C. R. Biologies 337 (2014) 625–634

Contents lists available at ScienceDirect

Comptes Rendus Biologies

www.sciencedirect.com

Molecular biology and genetics/Biologie et génétique moléculaires

Cytogenetic, cross-mating and molecular evidence of four cytological races of *Anopheles crawfordi* (Diptera: Culicidae) in Thailand and Cambodia

Atiporn Saeung [a,*], Visut Baimai [b], Sorawat Thongsahuan [c], Yasushi Otsuka [d], Wichai Srisuka [e], Kritsana Taai [a], Pradya Somboon [a], Wannapa Suwonkerd [f], Tho Sochanta [g], Wej Choochote [a]

[a] *Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*
[b] *Department of Biology and Centre for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[c] *Faculty of Veterinary Science (Establishment Project), Prince of Songkla University, Songkhla 90110, Thailand*
[d] *Research Center for the Pacific Islands, Kagoshima University, Kagoshima 890-8580, Japan*
[e] *Entomology Section, Queen Sirikit Botanic Garden, P.O. Box 7, Chiang Mai 50180, Thailand*
[f] *Office of Disease Prevention and Control No. 10, Department of Disease Control, Ministry of Public Health, Chiang Mai 50200, Thailand*
[g] *National Center for Malaria Control, Parasitology and Entomology, Phnom Penh 12302, Cambodia*

ARTICLE INFO

Article history:
Received 18 February 2013
Accepted after revision 1 August 2014
Available online 23 September 2014

Keywords:
Anopheles crawfordi
Metaphase karyotypes
Cross-mating experiments
ITS2
COI
COII

ABSTRACT

Twenty-nine isolines of *Anopheles crawfordi* were established from wild-caught females collected from cow-baited traps in Thailand and Cambodia. Three types of X (X_1, X_2, X_3) and four types of Y (Y_1, Y_2, Y_3, and Y_4) chromosomes were identified, according to differing amounts of extra heterochromatin. These sex chromosomes represent four metaphase karyotypes, i.e., Forms A (X_1, X_2, X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_2, Y_3) and D (X_2, Y_4). Forms C and D are novel metaphase karyotypes confined to Thailand, whereas forms A and B appear to be common in both Thailand and Cambodia. Cross-mating experiments between the four karyotypic forms indicated genetic compatibility in yielding viable progenies and synaptic salivary gland polytene chromosomes. The results suggest that the forms are conspecific and *A. crawfordi* comprises four cytological races, which is further supported by very low intraspecific variation (mean genetic distance = 0.000–0.018) of the nucleotide sequences in ribosomal DNA (ITS2) and mitochondrial DNA sequences (COI, COII).

1. Introduction

Anopheles crawfordi Reid, 1953 belongs to the Lesteri Subgroup of the Hyrcanus Group within the Myzorhynchus Series of the subgenus *Anopheles* [1]. So far, the distribution of this anopheline species has been recorded from India (Assam), Thailand, Cambodia, Vietnam, peninsular Malaysia and Indonesia (Sumatra) [2,3]. Even though *A. crawfordi* could be found abundantly as a proven outdoor-biter of humans in certain localities of eastern and southern Thailand, its status as a vector of pathogens of human diseases remains obscure and needs to be investigated more intensively [2]. However, our recent experiments indicate that this anopheline species could be an important vector of the filarial nematode, nocturnally subperiodic *Brugia malayi*, as determined by 80–85% susceptibility rates and an average of six L_3 larvae per infected mosquito [4]. These results were in agreement

* Corresponding author. Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail addresses: atsaeung@mail.med.cmu.ac.th, atiporn44@yahoo.com (A. Saeung).

http://dx.doi.org/10.1016/j.crvi.2014.08.001
1631-0691/

with previous investigation indicating that *A. crawfordi* could provide satisfactory susceptibility to *B. malayi* in Malaysia [5,6]. Additionally, *A. crawfordi* is considered an economic pest due to its vicious biting-behavior of cattle [1,2,5].

Cytogenetic investigations of *A. crawfordi* from two different localities in Thailand (eastern region: Chanthaburi Province; southern region: Phang Nga Province) were performed by Baimai et al. [7]. The results of their studies demonstrated that *A. crawfordi* exhibits genetic diversity at the chromosomal level, via a gradual increase in extra heterochromatin on the X and Y chromosomes. Two karyotypic variants (cytological forms), namely forms A (X_1, Y_1) and B (X_2, Y_2), were identified. The marked genetic variation of the X and Y chromosomes, as in other species of *Anopheles*, may indicate the existence of a species complex. The identical morphology or minimal morphological distinction among sibling species (isomorphic species) and subspecies (cytological races) within species complexes leads to difficulty in reliably identifying individual sibling species, which may differ in biological characteristics (e.g., microhabitats, resting and biting behaviors, sensitivity or resistance to insecticides, susceptibility or refractory character to pathogens, etc.) that may influence their vectorial capacity. Thus, inaccurate identification of individual members within a species complex may result in the failure to distinguish vector and non-vector species, and complicate vector control [8]. Although marked genetic variation at the chromosomal level of *A. crawfordi* has been observed, little is known about the genetics of chromosomal forms. This paper reports on the existence of two additional karyotypic forms of *A. crawfordi* and the results of cross-mating between the four karyotypic forms and comparisons of sequences for the second internal transcribed spacer (ITS2) of rDNA, and cytochrome *c* oxidase subunits I (COI) and II (COII) of mtDNA.

2. Materials and methods

2.1. Field collections and establishment of isoline colonies

Wild-caught, fully engorged females of *A. crawfordi* were collected from cow-baited traps at six locations in Thailand (Chiang Mai and Nan Provinces, northern region; Chumphon, Phang Nga, Trang and Songkhla Provinces, southern region), and two locations in Cambodia (Ratanakiri and Mondulkiri) (Fig. 1, Table 1).

