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Geographic distribution and genetic compatibility among six karyotypic forms of *Anopheles peditaeniatus* (Diptera: Culicidae) in Thailand

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Abstract. Fifty-three isolines of *Anopheles peditaeniatus* were established from individual wild-caught females collected from cow-baited traps in 17 provinces of Thailand. Three types of X (X_1, X_2, X_3) and 6 types of Y (Y_1,Y_2, Y_3, Y_4, Y_5, Y_6) chromosomes were determined based on different amounts of major block(s) of heterochromatin. These sex chromosomes comprised 6 karyotypic forms 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), E (X_1, X_2, X_3,Y_5) and F (X_2, X_3, Y_6). Form F is a new metaphase karyotype discovered in this study and is commonly found in all regions. Form A was found only in Lampang province, whereas Form E is widespread throughout the country. Forms B, C and D were obtained from the northern, northeastern, western and southern regions. Crossing experiments among the 11 isoline colonies representing the 6 karyotypic forms of *An. peditaeniatus* indicated genetic compatibility yielding viable progenies and complete synapsis of salivary gland polytene chromosomes through to the F_2-generations. The results suggested the conspecific nature of these karyotypic forms which were further supported by very low intraspecific variation (genetic distance = 0.000-0.003) of nucleotide sequences in ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII).

INTRODUCTION

Anopheles (*Anopheles*) *peditaeniatus* belongs to the Lesteri Subgroup, Hyrcanus Group of the Myzorhynchus Series. It is widely distributed in Asia including Thailand, Myanmar, Cambodia, Indonesia, Malaysia, the Philippines, Vietnam, Borneo, Celebes, China, India, Sri Lanka and Nepal (Reid, 1968; Scanlon *et al.*, 1968; Harrison & Scanlon, 1975). *Anopheles peditaeniatus* is considered a suspected vector of human malaria, *Plasmodium vivax*, in Thailand (Gingrich *et al.*, 1990; Rattanarithikul *et al.*, 2006). In addition, it has been incriminated as a secondary vector of Japanese encephalitis virus in China and India (Zhang, 1990; Kanojia *et al.*, 2003). However, its status as a vector of the Japanese encephalitis virus remains a crucial question in Thailand, although it is widespread throughout the country. Due to the vicious biting-behavior of *An. peditaeniatus* on cattle and its ability to transmit filariae of the genus *Setaria*, it is

considered an economic pest (Reid *et al.*, 1962; Reid, 1968; Harrison & Scanlon, 1975). Although *An. peditaeniatus* has never been incriminated as natural and/or suspected vector in endemic areas of filariasis caused by *Brugia malayi*, it has been demonstrated to be a good experimental vector for transmitting this filarial nematode (Wharton *et al.*, 1963). Our recent experiment has shown that *An. peditaeniatus* exhibited high potential as a vector to *B. malayi* (Narathiwat province, southern Thailand strain) (unpublished data).

Cytologically, Baimai *et al.* (1993) found that *An. peditaeniatus* from Chiang Mai, Phrae and Chanthaburi provinces, Thailand showed different forms of mitotic sex chromosomes due to extra block(s) of heterochromatin. Recently, Choochote (2011) reported crossing experiments and DNA sequence analyses of internal transcribed spacer 2 (ITS2) of ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI) and cytochrome *c* oxidase subunit II (COII) of mitochondrial DNA (mtDNA) among 8 allopatric strains (Chiang Mai, Nan, Udon Thani, Ubon Ratchathani, Kamphaeng Phet, Ratchaburi, Chon Buri and Chumphon provinces) which represented 4 karyotypic forms (B, C, D and E) of *An. peditaeniatus* in Thailand.

We report herein a new karyotypic form of *An. peditaeniatus*, crossing experiments among the 6 karyotypic forms and comparative DNA sequencing of the ITS2, COI and COII regions of 53 isolines obtained from different populations in Thailand.

MATERIALS AND METHODS

Field collections and establishment of isoline colonies

Wild-caught, fully engorged females of *An. peditaeniatus* were collected from cow-baited traps in 17 provinces of Thailand (Fig. 1, Table 1). A total of 53 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 in this study.

Metaphase karyotype preparation

Metaphase chromosomes were prepared from 10 samples of early 4th instar larval brains of F_1- and/or F_2-progenies of each isoline of *An. peditaeniatus* using the techniques previously described by Saeung *et al.* (2007). Identification of karyotypic forms followed the standard cytotaxonomic key of Baimai *et al.* (1993).

Crossing experiments

Eleven laboratory-raised isolines of *An. peditaeniatus* were selected arbitrarily from the 53 isolines representing the 6 karyotypic forms, i.e., Forms A (Lp3A), B (Nk1B, Ns4B), C (Cb3C, Ns8C), D (Ur5D, Ns5D), E (Cb5E, Tg3E) and F (Sb2F, Tg1F) (Table 2). These isolines were used for crossing experiments to determine post-mating reproductive isolation by employing the techniques previously reported by Saeung *et al.* (2007). The salivary gland polytene chromosomes of 4th instar larvae of F_1-hybrids from the crosses were investigated using the techniques described by Kanda (1979). Polytene chromosome arms were identified by comparing them with the euchromatic arms of mitotic karyotypes. The shortest chromosome is X; the autosomal long arms are designated as 2R and 3R, and short arms as 2L and 3L (White *et al.*, 1975).

