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Systematics of the *Anopheles barbirostris* species complex (Diptera: Culicidae: Anophelinae) in Thailand

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This study deals with five species of the Barbirostris Complex of *Anopheles* subgenus *Anopheles* that are known to occur in Thailand. Three new species of the complex, ***Anopheles dissidens* sp. nov.**, ***Anopheles saeungae* sp. nov.**, and ***Anopheles wejchoochotei* sp. nov.**, are characterized and compared with *Anopheles barbirostris* van der Wulp and *Anopheles campestris* Reid based on specimens of molecularly identified progeny broods. For practical purposes, the five species are essentially isomorphic and can only be unequivocally identified from diagnostic mitochondrial and ribosomal DNA sequences. Based on overall morphological similarity, *An. campestris* is considered to be a member of the Barbirostris Complex rather than a separate member of the Barbirostris Subgroup. The molecular data, mitotic karyotypes, bionomics, and distributions of the species are reviewed and discussed. It is concluded that integrated molecular epidemiological studies of the complex throughout the Oriental Region are needed to unambiguously elucidate the individual species and their relation to disease.

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ADDITIONAL KEYWORDS: Bayesian analysis – cryptic species – molecular systematics – morphological comparison – morphology – mosquitoes – mtDNA – new species – rRNA.

INTRODUCTION

Anopheles (*Anopheles*) *barbirostris* van der Wulp is the nominotypical member of a medically important group of 14 formally named species (Barbirostris Group) in the Oriental Region (Harbach, 2013). Twelve of these species are classified into subgroups, the Barbirostris and Vanus Subgroups. The Barbirostris Subgroup includes five species described by Reid (1962), including *Anopheles campestris* Reid and two nominal species of the Barbirostris Complex, *Anopheles barbirostris* and *Anopheles vanderwulpi* Townson & Harbach. Based on the results of cross-mating studies and analyses of mitochondrial and ribosomal DNA sequences (Saeung *et al*., 2007, 2008; Paredes-Esquivel *et al*., 2009; Suwannamit *et al*., 2009; Thongsahuan *et al*., 2009; Townson *et al*., 2013), the Barbirostris Complex is currently known to include at least four additional species. Three of these species are characterized, formally named, and contrasted with *An. barbirostris* in this paper. Based on overall morphological similarity, we also include and recognize *An. campestris* as a member of the Barbirostris Complex.

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MATERIAL AND METHODS

SPECIMENS AND MORPHOLOGY

Specimens of the new species described below were reared from progeny broods obtained from wild-caught blood-fed females collected in various locations in Thailand and Indonesia. The progeny broods were identified to species based on sequences for the *cytochrome c oxidase subunit I* (*COI*) of mitochondrial DNA (mtDNA) and the *internal transcribed spacer 2* (*ITS2*) of ribosomal DNA (rDNA) obtained using the procedures of Saeung *et al*. (2008), Paredes-Esquivel *et al*. (2009), and Suwannamit *et al*. (2009). Larvae were individually reared to provide adults with associated

larval and pupal exuviae. These specimens, individually reared specimens that comprise the type series of *An. campestris*, and specimens unequivocally identified from their *COI* sequence as *An. barbirostris* in the study of Townson *et al*. (2013), were used for the comparative anatomical study. Adults were studied using stereomicroscopy and simulated natural light. Larval and pupal chaetotaxy and dissected male genitalia were studied using bright-field microscopy. The morphological terminology used herein is defined in the Anatomical Glossary of the Mosquito Taxonomic Inventory (http://mosquito-taxonomic-inventory.info/). The symbols ♀, ♂, E, Le, Pe, P, and L used in the synonymies/literature summaries, *Material examined*, and *Type series* represent female(s), male(s), egg(s), larval exuviae, pupal exuviae, pupae, and fourth-instar larvae, respectively. An asterisk (*) following these symbols indicates that at least part of the life stage is illustrated in the corresponding publication.

Measurements (e.g. wing length) in millimeters (mm) and counts (e.g. number of setal branches) are given as a range followed by the mean or mode, respectively, in parentheses. Variation observed in anatomical features of adult females and male genitalia was analysed by one-way analysis of variance (ANOVA). Differences among the five species treated herein were compared using Scheffé's method. Data for thoracic setal groups were compared using the Kruskal–Wallis test. All data were analysed using SPSS v. 16.0 for Windows (Chicago, SPSS Inc.). Statistical significance was set at $P < 0.05$.

Cytotaxonomy

The cytotaxonomy of members of the Barbirostris Group is based on mitotic chromosomes found in the neural ganglia of fourth-instar larvae derived from isoline colonies. Metaphase karyotypes observed in the brain cells are characterized by the size and shape of the X and Y chromosomes. The karyotypic forms listed for the species described herein consist of combinations for the following chromosomal designations: X_1 (small metacentric chromosome), X_2 (medium submetacentric chromosome), X_3 (large submetacentric chromosome, same as X_2 but slightly longer), Y_1 (subtelocentric [acrocentric] chromosome), Y_2 (large submetacentric chromosome),Y_3 (large submetacentric or metacentric chromosome with large block of heterochromatin at the distal end of the long arm), Y_4 (medium metacentric chromosome), Y_5 (small metacentric chromosome), and Y_6 (large subtelocentric chromosome).

DNA extraction and amplification

DNA was extracted from individual adult female mosquitoes of progeny broods using a DNeasy Blood and Tissue Kit (QIAGEN). The ribosomal *ITS2* region and the mitochondrial *COI* gene were amplified using the primers and thermal profiles described in Saeung *et al*. (2007) and Suwannamit *et al*. (2009).

Sequence alignment and phylogenetic analyses

The DNA sequences were aligned using CLUSTAL W (Thompson, Higgins & Gibson, 1994) in BioEdit v. 7.0.5.3 (Hall, 1999). Gap sites were excluded from the following analysis. The Kimura two-parameter (K2P) model was employed to calculate genetic distances (Kimura, 1980), implemented in Molecular Evolutionary Genetics Analysis (MEGA) v. 6.0 (Tamura *et al*., 2013). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist *et al*., 2012) by using two replicates of 1 000 000 generations with the nucleotide evolutionary model. The best-fit model was chosen for each gene separately using the Akaike information criterion (AIC) in MrModeltest v. 2.3 (Nylander, 2004). The general time-reversible (GTR) with gamma distribution shape parameter (G) was the best-fit model for the *ITS2* and *COI* sequences. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% of trees as burn-in. Specimens used in the phylogenetic analyses, along with GenBank accession numbers for their *ITS2* and *COI* sequences, are listed in Table S1. *Anopheles* (*Cellia*) *dirus* Peyton & Harrison was used as outgroup in the analyses of both *ITS2* and *COI*.

SYSTEMATICS

Anopheles (*Anopheles*) *barbirostris* van der Wulp

Anopheles barbirostris van der Wulp, 1884 (♀). Holotype ♀: Mount Ardjoeno, Java, Indonesia (Nationaal Natuurhistorisch Museum, Leiden, Netherlands).

Anopheles martini Laveran, 1902 (♀). Type locality: near Pursat, Cambodia (Institut Pasteur, Paris). It is not possible to know for certain whether or not this nominal species is conspecific with *An. barbirostris* or another member of the Barbirostris Complex.

Anopheles barbirostris innominata Stoker & Waktoedi Koesoemawinangoen, 1949 (♀ ♂). Type locality: Sulawesi, Indonesia (type non-extent). This nominal form may be a distinct member of the Barbirostris Complex.

Anopheles (*Anopheles*) *barbirostris* in part of Bonne-Wepster & Swellengrebel, 1953 (Indonesia, ♀* ♂* L*); Reid, 1962 (Indonesia, Malaysia, Thailand, ♀* ♂ E* L P*, taxonomy); Reid, 1968 (Malaysia, Borneo, ♀* ♂* E L* P*, taxonomy, biology); Harrison & Scanlon, 1975 (Thailand, ♀* ♂* L* P*, taxonomy); Reid, Harrison & Atmosoedjono, 1979 (Indonesia, Malaysia, Thailand, ♀* L P* morphology, taxonomy).

Anopheles barbirostris in part (?) of Harrison, Rattanarithikul & Mongkolpanya, 1988 (Thailand, A L P morphology); Ndoen *et al*., 2010, 2011 (West Timor, Java, ecology, bionomics).

Anopheles barbirostris form A in part of Baimai, Rattanarithikul & Kijchalao, 1995 (Thailand, metaphase karyotype).

Anopheles barbirostris Clade I of Paredes-Esquivel *et al*., 2009 (Bornean Indonesia, Thailand, Vietnam, *COI* mtDNA, *ITS2* rDNA); Paredes-Esquivel & Townson, 2014 (Bornean Indonesia, Thailand, *ITS2* rDNA).

Anopheles barbirostris species A4 of Suwannamit *et al*., 2009 (Thailand, cross-matings, metaphase karyotype, *COI* and *COII* mtDNA, *ITS2* rDNA); Thongsahuan *et al*., 2011 (Thailand, *Plasmodium* susceptibility); Otsuka, 2011 (Thailand, *ITS2* rDNA).

Anopheles (*Anopheles*) *barbirostris* of Townson *et al*., 2013 (Javan Indonesia, ♀* ♂* L* P*, *COI* mtDNA and *ITS2* rDNA, taxonomy, bionomics, distribution).

Diagnosis

Females of *An. barbirostris* resemble other members of the Barbirostris Group in having entirely dark-scaled maxillary palpi with palpomeres 1 and 2 particularly shaggy and a tuft of black scales on abdominal sternum VII. The other sterna usually have a few scattered pale scales in both males and females. The thoracic pleura generally have pale scales associated with setae on the upper proepisternum, mesokatepisternum, and mesepimeron. The wing usually has a pale fringe spot at the apex of the cubitus and a few scattered pale scales on the proximal half of the costa. Larvae have bushy lateral palatal brushes and thoracic seta 1-P with long branches from near the base.

Anopheles barbirostris is very similar to other members of the Barbirostris Complex. Females of the complex are usually recognizable by the absence of a pale fringe spot at the apex of vein R_2, the presence of a pale fringe spot at the apex of vein R_{4+5}, extensive pale scaling on the cubitus, a few pale scales on the abdominal sterna, and narrow apical pale bands on tarsomeres of the foreleg. Pupae have a secondary cleft on the trumpet, setae 1,5-III–VII with numerous branches, seta 2-VII with few branches, and seta 9-II–VIII yellow to pale brown. Larval seta 3-C normally has more than 40 stiff, broom-like branches, palmate seta 1-II is well developed and pigmented, and the spiracular apparatus lacks an anterior median process. Sequences for the *COI* region of mtDNA distinguish *An. barbirostris* from other species of the Barbirostris Complex (see below).

Description

Female

Large, brownish-black mosquito with finely dappled wings and narrowly banded tarsi (Fig. 1). Measurements and statistical analysis of selected anatomical features compared with other species of the complex in Tables 1 and S2, respectively.

