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A new approach for the analysis of seroprevalence data: a mathematical analysis of the seroprevalence of *Toxoplasma gondii* infection in Hyogo prefecture (Japan) with an implication of periodic outbreaks among young children.

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Abstract: We propose a new approach for the analysis of seroprecvalence data. The seroprevalence date on *Toxoplasma gondii* infection published in 1997 at Kobe University Hospital were analyzed, with a simple mathematical model, $Y = \exp(-\lambda t)$, where Y, λ and t represent the percentage of seronegative people, annual infection rate and age, respectively. After calculating the mean annual infection rate (MAIR) on the basis of the above data, we determined the relationship between MAIR and the year of birth of the female participants in the study. Our present study indicates that MAIR for women born between 1940 and 1960 decreased over the years, and that infection rates correlated with the year of birth. Moreover, assuming that the historically declining trends of MAIR mainly reflect infection rate changes in childhood, we created a simulation of MAIR for the age under 5 years. This simulation demonstrated that MAIR for the age under 5 years decreased from around 4% for females born in 1940, to about 1% for those born in 1960. For women born after 1960, it implied that infection rates might have been cyclically fluctuating from 0% to 1%, with an approximate 10-year interval. Our analyses imply periodic outbreaks of *T. gondii* infection among young children in Hyogo prefecture. In Japan, it is difficult to make a sophisticated statistical analysis of seroprevalence of *T. gondii* mainly due to the lack of available data. Despite simplicity of our new approach, we believe the approach will be useful to grasp the current and the past situations of *T. gondii* infection even without enough data.
Keywords: *Toxoplasma gondii*, congenital toxoplasmosis, mathematical analysis, mean annual infection rate (MAIR), cyclical outbreaks.

INTRODUCTION

Toxoplasma gondii, an obligate intracellular parasitic protozoan, induces commonly benign or asymptomatic toxoplasmosis in healthy persons (Dubey and Beattie, 1988). However, it may have harmful effects on the fetus when pregnant women contract primary infection, although rare cases of congenital toxoplasmosis (CT) from patients with chronic infection have been reported (Dollfus *et al.,* 1988).

As we reported previously (Naoi and Yano, 2002), the risk of CT is dependent on annual infection rates before 15 years of age, as well as during the gestational period (equal to or older than 15 and younger than 45 years old). Hence, the historical trends of annual infection rates are crucial for evaluating the risk of CT currently faced by pregnant women. In 1997, Win *et al.* (1997) compared the seroprevalence data of *T. gondii* infection between 1981 and 1995. The study shows that less people under 60 acquired new *T. gondii* infections from 1981 to 1995, and that individuals under 40 contracted few *T. gondii* infections throughout their life. Furthermore, the data suggest that the incidence of *T. gondii* infection is relatively higher in children than adult women. Although these implications are informative, the study does not reveal historical trends of infection, since only two sets of seroprevalence data (taken in 1981 and 1995) are available.

Using a simple mathematical model, we devised a simulation of the historical trends of annual infection rates for females, based on the sampled data. We initially calculated the mean annual infection rates (MAIR) and examined the relationship between MAIR and the year of birth of the women taking part in the sampling study. Next, we calculated MAIR of women for the age under 5 years from 1940 to 1990, with the assumption that MAIR changes mainly re-

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flect infection rate trends at a relatively young age.

METHODE

In this study, we employed an exponential curve with a constant hazard, or $Y = \exp(-\lambda t)$ (1), mainly used for survival analysis. For survival analysis, Y, λ and t represent the percentage of people alive, annual mortality rate (which is constant) and years after interventions, respectively. By assuming that the annual infection rate is constant and the percentage of seropositivity is cumulative, Eq. (1) is applicable to the simulation of *T. gondii* infection, in which, Y, λ and t represent the percentage of seronegative people, annual infection rate and age, respectively.

By solving Eq. (1), we obtain the equation: $\lambda = (-t)^{-1}\ 1n\ Y$ (2), in which λ is defined as the mean annual infection rate (MAIR). In this study, we calculated MAIR for the female participants examined by Win *et al.*, (1997), using Eq. (2).

We simulated MAIR for the age under 5 years in the following manner: Consider a hypothetical case in which the percentage of seronegative women 30 years old in 1995 is Y^*. With the assumption that MAIR for the age between 5 to 30 years is λ_2, we obtain a hypothetical age, or t^*, as $t^* = (-\lambda_2)^{-1}\ 1n\ Y^*$ (3), which may be different from the real age, or 30 years old. The percentage of seronegative women at 5 years of age, or Y^{**} is defined as $Y^{**} = \exp\{-\lambda_2 [\ t^*-(30-5)]\} = \exp[-\lambda_2 (\ t^*-25)]$ (4). If we define MAIR for the age under 5 years as λ_1, we acquire $Y^{**} = \exp[-\lambda_2 (\ t^*-25)] = \exp(-5\lambda_1)$ (5).

By solving Eq. (5), λ_1 is calculated as $\lambda_1 = (-5)^{-1}\ 1n\ Y^{**}$ (6).

RESULTS

MAIR are presented in Table 1. The same data are illustrated in Fig. 1, in which historical downward trends are clearly indicated from 1940 to 1960. We analyzed data from 1940 to 1960 with a regression model to determine whether there is a correlation between the year of birth and MAIR. In addition, we evaluated statistical differences between the data of 1981 and 1995.

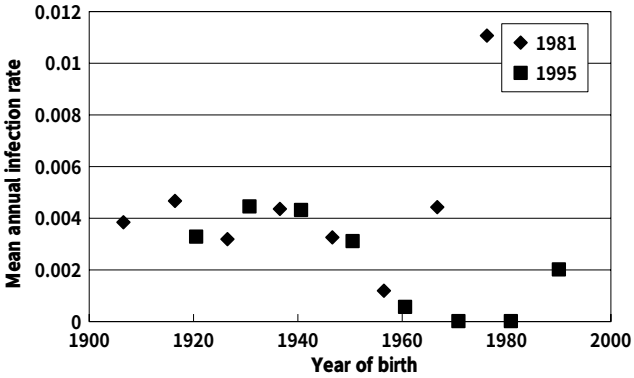

Figure 1 Longitudinal trends of MAIR (mean annual infection rate) of female in Hyogo prefecture.

The regression model

$MAIR = \alpha + \beta\ (\text{year of birth}) + \gamma D + \varepsilon$

D = 0 (for 1981), = 1 (for 1995).

We acquired the following results:

MAIR = 37.38-0.01904 (year of birth) + 0.02329D

D = 0 (for 1981), = 1 (for 1995).

R^2 =0.9676, adjusted R^2 = 0.8724, s = 0.03942

95% confidence interval for β: $-0.02977 < \beta < -0.08311$

Table 1 Seroprevalence of *T. gondii* in Kobe University Hospital

1981				1995			
age	MA	YOB (mean)	MAIR (%)	age	MA	YOB (mean)	MAIR (%)
1to9	5	1976	1.110	1to9	5	1990	0.201
10to19	14.5	1966.5	0.449	10to19	14.5	1980.5	0.000
20to29	24.5	1956.5	0.124	20to29	24.5	1970.5	0.000
30to39	34.5	1946.5	0.328	30to39	34.5	1960.5	0.059
40to49	44.5	1936.5	0.438	40to49	44.5	1950.5	0.313
50to59	54.5	1926.5	0.324	50to59	54.5	1940.5	0.433
60to69	64.5	1916.5	0.471	60to69	64.5	1930.5	0.446
70to79	74.5	1906.5	0.386	70to79	74.5	1920.5	0.334

(Calculated with the simple mathematical model, $Y = \exp(-\lambda t)$, Y = seronegativity, λ = mean annual infection rate, t = age)
MA: mean age, YOB: Year of birth, MAIR: mean annual infection rate in %

(statistically significant for $\beta < 0$, $p < 0.05$)

95% confidence interval for γ: $-0.1319 < \gamma < 0.1785$

n = 5, the birth year from 1940. 5 to 1960. 5 in Table 1.

The real value and the estimated value of MAIR based on the regression model are illustrated in Fig. 2. The results indicate that MAIR is negatively correlated with the year of birth. However, no statistically significant evidence of differences or a shift of curves, let alone historical decrease, were noted between the data of 1981 and those of 1995.

We presume that the historical downward trends in MAIR mainly reflect a decline in the incidence of *T. gondii* infection among younger children. As shown in Table 1 and Fig. 1, MAIR of children for the age under 5 years significantly decreased (from 1.11% in 1981 to 0.201% in 1995). However,our regression model analyses do not indicate a statistically valid reduction in MAIR from 1981 to 1995 for women born between 1940 and 1960 (age variation from 21 to 50 years). These results imply that MAIR changes historically during childhood rather than adulthood.

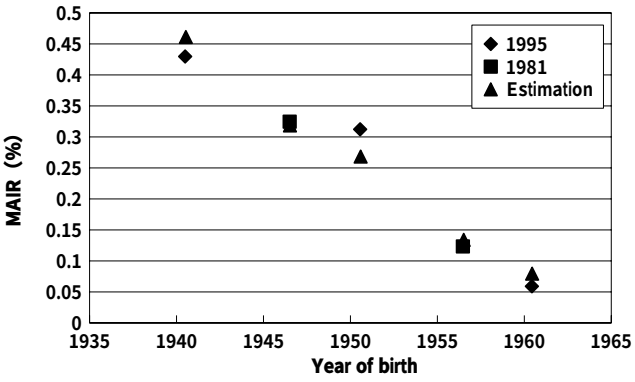

Figure 2 MAIR (mean annual infection rate): real vaues and estimations.

Table 2 Simulation of MAIR before 5 years of age

λ_2	1940.5	1946.5	1950.5	1956.5	1960.5
0	0.0471	0.0226	0.0279	0.00609	0.00404
0.0001	0.0462	0.0220	0.0271	0.00570	0.00345
0.0003	0.0452	0.0215	0.0263	0.00531	0.00286
0.0005	0.0422	0.0197	0.0239	0.00414	0.00109
0.0006	0.0412	0.0190	0.0231	0.00375	0.00050
0.0007	0.0402	0.0185	0.0223	0.00336	(<0)
λ_2	1966.5	1970.5	1976	1980.5	1990
0	0.0130	0	0.0111	0	0.00201
0.0001	0.0128	0	0.0111	0	0.00201
0.0003	0.0126	0	0.0111	0	0.00201
0.0005	0.0121	0	0.0111	0	0.00201
0.0006	0.0119	0	0.0111	0	0.00201
0.0007	0.0117	0	0.0111	0	0.00201

*λ_2: MAIR (mean annual infection rate) after 5 years of age.

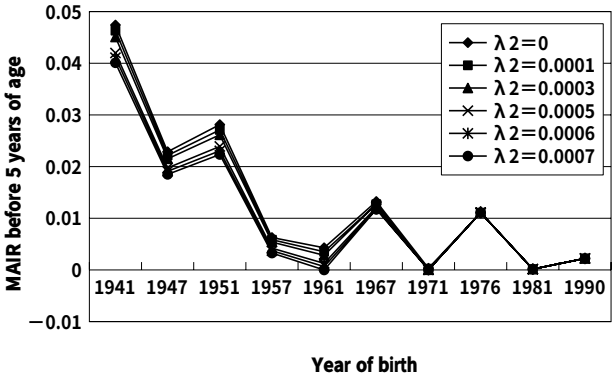

Figure 3 Simulation of MAIR for female before 5 years of age. λ2: Hypothetical value of MAIR after 5 years of age, varied between 0% and 0.07%.

We simulated MAIR of children for the age under 5 years, as explained in Methods, in which we varied λ_2 between 0% and 0.07% (Table 2 and Fig. 3). Figure 3 implies that MAIR for the age under 5 years basically decreased from 1940 until 1960, and may heve been fluctuating cyclically within a range of 0% to 1%, with an approximate 10-year interval, since 1960.

DISCUSSION

A number of reports demonstrate historically declining trends in the incidence of *T. gondii* infection in Europe (Ades and Nokes, 1993; Nokes *et al.*, 1997). In these reports, reasons for the decrease in incidence include increasing consumption of frozen meat, improvement in hygiene, and the increased use of processed and sterilized cat food, among others. In our present report, it is noteworthy that the term of MAIR reduction (from 1940 to 1960) includes 1945, the year of the end of World War II. Dramatic social changes related to the war as well as society reconstruction during the aftermath may have affected the decline in MAIR through this period.

An epidemiological study on *Helicobacter pylori* by Japanese scientists presents an interesting viewpoint on the correlation between the year of birth and incidence of infectious diseases in Japan (Asaka *et al.*, 1992). This study reported that seropositivity for *H. pylori* is higher in individuals born before 1950. They attributed this decline in seropositivity to the Westernization of Japan. Although many differences are prevalent between *H. pylori* and *T. gondii*, including species and infection routes, epidemiological studies on the bacteria may provide new perspectives for further research on the causes of historical reduction in

MAIR by the protozoan.

Based on our simulation, possible periodical outbreaks of *T. gondii* infection among young people are speculated. This speculation does not contradict our clinical experiences, which indicate periodic outbreaks of CT. Mothers are generally expected to be exposed to the same risk factors for *T. gondii* infection as their younger offspring as they care for them, and therefore *T. gondii* infection via contact with oocysts in cats'feces or contaminated soil is a significant threat for women as well as children. From the standpoint of risk management, public preventive measures, based on comprehensive research on infection routes for mothers and children, behavioral patterns and lifestyles are required.

From the viewpoint of strict statistical analysis, however, our simulation may face some criticism, mainly on the original data (Takahashi *et al.*, 1985; Win *et al.*, 1997), as follows:

(1) The seroprevalence data of *T. gondii* used in this simulation are of outpatients in a certain hospital, not of randomly selected samples from the general people in Kobe prefecture.
(2) The data of 1981 and those of 1995 are not prospective but cross-sectional data.
(3) For obtaining the data, self-identification was not executed.
(4) The number of samples is 100 by each age category in the 1995 survey, while it varies from 7 to 149 in the 1981 survey. Hence, for devising the simulation, interval estimation rather than point estimation will be preferred.

Currently in Japan, starkly different from the European nations, seroprevalence data of *T. gondii* infection are scarcely available, not to speak of those of randomly selected samples. While clinical cases of CT are reported in Japan, the recognition of the disease is still less prevalent even among medical practitioners here than in Europe. Therefore, new analytical methods, which are useful for researchers of CT to enrich their understanding about the disease even without enough data, are required. We believe that our new approach would be one of those methods. And we hope that our simulation, an example of analysis with MAIR, would be of use to find new perspectives for future epidemiological studies, albeit some controversies over original data and the way of estimation.

We believe that MAIR is an effective tool in mathematical analysis, which reflects the history of infection as a whole and presents an average of incidence in the past. Although in the current analysis, it is simply presumed that MAIR alterations mainly feflect changes during childhood, other assumptions based on comprehensive epidemiological studies in the future are also possible. On the basis of these presumptions, conclusions distinct to those of the present study may be drawn.

Current model analyses are made in an age-and time-dependent manner by European statisticians, based on several decades of seroprevalence data (Ades and Nokes, 1993). However, these kinds of data are not available in Japan. Although continuous sampling studies of seroprevalence are requested, it may take numerous more decades in Japan to obtain data extensive enough for similar statistical analyses as those in Europe. In the meantime, MAIR and related methods for mathematical speculations on *T. gondii* infection are effective, and the current procedures of choice in Japan.

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CHARACTERIZATION OF *VIBRIO CHOLERAE* O1 ISOLATED IN VIETNAM

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Abstract: *V. cholerae* O1 isolated in Vietnam in 1995 and 2000 were characterized. Most of the isolates in 2000 showed moderate resistance to tetracycline and chloramphenicol, and strong resistance to sulfamethoxazol-trimethoprim. The susceptibilities to ampicillin, erythromycin, nalidixic acid and ofloxacin were not very different from those of the isolates in 1995. *V. cholerae* O1 strains showing this kind of drug susceptibility pattern are unique to the seventh cholera pandemic, and they have become widely distributed in Southeast Asian countries. Genetic analysis of the strains from Vietnam by arbitrarily primed polymerase chain reaction suggested that they might be clonal derivatives. In prophage type, 6 of 20 strains (30%) in 1995 and 1 of 24 strains (4%) in 2000 belonged to Ubol type. Considering the current spreading of *V. cholerae* O1 with such unique susceptibility to the drugs, we recommend careful monitoring of the drug susceptibility of *V. cholerae* O1 throughout the world.
Key words: *Vibrio cholerae* O1, drug susceptibility, Vietnam

INTRODUCTION

It has been more than 40 years since the seventh cholera pandemic started in 1961. During that period, the responsible pathogen, *Vibrio cholerae* O1 El Tor has undergone some changes in its biological behavior. This pathogen was first distinguished from classical cholera vibrios based on its hemolytic property, and thereafter, some other unique properties of El Tor vibrios were described (Feeley, 1966). These unique properties include the following: El Tor vibrios are resistant to polymyxin B and cholera phage IV, produce acetoin from glucose (positive VP reaction), and agglutinate chicken erythrocytes when grown on nutrient agar plates. Soon after the beginning of this pandemic, the hemolytic property, as examined by Feeley and Pittman's routine method of testing (Feeley and Pittman, 1963), disappeared in most isolates, but it reappeared in African isolates in the 1980s (Iwanaga *et al.,* 1982). Cholera phage IV-sensitive El Tor vibrio appeared in Thailand in 1986 (Iwanaga *et al.* 1989). Drug resistant vibrios have appeared and disappeared (Ichinose *et al.,* 1986; Glass *et al.,* 1980; Mhalu *et al.,* 1979). Recently, the isolates in Laos were well characterized, and unique phenotypes such as polymyxin B sensitive and moderately resistant to tetracycline were found (Toma *et al.,* 1997; Phantouamath *et al.,* 2001). Polymyxin B sensitive strains were isolated in Southern Laos facing the border to the central area of Vietnam. However, general characterization of *V. cholerae* O1 in Vietnam has not been carried out yet. In this study, we characterized *V. cholerae* O1 isolated in Vietnam in 1995 and 2000 in comparison with the previously characterized strains in the neighboring country, Laos, in an attempt to understand the transmission of cholera through the border.

