a few slender setae on each side. Each side of 9th segment with a single slender seta. **Cocoon.** Simple, wall-pocket-shaped, compactly woven without open spaces in web, very thin, with anterior margin thickly woven, and extending ventrolaterally; anterodorsal projection absent; posterior 1/2 with floor neatly woven; individual threads visible; 4.0-4.2 mm long \times 3.0-3.4 mm wide.

Mature larva. Body length 6.0 mm. Body creamy white, with light brown dorsal markings on thorax and abdomen as follows: thoracic segment 1 with light brown transverse band, abdominal segments 3 and 4 each with light brown transverse band medially disconnected, abdominal segments 5-8 each with faint light brown transverse band (Fig. 45). Cephalic apotome yellow with posterior margin somewhat darkened; head spots distinct; 3 isolated spots below, and 2 larger spots behind, eye-spot region on lateral surface of head capsule also distinct; eye brow faint, not connected posteriorly to upper larger lateral spot. Cervical sclerite composed of 2 small rod-like pieces, not fused to occiput,

widely separated medially from each other. Antenna consisting of 3 segments and apical sensillum, longer than stem of labral fan; proportional lengths of 1st, 2nd and 3rd segments 1.0:0.9:1.1; segmentation between 1st and 2nd segments indistinct. Labral fan with ca. 33 main rays. Mandible (Fig. 46) with 2 usual mandibular serrations; comb-teeth composed of 3 teeth, 1st tooth largest, 2nd and 3rd teeth subequal in size to each other; supernumerary serrations absent. Hypostomium (Fig. 47) with a row of 9 apical teeth, median and corner teeth well developed; median tooth of 3 intermediate teeth on each side smallest; lateral serrations weakly developed apically; 4 or 5 hypostomal bristles lying parallel to, or slightly divergent posteriorly from, lateral margin on each side. Postgenal cleft (Fig. 48) small, shallow, round or nearly quadrate, and ca. 0.6 × as long as postgenal bridge. Gill histoblast with 4 filaments arising from medium-long basal common stalk. Thoracic cuticle bare. Abdominal cuticle bare except last segment moderately covered with colorless short setae on each side of anal sclerite. Rectal papilla of 3 lobes, each lobe with 16-20 finger-like secondary lobules. Anal sclerite of usual X-form, with posterior arms subequal in length to anterior ones; basal portion of arms widely sclerotized. Accessory sclerite absent. Ventral papillae large, conical, placed ventrally, then, well discernible when the larva is viewed laterally. Posterior circlet with ca. 72 rows of up to 14 hooklets per row.

TYPE SPECIMENS. Holotype, male, reared from pupa, collected at Timpohon (same locality as *S. guniki* sp. nov.), at alt. 1,970 m, upstream of the water reserve just above the Carson Fall, in Kinabalu National Park, Sabah State, Malaysia, 13. III.1998. Paratype 2 males, reared from pupa, 1 pharate male, and 1 mature larva, same data and date as holotype.

ECOLOGICAL NOTES. All the four pupae and 2 larvae collected were attached on glass leaves (except one pupa on slender tree stick) in water of a small clean stream (same stream in which *S. guniki* sp. nov. was collected). This species was collected together with *S. aeneifacies*, *S. guniki* sp. nov. and *S. nigripilosum*.

DISTRIBUTION. Sabah.

ETYMOLOGY. The species *borneoense* refers to the island name, Borneo.

REMARKS. The *feuerborni* species-group within the subgenus *Simulium* (*Nevermannia*) is a small and relatively homogenous taxon, consisting of 13 named species in the Ori-

ental and Palaearctic Regions (Crosskey and Howard, 1996; Crosskey, 1999; Takaoka and Saito, 2000).

S. borneoense sp. nov. is readily assigned to the *feuerborni* species-group by the characteristic male genitalia with a simple lamellate ventral plate, short inwardly-twisted styles, several parameral hooks, and a simple narrow median sclerite (Figs. 32-38).

It is worthwhile to note that the pupa of *S. borneoense* is easily distinguished from those of the nine known species within this species-group by the number of gill filaments per side, i.e., four (Fig. 43) in place of six.

The other four known species of the *feuerborni* species-group were described from adult males (and also females in one species) alone, and then, their pupal and larval stages have remained unknown. Among these, *S. fusciner-vis* was recorded from Sabah (Edwards, 1933), but it differs from the new species by having the reduced number of large eye facets, i.e., ca. 12 vertical columns and 12-14 horizontal rows (not 15 vertical columns and 17 horizontal rows), and paramere with 10 hooks (not eight); *S. bryopodium* described from Palawan Island, Philippines (Delfinado, 1971), also differs by having the subcosta bare (not hairy), ventral plate with basal arms straight (not diverged), and paramere with six hooks (not eight); *S. senile*, described from West Himalaya (Brunetti, 1911), is different by having the bicolored hind basitarsus and the style with no apical spine; *S. rufithorax*, described from a male and four females collected from India (Brunetti, 1911), has a reddish brown thorax according to the original description.

NOTES ON NEWLY RECORDED SPECIES

Simulium (*Gomphostilbia*) *dentistylum* Takaoka and Davies, 1995

Simulium (*Gomphostilbia*) *dentistylum* Takaoka and Davies, 1995: 51-55 (male, pupa and larva); Kuvangkadilok and Takaoka, 2000: 173.

SPECIMENS EXAMINED. 1 male, reared from pupa, collected from a stream (alt. ca. 490 m), crossing the road, near Entrance of Hot spring Park, Poring, Sabah, 12. III.1998; 4 mature and 1 immature larvae, collected from a small stream (alt. ca. 550 m) flowing inside Hot spring Park, Poring, Sabah, 12. III.1998.

ECOLOGICAL NOTES. The pupa of this species was collected from a trailing grass in a moderately-flowing stream with its width of 3-6 m, partially shaded. Five larvae of this species were collected from fallen leaves in a slowly-flowing shaded stream with its width of ca. 20 cm. The water temperature of the stream was 23-25 °C. This species

was collected together with *S. beludense*, *S. laterale*, *S. parahiyangum*, *S. sabahense* and *S. sheilae*, or with *S. sabahense* and *S. sheilae*.

DISTRIBUTION. Peninsular Malaysia, Sabah (new record), Thailand.

REMARKS. *S. dentistylum* was originally described from Peninsular Malaysia (Takaoka and Davies, 1995), and later it was also recorded from Thailand (Kuvangkadilok and Takaoka, 2000).

The male and its associated pupa, and larvae from Sabah agree well morphologically with the original descriptions except a difference of the leg coloration of the male. All the tibiae of the present male specimen are much darker than those of the holotype male in the original description, and also have a subbasal dark band. It is considered that the holotype male specimen from Peninsular Malaysia might have had a faded leg coloration when described since it had been preserved in ethanol for nearly 20 years. In fact, additional male specimens of *S. dentistylum* recently collected in Johor State, Peninsular Malaysia, have the darker tibiae with a dark subbasal band, as in the present male specimen (unpublished data). The revised description of the male, as well as the first description of the female of this species, will be given in a separate paper.

***Simulium (Gomphostilbia) parahiyangum* Takaoka and Sigit, 1992**

Simulium (Gomphostilbia) parahiyangum Takaoka and Sigit, 1992, 135-141 (female, male, pupa and larva); Takaoka and Davies, 1995: 50-51; Takaoka and Saito: 1996, 165; Takaoka *et al.*, 2000: 161.

SPECIMENS EXAMINED. 1 mature and 1 immature larvae, collected from a stream (alt. ca. 490 m), crossing the road, near Entrance of Hot spring Park, Poring, Sabah, 12. III.1998.

ECOLOGICAL NOTES. The larvae of this species were collected from trailing grasses in a moderately-flowing stream with its width of 3-6 m, partially shaded. The water temperature of the stream was 25 °C. This species was collected together with *S. beludense*, *S. dentistylum*, *S. laterale*, *S. sabahense* and *S. sheilae*.

DISTRIBUTION. Java, Sumatra, Thailand, Peninsular Malaysia, Sabah (new record).

REMARKS. *S. parahiyangum* was originally described from Java (Takaoka and Sigit, 1992), and it was also recorded

from Peninsular Malaysia (Takaoka and Davies, 1995), Thailand (Takaoka and Saito, 1996), and Sumatra (Takaoka *et al.*, 2000). The larva of this species is easily distinguished from other *Gomphostilbia* species by having the prominent dorsal protuberances on abdominal segments 1-5, several dark stout spines on the abdominal segments 6-8, and the deep postgenal cleft reaching the posterior margin of the hypostomium (Takaoka and Sigit, 1992).

***Simulium (Gomphostilbia) sheilae* Takaoka and Davies, 1995**

Simulium (Gomphostilbia) sheilae Takaoka and Davies, 1995: 60-65 (female, male, pupa and larva); Kuvangkadilok and Takaoka, 2000: 172-173; Takaoka *et al.*, 2000: 161.

SPECIMENS EXAMINED. 6 females, 5 males, all reared from pupae, collected from a small stream (alt. ca. 550 m) flowing in a forest in the Hot spring Park at Poring, Sabah, 12. III.1998.

ECOLOGICAL NOTES. The pupae and larvae of this species were collected from fallen leaves in a slowly-flowing stream with its width of ca. 20 cm, shaded. The water temperature of the stream was 23 °C. This species was collected together with *S. sabahense*.

DISTRIBUTION. Peninsular Malaysia, Sabah (new record), Sumatra and Thailand.

REMARKS. *S. sheilae* was originally described from Peninsular Malaysia, and was assigned to the *ceylonicum* species-group by Takaoka and Davies (1995). It was also recorded from Thailand (Kuvangkadilok and Takaoka, 2000) and Sumatra (Takaoka *et al.*, 2000).

This species is separated from other related species of the same species group by the enlarged oblong female sensory vesicle (ca. 0.7 times as long as 3rd maxillary palpal segment) and the almost brown male hind basitarsus (though basal 1/3 or a little less somewhat paler). The reared adult female and male specimens, as well as pupal and larval ones examined in this study, are morphologically almost the same as those originally described, with an exception that the scutum of the present male specimens is whitish pruinose on each shoulder, along both lateral margins and on the prescutellar area (not entirely whitish pruinose, as in the type male specimen).

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RESEARCH ACTIVITIES ON EMERGING AND REEMERGING DISEASES

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Abstract: This paper showed the trend in the number of paper published on emerging and reemerging diseases by using the list of the Medline, which is one of the most valuable databases in the clinical and biological medicine. The number of research papers published on the emerging and reemerging infections in that database decreased in 1997, in comparison with those between 1994 and 1996, in spite of the fact that the total number of the paper published has been increasing year by year. There is also a report showing that the number of the published papers of noticeable infectious diseases such as AIDS, Hepatitis B, Gonorrhoea, Pertussis and Tuberculosis declined by 15% or more in the USA from 1993 to 1995. It also became clear that USA is the highest in rank for the number of papers published and it was well ahead of the other countries. However, as for *Vibrio cholerae* O139, Human T-lymphotropic Virus type 1 and Plague, a lot of papers have been published by responding countries like India, Japan and Russia, respectively. This result might be showing that research activity of each country for each disease relates to the extent of their concern over each disease.

Key words: Emerging disease, Reemerging disease

INTRODUCTION

There was a paper titled "Infectious Diseases-A global health threat" published by US government in 1995, which showed the importance of arising awareness of the infectious disease. According to the WHO and CDC in Atlanta, United States, the term of "emerging and reemerging diseases" refers to diseases of infectious origin whose incidence in human has either increased within the past two decades or threatens to increase in the near future (Institute of Medicine, 1992). And CDC listed at least thirty diseases as representatives of emerging and reemerging diseases.

National opinion leaders generally had considered the threat of infectious diseases to be of only historical interest in 1960s and 1970s (Lederberg *et al.*, 1992; Garrett, 1994; Martine, 1996; Sande, 1996; Schwartz, 1997; Stephens *et al.*, 1998). However, the emergence of HIV or Ebola virus as well as the resurgence of Tuberculosis or Malaria has changed the political circumstances that surround the infectious diseases.

Infectious diseases also remain the leading courses of death not only in the developing countries but also in developed countries like USA (World Bank, 1993; McGinnis *et al.*, 1993). Further, emerging and reemerging infections

have been attracting greater attention from not only the public health viewpoint but also national security viewpoint in recent years. Under those circumstances, we had a strong interest in whether research activities on emerging and reemerging infections have changed or not, and how.

We thought that the number of paper published is one of the appropriate indicators to estimate their research activities on those infectious diseases.

METHODS

Medline on Internet was chosen for this survey because it has a broader coverage about biomedical studies. Papers written in English were also selected. There are 105,676 papers in total. The reason why papers written in other languages were excluded is that those might be inferior in quality to the international journals written in English and less reading worldwide. Medline is published by National Library of Medicine (NLM) in the United States and is the main database for secondary reference materials in the field of medicine. Papers were selected by the title including the name of pathogens and were categorized by country, in which corresponding institutions were located, even if studies were conducted through international col-

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Table 1 The number of published paper of emerging diseases

Pathogen	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	Total
HIV							108	977	2,035	2,267	2,853	3,244	3,482	3,787	3,728	3,610	3,856	2,514	32,461
Hepatitis C	9	11	11	9	18	10	16	19	24	66	285	528	747	879	1,106	1,379	1,213	904	7,234
<i>Helicobacter pylori</i>										7	213	409	390	589	564	809	913	589	4,483
HTLV-1		3	6	31	115	366	613	291	192	230	230	259	269	280	235	228	229	126	3,703
Rotavirus	97	122	126	182	166	139	167	181	204	197	199	164	151	155	152	123	161	66	2,752
<i>Borrelia burgdorferi</i>						12	39	53	73	118	106	131	167	194	164	145	143	97	1,442
<i>Campylobacter jejuni</i>	44	60	99	11	130	72	114	88	91	61	77	63	72	80	77	95	61	40	1,335
<i>Legionella pneumophila</i>	47	70	66	85	66	64	74	72	77	76	57	65	75	49	67	63	50	23	1,146
Hepatitis E	26	31	27	19	23	23	29	18	18	30	32	69	72	78	102	107	92	77	873
Prion			4	3	8	17	22	26	30	23	42	59	63	95	90	102	158	109	851
enterohemorrhagic <i>Escherichia coli</i> O157				2	1	6	12	20	15	26	17	22	22	42	48	65	80	92	470
Hantavirus				2	1	3	11	12	10	8	21	14	48	83	65	53	31		362
<i>Cryptosporidium parvum</i>						1	5	5	3	13	36	29	34	53	41	72	46		338
Ehrlichia	6	1	4	1		4	7	11	15	16	17	21	21	18	25	18	33	23	241
Ebola virus	10	6	10	10	1		3	1	1	3	6	2	7	8	5	45	30	16	164
<i>Vibrio cholerae</i> O139														13	40	51	38	16	158
Bartonella		1				1	1		2		2	3	6	4	7	46	52	31	156
Encephalitozoon	10	3	3		2	1	6	4	14	6		7	4	15	11	13	17	16	132
<i>Staphylococcus aureus</i> toxin shock			3		3	6	5	10	9	24	7	3	4	5	9	2	7	2	99
Cyclospora											1			6	13	20	26	18	84
HHV 6	1	1	3	3	2	1	1	1	3	6	5	10	11	5	9	3	7	5	77
<i>Enterocytozoon bienersi</i>											3	3	3	8	5	10	8	9	49
HHV 8			2		1	1	2	2		2		2		2	3	2	5	12	36
Guanarito															1	1	1		3
Sabia																	1	1	2
Total	250	309	364	356	538	724	1,223	1,790	2,820	3,171	4,173	5,121	5,609	6,394	6,597	7,043	7,306	4,863	58,651
No. of papers on Medline	263,310	265,496	275,406	287,713	297,353	249,485	333,854	258,200	378,147	369,645	376,690	375,903	377,414	207,871	388,433	398,231	403,323	405,173	6,011,647

Table 2 The number of published paper of reemerging diseases

Reemerging disease	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	Total
Tuberculosis	808	772	968	825	813	578	901	1,055	1,030	895	1,120	1,070	1,023	1,186	1,172	1,451	1,399	773	17,839
Malaria	221	271	325	368	376	367	387	494	555	688	568	596	624	606	617	577	580	396	8,616
Pertussis	55	86	89	122	141	183	216	240	341	312	309	342	280	261	255	235	238	188	3,893
Schistosomiasis	164	160	150	166	180	143	196	307	244	222	151	123	166	135	136	135	156	67	3,001
Leishmaniasis	85	83	107	122	134	117	184	195	163	146	188	174	186	239	226	226	224	94	2,893
Cholera	137	142	141	127	130	88	127	142	142	169	169	232	231	216	194	203	156	90	2,836
Rabies	121	143	171	104	122	93	150	140	197	122	135	125	145	145	144	129	141	73	2,400
Toxoplasmosis	122	111	114	127	136	78	118	113	126	143	158	135	171	138	138	134	121	68	2,251
Dengue	44	49	57	62	62	41	51	97	58	87	84	79	78	101	100	100	96	62	1,308
Plague	47	38	51	54	40	32	39	39	46	46	47	35	57	46	103	70	35	38	863
Echinococcus	22	32	35	34	41	22	47	52	55	44	60	58	56	61	52	52	42	43	808
Yellow fever	15	33	17	23	25	17	35	25	16	24	28	18	21	31	14	31	25	15	413
Diphtheria	1	1															1		3
Total	1,842	1,921	2,225	2,134	2,200	1,759	2,451	2,899	2,973	2,898	3,017	2,987	3,038	3,165	3,151	3,343	3,214	1,907	47,124
No. of papers on Medline	263,310	265,496	275,406	287,713	297,353	249,485	333,854	258,200	378,147	369,645	376,690	375,903	377,414	207,871	388,433	398,231	403,323	405,173	6,011,647

laboration.

The total ranking list was made by giving scores to the countries. In terms of the number of papers, the first, sec-

ond and third ranking countries were given five, three and one, respectively and all the scores for each country were added up at the end.

