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Cytogenetic, crossing and molecular evidence of two cytological forms of *Anopheles argyropus* and three cytological forms of *Anopheles pursati* (Diptera: Culicidae) in Thailand

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Abstract. Nine and 11 isolines of *Anopheles argyropus* and *Anopheles pursati*, respectively, were established from individual females collected from cow-baited traps, and the characteristics of metaphase chromosomes were investigated in their F_1-progenies. As determined by the different amounts of extra heterochromatin on sex chromosomes, 2 types of X (X_1, X_2) and Y (Y_1,Y_2), and 2 types of X (X_1, X_2) and 3 types of Y (Y_1, Y_2, Y_3) chromosomes were obtained from *An. argyropus* and *An. pursati*, respectively. These types of sex chromosomes comprised 2 [Forms A (X_1, Y_1) and B (X_1, X_2, Y_2)] and 3 [Forms A (X_1, X_2, Y_1), B (X_1, X_2, Y_2) and C (X_2, Y_3)] karyotypic forms of *An. argyropus* and *An. pursati*, respectively. All karyotypic forms acquired from *An. pursati* are new one that were discovered in this study, of which Forms A, B and C were found generally in Chiang Mai Province, while only 1 isoline of Form B was obtained in Ratchaburi Province. Form A was recovered from *An. argyropus* only in Ubon Ratchathani Province, whereas Form B from that species was found commonly in both Ubon Rathchathani and Nakhon Si Thammarat Provinces. Crossing experiments among the 2 and 3 isolines representing 2 and 3 karyotypic forms of *An. argyropus* and *An. pursati*, respectively, indicated genetic compatibility in yielding viable progenies and synaptic salivary gland polytene chromosomes through F_2-generations. The conspecific natures of these karyotypic forms in both species were further supported by very low intraspecific sequence variations (average genetic distance: *An. argyropus* = 0.003-0.007, *An. pursati* = 0-0.005) of ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII).

INTRODUCTION

Anopheles argyropus and *Anopheles pursati* belong to the subgenus *Anopheles* of the Hyrcanus Group and Myzorhynchus Series. *An. argyropus* is distributed widely in Thailand and other countries in Asia, i.e., India (Assam), Vietnam, Myanmar, Cambodia, Malaysia (Malaysian Peninsular) and Indonesia (Java and Sumatra). Regarding *An. pursati*, the distribution of this anopheline species has been recorded so far from Thailand, Vietnam, Cambodia and Malaysia (Malaysian Peninsular) (Reid, 1968; Scanlon *et al.*, 1968; Harrison & Scanlon, 1975; Knight & Stone, 1977; Oo *et al.*, 2006; Rattanarithikul *et al.*, 2006; Harbach, 2013). With regard to medical importance, these 2 anopheline species have never been incriminated as natural, suspected or

potential vectors of any human diseases. However, *An. pursati* was reported recently as a high potential vector for nocturnally subperiodic *Brugia malayi*, as determined by a 60% susceptibility rate and 3.83 (1-11) parasite load (Saeung *et al.*, 2013). Furthermore, these 2 anopheline species are considered as economic pests of livestock, due to their vicious and massive biting behavior when taking blood meals from cattle (Reid *et al.*, 1962; Reid, 1968; Harrison & Scanlon, 1975).

Regarding metaphase chromosome investigations, two karyotypic forms of *An. argyropus*, i.e., Forms A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2), were first reported from Chiang Mai and Phrae Provinces (northern Thailand), and Chiang Mai Province and Chanthaburi Province (eastern Thailand), respectively (Baimai *et al.*, 1993). These 2 karyotypic variants clearly appeared to result from a gradual increase in the extra heterochromatin on X and Y chromosomes. The genetic variation at the chromosomal level, within the taxon *Anopheles* species, potentially results in the existence of species complex and causes difficulty in identifying sibling species (isomorphic species) and/or subspecies (cytological forms/races) members of the complex that results from identical morphology or minimal morphological distinction. Additionally, those members of each complex may differ in biological characteristics (e.g., microhabitats, resting and biting behavior, sensitivity or resistance to insecticides, susceptible or refractory to pathogens, etc.), which can be used to determine their vectorial capacity (Subbarao, 1998; Choochote & Saeung, 2013). Thus, inaccurate identification of individual members within the taxon *Anopheles* species complex may result in failure to distinguish between a vector and non-vector species, and lead to complications and/or unsuccessful vector control-approaches. A recent good example was reported on *Anopheles barbirostris* complex in Thailand, which emphasized on the significance of *Anopheles* species complex status. These reports comprised 5 sibling species members (*Anopheles campestris*-like and *An. barbirostris* species A1, A2, A3 and A4), all of which exhibited identical morphology at the adult stage, and only the branch summation of seta 2-VI of pupal skins could be used to separate *An. campestris*-like from *An. barbirostris* species A1, A2, A3 and A4 (average summation of seta 2-VI: *An. campestris*-like = 22.40–24.50 branches; *An. barbirostris* species A1, A2, A3 and A4 = 9.2–16.40 branches) (Harrison & Scanlon, 1975; Saeung *et al.*, 2007, 2008; Suwannamit *et al.*, 2009; Thongsahuan *et al.*, 2009). Regarding distribution and biting behavior, *An. campestris*-like was found mostly in flat plain localities and it chose to bite humans, while *An. barbirostris* species A1, A2, A3 and A4 were rather confined in mountainous areas and they preferred to bite on cattle. Furthermore, *An. campestris*-like was a high potential vector for *Plasmodium vivax*, whereas *An. barbirostris* species A1, A2, A3 and A4 were very low potential vectors (Thongsahuan *et al.*, 2011).

