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Geographical versus ecological isolation of closely related black flies (Diptera: Simuliidae) inferred from phylogeny, geography, and ecology

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Abstract To investigate patterns of geographical and ecological separation among morphologically similar, closely related species of black flies, we integrated ecological, geographical, and phylogenetic information, based on multiple gene sequences, for 12 species in the subgenus *Gomphostilbia* in Thailand. Molecular characters supported the monophyly of the *Simulium ceylonicum* species group, but not of the *Simulium batoense* species group, suggesting that revisionary work is needed for the latter. Both ecological and geographical isolation of similar taxa were revealed. Stream velocity and altitude were among the principal ecological factors differing between closely related species. Most closely related species in the subgenus *Gomphostilbia* overlap geographically, suggesting the possibility of sympatric speciation driven by ecological divergence. Geographical isolation via dispersal also might have contributed to species divergence, while Pleistocene climate changes possibly influenced population genetic structure, demographic history, and speciation of some members of the subgenus.

Keywords *Gomphostilbia* · Phylogeny · *Simulium* · Speciation

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Introduction

Geographical isolation and ecological divergence contribute to species diversification. Ecological divergence has been suggested as an important factor promoting speciation (Orr and Smith 1998; Schluter 1998, 2001). Populations in different environments are under different selection regimes, which could result in genetic divergence leading to reproductive isolation (Mayr 1963). Alternatively, reproductive isolation could result from processes not linked directly to ecological divergence, such as genetic drift (Coyne and Orr 2004).

Integrating phylogenetic information with environmental factors and geographical distributions of closely related species can be used to indicate the relative evolutionary importance of ecological divergence and geographical isolation. For example, Graham et al. (2004) combined geographical distribution data, environmental geographic information system layers, environmental

niche models, and phylogentic information to investigate speciation processes in dendrobatid frogs. They concluded that ecological niche played a significant role in speciation because sister taxa typically differ ecologically regardless of their geographical distributions. Peterson et al. (1999) used ecological niche modeling to compare geographical distributions of sister taxa of birds, mammals, and butterflies in southern Mexico. Their results indicated that geographic isolation was the primary factor driving speciation, whereas ecological divergence evolved later.

Distributions of closely related species of black flies often are related to different ecologies (Adler 1988; Adler and McCreadie 1997; McCreadie and Adler 1998). For example, sibling species in the *S. vittatum* complex occupy streams with different thermal profiles (Adler and Kim 1984), and those in the African *S. damnosum* complex are associated with different forest types (Boakye et al. 1998), suggesting that ecological conditions play a significant role in the evolution of black flies. Ecological divergence, for example, has been suggested as a driving force in the speciation of black flies (Joy and Conn 2001; Joy et al. 2007; Rothfels 1989).

The species-rich subgenus *Gomphostilbia* contains nearly 10 % of all nominal black flies worldwide (Adler and Crosskey 2012), including more than 30 species in Thailand, one of which is a complex of seven cytoforms (Kuvangkadilok et al. 2008; Pramual and Wongpakam 2011). *Gomphostilbia* has been investigated morphologically (Takaoka and Choochote 2004), cytologically (Jitklang et al. 2008; Kuvangkadilok et al. 2008; Phasuk et al. 2005; Pramual and Wongpakam 2011), molecularly (Pramual et al. 2011a; b), and ecologically (Pramual and Kuvangkadilok 2009; Pramual and Wongpakam 2010). Previous phylogenetic studies indicate that this subgenus is monophyletic (Phayuhasena et al. 2010; Pramual et al. 2011a; Thanwisai et al. 2006), making it an ideal candidate for investigating the importance of ecology in black fly evolution.

Closely related species of the subgenus *Gomphostilbia* in Thailand are difficult to distinguish morphologically and often require chromosomal or molecular identification (Jitklang et al. 2008; Pramual et al. 2011a), raising the question of how these similar species occupy available habitat. Our objective, therefore, was to examine ecological divergence and geographical isolation by integrating phylogenetic information, based on multiple gene sequences, with geographical distributions and ecological conditions of the larval habitats of a set of closely related, nearly isomorphic species in the subgenus *Gomphostilbia* in Thailand.

Materials and methods

Samples, identifications, and ecological measurements

Larval black flies were collected throughout Thailand (Table 1) and fixed in Carnoy's solution (3:1, 95 % ethanol: glacial acetic acid) for cytogenetic and molecular analysis (Pramual et al. 2011a). Samples were identified morphologically (Takaoka and Choochote 2004), and cytologically (Jitklang et al. 2008; Kuvangkadilok et al. 2008). Ecological data for larval habitats, including altitude, conductivity, depth, pH, velocity, and width, were obtained from previous publications (Pramual and Kuvangkadilok 2009; Pramual and Wongpakam 2010) and unpublished data (C. Kuvangkadilok, P. Pramual, S. Jitklang, U. Tangkawanit).

DNA extraction, polymerase chain reaction, and sequencing

Treatment of larvae for molecular and cytogenetic studies followed the protocol of Pramual et al. (2005, 2011a). DNA was extracted from larval heads using a Genomic DNA extraction kit (RBC BioScience, Taiwan) and kept at −20°C. Polymerase chain reaction (PCR) was conducted for cytochrome oxidase II (COII), using primers TL2-J-3034 (5′-ATTATGGCAGAT TAGTGCA-3′) and TK-N-3785 (5′-GTTTAAGAGACCAGTACTTG-3′), and 18 S/ITS1, using primers 18 s/sd5′ (5′-TGGTGCATGGCCGTTCTTAG-3′) and 5.8 s/sd3′ (5′-GTCGATGTTCATGTGTCCTGC-3′) (Simon et al. 1994). We used the PCR conditions of Conflitti et al. (2010). PCR products were checked with 1 % agarose gel electrophoresis and cleaned using a HiYield™ Gel/PCR DNA Extraction Kit (RBC BioScience, Taiwan). Sequencing was performed at Macrogen sequencing service (Seoul, Korea), using the same primers as in the PCR. A total of 38 samples representing 12 species of the subgenus *Gomphostilbia* were sequenced for mitochondrial (COII) and nuclear genes (18 S/ITS1). Representative haplotypes were deposited with GenBank (Table 1). Previously published COI barcoding sequences (Pramual et al. 2011a) were also included in phylogenetic analyses. The final alignment and trees obtained have been deposited with TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S12315).