RESULT AND DISCUSSION

Trend in the number of research papers: In terms of emerging diseases, 58,552 papers had been published in Medline between 1980 and 1997, in total (Table 1). Human immunodeficiency virus (HIV), Hepatitis C virus (HCV) and *Helicobacter pylori* were the first, second and third in ranking for the number of papers, respectively. However, except for enterohemorrhagic *Escherichia coli* O157 and Human herpes virus 8 (HHV 8), the number of publication in 1997 is much fewer than the average number between 1994 and 1996. In recent year, enterohemorrhagic *E. coli* O157 endemic occurred in some developed countries and HHV 8 was shown to be associated with Kaposi's sarcoma.

Regarding the reemerging diseases, 47,124 papers were published in total in Medline between 1980 and 1997 (Table 2). The papers related to Tuberculosis take the first place and papers on Malaria take the second in number. The number of research papers listed as reemerging diseases decreased in 1997, compared with the average number of papers published between 1994 and 1996.

It is unknown whether the decreased number of the papers found may reflect reduced concern over emerging and reemerging diseases. The number of papers published reflects to some extents of concerning over those infectious diseases in the last several years. Thus, this result might be showing the decreasing of the concerning over emerging and reemerging diseases despite the fact that there are a lot of papers which advocate the importance of the research on emerging and reemerging diseases (Anthony, 1998; MMWR, 1998).

High-ranking countries in the number of papers: Regarding to the emerging diseases, USA was the highest in rank for the number of papers published except for HCV, Human T-lymphotropic Virus type 1 (HTLV-1), Ebola and *Vibrio cholerae* O139. Japan was the highest ranking for HCV and HTLV-1, and Russia was the highest for Ebola, and India was the highest for *V. cholerae* O139. UK is the second country for seven pathogens in terms of the number of paper published, and France and Japan were the second for five pathogens, and USA was the second for four, and Germany was the second for three. Excluding USA, UK, Japan, France and Germany, only Russia, India, Canada and Italy were seen in top three countries.

In terms of reemerging diseases, USA were also the highest in ranking for those except Leishmaniasis, Toxoplasmosis, Plague and Echinococcus. France was the highest in ranking for Leishmaniasis and Toxoplasmosis, and Russia was the highest for Plague, and New Zealand and Australia was the highest for Echinococcus. USA are also the second country for three pathogens in terms of the number of paper

Table 3 Total ranking of countries

	Emerging	(score)	Reemerging	(score)
1	USA	(117)	USA	(54)
2	Japan	(28)	France	(18)
3	UK	(24)	Russia	(12)
4	France	(22)	UK	(7)
5	Germany	(15)	NZ/Australia	(5)
6	Russia	(5)	India	(5)
7	India	(5)	Germany	(5)
8	Canada	(3)	Italy	(3)
9	Italy	(1)	Japan	(3)
10			Canada	(1)

published, and France, UK and Russia were the second for two pathogens. Japan was never seen in top two countries.

As for the *V. cholerae* O139, HTLV-1 and Plague, a lot of papers have been published by responding countries like India, Japan and Russia, respectively. This result might be showing that research activity of each country for each disease relates to the extent of their concern over each disease.

The total ranking was shown in Table 3. Regarding both emerging and reemerging diseases, USA were highest ranking and it was well ahead of the other countries. France and UK published papers in the field of both emerging and reemerging diseases were ranking within top four countries. On the other hand, Japan and Russia had tended to focus on emerging diseases and reemerging diseases, respectively. Although there is no clear reason why Japan and Russia had those tendencies, the paper on "Cholera" and "Plague" contribute Russia to the high ranking in the field of reemerging diseases.

ACKNOWLEDGMENT

We would like to thank Ms. Mayuko Kurashiki, Ms. Mari Mori, Ms. Eiko Tara and Mr. Masataka Yamaki for technical assistance and also thank Dr. Masahiro Kihara and Shigeru Katamine for invaluable discussion.

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CLINICAL AND PATHOLOGICAL FEATURES OF EXPERIMENTAL *ACANTHAMOEBA* KERATITIS IN RABBITS

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Abstract: Experimental *Acanthamoeba* keratitis was induced in 24 female Dutch rabbits to examine the clinical effects of infection in the eye and to study the usefulness of a new histopathological technique for evaluating rabbit models of this infection. One eye of each animal in groups A or B received an instillation of 1.3×10^4 (group A) or 1.3×10^5 (group B) amoeba cysts/eye; phosphate-buffered saline (PBS) solution was instilled into the contralateral eye as the control; animals in groups C and D received intrastromal injections of 5.0×10^3 (group C) or 1.5×10^4 (group D) cysts/eye in one eye and injection of an equal volume of PBS in the contralateral eye. Animals were observed daily for 5 to 84 days. Two rabbits in group D were killed on day 5 and enucleated eyes were embedded in paraffin and stained with hematoxylin-eosin or iodine-potassium iodide. In groups A and B, clinical signs of corneal injury disappeared by 4 hours after inoculation and signs of infection disappeared by day 2. In contrast, all eyes that had been injected with *Acanthamoeba* (groups C and D) developed severe keratitis, including keratoneuritis and corneal ulcer, followed by neovascularization or corneal perforation. Histologic examination showed infiltrates of leukocytes, lymphocytes, eosinophils, plasmacytes and spindle-shaped cells. The most extensive cell infiltration, and also exocytosis, liquefaction degeneration and intrastromal trophozoites, were seen in the limbic conjunctiva and palpebral conjunctiva. In addition, there was evidence of migration of inflammatory cells to the ciliary body and intravitreal space. This study showed that injection of *Acanthamoeba* into corneal stroma causes severe infection of the cornea and other eye tissues and that iodine-potassium iodide staining of paraffin embedded specimens is useful to detect *Acanthamoeba* trophozoites and cysts.

Key words: *Acanthamoeba* keratitis, Rabbit, Corneal ulcer, Keratoneuritis, Pathological feature, Iodine-potassium iodide staining

INTRODUCTION

The persistent and severe *Acanthamoeba* keratitis occurs occasionally in contact lens wearers and swimmers. Clinical findings in such cases include granular superficial corneal opacity, scleritis, discoid ulcer, pseudodendritis, stromatitis with ring infiltration. Thinning of cornea leading to corneal perforation may also occur when there is delay in accurate diagnosis and effective treatment of this condition (Wright *et al.*, 1985). However, accurate diagnosis and effective treatment for amoebic keratitis are difficult because amoebae are encysted in infected tissues (Osato *et al.*, 1991).

Rabbit models of *Acanthamoeba* keratitis have been used to study more effective techniques for diagnosis and

treatment of this condition. The first rabbit model was reported by Font and colleagues, who demonstrated that stromal keratitis and the corneal ulceration can be induced by the intrastromal injection of *A. polyphaga* after subconjunctival administration of corticosteroid for 4 days (Font *et al.*, 1981). They reported that corneas were culture-positive at intervals, and encysted amoebae have been observed (Font *et al.*, 1981). Another rabbit model was reported by Lin *et al.* (1989), who investigated the effectiveness of steroid treatment of *Acanthamoeba* keratitis. A rat model of *Acanthamoeba* keratitis was reported by Larkin and Easty (1990). They induced *Acanthamoeba* keratitis in Wistar rats by intrastromal inoculation of *A. polyphaga* cysts and reported that on histopathologic examination, sections of corneas showed liquefactive stromal necrosis and *Acanthamoeba*

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cysts in deep stroma (Larkin and Easty, 1990).

We undertook the present study to investigate the clinical and pathological features of several models of *Acanthamoeba* ocular infection, specifically the effects on tissues other than the cornea, such as the conjunctiva, ciliary body, and vitreous; and secondarily, to determine whether iodine-potassium iodide staining of paraffin-embedded sections might be useful in studying rabbit models of *Acanthamoeba* infection.

MATERIALS AND METHODS

Acanthamoeba isolated from a patient with *Acanthamoeba* keratitis were grown at 25 °C on 1% agar plate with an overlay of heat-killed *Escherichia coli* as a food source. On the basis of differences in the size and morphologic features of the cysts, the protozoan was identified as *Acanthamoeba polyphaga* (group 2) (Fig. 1) (Font *et al.*, 1981; Visvesvara, 1991).

Acanthamoeba cysts were instilled or injected into the eyes of 24 female Dutch rabbits that weighed 2.6 (standard deviation: 0.3) kg each. The rabbits were divided into four groups. Rabbits groups A (n=4) and B (n=4) received 9 linear latticed abrasions on the corneal epithelial layer, extending to the upper stromal layer. Then a suspension of cysts (1.3×10^4 amoebas/eye for group A or 1.3×10^5 amoebas/eye for group B) was instilled into one eye. Contra-lateral eyes of rabbits in groups A and B were instilled with an equivalent volume of phosphate buffered saline (PBS) solution. Rabbits in groups C (n=8) and D (n=8) received an injection of 5.0×10^3 cysts/kg body weight (b.w.) (20 μ l) (group C) or 1.5×10^4 cysts/kg b.w. (50 μ l) (group D); the injection was made intrastromally at the 2 o'clock position on the cornea, about 2.5 mm from the limbus. Contralateral eyes of rabbits in group C or D were injected with an equal volume (20 μ l or 50 μ l, respectively) of PBS.

Slit-lamp examinations of the anterior segments of all eyes of all rabbits were performed hourly for the first 5 hr and every day for up to 84 days after the inoculation.

Two rabbits in group D were killed and enucleated by intravenous injection of sodium pentobarbital on day 5, and all the other rabbits were sacrificed on day 84 after the inoculation. The enucleated eyes of rabbits were embedded in paraffin and sectioned at 3-5 μ m. Sections were then stained with hematoxylin-eosin (H-E) or iodine-potassium-iodide and examined by light microscopy.

RESULTS

1. Clinical features of *Acanthamoeba* instillation

(1) Group A and B

By 4 hrs after injury and instillation of *Acanthamoeba* in eyes in groups A and B, all signs of corneal injury had disappeared. On day 1 after the inoculation, hyperaemia and a few dot haemorrhage were seen in the conjunctivas of eyes that had received instillation of *Acanthamoeba*, but these manifestations were disappeared on day 2. Daily examination of eyes in groups A and B for the remainder of the 12-week study showed on signs of corneal disorder and no recurrence of conjunctivitis. There was no apparent difference between group A and B eyes in the clinical response to corneal injury and instillation of *Acanthamoeba* cysts.

(2) Group C and D

In inoculated eyes in groups C and D, corneal opacity occurred at the site of inoculation within minutes after inoculation and disappeared within 5 hrs after the inoculation. Simultaneously, hyperanemia and dot haemorrhages become evident in the palpebral conjunctiva. Cellular infiltration in an inoculated site of the cornea, keratoneuritis and corneal ulcer were seen in the area of inoculation on day 2 after inoculation in eyes in group C and on day 1 after inoculation in eyes in group D (Fig. 2). In rabbits in group C, the corneal lesions gradually increased in area until day 5 after the inoculation. On day 6, pannus appeared and corneal infiltration start to disappear. Small cystic lesions were found in the cornea on day 8, and these enlarged until day 14. The corneal neovascularization was noted at the limbic conjunctiva on day 14 and developed into a corneal ulcer. After day, 28, the cystic lesions in the corneas began to decrease in size and replaced by nubecula. By day 49, only small area of pannus and nubecula remained on the corneas of rabbits in group C (Fig. 3), and there were no signs of recurrent keratitis during the remainder of the 12-week period after instillation of *Acanthamoeba* in the eyes of rabbits in group C.

In rabbits group D, the corneal lesion appeared granular and turbid on day 2 after inoculation and had enlarged to involve half of the corneal surface. On day 4, a portion of the corneas in 2 eyes (2 rabbits) in group D was noticeably thinner (Fig. 4), and on day 7 perforation was noted at these site.

The significant clinical difference between rabbits in group C and those in group D was that corneal lesions in group C showed spontaneous resolution, while those in group D increased, resulting in corneal perforation.

2. Histological features of *Acanthamoeba* keratitis

In the 2 eyes of rabbits in group D that were killed on day 5 after inoculation of *Acanthamoeba*, histological ex-

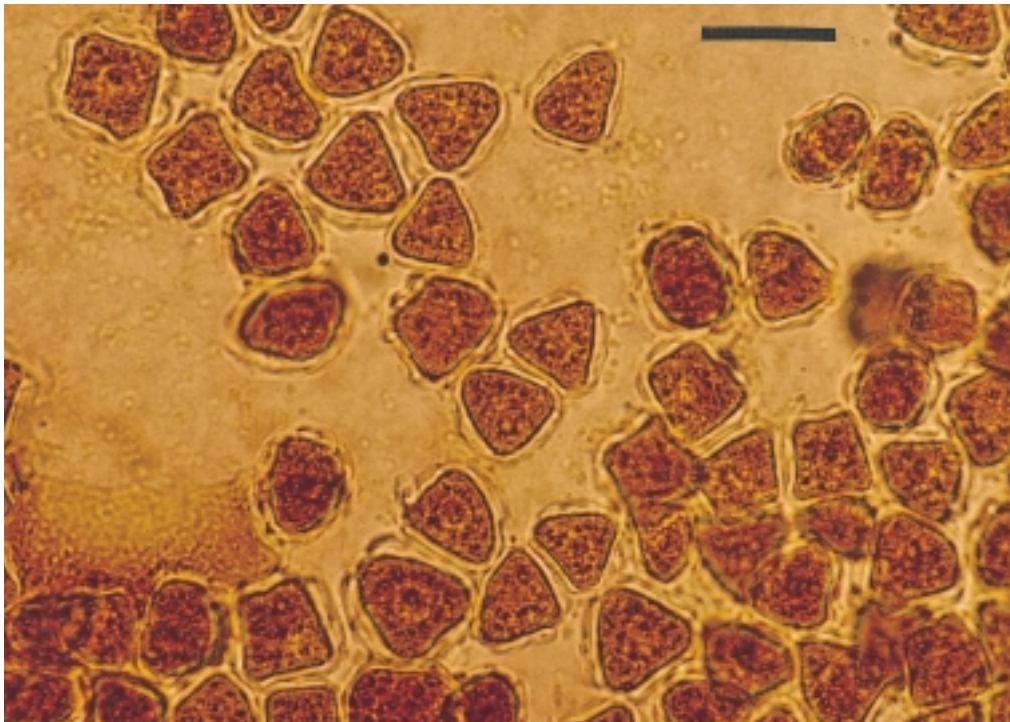


Figure 1 Iodine-potassium iodide stained *Acanthamoeba* cysts. Bar: 10 μ m

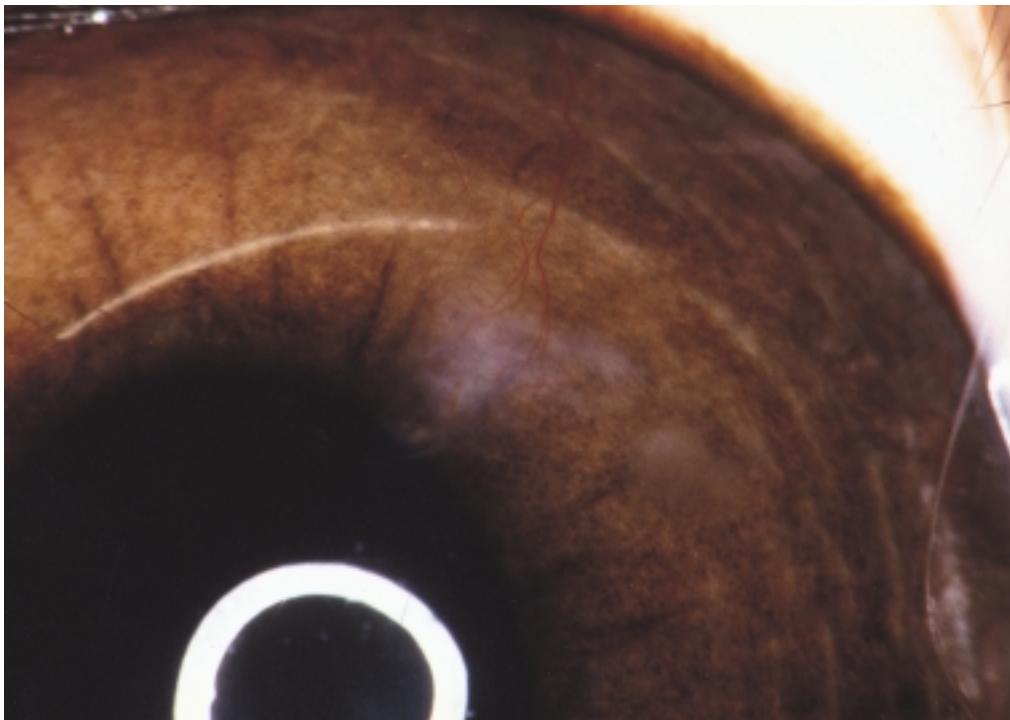


Figure 2 Biomicroscopic appearance of a rabbit cornea in group D, 1 day after inoculation of *Acanthamoeba*. A corneal ulcer, stromatitis and keratoneuritis have developed

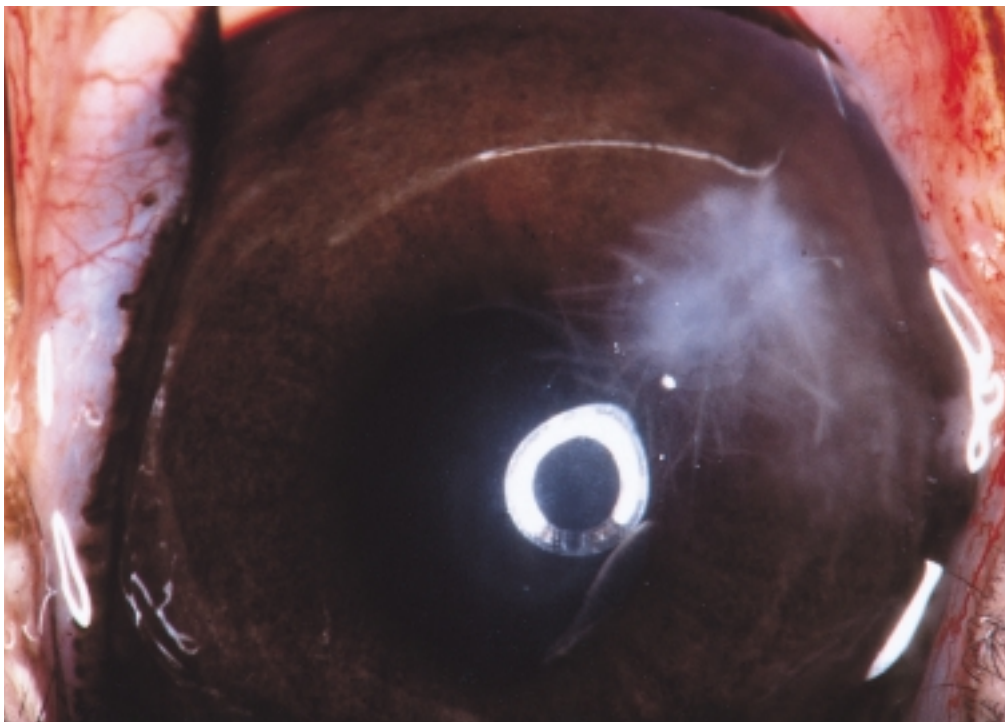


Figure 3 Biomicroscopic appearance of a rabbit cornea in group C at 7 week after inoculation of *Acanthamoeba*. The corneal ulcer has disappeared, and pannus and nubecula are seen.