Head: Vertex dark-scaled with patch of pale scales before interocular space; interocular space with mostly dark setae and narrow pale scales. Antenna about two-thirds length of proboscis; pedicel with scales on dorsolateral surface; flagellomere 1 with patch of scales, other flagellomeres without scales. Proboscis length about 2.45 mm, entirely dark-scaled, noticeably shaggy in proximal 0.5–0.7, labella also dark; forefemur/proboscis ratio 0.90–0.98 (mean 0.93). Maxillary palpus same length as proboscis, entirely dark-scaled, palpomeres 1 and 2 particularly shaggy.

Thorax: Scutum with longitudinal bare areas between rather broad lines of golden piliform scales on acrostichal, dorsocentral, and marginal areas; anterior promontory with whitish piliform scales medially; posterior segments of acrostichal and dorsocentral areas and entire prescutellar area with covering of golden piliform scales, scutal setae slightly darker and longer than scales; scutellum with golden piliform scales along bases of large golden to golden-brown setae in transverse posterior row. Mesopostnotum and postpronotum bare. Antepronotum with prominent patch of dark scales on dorsoanterior surface and rather sparse fine piliform scales posteriorly. Pleura with dark setae on upper proepisternum and golden to golden-brown setae on prespiracular area, prealar knob, upper and lower mesokatepisternal areas, and upper and middle mesepimeron; with four or five upper proepisternal setae, eight or nine prealar setae, five or six upper and four to six lower mesokatepisternal setae, and six to 15 upper and zero to two lower mesepimeral setae; scales usually accompany setae on upper proepisternum, mesokatepisternum, and mesepimeron; scales and setae often indistinct on middle of mesepimeron.

Wing: Pattern as illustrated, costa often with small humeral pale spot (at least on posterior margin of vein) and usually scattered pale scales between humeral crossvein and small subcostal pale spot, remainder of costa dark to preapical pale spot (sometimes absent); remigium usually with median pale scales; humeral crossvein with dark scales; vein R with scattered pale scales extending to sector pale spot; vein R_1 with apical pale spot adjoining preapical pale spot of costa and usually a line of interspersed pale scales at subcostal area; vein R_2 with subapical pale spot adjoining apical pale spot of R_1; vein R_3 usually with distinct postbasal and preapical pale spots; vein R_{4+5} with variable density of interspersed pale scales on median 0.80, length of pale-scaled area 1.64–2.00 mm (mean = 1.82 mm); vein M_1 with postbasal and preapical pale spots,

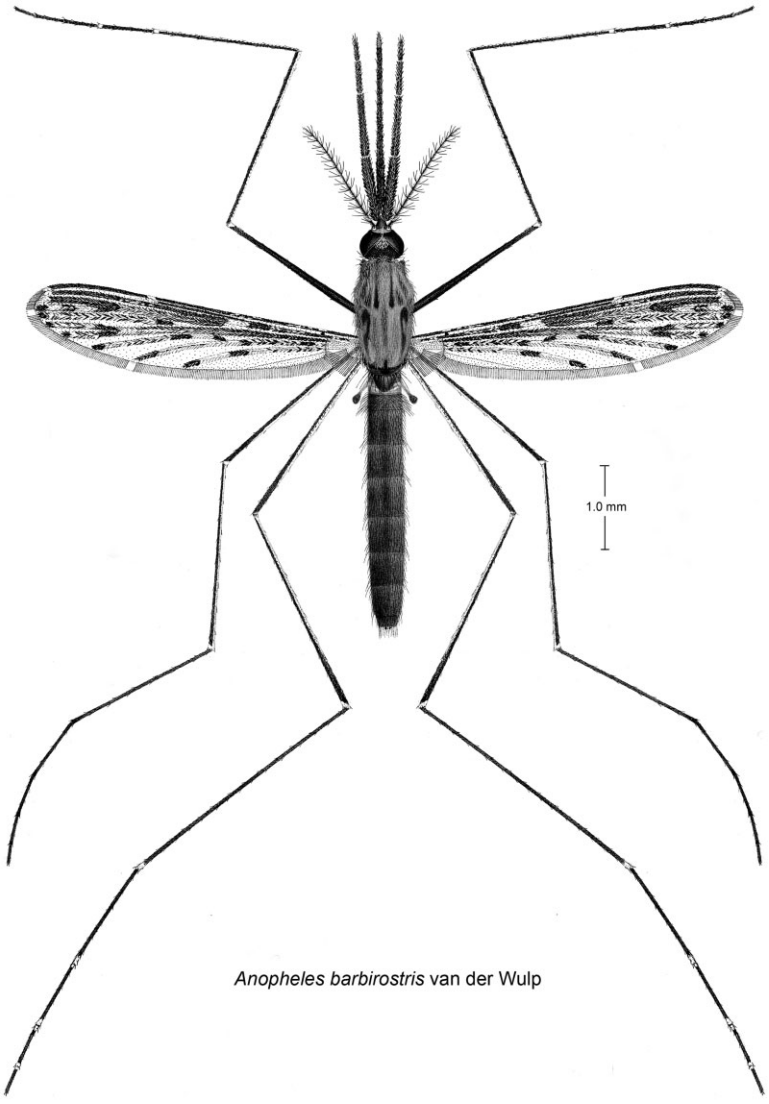

Figure 1. Female of *Anopheles barbirostris* van der Wulp (dorsal), modified from Townson *et al*. (2013).

sometimes with variable pale scaling in between; vein M_2 largely pale-scaled except distally; vein M_{3+4} with mostly pale scaling between basal and apical dark scaling; mediocubital crossvein with medial pale spot; vein CuA mainly pale-scaled with distinct short postbasal and apical dark spots, apex often with a few pale scales, postbasal dark spot usually separated by more than its length from medial dark spot on vein 1A; vein 1A largely pale-scaled with distinct medial and apical dark spots; narrow pale fringe spots at apices of veins R_{4+5} and CuA.

Halter: Integument of scabellum pale; integument of pedicel and capitellum dark and dark-scaled.

Legs: Mainly dark-scaled; coxae with small patches of pale scales; forefemur swollen towards base; all femora with a few pale scales at base; all tibiae narrowly pale-scaled at apex; foretarsomeres 1 and 2 with narrow apical pale marks or bands, midtarsus entirely dark-scaled, hindtarsomeres 1–4 with narrow apical pale bands or patches that sometimes cross the joints, apical band of hindtarsomere 3 0.06–0.10 mm (mean 0.08 mm), not extended across joint onto base of hindtarsomere 4.

Abdomen: Integument brownish black, sterna paler, especially basally on either side of midline; sterna II–VII with a median patch of pale scales, usually a few pale scales forming row along lateral margins and infrequently with scattered pale scales in between lateral rows and median patch, sternum VII with prominent posteromedian tuft of black scales, often a few dark scales in same position on sternum VI.

Table 1. Comparison of anatomical features observed in females of five species of the Barbirostris Complex in Thailand (means/modes in parentheses)

Morphological feature	*Anopheles barbirostris*	***Anopheles dissidens* sp. nov.**	***Anopheles saeungae* sp. nov.**	*Anopheles campestris*	***Anopheles wejchoochotei* sp. nov.**	*P*-value
Length of proboscis	2.16–2.60 (2.45)	1.60–2.04 (1.73)	1.84–2.12 (2.02)	2.12–2.40 (2.27)	1.92–2.24 (2.08)	0.000
Length of maxillary palpus	2.44–2.64 (2.54)	1.64–2.20 (1.78)	1.80–2.12 (2.00)	2.08–2.60 (2.39)	1.84–2.40 (2.15)	0.000
Length of flagellum	1.56–1.80 (1.70)	1.16–1.92 (1.34)	1.36–1.60 (1.46)	1.28–1.56 (1.43)	1.12–1.48 (1.31)	0.001
Length of wings	4.24–4.80 (4.58)	3.20–3.84 (3.49)	3.60–4.00 (3.84)	3.76–4.24 (4.03)	3.36–4.08 (3.72)	0.000
Width of wings	0.96–1.12 (1.04)	0.72–0.84 (0.79)	0.80–0.96 (0.89)	0.96	0.88–1.04 (0.96)	0.000
Length of vein R_2	1.04–1.12 (1.10)	0.70–0.92 (0.82)	0.82–0.98 (0.90)	1.08–2.04 (1.22)	0.80–0.96 (0.88)	0.001
Length of vein R_{4+5}	1.64–2.00 (1.82)	1.24–1.56 (1.38)	1.34–1.62 (1.51)	1.58–1.80 (1.67)	1.42–1.64 (1.54)	0.000
Length of forefemur	2.12–2.36 (2.27)	1.40–1.76 (1.63)	1.76–2.04 (1.91)	1.96–2.12 (2.07)	1.68–2.00 (1.83)	0.000
Forefemur/proboscis ratio	0.90–0.98 (0.93)	0.90–1.05 (0.94)	0.87–1.00 (0.94)	0.88–0.95 (0.91)	0.81–0.96 (0.88)	0.220
Upper proepisternal setae	4, 5 (4)	2–6 (3)	1, 2 (2)	3, 4 (3)	3–5 (4)	0.000
Prealar setae	0–9 (0)	2–9 (2)	0–9 (0)	4–7 (5)	0–8 (6)	0.208
Upper mesokatepisternal setae	5, 6 (5)	0–6 (2)	1–6 (2)	0–6	2–4 (4)	0.103
Lower mesokatepisternal setae	4–6 (4)	0–4 (3)	1–5 (2)	2–5 (3)	3–6 (4)	0.109
Upper mesepimeral setae	6–15	5–7 (5)	5–10 (7)	9–14	5–16 (8)	0.001
Lower mesepimeral setae	0–2 (0)	0	0–3 (0)	0–6 (0)	0–3 (0)	0.323

Male

Like female except as follows.

Head: Maxillary palpus usually completely dark-scaled, sometimes with indistinct apicolateral pale bands on palpomeres 4 and 5.

Wing: Scaling of veins posterior to radius not as dense as in female; small pale fringe spot often between apices of veins R_2 and R_{4+5}.

Abdomen: Tergum VIII with median patch of dark scales, sometimes with a few pale scales proximal to dark scales.

Genitalia: (Fig. 2C; Tables 2, S3) Lateral surface of gonocoxite with dark scales that become longer, more prominent, and black toward apex, proximal scaling less distinct with a few pale scales sometimes present at base; two parabasal setae borne on small protuberance at base of dorsomesal surface, lateral parabasal about twice as long as mesal one; inner seta on middle of ventromesal surface similar to lateral parabasal seta; ventral lobe of claspette small and ill-defined, with two simple setae borne on ventrolateral margin, the more ventromesal one longer than the other; dorsal lobe of claspette with club formed of four or five contiguous and distally fused setae; aedeagus with five or six pairs of leaflets, longest leaflet about half as long as aedeagus, bearing a blunt tooth at base and fine serration mainly on distal two-thirds of both edges, other large leaflets with serration on one or both edges.

Pupa

Lightly to moderately pigmented; positions and character of setae as figured (Fig. 2A, B); setae usually with ring of darker cuticle at base; numbers of branches in Table S4.