Data analysis

DNA sequences were aligned using CLUSTAL X (Thompson et al. 1997), with a final visual inspection. Phylogenetic analyses were conducted for the combined data set (COI, COII, and 18 S/ITS1), with sequences from *S. fenestratum*

Table 1 Black fly species, collection sites, and GenBank accession numbers for COI, COII, and 18 S/ITS1 sequences

Species	Locality	Collection date	GenBank accession number		
			COI	COII	18 S/ITS1
batoense species group					
S. angulistylum Takaoka and Davies 1995	Hinsamchan waterfall, Loei	14 October 2007	HM775239	JN547760	JN547775
	Khaopranarai waterfall, Ranong	13 December 2006	HM775236	JN547761	JN547775
	Pangsida waterfall, Srakeaw	27 November 2004	HM775237	JN547762	JN547775
S. decuplum Takaoka and Davies 1995	Huai Sai luaeng waterfall, Chiangmai	14 December 2004	HM775286	JN547758	JF505387
	Maetho, Chiangrai	9 January 2007	HM775284	JN547759	JF505387
S. gombakense Takaoka and Davies 1995	Mae klang waterfall, Chiangmai	9 July 2006	HM775247	JN547755	JN547773
	Mae klang waterfall, Chiangmai	9 July 2006	HM775250	JN547757	JN547773
	Ban Pha Mon, Chiangmai	14 December 2004	HM775249	JN547756	JN547772
S. siamense Takaoka & Suzuki, 1984	Huai Kaew Pang, Amnatchareon	23 February 2008	HM775226	JF916872	JF505387
	Huai Kayeng, Kanchanaburi	29 February 2004	HM775229	JN547751	JF505387
	Khaoyai National Park, Nakhon Ratchasima	25 July 2004	HM775230	JN547752	JF505387
	Mae La Noi, Mae Hong Son	11 December 2004	HM775231	JN547753	JF505387
	Huai Lao waterfall, Loei	9 February 2008	HM775225	JN547748	JF505387
	Ban Krangcamp, Petchaburi	16 August 2003	HQ738668	JN547750	JN547774
	Ban Kamkeaw, Amnatchareon	23 February 2008	HM775228	JN547749	JF505387
ceylonicum species group					
S. asakoae Takaoka and Davies 1995	Ban Pha Mon, Chiangmai	14 February 2004	HM775266	JN547730	JF505387
	Ban Nam Kad, Mae Hong Son	13 December 2004	HM775265	JN547729	JF505387
	Ban Na Ngew, Mae Hong Son	13 December 2004	HM775264	JN547728	JN547767
	Huai Toei waterfall, Loei	10 February 2008	HM775261	JN547727	JF505387
	Huai Toei waterfall, Loei	6 April 2008	HM775262	JN547731	JN547765
	Khaoyai National Park, Nakhon Ratchasima	8 August 2003	HM775263	JN547732	JN547766
S. inthanonense Takaoka and Suzuki, 1984	Huai Sai Leaung waterfall, Chiangmai	14 December 2004	HM775255	JN547741	JF505387
	Huai Sai Leaung waterfall, Chiangmai	14 December 2004	HM775256	JN547742	JF505387
	Huai Sai Leaung waterfall, Chiangmai	14 December 2004	HM775257	JN547743	JF505387
S. curtatum Jitklang et al. 2008	Siri Phum waterfall, Chiangmai	13 December 2002	HM775242	JN547744	JF505387
	Siri Phum waterfall, Chiangmai	13 December 2002	HM775243	JN547745	JF505387
	Siri Phum waterfall, Chiangmai	13 December 2002	HM775244	JN547746	JF505387
	Ban Khun Huai Hang, Chiangmai	14 December 2004	HM775246	JN547747	JN547771

Table 1 (continued)

Species	Locality	Collection date	GenBank accession number		
			COI	COII	18S/ ITS1
S. doisaketense Jitklang et al. 2008	Khun Korn waterfall, Chiangrai	8 December 1998	HM775267	JN547733	JF505387
	Khun Korn waterfall, Chiangrai	8 December 1998	HM775269	JN547734	JF505387
S. trangense Jitklang et al. 2008	Kham Toei waterfall, Kalasin	22 December 2007	HM775258	JN547739	JN547768
	Phu Pha Kham, Mukdahan	25 November 2009	HM775260	JN547740	JN547768
S. sheilae Takaoka and Davies 1995	Ngaw waterfall, Ranong	13 December 2006	HM775274	JN547738	JN547769
	Chong Sa Ngam, Sisaket	9 December 2007	HM775270	JN547735	JN547770
	Huai Yang waterfall, Prachuapkhirikhun	4 June 1999	HM775272	JN547737	JN547769
	Ban Sang Keaw, Sakonnakhon	22 December 2007	HM775271	JN547736	JN547770
varicorne species group					
S. chumpornense Kuvangkadilok and Takaoka 2000	Kapo waterfall, Chumporn	6 November 1999	HM775279	JN547763	JN547777
S. kuvangkadilokae Pramual and Tangkawanit 2008	Pha Chom Tawan waterfall, Nakhonratchasima	30 June 2007	HM775276	JN547764	JN547776

(accession number JF916884) and *S. takense* (accession number JF916877) as outgroups. Congruence between separate genes was tested using the partition homogeneity test (Farris et al. 1995) with 1,000 replicates implemented in PAUP* v. 4.10b (Swofford 2002). The results indicated no significant differences between the separate gene regions (P=0.970). To select the best-fit DNA substitution model for phylogenetic analysis, we used the program jModeltest v.0.1.1 (Posada 2008), based on the Akaike information criterion (AIC) algorithm. The best-fit model for combined sequences was the TIM2+I+G (Tamura and Nei 1993) with gamma shape parameter (G) of 0.7650 and proportion of invariable sites (I) of 0.6540.

The neighbor-joining (NJ) tree was calculated in PAUP*, based on the best-fit selected model. Bootstrap support was estimated using 1,000 replicates. Maximum parsimony analyses were performed in PAUP* (Swofford 2002), using a heuristic search with 1,000 random addition sequence replicates, TBR branch swapping, and MulTrees effect. Bootstrap support was estimated for 1,000 replicates. Phylogenetic relationships were analyzed by Bayesian methods using MRBAYES 3.04b (Huelsenbeck and Ronquist 2001). The best-fit model for Bayesian analysis was selected by hierarchical likelihood ratio tests implemented in MrModeltest (Nylander 2004). The general time-reversible (GTR) model (Rodriguez et al. 1990) with gamma distribution shape parameter of 0.8406 and proportion of invariable sites of 0.6625 was selected. Bayesian analysis was run for 2,000,000 generations, with a sampling frequency of 100 generations. Tracer version 1.5 (Rambaut and Drummond 2004) was used for visual inspection of the point where the log likelihood is stationary. Trees sampled before this point were discarded as burn-in. The remaining trees of two simultaneous runs were included in posterior probability calculations.