Regarding the above information, very little is known about the genetic proximities among 2 karyotypic variants of *An. argyropus*, and there is a complete lack of karyotypic information of *An. pursati* in a systematic direction. Therefore, this study is the first to report, 3 new karyotypic forms [Forms A (X_1, X_2, Y_1), B (X_1, X_2, Y_2) and C (X_2, Y_3)] of *An. pursati*, and determine the genetic proximity among 2 and 3 karyotypic variants of *An. argyropus* and *An. pursati*, respectively, by crossing experiments related to comparative DNA sequencing of the 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

Wild-caught, fully engorged female mosquitoes of *An. argyropus* and *An. pursati* were collected from cow-baited traps. The *An. argyropus* mosquitoes were obtained from Ubon Rathchathani Province in the

northeastern region and Nakhon Si Thammarat Province in the southern region of Thailand. The *An. pursati* mosquitoes were acquired from Chiang Mai Province in the northern region and Ratchaburi Province in the western region of Thailand. A total of 9 and 11 isolines of *An. argyropus* and *An. pursati*, respectively, were established successfully and maintained in our insectary, using the techniques described by Choochote & Saeung (2013). Exact species identification was performed using intact morphology of egg, larval, pupal and adult stages from the F_1-progenies of isolines, by following the standard keys (Reid, 1968; Harrison & Scanlon, 1975; Rattanarithikul *et al.*, 2006). These isolines were used for studies on the metaphase karyotype, crossing experiment and molecular analysis.

Metaphase karyotype preparation

Metaphase chromosomes were prepared from 10 samples of the early fourth-instar larval brains of F_1-progenies of each isoline in *An. argyropus* and *An. pursati*, using the techniques described by Choochote & Saeung (2013). Identification of karyotypic forms followed the standard cytotaxonomic systems of Baimai *et al.* (1993).

Crossing experiment

The 2 and 3 laboratory-raised isolines of *An. argyropus* and *An. pursati*, respectively, were selected arbitrarily from the stock isoline colonies. They were Form A (X_1, Y_1; Ur1A) and B (X_2, Y_2; Ns5B) of *An. argyropus*, and Form A (X_1, Y_1; Cm1A), B (X_2, Y_2; Rt1B) and C (X_2, Y_3; Cm7C) of *An. pursati* (Table 1). These isolines were used for crossing experiments in order to determine post-mating barriers by employing the techniques reported by Choochote & Saeung (2013).

DNA extraction and PCR amplification

Total genomic DNA was isolated from individual F_1-progeny adult female of each isoline of *An. argyropus* and *An. pursati* (Table 1) using DNeasy® Blood and Tissue Kit (QIAGEN, Japan). Primers for amplification of ITS2, COI, and COII regions followed previous studies by Saeung *et al.* (2007). The ITS2 region of the rDNA was amplified using primer ITS2A (5'-TGT GAA CTG CAG GAC ACA T-3') and ITS2B (5'-TAT GCT TAA ATT CAGGGGGT-3') (Beebe & Saul, 1995). Amplification of the 709 bp fragment of mitochondrial COI barcoding region was conducted 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.* (1994). 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') (Sharpe *et al.*, 2000). Each PCR reaction was carried out in total of 20 µl volume containing 0.5 U *Ex Taq* (Takara, Japan), 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 program consisted of initial denaturation at 94ºC for 1 minute, 30 cycles at 94ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 1 minute, and a final extension at 72ºC for 5 minutes. The amplification profile of COI and COII comprised initial denaturation at 94ºC for 1 minute, 30 cycles at 94ºC for 30 seconds, 50ºC for 30 seconds, and 72ºC for 1 minute, and a final extension at 72ºC for 5 minutes. 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, Japan). The PCR products were sequenced in both directions using the BigDye® V3.1 Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems of Life Technologies, Japan).