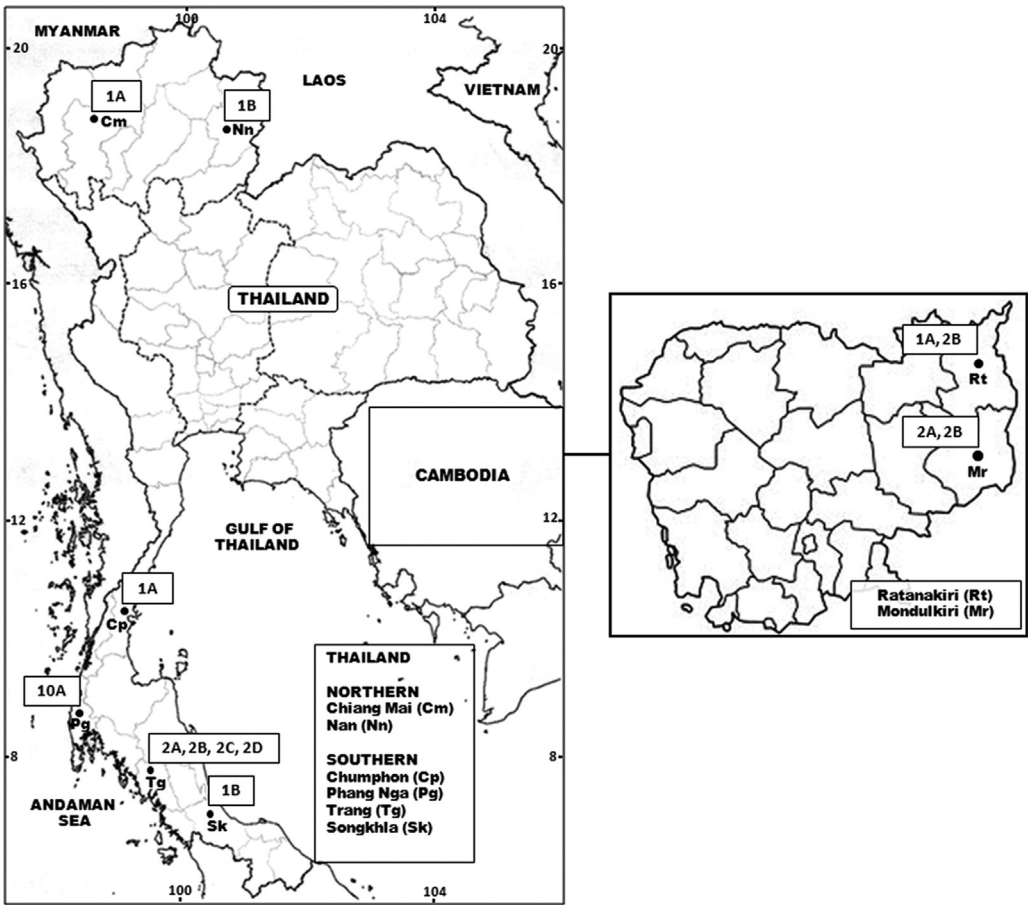

Fig. 1. Maps of Thailand and Cambodia showing eight provinces where specimens of *A. crawfordi* were collected and the number of isolines of the four karyotypic forms (A–D) detected in each location.

Table 1
Isolines of four karyotypic forms (A–D) of *A. crawfordi* and their GenBank accession numbers.

Location (geographical coordinate)	Code of isoline[a]	Karyotypic form	GenBank accession number			Reference
			ITS2	COI	COII	
Thailand						
Chiang Mai (18° 47′ N, 98° 59′ E)	Cm1A[a]	A (X_1, Y_1)	AB779131	AB779160	AB779189	This study
Nan (19° 21′ N, 100° 39′ E)	Nn1B[a]	B (X_1, Y_2)	AB779132	AB779161	AB779190	This study
Chumphon (10° 29′ N, 99° 11′ E)	Cp1A	A (X_3, Y_1)	AB779133	AB779162	AB779191	This study
Trang (07° 33′ N, 99° 38′ E)	Tg1B[a]	B (X_3, Y_2)	AB779134	AB779163	AB779192	This study
	Tg2C[a]	C (X_2, Y_3)	AB779135	AB779164	AB779193	This study
	Tg3A[a]	A (X_3, Y_1)	AB779136	AB779165	AB779194	This study
	Tg4D[a]	D (X_2, Y_4)	AB779137	AB779166	AB779195	This study
	Tg6B	B (X_2, Y_2)	AB779138	AB779167	AB779196	This study
	Tg8D	D (X_2, Y_4)	AB779139	AB779168	AB779197	This study
	Tg11A	A (X_2, Y_1)	AB779140	AB779169	AB779198	This study
	Tg12C	C (X_2, Y_3)	AB779141	AB779170	AB779199	This study
Phang Nga (08° 27′ N, 98° 31′ E)	Pg4A	A (X_1, Y_1)	AB779142	AB779171	AB779200	This study
	Pg5A[a]	A (X_2, Y_1)	AB779143	AB779172	AB779201	This study
	Pg6A	A (X_1, Y_1)	AB779144	AB779173	AB779202	This study
	Pg7A	A (X_2, Y_1)	AB779145	AB779174	AB779203	This study
	Pg8A	A (X_2, Y_1)	AB779146	AB779175	AB779204	This study
	Pg9A	A (X_2, Y_1)	AB779147	AB779176	AB779205	This study
	Pg11A	A (X_2, Y_1)	AB779148	AB779177	AB779206	This study
	Pg12A	A (X_1, Y_1)	AB779149	AB779178	AB779207	This study
	Pg14A	A (X_1, Y_1)	AB779150	AB779179	AB779208	This study
	Pg16A	A (X_2, Y_1)	AB779151	AB779180	AB779209	This study
Songkhla (07° 13′ N, 100° 37′ E)	Sk1B[a]	B (X_3, Y_2)	AB779152	AB779181	AB779210	This study
Cambodia						
Ratanakiri (13° 44′ N, 107° 0′ E)	Rt1A[a]	A (X_1, Y_1)	AB779153	AB779182	AB779211	This study
	Rt2B	B (X_2, Y_2)	AB779154	AB779183	AB779212	This study
	Rt3B	B (X_2, Y_2)	AB779155	AB779184	AB779213	This study
Mondulkiri (12° 27′ N, 107° 14′ E)	Mr1B[a]	B (X_2, Y_2)	AB779156	AB779185	AB779214	This study
	Mr2A	A (X_2, Y_1)	AB779157	AB779186	AB779215	This study
	Mr3A	A (X_1, Y_1)	AB779158	AB779187	AB779216	This study
	Mr4B	A (X_2, Y_2)	AB779159	AB779188	AB779217	This study
Vietnam						
	3.6	–	–	KF431868	KF431892	Ngo et al. [49]
	269	–	–	KF431873	KF431896	Ngo et al. [49]
	286	–	–	KF431865	KF431889	Ngo et al. [49]
	302	–	–	KF431881	KF431902	Ngo et al. [49]
A. belenrae	–	–	EU789794	–	–	Park et al. [38]
A. kleini	–	–	EU789793	–	–	Park et al. [38]
A. lesteri	–	–	EU789791	–	–	Park et al. [38]
	ilG1	–	–	AB733028	AB733036	Taai et al. [50]
A. paraliae	Sk1B	B (X_1, Y_2)	AB733487	AB733503	AB733519	Taai et al. [40]
A. peditaeniatus	RbB	B (X_3, Y_2)	AB539061	AB539069	AB539077	Choochote [31]
A. pullus	–	–	EU789792	–	–	Park et al. [38]
	–	–	–	AY444348	AY444347	Park et al. [35]
A. sinensis	i2ACM	A (X, Y_1)	AY130473	–	–	Min et al. [37]
	–	–	–	AY444351	–	Park et al. [35]
	i1BKR	B (X, Y_2)	–	–	AY130464	Min et al. [37]

[a] Used in crossing experiments.

A total of 29 isolines were established and maintained using the techniques described by Choochote and Saeung [9]. The isolines were identified as *A. crawfordi* based on the morphology of the egg, larval, pupal, and adult stages of F_1 progenies, using available keys [2,3,10]. The isolines were used for studies of the metaphase karyotype, cross-mating experiments and molecular analyses.

2.2. Metaphase karyotype preparation

Metaphase chromosomes were prepared from 10 early fourth-instar larval brains of F_1 progenies of each isoline, using techniques previously described by Saeung et al. [11]. Identification of karyotypic forms followed the standard cytotaxonomic systems of Baimai et al. [7].

