Karyotypic variation and geographic distribution of *Anopheles campestris*-like (Diptera: Culicidae) in Thailand

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Seventy-one isolines of Anopheles campestris*-like were established from wild-caught females collected from human-biting and animal-biting traps at 12 locations in Thailand. All isolines had an average branch summation of seta 2-VI pupal skins ranging from 20.3-30.0 branches, which is in the range of* An. campestris *(17-58 branches). They showed three different karyotypes based on the amount of extra heterochromatin in the sex chromosomes, namely Forms B (X_2, Y_2), E (X_1, X_2, X_3, Y_5) and a new karyotypic Form F (X_2, X_3, Y_6). Form B has been found only in Chaing Mai and Kamphaeng Phet populations, while Forms E and F are widely distributed throughout the species range. Genetic crosses between the 12 isolines, which were arbitrarily selected as representatives of* An. campestris*-like Forms B, E and F, revealed genetic compatibility that provided viable progeny through F_2 generations, suggesting a conspecific nature of these karyotypic forms. These results are supported by the very low intraspecies variation (genetic distance < 0.005) of ITS2, COI and COII from genomic DNA of the three karyotypic forms.*

Key words: *Anopheles campestris*-like - metaphase karyotype - crossing experiment - ITS2 - COI - COII

Anopheles (*Anopheles*) *barbirostris* belongs to the *Barbirostris* Subgroup of the Myzorhynchus Series and is widely distributed in Thailand and Southeast Asia (Reid 1968, Scanlon et al. 1968, Harrison 1980, Harbach 2004, Rattanarithikul et al. 2006). Normally, *An. barbirostris* and the closely related species, *Anopheles campestris,* can cause problems in species identification because of their similarity in external morphology. Accordingly, *An. barbirostris* was formerly considered a suspected vector of malaria and/or filariasis in Thailand (Iyengar 1953, Griffith 1955), while it has been incriminated as a natural vector of *Plasmodium vivax* and *Brugia malayi*, the causative agent of filariasis, in Indonesia (Atomosoedjono et al. 1976, Kirnowardoyo 1985). Recently, mosquitoes of the anthropophilic *An. barbirostris/campestris* complex were incriminated as potential natural vectors of *P. vivax* in the Aranyaprathet district, Sa Kaeo province (Limrat et al. 2001). Mosquitoes of this complex have played an important role in increasing cases of *P. vivax* infection in Thailand (Sattabongkot et al. 2004). Recent morphological, cytological, hybridization and molecular analysis have revealed that *An. campestris*-like and *An. barbirostris* are distinct species (Saeung et al. 2007). Furthermore, similar studies have shown that *An. barbirostris* s.l. is a cryptic species consisting of at least four sibling species, i.e., A1, A2, A3 (Saeung et al. 2008) and A4 (Suwannamit et al. 2009). The *An. barbirostris* species complex exhibited karyotypic variation due to different amounts of extra heterochromatin in the sex chromosomes. Likewise, our previous observations indicated that *An. campestris*-like had at least two karyotypic forms, i.e., Forms B (X_2, Y_2) and E (X_2, Y_5) (Saeung et al. 2007). Thus, it has been suggested that the acquisition of extra block(s) of heterochromatin played an important role in the chromosomal evolution of Oriental *Anopheles* (Baimai 1998). The crossing experiments between isolines of *An. campestris*-like Forms B and E showed no post-mating reproductive isolation. Comparative studies of nucleotide sequences of rDNA of ITS2 and mtDNA of COI and COII, among the isolines of *An. campestris*-like Forms B and E, revealed nearly identical and/or very low intraspecific variation (genetic distance < 0.005) (Saeung et al. 2007). Thus, crossing and molecular evidence support the conspecific relationships of the karyotypic forms of *An. campestris*-like mosquitoes.

This paper describes a new karyotypic form of *An. campestris*-like. We also present the results of crossing experiments and comparative DNA sequencing of the ITS2, COI and COII regions of the three karyotypic forms of *An. campestris*-like in Thailand.

MATERIALS AND METHODS

Field collections and establishment of isoline colonies - Wild-caught, fully engorged female mosquitoes of *An. campestris*-like were collected from human-baited and

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buffalo-baited traps during September 2006-December 2007 at 12 localities in Thailand (Fig. 1, Table I). A total of 71 isolines were successfully established and maintained in our insectary using the techniques described by Choochote et al. (1983) and Kim et al. (2003). These isolines were used for studies on metaphase karyotype, crossing experiments and molecular analysis.

Metaphase karyotype - Metaphase chromosomes were prepared from 10 samples of the early fourth-instar larval brains of F_1 and/or F_2-progenies of each isoline using the techniques previously described by Saeung et al. (2007, 2008). Identification of karyotypic forms followed the cytotaxonomic key of Baimai et al. (1995).

