Genetica (2006) 128:177–204
DOI 10.1007/s10709-005-5702-z

Molecular phylogeny of black flies (Diptera: Simuliidae) from Thailand, using ITS2 rDNA

Aunchalee Thanwisai[1], Chaliow Kuvangkadilok[1,]* & Visut Baimai[1,2]
[1]*Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, 10400, Thailand;* [2]*Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, 10400, Thailand; *Author for correspondence (Phone: +662-201-5274; Fax: +662-664-8706; E-mail: scckv@mahidol.ac.th)*

Received 30 July 2005 Accepted 2 December 2005

Key words: black flies, ITS2, molecular phylogeny, rDNA, *Simulium*

Abstract

The sequences of the second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA) were determined for 40 black fly species from Thailand, belonging to 4 subgenera of the genus *Simulium*, namely *Gomphostilbia* (12 species), *Nevermannia* (5 species), *Montisimulium* (1 species), *Simulium* sensu stricto (21 species), and an unknown subgenus with one species (*Simulium baimaii*). The length of the ITS2 ranged from 247 to 308 bp. All black fly species had high AT content, ranging from 71 to 83.8%. Intraindividual variation (clonal variation) occurred in 13 species, ranging from 0.3 to 1.1%. Large intrapopulation and interpopulation heterogeneities exist in *S. feuerboni* from the same and different locations in Doi Inthanon National Park, northern Thailand. Phylogenetic relationships among 40 black fly species were examined using PAUP (version 4.0b10) and MrBAYS (version 3.0B4). The topology of the trees revealed two major monophyletic clades. The subgenus *Simulium* and *Simulium baimaii* were placed in the first monophyletic clade, whereas the subgenera *Nevermannia* + *Montisimulium* were placed as the sister group to the subgenus *Gomphostilbia* in the second monophyletic clade. Our results suggest that *S. baimaii* belongs to the *malyschevi*-group or *variegatum*-group in the subgenus *Simulium*. The molecular phylogeny generally agrees with existing morphology-based phylogenies.

Introduction

Black flies (Diptera: Simuliidae) are a large group of medically and economically important insects in the suborder Nematocera of the order Diptera. According to the review of Crosskey and Howard (2004), 45 species of black flies have been recorded in Thailand based on morphological characters of larvae, pupae, and adults. An additional 20 species have been found since this review was published (Takaoka & Choochote, 2004a–c; Takaoka & Choochote, 2005a–g; Takaoka & Choochote, unpublished data). Adults and larvae of many species, for example in the subgenus *Gomphostilbia*, can be difficult to identify morphologically. Cytotaxonomic analyses of polytene chromosome banding patterns have been used to identify 18 species in Thailand (Kuvangkadilok et al., 1999a, b; Kuvangkadilok et al., 2003; Kuvangkadilok et al., unpublished data). However, the cytological technique has limitations. It is time consuming and typically necessitates samples in a particular stage of development (i.e., larvae). Hence, there is a need for an alternate method of identification for black flies. Recently, DNA-based technology has made available a wide range of molecular characteristics for systematic and phylogenetic studies of black flies (Xiong & Kocher, 1991; Post & Flook, 1992; Brockhouse et al., 1993; Tang et al., 1995; Krüger, Gelhaus & Garms, 2000; Joy & Conn, 2001). In Asia, the molecular evolution of black flies was first studied by Otsuka et al. (2001), who examined phylogenetic relationships in the subgenus *Nevermannia* and other subgenera, based on mitochondrial 16S rRNA gene sequences. Recently, Otsuka et al. (2003) reported the phylogenetic relationships of black flies in the subgenus *Himalayum* and other subgenera in the genus *Simulium*, using mitochondrial 16S rRNA gene sequences.

The utility of nuclear ribosomal DNA for studies of molecular evolution and phylogeny is widely accepted (Hillis & Dixon, 1991). The ribosomal DNA in insects, like in other eukaryotes, is composed of tandemly repeated units separated from each other by intergenic spacers (IGS), formerly called "nontranscribed spacers" (NTS). Each unit contains the coding genes for the 18S, 5.8S, and 28S ribosomal RNA in respective order, and spacers – an external transcribed spacer (ETS) and the internal transcribed spacers 1 (ITS1) and 2 (ITS2) (reviewed in Gerbi, 1985; Hillis & Dixon, 1991). The noncoding regions, ITS1 and ITS2, are located between the coding 18S, 5.8S, and 28S rDNA genes. The ITS1 separates the 18S small subunit from the 5.8S, whereas the ITS2 separates the 5.8S from the 28S large subunit. The coding regions of 18S, 5.8S, and 28S are highly conserved and commonly used to construct higher level phylogenies (e.g., Hillis & Dixon, 1991; Olsen & Woese, 1993; Miller, Crabtree & Savage, 1997; Nirmala, Hypsa & Zurovec, 2001; Shi, Chen & van Achterberg, 2005). In contrast, the noncoding regions of ITS1 and ITS2 are highly variable and evolve at a faster rate than do the coding regions (Schlötterer et al., 1994). The ITS2 sequence comparisons are popular for distinguishing closely related species (e.g., Porter & Collins, 1991; Paskewitz, Wesson & Collins, 1993; Cornel, Porter & Collins, 1996; Severini et al., 1996; Walton et al., 1999; Hackett et al., 2000), for differentiation of populations (Fritz et al., 1994; Marrelli et al., 1999), for study of divergence within and between species (Tang et al., 1996; Malafronte, Marrelli & Marinotti, 1999), and for reconstruction of evolutionary relationships (e.g., Wesson, Porter & Collins, 1992; Schlötterer et al., 1994; Cornel, Porter & Collins, 1996; Miller, Crabtree & Savage, 1996; Xu & Qu, 1997; Malafronte, Marrelli & Marinotti, 1999; Depaquit et al., 2000; Weekers, De Jonckheere & Dumont, 2001; Oliverio, Cervelli & Mariottini, 2002; Toma et al., 2002; Young & Coleman, 2004).

In Thailand, no work has been done on molecular evolution of black flies. In this study, we present and compare ITS2 sequences among 40 black fly species from Thailand and infer phylogenetic relationships at the species, species group, and subgenus levels.

Materials and methods

Black fly collection and species identification

Black fly larvae, pupae, and adults were collected from various localities in northern, northeastern, central and southern Thailand (Table 1). Larvae and pupae were removed with fine forceps from stones and trailing vegetation. Some pupae were identified and reared by putting them in vials tightly plugged with damp cotton wool. Some adults were collected on human bait, using an aspirator. All specimens except for *S. feuerborni* larvae were preserved in absolute ethanol. Larvae of *S. feuerborni* were preserved in Carnoy's fixative (absolute ethanol: acetic acid; 2:1). Identifications were based on the external morphology of larvae, pupae and adults according to Takaoka (1977), Takaoka (1979), Takaoka (2001), Takaoka and Suzuki (1984), Takaoka and Davies (1995), Takaoka and Saito (1996), Takaoka and Adler (1997), Takaoka and Kuvangkadilok (1999), Kuvangkadilok and Takaoka (2000) and Takaoka and Choochote (2005a, d). In addition to morphological identification, cytological criteria were used for identification of *S. feuerborni.* The head and thorax of each *S. feuerborni* larva were used for molecular work as described in Pramual et al. (2005). The remainder of the larva was used for salivary gland polytene chromosome preparation following the method of Rothfels and Dunbar (1953). Polytene chromosome banding patterns were read band by band using the standard map of *S. feuerborni* (Kuvangkadilok, Phayuhasena & Baimai, 1999a).

DNA extraction and PCR amplification

Genomic DNA was extracted from preserved specimens by the method described in Collins, Porter and Cope (1990). The rDNA ITS2 regions were amplified by PCR using two primers, CP17 (5′-GCGC

Table 1. Collection details of the forty *Simulium* species in the subgenera *Gomphostilbia*, *Nevermannia*, *Simulium*, and *Montisimulium* and an unknown subgenus from various localities in Northern, Northeastern, Central and Southern Thailand used in this study

Species	Specimen	Collection sites	Latitude/Longitude	Altitude (m)	Collection date
Subgenus *Gomphostilbia* *batoense*-group					
Simulium angulistylum Takaoka and Davies	Pupa	WTK: Wang Takhrai waterfall, Nakhon Nayok	14°19′ N/101°18′ E	240	June 1998
	Pupa	HLU: Huai Luang waterfall, Ubon Ratchathani	15°25′ N/105°30′ E	300	November 1998
	Larva	KHK: Khun Korn waterfall, Chiang Rai	19°50′ N/99°40′ E	500	December 1998
	Male Adult	MTU: Muang Tuad waterfall, Suratthani	8°45′ N/99°26′ E	120	June 1999
	Larva	MTU: Muang Tuad waterfall, Suratthani	8°45′ N/99°26′ E	120	June 1999
	Pupa	NGA: Ngao waterfall, Ranong	9°45′ N/98°37′ E	50	August 1999
	Pupa	NGA: Ngao waterfall, Ranong	9°45′ N/98°37′ E	50	August 1999
Simulium decuplum Takaoka and Davies	Larva	BMT: Ban Mae Tho, Chiang Rai	19°05′ N/99°24′ E	480	March 1999
	Larva	MTU: Muang Tuad waterfall, Suratthani	8°45′ N/99°26′ E	120	November 1999
Simulium duolongum Takaoka and Davies	Larva	MTU: Muang Tuad waterfall, Suratthani	8°45′ N/99°26′ E	120	May 2001
Simulium gombakense Takaoka and Davies	Larva	HSL: Huai Sai Luaeng waterfall, Doi Inthanon NP, Chiang Mai	18°31′ N/98°27′ E	950	December 2002
Simulium parahiyangum Takaoka and Sigit	Larva	MFA: Mork Fah waterfall, Chiang Rai	19°06′ N/98°46′ E	545	March 1999
Simulium siamense Takaoka and Suzuki	Larva	HSW: Haew Suwat waterfall: Nakhon Ratchasima	14°19′ N/101°21′ E	630	June 1998
	Pupa	HSW: Haew Suwat waterfall: Nakhon Ratchasima	14°19′ N/101°21′ E	630	June 1998
	Male Adult	STH: Sai Thong waterfall, Chaiyaphum	15°38′ N/101°23′ E	750	November 1998

Table 1. (*continued*)

Species	Specimen	Collection sites	Latitude/Longitude	Altitude (m)	Collection date
ceylonicum-group					
Simulium asakoae Takaoka and Davies	Pupa	RPJ: Royal Project, Doi Inthanon NP, Chiang Mai	18°33′ N/98°31′ E	1400	February 1997
	Larva	HKE: Huai Keaw waterfall, Chiang Mai	18°30′ N/98°34′ E	250	February 1998
	Larva	HKE: Huai Keaw waterfall, Chiang Mai	18°30′ N/98°34′ E	250	February 1998
	Larva	MSA: Mae Sa waterfall, Chiang Mai	18°32′ N/98°34′ E	400	February 1998
Simulium inthanonense Takaoka and Suzuki	Larva	BMT: Ban Mae Tho, Chiang Rai	18°34′ N/97°56′ E	417	March 1999
	Larva	SRP: Siri Phum waterfall, Doi Inthanon NP, Chiang Mai	18°32′ N/98°31′ E	1300	August 2001
	Larva	SRP: Siri Phum waterfall, Doi Inthanon NP, Chiang Mai	18°32′ N/98°31′ E	1300	August 2001
Simulium sheilae Takaoka and Davies	Female Adult	BRP: Boriphat waterfall, Songkhla	6°17′ N/100°01′ E	50	June 1999
	Larva	NGA: Ngao waterfall, Ranong	9°51′ N/98°37′ E	50	March 2000
Simulium sp. nr. *Sheilae*	Larva	NGA: Ngao waterfall, Ranong	9°51′ N/98°37′ E	50	July 2000
	Male Adult	NGA: Ngao waterfall, Ranong	9°51′ N/98°37′ E	50	May 2001
varicorne-group					
Simulium burtoni Takaoka and Davies	Larva	NNA: Nam Nao NP, Phethchabun	16°21′ N/101°21′ E	400	November 2000
Simulium chumpornense Takaoka and Kuvangkadilok	Female Adult	KPO: Ka Po waterfall, Chumporn	10°44′ N/99°12′ E	40	May 2001
Subgenus *Nevermannia*					
feuerborni-group					
Simulium feuerborni Edwards	Larva[a]	HSL: Huai Sai Luaeng waterfall, Chiang Mai	18°31′ N/98°27′ E	950	December 2002
	Larva[a]	RPJ: Royal Project, Doi Inthanon NP, Chiang Mai	18°33′ N/98°31′ E	1400	December 2002

	Larva[a]	RPJ: Royal Project, Doi Inthanon NP, Chiang Mai	18°33′ N/98°31′ E	1400	November 2003
	Larva[a]	RPJ: Royal Project, Doi Inthanon NP, Chiang Mai	18°33′ N/98°31′ E	1400	November 2003
	Larva[a]	RPJ: Royal Project, Doi Inthanon NP, Chiang Mai	18°33′ N/98°31′ E	1400	November 2003
	Larva[a]	RPJ: Royal Project, Doi Inthanon NP, Chiang Mai	18°33′ N/98°31′ E	1400	November 2003
Simulium sp. nr. *Feuerborni* 1[b]	Pupa	KHW: Khun Wang, Doi Inthanon NP, Chiang Mai	18°37′ N/98°30′ E	1362	May 2003
Simulium sp. nr. *Feuerborni* 2[b]	Larva[a]	TBT: Thung Bua Tong FP, Mae Hong Son	18°53′ N/98°05′ E	1500	June 2003
ruficorne-group					
Simulium aureohirtum Brunetti	Larva	NGA: Ngao waterfall, Ranong	9°51′ N/98°37′ E	50	March 2000
	Larva	NGA: Ngao waterfall, Ranong	9°51′ N/98°37′ E	50	March 2000
vernum-group					
Simulium caudisclerum Takaoka and Davies	Pupa	AKH: Ang Kha, Doi Inthanon NP, Chiang Mai	18°35′ N/98°28′ E	2400	December 2002
Subgenus *Simulium*					
griseifrons-group					
Simulium choochotei Takaoka and Kuvangkadilok	Larva	MTT: Mon Tha Tarn waterfall, Chiang Mai	18°48′ N/98°55′ E	800	December 2003
	Pupa	MTT: Mon Tha Tarn waterfall, Chiang Mai	18°48′ N/98°55′ E	800	December 2003
Simulium grossifilum Takaoka and Davies	Larva	NGA: Ngao waterfall, Ranong	9°51′ N/98°37′ E	50	March 2000
Simulium nigrogilvum Summers	Larva	WKW: Wang Kwai waterfall, Doi Inthanon NP, Chiang Mai	18°30′ N/98°40′ E	421	October 2000
Simulium rudnicki Takaoka and Davies	Larva	MYA: Mae Ya waterfall, Doi Inthanon NP, Chiang Mai	18°20′ N/98°20′ E	500	February 1998
	Larva	MYA: Mae Ya waterfall, Doi Inthanon NP, Chiang Mai	18°20′ N/98°20′ E	500	June 2000
malyschevi-group					
Simulium siripoomense Takaoka and Saito	Larva	WKW: Wang Kwai waterfall, Doi Inthanon NP, Chiang Mai	18°30′ N/98°40′ E	421	October 2000

Table 1. (*continued*)