2.3. Cross-mating experiments

The ten laboratory-raised isolines of *A. crawfordi* were selected arbitrarily from the 29 isoline colonies, which were representative of four karyotypic forms, i.e., form A [Cm1A (X_1, Y_1), Tg3A (X_3, Y_1), Pg5A (X_2, Y_1), Rt1A (X_1, Y_1)], form B [Nn1B (X_1, Y_2), Tg1B (X_3, Y_2), Sk1B (X_3, Y_2), Mr1B (X_2, Y_2)], form C [Tg2C (X_2, Y_3)], and form D [Tg4D (X_2, Y_4)] (Table 1). These isolines were used for cross-mating experiments to determine post-mating barriers by employing the techniques previously reported by Saeung et al. [11].

2.4. DNA extraction and PCR amplification

Total genomic DNA was isolated from individual F_1 progeny adult female of each isoline (Table 1) using the DNeasy® Blood and Tissue Kit (QIAGEN). Primers for amplification of the ITS2, COI, and COII regions followed previous studies by Saeung et al. [11]. The ITS2 region of rDNA was amplified using primers ITS2A (5′-TGT GAA CTG CAG GAC ACA T-3′) and ITS2B (5′-TAT GCT TAA ATT CAGGGGGT-3′) [12]. The 709-bp fragment of the mitochondrial COI barcoding region was amplified using the LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) primers of Folmer et al. [13]. The mitochondrial COII region was amplified using primers LEU (5′-TCT AAT ATG GCA GAT TAG TGC A-3′) and LYS (5′-ACT TGC TTT CAG TCA TCT AAT G-3′) [14]. Each PCR reaction was carried out in 20 μL containing 0.5 U *Ex Taq* (Takara), 1X *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer, and 1 μL of the extracted DNA. For ITS2, PCR consisted of initial denaturation at 94 °C for 1 min,

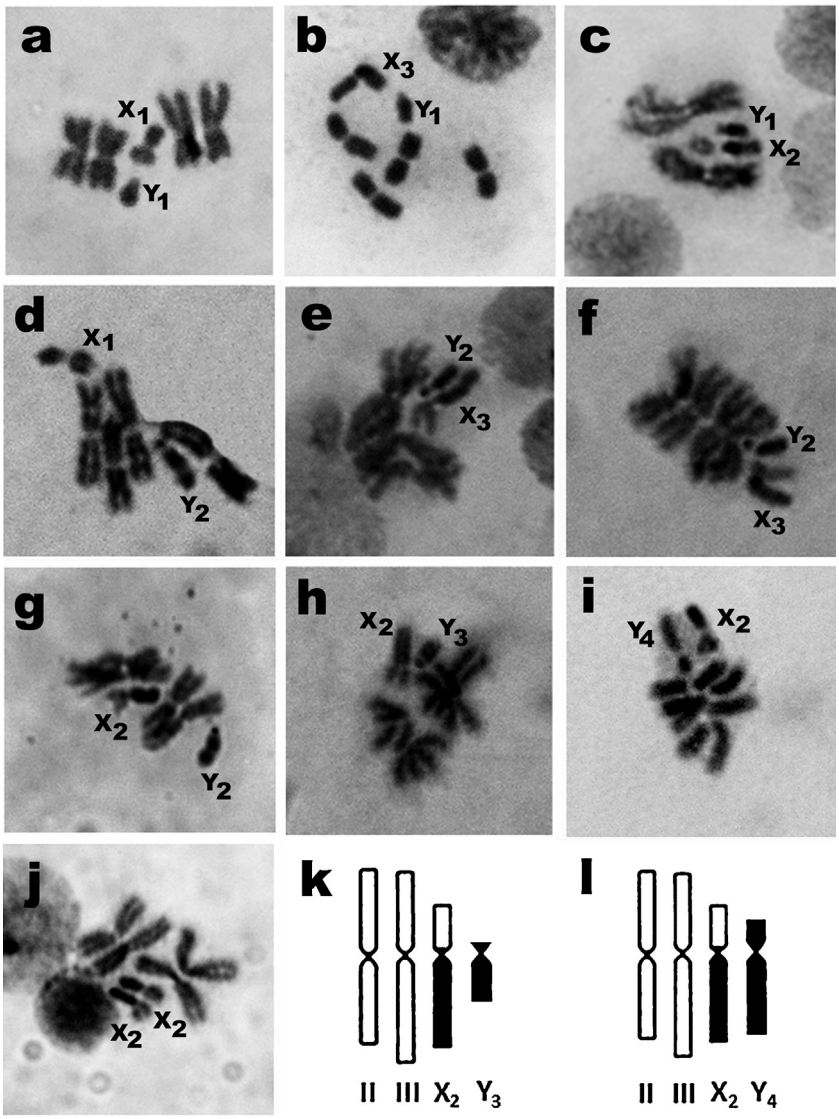

Fig. 2. Metaphase karyotypes of *A. crawfordi*: a: form A (X_1, Y_1: Chiang Mai); b: form A (X_3, Y_1: Chumphon); c: form A (X_2, Y_1: Trang); d: form B (X_1, Y_2: Nan);

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% agarose gels and stained with ethidium bromide. Finally, the amplicons were purified using the QIAquick® PCR Purification Kit (QIAGEN). The PCR products were sequenced in both directions using the BigDye® V3.1 Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems).

2.5. Sequencing alignment and phylogenetic analysis

Sequences were aligned using the CLUSTAL W multiple alignment program [15] and edited manually in BioEdit version 7.0.5.3 [16]. All positions containing gaps and missing data were excluded from the analysis. The Kimura two-parameter (K2P) model was employed to calculate

Table 2
Cross-mating experiments of 10 isolines of *A. crawfordi*.