Crossing experiments - The 12 laboratory-raised isolines of *An. campestris*-like were arbitrarily selected from the 30 isoline colonies as representatives of the three karyotypic forms, i.e., Form B (AKpB1), Form E (HCE6, AKkE4, AMkE1, AMsE3, HSkE3, ACpE6) and Form F (AUdF5, ACiF1, AAyF2, HCtF4, APkF1) (Table II). These isolines were used for crossing experiments, in order to determine post-mating reproductive isolation by employing the techniques previously reported by Saeung et al. (2007, 2008).

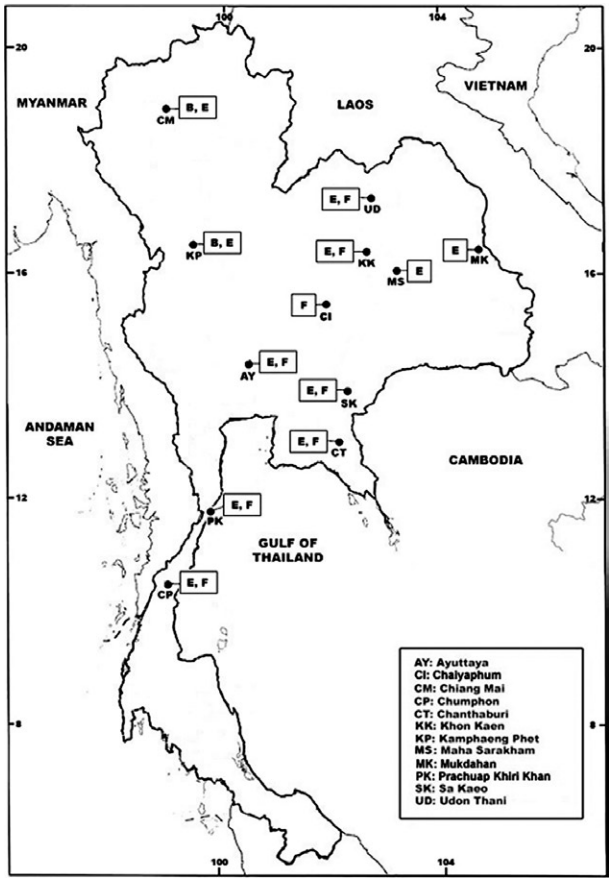

Fig. 1: map of Thailand showing distribution of *Anopheles campestris*-like Form B, E and F.

DNA extraction, amplification and sequencing - Individual feral and/or F_1-progeny adult females of each isoline were used for DNA extraction and amplification. Genomic DNA was extracted from a whole adult mosquito using a DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The amplification was done with primers and conditions, as described previously (Saeung et al. 2007, 2008). The rDNA ITS2, COI and COII regions were amplified by polymerase chain reaction (PCR) using the following primers: ITS2A, 5' -TGTGAACTGCAGGACACAT-3' and ITS2B, 5' -TATGCTTAAATTCAGGGGGT-3' for rDNA ITS2; LCO1490 (f), 5' -GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 (r), 5' -TAAACTTCAGGGTGACCAAAAAATCA-3' for COI; and LEU (f), 5' -TCTAATATGGCAGATTAGTGCA-3' and LYS (r), 5' -ACTTGCTTTCAGTCATCTAATG-3' for COII. PCR was carried out using 20 µL volumes containing 0.5 units of *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. The amplified products were electrophorised through a 1% agarose gel. PCR products of ITS2 were gel purified with the QIAquick® Gel Extraction Kit (Qiagen) and cloned into pCR2.1-TOPO (Invitrogen). Sequences of several clones from each isoline were determined. PCR products of COI and COII were purified with the QIAquick® PCR Purification Kit (Qiagen) and directly sequenced. Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit and run on a 3130 Genetic Analyzer (Applied Biosystems). The sequence data of this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB436074-AB436157 (Table I).

DNA sequence and phylogenetic analysis - For the ITS2 DNA region, three individual clones from each isoline were sequenced and aligned using the CLUSTALW multiple alignment program (Thompson et al. 1994). Gap sites were excluded from the following analysis. Genetic distances were estimated using the Kimura two-parameter method (Kimura 1980). Construction of neighbour-joining trees (Saitou & Nei 1987) and the bootstrap test, with 1,000 replications, were conducted using the MEGA version 4.0 program (Tamura et al. 2007) from the individual sequence of each isoline for all three DNA regions. The bootstrapping values, as percentages, are indicated above the branches of the tree. For the phylogenetic trees of COI and COII, *Anopheles gambiae* and *Anopheles pullus* were used as outgroups (NC_002084, AY444349, AY444350). The phylogenetic tree of ITS2 was constructed as an unrooted tree because an outgroup with easily aligned ITS2 was not available. The published data of *An. campestris*-like and *An. barbirostris* described by Saeung et al. (2007, 2008) were also used for phylogenetic analysis.