DNA extraction and amplification

One individual F_1-progeny adult female from each isoline of each *An. peditaeniatus* Form (A-F) was used for DNA extraction and amplification. Genomic DNA was extracted from each individual adult mosquito using DNeasy® Blood and Tissue Kit (Qiagen). The ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2), and the mitochondrial cytochrome *c* oxidase subunit I (COI) and subunit II (COII) were amplified using the primers described previously by Park *et al.* (2008b). The sequence data of this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB714987-AB715145. The ITS2, COI and COII sequences obtained from this study were also compared with deposited sequences available through GenBank (Table 1).

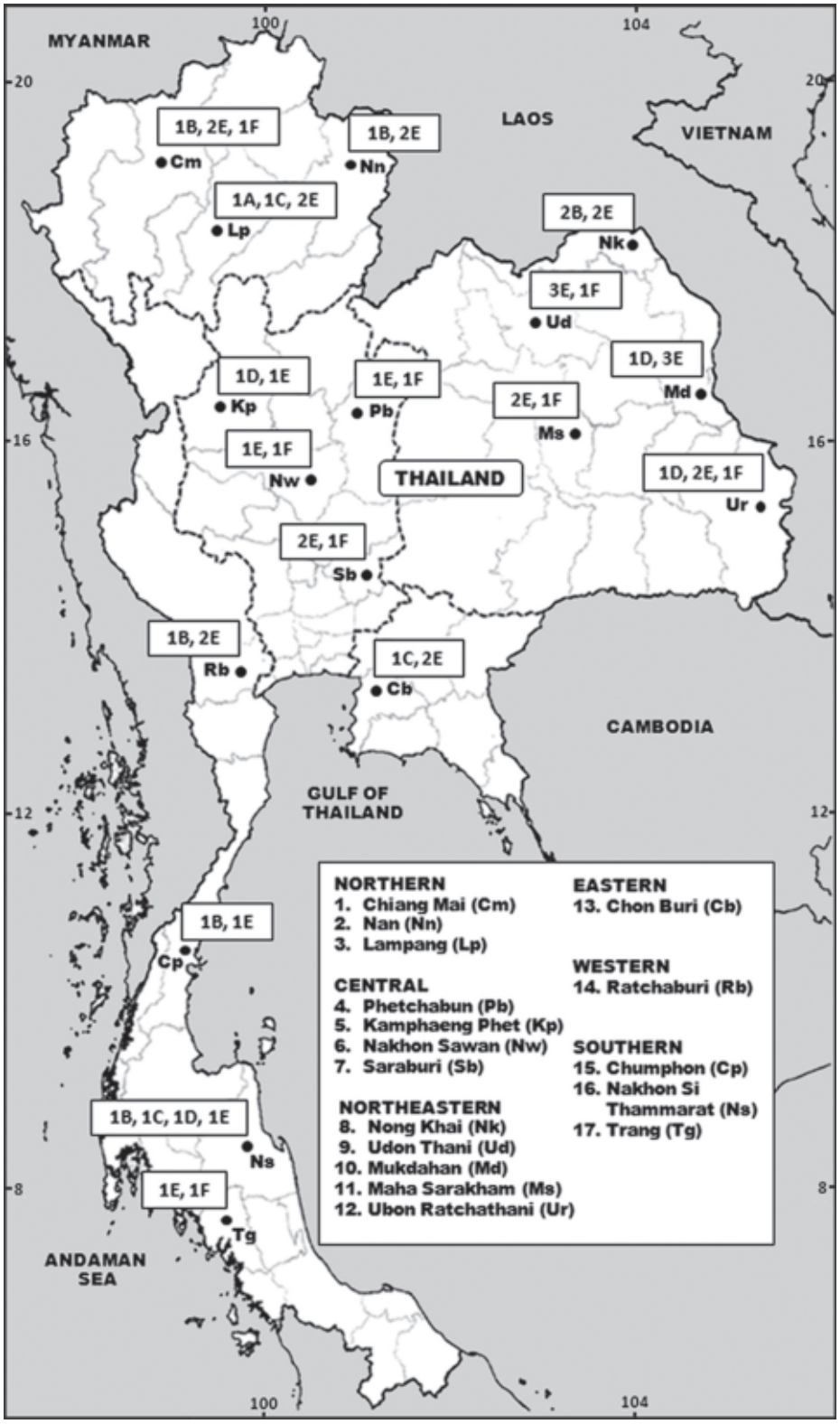

Figure 1. Map of Thailand showing 17 provinces where samples of *An. peditaeniatus* were collected and the number of isolines of the 6 karyotypic forms (A-F) detected in each location

Table 1. Locations (17 provinces of Thailand), code of isolines, karyotypic forms of *Anopheles peditaeniatus* and their GenBank accession numbers