Sequencing alignment and phylogenetic analysis

Sequences were aligned using the CLUSTAL W multiple alignment program (Thompson *et al.*, 1994) and edited manually in BioEdit version 7.0.5.3 (Hall, 1999). All positions containing gaps and missing data were excluded from the analysis. The Kimura two-parameter (K2P) model was employed to calculate genetic distances (Kimura, 1980). Using the distances, construction of neighbor-joining trees (Saitou & Nei, 1987) and the

Table 1. Locations, code of isolines, karyotypic forms of *Anopheles argyropus* and *An. pursati*, and their GenBank accession numbers

Location (Geograpical coordinate)	Code of isoline[a]	Karyotypic form	GenBank accession number			Reference
			ITS2	COI	COII	
An. argyropus						
Ubon Ratchathani	Ur1A[a]	A (X_1, Y_1)	AB826053	AB826073	AB826093	This study
(15° 312' N, 105° 352' E)	Ur2B	B (X_2, Y_2)	AB826054	AB826074	AB826094	This study
	Ur4B	B (X_2, Y_2)	AB826055	AB826075	AB826095	This study
Nakhon Si Thammarat	Ns5B[a]	B (X_2, Y_2)	AB826056	AB826076	AB826096	This study
(08° 292' N, 100° 02' E)	Ns8B	B (X_2, Y_2)	AB826057	AB826077	AB826097	This study
	Ns12B	B (X_2, Y_2)	AB826058	AB826078	AB826098	This study
	Ns19B	B (X_1, Y_2)	AB826059	AB826079	AB826099	This study
	Ns21B	B (X_2, Y_2)	AB826060	AB826080	AB826100	This study
	Ns24B	B (X_2, Y_2)	AB826061	AB826081	AB826101	This study
An. pursati						
Chiang Mai	Cm1A[a]	A (X_1, Y_1)	AB826062	AB826082	AB826102	This study
(18° 472' N, 98° 592' E)	Cm2C	C (X_2, Y_3)	AB826063	AB826083	AB826103	This study
	Cm4A	A (X_2, Y_1)	AB826064	AB826084	AB826104	This study
	Cm6B	B (X_2, Y_2)	AB826065	AB826085	AB826105	This study
	Cm7C[a]	C (X_2, Y_3)	AB826066	AB826086	AB826106	This study
	Cm9A	A (X_1, Y_1)	AB826067	AB826087	AB826107	This study
	Cm10A	A (X_1, Y_1)	AB826068	AB826088	AB826108	This study
	Cm11B	B (X_1, Y_2)	AB826069	AB826089	AB826109	This study
	Cm14C	C (X_2, Y_3)	AB826070	AB826090	AB826110	This study
	Cm15C	C (X_2, Y_3)	AB826071	AB826091	AB826111	This study
Ratchaburi (13° 212' N, 99° 222' E)	Rt1B[a]	B (X_2, Y_2)	AB826072	AB826092	AB826112	This study
An. belenrae	–	–	EU789794	–	–	Park *et al.*, 2008a
An. crawfordi	Sk1B	B (X_3, Y_2)	AB779152	AB779181	AB779210	Saeung *et al.*, 2014
An. kleini	–	–	EU789793	–	–	Park *et al.*, 2008a
An. lesteri	–	–	EU789791	–	–	Park *et al.*, 2008a
	ilG1	–	–	AB733028	AB733036	Taai *et al.*, 2013a
An. nigerrimus	Ur26A	A (X_3, Y_1)	AB778778	AB778791	AB778804	Songsawatkiat *et al.*, 2013
An. nitidus	Ur2D	D (X_3, Y_4)	AB777782	AB777803	AB777824	Songsawatkiat *et al.*, unpubl. data
An. paraliae	Sk1B	B (X_1, Y_2)	AB733487	AB733503	AB733519	Taai *et al.*, 2013b
An. peditaeniatus	Cm7B	B (X_2, Y_2)	AB714990	AB715043	AB715096	Saeung *et al.*, 2012
An. pullus	–	–	EU789792	–	–	Park *et al.*, 2008a
	–	–	–	AY444348	AY444347	Park *et al.*, 2003
An. sinensis	i2ACM	A (X, Y_1)	AY130473	–	–	Min *et al.*, 2002
	–	–	–	AY444351	–	Park *et al.*, 2003
	i1BKR	B (X, Y_2)	–	–	AY130464	Min *et al.*, 2002

[a] used in crossing experiments.

bootstrap test with 1,000 replications were performed with the Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 program (Tamura *et al.*, 2007). Bayesian analysis was conducted with MrBayes 3.2 (Ronquist *et al.*, 2012) 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 MrModeltest version 2.3 (Nylander, 2004). 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.

