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トリパノソーマ治療薬スラミンの抗ウイルス活性：日本脳炎ウイルス複製の阻害

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はじめに

熱帯地域における日本脳炎ウイルス（JEV）による感染例は多く，タイ，ベトナム，スリランカ，中国等のアジア諸国では今尚年間数千人の患者が発生している。日本脳炎は発症すれば致命率は高く，発症者の約1/3は死亡，1/3に後遺症を残し，治癒するのは1/3にすぎないということから，恐れられている感染症である（五十嵐　章，1993；保井　孝太郎，1991；宮本　勉，1992）。従って，有効な予防法及び治療法が必要とされている。JEV 感染の予防には不活化ワクチンがあり，一定の効果を有しているが，治療における有効な抗ウイルス剤は現在のところ知られていない。言い換えれば，日本脳炎の治療は対症療法にたよるしかない。現在，知られている抗ウイルス剤では，JEV 増殖を完全に抑制することは困難である。

スラミンは1920年頃から抗トリパノソーマの薬として用いられるようになった。現在本剤はアフリカトリパノソーマ症の治療薬に用いられている。スラミンの作用機作の詳細は明らかでないが，多陰イオン性の本剤には，その化学構造にかなりの特殊性があり，二つのメチル基はスラミンの生物活性と関わっているようである（Webster, 1992; Iton and Misu, 1986）。他方でスラミンは抗腫瘍活性があり，例えば前立腺癌，転移性副腎皮質癌，リンパ腫などに対して一定の効果があることが示唆された（Myeres *et al.*, 1992; Stein *et al.*, 1988; LaRocca *et al.*, 1990; Spigelman *et al.*, 1987; LaRocca *et al.*, 1992）。ウイルスに対する作用としてはスラミンがレトロウイルス逆転写酵素阻害剤であることが示されたことから，最近 AIDS 患者治療のために様々な臨床応用試験が行われている（Stein, 1993）。その作用機序としては DNA, RNA 合成酵素に対する阻害また蛋白との強い親和性が関与することが示唆されている（Basu and Modak, 1985）。こうした事実から，スラミンの JEV に対する抗ウイルス活性が期待されるが，この点について明確な報告はこれまでなかった。我々は，トリパノソーマの治療薬として知られるスラミンの JEV に対する抗ウイルス活性を検討した。

材料と方法

ウイルスと細胞：ウイルスは JEV（JaGAr-01 株）を用い，細胞は人肝癌由来株 HepG2，人神経芽腫細胞株 IMR-32 を用いた。培養液は HepG2 については D-MEM＋10%牛胎児血清（FCS），IMR-32 は ES（日水製薬）＋10% FCS を用いて培養を行った。ウイルス感染は m.o.i (multiplicity of infection)＝10のウイルス量で，37°C，60分間の吸着を行った後，上記培養液にて培養を行った。

スラミン処理：スラミン（金沢医大，西川克三教授より供与）（Fig. 1）は Bayer 社製のものを使用した。スラミンはウイルス吸着後に種々の濃度で添加した。培養48時間後に培養上清液および感染細胞を採取した。上清液は産生ウイルス量測定のためにプラーク法にかけた。細胞は10,000 rpm で10分間遠心後，沈澱物に TE　buffer（Tris-HCl 50mM, pH8.0, EDTA 1mM）を入れ，細胞懸濁液とした。その後，二つに分けて，一方は蛋白解析のためにウエスタンブロット法に，他は RNA 抽出を行い RT-PCR 法にかけた。

細胞変性（毒性）の判定：薬剤処理及びウイルス感染によって生じる細胞変性を形態的に観察し，その変性効果を定性的に判定した。さらに細胞増殖性に及ぼす影響を併せ検討するために，細胞をプレートにまき，各濃度で薬剤処理し，48時間後に細胞数を計測した。

ウイルス力価測定法：ウイルス力価は BHK 細胞を用い

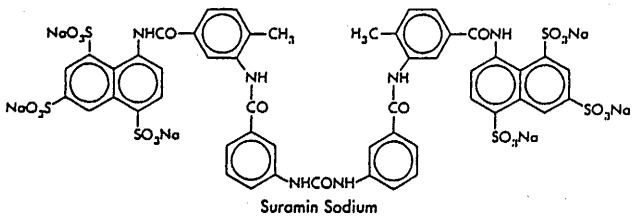

Figure 1　*Structure of suramin, shown as the hexasodium salt.*
The compound was first synthesized in 1916 and was known to be an active antitrypanosomal agent.

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たプラーク法によって測定した（Takegami, 1990）。方法の概略を以下に述べる。BHK細胞に感染させ，1%FCS, 0.6%Methylcellulose を含む MEM 培養液で，3日間の培養を行った。感染細胞を Methanol で固定した後，1%Crystal violet で染色した。

Western blot 法：得られた細胞抽出物（蛋白質量20μg）を10%の SDS-ポリアクリルアミドゲルにかけ電気泳動を行い(Laemmli, 1970)，更に PVDF 膜に転写し，ウエスタンブロット法を行った。一次反応においては特異的抗血清の抗E および抗 NS3 のウサギ血清（1：1000）を用いた（Takegami *et al.*, 1982a; Edward and Takegami, 1993)。二次反応は，パーオキシダーゼ標識抗ウサギ IgG を用いた。0.05%ジアミノベンチジンによる発色によって特異的バンドを検出した。蛋白量については PVDF 膜をデンシトメーターにかけ，その量の相対的変化を測定した。

RT-PCR 法：感染細胞から JEV-RNA をフェノール法によって抽出した。この JEV-RNA をテンプレートとしてアンチセンスプライマー（No. 42），ACCACCCGCGTCGGTGCCAA, 逆転写酵素を加えて，42℃にてcDNA合成を行い，さらにセンスプライマー(No. 41)，GTGTTTTGGGACACGCCATC, 及び Taq ポリメラーゼを添加し，DNA 増幅反応を20あるいは40サイクルで行った。産物は1.5%アガロースゲルを用いた電気泳動によって分析した。

in vitro RNA 合成法：JEV 感染細胞から得られた粗膜画分を用いて，in vitro での RNA 合成を行った。^{32}P-UTP とり込み量によって活性を測定したが，詳細は文献（Takegami and Hotta, 1989）に記載している方法に従った。in vitro RNA 産物は1%非変性アガロースゲルを用いた電気泳動によって分析した。

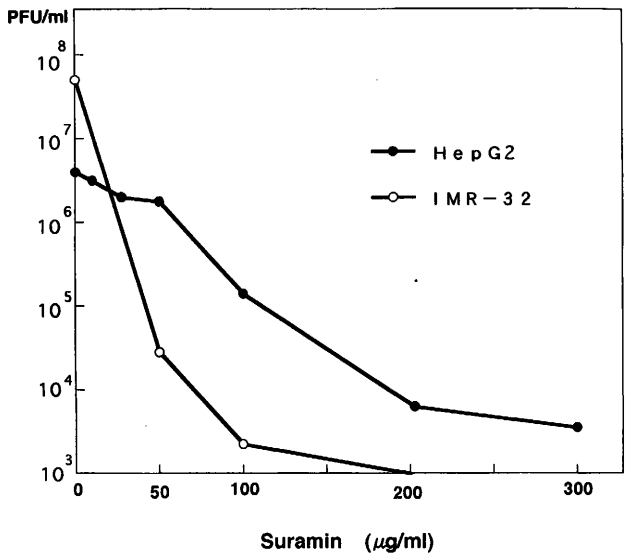

Figure 2 Inhibitory effect of suramin on JEV replication in IMR-32 and HepG2 cell lines.
HepG2 (—●—) and IMR32 (—○—) cells were infected with JEV at 10 m.o.i. and cultured for 48 hrs in the presence of suramin at various concentrations. Virus titers in the culture fluids were assayed by the plaque method, as described in the text.

結　果

スラミンによる JEV 増殖への影響：

スラミンの抗ウイルス活性を調べるために JEV 感染後培養液中にスラミンを種々の濃度で添加し，2日後にウイルス産生量を調べた。Fig. 2 に示されるよう，HepG2 における感染後48時間でのウイルス産生量は対照の5.6×10^6 PFU/ml に対し，50μg/ml，100μg/ml，200μg/ml の各濃度でのスラミン処理によってそれぞれ2.9×10^6PFU/ml，1.2×10^5PFU/ml，5.3×10^3PFU/ml の値となり，およそ0.1%にまでウイルス産生が抑制されていた。一方，この阻害効果は細胞によって異なり，神経芽腫細胞株 IMR-32 における JEV 増殖は50μg/ml のスラミン濃度でコントロールに比べ1/1,000にウイルス産生量が低下していた。なお，細胞に対するスラミンの変性作用は200μg/ml 濃度においては認められなかった。しかしながら300μg/ml で処理した場合は，HepG2 では明確な変性作用はみられなかったが，IMR-32 の方で細胞変性が認められた。細胞増殖への影響を調べた結果，細胞数を対照の半分にする濃度（IC50: Inhibitory Concentration）は IMR-32 で410μg/ml であり，HepG2 では955μg/ml であった。これはウイルス増殖抑制 (Fig. 2 参照) の IC50 が IMR-32 で25μg/ml，HepG2 では53μg/ml であることに比べ，16-18 倍の差がみられた。

JEV—特異蛋白質の生成：

ウイルス蛋白質生成への影響を調べるためにウエスタンブロット法を行った。クーマジーブルー（CBB）による染色では明確には JEV 蛋白質の同定はできなかったが，JEV 蛋白質特異的ウサギ抗血清（抗E及び抗 NS3）を用いたウエスタンブロット法によってウイルス特異的蛋白質として感染細胞に存在するE蛋白質（53KDa）と NS3 蛋白質（70 KDa）のそれぞれを同定した。Fig. 3 に示されるように，JEV 感染 HepG2 細胞における JEV 構造蛋白質 E はスラミン処理によって発現量減少が著しかった。他方，非構造蛋白質 NS3 についても若干の減少が認められたが，E蛋白質の減少に比べ差違は微量であった。200μg/ml のスラミン処理の場合はウイルス産生量が対照のそれの千分の1になっていたが，ウイルス蛋白質のレベルでも濃度依存性が認められ，200μg/ml でE蛋白量は対照の1/5以下に低下していた。他方 JEV 感染 IMR-32 細胞では，HepG2 の場合に比べスラミンの濃度によって顕著なウイルス蛋白の減少が見られた。Fig. 4 ではE蛋白発現量だけでなく NS3 発現量もスラミン濃度に対応して減少していることが示されているが，この場合もE蛋白質の方でより速い発現量低下が見られた。

スラミン処理細胞における JEV-RNA の検出及び in vitro RNA 合成への影響：

次に細胞内ウイルス RNA の変動を調べるためにRT-

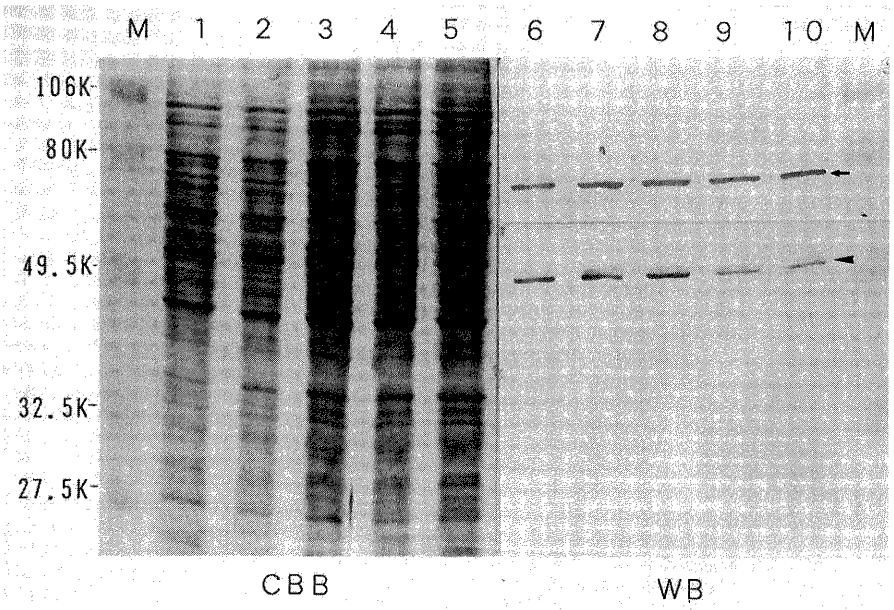

Figure 3 *Western blot analysis: Effect of suramin on expression of JEV-E and NS3 protein.*
HepG2 cells were infected with JEV and cultured in the presence of suramin at 0 (lanes 1 and 6), 50 (lanes 2 and 7), 100 (lanes 3 and 8), 200 (lanes 4 and 9) and 300 μg/ml (lanes 5 and 10), respectively. Protein samples were prepared from JEV-infected HepG2 cells and subjected to SDS-PAGE and transferred to the membrane, Immobilon P. Half of the membrane was stained with CBB (lanes 1-5), and other half (lanes 6-10) was reacted with the specific antisera, i.e. anti-NS3 (upper panel) and anti-E (bottom panel), respectively. Arrows indicate the JEV-specific proteins, NS3 and E. Lane M is size marker for proteins (Bio Rad).

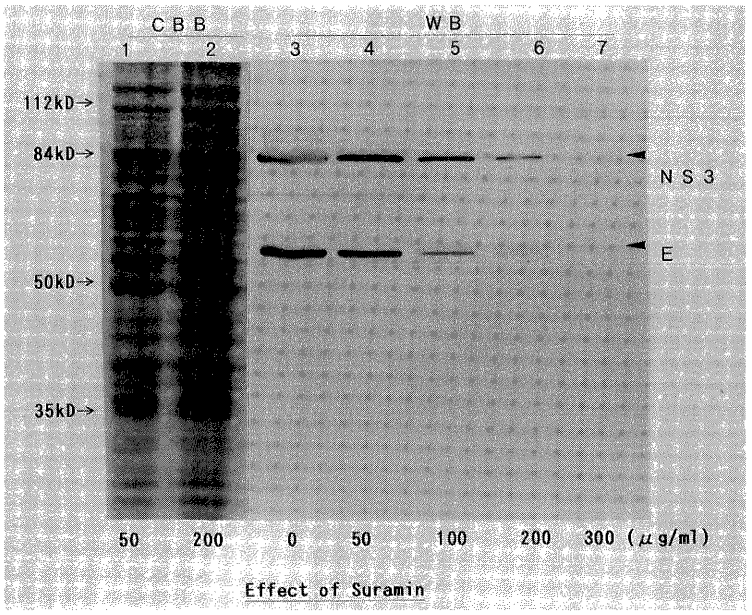

Figure 4 *Western blot analysis of JEV-E and NS3 proteins.*
IMR-32 cells were infected with JEV and cultured in the presence of suramin. Preparation of cell extracts and Western blot analysis were carried out as described in the text and legend to Fig. 3.