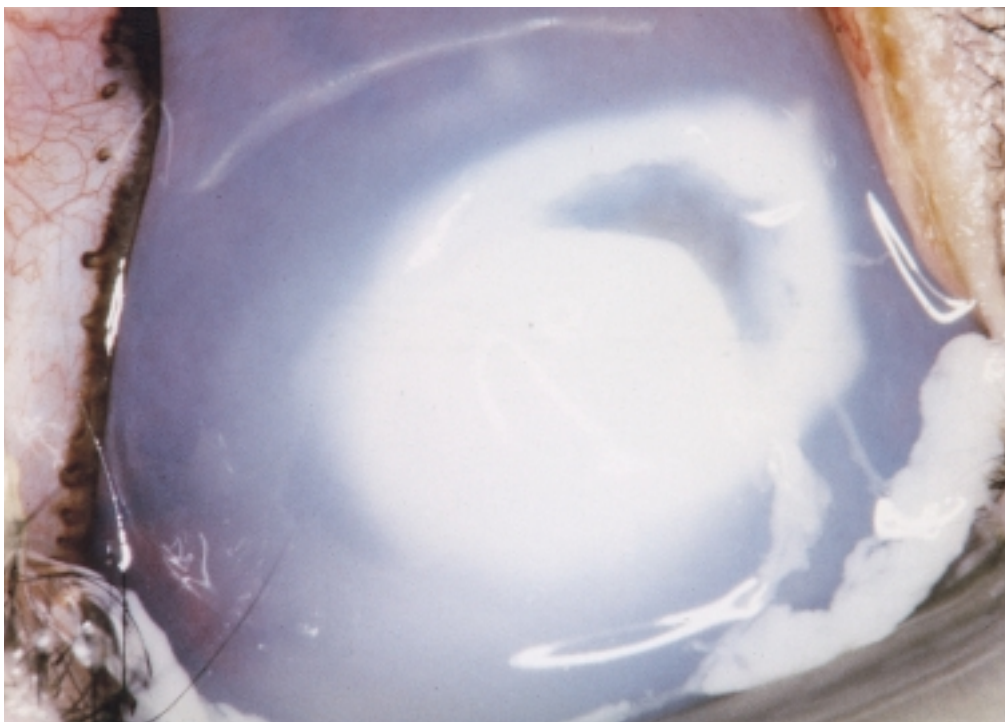


Figure 4 Biomicroscopic appearance of a rabbit cornea in group D on day 4 after inoculation of *Acanthamoeba*. Thinning of the cornea is evident.

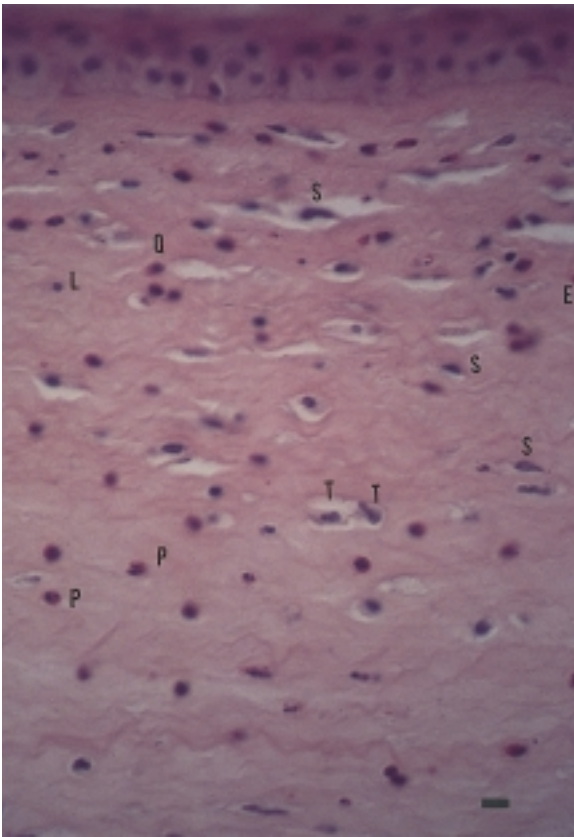


Figure 5 Histologic appearance of a rabbit cornea 5 days after inoculation with *Acanthamoeba*. The corneal stroma shows infiltration of cells, including polymorphonuclear leukocytes (P), lymphocytes (L), eosinophils (E), plasmacytes (Q), spindle-shaped cells (S) and trophozoites (T), surrounded by liquefaction products. Hematoxylin-eosin staining. Bar: 10 μ m.

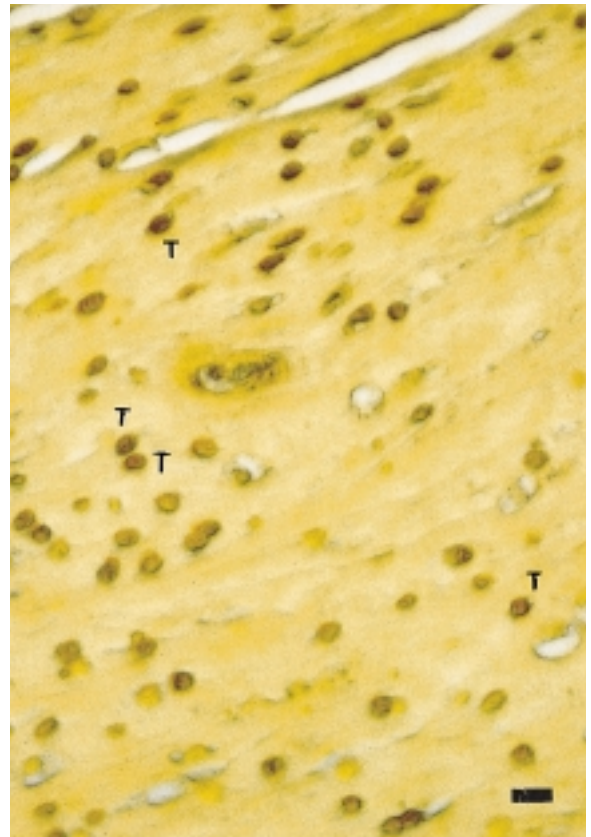


Figure 6 Histologic appearance of a rabbit cornea 5 days after inoculation with *Acanthamoeba*. Iodine-potassium iodide staining of the paraffin embedded section differentiates cysts from trophozoites (T). Bar: 20 μ m

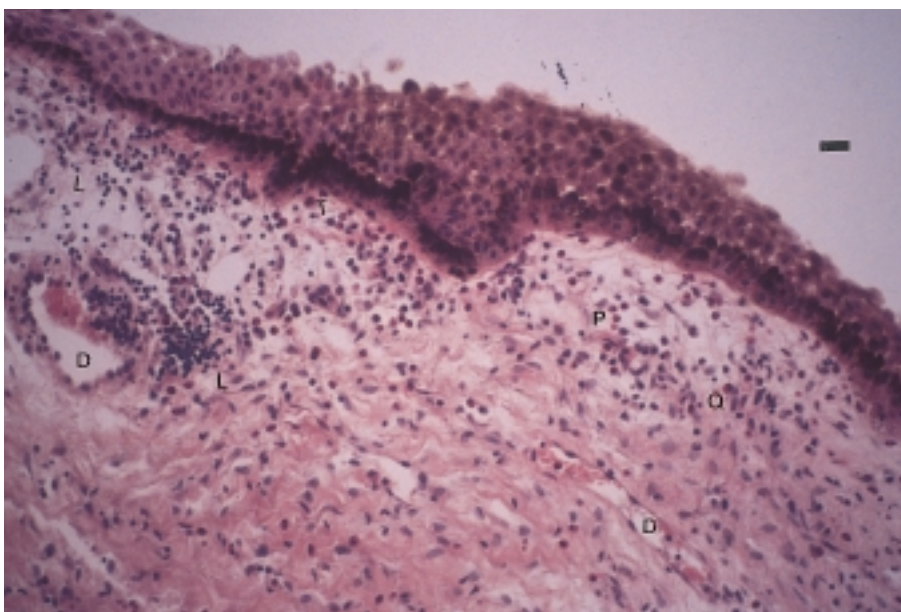


Figure 7 Histologic appearance of rabbit limbic conjunctiva 5 days after inoculation with *Acanthamoeba*. Infiltrates of lymphocytes (L), polymorphonuclear leukocytes (P) and plasmacytes (Q) and several trophozoites (T) are visible. Lymphocytes and plasmacytes may adhere to the walls of dilated blood vessels (D). Bar: 50 μ m

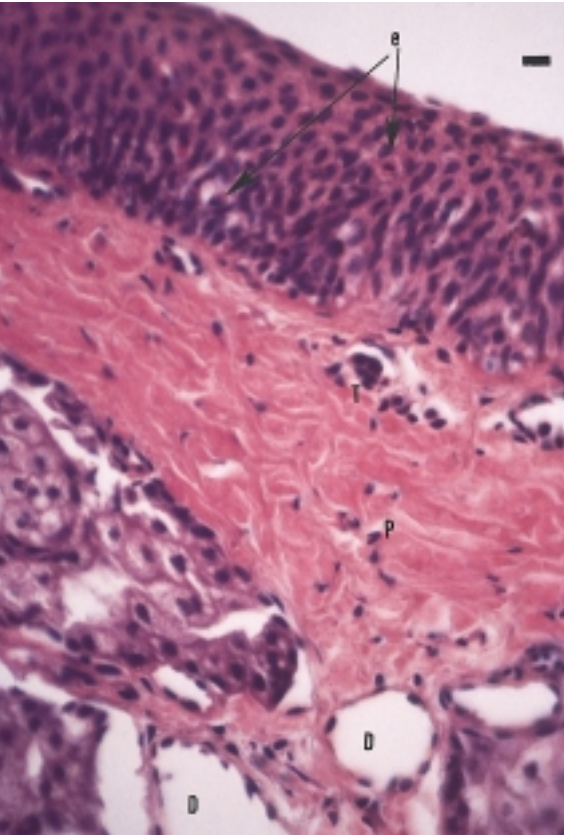


Figure 8 Histologic appearance of rabbit palpebral conjunctiva 5 days after inoculation of *Acanthamoeba*. Infiltration of inflammatory cells (P) into the palpebral conjunctiva, exocytosis (e) and migration of trophozoites (T), and dilated blood-vessels (D) are visible. Bar: 30 μ m

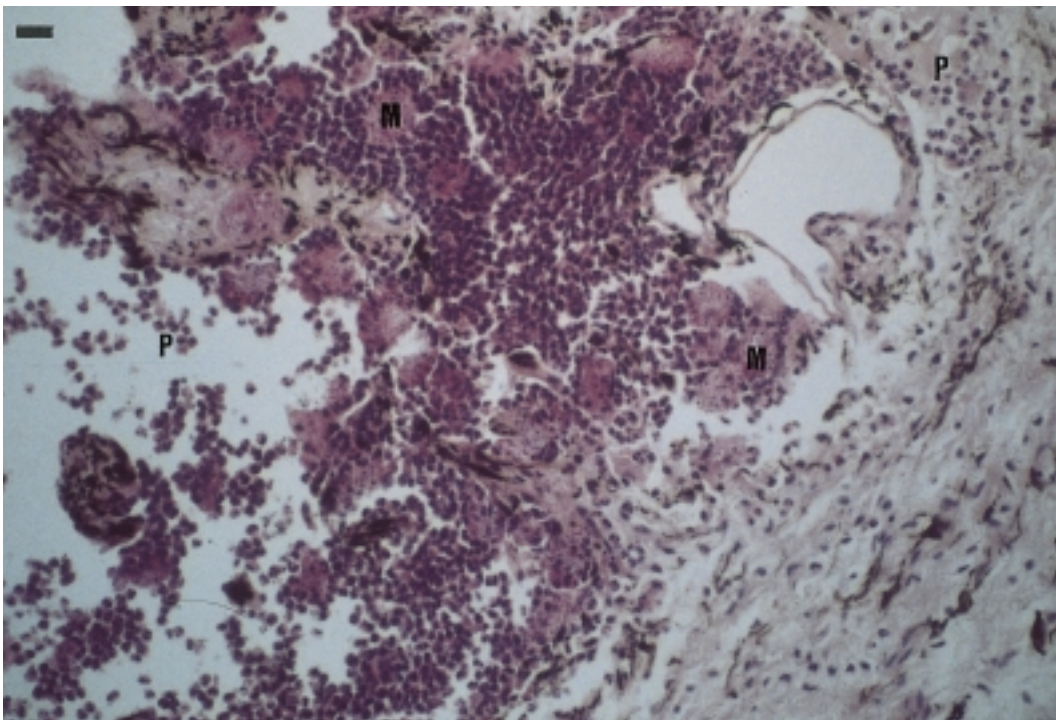


Figure 9 Histologic appearance of rabbit ciliary body 5 days after inoculation of *Acanthamoeba*. Polymorphonuclear leukocytic (P) infiltration and multinucleated giant cells (M) are visible in the ciliary body and intravitreal space. Bar: 50 μ m

amination showed remarkable cell infiltration in the corneal stroma, including polymorphonuclear leukocytes, lymphocytes, eosinophils, plasmacytes, spindle-shaped cells and trophozoites, surrounded by an area of liquefactive degeneration (Fig. 5).

On iodine-potassium iodide stained paraffin embedded sections of these eyes, it was possible to differentiate cysts from trophozoites (Fig. 6). Changes in the limbic conjunctiva 5 days after inoculation of *Acanthamoeba* cysts were infiltrations of lymphocytes, polymorphonuclear or eosinophilic leukocytes, plasmacytes and scattered trophozoites. Dilatation of intravascular spaces and neovascularization were also evident in the limbic conjunctiva of rabbits in group D 5 days after inoculation of *Acanthamoeba* cyste (Fig.7). In the palpebral conjunctiva, H-E staining showed in filtrations of inflammatory cells and trophozoites and exocytosis in the epithelial layer (Fig. 8). H-E staining showed polymorphonuclear leukocyte infiltrations in the ciliary body and the anterior vitreous, trophozoites in the ciliary body, and multinucleated giant cells in the intravitreal space (Fig. 9).

DISCUSSION

Acanthamoeba has a life cycle of trophozoite and dormant cyst stages.

Acanthamoeba keratitis was first described clinically more than 30 years ago, however, starting in 1985 the number of cases of amoebic keratitis reported has grown each year (Warhurst and Mann, 1988; Wright and Buckley, 1988; Wright *et al.*, 1985).

The pathogenesis of *Acanthamoeba* keratitis has been studied using a variety of experimental models. Culbertson *et al.* (1959) examined the results of intravenous injection or intranasal inoculation of *Acanthamoeba* organisms into mice and monkeys, and others have developed experimental models of amoebic keratitis in a variety of species, including rabbits, hamsters, swine and mice (Font *et al.*, 1981; Larkin and Easty, 1990; Klink *et al.*, 1994; Alizaeh *et al.*, 1995; Ruddell and Easty, 1995; John *et al.*, 1991). Larkin and Easty (1990) reported on the histological appearance of the cornea in eyes with *Acanthamoeba* keratitis. However, there is little information about the effects of *Acanthamoeba* infection on other eye tissues, including the conjunctiva, iris, intravitreal space and ciliary body.

In the present study, we created the first reported reproducible model of *Acanthamoeba* keratitis in rabbit eyes and documented changes in the cornea, limbic or palpebral conjunctiva, ciliary body and anterior vitreous after intrastromal injection of a suspension of *Acanthamoeba* cysts.

Our results show that intra-corneal injection of *Acanthamoeba* cysts leads to the development of small cystic lesions in the cornea that can be observed clinically by slit-lamp examination. In addition, paraffin-embedded sections of rabbit eyes obtained 5 days after inoculation showed the presence of inflammatory cell infiltrates and *Acanthamoeba* trophozoites in other parts of the eye, such as limbic conjunctivae, palpebral conjunctivae, ciliary body, as well as the intrastromal spaces in the cornea.

Differentiating between the cyst and the trophozoite stages of the *Acanthamoeba* life cycles helpful because almost all clinical specimen were found as the trophozoite stages of the *Acanthamoeba*. In our study, corneal smears and histologic sections stained with iodine-potassium iodide were useful for differentiating cysts from trophozoites. As a result, detailed studies of the *Acanthamoeba* life cycle in keratitis using anti-amoebic monoclonal antibodies are planned for the near future.

In patients, the course of *Acanthamoeba* keratitis is often prolonged in spite of intense treatment. Corneal curettage is the most effective treatment, especially when *Acanthamoeba* infection is confined to the cornea, because most anti-amoebic drugs are not very effective against encysted *Acanthamoeba*. The results of our study suggest that migration of *Acanthamoeba* trophozoites from the cornea to other parts of the eye, such as the palpebral or limbic conjunctiva, ciliary body and anterior vitreous, may help explain the difficulty of treating severe *Acanthamoeba* keratitis and the occurrence of complications such as endophthalmitis or panophthalmitis.

