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SEROEPIDEMIOLOGY OF DENGUE AND ASSESSMENT OF PUBLIC AWARENESS IN THE DOMINICAN REPUBLIC

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Abstract: Dengue fever (DF) is a major public health concern in the Dominican Republic. In recent years, several epidemics of DF have been reported to the Pan American Health Office (PAHO), but the extent of the epidemics has not been clearly understood yet. Therefore, we conducted a nationwide seroepidemiology of dengue (DEN) infection. At the same time, we conducted an interview survey to assess public awareness regarding the disease. The serum samples were collected at seven main cities in the Dominican Republic and screened for DEN antibody with a commercial ELISA kit. A total of 2007 serum specimens were examined. The prevalence of DEN antibody in the seven cities varied between 43.1 and 89.7%. Neutralization (N) test carried out on the ELISA-positive serum from Samana, one of the high antibody-prevalent cities, revealed that all the sera showed positive to at least two DEN serotypes. Geometric mean N titers against DEN-1, 2, 3 and 4 were 40.5, 463.7, 59.9 and 454.4 respectively. No difference in antibody prevalence was observed between males and females. It appeared that a high level of awareness regarding DF did little affect DEN prevalence. Strong, concrete public health strategies that motivate the local community to combat DF are required.

INTRODUCTION

The Dominican Republic is located at the Greater Antilles in the Caribbean Sea, on the east border of Haiti. The land area is 48,442km^2 and the population about 8.7million. In recent years, service industries have exceeded agriculture as the main industry due to the growth of tourism and the development of free trade zones.

Control and prevention of infectious diseases are the major public health problems in the Dominican Republic. Dengue fever/dengue hemorrhagic fever (DF/DHF) is a public health concern that, in severe cases, causes hemorrhagic diatheses and death due to hemorrhagic shock [1,2]. Four serotypes of dengue virus (DEN-1 to DEN-4) form a single antigenic complex. The main vector is Aedes aegypti, which is commonly found in tropical and subtropical areas including the Dominican Republic [3]. The first laboratory-confirmed dengue epidemic (DEN-3) in the Dominican Republic was in 1963 [4]. Since then, epidemics caused by multiple serotypes of DEN virus have been reported [5]. In 1988, four cases of DHF were reported for the first time in the country [4]. During a worldwide pandemic of DF/DHF in 1998, a total of 2,335 cases including 176 cases of confirmed DHF with 10 deaths were reported in the Dominican Republic [4]. In 1997, the Ministry of Public Health initiated the DEN Control Campaign through surveillance and eradication of *Aedes aegypti* and health education to the community. Although the campaign seemed effective in combating mosquitoes, the DEN epidemic persisted. In 2000, 3, 462 cases of DF, including 58 DHF with 6 deaths were reported. During this epidemic, all four serotypes of DEN virus were isolated [4].

In view of background, we conducted serological and interview surveys of DEN infection at the seven main cities in the Dominican Republic in order to determine the extent of DEN infection in the country and to assess public awareness regarding DF/DHF. In this paper, we report the results of the survey on DEN infection among a healthy population living in the seven main cities of the Dominican Republic. To our knowledge, this was the first nationwide survey of DEN infection in the Dominican Republic.

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MATERIALS AND METHODS

Interview survey and collection of serum specimens

During the period from September 2000 to July 2001, we conducted a survey of DEN together with a health checkup for local residents of seven main cities (Santo Domingo, S.P. de Macoris, La Romana, Bani, Samana, La Vega, and Santiago) in the Dominican Republic with the collaboration of the staff of local health centers, medical students and an NPO group (Fig. 1). Before initiating the health checkup, we explained the DEN survey project to the participants and others who wished to volunteer for the project, then proceeded to the interview survey and blood collection. The teaching staff of the epidemiology section at CEMADOJA did the interview in Spanish. The questionnaire consisted of demographic information, the location of water containers (including the presence or absence of lids) in and around their residence. The knowledge on DEN was evaluated by the questions "Have you ever heard of DF?" "Do you have any idea what transmits DF?" and "What would you do to avoid DF?"

Since no ethical code had been established at this point, the teaching staff explained the survey to the participants verbally, and only those who consented were subjected to the interview and blood sampling.

The serum specimens were stored at either −20 or −80C until use.

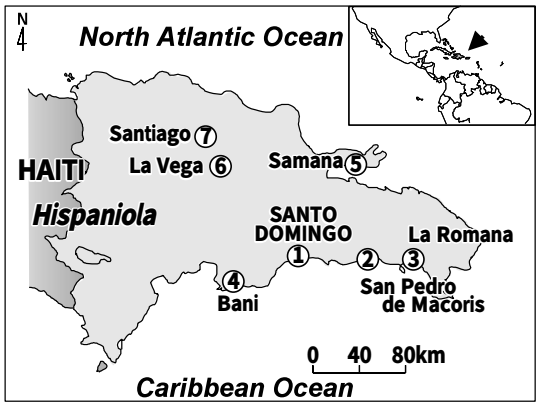

Figure 1. Map of the Dominican Republic.
The survey sites are indicated by numbers.

Detection of DEN antibody

Commercial ELISA kit (Microwell ELISA Dengue IgG, Diagnostic Automation Inc. California, U.S.A.) was used according to the manufacture's instructions. An antibody (Ab) index (titer) of 0.9 or over was considered positive.

Fifty-percent focus-reduction neutralization (N) test, using Vero cells, was carried out [6,7]. An N titer of 20 or over was considered positive.

Statistics

The χ^2 test was used to compare the Ab prevalence between different locations. It was also used to analyze the difference of Ab titer/prevalence among various settings. The unpaired-student's- t-test was used to compare the Ab titers between subjects with and without DEN-related knowledge. We used the Statistical Package for Biosciences (SPBS, Winesteem Institute of Community Medicine, Toda, Saitama, Japan).

RESULTS

Serum specimens

A total of 2007 serum specimens were collected. The number of serum specimens collected at the seven cities is shown in Table 1. Females predominated in six cities, but accounted for only 42.2% in one city (Bani) (overall female proportion was 72.2%). The age distribution of the volunteers varied from 16 to 97 years with a median age between 34 and 43 years.

Prevalence of DEN Ab

The prevalence of DEN Ab is presented with demographic data from each city in Table 1. The Ab prevalence varied from 43.1 to 89.7%, and southern cities seemed to have a lower prevalence than eastern and northern cities (south vs. east: χ^2=46.906, P<0.001; south vs. north: χ^2= 33.777, P<0.001). There was no statistical difference in Ab prevalence between males and females (P=0.097 by Mantel-Haenszel method).

Samana was one of the most highly prevalent areas (Ab prevalence of 86.0% by ELISA). In order to examine which Ab to DEN serotypes was predominant in this area, 50 Ab-positive sera were randomly selected and N test was performed. All the sera showed positive to at least two DEN serotypes. Geometric mean N titers against DEN-1, 2, 3 and 4 were 40.5, 463.7, 59.9 and 454.4 respectively.

Interview survey

A total of 1,988 volunteers responded to the inquiry. Most of them (1,986 individuals) stated that they had cisterns inside the house. Among these, only 255 (12.8%) stated that they placed a lid on the cistern (Table 2). Commonly found utensils containing water were vases and planters. When asked about DF, a majority of the volunteers (80.6%) answered "yes", meaning that they had some DEN-related knowledge. More people living in St. Domingo, the capital, knew about DEN (94.6%) than those in other cities (Table 3). There was no statistical correlation between DEN

Table 1. Demography and DEN antibody prevalence

	Total	South				East	North	
		St Domongo	S.P.Macoris	La Romana	Bani	Samana	La Vega	Santiago
Population (x103)	4,707	2,694	262	215	223	82	391	840
No.specimens	2,007	492	274	252	258	250	250	231
Age (years)								
Median	39	41.5	34	39	36	40	43	40
Range	16-97	19-97	17-71	18-85	19-80	19-76	16-85	19-78
Gender								
Male (%)	624 (31.1)	131 (26.6)	70 (25.5)	56 (22.2)	149 (57.8)	61 (24.4)	82 (32.8)	75 (32.5)
Female (%)	1,383 (68.9)	361 (73.4)	204 (74.5)	196 (77.8)	109 (42.2)	189 (75.6)	168 (67.2)	156 (67.5)
DEN-Ab positive								
Total (%)	1,369 (68.2)	327 (66.5)	118 (43.1)	226 (89.7)	115 (44.6)	215 (86.0)	169 (67.6)	199 (86.1)
Regional subtotal (%)			786 (61.6)			215 (86.0)		368 (76.5)
Male (%)	394 (63.1)	85 (64.9)	29 (41.4)	48 (85.7)	60 (40.3)	52 (85.2)	54 (65.9)	66 (88.0)
Female (%)	975 (70.5)	242 (67.0)	89 (43.6)	178 (90.8)	55 (50.5)	163 (86.2)	115 (68.5)	133 (85.3)
DEN-Ab negative								
Total (%)	638 (31.8)	165 (33.5)	156 (56.9)	26 (10.3)	143 (55.4)	35 (14.0)	81 (32.4)	32 (13.9)
Male (%)	230 (36.9)	46 (35.1)	41 (58.6)	8 (14.3)	89 (59.7)	9 (14.8)	28 (34.1)	9 (12.0)
Female (%)	408 (29.5)	119 (33.0)	115 (56.4)	18 (9.2)	54 (49.5)	26 (13.8)	53 (31.5)	23 (14.7)
Mean Ab index (SD)	1.5 (1.1)	1.5 (1.0)	0.8 (0.6)	2.1 (0.8)	0.9 (0.9)	1.6 (0.7)	1.4 (0.8)	2.4 (1.5)

Table 2. Results of interview survey on dengue

Total	1,988
Cistern inside house?	
“yes”	1,986
with lid	255
without lid	1,731
Any utensil containing water?	
vase and planter	1,090
old tire	238
empty bottle	92
other containers	315
no answer	253
Do you know DF?	
“yes”	1,603
“no”	385

Table 3. Results of interview survey and DEN antibody prevalence

Do you know dengue?	Total	South				East	North	
		St Domingo	sp Macoris	La Romana	Bani	Samana	La Vega	Sontiago
No.interviewe	1,988	479	273	250	257	249	249	231
Answered “yes”	1,602	453	221	194	207	177	193	157
(%)	(80.6)	(94.6)	(81.0)	(77.6)	(80.5)	(71.1)	(77.5)	(68.0)
Serodiangosis negative	52.5	153	122	18	119	25	66	22
(%)	(32.8)	(33.8)	(55.2)	(9.3)	(57.5)	(14.1)	(34.2)	(14.0)
Serodiagnosis positive	1,077	300	99	176	88	152	127	135
(%)	(67.2)	(66.2)	(44.8)	(90.7)	(42.5)	(85.9)	(65.8)	(86.0)
Answered “no”	386	26	52	56	50	72	56	74
(%)	(19.4)	(5.4)	(19.0)	(22.4)	(19.5)	(28.9)	(22.5)	(32.0)
Serodianosis negative	109	8	35	8	23	10	15	10
(%)	(28.2)	(30.8)	(67.3)	(14.3)	(46.0)	(13.9)	(26.8)	(13.5)
Serodiagnosis positive	277	18	17	48	27	62	41	64
(%)	(71.8)	(69.2)	(32.7)	(85.7)	(54.0)	(86.1)	(73.2)	(86.5)
P value (χ^2-test)	0.086	0.752	0.112	0.280	0.143	0.961	0.297	0.918

P value: The difference of Ab prevalence between “yes”and “no” was calculated.

-related knowledge and Ab prevalence. The difference in mean Ab index between those who answered “yes” and “no” was also not statistically different (1.5 vs. 1.6; P: 0.094 by unpaired- student’s-t-test).

DISCUSSION

The Dominican Republic has experienced several DEN epidemics in recent years. However, few virological or seroepidemiological analyses have been carried out. Therefore, we conducted a nationwide serological survey of DEN infection, together with virus isolation from DEN suspected cases. Since our survey was carried out during office hours on weekdays, more female (mainly homemakers) could participate in it. The Ab-positive rate varied from 43.1 to 89.7% with an overall rate of 68.2%. Bani and S.P. de Macoris appeared to be relatively low DEN endemic areas. Bani is well known for its mango plantations and the contributions of these make the city’s financial stability. The city of S.P. de Macoris, meanwhile, is the eastern center of the country and has a medical school and international baseball stadium where many foreign athletes gather. The health-administration systems of these cities should be evaluated in more detail.

The N titers to DEN-2 and DEN-4 appeared higher than DEN-1 and DEN-3, although it is not clear how many of these Abs represent prior DEN infection or cross-reactive response. It should be noted that during this period, DEN-2 was isolated from patients with unknown fever. These results were consistent with the report that multiple serotypes of DEN virus had circulated in the Dominican Republic for the past few years [4].

The government conducted community programs to promote DEN-related knowledge through televised public service announcements, posters, health education, etc. Our interview survey also showed that a considerable proportion of the volunteers (68.0-94.6%) had some knowledge of DF. Despite good public awareness, DF was still endemic in these areas. Our data showed that there was no direct association between the knowledge of DF and the prevalence of DEN antibody. In 2003, another DF/DHF epidemic occurred and 5,170 cases with 107 deaths were registered [8]. In nearby Puerto Rico, where DF is also a major health problem, a variety of programs have been conducted. These have resulted in an elevation of awareness, some behavioral changes, and a limited change in larval indices [9]. These findings indicate that more specific information about the behaviors that need to be changed should be provided along with political and financial support.

In the present study, an interview survey among adult and young adult populations was conducted to determine the level of the public awareness regarding DF prevention together with a serological survey. A serological survey on children, who are more susceptible to DEN but who were not included in the present study, will be conducted in a subsequent study. Recently, Ab to West Nile virus (WNV) was detected from domestic birds in the Dominican Republic [10]. Although no human case has been reported, all members of the genus Flavivirus, including WNV and DEN, share common antigenic sites [11], and thus care should be taken in interpreting the antibody-positive results of serological tests.

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TAXONOMIC NOTES ON THE *GRISEIFRONS* SPECIES-GROUP OF *SIMULIUM* (*SIMULIUM*) (DIPTERA: SIMULIIDAE) IN NORTHERN THAILAND

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Abstract: Two known and two new species of the *griseifrons* species-group of the subgenus *Simulium* (*Simulium*) in Northern Thailand are reported. The male, pupa and mature larva of *S.* (*S.*) *rudnicki* Takaoka and Davies, and the pupa and mature larva of *S.* (*S.*) *suchariti* Takaoka and Choochote, are described for the first time. Furthermore, two new simuliid species, *S.* (*S.*) *mediocoloratum* sp. nov. and *S.* (*S.*) *crocinum* sp. nov., both of which are very similar to *S.* (*S.*) *rudnicki*, are also described.
Key words: black fly, Simuliidae, *Simulium*, Thailand, new species, *griseifrons* species-group

In Thailand, the *griseifrons* species-group of the subgenus *Simulium* (*Simulium*) is represented by eight species [1]. During recent surveys on the immature stages of black flies in Northern Thailand, we collected *S. rudnicki* Takaoka and Davies [2], *S. suchariti* Takaoka and Choochote [3], and two new species, all of which are assigned to this species-group. The male, pupa and mature larva of *S. rudnicki*, and the pupa and mature larva of *S. suchariti* (only females of both species were so far known) are described here for the first time. The two new species are also described based on adult, pupal and larval specimens.

The terms for morphological features used here follow those of Takaoka [4]. Holotype and most paratype specimens of the new species will be deposited at the Department of Infectious Disease Control, Oita University.

Simulium (*Simulium*) *rudnicki* Takaoka and Davies, 1995

Simulium (*Simulium*) *rudnicki* Takaoka and Davies, 1995: 155 (female only).

