ภาคผนวก

ผลงานวิจัย

<u>โครงการย่อยที่ 1</u> [Studies on species diversity and distributional characteristics of black flies (Diptera: Simuliidae) in relation to ecological diversity in Thailand: ศ. ดร.เวช ชูโชติ]

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Simulium (*Nevermannia*) *chomthongense*, a new species of black fly (Diptera: Simuliidae) from Chiang Mai, Thailand

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Abstract. *Simulium* (*Nevermannia*) *chomthongense* sp. nov. is described from female, male, pupal and larval specimens collected from Doi Inthanon National Park and Doi Phahompok National Park, Chiang Mai, Thailand. This new species, first reported as *S.* (*Eusimulium*) sp. A, and later regarded as *S.* (*N.*) *caudisclerum* Takaoka & Davies, described from peninsular Malaysia, is distinguished from *S.* (*N.*) *caudisclerum* in the male by the number of enlarged upper-eye facets and the relative size of the hind basitarsus against the hind tibia and femur, and in the pupa by the relative length of the stalks of paired filaments against the common basal stalk and the color of the dorsal surface of abdominal segments 1–3 (or 4). Taxonomic and molecular notes are provided to separate this new species from four other known species of the *vernum* species-group, which share an accessory sclerite on the larval abdomen, a rare characteristic in this species-group.

INTRODUCTION

The *Simulium* (*Nevermannia*) *vernum* species-group, which consists of about 130 species, is widely distributed in the Holarctic Region and extends its distribution southward into the Oriental Region where 23 species are recorded (Adler & Crosskey, 2011). In Thailand, this species group is represented by one species, which was first reported as *Simulium* (*Eusimulium*) sp. A, based on a single mature larva collected from a stream at high elevation (1,700 m altitude) in Doi Inthanon National Park (Takaoka & Suzuki, 1984). Later, the larva of this species, under a different subgeneric name, *S.* (*Nevermannia*) sp. A, was noted to agree with that of *Simulium* (*N.*) *caudisclerum* Takaoka & Davies, described from Cameron's Highlands, peninsular Malaysia (Takaoka & Davies, 1995), in most morphological characteristics including the possession of an accessory sclerite just anterior to the posterior circlet of the abdomen, a characteristic rarely occurring in the *vernum* species-group. *Simulium* (*N.*) sp. A was identified tentatively as *S.* (*N.*) *caudisclerum* due to the lack of its adult and pupal specimens (Takaoka & Davies, 1995). The species name, *S.* (*N.*) *caudisclerum*, was first applied to larvae of the *vernum* species-group from Doi Inthanon National Park, when their polytene chromosomes were studied (Kuvangkadilok *et al.*, 1998, 1999). Although this taxonomic decision was followed in the list of black flies from Thailand (Takaoka & Choochote, 2004), and in a study of the molecular phylogeny of Thai black flies (Thanwisai *et al.*, 2006), detailed morphotaxonomic examination of the Thai specimens, in particular, the adult female, male and pupal specimens, remained to be carried out to confirm the species identity.

Recently, we had an opportunity to examine a sufficient number of adults (reared from pupae), pupae and larvae collected from Doi Inthanon National Park and Doi Phahompok National Park, in Chiang Mai, which had been thought to be those of *S.* (*N.*) *caudisclerum*, and to compare them with those of *S.* (*N.*) *caudisclerum* from peninsular Malaysia. As a result, the Thai specimens are shown to have several morphological characteristics differing from those of peninsular Malaysian *S.* (*N.*) *caudisclerum*. In this paper, the species of the *vernum* species-group in Chiang Mai is regarded as a new species and its female, male, pupa and mature larva are described.

The methods of collection, description and illustration, as well as terms for morphological features used here, follow those of Takaoka (2003). The type specimens are deposited at the Entomology Section, Queen Sirikit Botanic Garden, Chiang Mai, Thailand.

***Simulium* (*Nevermannia*) *chomthongense* Takaoka, Srisuka & Choochote sp. nov.**

Simulium (*Eusimulium*) sp. A: Takaoka & Suzuki, 1984: 12–13 (description of larva).
Simulium (*Nevermannia*) sp. A: Takaoka & Davies, 1995: 94.
[*Simulium* (*Nevermannia*) *caudisclerum* (not Takaoka & Davies, 1995): Kuvangkadilok *et al.*, 1998: 215–230; 1999: 197–207; Takaoka & Choochote, 2004: 189].

Description. **Female**. Body length 2.4–2.6 mm. ***Head***. Slightly narrower than width of thorax. Frons brownish-black, grey pruinose, not shiny, densely covered with whitish-yellow scale-like recumbent short hairs interspersed with few dark simple longer hairs along each lateral margin; frontal ratio 1.51–1.73:1.00:1.89–2.07; frons-head ratio 1.00:3.51–5.04. Fronto-ocular area well developed, narrow, directed dorsolaterally. Clypeus dark brown, grey pruinose, moderately covered with whitish-yellow hairs interspersed with few dark longer hairs on each side. Labrum 0.79–0.80 times as long as clypeus. Antenna composed of scape, pedicel and 9 flagellomeres, dark brown to brownish-black except scape, pedicel and base of 1st flagellomere medium brown; 1st flagellomere 1.49–1.70 times as long as 2nd one. Maxillary palp composed of 5 segments, light to medium brown, proportional lengths of 3rd, 4th, and 5th segments 1.00:0.75–0.87:1.48–1.59; 3rd segment (Fig. 1A) swollen; sensory vesicle (Fig. 1A) elongate (0.56–0.59 times as long as 3rd segment), with medium-sized opening. Maxillary lacinia with 12 inner and 12–16 outer teeth. Mandible with 30–33 inner and 13 outer teeth. Cibarium without any denticles. ***Thorax***. Scutum brownish-black except anterolateral calli ochreous, slightly shiny when illuminated at certain angle of light, densely covered with yellow scale-like recumbent hairs interspersed with several dark brown long upright hairs on prescutellar area. Scutellum medium brown, slightly shiny when illuminated at certain angle of light, moderately covered with yellow medium-long to long hairs mixed with several dark brown long upright hairs. Postnotum dark brown, slightly shiny when illuminated at certain angle of light and bare. Pleural membrane bare. Katepisternum dark brown to brownish-black, longer than deep, slightly shiny when illuminated at certain angle of light, and bare. ***Legs***. Foreleg: coxa dark yellow; trochanter light brown except base yellow; femur dark yellow except apical cap medium brown; tibia medium brown, with median large portion on outer surface greyish light brown; tarsus dark brown, with moderate dorsal hair crest; basitarsus moderately dilated, 7.89–9.17 times as long as its greatest width. Midleg: as in foreleg except coxa dark brown on anterolateral surface and brownish-black on posterolateral surface. Hind leg: coxa medium brown; trochanter light brown with base yellow; femur dark yellow with apical cap medium brown; tibia medium to dark brown except extreme base yellow, and medial large portion on outer surface greyish light brown; tarsus dark brown except basitarsus light to medium brown (though base dark brown) and basal 1/2 of 2nd tarsomere greyish; basitarsus (Fig. 1B) narrow, nearly parallel-sided, 7.15 times as long as wide, and 0.75–0.76 and 0.61 times as wide as greatest widths of tibia and femur,

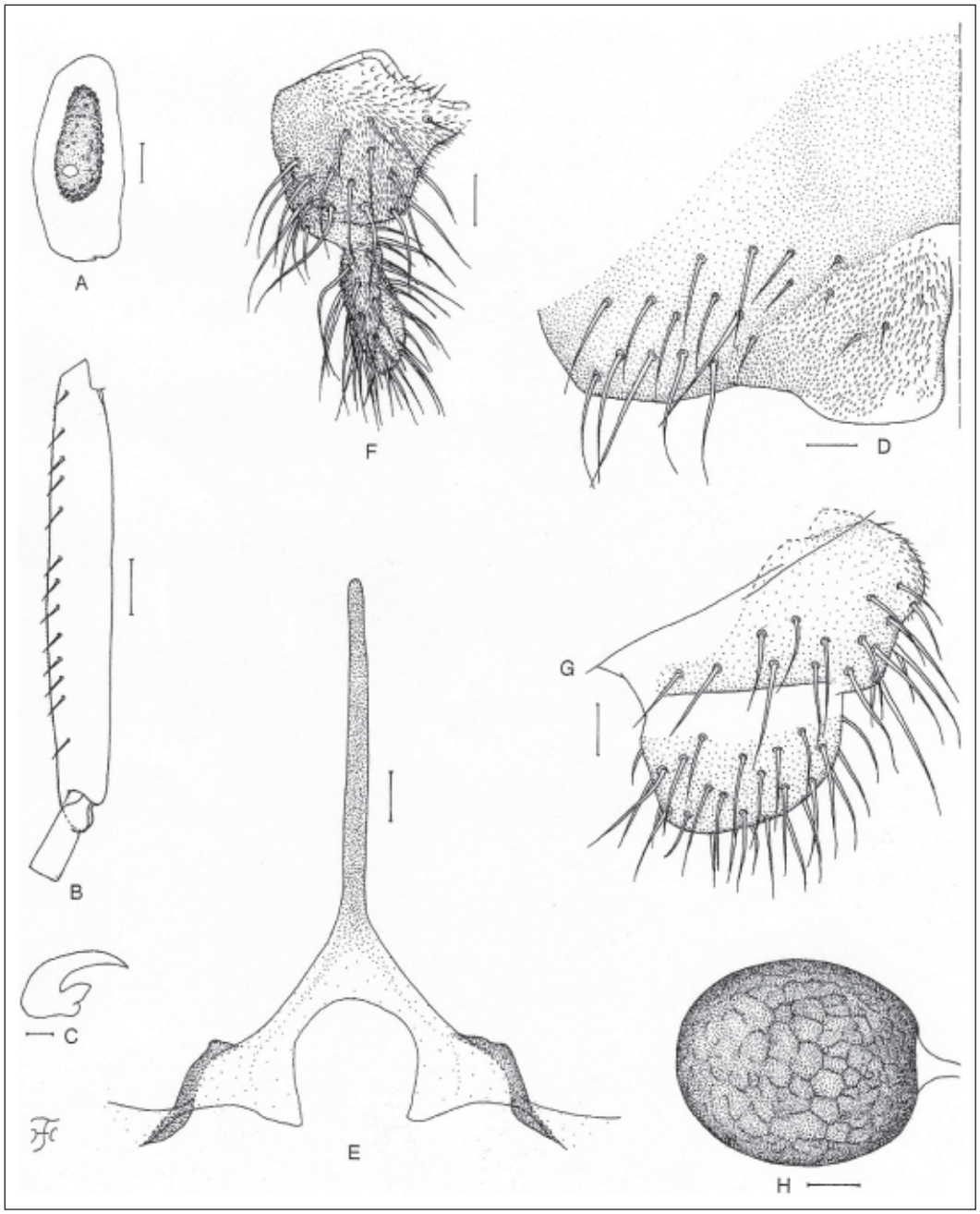

Figure 1. Female of *Simulium* (*Nevermannia*) *chomthongense* sp. nov. A, 3rd segment of left maxillary palp with sensory vesicle (front view); B, basitarsus and 2nd tarsomere of left hind leg showing calcipala and pedisulcus (outer view); C, claw (lateral view); D, sternite 8 and ovipositor valve (only right half shown) (ventral view); E, genital fork (ventral view); F & G, right paraprocts and cerci (F, ventral view; G, lateral view); H, spermatheca. Scale bars. 0.1 mm for B; 0.03 mm for A; 0.02 mm for D–H; 0.01 mm for C.

respectively; calcipala (Fig. 1B) nearly as long as width at base, and 0.50 times as wide as greatest width of basitarsus. Pedisulcus (Fig. 1B) shallow. Claw (Fig. 1C) with large basal tooth 0.44–0.47 times as long as claw.

Wing. Length 2.7–3.0 mm. Costa with dark brown spinules and yellow hairs though intermixed with few brown hairs basally.

Subcosta with light brown hairs except apical 1/5–2/5 bare. Hair tuft on stem vein dark brown. Basal portion of radius fully haired; R_1 with dark spinules and hairs; R_2 with hairs only. Basal cell absent. ***Haltere***. White except basal stem darkened. ***Abdomen***. Basal scale ochreous to light brown, with fringe of whitish-yellow hairs. Dorsal surface of abdomen dark brown to brownish-black except tergite 2 light brown, rest of segment 2 ochreous and tergites 7–9 light brown, moderately covered with dark short to long hairs intermixed with yellow short hairs; tergites of segments 2 and 7–9 wide and shiny when illuminated at certain angle of light, tergites 3–6 relatively narrow and dull. Ventral surface of abdomen mostly pale ochreous; sternal plate on segment 7 developed medially. ***Genitalia***. Sternite 8 (Fig. 1D) bare medially, with 10–14 medium-long to long yellow (except 3–7 longer hairs dark brown) hairs together with few slender short yellow hairs on each side. Ovipositor valves (Fig. 1D) triangular (though medioposterior corners rounded), thin, membranous, moderately covered with microsetae interspersed with 3 or 4 short slender yellow hairs; inner margins slightly concave medially, somewhat sclerotized, and somewhat separated from each other. Genital fork (Fig. 1E) of usual inverted-Y form, with slender stem; arms of moderate width medially; lateral plate of each arm strongly sclerotized along dorsolateral margin, and with thin lobe directed medioposteriorly. Paraproct in ventral view (Fig. 1F) nearly pentagonal, with 5–7 sensilla on unpigmented anteromedial surface; paraproct in lateral view (Fig. 1G) somewhat produced ventrally, 0.69 times as long as wide, with 12–20 medium-long to long hairs on ventral and lateral surfaces. Cercus in lateral view (Fig. 1G) short, rounded posteriorly, 0.48 times as long as wide. Spermatheca (Fig. 1H) ovoidal, 1.16 times as long as its greatest width, well sclerotized except duct and small area near juncture with duct unsclerotized, and with hexagonal patterns (though not well defined) on surface; internal setae absent; both accessory ducts slender, subequal in diameter to major one.

Male. Body length 2.5–3.0 mm. ***Head***. Wider than thorax. Upper eye medium brown, consisting of 15 or 16 vertical columns and 17 or 18 horizontal rows of large facets. Face dark brown, greyish-white pruinose. Clypeus brownish-black, whitish pruinose, moderately covered with golden yellow medium-long hairs (mostly directed upward) interspersed with few light brown longer hairs. Antenna composed of scape, pedicel and 9 flagellomeres, dark brown except base of 1st flagellomere yellow; 1st flagellomere elongate, 2.30 times as long as 2nd one. Maxillary palp light to medium brown, with 5 segments, proportional lengths of 3rd, 4th, and 5th segments 1.00:0.86:1.78; 3rd segment (Fig. 2A) not swollen; sensory vesicle (Fig. 2A) ellipsoidal, small, 0.22–0.25 times as long as 3rd segment, and with small opening. ***Thorax***. As in female except scutum with several golden-yellow long hairs as well as several medium brown ones on prescutellar area. ***Legs***. Color nearly as in female except following characteristics: fore coxa dark yellow to light brown, hind trochanter light brown on posterior surface, and basal 1/4 of hind 2nd tarsomere greyish. Fore basitarsus slightly dilated, 10.00–10.49 times as long as its greatest width. Hind basitarsus (Fig. 2B) enlarged, wedge-shaped, 3.59–3.90 times as long as wide, and 1.09–1.13 and 1.33–1.35 times as wide as greatest width of hind tibia and femur, respectively; calcipala (Fig. 2B) slightly shorter than basal width, and 0.22–0.27 times as wide as greatest width of basitarsus. Pedisulcus (Fig. 2B) well defined at basal 1/4 of 2nd tarsomere. ***Wing***. Length 2.6–3.0 mm; other characteristics as in female except subcosta without hairs. ***Abdomen***. Basal scale medium brown, with fringe of yellow long hairs. Dorsal surface of abdomen dark brown to brownish-black, moderately covered with yellow short hairs intermixed with dark brown short to medium-long hairs; segments 7 and 8 each with pair of slightly shiny lateral patches when illuminated at certain angle of light; ventral surface of segment 2 white, those of segments 3–6 ochreous except sternites medium brown, and those of other segments medium brown. ***Genitalia***. Coxite in ventral view (Fig. 2C)

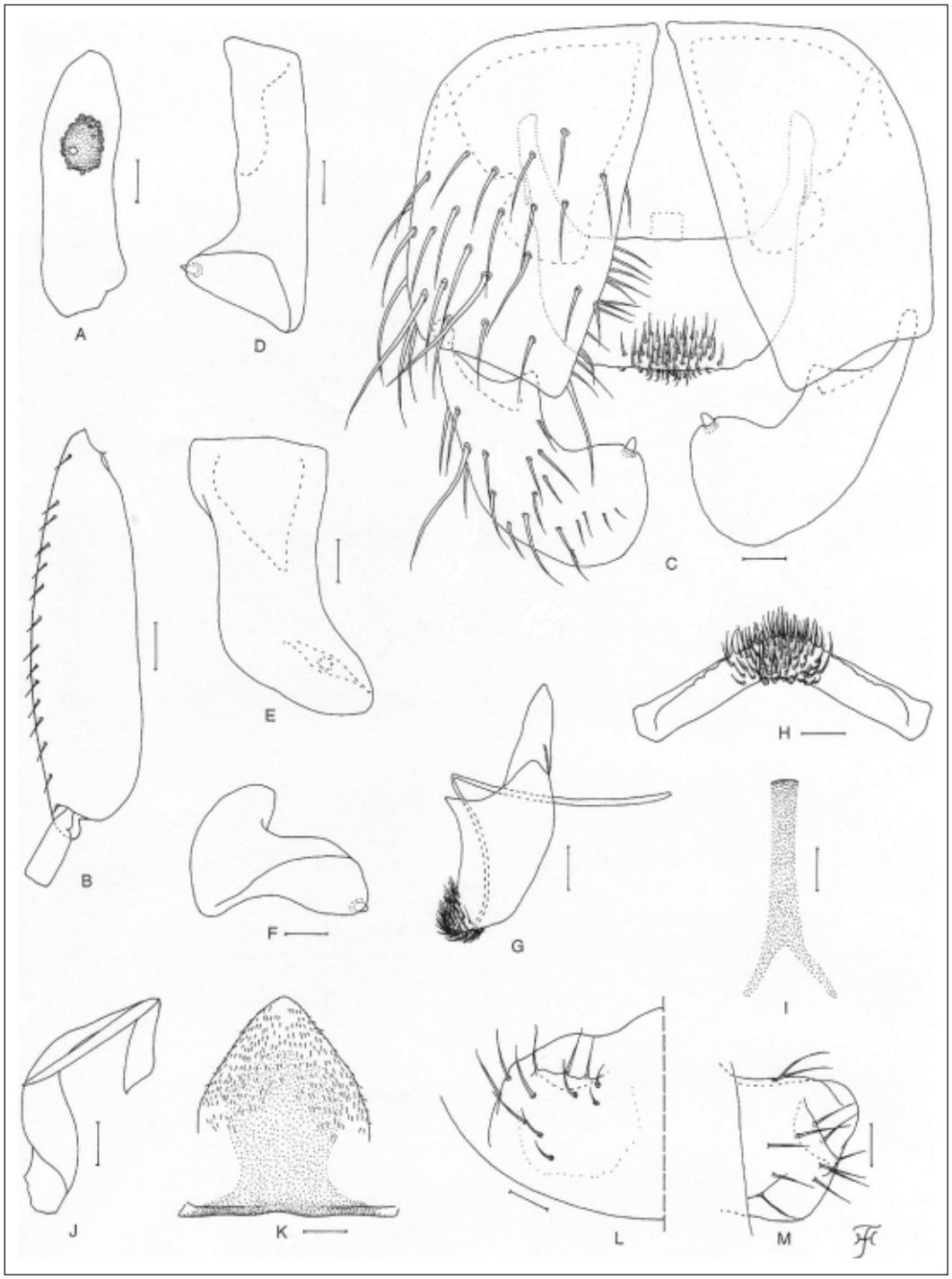

Figure 2. Male of *Simulium* (*Nevermannia*) *chomthongense* sp. nov. A, 3rd segment of right maxillary palp with sensory vesicle (front view); B, basitarsus and 2nd tarsomere of left hind leg showing calcipala and pedisulcus (outer view); C, coxites, styles and ventral plate (ventral view); D–F, right styles (D, lateral view; E, ventrolateral view; F, caudal view); G, ventral plate and median sclerite (lateral view); H, ventral plate (caudal view); I, median sclerite (caudal view); J, right paramere (caudal view); K, aedeagal membrane and dorsal plate (caudal view); L & M, 10th abdominal segments and cerci (right side; L, caudal view; M, lateral view). Scale bars. 0.1 mm for B; 0.02 mm for A & C–M.

nearly rectangular, 1.70 times as long as its greatest width. Style in ventral view (Fig. 2C) bent inward, nearly parallel-sided, rounded apically and with apical spine; style in lateral view (Fig. 2D) shorter than coxite (0.74 times as long as coxite), boot-shaped, with triangular apical lobe directed dorsomedially; style in ventrolateral view (Fig. 2E) straight up to apical 1/3, then curved inwardly, wide basally, nearly parallel-sided up to apical 1/3, and gradually narrowed toward apex; style in caudal view (Fig. 2F) widely depressed on posterior surface of medially directed apical lobe. Ventral plate in ventral view (Fig. 2C) with body transverse, 0.52 times as long as wide, with anterior margin nearly straight, most median portion of posterior margin nearly straight, though ragged, posterolateral corners rounded, darkened along anterior margin, and nearly bare except posteromedian portion of ventral surface densely covered with microsetae; basal arms of moderate length, directed forward, then slightly convergent apically, or somewhat divergent from base as shown in Fig. 2C; ventral plate in lateral view (Fig. 2G) with body gradually narrowed posteriorly; ventral plate in caudal view (Fig. 2H) with body appearing as shallow inverted-V shape, having similar width, posteroventral margin roughly undulate, and densely covered with microsetae medially on posterior surface. Median sclerite (Fig. 2G, I) club-shaped, narrow, with forked apex, and with base located in front of anterior margin of ventral plate. Parameres (Fig. 2J) large, each with small apical appendix directed forwardly, and with 1 distinct long and stout hook. Aedeagal membrane (Fig. 2K) moderately setose, dorsal plate well defined, broadly produced ventrally with round apex, though constricted subbasally. Ventral surface of abdominal segment 10 (Fig. 2L, M) with 2–5 distinct hairs near each posterolateral margin. Cercus (Fig. 2L, M) small, rounded, encircled with 10–13 hairs.

Pupa. Body length 3.0–3.5 mm. ***Head***. Integument light yellowish-brown, bare except lateral surfaces sparsely covered with small tubercles, and frons with few to about 20 round tubercles scattered near each lateral margin in some pupae; antennal sheath without any protuberances; face with pair of simple very long trichomes with coiled apices, and frons with 2 pairs of very long simple trichomes with coiled or uncoiled apices except 3 of 20 pupae examined which have different numbers of trichomes: 1 trichome only on right side though 2 trichomes on left side in 1 pupa; 3 trichomes on right side though 2 trichomes on left side in 1 pupa; 3 trichomes on each side in 1 pupa; among 3 trichomes on each side, additional trichome located dorsally little apart from other 2 trichomes and medium-long, about half as long as 2 other trichome). ***Thorax***. Integument light yellowish-brown, bare except dorsal and dorsolateral surfaces of posterior 2/3 sparsely covered with small round tubercles, with 3 simple very long dorsomedial trichomes with coiled or uncoiled apices, 2 simple anterolateral trichomes (1 very long with coiled apex, 1 long with uncoiled apex), 1 simple medium-long mediolateral trichome with uncoiled apex, and 3 simple ventrolateral trichomes with uncoiled apices (1 medium-long, 2 short), on each side. Gill (Fig. 3A,B) composed of 4 slender thread-like filaments, arranged in pairs, with medium-long common basal stalk having somewhat swollen transparent organ ventrally (often partially broken) at base; common basal stalk 0.76–0.79 times as long as interspiracular trunk; stalks of paired filaments 0.28 –0.96 times as long as common basal stalk; all filaments subequal in length and thickness to one another, though their lengths including their own stalk and common basal stalk varying from 4.2 mm to 5.7 mm by pupae; cuticle of all filaments with well-defined annular ridges and furrows, and densely covered with minute tubercles. ***Abdomen***. Dorsally, segments 1–3 (and often 4) dark greyish and without distinct tubercles; segment 1 with 1 long simple slender hair-like seta on each side; segment 2 with 1 short simple slender hair-like seta and 5 very short somewhat spinous setae submedially on each side; segments 3 and 4 each with 4 hooked spines and 1 very short somewhat spinous seta on each side; segments 5–8 each with spine-combs in transverse row and comb-like groups of minute spines on each side; segment 9 with

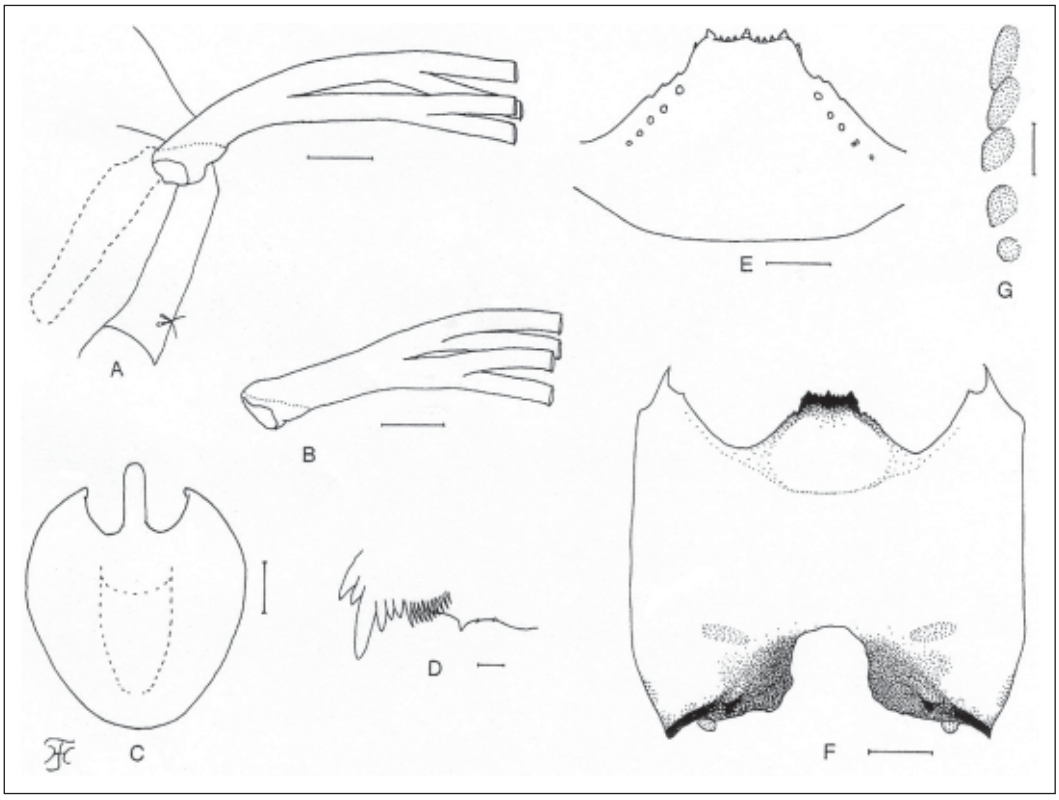

Figure 3. Pupa and larva of *Simulium* (*Nevermannia*) *chomthongense* sp. nov. A–C, pupa and D–G, larva. A, interspiracular trunk and basal portion of gill filaments showing longest stalks of paired filaments (right side; lateral view); B, basal portion of gill filaments showing shortest stalks of paired filaments (right side; lateral view); C, cocoon (dorsal view); D, mandible showing a minute supernumerary serration; E, hypostoma (ventral view); F, head capsule showing postgenal cleft (ventral view); G, accessory sclerite (left side; ventrolateral view). Scale bars. 1.0 mm for C; 0.1 mm for A, B & F; 0.05 mm for E; 0.02 mm for G; 0.01 mm for D.

pair of cone-like terminal hooks and comb-like groups of minute spines. Ventrally, segment 4 with 1 simple hook and few simple slender very short setae on each side; segment 5 with pair of bifid hooks submedially and few very short simple slender setae on each side; segments 6 and 7 each with pair of bifid inner and simple outer hooks somewhat spaced from each other and few very short simple slender setae on each side; segments 4–8 with comb-like groups of minute spines. Each side of segment 9 without grapnel-shaped hooklets. ***Cocoon*** (Fig. 3C). Wall pocket-shaped, moderately woven, widely extended ventrolaterally, appearing round when viewed dorsally; anterior margin thickly woven, with medium to long dorsal projection (up to 1.6 mm long) usually slightly curved downward; posterior 1/2 with floor roughly or moderately woven; individual threads partially visible; 3.6–4.5 mm long by 3.1–4.1 mm wide.

Mature larva. Body length 6.0–7.0 mm. Body creamy. Cephalic apotome whitish-yellow, though narrow area along posterior margin somewhat darkened; head spots moderately positive except anterior spot of posterolateral spots usually obscure. Lateral surface of head capsule whitish-yellow except eye-spot region whitish; eyebrow distinct; 2 relatively large spots and 1 smaller spot in front of posterior margin moderately positive; 2 small round spots below eye-spot region faintly to moderately positive or obscure. Ventral surface of head capsule whitish-yellow to yellow except darkened area near posterior margin on each side of postgenal cleft; horizontal long spot and

round spot on each side of postgenal cleft moderately positive. Antenna composed of 3 segments and apical sensillum, somewhat longer than stem of labral fan; proportional lengths of 1st, 2nd, and 3rd segments 1.00:1.02–1.04:0.67–0.73. Labral fan with 26–34 main rays. Mandible (Fig. 3D) with 3 comb-teeth, of which 1st is longest and 2nd and 3rd are subequal in length; mandibular serration composed of 2 teeth (1 medium-sized and 1very small); major tooth at obtuse angle against mandible on apical side; supernumerary serration usually absent, though rarely present (consisting of 1 very small tooth). Hypostoma (Fig. 3E) with row of 9 apical teeth; median and each corner tooth prominent, subequal in length to one another and much longer than 3 intermediate teeth on each side; lateral margin serrate; 5 or 6 hypostomal bristles per side lying parallel to lateral margin. Postgenal cleft (Fig. 3F) short, rounded apically, 0.65–0.75 times as long as postgenal bridge. Cervical sclerite composed of 2 dark yellow small oblong pieces, not fused to occiput, widely separated medially from each other. Thoracic cuticle bare. Abdominal cuticle almost bare except few posterior segments sparsely covered with colorless simple setae, and last segment densely covered with colorless simple setae on each side of anal sclerite. Rectal scales present. Rectal papilla compound, each of 3 lobes with 11–15 finger-like secondary lobules. Anal sclerite of usual X-form, with anterior arms 0.82–0.90 times as long as posterior ones, broadly sclerotized at base; accessory sclerite (Fig. 3G) composed of 3–6 sclerotized spots on each side. Last abdominal segment with pair of large conical ventral papillae. Posterior circlet with 80–84 rows of up to 15 hooklets per row.

Type specimens. Holotype male with associated pupal exuviae and cocoon (preserved in 80% ethanol), reared from pupa, collected from a stream (width 45 cm, depth 10 cm, stream-bed sandy and muddy, water temperature 10.9ºC, partially shaded, altitude 2,219 m, 18º33'19.4" N, 98º28'43.3" E) moderately flowing in a forest, Chomthong District, Kaew Mae Pan, Doi Inthanon National Park, Chiang Mai, Thailand, 23. I. 2012, by W. Srisuka. Paratypes: 3 females, 3 males, all with associated pupal exuviae and cocoons, and 12 mature larvae (all preserved in 80% ethanol), same data and date as those of the holotype; 3 females, 10 males, all with associated pupal exuviae and cocoons, and 5 mature larvae (all preserved in 80% ethanol), collected from a stream (Summit Stream 3) (width 40 cm, depth 10 cm, stream-bed sandy and muddy, partially shaded, altitude 1,799 m, 20º04'01.5" N, 99º08'36.9" E) moderately flowing in a forest, Fang District, Doi Phahompok National Park, Chiang Mai, Thailand, 22. IX. 2011, by W. Srisuka.

Biological note. The pupae and larvae of this new species were collected from dead tree leaves in the water.

Etymology. The species name *chomthongense* refers to the name of the district, Chomthong, in Doi Inthanon National Park, where this new species was collected.

Remarks. *Simulium* (*Nevermannia*) *chomthongense* sp. nov. is the same species as *S.* (*E.*) sp. reported based on the mature larva collected from Doi Inthanon National Park, Chiang Mai, Thailand (Takaoka & Suzuki, 1984), and later treated as *S.* (*N.*) *caudisclerum* described from Cameron's Highlands, Peninsular Malaysia (Kuvangkadilok *et al.*, 1998, 1999; Takaoka & Choochote, 2004; Thanwisai *et al.*, 2006). The present examination of the Thai specimens, which were so far regarded as *S.* (*N.*) *caudisclerum*, shows that *S.* (*N.*) *chomthongense* sp. nov. is almost indistinguishable in the adult female and larva from *S.* (*N.*) *caudisclerum*, but differs in the adult male and pupa from the latter species by having the following characteristics (characteristics of *S.* (*N.*) *caudisclerum* in parentheses): male upper-eye facets in 15 or 16 vertical columns and 17 or 18 horizontal rows (in 14 vertical columns and 15 horizontal rows), ratios of the hind basitarsus against the hind tibia and femur, 1.09–1.13 and 1.33–1.35 (0.9 and 1.0), body of the ventral plate somewhat narrowed posteriorly when viewed laterally (Fig. 2G) (nearly parallel-sided), lateral surfaces of the head of the pupa sparsely covered with small tubercles (bare), stalks of dorsal and ventral paired pupal gill filaments slightly to greatly

shorter than the common basal stalk (Fig. 3 A, B) (usually much longer, though very rarely shorter), and dorsal surfaces of pupal abdominal segments 1–3 (or 4) dark grey (yellow or transparent).

The result of the present morpho-taxonomic examination appears to be supported by the comparison of the sequences of the mitochondrial 16S rRNA gene (516 base pairs). The sequence of this gene of *S.* (*N.*) *chomthongense* sp. nov. (Accession number in Genbank: AB699899) differs from that of *S.* (*N.*) *caudisclerum* (Accession number in Genbank: AB699900) by seven base pairs, which are greater than the maximum difference of three base pairs usually seen as intraspecific variation (Otsuka *et al.*, Unpublished data). Further, the molecular phylogenetic analysis of the sequences of the mitochondrial 16S rRNA genes of several Oriental species of the *vernum* species-group shows that both species belong to different clusters which are, however, very close to each other: *S.* (*N.*) *chomthongense* sp. nov., together with *Simulium* (*Nevermannia*) *taulingense* Takaoka from Taiwan (Takaoka, 1979), in one cluster, and *S.* (*N.*) *caudisclerum*, together with *Simulium* (*Nevermannia*) *aberrans* Delfinado from the Philippines (Delfinado, 1969; Takaoka, 1983) and *Simulium* (*Nevermannia*) *yushangense* Takaoka from Taiwan (Takaoka, 1979), in another cluster (Otsuka *et al.*, Unpublished data).

Simulium (*N.*) *chomthongense* sp. nov. is placed in the *vernum* species-group within the subgenus *Nevermannia*, mainly by the shape of the male genitalia (Fig. 2C–M). Beside this new species and *S.* (*N.*) *caudisclerum*, the following four species are known to bear an accessory sclerite among species of the *vernum* species-group: *S.* (*N.*) *aberrans* from the Philippines (Takaoka, 1983), *Simulium* (*Nevermannia*) *ludingense* Chen, Zhang & Huang from Sichuan, China (Chen *et al.*, 2005), *S.* (*N.*) *yushangense* from Taiwan (Takaoka, 1979), and *Simulium* (*Nevermannia*) *zhangjiajiense* Chen, Zhang & Bi from Hunan, China (Chen *et al.*, 2004). However, this new species is easily distinguished from these four species by the following characteristics: from *S.* (*N.*) *aberrans* and *S.* (*N.*) *ludingense* by the cocoon with an anterodorsal projection (the cocoon is simple, without such a projection in the latter two species), from *S.* (*N.*) *yushangense* by the pupal gill filaments which are subequal in length and thickness to one another (one of two dorsal paired filaments is always longer and thicker than others in *S.* (*N.*) *yushangense*), and from *S.* (*N.*) *zhangjiajiense* by the greater number (17 or 18) of the horizontal rows of male upper-eye facets (13 horizontal rows in *S.* (*N.*) *zhangjiajiense*) and the ventral plate which is rectangular when viewed ventrally (Fig. 2C) (semicircular in *S.* (*N.*) *zhangjiajiense*).

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Simulium (*Asiosimulium*) *furvum*, a New Species of Black Fly (Diptera: Simuliidae) from Thailand

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Simulium (*Asiosimulium*) *furvum*, a New Species of Black Fly (Diptera: Simuliidae) From Thailand

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ABSTRACT *Simulium* (*Asiosimulium*) *furvum* sp. nov. (Diptera: Simuliidae) is described from female, male, pupal, and larval specimens collected from Maewa National Park, Lampang Province, Thailand. This new species represents the fourth member of the subgenus *Asiosimulium* Takaoka & Chochoote, one of two small black fly subgenera endemic in the Oriental Region. It is characterized by a pear-shaped spermatheca in the female; a ventral plate in the male with a laterally compressed median keel directed ventrally and with a deep notch posteromedially, and aedeagal membrane with stout spines; and by 22 gill filaments in the pupa. Taxonomic notes are provided to separate this new species from three known species, *Simulium* (*Asiosimulium*) *oblongum* Takaoka & Choochote and *Simulium* (*Asiosimulium*) *wanchaii* Takaoka & Choochote, both from Thailand, and *Simulium* (*Asiosimulium*) *suchitrae* Takaoka from Nepal.

KEY WORDS black fly, *Simulium*, *Asiosimulium*, new species, Thailand

Simulium (*Asiosimulium*) Takaoka & Choochote (Diptera: Simuliidae), one of two small black fly subgenera endemic in the Oriental Region, is represented by only three species, i.e., *Simulium* (*Asiosimulium*) *oblongum* Takaoka & Choochote and *Simulium* (*Asiosimulium*) *wanchaii* Takaoka & Choochote, both described from Thailand (Takaoka and Choochote 2005, 2006), and *Simulium* (*Asiosimulium*) *suchitrae* Takaoka from Nepal (Takaoka and Shrestha 2010). The phylogenetic relationships of this subgenus are not well resolved, although two molecular studies and one morphological study have been conducted. In multigene analyses using three mitochondrial genes (COI, ND4, and 16S rRNA) and two segments of the nuclear 28S ribosomal RNA (D1 to D2 and the D4 expansion regions), Phayuhasena et al. (2010) showed that among four other subgenera in Thailand, the subgenus *Asiosimulium* was positioned nearest to the subgenus *Simulium* Latreille, whereas Otsuka et al. (2007), using analysis of the sequences of the mitochondrial 16S rRNA gene, showed that the subgenus *Asiosimulium* had no sister relationship with any of eight other subgenera, although it was positioned next to the most basal subgenus *Wallacellum* Takaoka, forming a clade with other seven subgenera, i.e., *Daviesellum* Takaoka & Adler, *Gomphostilbia* Enderlein, *Hellichiella* Rubtsov, *Montisimulium* Rubtsov, *Morops* Enderlein, *Neveremannia* Enderlein, and *Simulium*. In a cladistic analysis of morphological characters, Takaoka (2012) showed that among 10 subgenera examined, the subgenus *Asiosimulium* has a closer relationship with a large clade composed of five subgenera, i.e., *Daviesellum*, *Gomphostilbia*, *Inseliellum* Rubtsov, *Morops*, and *Simulium*, than with four other subgenera, i.e., *Hebridosimulium* Grenier & Rageau, *Montisimulium*, *Neveremannia*, and *Wallacellum*.

The biting habit and medical or veterinary importance of the subgenus *Asiosimulium* remain to be investigated, although females of *S.* (*A.*) *wanchaii* were reported to be caught on a human attractant in Doi Pui National Park, Chiang Mai, Thailand (Takaoka and Choochote 2006). Until now, only one species of the subgenus *Gomphostilbia* and two species of the subgenus *Simulium* have been recorded to be vectors of different unnamed species of filariae of animal origin in Thailand (Fukuda et al. 2003, Takaoka et al. 2003, Ishii et al. 2008).

In a recent survey of larvae and pupae of black flies in Thailand, we collected a new species that has the following diagnostic characters of the subgenus *Asiosimulium* defined by Takaoka and Choochote (2005): in the female and male, both pleural membrane and katepisternum bare and the basal portion of the radial vein fully haired; in the female, the cibarium with numerous spinous processes and the claw with a large basal tooth; in the male, abdominal segments 5–7 each without a pair of shiny dorsolateral patches, the style shorter than the coxite, and the paramere lacking hooks; in the pupa, gill filaments of arborescent type, dorsal surface of abdominal segments 5–9 without spine combs, ventral surface of abdominal segments 5–7 each with a pair of simple hooks on each side, and lateral

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surface of abdominal segment 9 with grapnel-shaped hooklets; and in the larva, the postgenal cleft deep.

In this article, this new species is described on the basis of female, male, pupal, and larval specimens collected from Lampang Province, Thailand. The methods of collection, description, and illustration, as well as terms for morphological features used here, follow those of Takaoka (2003). The type specimens are deposited at the Entomology Section, Queen Sirikit Botanic Garden, Chiang Mai, Thailand.

Simulium (*Asiosimulium*) *furvum* Takaoka & Srisuka sp. nov.

Female. Body length 3.0 mm. *Head.* Slightly narrower than thorax. Frons brownish black, thinly whitish gray pruinose, dull, densely covered with whitish hairs interspersed with several dark longer and stouter hairs along each lateral margin; median suture on lower portion absent; frontal ratio 1.34:1.00:1.53. Frons: head ratio 1.00:4.22. Fronto-ocular area well developed, triangular, directed laterally, and somewhat upward. Clypeus brownish black, thinly whitish gray pruinose, dull, densely covered with whitish hairs (except narrow portions near upper margin bare) intermixed with dark longer and stouter hairs. Labrum 0.76 times as long as clypeus. Antenna composed of scape, pedicel, and nine flagellomeres, medium brown, except scape, pedicel, and base of first flagellomere yellow. Maxillary palp consisting of five segments, grayish brown, except third segment and anterior surface of fourth segment dark brown, proportional lengths of third, fourth, and fifth segments 1.00:0.88:1.40; third segment (Fig. 1A) not enlarged; sensory vesicle (Fig. 1A) roughly ellipsoidal, 0.24 times as long as third segment, with large opening apically. Lacinia with 12 or 13 inner and 14 or 15 outer teeth. Mandible with 27 inner and 16 outer teeth. Cibarium (Fig. 1B) moderately concave posterodorsally and with 111 spinous processes elaborately arranged in narrow space between inner and outer walls, somewhat apart from posterodorsal margin; these processes arising from inner wall. *Thorax.* Scutum medium-brown to brownish black, except anterolateral calli ochreous, thinly gray pruinose, faintly with dark narrow longitudinal vittae (one median and two submedian), slightly shiny when illuminated at certain angle of light, and densely covered with whitish or yellowish white short hairs and with several light-brown upright long hairs on prescutellar area. Scutellum dark brown, with many light-brown upright long hairs and with yellowish white short hairs. Postnotum dark brown, bare. Pleural membrane bare. Katepisternum longer than deep, brownish black, gray pruinose, shiny when illuminated at certain angle of light, and bare. *Legs.* Entirely medium brown to brownish black, except base of posterior surface of fore trochanter, posterior surface of hind trochanter, and extreme bases of all tibiae yellow. Fore basitarsus moderately dilated, 6.00 times as long as its greatest width, and with moderate dorsal hair crest. Hind basitarsus (Fig. 1C) nearly parallel-sided, although somewhat tapered near both ends, 5.81 times as long as its greatest width, 0.73 and 0.57 times as wide as greatest widths of tibia and femur, respectively; calcipala developed, short, as long as its width at base, and 0.38 times as wide as greatest width of basitarsus; pedisulcus developed. All claws (Fig. 1D) with large basal tooth 0.46 times as long as claw. *Wing.* Length 2.5 mm. Costa with two parallel rows of dark short spines as well as dark hairs. Subcosta with dark hairs, except near apex bare. Hair tuft on base of radial vein dark. Basal portion of radius fully haired. R1 with dark spinules and hairs. R2 with dark hairs only. Basal cell and basal median cell absent. *Abdomen.* Basal scale dark brown, with fringe of white long hairs. Dorsal and lateral surfaces of abdominal segments 2–9 brownish black to black; those of abdominal segments 2–4 densely covered with whitish short to medium-long hairs; those of abdominal segments 5–7 moderately or densely covered with whitish short hairs, mixed with dark short hairs; and those of abdominal segments 8 and 9 moderately covered with dark short hairs; tergites 2 and 6–8 shiny when illuminated at certain angle of light; ventral surface of abdominal segment 2 dark ochreous; those of segments 3–7 light to medium brown, except large median sternal plate on segment 7 dark brown; that of segment 8 dark brown. *Genitalia.* Sternite 8 (Fig. 1E) wide, bare medially but furnished with 11 or 12 short to medium-long hairs on each side. Ovipositor valve (Fig. 1E) nearly triangular; thin; membranous, except inner margin narrowly sclerotized; densely covered with microsetae interspersed with 10 or 11 short hairs; inner margins nearly straight or slightly convex, moderately separated from each other. Genital fork (Fig. 1F) inverted Y-shaped, with narrow well-sclerotized stem; each arm of moderate width, with stout projection directed anterodorsally near apex. Paraproct in ventral view (Fig. 1G) subquadrate, with ventral surface slightly depressed medially, having distinct process produced ventrally along anteromedial margin, and with four or five colorless sensilla on anteromedial surface; paraproct in lateral view (Fig. 1H) slightly wider than long, with numerous short hairs and few long hairs on lateral and ventral surfaces. Cercus in ventral view (Fig. 1G) elongate, as long as or slightly shorter than paraproct, with numerous short and medium-long hairs; cercus in lateral view (Fig. 1H) 1.26 times as long as width at base, gradually tapered toward apex, with rounded apex; cercus somewhat curved outwardly. Spermatheca (Fig. 1I) elongate, pear-like, strongly sclerotized, and pigmented, except duct unpigmented, without distinct reticulate surface pattern; surface of one side with several longitudinal ridges on apical two thirds, whereas surface of other side without such ridges; internal setae not discernible; accessory ducts subequal in diameter to each other and slightly larger than main duct.

Male. Body length 2.8 mm. *Head.* Nearly as wide as thorax. Holoptic. Upper eye medium brown, consisting of large facets in 16 vertical columns and 17 horizontal rows. Clypeus brownish black, moderately covered with whitish yellow hairs interspersed with dark longer hairs. Antenna composed of scape, pedicel, and nine flagellomeres, dark brown, except scape,

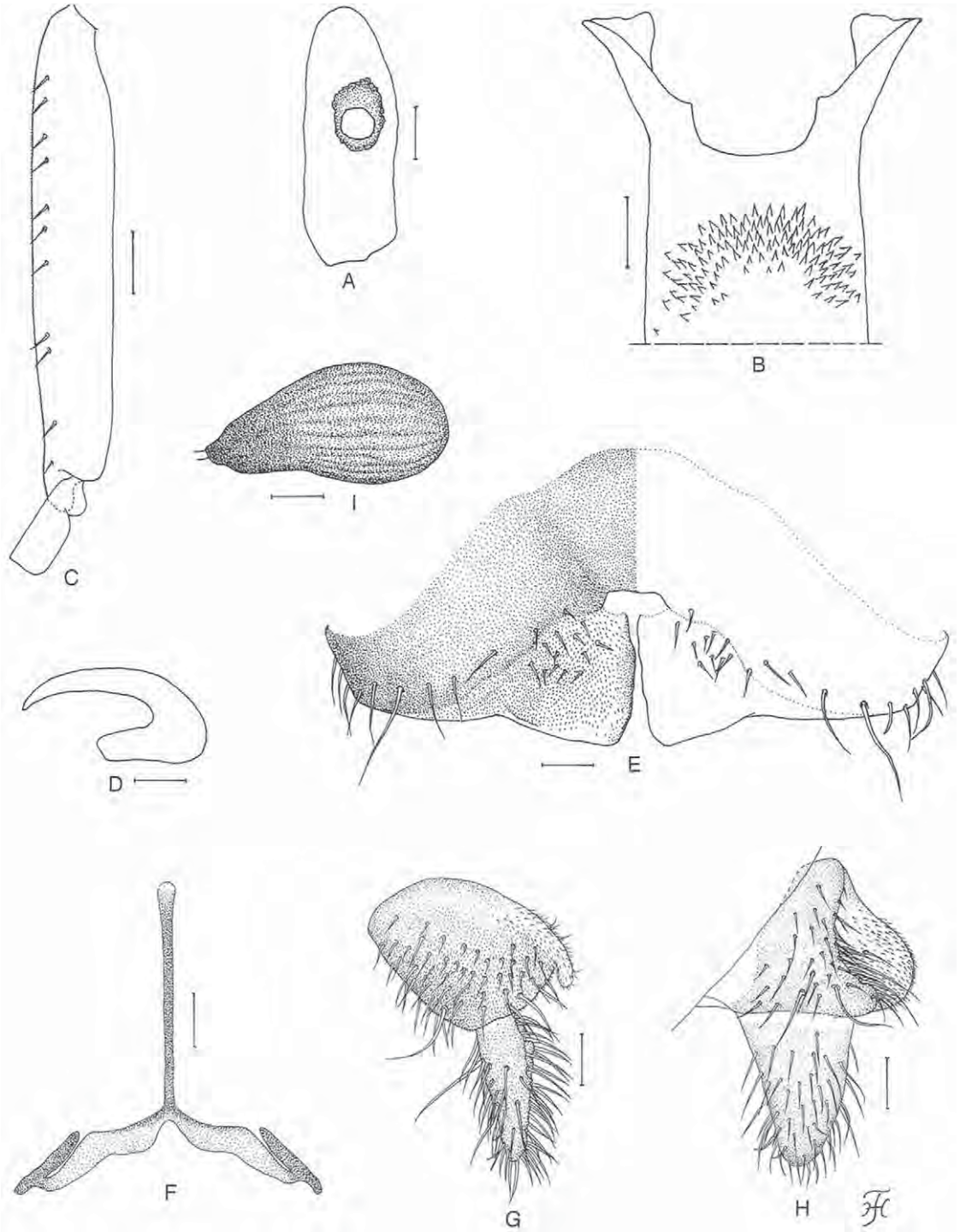

Fig. 1. Female of *S.* (*A.*) *furvum* sp. nov. (A) Third segment of maxillary palp with sensory vesicle (right side; front view). (B) Cibarium with many pointed processes elaborately arranged near posterodorsal margin (posterior view). (C) Basitarsus and second tarsomere of hind leg (left side; outer view). (D) Claw with large basal tooth (lateral view). (E) Eighth sternite and ovipositor valves (ventral view). (F) Genital fork (ventral view). (G and H) Paraprocts and cerci (right side; G, ventral view; H, lateral view). (I) Spermatheca. Scale bars = 0.1 mm (C), 0.04 mm (A, B and E–I), and 0.02 mm (D).

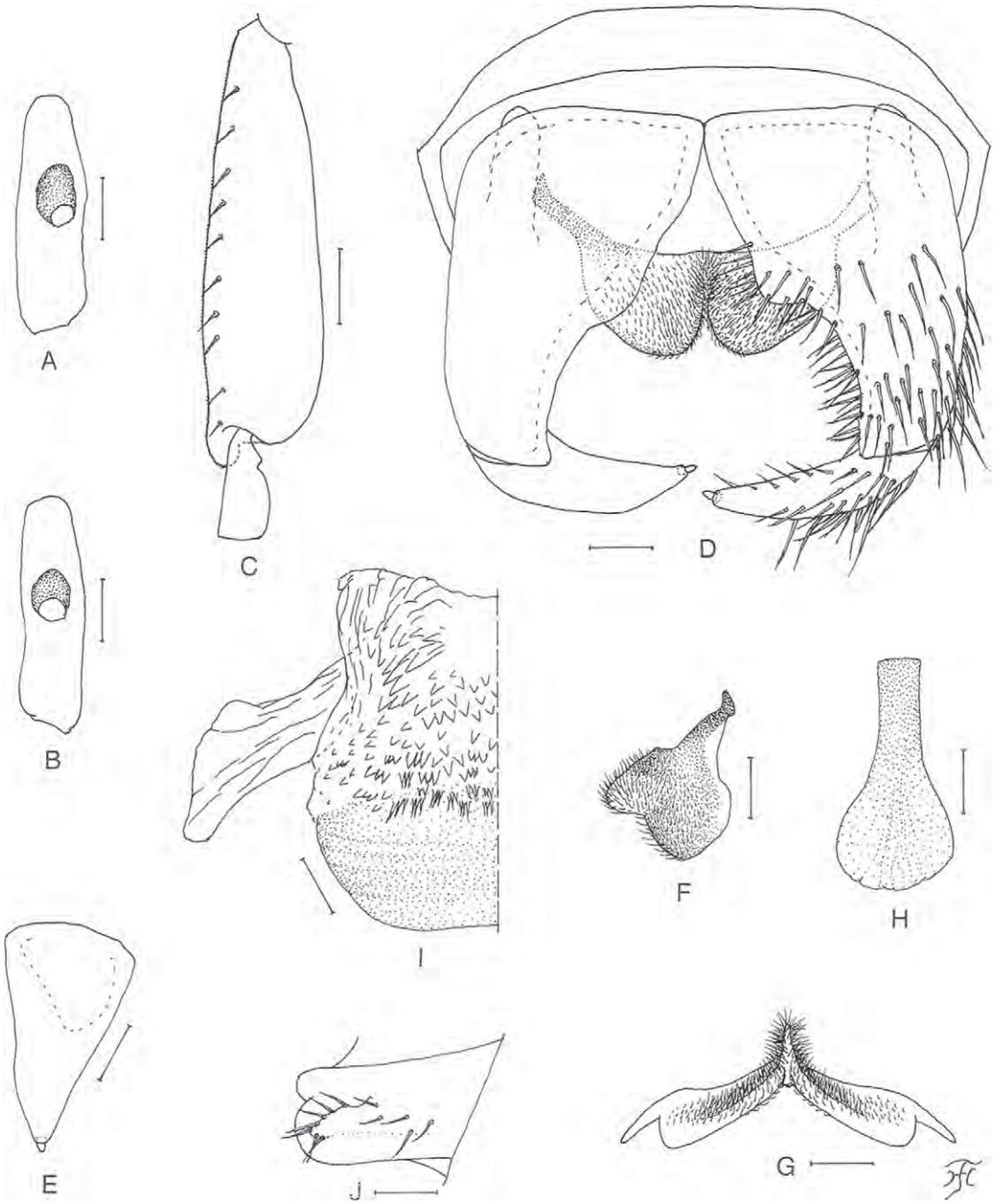

Fig. 2. Male of *S.* (*A.*) *furvum* sp. nov. (A and B) Third segments of maxillary palp with sensory vesicle bearing opening of slightly different sizes (A, right side; B, left side). (C) Basitarsus and second tarsomere of hind leg (left side; outer view). (D) Sternite of abdominal segment 9, coxites, and ventral plate (ventral view). (E) Left style (posterolateral view). (F and G) Ventral plate (F, lateral view; G, caudal view). (H) Median sclerite (ventral view). (I) Paramere, aedeagal membrane, and dorsal plate (caudal view). (J) Cercus (left side; lateral view). Scale bars = 0.1 mm (C), 0.04 mm (A, B, D–H and J), and 0.02 mm (I).

pedicel, and base of first flagellomere yellow; first flagellomere somewhat elongate, 1.60 times as long as second flagellomere. Maxillary palp composed of five segments, grayish brown, except third segment dark brown; proportional lengths of third, fourth, and fifth segments 1.00:0.90:1.32; third segment (Fig. 2A and B)

of moderate size; sensory vesicle (Fig. 2A and B) ellipsoidal, 0.22–0.26 times as long as third segment, with moderate or large opening. *Thorax.* Nearly as in female, except longitudinal vittae absent. *Legs.* Color nearly as in female. Fore basitarsus 7.2 times as long as its greatest width. Hind basitarsus (Fig. 2C) enlarged, wedge-shaped, 3.65 times as long as its greatest width, 0.96 and 0.91 times as wide as greatest widths of hind tibia and femur, respectively; calcipala well developed, slightly shorter than its width at base, and 0.26 times as wide as greatest width of basitarsus; pedisulcus weakly developed. *Wing.* Length 2.0 mm. Other characters as in female, except subcosta bare. *Abdomen.* Basal scale brownish black, with fringe of pale long hairs. Dorsal surface of abdomen dark brown to brownish black, moderately covered with pale hairs and dark hairs; tergite 2 with pair of dorsolateral shiny patches when illuminated at certain angle of light; other tergites without such shiny patches. Abdominal segment 9 in ventral view (Fig. 2D) with sternite in form of transverse thick bar. *Genitalia.* Coxite in ventral view (Fig. 2D) 1.61 times as long as its greatest width, with posterior half abruptly narrowed. Style in ventral view (Fig. 2D) short, 0.62 times as long as coxite, narrow, and with apical spine; style in posterolateral view (Fig. 2E) triangular, 1.71 times as long as its greatest width at base. Ventral plate in ventral view (Fig. 2D) with body lamellate, much shorter than wide, rounded posteriorly, deeply concave posteromedially, with anterior margin nearly straight, with median keel produced ventrally, and moderately covered with setae; basal arms short, divergent anterolaterally; ventral plate in lateral view (Fig. 2F) with short median keel produced ventrally; ventral plate in caudal view (Fig. 2G) with narrow median keel laterally compressed, and covered with setae on posterior surface. Median sclerite (Fig. 2H) plate-like, narrow, nearly parallel-sided on basal half, widened, and rounded on apical half. Aedeagal membrane (Fig. 2I) with simple stout spines; dorsal plate moderately sclerotized and broad, nearly twice as wide as long. Paramere (Fig. 2I) of moderate size, well sclerotized, without hook. Ventral surface of 10th abdominal segment without hair. Cercus (Fig. 2J) in form of narrow lobe, covered with 11 or 12 hairs.

Pupa. Body length 3.0–3.5 mm. *Head.* Integument dark yellow, moderately covered with round tubercles; frons with two simple short trichomes on each side; face with one simple medium-long trichome on each side; antennal sheath without any projection or tubercles. *Thorax.* Integument dark yellow, moderately covered with tubercles; thorax on each side with nine simple trichomes (two mediodorsally, two anterolaterally, two mediolaterally, and three ventrolaterally), all medium-long, except one of two anterolateral trichomes and all three ventrolateral trichomes short. Gill (Fig. 3A) of arborescent type, composed of 22 short to medium-long slender thread-like filaments arranged from dorsal to ventral as {(2+2)+2+2+(1+1+2)+[1+(1+2)]}+[(1+2)+(1+2)]; all filaments light to medium brown, variable in length, ranging from 0.8 to 2.0 mm in male pupa (from 1.0 to 2.6 mm in female pupa), with annular ridges and furrows and densely covered with minute tubercles; transparent organ at base of gill of normal size. *Abdomen.* Dorsally, all segments nearly transparent, except segment 9 yellowish; segment 1 smooth (without tubercles), with one short slender seta on each side; segment 2 sparsely with comb-like groups of minute spines, with one short slender seta, five short spinous setae (one of five spinous setae stout in female pupa) on each side; segments 3 and 4 each with four stout hooks and one or two short spinous setae on each side; segments 5–9 each with comb-like groups of minute spines, although sparsely on segment 5, and without spine-combs on each side; segment 9 with pair of small cone-shaped terminal hooks (Fig. 3B). Laterally, segments 2–4 each with three short spinous setae on each side; segment 9 with three grapnel-shaped hooklets on each side in male pupae (in female pupa, three simple hooklets on right side and one simple and one grapnel-shaped hooklet on left side). Ventrally, all segments nearly transparent, except segment 9 yellowish; segment 4 with pair of simple dark hooklets and few short setae on each side; segments 5–7 each with pair of simple stout dark hooks and few short setae on each side; segments 4–8 each with comb-like groups of minute spines on each side. *Cocoon.* Wall-pocket-shaped, moderately woven, with anterodorsal margin somewhat thickly woven, and slightly extended ventrolaterally: individual threads visible; 4.2–5.5 mm long by 2.8–2.9 mm wide.

Mature Larva. Body length 6.2–7.2 mm. Body grayish. Cephalic apotome whitish yellow, except narrow area along posterior margin dark brown; head spots distinct, dark-brown, except anterior spot of posterolateral spots on each side light brown; posterior spot of posterolateral spots on each side merged in dark brown background and posterior spot of mediolongitudinal spots sometimes connected posteriorly to dark-brown area along posterior margin (Fig. 3C). Lateral surface of head capsule yellow, except area above and posterior to eyespot region light to medium brown, with distinct dark-brown spots, i.e., two large spots and two small ones in front of posterior margin and two isolated small spots below eyespot region. Ventral surface of head capsule yellow, except median portions on each side of postgenal cleft and posterior portions along posterior margin widely light to medium brown, with medium-brown elongate and round spots on each side of postgenal cleft, although both spots sometimes merged into medium-brown background (Fig. 3D). Cervical sclerites composed of two yellow small elliptical pieces, not fused to occiput, widely separated medially from each other (Fig. 3C). Antenna consisting of three segments and apical sensillum, little shorter than stem of labral fan; proportional lengths of first, second, and third segments 1.00: 1.30:0.84. Labral fan with 45–49 main rays. Mandible (Fig. 3E) with mandibular serration consisting of two teeth (one tooth large and one tooth small); large tooth at right angle to mandible on apical side; comb-teeth composed of three or more teeth decreasing in size from first to third; supernumerary serrations ab-

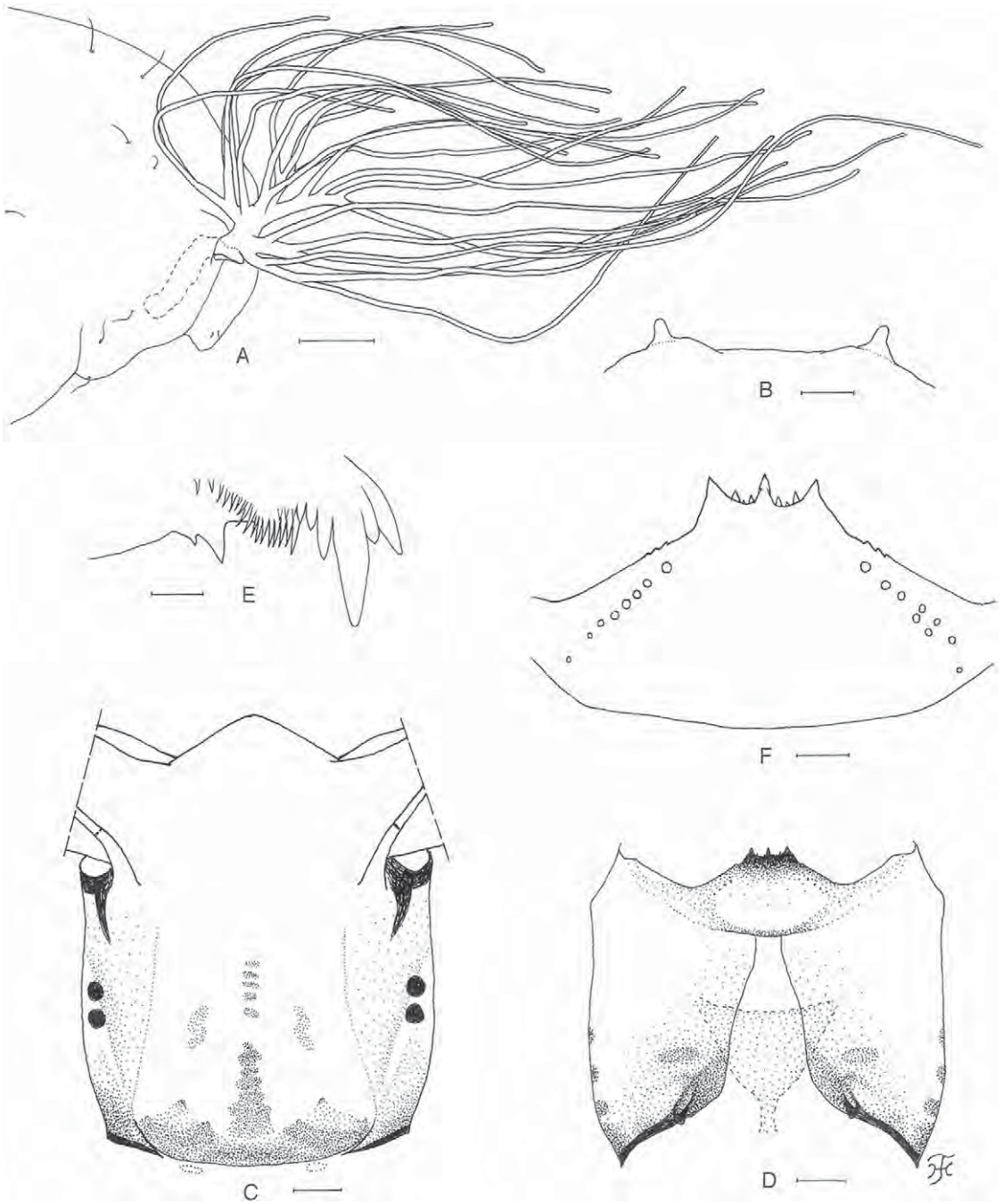

Fig. 3. Pupa and mature larva of *S.* (*A.*) *furvum* sp. nov. (A and B) Pupa. (C–F) Larva. (A) Anterior half of thorax and gill filaments (right side; outer view). (B) Terminal hooks (caudal view). (C) Head capsule showing distinct head spots on cephalic apotome (dorsal view). (D) Head capsule showing long postgenal cleft and subesophageal ganglion with gray-pigmented sheath (ventral view). (E) Apical portion of mandible. (F) Hypostoma. Scale bars = 0.2 mm (A), 0.1 mm (C and D), 0.04 mm (F), and 0.02 mm (B and E).

sent. Hypostoma (Fig. 3F) with nine apical teeth in row; median and corner teeth well developed; lateral serrations weakly developed anteriorly; seven to nine hypostomal bristles per side, lying slightly divergent posteriorly from lateral margin. Postgenal cleft (Fig. 3D) deep, reaching posterior margin of hypostoma; subesophageal ganglion with gray-pigmented sheath. Thoracic and abdominal cuticle bare, except both sides of anal sclerite moderately covered with simple colorless setae. Rectal scales not discernible. Rectal

organ compound, each of three lobes with 8–10 finger-like secondary lobules. Anal sclerite X-shaped, with anterior arms 0.9 times as long as posterior arms; anterior arms broadened, and space between arms widely sclerotized basally; two or three sensilla just posterior to basal juncture; accessory sclerite absent. Last abdominal segment somewhat expanded ventrolaterally forming bulge, ventral tip of which is visible as small ventral papilla when viewed from side. Posterior circlet with 80 rows of up to 15 hooklets per row.

Type Materials. HOLOTYPE: Female (with associated pupal exuviae and cocoon) (preserved in 80% ethanol) reared from pupa, Thailand: Maewa National Park, Thoen District, Lampang Province, 23-IX-2012, Wichai Srisuka. Paratypes: One male (with associated pupal exuviae and cocoon) (preserved in 80% ethanol) and eight mature larvae, same data as those of holotype.

Distribution. Thailand.

Etymology. The specific name *furvum* refers to the dark body including legs of the adults of this new species. The Latin adjective *furvus* means dark-colored.

Biology. The biting habit of females of *S.* (*A.*) *furvum* sp. nov. is unknown. The pupae and larvae of this new species were collected from grasses trailing in the water of a small stream slowly flowing on rocks (water temperature 27.6°C, exposed to sun, altitude 468 m) (17° 37′19.7″ N, 99° 18′49.0″ E). Associated species was *S.* (*A.*) *wanchaii* Takaoka and Choochote 2006.

Discussion. The subgenus *Asiosimulium* previously was represented by only three species, i.e., *S.* (*A.*) *oblongum*, *S.* (*A.*) *wanchaii*, both described from Thailand (Takaoka and Choochote 2005, 2006), and *S.* (*A.*) *suchitrae* from Nepal (Takaoka and Shrestha 2010). The male, pupa, and larva of *S.* (*A.*) *wanchaii* and the male of *S.* (*A.*) *suchitrae* are not known yet.

The female of *S.* (*A.*) *furvum* sp. nov. is distinguished from those of the three known species by the following characters (those of known species in parentheses): from *S.* (*A.*) *oblongum* by the cercus, which is nearly as long as the paraproct (Fig. 1H) (much longer than the paraproct) and the spermatheca pear-shaped (Fig. 1I) (ovoidal); from *S.* (*A.*) *wanchaii* by the spermatheca pear-shaped (ovoidal); and from *S.* (*A.*) *suchitrae* by the genital fork with a distinct projection on each arm (Fig. 1F) (with no projection), and the cercus medium-long, nearly as long as the paraproct (shorter than the paraproct).

The male of this new species is distinguished from that of *S.* (*A.*) *oblongum* by the smaller number of enlarged upper-eye facets in 16 vertical columns and 17 horizontal rows, the enlarged wedge-shaped hind basitarsus (Fig. 2C), the ventral plate with a deep notch posteromedially (Fig. 2D), and the aedeagal membrane with stout spines (Fig. 2I). The enlarged upper eye facets are in 19 vertical columns and 19 horizontal rows, the hind basitarsus is nearly parallel-sided, the ventral plate has a shallow notch posteromedially, and the aedeagal membrane is covered with minute spines in *S.* (*A.*) *oblongum* (Takaoka and Choochote 2005).

In the pupal stage, the smaller number of the gill filaments (22) distinguishes this new species from the two other related species, cf., 31–33 in *S.* (*A.*) *oblongum* and 28 in *S.* (*A.*) *suchitrae*. The pupa of this new species is also distinguished from that of *S.* (*A.*) *suchitrae* by the frons and thorax covered with tubercles and the absence of the bulbous transparent organ at the base of the gill.

The larva of *S.* (*A.*) *furvum* sp. nov. is easily distinguished from those of *S.* (*A.*) *oblongum* and *S.* (*A.*) *suchitrae* by the subesophageal ganglion with a gray-pigmented sheath (Fig. 3D).

In this study, only one female and one male, both emerged from pupae, were available for descriptions. A question may be raised of whether some morphological characters are normal or abnormal when only a single specimen is used. We think it unlikely that both adult specimens concerned are aberrant individuals with abnormal morphological characters. Among diagnostic adult characters of this new species, some are paired organs, and others are unpaired. In general, abnormal characters of paired organs are expressed asymmetrically, i.e., only left side or right side, in aberrant individuals. Such asymmetrical abnormalities were not recognized in female cerci (Fig. 1G and H), arms of the genital fork (Fig. 1F), enlarged upper eye facets, and male hind basitarsi (Fig. 2C). Among unpaired organs used as diagnostic characters of the new species, the pear-shaped spermatheca of the female (Fig. 1I) is judged as normal, as such a spermatheca was also reported in *S.* (*A.*) *suchitrae* from Nepal (Takaoka and Shrestha 2010); the ventral plate of the male genitalia with a deep notch posteromedially (Fig. 2D) also is judged as normal because it is interpreted as the more specialized type, probably evolved from the ventral plate, of which a posteromedian notch is shallow, as seen in *S.* (*A.*) *oblongum* (Takaoka and Choochote 2005); stout spines of the aedeagal membrane of the male genitalia (Fig. 2I) seem to be normal because such stout spines also were found in a few species of the subgenus *Wallacellum*, such as *Simulium* (*Wallacellum*) *marilogense* Takaoka and *Simulium* (*Wallacellum*) *ogonukii* Takaoka from the Philippines (Takaoka 2009).

The limited sample raises another question of whether it represents an extreme example of variation. The range of variation of quantitative diagnostic characters, such as the relative length ratio of the female cercus against its width and the number of male upper eye facets, as well as the number of pupal gill filaments, although expected to be small if any, will be determined in future studies.

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Simulium (*Nevermannia*) *khunklangense*, a new species of black fly (Diptera: Simuliidae) from Chiang Mai, Thailand

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Abstract

Simulium (*Nevermannia*) *khunklangense* **sp. nov.** is described from females, males, pupae and larvae collected in Doi Inthanon National Park, Chiang Mai, Thailand. This new species is placed in the *vernum* species-group of the subgenus *Nevermannia* and is similar to *S.* (*N.*) *chomthongense* Takaoka & Srisuka described from Doi Inthanon National Park, Thailand, but is distinguished in the male by the number of enlarged upper-eye facets and the relative width of the hind basitarsus against the hind tibia and femur, and in the pupa by the short common basal stalk of the gill and the cocoon with an anterodorsal bulge or a short anterodorsal projection. Taxonomic notes are provided to separate this new species from five other known species of the *vernum* species-group, which share an accessory sclerite on the larval abdomen, a rare characteristics in this species-group.

Key words: Diptera, Simuliidae, new spieces

Introduction

The *Simulium* (*Nevermannia*) *vernum* species-group, which consists of about 130 species, is widely distributed in the Holarctic Region and extends its distribution southward into the Oriental Region where 24 species are recorded (Adler & Crosskey 2013). In Thailand, this species-group is represented by one species, *S.* (*N.*) *chomthongense* Takaoka & Srisuka (Takaoka *et al.* 2012), which was first reported as *Simulium* (*Eusimulium*) sp. A (Takaoka & Suzuki 1984), and later tentatively identified as *S.* (*N.*) *caudisclerum* Takaoka & Davies originally described from Peninsular Malaysia (Takaoka & Choochote 2004).

Females of the *vernum* species-group are considered ornithophilic, as judged by the claws with a large basal tooth (Adler *et al*. 2004). In Asian countries, species of this group have not been investigated for their role as a vector of human and animal disease agents except one species, *S.* (*N.*) *uchidai* Takahasi, a common species in Japan, which was reported to be a vector of an unnamed filarial species, probably of a bird (Fukuda *et al*. 2005) and a potential vector of *Leucocytozoon lovati*, a haematozoan parasite of an endangered rock ptarmigan (Sato *et al*. 2009).

Recently, we collected another species of the *vernum* species-group, which is similar to *S.* (*N.*) *chomthongense* in many characters including an accessory sclerite on the larval abdomen, a rare character in this species-group, but it is distinguished from the latter species in the male by the number of upper-eye facets and the relative width of the hind basitarsus against the hind tibia and femur, and in the pupa by the short common basal stalk of the gill filaments relative to the interspiracular trunk, and the cocoon with a triangular anterodorsal bulge or a short anterodorsal projection.

In this paper, this species is described as new to science based on females, males, pupae and mature larvae collected in Doi Inthanon National Park, Chiang Mai, Thailand. Taxonomic notes are provided to separate this new

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species from five other known species of the *vernum* species-group, which share an accessory sclerite on the larval abdomen.

The methods of collection, description and illustration, as well as terms for morphological features used here, follow those of Takaoka (2003). The type specimens are deposited at the Entomology Section, Queen Sirikit Botanic Garden, Chiang Mai, Thailand.

Simulium (*Nevermannia*) *khunklangense* Takaoka & Srisuka sp. nov.

Description. **Female**. Body length 2.6–2.8 mm. ***Head***. Slightly narrower than width of thorax. Frons brownish-black, gray pruinose, not shiny, densely covered with whitish-yellow scale-like recumbent short hairs and lacking dark simple longer hairs along each lateral margin; frontal ratio 1.61–1.84:1.00:1.93–2.22; frons:head ratio 1.00:4.34–4.43. Fronto-ocular area well developed, narrow, directed dorsolaterally. Clypeus dark brown, gray pruinose, moderately covered with whitish-yellow hairs interspersed with few dark longer hairs near lower margin on each side (except medial portion of upper half bare). Labrum 0.79–0.86 times as long as clypeus. Antenna composed of scape, pedicel and 9 flagellomeres, dark brown to brownish-black except scape, pedicel and base of first flagellomere dark yellow to medium brown. Maxillary palp composed of 5 segments, light to medium brown, proportional lengths of third, fourth, and fifth segments 1.00:0.74–0.75:1.46– 1.57; third segment (Fig. 1A) swollen; sensory vesicle (Fig. 1A) elongate (0.59 times as long as third segment), with medium-sized opening. Maxillary lacinia with 11–14 inner and 15 or 16 outer teeth. Mandible with 34–36 inner and 14 or 15 outer teeth. Cibarium without denticles. ***Thorax.*** Scutum brownish-black except anterolateral calli ochreous, slightly shiny when illuminated at certain angle, densely covered with yellow scale-like recumbent hairs interspersed with several yellow medium-long to long hairs as well as dark brown long upright hairs on prescutellar area. Scutellum medium brown, slightly shiny when illuminated at certain angle, moderately covered with yellow medium-long to long hairs mixed with several dark brown long upright hairs. Postnotum dark brown, slightly shiny when illuminated at certain angle and bare. Pleural membrane bare. Katepisternum dark brown to brownish-black, longer than deep, slightly shiny when illuminated at certain angle, and bare. ***Legs***. Foreleg: coxa dark yellow; trochanter light brown except basal 1/2 of outer surface yellow; femur dark yellow except apical cap medium brown; tibia medium brown, with median large portion on outer surface grayish light brown; tarsus dark brown, with moderate dorsal hair crest; basitarsus moderately dilated, 8.13–8.25 times as long as its greatest width. Midleg: as in foreleg except coxa dark brown on anterolateral surface and brownish-black on posterolateral surface, and trochanter light brown. Hind leg: coxa medium brown; trochanter dark yellow except anterior surface light brown; femur dark yellow with apical cap medium brown and basal tip light brown on anterior surface; tibia medium to dark brown except extreme base yellow, and medial large portion on outer surface grayish light brown; tarsus dark brown except basitarsus light to medium brown (though base dark brown) and basal 1/2 of second tarsomere grayish; basitarsus (Fig. 1B) narrow, nearly parallel-sided, 6.77–6.85 times as long as wide, and 0.76 and 0.59 times as wide as greatest widths of tibia and femur, respectively; calcipala (Fig. 1B) nearly as long as width at base, and 0.46–0.5 times as wide as greatest width of basitarsus. Pedisulcus (Fig. 1B) well defined. Claw (Fig. 1C) with large basal tooth 0.48 times as long as claw. ***Wing***. Length 3.0 mm. Costa with dark brown spinules and hairs except basal portion covered with patch of yellow hairs. Subcosta with light brown hairs except apical 1/4 bare. Hair tuft on stem vein light to dark brown. Basal portion of radius fully haired; R_1 with dark spinules and hairs; R_2 with hairs only. Basal cell absent. ***Halter.*** White except basal stem darkened. ***Abdomen***. Basal scale ochreous to light brown, with fringe of whitish-yellow hairs. Dorsal surface of abdomen dark brown to brownish-black except segments 2 and 7–9 light brown (though base of segment 2 ochreous), moderately covered with dark short to long hairs and yellow short hairs; tergites of segments 3–6 relatively narrow, those of segments 2 and 7–9 wide and all dull. Ventral surface of abdomen mostly pale ochreous; sternal plate on segment 7 developed medially. ***Genitalia***. Sternite 8 (Fig. 1D) bare medially, with 12–17 short to long yellow hairs intermixed with 2 or 3 long dark brown hairs on each side. Ovipositor valves (Fig. 1D) triangular (though medioposterior corners rounded), thin, membranous, moderately covered with microsetae interspersed with 5 or 6 yellow short slender hairs (of which 1 hair usually medium-long) on each side; inner margins slightly concave medially, somewhat sclerotized, and somewhat separated from each other. Genital fork (Fig. 1E) of usual inverted-Y form, with slender stem; arms of moderate width medially; lateral plate of each arm strongly sclerotized along dorsolateral margin, and with thin lobe directed medioposteriorly. Paraproct in ventral

view (Fig. 1F) nearly pentagonal, with 4 or 5 sensilla on unpigmented anteromedial surface; paraproct in lateral view (Fig. 1G) somewhat produced ventrally, 0.74 times as long as wide, with 15 or 16 medium-long to long hairs on ventral and lateral surfaces. Cercus in lateral view (Fig. 1G) short, rounded posteriorly, 0.64 times as long as wide. Spermatheca (Fig. 1H) ovoidal, 1.16 times as long as its greatest width, well sclerotized except duct and small area near juncture with duct unsclerotized, and with hexagonal patterns (though not well defined) on surface; internal setae absent; both accessary ducts slender, subequal in diameter to major one.