Cephalothorax: Trumpet darkly pigmented, with thin uniform rim and deep secondary cleft; wings with blurred, lattice-like pattern of darker spots; antenna with incomplete dark rings at joints and dark apex; proboscis and fore- and midfemora and -tibiae with blurred rings of darker cuticle; sum of branches of pair of seta 12-CT = 2–5 (3), pair of seta 3-III = 11–16 (11).

Abdomen: Lightly to moderately pigmented. Branching of serially homologous seta 2 compared with other species of the Barbirostris Complex in Table 3. Seta 1-II with three to eight (five) branches, sum of branches of pair of seta 1-II = 6–13 (7); seta 2-II–VII well developed; sum of branches of pair of seta 3-III = 11–16 (11); seta 5-II with three to seven (four) branches; setae 1,5-III–VII strongly developed with numerous fine branches (fewer than actual number of branches shown

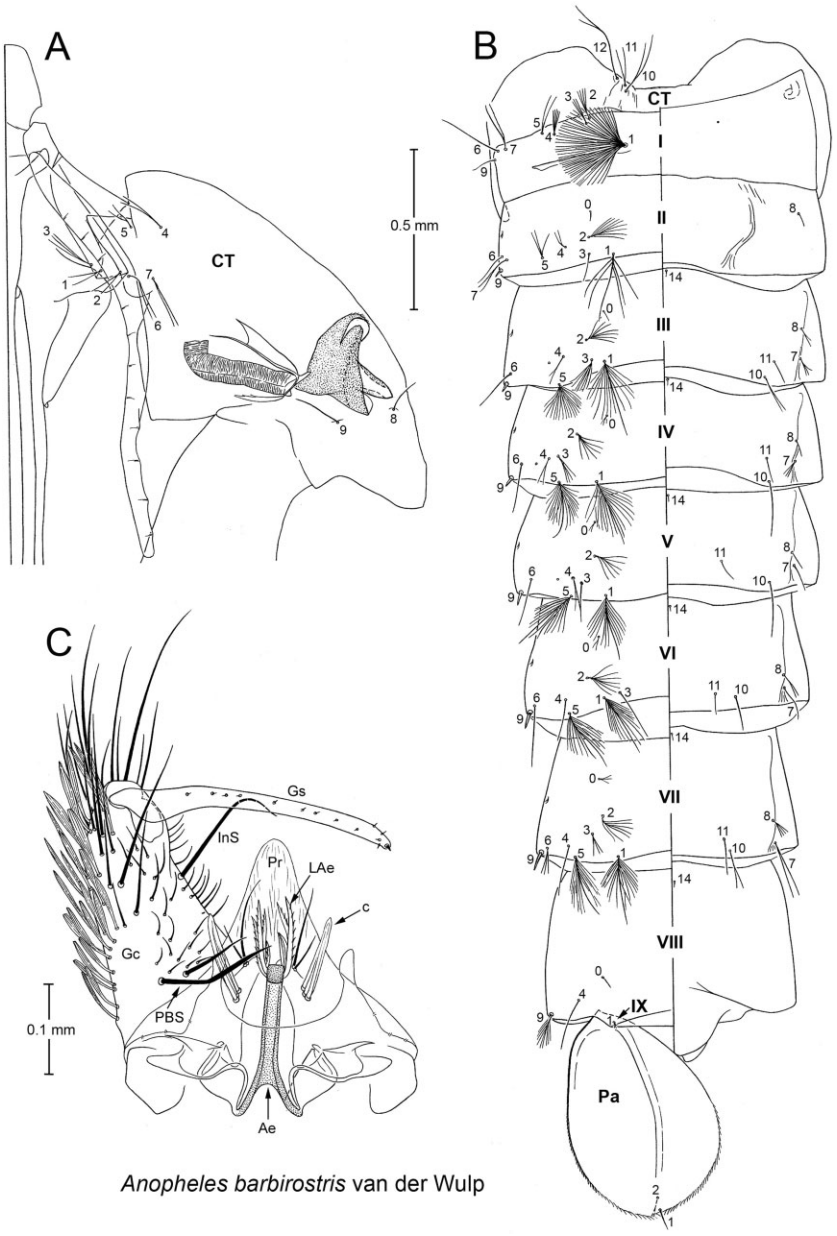

Figure 2. Pupa and male genitalia of *Anopheles barbirostris* van der Wulp (modified from Townson *et al*., 2013). A, pupa, left side of cephalothorax, dorsal to right. B, pupa, dorsal (left) and ventral (right) aspects of metathorax and abdomen. C, male genitalia, dorsal (tergal) aspect. Abbreviations: Ae, aedeagus; c, club on dorsal lobe of claspette; CT, cephalothorax; Gc, gonocoxite; Gs, gonostylus; InS, internal seta; LAe, leaflets of aedeagus; Pa, paddle; PBS, parabasal setae; Pr, proctiger; I–IX = abdominal segments I–IX; 1–14 = setal numbers for specified areas, e.g. seta 3-I.

in Fig. 2B), central branch usually distinctly longer than other branches; setae 1,5,9-IV–VII more darkly pigmented than integument; seta 8-II present or absent.

Paddle: With darkly pigmented base and spot of darker cuticle around insertions of setae 1,2-Pa; seta 1-Pa usually single with apex split into one to three elements.

Larva, fourth instar

Generally darkly pigmented; positions and character of setae as illustrated (Fig. 3); numbers of branches in Table S5.

Table 2. Comparison of characteristics of the male genitalia of five species of the Barbirostris Complex in Thailand (means/modes in parentheses)

Character	*Anopheles barbirostris*	***Anopheles dissidens* sp. nov.**	***Anopheles saeungae* sp. nov.**	*Anopheles campestris*	***Anopheles wejchoochotei* sp. nov.**	*P*-value
Length of gonocoxite	0.25–0.41 (0.30)	0.20–0.28 (0.26)	0.28–0.32 (0.30)	0.19	0.31–0.34 (0.32)	0.003
Length of distal parabasal seta	0.08–0.17 (0.13)	0.13–0.15 (0.14)	0.10–0.13 (0.13)	0.17	0.12–0.15 (0.13)	0.337
Length of internal seta	0.10–0.18 (0.14)	0.06–0.15 (0.12)	0.06–0.14 (0.11)	0.14	0.09–0.15 (0.13)	0.337
Length of gonostylus	0.29–0.38 (0.33)	0.27–0.32 (0.30)	0.29–0.32 (0.31)	0.3	0.31–0.33 (0.32)	0.656
Length of claspette	0.10–0.15 (0.12)	0.09	0.10–0.11 (0.11)	0.13	0.11–0.13 (0.13)	0.002
Length of claspette club	0.10–0.11 (0.10)	0.08–0.10 (0.09)	0.09–0.10 (0.10)	0.1	0.10–0.11 (0.11)	0.002
Setae of claspette club	4–6 (5)	4–6 (6)	4–7 (6)	4,5	3–5 (5)	0.360
Length of aedeagus	0.15–0.20 (0.17)	0.13–0.14 (0.13)	0.13–0.16 (0.15)	0.25	0.19–0.20 (0.20)	0.000
Leaflets of aedeagus	2–5 (3)	3–6 (4)	2–4 (3)	4	3–5 (3)	0.169

Table 3. Comparison of numbers of branches of seta 2-III–VII in pupae of five species of the Barbirostris Complex in Thailand (modes in parentheses)

	Anopheles barbirostris		***Anopheles saeungae* sp. nov.**		***Anopheles dissidens* sp. nov.**		***Anopheles wejchoochotei* sp. nov.**		*Anopheles campestris*	
Seta	No. of branches	Sum of pair	No. of branches	Sum of pair	No. of branches	Sum of pair	No. of branches	Sum of pair	No. of branches	Sum of pair
2-III	6–10 (8)	14–19 (15)	6–11 (7)	12–22 (12)	6–9 (7)	12–17 (14)	11–18 (11)	23–36 (29)	7–17 (10)	17–35 (21)
2-IV	5–8 (6)	9–15 (14)	3–7 (6)	9–14 (12)	5–7 (6)	10–14 (12)	7–16 (11)	17–31 (22)	8–23 (10)	17–41 (21)
2-V	5–8 (6)	9–15 (13)	5–8 (6)	10–15 (13)	4–8 (6)	10–15 (12)	7–13 (9)	16–25 (18)	7–16 (9)	16–29 (19)
2-VI	4–9 (7)	9–16 (14)	4–7 (5)	9–13 (12)	6–8 (6)	9–15 (12)	8–12 (8)	16–22 (19)	7–18 (9)	11–34 (19)
2-VII	3–8 (6)	8–15 (15)	5–9 (6)	10–15 (13)	5–8 (6)	10–15 (14)	5–13 (8)	12–25 (13)	6–15 (10)	16–28 (21)
Total	23–43 (33)	49–80 (71)	23–42 (30)	50–79 (62)	26–40 (31)	51–76 (64)	38–72 (47)	84–139 (101)	35–89 (48)	77–167 (101)

Head: About as wide as long; unevenly pigmented, with mottled pattern of darker cuticle, collar and dorsomentum darkly pigmented. Seta 2-C simple; seta 3-C broom-like; seta 4-C small, with one to three (two) branches; setae 6,7-C often with eight to 14 (ten, 13) branches; setae 8,9-C usually with five to 11 (eight, nine) branches.

Antenna: Moderately pigmented; mesal and ventral surfaces strongly spiculate; length about 0.28 times length of head.