Location (Geographical coordinate)	Code of isoline[a]	Karyotypic form	DNA Region	Genbank accession number			Reference
				ITS2	COI	COII	
An. peditaeniatus							
1. Chiang Mai	Cm2E	E (X_3, Y_5)	ITS2, COI, COII	AB714987	AB715040	AB715093	This study
(18° 47' N, 98° 59' E)	Cm3F	F (X_3, Y_6)	ITS2, COI, COII	AB714988	AB715041	AB715094	This study
	Cm4E	E (X_1, Y_5)	ITS2, COI, COII	AB714989	AB715042	AB715095	This study
	Cm7B	B (X_2, Y_2)	ITS2, COI, COII	AB714990	AB715043	AB715096	This study
2. Nan	Nn1E	E (X_3, Y_5)	ITS2, COI, COII	AB714991	AB715044	AB715097	This study
(18° 48' N, 100° 45' E)	Nn2B	B (X_2, Y_2)	ITS2, COI, COII	AB714992	AB715045	AB715098	This study
	Nn3E	E (X_3, Y_5)	ITS2, COI, COII	AB714993	AB715046	AB715099	This study
3. Lampang	Lp1E	E (X_3, Y_5)	ITS2, COI, COII	AB714994	AB715047	AB715100	This study
(17° 53' N, 99° 20' E)	Lp3A[a]	A (X_3, Y_1)	ITS2, COI, COII	AB714995	AB715048	AB715101	This study
	Lp4E	E (X_3, Y_5)	ITS2, COI, COII	AB714996	AB715049	AB715102	This study
	Lp5C	C (X_3, Y_3)	ITS2, COI, COII	AB714997	AB715050	AB715103	This study
4. Phetchabun	Pb5F	F (X_2, Y_6)	ITS2, COI, COII	AB714998	AB715051	AB715104	This study
(16° 25' N, 101° 08' E)	Pb9E	E (X_1, Y_5)	ITS2, COI, COII	AB714999	AB715052	AB715105	This study
5. Kamphaeng Phet	Kp2E	E (X_2, Y_5)	ITS2, COI, COII	AB715000	AB715053	AB715106	This study
(16° 50' N, 99° 04' E)	Kp5D	D (X_3, Y_4)	ITS2, COI, COII	AB715001	AB715054	AB715107	This study
6. Nakhon Sawan	Nw5E	E (X_2, Y_5)	ITS2, COI, COII	AB715002	AB715055	AB715108	This study
(15° 35' N, 100° 10' E)	Nw6F	F (X_3, Y_6)	ITS2, COI, COII	AB715003	AB715056	AB715109	This study
7. Saraburi	Sb2F[a]	F (X_3, Y_6)	ITS2, COI, COII	AB715023	AB715076	AB715129	This study
(14° 30' N, 100° 55' E)	Sb7E	E (X_3, Y_5)	ITS2, COI, COII	AB715024	AB715077	AB715130	This study
	Sb8E	E (X_2, Y_5)	ITS2, COI, COII	AB715025	AB715078	AB715131	This study
8. Nong Khai	Nk1B[a]	B (X_1, Y_2)	ITS2, COI, COII	AB715004	AB715057	AB715110	This study
(17° 50' N, 102° 46' E)	Nk2E	E (X_2, Y_5)	ITS2, COI, COII	AB715005	AB715058	AB715111	This study
	Nk4B	B (X_3, Y_2)	ITS2, COI, COII	AB715006	AB715059	AB715112	This study
	Nk6E	E (X_3, Y_5)	ITS2, COI, COII	AB715007	AB715060	AB715113	This study
9. Udon Thani	Ud2E	E (X_3, Y_5)	ITS2, COI, COII	AB715008	AB715061	AB715114	This study
(17° 24' N, 102° 47' E)	Ud3E	E (X_2, Y_5)	ITS2, COI, COII	AB715009	AB715062	AB715115	This study
	Ud5F	F (X_3, Y_6)	ITS2, COI, COII	AB715010	AB715063	AB715116	This study
	Ud6E	E (X_2, Y_5)	ITS2, COI, COII	AB715011	AB715064	AB715117	This study
10. Mukdahan	Md1E	E (X_2, Y_5)	ITS2, COI, COII	AB715012	AB715065	AB715118	This study
(15° 24' N, 103° 16' E)	Md2E	E (X_3, Y_5)	ITS2, COI, COII	AB715013	AB715066	AB715119	This study
	Md4D	D (X_3, Y_4)	ITS2, COI, COII	AB715014	AB715067	AB715120	This study
	Md5E	E (X_3, Y_5)	ITS2, COI, COII	AB715015	AB715068	AB715121	This study

Table 1. (continued)

