Evidence to Support a Conspecific Nature of Allopatric Cytological Races of Anopheles nitidus (Diptera: Culicidae) in Thailand

Siripan Songsawatkiat,¹ Visut Baimai,² Sorawat Thongsahuan,³ Yasushi Otsuka,⁴ Kritsana Taai,⁵ Chayanit Hempolchom,¹ Wichai Srisuka,⁵ Petchaboon Poolphol,⁶ Wej Choochote,¹ and Atiporn Saeung¹,⁷

¹Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
²Department of Biology and Centre for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
³Faculty of Veterinary Science (Establishment Project), Prince of Songkla University, Songkhla 90110, Thailand
⁴Research Center for the Pacific Islands, Kagoshima University, Kagoshima 890-8580, Japan
⁵Entomology Section, Queen Sirikit Botanic Garden, P.O. Box 7, Chiang Mai 50180, Thailand
⁶Office of Disease Prevention and Control No. 7, Department of Disease Control, Ministry of Public Health, Thailand, Ubon Ratchathani 34000, Thailand
⁷Corresponding author, e-mail: atiporn44@yahoo.com

Subject Editor: Igor Sharkhov

J. Insect Sci. 14(287): 2014; DOI: 10.1093/jisesa/ieu149

ABSTRACT. Metaphase karyotype investigation on two allopatric strains of Anopheles nitidus Harrison, Scanlon, and Reid (Diptera: Culicidae) was conducted in Thailand during 2011–2012. Five karyotypic forms, i.e., Form A (X1, Y1), Form B (X2, Y2), Form C (X3, Y3), Form D (X4, Y4), and Form E (X5, Y5) were obtained from a total of 21 isofemale lines. Forms A, B, and C were confined to Phang Nga Province, southern Thailand, whereas Forms D and E were restricted to Ubon Ratchathani Province, northeastern Thailand. Cross-mating experiments among the five isofemale lines, which were representative of five karyotypic forms of An. nitidus, revealed genetic compatibility by providing viable progenies and synaptic salivary gland polytene chromosomes through F2 generations. The results suggest that the forms are conspecific, and An. nitidus comprises five cytological races. The very low intraspecific sequence variation (average genetic distances = 0.002–0.008) of the nucleotide sequences in ribosomal DNA (internal transcribed spacer 2) and mitochondrial DNA (cytochrome c oxidase subunits I and II) among the five karyotypic forms were very good supportive evidence.

Key Words: Anopheles nitidus, metaphase karyotype, cross-mating experiment, rDNA, mtDNA

Anopheles (Anopheles) nitidus Harrison, Scanlon, and Reid (Diptera: Culicidae) is a foothill anopheline species that belongs to the Nigerrimus Subgroup and Hyrcanus Group of the Myzorhynchus Series and has a wide distribution range extending from India (Assam) to Vietnam, Cambodia, Thailand (a cosmopolitan species), Malaysia (Malaysian Peninsular and Sarawak), and Indonesia (Sumatra) (Reid 1968, Harrison and Scanlon 1975, Rattanarithikul et al. 2006, Harbach 2014). Although An. nitidus acts as a vicious biter of humans in some localities of Thailand, it has never been incriminated as a natural or suspected vector of any human diseases, unlike other species members of the Thai Anopheles hyrcanus group (e.g., Anopheles nigerrimus, Anopheles peditaeniatus, and Anopheles sinensis) that one suspected vectors of Plasmodium vivax (Baker et al. 1987, Harbach et al. 1987, Gingrich et al. 1990, Rattanarithikul et al. 1996); and An. nigerimus, a potentially natural vector of Wuchereria bancrofti in Phang Nga Province, southern Thailand (Division of Filariosis 1998)). Nevertheless, An. nitidus is considered an economic pest of cattle because of its vicious biting behavior (Reid et al. 1962, Reid 1968, Harrison and Scanlon 1975).