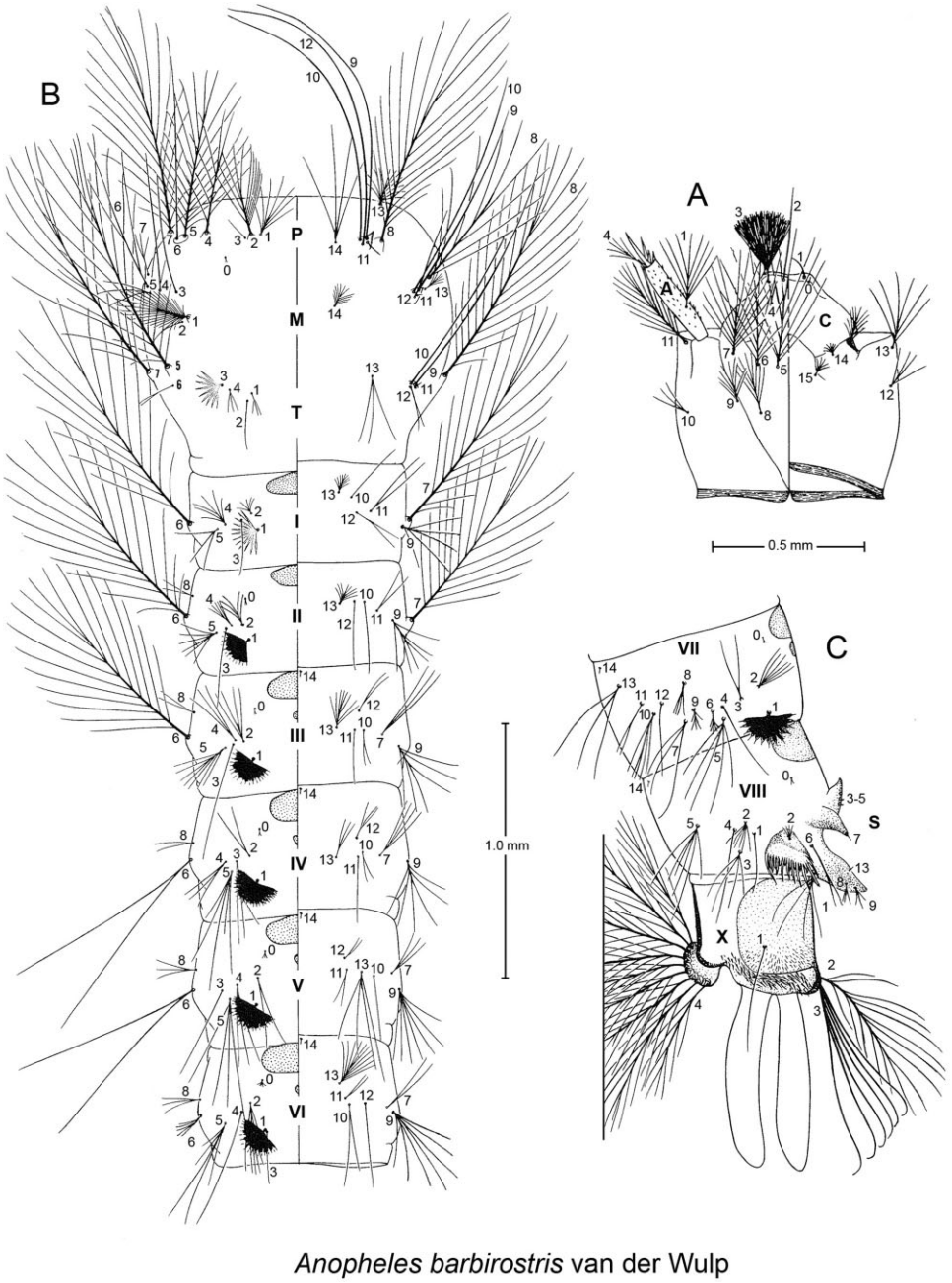

Figure 3. Fourth-instar larva of *Anopheles barbirostris* van der Wulp (modified from Townson *et al*., 2013). A, head, dorsal (left) and ventral (right) aspects of left side. B, thorax and abdominal segments I–VI, dorsal (left) and ventral (right) aspects of left side. C, abdominal segments VII, VIII, and X, left side. Abbreviations: A, antenna; C, cranium; P, prothorax; M, mesothorax; S, spiracular lobe; T, metathorax; I–VIII,X = abdominal segments I–VIII and X; 1–15 = setal numbers for specified areas, e.g. seta 5-C.

Thorax: Integument hyaline, smooth. Seta 1-P often with six to eight (seven) branches from near base; seta 11-P with one or two simple branches arising from short basal stem; sum of branches of pair of seta 13-P = 7–13 (11); seta 13-M with four to 11 (six) branches; seta 14-M commonly with eight to 14 (11) branches; seta 3-T palmate with pale lanceolate leaflets; seta 8-T normally with 13–26 (21) branches.

Abdomen: Integument smooth except for fine spicules on midventral areas of segments II–VIII. Seta 1-I similar to seta 3-T; seta 1-II–VII fully palmate, leaflets with blades darkly pigmented proximal to steplike margins of tapered terminus; seta 13-I often with five to 14 (nine) branches; seta 2-II generally with three to seven (five) branches; seta 5-III frequently with five to nine (seven) branches; sum of branches of pair of seta 10-III = 3–5 (3); seta 13-II with six to ten (eight) branches, seta 13-IV usually with three to seven (three) branches; seta 9-VII often with two to six (five) branches; pecten plate darkly pigmented, usually with eight or nine longer spines; seta 8-S with one to three branches, most often three; membrane posterior to saddle with numerous relatively long spicules; seta 1-X about as long saddle.

Mitotic karyotype

Two types of X chromosome (X_1, X_2) and one type of Y chromosome (Y_1) comprising a single karyotypic form ($X_1X_2Y_1$) have been identified in the early fourth-instar larval brains of *An. barbirostris* (Baimai *et al*., 1995; Suwannamit *et al*., 2009).

Cross-matings

Reciprocal cross-matings between *An. barbirostris*, *Anopheles dissidens* sp. nov., *Anopheles saeungae* sp. nov., and *Anopheles wejchoochotei* sp. nov. (as species A4, A1, A2, and *campestris*-like, respectively) conducted by Suwannamit *et al*. (2009) revealed strong reproductive isolation. The crosses yielded a few viable eggs that gave rise to F_1 larvae with asynaptic polytene chromosomes and F_1 adults with abnormally developed reproductive systems. Females had abnormal ovarian follicles and the accessory glands and testes of males were atrophied.

DNA sequence

Specimens identified as *An. barbirostris* are shown in Table S1, together with GenBank accession numbers for *ITS2* and *COI* sequences. The *ITS2* subunit for *An. barbirostris* yields a dominant product of 1637 bp. The seven interspecifically variable sites at bases 55, 100, 266, 389, 562, 565, and 607 that are unique for the *COI* gene of *An. barbirostris* are shown in Figure 4. The results of Bayesian analyses of *ITS2* and *COI* sequences are shown in Figures 5 and 6, respectively. Both trees show that *An. barbirostris* is well separated from the other species. Our *ITS2* sequence for *An. barbirostris* (BACm6) falls within a strongly supported Clade, Bayesian posterior probability (BPP) 100%, with two sequences (th1.3 and k2) previously reported by Paredes-Esquivel *et al*. (2009, as Clade I = *An. barbirostris s.s.* of Townson *et al*., 2013) (Fig. 5).

Bionomics

In general, *An. barbirostris* is a foothill mosquito. In Thailand, immature stages have been collected at elevations from sea level to 500 m a.s.l. and adults have been captured biting humans at elevations between 750 and 1400 m (Scanlon & Esah, 1965). Habitats of the immature stages include river and stream margins, river and stream pools, ditches with flowing and stagnant water, moats, lakes, permanent and temporary ground pools, flood pools, rice fields, wells, canals, marshes and swamps, ponds, rock pools, seepage springs, and buffalo and elephant footprints. Most habitats contain vegetation and are located in open sunny and partially shaded areas. Females are generally zoophilic but bite humans in the absence of their usual hosts. Adults have been found resting in animal shelters and inside and outside houses.

Both *Plasmodium falciparum* and *Plasmodium vivax* infections have been detected in mosquitoes identified morphologically as *An. barbirostris s.l.* in Sumatra and Sulawesi in the Greater Sundas (Bangs & Rusmiarto, 2007; Syafruddin *et al*., 2007), Flores, Adonara Island, and Timor-Leste in the Lesser Sundas (Bangs & Rusmiarto, 2007; Cooper *et al*., 2010), Sri Lanka (Amerasinghe *et al*., 1999), and Bangladesh (Alam *et al*., 2010). However, no oocysts or sporozoites of either *P. falciparum* or *P. vivax* were found in females of *An. barbirostris s.s.* (as *An. barbirostris* species A4) during experimental infection studies of Thai populations conducted by Thongsahuan *et al*. (2011). Future vector incrimination studies in other areas of the reported range of this species should include DNA applications to retrospectively unequivocally identify malaria infective females.

Distribution

Anopheles barbirostris is the most widespread member of the Barbirostris Complex. It is abundant and widely distributed in Thailand, but is not found at higher elevations or in heavily shaded forest. It is uncommon in the rice plains north of Bangkok where *An. campestris* also occurs. Literature records suggest that this species occurs widely in the Oriental Region from China southward to Indonesia and from Vietnam westward to India and Sri Lanka, but molecular data have only documented its presence in Indonesia (Java, South

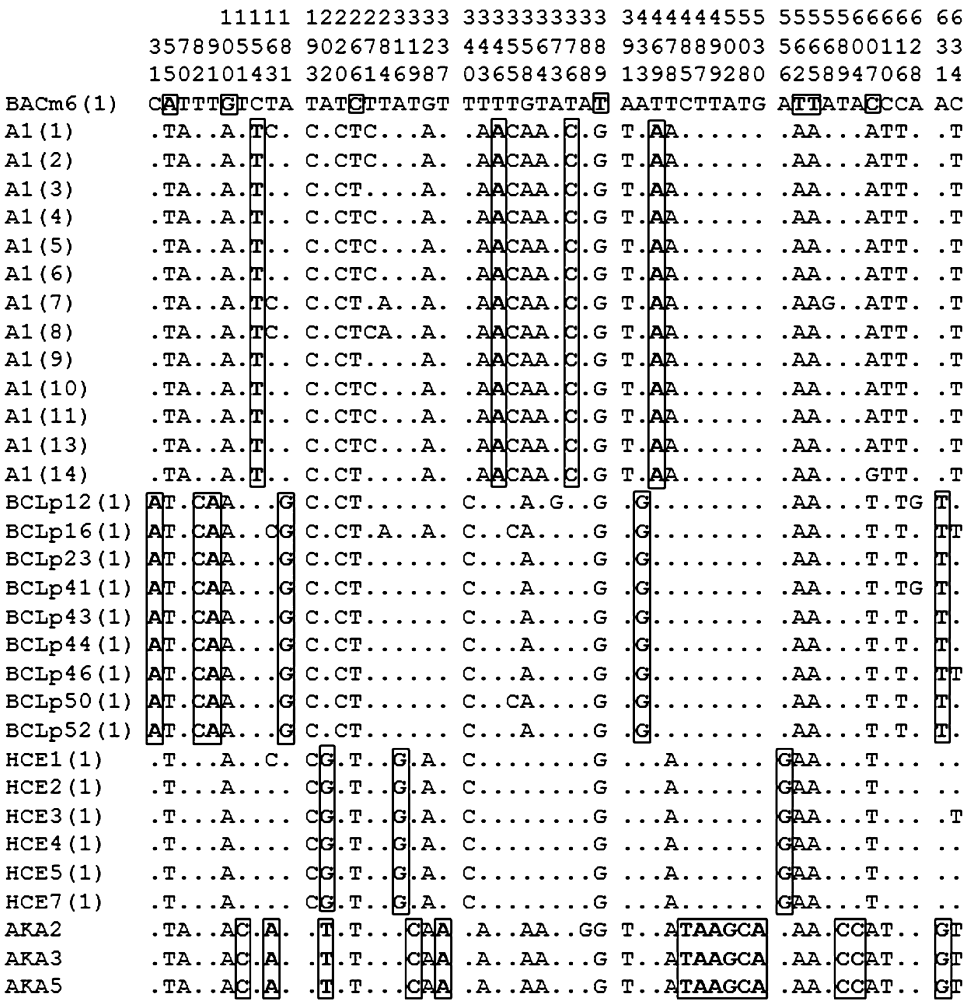

Figure 4. Alignment of the polymorphic sites along a 658-bp fragment of the mitochondrial *cytochrome c oxidase subunit I* gene in *Anopheles barbirostris* (one female), ***Anopheles dissidens* sp. nov.** (13 females), ***Anopheles saeungae* sp. nov.** (nine females), ***Anopheles wejchoochotei* sp. nov.** (six females), and *An. barbirostris* species A3 (three females) (Saeung *et al*., 2008). Codes for the specimens are shown in Table S1. Bases enclosed in boxes represent species-specific differences among the five species.