Location (Geographical coordinate)	Code of isoline[a]	Karyotypic form	DNA Region	Genbank accession number			Reference
				ITS2	COI	COII	
11. Maha Sarakham	Ms1E	E (X_2, Y_5)	ITS2, COI, COII	AB715016	AB715069	AB715122	This study
(15° 45' N, 103° 01' E)	Ms3E	E (X_2, Y_5)	ITS2, COI, COII	AB715017	AB715070	AB715123	This study
	Ms4F	F (X_3, Y_6)	ITS2, COI, COII	AB715018	AB715071	AB715124	This study
12. Ubon Ratchathani	Ur1F	F (X_3, Y_6)	ITS2, COI, COII	AB715019	AB715072	AB715125	This study
(15° 31' N, 105° 35' E)	Ur4E	E (X_2, Y_5)	ITS2, COI, COII	AB715020	AB715073	AB715126	This study
	Ur5D[a]	D (X_1, Y_4)	ITS2, COI, COII	AB715021	AB715074	AB715127	This study
	Ur6E	E (X_3, Y_5)	ITS2, COI, COII	AB715022	AB715075	AB715128	This study
13. Chon Buri	Cb3C[a]	C (X_3, Y_3)	ITS2, COI, COII	AB715029	AB715082	AB715135	This study
(13° 26' N, 101° 03' E)	Cb5E[a]	E (X_3, Y_5)	ITS2, COI, COII	AB715030	AB715083	AB715136	This study
	Cb8E	E (X_2, Y_5)	ITS2, COI, COII	AB715031	AB715084	AB715137	This study
14. Ratchaburi	Rb1B	B (X_3, Y_2)	ITS2, COI, COII	AB715026	AB715079	AB715132	This study
(13° 21' N, 99° 22' E)	Rb4E	E (X_2, Y_5)	ITS2, COI, COII	AB715027	AB715080	AB715133	This study
	Rb10E	E (X_2, Y_5)	ITS2, COI, COII	AB715028	AB715081	AB715134	This study
15. Chumphon	Cp2B	B (X_2, Y_2)	ITS2, COI, COII	AB715032	AB715085	AB715138	This study
(10° 29' N, 99° 11' E)	Cp7E	E (X_2, Y_5)	ITS2, COI, COII	AB715033	AB715086	AB715139	This study
16. Nakhon Si Thammarat	Ns1E	E (X_2, Y_5)	ITS2, COI, COII	AB715034	AB715087	AB715140	This study
(08° 29' N, 100° 0' E)	Ns4B[a]	B (X_2, Y_2)	ITS2, COI, COII	AB715035	AB715088	AB715141	This study
	Ns5D[a]	D (X_2, Y_4)	ITS2, COI, COII	AB715036	AB715089	AB715142	This study
	Ns8C[a]	C (X_3, Y_3)	ITS2, COI, COII	AB715037	AB715090	AB715143	This study
17. Trang	Tg1F[a]	F (X_2, Y_6)	ITS2, COI, COII	AB715038	AB715091	AB715144	This study
(07° 33' N, 99° 38' E)	Tg3E[a]	E (X_3, Y_5)	ITS2, COI, COII	AB715039	AB715092	AB715145	This study
Ratchaburi (13° 21' N, 99° 22' E)	RbB	B (X_3, Y_2)	ITS2, COI, COII	AB539061	AB539069	AB539077	Choochote, 2011
China	–	–	ITS2	AY129958	–	–	Ma & Xu, 2005
An. sinensis							
Korea	–	–	ITS2	EU789790	–	–	Park *et al.*, 2008a
	–	B (X, Y_2)	COI	–	AY444351	–	Park *et al.*, 2003
	i1BKR	B (X, Y_2)	COII	–	–	AY130464	Min *et al.*, 2002
An. lesteri							
Korea	–	–	ITS2	EU789791	–	–	Park *et al.*, 2008a
China	–	–	COI	–	EU699048	–	Yang & Ma, 2009
	–	–	COII	–	–	AY753146	Ma *et al.* (unpublished data)

Table 2. Crossing experiments among 11 isolines of *An. peditaeniatus*

Crosses (Female x Male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
Parental cross							
Lp3A x Lp3A	256 (123, 133)	86	218 (85.16)	207 (94.95)	192 (92.75)	91 (47.40)	101 (52.60)
Nk1B x Nk1B	281 (155, 126)	81	222 (79.00)	175 (78.83)	175 (100.00)	86 (49.14)	89 (50.86)
Ns4B x Ns4B	281 (150, 131)	83	228 (81.14)	217 (95.18)	217 (100.00)	111 (51.15)	106 (48.85)
Cb3C x Cb3C	374 (130, 244)	91	314 (83.96)	308 (98.09)	308 (100.00)	163 (52.92)	145 (47.08)
Ns8C x Ns8C	381 (212, 169)	94	343 (90.03)	333 (97.08)	333 (100.00)	176 (52.85)	157 (47.15)
Ur5D x Ur5D	261 (157, 104)	77	193 (73.95)	193 (100.00)	193 (100.00)	95 (49.22)	98 (50.78)
Ns5D x Ns5D	300 (148, 152)	100	282 (94.00)	265 (93.97)	259 (97.74)	120 (46.33)	139 (53.67)
Cb5E x Cb5E	385 (296, 89)	86	316 (82.08)	272 (86.08)	258 (94.85)	129 (50.00)	129 (50.00)
Tg3E x Tg3E	316 (169, 147)	81	246 (77.85)	234 (95.12)	229 (97.86)	108 (47.16)	121 (52.84)
Sb2F x Sb2F	299 (179, 120)	90	257 (85.95)	203 (78.99)	201 (99.01)	103 (51.24)	98 (48.76)
Tg1F x Tg1F	335 (220, 115)	79	251 (74.92)	206 (82.07)	206 (100.00)	107 (51.94)	99 (48.06)
Reciprocal cross							
Lp3A x Nk1B	332 (152, 180)	90	282 (84.94)	279 (98.94)	279 (100.00)	144 (51.61)	135 (48.39)
Nk1B x Lp3A	358 (201, 157)	95	322 (89.94)	306 (95.03)	291 (95.10)	142 (48.80)	149 (51.20)
Lp3A x Ns4B	360 (144, 216)	89	292 (81.11)	257 (88.01)	257 (100.00)	128 (49.80)	129 (50.20)
Ns4B x Lp3A	438 (330, 108)	80	342 (78.08)	291 (85.09)	291 (100.00)	131 (45.02)	160 (54.98)
Lp3A x Cb3C	331 (238, 93)	97	298 (90.03)	268 (89.93)	263 (98.13)	132 (50.19)	131 (49.81)
Cb3C x Lp3A	364 (181, 183)	100	338 (92.86)	314 (92.90)	298 (94.90)	146 (48.99)	152 (51.01)
Lp3A x Ns8C	360 (235, 125)	87	288 (80.00)	285 (98.96)	259 (90.88)	135 (52.12)	124 (47.88)
Ns8C x Lp3A	367 (210, 157)	84	301 (82.02)	301 (100.00)	289 (96.01)	142 (49.13)	147 (50.87)
Lp3A x Ur5D	425 (170, 255)	95	374 (88.00)	366 (97.86)	366 (100.00)	190 (51.91)	176 (48.09)
Ur5D x Lp3A	360 (187, 173)	88	302 (83.89)	266 (88.08)	263 (98.87)	126 (47.91)	137 (52.09)
Lp3A x Ns5D	400 (215, 185)	85	328 (82.00)	279 (85.06)	259 (92.83)	130 (50.19)	129 (49.81)
Ns5D x Lp3A	335 (127, 208)	89	281 (83.88)	267 (95.02)	267 (100.00)	139 (52.06)	128 (47.94)
Lp3A x Cb5E	377 (200, 177)	92	328 (87.00)	285 (86.89)	276 (96.84)	127 (46.01)	149 (53.99)
Cb5E x Lp3A	329 (200, 129)	85	263 (79.94)	208 (79.09)	206 (99.04)	105 (50.97)	101 (49.03)
Lp3A x Tg3E	318 (190, 128)	79	248 (77.99)	236 (95.16)	236 (100.00)	120 (50.85)	116 (49.15)
Tg3E x Lp3A	272 (189, 83)	82	212 (77.94)	189 (89.15)	189 (100.00)	94 (49.74)	95 (50.26)
Lp3A x Sb2F	335 (161, 174)	88	268 (80.00)	268 (100.00)	268 (100.00)	137 (51.12)	131 (48.88)
Sb2F x Lp3A	335 (96, 239)	88	281 (83.88)	258 (91.81)	240 (93.02)	125 (52.08)	115 (47.92)
Lp3A x Tg1F	303 (208, 95)	75	224 (73.93)	208 (92.86)	208 (100.00)	100 (48.08)	108 (51.92)
Tg1F x Lp3A	295 (189, 106)	97	271 (91.86)	238 (87.82)	238 (100.00)	112 (47.06)	126 (52.94)