Regarding cytogenetic investigations of An. nitidus by Baimai et al. (1993a), their results revealed that at least two types of X (X1, X2) and one type of Y chromosomes were obtained in two isofemale lines caught from Muang district, Phang Nga Province and Sadao district, Songkhla Province, southern Thailand. As emphasized by the above information, genetic proximity among the karyotypic variants of An. nitidus is obviously lacking. Thus, the main aim of this study was to determine whether the five karyotypic variants, from two allopatric populations of An. nitidus, exist as a single or distinct species by performing cross-mating experiments among them that relating to DNA sequence analyses of internal transcribed spacer 2 (ITS2) of ribosomal DNA, and cytochrome c oxidase subunits I (COI) and II (COII) of mitochondrial DNA (mtDNA).

Materials and Methods

Field Collections and Establishment of Isoline Colonies. Wild-caught, fully engorged female mosquitoes of An. nitidus were collected from cow-baited traps at two locations, i.e., Muang district, Phang Nga Province and Nachaluai district, Ubon Ratchathani Province in southeastern and northeastern Thailand, respectively (Fig. 1; Table 1). In total, 21 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 F1 progeny 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, cross-mating experiments, and molecular analyses.

Metaphase Karyotype Preparation. Metaphase chromosomes were prepared from 10 early fourth-instar larval brains of F1 progeny of each isolate, using techniques previously described by Saeung et al. (2007). Identification of karyotypic forms followed the standard cytogenetic systems of Baimai et al. (1993a).

Cross-Mating Experiments. The five laboratory-raised isolines of An. nitidus were selected arbitrarily from the 21 isolate colonies as representatives of the five karyotypic forms, i.e., Form A (Pg5A), B (Pg5B), C (Pg4C), D (Ur2D), and E (Ur5E) (Table 1). These isolines were used for cross-mating experiments to determine postmating barriers by employing the techniques previously reported by Saeung et al. (2007).

DNA Extraction and Amplification. Molecular analyses of three specific genomic loci (ITS2, COI, and COII) were performed to determine intraspecific sequence variation within An. nitidus. Individual F1 progeny adult female of each isolate of An. nitidus (Ur2D, Ur5E, Ur8E, Ur11D, Ur12D, Ur15D, Ur16E, Ur19D, Ur22E, Ur23E, Ur24D, Ur25E) were prepared from 10 early fourth-instar larval brains of F1 progeny of each isolate, using techniques previously described by Saeung et al. (2007).
Ur25D, Ur27D, Ur28E, Ur30E, Ur31D, Ur33E, Ur34D, Pg2A, Pg4C, and Pg5B; Table 1) was used for DNA extraction and amplification. Genomic DNA was extracted from each mosquito using DNeasy Blood and Tissue Kit (QIAgen, Japan). Primers for amplification of the ITS2, COI, and COII regions followed previous studies by Saeung et al. (2007). Each polymerase chain reaction (PCR) reaction was carried out in 20 μl containing 0.5 U Ex Taq (Takara, Japan), 1X Ex Taq DNA polymerase buffer, 2 mM of MgCl₂, 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 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 amplification profile of COI and COII 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% tris-acetate-EDTA agarose gels and stained with ethidium bromide. Finally, the PCR products were purified using the QIAquick PCR Purification Kit (QIAgen, Japan) and their sequences directly determined using the