Male. Body length 2.7 mm. ***Head***. Wider than thorax. Upper eye medium brown, consisting of 18 vertical columns and 19 or 20 horizontal rows of large facets. Face dark brown, grayish-white pruinose. Clypeus brownish-black, whitish pruinose, moderately covered with golden yellow medium-long hairs (mostly directed upward) interspersed with few light brown longer hairs near lower margin. Antenna composed of scape, pedicel and 9 flagellomeres, dark brown except base of first flagellomere yellow; first flagellomere elongate, 2.18 times length of second one. Maxillary palp light to medium brown, with 5 segments, proportional lengths of 3rd, 4th, and 5th segments 1.00:0.86:1.83; third segment (Fig. 2A) not swollen; sensory vesicle (Fig. 2A) ellipsoidal, small, 0.28 times as long as third segment, and with small opening. ***Thorax***. As in female. ***Legs***. Color nearly as in female except following characteristics: fore coxa dark yellow to light brown, mid trochanter medium brown except anterior surface pale, and basal 1/4 of hind second tarsomere grayish. Fore basitarsus slightly dilated, 11.2 times as long as its greatest width. Hind basitarsus (Fig. 2B) enlarged, spindle-shaped, 4.35 times as long as wide, and 1.0 and 1.1 times as wide as greatest width of hind tibia and femur, respectively; calcipala (Fig. 2B) slightly shorter than basal width, and 0.3 times as wide as greatest width of basitarsus. Pedisulcus (Fig. 2B) well defined at basal 1/4 of second tarsomere. ***Wing***. Length 2.6 mm; other characteristics as in female except subcosta without hairs and basal portion of costal vein covered with dark hairs mixed with yellow hairs. ***Abdomen***. Basal scale medium brown, with fringe of yellow long hairs. Dorsal surface of abdomen dark brown to brownish-black, moderately covered with yellow short hairs intermixed with dark brown short to medium-long hairs. ***Genitalia***. Coxite in ventral view (Fig. 2C) nearly rectangular, 1.90 times as long as its greatest width. Style in ventral view (Fig. 2C) bent inward, nearly parallel-sided, rounded apically and with apical spine; style in medial view (Fig. 2D) shorter than coxite (0.82 times as long as coxite), boot-shaped, with triangular apical lobe directed dorsomedially; style in ventrolateral view (Fig. 2E) straight up to apical 1/3, then curved inwardly, wide basally, narrowed to basal 2/5, nearly parallel-sided up to apical 1/5, and with rounded apex. Ventral plate in ventral view (Fig. 2C) with body transverse, 0.52 times as long as wide, with anterior margin slightly produced medioanteriorly, posterior margin with two shallow submedial notches, darkened along anterior margin, and nearly bare except posteromedian portion of ventral surface densely covered with microsetae; basal arms of moderate length, directed forward, somewhat divergent from base; ventral plate in lateral view (Fig. 2F) with ventral margin of body concave; ventral plate in caudal view (Fig. 2G) with body appearing as shallow inverted-V shape, having similar width, posteroventral margin roughly undulate, and densely covered with microsetae medially on posterior surface. Median sclerite (Fig. 2F, H) club-shaped, narrow, with forked apex, and with base located in front of anterior margin of ventral plate. Parameres (Fig. 2I) large, each with small apical appendix directed forwardly, and with 1 distinct long and stout hook. Aedeagal membrane (Fig. 2J) moderately setose, dorsal plate well defined, broadly produced ventrally with round apex, though constricted subbasally. Ventral surface of abdominal segment 10 (Fig. 2K) with 2–5 distinct hairs near each posterolateral margin. Cercus (Fig. 2K) small, rounded, encircled with 10–13 hairs.

Pupa. Body length 3.0–3.6 mm. ***Head***. Integument light yellowish-brown, bare, though frons with few to several tubercles; antennal sheath without any protuberances; face with pair of simple long trichomes with coiled apices, and frons with 2 simple long trichomes with coiled or uncoiled apices on each side in 2 pupae, or with 1 simple long trichome on left side and 2 simple long trichomes on right side in 2 pupae, or with 1 simple long trichome on right side, and 1 simple long trichome and 1 bifid long trichome on left side in 1 pupa. ***Thorax***. Integument light yellowish-brown, bare except dorsal and dorsolateral surfaces sparsely covered with small round tubercles, with 3 simple very long dorsomedial trichomes with coiled or uncoiled apices, 2 simple anterolateral trichomes (1 very long with coiled apex, 1 long with coiled or uncoiled apex), 1 simple medium-long mediolateral trichome with coiled or uncoiled apex, and 3 simple ventrolateral trichomes with uncoiled apices (1 medium-long, 2 short), on each side. Gill (Fig. 3A,B) composed of 4 slender thread-like filaments, arranged in pairs, with short common basal stalk having somewhat swollen transparent organ ventrally (often partially broken) at base; common basal stalk short, 0.50–0.65 times as long as interspiracular trunk; stalk of dorsal pair of filaments 0.56–1.32 times

as long as common basal stalk, and that of ventral pair of filaments 0.48–1.47 times as long as common basal stalk; all filaments subequal in length and thickness to one another, though their lengths including their own stalk and common basal stalk varying by individual pupae from 4.8 mm to 6.5 mm by pupae; cuticle of all filaments with well-defined annular ridges and furrows, and densely covered with minute tubercles. ***Abdomen***. Dorsally, segments 1–4 dark grayish and without distinct tubercles; segment 1 with 1 medium-long simple slender hair-like seta on each side; segment 2 with 1 short simple slender hair-like seta and 5 short somewhat spinous setae submedially on each side; segments 3 and 4 each with 4 hooked spines and 1 short somewhat spinous seta on each side; segments 5–8 each with spine-combs in transverse row on each side, and segments 6–9 each with comb-like groups of minute spines on each side; segment 9 with pair of cone-like terminal hooks. Ventrally, segment 4 with 1 simple hook and few simple slender short setae on each side; segment 5 with pair of bifid hooks submedially and few short simple slender setae on each side; segments 6 and 7 each with pair of bifid inner and simple outer hooks somewhat spaced from each other and few short simple slender setae on each side; segments 4–8 with comb-like groups of minute spines. Each side of segment 9 without grapnel-shaped hooklets. ***Cocoon*** (Fig. 3C). Wall pocket-shaped, moderately woven, widely extended ventrolaterally, appearing round when viewed dorsally; anterior margin thickly woven, with triangular medial bulge or a short mediodorsal projection (up to 0.7 mm long) (though 2 of 5 cocoons with anterior margin roughly woven and without anterodorsal bulge or projection–Fig. 3D); posterior 2/3 with floor roughly or moderately woven; individual threads invisible or partially visible; 4.3–5.0 mm long by 3.5–4.0 mm wide.

Mature larva. Body length 6.2–7.0 mm. Body creamy. Cephalic apotome whitish-yellow, though narrow area along posterior margin somewhat darkened; head spots moderately positive except anterior spot of posterolateral spots usually obscure. Lateral surface of head capsule whitish-yellow except eye-spot region whitish; eyebrow moderately positive; among spots in front of posterior margin, 2 relatively large spots moderately positive, and 2 small spots faintly positive; 1 or 2 small round spots below eye-spot region indistinct or faintly positive. Ventral surface of head capsule whitish-yellow to yellow except darkened area near posterior margin on each side of postgenal cleft; horizontal long spot and round spot on each side of postgenal cleft faintly or moderately positive. Antenna composed of 3 segments and apical sensillum, somewhat longer than stem of labral fan; proportional lengths of 1st, 2nd, and 3rd segments 1.00:1.07–1.11:0.68. Labral fan with 31–37 main rays. Mandible (Fig. 3E) with 3 comb-teeth, of which 1st tooth longest and 2nd tooth as long as or slightly longer than3rd one; mandibular serration composed of 2 teeth (1 medium-sized and 1 very small); major tooth at obtuse angle against mandible on apical side; supernumerary serration usually absent. Hypostoma (Fig. 3F) with row of 9 apical teeth; median and each corner tooth prominent, subequal in length to one another and much longer than 3 intermediate teeth on each side; lateral margin weakly serrate; 5 or 6 hypostomal bristles per side lying parallel to lateral margin. Postgenal cleft (Fig. 3G) short, somewhat pointed apically (though rarely rounded apically as shown in Fig. 3H), 0.69–0.92 times as long as postgenal bridge. Cervical sclerites composed of 2 light to medium brown small oblong pieces, not fused to occiput, widely separated medially from each other. Thoracic cuticle bare. Abdominal cuticle almost bare except few posterior segments sparsely covered with colorless simple setae, and last segment densely covered with colorless simple setae on each side of anal sclerite. Rectal scales present. Rectal papillae compound, each of 3 lobes with 8–14 finger-like secondary lobules. Anal sclerite of usual X-form, with anterior arms 0.89–1.00 times as long as posterior ones, broadly sclerotized at base; accessory sclerite (Fig. 3I) composed of 3–5 sclerotized spots on each side. Last abdominal segment with pair of large conical ventral papillae. Posterior circlet with 84–94 rows of up to 14 hooklets per row.

Type specimens. Holotype male with associated pupal exuviae and cocoon (Thailand QSBG-2012-254-1) (preserved in 80% ethanol), reared from pupa, collected from a stream (width 50 cm, depth 5.5 cm, stream-bed sandy and muddy, water temperature 17.5˚C, pH 6.3, partially shaded, altitude 1,611 m, 18˚31'15.6" N, 98˚29'59.5" E) moderately flowing in a forest (before Check Point 2), Chomthong District, Doi Inthanon National Park, Chiang Mai, Thailand, 27. XI. 2012, by W. Srisuka & S. Suriya. Paratypes: 2 females, with associated pupal exuviae and cocoons (QSBG-2012-265-1 & 2; same data as holotype except 14. XII. 2012, stream width 45 cm, water temperature 15.8 ˚C, PH 6.6, collected by W. Srisuka & R. Sawkord), 1 pharate female and 1 pharate male (QSBG-2012-265), same data as 2 female paratypes, and 8 mature larvae (QSBG-2012-254), same data and date as those of the holotype, all paratype specimens preserved in 80% ethanol.

Biological notes. The pupae and larvae of this new species were collected from dead tree leaves in the water. The only associated species was *Simulium* (*Gomphostilbia*) *inthanonense* Takaoka & Suzuki, 1984.

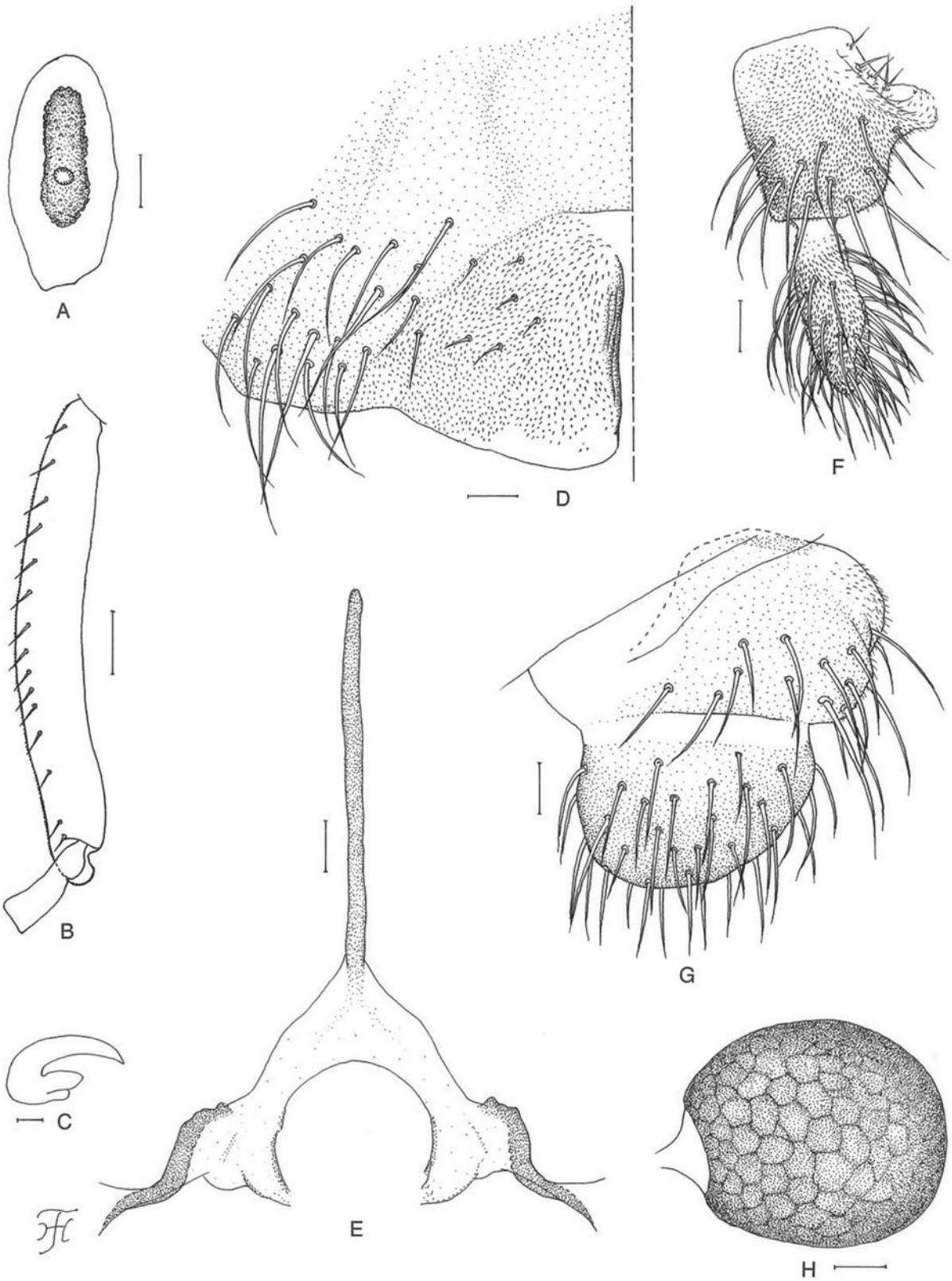

FIGURE 1. Female of *Simulium* (*Nevermannia*) *khunklangense* **sp. nov.** A, third segment of right maxillary palp with sensory vesicle (front view); B, basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view); C, claw (lateral view); D, sternite 8 and ovipositor valve (only right half shown) (ventral view); E, genital fork (ventral view); F & G, right paraprocts and cerci (F, ventral view; G, lateral view); H, spermatheca. Scale bars. 0.1 mm for B; 0.04 mm for A; 0.02 mm for D–H; 0.01 mm for C.

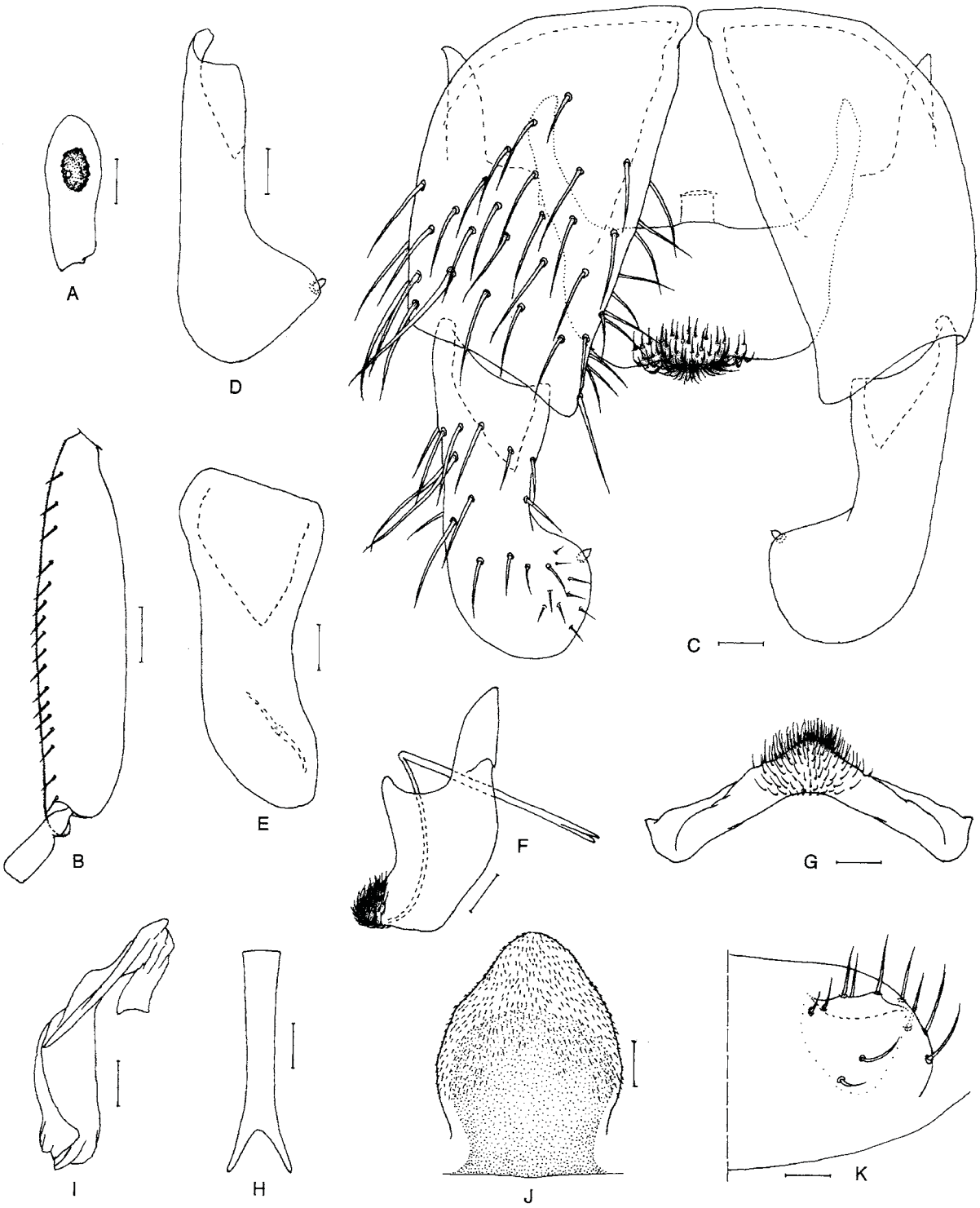

FIGURE 2. Male of *Simulium* (*Nevermannia*) *khunklangense* **sp. nov.** A, third segment of right maxillary palp with sensory vesicle (front view); B, basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view); C, coxites, styles and ventral plate (ventral view); D & E, right styles (D, medial view; E, ventrolateral view); F, ventral plate and median sclerite (lateral view); G, ventral plate (caudal view); H, median sclerite (caudal view); I, right paramere (caudal view); J, aedeagal membrane and dorsal plate (caudal view); K, abdominal segment 10 and cercus (left side; caudal view). Scale bars. 0.1 mm for B; 0.04 mm for A; 0.02 mm for C–K.

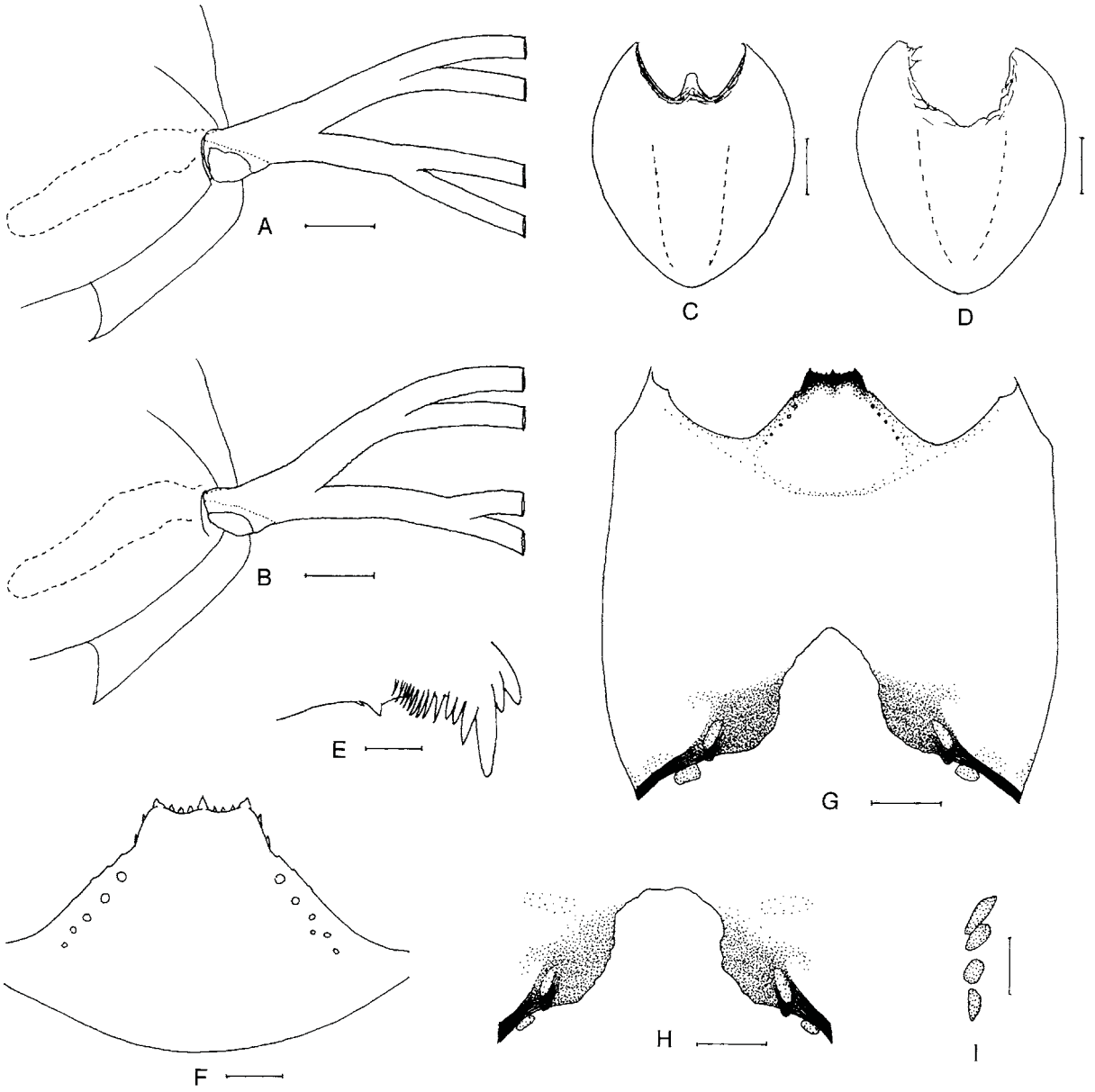

FIGURE 3. Pupa and larva of *Simulium* (*Nevermannia*) *khunklangense* **sp. nov.** A–D, pupa and E–I, larva. A & B, interspiracular trunk and basal portion of gill filaments showing different lengths of stalks of paired filaments relative to common basal stalk (right side; lateral view); C & D, cocoons (dorsal view; C, with short anterodorsal projection; D, without anterodorsal bulge or projection); E, mandible (lateral view); F, hypostoma (ventral view); G, head capsule showing postgenal cleft with pointed apex (ventral view); H, postgenal cleft with rounded anterior margin; I, accessory sclerite (left side; ventrolateral view). Scale bars. 1.0 mm for C & D; 0.1 mm for A, B, G & H; 0.04 mm for F; 0.02 mm for E & I.

Etymology. The species name *khunklangense* refers to the name of the village, Khunklang, in Doi Inthanon National Park, where this new species was collected.

Remarks. *Simulium* (*Nevermannia*) *khunklangense* **sp. nov.** is placed in the *vernum* species-group in the subgenus *Nevermannia*, mainly based on the shape of the male genitalia (Fig. 2C–K). Among the species of this group, this new species apparently is similar to the following six known species in bearing an accessory sclerite: *S.* (*N.*) *aberrans* Delfinado from the Philippines (Delfinado 1969; Takaoka 1983), *S.* (*N.*) *caudisclerum* from Peninsular Malaysia (Takaoka & Davies 1995), *S.* (*N.*) *chomthongense* from Thailand (Takaoka *et al*. 2012) , *S.* (*N.*) *ludingense* Chen, Zhang & Huang from Sichuan, China (Chen *et al*. 2005), *S.* (*N.*) *yushangense* Takaoka from Taiwan (Takaoka 1979), and *S.* (*N.*) *zhangjiajiense* Chen, Zhang & Bi from Hunan, China (Chen *et al*. 2004).

This new species appears to be most closely related to *S*. (*N*.) *chomthongense* among these species by sharing many adult female and larval characters, but it differs in the adult male and pupa from the latter species by having the following characteristics (characteristics of *S*. (*N*.) *chomthongense* in parentheses): male upper-eye facets in 18 vertical columns and 19 or 20 horizontal rows (in 15 or 16 vertical columns and 17 or 18 horizontal rows), ratio of the width of the male hind basitarsus against those of the hind tibia and femur, 1.0 and 1.1 (1.09–1.13 and 1.33–1.35), ratio of the length of the common basal stalk of the pupal gill filaments against that of the interspiracular trunk 0.50–0.65 (0.76–0.79), and the cocoon with a triangular anterodorsal bulge or a short anterodorsal projection (with a long anterodorsal projection).

The close relationship between *S*. (*N*.) *khunklangense* **sp. nov.** and *S*. (*N*.) *chomthongense* shown by morphological characters is also revealed by comparing the sequences of the mitochondrial 16S rRNA gene (516 base pairs) of both species (Otsuka et al. unpublished data). The difference in the sequences of this gene between *S*. (*N*.) *khunklangense* **sp. nov.** (Accession number in GenBank: AB820367) and *S*. (*N*.) *chomthongense* (Accession number in GenBank: AB699899) was only two base pairs.

Simulium (*N*.) *khunklangense* **sp. nov.** might have adapted to middle altitudes (about 1,600 m above sea level) with moderate water temperatures (16–18˚C), whereas *S*. (*N*.) *chomthongense* is confined to middle and higher altitudes (1,700–2,200 m above sea level) with low to moderate water temperatures (10–15˚C) in Doi Inthanon National Park (Takaoka *et al*. 2012). The distance of the type localities of both species is approximately 20 km.

This new species is distinguished in the male from the other five known species by the number of upper-eye facets (cf. about 22 horizontal rows in *S*. (*N*.) *aberrans,* 14 vertical columns and 15 horizontal rows in *S*. (*N*.) *caudisclerum*, 16 vertical columns and 15 horizontal rows in *S*. (*N*.) *ludingense*, about 17 horizontal rows in *S*. (*N*.) *yushangense*, and 17 vertical columns and 13 horizontal rows in *S*. (*N*.) *zhangjiajiense*); and in the pupa from *S*. (*N*.) *ludingense*, *S*. (*N*.) *yushangense* and *S*. (*N*.) *zhangjiajiense* by the gill filaments of equal thickness (cf. the dorsalmost filament somewhat thicker than three other filaments in the latter three known species), and from *S*. (*N*.) *aberrans* by the head and thoracic integument almost bare (cf. densely covered with tubercles in *S*. (*N*.) *aberrans*), and from *S*. (*N*.) *caudisclerum* by the short common basal stalk and the cocoon with a triangular anterodorsal bulge or a short anterodorsal projection (cf. the common basal stalk is medium-long, and the cocoon bears a long anterodorsal projection in *S*. (*N*.) *caudisclerum*).

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A New Species of *Simulium* (*gomphostilbia*) (Diptera: Simuliidae) from Thailand, with Keys to 11 Species of the *Simulium varicorne* Species-Group

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A New Species of *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) From Thailand, With Keys to 11 Species of the *Simulium varicorne* Species-Group

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ABSTRACT *Simulium* (*Gomphostilbia*) *piroonae* sp. nov. is described on the basis of females, males, pupae, and mature larvae collected in Mae Hong Son Province, Thailand. This new species is placed in the *chumpornense* subgroup of the *varicorne* species-group in the subgenus *Gomphostilbia* by having the antenna with eight flagellomeres, the pleural membrane bare, the female subcosta lacking hairs, and the pupal gill basally divided into two somewhat inflated branches. It is distinguished from all 10 known species of this group by the pupal gill with six filaments. Keys to identify 11 species of the *varicorne* species-group are provided for females, males, pupae, and mature larvae.

KEY WORDS blackfly, Simuliidae, Thailand, *Gomphostilbia*, taxonomy

The *Simulium varicorne* species-group, one of the 15 species-groups of *Simulium* (*Gomphostilbia*) redefined by Takaoka (2012), is characterized by adult antennae with seven or eight (rather than nine) flagellomeres. This group is small, consisting of 10 species, of which eight have been recorded in the Oriental Region and the remaining two in the Palearctic Region (Takaoka 2012, Adler and Crosskey 2013). In Thailand, this group is represented by four species: *Simulium burtoni* Takaoka & Davies, *Simulium chumpornense* Takaoka & Kuvangkadilok, *Simulium kuvangkadilokae* Pramual & Tangkawanit, and *Simulium novemarticulatum* Takaoka & Davies (Kuvangkadilok and Takaoka 2000, Takaoka and Choochote 2004, Pramual and Tangkawanit 2008, Takaoka et al. 2010). Biting habits and other biological aspects of these species remain unknown, although females of *S. burtoni* and *S. chumpornense* were captured using human attractants at low and medium altitudes in Doi Inthanon National Park in Chiang Mai (Choochote et al. 2005).

We collected a new species of the *varicorne* species-group in Mae Hong Son Province, Thailand, and we describe it on the basis of females, males, pupae, and mature larvae. Taxonomic notes are provided to distinguish this new species from related species, and keys to identify 11 species of this group are given for females, males, pupae, and larvae.

The methods of collection, description and illustration, as well as terms for morphological features, follow those of Takaoka (2003). The type specimens are deposited in the Entomology Section, Queen Sirikit Botanic Garden, Chiang Mai, Thailand.

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Simulium (*Gomphostilbia*) *piroonae* Takaoka & Srisuka sp. nov.

Description. Female. Body length 1.6 mm. *Head.* Nearly as wide as thorax. Frons brownish-black, dull, densely covered with yellowish-white scale-like recumbent short hairs interspersed with no or one or two dark unbranched longer hairs along each lateral margin; frontal ratio 1.33–1.40:1.00:1.73–1.75; frons: head ratio 1.00:4.30–4.54. Fronto-ocular area well developed, directed laterally, and slightly upward. Clypeus brownish-black, densely covered with yellowish-white scale-like short hairs interspersed with several dark unbranched longer hairs along lateral margin on each side. Labrum 0.59–0.65 times the length of clypeus. Antenna (Fig. 1A) composed of scape, pedicel and eight flagellomeres (of which fourth to eighth flagellomeres flattened), dark brown to brownish-black except scape and pedicel yellow, base of first flagellomere yellowish-white, rest of first flagellomere and third flagellomere medium to dark brown, and second and fourth flagellomeres medium to dark yellow. Maxillary palp composed of five segments, light brown, proportional lengths of third, fourth, and fifth segments 1.00:1.14–1.18:2.69–2.82; third segment (Fig. 1B) somewhat swollen apically; sensory vesicle (Fig. 1B) ellipsoidal, 0.25–0.30 times the length of third segment, with small opening. Maxillary lacinia with 8–10 inner and 11–14 outer teeth. Mandible with 18 teeth along inner margin and three to five teeth at some distance from apex along outer margin. Cibarium (Fig. 1C) with pair of short stout

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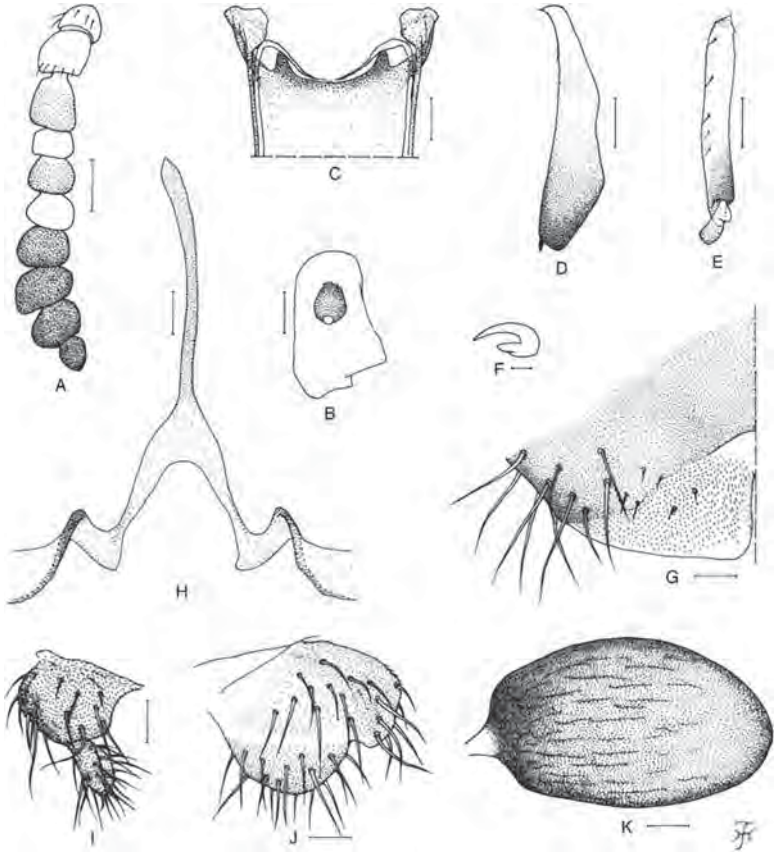

Fig. 1. Female of *S. piroonae* sp. nov. (A) antenna (left side; dorsal view); (B) third segment of maxillary palp with sensory vesicle (right side; front view); (C) cibarium; (D) hind tibia (left side; outer view); (E) hind basitarsus and second tarsomere (left side; outer view); (F) claw of hind tarsus (lateral view); (G) eighth sternite and ovipositor valves (right half; ventral view); (H) genital fork (ventral view); (I and J) paraprocts and cerci (right side; I, ventral view; J, lateral view); and (K) spermatheca. Scale bars = 0.1 mm for D and E; 0.05 mm for A; 0.02 mm for B, C, and G–K; 0.01 mm for F.

submedian projections directed dorsally on dorsal margin. *Thorax.* Scutum brownish-black (except anterolateral calli ochreous), shiny, gray pruinose with three longitudinal nonpruinose vittae (one medial and two submedial), densely covered with yellowish-white scale-like recumbent short hairs intermixed with brownish similar hairs. Scutellum dark brown, covered with yellowish-white short hairs and dark brown upright long hairs. Postnotum dark brown, bare, slightly shiny and gray pruinose when illuminated at certain angles. Pleural membrane bare. Katepisternum dark brown, longer than deep, moderately covered with yellowish fine hairs interspersed with dark brown hairs. *Legs.* Foreleg coxa and trochanter yellowish-white; femur light brown though apex and inner surface yellowish-white; tibia yellowish-white with basal tip medium brown, and subbasal and subapical light brown bands; tarsus brownish-black, with moderate dorsal hair crest; basitarsus somewhat dilated, 5.54–5.68 times as long as its greatest width. Midleg coxa medium brown with posterior surface dark brown; trochanter light brown except base yellowish-white; femur light brown though apex narrowly yellowish-white and somewhat paler band sub-apically; tibia whitish-yellow on basal two-fifths with light brown subbasal spot, and light to medium brown on apical three-fifths; tarsus yellowish-white except apical one-fourth or little less of basitarsus, apical one-third of second tarsomere and base of third tarsomere light brown. Hind leg coxa medium brown; trochanter yellowish; femur light brown with base yellowish-white, and apical cap medium brown (though tip yellowish-white); tibia (Fig. 1D) yellowish-white on basal one-half, faintly with light brown subbasal spot, and light to medium brown on apical one-half; tibia densely covered with whitish-yellow fine hairs on outer and posterior surface of basal three-fourths; tarsus whitish except base of basitarsus light brown and little less than apical one-third of basitarsus medium brown, apical one-half of second tarsomere and apical two-thirds of third tarsomere dark brown; basitarsus (Fig. 1E) narrow, nearly parallel-sided, 6.14–6.43 times as long as wide, and 0.53–0.58 and 0.50–0.66 times as wide as greatest width of tibia and femur, respectively; calcipala (Fig. 1E) 1.3 times as long as wide, and 0.5 times as wide as width of basi-

tarsus; pedisulcus (Fig. 1E) well marked. Hind tarsal claw (Fig. 1 F) with large basal tooth 0.52 times the length of claw. *Wing.* Length 1.5–1.6 mm. Costa with dark brown spinules and dark brown hairs except basal portion with patch of white hairs. Subcosta bare. Hair tuft on base of radial vein white. Basal portion of radius fully haired. Basal cell absent. *Halter.* White with stem yellowish. *Abdomen.* Basal scale light brown, with fringe of yellowish-white fine hairs. Dorsal surface of abdomen medium brown to brownish-black except little less than basal one-half lighter, moderately covered with yellowish-white short hairs interspersed with dark brown long hairs; tergites of segments 2 and 6–8 shiny; sternal plate on segment 7 undeveloped. *Genitalia.* Sternite 8 (Fig. 1G) bare medially, with 7–10 long stout hairs and few short setae on each side. Ovipositor valves (Fig. 1G) nearly triangular, with round medioposterior corners, thin, membranous, moderately covered with microsetae interspersed with one to three short setae; inner margins slightly sinuous, moderately sclerotized. Genital fork (Fig. 1H) of usual inverted Y-form, with narrow arms; arm folded medially. Paraproct in ventral view (Fig. 1I) rounded outwardly and tapered medially, with 15–18 long hairs on ventral and lateral surfaces, and with four or five sensilla on anteromedial surface; paraproct in lateral view (Fig. 1J) moderately produced ventrally beyond ventral margin of cercus. Cercus in lateral view (Fig. 1J) rounded posteriorly, 0.42–0.44 times as long as wide. Spermatheca (Fig. 1K) ellipsoidal, 1.62–1.93 times as long as wide, well sclerotized except duct unsclerotized (base of duct narrowly sclerotized), and with many fissures on surface; internal setae absent.

Male. Body length 1.7–2.0 mm. *Head.* Much wider than thorax. Upper eye consisting of 12 (rarely 11) vertical columns and 12 horizontal rows of large facets on each side. Face brownish-black, white pruinose, not shiny. Clypeus brownish-black, white pruinose, not shiny, densely covered with yellow scale-like short to medium-long hairs interspersed with several dark brown unbranched longer hairs. Antenna as in female except color pattern variable, that is, dark brown to brownish-black except scape and pedicel dark yellow to light brown, whole of second and fourth flagellomeres light brown, basal one-third of first flagellomere yellowish-white in most males, or dark brown to brownish-black except scape and pedicel light brown, apical two-thirds of first flagellomere and whole of second to fourth flagellomeres light to medium brown and basal one-third of first flagellomere yellowish-white in some males, or dark brown to brownish-black except scape and pedicel light to medium brown and basal one-third of first flagellomere yellowish-white in other males; first flagellomere elongate, 1.60 times length of second one. Maxillary palp as in female except proportional lengths of third, fourth, and fifth segments 1.00:1.09–1.17:2.53–2.56; third segment (Fig. 2A and B) somewhat widened apically; sensory vesicle (Fig. 2A and B) ellipsoidal (rarely globular), small, 0.18–0.22 times length of third segment, and with small opening. *Thorax.* As in female except scutellum covered with dark scale-like short hairs in place of golden yellow scale-like short hairs. *Legs.* Color and shape (except claw) nearly as in female except slight differences: fore basitarsus moderately dilated, 5.82–6.38 times as long as its greatest width; hind basitarsus 5.76–5.93 times as long as wide, and 0.56–0.57 and 0.46–0.51 times as wide as greatest widths of tibia and femur, respectively; calcipala slightly longer than wide, and 0.47–0.52 times as wide as greatest width of basitarsus. *Wing.* Length 1.3–1.5 mm; other features as in female. *Halter.* As in female. *Abdomen.* Basal scale light to medium brown, with fringe of yellow long hairs. Dorsal surface of abdomen medium brown to brownish-black except tergite of segment 2 yellow, covered with dark brown short to long hairs; segments 2 and 5–7 each with pair of shiny dorsolateral or lateral patches, of which those on segment 2 broadly connected in middle to each other. *Genitalia.* Coxite in ventral view (Fig. 2C) nearly rectangular, 1.27 times as long as its greatest width. Style in ventral view (Fig. 2C) slender, tapered toward apex, gently curved inward, with apical spine; style in ventrolateral view (Fig. 2D and E) somewhat wide basally (0.41 times as wide as its length), tapered toward basal two-fifths, then nearly parallel-sided or slightly widened, and with nearly triangular apical portion. Ventral plate in ventral view (Fig. 2C) transverse, 0.72 times as long as wide, widest at base, then slightly narrowed posteriorly, with anterior margin much produced anteromedially and posterior margin nearly straight, and densely covered with microsetae on ventral surface except near anterior margin bare; basal arms somewhat angulate basally, of moderate length, and slightly convergent apically or nearly parallel-sided; ventral plate in lateral view (Fig. 2 F) nearly flat, not produced ventrally, and with thick arms; ventral plate in end view (Fig. 2G) gently curved ventrally, with posterior surface moderately covered with microsetae medially. Median sclerite (Fig. 2 F and H) thin, plate-like, wide, connected to medial portion of ventral plate. Paramere (Fig. 2I) slender, with three distinct hooks (two large, one medium-sized) and several smaller ones at apex. Aedeagal membrane (Fig. 2I) moderately setose, not sclerotized. Ventral surface of abdominal segment 10 widely sclerotized basally, without distinct hairs near posterior margin on each side. Cercus (Fig. 2J and K) rounded, with eight hairs.

Pupa. Body length 1.8–2.0 mm. *Head.* Integument yellow, moderately covered with relatively large tubercles on most of frons and relatively smaller ones sparsely on face and moderately on ventrolateral portions of frons; relatively large tubercles each with tiny secondary projections (Fig. 3A) and relatively smaller tubercles with or without tiny secondary projections; antennal sheath without tubercle; frons with three pairs of unbranched long trichomes with uncoiled apices, all arising close together, and face with pair of unbranched long trichomes with uncoiled apices. *Thorax.* Integument yellow, moderately covered with various sizes of tubercles similar to those on frons on dorsal and dorsolateral surfaces of anterior one-half, moderately covered with small cone-shaped or round tubercles on dorsal surface of posterior one-half;

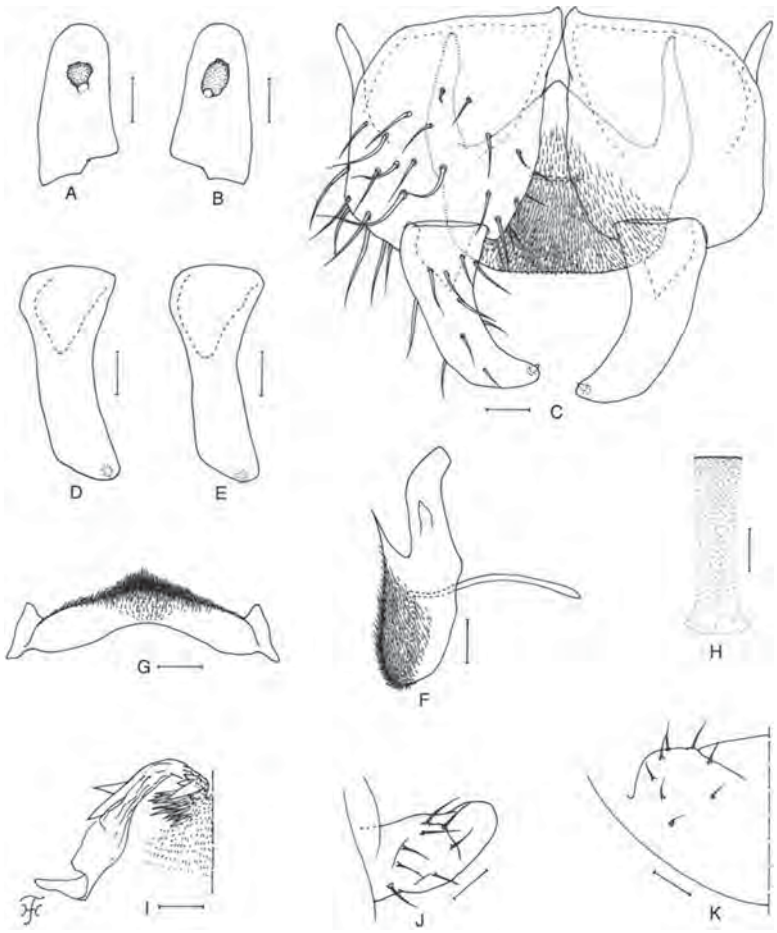

Fig. 2. Male of *S. piroonae* sp. nov. (A and B) third segments of maxillary palp showing sensory vesicle (front view; A, right side; B, left side); (C) coxites, styles and ventral plate (ventral view); (D and E) styles (right side; ventrolateral view); (F) ventral plate and median sclerite (lateral view); (G) ventral plate (end view); (H) median sclerite (anterior view); (I) paramere and aedeagal membrane (right side; posteroventral view); and (J and K) cerci (right side; J, lateral view; K, end view). Scale bars = 0.02 mm for A–K.

thorax with three long unbranched mediodorsal trichomes with or without coiled apices, two long unbranched anterolateral trichomes (one with coiled apex, one little shorter with uncoiled apex), one long simple posterolateral trichome with uncoiled apex and three unbranched ventrolateral trichomes with uncoiled apices (one long, two medium-long) on each side. Gill (Fig. 3B) composed of six filaments arranged as (2 + 2) + 2 filaments (though right gill of one pupa and left gill of another pupa each with arrangement of (1+[1 + 2]) + 2 filaments (Fig. 3C); common basal stalk short, with transparent basal fenestra ventrally; stalk of ventral pair of filaments long (0.4–0.8 mm), usually slightly longer and thinner than common stalk of dorsal and middle pairs of filaments; stalk of middle pair of filaments medium-long to long, usually longer than that of dorsal pair of filaments which is short to medium-long; all filaments light brown, subequal in length (1.6–2.1 mm when measured from base of gill to tips of filaments) and thickness to one another, with sharp transverse ridges forming reticulate patterns, in particular on stalks, though ridges becoming indistinct and only furrows remaining distinct on most of each filament, and densely covered with minute tubercles.

Abdomen. Dorsally, segments 1 and 2 whitish-yellow, sparsely covered with small tubercles; other segments almost transparent except segment 9 whitish-yellow; segment 1 with one medium-long unbranched seta on each side; segment 2 with one medium-long unbranched seta and five short setae sub-medially on each side; segments 3 and 4 each with four hooked spines and one short seta on each side; segment 5 lacking spine-combs; segments 6–9 each with spine-combs in transverse row and comb-like groups of minute spines on each side; segment 9 with pair of distinct conical terminal hooks (Fig. 3D). Ventrally, segment 4 with one unbranched hook and few short setae on each side; segment 5 with pair of bifid hooks submedially and few unbranched slender short setae on each side; segments 6 and 7 each with pair of bifid inner and

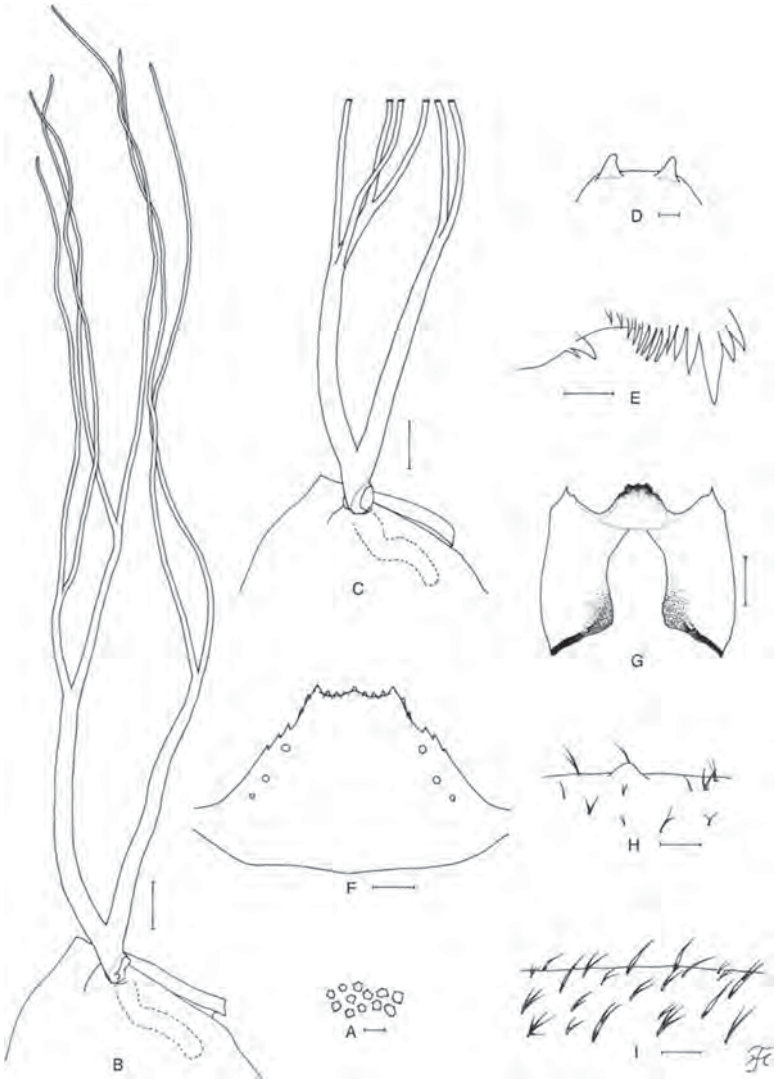

Fig. 3. Pupa and larva of *S. piroonae* sp. nov. (A–D) pupa; (E–I) larva. (A) tubercles with tiny secondary projections on frons; (B) gill filaments with interspiracular trunk (drawn in broken lines; right side; outer view); (C) gill filaments showing different arrangement (apical half not shown; right side; outer view); (D) terminal hooks (caudal view); (E) mandible; (F) hypostoma; (G) head capsule showing deep postgenal cleft (ventral view); (H) setae and protuberance on dorsal surface of thoracic segment 3 (lateral view); and (I) setae of various shapes on dorsal surface of abdominal segment 6 (lateral view). Scale bars = 0.1 mm for B, C, and G; 0.02 mm for F, H, and I; 0.01 mm for A, D, and E.

simple outer hooks somewhat spaced from each other and few simple slender short setae on each side; segments 4–8 with comb-like groups of minute spines. Each side of segment 9 with two or three grapnel-shaped hooklets. *Cocoon.* Wall-pocket shaped, moderately and neatly woven, extending ventrolaterally to varying extent; anterior margin somewhat thickly woven, often bulged anteriorly; floor roughly or moderately woven; individual threads invisible; 2.3–3.0 mm in length by 1.5–2.1 mm width.

Mature Larva. Body length 3.5–4.0 mm. Body characterized by transparent conical protuberances, that is, two pairs (one dorsal, one dorsolateral) on thoracic segments 1 and 2, and three pairs (one dorsal, one dorsolateral, and one lateral) on thoracic segment 3 and abdominal segments 1–5 (although lateral protuberances on thoracic segment 3 and abdominal segment 5 are tiny, probably overlooked). Body creamy, with markings as follows: thoracic segment 1 surrounded by gray wide band and anterior surface of thoracic proleg gray; thoracic segment 2 gray on dorsal and ventral surfaces; thoracic segment 3 gray on ventral surface; abdominal segments 1–4 each surrounded by gray wide band, although those on abdominal segments 2–4 disconnected dorsomedially; abdominal segments 5 and 6 each surrounded by gray band, although disconnected to varying extent laterally; abdominal segment 7 with gray areas on dorsal and

ventral surfaces, although disconnected dorsomedially; abdominal segment 8 widely gray on dorsal surface, although unpigmented anteromedially; gray areas on abdominal segments 6–8 often connected dorsolaterally; gray areas on ventral surface of abdominal segments 5–7 usually widely connected; most gray areas overlaid by reddish-brown pigment to varying extent on dorsal and dorsolateral surfaces but very rarely so on ventral surface. Head capsule uniformly covered with simple minute setae. Cephalic apotome whitish, although posterior one-half whitish-yellow in some larvae, narrow medial area along posterior margin somewhat darkened in several larvae, and broad area along posterior margin darkened in one larva; head spots faintly positive, although moderately positive in two larvae. Lateral surface of head capsule mostly whitish-yellow except eyespot region white; two large and one small spots in front of posterior margin faintly positive, or all or partially indistinct, although moderately positive in two larvae. Ventral surface of head capsule whitish-yellow, although slightly to moderately darkened along margins of postgenal cleft in several larvae, with dark brown area on each side of postgenal cleft; elongate spot on each side of postgenal cleft indistinct. Cervical sclerites composed of two small whitish-yellow to yellow rod-like pieces, not fused to occiput, widely separated from each other. Antenna unpigmented, composed of three segments and apical sensillum, much longer than stem of labral fan; proportional lengths of first, second, and third segments 1.00:0.80–1.00:0.91–1.09. Labral fan with 32–36 main rays. Mandible (Fig. 3E) with three comb-teeth, decreasing in length from first to third; mandibular serrations composed of two teeth (one large, one small); large tooth at acute angle to mandible on apical side. Hypostoma (Fig. 3 F) with row of nine apical teeth, median tooth and corner teeth prominent; lateral margins serrated apically; three hypostomal bristles in row, parallel to lateral margin on each side. Postgenal cleft (Fig. 3G) long, reaching posterior margin of hypostoma; subesophageal ganglion unpigmented, not visible. Pharate pupal gill with two long basal branches having four and two slender filaments, respectively. Thoracic cuticle and abdominal segments 1–4 sparsely or moderately covered with unbranched and bifid colorless minute setae (Fig. 3H) dorsally and dorsolaterally. Abdominal segments 5–9 densely covered with minute setae, of which relatively longer setae are dark at base, with basal half flattened, then bifid or not, tapered apically, and relatively shorter ones are nearly colorless or somewhat darkened at base, and with two to four branches (Fig. 3I), on dorsal, dorsolateral, and lateral surfaces including both sides of anal sclerite. Rectal scales absent. Rectal papillae compound, each of three lobes with seven to nine finger-like secondary lobules. Anal sclerite X-shaped, anterior arms 0.72–0.88 times the length of posterior ones; accessory sclerites absent; sensillum absent. Ventral papillae well developed, conical in shape. Posterior circlet of hooks with 58–64 rows of up to 10–12 hooks per row.

Type Materials. HOLOTYPE: Female (with associated pupal exuviae and cocoon), reared from pupa, THAILAND, Mae Hong Son Province, Pai District, 10–IV–2013, W. Srisuka, S. Suriya, and R. Sawcord. PARATYPES: 10 females, 16 males, (all with associated pupal exuviae and cocoon), reared from pupae, and 20 mature larvae, same data and date as those of holotype.

Biology. The pupae and larvae of this new species were collected from trailing grasses in a slow-flowing stream (width 1.7 m, depth 10 cm, streambed sandy, 31.1°C, pH 7.7, exposed to sun, altitude 451 m, 19°28′34.1″ N, 98°17′34.9″ E). Associated species were *Simulium chiangmaiense* Takaoka & Suzuki, *S. chumpornense*, *Simulium nakhonense* Takaoka & Suzuki, *Simulium nodosum* Puri, *Simulium siamense* Takaoka & Suzuki, and *Simulium siripoomense* Takaoka & Saito.

Etymology. The species name *piroonae* is in honor of Mrs. Piroon Wisuttipat, Mahasarakham University, who was a supervisor of W. Srisuka.

Discussion. *Simulium piroonae* sp. nov. is placed in the *varicorne* species-group of the subgenus *Gomphostilbia* based on the presence of eight antennal flagellomeres (Fig. 1A). It is further placed in the *chumpornense* subgroup by having the pleural membrane bare, the female subcosta lacking hairs, and the pupal gill basally divided into two somewhat inflated branches (Fig. 3B and C), as defined by Takaoka (2012).

The pupal gill consists of six filaments (Fig. 3B and C), which distinguishes the pupa of the new species from those of the other 10 known species of the *varicorne* species-group, which have 3, 8, or 12 gill filaments (Takaoka 2012). Apart from the difference in the pupa, *S. piroonae* sp. nov. is similar in the female to four known species of the *chumpornense* subgroup: *S. chumpornense*, *S. kuvangkadilokae* both described from Thailand, *Simulium tomae* Takaoka described from Sulawesi, and *Simulium varicorne* Edwards described from Sumatra and later recorded from Java and Peninsular Malaysia, in many characteristics including the bare subcosta, the color pattern of the antenna, and the configuration of the genitalia. The only difference is in the mandible, of which the outer margin has teeth in this new species and *S. varicorne* but lacks teeth in the other three known species (Edwards 1925; Kuvangkadilok and Takaoka 2000; Takaoka 2003; Takaoka and Srisuka 2010; H.T., unpublished data). The male of *S. piroonae* sp. nov. is also similar to those of *S. chumpornense*, *S. kuvangkadilokae*, and *S. varicorne* in sharing a similar color pattern of antennae and legs and a flat ventral plate, although there is a difference in the number of upper-eye enlarged facets, which consist of 15 vertical columns and 15 horizontal rows in *S. chumpornense* and 11 or 12 vertical columns and 12 or 13 horizontal rows in the other three species. The color of the antenna (i.e., dark yellow on scape and pedicel, medium brown on first, second and fourth flagellomeres [except base of first flagellomere yellowish-white] and dark brown on fifth to eighth flagellomeres), and the number of upper-eye enlarged facets of *S. chumpornense* are based on a male reared from a pupa collected together with *S. piroonae* sp. nov. The head of the male of *S. chumpornense*

was not described in the original description (Kuvangkadilok and Takaoka 2000). The larvae of *S. piroonae* sp. nov. and other three species of the *chumpornense* subgroup are similar to one another in sharing many characters including a deep postgenal cleft reaching the posterior margin of the hypostoma, protuberances on thoracic segments 1–3 and abdominal segments 1–5, and fine setae of similar shapes on the dorsal and dorsolateral surfaces of the abdomen. The female and larva of *S. varicorne* were unknown (Takaoka and Davies 1995) and information used here is based on females reared from pupae, and larvae collected from Peninsular Malaysia by H.T. The male and larva of *S. tomae* are unknown (Takaoka 2003).

Considering the many shared morphological characters, radiation might have occurred relatively recently in the *chumpornense* subgroup in a limited area of Southeast Asia ranging from Thailand through Peninsular Malaysia, Sumatra and Java to Sulawesi.

Interspecific differences in this subgroup are found mainly in the pupa, expressed in the number and arrangement of gill filaments. Relatively few such differences are expressed in the adults. The records from Java and Peninsular Malaysia of *S. varicorne* were based only on males (Crosskey 1973; Takaoka and Davies 1995, 1996), and the female pupa and larva of *S. varicorne* have remained unknown from the type locality of Sumatra (Edwards 1925). The pupa, thought to be that of *S. varicorne*, is known only from Peninsular Malaysia (Takaoka and Davies 1995). The species regarded as *S. varicorne* in Peninsular Malaysia and Java might be distinct from *S. varicorne* from Sumatra. Further study is needed to confirm whether *S. varicorne* is confined to Sumatra or extends to Peninsular Malaysia and Java, by collecting pupae from Sumatra and Java. *Simulium piroonae* sp. nov. is distinguished from other 10 known species of the *varicorne* species-group as shown in following keys.

Keys to 11 Species of the *varicorne* Species-Group of the Subgenus *Gomphostilbia*

Females*

1. Antenna consisting of scape, pedicel and seven flagellomeres 2
   Antenna consisting of scape, pedicel, and eight flagellomeres 3
2. Sensory vesicle 0.29–0.31 times the length of third maxillary palpal segment . . . . . *charlesi* Takaoka
   Sensory vesicle 0.21–0.25 times the length of third maxillary palpal segment . . . . . *novemarticulatum*
3. Pleural membrane haired . . . . . *trirugosum* Davies & Győrkős
   Pleural membrane bare 4
4. Subcosta haired 5
   Subcosta bare 7
5. Abdominal segments 5–8 shiny dorsally . . . . . *burtoni*
   Abdominal segments 6–8 shiny dorsally 6
6. Hind femur entirely darkened . . . . . *shogakii* Rubtsov
   Hind femur darkened on apical one-third. . . . . *synanceium* Chen & Cao
7. Mandible with teeth on outer margin . . . . . *piroonae* sp. nov. and *varicorne**
   Mandible without teeth on outer margin 8
8. Frons:head ratio 1.0:6.7. . . . . *tomae*
   Frons: head ratio 1.0:3.7–4.1 9
9. Hind basitarsus whitish on basal two-thirds, dark on rest . . . . . *chumpornense*
   Hind basitarsus whitish on basal one-half, dark on rest. . . . . *kuvangkadilokae*

*Information of the female of *S. varicorne* is based on specimens from Peninsular Malaysia.

Males**

1. Antenna consisting of scape, pedicel and seven flagellomeres 2
   Antenna consisting of scape, pedicel, and eight flagellomeres 3
2. Style slender, tapered apically. . . *novemarticulatum*
   Style broad, angulated . . . . . *charlesi*
3. Pleural membrane haired . . . . . *trirugosum*
   Pleural membrane bare 4
4. Ventral plate moderately produced ventrally, with width:height ratio 1.00:0.51–0.53 5
   Ventral plate not or slightly produced ventrally, with width:height ratio 1.00:0.29–0.34 7
5. Ventral plate in ventral view slightly tapered posteriorly . . . . . *burtoni*
   Ventral plate in ventral view slightly widened posteriorly. 6
6. Hind femur entirely darkened . . . . . *shogakii*
   Hind femur darkened on apical one-third. . . . . *synanceium*
7. Upper-eye with enlarged facets in 15 vertical columns and 15 horizontal rows . . . . . *chumpornense*
   Upper-eye with enlarged facets in 11 or 12 vertical columns and 12 or 13 horizontal rows 8
8. Hind tibia whitish on basal two-thirds, dark on rest . . . . . *varicorne*
   Hind tibia whitish on basal one-half, dark on rest . . . . *kuvangkadilokae* and *piroonae* sp. nov.

**The male of *S. tomae* is unknown. The information of the upper-eye enlarged facets of *S. chumpornense* is based on a male reared from a pupa collected from the type locality of *S. piroonae* sp. nov. in Thailand. The head of the male of this species was not described in the original description (Kuvangkadilok and Takaoka 2000).

Pupae

1. Gill with three somewhat inflated filaments . . . . . *trirugosum*
   Gill with 6–12 filaments 2
2. Gill with six filaments . . . . . *piroonae* sp. nov.
   Gill with 8 or 12 filaments 3
3. Gill with 12 slender filaments arising from two inflated trunks. . . . . *kuvangkadilokae*
   Gill with eight filaments 4
4. Gill composed of dorsal and middle triplets and ventral pair of filaments 5
   Gill basally divided into two branches, i.e., dorsal branch of six filaments and ventral branch of two filaments 8
5. Ventral pair of filaments subequal in length to other filaments of dorsal and middle triplets 6
   Ventral pair of filaments much longer than other filaments of dorsal and middle triplets 7
6. Terminal hooks much wider than long. . . . *burtoni*
   Terminal hooks cone-shaped, longer than wide. . . . . *shogakii* and *synanceium*
7. Inner filament of ventral pair much thicker and longer than outer filament. . . . . *charlesi*
   Inner filament of ventral pair as long as or slightly longer and as thick as or slightly thicker than outer filament. . . . . *novemarticulatum*
8. Dorsal branch composed of three pairs of filaments . . . . . *tomae*
   Dorsal branch composed of two triplets of filaments, or four individual and two paired filaments 9
9. Dorsal branch composed of two triplets of filaments. . . . . *varicorne*
   Dorsal branch composed of four individual filaments and two paired filaments . . . . . *chumpornense*

Larvae***

1. Abdomen with protuberances on dorsal surface 2
   Abdomen without protuberances on dorsal surface 7
2. Gill histoblast with six filaments. . . *piroonae* sp. nov.
   Gill histoblast with 8 or 12 slender filaments 3
3. Gill histoblast with 12 slender filaments arising from two enlarged trunks . . . . . *kuvangkadilokae*
   Gill histoblast with eight slender filaments 4
4. Gill histoblast with ventral pair of filaments subequal in length to six other filaments 5
   Gill histoblast with ventral pair of filaments much longer than six other filaments 6
5. Gill histoblast with dorsal group composed of two triplets of filaments . . . . . *varicorne*
   Gill histoblast with dorsal group composed of four individual filaments and two paired filaments . . . . . *chumpornense*
6. Stout spinous setae on dorsal surface of abdominal segments 5–8 unbranched. . . . . *charlesi*
   Stout spinous setae on dorsal surface of abdominal segments 5–8 bifid. . . . . *novemarticulatum*
7. Postgenal cleft elongate reaching posterior margin of hypostoma . . . . . *trirugosum*
   Postgenal cleft elongate but not reaching posterior margin of hypostoma . . . . . *burtoni* and *shogakii*

***The larvae of *S. shogakii*, *S. synanceium*, *S. tomae*, and *S. varicorne* are unknown (Bentinck 1955, Chen and Cao 1983, Takaoka and Davies 1995, Takaoka 2003). Information on larvae of *S. shogakii* and *S. varicorne* used in the key is based on specimens collected by H.T.

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Running title: A new black fly species from Thailand

A new species and species-group of *Simulium* (*Simulium*) (Diptera: Simuliidae) from Thailand

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ABSTRACT *Simulium* (*Simulium*) *atipornae* sp. nov. is described from females, males, pupae and larvae in Thailand. This new species is characterized in the female by the claw with a small subbasal tooth, ovipositor valve triangular with its inner margin nearly straight, in the male by the style with a short subbasal protuberance and ventral plate Y-shaped, with toothed posterior margin and in the pupa by the head and thoracic integument almost bare and gill with six filaments. Taxonomic notes are given to compare this new species with nine related species. A new species-group, the *christopheri* species-group, is proposed to accommodate *S. atipornae* sp. nov. and nine related species.

INTRODUCTION

Among the six subgenera of the genus *Simulium* Latreille in Thailand, the subgenus *Simulium* Latreille is the most abundant, represented by 43 (49.4%) of a total of 87 species (Adler and Crosskey 2013, Takaoka et al. 2013a,b). This subgenus is also rich in diversity, composed of 7 of the 12 species-groups in the Oriental Region, i.e., *griseifrons*, *malyschevi*, *multistriatum*, *nobile*, *striatum*, *tuberosum* and *variegatum* species-groups.

Females of three Thai species of the subgenus *Simulium* bit humans, i.e., *S. chamlongi* Takaoka & Suzuki, *S. nigrogilvum* Summers, and *S. nodosum* Puri, and those of the latter two species are reported to transmit two different kinds of filariae (Fukuda et al. 2003, Takaoka et al. 2003, Choochote et al. 2005).

Recently, we collected one new species of the subgenus *Simulium* in Thailand, which is not assignable to any of the 12 species-groups in the Oriental Region (Takaoka 2003, Takaoka and Saito 2007, Adler and Crosskey 2013).

We describe this new species based on females, males, pupae and mature larvae collected from Phetchabun Province in Thailand. Taxonomic notes are provided to separate this new species from nine other species, of which seven are unplaced to group in the world inventory of the family Simuliidae (Adler and Crosskey 2013). A new species-group is proposed to accommodate this new species and nine related species.

The methods of collection, description and illustration, as well as terms for morphological features used here, follow those of Takaoka (2003). The type specimens are deposited in the Entomology Section, Queen Sirikit Botanic Garden, Chiang Mai, Thailand.

***Simulium* (*Simulium*) *atipornae* Takaoka, Srisuka & Choochote sp. nov.**

Female. Body length 2.5–2.8 mm. ***Head***. Slightly narrower than thorax. Frons black, shiny, with several dark stout hairs along lateral margins; frontal ratio 1.26–1.27:1.00:1.25–1.33; frons-head ratio 1.00:3.95–4.13. Fronto-ocular area well developed, short, directed laterally, and round or pointed apically. Clypeus black, thinly white pruinose, shiny, moderately covered with dark brown medium-long hairs (though mediolongitudinal portion widely bare) interspersed with several dark brown longer curved hairs on each side of lower portion. Labrum 0.71–0.82 times length of clypeus. Antenna composed of scape, pedicel and nine flagellomeres, dark brown except scape, base of pedicel and base of first flagellomere yellow, and rest of pedicel medium brown.

Maxillary palp with five segments, light brown except first and second segments dark yellow and third segment dark brown; proportional lengths of third, fourth, and fifth segments 1.00:1.10–1.16:2.00–2.24; third segment (Fig. 1A) of normal size, with medium-sized ellipsoidal sensory vesicle (0.28 times length of third segment) having medium-sized opening. Maxillary lacinia with 12 inner and 13–15 outer teeth. Mandible with 31 or 32 inner and 13 outer teeth. Cibarium (Fig. 1B) with 73–85 minute processes near posterodorsal margin. ***Thorax***. Scutum black, shiny, densely covered with yellow recumbent short hairs interspersed with several dark brown long upright hairs on prescutellar area; when illuminated in front and viewed dorsally, scutum white pruinose, faintly with three longitudinal nonpruinose vittae (one narrow median vitta, two somewhat wider submedian vittae). Scutellum brownish-black, covered with dark brown upright long hairs as well as yellow short hairs. Postnotum brownish-black, shiny, whitish-gray pruinose, bare. Pleural membrane bare. Katepisternum brownish-black, longer than deep, bare. ***Legs***. Foreleg: coxa yellow; trochanter dark yellow to light brown, femur medium brown except basal one-third dark yellow to light brown; tibia whitish-yellow except apical one-fourth brownish-black, basal two-fifths of posterior portion light brown, and with whitish sheen widely on outer surface; tarsus brownish-black, with moderate dorsal hair crest; basitarsus greatly dilated, 5.94–6.13 times as long as its greatest width. Midleg: coxa dark brown; trochanter dark brown except basal one-third yellow; femur dark brown except base yellow and apical cap brownish-black; tibia yellow except apical one-third dark brown though outer surface of apical half light brown to dark brown, and with whitish sheen on posterior surface; tarsus dark brown except basal half of basitarsus yellow. Hind leg: coxa dark brown; trochanter yellow except anterior surface light brown; femur dark brown except base yellow and apical cap brownish-black; tibia (Fig. 1C) yellow on basal three-fifths, light to dark brown on rest, with whitish sheen on posterior surface; tarsus medium to dark brown except basal two-thirds of basitarsus, and basal half of second tarsomere whitish-yellow; basitarsus (Fig. 1D) nearly parallel-sided, 5.68–6.40 times as long as wide, and 0.71–0.73 and 0.56–0.65 times as wide as greatest widths of hind tibia and femur, respectively; calcipala (Fig. 1D) moderately developed, nearly as long as wide, and 0.4 times as wide as greatest width of basitarsus; pedisulcus (Fig. 1D) well developed at basal one-third of second tarsomere. Tarsal claw (Fig. 1E) with small subbasal tooth. ***Wing***. Length 2.5 mm. Costa with dark spinules and hairs; subcosta haired except apical one-fourth to one-third bare; basal section of radial vein bare; R_1 with dark brown spinules and hairs; R_2 with dark brown hairs; hair tuft on stem vein dark brown; basal cell absent. ***Abdomen***. Basal scale medium brown, with fringe of pale hairs. Dorsal

surface of abdomen dark brown except tergite of second segment light brown, with light to dark brown short hairs; tergites 2 and 6–8 shiny. Ventral surface of seventh segment with large weakly sclerotized sternal plate medially. ***Genitalia***. Sternite 8 (Fig. 1F) with 21–30 medium-long to long stout hairs (most are dark, few to several yellow) and few to several yellow short hairs on each lateral surface. Ovipositor valves (Fig. 1F) triangular, rounded posteromedially, membranous except inner margins moderately sclerotized, densely covered with microsetae (except narrow portion near posteromedial corner bare) interspersed with 3–7 yellow short hairs and 7–12 dark medium-long hairs; inner margins nearly straight or slightly sinuous, somewhat separated from each other. Genital fork (Fig. 1G) of inverted-Y form, with narrow well sclerotized stem; arms of moderate width, each with distinct projection directed anterodorsally. Paraproct in ventral view (Fig. 1H) nearly triangular, weakly pigmented on outer surface, not pigmented on narrow part of ventral surface along median margin and on most of anteromedial surface, with 25–32 pale and dark short to medium-long hairs on lateral and ventral surfaces, and with 9 or 10 short sensilla on anteromedial surface; paraproct in lateral view (Fig. 1I) 0.7 times as long as wide, and much protruding ventrally beyond ventral margin of cercus. Cercus in lateral view (Fig. 1I) short, 0.47 times as long as wide, with numerous medium-long hairs, and rounded posteriorly. Spermatheca (Fig. 1J) nearly ovoid or ellipsoidal, 1.08–1.31 times as long as greatest width, well sclerotized except small portion of junction with duct unsclerotized, with faintly defined reticulate patterns on its surface; internal setae present; accessory ducts subequal in thickness to each other, and slightly thicker than major duct.

Male. Body length 3.0–3.2 mm. ***Head***. Nearly as wide as thorax. Upper eye consisting of large facets in 21 or 22 vertical columns and in 22 or 23 horizontal rows. Clypeus black, thickly white pruinose and shiny when illuminated at certain angles, sparsely covered with dark brown hairs along and near lateral margins (most of central portion bare). Antenna composed of scape, pedicel and nine flagellomeres, brownish-black except base of first flagellomere yellow (though one male having a color pattern of antenna similar to that of female); first flagellomere elongate, 1.98–2.00 times length of second. Maxillary palp composed of 5 segments, light to medium brown except first and second segments yellow; proportional lengths of third, fourth, and fifth segments 1.00:1.16–1.21:2.13–2.24; third segment (Fig. 2A) of normal size; sensory vesicle (Fig. 2A) ellipsoidal, 0.22 times length of third segment, and with small opening. ***Thorax***. Scutum black, with whitish pruinose pattern, i.e., anterior pair of large spots on shoulders extending posteriorly along lateral margins and connected to large transverse spot entirely covering prescutellar area; scutum uniformly and moderately covered with

yellow recumbent short hairs interspersed with dark brown short hairs near anterior margin and long upright hairs on prescutellar area. Scutellum black, with several dark brown long upright hairs and dark short hairs. Postnotum black, bare. Pleural membrane bare. Katepisternum brownish-black, bare. ***Legs***. Foreleg: coxa dark yellow; trochanter medium brown; femur medium brown except apical cap dark brown; tibia dark brown except median outer surface widely white, and with large white sheen on outer surface; tarsus brownish-black, with moderate dorsal hair crest; basitarsus greatly dilated, 6.25–6.65 times as long as its greatest width. Midleg: coxa brownish-black; trochanter medium brown except base dark yellow; femur medium brown except apical cap dark brown; tibia medium brown with extreme base yellow and apical cap dark brown, and with white sheen on posterior surface; tarsus dark brown to brownish except base of basitarsus dark yellow to light brown. Hind leg: coxa brownish-black; trochanter dark yellow to light brown; femur dark brown except base dark yellow and apical cap brownish-black; tibia dark brown to brownish-black except extreme base yellow; tarsus dark brown except little less than basal half of both basitarsus and second tarsomere dark yellow; basitarsus (Fig. 2B) enlarged, spindle-shaped, 4.22–4.38 times as long as wide, and 0.76–0.84 and 0.71–0.77 times as wide as greatest width of hind tibia and femur, respectively; calcipala (Fig. 2B) developed, small, little shorter than its basal width, and 0.31 times as wide as greatest width of basitarsus; pedisulcus (Fig. 2B) well developed at basal two-fifths of second tarsomere. ***Wing***. Length 2.3–2.4 mm. Other characters as in female except subcosta bare. ***Abdomen***. Basal scale brownish-black, with fringe of dark brown long hairs. Dorsal surface of abdomen medium brown to brownish-black, moderately covered with dark brown short to medium-long hairs; segments 2, 6 and 7 each with pair of whitish pruinose spots dorsolaterally, those on segment 2 connected broadly to each other in middle. ***Genitalia***. Coxite in ventral view (Fig. 2C) nearly quadrate, 1.37 times as long as wide. Style in ventral view (Fig. 2C) elongate, slightly narrowed toward middle, then slightly widened to apex, inner margin gently concave, with subapical spine; style in ventrolateral view (Fig. 2D) 1.37 times length of coxite, 2.26 times as long as greatest width near apex; style in medial view (Fig. 2E) somewhat flattened ventro-dorsally, with short subbasal protuberance directed dorsomedially, covered with fine hairs; ventral plate in ventral view (Fig. 2C) Y-shaped, with narrow body and arms divergent from base; body bearing teeth in two parallel rows on posteroventral surface except ventral tip without teeth; ventral plate in lateral view (Fig. 2F) bearing serrated round posterior margin of body, with many short setae on anterior and lateral surface of body; ventral plate in end view (Fig. 2G) inverted-T-shaped, with many teeth in two vertical rows on posterior surface. Median sclerite (Fig.

2H) well sclerotized, plate-like, gradually widened, and with blunt apical tip. Paramere (Fig. 2I) with several short to medium-long hooks. Aedeagal membrane densely covered with minute setae; dorsal plate (Fig. 2J) small, moderately sclerotized. Abdominal segment 10 (Fig. 2K,L) with 4–11 short hairs (sometimes one hair is long) on each side of posterior surface. Cercus (Fig. 2K,L) small, rounded, with 11–13 hairs, of which 3 or 4 are long and others are short.

Pupa. Body length 2.8–3.3 mm. ***Head***. Integument yellow to dark yellow, bare; frons with two pairs of short trichomes with uncoiled apices; face with pair of medium-long trichomes with or without coiled apices, 2.1–2.9 times length of frontal trichomes; all trichomes unbranched. ***Thorax***. Integument yellow to dark yellow, bare except dorsal surface of posterior half of thorax sparsely covered with small tubercles; thorax on each side with two long trichomes (one with coiled apex, one with uncoiled apex, or both with coiled apices) anterodorsally (Fig. 3A), two trichomes (one long with or without coiled apex, one medium-long with uncoiled apex) anterolaterally (Fig. 3A), one medium-long trichome with uncoiled apex mediolaterally, and three trichomes with uncoiled apices (one long, two medium-long) ventrolaterally (Fig. 3A); all trichomes unbranced. Gill (Fig. 3A) with six thread-like filaments arranged as 2+2+2 from dorsal to ventral; dorsal and ventral pairs with short stalk, middle pair almost sessile; lowermost filament shortest (1.3–1.5 mm long) and other filaments subequal in length (1.7–2.0 mm); proportional thickness of 6 filaments from dorsal to ventral 1.29–1.39:1.23–1.29:1.15–1.21:1.08–1.14:1.23–1.27:1.00 when compared basally; all filaments medium brown, covered with annular ridges and furrows except basal short portion without such ridges and furrows and densely covered with minute tubercles on entire surface. ***Abdomen***. Dorsally, nearly transparent except segments 1 and 9 yellowish; segment 1 without tubercles, with one medium-long seta on each side; segment 2 without tubercle, with one medium-long seta and five short spinous setae, on each side; segments 3 and 4 each with four distinct hooks and one short spinous seta on each side; all setae and hooks unbranched; segments 5, 6 and 9 lacking spine-combs; segments 7 and 8 each with distinct spine-combs in transverse row; segments 6–9 each with comb-like groups of minute spines on each side; segment 9 without pair of terminal hooks. Ventrally, segments 3–8 transparent, each with comb-like groups of minute spines; segment 4 with one unbranched hooklet and few unbranched short setae on each side; segment 5 with pair of bifid stout hooklets submedially and few unbranched short setae on each side; segments 6 and 7 each with pair of bifid inner and unbranched outer stout hooklets somewhat separated from each other and few unbranched short setae on each side. Grapnel-shaped hooklets absent on each side of

segment 9. ***Cocoon*** (Fig. 3B). medium brown, wall-pocket shaped, with one or two large open windows and few to several small open spaces anterolaterally on each side (shape and size of open windows or spaces are variable by cocoons), tightly woven and not extending ventrolaterally; posterior half with floor; individual threads visible; 3.5–4.0 mm long by 1.4–1.7 mm wide.