Table 2. (continued)

Crosses (Female x Male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
F_1- hybrid cross							
(Lp3A x Nk1B)F_1 x (Lp3A x Nk1B)F_1	306 (162, 144)	93	272 (88.89)	245 (90.07)	235 (95.92)	115 (48.94)	120 (51.06)
(Nk1B x Lp3A)F_1 x (Nk1B x Lp3A)F_1	333 (215, 118)	83	263 (78.98)	250 (95.06)	250 (100.00)	120 (48.00)	130 (52.00)
(Lp3A x Ns4B)F_1 x (Lp3A x Ns4B)F_1	306 (137, 169)	88	260 (84.97)	250 (96.15)	245 (98.00)	118 (48.16)	127 (51.84)
(Ns4B x Lp3A)F_1 x (Ns4B x Lp3A)F_1	279 (156, 123)	93	246 (88.17)	231 (93.90)	226 (97.83)	115 (50.88)	111 (49.12)
(Lp3A x Cb3C)F_1 x (Lp3A x Cb3C)F_1	313 (214, 99)	98	288 (92.01)	248 (86.11)	246 (99.19)	116 (47.15)	130 (52.85)
(Cb3Cx Lp3A)F_1 x (Cb3C x Lp3A)F_1	367 (241, 126)	89	297 (80.93)	276 (92.93)	262 (94.93)	131 (50.00)	131 (50.00)
(Lp3Ax Ns8C)F_1 x (Lp3A x Ns8C)F_1	351 (198, 153)	90	281 (80.06)	244 (86.83)	244 (100.00)	129 (52.87)	115 (47.13)
(Ns8C x Lp3A)F_1 x (Ns8C x Lp3A)F_1	391 (135, 256)	89	317 (81.07)	276 (87.07)	262 (94.93)	123 (46.95)	139 (53.05)
(Lp3A x Ur5D)F_1 x (Lp3A x Ur5D)F_1	362 (233, 129)	82	290 (80.11)	281 (96.90)	281 (100.00)	141 (50.18)	140 (49.82)
(Ur5D x Lp3A)F_1 x (Ur5D x Lp3A)F_1	436 (201, 235)	90	373 (85.55)	332 (89.01)	325 (97.89)	150 (46.15)	175 (53.85)
(Lp3A x Ns5D)F_1 x (Lp3A x Ns5D)F_1	388 (222, 166)	85	318 (81.96)	315 (99.06)	312 (99.05)	162 (51.92)	150 (48.08)
(Ns5D x Lp3A)F_1 x (Ns5D x Lp3A)F_1	349 (167, 182)	97	311 (89.11)	302 (97.11)	302 (100.00)	148 (49.01)	154 (50.99)
(Lp3A x Cb5E)F_1 x (Lp3A x Cb5E)F_1	320 (230, 90)	84	262 (81.88)	223 (85.11)	223 (100.00)	120 (53.81)	103 (46.19)
(Cb5E x Lp3A)F_1 x (Cb5E x Lp3A)F_1	335 (221, 114)	90	288 (85.97)	262 (90.97)	254 (96.95)	119 (46.85)	135 (53.15)
(Lp3A x Tg3E)F_1 x (Lp3A x Tg3E)F_1	413 (172, 241)	88	326 (78.93)	313 (96.01)	313 (100.00)	156 (49.84)	157 (50.16)
(Tg3E x Lp3A)F_1 x (Tg3E x Lp3A)F_1	288 (116, 172)	92	242 (84.03)	203 (83.88)	199 (98.03)	105 (52.76)	94 (47.24)
(Lp3A x Sb2F)F_1 x (Lp3A x Sb2F)F_1	379 (212, 167)	79	292 (77.04)	277 (94.86)	277 (100.00)	141 (50.90)	136 (49.10)
(Sb2F x Lp3A)F_1 x (Sb2F x Lp3A)F_1	379 (202, 177)	87	311 (82.06)	246 (79.10)	246 (100.00)	123 (50.00)	123 (50.00)
(Lp3A x Tg1F)F_1 x (Lp3A x Tg1F)F_1	273 (169, 104)	85	218 (79.85)	207 (94.95)	188 (90.82)	98 (52.13)	90 (47.87)
(Tg1F x Lp3A)F_1 x (Tg1F x Lp3A)F_1	325 (181, 144)	90	263 (80.92)	258 (98.10)	245 (94.96)	120 (48.98)	125 (51.02)