Kalimantan), Thailand, and Vietnam (Suwannamit *et al*., 2009; Paredes-Esquivel *et al*., 2009; Townson *et al*., 2013; present study).

Material examined

Fifty-seven specimens (19 ♀, 9 ♂, 13 Le, 16 Pe) derived from four progeny broods: BACm6(1), JV52, JV58, and JV73 (the last three from the study of Townson *et al*., 2013). THAILAND, Chiang Mai Province, Chiang Dao District, Ban Wang Jom, buffalo-baited trap, offspring of female collected 23.xi.2013 (coll. Choochote *et al*.): 2 ♀LePe [BACm6(1)-2, -5]; 2 ♀Pe [BACm6(1)-100, -101], 3 ♂LePe [BACm6(1)-1, -3, -4], 1 ♂Pe [BACm6-102]. INDONESIA, Java, Malang Regency, East Java Province, Karangploso Subdistrict, Donowarin Village, Karang Hamlet (07°52′55″S, 112°35′02″E), 2280 ft, offspring of females collected 12.xi.2007 (coll. McAlister *et al*.): 6 ♀LePe [JV52-3, -4, -6, -8 to -10], 8 ♀ [JV52-14 to -17, JV58-1, -2, -4, -5], 2 ♂LePe [JV52-5, -7], 3 ♂ [JV52-11 to -13]; Semerang Regency, Central Java Province, Jambu Subdistrict, Kelurahan Village, Krajan Hamlet (07°17′03″S, 112°21′53″E), 1715 ft, offspring of female collected 14.xi.2007 (coll. McAlister *et al*.): 1 ♀ [JV73-4]. The specimens are deposited in the Natural History Museum, London (BMNH).

ANOPHELES (*ANOPHELES*) *DISSIDENS* SP. NOV.

Anopheles (*Anopheles*) *barbirostris* in part of Reid, 1962 (Thailand, ♀* ♂ E* L P*, taxonomy); Harrison & Scanlon, 1975 (Thailand, ♀* ♂* L* P*, taxonomy); Reid *et al*., 1979 (Thailand, ♀ L P morphology).

Figure 5. Bayesian consensus tree for *internal transcribed spacer 2* sequences. The tree is rooted on *Anopheles* (*Cellia*) *dirus*. Only Bayesian posterior probabilities greater than 50% are shown on the branches. The bar represents 0.1 substitutions per site.

Anopheles barbirostris in part (?) of Harrison *et al*., 1988 (Thailand, A L P morphology).

Anopheles barbirostris forms A (in part), B (in part), and C of Baimai *et al*., 1995 (Thailand, metaphase karyotypes).

Anopheles barbirostris Form A in part of Saeung *et al*., 2007 (Thailand, mitotic karyotype, cross-matings, *COI* and *COII* mtDNA, *ITS2* rDNA).

Anopheles barbirostris species A1 of Saeung *et al*., 2008 (Thailand, mitotic karyotype, cross-matings, *COI* and *COII* mtDNA, *ITS2* rDNA); Suwannamit *et al*., 2009 (Thailand, cross-matings, metaphase karyotype, *COI* and *COII* mtDNA, *ITS2* rDNA); Thongsahuan *et al*., 2011 (Thailand, *Plasmodium* susceptibility); Otsuka, 2011 (Thailand, *ITS2* rDNA).

Anopheles barbirostris Clade III of Paredes-Esquivel *et al*., 2009 (Thailand, *COI* mtDNA, *ITS2* rDNA); Paredes-Esquivel & Townson, 2014 (Sumatran Indonesia, Thailand, *ITS2* rDNA).

Diagnosis

Anopheles dissidens closely resembles but is generally smaller than other members of the Barbirostris Complex. Sequences for *COI* and *ITS2* are diagnostic and reliably distinguish *An. dissidens* from other members of the Barbirostris Complex, including the very closely related *An. vanderwulpi* (see below). Some potentially differential morphological features are denoted below, but for practical purposes *An. dissidens* is morphologically indistinguishable from other species of the complex.

Description

Female

Distinctly smaller than *An. barbirostris*, as indicated by measurements of anatomical features listed in Table 1: lengths of proboscis, maxillary palpus, antennal flagellum, wing, vein R_{4+5}, and forefemur statistically

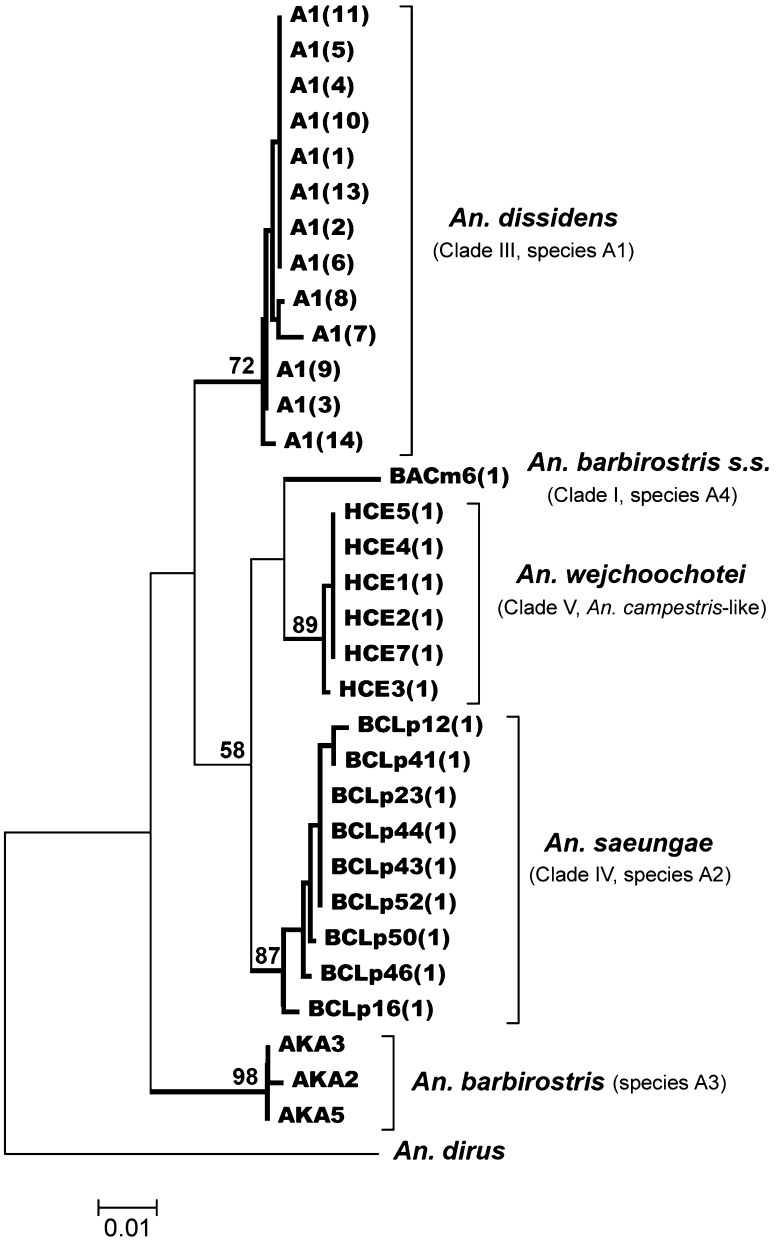

Figure 6. Bayesian consensus tree for *cytochrome c oxidase subunit I* sequences. Only Bayesian posterior probabilities greater than 50% are shown on the branches. The bar represents 0.01 substitutions per site.

significantly shorter (Table S2). Lower mesokatepisternal setae (zero to four) and upper mesepimeral setae (five to seven) usually less numerous than in *An. barbirostris* (Table 1); apical pale band of hindtarsomere 3 0.06–0.12 mm (mean 0.07 mm), extended across joint onto base of hindtarsomere 4 in 10% of females examined.

Male

As for *An. barbirostris* except generally smaller, as indicated by measurements of the gonocoxite, distal parabasal setae, internal seta, gonostylus, claspette club, and aedeagus listed in Table 2: lengths of claspette, claspette club, and aedeagus statistically significantly shorter (Table S3).

Pupa

As described for *An. barbirostris*; setal branching in Table S6; branching of seta 2 compared with other species of the complex in Table 3; differences include seta 2-V with one to three branches; sum of branches of pair of seta 12-CT = 4–7 (6, 7), pair of seta 3-III = 5–12 (7).

Larva, fourth instar
As described for *An. barbirostris*; setal branching in Table S7; differing in having seta 13-M with two to eight (three) branches; seta 8-S with three to eight (four) branches.

Mitotic karyotype
Three types of X chromosome (X_1, X_2, X_3) and four types of Y chromosome (Y_1, Y_2, Y_3, Y_4) comprising four karyotypic forms ($X_1X_2X_3Y_1$, $X_1X_2X_3Y_2$, $X_2X_3Y_3$, X_2Y_4) have been identified in the early fourth-instar larval brains of *An. dissidens* (Baimai *et al*., 1995; Saeung *et al*., 2007; Suwannamit *et al*., 2009).

Cross-matings
Reciprocal cross-matings between *An. dissidens* (as *An. barbirostris* species A1) and *An. saeungae* (as species A2) were conducted by Saeung *et al*. (2008). Only 21% (A1♀ × A2♂) and 13% (A2♀ × A1♂) of the eggs laid by the females gave rise to F_1 adults, with distorted sex ratios, abnormal ovarian follicles in females, and atrophied accessory glands and testes in males. Additionally, the salivary gland polytene chromosomes of the F_1 larvae exhibited partial asynapsis, particularly at the free ends of the chromosome arms. Suwannamit *et al*. (2009) obtained similar results from cross-matings between *An. dissidens* (as species A1) and *An. barbirostris* (as species A4).

DNA sequence
Specimens identified as *An. dissidens* are shown in Table S1, together with GenBank accession numbers for *ITS2* and *COI* sequences. The *ITS2* subunit for *An. dissidens* yields a dominant product of 1822 bp. The four interspecifically variable sites at bases 154, 346, 376, and 469 that are unique for the *COI* gene of *An. dissidens* are shown in Figure 4. The results of Bayesian analyses of *ITS2* and *COI* sequences are shown in Figures 5 and 6, respectively. Both trees show that *An. dissidens* is distinct from the other species of the Barbirostris Complex. Our *ITS2* sequences for *An. dissidens* (A1) fall within a strongly supported clade (Fig. 5, BPP 100%) with two sequences (th39.3 and bsk33) of *An. barbirostris* Clade III of Paredes-Esquivel *et al*. (2009).