RESULTS

Metaphase karyotypes

Cytogenetic observations of F_1-progenies of the 9 isolines of *An. argyropus* revealed different types of sex chromosomes, due to the addition of extra heterochromatin. There were 2 types of X (metacentric X_1 and submetacentric X_2) and 2 types of Y chromosomes (metacentric Y_1 and large submetacentric Y_2), which comprised 2 forms of metaphase karyotypes on the basis of Y chromosome configurations, i.e., Forms A (X_1, Y_1) and B (X_1, X_2, Y_2) (Table 1, Fig. 1a-f). Form A was recovered only in Ubon Ratchathani Province, northeastern region, whereas Form B was found commonly in both Ubon Rathchathani and Nakhon Si Thammarat Provinces, southern region. Likewise, 2 types of X (metacentric X_1 and submetacentric X_2) and 3 types of Y (metacentric Y_1, small submetacentric Y_2 and

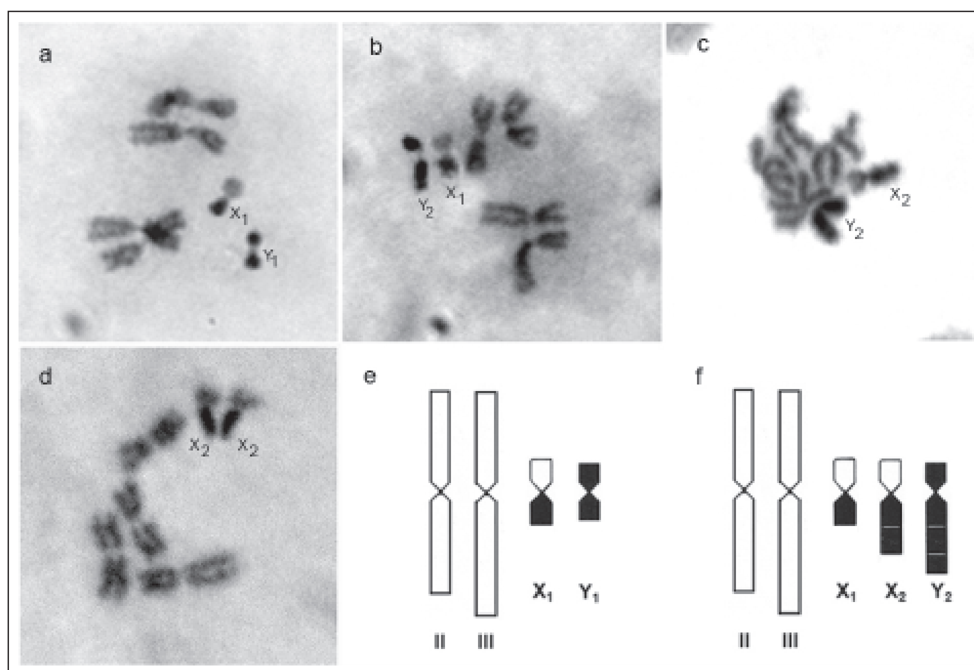

Figure 1. Metaphase karyotypes of *Anopheles argyropus*. (a) Form A (X_1, Y_1: Ubon Ratchathani Province); (b) Form B (X_1, Y_2: Nakhon Si Thammarat Province); (c) Form B (X_2, Y_2: Nakhon Si Thammarat Province); (d) Form B (homozygous X_2, X_2: Nakhon Si Thammarat Province); (e) Diagrams of representative metaphase karyotype of Forms A; (f) Diagrams of representative metaphase karyotype of Forms B.

large submetacentric Y_3) chromosomes of *An. pursati* were recovered from a total of 11 isolines. These types of X and Y chromosomes were designated as Forms A (X_1, X_2, Y_1), B (X_1, X_2, Y_2) and C (X_2, Y_3) (Fig. 2a-i). All karyotypic forms were found generally in Chiang Mai Province, while only 1 isoline obtained in Ratchaburi Province, western region, was X_2, Y_2 of Form B.

Crossing experiments

Table 2 shows details of hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal and F_1-hybrid crosses between the 2 isolines of *An. argyropus* representing Forms A and B. Table 3 shows these details on crossing experiments among the 3 isolines of *An. pursati* representing Forms A, B and C. All crosses yielded viable progenies through the F_2-generations. No evidence of genetic incompatibility 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 length of all autosomes and of X chromosome (Fig. 3a-c).