DESCRIPTION. **Male.** Body length 3.8−4.0 mm. ***Head.*** Slightly wider than thorax. Upper eye consisting of large facets in 24 horizontal and 22 or 23 vertical rows. Clypeus black, white pruinose, iridescent when illuminated, sparsely covered with dark brown hairs. Antenna composed of 2+9 segments, pale yellow on scape, pedicel, and base of 1st flagellar segment, dark yellow on the rest except 3 or 4 apical flagellar segments dark brown, (in 1 male, pale yellow on scape, pedicel, and base of 1st flagellar segment, light to medium brown on the rest of 1st flagellar segment, 2nd and 3rd flagellar segments, and dark brown on 4th to 9th flagellar segments); 1st flagellar segment elongate, about twice as long as 2nd one. Maxillary palp composed of 5 segments, proportional lengths of 3rd, 4th, and 5th segments 1.0:1.2: 2.8; 3rd segment (Fig. 1A) of normal size, with a small globular sensory vesicle having a very small opening. ***Thorax.*** Scutum black, with silvery iridescent pattern differing with angles of light: when illuminated anteriorly and viewed dorsally scutum shows subanteriorly a transverse pair of narrow silvery iridescent spots widely spaced in middle (distance between spots about one-third that of scutum); when illuminated laterally or posterolaterally and viewed dorsolaterally, the subanterior paired spots fade and are replaced by an anterior pair of narrow iridescent spots on shoulders which extend posteriorly along lateral margins in a wide band and connect to a large transverse posterior spot on prescutellar area; when illuminated anterolaterally and viewed dorsolaterally, scutum on each shoulder has a large anterior iridescent spot including anterior and subanterior narrow spots mentioned above, which is also contiguous to a broad lateral iridescent band along lateral border; scutum uniformly covered with yellow recumbent short hairs interspersed with dark long upright hairs on prescutellar area and dark short and medium-long hairs near anterior margin. Scutellum black except posterior margin brownish, with several dark long upright hairs as well as yellow short hairs. Postnotum black, silvery iridescent when illuminated, and bare. Pleural membrane bare. Katepisternum longer than deep, and bare. ***Legs.*** Foreleg: coxa pale yellow; trochanter

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and femur medium or dark yellow with narrow apical portion light brown on outer surface; tibia brownish black with basal 3/4 narrowly white on outer surface (white sheeny when illuminated); tarsus black, with moderate dorsal hair crest; basitarsus somewhat dilated, about 6.4 times as long as its greatest width. Midleg: coxa dark brown or blackish brown; trochanter pale yellow with apical 1/2 or a little more dark yellow to light brown; femur entirely yellow; tibia entirely yellow, with a broad white sheen on posterior surface when illuminated; tarsus brownish black except basal 1/2 or 2/3 yellow, though its border not well defined. Hind leg: coxa dark brown or brownish black; trochanter yellow; femur yellow except apical cap dark brown; tibia brownish black with base narrowly whitish yellow; tarsus brownish black except basal 1/2 of basitarsus and basal 1/2 of 2nd tarsal segment whitish yellow; basitarsus (Fig. 1B) somewhat widened from base toward apical 3/4, then somewhat narrowed toward apex, 4.5 times as long as its greatest width, and 0.8 times as wide as the greatest width of hind tibia, which is as wide as that of femur; calcipala of medium size, nearly as long as wide; pedisulcus distinct. ***Wing.*** Length 2.9–3.0 mm; costa with spinules and hairs; subcosta bare; basal section of vein R bare; R_1 with spinules and hairs; R_2 with hairs only; hair tuft at base of stem vein dark brown; basal cell absent. ***Abdomen.*** Basal scale blackish with a fringe of long dark hairs. Abdomen brownish black with dark hairs; segments 2, 5–7, each with a pair of silvery iridescent spots dorsolaterally, those on segment 2 broadly connected in middle. ***Genitalia*** (Fig. 1C–L). Coxite in ventral view nearly quadrate. Style elongate, spatulate ventrodorsally, 2.2 times as long as coxite, gradually narrowed from base to apical 1/3, then widened to rounded apex (widths at base and near apex subequal to each other and about 1.4 times as wide as the narrowest width at middle), with a slender subterminal spine; style with a prominent basal protuberance pointed dorsally and furnished at and near

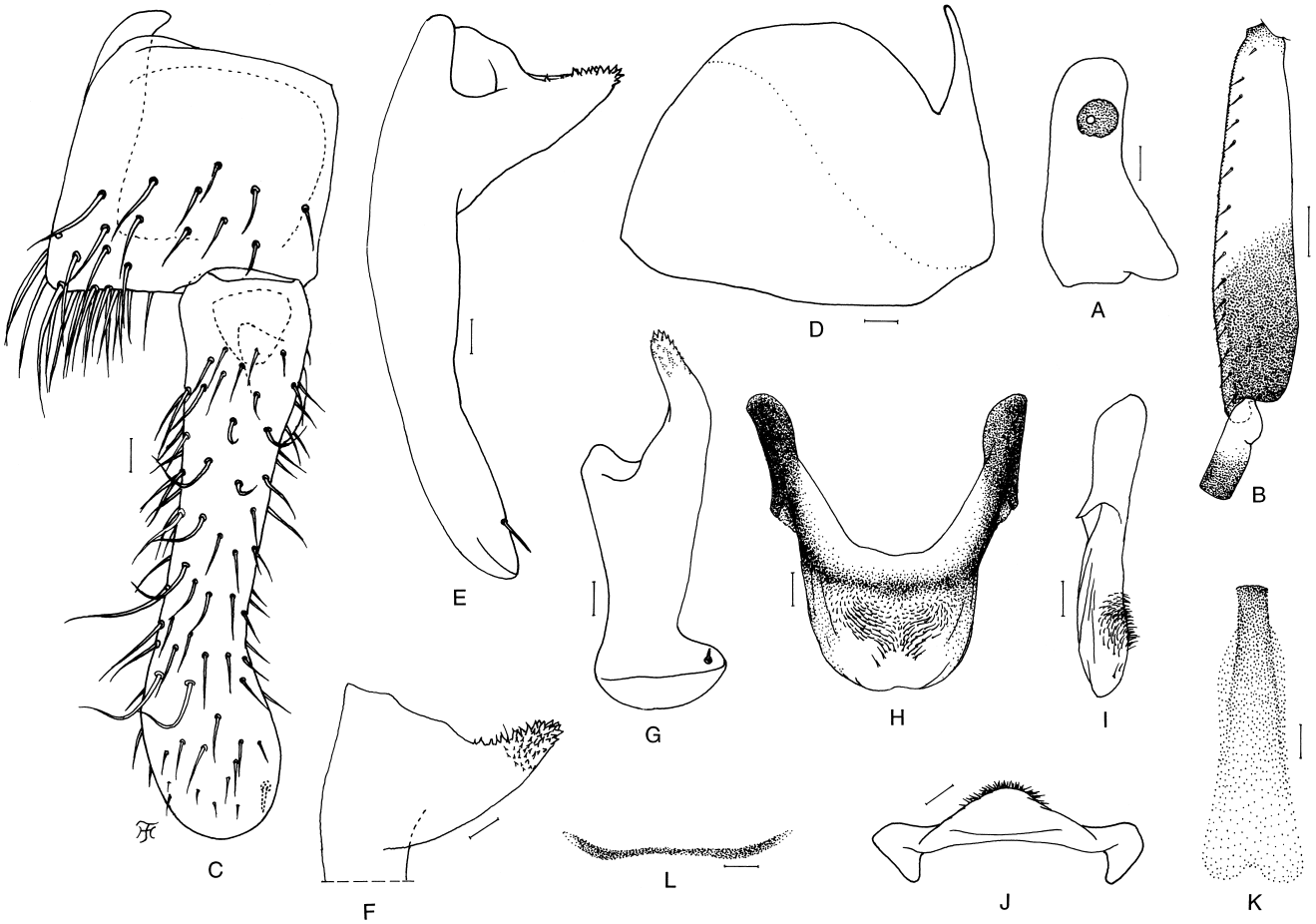

Fig. 1. Adult male of *Simulium* (*Simulium*) *rudnicki* Takaoka and Davies. A, 3rd segment of maxillary palp (right side, frontal view); B, hind basitarsus and 2nd tarsal segment (left side, outer view); C, coxite and style (right side, ventral view); D, coxite (left side, lateral view); E, style (left side, outer view); F, basal portion of style (right side, inner view); G, style (left side, end view); H–J, ventral plates (H, ventral view; I, lateral view; J, end view); K, median sclerite; L, dorsal plate. Scales. 0.02 mm for A, C–L; 0.1 mm for B.

apex with many conical spines on anterior and inside surfaces. Ventral plate in ventral view transverse, somewhat wider than its length, rounded posterolaterally, with posterior margin untoothed and somewhat concave medially, and covered with fine setae centrally on ventral surface; basal arms long and stout, curved outwardly and forwardly. Parameres wide basally, each with numerous parameral hooks. Median sclerite narrow, moderately widened toward apex. Aedeagal membrane densely covered with needle-like setae and with well-sclerotized narrow dorsal plate. Segment 10 without any distinct hair. Cercus rounded with 9−13 distinct hairs.

Pupa. Body length (excluding gill filaments) ca. 4.0 mm. ***Head.*** Integument yellowish brown, bare; face with a pair of long simple trichomes; frons with 2 pairs of long simple and bifid trichomes; antennal sheath bare. ***Thorax.*** Integument yellowish brown, bare on anterior 1/2 including a small raised area at base of gill, moderately covered with small cone-shaped tubercles dorsally on posterior 1/2; thorax anteriorly with 3 dorsal and 2 lateral pairs of long branched trichomes (split into 2−4), posteriorly with 1 lateral pair of long bifid trichomes, and ventrolaterally with 3 pairs of long simple or bifid trichomes. Gill (Fig. 2A) with 6 filaments arranged in sessile pairs; all filaments dark brown, subequal in length (1.5−2.2 mm) and thickness except inner filament of lower pair somewhat thinner than others; all filaments gradually tapered toward apical tip, with annular ridges and furrows throughout their length (though not marked near base), and densely covered with minute tubercles. ***Abdomen.*** Dorsally, segment 1 yellow with dark grey narrow portion along posterior margin, with 1 simple slender hair-like seta on each side; segment 2 dark grey on anterior 2/5, transparent on posterior 3/5, with 1 long simple hair-like seta (Fig. 2B), 4 short simple hooked spines of equal size (Fig. 2C) (much smaller than those on segments 3 and 4) and 1 short spinous seta (Fig. 2D) on each side; segments 3 and 4 each with 4 hooked spines along posterior margin on each side; segment 5 bare; segments 6, 7 and 9 each with a transverse row of comb-like groups of minute spines but lacking spine-combs on each side; segment 8 with a transverse row of spine-combs as well as a transverse row of comb-like groups of minute spines on each side; segment 9 lacking terminal hooks. Ventrally, segment 4 with 1 bifid hook, 1 slender medium-long seta and 2 short setae on each side; segment 5 with a pair of bifid hooks submedially on each side; segments 6 and 7 each with a pair of bifid inner and simple outer hooks somewhat separated from each other, on each side. Grapnel-like hooklets absent. ***Cocoon*** (Fig. 2E). Wall-pocket-shaped, with a large anterolateral window on each side; thin, film-like, individual threads not visible; anterior margin not well defined, often broken; about 4.5 mm long by 2.5 mm wide.

Mature larva. Body length 8.5−11.0 mm. Body color dark grey to greyish black. Abdomen gradually widened toward posterior tip, with the widest portion on segment 8, and abruptly narrowed to posterior tip. Cephalic apotome

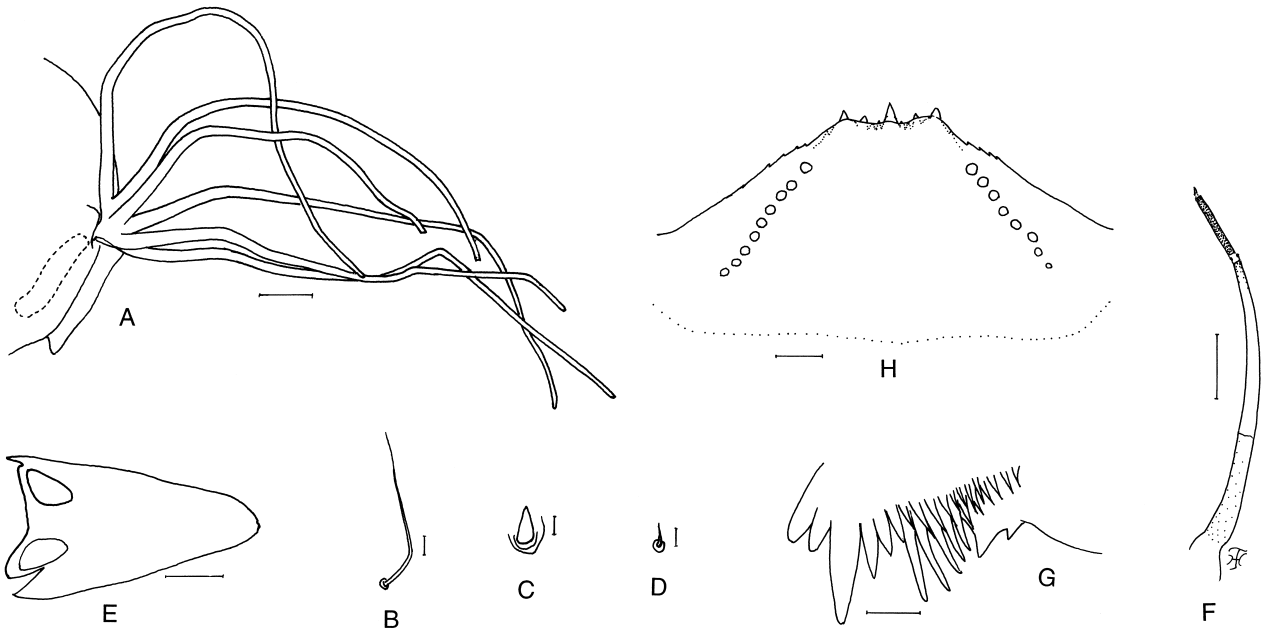

Fig. 2. Pupa and mature larva of *Simulium* (*Simulium*) *rudnicki* Takaoka and Davies. A, pupal gill filaments; B−D, hair-like seta, hooked seta and minute seta on the dorsal surface of the 2nd abdominal segment of pupa, respectively; E, cocoon (dorsal view); F, larval antenna (left side, dorsal view); G, larval mandible; H, larval hypostomium. Scales. 0.01 mm for B−D; 0.02 mm for G and H; 0.1 mm for F; 0.2 mm for A; 1.0 mm for E.

almost entirely medium to dark brown except narrow area along each lateral margin of anterior 1/3 pale; head spots distinctively positive except posterolateral ones usually merged into ground color (Plate 1A); lateral and ventral surfaces medium to dark brown except eye-spot region pale; spots around eye-spot region, and those on both sides of postgenal cleft faintly negative or merged into ground color (Plate 1E). Antenna (Fig. 2F) composed of 3 segments and apical sensillum, much longer than stem of labral fan; length ratio of segments (from base to tip) 1.0:1.1−1.2:0.5; all segments pale white to yellow except 3rd segment entirely brownish black, and apical small portion of 2nd segment somewhat darkened. Labral fan with 60−64 main rays. Mandible (Fig. 2G) with usual mandibular serration of 1 medium-sized tooth and 1 small one; main tooth at an obtuse angle apically to mandible; supernumerary serrations absent; comb-teeth decreased in length from 1st to 3rd. Hypostomium (Fig. 2H) with 9 apical teeth, of which median tooth slightly longer than each corner tooth; 3 intermediate teeth on each side shorter than corner teeth; lateral margins moderately serrate apically; 7−9 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Plate 1E) narrow, subtriangular, 1.7−1.9 times as long as postgenal bridge; lateral margins on posterior 1/2 nearly parallel-sided or slightly converged at base, and pointed apically; subesophagial ganglion well pigmented, wine-glass shaped. Thoracic cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short, colorless setae on each side of anal sclerite. Rectal scales present. Rectal organ of 3 lobes, each with 14−19 finger-like secondary lobules. Anal sclerite X-shaped, with broadened anterior arms about 0.7 times as long as posterior ones; 3−5 sensilla on the basal juncture area, and 6−12 sensilla just posterior to posterior arms. Ventral papillae absent. Posterior circlet with 144−160 rows of hooklets, with up to 20−22 hooklets per row.

SPECIMENS EXAMINED. 5 females, 5 males (all reared from pupae), 10 pupae, 5 pupal exuviae and 50 mature larvae, collected from a stream, Mae Klang Waterfall, Doi Inthanon National Park, Chiang Mai, Thailand, 31. XII. 2003, by W. Choochote.

ECOLOGICAL NOTES. The pupae and larvae of *S. rudnicki* were found in great numbers on the surface of the rocky stream-bed of a stream 5.0 m wide, with moderate to rapid flows, exposed to the sun. Water temperature was 19.5 ℃. Altitude was ca. 400 m. No other species was collected together with *S. rudnicki*.

DISTRIBUTION. Peninsular Malaysia and Thailand.

REMARKS. The male of *S. rudnicki* is very similar to *S. yongi* Takaoka and Davies, originally described from Malaysia [5] in most features including the genitalia, but is readily distinguished from the latter by the entirely yellow femur and tibia of the midleg, which are almost brownish black in *S. yongi* [5].

The pupa of *S. rudnicki* is also very similar to that of *S. yongi* [5] but differs in that it has the inner filament of the ventral pair not narrowed basally, and the small raised area of the thoracic integument near the base of the gill filaments without tubercles (Fig. 2A), and the cocoon with a large anterolateral window on each side (Fig. 2B). The larva of *S. rudnicki* has a dark cephalic apotome (Plate 1A) while that of *S. yongi* has a pale one [5].

