RESEARCH

Evidence to Support a Conspecific Nature of Allopatric Cytological Races of *Anopheles nitidus* (Diptera: Culicidae) in Thailand

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ABSTRACT. Metaphase karyotype investigation on two allopatric strains of *Anopheles nitidus* Harrison, Scanlon, and Reid (Diptera: Culicidae) was conducted in Thailand during 2011–2012. Five karyotypic forms, i.e., Form A (X_1, Y_1), Form B (X_1, Y_2), Form C (X_2, Y_3), Form D (X_1, X_3, Y_4), and Form E (X_1, X_2, X_3, Y_5) were obtained from a total of 21 isofemale lines. Forms A, B, and C were confined to Phang Nga Province, southern Thailand, whereas Forms D and E were restricted to Ubon Ratchathani Province, northeastern Thailand. Cross-mating experiments among the five isofemale lines, which were representative of five karyotypic forms of *An. nitidus*, revealed genetic compatibility by providing viable progenies and synaptic salivary gland polytene chromosomes through F_2 generations. The results suggest that the forms are conspecific, and *An. nitidus* comprises five cytological races. The very low intraspecific sequence variations (average genetic distances = 0.002–0.008) of the nucleotide sequences in ribosomal DNA (internal transcribed spacer 2) and mitochondrial DNA (cytochrome *c* oxidase subunits I and II) among the five karyotypic forms were very good supportive evidence.

Key Words: *Anopheles nitidus*, metaphase karyotype, cross-mating experiment, rDNA, mtDNA

Anopheles (*Anopheles*) *nitidus* Harrison, Scanlon, and Reid (Diptera: Culicidae) is a foothill anopheline species that belongs to the Nigerrimus Subgroup and Hyrcanus Group of the Myzorhynchus Series and has a wide distribution range extending from India (Assam) to Vietnam, Cambodia, Thailand (a cosmopolitan species), Malaysia (Malaysian Peninsular and Sarawak), and Indonesia (Sumatra) (Reid 1968, Harrison and Scanlon 1975, Rattanarithikul et al. 2006, Harbach 2014). Although *An. nitidus* acts as a vicious biter of humans in some localities of Thailand, it has never been incriminated as a natural or suspected vector of any human diseases, unlike other species members of the Thai *Anopheles hyrcanus* group (e.g., *Anopheles nigerrimus*, *Anopheles peditaeniatus*, and *Anopheles sinensis* that one suspected vectors of *Plasmodium vivax* [Baker et al. 1987, Harbach et al. 1987, Gingrich et al. 1990, Rattanarithikul et al. 1996); and *An. nigerrimus*, a potentially natural vector of *Wuchereria bancrofti* in Phang Nga Province, southern Thailand (Division of Filariasis 1998]). Nevertheless, *An. nitidus* is considered an economic pest of cattle because of its vicious biting behavior (Reid et al. 1962, Reid 1968, Harrison and Scanlon 1975).

Regarding cytogenetic investigations of *An. nitidus* by Baimai et al. (1993a), their results revealed that at least two types of X (X_1, X_2) and one type of Y chromosomes were obtained in two isoline colonies caught from Muang district, Phang Nga Province and Sadao district, Songkhla Province, southern Thailand. As emphasized by the above information, genetic proximity among the karyotypic variants of *An. nitidus* is obviously lacking. Thus, the main aim of this study was to determine whether the five karyotypic variants, from two allopatric populations of *An. nitidus*, exist as a single or distinct species by performing cross-mating experiments among them that relating to DNA sequence analyses of internal transcribed spacer 2 (ITS2) of ribosomal DNA, and cytochrome *c* oxidase subunits I (COI) and II (COII) of mitochondrial DNA (mtDNA).

Materials and Methods

Field Collections and Establishment of Isoline Colonies. Wild-caught, fully engorged female mosquitoes of *An. nitidus* were collected from cow-baited traps at two locations, i.e., Muang district, Phang Nga Province and Nachaluai district, Ubon Ratchathani Province in southern and northeastern Thailand, respectively (Fig. 1; Table 1). In total, 21 isolines were established successfully and maintained in our insectary using the techniques described by Choochote and Saeung (2013). Exact species identification was performed by using intact morphology of egg, larval, pupal, and adult stages from the F_1 progenies of isolines, following standard keys (Reid 1968, Harrison and Scanlon 1975, Rattanarithikul et al. 2006). These isolines were used for studies on the metaphase karyotype, cross-mating experiments, and molecular analyses.

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

Cross-Mating Experiments. The five laboratory-raised isolines of *An. nitidus* were selected arbitrarily from the 21 isoline colonies as representatives of the five karyotypic forms, i.e., Form A (Pg2A), B (Pg5B), C (Pg4C), D (Ur2D), and E (Ur5E) (Table 1). These isolines were used for cross-mating experiments to determine postmating barriers by employing the techniques previously reported by Saeung et al. (2007).

DNA Extraction and Amplification. Molecular analyses of three specific genomic loci (ITS2, COI, and COII) were performed to determine intraspecific sequence variation within *An. nitidus*. Individual F_1 progeny adult female of each isoline of *An. nitidus* (Ur2D, Ur5E, Ur8E, Ur11D, Ur12D, Ur15D, Ur16E, Ur19D, Ur22E, Ur23E, Ur24D,