Bionomics
Anopheles dissidens occurs in hilly country. Like all other species of the Barbirostris Complex, the immature stages of *An. dissidens* probably occur in stagnant or slowly running bodies of fresh water with vegetation, e.g. rice fields and associated waterways. Adult females are mainly zoophilic. Adults have been frequently collected in traps baited with water buffalo. Thongsahuan *et al*. (2011) found that 9.09% of females (as *An. barbirostris* species A1) were capable of developing sporozoites of *P. vivax* during experimental infection studies.

Distribution
Based on *COI*, *COII*, and *ITS2* sequences, *An. dissidens* is currently only definitely known to occur in Thailand (Chiang Mai, Mae Hong Son, Sa Kaeo, Tak, and Trat Provinces; Saeung *et al*., 2008; Paredes-Esquivel *et al*., 2009; present study).

Etymology
The specific name is taken from the Latin adjective *dissidens*, meaning differing or disagreeing, in reference to the sister relationship of the species with *An. vanderwulpi* Townson & Harbach (see below).

Type series
Two-hundred and twenty-six specimens (115 ♀, 65 ♂, 11 Le, 11 Pe, 24 L) derived from 13 molecularly identified progeny broods: A1(1)–A1(11), A1(13), and A1(14). *Holotype*, ♀ [A1(1)-6], with Le and Pe on microscope slide, offspring of female collected as follows: THAILAND, Chiang Mai Province, Chiang Dao District, Ban Wang Jom, buffalo-baited trap, 23.xi.2013, coll. Choochote *et al*. *Paratypes*, same data as holotype: 6 ♀LePe [A1(1)-2 to -4 and -9 to -11]; 108 ♀ [A1(2)-1, -2, -5 to -12; A1(3)-1 to -5, -8, -9; A1(4)-1, -3, -4, -6, -8 to -10; A1(5)-2, -4, -6; A1(6)-1, -5, -8, -9; A1(7)-1, -2, -4 to -12; A1(8)-1 to -7; A1(9)-2 to -4, -6 to -11; A1(10)-4 to -13; A1(11)-16 to -30; A1(13)-5, -7, -13 to -21; A1(14)-5, -8, -17, -18, -20 to -30]; 4 ♂LePe [A1(1)-1, -5, -7, -8]; 61 ♂ [A1(2)-3, -4; A1(3)-6, -7; A1(4)-2, -5,-7; A1(5)-1, -3, -5; A1(6)-2 to -4, -6, -7; A1(7)-3; A1(9)-1, -5; A1(10)-1 to -3; A1(11)-1 to -15; A1(13)-1 to -4, -6, -8 to -12; A1(14)-1 to -4, -6, -7, -9 to -16, -19]; 24 L [A1(1)-A, -B; A1(2)-A, -B; A1(3)-A, -B; A1(4)-A, -B; A1(5)-A, -B; A1(6)-A, -B; A1(7)-A, -B; A1(8)-A, -B; A1(10)-A, -B; A1(11)-A, -B; A1(13)-A, -B; A1(14)-A, -B]. The type series is deposited in BMNH.

ANOPHELES (*ANOPHELES*) *SAEUNGAE* SP. NOV.

Anopheles (*Anopheles*) *barbirostris* in part of Reid, 1962 (Thailand, ♀* ♂ E* L P*, taxonomy); Harrison & Scanlon, 1975 (Thailand, ♀* ♂* L* P*, taxonomy); Reid *et al*., 1979 (Thailand, ♀ L P morphology).

Anopheles barbirostris in part (?) of Harrison *et al*., 1988 (Thailand, A L P morphology).

Anopheles barbirostris forms A (in part) and B (in part) of Baimai *et al*., 1995 (Thailand, metaphase karyotype).

Anopheles barbirostris Forms A and B (in part) of Saeung *et al*., 2007 (Thailand, mitotic karyotype, cross-matings, *COI* and *COII* mtDNA, *ITS2* rDNA).

Anopheles barbirostris species A2 of Saeung *et al*., 2008 (Thailand, mitotic karyotype, cross-matings, *COI* and *COII* mtDNA, *ITS2* rDNA); Suwannamit *et al*., 2009 (Thailand, cross-matings, metaphase karyotype, *COI* and *COII* mtDNA, *ITS2* rDNA); Thongsahuan *et al*., 2011 (Thailand, *Plasmodium* susceptibility); Otsuka, 2011 (Thailand, *ITS2* rDNA).

Anopheles barbirostris Clade IV (unknown species) of Paredes-Esquivel *et al*., 2009 (Sumatran Indonesia, Thailand, *COI* mtDNA, *ITS2* rDNA); Paredes-Esquivel & Townson, 2014 (Sumatran Indonesia, Thailand, *ITS2* rDNA).

Diagnosis

Adult females of *An. saeungae* resemble the adult females of *An. campestris* in having a similar pattern of pale scales on the abdominal sterna, but the scaling of the wings is paler, as in *An. barbirostris*. This combination of characters, along with *COI* and *ITS2* sequences (see below), readily distinguish *An. saeungae* from other species of the Barbirostris Complex. Some additional potentially differential morphological characters are denoted below, but for practical purposes *An. dissidens* is nearly isomorphic with other species of the complex.

Description

Female

Overall similar to *An. barbirostris*, but generally smaller, as indicated by measurements of the proboscis, maxillary palpus, antennal flagellum, wing, and forefemur listed in Table 1: lengths of proboscis, maxillary palpus, wing, vein R_{4+5}, forefemur, and width of wing statistically significantly shorter (Table S2); apical pale band of hindtarsomere 3 0.04–0.18 mm (mean 0.10 mm), extended across joint onto base of hindtarsomere 4 in 20% of females examined.

Male

As described for *An. barbirostris* but generally smaller, as indicated by measurements of the gonocoxite, distal parabasal setae, internal seta, gonostylus, claspette club, and aedeagus listed in Table 2, but means of measurements not significantly different (Table S3).

Pupa

As described for *An. barbirostris*, indistinguishable; setal branching in Table S8; branching of seta 2 compared with other species of the complex in Table 3.

Larva, fourth instar

As described for *An. barbirostris*; setal branching in Table S9; differing in having seta 13-II with eight to 13 (eight) branches; sum of branches of pair of 13-P = 11–18, pair of seta 10-III = 4, 5 (4).

Mitotic karyotype

Three types of X chromosome (X_1, X_2, X_3) and two types of Y chromosome (Y_1, Y_2) comprising two karyotypic forms ($X_1X_2X_3Y_1$, $X_1X_2X_3Y_2$) have been identified in the early fourth-instar larval brains of *An. saeungae* (Baimai *et al*., 1995; Saeung *et al*., 2008; Suwannamit *et al*., 2009).

Cross-matings

The results of crosses between *An. saeungae* (as *An. barbirostris* species A2) and *An. dissidens* (as species A1) are summarized under *An. dissidens* above. Suwannamit *et al*. (2009) obtained similar results from cross-matings between *An. saeungae* (as species A2) and *An. barbirostris* (as species A4), i.e. incomplete hatching of eggs, asynaptic polytene chromosomes in larvae, reduced pupation, and abnormal reproductive organs in a few adults.

DNA sequence

Specimens identified as *An. saeungae* are shown in Table S1, together with GenBank accession numbers for *ITS2* and *COI* sequences. The *ITS2* subunit for *An. saeungae* yields a dominant product of 1678 bp. The six interspecifically variable sites of the *COI* gene at bases 31, 82, 91, 181, 433, and 631 that are unique for this species are shown in Figure 4. The results of Bayesian analyses of *ITS2* and *COI* sequences are shown in Figures 5 and 6, respectively. Both trees show that *An. saeungae* is a distinct member of the Barbirostris Complex. Our *ITS2* sequences for *An. saeungae* (BCLp) fall within a strongly supported clade (Fig. 5, BPP 100%) with two sequences (Btr7 and l14) of *An. barbirostris* Clade IV of Paredes-Esquivel *et al*. (2009).

Bionomics

Adult females of *An. saeungae* have been collected in buffalo-baited traps, but are likely to attack humans on occasions when preferred hosts are scarce. Thongsahuan *et al*. (2011) found that 6.67% of females (as *An. barbirostris* species A2) had developed sporozoites of *P. vivax* during experimental infection studies.

Distribution

Based on *COI*, *COII*, and *ITS2* sequences, *An. saeungae* is currently only definitely known to occur in Indonesia (Sumatra) and Thailand (Chanthaburi, Lampang, Phetchaburi, Ratchaburi, Sa Kaeo, Trat, Ubon Ratchaburi, and Udon Thani Provinces) (Saeung *et al*., 2008; Paredes-Esquivel *et al*., 2009; Suwannamit *et al*., 2009; present study).

Etymology

This species is named in honour of Dr Atiporn Saeung (Department of Parasitology, Faculty of Medicine, Chiang

Mai University, Chiang Mai, Thailand) for her many contributions to our knowledge of mosquitoes in Southeast Asia, especially her cytogenetic and molecular studies of the Barbirostris Group, which provided information for further studies of these economically important mosquitoes.

Type series

Two-hundred and ninety-seven specimens (135 ♀, 67 ♂, 36 Le, 35 Pe, 24 L) derived from nine molecularly identified progeny broods: BCLp12(1), BCLp16(1), BCLp23(1), BCLp41(1), BCLp43(1), BCLp44(1), BCLp46(1), BCLp50(1), and BCLp52(1). *Holotype*, ♀ [BCLp16(1)-2], with Le and Pe on microscope slide, offspring of female collected as follows: THAILAND, Lampang Province, Ko Kha District, Ban Don Tham, buffalo-baited trap, 7.xii.2013, coll. Choochote *et al*. *Paratypes*, same data as holotype: 20 ♀LePe [BCLp12(1)-3 to -6, -8, -10, -12; BCLp16(1)-1, -5 to -8, -12; BCLp23(1)-1, -3, -4, -8 to -11]; 1 ♀Le [BCLp12(1)-7]; 113 ♀ [BCLp12(1)-14, -16, -17, -21, -23, -25 to -29, -32; BCLp16(1)-13, -17, -18, -20, -22 to -49; BCLp23(1)-13, to -15, -17 -19; BCLp41(1)-1 to -3, -7, -8, -10, -11; BCLp43(1)-8 to -11; BCLp44(1)-2, -3, -5, -7, -9, -11 to -23, -100; BCLp46(1)-2, -3, -5, -7, -12, -14, -15; BCLp50(1)-1, -3, -9 to -11, -13, -14, -16 to -22; BCLp52(1)-6 to -19]; 14 ♂LePe [BCLp12(1)-1, -2, -9, -11; BCLp16(1)-3, -4, -9 to -11; BCLp23(1)-2, -5 to -7, -12]; 53 ♂ [BCLp12(1)-13, -15, -18 to -20, -22, -24, -30, -31; BCLp16(1)-14 to -16, -19, -21; BCLp23(1)-16, -18, -20 to -22; BCLp41(1)-4 to -6, -9; BCLp43(1)-1 to -7; BCLp44(1)-1, -4, -6, -8, -10; BCLp46(1)-1, -4, -6, -13, -16; BCLp50(1)-2, -4 to -8, -12, -15; BCLp52(1)-1 to -5]; 24 L [BCLp12(1)-A, -B; BCLp16(1)-A, -B; BCLp23(1)-A, -B; BCLp41(1)-A, -B; BCLp43(1)-A, -B; BCLp46(1)-A, -B; BCLp50(1)-A, -B, -C, -D, -E, -F; BCLp52(1)-A, -B,-C, -D, -E, -F]. The type series is deposited in BMNH.