Species	Specimen	Collection sites	Latitude/Longitude	Altitude (m)	Collection date
multistriatum-group					
Simulium chainarongi Kuvangkadilok and Takaoka	Larva	KLD: Keng Lam Duan waterfall, Ubon Ratchathani	15°10′ N/105°12′ E	140	November 1998
Simulium chaliowae Takaoka and Boonkemtong	Larva	NKH: Na Ku Ha waterfall, Phrae	18°06′ N/100°18′ E	550	June 2003
	Pupa	NKH: Na Ku Ha waterfall, Phrae	18°06′ N/100°18′ E	550	June 2003
Simulium fenestratum Edwards	Male Adult	PHQ: Park Headquarters, Doi Inthanon NP, Chiang Mai	18°30′ N/98°33′ E	1250	September 1999
	Pupa	BPF: Ban Pang Fan, Chiang Mai	19°00′ N/99°18′ E	625	December 2002
Simulium triglobus Takaoka and Kuvangkadilok	Larva	TTO: Ton Tong waterfall, Nan	18°29′ N/100°30′ E	389	December 1998
nobile-group					
Simulium nobile de Meijere	Larva	BPA: Bang Pae, Phuket	98°15′ N/98°40′ E	30	August 1999
	Female Adult	KPO: Ka Po waterfall, Chumphon	10°44′ N/99°12′ E	40	November 2000
Simulium nodosum Puri	Female Adult	BPF: Ban Pang Fan, Chiang Mai	19°00′ N/99°18′ E	625	March 1999
striatum-group					
Simulium chiangmaiense Takaoka and Suzuki	Larva	BTY: Ban Thung Yao, Chiang Rai	19°11′ N/99°27′ E	654	March 1999
Simulium nakhonense Takaoka and Suzuki	Female Adult	MTU: Muang Tuad waterfall, Suratthani	8°45′ N/99°26′ E	120	December 2000
Simulium quinquestriatum Shiraki	Male Adult	MTU: Muang Tuad waterfall, Suratthani	8°45′ N/99°26′ E	120	December 2000
tuberosum-group					
Simulium brevipar Takaoka and Davies	Larva	STI: Sai Tip waterfall, Uttaradit	17°44′ N/100°59′ E	1615	October 1998
Simulium rufibasis Brunetti	Male Adult	KMP: Kiew Mae Pan waterfall, Doi Inthanon NP, Chiang Mai	18°33′ N/98°29′ E	2300	June 2000
Simulium tani Takaoka and Davies	Male Adult	LSA: Lan Sang waterfall: Tak	16°46′ N/99°01′ E	280	January 1999
	Female Adult	MTU: Muang Tuad waterfall, Suratthani	8°45′ N/99°26′ E	120	November 2000
Siulium weji Takaoka	Larva	TTH: Tan Thong waterfall, Lampang	19°04′ N/99°43′ E	700	December 2002
Simulium doipuiense[c] Takaoka and Choochote	Female Adult	AKH: Ang Kha, Doi Inthanon NP, Chiang Mai	18°35′ N/98°28′ E	2400	December 2000

CGCGGTGTGAACTGCAGGACACATG-3′) and CP16 (5′-GCGGGTACCATGCTTAAATTTAGGGGGTA-3′) (Porter and Collins, 1991). Polymerase chain reaction (PCR) was carried out in 0.5 ml Eppendorf tubes using 50 μl volumes containing 1× reaction buffer, 2.0 mM $MgCl_2$, 0.5 mM of each dNTP, 0.5 μM of each primer, 1.25 units (0.25 μl) of *Taq* DNA polymerase (Promega), and 1 μl of DNA sample. The temperature profile was as follows: 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min and final extension at 72°C for 10 min. PCR products were checked on a 2% agarose gel containing 0.5 μg/ml of ethidium bromide (Sambrook, Fritsch and Maniatis, 1989). The size of PCR products was compared with the molecular marker 1 kb Plus DNA Ladder^{TM} (GIBCO).

Cloning and sequencing

The PCR products were purified using a GENECLEAN II kit (Bio 101). The purified DNA fragments were cloned into the pGEM-T Easy Vector (Promega). At least two independent clones from each sample were sequenced on an ABI Prism automated sequencer (PE Applied Biosystems). Additional clones were sequenced in cases where polymorphisms were encountered, and the prevalent nucleotide is given in each case.

Data analysis

ITS2 sequences were aligned using Clustal X version 1.81 (Thompson et al., 1997). The Kimura two-parameter distance method (Kimura, 1980) was used to calculate nucleotide sequence differences and distances using MEGA version 2.1 (Kumar et al., 2001). DNA sequence-based phylogenetic analyses were performed using both PAUP version 4.0b10 (Swofford, 2002) for maximum parsimony and neighbor-joining analyses and MrBAYES version 3.0B4 (Huelsenbeck & Ronquist, 2001) for Bayesian analysis (maximum likelihood analysis). A maximum parsimony analysis was performed to find the most parsimonious trees. Heuristic parsimony searches (Hillis, Moritz and Mable, 1996) were performed using 100 replicates of random addition sequences and the tree-bisection-reconnection (TBR) option for branch swapping, and followed by additional rounds of branch swapping on the resulting trees

Simulium sp. nr. *rufibasis*	Larva	AKH: Ang Kha, Doi Inthanon NP, Chiang Mai	18°35′ N/98°28′ E	2400	December 2000
variegatum-group					
Simulium chamlongi Takaoka and Suzuki	Female Adult	SRP: Siri Phum waterfall, Doi Inthanon NP, Chiang Mai	18°32′ N/98°31′ E	1300	September 1999
Subgenus *Montisimulium*					
Simulium merga[d] Takaoka and Choochote	Larva	AKH: Ang Kha, Doi Inthanon NP, Chiang Mai	18°35′ N/98°28′ E	2400	December 2000
Unknown subgenus					
Simulium baimaii Kuvangkadilok and Takaoka	Pupa	THY: Tham Yai waterfall, Phu Kradung NP, Loei	16°52′ N/101°46′ E	1100	October 2002

[a] Cytologically identified specimens.

[b] *S.* sp. nr. *feuerborni* 1 and 2 are the same species collected from different sites.

[c] Formerly *S.* (*S.*) sp. E reported by Takaoka and Suzuki (1984).

[d] Formerly *S.* (*M.*) sp. G reported by Kuvangkadilok, Boonkemtong and Phayuhasena, (1998, 1999b).

with restriction on the number of trees to one. Each base was treated as an unordered character with equal weights, with gaps treated as missing data. Statistical support for the phylogeny produced was determined by bootstrap re-sampling of 1000 replicate data sets. In the neighbor-joining analysis, a phylogenetic tree was produced based on the calculated Kimura two-parameter distance method, with 1000 bootstrap replications. The sequences of *Phlebotomus perniciosus* (AF205526; Muccio et al., 2000), *Chironomus annularius* (AJ296770; Koepf et al., unpublished data), and *Drosophila yakuba* (Z28416; Schlötterer et al., 1994) were used as outgroups for maximum parsimony and neighbor-joining analyses. For the Bayesian analysis, each run was performed using default starting parameters and comprised 2,000,000 generations. Bayesian posterior probabilities (P_{bay}) were calculated from majority-rule consensus of trees sampled every 100 generations once the Markov chain became stationary. The sequence of *Phlebotomus perniciosus* (AF205526; Muccio et al., 2000) was used as an outgroup for this analysis.

Results

DNA sequence analysis

The ITS2 sequences of 40 available Thai black fly species were PCR amplified using primers within the 5.8S and 28S coding genes. The remaining 25 species were not available at the time of the study and so, were excluded. The approximate boundaries of the ITS2 were defined by comparison with the 5.8S and 28S rDNA sequences of *Simulium vittatum* (U48383) (Miller, Crabtree and Savage, 1997). The length of the ITS2 of 40 black fly species ranged from 247 bp in *Simulium* (*G*) *inthanonense* to 308 bp in *S.* (*S.*) *choochotei.* The average percentages of base composition for the ITS2 sequence were (range in parentheses): A, 37.7% (34.5–40.6%); T, 39.1% (34.1–44.9%); G, 12.4% (8.3–15.9%); and C, 10.8% (7.7–14.4%). The ITS2 region of all species was AT rich, with a range of 71–83.8% (Table 2).

Intraspecific variation

Clonal variation within individuals was examined in 13 species belonging to the subgenera *Gomphostilbia* (6 species), *Nevermannia* (2 species), and *Simulium* (5 species). The sequence differences of all examined clones were due to base substitutions (transversions/transitions) and insertions and deletions of one, two, or three bases. In the subgenus *Gomphostilbia*, the ITS2 variability of the clones within individuals of three species in the *S. batoense*-group (*S. angulistylum*, *S. decuplum*, and *S. parahiyungum*) and three species in the *S. ceylonicum*-group (*S. asakoae*, *S. sheilae*, and *S.* sp. nr. *sheilae*) ranged from 0.3 to 0.7%. For the subgenus *Nevermannia*, clonal variation of *S. feuerborni* and *S. aureohirtum* was 0.7% and 1.1%, respectively. Five species of four species groups in the subgenus *Simulium*, i.e., *S. siripoomense* (*S. malyschevi*-group), *S. fenestratum* and *S. triglobus* (*S. multistriatum*-group), *S. nakhonense* (*S. striatum*-group), and *S. tani* (*S. tuberosum*-group), had ITS2 clonal variation, with a range of 0.4–0.8%.

Intrapopulation variation was found in *S. feuerborni* larvae from the Royal Project, Doi Inthanon National Park, Chiang Mai Province, with four different ITS2 spacer types being present. The ITS2 lengths of five individual larvae (larval numbers 1–5) varied from 273 bp in *S. feuerborni* 3 to 281 bp in *S. feuerborni* 1, 2, 4, and 5. Differences in sequences and lengths among four sequences occurred in two regions (data not shown). The most variable region was in the base, ranging from 23 to 40 due to three transitions (at positions 23, 27, and 30), six transversions (at positions 26, 32, 38, 39, and 40), four insertions/deletions (at positions 31, 33, 34, and 35) and two base repeats (TT) at positions 28 and 29. The second variable region occurred at positions 218 and 239 due to transitions and at positions 260–261 with 2 insertions/deletions. The intrapopulation variation among individuals of *S. feuerborni* from the Royal Project ranged from 0 to 2.2%.

In addition to intrapopulation variation, *S. feuerborni* collected from two near locations at Doi Inthanon National Park, Chiang Mai Province, namely the Royal Project and Huai Sai Luaeng waterfall, also had ITS2 sequence variations. Interpopulation variation in the ITS2 sequences among 12 clones of two *S. feuerborni* populations from the Royal Project and Huai Sai Luaeng waterfall (18 km apart) was large and confined to 20 positions (Figure 1): six transitions at positions 23, 27, 30, 218, 224, and 240; six

Table 2. The length and percent base composition of second internal transcribed spacer of forty black flies in the subgenera *Gomphostilbia*, *Nevermannia*, *Montisimulium*, and *Simulium* and an unknown subgenus

Species	Sequence length	Adenine (%)	Thymine (%)	Guanine (%)	Cytosine (%)	%AT
Subgenus *Gomphostilbia*						
batoense-group						
1. *S*. (*G*.) *angulistylum*	286	35.7	38.1	15.7	10.5	73.8
2. *S*. (*G*.) *decuplum*	294	38.1	37.1	13.9	10.9	75.2
3. *S*. (*G*.) *duolongum*	274	39.1	34.7	15.0	11.3	73.8
4. *S*. (*G*.) *gombakense*	251	36.7	34.3	15.9	13.1	71.0
5. *S*. (*G*.) *parahiyangum*	279	36.6	38.0	15.1	10.4	74.6
6. *S*. (*G*.) *siamense*	256	36.7	35.9	15.6	11.7	72.6
ceylonicum-group						
1. *S*. (*G*.) *asakoae*	249	40.6	35.3	12.4	11.6	75.9
2. *S*. (*G*.) *inthanonense*	247	37.7	35.6	14.6	12.1	73.3
3. *S*. (*G*.) *sheilae*	266	40.2	37.2	12.8	9.8	77.4
4. *S*. (*G*.) sp. nr. *sheilae*	266	40.2	38.0	12.4	9.4	78.2
varicorne-group						
1. *S*. (*G*.) *burtoni*	249	39.0	35.3	14.5	11.2	74.3
2. *S*. (*G*.) *chumpornense*	292	38.0	36.0	15.1	11.0	74.0
Subgenus *Nevermannia*						
feuerborni-group[a]						
1. *S*. (*N*.) *feuerborni* (HSL)	282	36.5	36.2	14.5	12.8	72.7
2. *S*. (*N*.) *feuerborni* 1 (RPJ)	281	37.0	35.6	14.2	13.2	72.6
3. *S*. (*N*.) *feuerborni* 2 (RPJ)	281	37.0	35.6	14.2	13.2	72.6
4. *S*. (*N*.) *feuerborni* 4 (RPJ)	281	35.6	37.4	14.6	12.5	73.0
5. *S*. (*N*.) *feuerborni* 5 (RPJ)	281	36.7	35.2	14.6	13.5	71.9
6. *S*. (*N*.) *feuerborni* 3 (RPJ)	273	35.9	35.9	15.0	13.2	71.8
7. *S*. (*N*.) sp. nr. *feuerborni* 1	276	36.6	36.6	14.5	12.3	73.2
8. *S*. (*N*.) sp. nr. *feuerborni* 2	286	37.8	35.7	13.6	12.9	73.5
ruficorne-group						
1. *S*. (*N*.) *aureohirtum*	266	36.1	36.5	14.7	12.8	72.6
vernum-group						
1. *S*. (*N*.) *caudisclerum*	270	37.4	34.1	14.1	14.4	71.5
Subgenus *Simulium*						
griseifrons-group						
1. *S*. (*S*.) *choochotei*	308	38.3	43.2	9.1	9.4	81.5
2. *S*. (*S*.) *grossifilum*	290	37.2	41.4	11.4	10.0	78.6
3. *S*. (*S*.) *nigrogilvum*	277	37.9	41.5	10.5	10.1	79.4
4. *S*. (*S*.) *rudnicki*	281	37.7	40.9	11.0	10.3	78.6
malyschevi-group						
1. *S*. (*S*.) *siripoomense*	271	38.7	38.4	11.8	11.1	77.1
multistriatum-group						
1. *S*. (*S*.) *chainarongi*	283	38.5	44.9	8.8	7.8	83.4
2. *S*. (*S*.) *chaliowae*	274	39.4	43.8	8.8	8.0	83.2
3. *S*. (*S*.) *fenestratum*	271	40.6	43.2	8.5	7.7	83.8
4. *S*. (*S*.) *triglobus*	278	38.5	45.3	8.3	7.9	83.8
nobile-group						

Table 2. (*continued*)

Species	Sequence length	Adenine (%)	Thymine (%)	Guanine (%)	Cytosine (%)	%AT
1. *S.* (*S.*) *nobile*	264	36.4	39.8	12.9	11.0	76.2
2. *S.* (*S.*) *nodosum*	278	36.0	42.4	11.5	10.1	78.4
striatum-group						
1. *S.* (*S.*) *chiangmaiense*	262	39.7	41.6	9.5	9.2	81.3
2. *S.* (*S.*) *nakhonense*	267	39.7	42.7	9.0	8.6	82.4
3. *S.* (*S.*) *quinquestriatum*	260	39.2	41.2	10.4	9.2	80.4
tuberosum-group						
1. *S.* (*S.*) *brevipar*	269	39.4	42.4	8.9	9.3	81.8
2. *S.* (*S.*) *rufibasis*	261	36.4	43.7	10.7	9.2	80.1
3. *S.* (*S.*) *tani*	271	36.5	44.6	10.0	8.9	81.1
4. *S.* (*S.*) *weji*	287	36.6	44.3	10.1	9.1	80.9
5. *S.* (*S.*) *doipuiense*	259	34.7	43.6	11.6	10.0	78.3
6. *S.* (*S.*) sp. nr. *rufibasis*	261	34.5	44.4	11.9	9.2	78.9
variegatum-group						
1. *S.* (*S.*) *chamlongi*	281	39.1	37.0	11.4	12.5	76.1
Subgenus *Montisimulium*						
1. *S.* (*M.*) *merga*	268	38.8	35.8	12.3	13.1	74.6
Unknown subgenus						
1. *S. baimaii*	291	38.8	39.5	11.3	10.3	78.3
Average	274.1	37.6	38.8	12.6	11.0	76.4

[a] *feuerborni*-group, six *S. feuerborni* larvae, one larva from Hui Sai Luaeng waterfall (HSL) and five larvae (larval numbers 1–5) from Royal Project (RPJ). *S.* sp. nr. *feuerborni* 1 and 2 are the same species collected from different sites.

transversions at positions 26, 32, and 38 (two different transversions), 39, and 40; seven insertions/deletions at positions 31, 33, 34, 35, 238, 260, and 261 and two base repeats (TT) at positions 28 and 29. Pairwise sequence comparisons of all clones ranged from 1.5 to 2.6%.