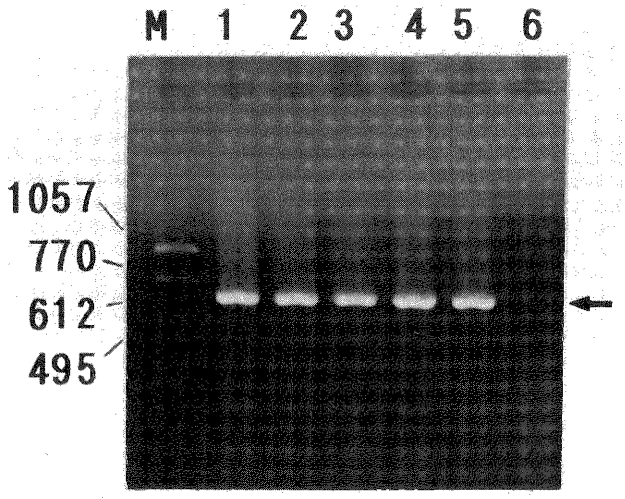

Figure 5 *Detection of JEV cDNA from JEV-infected cells by RT-PCR.*
HepG2 cells were infected with JEV and cultured in the presence of suramin. JEV-RNAs were extracted by the phenol method from the infected HepG2 cells at 48 hrs post infection and subjected to the RT-PCR method as described in the text. PCR products were analyzed by the electrophoresis using 1% agarose gel. Lanes 1-5 mean the treatment with suramin at the concentration 0, 50, 100, 200 and 300 μg/ml, respectively. Lane 6 means the uninfected cells treated with suramin at 300 μg/ml. Lane M is molecular size marker for DNA. Arrow indicates the specific PCR products.

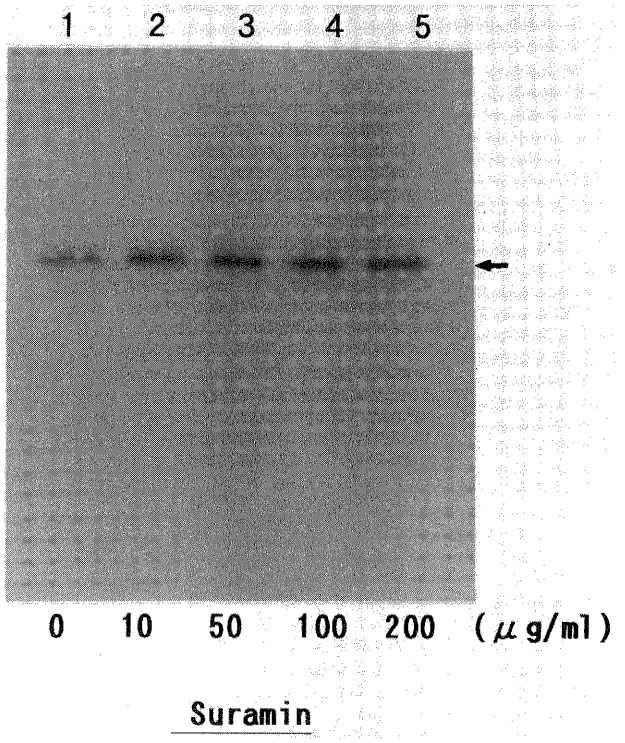

Figure 6 *Effect of suramin on in vitro JEV-RNA synthesis.*
In vitro RNA synthesis using membrane fraction was carried out as described previously (Takegami and Hotta, 1989). The crude membrane fractions were prepared from JEV-infected cells. The reaction was started by the addition of ^{32}P-UTP and incubated with suramin at various concentration. Arrow indicates ^{32}P-JEV-RNA synthesized *in vitro*.

PCR法によって(+)鎖ウイルスRNA量を相対的に検討した結果，HepG2細胞内においてJEVに特異的な増幅DNAバンドは300μg/mlまでのスラミン添加量による差はなかった(Fig. 5)。この結果はPCR増幅回数を20サイクルにした場合も同様で，対照に比べ差違は見られなかった。一方，Fig. 6はin vitroでのJEV-RNA合成系へスラミンを添加した場合であるが，この条件下でのRNA合成阻害は見られなかった。

考　察

スラミンによる抗ウイルス活性について2種類のJEV増殖系を用いて検討を行った。人肝臓細胞系として人肝癌由来株のHepG2，人ニューロン細胞系として人神経芽腫細胞株のIMR-32を用いて，培養細胞系におけるスラミンのJEVに対する抗ウイルス活性を調べたところ，JEV感染HepG2細胞培養系にスラミンを添加した場合は感染後48時間でのウイルス産生量は対照に対し，1/1000に低下し，顕著なウイルス増殖阻害が見られた。興味深いことにこの阻害効果は細胞によって異なり，神経芽腫細胞株IMR-32の方がHepG2よりも効果は顕著であった（Fig. 2)。このスラミンによるウイルス増殖阻害の作用の違いからJEV増殖阻害のプロセスにおける宿主細胞因子の関与が推定される。

ウイルス蛋白質の産生について，ウエスタンブロット法による解析ではJEV-E構造蛋白質とNS3非構造蛋白質共にスラミン処理によって発現量が減少していたが，特にE蛋白質の低下が著しかった（Fig. 3)。この事実は放出ウイルス量の低下（Fig. 2）と密接につながっていると考えられる。またHepG2の場合に比べIMR-32において顕著なウイルス蛋白質産生の低下が見られた。IMR-32におけるJEV増殖がスラミンによって強く阻害された事実と併せると，スラミンはその阻害過程においてIMR-32に存在すると推定されるウイルス複製促進因子の作用を阻害している可能性も考えられる。これらの事実からスラミンは宿主細胞に影響を及ぼし，更にウイルス複製関与宿主因子に作用し，その結果としてウイルス蛋白合成のプロセスに影響を及ぼしていると考えられる。特にウイルス粒子形成に必要なE蛋白質の産生が阻害されている事実（Fig. 3及び4）がウイルス産生阻害の大きな要因であろうと推定される。

ただし，E蛋白発現のみではウイルス産生阻害を説明することはできない，ウイルス粒子形成のプロセス，あるいは放出ウイルスの不活化にスラミンが作用している可能性もある。

スラミンはDNA及びRNA合成酵素を阻害することが示唆されているが，スラミン添加の条件下でPCR産物（JEV-DNA）はスラミン添加量による差がなかった（Fig. 5）。PCR産物量がプラトーに達していない増幅条件（20サイクル）においても差違はなかったこと，さらにin vitroでのJEV-RNA合成系を用いたRNA合成においてもスラミンの効果は見られなかったこと（Fig. 6）等の事実からスラミンによるJEV-RNA合成への影響は少ないと推定される。最近，培養細胞へのスラミンの作用として細胞増殖因子のbFGFの作用に影響を及ぼすことが報告されている（Middaugh *et al.*, 1992）。核酸合成阻害の他の作用をしている可能性は高い。

JEVはフラビウイルスに属する(+)鎖RNAウイルスである。ウイルス増殖が細胞内寄生によって宿主細胞の代謝系に依存しているため，抗ウイルス剤はしばしば宿主細胞の代謝阻害に関連することがあるが，本研究では200μg/mlのスラミン濃度で細胞に対するスラミンの毒性は認められなかった。この濃度は臨床のGuillain-Barre症候群に用いられている350μg/mlの血漿スラミン濃度（Lieberman *et al.*, 1990; Scher *et al.*, 1992; Cooper *et al.*, 1992）に比べ低い。このことからスラミンは細胞毒性の低い状態で組織培養系でのウイルス増殖を抑制していることが明らかとなった。本研究でスラミンはウイルス蛋白生成の過程を阻害することによってJEV複製を抑制している可能性が示唆されたが，その機序については宿主因子の関与を含め，動物実験および臨床的効果等で今後さらに研究を進める必要がある。またスラミンの抗ウイルス活性が他のウイルス増殖系でも見られるか否か，検討しなくてはならない。

結　語

ウイルス感染症の治療薬は，細菌感染症等と異なり開発がおくれている。日本脳炎の治療は対症療法にたよるしかなく，有効な抗ウイルス剤は現在のところ知られていない。我々は，トリパノソーマの治療薬として用いられているスラミンがDNA及びRNA合成酵素に影響を及ぼすことが示されていることから，スラミンのJEVに対する抗ウイルス活性を調査した。その結果(1)スラミンによるウイルス増殖の阻害および(2)ウイルス蛋白質生成に影響を及ぼしていること，特にE蛋白質の産生阻害が顕著であることが分かった。以上の事実から，スラミンはウイルス蛋白質生成の過程を阻害することによってJEV複製を抑制している可能性が示唆された。

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ANTIVIRAL ACTIVITY OF SURAMIN: INHIBITORY EFFECT ON JAPANESE ENCEPHALITIS VIRUS REPLICATION

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Abstract: Suramin, a polysulfonated naphthylurea, has been used for the treatment of trypanosomias and onchocerciasis, and is a potent inhibitor of nucleic acid polymerase including reverse transcriptase and DNA polymerase. This drug is used to examine an antiviral activity in the case of Japanese encephalitis virus (JEV) infection. Japanese encephalitis occurs in endemic and epidemic form over a wide area of Asia, at least tens of thousands of cases occur annually in East Asia. Although the vaccine against JEV is widely used, we have no antiviral drugs against JEV replication. Here we describe an antiviral activity of suramin on JEV replication in the cultured cells. In the presence of 50 μg/ml suramin, virus yields in human neuroblastoma cell line, IMR-32, reduced to 0.1% of control level. JEV growth in human hepatoma cell line, HepG2, was also inhibited, but inhibitory effect of suramin was lower than in IMR-32. The difference of inhibitory effects between host cells suggests that some host factors were involved in the process of inhibition of JEV growth. By Western blot analysis, it was clarified that expressions of JEV proteins, NS3 and E were markedly reduced by the treatment of suramin at 50-200 μg/ml. Especially the expression of E protein seems to be sensitive against suramin treatment. On the other hand, JEV-RNA level in the cells treated with suramin was not so different from control level, and *in vitro* JEV-RNA synthesis was also not inhibited by the addition of suramin. These results suggest that suramin inhibits virus replication through the influence to viral protein production, not to viral RNA synthesis.

Key Words: Suramin, Antiviral activity, Japanese encephalitis virus, Western blot analysis

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EPIDEMIOLOGICAL SURVEY OF CHAGAS DISEASE IN A RURAL AREA OF GUATEMALA; AN ASSOCIATION OF ECG ABNORMALITIES WITH SEROPOSITIVITY TO *TRYPANOSOMA CRUZI*

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Abstract: An epidemiological survey was done by examining 1084 inhabitants in a rural area of Guatemala to determine the prevalence of seropositivity to *Trypanosoma cruzi* as well as the association with chagasic cardiomyopathy. Results of the survey revealed that Chagas disease is endemic in this area because 77 (7.1%) of the inhabitants examined by an indirect hemagglutination test were seropositive to *T. cruzi*. The age-prevalence distribution showed that seropositivity increased with age. Analysis of electrocardiogram (ECG) revealed a significantly high frequency of ventricular conduction defects and arrhythmias among the seropositives. Ventricular conduction defects were observed in 18.2% of the seropositives and 1.7% of the seronegatives, and arrhythmias were 15.6% and 2.7%, respectively. In seropositive individuals, the most common alteration of ventricular conduction defects was right bundle branch block with or without fascicular block and that of arrhythmias was ventricular premature contraction. Among the seropositives, the prevalence of the above ECG abnormalities was low in the 40-59 age group, which may be accounted for the death due to chagastic cardiomyopathy. These results suggest that this study area is an endemic area to Chagas disease, and the infection is associated with ECG alterations.

INTRODUCTION

Chagas disease is a public-health problem in Central and South America and is a chronic parasitic disease caused by *Trypanosoma cruzi* (*T. cruzi*), involving cardiac, digestive and neurological lesions. The cardiomyopathy of chronic Chagas disease causes disability and mortality in the endemic area. Since the first report by Penalver (1953) in Guatemala, Chagas disease has been found in Chiquimula, Jalapa, El Progreso, Santa Rosa, and Zacapa in Guatemala (Aguilar *et al.*, 1993). The World Health Organization (WHO) reports a triatomine house infestation rate of 31% for *Triatoma dimidiata* and seropositivity of 13% in the blood bank in Guatemala (WHO, 1991). However, no report concerning Chagas disease based on a systematic study has been available in Guatemala.

We have been conducting an epidemiological survey of Chagas disease among inhabitants of a rural community in Guatemala since 1992. We report here the prevalence of *T. cruzi* and a relation between the seropositivity and ECG abnormalities among 1084 inhabitants in Santa Maria Ixhuatan, Department of Santa Rosa Guatemala. Results indicated that this area is an

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endemic area of Chagas disease and infection of *T. cruzi* is associated with ECG abnormalities in the inhabitants.

SUBJECTS AND METHODS

Subjects

This study area is Santa Maria Ixhuatan, Department of Santa Rosa, Guatemala which is located at an altitude of 1920 meters, 95 km south-east of Guatemala City, and most people living there are working in agriculture. In this survey, a total of 1084 people (7-89 yr, 401 males and 683 females) were examined.

Analysis of ECGs

ECGs were performed with a three-channel Fukuda Model Fx-3101 electrocardiograph (Fukuda Co, Tokyo, Japan) at paper speed 25 mm/sec. A 12-lead tracing and a 25-second V_1 rhythm strip were recorded. A modification of Minnesota Code (Maguire *et al.*, 1982) was used to classify the ECGs. The ECGs were analyzed independently by two physicians, followed by a cardiologist. Finally, abnormal ECGs were reviewed by another physician experienced in the use of the code at the end of this study and classified according to the criteria previously reported by Maguire, *et al* (1983).