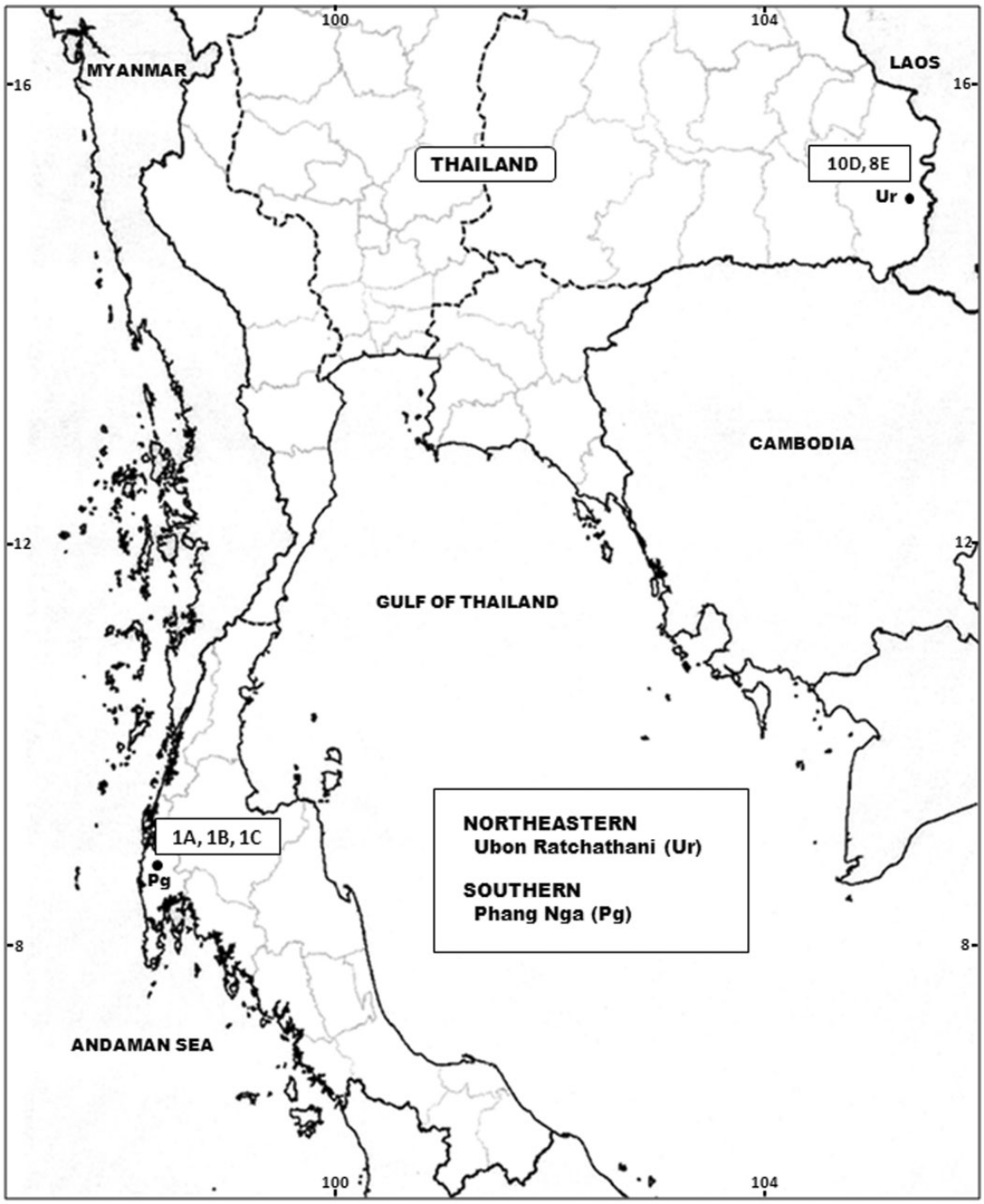

Fig. 1. Map of Thailand showing two provinces where specimens of *An. nitidus* were collected and the number of isolines of the five karyotypic forms (A–E) detected in each location.

Ur25D, Ur27D, Ur28E, Ur30E, Ur31D, Ur33E, Ur34D, Pg2A, Pg4C, and Pg5B; Table 1) was used for DNA extraction and amplification. Genomic DNA was extracted from each mosquito using DNeasy Blood and Tissue Kit (QIAgen, Japan). Primers for amplification of the ITS2, COI, and COII regions followed previous studies by Saeung et al. (2007). Each polymerase chain reaction (PCR) reaction was carried out in 20 µl containing 0.5 U *Ex Taq* (Takara, Japan), 1X *Ex Taq* DNA polymerase 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 94°C for 1 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. The amplification profile of COI and COII comprised initial denaturation at 94°C for 1 min, 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. The amplified products were electrophoresed in 1.5% tris-acetate-EDTA agarose gels and stained with ethidium bromide. Finally, the PCR products were purified using the QIAquick PCR Purification Kit (QIAgen, Japan) and their sequences directly determined using the

Table 1. Isolines of five karyotypic forms (A–E) of *An. nitidus* and their GenBank accession numbers