Interspecific variation

To compare the nucleotide sequence differences among the 40 *Simulium* species, pairwise sequence divergences among these species were calculated by using the Kimura two-parameter method (Table 3). The average pairwise nucleotide difference among 12 black fly species within the subgenus *Gomphostilbia* was 17.8%, ranging from 1 (between *S. siamense* and *S. gombakense*, *S. sheilae* and *S.* sp. nr. *sheilae*) to 26 (between *S. parahiyangum* and *S. asakoae*). For the subgenus *Nevermannia*, the pairwise nucleotide difference among 5 species was 9.8, ranging from 3 (between *S. feuerborni* and *S.* sp. nr. *feuerborni* 1, *S. feuerborni* and *S.* sp. nr. *feuerborni* 2) to 14 (between *S. feuerborni* 1 and *S. caudisclerum*). Among 21 black fly species in the subgenus *Simulium*, the mean pairwise nucleotide difference was 13.9, ranging from 1 (between *S. rudnicki* and *S. grossifilum*) to 23 (between *S. nodosum* and *S. rufibasis*, *S. nodosum* and *S. tani*, *S. nobile* and *S. brevipar*). Moreover, the pairwise nucleotide differences between *S. baimaii* (subgenus unknown) and the members of the subgenera *Gomphostilbia*, *Nevermannia*, and *Simulium* ranged from 24 to 29, 18 to 21, and 2 to 22, respectively.

Figure 1. Alignment of the internal transcribed spacer II region of twelve clones from six *Simulium feuerborni* larvae collected from Royal Project (RPJ) and Hui Sai Luaeng waterfall (HSL), Doi Inthanon National Park, Chiang Mai. A dot represents agreement with the concensus at that position and dashes indicate alignment gaps.

```
RPJ1L1^a     ATTTATCAATAGAACTGTTCTTTCATC--GAAAGATGCAAGACGCACGTGTCAACTATAA 58
RPJ1L2^a     ...........................--............................... 58
RPJ2L1^a     ...........................--............................... 58
RPJ2L2^a     ...........................--............................... 58
RPJ3L1^a     ......................C..AT--------..G...................... 52
RPJ3L2^a     ......................C..AT--------..G...................... 52
RPJ4L1^a     ......................C....--............................... 58
RPJ4L2^a     ......................C....--............................... 58
RPJ5L1^a     ..........................TTTA.T.....ATT.................... 60
RPJ5L2^a     ..........................TTTA.T.....ATT.................... 60
HSLL1^b      ...........................--........AT..................... 58
HSLL2^b      ...........................--........AT..................... 58
             ********************** **          **   ********************

RPJ1L1       GACTAGCTTTAATCGGCCAGACTTATATTTGATTAAATCAAGAGATGTGAAAAATCAAAT 118
RPJ1L2       ............................................................ 118
RPJ2L1       ............................................................ 118
RPJ2L2       ............................................................ 118
RPJ3L1       ............................................................ 112
RPJ3L2       ............................................................ 112
RPJ4L1       ............................................................ 118
RPJ4L2       ............................................................ 118
RPJ5L1       ............................................................ 120
RPJ5L2       ............................................................ 120
HSLL1        ............................................................ 118
HSLL2        ............................................................ 118
             ************************************************************

RPJ1L1       ACAGTTAACACATATCAATTTGATTGTGAAAGCCTATTTAATATTTTATATGAAACTATC 178
RPJ1L2       ............................................................ 178
RPJ2L1       ............................................................ 178
RPJ2L2       ............................................................ 178
RPJ3L1       ............................................................ 172
RPJ3L2       ............................................................ 172
RPJ4L1       ............................................................ 178
RPJ4L2       ............................................................ 178
RPJ5L1       ............................................................ 180
RPJ5L2       ............................................................ 180
HSLL1        ............................................................ 178
HSLL2        ............................................................ 178
             ************************************************************

RPJ1L1       ATTTCTTTGTAAATTGTATATGTAGATTATAAAACATAATATTGAATTTGGGCGATC-GA 237
RPJ1L2       .........................................................-.. 237
RPJ2L1       .........................................................-.. 237
RPJ2L2       .........................................................-.. 237
RPJ3L1       .....................................G...................-.G 231
RPJ3L2       .....................................G...................-.G 231
RPJ4L1       .........................................................-.. 237
RPJ4L2       .........................................................-.. 237
RPJ5L1       .....................................G...................-.G 239
RPJ5L2       .....................................G...................-.G 239
HSLL1        .....................................G.....A.............T.. 238
HSLL2        .....................................G.....A.............T.. 238
             ************************************* ***** ************* *

RPJ1L1       TCAATTTGATTCGACTGCATATTTGGATACTATAAATTATACAT 281
RPJ1L2       ............................................ 281
RPJ2L1       ............................................ 281
RPJ2L2       ............................................ 281
RPJ3L1       ...................--....................... 273
RPJ3L2       ...................--....................... 273
RPJ4L1       ............................................ 281
RPJ4L2       ............................................ 281
RPJ5L1       ...................--....................... 281
RPJ5L2       ...................--....................... 281
HSLL1        ............................................ 282
HSLL2        ............................................ 282
             *******************  ***********************
```

[a] ten clones from five RPJ larvae (two clones from each larva).

[b] two clones from one HSL larva.

Table 3. Kimura's two-parameter distances (above diagonal) and pairwise nucleotide differences (below diagonal) among 40 blackfly species and 3 outgroups species

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *S. parahiyangum*		0.20	0.16	0.18	0.12	0.21	0.16	0.17	0.25	0.24	0.16	0.18	0.24	0.24	0.23	0.23	0.20	0.23	0.26
2. *S. siamense*	21		0.19	0.20	0.13	0.01	0.15	0.16	0.20	0.12	0.19	0.10	0.18	0.22	0.17	0.20	0.21	0.22	0.25
3. *S. aungulistylum*	18	21		0.05	0.12	0.21	0.22	0.23	0.23	0.22	0.09	0.17	0.16	0.17	0.16	0.15	0.15	0.15	0.23
4. *S. decuplum*	20	21	6		0.12	0.21	0.22	0.23	0.22	0.20	0.09	0.17	0.15	0.16	0.16	0.15	0.17	0.13	0.23
5. *S. duolongum*	14	14	14	14		0.14	0.15	0.16	0.19	0.17	0.12	0.10	0.18	0.18	0.19	0.20	0.17	0.21	0.24
6. *S. gombakense*	22	1	22	22	15		0.16	0.17	0.20	0.12	0.20	0.10	0.20	0.23	0.18	0.21	0.22	0.22	0.26
7. *S.* sp. nr. *sheilae*	18	17	23	23	17	18		0.01	0.20	0.13	0.17	0.11	0.22	0.22	0.19	0.20	0.16	0.18	0.26
8. *S. sheilae*	19	18	24	24	18	19	1		0.22	0.14	0.18	0.12	0.23	0.23	0.20	0.21	0.17	0.19	0.27
9. *S. asakoae*	26	22	24	23	21	22	22	23		0.15	0.23	0.16	0.26	0.24	0.28	0.24	0.24	0.20	0.32
10. *S. inthanonense*	25	14	23	22	19	14	15	16	17		0.17	0.11	0.17	0.20	0.20	0.15	0.15	0.18	0.25
11. *S. chumpornense*	18	20	10	10	14	21	19	20	24	19		0.14	0.16	0.17	0.17	0.13	0.12	0.13	0.22
12. *S. burtoni*	20	11	19	19	11	12	13	14	18	13	16		0.20	0.20	0.20	0.17	0.16	0.17	0.24
13. *S. feuerborni*	25	20	18	17	19	21	23	24	27	19	18	21		0.02	0.02	0.11	0.11	0.15	0.19
14. *S.* sp.nr.*feuerborni*1	25	23	19	18	19	24	23	24	25	21	19	21	3		0.05	0.12	0.11	0.15	0.23
15. *S.* sp.nr.*feuerborni*2	24	19	18	18	20	20	21	22	29	21	19	21	3	6		0.11	0.11	0.14	0.19
16. *S. caudisclerum*	24	21	17	16	21	22	21	22	25	17	14	19	12	14	13		0.07	0.10	0.19
17. *S. aureohirtum*	22	22	17	19	19	23	18	19	25	17	14	18	13	13	13	8		0.13	0.19
18. *S. merga*	24	23	17	15	22	23	20	21	22	20	15	19	17	17	16	12	15		0.20
19. *S. fenestratum*	27	26	24	24	25	27	27	28	32	26	23	25	21	24	21	21	21	22	
20. *S. chaliowae*	25	24	22	22	23	25	25	26	30	24	21	23	19	22	19	19	19	20	2
21. *S. triglobus*	29	28	26	26	27	29	29	30	34	28	25	27	23	26	23	23	23	24	2
22. *S. chainarongi*	26	25	22	23	24	26	26	27	31	25	22	24	20	23	20	20	19	19	5
23. *S. nodosum*	29	30	30	30	28	31	26	27	29	27	26	30	22	23	24	23	22	24	18
24. *S. nobile*	32	32	29	29	29	33	28	29	31	29	25	30	25	26	27	26	25	22	17
25. *S. chiangmaiense*	26	26	21	20	25	27	19	20	29	24	21	24	19	20	17	19	16	15	14
26. *S. nakhonense*	29	28	24	23	26	29	22	23	31	27	22	26	23	24	21	23	18	18	15
27. *S. quinquestriatum*	29	25	24	23	27	26	21	22	30	24	23	25	21	24	19	21	19	18	14
28. *S. chamlongi*	25	25	26	25	24	26	23	24	27	22	22	25	17	18	19	16	17	21	14
29. *S . siripoomense*	24	26	25	24	23	27	22	23	26	23	21	24	18	17	20	17	16	20	15
30. *S.* sp. nr. *rufibasis*	24	27	23	25	25	28	28	29	34	29	23	30	22	25	20	23	21	23	14
31. *S. rufibasis*	24	28	23	25	25	29	30	31	34	31	23	30	24	27	22	25	23	23	12
32. *S. doipuiense*	23	27	22	24	24	28	29	30	33	30	22	29	23	26	21	24	22	22	14
33. *S. weji*	23	29	21	23	26	30	28	29	30	32	23	29	23	24	21	22	22	19	14
34. *S. tani*	24	28	22	24	25	29	28	29	30	31	22	28	25	26	23	24	22	20	14
35. *S. brevipar*	26	28	25	24	28	29	28	29	33	30	27	31	22	25	20	21	23	22	17
36. *S. rudnicki*	25	26	26	25	23	27	23	24	30	25	24	25	20	22	20	18	18	23	12
37. *S. grossifilum*	26	27	27	26	24	28	24	25	31	26	25	26	21	23	21	19	19	24	13
38. *S. choochotei*	25	25	22	22	24	26	21	22	28	25	21	24	20	21	18	18	18	17	12
39. *S. nigrogilvum*	25	24	24	23	23	25	23	24	27	22	21	25	16	19	18	16	17	21	10
40. *S. baimaii*	25	27	27	27	26	28	25	26	29	24	22	27	19	20	21	18	19	23	16
41. *C. annularius*	68	68	67	68	64	69	64	65	70	69	68	66	68	68	66	67	65	68	64
42. *P. perniciosus*	69	65	64	64	66	66	65	66	67	66	63	65	67	66	65	61	58	66	64
43. *D. yakuba*	66	68	61	65	66	69	65	65	70	69	64	69	69	68	68	67	66	68	62

All insertions/deletions removed from the data.

20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
0.24	0.28	0.25	0.28	0.32	0.25	0.28	0.28	0.24	0.23	0.23	0.23	0.22	0.22	0.23	0.25	0.24	0.25	0.24	0.24	0.24	1.01	1.06	0.96
0.23	0.27	0.24	0.30	0.32	0.25	0.27	0.24	0.24	0.25	0.26	0.28	0.26	0.28	0.28	0.27	0.25	0.26	0.24	0.23	0.26	1.03	0.92	1.02
0.20	0.25	0.20	0.30	0.28	0.19	0.23	0.23	0.25	0.24	0.22	0.22	0.20	0.19	0.20	0.24	0.25	0.26	0.20	0.23	0.26	1.00	0.89	0.81
0.20	0.25	0.22	0.30	0.28	0.18	0.22	0.22	0.24	0.23	0.24	0.24	0.23	0.22	0.23	0.23	0.24	0.25	0.20	0.22	0.26	1.01	0.90	0.92
0.22	0.26	0.23	0.27	0.29	0.24	0.25	0.27	0.23	0.22	0.24	0.24	0.23	0.25	0.24	0.27	0.22	0.23	0.23	0.22	0.25	0.92	0.96	0.95
0.24	0.29	0.25	0.31	0.34	0.26	0.29	0.25	0.25	0.26	0.27	0.29	0.27	0.30	0.29	0.28	0.26	0.27	0.25	0.24	0.27	1.07	0.95	1.06
0.24	0.28	0.25	0.25	0.27	0.17	0.21	0.20	0.22	0.20	0.27	0.30	0.28	0.27	0.27	0.27	0.22	0.23	0.20	0.22	0.24	0.90	0.92	0.92
0.25	0.30	0.26	0.26	0.28	0.18	0.22	0.21	0.23	0.22	0.28	0.31	0.30	0.28	0.28	0.28	0.23	0.24	0.21	0.23	0.25	0.92	0.95	0.92
0.30	0.35	0.31	0.28	0.31	0.28	0.31	0.30	0.26	0.25	0.35	0.35	0.33	0.30	0.30	0.33	0.30	0.31	0.27	0.26	0.28	1.08	0.98	1.07
0.23	0.27	0.24	0.26	0.28	0.23	0.26	0.23	0.20	0.22	0.29	0.31	0.30	0.32	0.31	0.30	0.24	0.25	0.24	0.20	0.23	1.07	0.96	1.08
0.19	0.24	0.20	0.25	0.24	0.19	0.20	0.22	0.20	0.19	0.22	0.22	0.20	0.22	0.21	0.26	0.23	0.24	0.19	0.19	0.20	1.04	0.87	0.89
0.22	0.26	0.23	0.30	0.30	0.23	0.25	0.24	0.24	0.23	0.30	0.30	0.28	0.28	0.27	0.31	0.24	0.25	0.23	0.24	0.26	0.96	0.92	1.04
0.17	0.22	0.18	0.20	0.24	0.17	0.22	0.19	0.15	0.16	0.20	0.23	0.22	0.22	0.24	0.20	0.18	0.19	0.18	0.14	0.17	1.04	0.99	1.04
0.20	0.25	0.22	0.22	0.25	0.18	0.23	0.23	0.16	0.15	0.24	0.26	0.25	0.23	0.25	0.24	0.21	0.22	0.20	0.17	0.18	1.05	0.95	1.00
0.17	0.22	0.18	0.23	0.26	0.15	0.19	0.17	0.17	0.18	0.18	0.20	0.19	0.19	0.22	0.18	0.18	0.19	0.16	0.16	0.19	0.97	0.92	1.00
0.17	0.22	0.18	0.22	0.25	0.17	0.22	0.19	0.14	0.15	0.22	0.24	0.23	0.20	0.23	0.19	0.16	0.17	0.16	0.14	0.16	1.00	0.81	0.98
0.17	0.22	0.17	0.20	0.24	0.14	0.16	0.17	0.15	0.14	0.19	0.22	0.20	0.20	0.20	0.22	0.16	0.17	0.16	0.15	0.17	0.92	0.75	0.95
0.18	0.23	0.17	0.23	0.20	0.13	0.16	0.16	0.19	0.18	0.22	0.22	0.20	0.17	0.18	0.20	0.22	0.23	0.15	0.19	0.22	1.01	0.95	1.01
0.02	0.02	0.04	0.16	0.15	0.12	0.13	0.12	0.12	0.13	0.12	0.10	0.12	0.12	0.12	0.15	0.10	0.11	0.11	0.09	0.14	0.89	0.90	0.84
	0.03	0.02	0.14	0.13	0.10	0.11	0.11	0.10	0.11	0.10	0.12	0.10	0.10	0.12	0.13	0.09	0.10	0.09	0.07	0.12	0.92	0.84	0.84
4		0.06	0.17	0.17	0.14	0.15	0.14	0.14	0.15	0.12	0.10	0.12	0.12	0.12	0.15	0.11	0.12	0.12	0.10	0.16	0.89	0.94	0.84
3	7		0.13	0.12	0.08	0.09	0.08	0.11	0.12	0.11	0.13	0.10	0.11	0.13	0.14	0.10	0.10	0.10	0.08	0.13	0.94	0.87	0.88
16	19	15		0.10	0.16	0.20	0.18	0.10	0.11	0.17	0.22	0.18	0.18	0.22	0.19	0.11	0.12	0.18	0.10	0.10	1.05	0.90	1.04
15	19	14	12		0.17	0.17	0.16	0.13	0.14	0.17	0.19	0.16	0.18	0.19	0.22	0.16	0.17	0.18	0.13	0.13	1.17	1.07	0.95
12	16	9	18	19		0.03	0.03	0.12	0.11	0.14	0.16	0.15	0.13	0.14	0.16	0.11	0.12	0.07	0.10	0.14	0.92	0.86	0.96
13	17	10	22	19	4		0.03	0.14	0.13	0.14	0.15	0.14	0.14	0.13	0.18	0.13	0.14	0.10	0.12	0.16	0.89	0.87	0.90
12	16	9	20	18	4	4		0.13	0.14	0.14	0.15	0.14	0.15	0.15	0.17	0.12	0.13	0.10	0.11	0.15	0.95	0.92	0.96
12	16	13	12	15	14	16	15		0.01	0.16	0.18	0.17	0.16	0.19	0.15	0.06	0.07	0.11	0.03	0.02	0.94	0.84	0.97
13	17	14	13	16	13	15	16	1		0.17	0.19	0.18	0.15	0.18	0.16	0.07	0.08	0.11	0.04	0.02	0.91	0.82	0.94
12	14	13	19	19	16	16	16	18	19		0.03	0.02	0.08	0.07	0.08	0.13	0.14	0.14	0.14	0.17	0.89	0.80	0.80
14	12	15	23	21	18	17	17	20	21	4		0.03	0.08	0.05	0.11	0.17	0.18	0.16	0.16	0.19	0.89	0.91	0.81
12	14	12	20	18	17	16	16	19	20	2	4		0.08	0.07	0.10	0.14	0.15	0.15	0.15	0.18	0.92	0.82	0.79
12	14	13	20	20	15	16	17	18	17	9	9	9		0.05	0.10	0.15	0.16	0.11	0.12	0.17	0.99	0.92	0.82
14	14	15	23	21	16	15	17	21	20	8	6	8	6		0.11	0.18	0.19	0.12	0.17	0.20	0.89	0.88	0.78
15	17	16	21	23	18	20	19	17	18	9	13	11	12	13		0.16	0.17	0.15	0.17	0.17	0.84	0.76	0.74
10	13	11	13	18	13	15	14	7	8	15	19	16	17	20	17		0.01	0.11	0.05	0.08	0.95	0.81	0.96
11	14	12	14	19	14	16	15	8	9	16	20	17	18	21	18	1		0.12	0.06	0.09	0.95	0.84	0.99
10	14	11	20	20	8	11	11	13	12	16	18	17	13	14	17	13	14		0.10	0.13	0.84	0.82	0.88
8	12	9	12	15	12	14	13	4	5	16	18	17	14	19	19	6	7	11		0.05	1.02	0.87	0.99
14	18	15	12	15	16	18	17	2	3	19	21	20	19	22	19	9	10	15	6		0.97	0.90	1.00
65	64	66	69	72	65	64	66	65	64	64	64	65	67	64	62	66	66	62	68	66		1.09	1.32
62	65	63	64	69	63	63	65	62	61	60	64	61	65	63	58	61	62	61	63	64	69		0.86
62	62	63	68	65	66	64	66	66	65	59	60	59	61	59	57	66	67	63	67	67	75	60	