Crosses (female × male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched *n* (%)	Pupation *n* (%)	Emergence *n* (%)	Total emergence *n* (%)	
						Female	Male
Parental cross							
Cm1A × Cm1A	309 (179, 130)	90	272 (88.03)	258 (94.85)	248 (96.12)	111 (44.76)	137 (55.24)
Nn1B × Nn1B	251 (141, 110)	87	208 (82.87)	200 (96.15)	200 (100.00)	88 (44.00)	112 (56.00)
Tg3A × Tg3A	395 (166, 229)	92	348 (88.10)	317 (91.09)	311 (98.11)	162 (52.09)	149 (47.91)
Tg1B × Tg1B	413 (234, 179)	79	326 (78.93)	293 (89.88)	287 (97.95)	149 (51.92)	138 (48.08)
Tg2C × Tg2C	314 (200, 114)	85	264 (84.08)	259 (98.11)	256 (98.84)	111 (43.36)	145 (56.64)
Tg4D × Tg4D	228 (123, 105)	83	185 (81.14)	183 (98.92)	183 (100.00)	93 (50.82)	90 (49.18)
Pg5A × Pg5A	326 (146, 180)	88	284 (87.12)	281 (98.94)	278 (98.93)	138 (49.64)	140 (50.36)
Sk1B × Sk1B	269 (103, 166)	97	261 (97.03)	256 (98.08)	251 (98.05)	118 (47.01)	133 (52.99)
Rt1A × Rt1A	254 (156, 98)	93	236 (92.91)	231 (97.88)	229 (99.13)	127 (55.46)	102 (44.54)
Mr1B × Mr1B	269 (175, 94)	88	237 (88.10)	232 (97.89)	230 (99.14)	112 (48.70)	118 (51.30)
Reciprocal cross							
Cm1A × Nn1B	360 (217, 143)	80	284 (78.89)	281 (98.94)	281 (100.00)	132 (46.98)	149 (53.02)
Nn1B × Cm1A	283 (105, 178)	93	252 (89.05)	252 (100.00)	252 (100.00)	111 (44.05)	141 (55.95)
Cm1A × Tg3A	232 (146, 86)	94	204 (87.93)	200 (98.04)	196 (98.00)	114 (58.16)	82 (41.84)
Tg3A × Cm1A	258 (129, 129)	92	235 (91.09)	230 (97.87)	228 (99.13)	108 (47.37)	120 (52.63)
Cm1A × Tg1B	269 (151, 118)	90	221 (82.16)	217 (98.19)	213 (98.16)	96 (45.07)	117 (54.93)
Tg1B × Cm1A	278 (113, 165)	93	256 (92.09)	246 (96.09)	239 (97.15)	126 (52.72)	113 (47.28)
Cm1A × Tg2C	320 (134, 186)	95	282 (88.13)	282 (100.00)	282 (100.00)	149 (52.84)	133 (47.16)
Tg2C × Cm1A	337 (179, 158)	96	313 (92.88)	285 (91.05)	242 (84.91)	117 (48.35)	125 (51.65)
Cm1A × Tg4D	280 (120, 160)	90	252 (90.00)	232 (92.06)	230 (99.14)	112 (48.70)	118 (51.30)
Tg4D × Cm1A	282 (113, 169)	88	240 (85.11)	200 (83.33)	196 (98.00)	102 (52.04)	94 (47.96)
Cm1A × Pg5A	255 (138, 117)	95	242 (94.90)	242 (100.00)	230 (95.04)	97 (42.17)	133 (57.83)
Pg5A × Cm1A	260 (160, 100)	96	247 (95.00)	232 (93.93)	216 (93.10)	111 (51.39)	105 (48.61)
Cm1A × Sk1B	296 (170,126)	95	281 (94.93)	281 (100.00)	275 (97.86)	138 (50.18)	137 (49.82)
Sk1B × Cm1A	333 (160, 173)	90	290 (87.09)	258 (88.97)	201 (77.91)	104 (51.74)	97 (48.26)
Cm1A × Rt1A	263 (145, 118)	94	247 (93.92)	230 (93.12)	230 (100.00)	121 (52.61)	109 (47.39)
Rt1A × Cm1A	277 (163, 114)	92	255 (92.06)	247 (96.86)	230 (93.12)	118 (51.30)	112 (48.70)
Cm1A × Mr1B	287 (109, 178)	87	227 (79.09)	209 (92.07)	209 (100.00)	102 (48.80)	107 (51.20)
Mr1B × Cm1A	308 (194, 114)	78	234 (75.97)	234 (100.00)	234 (100.00)	113 (48.29)	121 (51.71)
F_1-hybrid cross							
(Cm1A × Nn1B)F_1 × (Cm1A × Nn1B)F_1	320 (136, 184)	86	243 (75.94)	221 (90.95)	221 (100.00)	104 (47.06)	117 (52.94)
(Nn1B × Cm1A)F_1x (Nn1B × Cm1A)F_1	357 (168, 189)	91	300 (84.03)	267 (89.00)	267 (100.00)	134 (50.19)	133 (49.81)
(Cm1A × Tg3A)F_1 × (Cm1A × Tg3A)F_1	296 (169, 127)	80	216 (72.97)	216 (100.00)	207 (95.83)	101 (48.79)	106 (51.21)
(Tg3A xCm1A)F_1 × (Tg3A × Cm1A)F_1	325 (126, 199)	87	260 (80.00)	257 (98.85)	257 (100.00)	131 (50.97)	126 (49.03)
(Cm1A × Tg1B)F_1 × (Cm1A × Tg1B)F_1	235 (108, 127)	91	207 (88.09)	207 (100.00)	205 (99.03)	86 (41.95)	119 (58.05)
(Tg1B × Cm1A)F_1 × (Tg1B × Cm1A)F_1	252 (145, 107)	84	171 (67.86)	169 (98.83)	166 (98.22)	86 (51.81)	80 (48.19)
(Cm1A × Tg2C)F_1 × (Cm1A × Tg2C)F_1	318 (131, 187)	83	261 (82.08)	261 (100.00)	253 (96.93)	121 (47.83)	132 (52.17)
(Tg2C × Cm1A)F_1 × (Tg2C × Cm1A)F_1	354 (164, 190)	85	290 (81.92)	287 (98.97)	276 (96.17)	132 (47.83)	144 (52.17)
(Cm1A × Tg4D)F_1 × (Cm1A × Tg4D)F_1	263 (188, 75)	80	200 (76.05)	182 (91.00)	180 (98.90)	86 (47.78)	94 (52.22)
(Tg4D × Cm1A)F_1 × (Tg4D × Cm1A)F_1	250 (150, 100)	97	212 (84.80)	212 (100.00)	210 (99.06)	116 (55.24)	94 (44.76)
(Cm1A × Pg5A)F_1 × (Cm1A × Pg5A)F_1	265 (126, 139)	91	230 (86.79)	230 (100.00)	225 (97.83)	106 (47.11)	119 (52.89)
(Pg5A × Cm1A)F_1 × (Pg5A × Cm1A)F_1	250 (102, 148)	88	195 (78.00)	183 (93.85)	172 (93.99)	86 (50.00)	86 (50.00)
(Cm1A × Sk1B)F_1 × (Cm1A × Sk1B)F_1	336 (136, 200)	85	269 (80.06)	269 (100.00)	269 (100.00)	110 (40.89)	159 (59.11)
(Sk1B × Cm1A)F_1 × (Sk1B × Cm1A)F_1	320 (162, 158)	92	269 (84.06)	269 (100.00)	269 (100.00)	134 (49.81)	135 (50.19)
(Cm1A × Rt1A)F_1 × (Cm1A × Rt1A)F_1	227 (148, 79)	84	154 (67.84)	140 (90.91)	137 (97.86)	66 (48.18)	71 (51.82)
(Rt1A × Cm1A)F_1 × (Rt1A × Cm1A)F_1	235 (108, 127)	97	218 (92.77)	218 (100.00)	218 (100.00)	116 (53.21)	102 (46.79)
(Cm1A × Mr1B)F_1 × (Cm1A × Mr1B)F_1	268 (159, 109)	79	204 (76.12)	204 (100.00)	204 (100.00)	102 (50.00)	102 (50.00)
(Mr1B × Cm1A)F_1 × (Mr1B × Cm1A)F_1	245 (100, 145)	65	152 (62.04)	150 (98.68)	147 (98.00)	69 (46.94)	78 (53.06)

n: number.
[a] Two selective egg-batches of inseminated females from each cross.
[b] Dissection from 100 eggs.

genetic distances [17]. Using the distances, construction of neighbor-joining trees [18] and the bootstrap test with 1000 replications were performed with Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 [19]. Bayesian analysis was conducted with MrBayes 3.2 [20] by using two replicates of 1 million generations with the nucleotide evolutionary model. The best-fit model was chosen for each gene separately using the Akaike Information Criterion (AIC) in Mr Model test version 2.3 [21]. The general time-reversible (GTR) with gamma distribution shape parameter (G) was selected for ITS2, whereas the GTR + I + G was the best-fit model for COI and COII. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% trees as burn-in. Available sequences of the Hyrcanus Group were retrieved from GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for performing the phylogenetic analysis with our sequences (Table 1).