RESULTS

Morphological and karyotypic characters - Morphological investigations of F_1 and/or F_2-progenies of the 71 isolines showed an average summation of 20.3-30.0 seta

TABLE I

Locations, isoline colonies and karyotypic forms of *Anopheles campestris*-like and their GenBank accession numbers

Location (geograpical coordinates)	Code of isoline[a]	Karyotypic form	Length of ITS2 (bp)	Region	Genbank accession number		
					ITS2	COI	COII
Chiang Mai (18º47′N 98º59′E)	HCE6[b]	E	1,651	ITS2, COI, COII	AB331566	AB331583	AB331604
	HCB9	B	1,651	ITS2, COI, COII	AB331563	AB331582	AB331601
	HCmE12	E	1,651	ITS2, COI, COII	AB436074	AB436102	AB436130
	HCmE14	E	1,651	ITS2, COI, COII	AB436075	AB436103	AB436131
	HCmE15	E	1,651	ITS2, COI, COII	AB436076	AB436104	AB436132
	HCmB18	B	1,651	ITS2, COI, COII	AB436077	AB436105	AB436133
	HCmB20	B	1,651	ITS2, COI, COII	AB436078	AB436106	AB436134
Kamphaeng Phet (16º50'N 99º04'E)	AKpB1[b]	B	1,651	ITS2, COI, COII	AB436079	AB436107	AB436135
	HKpE1	E	1,651	ITS2, COI, COII	AB436080	AB436108	AB436136
Ayuttaya (14º01'N 101º02'E)	AAyF2[b]	F	1,651	ITS2, COI, COII	AB436081	AB436109	AB436137
	AAyF6	F	1,651	ITS2, COI, COII	AB436082	AB436110	AB436138
	AAyE7	E	1,651	ITS2, COI, COII	AB436083	AB436111	AB436139
Udon Thani (17º24′N 102º47'E)	AUdF3	F	1,651	ITS2, COI, COII	AB436084	AB436112	AB436140
	AUdF4	F	1,651	ITS2, COI, COII	AB436085	AB436113	AB436141
	AUdF5[b]	F	1,651	ITS2, COI, COII	AB436086	AB436114	AB436142
Khon Kaen (15º41'N 101º45'E)	AKkF1	F	1,651	ITS2, COI, COII	AB436087	AB436115	AB436143
	AKkE4[b]	E	1,651	ITS2, COI, COII	AB436088	AB436116	AB436144
	AKkE8	E	1,651	ITS2, COI, COII	AB436089	AB436117	AB436145
Maha Sarakham (15º45'N 103º01'E)	AMsE3[b]	E	1,651	ITS2, COI, COII	AB436090	AB436118	AB436146
	AMsE4	E	1,651	ITS2, COI, COII	AB436091	AB436119	AB436147
	AMsE5	E	1,651	ITS2, COI, COII	AB436092	AB436120	AB436148
Mukdahan (15º24'N 103º16'E)	AMkE1[b]	E	1,651	ITS2, COI, COII	AB436093	AB436121	AB436149
Chaiyaphum (15º481'N 101º30'E)	ACiF1[b]	F	1,651	ITS2, COI, COII	AB436094	AB436122	AB436150
Sa Kaeo (13º14′ N 101º51'E)	HSkF1	F	1,651	ITS2, COI, COII	AB436095	AB436123	AB436151
	HSkE2	E	1,651	ITS2, COI, COII	AB436096	AB436124	AB436152
	HSkE3[b]	E	1,651	ITS2, COI, COII	AB436097	AB436125	AB436153
Chanthaburi (12º37'N 102º07'E)	HCtE2	E	1,651	ITS2, COI, COII	AB436098	AB436126	AB436154
	HCtF4[b]	F	1,651	ITS2, COI, COII	AB436099	AB436127	AB436155
Prachuap Khiri Khan (11º48'N 99º49'E)	APkF1[b]	F	1,651	ITS2, COI, COII	AB436100	AB436128	AB436156
Chumphon (10º29'N 99º11'E)	ACpE6[b]	E	1,651	ITS2, COI, COII	AB436101	AB436129	AB436157

a: code of isoline: A: animal bait; H: human bait; *b*: used in crossing experiments.

2-VI branches, which is in the range of topotypic *An. campestris* (17-58 branches). Cytogenetic observations of F_1 and/or F_2-progenies of these isolines demonstrated three forms of metaphase karyotypes, i.e., Forms B (X_2, Y_2), E (X_1, X_2, X_3,Y_5) and F (X_2, X_3, Y_6) (Fig. 2). Form B has been detected only in Chiang Mai and Kamphaeng Phet. However, Forms E and F have been encountered in sympatry in most populations throughout Thailand (Fig. 1, Table I). Interestingly, the three karyotypic forms have been found in the mosquitoes collected from both

human-baits and animal-baits. Thus, it seems that there is no preferential host for these karyotypic forms.

The new metaphase karyotype, Form F, had submetacentric X_2 and X_3 chromosomes resembling those of Forms B and E. Nevertheless, the Y_6 chromosome had a large subtelocentric shape, which was quite different from the submetacentric Y_2 and the small metacentric Y_5 chromosomes of Forms B and E, respectively (Fig. 2).

Crossing experiments - Details of hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal and F_1-hybrid crosses among the 12 isolines of *An. campestris*-like Forms B, E and F are shown in Table II. All crosses yielded viable progeny through F_2 generations. No evidence of genetic incompatibility and/or post-mating reproductive isolation was observed among these crosses.