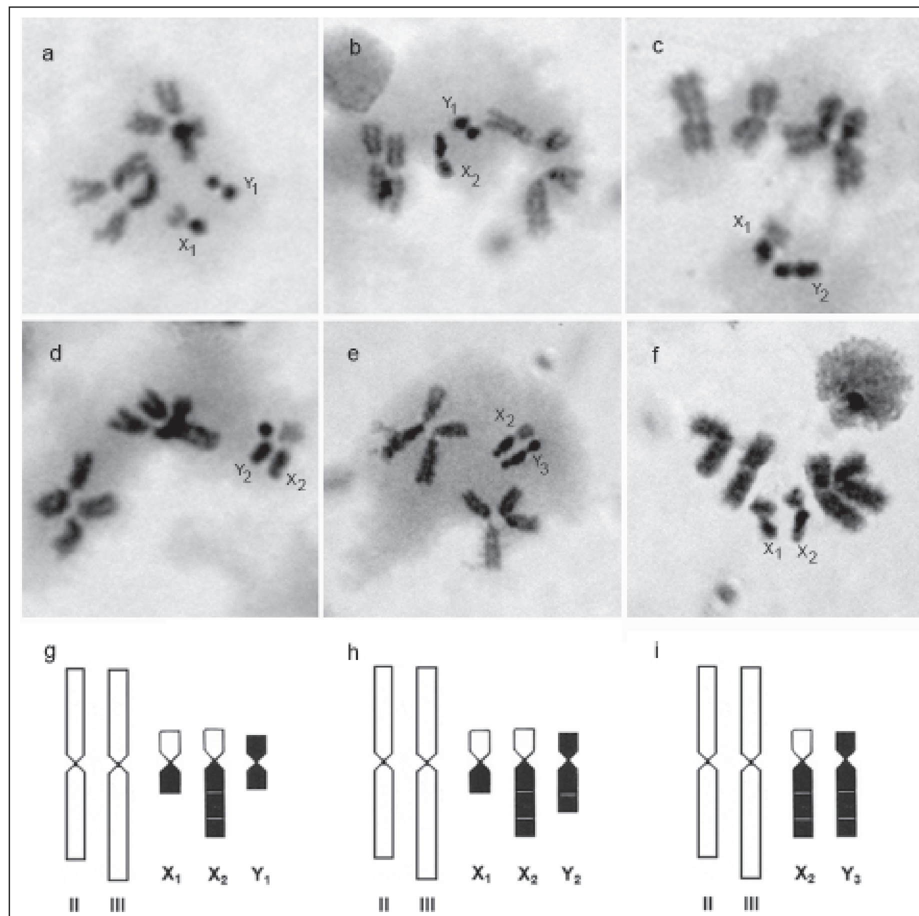

Figure 2. Metaphase karyotypes of *Anopheles pursati*. (a) Form A (X_1, Y_1: Chiang Mai Province); (b) Form A (X_2, Y_1: Chiang Mai Province); (c) Form B (X_1, Y_2: Chiang Mai Province); (d) Form B (X_2, Y_2: Ratchaburi Province); (e) Form C (X_2, Y_3: Chiang Mai Province); (f) Form B (heterozygous X_1, X_2: Chiang Mai Province); (g) Diagrams of representative metaphase karyotype of Forms A; (h) Diagrams of representative metaphase karyotype of Forms B; (i) Diagrams of representative metaphase karyotype of Form C.

Table 2. Crossing experiments among 2 isolines of *Anopheles argyropus*

Crosses (Female x Male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
Parental cross							
Ur1A x Ur1A	398 (245, 153)	81	314 (78.89)	273 (86.94)	268 (98.17)	123 (45.90)	145 (54.10)
Ns5B x Ns5B	279 (101, 178)	77	201 (72.04)	193 (96.02)	189 (97.93)	79 (41.80)	110 (58.20)
Reciprocal cross							
Ur1A x Ns5B	416 (240, 176)	83	295 (70.91)	292 (98.98)	272 (93.15)	135 (49.63)	137 (50.37)
Ns5B x Ur1A	267 (147, 120)	80	200 (74.91)	192 (96)	180 (93.75)	76 (42.22)	104 (57.78)
F_1- hybrid cross							
(Ur1A x Ns5B)F_1 x (Ur1A x Ns5B)F_1	308 (162, 146)	88	243 (78.90)	238 (97.94)	231 (97.06)	114 (49.35)	117 (50.65)
(Ns5B x Ur1A)F_1 x (Ns5B x Ur1A)F_1	324 (130, 194)	84	266 (82.10)	266 (100)	266 (100)	125 (46.99)	141 (53.01)