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DESCRIPTION OF TWO NEW SPECIES OF BLACK FLIES (DIPTERA: SIMULIIDAE) FROM SARAWAK, MALAYSIA

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Received February 27, 2001/Accepted April 8, 2001

Abstract: Two new black-fly species, *Simulium (Gomphostilbia) lehi* sp. nov. and *S. (G.) sarawakense* sp. nov., are described from adult flies reared from pupae, collected in Sarawak State, Malaysia. In addition, three known species of *Simulium* s.l., i.e., *S. (G.) sheilae*, *S. (Nevermannia) aureohirtum*, *S. (Simulium) laterale*, are newly recorded from Sarawak.

Key words: Simuliidae, Black fly, Malaysia, Sabah, New species, Fauna

Since Smart and Clifford (1969) recorded *Simulium sabahense* from Sarawak, Malaysia, no other black-fly species has been added to the simuliid fauna of this state.

In 1998, I made faunistic surveys on Simuliidae in several localities of Sarawak in Malaysia, and collected six species of *Simulium* s.l. consisting of *S. sabahense*, two new species and three newly recorded ones. I herein describe these new species.

The morphological features and terms used herein follow mostly those of Crosskey (1969), and partially those of Takaoka (1983). All type specimens of new species will be in due course deposited at the Natural History Museum, London, U.K.

DESCRIPTION OF NEW SPECIES

Simulium (Gomphostilbia) lehi sp. nov.

Female. Body length 1.8-2.0 mm. **Head.** As wide as thorax. Frons (Fig. 1) brownish black, semishiny, densely covered with yellowish white scale-like recumbent hairs, and with several dark stout hairs in vertical row along each lateral margin; frontal ratio 1.9:1.0:2.4; frons-head ratio 1.0:4.7. Fronto-ocular area (Fig. 2) well developed, narrow. Clypeus brownish black, semishiny, densely covered with yellowish-white scale-like recumbent hairs interspersed with 10-12 dark hairs on each side. Proboscis ca. 0.54 × as long as clypeus. Antenna composed of 2+9 segments, medium brown except scape, pedicel and base of 1st flagellar segment pale yellow. Maxillary palp composed of 5 segments, light brown, proportional lengths of 3rd, 4th and 5th segments 1.0:1.1:2.9; 3rd segment (Fig. 3) widened dis-

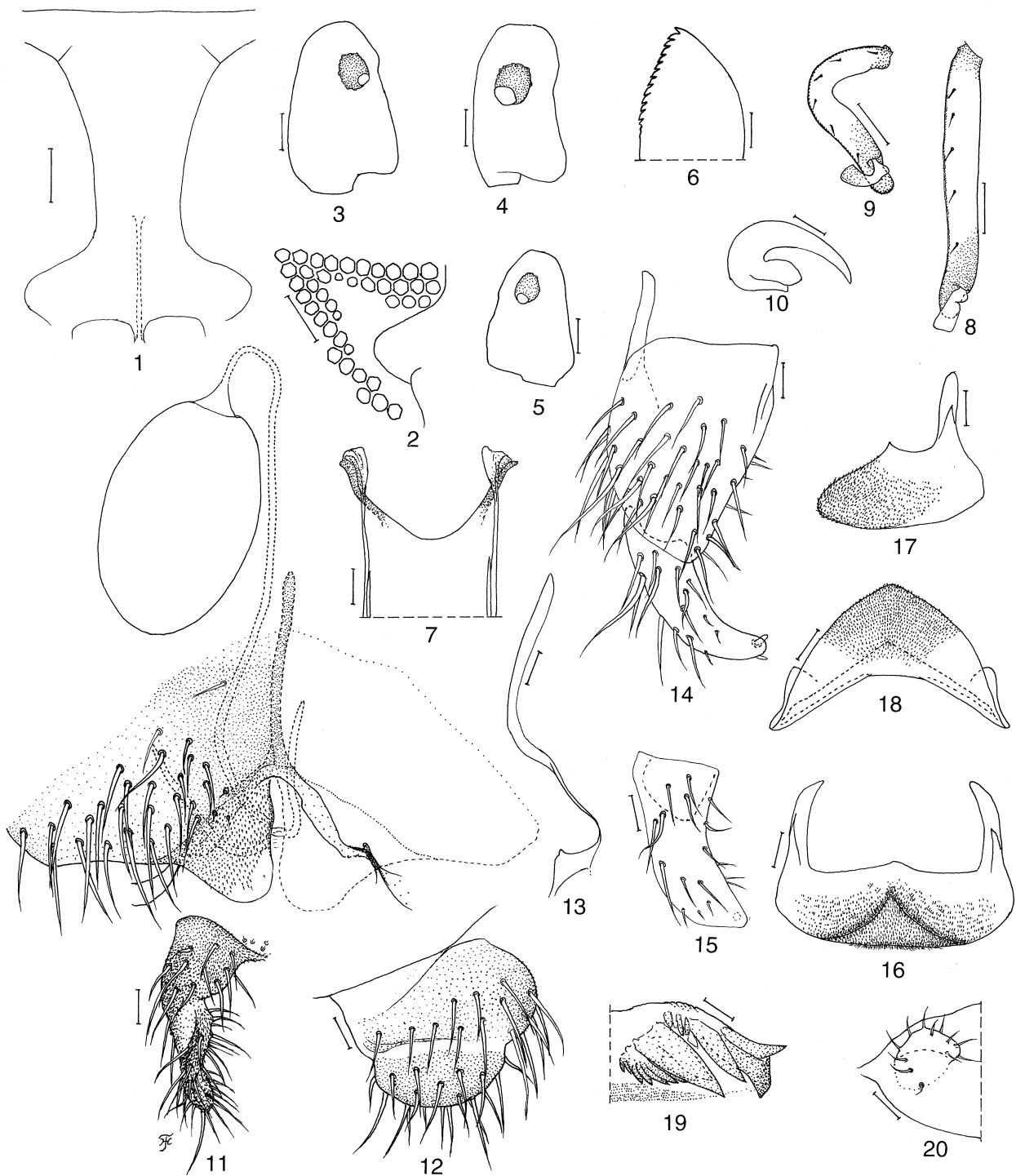
tally; sensory vesicle of small (or moderate in the other female, Fig. 4) size, nearly ellipsoidal, 0.21 × (or 0.26 × in the other female, Fig. 4) as long as 3rd segment, with medium (or large in the other female, Fig. 4) opening. Maxillary lacinia with 7 or 8 inner teeth and 9 or 10 outer teeth. Mandible (Fig. 6) with 12-14 small inner teeth and lacking outer ones. Cibarium (Fig. 7) smooth, without any processes or tubercles. **Thorax.** Scutum dark brown with anterolateral calli somewhat paler, subshiny, thinly white pruinose, with 3 faint longitudinal vittae (1 medial and 2 sub-medial), densely covered with yellowish-white scale-like recumbent hairs; scutellum medium brown, covered with short, yellowish-white scale-like hairs as well as long upright dark hairs along posterior margin. Postscutellum dark brown, bare. Pleural membrane bare. Katepisternum brownish black, longer than deep, with fine short dark hairs. **Legs** (Coloration appeared to be still incomplete). Foreleg: coxa pale yellowish white; trochanter and femur light brown; tibia light to medium brown; tarsus medium to dark brown, without distinct dorsal hair crest; basitarsus slightly dilated, ca. 6.7 × as long as its greatest width. Midleg: coxa dark brown; trochanter dark yellow; femur light brown; tibia medium brown with basal 2/5 dark yellow; tarsus medium brown with basal 2/3 of basitarsus, and base of 2nd tarsal segment dark yellow or light brown. Hind leg: coxa light brown, trochanter pale yellow; femur dark yellow or light brown with apical cap medium brown; tibia yellowish white on basal 3/4 and medium brown on distal 1/4; tarsus medium brown except basal 2/3 of basitarsus, basal 1/2 of 2nd tarsal segment yellowish white; basitarsus (Fig. 8) narrow, nearly parallel-sided, ca. 6.0 × as long as wide, and ca. 0.63 × and ca. 0.48 × as wide as greatest width of

tibia and femur, respectively; calcipala (Fig. 8) ca. $1.2\times$ as long as wide, and ca. $0.8\times$ as wide as distal portion of basitarsus. All femora, tibiae and parts of tarsus densely covered with dark (and also pale), scale-like hairs on outer and posterior surfaces. Claws (Fig. 10) each with large basal tooth, $0.47\times$ as long as claw. **Wing.** Length 1.6 mm. Costa with dark spinules as well as dark hairs except basal hair tuft pale. Subcosta haired except near apex bare. Hair tuft on stem vein pale. Basal portion of radius fully haired. **Abdomen.** Basal scale light brown, with fringe of pale hairs. Abdomen dark brown except base of 2nd segment paler, sparsely or moderately covered with short dark hairs; tergites of segments 2, 6, 7 and 8 subshiny; sternal plate on segment 7 undeveloped. **Genitalia** (Figs. 11-13). Sternite 8 bare medially, with 21-28 long hairs and a few short hairs on each side. Anterior gonapophyses nearly triangular, rounded posteromedially, thin, membraneous, covered densely with microsetae (though posteromedial corner somewhat widely bare), interspersed with 1 or 2 short setae; inner margins sinuous, narrowly sclerotized, moderately separated from each other. Genital fork of usual inverted-Y form; arms slender, folded medially, with a small projection directed forward. Paraproct somewhat produced ventrally, with 12-16 long hairs on outer surface, and with 4 or 5 sensilla on inside surface. Cercus ca. $0.5\times$ as long as wide, rounded posteriorly. Spermatheca ellipsoidal, ca. $1.5\times$ as long as wide, well sclerotized except near tubal juncture and tube unsclerotized, and with many fissures on surface; internal setae appear to be absent; both accessory tubes slender, subequal in diameter to major one.

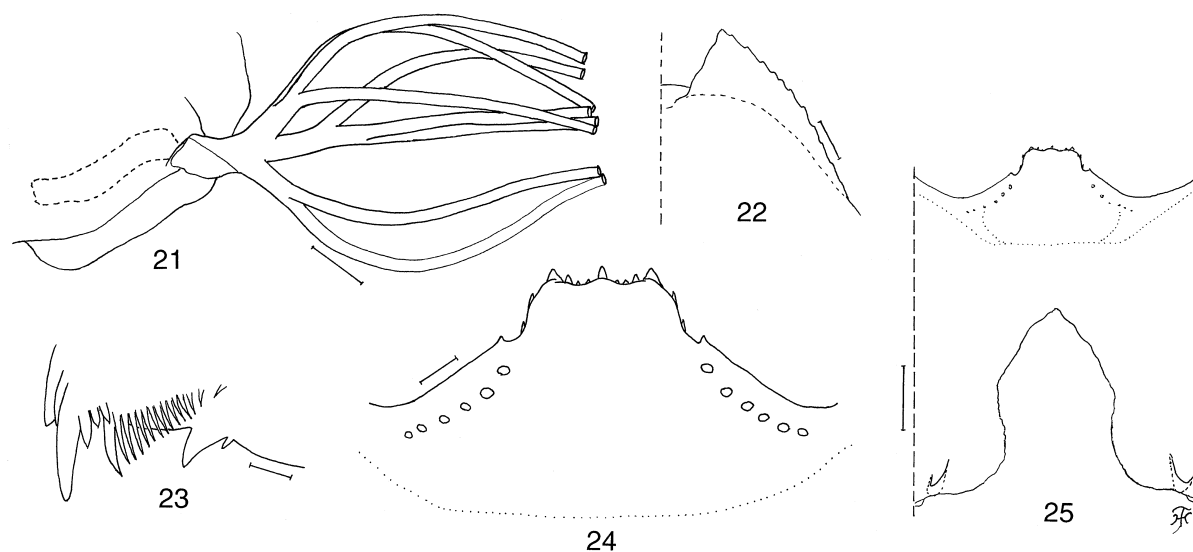
Male (dissected out of the pupa). Body length 2.0 mm. **Head.** Much wider than thorax. Upper eye consisting of 10 vertical columns and 11 horizontal rows of large facets. Face dark brown, white pruinose. Clypeus dark brown, thinly white pruinose, moderately covered with yellow simple and scale-like hairs (except median portion widely bare), intermixed with several dark longer simple hairs on each side. Antenna composed of 2+9 segments, dark brown. Maxillary palp with 5 segments, light to medium brown; 3rd segment (Fig. 5) widened apically; sensory vesicle ellipsoidal, $0.24\times$ as long as 3rd segment, with medium opening apically. **Thorax.** Scutum light brown, shiny, densely covered with yellow scale-like recumbent hairs. Scutellum pale, covered with yellow scale-like short hairs and several dark hairs near posterior margin. Postscutellum light brown, bare. Pleural membrane bare. Katepisternum as in female. **Legs.** Colorings (though incomplete) nearly as in female; fore basitarsus ca. $7.2\times$ as long as greatest width; hind basitarsus (still bent) (Fig. 9) slender, nearly parallel-sided like that of female. Calcipala (Fig. 9) en-

larged, ca. $1.6\times$ as long as wide. Pedisulcus well developed. **Wing.** As in female except subcosta bare; length not measurable. **Abdomen.** Basal scale light brown, with fringe of dark hairs. Dorsal surface of abdomen light brown, covered with dark hairs; segments 2, 5, 6 and 7 each with a pair of shiny dorsolateral patches. **Genitalia** (Figs. 14-20). Coxite in ventral view, subquadrate, ca. $1.5\times$ as long as wide. Style ca. $0.9\times$ as long as coxite, gradually tapered toward apex, curved inward, with apical spine (right style with additional spine near apex though somewhat smaller than main one). Ventral plate transverse, densely covered with microsetae on ventral surface (except along anterior and lateral margins rather broadly bare) and on posterior surface (except both lateral areas bare); basal arms directed forward, then converged apically. Parameres of moderate size, each with 3 large hooks (of which 1 is very wide) and ca. 10 smaller ones. Aedeagal membrane moderately covered with microsetae, weakly sclerotized along dorsal base forming dorsal plate. Median sclerite thin, plate-like, wide, with apex rounded. Ventral surface of 10th abdominal segment without short setae near posterior margin on each side. Cerci small, rounded, each with 12 or 13 short hairs.

Pupa. Body length ca. 2.2 mm. **Head.** Integument yellowish, densely covered with round tubercles; antennal sheath normal, with no spinous projections, and almost bare; face with 1 pair of simple long trichomes with coiled apex, and frons with 3 pairs of simple long trichomes with coiled apex; 3 frontal trichomes on each side arising close together, subequal in length to one another and slightly longer than facial one. **Thorax.** Integument yellowish, densely covered with round tubercles, with 3 pairs of simple long trichomes dorsally, 2 pairs of simple trichomes (1 long and 1 medium) anterolaterally, 1 pair of simple medium-long trichomes posterolaterally, and 3 pairs of simple trichomes (1 long and 2 medium) ventrolaterally; all long trichomes with coiled apex. Gill (Fig. 21) composed of 8 slender filaments, arranged in groups of 3+3+2 filaments from dorsal to ventral, with somewhat swollen transparent organ ventrally at base; stalks of all 3 groups of filaments as well as basal common stalk short; all filaments pale yellowish, subequal in length (ca. 2.0 mm) and thickness to one another; cuticular surface of filaments with distinct annular ridges and furrows gradually becoming indistinct apically, and densely covered with minute tubercles. **Abdomen.** Terga 1 and 2 pale yellow, almost bare; tergum 1 with 1 simple slender medium-long seta on each side; tergum 2 with 1 medium-long simple slender seta and 5 short setae (of which 2 or 3 spinous), submedially on each side; terga 3 and 4 pale, each with 4 hooked spines and 1 short somewhat spinous seta on each side; tergum 5 lacking



Figures 1-20 Morphological characters of *S. lehi* sp. nov. 1, female frons; 2, female fronto-ocular area; 3-5, 3rd segment of maxillary palp with sensory vesicle (3 and 4, female; 5, male); 6, apex of female mandible; 7, female cibarium; 8 and 9, basitarsus and 2nd tarsal segment of hind leg (8, female; 9, male); 10, female claw of hind leg; 11, sternite 8, anterior gonapophyses, genital fork, spermatheca, paraproct and cercus of female genitalia *in situ* (ventral view); 12, paraproct and cercus *in situ* (lateral view); 13, genital fork (lateral view); 14, coxite and style of male genitalia *in situ* (ventral view); 15, right style (ventrolateral view); 16-18, ventral plate (16, ventral view; 17, lateral view; 18, end view); 19, left paramere and aedeagal membrane (end view); 20, right cercus (end view). Scale bars: 0.1 mm for figs. 8 and 9; 0.05 mm for figs. 1 and 2; 0.02 mm for figs. 3-5, 7, and 11-20; 0.01 mm for figs. 6 and 10.



Figures 21-25 Morphological characters of *S. lehi* sp. nov. 21 and 22, pupa, and 23-25, larva. 21, right gill filaments (apical 1/2 omitted, lateral view); 22, right terminal hook (end view); 23, apical portion of mandible; 24, hypostomium (bristles omitted, ventral view); 25, ventral surface of head capsule showing hypostomium and postgenal cleft. Scale bars: 0.1 mm for fig. 21; 0.04 mm for fig. 25; 0.02 mm for fig. 24; 0.01 mm for figs. 22 and 23.

spine-combs; terga 6-9 each with distinct spine-combs in transverse row, together with comb-like groups of minute spines on each side; tergum 9 with 1 pair of distinct plate-like terminal hooks, extending laterally, and with serrated outer margin (Fig. 22). Sternum 4 with 1 simple hook (somewhat smaller than those on sternum 7) and a few simple minute setae on each side; sternum 5 with 1 pair of bifid hooks submedially on each side; sterna 6 and 7 each with 1 pair of bifid inner and simple outer hooks somewhat spaced from each other, and a few simple short minute setae on each side. Each side of segment 9 with 1 simple hooklet and 1 minute seta, but lacking grapple-like hooklets. **Cocoon.** Simple, wall-pocket-shaped, moderately woven without open spaces in webs; some cocoons with a narrow anteroventral collar; posterior 1/2 with floor roughly woven; individual threads distinct; 2.6-2.9 mm long \times 0.8-0.9 mm wide.