DNA sequences and phylogenetic analysis - DNA sequences were determined and analyzed for the ITS2, COI and COII regions of the 30 isolines of *An. campestris*-like Forms B, E and F. They all showed the same length for the ITS2 (1,651 bp), COI (658 bp) and COII (685 bp). The length of the three DNA regions of *An. campestris*-like Forms B and E obtained in this study agreed with that of the previous report (Saeung et al. 2007). Form F also showed no difference in length of the three DNA regions. To reveal the evolutionary relationship among the three karyotypic forms, neighbour-joining trees were constructed (Figs 3-5). Obviously, the average genetic distances within and between the three karyotypic forms of *An. campestris*-like exhibited no significant differences (0.001-0.004) in the three DNA regions (Table III). Hence, the 30 isolines were placed within a cluster of *An. campestris*-like. However, the trees for ITS2, COI and COII of these isolines of *An. campestris*-like Forms B, E and F were clearly different from those of the four sibling species of the *An. barbirostris* complex with strongly supported bootstrap probabilities (95-100%) (Figs 3-5).

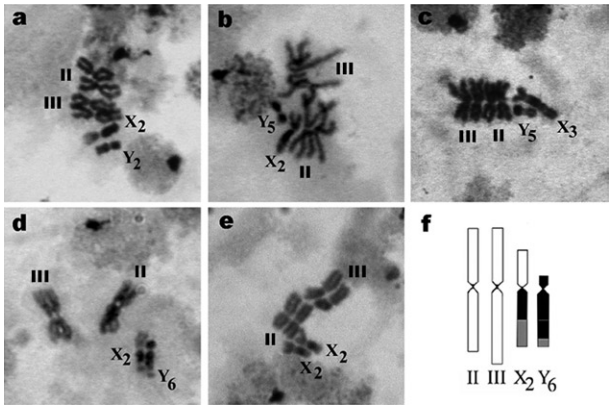

Fig. 2: metaphase karyotypes of *Anopheles campestris*-like Form B, E and F (a-e). Form B: a: Kamphaeng Phet strain, showing X_2, Y_2 chromosomes; Form E: b: Chumphon strain, showing X_2, Y_5 chromosomes; c: Sa Kaeo strain, showing X_3, Y_5 chromosomes; Form F: d: Udon Thani strain, showing X_2, Y_6 chromosomes; e: showing homozygous X_2, X_2 chromosomes; f: diagrams of representative metaphase karyotypes of *An. campestris*-like Form F.

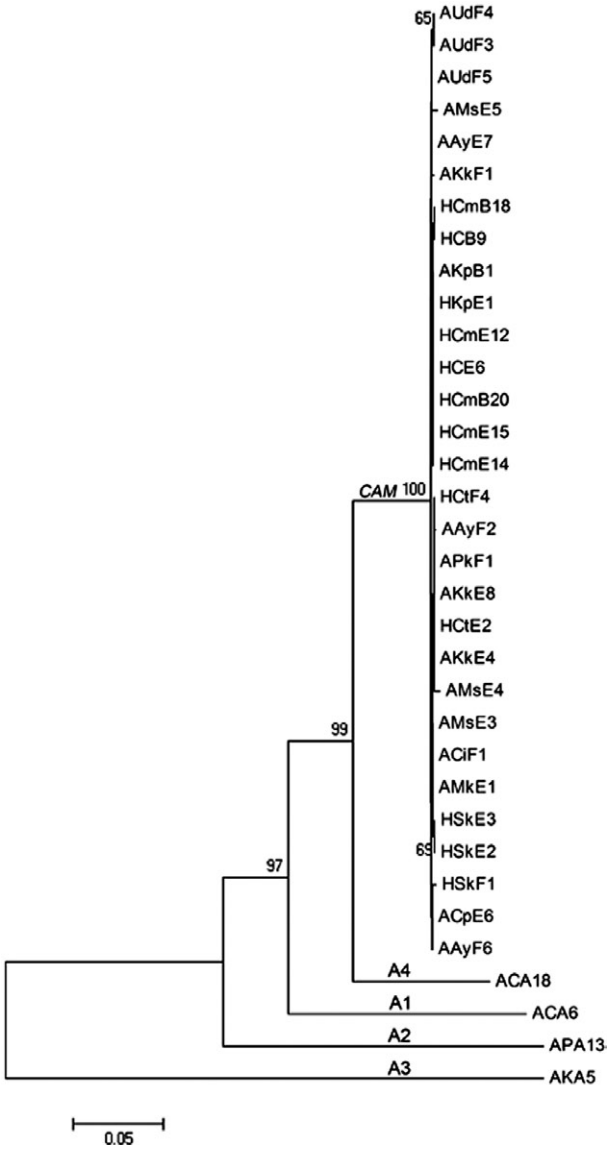

Fig. 3: a phylogenetic trees of *Anopheles campestris*-like Forms B, E and F (*CAM*) and *Anopheles barbirostris* species A1, A2, A3 and A4 based on molecular analysis of ITS2 sequences. The tree was generated by neighbor-joining analysis. Numbers on the nodes indicate probabilities based on 1,000 bootstrap replicates. A probability of more than 50% is shown. Branch lengths are proportional to genetic distance (scale bar).