To determine ecological divergence among closely related taxa, we used principal components analysis (PCA), reducing the number of stream variables into groups of independent components (PCs). PCs with eigenvalues greater than 1.0 were retained as variables. To interpret PCs, Spearman rank correlations were used to detect relationships between stream variables and PC scores (McCreadie et al. 2006). To determine if species differed significantly along environmental space, multivariate analysis of variance (MANOVA) was used in which species were assigned as a fixed factor and the PC score for each axis was the dependent variable (Graham et al. 2004).

Results

DNA sequence variation and phylogenetic relationships

The combined dataset was 1,875 bp (586 bp for COI, 697 bp for COII, and 592 bp for 18 S/ITS1), with 460 variable positions, of which 408 were parsimony informative. All three phylogenetic analyses revealed similar tree topologies; thus, only one of the ten maximum parsimony (MP) trees is

shown (Fig. 1), with tree length of 1,246 steps and consistency index (CI) of 0.491. The MP tree revealed two main clades (I and II) among the 12 species. Clade I comprised nine species in four subclades: *S. asakoae*, *S. doisaketense*, *S. inthanonense*, *S. curtatum* (subclade I-1), *S. sheilae*, *S. trangense* (subclade I-2), *S. gombakense*, *S. decuplum* (subclade I-3), and *S. siamense* (subclade I-4). Clade II consisted of *S. angulistylum*, *S. chumpornense*, and *S. kuvangkadilokae*.

The monophyletic *ceylonicum* group was derived within the subgenus. The *batoense* group was polyphyletic; one species was clustered with the *S. varicorne* group in Clade II, and three species belonged to Clade I, which included members of the *ceylonicum* group. Two species of the *varicorne* group (*S. chumpornense* and *S. kuvangkadilokae*) formed a monophyletic clade and were clustered with *S. angulistylum*, which currently is recognized as a member of the *batoense* group.

Three of the six species of the *ceylonicum* group were monophyletic. Four samples of *S. asakoae* formed a clade with strong bootstrap support (100 %) but two samples clustered with *S. doisaketense*. The clade of *S. asakoae* was sister to the remaining members of Clade I-1. *Simulium inthanonense* was monophyletic and sister to the clade comprised of individuals of *S. asakoae* and *S. doisaketense*. *Simulium curtatum* was monophyletic and sister to the clade that comprised *S. asakoae*, *S. doisaketense*, and *S. inthanonense*. *Simulium trangense* was monophyletic but in

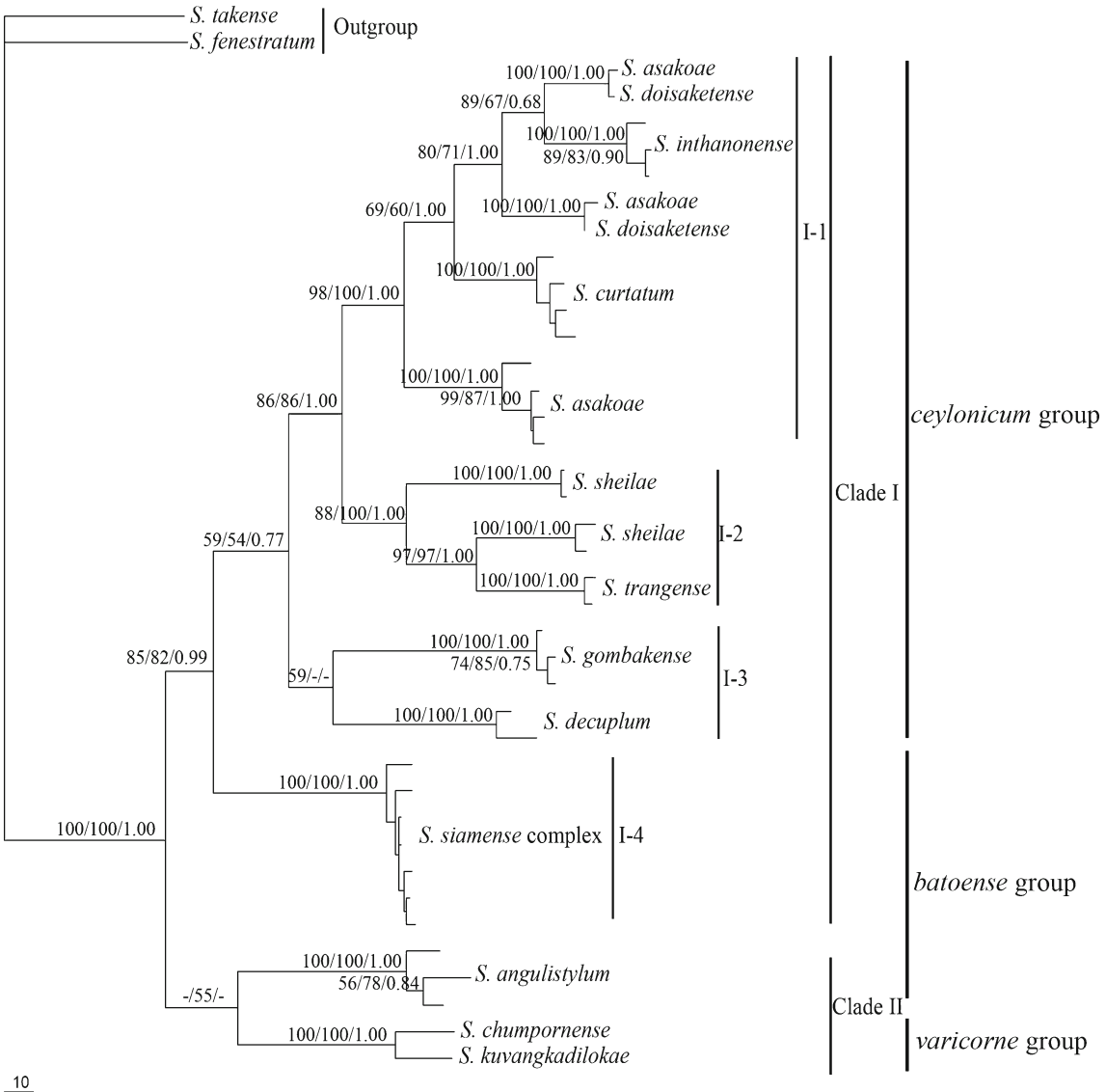

Fig. 1 Maximum parsimony tree for *Gomphostilbia* in Thailand, based on combined dataset of the cytochrome oxidase I, cytochrome oxidase II, and 18 S rRNA/ITS1 sequences. Bootstrap values for maximum parsimony/neighbor joining and posterior probability for the Bayesian analysis are shown above or near the branch. *Bar* Ten changes