Serological test for *T. cruzi*

Serum samples for serological examination were stored at −70°C. An indirect hemagglutination (IHA) test for antibodies to *T. cruzi* was performed with an IHA-kit (Sero-Immuno Diagnostics, Inc. Georgia, USA Lot# 1050). IHA titers equal to or greater than 1:64 were defined as positive.

Statistical analysis

Subjects were stratified by age and sex into 12 groups. The age groups are 0-19, 20-29, 30-39, 40-49, 50-59 and 60 or more. The prevalence of ECG abnormalities was compared between seropositivies and seronegatives by a Mantel-Haenszel (M-H) chi-square

Table 1 Age and sex-distribution of seropositives to *T. cruzi*

	Male			Female			Total		
	positive	%	examined	positive	%	examined	positive	%	examined
7-19	1	0.5	212	7	2.8	246	8	1.7	458
20-29	2	4.8	42	7	4.7	149	9	4.7	191
30-39	3	7.1	42	10	8.8	114	13	8.3	156
40-49	3	8.6	35	12	17.4	69	15	14.4	104
50-59	3	9.7	31	10	21.3	47	13	16.7	78
60-69	4	15.4	26	5	12.2	41	9	13.4	67
70-	4	30.8	13	6	35.3	17	10	33.3	30
Total	20	5.0	401	57	8.3	683	77	7.1	1084

Table 2 Abnormal ECG alterations and seropositivity

ECG alterations*	Seropositivity				Total
	+	%	−	%	
VCD	9	11.7	15	1.5	24
VCD+Arrythmia	3	3.9	1	0.1	4
VCD+Arrythmia+Abnormal P	1	1.3	0	0.0	1
VCD+Abnormal Q	1	1.3	1	0.1	2
Arrythmia	7	9.1	24	2.4	31
Arrythmia+Abnormal ST-T	0	0.0	1	0.1	1
Arrythmia+VH	0	0.0	1	0.1	1
Abnormal Q	0	0.0	12	1.2	12
VH	2	2.6	15	1.5	17
Abnormal P	2	2.6	6	0.6	8
Abnormal ST-T	0	0.0	13	1.3	13
A-V block	0	0.0	1	0.1	1
Total	25	32.5	90	8.9	115
Examined	77	100.0	1007	100.0	1084

*VCD: ventricular conduction defect;
VH: Ventricular hypertrophy.

Table 3 Characteristics of ventricular conduction defects and seropositivity

Ventricular conduction defects*	Seropositivity +	%	−	%	Total
RBBB	9	11.7	9	0.9	18
complete	1	1.3	5	0.5	
incomplete	4[a,b]	5.2	3[a]	0.3	
complete+LAFB	4	5.2	0	0.0	
complete+LPFB	0	0.0	1	0.1	
LBBB	3	3.9	6	0.6	9
complete	1[b]	1.3	2	0.2	
incomplete	2[b]	2.6	4	0.4	
Intravent. cond. def.	2	2.6	1	0.1	3
LAFB	0	0.0	1	0.1	1
Total	14[c]	18.2	17[a]	1.7	31
Examined	77	100.0	1007	100.0	1084

*RBBB: right bundle branch block; LAFB: left anterior fascicular block; LPFB: left posterior fascicular block; LBBB: left bundle branch block; Intravent. cond. def.: intraventricular conduction defects.
[a]; One case combined with atrial premature contraction (APC).
[b]; One case combined with ventricular premature contraction (VPC).
[c]; Four cases combined with APC or VPC.

test, controlling for age group and sex. Sexual difference was determined by a M-H chi-square test controlling for age group. The trend of the prevalence of seropositives and of ECG abnormalities with age was also tested by a M-H chi-square test.

RESULTS

The prevalence rate of IHA positive to *T. cruzi* is shown by sex and age in Table 1. Among the 1084 subjects examined in this study, 77 subjects (7.1%) consisting of 20 males (5.0%) and 57 females (8.3%), were seropositive to *T. cruzi*, and 1007 (92.9%) were seronegative. The age distribution of seropositivity to *T. cruzi* indicated that the prevalence increases significantly ($P<0.01$) with age. The prevalence of seropositivity tended to be higher in the females than in the males in the age group of 30 to 59. However, the difference was not significant.

Abnormal ECG alterations observed among inhabitants are summarized in Table 2. The overall prevalence of abnormal tracings was 3.50 times (odds ratio, 95% confidence limits=2.01−6.11, $P<0.001$) greater in the seropositive subjects than in the seronegative subjects, originating from a high prevalence of ventricular conduction defects and arrythmias in the seropositives. No sex-difference of the ECG abnormalities was observed in the seropositive individuals. No difference was observed in other ECG abnormalities, except the above two abnormalities, between the seropositives and the seronagatives.

Ventricular conduction defects were the most common alterations in the seropositive individuals (Table 3). The prevalence was 18.2% (14 out of 77) in the seropositive group and was 1.7% (17 out of 1007) in the seronegative group. The Mantel-Haenszel (M-H) overall odds ratio of ventricular conduction defects for the seropositives in relation to the seronegatives was estimated to be 8.73 (95%CI=4.17−18.26, $P<0.001$).

Table 4 Characteristics of arrythmias and seropositivity

Arrythmias*	Seropositivity +	%	−	%	Total
APC	3[a]	3.9	15[a]	1.5	18
VPC	8[b]	10.4	7	0.7	15
APC+VPC	1	1.3	1	0.1	2
AF	0	0.0	1	0.1	1
APC+AF	0	0.0	1	0.1	1
SVT	0	0.0	1	0.1	1
SA	0	0.0	1	0.1	1
Total	12[c]	15.6	27[a]	2.7	39
Examined	77	100.0	1007	100.0	1084

*APC: atrial premature contraction; VPC: ventricular premature contraction; AF: atrial fibrillation; SVT: supraventricular tachycardia; SA: sinus arrest.
[a]; One case combined with incomplete RBBB.
[b]; Three cases combined with incomplete RBBB, comlete IBBB and incomplete LBBB, respecitively.
[c]; Four cases combined with RBBB or LBBB.

Table 5 Age-distribution of ventricular conduction defects and/or arrythmias

Age	Seropositive				Seronegative			Total
	abnormal	%*	examined	%**	abnormal	%*	examined	
7-19	1	12.5	8	1.7	11[b]	2.4	450	458
20-29	2	22.2	9	4.7	7	3.8	182	191
30-39	4	30.8	13	8.3	3	2.1	143	156
40-49	2	13.3	15	14.4	3	3.4	89	104
50-59	2	15.4	13	16.7	6	9.2	65	78
60-	11[a]	57.9	19	19.6	13	16.7	78	97
Total	22[a]	28.6	77	7.1	43[b]	4.3	1007	1084

[a]; In four cases, ventricular conduction defects and arrythmias are combined.
[b]; In one case, ventricular conduction defect and arrythmia are combined.
*; prevalence of ECG abnormalities. **; prevalence of seropositives.

Among the ventricular conduction defects, right bundle branch block (RBBB) with or without fascicular block was strongly associated with seropositivity.

Another ECG abnormality observed in the seropositive individuals was arrhythmias, with 15.6% (12 cases) in the seropositives and 2.7% (27 cases) in the seronegatives (Table 4). The most common arrhythmia in the infected individuals was ventricular premature contraction. The odds of arrhythmias in the seropositives are 5.31 times greater than the seronegatives (95%CI=2.40−11.74, $P<0.001$). Four infected inhabitants had combined alterations of ventricular conduction defect and arrhythmia. Therefore, ECG alterations of ventricular conduction defects and/or arrhythmias which are characteristic of chagasic cardiomyopathy were present in 22 (28.6%) out of the 77 infected individuals, in contrast to 4.3% of the noninfected individuals.

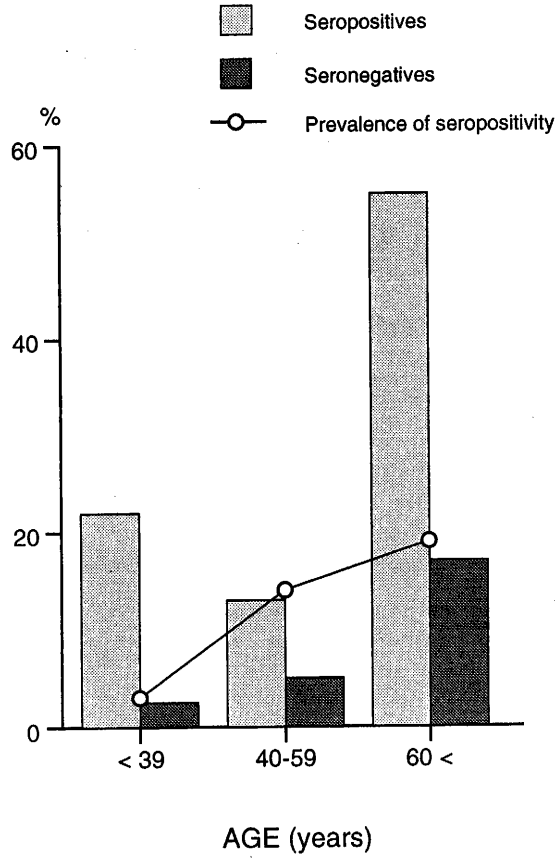

Figure 1 The prevalence of ventricular conduction defects and/or arrythmias in seropositives did not increase with age, whereas the seropositivity to *T. cruzi* increased with age.

The age-distribution showed that the prevalence of ventricular conduction defects and/or arrythmias was greater in the seropositives than in the seronegatives in respective age groups (Table 5). In the seronegative group, the prevalence of the ECG alterations increased with age ($P<0.001$). However, the prevalence did not increase with age in the seropositive group ($P>0.05$) despite as increase in the seropositivity with age. A high prevalence of the ECG abnormalities was observed in the age group of 60 and over. However, the prevalence was lower in the seropositives of the age group of 40-59 than in those of the group of 20-39 (Fig. 1).

DISCUSSION

Chagas disease is endemic in Latin America, from Southern Mexico to Central Argentina, and 18 million people are infected (WHO, 1991). The overall prevalence reported from countries in Latin America is around 2-8% (Schofield, 1985), although there are areas where more than 50% of the inhabitants are infected (Maguire *et al.*, 1983; Schofield, 1985; Pless *et al.*, 1992). An important vector, *Triatoma dimidiata,* is widely distributed in rural areas in Guatemala and it is easily observed in houses in this study area. Housing construc-

tion and housing conditions—mud-sick houses with cracks in the wall and dirt floor—observed in this study area are preferred by the vector as reported in other countries of Latin America (Zeledon *et al.*, 1975; WHO, 1991).

This is the first epidemiological survey of Chagas disease in Guatemala to determine the relation to ECG abnormalities. Several sensitive serological tests to determine anti-*T. cruzi* antibodies were used for indirect diagnosis of Chagas disease. A problem with these serodiagnostic assay is the occurrence of false positive results. This may occur with specimens from subjects having leishmaniasis, malaria and some other diseases (Nogueira & Coura, 1990). We used an indirect hemaggutination test for anti-*T. cruzi* antibodies in this epidemiological survey because the specific test for measuring a large number of samples is not available. This study indicates that Santa Maria Ixuatan, a rural area of Guatemala, is an endemic area to Chagas disease, because 7.1% of inhabitants was seropositive to *T. cruzi*. The prevalence rate is 11% in Costa Rica (Zeldon *et al.*, 1975) and less than 6% in Mexico (Schettino *et al.*, 1988), indicating that not much difference was observed among inhabitants in Central America. A report from Northeast Brazil (Maguire *et al.*, 1983) indicated that inhabitants are infected early in life and selective mortality due to chagastic cardiomyopathy accounts for the decline in the seropositivity rate among elderly inhabitants over 55 years. However, the prevalence rate of *T. cruzi* infection increased significantly with age in this area, and the same phenomenon is observed in Ecuador (Andrade *et al.*, 1978).

Chagas disease causes an interstitial myocarditis with dissociation and degeneration of myofibrils, and a good correlation between histopathological findings and ECG abnormalities has been demonstrated (Rosenbaum & Alvarez, 1955). Our survey indicated a strong association of ECG abnormalities with seropositivity to *T. cruzi*. In the seropositive individuals the most common ECG abnormalities were ventricular conduction defects, especially RBBB with or without anterior fascicular block, resulting from alterations in A-V conduction system which are unique to chagastic cardiomyopathy (Maguire *et al.*, 1982; Andrade *et al.*, 1978; Lima *et al.*, 1985; Kawabata *et al.*, 1987). Another ECG abnormality observed in the seropositives was arrhythmias. The most frequent type of arrhythmias was ventricular premature contraction. Both ventricular conduction defects and arrhythmias observed in this study are characteristic of inhabitants in the endemic area of Chagas disease (Maguire *et al.*, 1982; Andrade *et al.*, 1978; Lima *et al.*, 1985; Kawabata *et al.*, 1987; Pless *et al.*, 1992). In this study area, of 77 seropositive individuals examined, 22 (28.6%) had ventricular conduction defects and/or arrhythmias, suggesting a high occurrence of cardiomyopathy among the seropositive inhabitants. A similar frequency of the ECG alterations is observed among the infected individuals of other endemic countries (Maguire *et al.*, 1982; Zeledon *et al.*, 1975; Shettiono *et al.*, 1988; Andrade *et al.*, 1978; Lima *et al.*, 1985; Kawabata *et al.*, 1987; Pless *et al.*, 1992).

Age distribution of individuals with the ECG alterations is different among the countries examined (Maguire *et al.*, 1982; Andrade *et al.*, 1978). In highly endemic areas, selective mortality due to chagastic heart disease also causes the decline in older individuals with abnormal ECG (Maguire *et al.*, 1982). On the other hand, an other report indicated that there is a progressive increase in the number of patients with ECG abnormalities with age (Nogueira & Coura, 1990). In this study area, age distribution showed that the prevalence of the above ECG abnormalities did not relate with that of the seropositivity to *T. cruzi* in the seropositive group, and the prevalence was low in the seropositives of age group of 40 to 59. The decline in the ECG abnormalities in the middle aged seropositives may be accounted for the selective death due to chagastic cardiomyopathy.