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

Sequencing alignment and phylogenetic analysis

Sequences of ITS2, COI and COII were aligned using the CLUSTAL W multiple alignment program (Thompson *et al*., 1994). Gap sites were excluded from the following analysis. The Kimura two-parameter method was used to calculate genetic distances (Kimura, 1980). Construction of neighbor-joining trees (Saitou & Nei, 1987) and the bootstrap test with 1,000 replications were conducted with the MEGA version 4.0 program (Tamura *et al*., 2007).

RESULTS

Karyotypic characters

Cytogenetic observations of F_1- and/or F_2-progenies of the 53 isolines of *An. peditaeniatus* revealed different types of sex chromosomes due to the addition of extra block(s) of heterochromatin. There were 3 types of X (metacentric X_1, small submetacentric X_2 and large submetacentric X_3) and 6 types of Y chromosomes (very small telocentric Y_1, medium telocentric Y_2, large telocentric Y_3, very large telocentric Y_4, submetacentric Y_5 and medium metacentric Y_6) (Fig. 2). These types of X and Y chromosomes comprise 6 forms of mitotic karyotypes on the basis of Y chromosome configurations 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), E (X_1, X_2, X_3,Y_5) and F (X_2, X_3, Y_6). The number of isolines of these karyotypic forms occurring in different localities in 17 provinces of Thailand are shown in Fig. 1 and Table 1. Form A (X_3, Y_1) was very rare and has been detected only in Lampang province. On the other hand, Form E was quite common throughout the species' distribution in Thailand, while Forms B, C, D and F were found sporadically in several localities (Fig. 1).

Crossing experiments

Details of hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal and F_1-hybrid crosses among the 11 isolines of *An. peditaeniatus* representing Forms A-F are shown in Table 2. All crosses yielded viable progenies through to 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 4th instar larvae of F_1-hybrids from all crosses showed complete synapsis without inversion loops along the whole lengths of all autosomes and the X chromosome (Fig. 3).

DNA sequences and phylogenetic analysis

DNA sequences were determined and analyzed for the ITS2, COI and COII regions of the 53 isolines of *An. peditaeniatus* Forms A–F. They all showed the same length for the ITS2 (463 bp), COI (548 bp) and COII (672 bp) sequences. The evolutionary relationships among the 6 karyotypic forms using neighbour-joining trees were constructed (Fig. 4). The average genetic distances within and between the 6 karyotypic forms exhibited no significant difference in these DNA regions (genetic distance = 0.000-0.003). Hence, the 53 isolines were placed within a single species namely *An. peditaeniatus*. Additionally, these isolines showed little genetic distance difference (0.000-0.005) from *An. peditaeniatus* Form B from Ratchaburi province previously reported by Choochote (2011). However, the trees for ITS2, COI and COII of these isolines representing Forms A-F were clearly different from *An. sinensis* from Korea and *An. lesteri* from Korea and China with strongly supported bootstrap values (99-100%) (Fig. 4).

DISCUSSION

The first cytogenetic investigations of 27 isolines of *An. peditaeniatus* from 3 different localities in Thailand (Chiang Mai, Phrae and Chanthaburi provinces) were performed by Baimai *et al.* (1993). They showed that *An. peditaeniatus* exhibited karyotypic variation via a gradual increase of extra heterochromatin on X (X_1, X_2, X_3) and Y (Y_1, Y_2, Y_3, Y_4, Y_5) chromosomes. Recently,