the clade of *S. sheilae*, making the latter paraphyletic. *Simulium doisaketense* was not monophyletic; one individual clustered with an individual of *S. asakoae* sister to the clade of *S. inthanonense*. Another individual clustered with an individual of *S. asakoae* and was sister to the *S. inthanonense* and *S. asakokae*+*S. doisaketense* clades.

All members of the *batoense* group were monophyletic. *Simulium angulistylum* was monophyletic, with strong bootstrap support, but clustered with *S. chumpornense* and *S. kuvangkadilokae* of the *varicorne* group. *Simulium siamense* was monophyletic and the sister group of all other members of Clade I, which consisted of members of the *batoense* group (in part) and *ceylonicum* group. *Simulium gombakense* and *S. decuplum* were monophyletic sister species in MP analysis, albeit with low bootstrap support (59 %). This cluster was not resolved in NJ and Bayesian analyses.

Geographical and ecological patterns

Clade I-1: S. asakoae, S. curtatum, S. doisaketense, and S. inthanonense

This clade was represented by members of the *ceylonicum* group. Among the 37 sampling sites with at least one member of this clade, *S. asakoae* was found at 64.9 % (24 sites), *S. curtatum* at 21.6 % (8 sites), *S. doisaketense* at 16.2 % (6 sites), and *S. inthanonense* at 13.5 % (5 sites). Although their geographical ranges overlapped, these species rarely co-occurred. *Simulium asakoae* was found with *S. inthanonense* at two sites and with *S. curtatum* at one site. *Simulium doisaketense* was found with *S. asakoae* at one site. *Simulium asakoae* occurred over a wide range of altitudes, from 395 m to 1,615 m above sea level (a.s.l.) (Table 2). *Simulium doisaketense* also was found over a range of altitudes, but at lower elevations (304–870 ma.s.l.). *Simulium curtatum* and *S. inthanonense* were restricted to high-altitude streams (>1,100 ma.s.l.) (Table 2).

PCA revealed two principal components (PCs) with eigenvalues >1.0. These components explained 60.51 % of the total environmental variation among species (Table 3). PC-1 explained 33.75 % of the variation and was related significantly positively to stream width, depth, velocity, pH, and conductivity, and negatively to altitude. PC-2 explained 26.76 % of the variation and was related significantly positively to stream width and depth and negatively to pH and conductivity (Fig. 2). MANOVA analysis revealed significant separation of the species along both PCs (PC-1, $F_{3,\ 37}$= 6.389, P=0.001; PC-2, $F_{3,37}$=4.325, P=0.010).

Clade I-2: S. sheilae and S. trangense

Simulium sheilae was restricted to southern Thailand, whereas *S. trangense* was distributed widely in southern, central, and northeastern Thailand. Both species occupied a wide range of stream conditions (Table 2) but were not found above 600 m. Among the 13 sampling sites where at least one member of this clade was found, *S. trangense* occurred at 84.6 % (11 sites), *S. sheilae* at 38.5 % (5 sites), and both species at 23.1 % (3 sites).

PCA revealed three components that explained 91 % of the total variation in stream variables. PC-1 explained 49.49 % of the variation (Table 3), and was significantly positively related to pH and conductivity. PC-2 accounted for 24.80 % of the variation, and was related significantly positively to stream depth and negatively to altitude (Fig. 3). PC-3 accounted for 16.71 % of the variation but was not significantly related to stream variables. MANOVA analysis indicated that the species were not significantly different along PC-1 and PC-2 (PC-1, $F_{1,\ 14}$=0.430, P=0.525; PC-2, $F_{1,\ 14}$=0.050, P=0.827; PC-3, $F_{1,\ 14}$=1.142, P=0.258).

Table 2 Stream variables for larval habitats of 12 species in the black fly subgenus *Gomphostilbia* in Thailand

Species group	Species (n)[a]	Width (m) (min–max)	Depth (m) (min–max)	Velocity (m/s) (min–max)	pH (min–max)	Conductivity (μS/cm) (min–max)	Altitude (m) (min–max)
ceylonicum	*S. asakoae* (23)	1.17 (0.13–5.00)	0.11 (0.01–0.67)	0.42 (0.10–0.80)	7.60 (6.30–8.57)	79.35 (8.00–259.00)	900.82 (395.00–1,615.00)
	S. curtatum (7)	0.97 (0.20–2.50)	0.16 (0.03–0.53)	0.49 (0.20–0.76)	7.19 (6.43–8.20)	25.87 (10.10–50.00)	1,402.86 (1,250.00–1,615.00)
	S. doisaketense (6)	2.79 (1.50–3.75)	0.18 (0.05–0.30)	0.53 (0.23–0.96)	7.59 (7.20–8.40)	47.32 (27.20–130.00)	684.00 (304.00–870.00)
	S. inthanonense (5)	0.36 (0.20–0.87)	0.04 (0.02–0.05)	0.23 (0.03–0.50)	7.71 (7.36–8.20)	29.36 (10.10–50.00)	1,342.00 (1,125.00–1,615.00)
	S. sheilae (5)	1.69 (0.40–5.00)	0.10 (0.06–0.17)	0.64 (0.22–1.37)	6.51 (6.10–7.00)	26.29 (15.30–44.40)	162.40 (55.00–314.00)
	S. trangense (9)	2.24 (0.30–6.50)	0.17 (0.03–0.57)	0.49 (0.20–1.00)	6.90 (5.01–7.47)	33.02 (8.00–62.40)	221.44 (50.00–600.00)
batoense	*S. gombakense* (8)	2.55 (0.20–10.00)	0.12 (0.01–0.30)	0.48 (0.20–1.00)	7.53 (6.30–8.07)	64.20 (19.70–295.70)	618.63 (120.00–1,302.00)
	S. decuplum (19)	2.49 (0.30–5.33)	0.13 (0.01–0.28)	0.48 (0.29–1.10)	7.93 (7.20–8.80)	112.03 (7.80–280.33)	628.21 (50.00–1,261.00)
	S. siamense (13)	3.57 (0.37–15.00)	0.13 (0.02–0.30)	0.53 (0.30–0.80)	7.21 (5.70–8.13)	107.05 (15.00–324.93)	363.31 (156.00–700.00)
	S. angulistylum (11)	4.49 (0.40–25.00)	0.09 (0.01–0.21)	0.95 (0.38–1.60)	6.38 (5.50–7.25)	31.89 (6.00–184.00)	449.36 (110.00–1,153.00)
varicorne	*S. chumpornense* (4)	6.25 (3.00–15.00)	0.14 (0.06–0.20)	0.37 (0.24–0.50)	7.97 (7.20–8.40)	226.45 (20.00–495.50)	44.00 (40.00–48.00)
	S. kuvangkadilokae (10)	3.50 (0.32–20.00)	0.16 (0.04–0.33)	0.70 (0.33–1.02)	6.16 (5.50–7.49)	21.78 (1.00–43.70)	226.70 (132.00–415.00)