3. Results

3.1. Metaphase karyotypes

Cytological observations of F_1 progenies of 29 isolines of *A. crawfordi* demonstrated distinct types of sex chromosomes due to the addition of extra heterochromatin. There were three types of X (metacentric X_1, submetacentric X_2, and large submetacentric X_3) and four types of Y chromosomes (small telocentric Y_1, large subtelocentric Y_2, small subtelocentric Y_3, and submetacentric Y_4) (Fig. 2). These types of X and Y chromosomes comprised four forms of metaphase karyotypes on the basis of Y chromosome configurations, designated as forms A (X_1, X_2, X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_2,Y_3), and D (X_2, Y_4). The number of isolines of these karyotypic forms occurring in different localities in six and two provinces of Thailand and Cambodia, respectively, are illustrated in Fig. 1 and Table 1. Forms C and D are new metaphase karyotypes discovered in the present study. Forms A and B appeared to be common in both Thailand and Cambodia, whereas forms C and D were confined to Trang Province, southern Thailand.

3.2. Cross-mating experiments

Details of hatchability, pupation, emergence and adult sex ratio of parental, reciprocal and F_1-hybrid crosses among the 10 isolines of *A. crawfordi* representing forms A–D are shown in Table 2. All crosses yielded viable progeny through the F_2 generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses. The salivary gland polytene chromosomes of the fourth-instar larvae of F_1-hybrids from all crosses showed synapsis without inversion loops along the whole length of all autosomes and the X chromosome (Fig. 3).

3.3. DNA sequences and phylogenetic analysis

All sequences generated from the 29 isolines are available in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB779131-AB779217 (Table 1). The length of the ITS2 region ranged from 446 to 449 bp in seven and 22 isolines from Cambodia and Thailand, respectively. *A. crawfordi* from both provinces of Cambodia differed from *A. crawfordi* in Thailand by a deletion of T, C, and T at positions 21, 280, and 292, respectively. However, they all showed the same length for COI (658 bp, excluding primers) and COII (685 bp) sequences. The evolutionary relationships among the four karyotypic forms were determined using neighbor-joining (NJ) and Bayesian analysis. Both phylogenetic methods showed similar tree topologies, thus, only the Bayesian

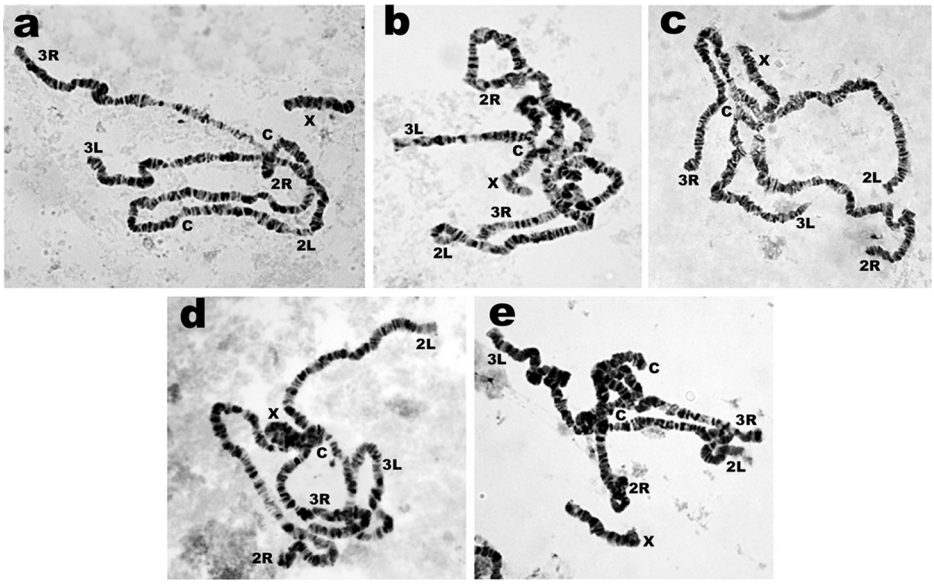

Fig. 3. Complete synapsis in all arms of salivary gland polytene chromosome of F_1-hybrid larvae of *A. crawfordi*: a: Cm1A female × Sk1B male; b: Cm1A female × Tg2 C male; c: Cm1A female × Tg4D male; d: Cm1A female × Rt1A male; e: Cm1A female × Mr1B male.

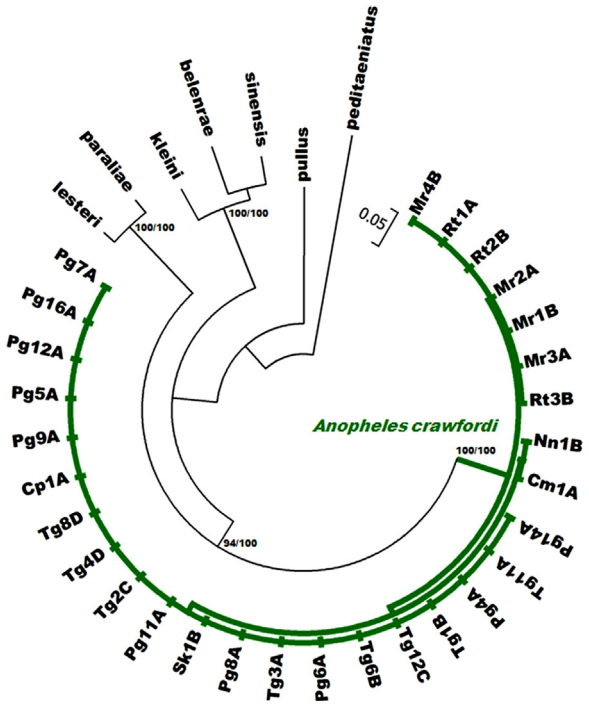

Fig. 4. Phylogenetic relationships of *A. crawfordi* from Thailand and Cambodia using Bayesian analysis based on ITS2 sequences compared with seven species of the Hyrcanus Group. Codes for the specimens are shown in Table 1. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 50% are shown. Bars represent 0.05 substitutions per site.

tree is shown in Figs. 4–6. All 29 isolines were placed within the same cluster and well separated from other species of the *Anopheles hyrcanus* group (*Anopheles belenrae*, *Anopheles kleini*, *Anopheles lesteri*, *Anopheles paraliae*, *Anopheles peditaeniatus*, *Anopheles pullus* and *Anopheles sinensis*). The mean intra-specific sequence divergences within (0.000–0.018) and between (0.000–0.016) the four karyotypic forms are not significantly different for the DNA regions (Table 3). In addition, COI and COII sequences of *A. crawfordi* from Vietnam (Table 1) formed the clade with our sequences with high support (NJ = 82–100%, BPP = 100%, Figs. 5–6). The low mean