Fig. 1. Map of Thailand showing two provinces where specimens of An. nitidus were collected and the number of isolines of the five karyotypic forms (A–E) detected in each location.
BioEdit version 7.0.5.3 (Hall 1999). Gap sites were excluded from the alignment program (Thompson et al. 1994) and edited manually in ITS2, COI, and COII were aligned using the CLUSTAL W multiple construction of neighbor-joining (NJ) trees (Saitou and Nei 1987) and the calculate genetic distances (Kimura 1980). Using the distances, con-following analysis. The Kimura two-parameter model was employed to obtained from this study were compared with published sequences AB777782–AB777844 (Table 1). The ITS2, COI, and COII sequences sequence data obtained have been deposited in the DDBJ/EMBL/lyzer (Applied Biosystems, www.appliedbiosystems.com). The BigDye V3.1 Terminator Cycle Sequencing Kit and 3130 genetic ana-
and distribution of heterochromatic block. Thus, the Y₁ chromosome is an apparently small telocentric, which represents the ancestral form (Fig. 2A). The Y₂ chromosome has a small subtelocentric or acrocentric that slightly differs from the Y₁ chromosome, which has a very small portion of the short arm present (Fig. 2B). Chromosome Y₃ has a large subtelocentric that obviously differs from the Y₂ chromosome in having an extra block of heterochromatin at the distal end of the long heterochromatic arm (Fig. 2C). The Y₄ chromosome is clearly submeta-centric, with the short arm ~one-third the length of the long arm (Fig. 2D and E). It appears to have derived from the Y₃ chromosome by means of adding an extra block of heterochromatin onto the short arm and transferring it to a submetacentric. Chromosome Y₅ had a small metacentric, which was quite different from chromosomes Y₁, Y₂, Y₃, and Y₄ by having an equal heterochromatic block on each arm (Fig. 2F and G). Based on uniquely different characteristics of Y chromosome from each isolate colony, they were designated as Form A (X₁, Y₁), Form B (X₁, Y₂), Form C (X₂, Y₃), Form D (X₁, X₃, Y₄), and Form E (X₁, X₂, X₃, Y₅). Forms A, B, and C were found in Phang Nga Province, and Forms D and E were obtained in Ubon Ratchathani Province.

Cross-Mating Experiments. Details of hatchability, pupation, emergence, and adult sex-ratio of parental, reciprocal, and F₁-hybrid crosses among the five isolines of *An. nitidus* Forms A, B, C, D, and E are listed in Table 2. All crosses yielded viable progenies through F₂ generations. No evidence of genetic incompatibility and/or postmating reproductive isolation was observed among these crosses. The salivary gland polytene chromosomes of the fourth-stage larvae from all crosses showed synapsis without any inversion loops along the whole length of all autosomes and the X chromosome (Fig. 4).

DNA Sequences and Phylogenetic Analysis. DNA sequences were determined and analyzed for the ITS2, COI, and COII of the 21 isolines of *An. nitidus* Forms A, B, C, D, and E. They showed various lengths of ITS2, at 480 bp in 18 isolines from Ubon Ratchathani Province and 481 bp in 3 isolines from Phang Nga Province. The *An. nitidus* from Ubon Ratchathani Province differed from that in Phang Nga Province by a deletion of T at position 421. They all showed the same length in COI (658 bp) and COII (685 bp). NJ and Bayesian trees were constructed to reveal the evolutionary relationship of the five karyotypic forms. Both phylogenetic methods showed similar tree topologies, thus only the Bayesian tree is shown in Figs. 5 and 6. The results

---

Fig. 2. Metaphase karyotypic forms of *An. nitidus*. Phang Nga Province (A–C) (A) Form A (X₁, Y₁), (B) Form B (X₁, Y₂), and (C) Form C (X₂, Y₃). Ubon Ratchathani Province (D–I) (D) Form D (X₁, Y₄), (E) Form D (X₁, Y₄), (F) Form E (X₁, Y₃), (G) Form E (X₂, Y₃), (H) Form E (homozygous X₂, X₂), and (I) Form E (heterozygous X₂, X₃).
showed that all sequences of *An. nitidus* Forms A, B, C, D, and E were monophyletic in both trees, with high support (NJ = 99–100%, BPP = 100%). The average genetic distances within the five karyotypic forms (21 isolines) of *An. nitidus* were 0.002, 0.008, and 0.006 for ITS2, COI, and COII sequences, respectively. Furthermore, all karyotypic forms of *An. nitidus* were well separated from other species members (*Anopheles belenrae*, *Anopheles crawfordi*, *Anopheles kleini*, *Anopheles lesteri*, *Anopheles paraliae*, *An. peditaeniatus*, *Anopheles pullus*, and *An. sinensis*) of the Hyrcanus Group (Figs. 5 and 6). The three published ITS2 sequences (GenBank accession numbers

![Fig. 3. Diagrams of representative metaphase karyotypes of Forms A, B, C, D, and E of *An. nitidus*.](image)