Anopheles (*Anopheles*) *campestris* Reid

Anopheles (*Anopheles*) *campestris* Reid, 1962 (♀* ♂* E P L*). Holotype ♀LePe: Rantau Panjang, Klang, Selangor, Malaysia (BMNH).

Anopheles (*Anopheles*) *campestris* of Reid, 1968 (peninsular Malaysia, southern Thailand, ♀* ♂* E P L*); in part (?) of Harrison & Scanlon, 1975 (southern Thailand only, ♀* ♂* L* P*, taxonomy); Reid *et al*., 1979 (Malaysia, Thailand, L P morphology).

Diagnosis

Adults of *An. campestris* generally have more darkly scaled wings and paler scaling on the abdominal sterna than the adults of *An. barbirostris*. The pupa and larva are normally distinguishable by having some setae with more numerous branches. DNA sequences are not available for this species, but the mitotic karyotype of larval brain cells (see below) is distinctive in having a telocentric Y chromosome.

Description

Female

Anatomical features compared with other species of the Barbirostris Complex in Table 1: wing statistically significantly shorter than wing of *An. barbirostris* (Table S3); forefemur/proboscis ratio 0.88–0.95 (mean 0.91); pleura with three or four upper proepisternal setae, four to seven prealar setae, three to six upper and two to five lower mesokatepisternal setae, and nine to 14 upper and zero to six lower mesepimeral setae; wing scaling generally darker than wing scaling of *An. barbirostris*; cubital vein often with more dark scales than pale scales; apex of wing frequently with bar of pale scaling extending posteriorly from preapical pale spot across veins R_1, R_2, R_{3+4}, and M_1; wing without pale fringe spot at apex of vein R_2; foretarsomere 3 usually without apical pale band, midtarsomeres usually without pale bands, apical pale band of hindtarsomere 3 0.08–0.10 mm (mean 0.08 mm), not extended across joint onto base of hindtarsomere 4 in 10% of females examined; sterna II–VII with numerous pale scales in median patch and lateral row, and scattered between median patch and lateral rows; posterior margin of sternum VI rarely with dark scales.

Male

Like female except for sexual differences and as follows: distal 0.5 of abdominal tergum VIII usually with central patch of dark scales and pale scales on lateral aspects; characters of genitalia listed in Table 2: lengths of gonocoxite and aedeagus statistically significantly shorter and longer, respectively (Table S3).

Pupa

As described for *An. barbirostris*; setal branching in Table S10; branching of seta 2 compared with other species of the complex in Table 3; differences include seta 1-II with five to 15 branches; sum of branches of pair of seta 1-II = 12–24, pair of seta 3-III = 15–19 (15).

Larva, fourth instar

As described for *An. barbirostris*; similar to *An. wejchoochotei*, setal branching in Table S11; differences include seta 14-P with four to eight (six) branches; seta 8-M with nine to 22 (11) branches; seta 2-T with one to three (one) branches; seta 13-II with nine to 19 (14) branches; seta 5-V with four to seven (five) branches; sum of branches of pair of seta 13-C = 18 or 19, pair of seta 7-P = 24–39, pair of seta 8-M = 22–31, pair of seta 2-I = 20–33, pair of 2-VIII = 16–20(16), pair of seta 5-IV = 8–11(11).

Mitotic karyotype
The mitotic karyotype of *An. campestris* is only known from a single male larva collected in Bangpa-in of Ayutthaya Province, located in the central plains area north of Bangkok (Baimai *et al*., 1995). The karyotype consists of a metacentric X chromosome, which differs from the metacentric X_1, and a telocentric Y chromosome, which is unique among the Y chromosomes of the Barbirostris Complex.

DNA sequence
Not available.

Bionomics
The immature stages are normally found in still, shaded bodies of fresh water containing some vegetation, including rice fields, marshes, swamps, ponds, ground, stream and flood pools, canals and ditches, pits, animal footprints, and wells (Harrison & Scanlon, 1975; Rattanarithikul *et al*., 2006). Adult females readily bite humans, more so than other members of the Barbirostris Complex, enter houses, and have been found infected with malaria protozoa. Females are important vectors of malaria in the western coastal plains of peninsular Malaysia (Reid, 1962) and are known to transmit the nematode *Brugia malayi* that causes lymphatic filariasis (Rattanarithikul *et al*., 2006).

Distribution
Anopheles campestris occurs in low-lying areas normally at elevations less than 200 m a.s.l. It reportedly occurs in Cambodia, China, Malaysia, Thailand, and Vietnam, but its occurrence is only confirmed for populations in peninsular Malaysia and in Thailand. It seems to be confined to the Korat Plateau, Chao Phrya River basin, and the coastal areas of Thailand (Reid, 1962; Harrison & Scanlon, 1975; Reid *et al*., 1979). Based on extensive surveys and detailed study of adults with associated larval and pupal exuviae, Harrison *et al*. (1988) showed that *An. campestris* does not occur in the plains area of Chiang Mai Province where the type locality of *An. wejchoochotei* resides. Current evidence suggests that *An. campestris* is unlikely to occur in China.

Etymology
The specific name is taken from the Latin adjective (*campestris*, *campestris*, *campestre*) meaning 'of fields' or 'plain-dwelling'. When Reid (1962) described this species, he stated 'This species appears to be largely confined to broad alluvial plains (thus the name *campestris*) along coasts and river deltas'.

Type series
Twenty-seven specimens (5 ♀, 4 ♂, 9 Le, 9 Pe) reared from eggs obtained from wild-caught females, with associated larval and pupal exuviae mounted on microscope slides. *Holotype*, ♀LePe (L.H. 75/10), mother collected MALAYSIA, Selangor, Klang, Rantau Panjang, 20.vii.1952, coll. J. A. Reid; *Allotype*, ♂LePe [L.H. 75/2], same data as holotype; *Paratypes*, ♀LePe [L.H. 75/21], same data as holotype; ♀LePe [377/1] and ♂LePe [377/4], same data but collected 31.iii1949; ♂LePe [L.H. 80/4], same data but collected 24.iii.1952; 2 ♀LePe [L.H. 81/7, /8]; ♂LePe [L.H. 81], same data but collected 3.viii.1952. The series is deposited in BMNH.

ANOPHELES (*ANOPHELES*) *WEJCHOOCHOTEI* SP. NOV.

Anopheles (*Anopheles*) *barbirostris* (*campestris*-like) in part (?) of Harrison & Scanlon, 1975 (northern Thailand, ♀* ♂* L* P*, taxonomy).

Anopheles barbirostris in part (?) of Harrison *et al*., 1988 (Thailand, A L P morphology).

Anopheles barbirostris form B in part of Baimai *et al*., 1995 (Thailand, metaphase karyotype).

Anopheles campestris-like Forms B and E of Saeung *et al*., 2007 (Thailand, mitotic karyotype, cross-matings, *COI* and *COII* mtDNA, *ITS2* rDNA).

Anopheles barbirostris Clade V (*An. campestris*) of Paredes-Esquivel *et al*., 2009 (Thailand, *COI* mtDNA, *ITS2* rDNA).

Anopheles campestris-like Form E of Suwannamit *et al*., 2009 (Thailand, cross-matings, metaphase karyotype, *COI* and *COII* mtDNA, *ITS2* rDNA).

Anopheles campestris-like Forms B, E, and F of Thongsahuan *et al*., 2009 (Thailand, cross-matings, mitotic karyotypes, *COI* and *COII* mtDNA, *ITS2* rDNA). Thongsahuan *et al*., 2011 (Thailand, *Plasmodium* susceptibility).

Anopheles campestris-like of Otsuka, 2011 (Thailand, *ITS2* rDNA).

Anopheles campestris of Paredes-Esquivel & Townson, 2014 (Thailand, *ITS2* rDNA).

Diagnosis
Anopheles wejchoochotei is morphologically similar to *An. campestris* but *ITS2* sequence data (not available for *An. campestris*) indicate that it is the sister species of *An. barbirostris* (Fig. 4). *Anopheles wejchoochotei* and *An. barbirostris* are distinguished and identified by differences in their *COI* and *ITS2* sequences (see below). Some potentially differential morphological characters are denoted below, but the two species are essentially isomorphic.

Description
Female
Anatomical features compared with other species of the Barbirostris Complex in Table 1, generally smaller than *An. barbirostris*, lengths of proboscis, maxillary palpus, antennal flagellum, wing, vein R_{4+5}, and

forefemur statistically significantly shorter (Table S2); similar to the female of *An. campestris*; apical pale band of hindtarsomere 3 0.06–0.10 mm (mean 0.09 mm), not extended across joint onto base of hindtarsomere 4 in 10% of females examined.

Male
Similar to the male of *An. campestris*; aedeagus with three to five pairs of leaflets; other characters of genitalia listed in Table 2, none statistically significantly different from those of *An. barbirostris* (Table S3).

Pupa
As described for *An. campestris*; setal branching in Table S12; branching of seta 2 compared with other species of the complex in Table 3; differences include seta 1-II with three to seven (four) branches; sum of branches of pair of seta 1-II = 7–13 (10), pair of seta 3-III = 8–13 (8).

Larva, fourth instar
As described for *An. campestris*; setal branching in Table S13; differences include seta 14-P with four to five (five) branches; seta 8-M with four to 11 (nine) branches; seta 2-T single; seta 5-V with three to five (three) branches; seta 13-II with four to 17 (12) branches; sum of branches of pair of seta 13-C = 11–16, pair of seta 7-P = 41–47, pair of seta 8-M = 15–19, pair of seta 1-II = 28–38, pair of seta 2-VIII = 10–17(10), pair of seta 5-IV = 6–8.

Mitotic karyotype
Three types of X chromosome (X_1, X_2, X_3) and three types of Y chromosome (Y_2, Y_5, Y_6) comprising three karyotypic forms (X_2Y_2, $X_1X_2X_3Y_5$, $X_2X_3Y_6$) have been identified in the early fourth-instar larval brains of *An. wejchoochotei* (Thongsahuan *et al*., 2009).

Cross-matings
Cross-matings of *An. wejchoochotei* (as *An. campestris*-like Form E) with *An. campestris*-like Forms B, E, and F produced fully fertile offspring, yielding high percentages of emergence. Backcrosses between the F_1 offspring and the respective parental strains yielded fertile F_2 progeny. However, crosses between *An. campestris*-like Form E and *An. barbirostris* Forms A (= *An. barbirostris*, *An. dissidens* and *An. saeungae*) and B (= *An. dissidens* and *An. saeungae*) failed to yield offspring (Saeung *et al*., 2007; Suwannamit *et al*., 2009).