Location, geographical coordinate	Code of isoline[a]	Karyotypic form	Region	GenBank accession number			Reference
				ITS2	COI	COII	
An. nitidus							
Ubon Ratchathani (15° 31′ N, 105° 35′ E)	Ur2D[a]	D (X_3, Y_4)	ITS2, COI, COII	AB777782	AB777803	AB777824	This study
	Ur5E[a]	E (X_2, Y_5)	ITS2, COI, COII	AB777783	AB777804	AB777825	This study
	Ur8E	E (X_1, Y_5)	ITS2, COI, COII	AB777784	AB777805	AB777826	This study
	Ur11D	D (X_3, Y_4)	ITS2, COI, COII	AB777785	AB777806	AB777827	This study
	Ur12D	D (X_1, Y_4)	ITS2, COI, COII	AB777786	AB777807	AB777828	This study
	Ur15D	D (X_3, Y_4)	ITS2, COI, COII	AB777787	AB777808	AB777829	This study
	Ur16E	E (X_1, Y_5)	ITS2, COI, COII	AB777788	AB777809	AB777830	This study
	Ur19D	D (X_1, Y_4)	ITS2, COI, COII	AB777789	AB777810	AB777831	This study
	Ur22E	E (X_2, Y_5)	ITS2, COI, COII	AB777790	AB777811	AB777832	This study
	Ur23E	E (X_3, Y_5)	ITS2, COI, COII	AB777791	AB777812	AB777833	This study
	Ur24D	D (X_3, Y_4)	ITS2, COI, COII	AB777792	AB777813	AB777834	This study
	Ur25D	D (X_1, Y_4)	ITS2, COI, COII	AB777793	AB777814	AB777835	This study
	Ur27D	D (X_1, Y_4)	ITS2, COI, COII	AB777794	AB777815	AB777836	This study
	Ur28E	E (X_3, Y_5)	ITS2, COI, COII	AB777795	AB777816	AB777837	This study
	Ur30E	E (X_1, Y_5)	ITS2, COI, COII	AB777796	AB777817	AB777838	This study
	Ur31D	D (X_3, Y_4)	ITS2, COI, COII	AB777797	AB777818	AB777839	This study
	Ur33E	E (X_2, Y_5)	ITS2, COI, COII	AB777798	AB777819	AB777840	This study
	Ur34D	D (X_3, Y_4)	ITS2, COI, COII	AB777799	AB777820	AB777841	This study
Phang Nga (08° 27′ N, 98 31′ E)	Pg2A[a]	A (X_1, Y_1)	ITS2, COI, COII	AB777800	AB777821	AB777842	This study
	Pg4C[a]	C (X_2, Y_3)	ITS2, COI, COII	AB777801	AB777822	AB777843	This study
	Pg5B[a]	B (X_1, Y_2)	ITS2, COI, COII	AB777802	AB777823	AB777844	This study
Hyrcanus Group	TR2	—	ITS2	HM488273	—	—	Paredes-Esquivel et al. (2011)
	TR3	—	ITS2	HM488272	—	—	Paredes-Esquivel et al. (2011)
	TR6	—	ITS2	HM488268	—	—	Paredes-Esquivel et al. (2011)
An. belenrae	—	—	ITS2	EU789794	—	—	Park et al. (2008a)
An. crawfordi	Pg4A	A (X_1, Y_1)	ITS2, COI, COII	AB779142	AB779171	AB779200	A.S., unpublished data
An. kleini	—	—	ITS2	EU789793	—	—	Park et al. (2008a)
An. lesteri	—	—	ITS2	EU789791	—	—	Park et al. (2008a)
	ilG1	—	COI, COII	—	AB733028	AB733036	Taai et al. (2013a)
An. paraliae	Sk1B	B (X_1, Y_2)	ITS2, COI, COII	AB733487	AB733503	AB733519	Taai et al. (2013b)
An. peditaeniatus	RbB	B (X_3, Y_2)	ITS2, COI, COII	AB539061	AB539069	AB539077	Choochote (2011)
An. pullus	—	—	ITS2	EU789792	—	—	Park et al. (2008a)
			COI, COII	—	AY444348	AY444347	Park et al. (2003)
An. sinensis	i2ACM	A (X, Y_1)	ITS2	AY130473	—	—	Min et al. (2002)
	—	—	COI	—	AY444351	—	Park et al. (2003)
	i1BKR	B (X, Y_2)	COII	—	—	AY130464	Min et al. (2002)

[a]Used in cross-mating experiments.

BigDye V3.1 Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems, www.appliedbiosystems.com). The sequence data obtained have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB777782–AB777844 (Table 1). The ITS2, COI, and COII sequences obtained from this study were compared with published sequences available in GenBank using the BLAST search (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 program (Thompson et al. 1994) and edited manually in BioEdit version 7.0.5.3 (Hall 1999). Gap sites were excluded from the following analysis. The Kimura two-parameter model was employed to calculate genetic distances (Kimura 1980). Using the distances, construction of neighbor-joining (NJ) trees (Saitou and Nei 1987) and the bootstrap test with 1,000 replications were performed with the MEGA version 6.0 program (Tamura et al. 2013). 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 combined COI and COII sequences. Bayesian posterior probabilities were calculated from the consensus tree after excluding the first 25% trees as burn-in.

Results

Metaphase Karyotype. Cytogenetic observations of F_1 progenies of the 21 isolines of *An. nitidus* revealed different types of sex chromosomes due to the addition of extra block(s) of heterochromatin. There were three types of X (small metacentric X_1, submetacentric X_2, and large submetacentric X_3) and five types of Y chromosomes (small telocentric Y_1, small subtelocentric Y_2, large subtelocentric Y_3, submetacentric Y_4, and small metacentric Y_5) (Figs. 2 and 3). The X_1 chromosome has a small metacentric 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. The X_3 chromosome has a large submetacentric 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 morphology between X_2 and X_3 chromosomes could be made easily in heterozygous females (Fig. 2I). Similar to the situation in the X chromosome, the Y chromosome also exhibited extensive variation in size and morphology, due to differing amounts