In conclusion, this epidemiological survey showed that Santa Maria Ixhuatan, Department of Santa Rosa, Guatemala, is an endemic area to Chagas disease, and the infection is associated with ECG alterations which may lead to death in middle aged inhabitants. An appropriate program to prevent *T. cruzi* infection is needed in Guatemala.

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THE GEOGRAPHICAL DISTRIBUTION OF THE GENUS *SIMULIUM* LATREILLE IN THE ORIENTAL AND AUSTRALASIAN REGIONS

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Abstract: The geographical distributions of the genus *Simulium* Latreille s.l. in the Oriental and Australasian Regions were mapped. The patterns of distribution were examined at the subgenus and species-group levels. Among the 11 subgenera recorded in these regions, five (*Byssodon, Eusimulium, Montisimulium, Nevermannia* and *Simulium* s.str.) were essentially Palaearctic, apparently penetrating to varying extents from the north to the Oriental Region and two of which were further extending their eastward ranges up to the Australasian Region; while the other six subgenera (*Gomphostilbia, Hebridosimulium, Himalayum, Inseliellum, Morops* and *Wallacellum*) were nearly endemic to the Oriental or Australasian Regions or both. All the endemic subgenera, except *Himalayum*, had their own center of development and distribution on the islands. Eight of 12 species-groups of *Simulium* s.str. were mostly confined to the Oriental Region, and five of which demonstrated a definite insular pattern in their distribution. The probable dispersal routes were inferred for several species-groups of the subgenera *Nevermannia* and *Simulium* s.str. The *Simulium* faunae of the Philippines, Sulawesi and the Maluku Islands present a mixture of the Oriental and Australasian elements but the faunal break is likely to be seen on both sides of the Weber's Line.

INTRODUCTION

Black flies (Diptera: Simuliidae) are one of the most important groups among the blood-sucking insects. This family includes serious pests of man and animals in many countries. Some species transmit several pathogens, such as filarioid nematodes of the genus *Onchocerca* to man and cattle. The family is widely distributed in all zoogeographical regions, being found almost anywhere if there is running water suitable as a habitat of the immature stages.

The Simuliidae in the Oriental Region were so far classified by Crosskey (1967, 1988) and Datta (1983), and their faunal feature was briefly described by Crosskey (1990). Recently we revised the species-groups within the two subgenera, *Gomphostilbia* Enderlein and *Simulium* Latreille s.str., of the genus *Simulium* Latreille s.l. (Takaoka and Davies, 1996) and provided a checklist of the Simuliidae in the Oriental Region (Takaoka and Davies, 1995), in which all the 246 species, but one tentatively assigned to the genus *Sulcicnephia* Rubtsov, were placed in the 11 subgenera of the genus *Simulium* s.l. and under three major subgenera were further classified into several species-groups.

The Oriental Region consists of two types of lands, continental (India, Indochina and south China) and insular (Philippines and Indonesia), and climatologically belongs for the most part to tropical and subtropical zones. This region is generally delimited on the north by the Himalayan mountain range and the Yangtse River in south China, on the west by the dry zone of Kashmir, and on the east by the so-called *Wallace's Line* or *Weber's Line*, both of which have been long reputed to separate it from the Australasian Region (Fig. 1). Gressitt (1956, 1961) analysing geographical distribution patterns of several insect families revealed that the Oriental elements stretched eastwardly far beyond these nominate lines, and included vast ranges of oceanic islands including New Guinea in the Oriental Region. He also discussed the faunal origins and affinities of most oceanic islands (Gressitt, 1961).

The distributional features of the genus *Austrosimulium* Smart in Australia and New Zealand, and the subgenus *Inseliellum* Rubtsov in Polynesia were already shown by Dumbleton (1963) and Craig (1983) respectively. However, no such attempts have been

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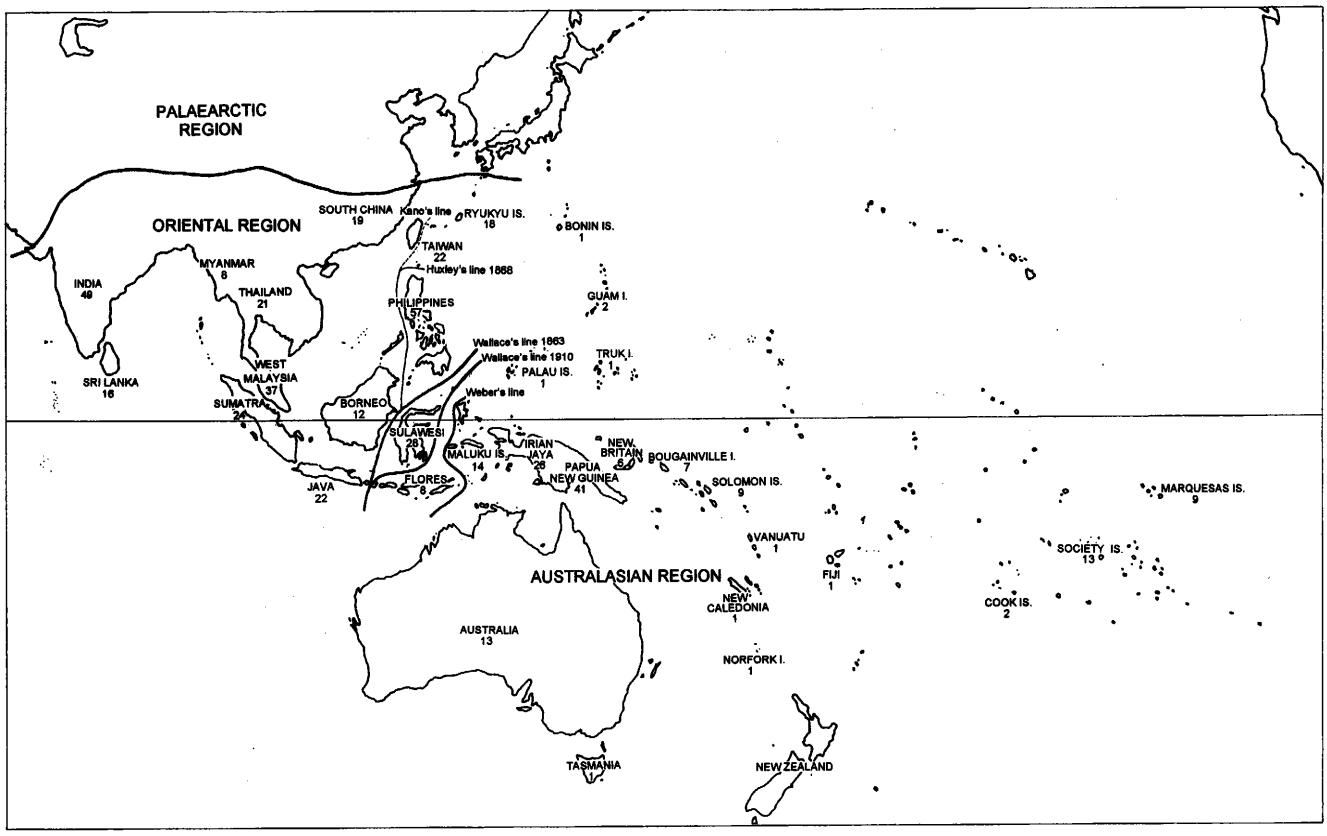

Figure 1 Map of the Oriental and Australasian Regions showing several zoogeographic borderlines. Numeral below locality name indicates the number of species of the genus *Simulium* s.l. so far found. Horizontal line indicates ecuator.

done in the Oriental Region, mainly due to the lack of the faunistic investigations, especially in the eastern part of Indonesia including Sulawesi Island (Celebes), the Maluku Islands (Moluccas), Irian Jaya and the Lesser Sunda Islands. In this paper the geographical distributions of the genus *Simulium* s.l. in the Oriental and Australasian Regions were outlined and drawn at subgenus and species-group levels, on the basis of the published and unpublished data including one recently obtained from our faunistic surveys in Indonesia and Peninsular Malaysia. Main information sources were Takaoka and Davies (1995) for the Oriental Region, Crosskey (1967, 1989), Takaoka and Suzuki (1995), Takaoka (1995) for the Australasian Region, Crosskey (1988) for both regions, and Craig and Craig (1986), Craig (1987) and Craig *et al.* (1995) for Polynesia.

I. DISTRIBUTIONS OF TRIBES AND GENERA (Fig. 2)

The family Simuliidae was divided into two subfamilies, i.e., Parasimuliinae and Simuliinae, of which the latter was further divided into two tribes, i.e., Prosimuliini and Simuliini (Crosskey, 1988). The majority of black fly species in this area were placed in the worldwide genus *Simulium* s.l. of the tribe Simuliini. This genus occurs in most areas of the Oriental and Australasian Regions and extends on the south up to Tasmania Island, on the northeast to the Micronesian Islands, and on the east as far as the Marquesas Islands. Another genus *Austrosimulium* is confined to south and east Australia and New Zealand (Dumbleton, 1963). In addition, two genera of the tribe Prosimuliini were reported: an unrevised "*Cnephia*" from Australia (9 spp.) and *Sulcicnephia* from Peninsular Malaysia. It should be remembered that the record of the latter genus was based on the tentative assignment of the unique species, i.e., *Su. unidens*, described from the pupa and larva, both of which have, though, many characters atypical for the genus *Sulcicnephia* mostly recorded from Central Asia (Takaoka and Davies, 1995). The male adult of *Su. unidens* recently obtained also differs in many morphological characters from most of the other *Sulcicnephia* species and presents more characters of tribe Simuliini than those of tribe Prosimuliini

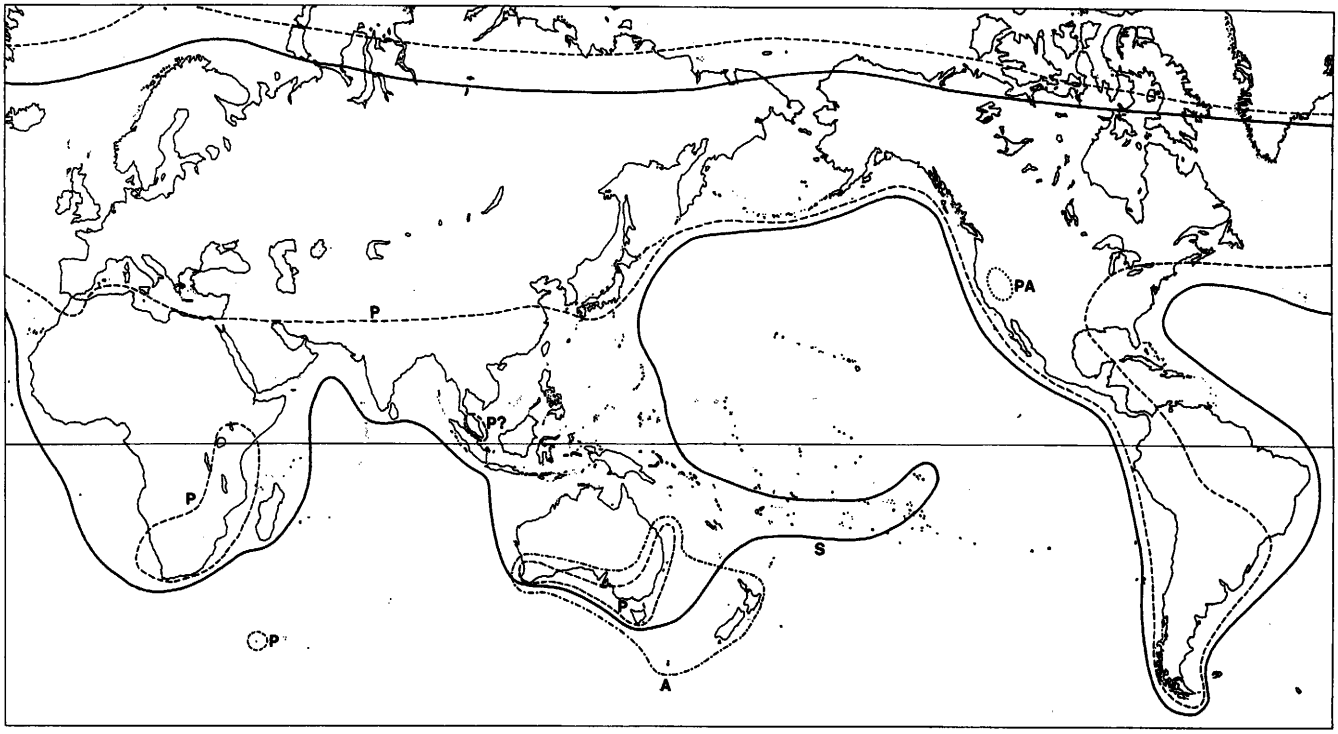

Figure 2 The world distributions of the supraspecific taxa of Simuliidae present in the Oriental and/or Australasian Regions. P, tribe Prosimuliini; S, genus *Simulium* s.l.; A, genus *Austrosimulium*; PA, subfamily Parasimuliinae (shown only for reference).

