Tropical Biomedicine 30(2): 238–249 (2013)

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

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Received 20 November 2012; received in revised form 20 February 2013; accepted 23 February 2013

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Field collections and establishment of isoline colonies

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

Metaphase karyotype preparation

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

Hybridization experiment

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

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

DNA extraction and amplification

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

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

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

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

[a] used in crossing experiments.

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

Sequencing alignment and phylogenetic analysis

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

RESULTS

Metaphase karyotype

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

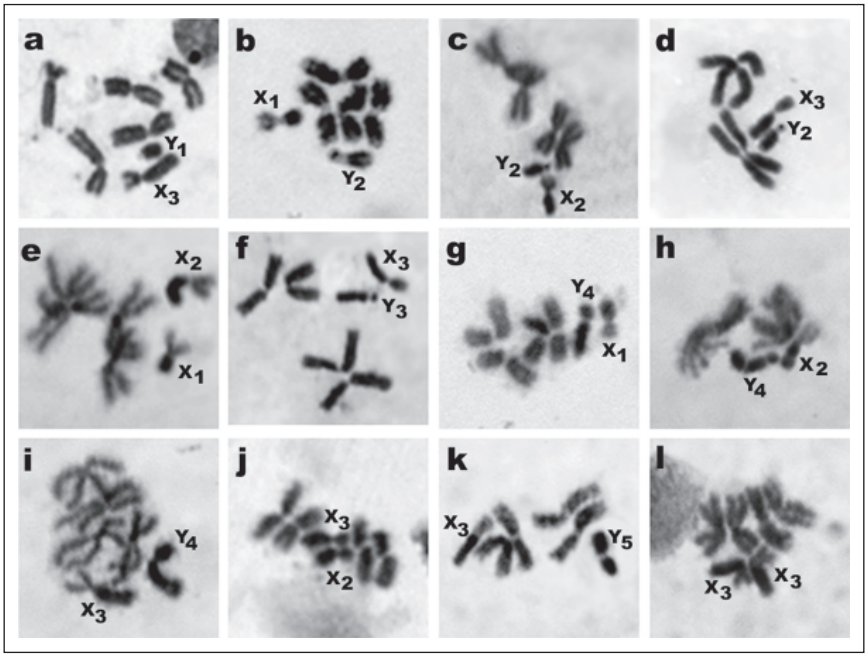

Figure 2. Metaphase karyotypic forms of *An. paraliae*. (a) Form A (X_3, Y_1), (b) Form B (X_1, Y_2), (c) Form B (X_2, Y_2), (d) Form B (X_3, Y_2), (e) Form B (X_1, X_2), (f) Form C (X_3, Y_3), (g) Form D (X_1, Y_4), (h) Form D (X_2, Y_4), (i) Form D (X_3, Y_4), (j) Form D (X_2, X_3), (k) Form E (X_3,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

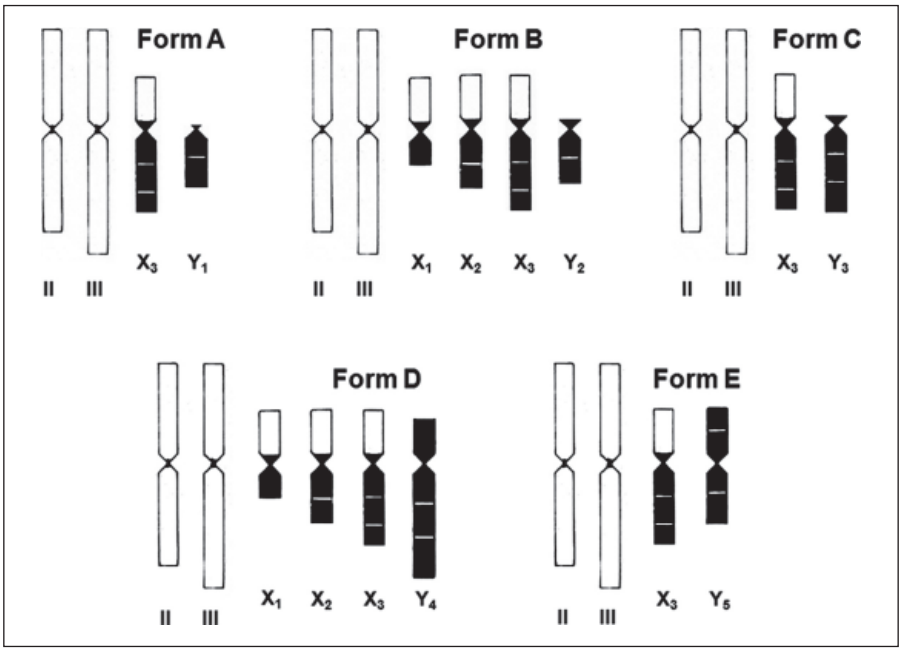

Figure 3. Diagrams of representative metaphase karyotypes of Forms A, B, C, D and E of *An. paraliae*