DISCUSSION

Comparative studies on metaphase chromosomes of anopheline mosquitoes in Thailand revealed at least three karyotypic forms in *An. barbirostris* s.l., i.e., Forms A (X_2, X_3, Y_1), B (X_1, X_2, X_3, Y_2) and C (X_2, X_3, Y_3) and one karyotypic form in *An. campestris* s.l. (X, Y) (Baimai et al. 1995). Recently, Saeung et al. (2007) reported two and three karyotypic forms of *An. campestris*-like and the *An. barbirostris* species complex, respectively. Moreover, the two karyotypic forms of *An. campestris*-like were detected in Chiang Mai, i.e., Forms B (X_2,

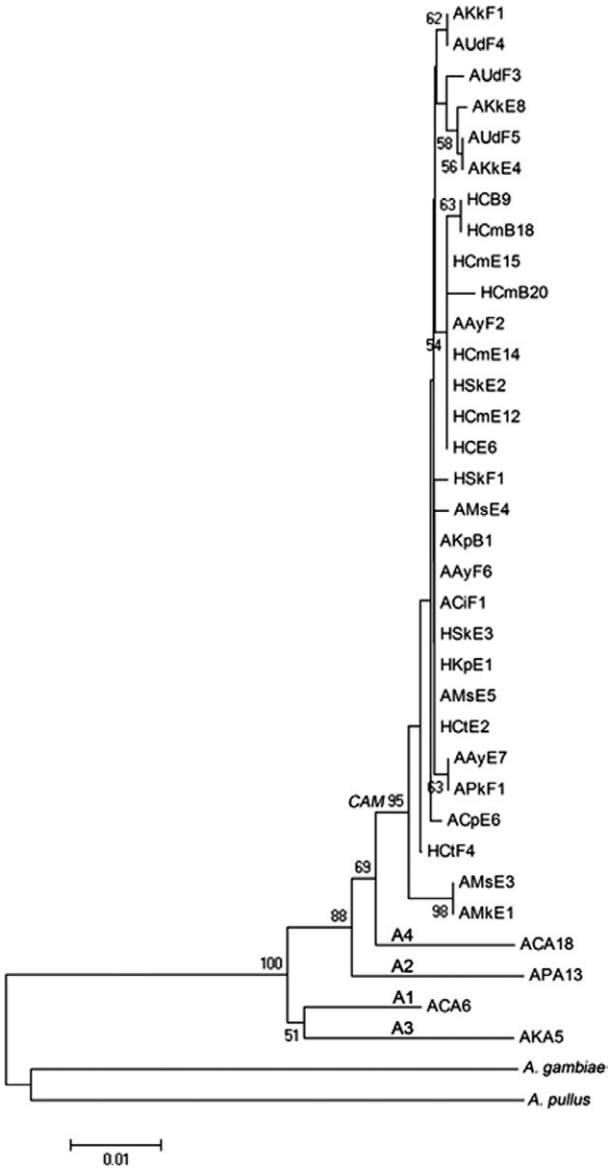

Fig. 4: a phylogenetic trees of *Anopheles campestris*-like Forms B, E and F (*CAM*) and *Anopheles barbirostris* species A1, A2, A3 and A4 based on molecular analysis of COI sequences. The tree was generated by neighbor-joining analysis. Numbers on the nodes indicate probabilities based on 1,000 bootstrap replicates. A probability of more than 50% is shown. Branch lengths are proportional to genetic distance (scale bar).