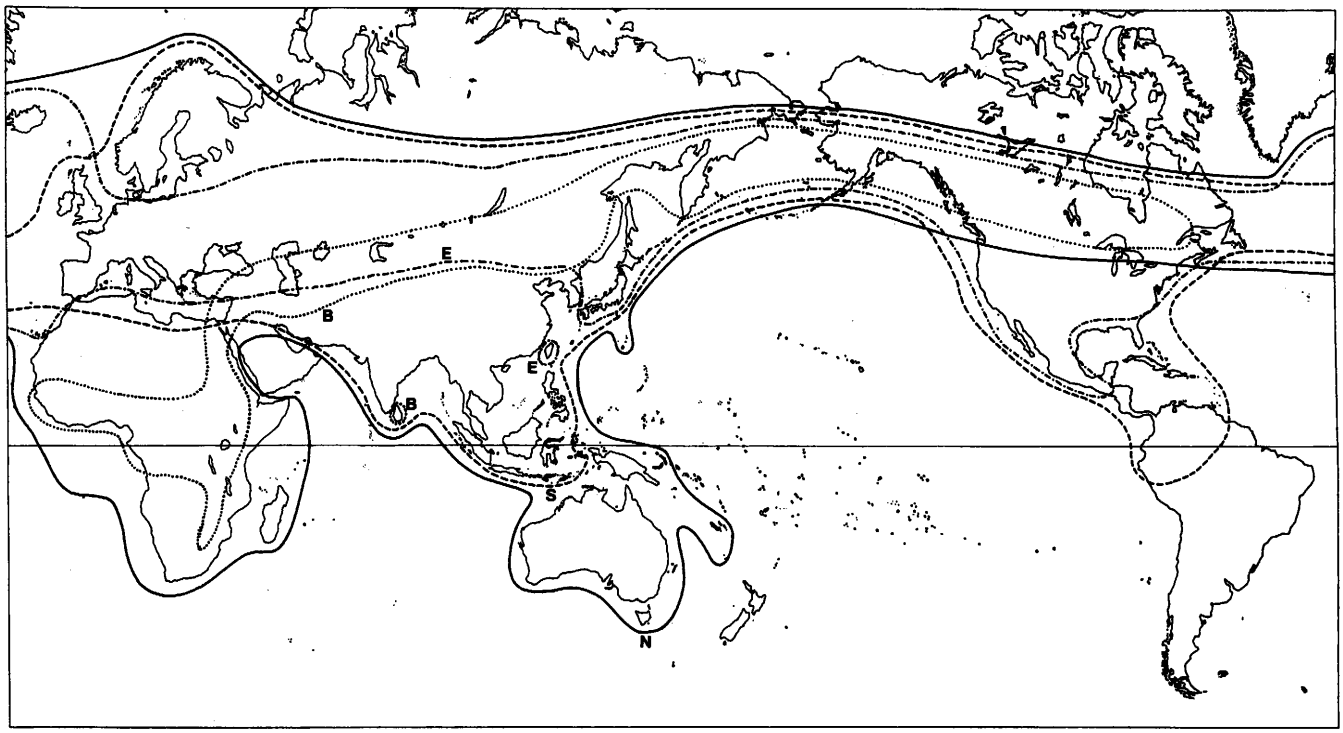

Figure 3 The distributions of four subgenera of *Simulium* s.l. in the Oriental and Australasian Regions. B, *Byssodon*; E, *Eusimulium*; N, *Nevermannia*; S, *Simulium* s.str.

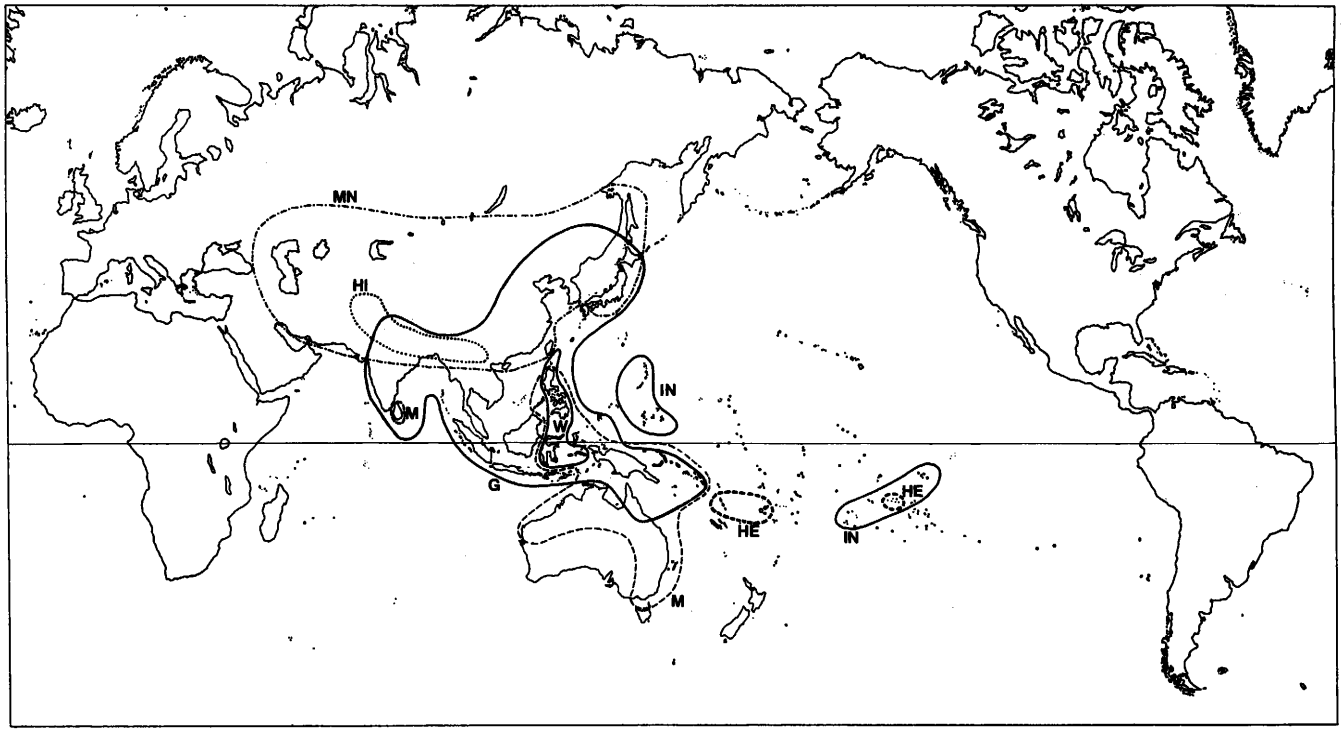

Figure 4 The distributions of seven subgenera of *Simulium* s.l. in the Oriental and Australasian Regions. HE, *Hebridosimulium*; HI, *Himalayum*; IN, *Inseliellum*; G, *Gomphostilbia*; M, *Morops*; MN, *Montisimulium*.

(unpublished data). Detailed taxonomic studies in future are certainly needed to prove that *Su. unidens* is assignable to the genus *Sulcicnephia* and also to the tribe Prosimuliini.

II. DISTRIBUTION OF GENUS *SIMULIUM* S.L.

IIa. Distributions at subgenus level (Figs. 3 & 4)

The *Simulium* fauna of these regions is represented by five main subgenera, *Gomphostilbia, Inseliellum*, *Morops* Enderlein, *Nevermannia* Enderlein and *Simulium* s.str. All but *Inseliellum* probably are not monophyletic, including certain heterogenous species-groups. There are six other minor subgenera of *Simulium* s.l. Four subgenera, i.e., *Nevermannia, Simulium* s.str., *Eusimulium* Roubaud and *Byssodon* Enderlein, are all cosmopolitan, of which first two occur widely in the Asiatic continent and extend eastwards to cross both the Wallace's and Weber's Lines as far as Norfork Island and the Maluku Islands, respectively, while the other two, *Eusimulium* and *Byssodon*, are poorly represented by two species, each in Taiwan and Shimokoshiki Island of the Ryukyu Islands, and by one species in Sri Lanka, respectively (Fig. 3). The remaining seven subgenera except *Montisimulium* Rubtsov (3 spp.) are almost endemic to this area, and all but *Himalayum* Lewis (2 spp.) occur for the most part on the islands (Fig. 4). *Gomphostilbia*, one of the dominant subgenera (64 spp.), occurs in vast areas of the Oriental Region and extends northward to Japan and eastward to the Solomon Islands; it is also represented in the Palau Islands (1 sp.), in Papua New Guinea (9 spp.) and in northern Australia (1 sp.). *Morops* centers on Papua New Guinea (33 spp.) and the Solomon Islands (14 spp.), and extends southwards into Australia (8 spp.) and westwards through the Maluku Islands (7 spp.) into north Sulawesi (1 sp.) and the Philippines (8 spp.); Sri Lanka has one representative. One Malaysian species was tentatively placed in the subgenus *Morops* (i.e., *S. (M.) gombakense*) by Takaoka and Davies (1995), but this was recently proved to belong to the subgenus *Gomphostilbia* on the basis of the adult morphology (unpublished data). *Inseliellum* occurs on the Polynesian Islands (23 spp.) with its western outposts in Guam Island (2 spp.) and Truk Island (1 sp.) of Micronesia. *Hebridosimulium* Grenier and Rageau, a small subgenus (3 spp.) endemic to South Pacific, has also a disjunct distribution (1 sp. each in Fiji, Vanuatu and Society Islands). *Wallacellum* Takaoka, close to *Morops*, has its main range in the Philippines (8 spp.) but extends northward up to Yonakuni Island (1 sp.) of the Ryukyus through Lan-yu Island (1 sp.), east

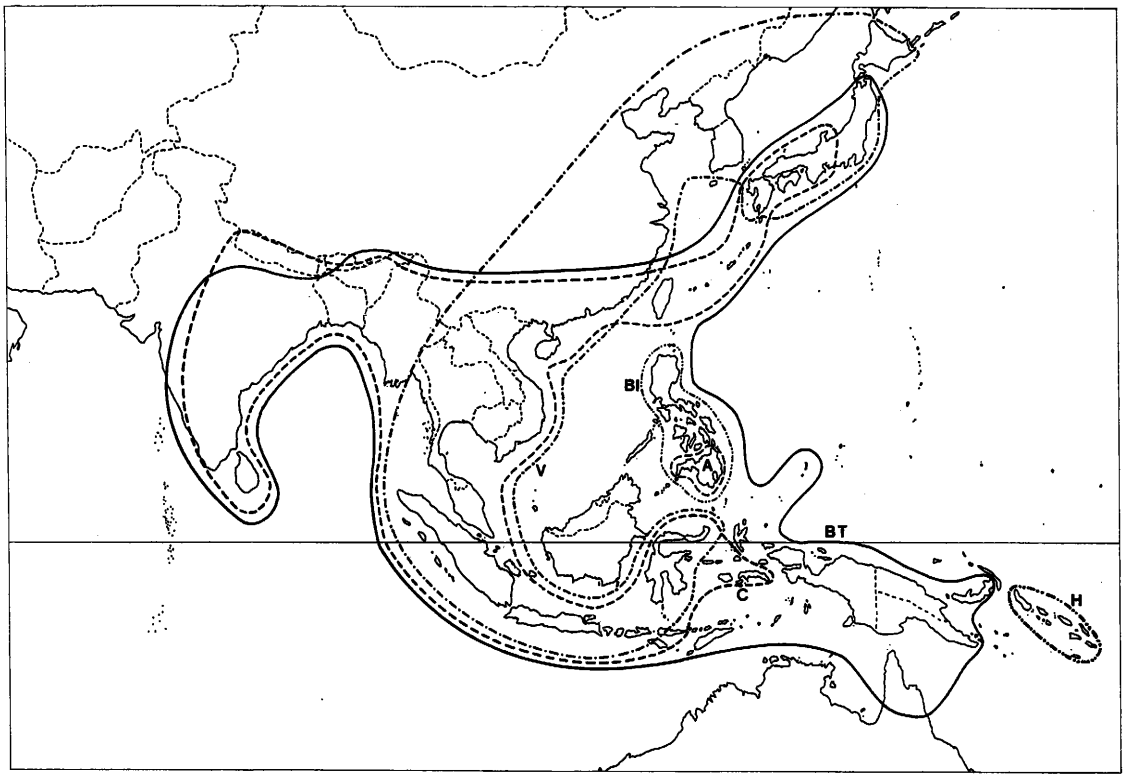

Figure 5 The distributions of six species-groups of the subgenus *Gomphostilbia*. A, *ambigens*-group; BI, *baisasae*-group; BT, *batoense*-group; C, *ceylonicum*-group; H, *hiroshii*-group; V, *varicorne*-group.

of Taiwan, and southward to Sulawesi Island (1 sp.); it is also represented in Seram Island (Ceram) (1 sp.) and Biak Island (1 sp.) (unpublished data).

IIb. Distributions at species-group level

IIb-1. Subgenus *Gomphostilbia* (Fig. 5)

There are five species-groups included so far in *Gomphostilbia* (Takaoka and Davies, 1996). Among these, three species-groups, i.e., *batoense*-, *ceylonicum*- and *varicorne*-groups, have a wide range in the Oriental Region, and are extending their northeast range up to Japan; while in Irian Jaya, Papua New Guinea and north Australia only *batoense*-group is represented by four, nine and one species, respectively. Seram Island of the Maluku Islands and Flores Island of the Lesser Sunda Islands each have one species of the *ceylonicum*-group, as well as one and two species of the *batoense*-group (unpublished data). The two other minor species-groups, i.e., *ambigens*-group (1 sp.) and *baisasae*-group (3 spp.), are confined to the Philippines.

In the map (Fig. 5), one more species-group, *hiroshii*-group, was added. This is provisionally proposed to accommodate *S.* (*G.*) *hiroshii* recently found in the Solomon Islands and Bougainville Island (Takaoka, 1994, 1995). The last three species-groups constitute a different element, presenting several characters which are atypical among *Gomphostilbia* (Takaoka, 1983, 1994). In particular *S.* (*G.*) *hiroshii* is an enigmatic species, of which the male genitalia are typical for *Morops*, and the female genitalia (including the spermatheca with internal setae) are very similar to those of the *ambigens*- and *baisasae*-groups.