Mature larva. Body length 5.8–6.6 mm. Body grayish light brown. Abdomen in lateral view gradually widened from segment 1 to segment 7, then narrowed to segment 9. Cephalic apotome whitish-yellow on anterior half, yellow to yellowish-brown on posterior half, often darkened medially between posterior margin of mediolongitudinal spots and posterior margin of cephalic apotome; head spots faintly positive though mediolateral spots almost always positive but others variable in intensity, appearing indistinct in some larvae; lateral surface of head capsule yellow except posterior one-third or little more light to medium brown and eyespot region white; eyebrow light brown; spots near posterior margin indistinct or somewhat negative; ventral surface of head capsule yellow except wide area along posterior margin light to medium brown, though medial area widely darkened in some larvae; elongate spots on each side of postgenal cleft indistinct or negative. Antenna composed of three segments and apical sensillum, slightly longer than stem of labral fan; length ratio of three segments (from base to tip) 1.00:1.21–1.40:0.60–0.90. Labral fan with 44–46 main rays. Mandible (Fig. 4A) with mandibular serrations composed of two teeth (one large and one small); main tooth at obtuse angle against mandible on apical side; comb-teeth composed of five teeth, first tooth longest, second and third teeth subequal in length, but third tooth wider than second; supernumerary serrations absent. Hypostoma (Fig. 4B) with nine anterior teeth, of which median tooth longest; followed by each corner tooth, three intermediate teeth on each side shortest; lateral margins weakly serrate apically; six or seven hypostomal bristles divergent posteriorly from lateral border on each side. Postgenal cleft (Fig. 4C), of medium-size, 2.10–2.75 times length of postgenal bridge; subesophageal ganglion unpigmented, indistinct. Cervical sclerites composed of two pairs of light brown pieces (anterior one elongate, posterior one ellipsoid), not fused to occiput. Histoblast of pharate pupal gill with six thread-like slender filaments. Thoracic and abdominal cuticle almost bare except dorsolateral and lateral area on each side of last abdominal segment moderately covered with short colorless setae. Rectal scales present. Rectal organ of three lobes, each with 19 or 20 finger-like secondary lobules. Anal sclerite X-shaped, with short broad anterior arms 0.67–0.80 times length of posterior ones and with forked apices; 6–18 sensilla on basal juncture area and 6–8 sensilla posterior to posterior arms. Last abdominal segment not bulged laterally and lacking ventral papillae. Posterior circlet with 82–88 rows of hooklets with up to 15 hooklets per row.

Type Materials. HOLOTYPE: Female (with its associated pupal exuviae and cocoon) (preserved in 80% ethanol) (QSBG-2013-159-33), collected from a fast-flowing stream (width 1.6 m, depth 18 cm, streambed gravelly and sandy, water temperature 18.0˚C, pH 4.55, shaded by a dense forest canopy, altitude 1,550 m, 16˚56'27.0" N, 101˚02'25.4" E), Man-Daeng, Lom Kao District, Phetchabun Province, Thailand, 15-VIII-2013, W. Srisuka. Paratypes: Four females, five males (all with their associated pupal exuviae and cocoons), one pupa and 12 mature larvae, same data and date as holotype.

Biology. The pupae and larvae of this new species were collected from grass leaves trailing in water. No other species was collected.

Etymology. The species name *atipornae* is in honor of Dr. Atiporn Saeung, Supervisor of W. Srisuka, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand, for her great contribution in taxonomy and ecology of black flies and analyses of the *Anopheles* species complexes in Thailand and neighboring countries.

Discussion. *Simulium atipornae* sp. nov. is characterized in the female by the claw with a small subbasal tooth (Fig. 1E), ovipositor valve triangular with its inner margin nearly straight, covered with many short to medium-long hairs (Fig. 1F), in the male by the style with a short subbasal protuberance (Fig. 2E) and ventral plate Y-shaped, with toothed posterior margin (Fig. 2C) and in the pupa by the head and thoracic integument almost bare and gill with six filaments (Fig. 3A).

According to keys for the 10 species-groups of the subgenus *Simulium* (two species-groups, *argentipes* and *ephemerophilum* species-groups, not included) in the Oriental Region (Takaoka 2003), *S. atipornae* sp. nov. is similar in the female to a small part of the *melanopus* species-group (*S. iridescens* De Meijere and *S. javaense* Takaoka and Hadi), and in the male to either the *malyschevi*, *melanopus* or *variegatum* species-group. However, as with all other species of the *melanopus* species-group, the females of *S. iridescens* and *S. javaense* differ from that of *S. atipornae* sp. nov. by the paraproct having the sclerotized inner plate. The males of the three species-groups differ from that of *S. atipornae* sp. nov. by the style lacking a short subbasal protuberance. The *argentipes* and *ephemerophilum* species-groups, of which females have claws each with a small subbasal tooth, cannot accommodate this new species because the groups have the ovipositor valve with its internal margin widely concave, style without a short subbasal protuberance (no information is available on this character of the *ephemerophilum* species-group), and pupal gill with eight or more filaments (Lewis 1973, Takaoka and Saito 2007). By having a combination of three characters, i.e., the female ovipositor valve with its inner margin nearly straight, claw with a small subbasal

tooth, and pupal gill with six filaments, *S. atipornae* sp. nov. cannot reliably be assigned to any of the currently recognized 12 species-groups in the Oriental Region.

On the basis of these three characters, this new species appears to be related to *S. christopheri* Puri (larva unknown) and *S. howletti* Puri (pupa and larva unknown), both described from India (Puri 1932a,b), and *S. acontum* Chen, Zhang & Huang from Sichuan Province, China (Chen et al. 2005). *Simulium christopheri* differs from this new species by having the femur of the female midleg dirty pale yellow with a black tip, male abdominal segment 5 with a pair of shiny spots (in addition to those on segments 2, 6 and 7), pupal gill filaments lacking annular ridges, presence of terminal hooks on the pupal last abdominal segment, and cocoon simple, wall-pocket-shaped, without anterolateral windows (Puri 1932a). *Simulium howletti* differs by having a greater number of hairs on the ovipositor valve (25–30 hairs) and male style gradually narrowed from the middle to the apex when viewed ventrally (Puri 1932b). *Simulium acontum* differs by having the female abdominal segments 5–8 shiny, reduced number of male upper-eye facets in 15 vertical columns and 15 horizontal rows, pupal gill with the ventral two filaments much thinner than the dorsal two filaments and postgenal cleft subequal in length to the postgenal bridge (Chen et al. 2005).

Simulium atipornae sp. nov. is similar to *S. triangustum* An, Guo and Xu from Tibet (An et al. 1995), *S. celsum* Takaoka and Davies, *S. nebulicola* Edwards, and *S. sumatraense* Takaoka and Sigit, the latter three from Indonesia (Takaoka and Davies 1996, Takaoka and Sigit 1997), in many characters including the female claw with a small subbasal tooth, ovipositor valve with the inner margin nearly straight, ventral plate Y-shaped with toothed posterior margin, and pupal gill with six filaments. However, these four species are distinguished from *S. atipornae* sp. nov. by the male style lacking a short subbasal protuberance, and/or cocoon simple, wall-pocket shaped and without anterolateral windows.

Simulium atipornae sp. nov. appears to be closely related to *S. fuscopilosum* Edwards described from females in Peninsular Malaysia (Edwards 1928) by sharing a combination of two characters: the claw with a small subbasal tooth and ovipositor with the inner margin nearly straight. Compared with the redescription of the female of *S. fuscopilosum* (Takaoka and Davies 1995), *S. atipornae* sp. nov. is distinguished by the greater number of minute processes on the cibarium (73–85 versus 55), mid and hind tibiae lacking a dark subbasal spot on the outer surface (Fig.1C), and the hind femur having a yellow portion basally.

The pupa of this new species is characterized by the head and thoracic integument almost bare, gill with six short slender filaments (Fig. 3A), abdomen with dorsal spine-

combs only on segments 7 and 8 and lacking terminal hooks on the last segment, and fenestrated cocoon (Fig. 3B). In this respect, the pupa of this new species is almost the same as that of *Simulium* sp. C, described from a pupa collected from Peninsular Malaysia, which remains unplaced to group (Takaoka and Davies 1995, Adler and Crosskey 2013). The similarity of the pupae of both species suggests the possibility that *S.* sp. C represents the pupa of *S. atipornae* sp. nov. or its related species including *S. fuscopilosum* which is still unknown.

The nine taxa compared with *S. atipornae* sp. nov. are unplaced to species-group (except *S. acontum* and *S. triangustum* which are in the *malyschevi* and *variegatum* species-groups, respectively) of the subgenus *Simulium* in the world inventory of the family Simuliidae (Adler and Crosskey 2013). We propose a new group, *Simulium christopheri* species-group, to accommodate *S. atipornae* sp. nov. and nine related taxa, i.e., *S. acontum*, *S. celsum*, *S. christopheri*, *S. fuscopilosum*, *S. howletti*, *S. nebulicola*, *S. sumatraense*, *S. triangustum*, and *S.* sp. C, with diagnostic characters as follows: cibarium with numerous tubercles, claw with a small subbasal tooth, ovipositor valves short, with inner margins nearly straight, not widely separated from each other (inner margins not widely concave as in the *variegatum* species-group and posterior margins not oblique as in most species of the *malyschevi* species-group), paraproct of normal form (anteromedian surface not sclerotized to form an internal plate as in the *melanopus* species-group) style with or without a short subbasal protuberance, ventral plate Y-shaped when viewed ventrally, with toothed posterior margin, pupal gill with six thread-like filaments, and cocoon wall-pocket-shaped, with or without anterolateral windows.

A phylogenetic analysis using the sequences of the mitochondrial 16S rRNA gene (517 base pairs) of *S. atipornae* sp. nov. and many other species belonging to the 10 species-groups of the subgenus *Simulium* shows that this new species and the *variegatum* species-group form a clade. The difference in the sequences of this gene between *S. atipornae* sp. nov. (Accession number in GenBank: AB #) and the closest species, *S. chamlongi* (from Thailand), in the *variegatum* species-group (Accession number in GenBank: AB #) was four base pairs (Otsuka et al. unpublished data). The validity of the new species-group and its phylogenetic relationship with other groups should be tested by future DNA analysis of related species that are included in the group.

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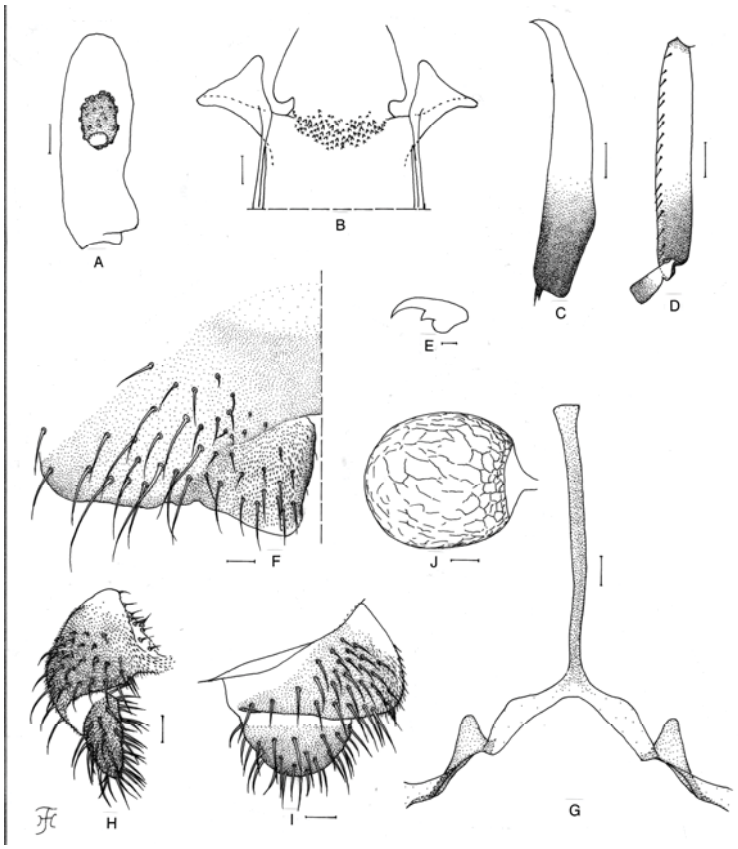

Fig. 1. Female of *Simulium atipornae* sp. nov. (A) Third segment of right maxillary palp showing sensory vesicle (front view). (B) Upper portion of cibarium showing numerous tubercles (front view). (C) Tibia of left hind leg (outer view). (D) Basitarsus and second tarsomere showing calcipala and pedisulcus (outer view). (E) Claw with small subbasal tooth. (F) Eighth sternite and ovipositor valve (right half, ventral view). (G) Genital fork (ventral view). (H) Paraproct and cercus (right side, ventral view). (I) Paraproct and cercus (right side, lateral view). (J) Spermatheca. Scale bars. 0. 1 mm for C and D; 0.02 mm for A, B and F-J; 0.01 mm for E.

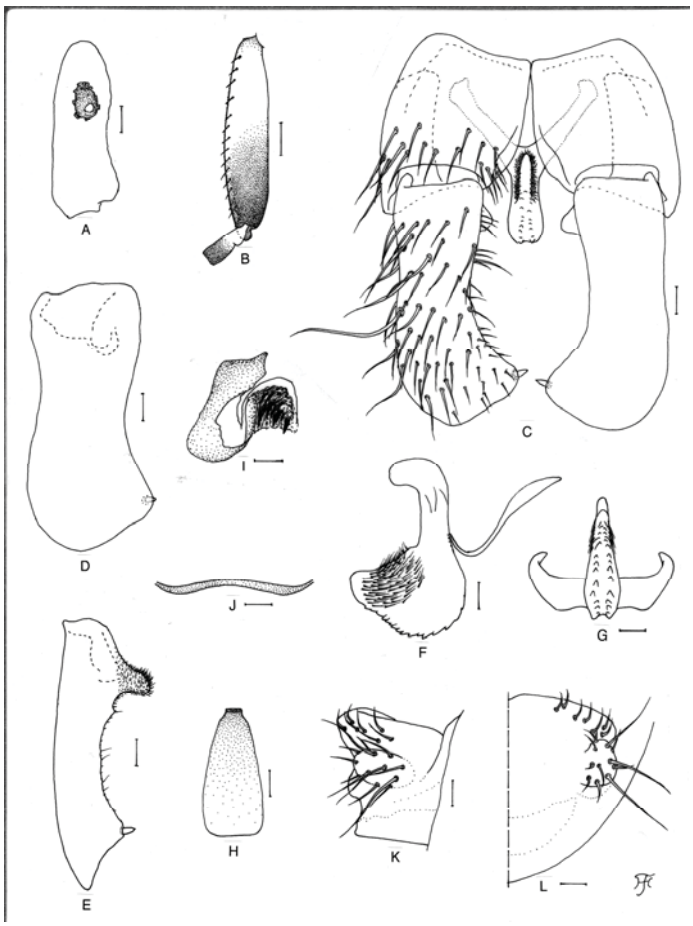

Fig. 2. Male of *Simulium atipornae* sp. nov. (A) Third segment of right maxillary palp showing sensory vesicle (front view). (B) Basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view). (C) Coxites, styles and ventral plate (ventral view). (D) Style (right side, ventrolateral view). (E) Style showing short basal protuberance (right side, medial view). (F) Ventral plate and median sclerite (lateral view). (G) Ventral plate (caudal view). (H) Median sclerite (posterodorsal view). (I) Paramere (right side, caudal view). (J) Dorsal plate (caudal view). (K) Tenth abdominal segment and cercus (left side, lateral view). (L) Tenth abdominal segment and cercus (left side, caudal view). Scale bars. 0.1 mm for B; 0.02 mm for A and C-L.

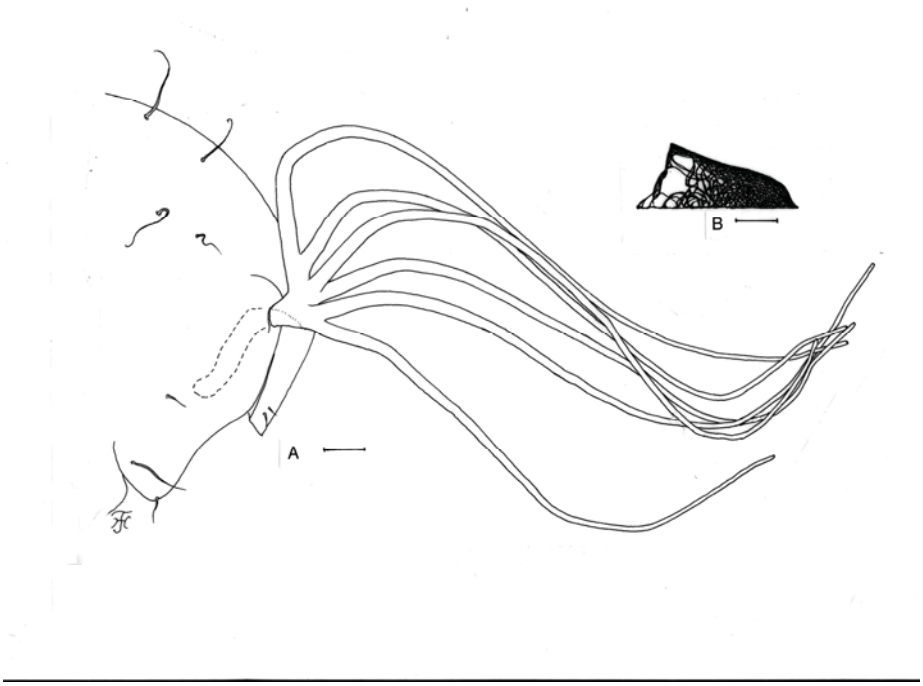

Fig. 3. Pupa of *Simulium atipornae* sp. nov. (A) Gill filaments and anterior part of thorax with trichomes (left side, outer view). (B) Cocoon (lateral view). Scale bars. 1mm for B, 0.1 mm for A.

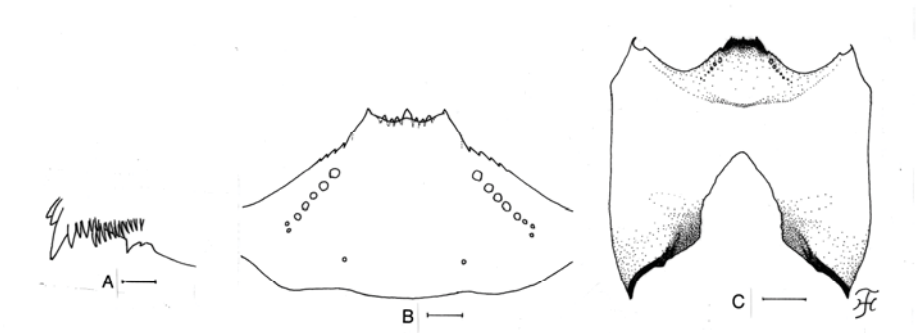

Fig. 4. Larva of *Simulium atipornae* sp. nov. (A) Mandible (left side, lateral view). (B) Hypostoma (ventral view). (C) Head capsule showing postgenal bridge (ventral view). Scale bars. 0.1 mm for C, 0.03 mm for B, 0.02 mm for A,

Running title: *A new species of black fly from Thailand*

***Simulium* (*Simulium*) *lomkaoense*, a new species of black fly (Diptera: Simuliidae) from Thailand**

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Abstract

Simulium (*Simulium*) *lomkaoense* sp. nov. is described from females, males, pupae and larvae in Thailand. This new species is assigned to the *malyschevi* species-group of the subgenus *Simulium*, and appears to be closely related to *Simulium baimaii* Kuvangkadilok & Takaoka from Thailand in sharing the similar shape of the female and male genitalia, pupal gill with two inflated filaments, and simple wall-pocket shaped cocoon. Taxonomic notes are given to compare this new species with *S. baimaii* and other related species. This represents the third species of the *malyschevi* species-group in Thailand.

Key words: black fly, *Simulium*, Thailand, new species

Introduction

The *Simulium* (*Simulium*) *malyschevi* species-group, which consists of 36 species, is mainly distributed in the Holarctic Region and extends its distribution southward into northern areas of the Oriental Region where four species are recorded (Adler & Crosskey 2013). In Thailand, where 87 species of black flies are recorded (Adler & Crosskey 2013, Takaoka *et al*. 2013a,b), this species-group is represented by two species, *S. baimaii* Kuvangkadilok & Takaoka and *S. siripoomense* Takaoka & Saito (Takaoka & Kuvangkadilok 1999, Takaoka & Saito 1996, Takaoka & Mulla 2000).

We collected one new species of the group, which appears to be closely related to *S. baimaii* in sharing the pupal gill with two inflated filaments, a rare character in the subgenus *Simulium*.

We describe this new species based on adult females, males, pupae and larvae, and taxonomic notes are given to separate it from related species.

The methods of collection, description and illustration, as well as terms for morphological features used here, follow those of Takaoka (2003). The type specimens are deposited in the Entomology Section, Queen Sirikit Botanic Garden, Chiang Mai, Thailand.

***Simulium* (*Simulium*) *lomkaoense* Takaoka & Srisuka sp. nov.**

Description. Female. Body length 2.5–2.7 mm. ***Head***. Narrower than thorax. Frons black, shiny, with several dark long stout hairs along each lateral margin and few similar hairs just above lower margin; frontal ratio 1.22–1.24:1.00:0.96–1.00; frons-head ratio 1.00:3.41–3.58. Fronto-ocular area well developed, short, directed laterally and slightly upward. Clypeus black, white pruinose (brightly iridescent when illuminated at certain angles), moderately covered with dark long stout hairs along lateral and ventral margins and middle portion of upper 2/3 widely bare. Labrum 0.68–0.69 times as long as clypeus. Antenna composed of scape, pedicel and nine flagellomeres, brownish-black except scape, pedicel, and base of first flagellomere yellow. Maxillary palp with 5 segments, medium brown except first and second segments grayish-yellow to light brown; proportional lengths of third, fourth, and fifth segments 1.00: 1.13–1.21:2.38–2.46; third segment (Fig. 1A) of normal size; sensory vesicle (Fig. 1A) of moderate size, (0.28 times length of third segment) having large opening near apex.

Maxillary lacinia with 12–14 inner and 14–16 outer teeth. Mandible with 27 or 28 inner and 13 outer teeth. Cibarium (Fig. 1B) with blunt median projection on posterior margin and with four to six minute processes on each side near posterior margin. ***Thorax*.** Scutum black, unpatterned, shiny, white pruinose when illuminated at certain angles, moderately covered with ochreous recumbent minute hairs intersmixed (deleted S ?) with many dark brown short hairs near anterior margin and several dark brown long upright hairs on prescutellar area. Scutellum black, white pruinose when illuminated at certain angles, covered with dark brown upright long hairs. Postnotum black, white pruinose when illuminated at certain angles, and bare. Pleural membrane bare. Katepisternum black, longer than deep, bare. ***Legs.*** Foreleg: coxa yellow; trochanter dark yellow; femur dark yellow on little less than basal 1/2 and dark brown on rest; tibia medium brown except median portion on inner surface light brown, brownish-black on apical cap and basal 5/6 on outer surface white, and with large white sheen on outer surface when illuminated at certain angles; tarsus black, with moderate dorsal hair crest; basitarsus greatly dilated, 3.92–4.14 times as long as its greatest width. Midleg: coxa brownish-black; trochanter medium brown except base yellow; femur dark brown with base light brown; tibia medium to dark brown with extreme base and basal half or little less of posterior surface yellowish-white (its border obliquely defined in lateral view), and with white sheen on posterior surface when illuminated at certain angles; tarsus yellowish-white except apical tip of basitarsus, apical 2/5 of second tarsomere dark yellow to light brown, third and fourth tarsomeres light brown and fifth tarsomere dark brown. Hind leg: coxa dark brown; trochanter yellow; femur dark brown except base yellow and apical cap brownish-black; tibia (Fig. 1C) medium to dark brown except base and basal half of posterior surface yellowish-white (its border obliquely defined in lateral view) and apical cap brownish-black; tarsus yellowish-white except apical 1/4 or little more of basitarsus medium brown and apical 1/3 of second tarsomere, apical 1/2 of third tarsomere and whole of fourth tarsomere light brown, and fifth tarsomere dark brown; basitarsus (Fig. 1D) parallel-sided, 5.45–6.17 times as long as wide, and 0.72–0.80 and 0.60–0.67 times as wide as greatest width of hind tibia and femur, respectively; calcipala (Fig. 1D) developed, small, little shorter than its basal width, and 0.4 times as wide as greatest width of basitarsus; pedisulcus (Fig. 1D) well developed at

basal 2/5 of second tarsomere; claw (Fig. 1E) with small subbasal tooth. ***Wing***. Length 2.3 mm. Costa with dark spinules and hairs; subcosta bare (except 1 female with 1 hair on each subcosta); basal section of radial vein bare; R_1 with dark spinules and hairs; R_2 with hairs; hair tuft on stem vein dark brown; basal cell absent. ***Halter.*** White except base light brown. ***Abdomen***. Basal scale dark brown, with fringe of long yellowish hairs. Dorsal surface of abdomen dark brown to brownish-black except tergite of second segment ochreous, with short dark hairs; tergite 2 brightly iridescent when illuminated at certain angles, and tergites 6–9 shiny.

Genitalia. Sternite 8 (Fig. 1F) bare medially, with 7–11 dark medium-long to long stout hairs and 1 or 2 yellow short hairs on each lateral surface. Ovipositor valve (Fig. 1F) flap-like, bluntly truncate posteriorly, forming oblique roof-like ridge parallel to posterior margin running obliquely, thin, membranous, covered with 6–8 dark medium-long or long hairs, 4–8 yellow short hairs and numerous microsetae; inner margins not sclerotized, moderately concave medially and widely separated from each other. Genital fork (Fig. 1G) of inverted-Y form, with narrow well sclerotized stem; arms of moderate width, each with distinct projection directed anterodorsally. Paraproct in ventral view (Fig. 1H) wide, with unpigmented shallow depression on ventral surface along anteromedial margin; anteromedial surface moderately sclerotized and with 5 or 6 sensilla; paraproct in lateral view (Fig. 1I) 0.82 times as long as wide, with unpigmented shallow depression narrowing ventrally along anteroventral margin, somewhat protruding beyond ventral margin of cercus, and with 11–14 hairs on ventral and lateral surface. Cercus in lateral view (Fig. 1I) short, rounded posteriorly, 0.56 times as long as wide, and with numerous short to medium-long hairs. Spermatheca (Fig. 1J) large, nearly ovoid, 1.19 times as long as wide, well sclerotized except portion of juncture with duct unsclerotized, with weakly defined surface patterns near base, and with internal setae; accessory ducts subequal in thickness to each other, and to major duct.

Male. Body length 2.6–3.0 mm. ***Head***. Nearly as wide as or little wider than thorax. Upper eye consisting of large facets in 22 or 23 vertical columns and in 23 or 24 horizontal rows. Clypeus brownish black, thickly white pruinose and brightly shiny when illuminated at certain angles, sparsely covered with dark brown hairs along and near lateral margins (most of central portion bare). Antenna composed of scape, pedicel and nine flagellomeres, brownish-black except scape and pedicel dark yellow to

medium brown, and base of first flagellomere yellow; first flagellomere elongate, 2.00–2.28 times length of second one. Maxillary palp composed of 5 segments, light to medium brown except first and second segments grayish-yellow; proportional lengths of third, fourth, and fifth segments 1.00: 1.19–1.32:2.73–2.76; third segment (Fig. 2A) of normal size; sensory vesicle (Fig. 2A) ellipsoidal, 0.23–0.25 times length of third segment, and with small opening. ***Thorax***. Scutum black, with whitish pruinose pattern (brightly iridescent when illuminated at certain angles), i.e., anterior pair of large spots on shoulders extending posteriorly along lateral margins and connected to large transverse spot entirely covering prescutellar area; scutum uniformly and moderately covered with ochreous recumbent short hairs interspersed with dark brown short hairs near anterior margin and dark brown long upright hairs on prescutellar area. Scutellum black, with several dark brown long upright hairs. Postnotum black, whitish pruinose (brightly iridescent when illuminated at certain angles) and bare. Pleural membrane bare. Katepisternum brownish-black, bare. ***Legs***. Foreleg: coxa dark yellow to medium brown; trochanter dark brown except base dark yellow; femur dark brown except apical cap brownish-black; tibia dark brown except median outer surface white and apical cap brownish-black, and with large white sheen on outer surface; tarsus black, with moderate dorsal hair crest; basitarsus greatly dilated, 4.46–4.57 times as long as its greatest width. Midleg: coxa brownish-black; trochanter dark brown except base light to medium brown; femur brownish-black; tibia brownish-black with extreme base yellowish-white, and with white sheen on posterior surface when illuminated at certain angles; tarsus yellowish-white except apical 1/5 to 1/4 of basitarsus, apical 1/2 of second tarsomere dark yellow to light brown, third and fourth tarsomeres light borwn and fifth tarsomere dark brown. Hind leg: coxa brownish-black; trochanter dark yellow except anterior surface light brown; femur brownish-black except base whitish-yellow; tibia brownish-black except base yellowish-white; tarsus dark brown except basal 2/3 (or little more or less) of basitarsus and basal 2/3 (or little less) of second tarsomere yellowish-white; basitarsus (Fig. 2B) enlarged, spindle-shaped, 4.55–4.61 times as long as wide, and 0.72–0.79 and 0.72–0.73 times as wide as greatest width of hind tibia and femur, respectively; calcipala (Fig. 2B) developed, small, little shorter than its basal width, and 0.30 times as wide as greatest width of basitarsus; pedisulcus (Fig. 2B) well developed. ***Wing***. Length 2.1–2.2 mm. Other characters as in female including bare subcosta. ***Halter.*** White except base light brown. ***Abdomen***. Basal scale brownish-black, with fringe of dark brown long hairs. Dorsal surface of abdomen brownish-black to black, moderately covered with dark brown short to medium-long hairs; segments 2, 6 and 7 each with pair of whitish pruinose spots (brightly iridescent when illuminated at

certain angles) dorsolaterally, those on segment 2 connected broadly to each other in middle. ***Genitalia***. Coxite in ventral view (Fig. 2C) nearly diamond-shaped; coxite in ventrolateral view (Fig. 2D) nearly quadrate 0.88 times as long as wide. Style in ventral view (Fig. 2C) elongate, with inner margin sinuous, with subapical spine; style in ventrolateral view (Fig. 2E) 1.53 times length of coxite, 2.90 times as long as greatest width near basal 1/4; style in medial view (Fig. 2F) flattened dorso-ventrally except basal 1/2 gently produced dorsally, which is densely covered with fine short hairs. Ventral plate in ventral view (Fig. 2C) Y-shaped, with narrow body; arms long and stout; ventral plate in lateral view (Fig. 2G) with body slightly curved ventrally, and with serrated posterior margin; ventral plate in end view (Fig. 2H) with body equilaterally triangular, bare, with 2–4 teeth in each of 2 vertical rows on posterior surface. Median sclerite (Fig. 2G, I) moderately sclerotized, plate-like, wide, with blunt apical tip having incision medially. Paramere (Fig. 2J) with wide base having several hooks apically. Aedeagal membrane densely covered with minute setae; dorsal plate (Fig. 2K) horizontal bar-like, weakly sclerotized (except medial portion widely unsclerotized). Abdominal segment 10 (Fig. 2L, M) without distinct hairs on each side of posterior surface. Cercus (Fig. 2L, M) small, rounded, with 7–11 hairs.

Pupa. Body length 2.7–3.2 mm. ***Head***. Integument including antennal sheaths ochreous, moderately covered with small round tubercles; antennal sheath produced outward medially along longitudinal axis forming long low ridge from base to apex, which, when viewed laterally, appearing to be divided into nine low ridges corresponding to flagellomeres of antenna, and each ridge covered with few round small tubercles; frons with two pairs of unbranched slender medium-long trichomes arising somewhat separated from each other; face with pair of unbranched medium-long trichomes. ***Thorax***. Integument ochreous, sparsely covered with small round tubercles except dorsal surface of posterior portion moderately covered with tubercles; thorax on each side with eight unbranched medium-long trichomes (two dorsomedially, two anterolaterally, one lateromedially, and three ventrolaterally) (Fig. 3A, B). Gill (Fig. 3A, B) composed of two inflated filaments arising from very short common basal stalk; two filaments lying in vertical plane, light to dark brown, gradually tapered toward apex, subequal in length (1.6–2.0 mm) and thickness to each other (though dorsal filament appearing to be slightly longer and thicker than ventral filament in some pupae); outer surface appearing to be irregularly wrinkled with transverse or oblique shallow furrows, and densely covered with minute tubercles, and without definite patterns except

numerous reticulate patterns near base of filaments. ***Abdomen***. Dorsally, segments 1 and 2, dark grayish; segment 1 with one unbranched slender medium-long seta on each side; segment 2 with one unbranched slender medium-long seta and four or five spinous short setae, on each side; segments 3–9 transparent; segments 3 and 4 each with four distinct unbranched hooks and one unbranched spinous short seta on each side; segments 5 and 6 lacking spine-combs; segments 7–9 each with distinct spine-combs in transverse row and comb-like groups of minute spines, on each side; segment 9 without terminal hooks. Ventrally, segments 3–9 transparent, each (except segment 9) with comb-like groups of minute spines; segment 4 with few slender minute setae on each side; segment 5 with pair of bifid stout hooks submedially and few unbranched short setae on each side; segments 6 and 7 each with pair of bifid inner and outer stout hooks somewhat separated from each other, and few unbranched short setae on each side.
Grapnel-shaped hooklets absent on each side of segment 9. ***Cocoon*** (Fig. 3C, D). Simple, wall-pocket shaped, ochreous, thickly woven with no spaces in webs, anterodorsal portion strongly produced forwardly appearing wide hood; posterior 2/3 with floor; individual threads invisible; 3.0–3.3 mm long by 1.4–1.6 mm wide.

Mature larva. Body length 5.5–6.2 mm. Body grayish. Cephalic apotome light yellow to dark yellow except little less than anterior half yellowish-white, and narrow area along posterior margin medium brown, with median dark portion slightly extended forward; head spots faintly positive, though posterolateral spots usually indistinct or faintly negative. Lateral surface of head capsule yellow except eye-spot region whitish; eyebrow not darkened or slightly darkened; spots below eye-spot region obscure, and large spots before posterior margin obscure or faintly negative. Ventral surface of head capsule yellow except each side of basal portion of postgenal cleft dark brown. Antenna composed of three segments and apical sensillum, slightly longer than stem of labral fan; length ratio of three segments (from base to tip) 1.00:1.10–1.26:0.76–0.83. Labral fan with 52–56 main rays. Mandible (Fig. 3E) with mandibular serrations composed of two teeth one medium-sized, one small) (blanket?); main tooth at obtuse angle against mandible on apical side; supernumerary serrations absent; comb-teeth decreasing in length from first to third. Hypostoma (Fig. 3F) with nine anterior teeth, of which corner teeth most prominent, longer than median tooth; three intermediate teeth on each side

small, subequal in length to one another; lateral margins weakly serrate apically; five or six hypostomal bristles divergent posteriorly from lateral border on each side. Postgenal cleft (Fig. 3G) large, rounded, 2.82–4.13 times length of postgenal bridge; subesophageal ganglion pigmented peripherily. Cervical sclerites composed of pair of yellow lod-like pieces, not fused to occiput. Histoblast of pharate pupal gill with 2 filaments. Thoracic and abdominal cuticle almost bare except last segment of abdomen moderately covered with short colorless setae on each side of anal sclerite. Rectal scales present. Rectal organ compound, each lobe with 12 or 13 finger-like secondary lobules. Anal sclerite X-shaped, with broad anterior arms 0.79–0.95 times length of posterior ones; two or three sensilla on base of anal sclerite and eight or nine sensilla posterior to posterior arms of anal sclerite; accessory sclerite absent. Last abdominal segment lacking ventral papillae. Posterior circlet with 82–84 rows of hooklets with up to 14–17 hooklets per row.

Type materials. HOLOTYPE: Male (with its associated pupal exuviae and cocoon) (preserved in 80% ethanol) (QSBG-2013-159-22), THAILAND: Phetchabun Province, Lom Kao District, Man-Daeng (16˚56'27.0" N, 101˚02'25.4" E), 15-VIII-2013, W. Srisuka. Paratypes: Four females, six males (all with their associated pupal exuviae and cocoons), one pupa and six mature larvae, same data and date as holotype.

Etymology. The specific name *lomkaoense* refers to the name of the district, where this new species was collected.

Biology. The pupae and larvae of *S. lomkaoense* sp. nov. were collected from leaves trailing in a fast-flowing stream (width 1.6 m, depth 18 cm, streambed gravelly and sandy, water temperature 18.0˚C, pH 4.55, shaded by a dense forest canopy, altitude 1,550 m). Associated species was *Simulium* (*Simulium*) sp.

Discussion. In the key to species-groups of the subgenus *Simulium* in the Oriental Region (Takaoka 2003), *S. lomkaoense* sp. nov. is assigned to the *malyschevi* species-group in having the ovipositor valve with the posterior margin oblique (Fig. 1F), female claw with a small subbasal tooth (Fig. 1E), male style without a basal or subbasal projection, and ventral plate of Y-shape, with toothed posterior margin (Fig. 2H). This new species is characterized by the pupal gill composed of two inflated filaments (Fig. 3A, B), a rare character in this species-group and in the subgenus

Simulium. In this respect, this new species is similar to *S. baimaii* described from Thailand (Takaoka & Chochoote 1999, 2006) (Takaoka & Kuvangkadilok 1999, Takaoka & Chochoote 2006 ?) , from which this new species is distinguished by the following characteristics (characteristics of *S. baimaii* in parentheses): in the female by lacking hairs on the subcosta (subcosta with hairs); in the male by the number of the enlarged upper-eye facets in 22 or 23 vertical columns and 23 or 24 horizontal rows (in 21 vertical columns and 21 horizontal rows),fore basitarsus greatly dilated, 4.46–4.57 times as long as its greatest width (moderately dilated, 5.5 times as long as its greatest width), hind basitrsus spindle-shaped, 4.55–4.61 times as long as wide (parallel-sided, 5.5 times as long as wide), in the pupa by the two gill filaments lacking a stalk (with a stalk), and in the larva by the subesophageal ganglion pigmented (not pigmented) .

The pupal gill with two filaments distinguish this new species from *S. siripoomense* from Thailand and other species of the *malyschevi* species-group from other countries, which have the pupal gill with six or more filaments.

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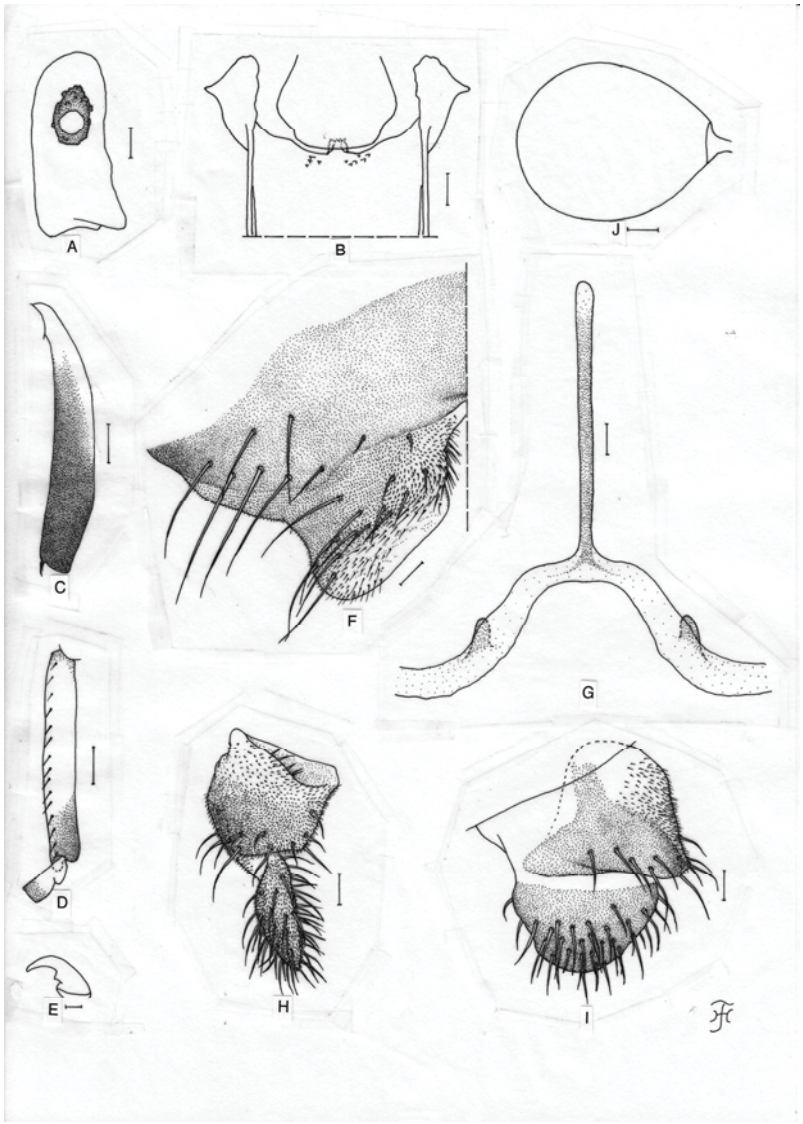

Fig. 1. Female of *Simulium* (*Simulium*) *lomkaoense* sp. nov. A, third segment of right maxillary palp with sensory vesicle (front view); B, upper portion of cibarium (front view); C, tibia of left hind leg (outer view); D, basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus? (outer view); E, claw; F, sternite 8 and ovipositor valve (only right half shown) (ventral view); G, genital fork (ventral view); H and I, right paraprocts and cerci (H, ventral view; I, lateral view); J, spermatheca. Scale bars. 0.1 mm for C and D, 0.02 mm for A, B andF –J. and 0.01 mm for E.

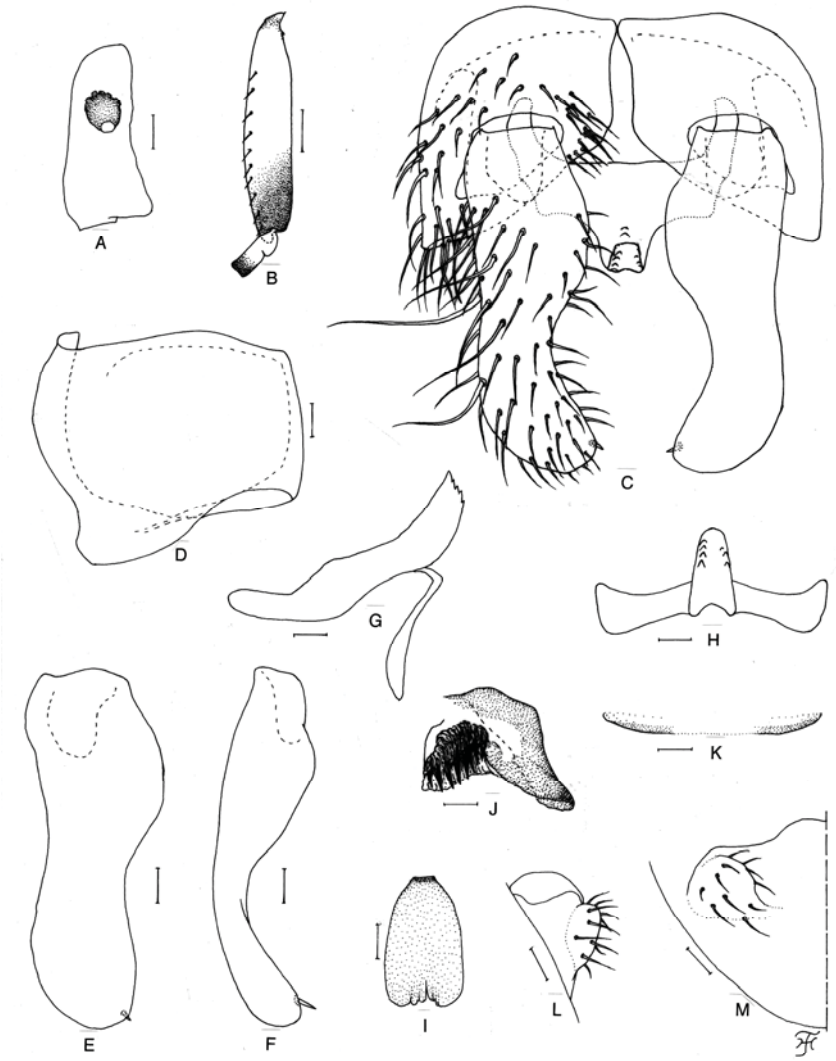

Fig. 2. Male of *Simulium* (*Simulium*) *lomkaoense* sp. nov. A, third segment of right maxillary palp with sensory vesicle (front view); B, basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view); C, coxites, styles and ventral plate (ventral view); D, right coxite (ventrolateral view); E and F, right styles (E, ventrolateral view; F, medial view); G, ventral plate and median sclerite (lateral view); H, ventral plate (caudal view); I, median sclerite (caudal view); J, left paramere (caudal view); K, dorsal plate (caudal view); L and M, tenth abdominal segments and cerci (right side; L, lateral view; M, caudal view). Scale bars. 0.1 mm for B; 0.02 mm for A and C–M.

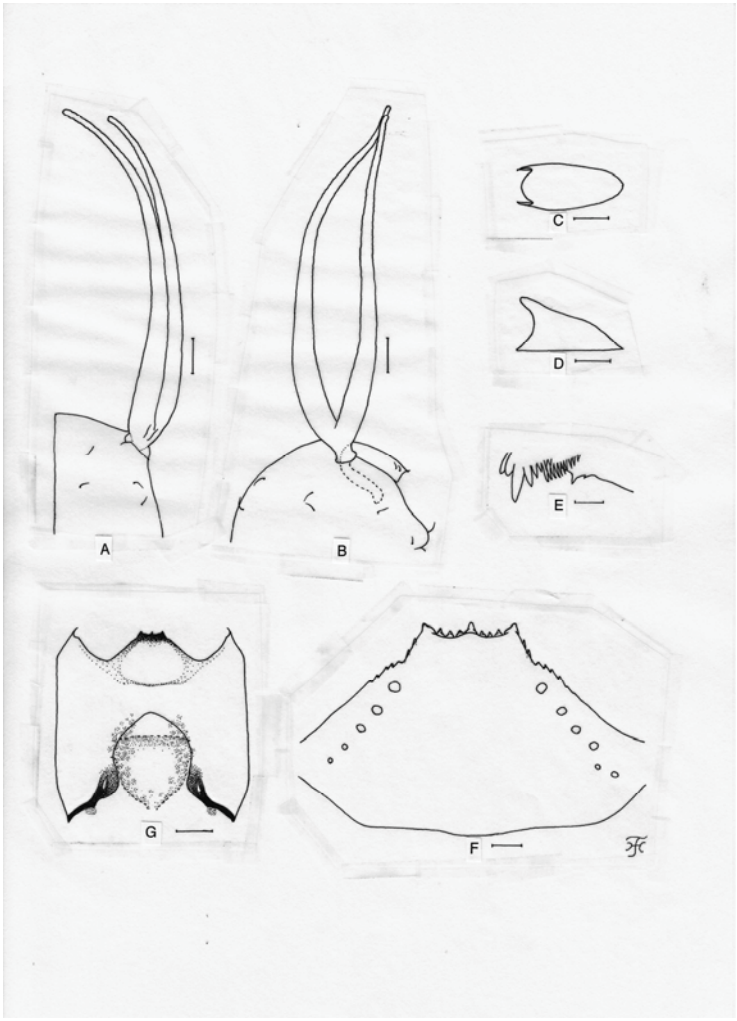

Fig. 3. Pupa and larva of *Simulium* (*Simulium*) *lomkaoense* sp. nov. A–D, pupa, and E–G, larva. A, anterior part of thorax (showing dorsomedial and anterolateral trichomes) with gill filaments (right half; dorsal view); B, anterior part of thorax (showing interspiracular trunk, dorsomedial, anterolateral and ventrolateral trichomes) with gill filaments (right,side; outer view); C and D, cocoons (C, dorsal view; D, lateral view); E, mandible; F, hypostoma (ventral view); G, head capsule showing postgenal cleft (ventral view). Scale bars. 1.0 mm for C and D; 0.1 mm for A, B and G; 0.02 mm for E and F.

2. Takaoka H, Srisuka W, Saeung A, **Choochote W**. *Simulium* (*Asiosimulium*) *furvum,* a new species of black fly (Diptera: Simuliidae) from Thailand. Journal of Medical Entomology 2013;50:493–500 (impact factor 2012 = 1.857).

Original Article

Molecular Analysis of Medically and Veterinary Important Muscid Flies (Diptera: Muscidae) in Thailand

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Abstract

We demonstrated the using of the internal transcribed spacer (ITS2) of ribosomal DNA as a tool for identification of medically and veterinary important Muscidae flies in Thailand. A total of 27 fly samples were collected from various regions of Thailand. Six fly species in three subfamilies including Azeliinae (*Hydrotaea spinigera*), Muscinae (*Musca domestica*, *M. sorbens*) and Stomoxyinae (*Stomoxys calcitrans*, *S. indicus* and *S. sitiens*) were identified base on morphological taxonomy. PCR amplicons of the ITS2 gene of these flies varied between 312-377 bp with A+T content of 76.6%. ITS2 sequences of the flies in this study were 93-100% identity to sequences in database and 21 samples were compatible with morphological identification, while sequences of 6 samples did not match any sequences in the database. The intra- and inter-specific divergence analysis results showed that the maximum of intra-specific (within species) variation (6.9%) was found in *M. domestica* while the minimum inter-specific (between species) variation (11.9%) was found in the sister grouped couple of *S. sitiens* and *S. indicus*. No overlapping between intra- and inter-specific divergences was found in all species of this study. The bootstrapped NJ tree constructed showed ability to classify each subfamily in to monophyletic clades. PCR-RFLP using *Xap*I restriction enzyme digestion was able to differentiate between the three *Stomoxys* species. Data obtained from this study would be valuable for both medical and veterinary entomologists for more accurate identification of important fly species. Therefore, it could be used for population dynamics studies and enrolled in integrated pest management control program.

Keywords: ITS2, molecular identification, Muscidae fly, PCR-RFLP, Thailand

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บทคัดย่อ

การวิเคราะห์ทางชีวโมเลกุลของแมลงวันในวงศ์ Muscidae ที่มีความสำคัญทางการแพทย์และสัตวแพทย์ในประเทศไทย

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ผู้วิจัยรายงานการใช้บริเวณ internal transcribed spacer (ITS2) ของ ribosomal DNA ในการจำแนกสายพันธุ์ของแมลงวันที่มีความสำคัญทางการแพทย์และสัตวแพทย์ในวงศ์ Muscidae ในประเทศไทย พบว่าแมลงวัน 27 ตัวอย่างที่เก็บจากพื้นที่ต่างๆ ในประเทศไทย ถูกจำแนกด้วยลักษณะทางสัณฐานวิทยาจำแนกเป็น 6 สปีชีส์ ใน 3 วงศ์ย่อย ประกอบด้วยวงศ์ Azeliinae (*Hydrotaea spinigera*), Muscinae (*Musca domestica*, *M. sorbens*) และ Stomoxyinae (*Stomoxys calcitrans*, *S. indicus* และ *S. sitiens*) ผลิตภัณฑ์พีซีอาร์ของ ITS2 มีขนาดตั้งแต่ 312-377 bp และมี A+T content เท่ากับร้อยละ 76.6 ผลการเปรียบเทียบลำดับนิวคลีโอไทด์ของ ITS2 ในการทดลองนี้กับฐานข้อมูลพบว่าตรงกับฐานข้อมูลร้อยละ 93-100 โดยที่แมลงวัน 21 ตัวอย่างมีผลการเปรียบเทียบตรงกับการจำแนกด้วยสัณฐานวิทยา ในขณะที่อีก 6 ตัวอย่างไม่มีข้อมูลลำดับนิวคลีโอไทด์ในฐานข้อมูล การวิเคราะห์ค่าความผันแปรภายในและระหว่างสปีชีส์พบว่าค่าความผันแปรภายในสปีชีส์สูงสุดพบในแมลงวัน *M. domestica* เท่ากับร้อยละ 6.9 ในขณะที่ค่าความผันแปรระหว่างสปีชีส์ต่ำสุดพบในคู่ของแมลงวัน *S. sitiens* กับ *S. indicus* เท่ากับร้อยละ 11.9 ทั้งนี้พบว่าไม่มีค่าความผันแปรภายในสปีชีส์มากกว่าค่าความผันแปรระหว่างสปีชีส์ของทุกสปีชีส์ในการทดลองนี้ แผนภูมิต้นไม้พันธุกรรมแบบ NJ method สามารถแบ่งวงศ์ย่อยของแมลงวันให้มีลักษณะเป็น monophyletic clade ได้ ผล PCR-RFLP โดยใช้เอ็นไซม์ *Xap*I สามารถบอกความแตกต่างระหว่างแมลงวัน *Stomoxys* ทั้ง 3 สปีชีส์ได้ ข้อมูลที่ได้จากการทดลองนี้จะเป็นประโยชน์สำหรับนักกีฏวิทยาทางการแพทย์และทางสัตวแพทย์ในการจำแนกสายพันธุ์แมลงวันที่มีความสำคัญทางการแพทย์และสัตวแพทย์ รวมถึงใช้ในการศึกษาความชุกของแต่ละสายพันธุ์ซึ่งจะเป็นข้อมูลสำคัญในการใช้ควบคุมแมลงวันแบบบูรณาการต่อไป

คำสำคัญ: ITS2 การจำแนกสายพันธุ์ด้วยอณูชีววิทยา แมลงวัน Muscidae, PCR-RFLP ประเทศไทย

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Introduction

Flies belong to the family Muscidae. They are of crucial importance to medicine and veterinary medicine because of their ability to transmit diseases to humans and animals. For example, stable flies (*Stomoxys* spp.), transmit diseases by biting. Larvae of some species cause myiasis in man and animals (Lane and Crosskey, 1993). Moreover, fly larvae can be found in corpses and, therefore, can be used as a forensic indicator for estimating Post-Mortem Interval (PMI) (Sukontason, 2007, Preativatanyou et al., 2010). Some adult flies of this family, especially house flies, are synanthropic which can cause annoyance and act as carriers of mechanical transmitting pathogens to human (Lane and Crosskey, 1993) and also transmit some pathogens biologically to animals (Iwasa, 1999).

Stomoxys flies (Musidae: Stomoxynae), known as stable flies, are blood sucking flies found worldwide especially in tropical zone, including

Thailand. These flies are the veterinary, medically and economically important flies due to both sexes feed on blood of large mammals, sometimes human. Their painful biting can be irritating and stressful to livestock resulting in significant decrease in weight and milk yield (Lane and Crosskey, 1993). Moreover, these species have been reported as mechanical vectors of pathogenic bacteria, *Mycobacterium* sp. (Fischer et al., 2001), *Bacillus anthracis* (Zumpt, 1973), and *Enterobacter sakazakii* (Mramba et al., 2007); and various virus, Surra disease and equine infectious anemia (EIA) virus (Veer et al., 2002) and Rift Valley fever virus (Turell and Knudson, 1987). The way to reduce problems from these flies is fly control. The most efficient of stable fly control is an integrated pest management (Williams et al., 1981) where surveillance is included. Results from surveys stable fly populations are not only data for prediction of the origins of outbreak and population dynamics but also for geographical patterns of insecticide resistance analysis. However, the important key for surveillance is accurate identification of stable fly species.

As described previously, identification of fly is essential for both epidemiological study and for control strategies. Accurate identification of fly larvae collected from corpses is can also help estimate the PMI more precisely. Although morphological identification of fly using taxonomic keys can identify flies at any stage (Tumrasvin et al., 1979, Greenberg and Kunich, 2002, Siriwattanarungsee et al., 2005, Sukontason et al., 2004, 2008[a,b]), this procedure is elaborate and, therefore, requires highly experienced. It also requires complete fly samples. However, the problem of morphological identification is there is no key covering all stages of fly species at present, especially in larval stage (Wells and Sperling, 2001, Wells et al., 2001). In order to solve the problems of taxonomic identification as previously described, molecular techniques such as nucleotide sequence analysis and PCR-RFLP are mostly chosen (Stevens and Wall, 2001). These techniques are fast, accurate and highly sensitive. Moreover, they can be performed even though the collected samples are damaged (Hajibabaei et al., 2007; Kress and Erickson, 2008).

Aims of this study were to analyze the nucleotide sequences of Muscid flies in Thailand. The internal transcribed spacer 2 (ITS2) between 5.8S and 28S ribosomal RNA (rRNA) gene region in the rRNA transcription unit of nuclear DNA was chosen for this study because this region is markedly different in length and sequence in each species of insect (Ratcliffe et al., 2003; Young and Coleman, 2004; Song et al., 2008[a]). Furthermore, it has been used as a DNA barcode for differentiation of some insect species such as mosquitoes (Torres et al., 2000; Van Bortel et al., 2000; Wilkerson et al., 2004; Li and Wilkerson, 2005), flies (Nelson et al., 2008; Ferreira et al., 2011) and black flies (Thanwisai et al., 2006). This study is the first molecular study in Muscid species in Thailand. Therefore, the nucleotide sequences from this study were accumulated to worldwide online database and will be valuable data for closely related fly specie identification in future study and useful for integrated pest management program of fly control.

Materials and Methods

Fly sampling and collection sites: Six muscid fly species in three subfamilies including Azeliinae (*Hydrotaea spinigera*), Muscinae (*Musca domestica*, *M. sorbens*) and Stomoxyinae (*Stomoxys calcitrans*, *S. indicus* and *S. sitiens*) were included in the study. *Stomoxys* flies were collected near a dairy farm in Nakhon Ratchasima province (North-eastern), Thailand by using carbon dioxide bait trapping. Muscidae flies were collected from various regions of Thailand including Chiang Mai (Northern), Nong Khai (North-eastern), Bangkok (Central), Ranong (Southern) and Phuket (Southern) (Fig 1) by using ground pig liver bait trapping. Species of all specimens were identified by using a morphologically taxonomic key (Zumpt, 1973, Crosskey and Lane, 1993; Masmeatathip et al., 2006) under a stereomicroscope (SZX9, Olympus, Japan) and then individual specimens were coded (Table 1) before being kept in a 1.5 ml microcentrifuge tube and preserved with 70% ethanol and stored at 4^0C until next step.

DNA extraction: Genomic DNA was extracted from thorax of individual adult by using DNA extraction kits (Invisorb® Spin tissue mini Kit, Invitek, Berlin, Germany), following the manufacturer's instructions. The extracted DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo-scientific, DE, USA) and stored at -20^0C until used.

Figure 1 Map of Thailand, showing the collecting sites in this study

PCR amplification: The primer set anneal specific to the ITS2 region used in this study was designed by Song et al. (2008[a,b]). This primer was designed by using a sequence of 3'-ends of 5.8S and 5'-ends of 28S rRNA gene region (Tautz et al., 1988). The forward primer sequences were 5'TGCTTGGACTACATA TGGTTGA3' and the reverse primer sequences were 5'GTAGTCCCATATGAGTTGAGGTT 3'.

PCR reaction was composed of 2 µl of 10X *Taq* buffer, 2 µl of 200 µM dNTP, 2 µl of 2.5 mM $MgCl_2$, 0.8 µl of 0.4 µM of each primer, 0.2 µl of *Taq* DNA polymerase (5U/µl; Invitrogen®, CA, USA) and 100 ng of DNA template, and DNase-free water to final volume of 20 µl. The PCR reactions were performed in a GeneAmp PCR system 2400 thermal cycler (Applied Biosystems®, Forster city, CA, USA) by using conditions as follows: initial denaturation step at 94^0C for 5 min, followed by 30 cycles of the amplification steps including 94^0C for 60 sec, 47^0C for 60 sec and 72^0C for 45 sec and finally extension at 72^0C for 10 min. The PCR amplicons were detected on a 1% agarose gel electrophoresis.

Cloning and Sequencing: PCR products were cloned into TA cloning vector, pTZ57R/T (InsTAclone™ PCR cloning kit; Fermentas, MD, USA) according to the manufacturer's protocol. The recombinant plasmid DNA was extracted by using FastPlasmid™ Mini kit (Eppendorf, Hamburg, Germany), following the manufacturer's instructions. Plasmid DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo-scientific, Wilmington, DE, USA). DNA sequencing was done by 1st BASE DNA sequencing services, Malaysia, using M13F (-20) primer (5' GTAAAACGACG GCCAGT 3'). Two recombinant colonies per specimens were chosen for DNA sequencing. Consensus sequence was then used for further analysis.

Sequence editing, Molecular identification and Accession numbers submission

BioEdit Sequence Alignment Editor Program Version 7.0.5.3 (Hall, 1999) was used to confirm the sequence electropherograms of both clones from each individual samples and to edit the ITS2 sequence where the 5'-ends and 3'-ends of each sequences were determined according to the sequences submitted by Tautz et al. (1988) and Song et al. (2008[b]).

The ITS2 sequences were compared with nucleotide sequence database collected in NCBI by optimizing highly similar sequences (megablast) using BLASTN 2.2.26+ (Basic Local Alignment Search Tool; http://www.ncbi.nlm.gov/ BLAST) (Zehner et al., 2004). Species were identified by considering the maximum percentage identities. All completed sequences obtained from this step were submitted to the NCBI database to be assigned the accession numbers.

Sequence variation and Phylogenetic Analysis

The consensus sequences of each species from each region were aligned with reference sequence and the percentage of intra-specific and inter-specific divergences were computerized using Clustal W version 2.0 (Thompson et al., 1994) implemented in the BioEdit Sequence Alignment Editor Program Version 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Program MEGA version 4.0.2 (http://www.megasoftware.net/; Tamura et al., 2007) was chosen to construct phylogenetic trees using the Neighbor-Joining method with the Kimura 2-parameter model (Tamura et al., 2007). The reliability of an inferred tree was tested by 1000 bootstrap and tree was rooted with *Drosophila melanogaster* (Song et al., 2008[b]).

Polymerase chain reaction-Restriction fragment length polymorphism; PCR-RFLP

The NEBcutter V2.0 web-based program (available at http://tools.neb.com/ NEBcutter2/index.php) was used to determine restriction enzymes sites with the sequences from each fly species. With the most appropriate patterns of digestion from the result of prediction (Table 3), *Xap*I restriction enzyme was chosen for RFLP in the reaction containing a final volume of 10 µl, which contained 1µl of 10X fast digest buffer (Fermentas® CA, USA), 1 µl of *Xap*I enzyme, 400 ng of PCR product and sterilized distilled water to final volume of 10 µl. The digested products were demonstrated on an 8% native polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized on a Gel Doc EQ system (Bio-Rad, CA, USA).

Results

Species identification and distribution of sarcophigid flies in Thailand

Among the 27 specimens collected from this study, 15 Muscid fly specimens consisted of 7 specimens of *M. domestica* (3 specimens from Chiang Mai, 2 specimens from Ranong and one specimen from Nong Khai and Phuket), 8 specimens of *M. sorbens* (4 specimens from Phuket, 2 specimens from Nong Khai and one specimen from Chiang Mai and Bangkok). There was a total of 9 specimens of Stomoxys flies, 3 specimens for *S. calcitrans* and *S. indicus* from Nakhon Ratchasima, 3 specimens of *S. sitiens* from Nakhon Ratchasima and 3 specimens of *H. spinigera* from Chiang Mai (Table 1).

PCR amplification

Genomic DNA extracted from thorax of fly samples in this study were appropriate for using to amplify by the primers. Size of PCR amplicons were different between species. There were 406-418 bp for *M. domestica*, 405 bp for *M. sorbens*, 437 bp for *H. spinigera*, 376-377 bp for *S. calcitrans*, 367-373 bp for *S. indicus* and 371 bp for *S. sitiens*. However, the sequence of PCR product in this step still contained a surplus of 5.8S and 28S rRNA gene which need to be trimmed out before the ITS2 analysis and comparison.

ITS2 Sequences analysis

DNA sequences length of ITS2 region, consistent with the length of amplified PCR products excluding 5.8S and 28S partial regions, from

individual fly samples in this study varied from 348 bp to 360 bp (mean 342 bp) for *Musca* species, 377 bp for *H. spinigera* and from 312 bp to 322 bp (mean 317 bp) for *Stomoxys* species. The average A+T content from all sequences was up to 76.6% (T= 37.9%, C= 10.3%, A= 38.7% and G= 13.1%). All completed sequences were submitted to the NCBI database and assigned the accession numbers as listed in Table 1

The results of the comparison of intra and inter-specific divergences showed that the percentage of intra-specific divergences ranged from 0.3-6.9 for *M. domestica*, 0.7-2.2 for *S.calcitrans* and 1.0-3.5 for *S. indicus* whereas no variation in *H. spinigera*, *M. sorbens* and *S. sitiens* was found (Table 2). When comparing each species to the same species from different countries, the percentage of sequence divergences were 0.6-6.4 and 2.8-3.7 for *M. domestica* and *S.calcitrans*, respectively, but no reference sequences of *H. spinigera, S. indicus* and *S. sitiens* in database for comparison were found.

Furthermore, the between species sequence comparison showed that the percentages of inter-specific divergences between *M. domestica* and *M. sorbens, H. spinigera*, *S. calcitrans, S. indicus*, *S. sitiens* were 19.2-21.2% (average 20.3%), 43.1-45.3% (average 44.3%), 31.3-34.5% (average 33.0%), 30.0-33.3% (average 31.5%) and 32.9-35.4% (average 34.1%), respectively. Percentages of the inter-specific divergences between *M. sorbens* and *H. spinigera*, *S. calcitrans, S. indicus, S. sitiens* were 46.4%, 32.5-33.0% (average 32.7%), 30.9-31.9% (average 31.3%) and 34.7%, in the order. The percentages of inter-specific variation between *H. spinigera* and *S. calcitrans*, *S. indicus*, *S. sitiens* were 41.9-42.2% (average 42.0%), 42.6-43.1% (average 42.8%) and 44.1%, respectively. When comparing *S. calcitrans* to *S. indicus* and *S. sitiens* the inter-specific divergences were 23.2-24.4% (average 23.8%) and 25.3-25.8 (average 25.6%), respectively. In the same way, the percentage of inter-specific variation between *S. indicus and S. sitiens* was 10.9-12.7% (average 11.9%).

Table 1 Muscid fly species in this study, their analyzed individual codes, source localities, sex, number of base pairs of ITS2 in each isolates, maximum identity (%) and accession number of their ITS2 sequences

Species based on Morphology with isolated codes	Sampling sites	Sex	Accession No. in each clones	ITS2 length (bp)	Species based on ITS2 sequence	% identity	References
Musca domestica							
Md1	Chiang Mai	♀	JQ811271-72	348	*M. domestica*	99	EU555401
Md2	Chiang Mai	♀	JQ811274-75	348	*M. domestica*	99	EU555401
Md3	Chiang Mai	♂	JQ811276-77	351	*M. domestica*	99	EU555401
Md4	Nong Khai	♀	JQ811278-79	359	*M. domestica*	99	EU555399
Md5	Phuket	♀	JQ811280-81	359	*M. domestica*	99	EU555399
Md6	Ranong	♀	JQ811282-83	348	*M. domestica*	99	EU555401
Md7	Ranong	♀	JQ811284-85	360	*M. domestica*	99	EF061807
Musca sorbens							
Ms1	Bangkok	♀	JQ811252-53	347	*M. sorbens*	100	EF061810
Ms2	Chiang Mai	♀	JQ811254-55	347	*M. sorbens*	100	EF061810
Ms3	Nong Khai	♀	JQ811258-59	347	*M. sorbens*	100	EF061810
Ms4	Nong Khai	♀	JQ811260-61	347	*M. sorbens*	100	EF061810
Ms5	Phuket	♀	JQ811263-64	347	*M. sorbens*	100	EF061810
Ms6	Phuket	♀	JQ811265-66	347	*M. sorbens*	100	EF061810
Ms7	Phuket	♀	JQ811267-68	347	*M. sorbens*	100	EF061810
MS8	Phuket	♀	JQ811269-70	347	*M. sorbens*	100	EF061810
Hydrotaea spinigera							
Hs1	Chiang Mai	♀	JQ811245-46	377	*S. calicitrans*	72	EU851201
Hs2	Chiang Mai	♀	JQ811248-49	377	*S. calicitrans*	72	EU851201
Hs3	Chiang Mai	♂	JQ811250-51	377	*S. calicitrans*	72	EU851201
Stomoxys calicitrans							
Sc1	Nakhon Ratchasima	♀	JQ811238-39	321	*S. calicitrans*	97	EF560191
Sc2	Nakhon Ratchasima	♀	JQ811240-41	322	*S. calicitrans*	98	EF560191
Sc3	Nakhon Ratchasima	♀	JQ811243-44	321	*S. calicitrans*	97	EF560191
Stomoxys indicus							
Si1	Nakhon Ratchasima	♀	JQ811229-30	318	*S. indicus*	93	EU851209
Si2	Nakhon Ratchasima	♂	JQ811233-34	313	*S. indicus*	95	EU851209
Si3	Nakhon Ratchasima	♂	JQ811236-37	312	*S. indicus*	95	EU851209
Stomoxys sitiens							
Ss1	Nakhon Ratchasima	♂	JQ811222-23	316	*S. indicus*	90	EU851257
					S. sitiens	82	EU851213
Ss2	Nakhon Ratchasima	♂	JQ811225-26	316	*S. indicus*	90	EU851257
					S. sitiens	82	EU851213
Ss3	Nakhon Ratchasima	♂	JQ811227-28	316	*S. indicus*	90	EU851257
					S. sitiens	82	EU851213

Table 2 Intra and inter-specific divergences of ITS2 sequence between different isolates of Muscid flies from each regions (%)

	Md1	Md2	Md3	Md4	Md5	Md6	Md7	MdA	MdB	MdC	Ms1	Ms2	Ms3	Ms5	MsA	Hs1	Sc1	Sc2	Sc3	ScA	Si1	Si2	Si3	Ss1
Md1	-																							
Md2	1.8	-																						
Md3	0.9	0.9	-																					
Md4	4.5	6.1	5.3	-																				
Md5	4.2	5.9	5.0	0.3	-																			
Md6	0.3	2.0	1.2	4.8	4.5	-																		
Md7	5.3	6.9	6.1	1.4	1.2	5.6	-																	
MdA	4.8	6.4	5.6	0.9	0.6	5.1	1.7	-																
MdB	1.8	3.5	2.6	5.6	5.3	2.1	4.8	5.9	-															
MdC	5.3	5.3	4.5	2.5	2.3	5.6	3.3	2.8	6.4	-														
Ms1	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	-													
Ms2	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	-												
Ms3	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	0	-											
Ms5	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	0	0	-										
MsA	20.0	19.8	19.2	20.5	20.8	20.3	21.2	20.8	20.0	20.2	0	0	0	0	-									
Hs1	43.8	43.1	43.5	45.3	45.0	44.0	45.3	45.0	43.8	44.8	46.4	46.4	46.4	46.4	46.4	-								
Sc1	32.2	31.3	31.9	34.2	33.9	32.2	34.1	33.9	32.2	33.7	32.7	32.7	32.7	32.7	32.7	41.9	-							
Sc2	32.5	31.6	32.2	34.5	34.2	32.5	34.4	34.2	32.5	33.9	33.0	33.0	33.0	33.0	33.0	41.9	0.7	-						
Sc3	32.3	31.4	32.0	34.3	34.0	32.3	34.2	34.0	32.3	33.7	32.5	32.5	32.5	32.5	32.5	42.2	2.2	2.2	-					
ScA	33.1	32.5	33.1	35.1	34.8	33.1	35.0	34.8	33.1	34.8	33.7	33.7	33.7	33.7	33.7	41.1	3.4	2.8	3.7	-				
Si1	31.2	30.7	31.2	33.3	33.0	31.2	33.0	33.0	31.0	33.0	31.9	31.9	31.9	31.9	31.9	43.1	23.7	23.9	23.2	24.1	-			
Si2	30.6	30.0	30.6	32.7	32.4	30.6	32.2	32.4	30.1	32.4	30.9	30.9	30.9	30.9	30.9	42.6	23.5	23.8	23.3	24.2	2.6	-		
Si3	30.6	30.0	30.6	32.7	32.4	30.9	32.2	32.4	30.1	32.4	31.2	31.2	31.2	31.2	31.2	42.8	24.1	24.4	23.9	24.8	3.5	1.0	-	
Ss1	33.5	32.9	33.5	35.4	35.2	33.5	34.9	35.2	33.2	35.2	34.7	34.7	34.7	34.7	34.7	44.1	25.6	25.8	25.3	26.2	10.9	12.1	12.7	-

Codes in this table are corresponding with Table 1

Md1-7, *Musca domestica*; MdA, *Musca domestica* from China 1 (EF061808); MdB, *Musca domestica* from China 2 (EU555394); MdC, *Musca domestica* from Brazil (EF560189); Ms1-5, *Musca sorbens*; MsA, *Musca sorbens* from China (EF061810); Hs1,*Hydrotaea spinigera*; Sc1-3, *Stomoxys calcitrans*; ScA, *Stomoxys calcitrans* from Brazil (EF560191); Si1-3*Stomoxys indicus*; Ss1, *Stomoxys sitiens*

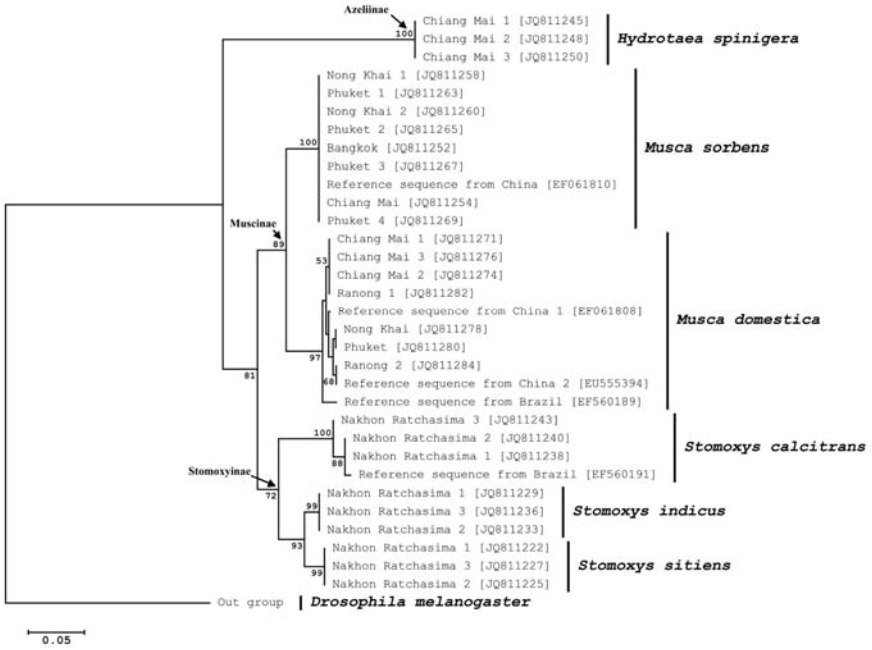

Figure 2 The 1000 bootstrapped Neighbor-Joining tree under the Kimura's 2-parmeter model based on ITS2 region sequence of four species of Muscidae flies and rooted with *D. melanogaster*.

Phylogenetic analysis

Consensus sequences of ITS2 region of Muscid flies obtained from this study together with sequences available from NCBI were used for constructing phylogenetic tree by using a 1,000 bootstraped Neighbor-Joining method under the Kimura 2-parameter and rooted with *D. melanogaster* (Song et al., 2008[b]) (Fig 2). The constructed phylogenetic tree demonstrated that ITS2 sequences could not only clearly separate Muscid flies in this study at subfamily level which consisted of clade of subfamily Azeliinae (bootstrap test 100%) consisting of *H. spinigera*, clade of subfamily Muscinae (bootstrap test 89%) consisting of *M. domestica* and *M. sorben*s and clade of subfamily Stomoxyinae (bootstrap test 72%) consisting of *S. calcitrans*, *S. indicus* and *S. sitiens*, but also separate Muscid flies deep to species level. Moreover, the same species from various regions in Thailand and other countries were clustered as individual clades with 97-100% bootstrap test. Although the clade of *M. domestica* and *S. calcitrans* were polyphyletic group, each was grouped together as well with 97-100% bootstrap supporting and clearly separated from their sister group (Fig 2).

PCR-RFLP

Based on the prediction of restriction site on ITS2 sequence using the NEBcutter program, *Xap*I restriction enzymes were appropriate for differentiation of Stomoxinae fly species in this study. The expected patterns of digested sequence in each species were summarized in Table 3. PCR-RFLP patterns of ITS2 fragment digested by enzyme *Xap*I were shown on an 8% native polyacrylamide gel electrophoresis (Fig 3-4).

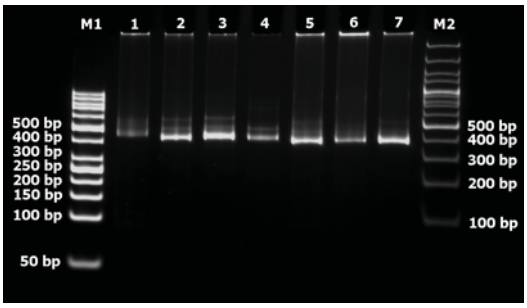

Figure 3 8% native polyacrylamide gel showed the undigested PCR products of ITS2 fragment. From left to right: lane M1 is 50 bp DNA standard marker; lanes 1–3 are PCR products from *S. indicus* (Si1, Si2 and Si3); lane 4 is PCR products from *S. sitiens* (Ss1); lanes 5-7 are PCR products from *S. calcitrans* (Sc1, Sc2 and Sc3); Lane M2 is 100 bp DNA standard marker.

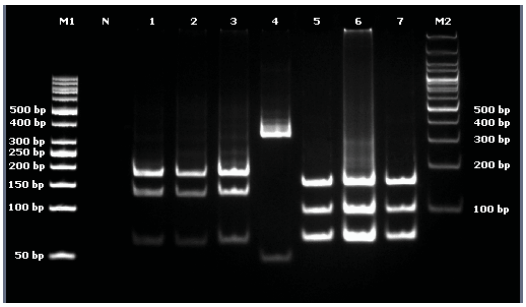

Figure 4 8% native polyacrylamide gel showed the different PCR-RFLP patterns of ITS2 fragment digested by enzyme *Xap*I. From left to right: lane M1 is 50 bp DNA standard marker; lane N is negative control; lanes 1–3 are digested PCR products from *S. indicus* (Si1, Si2 and Si3); lane 4 is digested PCR products from *S. sitiens* (Ss1); lanes 5-7 are digested PCR products from *S. calcitrans* (Sc1, Sc2 and Sc3); Lane M2 is 100 bp DNA standard marker.

Table 3 PCR-RFLP patterns among 3 species of Stomoxys flies in this study.

Flies species	Sample codes	*Xap*I digested patterns (bp)
Stomoxys indicus	Si1	179, 132, 62
	Si2	179, 127, 62
	Si3	179, 126, 62
Stomoxys sitiens	Ss1	322, 49
Stomoxys calcitrans	Sc1	154, 95, 65, 62
	Sc2	154, 95, 66, 62
	Sc3	154, 95, 65, 62

Discussion

This study determined the use of the ITS2 of rDNA region as a genetic marker to identify Muscid fly species. Twenty-seven fly samples collected from various regions of Thailand were identified based on morphology into 6 species consisting of *H. spinigera*, *M. domestica, M. sorbens*, *S. calcitrans*, *S. indicus* and *S. sitiens*. The ITS2 sequences of these 6 species were varies in length from 312 bp (*S. indicus*) to 377 bp (*H. spinigera*) average 341.45 bp. Moreover, these sequences had a high average of A+T content up to 76.6% (T= 37.9%, C= 10.3%, A= 38.7% and G= 13.1%). These high A+T content results are closely similar to recent reports on other flies such as *D. melanogaster* (80%) (Tautz et al., 1988) and Simuliidae (71-83.8%) (Thanwisai et al., 2006), but are quite different to some mosquito species which had a high G+C content such as *Anopheles crucians* (56.12%) (Wilkerson et al.,2004) and *Culex quinquefasciatus* (58%) (Severini et al.,1996). The high A+T content found in this study was caused by a repetitive region of A or T bases.

The results of comparison of ITS2 with reference sequences in NCBI indicated that the 21 morphology based identified samples of *M. domestica, M. sorbens*, *S. calcitrans* and *S. indicus* were matched with morphology identification with 93-100% identity. However, mismatching was found in 3 samples of *S. sitiens*, showing higher identity with *S. indicus* (90%) than *S. sitiens* (82%), because ITS2 sequence of *S. sitiens* in NCBI are partial sequences but *S. indicus* are completed sequences. Due to no reference sequence in *H. spinigera*, 3 sequences of these species were matched with a close species (*S. calcitrans*) with 72% identity.