[a] n represents number of sampling sites

Table 3 Results of principle complment ananlysis (PCA) and Spearman's rank correlations between stream variables and principal components (PCs) for phylogenetically derived clades

Variable	Min	Max	Mean ± SE	Principal components		
				PC-1	PC-2	PC-3
Clade I-1 (n=41) [a]						
Width	0.13	5.00	1.27±0.19	0.589*	0.628*	-
Depth	0.01	0.67	0.12±0.02	0.309	0.683*	-
Velocity	0.03	0.96	0.42±0.03	0.448*	0.456*	-
Altitude	304.00	1615.00	1008.61±61.07	−0.772*	0.004	-
pH	6.30	8.57	7.54±0.08	0.506*	−0.568*	-
Conductivity	8.00	259.00	59.44±8.91	0.708*	−0.428*	-
% Variation explained in PCA						
Proportion				33.75	26.76	-
Cumulative				33.75	60.51	-
Clade I-2 (n=14)						
Width	0.30	6.50	2.04±0.53	0.463	0.620	0.412
Depth	0.03	0.57	0.14±0.03	0.101	0.831*	0.388
Velocity	0.20	1.37	0.53±0.09	−0.637	−0.168	0.165
Altitude	50.00	600.00	200.36±42.27	−0.097	−0.670*	0.595
pH	5.01	7.47	6.76±0.17	0.783*	−0.040	0.135
Conductivity	8.00	62.40	30.62±4.51	0.841*	−0.132	0.392
% Variation explained in PCA						
Proportion				49.49	24.80	16.71
Cumulative				49.49	74.29	91.00
Clade I-3 (n=27)						
Width	0.20	10.00	2.51±0.44	0.799*	−0.213	-
Depth	0.01	0.30	0.13±0.02	0.758*	−0.418	-
Velocity	0.20	1.10	0.48±0.04	0.745*	−0.124	-
Altitude	50.00	1302.00	625.37±69.42	−0.372	−0.419	-
pH	6.30	8.80	7.81±0.10	0.222	0.838*	-
Conductivity	7.80	295.70	30.62±4.51	0.517*	0.681*	
% Variation explained in PCA						
Proportion				38.36	29.41	
Cumulative				38.36	67.77	
Clade II (n=25)						
Width	0.32	25.00	4.37±1.28	0.293	0.480	−0.263
Depth	0.01	0.33	0.13±0.02	0.313	−0.689*	−0.439
Velocity	0.24	1.60	0.76±0.07	−0.806*	0.025	0.335
Altitude	40.00	1153.00	295.44±58.12	−0.802*	−0.180	0.356
pH	5.50	8.40	6.55±0.18	0.662*	0.333	0.425
Conductivity	1.00	495.50	58.97±23.58	0.635*	0.018	0.228
% Variation explained in PCA						
Proportion				39.01	19.64	18.26
Cumulative				39.01	58.65	76.91

*P<0.01

[a] n represents number of sampling sites

Clade I-3: S. decuplum and S. gombakense

Simulium decuplum and *S. gombakense* were geographically widespread in Thailand over a broad range of stream conditions, and were among the few species inhabiting a wide range of altitudes (50–1,302 m a.s.l.) (Table 2). Of 27 sites where they were recorded, *S. decuplum* was found at 70.4 % (19 sites) and *S. gombakense* at 29.6 % (8 sites). Although these species overlapped geographically, they never occurred together.

PCA extracted two PCs with eigenvalues >1.00, which explained 67.77 % of the total variation in stream conditions (Table 3). PC-1 accounted for 38.36 % of the variation and was related significantly positively to stream width, depth,

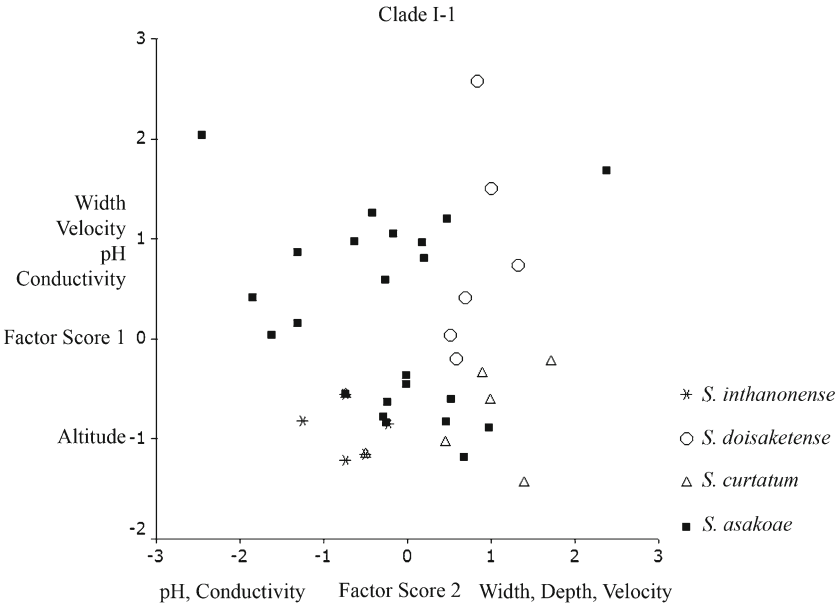

Fig. 2 Principal components analysis (PCA) of stream variables for four species in Clade I-1 of subgenus *Gomphostilbia* in Thailand. Factor score 1 (PC-1) explained 33.75 % of the total variation and was related positively to stream width, depth, velocity, pH, and conductivity and negatively to altitude. Factor score 2 (PC-2) explained 26.76 % of the total variation and was positively related to stream width, depth, and velocity and negatively to pH and conductivity. The four taxa of Clade I-1 were separated in environmental space along the two axes

velocity, and conductivity. PC-2 accounted for 29.41 % of the variation, which largely explained the chemical conditions of the streams, as this component was related significantly positively to pH and conductivity (Fig. 4). MANOVA analysis revealed no significant differences along PC axes (PC-1, $F_{1,\ 27}$=0.267, P=0.610; PC-2, $F_{1,\ 27}$=1.568, P=0.222).