MATERIALS AND METHODS

1. Bacterial strains:

Forty-four strains of *V. cholerae* O1 isolated from cholera patients in Vietnam were used. Twenty strains were isolated in 1995 and 24 were isolated in 2000. Several strains of Lao isolates in 2000 were used for the comparative studies. The strains H218 (biotype classic, serovar Ogawa) and UDT109 (non-O1 *V. cholerae*) were used as the indicator organisms for *kappa* phage and fs phages, respectively. Identification of *V. cholerae* O1 was made based on standard laboratory tests.

2. Hemolytic property:

The hemolytic property was examined using Feeley and Pittman's method (Feeley and Pittman, 1963) and sheep blood agar plate cultures with one point stabbing inocula-

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tion. Strains that appeared to be non-hemolytic based on these methods were further examined by using heart infusion broth (HIB) supplemented with 1% and 3% glycerol following the same procedures as in Feeley and Pittman's method.

3. Production of acetoin

The organisms were inoculated into VP-semisolid agar (Eiken) and cultured at 37℃ for 24 hours. Production of acetoin was then examined by pouring a few drops of 5% alpha-naphthol and 40% potassium hydroxide over the cultures, and assessing the development of red color.

4. Production of and sensitivity to phages:

To determine the prophage type of the isolates, *kappa* phage was examined as described by Takeya and Shimodori (1963). Production of the filamentous phages fs-1 and fs-2 was examined by using the indicator strain *V. cholerae* UDT109. *V. cholerae* O1 isolates were cultured in HIB (10 ml in 100-ml Erlenmeyer flasks) at 37℃ overnight with reciprocal shaking, and the culture supernatants were used as the samples for detection of fs-1 and/or fs-2. The indicator strain was cultured overnight in broth, and 0.1 ml of the culture fluid was mixed with 4 ml of soft agar medium (1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.6% agar, pH 7.2) and overlaid on a glass slide. Ten microlitters of this sample was dropped on to a layer of the indicator strain and incubated at 37℃ overnight. Sensitivity to the filamentous phages (fs-1 and fs-2), cholera phage IV, and El Tor phage 5 was examined according to the method described by Ikema and Honma (1998).

5. Drug susceptibility:

The minimum inhibitory concentration (MIC) of each drug was examined by the plate dilution technique. The drugs examined were ampicillin (Meiji), tetracycline (Nacarai), chloramphenicol (Wako), erythromycin (Dainippon), ofloxacin (Daiichi), nalidixic acid (Wako), sulfamethoxazol-trimethoprim (sulfamethoxazol: Wako, trimethoprim: Sigma) and polymyxin B (Pfizer). A series of heart infusion agar plates containing serial 2-fold dilutions of the drugs at concentration ranging from 100 μg/ml to 0.1 μg/ml were prepared. In the case of ofloxacin, the dilution series was extended to 0.0125 μg/ml. Sulfamethoxazol and trimethoprim (19:1) were mixed, and the concentration of the drug was expressed as the total drug weight / ml of the mixture. A dilution series from 640 μg/ml to 0.078 μg/ml of the drug combination was made in agar plates. A10-fold dilution of overnight broth culture was inoculated onto each plate using a Micro planter (Sakuma Co., model MITT # 00257), and incubated at 37℃ for 24 hours. The susceptibility was expressed as the MIC of each drug.

6. Arbitrarily primed polymerase chain reaction (AP-PCR)

The clonal variation of the isolates was evaluated based on the pattern of the products of AP-PCR obtained using a random primer AP42 (5'-CCGCAGCCAA-3') and AP46 (5'-GAGGACAAAG-3') as described by Akopyanz *et al.* (1992). The PCR conditions were 45 cycles of 94℃: 2 min, 38℃: 2 min, 72℃: 2 min, with 3 min of transitional time when raising the temperature from 38℃ to 72℃, followed by a final incubation at 72℃ for 10 min. The PCR products were electrophoresed on a 2% agarose gel for 2 hr at 50 V in an ice box. Then the gel was stained with ethidium bromide, and observed under a UV illuminator.

RESULTS

General properties of the isolates:

All isolates showed the following typical properties of *V. cholerae* O1: motile Gram-negative curved rods, yellow colonies on TCBS agar (sucrose fermentative), glucose fermentation without gas production, lactose non-fermentative, cytochrome oxidase positive, lysine decarboxylase positive and arginine hydrolase negative.

Hemolysis:

Although the hemolytic activity was generally very weak, 19 of 20 isolates (95%) in 1995 and 18 of 24 isolates (75%) in 2000 were hemolytic on sheep blood agar. When they were examined by Feeley and Pittman's method, 18 of 20 (90%) in 1995 and 12 of 24 (50%) in 2000 were hemolytic. The strains which did not show hemolysis in either method were cultured in heart infusion broth supplemented with 1% and 3% glycerol, but the hemolysis did not appear under these conditions, either.

Production of acetoin:

Examination of the production of acetoin in semi-solid VP medium (Eiken) revealed that 35% of isolates in 1995 and 62.5% of isolates in 2000 were positive.

Production of and sensitivity to phages:

The isolates in 1995 included 12 strains that produced *kappa* phage (Celebes type), 2 that did not produce the phage but was sensitive to it (Cured Celebes type) and 6 that neither produced nor were sensitive to it (Ubol type). The isolates in 2000 included 23 strains that produced *kappa* phage (Celebes type) and 1 that did not produce and was not sensitive to it (Ubol type). Most isolates (18 of 20) in 1995 were sensitive to filamentous phages fs-1 and/or fs-2. Three isolates produced fs-2, but none produced fs-1. None of the 24 isolates in 2000 produced fs phages, and 21 were not sensitive to them, while the other 3 isolates were sensitive only to fs-1 (Table 1).

Drug susceptibility:

The drug sensitivities of the isolates in 2000 were very different from those in 1995, as shown in Table 2 and Figure 1. Although the sensitivities of the isolates in 1995 and

Table 1 Production of and sensitivity to phages

Phage	20 isolates in 1995			24 isolates in 2000		
	production	sensitivity	No.	production	sensitivity	No.
kappa	+	-	12	+	-	23
	-	+	2	-	+	0
	-	-	6	-	-	1
fs-1	+	-	0	+	-	0
	-	+	17	-	+	3
	-	-	3	-	-	21
fs-2	+	-	3	+	-	0
	-	+	13	-	-	0
	-	-	4	-	-	24

Table 2 (A) Frequency distribution of MIC for 20 isolates in 1995

μg/ml	ABPC	TC	CP	EM	PLB	NA	OFLX	μg/ml	ST
0.0125≧							8	0.08	
0.025							7	0.156	
0.05							3	0.313	14
0.1						1	1	0.625	1
0.2		5				17	1	1.25	4
0.39		14				2		2.5	1
0.78			18					5	
1.56	4		2					10	
3.13	13	1		1				20	
6.25	3			7				40	
12.5				12	1			80	
25					5			160	
50					4			320	
100					2			640	
100<					8			640<	

(B) Frequency distribution of MIC for 24 isolates in 2000

μg/ml	ABPC	TC	CP	EM	PLB	NA	OFLX	μg/ml	ST
0.0125≧							4	0.08	
0.025							14	0.156	
0.05							6	0.313	1
0.1								0.625	
0.2						20		1.25	
0.39		1						2.5	
0.78			1			4		5	
1.56								10	
3.13	23	21						20	
6.25	1	2	20					40	
12.5			3	24				80	
25								160	
50								320	
100					3			640	
100<					21			640<	23

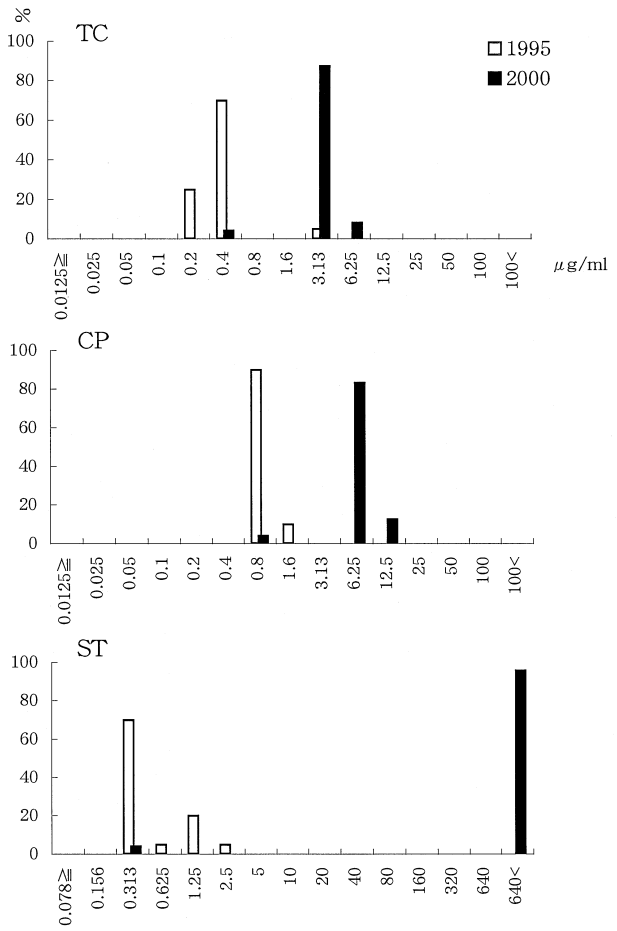

Fig. 1 Distribution of MIC of tetracycline (TC), chloramphenicol (CP) and sulfamethoxazol-trimethoprim (ST). The frequency is expressed by per cent.

2000 to erythromycin, ampicillin, nalidixic acid and ofloxacin were almost the same, the isolates in 2000 were moderately resistant to tetracycline and chloramphenicol, and highly resistant to sulfamethoxazol-trimethoprim. The MIC of tetracycline against the isolates in 1995 was 0.4 μg/ml or lower, except for 1 isolate against which it was 3.13 μg/ml, whereas the MIC against the isolates in 2000 was 3.13 or 6.25 μg/ml except for 1 isolate against which it was 0.4 μg/ml. Chloramphenicol showed almost the same pattern as that seen for tetracycline. The MIC of sulfamethoxazol-trimethoprim against the isolates in 1995 was in most case 0.313 μg/ml, but it was higher than 640 μg/ml against the isolates in 2000.

AP-PCR:

Chromosome analysis by AP-PCR using primers AP42 and AP46 was performed to determine the genetic diversity among the isolates from different areas and years. The PCR products revealed almost the same pattern, regardless of differences of phenotype, the year of isolation and the place of isolation, except in the case of 1 strain when the primer AP 46 was used. This strain was isolated in Vietnam in 1995. The AP-PCR patterns of representative isolates are shown in Figure 2.

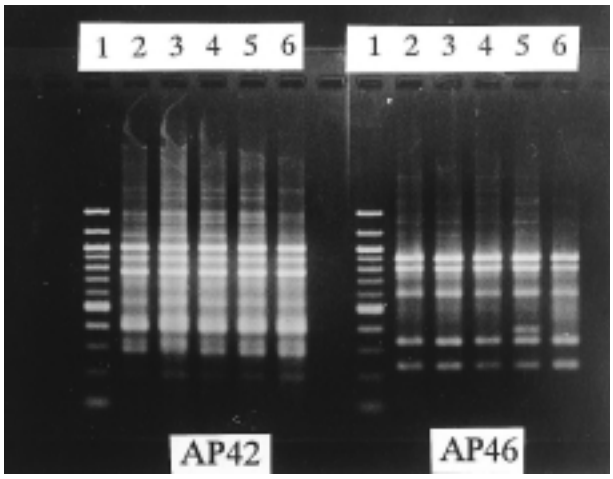

Fig. 2 AP-PCR pattern. Lane1:molecular marker (100-bp ladder), 2: strain 00Vie27 (Vietnam isolate in 2000), 3: strain 00LA4 (Lao isolate in 2000), 4: strain LA1 (Lao isolate in 2000, polymyxin B sensitive), 5: strain Vie47 (Vietnam isolate in 1995), 6: strain Vie156 (Vietnam isolate in1995). The left half of the panel shows the result obtained using primer AP42, and the right using AP46.

DISCUSSION

The general properties of *V. cholerae* O1 in Vietnam were clarified in this study. Although the isolates in 2000 were slightly different in hemolytic property, prophage type, and acetoin production from those in 1995, the changes of the drug- susceptibility patterns were notable. The characteristic drug susceptibility of the isolates in 2000 was that they were moderately resistant to tetracycline and chloramphenicol, and highly resistant to sulfamethoxazol-trimethoprim, while the susceptibilities to ampicillin, erythromycin, nalidixic acid and ofloxacin were not very different from those of the isolates in 1995. These features were very similar to those of the isolates in Laos after 1998 (Phantouamath *et al*., 2000), and also to those of the isolates in Thailand (unpublished data), except that polymyxin B sensitive strains were not found in Vietnam and Thailand. Since no plasmid was detected in the Lao isolates (Iwanaga *et al.* 1999) as well as in the Vietnam isolates (data not shown) and the phenotype of the Vietnam isolates was very similar to that of the Lao isolates, this specific susceptibility pattern may have been mediated by a chromosomal gene. Previously reported tetracycline-resistant *V. cholerae* O1 strains isolated in a cholera epidemic were highly resistant

to tetracycline as well as to other drugs; the resistance was mediated by a plasmid, and the resistant strains disappeared soon after the epidemic (Glass *et al.*, 1980). However, the chromosomally mediated resistance in organisms detected in the present study could be persistent in future epidemics. Indeed, such strains have spread in Southeast Asian countries since 1998.

Their indistinguishable phenotype and genotype imply that it is likely that the distribution and spread of these drug resistant cholera vibrios are due to the same clone. It is also suggested that the resistant strains arose from the mutation of previously sensitive strains and were not a new clone coming from some other source. One strain isolated in Vietnam in 1995 had a slightly different AP-PCR pattern (Fig. 2, lane 5 in AP46), and this strain was moderately sensitive to polymyxin B (MIC = 12.5 μg/ml), but its AP-PCR pattern was not identical to those of strains highly sensitive to polymyxin B isolated in Laos (Fig. 2, lane 4 in AP46). Therefore, the origin of the polymyxin B sensitive El Tor vibrios in Laos has still not been clarified.

In the present study of cholera due to organisms moderately resistant to tetracycline, clinical data about the prognosis of the patients treated with ordinary doses of tetracycline was not available, but it is likely that the diarrhea lasted longer than the diarrhea caused by sensitive organisms. If the use of tetracycline continues, there is a possibility that highly resistant clones will develop. Considering the current spreading of these organisms in Southeast Asian countries, careful monitoring of the drug susceptibility throughout the world is required.

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Monitoring of drug resistant *Staphylococcus aureus* in People's Democratic Republic of Lao

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Abstract: Drug susceptibility of *Staphylococcus aureus* has been examined in the past 9 years to monitor the appearance of drug resistant strains especially methicillin resistant *S. aureus* (MRSA). The pattern of drug susceptibility has been relatively constant, but *S. aureus* is gradually becoming resistant to ampicillin. One MRSA was isolated in 1996 from the nasal vestibulum of a non-infected patient at Mahosot Hospital but not isolated thereafter. In 2001, 2 strains of MRSA which belonged to different clones were first isolated from the infection foci of patients. One was isolated from Setthathirath Hospital, where many Japanese staff have been dispatched since 2000. Careful monitoring and epidemiological studies of MRSA are recommended.
Key words: *Staphylococcus aureus*, drug susceptibility, Laos

INTRODUCTION

Staphylococcus aureus is one of the most important pathogens throughout the world. Because *S. aureus* easily develop resistance to antibiotics when these drugs are used to treat infections, much attention has been focused on the appearance of drug-resistant strains. In the past 2 decades, the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) has been an especially hot issue in the clinical field. The proportion of MRSA among *S. aureus* isolates varies from country to country. One report showed that the proportion was 1.5% in the Netherlands but 25.1% in Belgium, a neighboring country, and 33.6% in France (Voss *et al*., 1994). In Thailand, which borders the People's Democratic Republic of Lao (Lao PDR or Laos), a high proportion of MRSA was reported (Wongwanich *et al*., 2000). It is a well-known fact that the proportion of MRSA among *S. aureus* isolates from hospitalized patients in Japan is over 50%.

Antibiotic availability was very restricted in Laos until the end of the 1980s and there was almost no problem of drug-resistant pathogens until then. Once hospitals become heavily contaminated with MRSA, eradication is almost impossible (Thompson *et al*., 1982). Therefore, prevention of the development of MRSA in the hospital is a critical problem. There must currently be an increased risk of developing drug-resistant pathogens in Laos, since the kinds of antibiotics available and their consumption were steadily increased from 1990 until the present. Because we anticipated the development resistant strains, we have been monitoring the drug-resistant *S. aureus* in Laos since 1993. In this communication, we described the results of 9 years of monitoring of drug-resistant *S. aureus* in Laos.

MATERIALS AND METHODS

Bacterial strains

S. aureus used in this study was isolated from clinical specimens submitted to Center for Laboratory and Epidemiology, Vientiane, Laos. The specimens included swabbed mateials (lesion of skin, throat, ear, or vagina), sputum, urine, aspirated fluid, etc. The number of isolates was 54 in 1993, 32 in 1995, 47 in 1996, 56 in 1997, 72 in 1998, 59 in 1999, 35 in 2000, and 61 in 2001 (416 in total).