Referring to a 1,000 bootstraped Neighbor-Joining with Kimura 2-parameter tree constructed based on ITS2 sequences, 27 samples of Muscid flies were separated into individual clade of each species with bootstrap supporting 97-100% as shown in Fig 2. In addition, this NJ tree could group flies species into 3 subfamilies consisting of Azeliine, Muscinae and Stomoxyinae with 72-100% bootstrap test. In species-level, ITS2 sequences from various regions in Thailand and other countries of *H. spinigera*, *M. sorbens*, *S. indicus* and *S. sitiens*, were each grouped into monophyletic clade. The paraphyly occurred in the clade of *M. domestica* and *S. calcitrans,* but no significance of variation pattern was found because it occur even in the same region. Although the paraphyly occurred in both clades, the NJ tree showed that all isolates of each species were grouped together into monophyletic clade by the high percentage of bootstrap (97% and 100%) and well separated from the sister group. These results indicated that NJ tree based on ITS2 could be used to identify 6 Muscid flies in this study at species level, but had low ability to differ same species from different regions.

According to intra-species variation analysis, the results showed no intra-species variations in *M. sorbens*, *H. spinigera* and *S. sitiens*. In contrast to *M. domestica, S. calcitrans* and *S. indicus*, intra-specific variations between Thai strains were 0.3-6.9%, 0.7-2.2% and1-3.5%, respectively. A maximum intra-species variation (6.9%) was found in *M. domestica* caused by indeling of short sequences (ATATTC, ATAATA, ACT). These results were similar when comparing to sequences from China (Accession no. EF061808) and Brazil (Accession no. EF560189). The results were also similar to recent report from Song et al. (2008[b]) which found that intra-specific divergence between *M. domestica* was high up to 5.34% and was caused by indeling of short sequences. Moreover, the intra-specific divergence between Thai and other country species (China and Brazil) ranges from 0-6.4% which causes a paraphyly in clade of *M. domestica* and divides Brazilian species out from Asian species. Although the intra-species variation was observed, each species was well grouped by the NJ tree into monophyletic clade and clearly separated from sister group.

The opportunity of species misidentification occurred when two species were similar or was a close species (Song et al., 2008[b]). Referring to phylogenetic tree and inter-specific divergence analysis as shown in Table 2, the closest species in this study was found in couple of *S. sitiens* and *S. indicus* with average inter-species divergence of 11.9%. Although the average inter-species divergence of this couple indicated that the ITS2 sequences were closely similar, the NJ tree showed that the monophyletic clade of each was clearly separated with 99% bootstrap supported.

According to a report from Wells and Stevens (2008), the intra- and inter-specific divergence are the important key for the differentiation of closely related species because if the inter-specific divergences are less than the intra-specific divergence, each clade of the sister group will be overlapped by phylogenetic tree construction. The results in this study showed no percentages of inter-specific divergence that were less than intra-specific divergence.

Identification of stable fly' species based on morphological characters by using taxonomic keys is based on body color, dorsal abdominal pattern, even in male genitalia (Masmeatathip et al., 2006). However, this procedure does not only require highly experienced person but also a complete fly samples. Moreover, some morphology still remains unclear for identification (Masmeatathip et al., 2006). The PCR-RFLP was used to demonstrate the use of molecular tool to differentiate three *Stomoxys* species in Thailand. The prediction of appropriate restriction

enzyme to differentiate these three species by using the NEB cutter web-based program resulted in the digestion patterns by *Xap*I (

Table), which can clearly discriminate these three species by the good resolution of different amount and length of DNA fragment as shown in Fig 4.

In conclusion, ITS2 gene region can be used as genetic marker to differentiate veterinary and medically important Muscid fly species in this study. The sequences of this region showed the ability to identify flies in this study deep to species level. PCR-RFLP can be used for screening of three *Stomoxys* species in this study. Although molecular tools were likely to be the effective method for differentiation between the fly species from this study, small sample size may not represent closely related species. Therefore, extensive survey for medically and veterinary important Muscids fly species in Thailand need to be investigated.

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Clinical report

Cutaneous myiasis caused by *Dermatobia hominis* in Thai travelers: first report in Thailand

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Background: Myiasis is the infestation with fly larvae in live vertebrate hosts. The disease has not been reported in Thailand.

Method and Results: We report the first case series of cutaneous myiasis caused by *Dermatobia hominis* in two Thai travelers who visited Brazil. Two of five travelers were infested with *D. hominis* larvae. Both presented with furuncular lesions. Surgical excision was performed for both patients and the larvae were removed. They were identified as second stage of *D. hominis.* Sequence data of both mitochondrial and nuclear genes of the larva were similar to previous reports from Brazil.

Conclusion: With increasing travel into endemic countries of *D. hominis*, physician should be aware of this parasitic infestation.

Keywords: *Dermatobia hominis*, imported case series, Myiasis, Thailand

Myiasis is the infestation with fly larvae in live vertebrate hosts. *Dermatobia hominis* (human botfly) is an obligate parasite that infests man and animals including live stock and other domestic animals [1]. Additionally, it is the most common cause of cutaneous myiasis in man [2]. *D. hominis* occurs in Central and South American countries. Adult *D. hominis* has a vestigial mouth, it cannot feed and can survive for only a few days. During this short period of life, adult females deposit packets of eggs on other arthropods (called phoretic hosts) which include haematophagous arthropods such as day-flying mosquitoes (*Psorophora*), flies (*Stomoxys*), ticks (*Amblyomma*) or event on non-blood feeding flies (*Sarcophaga* and *Musca*) [3]. Eggs are activated to hatch by the body heat of the vertebrate hosts when the phoretic host feeds or lands on a vertebrate animal [4]. The larvae then invade the vertebrate host either through a hair follicle, bite or by directly burrowing into the host's skin within five to ten minutes [3, 5]. After 6 to 12 weeks of full larval development, the mature larva emerges from the host's skin and drops to the ground for pupation. It finally develops into an adult fly and the cycle can then repeat [6]. This type of insect infestation can be found in Central and South American countries, e.g. Brazil. However, there is yet no previous report from Thai travelers who visited and returned infested. Herein, we report the first case series of imported cutaneous myiasis in Thailand from two travelers returning from Brazil. This study also demonstrates the use of internal transcribed spacer 2 (ITS2) and mitochondrial cytochrome oxidase (COI-COII) gene sequences to provide DNA-based identification and confirm whether they were indeed infested in Brazil.

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Case report

A 33 (case 1) and a 43 (case 2) year old men traveled to Brazil together. They noticed small papules on their left arm two days before they left Brazil to return to Thailand. One week after arrival, they observed that the lesions became enlarged and inflamed. After taking an oral antibiotic for a week, they found that the lesions did not improve and there was serosanguineous exudate excreting from a central pore of the lesion. They presented to a private hospital. The initial diagnosis was foreign body abscess.

Case 1

The furuncular lesion was located at the ventral side of the left forearm with a central pore. The size of the lesion was approximately 2×1.5 cm. Surgical excision was performed under local anesthesia (**Figure 1A**). The bottle-neck shaped larva, approximately 7×5 mm, was recovered. The posterior end of the larva was cut during the operation. Strong spines around the body of the larva could be observed macroscopically. The larva was identified as a second larval stage of *D. hominis* (**Figure 1B**). Molecular identification by sequence comparison is described below.

Case 2

The furuncular lesion was on the dorsal side of the left arm just above the elbow and approximately 1.5×1.5 cm in size (**Figure 2A**). Surgical excision was performed. A botfly larva was observed. The larva and soft tissue were removed (**Figure 2B**). The live larva was of bottle-neck shape, approximately 10×5 mm in size. The larva presented with many rows of backward projecting spines around the body (**Figure 3**). A prominent pair of cephalic hooks (**Figure 4A**) and posterior spiracles were seen (**Figure 4B**).

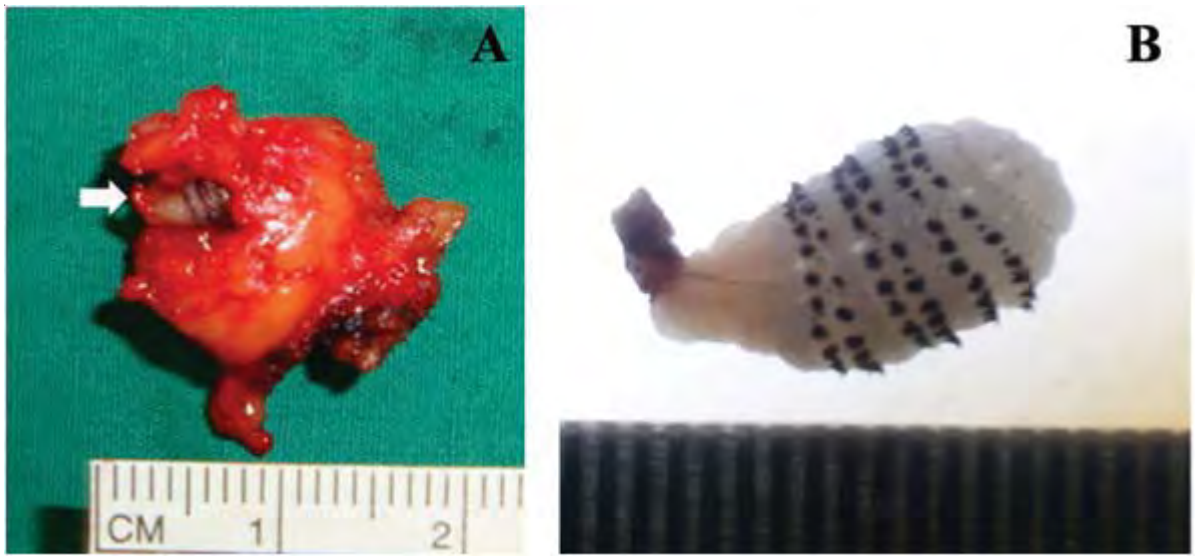

Figure 1. **A:** Surgical specimen from first case, a second stage larva of *D. hominis* (arrow). **B:** The larva of *D. hominis* from the first case. Tail of the larva was cut during the operation.

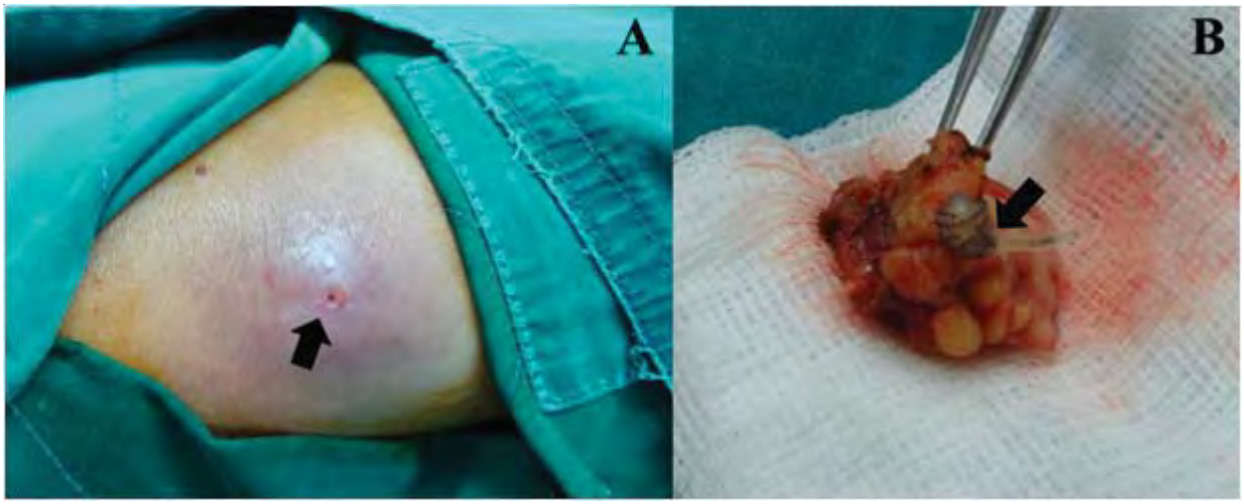

Figure 2. Furuncular lesion at ventral side of left forearm (**A**) with a central pore where serosanguineous exudates was drained (arrow). Surgical removal from second case (**B**), a live second stage larva of *D. hominis* (arrow).

Figure 3. Alive second larva of *D. hominis* from second case.

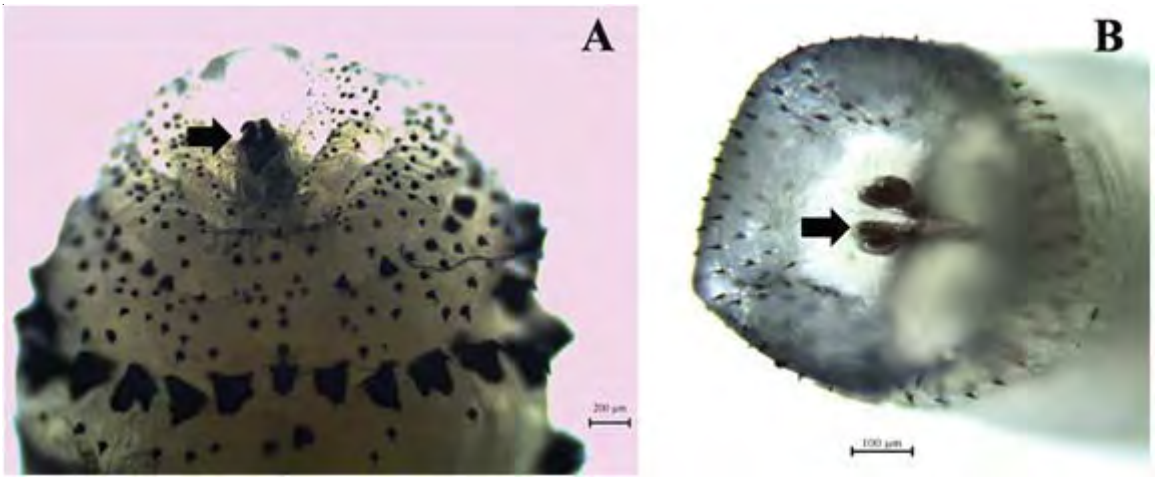

Figure 4. Anterior part of *D. hominis* larva (**A**), a pair of cephalic hooks (Arrow) and rows of backward projecting spines around the body. Posterior end of *D. hominis* larva (**B**), a pair of posterior spiracles (arrow).

Molecular Identification Methods

DNA extraction

Genomic DNA was extracted from the whole larva of the first case using QIAamp DNA mini kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. The eluted DNA was verified by spectrophotometer and diluted with 10 mM Tris-EDTA buffer, pH 8.0 to a concentration of 50 ng/µl and then stored at 4°C until the test.

PCR amplification and DNA sequencing

To retrieve sequence data of ITS2, two oligo sequences [7] as follows: 5'TGCTTGGACTACA TATGGTTGA3'and 5'GTAGTCCCATATGAGTTG AGGTT3' were synthesized for forward and reverse primers, respectively. For COI-COII, the forward primer 5'CAGCTACTTTATGAGCTTTAGG-3' and reverse primer 5'GAGACCATTACTTGCTTTCAG TCATCT-3' were designed from previous studies [8, 9]. The PCR reactions were carried out in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystem, Foster city, CA, USA) using the condition as follows: the initial denaturation (94°C for three minutes); followed by 30 cycles of 94°C for 45 seconds, 60°C (ITS2), or 56°C (COI-COII) for 45 seconds and 72°C for 1.0 minutes, and the final extension at 72°C for 10 minutes. The PCR amplification reaction was set up in a final volume of

25 µl, containing 150 ng of extracted DNA, 0.4 mM of each primer, 2.5 mM of $MgCl_2$, 200 mM of dNTPs and 1 unit of *Taq* DNA polymerase (Fermentas, USA).The PCR products were detected in 1% agarose gel (**Figure 5**) and then purified by using a Perfectprep Gel Cleanup kit (Eppendorf, Germany) following the manufacturer's instructions. Direct DNA sequencing was performed with the primer pairs described previously (1st BASE Laboratories, Malaysia).

Sequence analysis

Nucleotide sequences were prepared using Chromas Lite version 2.01 (http://www.technelysium.com.au) and aligned for maximum score comparison by the BLAST programme in NCBI website (http://www.ncbi.nlm.gov/BLAST). Two identified sequences with related data from this study were submitted and assigned for accession numbers as HQ215834 for ITS2 and HQ334260 for COI-COII in the GenBank database. Sequences were also aligned and compared with sequences data obtained from GenBank and sequence identity was calculated by using the Clustal W algorithm implemented in the BioEdit Sequence Alignment Editor v. 6.0.7 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (**Figures 6** and **7**).

Result and discussion

Although myiasis caused by *D. hominis* has been reported in many parts of the world, including as imported cases in the United States [1, 5, 10], United Kingdom [11], the Netherlands [2], Austria [12], Italy [13], and Japan [14], it was never reported from South East Asian countries. Here we describe two cases of Thai travelers infested by *D.hominis*. Both patients developed typical signs of furnicular myiasis on their extremities. Although the larvae obtained by surgical removal were identified by morphological appearance, molecular study provided more information of the source of infestation by comparison with sequence data in GenBank. The primer sets used in this study have been successfully used for identification of other fly species of the Order Diptera (suborder Brachycera) [7, 8, 9, 15]. This report demonstrated the use of these primer sets to amplify the ITS2 and COI-II genes of *D. hominis*, and obtained expected PCR products of approximately 645 bp and 1300 bp for the ITS2 and COI-II genes respectively (**Figure 5**). Sequence analysis of the ITS2 and COI-II genes by the BLAST programme compared with the GenBank database, found that the percentage of similarity was equal to 99% with expected value = 0.0 for both ITS2 and COI-COII sequences. Thus, it could be concluded that the species of this larva was

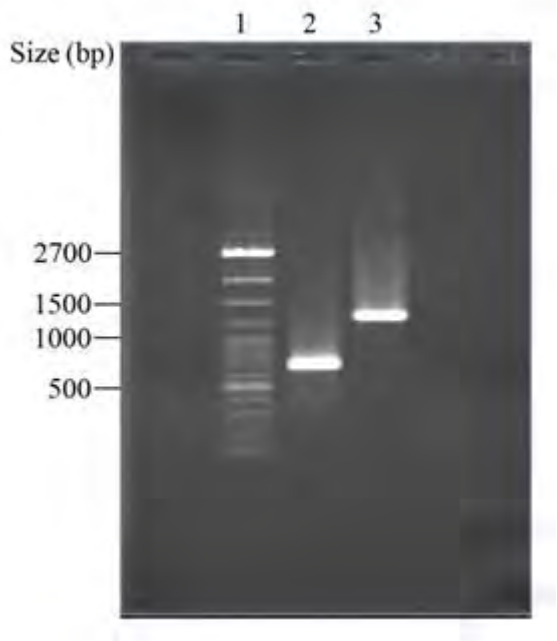

Figure 5. 1% agarose gel electrophoresis demonstrates the PCR products of amplified ITS2 and COI-II genes of *D. hominis*. Lane 1; 100bp marker (Fermentas, USA), lane 2, and lane 3; PCR products of amplified ITS2 and COI-II gene regions respectively.

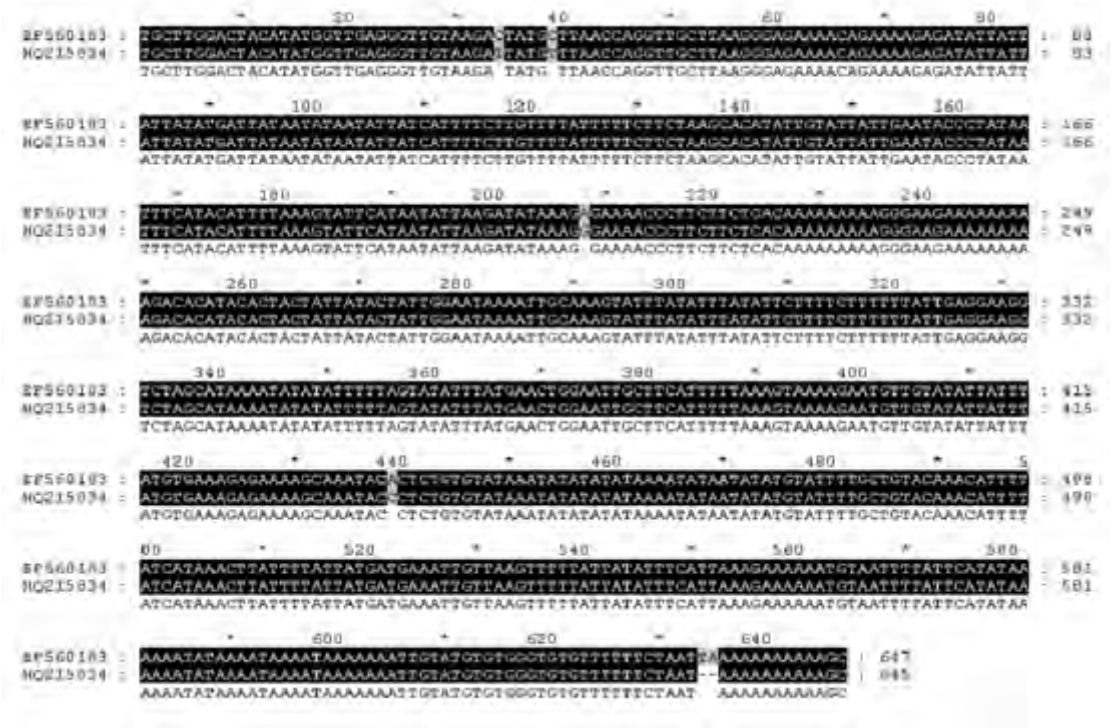

Figure 6. Nucleotide sequence alignment of *D. hominis* ITS2 gene from GenBank (EF560183) and *D. hominis* ITS2 gene from the patient (HQ215834).

D. hominis. This confirmed that this infection occurred in Brazil since the sequences are matched with GenBank sequence studied in Brazil (EF560183 for ITS2 and AY463155 for COI-COII) and confirm to the travel history to Brazil. Therefore, our data are the first report for Thai travelers infected with botfly myiasis and successfully show the application of molecular markers.

Myiasis is an infestation by fly larva in living mammals. Several fly species can cause furuncular myiasis in humans including *D. hominis* (human botfly) in Central and South American countries, *Cordylobia anthropophaga* (African tumbu fly) in Africa and *Hypoderma* spp. in North America, Europe, Africa and Asia [16]. *D. hominis* is an obligate parasite, all larval stages need to develop on a mammalian host, including humans. *Dermatobia*, *Hypoderma* and *Cordylobia* infection. They can cause skin lesions which may lead to misdiagnosis if this condition is not known to the local physician. Some studies stated that patients were usually given antibiotics or/and topical steroid cream without improvement [11, 14, 17]. The hallmark of this infection is a central pore and moving nodule. Apart from myiasis caused by *D. hominis* in the Americas, another common cause of furuncular myiasis is the tumbu fly (*C. anthropophaga*) which predominates in continental Africa south of the Sahara. It causes boil-like (furuncular) lesions, similar to myiasis caused by *D. hominis* [3]. The life cycle of the tumbu fly has been documented by Blacklock and Thompson [18]. This parasite has a different life cycle to *D. hominis*. The female deposits batches of 200-300 eggs on dry, shaded ground often laid them on laundry being hung out to dry. Larvae hatch one to three days after been deposited, and survive for 9 to 15 days without food. The larvae then wait for a mammal, often man, to use the clothing and invade skin. One of the authors, while working in West Africa, encountered one expatriate with almost a dozen mature larvae in his groin region, underneath his underwear. His native servant instructed him to cover the "blow hole" in the center of the lesions with Vaseline. Within hours, the larvae had partly come out of the opening and could be easily extracted. The lesions then healed without complications within one week. No surgery or further treatement was necessary. It is therefore prudent not to hang laundry to dry outside in Africa. If this cannot be avoided, it must be aggressively ironed with a very hot iron. Many tourists from Asia are now known to

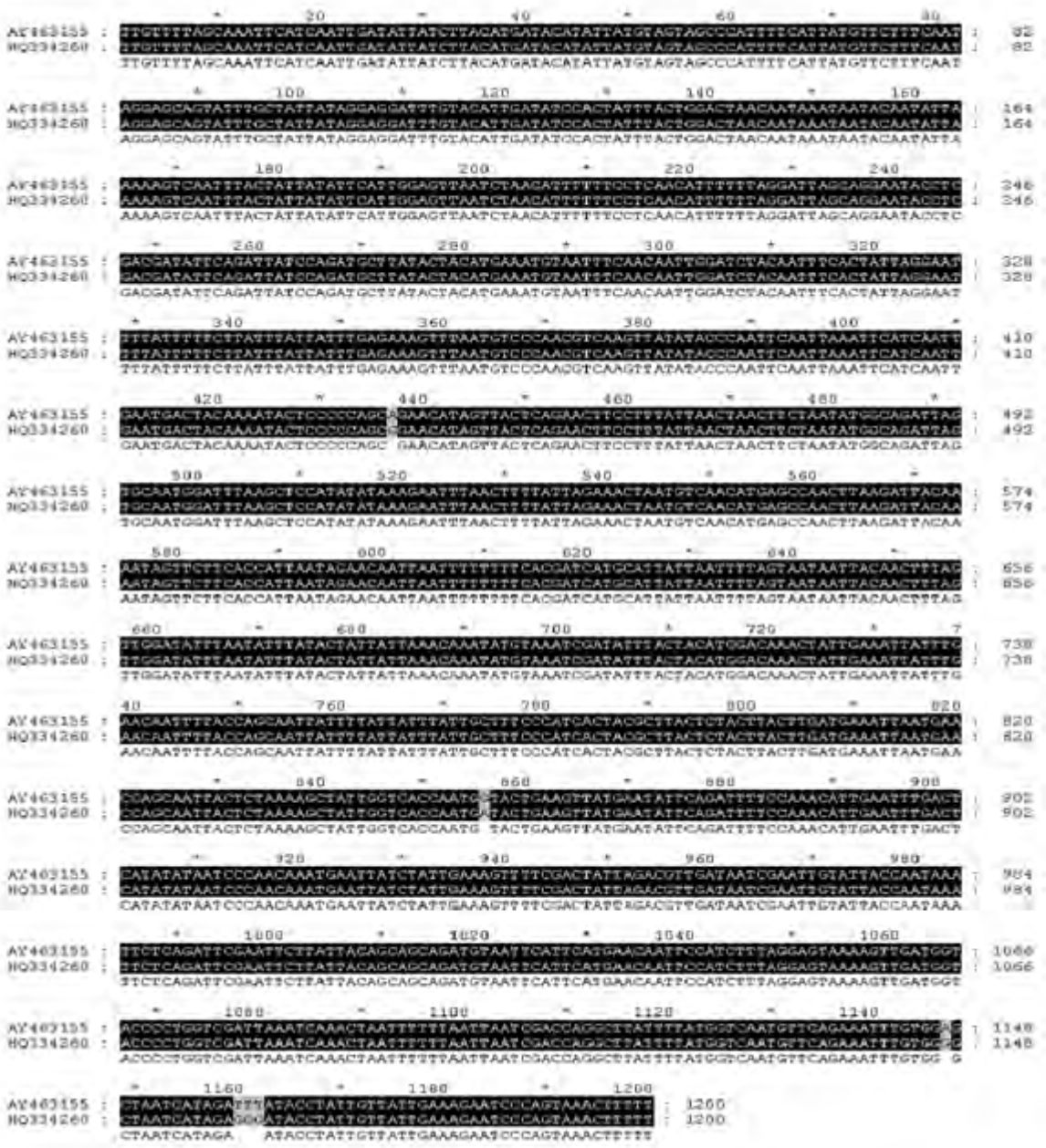

Figure 7. Nucleotide sequence alignment of *D. hominis* COI-COII gene from GenBank (AY463155) and *D. hominis* COI-COII gene from the patient (HQ334260).

travel to East and West Africa where tumbu fly myiasis is common. Although both bot fly and tumbu fly infestations show explicit furuncular lesions, differentiation between these two species is showed in **Table 1**.

Treatment of myiasis caused by *D. hominis* and *C. anthropophagi* is to remove the larvae from skin either by suffocating them using Vaseline and by squeezing the larvae out of the skin or by surgery. Prophylaxis for travelers who are going into endemic areas is to avoid insect bites by using repellants in case of bot fly, and avoiding hanging laundry to dry outside in case of tumbu fly. Obtaining a travel history can help to establish a provisional diagnosis and may avoid surgery. Thai travelers to risk zones of infestation should take precautions against insect exposure and avoid exposing washed closing and themselves to flies.

Table 1. Comparison between *D. hominis* (bot fly) and *Cordylobia anthropophaga* (tumbu fly).

Fly Species	Endemic area	Number of lesion/case	Mode of transmission	References
D. hominis (bot fly)	Central and South Americas	One to few furuncular lesions	Indirect (Phoretic hosts)	[12, 19, 20, 21]
Cordylobia anthropophaga (tumbu fly)	Continental Africa south of the Sahara	Multiple furuncular lesions	Indirect (Orifice of burrows, clothing)	[12, 22, 23, 24]

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Original Article

Discrimination between Tropical Bed Bug *Cimex hemipterus* and Common Bed Bug *Cimex lectularius* (Hemiptera: Cimicidae) by PCR-RFLP

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Abstract

Bed bugs, *Cimex hemipterus* and *Cimex lectularius,* are common blood-sucking ectoparasites of human and currently found in many countries around the world. In Thailand, both species have been found mostly in hotels in tourist attraction areas and the insecticide resistance of these insects was also documented. To date, identification of these two bed bug species is based on morphological taxonomy, a technique which requires expertise and in some instance is difficult especially for immature bed bugs or incomplete bed bug samples. In this study, we analyzed the *cytochrome c oxidase subunit I (COI)* gene of bed bugs, *C. hemipterus* and *C. lectularius* collected from various regions of Thailand. PCR-RFLP and phylogenetic analysis demonstrated that the *COI* gene could significantly differentiate between the two bed bug species. Moreover, the phylogenetic tree could separate clusters of insecticide resistant from insecticide susceptible *C. lectularius* strains. However, sequence analysis of *C. hemipterus* showed no significant intra-specific variation from different geographical regions of Thailand. Data obtained from this study will be valuable for epidemiological distribution of bed bugs in Thailand and subsequently for the most effective control of these insects.

Keywords: bed bug, *cytochrome c oxidase subunit I* gene, phylogenetic tree, PCR-RFLP

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บทคัดย่อ

การจำแนกตัวเรือดสายพันธุ์ *Cimex hemipterus* และ *Cimex lectularius* โดยวิธี PCR-RFLP

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ตัวเรือดชนิด *Cimex hemipterus* และ *Cimex lectularius* เป็นปรสิตภายนอก ที่ดูดเลือดรบกวนมนุษย์และพบได้ในหลายประเทศทั่วโลก ในประเทศไทยจะพบตัวเรือดทั้งสองชนิดนี้ในโรงแรมในพื้นที่ที่เป็นแหล่งท่องเที่ยวที่สำคัญและมีรายงานว่าตัวเรือดดังกล่าวมีการดื้อต่อสารเคมีกำจัดแมลง ปัจจุบันการจำแนกชนิดของตัวเรือดทั้งสองชนิดนี้อาศัยลักษณะทางสัณฐานวิทยา ซึ่งเทคนิคนี้ต้องการผู้เชี่ยวชาญและในบางกรณีเกิดความยุ่งยาก เช่นในระยะตัวอ่อน หรือตัวอย่างที่ไม่สมบรูณ์ รายงานนี้ได้ทำการศึกษายีนในตำแหน่งไซโตรโครม ซี ออกซิเดส หน่วยย่อยที่ 1 (*COI*) ในตัวเรือดชนิด *C. hemipterus* และ *C. lectularius* ที่เก็บตัวอย่างมาจากภูมิภาคต่างๆ ของประเทศไทย และพบว่าวิธี PCR-RFLP และ แผนภูมิต้นไม้ในยีนตำแหน่ง *COI* สามารถแยกความแตกต่างระหว่างตัวเรือดสองชนิดได้อย่างมีนัยสำคัญ นอกจากนี้แผนภูมิต้นไม้สามารถแยกตัวเรือดสายพันธุ์ *C. lectularius* กลุ่มที่ดื้อต่อสารเคมีกำจัดแมลงออกมาจากกลุ่มที่ไวต่อสารเคมีกำจัดแมลงได้ อย่างไรก็ตามพบว่าตัวเรือดสายพันธุ์ *C. hemipterus* มีความหลายหลายทางพันธุกรรมอย่างไม่มีนัยสำคัญในภูมิภาคต่างๆของประเทศไทย ข้อมูลที่ได้จากการศึกษานี้จะมีประโยชน์ในด้านระบาดวิทยาของตัวเรือดในประเทศไทยและช่วยให้การควบคุมตัวเรือดดำเนินไปอย่างมีประสิทธิภาพสูงสุด

คำสำคัญ: ตัวเรือด ยีนไซโตรโครม ซี ออกซิเดส หน่วยย่อยที่ 1 แผนภูมิต้นไม้ PCR-RFLP

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Introduction

Bed bugs (Hemiptera: Cimicidae) are important blood-sucking ectoparasites of human. Two major bed bug species feed on human blood; *Cimex hemipterus*, the tropical bed bug and *Cimex lectularius*, the common bed bug (Harlan et al., 2008). The first report of bed bug was from England in 1583 (Kemper, 1936). During the second half of the 20th century bed bugs were rare in North America and Western Europe (Ryan et al., 2002); however, in recent years they have increased in many parts of the world (Krueger and Paul, 2000; Bates, 2000; Potter et al., 2010; Criado et al., 2011). This may be due to the increase in human migration especially tourism industry and development of insecticides resistance of the insects (Romero et al., 2007; Krause-Parello and Sciscione, 2009). It is likely that the bed bugs were transported on clothes in luggages of travelers (Delaunay and Pharm, 2012).

Bed bugs require blood for development of nymphs to the next developmental stages (Johnson, 1941) and for reproduction of adults. Female bed bug produces 5-7 eggs per week with approximately 200–500 eggs in her lifetime, and adults can survive for up to a year without feeding (Pinto et al., 2007). Bed bugs live in cracks and crevices around bed or wooden furniture in hotels, hostels, private homes, trains, and cruise ships (Delaunay et al., 2011) and they can spread easily from shelter to shelter (Stephen et al. 2005). They are notorious as pests that crawl out at night to bite and feed on human blood. Although there has been no scientifically-based evidence showing that bed bugs transmit diseases (Dolling, 1991), people who are bitten may suffer from intense itch, inflammation, allergic symptoms and psychological effects (Usinger, 1966; O'Neill et al., 1997[a]; Doggett and Russell, 2009).

In Thailand, bed bugs had disappeared from the country for decades. Until recently, bed bugs were found in hotels in tourist attraction areas in different regions of the country and these bed bugs were resistant to various insecticides, especially those in the pyrethroid group (Tawatsin et al., 2011). In fact, *C. hemipterus* was resistant to DDT since 1970s (WHO, 1976) and *C. lectularius* showed resistance to bifenthrin and α-cypermethrin recently (Suwannayod et al., 2010).

Identification of bed bugs in Thailand has been based on insect morphology. Although this

procedure can identify adult stage easily, it is very difficult in immature stages or eggs (Kolb et al., 2009). Moreover, taxonomic identification requires highly experienced person and complete samples of bed bugs. Nowadays, molecular techniques have been developed for taxonomic identification such as nucleotide sequence analysis, phylogenetic tree, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Kress and Erickson, 2008). These techniques are fast, accurate and highly sensitive; moreover, it can be performed for species identification in immature stages, cast skins or incomplete samples of bed bugs from the fields. In this study, we demonstrate the utility of mitochondrial *cytochrome c oxidase* subunit I (*COI*) gene for discrimination by PCR-RFLP and phylogenetic analysis of these two main important human bed bugs. Comparisons of the *COI* gene among the bed bugs collected from different regions and between insecticide resistant and susceptibility strains were also reported.

Materials and Methods

Bed bug collections and rearing: Bed bugs, *C. hemipterus* and *C. lectularius*, in this study were collected from hotels in different parts of Thailand; Central (Bangkok), Northern (Chiang Mai and Phitsanulok), North-Eastern (Ubon Ratchathani), and Southern (Phuket and Krabi). Insecticide susceptible strain (from Tokyo) and insecticide resistant strain (from Chiba) of *C. lectularius* (provided by Dr. Mamoru Watanabe) were also used in this study. The bed bugs were identified using morphological keys described by Pratt and Stojanovich (1967). The insects were maintained in laboratory of the Biology and Ecology Section, National Institute of Health, Department of Medical Sciences, Thailand. Environmental conditions of the rearing room were set at 26-28^{0}C, 60-80% RH, and a photoperiod of 12 : 12 (L : D) hour. The bed bugs were reared in plastic cups covered with fine mesh chiffon cloth. A piece of cardboard (4 x 8 cm) was put inside the cups for the bed bugs to crawl up and insert their mouthparts through the mesh top to feed. For blood feeding, the bed bugs had access to artificial feeding unit for 30 minutes, using almost expired donated-blood received from Blood Bank, Thai Red Cross. This method was modified from that developed by Montes et al. (2002).

DNA extraction: Individual bed bug of each sample was lysed by lysis buffer and placed in liquid nitrogen for 1 minute and then ground with a sterile plastic pestle. Genomic DNA was isolated using DNA extraction kits: Invisorb® Spin Tissue Mini Kit (STRATEC Molecular GmbH, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 100 µl of elution buffer; the fraction of extracted DNA was spectrophotometrically quantitated using a Nanodrop 2000c (Thermo-scientific, USA). The extracted DNA samples were kept at -80^{0}C for long term storage.

PCR amplification: Sequences of the *COI* gene of *C. hemipterus* and *C. lectularius* were obtained from GenBank with Accession number GU985538.1 and GU985525.1, respectively (Balvin and Vilimova, 2010). The sequences were aligned using the multiple alignment programs ClustalX version 1.81 (Thompson et al., 1997). Degenerate oligonucleotide primers were designed as forward primer (5′ GMCAACCTGGCTCATTTATTG 3′) and reverse primer (5′ ATAAGTGTTGYTAWAGWARAGG 3′). Primers were synthesized by 1st BASE Oligonucleotide (Oligo) Synthesis services company (1st BASE Laboratories, Malaysia). The amplification reaction was set up in a final volume of 25 µl, containing approximately 100 ng of extracted DNA. Polymerase chain reactions (PCR) were performed in a GeneAmp PCR system 2400; Applied Biosystems®, USA. The reaction conditions are as follows: denaturation at 95^{0}C for 5 min, followed by 35 cycles of 95^{0}C for 1 min, 48^{0}C for 1 min, and 72^{0}C for 1 min and final extension at 72^{0}C for 7 min. Aliquots of the amplicons were analyzed on a 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized with Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

DNA sequencing and RFLP patterns prediction: The PCR amplicons were ligated into pGEM-T Easy Vector (Promega, USA). The ligated vectors were transformed into DH5α strain competent cells, and then the chimeric plasmids were screened by blue-white colony selection system. The suspected positive colonies were cultured and used for further plasmid DNA extraction by using Invisorb® Spin Plasmid Mini kit (STRATEC Molecular GmbH, Germany) following the manufacturer's instructions. Purified plasmids were sent to sequence by 1st BASE DNA sequencing services (1st base laboratories, Malaysia) using universal forward T7 primer. Nucleotide sequences were analyzed using BioEdit Sequence Alignment Editor Version 7.0.9.0 (Hall, 1999) and the consensus sequences were BLAST search (available at http://www.ncbi.nlm.gov/BLAST) for species identification. The nucleotide sequences of *COI* gene obtained from this study were submitted to the GenBank database. The resulting sequences were used for prediction of species-specific restriction sites by using NEBcutter V2.0 web-based program (available at http://tools.neb.com/NEBcutter2/index.php). From restriction prediction data, *Bfa*I restriction enzyme recognizes 5'...C↓T A G...3' sites were chosen for PCR-RFLP.

PCR-RFLP: The PCR products were digested in separate reaction with *Bfa*1 (Thermo-scientific, USA). The reaction mixture was incubated at 37^{0}C for 15 min followed by heat inactivation at 65^{0}C for 5 min. The restriction products were electrophoresed through 8% native polyacrylamide gel electrophoresis run at 100 V for 70 min (MiniProtein 3 cell; Bio-Rad®, USA), followed by ethidium bromide staining and visualized on a Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, CA, USA).

Sequence variation and Phylogenetic tree construction: The nucleotide sequences of each

species from various regions were aligned for variation positions. Phylogenetic tree were constructed by Maximum-likelihood method using Kimura's 2-parameter model implemented in MEGA© version 5.1 (Tamura et al., 2011). The reliability of an inferred tree was tested by 1000 bootstrap. *Triatoma dimidiata* (Kissing bug) accession no. JQ575031 as outgroup.

Results

In this study, 15 bed bugs were collected from 6 different regions of Thailand and 2 samples were provided from Japan. PCR amplicons of the *COI* gene from *C. hemipterus* and *C. lectularius* were approximately 580 bp in size (Fig 1). Amplified *COI* gene sequences obtained from this study varied from 576 to 581 bp. Consensus *COI* gene sequences of *C. hemipterus* and *C. lectularius* were blast in the GenBank database and showed the percentage identity range from 99 to 100. The nucleotide sequences showed maximum intra-specific variation approximately 0.8% in *C. hemipterus* and 0.6% in *C. lectularius*; nevertheless, the minimum inter-specific variation showed approximately 19.6% (data not shown). The nucleotide sequences of *COI* gene from the bed bugs were submitted to GenBank and accession numbers of JX826468 to JX826482 were assigned (Table 1).

The *COI* sequences of *C. hemipterus* collected from various parts of Thailand could be grouped into three groups (CH1, CH2 and CH3). The sequences variation was found at position 380 and deletion of three bases of *COI* gene between positions 445-447 were found in CH1 and CH3 (Fig 2). *COI* gene sequence of *C. lectularius* collected from Ubon Ratchathani was 100% identical to the insecticide susceptible *C. lectularius* from Tokyo isolated. Variations of *COI* gene sequences of *C. lectularius* between insecticide resistant and susceptible strains were found at position 96, 146 and 204 in this study (Fig 3).

Table 1 Collection site, isolated code, species, and accession number of each bed bug in this study

Location	Isolated code	Bed bug species	Accession no.
Chiang Mai	C.H.agri1	*C. hemipterus*	JX826468
	C.H.gate2	*C. hemipterus*	JX826469
	C.H.sarin3	*C. hemipterus*	JX826470
	C.H.cmu4	*C. hemipterus*	JX826471
	C.H.cmu1-5	*C. hemipterus*	JX826472
	C.H.cmu2-6	*C. hemipterus*	JX826473
Phuket	C.H.phu7	*C. hemipterus*	JX826474
Krabi	C.H.kb8	*C. hemipterus*	JX826475
Bangkok	C.H.bk 9-1	*C. hemipterus*	JX826476
	C.H.bk 10-2	*C. hemipterus*	JX826477
	C.H.12	*C. hemipterus*	JX826479
Phitsanulok	C.H.pl11	*C. hemipterus*	JX826478
Tokyo (susceptibility)	C.L.Tokyo	*C. lectularius*	JX826480
Chiba (resistance)	C.L.Chiba	*C. lectularius*	JX826481
Ubon Ratchathani	C.L.2	*C. lectularius*	JX826482

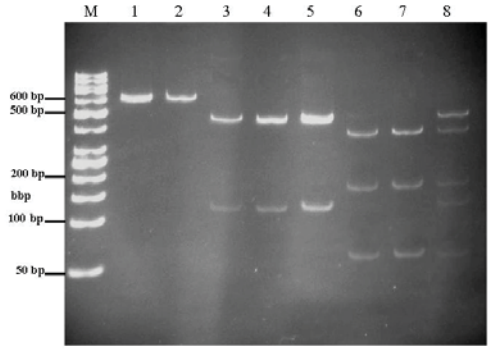

Figure 1 8% native polyacrylamide gel electrophoresis shows PCR-RFLP patterns of *COI* product digested with *Bfa*I restriction enzyme. Lane1-2: undigested PCR products amplified from *C. lectularius* and *C. hemipterus*, respectively. RFLP patterns of *C. lectularius*: 120 and 459 bp (lane 3-5); *C. hemipterus*: 57, 168, and 351 bp (lane 6-7) and mixed DNA of both species: 57, 120, 168, 351 and 459 bp (lane 8) from *Bfa*I digestion. Lane M: 25 bp DNA standard marker.

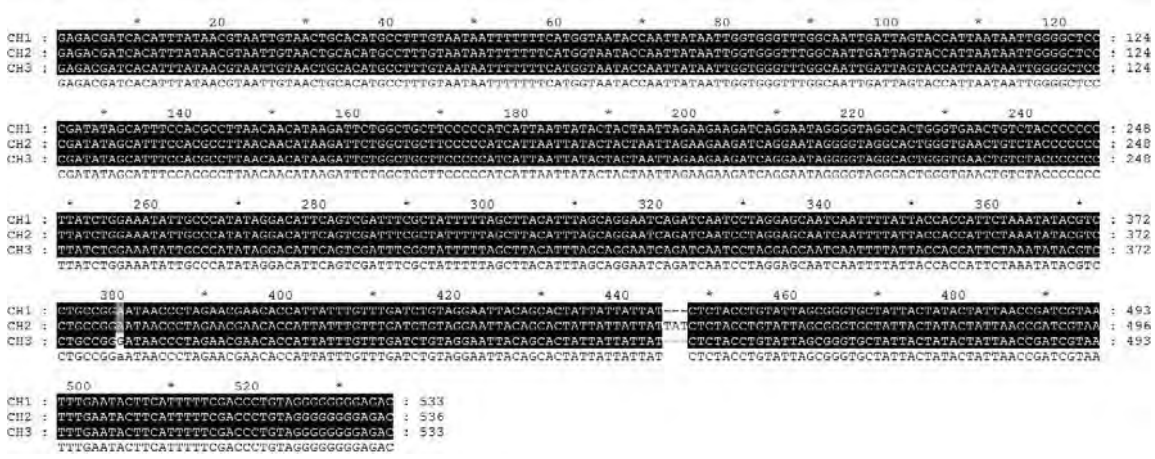

Figure 2 Nucleotide sequence comparison of *COI* genes of *C. hemipterus*, based on these sequences they can be classified into three groups: **CH1** represented sequence of *C. hemipterus* isolates C.H.sarin3, CMU4 and agri1 from Chiang Mai; **CH2** represented sequence of *C. hemipterus* isolates C.H.cmu2-6, gate 2 and C.H.cmu 1-5 from Chiang Mai, *C. hemipterus* isolates C.H.kb8 from Krabi, *C. hemipterus* isolate C.H.bk9-1 from Bangkok, and *C. hemipterus* isolates C.H.phu7 from Phuket; **CH3** represented sequence of *C. hemipterus* isolates C.H.bk10-2 and C.H.12 isolate from Bangkok and *C. hemipterus* isolate C.H.pl11 from Phitsanulok.

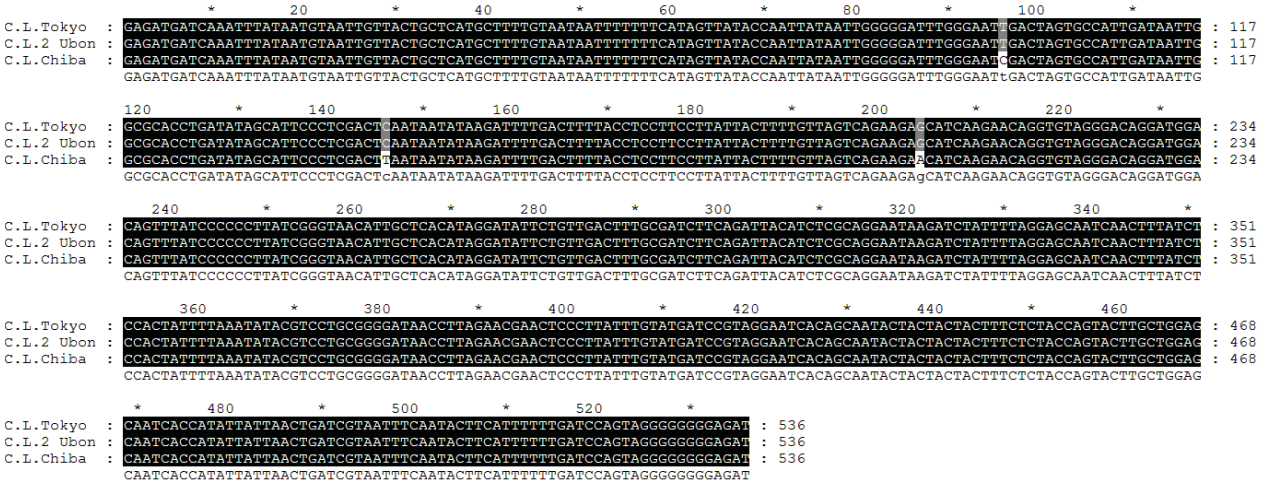

Figure 3 Nucleotide sequence comparison of *COI* genes of insecticide resistant and susceptible *C. lectularius strains*; C.L.Tokyo: *C. lectularius* isolate C.L.Tokyo (susceptibility); C.L.2 Ubon: *C. lectularius* isolate C.L.2 from Ubon Ratchathani; C.L.Chiba: *C. lectularius* isolate C.L.Chiba (resistant).

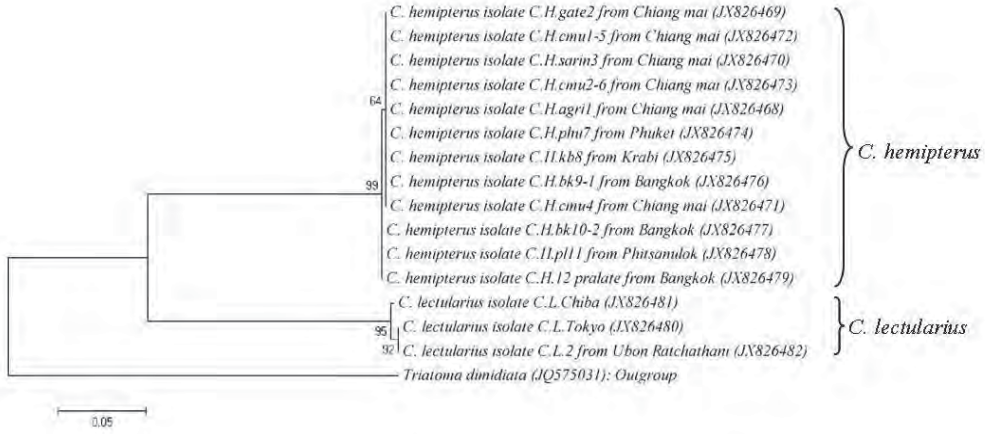

Figure 4 Maximum-likelihood trees were constructed using GTR + G + I evolution model of *COI* gene in 12 isolates of *C. hemipterus* and 3 isolates of *C. lectularius*. *T. dimidiata* accession no. JQ575031 sequences were used as outgroup.

RFLP pattern of these two species were predicted by the NEBcutter V2.0 web-based program and *Bfa*I restriction was selected. PCR products of each species were digested with the *Bfa*I restriction enzyme and the fragments separated by 8% native polyacrylamide gel electrophoresis. The results demonstrated the PCR product of approximate 579 bp in length for both species, *C. hemipterus* (57, 168, and 351 bp) and *C. lectularius* (120 and 459 bp) and mixed DNA of both species (57, 120, 168, 351 and 459 bp) (Fig 1).

The constructed phylogenetic tree could clearly separate two major clades of *C. hemipterus* and *C. lectularius* although they belonged to the same genus. However, all of the *C. hemipterus* isolates clustered together showing no significant difference between different regions because of minor nucleotide variations between the same species. On the contrary, in *C. lectularius,* the insecticide resistant strain from Chiba was separated from Tokyo (insecticide susceptibility) and Ubon Ratchathani strains by the phylogenetic construction (Fig 3).

Discussion

Nowadays, there are increasing reports of bed bug infestation and resistance to various insecticides has also been documented. Two species of bed bugs, *C. hemipterus* and *C. lectularius,* are found in Thailand and they become resistant to various insecticides (Tawatsin et al., 2011). Ghauri (1973) revealed that two species of bed bugs can be distinguished by looking at the first segment of the thorax, which expanded more laterally and of which the extreme margins are more flattened *in C. lectularius* than *C. hemipterus*. Several reports suggested that the type of insecticide resistance were different between bed bug strains (Karunaratne et al., 2007; Romero et al., 2007; Kilpinen et al., 2011). Therefore, taxonomic identification is important with necessary specialized taxonomic expertise. Molecular techniques are commonly used to apply in research labs worldwide for species identification such as sequence, phylogenetic tree analysis and PCR-RFLP in order to identify reliably and practically. This study used DNA-based identification by application of *COI* sequences for differentiation of bed bugs. *COI* is a mitochondrial gene which is conserved in arthropods,

species specific and has relatively high degree of genetic variation. We demonstrated the value of PCR-RFLP to differentiate two bed bug's species. This result showed that intra-specific polymorphism was not observed here by digestion with *BfaI* restriction enzyme as well as RFLP could be used for mix samples of two bed bugs. This benefit can help the survey of the bed bugs, when only cast skins or eggs as well as carcass damage of bed bugs are found. The PCR-RFLP can potentially lead to supersede taxonomic misidentification errors. In addition, phylogenetic tree showed the monophyletic clade in each species. According to intra-specific variation analysis, we found intra-variation in *C. hemipterus* (approximately 0.8%) because the sequence showed indeling of TAT base position, which could be confirmed by pick individual 10 colonies for sequencing. *C. hemipterus* can be grouped into 3 groups based on sequence variations. CH1 was found only in Chiang Mai, CH 2 was found in Chiang Mai, Bangkok, Krabi and Phuket, and CH3 was found in Bangkok and Phitsanulok (Fig 2). The study of *C. lectularius* indicated minor nucleotide variations between the insecticide resistant and susceptible strains in Japan as well as insecticide resistant isolates single branch of insecticide susceptible and *C. lectularius* in Thailand. This study is the first to report that the *COI* gene sequences were different between insecticide resistant (Chiba) and susceptible (Tokyo) strains of common bed bugs. *C. lectularius* collected from Ubon Ratchathani used in our study was susceptible to various insecticides (unpublished data) and the *COI* sequences were 100% identical to the insecticide susceptible isolates from Tokyo (Fig 3). Susceptible strain of *C. hemipterus* was unavailable in our laboratory, so it was not included in this study. Furthermore, this study investigated only 2 cosmopolite species, therefore, studies in other species such as *C. columbarius, C. pipistrelli, C. dissimilis,* and *Oeciacus* should be conducted as well as more collected samples from different geographical regions.

In conclusion, we demonstrated the ability of PCR-RFLP to discriminate between common bed bug and tropical bed bug. The sequence data obtained from the study showed minor variation between the same bed bug species. However, *COI* sequences of *C. lectularius* were different between insecticide resistant and insecticide susceptible strains. The sequence data from this study will be useful for epidemiological studies and for proper planning for effective bed bug control in the future.

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Short Report: Detection of *Leishmania siamensis* DNA in Saliva by Polymerase Chain Reaction

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Abstract. Polymerase chain reaction was used to detect *Leishmania siamensis* DNA from clinical samples collected from six leishmaniasis patients during 2011–2012. The samples used in this study came from bone marrow, blood, buffy coat, saliva, urine, and tissue biopsy specimens. Saliva was a good source for *L. siamensis* DNA by polymerase chain reaction. *L. siamensis* DNA was also found in saliva of an asymptomatic case-patient. Levels of *L. siamensis* DNA in saliva decreased until being undetectable after treatment. These levels could be used as a marker to evaluate efficacy of the treatment. A larger study is needed to evaluate this method as a screening and survey tool to study the silent background of *Leishmania* infection among the at-risk population.

Leishmaniasis is a neglected tropical diseases caused by an obligate intracellular protozoa belonging to the genus *Leishmania*. The disease is transmitted to vertebrate hosts by infected female sand flies taking a blood meal.[1] Leishmaniasis presents in three clinical forms; visceral, cutaneous, and mucocutaneous.[2] Clinical presentation of leishmaniasis depends on the species of *Leishmania* and the immunity of the host.[3]

Detection and species identification of the parasites is essential for prognostic and therapeutic reasons and surveys.[4] Several laboratory techniques have been used for diagnosis of *Leishmania* infection. They are microscopy, culture, immunologic techniques (enzyme-linked immunosorbent assay, direct agglutination test, and recombinant protein K39 dipstick test), and molecular techniques (polymerase chain reaction [PCR] and quantitative PCR).[5–7] In comparisons of microscopic examination, culture, and PCR in detecting *Leishmania* parasites, PCR has shown to have a significantly higher sensitivity than culture and microscopic examination (97%, 78%, and 76%[8–10] sensitivity, respectively). New cases of leishmaniasis caused by *L. siamensis,* a novel species of *Leishmania*, have been documented in patients in Thailand[11–17] and Myanmar (unpublished data). The infection was described in immunocompromised patients, mostly persons infected with human immunodeficiency virus (HIV).[11–17] Clinical presentations of these patients have included visceral,[11–13] diffuse cutaneous,[15] and overlapping diffuse cutaneous and visceral forms.[14]

With low prevalence of leishmaniasis in Thailand and Myanmar, screening tests for leishmaniasis such as enzyme-linked immunosorbent assay, direct agglutination test, and recombinant protein K39 dipstick test are not readily available. Moreover, sensitivity and specificity of these serologic tests for detection of *L. siamensis* infection have never been fully documented. Diagnosis of *L. siamensis* infection relies on microscopic examination, culture, and detection of parasite DNA by PCR.[11–15] Although microscopic examination and culture of *Leishmania* parasites have high specificity, they require experience and have low sensitivity. The PCR is commonly used to diagnose leishmaniasis caused by *L. siamensis*. Bone marrow, blood, buffy coat, tissue, saliva, and urine have been successfully used for detection of *L. siamensis* DNA by PCR.[14]

Saliva has shown to be a good source for *L. siamensis* DNA.[14] There are also reports of using saliva to identify other *Leishmania* species.[18–20] Collection of saliva is noninvasive and convenient for field studies. We describe a PCR to amplify the internal transcribed spacer1 (ITS1) gene of *L. siamensis* from six infected patients and compare it with specimens collected from patients and different clinical presentations. Details of clinical presentations and management of some patients enrolled in this study have been described.[14]

Bone marrow, blood or saliva was smeared on glass slides, and fixed with absolute methanol (Sigma-Aldrich, St. Louis, MO) for one minute. The slides were then stained with Giemsa (Sigma-Aldrich) in phosphate buffer, pH 7.2. Tissue biopsy specimens were stained with hematoxylin and eosin. Stained slides were then examined under a light microscope (Olympus, Tokyo, Japan).

Schneider's insect medium (Sigma-Aldrich) containing 20% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Sigma-Aldrich) was used for culturing *Leishmania* parasites. One hundred microliters of bone marrow, blood, or saliva was inoculated into 5 mL of culture media in a 25-cm^3 flask and incubated at 25 ± 2°C. Cultures were inspected for parasites every 24 hours by using an inverted microscope (Olympus).

Two hundred microliters of blood or bone marrow and 50 μL of buffy coat were used for DNA extraction by using a blood DNA extraction kit (Invisorb® Spin Blood Mini Kit (STRATEC Molecular, Berlin, Germany). Thirty milliliters of urine or 1 mL of saliva were centrifuged for 5 minutes at 5,000 × *g*, and the pellets were collected and used for further DNA extraction. Tissue specimens, urine pellets, and saliva pellets were used for DNA extraction by tissue DNA extraction (Invisorb® Spin Tissue Mini Kit) according to the manufacturer's instructions. Extracted DNA was eluted in 80 μL of elution buffer. Quantity and quality of the extracted DNA was determined by using a Nanodrop 2000c Apparatus (Thermo Scientific, Singapore). Extracted DNA samples were kept at –80°C for long-term storage. Blood, saliva, and urine were collected from three healthy uninfected persons and used for the PCR as negative controls.

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The PCR was performed in a final volume of 25 μL containing approximately 100 ng of extracted DNA, 10 μM of each primer, 25 mM of $MgCl_2$, 2 mM of dNTPs, and 1 unit of *Taq* DNA polymerase (Fermentas, Pittsburgh, PA). The primers were designed to anneal specifically to the ITS1 regions of ribosomal RNA (rRNA) of *Leishmania* parasites described by Spanakos and others:[21] (LeF: 5′-TCC GCC CGA AAG TTC ACC GAT A-3′ and LeR: 5′-CCA AGT CAT CCA TCG CGA CAC G-3′). The PCRs were performed in a PCR Mastercycler® Pro (Eppendorf, Hamburg, Germany) with conditions as follows: denaturation at 94°C for 4 minutes; followed by 40 cycles at 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute; and a final extension at 72°C for 7 minutes. Aliquots of the PCR amplicons were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized with Quantity One Quantification Analysis Software version 4.5.2 (Gel Doc EQ System; Bio-Rad, Hercules, CA). The extracted DNA samples from an uninfected persons and a no DNA template (double-distilled water) were used as negative controls in every PCR. Reactions in which either or both negative controls contained bands were discarded.

Confirmatory testing for PCR was performed by using another set of primers to amplify the partial small subunit (SSU) rRNA genes of the *Leishmania* parasite. The primer sequences (R221: 5′-GGT TCC TTT CCT GAT TTA CG-3′ and R332: 5′-GGC CGG TAA AGG CCG AAT AG-3′) and PCR conditions were described by Van Eys and others.[22]

The PCR amplicons were ligated into the pGEM-T Easy Vector (Promega, Madison, WI). Ligation reactions mixture was composed of 5 μL of 2× Rapid ligation buffer, 3 μL of PCR products, 1 μL pGEM-T Easy Vector, and 1 μL double-distilled water. The ligated vectors were transformed into DH5α competent cells and chimeric plasmids were screened by blue–white colony selection system. The suspected positive colonies were cultured and used for further plasmid DNA extraction by using the Invisorb® Spin Plasmid Mini Kit following the manufacturer's instructions. Purified plasmids were sequenced by 1st BASE DNA sequencing services (1st Base Laboratories, Kuala Lumpur, Malaysia) by using universal forward T7 primer. Nucleotide sequences were analyzed by using BioEdit Sequence Alignment Editor Version 7.0.9.0 (www.mbio.ncsu.edu/bioedit/bioedit.html), and consensus sequences were compared with available sequence data in a GenBank by BLAST search (www.ncbi.nlm.gov/BLAST).

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 385/55).

Patient 1 was a 46-year-old Thai man who was a rubber planter from southern Thailand. He has been given a diagnosis with HIV infection in 2003 and received boosted lopinavir and lamivudine. His CD4+ T cell count was 175 cells/mm^3, and serum virus levels were < 40 copies/mL. He also had Evans syndrome (an autoimmune disorder with destruction of erythrocytes, platelets, and leukocytes), a left knee ulcer, and hepatosplenomegaly. A bone marrow study showed *Leishmania* amastigotes within macrophages. Bone marrow, blood, discharge from the ulcer, saliva, and urine were cultured and DNA was extracted for PCR.

He improved after two weeks of intravenous amphotericin B deoxycholate (1 mg/kg/day), followed by 400 mg of itraconazole/day. A recurrence after two months of itraconazole therapy was re-treated with three weeks of intravenous liposomal amphotericin B (3 mg/kg/day), followed by 400 mg of itraconazole per day. Blood and saliva were collected for PCR three months after re-treatment and were negative. Details of this patient were reported by Chusri and others.[14]

Patient 2 was 30-year-old pet store owner from southern Thailand who had been given a diagnosis of HIV infection in 1999. The patient received tenofovir, lamivudine, and nevirapine. His CD4+ T-cell count was 111 cells/mm^3 and viral RNA was not detectable (< 40 copies/mL). He had multiple papules and plaques with ulceration and discharges. He also had anemia, thrombocytopenia, and hepatosplenomegaly. Bone marrow, papule, and ulcer biopsy specimens showed *Leishmania* amastigotes within macrophages. Bone marrow, blood, tissue biopsy specimens, and saliva and urine samples were used for culture and PCR. The patient received intravenous amphotericin B deoxycholate (1 mg/kg/day) for two weeks, followed by 400 mg of itraconazole per day. Blood and saliva were collected three months later and were negative for *Leishmania* and no recurrence was observed. This patient was reported by Chusri and others.[14]

Patient 3 was a 60-year-old man with diabetes who lived in Yangon, Myanmar and had not previously traveled abroad. He showed development of fever, multiple infiltrative skin lesions, and oral ulcers. A skin biopsy specimen indicated Sweet's syndrome or acute febrile neutrophilic dermatosis, which is a skin disease with fever and painful skin lesions that is commonly present on arms, neck, face, and back. He was

TABLE 1

Results of microscopic examination, culture, and polymerase chain reaction for detection of *L. siamensis* DNA from various clinical specimens collected under different conditions and different clinical presentations from six patients*

				Microscopic examination/culture for *Leishmania*			PCR detection of *L. siamensis* DNA						
Patient	Age (years)	Sex/nationality	Clinical presentation/host conditions	Blood smear	Skin biopsy	Blood/bone marrow culture	Bone marrow	Blood	Buffy coat	Saliva	Urine	Tissue	Reference
1	46	M/Thai	CL and VL/HIV infection and prednisolone therapy	+	N/A	+/+	+	+	+	+	+	N/A	Chusri and others[14]
2	30	M/Thai	CL and VL/HIV infection	+	+	–/+	+	+	+	+	+	+	Chusri and others[14]
3	60	M/Burmese	CL/DM and Prednisolone therapy	–	+	–/N/A	N/A	+	+	+	+	+	Unpublished data
4	22	F/Burmese	Asymptomatic/normal	–	N/A	–/N/A	N/A	-	+	+	–	N/A	Unpublished data
5	45	M/Thai	CL/HIV infection	–	+	–/N/A	N/A	+	+	+	–	+	Unpublished data
6	34	M/Burmese	CL/HIV infection	–	+	–/N/A	N/A	+	+	+	–	+	Unpublished data

*PCR = polymerase chain reaction; CL = cutaneous leishmaniasis; VL = visceral leishmaniasis; HIV = human immunodeficiency virus; NA = not available.

treated with systemic corticosteroids for two months without improvement. He came to Thailand for further evaluation. Multiple erythematous, shiny, infiltrative plaques; nodules on face, trunk, and extremities; and oral ulcers and white patches on the buccal mucosa were observed. Lymph nodes, liver, and spleen were not palpable. Complete blood counts, blood urea nitrogen levels, creatinine levels, and liver function test results were within normal limits. Results for antinuclear antibodies, antibodies against HIV, and C-reactive protein levels were unremarkable.

A new skin biopsy specimen of a trunk nodule showed diffuse histiocytic infiltrate and multinucleated giant cells in the upper and deep dermis. Many round-to-oval small organisms were present in histiocytes and fibrous stroma. They stained positive with Periodic Acid–Schiff (PAS) stain. Numerous small yeast-liked organisms were present in histiocytes and in the stroma. Some of these organisms were large and contained small basophilic dots in cytoplasm near nuclei stained positively with PAS stain but not with Gomori's methenamine silver stain and Giemsa. This finding suggested the presence of *Leishmania* parasites. Blood samples, tissue biopsy specimens, saliva and urine samples were used for culture and were negative. PCR testing of the ITS1 region of the rRNA gene in blood, skin biopsy specimen, urine, and saliva, and DNA sequencing identified *L. siamensis*. The patient was treated with intravenous amphotericin B for 40 days (total dose = 2.1 grams). After regression of the cutaneous lesions, he was discharged. When seen again two months later, he had gained weight but a few skin lesions were still present. He returned home and was lost to follow-up.

Patient 4 was the 22-year-old daughter of patient 3; she came to Thailand with her father. She was healthy and lived with her father in Yangon. Results of her physical examination were unremarkable. Saliva and urine samples were collected

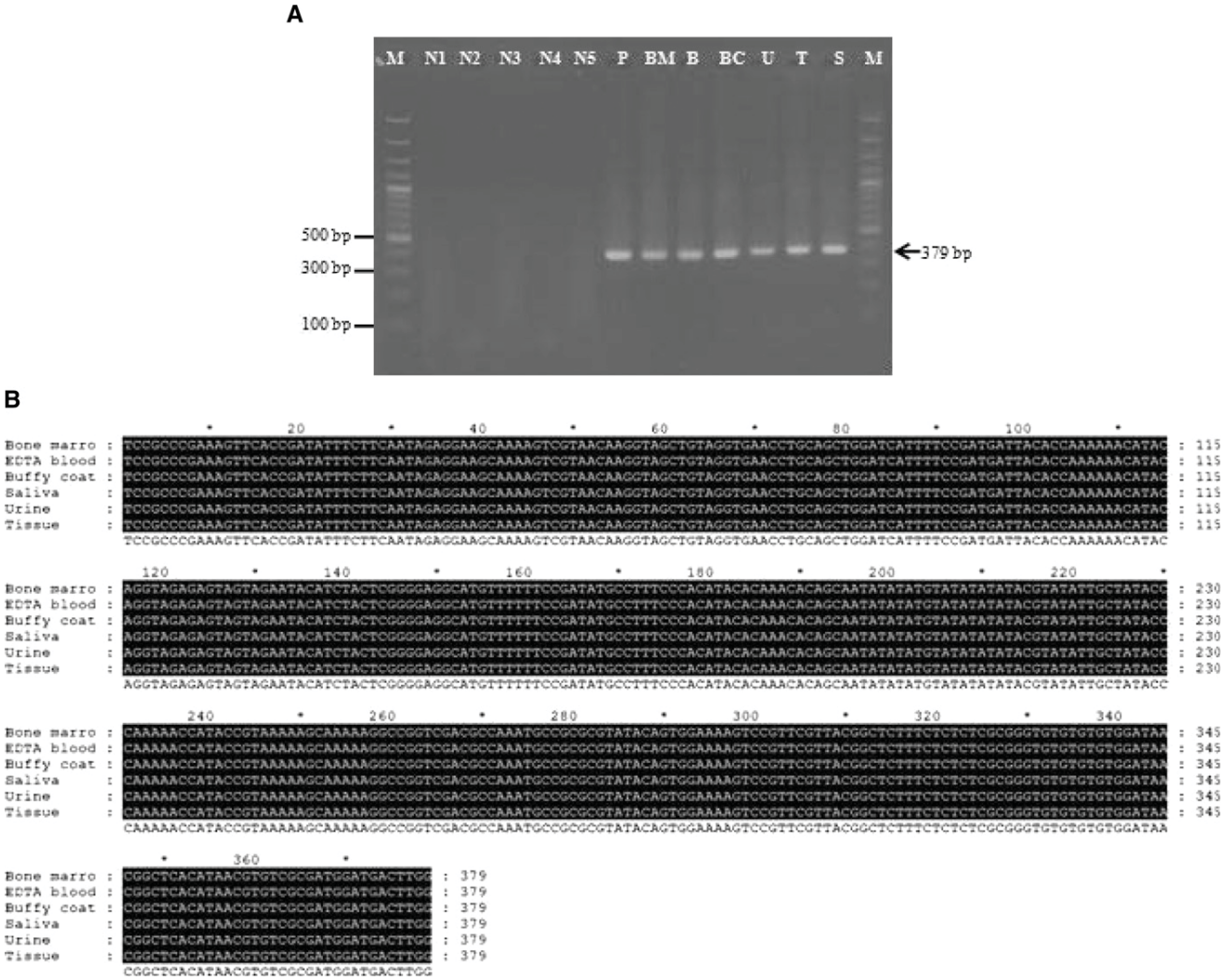

FIGURE 1. Polymerase chain reaction amplification of various sources of specimen of patient case 2 (**A**) were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Lane BM, bone marrow; lane B, Blood; lane BC, buffy coat; lane S, saliva; lane U, urine; lane T, tissue; lane M,: molecular mass marker (100 basepairs [bp]; lane, P, positive control containing extracted DNA from cultured *L. siamensis*; lane N1, negative control (no DNA template: double-distilled water); lanes N2–N5: negative control (DNA template from non- infected saliva, urine, blood, and buffy coat, respectively). Comparison of internal transcribed spacer 1 (ITS1) gene sequences amplified from various sources of specimen of the patient 2 (**B**), Amplified sequences of *L. siamensis* ITS gene from bone marrow, blood, saliva, urine, and tissue biopsy of patient 2 were assigned GenBank numbers KF227887–KF227892, respectively.

and used for culture; these samples were negative for *L. siamensis*. PCR testing of the ITS1 region of the rRNA gene in saliva DNA, followed by DNA sequencing, identified *L. siamensis*. Whole blood and buffy coat were then collected for *Leishmania* detection by culture, staining with Giemsa, and PCR. Only PCR identified *L. siamensis* in buffy coat. We planned to follow-up the patient for detection of *Leishmania* parasites without treatment of leishmaniasis but the patient was lost to follow-up.

Patient 5 was a 45-year-old Thai man living in Chiang Rai, Thailand. He has been given a diagnosis of HIV infection in 2005. He had a CD4+ T cell count of < 50 cells/mm^3. He was treated with stavudine, lamivudine, and nevirapine. In 2007, He showed development of lumpy skin lesions that were first not investigated. He was later hospitalized with fever, oral candidiasis, pancytopenia, pancreatitis, type 2 diabetes mellitus, epistaxis, perianal abscess, urinary retention, and abnormal liver function test results. Skin biopsy specimens from lesions present for more than five years showed epidermal hyperplasia, diffuse fibrosis, dilated blood vessels, and mild perivascular lymphohistiocytic infiltrates in the dermis. There were a few clumps of small parasites in histiocytes and extracellularly between collagen fibers in the upper dermis. They stained with Giemsa. Blood, tissue biopsy specimen, and saliva and urine samples were used for culture but were negative. A PCR of ITS1 region of the rRNA gene in blood, tissue biopsy specimen, and saliva DNA, followed by DNA sequencing, confirmed the presence of *L. siamensis*. The patient was then confirmed as having cutaneous leishmaniasis. He was severely debilitated and died of systemic bacterial infection without treatment of leishmaniasis.

Patient 6 was a 34-year-old Burmese man from Yangon who was seropositive for HIV for six years. He was treated with truvada, legalon, isoniazid, rifampicin, ethambutal, and pyrazinamide. Five months later, he was hospitalized with high fever and diarrhea. He was empirically treated with

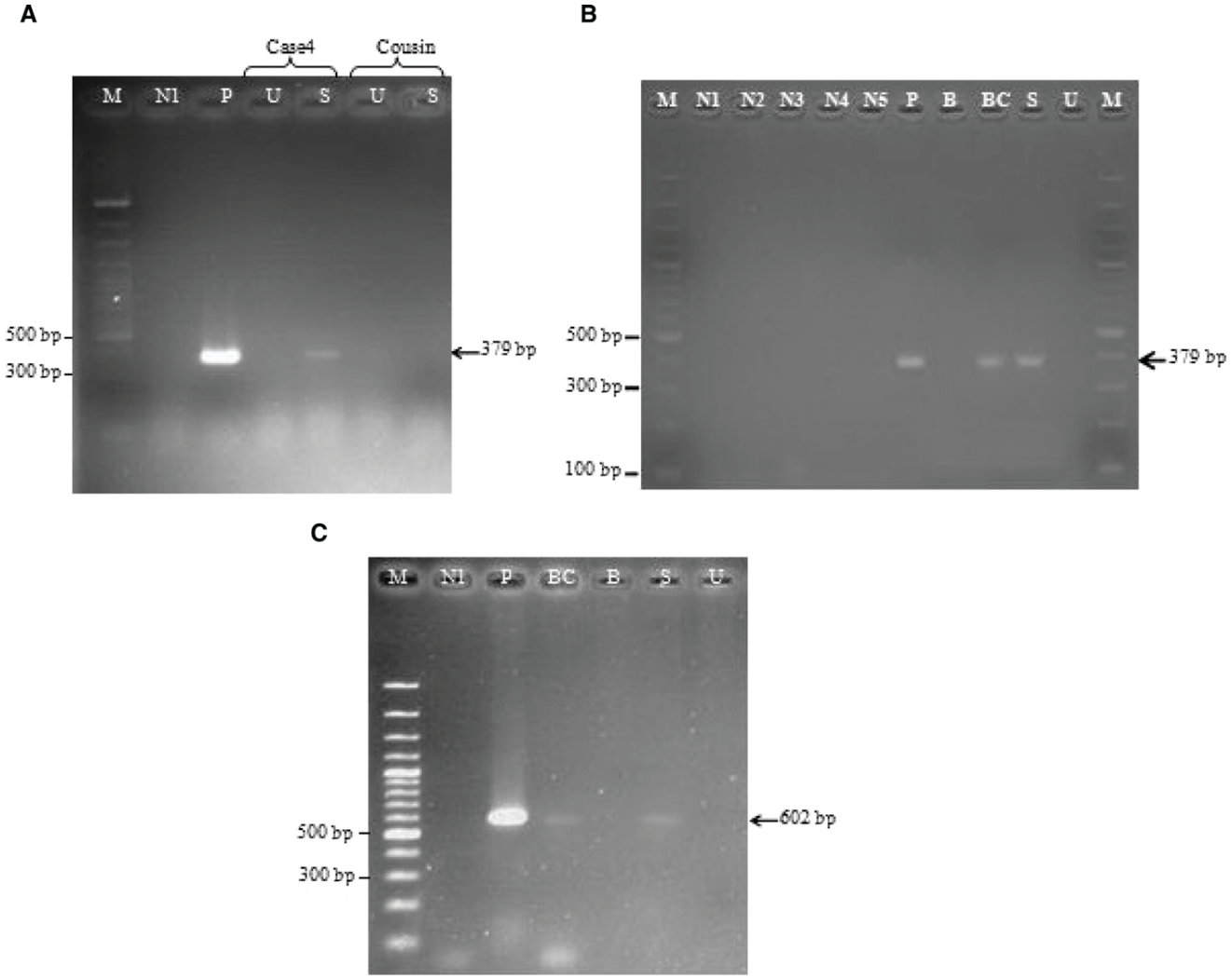

FIGURE 2. Polymerase chain reaction (PCR) amplification of the internal transcribed spacer 1 (ITS1) gene of *Leishmania* parasites of the first saliva and urine samples collected from case 4 and her cousin (**A**) and the second saliva, urine, blood, and buffy coat samples collected from case 4 (**B**), PCR amplification of case 4 by using primers annealed specifically to small subunit ribosomal RNA of *Leishmania* parasites (**C**). Lane B, blood; lane BC, buffy coat; lane S, saliva; lane U, urine; lane M, molecular mass marker (100 basepairs [bp]); lane P, positive control containing DNA from cultured *L. siamensis*; lane N1, negative control (no DNA template: double-distilled water); lanes N2–N5, negative control (DNA template from non-infected saliva, urine, blood, and buffy coat, respectively).

clarithromycin and moxifloxacin. He showed development of multiple, umbilicated, erythematous papules on his neck, arms, and chest wall. Skin biopsy specimens showed a moderately dense superficial and deep perivascular and interstitial histiocytic infiltrate. Many small yeast-like organisms were present in the cytoplasm of histiocytes and between collagen fibers. They stained with PAS and Giemsa, leading to diagnosis of cutaneous leishmaniasis. Blood, tissue, saliva, and urine samples were cultured. A PCR of the ITS1 region of the rRNA gene in blood, skin biopsy specimen, and saliva DNA, followed by DNA sequencing, identified *L. siamensis*. The patient was treated with liposomal amphotericin B and antituberculosis therapy was continued. Clinical recovery resulted within one month.

Specimens were obtained from patients with different clinical presentations of leishmaniasis (Table 1). Five of 6 patients were immunocompromised, only patient 4 was immunocompetent. Amastigotes of *L. siamensis* were detected in bone marrow and blood of patients 1 and 2, and in tissue biopsy specimens of patients 2, 3, 5, and 6 by microscopic examinations. Cultures were positive only for patients 1 and 2 (Table 1). Patients 3, 5, and 6 were treated with antifungal drugs before blood was collected for culture. *Leishmania* parasites in saliva were not detected in any cases by microscopy.

The PCR amplicon used in this study was 379 basepairs (Figure 1A). Nucleotide sequence analysis of all samples identified *L. siamensis* (Figure 1B). In cases where bone marrow or tissue biopsy specimens were available, both types of specimens were also positive by PCR (Table 1 and Figure 1A). Saliva and buffy coats were positive by PCR in all cases. Saliva and urine from an asymptomatic patient (patient 4) and her cousin was used for screening by PCR, but only patient 4 had a positive result (Figure 2A). Saliva and urine samples were collected from patient 4 again when she provided a blood sample, the PCR result was positive for saliva and buffy coat samples (Figure 2B). The PCR was also performed with another set of primers specific for the SSU rRNA gene for *Leishmania* parasite. DNA extracted from saliva and buffy coat of patient 4 was amplified by these primers (Figure 2C), and sequences of the amplified PCR products were 100% identical to the SSU rRNA gene of *L. siamensis* (GenBank accession no GQ226033).