Clade II: S. angulistylum, S. chumpornense, and S. kuvangkadilokae

Simulium chumpornense was restricted to southern and western Thailand, whereas *S. kuvangkadilokae* was found only in northeastern Thailand. *Simulium angulistylum* was widespread throughout the country. Among 25 sites with at least one member of this clade, *S. angulistylum* was found at 48 % (12 sites), *S. kuvangkadilokae* at 40 % (10 sites), and *S. chumpornense* at 16 % (4 sites). *Simulium angulistylum* and *S. kuvangkadilokae* co-occurred at one site, but no stream had all three species.

Simulium chumpornense and *S. kuvangkadilokae* inhabited streams of similar size, depth, velocity, and pH, but different conductivity and altitude (Table 2). *Simulium chumpornense* occurred at higher conductivity and lower altitude, whereas *S. kuvangkadilokae* was found in streams with lower conductivity but higher altitude (Table 2). *Simulium angulistylum* occupied a wide range of stream conditions from 110 m to 1,153 m a.s.l.

PCA revealed three PCs with eigenvalues >1.00 (Table 3). These PCs accounted for 76.91 % of the total variation in

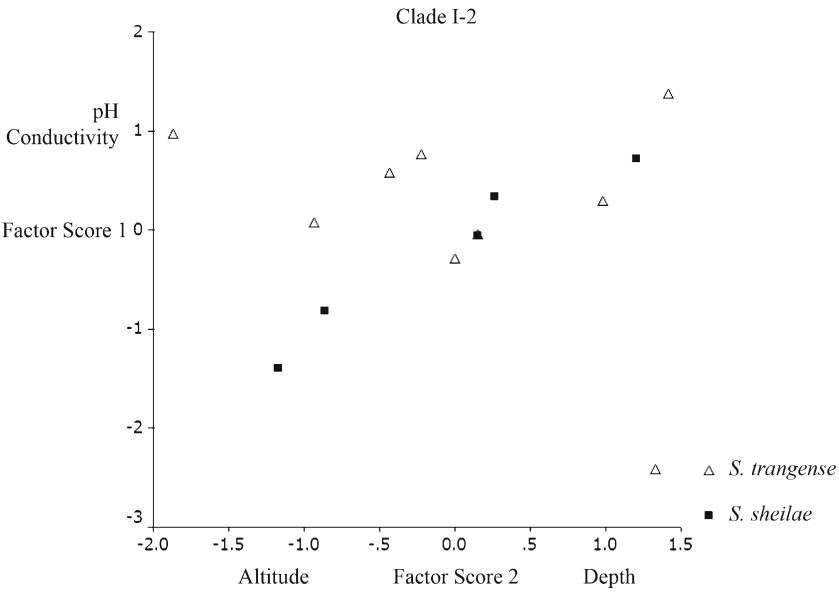

Fig. 3 PCA of stream variables for two species in Clade I-2 of subgenus *Gomphostilbia* in Thailand. Factor score 1 (PC-1) explained 49.49 % of the total variation and was positively related to water conductivity and pH. Factor score 2 (PC-2) explained 24.80 % of the total variation and was positively related to stream depth and negatively to altitude. The two taxa of this clade were not separated in environmental space

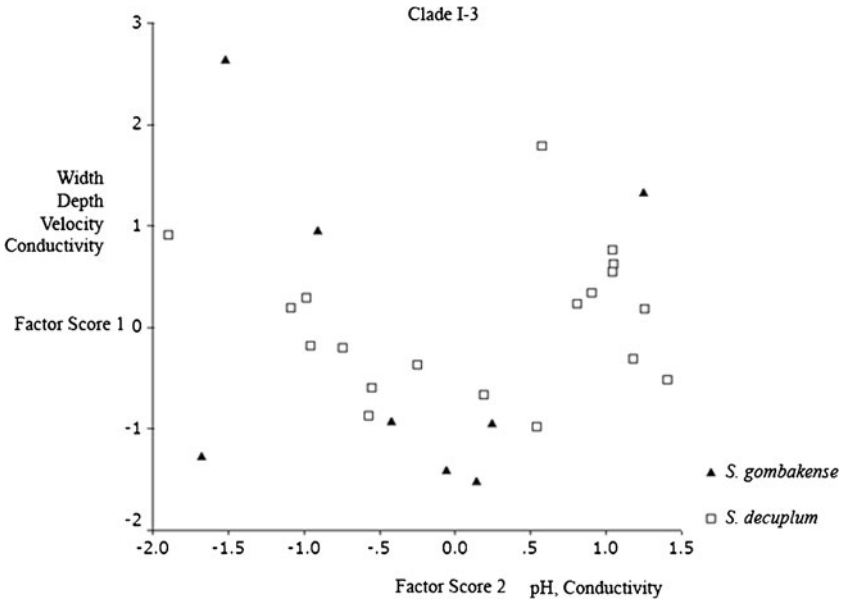

Fig. 4 PCA of stream variables for two species in Clade I-3 of subgenus *Gomphostilbia* in Thailand. Factor score 1 (PC-1) explained 38.36 % of the total variation and was related positively to stream width, depth, velocity, and conductivity. Factor score 2 (PC-2) explained 29.41 % of the total variation and was related positively to water conductivity and pH. The two taxa of this clade were not separated in environmental space

stream conditions among species. PC-1 accounted for 39.01 % and was related positively to stream chemistry (pH, conductivity) and negatively to physical conditions (velocity, altitude). PC-2 accounted for 19.64 % of the total variation and was related significantly negatively to stream depth (Fig. 5). PC-3 accounted for 18.26 % of the variation but showed no significant relationship with stream conditions. MANOVA indicated significant differences along PC-1 ($F_{1,\ 25}$=13.381, P<0.001) but not PC-2 ($F_{1,25}$=0.992, P=0.387) or PC-3 ($F_{1,25}$=3.226, P=0.059).