**Table 2. Cross-mating experiments of five isolines of *An. nitidus***

| Crosses (female x male) | Total eggs (number) | Embryonation rate | Hatched, n (%) | Pupation, n (%) | Emergence, n (%) | Total emergence, n (%) |
|-------------------------|---------------------|------------------|----------------|----------------|------------------|------------------------|
|                         |                     |                  |                |                |                  |                        |
| Parental cross          |                     |                  |                |                |                  |                        |
| Pg2A x Pg2A             | 244 (125, 119)      | 88               | 210 (86.06)    | 195 (92.86)    | 195 (100.00)     | 103 (52.82)            |
| Pg5B x Pg5B             | 277 (130, 147)      | 91               | 238 (85.92)    | 226 (94.96)    | 221 (97.79)      | 107 (48.42)            |
| Pg4C x Pg4C             | 283 (118, 165)      | 84               | 218 (77.03)    | 218 (100.00)   | 211 (96.79)      | 106 (50.24)            |
| Ur2D x Ur2D             | 292 (109, 183)      | 92               | 263 (90.07)    | 258 (98.10)    | 247 (95.74)      | 131 (63.04)            |
| Ur5E x Ur5E             | 301 (148, 153)      | 88               | 256 (85.05)    | 251 (98.05)    | 221 (88.05)      | 111 (50.23)            |
|                         |                     |                  |                |                |                  |                        |
| Reciprocal cross        |                     |                  |                |                |                  |                        |
| Pg2A x Pg5B             | 289 (147, 142)      | 94               | 260 (89.97)    | 257 (98.85)    | 239 (93.00)      | 117 (48.95)            |
| Pg5B x Pg2A             | 298 (158, 140)      | 90               | 220 (73.83)    | 202 (91.82)    | 198 (98.02)      | 97 (48.99)             |
| Pg2A x Pg4C             | 299 (131, 168)      | 92               | 260 (86.96)    | 231 (88.85)    | 226 (97.84)      | 112 (49.56)            |
| Pg4C x Pg2A             | 313 (162, 151)      | 80               | 225 (71.08)    | 218 (86.89)    | 209 (95.87)      | 112 (53.59)            |
| Pg2A x Ur2D             | 211 (103, 108)      | 86               | 172 (62.94)    | 159 (90.86)    | 159 (100.00)     | 64 (40.25)             |
| Ur2D x Pg2A             | 224 (111, 113)      | 91               | 201 (90.18)    | 196 (97.03)    | 171 (87.24)      | 81 (47.37)             |
| Pg2A x Ur5E             | 243 (118, 125)      | 87               | 207 (85.19)    | 207 (100.00)   | 197 (95.17)      | 100 (50.76)            |
| Ur5E x Pg2A             | 264 (139, 125)      | 91               | 235 (89.02)    | 235 (100.00)   | 204 (86.81)      | 108 (52.94)            |
|                         |                     |                  |                |                |                  |                        |
| F1-hybrid cross         |                     |                  |                |                |                  |                        |
| [(Pg2A x Pg5B)F1 x (Pg2A x Pg5B)F1] | 308 (118, 190) | 85           | 246 (79.87)    | 234 (95.12)    | 229 (97.86)      | 111 (48.47)            |
| [(Pg5B x Pg2A)F1 x (Pg5B x Pg2A)F1] | 312 (186, 126) | 87           | 250 (80.81)    | 235 (94.00)    | 225 (95.74)      | 110 (48.89)            |
| [(Pg2A x Pg4C)F1 x (Pg2A x Pg4C)F1] | 308 (147, 161) | 92           | 271 (87.99)    | 268 (98.89)    | 257 (95.90)      | 135 (52.53)            |
| [(Pg4C x Pg2A)F1 x (Pg4C x Pg2A)F1] | 329 (194, 135) | 80           | 250 (75.99)    | 230 (92.00)    | 225 (97.83)      | 115 (51.11)            |
| [(Pg2A x Ur2D)F1 x (Pg2A x Ur2D)F1] | 347 (157, 190) | 90           | 295 (85.01)    | 289 (97.97)    | 265 (91.70)      | 141 (53.21)            |
| [(Ur2D x Pg2A)F1 x (Ur2D x Pg2A)F1] | 287 (125, 162) | 90           | 250 (87.11)    | 222 (88.80)    | 220 (99.10)      | 112 (50.91)            |
| [(Pg2A x Ur5E)F1 x (Pg2A x Ur5E)F1] | 350 (167, 183) | 88           | 288 (80.00)    | 272 (97.14)    | 266 (97.79)      | 126 (47.37)            |
| [(Ur5E x Pg2A)F1 x (Ur5E x Pg2A)F1] | 339 (194, 145) | 84           | 268 (79.06)    | 263 (98.13)    | 242 (92.02)      | 124 (51.24)            |

*a*Two selective egg batches of inseminated females from each cross.