Detection of *L. siamensis* DNA in saliva pre-treatment and post-treatment was performed for patient 1. Blood and saliva were collected three months after treatment, but *L. siamensis* DNA was not detected in blood and saliva samples. Blood and saliva was collected two weeks after treatment from patient 3 and *L. siamensis* DNA was still detected (Figure 3).

Autochthonous leishmaniasis cases caused by *L. siamensis* have been reported in patients in Thailand and Myanmar. The prevalence of this disease has dramatically increased in past few years.[12,14,15] Most cases from Thailand have been reported in the southern region of the country[14], and cases in Myanmar patients were reported in Yangon; these cases were cutaneous, visceral, and asymptomatic cases (unpublished data). In this study, patients (except patient 4) were confirmed by demonstration of the parasites in blood smears, tissue biopsy specimens, or culture. The PCR was used to detect *Leishmania* DNA in bone marrow, blood, buffy coat, tissue, saliva, and urine. *L. siamensis* DNA was detected in the saliva of all 6 patients. Interestingly, in an asymptomatic leishmaniasis patient (patient 4), we were unable to detect

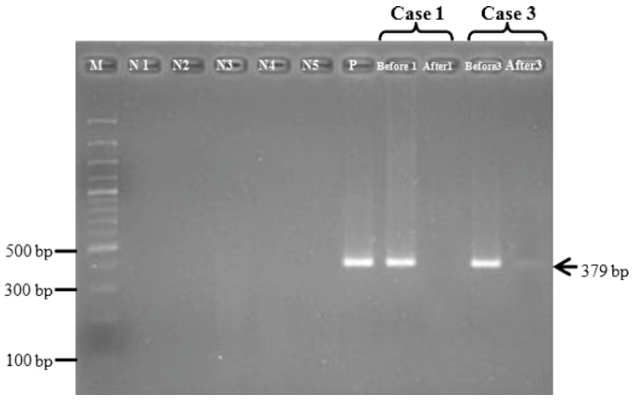

FIGURE 3. Changes in *L. siamensis* DNA in saliva after treatment. Lane M, molecular mass marker (100 basepairs [bp])); lane N1, negative control (no-DNA template: double-distilled water), lanes N2–N5: negative control (DNA template from non-infected saliva, urine, blood, and buffy coat, respectively); lane P, positive control; lane Before1, 3: pre-treatment of case 1 and case 3; lane After 1, 3: post-treatment of case 1 and case 3, respectively.

Leishmania by microscopic examination and culture, but *Leishmania* DNA was detected in saliva and buffy coat. There was only one patient in whom the PCR result was positive. However, this woman was asymptomatic and we could not detect *Leishmania* by other means.

To avoid DNA contamination in the PCR, the PCR were performed with all precautions suggested by Kwok and Higuchi.[23] There are several reports of viable *L. donovani* found in nasal, oral, and nasopharyngeal secretions,[20,24] but *L. siamensis* in this study was not detected in saliva by either microscopic examination or culture. This finding led to inappropriate treatment with antifungal agents before it was confirmed by PCR in buffy coat and saliva. Urine is another source for detection of *L. siamensis* DNA. Although there are several reports of renal involvement in patients with leishmaniasis,[12,25–27] the six patients had no evidence of renal disease. DNA extraction from urine requires 30 mL of urine, and we found that 50% of the patients in our series were negative for *Leishmania* DNA yet positive for DNA in saliva.

In regions in which the incidence of *L. siamensis* infection is low, immunologic diagnostic tests are not readily available. Demonstration of *Leishmania* by microscopic examination is the traditional test for diagnosis. It requires expertise to distinguish *Leishmania* from other pathogens such as *Histoplasma capsulutum* or *Penicillium manefeii*. Culture for *Leishmania* is available only in few laboratories. The current state of the art diagnosis of *L. siamensis* infection relies on PCR and nucleotide sequencing. These techniques are more sensitive than others,[6,9,28–31] and they can now be performed in most provincial and university hospitals in Thailand. Although use of traditional screening tests for this disease is being investigated, PCR could be used for survey and surveillance studies, including asymptomatic persons. Our report demonstrates that saliva is a good source of *L. siamensis* DNA, and that parasite DNA can also be found in asymptomatic patients. Furthermore, in symptomatic patients in whom leishmaniasis is a possibility, multiple studies on different samples by using PCR with sequencing are indicated.

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WOLBACHIA SUPERGROUPS A AND B IN NATURAL POPULATIONS OF MEDICALLY IMPORTANT FILTH FLIES (DIPTERA: MUSCIDAE, CALLIPHORIDAE, AND SARCOPHAGIDAE) IN THAILAND

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Abstract. Filth flies, belonging to suborder Brachycera (Family; Muscidae, Calliphoridae and Sarcophagidae), are a major cause of nuisance and able to transmit pathogens to humans and animals. These insects are distributed worldwide and their populations are increasing especially in sub-tropical and tropical areas. One strategy for controlling insects employs *Wolbachia*, which is a group of maternally inherited intracellular bacteria, found in many insect species. The bacteria can cause reproductive abnormalities in their hosts, such as cytoplasmic incompatibility, feminization, parthenogenesis, and male lethality. In this study we determined *Wolbachia* endosymbionts in natural population of medically important flies (42 females and 9 males) from several geographic regions of Thailand. *Wolbachia* supergroup A or B were detected in 7 of female flies using PCR specific for *wsp*. Sequence analysis of *wsp* showed variations between and within the *Wolbachia* supergroup. Phylogenetics demonstrated that *wsp* is able to diverge between *Wolbachia* supergroup A and B. These data should be useful in future *Wolbachia*-based programs of fly control.

Keywords: *Wolbachia*, fly control, medically important fly, *wsp*

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INTRODUCTION

Filth flies are classified in suborder Brachycera (Family; Muscidae, Calliphoridae and Sarcophagidae). These files are commonly known as house fly, blow fly and flesh fly (Monzon *et al*, 1991). Filth

flies are found worldwide especially in tropical and sub-tropical countries. It can cause a nuisance and transmit pathogens to humans and animals (Greenberg, 1971). Several strategies have been employed for controlling fly populations, including environmental management, biological and insecticidal control, but the fly population continues to increase, especially in sub-tropical and tropical areas.

Insecticides have been the most effective strategy used for fly control, but continuous applications of these chemicals have led to increase in resistance of the insects; moreover it contaminated the environment (Sirisuda *et al*, 2008). An alternative strategy for controlling fly population is biological control. However, traditional biological control using parasitoids or insect predators requires continuous release of these predators into the environment. Recently, *Wolbachia* bacteria from various insect species have investigated for their potential application for biological control of agricultural and medical importance arthropods (Moreira *et al*, 2009). Previous reports have shown that *Wolbachia* strain *w*MelPop isolated from *Drosophila* is able to invade and sustain themselves in the mosquito population and can reduce mosquito lifespan (Kambris *et al*, 2009).

Wolbachia are intracellular maternally inherited bacteria and are classified to Class Alphaproteobacteria, Order Rickettsiales. They are found mainly in arthropods including spiders, terrestrial crustaceans and insects, as well as in filarial nematodes (O'Neill *et al*, 1992; Werren, 1997). *Wolbachia* is classified into 12 supergroups, A-G and H-L, based on the *Wolbachia* surface protein (*wsp*) gene (Ros *et al*, 2009; Ravikumar *et al*, 2010), but the supergroup G-*wsp* is very similar to the sequences of supergroup A and B (Rowley *et al*, 2004; Baldo and Werren, 2007). Supergroup A and B are the most widely distributed in insects (Zhou *et al*, 1998; Ravikumar *et al*, 2010). Recently, *Wolbachia* classified based on 16S rRNA gene sequencing, showed two new *Wolbachia* supergroups M and N in aphids (Augustinos *et al*, 2011). Many studies reported that *Wolbachia* are found at least 20% of all insect species (Werren *et al*, 1995; Werren and Windsor, 2000; Hilgenboecker *et al*, 2008). Relationships between *Wolbachia* and their hosts have many forms ranging from reproductive parasitism to mutualistic symbiosis, which can induce reproductive alterations such as cytoplasmic incompatibility, male lethality, parthenogenesis, and feminization (Rousset *et al*, 1992). Some insect species are unable to produce offspring without *Wolbachia*, viz. bed bug (Hosokawa *et al*, 2010).

In Thailand, information regarding *Wolbachia* endosymbionts in medically important filth flies (Diptera: Muscidae, Calliphoridae, and Sarcophagidae) has never been investigated. In this study, we present preliminary data of *Wolbachia* infection in these medically important flies from different geographical populations in Thailand is not available using PCR-based detection of *wsp*.

MATERIALS AND METHODS

Fly collection and identification

Fifty-one adult fly samples were collected from various regions of Thailand: 9 from Chiang Mai (northern Thailand), 4 from Bangkok (central), 3 from Prajuab Kirikhan (central), 1 from Nakhon Ratchasima (north eastern), 4 from Nong Khai (north eastern), 20 from Phuket (southern), 6 from Ranong (southern), and 4 from Satun (southern) (Table 1).

Collected samples were identified based on morphological characteristics and molecular techniques as described by Preativatanyou *et al* (2010) and Bhakdeenuan *et al* (2012).

PCR amplification of wsp

Genomic DNA was isolated using Invisorb® Spin Tissue Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions. Each fly sample was ground in 200 µl of lysis buffer using sterile plastic pestle. Extracted DNA was stored in 50 µl of elution buffer, concentration determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Singapore) and kept at -20ºC until used.

Primers used were 136AF (5'-TGAAATTTTACCTCTTTTC- 3') and 691AR (5-'AAAAATTAAACGCTACTCCA-3') for amplification of *Wolbachia* supergroup A (550 bp) and primers 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and 522 R (5'-ACCAGCTTTTGCTTGATA-3') for supergroup B (450 bp) (Zhou *et al*, 1998). PCR, in a final volume of 25 µl, contained 100 ng of DNA, 10 µM each primer, 2.5 mM $MgCl_2$, 2 mM dNTPs and 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Thermocycling (Veriti; Applied Biosystems, Carlsbad, CA) conditions were as follows: 95ºC for 3 minutes; 40 cycles of 95ºC for 30 seconds, 50ºC for 30 seconds, and 72ºC for 30 seconds; and a final step at 72ºC for 7 minutes. Double-distilled water was used as negative control and DNA from bed bug was positive control. Amplicons were analyzed by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and recorded using Gel Doc EQ Quantity One quantification analysis software version 4.5.2 system (Bio-Rad, Hercules, CA).

Cloning and sequencing of *wsp* amplicons

PCR amplicons were ligated into pGEM-T Easy Vector (Promega, Madison, WI) and the recombinant plasmids were used to transform competent *Escherichia coli* DH5± strain. Transformed cells were cultured and recombinant plasmids were extracted using Invisorb® Spin Plasmid Mini kit (STRATEC Molecular, Berlin, Germany) following the manufacturer's instructions. Plasmids were sequenced by 1st Base Laboratories, Malaysia.

Sequence analysis and phylogenetic tree construction

Nucleotide sequences were analyzed by comparison with available sequence data in GenBank using BLAST search (http://www.ncbi.nlm.gov/BLAST). The nucleotide sequences generated in this study have been deposited in GenBank (Table 2). Sequences were aligned and the percentage of sequence similarity was calculated using BioEdit Sequence Alignment Editor Version 7.1.9 (Hall, 1999). Phylogenetic tree was constructed by Neighbor-joining method using Kimura's 2-parameter model implemented in MEGA© version 5.1 (Tamura *et al*, 2011). The reliability of an inferred tree was tested by 1000 bootstraps using *Wolbachia* endosymbiont of *Aprostocetus* spp accession no. HQ121415 as an outgroup.

RESULTS

Filth flies used in this study belonged to 3 families, namely, Muscidae, Calliphoridae and Sarcophagidae, and 15 fly species were identified using morphology and molecular techniques (Table 1). PCR-based detection of *wsp* showed *Wolbachia* in 7/51 (14%) of the collected samples (Table 2). No co-infection of *Wolbachia* supergroups A and B was detected (Fig 1). There were 4 samples with *Wolbachia*

Table 1
Flies collected from various regions of Thailand.

Family	Species	Province	Region of Thailand	Sex		Total (no.)
				Female (no.)	Male (no.)	
Muscidae	*Hydrotaea spinigera*	Chiang Mai	Northern	1	1	2
	Musca domestica	Ranong	Southern	2	1	3
		Phuket		1	0	1
		Satun		2	0	2
		Nong Khai	North easthern	1	0	1
	M. sorbens	Nong Khai	North easthern	2	0	2
		Bangkok	Central	1	0	1
		Phuket	Southern	3	0	3
Calliphoridae	*Chrysomya chani*	Chiang Mai	Northern	1	1	2
		Phuket	Southern	2	0	2
	C. megacephala	Prajuab Kiri Khan	Southern	2	0	2
		Phuket		1	0	1
		Ranong		1	1	2
		Satun		0	2	2
	C. pinguis	Chiang Mai	Northern	1	0	1
	Hemipyrellia ligurriens	Phuket	Southern	1	0	1
	H. pulchra	Chiang Mai	Northern	2	0	2
		Phuket	Southern	1	0	1
	Lucilia cuprina	Phuket	Southern	3	2	5
	L. porphyrina	Chiang Mai	Northern	1	0	1
Sarcophagidae	*Sarcophaga dux*	Bangkok	Central	2	0	2
		Prajuab Kiri Khan		1	0	1
		Phuket	Southern	4	0	4
	S. indica	Nakhon Ratchasima	Southern	1	0	1
	S. peregrina	Chiang Mai	Northern	1	0	1
	S. ruficornis	Nong Khai	North easthern	0	1	1
		Bangkok	Central	1	0	1
		Phuket	Southern	2	0	2
	S. scopariiformis	Ranong	Southern	1	0	1
Total				42	9	51

supergroup A, consisting of a female *M. sorbens* and *S. dux* from Bangkok, a female *C. megacephala* from Phuket, and a female *H. pulchra* from Chiang Mai (Fig 1A), whereas *Wolbachia* supergroup B were detected in 3 samples, consisting of a female *M. domestica* and *S. scopariiformis* from Ranong and a female *C. megacephala* from Prajuab Kiri Khan (Fig 1B).

Analysis of the mean G+C content of the partial *wsp* sequences revealed 37.90% of supergroup A and 35.50% of supergroup B. Both *Wolbachia* supergroup A and B partial *wsp* sequences did not show intra-variation of nucleotide sequences, but inter-variations among *Wolbachia* supergroup A showed the inter-species variation between *H. pulchra* from Chi-

Table 2
Wolbachia supergroup A and B in flies.

Species	Location	Sex	Supergroup	Clone no.	Code	GenBank accession no.
H. pulchra	Chiang Mai	Female	A	1	CM62_1	KC668275
H. pulchra	Chiang Mai	Female	A	3	CM62_3	KC668276
H. pulchra	Chiang Mai	Female	A	5	CM62_5	KC668277
M. sorbens	Bangkok	Female	A	1	BK7_1	KC668284
M. sorbens	Bangkok	Female	A	2	BK7_2	KC668285
M. sorbens	Bangkok	Female	A	3	BK7_3	KC668286
S. dux	Bangkok	Female	A	1	BK8_1	KC668278
S. dux	Bangkok	Female	A	2	BK8_2	KC668279
S. dux	Bangkok	Female	A	3	BK8_3	KC668280
C. megacephala	Phuket	Female	A	1	PK26_1	KC668281
C. megacephala	Phuket	Female	A	2	PK26_2	KC668282
C. megacephala	Phuket	Female	A	3	PK26_3	KC668283
S. scopariiformis	Ranong	Female	B	6	RN4_6	KC668287
S. scopariiformis	Ranong	Female	B	7	RN4_7	KC668288
S. scopariiformis	Ranong	Female	B	9	RN4_9	KC668289
M. domestica	Ranong	Female	B	1	RN8_1	KC668290
M. domestica	Ranong	Female	B	2	RN8_2	KC668291
M. domestica	Ranong	Female	B	3	RN8_3	KC668292
C. megacephala	Prajuab Kiri Khan	Female	B	1	PJ9_1	KC668293
C. megacephala	Prajuab Kiri Khan	Female	B	2	PJ9_3	KC668294
C. megacephala	Prajuab Kiri Khan	Female	B	3	PJ9_5	KC668295

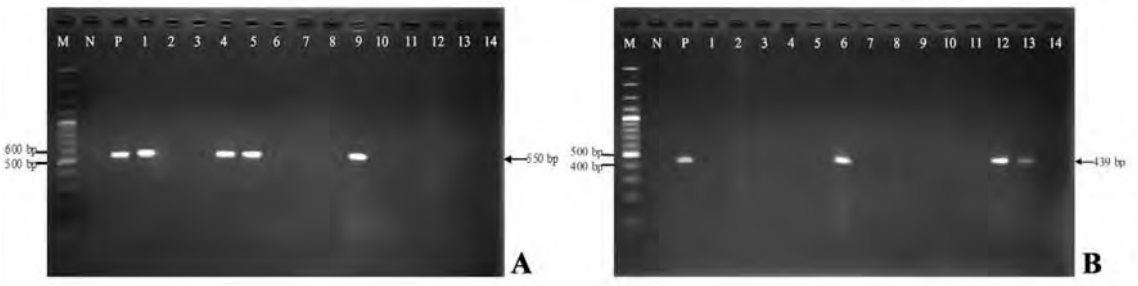

Fig 1–PCR amplicons of *wsp* specific to *Wolbachia* supergroup A (A) and supergroup B (B). Primers used and PCR condition are described in Materials and Methods. Lane M: 100 bp molecular weight markers, lane N: negative control, lane P: positive control *Aedes albopictus*, lane 1: *H. pulchra* from Chiang Mai (CM62), lane 2: *L. porphyrina* from Chiang Mai (CM95), lane 3: *S. ruficornis* from Nong Khai (NK 11), lane 4: *M. sorbens* from Bangkok (BK7), lane 5: *S. dux* from Bangkok (BK8), lane 6: *C. megacephala* from Prajuab Kiri Khan (PJ9), lane 7: *S. dux* from Prajuab Kiri Khan (PJ 15), lane 8: *M. domestica* from Phuket (PK5), lane 9: *C. megacephala* from Phuket (PK26), lane 10: *S. ruficornis* from Phuket (PK42), lane 11: *L. cuprina* from Phuket (PK61), lane 12: *S. scopariiformis* from Ranong (RN4), lane 13: *M. domestica* from Ranong (RN8), lane 14: *C. megacephala* from Ranong (RN15).

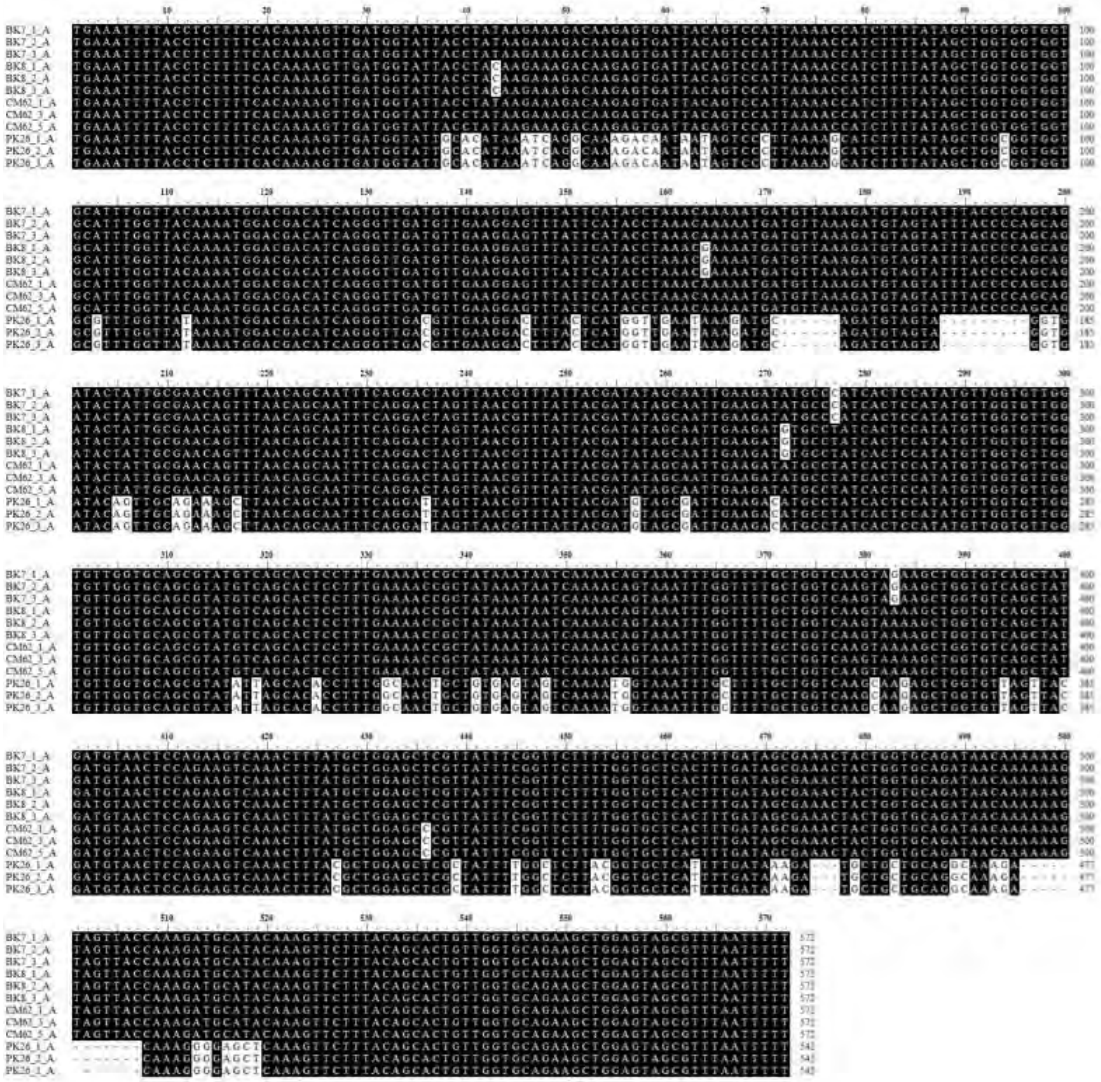

Fig 2–Comparison of partial *wsp* sequences of *Wolbachia* supergroup A from various sources of flies. *M. sorbens* from Bangkok (BK7)_1,_2, and _3 clones; *S. dux* from Bangkok (BK8)_1, _2, and _3 clones; *H. pulchra* from Chiang Mai (CM62)_1, _3, and _5 clones; *C. megacephala* from Phuket (PK26)_ 1, _2, and _3 clones.

ang Mai (CM62) and *C. megacephala* from Phuket (PK26) (20%), *S. dux* from Bangkok (BK8) (0.9%), and *M. sorbens* from Bangkok (BK7) (0.6%) (Fig 2). However, *Wolbachia* supergroup B did not show inter-variation in all positive isolates (Fig 3). Phylogenetic tree, constructed basing on the sequences obtained and that of *Wolbachia* sequence from *Aprostocetus* spp as outgroup clearly indicated that *Wolbachia* endosymbionts of flies in Thailand were divided into two major clades, with significant differences between supergroup A and B. *Wolbachia* supergroup A of *C. megacephala* from Phuket (PK26) was isolated from other supergroup A samples,

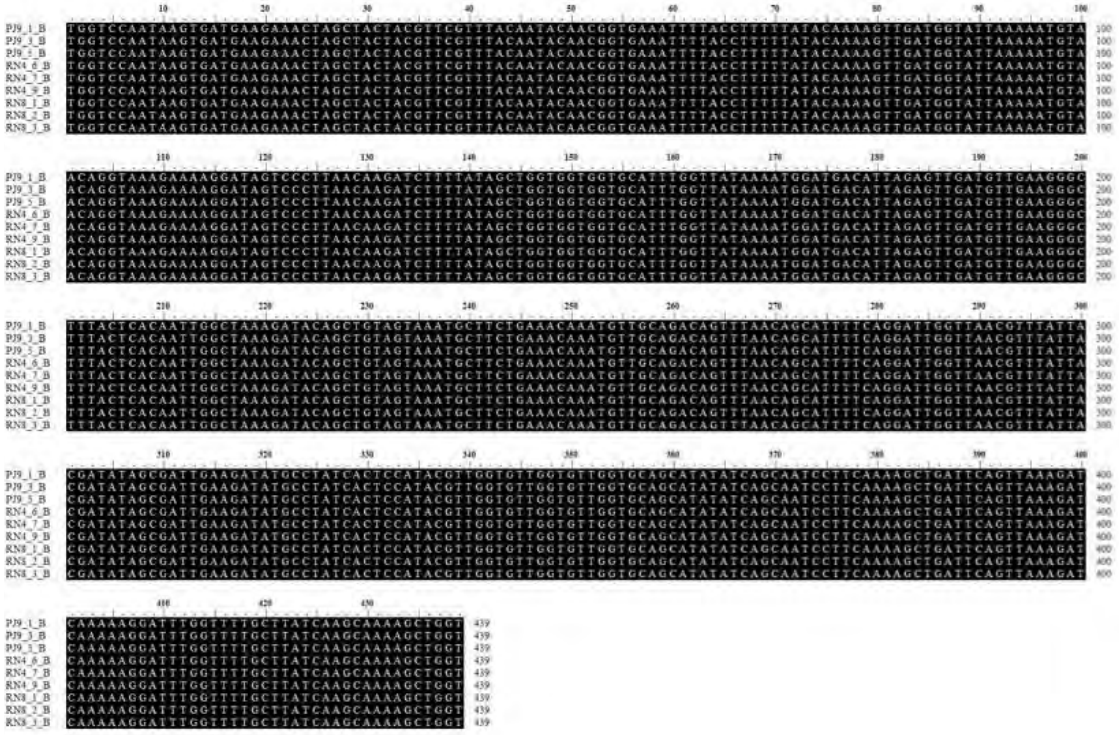

Fig 3–Comparison of partial *wsp* sequences of *Wolbachia* supergroup B from various sources of flies. *C. megacephala* from Prajuab Kiri Khan (PJ9)_1, _3, and _5 clones; *S. scopariiformis* from Ranong (RN4)_6, _7, and _9 clones; *M. domestica* from Ranong (RN8)_1, _2, and _3 clones.

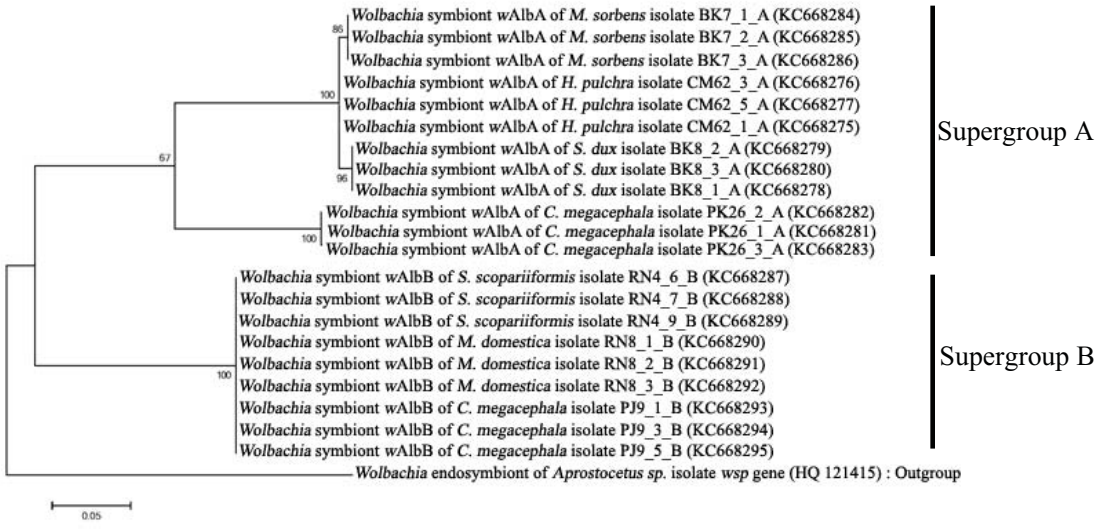

Fig 4–Phylogenetic tree of *Wolbachia* constructed from partial *wsp* sequences. Three isolates of each sample and *Wolbachia* endosymbiont of *Aprotocetus* spp as an outgroup were employed.

but *Wolbachia* supergroup B showed no differences among samples from various geographic areas and fly species (Fig 4).

DISCUSSION

This is the first survey study of *Wolbachia* endosymbionts in natural populations of medically important filth flies (Diptera: Muscidae, Calliphoridae, and Sarcophagidae) in several geographic regions of Thailand. The most common *Wolbachia* endosymbionts found in arthropods are classified into supergroup A and supergroup B (Zhou *et al*, 1998; Ravikumar *et al*, 2010). *Wolbachia* endosymbionts were detected in 14% of the samples, all being females with no specific host species or geographical regions. *Wolbachia* supergroup A was not specifically associated with any fly species or geographical regions, but supergroup B was found mostly in southern region of Thailand and there are no co-infections of *Wolbachia* supergroups A and B in any single fly sample. Baudry *et al* (2003) reported in the bird nest blow fly, *Protocalliphora sialia*, that *Wolbachia* endosymbionts in western North America are composed of two types of A supergroup, with some flies being singly and others doubly infected, but in eastern North America mostly of the flies are infected with only the B supergroup with both supergroups present in the Midwest. However it is not yet known whether the bacteria induce cytoplasmic incompatibility or other phenotypes in *Protocalliphora* (Baudry *et al*, 2003; Whitworth *et al*, 2007).

Marker genes used for detecting *Wolbachia* include ribosomal gene (16S rDNA) and a regulatory gene of the bacterial cell cycle (*ftsZ*) (Holden *et al*, 1993; Braig *et al*, 1998). PCR amplification was performed using *wsp*, which is capable of classifying *Wolbachia* into supergroups A and B (Zhou *et al*, 1998; Ravikumar *et al*, 2010).

Sequence analysis showed only slightly difference of mean G+C content between *Wolbachia* supergroup A and B. The low G+C content results are similar to those of *wsp* genes from other insects belonging to order Diptera, such as *Drosophila paulistorum* (mean G+C content of 38.03%) (Miller *et al*, 2010). There was 100% intra-specific similarity of the partial *wsp* sequences but 80-100% of inter-specific similarity of each supergroup. Phylogenetic analysis showed significant difference between *Wolbachia* supergroups. The NJ tree based on *wsp* was able to separate *Wolbachia* supergroups A and B in filth fly samples of this study. Moreover, *Wolbachia* supergroup A of the *C. megacephala* from Phuket (PK26) was clearly separated from other A supergroup. *Wolbachia* supergroup A of *S. dux* from Bangkok (BK8), *M. sorbens* from Bangkok (BK7), and *H. pulchra* from Chiang Mai (CM62) showed monophyletic clade and minor nucleotide variations in different regions and host. All *Wolbachia* supergroup B isolates clustered together and did not show differentiation between geographical regions and hosts.

This study is a preliminary survey of *Wolbachia* in medically important flies in Thailand. Extensive survey of *Wolbachia* infection in flies covering more areas of the country would provide valuable data for developing an effective *Wolbachia*-based fly control strategy.

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ORIGINAL PAPER

Scanning electron microscopy of *Anopheles hyrcanus* group (Diptera: Culicidae) eggs in Thailand and an ultrastructural key for species identification

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Abstract The eggs of *Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nigerrimus*, *Anopheles nitidus*, *Anopheles paraliae*, *Anopheles peditaeniatus*, *Anopheles pursati*, and *Anopheles sinensis* are described with the aid of scanning electron micrographs. Comparisons of the egg structure among the eight species showed that the eggs differed with respect to the following characteristics: the deck—complete (*An. argyropus*, *An. nigerrimus*, *An. paraliae*, *An. peditaeniatus*, and *An. sinensis*); variable (complete, split and incomplete decks found together within an egg batch/*An. crawfordi*); and division into an area at each end (*An. nitidus* and *An. pursati*). The ratios of the entire length per maximal deck width within the area covered by floats were 3.33–6.86 (*An. sinensis*), 8.78–18.20 (*An. peditaeniatus*), 13.67–22 (*An. nigerrimus*), 26.33–44.25 (*An. paraliae*), and 26.99–75.94 (*An. argyropus*). The numbers of float ribs were 21–27 (*An. peditaeniatus*) and 28–34 (*An. nigerrimus*), and the total numbers of anterior and posterior tubercles were 6–8 (*An. paraliae*) and 9–11 (*An. argyropus*). Exochorionic sculpturing was of reticulum type (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, and *An. sinensis*) and pure tubercle type (*An. pursati*). Attempts are proposed to construct a robust key for species identification based on the morphometrics and ultrastructures of eggs under scanning electron microscopy.

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Introduction

Anopheles (*Anopheles*) in the Hyrcanus Group comprises at least 27 species, which are distributed widely in the Oriental and Palearctic Regions, and several species of this group have been implicated as vectors of malaria, lymphatic filariasis, and Japanese encephalitis virus (Reid 1968; Rattanarithikul et al. 2006; Paredes-Esquivel et al. 2011; Harbach 2013).

In Thailand, eight species (*Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nigerrimus*, *Anopheles nitidus*, *Anopheles paraliae*, *Anopheles peditaeniatus*, *Anopheles pursati*, and *Anopheles sinensis*) of the Hyrcanus Group have been reported so far, with some of them being recorded also in the East, South, and other Southeast Asian countries (Harrison and Scanlon 1975; Rattanarithikul et al. 2006). Interestingly, *An. paraliae* has been considered recently as a synonymous species of *Anopheles lesteri* based on genetic compatibility from crossing experiments and very low intraspecific sequence variations of ribosomal (ITS2) and mitochondrial (COI and COII) DNA regions (Taai et al. 2013; Harbach 2013). Regarding medical importance, *An. nigerrimus*, *An. peditaeniatus*, and *An. sinensis* are considered as suspected vectors of malaria due to *Plasmodium vivax* (Baker et al. 1987;

Harbach et al. 1987; Gingrich et al. 1990; Frances et al. 1996; Rattanarithikul et al. 1996), while *An. sinensis* has been incriminated as a vector of *P. vivax* in China and Korea (Liu 1990; Lee et al. 2007) and *Plasmodium malariae* in Vietnam (Manh et al. 2010). Also, *An. peditaeniatus* is an incriminated vector of Japanese encephalitis virus in China and India (Mourya et al. 1989; Zhang 1990; Kanojia et al. 2003). In addition, *An. sinensis* and *An. nigerrimus* have been incriminated as a main vector and secondary or incidental vector, respectively, of *Wuchereria bancrofti* in Asia (Manguin et al. 2010). Recently, *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus*, and *An. pursati* have been reported as high potential vectors of *Brugia malayi* in Thailand (Saeung et al. 2013). Likewise, the eight species of the *Anopheles hyrcanus* group were considered also as an economic pest of cattle because of their vicious biting behavior and ability to transmit cervid filariae of the genus *Setaria* (Reid 1968; Harrison and Scanlon 1975).

The egg surface morphology of *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati*, and *An. sinensis* has been studied using light microscopy (Reid 1968). However, detailed description of egg surface topography under scanning electron microscopy (SEM) has been given only for *An. nigerrimus*, *An. paraliae*, *An. peditaeniatus*, and *An. sinensis* (Linley et al. 1995; Rueda et al. 2009). In view of the medical and veterinary importance of these eight anopheline species, this study aimed to describe the surface topography of their eggs more precisely and systematically and, in particular, construct a robust ultrastructural key for species identification under SEM. These descriptions are presented below.

Materials and methods

Mosquito species and locations

Eight mosquito species members of the Hyrcanus Group were collected in five provinces of western and southern Thailand, where malaria and filariasis are endemic due to *Plasmodium falciparum*, *P. vivax*, and *W. bancrofti*, respectively (Manguin et al. 2010; BVBD 2012). The province and species were as follows: (1) western region—*An. paraliae* and *An. pursati* (Ratchaburi Province; 13°30′ N, 99°54′ E) and (2) southern region—*An. argyropus* (Nakhon Si Thammarat Province; 08°29′ N, 100°0′ E), *An. crawfordi* (Trang Province; 07°33′ N, 99°38′ E), *An. nigerrimus* (Songkhla Province; 07°13′ N, 100°37′ E), *An. nitidus* and *An. peditaeniatus* (Phang Nga Province; 08°27′ N, 98°31′ E), and *An. sinensis* (Chumphon Province; 10°29′ N, 99°11′ E). Wild-caught, fully engorged females of these species members were collected from cow-baited traps.

Species identification and egg laying

Only the intact morphology of wild-caught, fully engorged female mosquitoes was used to perform species identification by following the standard illustrated keys of Rattanarithikul et al. (2006). After becoming gravid, the mosquitos were allowed to lay eggs individually in order to obtain an egg batch from each iso-female line using the techniques described by Choochote and Saeung (2013). To guarantee precise morphological species identification, feral egg-laid females from each iso-female line were used for molecular identification using the methods described by Hempolchom et al. (2013).

Scanning electron microscopy

Embryonated eggs or 36-h-old oviposited eggs of the eight species (five egg batches from each species) were placed in 2.5 % glutaraldehyde in phosphate buffer (PB, pH 7.4) at 4 °C for 24 h. Then, they were washed twice with PB at 10-min intervals, post-fixed in 1 % osmium tetroxide at room temperature for 1 h, and washed twice with PB at 10-min intervals. Subsequently, they were dehydrated by passing through an ethanol series of 35, 70, 80 % (10 min each), and 95 % (15 min, with two changes), followed by absolute ethanol (10 min, with two changes). Finally, they were dried with a critical point dryer, mounted on aluminum stubs, sputter-coated with gold, and viewed at 15 kV under a JEOL-JSM6610LV scanning electron microscope (Japan) (Junkum et al. 2004). The terms of morphological features used in this study followed those of Reid (1968) and Hinton (1968). Dimensions of the eggs (entire length, width including floats, maximal deck width within the area covered by floats; Fig. 1); numbers of anterior and posterior tubercles; numbers of float ribs; and the egg surface features of each species were given as means ± SD (range) based on ten samples of eggs (two eggs from each egg batch of five egg-laid iso-female lines).

Results

General morphological features

General morphological features and exochorionic sculpturing on eight species of the Thai Hyrcanus Group were generally similar in appearance. The eggs were boat-shaped, with the anterior or head end somewhat broader (Fig. 2a–d). Viewed laterally, the contour of the entire egg was more or less concave and, on occasions, slightly convex on the morphologically dorsal surface and convex on the ventral surface. In the middle region, each of the lateral sides was documented by a float, which was a folded longitudinal extension of the outer

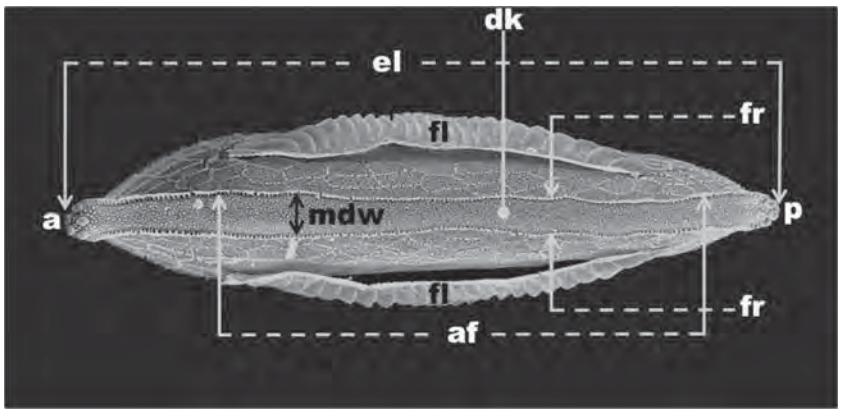

Fig. 1 Whole egg. Dorsal aspect of *An. peditaeniatus* (×180) showing measurement of morphometric parts. *a* anterior end, *p* posterior end, *el* entire length, *af* area covered by floats, *mdw* maximal deck width within the area covered by floats, *fl* float, *fr* frill, *dk* deck

chorionic sculpturing that consisted of approximately 21–34 ribs and separated by deep clefts. Viewed dorsally, there was a bare area known as the deck on the dorsal surface, which was surrounded by the two longitudinal bands of a sclerotized ridge-like texture (frill; Fig. 1). The deck could be complete (continuous for the entire length of the egg; Figs. 3a–e and 4a), incomplete (Fig. 4b, c), or divided into an area at each end (Fig. 4d, e). The inner surface of the frill was of a sclerotized, ridged-like texture and marked by picket-like ribs (Fig. 5a); the outer surface was smooth with a parallel brick-like texture along its entire length (Fig. 5b). Large-lobed tubercles on the dorsal surface of the egg ranged from 2 to 11 and from 2 to 8 in numbers at the anterior and posterior ends, respectively. They were rosette-shaped, giving rise to four to nine lateral lobes, and surrounded by a sclerotized ridge and raised border (Fig. 5c, d). The tubercles on either the deck or in areas covered by floats (observed from detached float specimens) were jagged irregularly and surrounded by other much smaller, irregular tubercles (Fig. 5e–g). The micropylar disc could be seen clearly down toward the anterior end (Fig. 5h) and was surrounded by a smooth collar, which had an irregular outer margin and five to seven spurs that extended radially toward the central orifice. One small central knob was seen clearly in unfertilized eggs (Fig. 5i). Two types of exochorionic sculpturing depended upon the species, i.e., (1) the reticulum type (Fig. 6a, b), which appeared as a more or less regular or irregular pentagonal or hexagonal reticulum formed by rows of prominent tubercles enclosing many smaller, less prominent and irregular base tubercles, which formed bonds with each other, and (2) the pure tubercle type (Fig. 6c, d), with tubercles seen all over the eggs that had an irregular base and less prominent surface, and they were arranged as an irregular cluster by forming bonds with each other. The non-forming bond tubercles resulted in the presence of irregular spaces, thus illustrating the outer chorionic sculpturing with cracking appearance.

Specific characteristics of each species

Species with complete decks

An. sinensis The deck width was extensive, with a scant area between the deck and floats (Fig. 3a). Dimensions: the entire length was 492.22±37.97 μm (400–533.33 μm), the width including the floats was 192.22±8.76 μm (172.22–200 μm), maximal deck width within the area covered by floats was

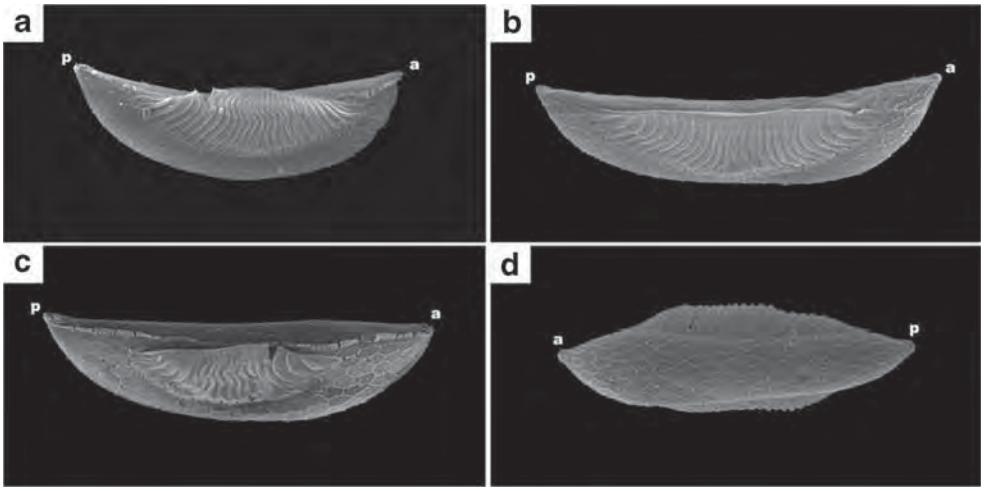

Fig. 2 Whole eggs. Lateral aspect (×180): **a** *An. sinensis*. **b** *An. peditaeniatus*. **c** *An. sinensis*. Ventral aspect: **d** *An. crawfordi*

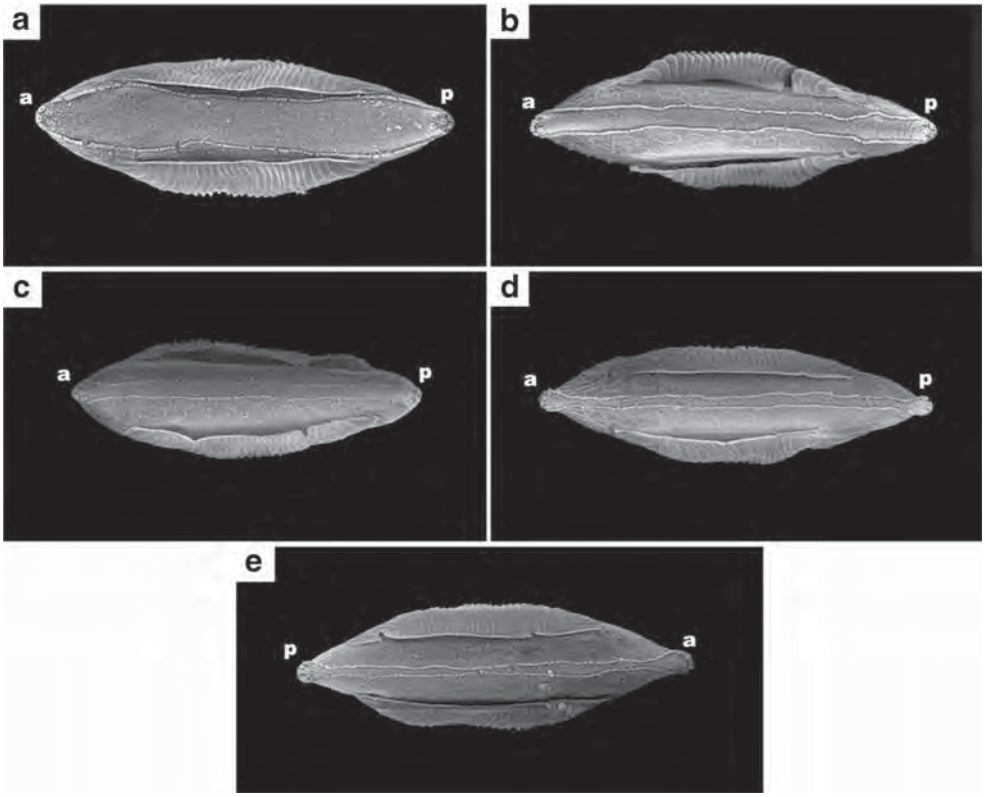

Fig. 3 Whole eggs. Dorsal aspects (×180). Complete deck: **a** *An. sinensis*. **b** *An. peditaeniatus*. **c** *An. nigerrimus*. **d** *An. paraliae*. **e** *An. argyropus*

112.78±25.67 μm (77.78–150 μm), and the entire length-to-maximal deck width ratio was 4.59±1.20 (3.33–6.86). Counts: the number of float ribs was 25.10±3.35 (21–30), of anterior tubercles was 8.20±1.32 (7–11), and of posterior

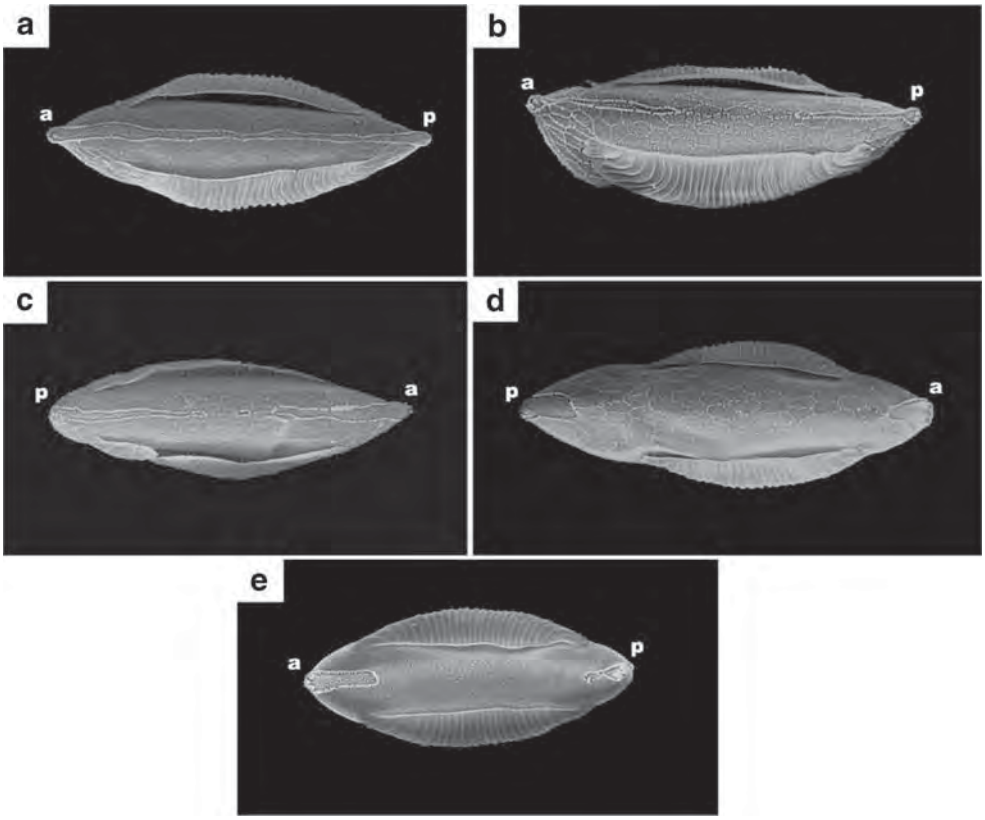

Fig. 4 Whole eggs. Dorsal aspects (×180). *An. crawfordi*: **a** Complete deck. **b** Split deck. **c** Incomplete deck. Deck was divided in the areas at each end of the eggs: **d** *An. nitidus*. **e** *An. pursati*

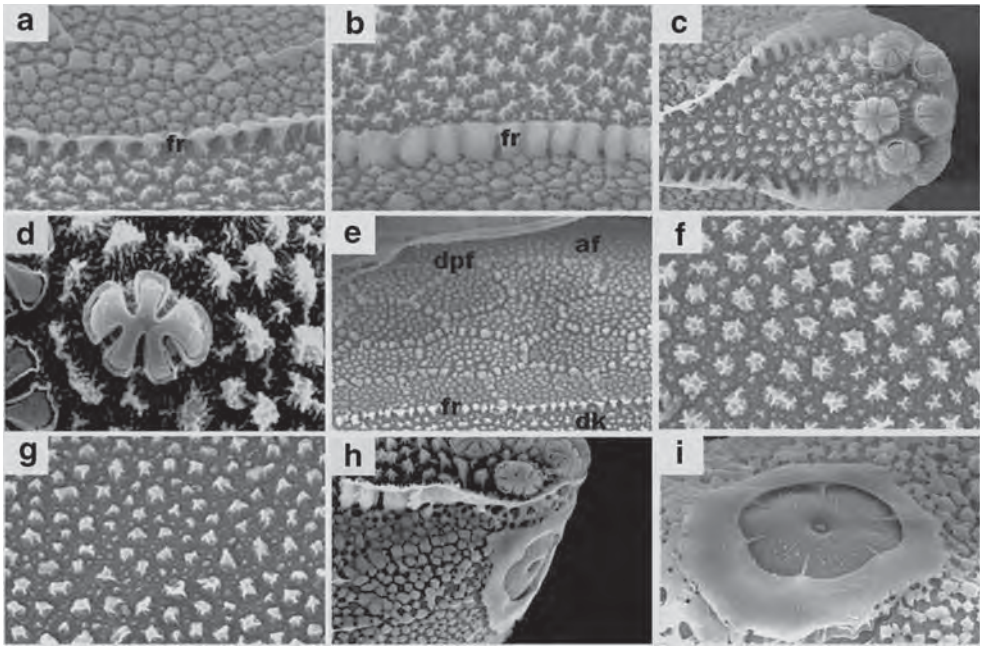

Fig. 5 *An. nigerrimus*: **a** Inner surface of the frill (*fr*; ×4,000). **b** Outer surface of the frill (*fr*; ×4,000). *An. nitidus*: **c** Anterior end, showing six large, rosette-shaped tubercles (×1,500). **d** Higher magnification of the large, rosette-shaped tubercle (×5,500). *An. peditaeniatus*: **e** Irregularly jagged tubercles on the deck (*dk*) and area covered by the float (*af*) as well as the reticulum type of exochrorionic sculpturing between the frill (*fr*) and detachment point of the float (*dpf*; ×1,400). **f** Higher magnification of the irregularly jagged tubercles on the deck (×5,500). **g** Higher magnification of the irregularly jagged tubercles on the area covered by the float (×5,500). *An. paraliae*: **h** Micropylar disc show down toward the anterior end (×1,500). **i** Higher magnification of the micropylar disc (×3,000)

tubercles was 6.30±1.06 (5–8), with the total number of anterior and posterior tubercles being 14.50±1.84 (13–19). Exochorionic sculpturing: the reticulum type measured approximately 12.43×30.99 μm and was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats.

An. peditaeniatus The deck was of tolerable width, with a fairly moderate area between the deck and float (Fig. 3b). Dimensions: the entire length was 508.61±29.70 μm (438.88–550 μm), the width including the floats was 163.94 ±20.66 μm (138.88–200 μm), maximal deck width within the area covered by floats was 45.57±8.61 μm (27.78–55.56 μm), and the entire length-to-maximal deck width ratio was 12.03±2.74 (8.78–18.20). Counts: the number of float ribs was 23.70±1.89 (21–27), of anterior tubercles was 5.90±0.74 (5–7), and of posterior tubercles was 5.10±0.88 (4–6), with the total number of anterior and posterior tubercles being 11±1.49 (9–13). Exochorionic sculpturing: the reticulum type measured approximately 17.13×26.88 μm and was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats.

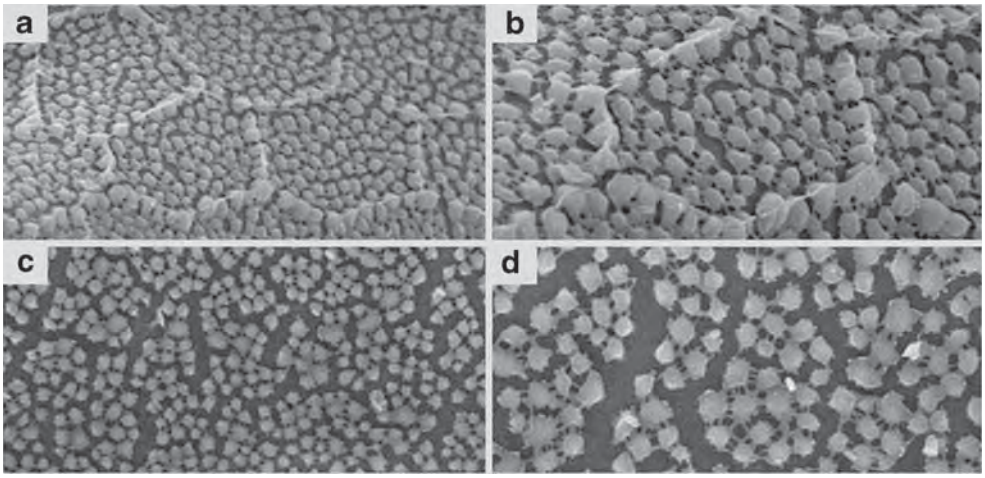

Fig. 6 *An. sinensis*: **a** Reticulum type of exochrorionic sculpturing of the ventral aspect of the egg at the middle region (×1,400). **b** Higher magnification of the reticulum type of exochrorionic sculpturing (×2,500). *An. pursati*: **c** Tubercle type of exochrorionic sculpturing of the ventral aspect of the egg at the middle region (×1,400). **d** Higher magnification of the tubercle type of exochrorionic sculpturing (×2,500)

An. nigerrimus The deck was of tolerable width, with a fairly moderate area between the deck and floats (Fig. 3c). Dimensions: the entire length was 469.44±35.74 μm (400–522.22 μm), the width including the floats was 166.11±12.95 μm (144.44–183.33 μm), maximal deck width within the area covered by floats was 27.78±4.54 μm (22.22–33.33 μm), and the entire length-to-maximal deck width ratio was 17.21±2.39 (13.67–22). Counts: the number of float ribs was 31.60±1.58 (28–34), of anterior tubercles was 6.10±0.74 (5–7), and of posterior tubercles was 5±0.82 (4–6), with the total number of anterior and posterior tubercles being 11.10±1.29 (9–13). Exochorionic sculpturing: the reticulum type measured approximately 17.50×27.80 μm and was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats. Remarkably, some specimens had sparse reticulum on the area between the deck and float.

An. paraliae The deck narrowed, with a broad area between the deck and float (Fig. 3d). Dimensions: the entire length was 463.06±23.06 μm (433.33–491.67 μm), the width including the floats was 161.39±9.84 μm (144.44–175 μm), maximal deck width within the area covered by floats was 12.22±2.34 μm (11.11–16.67 μm), and the entire length-to-maximal deck width ratio was 38.99±6.69 (26.33–44.25). Counts: the number of float ribs was 25.80±2.44 (23–31), of anterior tubercles was 3.30±0.48 (3–4), and of posterior tubercles was 3.60±0.52 (3–4), with the total number of anterior and posterior tubercles being 6.90±0.88 (6–8). Exochorionic sculpturing: the reticulum type measured approximately 17.50×25.63 μm and was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats. Remarkably, most of the specimens had sparse reticulum on the area between the deck and floats.

An. argyropus The deck narrowed, with a broad area between the deck and floats (Fig. 3e). Dimensions: the entire length was 459.17±23.64 μm (422.22–488.89 μm), the width including the floats was 165±5.86 μm (155.56–177.78 μm), maximal deck width within the area covered by floats was 9.99±2.99 μm (5.56–16.67 μm), and the entire length-to-maximal deck width ratio was 49.33±13.41 (26.99–75.94). Counts: the number of float ribs was 29.80±1.23 (28–32), of anterior tubercles was 5.70±0.82 (5–7), and of posterior tubercles was 4.30±0.48 (4–5), with the total number of anterior and posterior tubercles being 10±0.94 (9–11). Exochorionic sculpturing: the reticulum type measured approximately 17.55×30.72 μm and was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats. Remarkably, most of the specimens had sparse reticulum on the area between the deck and floats.

Species with variable decks

An. crawfordi The deck narrowed, with a broad area between the deck and floats. Complete, split, and incomplete decks were found together within an egg batch from each egg-laid iso-female line. Notably, the complete deck type could be found in more numbers than the other two types (Fig. 4a–c). Dimensions: the entire length was 460.28±26.52 μm (422.22–494.44 μm), the width including the floats was 201.11±33.11 μm (166.67–272.22 μm), maximal deck width within the area covered by floats was 8.06±3.81 μm (5.56–16.67 μm), and the entire length-to-maximal deck width ratio was 66.32±23.22 (29.33–88.93). Counts: the number of float ribs was 28.00±2.31 (26–31), of anterior tubercles was 3.10±0.57 (2–4), and of posterior tubercles was 2.70±0.48 (2–3), with the total number of anterior and posterior tubercles being 5.80±0.92 (4–7). Exochorionic sculpturing: the reticulum type measured approximately 23.60×28.53 μm and was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats. Remarkably, some specimens had sparse reticulum on the area between the deck and float.

Species with divided decks

An. nitidus The deck was divided into an area at each end of the egg (Fig. 4d) Dimensions: the entire length was 513.06±27.09 μm (461.11–555.56 μm), the width including the floats was 175.56±10.21 μm (166.67–194.44 μm), the deck lengths at the anterior and posterior ends were 111.12±26.06 μm (72.22–150 μm) and 73.89±12.01 μm (55.56–88.89 μm), respectively, and the maximal deck widths at the anterior and posterior ends were 32.22±5.74 μm (27.78–44.44 μm) and 25.00±3.93 μm (22.22–44.44 μm), respectively. Counts: the number of float ribs was 27.60±1.84 (25–30), of anterior tubercles was 6.10±1.20 (5–9), and of posterior tubercles was 5.30±0.82 (4–6), with the total number of anterior and posterior tubercles being 11.40±1.07 (10–13). Exochorionic sculpturing: the reticulum type measured approximately 14.56×30.88 μm and was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats.

An. pursati The deck was divided into an area at each end of the egg (Fig. 4e). Dimensions: the entire length was 393.61±13.49 μm (372.22–416.67 μm), the width including the floats was 163.06±2.64 μm (161.11–166.67 μm), the deck lengths at the anterior and posterior ends were 105±35.95 μm (72.22–166.67 μm) and 77.78±18.33 μm (55.56–111.11 μm), respectively, and the maximal deck widths at the anterior and posterior ends were 22.78±4.10 μm (16.67–27.78 μm) and 21.67±5.52 μm (11.11–27.78 μm), respectively. Counts: the number of float ribs was 27.10±1.52 (25–30), of anterior

tubercles was 4.40±0.52 (4–5), and of posterior tubercles was 4.00±0.47 (3–5), with the total number of anterior and posterior tubercles being 8.40±0.84 (7–10). Exochorionic sculpturing: the tubercle type was distributed throughout the entire egg surface, except on the micropylar area, deck, and areas covered by floats.

Discussion

SEM studies of all stages and specific organs of some dipteran insects have been used extensively since they provide descriptions more accurately and confidently than a conventional light microscope (Raybould et al. 1971; Amer and Mehlhorn 2006; Tangtrakulwanich et al. 2011; Klong-Klaew et al. 2012; Adham et al. 2013). Mosquito egg topography under SEM could be used robustly in characterizing and differentiating between species members of some groups (Iwaki and Choochote 1991; Iwaki et al. 1992, 1994; Linley 1989), some members of sibling species (Damrongphol and Baimai 1989; Sucharit et al. 1995), and strains within each taxon (Rodriguez et al. 1992).

In this study, the egg morphology under SEM of the eight anopheline species (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati*, and *An. sinensis*) was generally similar to the descriptions under a light microscope (Reid 1968) and the egg topography of the four species (*An. nigerrimus*, *An. paraliae*, *An. peditaeniatus*, and *An. sinensis*) under SEM (Linley et al. 1995; Rongsriyam et al. 1996; Rueda et al. 2009). Nonetheless, the deck variation of *An. sinensis* was recorded firstly from Japan in the East Asian region (Olsuru and Ohmori 1960), while it has never been recorded formerly from Malaysia (Reid 1968; Linley et al. 1995) or Thailand in the Southeast Asian region.

Differences in morphometrics and the detailed ultrastructure among the eggs in this study permit the construction of a key for identifying these eight anopheline species, as follows:

1. Deck, complete (Fig. 3a–e).....2
   Deck, divided into an area at each end of the egg (Fig. 4d, e).....6
   Deck, variable with complete, split, and incomplete decks found together within an egg batch (Fig. 4a–c).....*crawfordi*
2. Deck extensively wide, scant in a tiny area between the deck and float, with an entire length-to-maximal deck width ratio ranging from 3 to 7 (Fig. 3a).....*sinensis*
   Deck narrows to moderate width, with a fair area between the deck and float.....3
3. Entire length-to-maximal deck width ratio ranging from 8 to 22 (Fig. 3b, c).....4
   Entire length-to-maximal deck width ratio ranging from 26 to 76 (Fig. 3d, e).....5
4. Number of float ribs ranged from 21 to 27.....*peditaeniatus*
   Number of float ribs ranged from 28 to 34.....*nigerrimus*
5. Total number of anterior and posterior tubercles ranging from 6 to 8.....*paraliae*
   Total number of anterior and posterior tubercles ranging from 9 to 11.....*argyropus*
6. Exochorionic sculpturing throughout the entire egg was of the reticulum type (Fig. 4d).....*nitidus*
   Exochorionic sculpturing throughout the entire egg was of the tubercle type (Fig. 4e).....*pursati*

Overlapped morphological characteristics of adult females among the eight anopheline species (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati*, and *An. sinensis*) of the Hyrcanus Group have been documented. These led to the misidentification of adult females, particularly when using the traumatic scales of wild-caught specimens from epidemiology and vector control approaches. Some of the listed characteristics are, for example, highly variable hindtarsal banding (among *An. sinensis*, *An. crowfordi*, and *An. nigerrimus* and between *An. argyropus* and *An. peditaeniatus*); similar humeral crossvein with a patch of dark scales, remigium with dark scales, and midtarsi with narrow apical pale bands (between *An. argyopus* and *An. nigerrimus*); similar wing patterns (between *An. crawfordi* and *An. nitidus*); and similar narrow apical fringe spot on the wing and narrow tarsal bands (between *An. paraliae* and *An. pursati*; Reid 1953, 1968; Harrison and Scanlon 1975). In view of the limitation in using adult female characteristics, as mentioned before, at least two robust molecular assays (DNA barcoding and multiplex PCR) have been developed recently for accurately identifying the eight anopheline species (Wijit et al. 2013; Hempolchom et al. 2013). It is anticipated that the robust ultrastructural key proposed herein for identifying these anopheline species will be beneficial for use as an additional tool or when only this method is chosen.

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RESEARCH Open Access

Susceptibility of eight species members in the *Anopheles hyrcanus* group to nocturnally subperiodic *Brugia malayi*

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Abstract

Background: Filariasis, caused by *Brugia malayi*, is a public health problem in Thailand. Currently, at least two locations in southern Thailand are reported to be active endemic areas. Two and four *Mansonia* species are primary and secondary vectors, respectively, of the nocturnally subperiodic race, whereas, *Coquillettidia crassipes* is a vector of the diurnally subperiodic race. Although several *Anopheles* species have been incriminated extensively as natural and/or suspected vectors of *B. malayi*, little is known about vector competence between indigenous *Anopheles* and this filaria in Thailand. Thus, the susceptibility levels of eight species members in the Thai *An. hyrcanus* group to nocturnally subperiodic *B. malayi* are presented herein, and the two main refractory factors that affect them in different degrees of susceptibility have been elucidated.

Methods: *Aedes togoi* (a control vector), *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis* were allowed to feed artificially on blood containing *B. malayi* microfilariae, and dissected 14 days after feeding. To determine factors that take effect at different susceptibility levels, stain-smeared blood meals were taken from the midguts of *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. paraliae*, *An. sinensis* and *An. nitidus* immediately after feeding, and their dissected-thoraxes 4 days post blood-feedings were examined consecutively for microfilariae and L_1 larvae.

Results: The susceptibility rates of *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus*, *An. pursati*, *An. sinensis*, *An. paraliae* and *An. nitidus* to *B. malayi* were 70–95%, 70–100%, 80–85%, 50–65%, 60%, 60%, 10%, 5%, and 0%, respectively. These susceptibility rates related clearly to the degrees of normal larval development in thoracic muscles, i.e., *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. paraliae*, *An. sinensis* and *An. nitidus* yielded normal L_1 larvae of 93.15%, 96.34%, 97.33%, 23.60%, 15.38% and 0%, respectively.

Conclusions: *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus* and *An. pursati* were high potential vectors. *An. paraliae* and *An. sinensis* were low potential vectors, while *An. nitidus* was a refractory vector. Two refractory mechanisms; direct toxicity and/or melanotic encapsulation against filarial larval were involved in the refractoriness of development in the thoracic muscles of the mosquito.

Keywords: *Anopheles hyrcanus* group, *Brugia malayi*, Susceptibility level, Refractory factor, Thailand

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Background

Lymphatic filariasis, due to *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, is a major health problem in many tropical and sub-tropical countries. At present, 1.3 billion people worldwide are at risk of lymphatic filariasis infection, with approximately 120 million affected in 72 countries [1-4]. In Thailand, at least two endemic areas of lymphatic filariasis have been reported, i.e., *B. malayi* in the south and *W. bancrofti* on the southwest to northwest Thai-Myanmar border [5,6].

So far, at least two physiological races of *B. malayi*, i.e., nocturnally subperiodic and diurnally subperiodic have been discerned in southern Thailand. The nocturnally subperiodic race is located in endemic areas of five provinces, i.e., Nakhon Si Thammarat, Phattalung, Pattani, Yala and Narathiwat. These regions are rural and semi-forested, and *Mansonia uniformis* and *Ma. bonneae* are the primary vectors in open swamp and swamp-forested areas, respectively, whereas *Ma. dives*, *Ma. indiana*, *Ma. annulata* and *Ma. annulifera* are considered as secondary vectors. The endemic area for the diurnally subperiodic race is confined to Surat Thani province, and *Coquillettidia crassipes* is an important vector [6-8]. When comparing these six provinces, Narathiwat is the highest endemic area, with more than half of the filariasis cases reported there each year. This may result from suitable microhabitats or large areas of swamp for *Mansonia* breeding-places; the existence of cats as animal reservoir hosts; or local insurgence that is considered a main factor in bringing about control failure in this province [8,9]. Regarding control measures, the reduction of microfilariae in the peripheral blood of carriers interrupts the mosquito-transmitted cycle by using a microfilaricide (diethylcarbamazine, [DEC]), which was established in 2002 by the Division of Filariasis, Department of Communicable Disease Control, Ministry of Public Health, Thailand. Consequently, the provinces of Surat Thani and Narathiwat are considered active endemic areas of diurnally and nocturnally subperiodic *B. malayi*, respectively [10]. Despite the control program succeeding at satisfactory levels, as determined by the reduction of microfilaraemic cases to 0% in four provinces (Nakhon Si Thammarat, Phattalung, Pattani and Yala), the two active endemic areas (Surat Thani and Narathiwat provinces) are still regarded as a source of microfilaria. The transmitting cycle has the potential to generate infection not only in these two active endemic areas, but also in adjacent provinces, due to migration of microfilaraemic carriers and long-term settlements as well as inadequate control of animal reservoir-hosts. In addition, this endemic disease could re-emerge at any time, even in thoroughly controlled endemic regions, where the environmental factor(s) favors suitable conditions for the transmission-cycle. This was reported recently in other mosquito-borne diseases, e.g., re-emergence of malaria due to *Plasmodium vivax* in South Korea [11-13].

In southern Thailand, only one and six mosquito species, which have been incriminated as natural vectors of *B. malayi*, belong to the genera *Coquillettidia* and *Mansonia*, respectively. Besides, at least one anopheline species of the subgenus *Cellia* (*An. minimus*) and five of the subgenus *Anopheles* (*An. barbirostris*, *An. campestris*, *An. donaldi*, *An. lesteri* and *An. sinensis*) were reported and incriminated as natural and/or suspected vectors of this filarial nematode in southeast and/or east Asian regions [14]. This information clearly emphasizes that knowledge of the vector competence of *Anopheles* mosquitoes to *B. malayi* is lacking, particularly according to data on the susceptibility level of indigenous *Anopheles* species to a local strain of *B. malayi*. Hence, this study reports the susceptibility of eight species members of the indigenous Thai *An. hyrcanus* group (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis*) to nocturnally subperiodic *B. malayi* (Narathiwat province, southern Thailand strain). Additionally, the possible factor(s) affecting the different degrees in susceptibility of these anopheline species to nocturnally subperiodic *B. malayi* was elucidated.

Methods

Mosquito species and strains

As *B. malayi* is endemic, eight species members of the *An. hyrcanus* group were collected mainly in southern Thailand. This location comprised: (1) former endemic provinces [Chumphon (CP) and Nakhon Si Thammarat (NS)], and (2) provinces adjacent to former and/or current endemic provinces [Phang Nga (PG), Songkhla (SK), and Trang (TG)]. In addition, two provinces free from *B. malayi* infection in western [Ratchaburi (RB)] and northeastern [Ubon Ratchathani (UR)] Thailand were included in this study. The species and strains of the *An. hyrcanus* group were as follows: *An. argyropus* (NS strain), *An. crawfordi* (CP and TG strains), *An. nigerrimus* (UR, NS and SK strains), *An. nitidus* (UR and PG strains), *An. paraliae* (RB strain), *An. peditaeniatus* (CP and SK strains), *An. pursati* (RB strain) and *An. sinensis* (CP strain). Wild-caught, fully engorged females of the 8 *An. hyrcanus* species were collected from cow-baited traps and established successfully for many consecutive generations in the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand, using the techniques described previously [15,16]. These colonies were used for studies on susceptibility to nocturnally subperiodic *B. malayi* throughout the experiments. Regarding the control vector, autogenous *Ae. togoi* (Chanthaburi province, eastern Thailand) was selected as a proven efficient laboratory vector for a wide-range of genera and species of filarial nematodes, including the nocturnally subperiodic *B. malayi* [17,18].

Nocturnally subperiodic *B. malayi*

This filarial parasite originated from a 20-year-old women, who was a resident of Bang Paw district, Narathiwat province, southern Thailand. Domestic cats were later infected experimentally with the parasite, which was maintained at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, from 1982 to 1986, when it was transferred to Mongolian jirds (*Meriones unguiculatus*) and then maintained at the animal house of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand [19].

Preparation of blood containing *B. malayi* microfilariae

The jirds were intraperitoneally inoculated for at least 3 months with infective larvae of nocturnally subperiodic *B. malayi* [20] and anesthetized deeply with ethylene ether. The microfilariae were collected by injecting 3 ml of Hank's Balanced Salt Solution (HBSS, pH 7.2-7.4) into the peritoneal cavity before withdrawing by peritoneal washing. The 0.05 ml of peritoneal-washed-rich microfilariae was mixed with 10 ml of human-heparinized blood (10 units of heparin/ml of blood), taken from human volunteers who had signed the consent form. Then, the adjusted microfilarial density ranged from approximately 200 to 300 microfilariae (mf)/20 μl by using the human-heparinized blood for artificially feeding all of the mosquito species. The reason for adjusting microfilarial density in blood to range from 200 to 300 mf/20 μl was based on several proven experiments that yielded satisfactorily susceptible *Ae. togoi* to nocturnally subperiodic *B. malayi* (susceptibility rates: 70–95%). This agreed with experiments reporting susceptibility of *An. sinensis* to periodic *B. malayi*, i.e., using a microfilarial density of 5, 10, 20 and 50 mf/20 μl, with a susceptibility rate of 30, 65, 93 and 100%, respectively [21].

Infection of mosquitoes with *B. malayi* microfilariae

Five-day-old adult female *Ae. togoi*, *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis* fasted for 24 hrs and then were allowed artificial feeding simultaneously on blood-containing *B. malayi* microfilariae (microfilarial density = 312, 208, 256 and 283 mf/20 μl in experiment 1, 2, 3 and 4, respectively), using the techniques and apparatus previously described [22]. Fourteen days after feeding, all infected mosquitoes were dissected in normal saline solution and examined under a dissecting microscope. The number of mosquitoes with one or more infective stage larvae in any part of the body (head, thorax or abdomen) was recorded.

Determination of the possible factor(s) affecting the level of susceptibility

Five-day-old adult female mosquitoes, i.e., an efficient laboratory vector (*Ae. togoi*), high potential vectors [*An. peditaeniatus* (CP strain) and *An. crawfordi* (TG strain)], low potential vectors [*An. paraliae* (RB strain) and *An. sinensis* (CP strain)] and a refractory vector [*An. nitidus* (PG strain)] were allowed artificial feeding simultaneously on blood containing *B. malayi* microfilariae, as mentioned above. The infected mosquitoes were divided into 2 groups, i.e., (1) those with their midgut extracted immediately after full engorgement. The ingested blood meals were then made into thick blood films, dried out, de-hemoglobinized, fixed with methanol, stained with Giemsa (pH 7.2) and counted for microfilariae under a compound microscope; and (2) those with their thorax severed, torn in a drop of normal saline solution and examined under a compound microscope 4 days after feeding. The first stage (L_1) larvae were counted and scored as normal L_1 larvae if alive with intact morphology. The larvae were scored as melanized L_1 if they had evidence of a retained stage and melanotic encapsulation; and scored as degenerated L_1 if they demonstrated vacuolated internal organs without any evidence of melanotic encapsulation.

Ethical clearance

The protocols were approved by the Animal Ethics Committee of Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Results

Details of the infective rates and parasite loads of *Ae. togoi*, *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis* 14 days after feeding on blood containing *B. malayi* microfilariae are shown in Table 1. The 95%, 70%, 80% and 80% infective rates corresponded to an average of 19.05, 7.50, 10.56 and 11.81 infective (L_3) larvae per infected *Ae. togoi* in experiment 1, 2, 3 and 4, respectively, which indicated that all feeding experiments were under conditions of sufficient *B. malayi* microfilarial densities in infected blood.

The infective rates (IR) and average number of L_3 larvae per infected mosquito (AL_3) of *An. crawfordi* [experiment 1 (CP strain: IR = 85%, AL_3 = 6.24) and experiment 4 (TG strain: IR = 80%, AL_3 = 6.06)], *An. nigerrimus* [experiment 1 (NS strain: IR = 65%, AL_3 = 9.77; SK strain: IR = 65%, AL_3 = 6.69) and experiment 2 (UR strain: IR = 50%, AL_3 = 4.20)], *An. nitidus* [experiment 1 (PG strain: IR = 0%, AL_3 = 0%) and experiment 2 (UR strain: IR = 0%, AL_3 = 0%)], *An. argyropus* [experiment 2 (NS strain: IR = 60%, AL_3 = 2.92)], *An. pursati* [experiment 2 (RB strain: IR = 60%, AL_3 = 3.83)], *An. paraliae* [experiment 3 (RB strain: IR = 5%, AL_3 = 1.00)], *An. peditaeniatus* [experiment 3 (CP strain: IR = 100%, AL_3 = 7.75) and experiment 4 (SK strain: IR = 70%, AL_3 = 8.00)] and *An. sinensis* [experiment 4 (CP strain: IR = 10%, AL_3 = 1.50)]

Table 1 Infective rates and parasite loads of 8 species in the *An. hyrcanus* group after feeding on blood containing *B. malayi* microfilariae (microfilarial density = 312, 208, 256 and 283 mf/20 μl in experiment 1, 2, 3 and 4, respectively), with all mosquitoes dissected 14 days after feeding

Mosquito species	Infective rates (No.)*	Average No. L_3 per infected mosquito (range)+	L_3-distribution		
			% head (No.)	% thorax (No.)	% abdomen (No.)
Experiment 1					
Ae. togoi	95 (19/20)	19.05 (1–49)	59.39 (215)	21.27 (77)	19.34 (70)
An. crawfordi (CP)	85 (17/20)[a]	6.24 (1–27)[n]	52.83 (56)	38.68 (41)	8.49 (9)
An. nigerrimus (NS)	65 (13/20)[b]	9.77 (1–32)[o]	44.88 (57)	38.58 (49)	16.54 (21)
An. nigerrimus (SK)	65 (13/20)[c]	6.69 (1–15)[p]	45.98 (40)	19.54 (17)	34.48 (30)
An. nitidus (PG)	0 (0/20)[d]	-	-	-	-
Experiment 2					
Ae. togoi	70 (14/20)	7.50 (1–36)	83.81 (88)	9.52 (10)	6.67 (7)
An. argyropus (NS)	60 (12/20)[e]	2.92 (1–6)[q]	68.57 (24)	22.86 (8)	8.57 (3)
An. nigerrimus (UR)	50 (10/20)[f]	4.20 (1–9)[r]	69.05 (29)	16.66 (7)	14.29 (6)
An. nitidus (UR)	0 (0/20)[g]	-	-	-	-
An. pursati (RB)	60 (12/20)[h]	3.83 (1–11)[s]	67.39 (31)	19.57 (9)	13.04 (6)
Experiment 3					
Ae. togoi	80 (16/20)	10.56 (1–32)	73.37 (124)	16.57 (28)	10.06 (17)
An. paraliae (RB)	5 (1/20)[i]	1.00 (0–1)[t]	100.00 (1)	-	-
An. peditaeniatus (CP)	100 (20/20)[j]	7.75 (1–23)[u]	57.42 (89)	14.19 (22)	28.39 (44)
Experiment 4					
Ae. togoi	80 (16/20)	11.81 (2–28)	77.25 (146)	15.87 (30)	6.88 (13)
An. crawfordi (TG)	80 (16/20)[k]	6.06 (1–19)[v]	79.38 (77)	11.34 (11)	9.28 (9)
An. peditaeniatus (SK)	70 (14/20)[l]	8.00 (2–22)[w]	80.36 (90)	10.71 (12)	8.93 (10)
An. sinensis (CP)	10 (2/20)[m]	1.50 (1–2)[x]	100.00 (3)	-	-

*Fisher exact test: a, j, k vs. control, $P > 0.05$; b, c vs. control, $P < 0.05$.
*Chi-square test: e, f, h, l vs. control, $P > 0.05$; d, g, i, m vs. control, $P < 0.05$.
+ *t*-test (two-sided); q, r, s, u, w vs. control: $P > 0.05$; n, o, p, t, v, x vs. control: $P < 0.05$.

were mostly lower than those in *Ae. togoi*, an efficient control vector. This was the case in all experimental studies, except for the infective rate of *An. peditaeniatus* (100%), which was higher than that of *Ae. togoi* (80%) in experiment 3. Comparative statistical analyses of the infective rates and average number of L_3 larvae per infected mosquito were carried out between *Ae. togoi* and all *An. hyrcanus* species. The results revealed that the infective rates between *Ae. togoi* and *An. hyrcanus* species in experiment 1 [*An. crawfordi* (CP strain)], 2 [*An. argyropus* (NS strain), *An. nigerrimus* (UR strain) and *An. pursati* (RB strain)], 3 [*An. peditaeniatus* (CP strain)] and 4 [*An. crawfordi* (TG strain) and *An. peditaeniatus* (SK strain)], and average number of L_3 larvae per infected mosquito between *Ae. togoi* and *An. hyrcanus* species in experiment 2 [*An. argyropus* (NS strain), *An. nigerrimus* (UR strain) and *An. pursati* (RB strain)] and 3 [*An. peditaeniatus* (CP strain) and 4 (SK strain)] did not differ significantly ($P > 0.05$). It is noteworthy that all infective larvae obtained from the four experimental feedings were very active and found to distribute in all regions of the head, thorax and abdomen, and their behavior was similar, with more than 44% of infective larvae migrating from the thorax to the head and proboscis.