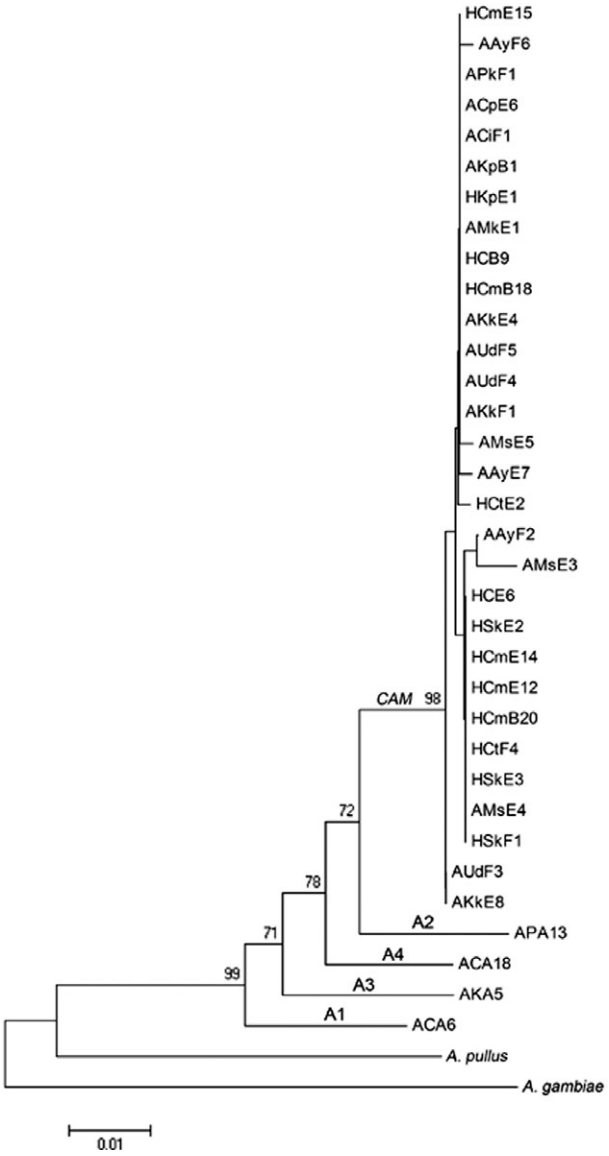

Fig. 5: a phylogenetic tree of *Anopheles campestris*-like Forms B, E and F (*CAM*) and *Anopheles barbirostris* species A1, A2, A3 and A4 based on molecular analysis of COII sequences. The tree was generated by neighbor-joining analysis. Numbers on the nodes indicate probabilities based on 1,000 bootstrap replicates. A probability of more than 50% is shown. Branch lengths are proportional to genetic distance (scale bar).

Y_2) and E (X_2, Y_5). The X_2, X_3, Y_6 chromosomes of *An. campestris*-like were new discoveries in this study. Particularly, the Y_6 chromosome was obviously different from the Y_2 and Y_5 chromosomes of *An. campestris*-like Forms B and E that were previously described.

Hybridization experiments for determining hybrid non-viability, sterility or breakdown are still useful criteria for biological species. Further, genetic incompatibility, including lack of insemination, embryonation, hatchability, larval survival, pupation, emergence, adult sex distortion, abnormal morphology and reproductive system are useful information to elucidate sibling species complexes in the Oriental *Anopheles* (Kanda et al. 1981, Baimai et al. 1987, Subbarao 1998). Nonetheless, a point worth noting is that an isoline colony established from the combinative characters of morphological, cytological (polytene and mitotic chromosomes) and/or molecular markers must be seriously considered. A laboratory-raised colony established from a naturally mixed population should be omitted because it may be a mixture of cryptic species or sibling species (Subbarao 1998). Despite the differences in metaphase karyotypes

TABLE II

Crossing experiments among the 12 isolines of *Anopheles campestris*-like Forms B, E and F