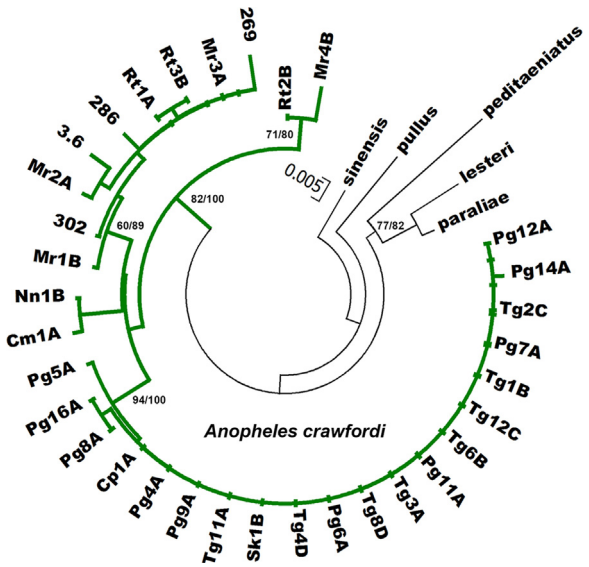

Fig. 5. Phylogenetic relationships of *A. crawfordi* from Thailand, Cambodia, and Vietnam using Bayesian analysis based on COI sequences compared with five species of the Hyrcanus Group. Codes for the specimens are shown in Table 1. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 50% are shown. Bars represent 0.005 substitutions per site.

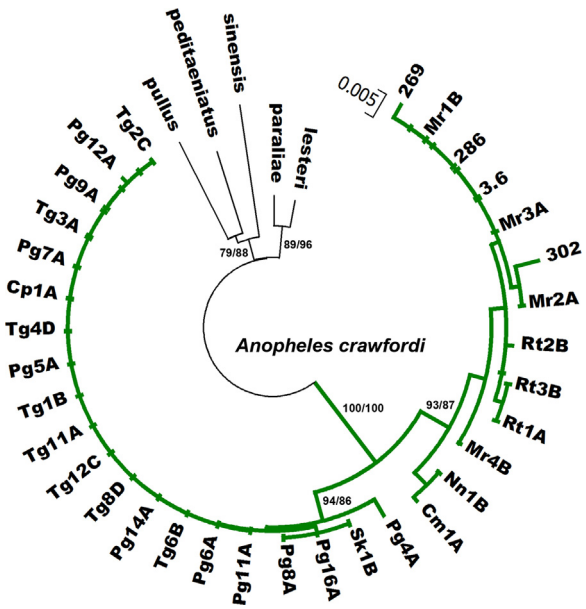

Fig. 6. Phylogenetic relationships of *A. crawfordi* from Thailand, Cambodia, and Vietnam using Bayesian analysis based on COII sequences compared with five species of the Hyrcanus Group. Codes for the specimens are shown in Table 1. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 50% are shown. Bars represent 0.005 substitutions per site.

genetic distance among specimens examined for COI (0.017) and COII (0.011) genes were good supportive evidence of a single species.

4. Discussion

Metaphase karyotypes of *A. crawfordi* from two different locations (eastern region, Chanthaburi Province; southern region, Phang Nga Province) in Thailand were investigated by Baimai et al. [7]. The results revealed karyotypic variation via a gradual increase of extra heterochromatin on the X (X_1, X_2) and Y (Y_1, Y_2) chromosomes, which gave rise to two karyotypic forms [forms A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2)]. These metaphase karyotypes could be distinguished based on size, shape, amount, and distribution of constitutive heterochromatin on the sex chromosomes. Likewise, the four distinct karyotypic forms [forms A (X_1, X_2, X_3, Y_1), B (X_1, X_2, X_3, Y_2), C (X_2, Y_3), and D (X_2, Y_4)] of *A. crawfordi* detected among the 29 isolines from six and two locations in Thailand and Cambodia, respectively, were due to the addition of extra heterochromatin on the sex chromosomes. Obviously, the above information indicated the possibility of a cytological mechanism for the karyotypic evolution of the Oriental *Anopheles* by gradually adding extra heterochromatin onto the arms of sex chromosomes, which is in keeping with hypothesis of Baimai [22]. Additionally, such chromosome distinction is very useful for the cytotaxonomic study of closely related species, especially sibling species and/or subspecies of *Anopheles*, as exemplified in other groups of Oriental anophelines [8,11,23–32].

Regarding the distribution of the four karyotypic forms of *A. crawfordi*, forms A and B appear to be common in all locations of both Thailand and Cambodia, whereas forms C and D are confined to Trang Province, southern Thailand. Remarkably, form A (10 isolines) was detected only in Phang Nga Province, whereas all karyotypic forms were obtained from eight isolines in Trang Province, despite these two provinces being separated by approximately 190 km. This is the first substantial evidence that supports the richness of ecological diversity in Trang Province, which seems to be the main key for supporting specific microhabitats that favor the karyotypic evolution of *A. crawfordi*.

Cross-mating experiments using isoline colonies of anopheline mosquitoes, which relate to results of cytology and molecular analysis to determine post-mating barriers, have proven to be an efficient technique for identifying sibling species and/or subspecies within *Anopheles* [8,11,23–32]. Regarding this matter, cross-matings among the four allopatric karyotypic forms of *A. crawfordi* were performed intensively. The absence of post-mating

Table 3
Mean intra-specific divergence of ITS2, COI and COII sequences of *A. crawfordi* Forms A, B, C and D from Thailand and Cambodia obtained using the Kimura two-parameter (K2P) model.

	ITS2	COI	COII
Within form			
A	0.009	0.010	0.008
B	0.014	0.018	0.012
C	0.000	0.000	0.000
D	0.000	0.000	0.000
Between forms			
A–B	0.014	0.016	0.011
A–C	0.005	0.006	0.005
A–D	0.005	0.006	0.005
B–C	0.014	0.015	0.011
B–D	0.014	0.015	0.011
C–D	0.000	0.000	0.000

reproductive isolation through F_2 generations strongly suggests that the four cytological races are conspecific. Low intra-specific sequence divergence (mean genetic distance = 0.000–0.018) of ITS2, COI, and COII of the four forms provides good supportive evidence. The maximum intra-species K2P values based on COI barcoding sequences obtained from this study were similar to that reported for *Anopheles pallidus* (0.0184) [33]. Kumar et al. [33] denoted that the K2P values were > 0.02 between different species for Culicidae. Our findings are in agreement with the results of cross-matings among karyotypic forms of other anophelines previously reported by several investigators, i.e., *Anopheles vagus* [34], *A. pullus* (= *Anopheles yatsushiroensis*) [35], *A. sinensis* [36–39], *Anopheles aconitus* [25], *Anopheles barbirostris* A1 and A2 [11,29], *Anopheles campestris*-like [30], *Anopheles peditaeniatus* [31,32], and *Anopheles paraliae* [40].