Phylogenetic analysis

The ITS2 sequences of 40 black fly species were aligned (Figure 2). The alignment of the ITS2 sequences resulted in a total 430 characters, including gaps. The sequences were deposited in GenBank under Accession numbers DQ 098997 to DQ 099036 and DQ 126003 to DQ 126006.

The phylogenetic trees of the 40 black flies, resulting from PAUP and MrBAYES analyses, are presented in Figures 3–5. The topology of the trees is quite similar. All trees showed two major monophyletic clades. Clade I consisted of all species belonging to the subgenus *Simulium* and *S. baimaii* (subgenus unknown), whereas the members of the subgenera *Gomphostilbia*, *Nevermannia*, and *Montisimulium* were placed in clade II. In the MP and NJ trees (Figures 3 and 4), clade I formed two groups in which the first group consisted of the *multistriatum*-group, *striatum*-group, and *S. choochotei* (the *griseifrons*-group), and it was a sister group to the clade of the *tuberosum*-group with a high bootstrap (99%) in the NJ tree but a weakly supported bootstrap (50%) in the MP tree. The monophyletic *tuberosum*-group consisted of six closely related species, namely *S.* sp. nr. *rufibasis*, *S. rufibasis*, *S. doipuiense*, *S. brevipar*, *S. weji* and *S. tani*. The second group consisted of the *malyschevi*-group, *variegatum*-group, *griseifrons*-group (*S. nigrogilvum*, *S. rudnicki*, and *S. grossifilum*), *nobile*-group, and *S. baimaii* (subgenus unknown). In contrast to the MP and NJ trees, *S. choochotei* (*griseifrons*-group) was placed as the sister taxon to the other species groups in the subgenus *Simulium* in the ML tree (Figure 5). The ML tree produced a trichotomy for the *tuberrosum*-group, *malyschevi*-group, *variegatum*-group, *nobile*-group, and *griseifrons*-group (except for *S. choochotei*) in the first group of clade I. On the other hand, the second group is composed of the *multistriatum* and *striatum*-groups. All trees indicated that *S. baimaii* is closely related to *S. siripoomense* (*malyschevi*-group) and *S. chamlongi* (*variegatum*-group), with high bootstrap values, and that the two species in the *griseifrons*-group (*S. rudnicki* and *S. grossifilum*) are closely related. Additionally, the *tuberosum* and *multistriatum*-groups formed monophyletic lineages in all trees.

In clade II, *S. merga* belonging to the subgenus *Montisimulium*, was placed in the same clade as subgenus *Nevermannia*, with a high bootstrap value of 99% in the NJ tree. The *S. merga–Nevermannia* clade is the sister group of the subgenus *Gomphostilbia*, with a strongly supported bootstrap in the MP tree (95%) and the ML tree (0.97) but weakly supported bootstrap (50%) in the NJ tree. Although the phylogenetic relationships among species in the *batoense*-group of the subgenus *Gomphostilbia* are unresolved in all trees, all three trees supported the close relationship between *S. siamense* and *S. gombakense* and between *S. angulistylum* and *S. decuplum*, with high bootstrap values. Moreover, in all trees the *ceylonicum*-group formed a monophyletic lineage and is a sister group to the *batoense–varicorne* clade, with strongly supported values in the MP (88%), the NJ (99%), and the ML trees (1.00). The *ceylonicum*-group consisted of four species, with close relationships between *S.* sp. nr. *sheilae* and *S. sheilae* and between *S. asakoae* and *S. inthanonense*. Additionally, all trees indicated a close relationship between the *varicorne* and *batoense*-groups because two species in the *varicorne*-group (i.e., *S. chumpornense* and *S. burtoni*) were placed in the clade of the *batoense*-group.

Discussion

The ITS2 sequences of the black flies in this study vary in length from 247 in *S.* (*G.*) *inthanonense* to 308 bp in *S.* (*S.*) *choochotei*, which are similar to the ITS2 sequences of other black flies and mosquitoes such as *Simulium vittatum* (259 bp) (Miller, Crabtree & Savage, 1997), *An. quadrimaculatus* complex (287–329 bp) (Cornel, Porter and Collins, 1996), *An. maculipennis* complex (280–312 bp) (Marinucci et al., 1999; Proft, Maier & Kampen, 1999), *An. hermsi* (305 bp), *An. occidentalis* (306 bp), and *An. freeborni* (310 bp) (Porter & Collins, 1991), and *Cx. pipiens* (297 bp) and *Cx. quinquefasciatus* (298 bp) (Severini et al., 1996). The ITS2 sequence of all black flies had a high AT content (71–83.8%). These percentages are similar to values observed in black flies and other insects such as *S. vittatum* (77.6%) and *Culicoides variipennis* (74.5%) (Miller, Crabtree & Savage, 1997), *Drosophila melanogaster* (80%) (Tautz et al., 1988), *D. yakuba* (79.1%), *D. simulans* (79.4%), and *Musca domestica* (74.9%) (Schlötterer et al., 1994), and *Phlebotomus perniciosus* (75.9%) (Muccio et al., 2000). In contrast, a high GC content was found in other anopheline and culicine mosquito species such as *An. dirus* A (69%) (Xu & Qu, 1997), *Ae. albopictus* (56.4%) (Wesson, Porter & Collins, 1992), and *Cx. pipiens* (58%) and *Cx. quinquefasciatus* (58%) (Severini et al., 1996), as well as in the dragonfly genus *Calopteryx* (68%) (Weekers, De Jonckheere & Dumont, 2001).

Variation between copies of the ITS2 within individuals was found in 13 species of three subgenera: *Gomphostilbia* (6 species), *Nevermannia* (2 species), and *Simulium* (5 species). Intraindividual variation of the ITS2 of all examined species, except *S. aureohirtum* (1.1%), was minimal ranging from 0.3 to 0.8%, as reported in some anopheline mosquitoes (*An. freeborni* and *An. hermsi*) (Porter and Collins, 1991), *An. gambiae* complex (Paskewitz, Wesson & Collins, 1993; Scott, Brogdon & Collins, 1993), *An. nuneztovari* (Fritz et al., 1994), *An. darlingi* (Malafronte, Marrelli & Marinotti, 1999), and *An. maculipennis* complex (Marinucci et al., 1999), as well as in many species of fruit flies (Schlötterer et al., 1994), some scallop species, *M. varia* and *P. maximus* (Insua et al., 2003), and frogs (Hillis & Davis, 1986). The high level of spacer variation was detected within populations (up to 2.2%) and between populations (up to 2.6%) of *S. feuerborni* even though the species identification was confirmed by cytological analysis prior to the molecular analysis. These results indicate that a large degree of intraspecific variability exits in the ITS2 of *S. feuerborni*. Similarly, the high levels of nucleotide diversity of the mitochondrial COI sequences within the populations of *S. tani*, *S. nakhonense*, and *S. quinquestriatum* collected from northern Thailand were 1.6, 1.7, and 1.8%, respectively (Pramual et al., 2005; Pramual et al., unpublished data). Five larvae from the Royal Project had four different sequences, with a range of 0–2.2%. In addition, comparison of ITS2 sequences between the Royal Project and Huai Sai Luaeng populations showed a high level of divergence ranging from 1.5 to 2.6%, although these locations are about 18 km apart in Doi Inthanon National Park. In contrast, intraspecific polymorphisms of the ITS2 sequence between populations of *An. nuneztovari* thousands of kilometers apart are small and are confined to only three regions of single-base repeats and simple repeat motifs (Fritz et al., 1994). The possibility of there being more than one locus of the rDNA transcription unit in *S. feuerborni*, as found in *Drosophila melanogaster* (on the X and Y chromosomes) (Yagura, Yagura & Muramatsu, 1979) cannot be ruled out but is less likely because the rDNA transcription unit of *Simulium* species is located at the nucleolar organizing region on one chromosome arm, as found in chromosome arm IL of *S. ornatipes* (Bedo, 1982). *Simulium feuerborni* prefers to breed in small, shallow streams about 30 cm wide, with trailing grasses and fallen leaves at the Royal Project and Hui Sai Luaeng waterfall. Such breeding sites dry up in the hot season of the year. A similar situation occurs at many shallow breeding sites of black flies in northern Thailand (Jitklang et al., unpublished data). It is most likely that the Royal Project and the Huai Sai Luaeng populations undergo local extinction and recolonization of unrelated females from the relatively small number of breeding sites that do not dry up during the dry season. Since the high levels of genetic diversity were detected within and between the populations of *S. feuerbori*, it is therefore possible that the populations of *S. feuerborni* consist of different cytoforms or cytospecies that occur in the same or different habitats as found in the populations of *S. tani* (Tangkavanit et al., unpublished data) and *S. siamense* (Lualon et al., unpublished data). Further studies of morphology, cytology, and mitochondrial and nuclear genes should provide insight into the existence of cytoforms or cytospecies in the *S. feuerborni* taxon.

Phylogenetic analyses using the maximum parsimony, neighbor-joining, and maximum-likelihood methods agreed that two major clades are well resolved. The subgenera *Nevermannia* and *Montisimulium* are closely related and are more closely related to the subgenus *Gomphostilbia* than to the subgenus *Simulium*, as shown