Crosses (female x male)	Total eggs (n)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence (%)	
						Female	Male
Parental cross							
HCE6 x HCE6	536 (60, 476)	88	456 (85.07)	357 (78.29)	336 (94.12)	162 (48.21)	174 (51.79)
AKpB1 x AKpB1	577 (286, 291)	85	433 (75.04)	312 (72.06)	303 (97.12)	154 (50.83)	149 (49.17)
AAyF2 x AAyF2	484 (186, 298)	71	335 (69.21)	256 (76.42)	242 (94.53)	142 (58.68)	100 (41.32)
AUdF5 x AUdF5	509 (145, 364)	89	387 (76.03)	348 (89.92)	306 (87.93)	156 (50.98)	150 (49.02)
AKkE4 x AKkE4	499 (171, 328)	95	455 (91.18)	400 (87.91)	320 (80.00)	164 (51.25)	156 (48.75)
AMkE1 x AMkE1	515 (202, 313)	84	443 (86.02)	363 (81.94)	316 (87.05)	143 (45.25)	173 (54.75)
AMsE3 x AMsE3	469 (186, 283)	75	352 (75.05)	299 (84.94)	250 (83.61)	110 (44.00)	140 (56.00)
ACiF1 x ACiF1	490 (231, 259)	86	397 (81.02)	381 (95.97)	355 (93.18)	153 (43.10)	202 (56.90)
HSkE3 x HSkE3	564 (263, 301)	87	474 (84.04)	431 (90.93)	328 (76.10)	180 (54.88)	148 (45.12)
HCtF4 x HCtF4	494 (226, 268)	82	346 (70.04)	327 (94.51)	278 (85.02)	131 (47.12)	147 (52.88)
APkF1 x APkF1	525 (197, 328)	93	389 (74.10)	319 (82.01)	290 (90.91)	130 (44.83)	160 (55.17)
ACpE6 x ACpE6	566 (272, 294)	94	521 (92.05)	454 (87.14)	427 (94.05)	217 (50.82)	210 (49.18)
Reciprocal cross							
HCE6 x AKpB1	473 (102, 371)	93	359 (75.90)	280 (77.99)	258 (92.14)	130 (50.39)	128 (49.61)
AKpB1 x HCE6	360 (87, 273)	74	220 (61.11)	216 (98.18)	190 (87.96)	96 (50.53)	94 (49.47)
HCE6 x AAyF2	467 (170, 297)	71	309 (66.17)	263 (85.11)	232 (88.21)	112 (48.28)	120 (51.72)
AAyF2 x HCE6	423 (207, 216)	92	360 (85.11)	266 (73.89)	218 (81.95)	97 (44.50)	121 (55.50)
HCE6 x AUdF5	398 (196, 202)	83	330 (82.91)	268 (81.21)	254 (94.78)	130 (51.18)	124 (48.82)
AUdF5 x HCE6	485 (184, 301)	96	378 (77.94)	318 (84.13)	309 (97.17)	151 (48.87)	158 (51.13)
HCE6 x AKkE4	402 (171, 231)	94	382 (95.02)	330 (86.39)	307 (93.03)	169 (55.05)	138 (45.95)
AKkE4 x HCE6	306 (119, 187)	62	189 (61.76)	136 (71.96)	117 (86.03)	63 (53.85)	54 (46.15)
HCE6 x AMkE1	499 (186, 313)	95	459 (91.98)	418 (91.07)	372 (89.00)	179 (48.12)	193 (51.88)
AMkE1 x HCE6	424 (138, 286)	86	344 (81.13)	337 (97.97)	317 (94.07)	139 (43.85)	178 (56.15)
HCE6 x AMsE3	440 (193, 247)	81	339 (77.05)	281 (82.89)	235 (83.63)	120 (51.06)	115 (48.94)
AAyF2 x HCE6	423 (207, 216)	92	360 (85.11)	266 (73.89)	218 (81.95)	97 (44.50)	121 (55.50)
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APkF1 x HCE6	403 (172, 231)	89	283 (70.22)	253 (89.40)	235 (92.89)	133 (56.60)	102 (43.40)
HCE6 x ACpE6	383 (33, 350)	90	306 (79.90)	301 (98.37)	247 (82.06)	114 (46.15)	133 (53.85)
ACpE6 x HCE6	473 (159, 314)	99	445 (94.08)	414 (93.03)	385 (93.00)	177 (45.97)	208 (54.03)
F_1 cross							
(HCE6 x AKpB1)F_1 x (HCE6 x AKpB1)F_1	407 (180, 227)	90	366 (89.93)	278 (75.96)	231 (83.09)	137 (59.31)	94 (40.69)
(AKpB1 x HCE6)F_1 x (AKpB1 x HCE6)F_1	399 (75, 324)	91	303 (75.94)	251 (82.84)	226 (90.04)	104 (46.02)	122 (53.98)
(HCE6 x AAyF2)F_1 x (HCE6 x AAyF2)F_1	529 (226, 303)	81	397 (75.05)	369 (92.95)	317 (85.91)	158 (49.84)	159 (50.16)
(AAyF2 x HCE6)F_1 x (AAyF2 x HCE6)F_1	541 (224, 317)	86	422 (78.00)	301 (71.33)	265 (88.04)	135 (50.94)	130 (49.06)
(HCE6 x AUdF5)F_1 x (HCE6 x AUdF5)F_1	485 (157, 328)	87	407 (83.92)	338 (83.05)	294 (86.98)	141 (47.96)	153 (52.04)
(AUdF5 x HCE6)F_1 x (AUdF5 x HCE6)F_1	438 (159, 279)	81	350 (79.91)	317 (90.57)	282 (88.96)	127 (45.04)	155 (54.96)

(HCE6 x AKkE4)F_1 x (HCE6 x AKkE4)F_1	459 (225, 234)	73	317 (69.06)	288 (90.85)	265 (92.01)	138 (52.08)	127 (47.92)
(AKkE4 x HCE6)F_1 x (AKkE4 x HCE6)F_1	400 (136, 264)	77	280 (70.00)	262 (93.57)	215 (82.06)	109 (50.70)	106 (49.30)
(HCE6 x AMkE1)F_1 x (HCE6 x AMkE1)F_1	420 (116, 304)	96	386 (91.90)	317 (82.12)	247 (77.92)	101 (40.89)	146 (59.11)
(AMkE1 x HCE6)F_1 x (AMkE1 x HCE6)F_1	489 (204, 285)	97	449 (91.82)	332 (73.94)	252 (75.90)	128 (50.79)	124 (49.21)
(HCE6 x AMsE3)F_1 x (HCE6 x AMsE3)F_1	439 (77, 362)	91	356 (81.09)	263 (73.88)	228 (86.69)	120 (52.63)	108 (47.37)
(AMsE3 x HCE6)F_1 x (AMsE3 x HCE6)F_1	507 (211, 296)	93	451 (88.95)	343 (76.05)	281 (81.92)	129 (45.91)	152 (54.09)
(HCE6 x ACiF1)F_1 x (HCE6 x ACiF1)F_1	493 (221, 272)	90	434 (88.03)	352 (81.11)	250 (71.02)	132 (52.80)	118 (47.20)
(ACiF1 x HCE6)F_1 x (ACiF1 x HCE6)F_1	491 (201, 290)	79	349 (71.08)	293 (83.95)	252 (86.01)	116 (46.03)	136 (53.97)
(HCE6 x HSkE3)F_1 x (HCE6 x HSkE3)F_1	443 (154, 289)	89	381 (86.00)	312 (81.89)	275 (88.14)	140 (50.91)	135 (49.09)
(HSkE3 x HCE6)F_1 x (HSkE3 x HCE6)F_1	531 (249, 282)	98	494 (93.03)	449 (90.89)	350 (77.95)	161 (46.00)	189 (54.00)
(HCE6 x HCtF4)F_1 x (HCE6 x HCtF4)F_1	374 (101, 273)	93	359 (95.99)	330 (91.92)	271 (81.12)	146 (53.87)	125 (46.13)
(HCtF4 x HCE6)F_1 x (HCtF4 x HCE6)F_1	424 (79, 345)	86	399 (94.10)	391 (97.99)	305 (78.01)	135 (44.26)	170 (55.74)
(HCE6 x APkF1)F_1 x (HCE6 x APkF1)F_1	506 (227, 279)	80	380 (75.10)	331 (87.11)	245 (74.02)	118 (48.16)	127 (51.84)
(APkF1 x HCE6)F_1 x (APkF1 x HCE6)F_1	531 (259, 272)	92	431 (81.17)	344 (79.81)	276 (80.23)	141 (51.09)	135 (48.91)
(HCE6 x ACpE6)F_1 x (HCE6 x ACpE6)F_1	545 (269, 276)	91	447 (82.02)	387 (86.58)	325 (83.98)	136 (41.85)	189 (58.15)
(ACpE6 x HCE6)F_1 x (ACpE6 x HCE6)F_1	397 (69, 328)	89	346 (87.15)	243 (70.23)	224 (92.18)	106 (47.32)	118 (52.68)