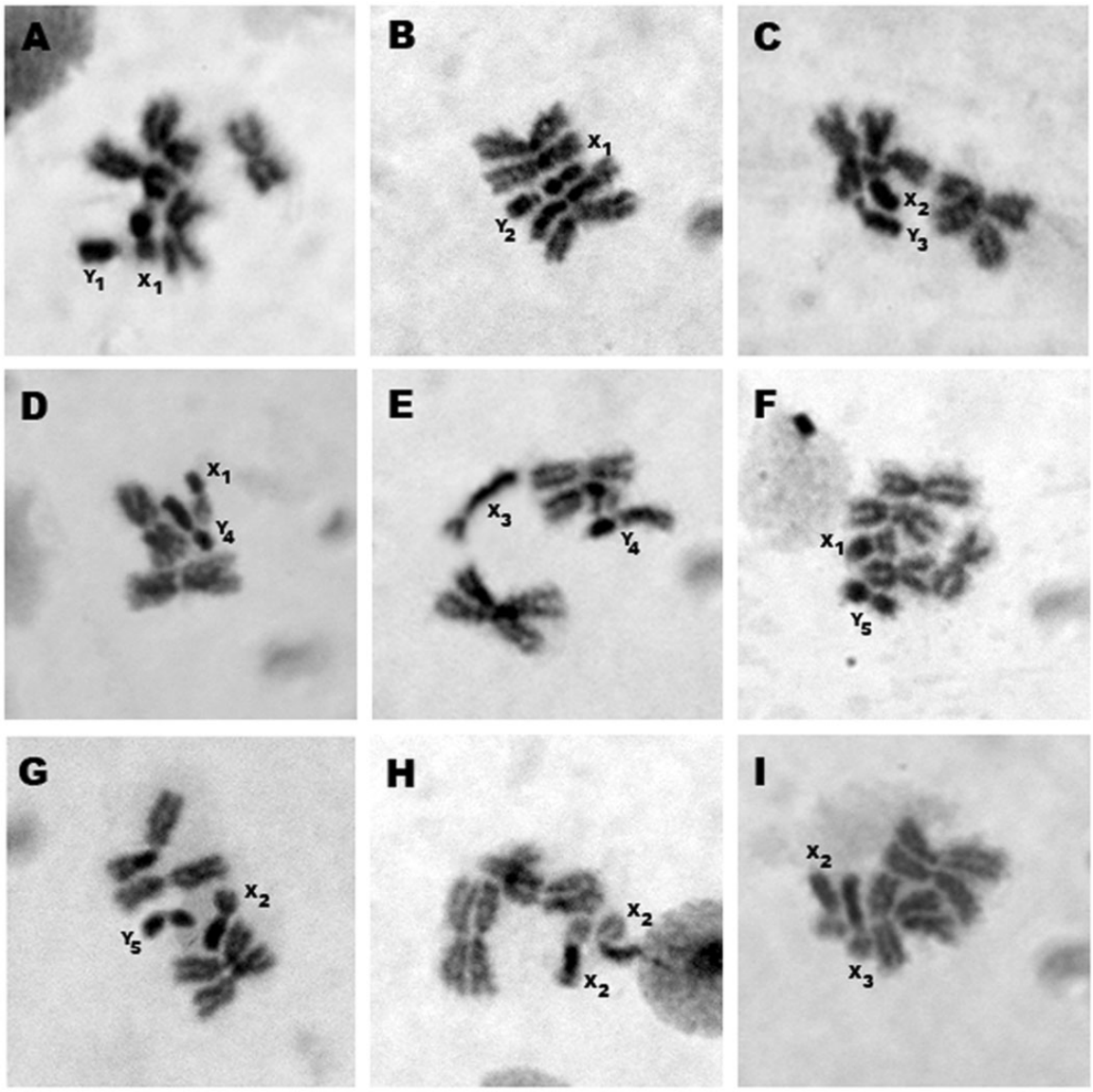

Fig. 2. Metaphase karyotypic forms of *An. nitidus*. Phang Nga Province (A–C) (A) Form A (X_1, Y_1), (B) Form B (X_1, Y_2), and (C) Form C (X_2, Y_3). Ubon Ratchathani Province (D–I) (D) Form D (X_1, Y_4), (E) Form D (X_3, Y_4), (F) Form E (X_1, Y_5), (G) Form E (X_2, Y_5), (H) Form E (homozygous X_2, X_2), and (I) Form E (heterozygous X_2, X_3).

and distribution of heterochromatic block. Thus, the Y_1 chromosome is an apparently small telocentric, which represents the ancestral form (Fig. 2A). The Y_2 chromosome has a small subtelocentric or acrocentric that slightly differs from the Y_1 chromosome, which has a very small portion of the short arm present (Fig. 2B). Chromosome Y_3 has a large subtelocentric 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. 2C). The Y_4 chromosome is clearly submetacentric, with the short arm ∼one-third the length of the long arm (Fig. 2D and E). 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. Chromosome Y_5 had a small metacentric, which was quite different from chromosomes Y_1, Y_2, Y_3, and Y_4 by having an equal heterochromatic block on each arm (Fig. 2F and G). Based on uniquely different characteristics of Y chromosome from each isoline colony, they were designated as Form A (X_1, Y_1), Form B (X_1, Y_2), Form C (X_2, Y_3), Form D (X_1, X_3, Y_4), and Form E (X_1, X_2, X_3, Y_5). Forms A, B, and C were found in Phang Nga Province, and Forms D and E were obtained in Ubon Ratchathani Province.

Cross-Mating Experiments. Details of hatchability, pupation, emergence, and adult sex-ratio of parental, reciprocal, and F_1-hybrid crosses among the five isolines of *An. nitidus* Forms A, B, C, D, and E are listed in Table 2. All crosses yielded viable progenies through F_2 generations. No evidence of genetic incompatibility and/or postmating reproductive isolation was observed among these crosses. The salivary gland polytene chromosomes of the fourth-stage larvae from all crosses showed synapsis without any inversion loops along the whole length of all autosomes and the X chromosome (Fig. 4).