*b*Dissection from 100 eggs; *n* = number.
HM488268, HM488272, and HM488273; Table 1), which were identified previously as the Hyrcanus Group, also were placed within the same clade of *An. nitidus* (Fig. 5).

**Discussion**

A cytogenetic investigation of *An. nitidus* in Thailand was documented first by Baimai et al. (1993a). The results indicated that this anopheline species exhibited genetic diversity at the chromosomal level via a gradual increase in the extra block(s) of constitutive heterochromatin in the X chromosome (X<sub>1</sub>, X<sub>2</sub>), whereas this event was not detected in the Y chromosomes, possibly due to the limited number of isolines used. Herein, the 21 *An. nitidus* isolines from two allopatric locations (Phang Nga Province, southern region; Ubon Ratchathani Province, northeastern region) in Thailand revealed three types of X (X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>) and five types of Y (Y<sub>1</sub>, Y<sub>2</sub>, Y<sub>3</sub>, Y<sub>4</sub>, Y<sub>5</sub>) chromosomes, which were designated as Form A (X<sub>1</sub>, Y<sub>1</sub>), Form B (X<sub>1</sub>, Y<sub>2</sub>), Form C (X<sub>2</sub>, Y<sub>3</sub>), Form D (X<sub>1</sub>, X<sub>2</sub>, Y<sub>4</sub>), and Form E (X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, Y<sub>5</sub>), depending upon the uniquely distinct characteristics of Y chromosomes. The five different karyotypic forms of *An. nitidus* found in this study were due clearly to the addition of extra block(s) of constitutive heterochromatin on sex chromosomes (X, Y), which is in keeping with Baimai’s (1998) hypothesis. Baimai et al. (1984a,b, 1988, 1993b) suggested that the quantitative differences in heterochromatin of mitotic chromosomes could be used as a genetic marker for further identification of cryptic (isomorphic) or closely related species, as exemplified in the population cytogenetic studies of the *Anopheles dirus* complex and the Maculatus Group. Interestingly, investigation of the 18 isolines from Ubon Ratchathani Province, northeastern region, revealed only two karyotypic forms (Form D: 10 isolines; Form E: 8 isolines), whereas that of the three isolines from Phang Nga Province, southern region, yielded three distinct karyotypic forms (Forms A, B, and C) in each isoline, even though these two allopatric locations were placed ~800 km apart. The climate of these two provinces is quite different, i.e., Ubon Ratchathani Province has a tropical wet and dry climate, whereas Phang Nga Province is located on the shore to the Andaman Sea, and has heavy rain. Our results are in accordance with Saeung et al. (2014).

![Fig. 4. Synapsis in all arms of salivary gland polytene chromosome of F<sub>1</sub>-hybrids fourth larvae of *An. nitidus*. (A) Pg2A female x Pg5B male; (B) Pg2A female x Pg4C male; (C) Pg2A female x Ur2D male; (D) Pg2A female x Ur5E male. Note: small common gap of homosequential asynapsis (arrow) was found on chromosome 2L, 2R, and 3R; 2L and 2R; and 3L from the crosses between Pg2A female x Pg5B male; Pg2A female x Pg4C male; and Pg2A female x Ur5E male, respectively.](image-url)
These authors showed that An. crawfordi Form A was detected only in Phang Nga Province, whereas Forms A, B, C, and D were found from eight isolines in Trang Province, which placed /C24 190 km apart. This phenomenon appeared to elucidate the difference in ecological diversity, which favored specific microhabitats for the karyotypic forms of An. nitidus. However, additional surveys are expected to obtain greater numbers of isolines from both provinces and/or other locations across six regions (northern, western, central, northeastern, eastern, and southern) of Thailand. This would bring about understanding of the population-genetic structure of this anopheline species.