DNA sequence
Specimens identified as *An. wejchoochotei* are shown in Table S1, together with GenBank accession numbers for *ITS2* and *COI* sequences. The *ITS2* subunit for *An. wejchoochotei* yields a dominant product of 1612 bp. The three interspecifically variable sites at bases 202, 316, and 556 of the *COI* gene that are unique for this species are shown in Figure 4. The results of Bayesian analyses of *ITS2* and *COI* sequences of *An. wejchoochotei* are shown in Figures 5 and 6, respectively. Both trees show that *An. wejchoochotei* is well separated from the other species of the Barbirostris Complex. Our *ITS2* sequences for *An. wejchoochotei* (HCE) fall within a strongly supported clade (Fig. 5, BPP 100%) with two sequences (bsk34 and csk10) of *An. campestris* (Clade V) of Paredes-Esquivel *et al*. (2009).

Bionomics
Suwannamit *et al*. (2009) found larvae of *An. wejchoochotei* (as *An. campestris*-like form E) in rice fields at 310 m above sea level in San Sai District of Chiang Mai Province. Adult females are known to attack and bite humans (the mothers of the broods that comprise the type series of this species were collected in human-baited traps). Limrat *et al*. (2001) and Apiwathnasorn *et al*. (2002) reported that either *An. barbirostris* or *An. campestris* is a probable vector of malaria in Sa Kaeo Province in eastern Thailand where high numbers of females were captured landing on humans both indoors and outdoors; however, no sporozoites of *P. vivax* developed in *An. wejchoochotei* females (as *An. campestris*-like Form E) from this province during experimental infection studies conducted by Thongsahuan *et al*. (2011). It is interesting to note, however, that 66.67 and 64.29% of females of Forms B and E, respectively, of this species from Chiang Mai Province did develop sporozoites of *P. vivax*.

Distribution
Based on *COI*, *COII*, and *ITS2* sequences, *An. wejchoochotei* is currently only definitely known to occur in Thailand (Ayuttaya, Chanthaburi, Chiang Mai, Chiyaphum, Chumphon, Kamphaeng Phet, Khon Kaen, Maha Sarakham, Mukdahan, Prachuap Khiri Khan, Sa Kaeo, and Udon Thani Provinces; Thongsahuan *et al*., 2009, as *An. campestris*-like; present study). However, because it is so widely distributed in Thailand, it is likely to occur in neighbouring countries.

Etymology
This species is named in honour of the late Prof. Dr Wej Choochote (Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand) for his many contributions to our knowledge of mosquitoes in Southeast Asia, especially his studies of the Barbirostris Group, which provided the taxonomic foundation for further studies of this medically important group of insects.

Type series
Two-hundred and fifty-two specimens (63 ♀, 41 ♂, 59 Le, 65 Pe, 24 L) derived from six molecularly

identified progeny broods: HCE1(1), HCE2(1), HCE3(1), HCE4(1), HCE5(1), and HCE7(1). *Holotype*, ♀ [HCE1(1)-15], with Le and Pe on microscope slide, offspring of female collected as follows: THAILAND, Chiang Mai Province, San Sai District, Ban Nong Chom, human-baited trap, 2.xi.2013, coll. Choochote *et al*. *Paratypes*, same data as holotype: 30 ♀LePe [HCE1(1)-1, -5, -9, -10, -12, -13, -16 to -20; HCE2(1)-9 to -13, -16 to -20; HCE3(1)-3, -6, -9, -16 to -20, -23]; 1 ♀Le [HCE1(1)-14]; 5 ♀Pe [HCE3(1)-2, -21, -22, -25, -26]; 26 ♀ [HCE4(1)-2 to -8, -10 to -16; HCE5(1)-1, -2, -5 to -8, -10; HCE7(1)-3, -5 to -7, -9]; 27 ♂LePe [HCE1(1)-2 to -4, -6 to -8, -11; HCE2(1)-1 to -8, 14, -15; HCE3(1)-4, -5, -7, -8, -10 to -12, -14, -15, -24]; 2 ♂Pe [HCE3(1)-1, -13]; 12 ♂ [HCE4(1)-1, -9; HCE5(1)-3, -4, -9, -11, -12; HCE7(1)-1, -2, -4, -8, -10]; 24 L [HCE1(1)-A, -B -C, -D, -E, -F; HCE2(1)-A, -B -C, -D, -E, -F; HCE3(1)-A, -B -C, -D, -E, -F; HCE4(1)-A, -B; HCE5(1)-A, -B; HCE7(1)-A, -B]. The type series is deposited in BMNH.

DISCUSSION

In addition to the five species characterized above, the Barbirostris Complex includes at least one additional species, i.e. *An. barbirostris* species A3 of Saeung *et al*. (2008). Molecularly identified progeny broods of this species were not available at the time of this study; hence, the species is not formally characterized and named herein. It has been suggested that this species may be conspecific with *An. campestris*, but this seems unlikely in view of the provenance of species A3 being a foothill-mountainous area of Kanchanaburi Province in western Thailand. Molecular data are not currently available for *An. campestris*, but it seems likely that this lowland species corresponds to the unique cytogenetic form (X Y) of Baimai *et al*. (1995) from the rice plains north of Bangkok. As noted by Paredes-Esquivel *et al*. (2009), species A3 has a much smaller *ITS2* amplicon than the corresponding region of their Clades I (*An. barbirostris*), II (*An. vanderwulpi*), III (*An. dissidens*), IV (*An. saeungae*), and V (*An. wejchoochotei*), suggesting that it is not closely related to the Barbirostris Complex. This is supported by the high level of average genetic distance (0.540–0.656) between *ITS2* sequences of *An. barbirostris* species A3 and the five species of the Barbirostris Complex (Table S14).

Mosquitoes from northern Thailand (Chiang Mai and Lampang Provinces) now identifiable as *An. wejchoochotei* were originally referred to as *An. campestris*-like by Harrison & Scanlon (1975) because they resembled *An. campestris* but their associated larval and pupal exuviae were clearly *An. barbirostris*. Based on detailed study of larval and pupal exuviae of adults reared from larvae collected in the plains of Chiang Mai Province, the lowland area that includes the type locality of *An. wejchoochotei*, Harrison *et al*. (1988) concluded that *An. campestris* does not occur in northern Thailand, and this is why *An. wejchoochotei* was referred to informally as *An. campestris*-like in recent chromosomal, cross-mating, and molecular studies (Saeung *et al*., 2007; Suwannamit *et al*., 2009; Otsuka, 2011). Although *An. campestris* is morphologically inseparable from *An. wejchoochotei*, the two forms are unlikely to be conspecific based on chromosomal and distributional evidence. *Anopheles campestris* exhibits a unique mitotic karyotype that is unlike that of the other members of the Barbirostris Complex (Baimai *et al*., 1995; Saeung *et al*., 2007; Thongsahuan *et al*., 2009, 2011) and is only known to occur in the central plains of Thailand and southward through coastal areas of southern Thailand and peninsular Malaysia. Until now, *An. campestris* has been classified as a member of the Barbirostris Subgroup of species (Harbach, 2004). Although its mitotic karyotype suggests that it is not as closely related as other members of the Barbirostris Complex, it is included as a member of the complex here based on morphological similarity.

The *ITS2* fragments amplified (exclusive of primers) for members of the Barbirostris Complex so far have shown length polymorphisms that are consistent within species: 1637 bp in *An. barbirostris*, 1822 bp in *An. dissidens* sp. nov., 1678 bp in *An. saeungae* sp. nov., 1727 bp in *An. vanderwulpi*, and 1612 bp in *An. wejchoochotei* sp. nov. These polymorphisms are mainly the result of differences of the number of repeats in the *ITS2* sequences (Otsuka, 2011). Sequence data are not currently available for specimens definitively identified as *An. campestris*. Based on *ITS2* sequence similarity, *An. barbirostris* is most closely related to *An. wejchoochotei*. The relationships of these species based on *ITS2* sequence are shown in Figure 5. *Anopheles barbirostris* is closer to *An. wejchoochotei* (approximately 82% similarity; average genetic distance = 0.114) than to *An. dissidens* (approximately 62% similarity; average genetic distance = 0.255). In comparison, *An. dissidens* and *An. vanderwulpi* share approximately 95% sequence identity (average genetic distance = 0.020, Table S14). The comparison of *COI* sequences between these two species was not possible as our *COI* sequences do not overlap with those of Paredes-Esquivel *et al*. (2009). However, the Bayesian tree for *COI* sequences generated in the study of Paredes-Esquivel *et al*. (2009) revealed that they are distinct species. In the present study, both the *ITS2* and *COI* trees showed similar topologies, with conspecific sequences falling within distinct, strongly supported clades. In addition, the pairwise distances between the *COI* sequences (Table S15) of *An. barbirostris*, *An. dissidens*, *An. saeungae*, and *An. wejchoochotei* range from 0.023 to 0.049, which are in agreement with the

threshold value (> 2%) for distinguishing species based on *COI* barcode sequences (Wijit *et al*., 2013).

Satoto (2001) identified two putative species, informally designated species X and W, among specimens from Sulawesi, Flores, and Java based on *COI* sequence. Townson *et al*. (2013) suggested that either species X or W may correspond to *An. barbirostris* and the other to *An. vanderwulpi*, and that another species of the complex may be present in Sulawesi. Considering the wide distribution and questionable malaria vector status of mosquitoes formerly identified as *An. barbirostris* in the Oriental Region, there is a great need for integrated molecular epidemiological studies of the Barbirostris Complex throughout the region to unambiguously elucidate the species and their relation to disease.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Provenance, codes, GenBank accession numbers, and sources of *internal transcribed spacer 2* (*ITS2*) and *cytochrome c oxidase subunit I* (*COI*) sequences for mosquitoes used in the phylogenetic analyses.

Table S2. Comparison of results of statistical analyses of selected anatomical features observed in females of five species of the Barbirostris Complex in Thailand.

Table S3. Comparison of results of statistical analyses of selected features of the male genitalia of five species of the Barbirostris Complex in Thailand.

Table S4. Numbers of branches for setae of pupae of *Anopheles barbirostris*.

Table S5. Numbers of branches for setae of larvae of *Anopheles barbirostris*.
Table S6. Numbers of branches for setae of pupae of ***Anopheles dissidens* sp. nov.**
Table S7. Numbers of branches for setae of larvae of ***Anopheles dissidens* sp. nov.**
Table S8. Numbers of branches for setae of pupae of ***Anopheles saeungae* sp. nov.**
Table S9. Numbers of branches for setae of larvae of ***Anopheles saeungae* sp. nov.**
Table S10. Numbers of branches for setae of pupae of *Anopheles campestris*.
Table S11. Numbers of branches for setae of larvae of *Anopheles campestris*.
Table S12. Numbers of branches for setae of pupae of ***Anopheles wejchoochotei* sp. nov.**
Table S13. Numbers of branches for setae of larvae of ***Anopheles wejchoochotei* sp. nov.**
Table S14. Average genetic distances between *internal transcribed spacer 2* (*ITS2*) sequences of species of the Barbirostris Complex obtained using the Kimura two-parameter (K2P) model.
Table S15. Pairwise distances between *cytochrome c oxidase subunit I* (*COI*) sequences of species of the Barbirostris Complex obtained using the Kimura two-parameter (K2P) model.