DNA Sequences and Phylogenetic Analysis. DNA sequences were determined and analyzed for the ITS2, COI, and COII of the 21 isolines of *An. nitidus* Forms A, B, C, D, and E. They showed various lengths of ITS2, at 480 bp in 18 isolines from Ubon Ratchathani Province and 481 bp in 3 isolines from Phang Nga Province. The *An. nitidus* from Ubon Ratchathani Province differed from that in Phang Nga Province by a deletion of T at position 421. They all showed the same length in COI (658 bp) and COII (685 bp). NJ and Bayesian trees were constructed to reveal the evolutionary relationship of the five karyotypic forms. Both phylogenetic methods showed similar tree topologies, thus only the Bayesian tree is shown in Figs. 5 and 6. The results

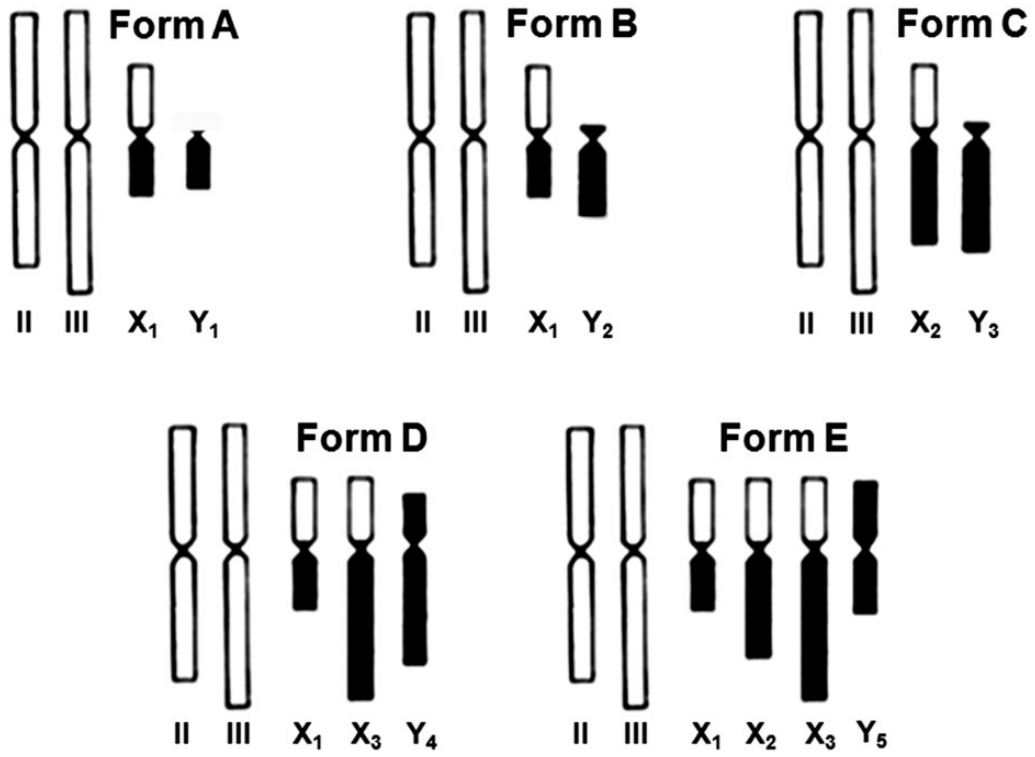

Fig. 3. Diagrams of representative metaphase karyotypes of Forms A, B, C, D, and E of *An. nitidus*.

Table 2. Cross-mating experiments of five isolines of *An. nitidus*

Crosses (female x male)	Total eggs (number)[a]	Embryonation rate[b]	Hatched, *n* (%)	Pupation, *n* (%)	Emergence, *n* (%)	Total emergence, *n* (%)	
						Female	Male
Parental cross							
Pg2A x Pg2A	244 (125, 119)	88	210 (86.06)	195 (92.86)	195 (100.00)	103 (52.82)	92 (47.18)
Pg5B x Pg5B	277 (130, 147)	91	238 (85.92)	226 (94.96)	221 (97.79)	107 (48.42)	114 (51.58)
Pg4C x Pg4C	283 (118, 165)	84	218 (77.03)	218 (100.00)	211 (96.79)	106 (50.24)	105 (49.76)
Ur2D x Ur2D	292 (109, 183)	92	263 (90.07)	258 (98.10)	247 (95.74)	131 (53.04)	116 (46.96)
Ur5E x Ur5E	301 (148, 153)	88	256 (85.05)	251 (98.05)	221 (88.05)	111 (50.23)	110 (49.77)
Reciprocal cross							
Pg2A x Pg5B	289 (147, 142)	94	260 (89.97)	257 (98.85)	239 (93.00)	117 (48.95)	122 (51.05)
Pg5B x Pg2A	298 (158, 140)	90	220 (73.83)	202 (91.82)	198 (98.02)	97 (48.99)	101 (51.01)
Pg2A x Pg4C	299 (131, 168)	92	260 (86.96)	231 (88.85)	226 (97.84)	112 (49.56)	114 (50.44)
Pg4C x Pg2A	313 (162, 151)	80	225 (71.88)	218 (96.89)	209 (95.87)	112 (53.59)	97 (46.41)
Pg2A x Ur2D	211 (103, 108)	86	175 (82.94)	159 (90.86)	159 (100.00)	64 (40.25)	95 (59.75)
Ur2D x Pg2A	224 (111, 113)	91	202 (90.18)	196 (97.03)	171 (87.24)	81 (47.37)	90 (52.63)
Pg2A x Ur5E	243 (118, 125)	87	207 (85.19)	207 (100.00)	197 (95.17)	100 (50.76)	97 (49.24)
Ur5E x Pg2A	264 (139, 125)	91	235 (89.02)	235 (100.00)	204 (86.81)	108 (52.94)	96 (47.06)
F_1-hybrid cross							
(Pg2A x Pg5B)F_1 x (Pg2A x Pg5B)F_1	308 (118, 190)	85	246 (79.87)	234 (95.12)	229 (97.86)	111 (48.47)	118 (51.53)
(Pg5B x Pg2A)F_1x (Pg5B x Pg2A)F_1	312 (186, 126)	87	250 (80.13)	235 (94.00)	225 (95.74)	110 (48.89)	115 (51.11)
(Pg2A x Pg4C)F_1 x (Pg2A x Pg4C)F_1	308 (147, 161)	92	271 (87.99)	268 (98.89)	257 (95.90)	135 (52.53)	122 (47.47)
(Pg4C x Pg2A)F_1 x (Pg4C x Pg2A)F_1	329 (194, 135)	80	250 (75.99)	230 (92.00)	225 (97.83)	115 (51.11)	110 (48.89)
(Pg2A x Ur2D)F_1 x (Pg2A x Ur2D)F_1	347 (157, 190)	90	295 (85.01)	289 (97.97)	265 (91.70)	141 (53.21)	124 (46.79)
(Ur2D x Pg2A)F_1 x (Ur2D x Pg2A)F_1	287 (125, 162)	90	250 (87.11)	222 (88.80)	220 (99.10)	112 (50.91)	108 (49.09)
(Pg2A x Ur5E)F_1 x (Pg2A x Ur5E)F_1	350 (167, 183)	88	280 (80.00)	272 (97.14)	266 (97.79)	126 (47.37)	140 (52.63)
(Ur5E x Pg2A)F_1 x (Ur5E x Pg2A)F_1	339 (194, 145)	84	268 (79.06)	263 (98.13)	242 (92.02)	124 (51.24)	118 (48.76)