Figure 2. Alignment of the internal transcribed spacer II region from 40 black fly species in the subgenera *Gomphostilbia*, *Nevermannia*, *Simulium*, and *Montisimulium*, and an unknown subgenus. A dot represents agreement with the consensus at that position and dashes indicate alignment gaps.

```
S.(G.)parahiyangum          ATTTATCAATAGAACTGTTCTTTTTTATTACTTTTA-GTAGTAACG--AA 47
S.(G.)siamense              ......................-------.T.....-A..A...------ 36
S.(G.)angulistylum          ......................A....A.G....A.TCGGCA..G.GT.. 50
S.(G.)decuplum              .................C....GC.C.AA...AA.TTT.GTGT.----.T 46
S.(G.)duolongum             ......................-------....-AC-TC.....------ 35
S.(G.)gombakense            ......................-------....--.-A.G-...------ 33
S.(G.)sp.nr.sheilae         ......T..............C-------.T.C--.-A.--.G.------ 32
S.(G.)sheilae               ......T..............C-------.T.C--.-A.--.G.------ 32
S.(G.)asakoae               ......................-------.....A.-A.T....------ 36
S.(G.)inthanonense          .....................---------...--.-A.--.G.------ 30
S.(G.)chumpornense          ......................A..ATA..T.C...TA..A.G.------ 44
S.(G.)burtoni               .....................---------...--.-AA--.GG------ 30
S.(N.)feuerborni[a]         ......................-------T.A.CG.AAG.TG.T-----. 38
S.(N.)sp.nr.feuerborni1     ......................-------T.A.CG.AAG.---------- 33
S.(N.)sp.nr.feuerborni2     ......................A------T.A.CG.AAG.TG.T-----. 39
S.(N.)caudisclerum          ......................----------.CGCAAG.A--------. 32
S.(N.)aureohirtum           ......................----------.CGCAAG.A--------. 32
S.(M.)merga                 ......................----------.CGCAAG.A--------. 32
S.(S.)fenestratum           ..........T...........--------T..--TAA.T-A..-----. 34
S.(S.)chaliowae             ..........T...........--------T..--TAA.TTA..-----. 35
S.(S.)triglobus             ..........T...........--------T----TAA.T---.-----. 30
S.(S.)chainarongi           ..........T...........--------T----TAA.T---.-----. 30
S.(S.)nodosum               ..........T............C-----G.A--------AG..-----. 32
S.(S.)nobile                ......T...T............C-----G.A--------AG..-----. 32
S.(S.)chiangmaiense         ......................--------T.A--TTA.T-A..-----. 34
S.(S.)nakhonense            ......................--------T.A--TTA.T-A..-----. 34
S.(S.)quinquestriatum       ......................--------T.----TACT-A-------. 30
S.(S.)chamlongi             .......................C-----G.A--------AG..-----. 32
S.(S.)siripoomense          .......................C-----G.A--------AG..-----. 32
S.(S.)sp.nr.rufibasis       .......................C-----TG----TAAA.AG.T-----T 36
S.(S.)rufibasis             .......................C-----TG----TAAA.AG.T-----T 36
S.(S.)doipuiense            .......................C-----CT----TATGGAA-------- 33
S.(S.)weji                  ..........T............C-----TTA..ATAA.TAG..-----. 40
S.(S.)tani                  .......................C-----T.A.--.AAA.AG..-----. 38
S.(S.)brevipar              ........................-----TT----TGAA.AA-------- 33
S.(S.)rudnicki              ........................-----GTC..------CA..-----. 34
S.(S.)grossifilum           ........................-----G.C..-.AAA.CA..-----. 39
S.(S.)choochotei            ....................C.AC-----.T.G--.AAA.TGTT-----T 38
S.(S.)nigrogilvum           ..........T............C-----G.A--------AG..-----. 32
S. baimaii                  .......................C-----G.A--------AG..-----. 32
                            ****** *** ****** **

S.(G.)parahiyangum          GACGTACGTGTCAACTATGAAGCTGATGTAAAAAT---------CAGAAT 88
S.(G.)siamense              ....C..............G......A......T.---------T....C 77
S.(G.)angulistylum          ..................A................---------...... 91
S.(G.)decuplum              ..................A.GTTG...........---------..A.C. 87
S.(G.)duolongum             ........................A.A......T.---------T..... 76
S.(G.)gombakense            ....C.....................A......T.---------.....C 74
S.(G.)sp.nr.sheilae         ........................A.A......T.---------T..T.. 73
S.(G.)sheilae               ........................A.A......T.---------T..T.. 73
S.(G.)asakoae               ..........................A......T.---------...... 77
S.(G.)inthanonense          ....C.....................A......T.---------...... 71
S.(G.)chumpornense          ..........................A......T.---------...... 85
S.(N.)feuerborni[a]         ....C.............A.GA..AGCT---TT.ATCG----GC....C. 81
S.(N.)sp.nr.feuerborni1     --................A.GA..CGCT---TT.ATCG----GC....C. 74
S.(N.)sp.nr.feuerborni2     ....C.............A.GA..AGCT---CT.ATCG----GC....C. 82
S.(N.)caudisclerum          ....C................A...T.TGGGTC.AACC----TT....G. 78
S.(N.)aureohirtum           ....................GA..AT---GGT..CAT--------...T. 71
S.(M.)merga                 ..................A.GA.AATCC---TT.ATTG----GAA...C. 75
S.(S.)fenestratum           ....CT...A.T......A.GAAAAT.A--.CTT.-----------TTC. 71
S.(S.)chaliowae             ....CT...A........A.GAAAAT.A--.CTT.-----------TTC. 72
S.(S.)triglobus             ....CT.T.A.T......A.GAAAA..G--..TT.ATTTTTGTT.TTTC. 78
S.(S.)chainarongi           ....CT...A........A.GAGAA..T--..TT.--------TTTTTC. 70
S.(S.)nodosum               ....CT.A............GAA--..--T...T.---------TT--C. 67
S.(S.)nobile                ....CT............A.GAA--..--T..TT.---------TT--C. 67
```

```
S.(S.)chiangmaiense           .........A........A.GAAAAT.A--.CTT.----------TT-C. 71
S.(S.)nakhonense              .........A........A.GAAAAT.A--.TTT.----------TTTC. 72
S.(S.)quinquestriatum         ....C....A........A.GAAAAT..--..TT.----------TT-C. 67
S.(S.)chamlongi               ....C....A............A--.AT.C.TTT.---------TC--C. 69
S.(S.)siripoomense            .........A..........GAA--..A.TGCTT.---------TC---. 68
S.(S.)sp.nr.rufibasis         ....CT.T..........ATGTA--------TTG.---------TTT.CA 69
S.(S.)rufibasis               ....C..T...T......ATGTA--------TTG.---------TTT.CA 69
S.(S.)doipuiense              ....CT.T..........ATGTA--------TTG.---------TTT.CA 66
S.(S.)weji                    .......T..........ATGT----------------------.TT.CA 68
S.(S.)tani                    .......T...T......ATGTT--------TCG.---------TTA.CA 71
S.(S.)brevipar                ....CT.T..........ATGAAGA.AT...TTT.---------TCT.CA 74
S.(S.)rudnicki                ....CT.A.A..........GAAG-.AA.T..TT.---------TTCT.. 74
S.(S.)grossifilum             ....CT.A.A..........GAAG-.AA.T..TT.---------TTCT.. 79
S.(S.)choochotei              .........A........A.T.A.AT.AGT..TT.-------TTT.TCA. 81
S.(S.)nigrogilvum             ....C....A..........GAA--..A.T.GTT.---------ATT.C. 71
S. baimaii                    ....C....A..........GAA--..A.T.CTT.---------TC---. 68
                                **  * * * ******

S.(G.)parahiyangum            CATATTTGATTAAATCAAGAGATATGAAAA--A-----TCGAATACGATC 131
S.(G.)siamense                ...........................-----------........AG.G 116
S.(G.)angulistylum            T................C............--T-----AT.....TAG.. 134
S.(G.)decuplum                T.............................--.-----.......TAG.T 130
S.(G.)duolongum               ..............................--.-----.......TAG.G 119
S.(G.)gombakense              ...........................-----------........AG.G 113
S.(G.)sp.nr.sheilae           ...........................-----------........T..A 112
S.(G.)sheilae                 ..............A............-----------........T..A 112
S.(G.)asakoae                 ...........................-----------.......TAC.T 116
S.(G.)inthanonense            ...........................-----------........AG.A 110
S.(G.)chumpornense            ..............................--.AAAAA.........G.A 133
S.(G.)burtoni                 ...........................-----------........AG.G 110
S.(N.)feuerborni[a]           T......................G......ATCAAATACA.TTA..ACAT 131
S.(N.)sp.nr.feuerborni1       T......................G......ATCAAATACA.TTAG.ACAT 124
S.(N.)sp.nr.feuerborni2       T......................G......ATCAAATACA.TTA..ACAT 132
S.(N.)caudisclerum            ..............................ATCAAATACA.TTAC.A-.A 127
S.(N.)aureohirtum             .................C............ATCAAATACA.TTAT.T-A. 120
S.(M.)merga                   T...........................---TCAAATACA.TTAC.A-.A 121
S.(S.)fenestratum             T......A........T...........----TT-AAA.T-C.A.TATGG 115
S.(S.)chaliowae               T...............T...........----TT-AAA.T-C.A.TATGG 116
S.(S.)triglobus               T......A........T....T......----TT-AAA.T-C.A.TATGG 122
S.(S.)chainarongi             T................T..........----TTTAAA.T-C.A.TATGG 115
S.(S.)nodosum                 ............................----TTAAAT..A..A.TTTGG 113
S.(S.)nobile                  T...........................----TT-----.A..A--TTGG 106
S.(S.)chiangmaiense           T................T..........----TACAAT.TTC.A.TATGG 117
S.(S.)nakhonense              T................T..........----TACAAT.TTC.A.TATGG 118
S.(S.)quinquestriatum         T................T..........----TACAAT.T-C.A.TATGG 112
S.(S.)chamlongi               ............................----.C-AAT..-..A.AAGGT 113
S.(S.)siripoomense            ............................----TT-ATTC--..A.AAGGT 111
S.(S.)sp.nr.rufibasis         T...............T...........----TAAAAA-----..T.G.G 110
S.(S.)rufibasis               T......A........T...........----TAAAAA-----..T.G.G 110
S.(S.)doipuiense              T...............C...........----TAAAAA-----..T.G.G 107
S.(S.)weji                    T...............T...........----TAAAAAA-A....T.G.G 113
S.(S.)tani                    T........C......T...........----TAAAAA-----..T.G.- 111
S.(S.)brevipar                T...............T...........----GAAAAAA-A.-A.TAG.- 117
S.(S.)rudnicki                ...........................C----.AAAAA.TC..ATATGGT 120
S.(S.)grossifilum             ...........................C----.AAAAA.TC..ATATGGT 125
S.(S.)choochotei              T............................TTTTCCAAA.T-C.A.TATGG 130
S.(S.)nigrogilvum             ............................-------AAT..A....--TGG 112
S. baimaii                    ............................----TT-ATTCGA..A.AAGGT 113
                               ****** * **** *  *** * ***

S.(G.)parahiyangum            ACAA-----TTTTTATTGTGTATGCCATTCA---ATACTTT-ATATAA-- 170
S.(G.)siamense                ..T.C----..GC..G.-A.A..CTTG....---...T..C-..C.-.-A 155
S.(G.)angulistylum            .------AC..GC.TG.-..A.GCTT.....---C......-......-- 171
S.(G.)decuplum                .T..CAAAC..GC.TG.-..A.GCTT.....---.......-......-- 173
S.(G.)duolongum               TAT.CA-AC..GC.TG.-AAA.GCTT.....---.......-......-- 161
S.(G.)gombakense              ..T.C----..GC..G.-A.A..CTTG....---...T..C-..C.-.-A 152
S.(G.)sp.nr.sheilae           GTTGCA---..G.AT---A...ACTT.....---...T...-...AT.-A 151
S.(G.)sheilae                 GTTGCA---..G.AT---A...ACTT.....---...T...-...AT.-A 151
S.(G.)asakoae                 G.-------..GCA.---A...GCAT.....---...T..C-..T.T.TA 152
S.(G.)inthanonense            ....C----..GC.TG.TA...GCTTG....---...TA..-..T.T.-A 151
S.(G.)chumpornense            ..C.TA-T-..A....--G.A.GCTT.....---.......-......-- 173
```

Figure 2. (Continued)

```
S.(G.)burtoni                 ..T.TA-T-..GC.TG.-A.A.GCTT.....---...T..A-..T.-T-A 151
S.(N.)feuerborni^a            .TC.ATTTGA.----.GTGAA.GC.T...T.---...T...-....G.-- 171
S.(N.)sp.nr.feuerborni1       .TC.ATTTGA.----.GTGAA.GC.T...T.---...T...-....G.-- 164
S.(N.)sp.nr.feuerborni2       .TC.ATTTGA.G-AT.GTGCA.AC.T...T.---...T...-....G.-- 175
S.(N.)caudisclerum            ..TTGCTTG.--------G.A.GC.T...T.---GC.T...-G.....-- 163
S.(N.)aureohirtum             ..TTGCTTG..-------G...GC.T...T.---.C.T...-....G.-- 157
S.(M.)merga                   ..TTGCTTG.G-------G.A.AC.T...T.---...T...--...G.-- 157
S.(S.)fenestratum             TTT-----A.C--ATA.TG...GC.T...T.---.G.T...-......-- 152
S.(S.)chaliowae               TTTT----A.C--ATA.TG...GC.T...T.---.G.T...-......-- 154
S.(S.)triglobus               TTTT----A.C--ATA.TG...GC.T...T.---.G.T...-......-- 160
S.(S.)chainarongi             TTT-----A.C--ATA.TG...GC.T...T.---.G.T...-......-- 152
S.(S.)nodosum                 T..T----ACC----.ATG...GC.T...TTT-T.G.T...-......-- 151
S.(S.)nobile                  T..T----ACC----.ATG...GC.T.C.T.---.G.T...-.A....-- 142
S.(S.)chiangmaiense           TTT-----A.C--.TA.TG...AC.T...T.---.G.T...-......-- 154
S.(S.)nakhonense              TTT-----A.C--.TA.TG...AC.T...T.---.G.T...-......-- 155
S.(S.)quinquestriatum         TTT-----A.C--.TA.TG...AC.T...T.---.G.T...-......-- 149
S.(S.)chamlongi               TT.T----C.C----A.AG...GC.T...T---A.G.T...-......-- 149
S.(S.)siripoomense            TT.T----C.C----A.TG...GC.T...TT--A.G.T...-......-- 148
S.(S.)sp.nr.rufibasis         TT.T----C.C--A..ATG...AC.T...T.---.G.T...-......-- 148
S.(S.)rufibasis               TT.T----C.C--A..ATG...AC.T...T.---.G.T...-......-- 148
S.(S.)doipuiense              TT.T----C.C--A..ATG...AC.T...T.---.G.T...-......-- 145
S.(S.)weji                    TT.T----C.C.CA..GTG...AC.T...T.---.G.T...-......-- 153
S.(S.)tani                    TT.T----C.C--A..ATG...AC.T...T.---.G.T...-......-- 149
S.(S.)brevipar                TT.T----C.C--A..ATG...AC.T...T.---.G.T...-......-- 155
S.(S.)rudnicki                TT.T----C..----A.GG...GC.T...T.---.G.T...-......-- 156
S.(S.)grossifilum             TT.T----C..----A.GG...GC.T...T.---.GGT...-......-- 161
S.(S.)choochotei              TTTTTATCA..--.TAATG...AC.T...T.---.G.T...T......-- 173
S.(S.)nigrogilvum             TTT.----TC.----.ATG...GC.T...T.---.G.T...-......-- 148
S. baimaii                    TT.T----C.C----A.TG...GC.A...TTTTA.G.T...-......-- 152
                                                  *      *          *

S.(G.)parahiyangum            TACTATCATTTCTTG----------------ATTGTAAGCGATGAATGTG 204
S.(G.)siamense                ..............A----------------......TTTA.-------- 181
S.(G.)angulistylum            ...............----------------..G.CT..A.-.....--. 202
S.(G.)decuplum                ...............----------------...TCT..........A.. 207
S.(G.)duolongum               .............A.----------------.....T.........ACAT 195
S.(G.)gombakense              ..............A----------------......TTTA.-------- 178
S.(G.)sp.nr.sheilae           ..............A----------------....C..TGA.AATG...A 185
S.(G.)sheilae                 ..............A----------------....C..TGA.AATG...A 185
S.(G.)asakoae                 CTA...........A----------------.......TGA.-------- 178
S.(G.)inthanonense            .T............A----------------.....C.TGA.-------- 177
S.(G.)chumpornense            ...............----------------..G.CT............A 207
S.(G.)burtoni                 ..............A----------------.....G---A.-------- 174
S.(N.)feuerborni^a            A.............T-------------------....ATTG.AT..--. 200
S.(N.)sp.nr.feuerborni1       A.T...........T-------------------....ATTG.AT..--. 193
S.(N.)sp.nr.feuerborni2       A.............T-------------------....ATTG.AT..A.. 206
S.(N.)caudisclerum            ..............T-------------------....ATT-.-T..--. 190
S.(N.)aureohirtum             ..............T-------------------....ATTG.-T..--. 185
S.(M.)merga                   ...A..........T-------------------T...A.A.A-...TAA 187
S.(S.)fenestratum             ..............AA------------TTT--AA.T.AT...A.T.--A 186
S.(S.)chaliowae               ..............AA------------TTTT.AA.T.AT...A.T.--A 190
S.(S.)triglobus               ..............AA------------TTT-----T.AT...ATT.CAA 193
S.(S.)chainarongi             ...A..........AAATAATT---ATTTTTT..TGT.AT...T..CT-A 198
S.(S.)nodosum                 ...A..........--TTT--T----AATTC.CAC..GAA..G--T.C-A 190
S.(S.)nobile                  ...A..........--ATTATT----CATAAG..CA.T.AT.G--.AT-A 183
S.(S.)chiangmaiense           ...A..........AG--------------------T.AAA-.ATTAAAT 183
S.(S.)nakhonense              ...A......C...AT--------------------T.AAA.CATTAAAT 185
S.(S.)quinquestriatum         ...A.......-..AT--------------------T.AAA.CATTAAAT 178
S.(S.)chamlongi               ..............TGATTCGTAAAAAAACC.A.T.G.AT...--.AC-A 196
S.(S.)siripoomense            ..............--ATT------AAAACC.--T...AT...T..AA-A 187
S.(S.)sp.nr.rufibasis         ...A..........A---ATT---------------G.AT.G.--..--A 176
S.(S.)rufibasis               ...A..........A---ATT---------------G.AT...--.A--A 176
S.(S.)doipuiense              ...A..........A---ATT---------------G.AT.G.--.A--A 173
S.(S.)weji                    ...A..........ATATATTA-----TTAT.A..CG.AT...--..T-A 195
S.(S.)tani                    ...A..........A--TATTT-----ATTT...--G.AT.G.--TA--A 186
S.(S.)brevipar                ...A..........ATTTATG---------------.TAT...--..--A 186
S.(S.)rudnicki                ..............--TTTGCT----ATCAC.A.T.G.AT...TT.AA-A 199
S.(S.)grossifilum             ..............--TTTGCT----ATTAC.A.T.G.AT...TT.AA-A 204
S.(S.)choochotei              ..............AGTTGTAA---AAACCAT---.T.AT...-TTAAAA 216
S.(S.)nigrogilvum             ..............--CTTTTTTCCAAAAAA.AAA.T.AT...--.AT-A 193
S. baimaii                    ..............--ATT------AAAACC.--T...AT...T..AA-A 191
```