Mature larva. Body length 4.3-5.1 mm. Body entirely greyish green. Cephalic apotome yellow, moderately covered with minute setae, with distinct dark usual head spots. Cervical sclerite composed of 2 small rod-like pieces, not fused to occiput, widely separated medially from each other. Antenna consisting of 3 segments and apical sensillum, longer than stem of labral fan; proportional lengths of 1st, 2nd and 3rd segments 1.0:1.0:0.5. Labral fan with ca. 38 main rays. Mandible (Fig. 23) with 2 usual mandibular serrations; comb-teeth composed of 3 teeth, 1st tooth largest of all, 2nd and 3rd ones subequal in size to each other; super-

numerary serrations absent. Hypostomium (Fig. 24) with a row of 9 apical teeth; median and corner teeth well developed; inner and median teeth of 3 intermediate teeth on each side subequal in size to each other, and somewhat shorter than outermost tooth; lateral serrations undeveloped; 6 hypostomal bristles lying parallel to lateral margin on each side. Postgenal cleft (Fig. 25) medium, arrow-head shaped, and ca. 2.9 \times as long as postgenal bridge. Thoracic and abdominal cuticle almost bare except last segment densely covered with colorless simple setae on each side. Rectal papilla of 3 lobes, each lobe with 7 or 8 finger-like secondary lobules. Anal sclerite of usual X-form, with posterior arms ca. 1.3 \times as long as anterior ones; basal portion of arms widely sclerotized. Accessory sclerite absent. Ventral papillae large, conical, placed ventrally, then, well discernible when the larva is viewed laterally. Posterior circlet with ca. 100 rows of up to 17 hooklets per row.

TYPE SPECIMENS. Holotype, female, reared from pupa, collected at Pueh, Sematan, Sarawak, Malaysia, 18.III.1998. Paratype 1 female, reared from pupa, 1 pharate male, 3 pupae, 8 mature larvae, same data and date as holotype; 1 pharate male, collected in Hot spring Park, at poring park, at Poring, Sabah, 12.III.1998.

ECOLOGICAL NOTES. Six pupae were collected, three from slender tree sticks, and the others from rocks, in water of a small clean shaded stream (0.5-3.0 m wide) flowing down on the rocky streambed in natural forest of Mt. Pueh.

One pupa of this species was collected from a slender stick in a slowly-flowing stream with its width of ca. 20 cm, shaded, at Hot spring Park in Poring. The water temperature was 23-25 °C. This species was collected together with *S. laterale*, *S. sabahense*, and *S. sarawakense* sp. nov. or with *S. sabahense* and *S. sheilae*.

DISTRIBUTION. Sabah and Sarawak.

ETYMOLOGY. The species *lehi* is given after Dr. Charles Leh, Curator, Zoology Department, Sarawak Museum, Kuching, Sarawak State.

REMARKS. This new species is assigned to the *batoense* species-group within the subgenus *Gomphostilbia*, defined by Takaoka and Davies (1996), by having 2+9 antennal segments, slender male hind basitarsus (Fig. 9), and 8-filamented pupal gill (Fig. 21).

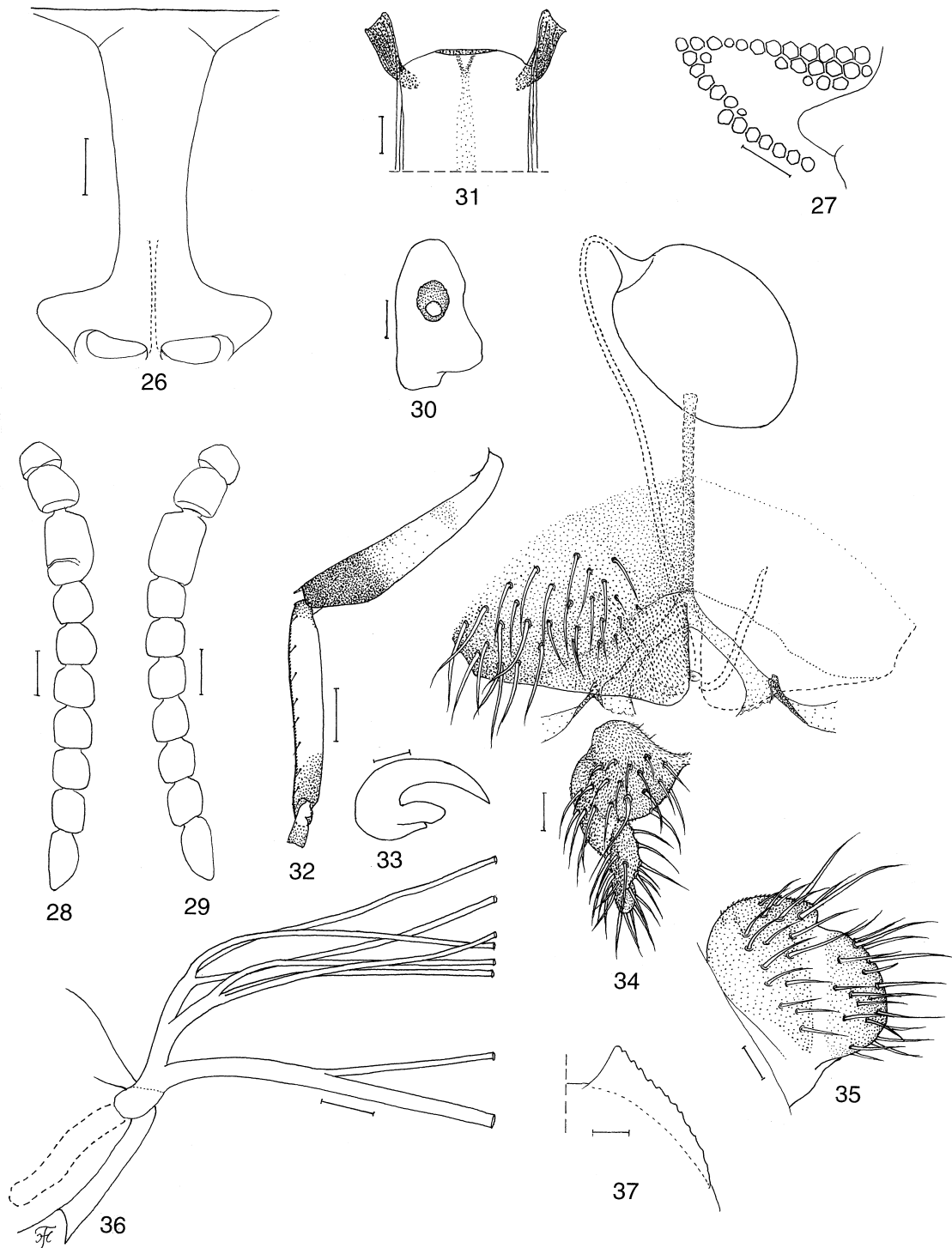
S. lehi is very similar to *S. miblosi* described from females and pupae collected from Mindanao Island, Philippines (Takaoka, 1983), by the mandible with serration only on inner margin (Fig. 6), simple cibarium (Fig. 7) and enlarged calcipala (Fig. 8) in the female, and the absence of the grapple-like hooklets on the last abdominal segment in the pupa. However, this species differs from the latter in the female by the reduced number of small teeth on inner margin of the mandible (12-14 versus ca. 24), of stout hairs on the eighth sternite (21-28 versus ca. 50 on each side), and in the pupa by the usual number of trichomes on the frons (3 versus 2 on each side).

The male of *S. lehi* is characterized by the very wide and high ventral plate (Figs. 16-18), and the paramere with one of the three major hooks very wide (Fig. 19), together with the reduced number of enlarged facets. The male of *S. rayohense*, described from Sabah (Smart and Clifford, 1969), has the similar ventral plate and the reduced number of enlarged facets. However, this known species differs from *S. lehi* by the usual short calcipala. The pupal gill filaments of *S. rayohense* are arranged in different manner, having the longer stalk of the ventral paired filaments; and the larval postgenal cleft of this known species is much deeper than that of *S. lehi*, according to the illustrations given by Smart and Clifford (1969).

***Simulium (Gomphostilbia) sarawakense* sp. nov.**

Female. Body length 2.0 mm. **Head.** Somewhat narrower than width of thorax. Frons (Fig. 26) narrow, dark brown, dull, covered moderately with yellowish-white scale-like recumbent hairs, interspersed with several dark simple long hairs along each lateral margin; frontal ratio 1.8:1.0:3.6;

frons-head ratio 1.0:5.8. Fronto-ocular area (Fig. 27) well developed, narrow. Clypeus dark brown, covered moderately with yellowish-white scale-like recumbent hairs, interspersed with several dark simple long hairs on each side. Proboscis ca. 0.67 × as long as clypeus. Antenna composed of 2+8 segments (this female appears to be an abnormal individual, showing the incomplete segmentation between the 1st and 2nd flagellar segments, since both of the 1st flagellar segments are unusually longer, and that of the right antenna has a transverse incision, as shown in Figs. 28 and 29; normal females of this species must have 2+9 antennal segments, since the pharate male examined shows usual 2+9 antennal segments on both sides), whitish yellow on basal 1/2 and gradually darkened toward apex, though its border not well defined. Maxillary palp composed of 5 segments, light to medium brown, proportional length of 3rd, 4th and 5th segments 1.0:1.2:3.3; 3rd segment somewhat swollen apically; sensory vesicle (Fig. 30) ellipsoidal, ca. 0.26 × as long as 3rd segment, with medium opening medially. Maxillary lacinia with 9 or 10 inner teeth and 12 or 13 outer ones. Mandible with ca. 20 inner teeth and ca. 10 outer ones. Cibarium (Fig. 31) medially forming round sclerotized plate folded forward from posterior margin, and with well sclerotized, medial longitudinal ridge with forked apex. **Thorax.** Scutum medium brown, with 3 faint dark longitudinal vittae (1 medial and 2 submedial), shiny, densely covered with yellowish-white scale-like recumbent hairs interspersed with a few dark upright long hairs on prescutellar area; scutellum medium brown, covered with yellowish short hairs as well as dark upright long hairs along posterior margin. Postscutellum medium brown, bare. Pleural membrane bare. Katepisternum medium brown, longer than deep, moderately covered with dark hairs. **Legs.** Foreleg: coxa whitish yellow; trochanter whitish yellow except outer surface slightly darker; femur light brown; tibia light brown except base whitish yellow; tarsus dark brown, with moderate dorsal hair crest; basitarsus slightly dilated, ca. 6.5 × as long as its greatest width. Midleg: coxa medium brown with posterior surface dark brown; trochanter whitish yellow; femur light brown except base yellow; tibia whitish yellow on basal 2/5 with slightly dark subbasal band, and medium brown on apical 3/5; tibia densely covered with whitish fine hairs on outer and posterior surfaces of basal 2/5; tarsus medium brown with basal 1/2 of basitarsus whitish yellow. Hind leg: coxa light brown, trochanter whitish yellow; femur light brown with base pale whitish yellow and apical cap medium brown; tibia (Fig. 32) whitish yellow basally, slightly darkened toward apical 1/3, with dark subbasal band, and medium to dark brown on apical 1/3; tibia densely covered with whitish fine hairs on outer and posterior surfaces of basal 2/3; tarsus brownish black



Figures 26-37 Morphological characters of *S. sarawakense* sp. nov. 26-35, female; 36 and 37, pupa. 26, frons; 27, fronto-ocular area; 28 and 29, antennae (dorsal view; 28, right; 29, left); 30, 3rd segment of maxillary palp with sensory vesicle; 31, cibarium; 32, tibia, basitarsus and 2nd tarsal segment of hind leg; 33, claw of hind leg; 34, sternite 8, anterior gonapophyses, genital fork, spermatheca, paraproct and cercus of genitalia *in situ* (ventral view); 35, paraproct and cercus *in situ* (lateral view); 36, basal portion of right gill filaments (lateral view); 37, terminal hook (end view). Scale bars: 0.1 mm for figs. 32 and 36; 0.05 mm for figs. 26-29; 0.02 mm for figs. 30, 31, 34 and 35; 0.01 mm for figs. 33 and 37.

except basal 3/4 of basitarsus, basal 1/2 of 2nd tarsal segment yellowish white; basitarsus narrow, nearly parallel-sided, ca. 6.7 × as long as wide, and ca. 0.67 × and ca. 0.55 × as wide as greatest width of tibia and femur, respectively; calcipala ca. 1.35 × as long as wide, and ca. 0.72 × as wide as apical portion of basitarsus. All femora, tibia and parts of tarsus also moderately covered with dark scale-like hairs. Claws (Fig. 33) each with large basal tooth, 0.51 × as long as claw. **Wing.** Length 1.7 mm. Costa with dark spinules as well as dark hairs. Subcosta with dark hairs except near apex bare. Hair tuft on stem vein yellowish. Basal portion of radius fully haired. Basal cell absent. **Abdomen.** Basal scale dark yellow, with fringe of pale fine hairs. Dorsal surface of abdomen dark brown except that of segment 2 yellow, moderately covered with short dark hairs; tergites of segments 2, 6, 7 and 8 shiny; sternal plate on segment 7 undeveloped. **Genitalia** (Figs. 34 and 35). Sternite 8 bare medially, with 22 or 23 long stout hairs and 4 or 5 short hairs on each side. Anterior gonapophyses nearly triangular, with round medioposterior corners, thin, membranous, covered moderately with microsetae, interspersed with no short setae; inner margins nearly straight or slightly sinuous, moderately sclerotized, and somewhat separated from each other. Genital fork of usual inverted-Y form, with arms of moderate width; arm moderately folded medially, with small projection directed anterodorsally. Paraproct moderately produced ventrally, with 22 long hairs on ventral and lateral surfaces, and with 3 sensilla on inside surface. Cercus ca. 0.5 × as long as wide, rounded. Spermatheca ellipsoidal, ca. 1.33 × as long as wide, well sclerotized except tube and small area of tubal base, and with many fissures on surface; internal setae appear to be absent; both accessory tubes slender, slightly larger in diameter than major one.

Male. Unknown.

Pupa. Body length 2.2 mm. **Head.** Integument yellow, moderately covered with round tubercles; antennal sheath normal, with no spinous projections, and almost bare; face with 1 pair of simple long trichomes with uncoiled apex, and frons with 3 pairs of similar trichomes; 3 frontal trichomes on each side arising close together, subequal in size to one another. **Thorax.** Integument yellow, moderately covered with round tubercles, and with 3 pairs of simple long trichomes with coiled or uncoiled apex dorsally, 2 pairs of simple trichomes (1 long and 1 medium) anterolaterally, 1 pair of medium-long simple trichomes posterolaterally, and 3 pairs of simple trichomes (2 medium and 1 short) ventrolaterally. Gill (Fig. 36) composed of 8 slender thread-like filaments, arranged in groups of 3+3+2 fila-

ments from dorsal to ventral; upper and middle triplets sharing short stalk; each triplet consisting of 2 paired filaments with very short stalk and 1 individual filament; lower pair with stalk of medium length; basal common stalk short, with somewhat swollen transparent organ ventrally; all filaments pale yellowish, subequal in length (1.2-1.6 mm) and thickness to one another except outer filament (ca. 3.0 mm long) of lower pair ca. 2.0 × length and thickness of the other filaments (when basal portion of each filament is compared); cuticular surface of apical 3/4 of stalk of lower pair and basal 1/2 of outer filament of lower pair with distinct annular ridges and furrows but those of other filaments and stalks smooth; cuticular surface of all filaments densely covered with minute tubercles. **Abdomen.** Tergum 1 mostly pale, bare, with 1 simple slender medium-long seta on each side; tergum 2 mostly pale, bare, and with 1 simple slender medium-long seta and 5 short setae on each side; terga 3 and 4 mostly pale, each with 4 hooked spines and 1 short seta on each side; tergum 5 lacking spine-combs; terga 6-9 each with distinct spine-combs in transverse row (though those on tergum 9 much smaller in size), together with comb-like groups of minute spines on each side; tergum 9 with 1 pair of distinct plate-like terminal hooks, extending laterally, and with serrated outer margin (Fig. 37). Sternum 4 with 1 simple or bifid hook and a few simple slender minute setae on each side. Sternum 5 with 1 pair of bifid or trifid hooks submedially and a few simple slender minute setae on each side; sterna 6 and 7 each with 1 pair of bifid or trifid inner and simple outer hooks somewhat spaced from each other, and a few simple slender minute setae on each side. Each side of segment 9 with 3 grapnel-like hooklets. **Cocoon.** Simple, wall-pocket-shaped, neatly and moderately woven, somewhat extending ventrolaterally; anterior margin not thickly woven; posterior 1/2 with floor roughly woven; individual threads visible; 2.5-2.8 mm long × 1.5-1.9 mm wide.

Mature larva. Unknown.

TYPE SPECIMENS. Holotype, female, reared from pupa, collected at Pueh, Sematan, Sarawak, Malaysia, 18.III.1998. Paratype 1 pupa, same data and date as holotype.

ECOLOGICAL NOTES. Two pupae were collected, one from a dead leaf, and the other from a rock, in water of a small clean shaded stream (0.5-3.0 m wide) flowing down on the rocky streambed in natural forest of Mt. Pueh. The water temperature was 25 °C. This species was collected together with *S. laterale*, *S. lehi* and *S. sabahense*.

DISTRIBUTION. Sarawak.

ETYMOLOGY. The species *sarawakense* is named after the name of state where this species was collected.