Drug susceptibility

The susceptibility was expressed as the minimum inhibitory concentration (MIC) of the following 5 drugs as examined by the plate dilution method. The drugs examined were methicillin (DMPPC, Banyu), oxacillin (MPIPC,

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Wako) as a substitute for methicillin, ampicillin (ABPC, Meiji), tetracycline (TC, Nacarai), erythromycin (EM, Dainippon), and cefdinir (CFDN, Fujisawa). A series of heart infuion agar plates containing serial 2-fold dilutions of the drugs at concentrations ranging from 100 μg/ml to 0.1 μg/ml or lower were prepared. A 10-fold dilution of an overnight broth culture of the *S. aureus* strains indicated in the text was inoculated onto each plate using a Microplanter (Sakuma Co. model MITP #00257) and incubated at 37℃ for 24 hours. The MICs of methicillin and oxacillin were determined using agar plates containing 2% NaCl and cultured at 30℃.

Detection of *mecA* gene

The *mecA* gene was detected by polymerase chain reaction (PCR). The PCR templates samples were prepared from the colonies on a nutrient agar plate by emulsifying a few colonies in distilled water and boiling them for 20 min. The PCR primers designed to amplify a 630 bp fragment derived from a portion of *mecA*, 5'-GAACCTCTGCTCAA CAAGTT-3' and 5'-GGATTTGCCAATTAAGTTTG-3', were used as described by Song *et al*. (1987). The PCR conditions consisted of 30 cycles of 94℃ for 1 minute, 52℃ for 1 minute, and 72℃ for 1 minute, and a final incubation at 72℃ for 10 minutes.

Detection of PBP2'

The expression of *mecA* was examined by detecting the gene product, PBP2'. A kit for the detection of PBP2', MRSA Screen (Denka Seiken Co., Tokyo) was used according to the instructions in the manual supplied with the kit.

Coagulase typing

Coagulase type was determined by neutralization test with the type-specific antiserum. A Coagulase Typing Immune Sera Kit (Denka Seiken Co. Tokyo) was used to classify the antigenic type of coagulase produced by the isolates. Coagulation inhibition with the type-specific anti-serum was examined using plastic microdilution plates as described by Tajima *et al*. (1992).

Detection of toxins

Production of Staphylococcal enterotoxins (SE) including the antigenic types A, B, C, D, and E was examined by using a SET-RPLA kit (Denka Seiken Co., Tokyo) for the detection and typing of staphylococcal enterotoxins. Toxic shock syndrome toxin-I (TSST-I) production was examined by using a TST-RPLA kit (Denka Seiken Co., Tokyo). The detection was performed according to the instructions given in the manuals supplied with each kit.

RESULTS

Drug susceptibilities

The MICs of ampicillin and methicillin gradually became higher during the past 8 years. This gradual change of drug susceptibility was shown by determining the cumulative distribution of MIC (Fig.1). The susceptibility of *S. aureus* to erythromycin varied from year to year. All isolates were highly sensitive to cefdinir throughout the period of monitoring (Fig. 1). The MIC of tetracycline became slightly lower in the past few years. Taken altogether, the MICs values reveal that the drug susceptibility pattern has not much varied so far. The raw data regarding MIC are presented in Table 1.

Among the isolates obtained in 2001, there were 3 strains against which the MIC of oxacillin was 12.5 or 100 μg/ml, while the MIC against the other isolates was lower than 3.13 μg/ml. The MICs of oxacillin, ampicillin, tetracycline, erythromycin and cefdinir against these 3 strains were: 12.5, 6.25, 12.5, 0.2, and 0.8 μg/ml, respectively, for strain 01LP32; 200, 200, 100, 6.25, 200 μg/ml, respectively, for strain 01LP40; and 12.5, 200, 25, 0.4, 0.8 μg/ml, respec-

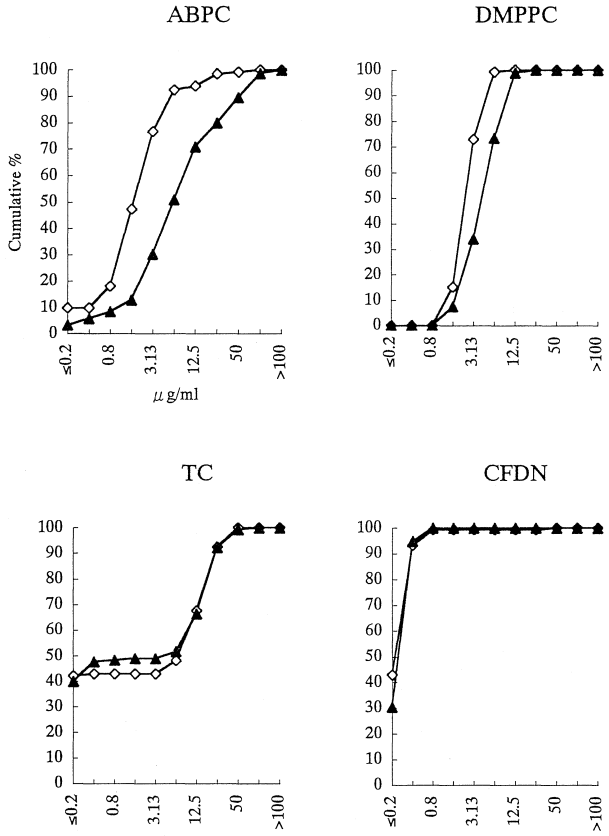

Fig. 1: Cumulative frequency distribution (%) of MIC.
—◇— : total of the isolates in 1993, 1995, and 1996
—▲— : total of the isolates in 1999, 2000, and 2001
Data for DMPPC do not include 2001

Table 1 MIC of each of the indicated drugs in the past 9 years
The frequency distribution of MIC is expressed as the percentage of total isolates in each year (The number of total isolates for each year is shown in the bottom line).

DMPPC

	93	95	96	97	98	99	0
≤0.2	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
2	28	3	9	0	4	10	3
3	65	59	49	2	52	41	3
6	7	38	40	64	44	46	29
13	0	0	2	32	0	3	62
25	0	0	0	0	0	0	3
50	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
>100	0	0	0	2	0	0	0
number	54	32	47	56	72	59	35

ABPC

	93	95	96	97	98	99	0	1
≤0.2	11	19	2	4	4	5	0	3
0	0	0	0	2	5	2	3	3
1	6	13	9	7	3	5	3	0
2	11	6	66	11	5	10	3	0
3	35	37	17	28	5	25	14	11
6	28	19	0	27	14	14	34	20
13	0	6	0	4	36	5	26	32
25	7	0	4	5	17	7	6	13
50	2	0	0	7	4	12	0	13
100	0	0	2	5	4	15	11	2
>100	0	0	0	0	3	0	0	3
number	54	32	47	56	72	59	35	61

TC

	93	95	96	97	98	99	0	1
≤0.2	42	28	51	0	3	24	45	51
0	0	0	2	30	31	12	3	7
1	0	0	0	0	30	2	0	0
2	0	0	0	0	0	2	0	0
3	0	0	0	0	3	0	0	0
6	0	22	0	0	1	3	6	0
13	24	25	11	4	0	17	20	10
25	28	25	21	25	19	26	17	30
50	6	0	15	36	13	14	9	0
100	0	0	0	5	0	0	0	2
>100	0	0	0	0	0	0	0	0
number	54	32	47	56	72	59	35	61

EM

	93	95	96	97	98	99	0	1
≤0.2	87	85	34	4	82	19	17	7
0	7	6	2	85	3	52	72	83
1	0	0	49	2	0	0	0	0
2	0	0	13	0	0	0	0	0
3	2	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	2
13	2	0	0	0	0	0	0	0
25	0	0	2	0	0	0	0	0
50	0	0	0	0	0	0	0	0
100	0	0	0	2	0	0	0	0
>100	2	9	0	7	15	29	11	8
number	54	32	47	56	72	59	35	61

CFDN

	93	95	96	97	98	99	0	1
≤0.2	26	34	68	16	14	42	9	31
0	68	50	30	61	79	58	74	66
1	6	13	2	21	7	0	17	3
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
50	0	3	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0
>100	0	0	0	2	0	0	0	0
number	54	32	47	56	72	59	35	61

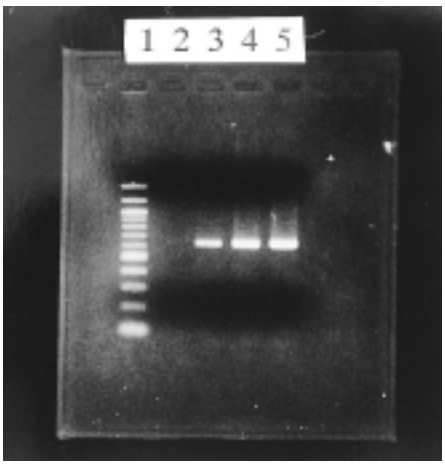

Fig.2: Detection of *mecA* gene by PCR
lane 1: Molecular size marker (100 bp ladder), lane 2: strain 01LP32, lane 3: strain 01LP40, lane 4: strain 01LP63, lane 5: *mecA*-positive *S. aureus* as control

tively, for strain 01LP63. The production of PBP2' and the presence of the *mecA* gene were examined in these 3 strains. Strain 01LP40 was isolated from the discharge from the middle ear of an 8-year-old female patient with otitis media (Friendship Hospital), and 01LP63 was isolated from the sputum of a 13-year-old male patient with a respiratory infection (Setthathirath Hospital). These 2 strains had the *mecA* gene, as demonstrated by the PCR (Fig. 2), and they expressed PBP2' as examined by the agglutination test. Strain 01LP32 was negative for both the gene and its product.

Characterization of MRSA strains 01LP40 and 01LP63

The biochemical characteristics of these MRSA strains were typical of *S. aureus*, and included fermentation of lactose, mannitol and maltose, and production of acetoin. The coagulase of 01LP40 did not belong to any of the 8 known types (I to VIII), and the strain did not produce enterotoxin or toxic shock syndrome toxin. However, 01LP63 produced enterotoxin type B and type D but did not produce toxic shock syndrome toxin. The coagulase of 01LP63 was classified as type II.

DISCUSSION

This is the first report of MRSA isolated from the infection foci of patients in Laos. Although we reported an isolate of MRSA from the nasal vestibulum of a noninfected patient in Laos in 1996 (Iwanaga *et al*., 1997), MRSA has not been found in the infection foci until this study. Considering the pattern of use of antimicrobial agents in Laos in the past, the incidence of MRSA is expected to be very low. However, recently, many Laotians have received medical care in neighboring Thailand, where MRSA is routinely isolated (Wongwanich *et al.*, 2000), and have then returned to Laos. Moreover, numerous antibiotics are imported and their use has increased. As a result, there is a high risk of the appearance of MRSA or other highly drug-resistant pathogens. The fact that 2 strains of MRSA appeared in 2 different hospitals warns us of the increase of MRSA in Laos.

Ampicillin and tetracycline have been the major antibiotics used in Laos, and this pattern of antibiotic use may explain why the susceptibility of *S. aureus* to ampicillin is gradually getting resistant. However, the susceptibility to tetracycline has not changed much, and may even have got sensitive in the past few years. Although it seems that the use of tetracycline decreased in Laos after 1998, when tetracycline resistant *Vibrio cholerae* O1 appeared, there is no reliable data on the rate of use of tetracycline. Therefore, it remains unknown why *S. aureus* has become more sensitive to tetracycline in the past few years.

The 2 strains of MRSA isolated here showed different phenotypes, implying that they belonged to different clones. One of the strains (01LP63) was isolated at a hospital which was donated by the Japanese government in 2000, and many Japanese staff members from a university hospital are working there under the name of International Cooperation. Since coagulase type Ⅱ is the major type of coagulase produced by MRSA in Japan (Matsumoto *et al.*, 1989; Kimura *et al.*, 1993), it is possible that 01LP63 was carried from Japan to Laos by the hospital staff. It should be clarified whether this MRSA strain was carried to Laos by Japanese staff or not. Regardless of whether it was or not, hospital staff members have to be careful to avoid the improper use of antibiotics in order to prevent the development of MRSA in Laos, and to avoid carrying MRSA from Japan to Laos. Careful monitoring of MRSA in Laos should be continued.

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TAXONOMIC NOTES ON THE *GRISEIFRONS* SPECIES-GROUP IN *SIMULIUM* (*SIMULIUM*) (DIPTERA: SIMULIIDAE) FROM THAILAND: DESCRIPTIONS OF TWO NEW SPECIES AND DESCRIPTION OF THE MALE, PUPA AND LARVA OF *S*. (*S*.) *DIGRAMMICUM* EDWARDS

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Accepted 28, May, 2002

Abstract: Two new black-fly species, *Simulium maenoi* sp. nov. and *S. choochotei* sp. nov. are described, based on the reared females, reared males, pupae and mature larvae collected in Chiang Mai Province, northern Thailand. In addition, the male, pupa and mature larva of *S. digrammicum* Edwards, 1928, originally described from Peninsular Malaysia and Thailand, are described for the first time. All these three species are assigned to the *griseifrons* species-group within *Simulium* (*Simulium*) Latreille s. str.
Key words: black fly, Simuliidae, *Simulium*, Thailand, new species, *griseifrons* species-group

The *griseifrons* species-group within the subgenus *Simulium* (*Simulium*) Latreille s. str. is characterized by the female scutum with five vittae, simple claws, the male style with a basal protuberance furnished with spinules, the ventral plate untoothed posteriorly, the pupal gill with six filaments per side, and the larval postgenal cleft deep, subtriangular or bullet-shaped (Takaoka and Davies, 1996). In the world list of black flies by Crosskey and Howard (1997), this species-group includes 10 species, all but one recorded from the Oriental Region. Apparently five other species placed in the *multistriatum* species-group by Crosskey and Howard (1997), and two more species left unplaced by Takaoka and Davies (1995; 1997) belong to the *griseifrons* species-group: i.e., *S. horokaense* Ono, 1980, *S. kawamurae* Matsumura, 1915, *S. konakovi* Rubtsov, 1956, *S. kurilense* Rubtsov, 1956, *S. subornatoides* Rubtsov, 1947 (all from Palaearctic Region), and *S. rudnicki* Takaoka and Davies, 1995, and *S. yongi* Takaoka and Davies, 1997 (both from Peninsular Malaysia and Thailand). In Thailand, the *griseifrons* species-group is represented by four species, *S. digrammicum* Edwards, 1928 (Edwards, 1928), *S. grossifilum* Takaoka and Davies, 1995, *S. rudnicki* and *S. yongi* (Kuvangkadilok and Takaoka, 2000), all originally described from Peninsular Malaysia.

During our recent surveys on aquatic stages of black flies in northern Thailand, we discoverd the pupae and larvae of *S. digrammicum*, which was so far known only from female specimens, and found two new species assignable to the *griseifrons* species-group.

These new species are here described on the basis of the reared females, reared males, pupae and mature larvae; in addition, the male, pupa and mature larva of *S. digrammicum* are described for the first time.

Collecting and rearing methods, as well as dissection of anatomical parts for description, were already mentioned in Takaoka (1983). The morphological features and terms used herein follow those of Crosskey (1969).

Holotype and most paratype specimens will be deposited at the Natural History Museum (BMNH), London, U.K.

***Simulium* (*Simulium*) *maenoi* Takaoka and Choochote, sp. nov.**

DESCRIPTION. **Female.** Body length ca. 2.5 mm. ***Head.*** Narrower than width of thorax. Frons black, white pruinose, shiny, widely bare except several dark stout hairs along each lateral margin; frontal ratio 1.4:1.0:1.2; frons-head ratio 1.0: 3.8. Fronto-ocular area (Fig. 1) moderately developed, subtriangular in shape. Clypeus black, white pruinose, shiny, moderately covered with dark stout hairs except upper portion bare medially. Proboscis ca. 0.8× as long as clypeus. Antenna composed of 2+9 segments, light to medium