Parasite loads dissected immediately and 4 days after feeding on blood containing *B. malayi* microfilariae in *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. paraliae*, *An. sinensis* and *An. nitidus* are detailed in Table 2. Investigative results on stain-smeared blood meals from the midguts of mosquitoes fed immediately, and fully engorged, indicated that all the mosquito species were successful in taking a considerable number of microfilariae from infected blood, with an average number of microfilariae per infected midgut of 25.20, 27.40, 22.00, 32.40, 26.40 and 25.80 in *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. paraliae*, *An. sinensis* and *An. nitidus*, respectively. Likewise, a satisfactory average number of 14.60, 16.40, 15.00, 17.80, 15.60 and 10.80 L_1 larvae were

Table 2 Parasite loads in *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. paraliae*, *An. sinensis* and *An. nitidus* dissected immediately and 4 days after feeding on blood containing *B. malayi* microfilariae (microfilarial density = 247 mf/20 μl)

Mosquito species	Average No. mf per infected midgut (range)*	Average No. L_1 per infected thorax (range)$^+$	% normal L_1 (No.)	% melanized L_1 (No.)	% degenerated L_1 (No.)
Ae. togoi	25.20 (26–65)	14.60 (14–30)	93.15 (68)	0 (0/73)	6.85 (5)
An. peditaeniatus (CP)	27.40 (22–94)	16.40 (12–47)	96.34 (79)	0 (0/82)	3.66 (3)
An. crawfordi (TG)	22.00 (15–41)	15.00 (8–25)	97.33 (73)	0 (0/75)	2.67 (2)
An. paraliae (RB)	32.40 (20–37)	17.80 (10–17)	23.60 (21)	47.19 (42)	29.21 (26)
An. sinensis (CP)	26.40 (23–72)	15.60 (13–36)	15.38 (12)	32.05 (25)	52.57 (41)
An. nitidus (PG)	25.80 (13–29)	10.80 (5–11)	0 (0/54)	94.44 (51)	5.56 (3)

*Dissected from 5 midguts; $^+$ Dissected from 5 thoraxes.

recovered in the thoracic muscles of *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. paraliae*, *An. sinensis* and *An. nitidus*, respectively. However, variations in degrees of normal and abnormal L_1 larval development in the thoracic muscles of six mosquito species were observed clearly. *Ae. togoi*, *An. peditaeniatus*, *An. crawfordi*, *An. paraliae*, *An. sinensis* and *An. nitidus* yielded normal, melanized and degenerated L_1 larvae of 93.15%, 0% and 6.85%; 96.34%, 0% and 3.66%; 97.33%, 0% and 2.67%; 23.60%, 47.19% and 29.21%; 15.38%, 32.05% and 52.57%; 0%, 94.44% and 5.56%, respectively (Figure 1).

Discussion

To incriminate a mosquito vector in an endemic area of filariasis, it is necessary to confirm the susceptibility rate in a laboratory-bred, clean mosquito colony, which has been fed on carrier blood containing microfilariae. By using this criterion, the susceptibility test in an experimental laboratory is an efficient classical tool when suspecting the potential vector of a certain mosquito species. Nonetheless, susceptibility alone does not imply an important role in the transmission of disease in nature, whereas a refractory one can rule out the significance of a vector entirely [14].

Investigation on the susceptibility of eight species members of the Thai *An. hyrcanus* group to nocturnally subperiodic *B. malayi* indicated that *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus* and *An. pursati* were high potential vectors. *An. paraliae* and *An. sinensis* were low potential vectors, while *An. nitidus* was a refractory vector. However, a crucial question regarding the susceptibility level determined in this study might be raised, due to the artificial feeding of mosquitoes on blood containing *B. malayi* microfilariae, which was not as natural as direct feeding on cat- and/or jird-infected *B. malayi*. Nevertheless, previous reports [23] confirmed that these two feeding techniques could be used robustly for routine screening of potential mosquito vectors of filarial parasites, since they did not differ significantly. This was despite the artificial feeding technique yielding slightly higher infective rates and parasite loads than the direct feeding method, presumably due to the effect of anticoagulant (10 units of heparin/1 ml of blood).

Among the five high potential vectors, *An. peditaeniatus*, *An. crawfordi* and *An. nigerrimus* were found to be abundant and widely distributed in Thailand and other countries [India (Assam, Bihar and Punjab), Sri Lanka, Bangladesh, China (Hainan Island), Myanmar, Cambodia, Vietnam, Malaysia (Malaysian Peninsular, Sabah and Sarawak), Indonesia (Java and Sumatra) and Brunei], and were proven as outdoor-biters of humans in certain localities of Thailand [24,25]. Regarding vector competence, *An. peditaeniatus* and *An. nigerrimus* have been incriminated so far as suspected vectors of *P. vivax* in Thailand [26-28], as well as *An. nigerrimus* as a potential natural vector of *W. bancrofti* in Phang Nga province, southern Thailand [8], and *An. peditaeniatus* as a secondary vector of Japanese encephalitis virus in China and India [29,30]. Beneficial results reported herein emphasize the potential role of *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus* and *An. pursati* in transmitting nocturnally subperiodic *B. malayi* in southern Thailand as well as other countries, in which these anopheline species and filarial parasite were found sympatrically and/or co-endemic with malaria and Japanese encephalitis. The list of these potential vector-species could be used as a promising guideline for the field approach to incriminate natural vectors in endemic areas of Brugian filariasis. Remarkably, *An. sinensis* has been incriminated as an important vector of nocturnally periodic *B. malayi* in China, Korea and Japan [14], but in this study, it was proven as a low potential vector of nocturnally subperiodic *B. malayi*. It is interesting to note that the *An. sinensis* strain from Korea and China was compatible genetically and/or nearly identical to that from Thailand, based on the crossing experiments and comparative sequence analyses of the ribosomal DNA (rDNA) internal transcribed spacer 2

(ITS2), and mitochondrial cytochrome *c* oxidase subunit I (COI) and subunit II (COII) [31]. This evidence appeared to support the high specificity between *B. malayi* physiological races and the *An. sinensis* vector.

It has been known for refractoriness of certain mosquito species towards filarial parasites to occur in the forgut (cibarial and pharyngeal amartures), midgut (fast blood coagulation) or thoracic muscles (direct toxicity and melanotic encapsulation) [32-34]. Regarding refractoriness in the thoracic muscle, large numbers of *B. malayi* and *B. pahangi* microfilariae exsheathed in refractory *Ae. albopictus* after gaining entry into the mosquitoes, and subsequently migrated to the thoracic muscles without further development [35,36]. The results revealed that the factor(s) in the thoracic muscles of *Ae. albopictus* conferred with the refractoriness. Evidence of refractoriness to *B. pahangi* microfilariae infection is of additional interest, as it could be induced in normally susceptible *Ae. tabu* by rearing female mosquitoes on sugar solution containing thoracic homogenate of refractory *Ae. malayansis* mosquitoes [33]. This result agreed with a subsequent study in that the high inhibition of *B. pahangi* larval development could be induced in the thoracic muscle of susceptible *Ae. togoi*. This was performed by intrathoracic injection of crude thoracic homogenate (CTH) from refractory *Ae. albopictus* into susceptible *Ae. togoi* prior to feeding on blood containing *B. pahangi* microfilariae [37]. Thus, these two pieces of evidence seem to reflect the inhibitory effect that might be due to direct toxicity of the homogenate on developing larvae. Furthermore, the melanization of immune responses in various insects against a wide-range of invading pathogens and parasites has been documented [34,38-40]. The immune response of mosquitoes is put into effect through the plasma components of both the hemolymph, i.e., the humoral response, and hemocytes, the cellular response [34]. The authors also suggested that the intracellular melanotic encapsulation of filarial developing stages, as observed in specific mosquito organs, may be caused by exposure to low-molecular-weight immune molecules, which are carried in the hemolymph (plasma) and can penetrate the basement membrane covering the cells of specific organs. This concept suggested that the

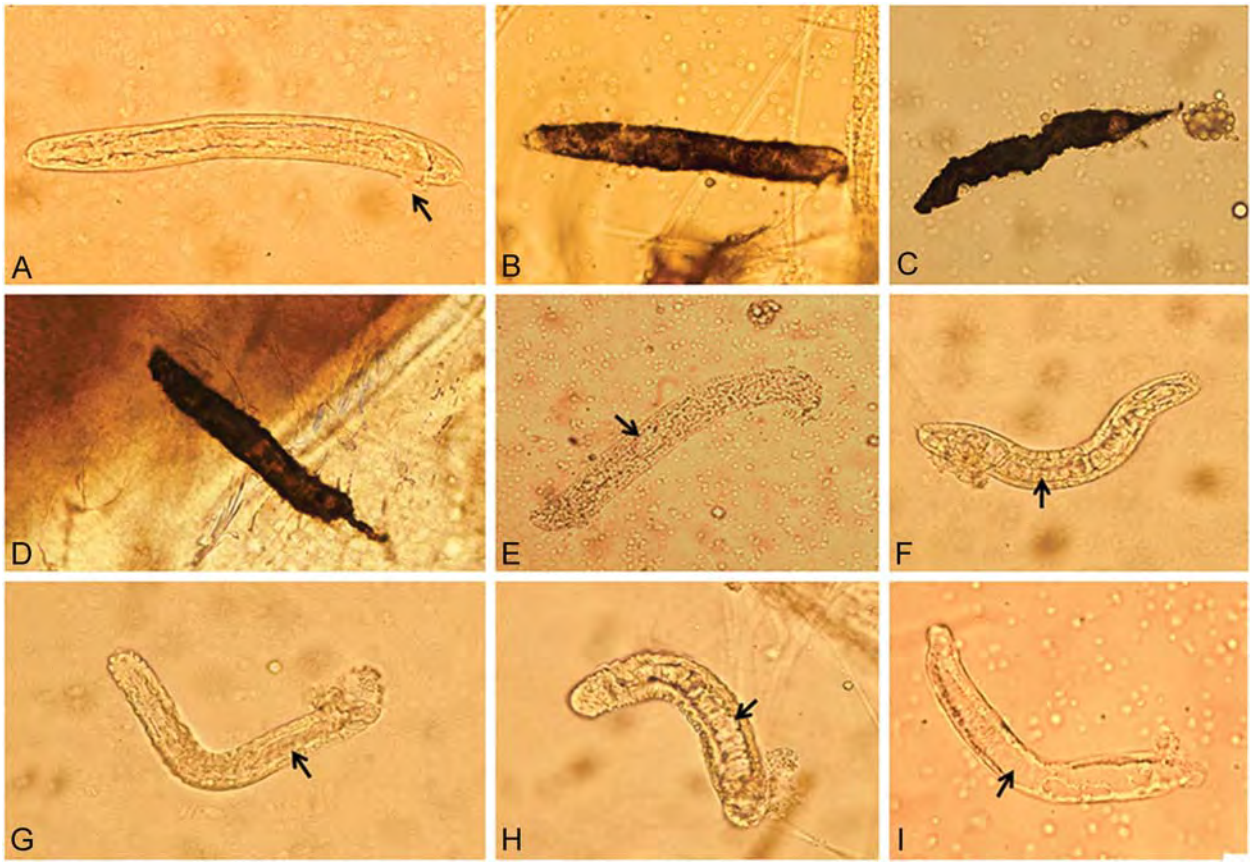

Figure 1 L_1 larvae recovered from thoracic muscles of mosquitoes 4 days after infected blood meals. (**A**) Normal live larva with intact cuticle and internal organs (small arrow: protuberance of anal plug at the anal pore) recovered from *Ae. togoi*. (**B**, **C**, **D**) Completely melanotic encapsulated larvae obtained from *An. paraliae*, *An. sinensis* and *An. nitidus*. (**E**, **F**, **G**, **H**, **I**) Degenerated and vacuolated internal organs (small arrow) acquired from *An. peditaeniatus*, *An. crawfordi*, *An. sinensis*, *An. paraliae* and *An. nitidus*, respectively.

same mechanisms controlling melanotic encapsulation reactions (immune response) extracellularly in the hemocoel also control them intracellularly in specific organs of the host in which the parasite develops. Subsequent evidence from using RNAi methodology to knockdown PAH (phenylalanine hydroxylase) expression in the mosquitoes, *Ae. aegypti* and *Armigeres subalbatus*, demonstrated that limitation in the amount of tyrosine, available for tyrosinase-mediated hydroxylation, significantly reduces the effectiveness of melanization reactions against inoculated filarial parasites [41]. Additionally, at least four specific enzymes [DCE (dopachrome conversion enzyme), DDC (dopa decarboxylase), PO (phenoloxidase) and TH (tyrosine hydroxylase)] were concerned in the biosynthesis of melanin [39]. Current studies on the possible factors affecting the difference in susceptibility levels of eight *An. hyrcanus* species to nocturnally subperiodic *B. malayi* revealed that at least two refractory mechanisms (direct toxicity and/or melanotic encapsulation) were involved in the refractoriness of thoracic muscles for parasite development. Variations in the percentages of melanotic encapsulation and degenerated L_1 larvae recovered in the thoracic muscles of *Ae. togoi* (0% and 6.85%), *An. peditaeniatus* (0% and 3.66%), *An. crawfordi* (0% and 2.67%), *An. paraliae* (47.19% and 29.21%), *An. sinensis* (32.05% and 52.57%) and *An. nitidus* (94.44% and 5.56%), were good supportive evidence.

Conclusions

Eight species members of the *An. hyrcanus* group, i.e., *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis* were tested for susceptibility to nocturnally subperiodic *B. malayi*. They were allowed to feed artificially on blood containing *B. malayi* microfilariae, and dissected 14 days after feeding. The susceptibility rates were 70-100%, 80-85%, 50-65%, 60%, 60%, 10%, 5% and 0% in *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus*, *An. pursati*, *An. sinensis*, *An. paraliae* and *An. nitidus*, respectively. As determined by levels of susceptibility, results indicated that *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus* and *An. pursati* were high potential vectors when compared with the control vector, *Aedes togoi*. *An. paraliae* and *An. sinensis* were low potential vectors, while *An. nitidus* was a refractory vector. In order to determine the possible factor(s) affecting different degrees of susceptibility, stained-smears of blood meals from midguts immediately after fully engorged and dissected-thoraxes 4 days post blood-feeding from the control vector (*Ae. togoi*), high potential vectors (*An. peditaeniatus* and *An. crawfordi*), low potential vectors (*An. paraliae* and *An. sinensis*) and refractory vector (*An. nitidus*) were examined for microfilariae and L_1 larvae, respectively. The results revealed that an appreciable number of microfilaria obtained in the ingested blood meals and L_1 larvae recovered in thoracic muscles was similar in appearance to those in all infected mosquitoes. Nonetheless, the marked variations in degrees of normal development of L_1 larvae in thoracic muscles were observed clearly from the four vector-groups, i.e., the control vector: 93.15%, high potential vectors: 96.34–97.33%, low potential vectors: 15.38–23.60% and refractory vector: 0%. At least, two refractory mechanisms, direct toxicity and melanotic encapsulation, were involved in the inhibition of L_1 larval development in thoracic muscles.

Competing interests

The authors declare no competing interests.

Authors' contributions

All the authors contributed significantly to this study. AS participated in the study design, field and laboratory experiments, data analysis and writing of the manuscript. CH, ST and KT carried out field and laboratory experiments. VB participated in data analysis, and criticized the manuscript. NJ and UC helped with data analyses. WC designed the experiments, carried out field and laboratory experiments, interpreted the results, and edited the manuscript. All authors read and approved the final version of the manuscript.

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ORIGINAL PAPER

Development of a facile system for mass production of *Brugia malayi* in a small-space laboratory

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Abstract *Brugia malayi* is one of the important lymphatic filarial nematodes that cause elephantiasis and disability in humans in the Asian region. Mass production at any stage of this nematode in both small laboratory animal hosts and mosquito vectors is still necessary in order to continue various research aspects. This study elucidated on the use of nonblood feeding or the autogenous *Ochlerotatus togoi* (Thailand strain) and male Mongolian jird (*Meriones unguiculatus*) system. This has brought about a low-cost and highly-effective procedure for the mass production of blood containing microfilariae, infective (L_3) larvae, and adults of *B. malayi* under nonanimal-blood-feeding insectary and small-space animal-house conditions. The highly-infective rates (human-heparinized blood, 86.67–93.33; swine-heparinized blood, 83.33–96.67; bovine-heparinized blood, 76.67–80; chicken-heparinized blood, 73.33–76.67) and parasite loads (human-heparinized blood, 10.58–12.36; swine-heparinized blood, 8.40–10.38; bovine-heparinized blood, 9.75–9.91; chicken-heparinized blood, 3.41–4.65) of autogenous *O. togoi* to *B. malayi* and high numbers of adults recovered from ten *B. malayi*-infected male jirds (total=327, 16–52) are good supportive evidence. In addition, all special techniques required for succeeding in the establishment of a facile system regarding these matters are detailed.

Introduction

Lymphatic filariasis, due to *Brugia malayi*, is still an important mosquito-borne human disease in many countries in the Asian region, especially India, Sri Lanka, Thailand, Malaysia, Indonesia, and the Philippines (World Health Organization 2011).

Acceptably, it is necessary to use blood containing microfilariae (mf), filarial developing stages, [especially infective (L_3) larvae] and adults as a starting point appropriate for each experiment in order to carry out various filarial researches, e.g., physiological, biochemical, pharmacological (Tompkins et al. 2011), pathological (Thirugnanam et al. 2012), immunological (Krushna et al. 2011), molecular, and diagnostic aspects. This has led to the search for reliable laboratory animal hosts and mosquito vectors for *B. malayi*. Regarding laboratory animal hosts, trials have been made by several investigators to transmit *B. malayi* to animals smaller than monkeys and cats (Denham and McGreevy 1977). The most successful attempts were carried out with Mongolian jirds (*Meriones unguiculatus*), and most of the original infections were initiated by inoculating L_3 larvae subcutaneously. In this type of infection, microfilaremia which developed in male jirds was much more susceptible to infection than that in females (Ash and Riley 1970; Ash 1973; el-Bihari and Ewert 1973; Choochote et al. 1991). However, if the L_3 larvae were inoculated intraperitoneally, they developed normally to the adult stage, free in the peritoneal cavity, and became a rich source of developing larvae, adult filariae, and microfilariae. Additionally, in this type of infection, female and male jirds yielded similar susceptibility but differed in adult worm recovery, i.e., 18 % (2–24 adults) and 32 % (14–57 adults) in females and males, respectively (McCall et al. 1973; Choochote et al. 1991). Regarding mosquito vectors, at least two mosquito species were used popularly in the mass production of L_3 larvae, i.e., *Stegomyia aegypti* (Liverpool strain) (MacDonald 1962) and *Ochlerotatus togoi* (Taiwan strain) (Ramachandran et al. 1963). However, an additional strain of autogenous *O. togoi* (Chanthaburi Province, eastern Thailand strain) was declared subsequently (Choochote et al. 1987; Riyong et al. 2000; Junkum et al. 2003), since it was highly susceptible to a wide

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range of genera and species of filarial nematodes, including *B. malayi*. Furthermore, this strain was bred easily and maintained on simple media (deionized tap water) and larval food (dog biscuit) in the laboratory. It was also a good artificial feeder on blood containing *B. malayi* microfilariae, readily mated in a 30-cm cubic cage and showed high survival rates.

To take advantage of the above information, this paper reports the development of a low-cost and highly-effective system for maintaining and/or mass-producing various stages of *B. malayi* by using autogenous *O. togoi* (Thailand strain) and male jird.

Materials and methods

Autogenous *O. togoi*

The origin of *O. togoi* was obtained from Koh Nom Sao, Chanthaburi Province, eastern Thailand. Larvae of this mosquito species were taken from their breeding places and in 1981, colonized in the insectary of the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok. Subsequently, this colony has been maintained continuously for several generations since 1983 in the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai. As stated by Choochote et al. (1987), this laboratory colony-strain of *O. togoi* exhibited autogenous behavior, thus, the autogenous colony was established by pooling first egg batches of nonblood-feeding adult females from a stock colony. The hatched larvae were reared to the next generation; nonblood-feeding adult females were allowed to oviposit eggs again, and these processes were repeated continuously to establish the autogenous colony. By following these protocols, more than 100 generations of autogenous *O. togoi* could be colonized successfully during the past decade. Additionally, the colony also has been proven as an efficient laboratory vector in the studies of filarial research aspects up to the present time (Riyong et al. 2000; Junkum et al. 2003; Saeung et al. 2013).

Nocturnally subperiodic *B. malayi*

Maintenance of B. malayi

This filarial parasite originated from a 20-year-old woman, who was a resident of Narathiwat Province, southern Thailand. Domestic cats were later infected subcutaneously with the parasite, which was maintained at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, from 1982 to 1986, when it was transferred to jirds by both intraperitoneal and subcutaneous inoculations and then maintained at the animal house of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Intraperitoneal inoculation

A total of 120 harvested L_3 larvae from *B. malayi*-infected *O. togoi* were suspended in 0.5 ml of Hank's balanced salt solution (HBSS; pH 7.2–7.4) and inoculated intraperitoneally into the right abdomen of a male jird using a tubercle syringe and 21G×1 in. (0.8×25 mm) needle. The use of HBSS for suspending L_3 larvae instead of 0.85 % normal saline solution (NSS) (Edeson and Wharton 1957) depended upon previous experiments declaring that it yielded higher susceptibility than NSS (Harinasuta et al. 1981).

Subcutaneous inoculation

Similar procedures to intraperitoneal inoculation were performed, except for 60 larvae (el-Bihari and Ewert 1973; Choochote et al. 1991) that were used to inoculate subcutaneously into the right inguinal region.

Preparation of blood containing B. malayi microfilariae and harvesting adults

Simply, the jird inoculated intraperitoneally with *B. malayi* L_3 larvae for at least 3 months was anesthetized deeply with ethylene ether. The microfilariae were collected by injecting 3 ml of HBSS into the peritoneal cavity using a 5-ml syringe and 21G×1 in. (0.8×25 mm) needle while slightly shaking the abdomen in order that the microfilariae mixed well with the HBSS before withdrawing the HBSS-rich-*B. malayi* microfilariae with the same instruments (Fig. 1a, b, c, d). The 0.05 ml of HBSS-rich-*B. malayi* microfilariae was mixed firstly with 5 ml of human-heparinized blood, which had been taken from human volunteers who had signed the consent form, and swine-, bovine-, or chicken-heparinized blood collected from slaughterhouses (10 units of heparin per milliliter of blood). All the blood was assessed for microfilarial density per 20 μl, which was used as a guide to calculate the proper added volume (ml) of a similar type of heparinized blood necessary for adjusting the microfilarial density in blood to range from about 300 to 400 mf per 20 μl. However, in real practice, after adding the calculated volume of heparinized blood, repeated additions of a small blood volume (0.10–1.00 ml) had to be performed until a satisfactory microfilarial density was obtained. The reason for using a microfilarial density ranging from about 300 to 400 mf per 20 μl was based on several proven experiments that yielded satisfactorily susceptible autogenous *O. togoi* to nocturnally subperiodic *B. malayi* (susceptibility rates, 80–95 %). After the withdrawal of HBSS-rich-*B. malayi* microfilariae, the abdominal cavity of the jird was operated on and rinsed several times with HBSS in order to recover any adult filariae (Fig. 1e, f).

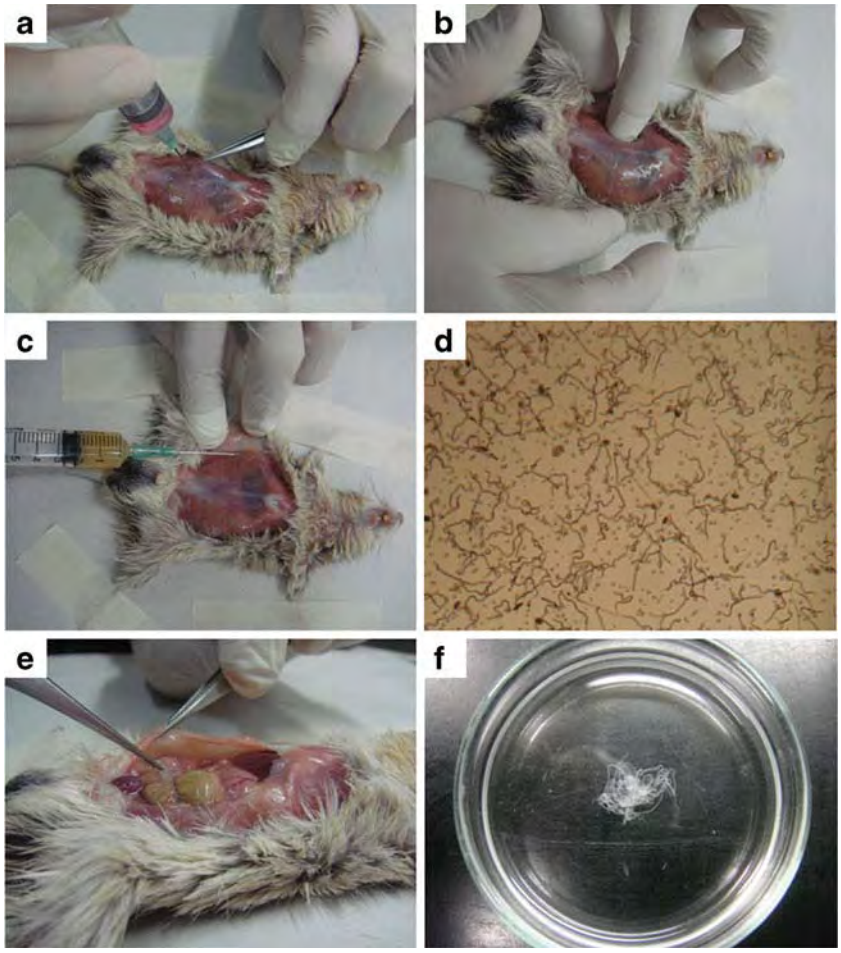

Fig. 1 Harvesting of microfilariae and adults from *B. malayi*-infected jird. **a** Injecting 3 ml of HBSS (pH 7.2–7.4) into the peritoneal cavity using a 5-ml syringe and 21G×1 in. (0.8×25 mm) needle. **b** Mixing the peritoneal fluid with HBSS by slightly pressing to the left and switching to the right abdomen and repeating at least ten times. **c** Withdrawing the injected HBSS with the same instruments. **d** Showing the HBSS-rich-*B. malayi* microfilariae. **e** Operating on the abdominal cavity of infected jird and picking up a white mass of adult filarial worms using forceps. **f** Showing massive adult filarial worms in 0.85 % of normal saline solution

Infection of autogenous O. togoi with B. malayi microfilariae

Five-day-old adult female autogenous *O. togoi* (F_{98} and F_{102}) fasted for 24 h and then were allowed artificial feeding on blood containing *B. malayi* microfilariae (microfilarial density in experiment 1=304, 346, 310, and 355 mf per 20 μl in human-, swine-, bovine-, and chicken-heparinized blood, respectively, and experiment 2=339, 328, 322, and 396 mf per 20 μl in human-, swine-, bovine-, and chicken-heparinized blood, respectively) using the techniques and apparatus described by Choochote and Saeung (2013). Fourteen days after feeding, all infected mosquitoes were dissected in 0.85 % normal saline solution and examined under a dissecting microscope. The number of mosquitoes with one or more infective stage larvae in any part of the body (head, thorax, and abdomen) was recorded (Fig. 2a, b).

Ethical clearance

The protocols were approved by Animal Ethics Committee of Faculty of Medicine, Chiang Mai University, Thailand.

Results and discussion

Prior to the study on physiological, biochemical, pharmacological (Tompkins et al. 2011), pathological (Thirugnanam et al. 2012), immunological (Krushna et al. 2011), molecular, and diagnostic aspects concerning lymphatic filarial *B. malayi*, the establishment of low-cost and highly-effective systems for maintaining and/or mass-producing various stages of this filaria (e.g., blood containing microfilariae, filarial developing stages, and adults) is the main key for success in operating appropriate experiments suited for each research target. Thus, extensive searches for suitably small laboratory animal hosts and efficient laboratory mosquito vectors have been documented widely during the past three decades (Denham and McGreevy 1977). Consequently, at least one small laboratory animal (Mongolian jird, *M. unguiculatus*) (Ash and Riley 1970; Ash 1973), and two mosquito species [*S. aegypti* (Liverpool strain) (MacDonald 1962) and *O. togoi* (Taiwan strain) (Ramachandran et al. 1963)] have been proposed for long-term use as an efficient laboratory definitive host and mosquito vectors, respectively. However, the drawback of these two mosquito species is their intensive need for animal-blood meals in each cycle of egg production. This

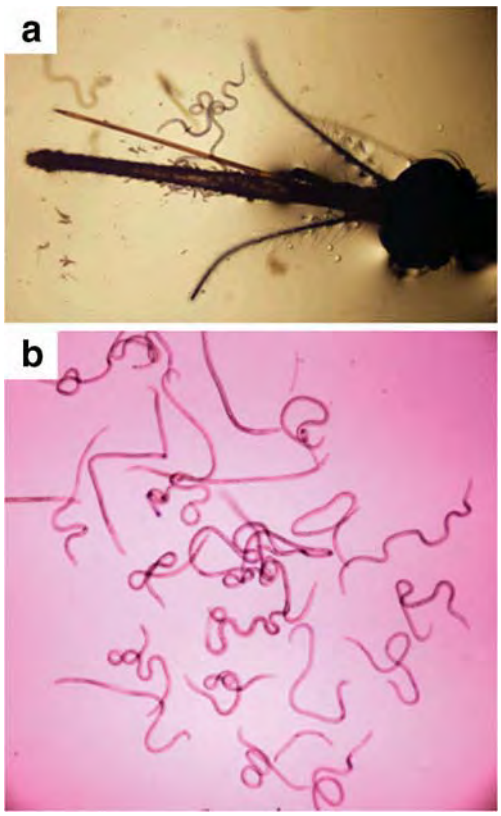

Fig. 2 Infective (L_3) larvae. **a** Showing infective (L_3) larvae penetrating into the labium of the proboscis, with some larvae trying to penetrate out. **b** Showing a cluster of harvested L_3 larvae in HBSS

leads to required space for an animal house and manpower to maintain white rats, golden hamsters, Guinea pigs, rabbits, etc., thus causing some unavailable laboratory facilities, particularly in research units that have limited space and manpower. It is fortunate that in this study, the discovery of an autogenous *O. togoi* (Thailand strain), which is easy to colonize and yielded high susceptibility to a wide range of genera and species of filariae (Choochote et al. 1987; Riyong et al. 2000; Junkum et al. 2003), has pointed the way to develop a low-cost and highly-effective system for the mass production of various stages of *B. malayi* in both small animal host and mosquito vector. Summarized systematic procedures for the mass production of microfilariae, L_3 larvae, and adults of *B. malayi* under nonanimal-blood-feeding insectary and small-space animal-house conditions are shown in Fig. 3. The space of the animal house as well as manpower for maintaining blood-source animals for the egg production of blood-feeding mosquitoes could be reduced in this system. The high-infective rates (IR), parasite loads (PL), number of L_3 larvae recovered from 30 *B. malayi*-infected *O. togoi* [human-heparinized blood: IR=86.67, PL=10.58, L_3=275 (experiment 1) and IR=93.33, PL=12.36, L_3=346 (experiment 2); swine-heparinized blood: IR=96.67, PL=10.38, L_3=301 (experiment 1) and IR=83.33, PL=8.40, L_3=210 (experiment 2); bovine-heparinized blood: IR=76.67, PL=9.91, L_3=228 (experiment 1) and IR=80, PL=9.75, L_3=234 (experiment 2); chicken-heparinized blood: IR=73.33, PL=3.41, L_3=75 (experiment 1) and IR=76.67, PL=4.65, L_3=107 (experiment 2)] (Table 1) and high numbers of adults recovered from ten infected male jirds (total=327, 16–52) were obvious supportive evidence (Table 2). Regarding *O. togoi* feeding on chicken-heparinized blood containing *B. malayi* microfilariae, rather low infective rates and parasite loads were yielded from both experiments. This method can still be used satisfactorily only when this type of heparinized blood is chosen. Remarkably, even though a large average number of ingested microfilariae recovered from stain-smeared blood meals from five infected midguts of immediately fed *O. togoi* and from fully engorged *O. togoi* fed on human- and chicken-heparinized blood containing *B. malayi* microfilariae in experiment 2 [human-heparinized blood, 33.60 (29–71); chicken-heparinized blood, 37.80 (32–126)], the average number microfilariae acquired 24 h after feeding differed markedly among five infected thoracic muscles [human-heparinized blood, 17.60 (12–44); chicken-heparinized blood, 7.20 (1–6)]. This clearly resulted from the low migration number of ingested microfilariae from the midguts to thoracic muscles, which appeared to be inhibited by some content factor(s) of chicken-heparinized blood.

The use of HBSS-rich-*B. malayi* microfilariae from peritoneal withdrawal of *B. malayi*-infected jirds is another beneficial point that has not been reported extensively or practically, in particular, the adjustment of appropriate microfilarial density (microfilarial density ranged from about 300 to 400 mf per 20 μl) that is suitable for target experiments. Approximately 2 ml + peritoneal withdrawal of HBSS-rich-*B. malayi* microfilariae could be prepared for at least 100 ml + of human-, swine-, bovine-, or chicken-heparinized blood containing *B. malayi* microfilariae, as 0.05 ml of HBSS-rich-*B. malayi* microfilariae can be prepared for approximately 10 ml of blood containing microfilariae. These microfilariae are very important in performing massive artificial feeding of *O. togoi* in order to obtain a large number of L_3 larvae for massive intraperitoneal and/or subcutaneous jird inoculations. However, many previous reports have declared the ability to perform direct mosquito feeding on peripheral blood of anesthetized jirds that were inoculated subcutaneously with *B. malayi* L_3 larvae (Nayar and Knight 1995; Michalski et al. 2010; Aliota et al. 2010). Nonetheless, at least two drawbacks from this type of feeding have been raised, i.e., (1) an approximately 2 ml of blood (measured from heart-punctured jirds) per 60–80 g of jird-weight could not be used for massive mosquito feeding directly on jirds, and (2) microfilarial density in the peripheral blood (microfilarial density ranged from about 1 to 198 mf per 20 μl) of infected jirds was erratic (Choochote et al. 1991; Nayar and Knight 1995; Michalski et al. 2010; Aliota et al. 2010).

It is also worth noting that the autogenous *O. togoi* colony is easy to maintain and mass-produce in a simple insectary at 27±2 °C and 70–80 % relative humidity, with fluorescent

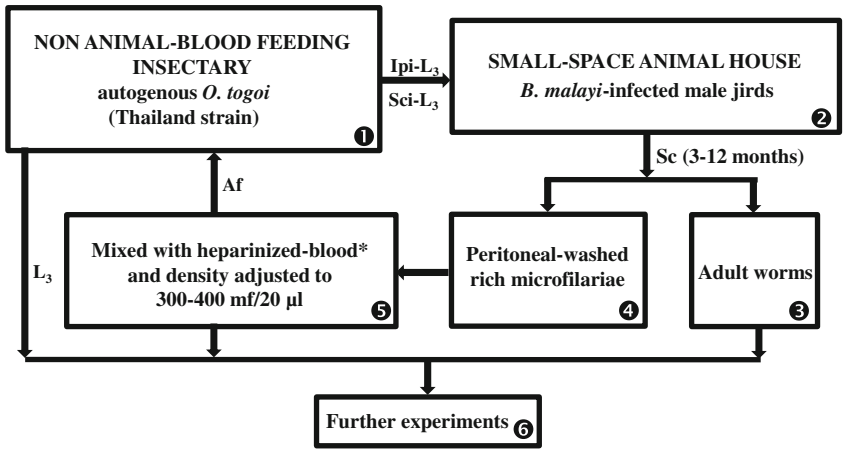

Fig. 3 Summarized systematic procedures from *1–6* for the mass production of microfilariae, infective (L_3) larvae, and adults of *B. malayi* under nonblood-feeding insectary and small-space animal-house conditions. *Ipi* intraperitoneal inoculation, *Sci* subcutaneous inoculation, *Sc* sacrifice, *Af* artificial membrane feeding

lighting for approximately 12 h per day. However, the rearing procedures in obtaining healthy adults are very important for providing high survival rates in *B. malayi*-infected *O. togoi* during development from L_1 to L_3 larvae, which takes approximately 14 days after an infected blood meal. Thus, throughout the larval rearing period, the number of larvae, rearing in rearing tray conditions and food feeding schedule were the most important factors for success in establishing a healthy *O. togoi* laboratory colony. The protocol for appropriate rearing condition is 300 L_1 instar larvae per 20×36×7.5 cm tray filled with 3,000 ml of deionized tap water. Stressful rearing conditions, e.g., overcrowding of larvae in a rearing tray and use of an inappropriate water medium and improper food-feeding schedule would lead to a rapid reduction or loss of a colony. Also, this would result in low-larval and pupal-survival rates, short life span of adult females and males, fewer or no eggs laid by gravid adult females, and low egg-hatchability. As mentioned previously, the food-feeding schedule was one of the most important factors for obtaining healthy larvae. Thus, 0.5 g of fine dog biscuit suspended in 10 ml of deionized tap water was added every 3 days for the first and second instar larvae, and this schedule was increased to every other day using 1.0 g of fine dog biscuit suspended in 10 ml of deionized tap water after most of the larvae had reached the third and fourth instars. However, in order to avoid scum forming and/or rotting in rearing water, due to unconsumed food and larval waste products, the water should be changed twice weekly or immediately whenever signs of scum and/or rotting are observed. By following these rearing procedures, the developmental time of one generation (from eggs to eggs) was approximately 29 days, comprising a duration of 2–4 (3),

Table 1 The infective rates and parasite loads of *O. togoi* (F_{98} and F_{102} in experiment 1 and 2, respectively) after feeding on human-, swine-, bovine-, and chicken-heparinized blood containing *B. malayi* microfilariae, with all mosquitoes dissected 14 days after feeding

Type of blood (no. of mf per 20 µl)	Infective rates (no.)	Average no. of L_3 per infected mosquito (range)	L_3 distribution			
			Percentage of head (no.)	Percentage of thorax (no.)	Percentage of abdomen (no.)	Total
Human blood						
Exp. 1 (304)	86.67 (26/30)	10.58 (1–36)	85.82 (236)	7.64 (21)	6.54 (18)	275
Exp. 2 (339)	93.33 (28/30)	12.36 (1–42)	79.19 (274)	14.16 (49)	6.65 (23)	346
Swine blood						
Exp. 1 (346)	96.67 (29/30)	10.38 (1–27)	68.44 (206)	16.94 (51)	14.62 (44)	301
Exp. 2 (328)	83.33 (25/30)	8.40 (1–30)	73.33 (154)	16.67 (35)	10 (21)	210
Bovine blood						
Exp. 1 (310)	76.67 (23/30)	9.91 (1–28)	85.09 (194)	7.89 (18)	7.02 (16)	228
Exp. 2 (322)	80 (24/30)	9.75 (1–21)	91.88 (215)	3.85 (9)	4.27 (10)	234
Chicken blood						
Exp. 1 (355)	73.33 (22/30)	3.41 (1–9)	88 (66)	10.67 (8)	1.33 (1)	75
Exp. 2 (396)	76.67 (23/30)	4.65 (1–17)	85.98 (92)	11.22 (12)	2.80 (3)	107

Table 2 The susceptibility of male Mongolian jirds to *B. malayi* after intraperitoneal inoculation with infective larvae; all animals were sacrificed 32 weeks after inoculation

Animal no.	No. of L_3 succeeded in inoculating	No. of adult recovered (%)	No. of female and male (%)	
			Female	Male
1	118	37 (31.36)	18 (48.65)	19 (51.35)
2	109	16 (14.68)	12 (75.00)	4 (25.00)
3	114	44 (38.60)	23 (52.27)	21 (47.73)
4	120	31 (25.83)	14 (45.16)	17 (54.84)
5	112	39 (34.82)	17 (43.59)	22 (56.41)
6	117	24 (20.51)	15 (62.50)	9 (37.50)
7	120	28 (23.33)	12 (42.86)	16 (57.14)
8	113	52 (46.02)	29 (55.77)	23 (44.23)
9	120	35 (29.17)	22 (62.86)	13 (37.14)
10	119	21 (17.65)	15 (71.43)	6 (28.57)

1–3 (2), and 10–16 (14) days of embryonic egg development, egg hatching, and larval and pupal development, respectively, as well as added durations of 2–4 (3), 3–5 (4), and 2–3 (3) days for pupal emergence, gravid-developed females, and egg-laid females, respectively. An additional benefit is that the deposited eggs on filter paper and kept in a plastic bag (Fig. 4) could be stored for at least 6 months in a refrigerator (8–10 °C), with embryonation and hatching rates of 79 and 75 %, respectively, when compared with 87 and 84 %, respectively, for 5-day-old eggs.

It is anticipated that the facile system for mass production of *B. malayi* in a small-space laboratory, as reported in this paper, will benefit other investigators, who are interested in various research aspects of filarial *B. malayi*. Finally, our laboratory can provide eggs of autogenous *O. togoi* (F_{100+}) massively to other investigators in order to facilitate the study of filariasis and/or other related aspects.

Fig. 4 Showing deposited eggs attached to filter paper and kept in a plastic bag before storing in a refrigerator (8–10 °C). The vital eggs could be kept for at least 6 months by keeping in this condition

Conclusion

This study has developed a facile system for the maintenance and mass production of *B. malayi* in a small-space laboratory by combining benefits of the autogenous *O. togoi* colony (Thailand strain) with HBSS-rich-*B. malayi* microfilariae obtained from peritoneal withdrawal of *B. malayi*-infected jirds. The 2 ml + of HBSS-rich-*B. malayi* microfilariae could be prepared for at least 100 ml + of blood containing *B. malayi* microfilariae (microfilarial density ranging from about 300–400 mf per 20 μl), which is the main key for performing massive artificial feeding of *O. togoi* on blood containing *B. malayi* microfilariae. The high susceptibility rates and parasite loads of autogenous *O. togoi* to *B. malayi* have led to the mass production of L_3 larvae. This is an important key in performing massive intraperitoneal inoculation of L_3 larvae into male jirds in order to mass-produce *B. malayi* developing stages and adults. The success in rearing an autogenous *O. togoi* colony for many consecutive generations (F_{100+}) has led to the complete reduction of animal-house space for maintaining blood-feeding animals that are necessary for producing blood-feeding mosquito eggs [*S. aegypti* (Liverpool strain) and *O. togoi* (Taiwan strain)].

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DNA barcoding for the identification of eight species members of the Thai Hyrcanus Group and investigation of their stenogamous behavior

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ABSTRACT

Eight species members of the Thai Hyrcanus Group were identified based on the intact morphology and molecular analysis (COI barcoding, 658 bp) of F_1-progenies. Five isofemale lines of each species were pooled in order to establish stock colonies. A stenogamous colony of each species was investigated by making 200 and 300 newly emerged adult females and males co-habit in a 30 cm cubic cage for one week. After ovipositon, the spermathecae of females were examined for sperms. The results revealed that *Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nitidus*, *Anopheles pursati*, *Anopheles sinensis*, *Anopheles nigerrimus*, *Anopheles paraliae* and *Anopheles peditaeniatus* yielded insemination rates of 0%, 0%, 0%, 31%, 33%, 42%, 50% and 77%, respectively. Continuous selection to establish stenogamous colonies indicated that *An. sinensis*, *An. pursati*, *An. nigerrimus*, *An. paraliae* and *An. peditaeniatus* provided insemination rates of 33–34%, 27–31%, 42–58%, 43–57% and 61–86% in 1, 2, 5, 6 and 20 generations of passages, respectively.

1. Introduction

The Hyrcanus Group Reid (Genus *Anopheles*, Subgenus *Anopheles*) is distributed widely from Europe to East and Southeast Asia, including some of the off-lying islands of the Indian and Pacific Oceans, and at least 27 species are reported within it [1]. In Thailand, eight species members of the Hyrcanus Group have been reported so far, i.e., *Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nigerrimus*, *Anopheles nitidus*, *Anopheles paraliae*, *Anopheles peditaeniatus*, *Anopheles pursati*, and *Anopheles sinensis* [2–4]. Among these, *An. nigerrimus*, *An. peditaeniatus* and *An. sinensis* are considered as suspected vectors of malaria due to *Plasmodium vivax* [5–9], while *An. sinensis* and *An. peditaeniatus* have been incriminated as vectors of *P. vivax* in China and Korea [10,11] and Japanese

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encephalitis virus in China and India [12–14], respectively. Even though *An. peditaeniatus* has been found abundantly and widely distributed throughout Thailand [3,15], its status as a vector of the Japanese encephalitis virus is still a cryptic question, which needs to be investigated more intensively. Recently, *An. sinensis* and *An. nigerrimus* have been incriminated as a main vector and secondary or incidental vector, respectively, of *Wuchereria bancrofti* in Asia [16]. In addition, *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus, An. argyropus* and *An. pursati* were reported as high potential vectors of nocturnally subperiodic *Brugia malayi* [17]. Likewise, the *Anopheles hyrcanus* group was also considered as an economic pest of cattle because of its vicious biting behavior and ability to transmit cervid filariae of the genus *Setaria* [2,3].

Establishment of anopheline colonies is the backbone of mosquito researches, and the inability to create a healthy colony of difficult-to-rear species is the principal cause behind every failure in research efforts. Very few research experiments concerning the Hyrcanus Group have been documented, during the past two decades, particularly those with a complete multidisciplinary approach (combination of related-aspects of morphology, cytology, molecular investigation, hybridization, susceptibility and refractory to pathogens, etc.), although eight species members of the Hyrcanus Group are found throughout Thailand and/or other Southeast Asian countries [2,3]. This might result from the lack of biological information and/or available laboratory-raised colonies, particularly the adaptive stenogamous colonies that are easy to maintain and mass produce, which reduces time, workload and manpower for artificially mating adult females with males. Hence, this paper reports establishment of a stenogamous colony of *An. peditaeniatus* that has existed for more than 20 successive generations, as well as promising possible stenogamous colonies of *An. paraliae* and *An. nigerrimus*, which are still being established and detailed. Furthermore, the utility of DNA barcoding, which is incorporated with the taxonomic key for exact identification of the eight species members, is present as well.

2. Materials and methods

2.1. Mosquito species and strains

Eight species members of the Hyrcanus Group were collected in five provinces of western and southern Thailand, where malaria and filariasis are endemic due to *Plasmodium falciparum* and *P. vivax*, and *W. bancrofti*, respectively [16,18]. The species and strains were as follows: *An. argyropus* (Nakhon Si Thammarat strain: 08°29′N, 100°0′E), *An. crawfordi* (Trang strain: 07°33′N, 99°38′E), *An. nigerrimus* (Songkhla strain: 07°13′N, 100°37′E), *An. nitidus* (Phang Nga strain: 08°27′N, 98°31′E), *An. paraliae* (Ratchaburi strain: 13°30′N, 99°54′E), *An. peditaeniatus* (Phang Nga strain: 08°27′N, 98°31′E), *An. pursati* (Ratchaburi strain: 13°30′N, 99° 54′E) and *An. sinensis* (Chumphon strain: 10°29′N, 99°11′E). Wild caught, fully engorged females of these species members were collected from cow-baited traps.

2.2. Species identification

Identification of wild caught females followed standard illustrated keys [2–4]. Subsequently, identification using intact morphology of eggs, larvae, pupal skins and adult females were performed intensively in F_1-progenies of iso-female lines.

2.3. Molecular investigation

In order to guarantee the exact morphological species identification, thus, individual F_1-progeny adult female of each iso-female line was performed for DNA extraction and amplification. Genomic DNA was extracted using DNeasy® Blood and Tissue Kit (QIAGEN, Japan). The LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) barcoding primers of Folmer et al. [19] were used to amplify the cytochrome *c* oxidase subunit I (COI) region of mitochondrial DNA (658 bp, excluding primers). Each PCR reaction was carried out in a 20-μL volume containing 0.5 U of *Ex Taq* (Takara, Japan), 1X of *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer, and 1 μL of the extracted DNA. The amplification profile comprised initial denaturation at 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. Lastly, the PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN, Japan) and their sequences directly determined using the BigDye® V3.1 Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems of Life Technologies, Japan). The sequence data obtained have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under the following accession numbers: *An. argyropus* (Nakhon Si Thammarat strain: AB781747-AB781751), *An. crawfordi* (Trang strain: AB781752-AB781756), *An. nigerrimus* (Songkhla strain: AB781757-AB781761), *An. nitidus* (Phang Nga strain: AB781762-AB781766), *An. paraliae* (Ratchaburi strain: AB781767-AB781771), *An. peditaeniatus* (Phang Nga strain: AB781772-AB781776), *An. pursati* (Ratchaburi strain: AB781777-AB781781) and *An. sinensis* (Chumphon strain: AB781782-AB781786). The newly COI sequences were also compared with those available in GenBank using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. *Anopheles gambiae* (accession number NC_002084) and *Anopheles braziliensis* (accession number DQ076238) were used as outgroup taxa [20,21]. Sequences were aligned with BioEdit version 7.0.5.3 [22]. Genetic distance was calculated using the Kimura two-parameter (K2P) model [23]. Using the distances, construction of neighbor-joining trees [24] and bootstrap test with 10,000 replications were performed with the MEGA version 4.0 program [25]. Bayesian analysis was conducted with MrBayes 3.2 [26] by using two replicates of 1 million generations with the nucleotide evolutionary model. The

best-fit model GTR + I + G were chosen using the Akaike Information Criterion (AIC) in MrModeltest version 2.3 [27]. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% trees as burn-in.

2.4. Rearing procedures

Mosquito rearing procedures for the Thai Hyrcanus Group (swamp-breeders) followed the detailed techniques described by Choochote and Saeung [28]. All of the experiments were performed in the simple insectarium at 27 ± 2 °C, 70–80% relative humidity, and illumination from a combination of natural daylight from a glass window and fluorescent lighting was provided for approximately 12 h a day.

2.5. Establishment of a stock colony

After exact species identification, based on the intact morphology of eggs, larvae, pupal skins and adult females, and molecular investigations in F_1-progenies, the stock colonies of the eight anopheline species were established by pooling five iso-female lines of each anopheline species that have been colonized consecutively for more than 10 generations. These stock colonies were used in the investigation of stenogamous behavior throughout the experiments.

2.6. Screening of stenogamous behavior and establishment of natural mating colonies

Mosquitoes of the ninth generation (F_9) were used to determine the natural mating ability in a standard $30 \times 30 \times 30$ cm cage. The reason for using this mosquito generation was based on the fact that any mosquito colony, colonized for more than eight consecutive generations, was of adaptive laboratory mosquito-strains, and easily maintained and mass produced for any experiments. Thus, 200 female and 300 male newly emerged mosquitoes of the ninth generation were introduced into the same cage to co-habit for one week following the former procedures of Sucharit and Choochote [29], and Choochote et al. [30]. This provided a density resting surface (or vertical resting surface per mosquito) of 7.20 [31]. Subsequently, the fasted females were allowed to feed on white rat. Five days after feeding, 20 gravid females were allowed to lay eggs for two days in an oviposited-plastic cup [28], and later, the spermathecae of females were investigated for the presence of sperms. All the eggs obtained from natural copulation in the $30 \times 30 \times 30$ cm cage were processed for hatching, larval and adult rearing, and use for establishing the next stenogamous colony. This process was performed repeatedly from generation to generation until the stenogamous colony was stable.

3. Results

3.1. Species identification

Species identification, based on molecular analyses (cytochrome *c* oxidase subunit I barcoding region, 658 bp), was generated from a total of 40 specimens of the F_1-progenies of the eight species, i.e., using one specimen from each of the iso-female lines of each species. The NJ and Bayesian trees revealed eight distinct clusters. Each species ($n = 5$) showed a low level of mean intra-specific genetic distances (0.001–0.009), whereas the mean inter-specific genetic distances were high (0.032–0.077) (Table 1). Among the species examined, the NJ tree revealed two major clades. Clade I comprised *An. crawfordi*, *An. paraliae*, *An. peditaeniatus*, and *An. sinensis* as well as a member of the Korean Hyrcanus Group, *Anopheles lesteri*. In this clade, the five species were separated from each other, with high bootstrap support (98–100%) (Fig. 1). The phylogenetic relationship revealed that *An. paraliae* was more closely related to *An. peditaeniatus* (mean genetic distances = 0.038) than that of *An. crawfordi* (mean genetic distances = 0.041). In addition, *An. paraliae* showed closer proximity to *An. sinensis* (mean genetic distances = 0.032) than that of *An. crawfordi* (mean genetic distances = 0.047). Remarkably, the low level of mean genetic distances (0.011) between *An. paraliae* and *An. lesteri* was observed in this study. Clade II comprised *An. argyropus*, *An. nigerrimus*, *An. nitidus* and *An. pursati*, which were monophyletic with high bootstrap support (99–100%). Within this clade, *An. nitidus* was more closely related to *An. pursati* (mean genetic distances = 0.039) than that of *An. nigerrimus* (mean genetic distances = 0.049). Additionally, *An. nitidus* was closely related to *An. argyropus* (mean genetic distances = 0.054) than that of other species. *Anopheles crawfordi* and *An. pursati* were most genetically diverse, with mean genetic distances of 0.077. The Bayesian tree showed almost similar tree topologies to the NJ tree (Fig. 2). Four species members, including *An. argyropus*, *An. nigerrimus*, *An. nitidus*, and *An. pursati*, were placed within the same clade, with high posterior probability (99%), and well separated from the remaining five species.

Table 1
Mean intra- and inter-specific genetic distances (K2P) in eight species members of the Thai Hyrcanus Group based on COI barcoding sequences.

Species	Mean intra-specific genetic distances ($n = 5$)	1	2	3	4	5	6	7
1. *An. argyropus*	0.004							
2. *An. crawfordi*	0.002	0.069						
3. *An. nigerrimus*	0.002	0.057	0.061					
4. *An. nitidus*	0.009	0.054	0.075	0.049				
5. *An. paraliae*	0.001	0.059	0.041	0.050	0.058			
6. *An. peditaeniatus*	0.005	0.069	0.057	0.067	0.059	0.038		
7. *An. pursati*	0.006	0.058	0.077	0.050	0.039	0.062	0.063	
8. *An. sinensis*	0.002	0.061	0.047	0.062	0.059	0.032	0.048	0.076

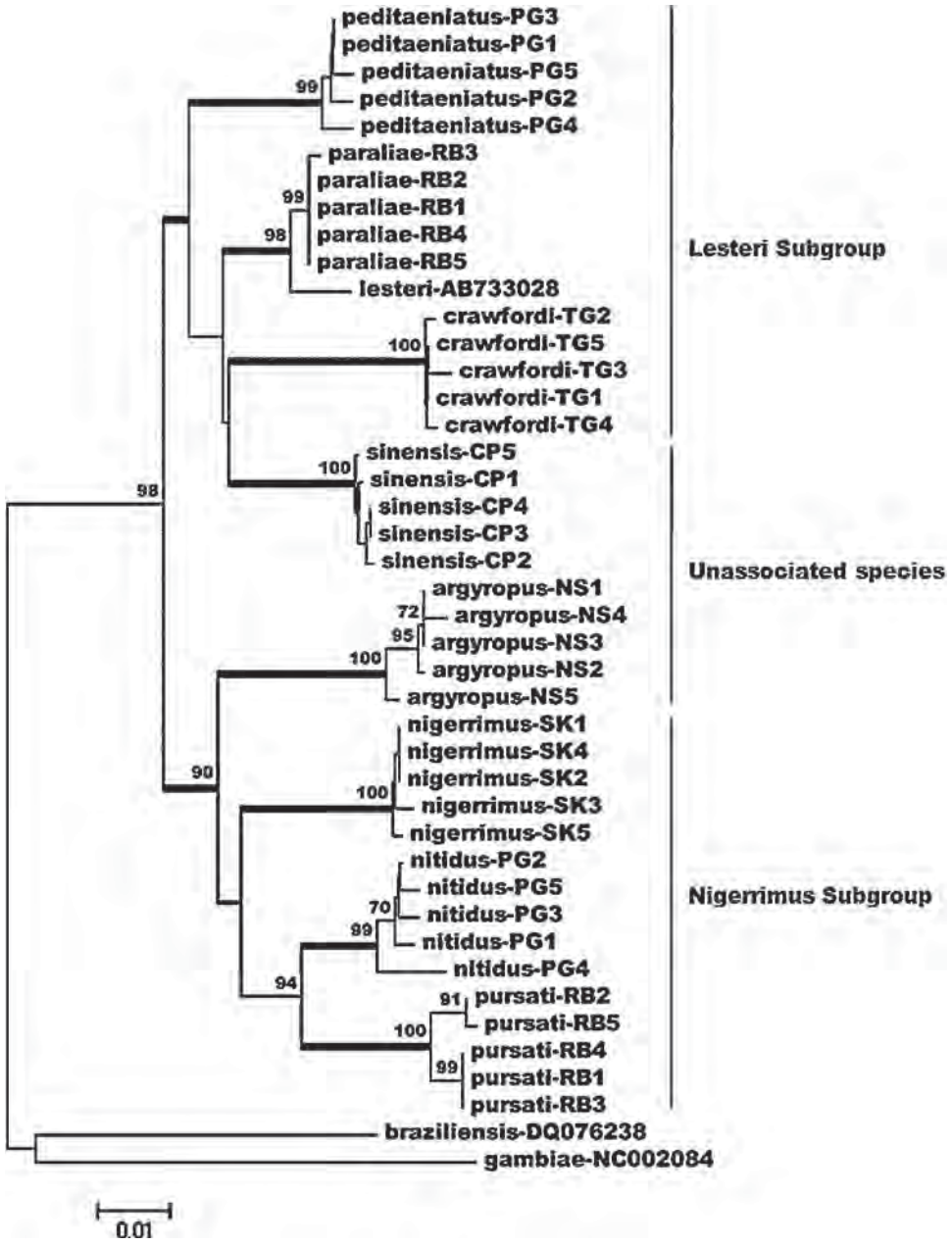

Fig. 1. Bootstrapped neighbor-joining tree (NJ) of the COI barcoding sequences of eight species members of the Thai Hyrcanus Group and a member of the Korean Hyrcanus Group, *Anopheles lesteri*. The *Anopheles gambiae* and *Anopheles braziliensis* were used as outgroups. Bootstrap values of higher than 70% are shown above the node. Bars represent 0.01 substitutions per site.

Anopheles paraliae were nested with *An. lesteri* with moderate support (posterior probability = 70%), whereas, *An. crawfordi*, *An. peditaeniatus*, and *An. sinensis* was placed into three distinct clusters with high support (posterior probability = 97–100%).

3.2. Establishment of a stock colony

Eight species members of the Hyrcanus Group, i.e., *An. argyropus* (F_{23}), *An. crawfordi* (F_{19}), *An. nigerrimus* (F_{15}), *An. nitidus* (F_{16}), *An. paraliae* (F_{16}), *An. peditaeniatus* (F_{29}), *An. pursati* (F_{12}) and *An. sinensis* (F_{11}) were reared successfully for many consecutive generations by using artificial mating techniques. The developmental time of one generation (from eggs to eggs) was more or less 28 days, comprising a duration of 3–4 (3), 8–14 (11) and 1–3 (2) days of egg, larval and pupal development, respectively, as well as added durations of 5-day-old adult females for blood feeding, 5 days for gravid-developed females, and 2 days for egg-laid females.

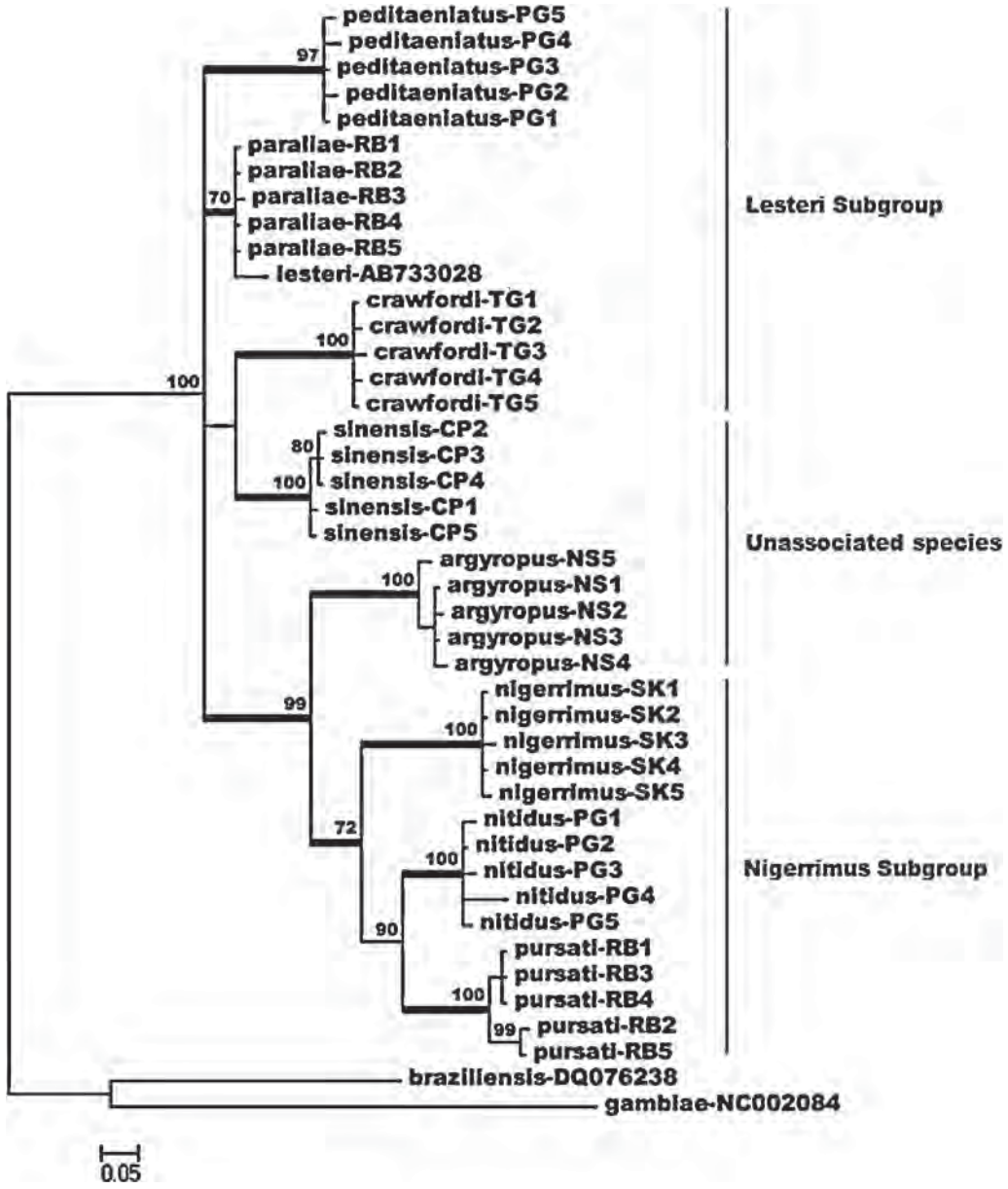

Fig. 2. Bayesian phylogenetic tree based on COI barcoding sequences of eight species members of the Thai Hyrcanus Group and a member of the Korean Hyrcanus Group, *Anopheles lesteri*. The *Anopheles gambiae* and *Anopheles braziliensis* were used as outgroups. The posterior probability of higher than 70% is shown above the node. Bars represent 0.05 substitutions per site.

3.3. Screening of stenogamous behavior and establishment of natural mating colonies

The results of induced natural copulation in a $30 \times 30 \times 30$ cm cage by co-habiting 200 and 300 newly emerged female and male F_9 mosquitoes for 1 week, indicated that only five species, i.e., *An. pursati*, *An. sinensis*, *An. nigerrimus*, *An. paraliae* and *An. peditaeniatus* could lay eggs successfully with insemination rates of 31%, 33%, 42%, 50% and 77%, respectively (Table 2). The remaining three species of *An. argyropus*, *An. crawfordi* and *An. nitidus* failed to copulate naturally and provided a 0% insemination rate at F_9, F_{10} and F_{11}. After selection, the stenogamous colony of *An. peditaeniatus* was colonized continuously for more than 20 generations, with insemination rates that ranged from 61–86%. As for *An. sinensis*, *An. pursati*, *An. nigerrimus* and *An. paraliae*, selection of the stenogamous colony is still in progress for 1, 2, 5 and 6 selected generations of passages, respectively, by yielding the insemination rates ranging from 33–34%, 27–31%, 42–58% and 43–57%, respectively.

4. Discussion

Prior to the study on biology, ecology and behavior of mosquito-borne disease vectors, accurate species

Table 2
The insemination rates of selective stenogamous colonies of *Anopheles peditaeniatus*, *Anopheles paraliae*, *Anopheles nigerrimus*, *Anopheles pursati* and *Anopheles sinensis*.

Mosquito species	Insemination rates of each selected generation of passage (F_{9-29})[a]																				
	F_9	F_{10}	F_{11}	F_{12}	F_{13}	F_{14}	F_{15}	F_{16}	F_{17}	F_{18}	F_{19}	F_{20}	F_{21}	F_{22}	F_{23}	F_{24}	F_{25}	F_{26}	F_{27}	F_{28}	F_{29}
An. peditaeniatus	77	81	75	69	62	83	86	76	61	72	68	64	67	85	66	71	80	72	76	74	79
An. paraliae	50	49	43	45	57	52	56	IP													
An. nigerrimus	42	44	51	46	54	58	IP														
An. pursati	31	27	30	IP																	
An. sinensis	33	34	IP																		

IP: in progress.
[a] Examined from 100 spermatecae.

identification is the main key for success in obtaining robust information. Consequently, ribosomal (ITS2) and mitochondrial (COI and COII) DNA have been used intensively and widely in the Oriental region for recognition of the species members of the Hyrcanus Group [1,32–42]. Nonetheless, information on COI barcoding sequences for identification of eight species of the Hyrcanus Group in Thailand is lacking. Thus, we initiated this approach to develop DNA barcodes for this species group. Our phylogenetic analyses here separated eight species members of the Thai Hyrcanus Group into eight distinct clusters. In accordance with Harbach [43] and Rattanarithikul et al. [9], *An. crawfordi*, *An. paraliae*, and *An. peditaeniatus* were classified into the Lesteri Subgroup, while *An. nigerrimus*, *An. nitidus* and *An. pursati* were classified into the Nigerrimus Subgroup. The *An. argyropus* and *An. sinensis* were categorized into the Unassociated Species. However, interestingly, *An. sinensis* and *An. argyropus* show closer relationship to the Lesteri and the Nigerrimus Subgroup, respectively (Figs. 1 and 2). Furthermore, the intra-specific genetic distances was less than 1%, while the inter-specific genetic distances was greater than the 2% value obtained from this study, which is in agreement with the threshold value for distinguishing species based on the COI barcode sequences [44–47]. In contrast to the previously reported of the Neotropical *Anopheles* (*Nyssorhynchus*) by Foster et al. [48], our results showed a non-overlapping of intra- and inter-specific genetic distances based on COI sequences. In addition, the COI sequences of *An. paraliae*, *An. peditaeniatus* and *An. sinensis* showed little difference (mean genetic distances = 0.002–0.005) from previous deposited-sequences in GenBank, i.e., *An. paraliae* (accession number AB733031) [49], *An. peditaeniatus* (accession numbers AB539069 and AB715091) [42], and *An. sinensis* (accession number AY444351) [33]. It was interesting to note that *An. lesteri* from Korea (accession number AB733028) showed a low level (0.011) of inter-specific genetic distances with our *An. paraliae*, which was congruent with the current results of Taai et al. [49], who suggested that these two morphological species were presumed conspecific, based on results of no post-mating reproductive isolation from hybridization experiments, together with the molecular data of low pairwise genetic distances of COI (0.007–0.017). In conclusion, our findings on mitochondrial DNA sequences of the COI barcoding region (658 bp) could be used for the identification of eight species members of the Thai Hyrcanus Group.

It has long been known that the anopheline mosquito has difficulty in copulating naturally under laboratory conditions, especially in a small space-cage, such as a $30 \times 30 \times 30$ cm cage. However, some species could succeed in copulating in a small space-cage, e.g., *Anopheles quadrimaculatus* [50,51], *An. gambiae* complex [52], *Anopheles earlei* [53], *An. sinensis* [54–56], *Anopheles farauti* No. 1 [57], *Anopheles albimanus* [58], *Anopheles subpictus* [59], *Anopheles balabacensis* (Perlis Form = *Anopheles dirus* B, recently as *Anopheles cracens*) [29,60], *Anopheles annularis* [30], *An. dirus* [61], *Anopheles barberi* [62], *Anopheles sergentii* [63], *Anopheles freeborni* [64], *Anopheles barbirostris* [65], *Anopheles minimus* [66], *Anopheles albitarsis* [67], *Anopheles maculatus* [68], *Anopheles aquasalis* [69], *Anopheles stephensi* [70] and *Anopheles pseudopunctipennis* [71]. Therefore, artificial mating techniques have been developed by previous investigators in order to solve the mating problems for maintaining laboratory colonies [72,73].

The mating behavior of anophelines in nature usually starts from the males, dancing in a swarm at dusk, often over the tops of bushes and other objects, whereas females may be seen to enter these swarms in small numbers. Each female is grabbed promptly by a male, which locates her through the hearing organs in his antennae, and then, the couple can be seen to fall out from the swarm [2]. In *An. maculatus*, swarming is 15–21 feet from the ground, which is rather high in comparison to other anopheline species [74]. Additional mating behavior results of *Anopheles stephensi* var. *mysorensis* in nature revealed approximately 400 copulations per swarm of 500–600 males, thus, confirming that anopheline females are mated as a result of entering swarms of males [75]. In laboratory conditions, the limited space in a $30 \times 30 \times 30$ cm cage appears to prevent swarming, and therefore causes failure of copulation. However, there are many species of *Aedes*, *Culex* and *Mansonia* mosquitoes that can copulate without males forming a swarm, and they mate easily in small spaces [74,76,77]. In this study, three species members of the Hyrcanus Group (*An. argyropus*, *An. crawfordi* and *An. nitidus*) failed to copulate naturally in a $30 \times 30 \times 30$ cm cage (experiment repeated three times). The success in selecting a stenogamous *An. peditaeniatus* colony strongly suggested that an artificially mated colony of this anopheline species could be adapted easily to natural copulation in a $30 \times 30 \times 30$ cm cage.

It has been documented that the behavioral polymorphism stenogamy/eurygamy of anophelines is

inherited and obviously controlled by one or more genes located on the Y-chromosome [78]. Additionally, the difference of male genitalia morphometry and frequency of clasper movements, which may be involved in the stenogamous behavior of mosquitoes, e.g., between stenogamous *An. balabacensis* (Perlis Form) and eurygamous *An. dirus* (Bangkok Colony Strain = *An. dirus* A, recently as *An. dirus* s.s.) [60], have been reported [29]. The genitalia of *An. balabacensis* (Perlis Form) are larger than that of *An. dirus* (Bangkok Colony Strain) and the former has a shorter duration of clasper movement and mating time than the latter species.

In conclusion, successful selection of the stenogamous colony of *An. peditaeniatus*, which yielded high insemination rates within a few generations, warrants intensive investigation of a specific stenogamy/eurygamy control mechanism(s) in this anopheline species group, and all the experiments are currently progressing.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Cytogenetic, hybridization and molecular evidence of four cytological forms of *Anopheles nigerrimus* (Hyrcanus Group) in Thailand and Cambodia

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ABSTRACT: Thirteen isoline colonies of *Anopheles nigerrimus* were established from individual wild-caught females collected from cow-baited traps at locations in Thailand and Cambodia. Three types of X (X_1, X_2, X_3) and 4 types of Y (Y_1, Y_2, Y_3, Y_4) chromosomes were recovered, according to differing amounts of extra heterochromatin. Four karyotypic forms were designed depending upon apparently distinct figures of X and Y chromosomes, i.e., Form A (X_1, X_2, X_3, Y_1), B (X_2, X_3, Y_2), C (X_1, Y_3), and D (X_3, Y_4). Forms C and D were new metaphase karyotypes discovered in this study. Form A appeared to be common in both Thailand and Cambodia. Forms B and D were found to be rather specific to southern and northeastern Thailand, respectively, whereas Form C was confined to Cambodia. Hybridization experiments among the eight isoline colonies, which were representative of four karyotypic forms of *An. nigerrimus*, demonstrated genetic compatibility in giving viable progenies and synaptic salivary gland polytene chromosomes through F_2-generations. These results elucidated the conspecific relationship, comprising four cytological forms within this taxon. Supportive evidence was confirmed further by very low intraspecific sequence variations (average genetic distance = 0.002-0.007) of the nucleotide sequences in ribosomal DNA [second internal transcribed spacer (ITS2)] and mitochondrial DNA [cytochrome *c* oxidase subunit I (COI) and subunit II (COII)]. ***Journal of Vector Ecology* 38 (2): 266-276. 2013.**

Keyword Index: *Anopheles nigerrimus*, metaphase karyotypes, hybridization experiments, ITS2, COI, COII.

INTRODUCTION

Anopheles (*Anopheles*) *nigerrimus* belongs to the Nigerrimus Subgroup and Hyrcanus Group of the Myzorhynchus Series, and is distributed widely in Thailand and other countries, i.e., India (Assam, Bihar and Punjab), Sri Lanka, Bangladesh, China (Hainan Island), Myanmar, Laos, Cambodia, Vietnam, Malaysia (Malaysian Peninsular, Sabah and Sarawak), Indonesia (Java and Sumatra), and Brunei (Reid 1968, Scanlon et al. 1968, Harrison and Scanlon 1975, Knight and Stone 1977, Harbach 2012). Regarding medical importance, *An. nigerrimus* was incriminated as a suspected vector of *Plasmodium vivax* in Thailand (Baker et al. 1987, Harbach et al. 1987, Gingrich et al. 1990, Rattanarithikul et al. 1996) and *P. falciparum* and *P. vivax* in Bangladesh (Alam et al. 2010, 2012). Recently, it was incriminated as a secondary or incidental vector of *Wuchereria bancrofti* in Asia (Manguin et al. 2010). Additional experiments indicated that this anopheline species could serve as a potential vector of the filarial nematode, nocturnally subperiodic *Brugia malayi*, as determined by 50-65% susceptibility rates and 4.20-9.77 average number of L_3 larvae per infected mosquito (Saeung et al. 2013).

Cytologically, two karyotypic forms of *An. nigerrimus*, Form A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), were obtained from Ubon Ratchathani and Ayutthaya Provinces, respectively (Baimai et al. 1993). These two karyotypic variants appeared to result from a gradual increase in the extra heterochromatin on X and Y chromosomes (Baimai 1998). Genetic variation at the chromosomal level, within the taxon *Anopheles*, potentially results in the existence of species complex and causes difficulty in exactly identifying sibling species (isomorphic species) and/or subspecies (cytological forms) members that result from identical morphology or minimal morphological distinction. Additionally, those members may differ in biological characteristics (e.g., microhabitats, resting and biting behaviors, sensitivity or resistance to insecticides, susceptible or refractory to pathogens, etc.), which can be used to determine their potential for transmitting pathogenic agents. Inability to identify individual members in the complexes of *Anopheles* vectors may result in the failure to differentiate between a vector and non-vector species, and result in unsuccessful vector control (Subbarao 1998, Van Bortel et al. 2001, Singh et al. 2010). Regarding the

above information, very little is known about the genetic proximities among karyotypic variants of *An. nigerrimus*. Thus, we first report two new karyotypic forms [C (X_1, Y_3) and D (X_3, Y_4)] of *An. nigerrimus* and determine the genetic proximity among its four karyotypic variants by hybridization experiments related to the comparative DNA sequencing of the second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI), and cytochrome *c* oxidase subunit II (COII) of mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Field collections and establishment of isoline colonies

Wild-caught, fully engorged female mosquitoes of *An. nigerrimus* were collected from cow-baited traps at four allopatric locations in Thailand (Lampang Province, northern region; Ubon Ratchathani Province, northeastern region; Songkhla and Nakhon Si Thammarat Provinces, southern region), and one location in Cambodia (Ratanakiri Province) (Figure 1, Table 1). A total of 13 isolines were established successfully and maintained in our insectary using the techniques described by Choochote and Saeung (2013). Exact species identification was performed by using intact morphology of egg, larval, pupal, and adult stages from the F_1-progenies of isolines, following standard keys (Reid 1968, Harrison and Scanlon 1975, Rattanarithikul et al. 2006). These isolines were used for studies on the metaphase karyotype, hybridization experiment, and molecular analysis.

Metaphase karyotype preparation

Metaphase chromosomes were prepared from ten samples of the early 4th-instar larval brains of F_1-progeny of each isoline, using techniques previously described by Saeung et al. (2007). Identification of karyotypic forms followed the standard cytotaxonomic systems of Baimai et al. (1993).

Hybridization experiment

The eight laboratory-raised isolines of *An. nigerrimus* were selected arbitrarily from the 13 isoline colonies, which were representative of four karyotypic forms, i.e., Form A [Lp1A (X_1, Y_1), Ur1A (X_2, Y_1), Ns3A (X_2, Y_1), Sk2A (X_2, Y_1), Rt4A (X_1, Y_1)], B [Ns1B (X_2, Y_2)], C [Rt2C (X_1, Y_3)], and D [Ur20D (X_3, Y_4)] (Table 1). These isolines were used for crossing experiments in order to determine post-mating barriers by employing the techniques previously reported by Saeung et al. (2007).

DNA extraction and amplification

Three molecular markers (ITS2, COI, COII) were used to determine intraspecific sequence variation within the taxon *An. nigerrimus*. DNA was extracted from individual adult female F_1-progeny of each isoline of *An. nigerrimus* using the DNeasy® Blood and Tissue Kit (QIAGEN). Primers for the amplification of ITS2, COI, and COII regions followed previous studies by Saeung et al. (2007). The ITS2 region of the rDNA was amplified using ITS2A and ITS2B primers (Beebe and Saul 1995). The universal LCO1490 and HCO2198 barcoding primers of Folmer et al. (1994) were used to amplify the 658 bp COI gene fragment. The mitochondrial COII region was amplified using LEU and LYS primers, as recommended by Sharpe et al. (2000). Each PCR reaction was carried out in a total volume of 20 µl containing 0.5 U *Ex Taq* (Takara), 1X *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer, and 1 µl of the extracted DNA. Regarding ITS2, the conditions for amplification consisted of initial denaturation at 94° C for 1 min, 30 cycles at 94° C for 30 s, 55° C for 30 s, and 72° C for 1 min, and a final extension at 72° C for 5 min. Thermal cycling conditions for COI and COII were initial denaturation at 94° C for 1 min, 30 cycles at 94° C for 30 s, 50° C for 30 s, and 72° C for 1 min, and a final extension at 72° C for 5 min. PCR products were visualized by ethidium bromide staining after electrophoresis in 1.5% agarose gel, and finally purified using the QIAquick® PCR Purification Kit (QIAGEN). Sequencing reactions were conducted in both directions using the BigDye® V3.1 Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems). The sequences obtained were submitted to the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB778774-AB778812 (Table 1). All sequence data generated from this study were compared with those available in GenBank, using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequencing alignment and phylogenetic analysis

The DNA sequence data were edited manually in BioEdit version 7.0.5.3 (Hall 1999) and aligned using CLUSTAL W (Thompson et al. 1994). Gap sites were excluded from the following analysis. The Kimura two-parameter (K2P) model was employed to calculate genetic distances (Kimura 1980), which were used to construct neighbor-joining trees (Saitou and Nei 1987) and bootstrap 1,000 replicates with the MEGA version 4.0 program (Tamura et al. 2007). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist et al. 2012) by using two replicates of one million generations with the nucleotide evolutionary model. The best-fit model was chosen for each gene separately using the Akaike Information Criterion (AIC) in MrModeltest version 2.3 (2004, program distributed by the author). The general time-reversible (GTR) with gamma distribution shape parameter (G) was selected for ITS2, whereas the GTR+I+G was the best-fit model for combining sequences of COI and COII. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% of trees as burn-in.