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

showed that all sequences of *An. nitidus* Forms A, B, C, D, and E were monophyletic in both trees, with high support (NJ = 99–100%, BPP = 100%). The average genetic distances within the five karyotypic forms (21 isolines) of *An. nitidus* were 0.002, 0.008, and 0.006 for ITS2, COI, and COII sequences, respectively. Furthermore, all karyotypic forms of *An. nitidus* were well separated from other species members (*Anopheles belenrae*, *Anopheles crawfordi*, *Anopheles kleini*, *Anopheles lesteri*, *Anopheles paraliae*, *An. peditaeniatus*, *Anopheles pullus,* and *An. sinensis*) of the Hyrcanus Group (Figs. 5 and 6). The three published ITS2 sequences (GenBank accession numbers

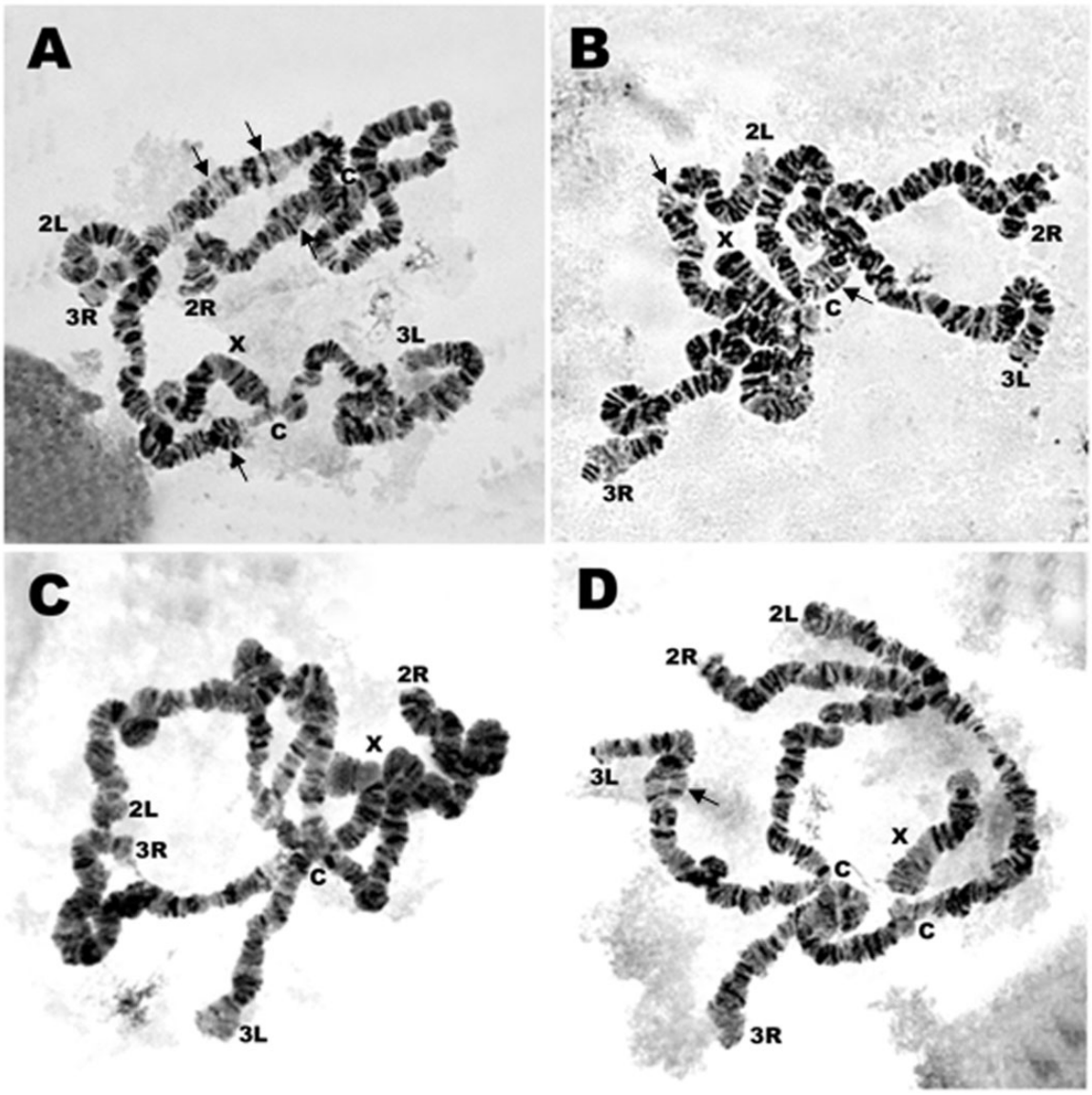

Fig. 4. Synapsis in all arms of salivary gland polytene chromosome of F_1-hybrids fourth larvae of *An. nitidus*. (A) Pg2A female x Pg5B male; (B) Pg2A female x Pg4C male; (C) Pg2A female x Ur2D male; (D) Pg2A female x Ur5E male. Note: small common gap of homosequential asynapsis (arrow) was found on chromosome 2L, 2R, and 3R; 2L and 2R; and 3L from the crosses between Pg2A female x Pg5B male; Pg2A female x Pg4C male; and Pg2A female x Ur5E male, respectively.