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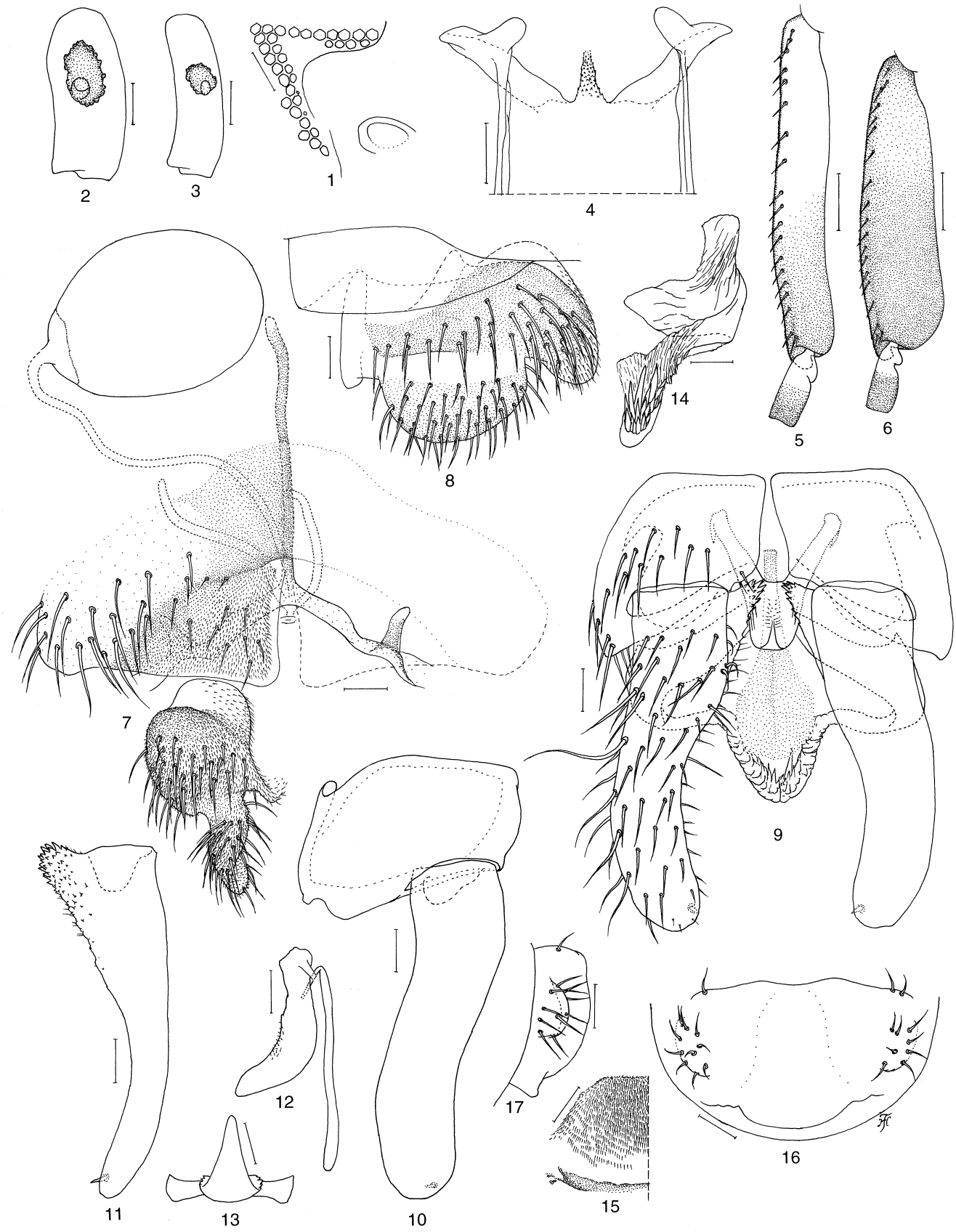
brown except scape, pedicel and most of 1st flagellar segment yellow; 1st flagellar segment ca. 1.5× as long as 2nd one. Maxillary palp medium brown, composed of 5 segments with proportional lengths from 3rd to 5th segments 1.0:1.1:2.3; 3rd segment (Fig. 2) of moderate size; sensory vesicle medium in size, ellipsoidal, with rugged surface, 0.37× length of 3rd segment, with large round opening apically. Maxillary lacinia with 13 or 14 inner and 14 or 15 outer teeth. Mandible with ca. 28 inner and 13 or 14 outer teeth. Cibarium (Fig. 4), at its posterior end, produced into pharynx as narrow projection covered with numerous indistinct minute tubercles. ***Thorax.*** Scutum dark brown, grey-pruinose, with 5 dark longitudinal unpruinose vittae (i.e., 1 narrow median, 2 rather broad submedian, and 2 rather broad lateral vittae), all united on prescutellar area when illuminated from dorsally and viewed from anterodorsally (this color pattern reversed when viewed from behind); scutum densely covered with yellow recumbent fine hairs, interspersed with long upstanding dark hairs on prescutellar area. Scutellum unpigmented, with long dark and short yellow hairs. Postscutellum light brown, shiny, without hairs. Pleural membrane bare. Ketepisternum longer than deep, and bare. ***Legs*** (coloration still incomplete, especially on femora and tibiae). Foreleg: coxa and trochanter whitish yellow; femur whitish yellow on basal 1/2, dark yellow on apical 1/2, with apical cap light brown; tibia whitish yellow except apical 1/5 medium brown; tarsus entirely medium brown; basitarsus, 2nd and 3rd tarsal segments moderately dilated; basitarsus ca. 5.5× as long as its greatest width. Midleg: coxa medium brown; trochanter light brown except base yellow; femur yellow somewhat darkened apically, and with apical cap light brown; tibia whitish yellow except apical 1/4 dark brown; tarsus dark brown except basal 1/2 of basitarsus whitish yellow. Hind leg: coxa dark brown; trochanter whitish yellow; femur and tibia whitish yellow with apical cap dark brown; tarsus medium brown except a little more of basal 1/2 of basitarsus and basal 1/2 of 2nd tarsal segment whitish yellow; basitarsus (Fig. 5) nearly parallel-sided, ca. 6.7× as long as wide, ca. 0.7× and ca. 0.6× as wide as hind tibia and femur, respectively; calcipala distinct but shorter than wide; pedisulcus distinct. All tarsal claws simple, without subbasal or basal tooth. ***Wing.*** Length 2.2 mm. Costa with spinules and hairs; subcosta haired except apical 1/5 bare; basal section of radial vein bare; hair tuft at base of stem vein dark brown; basal cell absent. ***Abdomen.*** Basal scale light brown with a fringe of long hairs; dorsal surface of 2nd segment pale yellow on anterior 3/4 with a pair of large dorsolateral whitish spots broadly connected to each other in middle; dorsal surface of posterior 1/4 of 2nd segment and those of other segments dark brown and with short hairs; tergites 3, 4 and 5 small, tergites 6-8 large and shiny. Ventral surface of abdominal segment 7 without any sternal plate or any hair clusters. ***Genitalia*** (Figs. 7 and 8). Sternite 8 well sclerotized, bare medially but with 18 or 19 long stout hairs as well as a few short slender hairs laterally on each side; anterior gonapophysis triangular in shape, membranous, covered with 8 or 9 short hairs as well as numerous microsetae; inner border slightly concave; narrow area along inner and posterior margins transparent, bare. Genital fork of inverted-Y form, with well sclerotized stem; arms slender, each with sclerotized apical bulge having a distinct projection directed anteriorly. Paraproct in ventral view nearly as long as wide, with distinct concavity on ventral surface along anterior margin; anteromedial surface of paraproct moderately sclerotized, and with a few short sensilla; paraproct much produced ventrally, coverd with 33-35 short stout hairs on ventral and lateral surfaces. Cercus in lateral view rounded posteriorly, ca. 0.5× as long as wide, covered with many short hairs. Spermatheca nearly ovoid, well sclerotized with no definite reticulate pattern, with minute internal setae; tube and large area around tubal base unsclerotized; accessary tubes subequal in diameter to each other, and somewhat larger than major one.

Male. Body length 2.5-2.8 mm. ***Head.*** Width slightly wider than thorax. Upper eye consisting of large facets in 19 or 20 horizontal rows and in 18-20 vertical columns. Clypeus brownish black, white pruinose, moderately covered with dark stout hairs. Antenna composed of 2+9 segments, dark brown except base of 1st flagellar segment somewhat pale; 1st flagellar segment elongated, ca. 2.2× as long as 2nd flagellomere. Maxillary palp composed of 5 segments, with proportional lengths from 3rd to 5th segments 1.0:1.2:2.5; 3rd segment (Fig. 3) of normal size; sensory vesicle small, globular or ellipsoidal, ca. 0.2× as long as 3rd segment, with small opening. ***Thorax.*** Scutum black, dull, uniformly and densely covered with golden-yellow recumbent fine hairs, interspersed with long upright hairs on prescutellar area; in certain angles of light, scutum with white-pruinose subquadrate spot on each shoulder, extending posteriorly forming a white-pruinose broad band along each lateral margin up to base of wing. Scutellum brownish black, with several upright dark hairs as well as golden-yellow fine hairs. Postscutellum brownish black, shiny, without hairs. Pleural membrane and katepisternum as in female. ***Legs.*** Foreleg: coxa whitish yellow; trochanter light to medium brown; femur light brown except apical cap medium brown; tibia brownish black except median large area of outer surface white and sheeny; tarsus entirely brownish black; basitarsus, 2nd and 3rd tarsal segments somewhat dilated (basitarsus ca. 6.8× as long as its greatest width). Midleg: coxa dark brown to brownish

black; trochanter dark brown except basal 2/5 yellow; femur light brown with basal 1/5 yellow and apical cap medium brown; tibia medium to dark brown except basal 1/2 (or a little more) whitish yellow; tarsus dark brown. Hind leg: coxa medium to dark brown; trochanter yellow; femur medium brown with basal 1/5 yellow and apical cap blackish brown; tibia dark brown to blackish brown except extreme base yellowish white; tarsus dark brown except basal 1/3 of basitarsus light brown, and a little less than basal 1/2 of 2nd segment yellowish white; basitarsus (Fig. 6) much enlarged, gradually widened apically, ca. 3.7× as long as its greatest width, and ca. 1.05× and ca. 1.13× as wide as hind tibia and femur, respectively. Calcipala distinct but small. Pedisulcus well marked. ***Wing.*** Length 2.0 mm. Other features as in female except subcosta bare. ***Abdomen.*** Basal scale brownish black with a fringe of dark long hairs. Dorsal surface brownish black except anterior 2/3 of 2nd segment light to medium brown, and with dark short hairs; segments 2, 5, 6 and 7 each with a pair of whitish-grey pruinose spots, those on segments 2, 5 and 6 situated dorsolaterally, those on segment 7 almost laterally; spots on segments 2 and 6 large and marked, while those on segment 5 small and somewhat indistinct (in 1 of 3 males examined, spots on segment 5 not discernible). ***Genitalia*** (Figs. 9-17). Coxite in ventrolateral view much shorter than wide, style in ventrolateral view elongate, much longer than coxite, ca. 3.2× as long as its greatest width near base, nearly straight and parallel-sided on basal 1/4 or a little more, but gently curved on apical 3/4, though nearly parallel-sided up to apex and slightly narrower than basal 1/4, and with a subterminal spine; style spatulate ventrodorsally, with basal protuberance directed dorsomedially, bearing numerous spinules on its surface. Body of ventral plate subquadrate though narrowed posteriorly, somewhat longer than wide, and having a long, narrow, ventrally-produced process with several short hairs basally on anteroventral surface; basal arms of ventral plate diverging from each other. Median sclerite moderately sclerotized, plate-like, widening apically, with rounded apex. Parameres broad basally, each with several hooks apically. Aedeagal membrane densely setose; dorsal plate moderately sclerotized, with medial portion somewhat narrowed. Abdominal segment 10 with 1-3 short hairs on each posterolateral corner. Cerci rounded, each with 8-10 short hairs.

Pupa. Body length 2.5-2.9 mm. ***Head.*** Integument dark yellow to yellowish brown, coverd moderately with large tubercles of various sizes (Fig. 18); large tubercles each with several minute projections on its surface (Fig. 19); frons with 2 long fan-like trichomes each with 6-9 long branches (Figs. 18 and 20) on each side; 2 frontal trichomes on each side situated close together; face with 1 medium-long trichome with 4 or 5 branches on each side (Fig. 18); antennal sheath with 9 moderately raised portions produced outwardly, corresponding to flagellar segments 1-9, similar to those of *S. digrammicum* (Fig. 70); outer surface of antennal sheath densely covered with small round tubercles except intersegmental spaces bare. ***Thorax.*** Integument dark yellow to yellowish brown, moderately covered with large tubercles of various sizes anteriorly, and with small conical tubercles posteriorly; large tubercles each with minute projections on its surface similar to those on frons; integument on each side with 5 long fan-like trichomes, each with 9-16 long branches anterodorsally and anterolaterally, 1 medium-long trichome with 4-6 branches posterolaterally, and 3 medium-long trichomes (2 simple or bifid, and 1 with 3-6 branches) ventrolaterally. Gill (Fig. 21) with 6 slender short filaments arranged in 3 pairs, all filaments arising directly from base, dorsal pair with very short stalk, and middle and ventral ones almost sessile; uppermost filament of dorsal pair longest (ca. 1.5 mm), ca. 1.3× as long as counter filament (ca. 1.2 mm), while 4 filaments of middle and ventral pairs subequal in length (0.9-1.1 mm) and thickness to one another, and slightly shorter than shorter filament of dorsal pair; uppermost filament thickest of all, ca. 1.4× as thick as counter filament of dorsal pair, ca. 2.1× as thick as 4 other filaments of middle and ventral pairs, and ca. 0.8× as thick as interspiracular trunk; 2 filaments of dorsal pair medium brown, while 4 other filaments pale yellow; cuticle of all filaments with well marked annular ridges and furrows which are becoming less marked toward apical tip, and densely covered with minute tubercles. ***Abdomen.*** Tergum 1 dark brown, with 1 simple long seta on each side; tergum 2 pale except areas near anterior margin dark brown, with 1 simple long seta and 5 short spinous setae submedially on each side; terga 3 and 4 each with 4 hooked spines and 1 spinous seta on each side; terga 5-9 lacking spine-combs except tergum 8 with spine-combs in transverse row on each side; tergum 9 with small conical terminal hooks (Fig. 22). Sternum 4 with 1 simple or bifid hooklet and a few minute setae on each side; sternum 5 with pair of simple or bifid hooks submedially and a few simple minute setae on each side; sterna 6 and 7 each with pair of bifid inner and simple outer hooks somewhat spaced from each other, and a few simple minute setae on each side. Grapnel-like hooklets absent. ***Cocoon*** (Figs. 23 and 24). Wall-pocket-shaped, tightly woven, with 1 or 2 small slit-like open spaces anterolaterally just behind anterior thick margin, on each side, and without ventrolateral flange on each side; individual threads invisible; 3.1-3.6 mm long × 1.5-1.7 mm wide.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Mature larva. Body length 5.9-6.4 mm. Body (Photo. 3) normal in shape, with thorax somewhat inflated, and abdomen gradually widened posteriorly up to posterior margin of segment 6, and then narrowed to posterior circlet; body color light bluish green. Cephalic apotome (Photo. 1) pale yellow with faint positive head spots. Lateral surface of head capsule pale yellow; spots behind and below eyespot region indistinct or faintly positive; ventral surface of head capsule (Photo. 2) pale yellow or darkly pigmented, with distinct positive spots on each side of postgenal cleft. Antenna composed of 3 segments and apical sensillum, much longer than stem of labral fan; length ratio of segments (from base to tip) 1.0:1.1-1.2:0.5-0.6. Labral fan with 30-36 main rays. Mandible (Fig. 25) with mandibular serrations composed of 1 large tooth and 1 small one, without supernumerary serrations; comb-teeth decreasing in length from 1st to 3rd. Hypostomium (Fig. 26) with 9 anterior teeth, median tooth longer than others; lateral margins weakly serrate apically; 6 or 7 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Photo. 2) deep, bullet-shaped, rounded apically, 1.9-2.9× as long as postgenal bridge. Cervical sclerite composed of 1 small, pale yellow, rod-like piece on each side in most larvae, or indiscernible in some larvae. Thoracic cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short colorless setae on each side of anal sclerite. Rectal papilla of 3 lobes, each with 8-12 finger-like secondary lobules. Anal sclerite X-shaped, with anterior arms broadened apically, which are ca. 0.6× as long as posterior ones. Last abdominal segment slightly bulged laterally but lacking ventral papillae. Posterior circlet with 118-126 rows of hooklets with up to 18-20 hooklets per row.

TYPE SPECIMENS. Holotype female, reared from pupa, Sai Yoi Waterfall, altitude 830 m, Chiang Mai Province, northern Thailand, 19.XII.2001, by W. Choochote. Paratypes 2 males, 4 pupae, same data as holotype; 1 pharate female, 1 pharate male, 16 pupae, 7 mature larvae, Monthatharn Waterfall, altitude 750 m, Chiang Mai Province, northern Thailand, 28.XI.2001, collected by W. Choochote.

ECOLOGICAL NOTES. Most of the pupae and larvae of *S. maenoi* were found to be attached to the surface of the stream-bed rocks in cascading waterfalls, and a few were collected from grass leaves trailing in the water. Water temperature was 16-19℃. This species was collected together with *S. brevipar*, *S. decuplum*, *S. fenestratum*, *S. grossifilum*, *S. rudnicki*, *S. rufibasis*, and *S. tani*. One of 28 immature larvae of this new species was infected with microsporidans.

ETYMOLOGY. The species name *maenoi* is given after Dr. Takashi Maeno, Professor emeritus, Department of Physiology, Shimane Medical University, Shimane, Japan, whom HT thanks for his advice and encouragement when he and HT were working at Kagoshima University in the 1970s.

REMARKS. *S. maenoi* sp. nov. is similar in the female to *S. digrammicum*, originally described from female specimens collected from Peninsular Malaysia and Thailand (Edwards, 1928). However, the female of *S. maenoi* differs from that of the latter species by the bare basal portion of the radial vein (cf., fully haired in *S. digrammicum*). The male, pupa and mature larva of *S. maenoi* are also different from those of *S. digrammicum* (described later in this paper) by the bare basal portion of the radial vein, and the wall-pocket-shaped cocoon, and the smaller number of posterior circlet rows (ca. 120), respectively (cf., in the latter species, the basal portion of the radial vein fully haired, the cocoon boot-shaped, and ca. 150 rows of posterior circlet, respectively).

This new species is distinguished from *S. griseifrons* Brunetti, 1911, from India (Puri, 1932) by the smaller number of setae on the anterior gonapophysis in the female, and the haired ventral plate in the male. The wall-pocket-shaped cocoon of *S. maenoi* easily separates it from *S. griseifrons*, which has the boot-shaped cocoon, like *S. digrammicum*.