***Simulium* (*Simulium*) *mediocoloratum* sp. nov.**

DESCRIPTION. **Female.** Body length 3.0-3.4 mm. ***Head.*** Narrower than width of thorax. Frons brownish black, shiny, not pruinose, with several dark stout hairs along lateral margins; frontal ratio 1.2−1.3:1.0:1.2; frons-head ratio1.0:4.0−4.3. Fronto-ocular area (Fig. 3A) well developed, triangular. Clypeus brownish black, shiny, with silvery iridescent pruinosity, moderately covered with dark stout hairs except medial portion of upper part bare. Labrum 0.62−0.67 times as long as clypeus. Antenna composed of 2+9 segments, brownish black except scape, pedicel, and base of 1st flagellar segment yellow when viewed posteriorly, or brownish black except scape, pedicel, and a few basal flagellar segments yellow to dark yellow when viewed anteriorly; each flagellar segment with a distinct pit apically on each lateral surface gradually becoming smaller in size toward apical tip except those on segment 9 located medially; each pit provided with numerous minute sensilla; each flagellar segment also with a small pit apically on dorsal surface except that on segment 9 located medially. Maxillary palp brownish black, with 5 segments, proportional lengths of 3rd, 4th and 5th segments 1.0:1.2−1.3:2.8−2.9; 3rd segment (Fig. 3B) not enlarged; sensory vesicle ellipsoidal, with rugged surface, 0.36−0.39 times length of 3rd segment, with medium to large round opening apically. Maxillary lacinia with 13 inner and 13 or 14 outer teeth. Mandible with 28−31 inner and 12−14 outer teeth. Cibarium (Fig. 3C) with a cluster of ca. 90 conical processes medially. ***Thorax.*** Scutum brownish black, shiny, whitish-grey pruinose (there appear to be faint scutal patterns formed by pruinose and non-pruinose portions differing by the angles of light: e.g. when illuminated dorsally and viewed laterally, 1 medial and 2 submedial narrow longitudinal whitish-grey pruinose vittae and 2 wide longitudinal whitish-grey pruinose bands along lateral margins connected to large whitish-grey pruinose portions on

shoulders), moderately covered with yellow recumbent short hairs as well as dark brown ones interspersed with long upright dark hairs on prescutellar area. Scutellum brownish black, with long dark hairs as well as yellow short hairs. Postnotum brownish black, shiny, whitish-grey pruinose, and bare. Pleural membrane bare. Katepisternum longer than deep, and bare. ***Legs.*** Foreleg (Fig. 3D): coxa pale yellowish-white; trochanter pale yellowish-white with outer surfaces somewhat darkened; femur yellowish on inside surface but light yellowish-brown to light brown on outer surface; tibia largely white except apical 1/5 dark brown and inner surface of apical 1/2 light to dark brown, and with a large white sheen on outer surface when illuminated; tarsus dark brown; basitarsus dilated, 5.5 times as long as its greatest width. Midleg (Fig. 3E): coxa dark brown; trochanter dark yellow or light yellowish-brown with base yellowish white; femur yellowish white to dark yellow (light brown in some females); tibia yellowish white except apical cap light to medium brown, with a large white sheen on posterior surface when illuminated; tarsus medium to dark brown except basal 3/5 or 3/4 of basitarsus yellowish white. Hind leg (Fig. 3F): coxa medium brown; trochanter yellowish white; femur light to medium brown with base pale yellowish-white and apical cap medium to dark brown; tibia light to dark brown with basal 1/3 and posterior surface of basal 2/3 white (its margin obliquely demarcated when viewed laterally), and with a large white sheen on posterior surface when illuminated; tarsus dark brown except a little more than basal 1/2 of basitarsus yellowish white and basal 1/2 of 2nd tarsal segment yellow; basitarsus

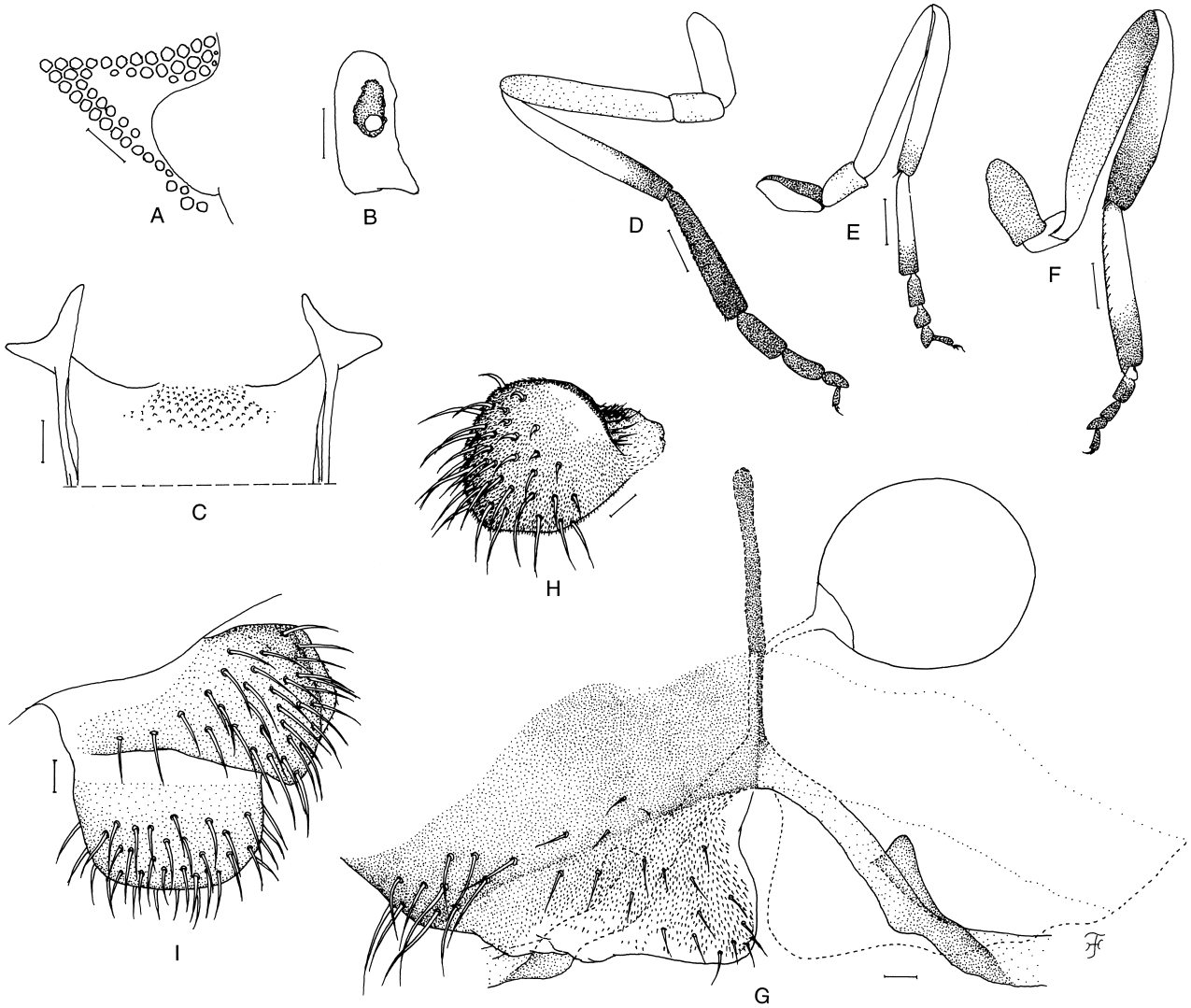

Fig. 3. Adult female of *Simulium* (*Simulium*) *mediocoloratum* sp. nov. A, fronto-ocular area (right side, frontal view); B, 3rd segment of maxillary palp (right side, frontal view); C, cibarium; D–F, foreleg, midleg and hind leg, respectively (all left side); G, 8th sternite, ovipositor valves, genital fork and spermatheca (ventral view); H, paraproct (right side, ventral view); I, paraproct and cercus (right side, lateral view). Scales. 0.02 mm for C and G–I; 0.05 mm for A and B; 0.2 mm for D–F.

nearly parallel-sided, 6.0 times as long as wide, and 0.8 and 0.7 times as wide as the greatest widths of hind tibia and femur, respectively; calcipala short, 0.9 times as long as its basal width and 0.4 times as wide as the greatest width of basitarsus; pedisulcus distinct at basal 1/3 of 2nd tarsal segment. Tarsal claws simple without any tooth. ***Wing.*** Length 2.8−3.0 mm; costa with dark spinules and hairs; subcosta fully haired; basal section of vein R bare; R_1 with spinules and hairs; R_2 with hairs only; hair tuft at base of stem vein dark brown; basal cell absent. ***Abdomen.*** Basal scale light to medium brown, with a fringe of pale hairs; segment 2 yellowish white except posterior 1/3 or 2/5 medium brown dorsally, with a dorsolateral pair of shiny whitish spots broadly connected in middle; remaining segments medium to dark brown dorsally, with dark hairs; tergites 6-8 shiny. ***Genitalia*** (Fig. 3G-I). Ventral surface of abdominal segment 7 without sternal plate. Sternite 8 well sclerotized, bare medially, with 3−9 pale short setae submedially near posterior border, and with 13 or 14 dark long hairs laterally on each side; ovipositor valve triangular in shape, membranous, expanded ventrally, each covered with 18−28 short setae as well as numerous microsetae except narrow portion along inner margin and rather wide portion along posterior margin bare and transparent; inner borders not sclerotized, narrowly separated from each other. Genital fork of inverted-Y form, with well sclerotized stem; arms rather wide, each with strongly sclerotized distal ridge having a distinct projection directed anterodorsally. Paraproct in ventral view rounded posterolaterally, with anteromedial surface strongly sclerotized, having about 20 short spinous setae on it; paraproct slightly produced ventrally along medial margin, covered with numerous stout hairs laterally and ventrally. Cercus rounded posteriorly, about half as long as wide, covered with numerous stout hairs. Spermatheca globular, well sclerotized (except small area of its juncture with duct unsclerotized), with reticulate surface pattern at least near base, with minute internal setae; both accessory ducts subequal in width to main one.

Male. Body length 3.0−3.2 mm. ***Head.*** Slightly wider than thorax. Upper eye consisting of large facets in 22 or 23 horizontal and 22 vertical rows. Clypeus black, white pruinose, iridescent when illuminated, covered with dark-brown long hairs along lateral margins (median large portion nearly bare). Antenna composed of 2+9 segments, dark brown except base of 1st flagellar segment pale; 1st flagellar segment elongate, 1.8 times as long as 2nd one; flagellar segments 1-8 each with 1 or 2 small pits apically on each lateral surface, which are not so developed as compared to those of female. Maxillary palp composed of 5 segments, proportional lengths of 3rd, 4th, and 5th segments 1.0:1.3:3.2; 3rd segment (Fig. 4A) of normal size, with a small, ellipsoidal sensory vesicle, 0.24−0.27 times length of 3rd segment, and with a small opening medially. ***Thorax.*** Scutum brownish black (3 narrow longitudinal black vittae visible in alcoholic specimen), with silvery iridescent pattern differing with angles of light, as in male of *S. rudnicki*; scutum uniformly covered with yellow (or bright brassy in some males) recumbent short hairs interspersed with dark long upright hairs on prescutellar area. Scutellum brownish black, silvery iridescent when illuminated, with several upright dark hairs as well as yellow or bright-brassy short hairs. Postnotum brownish black, silvery iridescent when illuminated, and bare. Pleural membrane bare. Katepisternum longer than deep, and bare. ***Legs.*** Foreleg: coxa whitish yellow; trochanter and femur medium brown with apical cap of femur dark brown; tibia dark brown to brownish black except outer surface of basal 3/4 white, and with a large white sheen on most of outer surface when illuminated; tarsus brownish black; basitarsus somewhat dilated, 6.4 times as long as its greatest width. Midleg: coxa brownish black; trochanter and femur medium brown; tibia light to medium brown except posterior surface of basal 1/2 white (its margin obliquely defined when viewed laterally) and with a white sheen on posterior surface when illuminated; tarsus dark brown to brownish black except basal 3/5 whitish yellow (though its border not well defined). Hind leg: coxa dark brown; trochanter yellow; femur medium brown except extreme base yellow and apical cap dark brown; tibia dark brown except extreme base yellowish white, and with a whitish sheen basally on posterior surface when illuminated; tarsus dark brown except a little less than basal 1/2 of basitarsus and basal 1/3 of 2nd tarsal segment yellowish; basitarsus (Fig. 4B) somewhat enlarged, spindle-shaped, 4.8 times as wide as its greatest width, and 0.8 times as wide as the greatest width of hind tibia, which is nearly as wide as that of hind femur; calcipala small, 0.9 times as long as wide and 0.3 times as wide as the greatest width of hind basitarsus; pedisulcus distinct. ***Wing.*** Length 2.8−2.9 mm; other characters as in female except subcosta completely bare. ***Abdomen.*** As in male of *S. rudnicki*. ***Genitalia*** (Figs. 4C−K). Coxite in ventral view nearly quadrate. Style elongate, spatulate ventrodorsally, 2.2 times as long as coxite, gradually narrowed from base to apical 1/2, then widened to rounded apex (width at base about 1.2 times that near apex and about 1.7 times the narrowest width at middle), with a slender subterminal spine; style with a prominent basal protuberance pointed dorsally and furnished at and near apex with many conical spines on anterior and inside surfaces. Ventral plate in ventral view transverse, somewhat wider than its length, rounded posterolaterally, with posterior margin untoothed and somewhat concave medially, and covered with fine setae cen-

trally on ventral surface; basal arms long and stout, curved outwardly and forwardly. Parameres wide basally, each with numerous parameral hooks. Median sclerite narrow, moderately widened from base to basal 1/3, then nearly parallel-sided, with small median incision on apex. Aedeagal membrane densely covered with needle-like setae, with well-sclerotized narrow dorsal plate. Segment 10 without any distinct hair. Cercus rounded with 10−16 distinct hairs.

Pupa. Body length (excluding gill filaments) 3.0−3.5 mm. ***Head.*** Integument yellowish brown, bare, with 1 facial pair of long, simple or bifid trichomes and 2 frontal pairs of long, bifid or trifid trichomes; antennal sheath bare. ***Thorax.*** Integument yellowish brown, bare on anterior 1/2 except a small raised area at base of gill densely covered with tubercles of irregular shapes, moderately covered with small cone-shaped tubercles on posterior 1/2; thorax anteriorly with 3 dorsal and 2 dorsolateral pairs of long branched trichomes (split into 2−5), posteriorly with 1 lateral pair of branched trichomes (split into 2−4) and ventrolaterally with 3 pairs of long trichomes (simple, bifid or trifid). Gill (Fig. 5A) with 6 filaments arranged in sessile pairs, and with medium-sized transparent protuberance at base; outer filaments of all pairs dark brown, subequal in thickness to one another but somewhat differing in length (1.5−2.0 mm), all gradually tapered from base to apex; inner filaments of all pairs medium brown, somewhat differing in length (1.0−1.5 mm), inner filaments of dorsal and middle pairs subequal in thickness to each other and gradually tapered toward apex after running with the same width or very slightly widened from base to basal 1/5 or 1/4 of their length, inner filament of lower pair apparently increasing its width from base to basal 1/5 or 1/4 of its length, then tapered toward apical tip; inner filament of each pair usually somewhat paler and shorter than its outer counter filament; all filaments with annular ridges and furrows throughout their length except near base, densely covered with minute tubercles. ***Abdomen.*** Dorsally, segment 1 light brown, with 1 simple long hair-like seta on each side; segment 2 light brown on anterior 1/2 or 1/3, with 1 simple long hair-like seta, 1 short spinous seta and 4 short simple hooked spines of equal size on each side; segments 3 and 4 each with 4 hooked spines along posterior margin on each side; segment 8 with a transverse row of

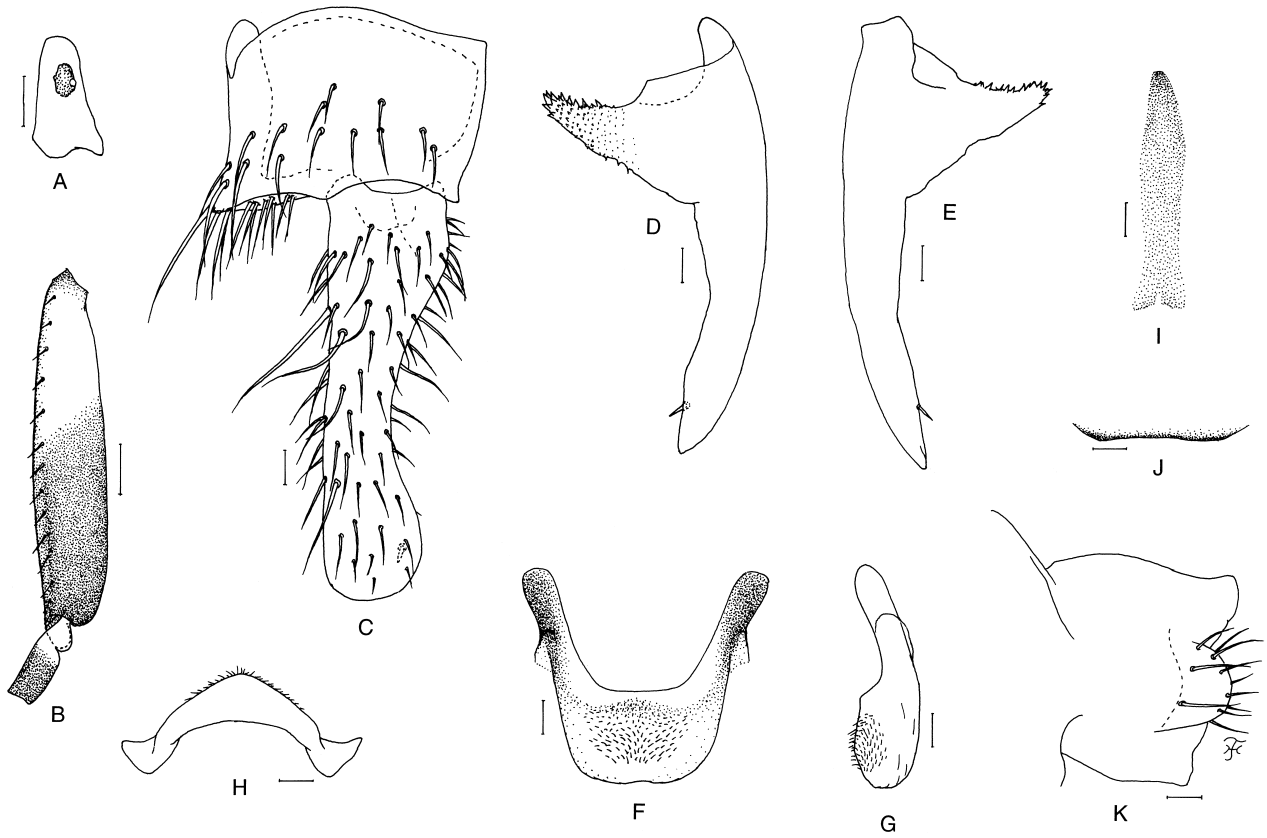

Fig. 4. Adult male of *Simulium* (*Simulium*) *mediocoloratum* sp. nov. A, 3rd segment of maxillary palp (right side, frontal view); B, hind basitarsus and 2nd tarsal segment (left side, outer view); C, coxite and style (right side, ventrolateral view); D and E, styles (both left side; D, inner view; E, outer view); F−H, ventral plates (F, ventral view; G, lateral view; H, end view); I, median sclerite; J, dorsal plate; K, abdominal tip showing cercus (lateral view). Scales. 0.02 mm for C−K; 0.05 mm for A; 0.1 mm for B.

spine-combs on each side; segment 5 bare; segments 6, 7 and 9 each lacking spine-combs, with a transverse row of comb-like groups of minute spines on each side; segment 9 lacking terminal hooks. Ventrally, segment 4 with 1 simple hook and a few short setae on each side; segments 5−7 each with a pair of simple or bifid inner and simple outer hooks on each side. Grapnel-like hooklets absent. ***Cocoon.*** Wall-pocket-shaped, pale yellow, thin, with anterior margin irregular in form, usually appearing to have a broad anterodorsal projection when viewed dorsally (Fig. 5B,C); some cocoons have a fragment of thread of various lengths directed downward from each side of the apex of the anterodorsal projection (Fig. 5D), suggesting that there may be a large anterolateral window on each side; cocoon usually somewhat extending ventrolaterally; 3.0−3.5 mm long by 2.0−2.2 mm wide.