IIb-2. Subgenus *Morops* (Fig. 6)

Of the eight species-groups of *Morops* defined by Crosskey (1967) and Colbo (1976), two species-group, i.e., *farciminis*-group (8 spp.) and *oculatum*-group (7 spp.), are confined to Irian Jaya and Papua New Guinea; another species-group, *papuense*-group (1 sp.), is also distributed in Irian Jaya and Papua New Guinea but extends eastwards into the Solomon Islands and westwards as far as Seram Island; a similar pattern is shown for the *clathrinum*-group (ca. 20 spp.) which has, though, a weak representation in north Australia (1 sp.) and extends a little further westward as far as Halmahera Island. On the other hand three small groups, i.e., *faheyi*-group (3 spp.), *lawnhillense*-group (1 sp.) and *mackerrasorum*-group (1 sp.), have their limited distri-

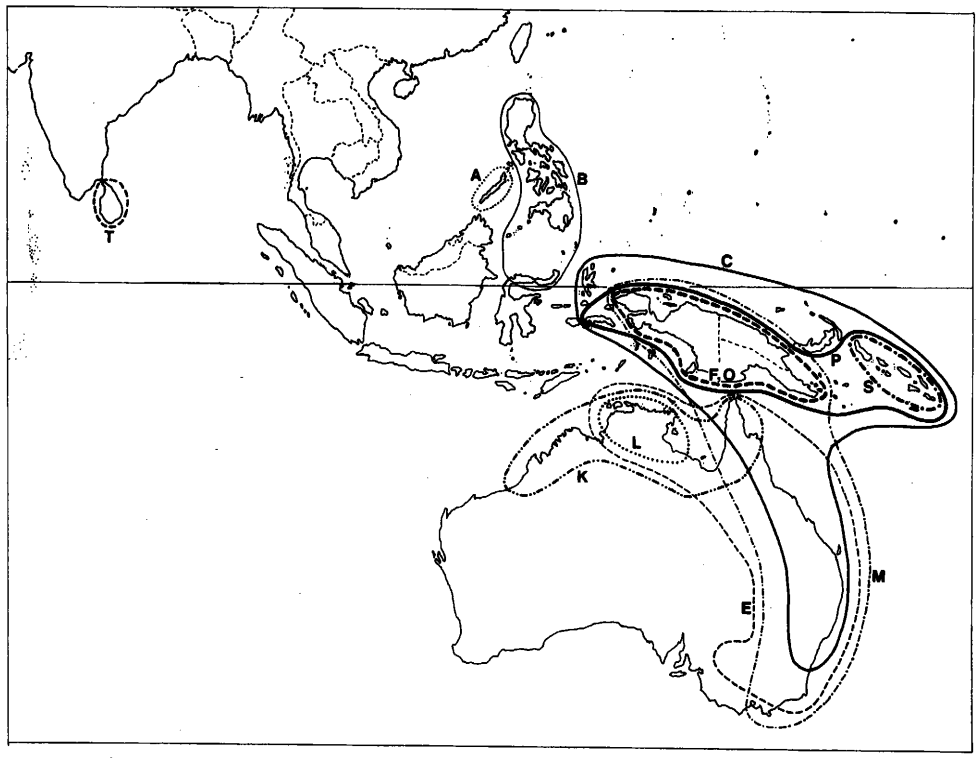

Figure 6 The distributions of 12 species-groups of the subgenus *Morops*. A, *alienigenum*-group (provisional name); B, *banauense*-group (provisional name); C, *clathrinum*-group; E, *faheyi*-group; F, *farciminis*-group; K, *mackerrasorum*-group; L, *lawnhillense*-group; M, *melatum*-group; O, *oculatum*-group; P, *papuense*-group; S, *sherwoodi*-group; T, *trirugosum*-group (provisional name).

bution only in Australia. The *melatum*-group (7 spp.) occurs in both Papua New Guinea (also Irian Jaya) and Australia although it is more abundant in Papua New Guinea.

The *sherwoodi*-group (6 spp.), recently proposed by Takaoka (1995), is limited to the Solomon Islands and Bougainville Island. In the Oriental Region, eight and one *Morops* species were reported from the Philippines and Sulawesi, respectively, of which one (from Palawan Island) was placed in the *alienigenum*-group (provisional name) and the others were in the *banauense*-group (provisional name); one more species found in Sri Lanka (Davies and Györkös, 1988) was also provisionally treated to represent the different group (*trirugosum* group) by itself. The last four species-groups possess the male genitalia with parameral hooks, which are atypical among *Morops* (Takaoka, 1983, 1995; Davies and Györkös, 1988).

IIb-3. Subgenus *Nevermannia* (Fig. 7)

There are three species-groups of *Nevermannia* in these regions, which are much different morphologically from each other. The *vernum*-group, of an apparently Holarctic origin, is widely distributed in north India, Indochina, south China, and adjacent continental islands, such as Taiwan and Java (2 spp. each) and also occurs on the oceanic islands (*S.* (*N.*) *aberrans* in north Luzon and *S.* (*N.*) *bonninense* in Hahajima Island of the Bonin Islands). The *feuerborni*-group is a small group comprising 12 species, of which 10 species are distributed in the Oriental Region, one species, *S.* (*N.*) *sasai*, is in the Palaearctic Region and one species, *S.* (*N.*) *mie*, is in both regions. In Southeast Asia, its range extends eastward to Bali Island (1 sp.), Sulawesi Island (1 sp.) and Mindanao Island (1 sp.). The *ruficorne*-group, which has a wide distribution in the Ethiopian, Palaearctic, Oriental and Australasian Regions, includes only six species in the latter two regions, of which *S.* (*N.*) *aureohirtum* and *S.* (*N.*) *ornatipes*, both autogenous in ovarian development (Hunter, 1977; Takaoka, 1989), are widely distributed in the Oriental and Australasian Regions, respectively. The ranges of both species are separated from each other by the strait between Irian Jaya and the Maluku Islands, though in the latter islands only Halmahera is inhabited by *S.* (*N.*) *aureohirtum*; this species also exists in Flores Island of the Lesser

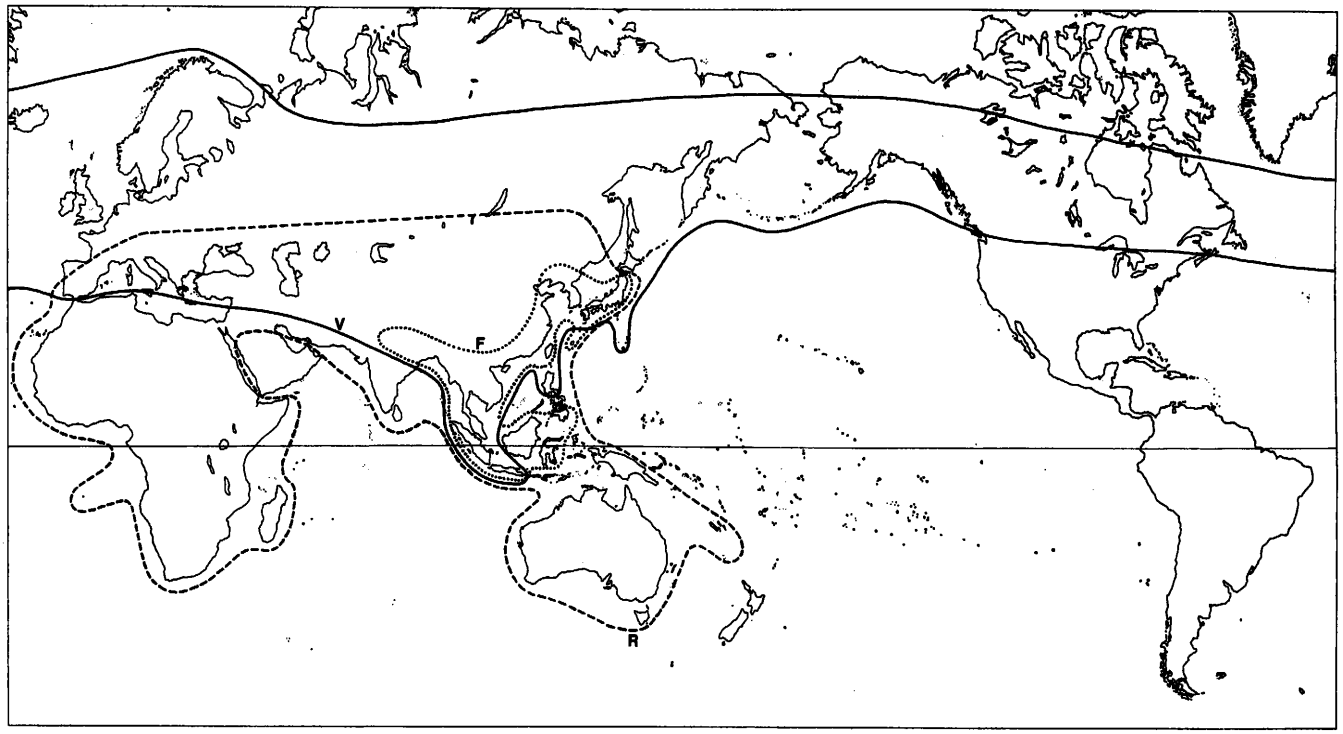

Figure 7 The distributions of three species-groups of the subgenus *Nevermannia*. F, *feuerborni*-group; R, *ruficorne*-group; V, *vernum*-group (not confirmed from Sumatra, though included).

Sunda Islands (unpublished data). The other four species of the *ruficorne*-group have a restricted range: *S.* (*N.*) *angustitarse* in south China, *S.* (*N.*) *glatthaari* in Sumatra, *S.* (*N.*) *neornatipes* in New Caledonia, and *S.* (*N.*) *norfolkense* in Norfolk Island.

IIb-4. Subgenus *Simulium* s.str. (Figs. 8, 9 & 10)

Twelve species-groups of the subgenus *Simulium* s. str. are known in the Oriental Region but not in the Australasian Region except one species of the *melanopus*-group recorded from Seram of the Maluku Islands (unpublished data), just east of Weber's Line redrawn in 1904. The *tuberosum*- and *malyschevi*-groups are generally distributed in the Holarctic Region (Fig. 8). The *malyschevi*-group is represented by three species in south China, one of which occurs also in northeast India. The *tuberosum*-group (13 spp.) is widely distributed not only in the Asiatic mainland but also in the continental islands, such as Sumatra (2 spp.), Java (1 sp.), Borneo (2 spp.), Palawan Island (1 sp.) and the Ryukyu Islands (1 sp.). The *ornatum*- and *variegatum*-groups, which are morphologically very close to each other, are widely distributed in the Palaearctic Region, of which the *ornatum*-group has only two species in the Oriental Region, one in south China and the other in northwest India; on the other hand, the *variegatum*-group has a wide range mostly in the mainland of the Oriental Region, where it is represented by 13 species (Fig. 8).

The three species-groups, i.e., *griseifrons*-group (9 spp.), *striatum*-group (14 spp.) and *multistriatum*-group (9 spp.), which are apparently allied to one another, are widely distributed in the mainland and adjoining continental islands of the Oriental Region (Fig. 9), of which the *griseifrons*- and *striatum*-groups are spreading both northeastwards and northwestwards into the Palaearctic Region. The *multistriatum*-group extends only northwestwards into Central Asia. Both the *multistriatum*- and *striatum*-groups are well represented in India by five and six species, respectively.

All the other five species-groups have their ranges only on the islands except the *nobile*-group, which, in addition to its probable distribution center on the Philippines (6 spp.), is also represented by two species in the Asiatic mainland (1 sp. in northeast India and 1 sp. from northeast India to south China through Thailand), and also by one species in Taiwan (Fig. 10). The *melanopus*-group is mostly distributed in the Philippines (14 spp.); it is also represented in Sulawesi (4 spp.), Borneo (3 spp.), Peninsular Malaysia (1 sp.), and the

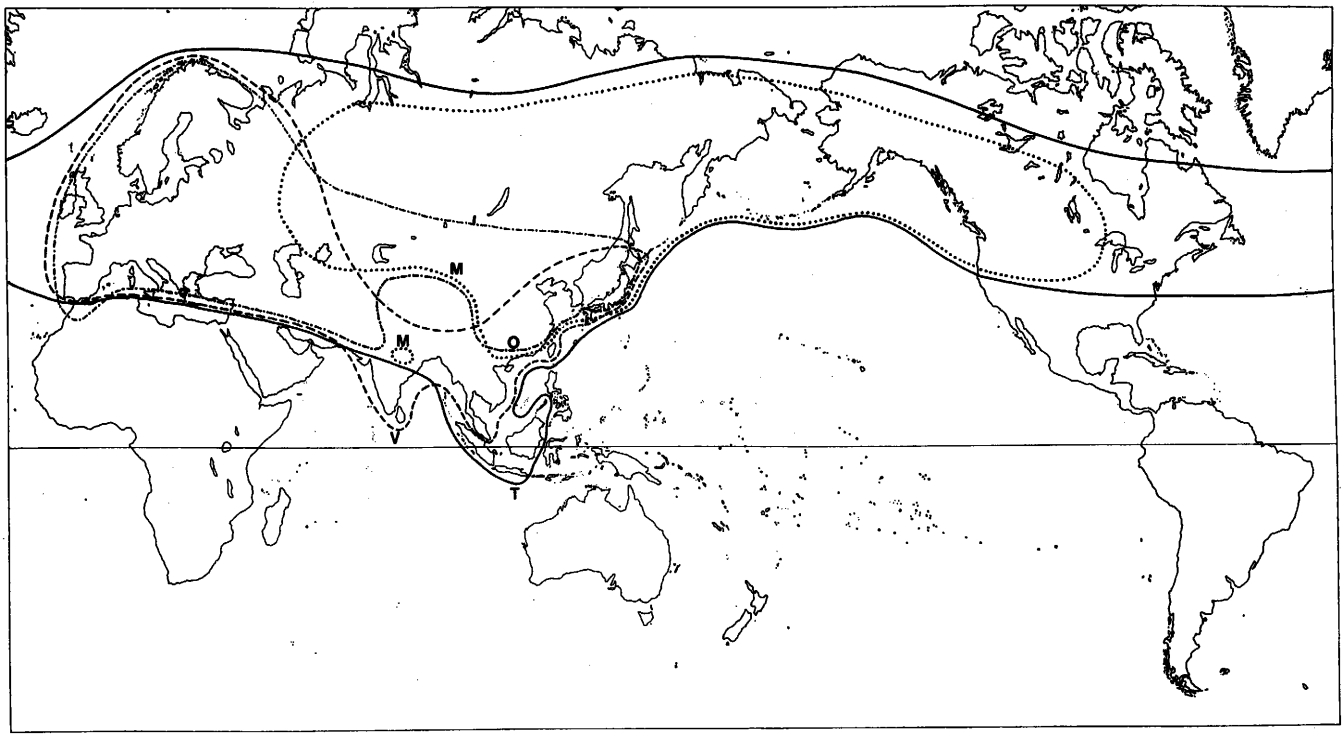

Figure 8 The distributions of four species-groups of the subgenus *Simulium* s.str. M, *malyschevi*-group; O, *ornatum*-group; T, *tuberosum*-group; V, *variegatum*-group.

Sunda Islands from Sumatra to Flores (3 spp.). The one undescribed species of this species-group was found on Seram Island of the Maluku Islands, representing the easternmost distribution of the subgenus *Simulium* s. str., as already noted. In the Sunda Islands (Sumatra, Java and Flores) there are two small species-groups, i.e., *eximium*-group (3 spp.) and *celsum*-group (provisional name, 2 spp.). The most striking finding is the new species-group (yet unnamed) found only on Sulawesi Island, which consists of 11 new species (unpublished data).