S. maenoi seems to be most related to *S. japonicum* Matsumura, 1931 from Japan, Korea, Siberia and China (Bentinck, 1955; Takaoka, 1977) by sharing the fenestrate cocoon, the similar arrangement of the six pupal gill filaments, and the multibranched trichomes on the pupal head

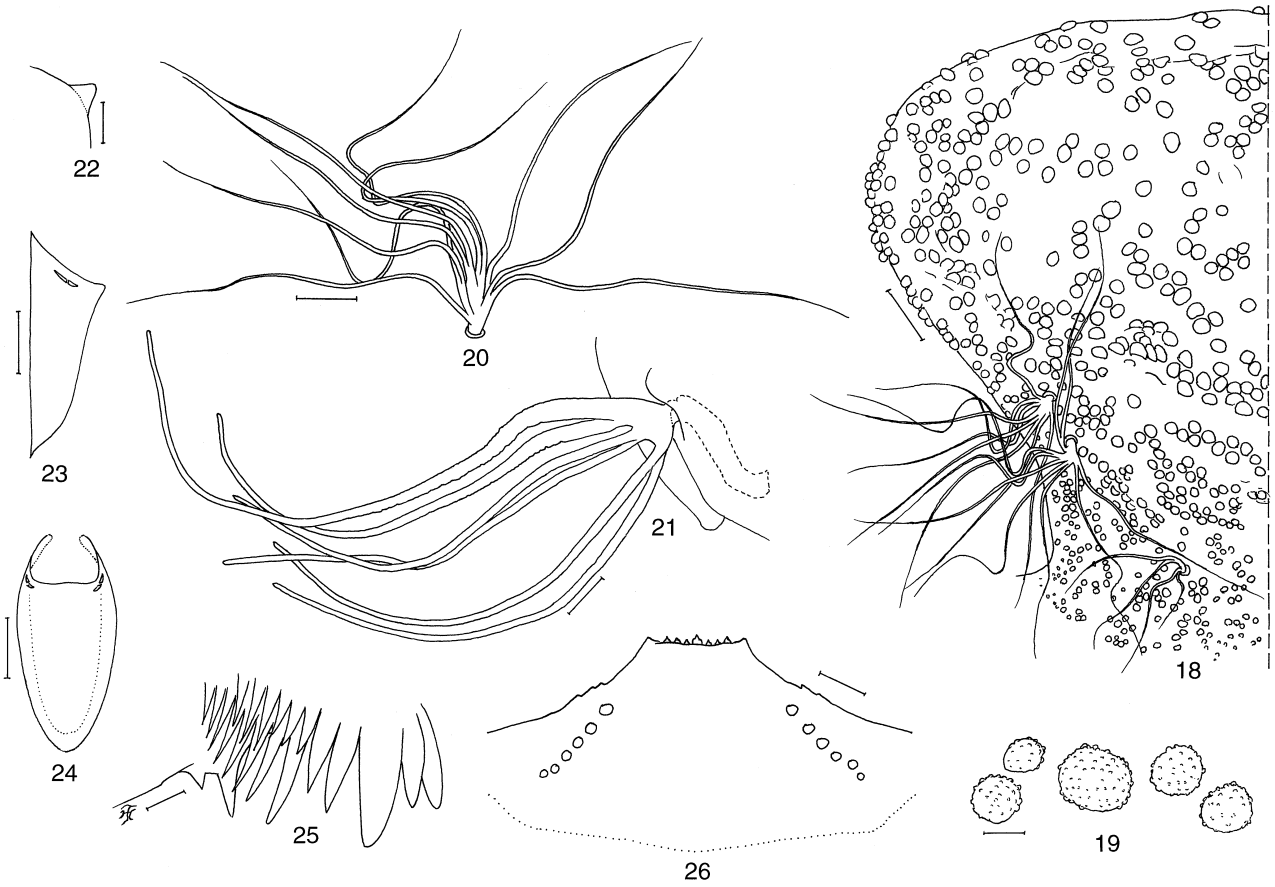
Figures 1-17. Morphological features of adults of *S. maenoi* sp. nov. 1, female fronto-ocular area; 2 and 3, 3rd segments of maxillary palp with sensory vesicle (2, female; 3, male); 4, female cibarium; 5 and 6, basitarsi and 2nd tarsal segments of hind leg (left side and outside view; 5, female; 6, male); 7, sternite 8, anterior gonapophyses, genital fork, right paraproct, right cercus (left paraproct and cercus omitted) and spermatheca of female genitalia *in situ* (ventral view); 8, right paraproct and cercus (lateral view); 9, coxites, styles, ventral plate, median sclerite and parameres of male genitalia *in situ* (ventral view); 10, right coxite and style (ventrolateral view); 11, left style showing a spiny basal protuberance (medial view); 12, ventral plate and median sclerite (lateral view); 13, ventral plate (end view); 14, right paramere (dorsal view); 15, aedeagal membrane and dorsal plate (end view); 16 and 17, 10th abdominal segments and cerci (16, end view; 17, lateral view). Scale bars. 0.1 mm for figs. 5 and 6; 0.05 mm for fig. 1; 0.03 mm for figs. 2-4 and 7-17.

and thorax. However, this new species is distinguished from the latter species by the following characters (*S. japonicum* in parenthesis): the female cibarium without tubercles near its posterior margin, and with a dorsally-directed projection furnished with numerous tubercles (cibarium with numerous tubercles near its posterior margin but lacking any dorsally-directed projection), the male ventral plate with a posteriorly-narrowed body (ventral plate with a parallel-sided body), spine-combs present only on the pupal abdominal segment 8 (spine-combs present on the pupal abdominal segments 8 and 9), the pupal gill filaments of middle and ventral pairs sessile (gill filaments of all 3 pairs sessile), the cocoon with a small, slit-like window on each side (cocoon with a large anterolateral window on each side), the larval cephalic apotome with faint positive spots (larval cephalic apotome with distinct spots), and the bullet-shaped postgenal cleft (postgenal cleft subtriangular).

This new species seems to be also related to *S. biforaminiferum* Datta, 1974, described from pupae and larvae, collected from west Bengal (Datta, 1974) and left unplaced to any species group by Crosskey and Howard (1997), by sharing the fenestrate cocoon, the similar arrangement of the six pupal gill filaments, and the multibranched trichomes on the pupal head and thorax, and the bullet-shaped larval postgenal cleft. However, *S. maenoi* is easily distinguished from the latter species by the following pupal characters (*S. biforaminiferum* in parenthesis): fan-like trichomes on the head and thorax (brush-like trichomes), spine-combs present only on the abdominal segment 8 (spine-combs present on the abdominal segments 7, 8 and 9), and the cocoon with small, slit-like windows (the cocoon with large, elliptical windows).

Simulium (*Simulium*) *choochotei* Takaoka, sp. nov.

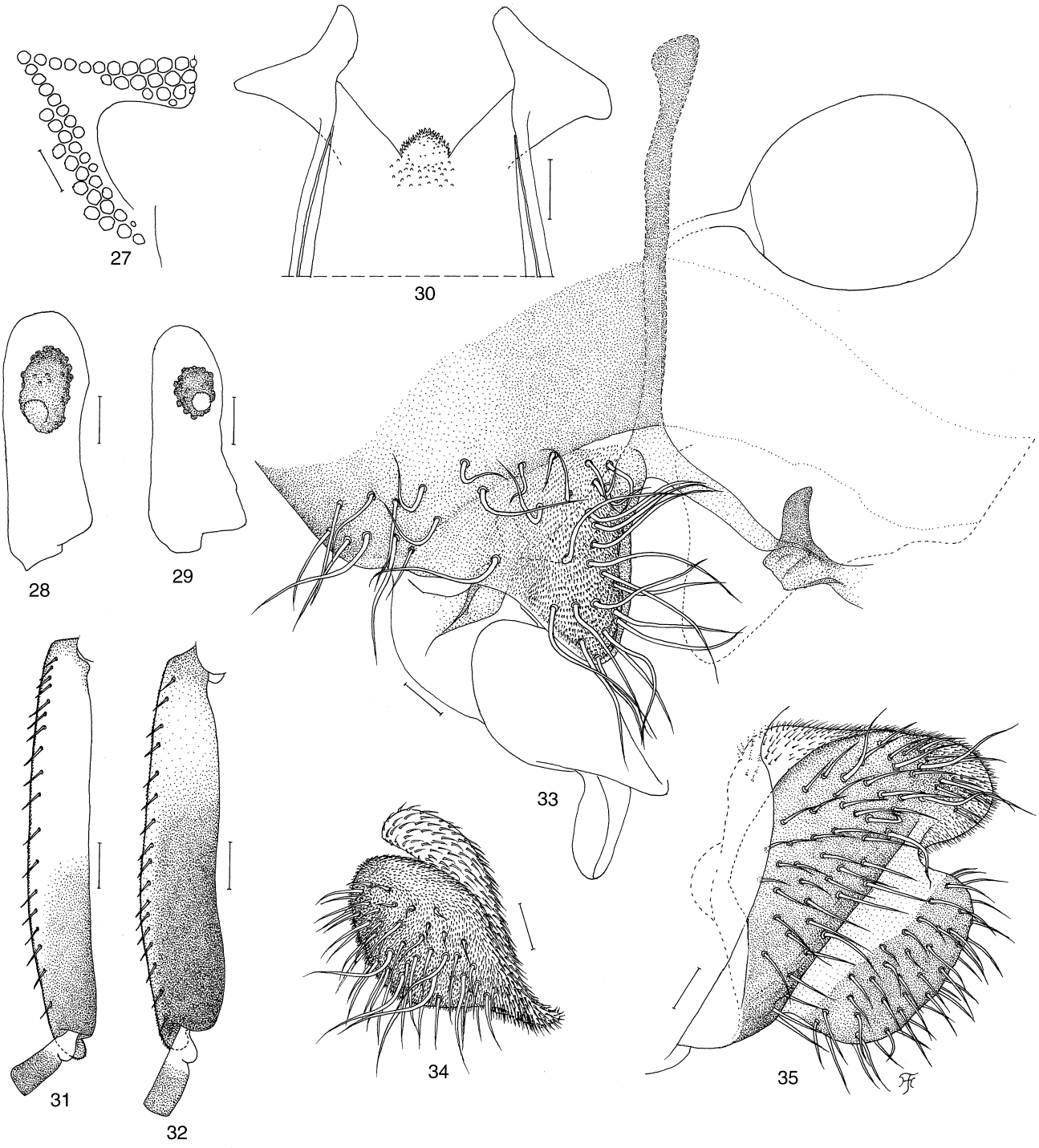
DESCRIPTION. **Female.** Body length 3.4-4.0 mm. ***Head.*** Narrower than width of thorax. Frons black, white pruinose, shiny, widely bare except several dark stout hairs along each



Figures 18-26. Morphological features of the pupa and larva of *S. maenoi* sp. nov. 18, frons and part of face of pupa (right half); 19, tubercles with secondary projections on frons; 20, frontal trichome; 21, pupal gill filaments (left side, lateral view); 22, terminal hook (lateral view); 23 and 24, cocoons (23, lateral view; 24, dorsal view); 25, apical part of larval mandible; 26, larval hypostomium. Scale bars. 1.0 mm for figs. 23 and 24; 0.1 mm for fig. 21; 0.05 mm for fig. 18; 0.03 mm for figs. 20 and 26; 0.02 mm for fig. 22; 0.01 mm for figs. 19 and 25.

lateral margin and a few hairs near antennal bases; frontal ratio 1.4:1.0:1.6; frons-head ratio 1.0:5.2. Fronto-ocular area (Fig. 27) moderately developed, subtriangular in shape. Clypeus black, white pruinose, shiny, moderately covered with dark stout hairs except median portion bare longitudinally. Proboscis ca. 0.58× as long as clypeus. Antenna composed of 2+9 segments, dark brown except scape, pedicel and most of 1st flagellar segment yellow when viewed from ventrally (though dorsal surface of 1st flagellar segment dark brown on apical 1/2 or more); 1st flagellar segment ca. 1.9× as long as 2nd one. Maxillary palp dark brown, composed of 5 segments with proportional lengths from 3rd to 5th segments 1.0:1.0:2.3; 3rd segment (Fig. 28) of moderate size; sensory vesicle medium in size, ellipsoidal, with rugged surface, 0.33× length of 3rd segment, with medium round opening apically. Maxillary lacinia with 11 or 12 inner and 16-18 outer teeth. Mandible with ca. 30 inner and 16 or 17 outer teeth. Cibarium (Fig. 30) with ca. 36 minute tubercles near its posterior margin, and at its posterior end, produced into pharynx as round medial projection covered with numerous minute tubercles. ***Thorax.*** Scutum brownish black, shiny, grey pruinose, with 5 dark longitudinal unpruinose vittae (i.e., 1 narrow median, 2 rather broad submedian, and 2 rather broad lateral vittae), all united on prescutellar area when illuminated from dorsally and viewed from anterodorsally (this color pattern reversed when viewed from behind); scutum densely covered with yellow recumbent fine hairs, interspersed with long upstanding dark hairs on prescutellar area. Scutellum pale, with long dark marginal hairs as well as short yellow hairs. Postscutellum medium brown, shiny, without hairs. Pleural membrane bare. Katepisternum longer than deep, and bare. ***Legs.*** Foreleg: coxa yellowish white; trochanter light brown except base yellow; femur dark yellow, with apical cap light brown; tibia whitish yellow except base light brown, and a little less than apical 1/2 medium brown to blackish, though surface of inner margin somewhat darkened even along basal 1/2; tarsus entirely black; basitarsus, 2nd and 3rd tarsal segments moderately dilated; basitarsus ca. 6.5× as long as its greatest width. Midleg: coxa dark brown; trochanter light brown except basal 1/2 yellow; femur yellow or dark yellow, with apical cap light brown; tibia whitish yellow except a little less than apical 1/2 dark brown to black; basitarsus dark brown to brownish black except basal 2/5 dark yellow or light brown, though its border not well defined; other tarsal segments black. Hind leg: coxa dark brown; trochanter yellow; femur yellow on basal 1/3, dark yellow on apical 2/3, with apical cap medium brown, tibia yellowish white on basal 3/5 (or a little more), dark brown to brownish black on apical 2/5; tarsus brownish black except a little more than basal 1/2 of basitarsus and basal 1/2 of 2nd tarsal segment whitish yellow; basitarsus (Fig. 31) nearly parallel-sided, ca. 7.2× as long as wide, ca. 0.7× and ca. 0.6× as wide as hind tibia and femur, respectively; calcipala distinct, nearly as long as wide; pedisulcus distinct. All tarsal claws simple, without subbasal or basal tooth. ***Wing.*** Length 3.0-3.1 mm. Costa with spinules and hairs; subcosta haired except near apex bare; basal section of radial vein fully haired; hair tuft at base of stem vein dark brown; basal cell absent. ***Abdomen.*** Basal scale medium brown with a fringe of dark long hairs; dorsal surface of 2nd segment pale yellow on anterior 3/5 with a pair of large dorsolateral whitish spots broadly connected to each other in middle; dorsal surface of posterior 2/5 of 2nd segment and those of other segments brownish black except tergites 3-5 pale yellow, and with short hairs; tergites 3, 4 and 5 small, tergites 6-8 large and shiny. Ventral surface of abdominal segment 7 with a large round sternal plate medially. ***Genitalia*** (Figs. 33-35). Sternite 8 well sclerotized, bare medially but with 13 or 14 long stout hairs on each side; anterior gonapophysis somewhat produced posteriorly and rounded ventrally, with round posteromedial corner, membranous basally and laterally, but well sclerotized along inner margin, covered with 22 or 23 long stout hairs as well as numerous microsetae; inner border slightly concave; area along posterior margin narrowly transparent, bare. Genital fork of inverted-Y form, with well sclerotized stem; arms slender, each with sclerotized apical bulge having a distinct projection directed anteriorly. Paraproct in ventral view ca. 1.4× as wide as long, and with deep concavity along anterior margin; anteromedial surface of paraproct moderately sclerotized, and with 7 or 8 sensilla; paraproct much produced ventrally, covered with ca. 60 short and long stout hairs on ventral and lateral surfaces. Cercus in lateral view subquadrate, ca. 0.6× as long as wide, covered with many short hairs. Spermatheca nearly ovoid, well sclerotized with no definite reticulate pattern, with minute internal setae; tube and large area around tubal base unsclerotized; accessary tubes subequal in diameter to each other, and also subequal to major one.

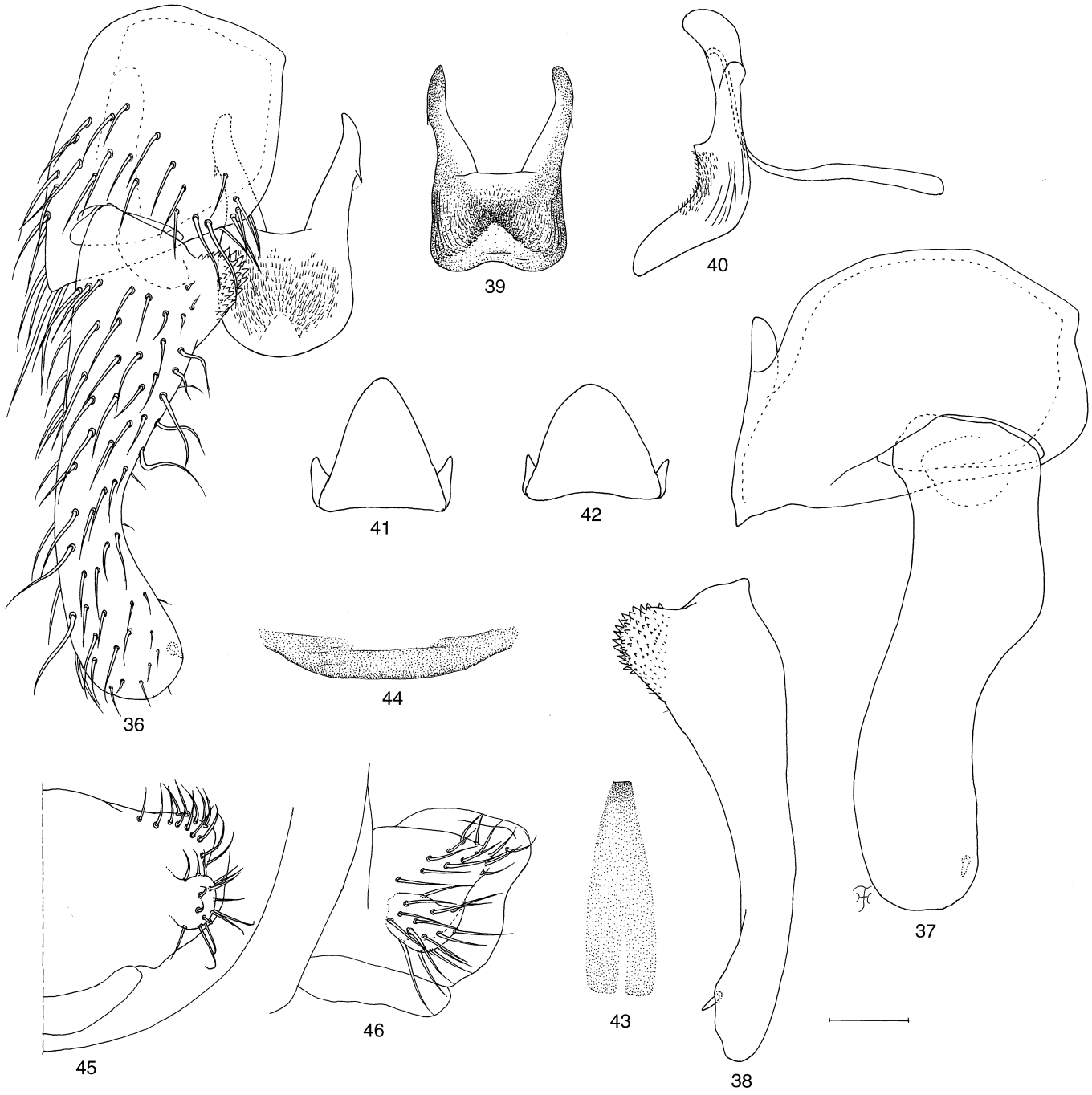
Male. Body length 3.6-4.2 mm. ***Head.*** Width slightly wider than thorax. Upper eye consisting of large facets in 20 horizontal rows and in 17 vertical columns. Clypeus brownish black, white pruinose, moderately covered with dark stout hairs. Antenna composed of 2+9 segments, dark brown except scape, pedicel and basal 1/2 of 1st flagellar segment yellow; 1st flagellar segment elongate, ca. 2.2× as long as 2nd flagellomere. Maxillary palp composed of 5 segments, with proportional length from 3rd to 5th segments 1.0:1.1:2.6; 3rd segment (Fig. 29) of normal size; sensory vesicle small, ellipsoidal, ca. 0.23× as long as 3rd