Mature larva. Body length 7.0−8.0 mm. Body color usually dark greyish on thorax and light to dark brown or blackish brown on abdomen (darkened posteriorly). Abdomen gradually widened toward segment 7, then narrowed to posterior tip when viewed laterally; segments 6 and 7 appear to be equal in width to each other when viewed dorsally. Cephalic apotome (Plate 1B) pale yellow on anterior 1/2 or less, light to medium brown on the rest (except narrow portion along each lateral margin usually pale down to level of posterolateral spots), with a small dark area in middle along posterior border; anteromedial longitudinal spot faintly positive or negative, or sometimes merged into ground color, posteromedial spot usually positive or rarely merged into ground color; anterior 1/2 of mediolateral spot on each side usually negative or merged into ground color, and posterior 1/2 usually positive; posterolateral spots usually merged into ground color, and rarely anterior one of these spots negative; lateral and ventral surfaces variable in color by populations; eg. in most larvae collected from Monthatharn Waterfall, nearly yellowish with spots merged into ground color, or in most larvae collected from Siribhume Waterfall, light to medium brown except eye-spot region yellowish and medial narrow area of postgenal bridge somewhat paler than ground color, with spots faintly or moderately negative (Plate 1F). Antenna composed of 3 segments and apical sensillum, much longer than stem of labral fan; length ratio of segments (from base to tip) 1.0: 1.2:0.4−0.5; all segments pale white to yellow except 3rd segment entirely brownish black and apical small portion of 2nd segment somewhat darkened, as in *S. rudnicki.* Labral fan with about 52 main rays. Mandible (Fig. 5E) with usual mandibular serration of 1 medium-sized tooth and 1 small one; main tooth at an obtuse angle to mandible on apical side: supernumerary serrations absent; comb-teeth decreased in length from 1st to 3rd. Hypostomium (Fig. 5F) with 9 apical teeth, of which median tooth and each corner tooth subequal in length to each other, and longer than others; lateral margins moderately serrate apically; 8 or 9 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Plate 1F) narrow, subtriangular or bullet-shaped, 1.6−2.3 times as long as postgenal bridge; lateral margins on posterior 1/2 nearly parallel-sided or slightly diverged at base, and pointed apically; subesophagial ganglion well pigmented, wine-glass-shaped. Thoracic

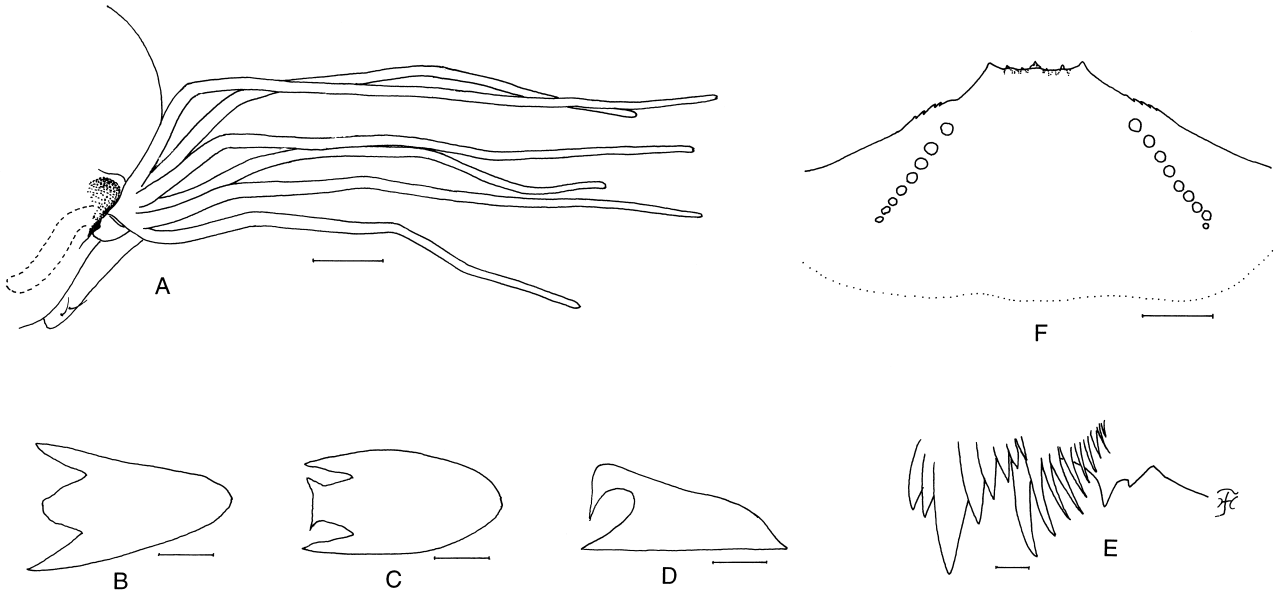

Fig. 5. Pupa and mature larva of *Simulium* (*Simulium*) *mediocoloratum* sp. nov. A, pupal gill filaments; B−D, cocoons (B and C, dorsal view; D, lateral view); E, larval mandible; F, larval hypostomium. Scales. 0.01 mm for E; 0.05 mm for F; 0.2 mm for A; 1.0 mm for B−D.

cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short colorless setae on each side of anal sclerite. Rectal scales present. Rectal organ of 3 lobes, each with 13−17 finger-like secondary lobules. Anal sclerite X-shaped, with broadened anterior arms about 0.7 times as long as posterior ones; 5−11 sensilla on basal area of anal sclerite, and 8−13 sensilla just posterior to posteior arms. Ventral papillae absent. Posterior circlet with 146−150 rows of hooklets, with up to 20 hooklets per row.

TYPE SPECIMENS. Holotype female, reared from pupa collected from a cascading stream, Monthatharn Waterfall, Chiang Mai, Thailand, 31. XII. 2001, by W. Choochote. Paratypes: 8 females, 11males, reared from pupae, 5mature larvae, in alcohol, same data as holotype; 5 pupae and 20 mature larvae, collected from a small stream, Siribhume Waterfall, Doi Inthanon National Park, Chiang Mai, 15. II. 2004, by W. Choochote.

ECOLOGICAL NOTES. The pupae and larvae of *S. mediocoloratum* were found on the surface of the stream-bed rock in two streams: one in Monthatharn Waterfall (ca. 750 m in altitude) was a cascading stream, exposed to the sun or partially shaded, and water temperature was 17 ℃, and the other in Siribhume Waterfall (ca. 1,500 m in altitude) was a small, moderately-flowing stream 0.5m wide, exposed to the sun and its water temperature was 18.5 ℃.

Simulium mediocoloratum was collected together with many other species in the two streams.

Mermithid and microsporidan infections were found in five and one of the 47 immature larvae of *S. mediocoloratum* collected from Monthatharn Waterfall, respectively.

ETYMOLOGY. The specific name *mediocoloratum* refers to the female of this species which is intermediate in the leg color between *S. rudnicki* and *S. yongi*.

DISTRIBUTION. Thailand.

REMARKS. *Simulium mediocoloratum* sp. nov. is assigned to the *griseifrons* species-group by the combination of the following characters: tarsal claws simple in the female; style with a prominent basal protuberance (Fig. 4D,E) and ventral plate transverse, without toothed margins (Fig. 4F) in the male; and gill with six filaments per side in the pupa (Fig. 5A).

The female of *S. mediocoloratum* resembles that of *S. rudnicki* in many characters including the antenna with pits and the ovipositor valve with transparent inner and posterior margins and is barely differentiated from the latter by the mid tibia yellowish white except its apical cap light to medium brown (Fig. 3E) (cf. entirely yellow in *S. rudnicki*). On the other hand, this new species is readily distinguished from *S. rudnicki* in the male by the medium-brown mid femur and the style with the narrowest portion medially (Fig. 4C), in the pupa by the inner filament of the ventral pair narrowed basally as well as the small raised area near the gill base tuberculate (Fig. 5A), and in the larva by the cephalic apotome darkened only on the posterior 1/2 (Plate 1 B).

This new species is also very similar to *S. yongi* [5] but differs from the latter in that it has the more slender fore basitarsi in both sexes, the cibarium with about 90 conical processes (Fig. 3C), the mid femur entirely yellow (Fig. 3E) in the female, the ventral plate somewhat bent ventrally (Fig. 4G) in the male, the cocoon appearing to have an anterodorsal projection (Fig. 5B,C), and the larval cephalic apotome pale on the anterior 1/2 (Plate 1B).

The male genitalia suggest that this new species is closely related to *S. ufengense* Takaoka, described from Taiwan [6], and *S. fuzhouense* Zhang and Wang, from Fujian, south China [7]. However there are differences in the leg colors, the arrangement of the pupal gill filaments, and the cocoon. In the latter two species, the femora of the male legs are largely yellowish, four of six pupal gill filaments are distinctively more slender than the remaining two filaments, and the cocoon has a thick anterodorsal margin.

Simulium taipokauense Takaoka, Davies and Dudgeon (only the male adult is known) from Hong Kong [8] shows some similarities to *S. mediocoloratum* but differs in that it has the much longer basal protuberance of the style and the saddle-shaped ventral plate with a nipple-like median process.

***Simulium* (*Simulium*) *crocinum* sp. nov.**

DESCRIPTION. **Female.** Body length 3.0 mm. ***Head.*** Nearly as in female of *S. mediocoloratum* except following characters. Frontal ratio 1.3:1.0:0.9; frons-head ratio 1.0:3.7. Fronto-ocular area (Fig. 6A) well developed, triangular. Clypeus moderately covered with dark long hairs except median portion largely bare. Labrum 0.56 times as long as clypeus. Maxillary palp brownish black, with 5 segments, proportional lengths of 3rd, 4th, and 5th segments 1.0:1.1:2.6; 3rd segment (Figs. 6B, C) not enlarged; sensory vesicle ellipsoidal or oblong, with rugged surface, 0.38−0.43 times length of 3rd segment, with a large opening. Maxillary lacinia with 12 or 13 inner and 15 or 16 outer teeth. Mandible with 28−30 inner and 15 outer teeth. Cibarium (Fig. 6D) with a cluster of about 56 conical processes medially. ***Thorax.*** Nearly as in female of *S. mediocoloratum* except scutum almost black and scutellum dark brown. ***Legs.*** Fore-

leg (Fig. 6E): coxa pale yellowish-white; trochanter pale yellowish-white with outer surfaces somewhat yellowish; femur yellow with extreme apex somewhat darkened; tibia pale yellowish-white except apical 1/5 brownish black, with a large white sheen on outer surface when illuminated; tarsus black; basitarsus dilated, 5.4 times as long as its greatest width. Midleg (Fig. 6F): coxa dark brown; trochanter yellow with apical 1/2 somewhat darkened; femur entirely yellow; tibia yellowish-white to yellow, with a large white sheen on posterior surface when illuminated; tarsus medium to dark brown except basal 2/3 of basitarsus and base of 2nd segment yellowish white. Hind leg (Fig. 6G): coxa dark brown; trochanter yellow; femur yellow with apex medium brown narrowly; tibia yellowish-white to yellow with apical cap dark brown on inner surface (Fig. 6H) but there is a broad light brown area extending forwardly from dark apical cap to a little less than basal 1/2 on outer surface (Fig. 6 G), and with a large white sheen on posterior surface when illuminated; tarsus brownish black except basal 3/5 of basitarsus and basal 1/3 of 2nd tarsal segment yellowish white; basitarsus (Fig. 6I) nearly parallel-sided, 5.7 times as long as wide, and 0.7 and 0.6 times as wide as the greatest widths of hind tibia and femur, respectively; calcipala short, 0.8 times as long as its basal width and 0.4 times as wide as the greatest width of basitarsus; pedisulcus distinct at basal 1/3 of 2nd tarsal segment. Tarsal claws simple without any tooth. ***Wing.*** Length 2.8 mm; other characters as in female of *S. mediocoloratum.* ***Abdomen.*** Basal scale medium brown with a fringe of pale hairs; dorsal surface of abdomen dark brown to brownish black except anterior 1/2 medium brown and with a dorsolateral pair of shiny whitish spots broadly connected in middle, tergites 6−8 shiny, covered with dark short hairs. ***Genitalia*** (Fig. 6J−L). Ventral surface of abdominal segment 7 with a pair of weakly-developed sternal plates. Sternite 8 well sclerotized, bare medially, with 5−8 pale short setae submedially near posterior border, and with 24 or 25 dark long hairs laterally on each side; ovipositor valve triangular in shape, membranous,

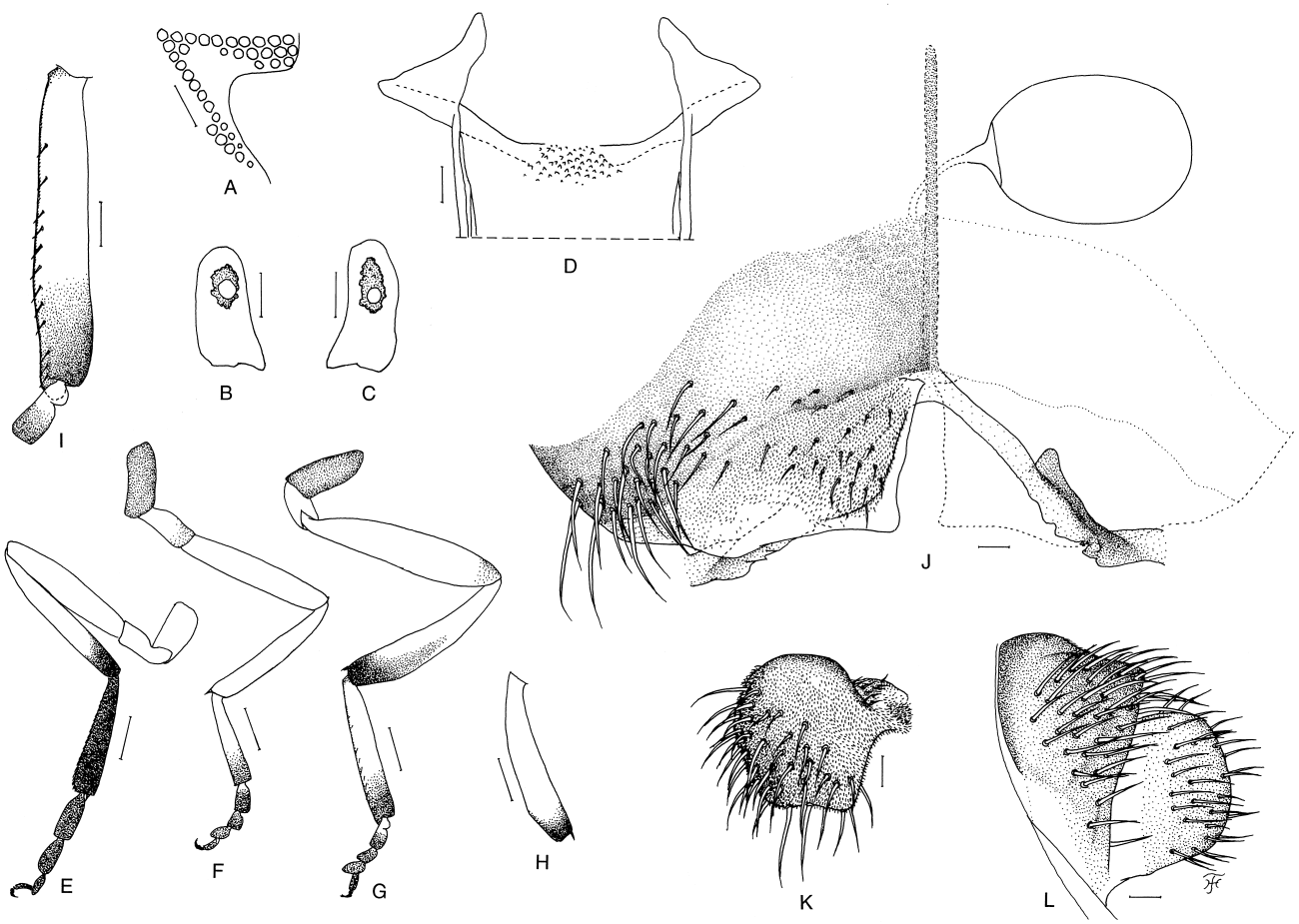

Fig. 6. Adult female of *Simulium* (*Simulium*) *crocinum* sp. nov. A, fronto-ocular area (right side, frontal view); B and C, 3rd segments of maxillary palp (B, right side; C, left side; both frontal view); D, cibarium; E-G, foreleg, midleg and hind leg, respectively (all left side); H, hind tibia (left side, inside view); I, hind basitarsus and 2nd tarsal segment (outer view); J, 8th sternite, ovipositor valves, genital fork and spermatheca (ventral view); K, paraproct (right side, ventral view); L, paraproct and cercus (right side, lateral view). Scales. 0.02 mm for D and J−L; 0.05 mm for A−C; 0.1 mm for I; 0.2 mm for E−H.

expanded ventrally, each covered with about 20 short setae as well as numerous microsetae except narrow portion along inner margin and rather wide portion along posterior margin bare and transparent; inner borders not sclerotized, moderately separated from each other. Genital fork of inverted-Y form, with well sclerotized stem; arms rather wide, each with strongly sclerotized distal ridge having a distinct projection directed anterodorsally. Paraproct in ventral view rounded posterolaterally, with anteromedial surface moderately sclerotized, having about 10 short spinous setae as well as numerous microsetae on it; paraproct slightly produced ventrally along medial margin, covered with numerous stout hairs laterally and ventrally. Cercus rounded posteriorly, about 0.6 times as long as wide, covered with numerous stout hairs. Spermatheca ellipsoidal, well sclerotized (except small area of its juncture with duct unsclerotized), with reticulate surface pattern at least near base, with minute internal setae; both accessory ducts subequal in width to main one.