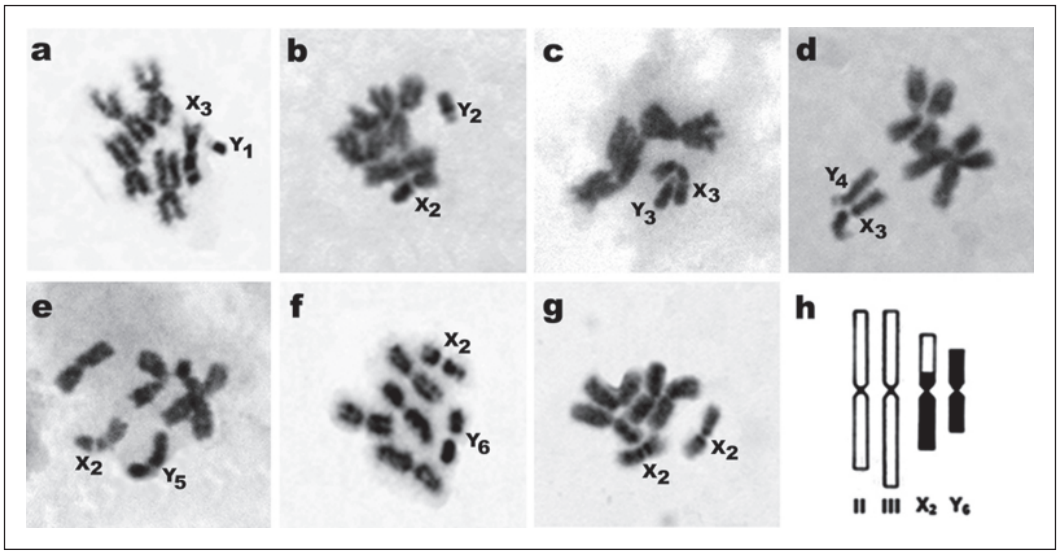

Figure 2. Metaphase karyotypic forms of *An. peditaeniatus*. (a) Form A (X_3, Y_1: Lampang); (b) Form B (X_2, Y_2: Chumphon); (c) Form C (X_3, Y_3: Chon Buri); (d) Form D (X_3, Y_4: Mukdahan); (e) Form E (X_2, Y_5: Nakhon Sawan); (f) Form F (X_2, Y_6: Trang); (g) Form F (X_2, X_2: Trang); (h) diagrams of representative metaphase karyotype of Form F

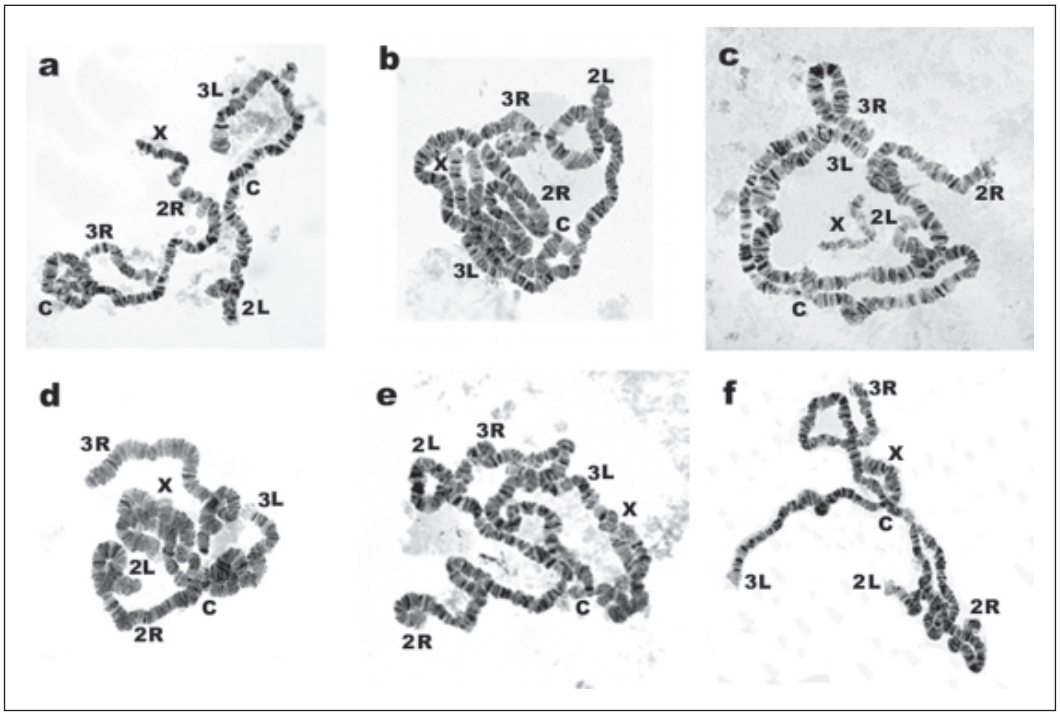

Figure 3. Complete synapsis in all arms of salivary gland polytene chromosome of F_1-hybrids of *An. peditaeniatus*. (a) Lp3A female x Nk1B male; (b) Lp3A female x Cb3C male; (c) Lp3A female x Ur5D male; (d) Lp3A female x Tg3E male; (e) Lp3A female x Sb2F male; (f) Lp3A female x Tg1F male