Figure 2. (Continued)

```
S.(G.)parahiyangum          T-TGTTATA---GAGTAAA-TGTAATTGAATGGCGTTGAT--AAA--AAT 245
S.(G.)siamense              --..G...T---T....TG-.TAG.....-AT.A.A..GGCAGC.--... 222
S.(G.)angulistylum          .A.A.....GAGT.A.TGTACTG...AAGTGTTA.A...GTC..T-TG.- 250
S.(G.)decuplum              .A.A...T.GAGT.A.TGTACTG...AAGTGATA.A...GTT...GTG.A 257
S.(G.)duolongum             G-......TA--T..AGT.AC........-AT.A..G.CATTGT.--.G. 239
S.(G.)gombakense            --..G...T---C....TG-.--G.....-AT.A.A...GCGGC.--... 217
S.(G.)sp.nr.sheilae         .-.A.ATAT---TT.....AAA.......A.T.A..GA.ACA.C.--..G 229
S.(G.)sheilae               .-.A.ATAT---TT...G.AAA.......A.T.A..GA.ACA.C.--..G 229
S.(G.)asakoae               --AAAGTAT---..T.G..T.TG...AA.AGCAAAAA.CAATGC.--T.C 221
S.(G.)inthanonense          --.A.G.AT---..A....A.TG......-GC.AAGCA..--GC.--... 217
S.(G.)chumpornense          .A.TA..GGG--T.ACGT.A.TG...A.GTG.TACA.AC.AA.TG--C.. 253
S.(G.)burtoni               --..AA.AT---.TA.G..-.TG......TAT.A..GACAACGC.--.C. 216
S.(N.)feuerborni[a]         .AGA.....AA-ACA.G.-----T...A.--ATTTGG.CGATCTG---.. 239
S.(N.)sp.nr.feuerborni1     .AGA.....AA-ACA.G.-----T.....--ATTTGG.CGAT..GCAT.. 235
S.(N.)sp.nr.feuerborni2     .AGA.....AA-ACA...-----T...A.--ATTTGG.TGATCTG---.. 245
S.(N.)caudisclerum          .A.A.....AA-.TAACG-----TGCCA.--ATA.G...AAA.C.--.G. 230
S.(N.)aureohirtum           .AGA.....AAA..AAC.-----CG....--ATATG-.TGAATC.--.G. 225
S.(M.)merga                 .G.A.A..CAT--.TAT.-----TG..CT--ATA.G....AACC.--.G. 226
S.(S.)fenestratum           .G.A....TTTAA.A-CT-TATATC..A---AATAG..TGTA.T.TG.TA 231
S.(S.)chaliowae             .G.A....TTTAA.A-CT-TATATC..A---AATAG..TGTA.T.TG.TA 235
S.(S.)triglobus             .G.A....TTTAA.A-CT-TATATC..ATAAAATAG..TGTA.T.TG.TA 241
S.(S.)chainarongi           .G.A....TTTAA.AACT-TATATC..A---AATAG..TGTT.T.TG.TA 244
S.(S.)nodosum               ATGA..T.TTAGA.A-.T--.-...A-A---ATTTG..CGT-.T.GG..A 231
S.(S.)nobile                AG.A....CTAAAGA-.T--A-.GC..A---A.TAG..CGT-.T.GT.TG 225
S.(S.)chiangmaiense         GG.A.....T-GA.CC.T---AATC..A---CTTAG..TGTA.T.TG.TA 226
S.(S.)nakhonense            GG.A.....T-AAGT..T-A.AATC..A.--TATAG..TGTA.T.TG.TA 231
S.(S.)quinquestriatum       GG.A.....T-AAGT..T---AGTC..A---TCTAG..TGTA.T.TG.TA 221
S.(S.)chamlongi             .G.A....TTTAA-A-CC--.A.GC..A---AATAG..CGTT.TGAGCTA 239
S.(S.)siripoomense          .G.A....TTAAAGA-..--...CC.GA---AAT.G..CGTA.TGAG.TA 231
S.(S.)sp.nr.rufibasis       GG.A....TTTAA.CATT--.T-TC..A---AATAG..TGT-GT.TTGGG 219
S.(S.)rufibasis             AG.A....TTTAA.CATT--.T-TC..A---AATAGC.TGT-GT.TTGGG 219
S.(S.)doipuiense            AG.A....TTTAA.CATT--.T-TC..A---AATAG..TGT-GT.TTG.G 216
S.(S.)weji                  AG.A....TTTAA.A.TT--..-TC..A---AATAGC.TGT-.T.TTG.G 238
S.(S.)tani                  .G.A....TTTAA.C.TT--..-TC..A---AATAGC.TGT-.T.TTG.G 229
S.(S.)brevipar              AG.A....TTTAA.CATT--.TATC..A---AATAG..TGT-.T.TTG.A 230
S.(S.)rudnicki              .G.A....TTTAA.ACGC--.A.-C..A---AATAGA.TGTAGT.AG.TA 243
S.(S.)grossifilum           .G.A....TTTAA.ACCG--AA.TC..A---AATAG..TGTAGT.AG.TA 249
S.(S.)choochotei            .G.A....TTTAT.C..T.TATATC..A.A.T.T-G..TATATT.TG.TA 265
S.(S.)nigrogilvum           .G.A....TTTAA.A-.T--.A.TC..A---.ATAG..CGT-TTTAAG.A 236
S. baimaii                  .G.A....TTAAAGA-..--...CT.GA---AATAG..CGTA.TGAG.TA 235


S.(G.)parahiyangum          TCTA---ATT------------GTATTTGAAAT-----------ACTAAA 269
S.(G.)siamense              AACGA--...------------..G..CA....-----------..A-.. 246
S.(G.)angulistylum          --.TTTTGC.A----------TA.T.....-..-----------....T. 276
S.(G.)decuplum              AT.TTCA.C.A----------TA...C-.T-..-----------...... 284
S.(G.)duolongum             .-..A--.C.A----------TA...CG..T.C-----------.AAT.. 265
S.(G.)gombakense            AACGA--...------------.....CA....-----------..G-.. 241
S.(G.)sp.nr.sheilae         CA..T--...T----------A......A.T.C-----------TTA... 256
S.(G.)sheilae               CAC.T--...T----------A......A.T.C-----------TTA... 256
S.(G.)asakoae               AA..T--TC.------------T...ACT...----------------.. 241
S.(G.)inthanonense          AA.----...------------.....CA.T.C-----------TTG-.. 239
S.(G.)chumpornense          .T.TGTG.C.G----------TA.T.CG.....-----------....T. 282
S.(G.)burtoni               .TA.A--.C.------------...A.CA.-..-----------...-.. 239
S.(N.)feuerborni[a]         CAATTTG..------TCGACTGCATA..TGG..-----------....T. 272
S.(N.)sp.nr.feuerborni1     CAATTTG..------TCGACTGAA--..TGG..-----------....T. 266
S.(N.)sp.nr.feuerborni2     CAATTCG..------TCGACTGAA--..TGG..-----------....T. 276
S.(N.)caudisclerum          CAATTTA.-------CCAATTGCA--..TG...-----------....T. 260
S.(N.)aureohirtum           ATATTGA.A------TTGACTGTC--..TGC..-----------....T. 256
S.(M.)merga                 CAATTGAT.A-----ACGACTGAA--..TGT..-----------....T. 258
S.(S.)fenestratum           --A.TAGT.AAAG-------AAT.TAAAA....-----------....T. 261
S.(S.)chaliowae             --A.TAGT.AAAG-------AAT.TAAAA..-.-----------....T. 264
S.(S.)triglobus             --A.TA-----AG-------AAT.TAA.A....-----------....T. 266
S.(S.)chainarongi           --A.TAT..AAAG-------AAT.TAAAAT.-.-----------....T. 273
S.(S.)nodosum               AGATTGATA-------------T.T...TG...TTTTTTTTTAA.T.... 268
S.(S.)nobile                .GACTGTTA-------------T.T----G...TTTTT----AT....T. 254
S.(S.)chiangmaiense         --A.TAA.A.-GA-------AAT.TG..A---.-----------....T. 252
S.(S.)nakhonense            -TACTAA..--GA-------AAT.TG..A---.-----------....T. 257
```

Figure 2. (Continued)

```
S.(S.)quinquestriatum          A.A.TAA..AGGA-------AAT.G...A---.-----------....T. 250
S.(S.)chamlongi                AA..ATAGAA------------T.T...TG...T--AT----AT...... 271
S.(S.)siripoomense             AAC.TTAT.---------------T...TG...TCAAA-----T....T. 261
S.(S.)sp.nr.rufibasis          AT.TTTTT..-----TACCAAAT.T..--....-----------....T. 251
S.(S.)rufibasis                AT.TTTTT..-----TACCAAAT.T..--....-----------....T. 251
S.(S.)doipuiense               AT.TTTTT..A----TGCCAAAT.T..--....-----------....T. 249
S.(S.)weji                     AT.TATTT..AAATTTGCCAATT.T...T....-----------....T. 277
S.(S.)tani                     ATA.TTTT..-------TTATTT.TA..A....-----------....T. 261
S.(S.)brevipar                 .AAAATTTC----------ATTT.T.C.T....-----------..C.T. 259
S.(S.)rudnicki                 AA..TTTGGAA-----------T.T...A....-----------....T. 271
S.(S.)grossifilum              AA.TTTTTGGAAT--------TT.T...TG...-----------....T. 280
S.(S.)choochotei               -AACTCA..GAATT-----TGAT.CAAAA.T..-----------....T. 298
S.(S.)nigrogilvum              AAAC-GAT.-------------T.T...TG...TTAT-----AT....T. 267
S. baimaii                     AGCGTAAT.AATTTTTATTTTTT.T...TG...TCAAA----AT...... 281


S.(G.)parahiyangum             TTGTATACAT-- 279
S.(G.)siamense                 A.........-- 256
S.(G.)angulistylum             AAT.......-- 286
S.(G.)decuplum                 GAT.......-- 294
S.(G.)duolongum                A-........-- 274
S.(G.)gombakense               A.........-- 251
S.(G.)sp.nr.sheilae            G.........-- 266
S.(G.)sheilae                  G.........-- 266
S.(G.)asakoae                  G.A..C.T---- 249
S.(G.)inthanonense             G.A..C.T---- 247
S.(G.)chumpornense             AAT.......-- 292
S.(G.)burtoni                  AA........-- 249
S.(N.)feuerborni[a]            GAT.......-- 282
S.(N.)sp.nr.feuerborni1        AAT.......-- 276
S.(N.)sp.nr.feuerborni2        AAT.......-- 286
S.(N.)caudisclerum             AAT.......-- 270
S.(N.)aureohirtum              AAT.......-- 266
S.(M.)merga                    AAT.......-- 268
S.(S.)fenestratum              AAT.......-- 271
S.(S.)chaliowae                AAT.......-- 274
S.(S.)triglobus                AAT....T.CAT 278
S.(S.)chainarongi              AAT.......-- 283
S.(S.)nodosum                  ..T.T.....-- 278
S.(S.)nobile                   AAT.......-- 264
S.(S.)chiangmaiense            AAT.......-- 262
S.(S.)nakhonense               AAT.......-- 267
S.(S.)quinquestriatum          AAT.......-- 260
S.(S.)chamlongi                AAT.......-- 281
S.(S.)siripoomense             AAT.......-- 271
S.(S.)sp.nr.rufibasis          AAT.......-- 261
S.(S.)rufibasis                AAT.......-- 261
S.(S.)doipuiense               AAT.......-- 259
S.(S.)weji                     AAT.......-- 287
S.(S.)tani                     AAT.......-- 271
S.(S.)brevipar                 AAT.......-- 269
S.(S.)rudnicki                 AAT.......-- 281
S.(S.)grossifilum              AAT.......-- 290
S.(S.)choochotei               AAT.......-- 308
S.(S.)nigrogilvum              AAT.......-- 277
S. baimaii                     AAT.......-- 291
                                   *  *
```

[a] The ITS2 sequence of the *S. feuerborni* larva from Hui Sai Luaeng waterfall

Figure 2. (Continued)

in all trees. This finding is consistent with existing morphological and behavioral data. Adult females of the subgenera *Nevermania*, *Montisimulium*, and *Gomphostilbia* are ornithophilic (bird feeders), with toothed claws adapted for movement through feathers (Adler, Currie and Wood, 2004). Moreover, the larvae of these subgenera have ventral tubercles (Takaoka & Davies, 1995). On the other hand, adult females of the subgenus *Simulium* are more typically mammalophilic, with toothless claws (Crosskey, 1990) and no ventral tubercles (Takaoka & Davies, 1995).

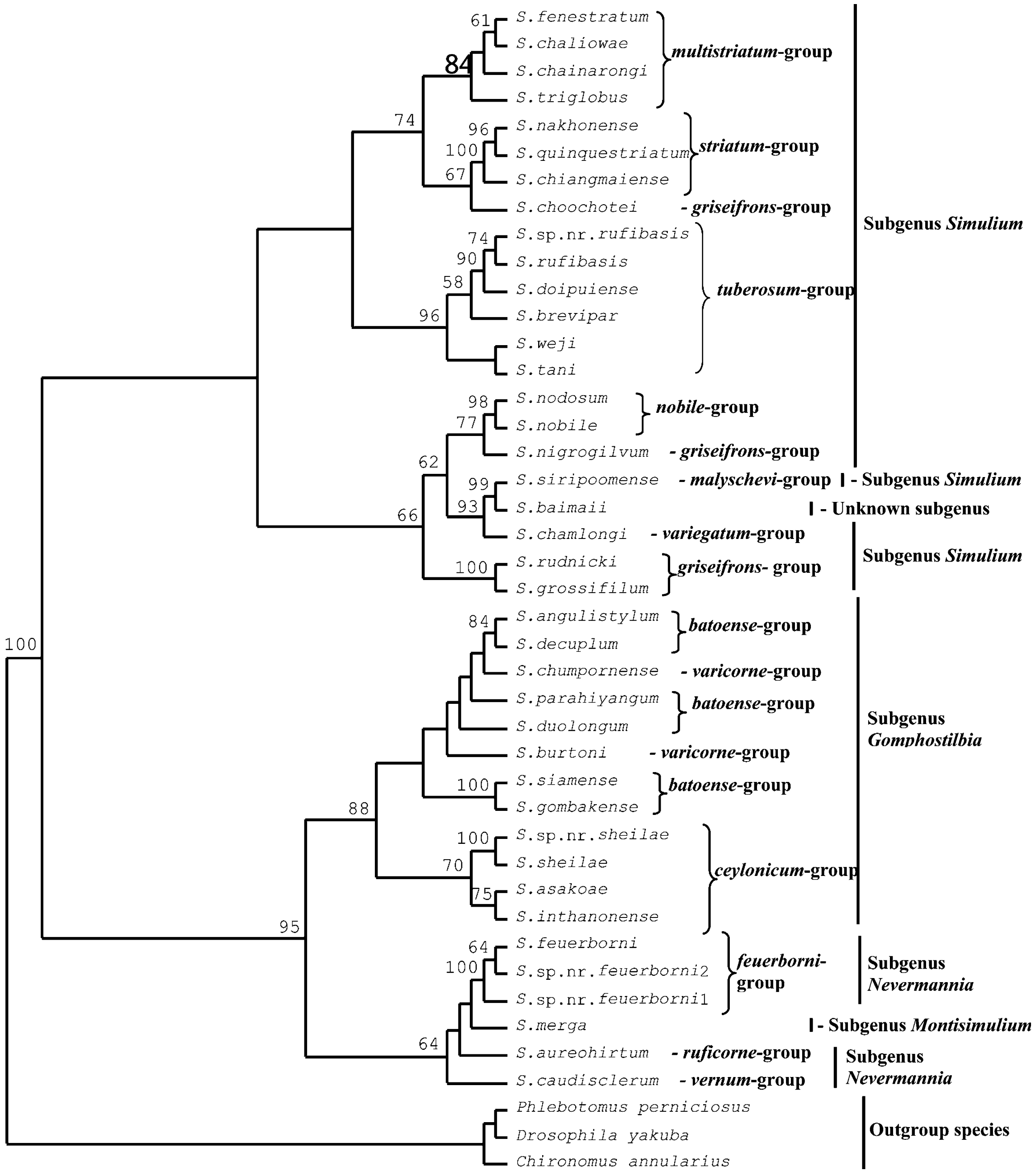

Figure 3. Phylogenetic tree based on rDNA ITS2 sequences generated by maximum parsimony method (PAUP). The tree is the 50% majority rule consensus of 1000 bootstrap replications. Numbers above branches are bootstrap percentages for clades supported above the 50% level. Tree length 1559 with consistency index (CI) = 0.4638, retention index (RI) = 0.6158, and rescaled consistency index (RC) = 0.2856.