Cross-mating experiments using anopheline isoline-colonies, relating to information on cytology and molecular analysis to determine postmating barriers, have been proven so far as an effective classical technique for recognizing sibling species and/or subspecies (cytological races) within Anopheles (Kanda et al. 1981; Baimai et al. 1987; Subbarao 1998; Junkum et al. 2005; Somboon et al. 2005; Saenng et al. 2007, 2008; Thongwat et al. 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009; Choochote 2011). Cross-mating experiments among the five karyotypic forms of An. nitidus showed no postmating reproductive isolation. They yielded viable progenies through F2 generations and synaptic salivary gland polytene chromosomes, along the entire length of autosomes and the X chromosome. Thus, our results indicated that the five karyotypic forms were conspecific. Quantitative changes in constitutive heterochromatin in mitotic chromosomes of An. nitidus observed in this study were likely intraspecific chromosomal variation, which may lead to interspecific difference in the process of speciation. Our results are agreed with previous cross-mating experiments among sympatric and/or allopatric karyotypic forms of other anopheline species, i.e., Anopheles 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), Anopheles aconitus (Junkum et al. 2005), Anopheles barbirostris A1 and A2 (Saenng et al. 2007, Suwannamit et al. 2009); Anopheles campestris-like (Thongsahuan et al. 2009), An. peditaeniatus (Choochote 2011, Saenng et al. 2012), and An. paraliae (Taai et al. 2013b).

Furthermore, this study incorporated a nuclear DNA and mtDNA sequence to increase the exact identification of this species from other species members of the Hyrcanus Group (Min et al. 2002; Park et al. 2003, 2008a; Choochote 2011; Taai et al. 2013a). The monophyletic
trees and very low intraspecific sequence variations (average genetic distances = 0.002–0.008) of the ITS2, COI, and COII of the five karyotypic forms are good supportive evidence, which confirms that these forms represent a single species of *An. nitidus*. It is interesting to note that the three specimens (TR2, TR3, and TR6) collected from Trat Province, eastern Thailand, and identified as the Hycranus Group by Paredes-Esquível et al. (2011), based on ITS2 sequences, were clustered together with five karyotypic forms of *An. nitidus*, and are presumed to belong to that species.

In conclusion, this is the first report to clarify the species status of five karyotypic variants of *An. nitidus* collected from two locations in Thailand by using multidisciplinary approaches (cytogenetic investigations, cross-mating experiments, and molecular analyses) and indicate that these forms are of the same species.

Acknowledgments

This work was supported by The Thailand Research Fund to W.C. and A.S. (TRF Senior Research Scholar: RTA5480006), the Royal Golden Jubilee Ph.D. Program to Wej Choochote (W.C.), Atiporn Saeung (A.S.) and Siriporn Songwatakit (S.S.) (PHD/0356/2552), and Faculty of Medicine Research Fund, Chiang Mai University, Chiang Mai, Thailand.

References Cited

Baimai, V. 1998. Heterochromatin accumulation and karyotypic evolution in some dipteran insects. Zool. Stud. 37: 75–88.

Baimai, V., R. G. Andre, and B. A. Harrison. 1984a. Heterochromatin variation in the sex chromosomes in Thailand populations of *Anopheles dirus* A (Diptera: Culicidae). Can. J. Genet. Cytol. 26: 633–636.

Baimai, V., C. A. Green, R. G. Andre, B. A. Harrison, and E. L. Peyton. 1984b. Cytogenetic studies of some species complexes of *Anopheles* in Thailand and Southeast Asia. Southeast Asian J. Trop. Med. Public Health 15: 536–546.

Baimai, V., R. E. Harbach, and U. Kijchalao. 1988. Cytogenetic evidence of the fifth species within the taxon *Anopheles dirus* (Diptera: Culicidae) in Thailand. J. Am. Mosq. Control Assoc. 4: 333–338.

Baimai, V., R. G. Andre, B. A. Harrison, U. Kijchalao, and L. Panthusiri. 1987. Crossing and chromosomal evidence for two additional sibling species within the taxon *Anopheles dirus* Peyton and Harrison (Diptera: Culicidae) in Thailand. Proc. Entomol. Soc. Wash. 89: 157–166.