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

of *An. campestris*-like Forms B, E and F, either from sympatric or allopatric populations, the present studies revealed no post-mating reproductive isolation among the three karyotypic forms. This is in contrast to the case of the *An. barbirostris* complex in which all four sibling species exhibited distinct metaphase karyotypes, particularly the sex chromosomes (Saeung et al. 2008, Suwannamit et al. 2009).

Molecular investigations of some specific genomic markers, e.g., rDNA (ITS1, ITS2, D3) and mtDNA (COI and COII) have been used extensively as a supportive tool to determine and/or characterise sibling species or cryptic species of anopheline mosquitoes (Mitchell et al. 1992, Sharpe et al. 2000, Min et al. 2002, Park et al. 2003, Junkum et al. 2005, Saeung et al. 2007, 2008). The molecular evidence of very low intraspecies variation (genetic distance < 0.005) of ITS2 of rDNA and COI and COII of mtDNA among the 30 isolines of *An. campestris*-like Forms B, E and F strongly supports a conspecific nature of these karyotypic forms. Therefore, we can confidently conclude that *An. campestris*-like Forms B, E and F represent intraspecies karyotypic variation due to the gain of heterochromatin in sex chromosomes in Thai populations. Similar results have been reported in other Asian anopheline mosquitoes, e.g., *Anopheles sinensis* Forms A and B (Choochote et al. 1998, Min et al. 2002), *Anopheles vagus* Forms A and B (Choochote et al. 2002), *An. pullus* Forms A and B (Park et al. 2003), *Anopheles aconitus* Forms B and C (Junkum et al. 2005) and South American anopheline mosquitoes, e.g., *Anopheles darlingi* and *Anopheles nuneztovari* (Rafael & Tadei 1998, 2000) and *Anopheles albitarsis* (Rafael et al. 2005). Such heterochromatin variation in sex chromosomes is a general phenomenon in *Anopheles* and some dipteran insects (Baimai 1998). Moreover, the results in phylogenetic analysis based on the rDNA ITS2 sequences clearly support previous findings, suggesting that *An. campestris*-like is more closely related to *An. barbirostris* species A4 than to species A1, A2 and A3 (Suwannamit et al. 2009). In this study, the genetic distances between *An. campestris*-like and *An. barbirostris* species A2 are close to those between *An. campestris*-like and *An. barbirostris* species A4. Therefore, our study suggested that *An. campestris*-like is more closely related

TABLE III

Average genetic distance within and between the *Anopheles campestris*-like Forms B, E and F for the ITS2, COI and COII regions

	ITS2	COI	COII
Within Form			
B	0.001	0.004	0.001
E	0.002	0.004	0.002
F	0.002	0.003	0.002
Between Forms			
B-E	0.002	0.004	0.001
B-F	0.002	0.004	0.001
E-F	0.002	0.003	0.002

to *An. barbirostris* species A2 and A4 than to species A1 and A3 for COI and COII. Additionally, the crossing experiments also supported molecular evidence because the reciprocal crosses between *An. campestris*-like Form E and *An. barbirostris* species A4 yielded F_1-hybrids in both directions, with lower degrees of asynaptic polytene chromosomes than those for the crosses A1 x A4, A2 x A4 and A3 x A4 (Suwannamit et al. 2009). Further detailed investigation of population biology for these sibling species may shed some light on speciation processes of these anopheline mosquitoes in Thailand.

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