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

Table 3. Crossing experiments among 3 isolines of *Anopheles pursati*

Crosses (Female x Male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched n (%)	Pupation n (%)	Emergence n (%)	Total emergence n (%)	
						Female	Male
Parental cross							
Cm1A x Cm1A	254 (132, 122)	81	199 (78.35)	172 (86.43)	167 (97.09)	76 (45.51)	91 (54.49)
Rt1B x Rt1B	237 (128, 109)	78	184 (77.64)	165 (89.67)	158 (95.76)	78 (49.37)	80 (50.63)
Cm7C x Cm7C	249 (113, 136)	75	187 (75.10)	183 (97.86)	181 (98.91)	92 (50.83)	89 (49.17)
Reciprocal cross							
Cm1A x Rt1B	236 (110, 126)	82	194 (82.20)	165 (85.05)	159 (96.36)	70 (44.03)	89 (55.97)
Rt1B x Cm1A	242 (124, 118)	72	172 (71.07)	163 (94.77)	148 (90.79)	78 (52.70)	70 (47.30)
Cm1A x Cm7C	221 (113, 108)	86	188 (85.07)	169 (89.89)	160 (94.67)	81 (50.63)	79 (49.37)
Cm7C x Cm1A	261 (119, 142)	88	206 (78.93)	202 (98.06)	196 (97.03)	97 (49.49)	99 (50.51)
Rt1B x Cm7C	234 (104, 130)	92	211 (90.17)	198 (93.84)	190 (95.96)	93 (48.95)	97 (51.05)
Cm7C x Rt1B	284 (167, 117)	87	233 (82.04)	226 (97.00)	221 (97.79)	112 (50.68)	109 (49.32)
F_1- hybrid cross							
(Cm1A x Rt1B)F_1 x (Cm1A x Rt1B)F_1	264 (112, 152)	81	214 (81.06)	214 (100)	201 (93.93)	90 (44.78)	111 (55.22)
(Rt1B x Cm1A)F_1 x (Rt1B x Cm1A)F_1	220 (118, 102)	94	205 (93.18)	205 (100)	201 (98.05)	94 (46.77)	107 (52.23)
(Cm1A x Cm7C)F_1 x (Cm1A x Cm7C)F_1	255 (131, 124)	90	217 (85.10)	174 (80.18)	170 (97.70)	94 (55.29)	76 (44.71)
(Cm7C x Cm1A)F_1 x (Cm7C x Cm1A)F_1	231 (103, 128)	85	189 (81.82)	157 (83.07)	151 (96.18)	72 (47.68)	79 (52.32)
(Rt1B x Cm7C)F_1 x (Rt1B x Cm7C)F_1	286 (109, 177)	89	249 (87.06)	242 (97.19)	240 (99.17)	110 (45.83)	130 (54.17)
(Cm7C x Rt1B)F_1 x (Cm7C x Rt1B)F_1	212 (103, 109)	93	197 (92.92)	177 (89.85)	175 (98.87)	80 (45.71)	95 (54.29)

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

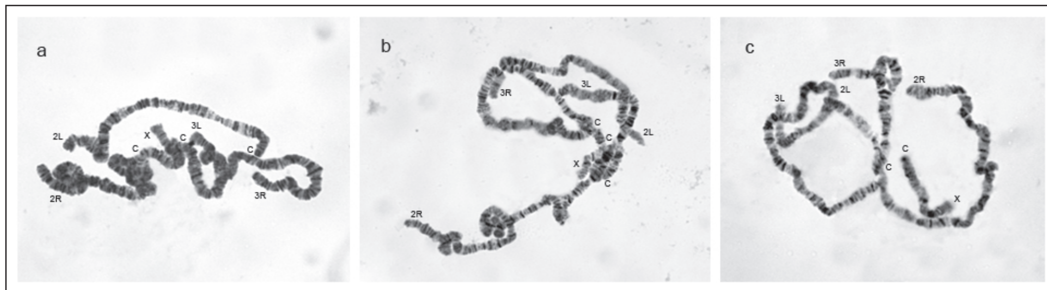

Figure 3. Synapsis in all arms of salivary gland polytene chromosome of F_1-hybrids 4th larvae of *Anopheles argyropus* and *An. pursati*. (a) *An. argyropus*: Ur1A female x Ns5B male. (b) *An. pursati*: Cm1A female x Rt1B male; (c) *An. pursati*: Cm1A female x Cm7C male.