Male. Body length 3.0−3.3 mm. ***Head.*** Slightly wider than thorax. Upper eye consisting of large facets in 20 horizontal and 22 vertical rows. Clypeus black, white pruinose, iridescent when illuminated, sparsely covered with dark brown hairs (median portion largely bare or with a few hairs). Antenna composed of 2+9 segments, dark yellow to light yellowish-brown on scape, pedicel, and a few basal flagellar segments, and the rest medium to dark brown; 1st flagellar segment elongate, 1.8 times as long as 2nd one. Maxillary palp composed of 5 segments, proportional lengths of 3rd, 4th, and 5th segments 1.0:1.1:2.3; 3rd segment (Fig. 7A) of normal size, with an ellipsoidal sensory vesicle having a small opening apically; sensory vesicle 0.25 times as long as 3rd segment. ***Thorax.*** Nearly as in male of *S. mediocoloratum.* ***Legs.*** Foreleg (Fig. 7B): coxa pale yellow; trochanter yellow with outer and posterior portions somewhat darkened; femur yellow on inside surface but dark yellow or light brown on outer surface; tibia medium brown to brownish black with basal 3/4 narrowly white on outer surface (white sheeny when illuminated); tarsus black, with moderate dorsal hair crest; basitarsus somewhat dilated, about 5.3 times as long as its greatest width. Midleg (Fig. 7C): coxa dark brown or blackish; trochanter medium brown with base yellow; femur entirely yellow; tibia entirely yellow, with a white sheen widely on posterior surface when illuminated; tarsus brownish black except basal 2/3 of basitarsus yellow (though its border not well defined) and extreme base of 2nd segment pale yellow. Hind leg (Fig. 7D): coxa dark brown; trochanter yellow; femur yellow except apical cap medium brown; tibia dark brown to brownish black with base whitish yellow (in another male, anterior surface of tibia pale more extentively from base to near apical cap); tarsus brownish black except a little more than basal 1/2 of basitarsus whitish yellow and basal 1/3 of 2nd tarsal segment dark yellow; basitarsus (Fig. 7E) somewhat widened from base toward apical 3/4, then somewhat narrowed toward apex, 4.3 times as long as its greatest width, and 0.8 times as wide as the greatest width of hind tibia which is as wide as that of femur; calcipala of medium size, nearly as long as wide; pedisulcus distinct. ***Wing.*** Length 2.6 mm; other characters as in male of *S. rudnicki.* ***Abdomen.*** As in male of *S. rudnicki.* ***Genitalia*** (Fig. 7F−N). Coxite in ventral view nearly quadrate. Style elongate, spatulate ventrodorsally, 1.7 times as long as coxite, gradually narrowed from base to apical 1/3, then slightly widened to rounded apex, width near apex 0.75 times as wide as basal width, and about 1.4 times as wide as the narrowest width (in another male, style gradually narrowed from base to middle, then slightly widened toward apex, width near apex 0.85 times as wide as basal width, and about 1.3 times as wide as the narrowest width, as shown in Fig. 7G), with no subterminal spine (though right style of 1 male bears a slender hair-like spine in place of usual needle-like subterminal spine, as shown in Fig. 7G); style with a prominent basal protuberance pointed dorsally and furnished at and near apex with several conical spines mostly on anterior surface. Ventral plate in ventral view transverse, somewhat wider than its length, rounded posterolaterally, with posterior margin untoothed and slightly concave medially, and covered with fine setae on anterior 1/2 of ventral surface; basal arms long and stout, directed forwardly and somewhat outwardly. Parameres wide basally, each with numerous parameral hooks. Median sclerite in end view widened from base to basal 1/3, somewhat narrowed to apical 1/3, then widened toward apex, plate-like, and well sclerotized except apical portion. Aedeagal membrane densely covered with needle-like setae and with well-sclerotized narrow dorsal plate. Segment 10 with 2 distinct hairs on each lateral side (in another male, 1 distinct hair on right lateral side and no hair on left lateral side). Cercus rounded with 7−10 distinct hairs.

Pupa. Body length (excluding gill filaments) about 3.0 mm. ***Head.*** Integument medium to dark brown, bare; face with a pair of long simple or bifid trichomes; frons with 2 pairs of long bifid trichomes somewhat separated from each other; antennal sheath bare. ***Thorax.*** Integument medium to dark brown, bare on anterior 1/2 (except a few small raised areas at base of gill densely covered with tubercles), moderately covered with small cone-shaped tubercles dorsally on posterior 1/2; thorax anteriorly with 3 dorsal and 2 lateral pairs of long simple and/or branched trichomes (split into 2−4), posteriorly with 1 lateral pair of long bifid or trifid trichomes, and ventrolaterally with 3 pairs of long simple

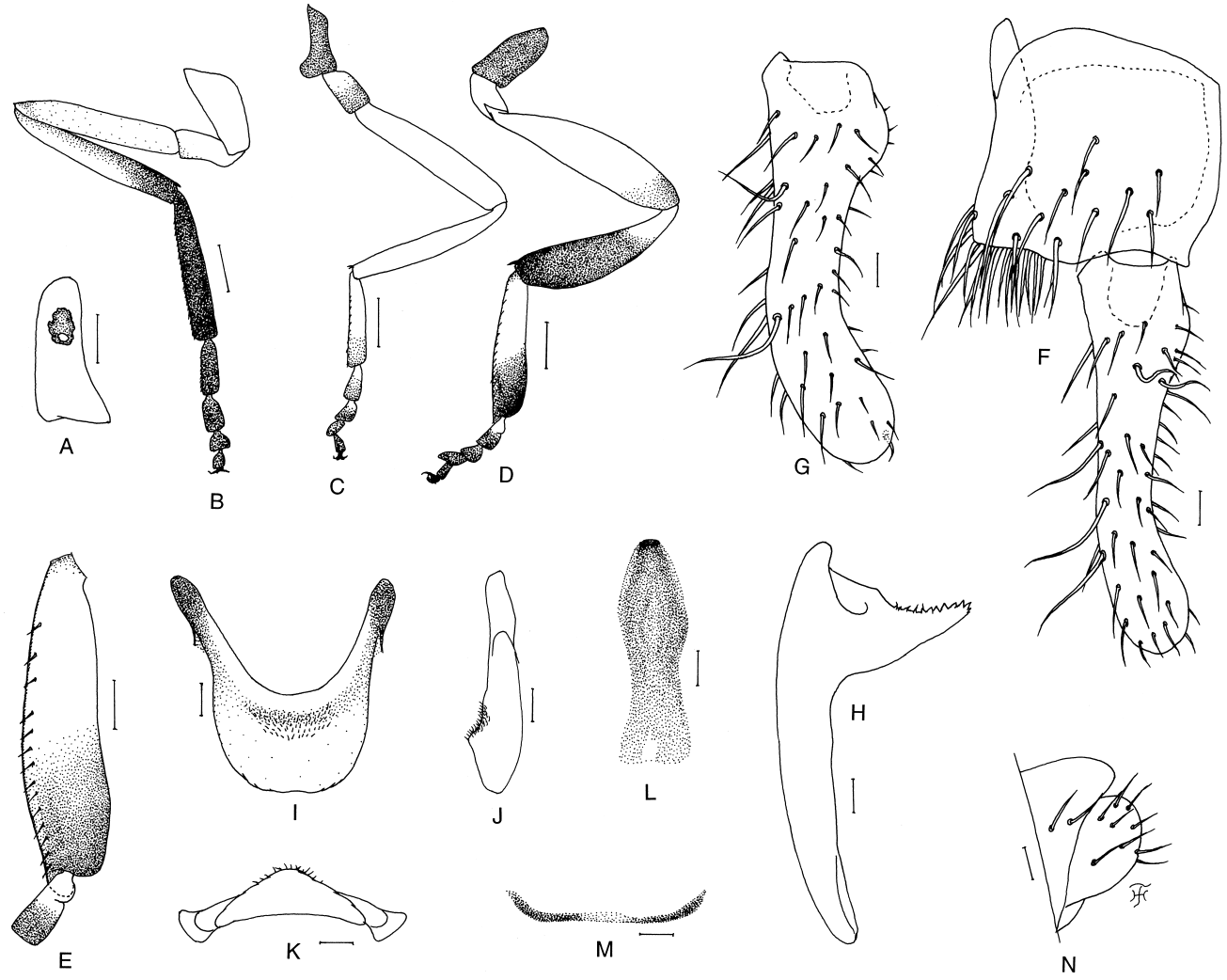

Fig. 7. Adult male of *Simulium* (*Simulium*) *crocinum* sp. nov. A, 3rd segment of maxillary palp (right side, frontal view); B–D, foreleg, midleg and hind leg, respectively (all left side); E, hind basitarsus and 2nd tarsal segment (left side, outside view); F, coxite and style (right side, ventrolateral view); G and H, styles (G, right side, of different male, ventrolateral view; H, left side, outer view); I–K, ventral plates (I, ventral view; J, lateral view; K, end view); L, median sclerite; M, dorsal plate; N, abdominal tip showing cercus (lateral view). Scales. 0.02 mm for F–N; 0.05 mm for A; 0.1 mm for E; 0.2 mm for B–D.

and/or bifid trichomes. Gill (Fig. 8A) with 6 filaments arranged in sessile pairs, shortened in length from dorsal to ventral, outer filament of dorsal pair longest of all (1.0–1.5 mm long); usually outer filament of each pair somewhat longer than its counter inner filament; outer filament of dorsal pair slightly thicker than outer filament of middle pair, which is much thicker than the other filaments; inner filament of dorsal pair usually somewhat thicker than inner filament of middle pair and both inner and outer filaments of ventral pair; outer filaments of dorsal and middle pairs dark brown to brownish black at least basal 1/3, then gradually become paler toward apex, basal portions of these two filaments much darker than the other filaments which are uniformly light brown; outer filaments of dorsal and middle pairs gradually tapered toward apical tip, all inner filaments and outer filament of ventral pair nearly parallel-sided along basal 1/3 or 1/4, then tapered toward apical tip; all filaments with annular ridges and furrows throughout their length, and densely covered with minute tubercles; gill with medium-sized transparent protuberance at base. ***Abdomen.*** Dorsally, segment 1 greyish light-brown, with 1 medium-long simple hair-like seta on each side; segment 2 grey on anterior 2/5, transparent on posterior 3/5, with 1 medium-long simple hair-like seta, 4 short simple hooklets of equal size (much smaller than those on segments 3 and 4) and 1 short spinous seta on each side; segments 3 and 4 each with 4 hooked spines and 1 short simple hooklet along posterior margin on each side; segments 5–9 each with a transverse row of comb-like groups of minute spines (though those on segment 5 very weakly developed) and lacking spine-combs on

each side except segment 8 with a transverse row of distinct spine-combs; segment 9 lacking terminal hooks. Ventrally, segment 4 with 1 simple hook, 1 simple hooklet and 2 short setae on each side; segment 5 with a pair of simple hooks submedially on each side; segments 6 and 7 each with a pair of simple (or bifid) inner and simple outer hooks somewhat separated from each other, on each side. Grapnel-like hooklets absent. ***Cocoon*** (Fig. 8B,C). Wall-pocket-shaped, with a medium to large anterolateral window on each side; thick, individual threads not visible; anterior margin well defined; cocoons attached on sticks and grass leaves thickly woven and well pigmented but those on rock surfaces usually thinly woven, less pigmented and sometimes nearly transparent; 3.2−3.6 mm long by 1.7−2.0 mm wide.

Mature larva. Body length 6.5−7.0 mm. Body color light to medium brown. Abdomen gradually widened toward segment 7, then narrowed to posterior tip when viewed laterally; segments 6 and 7 appear to be equal in width to each other when viewed dorsally. Cephalic apotome (Plate 1C) pale white on anteior 1/2 and pale to dark yellow on posterior 1/2 with a small dark area in middle along posterior border, with mediolateral spots and posteromedial longitudinal spot always distinctively positive (and rarely anteromedial longitudinal spot and posterolateral spots faintly positive); lateral surface yellowish brown to dark brown except eye-spot region and its dorsal area pale white to yellow; spots around eye-spot region faintly to distinctively negative; ventral surface (Plate 1G) light to dark brown with elongate spot on each side of postgenal cleft negative. Antenna composed of 3 segments and apical sensillum, much longer than stem of labral fan; length ratio of segments (from base to tip) 1.0:1.2:0.4; all segments pale white to yellow except 3rd segment entirely brownish black and apical small portion of 2nd segment darkened to some extent. Labral fan with 40−44 main rays. Mandible (Fig. 8D) with usual mandibular serration of 1 medium-sized tooth and 1 small one, without supernumerary serrations; main mandibular tooth at an obtuse angle to the mandible on apical side; comb-teeth decreasing in length from 1st to 3rd. Hypostomium (Fig. 8E) with 9 apical teeth, of which median tooth and each corner tooth subequal in size, longer than others; lateral margins moderately serrate apically; 8−10 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Plate 1G) bullet-shaped, somewhat pointed apically, 1.9−2.3 times as long as postgenal bridge; subesophagial ganglion well pigmented, wine-glass shaped. Cervical sclerite composed of a pair of small rod-like pieces, widely separated in middle from each other, not fused to occiput. Thoracic cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short colorless setae on each side of anal sclerite; rectal scales present (indistinct, then often overlooked). Rectal organ of 3 lobes, each with 19−22 finger-like secondary lobules. Anal sclerite X-shaped, with broadened anterior arms about 0.7 times as long as posterior ones; 16−18 sensilla just posterior to posterior arms. Ventral papillae absent. Posterior circlet with about 136 rows of hooklets, with up to 18−22 hooklets per row.

TYPE SPECIMENS. Holotype female, with associated pupal exuvia and cocoon, collected from a small stream, Siribhume waterfall, Doi Inthanon National Park, Chiang Mai, Thailand, 28. II. 2004, by W. Choochote. Paratypes: 2 males (reared from pupae), and 13 pupae, same data and date as holotype; 11 pupae and 6 mature larvae, same data as holotype but date, 15. II. 2004.

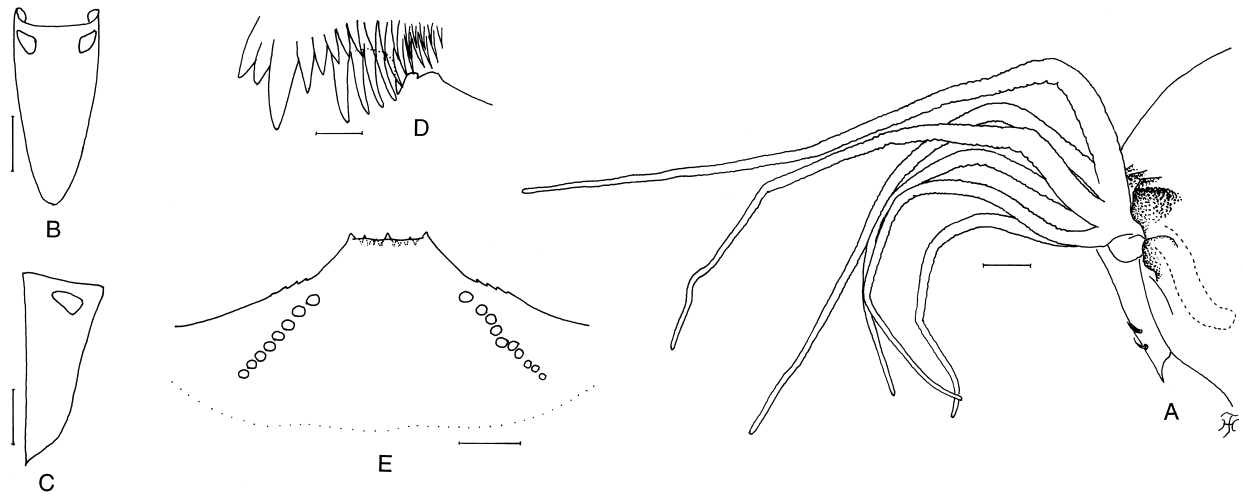

Fig. 8. Pupa and mature larva of *Simulium* (*Simulium*) *crocinum* sp. nov. A, pupal gill filaments; B and C, cocoons (B, dorsal view; C, lateral view); D, larval mandible; E, larval hypostomium. Scales. 0.02 mm for D; 0.05 mm for E; 0.1 mm for A; 1.0 mm for B and C.

ECOLOGICAL NOTES. The pupae and larvae of *S. crocinum* sp. nov. were found on the rock surface of a moderately flowing mountainous stream 0.5 m wide, exposed to the sun. A few pupae were also collected on trailing grasses and slender sticks in the fast flowing water. Water temperature was 18.5 ℃. Altitude was ca. 1,500 m.

This species was collected together with *S.* (*S.*) *rufibasis* Brunetti, *S.* (*S.*) *mediocoloratum* sp. nov., *S.* (*Gomphostilbia*) *inthanonense* Takaoka and Suzuki, *S.* (*Nevermannia*) *feuerborni* Edwards and *S.* (*Montisimulium*) sp.

ETYMOLOGY. The specific name *crocinum* refers to the yellowish legs of both sexes of this new species. The Latin word *crocinus* means yellow.

DISTRIBUTION. Thailand.

REMARKS. *Simulium crocinum* sp. nov. is assigned to the *griseifrons* species-group, as in the previous species.