REMARKS. This new species is assigned in the *batoense* species-group by the slender male hind basitarsus (dissected out of the pharate male) as well as the usual 2+9 segments of the adult antennae. The pupa of *S. sarawakense* is characterized by the gill composed of one long and seven short filaments which are about half the length and thickness of the longer filament (Fig. 36). The similar arrangement of the pupal gill filaments, coupled with plate-like terminal hooks with a serrated outer margin, has been seen in *S. epistum* Delfinado and *S. luzonicum* Takaoka, both from Philippines (Takaoka, 1983). However, stalks of upper and middle triplets of these two species share a very short common stalk. This new species is distinguished in the female from these known species by the narrower frons and smaller sensory vesicle. The frons-head ratio is 1:5.8 in this new species, while that is 1:5.2 in *S. epistum* and 1:4.3 in *S. luzonicum*; and the relative length of sensory vesicle to the third segment is 0.26 in this new species but it is 0.4 in the other two species. The pupal gills of *S. rayohense* Smart and Clifford described from Sabah, seem to be similar to those of *S. sarawakense*, but seven shorter filaments of this known species are much longer (i.e., 2.0-2.3 mm), according to the illustration given by Smart and Clifford (1969). The pupa of *S. pegalanense* Smart and Clifford, described from Sabah, also differs from that of this new species by the terminal hooks not serrated. In the pupal gills of *S. pegalanense*, it is not the outer filament of the ventral pair but the counter inner filament that is somewhat thicker and longer than its counter filament and six other filaments of upper and middle triplets. This was confirmed with slide-mounted type specimen.

NOTES ON OTHER SPECIES NEWLY RECORDED FROM SARAWAK

Simulium (Gomphostilbia) sheilae Takaoka and Davies, 1995

Simulium (Gomphostilbia) sheilae Takaoka and Davies, 1995: 60-65 (female, male, pupa and larva); Kuvangkadilok and Takaoka, 2000: 172-173; Takaoka *et al.*, 2000: 161.

SPECIMENS EXAMINED. 1 female, 1 male, both reared from pupae, and 2 larvae, collected at Saya Bakti, 10 km from Kuching, 17.III.1998; 6 females, 5 males, all reared from pupae, and 10 larvae, collected from Jau R., at Santobung, 30 km from Kuching, 17.III.1998; 2 males, reared from pupae, 2 larvae, collected at Damai Look-out Point, 17.III.1998; 1 male, reared from pupa, and 3 larvae, collected

at Lundu, in Gunung Gading National Park, 18.III.1998.

ECOLOGICAL NOTES. The pupae and larvae of this species were collected from fallen leaves and trailing grasses in slowly-flowing lowland streams 0.1-4.0 m wide, exposed to the sun or partially shaded. The water temperature of the stream was 26 or 27 °C. This species was collected together with *S. sabahense*.

DISTRIBUTION. Peninsular Malaysia, Sabah, Sarawak (new record), Sumatra and Thailand.

REMARKS. *S. sheilae* was originally described from Peninsular Malaysia, and was assigned to the *ceylonicum* species-group by Takaoka and Davies (1995). It was also recorded from Thailand (Kuvangkadilok and Takaoka, 2000), Sumatra (Takaoka *et al.*, 2000) and Sabah (Takaoka, 2001).

The reared adult female and male specimens, as well as pupal and larval ones examined in this study, are morphologically almost the same as those originally described, with an exception that the scutum of the present male specimens is whitish pruinose on each shoulder, along both lateral margins and on the prescutellar area (not entirely whitish pruinose, as in the type male specimen). The same difference in the male scutal pattern has been noted for specimens of this species collected from Sabah (Takaoka, 2001).

Simulium (Nevermannia) aureohirtum Brunetti, 1911

Simulium aureohirtum Brunetti, 1911: 283-288 (male); Edwards, 1934: 134-137 (female, pupa and larva).

Simulium (Nevermannia) aureohirtum: Ogata, 1956: 61-62; Ogata, 1966: 129; Crosskey, 1988: 459; Takaoka and Roberts, 1988: 194-195; Crosskey and Lowry, 1990: 204; Takaoka and Davies, 1995: 87-88; Takaoka and Davies, 1996: 5-6. Crosskey and Howard, 1997: 47.

Simulium aureohirtum: Oriei, Uemoto and Onishi, 1969: 1-13 (larva).

Simulium (Eusimulium) aureohirtum: Puri, 1933: 1-7 (female, male, pupa and larva); Ogata and Sasa, 1954: 325; Ogata, Sasa and Suzuki, 1956: 73; Crosskey, 1973: 423; Takaoka, 1976: 170-171; Takaoka, 1979: 382-384 (female, male, pupa and larva); Datta, 1983: 225; Takaoka and Suzuki, 1984: 11-12.

Simulium (Eusimulium) tuaranense Smart and Clifford, 1969: 40-43. Syn. by Crosskey, 1973.

Simulium (Eusimulium) philippinense Delfinado, 1962: 47-62. Syn. by Takaoka, 1983.

SPECIMENS EXAMINED. 1 female reared from pupa, and 1 larva, collected from Bunga R., Kuching, Sarawak, 16.III.1998.

ECOLOGICAL NOTES. The one pupa and one larva of this species were collected from grass leaves trailing in the water of a stream with width of 2-5 m, running in a cacao plantation, exposed to the sun. The water temperature was 26 °C. Many pupae and larvae of *S. sabahense* were collected together with this species.

DISTRIBUTION. Indonesia, Japan, Malaysia (Peninsular Malaysia, Sabah, and Sarawak (new record)), Nepal, Pakistan, Philippines, Sri Lanka, Taiwan and Thailand.

REMARKS. This species belongs to the *ruficorne* species-group of the subgenus *Nevermannia* (Crosskey, 1969) and is known to be widely distributed in the Oriental Region and parts of the Palaearctic Region (Crosskey and Howard, 1997). The female, its associated pupa and larva collected from Sarawak agree in morphological characters with the redescription of this species given by Takaoka (1979).

***Simulium (Simulium) laterale* Edwards, 1933**

Simulium laterale Edwards, 1933: 256 (female).

Simulium (Simulium) laterale: Smart and Clifford, 1969: 22-26 (female, male, pupa and larva); Takaoka, 1983: 164 (only female genitalia illustrated).

SPECIMENS EXAMINED. 1 male reared from pupa, 2 pupae and 5 mature larvae, collected at Pueh, Sematan, Sarawak, 18.III.1998.

ECOLOGICAL NOTES. Pupae and larvae were attached on stones and rocks in water of a small clean shaded stream 0.5-3.0 m wide, flowing down on the rocky streambed in a natural forest of Mt. Pueh. The water temperature was 25 °C. This species was collected together with *S. lehi*, *S. sabahense* and *S. sarawakense*.

DISTRIBUTION. Sabah and Sarawak (new record).

REMARKS. This species was originally described from female specimens collected at Lumu Lumu, Sabah, by Edwards (1933). The male, pupa and larva were described later by Smart and Clifford (1969). Takaoka (1983) examined the female genitalia of one of the syntype specimens of *S. laterale* preserved at The Natural History Museum, London, and assigned this to the *melanopus* species-group of the subgenus *Simulium* s. str. The male, pupal and larval specimens collected from Sarawak agree with the descriptions of this species given by Smart and Clifford (1969), except the difference of leg colorings: fore coxa white (not light brown), fore tibia dark brown with a wide whitish area

on outer surface (not entirely dark brown); mid tibia dark brown with a basal tip white (not entirely dark brown), and basitarsus almost white with an apical tip dark brown (not lighter brown); hind tibia dark brown with a basal small area white (not entirely dark brown), and basitarsus white on the basal 1/2, dark brown on the apical 1/2 and on a basal tip (not dark brown with the basal 1/3 light brown).

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COMPARATIVE ORAL SUSCEPTIBILITY OF DENGUE VECTOR MOSQUITOES FROM JAPAN AND SOUTHEAST ASIA TO TWO DENGUE-1 VIRUS STRAINS

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Abstract: Five geographic strains of *Aedes albopictus* from Japan and Southeast Asia and three strains of *Aedes aegypti* from Indonesia and Pakistan were compared for their susceptibility to oral infection with the human virus, dengue-1 Mochizuki strain (isolated in 1943, Japan) and A88 strain (isolated in 1988, Indonesia). Female mosquitoes, aged 3-4 days, were infected with a virus-erythrocyte-sucrose suspension. After 14 days of incubation at 25-30 °C, viral infection in mesenteron (midgut) or in head-thorax (salivary gland) of each individual mosquito was identified by the reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of dengue-1 virus envelope gene. Although the results of susceptibility varied in some extent in different strains of mosquitoes and viral strains, the oral susceptibility to both dengue-1 virus strains was not significantly different among dengue vector mosquitoes from Japan and Southeast Asia.

Key words: Susceptibility, Vector mosquitoes, Geographic strains, Dengue-1 virus, Oral infection

INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) have become a global public health problem in recent years (WHO, 2000). In Japan there are 42 confirmed cases of DF 1988 (Yamada *et al.*, 1999), most of them infected when visiting Southeast Asian countries.

Aedes aegypti, which is found in most parts of the tropics and subtropics, has been considered to be main vector of DF/DHF. Another important vector, *Aedes albopictus* is able to thrive in both cold and hot climates. The latter species is commonly found in Japan and has been implicated as a vector of DF epidemics in Japan during World War II (Sabin, 1952; Hotta, 1998).

There are some studies on mosquito susceptibility to oral infection with dengue viruses using Asian strains of *Ae. aegypti* and *Ae. albopictus* (Gubler and Rosen, 1976; Gubler *et al.*, 1979; Rosen *et al.*, 1985; Tardieux *et al.*, 1992). However *Ae. albopictus* from Tokyo, Japan is the only one strain that has been tested for its vector competence to dengue virus (Boromisa *et al.*, 1987). In this report, the similarity in vector competence of this species from Japan with that from North America support the possibility

that this mosquito was introduced in North America with the shipment of used tires from Northern Asia (Hawley *et al.*, 1987). The development of transportation systems in the world has stimulated the global spread of dengue via travelers and trade.

The immunofluorescence technique was used for detection of virus in most of previous investigation on vector competence. Recently, several studies described the sensitivity and rapid generation of results of polymerase chain reaction (PCR) or reverse transcriptase-PCR (RT-PCR) assay for detecting and typing dengue viral RNA extracted from mosquitoes (Tardieux and Poupel, 1990; Tardieux *et al.*, 1992; Chungue *et al.*, 1993; Chow, *et al.*, 1998). The PCR method that can detect the dengue virus in infected mosquitoes from extremely small amounts of sample was applied in this study.

The dengue-1 viruses (DEN-1) used in this study are Mochizuki and A88 strains. Mochizuki strain was isolated from the blood of a DF patient during an epidemic of dengue fever in Nagasaki, Japan in 1943 (Kimura and Hotta, 1994) and was found to have lost its pathogenicity for human (Hotta, 1952). The A88 strain was isolated from the blood of a DHF patient in an outbreak of DHF in Jakarta, Indonesia in 1988 (Fujita *et al.*, 1997).

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Ae. albopictus strains from different areas of Japan were tested for their susceptibility to infection with both DEN-1 Mochizuki and A88 strains and then compared with the susceptibility of *Ae. albopictus* and *Ae. aegypti* from epidemic areas of Southeast Asia. Mochizuki and A88 strains were also compared for their abilities to infect orally and replicate in mosquito vectors.

MATERIAL AND METHODS

Mosquito strains:

The origin of *Ae. albopictus* (5 strains) and *Ae. aegypti* (3 strains) and the number of generations used for oral infection are shown in Table 1. All mosquito strains were colonized in our insectary room from field collected eggs in different areas of Japan and Southeast Asia. The mosquitoes were reared in plastic pans (33.5 cm × 25 cm × 11.5 cm high) containing 3 liters of aged tap water with a density of 100-200 larvae per container. The larvae were fed a mixture of yeast extract with a liver and vegetables powder and maintained at 25 °C, 70% relative humidity and a 14:10 hr light: dark photoperiod. Adult colony mosquitoes were provided with 10% sucrose solution.

Preparation of virus as source of virus solution:

DEN-1 Mochizuki and A88 strains used in this study were prepared by growth in Vero cell cultures. A confluent 25 cm² tissue culture of Vero cell monolayer was infected at a multiplicity of infection of 0.01 PFU/cell with addition of 5 ml of Eagle's essential medium (E-MEM) containing 5% heat-inactivated fetal calf serum (FCS). After 7 days of incubation at 37 °C, cells were scraped into the medium, centrifuged at 2,000 rpm for 5 min, and then re-suspended in 500-600 µl of the infected supernatant culture medium as source of viral suspension.

Oral infection of mosquito strains:

The 3-4 days old female mosquitoes were placed in a small cylindrical cardboard container and deprived of sucrose solution 26-36 hr prior to the infection meal, and then allowed to feed on a hanging drop virus solution. Virus solution was prepared by mixing equal parts of DEN-1 virus suspension, washed human red blood cells and 10% (final concentration) sucrose. Adenosine triphosphate (ATP) was added at a final concentration of 5×10^{-3} M as a phagostimulant (Rutledge *et al.*, 1964). The drop of virus solution was warmed at 40 °C for 5-10 min and placed on a fine mesh covering a small cylindrical cardboard container containing mosquitoes. In each experiment, a small aliquot of virus solution was held at room temperature during mosquito feeding (1 to 2 hr) and subsequently titrated by plaque assay in Vero cell monolayers. Mosquito strains to be compared were fed the same mixture at the same time. Mosquitoes that took at least three-fourth of full blood meal were selected and held for 14 days at 30 °C or 25 °C and a 76% relative humidity in a desiccator. The infected mosquitoes were killed by freezing and the virus was detected in mesenteron (midgut) and thorax-head (salivary gland) by RT-PCR amplification of envelope gene. The experiments using viruses and infected mosquitoes were conducted in P3 level laboratory of Animal Research Center, Toyama Medical and Pharmaceutical University.

Sample preparation for virus assay:

Mesenteron and thorax-head were removed from individual mosquito and triturated by a pellet mixer in 150 µl of phosphate-buffered saline with 30% FCS. Triturated samples were centrifuged at 10,000 rpm at 4 °C for 5 min. Two-third of the supernatant fluid was diluted, filtered through 0.45 µm syringe filter unit and titrated by plaque assay on Vero cell monolayers and the remaining fluid was treated for RNA extraction. The virus titer was calculated as

Table 1 Origin of *Aedes albopictus* and *Aedes aegypti* tested for oral infection with dengue virus type 1 (DEN-1) Mochizuki and A88 strains

Species/strains	Geographic origin	Year of colonization	Number of generations
<i>Ae. albopictus</i>			
Toyama	Toyama, Japan	1992	F ₈₈
Awajishima	Hyogo, Japan	1998	F ₂₀
Kojima	Okayama, Japan	1996	F ₃₈
Thailand	Bangkok, Thailand	1986	F ₁₃₇
Makassar	South Sulawesi, Indonesia	1996	F ₃₀
<i>Ae. aegypti</i>			
Timor	West Timor, Indonesia	1999	F ₁₀
Surabaya	East Java, Indonesia	1986	F ₁₀₃
Pakistan	Karachi, Pakistan	1990	F ₈₁

plaque forming units (PFU) per mosquito based on the estimation of blood meal size of *Ae. albopictus* (2 μ l) as previously reported (Konishi and Yamanishi, 1984).

RT-PCR amplification for viral detection in infected mosquitoes:

Viral RNA was extracted from triturated sample using Isogen (Nippon Gene). The pellet was dried up and suspended in 10 μ l of sterile water. RNA reverse transcription was carried out at 42 $^{\circ}$ C for 60 min in a final volume of 20 μ l (5 μ l of RNA template, 25 pmol of primer, 10 units of RNase inhibitor, 10 mM of dNTP and 2.5 units of reverse transcriptase). The reaction mixture also was held at 95 $^{\circ}$ C for 5 min and then quickly chilled in ice. For PCR, 20 μ l of cDNA was added with 25 pmol of sense primer, 25 pmol of complementary primer, 10 mM of dNTP and 1.25 units of Taq polymerase in a final volume of 50 μ l. The reaction mixture was overlaid with mineral oil and amplification was performed in a Perkin Elmer thermal cycler. Each of the 30 cycles performed comprised a 1 min denaturation at 94 $^{\circ}$ C, 1 min annealing at 60 $^{\circ}$ C and 2 min elongation at 72 $^{\circ}$ C.

The cDNA primer, which was also the PCR complementary primer: D1-NS1N/2439, a 20-mer (5'-ACTGGAAAGGCAGAGAACTC-3') and the PCR sense primer: D1-ES/1552, a 20-mer (5'-GCTCGTCCACAAACAATGG-3') were synthesized based on the known nucleotide sequences of dengue-1 Nauru strain (Puri *et al.*, 1998). The PCR was carried out to amplify a 900 bp target sequence of the envelope gene. PCR product samples were electrophoresed on 1.5% agarose gel in Tris-Borate-EDTA (TBE) buffer and the bands were visualized by ethidium bromide staining. To ensure that cross contamination did not occur between samples, a negative control was made without RNA for each PCR experiment.

RESULTS

Oral susceptibility of mosquito strains to DEN-1:

For the experiment of the oral susceptibility to DEN-1 Mochizuki and A88 strains, 3 different viral concentrations were used to infect mosquito strains. The proportions of mosquitoes infected with Mochizuki and A88 strains at 30 $^{\circ}$ C incubation were estimated based on the PCR results (Tables 2 and 3). *Ae. albopictus* Kojima strain was the only one strain orally susceptible to DEN-1 Mochizuki infection after ingestion of low viral dose (1.56 $\times 10$ PFU/mosquito) (Table 2). Most of *Ae. albopictus* strain were infected after ingestion of higher viral doses (1.98 $\times 10^2$ and 7.9 $\times 10^2$ PFU/mosquito). However, all of mosquito strains showed low susceptibility to DEN-1 Mochizuki strain even with a higher viral dose (7.9 $\times 10^2$ PFU/mosquito). *Ae. aegypti* strains could be infected with DEN-1 Mochizuki strain within 7 days incubation at 30 $^{\circ}$ C (data not shown), but the infection rate decreased after 14 days incubation, therefore the infections were not observed.