III. SUMMARIZED DISTRIBUTION PATTERNS AND POSSIBLE DISPERSAL ROUTES OF SOME SUBGENERA AND SPECIES-GROUPS

As shown in Figs. 2-4, the genus *Simulium* s.l. as a whole showed a rather simple pattern of distribution, with more subgenera in the Asiatic mainland, progressively diminishing its faunal variety toward the east to the South Pacific, reflecting the geographical situations. The similar tendency was already reported in many other groups of insects (Gressitt, 1961).

The more definite patterns of distribution were demonstrated at the subgenus and species-group levels. The 11 subgenera of the genus *Simulium* s.l. recorded in these regions are divided into five groups on the basis of their distribution patterns: the first group is an essentially Holarctic or Palaearctic, apparently is penetrating to varying extents from the north to the Oriental Region (eg., *Eusimulium*, *Byssodon* and *Montisimulium*, all confined to the mainland or nearby continental islands), and further extending their eastward ranges up to the Australasian Region (*Simulium* s.str. and *Nevermannia*) ; the second is confined to the Asiatic mainland (*Himalayum*); the third has its distribution center on the continental islands (eg., the Sunda Islands) and is widely extending northwards into the Asiatic mainland and eastwards beyond the Weber's Line (*Gomphostilbia*) ; the fourth is almost the same as the third except its distribution center on New Guinea and its westward and southward dispersals (*Morops*); the fifth is characterized by an exclusively insular distribution (*Wallacellum*, *Inseliellum* and *Hebridosimulium*). The last five subgenera are morphologically similar to one another, and certainly must have derived from a common ancestor, but their phylogenetic relationship has not been elucidated yet. None of the subgenera has been attempted to reconstruct its phylogeny, except *Inseliellum* which is presently under study by Dr. D. A. Craig (pers. commun.).

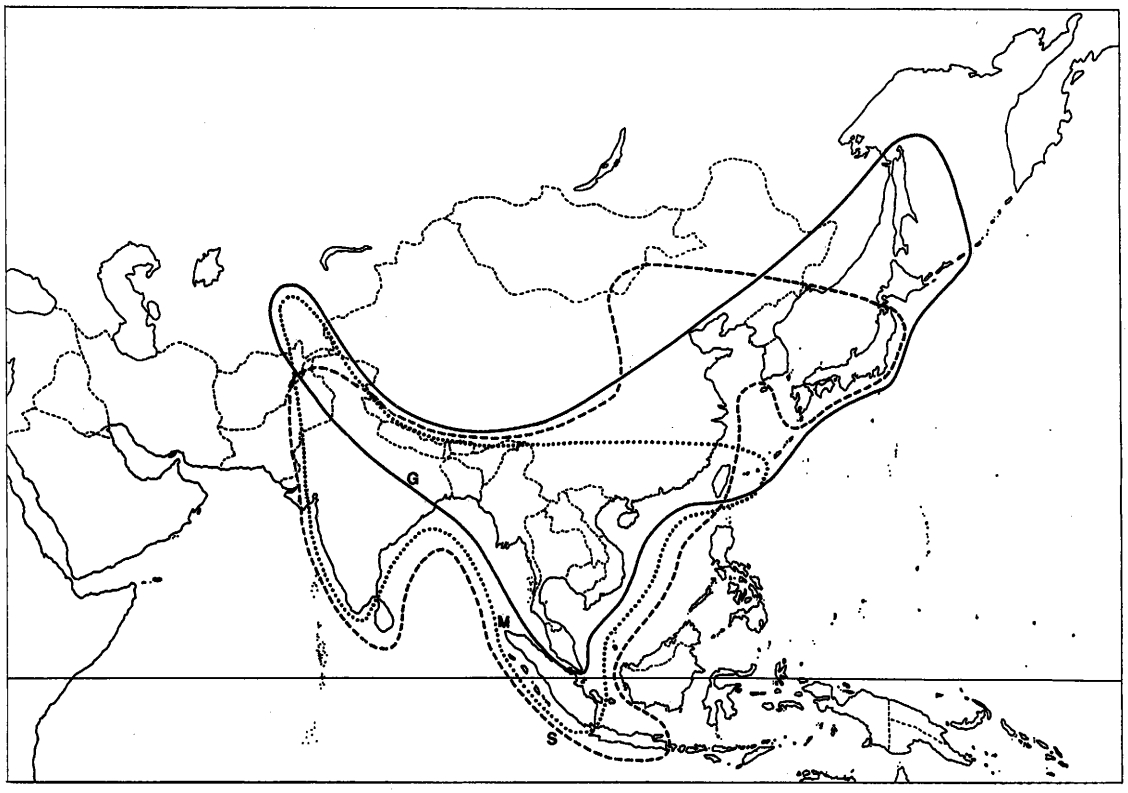

Figure 9 The distributions of three species-groups of the subgenus *Simulium* s.str. G, *griseifrons*-group; M, *multistriatum*-group; S, *striatum*-group.

The subgenus *Simulium* s.str., though generally regarded as a cosmopolitan, is heterogenous in element, including eight species-groups whose ranges are mostly in the Oriental mainland and/or islands. They also exhibit two different distribution patterns, i.e., center of distribution being on the continent and/or continental islands in one group (eg., *griseifrons, multistriatum, striatum*, *eximium*, and *celsum*), and on the oceanic islands in the other (*nobile, melanopus*, and the unnamed group on Sulawesi). All these species-groups apparently had developed in tropical Asia. The first group may be inferred to have been inferior to the second group in over-sea dispersal abilities or in adaptabilities to insular environments. In the second group, supposedly the local speciation of the *nobile*-group had taken place on each island of the Philippine Archipelago and a few of its descendants had then undergone their westward and northward movements to reach northeast India, south China and Taiwan. This hypothesis on dispersal may be supported by the morphological characters, such as the variation in number and shape of the pupal gill filaments in the *nobile*-group, assuming that the pupal gill filaments tend to decrease in number and to become inflated in the process of the specialization. The similar dispersal from the Philippines might have been done also by the *melanopus*-group, according to the morphological changes in the female genitalia, particularly, in the paraprocts (Takaoka, 1983). The unnamed species-group found in Sulawesi is different from the former two species-groups by presenting a remarkable intra-island speciation.

The difference in colonization of oceanic islands by these species-groups (also by the three insular subgenera) may have been caused not only by the difference in the over-sea dispersal abilities of the adults and the adaptabilities of the immature stages to the various insular environments but also by the difference in the time of their ancester's arrival(s) and various geological events on each island in the past.

Among the species-groups widely distributed in and outside the Oriental Region, the *ruficorne*-group of *Nevermannia* seems to have well conformed to warm climate conditions and also to very slow-flowing waters since it was often found to inhabit lowland ditches with the water temperature of 30 °C or more in paddy fields where no other black fly species usually could live

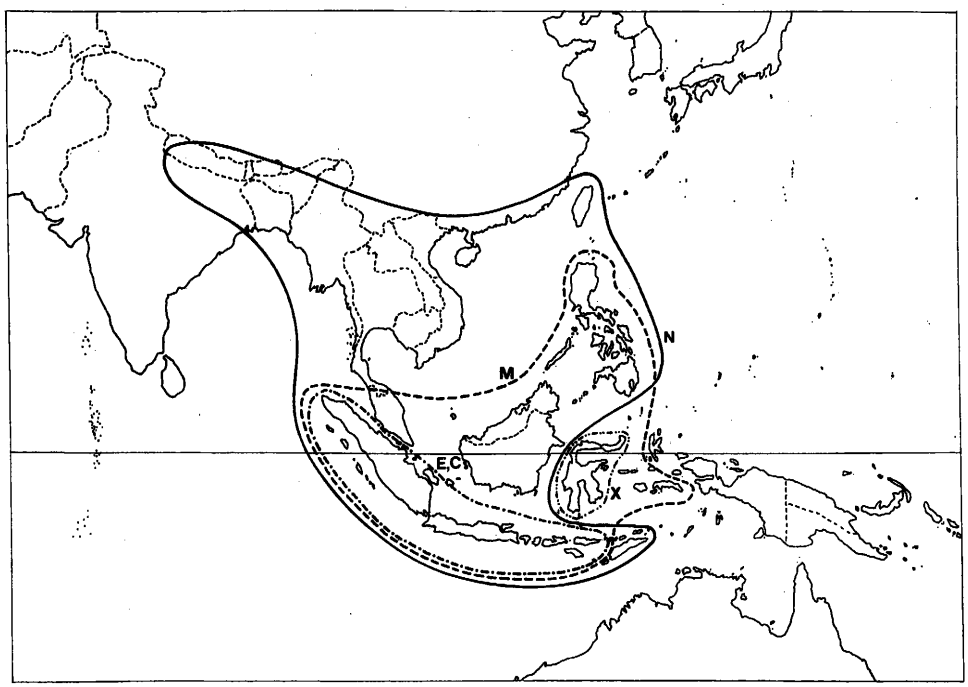

Figure 10 The distributions of five species-groups of the subgenus *Simulium* s.str. C, *celsum*-group (provisional name); E, *eximium*-group; M, *melanopus*-group; N, *nobile*-group; X, unnamed group.

(unpublished data). Interestingly *S.* (*N.*) *glatthaari* and *S.* (*N.*) *aureohirtum* in the Oriental Region have eight and six pupal gill filaments per side respectively, while all the other species in the Palaearctic, Ethiopian and Australasian Regions have four gill filaments. As inferred for the *nobile*-group, it may be speculated that the *ruficorne*-group had originated in Southeast Asia (probably in or near Sumatra) and had migrated both eastwards and westwards. On the other hand, the *vernum*-group of *Nevermannia* inhabits only high mountains over 1000 m in altitude with cool climate conditions in Peninsular Malaysia, Java, Taiwan and Philippines (Takaoka and Davies, 1995, 1996; Takaoka, 1979, 1983). The relic distribution of this species-group may be explained by their influx into Southeast Asia from the north at the Pleistocene glaciations and their subsequent retreat northwards, leaving several species only at the high elevations where the climate was cool enough to support their breeding. This species-group would be predicted to be found also at the Mt. Kinabaru (alt., ca. 4100 m) in north Borneo and at the Mt. Kerinci and others (alt., over 3000 m) in Sumatra if explored.

The *tuberosum*-group of *Simulium* s. str., which seems to be less susceptible to the high temperature conditions, is distributed not only on the mainland but also on the continental islands, such as Sumatra, Java, Borneo and Palawan, all of which are situated on the Sunda continental shelf delimited by the contour of 180 m below sea surface. The colonization of these islands in tropical Asia by this species-group might have occurred also during the Pleistocene glacial periods when these islands had been connected. The relatively poor representation on each continental island by these two species-groups, only one or two endemic species (Takaoka and Davies, 1995), might have been due to the shortage of time to allow subsequent local speciation.

IV. THE *SIMULIUM* FAUNA AND THE WALLACE'S AND WEBER'S LINES

The Wallace's and Weber's Lines are the zoogeographical boundaries separating the Oriental from the Australasian Region. The significance of these lines differs by the different groups of animals (Mayr, 1944). The modified Wallace's Line (Huxley's Line) united by the Kano's Line coincides well with the western edge of the range of the insular subgenus *Wallacellum* (Figs. 1 & 4), and the Wallace's Line 1910 corresponds to the eastern edge of the *feuerborni*-group range (Figs. 1 & 7). However the more drastic change of the *Simulium* fauna is likely to be seen on both sides of the Weber's Line. In the Philippines the Oriental elements (72% or 41 of 57 total spp., i.e., *Nevermannia* 4 spp., *Gomphostil-*

bia 13 spp., and *Simulium* s.str. 24 spp.) outnumber the Australasian element (14%, *Morops* 8 spp.) (Takaoka, 1983). The Sulawesi fauna of *Simulium* s. l. is also characterized by the predominance of the Oriental elements (92.8% or 26 of 28 total spp., i.e., *Nevermannia* 2 spp., *Gomphostilbia* 9 spp. and *Simulium* s. str. 15 spp.) and the impoverishment of the Australasian element (3.5%, *Morops* 1 sp.) (Takaoka and Roberts 1988; unpublished data). The fauna of Flores Island of the Lesser Sunda Islands is all represented by the Oriental elements (*Nevermannia* 1 sp., *Gomphostilbia* 4 spp. and *Simulium* s.str. 3 spp.) (unpublished data). On the other hand, in the Maluku Islands, just east of the Weber's Line, the Australasian element (50% or 7 *Morops* spp. of 14 total spp.) somewhat predominates the Oriental elements (43%, i.e., *Nevermannia* 1 sp., *Gomphostilbia* 4 spp., and *Simulium* s.str. 1 sp.) (unpublished data).

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Short communication

CONTAMINATION OF SOIL WITH PARASITE EGGS AND OOCYSTS IN AND AROUND KUALA LUMPUR, MALAYSIA

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Attempts to isolate parasite eggs from the soil have been made in many countries in the world. The soil samples collected from the surrounding environment in developed countries like Germany, England, Australia, Japan, or America have revealed the presence of *Toxocara* eggs (Schantz, 1989). *Toxocara* that causes the larva migrans in human has attracted considerable attention during recent years as an important public health problem. *Toxocara* infection in humans occurs primarily by ingestion of embryonated eggs in soil contaminated by feces of dogs and cats (Schantz and Stehr-Green, 1988). On the other hand, in many countries located in the tropical areas, little attempt has been made to detect parasite eggs from soil. Uga *et al.* (1995) examined the soil around houses in slum areas of Surabaya, Indonesia, and have found the eggs of *Ascaris lumbricoides* and *Trichuris trichura* including those of *Toxocara* sp. These reports suggest that the soil contamination in developed countries is caused by pet feces, while that in developing countries by both human and pet animal feces.