The female of this new species resembles those of *S. rudnicki* and *S. mediocoloratum* in many characters including the antenna with pits and the ovipositor valve with transparent inner and posterior margins (Fig. 6J), but is differentiated from the latter two species by the dorsal surface of the anterior 1/2 of the second abdominal segment medium brown (cf. white or yellowish white in *S. rudnicki* and *S. mediocoloratum*), the fore tibia pale yellowish-white except apical 1/5 brownish black (cf. usually dark portion extending from the apical cap to basal 1/2 or more along the inside margin in *S. rudnicki* and *S. mediocoloratum*), the hind tibia yellowish-white to yellow except the apical cap dark brown at least on the inside surface though the outer surface with an excess light brown portion extending from the apical cap to a little less than basal 1/2 (Fig. 6G) (cf. hind tibia light to dark brown or brownish black except basal 1/3 and posterior surface of basal 2/3 white in *S. rudnicki* and *S. mediocoloratum*), and the ellipsoidal spermatheca (Fig. 6J) (cf. globular in the other two species). The female of this species is also distinguished from *S. mediocoloratum* by the entirely yellowish-white mid tibia.

The male of *S. crocinum* is very similar to that of *S. rudnicki*, but is barely distinguished by the hind tibia dark brown to brownish black with basal 1/4 or 1/5 whitish yellow (Fig. 7D) (cf. only the extreme base of hind tibia white in *S. rudnicki*) and the style without subterminal spine (Fig. 7F) (subterminal spine present in *S. rudnicki*). On the other hand, this new species is readily separated in the immature stages from *S. rudnicki* and *S. mediocoloratum*: in the pupa by the gill with two filaments much thicker than the other four filaments (Fig. 8A) and the light to medium brown, thickly-woven cocoon with an anterolateral window on each side (Fig. 8B,C), and in the larva by the cephalic apotome with its ground color mostly pale (Plate 1C).

The male genitalia, leg colors and the pupal gill filaments of *S. crocinum* are very similar to those of *S. ufengense* Takaoka, described from Taiwan [6], and of *S. fuzhouense* Zhang and Wang, from Fujian, south China [7]. However, this new species is easily separated from these two species by the fenestrated cocoon (Fig. 8B,C) (cf. cocoons simple without lateral windows in *S. ufengense* and *S. fuzhouense* [6, 7]).

Simulium taipokauense from Hong Kong [8] shows some similarities to *S. crocinum* but differs in that it has the much longer basal protuberance of the style and the saddle-shaped ventral plate with a nipple-like median process.

Simulium (*Simulium*) *suchariti* Takaoka and Choochote, 2004

Simulium (*Simulium*) *suchariti* Takaoka and Choochote, 2004: 31−33 (female only).

DESCRIPTION. **Pupa.** Body length (excluding gill filaments) 3.5−4.0 mm. ***Head.*** Integument yellowish brown, densely and elaborately covered with tubercles of various sizes (Fig. 9A); all tubercles but small ones with several minute projections on their surface; antennal sheath also densely covered with tubercles; face with a pair of medium-long trifid or quadrifid trichomes (Fig. 9B); frons with 2 pairs of medium-long branched (split into 4−6) trichomes slightly shorter than facial ones (Fig. 9C); 2 frontal trichomes on each side arising close together. Thorax. Integument yellowish brown, moderately covered with tubercles of various sizes (Fig. 9D) (similar to those on head but most are much larger) on anterior 1/2 including areas near base of gill, and moderately covered with small cone-shaped tubercles dorsally on posterior 1/2; thorax anteriorly with 3 dorsal and 2 lateral pairs of very long fan-like trichomes each with 20−31 branches (Fig. 9E) (about twice as long as facial trichomes), posteriorly with 1 lateral pair of medium-long trichomes each with 7-10 branches (Fig. 9 F), and ventrolaterally with 3 pairs of long and medium-long branched (split into 2−7) trichomes. Gill (Fig. 9G) with 6 filaments arranged in pairs; all pairs almost sessile except dorsal pair with very short stalk; all filaments usually pale, gradually decreasing in length and thickness from dorsal to ventral; outer filament of dorsal pair longest (1.8−2.0 mm) and thickest of all, and inner filament of lower pair shortest (0.5−0.8 mm) and thinnest (about half times as thick as the thickest dorsalmost filament); all filaments gradually tapered toward apical tip, with annular ridges and furrows throughout their length, and densely covered with minute tubercles. ***Abdomen.*** Dorsally, segment 1 light yel-

low with narrow pale portion along posterior margin, with 1 bifid or trifid (or quadrifid) medium-long seta on each side; segment 2 almost pale, nearly transparent, with 1 simple or bifid medium-long seta, 1 short minute seta and 4 short simple spines of equal size on each side; segments 3 and 4 each with 4 hooked spines along posterior margin on each side; segment 5 bare; segments 6–9 each with a transverse row of comb-like groups of minute spines on each side; segment 8 with a transverse row of distict spine-combs on each side; segment 7 sometimes with a transverse row of spine-combs, though much fewer in number and smaller in size than those on segment 8; segment 9 lacking terminal hooks. Ventrally, segment 4 with 1 simple hook (similar in size to those on segments 5–7) and a few simple or bifid short setae on each side; segment 5 with a pair of simple or bifid hooks submedially and a few simple or bifid hooklet-like setae on each side; segments 6 and 7 each with a pair of bifid inner and simple or bifid outer hooks somewhat separated from each other and a few simple or bifid setae on each side. Grapnel-like hooklets absent. ***Cocoon*** (Fig. 9H–J). Wall-pocket-shaped, with a large anterolateral window (in some cocoons 1 of 2 windows is small, or even absent, though the window on the other side is large; in other cocoons, window is divided into 2 or 3 spaces of various sizes) on each side; ventolateral tips of anterior margin approaching to each other anteroventrally, sometimes connected to each other; cocoon thin and usually pale; individual threads partially visible; 5.0–5.2 mm long by 1.8–2.0 mm wide.

Mature larva. Body length 7.5–8.0 mm. Body color greyish. Abdomen gradually widened toward posteriorly, with the widest portion on segment 7. Cephalic apotome (Plate 1D) almost entirely pale yellow except a small somewhat dark area in middle along posterior border (though mediolateral spots and anterior ones of posterolateral spots very faintly positive in 1 of 3 larvae examined); lateral surface entirely pale yellow without any dark spot; ventral surface (Plate 1H) somewhat darkened with elongate spot on each side of postgenal cleft usually faintly negative. An-

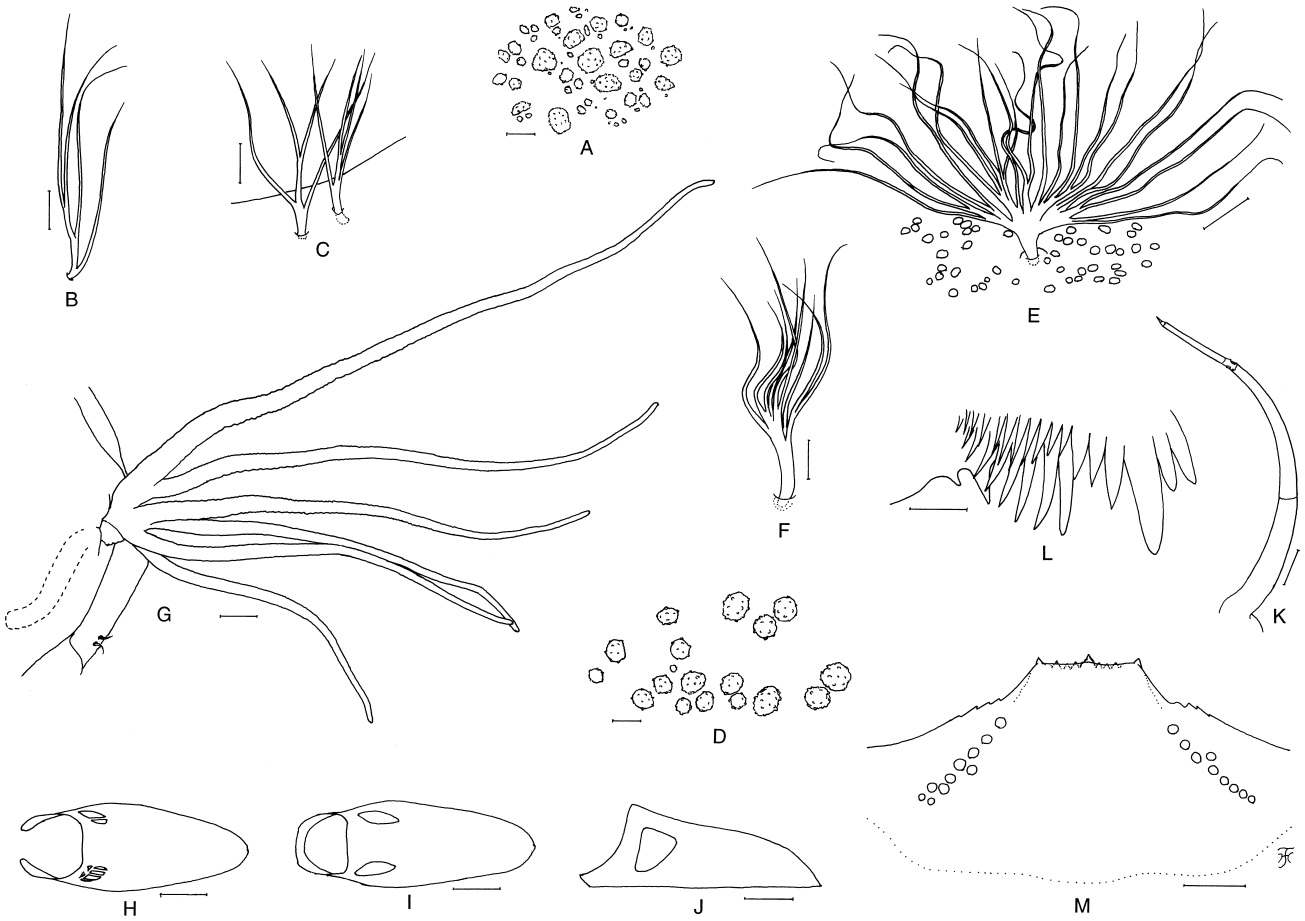

Fig. 9. Pupa and mature larva of *Simulium* (*Simulium*) *suchariti* Takaoka and Choochote. A–G, pupa; K–M, larva. A, tubercles on frons; B, trichome on face; C, 2 trichomes on frons (right side); D, tubercles on dorsal surface of thorax; E, trichome and tubercles near its base on dorsal surface of thorax; F, trichome on posterolateral surface of thorax; G, gill filaments; H–J, cocoons (H and I, dorsal view; J, lateral view); K, antenna (left side, dorsal view); L, mandible; M, hypostomium. Scales. 0.01 mm for A and D; 0.02 mm for B, C, F and L; 0.05 mm for E, K and M; 0.1 mm for G; 1.0 mm for H–J.

tenna (Fig. 9K) composed of 3 segments and apical sensillum, much longer than stem of labral fan; length ratio of segments (from base to tip) 1.0:1.6:0.7; all segments pale or yellow. Labral fan with about 40 main rays. Mandible (Fig. 9L) with usual mandibular serration of 1 medium-sized tooth and 1 small one, without supernumerary serrations (though left mandible of 2 larvae with 1 additional small tooth); main mandibular tooth at an obtuse angle to the mandible on apical side; 1st comb-tooth longest and thickest, followed by 2nd and 3rd teeth of similar size. Hypostomium (Fig. 9M) with 9 apical teeth, of which median tooth subequal in size to, or a little larger than, each corner tooth, and intermediate teeth small; lateral margins moderately serrate apically; 7−10 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Plate 1H) bullet-shaped, rounded apically, 1.8−2.0 times as long as postgenal bridge; subesophagial ganglion not visible. Cervical sclerite composed of a pair of small rod-like pieces, widely separated in middle from each other, not fused to occiput. Thoracic cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short colorless setae on each side of anal sclerite; rectal scales present. Rectal organ of 3 lobes, each with about 17 finger-like secondary lobules. Anal sclerite X-shaped, with broadened anterior arms about 0.64 times as long as posterior ones; 10−20 sensilla just posterior to posterior arms. Ventral papillae absent. Posterior circlet with about 120 rows of hooklets, with up to 18−20 hooklets per row.

SPECIMENS EXAMINED. 2 pharate females, 8 pupae, 1 pupal exuvia and 5 mature larvae, all collected from a small forest stream, Ang Ka, Doi Inthanon National Park, Chiang Mai, Thailand, 10. XII. 2003, by W. Choochote.

ECOLOGICAL NOTES. The pupae and larvae of *S. suchariti* were found on the surface of the rocky stream-bed of a shaded stream 0.3 m wide, slow-flowing in natural forest. Water temperature was 6.5 ℃. Altitude was 2,565 m.

This species was collected together with *S.* (*S.*) *setsukoae* Takaoka and Choochote, *S.* (*Montisimulium*) sp. and *S.* (*Nevermannia*) *caudisclerum* Takaoka and Davies.

DISTRIBUTION. Thailand.

REMARKS. *Simulium suchariti* differs from the three related species treated above in the pupa by the head and thoracic integuments moderately or densely covered with tubercles as well as the arrangement of the gill filaments (Fig. 9A,G), and in the larva by the pale cephalic apotome (Plate 1D), the antenna with the third segment not darkened compared to the first and second segments (Fig. 9K) and the subesophageal ganglion not visible (Plate 1H).

The pupa and larva of *S. suchariti* are similar to those of *S. maenoi* Takaoka and Choochote, described from Thai-

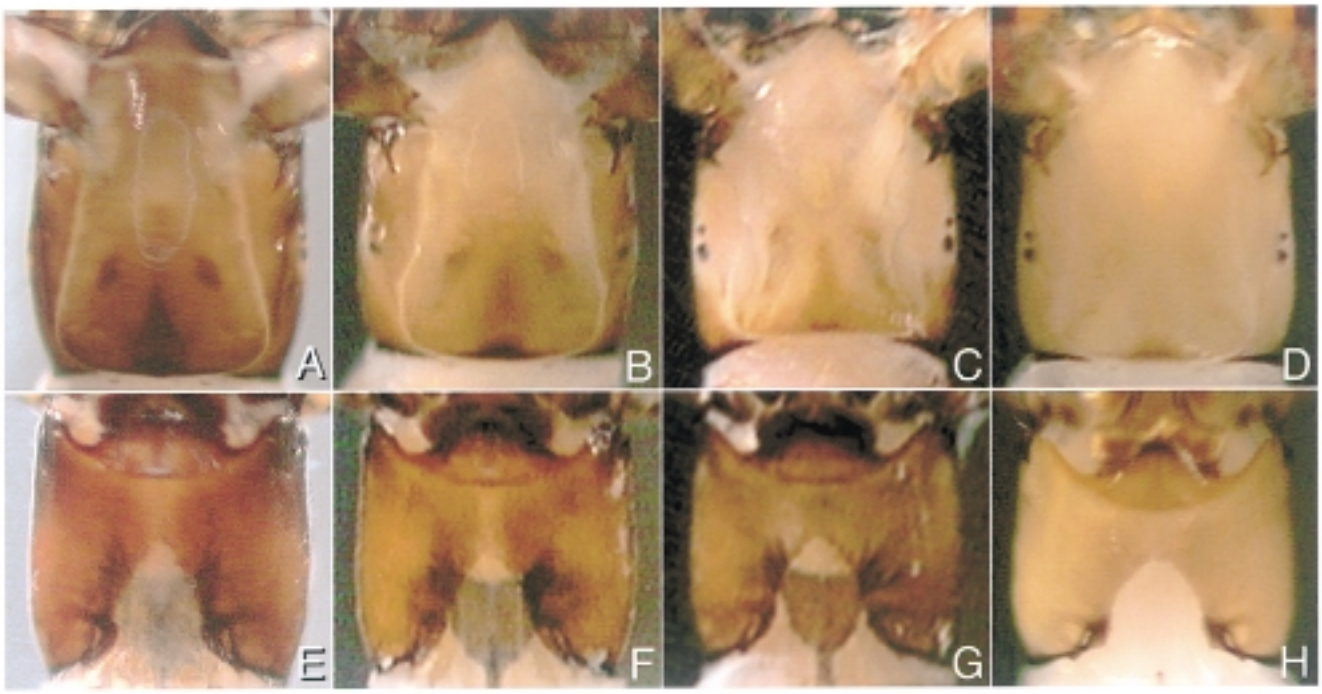

Plate 1. Larval head capsules of four *Simulium* (*Simulium*) species. A and E, *S. rudnicki* Takaoka and Davies; B and F, *S. mediocoloratum* sp. nov.; C and G, *S. crocinum* sp. nov.; D and H, *S. suchariti* Takaoka and Choochote. A−D, dorsal view; E−H, ventral view.

land [9] in the arrangement of the pupal gill filaments (Fig. 9G), and the pale larval cephalic apotome (Plate 1D) and the shape of the larval body. However *S. suchariti* is readily distinguished from *S. maenoi* in the pupa by the absence of the terminal hooks, head integument with tubercles of different sizes (Fig. 9A), and dorsal trichomes of the thorax with 20-31 branches (Fig. 9E), and the cocoon with a large anterolateral window on each side (Fig. 9J), and in the larva by the pale ventral surface of the head capsule (Plate 1H).

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CURRENT STATUS OF RESPIRATORY DISEASES SUFFERED BY JAPANESE PEOPLE LIVING IN SOUTHEAST ASIA

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Abstract: We gathered and analyzed date on respiratory diseases suffered by Japanese adults treated in Ram Hospital in Chiang Mai and Subang Jaya Medical Center in Kuala Lumpur. In both hospitals, the percentages of patients undergoing treatment for respiratory diseases was the greatest. Of these, relatively mild symptoms such as upper respiratory tract infection account for the majority of the diseases, but also included are instances of lower respiratory tract infection or chronic respiratory diseases such as chronic sinusitis and COPD/bronchial asthma. In Kuala Lumpur, we conducted a questionnaire-based survey targeted on Japanese people living there, in order to detemine the currnt status of respiratory deseases. The date showed that many Japanese had symptoms of respiratory diseases and felt that air pollution was serious.