RESULTS

Metaphase karyotypes

Cytogenetic investigations of F_1-progenies of the 13 isolines of *An. nigerrimus* revealed different types of sex chromosomes due to the addition of extra heterochromatin. There were three types of X (submetacentric X_1, large submetacentric X_2, and small metacentric X_3) and four types of Y chromosomes (large subtelocentric Y_1, submetacentric Y_2, small telocentric Y_3, and small subtelocentric Y_4). The

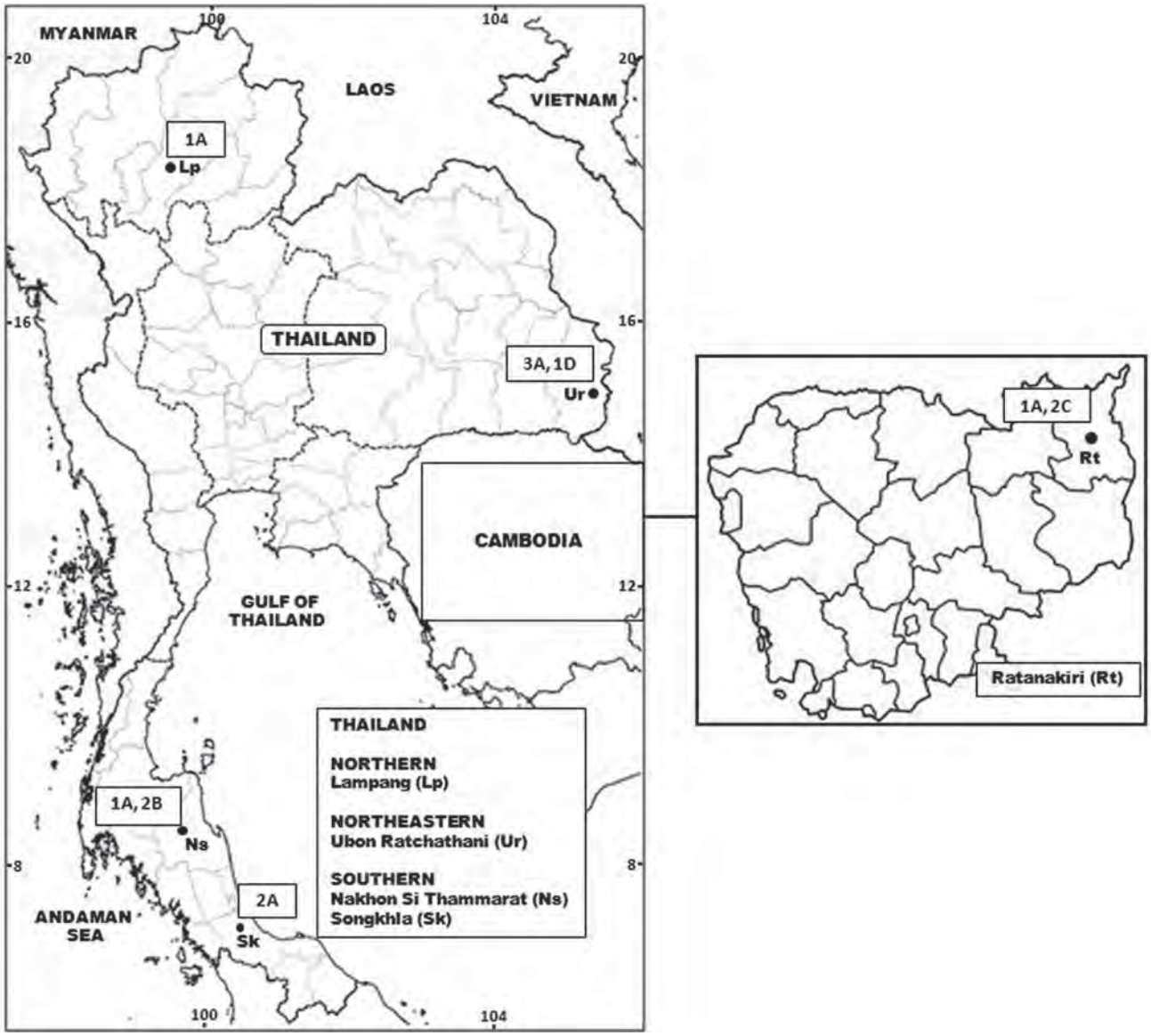

Figure 1. Map of Thailand and Cambodia showing five provinces where samples of *An. nigerrimus* were collected and the number of isolines of the four karyotypic forms (A-D) detected in each location.

X_3, Y_3, and Y_4 chromosomes were discovered in the present study. Based on the figures of X_3 and Y_3 chromosomes, they were probably represented the ancestral forms of X and Y chromosomes, respectively (Figure 2). These types of X and Y chromosomes formed four karyotypic forms on the basis of X and Y chromosome configurations, which were designated as Forms A (X_1, X_2, X_3, Y_1), B (X_2, X_3, Y_2), C (X_1, Y_3), and D (X_3, Y_4). Forms C and D were new karyotypic forms discovered in the present investigation. The number of isolines of these karyotypic forms occurring in different locations of Thailand and Cambodia are demonstrated in Figure 1 and Table 1. Form A appeared to be common in both Thailand and Cambodia. Forms B and D were found to be rather specific to southern and northeastern Thailand, respectively, whereas Form C was confined somewhat to Cambodia.

Hybridization experiment

The parental, reciprocal, and F_1-hybrid crosses among the eight isolines of *An. nigerrimus* yielded viable progenies through the F_2-generations. The hatchability, pupation, emergence rates, and ratio of adult female/male of parental, reciprocal, and F_1-hybrid crosses were 79.06-94.05%, 91.14-100%, 94.64-100%, and 0.81-1.12; 71.97-92.01%, 85.07-100%, 87.23-100%, and 0.89-1.14; 67.86-92.97%, 87.00-100%, 89.82-98.17%, and 0.71-1.27, respectively. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses. The salivary gland polytene chromosomes of the 4th instar larvae of F_1-hybrids from all crosses showed synapsis without inversion loops along the whole length of all autosomes and the X chromosome (Figure 3).

Table 1. Locations in Thailand and Cambodia, code of isolines, four karyotypic forms (A-D) of *An. nigerrimus* and their GenBank accession numbers.

Location (Geograpical coordinate)	Code of isoline[a]	Karyotypic form	GenBank accession number			Reference
			ITS2	COI	COII	
Thailand						
Lampang (17° 53′ N, 99° 20′ E)	Lp1A[a]	A (X_1, Y_1)	AB778774	AB778787	AB778800	This study
Ubon Ratchathani (15° 31′ N, 105° 35′ E)	Ur1A[a]	A (X_2, Y_1)	AB778775	AB778788	AB778801	This study
	Ur7A	A (X_2, Y_1)	AB778776	AB778789	AB778802	This study
	Ur20D[a]	D (X_3, Y_4)	AB778777	AB778790	AB778803	This study
	Ur26A	A (X_3, Y_1)	AB778778	AB778791	AB778804	This study
Nakhon Si Thammarat (08° 29′ N, 100° 0′ E)	Ns1B[a]	B (X_2, Y_2)	AB778779	AB778792	AB778805	This study
	Ns2B	B (X_3, Y_2)	AB778780	AB778793	AB778806	This study
	Ns3A[a]	A (X_2, Y_1)	AB778781	AB778794	AB778807	This study
Songkhla (07° 13′ N, 100° 37′ E)	Sk2A[a]	A (X_2, Y_1)	AB778782	AB778795	AB778808	This study
	Sk3A	A (X_1, Y_1)	AB778783	AB778796	AB778809	This study

Table 1. Continued.

Location (Geograpical coordinate)	Code of isoline[a]	Karyotypic form	GenBank accession number			Reference
			ITS2	COI	COII	
Cambodia						
Ratanakiri (13° 44′ N, 107° 0′ E)	Rt2C[a]	C (X_1, Y_3)	AB778784	AB778797	AB778810	This study
	Rt3C	C (X_1, Y_3)	AB778785	AB778798	AB778811	This study
	Rt4A[a]	A (X_1, Y_1)	AB778786	AB778799	AB778812	This study
Kalimantan, Indonesia						
Hyrcanus Group	K13	-	HM488261	-	-	Paredes-Esquivel et al. 2011
	K22	-	HM488263	-	-	Paredes-Esquivel et al. 2011
	K26	-	HM488267	-	-	Paredes-Esquivel et al. 2011
An. belenrae	-	-	EU789794	-	-	Park et al. 2008a
An. crawfordi	Sk1B	B (X_3, Y_2)	AB779152	AB779181	AB779210	Saeung et al. unpublished data
An. kleini	-	-	EU789793	-	-	Park et al. 2008a

Table 1. Continued.

Location (Geograpical coordinate)	Code of isoline[a]	Karyotypic form	GenBank accession number			Reference
			ITS2	COI	COII	
An. lesteri	-	-	EU789791	-	-	Park et al. 2008a
	ilG1	-	-	AB733028	AB733036	Taai et al. 2013a
An. nitidus	Ur2D	D (X_3, Y_4)	AB777782	AB777803	AB777824	Songsawatkiat et al. unpublished data
An. paraliae	Sk1B	B (X_1, Y_2)	AB733487	AB733503	AB733519	Taai et al. 2013b
An. peditaeniatus	RbB	B (X_3, Y_2)	AB539061	AB539069	AB539077	Choochote 2011
An. pullus	-	-	EU789792	-	-	Park et al. 2008a
	-	-	-	AY444348	AY444347	Park et al. 2003
An. sinensis	i2ACM	A (X, Y_1)	AY130473	-	-	Min et al. 2002
	-	-	-	AY444351	-	Park et al. 2003
	i1BKR	B (X, Y_2)	-	-	AY130464	Min et al. 2002

a: used in crossing experiments.

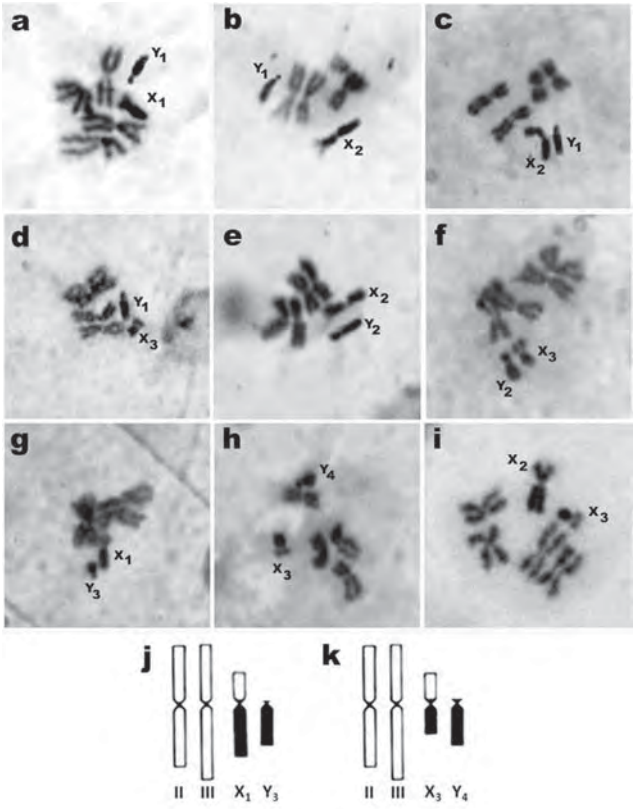

Figure 2. Metaphase karyotypic forms of *An. nigerrimus*. (a) Form A (X_1, Y_1: Lampang); (b) Form A (X_2, Y_1: Ubon Ratchathani); (c) Form A (X_2, Y_1: Songkhla); (d) Form A (X_3, Y_1: Ubon Ratchathani); (e) Form B (X_2, Y_2: Nakhon Si Thammarat); (f) Form B (X_3, Y_2: Nakhon Si Thammarat); (g) Form C (X_1, Y_3: Ratanakiri); (h) Form D (X_3, Y_4: Ubon Ratchathani); (i) Form A (heterozygous X_2, X_3: Ubon Ratchathani); diagrams of representative metaphase karyotype of Form C (j) and Form D (k).

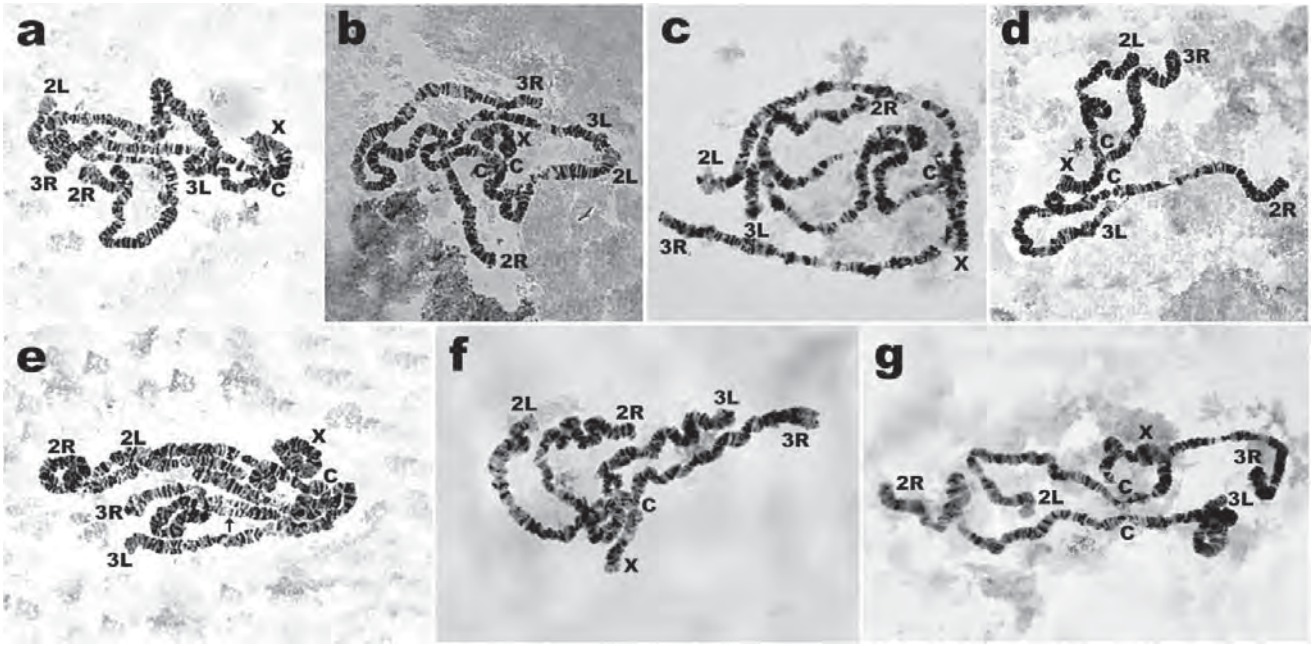

Figure 3. Synapsis in all arms of salivary gland polytene chromosome of F_1-hybrid larvae of *An. nigerrimus*. (a) Lp1A female x Ur1A male; (b) Lp1A female x Sk2A male; (c) Lp1A female x Ns3A male; (d) Lp1A female x Rt4A male; (e) Lp1A female x Ns1B male, note: small gap of homosequential asynapsis was found on chromosome 3R (small arrow); (f) Lp1A female x Rt2C male; (g) Lp1A female x Ur20D male.

DNA sequences and phylogenetic analysis

Sequences generated from 13 isolines of *An. nigerrimus* Forms A-D from Thailand and Cambodia showed the same length as those of ITS2 (508 bp), COI (658 bp), and COII (685 bp). Comparison of ITS2 sequences of ten and three isolines from Thai and Cambodian *An. nigerrimus*, respectively, were performed. Among them, eight were identical and the remaining five (Ns1B, Ns2B, Ns3A, Sk2A and Sk3A) from the southern region of Thailand shared the same nucleotide sequences, but they differed from the other eight by only two nucleotide substitutions (A↔G at position 70 and T↔G at position 481). The evolutionary relationships were constructed among the four karyotypic forms using neighbor-joining and Bayesian trees. Both phylogenetic methods showed similar tree topologies, thus, only the Bayesian tree was shown for all regions (Figures 4 and 5). The average genetic distances within and between the four karyotypic forms exhibited no significant difference in three DNA regions (0.002-0.007) of both Thai and Cambodian populations. Therefore, the 13 isolines were placed within a single species, namely *An. nigerrimus*. Additionally, three ITS2 retrieved sequences (428 bp) from GenBank, previously identified as the Hyrcanus Group by Paredes-Esquivel et al. (2011), were grouped together with four karyotypic forms of *An. nigerrimus* (average genetic distances = 0.003). These ITS2 sequences were nearly identical to our sequences, and only two nucleotide substitutions were found among them. In support, the phylogenetic trees for ITS2, COI, and COII of these isolines, representing Forms A-D, were clearly different from other species of the Hyrcanus Group (Figures 4 and 5).

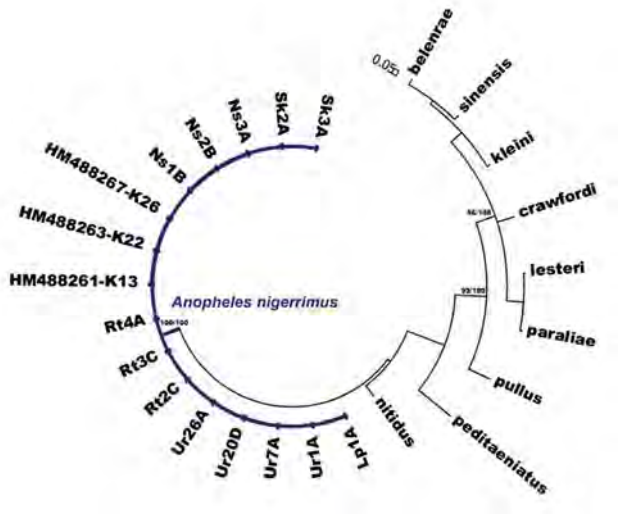

Figure 4. Bayesian phylogenetic relationships among the 13 isolines of *An. nigerrimus* from Thailand and Cambodia based on ITS2 sequences compared with nine species of the Hyrcanus Group and three Hyrcanus-group specimens from Kalimantan, Indonesia (Paredes-Esquivel et al. 2011). Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 70% both on bootstrap values and posterior probabilities are shown. Branch lengths are proportional to genetic distance (scale bar).

DISCUSSION

Metaphase chromosome investigations of four *An. nigerrimus* isolines from two allopatric locations (two isolines/each location) in Thailand (Nachaluai District, Ubon Ratchathani, and Bangpa-in District, Ayutthaya Provinces) were performed first by Baimai et al. (1993). The results revealed that this anopheline species exhibited karyotypic variation via a gradual increase in the extra heterochromatin on X (X_1, X_2) and Y (Y_1, Y_2) chromosomes. In this study, a total of 13 *An. nigerrimus* isolines obtained from four and one locations in Thailand and Cambodia, respectively, demonstrated three types of X (X_1, X_2, X_3) and four types of Y (Y_1, Y_2, Y_3, Y_4) chromosomes, thus forming four karyotypic forms, which were designated as Form A (X_1, X_2, X_3, Y_1), B (X_2, X_3, Y_2), C (X_1, Y_3), and D (X_3, Y_4). The newly discovered Forms C and D from Ratanakiri, Cambodia, and Ubon Ratchathani, northeastern Thailand, were based on the unique characteristics of small telocentric Y_3 and small subtelocentric Y_4 chromosomes, respectively, which were clearly different from the former two types of Y chromosomes (large subtelocentric Y_1, submetacentric Y_2) previously reported by Baimai et al. (1993). Apparently, the four distinct karyotypic forms of *An. nigerrimus* were due to the gradual addition of extra heterochromatin on sex chromosomes. Thus, the accumulation of heterochromatin in the genome elucidates the possible cytological mechanism for karyotypic evolution

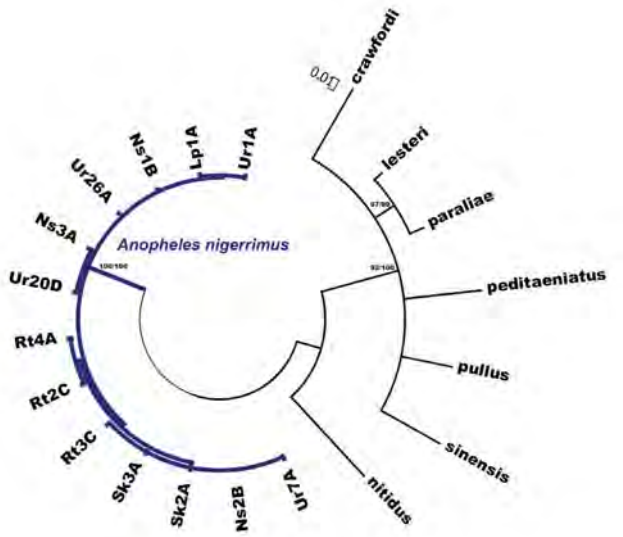

Figure 5. Bayesian phylogenetic relationships among the 13 isolines of *An. nigerrimus* from Thailand and Cambodia, based on combined sequences of COI and COII, compared with seven species of the Hyrcanus Group. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 70% both on bootstrap values and posterior probabilities are shown. Branch lengths are proportional to genetic distance (scale bar).

of Oriental anophelines as proposed by Baimai (1998). Regarding the distribution of *An. nigerrimus* cytological forms, it is worth noting that a new karyotypic Form C was detected in only two isoline colonies from Ratanakiri, Cambodia, whereas Form A was common in both Thailand and Cambodia. Interestingly, Form B and D were recovered specifically in Nakhon Si Thammarat Province, southern region and Ubon Ratchathani Province, northeastern region of Thailand, respectively. However, additional surveys are needed in order to obtain greater numbers of isolines from both countries, and this would bring about understanding of the exact distribution pattern of *An. nigerrimus* cytological forms.

Hybridization experiments using isoline colonies of *Anopheles* mosquitoes, which relate to data of cytogenetic and molecular investigations to elucidate post-mating barriers, have been proven to be robust traditional techniques for recognizing sibling species and/or subspecies members within the taxon *Anopheles* (Kanda et al. 1981, Baimai et al. 1987, Subbarao 1998, Junkum et al. 2005, Somboon et al. 2005, Saeung et al. 2007, 2008, Thongwat et al. 2008, Suwannamit et al. 2009, Thongsahuan et al. 2009, Choochote 2011, Saeung et al. 2012). The genetic diversity at the chromosomal level of *An. nigerrimus* found in this study warrants intensive hybridization experiments among the four karyotypic forms. The results showed no post-mating reproductive isolation. All crosses yielded viable progenies through F_2-generations and synaptic salivary gland polytene chromosomes, suggesting conspecific nature, comprised four cytological forms within this taxon. The low intraspecific sequence variations (average genetic distance = 0.002-0.007) of the nucleotide sequences in ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII) of the four karyotypic forms in both Thai and Cambodian *An. nigerrimus* populations were good supportive evidence. The present results are in accordance with hybridization experiments among karyotypic forms of other *Anopheles* species, including *An. vagus* (Choochote et al. 2002), *An. pullus* (= *An. yatsushiroensis*) (Park et al. 2003), *An. sinensis* (Choochote et al. 1998, Min et al. 2002, Park et al. 2008b), *An. aconitus* (Junkum et al. 2005), *An. barbirostris* species A1 and A2 (Saeung et al. 2007, Suwannamit et al. 2009), *An. campestris*-like taxon (Thongsahuan et al. 2009) and *An. peditaeniatus* (Choochote 2011, Saeung et al. 2012).

Misidentification of species can lead to failure in controlling target vectors, especially the sibling species and/or subspecies members of *Anopheles* species complexes in sympatric areas. Several studies reported misidentification of malaria vectors due to overlapping and/or variations based on morphological characters (Van Bortel et al. 2001, Singh et al. 2010). Paredes-Esquivel et al. (2011) reported that field workers had misidentified mosquitoes of the Hyrcanus Group as belonging to species of the Barbirostris Group. In order to overcome unresolved taxonomic questions on members of the *An. hyrcanus* group, the evidence from molecular markers was combined with morphological and hybridization experiments that identified the exact species status of four karyotypic forms of *An. nigerrimus* for the first time in two different geographical localities. Furthermore, three ITS2 published sequences of specimens from south Kalimantan, Indonesia (K13: GenBank accession number HM488261, K22: GenBank accession number HM488263, K26: GenBank accession number HM488267) were identified as the Hyrcanus Group by Paredes-Esquivel et al. (2011) and retrieved and compared with sequences of this study. It was interesting to note that these sequences were placed within the same clade of the Thai and Cambodian *An. nigerrimus* in the phylogenetic tree and the low level of intra-specific divergence (0.001-0.006) was found among them. This study confirms the presence of *An. nigerrimus* in Kalimantan, Indonesia, which corresponds to previous findings by O'Connor (1980).

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ORIGINAL RESEARCH PAPER

Development of a multiplex PCR assay for the identification of eight species members of the Thai Hyrcanus Group (Diptera: Culicidae)

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Abstract The Hyrcanus Group comprises important vectors of malaria because of *Plasmodium vivax* Grassi and Feletti and filariasis caused by *Brugia malayi* Brug in many countries of South, Southeast and East Asian regions. In Thailand, eight species members (*Anopheles argyropus* Swellengrebel, *An. crawfordi* Reid, *An. nigerrimus* Giles, *An. nitidus* Harrison, Scanlon & Reid, *An. paraliae* Sandosham, *An. peditaeniatus* Leicester, *An. pursati* Laveran and *An. sinensis* Wiedemann) of the Hyrcanus Group have been recognized. Due to morphological overlap, adult females of the Hyrcanus Group in Thailand have been misidentified markedly among the eight species, particularly when using the traumatic scales of wild-caught specimens from epidemiology and control approaches. Therefore, this study first developed a simple and robust multiplex PCR assay, based on second internal transcribed spacer sequences of ribosomal DNA, for differentiating the eight species members of the Thai Hyrcanus Group.

Keywords *Anopheles* · Hyrcanus Group · ITS2 · Multiplex PCR · Thailand

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Introduction

The Hyrcanus Group in the Myzorhynchus Series of the subgenus *Anopheles* comprises a large number of species that occur widely in Asia (Harbach 2013; Harrison and Scanlon 1975; Reid 1968; Scanlon et al. 1968). At least eight species of this group, i.e., *Anopheles argyropus* Swellengrebel, *An. crawfordi* Reid, *An. nigerrimus* Gilles, *An. nitidus* Harrison, Scanlon & Reid, *An. paraliae* Sandosham, *An. peditaeniatus* Leicester, *An. pursati* Laveran and *An. sinensis* Wiedemann, have been recorded in Thailand (Harrison and Scanlon 1975; Rattanarithikul et al. 2006; Reid 1968). Among these, *An. nigerrimus*, *An. peditaeniatus* and *An. sinensis* are considered as suspected vectors of *Plasmodium vivax* Grassi & Feletti in Thailand (Baker et al. 1987; Frances et al. 1996; Gingrich et al. 1990; Harbach et al. 1987; Rattanarithikul et al. 1996), while *An. sinensis* and *An. peditaeniatus* have been incriminated as natural vectors of *P. vivax* in China and Korea (Chai 1999; Liu 1990; Mourya et al. 1989; Ree et al. 2001; Whang et al. 2002) and Japanese encephalitis virus in China and India (Kanojia et al. 2003; Zhang 1990), respectively. Recently, *An. nigerrimus* was incriminated as a suspected vector of *P. falciparum* Welch and *P. vivax* in Bangladesh (Alam et al. 2010, 2012) and as a secondary or incidental vector of *Wuchereria bancrofti* Cobbold in Asian regions (Manguin et al. 2010). Additionally, at least

five species, i.e., *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. peditaeniatus* and *An. pursati*, are currently reported to be high-potential vectors for nocturnally subperiodic *Brugia malayi* Brug (Narathiwat Province, southern Thailand strain), as determined by 60–100 % susceptibility levels (Saeung et al. 2013).

Members among the eight species of the Thai Hyrcanus Group exhibit overlapping morphological characteristics in the adult stages, which lead to the misidentification of adult females, particularly when using the traumatic scales of wild-caught specimens from epidemiology and vector control approaches (Harrison and Scanlon 1975; Reid 1953, 1968).

In view of the above information, robust molecular assays are needed to identify the eight species members of the Thai Hyrcanus Group. Sequence comparisons of the second internal transcribed spacer (ITS2) of ribosomal DNA and cytochrome *c* oxidase subunit I and II (COI and COII) of the mitochondrial DNA have been used widely and effectively to distinguish other species members of the Hyrcanus Group (Choochote 2011; Djadid et al. 2009; Li et al. 2005; Min et al. 2002; Parades-Esquivel et al. 2011; Park et al. 2003; Saeung et al. 2012; Taai et al. 2013; Wilkerson et al. 2003). Recently, Joshi et al. (2010) designed multiplex primers to identify six species members of the Korean Hyrcanus Group. To date, there has been no report on the utility of a multiplex PCR assay for identifying the eight species members of the Thai Hyrcanus Group. This study described the development of species-specific primers for a single multiplex PCR in order to distinguish these species members based on ITS2 sequences. The advantage of using the second internal transcribed spacer (ITS2) of ribosomal DNA is that it has been represented in multiple copies throughout the genome in mosquitoes and leads to a high amplification signal (Garros et al. 2004). Due to the high inter-specific and low intra-specific variability of this region, it useful for development into PCR-based diagnostic tools that distinguish between cryptic and isomorphic species (Li and Wilkerson 2007; Norris 2002). Furthermore, these ITS2 sequences were used to study the phylogenetic relationships among the species. The novel multiplex PCR assay was evaluated using specimens of the eight species members of the Hyrcanus Group from different areas in Thailand and Cambodia.

Materials and methods

Mosquitoes

Eight species members of the Hyrcanus Group were collected in five provinces in Thailand. The species and strains were as follows: *An. argyropus* (Nakhon Si Thammarat strain), *An. crawfordi* (Chumphon strain), *An. nigerrimus* (Ubon Ratchathani strain), *An. nitidus* (Phang Nga strain), *An. paraliae* (Songkhla strain), *An. peditaeniatus* (Chiang Mai strain), *An. pursati* (Chiang Mai strain) and *An. sinensis* (Chiang Mai strain). Wild-caught, fully engorged females of these species members were collected from cow-baited traps. Identification of wild-caught females followed standard illustrated keys (Harrison and Scanlon 1975; Rattanarithikul et al. 2006; Reid 1968). Subsequently, identification using intact morphology of eggs, larvae, pupal skins and adult females were performed intensively in F_1-progenies of iso-female lines (isolines).

DNA extraction, amplification and sequencing of the ITS2 region

Genomic DNA was extracted from individual adult female mosquitoes using the DNeasy® Blood and Tissue Kit (QIAGEN, Japan). The rDNA ITS2 region was amplified using primers on conserved regions of 5.8S and 28S rRNA (Beebe and Saul 1995). Primers were as follows: ITS2A: 5′-TGTGAACTGCAGGACACAT-3′ and ITS2B: 5′-TATGCTTAAATTCAGGGGGT-3′. PCR was carried out using 20-µl volumes containing 0.4 U Phusion® High-Fidelity DNA polymerase (New England BioLabs, Japan), 1× Phusion® HF buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer and 1 µl of the extracted DNA. The amplification profile comprised initial denaturation at 98 °C for 30 s, 30 cycles at 98 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. The amplified products were subjected to electrophoresis on 1.5 % agarose gel. The PCR products were gel purified with the QIAquick® Gel Extraction Kit (QIAGEN, Japan) and cloned by the Mighty Cloning Reagent Set (Blunt End) (Takara, Japan). Finally, sequences of at least 12 clones of each isoline were determined using the BigDye® Terminator Cycle Sequencing Kit and run on a 3130 genetic analyzer (Applied Biosystems of Life Technologies, Japan).

Primer design and species-specific PCR

The ITS2 sequences of eight individuals (8 isolines) from the eight species were investigated for designing species-specific reverse primers. The sequences were aligned using BioEdit (Hall 1999). Primers were designed manually and by using the Primer3 Input (version 0.4.0) program (Rozen and Skaletsky 2000). The universal ITS2 forward primer (ITS2A) and each species-specific reverse primer were tested with the control samples in order to check the length of the amplified fragments and assess primer specificity. PCR was carried out using 20-µl volumes containing 0.5 U Ex *Taq* (Takara, Japan), 1× Ex *Taq* buffer, 2 mM of

$MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer and 1 μl of the extracted DNA. Conditions for the amplifications were initial denaturation at 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 5 min. The amplified products were subjected to electrophoresis on 1.5 % agarose gel stained with ethidium bromide.

Multiplex PCR

The reaction mixtures were prepared from 20-μl volumes that contained 0.5 μM of all nine primers (ITS2 forward and eight species-specific reverse primers). PCR reaction mixture and conditions for amplifications were performed in the same way as the species-specific PCR method. The PCR products were subjected to electrophoresis on 2 % agarose gel stained with ethidium bromide.

Validation of the assay

All 84 isolines of the 8 species members of the Hyrcanus Group collected from different locations in Thailand and Cambodia were tested in order to determine the reliability of the novel multiplex PCR assay (Table 2). Furthermore, in order to verify the correction of the species status, phylogenetic relationships of these sequences were constructed by using the neighbor-joining (NJ) (Saitou and Nei 1987) and Bayesian analysis. For NJ analysis, genetic distances were calculated using the Kimura two-parameter (K2P) model (Kimura 1980) and bootstrap test, with 10,000 replications performed with the MEGA version 4.0 program (Tamura et al. 2007). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist et al. 2012) by using two replicates of 1.5 million generations with the nucleotide evolutionary model. The best-fit model was chosen using the Akaike information criterion (AIC) in MrModeltest version 2.3 (Nylander 2004). The general time-reversible (GTR) model with the gamma distribution shape parameter (G) was selected. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25 % of the trees as burn-in. *An. gambiae* Giles (GenBank accession no. EU104648) was used as outgroup (Choi and Townson 2012).

Results

Sequencing of the ITS2 region

The complete ITS2 sequence length of each species was 448 bp for *An. paraliae*, 449 bp for *An. crawfordi*, 457 bp for *An. peditaeniatus*, 469 bp for *An. sinensis*, 472 bp for *An. argyropus*, 481 bp for *An. nitidus*, 499 bp for *An. pursati* and 508 bp for *An. nigerrimus.* All sequences have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under the following accession numbers: *An. argyropus* (AB826113), *An. crawfordi* (AB826114), *An. nigerrimus* (AB826115), *An. nitidus* (AB826116), *An. paraliae* (AB826117), *An. peditaeniatus* (AB826118), *An. pursati* (AB826119) and *An. sinensis* (AB826120).

Primer design

Primer names, sequences, size of the PCR product and respective melting temperatures (T_m) are shown in Table 1. Eight species-specific reverse primers were designed, based on nucleotide alignment of the complete ITS2 region, for distinguishing the eight species members of the Hyrcanus Group, and the species-primer sites are shown in Fig. 1. The universal forward primer (ITS2A) is located on the conserved 5.8S rRNA gene, whereas the species-specific reverse primers were within the ITS2 region.

Table 1 Information of the primers designed for the multiplex PCR assay in this study

Species	Primer name	Sequences (5′-3′)	Product size (bp)	C + G %	T_m (°C)	References
Universal forward primer	ITS2A	TGTGAACTGCAGGACACAT		47.37	60.34	Beebe and Saul (1995)
Species-specific reverse primers						
An. argyropus	Arg	AAACCTTCGGACCTTCGC	379	55.56	64.09	This study
An. crawfordi	Craw	TTGTTCGTCTTCACGGTTCTT	230	42.86	63.49	This study
An. nigerrimus	Niger	TCTTCTCACGCCATAAACAA	185	40.00	60.81	This study
An. nitidus	Niti-2	CCCAGATTCCAACTGTATCTCT	510	45.45	60.90	This study
An. paraliae	Par-3	TCAGTTTCCGTTTCCAGTCTC	416	47.62	63.11	This study
An. peditaeniatus	Ped-2	CGGCGTAGGTTATTGTCTCTC	123	52.38	62.46	This study
An. pursati	Pst	AATGGCCGTGGTTTTGTCTA	566	45.00	64.07	This study
An. sinensis	Sinen-2	AATATACTTCCTCCGCCGTC	279	50.00	61.74	This study

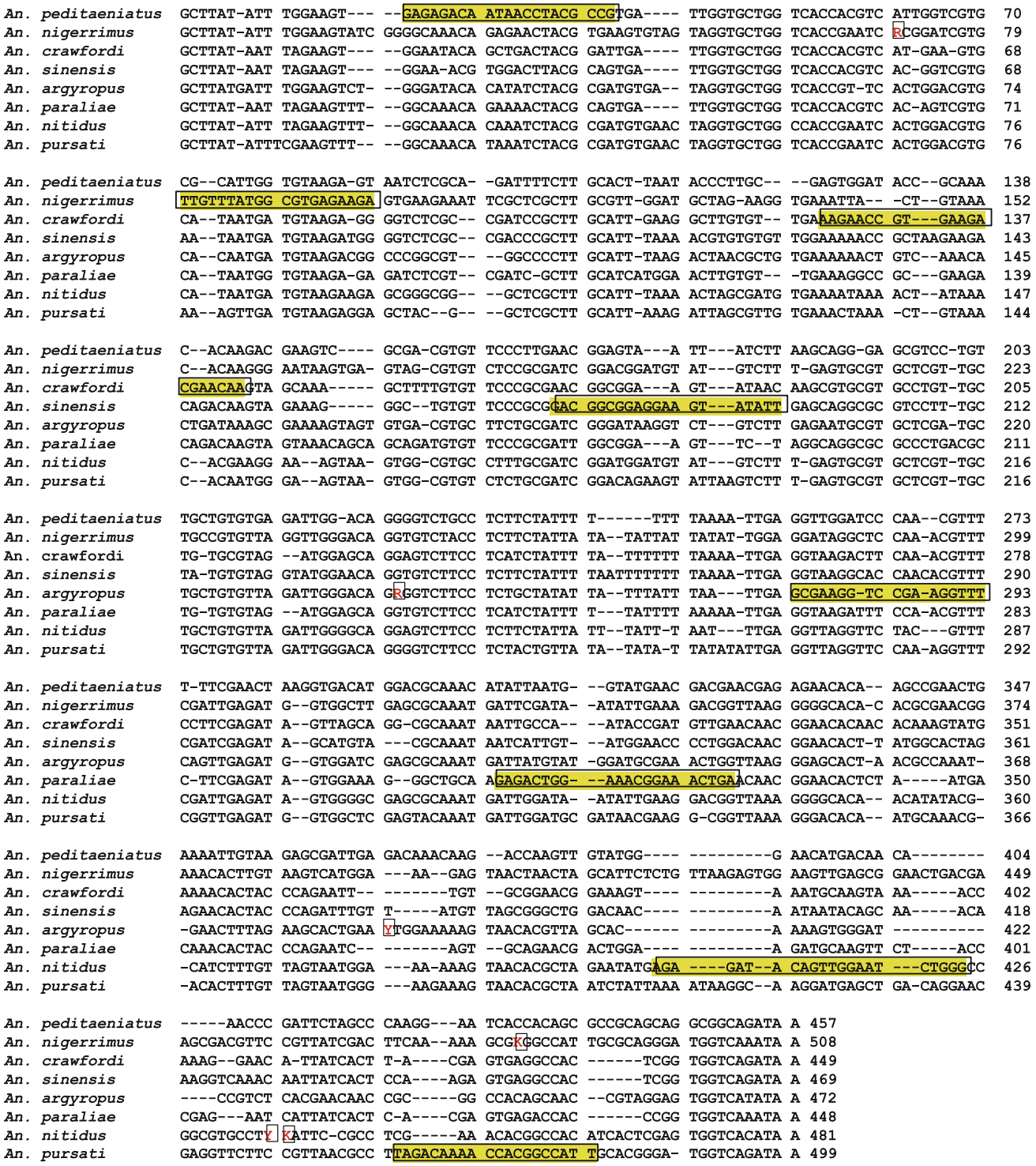

Fig. 1 Alignment of ITS2 sequences of eight species members of the Thai Hyrcanus Group. The primer selection sites are shaded in color in *black boxes*. Of 84 isolines, polymorphisms found in some isolines of *An. argyropus*, *An. nigerrimus* and *An. nitidus* as presented with the IUPAC nucleotide code in small, colored *black boxes*. K = G or T, R = A or G and Y = C or T

Multiplex PCR

The novel species-specific reverse primers that combined with the universal forward primer (ITS2A) could differentiate among all eight species members of the Hyrcanus Group simultaneously. This multiplex PCR assay provided 123 bp for *An. peditaeniatus*, 185 bp for *An. nigerrimus*, 230 bp for *An. crawfordi*, 279 bp for *An.*

sinensis, 379 bp for *An. argyropus*, 416 bp for *An. paraliae*, 510 bp for *An. nitidus* and 566 bp for *An. pursati* (Table 1; Fig. 2).

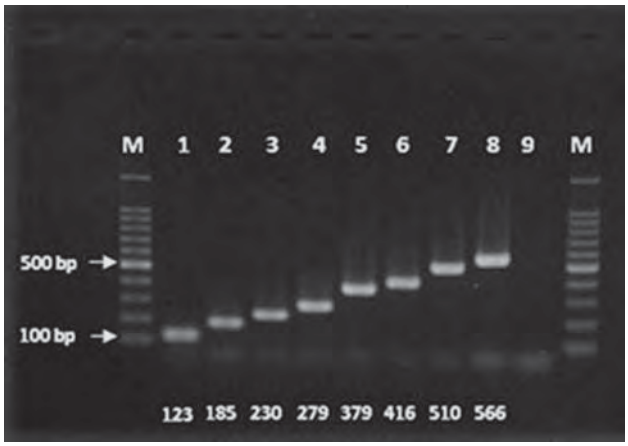

Fig. 2 PCR products from the multiplex PCR assay run on 2 % agarose gel. *Lane 1*: *An. peditaeniatus*; *lane 2*: *An. nigerrimus*; *lane 3*: *An. crawfordi*; *lane 4*: *An. sinensis*; *lane 5*: *An. argyropus*; *lane 6*: *An. paraliae*, *lane 7*: *An. nitidus*, *lane 8*: *An. pursati*; *lane 9*: negative control. The 100-bp molecular weight ladders are loaded in lane M

Validation of the multiplex PCR assay

A total of 84 specimens (1 specimen from each isoline), collected from different locations and previously identified by morphology, were tested with this assay (Table 2). All species-specific reverse primers could be annealed and amplified, and they provided the specific band for each species (Fig. 2). Furthermore, we determined the sequences of the 84 specimens to confirm whether base substitution occurs at the annealing site of all species-specific reverse primers, which were designed in this study. Consequently, although polymorphisms were found in some isolines of *An. argyropus*, *An. nigerrimus* and *An. nitidus*, no polymorphism was detected at the annealing sites (Fig. 1). The NJ and Bayesian trees had a similar topology and revealed eight distinct monophyletic clades with a highly supportive bootstrap value of NJ (99 %) and Bayesian posterior probabilities (99–100 %) (Fig. 3, only Bayesian tree was shown). They showed a low level of mean genetic distance within species (0.000–0.013), whereas the mean genetic distance was high (0.186–0.655) between species.

Table 2 Information on specimens of the eight species members of the Hyrcanus Group collected from different locations in Thailand and Cambodia

Species	Location	Number of isoline	GenBank Accession number
An. argyropus	Ubon Ratchathani, Thailand	2	AB839773–AB839774
	Nakhon Si Thammarat, Thailand	6	AB839775–AB839780
An. crawfordi	Chiang Mai, Thailand	1	AB839781
	Chumphon, Thailand	1	AB839782
	Trang, Thailand	2	AB839783–AB839784
	Phang Nga, Thailand	10	AB839785–AB839794
	Songkhla, Thailand	1	AB839795
	Ratanakiri, Cambodia	4	AB839796–AB839799
	Mondulkiri, Cambodia	4	AB839800–AB839803
An. nigerrimus	Ubon Ratchathani, Thailand	4	AB839804–AB839807
	Nakhon Si Thammarat, Thailand	3	AB839808–AB839810
	Songkhla, Thailand	2	AB839811–AB839812
	Ratanakiri, Cambodia	3	AB839813–AB839815
An. nitidus	Ubon Ratchathani, Thailand	18	AB839816–AB839833
	Phang Nga, Thailand	3	AB839834–AB839836
An. paraliae	Nakhon Si Thammarat, Thailand	1	AB839837
	Songkhla, Thailand	2	AB839838–AB839839
An. peditaeniatus	Chiang Mai, Thailand	1	AB839840
	Lampang, Thailand	1	AB839841
	Ubon Ratchathani, Thailand	1	AB839842
	Nakhon Si Thammarat, Thailand	2	AB839843–AB839844
	Trang, Thailand	1	AB839845
An. pursati	Chiang Mai, Thailand	10	AB839846–AB839855
An. sinensis	Chiang Mai, Thailand	1	AB826121
Total		84	

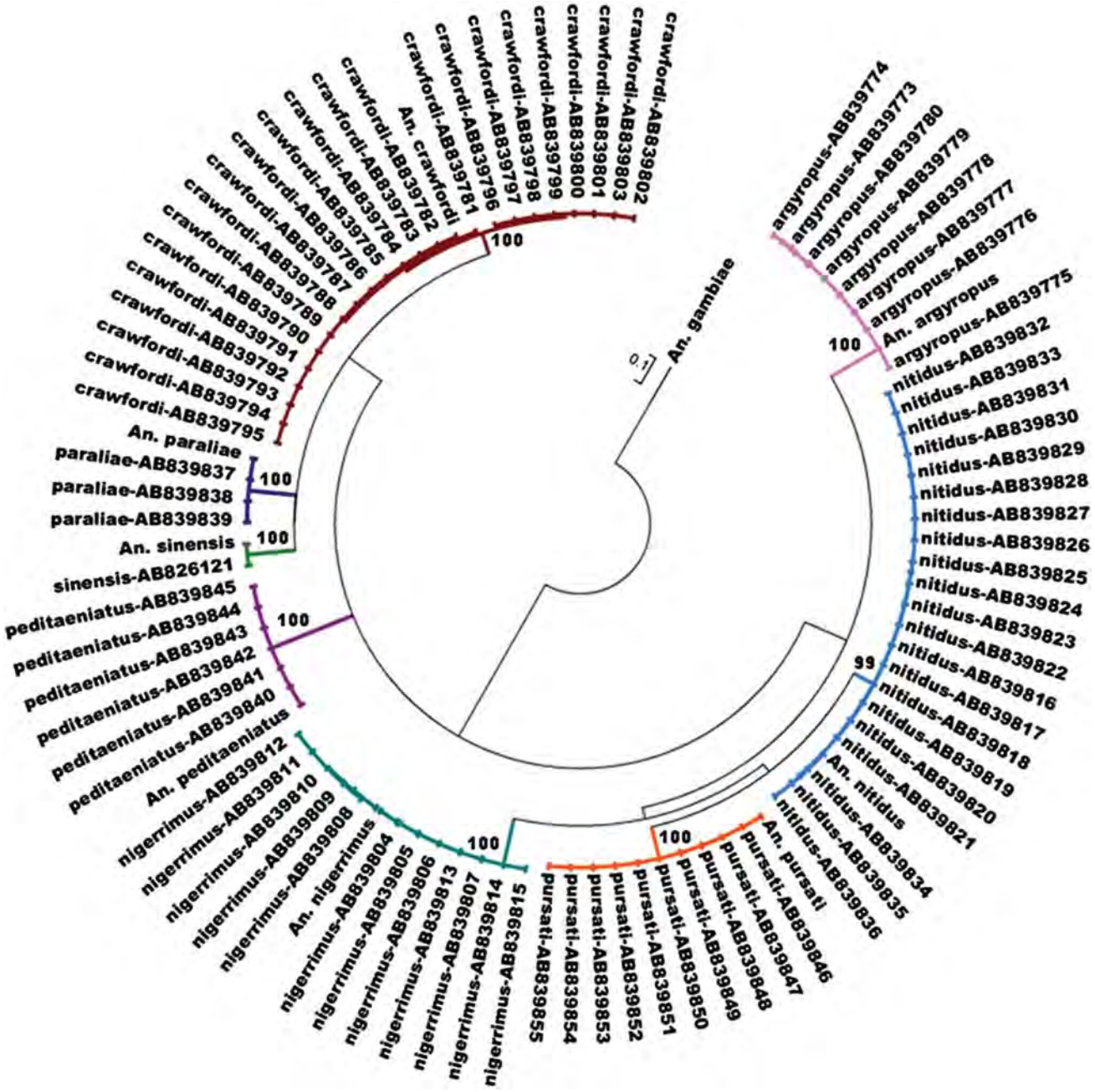

Fig. 3 Phylogenetic relationships among the eight species members of the Thai and Cambodian Hyrcanus Group using Bayesian analysis based on ITS2 sequences. The Bayesian posterior probabilities of higher than 70 % are shown. *Bars* represent 0.1 substitutions per site

Discussion

Accurate identification of anopheline vector species is an important key for planning an effective control program. It also is necessary for resolving taxonomic problems in order to recognize new species and establish species distribution (Parades-Esquivel et al. 2011). Members of the Thai Hyrcanus Group are difficult to distinguish morphologically, particularly when they occur sympatrically. Some of the listed characteristics are, for example, highly variable hindtarsal banding (among *An. sinensis*, *An. crowfordi* and *An. nigerrimus*, and between *An. argyropus* and *An. peditaeniatus*); similar humeral crossvein with a patch of dark scales, remigium with dark scales and midtarsi with narrow apical pale bands (between *An. argyopus* and *An. nigerrimus*); similar wing patterns (between *An. crawfordi* and *An. nitidus*); and a similar narrow apical fringe spot on the wing and narrow tarsal bands (between *An. paraliae* and *An. pursati*) (Harrison and Scanlon 1975; Reid 1953, 1968). Regarding PCR-based species identification in the

Hyrcanus Group, Parades-Esquivel et al. (2009) reported mosquitoes of the Hyrcanus Group being misidentified by field workers as those in the Barbirostris Group. Recently, Parades-Esquivel et al. (2011) investigated specimens of the Hyrcanus Group that were initially identified by field staff and subsequently identified based on DNA sequences. The results revealed that 22 specimens had been misidentified as members of the Barbirostris Group. For correct identification, molecular markers are used as a powerful tool. The multiplex PCR assay has been developed successfully for identifying anopheline species based on ITS2 [e.g., *An. minimus* A Theobald, *An. mininus* C (=*harrisoni*) Harbach & Manguin, *An. aconitus* Dönitz, *An. pampanai* Büttiker & Beales, *An. varuna* Iyengar and *An. jeyporiensis* James (Phuc et al. 2003); *An. minimus* A, *An. mininus* C, *An. aconitus*, *An. varuna*, *An. pampanai*, *An. leesoni* Evans, *An. funestus* Giles, *An. vaneedeni* Gillies & Coetzee, *An. rivulorum* Leeson and *An. parensis* Gillies (Garros et al. 2004); *An. punctulatus* Dönitz, *An. koliensis* Owen, *An. farauti* 1 Laveran, *An. hinesorum* Schmidt, *An. farauti* 4 Foley et al. (1993) (Henry-Halldin et al. 2011)] and 28S regions (D1, D2 and D3) [*An. culicifacies* Giles complex (species A, B, C, D and E) (Raghavendra et al. 2009); *An. annularis* van der Wulp, *An. philippinensis* Ludlow and *An. pallidus* Theobald (Swain et al. 2009); *An. belenrae* Rueda, *An. kleini* Rueda, *An. lesteri* Baisas & Hu, *An. pullus* Yamada, *An. sinensis* and *An. sineroides* Yamada (Joshi et al. 2010)]. This study used universal primers combined with eight species-specific reverse primers to amplify eight species members of the Thai Hyrcanus Group (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. peditaeniatus*, *An. pursati* and *An. sinensis*) in a one-step reaction. The amplified PCR products showed a specific band of each species, which was seen easily on agarose gel. This assay showed its reliability because results of the PCR species identification of 84 specimens were concordant with those of the original morphological results. In addition, the phylogenetic tree has supported the presence of eight distinct monophyletic clades. The high level of mean genetic distance (0.186–0.655) between species also was strong supportive evidence confirming a different species-specific status.

In conclusion, this study was the first to develop the multiplex PCR assay for identifying the eight species members of the Thai Hyrcanus Group. This assay is simple, rapid, sensitive and specific. Furthermore, it also could identify *An. crawfordi* and *An. nigerrimus* strains from Cambodia. Therefore, it would be a very effective assay in identifying field-collected Hyrcanus Group mosquitoes not only in Thailand but also in other countries.

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Geographic distribution and genetic compatibility among six karyotypic forms of *Anopheles peditaeniatus* (Diptera: Culicidae) in Thailand

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Abstract. Fifty-three isolines of *Anopheles peditaeniatus* were established from individual wild-caught females collected from cow-baited traps in 17 provinces of Thailand. Three types of X (X_1, X_2, X_3) and 6 types of Y (Y_1,Y_2, Y_3, Y_4, Y_5, Y_6) chromosomes were determined based on different amounts of major block(s) of heterochromatin. These sex chromosomes comprised 6 karyotypic forms designated as Forms A (X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_3, Y_3), D (X_1, X_2, X_3, Y_4), E (X_1, X_2, X_3,Y_5) and F (X_2, X_3, Y_6). Form F is a new metaphase karyotype discovered in this study and is commonly found in all regions. Form A was found only in Lampang province, whereas Form E is widespread throughout the country. Forms B, C and D were obtained from the northern, northeastern, western and southern regions. Crossing experiments among the 11 isoline colonies representing the 6 karyotypic forms of *An. peditaeniatus* indicated genetic compatibility yielding viable progenies and complete synapsis of salivary gland polytene chromosomes through to the F_2-generations. The results suggested the conspecific nature of these karyotypic forms which were further supported by very low intraspecific variation (genetic distance = 0.000-0.003) of nucleotide sequences in ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII).

INTRODUCTION

Anopheles (*Anopheles*) *peditaeniatus* belongs to the Lesteri Subgroup, Hyrcanus Group of the Myzorhynchus Series. It is widely distributed in Asia including Thailand, Myanmar, Cambodia, Indonesia, Malaysia, the Philippines, Vietnam, Borneo, Celebes, China, India, Sri Lanka and Nepal (Reid, 1968; Scanlon *et al*., 1968; Harrison & Scanlon, 1975). *Anopheles peditaeniatus* is considered a suspected vector of human malaria, *Plasmodium vivax*, in Thailand (Gingrich *et al*., 1990; Rattanarithikul *et al*., 2006). In addition, it has been incriminated as a secondary vector of Japanese encephalitis virus in China and India (Zhang, 1990; Kanojia *et al*., 2003). However, its status as a vector of the Japanese encephalitis virus remains a crucial question in Thailand, although it is widespread throughout the country. Due to the vicious biting-behavior of *An. peditaeniatus* on cattle and its ability to transmit filariae of the genus *Setaria*, it is

considered an economic pest (Reid *et al.*, 1962; Reid, 1968; Harrison & Scanlon, 1975). Although *An. peditaeniatus* has never been incriminated as natural and/or suspected vector in endemic areas of filariasis caused by *Brugia malayi*, it has been demonstrated to be a good experimental vector for transmitting this filarial nematode (Wharton *et al.*, 1963). Our recent experiment has shown that *An. peditaeniatus* exhibited high potential as a vector to *B. malayi* (Narathiwat province, southern Thailand strain) (unpublished data).

Cytologically, Baimai *et al.* (1993) found that *An. peditaeniatus* from Chiang Mai, Phrae and Chanthaburi provinces, Thailand showed different forms of mitotic sex chromosomes due to extra block(s) of heterochromatin. Recently, Choochote (2011) reported crossing experiments and DNA sequence analyses of internal transcribed spacer 2 (ITS2) of ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI) and cytochrome *c* oxidase subunit II (COII) of mitochondrial DNA (mtDNA) among 8 allopatric strains (Chiang Mai, Nan, Udon Thani, Ubon Ratchathani, Kamphaeng Phet, Ratchaburi, Chon Buri and Chumphon provinces) which represented 4 karyotypic forms (B, C, D and E) of *An. peditaeniatus* in Thailand.

We report herein a new karyotypic form of *An. peditaeniatus*, crossing experiments among the 6 karyotypic forms and comparative DNA sequencing of the ITS2, COI and COII regions of 53 isolines obtained from different populations in Thailand.

MATERIALS AND METHODS

Field collections and establishment of isoline colonies

Wild-caught, fully engorged females of *An. peditaeniatus* were collected from cow-baited traps in 17 provinces of Thailand (Fig. 1, Table 1). A total of 53 isolines were successfully established and maintained in our insectary using the techniques described by Choochote *et al.* (1983) and Kim *et al.* (2003). These isolines were used in this study.

Metaphase karyotype preparation

Metaphase chromosomes were prepared from 10 samples of early 4th instar larval brains of F_1- and/or F_2-progenies of each isoline of *An. peditaeniatus* using the techniques previously described by Saeung *et al.* (2007). Identification of karyotypic forms followed the standard cytotaxonomic key of Baimai *et al.* (1993).

Crossing experiments

Eleven laboratory-raised isolines of *An. peditaeniatus* were selected arbitrarily from the 53 isolines representing the 6 karyotypic forms, i.e., Forms A (Lp3A), B (Nk1B, Ns4B), C (Cb3C, Ns8C), D (Ur5D, Ns5D), E (Cb5E, Tg3E) and F (Sb2F, Tg1F) (Table 2). These isolines were used for crossing experiments to determine post-mating reproductive isolation by employing the techniques previously reported by Saeung *et al.* (2007). The salivary gland polytene chromosomes of 4th instar larvae of F_1-hybrids from the crosses were investigated using the techniques described by Kanda (1979). Polytene chromosome arms were identified by comparing them with the euchromatic arms of mitotic karyotypes. The shortest chromosome is X; the autosomal long arms are designated as 2R and 3R, and short arms as 2L and 3L (White *et al.*, 1975).

DNA extraction and amplification

One individual F_1-progeny adult female from each isoline of each *An. peditaeniatus* Form (A-F) was used for DNA extraction and amplification. Genomic DNA was extracted from each individual adult mosquito using DNeasy® Blood and Tissue Kit (Qiagen). The ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2), and the mitochondrial cytochrome *c* oxidase subunit I (COI) and subunit II (COII) were amplified using the primers described previously by Park *et al.* (2008b). The sequence data of this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB714987-AB715145. The ITS2, COI and COII sequences obtained from this study were also compared with deposited sequences available through GenBank (Table 1).

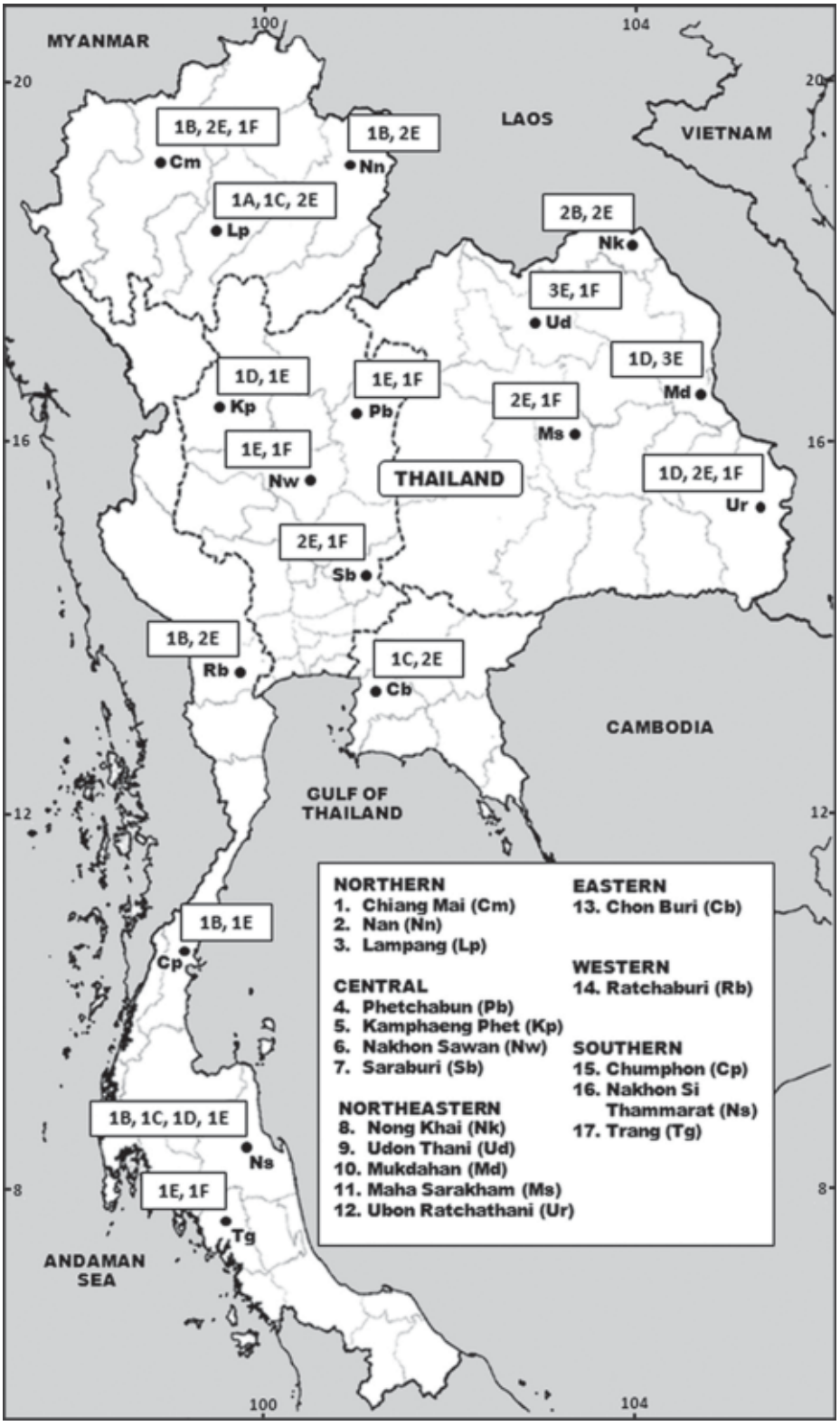

Figure 1. Map of Thailand showing 17 provinces where samples of *An. peditaeniatus* were collected and the number of isolines of the 6 karyotypic forms (A-F) detected in each location

Table 1. Locations (17 provinces of Thailand), code of isolines, karyotypic forms of *Anopheles peditaeniatus* and their GenBank accession numbers

Location (Geographical coordinate)	Code of isoline[a]	Karyotypic form	DNA Region	Genbank accession number			Reference
				ITS2	COI	COII	
An. peditaeniatus							
1. Chiang Mai	Cm2E	E (X_3, Y_5)	ITS2, COI, COII	AB714987	AB715040	AB715093	This study
(18° 47' N, 98° 59' E)	Cm3F	F (X_3, Y_6)	ITS2, COI, COII	AB714988	AB715041	AB715094	This study
	Cm4E	E (X_1, Y_5)	ITS2, COI, COII	AB714989	AB715042	AB715095	This study
	Cm7B	B (X_2, Y_2)	ITS2, COI, COII	AB714990	AB715043	AB715096	This study
2. Nan	Nn1E	E (X_3, Y_5)	ITS2, COI, COII	AB714991	AB715044	AB715097	This study
(18° 48' N, 100° 45' E)	Nn2B	B (X_2, Y_2)	ITS2, COI, COII	AB714992	AB715045	AB715098	This study
	Nn3E	E (X_3, Y_5)	ITS2, COI, COII	AB714993	AB715046	AB715099	This study
3. Lampang	Lp1E	E (X_3, Y_5)	ITS2, COI, COII	AB714994	AB715047	AB715100	This study
(17° 53' N, 99° 20' E)	Lp3A[a]	A (X_3, Y_1)	ITS2, COI, COII	AB714995	AB715048	AB715101	This study
	Lp4E	E (X_3, Y_5)	ITS2, COI, COII	AB714996	AB715049	AB715102	This study
	Lp5C	C (X_3, Y_3)	ITS2, COI, COII	AB714997	AB715050	AB715103	This study
4. Phetchabun	Pb5F	F (X_2, Y_6)	ITS2, COI, COII	AB714998	AB715051	AB715104	This study
(16° 25' N, 101° 08' E)	Pb9E	E (X_1, Y_5)	ITS2, COI, COII	AB714999	AB715052	AB715105	This study
5. Kamphaeng Phet	Kp2E	E (X_2, Y_5)	ITS2, COI, COII	AB715000	AB715053	AB715106	This study
(16° 50' N, 99° 04' E)	Kp5D	D (X_3, Y_4)	ITS2, COI, COII	AB715001	AB715054	AB715107	This study
6. Nakhon Sawan	Nw5E	E (X_2, Y_5)	ITS2, COI, COII	AB715002	AB715055	AB715108	This study
(15° 35' N, 100° 10' E)	Nw6F	F (X_3, Y_6)	ITS2, COI, COII	AB715003	AB715056	AB715109	This study
7. Saraburi	Sb2F[a]	F (X_3, Y_6)	ITS2, COI, COII	AB715023	AB715076	AB715129	This study
(14° 30' N, 100° 55' E)	Sb7E	E (X_3, Y_5)	ITS2, COI, COII	AB715024	AB715077	AB715130	This study
	Sb8E	E (X_2, Y_5)	ITS2, COI, COII	AB715025	AB715078	AB715131	This study
8. Nong Khai	Nk1B[a]	B (X_1, Y_2)	ITS2, COI, COII	AB715004	AB715057	AB715110	This study
(17° 50' N, 102° 46' E)	Nk2E	E (X_2, Y_5)	ITS2, COI, COII	AB715005	AB715058	AB715111	This study
	Nk4B	B (X_3, Y_2)	ITS2, COI, COII	AB715006	AB715059	AB715112	This study
	Nk6E	E (X_3, Y_5)	ITS2, COI, COII	AB715007	AB715060	AB715113	This study
9. Udon Thani	Ud2E	E (X_3, Y_5)	ITS2, COI, COII	AB715008	AB715061	AB715114	This study
(17° 24' N, 102° 47' E)	Ud3E	E (X_2, Y_5)	ITS2, COI, COII	AB715009	AB715062	AB715115	This study
	Ud5F	F (X_3, Y_6)	ITS2, COI, COII	AB715010	AB715063	AB715116	This study
	Ud6E	E (X_2, Y_5)	ITS2, COI, COII	AB715011	AB715064	AB715117	This study
10. Mukdahan	Md1E	E (X_2, Y_5)	ITS2, COI, COII	AB715012	AB715065	AB715118	This study
(15° 24' N, 103° 16' E)	Md2E	E (X_3, Y_5)	ITS2, COI, COII	AB715013	AB715066	AB715119	This study
	Md4D	D (X_3, Y_4)	ITS2, COI, COII	AB715014	AB715067	AB715120	This study
	Md5E	E (X_3, Y_5)	ITS2, COI, COII	AB715015	AB715068	AB715121	This study

Table 1. (continued)

Location (Geographical coordinate)	Code of isoline[a]	Karyotypic form	DNA Region	Genbank accession number			Reference
				ITS2	COI	COII	
11. Maha Sarakham	Ms1E	E (X_2, Y_5)	ITS2, COI, COII	AB715016	AB715069	AB715122	This study
(15° 45' N, 103° 01' E)	Ms3E	E (X_2, Y_5)	ITS2, COI, COII	AB715017	AB715070	AB715123	This study
	Ms4F	F (X_3, Y_6)	ITS2, COI, COII	AB715018	AB715071	AB715124	This study
12. Ubon Ratchathani	Ur1F	F (X_3, Y_6)	ITS2, COI, COII	AB715019	AB715072	AB715125	This study
(15° 31' N, 105° 35' E)	Ur4E	E (X_2, Y_5)	ITS2, COI, COII	AB715020	AB715073	AB715126	This study
	Ur5D[a]	D (X_1, Y_4)	ITS2, COI, COII	AB715021	AB715074	AB715127	This study
	Ur6E	E (X_3, Y_5)	ITS2, COI, COII	AB715022	AB715075	AB715128	This study
13. Chon Buri	Cb3C[a]	C (X_3, Y_3)	ITS2, COI, COII	AB715029	AB715082	AB715135	This study
(13° 26' N, 101° 03' E)	Cb5E[a]	E (X_3, Y_5)	ITS2, COI, COII	AB715030	AB715083	AB715136	This study
	Cb8E	E (X_2, Y_5)	ITS2, COI, COII	AB715031	AB715084	AB715137	This study
14. Ratchaburi	Rb1B	B (X_3, Y_2)	ITS2, COI, COII	AB715026	AB715079	AB715132	This study
(13° 21' N, 99° 22' E)	Rb4E	E (X_2, Y_5)	ITS2, COI, COII	AB715027	AB715080	AB715133	This study
	Rb10E	E (X_2, Y_5)	ITS2, COI, COII	AB715028	AB715081	AB715134	This study
15. Chumphon	Cp2B	B (X_2, Y_2)	ITS2, COI, COII	AB715032	AB715085	AB715138	This study
(10° 29' N, 99° 11' E)	Cp7E	E (X_2, Y_5)	ITS2, COI, COII	AB715033	AB715086	AB715139	This study
16. Nakhon Si Thammarat	Ns1E	E (X_2, Y_5)	ITS2, COI, COII	AB715034	AB715087	AB715140	This study
(08° 29' N, 100° 0' E)	Ns4B[a]	B (X_2, Y_2)	ITS2, COI, COII	AB715035	AB715088	AB715141	This study
	Ns5D[a]	D (X_2, Y_4)	ITS2, COI, COII	AB715036	AB715089	AB715142	This study
	Ns8C[a]	C (X_3, Y_3)	ITS2, COI, COII	AB715037	AB715090	AB715143	This study
17. Trang	Tg1F[a]	F (X_2, Y_6)	ITS2, COI, COII	AB715038	AB715091	AB715144	This study
(07° 33' N, 99° 38' E)	Tg3E[a]	E (X_3, Y_5)	ITS2, COI, COII	AB715039	AB715092	AB715145	This study
Ratchaburi (13° 21' N, 99° 22' E)	RbB	B (X_3, Y_2)	ITS2, COI, COII	AB539061	AB539069	AB539077	Choochote, 2011
China	–	–	ITS2	AY129958	–	–	Ma & Xu, 2005
An. sinensis							
Korea	–	–	ITS2	EU789790	–	–	Park *et al.*, 2008a
	–	B (X, Y_2)	COI	–	AY444351	–	Park *et al.*, 2003
	i1BKR	B (X, Y_2)	COII	–	–	AY130464	Min *et al.*, 2002
An. lesteri							
Korea	–	–	ITS2	EU789791	–	–	Park *et al.*, 2008a
China	–	–	COI	–	EU699048	–	Yang & Ma, 2009
	–	–	COII	–	–	AY753146	Ma *et al.* (unpublished data)

Table 2. Crossing experiments among 11 isolines of *An. peditaeniatus*

Crosses (Female x Male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
Parental cross							
Lp3A x Lp3A	256 (123, 133)	86	218 (85.16)	207 (94.95)	192 (92.75)	91 (47.40)	101 (52.60)
Nk1B x Nk1B	281 (155, 126)	81	222 (79.00)	175 (78.83)	175 (100.00)	86 (49.14)	89 (50.86)
Ns4B x Ns4B	281 (150, 131)	83	228 (81.14)	217 (95.18)	217 (100.00)	111 (51.15)	106 (48.85)
Cb3C x Cb3C	374 (130, 244)	91	314 (83.96)	308 (98.09)	308 (100.00)	163 (52.92)	145 (47.08)
Ns8C x Ns8C	381 (212, 169)	94	343 (90.03)	333 (97.08)	333 (100.00)	176 (52.85)	157 (47.15)
Ur5D x Ur5D	261 (157, 104)	77	193 (73.95)	193 (100.00)	193 (100.00)	95 (49.22)	98 (50.78)
Ns5D x Ns5D	300 (148, 152)	100	282 (94.00)	265 (93.97)	259 (97.74)	120 (46.33)	139 (53.67)
Cb5E x Cb5E	385 (296, 89)	86	316 (82.08)	272 (86.08)	258 (94.85)	129 (50.00)	129 (50.00)
Tg3E x Tg3E	316 (169, 147)	81	246 (77.85)	234 (95.12)	229 (97.86)	108 (47.16)	121 (52.84)
Sb2F x Sb2F	299 (179, 120)	90	257 (85.95)	203 (78.99)	201 (99.01)	103 (51.24)	98 (48.76)
Tg1F x Tg1F	335 (220, 115)	79	251 (74.92)	206 (82.07)	206 (100.00)	107 (51.94)	99 (48.06)
Reciprocal cross							
Lp3A x Nk1B	332 (152, 180)	90	282 (84.94)	279 (98.94)	279 (100.00)	144 (51.61)	135 (48.39)
Nk1B x Lp3A	358 (201, 157)	95	322 (89.94)	306 (95.03)	291 (95.10)	142 (48.80)	149 (51.20)
Lp3A x Ns4B	360 (144, 216)	89	292 (81.11)	257 (88.01)	257 (100.00)	128 (49.80)	129 (50.20)
Ns4B x Lp3A	438 (330, 108)	80	342 (78.08)	291 (85.09)	291 (100.00)	131 (45.02)	160 (54.98)
Lp3A x Cb3C	331 (238, 93)	97	298 (90.03)	268 (89.93)	263 (98.13)	132 (50.19)	131 (49.81)
Cb3C x Lp3A	364 (181, 183)	100	338 (92.86)	314 (92.90)	298 (94.90)	146 (48.99)	152 (51.01)
Lp3A x Ns8C	360 (235, 125)	87	288 (80.00)	285 (98.96)	259 (90.88)	135 (52.12)	124 (47.88)
Ns8C x Lp3A	367 (210, 157)	84	301 (82.02)	301 (100.00)	289 (96.01)	142 (49.13)	147 (50.87)
Lp3A x Ur5D	425 (170, 255)	95	374 (88.00)	366 (97.86)	366 (100.00)	190 (51.91)	176 (48.09)
Ur5D x Lp3A	360 (187, 173)	88	302 (83.89)	266 (88.08)	263 (98.87)	126 (47.91)	137 (52.09)
Lp3A x Ns5D	400 (215, 185)	85	328 (82.00)	279 (85.06)	259 (92.83)	130 (50.19)	129 (49.81)
Ns5D x Lp3A	335 (127, 208)	89	281 (83.88)	267 (95.02)	267 (100.00)	139 (52.06)	128 (47.94)
Lp3A x Cb5E	377 (200, 177)	92	328 (87.00)	285 (86.89)	276 (96.84)	127 (46.01)	149 (53.99)
Cb5E x Lp3A	329 (200, 129)	85	263 (79.94)	208 (79.09)	206 (99.04)	105 (50.97)	101 (49.03)
Lp3A x Tg3E	318 (190, 128)	79	248 (77.99)	236 (95.16)	236 (100.00)	120 (50.85)	116 (49.15)
Tg3E x Lp3A	272 (189, 83)	82	212 (77.94)	189 (89.15)	189 (100.00)	94 (49.74)	95 (50.26)
Lp3A x Sb2F	335 (161, 174)	88	268 (80.00)	268 (100.00)	268 (100.00)	137 (51.12)	131 (48.88)
Sb2F x Lp3A	335 (96, 239)	88	281 (83.88)	258 (91.81)	240 (93.02)	125 (52.08)	115 (47.92)
Lp3A x Tg1F	303 (208, 95)	75	224 (73.93)	208 (92.86)	208 (100.00)	100 (48.08)	108 (51.92)
Tg1F x Lp3A	295 (189, 106)	97	271 (91.86)	238 (87.82)	238 (100.00)	112 (47.06)	126 (52.94)

Table 2. (continued)

Crosses (Female x Male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
F_1- hybrid cross							
(Lp3A x Nk1B)F_1 x (Lp3A x Nk1B)F_1	306 (162, 144)	93	272 (88.89)	245 (90.07)	235 (95.92)	115 (48.94)	120 (51.06)
(Nk1B x Lp3A)F_1 x (Nk1B x Lp3A)F_1	333 (215, 118)	83	263 (78.98)	250 (95.06)	250 (100.00)	120 (48.00)	130 (52.00)
(Lp3A x Ns4B)F_1 x (Lp3A x Ns4B)F_1	306 (137, 169)	88	260 (84.97)	250 (96.15)	245 (98.00)	118 (48.16)	127 (51.84)
(Ns4B x Lp3A)F_1 x (Ns4B x Lp3A)F_1	279 (156, 123)	93	246 (88.17)	231 (93.90)	226 (97.83)	115 (50.88)	111 (49.12)
(Lp3A x Cb3C)F_1 x (Lp3A x Cb3C)F_1	313 (214, 99)	98	288 (92.01)	248 (86.11)	246 (99.19)	116 (47.15)	130 (52.85)
(Cb3Cx Lp3A)F_1 x (Cb3C x Lp3A)F_1	367 (241, 126)	89	297 (80.93)	276 (92.93)	262 (94.93)	131 (50.00)	131 (50.00)
(Lp3Ax Ns8C)F_1 x (Lp3A x Ns8C)F_1	351 (198, 153)	90	281 (80.06)	244 (86.83)	244 (100.00)	129 (52.87)	115 (47.13)
(Ns8C x Lp3A)F_1 x (Ns8C x Lp3A)F_1	391 (135, 256)	89	317 (81.07)	276 (87.07)	262 (94.93)	123 (46.95)	139 (53.05)
(Lp3A x Ur5D)F_1 x (Lp3A x Ur5D)F_1	362 (233, 129)	82	290 (80.11)	281 (96.90)	281 (100.00)	141 (50.18)	140 (49.82)
(Ur5D x Lp3A)F_1 x (Ur5D x Lp3A)F_1	436 (201, 235)	90	373 (85.55)	332 (89.01)	325 (97.89)	150 (46.15)	175 (53.85)
(Lp3A x Ns5D)F_1 x (Lp3A x Ns5D)F_1	388 (222, 166)	85	318 (81.96)	315 (99.06)	312 (99.05)	162 (51.92)	150 (48.08)
(Ns5D x Lp3A)F_1 x (Ns5D x Lp3A)F_1	349 (167, 182)	97	311 (89.11)	302 (97.11)	302 (100.00)	148 (49.01)	154 (50.99)
(Lp3A x Cb5E)F_1 x (Lp3A x Cb5E)F_1	320 (230, 90)	84	262 (81.88)	223 (85.11)	223 (100.00)	120 (53.81)	103 (46.19)
(Cb5E x Lp3A)F_1 x (Cb5E x Lp3A)F_1	335 (221, 114)	90	288 (85.97)	262 (90.97)	254 (96.95)	119 (46.85)	135 (53.15)
(Lp3A x Tg3E)F_1 x (Lp3A x Tg3E)F_1	413 (172, 241)	88	326 (78.93)	313 (96.01)	313 (100.00)	156 (49.84)	157 (50.16)
(Tg3E x Lp3A)F_1 x (Tg3E x Lp3A)F_1	288 (116, 172)	92	242 (84.03)	203 (83.88)	199 (98.03)	105 (52.76)	94 (47.24)
(Lp3A x Sb2F)F_1 x (Lp3A x Sb2F)F_1	379 (212, 167)	79	292 (77.04)	277 (94.86)	277 (100.00)	141 (50.90)	136 (49.10)
(Sb2F x Lp3A)F_1 x (Sb2F x Lp3A)F_1	379 (202, 177)	87	311 (82.06)	246 (79.10)	246 (100.00)	123 (50.00)	123 (50.00)
(Lp3A x Tg1F)F_1 x (Lp3A x Tg1F)F_1	273 (169, 104)	85	218 (79.85)	207 (94.95)	188 (90.82)	98 (52.13)	90 (47.87)
(Tg1F x Lp3A)F_1 x (Tg1F x Lp3A)F_1	325 (181, 144)	90	263 (80.92)	258 (98.10)	245 (94.96)	120 (48.98)	125 (51.02)

a: two selective egg-batches of inseminated females from each cross; *b*: dissection from 100 eggs; n = number

Sequencing alignment and phylogenetic analysis

Sequences of ITS2, COI and COII were aligned using the CLUSTAL W multiple alignment program (Thompson *et al.*, 1994). Gap sites were excluded from the following analysis. The Kimura two-parameter method was used to calculate genetic distances (Kimura, 1980). Construction of neighbor-joining trees (Saitou & Nei, 1987) and the bootstrap test with 1,000 replications were conducted with the MEGA version 4.0 program (Tamura *et al.*, 2007).