In recent years, in the capital cities and their suburbs in many developing countries in the tropics, a systematic developmental process is underway to establish an environment wherein the inhabitants can live comfortably in modern facilities. It is not exceptional in and around Kuala Lumpur, the capital city of Malaysia. In Kuala Lumpur, many big modern parks like those seen in America and Europe have been set up. However, at the same time, in rural areas of this country, there is a high infection rate of human parasites and subsequent soil contamination has been suggested (Lai, 1992; Rahman, 1994). Whether soil contamination by the parasite eggs occurred in the newly developed modern parks has not been reported. In this study, we examined the present situation of soil contamination by parasite eggs in modern parks located in Kuala Lumpur city and its suburb.

Surveys were carried out on two occasions: December, 1994 (rainy season), and July, 1995 (dry season). In this study, the survey was done in three areas, and five parks in each area were examined, i.e., 15 parks in total. Those areas were Kepong area including Parks 1-5 located north-west of the city, Petaling Jaya area including Parks 6-10 in the south-west, and Serdang area including Parks 11-15 located south in the suburb. Soil samples were collected from 4-6 places of each park in the respective areas (89 samples were collected in each season; 178 samples in all). Collected samples were divided into four types (sand, loam, clay, and others) according to their character so as to clarify the relationship between the quality of soil and the contamination rate. The parasite eggs were detected by the centrifugal flotation technique described previously for the recovery of eggs from sand (Uga *et al.*, 1993).

Throughout the study, three species of helminth eggs (*Trichuris vulpis, Toxocara canis,* Unidentified nematoda) and four species of protozoan oocysts (*Isospora* sp., *Wenyonella* sp., *Hoarella* sp. *Octosporella* sp.) were detected (Table 1). Prevalence of parasites was 42% in the rainy season, and 24% in the dry season, suggesting parasite infections many occur more in the rainy season than in the dry season. Similar observation has also been made in Indonesia (Uga *et al.*, 1995). The contamination rate of soil with *T. vulpis* eggs was not changed either in the rainy or dry season. Because of

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Table 1 Species of soil-transmitted parasite eggs and oocysts recovered from soil in Kuala Lumpur, Malaysia

Species	No. of sample	Dec. 1994		Jul. 1995	
		No. of positive	(%)	No. of positive	(%)
Trichuris vulpis	89	4	4	4	4
Toxocara canis	89	0	0	1	1
Unidentified nematoda eggs	89	13	15	12	13
Isospora sp.	89	3	3	1	1
Wenyonella sp.	89	13	15	0	0
Oocysts#	89	8	9	7	8
Total	89	37*	42	21*	24

*More than two species of parasite were found in one specimen.
#Either the oocysts of *Hoarella* sp. or *Octosporella* sp.

the close morphological similarity between the eggs of *T. canis* and *T. cati,* little attempt has been made to differentiate between them. Scanning electron microscopic observation, however, has revealed a difference in surface structure of *T. canis* and *T. cati* eggs. They can also be distinguished by light microscopy (Uga *et al.*, 1993). Based on these previous observations, the *Toxocara* eggs detected in this study were identified as *T. canis.* The unidentified nematoda eggs shown in Table 1 were oval, embryonated, and measuring 36×71 μm in size, with a transparent egg shell. We are unable to determine the type of animal discharging these eggs or whether they were infective to human. As to *Wenyonella* sp., it seemed that the oocysts were less resistant to dryness, as its prevalence was 15% in the rainy season and 0% in the dry season, showing a significant difference. The definitive host of *Wenyonella* sp. is ducks. In the parks where such oocysts were detected, a pond was located at the center with ducks being raised, which might have been the source of contamination, but the possibility of other avian species cannot be excluded. The oocysts detected in this study were identified as *Hoarella* sp. or *Octosporella* sp. on the basis of their size, number of sporocysts, and number of sporozoites. Since the *Hoarella* sp. or *Octosporella* sp. are reported only from sauropsids, no further analyses were made.

Of the total soil samples collected, 53% were loam type followed by clay type (22%), sand type (18%), and other types (7%) with a parasite egg detection rate of 28, 25, 19, and 17%, respectively. These findings were not related with the type of soil and their contamination rate by parasite eggs.

Among the parasites recovered through this study, there were two species known to infect human beings, namely, *T. vulpis* and *T. canis,* dogs being the definitive host of both nematodes. No parasite that has human beings as the definitive host was recovered, indicating no contamination by human feces occurred in these areas. On the contrary, Mohammod *et al.* (1988) reported that soil-transmitted helminths were widely distributed in the urban slums of Kuala Lumpur. Furthermore, recent studies done by Lai (1992) and Rahman (1994) have clarified the existence of many species of human parasites among the people living in the rural areas in Malaysia. Lai (1992) has also found eggs of *A. lumbricoides* and *T. trichura* in 10% of the dirt of the children's nails. With the importance of *T. vulpis* and *T. canis* in the causation of zoonosis, we analyzed the data further in detail (Table 2). Out of 15 parks studied, these two parasites were found in six parks (40%; Parks 1, 7, 9, 11, 12, and 13). Throughout the study, the Serdang area revealed the highest rate of contamination, especially from Park 11 in this area. *T. vulpis* eggs were also detected from adjacent Parks 12 and 13 indicating the higher intensity of contamination in Serdang area compared to other two areas. The reason for the higher contamination rate in this area appears to be due to many dogs kept in the Chinese community. Oikawa (1995) reviewed the study on a survey of dog and cat parasites in Malaysia. Subsequently, 12 species of parasites were reported and many of those were known to be a cause of zoonosis or larva migrans. Since

Table 2 Contamination rate of different parks by *T. vulpis* and *T. canis* eggs

Parsites	1994		1995	
	Park no.	No. of eggs found	Park no.	No. of eggs found
Trichuris vulpis	1	3	9	1
	7	1	11	1
	9	1	12	3
	11	105	13	1
Toxocara canis	—	—	11	2

contamination of soil by pet parasites might increase further, proper control measures in public areas to avoid zoonotie diseases should be taken.

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Research Note

LEG COLOR VARIATION OF FEMALE *SIMULIUM AOKII* (DIPTERA: SIMULIIDAE)

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Abstract: Color variations of legs (in particular hind femora and tibiae) were observed in female *Simulium aokii* (Takahasi, 1941) caught monthly at cattle shed in Oita, southwest Japan. Female flies were collected from November to April but not during warm and hot months (May to October). Female flies with legs darker than normal began to appear in the mid autumn (November) and increased in relative abundance up to the mid winter (January), and then decreased gradually until the mid spring (April). The intensity of darkness on both hind femora and tibiae varied similarly, with the darkest in January. During these cool or cold months, there was a positive correlation between the relative size of dark area on the tibia and the wing length. From these results the species status of *S. aokii* was discussed in relation to *S. oitanum* originally described from Oita.

INTRODUCTION

Coloration of adult blackflies is usually stable and is used as a character for species identification. There have been only a few reports on the color variations of adult simuliids (Garms, 1978; Garms *et al.*, 1982).

When working on the transmission of bovine *Onchocerca* spp. in Oita, Kyushu, Japan, we collected females of *Simulium aokii,* of which legs were darker to varying extent than those originally described (Takahasi, 1941). This is one of the common blackfly species in Oita, is abundant from autumn to spring (Takaoka, 1994) and is known as a potential vector of bovine *Onchocerca* sp. in Oita (Takaoka and Bain, 1990).

We examined whether the occurrence of the female *S. aokii* with darker legs is related to the cold months and also to the fly's body size.

The taxonomic status of *S. aokii* was also discussed in relation to *S. oitanum* described from Oita (Shiraki, 1935) which had been long thought, by some taxonomists, to be a senior synonym of the former species.

MATERIALS AND METHODS

The study was conducted at Imanaga cattle shed in the western part of Oita City, Oita Prefecture. The detailed information on the cattle shed, its locality and environment were already given in Takaoka (1994). The collections were made twice per month from February 1993 to April 1995. The time used for one collection was 1-3 hrs in the afternoon (15:00-18:00 hr). Female flies landing or resting on the inside surface of glass windows of the cattle shed were collected by an aspirator, and preserved in 80% ethanol. After identification to species using the keys of Takaoka (1977), the wings of all *S. aokii* females were individually measured under dissecting microscope. The wing length was represented by the distance from arculus to the wing tip. The values were used as an index of body size. The coloration of hind femora and tibiae of each fly was recorded, and depending on the relative length of dark area(s) against the total length of segment, the flies were divided into four types (Fig. 1): for tibiae, 20-40% darkness to type I, 41-60% to type II, 61-80% to type III, 81-100% to type IV; for femora, 20-35% to type I, 36-50% to type II, 51-65% to type III and 66-80% to type IV.

RESULTS

The results of collections are presented in Table 1. Female *S. aokii* were collected from November to April but not from May to October. Fig. 2 shows the frequency distribution of four types of flies based on the

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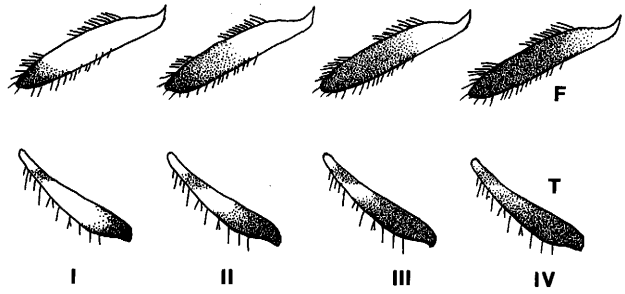

Figure 1 Color variations of hind femora and tibiae of female *Simulium aokii*. Numbers show type of color depending on the darkness. F and T mean femora and tibiae, respectively.

Table 1 Results of monthly collections of female *Simulium aokii* at Imanaga cattle shed in Oita city, Japan. Data obtained in 1993-1995 were combined.

Month	No. collections	No. flies caught	Air temp. (°C)
Jan.	4	19	6-10
Feb.	4	110	9-12
Mar.	4	63	12-14
Apr.	4	7	15-19
May	2	0	18-21
Jun.	2	0	22-28
Jul.	2	0	25-30
Aug.	2	0	28-33
Sep.	4	0	20-26
Oct.	4	0	17-22
Nov.	4	29	16-19
Dec.	4	22	6-11

coloration of the hind tibiae. It was clearly shown that the females with darker tibiae (types II-IV together) increased in relative abundance from November to January and then decreased until April, occupying 70-100% of the total catches during the winter months (December to March). The females with type III tibiae were the most abundant in January, February and March. The frequency distribution of four types based on the coloration of the femora showed a similar pattern (Fig. 3).

The coloration of tibiae had a significant positive correlation with the wing length ($Y=2.62+0.0087X$; X: % darkness of tibia, Y: wing length; $r=0.73$, $df=248$, $P<0.01$).

The average wing length (mm) was 2.95 ± 0.11 for type I ($n=50$), 3.03 ± 0.10 for type II ($n=63$), 3.24 ± 0.17 for type III ($n=107$), and 3.39 ± 0.16 for type IV ($n=29$).

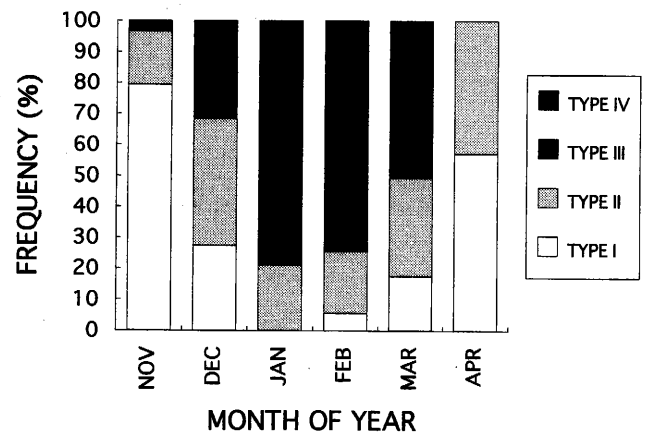

Figure 2 Frequency distribution of four types of female *Simulium aokii* based on tibial coloration.

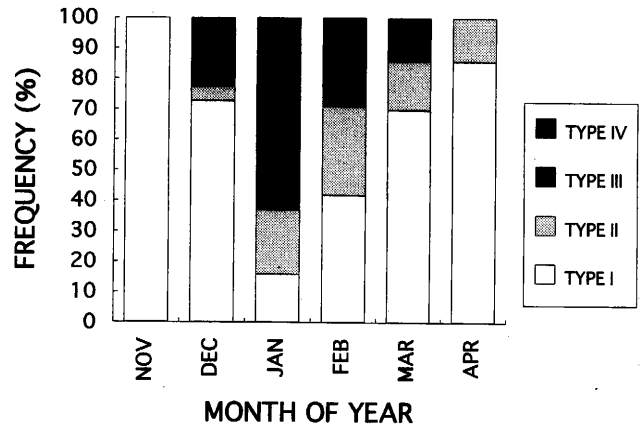

Figure 3 Frequency distribution of four types of female *Simulium aokii* based on femoral coloration.

DISCUSSION

It was found that the leg color variation of *S. aokii* in Oita was seasonal; flies with darker legs dominantly occurred in mid winter but rarely in autumn and spring.

In our observations, the leg color variation was also found to be related to the wing length (then, body size). The leg coloration became darker as the wing length became longer. It might be attributed to the sufficient nutrition during the prolonged larval development under low water temperatures, although it is not known why excess melanin is produced only in the larger adult flies.

Our results suggested that variations found in the leg coloring of female *S. aokii* were probably intraspecific, because of their continuous changes although flies were roughly classified into four types dependent on the coloration. The possibility that our material was composed of two or more species with different leg colors would be low, since no siblings were

detected so far in the populations of *S. aokii* in Oita according to our cytotaxonomic study of larval polytene chromosomes (Hadi *et al.*, 1996).

According to the original description (Takahasi, 1941) *S. aokii* was distinguished from *S. oitanum* in the female by the pale mid and hind femora. As shown in the present study, the females of *S. aokii* grouped into type III and IV for femora were indistinguishable from those of *S. oitanum*. This may support the claim that *S. aokii* and *S. oitanum* are the same species. However we hesitate to make such a conclusion. It is because there is a discrepancy in seasonal occurrence between these two species. It was in August that type female specimens of *S. oitanum* were collected (Shiraki, 1935) when adult *S. aokii* were almost nil in Oita (Takaoka, 1994). If the collection date given by Shiraki was not erroneously noted the two species will be regarded to be distinct from each other. At present, type specimens of *S. oitanum* can not be located, and no additional new specimen is available in Oita for comparative studies.

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