In contrast to Mochizuki strain, most of mosquito strains were susceptible to DEN-1 A88 infection even with low viral dose (8.55 PFU/mosquito) (Table 3). The susceptibility to A88 infection increased in *Ae. albopictus* Toyama strain as same as that of *Ae. aegypti* Timor and Pakistan strains after ingestion of high viral dose (2.16 $\times 10^3$ PFU/mosquito). *Ae. albopictus* Kojima, Thailand, and Makassar strains showed same or similar proportions of infection with 3 different infectious doses. In general, mosquito strains incubated at 30 $^{\circ}$ C were more susceptible to DEN-1 A88 than to Mochizuki strain as shown in Table 4.

The above data were statistically analyzed by the multiple comparison Bonferroni test (SPSS 10.0J for Windows base system) and no significant differences were found

Table 2 Proportion of *Ae. albopictus* and *Ae. aegypti* strains orally infected with different doses of DEN-1 Mochizuki strain after 14 days of incubation at 30 $^{\circ}$ C

Species/strains	Proportion of mosquitoes infected (n)		
	Virus ingested (PFU/mosquito)		
	1.56 $\times 10$	1.98 $\times 10^2$	7.9 $\times 10^2$
<i>Ae. albopictus</i>			
Toyama	0.00(3)	0.75(4)	0.33(3)
Awajishima	0.00(3)	0.25(4)	0.33(3)
Kojima	0.33(3)	0.25(4)	0.00(3)
Thailand	0.00(3)	0.00(4)	0.67(3)
Makassar	0.00(3)	NT	0.00(3)
<i>Ae. aegypti</i>			
Timor	0.00(3)	0.25(4)	0.00(3)
Surabaya	0.00(3)	0.00(4)	NT
Pakistan	0.00(3)	0.00(4)	NT

NT=not tested.

Table 3 Proportion of *Ae. albopictus* and *Ae. aegypti* strains orally infected with different doses of DEN-1 A88 strain after 14 days of incubation at 30 °C

Species/strains	Proportion of mosquitoes infected (n)		
	Virus ingested (PFU/mosquito)		
	8.55	1.48 × 10 ²	2.16 × 10 ⁰
<i>Ae. albopictus</i>			
Toyama	0.33(3)	0.25(4)	0.67(3)
Awajishima	0.33(3)	0.75(4)	0.00(3)
Kojima	0.00(3)	0.25(4)	0.00(3)
Thailand	0.33(3)	0.50(2)	0.33(3)
Makassar	0.67(3)	NT	0.67(3)
<i>Ae. aegypti</i>			
Timor	0.00(2)	0.25(4)	0.67(3)
Surabaya	1.00(2)	0.50(4)	NT
Pakistan	0.33(3)	0.00(4)	0.67(3)

NT=not tested.

Table 4 Comparative susceptibility of *Ae. albopictus* and *Ae. aegypti* strains orally infected with DEN-1 Mochizuki and A88 strain at 30 °C

Species/ strains	No. (%) of mosquitoes infected with DEN-1	
	Mochizuki*	A88**
<i>Ae. albopictus</i>		
Toyama	0/30 (0.0)	6/30 (20.0)
Kojima	1/11 (9.1)	2/15 (13.3)
Makassar	1/29 (3.4)	3/29 (10.3)
<i>Ae. aegypti</i>		
Timor	0/18 (0.0)	1/18 (5.6)
Surabaya	1/9 (11.1)	2/13 (15.4)
Pakistan	0/12 (0.0)	1/16 (6.3)

* Virus ingested was 4.0 × 10 PFU/mosquito ** 6.0 × 10 PFU/mosquito

among oral susceptibility of mosquitoes to Mochizuki or A88 strains.

Comparison of mesenteronal and head-thorax (salivary gland) infections of mosquito strains:

The comparison of mesenteronal and disseminated (head-thorax) infections of mosquito strains with DEN-1 Mochizuki and A88 strains after 14 days of extrinsic incubation at 25 °C are shown in Figs. 1 and 2, respectively. In Fig. 1, *Ae. albopictus* Kojima strain showed the highest proportion of infection with Mochizuki strain in the mesenteron (14/17) and near half of them (7/17) showed disseminated infection. Toyama strain also showed high proportion of disseminated infection in contrast to Awajishima strain, in which disseminated infection was not detected. The same proportion of mesenteronal and disseminated infection was observed in *Ae. albopictus* Thailand strain and *Ae. aegypti* Timor strain. Infection did not occur in *Ae. aegypti* Surabaya and Pakistan strains.

Fig. 2 shows that all *Ae. albopictus* strains were infected orally with A88 strain except Awajishima strain. However, not all of them developed disseminated infection. Surabaya strain, among *Ae. aegypti* species, had higher mesenteronal infection with A88 strain than other strains, but the infected mosquitoes did not develop disseminated infection. Timor strain had the same proportion of mesenteronal and disseminated infection, while Surabaya strain did not show disseminated infection although it showed relatively high proportion of mesenteronal infection.

From the above results *Ae. albopictus* strains tended to be more susceptible to oral infection with either DEN-1 Mochizuki or A88 strain than *Ae. aegypti* strains.

Mesenteronal escape barrier of mosquito strains:

Based on the data of disseminated infection (Figs. 1 and 2), the mesenteronal escape barrier was estimated as shown in Fig. 3. The existence of mesenteronal escape barrier means that virus multiplies only in mesenteron and

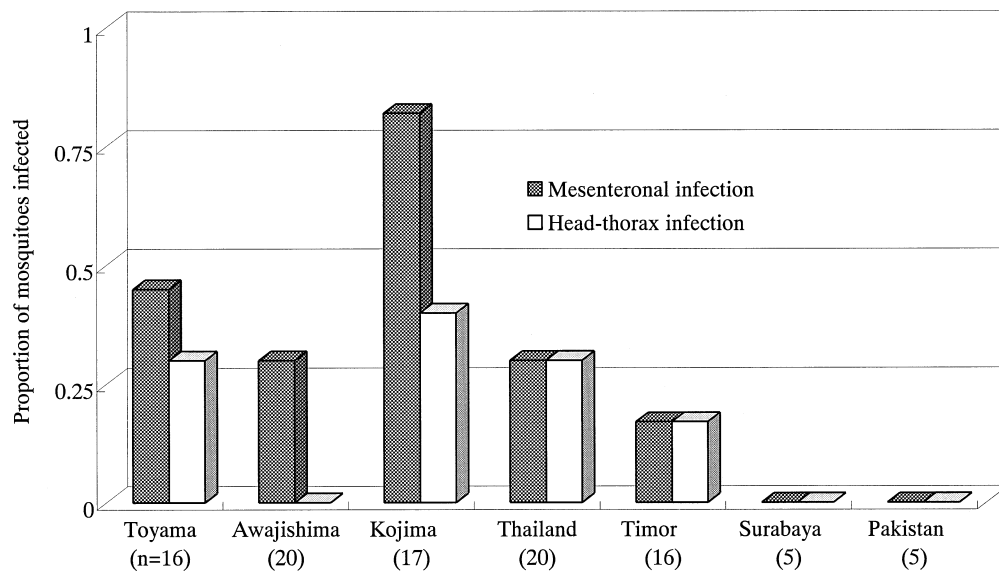


Figure 1 Proportions of mesenteronal (midgut) and disseminated (head-thorax) infections of *Ae. albopictus* and *Ae. aegypti* strains orally infected with DEN-1 Mochizuki strain after 14 days incubation at 25 °C. The amount of virus ingested was 1.3×10^3 PFU/mosquito.

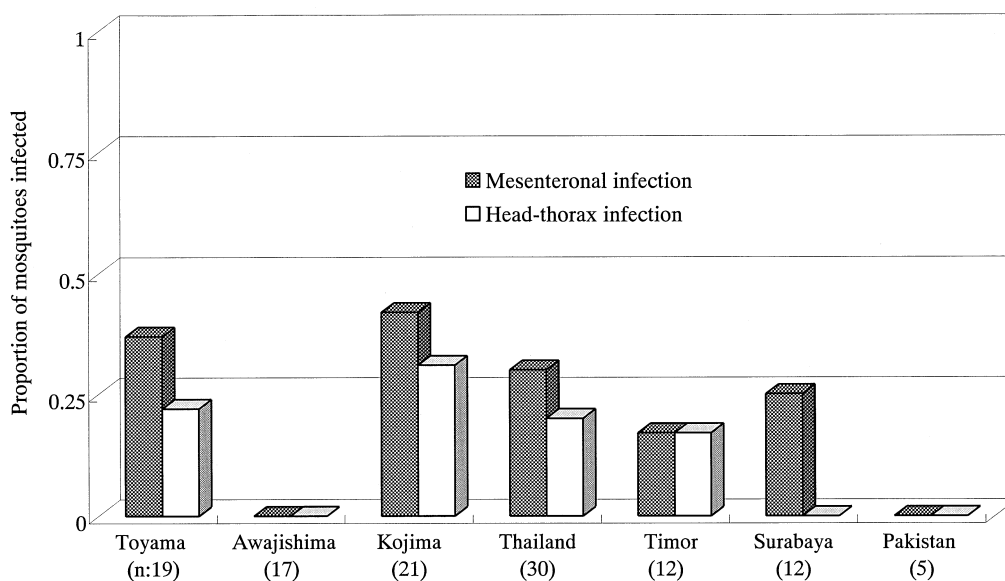


Figure 2 Proportions of mesenteronal (midgut) and disseminated (head-thorax) infections of *Ae. albopictus* and *Ae. aegypti* strains orally infected with DEN-1 A88 strain after 14 days incubation at 25 °C. The amount of virus ingested was 1.1×10^3 PFU/mosquito.

does not invade tissues regardless of the length of extrinsic incubation (Kramer *et al.*, 1981). *Ae. albopictus* from Japan especially Kojima strain showed the highest mesenteronal escape barrier to infection with Mochizuki strain (Figs. 1 and 3). *Ae. aegypti* Surabaya strain showed high mesenteronal escape barrier to infection with A88 strain (Figs. 2 and 3). *Ae. albopictus* Toyama strain showed the same

mesenteronal escape barrier to infection with both Mochizuki and A88 strains.

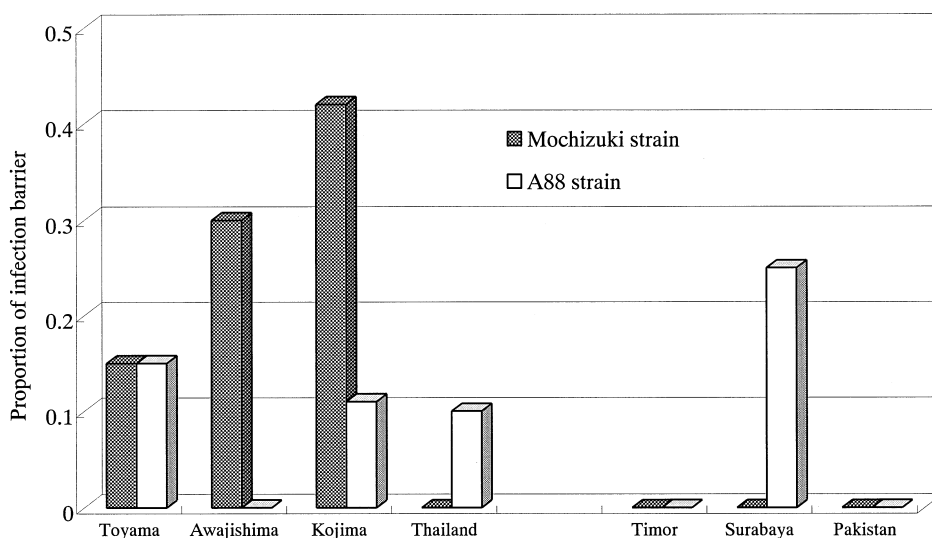


Figure 3 Proportions of mesenteron escape barrier* of *Ae. albopictus* and *Ae. aegypti* strains orally infected with DEN-1 Mochizuki and A88 strains.

* Barrier: proportion of (mesenteron-head thorax) infection

Viral titers in mesenteron of mosquito strains:

The viral titer of DEN-1 A88 strain (2.16×10^3 PFU/mosquito) in mesenteron of *Ae. albopictus* were measured after 7, 14 and 21 days in one mosquito of each Toyama and Makassar strains. The titers in Makassar strain decreased slightly after 7 days (3.41×10^2 PFU/mosquito), 14 days (2.1×10^2 PFU/mosquito) and 21 days (4.8×10^2 PFU/mosquito) of extrinsic incubation at 30 °C. The titers in Toyama strain were 2.36×10^2 PFU/mosquito after 7 days and remained constant after 14 days (1.41×10^2 PFU/mosquito) and 21 days (1.30×10^2 PFU/mosquito).

DISCUSSION

A RT-PCR method was applied with some modifications of the method of Sambrook *et al.* (1989) for rapid and sensitive detection of viral RNA in infected mosquitoes, while the viral titer was detected with the method of plaque assay. Since this method can detect rapidly a few doses of infected materials, it is expected to be a useful tool for early warning monitoring system for dengue outbreaks in the future. The sensitivity and specificity of PCR method for viral detection allowed a comparison of susceptibility of mosquitoes to dengue viruses in the present study. For this purpose, low viral doses (about 1/1,000 of the viral concentrations reported so far) were used to infect the mosquitoes orally. In the preliminary study, various infectious meals and conditions were tested for oral infection. The blood meal prepared with the mixture of infected cells and their culture media was more effective to infect the mosquitoes

than the meal prepared with the infected cell culture supernatant. This result led us to add cells in infective meal.

The susceptibility is also dependent to the serotype of dengue virus. The oral threshold of infection for DEN-1 has been reported to be higher than that for other dengue serotypes (Gubler and Rosen, 1976; Gubler *et al.*, 1979). The high concentration of virus in the blood meal is necessary to overcome this threshold, but it varies for different virus strains in the same mosquito species and for the same virus in different species or different geographic strains of the same species as reviewed by Hardy *et al.* (1983).

Colonization of mosquito strains in the laboratory may have some influences over susceptibility. This possibility was shown by the proportions of orally infected mosquitoes that were higher in Timor strain (F8) than Surabaya (F107) or Pakistan strains (F81) of *Ae. aegypti*. The influence of colonization was not investigated in the present study but concerning this, Lorenz *et al.* (1984) correlate the differences of susceptibility with genetic variation at the malate dehydrogenase locus.

There was a tendency for a high susceptibility at high viral concentrations, i.e., it seemed to possess a dose-dependent mesenteron (midgut) barrier as discussed by Gubler and Rosen (1976), Gubler *et al.* (1979) and Kramer *et al.* (1981). The dissemination barriers and infections virus titer in mosquito determine vector competence for dengue virus (Bosio *et al.*, 1998). In this experiment not all infected mosquitoes developed disseminated infection, which indicates the existence of a midgut escape barrier. According to Kramer *et al.* (1981), there are two dissemination

barriers (mesenteron escape barrier and salivary gland infection barrier) in *Culex tarsalis* infected with Western equine encephalomyelitis virus. For dengue infection the midgut basal lamina thickness in mosquito strains influences the viral dissemination (Thomas *et al.*, 1993). On the other hand, Rosen *et al.* (1985) reported that dengue virus usually replicate to about the same extent in orally infected mosquitoes as well as in the parenterally infected specimens of the same species.

Mosquitoes were more susceptible to DEN-1 Mochizuki strain than to A88 strain after incubation at 25 °C, but inversely at the elevated temperature, 30 °C. The effect of extrinsic incubation temperature on the viral susceptibility of mosquitoes has been reported by Kramer *et al.* (1983). The different abilities of DEN-1 Mochizuki (human attenuated virus) and A88 strains to infect mosquitoes orally may be related to their genetic differences (Ishak *et al.*, 2001). In an experiment using dengue-2 vaccine virus Miller *et al.* (1982) showed that it was markedly less efficient in its ability to infect mosquitoes orally.

Boromisa *et al.* (1987) reported that the susceptibility of *Ae. albopictus* from Japan (Tokyo strain) to dengue 1 virus was not significantly different to that from Malaysia (Southeast Asia), but its transmission rate was lower than Malaysia strains. Similarly, from the present results it was concluded that Japanese strains of *Ae. albopictus* are susceptible to DEN-1 in almost the same extent as *Ae. albopictus* and *Ae. aegypti* from Southeast Asian countries.

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PHYLOGENETIC ANALYSES OF A BLACKFLY SUBGENUS *SIMULIUM* (*NEVERMANNIA*) BASED ON MITOCHONDRIAL 16S RIBOSOMAL RNA GENE SEQUENCES

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Abstract: Nucleotide sequences of a subregion of the mitochondrial 16S ribosomal RNA gene of 10 species of a blackfly subgenus *Simulium* (*Nevermannia*), which include four species of *feuerborni* species-group, two species of *ruficorne* species-group, three species of *vernum* species-group and an ungrouped species (*S. konoii*), were determined. Phylogenetic analyses of the sequences of the *Nevermannia* species and other species of related subgenera of *Simulium* s.l. showed that the *feuerborni* and *vernum* species-groups were closely related, but the *ruficorne* species-group and *S. konoii* were not. Variations between the *ruficorne* species-group and other *Nevermannia* species were larger than those between *Nevermannia* species (excluding the *ruficorne* species-group) and other subgenera species. These molecular data suggest that revision of the definition of the subgenus *Nevermannia* is needed.

Key words: Black fly, *Simulium*, *Nevermannia*, Phylogeny, Mitochondrial rRNA

INTRODUCTION

A blackfly subgenus *Simulium* (*Nevermannia*) is distributed worldwide. In Asia, there are 3 species-groups (i.e. *feuerborni*, *ruficorne* and *vernum* species-group), and some ungrouped species (Crosskey and Howard, 1997). To investigate the relationship within subgenus *Nevermannia* species and between subgenus *Nevermannia* and other subgenera, we analyzed sequence variations in a subregion of the mitochondrial 16S ribosomal RNA (rRNA) gene of 10 species of subgenus *Nevermannia* and related species.