When *S. angulistylum* was omitted from analysis, PCA revealed three PCs that explained 81.68 % of the total variation in stream conditions. PC-1 accounted for 41.93 % of the variation and was positively related to stream width and significantly negatively related to stream velocity and altitude. PC-2 accounted for 21.44 % of the variation and was related significantly positively to stream depth. PC-3 accounted for 18.31 % of the variation but was not significantly related to the stream variables. MANOVA revealed significant differences along PC-1 ($F_{1,14}$=48.128, P<0.001) but not PC-2 ($F_{1,14}$=0.128, P=0.727) or PC-3 ($F_{1,14}$=0.393, P=0.543).

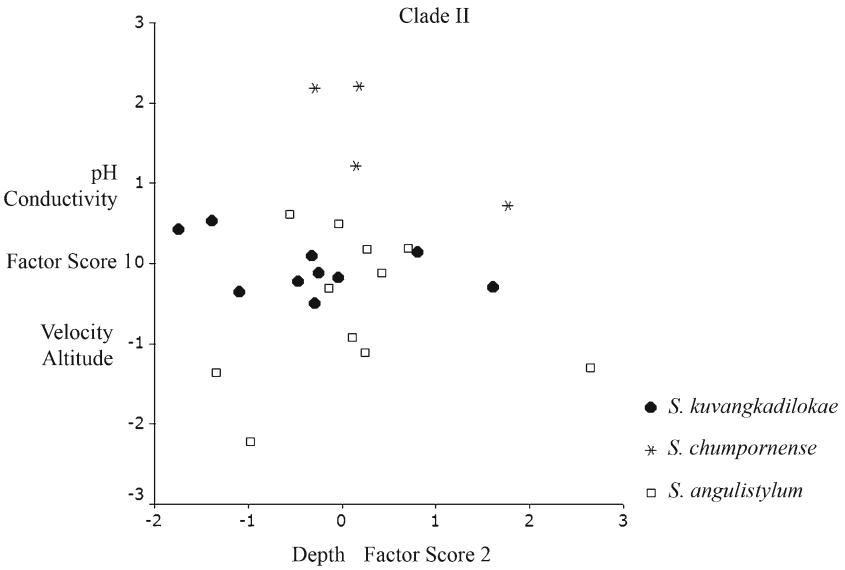

Fig. 5 PCA of stream variables for three species in Clade II of subgenus *Gomphostilbia* in Thailand. Factor score 1 (PC-1) explained 39.01 % of the total variation and was related positively to stream width and related negatively to velocity and altitude. Factor score 2 (PC-2) explained 19.64 % of the total variation and was related positively to stream depth. *Simulium chumpornense* was distributed separately from other taxa of the clade along the PC-1 axis

Discussion

Phylogenetic relationships of *Gomphostilbia* in Thailand

Previous phylogenetic analyses have shown that *Gomphostilbia* in Thailand is a monophyletic group but placement of the species into species groups needs revision (Phayuhasena et al.

2010; Pramual et al. 2011a;; Thanwisai et al. 2006). Our results confirm the monophyly of the *ceylonicum* group, in accord with previous morphological (Takaoka and Choochote 2004), chromosomal (Jitklang et al. 2008), and molecular studies (Phayuhasena et al. 2010; Pramual et al. 2011a; Thanwisai et al. 2006). As with previous studies (Phayuhasena et al. 2010; Pramual et al. 2011a; Thanwisai et al. 2006), we also found that the *batoense* group is not monophyletic. Four species of the *batoense* group in our study formed three clades. *Simulium decuplum* and *S. gombakense* were the sister clade to the *ceylonicum* clade, in agreement with the phylogeny based on the COI barcode gene (Pramual et al. 2011a). Morphologically, females of *S. gombakense* and *S. decuplum* have an enlarged sensory vesicle (Takaoka and Davies 1995; Takaoka et al. 2010), which also is found in four species of the *ceylonicum* group (*S. curtatum*, *S. inthanonense*, *S. sheilae*, and *S. trangense*) and one species of the *batoense* group (*S. parahiyangum*). Although our analysis did not include *S. parahiyangum*, Phayuhasena et al. (2010) found that this species is sister to *S. gombakense* and that these two species are the sister clade of the *ceylonicum* group. We therefore suggest a transfer of *S. decuplum* and *S. gombakense* to the *ceylonicum* species group, in which case the principal morphological criteria currently used to define the species groups of *Gomphostilbia* (Takaoka et al. 2011) would no longer apply universally.

Simulium angulistylum was clustered with the *varicorne* group, in agreement with analyses based on ITS2 (Thanwisai et al. 2006) and COI (Pramual et al. 2011a). However, a close relation between *S. angulistylum* and the *varicorne* group was not observed in the multiple gene analysis of Phayuhasena et al. (2010). Given the different phylogenetic analyses and lack of shared morphological characters between *S. angulistylum* and the *varicorne* group, placement of this species in a group needs further study.

Patterns of geographical isolation and ecological divergence among closely related species

Our results revealed three patterns of geographical isolation and ecological divergence for closely related, nearly isomorphic taxa: (1) species overlap geographically but are isolated by habitat and are ecologically divergent (Clade I-1), (2) species overlap geographically but are isolated by habitat while having similar larval ecologies (Clades I-2 and I-3), and (3) species are geographically isolated and ecologically divergent (Clade II).

PCA indicated that five stream variables (altitude, stream conductivity, depth, width, and velocity) differ among the members of clade I-1, and combinations of altitude and stream velocity reveal ecological distinction of the species. *Simulium curtatum* and *S. inthanonense* are found at high altitudes (>1,000 m a.s.l.) but occupy streams of different velocity, averaging about twofold greater for *S. curtatum* (0.49 m/s) than for *S. inthanonense* (0.23 m/s). Species distributions of black flies often are related to stream velocity (Palmer and Craig 2000; Zhang and Malmqvist 1996). Morphological adaptation to different stream velocities could drive adaptive radiation (Zhang 2006). Thus, habitat isolation between *S. inthanonense* and *S. curtatum* might be a result of adaptation to exploit different stream velocities. Further morphological analyses could be used to test this hypothesis. *Simulium doisaketense* inhabits lower altitudes, relative to other members of Clade I-1, and is not found above 900 m. *Simulium inthanonense* and *S. curtatum* are found at high elevations, whereas *S. asakoae* occurs over a wide range of altitudes (304–1,615 m a.s.l.). The results are consistent with previous studies showing that elevation is associated with species distributions (Jitklang et al. 2008; Kuvangkadilok et al. 1999).