Until now, numerous studies have used ribosomal and mitochondrial DNA markers for phylogenetic analysis to determine the relationships among sibling species and/or subspecies of *Anopheles* [11,27,29,30,41–48]. Recently, Ngo et al. [49] reported that *Anopheles dangi* is deemed to be a synonym of *A. crawfordi* based on low mean genetic distance (0.006) of COI, COII and Cyt-b genes of mtDNA and the D3 gene of rDNA derived from specimens collected in south-central Vietnam. However, there have been no reports of evolutionary relationships among different karyotypic forms of *A. crawfordi*. Thus, our report is the first on the phylogenetic relationships among four karyotypic forms of Thai and Cambodian *A. crawfordi* populations. The comparison of our COI and COII sequences with those reported from Ngo et al. [49] were also performed in this study. Both Bayesian trees revealed that they are the same species. This study provides important information on the distribution of this species across different geographic regions, and highlights that the four karyotypic forms represent a single species. In addition, this is the first multidisciplinary approach based on cytological markers and DNA sequences to investigate different populations of *A. crawfordi*.

Disclosure of interest

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

Acknowledgements

This work was supported by the Thailand Research Fund (TRF Senior Research Scholar: RTA5480006) and the Diamond Research Grant of Faculty of Medicine, Chiang Mai University to Wej Choochote and Atiporn Saeung. The authors would like to thank Dr. Wattana Navacharoen, Dean of the Faculty of Medicine, Chiang Mai University, for his interest in this research.

References

[1] R.E. Harbach, *Anopheles* classification, Mosquito taxonomic inventory, 2014 http://mosquito-taxonomic-inventory.info/node/11358 [Accessed 10 July 2014].
[2] J.A. Reid, Anopheline mosquitoes of Malaya and Borneo, Stud. Inst. Med. Res. Malaysia 31 (1968) 1–520.
[3] B.A. Harrison, J.E. Scanlon, Medical entomology studies II. The subgenus *Anopheles* in Thailand (Diptera: Culicidae), Contrib. Am. Entomol. Inst. 12 (1975) 1–307.
[4] A. Saeung, C. Hempolchom, V. Baimai, S. Thongsahuan, K. Taai, N. Jariyapan, U. Chaithong, W. Choochote, Susceptibility of eight species members of *Anopheles hyrcanus* group to nocturnally subperiodic *Brugia malayi*, Parasit. Vectors 6 (2013) 5.
[5] J.A. Reid, T.A. Wilson, Ganapathipillai, Studies on filariasis in Malaya: The mosquito vectors of periodic *Brugia malayi* in North-West Malaya, Ann. Trop. Med. Parasitol. 56 (1962) 323–336.
[6] R.H. Wharton, A.B.G. Laing, W.H. Cheong, Studies on the distribution and transmission of malaria and filariasis among aborigines in Malaya, Ann. Trop. Med. Parasitol. 57 (1963) 235–254.
[7] V. Baimai, R. Rattanarithikul, U. Kijchalao, Metaphase karyotypes of *Anopheles* of Thailand and Southeast Asia: I. The *hyrcanus* group, J. Am. Mosq. Control Assoc. 9 (1993) 59–67.
[8] S.K. Subbarao, Anopheline species complexes in South-East Asia, WHO, Tech. Pub. Ser. 18 (1998) 1–82.
[9] W. Choochote, A. Saeung, Systematic techniques for the recognition of *Anopheles* species complexes, in: S. Manguin (Ed.), *Anopheles* mosquitoes – New insights into malaria vectors, InTech, Rijeka, Croatia, 2013, pp. 57–79.
[10] R. Rattanarithikul, B.A. Harrison, R.E. Harbach, P. Panthusiri, R.E. Coleman, Illustrated keys to the mosquitoes of Thailand IV. *Anopheles*, Southeast Asian J. Trop. Med. Public Health 37 (suppl. 2) (2006) 1–128.
[11] A. Saeung, Y. Otsuka, V. Baimai, P. Somboon, B. Pitasawat, B. Tuetun, A. Junkum, H. Takaoka, W. Choochote, Cytogenetic and molecular evidence for two species in the *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand, Parasitol. Res. 101 (2007) 1337–1344.
[12] N.W. Beebe, A. Saul, Discrimination of all members of the *Anopheles punctulatus* complex by polymerase chain reaction-restriction fragment length polymorphism analysis, Am. J. Trop. Med. Hyg. 53 (1995) 478–481.
[13] O. Folmer, M. Black, W. Hoeh, R. Lutz, R. Vrijenhoek, DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates, Mol. Mar. Biol. Biotechnol. 3 (1994) 294–299.
[14] R.G. Sharpe, R.E. Harbach, R.K. Butlin, Molecular variation and phylogeny of members of the Minimus group of *Anopheles* subgenus *Cellia* (Diptera: Culicidae), Syst. Entomol. 25 (2000) 263–272.
[15] J.D. Thompson, D.G. Higgins, T.J. Gibson, 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 (1994) 4673–4680.
[16] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucl. Acids. Symp. Ser. 41 (1999) 95–98.
[17] M. Kimura, Simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences, J. Mol. Evol. 16 (1980) 111–120.
[18] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[19] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: Molecular Evolutionary Genetics Analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[20] F. Ronquist, M. Teslenko, P. van der Mark, D.L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M.A. Suchard, J.P. Huelsenbeck, MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space, Syst. Biol. 61 (2012) 539–542.
[21] J.A.A. Nylander, MrModeltest v2. Program distributed by the author, Evolutionary Biology Centre, Uppsala University, Sweden, 2004.
[22] V. Baimai, Heterochromatin accumulation and karyotypic evolution in some dipteran insects, Zool. Stud. 37 (1998) 75–88.
[23] T. Kanda, K. Takai, G.L. Chiang, W.H. Cheong, S. Sucharit, Hybridization and some biological facts of seven strains of the *Anopheles leucosphyrus* group (Reid, 1968), Jpn. J. Sanit. Zool. 32 (1981) 321–329.
[24] V. Baimai, R.G. Andre, B.A. Harrison, U. Kijchalao, L. Panthusiri, 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 (1987) 157–166.
[25] A. Junkum, N. Komalamisra, A. Jitpakdi, N. Jariyapan, G.S. Min, M.H. Park, K.H. Cho, P. Somboon, P.A. Bates, W. Choochote, Evidence to support two conspecific cytological races on *Anopheles aconitus* in Thailand, J. Vector. Ecol. 30 (2005) 213–224.
[26] P. Somboon, D. Thongwat, W. Choochote, C. Walton, M. Takagi, Crossing experiments of *Anopheles minimus* species C and putative species E, J. Am. Mosq. Control Assoc. 21 (2005) 5–9.