Figures 27-35. Morphological features of adults of *S. choochotei* sp. nov. 27, female fronto-ocular area; 28 and 29, 3rd segments of maxillary palp with sensory vesicle (28, female; 29, male); 30, female cibarium; 31 and 32, basitarsi and 2nd tarsal segments of hind leg (left side and outside view; 31, female; 32, male); 33, sternite 8, anterior gonapophyses, genital fork, right paraproct, right cercus (left paraproct and cercus omitted) and spermetheca of female genitalia *in situ* (ventral view); 34, right paraproct (ventral view); 35, right paraproct and cercus (lateral view).
Scale bars. 0.1 mm for figs. 31 and 32; 0.05 mm for fig. 27; 0.03 mm for figs. 28-30 and 33-35.

segment, with opening of medium size. ***Thorax.*** Scutum brownish black, slightly shiny, uniformly and densely covered with golden-yellow recumbent fine hairs, interspersed with long upright hairs on prescutellar area; scutum with large white-pruinose spot on each shoulder, extending posteriorly forming a white-pruinose broad band along each lateral margin up to base of wing; in certain angle of light, large white-pruinose spot present on prescutellar area. Scutellum brownish black, with several upright dark hairs as well as golden-yellow fine hairs. Postscutellum brownish black, shiny, without hairs. Pleural membrane and katepisternum as in female. ***Legs.*** Foreleg: coxa yellowish white; trochanter medium brown except basal 1/2 of inner surface yellow; femur light brown except apical cap medium to dark brown; tibia brownish black with median large area of outer surface slaty and sheeny in certain angle of light; tarsus entirely brownish black; basitarsus, 2nd and 3rd tarsal segments somewhat dilated (basitarsus ca. 7.3× as long as its greatest width). Midleg: coxa brownish black; trochanter brownish black except basal 2/5 yellow; femur light brown with apical cap brownish black; tibia dark brown to brownish black except extreme base yellowish white; tarsus brownish black though basal portion of basitarsus dark brown. Hind leg: coxa brownish black; trochanter yellow; femur light brown with basal 1/4 yellow and apical cap brownish black; tibia brownish black except extreme base yellowish white; tarsus brownish black except basal 1/3 or a little more (and basal 1/2 or more along ventral margin) of basitarsus dark yellow or light brown, and basal 1/3 of 2nd segment yellowish white; basitarsus (Fig. 32) somewhat enlarged, slightly widened from base to apical 1/4, then slightly narrowed toward apex, ca. 4.6× as long as its greatest width, and ca. 0.9× and ca. 1.0× as wide as hind tibia and femur, respectively. Calcipala distinct but small. Pedisulcus well marked. ***Wing.*** Length 3.0-3.1 mm. Other features (including the haired basal portion of radial vein) as in female except subcosta bare. ***Abdomen.*** Basal scale brownish black with a fringe of long dark hairs. Dorsal surface brownish black except anterior 2/3 of 2nd segment light to medium brown, and with dark short hairs; segments 2, 5, 6 and 7 each with a pair of large whitish-grey pruinose spots (in addition, segments 3 and 4 each often with a pair of similar dorsal spots though much smaller and not shining); those on segments 2, 5 and 6 situated dorsolaterally, while those on segment 7 almost laterally. ***Genitalia*** (Figs. 36-46). Coxite in ventrolateral view shorter than wide; style in ventrolateral view elongate, longer than coxite, ca. 3.2× as long as its greatest width near base, nearly parallel-sided, though apical 2/3 gently curved and slightly narrower than basal 1/3, and with a subterminal spine; style spatulate ventrodorsally, with basal protuberance directed dorsomedially, bearing numerous spinules on its surface. Body of ventral plate nearly quadrate, slightly shorter than wide, and having a long ventrally-produced process with short hairs basally on ventral surface, but its apex and posterior surface entirely bare; basal arms of ventral plate nearly parallel-sided or slightly diverging from each other. Median sclerite long, plate-like, narrow basally, gradually widened toward apex, and moderately sclerotized except median portion of apex unsclerotized or weakly sclerotized. Parameres broad basally, each with several hooks apically, as in *S. maenoi.* Aedeagal membrane densely setose; dorsal plate strongly sclerotized. Abdominal segment 10 with 13-16 short hairs on each posterolateral corner. Cerci rounded, each with 14-18 short hairs.

Pupa. Body length ca. 4.0 mm. ***Head.*** Integument dark yellow to yellowish brown, densely covered with tubercles all over, even on antennal sheaths, which have no outwardly-raised portions; tubercles on frons somewhat larger than others on face and antennal sheaths, and with several minute projections on its surface (Fig. 47); frons with 2 long trichomes each with 2-4 long branches (Fig. 48) on each side; 2 frontal trichomes on each side situated close together; face with 1 long trichome with 3 or 4 long branches (Fig. 49) on each side. ***Thorax.*** Integument dark yellow to yellowish brown, densely covered with tubercles all over; relatively larger tubercles on anterior surface each with minute projections on its surface similar to those on frons; integument on each side with 5 very long fan-like trichomes, each with 5-8 long branches (Fig. 50) anterodorsally and anterolaterally, 1 long trichome with 2-4 branches posterolaterally, and 3 long trichomes (each with 2-4 branches, or rarely no branch) ventrolaterally. Gill (Figs. 51 and 52) with 6 somewhat inflated short filaments arranged in 3 pairs, all arising from base; middle pair almost sessile and dorsal and ventral pairs with short stalk; inner filament of dorsal pair directed inward and upward, and suddenly curved outward or forward or backward, outer filament of dorsal pair directed upward and forward; 2 filaments of middle pair directed forward and then somewhat upward; 2 filaments of ventral pair directed downward and then forward, though inner filament usually directed somewhat inward; all filaments dark brown, subequal in length (average 1.8 mm, range 1.3-2.5 mm) and in thickness to one another; cuticle of all filaments with well-marked annular ridges and furrows, ridges elaborately forming reticulate patterns and densely covered with minute tubercles, somewhat larger ones on ridges and smaller ones on interridge spaces (Fig. 53). ***Abdomen.*** Tergum 1 yellow, with 1 simple long seta on each side; tergum 2 pale, with 1 simple medium-long seta and 5 short spines submedially on each



Figures 36-46. Male genitalia of *S. choochotei* sp. nov. 36, right coxite, right style and ventral plate *in situ* (ventral view); 37, right coxite and style (ventrolateral view); 38, left style (medial view); 39-42, ventral plates (39, ventral view; 40, lateral view, with median sclerite; 41 and 42, of different males, end view); 43, median sclerite (end view); 44, dorsal plate (end view); 45 and 46, 10th abdominal segments and cerci (45, end view; 46, lateral view). Scale bar. 0.05 mm for figs. 36-46.

side; terga 3 and 4 each with 4 hooked spines and 1 simple spine on each side; terga 5-9 lacking spine-combs except tergum 8 with well developed spine-combs in transverse row on each side (tergum 8 broadly yellow transversely covering bases of spine-combs); terga 6-9 each with comb-like groups of minute spines in transverse rows on each side: tergum 9 yellow entirely, and sparsely tuberculate at bases of terminal hooks, which are very large and directed posteriorly (Figs. 54 and 55). Segments 2-5 each with 3 short simple spines on each lateral surface. Sternum 4 with 2 simple or bifid hooklets and a few medium-long spinous setae on each side; sternum 5 with pair of simple or bifid hooks submedially and a few simple medium-long spinous setae on each side, sterna 6 and 7 each with pair of simple inner and simple outer hooks somewhat spaced from each other, and a few simple medium-long spinous setae on each side. Grapnel-like hooklets absent. ***Cocoon*** (Fig. 56). Boot-shaped, loosely woven, with many small open spaces in webs; wall around opening very high, being ca. 3.0 mm; individual threads visible; ca. 6.0 mm long × ca. 1.8 mm wide.

Mature larva. Body length 9.6-11.2 mm. Body (Photo. 6) characteristic in shape, being gradually widened posteriorly up to abdominal segment 8, and then sharply narrowed to posterior circlet; body color entirely dark greyish brown. Cephalic apotome (Photo. 4) light to medium brown except anterior 2/5 usually dark brown, though narrow portion along posterior margin dark brown, and lateral portion between mediolateral and posterolateral spots on each side usually medium to dark brown; head spots usually indistinct or appearing negative except posterior one of median longitudinal spots and posterior 1/2 of mediolateral spots usually dark brown, and anterior one of the same spots rarely positive. Lateral and ventral surfaces of head capsule (Photo. 5) medium to dark brown with faint to marked negative spots. Antenna composed of 3 segments and apical sensillum, much longer than stem of labral fan; length ratio of segments (from base to tip) 1.0:1.4:0.5. Labral fan with ca. 50 main rays. Mandible (Fig. 57) with mandibular serrations composed of 1 large tooth and 1 small one, without supernumerary serrations; comb-teeth decreasing in length from 1st to 3rd. Hypostomium (Fig. 58) with 9 anterior teeth, median tooth longer than others; lateral margins weakly serrate apically; 5 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Photo. 5) deep, subtriangular, though nearly parallel-sided near base, pointed apically, 2.3-3.0× as long as postgenal bridge. Cervical sclerite (Fig. 59) composed of 2 light brown pieces, of which 1 is stick-like, appearing fused to occiput, and the other is elliptical, fused or unfused to the stick-like piece, on each side. Thoracic cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short colorless setae on each side of anal sclerite. Rectal papilla of 3 lobes, each with 14-18 finger-like secondary lobules. Anal sclerite X-shaped, with anterior arms broadened and bifurcated apically, which are ca. 0.7× as long as posterior ones. Last abdominal segment slightly bulged laterally but lacking ventral papillae. Posterior circlet with ca. 210 rows of hooklets with up to 25 hooklets per row.

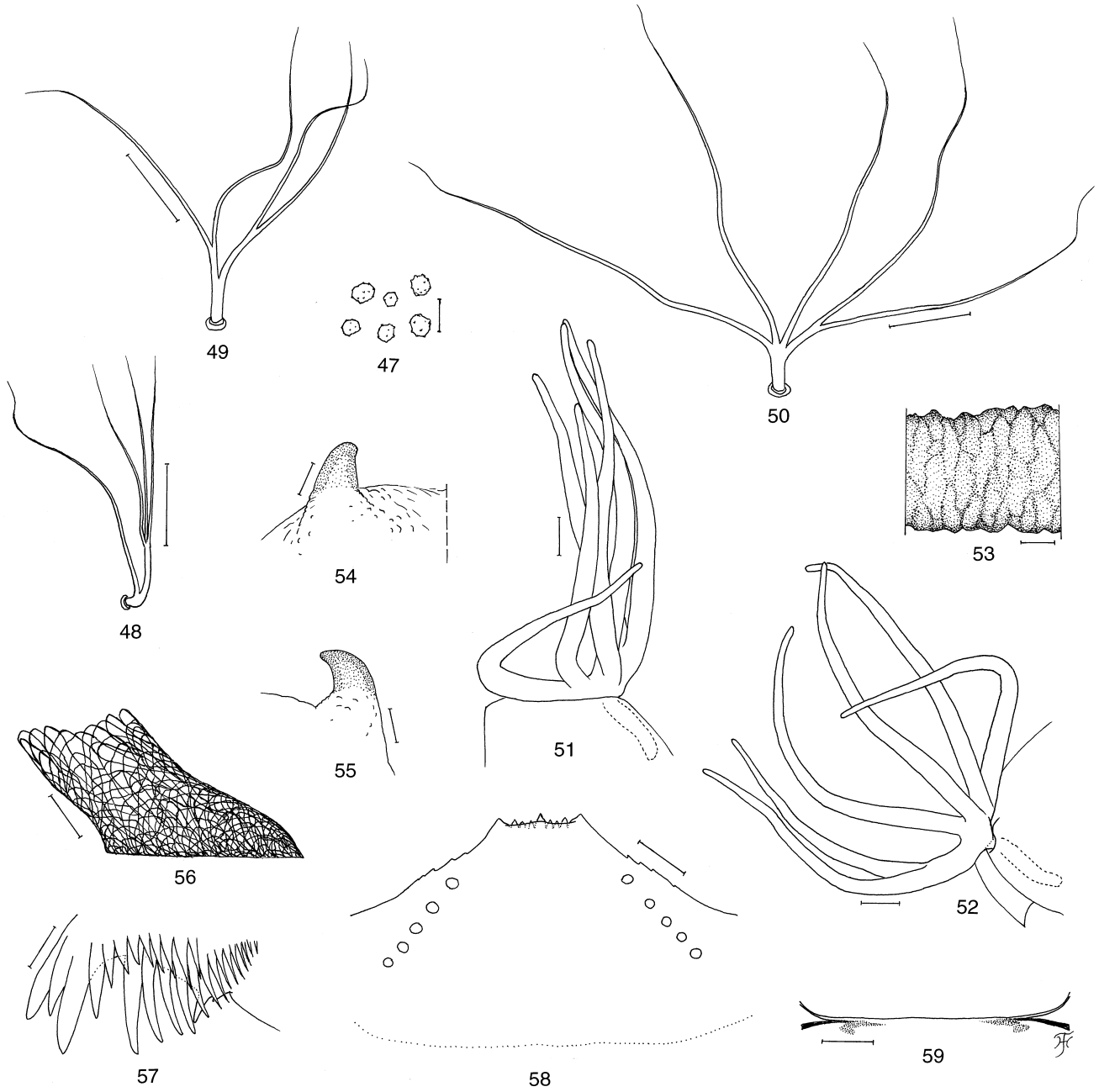
TYPE SPECIMENS. Holotype female, reared from pupa, Monthatharn Waterfall, altitude 750 m, Chiang Mai Province, northern Thailand, 28.XI.2001, collected by W. Choochote. Paratypes 7 females, 7 males, 3 pupae, 49 pupal exuviae and 4 mature larvae, same data as holotype except the date 19.XII.2001.

ECOLOGICAL NOTES. The pupae and larvae of *S. choochotei* were found to be attached to the surface of the stream-bed rock in shaded cascading waterfalls. Water temperature was 16 or 17℃. This species was collected together with *S. brevipar*, *S. decuplum*, *S. fenestratum*, *S. grossifilum*, *S. maenoi*, *S. rudnicki*, *S. rufibasis*, and *S. tani.*

ETYMOLOGY. The species name *choochotei* is given after Mr. Wej Choochote, Associate professor, Department of Parasitology, Chiang Mai University, Chiang Mai, Thailand, who discovered this new species.

REMARKS. *S. choochotei* sp. nov. is characterized by its large body. It seems to be most related to *S. tenuitarsus* Puri, 1933, originally described from the male and pupal specimens collected from Bengal Terai, India (Puri, 1933), by having the similar shape of the male genitalia, the similar coloration of the antenna, the haired basal portion of the radial vein, multibranched trichomes on the head and thorax of the pupa and the boot-shaped cocoon. However, this species is easily distinguished from the latter species by the somewhat inflated pupal gill filaments (Figs. 51 and 52). The cocoon and the male ventral plate of *S. kawamurae* from Japan (Bentinck, 1955) are similar to those of *S. choochotei*, but both species are different from each other by many other characters including female genitalia, male style, and pupal gill filaments.

S. choochotei differs from *S. digrammicum*, *S. grossifilum*, *S. maenoi*, *S. rudnicki* and *S. yongi* in the female by the presence of numerous long stout hairs on the anterior gonapophysis (Fig. 33), haired basal portion of the radial vein (except *S. digrammicum*), the cibarium with a round dorsally-directed projection (Fig. 30); in the male by the



Figures 47-59. Morphological features of the pupa and larva of *S. choochotei* sp. nov. 47, tubercles with secondary projections on frons; 48, frontal trichome; 49, facial trichome; 50, thoracic trichome; 51 and 52, pupal gill filaments (51, right side, dorsal view; 52, left side, lateral view); 53, middle part of pupal gill filament; 54 and 55, terminal hooks (54, end view; 55, lateral view); 56, cocoon (lateral view); 57, apical part of larval mandible; 58, larval hypostomium; 59, cervical sclerite of larval head. Scale bars. 1.0 mm for fig. 56; 0.2 mm for figs. 51 and 52; 0.1 mm for fig. 59; 0.05 mm for figs. 48-50 and 58; 0.02 mm for 53-55 and 57; 0.01 mm for fig. 47.

shape of the ventral plate (Fig. 39) and the 10th abdominal segment with numerous hairs on each posterolateral corner (Figs. 45 and 46); in the pupa by the somewhat inflated gill filaments (Figs. 51 and 52), and the shape of the cocoon (Fig. 56), and in the larva by the shape and size of the larva (Photo. 6).