In the clade with the subgenus *Simulium*, the molecular data indicate that the *multistriatum*-group is more closely related to the *striatum*-group than to the *tuberosum*-group. The morphological data support this result. The number of gill filaments for species in the *multistriatum*-group and *striatum*-group are 8 and 10, respectively. In contrast, the *tuberosum*-group has 6 gill filaments.

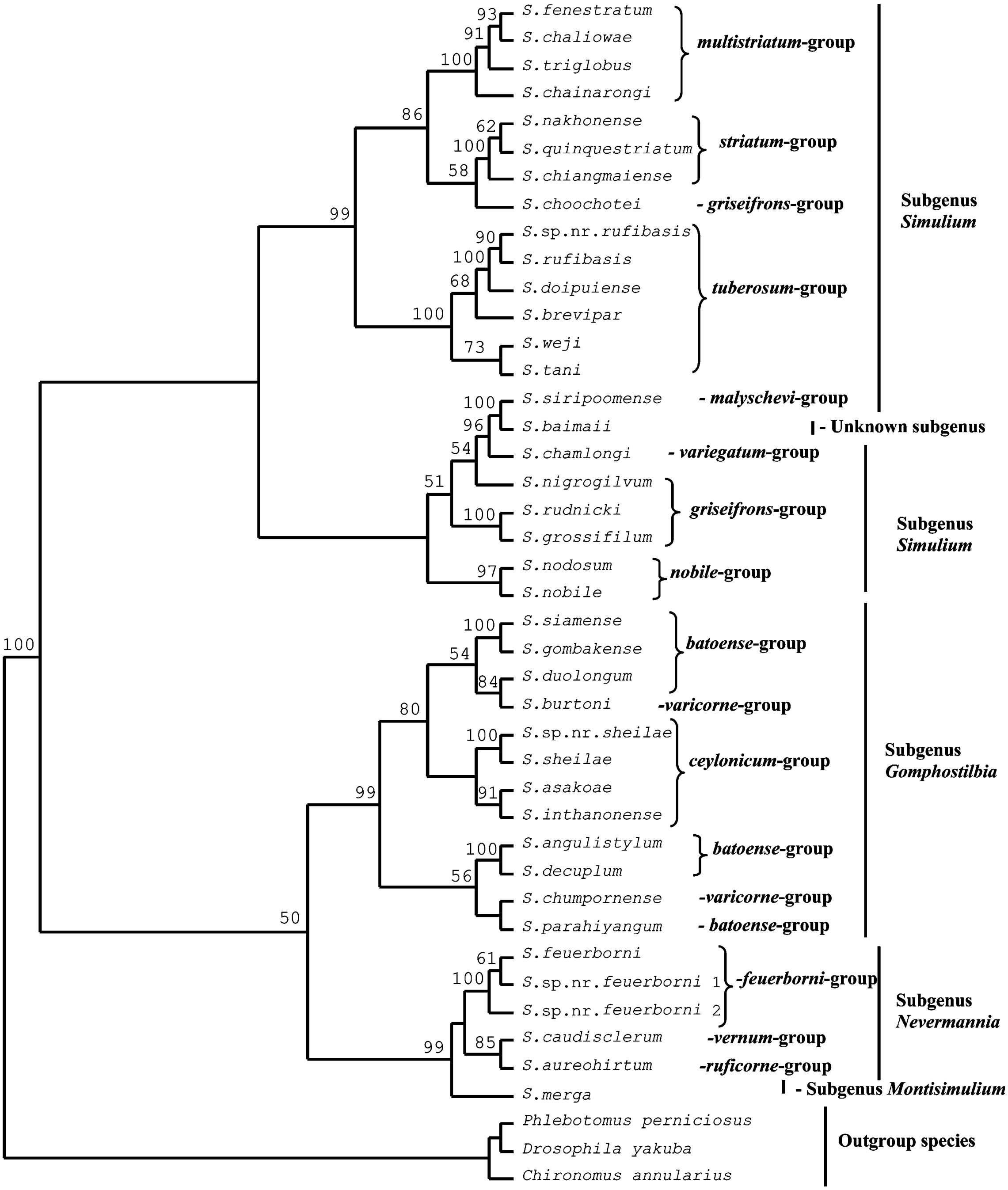

Figure 4. Phylogenetic tree based on rDNA ITS2 sequences generated by neighbor-joining method (PAUP). The tree is the 50% majority rule consensus of 1000 bootstrap replications. Numbers above branches are bootstrap percentages for clades supported above the 50% level.

Similarly, the *nobile*-group, *malyschevi*-group, *variegatum*-group, and *griseifrons*-group, which are more closely related to the *tuberosum*-group than to the *multistriatum*-group and *striatum*-group, also have 6 gill filaments (Takaoka and Davies, 1995). In the ML tree, the five species groups with 6 gill filaments were placed in the same clade, separated from the clade with the

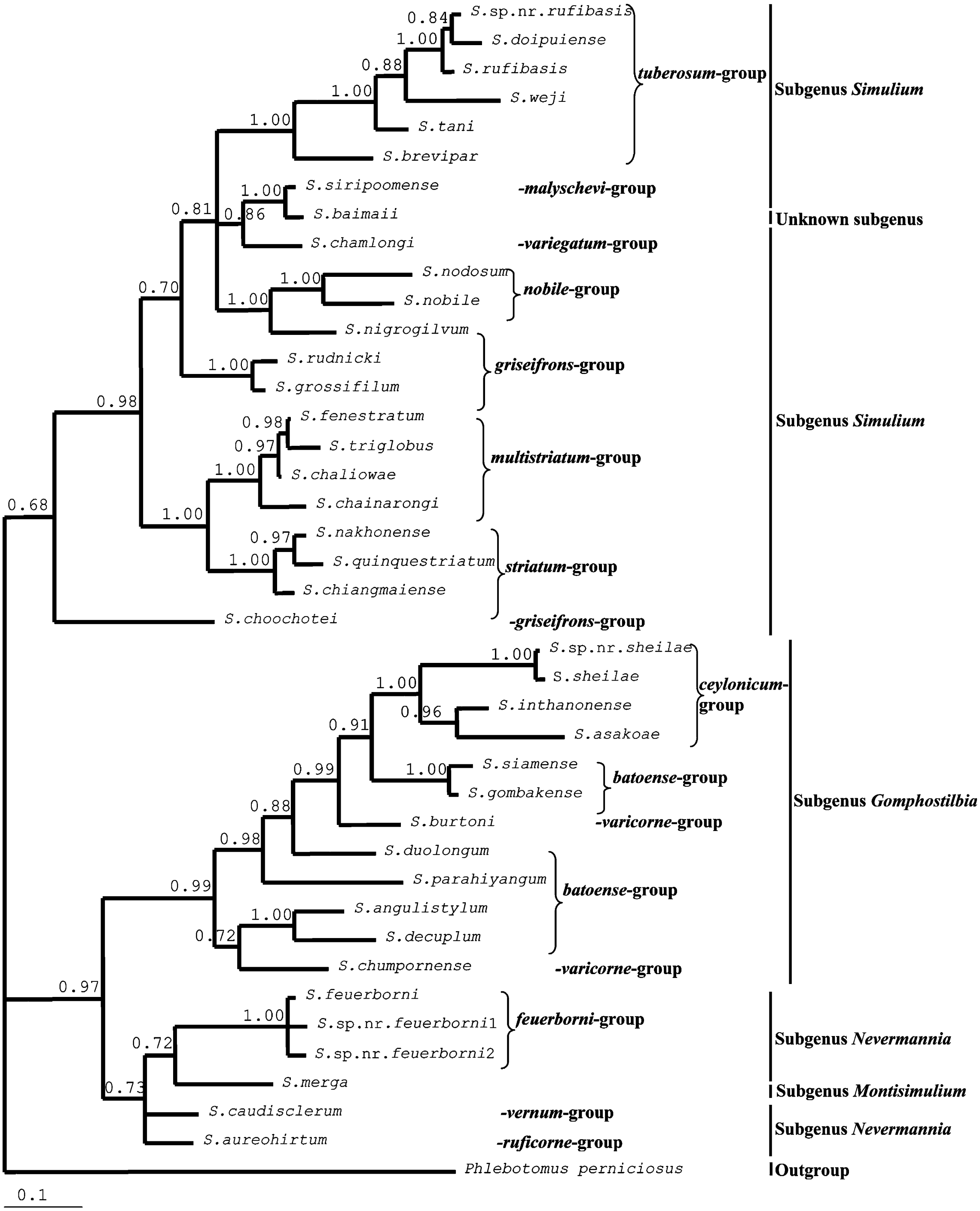

Figure 5. Phylogenetic tree based on rDNA ITS2 sequences with equal weight using maximum likelihood method (MrBAYES). Numbers at nodes are Bayesian posterior probabilities.

multistriatum-group (10 gill filaments) and *striatum*-group (8 gill filaments). The relationships among the four member species in the *griseifrons*-group, namely *S. choochotei*, *S. nigrogilvum*, *S. rudnicki*, and *S. grossifilum*, are unresolved because *S. choochotei* was placed in the clade with the *striatum*-group in the MP and NJ trees, but was the sister taxon to the remaining species in the ML tree. This study does not support the placement of *S. choochotei* in the *griseifrons*-group of the previous morphological study of Takaoka and Choochote (2002). Some morphological characters of the female, male, pupa, and larva of *S. choochotei* differ from those of the other member species by the presence of numerous long stout hairs on the anterior gonapophysis, a haired basal portion of the radial vein, and a cibarium with a round dorsally directed projection in the female; in the male by the shape of the ventral plate and the 10th abdominal segment with numerous hairs on each posterolateral corner; in the pupa by the somewhat inflated gill filaments and the shape of the cocoon; and in the larva by the shape and size of the body (Takaoka & Choochote, 2002). In the present study, *S. nigrogilvum* was placed in the clade with the *nobile*-group instead of the *griseifrons*-group, with high bootstrap values in the MP and ML trees. Previous studies by Takaoka and Suzuki (1984) indicated that *S. nigrogilvum* belonged to the subgenus *Himalayum* Lewis. Recently *S. nigrogilvum* was assigned to the *griseifrons*-group of the subgenus *Simulium* based on mitochondrial 16S rDNA and morphological data (Otsuka et al., 2003). The present study supports the previous report of Otsuka et al. (2003) that *S. nigrogilvum* belongs to the subgenus *Simulium* because nucleotide differences between *S. nigrogilvum* and the members of the subgenus *Simulium* ranged from 4 to 19, falling within the range of nucleotide differences among species in the subgenus *Simulium* (1–23). Further morphological, cytological, and molecular analyses are needed to determine if *S. nigrogilvum* and *S. choochotei* belong to the *griseifrons*-group. The present study indicates that *S. baimaii*, previously unplaced to subgenus, is closely related to *S. siripoomense* (*malyschevi*-group) and *S. chamlongi* (*variegatum*-group), although the morphological characters of *S. baimaii* larva differ from those of *S. siripoomense* and *S. chamlongi*. *Simulium baimaii* might belong to the *malyschevi*-group, *variegatum*-group, or other species groups in the subgenus *Simulium*. Additional morphological study of adults and cytological study of larvae might be useful in resolving the taxonomic status of *S. baimaii*. The six closely related species in the *S. tuberosum*-group (i.e., *S.* sp. nr. rufibasis, *S. rufibasis*, *S. doipuiense*, *S. brevipar*, *S. weji*, and *S. tani*) were placed together in one monophyletic clade. This result is consistent with the morphological and cytological data. The larvae of *S.* sp. nr. *rufibasis* and *S. rufibasis* are morphologically similar, with identical chromosomal banding patterns (Kuvangkadilok, unpublished data). They are probably sibling species. *S. doipuiense* is closely related to *S. rufibasis* by having a pair of submedian clusters of long hairs on the seventh sternite of females and identical pupae (Takaoka & Suzuki 1984). The adults of the members of the *tuberosum*-group are distinguished by minor differences. For example, the adults of *S. doipuiense* differ from those of *S. rufibasis* by the clearer leg coloration, with all tibiae being more widely paler (Takaoka & Suzuki, 1984; Takaoka, personal communication). The *S. multistriatum*-group consists of four species of which three (*S. chaliowae*, *S. chainarongi*, and *S. triglobus*) were described as new species in Thailand (Takaoka & Kuvangkadilok, 1999). The pupae of the monophyletic *multistriatum*-group have 8 gill filaments but they differ in shape. The pupae of *S. chaliowae* and *S. chainarongi* have shoe-shaped cocoons with an anterior collar of moderate height, whereas *S. triglobus* has a corbicular cocoon (Takaoka & Kuvangkadilok, 1999) and *S. fenestratum* has a boot-shaped cocoon with a large elongate window on either side (Takaoka, 1977). However, most characters of females and males of these species are similar. For example, the male of *S. chaliowae* is similar to that of *S. fenestratum* by having the hind basitarsus almost entirely darkened and the ventral plate hairy on the ventral surface. Additionally, most characters of genitalia of females and males of *S. chaliowae* and *S. chainarongi* are similar. The most striking character of *S. triglobus* females is the presence of three spermathecae (Takaoka and Kuvangkadilok, 1999), as opposed to the single typical one found in nearly all other black fly species (Crosskey, 1990).

In the subgenus *Gomphostilbia*, the relationships within the *batoense*-group and the *varicorne*-group are unresolved, as evidenced by unstable branching and low bootstrap values. The *batoense*-group and the *varicorne*-group do not form independent monophyletic clades. This result is consistent with morphological data that larvae and pupae of most species in these groups are not clearly distinguished from each other. Members of the *S. batoense* and *S. ceylonicum* species groups can be distinguished by their larval chromosomal banding patterns (Kuvangkadilok et al., 2003). Additionally, intraspecific variation in morphological characters and inversion polymorphisms apparently exist in populations of *S. asakoae* (Jitklang et al., unpublished data) and *S. siamense* (Lualon et al., unpublished data). Although *S. chumpornense* and *S. burtoni* were assigned to the *varicorne*-group, they are not closely related. *S. chumpornense* and *S. burtoni* are more likely closely related to the *batoense*-group than to the *ceylonicum*-group because they were placed in the clade with the *batoense*-group. In the monophyletic *ceylonicum*-group, *S.* sp. nr. *sheilae* is closely related to *S. sheilae*, as well as to *S. inthanonense* and *S. asakoae*. The larval morphology of *S.* sp. nr. *sheilae*, except for the size of the stalk of the gill histoblast is similar to that of *S. sheilae*. They were not easily distinguished from each other when they occurred in the same breeding sites (Kuvangkadilok et al., unpublished data). *S.* sp. nr. *sheilae* is possibly conspecific to *S. sheilae*, with high intraspecific variation in the pattern of gill filaments. The phylogenetic relationships among the members of the subgenus *Gomphostilbia* need further study to clarify their taxonomic status.

In conclusion, the ITS2 molecular data produced an informative phylogeny of the genus *Simulium*. The molecular data are consistent with the morphological data for supporting the monophyly of the subgenus *Simulium* + *S. baimaii* (subgenus unknown) and *Nevermannia* + *Montisimulium* + *Gomphostilbia*, as well as the *tuberosum* and *multistriatum*-groups in the subgenus *Simulium* and the *ceylonicum* group in the subgenus *Gomphostilbia*. In contrast, the phylogenetic relationships among members of the *griseifrons*-group (subgenus *Simulium*) and the *batoense* and *varicone*-groups (subgenus *Gomphostilbia*) are not resolved, and further analyses are needed. However, the ITS2 phylogeny obtained by maximum parsimony, neighbor-joining and maximum-likelihood analyses generally agreed with the relationships based on previous morphological criteria.

Acknowledgements

We thank Professor Peter H. Adler for his valuable review and comment on the first draft of the manuscript, Dr. John Milne for review of the revised manuscript, Dr. Charles H. Porter for providing the CP16 and CP17 primers and Mr. Prirot Pramual for suggesting the computer program. We also thank two anonymous reviewers for their valuable comments. This work was financially supported by the Thailand Research Fund (RDG4530034).

References

Adler, P.H., D.C. Currie & D.M. Wood, 2004. The Black Flies (Simuliidae) of North America. Cornell University Press, Ithaca, New York.

Bedo, D.G., 1982. Patterns of polytene-chromosome replication in *Simulium ornatipes* (Diptera: Simuliidae). Genetica 59: 9–21.