HM488268, HM488272, and HM488273; Table 1), which were identified previously as the Hyrcanus Group, also were placed within the same clade of *An. nitidus* (Fig. 5).

Discussion

A cytogenetic investigation of *An. nitidus* in Thailand was documented first by Baimai et al. (1993a). The results indicated that this anopheline species exhibited genetic diversity at the chromosomal level via a gradual increase in the extra block(s) of constitutive heterochromatin in the X chromosome (X_1, X_2), whereas this event was not detected in the Y chromosomes, possibly due to the limited number of isolines used. Herein, the 21 *An. nitidus* isolines from two allopatric locations (Phang Nga Province, southern region; Ubon Ratchathani Province, northeastern region) in Thailand revealed three types of X (X_1, X_2, X_3) and five types of Y (Y_1, Y_2, Y_3, Y_4, Y_5) chromosomes, which were designated as Form A (X_1, Y_1), Form B (X_1,Y_2), Form C (X_2, Y_3), Form D (X_1, X_3, Y_4), and Form E (X_1, X_2, X_3, Y_5), depending upon the uniquely distinct characteristics of Y chromosomes. The five different karyotypic forms of *An. nitidus* found in this study were due clearly to the addition of extra block(s) of constitutive heterochromatin on sex chromosomes (X, Y), which is in keeping with Baimai's (1998) hypothesis. Baimai et al. (1984a,b, 1988, 1993b) suggested that the quantitative differences in heterochromatin of mitotic chromosomes could be used as a genetic marker for further identification of cryptic (isomorphic) or closely related species, as exemplified in the population cytogenetic studies of the *Anopheles dirus* complex and the Maculatus Group. Interestingly, investigation of the 18 isolines from Ubon Ratchathani Province, northeastern region, revealed only two karyotypic forms (Form D: 10 isolines; Form E: 8 isolines), whereas that of the three isolines from Phang Nga Province, southern region, yielded three distinct karyotypic forms (Forms A, B, and C) in each isoline, even though these two allopatric locations were placed ~800 km apart. The climate of these two provinces is quite different, i.e., Ubon Ratchathani Province has a tropical wet and dry climate, whereas Phang Nga Province is located on the shore to the Andaman Sea, and has heavy rain. Our results are in accordance with Saeung et al. (2014).

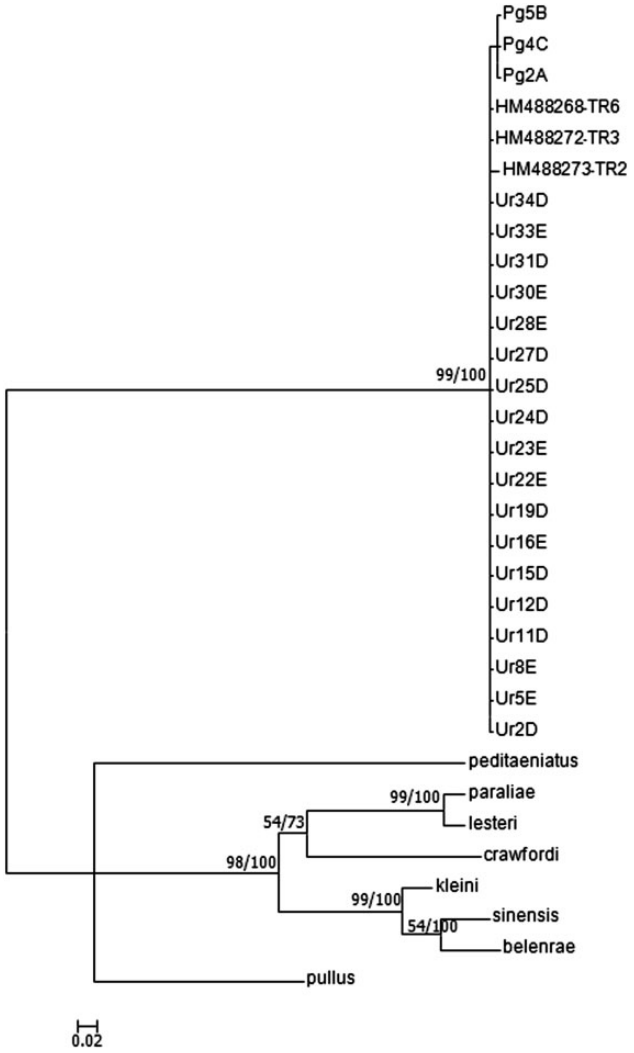

Fig. 5. Phylogenetic relationships of the five karyotypic forms of *An. nitidus* using Bayesian analysis based on ITS2 sequences compared with three specimens from Trat Province (Paredes-Esquivel et al. 2011) and eight species of the Hyrcanus Group. Codes for the specimens are listed in Table 1. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values >50% are shown. Branch lengths are proportional to genetic distance (scale bar).