The second pattern that emerged from our analyses is that closely related species overlap geographically but are isolated by habitat and have similar ecologies. Similar ecological niches among closely related species have been attributed to historical constraints, with species tending to retain their ancestral niche (Weins 2004). Four factors prevent ecological niche divergence of closely related species, viz., natural selection, pleiotropy, gene flow, and lack of variability (Weins 2004), but identifying the responsible factor(s) can be difficult. *Simulium gombakense* and *S. decuplum* (Clade I-3) overlap geographically, but do not co-occur in streams. *Simulium sheilae* and *S. trangense* (Clade I-2) also geographically overlap; although they are not isolated at the habitat scale, they rarely coexist. These two species are nearly identical morphologically, and *S. trangense* was confirmed and described as a distinct species only after chromosomal study revealed fixed-banding differences (Jitklang et al. 2008). What prevents similar species from coexisting if they overlap geographically and prefer the same ecological niche? One possibility is interspecific competition. Species that use the same ecological niche potentially compete for resources (Morin 1999). Given that competition decreases fitness, natural selection should favor traits that reduce interspecific competitive interactions. We hypothesize that habitat separation of these species is related to oviposition preferences. If a female lays eggs in the same stream as that of another species with a similar ecological niche, its fitness might be compromised by larval competition. Natural selection, therefore, should favor females that lay eggs in streams without competitors.

The third pattern involves closely related species that are geographically isolated and ecologically divergent. *Simulium kuvangkadilokae* and *S. chumpornense*, which have nearly identical larvae, are possible sister species (Pramual and Tangkawanit 2008), but *S. kuvangkadilokae* is found in northeastern Thailand, whereas *S. chumpornense* is found in southern and western Thailand. Both physical

and chemical factors of the streams differ significantly between these species. Geographical isolation of the species could be due to vicariance or dispersal. However, no vicariance event is apparent that could result in separation of *S. chumpornense* from *S. kuvangkadilokae*. Although a seaway putatively existed during the Neogene period (Woodruff 2003), it was located at a latitude lower than the current distribution of these species. Geographic isolation of *S. chumpornense* and *S. kuvangkadilokae* is therefore more likely the result of dispersal. Phylogeographic study of another group of black flies, the *S. tani* complex, indicated colonization of the eastern and southern areas from northern populations through the western forest corridor (Pramual et al. 2005). *Simulium chumpornense* is found in the western and southern regions, and we hypothesize that the ancestral population colonized these regions by dispersal. A phylogeographic approach could be used to test this hypothesis. Among the stream variables that differ significantly between *S. kuvangkadilokae* and *S. chumpornense*, altitude, conductivity, and stream velocity are markedly divergent. This situation resembles that of Clade I-1, suggesting that these factors could have played an important role in population isolation leading to species divergence.

A model of black fly speciation driven by chromosomal rearrangements, coupled with biological divergence, was proposed by Rothfels (1989). According to this model, closely related species differ in chromosomal inversions, and speciation results from different co-adapted gene systems leading to divergence in life cycle, host choice, or niche preference. Our results support this model; closely related species occupy different ecological niches. Previous studies have shown extensive chromosomal polymorphisms in many species of *Gomphostilbia*, which could be partitioned into distinct cytoforms (Jitklang et al. 2008; Kuvangkadilok et al. 2008). Ecological differentiation among cytoforms also has been demonstrated (Jitklang et al. 2008; Kuvangkadilok et al. 2008; Pramual and Wongpakam 2011), suggesting that chromosome inversions play a role in ecological preference. The Rothfels (1989) model also suggests that sympatric speciation is probable, based on empirical evidence that sibling species tend to be sympatric. In our study, 8 of the 12 species overlap geographically. These species are ecologically divergent, suggesting that adaptation to different ecological niches could have promoted speciation.

Pleistocene climate and environmental changes also could have facilitated population divergence leading to speciation or perhaps aiding chromosome-mediated speciation of some members of *Gomphostilbia*. These Pleistocene changes promoted population fragmentation and speciation in a number of different organisms (e.g., Carstens and Knowles 2007; Johnson and Cicero 2004; Knowles 2001). Although the effects of Pleistocene glaciations might have been less severe in the tropics than in the temperate regions, the environmental changes promoted speciation (Haffer 1969; Mayr and O'Hara 1986) and in several Southeast Asian mainland species, influenced genetic structure and diversity (e.g., Cannon and Manos 2003; Morgan et al. 2010; O'Loughlin et al. 2008). The dry conditions produced during glaciations reduced running-water habitats, although streams persisted in high mountainous areas (Gathorne-Hardy et al. 2002). Pleistocene glaciations, thus, could have driven population fragmentation of black flies, as demonstrated by the phylogeographic study of the *S. tani* complex (Pramual et al. 2005). Climate changes during the Pleistocene also played a significant role in the demographic history of simuliids. Recent population expansions, for example, have been found in the *S. tani* (Pramual et al. 2005) and *S. siamense* complexes (Pramual et al. 2011b). Geographical isolation of *S. chumpornense* and *S. kuvangkadilokae* also might have been a result of Pleistocene effects, the geographic distributions of these species reflecting the historical dispersal route of the *S. tani* complex (Pramual et al. 2005).

Integrating information on geographical distributions, larval ecology, and phylogeny enabled us to infer factors contributing to habitat exploitation of black flies. Both geographical isolation and ecological divergence are found among similar *Gomphostilbia* species. Most closely related species overlap geographically but are ecologically divergent, suggesting that ecological shifts could have been involved in species divergence in *Gomphostilbia*, although allopatric speciation driven by Pleistocene climate changes also could have contributed to speciation. If further evidence, such as broader phylogenetic study, supports the finding that some of the species pairs in our study (e.g., *S. kuvangkadilokae* and *S. chumpornense*) are true sister species, speciation driven by adaptation to different ecological niches might have been a prevalent mode in the subgenus *Gomphostilbia*.

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Conflict of interest The authors declare that they have no conflict of interest.

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