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

Hybridization experiment

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

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

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

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

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

DNA sequences and phylogenetic analysis

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

DISCUSSION

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

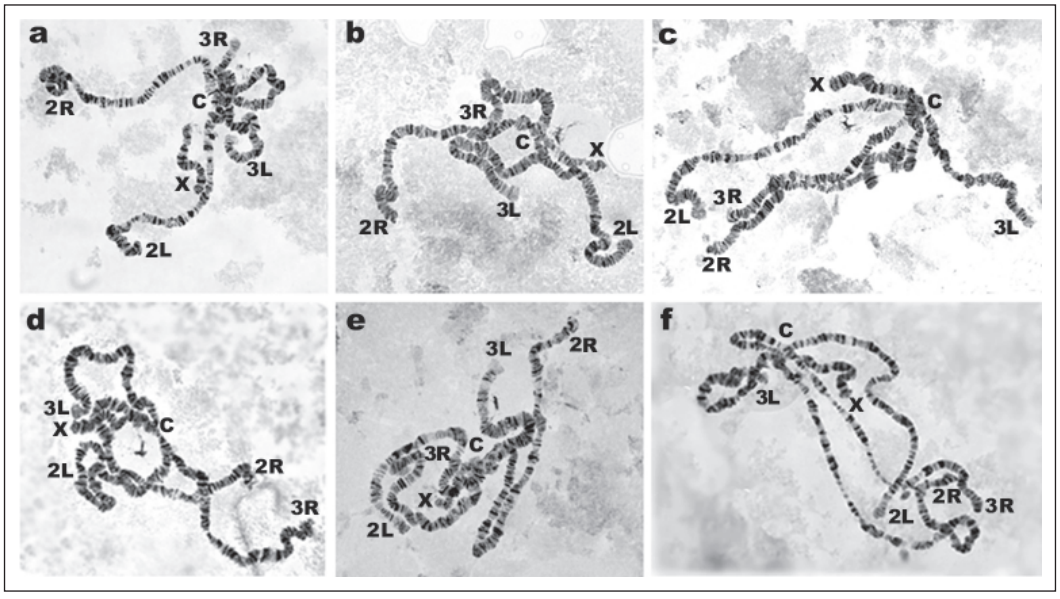

Figure 4. Complete synapsis in all arms of salivary gland polytene chromosomes of F_1-hybrids of *An. paraliae*. (a) Sk3A female x Ns1B male; (b) Sk3A female x Rt4B male; (c) Sk3A female x Ch1C male; (d) Sk3A female x Rt7D male; (e); Sk3A female x Rt8D male; (f) Sk3A female x Rt5E male

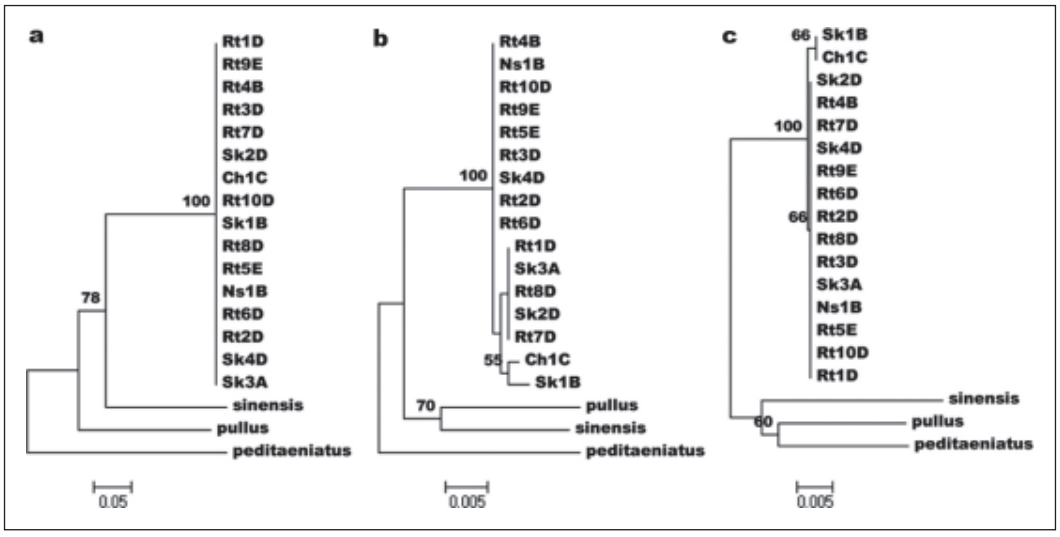

Figure 5. Neighbour-joining (NJ) trees inferred from sequences of 3 loci (a) ITS2, (b) COI and (c) COII of the 16 isolines of *An. paraliae* compared with *An. peditaeniatus*, *An. pullus* and *An. sinensis*. Numbers on branches are bootstrap values (%) after 1,000 replications. Bootstrap values under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

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

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

Acknowledgements. This work was supported by The Thailand Research Fund to W. Choochote and A. Saeung (TRF Senior Research Scholar: RTA5480006; TRF Advanced Research Scholar: BRG5380021), the Royal Golden Jubilee Ph.D. Program to W. Choochote and K. Taai (PHD/0297/2551) and Faculty of Medicine Endowment Fund, Chiang Mai University, Chiang Mai, Thailand.

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