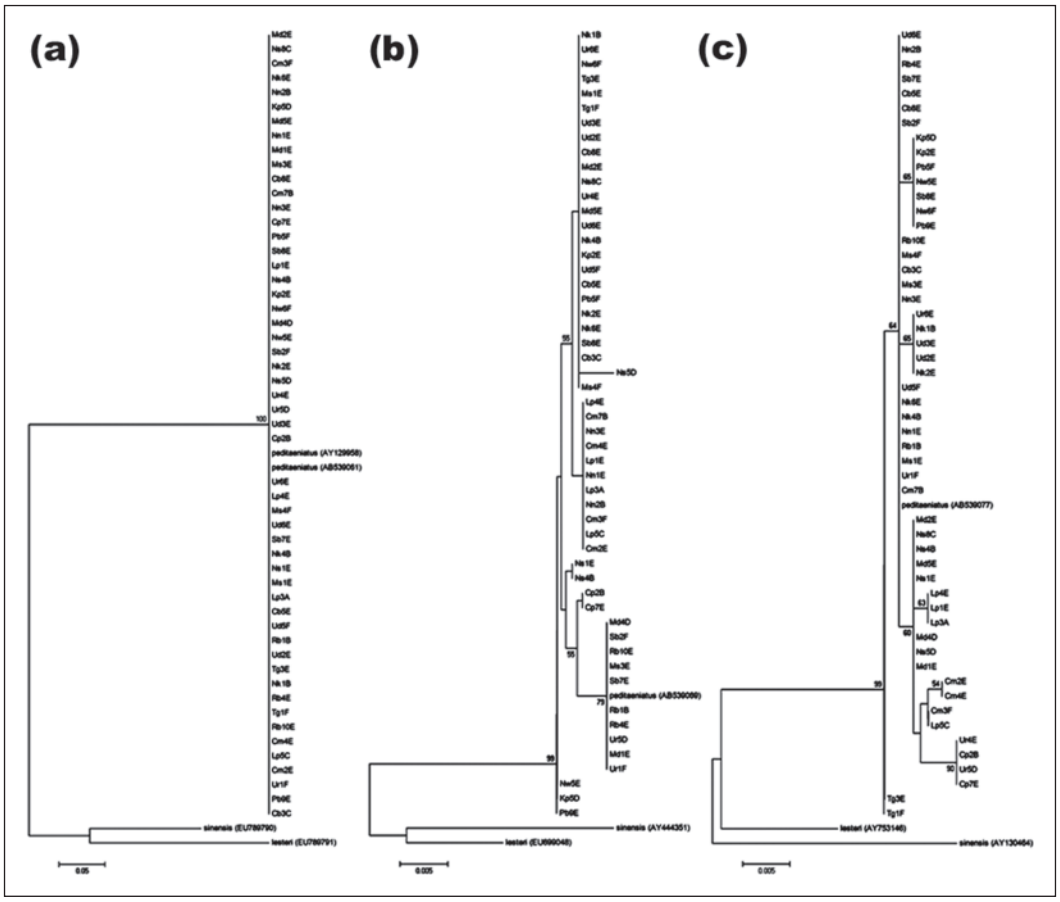

Figure 4. Phylogenetic relationships among the 53 isolines of *An. pediataeniatus* based on molecular analysis compared with *An. sinensis* and *An. lesteri*. (a) ITS2; (b) COI; (c) COII. The trees were generated by neighbor-joining analysis. Numbers on branches are bootstrap values (%) after 1,000 replications. Bootstrap values under 50% are not shown. Branch lengths are proportional to genetic distance (scale bar)

Choochote (2011) studied mitotic karyotypes of *An. peditaeniatus* from 8 different localities in Thailand (Chiang Mai, Nan, Kamphaeng Phet, Udon Thani, Ubon Ratchathani, Chon Buri, Ratchaburi and Chumphon provinces) and demonstrated 2 types of X (X_2, X_3) and 4 types of Y (Y_2, Y_3, Y_4, Y_5) chromosomes forming 4 karyotypic forms tentatively designated as Forms B (X_2, X_3, Y_2), C (X_3, Y_3), D (X_3, Y_4) and E (X_2, X_3,Y_5). In this study, we have detected 3 types of X (X_1, X_2, X_3) and 6 types of Y (Y_1, Y_2, Y_3, Y_4, Y_5, Y_6) chromosomes forming 6 karyotypic forms, i.e., 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). The newly discovered Form F in this study was based on the medium metacentric Y_6 chromosome which was obviously different from the other 5 types previously reported by Baimai *et al.* (1993) and Choochote (2011). Clearly, the 6 distinct karyotypic forms of *An. peditaeniatus* were due to a gain in extra heterochromatin within sex chromosomes. The phenomenon of accumulation of heterochromatin in the genome has played an important role in karyotype evolution, at least in dipteran insects (Baimai, 1998). In addition, such a chromosome difference is very useful for cytotaxonomic study of closely related species particularly sibling species as exemplified in the *Anopheles dirus* complex (Baimai, 1988) and other groups of *Anopheles* (Kanda *et al.*, 1981; Baimai *et al.*, 1987;

Subbarao, 1998; Junkum *et al*., 2005). In this study, we found an ancestral Form A (X_3, Y_1) in only one isoline from Lampang province, while Forms B, C, D, E and F were widespread in Thailand.

Crossing experiments using isoline colonies of anopheline mosquitoes to determine post-mating reproductive compatibility have proven to be efficient techniques for recognition of sibling species within the Oriental *Anopheles* (Kanda *et al*., 1981; Baimai *et al*., 1987; Subbarao, 1998; Junkum *et al*., 2005). In this regard, intensive crossing experiments among the 6 allopatric karyotypic forms of *An. peditaeniatus* showed no post-mating reproductive isolation. Hence, these results strongly suggested a conspecific nature of these karyotypic forms of *An. peditaeniatus*. Identical and/or very low intraspecific sequence variations (genetic distance = 0.000-0.003) of ITS2, COI and COII of the 6 karyotypic forms provided good supportive evidence. Thus our findings are in agreement with the results of hybridization experiments among the 4 karyotypic forms of *An. peditaeniatus* in Thailand previously reported by Choochote (2011). Similar studies on other anopheline species have been reported, e.g., *Anopheles vagus* (Choochote *et al*., 2002), *Anopheles pullus* (= *An. yatsushiroensis*) (Park *et al*., 2003), *Anopheles sinensis* (Choochote *et al*., 1998; Min *et al*., 2002; Park *et al*., 2008b), *Anopheles aconitus* (Junkum *et al*., 2005), *Anopheles barbirostris* species A1 and A2 (Saeung *et al*., 2007; Suwannamit *et al*., 2009), and an *Anopheles campestris*-like taxon (Thongsahuan *et al*., 2009). Thus, karyotypic variation based on extra heterochromatin in sex chromosomes seems to be a general phenomenon within the Oriental *Anopheles*.

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