DNA sequences and phylogenetic analysis

The ITS2, COI and COII sequences were available in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB826053-AB826112 (Table 1). The length of ITS2 was 472 bp and 499 bp in *An. argyropus* and *An. pursati*, respectively. No intraspecific ITS2 sequence variation was observed among the 11 isolines of *An. pursati*, whereas 4 base substitutions (A↔G at position 242 and 289, C↔T at position 388, A↔C at position 435) were found among the 9 isolines of *An. argyropus*. The analysis of COI (658 bp) among the 9 isolines of *An. argyropus* revealed 13 base substitutions, while 7 base substitutions were obtained among the 11 isolines of *An. pursati*. The analysis of COII (685 bp) among the 9 isolines of *An. argyropus* showed 8 base substitutions, whilst 2 base substitutions derived from the 11 isolines of *An. pursati*. All the substitutions were not specific to karyotypic forms. The evolutionary relationships among the karyotypic forms of *An. argyropus* and *An. pursati* were determined using neighbor-joining (NJ) and Bayesian analysis (BA). Both phylogenetic methods showed the same tree topologies, therefore, only the Bayesian tree result was shown for all DNA regions (Fig. 4-6). The 9 isolines of *An. argyropus* were grouped as a monophyletic clade, with high branch support in all DNA regions (100% in NJ, 98-100% in BA). Likewise, all 11 isolines of *An. pursati* were placed within the same clade, with high branch support in all DNA regions (99-100% in NJ, 100% in BA). The average genetic distances within 2 and 3 karyotypic forms of *An. argyropus* and *An. pursati* were 0.003 and 0, 0.007 and 0.005, and 0.004 and 0.001, based on ITS2, COI and COII sequences, respectively. The phylogenetic tree revealed that *An. pursati* was more closely related to *Anopheles nitidus* and *Anopheles nigerrimus* than to *An. argyropus* based on ITS2 and COI sequences. However, both species were well separated from other species members of the Hyrcanus Group in all DNA regions.

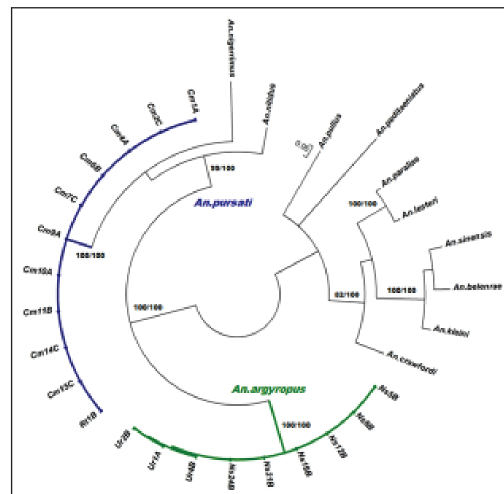

Figure 4. Bayesian phylogenetic relationships among the 9 isolines of *Anopheles argyropus* and 11 isolines of *An. pursati* based on ITS2 sequences compared with 10 species members of the Hyrcanus Group. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities. Only the values higher than 70% both on bootstrap values and posterior probabilities are shown. Branch lengths are proportional to genetic distance (scale bar).

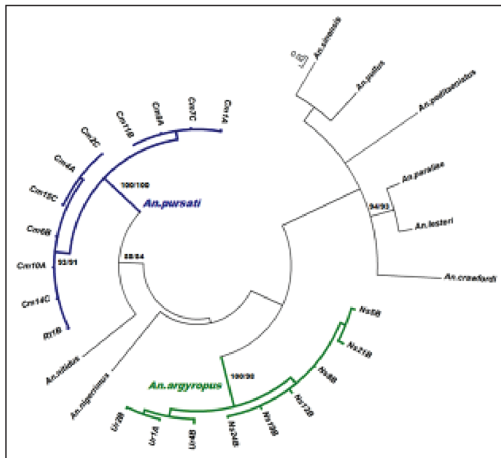

Figure 5. Bayesian phylogenetic relationships among the 9 isolines of *Anopheles argyropus* and 11 isolines of *An. pursati* based on COI sequences compared with 8 species members of the Hyrcanus Group. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities. Only the values higher than 70% both on bootstrap values and posterior probabilities are shown. Branch lengths are proportional to genetic distance (scale bar).

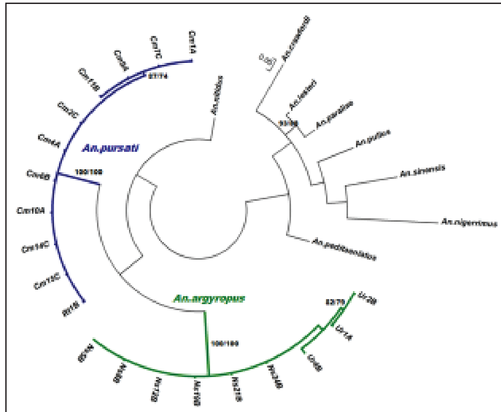

Figure 6. Bayesian phylogenetic relationships among the 9 isolines of *Anopheles argyropus* and 11 isolines of *An. pursati* based on COII sequences compared with 8 species members of the Hyrcanus Group. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities. Only the values higher than 70% both on bootstrap values and posterior probabilities are shown. Branch lengths are proportional to genetic distance (scale bar).