MATERIALS AND METHODS

Materials used in this study and their origin are listed in Table 1. Total DNA was extracted from single larva using the crude STE boiling method (O'Neill *et al.*, 1992). Polymerase chain reactions (PCR) were performed in a 50 μ l reaction mixture using 2 μ l of the DNA solution. The primers (primer A, 5'-CGCCTGTTTATCAAAAACA-T-3'; primer B, 5'-CTCCGGTTTGAAGTCAGATC-3') were used to amplify the mitochondrial 16S rRNA region as described by Xiong and Kocher (1991). The reaction mix-

ture contained 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 250 μ M dNTPs, 2.5 units of *Tag* DNA polymerase and 50 pM of each of the primers. The thermal cycling conditions were 35 cycles of denaturation at 95 $^{\circ}$ C for 1 min, annealing at 50 $^{\circ}$ C for 2 min, extension at 72 $^{\circ}$ C for 2 min and a final extension at 72 $^{\circ}$ C for 10 min. PCR products were purified by using the QIAquick PCR purification kit (Qiagen), and cloned into pGEM-T Easy vector (Promega). At least 4 independent clones from each blackfly sample were sequenced to identify polymerase error using the *fmol* DNA sequencing system (Promega). Sequences were deposited in DDBJ/EMBL/GenBank databases under accession numbers AB056728-AB056747.

The sequences were aligned by using the program CLUSTAL W ver. 1.7 (Thompson *et al.*, 1994). Sites containing alignment gaps were removed in the following analyses. The number of nucleotide substitution per site was estimated between each pair of the sequences, using Jukes-Cantor methods (Jukes and Cantor, 1969). Construction and bootstrap probability estimation of the neighbor-joining tree (Satiou and Nei, 1987) were performed by PHYLIP 3.57c (Felsenstein, 1995).

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Table 1 Materials used in this study

Genus	Subgenus	Species-group	Species	Locality	GenBank Acc.	
<i>Simulium</i>	<i>Nevermannia</i>	<i>feuerborni</i>	<i>S. feuerborni</i> Edwards	Java, Indonesia	AB056728	
			<i>S. mie</i> Ogata & Sasa	Peninsular Malaysia	AB056729	
				Kanagawa, Japan	AB056730	
				Nagano, Japan	AB056731	
			<i>S. saitoi</i> Takaoka	Yakushima, Japan	AB056732	
				Kanagawa, Japan	AB056733	
				Hakone, Japan	AB056734	
			<i>ruficorne</i>	<i>S. aureohirtum</i> Brunetti	Indonesia	AB056735
				<i>S. ornatipes</i> Skuse	Peninsular Malaysia	AB056736
		Irian Jaya, Indonesia			AB056737	
		<i>vernum</i>	<i>S. bonninense</i> Shiraki	Ogasawara, Japan	AB056738	
			<i>S. subcostatum</i> Takahasi	Kanagawa, Japan	AB056739	
				Oita, Japan	*	
		<i>Gomphostilbia</i>	ungrouped	<i>S. uchidai</i> Takahasi	Oita, Japan	AB056740
				<i>S. konoii</i> Takahasi	Yakushima, Japan	†
				<i>S. palauense</i> Stone	Tochigi, Japan	AB056741
				<i>S. whartoni</i> Takaoka & Davies	Palau	AB056742
				<i>S. farciminis</i> Smart & Clifford	Peninsular Malaysia	AB056743
		<i>Morops</i>		<i>S. farciminis</i> Smart & Clifford	Irian Jaya, Indonesia	AB056744
<i>Simulium</i> s.str.		<i>S. aokii</i> Takahasi	Oita, Japan	AB056745		
		<i>S. quinquestriatum</i> Shiraki	Oita, Japan	AB056746		
		<i>P. kiotoense</i> Shiraki	Oita, Japan	AB056747		

* Sequence of *S. subcostatum* from Oita, Japan was identical to that from Kanagawa, Japan

† Sequence of *S. uchidai* from Yakushima, Japan was identical to that from Oita, Japan

RESULTS

We determined the mitochondrial 16S rRNA region of 10 *Nevermannia* species including three species-groups (*feuerborni*, *ruficorne*, *vernum*) and an ungrouped species (*S. konoii* Takahasi), five species of other subgenera and *Prosimulium kiotoense* Shiraki, and aligned (Fig. 1). All of the *Nevermannia* species had 516 bases in this region. As for the five species (*S. feuerborni* Edwards, *S. mie* Ogata and Sasa, *S. aureohirtum* Brunetti, *S. subcostatum* Takahasi, *S. uchidai* Takahasi), we determined the sequences of two or three samples from different localities. *S. subcostatum* and *S. uchidai* did not have any intraspecific variations, but *S. feuerborni*, *S. mie* and *S. aureohirtum* had. These intraspecific variations were not larger than the interspecific variations.

To study relationships between *Nevermannia* species and between *Nevermannia* and other subgenera, a neighbor-joining tree was constructed based on the estimated *d* values (the number of the nucleotide substitutions per site) between each pair of the samples (Fig. 2). *P. kiotoense* was used as an outgroup. The three species-groups of *Nevermannia* were separated into different clusters with high bootstrap probabilities. The *feuerborni* and *vernum* species-

groups were clustered, but the *ruficorne* species-group was placed in a distinct cluster. One of the objectives of this study was to determine the relationship of the ungrouped species, *S. konoii*, to the known species-groups. But *S. konoii* was not related to any species-groups of *Nevermannia* in the tree.

Table 2 summarizes the average *d* values among species-groups of *Nevermannia* and other subgenera. The average *d* values between the *ruficorne* species-group and the other species-groups of *Nevermannia* were higher than those between the species-groups (without the *ruficorne* species-group of *Nevermannia*) and other subgenera, and were approximately the same level as those between the *ruficorne* species-group and other subgenera.

DISCUSSION

Our phylogenetic analyses of subgenus *Nevermannia* based on the mitochondrial 16S rRNA gene sequences showed that the *feuerborni* and *vernum* species-groups were closely related, but the *ruficorne* species-group and the ungrouped species, *S. konoii*, were not. The *ruficorne* species-group was largely divided from other *Nevermannia* species and other subgenus species. The *ruficorne* species-

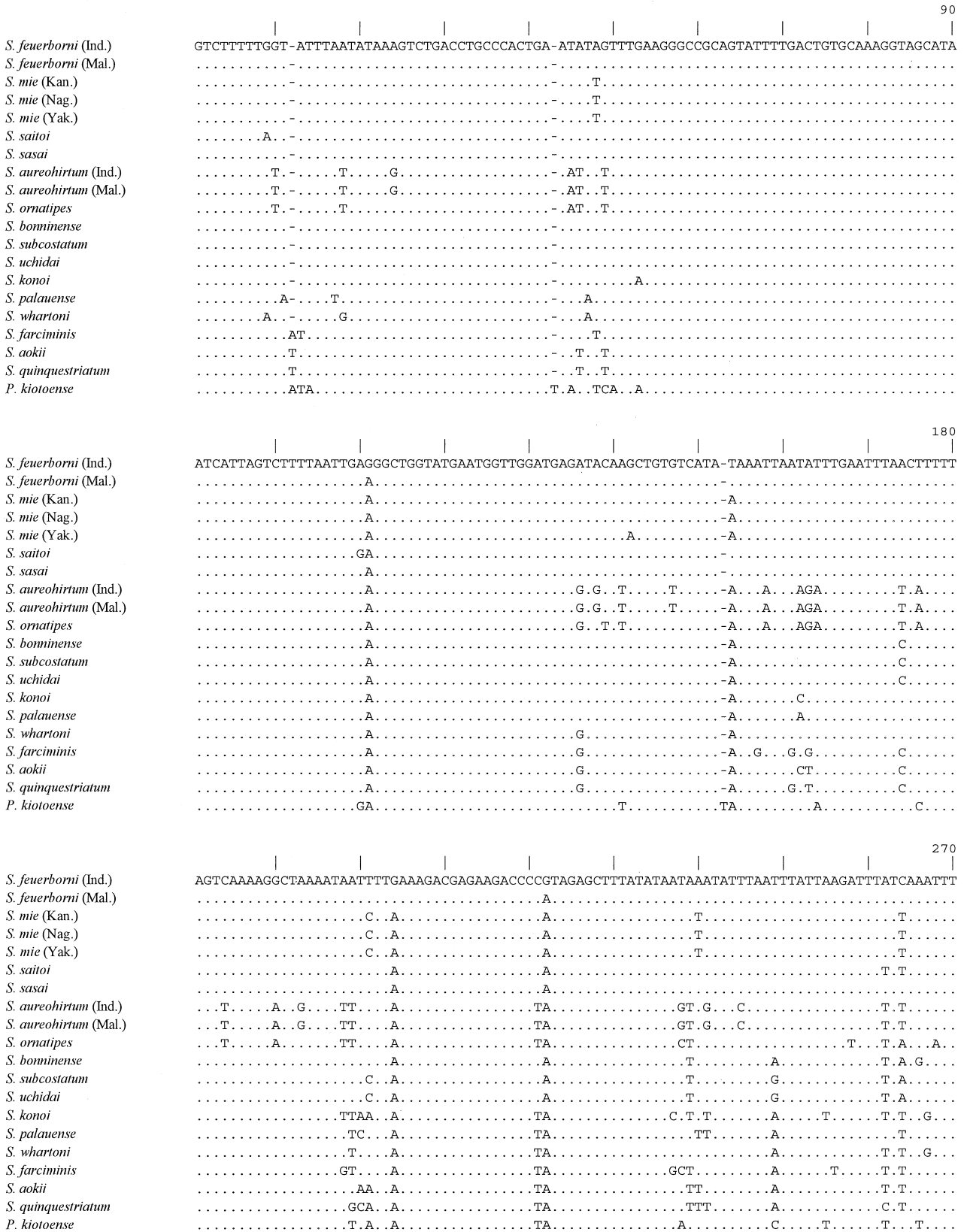


Figure 1-1 DNA alignment of mitochondrial 16S rRNA region for the 16 species. A period indicates the site identical to *S. feuerborni* (Indonesia); a dash indicates a gap site.

	AGTTATTTTATTATATTTTGTGGGGTGACAATAAAATTTATAAAACTTTTATTTTATTTAACATTAATTTATGATTAATGATCCAGT
<i>S. feuerborni</i> (Ind.)G.....A.....
<i>S. feuerborni</i> (Mal.)G.....G.....
<i>S. mie</i> (Kan.)G.....G.....
<i>S. mie</i> (Nag.)G.....G.....
<i>S. mie</i> (Yak.)G.....G.....
<i>S. saitoi</i>G.....T.....
<i>S. sasai</i>
<i>S. aureohirtum</i> (Ind.)AT.....C.....G.....
<i>S. aureohirtum</i> (Mal.)AT.....C.....G.....
<i>S. ornatipes</i>AT.....C.....T.....GC.....
<i>S. bomminense</i>	AA.....AA.....G.....
<i>S. subcostatum</i>	A.....AA.....G.....GC.....
<i>S. uchidai</i>	A.....AA.....
<i>S. konoj</i>	A.....G.....A.....
<i>S. palauense</i>	A..T..G.....T.....T..T.....
<i>S. whartoni</i>	A..T..G.....A..T.....G.....T.....
<i>S. farciminis</i>	A..G..AAGC.....AT.....G.....
<i>S. aokii</i>	A.....A..C.....T.....A..T.....
<i>S. quinquestriatum</i>	A.....C.....C.....A.....T.....
<i>P. kiotoense</i>	A.....A..T.....TA..T.....C.....

	TTTATTGATTATAAATTTAAGTTACCTTGGGGATAACAGCGTAATTTTTTTTGAGAGTTCTTATCGACAAAAAAGATTGCGACCTCGATG
<i>S. feuerborni</i> (Ind.)
<i>S. feuerborni</i> (Mal.)
<i>S. mie</i> (Kan.)
<i>S. mie</i> (Nag.)
<i>S. mie</i> (Yak.)
<i>S. saitoi</i>
<i>S. sasai</i>
<i>S. aureohirtum</i> (Ind.)A.....
<i>S. aureohirtum</i> (Mal.)A.....T.....
<i>S. ornatipes</i>A.....
<i>S. bomminense</i>
<i>S. subcostatum</i>
<i>S. uchidai</i>
<i>S. konoj</i>A.....
<i>S. palauense</i>A.....
<i>S. whartoni</i>A.....
<i>S. farciminis</i>A.....C.....T.....
<i>S. aokii</i>A.....
<i>S. quinquestriatum</i>A.....
<i>P. kiotoense</i>A.....A.....

	TTGGATTAAGAGTAATTTTGGGGTGTAGAAGTTCAAAGTTTAAGTCTGTTTCGACTTTTAAATTCCTTACAT
<i>S. feuerborni</i> (Ind.)
<i>S. feuerborni</i> (Mal.)
<i>S. mie</i> (Kan.)A.....
<i>S. mie</i> (Nag.)A.....G.....
<i>S. mie</i> (Yak.)A.....G.....
<i>S. saitoi</i>
<i>S. sasai</i>
<i>S. aureohirtum</i> (Ind.)
<i>S. aureohirtum</i> (Mal.)
<i>S. ornatipes</i>
<i>S. bomminense</i>
<i>S. subcostatum</i>
<i>S. uchidai</i>
<i>S. konoj</i>
<i>S. palauense</i>
<i>S. whartoni</i>
<i>S. farciminis</i>
<i>S. aokii</i>
<i>S. quinquestriatum</i>
<i>P. kiotoense</i>T.....

Figure 1-2

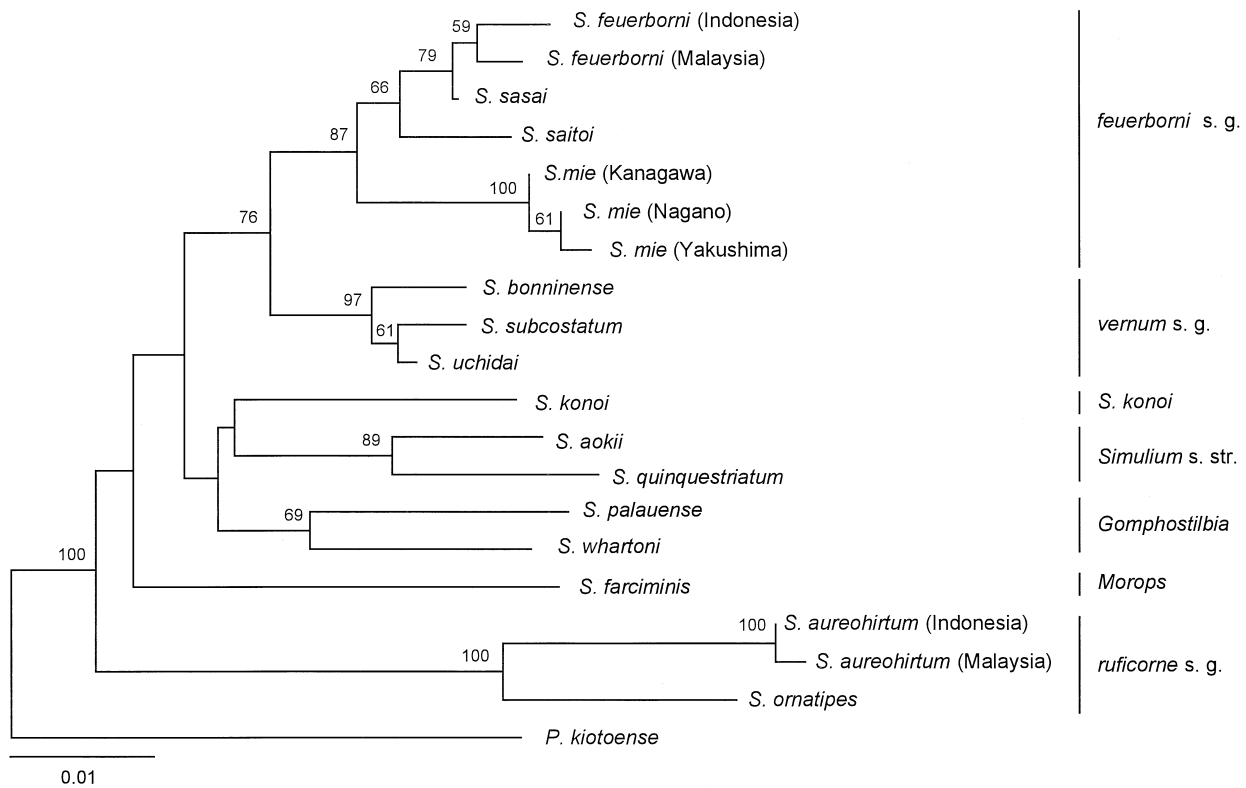


Figure 2 The neighbor-joining tree of the mitochondrial 16S rRNA region for the 16 species. Numbers on the nodes indicate probabilities based on 1,000 bootstrap replicates. The probability of more than 50% is shown. Branch lengths are proportional to genetic distance (scale bar).

Table 2 The average number of nucleotide substitutions per site among the species-groups of *Nevermannia* and other subgenera

	2	3	4	5	6	7
1 <i>feuerborni</i> s.g.	0.076	0.029	0.045	0.043	0.059	0.050
2 <i>ruficorne</i> s.g.		0.070	0.072	0.080	0.075	0.071
3 <i>vernum</i> s.g.			0.040	0.044	0.045	0.043
4 <i>S. konoï</i>				0.042	0.054	0.041
5 <i>Gomphostilbia</i>					0.054	0.045
6 <i>Morops</i>						0.056
7 <i>Simulium</i> s.str.						

group and the ungrouped species, *S. konoï*, have morphological characters which depart from the other species of *Nevermannia*. One of such characters in the *ruficorne* species-group is the male genitalia with ventral plate with a distinct median keel (Crosskey, 1969). On the other hand, *S. konoï* has a female adult cibarium with a distinctive armature consisting of several oblique rows of denticles on each side (Bentinck, 1955); its larval antennae have a few unpigmented annulations on the second segment (unpublished data). These molecular and morphological data taken into consideration, revision of the definition of the subgenus

Nevermannia may be needed.

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