It is important for Japanese people living in Asia to be aware of preventative measures to prevent respiratory diseases, such as those caused by air pollution and infection.

Key words: Japanese people living abroad;Respiratory diseases; Air pollution

INTRODUCTION

During the post-war era, the number of Japanese traveling and living abroad long-term increased dramatically. According to statistics from the Ministry of Justice, those who went abroad in 2000 exceeded 17 million; roughly double that of 10 years ago. Also, according to statistics from the Ministry of Foreign Affairs, long-term overseas residents (three months or more) reached an all-time high of 540,000 in 2001. Particularly, the percentage of Japanese staying long-term in the Asian region has reached about 20% of the world total, and is increasing yearly.

In the past, health problems for Japanese people living abrond consisted mainly of infection, such as malaria and diarrhea. Recently, however, respiratory diseases have been increasing. Respiratory organs are sensitive to external influences, especially to changes in temperature and humidity. These are the main causes of respiratory diseases.

Also, rapid economic development, with its accompanying air pollution, is becoming serious in Asia. Reports from WHO rank cities in Asia, including those in China, India ets., as having the worst air pollution problems [1]. Many reports have alreadey linked the air pollution problem to respiratory diseases [2]. An effective response to this problem is needed because increasing numbers of Japanese people are going to live in the Asian region.

To date, research on diseases suffered by Japaneses, European and American people living abroad has been conducted frequently [3] [4].As a result, it is clear that there are many cases of respiratory diseases among those treated in local medical facilities. For example, research done by us in 2003 at two medical facilities in Southeast Asia (Thailand and Malaysia) on Japanese adults showed that the largest number of cases encountered were respiratory diseases [5]. Therefore, this time we concentrated on analyzing the data from these two facilities in order to learn more clearly the extent of respiratory diseases. We also analyze date from questionnaires given in Kuala Lumpure, Malaysia to learn the extent of respiratory diseases suffered by Japanese living there.

On the basis of these analyses, we describe the current status of respiratory diseases afflicting Japanese people liv-

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ing in Southeast Asia and the preventative measures used to combat these health problems.

METHODS

(1)Survey on diseases under medical treatment in Kuala Lumpure, Malaysia and Chiang Mai, Thailand.

The medical facilities covered by this research are: Ram Hospital in Chiang Mai, Thailand and Subang Jaya Medical Center in Kuala Lumpur, Malaysia. Ram Hospital, opened in 1993, is a private hospital with 300 beds, used by most of the Japanese people living in the city of Chiang Mai (Japanese population approximately 1,000). Subang Jaya Medical Center, opened in 1985, is a private hospital with 400 beds, located in the Subang Jaya area in the suburbs of Kuala Lumpur (Japanese population approximately 6,000). There are other hospitals in the city, such as Pantai Medical Center, Japan Medicare Clinic and Gleneagles Medical Center, each of which is used by many Japanese people.

The research spanned two years from January 2000 to December 2001. We gathered information including age, gender and diseases of Japanese being treated as out patients. To protect privacy, we gave patients ID numbers instead of using names. The number shown for each month represents the total number of treatments (some patients were counted more than once). The diseases treated are numbered 1 to 21 in accordance with the International Classification of Diseases (ICD-10). While most of patients coverd are long-term residents, the date also includes a number of short-stay tourists.

(2)Questionnaire survey in Kuala Lumpur, Malaysia

Questionnaires were given to adults in Kuala Lumpur, Malaysia when they came for a health consultation conducted in February 2003 by us. The contents of the questionnaire consisted of the followin items: ① Do you have symptoms of respiratory disease? ② If so, what are the symptoms? ③ When did you begin to feel the symptoms? ④ Do you feel that air pollution is serious? ⑤ Do you feel that air pollution is harmful to your health? ⑥ What do you think is the cause of the air pollution?

RESULTS

1） Diseases suffered by Japanese patients treated in Ram Hospital

Out of a total of 5,379 Japanese patients, 4,315 were adults (16 and over). The 30 to 39 age group was the largest, with 1,217 patients, followed by 20 to 29 with 937, and 40 to 49 with 773. By gender, 2,482 were male and 1,332 were female (1unknown). The average monthly number of treatments was 180, with more during the dry season from November to February and the rainy season from July to August. The fewest number of treatments occurred durting the hottest month of April.

Over the two-year period of the study, we noted that the largest number (739 patients representing 17.1%) suffered respiratory diseases, followed by digestive tract diseases, infections, injuries and skin diseases.

Out of these respiratory diseases, upper respiratory tract infection such as common cold and laryngopharyngitis accounted for more than half, (59.4%) of the total, followed by bronchitis (13.9%), tonsilitis (10.8%), chronic sinusitis (7.4%), chronic obstructive pulmonary disease (COPD) / bronchial asthma (4.2%) (Table 1). The number of patients treated on a monthly basis shows certain unevenness between 2000 and 2001, though the trend was high in the dry season from November to February (Figure 1).

Table 1. Number of respiratory diseases

	Ram Hospital 2000	2001	Total (%)	Subang Jaya Medical Center 2000	2001	Total (%)
⑴Upper Respiratory Tract Infection						
Common Cold	132	116	248 (33.5%)	120	144	264 (51.3%)
Laryngopharyngitis	98	94	192 (25.9%)	45	37	82 (15.9%)
Tonsilitis	36	44	80 (10.8%)	7	7	14 (2.7%)
⑵Lower Respiraory Tract Infection						
Bronchitis	44	59	103 (13.9%)	8	1	9 (1.2%)
Pneumonia	0	0	0	0	0	0
⑶Chronic Sinusitis	41	14	55 (7.4%)	15	15	30 (5.8%)
⑷COPD/Bronchial asthma	15	16	31 (4.2%)	21	13	34 (6.6%)

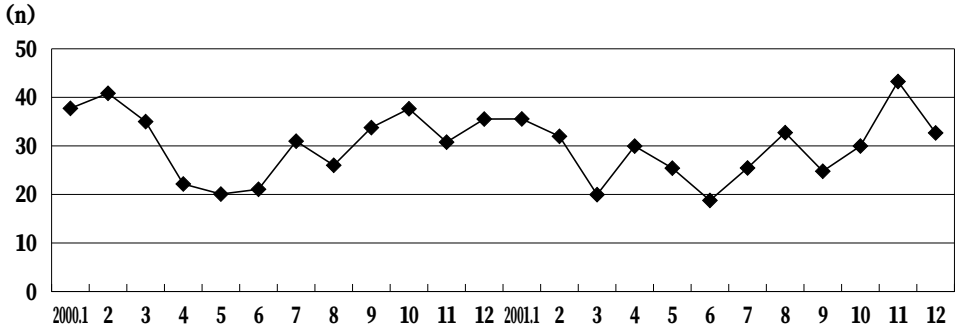

Figure 1. Ram Hospital
The monthly trend of respiratory disease patients

2）Diseases suffered by Japanese patients treated in Subang Jaya Medial Center

Out of a total of 7,313 patients, 4,570 were adults. The 30 to 39 age group was the largest, with 2,066 patients, followed by 40 to 49 with 1,066 and 20 to 29 with 630. By gender, 2,403 were male and 2,165 were female (two unknown). The average monthly number of treatments was 190, with more during the relatively dry season from May to July.

Over the two-year period of the study, we noted that the largest nuver (514 patients representing 11.2%) suffered respiretory diseases, followed by occular diseases, various clinical symptoms, skin diseases and muscle and skeletal diseases.

Out of these respiratory deiseases, acute upper respiratory tract infection such as common cold and laryngopharyngitis accounted for about 67% of the total, follwed by COPD / bronchial asthma (6.6%), chronic sinustitis (5.8%), tonsilitis (2.7%), bronchitis (1.2%) (Table 1). The monthly trend was high for treatments occurring during the dry season from May to July and in December (Figure 2).

Table 2. Result of questionnaire survey in Kuala Lumpur

①Do you have symptoms of respiratory disease?	
yes	27 (42.9%)
no	36 (57.1%)
②If so, what are the symptoms?	
sticking sputum	19 (30.2%)
coughing	18 (28.6%)
shortness of breath	17 (27.0%)
nasal secretion and congestion	10 (15.9%)
sore throat	7 (11.1%)
hoarse voice	6 (9.5%)
wheezing	3 (4.8%)
③When did you begin to feel the symptoms?	
before arrival	9 (33.3%)
after arrival	4 (14.8%)
not sure or did not answer	5 (18.5%)
④Do you feel that air pollution is serious?	
yes	36 (57.1%)
no	24 (38.1%)
⑤Do you feel that air pollution is harmful to your health?	
yes	18 (28.6%)
no	38 (60.3%)
⑥What do you think is the cause of the air pollution?	
industrial gas	8 (12.7%)
automobile exaust	26 (41.3%)
climate	6 (9.5%)
Enviromental sanitation	2 (3.2%)
Haze (smog from forest fire)	27 (42.9%)

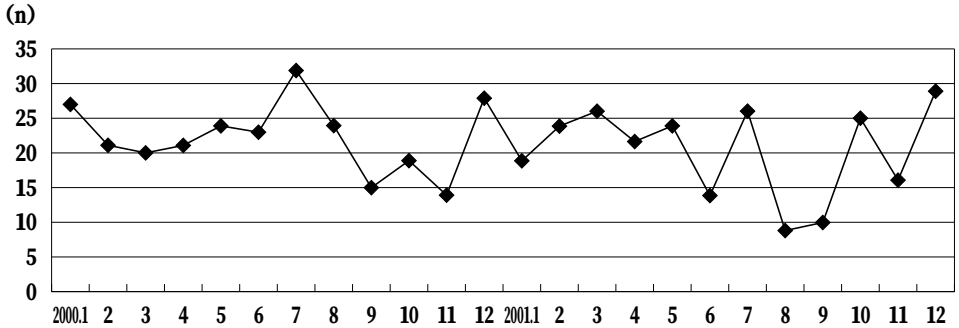

Figure 2. Subang Jaya Medical Center
The monthly trend of respiratory disease patients

3）Results of the questionnaire survey in Kuala Lumpure (Table2)

The total number of responders was 63, of whom 37 were male and 26 female. The average age was 55 and 47 of these had live there for two years or more.

Those who had symptoms of respiratory disease numbered 27, a high proportion of the total (42.9%). Of these 27, many suffered from light symptoms of upper tract infection, more specifically, 19 from sticking sputum, 18 from coughing, 17 from shortness of breath, 10 from nasal secreation and congestion, seven from sore throat, six from hoarse voice and three from wheezing or stridor. Also among the same 27, four indicated that symptoms began after their arrival and nine before, while 14 were not sure or did not answer at all.

More than half (36 responders, representing 57.1%) responded that air pollution was serious. On the other hand, only 18 responders (28.6%) indicated awareness that air pollution causes health problems. As to the causes of air pollution, eight mentioned industrial gas emission (12.7%), 26 automobile exhaust (41.3%), 27 haze (42.9%), and six climate (9.5%).

DISCUSSION

Since respiratory organs are in direct contact with the outside, they are affected by factors such as climate and pollution and can also be the entry point for viruses and bacteria. In 2003, the SARS epidemic ravaged Asia, and other infectious respiratory disease epidemics like influenza have been occurring continuously. Clearly, we must stress the importance of measures to prevent respiratory diseases among Japanese people living abroad.

The occurrence of respiratory diseases is common among travelers [3]. Steffen et al. estimated the rate of acute respiratory tract infections with fever to be 1.261 per 100,000 travelers for a stay of one month in a developing country [6].

Also, we see that many Japanese living abroad are being treated for respiratory diseases at overseas medical facilities [4]. Medical personnel of the Japanese Ministry of Foreign Affairs analyzed diseases being treated at Japanese embassies (178,014 cases from 1989 to 1998). The results indicated that respiratory diseases were the most prevalent, accounting for 31.7% of the total. Moreover, research conducted by the Japan Overseas Cooperation Volunteers on Japanese people living in developing countries indicates that respiratory diseases rank high among diseases under medical treatment[7]. In our previousu study on two medical facilities in Southeast Asia, we saw that respiratory diseases were the most prevalent, far exceeding infectious diseases and digestive tract diseases [5].

From the results of this latest research, we can see that instances of respiratory diseases increase during the dry season. At Ram Hospital in Chiang Mai, Thailand, the climate is tropical monsoon. The seasons are broadly divided into the dry season mainly from November to May, and the rainy season from June to October. The cold season is from November to February, while the hot season is from March to May. The average yearly temperature is around 25 degrees, and sometimes exceeds 40℃ during the hottest season.

Subang Jaya Medical Center is located in Kuala Lumpur, the capital of Malaysia. The climate of Malaysia, though different on the east and west sides of the Malay Peninsula, is generally that of a tropical rain forest. While temperature and humidity are high all year, the dry season is from May to September when precipitation is relatively low and the rainy season is from October to February when there is an abundant precipitation.

The number of cases of respiratory diseases at both facilities tends to increase during the dry season, though some difference is evident between 2000 and 2001. Relatively mild symptoms such as upper respiratory tract infection account for the majority of the respiratory diseases, but these also include instances of lower respiratory infection and chronic respiratory diseases such as COPD/bronchial asthma.

When the air is dry, the effectiveness of mucous membranes in the air passages diminishes. Also, dust and automobile exhaust are stirred up, causing an increase in respiratory diseases, mainly in the respiratory tract. We also know that many infectious viral respiratory diseases spread when temperature and humidity are low. Even in Japan, respiratory diseases caused by influenza or RS virus spread more readily during the winter dry period, and there are many cases of upper respiratory tract infection. Therefore, we feel that climactic factors, such as changes in temperature and humidity greatly affect the development of respiratory diseases in Japanese people living abroad.

Air pollution is another serious factor recently in the Asian region. Air pollutants such as sulfur oxides, nitrogen oxides, and suspended particulate matter (SPM) are known to cause damage to health. These substances can cause bronchitis, bronchial asthma, COPD and other respiratory diseases. SPM is a particle of 10 μm (PM 10) or less that floats in the atmosphere. Recent studies have reported a correlation between particulate air pollution and daily mortality rate. Especially particles of 2.5 μm (PM 2.5) contribute to the mortality related to respiratory diseases [2]. According to WHO, Asian cities such as those in China and India have acute air pollution levels, with the density of air pollutants

greatly exceeding Japanese standards. In 2002 during the health consultation in India, our questionnaire survey showed that many Japanese people living in India were aware of the health problems associated with air pollution, respiratory diseases in particular [8].

Near Malaysia, where Subang Jaya Medical Center is located, large-scale forest fires create smoke pollution (called “haze”) during the dry season, causing respiratory diseases to residents of the surrounding areas. Every year in this region, fires are set to clear forests for agriculture. During abnormally dry weather, fires can rage out of control. Large-scale smoke pollution occurred in 1991 and 1997, causing health problems for many inhabitants. Reports by Takeuchi in 2000 showed that many Japanese people living in Malaysia suffered from symptoms of respiratory disease, mainly those of upper respiratory tract infection. And many were concerned about the lack of information and effectiveness of local medical treatment [9].

In Kuala Lumpur our questionnaire survey showed that many Japanese living there had symptoms of respiratory diseases. Most Japanese people knew of haze, and many felt that the air pollution problem was serious even though the survey showed that relatively few of them were aware of the health problems caused by air pollution.

As more and more Japanese people migrate to Asian regions, the need for education in prevention of environmental diseases is becoming critical. Air pollution, temperature and humidity fluctuations, newly emerging infections and other factors are increasing. Although respiratory diseases caused by changes in weather and air pollution are apt to be overlooked because the early-stage symptoms are relatively light, adequate preventative measures are required in view of the fact that irreversible damage to health can occur with long-term exposure.

Based on the results of this research, we will continue to develop and issue preventative measures, and continue our research efforts in the Asian region.

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A PILOT FIELD TRIAL OF AN *IN VITRO* DRUG SUSCEPTIBILITY TEST USING THE ANAEROPACK® MALARIA CULTURE SYSTEM ON THE THAI-MYANMAR BORDER

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Abstract: The AnaeroPack® malaria culture system with a portable thermostat incubator was evaluated in a field laboratory on the Thai-Myanmar border conducting *in vitro* drug susceptibility tests on blood samples from 5 Karen children infected with *P. falciparum.* Only one isolate was susceptible to chloroquine; the others were highly resistant. The IC_{50} value of an isolate was only resistant to mefloquine, whereas the values of the 3 patients who presumably showed recrudescence were slightly elevated in the susceptible ranges. These results suggested that chloroquine should no longer be used for *P. falciparum* malaria in this geographic area, and that mefloquine should be carefully monitored for its *in vivo* effectiveness. In this study, the AnaeroPack® malaria culture system with portable thermostatic incubator is a powerful and useful mobile tool, which aids in providing detailed evidence-based distribution data concerning of drug resistant malaria in the field.
Key words: AnaeroPack®, Drug susceptibility test, *Plasmodium falciparum*

INTRODUCTION

Since chloroquine-resistant *Plasmodium falciparum* was first reported in 1959 in Thailand, it has developed resistance to all commonly used drugs [1]. The spread of multi-drug resistant *P. falciparum* is now a major public health problem worldwide, with prophylactic and therapeutic implications [2]. Evidence-based detection of drug-resistant parasites is important for the accurate evaluation of susceptibility to antimalarial drugs. However, isolation of fresh parasites for *in vitro* drug susceptibility testing is often difficult in the field because of the precise experimental conditions needed for the test. *In vivo* susceptibility tests are frequently conducted, but they sometimes fail because of difficulties in following up the patients. In fact, it is sometimes difficult to determine whether an adequate drug concentration has been successfully achieved in the patients' blood. Therefore, *in vitro* tests are indispensable to determine the exact degree of resistance acquired by the parasites.