***Simulium* (*Simulium*) *digrammicum* Edwards, 1928**

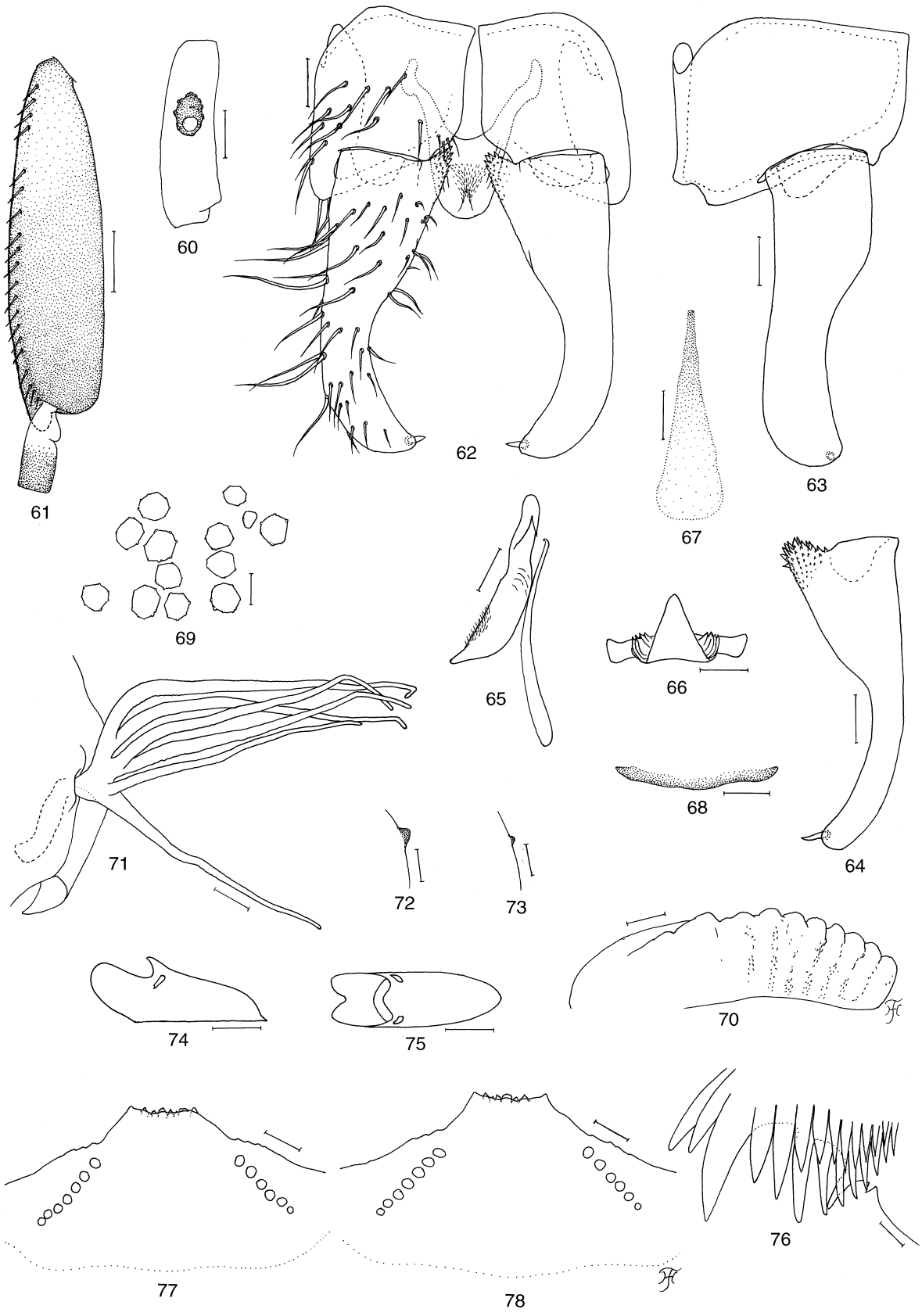
Simulium digrammicum Edwards, 1928: 61-62 (female).

Simulium (*Simulium*) *digrammicum*: Takaoka and Suzuki, 1984: 41; Crosskey and Lowry, 1990: 210; Takaoka and Davies, 1995: 102-105 (female).

This species was described from female specimens collected in Peninsular Malaysia, together with a single female specimen collected from Nakon Sri Tamarat, Khao Lung Province, in Thailand (Edwards, 1928). The female lectotype and paralectotype specimens of *S. digrammicum* have been examined and redescribed by Takaoka and Davies (1995). The reared male, pupa and mature larva of this species are here described for the first time based on the specimens collected in northern Thailand.

DESCRIPTION. **Male.** Body length 2.8 mm. ***Head.*** Width slightly wider than thorax. Upper eye consisting of large facets in 21 horizontal rows and in 21 or 22 vertical columns. Clypeus brownish black, white pruinose, moderately covered with dark stout hairs except median portion widely bare. Antenna composed of 2+9 segments, dark brown except base of 1st flagellar segment yellow; 1st flagellar segment much elongated, ca. 2.7× as long as 2nd flagellomere. Maxillary palp composed of 5 segments, with proportional lengths from 3rd to 5th segments 1.0:1.0:2.2; 3rd segment (Fig. 60) of normal size; sensory vesicle of medium size, ellipsoidal, ca. 0.23× as long as 3rd segment, with opening of medium size. ***Thorax.*** Scutum brownish black, uniformly and densely covered with golden-yellow recumbent fine hairs, interspersed with long upright hairs on prescutellar area; in certain angles of light scutum with large white-pruinose shiny spot on each shoulder, extending posteriorly forming a broad white-pruinose band along each lateral margin up to base of wing. Scutellum medium brown, with several upright dark hairs as well as golden-yellow fine hairs. Postscutellum dark brown, shiny, without hairs. Pleural membrane and katepisternum as in female of *S. maenoi*. ***Legs.*** Foreleg: coxa yellowish white; trochanter dark brown except base of inner surface yellow; femur medium brown; tibia medium brown with median large area light brown, and outer surface of basal 3/4 whitish, and sheeny in certain angle of light; tarsus entirely brownish black; basitarsus, 2nd and 3rd tarsal segments somewhat dilated (basitarsus ca. 7.1× as long as its greatest width). Midleg: coxa dark brown; trochanter dark brown except base yellow; femur medium to dark brown; tibia yellowish white on basal 1/2, medium to dark brown on apical 1/2; tarsus brownish black though basal portion of basitarsus dark brown. Hind leg: coxa dark brown; trochanter dark yellow or light brown except basal 1/2 yellow; femur medium brown with basal 1/5 yellow and apical cap dark brown; tibia dark brown except extreme base yellow; tarsus light to medium brown except basal 1/2 of 2nd tarsal segment pale white; basitarsus (Fig. 61) much enlarged, gradually widened from base to apical 1/4, then slightly narrowed toward apex, 3.7× as long as its greatest width, and 0.96× and 1.14× as wide as hind tibia and femur, respectively. Calcipala distinct but small. Pedisulcus well marked. ***Wing.*** Length 2.3 mm. Costa with spinules and hairs; subcosta bare; basal section of radial vein fully haired; hair tuft at base of stem vein dark brown; basal cell absent. ***Abdomen.*** Basal scale brownish black with a fringe of long dark hairs. Dorsal surface medium to dark brown, and with dark short hairs; segments 2, 6 and 7 each with a pair of large whitish-grey pruinose (iridescent in certain lights) spots; those on segments 2 and 6 situated dorsolaterally, while those on segment 7 almost laterally. ***Genitalia*** (Figs. 62-68). Coxite in ventrolateral view much shorter than wide; style in ventrolateral view elongate, much longer than coxite, ca. 3.1× as long as its greatest width near base, straight and nearly parallel-sided on basal 1/3, then abruptly narrowed toward apical 1/3 (0.6× as wide as basal 1/3), and again nearly parallel-sided up to near apex, though inner margin of apical 2/3 gently curved, and with a terminal spine; style spatulate ventrodorsally, with basal protuberance directed dorsomedially, bearing numerous spinules on its surface. Body of ventral plate longer than wide, narrowed posteriorly when viewed ventrally, and having a ventrally-produced process with short hairs basally on ventral surface, but its apex and posterior surface entirely bare; basal arms of ventral plate widely diverging from each other. Median sclerite long, plate-like, narrow basally, gradually widening toward apex, and moderately sclerotized. Parameres broad basally, each with several hooks apically, as in *S. maenoi.* Aedeagal membrane densely setose; dorsal plate strongly sclerotized. Abdominal segment 10 with 1 or 2 short hairs on each posterolateral corner. Cerci rounded, each with 10 or 11 short hairs.

Pupa. Body length ca. 2.8 mm. ***Head.*** Integument dark yellow, moderately covered with tubercles of various sizes; large tubercles each with several minute projections peripherily on its surface (Fig. 69), but smaller tubercles with no such projections; frons with 2 long fan-like trichomes each with 9 or 10 long branches (similar to those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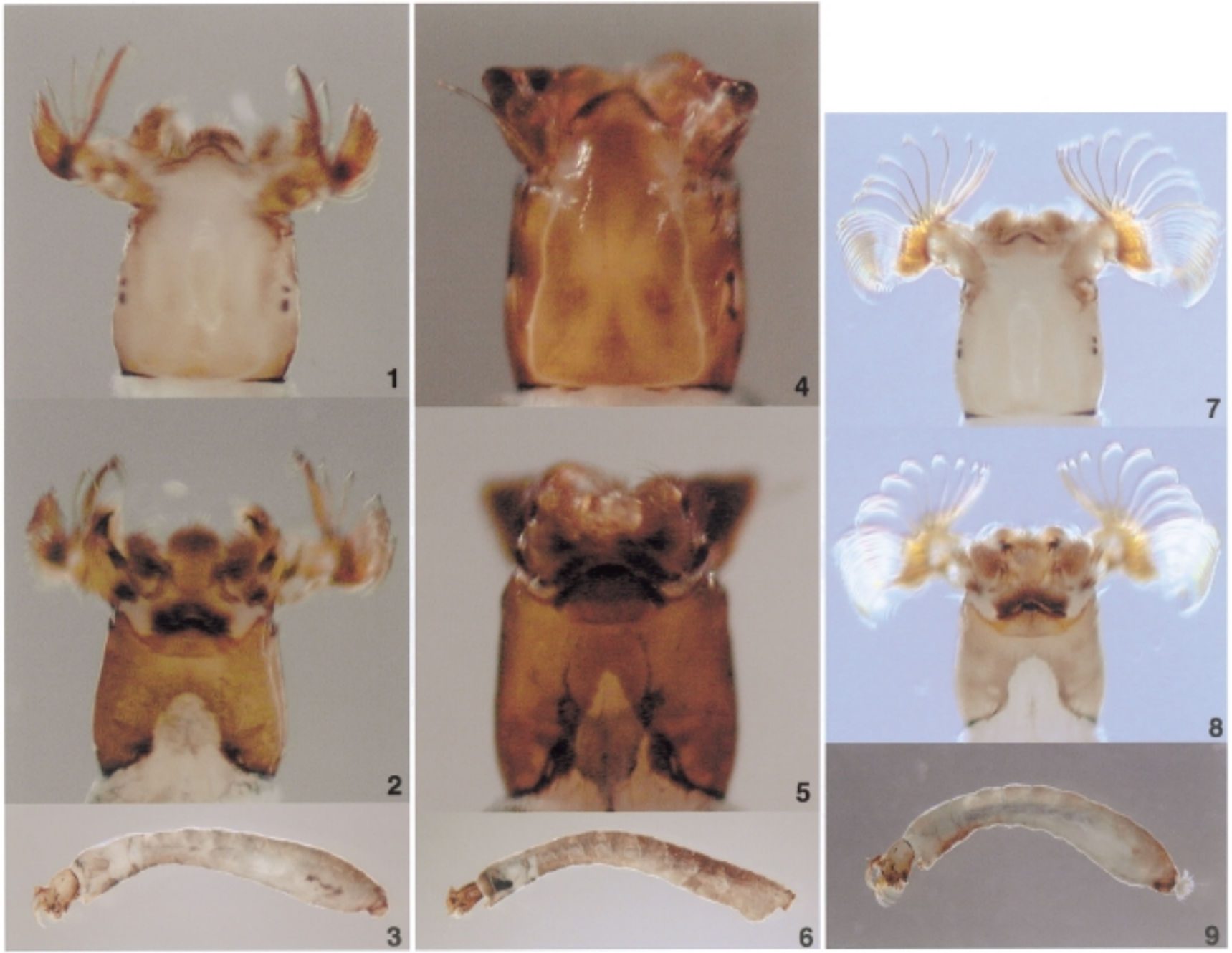
of *S. maenoi*) on each side; 2 frontal trichomes on each side situated close together; face with 1 long fan-like trichome with 7-11 long branches (similar to those on frons) on each side; antennal sheath (Fig. 70), with 9 moderately raised portions produced outwardly, corresponding to flagellar segments 1-9; outer surface of antennal sheath sparsely covered with small round tubercles. ***Thorax.*** Integument dark yellow, moderately or sparsely covered with tubercles of various sizes anteriorly, and with small conical tubercles posteriorly; most of large tubercles on anterior surface each with minute projections on its surface similar to those on frons, but smaller tubercles with no such projections; integument on each side with 5 long fan-like trichomes, each with 10-14 long branches (similar to those of *S. maenoi*) anterodorsally and anterolaterally, 1 medium-long stout trichome with 4 or 5 branches posterolaterally, and 3 trichomes (1 short slender simple or bifid, 1 medium-long stout simple, and 1 medium-long stout trifid) ventrolaterally. Gill (Fig. 71) with 6 slender short filaments arranged in 3 pairs; dorsal and middle pairs arising from very short common stalk and each pair with short stalk, 2 filaments of ventral pair directly arising from base of gill and almost sessile; all filaments subequal in length (0.7-0.9 mm) and thickness to one another, except outer filaments of middle and ventral pair somewhat thinner than others, and inner filament of ventral pair somewhat thicker basally than others; all filaments directed forwards except inner filament of ventral pair directed downward and forward; cuticle of all filaments pale yellow, with well marked annular ridges and furrows, and densely covered with minute tubercles. ***Abdomen.*** Tergum 1 pale yellow, with 1 simple medium-long seta on each side; tergum 2 pale, with 1 simple medium-long seta, 1 simple short seta and 4 short spinous setae (in 1 pupa, 4 of 8 spinous setae bifid apically) submedially on each side; terga 3 and 4 each with 4 hooked spines and 1 simple short seta on each side; terga 5-9 lacking spine-combs except tergum 8 with spine-combs (consisting of 6-9 spines) in transverse row (in 2 of 3 pupae, tergum 8 with only 1 small conical spine, not forming transverse spine-combs) on each side; tergum 9 with small conical terminal hooks (Fig. 72) [in 1 of 3 pupae, terminal hooks weakly-developed (Fig. 73)]. Sternum 4 with 1 simple slender hooklet and a few minute setae on each side; sternum 5 with pair of simple or bifid hooks submedially and a few simple minute setae on each side; sterna 6 and 7 each with pair of simple or bifid inner and simple outer hooks somewhat spaced from each other, and a few simple minute setae on each side. Grapnel-like hooklets absent. ***Cocoon*** (Figs. 74 and 75). Boot-shaped, tightly woven, with relatively high wall around opening except anteromedian portion with deep notch; anterodorsal margin thickly woven forming a short bulge; cocoon with small lateral window on each side; individual threads invisible; 3.7-4.0 mm long × 1.1-1.4 mm wide.

Mature larva. Body length 5.0-5.5 mm. Body (Photo. 9) normal in shape, with thorax somewhat inflated, and abdomen gradually widened posteriorly up to segments 6 and 7, and then narrowed to posterior circlet; body color pale creamy. Cephalic apotome (Photo. 7) pale creamy; head spots indistinct. Lateral surface of head capsule pale creamy; spots behind and below eye-spot region indistinct; ventral surface of head capsule (Photo. 8) pale creamy or yellow, though somewhat darkened around postgenal cleft; spots on each side of postgenal cleft negative. Antenna composed of 3 segments and apical sensillum, somewhat longer than stem of labral fan; length ratio of segments (from base to tip) 1.0:1.4:0.6. Labral fan with 38-40 main rays. Mandible (Fig. 76) with mandibular serrations composed of 1 large tooth and 1 small one, without supernumerary serrations; 1st tooth of comb-teeth shorter than 2nd one but longer than 3rd one. Hypostomium (Figs. 77 and 78) with apparently abnormal row of apical teeth; lateral margins weakly serrate apically; 6 or 7 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Photo. 8) deep, bullet-shaped, rounded apically, ca. 3.5× as long as postgenal bridge. Cervical sclerite composed of small, pale yellow, rod-like piece on each side. Thoracic cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short colorless setae not only on each side of anal sclerite but also on each lateral bulge, and sparsely on dorsal surface just before rectal papilla. Rectal papilla of 3 lobes, each with 14-17

Figures 60-78. Morphological features of the male, pupa and larva of *S. digrammicum* Edwards. 60, 3rd segment of male maxillary palp with sensory vesicle (right side; front view); 61, basitarsus and 2nd tarsal segment of male hind leg (left side and outside view); 62, coxites, styles and ventral plate *in situ* (ventral view); 63, right coxite and style (ventrolateral view); 64, left style (medial view); 65, ventral plate and median sclerite (lateral view); 66, ventral plate (end view); 67, median sclerite (ventral view); 68, dorsal plate (end view); 69, tubercles with secondary minute projections on pupal frons; 70, left antennal sheath of pupa; 71, pupal gill filaments (right side, lateral view); 72 and 73, pupal terminal hooks of different size (lateral view); 74 and 75, cocoons (74, lateral view; 75, dorsal view); 76, apical part of larval mandible; 77 and 78, larval hypostoma showing abnormal anterior teeth. Scale bars. 1.0 mm for figs. 74 and 75; 0.1 mm for figs. 61 and 71; 0.05 mm for fig. 70; 0.03 mm for figs. 60, 62-68, 77 and 78; 0.02 mm for figs. 72 and 73; 0.01 mm for figs. 69 and 76.