RESULTS

Karyotypic characters

Cytogenetic observations of F_1- and/or F_2-progenies of the 53 isolines of *An. peditaeniatus* revealed different types of sex chromosomes due to the addition of extra block(s) of heterochromatin. There were 3 types of X (metacentric X_1, small submetacentric X_2 and large submetacentric X_3) and 6 types of Y chromosomes (very small telocentric Y_1, medium telocentric Y_2, large telocentric Y_3, very large telocentric Y_4, submetacentric Y_5 and medium metacentric Y_6) (Fig. 2). These types of X and Y chromosomes comprise 6 forms of mitotic karyotypes on the basis of Y chromosome configurations designated as Forms A (X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_3, Y_3), D (X_1, X_2, X_3, Y_4), E (X_1, X_2, X_3,Y_5) and F (X_2, X_3, Y_6). The number of isolines of these karyotypic forms occurring in different localities in 17 provinces of Thailand are shown in Fig. 1 and Table 1. Form A (X_3, Y_1) was very rare and has been detected only in Lampang province. On the other hand, Form E was quite common throughout the species' distribution in Thailand, while Forms B, C, D and F were found sporadically in several localities (Fig. 1).

Crossing experiments

Details of hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal and F_1-hybrid crosses among the 11 isolines of *An. peditaeniatus* representing Forms A-F are shown in Table 2. All crosses yielded viable progenies through to the F_2-generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses. The salivary gland polytene chromosomes of the 4th instar larvae of F_1-hybrids from all crosses showed complete synapsis without inversion loops along the whole lengths of all autosomes and the X chromosome (Fig. 3).

DNA sequences and phylogenetic analysis

DNA sequences were determined and analyzed for the ITS2, COI and COII regions of the 53 isolines of *An. peditaeniatus* Forms A–F. They all showed the same length for the ITS2 (463 bp), COI (548 bp) and COII (672 bp) sequences. The evolutionary relationships among the 6 karyotypic forms using neighbour-joining trees were constructed (Fig. 4). The average genetic distances within and between the 6 karyotypic forms exhibited no significant difference in these DNA regions (genetic distance = 0.000-0.003). Hence, the 53 isolines were placed within a single species namely *An. peditaeniatus*. Additionally, these isolines showed little genetic distance difference (0.000-0.005) from *An. peditaeniatus* Form B from Ratchaburi province previously reported by Choochote (2011). However, the trees for ITS2, COI and COII of these isolines representing Forms A-F were clearly different from *An. sinensis* from Korea and *An. lesteri* from Korea and China with strongly supported bootstrap values (99-100%) (Fig. 4).

DISCUSSION

The first cytogenetic investigations of 27 isolines of *An. peditaeniatus* from 3 different localities in Thailand (Chiang Mai, Phrae and Chanthaburi provinces) were performed by Baimai *et al.* (1993). They showed that *An. peditaeniatus* exhibited karyotypic variation via a gradual increase of extra heterochromatin on X (X_1, X_2, X_3) and Y (Y_1, Y_2, Y_3, Y_4, Y_5) chromosomes. Recently,

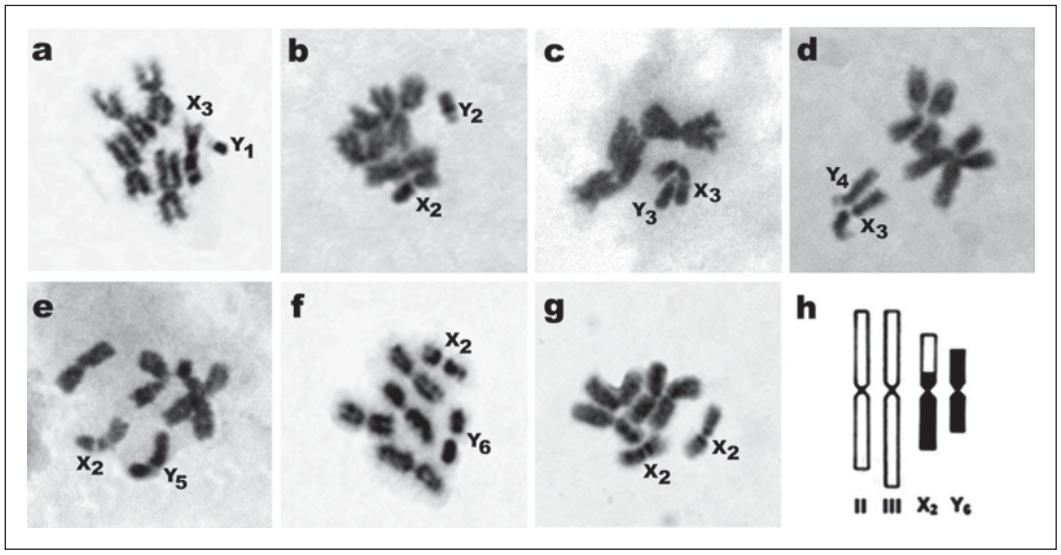

Figure 2. Metaphase karyotypic forms of *An. peditaeniatus*. (a) Form A (X_3, Y_1: Lampang); (b) Form B (X_2, Y_2: Chumphon); (c) Form C (X_3, Y_3: Chon Buri); (d) Form D (X_3, Y_4: Mukdahan); (e) Form E (X_2, Y_5: Nakhon Sawan); (f) Form F (X_2, Y_6: Trang); (g) Form F (X_2, X_2: Trang); (h) diagrams of representative metaphase karyotype of Form F

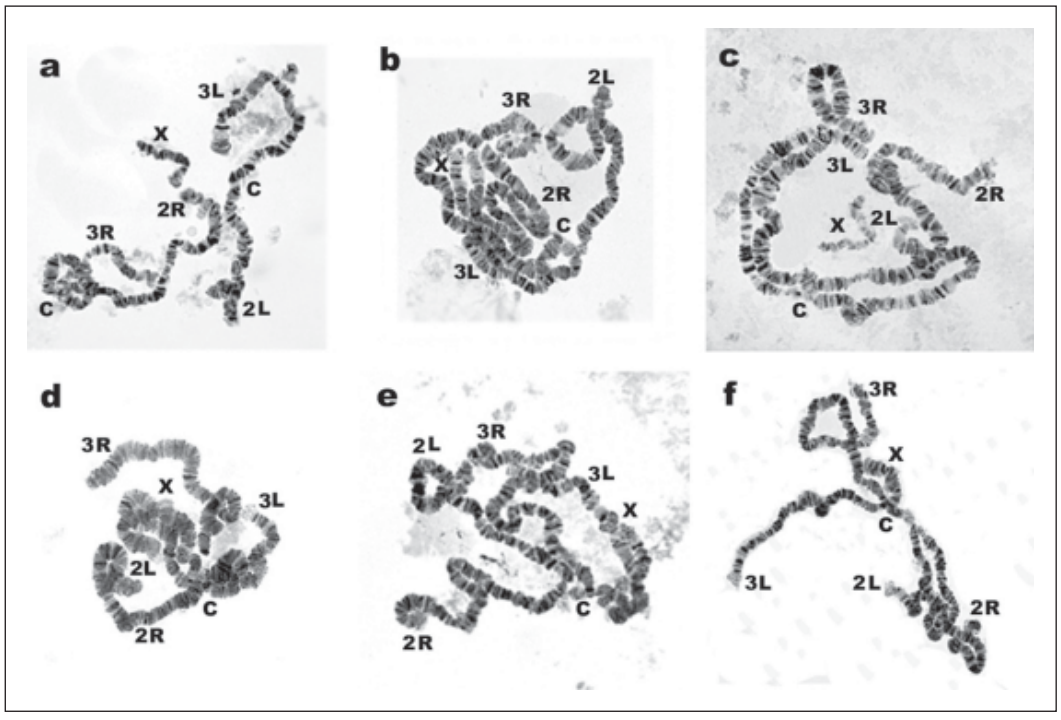

Figure 3. Complete synapsis in all arms of salivary gland polytene chromosome of F_1-hybrids of *An. peditaeniatus*. (a) Lp3A female x Nk1B male; (b) Lp3A female x Cb3C male; (c) Lp3A female x Ur5D male; (d) Lp3A female x Tg3E male; (e) Lp3A female x Sb2F male; (f) Lp3A female x Tg1F male

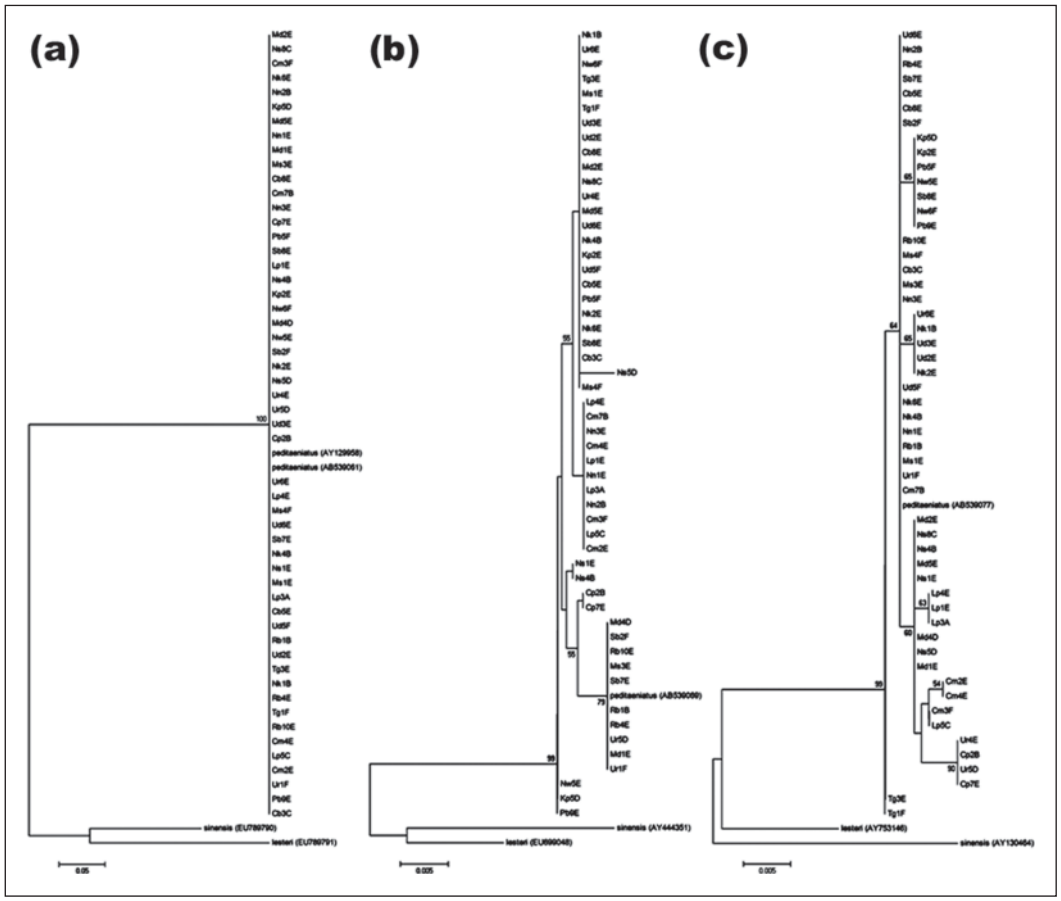

Figure 4. Phylogenetic relationships among the 53 isolines of *An. pediataeniatus* based on molecular analysis compared with *An. sinensis* and *An. lesteri*. (a) ITS2; (b) COI; (c) COII. The trees were generated by neighbor-joining analysis. Numbers on branches are bootstrap values (%) after 1,000 replications. Bootstrap values under 50% are not shown. Branch lengths are proportional to genetic distance (scale bar)

Choochote (2011) studied mitotic karyotypes of *An. peditaeniatus* from 8 different localities in Thailand (Chiang Mai, Nan, Kamphaeng Phet, Udon Thani, Ubon Ratchathani, Chon Buri, Ratchaburi and Chumphon provinces) and demonstrated 2 types of X (X_2, X_3) and 4 types of Y (Y_2, Y_3, Y_4, Y_5) chromosomes forming 4 karyotypic forms tentatively designated as Forms B (X_2, X_3, Y_2), C (X_3, Y_3), D (X_3, Y_4) and E (X_2, X_3,Y_5). In this study, we have detected 3 types of X (X_1, X_2, X_3) and 6 types of Y (Y_1, Y_2, Y_3, Y_4, Y_5, Y_6) chromosomes forming 6 karyotypic forms, i.e., Forms A (X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_3, Y_3), D (X_1, X_2, X_3, Y_4), E (X_1, X_2, X_3,Y_5) and F (X_2, X_3, Y_6). The newly discovered Form F in this study was based on the medium metacentric Y_6 chromosome which was obviously different from the other 5 types previously reported by Baimai *et al.* (1993) and Choochote (2011). Clearly, the 6 distinct karyotypic forms of *An. peditaeniatus* were due to a gain in extra heterochromatin within sex chromosomes. The phenomenon of accumulation of heterochromatin in the genome has played an important role in karyotype evolution, at least in dipteran insects (Baimai, 1998). In addition, such a chromosome difference is very useful for cytotaxonomic study of closely related species particularly sibling species as exemplified in the *Anopheles dirus* complex (Baimai, 1988) and other groups of *Anopheles* (Kanda *et al.*, 1981; Baimai *et al.*, 1987;

Subbarao, 1998; Junkum *et al*., 2005). In this study, we found an ancestral Form A (X_3, Y_1) in only one isoline from Lampang province, while Forms B, C, D, E and F were widespread in Thailand.

Crossing experiments using isoline colonies of anopheline mosquitoes to determine post-mating reproductive compatibility have proven to be efficient techniques for recognition of sibling species within the Oriental *Anopheles* (Kanda *et al*., 1981; Baimai *et al*., 1987; Subbarao, 1998; Junkum *et al*., 2005). In this regard, intensive crossing experiments among the 6 allopatric karyotypic forms of *An. peditaeniatus* showed no post-mating reproductive isolation. Hence, these results strongly suggested a conspecific nature of these karyotypic forms of *An. peditaeniatus*. Identical and/or very low intraspecific sequence variations (genetic distance = 0.000-0.003) of ITS2, COI and COII of the 6 karyotypic forms provided good supportive evidence. Thus our findings are in agreement with the results of hybridization experiments among the 4 karyotypic forms of *An. peditaeniatus* in Thailand previously reported by Choochote (2011). Similar studies on other anopheline species have been reported, e.g., *Anopheles vagus* (Choochote *et al*., 2002), *Anopheles pullus* (= *An. yatsushiroensis*) (Park *et al*., 2003), *Anopheles sinensis* (Choochote *et al*., 1998; Min *et al*., 2002; Park *et al*., 2008b), *Anopheles aconitus* (Junkum *et al*., 2005), *Anopheles barbirostris* species A1 and A2 (Saeung *et al*., 2007; Suwannamit *et al*., 2009), and an *Anopheles campestris*-like taxon (Thongsahuan *et al*., 2009). Thus, karyotypic variation based on extra heterochromatin in sex chromosomes seems to be a general phenomenon within the Oriental *Anopheles*.

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Metaphase karyotypes of *Anopheles paraliae* (Diptera: Culicidae) in Thailand and evidence to support five cytological races

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Abstract. Sixteen isoline colonies of *Anopheles paraliae* were established from wild-caught females collected from cow-baited traps at 4 locations in Thailand. They showed 3 types of X (X_1, X_2, X_3) and 5 types of Y (Y_1, Y_2, Y_3, Y_4, Y_5) chromosomes based on the number and amount of major block(s) of heterochromatin present in the heterochromatic arm, and were designated as Forms A (X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_3, Y_3), D (X_1, X_2, X_3, Y_4) and E (X_3, Y_5). Form A was found in Songkhla Province, Form B was obtained in Ratchaburi, Nakhon Si Thammarat and Songkhla Provinces, Form C was acquired in Chanthaburi Province, Form D was recovered in Ratchaburi and Songkhla Provinces, and Form E was encountered in Ratchaburi Province. Hybridization experiments among the 7 isoline colonies, which represented the 5 karyotypic forms of *An. paraliae*, revealed genetic compatibility in providing viable progenies and synaptic salivary gland polytene chromosomes through F_2-generations, and thus suggest the conspecific nature of these karyotypic forms. These results were supported by the very low intraspecific sequence divergence (mean genetic distance = 0.000–0.002) of the nucleotide sequences in ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII) of the 5 forms.

INTRODUCTION

Anopheles paraliae Sandosham belongs to the Hyrcanus Group of the Myzorhynchus Series, of the subgenus *Anopheles*, and is distributed widely along coastal regions of southern Thailand, Malaysia (Malaysian peninsular, Sabah and Sarawak), Brunei and Vietnam (Reid, 1968; Harrison & Scanlon, 1975; Rattanarithikul *et al*., 2006). Originally, *An. paraliae* was considered a subspecies of *Anopheles lesteri* Baisas & Hu, but was elevated to species status by Harrison *et al.* (1991). Regarding 8 species members (*Anopheles argyropus* Swellengrebel, *Anopheles crawfordi* Reid, *Anopheles nigerrimus* Giles, *Anopheles nitidus* Harrison, Scanlon and Reid, *An. paraliae*, *Anopheles peditaeniatus* Leicester, *Anopheles pursati* Laveran, and *Anopheles sinensis* Wiedemann) of the Thai Hyrcanus Group; *An. nigerrimus*, *An. peditaeniatus* and *An. sinensis* are considered as suspected vectors of *Plasmodium vivax* in Thailand (Baker *et al*., 1987; Harbach *et al*., 1987; Gingrich *et al*., 1990; Frances *et al*., 1996;

Rattanarithikul *et al*., 1996), while *An. sinensis* has been incriminated as natural vector of *P. vivax* in China and Korea (Mourya *et al*., 1989; Liu, 1990; Chai, 1999; Ree *et al*., 2001; Whang *et al*., 2002), and *Wuchereria bancrofti* and *Brugia malayi* in China (Sasa, 1976), and *An. peditaeniatus* as a secondary vector of Japanese encephalitis virus in China and India (Zhang, 1990; Kanojia *et al*., 2003). In addition, *An. nigerrimus* was incriminated recently as a potentially natural vector of *Wuchereria bancrofti* in Phang Nga Province, southern Thailand (Division of Filariasis, 1998). As for *An. paraliae*, although it has never been incriminated as a natural and/or suspected vector of any human-diseases, it and other seven species members are considered economic pests of cattle because of their vicious biting-behavior and their ability to transmit cervid filariae of the genus *Setaria* (Reid *et al*., 1962; Reid, 1968; Harrison & Scanlon, 1975).

Regarding metaphase karyotypes, extensive investigations have been performed on 6 species of the Hyrcanus Group. The results demonstrated that these anopheline species exhibited genetic diversity at the chromosomal level, resulting in marked karyotypic variations via a gradual increase in the extra block(s) of heterochromatin on X and Y chromosomes. These findings were *An. sinensis* Forms A (X, Y_1) and B (X, Y_2), *An. nigerrimus* Forms A (X_1, Y_1) and B (X_2, Y_2), *An. crawfordi* Forms A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), *An. argyropus* Forms A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), *An. peditaeniatus* Forms A (X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_3, Y_3), D (X_1, X_2, X_3, Y_4), E (X_1, X_2, X_3,Y_5) and F (X_2, X_3, Y_6), and 2 types of X (X_1, X_2) and 1 type of Y chromosomes in *An. nitidus* (Baimai *et al*., 1993; Choochote, 2011; Saeung *et al*., 2012). The marked genetic variation at the chromosomal level of each species, potentially results in the existence of a species complex and complicates identification of potential vector species within the complex [sibling species and/or subspecies members (cytological races)], because of nearly identical morphology or minimal morphological distinction (Subbarao, 1998). As seen from the information above, there is obviously a lack of cytogenetic evidence for *An. paraliae*, that indicates the genetic proximity among karyotypic variants within the taxon. Thus, we report the metaphase karyotypes of *An. paraliae* and determine the role of karytoypic variants in generating post-mating barriers. This was done by hybridization experiments among karyotypic forms verified by DNA sequence analyses of second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI) and cytochrome *c* oxidase subunit II (COII) of mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Field collections and establishment of isoline colonies

Samples of fully engorged females of *An. paraliae* were collected from cow-baited traps in 4 provinces of Thailand (Fig. 1, Table 1). A total of 16 isolines were established and maintained in our insectary at Chiang Mai University using the techniques described by Choochote *et al*. (1983) and they were used for studies on metaphase karyotype, hybridization experiments and molecular analysis.

Metaphase karyotype preparation

Metaphase chromosomes were prepared from 10 samples of the early fourth-instar larval brains of F_1- and/or F_2-progenies of each isoline, using techniques previously described by Saeung *et al*. (2007). Identification of karyotypic forms followed the standard cytotaxonomic systems of Baimai *et al*. (1993).

Hybridization experiment

Seven isolines of *An. paraliae* were arbitrarily selected from the 16 laboratory-raised colonies representing the 5 karyotypic forms, i.e., Forms A (Sk3A), B (Ns1B, Rt4B), C (Ch1C), D (Rt7D, Rt8D) and E (Rt5E) (Table 1). They were used for hybridization experiments to determine post-mating reproductive isolation employing the techniques of Saeung *et al*. (2007). The salivary gland polytene chromosomes of

F_1-hybrids from these crosses were examined using the techniques described by Kanda (1979).

DNA extraction and amplification

An individual F_1 adult female from each isoline of *An. paraliae* (Ch1C, Rt1D, Rt2D, Rt3D, Rt4B, Rt5E, Rt6D, Rt7D, Rt8D, Rt9E, Rt10D, Ns1B, Sk1B, Sk2D, Sk3A, Sk4D) was used for DNA extraction and amplification. Molecular analysis of 3 specific genomic loci [second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI) and cytochrome *c* oxidase subunit II (COII) of mitochondrial DNA (mtDNA)] was performed to determine intraspecific sequence variation. Genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (QIAGEN). The primers used for PCR amplification and sequencing were those reported by Saeung *et al*. (2007). Amplifications were performed in a total of 20 µl volumes containing 0.5 U *Ex Taq* (Takara), 1X *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer, and 1 µl of the extracted DNA. For ITS2, the conditions for amplification consisted of initial denaturation at 94ºC for 1 min, 30 cycles at 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1 min, and a final extension at 72ºC for 5 min. The amplification profile of COI and COII comprised initial denaturation at 94ºC for 1 min, 30 cycles at 94ºC for 30 sec, 50ºC for 30 sec, and 72ºC for 1 min, and a final extension at 72ºC for 5 min. The amplified products were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Finally, the PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN) and their sequences directly determined using the BigDye® Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems). The sequence data

Figure 1. Map of Thailand showing 4 provinces where samples of *Anopheles paraliae* were collected and the numbers of isolines of the 5 karyotypic forms (A-E) were detected

Table 1. Locations in 4 provinces of Thailand, code of isolines, 5 karyotypic forms (A-E) of *Anopheles paraliae* and their GenBank accession numbers

Location (Geograpical coordinate)	Code of isoline[a]	Karyotypic form	DNA Region	GenBank accession number			Reference
				ITS2	COI	COII	
An. paraliae							
Chanthaburi (12° 38'N, 102° 12'E)	Ch1C[a]	C (X_3, Y_3)	ITS2, COI, COII	AB733475	AB733491	AB733507	This study
Ratchaburi (13° 30'N, 99° 54'E)	Rt1D	D (X_3, Y_4)	ITS2, COI, COII	AB733476	AB733492	AB733508	This study
	Rt2D	D (X_3, Y_4)	ITS2, COI, COII	AB733477	AB733493	AB733509	This study
	Rt3D	D (X_3, Y_4)	ITS2, COI, COII	AB733478	AB733494	AB733510	This study
	Rt4B[a]	B (X_2, Y_2)	ITS2, COI, COII	AB733479	AB733495	AB733511	This study
	Rt5E[a]	E (X_3, Y_5)	ITS2, COI, COII	AB733480	AB733496	AB733512	This study
	Rt6D	D (X_3, Y_4)	ITS2, COI, COII	AB733481	AB733497	AB733513	This study
	Rt7D[a]	D (X_2, Y_4)	ITS2, COI, COII	AB733482	AB733498	AB733514	This study
	Rt8D[a]	D (X_1, Y_4)	ITS2, COI, COII	AB733483	AB733499	AB733515	This study
	Rt9E	E (X_3, Y_5)	ITS2, COI, COII	AB733484	AB733500	AB733516	This study
	Rt10D	D (X_3, Y_4)	ITS2, COI, COII	AB733485	AB733501	AB733517	This study
Nakhon Si Thammarat (08° 29'N, 100° 0'E)	Ns1B[a]	B (X_3, Y_2)	ITS2, COI, COII	AB733486	AB733502	AB733518	This study
Songkhla (07° 13'N, 100° 37'E)	Sk1B	B (X_1, Y_2)	ITS2, COI, COII	AB733487	AB733503	AB733519	This study
	Sk2D	D (X_1, Y_4)	ITS2, COI, COII	AB733488	AB733504	AB733520	This study
	Sk3A[a]	A (X_3, Y_1)	ITS2, COI, COII	AB733489	AB733505	AB733521	This study
	Sk4D	D (X_3, Y_4)	ITS2, COI, COII	AB733490	AB733506	AB733522	This study
An. sinensis	i2ACM	A (X, Y_1)	ITS2	AY130473	–	–	Min *et al.*, 2002
	–	B (X, Y_2)	COI	–	AY444351	–	Park *et al.*, 2003
	i1BKR	B (X, Y_2)	COII	–	–	AY130464	Min *et al.*, 2002
An. pullus	–	A (X_1, X_2, Y_2)	ITS2, COI, COII	AY444345	AY444348	AY444347	Park *et al.*, 2003
An. peditaeniatus	RbB	B (X_3, Y_2)	ITS2, COI, COII	AB539061	AB539069	AB539077	Choochote, 2011

[a] used in crossing experiments.

of this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB733475-AB733522. The ITS2, COI and COII sequenced obtained from this study were also compared with sequences available in GenBank (Table 1) using the Basic Local Alignment Search Tool (BLAST) available at (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequencing alignment and phylogenetic analysis

Sequences of ITS2, COI and COII were aligned using the CLUSTAL W multiple alignment programme (Thompson *et al.*, 1994) and edited manually in BioEdit version 7.0.5.3 (Hall, 1999). Gap sites were excluded from the following analysis. Genetic distances were estimated with the Kimura two-parameter method (Kimura 1980). Construction of neighbour-joining trees (Saitou & Nei, 1987) and the bootstrap test with 1,000 replications were conducted with the MEGA version 4.0 programme (Tamura *et al.*, 2007).

RESULTS

Metaphase karyotype

Cytogenetic observation of F_1- and/or F_2-progenies of the 16 isolines which were represented in 4 locations across 3 regions (western, eastern, southern) in Thailand, demonstrated that *An. paraliae* has the typical chromosome number of 2n=6, consisting of two pairs of autosomes (submetacentric and metacentric) and one pair of heteromorphic sex chromosomes (XX in females and XY in males). Based on the number and amount of major block(s) of the heterochromatin present in the heterochromatic arm of the sex chromosomes, 3 types of X (X_1, X_2, X_3) and 5 types of Y (Y_1, Y_2, Y_3, Y_4, Y_5) chromosomes were obtained in this investigation (Fig. 2 and 3). The X_1 chromosome has a small metacentric shape with one arm euchromatic, and the opposite one totally heterochromatic. The X_2 chromosome is different from the X_1 chromosome in having an extra block of heterochromatin in the heterochromatic arm, making it a long arm of submetacentric

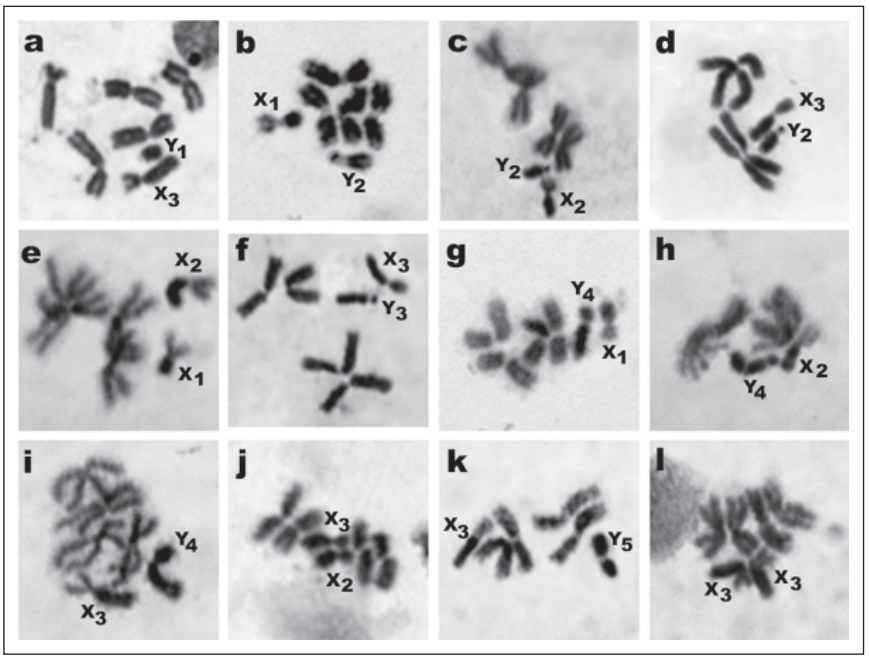

Figure 2. Metaphase karyotypic forms of *An. paraliae*. (a) Form A (X_3, Y_1), (b) Form B (X_1, Y_2), (c) Form B (X_2, Y_2), (d) Form B (X_3, Y_2), (e) Form B (X_1, X_2), (f) Form C (X_3, Y_3), (g) Form D (X_1, Y_4), (h) Form D (X_2, Y_4), (i) Form D (X_3, Y_4), (j) Form D (X_2, X_3), (k) Form E (X_3, Y_5), (l) Form E (X_3, X_3)

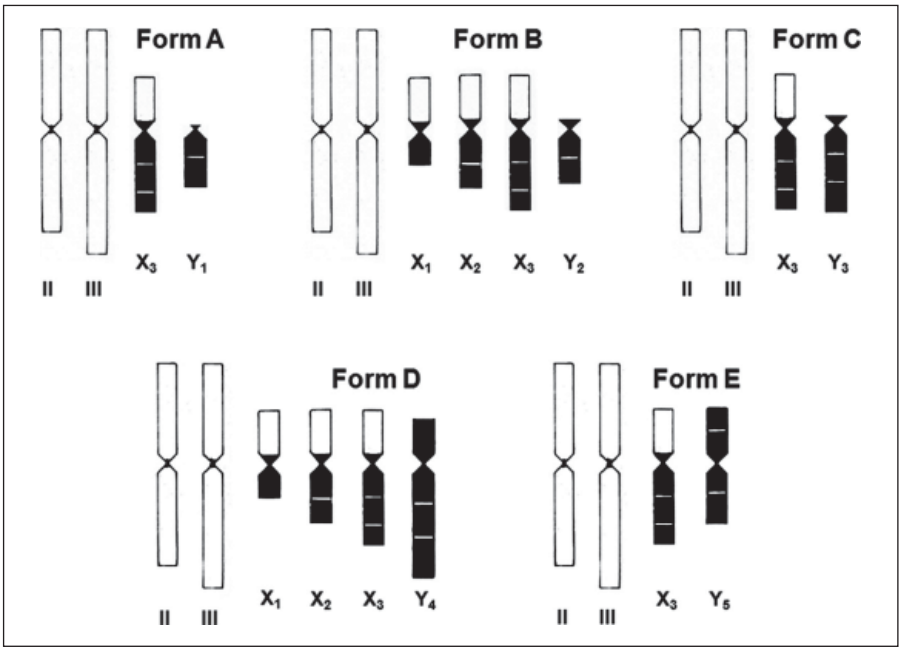

Figure 3. Diagrams of representative metaphase karyotypes of Forms A, B, C, D and E of *An. paraliae*

configuration. The X_3 chromosome has a large submetracentric shape that was slightly different from the X_2 chromosome in having an extra block of heterochromatin at the distal end of the long heterochromatic arm. A good comparison of the size and shape between X_2 and X_3 chromosomes can be seen in heterozygous females (Fig. 2j). Similar to the situation in the X chromosome, the Y chromosome also exhibited extensive variation in size and shape, due to differing amounts and distribution of heterochromatic block. Thus the Y_1 chromosome is a small telocentric figure, which probably represents the simple or ancestral form of the Y chromosome (Fig. 2a). The Y_2 chromosome has a small subtelocentric or acrocentric shape that slightly differs from the Y_1 chromosome which has a very small portion of the short arm present (Fig. 2b-d). Chromosome Y_3 has a large subtelocentric shape that obviously differs from the Y_2 chromosome in having an extra block of heterochromatin at the distal end of the long heterochromatic arm (Fig. 2f). The Y_4 chromosome is clearly submetacentric, with the short arm approximately 1/3 the length of the long arm. It appears to have derived from the Y_3 chromosome by means of adding an extra block of heterochromatin onto the short arm, and transferring it to a submetacentric configuration (Fig. 2g-i). Chromosome Y_5 has a medium metacentric configuration, which is slightly shorter than that in the Y_4 chromosome. It could have arisen from the ancestral Y_1 chromosome simply through addition of 2 extra blocks of heterochromatin onto the opposite short arm (Fig. 2k). Hence, 5 karyotypic forms were recognized based on unique characteristics of the X and Y chromosomes and they were designated as Forms A (X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_3, Y_3), D (X_1, X_2, X_3, Y_4) and E (X_3, Y_5). These karyotypic forms were detected in different locations as shown in Fig. 1 and Table 1.

Hybridization experiment

Details of hatchability, pupation, emergence and adult sex ratio of parental, reciprocal and F_1-hybrid experiments among the 7 isolines of *An. paraliae* representing Forms A-E are shown in Table 2. All crosses gave viable progenies through the F_2-generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses. The salivary

Table 2. Crossing experiments among 7 isolines of *An. paraliae*

Crosses (Female x Male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
Parental cross							
Sk3A x Sk3A	316 (158, 158)	84	231 (73.10)	212 (91.77)	208 (98.11)	120 (57.69)	88 (42.31)
Ns1B x Ns1B	319 (162, 157)	83	258 (80.88)	248 (96.12)	199 (80.24)	121 (60.80)	78 (39.20)
Rt4B x Rt4B	372 (150, 222)	84	305 (81.99)	305 (100.00)	305 (100.00)	143 (46.89)	162 (53.11)
Ch1C x Ch1C	394 (187, 207)	82	315 (79.95)	277 (87.94)	265 (95.67)	151 (56.98)	114 (43.02)
Rt7D x Rt7D	285 (167, 118)	92	242 (84.91)	242 (100.00)	220 (90.91)	116 (52.73)	104 (47.27)
Rt8D x Rt8D	299 (190, 109)	85	245 (81.94)	213 (86.94)	196 (92.02)	83 (42.35)	113 (57.65)
Rt5E x Rt5E	309 (147, 162)	81	226 (73.14)	212 (93.80)	208 (98.11)	106 (50.96)	102 (49.04)
Reciprocal cross							
Sk3A x Ns1B	346 (186, 160)	73	242 (69.94)	213 (88.02)	194 (91.08)	97 (50.00)	97 (50.00)
Ns1B x Sk3A	359 (158, 201)	75	266 (74.09)	231 (86.84)	204 (88.31)	106 (51.96)	98 (48.04)
Sk3A x Rt4B	339 (157, 182)	89	295 (87.02)	289 (97.97)	274 (94.81)	162 (59.12)	112 (40.88)
Rt4B x Sk3A	391 (194, 197)	90	344 (87.98)	310 (90.12)	292 (94.19)	110 (37.67)	182 (62.33)
Sk3A x Ch1C	344 (189, 155)	90	299 (86.92)	299 (100.00)	275 (91.97)	121 (44.00)	154 (56.00)
Ch1C x Sk3A	331 (206, 125)	96	314 (94.86)	314 (100.00)	308 (98.09)	135 (43.83)	173 (56.17)
Sk3A x Rt7D	348 (158, 190)	88	306 (87.93)	278 (90.85)	272 (97.84)	131 (48.16)	141 (51.84)
Rt7D x Sk3A	325 (125, 200)	90	260 (80.00)	260 100.00)	260 (100.00)	130 (50.00)	130 (50.00)
Sk3A x Rt8D	347 (157, 190)	84	232 (66.86)	204 (87.93)	200 (98.04)	93 (46.50)	107 (53.50)
Rt8D x Sk3A	305 (147, 158)	82	247 (80.98)	247 (100.00)	237 (95.95)	117 (49.37)	120 (50.63)
Sk3A x Rt5E	353 (167, 186)	86	297 (84.13)	273 (91.92)	273 (100.00)	134 (49.08)	139 (50.92)
Rt5E x Sk3A	376 (194, 182)	82	293 (77.92)	293 (100.00)	293 (100.00)	126 (43.00)	167 (57.00)
F_1- hybrid cross							
(Sk3A x Ns1B)F_1 x (Sk3A x Ns1B)F_1	309 (109, 200)	78	229 (74.11)	199 (86.90)	192 (96.48)	89 (46.35)	103 (53.65)
(Ns1B x Sk3A)F_1 x (Ns1B x Sk3A)F_1	308 (194, 114)	98	274 (88.96)	186 (67.88)	186 (100.00)	129 (69.35)	57 (30.65)
(Sk3A x Rt4B)F_1 x (Sk3A x Rt4B)F_1	350 (155, 195)	93	301 (86.00)	256 (85.05)	250 (97.66)	117 (46.80)	133 (53.20)
(Rt4B x Sk3A)F_1 x (Rt4B x Sk3A)F_1	383 (220, 163)	94	326 (85.12)	297 (91.10)	293 (98.65)	140 (47.78)	153 (52.22)
(Sk3A x Ch1C)F_1 x (Sk3A x Ch1C)F_1	345 (150, 195)	76	228 (66.09)	228 (100.00)	221 (96.93)	93 (42.08)	128 (57.92)
(Ch1C x Sk3A)F_1 x (Ch1C x Sk3A)F_1	248 (118, 130)	80	169 (68.14)	169 (100.00)	169 (100.00)	73 (43.20)	96 (56.80)
(Sk3A x Rt7D)F_1 x (Sk3A x Rt7D)F_1	406 (220, 186)	73	252 (62.07)	244 (96.83)	224 (91.80)	98 (43.75)	126 (56.25)
(Rt7D x Sk3A)F_1 x (Rt7D x Sk3A)F_1	351 (189, 162)	76	253 (72.08)	253 (100.00)	248 (98.02)	137 (55.24)	111 (44.76)
(Sk3A x Rt8D)F_1 x (Sk3A x Rt8D)F_1	391 (209, 182)	67	242 (61.89)	242 (100.00)	240 (99.17)	120 (50.00)	120 (50.00)
(Rt8D x Sk3A)F_1 x (Rt8D x Sk3A)F_1	334 (148, 186)	72	230 (68.86)	223 (96.96)	216 (96.86)	115 (53.24)	101 (46.76)
(Sk3A x Rt5E)F_1 x (Sk3A x Rt5E)F_1	403 (209, 194)	92	363 (90.07)	341 (93.94)	319 (93.55)	145 (45.45)	174 (54.55)
(Rt5E x Sk3A)F_1 x (Rt5E x Sk3A)F_1	405 (220, 185)	87	344 (84.94)	289 (84.01)	282 (97.58)	189 (67.02)	93 (32.98)

[a] two selective egg-batches of inseminated females from each cross. [b] dissection from 100 eggs; n = number.

gland polytene chromosomes of the hybrid larvae from all crosses showed complete synapsis without inversion loops in all chromosome arms (Fig. 4).

DNA sequences and phylogenetic analysis

DNA sequences of the ITS2, COI and COII of the 16 isolines of *An. paraliae* Forms A-E were analysed. They all showed the same lengths for ITS2 (448 bp), COI (658 bp) and COII (685 bp) sequences. All 5 karyotypic forms showed completely identical ITS2 sequences. Neighbour-joining (NJ) trees were constructed in order to determine genetic relationships among the 5 karyotypic forms (Fig. 5). The 16 isolines were monophyletic with high support in NJ tree (bootstrap values 100%). Obviously, the mean genetic distance within and between the 5 karyotypic forms exhibited no significant difference (0.000-0.002) in these DNA regions. However, the trees for ITS2, COI and COII of these isolines (Forms A-E) were clearly different from the 3 species of the Hyrcanus Group, i.e., *An. peditaeniatus*, *Anopheles pullus* Yamada and *An. sinensis*, with strongly supported bootstrap values (100%) (Fig. 5).

DISCUSSION

Investigations on metaphase karyotypes of the 16 *An. paraliae* isolines from 4 locations in 3 regions (western, eastern, southern) across Thailand indicated that typical metaphase karyotypes (2n=6) consist of two pairs of autosomes (submetacentric and metacentric) and one pair of heteromorphic sex chromosomes. These metaphase karyotypes can be distinguished on the basis of size, shape, amount and distribution of constitutive heterochromatin, similar to those of 6 species (*An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. peditaeniatus* and *An. sinensis*) of the *Hyrcanus* Group previously reported (Baimai *et al.*, 1993; Choochote, 2011; Saeung *et al.*, 2012). The 5 distinct karyotypic variants: Forms A (X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_3, Y_3), D (X_1, X_2, X_3, Y_4) and E (X_3, Y_5), of *An. paraliae* are due to additional extra block(s) of heterochromatin on sex chromosomes (X, Y), which means this study is keeping with Baimai's hypothesis that is an important mechanism in the speciation process of Oriental anophelines (Baimai, 1998). It also

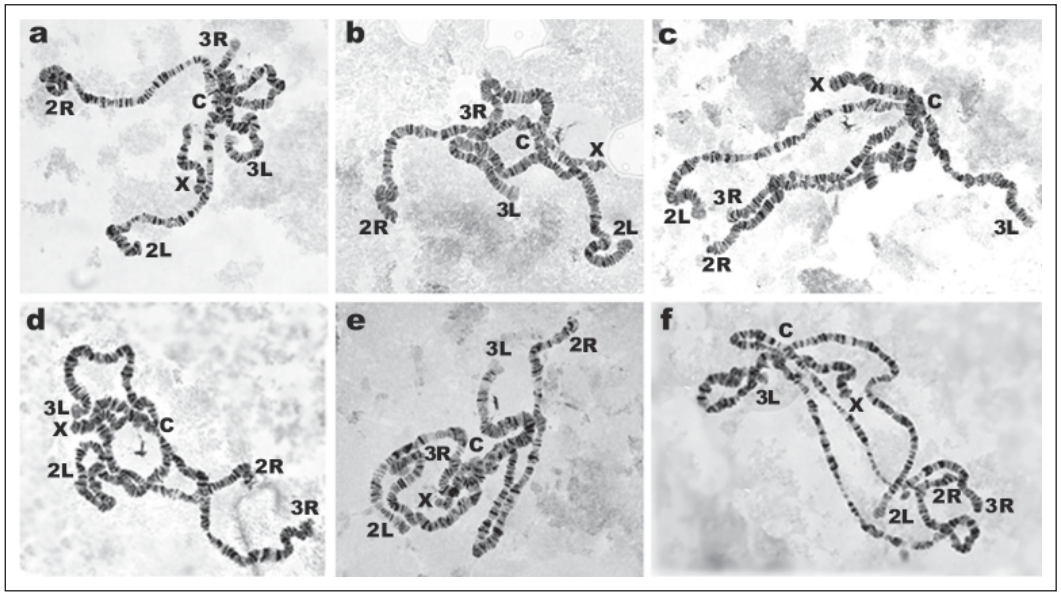

Figure 4. Complete synapsis in all arms of salivary gland polytene chromosomes of F_1-hybrids of *An. paraliae*. (a) Sk3A female x Ns1B male; (b) Sk3A female x Rt4B male; (c) Sk3A female x Ch1C male; (d) Sk3A female x Rt7D male; (e); Sk3A female x Rt8D male; (f) Sk3A female x Rt5E male

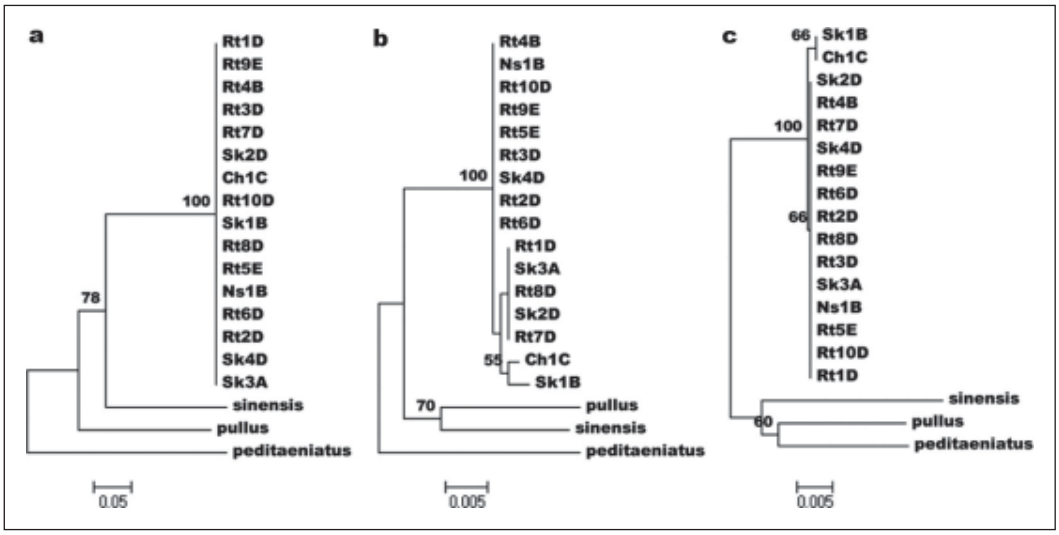

Figure 5. Neighbour-joining (NJ) trees inferred from sequences of 3 loci (a) ITS2, (b) COI and (c) COII of the 16 isolines of *An. paraliae* compared with *An. peditaeniatus*, *An. pullus* and *An. sinensis*. Numbers on branches are bootstrap values (%) after 1,000 replications. Bootstrap values under 50% not shown. Branch lengths are proportional to genetic distance (scale bar)

could be used effectively as a primary genetic marker for further recognitions of sibling species and/or subspecies members within the taxon *Anopheles*.

Hybridization experiments using isoline colonies of *Anopheles* mosquitoes together with data of cytology and molecular analysis to determine post-mating barriers have been proven so far as robust systematic procedures for clarifying sibling species and subspecies members within the taxon *Anopheles* species (Kanda *et al.*, 1981; Baimai *et al.*, 1987; Subbarao, 1998; Junkum *et al.*, 2005). The markedly distinct characteristics of X (X_1, X_2, X_3) and Y (Y_1, Y_2, Y_3, Y_4, and Y_5) chromosomes among the 5 karyotypic forms of *An. paraliae* warrant intensive determination of post-mating barriers. Accordingly, hybridization experiments were carried out among the 5 karyotypic forms, relating to their comparative DNA sequences of ITS2, COI, and COII in order to determine the degree of genetic proximity. The results of no post-mating reproductive isolation by yielding viable progenies through F_2-generations and synaptic salivary gland polytene chromosomes indicate a conspecific nature, comprising 5 cytological races within this taxon. The very low intra-specific sequence divergence (mean genetic distance = 0.000-0.002) of the nucleotide sequences of the ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII) of the 5 karyotypic forms were strong supportive evidence. Similar results have been reported in *Anopheles vagus* Forms A and B (Choochote *et al.*, 2002), *An. pullus* Forms A and B (=*Anopheles yatsushiroensis*) (Park *et al.*, 2003), *An. sinensis* Forms A and B (Choochote *et al.*, 1998; Min *et al.*, 2002; Park *et al.*, 2008), *Anopheles aconitus* Forms B and C (Junkum *et al.*, 2005), *Anopheles barbirostris* species A1 (Forms A, B and C) and species A2 (Forms A and B) (Saeung *et al.*, 2007; Suwannamit *et al.*, 2009), *Anopheles campestris*-like Forms B, E, and F (Thongsahuan *et al.*, 2009), and *An. peditaeniatus* Forms A, B, C, D, E and F (Choochote, 2011; Saeung *et al.*, 2012). Additionally, this is the first report of crossing experiments and molecular investigations of *An. paraliae* using karyotypic markers. In an investigation now in progress we will use additional isolines to elucidate the population-genetic structure of karyotypic forms of *An. paraliae* in Thailand and/or neighbouring countries.

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Genetic compatibility between *Anopheles lesteri* from Korea and *Anopheles paraliae* from Thailand

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To assess differentiation and relationships between Anopheles lesteri *and* Anopheles paraliae *we established three and five iso-female lines of* An. lesteri *from Korea and* An. paraliae *from Thailand, respectively. These isolines were used to investigate the genetic relationships between the two taxa by crossing experiments and by comparing DNA sequences of ribosomal DNA second internal transcribed spacer (ITS2) and mitochondrial DNA cytochrome* c *oxidase subunit I (COI) and subunit II (COII). Results of reciprocal and F_1-hybrid crosses between* An. lesteri *and* An. paraliae *indicated that they were compatible genetically producing viable progenies and complete synaptic salivary gland polytene chromosomes without inversion loops in all chromosome arms. The pairwise genetic distances of ITS2, COI and COII between these morphological species were 0.040, 0.007-0.017 and 0.008-0.011, respectively. The specific species status of* An. paraliae *in Thailand and/or other parts of the continent are discussed.*

Key words: *Anopheles lesteri* - *Anopheles paraliae* - crossing experiments - second internal transcribed spacer - cytochrome *c* oxidase subunit I - cytochrome *c* oxidase subunit II

The *Anopheles hyrcanus* Group has a wide range of distributions extending from Iberia in Europe to East and Southeast Asia, including some of the off-lying islands of the Indian and Pacific Oceans. Up until now, at least 27 species have been reported within this group (Harrison & Scanlon 1975, Harbach 2010). It is well known that some species of the Hyrcanus Group are involved in transmission of human diseases, particularly in the Oriental and contiguous parts of the eastern Palaearctic regions. For example, human malaria *Plasmodium vivax* was detected in *Anopheles sinensis*, *Anopheles lesteri*, *Anopheles kleini*, *Anopheles pullus* and *Anopheles belenrae* (Harrison 1973, Ree et al. 2001, Whang et al. 2002, Ma & Xu 2005, Lee et al. 2007, Joshi et al. 2009, 2011, Rueda et al. 2010). Moreover, *Brugia malayi* was found in *An. sinensis* and *An. lesteri* (Sasa 1976) while *Anopheles peditaeniatus* was infected with Japanese encephalitis virus (Zhang 1990, Kanojia et al. 2003). In addition, some members of the Hyrcanus Group have also been considered as economic pests of cattle because of their vicious biting-behaviour and ability to transmit cervid filariae of the genus *Setaria* (Reid et al. 1962, Reid 1968, Harrison & Scanlon 1975).

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An. lesteri has been found in the Philippines (type locality) and the Palaearctic regions (China, Korea and Japan) whereas *Anopheles paraliae* has been detected in the coastal areas of Peninsular Malaysia, Sabah, Sarawak, Brunei, Vietnam and Thailand. However, the taxonomic ambiguity of *An. paraliae* was raised as early as 1959. Morphologically, *An. paraliae* has a narrower apical fringe spot on the wing compared with that of *An. lesteri*, but their immature stages can not be distinguished from each other. Consequently, *An. paraliae* was considered to be a subspecies, *An. lesteri paraliae*, by earlier authors (Sandosham 1959, Reid 1963, 1968, Harrison & Scanlon 1975). Nevertheless, this subspecies was elevated subsequently to species status, i.e., *An. paraliae*, based on distinct characteristics of the adult wings and immature habitats (brackish and/or peaty water) (Harrison et al. 1991). Yet there is no evidence of genetic differences between *An. lesteri* and *An. paraliae*. This article presents the results of crossing experiment and cytogenetic study of these two species and comparative DNA sequence analyses of the second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA), the cytochrome *c* oxidase subunit I (COI) and subunit II (COII) of mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Collection sites - Samples of *An. lesteri* from Korea were caught in a vinyl tent in a rice paddy field of the district of So Rae, Incheon City, northern of the province of Gyeonggi. The *An. paraliae* specimens from Thailand were obtained by using cow-baited traps in three localities, i.e., district of Damnoen Saduak, province of Ratchaburi, district of Pak Panang, province of Nakhon

Si Thammarat and district of Hat Yai, province of Songkhla (Table I). Species identification using F_1-progeny of each iso-female line followed the keys of Rueda et al. (2005) and Rattanarithikul et al. (2006). The distinctive characteristics of wings to separate *An. lesteri* from *An. paraliae* are illustrated in Fig. 1.

Establishment of iso-female lines - Three and five iso-female lines of *An. lesteri* (ilG1, ilG2, ilG3) and *An. paraliae* (ipR1, ipR2, ipN1, ipS1, ipS2), respectively, were established successfully using the methods of Choochote et al. (1983) and Kim et al. (2003). They have been maintained in colonies for more than five consecutive generations in our laboratory and they were used for crossing experiments and comparative DNA sequence analyses.

Crossing experiments - One iso-female line (ilG1) of *An. lesteri* and three iso-female lines (ipR1, ipN1, ipS1) of *An. paraliae* were arbitrarily selected for crossing experiments to determine post-mating reproductive isolation by employing the techniques previously reported by Saeung et al. (2007). Study on salivary gland polytene chromosomes of 4th instar larvae of F_1-hybrids from the crosses followed the techniques of White et al. (1975) and Kanda (1979).

DNA extraction and amplification - Individual F_1-progeny adult females of each iso-female line of *An. lesteri* (ilG1, ilG2, ilG3) and *An. paraliae* (ipR1, ipR2, ipN1, ipS1, ipS2) were used for DNA extraction and amplification. Molecular analysis of ITS2, COI, COII was performed to determine intraspecific sequence variation in *An. lesteri* and *An. paraliae*. Genomic DNA was extracted from adult mosquitoes using the DNeasy® Blood and Tissue Kit (Qiagen). Primers for amplification of ITS2, COI and COII regions followed the methods of Saeung et

TABLE I

Locations, code of iso-female lines of *Anopheles lesteri* and *Anopheles paraliae* and their GenBank accessions

Location	Code of isoline[a]	Length of ITS2 (bp)	GenBank accession: DNA region	ITS2	COI	COII	Reference
An. lesteri							
Korea: Gyeonggi	ilG1[a]	448	ITS2, COI, COII	AB733020	AB733028	AB733036	This paper
Gyeonggi	ilG2	448	ITS2, COI, COII	AB733021	AB733029	AB733037	This paper
Gyeonggi	ilG3	448	ITS2, COI, COII	AB733022	AB733030	AB733038	This paper
	-	448	ITS2	EU789791	-	-	Park et al. (2008)
Japan	-	448	ITS2	AB159606	-	-	K Sawabe et al., unpublished observations
Philippines	-	438	ITS2	AY375469	-	-	Wilkerson et al. (2003)
An. lesteri (= *Anopheles anthropophagus*)							
China	-	448	ITS2	AY803792	-	-	Ma and Yang (2005)
	-	438	ITS2	AY375467	-	-	Wilkerson et al. (2003)
An. paraliae							
Thailand: Ratchaburi	ipR1[a]	448	ITS2, COI, COII	AB733023	AB733031	AB733039	This paper
Ratchaburi	ipR2	448	ITS2, COI, COII	AB733024	AB733032	AB733040	This paper
Nakhon Si Thammarat	ipN1[a]	448	ITS2, COI, COII	AB733025	AB733033	AB733041	This paper
Songkhla	ipS1[a]	448	ITS2, COI, COII	AB733026	AB733034	AB733042	This paper
Songkhla	ipS2	448	ITS2, COI, COII	AB733027	AB733035	AB733043	This paper
Anopheles sinensis							
Thailand	-	469	ITS2	AY130473	-	-	Min et al. (2002)
Korea	-	469	ITS2, COI, COII	EU789790	GQ265918	AY130464	Min et al. (2002), Park et al. (2008), Joshi et al. (2009)
Anopheles peditaeniatus							
Thailand	-	463	ITS2, COI, COII	AB539061	AB539069	AB539077	Choochote (2011)

a: used in crossing experiments; COI: cytochrome *c* oxidase subunit I; COII: cytochrome *c* oxidase subunit II; ITS2: second internal transcribed spacer.

al. (2007). Polymerase chain reaction (PCR) reaction was performed in total 20 µL volume containing 0.5 U *Ex Taq* (Takara), 1X *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer and 1 µL of the extracted DNA. For ITS2, the conditions for amplification consisted of initial denaturation at 94ºC for 1 min, 30 cycles at 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 1 min and a final extension at 72ºC for 5 min. The amplification profile of COI and COII comprised initial denaturation at 94ºC for 1 min, 30 cycles at 94ºC for 30 sec, 50ºC for 30 sec and 72ºC for 1 min and a final extension at 72ºC for 5 min. The amplified products were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Finally, the PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and their sequences directly determined using the BigDye® Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems). The sequence data of this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accessions AB733020-AB733043. The ITS2, COI and COII sequences obtained from this study were also compared with deposited sequences available through GenBank (Table I).

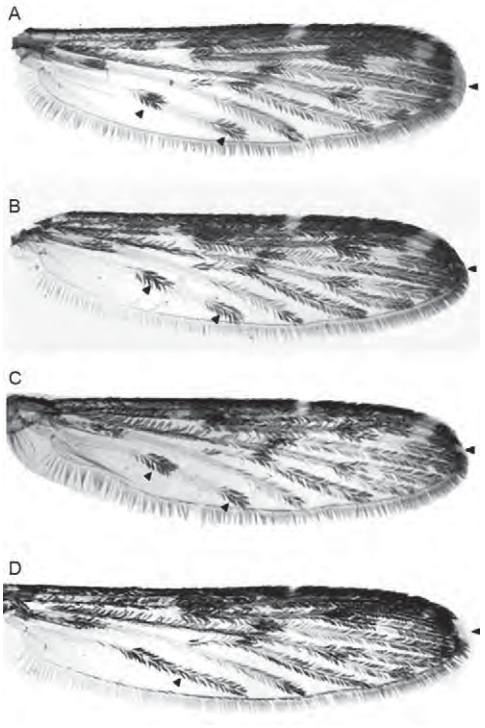

Fig. 1A: wing of *Anopheles lesteri* from Korea showing wide pale fringe spot extending from tip of vein R_1 to R_{4+5} and two dark spots on anal vein (1A); B-D: wings of *Anopheles paraliae* from Thailand showing very narrow pale fringe spot at tip of vein R_2, and two dark spots on 1A similar to that of *An. lesteri* (B), narrow fringe spot at tip of vein R_2 and two dark spots on 1A (C) and moderated fringe spot extending from tip of vein R_{1-3} and one dark spot on 1A (D).

Sequencing alignment and phylogenetic analysis - Sequences of ITS2, COI and COII were aligned using the CLUSTALW multiple alignment program (Thompson et al. 1994). Gap sites were excluded from the following analysis. The Kimura two-parameter method was used to calculate genetic distances (Kimura 1980). Construction of neighbour-joining (NJ) trees (Saitou & Nei 1987) and the bootstrap test with 1,000 replications were performed with the MEGA version 4.0 program (Tamura et al. 2007). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist et al. 2012) by using two replicates of one million generations with the nucleotide evolutionary model, GTR+I, which was selected by MrModeltest version 2.3 (Evolutionary Biology Centre, Uppsala University, 2004) as the best-fit model for ITS2, COI and COII. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% trees as burnin.

RESULTS

Crossing experiments - Details of hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal and F_1-hybrid crosses between *An. lesteri* from Korea and *An. paraliae* from Thailand are shown in Table II. All crosses yielded viable progenies through the F_2-generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses (repeated twice: experiments 2 and 3, data are not shown). The salivary gland polytene chromosomes of F_1-hybrid larvae from all crosses showed complete synapsis without inversion loops in all chromosome arms (Fig. 2).

Sequence analysis of ITS2, COI and COII regions - The level of genetic distance and number of base substitutions between sequences of the three regions are presented in Tables III-V. Analysis of the ITS2 sequence revealed no intraspecific sequence variation among the three and five iso-female lines of *An. lesteri* and *An. paraliae*, respectively. Comparison of ITS2 sequences indicated that *An. lesteri* differed from *An. paraliae* by 16 base substitutions (pairwise distance = 0.040). In addition, three iso-female lines of *An. lesteri* from Korea were identical with *An. lesteri* from China (= *Anopheles anthropophagus*) (AY803732, AY375467), Japan (AB159606) and Korea (EU789791), but they differed from those of the Philippines (AY375469) by three base substitutions (pairwise distance = 0.007). The average percentages of base composition for the ITS2 sequence of the eight iso-female lines (3 of *An. lesteri* from Korea and 5 of *An. paraliae* from Thailand) were A: 29.9% (29.2-30.5%), T: 24.2% (23.6-24.9%), G: 25.2% (25-25.4%) and C: 20.8% (20.6-20.9%). Percentage of GC content was 46% in *An. lesteri* (448 bp) and 45% in *An. paraliae* (448 bp). All eight sequences differed markedly from *An. sinensis* (pairwise distance = 0.321-0.338) and *An. peditaeniatus* (pairwise distance = 0.550-0.566) (Table III). The analysis of COI (658 bp) among the eight iso-female lines revealed four-nine base substitutions (pairwise distance = 0.007-0.017). On the contrary, *An. lesteri* and *An. paraliae* showed significant differences

TABLE II

Crossing experiments among the four iso-female lines of *Anopheles lesteri* and *Anopheles paraliae*

Crosses (female x male)	Total eggs (n)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
Parental cross							
ilG1 x ilG1	345 (155, 190)	65	221 (64.05)	221 (100)	221 (100)	111 (50.23)	110 (49.77)
ipR1 x ipR1	352 (194, 158)	84	296 (84.09)	245 (82.77)	240 (97.96)	98 (40.83)	142 (59.17)
ipN1 x ipN1	380 (190, 190)	86	327 (86.05)	327 (100)	327 (100)	137 (41.90)	190 (58.10)
ipS1 x ipS1	344 (194, 150)	73	186 (54.07)	171 (91.93)	165 (96.49)	61 (36.97)	104 (63.03)
Reciprocal cross							
ilG1 x ipR1	382 (131, 251)	97	332 (86.91)	289 (87.05)	282 (97.57)	169 (59.93)	113 (40.07)
ipR1 x ilG1	393 (187, 206)	98	334 (84.99)	334 (100)	317 (94.91)	190 (59.94)	127 (40.06)
ilG1 x ipN1	402 (182, 220)	60	233 (57.96)	233 (100)	233 (100)	116 (49.79)	117 (50.21)
ipN1 x ilG1	263 (109, 154)	57	147 (55.89)	147 (100)	147 (100)	59 (40.14)	88 (59.86)
ilG1 x ipS1	309 (200, 109)	46	117 (37.86)	107 (91.45)	105 (98.13)	50 (47.62)	55 (52.38)
ipS1 x ilG1	308 (118, 190)	44	114 (37.01)	111 (97.37)	111 (100)	47 (42.34)	64 (57.66)
F_1-hybrid cross							
(ilG1 x ipR1)F_1 x (ilG1 x ipR1)F_1	352 (194, 158)	94	320 (90.91)	320 (100)	314 (98.13)	157 (50)	157 (50)
(ipR1 x ilG1)F_1 x (ipR1x ilG1)F_1	341 (186, 155)	88	290 (85.04)	261 (90)	258 (98.85)	113 (43.80)	145 (56.20)
(ilG1 x ipN1)F_1 x (ilG1 x ipN1)F_1	324 (157, 167)	67	201 (62.04)	201 (100)	201 (100)	101 (50.25)	100 (49.75)
(ipN1 x ilG1)F_1 x (ipN1 x ilG1)F_1	347 (197, 150)	58	180 (51.87)	180 (100)	175 (97.22)	85 (48.57)	90 (51.43)
(ilG1 x ipS1)F_1 x (ilG1 x ipS1)F_1	347 (190, 157)	65	215 (61.96)	213 (99.07)	209 (98.12)	97 (46.41)	112 (53.59)
(ipS1 x ilG1)F_1 x (ipS1 x ilG1)F_1	348 (158, 190)	60	174 (50)	167 (95.98)	164 (98.20)	77 (46.95)	87 (53.05)

a: two selective egg-batches of inseminated females from each cross; *b*: dissection from 100 eggs.

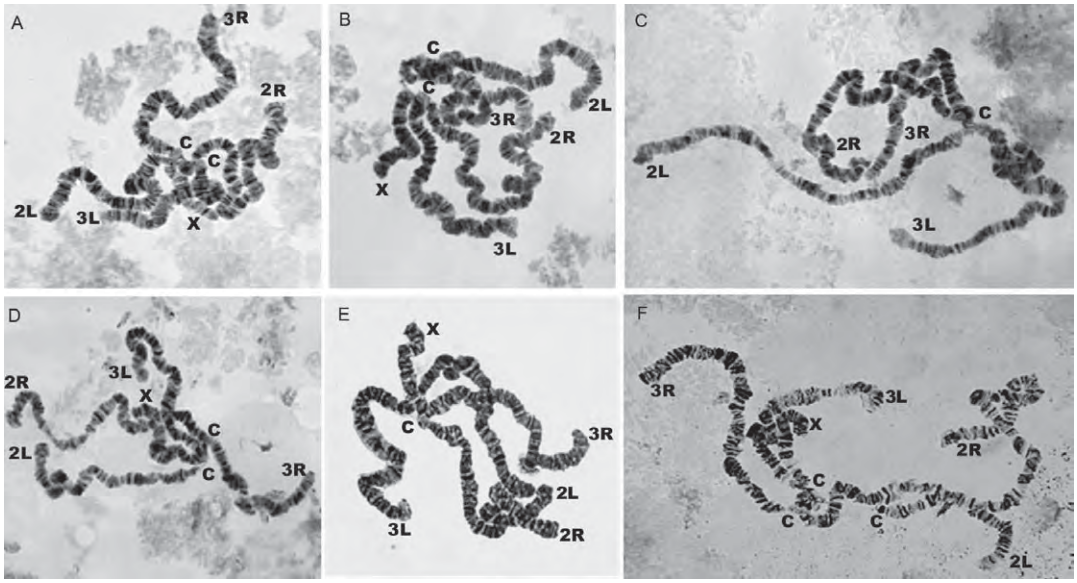

Fig. 2: complete synapsis in all arms of salivary gland polytene chromosome of F_1-hybrid larvae of crosses between *Anopheles lesteri* and *Anopheles paraliae*. A: ilG1 female x ipR1 male; B: ipR1 female x ilG1 male; C: ilG1 female x ipN1 male; D: ipN1 female x ilG1 male; E: ilG1 female x ipS1 male; F: ipS1 female x ilG1 male.

from *An. sinensis* (pairwise distance = 0.034-0.042) and *An. peditaeniatus* (pairwise distance = 0.037-0.041) (Table IV). The analysis of COII (685 bp) among the eight iso-female lines revealed five-seven base substitutions (pairwise distance = 0.008-0.011). These two species also showed significant differences from *An. sinensis* (pairwise distance = 0.039) and *An. peditaeniatus* (pairwise distance = 0.031-0.036) (Table V).

Phylogenetic analysis - The NJ and Bayesian trees of *An. lesteri*, *An. paraliae*, *An. sinensis* and *An. peditaeniatus* were constructed based on the ITS2, COI and COII sequences (Fig. 3). For ITS2, *An. lesteri* (n = 8) and *An. paraliae* (n = 5) were clustered in each monophyletic and well separated from *An. sinensis* and *An. peditaeniatus* with high bootstrap values (93-100%) in both NJ and Bayesian trees. The trees indicated that *An. lesteri* was more closely related to *An. paraliae* (average genetic distances = 0.038) than to the other species. Further, lower sequence divergences (0.000-0.002) were found within the population of each species. For COI and COII, the trees showed that *An. lesteri* was more closely related to *An. paraliae* than to the other species with low level of average genetic distances (0.008-0.011) for both regions, while very low genetic distances (0.003-0.005) were obtained within the population of each species.

DISCUSSION

Crossing experiments using iso-female lines of closely related species of the Oriental *Anopheles* have proven to be a robust systematic procedure for clarifying species status, for example, *Anopheles minimus* and *Anopheles aconitus* (Harrison 1980, Sucharit & Choochote 1982), *Anopheles annularis* and *Anopheles philippinensis* (Choochote et al. 1984), *Anopheles nivipes* and *An. philippinensis* (Klein et al. 1984) and *An. minimus* and *Anopheles flavirostris* (Somboon et al. 2000). These methods are useful for solving taxonomic problems of some sibling species complexes, e.g., *Anopheles dirus* (Baimai et al. 1987), *Anopheles maculatus* (Thongwat et al. 2008), *An. minimus* (Somboon et al. 2001, 2005, Choochote et al. 2002) and *Anopheles barbirostris* (Saeung et al. 2007, 2008, Suwannamit et al. 2009). Likewise, the status of subspecies or cytological races of *Anopheles* can be elucidated by the same approach of cytogenetic study as exemplified in *An. pullus* (= *Anopheles yatsushiroensis*) (Park et al. 2003), *Anopheles vagus* (Choochote et al. 2002), *An. aconitus* (Junkum et al. 2005), *An. sinensis* (Choochote et al. 1998, Min et al. 2002, Park et al. 2008), *An. barbirostris* species A1 (Saeung et al. 2007, Suwannamit et al. 2009), *Anopheles campestris*-like taxon (Thongsahuan et al. 2009) and *An. peditaeniatus* (Choochote 2011). Our findings in this study showed no post-mating reproductive isolation between *An. lesteri* from Korea and *An. paraliae* from Thailand. These results were clearly supported by cytological evidence and DNA analysis. Thus, complete synapsis of salivary gland polytene chromosomes without inversion loops along the entire lengths of all chromosome arms was observed in the F_1-hybrid larvae between *An. lesteri* and *An. paraliae* which strongly indicated genetic compatibility between them.

Analysis of ITS2 sequences of *An. lesteri* from Korea (ilG1, ilG2, ilG3) revealed identical sequences to *An. lesteri* from China (= *An. anthropophagus*), Japan and Korea (genetic distance = 0.000), although they showed little difference from those of the Philippines (genetic distance = 0.007) (Wilkerson et al. 2003, Ma & Yang 2005, Park et al. 2008, K Sawabe et al., unpublished observations). Our results were in agreement with those previously reported by Ma and Xu (2005). Moreover, the low level of pair-

TABLE III

Genetic distance and number of nucleotide substitutions in second internal transcribed spacer sequences among *Anopheles lesteri*, *Anopheles paraliae*, *Anopheles sinensis* and *Anopheles peditaeniatus*

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 ilG1	-	0	0	16	16	16	16	16	0	0	0	0	3	107	108	163
2 ilG2	0.000	-	0	16	16	16	16	16	0	0	0	0	3	107	108	163
3 ilG3	0.000	0.000	-	16	16	16	16	16	0	0	0	0	3	107	108	163
4 ipR1	0.040	0.040	0.040	-	0	0	0	0	16	16	16	16	17	110	111	160
5 ipR2	0.040	0.040	0.040	0.000	-	0	0	0	16	16	16	16	17	110	111	160
6 ipN1	0.040	0.040	0.040	0.000	0.000	-	0	0	16	16	16	16	17	110	111	160
7 ipS1	0.040	0.040	0.040	0.000	0.000	0.000	-	0	16	16	16	16	17	110	111	160
8 ipS2	0.040	0.040	0.040	0.000	0.000	0.000	0.000	-	16	16	16	16	17	110	111	160
9 anthC (AY803792)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	-	0	0	0	3	107	108	163
10 anthC (AY375467)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	0.000	-	0	0	3	107	108	163
11 lesJ (AB159606)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	0.000	0.000	-	0	3	107	108	163
12 lesK (EU789791)	0.000	0.000	0.000	0.040	0.040	0.040	0.040	0.040	0.000	0.000	0.000	-	3	107	108	163
13 lesP (AY375469)	0.007	0.007	0.007	0.042	0.042	0.042	0.042	0.042	0.007	0.007	0.007	0.007	-	105	106	163
14 sinK (EU789790)	0.321	0.321	0.321	0.334	0.334	0.334	0.334	0.334	0.321	0.321	0.321	0.321	0.314	-	3	154
15 sinT (AY130473)	0.325	0.325	0.325	0.338	0.338	0.338	0.338	0.338	0.325	0.325	0.325	0.325	0.318	0.007	-	155
16 pedT (AB539061)	0.566	0.566	0.566	0.550	0.550	0.550	0.550	0.550	0.566	0.566	0.566	0.566	0.567	0.520	0.525	-

above triangle: number of nucleotide substitutions; below triangle: genetic distance.

TABLE IV

Genetic distance and number of nucleotide substitutions in cytochrome *c* oxidase subunit I sequences among *Anopheles lesteri*, *Anopheles paraliae*, *Anopheles sinensis* and *Anopheles peditaeniatus*

Taxon	1	2	3	4	5	6	7	8	9	10
1 ilG1	-	1	2	7	6	6	8	7	22	22
2 ilG2	0.002	-	3	8	7	7	9	8	21	21
3 ilG3	0.004	0.006	-	5	4	4	6	5	20	22
4 ipR1	0.013	0.015	0.009	-	1	1	5	0	19	21
5 ipR2	0.011	0.013	0.007	0.002	-	0	4	1	18	20
6 ipN1	0.011	0.013	0.007	0.002	0.000	-	4	1	18	20
7 ipS1	0.015	0.017	0.011	0.009	0.007	0.007	-	5	22	20
8 ipS2	0.013	0.015	0.009	0.000	0.002	0.002	0.009	-	19	21
9 sinK (GQ265918)	0.042	0.040	0.038	0.036	0.034	0.034	0.042	0.036	-	29
10 PedT (AB539069)	0.041	0.039	0.041	0.039	0.037	0.037	0.037	0.039	0.055	-

above triangle: number of nucleotide substitutions; below triangle: genetic distance.

wise distance (0.040) detected between *An. lesteri* from Korea and *An. paraliae* from Thailand, based on ITS2 sequences, was in accordance with previous reports of different groups of *Anopheles*, e.g., the *Anopheles gambiae* complex (0.4-1.6%) (Paskewitz et al. 1993), *Anopheles dunhami* and *Anopheles nuneztovari* (mean genetic distance = 0.025) (Ruiz et al. 2010), *Anopheles fluviatilis* S and *An. minimus* C (pairwise distance = 0.036) (Singh et al. 2006), *Anopheles kunmingensis* and *Anopheles liangshanensis* (pairwise distance = 0.0381) and *An. pullus* (= *An. yatsushiroensis*) and *Anopheles junlianensis* (pairwise distance = 0.03081) (Hwang 2007). Currently, Calado et al. (2008) showed that *An. nuneztovari* A is not conspecific with *An. nuneztovari* B/C based on COI sequences (genetic distance = 0.00818-0.02071) and *An. dunhami* has been reported as new record in the Brazilian Amazon by comparing sequences with those of *An. nuneztovari* A (genetic distance = 0.01436-0.03343). Similarly, comparative sequences for COI and COII between *An. lesteri* and *An. paraliae* revealed low average genetic distance between them (0.008-0.011). Despite such low genetic distances, phylogenetic trees seem to

TABLE V

Genetic distance and number of nucleotide substitutions in cytochrome *c* oxidase subunit II sequences among *Anopheles lesteri*, *Anopheles paraliae*, *Anopheles sinensis* and *Anopheles peditaeniatus*

Taxon	1	2	3	4	5	6	7	8	9	10
1 ilG1	-	1	0	5	5	5	6	5	25	20
2 ilG2	0.002	-	1	6	6	6	7	6	25	20
3 ilG3	0.000	0.002	-	5	5	5	6	5	25	20
4 ipR1	0.008	0.009	0.008	-	0	0	5	0	25	22
5 ipR2	0.008	0.009	0.008	0.000	-	0	5	0	25	22
6 ipN1	0.008	0.009	0.008	0.000	0.000	-	5	0	25	22
7 ipS1	0.009	0.011	0.009	0.008	0.008	0.008	-	5	25	23
8 ipS2	0.008	0.009	0.008	0.000	0.000	0.000	0.008	-	25	22
9 sinK (AY130464)	0.039	0.039	0.039	0.039	0.039	0.039	0.039	0.039	-	29
10 PedT (AB539077)	0.031	0.031	0.031	0.034	0.034	0.034	0.036	0.034	0.045	-

above triangle: number of nucleotide substitutions; below triangle: genetic distance.

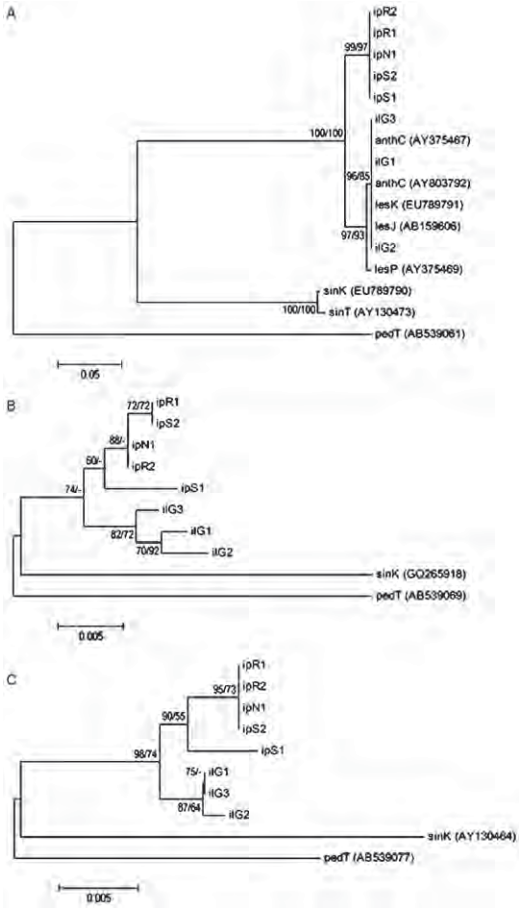

Fig. 3: neighbour-joining (NJ) trees inferred from sequences of three loci. A: second internal transcribed spacer; B: cytochrome *c* oxidase subunit I (COI); C: COII of *Anopheles paraliae*, *Anopheles lesteri*, *Anopheles sinensis* and *Anopheles peditaeniatus*. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). A hyphen (-) shows that the branch did not appear in majority rule (50%) consensus trees of Bayesian analysis. Branch lengths are proportional to genetic distance (scale bar).

indicate that *An. lesteri* and *An. paraliae* were well separated from each other with NJ and Bayesian analyses for three regions, except for the Bayesian tree of COI. Although these two species were distinguished apparently by DNA sequence analysis, they obviously showed genetic compatibility by crossing experiments. Controversy over taxonomic problems with respect to full-fledged species, sibling species and subspecies within a taxon of *Anopheles* has occurred when only data of comparative DNA sequence analyses of certain specific genomic regions were used as first hand criteria for separating them. For example, *An. fluviatilis* S was considered a synonym of *An. minimus* C based on comparison of the D3 domains of 28S (28S-D3) (Harbach 2004, Garros et al. 2005, Chen et al. 2006). However, Singh et al. (2006) carried out molecular analysis on ITS2 and D2-D3 domains of 28S rDNA regions of *An. fluviatilis* S and *An. minimus* C. The authors suggested that these *Anopheles* species did not deserve synonymous status. Hence, crossing experiments between *An. fluviatilis* S and *An. minimus* C using iso-female lines are essential prior to a definite conclusion as to their conspecificity. Our studies using crossing experiments between *An. lesteri* from Korea and *An. paraliae* from Thailand together with data on species distributions, morphological variants, cytology and comparative DNA sequence analyses have clearly indicated that they are conspecific within the taxon *An. lesteri*. Additionally, the population genetic structure will be studied further in order to evaluate the gene flow among *An. lesteri* and *An. paraliae* populations before definitely concluding that *An. lesteri* is a synonym of *An. paraliae*.

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