[27] A. Saeung, V. Baimai, Y. Otsuka, R. Rattanarithikul, P. Somboon, A. Junkum, B. Tuetun, H. Takaoka, W. Choochote, Molecular and cytogenetic evidence of three sibling species of the *Anopheles barbirostris* Form A (Diptera: Culicidae) in Thailand, Parasitol. Res. 102 (2008) 499–507.
[28] D. Thongwat, K. Morgan, M.S. O'loughlin, C. Walton, W. Choochote, P. Somboon, Crossing experiment supporting the specific status of *Anopheles maculatus* chromosomal form K, J. Am. Mosq. Control Assoc. 24 (2008) 194–202.
[29] S. Suwannamit, V. Baimai, Y. Otsuka, A. Saeung, S. Thongsahuan, B. Tuetun, C. Apiwathnasorn, N. Jariyapan, P. Somboon, H. Takaoka, W. Choochote, Cytogenetic and molecular evidence for an additional new species within the taxon *Anopheles barbirostris* (Diptera: Culicidae) in Thailand, Parasitol. Res. 104 (2009) 905–918.
[30] S. Thongsahuan, V. Baimai, Y. Otsuka, A. Saeung, B. Tuetun, N. Jariyapan, S. Suwannamit, P. Somboon, A. Jitpakdi, H. Takaoka, W. Choochote, Karyotypic variation and geographic distribution of *Anopheles campestris*-like (Diptera: Culicidae) in Thailand, Mem. Inst. Oswaldo Cruz. 104 (2009) 558–566.
[31] W. Choochote, Evidence to support karyotypic variation of the mosquito, *Anopheles peditaeniatus* in Thailand, J. Insect Sci. 11 (2011) 10.
[32] A. Saeung, V. Baimai, S. Thongsahuan, G.S. Min, M.H. Park, Y. Otsuka, W. Maleewong, V. Lulitanond, K. Taai, W. Choochote, Geographic distribution and genetic compatibility among six karyotypic forms of *Anopheles peditaeniatus* (Diptera: Culicidae) in Thailand, Trop. Biomed. 29 (2012) 613–625.
[33] N.P. Kumar, A.R. Rajavel, R. Natarajan, P. Jambulingam, DNA barcodes can distinguish species of Indian mosquitoes (Diptera: Culicidae), J Med Entomol. 44 (2007) 1–7.
[34] W. Choochote, A. Jitpakdi, K.L. Sukontason, U. Chaithong, S. Wongkamchai, B. Pitasawat, N. Jariyapan, T. Suntaravitun, E. Rattanachanpichai, K. Sukontason, S. Leemingsawat, Y. Rongsriyam, Intraspecific hybridization of two karyotypic forms of *Anopheles vagus* (Diptera: Culicidae) and the related egg surface topography, Southeast Asian J. Trop. Med. Public Health 33 (suppl. 3) (2002) 29–35.
[35] S.J. Park, W. Choochote, A. Jitpakdi, A. Junkum, S.J. Kim, N. Jariyapan, Evidence for a conspecific relationship between two morphologically and cytologically different Forms of Korean *Anopheles pullus* mosquito, Mol. Cells 16 (2003) 354–360.
[36] W. Choochote, A. Jitpakdi, Y. Rongsriyam, N. Komalamisra, B. Pitasawat, K. Palakul, Isoenzyme study and hybridization of two forms of *Anopheles sinensis* (Diptera: Culicidae) in Northern Thailand, Southeast Asian J. Trop. Med. Public Health. 29 (1998) 841–847.
[37] G.S. Min, W. Choochote, A. Jitpakdi, S.J. Kim, W. Kim, J. Jung, A. Junkum, Intraspecific hybridization of *Anopheles sinensis* (Diptera: Culicidae) strains from Thailand and Korea, Mol. Cells 14 (2002) 198–204.
[38] M.H. Park, W. Choochote, A. Junkum, D. Joshi, B. Tuetan, A. Saeung, J.H. Jung, G.S. Min, Reproductive isolation of *Anopheles sinensis* from *Anopheles lesteri* and *Anopheles sineroides* in Korea, Genes & Genomics 30 (2008) 245–252.
[39] M.H. Park, W. Choochote, S.J. Kim, P. Somboon, A. Saeung, B. Tuetan, Y. Tsuda, M. Takagi, D. Joshi, Y.J. Ma, G.S. Min, Nonreproductive isolation among four allopatric strains of *Anopheles sinensis* in Asia, J. Am. Mosq. Control Assoc. 24 (2008) 489–495.
[40] K. Taai, V. Baimai, S. Thongsahuan, A. Saeung, Y. Otsuka, W. Srisuka, P. Sriwichai, P. Somboon, N. Jariyapan, W. Choochote, Metaphase karyotypes of *Anopheles paraliae* (Diptera: Culicidae) in Thailand and evidence to support five cytological races, Trop. Biomed. 30 (2013) 238–249.
[41] R.C. Wilkerson, P.G. Foster, C. Li, M.A. Sallum, Molecular phylogeny of neotropical *Anopheles* (*Nyssorhynchus*) *albitarsis* species complex (Diptera: Culicidae), Ann. Entomol. Soc. Am. 98 (2005) 918–925.
[42] I. Dusfour, J.R. Michaux, R.E. Harbach, S. Manguin, Speciation and phylogeography of the Southeast Asian *Anopheles sundaicus* complex, Infect. Genet. Evol. 7 (2007) 484–493.
[43] K. Morgan, S.M. O'Loughlin, F. Mun-Yik, Y.M. Linton, P. Somboon, S. Min, P.T. Htun, S. Nambanya, I. Weerasinghe, T. Sochantha, A. Prakash, C. Walton, Molecular phylogenetics and biogeography of the Neocellia Series of *Anopheles* mosquitoes in the Oriental Region, Mol. Phylogenet. Evol. 52 (2009) 588–601.
[44] C. Paredes-Esquivel, M.J. Donnelly, R.E. Harbach, H. Townson, A molecular phylogeny of mosquitoes in the *Anopheles barbirostris* Subgroup reveals cryptic species: implications for identification of disease vectors, Mol. Phylogenet. Evol. 50 (2009) 141–151.
[45] N. Nanda, O.P. Singh, V.K. Dua, A.C. Pandey, B.N. Nagpal, T. Adak, A.P. Dash, S.K. Subbarao, Population cytogenetic and molecular evidence for existence of a new species in *Anopheles fluviatilis* complex (Diptera: Culicidae), Infect. Genet. Evol. 13 (2013) 218–223.
[46] B. Chen, R.E. Harbach, R.K. Butlin, Molecular and morphological studies on the *Anopheles minimus* group of mosquitoes in southern China: taxonomic review, distribution and malaria vector status, Med, Vet. Entomol. 16 (2002) 253–265.
[47] B. Chen, R.K. Butlin, R.E. Harbach, Molecular phylogenetics of the Oriental members of the Myzomyia Series of *Anopheles* subgenus *Cellia* (Diptera: Culicidae) inferred from nuclear and mitochondrial DNA sequences, Syst. Entomol. 28 (2003) 57–69.
[48] C. Garros, R.E. Harbach, S. Manguin, Morphological assessment and molecular phylogenetics of the Funestus and Minimus groups of *Anopheles* (Cellia), J. Med. Entomol. 42 (2005) 522–536.
[49] C.T. Ngo, R.E. Harbach, C. Garros, D. Parzy, H.Q. Le, S. Manguin, Taxonomic assessment of *Anopheles crawfordi* and *An. dangi* of the Hyrcanus Group of subgenus *Anopheles* in Vietnam, Acta. Trop. 128 (2013) 623–629.
[50] K. Taai, V. Baimai, A. Saeung, S. Thongsahuan, G.S. Min, Y. Otsuka, M.H. Park, M. Fukuda, P. Somboon, W. Choochote, Genetic compatibility between *Anopheles lesteri* from Korea and *An. paraliae* from Thailand, Mem. Inst. Oswaldo Cruz 108 (2013) 312–320.