DISCUSSION

Cytogenetic investigations of 17 *An. argyropus* isolines from 3 different localities in Thailand (Chiang Mai and Phrae Provinces, northern region; Chanthaburi Province, eastern region) were performed firstly by Baimai *et al.* (1993). The results demonstrated that this anopheline species exhibited karyotypic variation via a gradual increase of extra heterochromatin on X and Y chromosomes, and forming 2 karyotypic forms [Forms A (X_1, X_2, Y_1) and B (X_1, X_2, Y_2)]. In the present study, similar results of 2 karyotypic forms have been obtained by examining 9 isolines from 2 different locations (Ubon Ratchatani Province, northeastern region; Nakhon Si Thammarat Province, southern region). Remarkably, the Form A (X_2, Y_1), reported by Baimai *et al.* (1993), was not detected in any isoline colonies, as the limitation in number of samples appeared to be used in the current study. Regarding *An. pursati*, the 3 new karyotypic forms [Forms A (X_1, X_2, Y_1), B (X_1, X_2, Y_2) and C (X_2, Y_3)] were recovered from 11 isolines in 2 different localities (Chiang Mai Province, northern region; Ratchaburi Province, western region). Apparently, these distinct karyotypic forms were caused by the gradual addition of extra heterochromatin on sex chromosomes.

According to the genetic diversity at the chromosomal level of the *An. argyropus* [Forms A (X_1, Y_1) and B (X_1, X_2, Y_2)] and *An. pursati* [Forms A (X_1, X_2, Y_1), B (X_1, X_2, Y_2) and C (X_2, Y_3)] found in this study, crossing experiments among the karyotypic variants of *An. argyropus* and *An. pursati* were performed intensively by following robust systematic procedures as documented by Choochote & Saeung (2013). The results showed no post-mating reproductive isolation. All crosses yielded viable progenies through F_2-generations and synaptic salivary gland polytene chromosomes, suggesting the conspecific nature of these karyotypic variants, which comprised 2 and 3 cytological forms within the taxon *An.*

argyropus and *An. pursati*, respectively. The low intraspecific sequence variations [average genetic distance = 0.003-0.007 (*An. argyropus*) and 0-0.005 (*An. pursati*)] of the nucleotide sequences in ribosomal DNA (ITS2) and mitochondrial DNA (COI and COII), all isolines of *An. argyropus* and *An. pursati* were placed within each monophyletic clade and well separated from the other 10 species members of the Hyrcanus Group. Acceptably, *Anopheles hyrcanus* group comprises 26 species that occur widely in Asia. But in Thailand, at least 8 species members of this group i.e., *An. argyropus*, *Anopheles crawfordi*, *An. nigerrimus*, *An. nitidus*, *Anopheles paraliae*, *An. pursati*, *Anopheles peditaeniatus*, and *Anopheles sinensis* (Harrison & Scanlon, 1975; Rattanarithikul *et al.*, 2006) are recorded. Among these, they are also found in South East Asia regions, even *An. sinensis* is the cosmopolitan species distributed widely. In addition, the DNA sequences in present study were not only compared with Thai species but also 4 species, *Anopheles belenrae*, *Anopheles kleini*, *Anopheles lesteri* and *Anopheles pullus*, from East Asia regions (Min *et al.*, 2002; Park *et al.*, 2003, 2008a). This was based on neighbor-joining (NJ) and Bayesian analyses (BA), which acted as good supportive evidence. It is interesting to note that the differences in the amount and distribution of heterochromatin observed from both anopheline species were not resulted in the evolution divergence as in, for example, *Drosophila kikkawai* complex, *Anopheles dirus* complex, *Anopheles maculatus* group and *Bactocera dorsalis* complex, as stated by Baimai (1998). The present results are in accordance with crossing experiments among karyotypic forms of other *Anopheles* species, i.e., *Anopheles vagus* Forms A and B (Choochote *et al.*, 2002), *An. pullus* (= *Anopheles yatsushiroensis*) Forms A and B (Park *et al.*, 2003), *An. sinensis* Forms A and B (Choochote *et al.*, 1998; Min *et al.*, 2002; Park *et al.*, 2008b), *Anopheles aconitus* Forms B and C (Junkum *et al.*, 2005), *An. barbirostris* species A1 (Forms A, B and C) and A2 (Forms A and B) (Saeung *et al.*, 2007, Suwannamit *et al.*, 2009), *An. campestris*-like Forms B, E and F (Thongsahuan *et al.*, 2009), *An. peditaeniatus* Forms A, B, C, D, E and F (Choochote, 2011; Saeung *et al.*, 2012), *An. nigerrimus* Forms A, B, C and D (Songsawatkiat *et al.*, 2013) and *An. paraliae* Forms A, B, C, D and E (Taai *et al.*, 2013b).

In conclusion, this is the first report of 3 new karyotypic forms of *An. pursati* in Thailand and our results of crossing experiment and DNA sequencing also indicated the conspecific cytological race among 2 and 3 karyotypic forms of *An. argyropus* and *An. pursati*, respectively.

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