Brockhouse, C.L., C.G. Vajime, R. Marin & R.M. Tanguay, 1993. Molecular identification of onchocerciasis vector sibling species in black flies (Diptera: Simuliidae). Biochem. Biophys. Res. Commun. 194: 628–634.

Collins, F.H., C.H. Porter & S.E. Cope, 1990. Comparison of rDNA and mtDNA in the sibling species *Anopheles freeborni* and *A. hermsi*. Am. J. Trop. Med. Hyg. 42: 417–423.

Cornel, A.J., C.H. Porter & F.H. Collins, 1996. Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. J. Med. Entomol. 33: 109–116.

Crosskey, R.W., 1990. The Natural History of Blackflies . John Wiley and Sons, London.

Crosskey, R.W. & T.M. Howard, 2004. A Revised Taxonomic and Geographical Inventory of World Blackflies (Diptera: Simuliidae) . The Natural History Museum, London.

Depaquit, J., H. Ferté, N. Léger, R. Killick-Kendrick, J.-A. Rioux, M. Killick-Kendrick, H.A. Hanafi & S. Gobert, 2000. Molecular systematics of the phlebotomine sandflies of the subgenus *Paraphlebotomus* (Diptera, Psychodidae, *Phlebotomus*) based on ITS2 rDNA sequences. Hypotheses of dispersion and speciation. Insect Mol. Biol. 9: 293–300.

Fritz, G.N., J. Conn, A. Cockburn & J. Seawright, 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). Mol. Biol. Evol. 11: 406–416.

Gerbi, S.A., 1985. Evolution of ribosomal DNA, pp. 419–517 in Molecular Evolutionary Genetics, edited by R.J. Maclntyre. Plenum, New York.

Hackett, B.J., J. Gimnig, W. Guelbeogo, C. Costantini, L.L. Koekemoer, M. Coetzee, F.H. Collins & N.J. Besansky, 2000. Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *An. rivulorum*, and uncover a cryptic taxon. Insect Mol. Biol. 9: 369–374.

Hillis, D.M., C. Moritz & B.K. Mable, 1996. Molecular Systematic. 2nd ed. Sinauer, Sunderland, MA.

Hillis, D.M. & S.K. Davis, 1986. Evolution of ribosomal DNA: fifty million years of recorded history in the frog genus *Rana*. Evolution 40: 1275–1288.

Hillis, D.M. & M.T. Dixon, 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol. 66: 411–453.

Huelsenbeck, J.P. & F. Ronquist, 2001. MrBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755.

Insua, A., M.J. López-Piñón, R. Freire & J. Méndez, 2003. Sequence analysis of the ribosomal DNA internal transcribed spacer region in some scallop species (Mollusca: Bivalvia: Pectinidae). Genome 46: 595–604.

Joy, D.A. & J.E. Conn, 2001. Molecular and morphological phylogenetic analysis of an insular radiation in Pacific black flies (*Simulium*). Syst. Biol. 50: 18–38.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Krüger, A., A. Gelhaus & R. Garms, 2000. Molecular identification and phylogeny of East African *Simulium damnosum* S.L. and their relationship with West African species of the complex (Diptera: Simuliidae). Insect Mol. Biol. 9: 101–108.

Kumar, S., K. Tamura, I.B. Jakobsen & M. Nei, 2001. MEGA 2.1: Molecular Evolutionary Genetics Analysis Software. Arizona State University, Tempe, Arizona, USA.

Kuvangkadilok, C. & H. Takaoka, 2000. Taxonomic note on Simuliidae (Diptera) from Thailand: description of a new species and new species distributional records of nine known species. Jpn. J. Trop. Med. Hyg. 28: 167–175.

Kuvangkadilok, C., C. Boonkemtong & S. Phayuhasena, 1998. C-banding in polytene chromosomes of six *Simulium* species (Diptera: Simuliidae) from Doi Inthanon national Park, northern Thailand. J. Sci. Soc. Thailand 24: 215–230.

Kuvangkadilok, C., S. Phayuhasena & V. Baimai, 1999a. Population cytogenetic studies on *Simulium feuerborni* Edwards (Diptera: Simuliidae) from northern Thailand. Genome 42: 80–86.

Kuvangkadilok, C., C. Boonkemtong & S Phayuhasena, 1999b. Larval polytene chromosomes of five *Simulium* species (Diptera: Simuliidae) from Doi Inthanon national Park, northern Thailand. Cytologia 64: 197–207.

Kuvangkadilok, C., C. Boonkemtong, S. Phayuhasena & V. Baimai, 2003. Larval polytene chromosome of black flies (*Simulium*) of Thailand. I. Comparison among five species in the subgenus *Gomphostilbia* Enderlein. Genetica 118: 69–81.

Malafronte, R.S., M.T. Marrelli & O. Marinotti, 1999. Analysis of ITS2 DNA sequences from Brazilian *Anopheles darlingi* (Diptera:Culicidae). J. Med. Entomol. 36: 631–634.

Marinucci, M., R. Romi, P. Mancini, M. Di Luca & C. Severini, 1999. Phylogenetic relationships of seven Palearctic members of the *maculipennis* complex inferred from ITS2 sequence analysis. Insect Mol. Biol. 8: 469–480.

Marrelli, M.T., R.S. Malafronte, C. Flores-Mendoza, R. Laurenco-de-Oliveira, J.K. Kloetzel & O. Marinotti, 1999. Sequence analysis of the second internal transcribed spacer of ribosomal DNA in *Anopheles oswaldoi* (Diptera: Culicidae). J. Med. Entomol. 36: 679–684.

Miller, B.R., M.B. Crabtree & H.M. Savage, 1996. Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. Insect Mol. Biol. 5: 93–107.

Miller, B.R., M.B. Crabtree & H.M. Savage, 1997. Phylogenetic relationships of the Culicomorpha inferred from 18S and 5.8S ribosomal DNA sequences (Diptera: Nematocera). Insect Mol. Biol. 6: 105–114.

Muccio, T.D., M. Marinucci, L. Frusteri, M. Maroli, B. Pesson & M. Gramiccia, 2000. Phylodenetic analysis of *Phlebotomus* species belonging to the subgenus *Larroussius* (Diptera, Psychodidae) by ITS2 rDNA sequences. Insect Biochem. Mol. Biol. 30: 387–393.

Nirmala, X., V. Hypsa & M. Zurovec, 2001. Molecular phylogeny of Calyptratae (Diptera: Brachycera): the evolution of 18S and 16S ribosomal rDNA in higher dipterans and their use in phylogenetic inference. Insect Mol. Biol. 10: 475–485.

Oliverio, M., M. Cervelli & P. Mariottini, 2002. ITS2 rRNA evolution and its congruence with the phylogeny of muricid neogastropods (Caenogastropoda, Muricoidea). Mol. Phylogenet. Evol. 25: 63–69.

Olsen, G.J. & C.R. Woese, 1993. Ribosomal RNA: a key to phylogeny. FASEB J. 7: 113–123.

Otsuka, Y., C. Aoki, K. Saito, U.K. Hadi, H. Suzuki & H. Takaoka, 2001. Phylogenetic analyses of a blackfly subgenus *Simulium* (*Nevermannia*) based on mitochondrial 16S ribosomal RNA gene sequences. Jpn. J. Trop. Med. Hyg. 29: 261–266.

Otsuka, Y., H. Takaoka, C. Aoki & W. Choochoti, 2003. Phylogenetic analysis of the subgenus *Himalayum* within the genus *Simulium* s.l. (Diptera: Simuliidae) using mitochondrial 16S rRNA gene sequences. Med. Entomol. Zool. 54: 113–120.

Paskewitz, S.M., D.M. Wesson & F.H. Collins, 1993. The internal transcribed spacer of ribosomal DNA in five members of the *Anopheles gambiae* species complex. Insect Mol. Biol. 2: 247–257.

Porter, C.H. & F.H. Collins, 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 45: 271–279.

Post, R.J. & P.K. Flook, 1992. DNA probes for the identification of members of the *Simulium damnosum* complex (Diptera: Simuliidae). Med. Vet. Entomol. 6: 379–384.

Pramual, P., C. Kuvangkadilok, V. Baimai & C. Walton, 2005. Phylogeography of the black fly *Simulium tani* (Diptera: Simuliidae) from Thailand as inferred from mtDNA sequences. Mol. Ecol. 14: 3989–4001.

Proft, J., W.A. Maier & H. Kampen, 1999. Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. Parasitol. Res. 85: 837–843.

Rothfels, K.H. & R.W. Dunbar, 1953. The salivary gland chromosomes of black fly *Simulium vittatum* Zett. Can. J. Zool. 31: 226–241.

Sambrook, J., E.F. Fritsch & T. Maniatis, 1989. Molecular Cloning: a Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Schlötterer, C., M.T. Hauser, A. Von Haeseler & D. Tautz, 1994. Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. Mol. Biol. Evol. 11: 513–522.

Scott, J.A., W.G. Brogdon & F.H. Collins, 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am. J. Trop. Med. Hyg. 49: 520–529.

Severini, C., F. Silvestrini, P. Mancini, G. La Rosa & M. Marinucci, 1996. Sequence and secondary structure of the rDNA second internal transcribed spacer in the sibling species *Culex pipiens* L. and *Cx. quinquefasciatus* Say (Diptera: Culicidae). Insect Mol. Biol. 5: 181–186.

Shi, M., X.-X. Chen & C. van Achterberg, 2005. Phylogenetic relationships among the Braconidae (Hymenoptera: Ichneumonoidea) inferred from partial 16S rRNA, 28S rDNA D2, 18S rDNA gene sequences and morphological characters. Mol. Phylogenet. Evol. 37: 104–116.

Swofford, D.L., 2002. PAUP (version 4.0b10) . Sinauer Associates, Sunderland, Massachusetts.

Takaoka, H., 1977. Studies on black flies of the Namsei Islands, Japan (Diptera: Simuliidae). III. In six species of the subgenus *Simulium latreille*. Jpn. J. Sanit. Zool. 28: 193–217.

Takaoka, H., 1979. The black flies of Taiwan (Diptera: Simuliidae). Proc. Insects 20: 365–403.

Takaoka, H., 2001. *Simulium* (*Simulium*) *weji* sp. nov. (Diptera: Simuliidae) from Thailand. Jpn. J. Trop. Med. Hyg. 29: 349–354.

Takaoka, H. & P.H. Adler, 1997. A new subgenus, *Simulium* (*Daviesellum*) and a new species, *S.* (*D.*) *courtneyi*, (Diptera: Simuliidae) from Thailand and Peninsular Malaysia. Jpn. J. Trop. Med. Hyg. 25: 17–27.

Takaoka, H. & W. Choochote, 2002. Taxonomic notes on the *griseifrons* species-group in *Simulium* (*Simulium*) (Diptera: Simuliidae) from Thailand: descriptions of new species and description of the male, pupa and larva of *S.* (*S.*) *digrammicum* Edwards. Jpn. J. Trop. Med. Hyg. 30: 115–132.

Takaoka, H. & W. Choochote, 2004a. A list and keys to black flies (Diptera: Simuliidae) in Thailand. Trop. Med. Hlth. 32: 189–197.

Takaoka, H. & W. Choochote, 2004b. Two new species of *Simulium* (*Simulium*) (Diptera: Simuliidae) from Thailand. Trop. Med. Heth. 32: 31–36.

Takaoka, H. & W. Choochote, 2004c. Taxonomic notes on the *griseifrons* species-group of *Simulium* (*Simulium*) (Diptera: Simuliidae) in northern Thailand. Trop. Med. Hlth. 32: 311–327.

Takaoka, H. & W. Choochote, 2005a. Two new species of *Simulium* (*Montisimulium*) (Diptera: Simuliidae) from northern Thailand. Med. Ent. Zool. 56: 21–31.

Takaoka, H. & W. Choochote, 2005b. A new subgenus and a new species of *Simulium* s.l. (Diptera: Simuliidae) from Thailand. Med. Ent. Zool. 56: 33–41.

Takaoka, H. & W. Choochote, 2005c. A new species of *Simulium* (Diptera: Simuliidae) from Thailand. Med. Ent. Zool. 56: 43–47.

Takaoka, H. & W. Choochote, 2005d. Two new species of *Simulium* (Diptera: Simuliidae) from northern Thailand. Med. Ent. Zool. 56: 99–110.

Takaoka, H. & W. Choochote, 2005e. Two new species of *Simulium* (Diptera: Simuliidae) from northwestern Thailand. Med. Ent. Zool. 56: 123–133.

Takaoka, H. & W. Choochote, 2005f. A new species of *Simulium* (*Simulium*) from northern Thailand (Diptera: Simuliidae). Trop. Med. Heth. 33: 95–101.

Takaoka, H. & W. Choochote, 2005g. Two new species of the *griseifrons* species-group of *Simulium* (*Simulium*) (Diptera: Simuliidae) in northern Thailand. Med. Ent. Zool. 56: 219–235.

Takaoka, H. & D.M. Davies, 1995. The Black Flies (Diptera: Simuliidae) of West Malaysia. Kyushu University Press, Fukuoka.

Takaoka, H. & C. Kuvangkadilok, 1999. Four new species of black flies (Diptera: Simuliidae) from Thailand. Jpn. J. Trop. Med. Hyg. 27: 497–509.

Takaoka, H. & K. Saito, 1996. A new species and new records of black flies of black flies (Diptera: Simuliidae) from Thailand. Jpn. J. Trop. Med. Hyg. 24: 163–169.

Takaoka, H. & H. Suzuki, 1984. The black flies (Diptera: Simuliidae) from Thailand. Jpn. J. Sanit. Zool. 35: 7–45.

Tang, J., L. Toé, C. Back, P.A. Zimmermann, K. Pruessand & T.R. Unnasch, 1995. The *Simulium damnosum* species complex: phylogenetic analysis and molecular identification based upon mitochondrially encoded gene sequences. Insect Mol. Biol. 4: 79–88.

Tang, J., L. Toé, C. Back & T.R. Unnasch, 1996. Intra-species heterogeneity of the rDNA internal transcribed specer in the *Simulium damnosum* (Diptera: Simuliidae) complex. Mol. Biol. Evol. 13: 244–252.

Tautz, D., J.M. Hancock, D.A. Webb, C. Tautz & G. Dover, 1988. Complete sequences of the rRNA genes of *Drosophila melanogaster*. Mol. Biol. Evol. 5: 366–376.

Thompson, J.D., T.J. Gibson, F. Plewnniak, F. Jeanmougin & D.G. Higgins, 1997. The clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analyses tools. Nucleic Acid Res. 24: 4876–4882.

Toma, T., I. Miyagi, M.B. Crabtree & B. Miller, 2002. Investigation of the *Aedes* (*Stegomyia*) *flavopictus* complex (Diptera: Culicidae) in Japan by sequence analysis of the internal transcribed spacers of ribosomal DNA. J. Med. Entomol. 13: 461–468.

Walton, C., M. Handley, C. Kuvangkadilok, F.H. Collins, R.E. Harbach, V. Baimai & K.R. Butlimn, 1999. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. Med. Vet. Entomol. 13: 24–32.

Weekers, P.H.H., J.F. De Jonckheere & H.J. Dumont, 2001. Phylogenetic relationships inferred from ribosomal ITS sequences and biogeographic patterns in representatives of the genus *Calopteryx* (Insect: Odonata) of the West Mediterranean and adjacent West European zone. Mol. Phylogenet. Evol. 20: 89–99.

Wesson, D.M., C.H. Porter & F.H. Collins, 1992. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). Mol. Phylogenet. Evol. 1: 253–269.

Xiong, B. & T.D. Kocher, 1991. Comparison of mitochondrial DNA sequences of seven morphospecies of black flies (Diptera: Simuliidae). Genome 34: 306–311.

Xu, J.N. & F.Y. Qu, 1997. Ribosomal RNA differences between species A and D of the *Anopheles dirus* complex of mosquitoes from China. Med. Vet. Entomol. 11: 134–138.

Yagura, T., M. Yagura & M. Muramatsu, 1979. *Drosophila melanogaster* has different ribosomal RNA sequences on X and Y chromosomes. J. Mol. Biol. 133: 533–547.

Young, I. & A.W. Coleman, 2004. The advantages of the ITS2 region of the nuclear rDNA cistron for analysis of phylogenetic relationships of insects: a *Drosophila* example. Mol. Phylogenet. Evol. 30: 236–242.