Baimai, V., R. Rattanarithikul, and U. Kijchalao. 1993a. Metaphase karyotypes of *Anopheles* of Thailand and Southeast Asia I: The hycranus group. J. Am. Mosq. Control Assoc. 9: 59–67.

Baimai, V., R. Rattanarithikul, U. Kijchalao, and C. A. Green. 1993b. Metaphase karyotypes of *Anopheles* of Thailand and Southeast Asia. II. The maculatus group, Neocellia series, subgenus *Cellia*. Mosq. Syst. 25: 116–123.

Baker, E. Z., J. C. Beier, S. R. Meek, and R. A. Wirtz. 1987. Detection and quantification of *Plasmodium falciparum* and *P. vivax* infections in Thai Kampucheian *Anopheles* (Diptera: Culicidae) by enzyme linked immunosorbent assay. J. Med. Entomol. 24: 357–541.

Choochote, W. 2011. Evidence to support karyotypic variation of the mosquito, *Anopheles peditaeniatus* in Thailand. J. Insect Sci. 11: 10.

Choochote, W., and A. Saeung. 2013. Systematic techniques for the recognition of *Anopheles* species complexes, pp. 57–79. In S. Manguin (ed.), *Anopheles in Asia*. J. Am. Mosq. Control Assoc. 27: 1016–1026.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95–98.

Harbach, R. E. 2014. Mosquito taxonomic inventory. *Anopheles* classification. (http://mosquito-taxonomic-inventory.info/anophelesclassification) (accessed 1 June 2014).

Harbach, R. E., J. B. Gingrich, and L. W. Pang. 1987. Some entomological observations on malaria transmission in a remote village in northwestern Thailand. J. Am. Mosq. Control Assoc. 3: 296–301.

Harrison, B. A., and J. E. Scanlon. 1975. Medical entomology studies II. The subgenus *Anopheles* in Thailand (Diptera: Culicidae). Contrib. Am. Entomol. Inst. 12: 78.

Junkum, B. Tuetun, H. Takaoka, and W. Choochote. 2008. Reproductive isolation of *Anopheles sinensis* from *Anopheles leucosphyrus* group (Reid, 1968). Jpn. J. Sanit. Zool. 32: 321–329.

Kimura, M. 1980. Simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Min, G. S., W. Choochote, A. Jitpakdi, S. Kim, W. Kim, J. Jung, and A. Saeung. 2002. Intraspecific hybridization of *Anopheles sinensis* (Diptera: Culicidae) strains from Thailand and Korea. Mol. Cells 14: 198–204.

Nylander, J. A. A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

Paredes-Esquível, C., R. E. Harbach, and H. Townsend. 2011. Molecular taxonomy of members of the *Anopheles hycranus* group from Thailand and Indonesia. Med. Vet. Entomol. 25: 348–352.

Park, S. J., W. Choochote, A. Jitpakdi, A. Junkm, S. Kim, and N. Jariyapan. 2003. Evidence for a conspecific relationship between two morphologically and cytologically different forms of Korean *Anopheles pullus* mosquito. Mol. Cells 16: 354–360.

Park, M. H., W. Choochote, A. Junkm, D. Joshi, B. Tuetan, A. Saeung, J. H. Jung, and G. S. Min. 2008a. Reproductive isolation of *Anopheles sinensis* from *Anopheles leucosphyrus* and *Anopheles sinensis* in Korea. Genes Genomics 30: 245–252.

Park, M. H., W. Choochote, S. J. Kim, P. Somboon, A. Saeung, B. Tuetan, Y. Tsuda, M. Takagi, D. Joshi, Y. J. Ma, et al. 2008b. Nonreproductive isolation among four allopatric strains of *Anopheles sinensis* in Asia. J. Am. Mosq. Control Assoc. 24: 489–495.

Rattanarithikul, R., E. Konishi, and K. J. Linthicum. 1996. Detection of *Plasmodium vivax* and *Plasmodium falciparum* circumsporozoites antigen in anopheline mosquitoes collected in southern Thailand. Am. J. Trop. Med. Hyg. 54: 114–121.