Photographs 1-9. Whole bodies and head capsules of mature larvae. 1, 4 and 7, dorsal views of head capsules; 2, 5 and 8, ventral views of head capsules; 3, 6 and 9, whole bodies of larvae (lateral view; body length: 3, 6.2 mm; 6, 11.0 mm; 9, 5.0 mm). 1-3, *S. maenoi* sp. nov.; 4-6, *S. choochotei* sp. nov.; 7-9, *S. digrammicum* Edwards.

finger-like secondary lobules. Anal sclerite X-shaped, with anterior arms broadened apically, which are ca. 0.6× as long as posterior ones. Last abdominal segment moderately bulged laterally but lacking ventral papillae. Posterior circlet with ca. 150 rows of hooklets with up to 23 hooklets per row.

SPECIMENS EXAMINED. 1 female and 1 male, both reared from pupae, 1 pupal exuvia, 2 mature larvae, at Siriphum Waterfall, altitude 1,265 m, Chiang Mai Province, northern Thailand, 12.VI.2001, by H. Takaoka and W. Choochote.

ECOLOGICAL NOTES. The pupae and larvae of *S. digrammicum* were found to be attached on grass stalk or leaf in a shaded stream 2-5 m wide. Water temperature was 18 ℃. This species was collected together with *S. asakoae*, *S. chamlongi*, *S. decuplum*, *S. fenestratum*, *S. feuerborni*, *S. inthanonense*, *S. rufibasis*, and *S. sheilae.*

REMARKS. *S. digrammicum* was originally described from female specimens collected from Cameron's Highlands, Pahang, Malaysia (Edwards, 1928), but was later treated as a junior synonym of *S. griseifrons* from India by Puri (1932), and this course was followed by Crosskey (1973 and 1988). Takaoka and Davies (1995), after examining the type specimens of both species preserved in the Natural History Museum, London, reported that the female of *S. digrammicum* is separable from that of *S. griseifrons* by the shape of the anterior gonapophysis and the number of short hairs on it.

In this study, we obtained two reared adults of this species, one female and one male, in northern Thailand, and confirmed its identity by comparing the female characters including the genitalia, with those of *S. digrammicum* given by Takaoka and Davies (1995). It is however noted that the Thai female specimen shows a few differing characters as follows (characters of the Malaysian specimens in parenthesis): Frons-head ratio 1:4.1 (1:3.6); proportional lengths of 3rd, 4th and 5th segments of maxillary palp 1.0:1.0:2.1 (1.0:1.2:2.4); cibarium with a few tubercles, though very tiny, near posterior margin (bare); cercus round posteriorly when viewed from side (nearly straight).

The male and pupa of this species are very similar to those of *S. griseifrons* described by Puri (1932). However there are several distinct differences in the male characters between the two species. For instance, *S. digrammicum* is easily distinguished from *S. griseifrons* by the entirely darkened femora and the haired ventral plate. On the contrary, the pupae of both species are alike except the shape of the cocoon, of which the anterior collar has a deep notch medially in this species [c.f., the anterior collar does not have such a notch in *S. griseifrons,* according to the figure given by Puri (1932)].

S. digrammicum is distinguished from the other related species (including two new species described here) of the *griseifrons* species-group by its unique shape of the cocoon (Figs. 74 and 75) and the long second comb-tooth of the larval mandible (Fig. 76).

ACKNOWLEDGEMENTS

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Etiology of Diarrhoea Among Adult Patients During the Early Monsoon Period in Kathmandu, Nepal

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Abstract: One hundred and eighty-one patients with acute diarrhoea attending the gastroenteritis ward of Sukra Raj Tropical and Infectious Disease Hospital (STIDH) were investigated during the early monsoon, April to May 2001. Bacterial pathogens were isolated in 33% of the patients. Enteropathogenic *Escherichia coli* was isolated in 8.28%, *Shigella* species in 13.25% and *Vibrio cholerae* 01 in 1.1% of the patients. Mixed infections with bacterial pathogens, helminths and protozoan parasites were commonly observed in the study. *Trichuris trichiuria* was detected in 27.6%, hookworms in 12.7% and *Ascaris lumbricoides* in 11.04%. *Entamoeba histolytica/dispar* and *Giardia lamblia* were observed in 12.7% and 7.73% of the patients, respectively. A large number of *Cryptosporidium* (7.73%) and *Cyclospora* species (3.86%) usually present in immunocompromised patients were also detected in acute diarrhoeal cases. The results showed that a wide range of bacterial pathogens was isolated from the inhabitants of Kathmandu, Nepal prior to the monsoon. These findings indicate that the bacterial pathogens, especially diarrhoeaginic *E. coli* and *Shigella*, and protozoan parasites, need to be given additional attention in the diagnosis and treatment of acute diarrhoea.

Key words: Acute diarrhoea, etiology, bacterial pathogens, helminthic parasite, Nepal

INTRODUCTION

Diarrhoea is the most common illness among children in the developing world. It is the primary cause of mortality and morbidity and the highest risk occurring within the first 5 years of life (Bern *et al.*, 1992). However, it is also a serious public health problem among adults, causing significant levels of morbidity during early summer and the rainy season in developing countries like Nepal (WHO, 1992; Bern *et al.*, 1992). The incidence of diarrhoeal diseases in Nepal tends to rise sharply during warm summer months with the peak incidence occurring in July and August. Food borne gastroenteritis does increase in April and May each year, followed by water-borne gastroenteritis and cholera in the beginning of rainy season (WHO, 1992). Sukraraj Tropical and Infectious Disease Hospital (STIDH) is the only infectious disease hospital receiving adult patients with diarrhoea in Kathmandu. A total of 4497 patients with diarrhoea were admitted in the year 2000 in STIDH. Although several studies have been conducted in the past, there are no clear figures on the etiology of diarrhoea in the adult population (WHO, 1992; Hoge *et al.*, 1995; Sherchand and Shrestha, 1996). Despite a number of individual hospitals setting up their own guidelines at present, there is still no established national protocol for the management of diarrhoeal diseases. In this situation, etiological study of diarrhoeal disease is very important for the provision of base-line information on the causes of diarrhoeal disease and preparation of a na-

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tional protocol for the management of diarrhoea.

The present study was initiated to establish the etiological agent responsible for acute diarrhoea in the adult population with samples collected from inhabitants of the Kathmandu Valley.

Materials And Methods

Patients attending the STIDH those with acute diarrhoea were enrolled in the study. Criteria for the exclusion from the study included patient's administration of antibiotics and systemic illness (heart disease, enteric fever, leshmaniasis, central nervous system manifestations). The hospital staff completed a set of questionnaires after obtaining written consent from the patients. Demographic profile was recorded, general physical examination was performed and severity of dehydration was assessed. Stool samples were collected, and appropriate treatment was started based on the degree of dehydration. Samples were subjected to routine parasitological and bacteriological examination. Treatment was reviewed after receiving the laboratory reports for the stool test.

Parasitological test

The stool samples were collected in the morning from 181 patients older than 14 years in STIDH and were examined. Direct smears were prepared with normal saline, iodine, and sucrose solution. Two and half percent of potassium dichromate solution and modified Ziehl Nelsen staining were applied to differentiate protozoan parasites into mainly *Cryptosporidium*, *Isospora* and *Cyclospora* (Casemore, 1991). A formal ether concentration method was also used to identify other intestinal parasites.

Bacteriological test

Samples received in the laboratory were cultured immediately to identify pathogenic microorganism such as *Vibrio cholerae, Salmonella* species, *Shigella* Species and *Escherichia coli* using standard bacteriological method (Mackie and McCartney, 1996). Stool samples were inoculated onto thiosulphate citrate bile salt sucrose (TCBS) Agar, MacConkey agar, eosin methylene blue (EMB) agar as well as SS agar, and into enrichment media such as Selenite F, Rappaport and Alkaline peptone water (pH 8.5). After 24 hours of incubation at 37℃, Rappaports and Selenite F broth cultures were inoculated onto MacConkey and SS agar, respectively. Alkaline peptone water was subcultured onto TCBS agar after 12 hours and incubated overnight at 37℃. Typical characteristic vibrio like colonies seen on TCBS agar were subcultured onto nutrient agar and identified by biochemical test. Biotypes of *V. cholerae* were determined using Voges-Proskauer (VP), polymyxin B sensitive and haemolysin production test (Blood agar). Biochemically confirmed *V. cholerae* was subjected for serotyping. Serotyping was carried out with the use of diagnostic antiserum raised against *V. cholerae* manufactured by Denka Seiken, Japan as per manufacturer's protocol.

Both lactose and non-lactose fermenting colonies from MacConkey and SS Agar were subjected to further identification. Biochemical and serological tests such as TSI, SIM, VP, dycarboxylase, simons citrate medium. Diagnostic antiserum (Denka Seiken Co. LTD Tokyo, Japan) was also used for serotyping of enteropathogenic *E. coli*, *Salmonella* and *Shigella* species. Skirro's medium was used for the isolation of *C. jejuni* and suspected colinies were further examined by gram stain. The strains identified as above were also confirmed by using API 20E *in vitro* diagnostic biochemical kit manufactured by bioMerieux SA 69280 Marcy/Etoile-France.

Statistical methods

Differences between valuse were examined using Student's t-test analysis was carried out using SPSS version 10, Statistical Analysis Software, 2000.

Results

A total of 181 patients aged 14 years and above attending the STIDH between April and May with acute diarrhoea was included in this study (Table 1). The patients recorded, an average, 10-15 loose motions before coming to the hospital. The patients reported an average of 11 hours of loose motion before coming to the hospital. Of those 60% of the cases were female (P<.0001) and the highest numbers (40%) were in the age group between 20-29 for both males and females (Fig. 1). The main symptoms associated with diarrhoea were combination of abdominal pain (70%), vomiting (62%), and fever (15%) with moderate to severe dehydration (Fig. 2).

Since the etiology of diarrhoea has not been studied in adult population, the prevalence of all the enteropathogens including *Cryptosporidium* and *Cyclospora* was also determined. Bacterial pathogens were isolated from 33% of the patients (Table 1). Among them enteropathogenic *E. coli* (EPEC) were isolated from 8.28%, *Shigella* species from 13.25% (*Shigella dysenteriae* in 5%, *S. flexneri* in 4.41% *S. boydi* in 2.2% *S.sonnie* in 1.65%). The result also showed that EPEC was the most commonly isolated single bacterial pathogen, followed by *S. dysenteriae* 5% and *Salmonella* species was isolated in 4.41% of the cases tested.

The result showed that 40% of the patients were infected with more than one organism. Protozoal pathogens

Table. 1 Enteric pathogens identified among 181 patients of diarrhoea from April and May, 2001 in STIDH, Teku, Kathmandu

Serial Number	Enteropathogens	Patients (n＝181)	Percentage
1	Enteropathogenic *Escherichia coli*	15	8.28
2	Enterotoxigenic *Escherichia coli*	5	2.76
3	Enteroinvasive *Escherichia coli*	4	2.20
4	Enterohaemorrhagic *Escherichia coli*	2	1.10
5	*Shigella dysenteriae*	9	5.0
6	*Shigella boydi*	4	2.2
7	*Shigella flexneri*	8	4.41
8	*Shigella sonnei*	3	1.65
9	*Salmonella species*	8	4.41
10	*Vibrio cholerae* 01	2	1.1
11	*Camphylobacter jejuni*	1	0.55
12	*Entamoeba histolytica/ E. dispar*	23	12.7
13	*Cyclospora species*	7	3.86
14	*Cryptosporidium*	14	7.73
15	*Blastocystis hominis*	14	7.73
16	*Ascaris lumbricoides*	20	11.04
17	*Giardia lamblia*	14	7.73
18	*Trichuris trichiuria*	50	27.6
19	Hookworm	23	12.7
20	*Hymenolepsis nana*	2	1.1
21	*Chilomastix mesnili*	3	1.65
22	*Dientamoeba fragilis*	1	0.55
23	Trichomonas	1	0.55
24	Strongyloides	3	1.65
25	*Isospora belli*	1	0.55
26	*Iodamoeba butschlii*	3	1.65

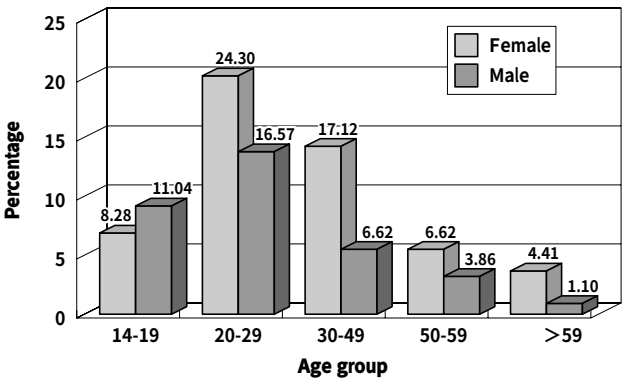

Fig.1: Percentage of sex and age distribution of patients with acute diarrhoea.

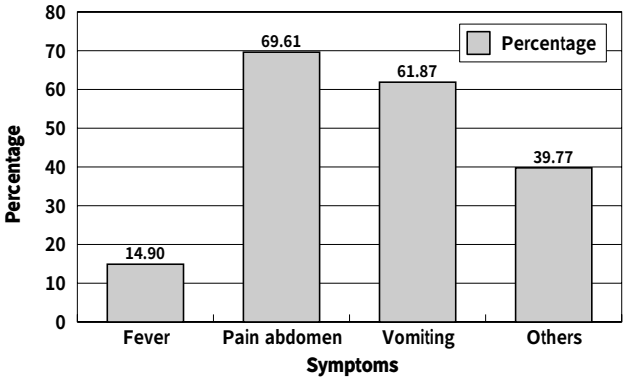

Fig.2: Percentage of other symptoms associated with diarrhoea on admission to hospital in Kathmandu, Nepal, 2001.

E. histolytica/E. dispar, G. lamblia, Cryptospridium and *Cyclospora* were detected in 31% of the patients. Of these 12.7% were infected with *E. histolytica/E. dispar*, 3.86% with *Cyclospora* and 7.73% each with *G. lamblia* and *Cryptosporidium*. It was also observed that *T. trichiuria* infection was the predominant helminthic infection (27.6%) among the patients. On the other hand, 13% of the patients were infected with hookworm and 11.04% with *A. lumbricoides*. The symptoms associated with *Blastocystis hominis* are similar to those associated with *G. lamblia* both of them isolated in 7.73% of the patients. *Hymenolepsis nana* and *Isospera belli* were detected in 1.1 and 0.55% of the patients, respectively.

Although *V. cholerae* 01 is the major cause of acute watery diarrhoea during the rainy season, it was only isolated 1.1% in our study period, which was conducted during early part of the rainy season. On the other hand rare pathogens like *Chilomastix mesnili, Iodamoeba butschlii* were also detected in the range of 1% and *Strongyloides* was detected in 1.65% of the cases.

DISCUSSION

This paper is one of the first studies to examine the etiology of diarrhoea during warm early monsoon among the adult Nepalese population. This study confirms the importance of *E. coli* species, *Shigella species, E. histoytica, G. lamblia* and *Cryptosporidium* as the causative agents of acute diarrhoea in Nepal. Hoge *et al.* (1995) reported similar observation among children under five years. Our results indicate that *Cryptosporidium* and *Cyclospora* species are also important pathogens in Kathmandu. The low prevalence of cholera in this study is most likely due to the season during which our sampling was undertaken. It is important that an etiological study should be continued for a complete year irrespective of age of the patients.

Diarrhoea is usually classified as either inflammatory or non-inflammatory disease since such a classification has therapeutic significance. The other categories of diarrhoegenic *E. coli*, (not isolated in this study) like enteroaggregative *E. coli*, have also been shown to be an important causal agents of acute diarrhoea in developing countries (Bouckenooghe *et al.*, 2000). More extensive study is required to establish their role in the Nepalese context. Inflammatory diarrhoeas that may be associated with higher mortality can be studied by stool culture. Several inflammatory markers like faecal leucocytes, occult blood, and fecal lactoferrin could be used as a predictor of invasive pathogen in enteric inflammation (Huicho *et al.*, 1996).

The source of drinking water, knowledge and attitude of the patients and the family members were assessed in this study. As shown in Fig. 3, 80% of the patients reported to use their tap water as a source of drinking water, has low chlorine concentration and which is polluted with high numbers of coliform in Kathmandu (WHO, 1992). Only 23% family's boil their drinking water and the rest of the people drink water without boiling which could be one of the major predisposing factors for diarrhoea. We attempted to access the knowledge regarding the etiology of diarrhoea during the study. Forty-five percent of the patients still believe that diarrhoeal diseases are not communicable. Contaminated public water supplies probably contributed significantly to sustained transmission of disease in the Kathmandu Valley. Health education and improvement of sanitation are necessary for prevention. Increased administration of oral rehydration solutions is vital in order to decrease the morbidity and mortality from acute diarrhoea. The present study indicates that there is a seasonal variation on the etiology of diarrhoea in Kathmandu Valley, which should be confirmed by more extensive longitudinal study.

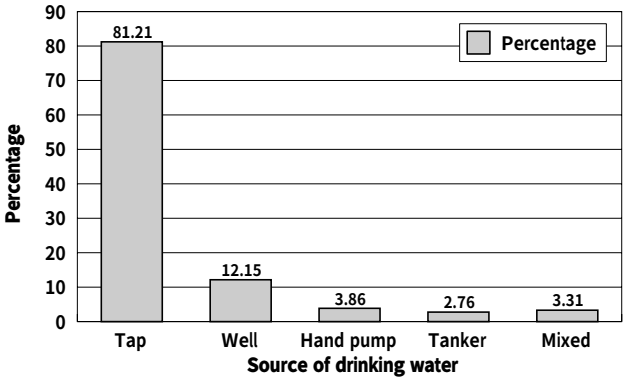

Fig.3: Source of drinking water in the inhabitants of Kathmandu, Nepal

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