We previously reported that the AnaeroPack® gas system can be used for the continuous cultivation of both laboratory strains and fresh isolates of *P. falciparum* from patients [3]. This gas system is safer, simpler, and easier to use than the candle jar method. In this study, we evaluated the AnaeroPack® malaria culture system with a portable thermostat incubator in a field laboratory on the Thai-Myanmar border to conduct *in vitro* drug susceptibility tests on *P. falciparum.* The feasibility of the system and the results of the tests are described in this report.

The study was conducted at Rajanagarindra Tropical Disease International Center, a field laboratory center of the Faculty of Tropical Medicine, Mahidol University, located in Suan Phung, Rachaburi, about 200 km west of Bangkok. The area was inhabited by Karen as well as Thai people, and in 2001 more than 6,000 people were subjected to microscopic diagnosis of malaria in the center with about 1,000 positive cases found. Malaria is endemic throughout the year in this area with the peak season occurring around May and June. Mefloquine is currently the first drug of

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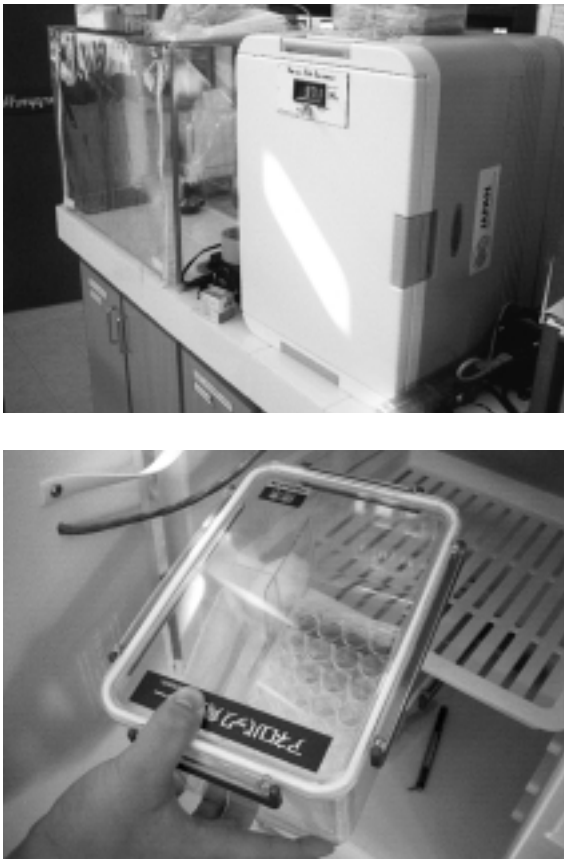
choice for the treatment of uncomplicated malaria, and artesunate, a qinghaosu derivative, is administered in complicated cases.

The AnaeroPack® CO_2 (Mitsubishi Gas Co., Tokyo, Japan) is a foil-packed paper sachet that on exposure to air immediately absorbs atmospheric O_2 and simultaneously generates CO_2 until a condition of 15% O_2 and 5% CO_2 is attained. The microaerophilic atmosphere produced within a sealed jar (AnaeroPack® Kakugata jar, SUGIYAMA-GEN Co., Ltd., Tokyo, Japan) can be maintained for at least 24 hours. A portable thermostat incubator (SUGIYAMA-GEN Co., Ltd.) was carried to the laboratory, and the temperature inside the incubator was adjusted to 37℃. During *P. falciparum* cultivation, the sachet inside the jar was replaced with a new sachet every day when the culture medium was changed (Fig. 1).

The *in vitro* drug susceptibility test used in this study was a modified semi-micro test described previously [4, 5]. The following procedures were conducted right after the sampling in the field laboratory center. Briefly, blood samples were washed 3 times with RPMI 1640 and resuspended in RPMI 1640 (GIBCO BRL), pH 7.4, supplemented with 10% human serum (from non-immune Japanese donors without a previous history of malaria; Blood Type A) and 25 μg/ml gentamicin (Sigma, Saint Louis, Mo), 25 mM HEPES, and sodium bicarbonate at a hematocrit of 5%. Five hundred microliters of the erythrocyte suspension were placed in each well of a tissue culture plate (24-well flat bottom, Corning Costar, NY). Chloroquine diphosphate was purchased from Sigma. Mefloquine hydrochloride was kindly provided by Hoffman-La Roche Ltd., Basel. Twenty microliters of chloroquine diphosphate or mefloquine was added to each well (for chloroquine to create a series of doubling dilutions from 20 to 10240 nM, and for mefloquine to create a series of 10 times dilutions from 0.01 to 1000 nM dissolved in distilled water). To monitor parasite growth, 6 wells per plate served as controls without antimalarials. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from each well, and we counted the number of erythrocytes microscopically in the control smears until encountering 50 schizonts. We defined the schizonts as parasites that have both dark brown pigment and more than 3 nuclei [6]. The effect of antimalarials on parasite growth was evaluated by observing the decreased number of schizonts per equal number of erythrocytes counted previously in the control cultures. The percentage of growth inhibition effect was calculated as follows: test well schizont count/control well schizont count (50) x 100. Fifty percent growth inhibitory concentration (IC_{50}) was calculated from the inhibition curve obtained from the values by a statistical probit method.



(A) (B)

Figure 1. AnaeroPack® culture system. (A) the portable incubator and (B) the AnaeroPack® Kakugata jar with a culture plate inside.

Blood samples were obtained from 5 Karen children age 5 to 13 years (Table 1). The guardians gave written consent to this study, which had been approved by the Ethical Committee of Mahidol University. This survey research also followed the ethical guidelines for epidemiological studies by the Ministry of Education, Culture, Science and Technology and Ministry of Health, Labour and Welfare of Japan. Three of the 5 patients had a history of *P. falciparum* malaria within 1 month, and they had received mefloquine at that time. The parasitemias of the patients were relatively low, 0.05-0.20%. The incubation period (the time needed for the tested isolates to grow to schizonts) ranged from 48 to 96 hrs. The IC_{50} values of the isolates for chloroquine varied from 91 to 402 nM, with a geometric mean of 273 nM and SD of 90 nM. Only one isolate, from patient B, was susceptible to chloroquine; the others were highly resistant. The IC_{50} values of the isolates for mefloquine varied from 4-52 nM, with a geometric mean of 24 nM and SD of 13 nM. The IC_{50} value of the isolate from patient A was only resistant to mefloquine, whereas the values of the 3 patients who

Table 1 Profiles of the *P. falciparum* isolates from 5 patients in Suan Phung

No.	Age	Sex	% Parasitemia	Day of recrudescence	Chloroquine		Mefloquine	
					IC_{50} (nM)	Judgment*	IC_{50} (nM)	Judgment**
A	12	female	0.30	none	231	Resistant	52	Resistant
B	13	male	0.05	D29	91	Susceptible	18	Susceptible
C	8	male	0.20	D24	358	Resistant	27	Susceptible
D	12	male	0.11	none	285	Resistant	4	Susceptible
E	5	female	0.11	D22	402	Resistant	17	Susceptible

* Threshold of IC_{50} for chloroquine resistance is 114 nM [5].
** Threshold of IC_{50} for mefloquine resistance is 40 nM [9].

presumably showed recrudescence were slightly elevated in the susceptible ranges. These results suggested that chloroquine should no longer be used to treat *P. falciparum* malaria in this geographic area, and that mefloquine should be carefully monitored for its *in vivo* effectiveness.

The World Health Organization's test with the candle jar is the standard method used to cultivate parasites for drug susceptibility testing in the field [7]. This method is sometimes difficult to carry out under field conditions. Our study found the AnaeroPack® system to be an effective alternative method for cultivation of malaria parasites and for *in vitro* drug susceptibility testing in the field. The AnaeroPack® gas system does not need a catalyst, water, or hydrogen for the activation of gas production, and the gas jars or incubator chambers are lightweight and portable. Our findings were consistent with other reports that the AnaeroPack® culture system produced acceptable results for the growth of malaria parasites in the laboratory application. The IC_{50} values attained with the AnaeroPack® CO_2 were similar to those attained by the candle jar method [3, 4, 8]. In addition, the thermostatic incubator of AnaeroPack® malaria culture system can now be powered by a car battery. The AnaeroPack® malaria culture system with portable thermostatic incubator is a powerful and useful mobile tool that helps to provide detailed evidence-based distribution data concerning drug resistant malaria in the field.

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SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* IN VIETNAM

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Abstract: Shiga-toxin producing *Escherichia coli* isolated in Vietnam were examined. The 9 isolates included 5 from 400 diarrheal patients and 4 from 42 cows in 2003. No isolate carried *eae* gene. All human isolates except one and one of four animal isolates carried both *stx*1 and *stx*2. One of the human isolates could not be definitely identified as a Shiga-toxin producing *Escherichia coli*, because it showed positive for PCR using a common primer set for *stx* gene family and also positive for *stx*2d subtype, but negative for *stx*1, *stx*2 and for expression of the toxin. The serotypes of the isolates were various, but major serogroups such as O157, O26 and O111 were not found.

Enterohemorrhagic *Escherichia coli*(EHEC) has been recognized as a distinct class of diarrheagenic *E. coli* since 1982, when outbreaks of hemorrhagic colitis were observed in the United States and Canada [1, 2]. Prior to these outbreaks, in 1980, verocytotoxin-producing *E. coli* was reported in classical enteropathogenic *E. coli*, EPEC [3]. In the early 1980s, O'Brien *et al*. investigated an *E. coli* O26 strain and purified a toxin closely related to Shiga-toxin of *Shigella dysenteriae* type1 [4, 5]. EHEC was once called Verotoxin producing *E. coli*(VTEC), and recently it is commonly called Shiga-toxin-producing *E. coli*(STEC). Readers in the other research fields thus have to keep in mind that the terms EHEC, VTEC and STEC are basically the same. We use the term STEC hereafter. After the initial outbreaks of STEC infection in 1982, STEC attracted worldwide attention because the illness is clinically severe and can be followed by serious sequels such as hemolytic uremic syndrome (HUS) and central nervous disorder. Outbreaks involving hundreds of individuals cause a sensation, but sporadic infections are frequently overlooked unless the illness is serious. Nevertheless, the sporadic infection represents the major impact of this pathogen. The epidemiology of the illness and the distribution of STEC are well studied in industrialized countries [6], but there are not many reports from the developing world. STEC strains were actively sought in a study on Bangladeshi children, but no child was infected with STEC [7]. In Lao People's Democratic Republic, 880 diarrheal patients randomly collected in1996 and 1997 were examined, but STEC with O111 serogroup was detected in only1 patient. Also, 1 strain without *eae* gene was detected among 278 patients examined in 2002 [8, 9]. Detection of STEC in Thailand was also reported but serotype O157:H7 strains were not found [10]. In the present study, we intensively examined human diarrheal stools and cattle stools for STEC in Vietnam.

E. coli strains were isolated from human diarrheal stools and cow's stools. The human diarrheal stools were collected from 400 patients in Nam Dinh province, and the cow stools were collected from 42 animals at a stock farm in a suburb of Hanoi in 2003. The isolated *E. coli* were first examined by multiplex PCR as described by Toma *et al*. [11]. *stx* positive strains were examined again using a single primer set (v1/v5 and v3/v4) to determine the toxin type. Primer set v1 (5'AGTTAATGTGGTGGCGAA3') and v5 (5'GACTCTTCCATCTGCCG3') was used to amplify 811 base pairs of stx_1. Primer set v3 (5'TTCGGTATCCTATTCCCG

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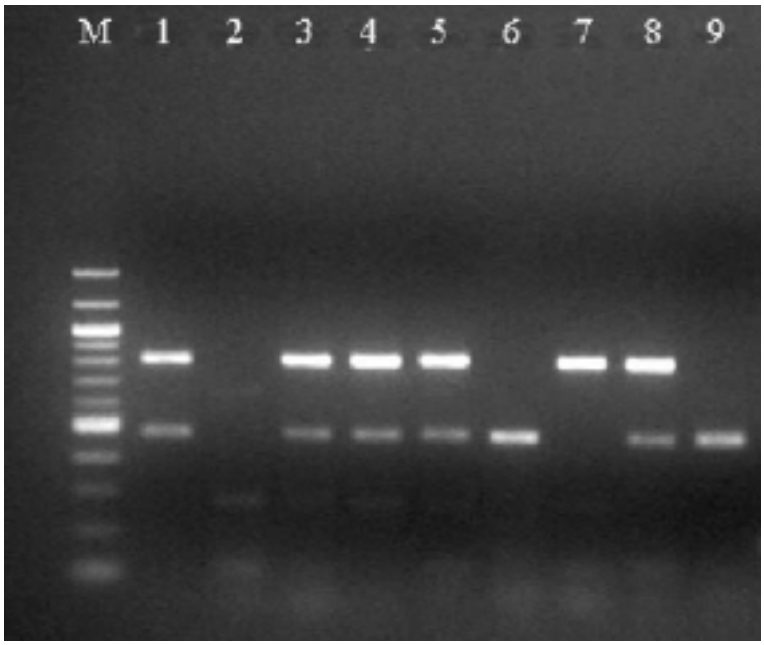
3') and v4 (5'TCTCTGGTCATTGTATTA3') was used to amplify 471 base pairs of stx_2 and its variants [12]. Further classification of the stx_2 gene family was performed using primers reported by Wang *et al.* [13]. DNAs were extracted from the organisms as described by Yokoyama [14]. The PCR mixture used with a single primer set consisted of a total volume of 30 µl containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.75 U of *Taq* DNA polymerase (Toyobo, Osaka, Japan), 0.2 mM deoxynucleoside triphosphate, a 0.25 µM concentration of each primer and 3 µl of the DNA template. The PCR program was 95℃ for 1 min, 52℃ for 1 min and 72℃ for 1 min, for 25 cycles. *stx* gene positive *E. coli* were examined for production of Shiga toxin using reversed passive latex agglutination kit VTEC-RPLA (DENKA-SEIKEN Co. LTD., Tokyo). The organisms were cultured in CAYE medium as indicated in the manual attached to the kit. O:H serotyping of the strains was performed by bacterial agglutination as described previously by Orskov F., and Orskov I. [15].

STEC was detected in 5 of the 400 patients examined (1 of the 5 was questionable). Therefore, the frequency of the definite isolation of STEC was 4/400 (1.0%). STEC was also detected in 4 of the 42 cows examined (9.5%). Serotype O157:H7 was not found. Three of the 9 isolates belonged to serotype O44:H16. In one isolate, O-antigen was untypable but H8; the other 5 isolates belonged to serotypes O8:H8, O100:H40, O159:H21, O55:H11 and O2:H42 (Table1). The toxin type was variable and all 9 isolates were negative for the *eae* gene. PCR for toxin typing showed that the 4 human isolates (O1-A, O3-D, O4-C, O6-B) carried both stx_1 and stx_2 and that the one questionable isolate was positive for primer set VTcom-u / VTcom-d, common to *stx*1 and *stx*2, but did not react with the primer set v1/v5 and v3/v4 specific to *stx*1 and *stx*2, respectively. Nevertheless, using the primers for detecting various genes belonging to the *stx*2 family reported by Wang *et al.,* this isolate (O2-C) produced a relevant amplicon compatible with *stx*2d. Moreover, it did not produce the toxin when examined by RPLA. Since the details are not available at present, it is questionable whether this isolate can be regarded as STEC. Among the animal isolates, one (93-6) carried both stx_1 and stx_2, and the other three carried one of them (Table1 and Fig.1). The production of toxins was compatible with the genotype, except for the one questionable isolate.

To the best our knowledge, this is the first study on STEC isolated in Vietnam and the first to suggest that STEC strains have already spread in this country. However,

Figure 1



Lanes, M:100-bp DNA ladder (upper broad band indicates 1000 bp and lower broad band indicates 500 bp), 1:O1-A, 2:O2-C, 3:O3-D, 4:O4-C, 5:O6-B, 6:5-5, 7:18a., 8:93-6, 9:2245-2 Primer sets v1/v5 and v3/v4 were used to detect 811 bp product of *stx1* and 471 bp product of *stx2*, respectively. Lane2 (questionable strain O2-C as mentioned in the text)

Table 1

No.	strains	PCR				RPLA			serotype
		stx	*stx*1	*stx*2	Sub.	*eae*	Stx-1	Stx-2	
1	O1-A	+	+	+	2c, 2d	−	+	+	O44:H16
(2)∗	(O2-C)∗	(+)∗	(−)∗	(−)∗	(2d)∗	(−)∗	(−)∗	(−)∗	(O8:H8)∗
3	O3-D	+	+	+	2	−	+	+	O100:H40
4	O4-C	+	+	+	2	−	+	+	OUT:H8
5	O6-B	+	+	+	2c	−	+	+	O159:H21
6	5-5	+	−	+	2c, 2d	−	−	+	O44:H16
7	18a	+	+	−	−	−	+	−	O55:H11
8	93-6	+	+	+	2c	−	+	−	O2:H42
9	2245-2	+	−	+	2d	−	−	+	O44:H16

No. 1-5: human isolates, No. 6-9: cattle isolates, OUT: O-antigen untypable, Products from the primer set VTcom-u and VTcom-d (*stx*), v1 and v5 (*stx*1), v3 and v4 (*stx*2), subtypes of *stx*2 family identified using primer sets described by Wang *et al*. (12). ( )∗: to be discussed

it is unknown whether these STEC originally developed in Vietnam or came from somewhere else. Serotypes of STEC in Vietnam are different from those in neighboring countries [8, 9, 10], and the organisms did not carry *eae* gene. Since the clinical data were not obtained in the present study, the pathogenicity of these isolates remains unclear. But the fact that the isolates lacked an important pathogenic gene (*eae*) indicates the necessity for further studies on the clinical findings and pathogenicity of these organisms. The adhesive factors encoded outside of the locus enterocyte effacement are now under intensive investigation in our laboratory.

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