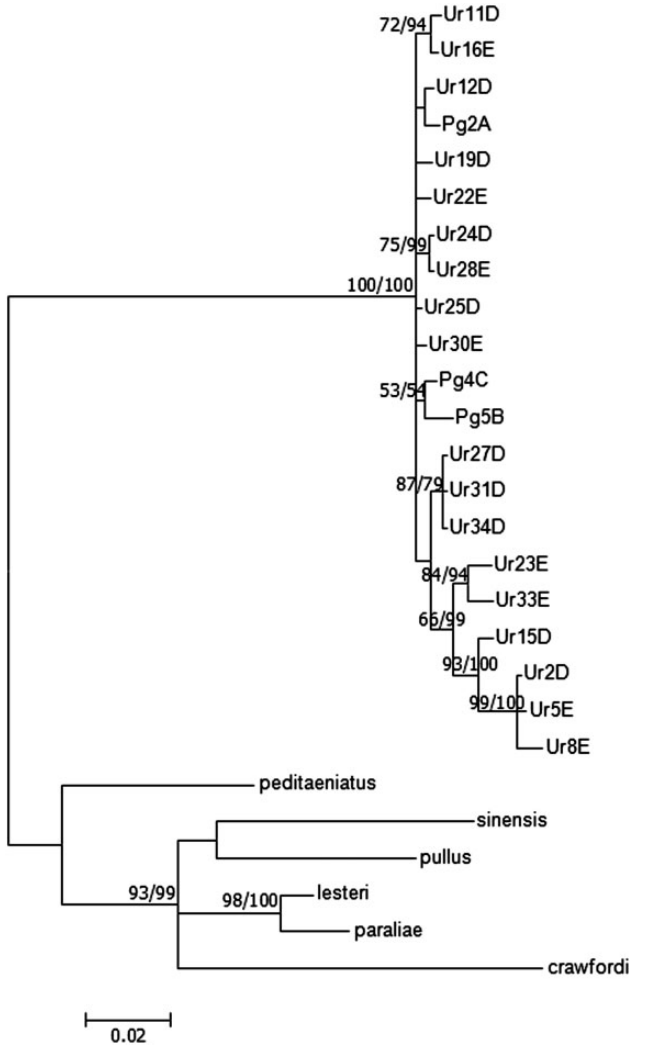

Fig. 6. Phylogenetic relationships among the five karyotypic forms of *An. nitidus* using Bayesian analysis based on combined COI and COII sequences compared with six species of the Hyrcanus Group. Codes for the specimens are listed in Table 1. Numbers on branches are bootstrap values (%) of NJ analysis and Bayesian posterior probabilities (%). Only the values higher than 50% are shown. Branch lengths are proportional to genetic distance (scale bar).

These authors showed that *An. crawfordi* Form A was detected only in Phang Nga Province, whereas Forms A, B, C, and D were found from eight isolines in Trang Province, which placed ~190 km apart. This phenomenon appeared to elucidate the difference in ecological diversity, which favored specific microhabitats for the karyotypic forms of *An. nitidus*. However, additional surveys are expected to obtain greater numbers of isolines from both provinces and/or other locations across six regions (northern, western, central, northeastern, eastern, and southern) of Thailand. This would bring about understanding of the population-genetic structure of this anopheline species.

Cross-mating experiments using anopheline isoline-colonies, relating to information on cytology and molecular analysis to determine postmating barriers, have been proven so far as an effective classical technique for recognizing sibling species and/or subspecies (cytological races) within *Anopheles* (Kanda et al. 1981; Baimai et al. 1987; Subbarao 1998; Junkum et al. 2005; Somboon et al. 2005; Saeung et al. 2007, 2008; Thongwat et al. 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009; Choochote 2011). Cross-mating experiments among the five karyotypic forms of *An. nitidus* showed no postmating reproductive isolation. They yielded viable progenies through F_2 generations and synaptic salivary gland polytene chromosomes, along the entire length of autosomes and the X chromosome. Thus, our results indicated that the five karyotypic forms were conspecific. Quantitative changes in constitutive heterochromatin in mitotic chromosomes of *An. nitidus* observed in this study were likely intraspecific chromosomal variation, which may lead to interspecific difference in the process of speciation. Our results are agreed with previous cross-mating experiments among sympatric and/or allopatric karyotypic forms of other anopheline species, i.e., *Anopheles vagus* (Choochote et al. 2002), *An. pullus* (= *An. yatsushiroensis*) (Park et al. 2003), *An. sinensis* (Choochote et al. 1998, Min et al. 2002, Park et al. 2008b), *Anopheles aconitus* (Junkum et al. 2005), *Anopheles barbirostris* A1 and A2 (Saeung et al. 2007, Suwannamit et al. 2009); *Anopheles campestris*-like (Thongsahuan et al. 2009), *An. peditaeniatus* (Choochote 2011, Saeung et al. 2012), and *An. paraliae* (Taai et al. 2013b).

Furthermore, this study incorporated a nuclear DNA and mtDNA sequence to increase the exact identification of this species from other species members of the Hyrcanus Group (Min et al. 2002; Park et al. 2003, 2008a; Choochote 2011; Taai et al. 2013a). The monophyletic

trees and very low intraspecific sequence variations (average genetic distances = 0.002–0.008) of the ITS2, COI, and COII of the five karyotypic forms are good supportive evidence, which confirms that these forms represent a single species of *An. nitidus*. It is interesting to note that the three specimens (TR2, TR3, and TR6) collected from Trat Province, eastern Thailand, and identified as the Hyrcanus Group by Paredes-Esquivel et al. (2011), based on ITS2 sequences, were clustered together with five karyotypic forms of *An. nitidus*, and are presumed to belong to that species.

In conclusion, this is the first report to clarify the species status of five karyotypic variants of *An. nitidus* collected from two locations in Thailand by using multidisciplinary approaches (cytogenetic investigations, cross-mating experiments, and molecular analyses) and indicate that these forms are of the same species.

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