Rattanarithikul, R., B. A. Harrison, R. E. Harbach, P. Panthusiri, and R. E. Coleman. 2006. Illustrated keys to the mosquitoes of Thailand IV. *Anopheles*. Southeast Asian J. Trop. Med. Public Health 37: 1–128.

Reid, J. A. 1968. Anopheline mosquitoes of Malaya and Borneo. Stud. Inst. Med. Res. Malaysia 31: 1–520.

Reid, J. A., T. Wilson, and A. Ganapathipillai. 1962. Studies on filariasis in Malaya: the mosquito vectors of periodic *Brugia malayi* in North-West Malaysia. Ann. Trop. Med. Parasitol. 56: 323–336.

Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Hueslenbeck. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61: 593–542.

Saeung, A., Y. Otsuka, V. Baimai, P. Somboon, B. Pitasawat, B. Tuetan, A. Junkm, H. Takaoka, and W. Choochote. 2007. Cytogenetic and molecular evidence for two species in the *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand. Parasitol. Res. 101: 1337–1344.

Saeung, A., V. Baimai, Y. Otsuka, R. Rattanarithikul, P. Somboon, A. Junkm, B. Tuetan, H. Takaoka, and W. Choochote. 2008. Molecular and cytogenetic evidence of three sibling species of the *Anopheles barbirostris* Form A (Diptera: Culicidae) in Thailand. Parasitol. Res. 102: 499–507.

Saeung, A., V. Baimai, S. Thongsahuan, Y. Otsuka, W. Srisuka, K. Taai, P. Somboon, W. Suwankerd, T. Sochanta, and W. Choochote. 2014. Cytogenetic, cross-mating and molecular evidence of four cytological races
of *Anopheles crawfordi* (Diptera: Culicidae) in Thailand and Cambodia. C. R. Biologies 337: 625–634.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Somboon, P., D. Thongwat, W. Choochote, C. Walton, and M. Takagi. 2005. Crossing experiments of *Anopheles minimus* species C and putative species E. J. Am. Mosq. Control Assoc. 21: 5–9.

Subbarao, S.K. 1998. Anopheline species complexes in South-East Asia. World Health Organ. Tech. Pub. Searo 18: 1–82.

Suwannamit, S., V. Baimai, Y. Otsuka, A. Saeung, S. Thongsahuan, B. Tuetun, C. Apiwathnasorn, N. Jariyapan, P. Somboon, H. Takaoka, et al. 2009. Cytogenetic and molecular evidence for an additional new species within the taxon *Anopheles barbirostris* (Diptera: Culicidae) in Thailand. Parasitol. Res. 104: 905–918.

Taaí, K., V. Baimai, A. Saeung, S. Thongsahuan, G. S. Min, Y. Otsuka, M. H. Park, M. Fukuda, P. Somboon, and W. Choochote. 2013a. Genetic compatibility between *Anopheles lesteri* from Korea and *Anopheles paralae* from Thailand. Memórias do Instituto Oswaldo Cruz 108: 312–320.

Taaí, K., V. Baimai, S. Thongsahuan, A. Saeung, Y. Otsuka, W. Srisuka, P. Sripichai, P. Somboon, N. Jariyapan, and W. Choochote. 2013b. Metaphase karyotypes of *Anopheles paralae* (Diptera: Culicidae) in Thailand and evidence to support five cytological races. Trop. Biomed. 30: 238–249.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Thongsahuan, S., V. Baimai, Y. Otsuka, A. Saeung, B. Tuetun, N. Jariyapan, S. Suwannamit, P. Somboon, A. Jitpakdi, H. Takaoka, et al. 2009. Karyotypic variation and geographic distribution of *Anopheles campestris*-like (Diptera: Culicidae) in Thailand. Memórias do Instituto Oswaldo Cruz 104: 558–566.

Thongwat, D., K. Morgan, M. S. O’loughlin, C. Walton, W. Choochote, P. Somboon. 2008. Crossing experiment supporting the specific status of *Anopheles maculatus* chromosomal form K. J. Am. Mosq. Control Assoc. 24: 194–202.

Received 3 February 2013; accepted 5 September 2014.